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The Cassava Biotechnology Network

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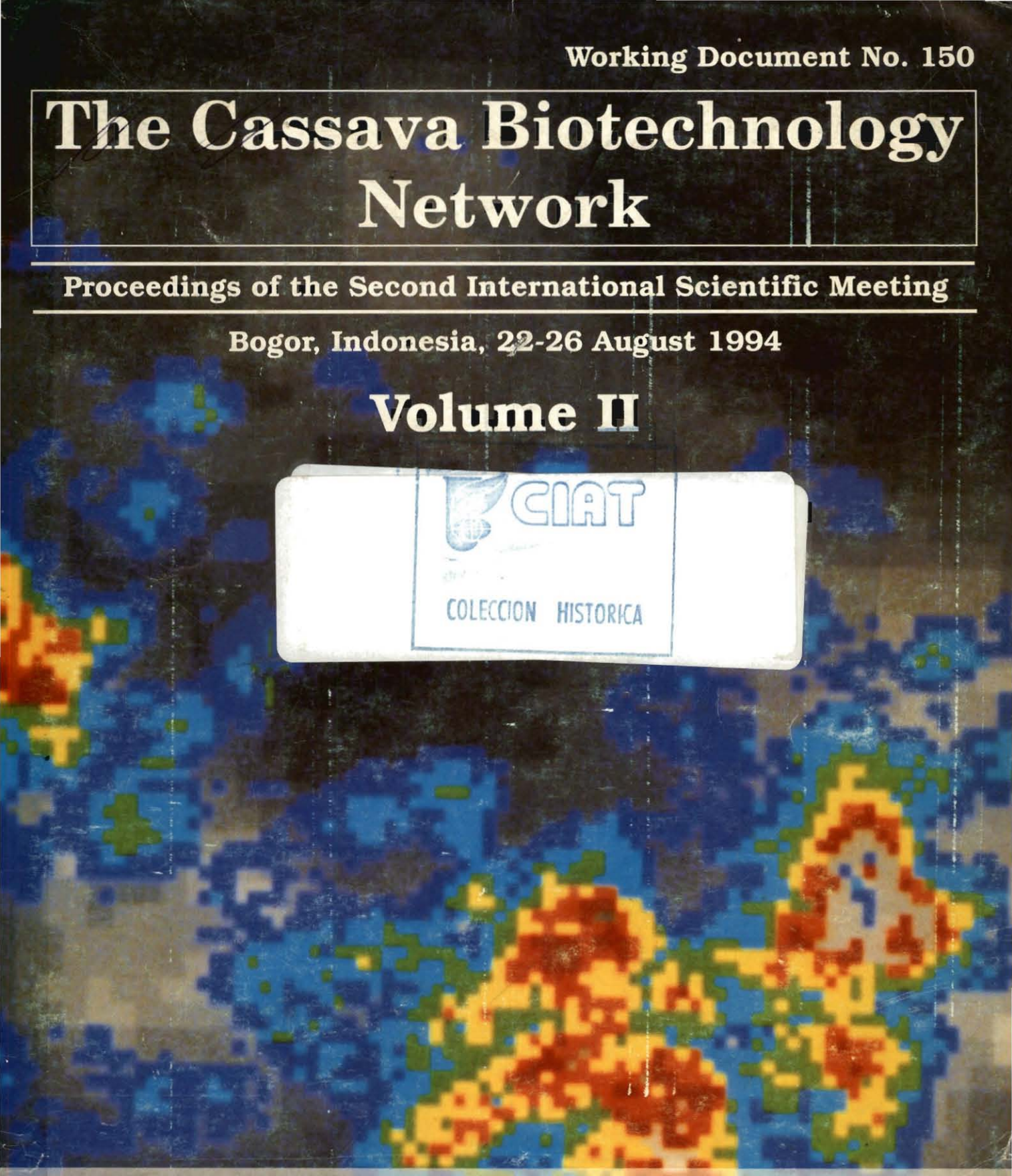
Volume II



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BIOCHEMISTRY AND MOLECULAR BIOLOGY OF CYANOGENESIS

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Hydrogen cyanide (HCN) is released when tissues in cassava are mechanically damaged. Two structurally related glucosides (linamarin and lotaustralin) undergo a sequential enzymatic breakdown caused by a β-glucosidase (linamarase) and an α-hydroxynitrilase (HNL). Recently, genes involved in cyanogenesis have been cloned from cassava, and from white clover, *Sorghum*, and black cherry. We summarize information so far obtained on the enzymes involved in cyanogenesis in cassava, including their structure, mechanism of action, and control of synthesis of linamarase. We also discuss the biosynthesis of cyanoglucosides and studies of HNL in relation to cyanogenesis in other species. Knowledge gaps will be highlighted. We also discuss the extent to which existing molecular and biochemical data can be used to devise strategies for reducing the hazard of HCN toxicity in cassava products, without compromising the protective function of cyanogenesis in this crop. We outline the work still needed to be done to further understanding of cyanogenesis in cassava.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is cyanogenic, that is, hydrogen cyanide (HCN) is released from damaged tissues in any part of the plant. Cassava food products must be extensively processed to remove HCN and its toxic precursors (Nambisan and Sundaresan, 1985). HCN in cassava tissues has been medically proven to be a potential health hazard for consumers if the plant is inadequately processed, particularly if the diet is poor and calories are mostly obtained from cassava (Howlett et al., 1990; Banea et al., 1992; Mlingi et al., 1992; Tylleskar et al., 1992). Cyanogenesis has been reported in over 3,000 plant species, although the source of the HCN has been identified in only about 300 (Poulton, 1990). In cassava, the cyanogenic glucosides, whose breakdown results in HCN production, were identified in 1968 by Nartey (1968). Although cassava cultivars differ in the level of HCN produced when tissues are damaged, no acyanogenic cultivar has been found. The genetic basis of this variation is now known.

The physiological role of cyanogenic compounds in plants is still a matter of debate. The production of HCN during tissue damage is widely believed to act as a deterrent to predation by small herbivores. The best experimental data testing this hypothesis come from studies in the polymorphic white clover (Hughes, 1991). Even so the picture is not clear. Evidence indicates that some species of slugs and snails will selectively eat the acyanogenic morph of white clover, but many insects and other species of slugs do not discriminate between cyanogenic and acyanogenic

plants. The polymorphism in white clover argues against a role in primary metabolism and the observed selective advantage of the acyanogenic morph in some environments suggests a metabolic or physiological cost to cyanogenesis. Indeed, in rubber and barley, increased levels of cyanogenesis may make the plants less resistant to fungal infection (Kieberei et al., 1989; Pourmohensi and Ibenthal, 1991). In cassava, the correlation between cyanoglucoside content and resistance to pests has been clearly demonstrated only for the burrowing bug, *Cyrtomenus bergi* (Bellotti and Arias, 1993).

CASSANOVA is a project to study the basics of the biochemistry and molecular biology of cyanogenesis in cassava. It is supported by the European Commission for Science and Technology through the Development Programme of the University of Newcastle, UK; the Royal Agricultural and Veterinary University, Denmark; Mahidol University, Thailand; and Oyo State University of Technology, Nigeria. This paper summarizes the progress so far made by the CASSANOVA project and other research groups, and relates cyanogenesis in cassava to cyanogenesis in other species. The paper focuses on the project's molecular characterization of the catabolic enzymes involved in HCN production during tissue damage and food processing.

BIOCHEMISTRY OF CYANOGENESIS

Cassava synthesizes and stores two structurally related cyanoglucosides (linamarin and lotaustralin) produced from the amino acids valine and isoleucine. These cyanoglucosides accumulate in the leaves and roots of the plant at ratios of about 93 parts of linamarin to 7 parts of lotaustralin (Nartley, 1968). During tissue damage, the cyanoglucosides become exposed to two catabolic enzymes that sequentially break them down to glucose, a ketone, and HCN. The first catabolic enzyme, cyanogenic β-glucosidase (linamarase), hydrolizes the cyanoglucoside to glucose and a cyanohydrin (α-hydroxynitrile). The second enzyme, α-hydroxynitrile lyase, breaks the cyanohydrin down to a ketone and HCN. Although the possibility of a turnover of cyanoglucosides in undamaged tissue has been suggested, the cyanoglucosides and catabolic enzymes are believed to be spatially separated in the intact tissue so that HCN is produced only after damage. The production of HCN from valine via linamarin is shown in Figure 1.

The cyanoglucosides (linamarin and lotaustralin) are produced by several plant species from widely different plant groups, as in the following list:

List of plants producing the cyanoglucosides:

1-cyano-1-methylethyl β-D-glucopyranoside (linamarin), and
R-1-cyano-1-methylpropyl β-D-glucopyranoside (lotaustralin).

Trifolium repens L. (white clover)
Manihot esculenta Crantz (cassava)
Linum usitatissimum L. (flax)
Phaseolus lunatus L. (lima bean)
Hevea braziliensis L. (rubber)
Lotus corniculatus L. (bird's-foot trefoil)

Cassava and rubber are members of the Euphorbiaceae; white clover, lima bean, and bird's-foot trefoil are members of the Leguminoseae; and flax belongs to the Linaceae. Both white clover and bird's-foot trefoil are polymorphic for cyanogenesis, that is, they have both cyanogenic and acyanogenic plants. This polymorphism has been studied most thoroughly in white clover (Hughes, 1991), where acyanogenic plants may lack the cyanoglucosides, linamarase, or both. The presence or absence of cyanoglucosides and linamarase is determined by two simply inherited genetic loci (Hughes, 1991).

Biochemical and physiological studies on cyanogenesis in the species itemized in the list above have shown both similarities and differences in the details of the process. For example, the seeds of both rubber and flax contain cyanoglucosides, whereas the seeds of white clover and cassava do not. During germination in rubber (Selmar et al., 1988) and flax (Smith et al., 1990), linamarin and lotaustralin, which are monoglucosides, are further respectively glycosylated to the diglucosides linustatin and neolinustatin. In rubber, linustatin is transported out of the endosperm to the growing seedling, where it is broken down to noncyanogenic compounds (Selmar et al., 1988). Thus, although cyanoglucosides are present in the seeds of rubber, they are also synthesized *de novo* during germination.

In the past few years, rapid advances have been made in the understanding of the proteins involved in cyanogenesis, and some genes have been cloned.

BIOSYNTHESIS OF CYANOGLUCOSIDES

A biosynthetic pathway for cyanoglucosides was first proposed on the basis of *in vivo* tracer experiments and has subsequently been confirmed with *in vitro* work. To summarize, the pathway involves first converting the precursor amino acid by membrane-bound enzymes to a hydroxynitrile (the cyanohydrin) via an oxime intermediate. The hydroxynitrile is then glycosylated by uridine diphosphate (UDP) glucosyltransferase, which behaves as a soluble enzyme. To date, the most detailed information on cyanogenic glucoside biosynthesis is for *Sorghum* (Moller and Poulton, 1993), where the conversion of L-tyrosine to p-hydrophenylacetaldehyde oxime is carried out by cytochrome $P_{450, TYR}$ (Sibbesen et

al., n.d.). This protein has been purified to homogeneity from isolated microsomal membranes.

A microsomal system has been isolated from cotyledons of young etiolated cassava seedlings (Koch et al., 1992), which converts valine and isoleucine to their respective cyanohydrins. This cyanohydrin synthesis is photo-reversibly inhibited by carbon monoxide, demonstrating the involvement of cytochrome P_{450} in the hydroxylation processes in cassava. In cyanohydrin biosynthesis, the oxime is the intermediate most commonly and easily detected *in vivo* (Hughes and Conn, 1976) and metabolized *in vitro* (Collinge and Hughes, 1982). This is partly a consequence of the two N-hydroxylation reactions in the conversion of amino acid to oxime being carried out by a single enzyme, cytochrome P_{450} (Sibbesen et al., n.d.).

The final step in cyanoglucoside biosynthesis is the glycosylation of the cyanohydrin by a soluble uridine diphosphoglucose (UDPG) glucosyltransferase. A wide range of secondary plant compounds are glycosylated, commonly by a UDPG-glucosyltransferase, to produce a stable water-soluble compound.

Given the reported specificity of these enzymes and the large number of potential substrates, a wide range of UDPG-glucosyltransferase enzymes can be expected to occur within a single species (Harborne, 1988). Cassava UDPG-glucosyltransferase has been partially purified (Mederacke and Hughes, unpublished), and its activity studied in different tissues during seed germination.

A heterologous probe was used to isolate six different UDPG-glucosyltransferase genes from a cassava cotyledon cDNA library (Hughes and Hughes, n.d.). Their pattern of expression has been studied in the tissues of germinating cassava seedlings. The identification of the cyanoglucoside biosynthetic UDPG-glucosyltransferase among these genes awaits either further purification of the enzyme to produce peptide sequence data or the analysis of the substrate specificity of the proteins encoded by these genes. For the latter, two genes have been put into bacterial expression vectors to allow purification of the expressed protein.

Unlike white clover (Hughes, 1991), cyanoglucosides are found in cassava roots. In young cassava seedlings, active microsomes were produced only from cotyledons and petioles, despite the presence of cyanoglucosides in hypocotyls and roots (Koch et al., 1992). This finding supports those of several physiological studies that suggested that cyanoglucosides are transported from leaves to roots (Makame et al., 1987) but were at odds with the presence of acetone cyanohydrin UDPG-glucosyltransferase activity in cassava seedling roots (Mederacke and Hughes, unpublished). Recently, McMahon & Sayre (these proceedings) have demonstrated the synthesis of linamarin *in vivo* in roots.

CATABOLISM OF CYANOGLUCOSIDES

Linamarase

The cassava gene for linamarase was cloned as a cDNA in 1992 by Hughes et al., and the protein purified by many groups (Eksittikul and Chulavatnatol, 1988; Mkpong et al., 1990; Hughes et al., 1992). The native active enzyme exists as multimeric aggregates of a 70,000-Mr glycoprotein subunit. About 7% of the molecular mass is N-linked oligosaccharide molecules (Hughes et al., 1992). The protein has more than a 70% similarity of amino acid sequence with the cloned white-clover linamarase, and considerable homology to other group A β-glycosidases—the closest nonplant enzyme being that from *Agrobacterium* spp. (Hughes, 1993). These glycosidases are retaining β-glycosidases and, in this type of enzyme, hydrolysis probably occurs in a double-displacement mechanism that involves a catalytic diad (an acid catalyst and a nucleophile, which stabilizes a transition state). Affinity labelling of the *Agrobacterium* β-glucosidase with the irreversible inhibitor, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucose, identifies the glutamate residue in the peptide, I/VTENG, as the catalytic nucleophile (Withers et al., 1990). This peptide is highly conserved and present in cassava linamarase.

Keresztessy et al. (n.d.), by using the irreversible inhibitor, N-bromoacetyl-β-D-glucopyranosylamine, directly identified the glutamate, Glu-198, as the cassava-linamarase, active-site, carboxylate group, with an acid catalytic function. This glutamate lies within another highly conserved peptide, FNEP. Keresztessy et al. (n.d.) also showed, through kinetic studies, that a functional histidine residue is present in the active center. Recently, Tolley et al. (1993) crystallized the closely related cyanogenic β-glucosidase (linamarase) from white clover. On analyzing this substance, the active center was found to contain glutamate residues from I/VTENG and FNEP arranged in an appropriate configuration. The active center also contains a conserved histidine residue.

Cassava linamarase has been expressed as an active protein in the bacterium, *E. coli* (Keresztessy et al., unpublished). Because we can produce the enzyme from cloned DNA and understand the structure of the enzyme's active site we can redesign the enzyme to improve the efficiency of cyanoglucoside degradation during cassava processing.

Southern blot analysis of the cassava genome indicates that it contains more than one gene for linamarase. Restriction fragment length polymorphism (Hayson et al., n.d.) shows that considerable variation exists in these genes between cultivars. Sequence analysis of the genomic linamarase DNA is only preliminary, but indicates a complicated structure that has, so far, seven introns (Liddle et al., unpublished). We know that the expression of the linamarase genes is restricted to the latex vessels of the plant, where it is extremely high (Pancoro and Hughes, 1992).

α-Hydroxynitrile lyase

The second enzyme involved in the breakdown of cyanoglucosides is α-hydroxynitrile lyase (HNL). This enzyme has been purified from cassava leaves and characterized by Hughes et al. (1994). Although it is not present in the latex, its exact tissue location is not known. Cassava HNL has been cloned as cDNA (Hughes et al., 1994). Comparison of the known enzyme N-terminal amino acid sequence with the cDNA coding sequence indicates that no signal peptide is found on the nascent polypeptide, suggesting a cytosolic location. But some evidence exists for S-S bonds in the native protein, which argues against this interpretation of the location.

HNL proteins have been purified and characterized from a number of cyanogenic species (Table 1). When the structure and physical properties of these enzymes are compared, they are seen to form a highly heterogeneous group, unlike the cyanogenic β-glucosidases. The HNL from *Prunus serotina* (black cherry) has also been cloned (Cheng and Poulton, 1993), but this enzyme shows no homology with cassava HNL, at either the DNA or amino acid level. This heterogeneity of HNL enzymes indicates that care must be taken when making conclusions about cyanogenesis in one species based on work carried out in another.

Studies on the chromosomal organization of the HNL gene in cassava are preliminary, but Southern blot analysis of genomic DNA indicates that the gene is probably a single-copy type. Sequence analysis shows that it has two introns (Hughes and Hughes, unpublished).

LOCALIZATION

Spatial separation of cyanoglucosides and catabolic enzymes is a key feature of cyanogenesis. Knowing the locations of the components is vital for developing strategies to redesign the cassava plant so that the potential toxicity of its food products is eliminated without compromising the possibly protective function of cyanogenesis. We know very little about the localization of cyanoglucosides in cassava. Earlier work suggesting that their synthesis is limited to leaves is questionable as they also appear to be synthesized in root tissues. Judging from studies in other cyanogenic plants, we can expect them to be stored within vacuoles (Saunders and Conn, 1978). The cyanogenic β-glucosidase (linamarase) is localized in latex vessels (Pancoro and Hughes, 1992), but the location of HNL is not known.

The best studies of localization of cyanogenic components are in *Sorghum* and *Prunus*. In both these species, although characterized by intracellular compartmentalization, they are also characterized by tissue level compartmentalization. Thus, in *Sorghum*, the cyanoglucosides are found in the

vacuoles of the leaf epidermal cells, and the β-glucosidase and HNL are located in the underlying mesophyll cells (Saunders and Conn, 1978; Kojima et al., 1979). In *Prunus domestica* (plum) seeds, two cyanogenic glycosides are produced: the diglucoside, amygdalin, and the monoglucoside, prunasin. This species also produces two cyanogenic glycosidases, amygdalin hydrolase (AH) and prunasin hydrolase (PH). AH and PH are located in protein bodies within specific cells in the procambium, whereas the glycosides occur only in the cotyledonary parenchyma and are absent from the procambium (Poulton and Li, 1994).

The presence of linamarase and absence of HNL and cyanoglucosides in cassava latex cells suggest that similar tissue level compartmentalization also occurs in this species. Knowledge of the molecular controls of expression in these genes is important for reprogramming expression to produce a plant in which cyanoglucoside degradation is faster, thus, making both HCN release during processing more efficient and, at the same time, the plant more cyanogenic.

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Table 1. Comparing α-hydroxynitrile lyase enzymes from six cyanogenic species.

Species	Cyanoglucoside (precursor)	α-Hydroxynitrile lyase		
		Glyco-protein	Lavin group	Subunit molecular mass
Cassava	Linamarin (valine) Lotaustralin (isoleucine)	X	X	28,500 (homotrimer)
Hevea	Linamarin (valine) Lotaustralin (isoleucine)	†	†	†
Flax	Linamarin (valine) Lotaustralin (isoleucine)	X	X	42,000 (homodimer)
White clover	Linamarin (valine) Lotaustralin (isoleucine)	‡	‡	‡
Sorghum	Dhurrin (tyrosine)	✓	X	22,000 + 33,000 (heterotetramer)
Prunus (black cherry)	Prunasin (phenylalanine)	✓	✓	58 to 60,000 (monomer)

† = Not characterized.
‡ = None identified.

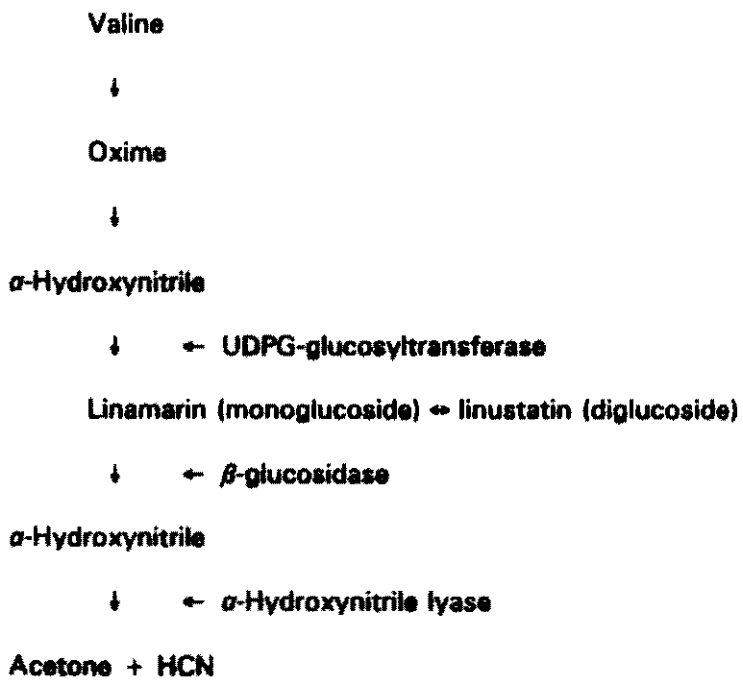

Figure 1. Production of HCN from valine, via linamarin.

MANIPULATING CASSAVA CYANOGENESIS IN TISSUE CULTURE BY USING EXTRINSIC FACTORS

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Together with genetics, environmental factors exert strong influence on the cyanide content of cassava roots. To create acyanogenic varieties of cassava, genetic engineering still requires much more investment. But better understanding of the extrinsic factors that control cyanogenesis may result in alternatives that are practical to apply for managing the cyanide content of cassava roots. To this end, cassava plantlets in tissue culture were first shown to accumulate linamarin during the aseptic growth. This system was then employed to search for and evaluate different extrinsic agents that could interfere with linamarin synthesis. Based on the molecular knowledge of linamarin biosynthesis, several inhibitors of glucosyltransferase and cytochrome P_{450} were identified and tested in tissue culture for specific suppressive effects on the linamarin level of cassava plantlets. Field trials of these compounds could lead to a practical agro-management of the cassava cyanide problem.

INTRODUCTION

Despite years of research and breeding, no cyanide-free cassava variety is available. Thus, chronic cyanide toxicity remains a health threat to 500 million poor people who subsist on cassava. Although food processing effectively removes most of the toxic cyanide, it adds to the cost of this staple food of the poor.

Cassava cyanide exists in three forms: the stable cyanogenic glucosides (linamarin and lotaustralin), the unstable cyanohydrins (acetone and butanone cyanohydrins), and the free HCN. Many advances have been made toward understanding the biology and metabolism of cyanogenic glycosides (Conn, 1991; Nahrstedt, 1992). Properties of the key enzyme (Eksittikul and Chulavatnatol, 1988; Koch et al., 1992) and the genes involved (Hughes et al., 1992; Pancoro and Hughes, 1992) have also been described. Genetic engineering may soon create a cyanide-free cassava variety.

However, before undertaking an expensive mission to produce an acyanogenic variety, cassava needs to be tested for survival under acyanogenic conditions: cyanogenic glucosides are known to serve as sources of nitrogen in plants (Selmar et al., 1988; Blumenthal-Goldschmidt et al., 1963). One way to manipulate the level of cyanogenic glucosides *in vivo* is to use specific inhibitors.

In this study, tissue culture of cassava plantlets has been chosen to avoid multiple variables that normally prevail in the field. As the biosynthetic pathway for linamarin is known, choices of suitable inhibitors can be made and used to lower the linamarin content in plantlets in tissue culture.

MATERIALS AND METHODS

Plant materials and chemicals

Plantlets of the cassava cultivar Rayong 2 were obtained from the Yupa Mongkolsuk Tissue Culture Laboratory of the Kasetsart University. Chlorobutanol, coumarin, and chlorpromazine were purchased from Sigma. Isobutyraldehyde and 2-methylbutyraldehyde were obtained from Aldrich. All other chemicals were of analytical grade.

Cassava plantlet propagation

Stem sections with lateral buds were cut from cassava plantlets and used for further propagation by plant tissue culture as described by Roca (1984). Leaves and petioles were removed, and the stems with lateral buds cut into 1-cm pieces and placed in sterile tissue culture bottles containing Murashige and Skoog's (MS) medium (Roca, 1984). The bottles were kept in a plant tissue culture room at 28-30 °C, with a photoperiod of 8 h of light and 14 of darkness. The light was supplied by a set of daylight and red-light lamps placed in alternate positions. The light intensity was 3,500 lux. Stems from 3-month-old plantlets were used for subculturing and propagation.

Linamarin extraction and assay

Linamarin was extracted from cassava tissues by using boiling 80% ethanol (Nambisan and Sundaresan, 1984). The linamarin content was determined by using linamarase (Eksittikul, 1986).

Treating plantlets in tissue culture with test compounds

To test if a compound was suppressing the linamarin level in cassava plantlets in tissue culture, the compound was first filtered through a sterile millipore membrane (pore size was 0.4 micron). With a sterile serological pipette, the filtered solution (0.5 ml) was added directly and aseptically on the surface of an MS agar in a culture bottle in which cassava plantlets were grown for 2 months. The bottle was closed and kept for 10 days. During this period, any abnormality in the appearance

and color of the plantlets was noted. After 10 days of treatment with each compound, the stem length was measured and linamarin was extracted and determined separately from leaves and petioles.

RESULTS

Linamarin synthesis and growth of cassava plantlets in tissue culture

Cassava plantlets were maintained in tissue culture for 6 months. During the period, the stem grew from 2-3 cm in the first month through 15-20 cm in the fourth month to 20-25 cm in the sixth month. The cassava plantlets accumulated linamarin during growth under the aseptic conditions (Table 1). Linamarin concentrated mainly in leaves and petioles, levels being much lower in stems and roots. The roots of cassava plantlets under tissue culture, however, do not form tubers. Because the MS medium, a growth medium, contained 30 mM glycine as the only amino acid and valine is the precursor of linamarin, glycine was replaced by valine to see if linamarin content could be enhanced. When 30 μM or 300 μM valine was used instead of 30 μM glycine in the MS medium, neither linamarin levels nor growth (stem length) was affected.

Effects of inhibitors of glucosyltransferase on linamarin content and growth of cassava plantlets in tissue culture

Several analogs of acetone cyanohydrin were found to inhibit glucosyltransferase, which catalyzes the last step in the biosynthesis of linamarin (Sanghirun, 1993). These were tested to see if any could lower the linamarin content in the plantlets. Among three analogs tested, two showed a suppressive effect on the linamarin content without any growth abnormality (Table 2): 0.35 mM 2-methylbutyraldehyde and 0.5 mM chlorobutanol. However, 1 mM isobutyraldehyde did not affect linamarin content or plantlet growth.

Effect of inhibitors of cytochrome P_{450} on linamarin content and growth of cassava plantlets in tissue culture

Two inhibitors of cytochrome P_{450}, coumarin and chlorpromazine, were found to inhibit linamarin synthesis (Sanghirun, 1993) because cytochrome P_{450} is involved in the microsomal oxidation of valine into acetone cyanohydrin (Koch et al., 1992). In plantlets in tissue culture, 0.5 mM coumarin was found to lower linamarin content without affecting plantlet growth (Table 2), but 02 mM chlorpromazine caused abnormality in growth without affecting linamarin content.

Because methanol was needed to dissolve the test compounds, a control was carried out to show that 0.8% methanol did not affect either the linamarin content or growth (Table 2).

DISCUSSION

The finding that cassava plantlets can form and accumulate linamarin under aseptic conditions (Table 1) indicates that the cyanogenic glucoside can be made in the absence of external pathogens. The finding also allows tests to be performed on the effects of extrinsic compounds on linamarin levels. Although valine is the known precursor for the linamarin synthesis, the accumulation of linamarin cannot be enhanced by an excess of the amino acid.

The observations that linamarin contents in cassava plantlets can be significantly suppressed without growth abnormality in the presence of an inhibitor (2-methylbutyraldehyde, chlorobutanol, or coumarin [Table 2]) implies that these compounds may be useful in the field to suppress linamarin levels in cassava. However, field testing must be carried to verify this suggestion because field cassava plants form tubers, whereas plantlets in tissue culture do not.

Because, under each suppressive condition, some residual amount of linamarin remains, to conclude that linamarin-free cassava plantlets will grow normally is not yet possible. This is a major limitation associated with this type of experiment. At higher concentrations of each inhibitor, growth becomes abnormal (Table 2), possibly as a result of side effects from the inhibitor. For example, 2-methylbutyraldehyde can be a substrate of cytochrome P_{450} oxidases to form olefinic compounds (Roberts et al., 1991). Coumarin may inhibit other mixed-function oxidases requiring P_{450} (Goeger and Anderson, 1991). Chlorobutanol may alter CA^{2+} transport (Habara and Kanno, 1993).

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Table 1. Linamarin content and plantlet length during growth of cassava plantlets in tissue culture.

Cassava	Linamarin (μmol/g fresh wt)	
	2 months old	3 months old
Leaf and petiole	2.33 ± 2.25	3.25 ± 4.07
Stem	0.46 ± 0.66	0.91 ± 1.21
Root	0.08 ± 0.25	0.21 ± 0.37
Length (cm)	8.04 ± 4.16	14.47 ± 5.95

Table 2. Stem lengths and linamarin contents of cassava plantlets in the presence of a test compound. Cassava plantlets 2 months old in tissue culture were treated with each test compound and allowed to grow for 10 days before stem length and linamarin content were determined.

Test compound	Concentration (mM)	n	Stem length (cm)		Linamarin content (mmol/g fresh wt)			
					Leaves		Petioles	
			x ± SD	"t" test	x ± SD	"t" test	x ± SD	"t" test
2-Methyl-butyraldehyde	0.2 (in 0.2% CH_3OH)	10	12.85 ± 2.20	P>0.05	4.75 ± 2.60	P>0.05	2.26 ± 1.37	P>0.05
	0	10	13.06 ± 2.06		5.93 ± 1.83		3.34 ± 2.04	
	0.35 (in 0.35% CH_3OH)	9	10.66 ± 1.09	P>0.05	0.91 ± 0.72	P<0.01	0.60 ± 0.36	P<0.01
	0	9	10.30 ± 1.8		2.68 ± 1.56		2.85 ± 2.16	
	0.40* (in 0.40% CH_3OH)	10	11.37 ± 1.42	P>0.05	1.06 ± 0.74	P<0.01	0.76 ± 0.52	P<001
	0	10	10.50 ± 1.53		5.74 ± 3.90		4.82 ± 3.50	
Iso-butyraldehyde	0.5 (in 0.2% CH_3OH)	10	10.26 ± 1.74	P>0.05	3.92 ± 2.33	P>0.05	3.11 ± 1.42	P>0.05
	0	10	11.97 ± 1.55		4.39 ± 2.60		3.68 ± 2.17	
	1.0 (in 0.2% CH_3OH)	10	11.40 ± 1.88	P>0.05	4.93 ± 2.89	P>0.05	3.11 ± 1.42	P>0.05
	0	10	11.59 ± 2.12		4.75 ± 2.91		3.68 ± 2.17	
	2.0* (in 0.4% CH_3OH)	10	11.48 ± 1.48	0.01<P<0.05	2.33 ± 1.55	P>0.05	2.82 ± 1.26	P>0.05
	0	10	14.20 ± 1.89		3.57 ± 2.76		3.32 ± 2.43	
Chlorobutanol	0.5 (in 0.21% CH_3OH)	10	11.54 ± 1.66	P>0.05	0.79 ± 0.36	0.01<P<0.05	1.59 ± 0.63	P>0.05
	0	10	11.66 ± 1.84		2.67 ± 2.68		2.95 ± 2.48	
	1.0* (in 0.42% CH_3OH)	10	11.00 ± 1.69	0.01<P<0.05	2.65 ± 1.47	P>0.05	♣2.62 ± 1.32	P>0.05
	0	10	13.43 ± 1.78		2.63 ± 1.61		2.55 ± 1.53	

(Continued)

Table 2. (Continued).

Test compound	Concentration (mM)	n	Stem length (cm)		Linamarin content (mmol/g fresh wt)			
					Leaves		Petioles	
			x ± SD	"t" test	x ± SD	"t" test	x ± SD	"t" test
Coumarin	0.5 (in 0.4% CH_3OH)	10	10.27 ± 1.39	P>0.05	0.94 ± 0.54	P<0.01	1.44 ± 1.14	P>0.015
	0	10	9.29 ± 1.38		4.21 ± 2.65		4.48 ± 3.11	
	1.0* (in 0.8% CH_3OH)	9	8.35 ± 1.49	0.01<P>0.05	1.48 ± 1.85	0.01<P<0.01	1.48 ± 0.90	0.01<P<0.05
	0	9	10.55 ± 1.72		4.29 ± 1.97		3.64 ± 1.48	
Chlor-promazine	0.2*	10	11.13 ± 1.20	0.01<P>0.05	2.09 ± 0.80	P>0.05	2.24 ± 1.38	P>0.05
	0	10	13.35 ± 1.89		2.39 ± 1.29		2.28 ± 1.24	
	0.5**	10	8.08 ± 0.96	P>0.01	2.62 ± 1.79	P>0.05	3.18 ± 1.06	P>0.05
	0	10	11.78 ± 1.76		3.40 ± 1.95		3.33 ± 2.47	
	1.0**	10	7.76 ± 0.84	P<0.05	2.62 ± 1.79	P>0.05	3.18 ± 1.06	P>0.05
	0	10	11.03 ± 1.76		3.40 ± 1.95		3.33 ± 2.47	
Methanol	0.8%	11	13.17 ± 1.43	P>0.05	3.81 ± 2.40	P<0.05	2.29 ± 0.93	P>0.05
	0	12	12.12 ± 0.83		3.34 ± 1.61		2.38 ± 0.97	

* Slight retardation of growth: Slightly smaller stems and folded leaves were found in some treated plantlets.
** Severe retardation of growth: Smaller stems and withered leaves were found.
♣ n = 9.

LINAMARASE ACCUMULATION IN CASSAVA LEAVES (*MANIHOT ESCULENTA* CRANTZ)

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Since the cassava (*Manihot esculenta* Crantz) is one of the most widely consumed foods in the tropics, we have to consider the toxicity of hydrogen cyanide (HCN) produced by cyanogenic glucosides hydrolyais. In order to contribute to the understanding of the cyanogenesis process in cassava it is necessary to establish a correlation between the linamarase enzimatic activity in the leaves during maduration and the cyanogenic glucosides content in the root parenchyma. Four cultivars were analyzed. The methods used in the analysis were described by Ikediobi, Ongia and Eluwah (Agric. Biol. Chem. 44(12)2803-2809, 1980). The correlation between enzymatic activity in young, fully expanded leaves (1-2 weeks old) and the content of cyanide (CN) in root parenchyma is significantly higher with a correlation of 0.99 and one lower probability of 1 per one thousand ($P < 0.01$). In mature leave (12-14 weeks old) the estimated correlation coefficient is not significant to the level of 70% ($P > 0.7$). The results suggest that enzyme accumulation in the leaves occurs later in the "bitter" cassava plant, with high cyanogenic content in root parenchyma than in plants with low content of CN in the root parenchyma. This can be interpreted as a later linamarase accumulation of the cassava leaves.

INTRODUCTION

One of the most consumed foods by the people from the tropics is the cassava (*Manihot esculenta* Crantz). Around 500 million people from 30 tropical countries have in it an important daily supply (Cock, 1992), the world production is 161.5 million tons as has been estimated by FAO for 1991. From which 33,4 million were produced by Latin America (CIAT, 1991; FAO, 1992). It can be cultivated in poor soils and can tolerate adverse natural factors.

In this crop, it is so important to have a good knowledge about the cyanogenesis, the process of cyanide production and liberation. This process occurs when the plant tissue is destroyed or injured and the precursory cyanogenic glucosides are hydrolysed. The broken of the differential partitioning of the enzyme and the precursor substrate makes possible the hydrolysis process (Poulton, 1988). The main cyanogenic glucosides are linamarine (2-hydroxylsobutyronitril-a-D-glucopyranoside) and lotaustraline (2-hydroxy-2-methilbutyronitril-b-D-glucopyranoside), with a 93 and 7% of the total cyanogenic matter in the extracts (Nartey, 1968).

Linamarase exposed to the action of b-glucosidase, when roots are sliced for processing, hydrolyses into glucose and cyanohydrin-acetone. The last can dissociate into a cetone and cyanidric acid, either under the action of an a-hydroxynitril liase or when the pH is equal or less than 5.

A large amount of linamarine root content completely hydrolyses during the preparation process and the cyanidric acid volatilizes. However some amount could be hydrolyzed after consumption of the processed roots, inside the human or animal body.

Coursey (1973) provides the following toxicity relation of cyanide in cassava:

- Non toxic < 50 ppm
- Moderate toxic 50-100 ppm
- Dangerous toxic > 100 ppm

The glucoside and cyanhydric acid content is different according to the plant organ.

Montaldo (1985) mentions a 1973 Bruijn's table, with the glucoside concentration according to the plant part. This concentration is so variable with the highest concentration in fully developed leaves.

MATERIALS AND METHODS

The cassava cultivars employed in this experiment were: Gancho, Collita, Rama Negra and Yuca Brava, recommended by the Roots and Tuber Research Programme of the "El Vallecito" Agricultural Research Institute of the "Gabriel René Moreno" Autonomous University of Santa Cruz de la Sierra, Bolivia.

For the enzymatic activity determination two kinds of leaves were used, recently expanded leaves (1 or 2 weeks) and mature leaves (12 or 14 weeks). Collected in the early morning and once separated from the plant, they were conserved under cold conditions (less than 0 °C) until their use. The cyanogenic glucosides were determined on the root parenchyma of the same plants. The root harvest always was done on plants with the same maturity stage. The root samples were obtained at the same day of the analysis.

The determination method is described by Ikediobi et al. (1980).

Enzyme activity determination in leaves

- From the leaves fractioned into small pieces, a 25 g sample were weighed and it was well homogenized.

- The leaves were homogenized in 75 ml of buffer acetate (pH 5.5) under cold conditions. The buffer was previously cold stored.

- The mixture was vacuum filtered into a cold recipient, with filters Wattman #1 to retain celulose residues from the cell wall.
  This filtrate which contains enzymes and substrate, was stored at 4 °C, to slow down any reaction that could occur.

- 1 ml of buffer phosphate (0.01 M, pH 6.8), with 62 mg of p-nitrophenyl-b-glucopyranoside and 100 ml of the enzyme preparation was added, mixed and incubated for 1 h at 37 °C in Maria's bath.

- To stop the reaction 2 ml of borate buffer, 0.2 M (pH 9.8) was added.

- The mixture was raised with distilled water up to 4 ml.

- The transmittance was measured at 400 mm level and to calculate the absorbance.

- An activity unit (V) of linamarase is defined as the enzyme amount which can produce in 1 min of digestion a change of 0.001 in the absorbance measured at 400 mm under the described conditions.

- From the absorbance in thousands obtained at 400 mm, the number of units is multiplied by the dilution to obtain the number of units present in the reaction sample with the pnp-glucoside. This activity is divided into the volume of the extract used and the incubation time to obtain the activity of the extract of the raw enzyme. The activity in the extract was normalized by grame of fresh tissue, multiplying by the volume of buffer used in the extraction and dividing into the leaves fresh weight.

Determination of the CN^- content in the root parenchyma

The semipurified enzyme obtained from the root cortex was used for the determination of the cyanogenic glucosides, it was prepared as follows:

- Small portions from the cortex of cassava roots were fractioned.

- A weight of 100 g were homogenized under cold conditions in 300 ml of acetate buffer (pH 5.5).

- The mixture was vacuum filtered into a cold container.

- The filtrate was used for a second sample of 100 g of cortex and this was used again for the extraction of a third sample of 100 g. This is done to obtain a greater enzyme activity per volume unit of buffer extraction.

- To precipitate the proteins of the extract, 2.3 volumes of cold acetone were added to the filtrate and the mixture were shaked 2 h over ice in many 2 min intervals. For a better separation the precipitate were centrifuged at 500 rpm per grame during the 5 min.

- The greenish precipitate was collected by decantation and was extracted three successive times with portions of 10 ml of buffer acetate 0.1 M (pH 5.5) in which the enzyme was solubilized.

- The extract was centrifuged at 500 rpm per grame during 5 min and the floating material was dialized in cold with buffer acetate 0.1 M (pH 5.5).

- The semipurifed enzyme obtained was evaluated and was diluted in buffer acetate to obtain the required activity in the determination of the cyanogenic glucosides in the root.

The procedure used to evaluate the activity of the enzyme preparation is similar to the one described for leaves.

- The absorbance was calculated from the transmittance lecture obtained at 400 nm. The thousand units of absorbance were obtained from the described conditions. The number of units calculated from the absorbance were multiplied by the dilution to obtain the number of units present in the reaction sample with the pnp-glucoside. This activity thus obtained was divided into the volume of extract used and the incubation time to obtain the extract activity of the semipurifled enzyme.

- This activity value was used in the calculation of the volume of extract added in the reaction with the extract of root parenchyma for the CN^-.

- Until its use, the enzyme solution was cold preserved to avoid its degradation.

Preparation of the root parenchyma extract

- 25 g of cassava fresh root parenchyma were homogenized under cold conditions in 75 ml of HCl 0.1 M for 3 min.

- The homogenized was vacuum filtrated, the resulting filtrate was adjusted to pH 6.8 with KOH.

- The filtrate was centrifuged in cold at 500 rpm per grame for 3 min and the floating was collecting.

- The sample was maintained cold until its analysis.

Quantitative determination of the CN^-

- 0.5 ml from the root parenchyma extract was put together with 50 ml of semipurified enzyme solution, with a minimum activity of 250 units. This is an excess of enzyme to guarantee that the reaction will take place in a short time.

- It was raised to 2 ml with buffer phosphate 0.2 M (pH 6.8), to take the reaction in the most favorable condition for the enzyme.

- It was incubated at room temperature in a closed test tube for 10 min, to help the reaction of the enzyme-substrate complex.

- From this point the samples were processed and the standard solutions were simultaneously prepared using known concentrations of KCN.

- 5 ml of alcaline picrate were added to each test tube.

- They were incubated 20 min in water bath at 95 °C to favor the reaction that makes the cyano-picrate complex with an orange color, and whose intensity is a function of the concentration of CN^- is a constant picrate concentration has been maintained. After that, they were leaved in rest until they reached the room temperature.

- The standard solutions and the leaf samples were diluted 11 times (0.5 ml of solution with 1 of water).

- The transmittance was read at 490 nm, and was transformed to absorbance units. Absorbance is used to obtain a straight line as a function of the concentration. This makes easy the interpretation of the obtained values of the samples on the line made with the values from the standard solutions.

- Using the CN^- concentrations from the standard solutions expressed in mg/ml (ppm) as an independent variable and the absorbance values obtained at 4909 nm, a linear regression is fixed using the minimum quadratic method (Snedecor and Cochran, 1968). In this event the absorbance at 490 nm is a

linear function of the CN^- concentration, only if the picrate is maintained constant and in excess in order not to be a limitation for the reaction.

RESULTS

Activity of the enzyme in leaves

Table 1 shows the enzymatic activities in the recently expanded leaves and in the mature leaves for the four cultivars studied. The first column shows the cultivar name, the second and third columns show the activity on recently and mature leaves respectively.

CN^- concentration on the root parenchyma

The activity of the semipurifeid enzyme from the root cortex, in the hydrolisis of the p-nitrophenyl-b-glucopyranoside was evaluated. The transmittance obtained were 16%, which is equivalent to an absorbance of 0.795. This absorbance corresponds to 795U. Considering the dilutions made for the readings, the buffer volume used in the reaction with the pnp-gluco-piranoside and the incubation time, the enzymatic activity obtained was 54,325U/ml-min.

For the CN^- determination a volume of 50 ml of the semipurifeid enzyme solution was used, with an equivalent of 2716U. This activity excess makes sure a complete reaction (Ikediobi et al., 1980).

Standard curve for the CN^- determination

Table 2 shows the results of the regression between the data of the CN^- concentration of the standard solutions used, and the absorbance values measured. The values from the Y intersection, when X = 0 and the regression coefficient (DY/DX) make possible to calculate the CN^- concentrations in the root parenchyma based on the absorbance values obtained from the samples.

The intersection on the Y axis, -0,0083 ± 0,342, does not differ from zero. The slope indicates that for a ppm increment of CN^-, an absorbance increment of 0,049 ± 0,001 takes place.

Table 3 shows the results of the cyanogen content determinations from the root parenchyma. The first column shows the cultivar name, the second and third columns show the transmittance values read on the processed samples and the calculated absorbance values respectively; the fourth column shows the CN^- concentrations in the extracts; and the last column show the cyanogen concentration as CN^- mg of CN^-/g of fresh weight of root parenchyma.

DISCUSSION

A correlation analysis was conducted to know the interaction between the enzymatic activities in leaves and the cyanogenic content in roots. The correlation or association degree between this two variables van be mathematically expressed by the correlation coefficient (r). Because the data used in the calculation of the correlation coefficients are samples from a population, the r obtained is an estimation of the correlation. When the number of analyzed data is limited, as normally occur in biology, a significance test for the estimated correlation coefficient becomes necessary (Paterson, 1939).

The regression is a concept close related to the correlation and permits predict the changes in one variable knowing the changes occurred in the other. As in the previous statement it is also necessary to test the significance of the regression coefficient (Paterson, 1939).

Table 4 shows the results of the correlations and the regressions obtained from the data of the enzymatic activity on the cyanogenic concentrations in the root parenchyma.

The estimated correlation coefficient between the enzymatic activity in the leaves recently expanded and the CN^- content in the root parenchyma is highly significative, with a probability of less than 1 per thousand (p 0,001) (Fisher, 1950). In the mature leaves the estimated correlation coefficient is not significative, at a 70% level ($p > 0,7$) (Fisher, 1950).

Mkpong et al. (1990) did not find difference in the cyanide content using mild and bitter cassava varieties.

The evidence exposed suggests that the enzyme accumulation in the mature leaves is independent of the cyanogen content in the mature roots.

However it appears that the enzyme activity in the leaves is reached later on plants that must to mature a high content of cyanogen compounds in the roots than plants with lower CN^- content in the root parenchyma. On bitter cassava varieties, the enzymatic activity is lower on recently expanded leaves than in mature leaves, the accumulation is completed during ripening. In mild cassava varieties the recently expanded leaves already have the same enzymatic activities than in mature ones. This contribution to the cyanogenesis process could have a practical use in the recognition of bitter or mild varieties using enzymatic analysis on the leaves of plants from the 12th week, when it is far yet from the root ripening. In this moment already exists a difference in the enzymatic activity between newly expanded and mature leaves on bitter varieties. This cannot be detected on the leaves of mild varieties. By this way it could be planned in advance the future use of the produced material.

This is of a special importance when working is done on plants which are in an edafo-climatic adaptation process out of their natural environment, because the cyanogenic content in the parenchyma depends on the external conditions (Ferrero and Villegas, 1992; Villegas et al., 1993).

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Table 1. Enzyme activity in leaves.

Variety	Activity in leaves recently expanded (U * 100/g min)	Activity in leaves matures (U* 100/g min)
Gancho	139	131
	139	148
	139	148
Collita	143	75
	141	71
	143	73
Rama Negra	137	127
	139	172
	135	175
		135
		110
Yuca Brava	73	101
	72	112
		123

Table 2. Regression between absorbance and concentration of CN^-.

Parameter	Value
Coefficient of correlation (r)	0,9942
No. of observations (n)	18
Grades of release (n-2)	16
Intersection in Y when X = 0	-0,0083
Estimation Standard Error of Y	0,0342
Coefficient of regression ($\Delta Y/\Delta X$)	0,0049
Standard Error of the Coefficient of regression	0,0001

Table 3. Concentration of CN^- in root parenchyma.

Variety	Transmittance	Absorbency	CN^- extract	CN^- parenchyma
Gancho	84.5	0.073	16.6	18.1
	81.0	0.092	20.3	22.2
	84.0	0.076	17.1	18.7
Collita	82.0	0.086	19.3	21.0
	83.0	0.081	18.2	19.8
	84.0	0.076	17.1	18.7
Rama Negra	82.0	0.086	19.3	21.0
	72.0	0.143	30.8	33.6
	79.0	0.102	22.6	24.6
Yuca Brava	44.0	0.357	74.4	81.1
	32.0	0.495	102.5	111.9
	18.0	0.745	153.4	167.4

Table 4. Correlation between the enzyme activity in leaves, and the CN^- content in root parenchyma.

	Leaves recently expanded	Leaves mature
Coefficient of correlation (r)	0.99	0.10
No. of observations (n)	11	14
Grades of release (n-2)	9	12
Intersection in Y when X = 0	154.4	125.6
Estimation Standard Error of Y	1.8	34.9
Coefficient of regression ($\Delta Y/\Delta X$)	-0.68	-0.09
Standard Error of the Coefficient of regression	0.01	0.23

A MOLECULAR STUDY OF CYANOGENIC α-HYDROXYNITRILE LYASE FROM CASSAVA (*MANIHOT ESCULENTA*)

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All parts of the cassava plant are cyanogenic, that is, hydrogen cyanide (HCN) is produced when tissues are damaged. Cyanogenic glucosides are synthesized from the precursor amino acids valine and isoleucine. HCN is released after the sequential action of two catabolic enzymes, a β-glucosidase and an α-hydroxynitrile lyase (HNL). HNL protein from young cassava leaves was purified to homogeneity. The native protein is a homotrimer with a subunit relative molecular mass of 28,500 on SDS-PAGE. The active protein is not glycosylated and does not contain a flavin group. N-terminal and internal peptide sequences, obtained from HNL digested with the endoproteinase Glu-C, were used to design degenerate oligonucleotide primers for PCR with ss cDNA, using purified mRNA from cotyledons as template. The resulting DNA fragment was used to probe a cassava cotyledon cDNA library. The nucleotide sequence of an isolated cDNA clone was shown to contain derived amino acid sequences identical to those obtained from the purified protein.

INTRODUCTION

The release of hydrogen cyanide (HCN) from damaged tissues (cyanogenesis) has been described in more than 3,000 plant species, including those of several crops (Conn, 1981). The character is widely accepted as a defence mechanism against predation. HCN is produced when stored cyanoglucosides are broken down through the action of one or more β-glucosidases to produce glucose and a relatively unstable α-hydroxynitrile (cyanothydrin) (Poulton, 1990). In many plants, the breakdown of cyanohydrin to a ketone or aldehyde and HCN is catalyzed by the enzyme α-hydroxynitrile lyase (HNL) (Poulton, 1990). The importance of this enzyme in HCN release has been demonstrated in *Hevea* (Selmar et al., 1989). α-Hydroxynitrile lyases from almond and sorghum have been studied as biocatalysts for the production of optically active α-hydroxynitriles with potential uses in the fine chemical and pharmaceutical industries (Niedermeyer and Kula, 1990; Smitskamp-Wilms et al., 1991).

The HNLs characterized from plants fall into two groups: the first, consisting of those isolated from species in the *Rosaceae*, have (R)-(+)-mandelonitrile as the natural substrate. They are monomeric glycoproteins (Xu et al., 1986; Yemm and Poulton, 1986) and contain a flavin prosthetic group (Gerstner and Pfeil, 1972; Xu et al., 1986) whose role is not yet understood. Cheng and Poulton (1993) have recently published the sequence of a cDNA clone of (R)-(+)-mandelonitrile lyase from *Prunus serotina*.

The HNLs in the second group do not contain flavins and vary in structure. The HNL from *Ximenia americana* has (S)-(-)-mandelonitrile as the natural substrate and is a monomeric glycoprotein (Kuroki and Conn, 1989). Sorghum produces 4-hydroxy-(S)-mandelonitrile and, in this monocotyledonous species, HNL is a multimeric glycoprotein (Wajant and Mundy, 1993; Wajant et al., 1993). Many plants, including flax, *Hevea*, white clover, and cassava, produce the two aliphatic cyanohydrins, acetone cyanohydrin and R-2-cassava cyanohydrin. The HNLs from *Hevea* (Selmar et al., 1989) and flax (Xu et al., 1988) have been purified and the structure of the flax enzyme reported to be a homodimer with a subunit molecular weight of 42,000 M_r. The flax enzyme was also shown not to be a glycoprotein (Xu et al., 1988).

Interestingly, the moth *Zygaena trifollii*, which feeds on plants that produce aliphatic cyanohydrins, is cyanogenic, producing both acetone cyanohydrin and R-2-butanone cyanohydrin. The HNL from larval haemolymph is a flavin-bound protein, the native form of which is a homodimer with a relative molecular mass of 145,000 (Muller and Nahrstedt, 1990).

The tropical root crop cassava (*Manihot esculenta* Crantz) is a staple food crop for about 500 million people with an estimated annual production of 150 million tons of fresh roots (Hershey, 1993). The cyanogenic properties of its roots and leaves form a potential health hazard to its consumers. In this document, we report the purification and characterization of HNL isolated from young cassava leaves.

METHODOLOGY AND RESULTS

Purification and physical characterization

α-Hydroxynitrilase was purified from the acetone powders of young cassava leaves collected from plants growing at 26 °C in a growth cabinet with 12 h of daylight. Three purification steps routinely gave a single Coomassie blue band on SDS-PAGE; these steps were molecular exclusion chromatography, using Sephacryl S-300 (Pharmacia); ion exchange chromatography, using DEAE Sepharose (Pharmacia); and FPLC, using Mono Q (Pharmacia). A typical purification is illustrated in Table 1. The ease with which the enzyme can be purified is a measure of both its abundance in young leaf tissue and its stability. Purified protein was stored at 4 °C for as long as 12 months without significant loss of activity.

The Sephacryl S-300 column was calibrated with alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 29,000), and cytochrome C (M_r 12,400). The elution of HNL indicated a native molecular weight of 92,000. SDS-PAGE of the purified enzyme gave a single subunit with an M_r of 28,500, indicating that, in the active form, the enzyme is a trimer. When

SDS-PAGE is used to analyze HNL samples that have not been denatured with mercaptoethanol, two Coomassie bands are produced, one at M_r 28,500 and a faster migrating band with an apparent M_r of 24,000. This result is consistent with the retention of S-S bonds in a portion of the material in the absence of mercaptoethanol, and indicates the involvement of these bonds in the folded structure of the native protein.

IEF (PhastSystem, Pharmacia) revealed three isoforms: a major isoform with an isoelectric point of pI 4.4, and two minor isoforms with isoelectric points of pI 4.1 and pI 4.6. Because the material for isolating the enzyme was collected from several plants, the existence of isoforms could result from allelic variation among plants. Alternatively, each plant may produce more than one isoform—multiple forms of HNL have been reported in black cherry (Yemm and Poulton, 1986).

The sensitive Glycotrack carbohydrate detection system (Oxford Glycosystems), which can detect nanograms of sugar residues, was used to test for oligosaccharide residues in partially purified HNL, with cassava linamarase (β-glucosidase) as a known glycoprotein control. SDS-PAGE and Western blot analysis for the presence of carbohydrates clearly showed that cassava α-hydroxynitrile lyase is not a glycoprotein.

Purified cassava HNL is not yellow, and absorption of the protein between 700 and 300 nm did not peak between 375 nm and 445 nm, indicating that, like the aliphatic HNL from flax (Xu et al., 1988), the cassava enzyme is not a flavoprotein.

Peptide sequencing

Following denaturation, purified HNL was digested with the endoproteinase Glu-C, and the products separated by SDS-PAGE (Figure 1a). The separated peptides were electroblotted onto Hyperbond membrane and the excised bands sequenced on a Beckman LF3000 gas phase protein microsequencer. The sequence data are shown in Figure 1b.

cDNA cloning and sequencing

Degenerate oligonucleotide primers based on the N-terminal and internal peptide sequences shown in Figure 1 were designed as follows: alternative nucleotides at positions of two- or three-base degeneracy, and the neutral nucleotide inosine at positions of four- or six-base degeneracy. Thus, the overall degeneracy was six-fold for the sense primer and eight-fold for the antisense primer.

SENSE PRIMER:

Nucleotide sequence: 5′ C A T/C G G I G C I T G G A T T/C/A T G G C A 3′
Peptide sequence: H G A W I W H

ANTISENSE PRIMER:

Nucleotide sequence: 5′ C C T/C T T I C G/T C A T I A C C A T C/T T T 3′
Peptide sequence: G K R M V M K

The antisense primer set was used to generate a cDNA strand from mRNA, using M-MLV reverse transcriptase and then, without intermediate purification, a PCR reaction that involved both oligonucleotide sets as primers. The five cycles, including a 37 °C-annealing step, followed by a 5-sec/°C ramp to the 72 °C elongation step, were essential for obtaining distinct PCR products from these sequential reactions.

The size of the peptide bands on SDS-PAGE after the HNL was digested with endoproteinase Glu-C suggested that the distance between the two peptide sequences was about 400 nucleotides. Three discrete bands were obtained from the PCR reaction, at sizes 0.6 Kb, 0.5 Kb, and 0.3 Kb. These were cloned into the modified Eco RV-T site of the plasmid vector pGemT, which is designed to use the additional adenosine residue attached to the 3′ end of PCR chains with Taq polymerase. The three subcloned fragments were sequenced, using plasmid-specific primers.

All three fragments contained variations of the degenerate oligonucleotide sequences, but the 0.5 Kb subclone also contained the nucleotide sequence that corresponds to the known additional 16 amino acid residues that follow the N-terminus (sense) primer and two amino acid residues that procede the internal (antisense) primer. This clone was subsequently used as a probe to screen an existing cassava cotyledon cDNA library (Hughes et al., 1992).

Ten positive λGT10 recombinant clones were obtained from a screen of 15,000 plaques (1:1,500), indicating a relatively high level of expression of the HNL gene at this stage of development. All the positive clones were amplified by PCR with GT10-specific primers. The four largest were selected for sequencing after subcloning into the plasmid vector pGem5Zf(-). The sequence of the largest clone (pHNL10, 1,021 bp) has been deposited with the GenBank/EMBL data libraries under Accession No. Z29091. The open reading frame codes for a protein of 258 amino acid residues with a predicted relative molecular mass of 29,481. Transcript size of the HNL gene was estimated from Northern blotting to be 1.2 Kb. This sequence is therefore not a full length transcript but contains the whole of the coding region of the active protein.

DISCUSSION

α-Hydroxynitrile lyase proteins have been purified from several different cyanogenic species (Selmar et al., 1989; Wajant and Mundry, 1993; Xu et al., 1986; Xu et al., 1988; Yemm and Poulton, 1986). The N-terminus (Wu and Poulton, 1991) and a cDNA sequence have been published for the FAD-containing hydroxynitrile lyase from *Prunus serotina* (Cheng and Poulton, 1993). This document reports for the first time the isolation and characterization of a cDNA clone corresponding to the mRNA of the second class of α-hydroxynitrile lyase enzymes.

No significant homology exists among the derived amino acid sequences of the HNL clones from cassava and black cherry, thus confirming, at the molecular level, the distinction made at the protein level between the two broad classes of α-hydroxynitrile lyases. A cassava clone's identity is confirmed by the presence, in the derived amino acid sequence, of 36 N-terminus and 24 internal amino acid residues obtained by peptide sequencing. The calculated molecular weight of the predicted protein, M_r 29,481, is believed to correspond with the estimated value of 28,500 on SDS-PAGE, because we consistently found that molecular weight values obtained from SDS-PAGE were lower than values calculated from DNA sequences.

Searches in the GenBank (May 1992) and EMBL (June 1992) DNA sequence databases and in the NBRF and SwissProt (May 1992) protein sequence databases showed no significant homology to other reported genes. The cassava enzyme, like those from *Hevea* and *Linum*, is not a glycoprotein and does not contain a flavin prosthetic group. No sequence data are available for the cyanogenic hydroxynitrile lyases from *Hevea* and *Linum*, which have the same substrates as cassava. Interestingly, we were unable to detect HNL activity in cyanogenic white clover, despite the clear similarities in cyanogenic pathways and the strong homology of the cyanogenic β-glucosidase genes of white clover and cassava.

Konzo is a form of tropical myelopathy, and is caused by eating insufficiently processed cassava products when the diet is deficient in sulfur-containing compounds. A study in Zaire by Tylleskar et al. (1993) separately measured the cyanoglucoside, cyanohydrin, and HCN levels in local cassava meals. They showed that, although the cyanoglucoside and free HCN levels of flours produced by a short processing method were below 20 mg HCN equivalents per kg dry weight, cyanohydrin levels could be over 100 mg HCN equivalents per kg dry weight.

The study also showed that cyanohydrins are the main source of cyanide from insufficiently processed cassava. The authors inferred that the nonenzymatic conversion of acetone cyanohydrin to volatile HCN stops during the first day of soaking, when the pH falls as lactic acid fermentation stabilizes the cyanohydrin (Cooke, 1978). Because the reaction catalyzed by HNL may be the rate-limiting step in detoxifying cyanogenic glucosides in cassava processing, the ability to manipulate the cassava HNL gene is of potential significance in producing less toxic cassava food products.

The data in this report, together with additional kinetic information and details of materials and methods, have been published in Hughes et al., 1994.

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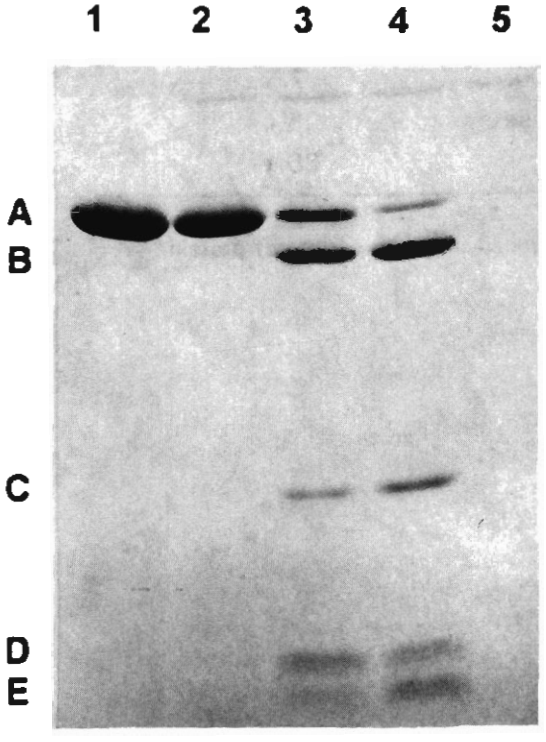
Table 1. Purification of *α*-hydroxynitrile lyase from young cassava leaf tissues.

Step	Fraction	Specific activity[a]	Purification factor
1	Crude	0.13	
2	Sephacryl-S300	7.5	x 57.7
3	DEAE Sepharose	25.4	x 195.4
4	MonoQ	36.1	x 277.7

a. Specific activity: *μ*moles HCN produced in 1 min per mg of protein.

(a)



(b)

A: (undigested) V.T.A.H.F.V.L.I.H.T.I.C.H.G.A.W.I.W
H.K.L.K.P.A.L.E.R.A.G.H.K.V.T.A.L.D

B: (N-terminal)

C: (N-terminal)

D: (internal) L.A.K.M.V.M.R.K.G.S.L.F.Q.N.L.A.Q
R.P.K.F.T.E

E: mixture

Figure 1. Peptide sequencing of cassava α-hydroxynitrile lyase (HNL). (a) SDS-PAGE of endoproteinase Glu-C digestion of HNL: track 1, undigested purified HNL; track 2, Glu-C 0′ digestion of HNL; track 3, Glu-C 30′ digestion of HNL; track 4, Glu-C 60′ digestion of HNL; track 5, endoproteinase Glu-C < M_r 29,000 molecular weight marker. (b) Amino acid sequence of HNL peptide fragments shown in Figure 4A.

REGULATION OF CYANOGENIC POTENTIAL IN CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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The food safety of cassava is compromised by the presence of cyanogenic glycosides, of which linamarin is predominant. It has been generally assumed that all cyanogenic glycosides present in cassava are produced in the leaves and subsequently transported to other organs. Recently, we have determined that linamarin can be synthesized in both roots and leaves of cassava. The potential for roots to synthesize linamarin suggests that their linamarin content may not be determined solely by transport of cyanogenic glycosides from the top of the plant. We have also determined that rates of linamarin synthesis are highest in leaves of young, high-cyanogenic plants and decrease in an age-dependent manner. *In vitro* labeling studies indicate that leaf protoplasts are capable of synthesizing linamarin from ^{14}C-valine, but in a pattern which differs from whole leaves. Fractionation of leaf protoplasts has indicated that linamarin can also be synthesized from valine by isolated vacuoles, where it is then sequestered.

INTRODUCTION

Cassava accumulates cyanogenic glycosides in all organs of the plant (Balagopalan et al., 1988). But there are organ and cultivar-dependent differences in the levels of cyanogenic glycosides. Linamarin is the predominant cyanogenic glycoside in cassava. It has been shown that leaves typically have significantly higher linamarin content than roots on a fresh-weight basis (Mkpong et al., 1990). Also, cassava cultivars may vary 5-fold in the levels of cyanogenic glycosides in roots (Balagopalan et al., 1988). Koch et al. (1992) recently proposed a pathway for linamarin synthesis. The first dedicated precursor for linamarin synthesis is valine. Valine is converted to acetone cyanohydrin in a step-wise manner, possibly catalyzed by two membrane-bound cytochrome P_{450}'s (B. Møller, personal communication). Acetone cyanohydrin is subsequently converted to linamarin by glycosylation. Cyanogenesis results when linamarin is hydrolyzed to acetone cyanohydrin and glucose by a specific β-glucosidase, linamarase. The acetone cyanohydrin subsequently decomposes or is metabolized to acetone and hydrogen cyanide (White et al., n.d.; Selmar et al., 1988).

The physiological and biochemical causes for the varietal differences in root linamarin content are poorly understood. Previous grafting and girdling experiments have led to the conclusion that the linamarin present in the roots was due to synthesis in the leaves and subsequent transport (DeBruijn, 1973; Makame et al., 1987). But the model for transport to the roots is complicated by linamarase being

present in the apoplast of leaves (Mkpong et al., 1990). Alternate pathways for linamarin transport have been suggested. These include either symplastic transport, or conversion of linamarin to the glucoside—linustatin—a compound not hydrolyzed by linamarase. But we have not detected linustatin synthesis using radiolabeled valine in cassava (McMahon and Sayre, 1993). In addition, we have found that roots are capable of synthesizing linamarin, suggesting that cyanogenic potential in roots may not depend upon synthesis and transport from the leaves.

We have previously demonstrated differential linamarin synthesis in cassava leaves from low- and high-cyanogenic cultivars using a radioactive precursor to linamarin, valine. High-cyanogenic leaves synthesized linamarin at substantially higher rates than low-cyanogenic leaves in young plants (49 days old) (McMahon and Sayre, 1993). Leaves of older plants of both varieties ($\geq$ 83 days old) were not able to synthesize measurable quantities of linamarin. However protoplasts, isolated from leaves of older plants were capable of synthesizing linamarin but the rate of synthesis was higher in low-cyanogenic varieties than in high-cyanogenic varieties. These results may be due in part to an age-dependent metabolic activity, or a change in source-sink relationships. In this report, we compare results from *in vitro* labeling experiments using intact leaves and leaf cell fractions from young plants.

MATERIALS AND METHODS

Protoplast Isolation

Protoplasts were isolated from leaves using a previously described technique (Szabados et al., 1987). Leaves were cut into 1 x 3 mm pieces and incubated 12-16 h in protoplast isolation buffer (7 mM $CaCl_2$, 0.7 mM KH_2PO_4, 3 mM 2-(N-morpholino)ethanesulfonic acid [MES], 0.6 M mannitol, 1.5% (w/v) Onozuka cellulase, and 0.4% (w/v) Macerozyme, pH 5.6). Protoplasts were filtered through 74μ nylon mesh, pelleted at 100 x g for 4 min, and washed twice in protoplast wash buffer (7 mM $CaCl_2$, 0.7 mM KH_2PO_4, 3 mM MES, 0.6M mannitol, pH 6.5). Protoplasts were resuspended in wash buffer for further use.

Vacuole Isolation

Vacuoles were isolated using a modification of a 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfate (CHAPS) isolation method from Matoh et al. (1987). Protoplasts were incubated with 0.5% (w/v) Neutral red and 1 mM CHAPS in protoplast wash buffer for 30 min with gentle agitation. Cell lysate was layered over a continuous Ficoll-400 gradient in vacuole isolation buffer (0.6 M mannitol, 5 mM EDTA, 0.1% (w/v) bovine serum albumin [BSA] and 25 mM 2-(N-morpholino) propanesulfonic acid [MOPS], pH 7.5) in concentrations ranging from 3% to 6%

Ficoll. Vacuoles were separated by centrifugation at 500 x g for 30 min at 15 °C using a Sorvall HB-4 rotor. Vacuoles were isolated from a band near the top of the gradient and concentrated via centrifugation in a microfuge at 500 x g for 15 min.

^{14}C-valine labeling and glycoside analysis

Protoplasts were labeled in 0.5 mM valine (3 μCi/μmol) in protoplast wash buffer with gentle agitation. Samples were taken from the labeling chamber at 1 h intervals. Vacuole samples were treated in a manner similar to protoplasts. Soluble compounds were extracted from frozen protoplasts or vacuoles in boiling 80% methanol, followed by two extractions with chloroform (Brimer and Dalgraad, 1984). Following lyophilization of the aqueous phase, the extracts were resuspended in 50% methanol. Samples were loaded onto thin-layer chromatography plates and chromatographed using a solvent system of methanol:chloroform:15% NH_4OH (2:2:1) (Selmar et al., 1988). TLC plates were exposed to a phosphorimaging screen (Molecular Dynamics) and levels of ^{14}C-linamarin were analyzed on a phosphorimager. The location of the linamarin on the thin-layer chromatograph was previously verified using linamarin standards and a colorimetric p-anisaldehyde reaction (Zitnak et al., 1977).

Enzyme assays

Assays for the presence of linamarin in protoplasts and vacuoles were performed by extracting water-soluble compounds (see above) and incubation with purified linamarase. Cyanide released was assayed using a Spectroquant kit. Protoplast and vacuolar extracts for acid phosphatase and α-mannosidase were performed using the method of Boller and Kende (1979). Glucose-6-phosphate dehydrogenase assays were performed by the method of Li et al. (1989).

RESULTS AND DISCUSSION

Several studies have demonstrated that the steady-state levels of linamarin in leaves of different cassava varieties are similar (Mkpong et al., 1990; DeBruijn, 1973; Nartey, 1968). But unlike leaves the linamarin content of roots may vary several fold between different cassava varieties (Balagopalan et al., 1988). The biochemical and physiological basis for the varietal differences in root linamarin content are not well characterized. Further, the origin of linamarin, which is sequestered in roots, remains controversial. It has generally been assumed that linamarin is synthesized only in leaves and stems and is transported to roots; but the localization of the cyanogenic enzymes (linamarase and hydroxynitrile lyase) in the cell walls precludes apoplastic transport of linamarin to the roots (White et al., n.d.).

Previously, we reported that linamarin can be isotopically labeled in leaves by exogenous application of ^{14}C valine. Further, we demonstrated that the rates of ^{14}C linamarin synthesis varied in an age-dependent manner (for leaves of the same position from the top of the plant)(McMahon and Sayre, 1993). Leaves from plants older than 83 days did not synthesize measurable quantities of linamarin regardless of whether they were from high (CM996-6) or low (HMC-1) cyanogenic potential (CN_p) varieties. In the present study, we compare rates of linamarin synthesis in young leaves and roots from low and high CNp cassava varieties. Figure 1 shows leaves of the high CNp variety (CM 996-6) synthesized linamarin at rates approximately 3-fold higher than those of the low CNp variety (HMC-1). The high CNp variety synthesized linamarin at a rate of 4.73×10^{-4} ($\pm$ 3.7) μmol/gfw/h while the rate for the low CNp variety was 1.61 x 10-4 ($\pm$ 2.0) μmol/gfw/h. The leaf steady-state linamarin levels are likely to be similar, so it is apparent that leaves capable of higher rates of synthesis must either be exporting linamarin to other plant tissues, metabolizing linamarin to some other form; or that there is a barrier to labeling of whole leaves associated with reduced delivery of valine to the cytoplasm in the low CNp variety (Figure 2).

Figure 3 shows an autoradiography of ^{14}C-linamarin labeled leaf extracts, separated by thin-layer chromatography (TLC) from leaves of high and low CNp variety plants. It is apparent that there is another heavily labeled compound (with ^{14}C-valine) which is also detectable with p-anisaldehyde suggesting it contains a carbohydrate moiety. This compound has not been identified to date, but the possibility exists that there may be another storage and/or transportable form of linamarin. Interestingly, ^{14}C-valine labeled protoplasts do not synthesize this unknown compound. This result could be attributed to the absence of a cell wall localized enzyme (not present in protoplasts), or altered metabolic activity in isolated protoplasts versus intact leaves, or altered source-sink relationships.

We have also observed quantitative differences in the pattern of ^{14}C labeling between intact leaves and protoplasts. As Table 1 shows, the rates of linamarin synthesis for leaf protoplasts of low and high CNp varieties were more similar than those for intact leaves. In contrast to whole leaves, the rate of linamarin synthesis in low CNp protoplasts was higher (2-fold) than that of the high CNp varieties. These results show that rates of ^{14}C-linamarin synthesis in leaves are not correlated with root linamarin steady-state levels.

In contrast to earlier studies indicating that cassava roots do not synthesize linamarin (Balagopalan et al., 1988), we have observed linamarin synthesis in roots fed radioactive valine at rates equivalent to those of leaves of low CNp varieties. Younger roots (49 days old), less swollen than older roots (8 months), had linamarin synthesis rates 10-fold greater than older roots (Figure 1). But there were no differences in ^{14}C-linamarin synthesis in low and high CN_p roots, suggesting that varietal differences in root linamarin content cannot be attributed to varietal differences in root linamarin synthesis rates. These observations are complicated

by those of Makame et al. (1987) who demonstrated in grafting experiments, where high CN_p tops were grafted to low CNp roots, that linamarin content of the roots was not significantly altered. Our results and those of Makame et al. (1987) do not allow us to determine whether leaf or root linamarin synthesis determines root steady-state linamarin content. In addition, it is apparent that age-dependent factors may also regulate overall linamarin content.

A second question concerning linamarin metabolism is the sub-cellular site(s) of synthesis and storage. We previously demonstrated that the cyanogenic enzymes linamarase and hydroxynitrile lyase are apoplastic, or located in the cell wall of leaves (White et al., n.d.). A symplastic site of linamarin storage is required to prevent its metabolism to HCN. We have fractionated cassava protoplasts and determined the linamarin content of vacuoles using a cyanide detection assay (Table 2). We observed a 6-fold higher content of linamarin in vacuoles than in protoplasts. The basis for this is unknown at present but may be attributed to fusion of vacuoles after detergent treatment.

Although our data indicate the vacuole is the site of storage, the subcellular site of linamarin synthesis is still unknown but the endoplasmic reticulum has been suggested as a likely site of synthesis (B. Møller, personal communication). To investigate whether vacuoles were capable of linamarin synthesis, we incubated vacuoles with ^{14}C-valine. Assays for vacuolar and cytoplasmic markers indicate that our vacuole fractions are enriched for vacuolar enzymes and are not substantially contaminated with cytosolic components (Table 3). We observed that vacuoles were capable of synthesizing linamarin at rates 100-fold greater than for protoplasts (Table 1). Significantly, the addition of cytoplasmic extract (100,000 x g supernatant) did not increase the rates of linamarin synthesis, indicating that soluble factors were not required for linamarin synthesis. Preliminary studies with two inhibitors of endomembrane transport, brefeldin A and monensin, also indicated that these inhibitors had little or no effect on linamarin synthesis. These findings do not rule out a microsomal contribution to linamarin synthesis. Overall, our results are consistent with a vacuolar site of linamarin synthesis and storage.

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Table 1. ^{14}C-Linamarin synthesis in cassava leaf protoplasts and vacuoles.

	μmol x 10^5/10^6 cells or vacuoles/h	S.D.
Protoplasts (49 days old)		
High CNp (CM 996-6)	0.043	0.0
Low CNp (HMC-1)	0.100	0.007
Vacuoles (CM 996-6)		
+ cytoplasmic extract[a]	3.06	0.63
- - cytoplasmic extract	4.10	0.53

a. Cytoplasmic extract equivalent to one cell vol./vacuole added.

Table 2. HCN equivalents in protoplasts and vacuoles.

Source	μmol HCN/10^6 cells or vacuoles	S.D.
Cassava	0.025	0.012
Protoplasts	0.162	0.077
Vacuoles[a]		
Sorghum[a]		
Protoplasts	1.09	0.19
Vacuoles	1.13	0.45

a. Saunders and Conn (1978).

Table 3. Marker enzyme activity for protoplasts and vacuoles from cassava.

Enzyme	Activity (nmol/min/mg protein)				Enrichment in vacuoles
	Protoplasts	S.D.	Vacuoles	S.D.	
Acid phosphatase	14.7	3.6	56.0	25.0	3.8
α-mannosidase	10.6	1.2	48.0	4.1	4.5
Glucose-6-phosphate dehydrogenase	4.6	0.9	0.9	0.5	0.2

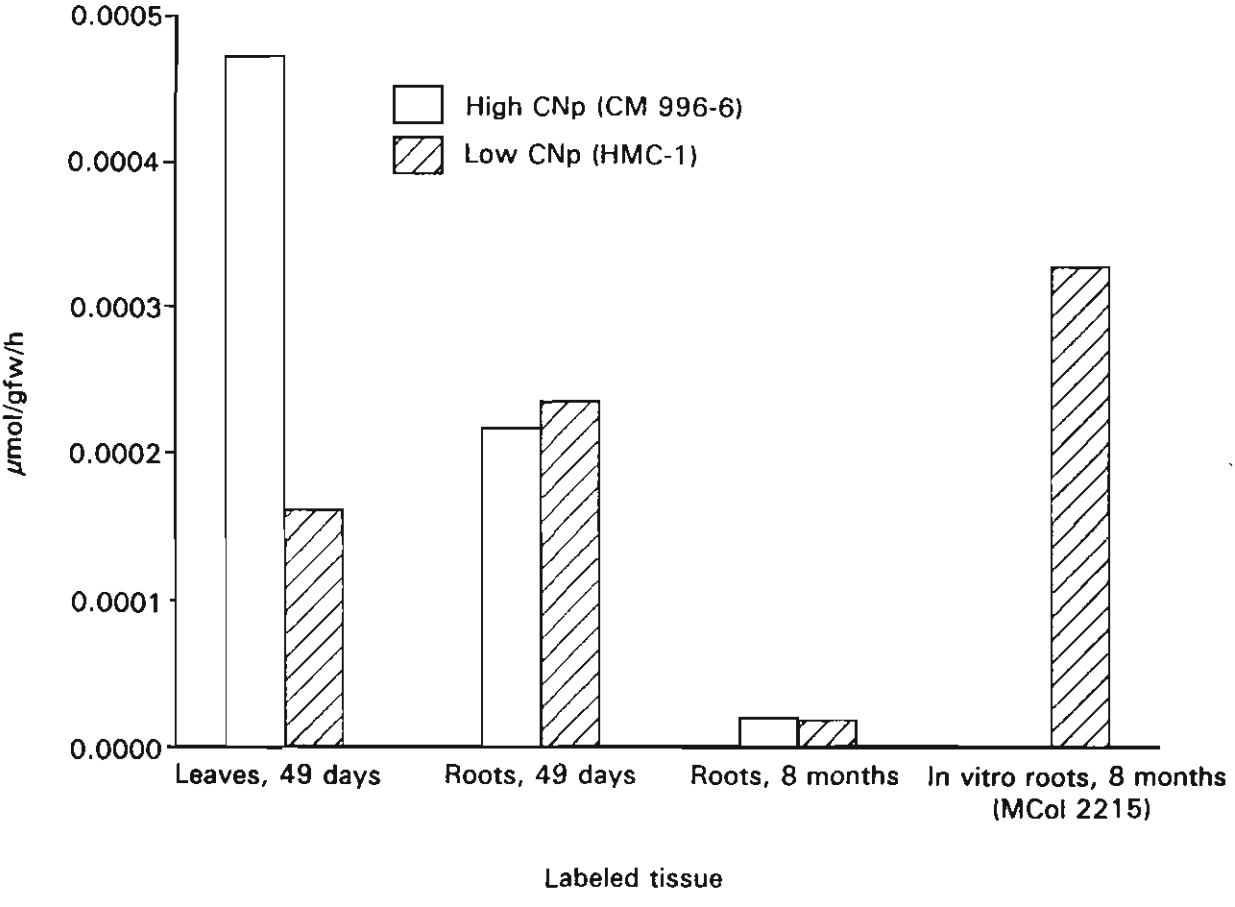

Figure 1. ^{14}C-linamarin synthesis in high and low CNp varieties of cassava.

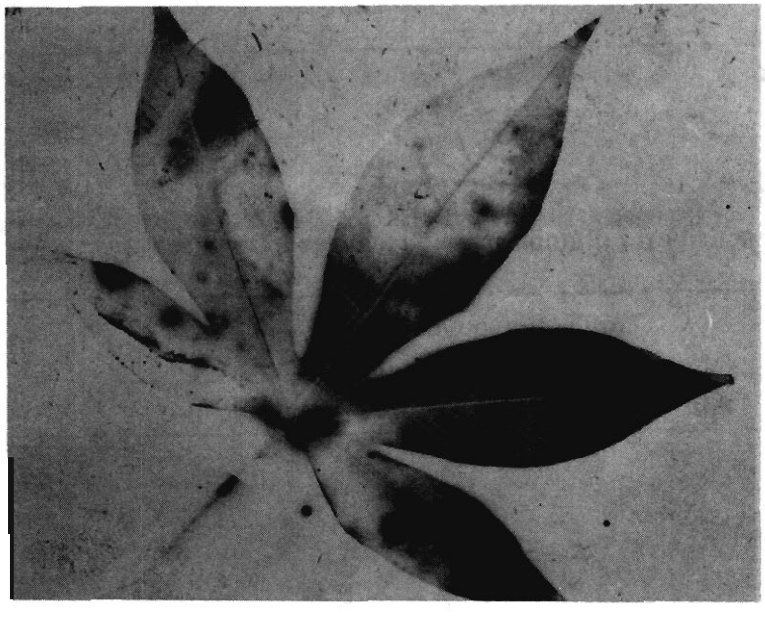

Figure 2. Autoradiography of cassava leaf exposed to ^{14}C-valine.

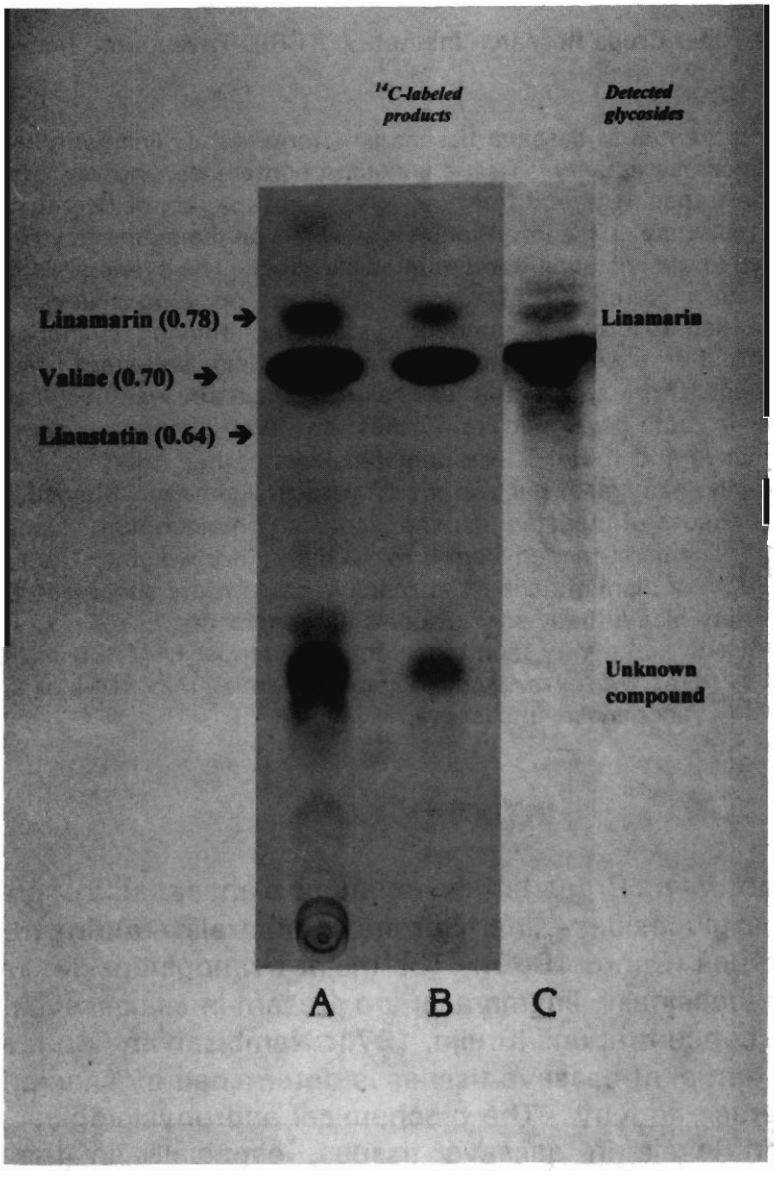

Figure 3. Thin-layer chromatography images of ^{14}C labeled extracts: A = high CNp leaf extract; B = low CNp leaf extract; C = phosphorimage of low CNp protoplast extract.

CYANOGENIC POTENTIAL OF CULTURED CELLS, TISSUES, PLANTS AND SEEDLINGS OF CASSAVA

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The cyanogenic potential of cassava tissues is determined by linamarin levels and endogenous linamarase activity. Tissue linamarin content depends on the rate of biosynthesis, the transport to and from the tissue, and the rate of degradation and conversion to asparagine. Little information is available on the extent to which these processes influence the linamarin content in edible roots. The cyanogenic potential of *in vitro* cassava cultures and of cassava seedlings, at various developmental stages, was studied to develop a model system for studying cyanogenesis in cassava. Callus cultures were developed from anther, leaf, and stem tissues, and rooting was induced from anther and leaf callus. Linamarin was not detected in callus tissue nor in roots developed from callus; however, linamarase was present in both callus tissue and roots. Cells prepared from anther callus in suspension cultures elaborated linamarase, but did not synthesize linamarin. Shoot (including leaves) and root tissues of meristem-derived plantlets contained both linamarin and linamarase. Root linamarin ranged from 5 to 30 mg/kg dry weight. The presence of linamarin in roots of plantlets and its absence in callus roots suggested that leaf tissue is the primary biosynthetic site. Studies on germinating seeds also revealed that cotyledon tissue had a very high biosynthetic potential (> 1000 mg CN/kg). These different systems, with varying cyanogenic potential, may serve as effective tools for studying cyanogenesis in cassava.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a cyanogenic plant capable of producing HCN from two cyanogenic glucosides—linamarin and lotaustralin—during the mechanical disruption of its tissues (Conn, 1969). The major cyanoglucoside, linamarin, and the endogenous β-glucosidase, linamarase, are present in all cassava plant tissues, although in varying concentrations (Bruijn, 1971; Nambisan and Sundaresan, n.d.). The cyanogenic potential of cassava tissues is determined by linamarin levels and endogenous linamarase activity. The biochemical and physiological mechanisms regulating linamarin levels in cassava tissues, especially in the edible root parenchyma, are not clearly understood. Although the biosynthesis of cyanoglucosides from the precursors L-valine and L-isoleucine has been demonstrated in etiolated seedlings and in young leaves (Bediako et al., 1980; Bokanga et al., 1992; Koch et al., 1992; Nartey, 1963, 1973), little information is available on the relative biosynthesis of linamarin in different tissues.

The lack of correlation between the rate of incorporation of ^{14}C-valine into ^{14}C-linamarin and endogenous linamarin levels indicates that other factors play a role

seedling increased rapidly. The linamarase activity in seedling tissues (cotyledon, root, hypocotyl, leaves) was high at all stages of development (data not shown).

The results indicate that undifferentiated callus tissue—whether derived from anthers, leaves or stems—lacks the potential to synthesize linamarin. But linamarase activity was presnt in all cultures. Anther cells in suspension cultures elaborate only linamarase, and therefore could adequately serve as a acyanogenic model system for studying the localization and secretion of this enzyme. The absence of linamarin in callus-derived roots and its presence in roots of meristem-derived plantlets indirectly suggests that the cassava leaf is a primary biosynthetic site that transports linamarin to the roots. Studies on the cyanogenic potential of seedling tissues indicated that cotyledon tissue (which later develops into leaves) is also a major biosynthetic site for linamarin.

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Table 1. Cyanogenic potential of callus cultures.[a]

Tissue	Linamarin (mg CN/kg dry wt.)	Linamarase (µg CN/15 min/g dry wt.)
Anther callus	Nil	15 - 30
Leaf callus	—	18 - 35
Stem callus	—	15 - 25
Roots from anther callus	—	40 - 80
Anther callus cell suspension	—	0.95[b]

a. Values expressed as a range of 5 independent samples.
b. Fresh weight basis.

Table 2. Cyanogenic potential of meristem-derived plantlets.[a]

Cassava variety	Linamarin (mg CN/kg dry wt.)		Linamarase (µg/CN 15 min/g dry wt.)	
	Shoot	Root	Shoot	Root
M4	300 ± 20	5 - 50	90 ± 10	65 ± 5
H 1687	325 ± 50	5 - 30	65 ± 5	50 ± 7
H 226	250 ± 20	20 - 35	100 ± 20	65 ± 8

a. Average value of 5 values ± SE.

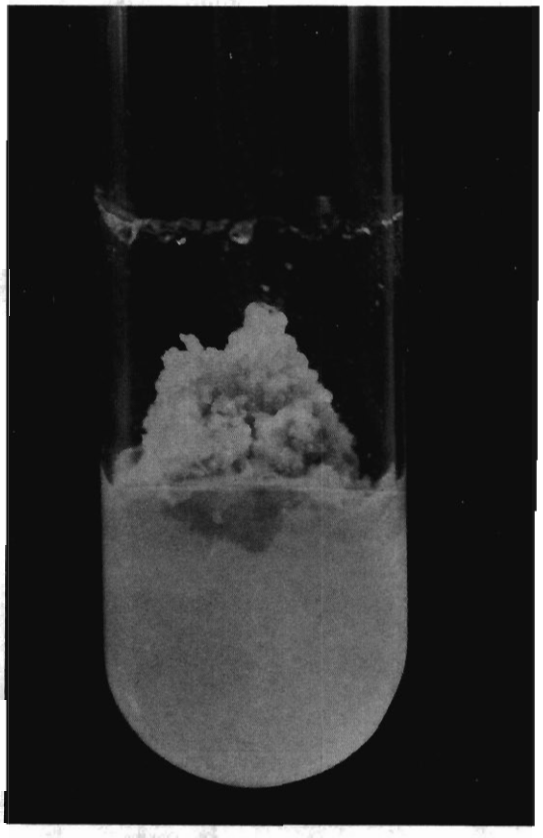

Figure 1. Callus produced from cassava leaf explants on MS + 2,4-D medium.

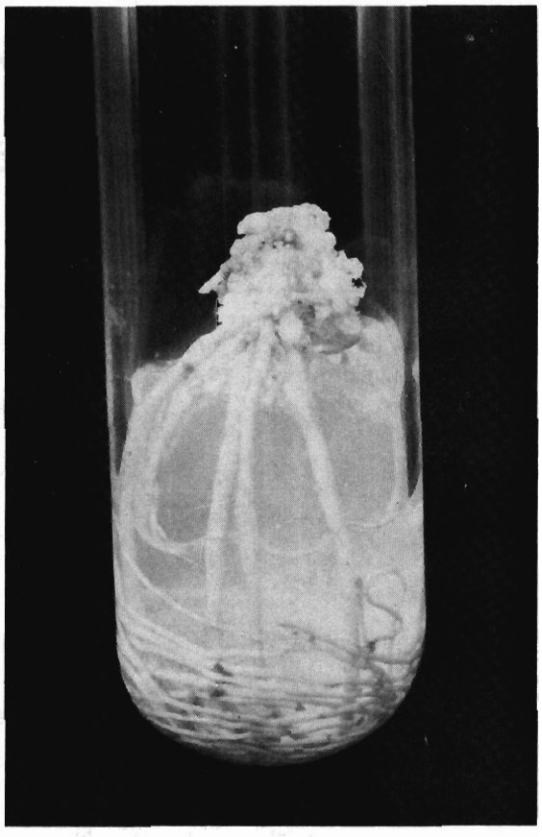

Figure 2. Thick roots induced from anther callus on MS + NAA medium.

PRODUCTION OF LINAMARASE FROM THE YEAST *HANSENULA ANOMALA*

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Of 131 yeast strains from fermented cassava, 101 strains showed β-glucosidase activities. Forty-two strains showed linamarase activity when grown on a medium containing linamarin, and 12 strains showed activity without linamarin. The yeast strain identified as *Hansenula anomala* (β-1-14 strain) was found to produce highly active linamarase in liquid medium containing 0.5% glucose, 0.5% bactopeptone, 0.3% malt extract, 0.3% yeast extract, and 40 ppm KCN. Linamarase production was 3.75 units/g fresh weight. Intracellular protein—linamarase of *H. anomala*—was precipitated with 30% to 60% ammonium sulphate saturation. Concentrated proteins were separated by ion-exchange column chromatography on DEAE-Toyopearl 650 M and Toyopearl HW-55 gel filtration. The enzymes had a 61.5-fold increase in purity and a 41.2% increase in recovery. The purified enzyme had a molecular weight of approximately 110,000 daltons. The Km value for linamarin was 2.94, optimal activity occurring at 40 °C and pH 6.5. The enzyme was stable at temperatures ranging between 20 and 45 °C.

INTRODUCTION

Previous papers have reported on cyanogenic glucosides in cassava and the release of free cyanide by the breakdown of bound cyanide in the fermentation of fresh cassava (Chantian and Pichyangkura, 1984; Ikediobi et al., 1985; Ketiker et al., 1978; Okafor, 1977; Okafor and Ejiofor, 1990; Wood, 1966). The interaction of the linamarase released from cassava root cells and microbes play an important role in decreasing the cyanogenic glucosides in fermented cassava, such as fermented food, gari, and animal feeds (Giraud et al., 1992; Ketiker et al., 1978; Okafor, 1977; Okafor and Ejiofor, 1990; Tinay et al., 1984). Yeast, lactic acid bacteria, and fungi were reported to produce linamarase (Abalaka and Garba, 1989; Chantian and Pichyangkura, 1984; Ketiker et al., 1978; Okafor, 1977). Abalaka and Garba (1989) cultivated isolated linamarase fungi in liquid medium, for later reinoculation in gari. The fermentation process of cyanogenic glucosides was reduced to 36 h for completed undesirable cyanogenic compounds. Fermented cassava is not only used as food for humans, but also as a major animal feed after a controlled fermentation process to decrease cyanogenic the compounds present. Selected strains of linamarase yeasts were screened and cultivated in an optimal medium to produce linamarase. Extraction and purification were performed in ion-exchange column chromatography, and molecular weight was determined by gel filtration. Several linamarase characteristics were identified.

MATERIALS AND METHODS

Linamarin was prepared according to the method of Kinoshita et al. (1988). Isolated yeast colonies from fermented cassava were screened on solid medium to which extracted linamarin was added. β-glucosidase activity occurred in yeast colonies flooded with p-nitrophenly β-D-glucoside (Okafor and Ejiofor, 1990; Pichyangkura et al., 1973), with a yellow color present around the colonies. Linamarase yeast was identified as the strain presenting the highest activity. Yeast was grown in an optimal medium consisting of 5 g glucose, 5 g bactopeptone, 3 g malt extract, 3 g yeast extract, and 40 ppm of potassium cyanide (KCN) per liter. Optimal cultivation conditions consisted of a shaking speed of 150 rpm for 48 h at pH 6 and 30 °C. Yeast cells were produced by centrifugating 5 liters of medium.

Activities of the enzyme linamarase were determined according to Cooke's method (Cooke, 1978a). Chloromin T and pyridine-pyrazolone reagents were used to prepare fresh samples.

The intracellular enzyme linamarase was obtained by submitting cells to French pressure (8000 psi), suspension in 0.01 M phosphate buffer at pH 6, and centrifugation of 5000 g discarded cell debris. Proteins were precipitated in ammonium sulphate 30%-60% saturation, and dialyzed against 0.01 M phosphate buffer at pH 6. After balancing in DEAE-ion exchange (Toyopearl-650 M) gel in a 70 x 1.6 cm column, proteins were loaded and eluted by 0-0.5 M sodium chloride gradient. Protein solution fractions were determined by optical density 280 nm, and linamarase activity was found. Linamarase activity fractions were pooled and loaded on gel filtration (Toyopearl HW-55) in a 70 x 2.5 cm column. Proteins were detected in the fractions at 280 nm, and linamarase activity was determined. Linamarase molecular weight was determined by comparing the molecular weights of aldolase, cytochrome C, catalase, albumin, blue dextran, and potassium dichromate. The Km value was determined with used linamarin (sigma). The optimal temperature and pH of linamarase were characterized.

RESULTS AND DISCUSSION

When 131 isolated yeast strains were screened, 101 strains gave positive β-glucosidase activities, 42 strains were grown on the medium containing potassium cyanide, and 12 strains were able to grow without cyanide. The strain that produced the highest linamarase activities was identified as *Hansenula anomala*. This strain needed the cyanogenic compound to induce linamarase formation. The optimal medium, containing 40 ppm of potassium cyanide, was used to cultivate 5 liters of yeast, yielding 89.02 g of wet cells.

Intracellular enzymes, linamarase and proteins were precipitated and loaded on DEAE-ion exchange. Results are shown in Figure 1.

Linamarase eluted on 0.19-0.21 M sodium chloride, indicating that linamarase belongs to negatively charged proteins in the same group as cassava linamarase (Cooke, 1978b) and *Leuconostoc mesenteroides* (Okafor and Ejiofor, 1990). The fractions pattern only showed one peak of linamarase activity. It was similar to linamarase from *Fusarium equiseti* (Abalaka and Garba, 1989; Ikediobi et al., 1985), but different from cassava linamarase (Cooke, 1978b; Yeoh Hock-Hin, 1989) and the bacteria *L. mesenteroides* which showed two peaks of activity (Giraud et al., 1992).

Active proteins were submitted to gel filtration to separate proteins depending on their molecular size. The activity of linamarase protein was determined and represented at tube numbers 57-66 in Figure 2. The purity increased 61.5 times (Table 1).

Molecular weight of yeast linamarase was determined by gel filtration and compared with known values (Figure 3).

The molecular weight of linamarase from the yeast *H. anomala* was estimated at 110,000 daltons, based on the comparison with known molecular weights, and Km values at 0.36 (Figure 4). The Km value is shown in Figure 5.

Optimal conditions for enzyme activity were 40 °C and pH 6.5. Enzymes were stable in the temperature range of 20-45 °C as shown in Figures 6 and 7.

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Table 1. Linamarase from *Hansenula anomala* (β-1-14) during extraction and purification.

Purification steps	Total volume (ml)	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Amount of enzyme (%)	Increase in purity (times)
Extraction of crude enzymes	210	203.7	687.1	0.3	100	1
% $(NH_4)_2SO_4$ 30-60% saturation	46	138.5	100.1	1.4	68	4.8
Ion-exchange chromatography on DEAE-Toyopearl	19	128.6	14.9	8.6	63.1	29.8
Gel filtration chromatography on Toyopearl HW-55	16.6	83.9	4.7	17.8	41.2	61.5

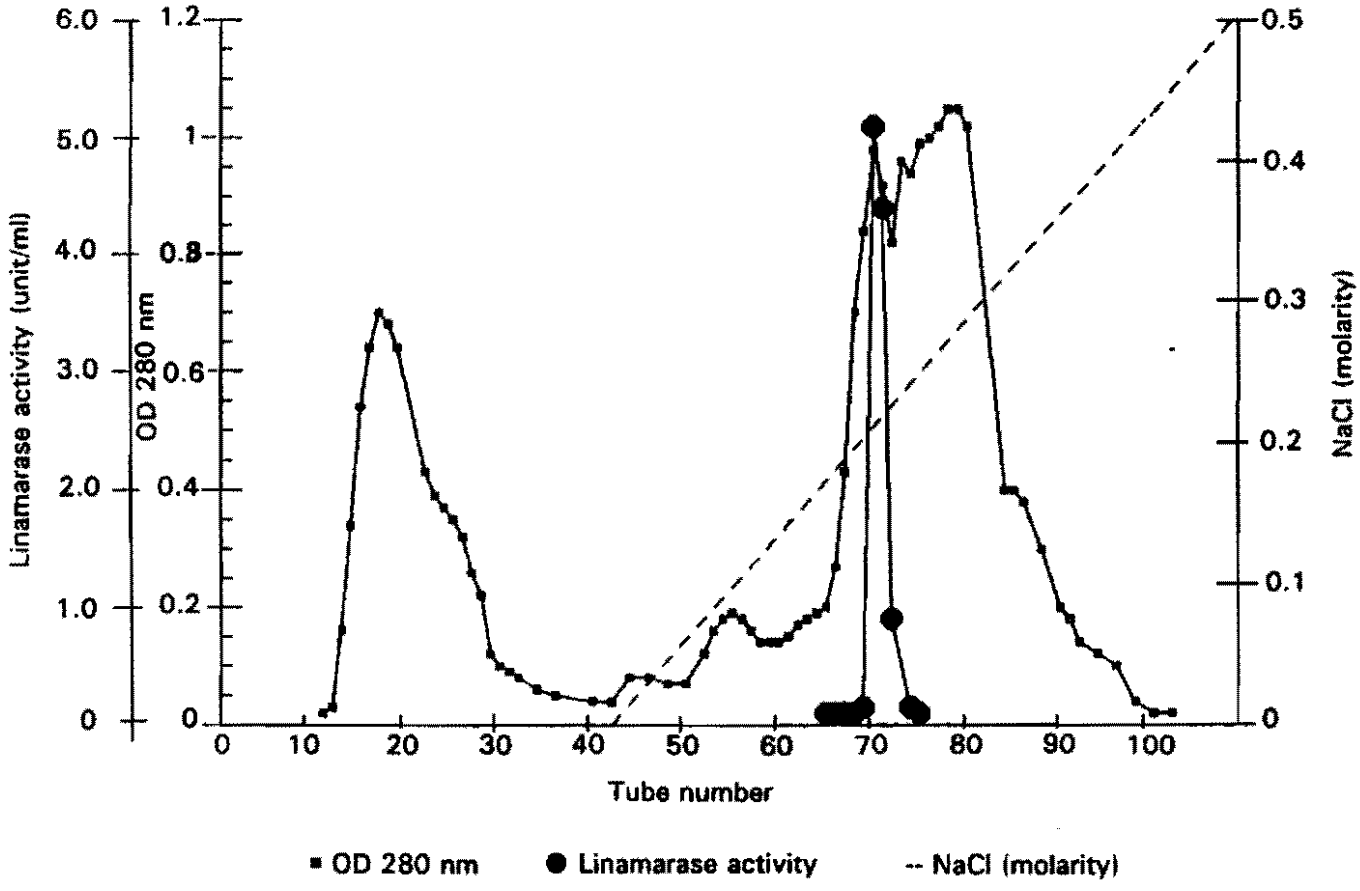

Figure 1. DEAE-ion exchange chromatography of linamarase eluted from the yeast *Hansenula anomala* (ß-1-14) on 0.01 M phosphate buffer at pH 6.0 and 0-0.5 M NaCl.

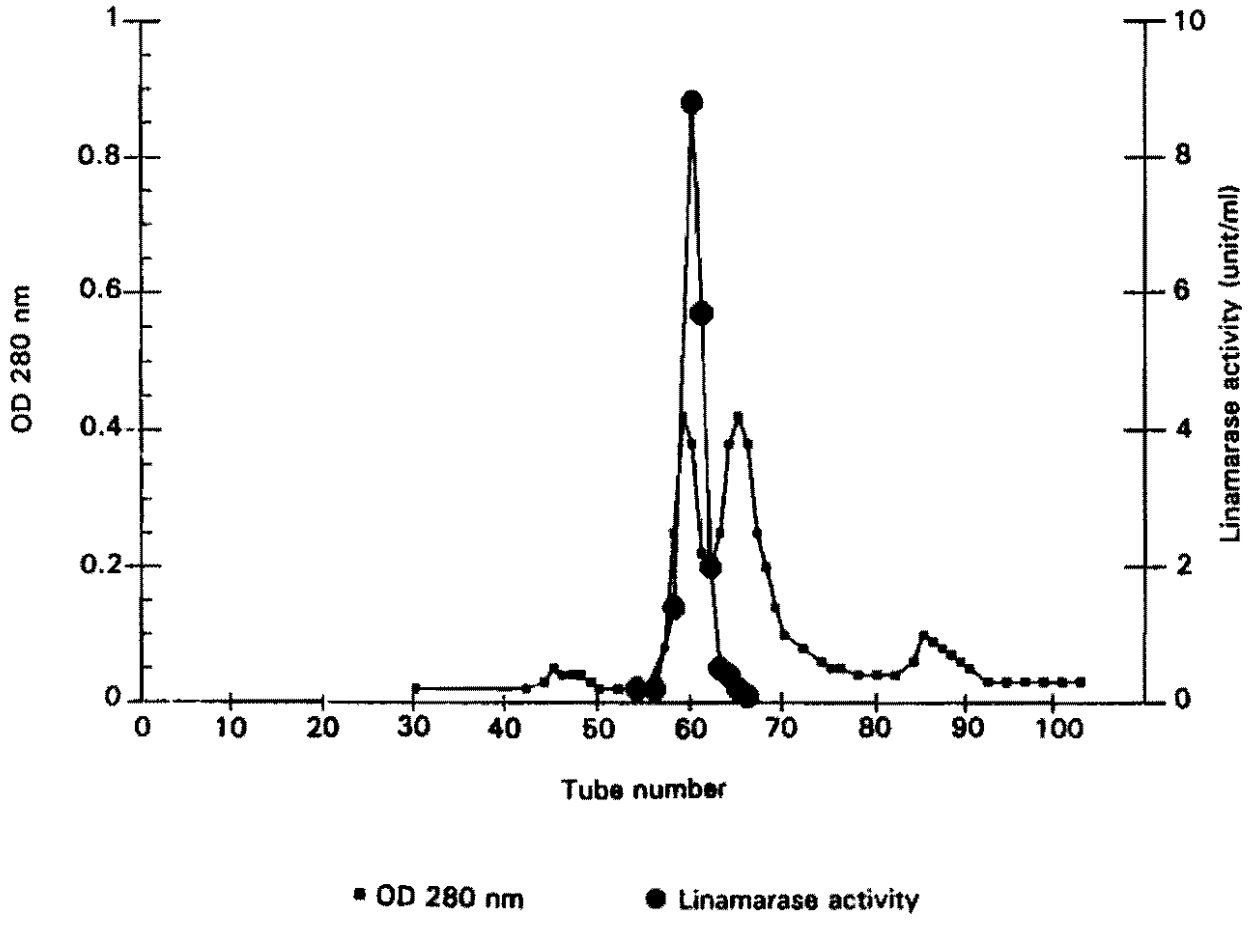

Figure 2. Gel filtration chromatography of linamarase from the yeast *Hansenula anomala* (ß-1-14) presenting activity at tube numbers 57-66 with only one peak of protein.

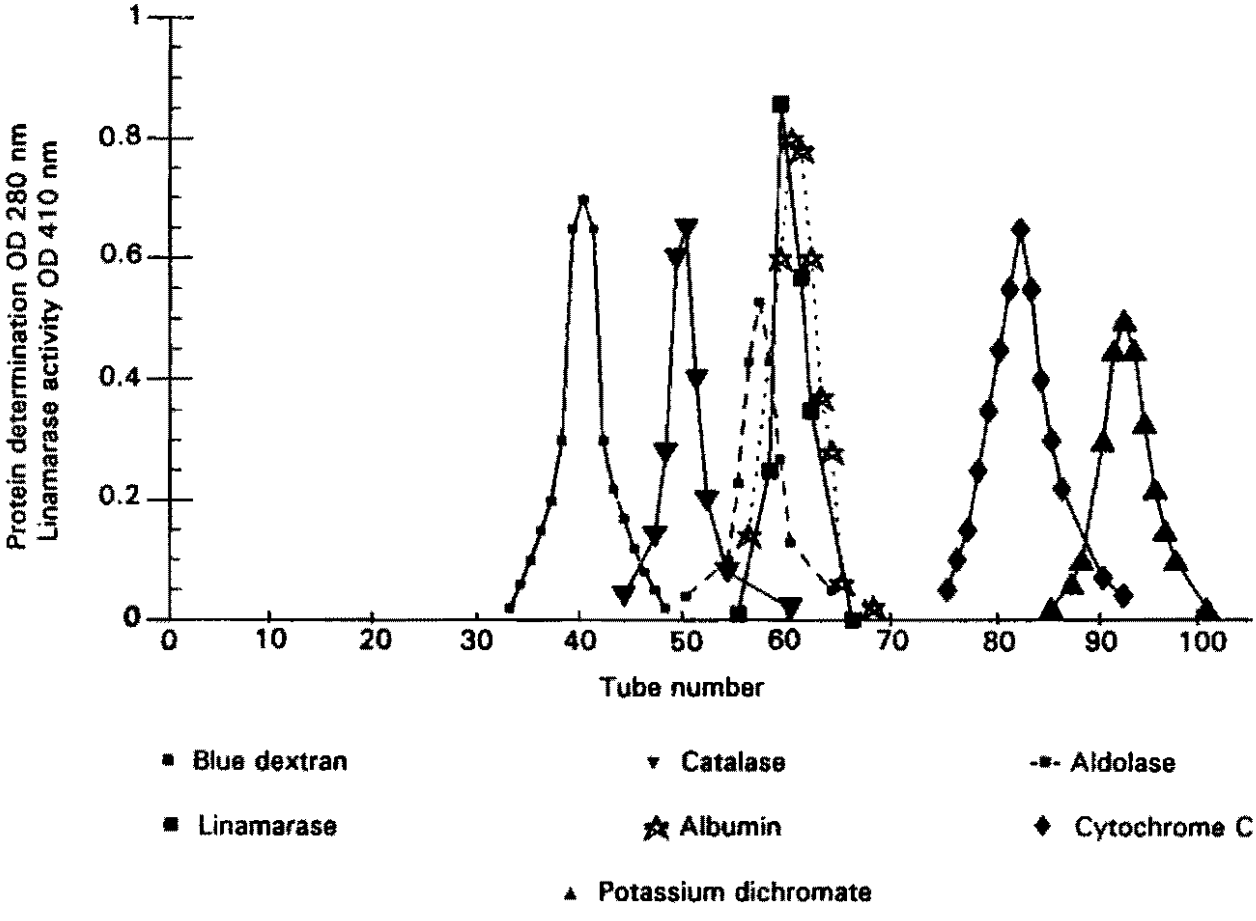

Figure 3. Gel filtration chromatography to determine the molecular weight of the enzyme linamarase compared with the standard molecular weights of aldolase, catalase, cytochrome C, potassium dichromate, albumin, and blue dextran.

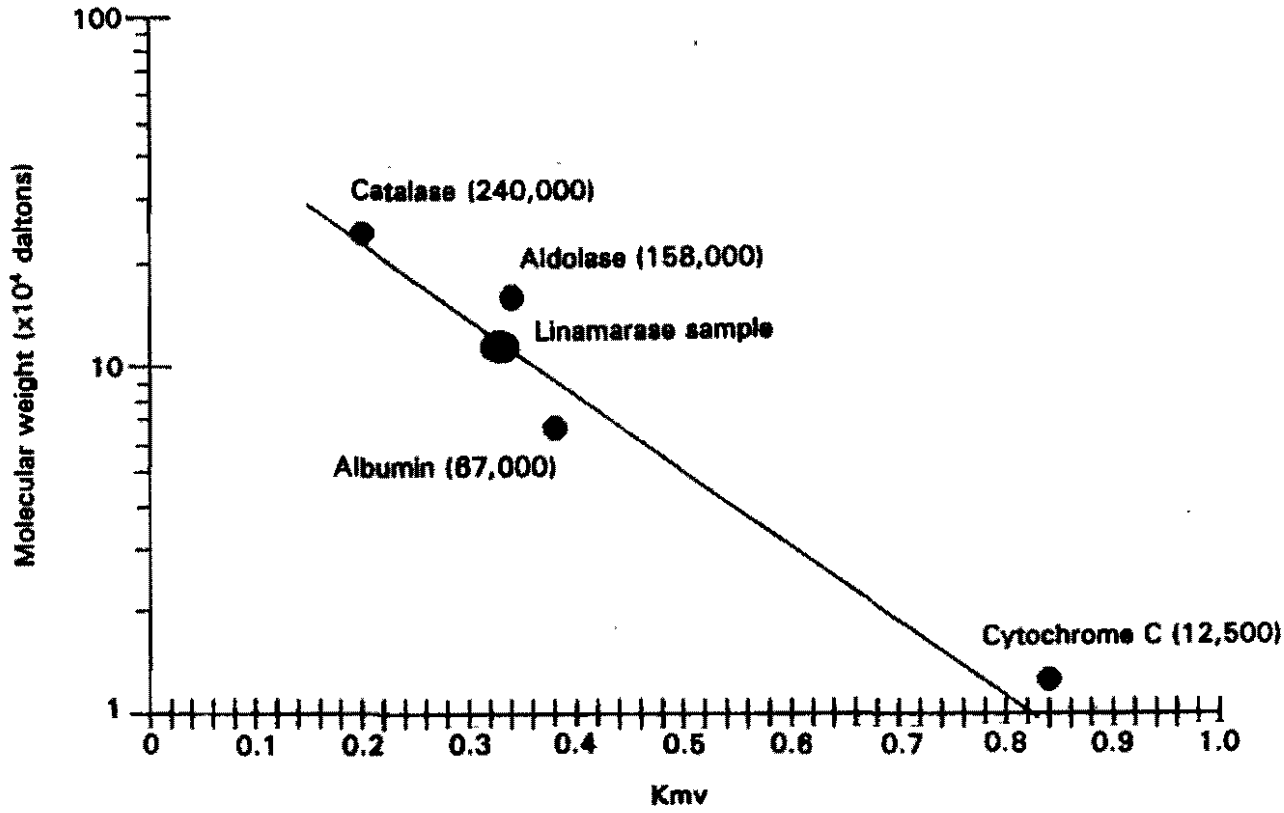

Figure 4. The relationship between logarithm, molecular weight, and Km value of linamarase from *Hansenula anomala* (ß-1-14), compared with several standard molecular weights.

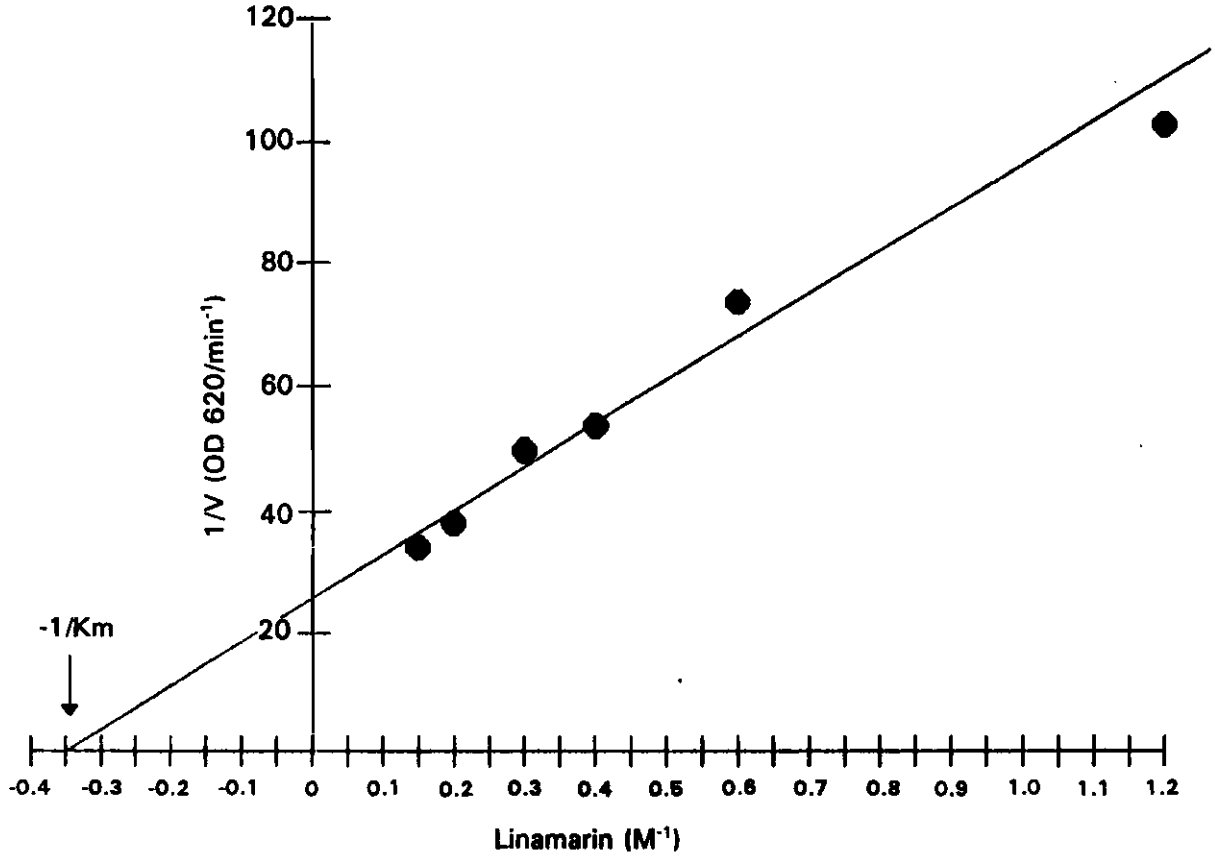

Figure 5. Km value of linamarase from *Hansenula anomala* yeast.

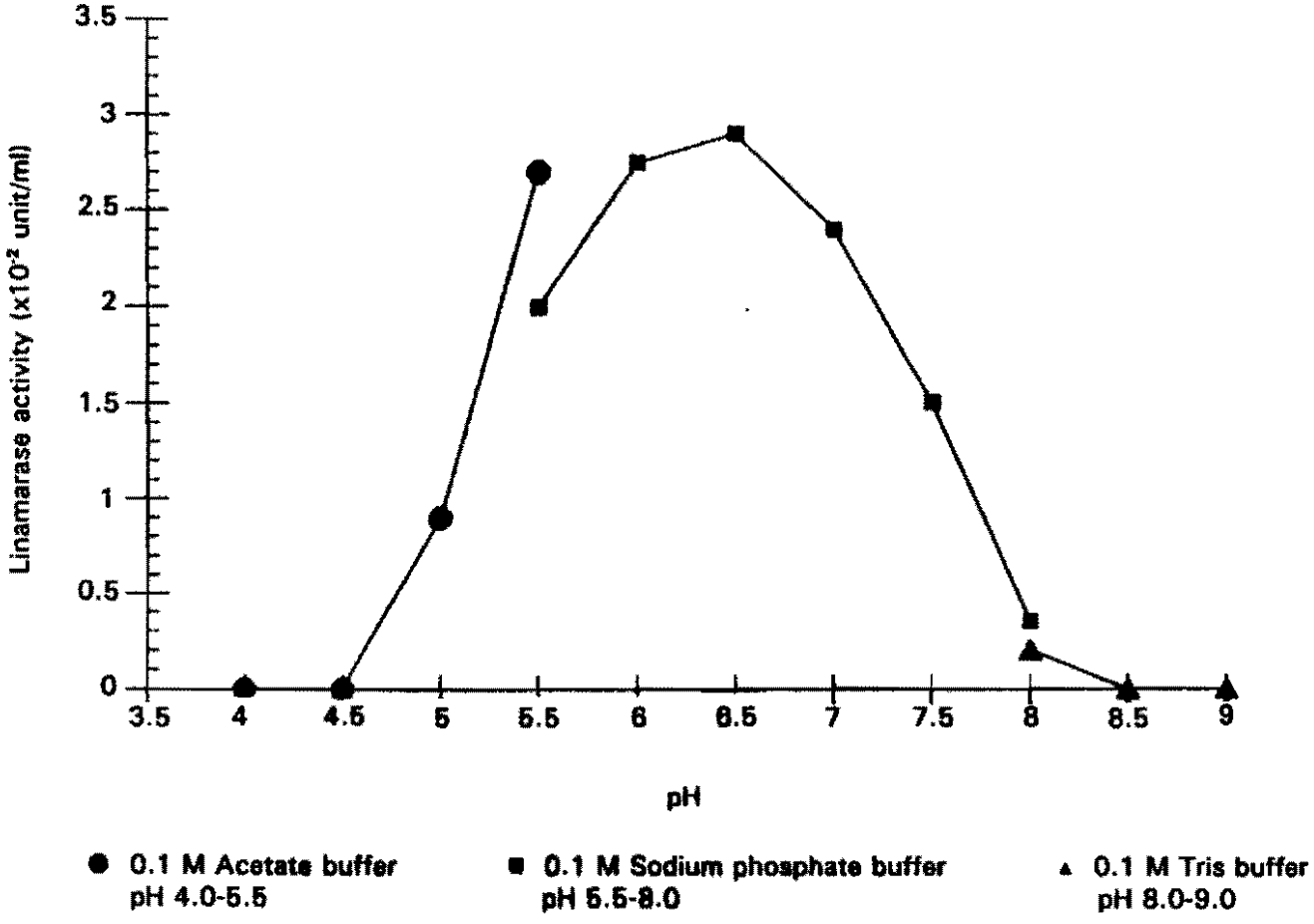

Figure 6. Effects of pH on the linamarase activities on different kinds of buffer at 30 °C and 30 minutes incubation time.

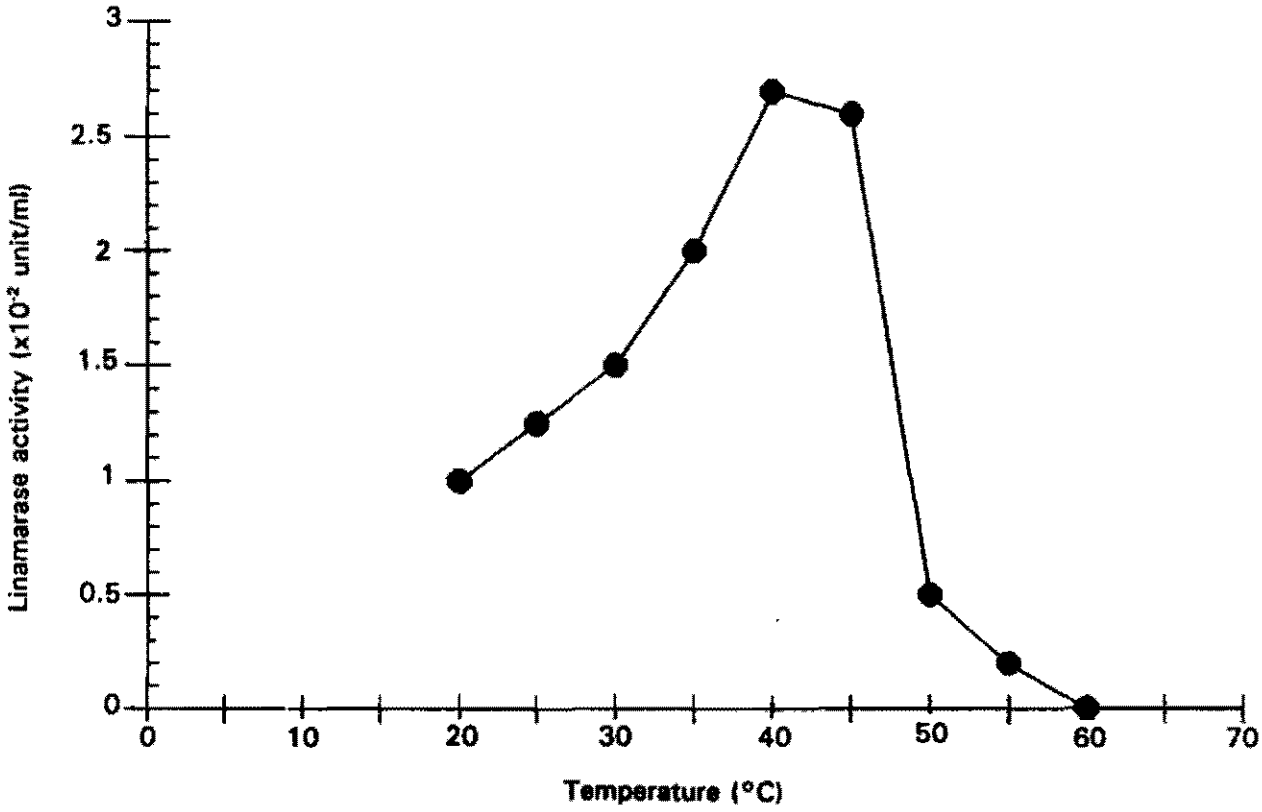

Figure 7. Effects of temperature on linamarase activities in 0.1 M phosphate buffer at pH 6.5, at temperatures ranging from 20 to 60 °C, incubation time 30 minutes.

RESIDUAL CYANIDE IN PROCESSED CASSAVA CHIPS RELATED TO STORAGE INSECTS

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This laboratory study studied the effect of the cyanogens present in dried cassava chips on the storage insects *Araecerus fasciculatus* (DeGeer) and *Sitophilus oryzae* (L.). The effect of parboiling on reducing insect infestation was also studied. Low-cyanide cultivars M4 and CE 501 and high-cyanide cultivars 76-9, S 300, and CE 512 were used; HCN content in the former ranged from 145 to 168 mg/kg DM, and in the latter, from 202 to 291. Sun-dried chips from low-cyanide cultivars had 77-83 mg HCN kg^{-1} DM, while those from high-cyanide cultivars had 125-164. Parboiled chips retained more cyanide than sun-dried chips. Both free-choice and no-choice tests were used to study the effect of the residual cyanide in processed chips on the feeding behavior and population buildup of storage pests. Sun-dried chips of CE 512, with higher residual cyanide, were the least preferred by pests; M4 chips, with low cyanide, were preferred the most. Parboiled chips were least preferred by both insects because of their hardness. In the free-choice test, from 21 to 26 adult progeny of *A. fasciculatus* developed from low-cyanide chips, whereas 6 to 16 developed from high-cyanide chips. No adults emerged from parboiled chips. Adult emergence values for *S. oryzae* ranged from 13 to 20 in low-cyanide cultivars and from 2 to 7 in high-cyanide cultivars. In no-choice tests, apparent weight loss caused by *A. fasciculatus* was significantly higher (7.0%-17.8%) in plain chips compared to parboiled chips (1.1%-2.5%). Maximum weight loss in plain chips occurred in variety M4 and minimum weight loss in CE 512. Weight loss caused by *S. oryzae* was also significantly higher in plain chips of low-cyanide cultivars.

INTRODUCTION

Cassava roots deteriorate rapidly within 3 to 10 days after harvest (Booth, 1976). Biochemical changes and microbial infestation spoil the roots and make them unfit for consumption (Balagopalan et al., 1988). To reduce perishability, the roots were sun-dried and processed into chips and flour immediately after harvest. In South India, cassava is also stored as parboiled chips (cooked in boiling water for 10 minutes and then sun-dried). Stored cassava is susceptible to a wide range of storage pests that cause considerable losses converting chips into dust and frass (insect dust). Over two dozen insect species are reported to infest dry cassava (CTCRI, 1992; Ingram and Humphries, 1972; McFarlane, 1982; Parker et al., 1981).

The major insect species infesting dry cassava chips in India are *Araecerus fasciculatus*, *Dinoderus bifoveolatus*, *Sitophilus oryzae*, *Rhyzopertha dominica*, and *Lasioderma serricorne*. *Tribolium castaneum* is a major pest infesting cassava flour.

Reports indicate that cultivars vary in their susceptibility to insect infestation. Pillai (1977) found two cassava varieties that were less susceptible to *A. fasciculatus*. There are also indications that insect pests attack bitter (high cyanide) varieties less (Kerr, 1941; Rajaguru, 1973; Rajamma and Premkumar, 1993; Rajamma et al., 1994). The harder texture of parboiled chips, due to the partially gelatinized starch, make them less susceptible to insect damage during storage (CTCRI, 1986; Hirandani and Advani, 1955; Rajamma and Premkumar, 1993).

The effect of residual cyanide in processed cassava chips on the feeding behavior and population buildup of two major pests of cassava chips—*A. fasciculatus* and *S. oryzae*—was studied.

MATERIALS AND METHODS

Preparing samples

Two low-cyanide cultivars (M4 and CE 501) and three high-cyanide cultivars (76-9, S 300, and CE 512) were grown under similar conditions and their roots harvested at 12 months and subjected to two types of drying treatments: (1) peeling and sun drying, and (2) peeling, parboiling, and sun drying. Peeled roots were cut into round chips, 0.75 cm thick. Part of the chips was directly sun-dried and the other was parboiled in boiling water (steeped) for 10 minutes (1:3 w/v), then drained and sun-dried. Chips were then heated in an oven overnight, at 60 °C, to eliminate any insect infestation that might have occurred during sun drying. After reaching room temperature, the chips were stored in plastic bottles and sprinkled with water until a moisture content of 14% was reached. Sample moisture content was determined by heating the chips in an oven, preheated to 130-133 °C, for 3 h. Sample cyanide content was analyzed by combining the methods of O'Brien et al. (1991) and Nambisan and Sundaresan (1984).

Origin of experimental insects

Araecerus fasciculatus and *S. oryzae* were collected from local cassava storehouses, and a mass culture of both was maintained on a susceptible cassava cultivar, H 1687, in the laboratory at room temperature and humidity.

Resistance tests

Choice and non-choice tests were conducted separately for each insect species under room conditions.

Choice test

To establish insect preferences, *A. fasciculatus* and *S. oryzae* were offered plain and parboiled chips of both low- and high-cyanide cultivars. Twenty-five grams of each type of chip were placed in petri dishes, arranged in a circle on top of a plastic tray. In the middle of the tray, in a separate dish, 100 adult insects of *A. fasciculatus* and *S. oryzae* were released. The tray was then covered with a cloth, which was closed shut with a rubber band. The trial was replicated twice for each insect species. The insects were removed after 1 week, and the samples were placed in plastic containers covered with a cloth and closed shut with a rubber band. After about 30 days, the samples were examined daily for the emergence of F1 adults. Any adults were removed and counted. Preference was assessed based on the number of F1 adult progeny and the weight of frass formed within 12 weeks.

Non-choice test

The no-choice test involved 10 treatments (5 cultivars x 2 processing methods) and three replications for each insect species. A control replicate was also provided for each treatment to monitor weight changes due to fluctuations in humidity. In the case of *A. fasciculatus*, each replicate contained 75-g chips and 40 adults, and in the case of *S. oryzae*, 50-g chips and 30 adults. Cloth-covered plastic containers were used for culturing the insects. Insects were not introduced into the control replicates. The samples were weighed at the beginning of the test and at 9 weeks; the difference in weight was considered the apparent weight loss. In *S. oryzae*, samples were also weighed at 14 weeks since feeding was poor at 9 weeks. The total adult population was also recorded at the end of the experiment. The amount of frass formed was also weighed by sieving through a 1-mm sieve. Susceptibility was assessed on the basis of weight loss, frass weight, and insect population growth.

Developmental period

The developmental period of both insects on plain chips was also studied under room conditions. Twenty adult insects were allowed to lay eggs for 5 days on chips (50 g) of each cultivar, placed in 250-ml plastic containers. There were four replications for each insect species and cultivar. After 5 days, adult insects were removed and kept for development, and then newly emerged adults were counted daily and also removed. The minimal time needed for insect development was calculated from the third day after insect release until the first day of emergence.

RESULTS

Biochemical characteristics of chips

The total cyanide contents of fresh, plain dried, and parboiled chips are presented in Figure 1. Cyanide content in fresh cassava roots ranged from 145.1 to 291.1 mg kg^{-1} DM, while its values in plain dried chips were 77.1 to 163.5 mg kg^{-1} DM and in parboiled (dried) chips from 99.7 to 279.7 mg kg^{-1} DM. Cyanide content is considerably reduced in plain (sun-dried) chips compared with parboiled chips. Among the cultivars studied, CE 512 showed the highest cyanide content in both plain dried and parboiled chips. Table 1 indicates the initial moisture contents of the chips used for the different resistance tests, as well as the frass formed by both insects.

Figure 2 shows the adult progeny that developed. Adult emergence and frass weight were higher in the low-cyanide cultivars M4 and CE 501; M4 was preferred the most and CE 512, with the highest cyanide content, the least. No adult *A. fasciculatus* progeny developed in parboiled chips, while very few individuals of *S. oryzae* emerged from chips of low-cyanide cultivars.

Non-choice tests

The weight losses due to feeding by *A. fasciculatus* and frass weight by this pest in non-choice tests, after 9 weeks, are presented in Table 2. Apparent weight losses caused by *A. fasciculatus* were significantly higher in plain chips compared to parboiled chips. Among plain chips, weight loss was highest (17.8%) in M4 compared to other cultivars, and lowest in CE 512. Weight loss in parboiled chips caused by *A. fasciculatus* was negligible in all cultivars (1.1% to 2.5%). Frass weight was highest in plain chips of M4 and lowest in CE 512. Figure 3 indicates final adult population (including the initial population). Adult population was highest in plain chips of M4 (143 individuals) and lowest in CE 512 (58 individuals). Population growth was nil in parboiled chips of all cultivars, except for a slight increase in M4.

Apparent weight loss due to *S. oryzae*, at the end of 9 and 14 weeks, is shown in Table 3. Among plain chips, M4 had the highest weight loss (6.97% at 9 weeks increasing to 18.97% at 14 weeks), followed by CE 501 (2.43% at 9 weeks increasing to 5.92% at 14 weeks). Weight loss in the 3 high-cyanide cultivars was negligible, being similar up to 9 weeks; however, a significant loss was noted in cultivar 76-9 at 14 weeks. Parboiled chips of M4 had significantly higher weight loss (2.75 at 9 weeks and 6.73 at 14 weeks), being negligible in those of the other four cultivars. Frass weight at the end of 14 weeks (Table 3) was also significantly higher in M4, being lowest in CE 512. Figure 3 indicates the final adult population of *S. oryzae* (including the initial population). Plain chips of CE 512

had the lowest *S. oryzae* population (35 individuals) while those of M4 had the highest (227). Likewise, *S. oryzae* population was highest in parboiled chips of M4 (112) and lowest in CE 512 (32).

Developmental period

Figure 4 indicates the minimal time required for development (from egg to first emergence of adults). High-cyanide cultivars prolonged the development period of both *A. fasciculatus* and *S. oryzae*. Regarding *A. fasciculatus*, the developmental period was shortest in M4 (38 days) and longest in CE 512 (59 days). *S. oryzae* took 42 days to develop in M4 and 57 days in CE 512.

DISCUSSION

When *A. fasciculatus* was given free choice of the different types of chips, it preferred plain chips and rejected parboiled chips. Plain chips from low-cyanide cultivars, especially cultivar M4, were preferred to those from high-cyanide cultivars. *S. oryzae* also preferred plain chips of low-cyanide cultivars, and parboiled chips of M4. Although M4 and CE 501 have similar cyanide content, M4 was preferred over CE 501, perhaps because roots of CE 501 have a fibrous texture.

In non-choice tests, M4 was the cultivar presenting the highest infestation; high-cyanide cultivars had the lowest infestation. Earlier research had already reported the role that cyanide plays in reducing insect infestation in cassava chips (Kerr, 1941; McFarlane, 1982; Rajaguru, 1973). Rajamma and Premkumar (1993) found that cassava cultivar 76-9 was the least infested by *A. fasciculatus* and *S. oryzae* among six varieties tested. They also found that parboiled chips were attacked less; however, a choice test was not conducted in their study. Rajamma et al. (1994) found that *Rhyzopertha dominica* cultured on chips and *Tribolium castaneum* on flour, but were reluctant to multiply on high-cyanide varieties. Parker and Booth (1979) attempted to correlate the cyanide content of cassava chips with resulting insect population and damage, but no conclusive results were obtained.

The present study confirmed the reducing effect that residual cyanide in processed cassava has on insect infestation, as well as the effect of parboiling on reducing insect infestation.

ACKNOWLEDGMENT

Acknowledgment is due to the Director of the Central Tuber Crops Research Institute, Trivandrum, for providing the facilities for this research work.

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Table 1. Frass weight due to feeding by *Araecerus fasciculatus* and *Sitophilus oryzae* in choice tests.

Cultivars	Initial moisture content (%)[a]		Frass weight (g)			
			A. fasciculatus		*S. oryzae*	
	Plain	Parboiled	Plain	Parboiled	Plain	Parboiled
M4	14.0	13.9	13.83	0.02	0.25	0.03
CE 501	14.1	13.8	11.08	0.02	0.10	0.01
76-9	14.3	14.0	6.67	0.03	0.05	0.01
S 300	14.1	14.1	4.89	0.01	0.03	0.00
CE 512	14.5	13.7	1.79	0.02	0.02	0.00

a. Wet basis.

Table 2. Apparent weight loss and frass weight due to feeding by *Araecerus fasciculatus* during 9 weeks in non-choice tests[a].

Cultivars	Apparent weight loss (%)		Frass weight (g)	
	Plain	Parboiled	Plain	Parboiled
M4	17.76 e	2.51 a	47.49 e	0.26 a
CE 501	11.71 d	1.86 a	28.53 d	0.11 a
76-9	9.11 bc	1.07 a	26.07 c	0.08 a
S 300	10.15 cd	1.20 a	23.91 c	0.10 a
CE 512	7.02 b	1.13 a	10.97 b	0.12 a

a. Vertical values followed by different letters are significantly different ($P < 0.05$) for each parameter, as determined by ANOVA.

Table 3. Apparent weight loss and frass weight due to feeding by *Sitophylus oryzae* in non-choice tests[a].

Cultivars	Apparent weight loss (%)				Frass weight (g)	
	9 weeks		14 weeks		14 weeks	
	Plain	Parboiled	Plain	Parboiled	Plain	Parboiled
M4	6.97 c	2.75 b	18.97 d	6.73 b	6.00 e	0.60 bc
CE 501	2.43 b	0.11 a	5.92 b	0.72 a	1.45 d	0.04 a
76-9	1.03 a	0.17 a	3.59 c	0.79 a	0.75 c	0.04 a
S 300	0.31 a	0.23 a	1.17 a	1.36 a	0.31 c	0.02 a
CE 512	0.07 a	0.07 a	1.07 a	0.77 a	0.19 ab	0.01 a

a. Vertical values followed by different letters are significantly different ($P < 0.05$) for each interval of feeding, as determined by ANOVA.

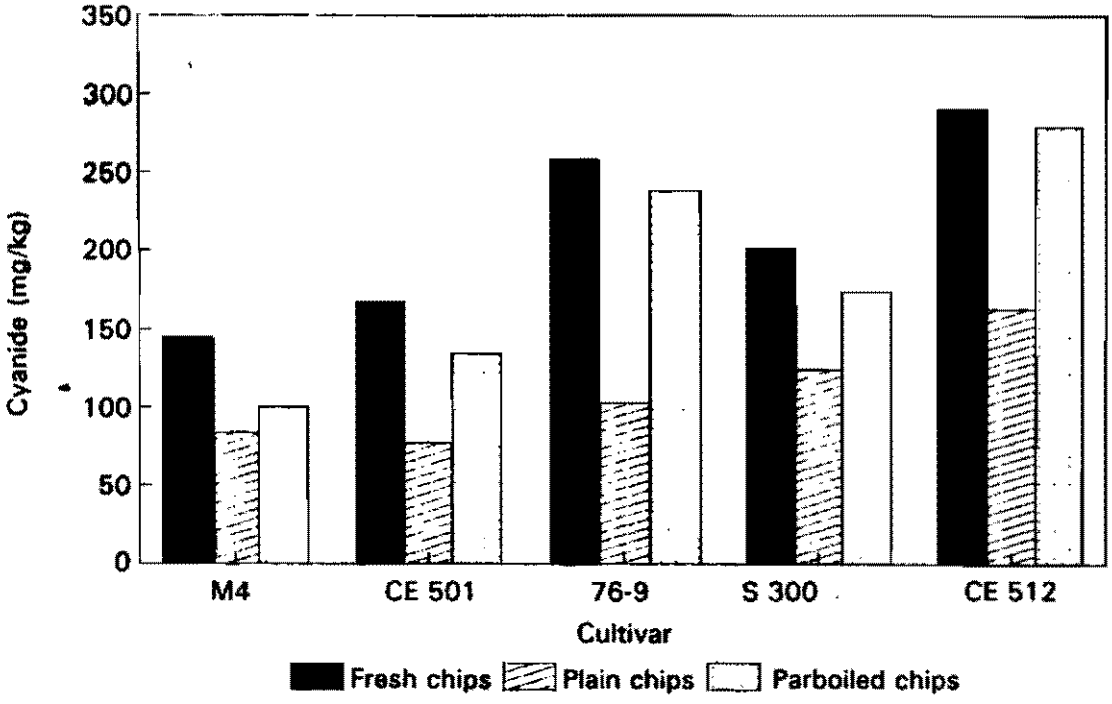

Figure 1. Cyanide content on different types of chips of five cassava cultivars.

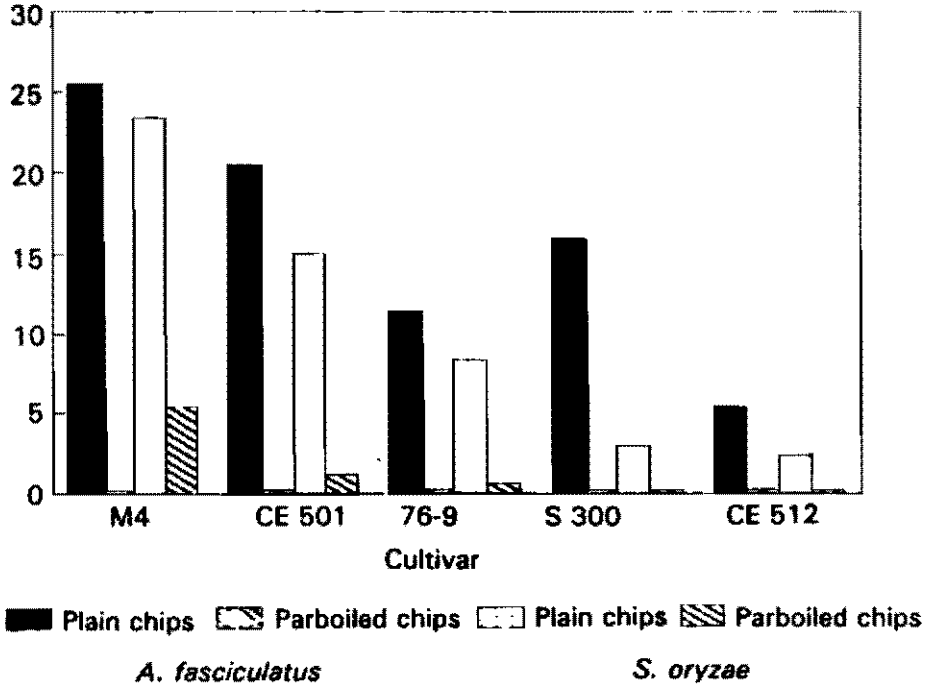

Figure 2. Number of adult progeny of *Araecerus fasciculatus* and *Sitophilus oryzae* on different types of chips in choice tests.

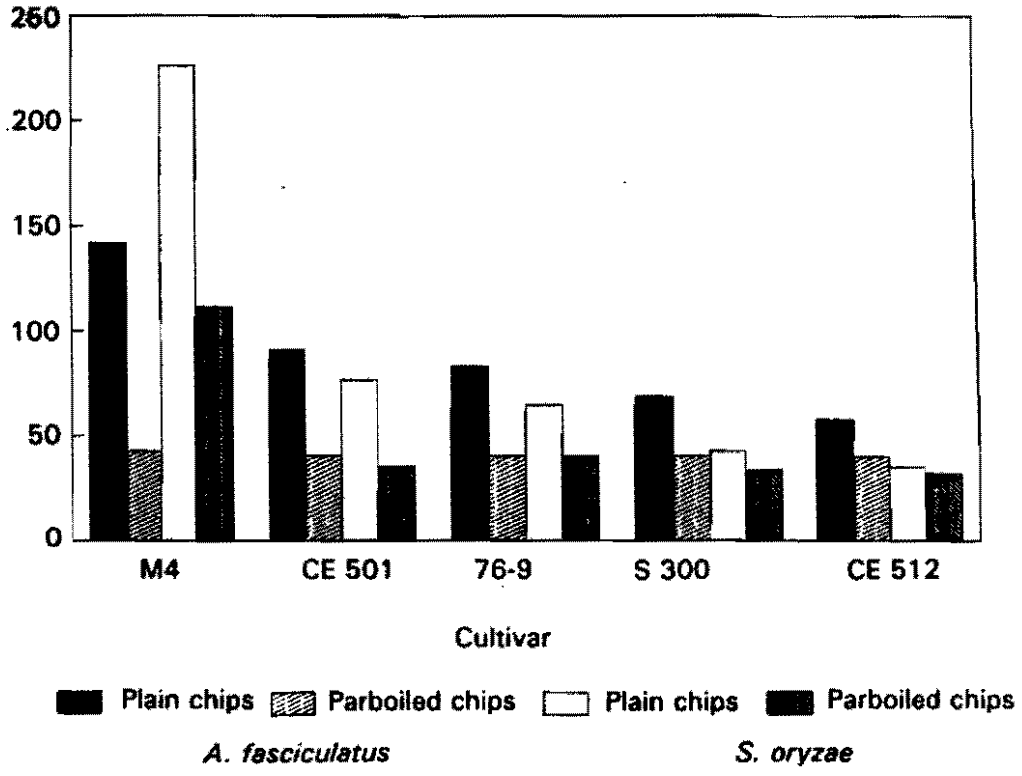

Figure 3. Population of *Araecerus fasciculatus* and *Sitophilus oryzae* on different types of chips in non-choice tests.

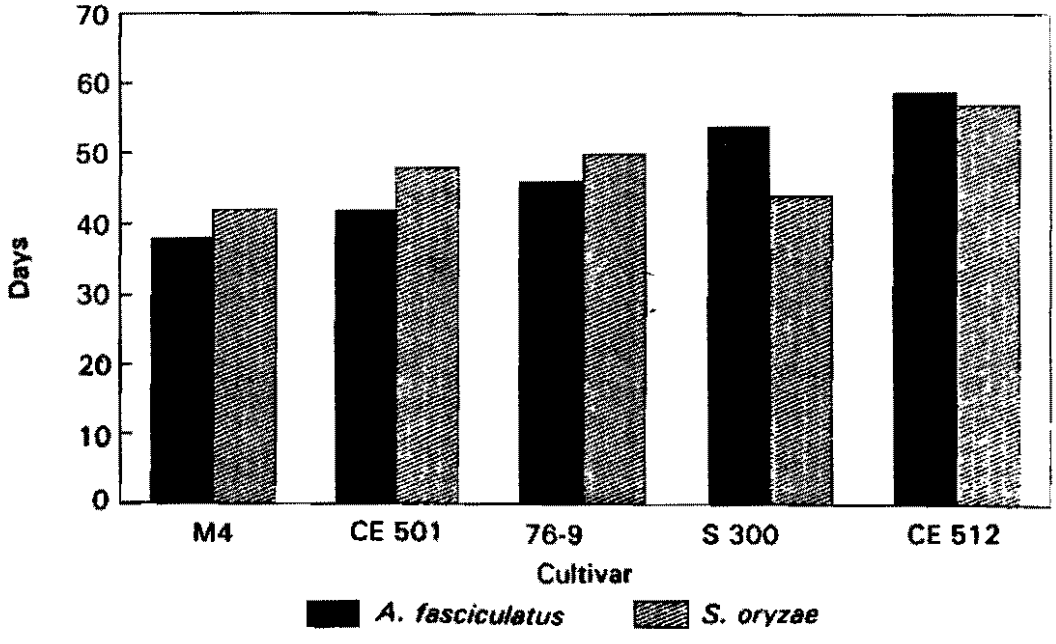

Figure 4. Minimum developmental period (days) for *Araecerus fasciculatus* and *Sitophilus oryzae* on five different cassava cultivars.

BIOTECHNOLOGY APPLICATIONS IN CASSAVA RESEARCH AND DEVELOPMENT

Biotic and Abiotic Stresses on Cassava Production

CASSAVA VIRUSES IN THE OLD WORLD

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Virus diseases are a major cause of crop loss in cassava, especially in Africa, and reliable methods are needed to detect, identify and quantitatively assay their causal agents. The most serious disease, mosaic, is caused by each of three whitefly-transmitted geminiviruses that have essentially nonoverlapping distributions: African cassava mosaic virus (ACMV) in Africa west of the Rift Valley, East African (EACMV) in East Africa and Madagascar, and Indian (ICMV) in the Indian subcontinent. These distributions are thought to reflect the different routes of cassava introduced from South America and the subsequent acquiring of different indigenous geminiviruses from other plant species in the three regions. The three viruses can be detected and distinguished by their patterns of reactions in ELISA with panels of monoclonal antibodies prepared against ACMV and ICMV, and by their reactions with probes for viral DNA. They can be detected in plants and individual vector whiteflies, *Bemisia tabaci*, by the polymerase chain reaction (PCR), and distinguished by the pattern of fragments obtained by cleaving the PCR products with restriction endonucleases. Resistance-breeding programs and ecological studies should take into account that results for one of the viruses may not apply to the other two. Other viruses of lesser but largely unknown importance include cassava brown streak virus in East Africa, cassava Ivorian bacilliform virus in Ivory Coast, cassava virus Q in widely separated parts of Africa, and Kumi viruses A and B in Uganda. The first three of these can be detected by ELISA.

INTRODUCTION

In recent years, research on the mosaic disease of cassava found in the Old World has greatly increased knowledge about the causal viruses and also, incidentally, has led to the discovery of several other sap-transmissible cassava viruses whose properties are now known in different degrees of detail. In this paper, we summarize the evidence that mosaic disease is associated with infection by any one of three distinct whitefly-transmitted geminiviruses. We also describe modern methods for distinguishing these three viruses and for detecting them in plants and vector whiteflies. In addition, we briefly summarize information on the detection of other Old World cassava viruses and their characteristic properties.

CASSAVA GEMINIVIRUSES

In 22 countries in Africa or the Indian subcontinent, tests on more than 150 geminivirus isolates from cassava indicate that they can be assigned to three clusters. In tests with panels of monoclonal antibodies raised against virus

particles, the isolates have different patterns of reactivity characteristic for each cluster and vary little among isolates in the same cluster. Representative isolates from the different clusters also differ in biological properties and in the nucleotide sequences of their genomic DNA. These correlated differences have led to the conclusion that the isolates represent three distinct geminiviruses: African cassava mosaic (ACMV), East African cassava mosaic (EACMV) and Indian cassava mosaic (ICMV) viruses (Hong, Robinson and Harrison, 1993; Swanson and Harrison, 1994).

ACMV

We have found ACMV in samples from Angola, Benin, Burkine Faso, Burundi, Cameroon, Chad, Congo, Ghana, Ivory Coast, Mozambique, Nigeria, Senegal, South Africa, Uganda, Zambia and the western parts of Kenya and Tanzania (Swanson and Harrison, 1994). Most isolates can be easily transmitted by inoculation of sap to *Nicotiana benthamiana* and *N. clevelandii*, inducing systemic mosaic, stunting and leaf curling. The type isolate (isolate 844 from western Kenya) multiplied better at about 23 °C than at higher or lower temperatures (Robinson et al., 1984). The genome of this isolate consists of two molecules of circular, single-stranded DNA (Harrison et al., 1977). The nucleotide sequences of these molecules contain six open reading frames encoding proteins of at least 10 K, four in DNA-A and two in DNA-B (Stanley and Gay, 1983). Transcription is bidirectional, with one open reading frame in each DNA segment being translated from RNA molecules of the same sense as the DNA in virus particles (+ sense) and the others being translated from complementary-sense RNA molecules. The virus coat protein (about 30 K) is encoded by + sense DNA-A. The nucleotide sequences of the two DNA molecules are different except for a non-coding stretch of about 200 nucleotides (the "common region") which is shared (Stanley and Gay, 1983).

ICMV

ICMV occurs in India and Sri Lanka (Swanson and Harrison, 1994). Most isolates are transmissible to *N. benthamiana* by inoculation with sap from cassava but somewhat less readily than ACMV. Symptoms in cassava and *N. benthamiana* are essentially the same as those of ACMV but ICMV multiplies best at about 30 °C, and only reaches relatively low concentrations at 23 °C (Harrison et al., 1987). The genome of ICMV, like that of ACMV, consists of two circular, single-stranded molecules of DNA containing six main open reading frames (Hong et al., 1993). But the putative proteins encoded by the DNA of an Indian isolate of ICMV have only 59%-78% (DNA-A products) or 29%-42% (DNA-B products) amino acid sequence identity with the comparable proteins of ACMV. Further, the common region of ICMV (about 400 nt) is much longer than that of ACMV and is largely different from it in nucleotide sequence (Hong et al., 1993). Because of the sequence similarity between DNA-A of the two viruses, cDNA probes derived from ACMV DNA-A

detect ICMV DNA-A, whereas a full-length probe for ACV DNA-B does not detect ICMV (Harrison et al., 1987), reflecting the much smaller nucleotide sequence similarity in DNA-B. This degree of relationship is typical for whitefly-transmitted geminiviruses in general. Indeed ICMV seems no more closely related to ACMV than to some other geminiviruses. Further, ICMV and ACMV were unable to form pseudo-recombinants by associating DNA-A of one virus with DNA-B of the other, emphasizing their genetic distinctness (Frischmuth et al., 1993).

EACMV

EACMV occurs in Malawi, Madagascar, Zimbabwe and the coastal regions of Kenya and Tanzania (Swanson and Harrison, 1994). Symptoms induced in cassava are indistinguishable from those of ACMV and ICMV, but EACMV is much the most difficult of the three to transmit to *N. benthamiana* by inoculation with sap and it can hardly be maintained in this species, especially at temperatures below 30 °C (Robinson et al., 1984). Examining the partial nucleotide sequence of DNA-A of a Malawian isolate of EACMV shows that its common region and coat protein gene are substantially different from the corresponding sequences of ACMV and ICMV (Hong et al., 1993). Also, the full-length cloned cDNA probe for ACMV DNA-B failed to detect EACMV whereas an ACMV DNA-A probe did react with EACMV DNa. These results all point to the distinctness of EACMV from ACMV and ICMV.

DETECTION AND DIFFERENTIATION OF CASSAVA GEMINIVIRUSES BY ELISA

In early serological tests for ACMV, the virus was readily detected in leaf extracts by double antibody sandwich ELISA (DAS-ELISA; Sequeira and Harrison, 1982) and immunosorbent electron microscopy (Roberts et al., 1984) with polyclonal antibodies. In tests on cassava, best results were obtained when symptom-bearing leaves were used as the virus source (Fargettet et al., 1987). But these tests do not distinguish ACMV, EACMV and ICMV because of their serological relationship. The three viruses can be detected and differentiated by triple antibody sandwich ELISA (TAS-ELISA) with selected monoclonal antibodies (Thomas et al., 1986) that have different specificities. Table 1 shows examples of the reactions of seven of the most useful monoclonal antibodies. TAS-ELISA has also proved to be more sensitive than DAS-ELISA with polyclonal antibodies for detecting the viruses because the specific reactions are often stronger and background non-specific reactions usually weaker. In tests with more than 200 samples, the antibodies listed in Table 1 performed consistently and no difficulty was experienced in allocating each virus isolate to one of the three viruses except when only poor quality leaf material was available for the tests.

Some African and Indian laboratories have already adopted TAS-ELISA for work on cassava geminiviruses. But it uses polyclonal antibodies, which are in

limited supply, for coating the wells of microtitre plates. Further work is therefore planned to develop an ELISA-based procedure that relies solely on monoclonal antibodies, that could be produced in large amounts and distributed for use in laboratories in many countries.

GEMINIVIRUS DETECTION IN PLANTS AND WHITEFLIES BY THE POLYMERASE CHAIN REACTION (PCR)

PCR is proving useful for detecting a wide range of geminiviruses in plant tissues (Rojas et al, 1993; Deng et al., 1994). To date, most emphasis has been given to tests with primers representing conserved sequences in the geminivirus genomic DNA. For example, Deng et al. (1994) found that using primers, chosen to amplify the DNA sequence lying between the middle of the common region and the middle of the coat protein gene, resulted in detecting all 13 whitefly-transmitted geminiviruses tested, including ACMV, EACMV and ICMV.

To identify individual viruses, two approaches have been used. The first uses primers specific for the virus in question, namely those representing regions of the vital genome conserved among strains of the same virus but not among different geminiviruses. Primers derived from DNA-B promise to be suitable for this purpose. The second approach involves cleaving with restriction endonucleases the DNA fragment amplified by the primers representing conserved sequences. The pattern of sizes of the products, separated by gel electrophoresis, indicates the identity of the virus.

These procedures are so sensitive that they can be applied successfully to tiny samples or samples with very low virus concentration. For example, seven geminiviruses have been detected in single viruliferous whiteflies (*Bemisia tabaci*) and distinguished by analysis of the products of restriction endonuclease digestion of the amplified DNA sequence (Dent et al., 1994). But PCR is technically more demanding than TAS-ELISA and, as currently practiced, less suitable for quantitative or large-scale tests.

OTHER VIRUSES IN OLD WORLD CASSAVA

Any scheme for production of superior stocks of a vegetatively propagated crop, such as cassava, should take precautions to ensure that they are not infected by any virus, however slight its effects, to avoid the large scale dissemination of such a pathogen. In the Old World, as elsewhere, information is therefore required on which viruses other than geminiviruses occur in cassava, on their properties and on methods for the detection. We have encountered six such viruses (Table 2), that have been studied in different degrees of detail. All are transmissible to indicator plants by inoculation with sap from infected cassava leaves.

Cassava brown streak disease occurs in East Africa and Malawi. It is associated with flexuous filamentous particles resembling those of carla viruses (Lennon et al., 1986; Bock, 1994) but experimental hosts, unlike carla virus-infected plants, also contain pinwheel virus inclusion bodies. This suggests that affected cassava is infected either with a novel type of virus or with a complex of two dissimilar viruses (Lennon et al., 1986). Work is in progress to resolve this enigma. Leaf symptoms of brown streak develop only in older leaves, in association with increasing virus content. Detection tests should therefore be done on mature leaftissue and may fail when young leaves from infected plants are used. In Malawi, brown streak occurs combined with EACMV infection, giving rise to a more serious disease than either component alone.

Cassava Ivorian bacilliform virus has been found only in northwest Ivory Coast, in plants also containing ACMV (Fargette et al., 1991). Its effects on cassava are unknown. The bacilliform particles are of three predominant lengths of comparable, although different, sizes to those of alfalfa mosaic virus. But little if any serological relationship exists between the two viruses.

Cassava virus Q is the provisional name given to a virus with squat bacilliform particles (Aiton et al., 1988) that is found in cassava from Cameroon, Ivory Coast, Malawi and Zambia, where it is apparently associated with leaf fleck symptoms. The virus particles vary somewhat in length, as can be seen by immunosorbent electron microscopy of leaf extracts.

Kumi A and Kumi B are two recently discovered viruses occurring in cassava in one district in Uganda. Kumi A virus causes systemic necrotic ringspot symptoms, followed by recovery, in *Nicotiana tabacum* cv. Samsun NN. It has small bacilliform particles but seems to be serologically unrelated to cassava Ivorian bacilliform virus. Kumy B virus has isometric particles of about 30 nm diameter and causes extensive chlorotic ring and line patterns in systemically infected *Nicotiana benthamiana* leaves (B. D. Harrison and I. M. Roberts, unpublished data).

Finally, cassava common mosaic virus, a potex virus (Costa and Kitajima, 1972), has been found in cassava from Africa, but only rarely, and has probably been imported from South America in clonal planting material.

These six viruses can all be detected by inoculation of sap to indicator plants, such as *N. benthamiana, N. occidentalis* and *Chenopodium quinoa*. All except the two Kumi viruses, for which antiserum is not yet available, can be identified by ELISA with polyclonal antibodies. Immunosorbent electron microscopy is also helpful for identifying some of the viruses (Table 3). The two Kumi viruses are characterized by the symptoms they induce in *N. tabacum* and *N. benthamiana*.

DISCUSSION

It is now clear that Old World cassava is subject to infection by three different whitefly-transmitted geminiviruses: ACMV, EACMV and ICMV. The three viruses have different, largely non-overlapping geographical distributions and are apparently not found in the New World. We suggest that they were endemic in other plant species in three different regions of the Old World before cassava was introduced from South America in the 17th to 18th centuries. Their current distributions reflect the geographical expansion of cassava growing, following the three main points of the crop's introduction: (a) to West Africa, then along the coast and across the continent to the north and east; (b) to East Africa and Madagascar and then inland towards the west; and (c) to India and Sri Lanka (Harrison and Robinson, 1988).

Good progress has been made in devising serological tests, based on ELISA, with monoclonal antibodies, that will detect and differentiate the three viruses. The tests have already been introduced in a few African and Indian laboratories and, with some further development, should soon be suitable for large scale use. It is important that this be done because results obtained with one of the viruses cannot be assumed to apply to the others. For example, a cassava genotype resistant to one of the viruses may not be resistant to all. Also, naturally occurring alternative hosts may differ in importance as reservoirs of the different viruses, which may also differ in vector transmissibility by different *Bemisia tabaci* biotypes or, perhaps, *Bemisia* species. As regards breeding programs, cultivars that have been selected for resistance to ACMV in the large cassava breeding program at the International Institute of Tropical Agriculture (IITA), Nigeria, seem likely to have a measure of resistance to EACMV. This is because the IITA breeders have made extensive use of sources of resistance identified in earlier breeding programs in East Africa. But this expectation needs to be tested critically and comparable tests of novel types of resistance, such as transgenic resistance based on the introduction of viral genetic material into the cassava genome, will be especially important.

Finally, failure to ensure that geminivirus-free cassava stocks are also free from other viruses should not compromise the potential value of their large scale propagation and introduction.

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Table 1. Differentiation of cassava geminiviruses by triple antibody sandwich triple antibody sandwich (TAS)-enzyme-linked immunasorbent assay (ELISA) with monoclonal antibodies.[a]

Virus[b]	Monoclonal antibody[c]						
	SCR 20	SCR 17	SCR 23	SCR 21	SCR 33	SCR 52	SCR 60
ACMV	+	+	+	+	+	0	0
EACMV	+	+	+	0	0	0	0
ICMV	+	0	0	0	0	+	+

a. + = strong reaction, 0 = no reaction.

b. ACMV = African cassava mosaic virus; EACMV = East African cassava mosaic virus; ICMV = Indian cassava mosaic virus.

c. Monoclonal antibodies SCR 52 and SCR 60 were raised against particles of ICMV; the other antibodies were raised against ACMV particles.

Table 2. Viruses occurring in cassava in the Old World.

Virus	Known distribution	Particle shape and size (nm)	Method of spread
African cassava mosaic	W., C. and S. Africa	Geminate 30 x 20	*Bemisia tabaci*
East African cassava mosaic	E. Africa, Zimbabwe	Geminate 30 x 20	*B. tabaci*
Indian cassava mosaic	India, Sri Lanka	Geminate, 30 x 20	*B. tabaci*
Cassava brown streak	E. Africa, Malawi	Filamentous, 630 x 13	*B. tabaci*
Cassava Ivorian bacilliform	Ivory Coast	Bacilliform, 40-85x18	Unknown
Cassava Q	W. Africa, Malawi, Zambia	Bacilliform, 30 x 20	Unknown
Kumi A	Uganda	Bacilliform, 50 x 18	Unknown
Kumi B	Uganda	Isometric, 30	Unknown
Cassava common mosaic	Introduced from S. America	Filamentous, 495 x 13	Contact

Table 3. Methods of detecting and identifying African cassava viruses other than geminiviruses.[a]

Virus	ELISA[b]	ISEM[c]	Test plants[d]
Cassava brown streak	+	0	CQ, NB
Cassava Ivorian bacilliform	+	+	CA, CQ
Cassava Q	+	+	NB, NO
Kumi A	nt	nt	CQ, NB, NT
Kumi B	nt	nt	NB

a. + = suitable, 0 = not suitable, nt = not tested.
b. ELISA = enzyme-linked immunosorbent assay.
c. ISEM = immunosorbent electron microscopy.
d. CA = *Chenopodium amaranticolor*; CQ = *C. quinoa*; NB = *Nicotiana benthamiana*; NB = *N. occidentalis*; NT = *N. tabacum* Samsun NN.

CASSAVA BACTERIAL BLIGHT: PATHOGENICITY AND RESISTANCE AND IMPLICATIONS IN DEVELOPMENT OF RESISTANT LINES

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Xanthomonas campestris pv. *manihotis* (*Xcm*) mainly invades xylem vessels and is present in advance of symptoms. Initial parasitism appears to be biotrophic because leaf necrosis is slow, electrolyte leakage increases only after 2 days and host suspension-cultured cells are killed far more slowly (3-6 days) than by necrotrophs (within 1 day). Three bacterial products are potential determinants of pathogenicity. Copious extracellular polysaccharide (xanthan) is evident ultrastructurally in vessels and probably causes the characteristic water stress and possibly suppression of host defences. Pectate lyase is produced as a single form of isoelectric point 9, kills cassava cells at high dilution (1/1000 culture fluids) and reproduces leaf symptoms; it is highly induced by cassava cell walls. A toxin has been detected *in planta* by others, but production of this methionine metaboline (MTPA) requires at least 50 $\mu g ml^{-1}$ of this amino acid and free methionine in cassava leaves is 0.1 $\mu g/g$ fresh wt. Resistance of certain field-resistant lines (MNGA1, M Ven 77) to virulent isolates from Niger and Brazil appeared incomplete as although there was a delay of 2-4 days in leaf symptoms and 1 d in electrolyte loss compared with susceptible lines (e.g., M Col 113, M Col 22), by 12 days symptoms and bacterial growth were not significantly different. Resistance remained stable only with a low inoculum of 10^2 cells ml or less. This may be effective against the low numbers spread by rain splash but is likely to be overcome by potentially high inoculum on propagation tools. Co-culture of embryogenic cassava cells and *Xcm* has potential for regenerating resistant plants from cells surviving challenge with *Xcm* or its products and may facilitate development of cultivars with more effective disease resistance.

INTRODUCTION

Bacterial blight (CBB) caused by *Xanthomonas campestris* pv. *manihotis* (*Xcm*) remains one of the most serious diseases of cassava worldwide and is often a constraint to production. The pathogen is spread by rain splash and insects, as well as during vegetative propagation from stem cuttings. Symptoms appear as angular leaf spots followed by systemic infection and consequent wilting. Practica., long-term disease control can be achieved only through resistant varieties.

There is a paucity of information on mechanisms of bacterial pathogenicity and host resistance in CBB. This paper describes some of our initial work on host-parasite interactions as a prelude to a study on mechanisms of and selection for disease resistance to *Xcm*.

RESULTS AND DISCUSSION

Isolate pathogenicity and movement

Eight *Xcm* isolates from Colombia, Brazil, Zaire, Nigeria, Cameroon and Niger were compared for virulence under controlled environmental conditions (28 °C, 70%-80% RH) by several inoculation techniques; i.e., stem stab, leaf clip or leaf infiltration. All except one isolate from Colombia with a slow *in vitro* growth rate were highly virulent and caused typical, severe disease symptoms. Others have found *Xcm* isolates differ in aggressiveness, but there is no evidence of race-cultivar interactions (Maraite and Meyer, 1975).

The lower limit for inoculation was ≤ bacterial cells and in infected leaves *Xcm* multiplied to reach 10^{10} colony forming units (cfu) per gram fresh weight following leaf infiltration and 10^{8} cfu per gram following leaf clip. Invasion preceded symptom development and was mainly vascular as evidenced by the rapid rate of invasion of leaves and stems. *Xcm* had moved 3 cm from the point of inoculation in stems, both upwards and downwards, after only 5 h and had reached 9 cm within 8 days (Figure 1). In the leaves *Xcm* was present in lobes either side of the inoculated lobe after only 4 h, which could only be achieved by vascular transmission. This confirms earlier observations on the systemic spread of *Xcm* (Lozano, 1986; Maraite and Meyer, 1975). Bacterial movement from leaves to stems was restricted because infected leaves and petioles generally abscissed before stem invasion, in agreement with findings of Maraite and Meyer (1975).

Transmission electron microscopy confirmed that *Xcm* mainly invades xylem vessels. Following leaf infiltration stomates, bacteria were found exclusively in vascular tissue. In stems inoculated by puncture, bacteria-colonized xylem vessels and intercellular spaces of vascular bundles; no other stem tissues were invaded.

Host-parasite interactions

Initial parasitism appears to be biotrophic because leaf necrosis is slow to develop and form leaf discs incubated with *Xcm* cells electrolyte leakage, indicative of cellular membrane damage, was evident only after 2 days; whereas the incompatible pathogen *Erwinia amylovora* caused rapid ion loss, presumably as a result of inducing hypersensitivity (Figure 2). The CBB pathogen is very slow at inducing hypersensitivity in tobacco leaves; this localized resistance response is usually visible by 4-6 h for erwinias and pseudomonads but requires ca. 24 h with *Xcm*.

Co-culture of *Xcm* cells with suspension-cultured cassava cells which involves synchronous inoculation, allows more detailed examination of the interaction. Possible applications of this technique for selection of disease-resistant

lines are discussed later. Suspension cultures of cultivar M Col 113 were killed by *Xcm* and susceptibility increased during the cel cycle; thus at lag (5 days), early log (10 days) and late log phase (20 days) 80% killing took 6, 4, 5 and 3 days, respectively (Figure 3). This is much slower than in comparable experiments with necrotrophic pathogens and their corresponding host cells such as *Erwinia amylovora* and apple cells and *Erwinia carotovora* subsp. *carotovora* and carrot cells, which both lose viability within 24 h (e.g., Cooper et al., 1990). Host and bacterial cells need to be in contact or in very close proximity because separation of the cell types by dialysis membrane protects cassava cells from being killed by *Xcm*.

Some of the genes required by gram negative bacteria for pathogenicity (p) and hypersensitivity (hr) are clustered in a *hrp* region 25-40 kb. *Hrp* genes of *Xcm* are highly homologous to those of *P. solanacearum*, and some appear to play a role in transport of virulence proteins (Brown et al., 1993; Bonas et al., 1993; Seal et al., 1990). The genetics and functions of pathogenicity loci of *Xcm* have yet to be investigated.

Pathogenicity determinants

Xcm must enter, obtain nutrients from host cells presumably by damaging them, multiply and spread in host tissues. In the light of past work on xanthomonads and other pathogenic bacteria, we investigated the possible involvement in disease of toxins, cell wall-degrading enzymes and extracellular polysaccharide(s) from. Studies were centered mainly on isolates 3194 (Niger) and 2967 (Brazil).

Toxin

Perreaux et al. (1982) extracted a toxin, 3 methyl thiopropionic acid (MTPA) from *Xcm* cultures. This metabolite of methionine (converted by transamination and decarboxylation) was later detected by GLC in infected cassava leaves (Perreaux et al., 1985. We assayed for toxicity by infiltrating cassava leaves to cause visible symptoms and by adding to cassava suspension cells and determining loss of viability. No toxicity was found from a very wide range of culture conditions and media; moreover, fluids from co-culture of *Xcm* and host cells were not toxic. Toxicity was detected, however, in supernatants from Watanabe medium, which contains 500 μg/ml methionine whereas *Xcm* requires only 5 μg/ml for growth. No toxicity was detectable if the methionine concentration was $\leq$ 50 μg/ml. Levels of free methionine in uninfected or in infected cassava leaves were $<$ 0.1 μg/g fresh weight and presumably would not therefore, allow production of the toxin *in planta*. Transmission electron microscopy (TEM) did not show membrane damage at a distance from the pathogen as might have been expected with a small, diffusible toxin like MTPA (Cooper, 1981); however, total methionine (free and bound in proteins) in leaves was ca. 25 μg/g. It is possible that some of this could be released by proteolytic enzymes of *Xcm* to give rise to toxin production. All

eight *Xcm* isolates produced protease on casein-agar, but in liquid culture extracellular protease activity was low. The evidence based on defective mutants for proteases in pathogenicity of other *X. campestris* pathovars is conflicting (Dow et al., 1990). This aspect needs further investigation with *Xcm*.

Cell wall-degrading enzymes

The symptoms caused by phytopathogenic *Xanthomonas* spp. are unlike those induced by pectolytic soft rot *Erwinia* and *Pseudomonas* spp; however, pectate lyase (PL) and pectin methylesterase (PME) have been found in xanthomonads (e.g., Dow et al., 1989) including *Xcm* (Ikotun, 1984).

The only pectic enzyme detected (of PL, PME, pectin lyase and polygalacturonase) in cultures of 4 isolates grown on cassava cell walls as sole source of carbon was PL. Activities were low (ca 0.1 U[1]. PL production is strictly controlled by induction (by galacturonides) and by catabolite repression (Cooper, 1983). Significantly, highest production occurs on cassava cells walls (with which *Xcm* will be in close contact *in planta*). Carbon sources and relative activities were glucose 0, xylan 0, polygalacturonan 2.5, cell walls 8.

The enzyme exists as a single form of isoelectric point (pI) 9. Endopolygalacturonide hydrolases and lyases are unique among polysaccharidases in causing death of plant cells (Cooper, 1983). PL killed cells in cassava stem sections and suspension-cultured cells at very low activities (.017 U) (Figure 4). It also produced lesions when infiltrated into leaves, whereas equivalent fluids based on glucose cultures were inactive in all three bioassays.

However, we could not detect PL in lesions and significant host cells wall-degradation by *Xcm* was not apparent by TEM. This contrasts with the report of massive wall breakdown by *Xcm* by Ikotun (1984). Also, isolates that produced low PI levels were as pathogenic as the high producer; and low PL activities are sufficient to damage host cells (see Figure 4) and may facilitate vascular colonization by degrading pit membranes at vessel ends. Molecular genetic analysis is required to reveal the role of PL in CBB as performed with some other *X. campestris* pathovars (e.g., Dow et al., 1989) and other pathogens (Durrands and Cooper, 1988).

Extracellular polysaccharide (EPS)

EPS is a prerequisite for pathogenicity of most bacterial pathogens and probably serves numerous functions such as water absorption, ion chelation, suppression or negation of host defences (Coplin and Cook, 1990). Massive

1. 1U = μmol galacturonide released/ml/min).

production of EPS (or xanthan: Sutherland, 1993) was evident ultrastructurally during host invasion by *Xcm*. Infected vessels were completely occluded by EPS, which was clearly visible even without specific staining. The equivalent polymer of *X. campestris* pv. *vesicatoria* has recently been visualized immunocytochemically in infected plants by Brown et al. (1993). In view of the very high susceptibility of xylem flow to even trace amounts of macromolecular polysaccharides (e.g., Van Alfen et al., 1983), EPS is likely the main cause of water stress, which characterizes CBB. Also the movement of bacteria *en masse* may be facilitated by the absorption of water and expansion by the EPS matrix (Schouten, 1988). Electron micrographs frequently revealed the apparent exertion of physical pressure by *Xcm* surrounded by its EPS on (and through) pit membranes between vessels.

Disease resistance or tolerance

Field-resistant lines from CIAT (MNGA1 and M Ven 77) were compared with susceptible lines (M Col 113 and M Col 22) following inoculation with 10^8 cells/ml of *Xcm* by the three methods described. With leaf infiltration only, there was a significant delay by 2-4 days in appearance of symptoms in resistant lines compared to susceptible lines. However, eventually (> 12 days) symptoms were equally severed and, throughout, bacterial populations were not significantly different. Resistance (or tolerance) was apparent only at very low inoculum levels of $\leq 10^2$ cells/ml when symptoms remained as localized chlorosis; and there was no wilting, compared with the delayed but eventually full range of symptoms with the two susceptible lines (Table 1). Also, from leaf discs incubated with 10^8 cells/ml of *Xcm* electrolyte loss began later and was significantly ($p < 0.05$) slower over 4 days from both resistant cultivars compared with susceptible cultivars (Figure 2).

Perhaps in the field this type of resistance could be effective against low numbers of *Xcm* cells spread by rain splash, but it is likely to be overcome by occasionally high inoculum on tools during vegetative propagation.

These data emphasize several points: (i) the importance of choosing a suitable method of inoculation; (ii) choosing a suitable inoculum level to reveal potentially useful resistance; (iii) the possible influence of environment on resistance. According to IITA and CIAT, resistance to CBB is multigenic (Lozano, 1986); and multigenic resistance to some other *X. campestris* pathovars on bean and cotton is influenced by environment and may be ineffective in some regions (Mew and Natural, 1993). These cultivars which were selected for resistance in Colombia using high inoculum were vulnerable under different environmental conditions used here as optimal for disease development. This suggests that new forms of resistance must continue to be sought and that new cultivars will require testing in the areas in which they are to be released.

Future work

In contrast to many other crop species of which components of potential disease resistance are known (e.g., Dixon et al., 1987), cassava has been virtually ignored. Criteria for resistance selection might be provided through this knowledge. The only example from Euphorbiaceae studied in the context of resistance is the phytoalexin from *Ricinus communis* (Sitton and West, 1975). Likewise, the nature and control of pathogenicity by *Xcm* has received little critical attention to date. Identification of pathogenicity factors is justified because they may be used for *in vitro* selection of disease resistance (see below).

Now that we are able to regenerate cassava plants routinely from embryogenic suspension culture (Taylor and Henshaw, *this meeting*), there exists a rare opportunity to use *Xcm* or its pathogenicity determinants as selection agents so that any host cells surviving the challenge may be disease resistant or tolerant. Using a similar approach there has been success with embryogenic suspension cultures giving disease resistant alfalfa, rape and celery (van den Bulk, 1991). Resistance selection to *X. campestris* pvs. *pruni* and *oryzae* involved exposing cells to bacteria in one case and to crude culture fluids in another (Sun et al., 1986; Hammerschlag, 1988). We have already established protocols for co-culture between cassava cells and *Xcm* and will investigate the possibility of regenerating novel disease-resistant lines. In combination with the overdue study of biochemistry and physiology of cassava diseases resistance, it is hoped that disease resistance selection will be given some new directions. Identification of disease resistance-related gene products or even absence of certain gene products could aid future gene transfer or manipulation in cassava, in line with more developed crop species (Nester and Verma, 1993).

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Table 1. The effect of inoculum concentration of *Xcm* (isolate 2967) on disease induced in leaves of cassava cv. M Col 113 (S), MNGA1 and M Ven 77 (R).

Cultivar	Day	Symptoms induced by different concentrations						
		10^7	10^6	10^5	10^4	10^3	10^2	10
M Col 113	8	3	3	3	2	2	0	0
MNGA 1		2	2	2	1	1	0	0
M Ven 77		2	2	2	1	1	0	0
M Col 113	15	5	5	5	5	5	2	2
MNGA 1		4	4	4	4	4	1	1
M Ven 77		4	4	4	4	4	1	1
M Col 113	18	5	5	5	5	5	5	5
MNGA 1		5	5	5	5	5	1	1
M Ven 77		5	5	5	5	5	1	1

Disease index: 0 = no symptoms.
1 = >0 <20% of the infected lobe affected.
5 = 80%-100% of the leaf affected.

There were six replicate leaves for each concentration for each cultivar.

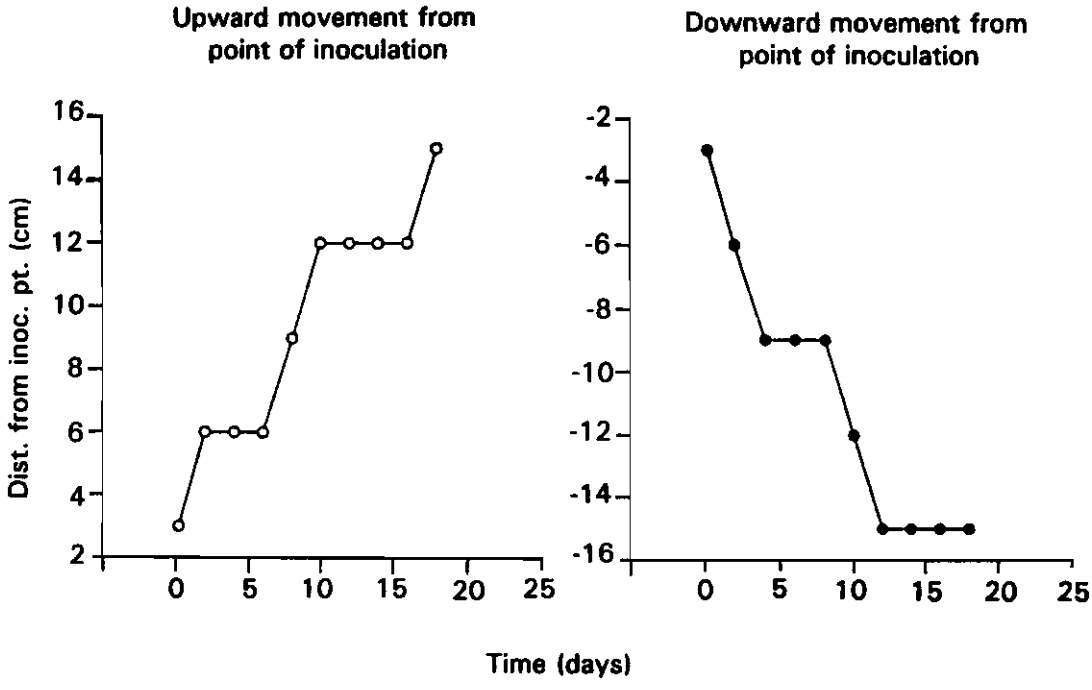


Figure 1. Movement of *Xcm* isolate 2967 in the stems of susceptible cassava cv. M Col 22 inoculated by stem injection.

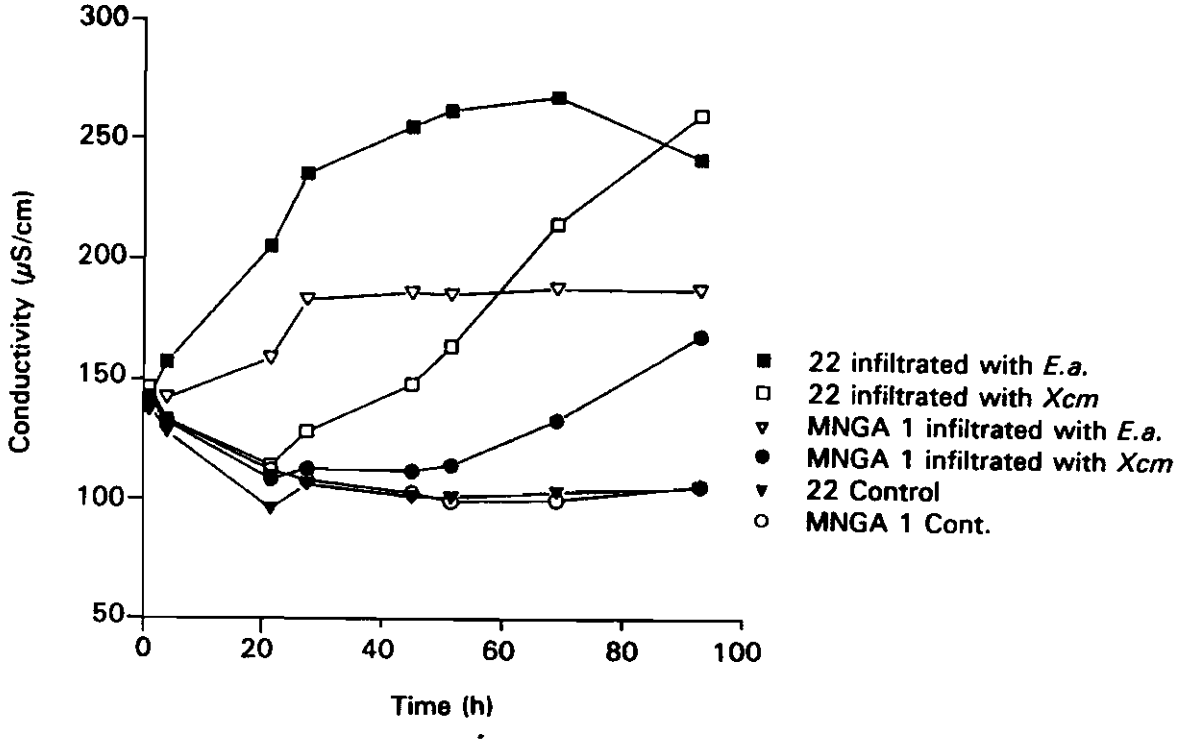


Figure 2. Effects of *X. campestris* pv. *manihotis* (isolate 2967) and *E. amylovora* on electrolyte leakage from leaf discs of susceptible (M Col 22) and resistant (MNGA 1) cassava cultivar.

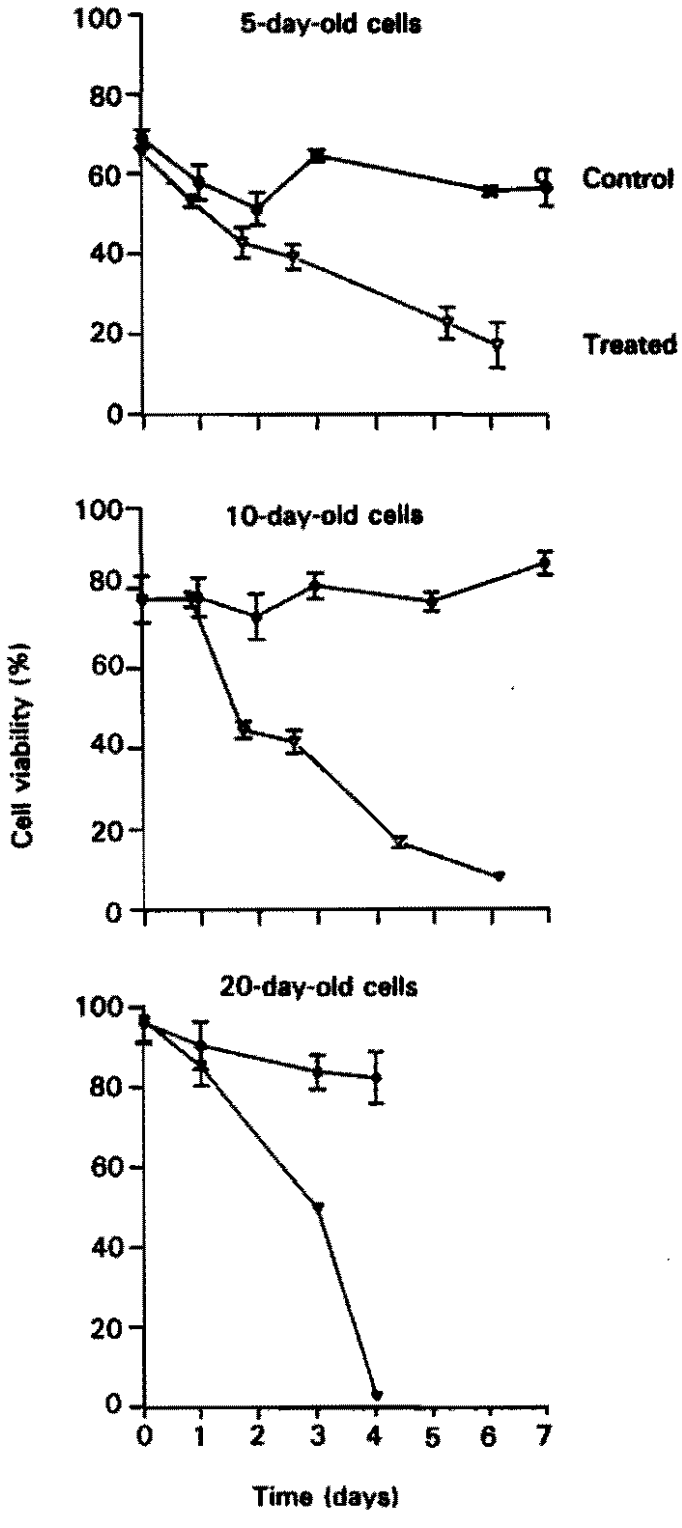
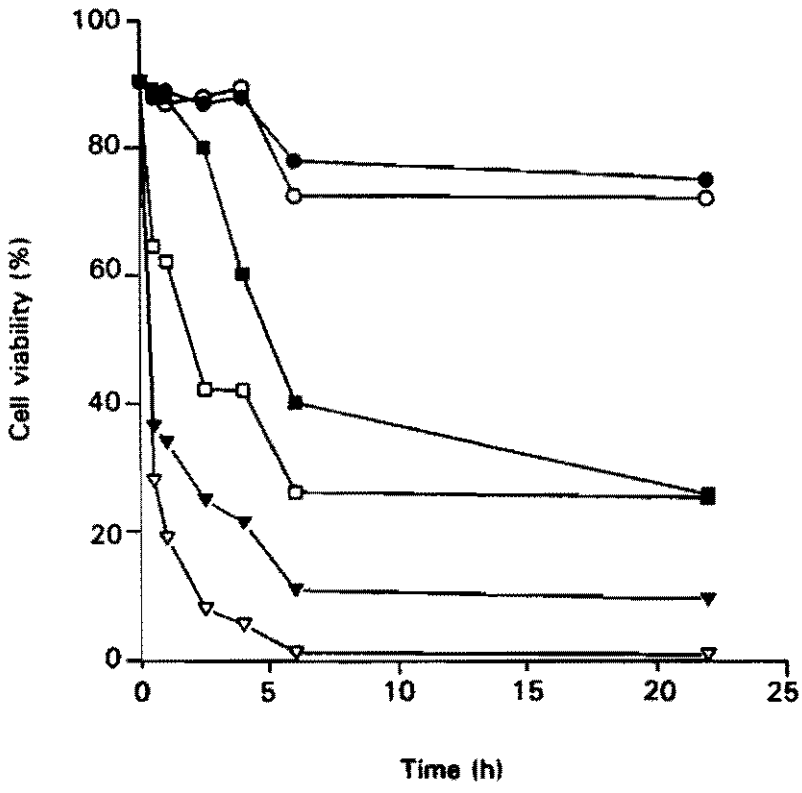


Figure 3. Co-culture of suspension-cultured cells of cv. M Col 113 and *X. campestris* pv. *manihotis*. Cassava cells were inoculated with 5×10^7 cells/ml of *Xcm* when 5, 10 or 20 days into the cell cycle, and viability was determined with fluorescein diacetate.



PGL activity of undiluted culture fluid 15.80 μmol/ml/min

Suspension-cultured cassava cells were suspended in:

- o Control (culture fluids from bacteria grown in glucose)
- ■ 1/1000 dilution of the supernatant from *Xcm* cultures with host cell walls
- ¤ 1/100 dilution of the supernatant
- ▼ 1/10 dilution of the supernatant
- ▽ Undiluted supernatant
- ● Heat-treated supernatant

Figure 4. Toxicity of pectate lyase from *X. campestris* pv. *manihotis* to cassava cells.

POTENTIAL BIOCHEMICAL MECHANISMS USED BY CONGOLESE CASSAVA TO RESIST MEALYBUG

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A pest control program in the Congo, studied the resistance of cassava (*Manihot esculenta*) to the mealybug (*Phenacoccus manihoti*). In particular, the feeding behavior of *P. manihoti* and cassava's defensive biochemical mechanisms against the pest were studied. The mealybug's feeding behavior was analyzed with an electrical penetration graph technique and light microscopy; the biochemical mechanisms with chromatography. As do other homopteras, *P. manihoti* feeds mainly on phloem sap. Its mouth parts (or stylets) enter extracellular pathways in the phloem before penetrating the cells themselves. Our previous studies showed that pre-phloem interactions with the mealybug's stylets are most important for host-plant acceptance, and that early plant rejection may result from delayed phloem feeding. Field experiments showed the occurrence of a putative defensive response of cassava against the mealybug: phenolic acid levels decreased significantly during the dry season, precisely when the insect population was at its maximum. However, we also observed a significant increase in glycosyl flavonoid levels that same season, although the infestation factor induces a significant decrease in the more susceptible cassava genotype. Overall, our results suggest that cassava resistance is partial and probably polygenic, implying the use of several resistance mechanisms.

INTRODUCTION

The cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Homoptera: Pseudococcidae) is an oligophagous insect that feeds mainly on cassava, *Manihot esculenta* Crantz (Euphorbiaceae) (Matile-Ferrero, 1976). During the early 1970s, *P. manihoti* was accidentally introduced into Africa, causing losses of cassava crops and reducing the quality of planting material (Herren, 1981). In 1982, biological control was initiated by successfully introducing the exotic South American parasitoid *Epidinocarsis lopezi* De Santis (Hymenoptera: Encyrtidae) (Herren, 1987). A strategy for enhancing the parasitoid's efficacy would be to introduce resistant varieties of cassava. For example, resistant cassava increases the pest's development time, thus extending the period of access for *E. lopezi* (van Emden and Wearing, 1965; Starks et al., 1972; Dreyer and Campbell, 1987; Auclair, 1988).

Host-plant resistance (HPR) is a tool for pest control, and is especially useful in developing countries, where chemical control is expensive and difficult to achieve in food crops. HPR genetics is central to breeding programs, but an understanding of the underlying biological mechanisms of resistance or tolerance is also essential

when dealing with questions such as durability, resistance pyramiding, pest variability, or the need for biological or chemical markers of resistance.

Resistant cassava varieties have been previously evaluated in the Congo (Tertuliano et al., n.d.), but no relationship could be found between resistance and leaf content of primary compounds (amino acids and sugars). Other biochemical factors may therefore be involved in resisting the pest (Tertuliano and Le Rü, 1992). Secondary compounds, in addition to nutritional functions, often play an important part in plant resistance to pests (Fraenkel, 1969; Kogan, 1977; Pickett et al., 1992). Numerous data on aphids have illustrated the effects of alkaloids (Dreyer et al., 1985; Smith, 1966; Wink and Witte, 1991), phenolic acids, and flavonoids (Dreyer and Jones, 1981; Leszczynski et al., 1985; McFoy and Dabrowski, 1984; Todd et al., 1971) and cyanide compounds (Dreyer and Jones, 1981; Schoonhoven and Derksen-Koppers, 1976). For cassava, in those varieties tested, chemical analysis of some secondary plant substances did not detect alkaloids (Calatayud et al., n.d.b), but did find phenolic compounds and cyanogenic glucosides.

Populations of cassava pests in the Congo spread out in abundance every year during the dry season. Their numbers multiply from fewer than 10 individuals per stem (a common number in the rainy season) to about 100 within 7-10 weeks. These dramatic increases occur during important modifications in cassava physiology (cessation of the plant's development and rising of the sap) imposed on the plant by drought in the dry season (Le Rü et al., 1991; Calatayud et al., n.d.c). The abundance of mealybugs could have its origin in modifications of the trophic quality of cassava, expressed as a temporary alteration in resistance against the insect in the dry season.

This study was carried out in two parts: the first in the Congo, on the feeding behavior of *P. manihoti* on cassava. The pathways followed by the insect's stylets through leaf tissue was examined, using electrical penetration graphs (EPGs) and light microscopy. This first study of a coccid by EPG was based on existing background work on aphids (Tjallingii, 1988).

The second part of the study clarified the possible role of the phenolic compounds present in cassava leaves in the biochemical mechanisms that the plant uses to resist mealybugs.

MATERIALS AND METHODS

Plants

For light microscopy and standard EPGs on cassava, we used the M'pembe cultivar as a reference; it is the major cultivar grown in the Pool area of the Congo and is commonly infested with *P. manihoti* in the field. We did not include this cultivar in

other studies presented here because it was used for mass rearing the insects used in the experiment, and thus bore a special host status.

Two other cassava genotypes, with different resistance to mealybugs were used; they were cv. MM79 and the Faux Caoutchouc hybrid *M. esculenta* x *M. glaziovii* Mull. Arg. The hybrid was significantly more resistant to the insect than cv. MM79 (Tertuliano et al., 1993).

Insects

Phenacoccus manihoti reproduces by lytokous parthenogenesis, and the clone we used was initially collected in 1985 from cassava in a local garden in Brazzaville. Since then, a culture of *P. manihoti* has been maintained on cv. M'pembe in the laboratory at 22-32 °C and photoperiod 12 h light to 12 h darkness.

Mealybug feeding behavior

Light microscopy

'M'pembe' plants were infested with adult mealybugs. Infested leaves were then cut and fixed in Halmi fixative (Gabe, 1968), rehydrated, and embedded in paraffin. Cross sections were cut at 6 μm on an LKB microtome, dehydrated, stained with safranine-methyl blue (Locquin and Langeron, 1978), and viewed with a Leitz Dialux 20 light microscope.

Electrical monitoring

Stylet penetration was monitored by a single-channel DC-EPG system (Tjallingii, 1988), with an input resistance of 10^9 ohm (Model 'EPG 86' summer course, Van de Pers Inc., Netherlands). Mealybugs were placed in a plastic petri dish for 1-2 min. A gold wire (2-3 cm, Ø 25 μm) was then fixed on the insect's dorsum with a water-based silver paint. The insect was connected to the amplifier before being placed on the upper side of a leaf and experiments were carried out in a Faraday cage, at temperatures between 21 and 23 °C.

For reference EPGs (see EPG set up in Figure 1), we used cv. M'pembe and a data acquisition system that consisted of a PC-based Kontron HPLC devise (MT450 software and digitizing card), and converted and stored the (-5, +5V) EPG signal at a 100-Hz sampling rate. The stored signal was then transferred to MacIntosh-based EPG analysis software (MacStylet).

Reaction of cassava to mealybug attack

Plant extracts ("extracellular fluids")

A mixture of phloem sap (sugars were 80% sucrose, data not shown) and intercellular fluid from leaves was sampled by a centrifuging method modified from Rohringer et al. (1983). The top second and third leaves were cut without their petioles, washed in distilled water, and wiped dry. They were then wrapped in nylon muslin (0.05 mm) and centrifuged at 4,000 g for 20 min. in a Sorvall SS 34 rotor. All extracts were freeze-dried, weighed (± 0.1 mg), and stored at -20 °C until required.

Chemical analyses

The units used throughout this work (mg/g of dry weight), although not representing actual concentrations in the plant, are a good comparative index as they reflect the relative "investment" in secondary chemistry as compared with the total solutes present in a sample. In some instances they may provide a better estimate than true concentration data, which may vary because of dilution effects caused by artefacts or natural physiological responses.

Extracts were dissolved in 250 µl of 50% methanol and centrifuged at 15,000 g for 5 min. to remove solids; 20 ml were injected on a C18 RP-HPLC column with UV detection at 320 nm (Spherisorb S50DS2, 4.6 x 250 mm, from Prolabo, FRA). Isocratic elution with a mobile phase of aqueous acetonitrile (23.4% acetonitrile, 2% acetic acid) was performed at a flow rate of 0.8 ml/min. Attribution of each peak to either flavonoid or phenolic acid families was realized by comparison with the retention times of standard phenolic compounds, and by comparing isocratic and gradient HPLC profiles where peak collection was performed, if needed, for UV spectrum characterization. Quantification was subsequently expressed either as þ-coumaric acid equivalent or as rutin (these two compounds have been identified in our cassava extracts and only comparative values were of interest).

Plot used

The study was conducted on 700 m^2 of land that had been fallow for about 10 years, from October 1990 to December 1991, in Brazzaville. The main vegetation was *Chromolaena odorata* L. (Asteraceae). The soil, typical in the region (Denis and De Champ, 1970), was ferralitic, strongly desaturated, with a sablo-argilous texture at the surface and an argilous texture at depth. The pH was between 4.9 and 5.6. Chemical data on the soil were provided by ORSTOM's soil chemistry laboratory in Brazzaville. The lack of organic matter and mineral components (data not shown) was balanced by N-P-K (15-15-15) fertilization before the experiment. Monthly mean air temperature during the study was 25.7 °C

(ranging from 23.0 to 27.2 °C), and rainfall was 1,653 mm, with a long dry season from June to September, and a short dry season from January to February (Figure 2). Climatic data were provided by the ORSTOM meteorological station at Brazzaville.

Stakes, 20 cm long, of the two cassava genotypes under study were planted vertically in random fashion during November 1990. The field was cultivated, following a traditional method used in the Congo: weeding at the end of the dry season and plowing with a hoe at the beginning of the rainy season. The upkeep of the plot involved two weedings and two hoeings during the third and sixth months of culture. Mite populations were controlled every 3 months.

Infestation by mealybugs

Taking into account the characteristics of the pest's population dynamics, the plants were infested in four periods: January-February (short dry season), April-May (end of short rainy season), July-August (long dry season), and October-November (beginning of long rainy season). Twenty plants were artificially infested on the second and third leaves, under the apex, with 200 neonate larvae from our culture of *P. manihoti* in the laboratory and 20 other plants remained uninfested (control). Infestation took 1 month, and all infested plants were examined twice a week. Mealybugs were added or removed when necessary to maintain a constant population. For each infestation period we used only plants that had never been infested. After the month of infestation, analyses of phenolic compounds of dry extracts for each genotype were carried out on infested and uninfested leaves of the same foliar level.

Statistical analysis

For the analysis of variance, factors "genotype," "infestation," and "season" were considered as fixed. Phenolic acid contents were found to be homogeneously distributed among factors, as tested with the Kolmogorov-Smirnov test (homoscedasticity hypothesis for ANOVA). For each significant factor ($P<0.05$), the means were compared, using Fisher's PLSD multiple range test (Table 1). These statistics were completed with the Statview software (Abacus Concept, USA).

RESULTS AND DISCUSSION

Mealybug feeding behavior

The pathways followed by mealybug stylets through leaf tissue showed strong similarity with those realized by phloemophagous aphids (Calatayud et al., n.d.b). The EPG showed that at the beginning of stylet penetration, electrical contact was

established between the mouthparts and plant tissues (Figure 3a). We followed the stylets' pathway (Figure 3b) by using microscopy, and saw inter- and intracellular positions of the stylets. Through the EPG, we visualized these positions with the C pattern (extracellular pathway activities, including salivary secretion) and pd pattern (short intracellular punctures within pathways, characterized by sudden drops in the voltage level, presumably caused by the transmembrane potential of a plant cell). After about 2 h of following stylet pathways (Figure 3c), we observed a drop in the voltage level (as in the pd), which was maintained throughout the whole pattern, indicating a sustained intracellular position of stylet tips, but the membrane potential remained intact. According to microscopic observations, this ultimate pattern (E) represents a puncture in the phloem sieve element[1], with sap ingestion, somewhat comparable with those of aphid EPGs.

In previous work, we confirmed the typical phloem-feeding behavior of *P. manihoti*, which has an aphid-like predominance of extracellular pathways[2]. We also observed that pre-phloem interactions are most important for host-plant acceptance, and early plant rejection may be because of a delay in finding phloem. In contrast to phenolic acids, flavonoids were not involved in the initial interaction between the mealybug and its host. Phenolic acids did not facilitate the insect's search for phloem (Calatayud et al., n.d.b). These results led us to use an intercellular and phloem-fluid extraction method in the field experiment.

Reaction of cassava to mealybug attack

In previous work, cyanogenic glucosides and three glycosyl flavonoids (including rutin) were found to be translocated in cassava phloem sap and consumed by the mealybug. A relationship exists between the foliar concentrations of these compounds and the expression of resistance to *P. manihoti*: mealybug infestation is followed by an obvious increase in rutin levels, but, apparently, no modifications in cyanide content occur. The best correlation between resistance and secondary compounds was with rutin, which was therefore characterized as unfavorable for *P. manihoti* (Calatayud et al., n.d.a).

Role of phenolic acids and glycosyl flavonoids in resistance to mealybug attack

Independently of infestation and genotype factors, the lowest values of phenolic acid content were registered during the long dry season (July-August) (<0.8 mg/g

1. In xylem sieve elements, we cannot observe drops in the voltage level (Calatayud et al., n.d.b) because xylem cells do not have membranes.

2. Observations also valid with cv. MM79 and the Faux Caoutchouc hybrid used in our field experiment.

of dry weight for cv. MM79 and hybrid Faux Caoutchouc) (Figure 4 and Table 1), precisely when mealybugs were at their most abundant. The highest values were seen during the short rainy season (April- May) (>1.5 mg of dry weight for cv. MM79 and hybrid Faux Caoutchouc) when insect populations were low.

Seasonal changes in phenolic acid content did not depend on either the infestation or genotype factor (P = 0.1138 and 0.0606, Table 1), an interesting result in view of the role phenolic acids play in cell-wall structures—as precursors of lignins, cutins/suberins, or phenolic-coupled pectins, any of which could interact with the salivary oxidizing enzymes of the insect (Fry, 1983; Goodman, 1986).

In contrast, seasonal changes in glycosyl flavonoids (Figure 5 and Table 1) depended on the infestation factor (P = 0.0029). For the susceptible cv. MM79, infestation induced significant decreases in the level of these compounds, from 14.5 to 7.7 mg/g of dry weight, during the long dry season (July-August), when mealybugs were at their most abundant. In contrast, for the resistant hybrid Faux Caoutchouc, infestation induced significant increases in the level of glycosyl flavonoids, regardless of season, except during June-February. This induced response was higher at the start of the long rainy season (October-November), precisely when pest numbers decreased significantly, from 17.3 to 9.5 mg/g of dry weight. These responses to infestation, depending on genotype, were essentially caused by rutin levels, the major glycosyl flavonoid compound found in these extracts (Calatayud et al., n.d.a).

For both genotypes, when uninfested, seasonal levels of phenolic acids change in the opposite direction to the seasonal levels of glycosyl flavonoids, even though phenolic acids (especially þ-coumaric acid) are precursors of flavonoids, which frequently enable plants to resist insects. Rutin may have that function in *P. manihoti-M. esculenta* interactions. Our results, however, do not illustrate this biochemical link because of the extraction method that we used. Because it involves mainly phloem sap, the level of phenolic acids, which are present mainly in intracellular fluids, is lower than the level of glycosyl flavonoids, that is, of rutin translocated by phloem sap.

To conclude, cassava's resistance to mealybugs is partial and probably polygenic, implying the use of several resistance mechanisms. For example,

- The resistance observed in those cassava cultivars studied never induced 100% mortality in the pest population (Tertuliano et al., n.d.).

- Independent of genotype and infestation factors, the phenolic acid levels, which prevent the insect's mouth parts from reaching the phloem (Calatayud et al., n.d.a), decreased significantly in the long dry season when the pest was at its most abundant.

- Glycosyl flavonoid levels, represented mainly by rutin—an unfavorable substance for *P. manihoti* (Calatayud et al., n.d.a)—decrease significantly during the dry seasons when the pest is most abundant, especially in the susceptible genotype. Levels in uninfested plants remain high.

- For each genotype studied, the cyanide content, which can be either phagostimulating or toxic for *P. manihoti* (Calatayud et al., n.d.a), increased in the dry seasons (Calatayud et al., n.d.c).

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Table 1. Seasonal mean contents (mg/g of dry weight) of phenolic acids (PA) and glycosyl flavonoids (GF) in "extracellular fluids" for two cassava genotypes, infested and uninfested by mealybugs (*Phenacoccus manihoti*). Results of a 3-way ANOVA (genotype, infestation, and period factors).*

Periods	cv. MM79		Hybrid Faux Caoutchouc		cv. MM79		Hybrid Faux Caoutchouc	
	Uninfested	Infested	Uninfested	Infested	Uninfested	Infested	Uninfested	Infested
Jan.-Feb.	0.9 ± 0.2 a	1.1 ± 0.2 b	0.7 ± 0.2 ab	1.0 ± 0.2 b	7.1 ± 0.7 a, u	8.6 ± 1.3 a, u	8.5 ± 0.8 a, u	9.7 ± 1.5 a, u
April-May	1.6 ± 0.2 b	2.1 ± 0.3 c	1.9 ± 0.2 c	2.1 ± 0.3 c	9.3 ± 2.1 ab, u	12.1 ± 1.8 b, u	9.3 ± 1.9 a, u	16.8 ± 5.9 b, v
July-Aug.	0.7 ± 0.1 a	0.3 ± 0.04 a	0.3 ± 0.04 a	0.4 ± 0.1 a	14.5 ± 1.6 c, v	7.7 ± 1.2 a, u	12.6 ± 1.3 b, u	17.6 ± 3.2 b, v
Oct.-Nov.	1.3 ± 0.1 b	1.6 ± 0.1 bc	1.6 ± 0.1 bc	0.6 ± 0.1 ab	12.9 ± 1.2 bc, u	17.6 ± 1.9 c, u	9.5 ± 1.2 a, u	17.3 ± 2.9 b, v

ANOVA factors		
Genotype (A)	0.0606	0.1404
Period (B)	0.0001	0.0001
A x B	0.0248	0.1929
Infestation (C)	0.1138	0.0029
A x C	0.9856	0.0140
B x C	0.3439	0.0401
A x B x C	0.4134	0.1634

* Means (± SE) followed by the same letter are not significantly different at the 5% level (Fisher's PLSD test, following ANOVA). Number of cassava plants for each category = 40. Letters a, b, c = comparison for period factor (down column); letters u, v = comparison for infestation factor (along row).

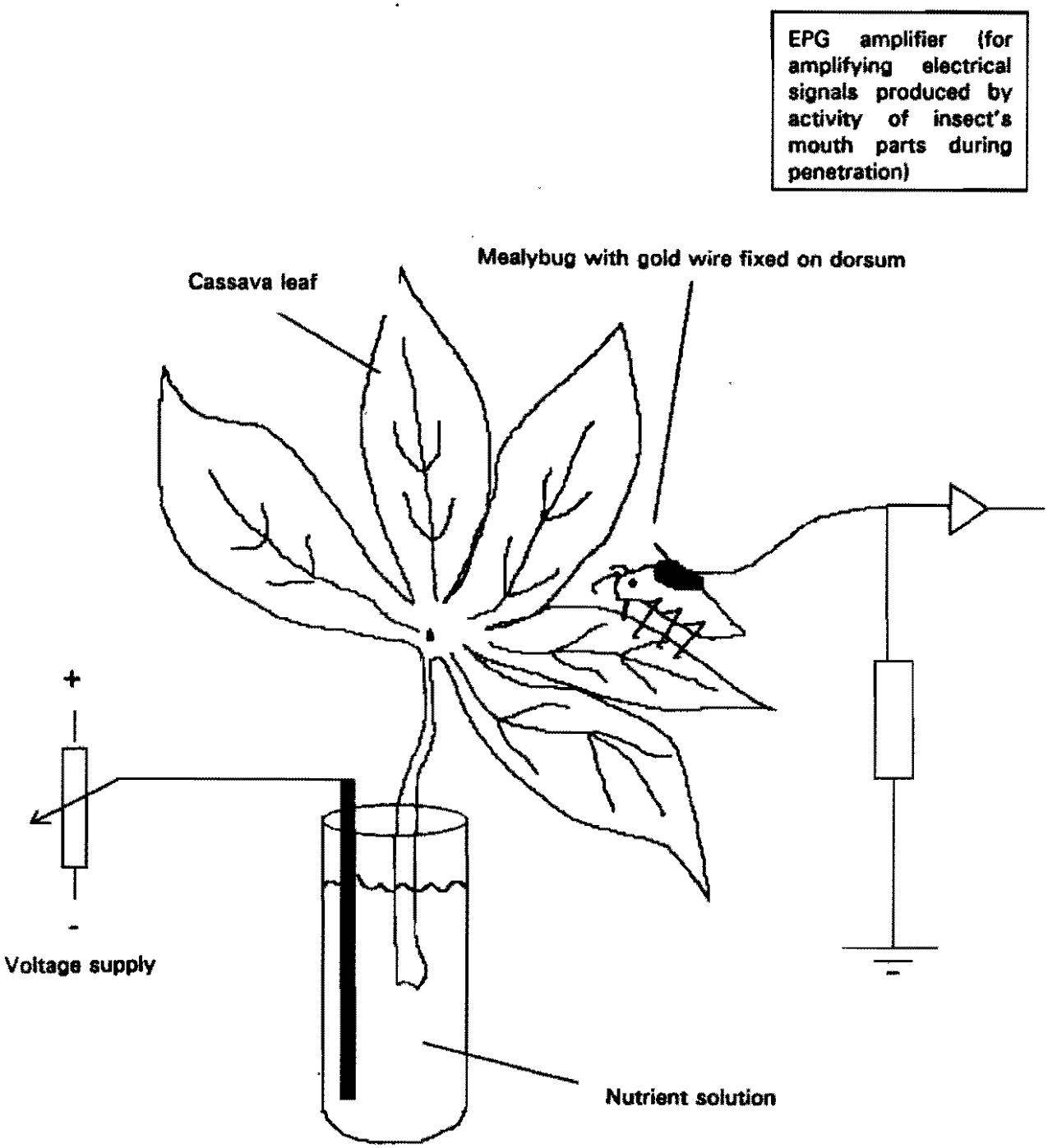

Figure 1. Set up of the EPG primary circuit. The EPG amplifier is connected to a data acquisition system (PC-based Kontron).

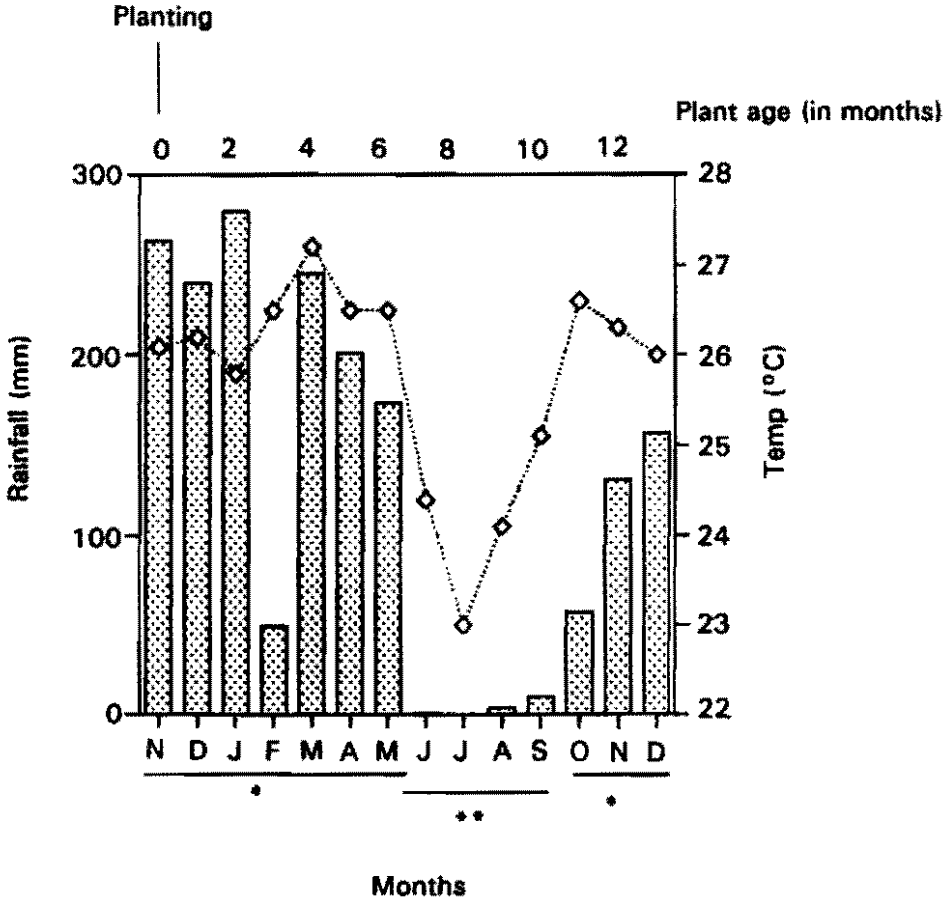

Figure 2. Meteorological conditions (monthly rainfall and mean air temperature) recorded throughout the study period. (* = rainy season; ** = dry season; —◇— = mealybug (*Phenacoccus manihoti*) population.)

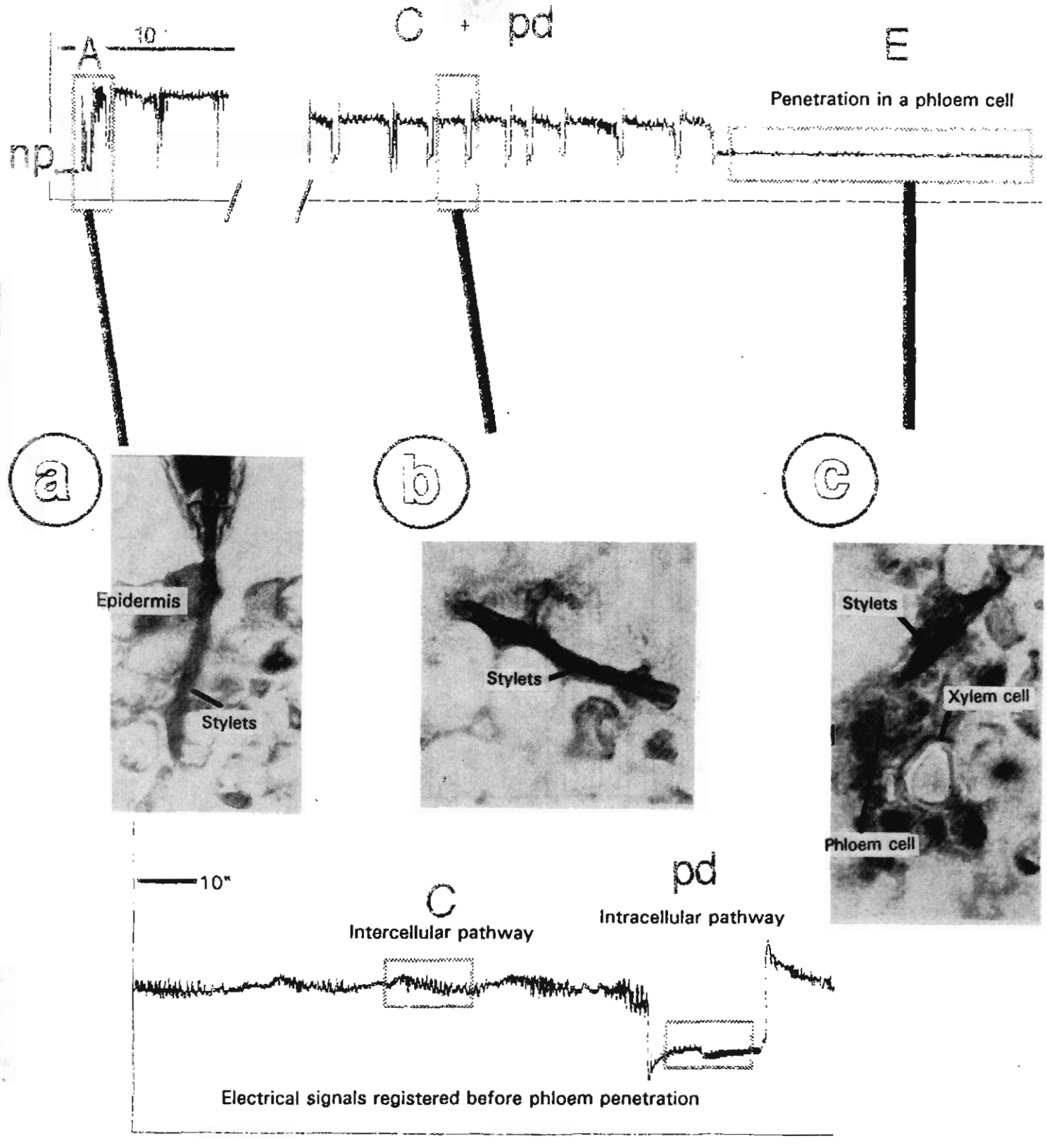

Figure 3. Relationships between position of stylets in leaf tissues and electrical signals registered by EPGs (np = nonpenetration and A = start of stylet penetration).

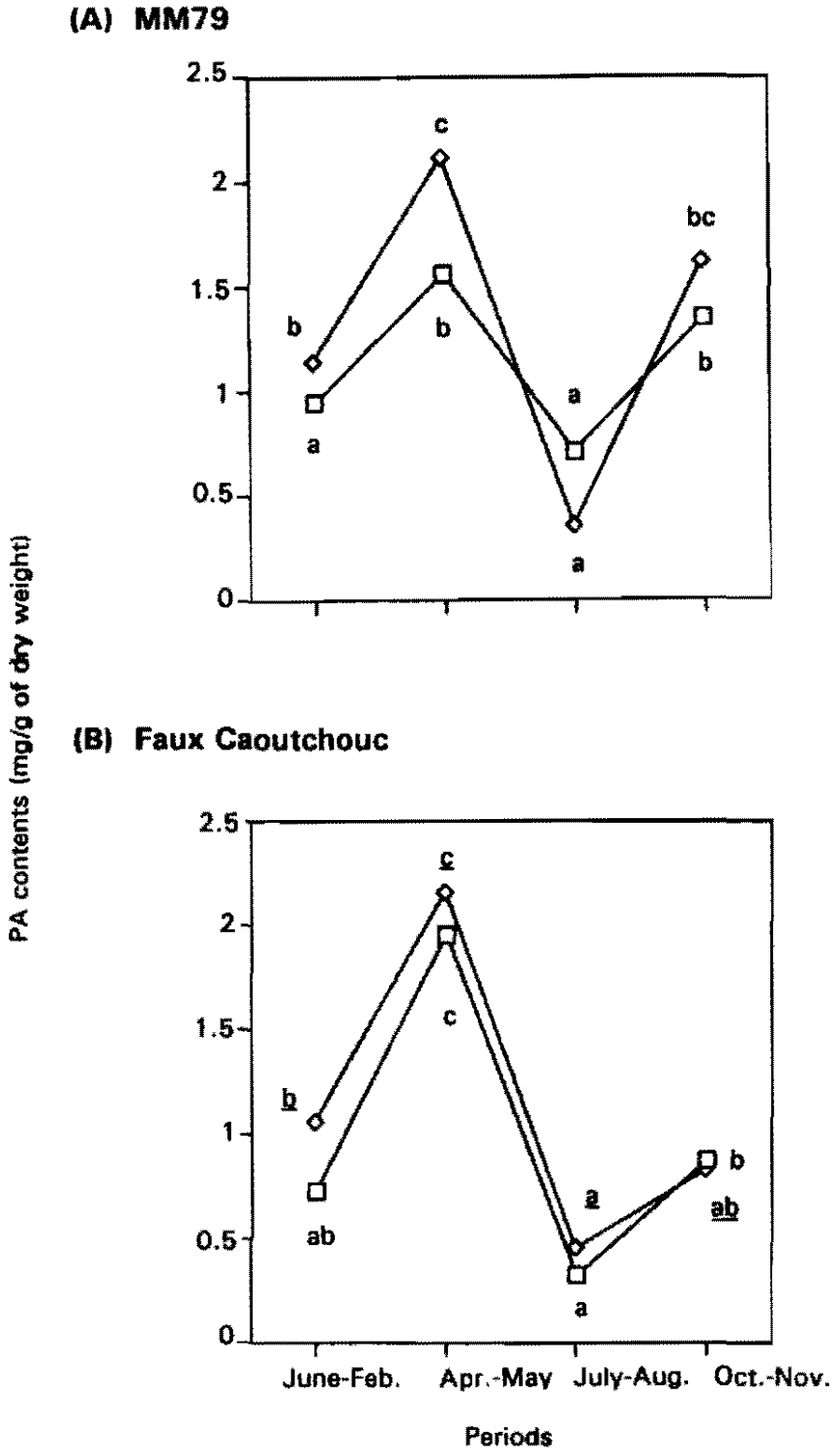

Figure 4. Seasonal mean contents of phenolic acids (PA) in "extracellular fluids" for two cassava genotypes infested (—◇—) and uninfested (—□—) by mealybug (*Phenacoccus manihoti*). Number of cassava plants = 40. Means followed by the same letter are not significantly different at 5% level (Fisher's PLSD test, following ANOVA). Letters a, b, c = comparison for period factor.

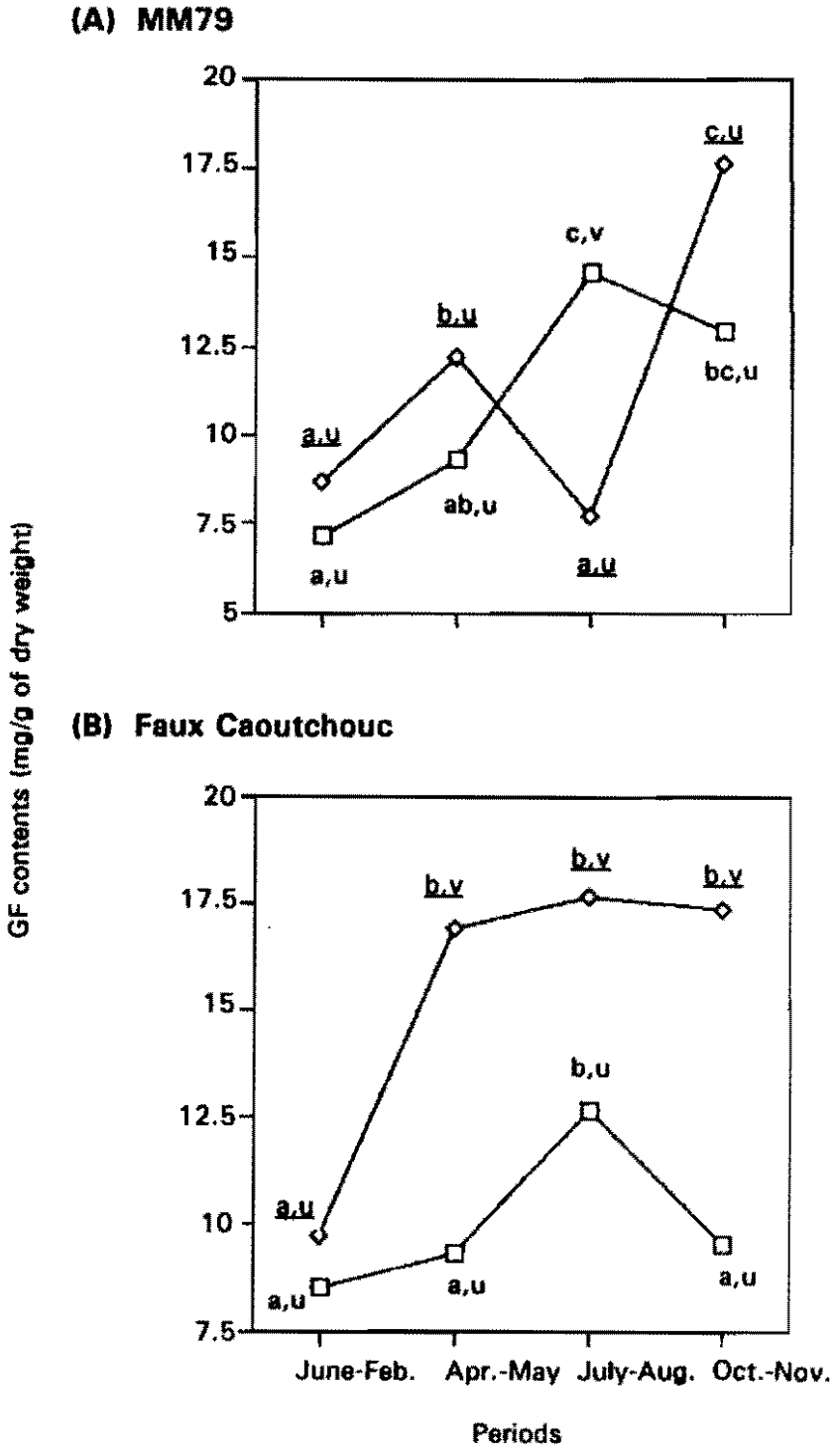

Figure 5. Seasonal mean contents (mg/g of dry weight) of glycosyl flavonoids (GF) in "extracellular fluids" for two cassava genotypes, infested (—◇—) and uninfested (—□—) by mealybugs (*Phenacoccus manihoti*). Number of cassava plants = 40. Means followed by the same letter are not significantly different at the 5% level (Fisher's PLSD test, following ANOVA). Letters a, b, c = comparison for period factor; letters u, v = comparison for infestation factor.

THE RESPONSE OF A POLYPHAGOUS PEST (*CYRTOMENUS BERGI* FROESCHNER) TO CASSAVA CULTIVARS WITH VARIABLE HCN CONTENT IN ROOT PARENCHYMA AND PEEL

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The hypothesis that HCN in cassava is a defence mechanism against arthropod pests is suggestive, but quality and quantity of empirical data is unsatisfactory and scarce. The burrowing bug (*Cyrtomenus bergi* Froeschner: Cydnidae: Hemiptera) is recorded as a major pest on cassava in the neo-tropics since 1980. Most major cassava pests are leaf feeders whereas *C. bergi* feeds on cassava roots, introducing soil-borne pathogens which cause rot lesions on the parenchyma. It is a serious pest on cassava in Colombia and Panama. Whereas most major cassava pests are specialists that have co-evolved with cassava and feed readily on leaves with high HCN content, *C. bergi* is polyphagous feeding on many food crops as well as weeds. As a generalist that has not co-evolved with cyanogenic host plants, it can serve as a model in the study of HCN levels in cassava roots on non-specialists. Root damage caused by *C. bergi* was evaluated on 125 cultivars from the cassava CIAT core collection and compared to HCN level in root parenchyma and peel. Severe damage did not occur on clones with high HCN level in the parenchyma and approximately 400 ppm (fresh root parenchyma) was the upper limit to *C. bergi* feeding. Nevertheless many low HCN clones had low levels of damage indicating other possible mechanisms responsible for resistance to *C. bergi* in low HCN clones. These mechanisms should be investigated. High HCN levels in the root peel, however, did not prevent pest attack, indicating that high HCN content in the root peel will not protect against sucking pests and may have little effect on feeding pests.

INTRODUCTION

To archieve food safety by avoiding human HCN poisoning in cassava growing areas (Mayanbu, 1993) much effort is being expended to create low cyanide or even cyanide free varieties in cassava (Cooke and Coursey, 1981; McKey and Beckerman, n.d.). But to achieve food security, the role of cyanide as a secondary compound in cassava must be considered. Cassava is highly suitable for subsistence agriculture in America, Africa and Asia, but will selection for low or even cyanide free varieties increase susceptibility for pests and theft, with the result of cassava losing its advantage of food security in areas where no other crop alternatives are available ?

A literature review shows that a number of pests on white clover, sorghum, bracken fern, peach and cassava appear to be deterred from feeding due to HCN content in the host plant (Bellotti and Riis, n.d.). Also mammals such as wild boars

are selective showing preference for low HCN cassava varieties and leaving high HCN varieties untouched (McKey and Beckerman, n.d.). On a visit to cassava gardens of amazonian Tatuyo indians, Wilson, Univ. of Colorado Anthropology Dept. (personal communication) observed that all the sweet varieties had disappeared due to theft by a wild rodent *Dacyprota* sp. There are strong indicators that HCN in cassava plays a role as a protectant against herbivores and that high HCN varieties are generally less susceptible to attack from generalist feeders.

Glycosinolates (Berenbaum, 1986) and cyanide (Price, 1984) are considered qualitative (toxic) defenses. They are plant compounds of low molecular weight that are easily transported within the plant. They are associated with specific biochemical properties and can be detoxified by adapted insects. This is in contrast to quantitative defenses, e.g. tanins, that are more difficult to counteradapt to or detoxify as they are large amorphous compounds that exerts their effect via non-specific binding. This theory fits well with cassava as most major cassava pests are specialists, e.g. cassava hornworm (*Erinnyis ello*) and cassava green mite (*Mononychellus tanajoa*), that have co-evolved with cassava and feed readily on leaves with high HCN content. According to the literature, only two pests of cassava, which are both polyphagous (generalists), are reported to be deterred from feeding due to leaf and root HCN content (Bellotti and Riis, n.d.). Bernays et al. (1977) and Schaefers (1979) state that growing cassava is not a very acceptable food for the grasshopper *Zonocerus variegatus* due to the relatively large amounts of HCN produced in the leaves. Cassava roots contain variable amounts of HCN and the burrowing bug *Cyrtomenus bergi* (Cydnidae: Hemiptera) prefers feeding on low HCN rather than high HCN roots (Arias and Bellotti, 1985; Bellotti et al., 1988; García and Bellotti, 1980) and show a higher oviposition rate, survival rate and longer adult longevity while feeding on low HCN than on high HCN roots (Bellotti and Arias, 1993; CIAT, 1993; Riis, 1990).

Plants under stress conditions are more sensitive to herbivore damage. Induced defence mechanisms as a respond to stress and damage are reported frequently in the literature (Norris, 1988). Stress situations in cassava due to drought (El-Sharkawy, 1993) and disease infection can raise the cyanide content (Fry and Myers, 1981). Cassava stressed by the green mite, *Mononychellus tanajoa*, and the mealybug, *Phenacoccus manihoti*, demobilized cyanogen from the leaves to root tissue, specifically to the parenchyma rather than the peel, without changing the total cyanide content of the plant (Ayanru and Sharma, 1984-85). McKey and Beckerman (n.d.) postulate that the advantage of cassava as an economical crop in harsh and marginal conditions (El-Sharkawy, 1993) is directly due to the antiherbivore defenses of the crop.

Cyrtomenus bergi is recorded as a major pest on cassava in the neo-tropics since 1980, especially in Panama and Colombia. It feeds on cassava and many other food crops as well as weeds (Riis, 1990). In contrast to most major cassava pests which are leaf feeders (Bellotti and Schoonhoven, 1978), *C. bergi* feeds

directly on cassava roots, introducing soil-borne pathogens which cause rot lesions on the parenchyma, and which are not detectable until roots are peeled (García and Bellotti, 1980). Both juvenile and adult stages cause damage. Middlemen will not buy roots when more than 20% of roots are found infected by random checks (Bellotti et al., 1988).

The hypothesis that HCN in cassava is a defence mechanism against arthropod pests is suggestive and the effect of a hypothetically cyanide-free cassava variety to pest pressure within this crop is a subject of speculation. As a generalist that has not co-evolved with cyanogenic host plants, *C. bergi* can serve as a model in the study of the effect of HCN levels in cassava roots on non-specialists.

METHODS

Root damage caused by *C. bergi* was evaluated on 125 cultivars from the CIAT cassava core collection and compared to the HCN level in root parenchyma and peel. Cultivars were screened for damage by placing one root in a container with five adults. The same insect populations were used for a series of cultivars, an unfortunate procedure that may have influenced results due to differences in feeding histories between treatments. Damage was evaluated on a 0-5 scale with 0 indicating no damage, 1 indicating 1%-20% of the parenchyma surface covered with rot spots, 2 indicating 21%-40%, 3 indicating 41%-60%, 4 indicating 61%-80% and 5 indicating 81%-100%. Damage levels above 1 are considered of commercial importance. The damage level was compared to recent HCN measurements carried out by the Cassava Utilization Unit at CIAT using the enzymatic rapid assay. Unfortunately, these measurements were taken from different harvests. Statistical models tested in SAS, including correlation analysis, ANOVA and chisquare, failed to describe data, even when logarithmicly transformed. Data are therefore presented graphically. Oviposition, survival and adult longevity of *C. bergi* were also compared while feeding on maize, on a low and on a high HCN cassava variety.

RESULTS AND DISCUSSION

Severe damage by *C. bergi* only occurs on clones with low HCN levels in the parenchyma and *not* on high HCN clones indicating a preference for low HCN clones, cf. Figure 1. The upper limit to *C. bergi* feeding was approximately 400 ppm (fresh root parenchyma), indicating that *C. bergi* distinguishes between sweet and bitter varieties at a higher level than that of humans. Nevertheless many low HCN clones had low levels of damage indicating other possible mechanisms responsible for resistance to *C. bergi* in low HCN clones. These mechanisms should be investigated. High HCN levels in the root peel, however, did not prevent pest

attack, cf. Figure 2, indicating that high HCN content in the root peel will *not* protect against sucking pests and may have little effect on feeding pests.

Comparing oviposition, nymphal survival and adult longevity of *C. bergi* on maize, on a low HCN cassava clone (var. MCol1468, 44 ppm HCN in fresh root parenchyma) and on a high HCN cassava clone (var. MCol1684, 275 ppm in fresh root parenchyma), maize was highly preferred over the low HCN clone which was preferred over the high HCN clone, cf. Figure 3, with significant difference at the 0.01% level. This indicates that *C. bergi* has a *higher* potential for oviposition and survival than demonstrated when feeding on a low HCN cassava variety. It can be speculated that this effect is due to the HCN content, to better nutritional value in maize or an interaction of both. It also leads to speculation on potential pest damage in a hypothetically cyanide-free cassava clone.

Generally, cassava is considered to be relatively resistant to pests. The reason for the appearance of *C. bergi* as a "new" pest in cassava in Panama and Colombia in the beginning of the 1980s might be explained by the intensified cultivation of cassava during the last decades (Cock, 1982). Strong et al. (1984) also explain that changes in abundance of local host plants and their distribution can influence the local diversity of herbivore insect communities. With the creation of more simplified ecosystems, many host plant resistance factors may become neutralized by rapid evolution of pests. The case of *C. bergi* is likely to fit that theory.

In the attempt of preventing human health hazards caused by HCN content in cassava roots, partitioning HCN between leaves and roots should be considered, reducing HCN level in leaves and root peel apparently give only partial or even poor protection against root feeders as in the case of *C. bergi*. Vigilance of increased pest attack and new pest problems, will be required especially for root pests, demanding investigation of pest control by other means. The present investigation provides some evidence that other protective mechanisms to *C. bergi* feeding may exist in cassava.

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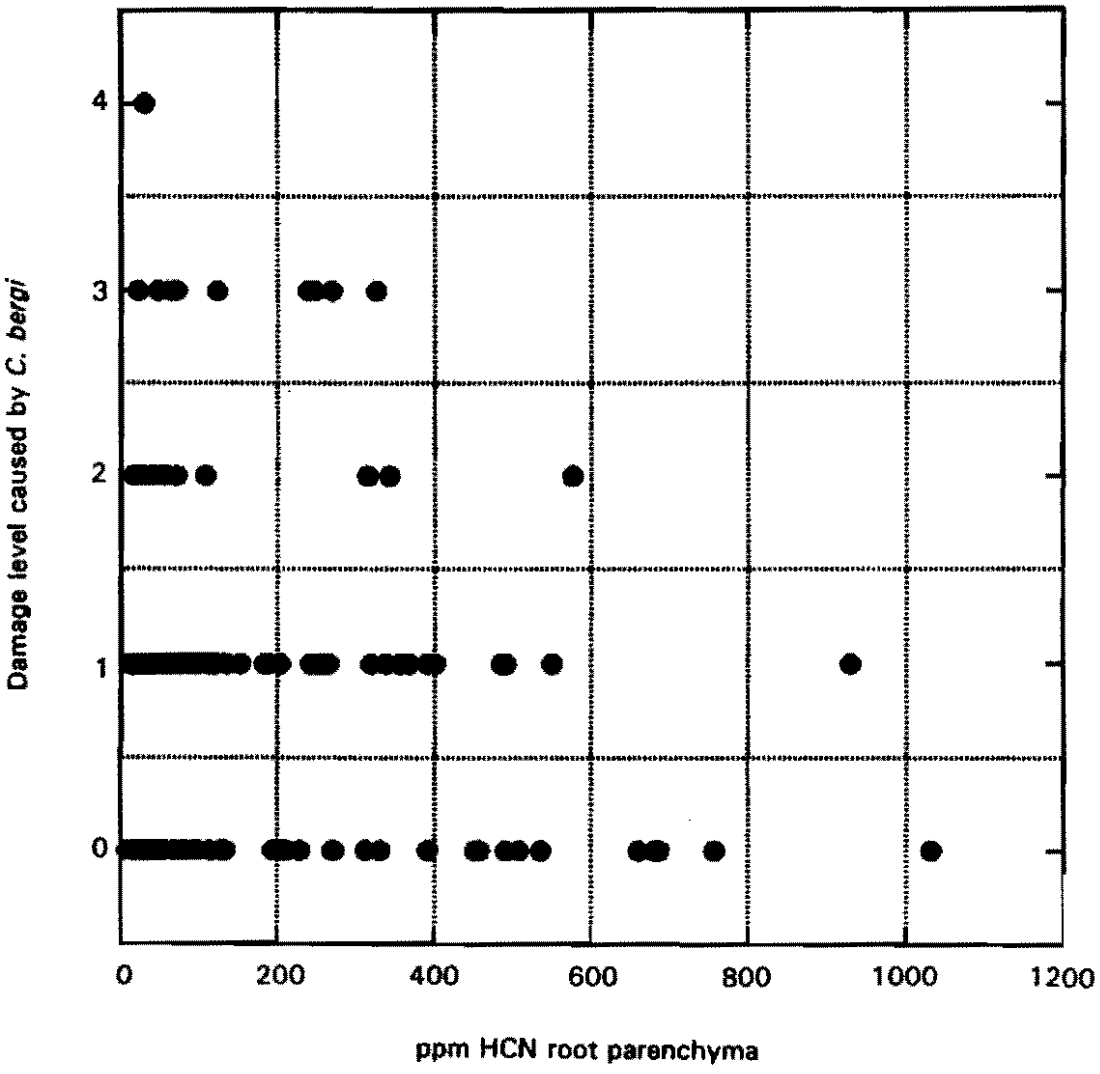

Figure 1. Relation between HCN content in fresh root parenchyma and damage levels (0 = no damage) caused by *Cyrtomenus bergi* on 125 cultivars.

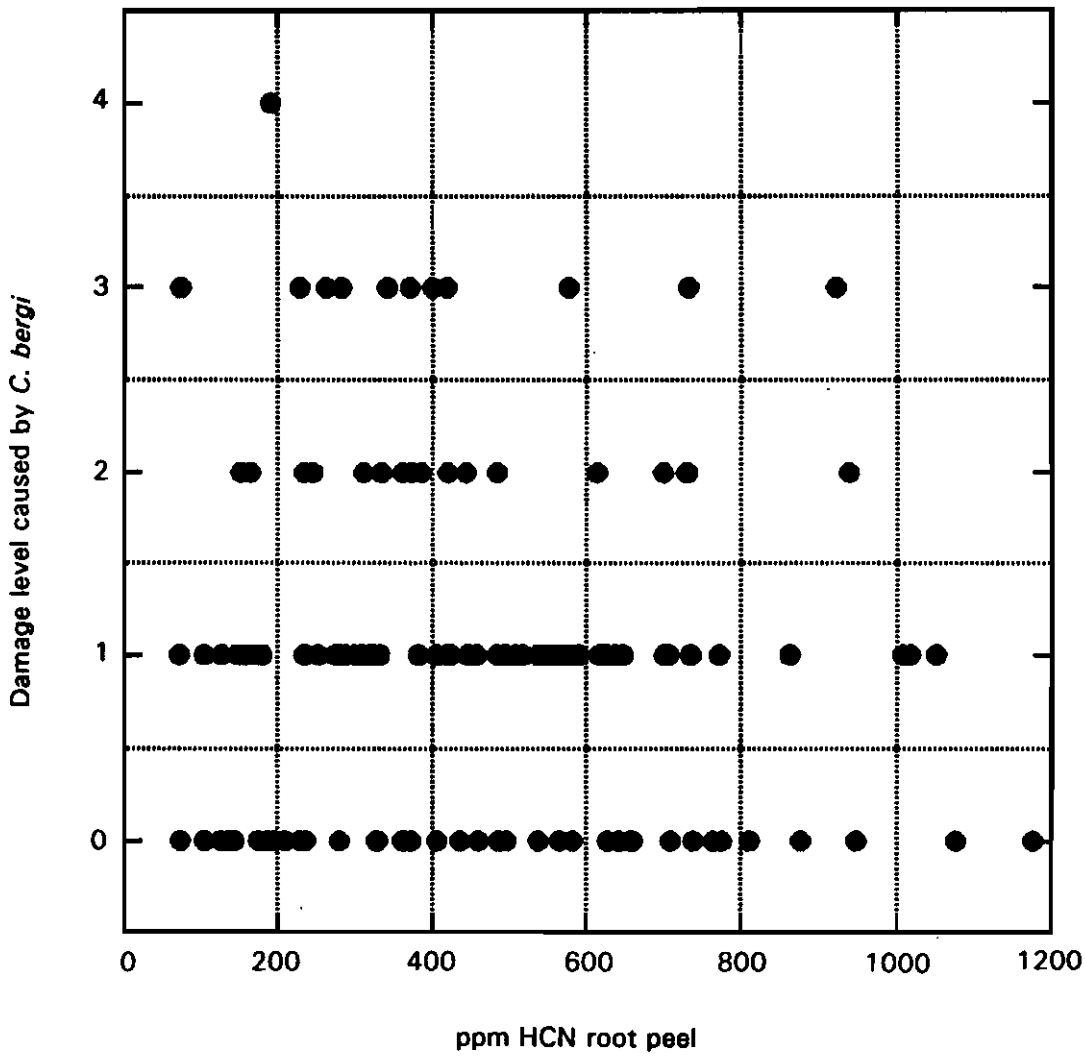

Figure 2. Relation between HCN content in fresh root peel and damage levels (O = no damage) caused by *Cyrtomenus bergi* on 125 cassava cultivars.

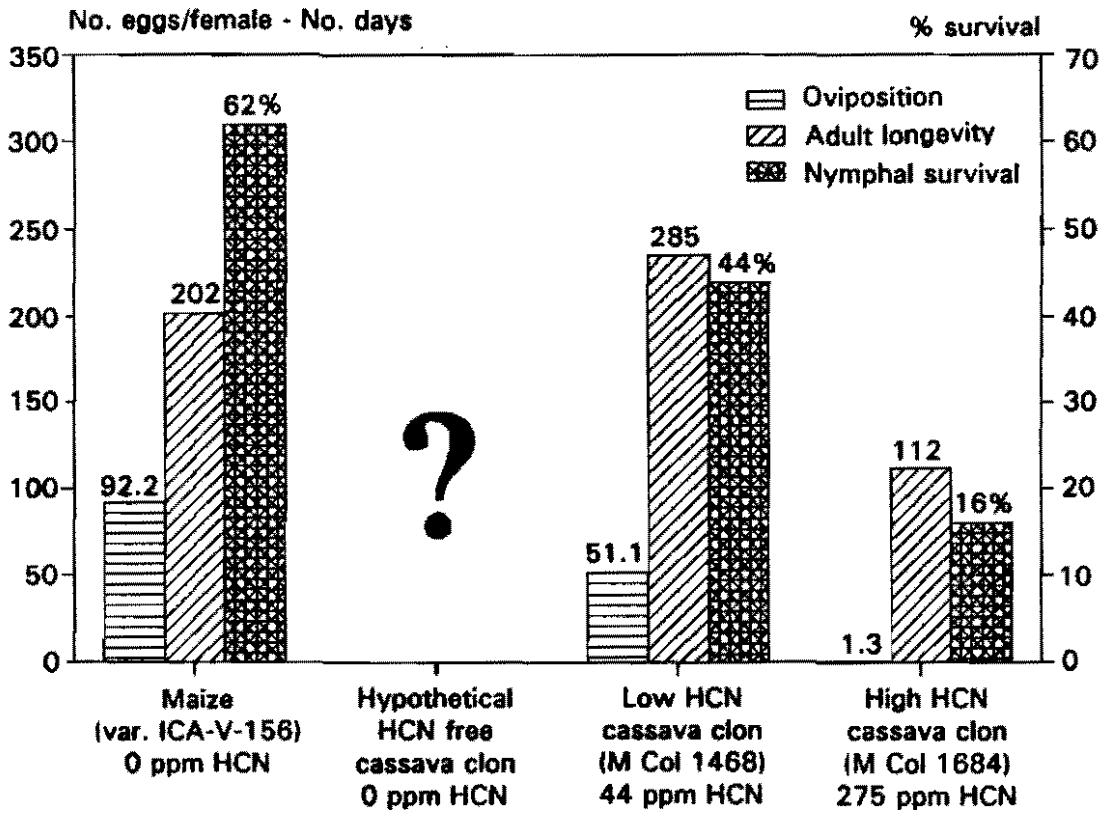

Figure 3. Oviposition (eggs per female), nymphal survival and adult longevity of *Cyrtomenus bergi* while feeding on maize, on a low and on a high HCN cassava variety (HCN measured in fresh root parenchyma) were significantly different at the 0.01% level with maize preferred over the low HCN clone which was preferred over the high HCN clone. How would a hypothetically cyanide-free cassava clone affect the infestation of *C. bergi* ?

PRELIMINARY STUDIES ON BIVALENT CECROPIN GENE EXPRESSION VECTOR CONSTRUCTION AND TRANSFERRING INTO CASSAVA

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The present study tried to establish Cassava Bacterial Blight (CBB)-resistant, transgenic cassava strains using biotechnology. The study included: effects of cecropin on the CBB causal agent; tissue and cell culture of cassava plants; establishment of a transformation system; construction of a cecropin D + cecropin B bivalent gene expression vector; and transformation of the bacterial resistance gene into the cassava genome. Experiments showed that cecropin was quite effective on 14 major bacterial causal agents of diseases of tropical crops, including CBB of cassava. Tissue culture with young embryos and shoots as explants could offer intact regenerated plantlets. Cecropin D and cecropin B genes were enzyme-cut and connected to an expression vector with two sets of promoter and terminator. Transference of the genes into cassava is still under way.

INTRODUCTION

Cassava is a staple crop in south China. The production area of the crop totalled 450,000 ha, with a total fresh root yield of 5 million tons. Cassava bacterial leaf blight (CBB) has been found the most problematic among all the diseases and pests of the crop. When epidemic, CBB infected over 90% of cassava plants, resulting in a yield loss of 50%. Chemical control has so far not given satisfactory results. Research with isolates identified the causal agent as *Xanthomanas campestris* pv. *Manihotis*. The present study tried to transfer CBB-resistant genes by using molecular biotechniques to obtain CBB resistant transgenic cassava varieties.

MATERIALS AND METHODS

Induction of cecropins in cecropia pupae

E. coli K_{12} D_{31} was microinjected into the diapausing pupae of the cecropia moth (*Hyalaphora cecropia*) and cultured at 25 °C for 4 to 5 days. Immune hemolymph was subject to CM-Sepharose chromatography and the resultant eluate (containing Cecropin D and Cecropin B) assayed with a DU-Spectrophotometer for cecropin activity.

Antibacterial assay of cecropin-containing immune hemolymph eluate on 15 bacterial diseases of tropical crops

The agar pores diffusion method was employed to inject 5 μl of cecropin-containing hemolymph eluate through agar pores to the media, using 15 bacterial species in individual culture.

Establishment of an *in-vitro* culture system

The cold-tolerant, infertility-insensitive and high-yielding local cassava cultivar, SCATC-124, was used as experimental material. Explants were taken from shoot-tips, disinfected with 75% alcohol for 30 seconds, followed by 2% $HgCl_2$ for another 8 to 10 minutes and rinsed with four changes of distilled water. They were then cut into slices which were inoculated onto MS (Murashige and Skoog, 1962) + BA (benzyl adenine) 0.2 mg/l + NAA (naphthalenacetic acid) 0.1 mg/l + sugar 2% + agar 4.5% to promote the formation of callus. On germination of apical and adventitious buds, the aseptic cultures were used as explants to be inoculated onto the same culture medium for obtaining large amounts of cassava callus. In addition, inoculation was made using young embryos from seed of SCATC-124 as explants on MS + 2,4-D (dichlorophenoxyacetic acid) 0.25 mg/l + NAA 0.15 mg/l + BA 0.03 mg/l + GA_3 (gibberellic acid) 0.2 mg/l.

Construction of a bivalent gene expression vector

Plasmids used

The principal properties of the plasmids used in the experiment are given below. The *E. coli* receptor of all plasmids was HB 101.

Plasmid	Principal properties	Source
pBin19	MCS(in LacZ'),LacZ',neo,Kan^R,LB,RB	Purchased
pCo24	CaMV 35Sp-MCS-NOS_T,neo(NOSp-NPT-II-PCS_T),Spc^R	Purchased
pCo25	CaMV 35Sp-D-NOS_T,neo,Spc^R	Constructed at CATAS
CB	B,MCS,Amp^R	Donation (Chinese)

Enzymes and biochemical agents

All the enzymes and biochemical agents used were purchased from Promega Co. and Biolabs Co.

METHOD

For extraction of large and small quantities of plasmids procedures from "Molecular Cloning—A Laboratory Manual" (Sambrook et al., 1989) were followed. All conditions for enzyme digestion reactions followed those proposed by the suppliers. The method used for low melting point (LMP) agarose recovery for DNA fragments also followed that described in "Molecular Cloning—A Laboratory Manual" (Sambrook et al., 1989). The preparation and transformation of *E. coli* competent cells followed that described by A. Nishimura et al.

The construction strategy of the plant bivalent expression vector was as follows. First, BamH I was used to digest CB for recovery of 156 bp DNA fragments (Figure 1), which was then ligated with the plasmid pCo24, digested by Bgl II and dephosphorized with calf intestinal alkaline phosphatase (CIP). Screening and identification of the recombinant plasmid were done with restriction enzymes. The resultant Cecropin B gene was positively inserted into the recombinant pSB13 at the downstream side of CaMV 35S promoter (Figure 2).

Second, digestion of pCo25 was made with BamH I. Recovery of 2.0 kb DNA fragments containing CaMV 35Sp-D-NOS$_T$ was done with 1% LMP agarose and spliced with T4 DNA polymerase. The spliced DNA fragments were ligated with the vector pBin19 that had been digested with Sma I and dephosphorized with CIP. Employment of restriction enzymes for assay was then carried out to yield the combinant pTD1 capable of expressing Cecropin D gene in plants (Figure 3).

Digestion of pSB13 was made with BamH I, and recovery of 2.0 kb DNA fragments (containing CaMV 35Sp-B-NOS$_T$) achieved with 1.0% LMP agarose, before ligating with pTD1 that had been digested with BamH I and dephosphorized with CIP. Assay and identification of the recombinant plasmid pDB3 was made with restriction enzymes (Figure 4). The pBD3 was found able to express both the Cecropin B and Cecropin D genes and to possess the selective marker, neo, in plant cells and tissue culture. Originated from T-DNA, LB (left border sequence, 25 bp) and RB (right border sequence, 20 bp) are conducive to the integrity and expression of the target gene fragments in host chromosomes. Figure 5 shows the entire sequence.

Gene transferring

Two methods were employed in gene transferring operations. In the first case, *Agrobacterium* was cocultured with cassava explants, while in the second, the gene-gun bombardment method was used. Sections of aseptic plantlet shoots from tissue culture were used as explants to be infected with cecropin D, cecropin B, and cecropin D + B bacterial solutions. They were then cocultured with carrot suspension cells before transferring onto plantlet-induction medium for further culture. Gene gun bombardment was made with a gunpowder-type gun in a vacuum. The explants used were dominated by calli.

RESULTS AND DISCUSSION

The cecropins showed good activity in antibacterial assays. Experiments on bacterial inhibition showed obvious antibacterial effects, typical evidence including:

a) *Xanthomonas campetris* pv. *manihotis* (Cassava): growth inhibition zone diameter of 17.4 mm;

b) *Zanthomonas campestris* pv. *oryzicola* (Rice): growth inhibition zone diameter of 29.4 mm;

c) *Erwinia caratovora* pv. *corotovora* (Vanilla): growth inhibition zone diameter of 17.4 mm;

d) *Pseudomonas solanaceram (pogostemum)* (Eucalyptus): growth inhibition zone of 19.5 mm;

e) *Pseudomonas rubrilieneana* (Sugar cane): growth inhibition diameter of 14.2 mm;

f) *Pseudomonas syringae* pv. *garcae* (Coffee): growth inhibition zone of 32.1 mm.

E. coli K_{22} D_{31} was used as control bacterium and had an effective colony inhibition diameter of 25 mm. In the control, the central point of the culture dish received a small amount of aseptic water to give an inhibition diameter of zero. It can be seen from the above that cecropin has marked bactericidal effects on the CBB causal agent, and the above-listed *Pseudomonas* and *Xanthomonas*.

With cassava shoot-tips as explant, tissue culture led to the initial formation of callus at day 15, and shoots at day 25. Multiplication with aseptic shoot segments was possible at day 30 after culture. Tissue culture with young embryos as explants gave callus at day 7 and clustered buds at day 50.

Construction of the bivalent gene (Cecropin D + Cecropin B) expression vector was successful. The genes of Cecropin D and Cecropin B were obtained. Cecropin D had a nucleotide sequence with length of 122 bp and Cecropin B 144 bp. They expressed themselves quite well in *E. coli*. The polypeptide was composed of 35-43 amino acid residues. It was lethal to bacteria, causing a collapse of bacterial cell membrane. The two genes were digested by restriction enzymes from their vectors before inserting into the same expression vector for enhancing the expression and CBB-resistance efficiency of the transgenic cassava varieties. Cloning into the same vector may enhance the expression ability of the cecropin genes and lead ultimately to the production of effective CBB-resistant transgenic cassava strains.

These genes have been induced into a number of materials and evaluation is to be made.

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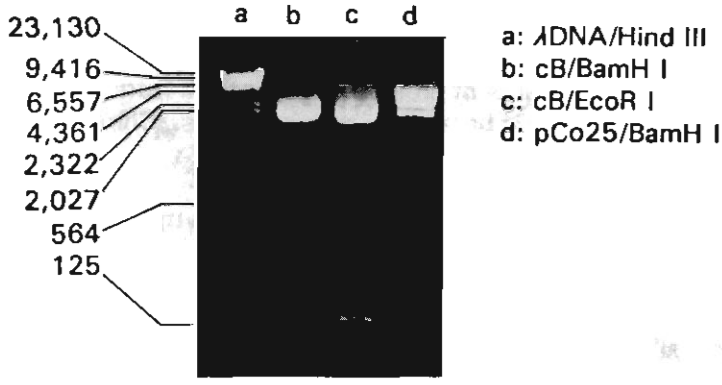

Figure 1. Splicing of Cecropin B and D genes fragments.

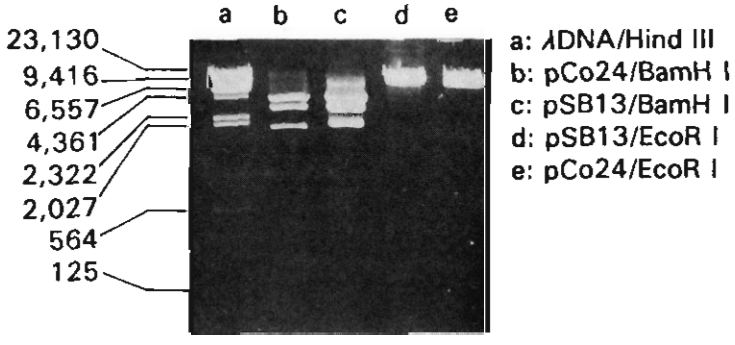

Figure 2. Assay of plasmid pSB13 with restriction enzymes.

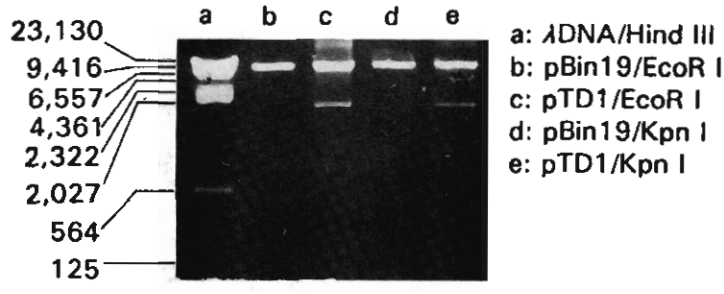

Figure 3. Assay of plasmid pTD1 with restriction enzymes.

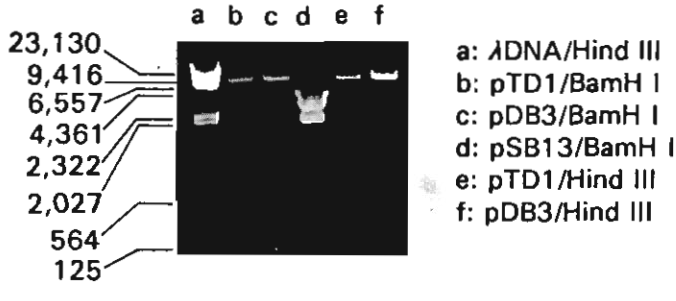

Figure 4. Assay of pDB3 with restriction enzymes.

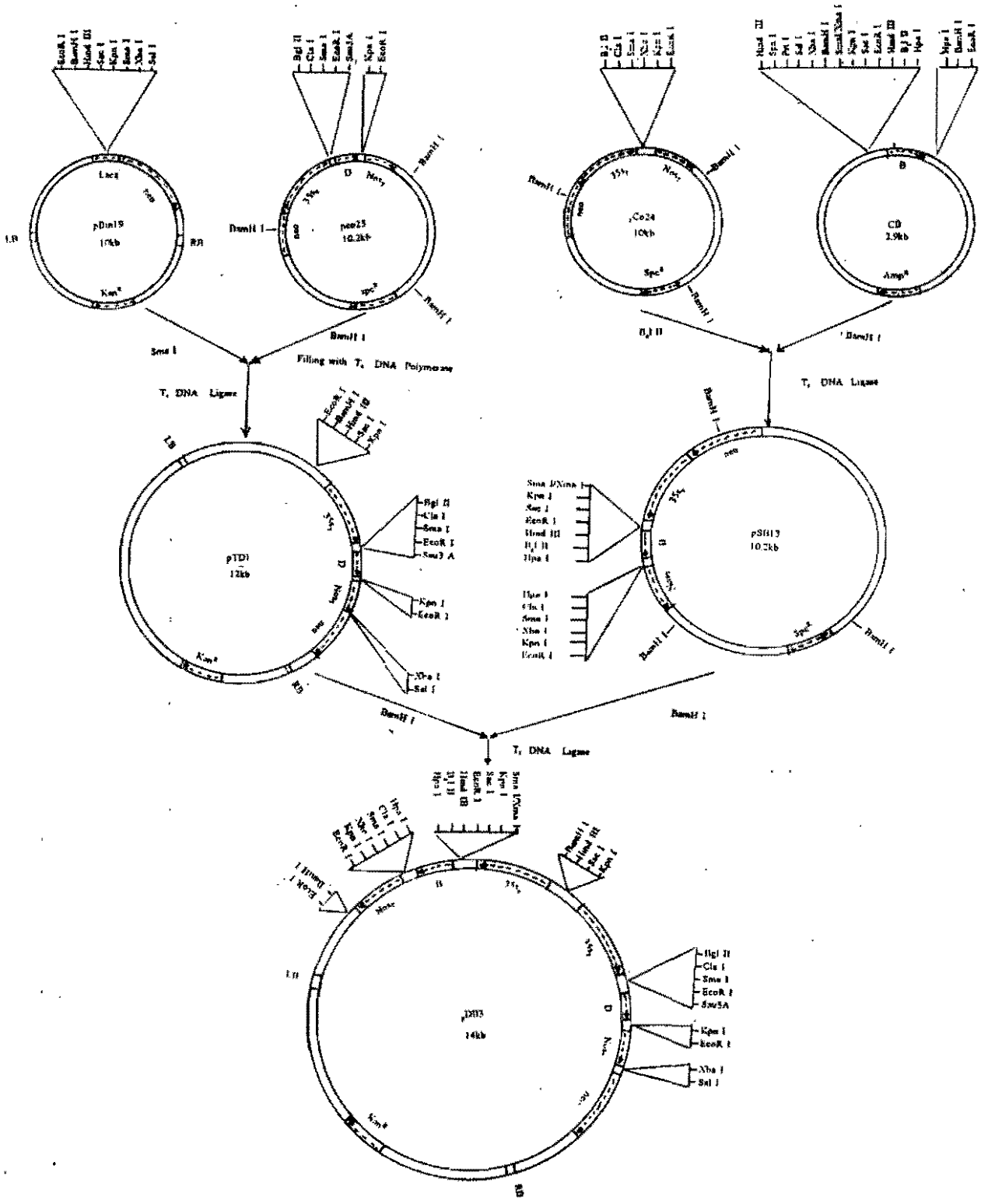

Figure 5. Procedures of construction of Cecropin B and D bivalent gene vector.

THE AFRICAN CASSAVA MOSAIC VIRUS (ACMV): A THREAT TO FOOD SECURITY IN AFRICA

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The concept of food security and the role of cassava in ensuring basic food are discussed. The impact of African cassava mosaic virus (ACMV) on food security in Africa as well as the need for action to contain this disease are presented. The devastating situation in Uganda is outlined.

INTRODUCTION

Food security and how to achieve it have been widely discussed. Reutlinger and Pellekani (1986) considered food security as the ability to meet target consumption levels in the face of fluctuating production, prices and incomes, and to ensure its absolute availability at any price. Hussain (1986) defines food security as: the objective that ensures all people at all times have both physical and economic access to the basic food they need in terms of adequate food supplies; stability in the flow of supplies; and access to available supplies by those who need them. The Food and Agricultural Organization (FAO, 1991) stipulates that the food must be available throughout the year to sustain household energy, health and nutrition. The author considers the availability of food and the ability to organize it as the basic elements of food security vis-à-vis lack of access to enough food—a situation amounting to food insecurity.

Many organizations and governments have talked about national food security. Famine and food insecurity tend to be associated with rural households and the urban poor. These are the most vulnerable to high food prices and limited access to food because of either low income or low household food production. This paper examines the role of cassava in providing food to the majority of African farmers who are peasants, poor and disadvantaged compared to the few rich ones. The threat posed by the African cassava mosaic virus (ACMV) towards achieving this security and the role of biotechnology in ACMV research are also discussed.

IMPORTANCE OF CASSAVA IN HOUSEHOLD FOOD SECURITY IN SUB-SAHARAN AFRICA

Cassava is grown in most parts of Africa, where it gives a higher calorie yield per hectare than the main cereal crops and can potentially supply calories at

considerably lower resource costs. On the basis of current average yields in the region, cassava produces about two and half times as many calories per hectare as maize; twice as many as yams, and one and half times as many as sweet potatoes. It accounts for more than one half of the by-product weight of total staples in sub-Saharan Africa (FAO, 1986).

In most cassava producing countries, production has been increasing over the last few decades. Most of the increase is attributed to the decline in production of more traditional food crops such as maize, bananas and finger millet. The increasing production has been due to cassava's ability to offer food security to farm households, its high yield and ability to do well in marginal environments such as drought conditions, poor soil fertility, low inputs, and high population pressure. Cassava is popular with farmers in Africa because: it is amenable to mixed cropping and food systems; it can stay in the ground for long periods; it attracts high market demands and good prices; and it is able to displace other food crops.

Though cassava is mostly a subsistence crop in Africa, increasingly farmers are trading it close to big cities. Bags of gari for sale are now common in Nigeria (Hahn et al., 1987). In Uganda, truckloads of cassava are shipped to urban markets every day (G. W. Otim-Nape, unpublished data) while in Kinshasha, Zaire, loads of "chekwang" and fresh cassava leaves are a common sight. Cassava also provides more calories for the money than any competing or complementary crop.

In Nigeria, annual quantities available for consumption increased from 7.7 million tons from 1965 to 1974, to 9.1 million tons from 1975 to 1984 (an annual increase of 1.2%). But population increase reduced the quantity available per individual by 15% annually (FAO, 1986). On average, cassava provides 47% of daily calories for each individual in Nigeria (FAO, 1986). Calorie yield per unit of labor is also as high as 138 million joules (Chandra et al., 1976).

PRODUCTION CONSTRAINTS

There are several pests and diseases that seriously decrease cassava growth and yield. The most important of these are the cassava mealybug (*Phenacoccus manihoti* Mat.-Ferr.), green spider mites (*Mononychellus tanajoa*), the African cassava mosaic virus disease (ACMD) and bacterial blight (CBB) caused by *Xanthomonas campestris* pv *manihotis* (Berther & Bonder) Dye. Of these, ACMD is currently the most serious.

The African cassava mosaic virus disease and its devastation in Uganda

The ACMD, now known to be caused by a whitefly-borne geminivirus (ACMV), was first reported in 1894 in Tanzania (Warburg, 1984). It now occurs in all the

cassava-growing countries in Africa and neighboring islands but there is little information on its incidence or production losses in the different countries. Nevertheless, various estimates of disease prevalence have been made based on general experience or limited surveys. Bock (1983) noted that the ACMV incidence was generally high in coastal and western Kenya, where it exceeded 80% in some districts and approached 100% on some individual farms. Infection was even greater in a sample of 10 farms assessed in Ghana, where the mean incidence was 96%. Virtually all cassava plants except those selected especially and propagated for experiments (D. Fargette, personal communication) were infected with ACMV in Cote d'Ivoire; whereas in Zaire mean ACMV incidence was 87%, in Ghana 85%, in Nigeria 82%, and in Cote d'Ivoire 82% (Nweke, personal communication). Table 1 shows the relative importance of ACMD in African countries.

In Uganda, since report of severe epidemics of the ACMD transmitted by the whitefly (*Bemisia tabaci*) was first reported in Buruli County, Luwero District, in 1988, more and serious cases of epidemics have been reported in northern, eastern and western regions of the country. The epidemic, which spreads in a front, moves southward at a rate of about 15 to 20 km/year. Areas dominated by plants infected through whitefly transmissions characterize the front. The lower leaves of the whitefly-infected plants appear healthy, while the leaves above points of infection show severe symptom expression, are severely reduced in size and markedly distorted and misshapen. This gives infected plants a paintbrush- like appearance. The plants harbor numerous adult whitefly populations on young shoots and large nymphal populations on lower surfaces of the apparently healthy lower leaves. Some 15 to 20 km from the fronts, all plants show severe ACMD symptoms arising from infections due to infected cuttings, are severely stunted and produce no or very poor root yields. Discouraged farmers abandon growing cassava. Annually, over 150,000 ha equivalent to over 2.2 million tons (US$440 million) of fresh cassava roots are being lost this way. Also, over 500 local genetic materials are threatened with extinction unless special measures are taken to protect the germplasm currently being conserved. The cause of the recent epidemics is being investigated but a new biotype of *B. tabaci* is suspected.

The effects on yields

The degree of symptom expression on the plants also determines the magnitude of yield loss in cassava. Fauquet and Fargette (1990) found negative correlations between yield and severity of symptoms caused by ACMV. Thresh et al. (1994) found that decreases in yields were positively correlated with severity and the extent of the symptoms expressed.

The stage of growth when a plant is infected and the degree of symptom severity in plants infected by ACMV determines the extent of yield loss. In Zanzibar, plants infected as cuttings produced only 5.5% to 44.4% the yields of healthy controls (Briant and Johns, 1940). Bock and Guthrie (1978) found that

plants infected as cuttings produced 14% to 56% the yields of healthy controls. Yields of 25.3% to 76.1% of healthy controls were also reported for some cassava varieties in Kenya (Seif, 1982) and Nigeria (Terry and Hahn, 1980). Similar results were obtained in Cote d'Ivoire (Fargette et al., 1988) where ACMV caused a 37% yield loss in trials with one of the main locally grown varieties. Assuming that 70% to 80% of all plants in Africa are infected and on average sustain a 405 to 50% yield loss, current production in Africa is decreased by 28.5 to 48.9 million tons; actual production was estimated at 73.3 million tons in 1990. In Uganda all ACMV-infected fields in three-quarters of the country subsequently produced no yields; consequently farmers in those areas stopped growing the crop altogether.

Impact of ACMV on food security in Africa

Considering the importance of cassava as a staple food crop in Africa and the high ACMV incidence and its severity on infected plants in Africa, the impact of ACMV on food production in Africa is considerable.

Reduction in food availability. The ACMV is one of the major factors reducing availability of food at the rural and urban household levels in Africa. Examination of production figures indicates that in large parts of Africa, production per unit area of land declines partly on account of soil depletion but also because of political instability and devastating crop pests and diseases.

With annual losses of 28% to 40% of a crop providing the main dietary energy requirements, the consequences can be grave. Hunger and malnutrition—as witnessed in eastern Uganda in 1994—can become rampant; growth and reproduction can be retarded. People's health, learning capacity, activity and overall quality of life and well-being are seriously undermined.

In sub-Saharan Africa for instance, both the number and prevalence of underweight children have increased to about 30%. Among the poor, women usually have low status and work long hours; many marry when young and tend to have underweight children and more of these children tend to die in infancy because of poor nourishment (FAO, 1992a). Those who survive remain vulnerable to malnutrition and infectious diseases, which can handicap them for the rest of their lives (FAO, 1992a). For a growing continent such as Africa, such a loss in human potential has socioeconomic consequences that cannot be tolerated.

Dependence on food aid. Crop losses from some devastating diseases such as ACMV imply that the continent cannot produce enough food to sustain its population. Consequently it has to depend more on food aid to be able to keep the population surviving. For a long time, Africa has continued to be the greatest recipient of food aid in the World (FAO, 1992b).

Reduced incomes and rural poverty. Rural families who grow cassava generate incomes from four sources: harvest and sale of cassava roots, sale of

processed cassava; rent from cassava processing equipment; and working in cassava-related jobs for other families (Table 2). Destruction of cassava by ACMV implies loss of income, accompanied by poverty.

Cassava is the richest source of energy for rural households in Africa. As already mentioned, loss of 28% to 40% of the energy source has serious implications on the socioeconomic lives of the rural people. Because they are malnourished and less energetic, their economic activity and income generation capacity is greatly reduced. It was estimated that 64% of total rural African population were poor in 1980 and 57% in 1987. This was also the highest in the world (FAO, 1992b).

THE NEED FOR ACTION

Research on ACMV

Tables 3 and 4 summarize a century of research carried out on ACMV and the disease it causes in Africa. It is evident that, compared with many other viral diseases, research on ACMD has an unusually long history and workers in several countries have contributed toward an understanding of the virus and the disease. The overall research effort has been considerable, and ACMD has received more attention than any other virus disease of an African food crop (Thresh et al., 1994); and yet the disease still causes heavy losses in Africa and is responsible for famine, poverty and starvation in a number of countries. Why is this so? In order to answer this, it is necessary to consider the following questions:

Is there sufficient research on ACMD?

Is there an appropriate balance of effort on ACMD in relation to other cassava pests and diseases?

Is the current prevalence of ACMD due to a lack of effective control measures or to a failure to adopt those already available?

Is ACMD becoming increasingly prevalent and more difficult to control because of changes in climate or cropping practices, and as cultivation of cassava increases and extends to new areas?

What are the prospects for achieving satisfactory control of ACMD on a suitably large scale?

These questions (Thresh e al., 1994) call for serious considerations of the problems and emphasize the need for more intensified research into the problem.

Farmer know-how

The ACMV has lived with the farmers for the last 100 years (Tables 3 and 4). It is therefore expected that traditional farmers must have generated a wealth of knowledge and practices of living with the problem. This knowledge and practice must be understood in order to design sustainable control strategies. A study of methods of past management of ACMV in traditional agriculture, and research on improving their use would realistically improve systems of ACMV management by traditional peasants in Africa.

Sociocultural factors

It must be recognized that to develop or import technological change in defiance of the sociocultural background of African peasant farmers' practices is a recipe for disaster. Traditional agriculture systems are finely tuned and adapted, both biologically and socially, to counter the pressure of what are often harsh and minimal environments. They often represent centuries of adaptive evolution in which most factors have been incorporated to face tremendous odds. Methods in controlling ACMV must therefore address farmers' sociocultural and economic circumstances.

Integrated perspective

Studies on ACMD should be looked at in the context of the farming system and the environment. Such studies should consider ACMV in relation to other diseases and pests and their interactions; to farmers perceptions; to cassava in the cropping systems; and to the ecological environment.

The role of biotechnology

Recent advances in biotechnology have opened opportunities to understand molecular and genetic aspects of organisms. Biotechnology would be useful to detect possible differences in whitefly biotypes, ACMV strains and developing genetically- engineered plants, resistant to the virus and the vector.

Intensified efforts in technology transfer

Although a great deal remains to be done, a lot can be achieved with existing technologies. In any efforts to control ACMD and to improve food security in Africa, the following must be given top priority: adapting and intensified transferring of technologies through strengthening research-extension and farmer linkages;

on-farm trials and training of extension staff, opinion leaders and farmers; ACMV awareness campaigns; and involvement and use of Non governmental organizations (NGOs) in the technology transfer process.

Policies and donor support

Development of policies supportive of cassava and increased donor support to cassava research and development and transfer of technologies for controlling ACMV are a must if this ever-worsening situation is to be curtailed.

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Table 1. Cassava in agriculture and nutrition

Country	Rank of cassava among food crops	Mean yield/ha (t)
Benin	1 (area planted with food crops 15%)	7
Burundi	2 (area planted with food crops 25%)	11
Cameroon	n.n.	6
Cent.Afr.Rep.	1 (by vol.)	12
Congo	1 (70% of cultivated area)	10
Ghana	3 (by vol.)	10
Ivory	2 (by vol.)	5
Kenya	14 (by area under cultivation)	6-23
	3 (by vol.)	
Liberia	2 (by vol.)	9
Malawi	2 (by vol.)	8
Nigeria	2 (by vol.)	10
Rwanda	2 (among tuber crops)	20
Senegal	3 (by vol.)	3
Sierra Leone	2	4
Tanzania	4	13
Togo	1 (by vol. in the South with maize)	10
Uganda	1 (40% of cultivated area)	6
Zaire	1 (by area and volume)	7

SOURCE: Alaux and Fauquet (1990).

Table 2. Estimated average income generated from 5.89 t by a cassava-growing household in Oyo LGA, 1985. (Nigerian currency—nairas [N].)

	N
Gross income:	
Sale of cassava roots (4.06 t @ N380)	1543
Sale of processed gari (2.193/t @ N1500)	3289
Rent from cassava processing equipment	162
Miscellaneous income from cassava-related jobs	739
Value of home-consumed cassava (1.83 t @ N380)	695
Total gross income	6428
Less cost of goods sold (COGS):	
Cassava cuttings	177
Losses due to deterioration of roots and gari (15%)	1035
Total COGS	1212
Gross margin	5216
Less operating expenses:	
Labor	
Land preparation (N60.25 x 5.89 t)	355
Production (N60.98 x 5.89 t)	359
Processing (N184.59 x 5.89 t)	1087
Marketing (N5.00 x 39.67 days)	198
Total labor	1999
Land	50
Machine grating	130
Transport	707
Miscellaneous expenses	118
Total operating expenses	3004
Net operating margin	2212
Less fixed expenses	
Depreciation on production tools	
Processing equipment	145
Net profit from cassava/household	2067

SOURCE: United Nations Children's Fund (UNICEF)/International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria/University of Ibadan (UI) Field Survey, 1986.

Table 3. Chronology of important developments in research on African Cassava Mosaic Virus (ACMV).

1975	Virus first isolated (Kenya; Bock, 1975)
1976	Polyclonal antiserum produced (Kenya; Bock and Guthrie, 1976)
1977	Virus shown to contain DNA (UK; Harrison et al., 1977)
1983	Bipartite genome demonstrated (UK; Stanley and Gay, 1983) Nucleotide sequence determined (UK; Stanley and Gay, 1983) Virus shown to cause mosaic (Kenya; Bock and Woods, 1983)
1984	Detected by nuclei acid hybridization (UK; Robinson et al., 1984)
1986	Monoclonal antibodies produced (UK; Thomas et al., 1986)
1987	'East' and 'West' strains of ACMV distinguished serologically (UK; Harrison et al., 1987)
1988	Agroinfection demonstrated (UK; Morris et al., 1988)
1989 to present	International cassava-trans project (Fauquet and Beachy, 1989)
1993	Three distinct mosaic geminiviruses distinguished serologically (Hong et al., 1993).

Table 4. Chronology of research on African Cassava Mosaic Disease (ACMD) and the whitefly vector, *Bemisia tabaci*.

1894	ACMD first reported (Tanzania; Warburg, 1984)
1926	First reported in W. Africa (Golding, 1936)
1930s	Comprehensive studies: Tanzania
1931	Effect on yield first evaluated (Congo; Muller, 1931)
1932	First whitefly transmission (Congo; Ghesquiere, 1932)
1932-1939	Resistance breeding (Nigeria/Ghana)
1934-1960	Resistance breeding (E. Africa)
1940	Cassava breeding (Madagascar)
1950s	Research in Nigeria
1972-present	IITA[a] Root and Tuber Improvement Program
1970s-1980s	ODA[b] project (Kenya)
1979-1989	ORSTOM[c] project (Cote d'Ivoire)
1980s-present	Research in Uganda
1990	Cassava-restricted biotype of *B. tabaci* distinguished (Burban et al., 1992)

a. International Institute of Tropical Agriculture, Ibadan, Nigeria.
b. Overseas Development Administration, UK.
c. Office de la Recherche Scientifique et Technique d'Outre-Mer, France.

SOURCE: Thresh et al., 1994.

GENETIC ENGINEERING APPROACHES TO MINERAL NUTRITION

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This presentation reviews potential ways to improve mineral nutrition of cassava by direct genetic engineering of its genome, using P nutrition as a specific example. We will assume that a gene transfer system can be successfully developed for cassava, then consider ways that individual nutritional traits might be addressed by genetic manipulation. In a general sense, the efficiency of P nutrition could be improved by increasing the plants ability to take up P from the soil or by increasing overall yield for a given uptake of P. Ways of achieving both approaches by genetic manipulation will be considered. The major current obstacle to such genetic manipulation is the lack of information about the biochemistry, physiology, and molecular genetics of nutrition. We require a much better understanding of the critical steps in P uptake, transport, and storage, and bout the plant genes that execute these steps, before we can consider intelligent engineering of nutrition efficiency. However, rapid progress is now being made in the area of plant gene isolation, and powerful techniques for studying the mechanism of gene action are becoming available. Over the next few years we can expect to be able to undertake genetic manipulation of plant nutritional characters.

INTRODUCTION

It has become abundantly clear over the past 25 years that the uptake and transport of mineral nutrients in plants is under genetic control (Epstein, 1972). The uptake of nutrients from the soil to the root, their transport and distribution throughout the plant, and movement of nutrients between the cytoplasm and various organelles within cells, are all executed by gene products. Once the function of these genes is sufficiently well understood and the genes are available, individual steps in uptake and transport will be able to be manipulated.

Plant nutrition covers a continuum from nutrient deficiency, through sufficiency and optimum status, to toxicity. The simplest nutritional problem to address is toxicity, and it is here that early progress is being made using gene transfer. For example, recent work has been successful in genetic manipulation of plant salt tolerance by engineering a mannitol biosynthesis gene (Tarczynski et al., 1993). As our understanding of nutrition and nutritional genes improves, the more complex issues of overcoming deficiency and increasing overall nutrient efficiency will be able to be tackled.

Our goal in this presentation is to create an awareness of what might be achieved by engineering approaches to mineral nutrition, and to consider a potential application of this approach in a crop such as cassava. Gene transfer is an extremely powerful tool that opens up the possibility of introducing nutritional efficiency genes from any organism (other plants, fungi, bacteria, or animals) into cassava. To assess such a wide range of options, it is necessary to step back and adopt a somewhat different vision of nutrition, one that focuses on the role of individual genes in the overall process, and critically assesses what the limiting steps in the process are.

Mineral nutrition issues in cassava

Nutritionally, cassava is known as an extremely efficient plant that is capable of growth on soils of extremely low fertility, especially acid soils. In terms of genetic manipulation, then, one good reason for studying cassava is to investigate the genes that are responsible for this efficiency, in order that they might be cloned and introduced into other, less efficient crops. In addition, however, there are a number of nutritional issues in cassava that might be drain from soils. Cassava contains high concentrations of K, and repeated cropping drains the soils of available K which must be replenished. Another nutritional issue is P uptake efficiency, which remains a limit on productivity in certain growing areas (especially S. America). The other major macronutrients (N, S, Mg and Ca) appear not to be a limit on cassava production in most production areas (Cock, 1985).

Here we will review approaches that might be taken to improve mineral nutrition in cassava by direct genetic engineering of the plant. A crucial requirement for genetic engineering of cassava is a good gene transfer system. Progress in this research is considered elsewhere in this volume, and for the purposes of this discussion, we will assume that a gene transfer system is available in cassava.

We will consider P uptake efficiency as a specific example of macronutrient engineering. The general process of uptake, transport and storage are substantially similar for different nutrients, and most of our comments are generally applicable to other macronutrients.

How might P efficiency be engineered?

In a general sense, there are two ways in which changing the plant could improve the efficiency of P nutrition. For example, one can image increasing the plants ability to take up P from the soil (eg) improving P scavenging efficiency, which would lead to improved a reduce uptake of P, either by redistributing (spatially or temporally) the P within the plant more efficiency, or by reducing the overall requirement for P in plant tissues.

The process of P uptake can be envisaged as occurring in a number of separate steps. (a) First there is the passage of the P ion into solution. Most P in the soil is not present as the inorganic P ion (a typical soil concentration is 3 uM), in organic form (eg phytic acid), or within soil microorganisms. (b) the next step is movement of the P ion (by diffusion) or the root (by growth) so that the P ion and root surfaces come into contact. (c) Next, the P ion (negatively charged) must diffuse through the cell wall (also negatively charged) to the P transporter located in the plasmalemma at the surface of the cytoplasm. (d) The phosphate ion is then pumped by the transporter through the plasmalemma into the cytoplasm.

Once it has entered a cell of the plant, it can follow a number of paths. (e) Some can pass back through the plasmalemma into the apoplast by effux (passive or active), and be lost to the system. (f) Some can be used directly in ester synthesis (to P-lipid, RNA, glucose-6-P, etc.) in the cytoplasm. (g) Some can be transferred, possibly by the action of another transporter located in the tonoplast, into the cell vacuole for storage (a typical concentration there is 5 mM). (h) Some can be moved through the plasmodesmata into adjacent cells. (i) Some can be unloaded in a directed way through the plasmamembrane into another part of the extracellular space (such as the lumen of the xylem). (j) Some can be unloaded through the plasmodesmata and plasmalemma into the conducting pathway of the sieve tubes, which are probably best interpreted as highly specialized, highly elongated intracellular spaces (that is, still bounded by a membrane).

(k) Once within the xylem, P can be transported long distances passively as part of the water flow. (1) Once within the phloem, it can be moved distances, passively or possibly actively, as part of the carbohydrate stream. (m) Throughout the xylem and phloem transport paths, adjacent cells can initiate a new cycle of accumulation and allocation, as in (c, d, e, f, g, and h). (n) Mycorhizas add to the complexity. In effect they represent a shunt acting in parallel with the root hair system in carrying out their own version of steps b, c, d, e, f, g, and i. (o) In times of P stress, or in senescing parts of the plant, P can be recovered from intracellular storages (eg the vacuole), and remobilized into growing of the plant.

Each of the steps above can be modulated by plant genes. The involvement is direct for some steps, such as the active transport of P across a membrane by a protein (d, e, g, i, j, m, m, and o), or the utilization of P within cells (f). It is also easy to imagine how P uptake can be affected by genes that affect root morphology, root hair length, or mycorhizal interactions. But plant genes can also affect such processes as P solubility or the rate of diffusion. For example, most plants secrete acid phosphatases, which are able to cleave a range of organic phosphate forms and make them available for transport and uptake (see Goldstein, 1992). Another example is provided by yellow lupin, which actively secretes citric acid into the rhizosphere, improving P solubility and diffusion of the inorganic PO_4 through the soil particles to the root surface (Gardner et al., 1983). In all there are

likely to be dozens, perhaps hundreds of genes with the potential to affect the complex processes of uptake, transport, usage, storage and mobilization of P in the plant.

Which gene or genes should be manipulated? To make the largest gains in P efficiency, the limiting steps in these processes need to be defined. We need to determine the effects of the individual genes on each step of the process. We further need to establish what genes are available in other organism that might give significant improvements. Clearly, these requirements would be a tall order even for a simple pathway. When we are looking at a complicated physiological process such as P efficiency (even without considering the interactions which must occur between P and other nutrients), the problem cas seen overwhelming. Discovering this information will require the combined application of genetic, biochemical and physiological approaches, as well as molecular techniques.

Our lack of understanding of gene action is probably the largest obstacle to any genetic engineering project in plants. The complexity of mineral nutrition makes the issue particularly problematic. In the following two sections, we will consider how we might clone some of the genes that affect P uptake, transport and storage, and then how we can investigate what the critical limiting steps in these processes are.

Cloning plant genes involved in P uptake and transport

There are at least five ways in which P uptake or transport genes might be cloned.

(i) The most straightforward approach to gene cloning is to purify the protein that carries out a given biochemical step. For example, a secreted acid phosphatase could be purified on the basis of assaying fractioned cell proteins for phosphatase activity. Once the purified protein is sequenced. The gene that encodes the protein can be identified in cDNA libraries by using an oligonucleotide probe.

(ii) Another approach uses the fact that plants grown in low P increase expression of a number of genes (Goldstein, 1992). The induced genes are presumed to improve the plants' capability to obtain P from soil or to improve the distribution of P in their tissues under P-starvation conditions. To date two types of gene have been identified that are induced by low P (and neither was isolated by virtue of this attribute): RNases which are presumed to be involved in scavenging organic P from the cell wall and vacuole (Taylor et al., 1993; Howard et al., 1994), and a vegetative storage protein that has acid phosphatase activity (Staswyck, 1990). Recently Matsumoto and coworkers from Japan have isolated four additional genes induced in low P: glutathione-S-transferase, an auxin-induced gene, and two genes (Ezaki et al., 1994). Other groups are actively involved in cloning low-P induced plant genes: eg Lefebvre and coworkers (see Duff et al., 1991).

Genes induced under P deprivation have been characterized in microorganisms (see Torriani-Gorini et al., 1987). In *E. coli* the P regulon consists of at least 20 genes, while in yeast at least 14 P-induced genes have been isolated. In yeast, one of the genes that regulates the P induction process also regulates the cell cycle (Kaffman et al 1994). Since the major end use of P in the cell is in DNA and RNA, it is perhaps not surprising that cell division and P uptake are linked.

(iii) Another way of cloning plant P genes is to isolate genes in which mutations cause disrupted P uptake or P transport. This cloning can best be accomplished in species for which the genome is small and the genetic are good—*Arabidopsis* is the best example. Two mutations in phosphate uptake/transport genes have been isolated in this species: pho1 appears to be involved in loading P into the xylem (the mutants are P-deficient; Poirier et al., 1991; Delhaize, 1994), while pho2 may be involved in P export out of leaves (the mutants accumulate P; Delhaize, 1994). A cell line of tomato was selected that shows constitutive expression of the high affinity P uptake system. This line showed enhanced P use efficiency in culture (Goldstein, 1991). An arsenate-tolerant grass, *Holcus lanatus*, has also been identified that does not induce the high-affinity P uptake system (Meharg, 1994).

The mutants provide valuable information about gene function, and aid in the identification of critical steps in the pathway, in addition to providing a method of cloning the gene.

(iv) It is also possible to clone genes via functional expression in other systems. For example, plant genes can be expressed in yeast to try to complement deficiences in yeast mutant strains. Mutants are available in the yeast genes for acid phosphatases, P uptake and various P regulatory genes. Complementation of these mutations using expression libraries is an attractive approach to cloning the functionally equivalent plant genes. The approach has already been used for genes encoding potassium channels (Sentenac et al., 1992; Anderson et al., 1992), as well as transporters for sucrose (Saucer and Stadler, 1993), NH4 and amino acids (Ninneman et al., 1994). Screening is underway to isolate phosphate transporter genes (Rreismejer et al., 1994). Complementation appears to be particulary suited to proteins that are functionally conserved, which appears to be the case for nutrient transport genes.

(v) Taking advantage of homology between species is another way of cloning plant genes, and the conservation seen in transport proteins augurs well for this approach. The most direct method is to use P genes cloned from (for example) filamentous fungi as probes against plant cDNA libraries. If the regions of homology are short, oligonucleotides complementary to conserved regions in P genes can be used to prime PCR amplifications from plant nucleic acids. Finally, the random sequencing of expressed genes from species such as *Arabidopsis* and rice is rapidly

resulting in a series of clones identified purely on the basis of their homology, and some of these are likely to be transport genes, perhaps including P transportes.

The rapid rate of current progress suggests that over the next few years a number of genes involved in P uptake and transport will become available.

Identification of critical steps in nutrient efficiency

Rather circuitously and ironically, one of the most powerful tools for identifying the biological function of a gene transfer itself. Both under- and over-expression of individual genes is possible to make pairs of plant lines that are genetically except for a difference in the expression level of a single gene. Such plant lines are one of the most powerful tools of modern plant physiology.

As an example, imagine we have isolated a gene with homology to a known (say yeast) phosphate transporter, and discovered that mRNA from this gene is induced in mesophyll cells by low P treatments. It should be possible to create plant lines that have high constitutive levels of this protein, and lines that are unable to induced it in low P. Careful examination of the behavior of P in these lines could establish the conclusion that the unknown gene functions in translocating Pi from the vacuole to the cytoplasm in leaf mesophyll cells as a means of phosphate scavenging under low P conditions. Moreover, the high and low expressing lines would establish whether the gene is a limiting step in P efficiency. This information is critical to deciding whether the enzyme should be a target for genetic manipulation, and whether introducing a new enzyme with altered properties might increase overall P efficiency.

Gene transfer is not limited to the use of genes that occur within a species—any gene from any source will do, as mentioned above. This power has several advantages at various stages of the process. For example, imagine that we are attempting to increase the capability of cassava to scavenge organic P from the soil. One option is to express an improved acid phosphatase that is secreted into the soil rhizosphere. What specific improvements would be sought? The characteristics of the endogenous cassava acid phosphatase enyzme could be assessed by expressing it in an amenable organism such as yeast. The biochemical function of the cassava gene could be studied by purifying it from yeast, and by characterising the growth of the yeast strain on various organic P sources. A range of acid phosphatase genes are now available from fungi and bacteria. Heterologous expression of these genes in yeast would allow their enzyme activity to be compared directly. An enzyme which cleaved some substrates more efficiently than the endogenous plant enzyme would be a potential candidate for introduction into transgenic cassava. The resulting plant would be expected to degrade a wider range of organic P sources, increasing overall P utilization. Similar arguments could apply to genes that control any other stage of P uptake, storage, or transport.

Prospects

Genetic engineering offers considerable promise for improvement of plant crops generally, and we have outline ways in which a trait such as P efficiency in cassava might be addressed using this technology. Currently the major obstacle to genetic manipulation of nutritional efficiency is lack of information about the plant genes involved in nutrient uptake, transport, and storage. In a few cases where the pathways or genes for certain steps have been identified, knowledge of the critical steps that are limiting in these processes remains unknown. Clearly a large body of data (biochemical, physiological, as well as molecular genetic) is required before we can undertake intelligent engineering of nutrient efficiency. The good news is that very rapid progress is now being made in the area of gene isolation, and that techniques for attacking these problems are available. In the next few years we can expect to be able to undertake genetic engineering of mineral nutrition in crops like cassava.

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EFFECT OF FLOODING ON CASSAVA PERFORMANCE

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One of the problems when planting cassava on peat is the seasonal flooding of the fields. The aims of the studies were to determine the growth stage of cassava most susceptible to flooding, and the maximum duration of flooding tolerable to the crop at this stage. The studies were carried out in fibre-glass lysimeters where the water-table was set at 15 cm depth (except when flooding was imposed). The studies were carried out on the variety Black Twig (harvested at 9 months), and the early variety MM 92 (harvested at 6 months). A completely randomized design (four replications) was used to test flooding at four growth stages and to test four flooding durations against an unflooded control. With Black Twig, flooding between three and a half and 5 months reduced root yield by 47.5%-80%. Flooding at three and a half months did not significantly depress Black Twig's yield even for 4 days, although a 25% reduction was noted. MM 92 did not tolerate more than 3 days' flooding at three and a half months without yield depression. Flooding for 4 days at one, 4 and 5 months reduced yield significantly from the control (80% reduction at 5 months). Thus, for a late variety, if flooding occurs between root initiation and early root bulking, it is advisable to replant the crop; at other times, the standing crop may be left to recover. An early variety is less tolerant to flooding at any stage. Drainage infrastructure for peat must be efficient enough to remove surface water within three days of flooding.

INTRODUCTION

Malaysia has 2.7 million hectares of tropical woody peatland (Mutalib et al., 1992). Cassava (*Manihot esculenta* Crantz) adapts well to drained peat (Chew, 1977; Tan and Chan, 1989), resulting from the felling of peatland forest and the establishment of drainage infrastructure. On peat, the problem is seldom one of insufficient moisture. Instead, during the rainy seasons, the problem of flooding or waterlogging is often encountered. This means that the drainage infrastructure must be efficient enough for precise water management so that excess water may be drained before it jeopardizes crop growth and adversely affects cassava yield.

It is therefore necessary to establish the tolerance limits of cassava to flooding or waterlogging so that drainage will be timely to save a standing crop. At the same time, such information is important in deciding whether or not a crop should be replanted or can be salvaged.

The aim of the studies reported was to determine the effects of flooding on the performance of cassava planted on peat. Specifically, to establish the critical growth stage when the crop is most sensitive to flooding, and to determine the duration of flooding tolerable to cassava at this stage.

MATERIALS AND METHODS

The studies were carried out in lysimeters for more precise water-table control than under field conditions. Fiberglass lysimeters, 50 cm in diameter and 150 cm in height, were used. Each lysimeter had a series of overflow pipes spaced at 15 cm intervals down the whole height, the first starting 15 cm from the rim. Using peat extracted from the field, the lysimeters were filled in layers corresponding in sequence to that in the field. In the earlier part of the first experiment, the lysimeters were placed in a glasshouse, but by the third month the cassava plants had almost reached the height of the glasshouse roof, and had to be moved out to an open area where they remained. Each lysimeter was planted with one cassava cutting (20 cm length) vertically orientated in a central position. The cuttings were soaked for ten minutes in 2% $CuSO_4.5H_2O$ solution before planting, to supply the subsequent plants with Cu which is intrinsically deficient in peat. Fertilizers at 20 g N, 3 g P_2O_5 and 16 kg K_2O per plant were applied by banding at planting. Weeds were kept in check by hand.

An earlier study of the effect of water-table on cassava performance has shown that optimum crop performance was recorded at a static water-table of 15 cm from the peat surface (Tan and Ambak, 1989). So the studies were carried out at this water-table, with all other overflow pipes plugged. When flooding treatment was imposed, the first overflow pipe was also plugged to allow surface flooding. All lysimeters were watered daily except when flooding treatments were imposed.

A total of five sets of experiments were carried out, starting with the late-maturing variety Black Twig, then switching over to the early 6-month variety MM 92. In all the experiments, a completely randomized design was used with each treatment replicated four times. The first study attempted to determine the stage of crop growth most affected by flooding. The treatments included flooding (for a duration of four days) at one, three and a half, 5 and 8 months after planting, as well as a control where the water-table was maintained at 15 cm without any flooding treatment throughout the duration of the study. This experiment was repeated after the crop was harvested at 9 months when data on fresh root yield and dry matter content were collected. Dry matter content was determined by oven-drying root samples at 70 °C to constant weight.

The third experiment (again with Black Twig) attempted to determine the effect of varying durations of flooding at the critical growth stage established in one of the earlier experiments, which was three and a half months after planting. The treatments included a control (no flooding) and flooding for 1, 2, 3 and 4 days.

The fourth experiment basically repeated the third, using the early variety MM 92. The experiment was harvested after 6 months.

In the fifth experiment, the critical growth stage of MM 92 sensitive to flooding was studied, with flooding at 1, 3, 4 and 5 months after planting. An unflooded control was included, and the crop harvested after 6 months.

RESULTS

Late variety Black Twig

Results from the first experiment showed that the most critical growth stage to flooding for the late variety Black Twig was three and a half months after planting. Fresh root yield was reduced by as much as 80.8% compared to the control (Figure 1). Root dry matter content was not significantly reduced by flooding when compared to the control. Indeed, flooding at 5 months increased dry matter content significantly from 27.3% to 36.3% (Figure 2).

But in the second experiment the critical growth stage was found to be at 5 months when fresh root yield was reduced by 47.5% compared to the control (Figure 1). In this case, flooding had no significant effect on dry matter content (Figure 2).

In the third experiment, no significant effects were detected on the yield and dry matter content of Black Twig due to flooding for 1 to 4 days at the growth stage of three and a half months. Yet flooding for 4 days reduced fresh root yield by 25.8% (Figure 3).

Early variety MM 92

In the fourth experiment, the fresh root yield of early variety MM 92 was reduced significantly (23.8%) compared to the control when flooding lasted for 4 days, but not when from 1 to 3 days (Figure 4). However, root dry matter content was not affected even when flooding was for 4 days.

Experiment 5 showed that for MM92 flooding at any growth stage (except at 3 months) led to a decline in yield. The worst effects occurred when flooding was imposed at 5 months when yield was only 18.9% that of the control (Figure 5). Again, dry matter content was not affected by flooding at any of the stages of growth studied.

DISCUSSION

Generally, it seems that flooding in peat does not affect cassava root dry matter content, regardless of the stage of crop growth or duration of flooding. This is in line with results obtained by Tan and Ambak (1989) who found that the water-table in peat did not affect dry matter content.

It appears that for Black Twig, if the field is flooded between three and a half and 5 months after planting (coinciding with storage root initiation and early root bulking—Wholey and Cock (1974), the management decision is to pull out the existing stand and replant, since flooding at this stage of growth severely and significantly affects yields. If flooding occurs earlier or later than this stage, it may be worthwhile keeping the existing crop till harvest, since results show that the crop seems able to recover from the effects of flooding with a minor degree of yield loss.

For Black Twig also, flooding for as long as 4 days does not affect yields too badly. Thus, a system of drainage has to be devised, able to remove excess water from the field within this time.

On the other hand, early variety MM 92 is able to withstand only 1 to 3 days of flooding, since yield is severely affected when flooding lasts 4 days. Flooding jeopardizes yields when it occurs at 1 month (during germination and early establishment), or at 4 and 5 months after planting (after storage root initiation and during root bulking). For some unexplained reason, flooding at 3 months (onset of storage root initiation) did not seem to depress yield significantly. Thus, unless the field floods at 3 months, it is necessary to replant when flooding occurs for as long as 4 days. If flooding occurs at 5 months, the management decision may be to harvest as soon as field conditions allow so as to salvage whatever yield is present before the roots rot. Root rot is the main cause of serious yield loss if harvest is delayed to 6 months, the normal time to harvest the variety MM 92.

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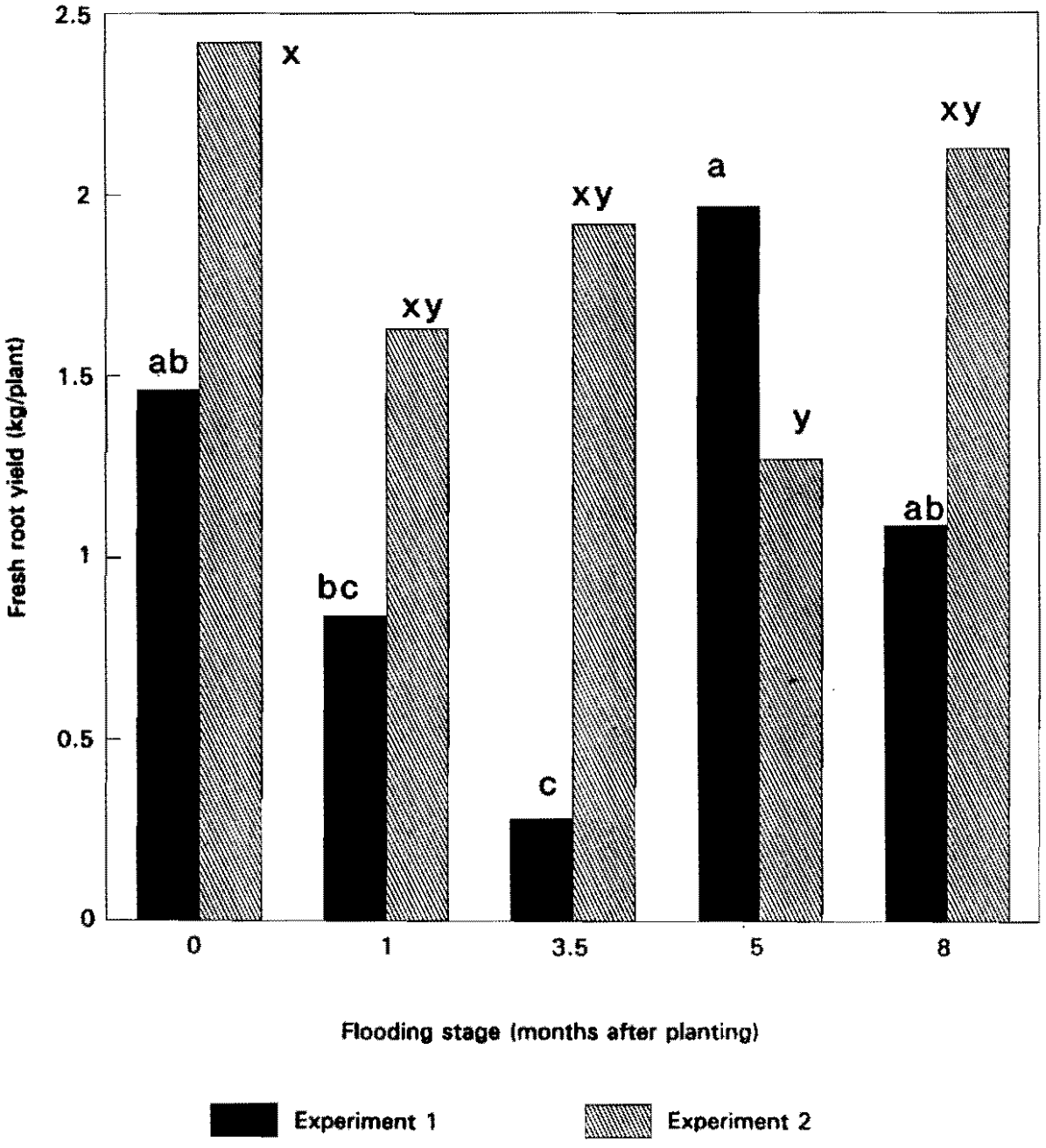

Figure 1. Effect of flooding at different stages of crop growth on fresh root yield of cassava variety Black Twig.

[Bars bearing the same letter are not significantly different from one another according to L.S.D. test at 5% level of probability]

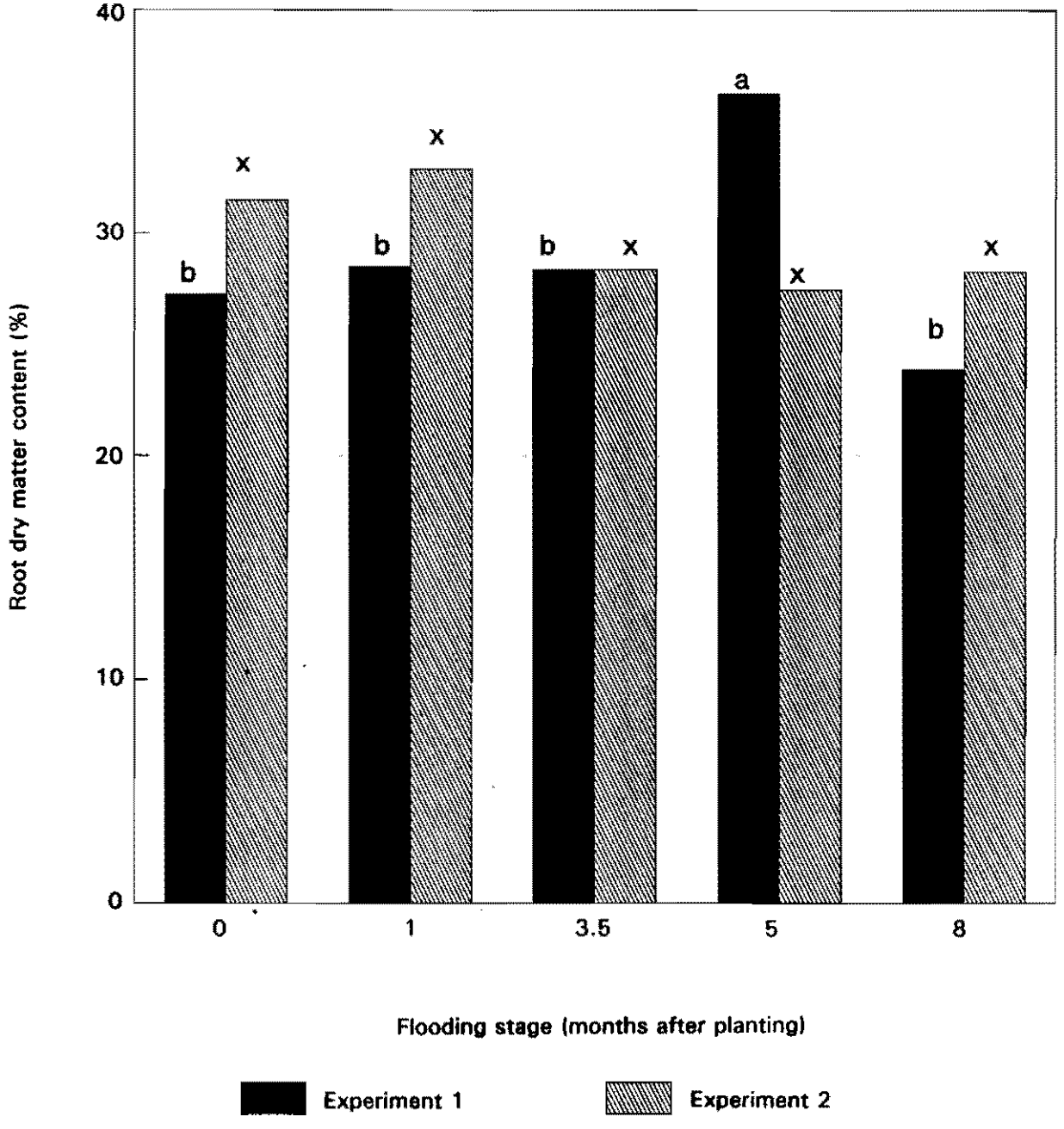

Figure 2. Effect of flooding at different stages of crop growth on root dry matter content of cassava variety Black Twig.

[Bars bearing the same letter are not significantly different from one another according to L.S.D. test at 5% level of probability]

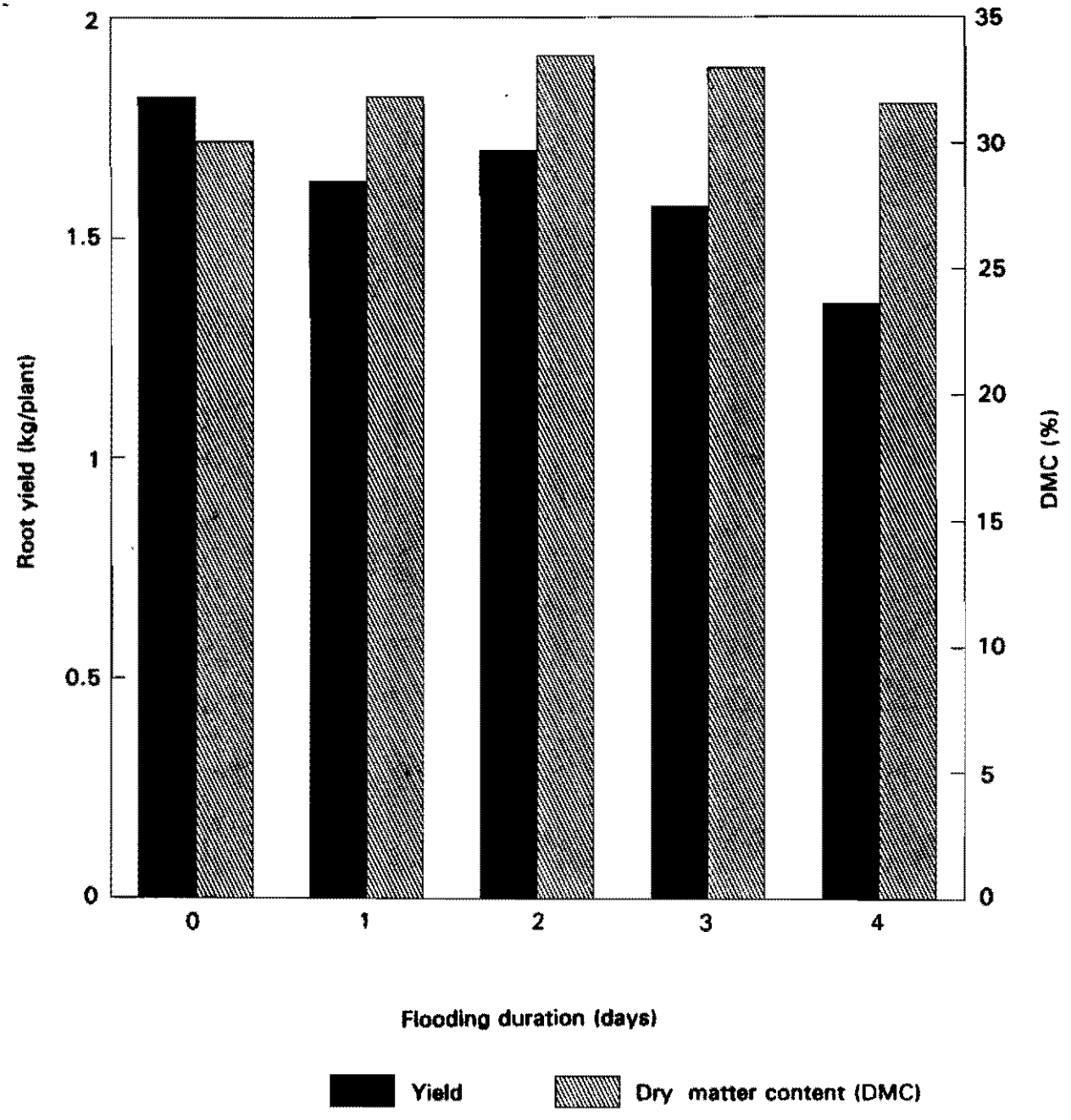

Figure 3. Effect of duration of flooding on fresh root yield and dry matter content (DMC) of cassava variety Black Twig.

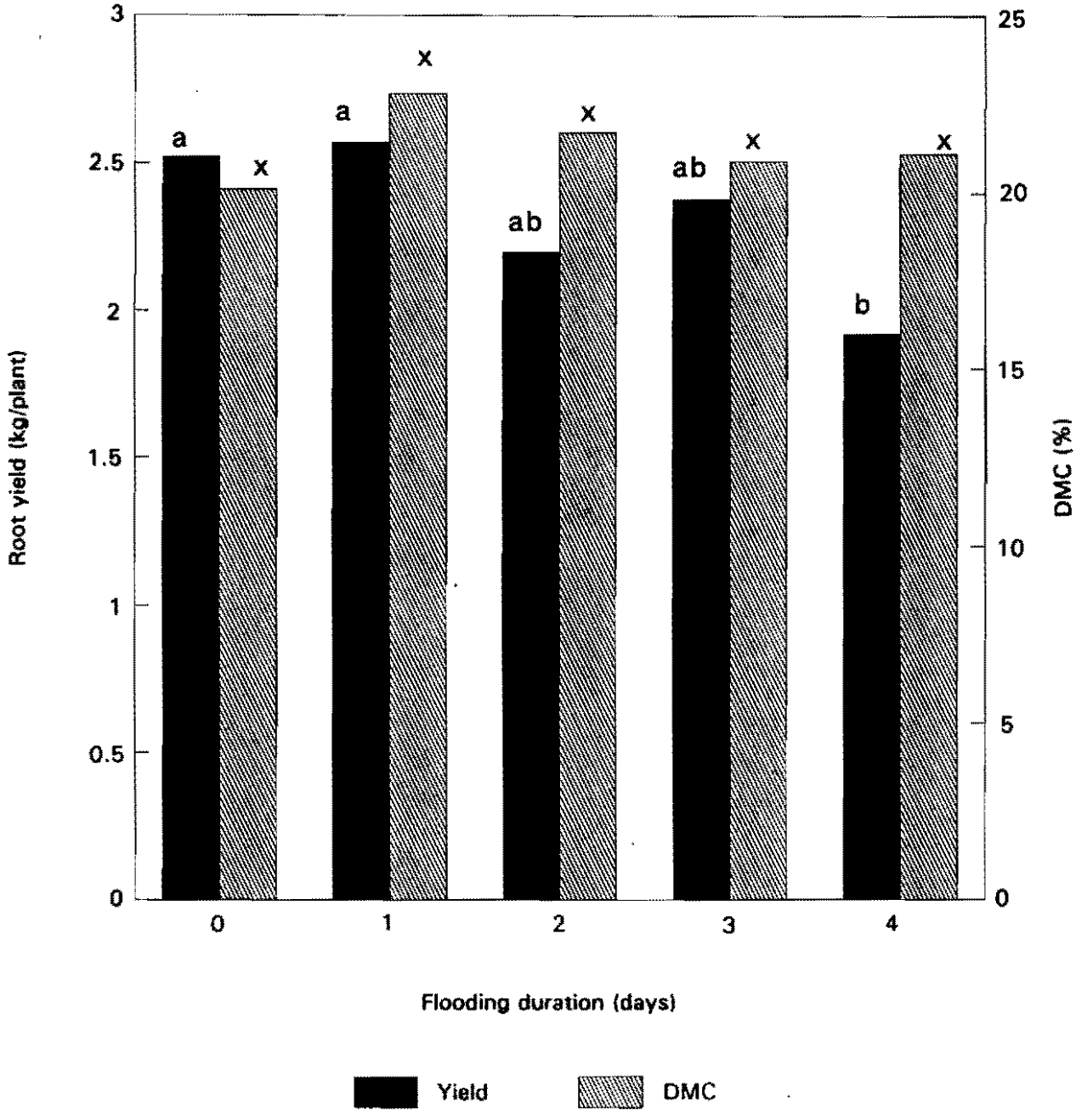

Figure 4. Effect of duration of flooding on fresh root yield and dry matter content (DMC) of cassava variety MM 92.

[Bars bearing the same letter are not significantly different from one another according to L.S.D. test at 5% level of probability]

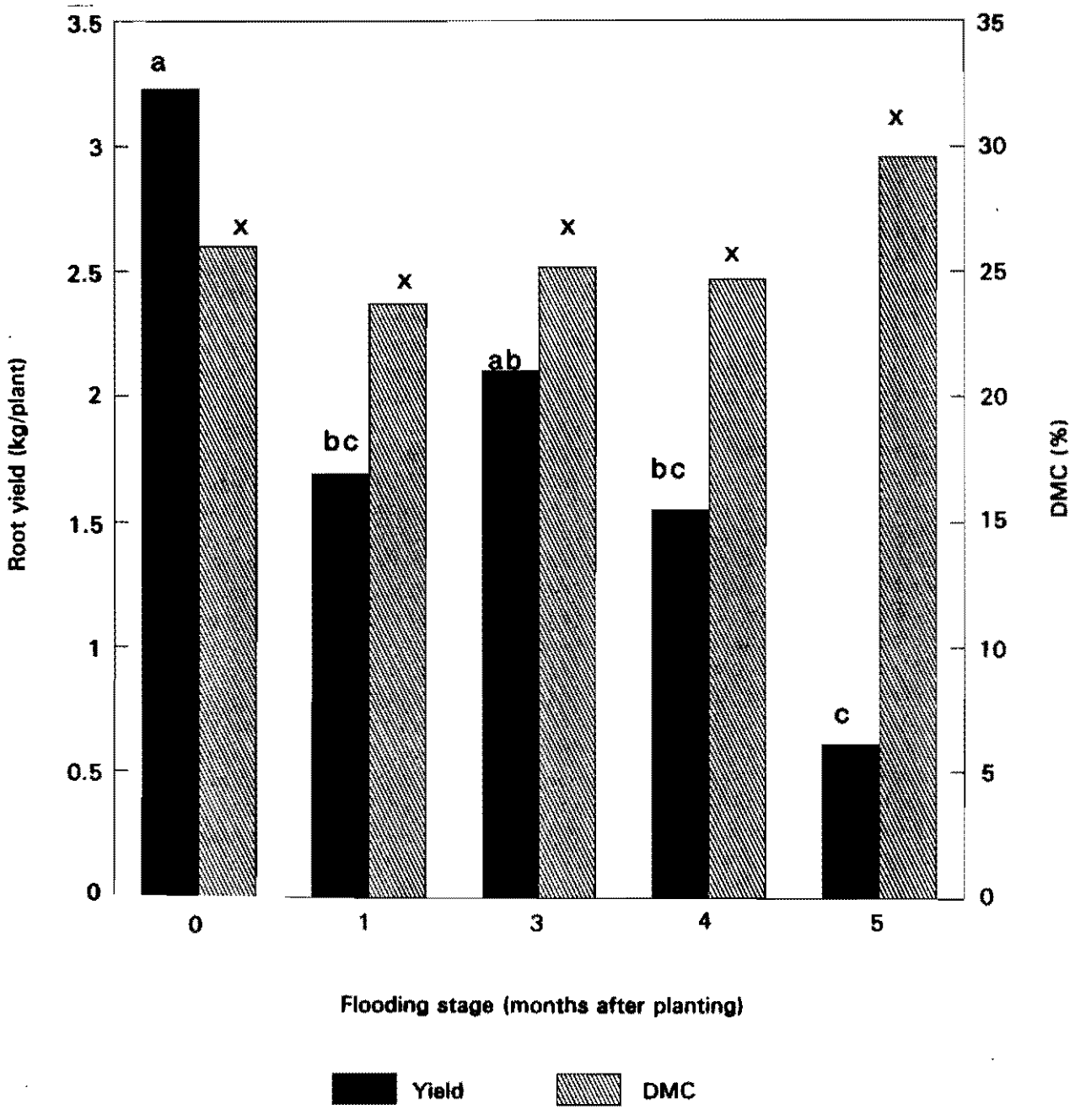

Figure 5. Effect of flooding at different stages of crop growth on fresh root yield and dry mater content (DMC) of cassava variety MM 92.

(Bars bearing the same letter are not significantly different from one another according to L.S.D. test at 5% level of probability)

A REVIEW ON PRODUCTION AGRONOMY AND CYANOGENESIS

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Cassava (*Manihot esculenta* Crantz) has been grossly underresearched and underdeveloped in terms of the use of a production agronomy approach to manage its cyanogenic potential (CNP) to safe dietary levels. Most of the preharvest management efforts to date have been on breeding for low CNP genotypes and including such low CNP genotypes in the cropping systems. The use of low CNP genotypes alone is insufficient due to the strong genotype x environment interaction effect on CNP. In a holistic approach to reduce CNP levels, agronomic management alternatives offer strengthening tools to the use of low CNP genotypes, use of processing technologies and other nutritional aspects. This paper aims to analyze the agronomic research information based on reducing CNP levels through cultural management practices and the noted complexities of cyanogenesis viz-a-viz crop growth patterns. This includes limitations on the use of agronomic approaches, and investigating the potential areas where low levels of CNP at field level can be achieved with reduced abiotic and biotic stresses. Indirectly the identification of morphological, physiological and biochemical traits associated with low CNP can enhance biotechnological approaches to CNP amelioration. Some of the preharvest agronomic practices that alter CNP levels are: planting date, with respect to cropping season and rainfall in particular; crop age at harvest; use of organic manure, soil additives and nutrients; plant density and spacing; mulching, wounding, and water management practices. Because of the perceived insect and macro-pest resistances associated with high CNP levels and drought tolerance, an educated manipulation of cultural and management practices can offer the plasticity required to use both low and high CNP genotypes in different environments. There are large variations in cassava agroecological and socio-economic factors, and a dependence on cassava as a drought-related, famine-relief food. These factors require agroecology and site-specific recommendations on the use of various management practices rather than global recommendations to change current farming practices in order to achieve safe CNP levels.

INTRODUCTION

Cassava, an important root and tuber crop in sub-Saharan Africa, is constrained from wider use, due to its cyanogenic glucosides that can be converted to the potentially toxic, hydrocyanic acid (HCN) (Rosling, 1988). All cassava tissues contain cyanogenic glucosides, and an acyanogenic genotype is yet to be found (Bokanga, n.d.). According to the Collaborative Study on Cassava in Africa (COSCA) (Nweke et al., 1994), high cyanogenic potential (CNP) is a reason for abandoning genotypes in villages that cultivate both bitter and sweet types. In low population density villages, high CNP is related to a greater frequency of abandoning

genotypes. Cassava is one of the most drought tolerant crops (Hahn, 1989). A large proportion of cassava is grown in marginal and low fertile soils, in humid to semiarid agroecozones. The relative frequency of genotypes abandoned due to high CNP is higher outside than within the humid agroecozone. High CNP is cited as a reason for abandoning genotype cultivation in Cote d'Ivoire, Kenya, Ghana, Malawi and Zambia (Nweke et al., 1994). In Zaire, where cassava processing is relatively advanced, high CNP is not cited as a reason for abandoning cassava genotypes. This suggests that for most African cassava growing countries or regions, where the processing sector is weak, high CNP is a major problem in farmers' adopting cassava varieties and for long-term use of a specific genotype.

The purpose of this paper is to review our knowledge base on production agronomy implications for cyanogenesis. It has been recognized that the greater potential for increasing cassava productivity is by improving resistance to diseases in low CNP genotypes (Hahn, 1988). Cassava is a widely adapted crop but there is a strong environmental effect on the CNP expression of individual genotypes and a weaker genotype x environment (G x E) interaction effect (TRIP, 1993a; Bokanga, 1994; Bokanga et al., 1994). Environmental factors (soil and weather conditions) during the growing season contribute to the variation in CNP among and within a genotype and in various plant parts (Bolhuis, 1954; de Bruijn, 1973). Crop management practices also have a profound effect on CNP levels. The ensuing variations in CNP due to stress factors (i.e., abiotic and biotic stresses and G x E effects of CNP) are important considerations for the widespread use of fresh cassava. For lack of a standardized methodology for CNP determination and the manner in which analytical quantities are presented, reviewing the literature on CNP is a major task. Quantitative data on CNP of various genotypes have improved since the development of an automated enzymic assay (Rao and Hahn, 1984).

At a global cassava workshop held 10 years ago, the relationship between CNP and production was summarized as follows (Delange and Ahluwalia, 1983):

> "It was pointed out that CNP content is a function of both the genetic composition and environment, and that availability of a genotype with a consistently low CNP may not be possible, owing to difficulties in ensuring constant production conditions. Furthermore, some of the research conducted in Africa indicates a positive association between insect resistance and plant CNP. This association has not yet been found in other parts of the world, and there is no clear association between CNP and disease resistance. Until the role of CNP in production is better understood, it is not possible to suggest that genetically reducing or removing CNP is the most important area of research."

At the end of the workshop, it was concluded that agricultural research should continue to select low-cyanide genotypes, bearing in mind the possible

effects of low CNP on resistance to diseases and pests, and on production. In an attempt to breed for acyanogenic genotypes, Cock (1983) mentioned the possibility of developing haploids through anther culture techniques to better expose low-cyanide genotypes, and he suggested that, when a trait needs to be deleted rather than added, a combination of anther culture and mutation breeding may be important. Some progress has been made in breeding for low CNP (TRIP, 1993a).

A holistic approach to CNP management within safe levels include preharvest (genetic and agronomic) and postharvest strategies (processing). Using low-CNP genotypes and agronomic manipulations is more effective than processing, because of toxic effects on humans. During processing and fermentation to remove CNP, several nutrients (especially vitamin C), minerals, and proteins are removed (Bokanga et al., 1988). A health hazard of cyanogenic cassava processing (i.e., gari frying), is the smoke and fumes containing hydrogen cyanide (HCN) that affect small children and women processors (Gebremeskel et al., 1989). Yet the merits of processing to reduce CNP far outweigh the defects. Postharvest strategies to manage CNP are not discussed in this paper. Using feedback from different national programs, data (including food samples from various study areas), and COSCA reports, IITA has grouped the major qualities determining consumer acceptance of cassava as follows: organoleptic properties of taste, appearance, texture (mealiness) after cooking, CNP and keeping quality/shelf life (IITA, 1992). The CNP is only one quality trait among several for genetic and agronomic manipulations.

PREHARVEST TECHNOLOGIES TO MANAGE CNP AT SAFE LEVELS

Cassava physiology and CNP

A critical look was taken at the influence of physiological age, plant organ and morphology on CNP as described below. It is suggested that CNP modulation by cultural methods is complex due to the interaction of physiological factors on cultural practices.

Crop age and CNP

Genotypes grown under similar edapho-climatic conditions differ widely in root CNP at any given age (Gómez et al., 1984). In addition to genotypic differences, other factors are noted to affect tissue CNP (Bolhuis, 1954; de Bruijn, 1971). Genotype and plant age are normally considered two important factors affecting root yield and quality (Cooke and De la Cruz, 1982). Information on this subject, even with three decades of research, is not substantial. Analysis of roots harvested at various dates showed that CNP level depended on genotype and plant age at harvest (Hernández and Guillen, 1984). Starch percentages tended to rise with increasing plant age while flavor showed no direct dependence on the CNP content. Foliage of four

genotypes was evaluated at ages 6, 8, 10 and 12 months (Gómez and Valdivieso, 1984). Leaf protein and fiber contents varied most with age and not CNP. Studies on the effect of plant age (9-12 months) on root CNP indicate that parenchyma CNP is the most stable parameter and is almost unaffected by plant age (Gómez, 1983). The CNP levels in fresh chips decreased progressively from the 9th to 12th month of age in a manner similar to the trend observed with respect to root cortex CNP (CIAT, 1981). Plant age has some effect on root cortex CNP. Distribution of CNP was measured after 2, 4, 6 and 10 months of growth in genotypes Ce22 (with high CNP and bitter roots) and M4 (with low CNP and non-bitter roots) during two consecutive seasons (Indira and Ramanujam, 1987). In Ce22, CNP increased with time in leaves and roots, and tended to increase in stems. In M4, root CNP showed a marked decrease after 6 months. The contrasting behavior of genotypes was attributed to the drought susceptibility of M4 that leads to leaf shedding with a consequent lowering of translocation of glucosides from leaves to roots. Harvesting at 6 to 9, and 10 to 12 months after planting (m.a.p.) in two genotypes showed that root CNP increased up to 8 m.a.p. while root yield and fiber content increased up to 12 m.a.p. (Ashokan et al., 1984).

Plant organs and CNP

Studies on relative CNP content in different plant parts have indicated that it is highest in root cortex and lowest in the parenchyma region (Narinesingh et al., 1988). In two genotypes, it was noted that the highest CNP was in root tissue with leaves having intermediate levels. The CNP in peel was high, but diminished with age from the 9th to 12th m.a.p.

The average leaf CNP of 4-month old genotypes (Isunkankiyan, LCN 6051 and TMS 30555), grown in plastic bags, was 57.8 mg/100g fresh weight (fw) in the range 57.4-149.7 mg/100g fw from the top third of stems, 35.4 mg/100g fw in the range 44.3-82.3 mg/100g fw from the middle third, and 75.5 mg/100g fw in the range 20.5-41.5 mg/100g fw from the lower third (Lutaladio and Wahua, 1984). High CNP of uppermost leaves was ascribed to their greater metabolic activity arising from youth and high exposure to sunlight. Leaf CNP, P and K contents followed a similar trend but Mg and Ca contents decreased acropetally. Variability between plants was greater in tall, non-branching than in branched genotypes.

Morphological and physiological traits and CNP

There is often a belief among African farmers that bitterness in cassava is associated with high yield (Mahungu, 1987). Holleman and Aten (1956) reported a positive correlation between root CNP and root yield and pointed out the difficulty in breeding cassava for low cyanide and high yield. But since then this has been

disputed, illustrating no genetic barrier for breeding clones with high yields and low CNP (Cooke et al., 1978; Sadik and Hahn, 1973; Mahungu, 1987; TRIP, 1993a and 1993b).

Several studies have also indicated significant associations between morphophysiological traits and CNP (Table 1). Local genotypes from the southern humid forest zone of Nigeria are usually nonbranching with totally pinkish petioles. All indigenous sweet varieties are characterized by pinkish rind, distinguishing them from the milk-colored rind of bitter roots (Okeke and Oti, 1989). In most African countries, sweet genotypes and those with light yellowish and red petiole leaves are preferred, in the belief that such leaves require less palm oil; the preference may also be based on custom and experience. Ene et al. (1989) reported a high correlation between rind color and CNP where pink roots were low in CNP. Indigenous low-cyanide genotypes belong to three morphological types: short and sparsely branching types, with buff-colored stems, common in the northern savanna; medium to tall types (90-100 cm) with few or no branches, pink at both ends of the petiole and common in the middle belt of Nigeria; and tall (2m), usually non-branching type with completely pink petioles, found in the rain forest zone of southern Nigeria (Oti and Ene, 1992). Peel of roots of all indigenous low-CNP genotypes is pinkish and peel of high-CNP genotypes, cream-colored. But characterization of large cassava germplasm collections at IITA show that such correlations are dependent on the origin and composition of the genotypes tested (Table 1; TRIP, 1993a). Among the IITA elites, several clones with white peel and low CNP exist (Almazan, 1989).

Crop ecology, abiotic and biotic stresses and CNP

Root CNP is a highly variable trait, subject to environmental influence (Jones, 1959). Drier soils appear to favor high CNP levels. The first mention of G x E effects of CNP was observed by de Bruijn (1971):

> "that different genotypes do not react in the same way to changing ecological conditions with regard to CNP content."

Dufour (1988) reported that 13 "bitter varieties" commonly grown by Colombian indigenous people had significantly higher CNP levels than reported for the same genotypes in other areas. Yellow-fleshed cassava varieties were found to have more free CNP than white-fleshed varieties in that region.

Water stress and CNP

Water stress effects that enhance root CNP are frequently reported (de Bruijin, 1973; CIAT, 1991; Bokanga et al., 1994), but little is known on its physiological

role in synthesizing, translocating and depositing CNP. Water stress increased total cyanide in a local and improved genotype (Bokanga et al., 1994).

The ability of cassava genotypes to maintain low CNP under stress is of paramount importance, particularly in areas where fresh cassava is used for human consumption, such as the drought-prone areas of sub-Saharan Africa and Southeastern Brazil. Such low-CNP genotypes have been identified (Bokanga, 1994; CIAT, 1991). Growth stages at which water stress occurs, appear to have an effect on the CNP production (Bokanga et al., 1994). Due to this differential sensitivity, manipulation of planting date appears to be useful in maintaining low CNP. In a field trial conducted at Ibadan (humid forest/moist savanna transition zone), in which the planting time ranged from May to September at monthly intervals, CNP was shown to vary 2-fold with planting date (Kayode, 1983). A positive and significant correlation between root yield and CNP, and between CNP and starch content was noted, irrespective of the planting date. Genotypes with low CNP (CM1335-4 and CM9222-2) when stressed at mid season (100-183 days after planting) at Quillichao, Colombia, continued to have low CNP (123-190 ppm on dry basis) at harvest (CIAT, 1991).

Soil nutrition traits and CNP

In general, nitrogen fertilizers increase yields and CNP. Applying nitrogenous fertilizers as a foliar spray is shown to reduce root CNP to some extent (Sinha, 1969). Nitrogen fertilization significantly raised the CNP levels in leaves and storage roots of four cassava genotypes—TMS 30001, TMS 30211, TMS 30395 and TMS 30572 (Obigbesan, 1984). Increased CNP in roots with farm yard manure application have been recorded (Kurian et al., 1975). Long term effect (10-year average) on CNP for N fertilizer and farmyard manure (FYM) was positive (Kabeerathumma et al., 1988). The FYM and N alone, or in combination, tended to increase root CNP. A recent study has confirmed that applying nitrogenous fertilizers increases CNP while K fertilizer decreases CNP (Wheeler and Dahniya, 1994).

Cassava is able to extract sufficient nutrients for root production from soils that are very infertile (Van der Zaag et al., 1979). Some varieties have been shown to produce high yields when the soil P is as low as 0.025 ppm or 0.002 ppm in solutions. It was shown that by forming mycorrhizal associations, cassava can effectively grow in such low P soils and increase the absorption of P, K, S and, to a lesser extent, Zn.

Early literature tends to suggest that applying fertilizer, K fertilizer in particular, influences root quality, via an increase in dry matter and starch content and a decrease in CNP (Howeler, 1985). Low soil K increases CNP but P, Mg and Ca produce conflicting results. A poor soil will be low in K and therefore will increase CNP in cassava. A marked decrease in CNP from 270 to 160 mg kg^{-1} in

the fresh tuberous roots was noted with the application of 112 kg K Ha^{-1} (Obigbesan, 1973). But field trials conducted later showed no significant effect of K on CNP of tuberous roots (Obigbesan, 1977). Studies done in India (CTCRI, 1975) report a marked decrease in CNP of tuberous roots with the application of K fertilizer. Field studies have also shown that adding wood ash (rich in potash) lowers the CNP of tuberous roots (Indira, 1979). It has been shown that the physiological age of the tissue and plant organ used has a marked effect on the critical K concentration (Bates, 1971; Spear et al., 1979). Ash or K alone or in combination was shown to decrease CNP (Kabeerathumma et al., 1988). Obigbesan (1973) and Nair et al. (1980) reported the effect of K on the reduction of CNP. Bolhius (1954) also found that K fertilizer increased the linamarin content of cassava roots. Several other studies indicate a reduction in CNP by fertilization of K (Ramanathan et al., 1980; Pushpadasa and Aiyer, 1976; Ashokan and Sreedharan, 1977; Normanha, 1969).

Biotic stresses and CNP

Several associations have been reported between cassava pests and foliar diseases and CNP. Cassava CNP gives it relative immunity from most insect pests (Wood, 1966). A positive correlation was noted between leaf CNP and density of infestation by *M. tanajoa* in 23 cassava genotypes (Yen et al., 1984). The CNP of leaves, stems, and roots of mite- and mealy bug-infested plants of five genotypes were high relative to control plants sprayed with monocrotophos acaricide (Ayanru and Sharma, 1984). Overall mean total CNP of entire plants, sprayed and unsprayed, were similar, but considerable differences were noted in individual tissues. Resistance to *Meloidogyne incognita* and *M. javanica* was not related to CNP in plant parts of 11 genotypes according to a laboratory study (Freitas and Moura, 1986). Intercropping cassava and sun hemp was noted to be the most efficient mode of control of *Cyrtomenus bergi* in both low- and high-CNP genotypes (Castaño et al., 1987). Lal and Maini (1977) noted an association between rat damage and CNP. Akoroda et al., (1994) noted that the desired cassava clones should have high yields of good quality but must be unpalatable to wild animals and also deter pests for most of the over 18 months of crop growth in fields. It was suggested that clones with high root CNP (19-25 mg HCN/100g of fresh root) be introduced, which will not be dangerous to humans after processing. Amadou (1988) noted that low CNP and sweet cassava clones accepted by farmers in on-farm trials (clones 658, 228 and 844) could not be widely extended due to greater susceptibility to animal damage.

Conflicting associations between cassava diseases and CNP is shown in Table 1. In the early 80s the association between CNP and mosaic (CMD) in leaves was shown and leaves with more chlorotic spots due to CMD were lower in CNP (Mahungu and Hahn, 1981). This was postulated to be one reason why most Africans who consume cassava leaves as a vegetable prefer leaves affected by CMD (Hahn, 1983) which are claimed to be sweet leaves.

Locational effects and CNP

Performance of genotypes from one location to another can alter CNP due to differences in climatic and soil characteristics. Data have shown that G x E interactions exist for dry cassava yield and its components (TRIP, 1993a) and root CNP (Bokanga et al., 1994). Stability characteristics of CNP across locations are being investigated at IITA. Field trials were conducted in six locations, representing humid forest zone (Onne and Uyo), humid forest and southern Guinea savanna transition zone (Ibadan and Ubiaja) and southern Guinea savanna zone (Ilorin and Mokwa) of Nigeria, and in Ghana, using ten genotypes (Bokanga et al., 1994). Genotype and location (L) effects for root CNP at 12 months after planting were significant, whereas the G x L effect was not. In each location, ranking of the genotypes from the lowest CNP to the highest was fairly well conserved (TRIP, 1993b). To test for a possible dilution effect of the concentration of cyanogenic glucosides per unit dry weight, total CNP yield was calculated. It had the same trend as CNP. In one location, genotype TME1 (with lowest CNP), was also the highest yielder, contradicting the opinion that only high-CNP genotypes can produce high yields. Lowest CNP levels were in Onne (the high rainfall site) while the highest CNP was in Ubiaja (in transition zone). A low-CNP genotype, TME1, recorded higher levels of CNP in drier sites compared to the high rainfall sites (Bokanga et al., 1994). In another field trial for 19 genotypes, the previous season's findings, that there were wide variations in CNP due to locational effects whereas G x L interaction effects were not significant, was confirmed. Moreover, a year-year effect was also noted. CNP levels averaged over two seasons indicated that CNP were higher in Onne than in Ilorin (a low rainfall site). Onne soils are acidic but high in P and a soil nutrient stress effect combined with excess soil humidity conditions created by continuous rains and lowered drainage may have contributed to plant stress and CNP.

EFFECTS OF CULTURAL AND MANAGEMENT PRACTICES ON CNP

Depending on abiotic stresses experienced by cassava in a given environment, cultural practices, such as date of planting in relation to the rainfall distribution, irrigation, mulching, and use of fertilizers, may be used to reduce root CNP levels of low-CNP genotypes. Other cultural practices, such as stem-bark ringing and stem pruning 1 week before harvest, have been suggested as effective in lowering CNP (Ramanujam and Indira, 1984). Mukibat cassava (a graft or bud of a scion of *M. glaziovii* on to a stock of *M. esculenta*) has relatively higher CNP than conventional cassava (Guritmo et al., 1984). CNP level of Mukibat cassava varies depending on factors like fertilizer use, genotype, density, and spacing. Nitrogen fertilizers increased CNP while P and K applications tended to reduce CNP in Mukibat cassava. Wider spacing appears to increase CNP. An interesting question is whether, and at what cost, a high CNP genotype can be made to express a low CNP level, by growing it under conditions unfavorable to high CNP expression (Bokanga et al., 1994).

Wounding

Mechanical root injury may influence CNP. When roots were cut into blocks and incubated under laboratory conditions, blocks showed more widespread and even symptoms of physiological deterioration than those under natural conditions, while total cyanide content was greatest in the cortex and decreased markedly towards the center of the root (Kojima et al., 1983). The CNP increased in all tissues after 3-day incubation, the increase being greatest in white parenchymal tissue next to cortex and in which no physiological deterioration symptoms appeared. Linamarase activity showed a radial gradient similar to that of total cyanide and deceased in all tissues after 3-day incubation, showing a clear reciprocal relationship with changes in total cyanide. Free cyanide constituted a very small portion of total cyanide and did not change markedly.

Planting season and CNP

Seasonal changes in root CNP were noted on ten clones, at 3-month intervals, for first and second rainy season planting in Ibadan (Bokanga et al., 1994). High CNP level at 4 m.a.p. dropped twofold at 9 m.a.p., after a short, dry season at 7-8 m.a.p. After a lag period, cyanogenic yield increased steadily throughout the growth cycle. During the dry period (9-12 m.a.p.), CNP levels were maintained. During regrowth period, CNP increased and then dropped with second-year rains. High CNP levels coincided with the vegetative growth phases: first, or regrowth, when leaf formation was high and plants had a high leaf area index (LAI); as the tuberous root growth became the dominant sink (Ekanayake, 1993). The CNP levels had a parallel course of increment to plant growth pattern, determined mostly by rainfall. Unlike the typical response to drought stress, seasonality in CNP was dependent on flow of assimilates to roots; a trend which was supported by the starch yield (Bokanga et al., 1994).

Irrigation and mulching on CNP

Irrigation mitigating drought stress was shown to influence CNP. Water stress occurred 3-m.a.p. and was negated by irrigation in control plots. The CNP for 10 genotypes on fresh weight basis was significantly higher in nonirrigated plots than in irrigated (Bokanga et al., 1994). Differences between irrigated and nonirrigated treatments for total CNP yield were significant, except for three genotypes, while a fourth had a reversed effect. Subsequent testing using water table depth to create water-deficit stress in a Sudan savanna site indicated a reversal in CNP trend for the same genotypes (Bokanga et al., 1994), while a repeat trial supported the first year results. Some G x E effects were also observed (Ekanayake et al., unpublished data, 1994). Under transient water table conditions, at a hydromorphic site, valley slopes significantly influenced CNP levels; extremes

of water stress, too wet and too dry conditions led to high CNP (Bokanga et al., 1994), CNP levels were reduced partly by mulching and mid-season irrigation. The CNP was reduced by mulching which is presumed to enhance early vigor by reduced water stress and soil temperatures but not with irrigation under hydromorphic conditions.

CONCLUSIONS

An earlier paper dealt with the need to use low CNP genotypes, the use of environments unsuitable for the expression of high CNP, and the presence of G x E interactions on CNP (Bokanga et al., 1994). Main areas of analysis in this review indicate the differential performance of cassava genotypes under various cultural management practices and in stressed environments due to soil factors, moisture regimes or agronomy and biotic factors. The effect of location as dominated by abiotic stresses, G x E effects on CNP yields, genotypic variations in CNP, and seasonal differences modifying CNP were highly evident. Use of cultural management modifying CNP, though observed in several studies, need more confirmatory analysis in terms of their farmer and end-user adoption and socio-economic factors. Also, since cassava is cultivated both as a monocrop and an intercrop, G x cropping systems effect on CNP has to be analyzed. For most cassava farmers, cultural practices such as irrigation, fertilization, and mulching, may not be useful. However, combinations of low-CNP genotypes and cultural practices can offer short-term alleviation of cassava's high CNP problem, while the usefulness of improved processing technologies cannot be underestimated.

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Table 1. List of published associations between tuberous root cyanogenic potential (CNP) and morphological traits, physiological traits, root quality traits, disease and pest ratings, yield and yield components.

Trait	Population characteristics	Type of relationship	References
Morphological traits			
Stem color	107 genotypes	NS	Rogers, 1965
Leaf color	101 genotypes; 3 seasons	P=0.01	Dixon et al., unpub.
Petiole color	101 genotypes; 3 seasons	P=0.05	Dixon et al., unpub.
Yield and yield components			
Root yield	67 genotypes	P=0.05	de Bruijn, 1973
	45 genotypes	P=0.05	Mahungu, 1994
	4 populations of >20 genotypes	NS	Mahungu, 1994
	10 genotypes; 4 sites	P=0.05	Dixon et al., unpub.
Root number	42 genotypes	P=0.05	Mahungu, 1994
	25 genotypes	P=0.05	Mahungu, 1994
	3 populations of >20 genotypes	NS	Mahungu, 1994
	10 genotypes; 4 sites	NS	Dixon et al., unpub.
Root size	5 populations of >20 genotypes	NS	Mahungu, 1994
Root quality traits			
Dry matter (%)	67 genotypes	P=0.01	De Bruijn, 1973
	35 genotypes	P=0.05	Mahungu, 1994
	4 populations of >20 genotypes	NS	Mahungu, 1994
Root starch (%)	5 populations of >20 genotypes	NS	Mahungu, 1994
Root color	101 genotypes; 3 seasons	P=0.05	Dixon et al., unpub.
Taste	101 genotypes; 3 seasons	P=0.05	Dixon et al., unpub.
Poundability	101 genotypes; 3 seasons	P=0.001	Dixon et al., unpub.
Biochemical traits			
Leaf CNP	26 genotypes	P=0.01	Moh, 1976b
	2 populations of 70 and 14 genotypes	NS	Bokanga, 1994a
	108 genotypes	P=0.05	Cooke et al., 1978
	4 populations	P=0.05	Mahungu et al., 1992
	2 populations	NS	Mahungu et al., 1992
	1 population	P=0.05	Hahn, 1988
Leaf sugar	–	NS	Hahn et al., 1987
Diseases			
Mosaic	5 populations of >20 genotypes	NS	Mahungu, 1994
:severity	101 genotypes; 3 seasons	P=0.001	Dixon et al., unpub.
:incidence	101 genotypes; 3 seasons	P=0.001	Dixon et al., unpub.
Bacterial blight	5 populations of >20 genotypes	NS	Mahungu, 1994
Leaf CNP and diseases			
Mosaic	35 genotypes	P=0.01	Mahungu et al., 1992
	5 populations of >20 genotypes	NS	Mahungu et al., 1992
Bacterial blight	25 genotypes	P=0.01	Mahungu et al., 1992
	5 populations of >20 genotypes	NS	Mahungu et al., 1992

SPECIAL REPORT
CASSAVA SAFETY: LESSONS FROM AN INTERDISCIPLINARY WORKSHOP

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The International Workshop on Cassava Safety was held at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, March 1-4, 1994, to review the state of knowledge on issues of cyanide in cassava. The meeting resolved that the biochemical pathway for the synthesis of cyanogenic glucosides in cassava was fairly well understood, but that the physiological processes controlling their accumulation in various tissues were still unknown. Effective processing has been recognized as the most efficient way of controlling cyanogenesis in the short term. The dynamics of cyanogen removal from cassava and the factors controlling it have been elucidated. These advances were made possible by the development of new analytical methods for determining cyanogenic glucosides and their breakdown products. Advances in the molecular biology of cyanogenesis, combined with conventional plant breeding, now make it possible to develop powerful approaches to optimize the levels and distribution of cyanogenic glucosides in cassava. Agronomic research has shown that environmental factors can be as important as genetic factors in determining the levels of cyanogenic glucosides in cassava roots. The understanding of causal relationships between cassava cyanogenesis and associated human diseases has improved. Of special importance was the identification of similar underlying causes of the reported outbreaks of paralytic diseases and acute poisoning attributed to cyanogens in cassava. These outbreaks occurred in socio-economically deprived communities that relied on cassava for food security and which, as a result of food shortage, war or poverty, made shortcuts in their traditional processing methods. New socioeconomic findings emphasize the importance of cassava processing—not only for the sake of safety but also for expanding cassava production by improving shelf life, facilitating transport and introducing consumer-specific taste and texture in cassava products.

INTRODUCTION

For thousands of years, people living in the Amazon Basin have been consuming cassava (*Manihot esculenta* Crantz) despite the potential toxicity of this crop. Since its introduction to Africa about 400 years ago, cassava has spread considerably and is now established as one of the most important crop between the tropics in Africa. It is consumed to varying degrees from Senegal to Mozambique and from Ethiopia to Angola.

The importance of cassava as a staple crop is now well established. In the tropical world, cassava ranks fourth in importance after rice, wheat and maize. The presence of varying amounts of cyanogenic glucosides and their breakdown products (cyanohydrins and hydrogen cyanide), in cassava food products has been a cause of concern because of their possible effects on health. The relationship between cassava consumption and health—particularly the thyroid function—was reviewed in two workshops sponsored by the International Development Research Centre, Ottawa, Canada, in 1973 (Nestel and MacIntyre, 1973) and in 1982 (Delange and Ahluwalia, 1983).

Since the last workshop, many advances have been made in the understanding of cyanogen removal during processing and in improving analytical methods; in understanding the causal relationship between cassava cyanogenesis and human diseases, especially the understanding of the factors underlying toxic effects; in elucidating the genetic basis of the synthesis of cyanogenic glucosides; and in understanding the socioeconomic mechanisms influencing cassava production.

There are several reasons why a new workshop was needed at this time:

- The increased importance of cassava in agricultural and economic development, as well as in food security, particularly in Africa.

- Reports of outbreaks of a new paralytic disease, konzo, and of acute poisoning, both of which have been attributed to cyanide exposure from insufficiently processed cassava roots.

- The need to understand the biological and social role of cyanogenesis in cassava and to consider how recent advances in various scientific disciplines could be used to expand cassava production.

- The need for a better understanding of safety issues when developing and promoting new cassava cultivars in communities under economic or ecological stress.

The Working Group on Cassava Safety (WOCAS) was formed under the umbrella of the International Society for Tropical Root Crops (ISTRC), during a meeting of the Society's Africa Branch in Kampala, Uganda in 1992. The aims of WOCAS are to provide recommendations for promoting safe cassava based on current knowledge, to identify research needs and develop research strategies, to identify people working in this field and facilitate exchange of information and experiences. Considering that reported toxic effects of cassava are relatively rare in relation to its wide use as a staple, we decided that cassava safety was a better working concept than toxicity; hence the name of the working group and of the workshop. The first activity of WOCAS was the organization of a workshop, the proceedings of which represent the first output.

The workshop aimed to take stock of the present state of knowledge on safety issues related to cyanogenesis in cassava and to disseminate this information more widely among researchers in the field. It was organized around seven main themes: biology of cyanogenesis; analytical methods; agronomic research; cassava processing and cyanogen removal; livestock feeds; human health and nutrition; socioeconomic aspects. Leading researchers in each field were invited to prepare discussion papers. The session chairperson and speakers were responsibility summarizing the major findings of their respective session and formulate any consensus and recommendations that emerged during the debates. These summaries and recommendations were debated in a final plenary session. After the workshop, WOCAS members met to crystallize the ideas that emanated from the final plenary session into the set of recommendations reproduced here.

WORKSHOP SUMMARY AND RECOMMENDATIONS

Biology of cyanogenesis

1. Conventional plant breeding for cassava cultivars, with low or high cyanogenic potential, that satisfy user needs and preferences in diverse socioeconomic and agroecological conditions can be effectively complemented with biotechnological approaches. Current knowledge on synthesis, degradation, transport and regulation of cyanogenic glucosides in cassava provides the possibility of developing new approaches and tools for optimizing the content and distribution of cyanogenic glucosides and associated enzymes in cassava.

2. Three genes coding for key enzymes controlling the biosynthesis and degradation of cyanogenic glucosides have already been isolated and cloned: The gene for cytochrome P450, the enzyme catalyzing the rate-limiting conversion of the parent amino acid to the corresponding oxime in the biosynthetic pathway, the gene for linamarase, which catalyzes the degradation of linamarin to acetone cyanohydrin; and the gene for hydroxynitrile lyase, which catalyzes the degradation of acetone cyanohydrin to hydrogen cyanide and acetone. Important genes that have yet to be isolated include those coding for glucosyltransferase, which converts linamarin to its transport form linustatin; simultaneous diglucosidase, which would split linustatin in the first step of its metabolism to a noncyanogenic compound; and root- and leaf-specific promoters effective in cassava.

3. An efficient transformation system for cassava is urgently needed in order to introduce already available relevant genes. Progress in cassava transformation and regeneration (not reviewed at this meeting) appears likely to permit transgenic cassava plants, using currently and shortly available genes, to be ready for controlled testing within 2-3 years.

4. The presently observed demand among cassava farmers, processors and consumers for cassava cultivars with a high content of cyanogenic glucosides and/or bitter taste may reflect a tight genetic coupling of the cyanogenic character and bitterness to other beneficial characteristics. Despite this tight coupling, these associations may be broken by continued traditional breeding using better analytical methods (e.g., antibodies and cDNA probes) enabling the desired cultivars to be selected with a higher degree of accuracy.

5. The control of cyanogenesis could be achieved by:

   - Transforming cassava with the introduction of an anti-sense construct of cytochrome P450 under the control of a strong constitutive promoter to produce acyanogenic plants

   - Inserting tissue-specific promoters or developmentally controlled promoters in front of the cytochrome P450 gene to limit production of linamarin to certain tissues and at specific periods of plant growth

   - Introducing a strong promoter in front of the linamarase gene to increase the breakdown of linamarin during processing

   - Preventing linamarin conversion to the transport metabolite linustatin

   - Increasing the conversion of linustatin to asparagine instead of its conversion back to linamarin. This last approach may result in the accumulation of protein nitrogen in the roots.

The plants so obtained would constitute ideal research material for specifically testing the relationship, if any, between cyanogenic glucoside content and desired properties such as starch quality, insect resistance, bitterness and others.

6. Plant breeding can be used to transfer the desired cyanogen metabolism into appropriate cultivars. When genetic transformation of cassava has been extended to a wider range of genotypes, it may be possible to confer the desired cyanogenesis phenotype on cultivars improved for quantitative traits (i.e., for those more difficult to define genetically), by using transformation of elite selections. This option would relieve the plant breeder of a set of selection objectives for cyanogenesis and thereby permit faster progress for quantitative traits such as yield and environmental adaptation.

7. Molecular biology research may produce experimental data within 5 years to answer some of the questions that cannot be answered otherwise; however, results will not likely be transferable to cassava farmers in the next 10 years. It is important therefore that these efforts be combined with continued and

enlarged efforts to expand our knowledge of effective and practical processing techniques to reduce cyanogen levels in cassava products.

8. In the long term, molecular biology can offer more than mechanisms for removal of cyanogenic glucosides. Combined with plant breeding and other disciplines, it provides a new, potentially powerful approach. It can circumvent the loss of any desirable functions that may be found to be associated with cyanogens by introducing nutritionally less problematic factors including plant protective agents and quality factors.

9. Similar to the large resources being spent for research on temperate crops by industrialized countries, it is imperative to strengthen basic research on cassava with respect to nutritive value, productivity, tolerance to biotic and abiotic stresses, etc. if this crop is to provide an economic as well as food security resource to tropical countries.

Analytical methods

1. There is extensive variation in the levels of cyanogenic glucosides and linamarase activity within the same tissue of a cassava plant. In addition, variations occur among cultivars, and also among different plants of the same cultivar in apparently similar environments, and among plants from the same cultivar in different environments. Sampling procedures are therefore critical for statistically valid analysis of results, no matter which chemical methods are used. There is a need to standardize sampling protocols, depending on the kind of the material and the purpose of the measurements. Investigators should be aware that handling and storage of the fresh and processed cassava collected for analysis, as well as extracts obtained from these, must be standardized and validated as considerable losses may occur. Simple, mobile equipment for homogenization and extraction needs to be developed.

2. Linamarin and, to a lesser extent, lotaustralin are the cyanogenic glucosides found in the cassava plant. It is only when the tissue is damaged—mainly by mechanical or microbial action—that the cyanogenic glucosides can decompose to cyanohydrins, which may further hydrolyze to hydrogen cyanide. At harvest intact cassava tissues contain only cyanogenic glucosides; however, processed products may contain varying amounts of cyanogenic glucosides, cyanohydrins and hydrogen cyanide. The development of analytical methods for separate determination of these three types of cyanogenic compounds have advanced the understanding of the dynamics of cyanogen removal during processing. Simplistic reference to cyanide or total cyanide content in cassava continues to hamper this understanding and should be avoided.

3. Different chemical assay methods are needed as no single technique serves all requirements. The method should be chosen depending on resources available and the objectives of the analysis. Many developing countries laboratorieswith limited resources require robust, low-cost and simple methodologies. Simple, specific and relatively sensitive methods for use in field surveys are much needed. Of importance for both qualitative and quantitative techniques is reproducibility. Particularly important in enzyme-mediated methods is the standardization of pH and thus the use of effective buffer systems.

4. Autolytic methods, which rely on an endogenous enzyme for glucoside hydrolysis, are unreliable for processed products in which the endogenous enzyme may have been inactivated. The use of exogenous linamarase is recommended but is currently constrained by high costs. One alternative is to produce crude linamarase preparations from cassava leaves or root cortex. Alternative sources of low-cost but effective enzymes should be explored. The immobilization of linamarase to allow its repeated use provides an alternative approach.

5. HPLC techniques allow the separate measurement of cyanogenic glucosides and cyanohydrins, but are costly and complicated. Potentiometric methods using cyanide electrodes have limited sensitivity and reproducibility.

6. The safe handling of reagents use to estimate cyanogens needs to be taken into consideration. The pyrazalone/pyridine reagent used as a color reagent is a highly toxic and volatile. Its use requires appropriate safety equipment, which may not be readily available in developingcountries. The reagent has to be prepared daily. The use of isonicotinate and 1,3-dimethyl barbiturate as a color reagent is a better alternative. Picrate and tetrabase can be used in qualitative and semiquantitative assays although their potential health hazard should be investigated.

7. Interfering compounds occur in samples rich in oils, fats, proteins and phenolic compounds, resulting in significant problems in extraction, recovery and colorimetric estimation. The importance of estimations in the prepared foods for human consumption (which may contain high levels of added oils and proteins) makes it necessary to solve this problem.

Agronomic research

1. Cassava cultivars show a very wide range of cyanogenic glucosides levels in the storage roots. New findings in the health and food sciences now call for a revision of the safe levels established more than 40 years ago.

2. Cyanogenic potential is a heritable trait. The polygenic and recessive nature of its inheritance, coupled with inadequate sampling strategies, has slowed progress in conventional breeding for this trait. Recent improvements in sampling methodology will facilitate more rapid progress. There is a very large environmental influence on the expression of cyanogenic potential in cassava. A thorough review of existing knowledge and a focus on identifying genotypes with greater stability over environments and at key developmental stages are essential. The contribution of field cultural practices in modulating cyanogenesis also needs to be addressed.

3. There is preference for bitter or potentially toxic cultivars in some cassava-growing communities. Observational studies indicate that bitterness or toxicity may play an important role in the prevention of damage to the crop by animals that feed on the roots. Current evidence suggests a good correlation between cyanogenic potential and bitterness; however, some cultivars with extreme expression of either trait do not follow the general trend. The compound responsible for bitterness needs to be identified and the reasons for preference of bitter or toxic cultivars in some farming communities needs to be established in order to determine the value of developing cultivars with a low cyanogenic potential and a bitter taste should the latter turn out to be the factor deterring animals from feeding on the crop.

4. It remains to be established whether acyanogenesis is a viable option for addressing cassava safety. A recent study has suggested a role for cyanogenic glucosides in resistance to pests; however, further investigation should be conducted. The possibility of partitioning the cyanogenic glucosides into nonedible plant parts while maintaining pest resistance needs to be explored.

5. Available data on relationships between cyanogenic potential and morphological/agronomic traits show many inconsistencies. Molecular markers to be used for detecting genotypes for low or high cyanogenic potential are needed to accelerate breeding efforts in this area.

Cassava processing

1. Cassava processing can reduce the cyanogenic content of roots and leaves of even the most potentially toxic cultivars to safe levels. There is, however, a myriad of processing methods, but they are not equally effective in achieving cyanogen reduction. The effectiveness of several processing techniques needs to be verified for different cassava cultivars.

2. Most of the principles of cyanogen removal during processing are now understood. Our current knowledge indicate that plant cells contain cyanogenic glucosides, mainly linamarin. Disintegration of the cells brings linamarin into contact with the endogenous enzyme linamarase, resulting in hydrolysis of linamarin into glucose and acetone cyanohydrin. Acetone cyanohydrin breaks down into acetone and hydrogen cyanide through the action of the enzyme hydroxynitrile-lyase or spontaneously at increased rates at higher pH's. The latter pathway appears to be the principal one. The volatile HCN (boiling point 25.7 °C) can escape into the air.

3. Effective cyanogen reduction is achieved in two steps: First, the hydrolysis of glucosides is facilitated through disintegration of cells, which is achieved by grating, crushing, microbial fermentation, enzymic action or a combination of these. In the second step, high pH facilitates the spontaneous breakdown of cyanohydrin. Higher temperatures achieved through heating and reducing moisture contents during drying also influence this effect. The factors determining cyanohydrin stability require further clarification.

4. Processing methods that involve effective disintegration followed by heating or drying result in the highest removal of cyanogens. Examples include mechanical grating followed by roasting in the production of *gari* and *farinha*, and microbial fermentation followed by drying or steaming as in the production of *lafun* and *chickwangue*. Incomplete disintegration will result in residual cyanogens, particularly linamarin; and incomplete drying or heating may result in residual cyanohydrin. Whether reduced linamarase activity may be a limiting factor in the removal of cyanogens in some cultivars is not known. Similarly, the role played by hydroxynitrile lyase requires further elucidation.

5. Direct sun drying of whole fresh roots achieves only partial removal of glucosides. Slower drying extends the effect of linamarase activity but simultaneously allows microbial growth. Chipping of fresh roots involving extensive mechanical tissue damage will facilitate glucoside breakdown, but slicing of roots with minimal tissue damage followed by rapid drying will result in a high retention of glucosides. In sundried cassava pieces, an inverse relationship seems to exist between cyanogen and microbial content. Possibilities for optimizing cyanogen removal while minimizing microbial contamination during sun-drying should be further explored. The issue of mycotoxin contamination in sun-dried cassava, as well as in cassava molded on purpose needs to be addressed. Studies have identified mycotoxins in some sun-dried root products, but so far not in purposely molded cassava.

6. Ineffective processing methods found in some communities can lead to cassava products with high residual cyanogen levels. Appropriate improvements to these methods may reduce levels to within safe limits. It

is recognized that in communities where intoxications due to cassava consumption occur, this minimalist intervention represents a powerful approach to solving the problem.

7. As commercial cassava processing intensifies and the scale of cassava operations increases, safety issues become of critical importance. For example, inhalation of hydrogen cyanide vapors from roasting cassava and other occupational hazards should be minimized by good ventilation and accident prevention. Disposal of effluents from cassava processing is expected to become an increasing problem. Additional problems may arise from high biological oxygen demands (BOD) rather than from cyanogens due to high solid contents in the effluents.

8. There is a lack of detailed knowledge about why people process cassava the way they do and the factors leading to this. Recommendations regarding processing should therefore take into account the quality characteristics of the raw materials and the end products as they relate to the wider socioeconomic and cultural environment. The relationship between sensory characteristics (i.e., bitterness), and cyanogenic content as they relate to cassava processing needs further elucidation.

Cassava in livestock production

1. Effective processing techniques for removal of cyanogens exist for preparing of dried cassava chips for animal feeding. A total cyanogen level of less than 100 mg HCN equivalents/kg dry cassava for inclusion in balanced compounded animal feed is economically acceptable in intensive livestock production systems.

2. It has been established that cyanogens in feeds can increase the requirement for sulfur compounds, iodine, zinc, copper and selenium. Optimal levels of these compounds per unit cyanogen need to be determined for various livestock species.

3. Sporadic deaths attributed to cyanogens in cassava have been reported in various livestock production systems. Cassava roots, leaves and wastes are often used as components of livestock feed in rural farming communities. It is therefore necessary to substantiate these claims and develop safe handling strategies to incorporate cassava into livestock feed, particularly in smallholder livestock systems.

4. Problems of cassava toxicity in livestock also appear to be due to microbial contamination as a result of poor handling and humid climate. Efforts to

improve the safety of cassava-based feed should also address its microbial quality.

Human health and nutrition

1. Hydrogen cyanide is rapidly lost during processing and it probably does not constitute the main source of dietary cyanide exposure from insufficiently processed cassava. The main sources appear to be residual linamarin and acetone cyanohydrin that are broken down in varying degrees to cyanide in the human body. A substantial proportion of ingested linamarin is absorbed from the gut and excreted unchanged in the urine; thus the dietary cyanide exposure can be considerably lower than that expected from the total amount of cyanogens ingested. Cyanide release from linamarin in the gut may depend on whether active are present in ingested from cassava or foods simultaneously consumed provides. The presence in the gut of active a-glucosidases from cassava, other foods, or from gut microflora will influence the release of cyanide from ingested linamarin.

2. Although there are few published reports, it is reasonable to conclude that dietary cyanide exposure from insufficiently processed cassava can cause acute poisoning. Such acute poisonings occur when food shortage and social instability induce shortcuts in established processing methods. They may also occur when cassava cultivars with high glucoside levels are rapidly introduced into communities lacking efficient processing methods. Hospitals expected to receive such cases should be provided with rapid analytical methods and cyanide antidotes. This can save patients, verify the cause of the intoxication and thereby avoid unnecessary sensationalism. The importance of gari in West Africa and the attribution of acute poisonings to shortcuts in gari processing in Nigeria provide a strong justification to study whether shortcuts in gari processing can yield products with lethal cyanogen levels.

3. It is well established that the thiocyanate load resulting from dietary cyanide exposure from cassava can aggravate iodine deficiency disorders (IDD), expressed mainly as goiter and cretinism. This aggravating effect occurs only in populations with low iodine intake and is of secondary importance to the global IDD problem. Supplementation and fortification with iodine now receives high international priority and can counteract the effect of thiocyanate from cassava on the thyroid gland.

4. The evidence is strong, although not conclusive, for a causal role of cyanide exposure from cassava in the paralytic diseases konzo and tropical ataxic neuropathy (TAN). The pathogenic mechanisms are unknown. These diseases occur only in populations with severe socioeconomic problems,

monotonous diet and food insecurity. The acute onset of konzo is attributed to several weeks of high cyanide exposure due to shortcuts in cassava processing and concomitant low protein intake, which reduces the rate of cyanide to thiocyanate conversion. The gradual onset of TAN is linked to several years of moderate cyanide exposure combined with low intake of protein and some B vitamins.

5. The proposed association between dietary cyanide exposure and malnutrition-related diabetes as well as tropical pancreatitis remain speculative as no epidemiological data support a causal role of cyanide exposure. The suggested aggravating role of cyanide exposure from cassava in protein-energy malnutrition still lacks supporting data.

6. Animal models can further elucidate the mechanisms involved and clarify the causal factors of the diseases associated with cyanide exposure from cassava. Long-term follow-up studies of populations known to have had high dietary cyanide exposure in combination with various dietary deficiencies can provide new information on safe cyanogen levels in cassava products. The cyanogenic potential of cassava cultivars, together with residual levels of cyanogenic compounds in cassava products, linamarin intake and cyanide exposure should be studied in cassava-consuming communities where no related diseases are found. Such studies will advance the understanding of safety limits for cyanogens in the diet.

7. Future studies are facilitated by the recent development of new sensitive, specific and rapid analytical methods for blood or urine levels of linamarin, cyanide, thiocyanate and the alternative cyanide metabolites amino-thiazoline-carboxylic acid and cyanate.

8. Given the crop's sustainable production on marginal land and under drought conditions, it is crucial for food security in areas where toxic effects are reported. Affected populations state that bitter and potentially toxic cultivars provide the best food security. Given the constraints to agriculture in such areas, these cultivars may paradoxically have an overall positive effect on human survival. Prevention of toxicity should not be attempted through banning of certain cultivars but through positive actions such as the introduction of new cultivars and the promotion of effective processing.

9. The majority of the 400 million people who consume cassava daily is not at risk of the diseases described above. From a public health perspective, the linkages between cassava and these toxico-nutritional diseases are similar to the linkages between monotonous rice and maize diets, and the nutritional diseases beri-beri and pellagra, respectively. The main reason for public health concern regarding cassava-related diseases is that the underlying

causes—severe social instability, agroecological crises and food insecurity—are becoming more common in parts of Africa.

10. Human diseases linked to cassava cyanogenesis are entirely preventable. Preventive actions include the promotion of effective processing, iodine supplementation and dietary improvements. The diseases can also be prevented by measures against underlying factors such as food shortage, socioeconomic deterioration and market distortions for cassava. Introduction of high-yielding cassava cultivars with low glucoside levels may be long-term prevention in farming and food systems where cyanogenesis is not indispensable for food security. However, promotion of such cultivars should be done only when proven to perform well under stress in the local farming system conditions.

11. A cyanogen level of 300 mg HCN equivalent/kg dry weight (10 mg/100 g wet weight) has been used as the upper limit for "low cyanide" in breeding programs since 1954. This level is 30 times higher than the 10 mg HCN equivalent/kg, dry weight defined by FAO/WHO as the safe level for cassava products in *codex alimentarius*. A revision of these levels should be made based on a conceptual framework relying on new current knowledge from several disciplines. The estimates should be based on cyanide detoxification rates in humans, necessary safety margins for natural toxins, degree of cyanide release from ingested cyanogens, expected daily consumption and degree of cyanogen removal during processing. Theoretical levels should be compared with empirical measurements of the content of cyanogenic compounds in processed and fresh products consumed without effects by human populations according to general principles for natural substances in food.

Socioeconomic considerations

1. For the majority of cassava consumers, cyanide intoxication is not a concern. In some communities, particularly those facing nutritional deficiency and economic hardship, long-term exposure to dietary cyanide from cassava has been reported to be an aggravating factor for diseases attributed to chronic cyanide intoxication. In situations of war, social distress, drought or economic instability, populations may be forced to survive on cassava as the sole food for extended period of time as cassava is usually the only food that remains. The shortage of food may lead to shortcuts in processing methods to obtain food more quickly. Such shortcuts result in high residual cyanogen levels which cause acute intoxications among consumers. In cases of cassava-related intoxications, intervention strategies should recognize social, cultural and economic peculiarities in order to find appropriate approaches and for effective implementation.

2. Problems of cassava intoxications are linked to situation of economic depravation. Exploration and development of transnational and multiregional markets is a mechanism for enhancing local economies. The ability of cassava products to enter new markets will depend on product quality with respect to convenience, performance and safety. Building of rural infrastructure and amplifying trade relationships between and among various indigenous communities are part of the development of the cassava market.

3. Cassava varietal dissemination is currently largely a local farmer-initiated event. Therefore, we need to understand better the local rationale for preference, in particular regarding adoption of new cultivars. The rate of introducing germplasm of improved cultivars with the desirable characteristics, as defined by local farmers, should be increased. Cassava cultivars with higher levels of cyanogenic glucosides than those already used should never be introduced without vigorous simultaneous promotion of appropriate processing methods. New cultivars with very low levels of cyanogenic glucosides that hold promise of good performance in areas affected by toxic effects from cassava should be introduced immediately in those areas as a matter of priority. Varietal characteristics should be linked with particular processing methods.

4. A broad diversity of ecologically, socioculturally and technologically appropriate cassava processing techniques should be evaluated in a range of socioeconomic and ecological settings and disseminated. As many traditional forms of cassava processing are gender skewed (toward women and children), it is important that labor-saving technologies should be promoted to reduce women's workload and increase productivity without compromising their access to income.

5. The development and consumption of supplementary foods—both indigenous and introduced—in conjunction with various cassava food products should be promoted. We recommend the exploration of new uses of cassava to improve the economy of cassava-growing communities.

6. Cassava safety can best be improved by distributing desirable cultivars, promotion of effective processing techniques and diversification of markets for this root crop.

CONCLUDING REMARKS

Several topics could not be settled at the workshop and require further study. The reason for the use of bitter and toxic cultivars in communities, where the risk of intoxication is great, remain unclear. The levels of cyanogenic glucosides in fresh cassava roots currently used by plant breeders as target for developing cassava

genotypes with low cyanogenic potential are not in agreement with the understanding of safety limits for cyanogens in cassava. An approach to be followed to establish safe levels of cyanogenic glucosides has been proposed by Hans Rosling. It takes into consideration variables such as the toxic cyanide exposure rate in humans, the cyanide uptake from the gut, the level of daily cyanogen ingestion and the cyanogen content of consumed products while recognizing the factors controlling these parameters.

It has been hypothesized that long-term exposure to sub-clinical amounts of cyanogens from cassava-based diets may influence human biological fitness and microevolution. Evidence to support or reject this hypothesis is currently limited.

The relationship between bitterness of fresh cassava roots and their total cyanogen content needs further clarification. Although the correlation coefficient between the two is high, there is a need to establish whether there is a cause-effect relationship.

Although some progress has been made on the understanding of the role of cyanogenic glucosides in resistance to pests, an irrevocable proof has not been obtained. The development of acyanogenic cultivars by genetic engineering techniques that enable the silencing of gene(s) coding only for the biosynthesis of cyanogenic glucosides may provide such a proof by demonstrating that a cultivar that was otherwise resistant to pests becomes sensitive when it no longer produces cyanogenic glucosides.

The terminology used in the scientific literature when reporting the concentration of various cyanogenic compounds found in cassava is very diverse, often confusing and sometimes misleading. An agreement could not be reached during the meeting. Afterwards, advice was sought from the International Union of Pure and Applied Chemistry (IUPAC). Based on the IUPAC's suggestions and current state of knowledge and the desire to foster a better understanding of safety issues in cassava, the following recommendations on terminology are being made. Firstly, it should be recognized that intact and fresh cassava tissues contain mainly the cyanogenic glucosides linamarin and lotaustralin. Processed or damaged tissues may contain varying amounts of cyanogenic glucosides, cyanohydrins and hydrogen cyanide. The recommended analytical procedure can determine the total amount of all three compounds (Fraction A), the total amount of cyanohydrins and hydrogen cyanide (Fraction B) or the amount of hydrogen cyanide (Fraction C). Fraction A should be referred to as "total cyanogen content," fraction B as "non-glucosidic cyanogen content" and Fraction C as "hydrogen cyanide content." The "cyanogenic glucoside content" is obtained by subtracting fraction B from fraction A, while the "cyanohydrin content" is obtained by subtracting fraction C from fraction B. The recommended unit to be used is "mg HCN equivalent/kg," indicating whether data were calculated on a fresh or dry matter basis. The potential for a sample to produce HCN (expressed as total amount of HCN equivalent/weight of

sample) has been called HCN potential, HCN-releasing potential, cyanide potential or cyanogenic potential. The last one is preferred. Abbreviations such as HCNp, CNp or CNP are discouraged.

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BIOTECHNOLOGY APPLICATIONS IN CASSAVA RESEARCH AND DEVELOPMENT

Asia Overview

ASIAN CASSAVA MARKET DYNAMICS: OPPORTUNITIES FOR BIOTECHNOLOGY

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INTRODUCTION

During the 1970s the cassava-growing areas of Latin America and Africa experienced relatively few changes. In Latin America, traditional fresh cassava consumption steadily declined in several countries. In Africa, consumption of traditional processed products increased with population growth. But in South East Asia, this decade saw the start of an export-led cassava boom, especially in Thailand and Indonesia where significant investments were made to create a large-scale cassava drying and pelletizing industry.

During the 1980s, African cassava production and consumption continued to increase, while Latin America saw a slight upswing caused by product and market diversification, strengthening local demand for cassava roots as raw materials. This decade proved to be the golden years for the cassava industry in South East Asia. But, at the same time, European Union (EU)* government policies began putting pressure on exporting countries towards a longer-term reduction of import volumes. This, and further General Agreement on Tariffs and Trade (GATT) negotiations during the early 1990s, has led to the current situation where export volumes of traditional cassava pellets to the EU are expected to be reduced by at least 30%-40% in the coming years. Internal EU feed grain prices have been reduced to such a level that Thai export margins have eroded to near-zero profit. The Asian response during the last few years has been a move towards increased cassava product and market diversification. The shift has been mostly from pellet and native starch towards modified starches and from mainly exports to increasing domestic use. Largely similar trends can be seen in Indonesia, China** and Vietnam (Bottema and Henry, 1992).

From a global perspective, Asia is leading market dynamics in the cassava sector, followed by Latin America. Africa, although steadily increasing cassava production levels, does not show significant changes in cassava utilization for the near future.

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* Previously the European Economic Community (EEC).

** In this paper, China refers to The People's Republic of China.

In several countries, cassava use has been undergoing significant changes during the last 15 years, and global production levels have steadily increased in most countries. This has mainly been due to increases in planted area and, to a lesser extent, to productivity improvements. There has been significant adoption of technologies in several regions (Polson and Spencer, 1992; Henry and Gottret, n.d.; Henry et al., 1994a). But compared with primary crops like rice, wheat and maize, cassava Research and Development (R&D) impact has been slower and much less.

Henry and Gottret discussed in detail the major areas of constraints hindering a faster technology transfer and adoption process. These include biological, technical, socio-economical, political and institutional constraints. In addition, the orphan-crop syndrome as mentioned by Persley (1990) tries to capture the fact that cassava suffers from a significant underinvestment of national and international R&D resources.

The advent of biotechnology to agricultural research has opened new paths to advance the development of technological solutions. In 1991, the founding of the Cassava Biotechnology Network (CBN) represented a significant step. This unified, integrated and collaborative approach strengthens global cassava R&D towards generating a greater, much needed impact. But to optimize these efforts' effectiveness, client needs and opportunities must be well identified and carefully targeted.

Hence, this paper aims first to analyze cassava sector constraints, opportunities and market trends. This will include selected countries in South East Asia: Thailand, Indonesia, Vietnam and China. Second, it aims to make cross-country comparisons to identify common denominators for the areas of interest. Last, it uses the comparisons' results for discussing implications for cassava biotechnology opportunities in South East Asia and recommending future actions.

THAILAND

There are two unique features of the Thai cassava industry: (1) it is an export-driven industry, dominated by one market (the EU) and one usage (cassava products for animal feed; and (2) it is the greatest exporter of cassava products in the world. The industry's growth was slowed down by the agreement signed by the EU and Thailand on Cassava Production, Marketing and Trade in July, 1982. Under this agreement, the maximum Thai export of cassava products for animal feed to the EU was set at 5 million tons per annum.

Recent developments in international trade policy, measures such as the Common Agricultural Policy (CAP) reforms and the conclusion of GATT, are expected to have a strong negative impact on the Thai export of cassava products

such as animal feed. However, these developments toward freeing trade are envisaged to be in favor of the cassava starch markets for Thailand. So those countries with high protection levels for domestic starch markets, like Japan, Korea and European countries, will eventually open up trade for Thai cassava starch imports.

There is no solid evidence on the advantage of cassava starch compared to other starches. Cassava starch producers believe that it is the cheapest starch technically able to substitute for other starches. If this is so, all those concerned will be interested to know the uses and desirable attributes of cassava roots needed to meet future demand in the industry.

Production and cost of production

From 1984 to 1993, the total harvested cassava area increased from 8,345 to 8,988 thousand rais (6.25 rai = 1 ha) equal to an annual growth rate of 0.828%. Total production increased from 20 to 20.2 million tons (0.121% growth rate). However, the average yield decreased from 2,395 to 2,248 kg/rai or a decrease of 0.707% per annum (Table 1).

Overall, the North-Eastern region produces more than 60% of total root supplies, and the Central Plain region, including provinces in the Eastern seaboard, produces about 30%, while the Northern region produces the rest. In terms of production by province, Nakhon Ratchasima or Korat, the large cassava producing province, produces more than 4 million tons per year, followed by the provinces of Chaiyaphum, Khon Kaen, Kalasin and Udon Thani. Chon Buri (original center of the cassava industry) and Rayong are the two major cassava producing provinces on the eastern seaboard and each produces about 1 million ton per annum.

The national average production cost was about 0.51 baht/kg or US$ 20.4 per ton (25 baht = 1 US$) in 1991-92. The total cost per rai was 1,137.26 baht of which 87% was variable cost and 13% fixed cost.

In 1992, the detailed data on cost of production from the Thailand Development Research Institute (TDRI) (1993) showed that the total labor cost for planting, weeding and harvesting was 50% of the overall total. The cost of production ranged from 0.59 to 0.99 baht per kg with quite a wide range of yield per rai from 1,400 to 2,800 kg (Table 2).

Use of cassava products: past, present and future

In general, cassava products are used either in the form of starch or as products for animal feed such as pellets, chips, meal and wastes. Starch is used for human

consumption, in food industries as monosodium glutamate (MSG), sweeteners, pearl, etcetera and in non-food industries (paper, textile, plywood, pharmaceutical etc.).

The availability and price of high protein feed ingredients substituting for feed grains determines the use of cassava products for animal feed. The price of the cassava product itself is thus only one of the determining factors. Further, adequate and timely supply of the feed ingredients are key determinants.

Additional non-price factors indicating the potential use of cassava as animal feed are domestic infrastructure and the livestock industry's structure whether backyard or commercial systems.

Thailand exports both cassava products and grain such as maize and sorghum for feed and imports high protein feed ingredients such as soybean meal and other protein oil cakes. Therefore, cassava products are still not being used sufficiently for domestic feeds. This is because the price difference between cassava, mixed with high protein feeds, and maize is not great enough to compensate for the added cost of using other feed supplement ingredients, especially in poultry feed. Also, meat packers are reluctant to buy pigs fed on cassava products because of the texture and white color of the resulting pig meat. Meat packers claim that the domestic pig meat market is dominated by red rather than white meat.

The domestic use of cassava starch was dominated by the MSG industry in the mid 1970s and 1980s. It is expected that a major cassava starch consumption industry in the years to come will be the sweetener industry. But the modified starch industry is another fast growing one which currently consumes about 20% of total domestic starch supplies. In fact, most modified starch is exported to Japan for the paper industry. At present, domestic starch processors and traders believe that future exports of cassava starch will shift from the current, native starch to modified starch.

Table 3 shows the total annual use of cassava products for 1982 and 1991 and estimated for 2001. In terms of percentage on a root equivalent base, domestic consumption of starch increased from 3.5% in 1982 to 11.9% in 1991 and will be 24.8% by 2001. Starch exports also increased from 8.2% in 1982 to 16.5 in 1991 and will be 29.5% by 2001. But exports of animal feed products decreased from 87.9% in 1982 to 69.8% in 1991 and will be 41.8% by 2001.

It is noteworthy that domestic consumption of cassava for animal feed shows an increasing trend. Also that future use of cassava as animal feed is very sensitive to root price, while that of starch can still afford to pay a much higher price. Further, the 2001 scenario shows that exports of animal feed products, particularly to the EU market, will decrease to 4 million tons provided that the price of root at Korat is about US$ 21.6 or 540 bt. per ton.

Constraints

The constraints faced by the Thai cassava industry can be identified for three areas as follows.

Production/supply

Cassava production is facing about the same constraints as faced by the Thai agriculture sector in general. First, the widely prevailing land constraint, due mainly to the rapid economic restructuring toward more non-agricultural sectors accelerating the urbanization process. National forest reserves are already endangered and it is no longer possible to further clear new land for agricultural production as before. The competition for land use among economic sectors and within agriculture will push cassava production into more marginal land and further north, into the northeastern region.

The second constraint is availability of labor. At present, the rapid growth rate of sectors other than agricultural has pushed up wages and induced labor to shift away from agriculture. Also, the increasing labor demand in the newly industrialized countries in Asia and countries in the Middle East attracts a great number of Thai farmers to seek higher paid employment abroad. As a result, large rural areas are faced with labor shortages for agricultural activities.

Processing

The future direction of cassava industrial development is toward modified starch but there is still a lack of research on modified starch technologies. In fact, some of the modified starch technologies are imported from abroad. But the development of the industry has to be self-reliant and self-sufficient in basic know-how and technology as well as research capability to ensure that Thailand continues competing in the world market.

Since the processing industry has become more specialized and increased in both size and scale of production, toxic waste generated by factories will be an environmental problem. The rapid urbanization of provincial towns has created a situation where some factories are now located near towns and within municipal district controls, thus having to comply with their environmental regulations. Those factories outside municipal districts will also be facing stricter environmental regulations in years to come.

This directly implies that stricter environmental regulations will increase processing costs unless new technology for waste treatment is available comparatively cheaply. However, new technologies for toxic waste treatment may also generate income for factories, if such technologies can produce new value-added products from the polluted waste.

Markets

At present, Thai starch exports are still facing both high import tariffs and non-tariff protection barriers imposed by importing countries like Japan. It is hoped that, under the latest GATT resolutions, these trade barriers will gradually decrease and eventually be eliminated.

Potential for cassava research and development

It is quite clear that the real production costs of cassava in Thailand will increase, unless the yield per rai can be significantly improved and some labor-intensive activities in growing cassava can be mechanized. Therefore, research on varietal improvement should be aimed at yield, weeding and harvesting aspects. The appearance and color of the root should also be considered in line with processors' requirements such as dry matter content, texture, color and size, particularly for starch processing usage.

Research and development are greatly needed in the area of modified starch technology, especially to develop cost-saving technologies and new products. Moreover, waste treatment technologies and those which include new by-products are also crucial to the sustainable development of the industry.

Future research on cassava based on the use of the root can be classified into (a) industrial use and (b) direct food consumption. For industrial use, criteria and research priorities are very much in line with those discussed above. In the case of future research for food usage, the criteria and research priorities are rather different.

INDONESIA

In Indonesia, cassava is the third most important annual food and industrial crop after rice and maize. The area planted to cassava has been relatively stable since the early 1970s. The consumption of cassava as a food is concentrated in Java. All Indonesian cassava consumed as food fluctuates around 60 kg/year but there is significant regional variation. Indonesia produces about 15 million tons of cassava yearly. The domestic market is the most important one, where cassava is processed in gaplek, pellets for animal feed and starch. An extensive small-scale and cottage industry of cassava operates. It is very difficult to estimate the distribution of cassava processed for starch for the domestic market.

Cassava is usually grown on upland at the beginning of the wet season as a monoculture or in mixed cropping sequences. About 1.4 million hectares of land, distributed all over the country, is planted yearly to cassava. All Indonesian

productivity has increased a little in the past few years to around 10 t/ha. About 65% of total production comes from Java, while the contribution from other islands has recently increased.

Production aspects

Yields vary between 5 t/ha in the Eastern Islands to over 20 t/ha in irrigated cropping patterns in West Java. This productivity variation is accompanied by one in cost of production, dried cassava costs varying in 1989 between about Rp 90/ka in East Java to over Rp 200 in the Eastern Islands (Roche, 1992). It is self-evident that, regarding export and inter-archipelago trade, regional potentials are highly specific and depend largely on local agroclimatic conditions.

In Indonesia, cassava is usually harvested from late July to September with optimum harvesting time depending on the variety and generally being done by hand. Cassava deteriorates rapidly after harvest and is very bulky, making transportation difficult and expensive. Usually farmers process (peel and dry) the cassava directly or take it home for further processing or sale. Seasonality of supply greatly constrains the private sector in using large-scale technology for processing concerns.

Processing aspects

There are signs that smaller, highly localized businesses are the major users of year-round supply cassava and that their products remain restricted to the local and regional food market. Because a relatively small area of land is likely to receive water during the dry months, year-round processing of cassava at this point has served only small-scale processors.

In 1991, in addition to larger-scale factories processing some 3.5 million tons of cassava per year primarily for export, of a total of 122,860 small industries, 38,271 were active in food processing of which approximately 10,000 were in urban areas and 28,000 in rural areas (CBS, 1991). In the cottage industries (2,350,984 in 1991), food processing was the main activity of 833,228 establishments (CBS, 1991). Cassava processing is bound to take up a proportion, perhaps 10%, of these small-scale and cottage industries, concentrating around towns and cities. The actual volume of processed cassava in this subsector is unknown, but one can assume that cassava processing for the domestic market is growing steadily. Various scenarios of higher and lower rice prices still show growth in cassava demand (Altemeier and Bottema, 1991).

Policy aspects

A perception in the mid 1980s of the growing importance of cassava as food, feed, raw material for industry and export commodity led to government (national and regional) establishing policies to increase cassava production. A nucleus estate small holder (NES) system for food crops was launched in 1984-85 by decree of the Minister of Agriculture and Director General of Food Crops. In principle, the NES system is a collaboration between a processing firm as manager/guide and farmers. For cassava, the nucleus estate is the tapioca or pellet factory. In East Java, the system does not function as expected (Pakpahan et al., 1993), whereas in Lampung it has increased cassava yield to a continuously attained 25-30 t per hectare (Rusastra 1988).

Other policies are directed to meet the export quota. These were not met in the period 1983-1987 but were achieved in the late 1980s through a supervised mechanism of licensing export quotas, involving the cassava exporters association. Because gaplek exports rapidly expanded over the last 5 years, Indonesia's quota within the EC has been binding since 1988. In August 1989, the Indonesian government introduced a 2-for-1 system under which exporters were permitted to ship two tons of gaplek (either pellets or chips) to the EC if one ton was also exported to non-EC countries. Indonesia's export supply declined by about 10% in 1990, while f.o.b. prices rose. The 2:1 regulation was allowed to lapse in early 1991. At present the potential for exports seems to be more in Asian markets. Although cassava exports have been contributing to growth, the future may show a dampened demand.

Cassava sector constraints

In view of the large variation in agroclimatic conditions, it is not surprising that the greatest needs for cassava improvement concern adapted varieties, planting materials, identification of local markets and improved use. The red spider mite is important among cassava pests and bacterial blight among its diseases, with bacterial wilt and leaf spot also common. Cassava production systems in Indonesia vary and this is reflected in the number of encountered cassava-cropping patterns (12). Some of these concern inter- and relay-cropping, reflecting on the importance of cassava as a local food in Java.

The problems to be solved in cassava marketing concern cassava's inherent characteristics of bulkiness and perishability, which tie it to localized processing and use. All the various stages of product transformation cannot be detailed here. But cassava's two main characteristics, perishability upon harvest and bulkiness, influence both the structure of the markets for cassava as a food and for industrial use. Seasonality affects the collection, transport and transactions as well as

production and processing technology. Seasonality of supply is thus directly transferred to the market, and induces preprocessing.

There have been recent attempts to develop alternative uses of cassava, such as cassava flour, in Java and Lampung. The general idea was that farm- and village-level processing and drying would add value to cassava in the local community and create its own demand. But domestic wheat flour already uses starch produce in various quality classes, and explorative research shows that cassava flour so far finds some demand in small food processing establishments on Java (Damardjati et al., 1994).

The campaign-structured collection of cassava for food and feed brings about a range of inefficiencies in the market: quality loss due to transport and bulking time; and, because of synchronical supply, peak localized price trends that affect farm productivity. Cheap drying of cassava at farm-level would diminish the seasonality of production. In the rainy parts of Indonesia, off-season rain constrains sun drying year-round.

The available price information over the long term indicates more or less regional price cycles. This shows that the relation between annual investment, planned production and returns is not optimal. The present structure of the cassava market reveals multi-year cycles in the price of cassava, that behave differently in the various production areas and market segments of Java. This confirms the structuring impact of cassava's perishability and its price/bulk ratio economies of scale:

As yet there is little information that confirms regional arbitrage in the trade of dried cassava in the archipelago and surrounding consumer centers. One would expect that such arbitrage would have increased in recent years, also because the export of cassava from Indonesia supplies a number of users in Asia and Europe. The question is whether better integrating the regional market for cassava and forming a more diverse one would lead to a beneficial competition and a transfer of price signals in the local collection markets.

Table 4 shows a hypothetical connection of the temporal pattern of cassava production and the various market segments. In looking at adapted varieties for the various agroclimatic zones, there seems to be an argument for looking at the temporal production pattern of cassava in Indonesia. It may be possible to develop varieties that run over an extended time and that would stabilize monthly input flows under supervised or managed-production conditions. The conclusion regarding the role of biotechnology in cassava research concerns the selection of material to be adapted to the wide range of agroclimatic conditions, including long and short duration varieties.

VIETNAM

Although supplies of Vietnamese cassava root supplies (2.5 million tons) in 1991-93 make up only 5% of total Asian supplies, during recent years the cassava sector has been quite dynamic. Figures 1, 2 and 3 show that, during the initial postwar period, cassava plantings increased dramatically. Although it has to be noted that there are reasons to believe that the Food and Agricultural Organization (FAO) cassava statistics for Vietnam during and after the war may not be very reliable, the general trends for these periods still hold. But at the end of the 1970s there was an increased emphasis on rice and decreased cassava plantings. Then, starting with the "doi moi" or opening of the economy at the beginning of the 1990s, increased interest in cassava returned and plantings picked up significantly (at a 3.5% annual growth rate).

It serves to discuss this latter trend in more detail. To a large extent, the upward, cassava-planting trend has also been induced by policy. In the early 1990s, cheap soviet inputs for agriculture (including fertilizers and gasoline) were withdrawn and national rice self-sufficiency emphasized. Thus roots and tubers were targeted for supplying increased raw material to processing industries, partially substituting for rice. Rice production costs increased relative to cassava, making it more competitive.

Further, the opening of the economy offered opportunities for investment of foreign capital. Ha et al. (1994) analyzed current usage and future potential of cassava starch in Vietnam and concluded that there exists a very elastic market for it. They also show that, during the last few years, several large foreign companies (Japan, Taiwan, France) have begun producing MSG. The majority of these base production on local cassava starch. This has been and will continue to be a major boast for both root production and starch processing.

Figure 3 shows that during the last decade, cassava yields have fluctuated between 8 and 9 ton/ha. But since 1990, average yields have steadily increased at an annual rate of 1.3%. Ngoan et al. (1992) showed that Vietnam has a wide range of traditional cassava varieties. Kazuo Kawano (personal communication, 1994) also notes that there is a large yield potential through the introduction of improved (mainly Thai) varieties as shown over several years of local yield trials. By spontaneously adopting experimental varieties, farmers near Hung Loc (near Ho Chi Minh City) are currently achieving over 20 ton/ha using traditional practices (H. Kim, personal communication, 1994).

Production and production cost aspects

Total cassava planted areas are similar for North and South Vietnam. The major production areas are in the coastal and mountain regions. Most farms growing

cassava are less than 1 ha in size, with an average cassava planted area of 0.27 ha (Binh et al., 1992). Yields vary significantly between regions, 16-20 ton/ha in provinces like Bac Thai, Ha Son Binh and Tay Ninh and about 6 ton/ha in highland areas in Central Vietnam. The national average cassava yield from a 1991 farm survey (Henry, 1992a) shows around 12 tons, while FAO statistics report around 9 tons/ha (FAO, 1994).

Vietnamese cassava production is highly labor intensive. Binh et al. (1992) report an average of 240 man-days per cycle per ha, with three quarters of this coming from family labor. On average, labor constitutes 55% of total production costs and fertilizer another 15%. In 1991, average total production costs were reported at US$154/ha, which translates into a cost of approximately US$12/ton of roots. Average cost per ton of roots show very similar for both North and South Vietnam (Binh et al., 1992).

Use and processing costs

Cassava use varies significantly in Vietnam. Typically, isolated mountainous areas show a higher degree of on-farm consumption than cassava areas near (semi) urban processing centres. Based on the 1991 survey, the calculated average indicates that approximately 35% of cassava production is consumed on-farm (mostly by pigs), while 65% is further processed (on-farm) and/or directly sold to serve as raw material for processing centres elsewhere (Binh et al., 1992).

In Vietnam, a large variety of cassava-based products are produced both on and off-farm (Nghiem, 1992). These products include chips, flour, wet and dry starch, maltose, glucose, alcohol, etc. Some of these serve as raw material for further processing into noodles, glue, paint, paper and cardboard, candy, pastry, MSG, etc.

Contrary to Thailand, in Vietnam most cassava processing takes place in small (family cottage industry) to medium scale units. It is common to see entire communities or villages dedicated to producing one or two cassava-based products. As such, a village can benefit from scale economies regarding raw material supplies and commercialization. In 1991, several large-scale starch factories were reported to exist but most of these state-owned units relied on very traditional technologies and functioned at low capacity (Nghiem, 1992). Also very different from Thailand, is the fact that, as well as fresh roots, chips are widely used in starch processing. This is also very common in neighbouring China.

Vietnamese starch processing at village-level is based on traditional technologies, generating mostly low quality starch (grade III). In 1991, cost price (in the village) of this starch was approximately US$150/ton. If this type of starch is transported to major cities and upgraded for export starch (grade I) the cost

increases to approximately US$200/ton. At village level, raw material cost represents an average of 80%-86% of total starch costs, while labor constitutes 5%-12% (Binh et al., 1992).

Cassava sector constraints

In a study survey of rural households (Henry, 1992a), cassava farmers and processors were asked to identify and rank the major constraints to their production, marketing and processing systems. The results show that cassava farmers (overall) are mainly concerned about (low) soil fertility and market-related aspects (Henry et al., 1994b). To a large extent, the soil fertility constraint confirms the idea that Vietnamese cassava production is shifting increasingly to more marginal, fragile production zones. It is argued that increasing urban economic development is moving semi-urban and urban agriculture systems toward higher value crops, e.g., fruit, vegetables, so pushing root and tuber crops to cheaper, more marginal lands (this trend can also be observed in Thailand, China and Colombia). Especially in hilly and mountainous zones, soil erosion and depletion is a significant problem. One of the more visible short-term effects is the relatively low yields in these zones.

In the market-related group of constraints, farmers' perceptions of significant "marketing problems" are related to demand, both low and fluctuating, at farm-level. Further, farmer perception of cassava's low profitability is related to yield, factor efficiency and input/output prices.

In Vietnam, function analysis of cassava production has also shown overall the negative influence of land, labor and credit constraints on production and productivity. However, specific constraints must be evaluated by province or county to be valid (Henry et al., 1994b).

Binh et al. (1992) found perceptions of constraints on cassava processing ranged widely depending on type of product and region. Constraints include raw material supplies, product quality, market organization (three highest ranked), and demand, labor and capital. It must be noted that processors mentioned neither low efficiency because of traditional processing technologies, nor potential danger from toxic processing waste dumped in open waters. These more expert views complement farmer perceptions in this needs assessment which is client-oriented.

Binh et al. (1992) based a market survey on interviews throughout each cassava-based, product channel. This showed, among other items, that the number of pricing points (middlemen) is very high while, in contradiction, marketing margins are not equally high for most products. There is an obvious lack of and poor quality in information systems on public pricing. This offers intermediaries the opportunity to make sizable profits. Supply and demand of regional products are not balanced,

resulting in large price fluctuations and market inefficiencies at a cost to producers, processors and consumers. This lack of information plus a slow and inefficient export system often erodes the costprice edge for export opportunities. While up to a few years ago, state-owned export companies controlled all exports, lately an increasing amount of products (including cassava-based) are exported through privately owned companies. As such, it can be argued that the former export marketing inefficiencies will decrease in time.

CHINA

Relatively little cassava data exists for China. Moreover, Stone (1987) argues that existing FAO data may not be very reliable nor consistent. Also, most existing information is written in mandarin and often only published as "grey literature" making public access difficult. But since the late 1980s, various international institutes (Natural Resources Institute [NRI], Centro Internacional de Agricultura Tropical [CIAT], Centro Internacional de al Papa [CIP], etc.) have significantly increased data collection and information gathering for specific crops and/or agricultural systems. Improved Chinese economic development and statistical services have also significantly boosted the quantity and quality of basic agricultural data bases and more information has become available in the English language.

Based on Chinese municipal data, Shu Ren and Henry (1993) noted that cassava production in China is concentrated in the southern (subtropical) provinces of Guangxi, Guangdong, Hainan, Fujian and Yunan. The current total cassava area fluctuates between 400-440,000 ha. Between 85% and 90% of this lies in the provinces of Guangdong and Guangxi. Total Chinese cassava root production is estimated at 4.2 to 4.4 million tons (Shu Ren and Henry, 1993). This contrasts sharply with the FAO estimate of 3.4 million tons (FAO, 1994). While the absolute numbers differ, both series show similar trends. It is noteworthy that there has been a significant recovery in cassava plantings since 1990. To a major extent this has been due to political changes towards a freer market system bringing about renewed interest in cassava processing, especially in native and modified starches. As such, these developments are similar to those in neighbouring Vietnam.

Production and costs

In most growing areas, cassava is generally planted on hillsides and the less fertile lands further away from homesteads. Also, cassava is planted on "new" or "waste" lands. Cassava is often intercropped with young rubber, fruit or medicinal trees (Henry and Howeler, n.d.).

Yields differ significantly in China. Shu Ren and Henry (1993) noted 1988-89 cassava yields varying from 8.1 ton/ha in Guangxi to 14.7 in Hainan. Figure 4 shows historic cassava yields for Guangdong and Guangxi provinces, both of which have significantly increased yields. The annual growth rate for Guangdong was 8% and 4.7% for Guangxi, during the 1972-91 period. Yield data from FAO only shows an annual 2% growth since 1990 for aggregate Chinese cassava yields. Whatever the case, cassava yields have been steadily increasing.

Cassava production in China takes place on small family plots which, depending on the family size, range from 1 to 10 mu (1 ha = 15 mu). Cassava production is labor intensive. Henry and Howeler (n.d.) found that total labor needs, including chipping, drying and transport range between 8-16 man-days/mu or 120-240 man-days/ha. This may be as high as 360 man-days/ha when opening new (or after fallow) upland plots. But compared to rice or peanut cultivation, cassava requires less than half of the labor needs per cycle, or between 15%-20% on a per-month basis (Henry and Howeler, n.d.).

It is hard to find reliable information on cassava income or profit. But it can be noted that farmers will most often reply that cassava-growing in general is less profitable than rice, maize, peanuts, sugar cane, rubber and fruit trees. Yet farmers still like to grow cassava as it serves many purposes for both the household and the market, requires relatively less labor, grows well on marginal lands and dry chips can be stored.

Utilization and processing

According to a Rapid Rural Appraisal (RRA) conducted in 1994, aggregate cassava use in China is divided as follows: on-farm human consumption 2%-4%; on-farm animal feeding 20%-30%; on-farm chipping for sales 30%-40%;and fresh root sales to processing industry 30%-40% (Henry and Howeler, n.d.). This contrasts sharply with earlier observations (Stone, 1987) showing that the majority (60%-80%) of cassava in China was fed on-farm to pigs. Some significant changes have occurred during the last decade. First, on-farm cassava consumption for food has drastically decreased, given increased supplies of rice, preferred as a basic staple (Henry and Howeler, n.d.). Second, with a strengthening demand for cassava as a raw material for the processing industry, direct on-farm sales of fresh and dried cassava have increased at a cost to the share allocated for on-farm pig feeding. While this is an overall, general tendency, in isolated areas, far removed from processing centers, the share for on-farm pig feeding is significantly higher.

During the late 1980s, the cassava processing industry increased in scale and level of production due to changes in Chinese government policies. Also, a gradual change from government-controlled to private enterprise has been occurring. Small, household-level, starch processing has been decreasing making place for large-scale,

private, processing factories. State-run factories are looking for private (often foreign) capital to replace traditional processing technology and venture into product diversification.

Shu Ren and Henry (1993), estimated total Chinese (native) cassava starch production in 1992 at approximately 320,000 ton, accounting for a quarter of Chinese starch production. As such, cassava starch processing used about 1.6 million tons of fresh roots. Part of the high quality (grade I) is destined for exports. While China still holds an EU export quota, annual cassava chip export volumes fluctuate between 300,000 and 600,000 tons. The future potential for this product and market seems to be rather low, given the decreasing prices of EU feedgrains and the little advantage China holds producing and exporting low quality chips (compared to, for example, Thailand).

In China, the current and future trend in cassava products is towards further processed or modified starches. Shu Ren and Henry (1993) note that in the mid 1980s, fructose and crystalline fructose based on cassava (for the pharmaceutical industry) started to be produced on a larger scale. Also, sorbitol (to make vitamin C), mannitol (for certain medicines), and maltol (a sweetener) productions have come on stream. Cassava is also being increasingly used in manufacturing fermented products such as alcohol, MSG and citric acid. The majority of domestic MSG is produced from molasses but the share of cassava-based MSG is steadily increasing, several new factories being built in South China, with the help of foreign capital. Although still relatively small, the production of denatured starches, such as starch phosphate esters and co-polymerized starch, has been receiving growing interest.

Cassava sector constraints

Since little information on the needs of clients exists for the sector, in 1992, a Chinese strategic cassava research planning exercise was proposed (Henry, 1992b). It aims to develop a national cassava R&D agenda, around which the various Chinese research and teaching institutions working on cassava can be integrated in a concerted and efficient manner. As a first activity an RRA was made which included assessing (and ranking) major constraints at each level of the cassava sector (Henry and Howeler, n.d.). Preliminary results, based on cassava farmers' perceptions, show that generally, major problems include: low profitability, high labor needs (especially in upland areas and for chipping and drying activities), low and fluctuating product prices, lack of capital (and credit), low yields (in Hainan declining due to soil problems), and high transport costs. It must be noted that these are aggregated problems, while each area has different constraints and rankings.

Expert opinions from researchers, extensionists and village leaders offered further clarifications. First, in general, fresh root and dry chip prices were not particularly low relative to the price of rice and own price in time. Further, root and chip prices are being pushed up in many areas due to increasing demand from the processing industries. In several production zones, especially in Hainan province, farmers would not consider soil fertility and erosion losses as a major problem, yet these were often one of the main causes for declining yields (Henry and Howeler, n.d.).

At the cassava processing level, perceptions of major problems were quite consistent between areas and products. State- or municipal-controlled factories generally have significant, chronic problems of low profitability or, in many cases, losses. Other high-priority problems include: supplies of raw material being insufficient resulting in under-capacity production; lack of investment and operational capital; faster increase of input than output prices; and little government collaboration (policies, credit, technology and R&D).

Further, expert opinion identified additional problems in the toxic, processing-waste disposal that is often unchecked in open waters, and in low processing conversion rates due to old and/or malfunctioning processing technologies.

CROSS-COUNTRY COMPARISONS

In this section, key sector parameters of cassava will be compared between the various countries. Table 5 shows a cross-country comparison of current cassava yields, prices and costs. Thailand may still consistently have the highest yields while China and Vietnam show the lowest root, chip and starch prices. It is also of interest to note that Indonesia consistently shows relatively higher prices. Based on these few data, and given the fact that both China and Vietnam produce and process cassava still at relatively low levels of technology, it could be argued that these two countries can further develop a costprice edge by adopting future technology.

Table 6 shows a similar comparison but of cassava trends. It can be seen that China shows the strongest trends regarding area and yield growth. Increased cassava supplies have been mainly generated through increased productivity and to a lesser extent through area expansion. As regards expected product trends for the future, it is obvious that they move away from chips and pellets and towards starch, especially modified starches (Thailand and China). Moreover, while during the last 20 years the trend was towards cassava product export markets, future trends will be to increasingly emphasize domestic markets. Also it can be noted that in general, there will be a significant emphasis on product diversification.

These trends form the base from which cassava sector constraints must be analyzed. Table 7 attempts to summarize, in a qualitative fashion, the major constraints by production and processing sectors. In most of the countries, the issue of seasonality/price fluctuations/market demand is viewed as a major problem. Most Asian farmers will also mention low profitability but this problem ought to be seen as an effect rather than a constraint. Also, Asian farmers in several countries and zones, see soil fertility problems, including erosion, as a major problem. This issue seems to be a dichotomy, since farmers in general like the crop as it still performs well on marginal soils, etcetera. The increasing shift towards more marginal sites for cassava production and low levels of fertilizer application contribute significantly to the problem.

Basic production factors such as labor and land have become major constraints in a labor-intensive production system like cassava. This is especially true in Asian countries with an accelerated economic and urban growth. Rural labor wages have been under pressure from rising industrial and urban wages. So farmers, especially those near industrial zones (i.e., Rayong and Guangdong provinces), and/or semi-urban zones, point high labor cost out as a major constraint. A partial answer has been increased mechanization and herbicide use, where possible.

Like low profitability, low yield when mentioned as a problem, is an effect of other constraints, rather than a problem by itself. Nevertheless, farmers see it as a problem in most countries.

In the chip and pellet processing sector, export-oriented countries like Thailand and Indonesia stand out for the importance assigned to market problems. This is directly related to eroding feedgrain prices in the EU. In Thailand, this fact further relates to subsequent problems of raw material supplies for pellet factories, as starch factories can offer better root prices, because of their more favorable profit margins and stronger market demand.

In China and Vietnam, on-farm chipping technology is very traditional, and offers opportunity for improvement, especially since farmers point out that chipping and drying is too labor intensive. Traditional technologies directly influence chip quality, which is low and variable. Starch factory owners, who rely on chips as raw material between cassava harvest periods, often mention this fact.

The cassava starch industry in South East Asia shows similar, almost universal, problems of raw material supplies and availability of capital. This is also found in Colombia, Brazil, the Philippines and India. Moreover, in countries like Vietnam and China, the majority of factories (small and large) suffer from low technology levels (outdated), with subsequent low conversion rates and quality, finally translating into profitability problems. Another universal problem, already discussed in the section on Thailand, is toxic processing waste disposal. Although

factory managers seldom mention it as a problem, it is dangerous to the environment and to mankind. In countries like Thailand and Indonesia, where environmental laws are more clearly defined and more strictly enforced, in several (more urban) zones, waste disposal already bears an additional cost. In other countries, it is only a matter of time for the legal apparatus to catch up with serious infractions of the laws. Factory managers ought to see this as an opportunity for developing value-adding by-products rather than as a cost-adding problem.

CONCLUSIONS AND IMPLICATIONS FOR BIOTECHNOLOGY R&D

The "best" opportunities for cassava biotechnology in South East Asia need to be found. It may seem inappropriate for a largely socio-economic group of authors to tackle such an issue. But let it suffice as an attempt to indicate general areas of opportunities. This can then serve for a more specialist audience, for example the CBN, to discuss the propositions in more detail and make the necessary recommendations.

Seasonality

The issue here is that peak harvest seasons sharply depress prices and, during the off-season, generate under-supplies at high prices. The aim would be to attempt smoothing out peaks in both supply and price throughout the year and between years. This can be tackled through biotechnology using two approaches. First, the development of early maturing varieties can be accomplished with traditional breeding efforts, since there exists a large enough variation in the existing cassava germplasm collection. But biotechnology can be used in the varietal multiplication phase. Given the inherent low multiplication rate (10 to 12 plants from one) of vegetatively propagated cassava, micro-propagation offers an alternative as has been shown by Liu et al. (1990). However, larger scale socio-economic feasibility studies are needed to validate this method.

Second, the issue of cassava's post-harvest deterioration (PHD) can be tackled with biotechnology. To alter the PHD rate, two significant obstacles need to be overcome, i.e., genetic transformation and gene cloning. Once successful, the expected final product of this research could be cassava varieties having a fresh root shelf-life of from one up to several months, instead of a few days. This would broaden harvest and supply periods and drastically reduce losses.

Toxic processing waste

Research to resolve this constraint needs to be conducted along different lines. The issue of detoxification can be approached with microbial biotechnology. This

includes the breaking down or metabolizing of active toxins like cyanogenic components through fermentation processes with microorganisms.

As well as the direct toxic components in the processing wastes, a certain amount of starch will be present. This starch needs to be captured by improving processing techniques and allowing adequate sedimentation. It is envisioned that the fermentation process can increase protein content of waste material and so generate, once dried and milled, an excellent animal (fish or pig) feed ingredient.

Besides the detoxification and neutralizing process, researchers need to aim at optimizing the value-adding of the waste product. This involves a phase of product and market testing to develop a useful product that enjoys a strong demand and fetches a good price. Thus, through biotechnology and other research approaches, a problem and danger to humankind and the environment can be transformed into a value-added by-product, potentially offsetting additional costs or even generating more economic gain.

Labor constraint

Opportunities to reduce high labor needs in cassava production have been a constant challenge to research. Mechanization and using herbicides can theoretically substitute, in part, for labor. This would decrease labor used in preparing land and weeding. But labor for harvesting can be sometimes as high as 50%-60% of total labor needs. Research can aim at altering conventional cassava plant architecture and root morphology to develop a cassava plant and root system that could be mechanically harvested with ease. Direct efforts would basically involve traditional breeding, since there exists a large genetic variation of the desirable traits. Indirectly, biotechnology can serve through the use of molecular markers. This method, by tagging traits, is a fast way of screening a germplasm collection for the presence of desirable characteristics. Currently however, the economic cost of this technique is high and as such forbids its extensive and frequent use for traits such as plant morphology, that can be easily selected. Further research is needed to develop more cost-effective techniques.

In addition to these biological research activities, altering plant architecture for increased mechanization invites an extensive array of complementing socio-economic research activities. Questions like: "Will it only benefit large-scale cassava farmers or small-scale ones too?" and: "What will happen to the subsistence character of cassava?" are only a few issues needing investigation in an ex-ante fashion.

Varietal diffusion and multiplication

The majority of constraints needing to be resolved, through higher yielding, or increased starch content, or "dwarf" varieties, will involve a multiplication and diffusion phase. Ultimately, research only pays off if a fast, high level of adoption rate can be achieved. The base multiplication remains a slow, expensive process that significantly slows down transfer and the subsequent process of adoption (Henry and Gottret, n.d.). Biotechnology has the opportunity to alleviate these constraints. Micropropagation methods have already been proposed as a potential alternative. True Cassava Seed (TCS) offers another means of tackling the above and other problems. This would not only be relevant for the Asian cassava sector but also benefit global, more generic cassava constraints. Perhaps current and future strong interest in cassava varietal solutions in Asia, plus the relatively strong national cassava programs (and funds), would make this continent appropriate for targeting initial research projects related to TCS.

A final point relates to biotechnology vis-à-vis traditional biological research. The current exercise aims at reviewing cassava trends and constraints, the review then serving as the demand side of technological needs. The supply side consists of a wide array of technological options, including biotechnology tools and applications. Marketing of technology components is done through technology transfer agents. Certain sector problems, needs or opportunities can potentially be solved through traditional research approaches, others only through biotechnology, others again through a combination of the two, or one of the two depending on such constraints as relative costs, urgency, and institutional capacities. Often biotechnology represents an option. It can be cheaper, or faster, or in some cases the only option. In this section it has been noted several times that a certain biotechnology option was theoretically feasible but that further socio-economic validation was still needed. Such validation clearly earns a place on the biotechnology priority agenda. Thus these conclusions may serve CBN collaborators in their deliberations on what, where and how to target cassava biotechnology in such a way as to optimize both available resources and expected impact on the cassava sector.

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Table 1. Cassava in Thailand: Area, production, yield, farm-gate price and farm value, 1984-1993.

Year	Planted area	Harvested area	Production 1,000 m	Yield/ rai kg	Farm price Baht/kg	Farm value million Baht
	1,000 rais					
1984	8,780	8,345	19,985	2,395	0.66	13,190.3
1985	9,230	8,603	19,263	2,239	0.40	7,705.1
1986	7,748	7,528	15,255	2,026	0.78	11,898.8
1987	8,820	8,567	19,554	2,283	0.89	17,403.2
1988	9,879	9,668	22,307	2,307	0.61	13,607.3
1989	10,136	9,957	24,264	2,437	0.56	13,587.8
1990	9,562	9,297	20,701	2,227	0.62	12,834.3
1991	9,323	8,960	19,705	2,199	0.83	16,355.2
1992	9,323	9,066	20,356	2,245	0.77	15,673.9
1993	9,100	8,988	20,203	2,248	0.66	13,333.9

SOURCE: MAC, 1993.

Table 2. Cassava root costs of production in Chon Buri and Rayong: Local variety planted on sandy and good drainage soil in 1992.

Item	Baht/rai	%
Land preparation	260.00	15
Planting	115.00	6
Crop caring (weeding)	405.00	23
Fertilizing	295.00	17
Harvesting (labor)	358.75	21
Land rent	200.00	11
Interest on working capital	106.25	6
Total cost	1,740.00	100
Yield (kg/rai)	2,272.00	
Yield range (kg/rai)	2,800-1,400	
Average cost (B/kg)	1.07	
Min-max ave cost (B/kg)	0.57-0.99	

SOURCE: TDRI, 1993.

Table 3. Thai historic and projected cassava utilization, 1982-2001.

Item		1982		1991		2001*		
		1000 MT	%	1000 MT	%	1000 MT	%	P root in Korat[b] (Bt/MT)
Domestic consumption:								
Starch[c]	- product	178	(3.5)	511	(11.9)	1,185	(24.8)	> 905
	- root[d]	892		2,556		5,923		
Animal feed	- product	4	(0.0)	150	(1.8)	276	(2.9)	876
	- root	10		375		690		
Fructose	- product					44	(0.9)	> 905
	- root					221		
Export:								
Animal feed	- product	8,928	(87.9)	5,977	(69.8)	4,000	(41.8)	540
	- root	22,320		14,943		10,000		
Starch	- product	425	(8.4)	707	(16.5)	1,409	(29.5)	905
	- root	2,128		3,536		7,047		
Total	- root	25,350	100	21,410	100	23,881	100	

a. Projected.
b. Subsequent root price per ton at farm-gate.
c. Starch excluding fructose.
d. In root equivalent.

SOURCE: TDRI, 1992; TTTA (various years).

Table 4. Hypothetical relations of seasonality, scale of production, and collection and use.

Overall seasonality	Scale processing	Major use	Proportion cassava (%)
Campaign			
Collection/processing	Medium/large	Tapioca/chips/pellets	72
Year-round			
Collection/processing	Small	Tapioca, food, snacks	28

Table 5. Cross-country comparison of current cassava yields, prices and costs.

	Vietnam	China	Thailand	Indonesia
Average yield (t/ha)	10-12	10-14	13-14	12
Root price (US$/t)	20-25	20-25	20-30	10-50
Chip price (US$/t)	70-85	70-85	60-94	100-120
Starch price (US$/t)	120-170	120-170	124-194	230-320
Starch processing cost (US$/t)	12-25	20-40	14-36	–

Table 6. Cross-country comparison of historic and future cassava trends.[a]

Historic trend (10 years)	Vietnam	China	Thailand	Indonesia
Area (ann. %)	0	2-3	1	0
Yield (ann. %)	2.6	4-8	0	2.2
Future trend				
Chip/pellet	?	?	–	+
Native starch	+ +	+ +	+	0
Modified starch	+	+ +	+ +	?
Utilization trend				
Domestic	+	+	+	+
Export	0	+ +	+	0

a. + + = strong trend, + = intermediate trend, 0 = no change, ? = unknown.

Table 7. Cross country comparison of the relative importance of major cassava sector constraints.[a]

	Vietnam	China	Thailand	Indonesia
Production				
seasonality/prices	••	••	•	•••
profitability	•	•	•	—
soil fertility	••	•	••	(•)
yield	•(•)	•	•	(•)
labor	•	••	•••	•
Processing				
chip/pellet				
RM[b] supplies	••	—	••	—
technology	••	(••)	—	••
quality	••	••	—	••
profitability	—	•	••(•)	—
market	—	—	•••	••
starches				
technology	(•••)	(••)	—	?
quality	••	•	—	—
RM supplies	•	•••	••	••
profitability	•	•	—	—
market	—	—	••	—
capital	•	••	—	•
waste disposal	(••)	(••)	(••)	(••)

a. ••• = high; •• = medium; • = low importance; — = no relative importance; ? = not known; ( ) = an opportunity, not a user perception's problem.

b. RM = raw material.

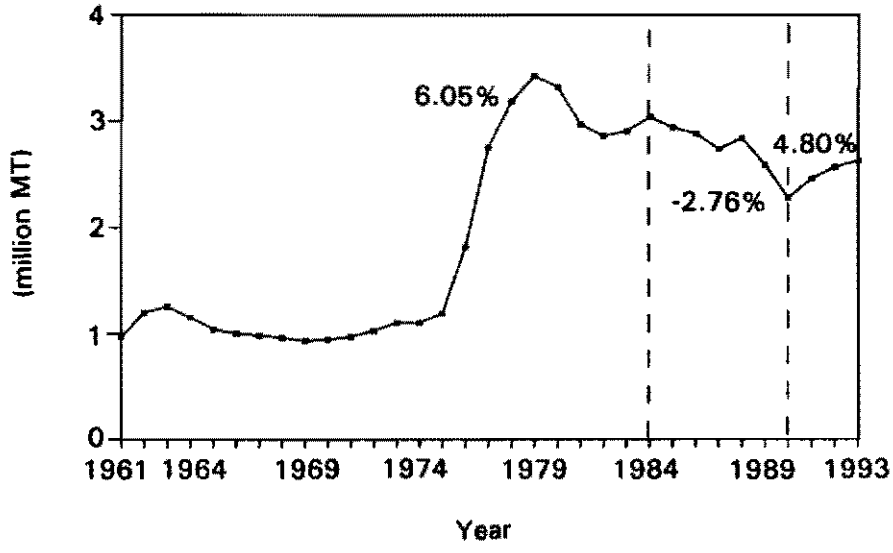

Figure 1. Vietnam cassava production and growth, 1961-1993. Percentages refer to annual growth rates for 1961-1984, 1984-1989 and 1989-1993.

SOURCE: FAO, 1994.

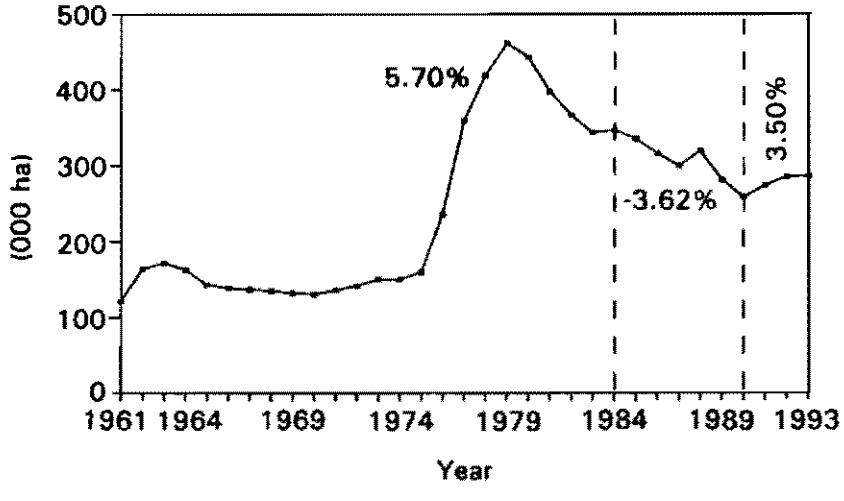

Figure 2. Vietnam cassava area and growth, 1961-1993. Percentages refer to annual growth rates for 1961-1984, 1984-1989 and 1989-1993.

SOURCE: FAO, 1994.

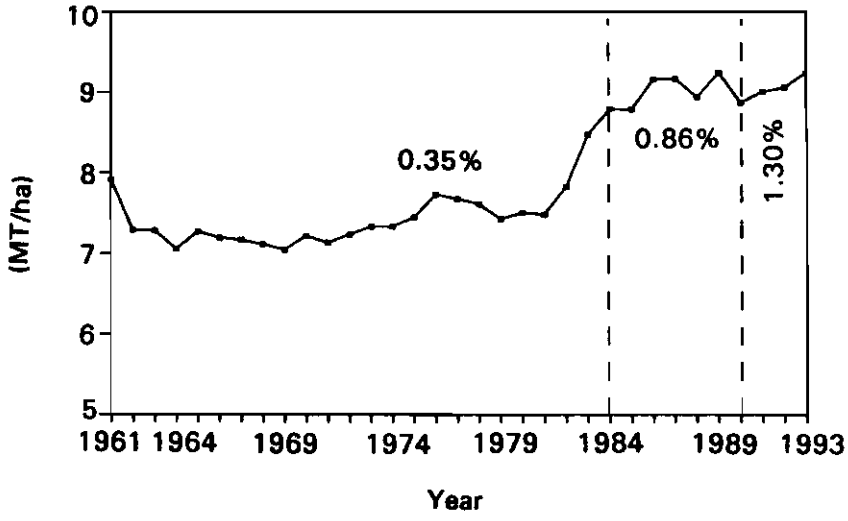

Figure 3. Vietnam cassava yield and growth, 1961-1993. Percentages refer to annual growth rates for 1961-1984, 1984-1989 and 1989-1993.

SOURCE: FAO, 1994.

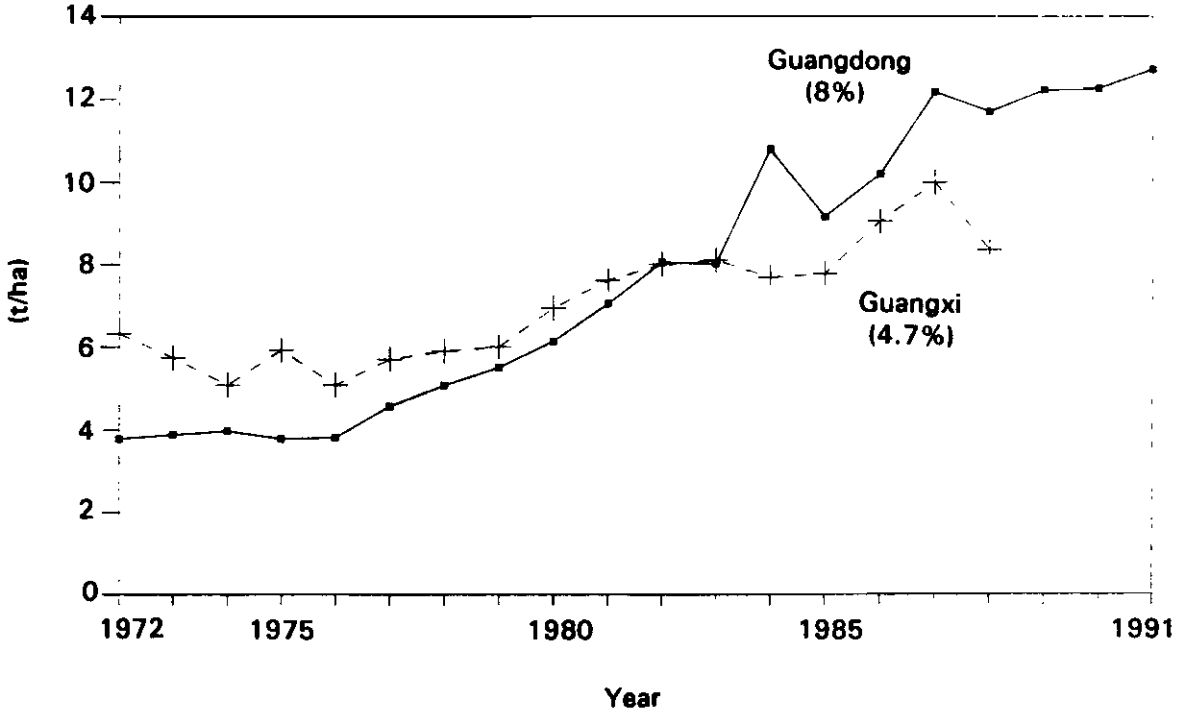

Figure 4. Average cassava yields and annual growth rates (%) in Guangdong and Guangxi provinces, People's Republic of China (1972-1991).

SOURCE: Shuren and Henry, 1993.

DETOXIFICATION OF CASSAVA CYANOGENS DURING PROCESSING: AN OVERVIEW OF RESEARCH AND COMMERCIAL PRACTICES IN ASIA

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Cassava processing practices vary widely among Asian countries, and are influenced by the pattern of use in each country. In countries like India, Indonesia, and the Philippines, where most cassava is used as food or feed, processing practices aim at maximum removal of cyanogens. Cassava is a more commercial crop in Thailand, China, and Malaysia and, hence, little emphasis is given to cyanogen removal. Despite differences in processing for various products, the result is the same: an interaction between glucosidases and cyanogens that releases free cyanide, which, being volatile, is rapidly lost. Processing techniques generally adopted throughout Asia are drying, boiling, and fermentation. Sun-drying is the most ubiquitous, and removes 50% to 70% of cyanogens. Residual cyanide in cooked cassava depends on such factors as period of boiling, volume of water, and chip size. As much as 50% to 72% of cyanogens can be eliminated through cooking. Products from fermented cassava are mostly consumed in Indonesia and the Philippines. Fermentation is highly effective in detoxifying cassava. The retting permits rapid hydrolysis of linamarin, possibly complemented by microorganisms releasing beta-glucosidases. As much as 94% of cyanogens can be removed when cassava roots are fermented for 72 h, changing the steeping water at 48 h, and then sun-dried. High concentrations of cyanogens deter effective exploitation of cassava leaves and peelings for animal feed. If leaves are cut and left to wilt for 16 to 24 h before drying, most of the cyanogens will be eliminated. Fermentation, followed by sun-drying, effectively detoxifies cassava peelings, removing about 97% of cyanogens. Although cyanogen removal is vital for the safe use of cassava, the benefits obtained must balance against the loss of nutrients that occurs in certain processes.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the primary or secondary staple for about 500 million people in tropical countries (Cock, 1985). It is cyanogenic, containing linamarin and lotaustralin in almost all parts of the plant. Although the edible tuber-like root generally contains low levels of cyanogens, levels vary according to variety. The starchy roots are used for both human consumption and animal feed, while peelings and leaves are mostly used only as animal feed. Cassava roots are consumed after boiling, baking, or frying in most Asian countries, whereas fermented products are popular in Africa, Latin America, Indonesia, and the Philippines (Coursey, 1973; Lancaster et al., 1982). Traditional processing practices reduce cyanide, but leave small amounts of residual cyanide (Cooke and Maduagwu, 1978; Padmaja, n.d.). Toxication from the consumption of insufficiently

processed cassava occurs during famines or when cassava products are consumed by a chronically malnourished population (Rosling et al., 1992).

THE IMPORTANCE OF PROCESSING CASSAVA

Cassava roots are traditionally processed by several methods in Asia, varying among countries according to the pattern of use in a given country. In countries where roots are mostly used as food or feed, more effort is given to cyanogen removal. Cassava is a commercial crop in Thailand, China, and Malaysia, where less importance is given to cyanogen removal. Residual cyanide in processed cassava exists as glucoside, cyanohydrin, or free cyanide. The three forms probably exist as an easily dissociable complex. Rosling et al. (1992) observed that residual cyanohydrins in processed cassava were the major source of cyanide in the diet. Processing is thus vital for ensuring the safe consumption of cassava products.

ASIAN CASSAVA PROCESSING PRACTICES AND CYANOGEN DETOXIFICATION

Cassava roots

Table 1 shows common cassava-root processing practices in Asia. Drying is the most ubiquitous method; it improves the shelf life of roots and eliminates cyanogens to an appreciable degree. Nambisan and Sudaresan (1985) compared, on a percentage basis, the efficacy of sun-drying and oven-drying in removing cyanogens from thick (10 mm) and thin (3 mm) chips. They found that sun-drying eliminated more cyanogens than oven-drying because of lengthier contact between linamarase and glucosides. Thick chips retained less cyanide than did thin chips.

Varietal variations significantly affect cyanogen removal during any processing method. The effect of certain moist and dry heat methods, simulating common Philippine cassava-processing practices, was studied by Fukuba et al. (1984). About 55% to 72% of cyanogen was removed from three varieties during 10 minutes of boiling: only 27% was removed from 'Hawaiian 4', compared with 72% for 'Carmen Singapore'. However, 72% of cyanogens were eliminated from 'Hawaiian 4' during 90 seconds of microwave cooking, compared with 44% from 'W 238' (Table 2). Such variations may result principally from differences in the quantity of linamarase in roots and/or in the thermal stability of linamarase isozymes in various cultivars.

Unfortunately, most studies concentrate on quantifying cyanogens in fresh and processed roots, paying little attention to linamarase activity in processing. Dry heat treatments, such as baking and frying, are least effective in removing cyanogen (Table 2). Factors that affect cyanide detoxification during boiling are

length of heating and chip size. Fukuba et al. (1984) observed remarkable variation in cyanogen removal when chips were put to cook in boiling water, compared with chips put in cold water and the temperature slowly raised to boiling. More cyanogen was removed in the latter case (80%) than in the former (70%). More contact time (between linamarase and glucosides) before the enzymes became inactivated thus helps ensure more efficient cyanogen detoxification.

Modifying processes to increase cyanogen removal

Parboiling of cassava roots is practiced mostly in India to improve the shelf life of processed cassava. Conventionally, cassava chips are added to boiling water, cooked for 10 minutes, drained, and then sun-dried. "Yuca rava" and "porridge" are two novel food products made from parboiled cassava chips at the Central Tuber Crops Research Institute (CTCRI) (Padmaja et al., 1994a). When high cyanide cultivars (e.g., H 165) were used to make these products, high residual cyanide (130-150 mg of cyanide per kilogram of dry matter) resulted. The process was modified by grating the roots, exposing the gratings to room temperature for 4 h, and parboiling by adding the gratings to cold water and slowly heating. The resulting product had only 30-40 mg of cyanide per kilogram of dry matter.

In the Philippines, cassava roots are processed into pastries and cakes. These products contain 27 to 84 mg/kg cyanide (Fukuba et al., 1984). "Suman" is a traditional food made from cassava by grating peeled roots, expressing the juice from the pulp, adding sugar to the residue, and steaming it. "Suman" so prepared contains 109 mg/kg cyanide. The traditional process was modified by soaking the pressed residue in water for 30 minutes, squeezing out the water, and adding sugar and steaming. This slight modification gave a product with 42 mg/kg cyanide.

In Indonesia, residual cyanide in cassava flour and shredded cassava can be reduced by introducing soaking and pressing steps in flour preparation. Soaking peeled roots in 0.1% benzoate or sulfite reduces cyanide in the resultant flour (Widowati et al., 1992).

Fermentation

Microbial fermentation of cassava roots improves the shelf life of flour because of the organic acids formed during the process. It also improves the textural qualities of the flour and reduces the level of cyanogens (Arihantana and Buckle, 1987; Ayernor, 1985; Bokanga et al., 1988; Moorthy et al., 1993; Padmaja et al., 1993). Although traditional products made from fermented cassava are popular in Africa, they do not form a significant part of Asian diets, except in Indonesia, Malaysia, and the Philippines.

One factor deterring the use of fermented-cassava products is the obnoxious smell emanating from the vats where roots are fermented. The CTCRI undertook

studies to control the smell, and so make products from fermented cassava more acceptable (George et al., 1991). A fermentation inoculum was made from palm wine and yogurt, and contained *Lactobacillus* sp., *Streptococcus* sp., *Corynebacteria* spp., and yeast. It fermented cassava roots within 72 h, imparting a flavor to the product.

The inoculum's ability to detoxify cassava was also studied (Padmaja et al., 1993) in cultivars with either low or high cyanide contents. Compared with unfermented roots (incubated at room temperature with an antimicrobial combination), fermented roots had many fewer bound glucosides. Whether the fermentation microorganisms induce cyanogen hydrolysis by softening roots or complement hydrolysis by releasing beta-glucosidases is not yet resolved (Ikediobi et al., 1985; Padmaja et al., 1993; Westby and Choo, 1994).

Our studies showed that, *in vitro*, beta-glucosidase activity in fermented cassava roots is enhanced, compared with that of unfermented roots. The enzyme is evidently extracted in full from fermented as well as unfermented roots during the *in vitro* assay. The high *in vitro* activity of beta-glucosidase in fermented roots thus reinforces the view that microbial beta-glucosidases also complement linamarin hydrolysis. We concluded that 72 h fermentation alone was not sufficient to detoxify roots with high cyanide contents.

In subsequent studies, we modified the process by changing the steeping water at 48 h, and sun-drying the fermented product. As much as 94% of cyanogens could be removed from roots with high cyanide contents (G. Padmaja and Mathew George, unpublished data). In contrast, only 70%-74% of cyanogens were removed from unfermented roots (Table 3). In unfermented roots, incubation contributed only 30%-32% to removing cyanogens and the remaining 40%-42% was eliminated during sun-drying. But, for fermented roots, sun-drying played only a minimal role in cyanogen elimination, with fermentation removing almost all (Table 3). Fermentation is the most effective processing technique for detoxifying roots with high cyanide contents.

"Tapé ketela" is a traditional Indonesian food prepared from fermented cassava roots. Arihantana and Buckle (1987) studied the effect of fermentation, using a ragi inoculum, on cyanogen removal in the making of "tapé." They found that, when steamed cassava was fermented with 0.3% (w/w) ragi inoculum, 72%-80% of cyanogens were eliminated, whereas from steamed cassava alone, only 44%-62% of cyanogens were removed.

In Malaysia, "tapai ubi" is made by fermenting, in the solid, peeled and cooked roots inoculated with a ragi inoculum (Isa and Khatijah, 1992).

Cassava leaves

Consumption of protein-rich cassava leaves can significantly improve the nutrition of people subsisting primarily on cassava. Leaves are used as a vegetable in many African countries and in the Philippines. In most Asian countries, however, leaves are used for animal feed. Unlike the roots, leaves contain high levels of cyanogens, needing effective processing for their safe use as feed.

Drying is the most widely practiced technique for cyanogen removal. Padmaja (1989) studied the effect of drying temperatures on cyanogen removal in the leaves of five cassava cultivars. Maximum cyanogen removal (78%-86%) occurred at 60 °C. First wilting whole leaf blades enhances cyanogen removal (Table 4). When wilted leaves were chopped just before drying, less cyanogen was eliminated, because of rapid drying. In contrast, when chopped leaves were left to wilt for 3 days before sun-drying, as high as 96% of cyanogens were removed (Ravindran, 1993).

Fukuba et al. (1984) studied the degree of cyanogen removal from cassava leaves during traditional Philippine cooking. Variety appears to affect cyanogen removal more in processes like boiling and blanching, which involve shorter contact time between linamarase and glucoside than in processes like drying, where linamarase can act over more time.

Cassava peelings

Cassava peel is a fibrous, low-energy byproduct of cassava processing. It can be effectively used as animal feed. FAO (1985) estimates production to range from 13 to 26 million metric tonnes per year. Peelings contain more cyanide than does pulp (Dufour, 1988; Padmaja et al., 1993; Tewe et al., 1976). Cyanogen removal from peelings has been attempted mostly in Africa and, to a limited extent, in India (Padmaja et al., 1993). We found that fermenting cassava peelings, followed by sun-drying, can eliminate as much as 97% of cyanogens, whereas sun-drying alone removes only 73%-80% of cyanogens (G. Padmaja and Mathew George, unpublished data) (Table 5).

NUTRITIONAL TRADE-OFFS IN CASSAVA PROCESSING AND COSTS OF REMOVING CYANOGENS

The safety limit for cyanogens in cassava products has been set at 10 mg of cyanide equivalents per kilogram of dry weight (Codex Alimentarius Commission, 1989). To achieve this target means strict adherence to rigorous processing practices, involving elaborate steps.

Those processing techniques, such as boiling, soaking, and fermentation, that involve steeping roots are likely to leach out soluble nutrients like amino acids, proteins, minerals, and vitamins. Drying, roasting, baking, and frying can cause a high loss of heat labile vitamins. Because of the need to maintain low contents of residual cyanide in processed cassava, the compromise between the loss of nutrients and cyanogen removal often becomes difficult.

Asian literature on the loss of nutrients during cassava processing is scarce. When we compared flour made from sun-dried cassava with that from fermented cassava, we found a 32% reduction in crude protein, a 76% reduction in ash content, and significant reductions in all amino acids in flour from fermented cassava (Padmaja et al., 1994b). Our study showed that products made from fermented cassava must be fortified to upgrade their food value.

Table 6 compares nutritional and other advantages or disadvantages of Asian cassava-processing practices. The more cyanogens removed by a process, the higher the loss of nutrients and processing costs. However, the health hazards involved in consuming insufficiently processed cassava for populations subsisting on cassava demand that cyanogen removal be given priority over costs.

Table 7 ranks Asian cassava-processing practices according to processing costs and effectiveness in removing cyanogens. For cassava roots with low and medium cyanide contents, boiling and drying are probably the most effective processing methods in that they are not costly and are reasonably effective in eliminating cyanogens. Fermentation is more effective for peelings and roots with high cyanide contents.

Cassava consumers must be made aware of the need for rigorous processing practices for roots with high cyanide contents, simultaneously with the need to balance cassava products with proteins and minerals. Genetic manipulation leading to acyanogenic cultivars that retain the plant's genetic architecture for resistance against pests and diseases may be the only permanent solution to cassava toxicity. But the possibility of evolving such cultivars in the near future seems remote. Hence, postharvest processing emerges as the most practical approach to combat cassava toxicity, especially in developing Asian countries.

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Table 1. Common cassava-processing practices in Asia.

Country	Processing practices[a]
India	Boiling, baking, frying, drying
Indonesia	Boiling, drying, fermentation
Philippines	Boiling, frying, steaming, baking, fermentation, drying
Malaysia	Boiling, baking, drying, frying, fermentation
Sri Lanka	Boiling, drying, frying
Vietnam	Boiling, drying, baking, latex-free cassava pellets
Thailand	Drying for flour and starch
China	Drying for flour and starch

a. Drying is either sun-drying or oven-drying.

Table 2. Quantity of cyanogen removed (%), according to type of heat treatment.

Cassava cultivar	Treatment						
	Moist heat[a]		Dry heat[a]		Moist heat[b]	Dry heat[b]	
	10 min. boiling	10 min. boiling after 15-24 h soaking	10 min. steaming	90 sec. microwave cooking	30 min. boiling	20 min. baking at 110 °C	5 min. frying
Carmen Singapore	72	79	37	47			
W 238	62	74	27	44			
Bogor 397	55	67	78	62			
Hawaiian 4	27	45	22	72			
H 165					45	13	11
H 2304					47	15	9
H 1687					47	15	15

a. Reproduced from Fukuba et al., 1984.
b. Computed from Nambisan and Sundaresan, 1985.

Table 3. Contribution of incubation or fermentation (72 h) versus sun-drying toward cyanogen removal from cassava roots.

Cassava cultivar	Treatment[a]	Percentage of cyanogens removed	Contribution (%) by:	
			Incubation or fermentation	Sun-drying
H 97	T1	71	31	40
	T2	92	92	<1
	T3	90	90	<1
	T4	30		30
H 165	T1	74	32	42
	T2	93	93	<1
	T3	93	93	<1
	T4	44		44

a. T1 = Roots incubated with an antimicrobial combination containing thiomersal, streptomycin, and penicillin.
T2 = Fermented with a mixed culture of inoculum with water changed at 24-h intervals.
T3 = Fermented with water changed at 48 h only.
T4 = Sun-dried only.

SOURCE: G. Padmaja and Mathew George, unpublished data.

Table 4. Effect of wilting and drying on cyanogen removal from cassava leaves.

Cassava cultivar	Percentage of cyanogens removed			
	Wilted whole leaves			Wilted chopped leaves
	16 h, oven-dried at 60 °C[a]	Chopped, oven-dried at 60 °C[a]	3 days, sun-dried[b]	3 days, sun-dried
M 4	87	77		
H 1687	89	80		
H 165	89	86		
H 226	89	87		
H 2304	92	80		
Unspecified			94	96

a. Reproduced from Padmaja, 1989.
b. Reproduced from Ravindran, 1993.

Table 5. Contribution of incubation or fermentation (120 h) versus sun-drying to cyanogen removal from cassava peelings.

Cassava cultivar	Treatment*	Percentage of cyanogens removed	Contribution (%) by:	
			Incubation or fermentation	Sun-drying
H 97	T1	94	74	19
	T2	97	87	9
	T3	97	87	10
	T4	81		81
H 165	T1	90	65	25
	T2	98	86	12
	T3	97	85	12
	T4	73		73

a. T1 = Incubated (120 h) with antimicrobial combination.
T2 = Fermented (120 h) with water changed at 24-h intervals.
T3 = Fermented (120 h) with water changed at 48-h intervals.
T4 = Sun-drying only.

SOURCE: G. Padmaja and Mathew George, unpublished data.

Table 6. Nutritional and other advantages or disadvantages of Asian cassava-processing practices.

Process	Advantages	Disadvantages
Drying (fresh cassava chips)	Appreciable reduction in cyanogens	Insect infestation and mild loss of vitamins
Drying (parboiled chips)	Moderate reduction in cyanogens; high shelf life; least insect infestation	Possible leaching of nutrients during parboiling and loss of vitamins; high residual cyanide
Boiling	Appreciable reduction in cyanogens	Mild loss of vitamins and soluble nutrients
Baking	Minimal loss of nutrients	Very high residual cyanide
Frying	Good storability; consumer acceptability	Very high residual cyanide
Fermentation	Most effective for cyanogen removal	Loss of nutrients; fortification needed

Table 7. Comparative assessment of Asian cassava-processing practices (based on operational costs and effectiveness in cyanogen removal).

Cost intensiveness (operational costs not on absolute money terms)	Process	Effectiveness of cyanogen removal
Least intensive	Sun-drying and oven-drying	Moderate
Slightly intensive	Drying (parboiled chips)	Slight
Least intensive	Boiling	Moderate
Slightly intensive	Baking	Least
Slightly intensive	Frying	Least
Most intensive	Fermentation (mixed culture inoculum; modified process)	Most

BIOTECHNOLOGY APPLICATIONS IN CASSAVA RESEARCH AND DEVELOPMENT

Cassava Processing, Quality and New Products

CASSAVA STARCH, STRUCTURE, PROPERTIES AND IMPLICATIONS FOR CONTEMPORARY PROCESSING

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INTRODUCTION

Cassava is one of the most important sources of metabolic energy for 500 million people in the tropics. Starch is the major component and source of calories constituting from 73.7% to 84.9% of the dry root weight. The production of cassava is no longer a cottage industry, by means of large-scale operations, Thailand, Brazil, the Philippines and Indonesia produce 80% of the world's starch. This paper briefly describes the important features of the starch granule and cassava starch in particular, and possible opportunities for its exploitation.

Generalized structure of starch granules

The starch granule, depending on its source, is an organized agglomerate of a substantially linear α-1,4-polyglucan (amylose) and a larger, highly branched molecule with linear α-1,4-polyglucan chains (DP<100), linked α-1,6 to other chains. The granules also possess lipid and phosphorus (<2.0%), the amounts of both depending on the species and source. On heating in excess water (>4:1 H_2O:starch), the granules lose their order over a characteristic temperature range for cassava (~59-60 °C) and swell. At reduced water contents, the order is also lost but only on heating to higher temperatures.

Investigations into the macromolecular components have shown that these are organized with distinct architecture. In general the polymer chains are radially oriented. The effect of diurnal variations in light and/or temperature results in annular differences in density of the carbohydrate substance, and in some starches (e.g., potato starch) this shows clearly under the optical microscope. The radial distances between such rings is typically 100-400 nm and the structure that is envisaged is composed of a series of crystallites (Figure 1). Figure 2 shows a more detailed picture of such crystallites. They consist of domains of parallel-packed double-helices but also with disordered regions within or between the crystallites which may contain amylose or non organized regions of amylopectin chains. A possible location for the lipid is also indicated (Blanshard, 1987). The dimensions of the crystallites within the starch granule seem to be remarkably invariant (Blanshard et al., 1984).

Distinctive features of cassava starch

At the phenomenological level cassava starch offers four positive qualities: the clarity of the gel, superior to all starches other than potato (Craig et al., 1989); excellent thickening—i.e., swelling capacity—(but again inferior to potato starch (Figure 3); a desirable neutral flavour advantageous for delicately flavoured products; and the production of good textural quality, puffed half-products (e.g., keropok) in hot oil.

On a more precise scientific level, a number of properties, as a profile, distinguish cassava from other starches.

Cassava starch is highly susceptible to oxidative depolymerization in the presence of sulphur dioxide

In earlier studies, Mitchell et al. (1991) found that thermal processing degraded galactomannans, resulting in a substantial fall in viscosity. The addition of appropriate antioxidants could prevent this. But contrary to expectations, sulphur dioxide at concentrations < 100 ppm (0.01%) seemed to facilitate cassava starch degradation, whereas at higher levels this process was reversed (Figure 4) (Mat Hashim et al., 1992). The tuber and root starches—potato and sago—paralleled cassava starch, while the cereal starches were more resistant. Heating the tuber and root starches to 121 °C with 0.01% SO_2 resulted in their total solubilization, but not so the cereal starches. Adding the anti-oxidant, propyl gallate, removed this effect. The presumption is that, in the presence of oxygen and small quantities of metal ions, SO_2 acts as a proxidant promoter of free radicals, while at higher levels it exercises a reducing function with normal concentrations of aqueous O_2. Bubbling O_2 through the system extends the proxidant role to higher SO_2 concentrations. But clearly the SO_2 is promoting intragranular breakdown of the macromolecules which then readily perfuse into the surrounding medium.

Native cassava starch is more readily digested *in vivo* and *in vitro* than native potato starch

G. Norton and co-workers have shown in extensive studies (personal communication, 1994) that native potato starch is resistant to digestion within the stomach and ileum of the rat, whereas cassava starch is 99% to 100% digested. In this connection it is interesting that potato starch has a B X-ray pattern, while cassava starch has a C_A.

Cassava starch, by repute, is particularly effective in the expansion of starch products such as kerapok

The expansion of gelatinized cassava starch produces kerapok. Boiling the sample in water (followed by drying), or extrusion brings about the prior

gelatinization. If for simplicity's sake we consider the expansion of one cell from a nucleus, then an equation can be written in the form:

$$P_b - P_a = \frac{2\sigma}{R} + 4\eta a \frac{\dot{R}}{R}$$

P_b is the vapour pressure inside the gel, P_a that outside. The rate $\dot{R}$ is a function of surface tension (having a minimal effect except at very small values of R) and the viscosity of the surrounding medium. Thus, $\dot{R}$ may well reflect the structure, composition, hydration and degree of disintegration of the granular structure. Equally important is the dependence of the mechanical properties on the water content, both in terms of minimizing shrinkage and of providing the desired organoleptic qualities of the expanded product.

Variation in starch properties between and within cassava cultivars

It is also important to establish whether there is significant variation among different cultivars, whether environment exercises an effect, and what is the basis for such differences. It is well known that the organoleptic quality of whole, cooked cassava roots may differ considerably depending on the cultivar, the environment/season, and cultivation (e.g., use of pre-harvest pruning). But, surprisingly, Asaoka et al. (1991, 1992, 1993) were unable to find any significant structural or physicochemical differences in the extracted starch that would account for the substantial differences in root quality.

If differences do exist in the behaviour of the starches in a given process, they must have a structural basis. This may lie in the differences in the fine structure of the macromolecules themselves, the relative concentration of the molecules in the granule, the uniformity (or otherwise) of their distribution, and the architecture and macromolecular organization.

The results of Asaoka et al. and further studies by A. Fernández (personal communication, 1994) have shown some differences in amylose content but only minimal differences in x-ray crystallinity among different cassava starch samples. Ong and Blanshard (n.d.) correlated the fine structure of rice amylopectin with the organoleptic texture of parboiled rice. In comparison, the amylopectins of 30 cassava starch cultivars have shown a remarkable lack of variation. In parallel studies of the starches of these cultivars, the swelling behaviour and the resulting gel strengths may be very different (Figure 5). The sample M4 is a particularly clear example of the variation, grown in two locations with identical amylopectins but behaving differently on gelatinization in terms of swelling volume and solubility (Table 1). It is possible that the other major component (amylose) may differ in

structure sufficiently among different cultivars, or different environments may moderate it, for it to the cause of these substantial differences in behaviour. Further research should elucidate this point. An explanation perhaps more difficult to establish is the possibility that the macro-architecture within the granule may be different. Just as two houses may be of similar size with the same percentage of bricks and roofing tiles but of very different construction, so also the architecture of the starch granule may differ substantially, depending on genetic or environmental factors prevailing during its development.

The potential for genetic manipulation is, of course, substantial; and the changes accomplished in maize, plus the experience gained in the more similar potato tuber, suggest that significant modifications can be induced.

Contemporary processing possibilities

At the start of this section it is important to note at least two factors. First we need to evaluate if cassava starch in terms of its production, extraction and properties can compete with maize and potato starches on world markets. This, of course, can radically change over a relatively short period. Second we need to assess the technical possibilities of modifying cassava starch to fulfil other objectives and determine if this is economically feasible. In those countries where cassava starch is a valuable natural resource, there may be many good reasons why it should be used relative to other materials. But it should be emphasized that in much of the scientific and patent literature, cassava starch is regarded as not much distinct from maize or potato starches.

The modification of starches for food purposes

Chemical methods

The chemical modification of starches through a process of addition of different groups is principally believed to occur on the surface of the starch granules. The effects are frequently substantial and important in food processing.

Cross-linking agents

Cross-linking agents vary in character but must be bifunctional. They include such materials as adipic anhydride, sodium trimetaphosphate and glyoxal. The resultant effects include stabilization of viscosity behaviour, the inhibition of gel formation and resistance to damage by acid, heat, or shear forces. Mancepun and Sirirojana (1993), for example, employed sodium rimetaphospate at pH 10 for cross-linking cassava starch and were able to optimize the reaction time to yield a product with a more stable viscosity and an increased pasting temperature. The product has

proved effective in replacing 50% of mung bean starch (which is x5 the cost of cassava starch) in Grade A mung bean noodles.

Substitution agents

Substitution often involves esterification or etherification and leads to the substitution of a few hydroxyl groups in the starch granule resulting in important changes in properties like improved clarity, viscosity, and freeze-thaw stability, and reduced syneresis. When substitution is by hydrophobic groups, emulsifier properties may result. Richards and Bauer (1977) disclosed a method of making lipophilic modified starch derivatives, e.g., the n-octenyl succinate, suitable for encapsulating fats and oils and producing stable emulsions. Trzasko et al. (1985) more recently developed the use of a reagent with a linear hydrocarbon chain, where the number of carbons is ≥ 12 (e.g., tetradecenyl succinic anhydride) and, with a starch of amylose content $\geq 17\%$, yields a gel that is reversible either by heating to 70 °C or by changing the pH (temporarily) to pH ≥ 13. Cooling or adjusting pH to 1-10 results in redevelopment of the gel.

Rapaille et al. (1988) demonstrated how a careful balancing of substitution (by acetylation) and cross-linking (either by adipic anhydride or sodium trimetaphosphate) can provide starches with a range of properties not only able to withstand the rigorous temperature, pH and shearing conditions of UHT processing but also giving the desired textural qualities and freeze-thaw stability critical in UHT milk desserts. Although the study was performed with maize starches, the general principles clearly apply to starches from other sources.

More recently, the potential of using the barrel of an extruder as a chemical reactor during extrusion has been explored in contrast to conventional methods of cross-linking and substitution, where the process takes place at the granule surface. Della Valle et al. (1991) investigated the production of a cationic starch using a pilot-scale twin-screw Clextral BC-45 extruder as a reactor, with 3-chloro-2-hydroxypropyl-trimethyl ammonium chloride as the reagent, and sodium hydroxide as the catalyst. Analysis of the extrudate by ion exchange chromatography showed that while conventionally prepared commercial samples had up to 25% of substituted macromolecules with a DS of 0.055, the extruded product had up to 75% substituted macromolecules with a DS of 0.09 (Figure 6). This gave dramatic reductions in hot paste consistency.

Biochemical methods

Enzymic methods for breakdown and conversion of starches to glucose and fructose and their associated syrups (containing in addition a whole range of malto-oligosaccharides) are well known and the basis for substantial industries. Casey (1977) gives a convenient history of these developments. Kimura et al. (1986) reported techniques for producing specific oligosaccharides using immobilized amylases from different sources. Kafer et al. (1987) described the production of

starch hydrolysates containing β-limit dextrins using a carefully designed protocol with successive treatments by β-, α- and β-amylase. The resultant product contains 44% β-limit dextrin, 44.5% maltose and 1.5% maltotriose and has properties promoting texture development and the stabilization of frozen and dried food systems, as well as contributing as a sweetener.

Starch-based fat substitutes has been a particularly active area of development. Maltodextrins are particularly useful in this area. N-oil (a tapioca dextrin), one of the first to be developed, can be used to partially replace butter fat in rich-tasting frozen deserts. As the energy value of fats is approximately 38 kJ/g, and that of maltodextrins only 16.5 kJ/g, when a maltodextrin is used at the frequently recommended concentration of 20% the energy contribution is only 3.3 kJ/g which is a notable reduction in the energy content of a diet.

Subtle modifications of starch structure can be introduced by either enzymic or heat treatments, for example α-1,3- and β-1,4- linkages that are indigestible rather than the conventional α-1,4- and α-1,6- that make the molecules less accessible to digestion within the stomach but do provide "resistant starch" for bacterial breakdown in the colon (Ohkuma et al., 1991).

Physical methods

Chiu (1982) described the preparation of a drum-dried and subsequently heat-treated tapioca starch dispersible in cold water but thereafter without further significant heating rapidly forming a gel, whose strengths are controlled by the heating regime adopted in starch preparation.

The modification of starches for non food uses

There are enormous commercial uses for starches in the industrial sector. Some have been in existence for many years, others are rapidly developing. Some of these are briefly surveyed below.

Biodegradable plastics

In Europe some 199 million tons of municipal solid waste is produced each year, of which 5%-10% by weight is synthetic plastic, but this forms some 25%-30% by volume posing severe problems for landfill disposal. So there is a strong motivation to develop biodegradable packaging. Areas that have been explored include encapsulation (Eith et al., 1984; Wittwer and Tomka, 1984; Carr et al., 1990) and destructured starch by itself (employing sodium, potassium or calcium hydroxides and plasticizers such as glycerol, PEG—MW 220 to 4000—, ethylene glycol and sorbitol, Bastioli et al., 1990) or with other materials e.g., Zein for packaging materials and disposable bags (Cole and Daumesnil, 1988).

The fact that a number of companies have produced loose-fill materials for packaging based on potato or maize starch suggests that this is an area that can be explored with cassava starch.

Detergents

Koch et al. (1993) outline the potential of starch derivatives as the so-called "builders" and "co-builders" in detergents. These materials complex Ca^{2+} and Mg^{2+} ions responsible for the temporary hardness of water. If these are not removed, on the industrial scene they are responsible for insoluble carbonates which have a deleterious effect both on the fabrics and on metal surfaces through the formation of scaling. To lessen this effect the initial use of polycarboxylic acids derived from copolymers of acrylic and maleic acids was limited because they are non-biodegradable. This problem has been overcome, however, where these units have been graft copolymerized onto a starch backbone (Yamaguchi et al., 1990) (Figure 7). An alternative approach has been to induce oxidation cleavage of the C2-C3 bond in the glucopyranose ring which yields a product able to form a chelate type structure with divalent cations such as Ca^{2+} or Mg^{2+} (Floor et al., 1989).

Super-absorbent polymers (SAPs)

The combination of the absorption properties of polyacrylates with the hydrophilic properties of starches, by a process of grafting partially neutralized acrylic acid onto the backbone of the starch molecule followed by cross-linking, has produced an expanded type of structure with remarkable absorbent properties. These materials can be used in high-value health care products, e.g., pads for geriatric incontinence as well as baby diapers.

CONCLUSION

Cassava starch is an important member of the various starches on the world market. Expansion of its use depends on the ability to produce a high-quality product at competitive prices, exploit its particular characteristics, and develop high-value derivatives which are widely recognized for their commercially advantageous properties.

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Table 1. Swelling and solubility of cassava starch. M4 Starch.

	India	Malaysia
Swelling volume (ml/100 ml)	33.3	24.3
Solubility (% d.b.)	30.4	53.3

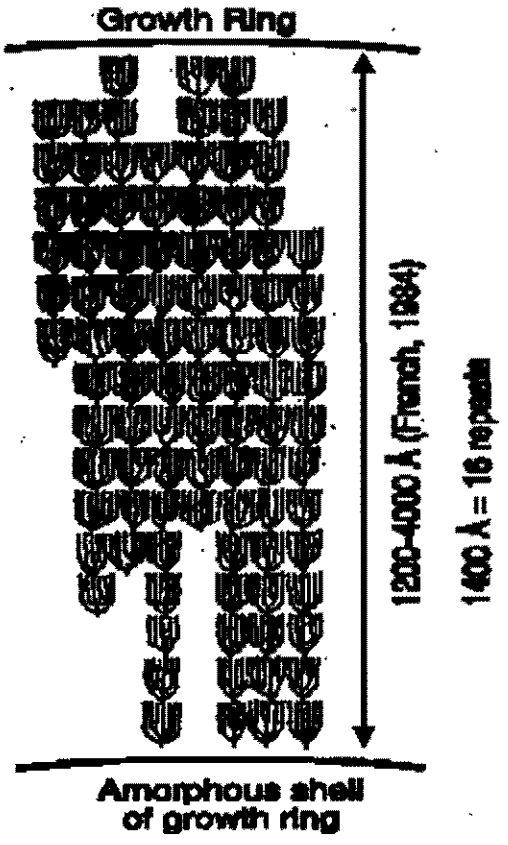


Figure 1. Series of crystallites within starch granule.

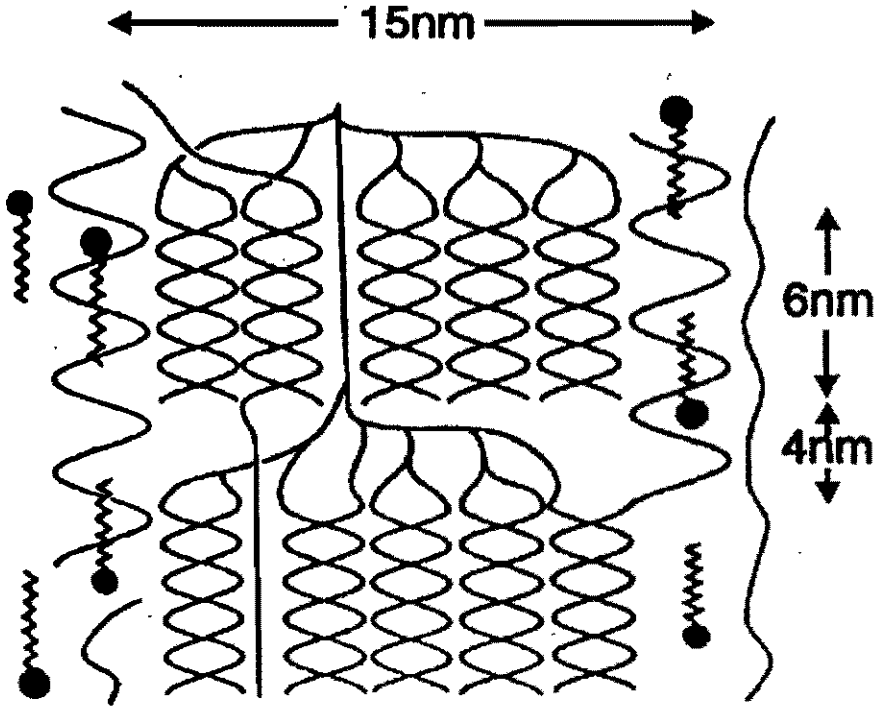


Figure 2. Detail of crystallite within starch granule.

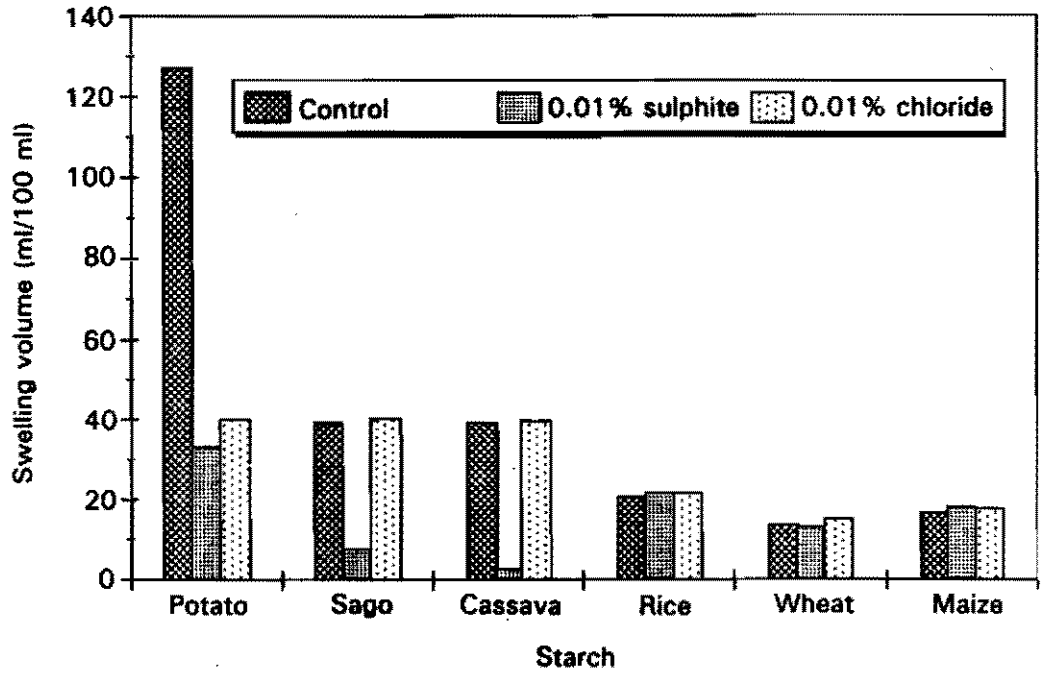


Figure 3. Swelling capacity of cassava starch compared with other starches.

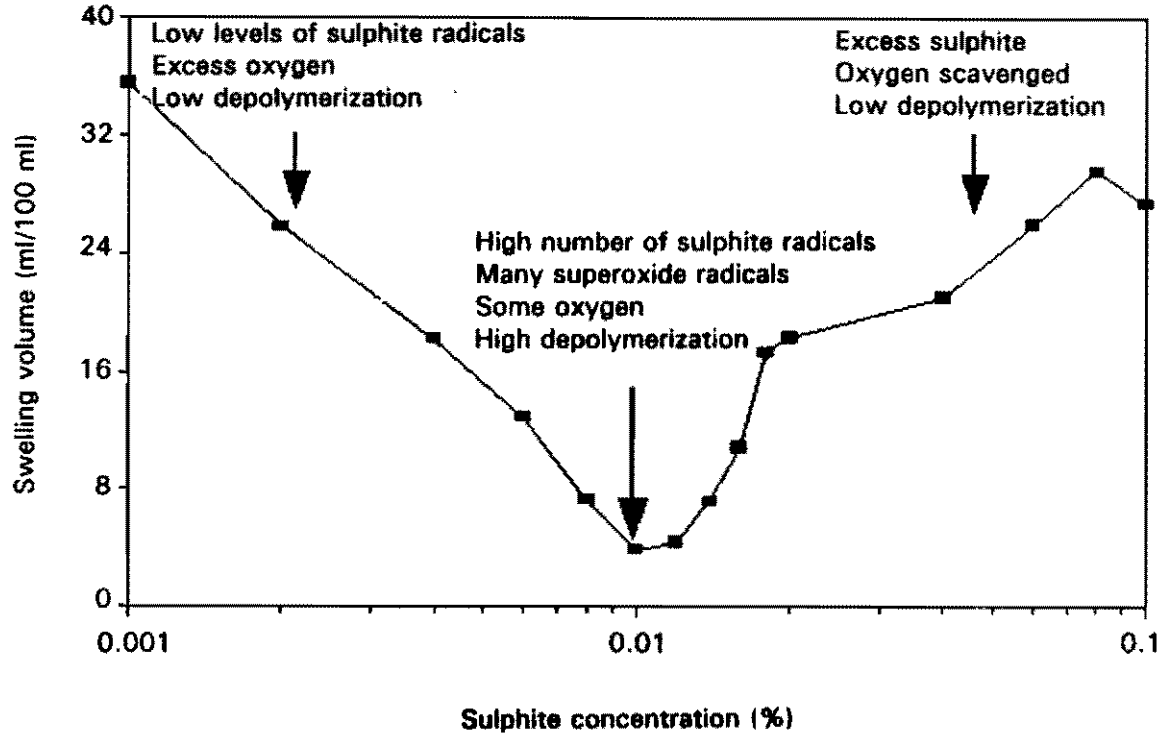
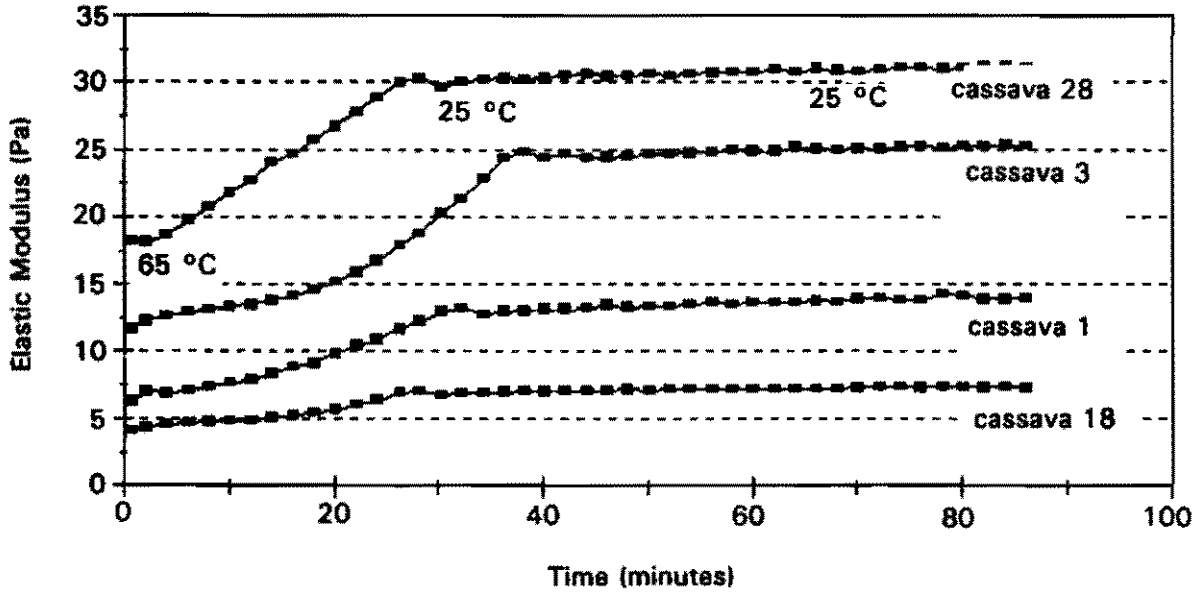


Figure 4. Cassava starch susceptibility to oxidative depolymerization in the presence of sulphur dioxide.



Pastes examined in Bohlin Rheometer in oscillatory mode, the cooling rate was 1.5 C/min from 65 to 25 °C; subsequently the samples were held at 25 °C for 1 h.

Figure 5. The change of Elastic Modulus of 6% cassava starch pastes upon cooling.

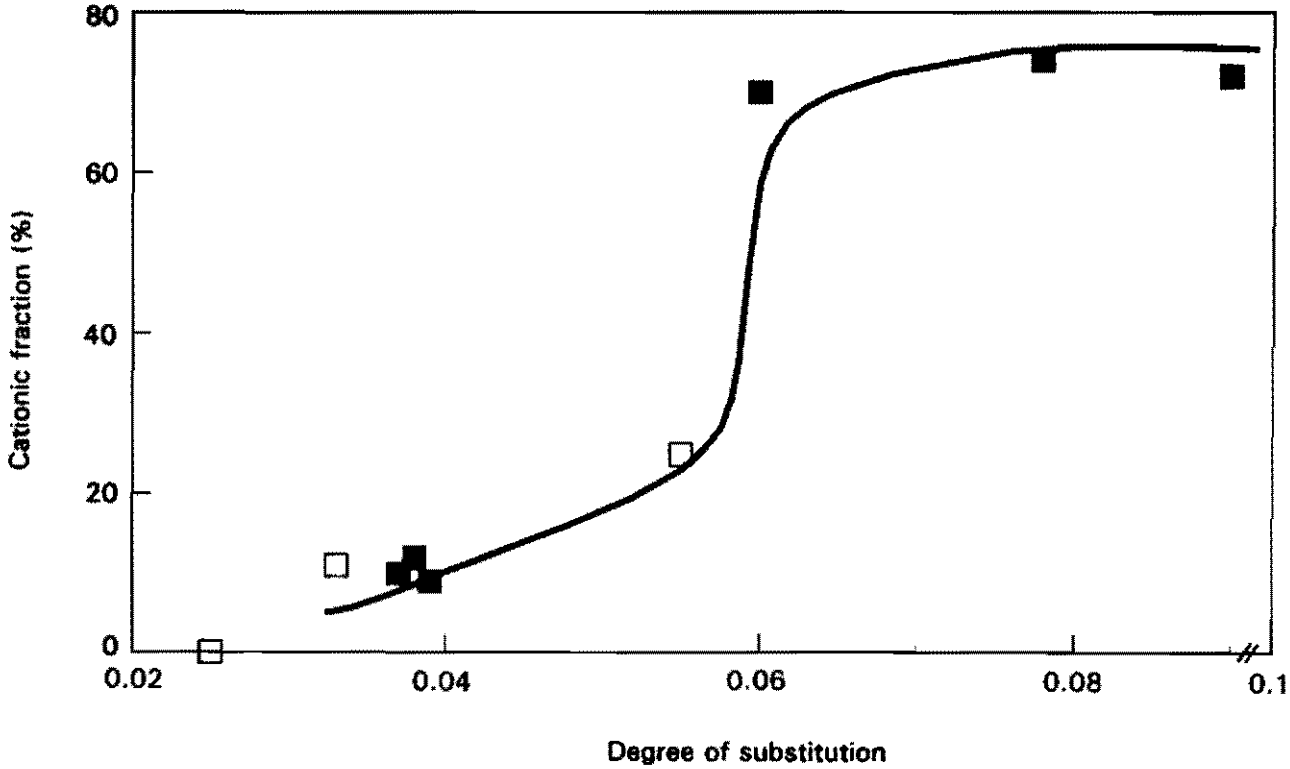


Figure 6. Cationic fraction (determined by GPC) as a function of substitution for extruded (■) and commercial (□) starches.

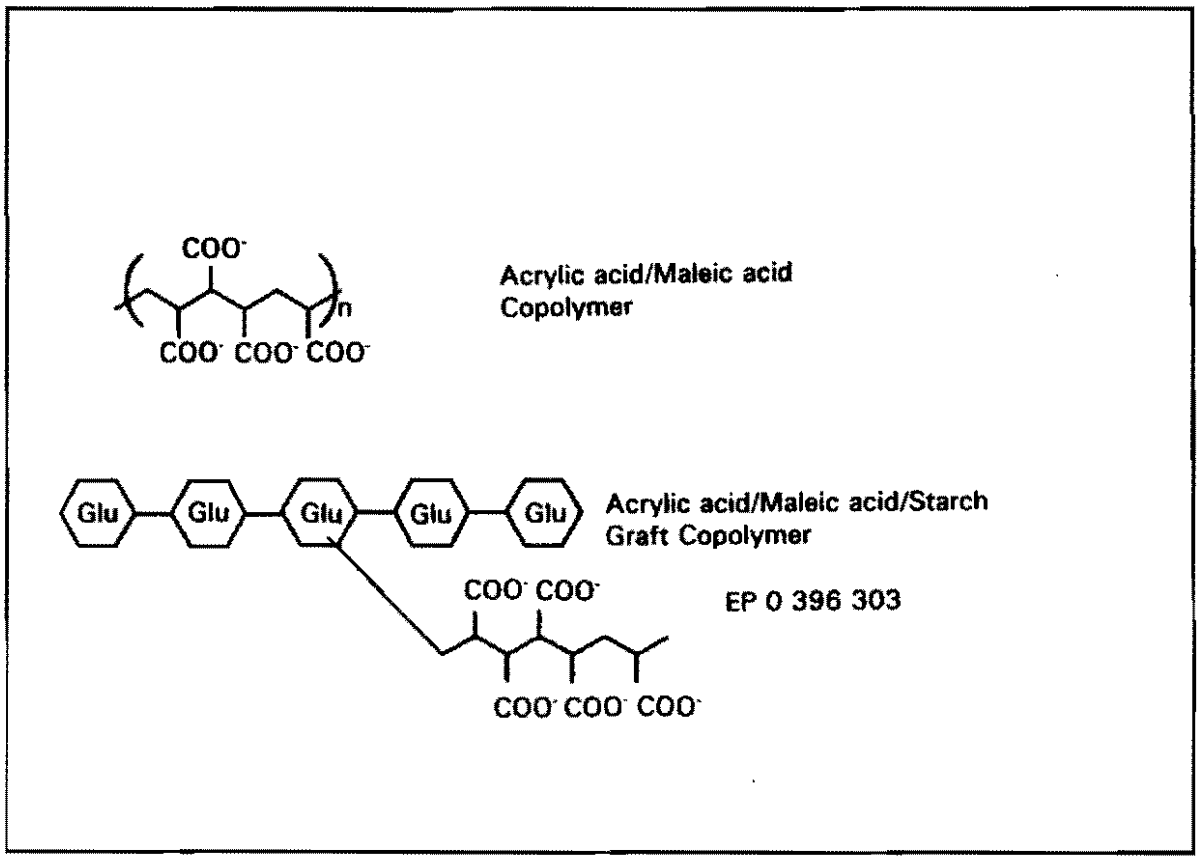


Figure 7. Graft copolymerization onto a starch backbone: polycarbozylate-copolymers.

CLONING AND CHARACTERIZATION OF CASSAVA GENES INVOLVED IN STARCH BIOSYNTHESIS

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Screening of a thickened-root specific cassava cDNA library resulted in several full length cDNA clones of cassava genes involved in starch biosynthesis being cloned. These cDNA clones code for the enzymes Adenosine Diphosphate Glucose Pyrophosphorylase (AGPase) B, S2 and S3 subunits as well as for Granule Bound Starch Synthase II (GBSSII). The complete sequence for AGPase B has been obtained whilst the sequencing and characterization of the other cDNA clones is in progress. The sequence of the AGPase B gene of cassava shares some 68% identity with the potato AGPase B. Analysis of the genomic DNA of allotetraploid cassava has revealed that this is a low copy number gene. As well as helping us fully understand the process of starch biosynthesis, the cloning of these cassava starch genes gives us the opportunity to modify plants genetically to produce specific, novel starches for particular purposes.

INTRODUCTION

During the process of photosynthesis, carbohydrates are produced in mature leaves and transported to sites of carbohydrate demand. These sites include utilization sinks, such as meristematic tissue where the carbohydrates are subjected to respiration or are used in the biosynthesis of other cellular components, and storage sinks, such as roots and seeds where reserve carbohydrates are stored as starch in the form of osmotically inactive, water insoluble granules in amyloplasts and chloroplasts. In cassava (*Manihot esculenta* Crantz) plants most of the starch is stored within amyloplasts in the thickened roots.

In common with other plant starches, cassava starch consists of about 20% amylose and 80% amylopectin (Rickard et al., 1988). The key step in starch biosynthesis in plants is the formation of ADP glucose from glucose-1-phosphate and ATP by the enzyme ADP glucose pyrophosphorylase (AGPase). Starch synthase is responsible for the formation of amylose by adding ADP glucose to an alpha 1,4 glucosyl chain to form a linear molecule. Starch branching enzyme is responsible for the formation of amylopectin by the cleavage of a 1,4 glucan chain and the formation of 1,6 cross linkages. Preiss (1991) identified several isoforms of these enzymes in various plant species.

Cassava starch is important as a component of human diet in the tropics and as feed stock (Jennings and Hershey, 1985). It is also becoming extremely important in the brewing and fermentation industry, and the paper and textile industries (Cooke and Cock, 1989). Thus the manipulation of the physicochemical properties of cassava starch has become an important consideration, one for which we need to understand the processes leading to starch formation and breakdown. The molecular identification and characterization of genes involved in starch biosynthesis in cassava is our major goal. This will provide us with invaluable tools both for analyzing the process of starch biosynthesis and for modifying the quality and quantity of starch produced by cassava plants as we and others have previously shown for potato. In potato, the use of genes encoding the aforementioned enzymes, either in sense or antisense orientation, has led to the production of plants with altered starch composition and content.

MATERIALS AND METHODS

Plant material and bacterial strains

We used cassava genotype M Col22 and Zimbabwean lines in this study. We maintained *in vitro* plant material and multiplied it on basal medium, containing Murashige and Skoog salts and vitamins + 0.5% (w/v) agar (pH 5.7), and 30 g/l sucrose. Irradiance was 40 μmol m^{-2} s^{-1} in the growth chamber. We maintained day temperatures at 28 °C and night temperatures at 24 °C. We cultured and transformed *Escherichia coli* strains DH5 alpha and Y1090 using standard techniques (Maniatis et al., 1982).

Screening of a cDNA library

We screened a cassava M Col22 thickened-root specific cDNA library in lambda gt11 was screened by plaque hybridization for the genes ADP Glucose pyrophosphorylase B and S subunits, and Granule Bound Starch Synthase II. We used as probes the cloned genes from potato and maize, labelled with [32P] dCTP by the random primer labelling technique (Feinberg and Vogelstein, 1983). We screened approximately 2 x 10^5 pfu for each probe at the first round. We hybridized the blots overnight at 60 °C and washed them three times with 2xSSPE (3M NaCl, 0.2 M sodium phosphate) and 0.1% Sodium Dodecyl sulphate (SDS) at 60 °C, for 30 minutes each time. We carried out autoradiography at -80 C within intensifying screens. We cloned the positive cDNAs obtained into the EcoRI site of pUC19.

Isolation of DNA

We prepared minipreps of DNA from recombinant lambda gt11 from cultures of *E. coli* (Y1090) infected with individual plaques. We grew these at 43 °C for 6 h on L agar with 50ug/ml ampicillin and 10mM $MgSO_4$. We carried out minipreps and large scale preparations of plasmid DNA according to Maniatis et al. (1982). We used the method of Dellaporta et al. (1983) to carry out genomic DNA isolation from cassava.

Southern and northern hybridization

We electrophoresed cassava DNA digested with various enzymes on 0.8% agarose gels and blotted it on to nitrocellulose. We used formaldehyde and formamide to denature total RNA from plant organs. We size fractionated it in denaturing 1.4% (w/v) agarose gels, then transferred the RNA to Hybond-N (RPN203N, Amersham, UK). We carried out hybridization with isolated cassava cDNA was carried out at 60 °C for 16 h. We washed the filters twice with 2XSSPE +0.1% SDS at 65 °C.

Sequencing

Using the dideoxy method (Sanger et al., 1977) we sequenced the clones and subclones of isolated cassava cDNAs.

RESULTS AND DISCUSSION

Cloning and sequencing of AGPase B

Primary screening of 2 x 10^5 pfu with the AGPase B from potato resulted in the isolation of six positive plaques (Figure 1). After the second round of screening, four out of the six positive clones still gave a strong signal under very stringent conditions of hybridization and washing. We then isolated and characterized these four positive cDNA clones using restriction analysis. Sizes of the cDNA inserts ranged from 1.5kb (clone B55-1) to about 2.0kb (B45-1). We cloned these cDNAs into pUC18 and characterized them by restriction analysis. We sequenced the largest clone after subcloning into pUC18. The entire AGPAse B cDNA of cassava has now been sequenced. Initial results show that the AGPase B cDNA of cassava shares up to 68% sequence homology with the corresponding gene from potato. Figure 2 shows the alignment of a subclone of the AGPase B (clone E34FOR) cDNA of cassava against the potato AGPase B cDNA.

Characterization of AGPase B by hybridization to genomic DNA of cassava M Col 22 has shown that there are few copies of this gene present in the cassava

genome. Hybridization to cassava RNA blots reveals that the gene is well expressed in the tubers. Currently we are making an antisense construct of AGPase B. We will use this to transform potato plants (and possibly cassava plants) in order to examine the effect on starch composition and quantity when introducing a cassava AGPase B gene in normal potato plants.

Screening of the cassava cDNA library for AGPase S2 and S3

The screening for AGPase S2 revealed 6 and plaques and for AGPase S3 revealed 14 which hybridized with the corresponding potato genes used as probes. Following purification through three further rounds of stringent hybridization and washing we obtained six positive cDNA clones of S2 and five positive cDNA clones of S3. We characterized these by restriction analysis. We sequenced the full length CDNA clone, pT266 for S2 and that of pT345 for S3. Analysis and further characterization of these AGPase S2 and S3 genes is in progress.

Screening the cDNA library for Granule Bound Starch Synthase II

Screening of the cassava cDNA library for GBSSII with the appropriate cDNA probes from potato led to two clones. These gave a very strong signal under stringent hybridization conditions, being isolated after two rounds of screening.
They have now been isolated and cloned into pUC19. Sequencing and further characterization of these cassava cDNA clones is in progress.

In our laboratory we have now isolated and cloned several cassava genes involved in the process of starch biosynthesis. These are the genes coding for AGPase B and S subunits, Branching Enzyme, GBSS and their isomorphs. We now have the tools to genetically manipulate plants to produce starches that are tailor-made to meet the needs of both domestic and industrial users. We also have the means of understanding how starch biosynthesis occurs in plants.

The availability of a reproducible transformation and regeneration protocol is important in order to carry out this work. At present it is not available for cassava. So these genes have been transformed into potato which both meets criteria of producing starch and has an efficient transformation and regeneration system. Salehuzzaman et al. (1993) have already shown that when potato plants are transformed with a cassava GBSS cDNA in antisense orientation they produce almost completely amylose free starch. Thus, by cloning the other starch genes, we have opened more options and opportunities to alter starch structures and produce granular starches with new physical properties and uses. In addition, the introduction of AGpase B in sense orientation in starch producing plants will lead to increased expression of this key enzyme of starch biosynthesis and to increased yield of starch as shown for bacterial AGPase in potato (Stark et al., 1992). Plants

with an AGPase introduced in antisense will have a reduced capacity to produce starch and may accumulate other storage products such as lipids, sucrose and fructose in their storage compounds which may be of great nutritional benefit for resource-poor communities.

Altering starches through the genetic manipulation of plants will also limit the amount of post-harvest modification of starches that is currently carried out. This will considerably reduce the amount of chemical pollutants released. The need for the development of new starches through genetic modification of plants is thus of great importance.

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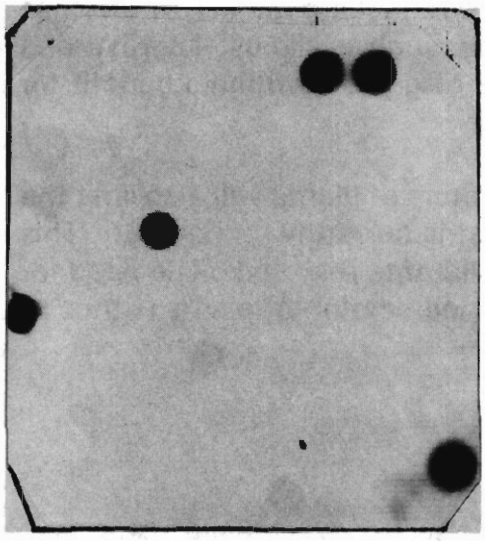
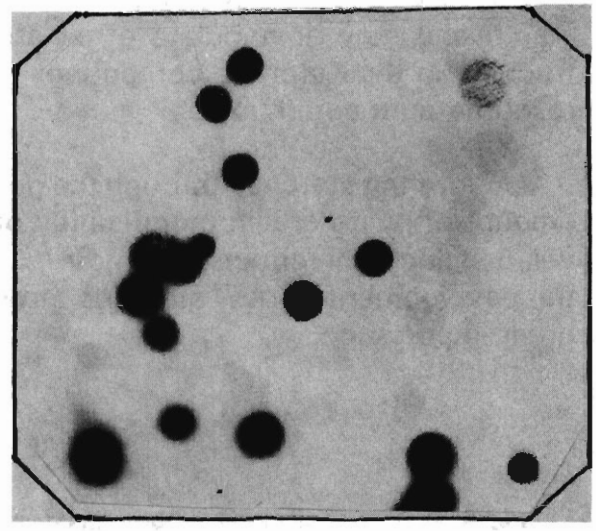
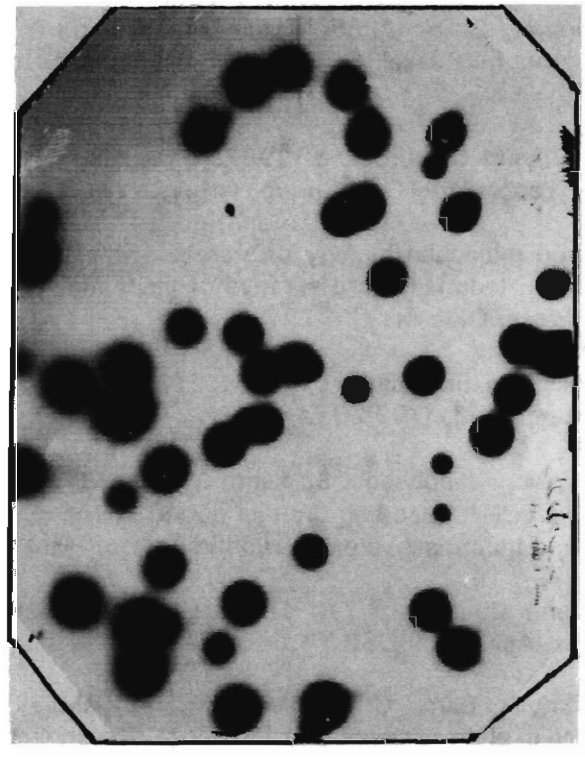


Primary screening of 2 x 10^5 plaques revealed a few positive ones.



Secondary screening of one of the positive plaques from primary screening.



Tertiary screening for the isolation of single positive clones.

Figure 1. The stages in the screening of a cassava cDNA library for the AGPase B gene using a [32P] dCTP labelled potato AGPase B cDNA as a probe.

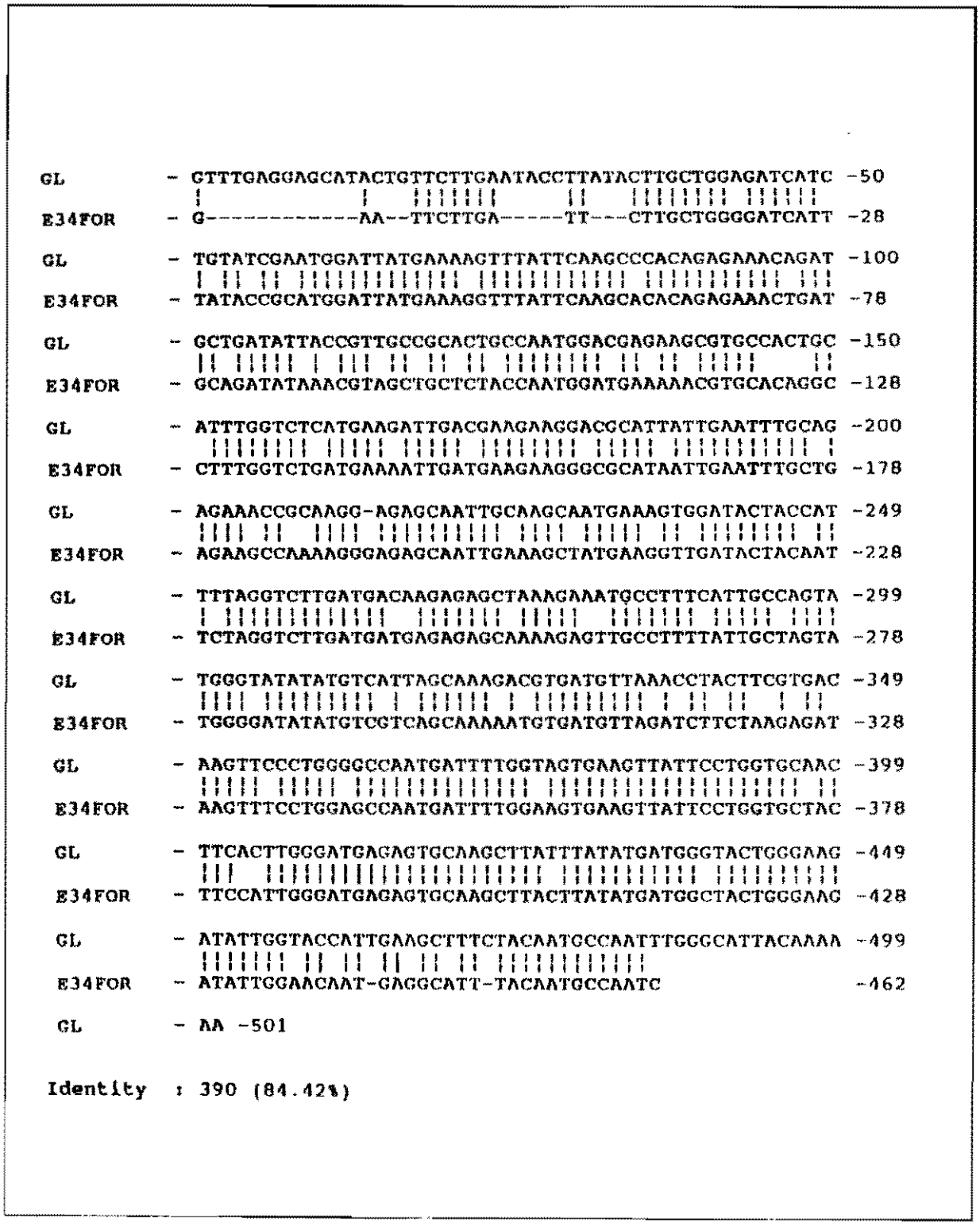


Figure 2. The sequence alignment of a subclone of the AGPase B of cassava (clone E34FOR) with the potato AGPase B cDNA.

RECENT DEVELOPMENTS IN CASSAVA UTILIZATION IN THAILAND

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Most of the cassava root production in Thailand is exported as chips and pellets to European Community countries. Given the EC's agricultural policy, surplus of cassava roots is unavoidable. The present utilization of cassava roots in Thailand is reviewed. Most of the direct and indirect consumption of cassava roots is in starch form. The existing industries using cassava starch as a raw material are listed. Ethanol and single-cell protein (SCP) production are also illustrated. Recent developments of cassava utilization are directed toward the production of food coloring agents (red and yellow) from *Monascus* sp. on cassava medium and research based on graft copolymerization of cassava starch with some monomers to produce high, water absorbing polymers (HWAPs) and biodegradable plastic.

INTRODUCTION

Cassava is regarded as one of the most important crops to the Thai economy, with an estimated 1.5 million hectares (9.3 million rais) of plantation area producing 20 million tons of fresh cassava roots. About 70% of the fresh roots is processed into pellets and chips for exportation to EC countries. Because of the EC's common agricultural policy (CAP) to reduce the current subsidy for several feed grains, a surplus of cassava roots is expected in the future. So use of cassava is an important topic to be discussed at the national level.

DIRECT CONSUMPTION

Cassava roots

Only sweet varieties of cassava are consumed directly as food. Their cultivation produces less than 10 t/yr. Some convenient items produced from cassava have been introduced to the market and are well accepted. But it should be noted that the low cyano-compound cassava shows high potential for human consumption.

Cassava starch

Local consumption of cassava starch was about 500,000 t/yr in 1991 (Table 1). Household consumption accounted for the highest figure. Before 1974, there were 96 cassava starch factories registered by the Ministry of Industry. From 1974 to

1975, many factories improved the separation system. Since then, only 52 factories (including those for modified starch) are active. In 1994-95, about 10 million tons of fresh roots will be milled to produce 2 million tons of starch, of which 60% should go to the export market.

INDUSTRIAL APPLICATIONS

Monosodium glutamate (MSG)/lysine

In industry, the highest volume of cassava starch is consumed by the MSG industry (four manufacturers) and the Lysine (only one). Starch consumption is in the proportion of 80:20 for the MSG:Lysine industries.

Glucose/fructose/sorbitol

There are seven factories manufacturing glucose syrup (two also produce sorbitol) and two large international sorbitol producers (UENO Co., Ltd., Japan, and Lucky Chemical Co., Ltd., Korea). Table 1 shows the 1991 production. There is only one factory producing about 150,000 tons of high fructose syrup (42% fructose) per year (from 70,000 to 80,000 tons of fresh roots).

The enzymes (α-amylase, glucoamylase and glucoisomerase) are imported at a high cost. Experiments are being conducted to produce these enzymes. The fluidized bed technology is supposed to reduce retention time of isomerization from 66 hours at 60 °C to about 6 hours.

Food/sago industry

The applications of cassava starch in the food industry are widely known, especially as canned products. The properties of cassava starch as binding and thickening agents play important roles in many products such as ice-cream, noodles, and puddings. It is also used as a filler in wheat flour to control the protein content. About 30% of cassava starch can be used as filler with wheat flour for many applications.

Paper/textile/plywood

Cassava starch has the properties of gel-formation and retrogradation. For this reason it is used in the paper industry for surface treatment (sizing). In the textile industry it is used for yarn treatment and in the plywood industry for its binding properties.

Citric acid

There are only two factories manufacturing citric acid in Thailand. One uses cassava pulp from starch factories as raw material (about 5-6 t/day) for its solid state (surface) fermentation. The other, recently established, uses cassava chips as raw material for its submerged fermentation process. About 40 t of chips produces 6 t of citric acid per day.

Ethanol

Fermentation of molasses produces alcohol for domestic use. The Thai Institute of Science and Technology, Bangkok, has a pilot plant producing about 1500 l of 99.5% ethanol per day, using cassava root (9 t/day) as raw material. Many attempts have been made to use the root as raw material for this fermentation, but the disadvantage lies in the conversion cost. Table 2 shows the production cost based on the calculation of manufacturing 150,000l (99.5%) ethanol per day by the low boiling method.

SCP/protein-enriched feeds

SPC production using cassava roots or starch as raw material has been done only at the experimental level. There is no commercial approach as cheap protein sources are still available, e.g., fish meal at 10 to 14 baht per kg. Molasses is more suitable than cassava for SCP, ethanol and baker's yeast production. Protein enrichment for feeds has also been studied. Experiments are still at the university—farm scale (Kasetsart University). The aim is to develop this enrichment, using yeast with good amylase activity and high nutritive value such as high lysine content.

RECENT DEVELOPMENTS

Food colorants

The production of natural colorants is of interest, especially from microorganisms. Two red molds, with sufficient activity to produce red and yellow pigments extracellularly from cassava medium, were selected from 300 isolates at the Department of Microbiology, Kasetsart University (1982). They were tentatively identified as *Monascus kaoliang* KB13 and *Monascus barkari* KB10.

Pigment production from both strains using mutagenesis has been improved. Results showed that a mutant strain (10M16) of KB13 increased red pigmentation by 100%, while strain 10M10.2 (a repeated mutant of 10 M16), could change red

pigmentation to yellow at any pH (pH 2.5 to 7.0) and also improve yield up to 500% over that obtained from *M. barkari* KB10. Moreover, it was found that both potent cassava-utilizing *Monascus* mutants could produce red or yellow under either submerged or solid-state cultivation. Their glucoamylase synthesis has also been improved by the mutation.

These water-solubilized pigments could be applied to various kinds of food, drink and pharmaceutical products such as tomato ketchup, surimi, Yen tao fo sauce, pickles, jellies, syrup, milk, ice-cream, phracetamal tablets and syrup. The pigment mixtures enhance attractive colors. Sephadex LH-20 was found to be the best in purifying the pigments. The core chemical formula of the pigment structure is C_{22} H_{28} O_5 with a molecular weight of about 372 kd. Its main structure, as determined by spectroscopy analysis, is a monascin-ankaflavin-monascorubrin skeleton.

Cost evaluation was done using the data obtained from a 90-l fermentor. The concentrated red pigment (500 U/ml, A330) was about 328 baht/l; the yellow, 545 baht/l.

Starch-based plastic

The graft copolymerization of cassava starch and anomers is of interest. The generating of free radicals on cassava starch using ceric iron, manganic ion and hydrogen peroxide-ascorbic acid initiation has been successful. The production of a high water-absorbing polymer (HWAP) by saponification of these polymers has also been reported. The graft copolymerization of cassava starch with methyl/acrylate has been applied as a filler to polyethylene (high density) during plastic/blow film, resulting in biodegradable plastic. Research on this copolymerization continues.

CONCLUSION

In the near future, Thailand will face surplus production of cassava roots. The marketing of cassava starch cannot totally solve the problem. Industrial uses of cassava have to be extensively studied and promoted. Recent developments in cassava use in Thailand are the production of food coloring agents from cassava starch, and the production of starch-based plastic, such as HWAP and biodegradable plastic.

Table 1. Cassava starch utilization in Thailand.

	Tons	%
Household consumption	134,908	26
Monosodium glutamate/lysine	97,977	19
Glucose syrup	76,375	15
Paper	47,557	9
Food	33,752	7
Sago	32,060	6
Textile	14,577	3
Plywood	6,700	1
Other	67,798	14
Total	511,704	100

SOURCE: The Thai Taploca Flour Industries Trade Associations.

Table 2. Breakdown of ethanol production costs from cassava, based on the manufacture of 150,000l (99.5%) ethanol/day.

	Cost (%)
Cassava	35.51
Enzyme/chemical	10.41
Fuel	10.50
Electricity	10.82
Labor	2.82
Management	7.43
Others	2.11

SOURCE: Thailand Institute of Science and Technology.

CASSAVA FERMENTATION: CASSAVA SOUR STARCH IN LATIN AMERICA

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Indigenous food fermentations are one of the oldest forms of biotechnology. Fermented foods play an important role in human nutrition on a global level especially in tropical regions. Indeed effective, well-managed fermentation processes facilitate the conservation of agricultural products without requiring energy inputs, whilst improving the digestibility and nutritional quality of certain foods. Also of importance are the organoleptic characteristics produced in foods as a result of fermentation. Cassava, a widely distributed crop, is an important calorie-source for about 500 million people. One of cassava's problems is that certain varieties of the crop naturally contain toxic levels of cyanogenic glucosides. Cassava-consuming peoples have developed fermentation techniques to reduce this toxicity to safe levels. This article first describes a number of different fermented cassava foods, then provides a more detailed analysis of a particular example, that of cassava starch fermentation in Latin America. This fermented starch, known as sour starch, is used in the production of typical cheese breads. Lactic fermentation of cassava starch is essential for the acquisition of its breadmaking capacity. The microbial flora involved and the physicochemical changes undergone in processing are detailed here. This traditional fermentation is still poorly controlled at the farm level. Some cassava processors have improved fermentation by inoculating starch with a starter culture taken from previous fermentations. But this practice still produces sour cassava starch of irregular quality. The present study aims at optimizing cassava starch fermentation and developing starter inocula. These starter cultures will facilitate the standardization of product quality and shorter fermentation time. Amylolytic Lactic Acid Bacteria (ALAB) were isolated from traditionally fermented cassava starch. The genetic diversity of 60 purified isolates was evaluated in terms of starch hydrolysis, metabolism of organic acids and protein profile. A selected strain has been tested as experimental inoculum in a cassava starch production unit. Results are very promising as ALAB 20 gave faster obtention of breadmaking capacity than natural fermentation, although the final breadmaking value was the same. As these starter cultures were isolated in their natural ecosystem, they are sustainable and easy to use at the farm level. The improvement of cassava sour starch quality will provide greater market opportunities for this product and bring benefits both to small scale cassava processors and to producers.

INTRODUCTION

Indigenous food fermentations are one of the oldest forms of biotechnology. Food fermentation is a very old and traditional technique used in many parts of the world (Cook, 1994). Many agricultural products involve fermentation techniques, leading to a wide variety of fermented foods, such as dairy products (cheese, yoghurt,

kefir), cereals (rice, sorghum, maize), legumes (soybean), roots and tubers (cassava), meat (salami, ham), fish (fish sauces),fruits and vegetables (grapes, pickles, sauerkraut, olives) and miscellaneous (mushrooms, coffee, cocoa, tea) (Campbell-Platt, 1994). Fermented foods can serve as main-course meals, others are beverages or highly prized food condiments. They add variety to the diet and contribute greatly to the general nutrition of large populations (Odunfa, 1985).

Fermentation provides a way of preserving food products, enhancing nutritive value (improved digestibility, bioenrichment in proteins and vitamins), destroying undesirable factors, making a safer product, salvaging material otherwise not for human consumption and reducing the amount of energy required for cooking. Fermentation also improves the appearance, texture and flavor of some foods, according to local consumer tastes. It is then closely related to psychological and social habits (Stanton, 1985; Fook-Min Yong, 1992; Paredes López, 1992). It should be remembered that production of fermented foods in tropical regions is still largely a traditional family art done at home. Consequently, production has not increased substantially, quality is irregular, and the shelf life of many of these fermented products is very short (Odunfa, 1985).

Fermentation involves the action of microorganisms and/or enzymes which cause desirable biochemical changes and significant modification to the food. Enzymes are organic substances acting as catalysts of the all-chemical changes occurring during fermentation (Aubert, 1985). During fermentation, microorganisms break down carbohydrates, proteins and lipids present in the raw materials to be fermented by releasing hydrolytic enzymes into the medium. The breakdown products (e.g., fatty acids, amino acids and simple sugars) thus liberated are converted into microbial structural components, secondary metabolites and odoriferous volatile molecules (Fook-Min Yong, 1992; Paredes López, 1992).

Fermented foods may be divided into two categories: submerged deep fermentation (SDF), and solid state fermentation (SSF). In SDFs, microbial activity occurs at a relatively low biomass concentration in the liquid phase, while in SSFs, microbial growth and product formation occur (either spontaneously or by adding inoculum) on the surfaces of solid substrates. SDF technique is mainly used in industrialized countries for the preparation of bulk industrial enzymes, while artisanal food fermentations in developing countries often involve solid state fermentation. In many regions of the world, traditional SSFs of legumes, cereals and starchy substrates have been associated with the activity of lactic acid bacteria. But for many indigenous food fermentations, mixed fungal-bacterial, fungal-yeast and yeast-bacterial combinations may occur. These complex microbial interactions play an important role in the nutritional, safety and sensory characteristics of the end product (Paredes López, 1992).

Cassava is one of the most important staple food crops grown in tropical regions. This widely distributed starchy root is an important calorie source for

about 500 million people. Cassava naturally contains cyanogenic glucosides such as linamarin and lotaustralin, responsible for chronic toxicity (e.g., goiter and cretinism). Bitter varieties of cassava contain higher levels of cyanogenic glucosides than sweet ones. This paper first describes a number of fermented cassava foods and gives a review of the main research work carried out on cassava fermentation. It then provides a more detailed analysis of a particular example, that of cassava starch fermentation in Latin America, with an emphasis on fermentation optimization and starter culture development.

REVIEW OF CASSAVA FERMENTATION. MAIN RECENT RESEARCH

As a means of reducing cassava toxicity to safe levels, cassava-consuming peoples have developed fermentation techniques. Moreover, cassava fermentation has many advantages. It gives specific organoleptic characteristics (desired aromas and sour taste), allows preservation and easier handling of the raw material and ensures protection of the environment (by cassava waste treatment).

Root fermentation plays an important role in detoxifying bitter cassava varieties. It contributes to the release of the endogenous linamarase from the plant tissues. This enzyme is involved in the breakdown and hydrolysis of cyanogenic glucosides, hydrogen cyanide release, and thus product detoxification (Oyewole, 1992; Campbell-Platt, 1994). Fermentation in water leads to faster tissue disintegration and appears to be more efficient for reducing both free and residual cyanide in cassava roots and products (Bokanga, 1992; Hahn, 1992). Some authors believe that during cassava fermentation, enzymatic detoxification may override the microbial action. Indeed, although high linamarase lactic bacteria are involved in cassava retting (soaking in water), it appears that the degradation of cyanoglucosides largely depends on the endogenous linamarase of cassava roots (Brauman et al., 1994). Aidoo (1992) reported that traditional cassava fermentation by itself is an unreliable detoxification method as it does not achieve total cyanide elimination. Sokari (1992) noted that adding water to the grated cassava reduced linamarin content up to 99% with little or no change in the pH of mash. This author states that fermentation has nothing to do with detoxification and that linamarin breakdown is essentially a hydrolytic process which is catalyzed by the endogenous linamarase and enhanced by adding water. For Sokari (1992), fermentation is only involved in flavor development. Due to these divergent points of view, more studies are needed to clarify the role of identified microorganisms in cassava fermentation and their real contribution to detoxification.

Much research carried out on traditional cassava fermented foods has allowed better understanding of their fermentation mechanisms. Here two of these fermented foods, *gari* (solid state fermentation) and *chikwangue* (submerged deep fermentation), are described with emphasis on current knowledge about their respective fermentation.

Gari, a popular West African staple food, is a granular, coarse cassava flour. Gari is obtained by washing, peeling and grating cassava roots. An inoculum of 3-day-old cassava juice or fermented mash liquor is added. The pulp is placed in cloth or jute bags and squeezed under heavy stones. Excess water is drained off and the pulp undergoes anaerobic lactic fermentation for two to four days. The fermented pulp is then sieved, roasted and dried to about 10% water content. The organic acids produced (lactic, acetic, propionic, succinic and pyruvic) give to gari its desired sour flavor and characteristic aroma (Steikraus, 1992). The main microorganisms involved in gari fermentation are *Corynebacteria manihot*, *Geotrichum candida*, *Lactobacillus plantarum* and *Streptococcus* sp. (Steinkraus, 1983; Hahn, 1992). Gari can be eaten either dry or soaked in cold water with sugar, it may also be added to boiled water in which it increases in volume, due to its starch pregelatinization during roasting. Another similar product, farinha de mandioca, is well known in Latin America, especially in Brazil. Research work was conducted on gari detoxification using different *Brevibacterium* and *Lactobacillus* strains. This detoxification was based on strain microbial enzymatic systems such as beta glucosidase, nitrile hydratase and amidase. Results show that some genes of the tested enzymatic systems could be cloned and transferred to the lactic bacteria naturally involved in traditional cassava fermentation (Dufour and Griffon, 1992).

Another traditional cassava fermented food is *chikwangue*, a very stiff paste, processed as follows. Cassava roots are peeled, steeped in water for 3-5 days to ferment and become soft. The fermented pulp is taken out and the fibers are removed. The pulp is then heaped on racks for further fermentation and pressed with heavy objects to drain off excess liquid. Next it is ground on a stone or pounded in a mortar to make it finer. The fine pulp is wrapped in plantain leaves and tied firmly with fibers. These are steamed in pots (Hahn, 1992). Chikwangue fermentation is then a submerged deep fermentation occurring through retting (soaking in water). Recent studies show that retting is a two-stage anaerobic lactic fermentation process; the first stage managed by heterofermentative *Leuconostoc mesenteroides* and the second performed by homofermentative amylolytic *Lactobacillus plantarum*. Yeasts, such as *Candida*, appear at the end of fermentation. In addition, lactic acid and ethanol are produced in large quantities. Cassava retting also leads to root softening, due to enzymatic action of pectinases (Dufour and Griffon, 1992). Many studies focus on retting optimization in terms of fermentation duration and temperature effect. On the other hand, "dry retting" technique was tested as an alternative for reducing the quantities of water needed for cassava soaking. "Dry retting" consists of fermenting cassava pieces in bags for 4 days and then immersing them in water for 24 hours. This technique is proved to give the same organoleptic characteristics as traditional soaking in water (Dufour and Griffon, 1992).

As cassava fermentation is mainly concerned with *lactic bacteria*, many recent works deal with the metabolism, physiology, genetics and amylolytic activity

of lactic strains isolated from the natural cassava fermentations. Other studies focus on the functional properties of these bacteria such as their role in the acidification process and their interaction with yeasts resulting in specific aromas. Research work on lactic bacteria aims at developing suitable starter cultures for better fermentation control (Dufour and Griffon, 1992).

Cassava is frequently denigrated because of its low protein content. A number of methods have been developed to improve its protein and vitamin content, based on the fungal growth on starch materials, including cassava starch. Stanton and Wallbridge (1969) developed a fermentation process with *Rhizopus* to upgrade cassava and give a product significantly higher in protein. The symbiotic action of *Trichoderma ressei* and *Saccharomyces cerevisiae* gave highly (51%) protein-enriched cassava starch (Kennedy et al., 1987). Harris (1970) reported gamma linolenic acid from *Rhizopus* fermented cassava flour. Currently, work is being conducted on anaerobic solid state fermentation with *Rhizopus oryzae,* for the *bioenrichment* of cassava flours with proteins, lactic and fumaric acids. These enriched flours can find use in bakery products (Ramírez, 1993).

Cassava starch has been extensively used as a starting compound for the chemical industry and as a substrate for *ethanol* production, especially in Brazil. Production of ethanol was then devoted either for alcoholic beverages or for use as fuel in cars. Although cassava is a potentially viable, alternative raw material for ethanol production through fermentation processes, Brazilian "alcohol" projects were not very successful due to the high capital costs in fermentation plants (Kennedy et al., 1987).

Cassava processing is very polluting in terms of solid wastes (peels, fibers) and waste waters. The biological upgrading of cassava wastes may be achieved with anaerobic fermentation techniques for a safer and less polluted environment. For solid cassava wastes, many research lines are emerging. The first concerns the production of a biogas, such as *methane,* by the action of specific anaerobic methanogenic microorganisms. Solid cassava wastes may also be converted into *ethanol* by the amylolytic yeast *Schwaniomyces castelli* or into *lactic acid* by the action of amylolytic *Lactobacillus plantarum* (Alazard, 1993). Another promising fermentation is the conversion of cassava solid wastes into high-value lipidic biomass by the action of *Trichosporum* sp. which is resistant to high cyanide contents (Wosiacki et al., 1994). Cassava waste waters may undergo an anaerobic biofiltration on natural supports such as bamboo, straw or cassava fibre for *biocompost* production (Gotin et al., 1994). Recovery of cassava wastes is an innovative and crucial way to reduce the environmental pollution and to add value to this starchy root crop. It should be remembered that all residues of cassava waste fermentations cited above could be used as feed materials.

SOUR CASSAVA STARCH IN LATIN AMERICA

Sour cassava starch is a traditional product in Latin America (Colombia, Brazil, Paraguay and Argentina); it is small scale, produced by a highly developed rural agroindustry in these countries. Sour cassava starch is used in the preparation of typical cheese breads, such as "pandebono" and "pan de yuca" in Colombia, or "pao de queijo" in Brazil. The originality of this product is its ability to expand and to make bread despite the fact that it is entirely composed of starch and does not contain gluten, responsible in classic breadmaking with wheat flour for the formation of a network permitting gas retention. Moreover, bread production from cassava sour starch involves no yeast fermentation before cooking.

Processing of sour cassava starch consists of root washing, peeling and grating. Starch "milk" is extracted from the pulp by sieving under running water and let to sediment into tanks. Sedimented starch is allowed to ferment for 20 to 30 days and then sun-dried. Residues of starch extraction are used for animal feed (Camargo et al., 1988; Chuzel, 1990). Recent studies carried out in Colombia (Brabet and Dufour, 1993; Larsonneur, 1993) have shown that fermentation and sun-drying are the key stages for the acquisition of sour starch breadmaking capacity. Traditional fermentation is still poorly controlled at the farm level. Some cassava processors have improved fermentation by inoculating starch with a starter culture taken from previous fermentations. But this practice still produces sour cassava starch of irregular quality. The study presented below aims first at understanding and optimizing natural cassava starch fermentation and then at developing starter inocula for upgrading product quality and reducing fermentation time.

MATERIALS AND METHODS

For starter culture study, 60 amylolytic lactic acid bacteria (ALAB) were isolated from traditionally fermented cassava starch. The genetic diversity of these purified isolates was evaluated in terms of starch hydrolysis, metabolism of organic acids and protein profile. The strain (ALAB 20) identified as *Lactobacillus crispatus* using the API 50 CH system, selected for its great amylase-producing ability, was tested as experimental inoculum in a traditional cassava production site (La Agustina, Cauca department, Colombia). The inoculum was progressively poured into the sedimentation tank and vigorously mixed with the arriving starch milk, the latter being extracted from the coded CM 523-7 cassava clones of the CIAT international germplasm. At the same time, natural fermentation was followed as reference in the same production unit.

To study either natural or starter culture fermentations, samples of starch were taken in a sterile manner from the fermentation tank using a geologist auger. Starch "milk" (unfermented starch) was collected immediately after extraction. The

other samples were taken at different time intervals, i.e., after 3, 7, 9, 13, 19, 26 and 33 days of fermentation. All samples first underwent microbiological analyses and then were sun-dried for 8 hours as for traditional sour starch drying before further physicochemical measurements. *Microbiological analyses* consisted of classic microbial counts, such as aerobic and anaerobic total floras, yeasts and molds. *Physicochemical measurements* (on the supernatant of a centrifugated 10% w/w aqueous starch suspension)were *pH, total acidity* (determined by NaOH titration of the previous supernatant), *lactic content* (by HPLC method), *pasting properties* (Brabender apparatus) and *breadmaking potential* (by the measurement of bread specific volume according to Vanhamel et al., 1991).

RESULTS AND DISCUSSION

Figure 1 shows that natural cassava sour starch fermentation is predominantly of a lactic type, with (at 0 fermentation day) approximately 10^7 colony forming units (CFU) per gram of starch dry matter. Some yeasts and molds are also present (10^5 CFU per gram of starch dry matter). Lactic bacteria multiply during the first 5 days to attain an optimal level (10^9 CFU per gram of starch dry matter) and do not multiply any more between this point and the end of fermentation (30 days). This phenomenon is probably linked with the nutritional deficiencies (essentially amino acids and vitamins) of the culture medium which consists of almost 100% of native cassava starch. Yet these bacteria, though they undergo no further division, continue to metabolize the carbohydrates and produce lactic acid at a constant rate until the end of the fermentation.

According to bacterial growth, the pH of starch falls during fermentation from 6.8 to a final 3.7; this last value being close to the pKa of lactic acid (3.5), while total acidity and lactic acid content respectively increase and are almost equal (Figure 2). This confirms that the acidity of the medium is mainly related to the production of lactic acid.

Figure 3 shows the evolution of pasting properties during sour starch natural fermentation. Temperatures corresponding to starch pasting (62.5 °C) and maximal viscosity (70 °C) are the same for unfermented and fermented starches. However, the maximal viscosity value decreases and the "viscosity falls after the peak" increases with fermentation time. These rheological changes could be explained by the decrease of starch molecule size, due to the bacterial enzymatic breakdown (Camargo et al., 1988). Acquisition of breadmaking potential during fermentation is shown in Figure 4. The specific loaf volume increased from 2.5 to 5.5 $cm^3\ g^{-1}$. This confirms that lactic fermentation of cassava starch is essential for the acquisition of its breadmaking capacity.

Figure 5 shows the evolution of anaerobic flora on MRS and MRS-starch media, for natural fermentation and Figure 6 that for starter inoculated fermentation.

The same tendency as explained above (Figure 1), is noted—an increase of the anaerobic flora during the first days of fermentation up to an optimal level (around 10^9 CFU per gram of starch dry matter) and a stabilization until the end of fermentation. Inoculated starch with the ALAB 20 strain shows similar evolution of the microfloras as naturally fermented ones. Hydrolysed zones on MRS-starch are revealed by contact with iodine vapors; they confirm the amylolytic activity of lactic bacteria involved in cassava starch fermentation. Evolution of the amylolytic bacteria follows the same tendency as anaerobic floras (Figs. 5 and 6). But the proportion of amylolytic bacteria compared to the total flora on MRS-starch medium seems to increase more rapidly in inoculated starch (100% after 10 days fermentation) than in naturally fermented, about 45% for the same fermentation time (Figure 7). This could be explained by the fact that flora is heterogenous in the natural fermentation whereas the ALAB 20 strain is predominant in the starter inoculated fermentation (Brabet et al., 1994).

Figure 8 shows the changes of pH and Figure 9 those of lactic acid content. In the inoculated starch, acidification is more important in the first five days of fermentation but pH value finally stabilizes at 3.5 (of value lactic acid pK_a) in both natural and starter inoculated fermentations. Similarly, lactic acid production during the first days of fermentation is higher for inoculated starch than for naturally fermented, although the final lactic acid content (after 20 days) is higher for natural fermentation.

Figure 10 shows the evolution of breadmaking potential for natural and starter inoculated fermentations. Inoculation of cassava starch with ALAB 20 allowed a faster acquisition for an identical breadmaking capacity. Final specific loaf volume is reached 10 days earlier than in natural fermentation.

In conclusion, inoculation trials in fermentation tanks on the production site, using the ALAB 20 strain as a starter culture, has made possible a considerable reduction in fermentation time for the production of an identical breadmaking capacity (10 to 12 days instead of 30). This starter culture could allow a more regular quality for cassava sour starch. Further studies will consist of assessing the genetic diversity, the physiology and the amylolytic activity of our (ALAB) collection. As these potential starter cultures were isolated in a cassava natural ecosystem, they are sustainable and easy to use at the farm level for improving cassava sour starch quality. This will provide greater market opportunities for this product and brings benefits both to small-scale cassava processors and to producers.

CONCLUSION AND PROSPECTS

Many prospects are now emerging for biotechnology contribution to cassava fermentation. The wide potential of Lactic Acid Bacteria (LAB) opens the way to

various applications, such as *starter cultures* (for improving and standardizing the product quality), *polysaccharide production* (for use as breadmaking additives and texture improvers), *bacteriocin production* (these compounds being effective against food-borne pathogens). Amylolytic LAB could also be used for elaboration of *fermented beverages and gruels*. These low-cost and hygienic products show high nutritional and dietetic value. They can act as dairy substitutes and weaning foods, especially in isolated, rural areas of the tropics. Amylolytic LAB can also serve to produce other kinds of fermented starchy beverages, such as highly energetic, vitamin-enriched drinks for sportsmen.

Enzymatic degradation of cassava starch is another facet of biotechnology contribution to cassava fermentation. The participation of many enzymes in food fermentation processes makes them of great interest for further development (with or without microbial action). Cassava starch could be degraded by specific enzymes for glucose and maltose syrup production. These syrups are of great value for sweet and confectionery products. Moreover, maltose syrups could act as malt substitutes for breweries allowing a reduction of malt importation in many developing countries. Another promising molecule, *cyclodextrin*, is currently emerging. Cyclodextrin is produced by a specific enzymatic degradation of starch. As this molecule may form inclusion complexes, it is of interest for encapsulation of flavors, drugs, chemicals, dyes, vitamins and for emulsion stabilization. It presents a large industrial potential for cosmetic, pharmaceutical, food and agrochemical uses.

Finally, fermented *cassava sour starch* deserves more attention. Sour starch technology could be transferred and adapted to other cassava-consuming countries. Sour starch shows a very attractive industrial potential. Indeed, its breadmaking capacity allows it to replace wheat and thus to reduce wheat importations in developing countries. It could also serve as breadmaking mixes for gluten-free products, which are essential for people allergic to wheat gluten. Moreover, the expansion property of sour cassava starch makes it of interest for the elaboration of expanded snack foods, without the use of extrusion equipment.

So, a better knowledge and a strict control of cassava fermentations can improve the quality of traditional fermented foods, help in elaborating new cassava products and reduce environmental pollution. Contribution of biotechnology to cassava upgrading will undoubtedly open the way to innovative uses and to larger market opportunities, either at the national level or for export. "Modern biotechnology", such as recombinant DNA techniques and gene transfers, may help towards a better understanding of taxonomy, physiology and metabolism of the microorganisms involved in cassava fermentations. Nevertheless, developing countries need to acquire a better understanding of the "old biotechnology" before efficiently implementing the new one; "old biotechnology" consisting of direct control of the physicochemical environment of the fermentation process, as well as mutagenesis and selection of superstrains.

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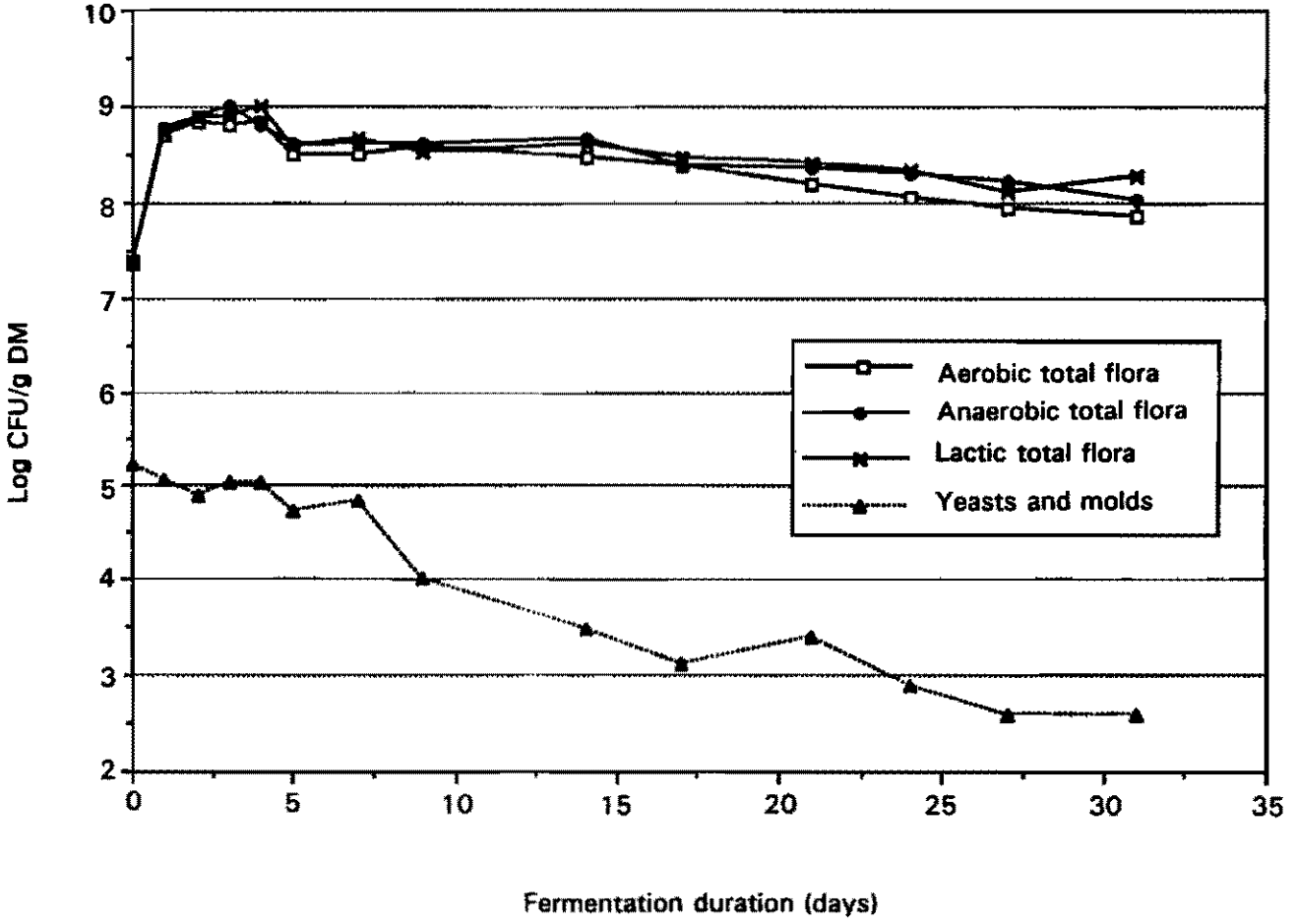

Figure 1. Evolution of microflora during natural fermentation of sour cassava starch.

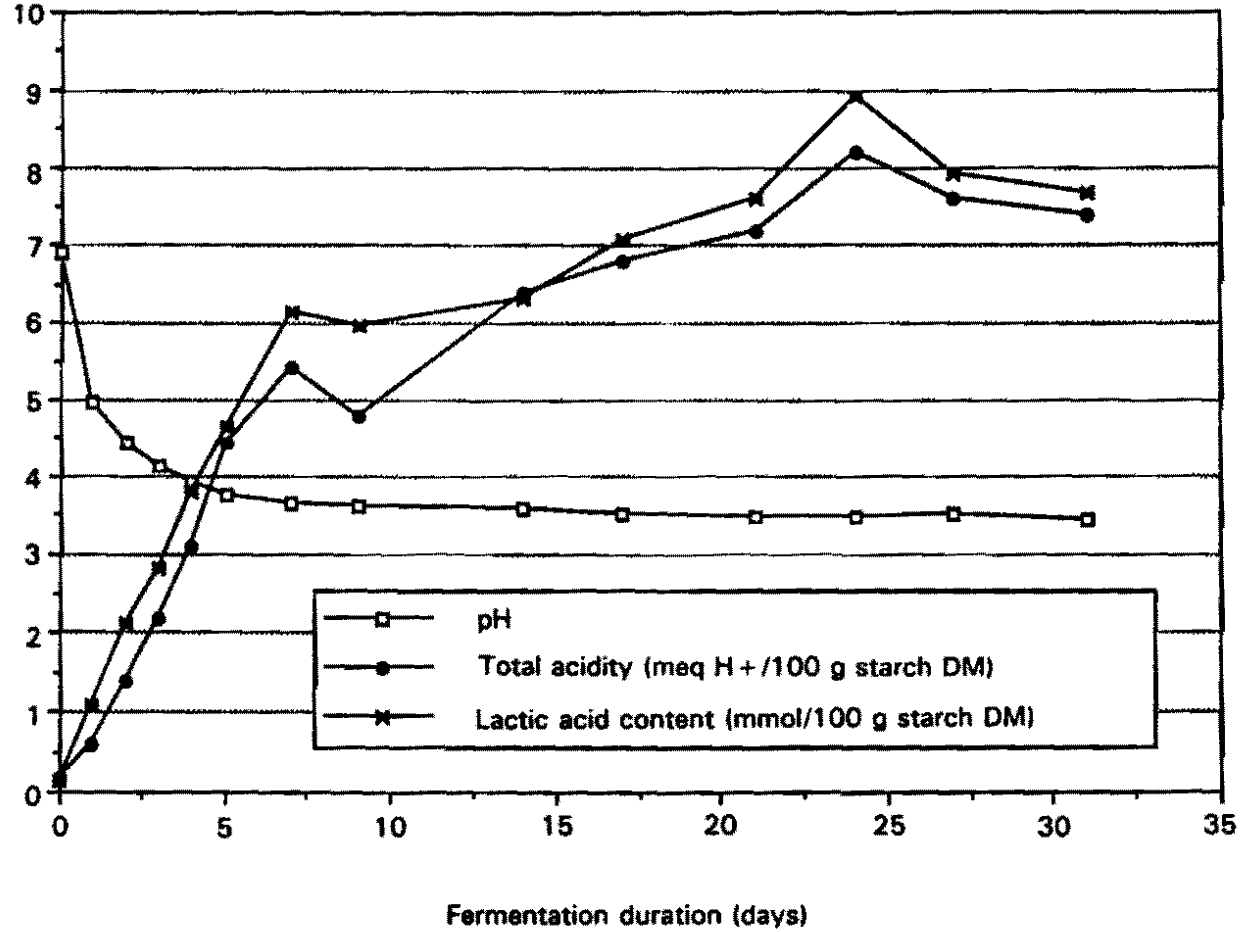

Figure 2. Evolution of pH, total acidity and lactic acid content during natural fermentation of sour cassava starch.

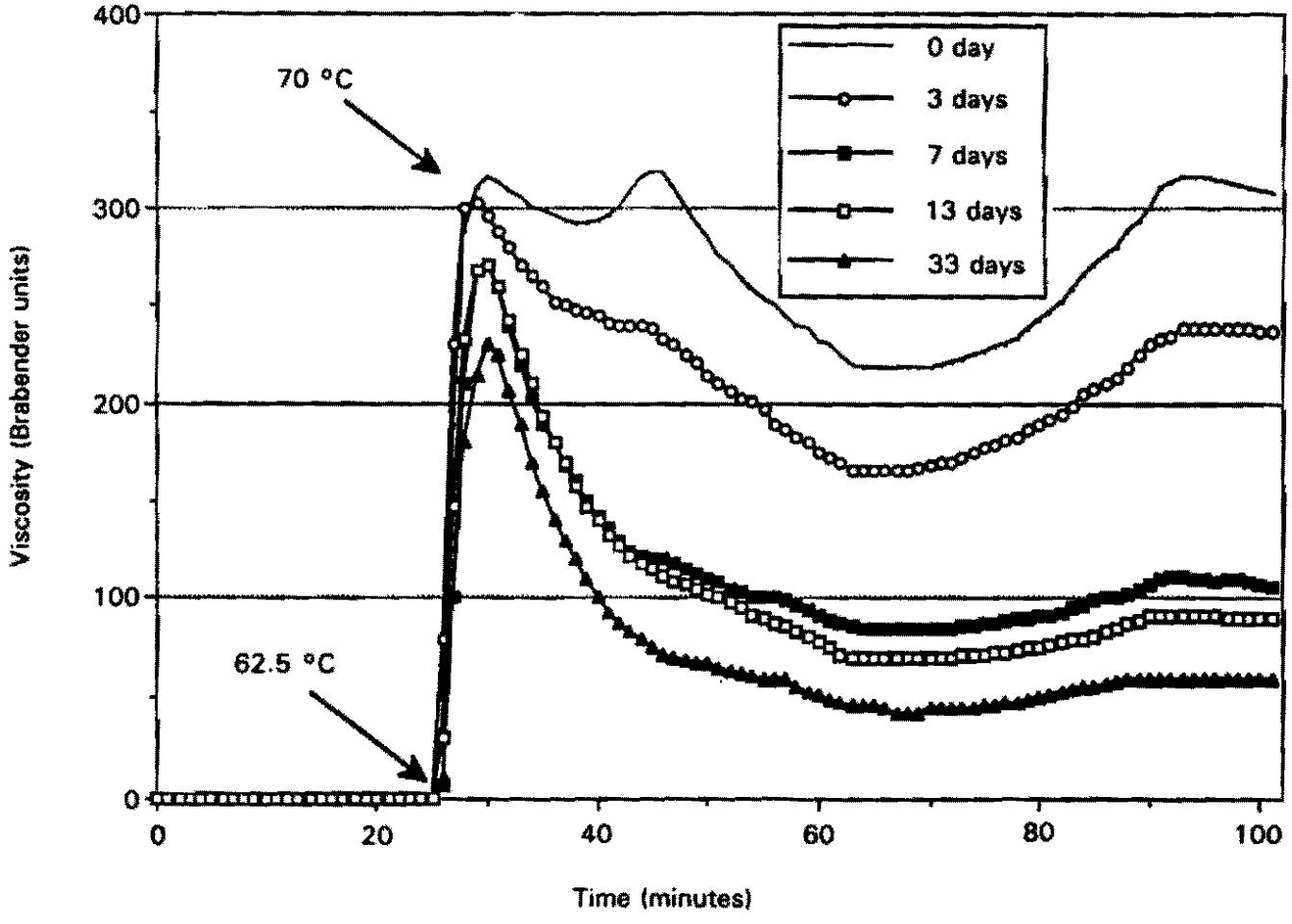

Figure 3. Evolution of pasting properties during natural fermentation of sour cassava starch.

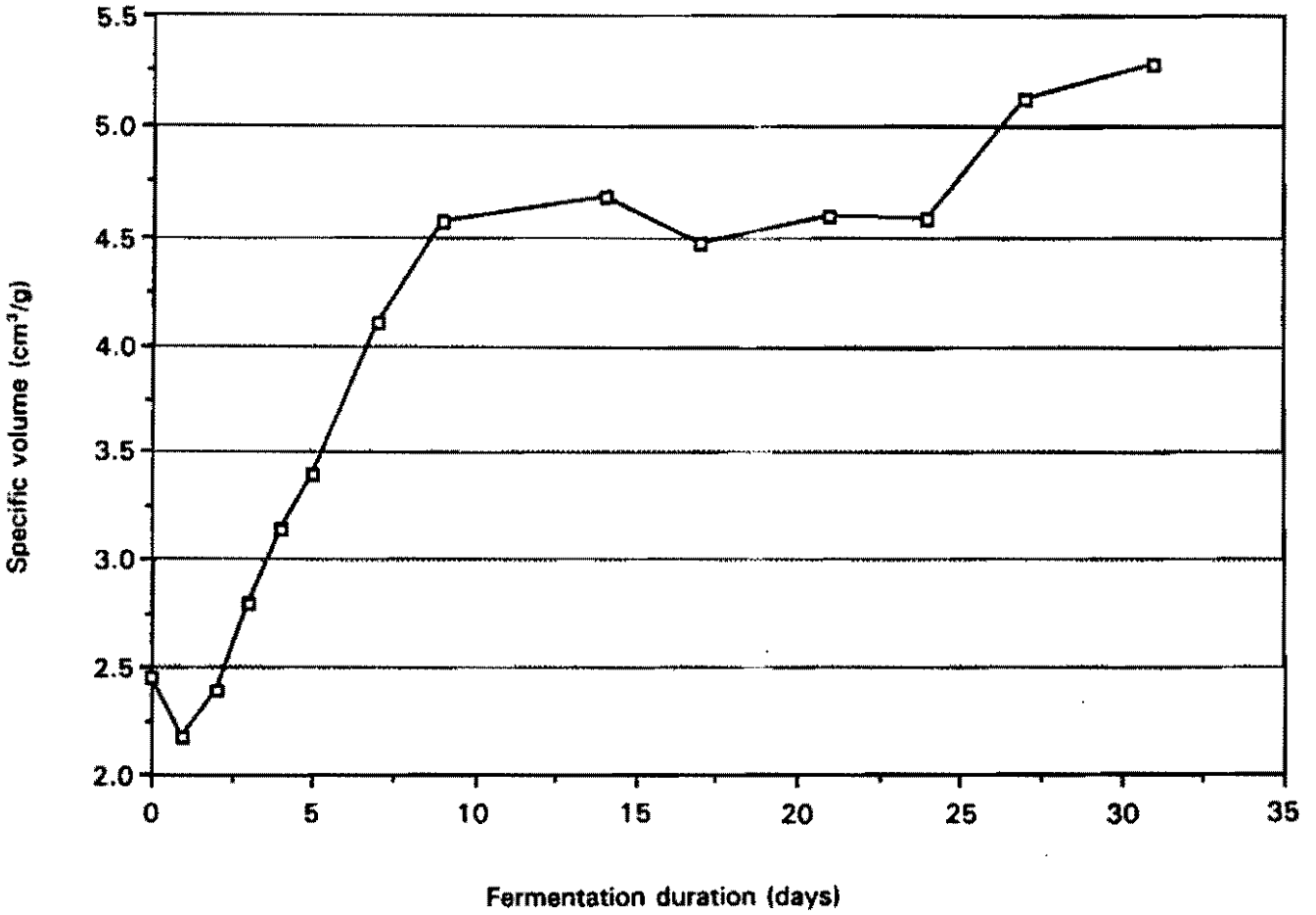

Figure 4. Evolution of breadmaking capacity during natural fermentation of sour cassava starch.

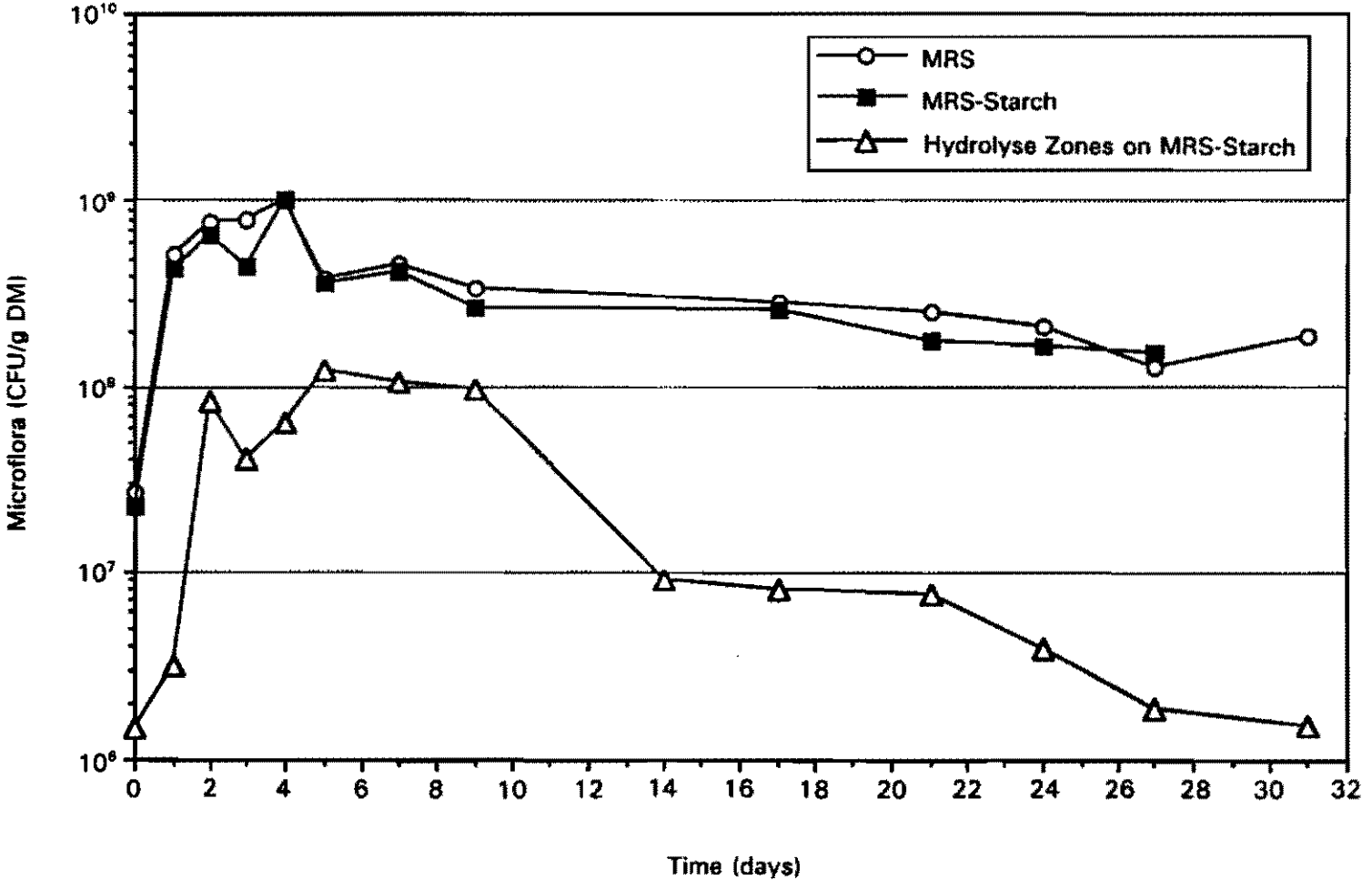

Figure 5. Changes in anaerobic microflora on MRS and MRS-starch during natural fermentation.

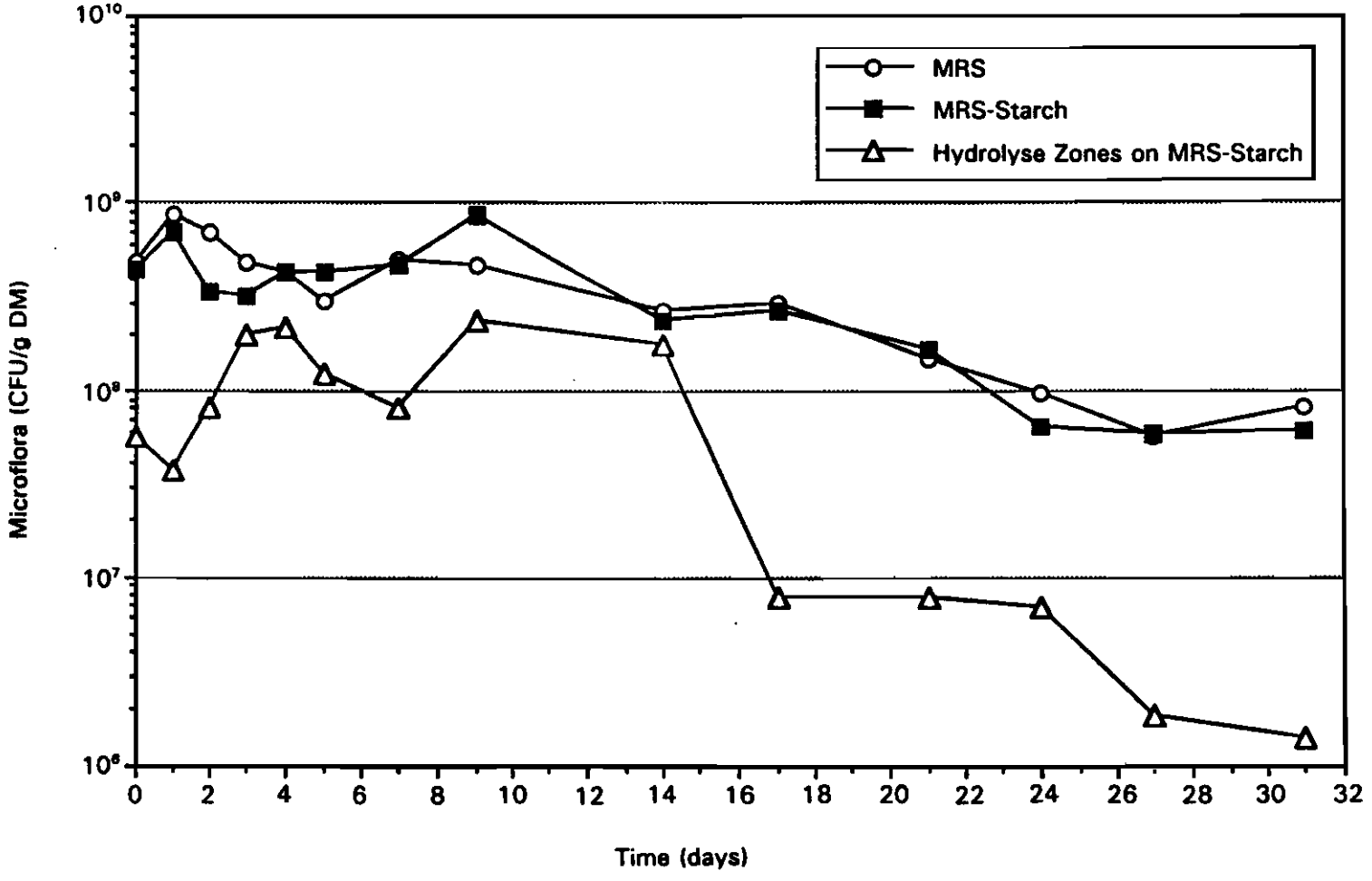

Figure 6. Changes in anaerobic microflora on MRS and MRS-starch during inoculated fermentation (ALAB 20).

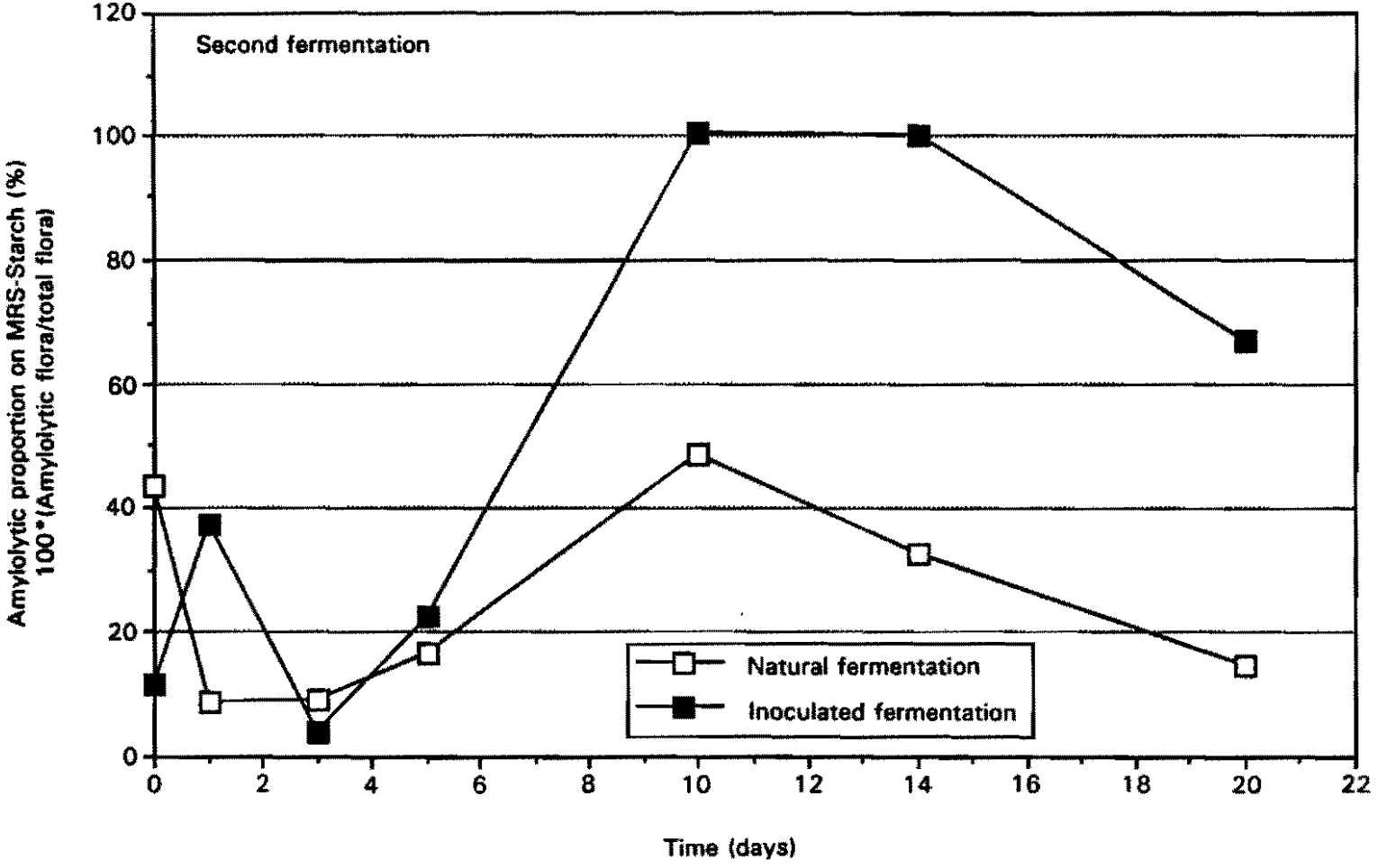

Figure 7. Changes in amylolytic flora as proportion of total flora on MRS-cassava starch.

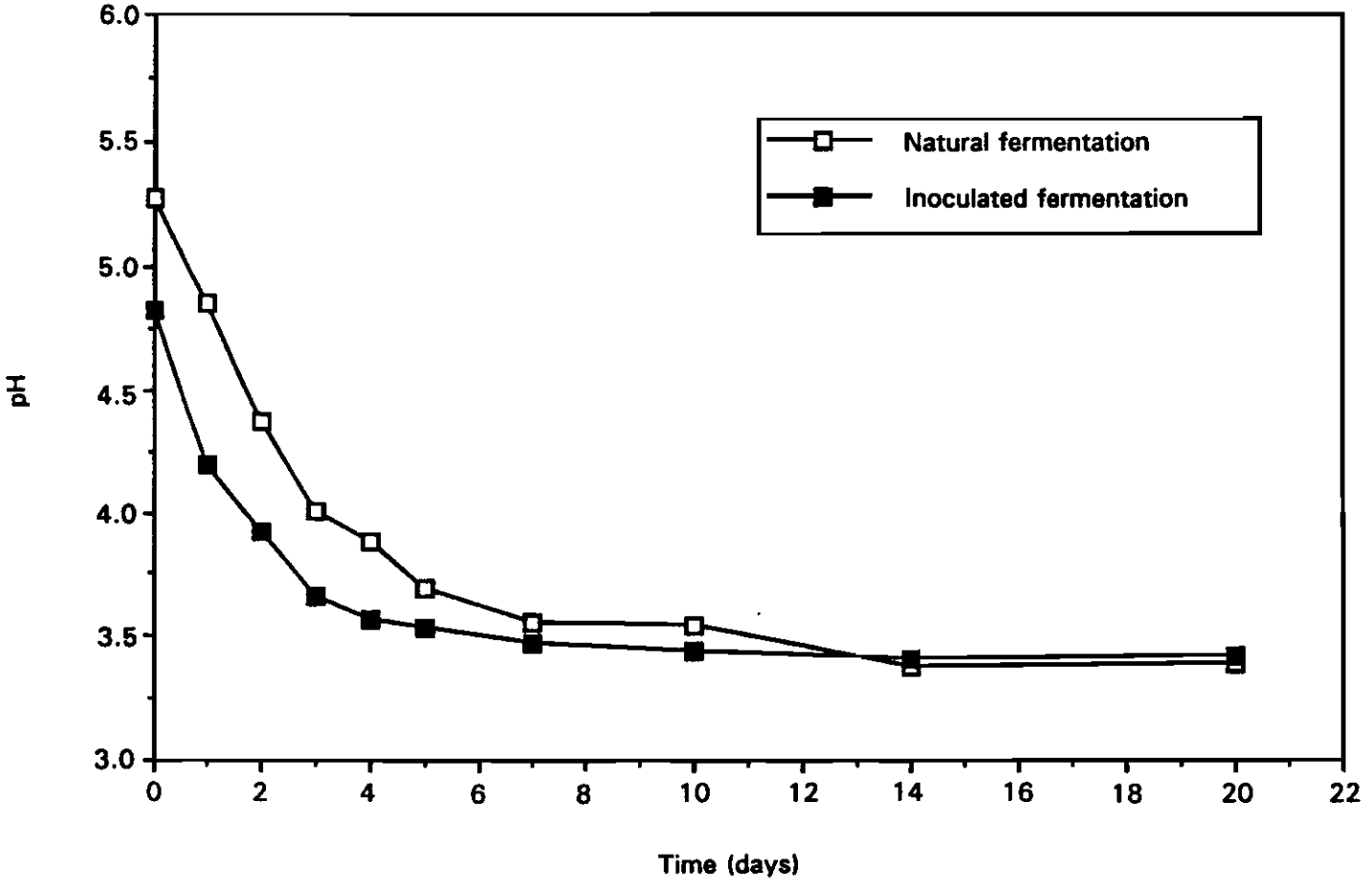

Figure 8. Changes in pH during cassava starch fermentation.

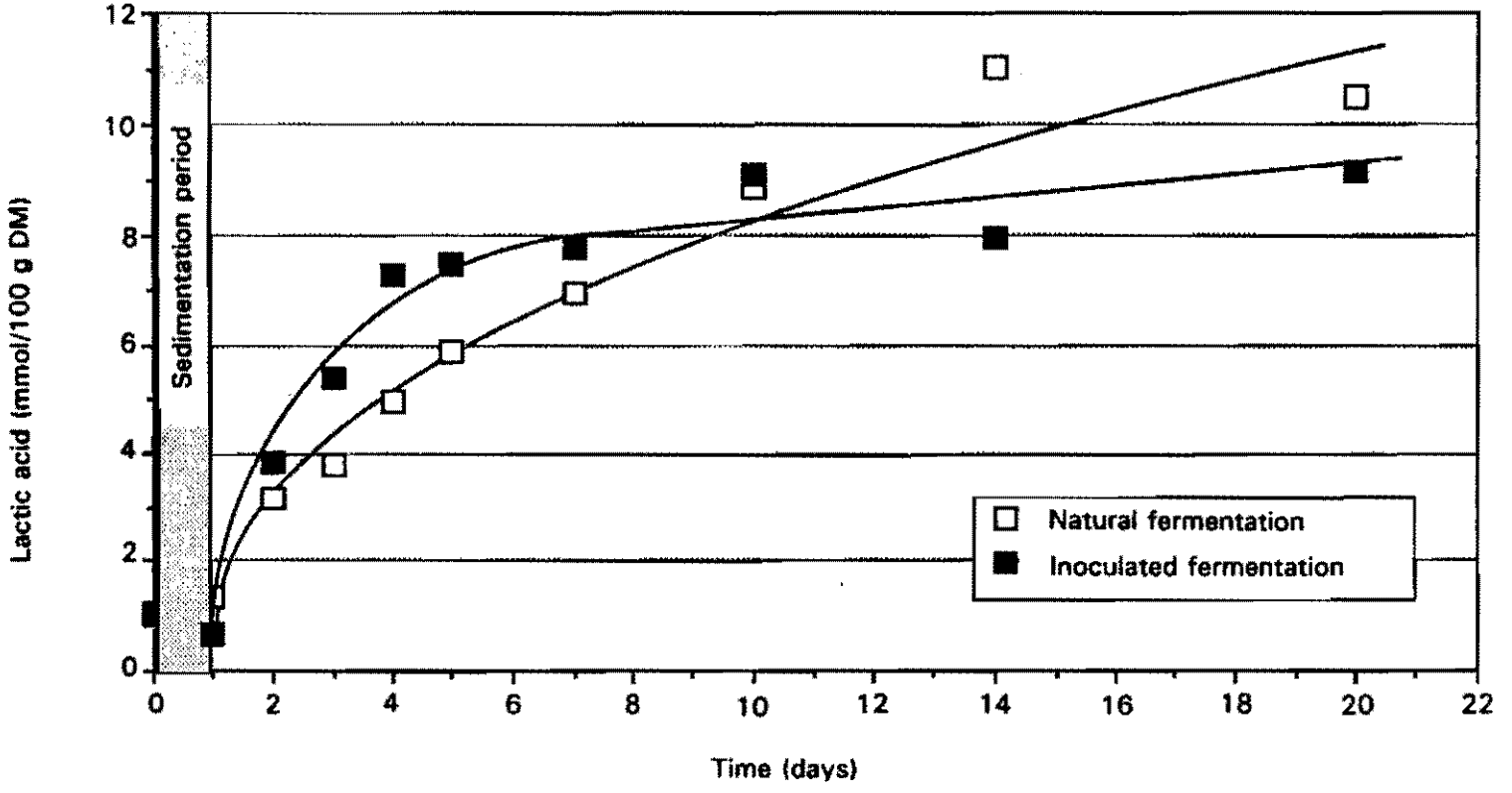

Figure 9. Changes in lactic acid content during cassava starch fermentation.

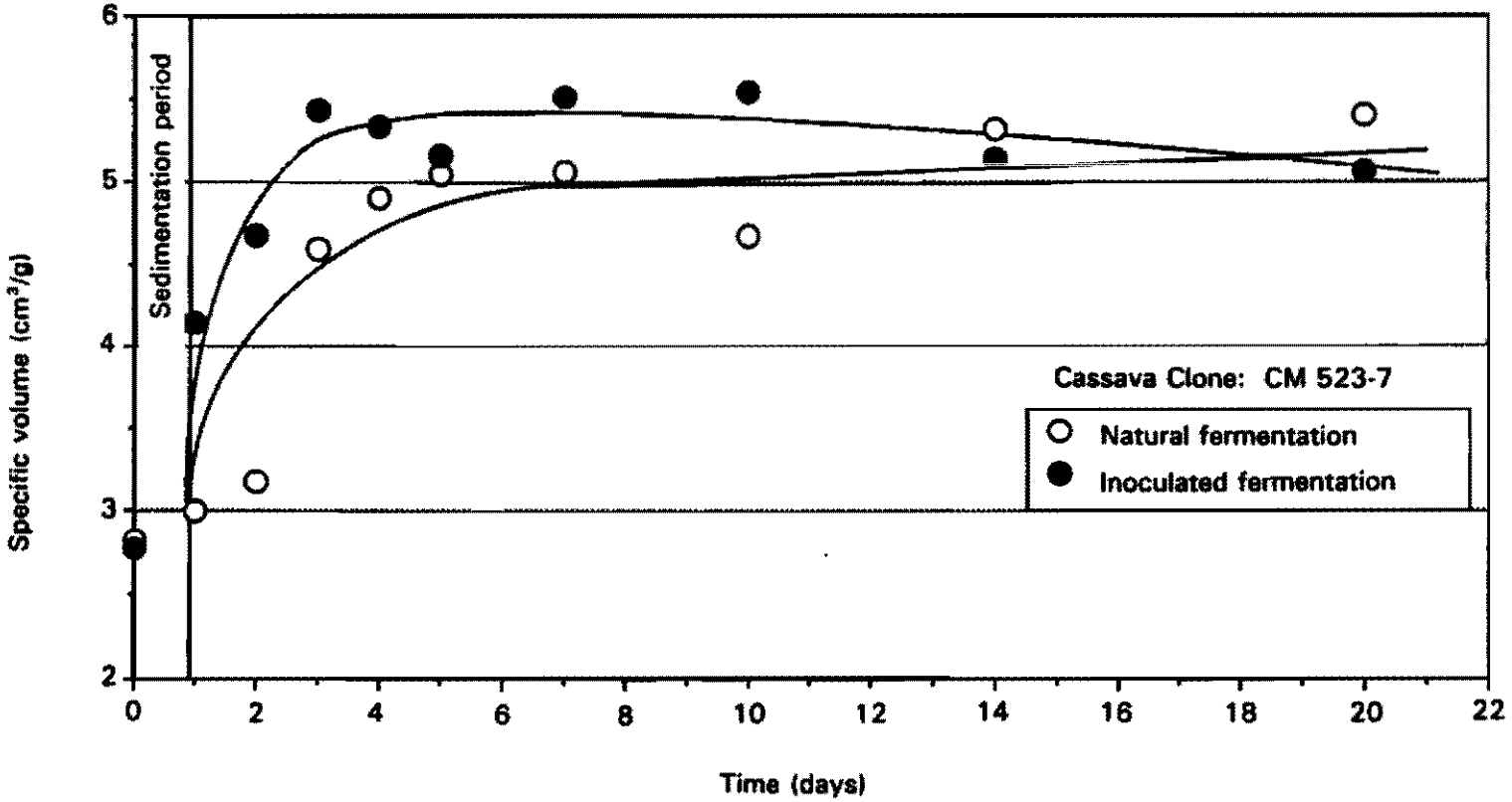

Figure 10. Evolution of cassava starch breadmaking capacity during fermentation.

ENRICHING CASSAVA PROTEIN, USING SOLID STATE FERMENTATION

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Major problems associated with high yielding cassava varieties (bitter-type) are the high cyanide, and low protein content. Simultaneous bioconversion of carbohydrate into protein and reduction of cyanide in cassava meat by *Aspergillus niger* were carried out using a batch and repeated batch-solid state fermentation (SSF) process at laboratory and bench-scale production. A stationary tray bioreactor was designed and equipped with accessories such as air flow meter, air filter and humidifier. The optimization of process conditions (tray location inside bioreactor, moisture content, pH, initial biomass concentration) was conducted by a batch and repeated batch SSF process. The protein concentration in the final product was 27.5% (dry basis, d.b.) at the optimized process conditions in a batch SSF for 72 h of fermentation time. The cycle time and feed amount of repeated batch SSF process were also optimized. The protein concentration of 32% (d.b.) in the product was obtained after 48 h of cycle time of feeding fresh substrate-fermentation medium. This subsequently resulted in cost savings for the preparation of inoculum, and energy, and an increase in protein. The essential amino acids of lysine and methionine increased to 198.5 and 107.4 mg/100 g sample (d.b.). Total cyanide remaining in the fermented cassava of bitter variety was 19% (d.b.). The good quality, final product could be used as feed for fish, poultry and swine.

INTRODUCTION

Cassava grows abundantly in many tropical countries in the world. The dry matter of cassava roots contains about 90% carbohydrate and only 2%-4% crude protein (Grace, 1971). This root crop, because of its low protein content, must be supplemented with protein concentrate if it is to be used as food or feed.

Modern technology helped develop the use of microorganisms for the production of dietary protein supplement from carbohydrates to provide both industrialized and developing countries with these new and valuable protein sources. Many researchers have studied protein enrichment of starchy materials such as cereals, cassava, banana and other crops over the past 20 years (Hesseltine, 1972; Barjracharya and Mudgett, 1979). The low protein content of cassava can be quantitatively and qualitatively improved to a large degree by submerged fermentation (Azoulay et al., 1980; Gregory et al., 1976). But a more attractive, alternative method is a solid state fermentation (SSF), which deals with the use of water-insoluble materials, at low moisture content, for microbial growth and

metabolism (Moo-Young et al., 1983). The SSF process does not involve a separation step, so the final product consists of the whole fermented culture which is a mixture of microbial biomass and residual raw material (Senez, 1987).

This study was conducted to optimize the fermentation conditions for protein enrichment of cassava using batch and repeated batch processes of SSF in a stationary tray bioreactor.

MATERIALS AND METHODS

Materials

The sweet and bitter varieties of cassava tubers (*Manihot esculenta*) were used. The sweet variety cassava was purchased from the Junction market at Los Baños, Laguna, while the Matling Industrial and Commercial Corporation plantation, Malabang, Lanao Del Sur, Philippines donated the bitter variety. The cassava was peeled and cut to approximately 0.5 cm in size and then oven dried 70 °C overnight until its final moisture content reached 7.5% (dry basis, d.b.).

The dried cassava was granulated using a Wiley mill with different size screen (2-3 mm, Internal Diameter). The granulated cassava was stored in a jar or plastic bag at room temperature. Cassava granules of the sweet variety have the following composition (d.b.): protein, 1.50%; lipid, 1.00%; starch, 82.10%; and free sugars, 2.60%.

Agar, dextrose and soluble starch were obtained from Difco. Merck supplied other reagents, all of analytical grade, such as K_2HPO_4, K_2SO_4, and NaOH. Urea and $(NH_4)_2SO_4$ are of technical grade.

Microorganisms

Aspergillus niger BIO-3104 was selected among the 10 strains of *Aspergillus* which are best protein producers in SSF. The strains were subcultured on potato dextrose agar (PDA) test tube slants containing: 25 g potato, 10 g dextrose and 10 g agar per liter; and were incubated at 37 °C for three days. The stock cultures were maintained at refrigerated temperature.

Preparation of inoculum

A suspension of spores was prepared from mold cultures grown on 20 ml PDA slants, aged three days. Five ml of sterile fermentation medium was added to the culture slants and the spores scraped with a loop. The spore suspension was then poured into an empty sterile flask and vigorously mixed using a Vortex mixer.

Further dilution was made to give appropriate optical density as measured on a Shimadzu double-beam spectrophotometer at 510 nm. Two methods were used for calibration of spore densities: dried weight of cell biomass, and viable cell counts using the plating method with PDA medium.

Solid substrate fermentation medium

The nutrition solution used for SSF contained the following components per 100 g dry substrate: 8 g $(NH_4)_2SO_4$; 2 g urea; 4 g K_2HPO_4 and 91.85 ml distilled water to obtain a 50% moisture content. The pH of the medium was adjusted to selected values by addition of 6 M H_3PO_4 and was sterilized at 121 °C for 20 min.

Experimental studies for solid state fermentation

Figure 1 shows a schematic diagram of the experimental set up using either a cabinet tray or a diamond-shaped tray bioreactor. The SSF process was carried out by inoculating mold solution into cassava granules inside the bioreactor. The bioreactor was supplied with 0.257 L/min air which passed through an air filter, then to the 4 N NaOH solution to remove the carbon dioxide, and to saturated K_2SO_4 solution to maintain the relative humidity of air at 97%. To drive out all the CO_2 from the bioreactor, the nitrogen gas was introduced before fermentation started. The output gasses were bubbled through a 2N NaOH solution to absorb CO_2.

In a batch fermentation, the tray location inside the bioreactor and process parameters such as moisture content (30, 40, 50 and 60%), pH (2, 3.5, 5) and initial biomass concentration (5.97, 6.88, 7.99 and 12.05 g) were studied.

In a repeated batch fermentation, the cycle time and the amount of feed added were studied. The optimum conditions from the batch fermentation process were used in determining the effect of cycle time of the fed batch (72, 48 and 24 h) using substrate inoculum. Removal of one-half of the fermented product and addition of the same amount of fresh substrate was done. The effect of feed ratio, or the amount of feed added (2/3, 1/2, 1/3 and 1/5 of substrate fermented), was conducted at 30 °C temperature, 0.257 L/min air flow rate, 50% initial moisture content, 12.05 g biomass/100 g dry substrate and pH 5 of fermentation medium. Repeated batch was done after 48 h of fermentation.

The bench scale production of protein-enriched cassava was also carried out using a minimum of 8 kg raw material loaded on each tray, by batch and repeated batch processes. The bench scale fermentation system was scaled up from the experimental set up (Figure 1). The optimized fermentation conditions at laboratory scale were also applied for the bench scale process.

The repeated batch process at bench scale was done by removing two-thirds of the fermented product and adding the same amount of substrate plus fermentation medium. The remaining one-third of fermented product served as the inoculum.

All substrates and the fermentation medium were sterilized at 121 °C for 15 min and cooled to room temperature prior to inoculation. The rate of inoculation was 20% (v/w) of substrate.

Analytical methods

Determination of dry weight. Fermented cassava product was dried at 100° until its weight remained constant.

Determination of biomass concentration. Twenty ml of the prepared mold solution was centrifuged at 2,000 rpm for 15 min. The biomass was separated and dried at 70 °C until a constant weight was achieved.

Determination of soluble protein. A two g sample was diluted with 50 ml acetate buffer and then vigorously stirred for 20 min using a magnetic stirrer. The diluted sample was filtered using Whatman #541 paper. One ml of 1 N NaOH was added to 2 ml of filtrate, heated in a boiling water bath for 5 min, cooled and then 1 ml of 1 N HCl solution added. The protein content of the solution was determined using the Lowry et al. (1951) method with Bovine Serum Albumin as standard.

Determination of total protein. Total protein was determined by the Kjeldahl method (AOAC, 1980). Protein content was obtained by multiplying the quantity of nitrogen by 6.25.

Determination of amino acids. The protein in the fermented product was hydrolyzed using 6 N HCl in a sealed test tube containing nitrogen gas. The amino acid composition was determined using a Technicon-Amino Acid Analyzer at Korea Food Research Institute, Bundang-ku, Kyonggi-do, Republic of Korea.

Determination of starch. To a two g sample were added 19 ml distilled water, 0.1 ml glucoamylase enzyme solution (Novo Industri, Denmark). The solution was then incubated for 48 h at 55 °C with mixing by magnetic stirrer. The starch content in the sample was determined by the free reducing sugar analysis using Somogyi's (1952) method. Absorbance was measured at 520 nm using the "spectrophotometer".

Determination of cyanide. The free and total cyanide concentration of raw and fermented samples were determined using the calorimetric method of Ikediobi et al. (1980). Linamarase used in the hydrolysis of cyanogenic glucoside

compounds was extracted following the acetone precipitation method of Cooke et al. (1978). The absorbance was read at 490 nm using a spectrophotometer.

RESULTS AND DISCUSSION

Batch fermentation

Effect of tray location inside bioreactor

Figure 2 shows the total protein in the product of cassava SSF carried out in four different tray locations inside the bioreactor. Results showed no significant differences in the total protein yield on the different location of tray levels in the bioreactor. The same trend in soluble protein, free reducing sugars, and starch contents was also obtained. It is observed that the density of filamentous fungi which grew was apparently the same among four trays.

Effect of initial moisture content

Figure 3 shows the results of an optimization study for moisture content in cassava SSF with *A. niger* using the stationary tray bioreactor (STB). A maximum protein of 26% was obtained after 72 h of fermentation where substrate contained 50% initial moisture content. But after 96 h fermentation, the protein content in the product reached 31% with substrate containing 60% initial moisture content, and reached 15% with that containing 40% initial moisture content. The substrate with 30% initial moisture content reached only 7.7% protein content at 96 h. These differences could be attributed to problems concerning moisture regulations of solid state systems. These facts are also supported by the changes of starch, free reducing sugars and pH in the SSF during the time course of fermentation process.

Effect of initial pH

To inhibit bacterial development, the pH value of the medium is generally critical in the SSF process. Figure 4 shows the optimal pH value for mycelial growth (as protein content) on cassava substrate. It was observed that *A. niger* grew more rapidly on lower pH values. At low pH values 2 to 3.5, maximum protein content of 25 to 26% was reached after 72 h, while protein of 13.5% was obtained at pH 5 after 120 h of fermentation. This is a slow conversion process which is almost one-half of the conversion using low pH medium.

Effect of initial biomass concentration

An increase in inoculated, initial biomass concentration does not imply an increase in protein yield (Figure 5). The protein content values reached only 15%

after 72 h of fermentation. These results suggest that protein enrichment is not synonymous with biomass generation. They may be due to the 5.97% (w/w, d.b.) biomass concentration, which has very high initial spores, used in the fermentation medium. Certain geometric limitations are encountered if the added amount of inoculum is large. With the limited available nutrient supply, more biomass inoculated means faster exhaustion of nutrients supply and spores tend to overcrowd and compete. These facts resulted in earlier sporulation which hindered protein enrichment of cassava.

Bench scale production

Protein enrichment of cassava at bench scale was also conducted using the SSF process. The protein content in the fermented cassava reached 26%-28% (d.b.), while the carbohydrate remaining was 54%-51% (d.b.) of three averages of results at bench scale process (Figure 6). Results obtained of protein concentration are very similar compared to laboratory scale.

Repeated batch fermentation

Effect of feed amount

Figure 7 shows the optimal amount of feed for the repeated batch process using the STB as expected. Removing one-third of the fermented product and adding the same amount of fermentation medium increased the protein yield to 10.8%, compared to 8% for those fed by one-half and two-third ratios, and 6% to that fed with one-fifth ratio, 24 h immediately after repeated batch to time interval of 48 h. Addition was done in the second cycle time after 48 h of fermentation with fresh medium (pH 5.0) which explains the low values of protein yield observed.

The effect of different proportions of fermentation medium added is related to the effect of biomass concentration. Too large an amount of fermented inoculum left on the tray and too little additional substrate causes early exhaustion of added nutrients and the accumulation of repressors thus causing sporulation. On the other hand, too little inoculum left on the tray exhibits minimum generation during the early stage of fermentation causing a slower phase in protein enrichment of the available substrate.

Effect of time intervals of repeated batch

Figure 8 shows the effect of repeated batch fermentation, with or without inoculum, at time intervals of 24, 48, and 72 h. The results showed that addition of fermentation medium without inoculum almost equaled that of additional fermentation medium with inoculum. Removing one-half of the fermented product and adding fresh fermentation medium into each tray to maintain a constant layer

thickness further increased the total protein yield after 72 h. A 32.5% to 33.5% protein content was obtained at 120 h when repeated batch was performed on the fermented product at a time interval of 48 h. Another repeated batch was carried out at this time after only a 24 h time interval, and an increasing trend in protein production was observed (Figure 7). If this experiment is extended to its peak protein yield and another repeated batch process performed, then a higher conversion of about 40 to 45% could be expected. This fact confirms the previous results in liquid culture that repeated batch cultivation is better than batch cultivation.

Bench scale production

Protein enrichment of cassava was also carried out at bench scale using repeated batch-SSF process in a cabinet tray bioreactor. Figure 9 shows that the protein concentration in the fermented products was 26.5% in the first cycle and 28.5% in the second cycle in a repeated batch fermentation. This was done by the removal of 2/3 (w/w) fermented product and then the same amount of fresh substrate-fermentation medium was added and mixed well. Results confirmed the protein concentration obtained in SSF process at laboratory scale and that repeated batch fermentation has advantages in increasing the protein concentration of the fermented product. Thus, the repeated batch fermentation also saves energy for sterilization of the bioreactor, and labor cost.

Amino acid composition

With the increase in protein it is presumed that there is an increase of the amino acid concentration in the fermented cassava (Table 1). Results show that all 10 essential amino acids (EAA) are present in the product. Lysine and methionine, usually limiting essential amino acids in the feed, increased to 198.51% and 76.17%, respectively. The lysine concentration required is 1 to 1.2 kg/t in feed ration. Therefore, the protein-enriched cassava could be used to substitute feed concentrates such as soybeans.

Cyanide content

The concentration of total and free cyanide in the raw and fermented sample is shown in Table 2. In the bitter variety, the cyanide concentration in the fermented sample was reduced to 25.6% of unfermented sample in free cyanide and 19% in total cyanide. The reductions of 74.4% and 81% in free and total cyanide are attributed to the SSF process. It may be that the hydrolysis of polysaccharides in cassava starch by enzymes released by *A. niger* affected the cyanogenic glucoside-compounds and reduced more free cyanide in the fermented product than expected.

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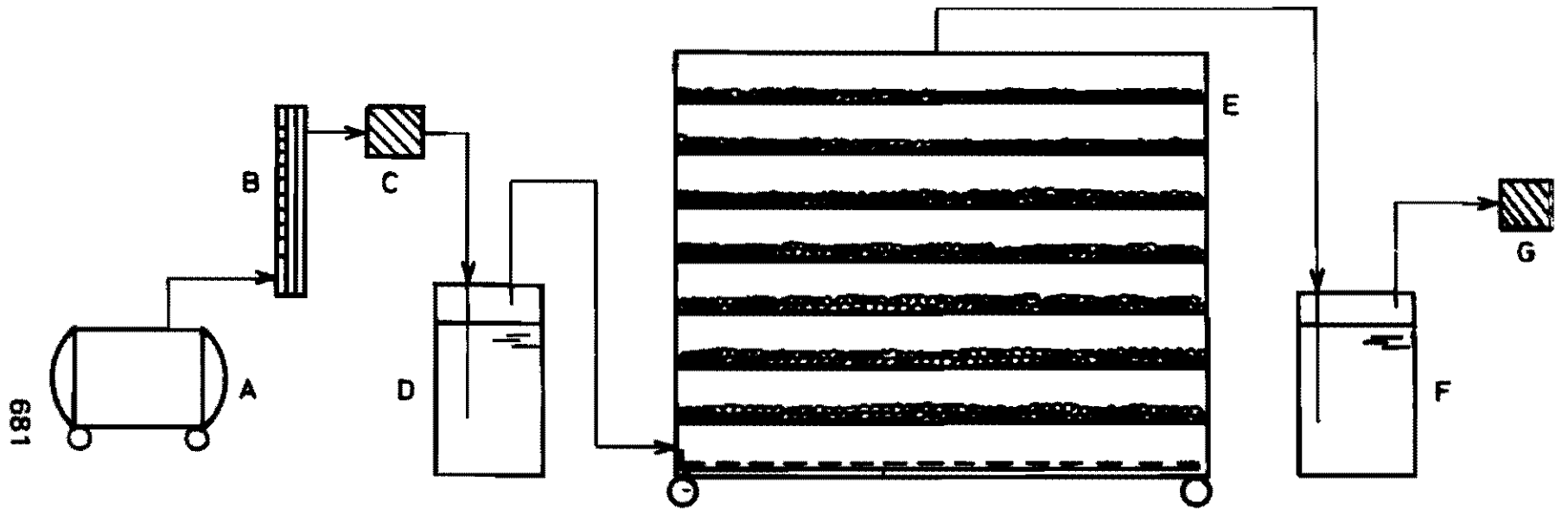
Table 1. Amino acid profile of fermented and unfermented cassava meal.

Composition	Fermented cassava meal	Unfermented cassava meal[a]
A. Protein (%)[b]	33	2.87
B. Amino acid (mg/100 g sple)		
Arginine[c]	162.73	75.00
Theonine[c]	114.25	13.00
Leucine[c]	213.83	15.00
Isoleucine[c]	143.40	10.00
Valine[c]	223.63	14.00
Lysine[c]	198.51	16.00
Histidine[c]	107.39	11.00
Methionine[c]	76.17	3.00
Tryptophan[c]	100.71	—
Phenylalamine[c]	176.21	9.00
Tyrosine	100.71	3.60
Cystine	—	1.00
Asparagine	231.25	—
Serine	114.25	—
Glutamine	—	—
Proline	213.70	—
Glycine	145.09	—
Alanine	192.57	—

a. Nutrient composition of some Phil. Feed stuff (UPLB Dept. of Animal Sci., CA).
b. Dry basis.
c. Essential amino acid.

Table 2. Cyanide content in raw and fermented cassava.

Cyanide content (%)	Total cyanide	Free cyanide
Bitter variety		
Unfermented	100	100
Fermented	19.0	25.6
Sweet variety		
Unfermented	100	100
Fermented	45.20	41.40



A = air compressor
B = air flowmeter
C = air filter
D = saturated K_2SO_4 solution
E = bench-scale bioreactor
F = 2N NaOH solution
G = air filter

Figure 1. Schematic diagram of experimental set up for solid-state fermentation.

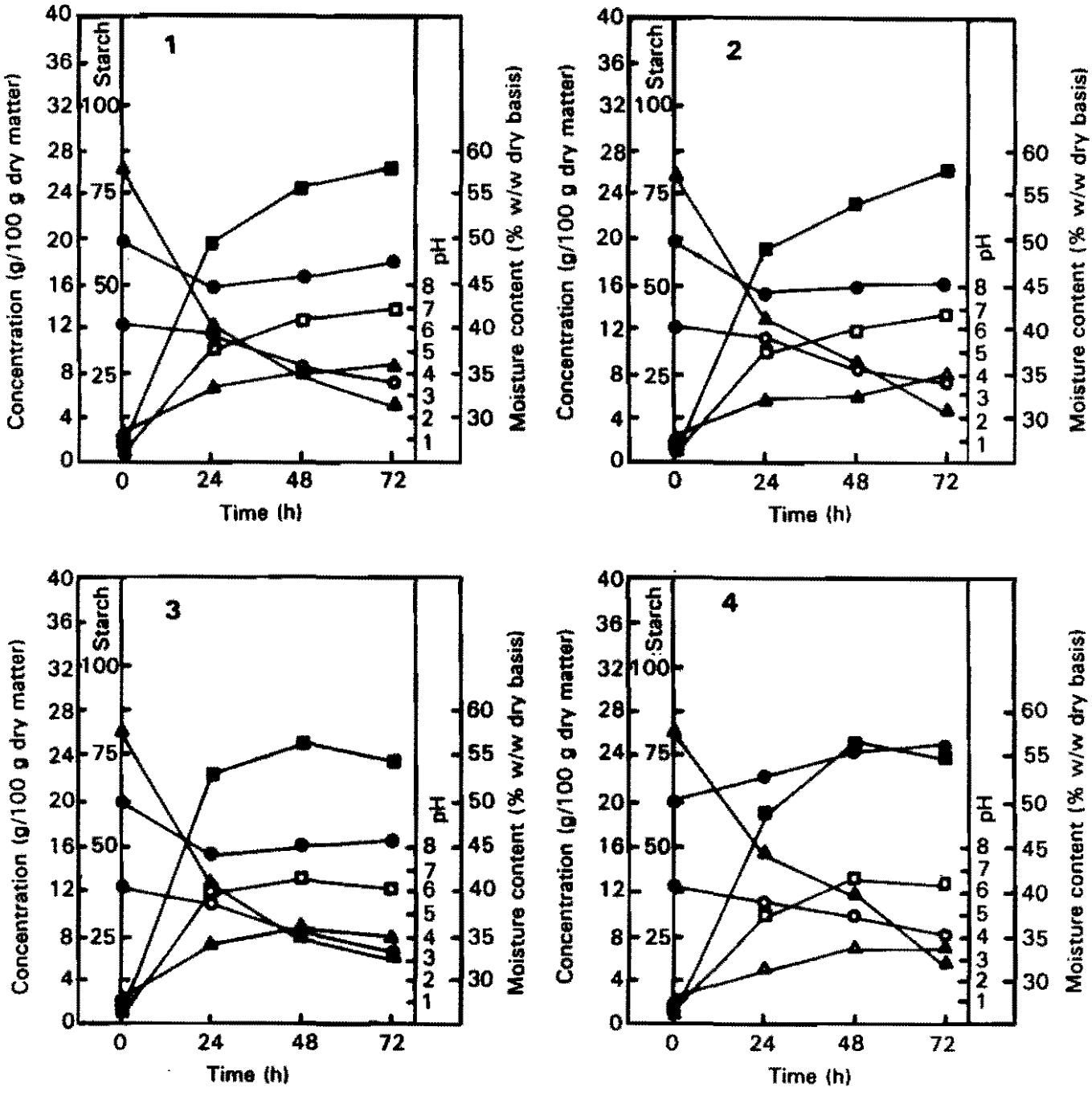


Figure 2. Effect of tray location inside bioreactor on moisture, pH, protein and starch concentration in solid state fermentation of cassava at 30 °C, 0.257 L/min air flow rate, 0.25 cm substrate layer thickness, initial pH 6.0, and 50% initial moisture content for tray: 1, 2, 3 and 4. Concentration: △——△ = free sugar; ▲——▲ = starch; □——□ = soluble protein; ■——■ = total protein. ●——● = Moisture content; ○——○ = pH.

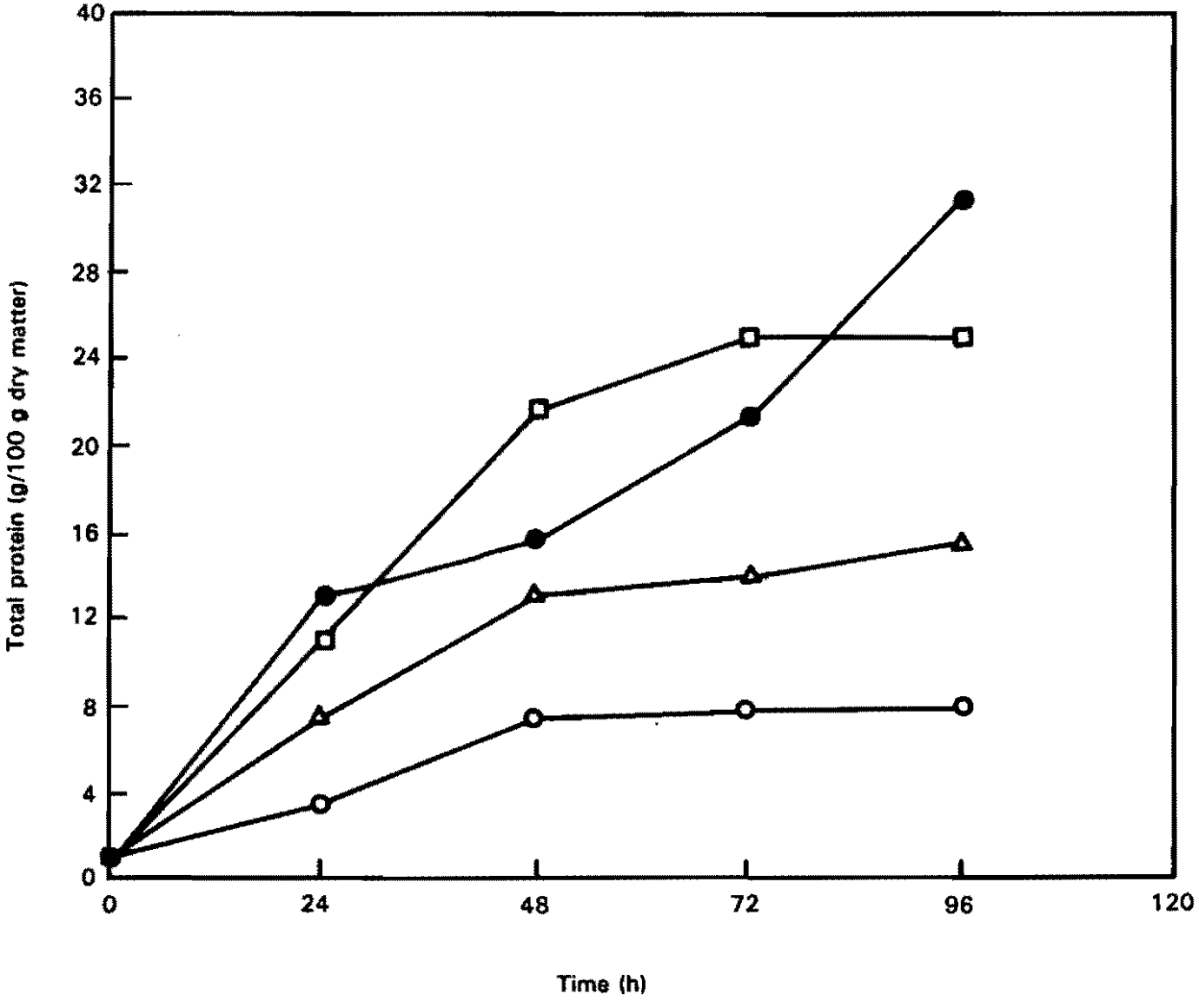


Figure 3. Effect of initial moisture content on protein content of protein enriched cassava at 30 °C, 0.257 L/min air flow rate, 0.25 cm substrate layer thickness and pH 3.5. Initial moisture content (% w/w dry basis): ●——● = 60; □——□ = 50; △——△ = 40; ○——○ = 30.

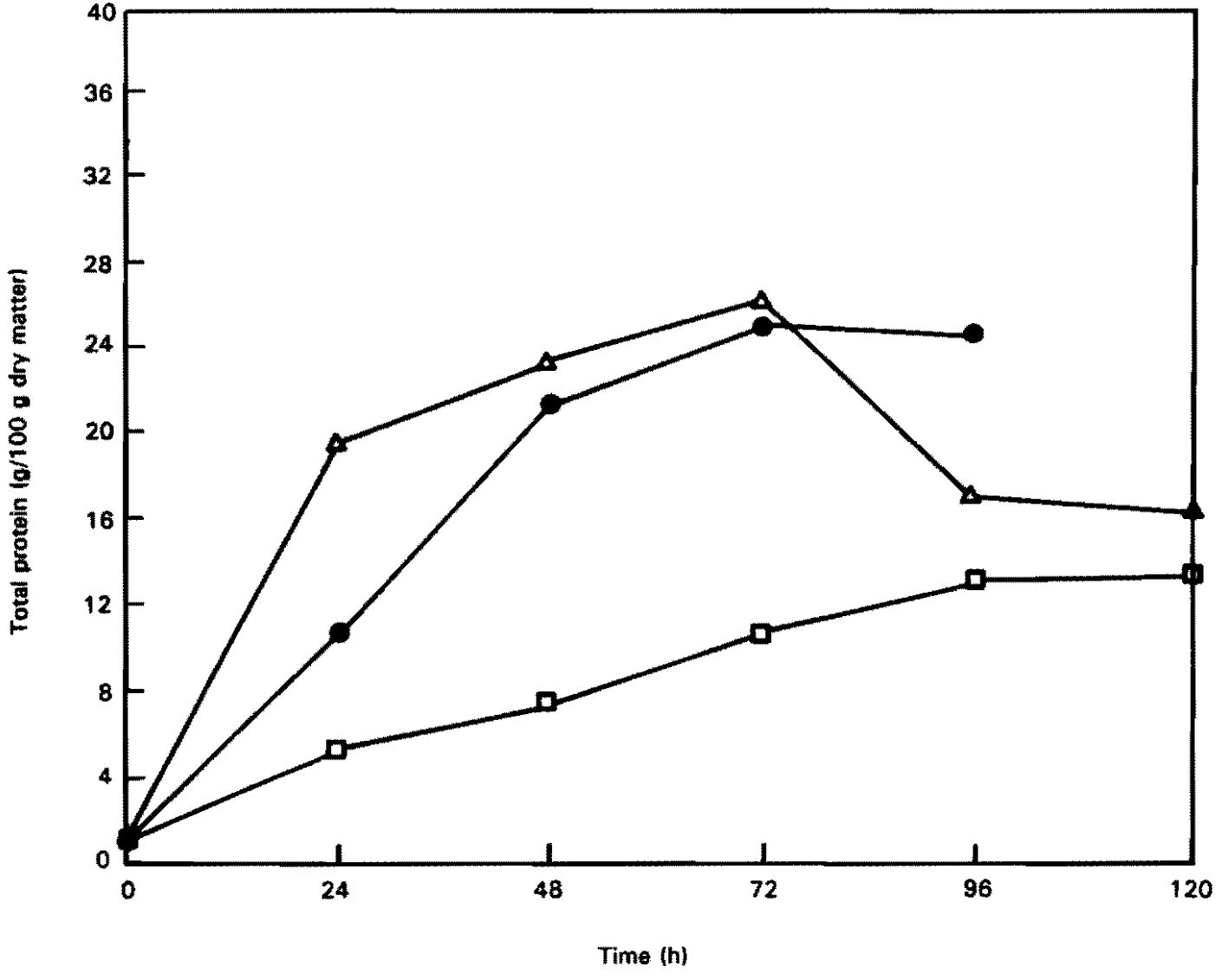


Figure 4. Effect of initial pH on protein content of protein enriched cassava at 30 °C, 0.257 L/min air flow rate, 50% initial moisture content and 0.25 cm substrate layer thickness. Initial pH: ●——● = 3.5; □——□ = 5.0; △——△ = 2.0.

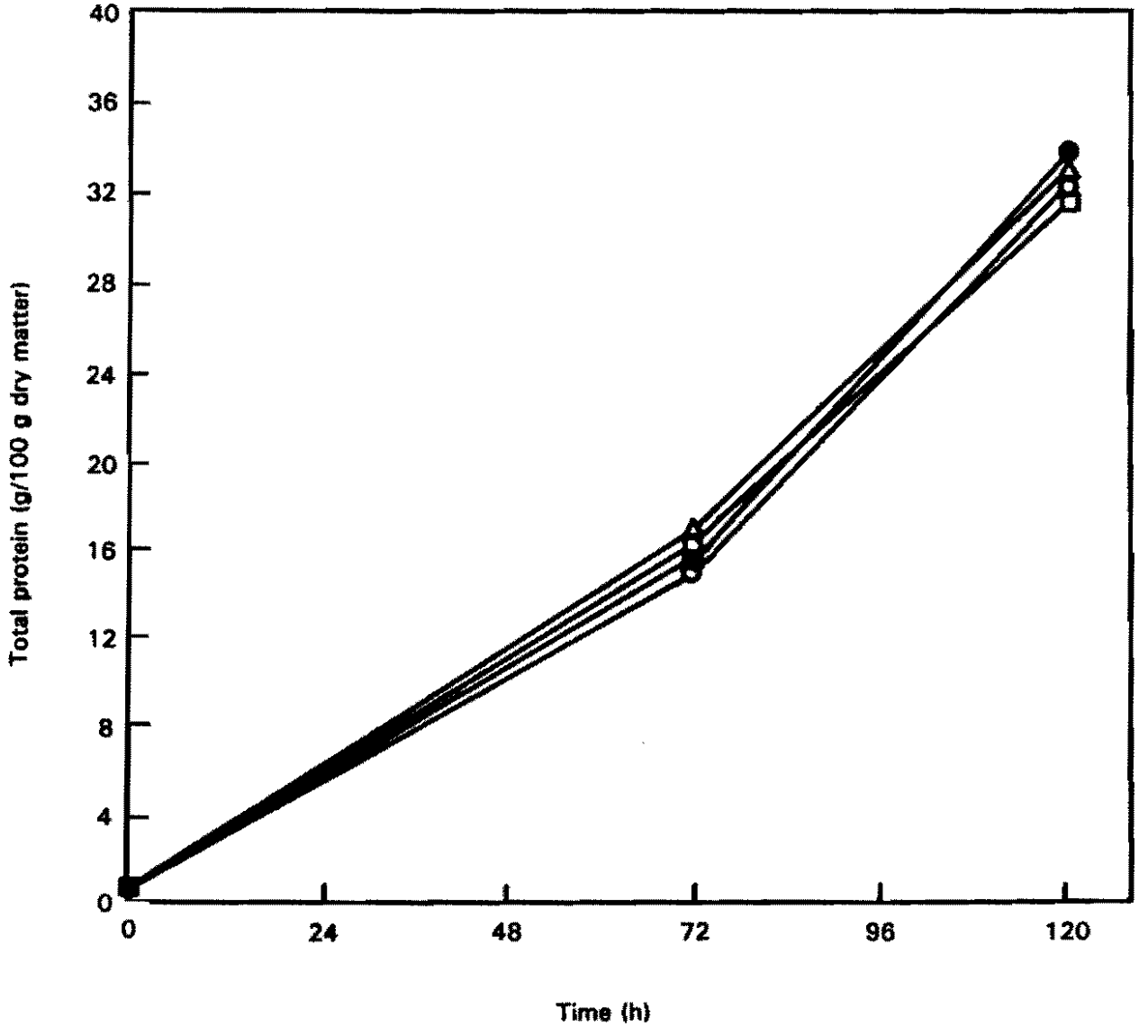


Figure 5. Effect of biomass concentration on protein content of protein enriched cassava at 30 °C, 0.257 L/min air flow rate, 50% initial moisture content, 0.25 cm substrate layer thickness and pH 3.5. Biomass concentration (% w/w dry basis): ●——● = 12.05; □——□ = 7.99; ▲——▲ = 6.88; ○——○ = 5.97.

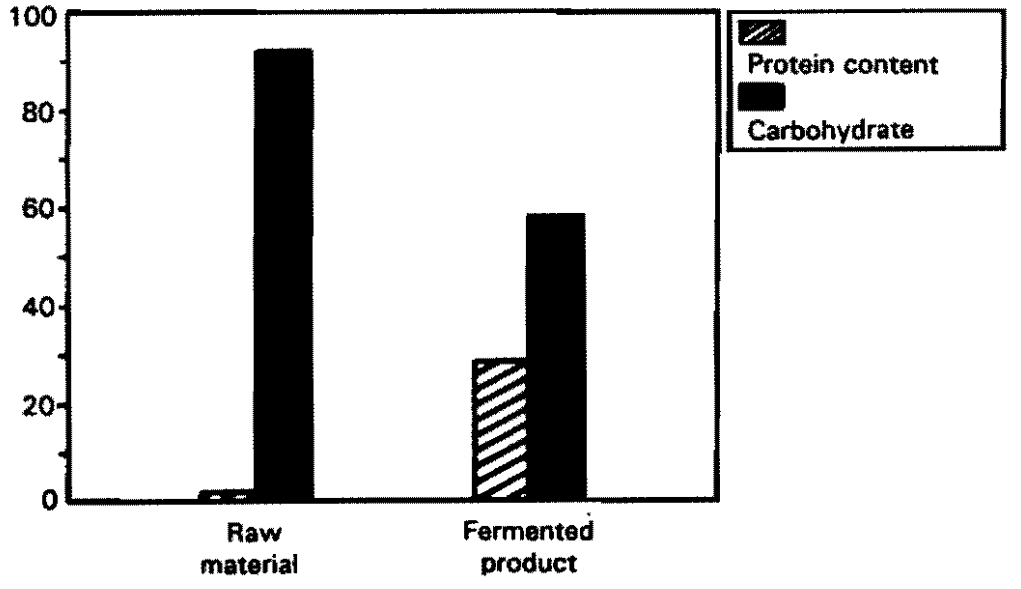


Figure 6. Changes in protein and carbohydrate of raw and fermented cassava at bench scale process using solid state fermentation.

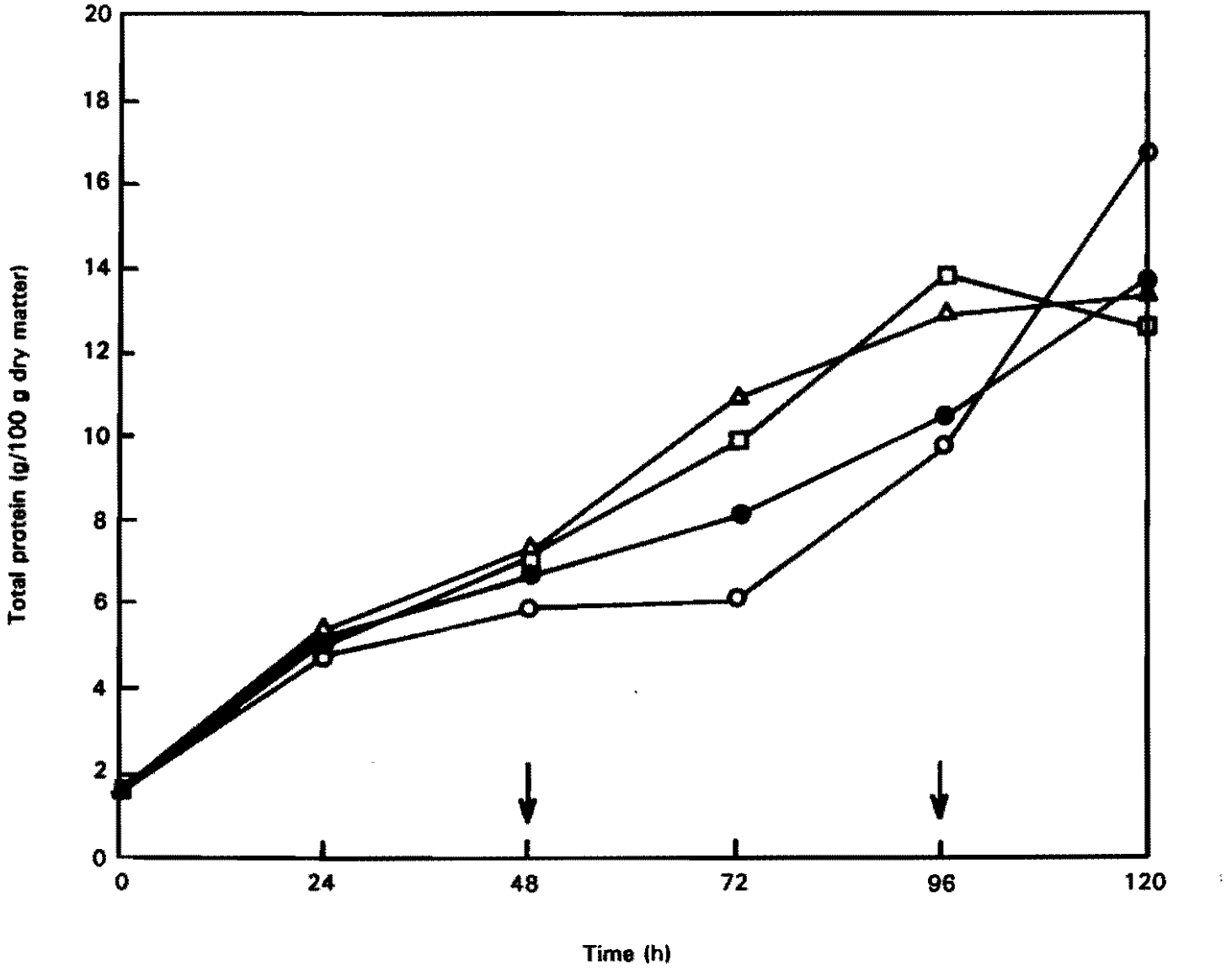


Figure 7. Effect of feed amount on protein produced in repeated batch of solid state fermentation of cassava at 30 °C, 0.257 L/min air flow rate, 50% moisture content, 0.25 cm substrate layer thickness and pH 5. Additional feed amount: ●——● = 2/3; △——△ = 1/3; ○——○ = 1/5; □——□ = 1/2 feeding time of repeated batch.

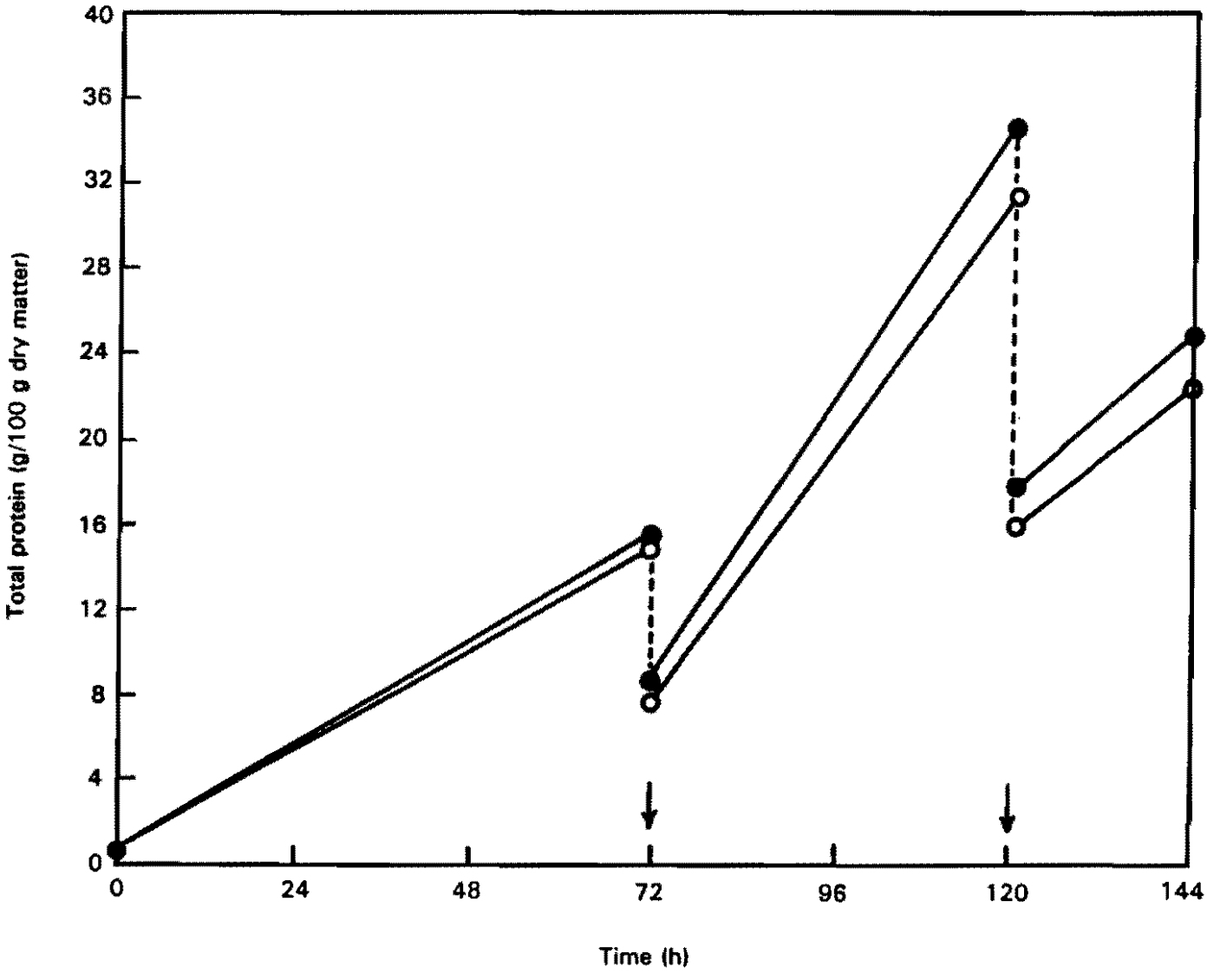


Figure 8. Comparison of two modes of repeated batch addition: ●——● = mold (12.05 g biomass/100 g dry substrate) and substrate fermentation medium; ○——○ = substrate fermentation medium.

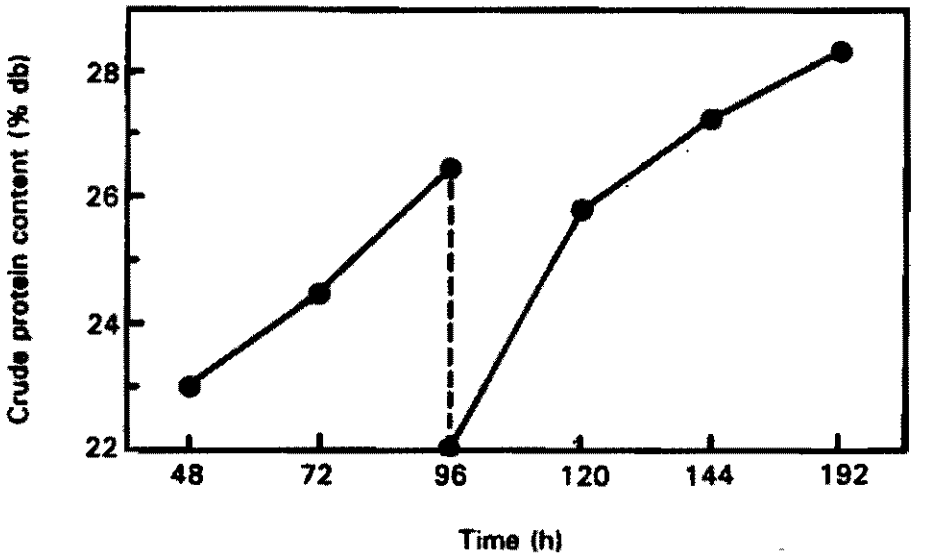


Figure 9. Changes in crude protein content of cassava during repeated batch fermentation where 2/3 of fermented product was removed and the same amount of fresh material was added after 96 h.

BIOTECHNOLOGY FOR THE VALUE ADDITION OF WASTE WATERS AND RESIDUES FROM CASSAVA PROCESSING INDUSTRIES

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Waste waters from starch and sago factories, if treated properly, could be recycled for irrigation and aquaculture besides improving the quality of life around processing factories. Residues could be processed to value-added products using conventional and new biotechnological methods. By incubation and filtration, cyanide concentration in waste water was brought down to 0.6 µg/ml from an initial concentration of 28.45 µg/ml. Cyanide-degrading microorganisms indicated the possibility of developing a consortium of microorganisms to remove cyanide from waste waters and the environment of cassava processing industries. Co-culturing experiments with *Endomycopis* fibuliger and *Candida utilis* showed that the BOD level of effluents could be reduced to 5.1 g/l from an initial value of 38.0, and the COD level to 6.7 g/l from 20.0. The concomitant SCP production was 20 g/100 ml. Solid-state fermentation with fungal cultures could elevate the total protein content of the residues. It was possible to separate food-grade industrial starch from the residues of cassava processing industries by treating with pectinase and cellulase. The starch recovery was 7.1%, 15.9% and 37.0% when the concentration of enzymes was 0.2%, 0.3% and 0.4% respectively. Saccharification of residues with dilute hydrochloric acid, amylase enzymes (singly and in combination with cellulase and pectinase), resulted in fermentable sugars ranging from 5.43% to 10%. Results of the experiments showed possibilities of application of biotechnology for recycling waste waters and utilization of residues from cassava processing industries for value added products.

INTRODUCTION

On average, the starch/sago industry generates waste waters in the range of 16-20 m^3/ton of starch/sago produced. The effluent discharge is intermittent and the waste water is acidic in nature with high organic content (Balagopalan et al., 1988). Normally effluents are discharged to the land or river outside factory premises without any pretreatment. The peeled cassava skin and residues after starch extraction constitute the solid waste. The residues, including fibers, knots etc. is known as "Thippi". Peelings amount to 50-60 kg/ton of roots peeled and Thippi to about 55-70 kg/ton of roots processed. Conventional biotechnological methods are found to be efficient in the treatment of organic effluents where different microorganisms are involved in the oxidation process of organic matter. The probability for exploitation of naturally occurring microorganisms for cyanide degrading enzymes were explored (Padmaja and Balagopalan, 1985). Introducing and developing a consortium of specific microorganisms and enzyme systems in the

treatment is a viable way of reducing pollution accompanying the production of single-cell proteins (Manilal et al., 1991). So attempts were made to develop technologies to eliminate cyanide, reduce BOD and COD, and reuse waste waters. The residues were also processed to recover starch, enrich protein and bioconvert to ethanol.

TREATMENT AND VALUE ADDITION OF WASTE WATERS FROM CASSAVA PROCESSING INDUSTRY

Elimination of cyanide and reduction of biological oxidation demand (BOD)

Materials and methods

Cassava starch/sago factory effluent from a small-scale starch factory were collected from the point of discharge and divided into two samples. One sample was exposed to sunlight (6 h) and the other to the laboratory atmosphere. The initial concentration of total cyanide and BOD was estimated using the standard procedure. The effluents were incubated for 24, 48 and 72 h. At the end of each incubation period, the effluents were filtered through sand, gravel and charcoal columns and three cycles of filtration with the same effluent were attempted. At the end of each filtration, samples were collected and total cyanide and BOD estimated.

Results and discussion

A drastic reduction in the total cyanide and BOD was observed in the effluents after different incubation times and filtration through different columns (Tables 1 and 2). Samples exposed to the sun showed a more pronounced reduction in cyanide concentration. Concentration of cyanide could be reduced to 0.15 mg/l from an initial concentration of 28.45 mg/l after incubating 24 h during the first cycle of filtration. The corresponding value for the samples exposed under laboratory conditions was 0.62 mg/l. Incubating the samples for more than 24 h and further repeating filtration did not help eliminate the cyanide. This was perhaps because of cyanide being released from the adsorbed charcoal columns saturated with it.

Terrestrial plants containing cyanoglucosides release free HCN when their cellular structure is disrupted (Leduc, 1981). During the starch extraction process cyanide is released from the disrupted cells and becomes a major part of the effluents discharged. Cyanide's deleterious effects in the ecosystem have been discussed in detail (Doudoroff, 1976; Dixon and Leduc, 1981).

The BOD of the effluents could also be reduced from an initial value of 2400 mg/l to less than 100 mg/l. Prolonged exposure to sunlight seems to be advantageous in reducing BOD. Reasons for the reduction of BOD may be

sedimentation of carbohydrates and starch breakdown by proliferating microorganisms during the prolonged incubation (Manilal et al., 1991). The columns used for filtration also helped reduce the solids present in the effluents which lowered BOD.

Role of specific microorganisms in the detoxification of cyanide from cassava starch factory effluents

Various types of microorganisms have been found to proliferate in the Cassava starch factory effluents (Balagopalan and Maini, 1976; Manilal et al., 1983; Noparathnarapom, 1987). In our laboratory, we made a detailed study of the role of cyanide degrading fungus, *Rhizopus oryzae*, and documented it (Padmaja and Balagolpalan, 1985; Ray et al., 1991). High levels of extracellular rhodanese was produced during the exponential growth phase of the fungus and the optimum pH was 4.6 to 5.4 and temperature 30 to 35 °C. Potassium cyanide (KCN) at 0.025 and 0.5 mμ levels was found to induce rhodanese synthesis and beyond this level it completely suppressed cell growth. These investigations revealed the potential of *R. oryzae* to detoxify cyanoglucosides in waste water.

Co-culturing experiments for treating cassava starch factory effluents with concomitant scp production

An attempt was made at CTCRI to treat the cassava starch factory effluents for SCP production by submerged fermentation of three organisms: *Candida utilis*, *Endomycopsis fibuliger* and *E. magnusi* (Manilal et al., 1991). Starch is the major constituent of starch factory effluents so its removal is important in reducing pollution. But *C. utilis* does not degrade starch and so must be co-cultured with amylolytic yeasts such as *E. fibuliger* and *E. magnusi*. The results of mixed cultivation (Tables 3 and 4) showed that, supported by a rapid growth of *C. utilis*, the production of biomass protein was higher in the mixed culture with *E. fibuliger*, which proved to be best for bioconverting carbohydrates into yeast protein. Microbial biomass has been produced from other starch materials using mixed cultures of yeasts (Jarl, 1969; Lemmel et al., 1979; Passari et al., 1989).

TREATMENT AND VALUE ADDITION OF RESIDUES FROM CASSAVA PROCESSING INDUSTRY

Enzymatic separation of starch from the residues of cassava processing industries

Thippi produced from cassava processing industries is currently disposed of as animal feed. It contains about 50% to 60% starch on dry weight basis and the starch granules are located in the lignocellulosic and pectic matrix of the cells and

so cannot be easily extracted. An attempt was therefore made to separate starch from Thippi. To do this a novel method (Kallabinski and Balagopalan, 1991), involving maceration of the tissues and disintegration of cell wall by cellulolytic and pectinolytic enzymes, was attempted.

Materials and methods

Starch/sago factory residues were pulverized in a warring blender with thrice the volume of water and transferred into 500 ml Erlenmeyer flasks. Pectinase and cellulase enzymes at 0.2%, 0.3% and 0.4% concentration (w/w) were added to the slurry and incubated for 18 h at 30 ± 1 °C in static conditions with occasional stirring (pectic enzyme Rohament PC from Roham Industries, Germany, and cellulase enzyme celluclast from Novo Nordisk, Denmark). Starch was then recovered, following standard procedures of filtering through 80 mesh sieves.

Results and discussion

The present study showed it is possible to recover a higher quantity of bright colored starch from the residues of cassava processing industries (Table 5). In enzyme treatment at 0.2% concentration, the weight of starch recovered was 71.4 g from one kg of residue. The quantity gradually increased to 159.2 g when the concentration of enzymes was increased to 0.3% and to 370 g at 0.4%. The corresponding value for the control experiments where no enzyme was used was only 33.94 g. The brabender viscographic studies of starch recovered from treated and untreated samples showed no difference in the rheological properties. Padmanabhan and Losane (1992) made similar observations in the case of dried cassava chips subjected to enzymatic treatment.

Solid state fermentation of residues from cassava processing industries for protein enrichment

Balagopalan and Padmaja (1988) developed a solid sate fermentation process for the protein enrichment of cassava starch factory residues using the fungus *Trichoderma pseudokonigii* Rifai. Results showed that using this process was possible to convert the substrate, using minimum nutrients, to a protein enriched animal feed (Figure 1).

Padmaja and Balagopalan (1990) studied *T. pseudokoningii* enriched cassava waste: flour mix (50:50) as an energy source in broiler rations, using three levels of feed inclusion—50%, 55% and 60%.

Growth performance and percentage carcass yields showed that use of up to 60% of the enriched material did not adversely affect the performance of birds. But the cumulative feed intake was less for the test birds which led to the reduction

in overall feed use. This study clearly shows the potential that exists for commercial broiler farming to switch from conventional feeds to a feed based on cassava waste (Figure 2).

Saccharification and fermentation of residues of residues of cassava processing industries for ethanol production

There has been little past interest in recovering ethanol or other commodity chemicals from residues of cassava starch processing industries. An attempt was therefore made to saccharify the residues with acids and enzymes and ferment them to produce ethanol.

Materials and methods

Residues from cassava processing industries were subjected to saccharification with 0.1 N HCl. The residue was first cooked for two and a half hours to release the starch granules bound to the lignocellulosic compounds of the roots at 1 atmosphere with 0.1 N HCl. After saccharification, the slurry was cooled and filtered to discard the residues. The sugar solution was then neutralized with ammonium hydroxide and the pH was adjusted to 5.1. The neutralized solution was subjected to fermentation with *Saccharomyces cerevisiae* for 48 h (1.0-1.1 g | yeast | 100 ml hydrolysate).

Termamyl, a heat stable amylase enzyme, was added to the residue at the rate of 0.1% and the residue was heated to 60 °C for 30 min. After gelatinization and cooling, the enzyme amyloglucosidase at 0.3 percent was added and the slurry incubated for 48 h. The resultant sugar solution was filtered and subjected to fermentation for 48 h.

Another batch of gelatinized slurry obtained after treatment with Termamyl (0.1%) was saccharified with cellulase, 0.2% (celluclast), pectinase 0.2% (Rohm PC) and amyloglucosidase 0.3% for 48 h. The sugar solution was filtered and subjected to fermentation for 48 h.

Results and discussion

Results given in (Table 6) showed that maximum saccharification efficiency was obtained with 0.1 N HCl and 10% sugar was recorded in the filtered solution. In the case of termamyl and amyloglucosidase the conversion was only 5.43%, whereas in treatments with mixed enzymes including termamyl, amyloglucosidase, cellulase and pectinase, the conversion was 8.06%. Lonsane and Ghildyal (1992) recorded a yield of 55 kg of ethanol from one ton of residue by enzyme saccharification. The yield of sugar obtained in the present study is much higher and acid saccharification appears a better process for ethanol production from

residues because the conversion of starch to sugar was higher. Two of the disadvantages of the use of acids in the saccharification process are: the formation of secondary reversion reactions setting a limit to the yield of glucose that may be obtained; and the formation of high inorganic salts due to pH adjustment, corrosion of machinery etc.

CONCLUSION

The total cyanide and BOD concentration of cassava starch, factory processing industries can be reduced by incubation and filtration through sand, soil and charcoal. The potential of *Rhizopus oryzae* for producing rhodanese to apply cyanide detoxification in the environment has to be studied in detail. Co-culturing of microorganisms resulted in reducing BOD and COD in the effluents with concomitant SCP production. Industries can increase income by adding cassava starch factory residues to animal feed and to commodity chemicals like ethanol, citric acid, vitamin C and liquid glucose. The impact on the environment of waste waters and residues of cassava processing industries has to be investigated. There are possibilities for recycling the treated waste waters for aquaculture.

ACKNOWLEDGMENTS

The authors wish to thank the Director, CTCRI, for encouraging and supporting the investigations. Thanks are due to Smt. K.R. Lakshmi, Scientist, CTCRI for the preparation of computer graphics. Thanks are also due to Dr. Ann Marie Thro, Co-ordinator, CBN, for financial support given to present the paper.

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Table 1. Total cyanide in cassava starch factory waste waters filtered through gravel, sand and charcoal columns. (All values expressed in mg/l.). Initial cyanide: 28.45 mg/l.

Incubation period	Cycle	Exposed to sunlight	Not exposed to sunlight
24 h	O	7.95	25.57
	I	0.15	0.60
	II	3.45	9.05
	III	2.60	7.82
48 h	O	6.32	22.52
	I	0.30	4.22
	II	2.05	9.55
	III	0.95	6.25
72 h	O	6.72	19.37
	I	0.75	2.30
	II	2.52	8.50
	III	9.87	4.90

Table 2. BOD in cassava starch factory waste waters filtered through gravel, sand and charcoal columns. (All values expressed in mg/l). Initial BOD: 2400 mg/l.

Incubation period	Cycle	Exposed to sunlight	Not exposed to sunlight
24 h	O	120	480
	I	200	280
	II	360	200
	III	160	360
48 h	O	200	360
	I	960	640
	II	80	240
	III	240	20
72 h	O	360	120
	I	–	120
	II	40	120
	III	120	80

Table 3. Profile of mixed culturing of *C. utilis* and *E. fibuliger* in the cassava starch factory effluent.

Time (h)	Starch (%w/v)	Reducing sugars (%w/v)	Cell number* ($x10^{-6}$/ml)	Cell number+ ($x10^{-6}$/ml)	Biomass protein (g/100 ml effluent	COD[a] (g/l)	BOD[a] (g/l)
0	3.2	0.10	40	37	9	38.0	20.0
24	2.1	0.16	77	48	14	9.5	7.4
48	1.8	0.58	220	127	17	12.2	11.0
72	1.8	0.20	206	123	18	10.3	7.0
96	1.7	0.25	288	132	20	7.4	5.5
120	1.5	0.16	281	119	20	6.7	5.1

* *C. utilis*.
\+ *E. fibulger*.

a. COD = Chemical Oxygen Demand; BOD = Biochemical Oxygen Demand.

Table 4. Profile of mixed culturing of *C. utilis* and *E. magnusi* in the cassava starch factory effluent.

Time (h)	Starch (%w/v)	Reducing sugars (%w/v)	Cell number* ($x10^{-6}$/ml)	Cell number+ ($x10^{-6}$/ml)	Biomass protein (g/100 ml effluent	COD[a] (g/l)	BOD[a] (g/l)
0	3.3	0.10	40	38	5	38.5	20.5
24	3.2	ND[b]	62	38	7	3.9	3.6
48	3.2	ND	82	39	9	4.0	3.9
72	3.2	ND	85	40	10	4.0	3.9
96	2.7	0.15	134	652	12	9.3	5.5
120	1.7	0.81	134	85	12	14.5	12.2

* *C. utilis*.
\+ *E. fibulger*.

a. COD = Chemical Oxygen Demand; BOD = Biochemical Oxygen Demand.
b. ND = Not detected.

Table 5. Enzymatic separation of starch from cassava starch factory residues.

Concentration of enzymes[a] (%)	Weight of starch (g)	Weight of residues (g)	Percentage of starch
0.2	71.4	770.0	84.12
0.3	159.2	650.0	80.90
0.4	370.0	410.0	84.74
Control	33.9	870.0	84.90

a. Enzymes: Celluclast (Celluclast); Pectinase (Rohament PC).

Table 6. Saccharification of cassava starch factory residues

	Percentage of sugar	Residues recovered (g)
Acid hydrolysis[a]	10.00	137.0
TA + AMG[b]	5.43	290.0
TA + AMG + PECT + CL	8.06	143.0

a. Acid concentration: 0.1 N.

b. TA = Termamyl; AMG = Amyloglucosides; PECT = Pectinase; CL = Cellulase. Enzyme concentration: TA: 0.1 %; AMG: 0.3%; CL: 0.2%; PECT: 0.2%.

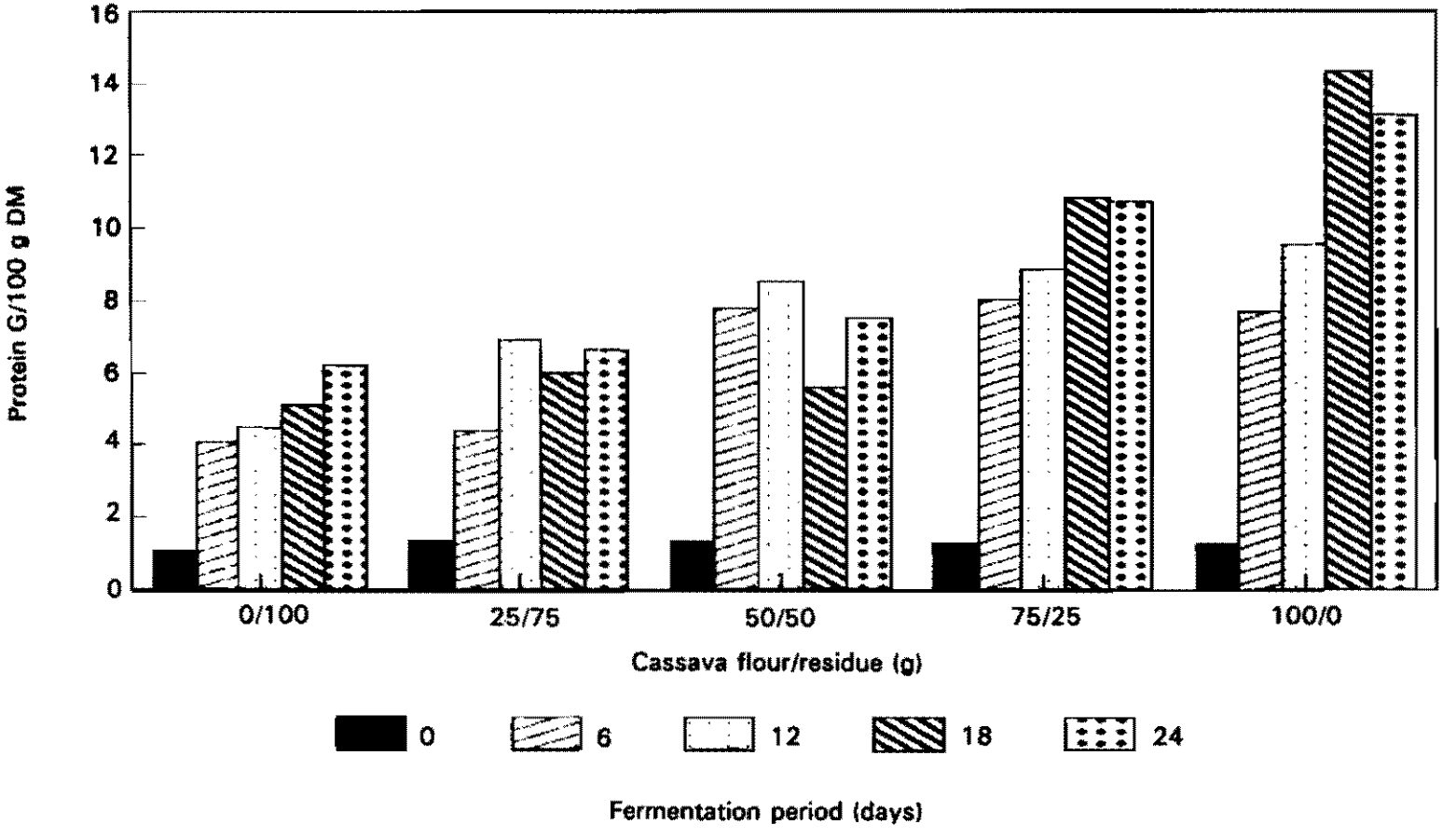

Figure 1. Protein build up during solid state fermentation of cassava with *Trichoderma pseudokoningii* Rifai.

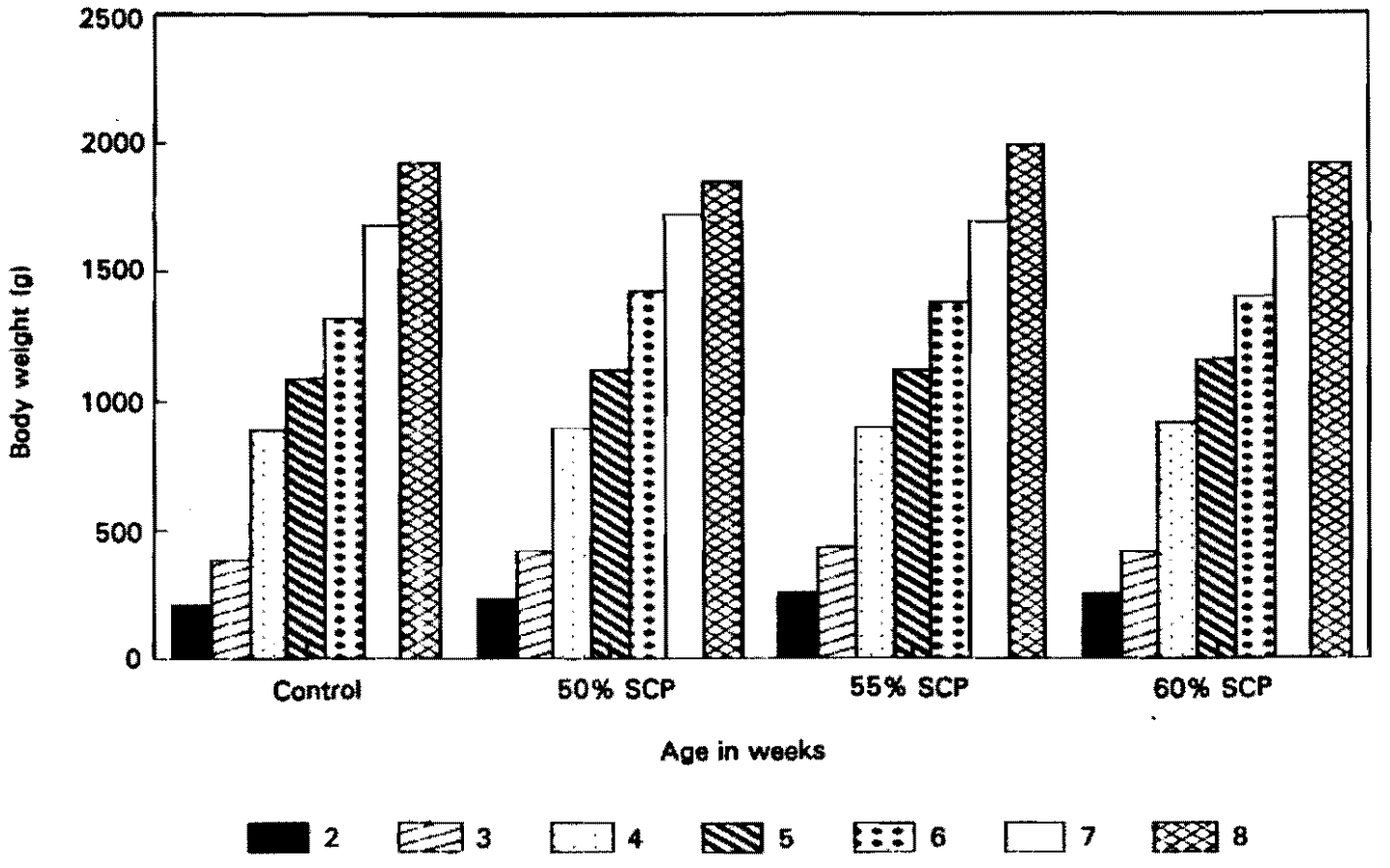

Figure 2. Growth performance of broilers fed with SCP enriched cassava feed.

MICROORGANISMS INVOLVED IN *GROWOL* (SUBMERGED FERMENTED CASSAVA) PRODUCTION

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Growol is a traditional Indonesian food made by submerged fermentation process. The research aims were to identify the types and roles of microorganisms present during the fermentation of *growol*, and its physicochemical properties. Sweet cassava was peeled and soaked in a clay pot with excess well water. Fermentation was conducted outdoors for 2 to 5 days. Types and total of microorganisms were determined using spread and standard plate count methods. The isolate was purified using the four streak plate method. During 5 days of fermentation, the microorganisms found were: *Streptococcus* sp., *Coryneform* group, Yeast, *Enterobacteriaceae* sp., *Bacillus* sp., *Acinetobacter* sp., *Lactobacillus* sp., and *Moraxella* sp. Microbial growth was affected by substrate changes, such as pH and soluble oxygen, during fermentation. It was concluded that fermentation of *growol* can be classified as heterofermentation. Microorganism types and total microorganism growth, play an important role in texture and flavor forming.

INTRODUCTION

Traditional food fermentation processes use microbial growth to produce a food product, more attractive and consumer preferred than in its original form (Kosaki, 1979). Some traditional fermented foods are popular in Indonesia, such as: *kecap* (soy sauce), *tempe* (fermented soy cake); *Terasi* (fermented fish cake); *Oncom* (fermented peanut cake); and *tape, brem* and *growol* (fermented cassava).

Growol is a traditional fermented product made from cassava (*Manihot utilisma*) and is a well-known staple food in Central Java, especially in the Kulon Progo district. It is classified as a traditional product because the process has been known for generations. This food is processed using simple equipment and with limited knowledge of fermentation techniques, but the product has a good taste, is safe, typical and enjoyed by the community in Kulon Progo district.

Lie (1983) stated that success in traditional food production is because producers keep the process they learned from their ancestors. In Indonesia, traditional food is still a small-scale industry or mostly home industry. Because of its locally characteristic taste, widespread consumer acceptance does not as yet exist. The demand remains very limited, so production on a commercial scale has still not been developed.

ROLE OF *GROWOL* IN INDONESIAN DIET

At first, *growol* was processed for ordinary needs. Usually it is done by housewives in villages. It is also practiced in an effort to lengthen cassava storage period, especially during peak harvesting season. The product began to develop when urban communities began to accept it. Consumer preference areas are Kulon Pro-go district and around Yogyakarta, Muntilan and Purworejo districts (Rascana, 1986).

Growol is a source of carbohydrate which can substitute for rice as a staple food. Usually, farmers take some *growol* for a meal (lunch) when they go to the field. It is different from other cassava products (boiled cassava, pasta cassava), in that farmers can consume as much as they need without getting stomach ache. *Growol* is a preserved cassava form with a storage life of more than 1 month under good conditions. Resteaming *growol* every week is a traditional way to extend its shelf life.

GROWOL PROCESSING

Methods of fermentation

Fresh cassava is peeled, cut and soaked in well water (completely covered) in a clay pot or plastic bowl. During fermentation, all cassava should be submerged in water. Fermentation is conducted outdoors, for 2 to 5 days, depending on the producer's habit, or until the cassava is soft and floating. Figure 1 shows the procedure for making *growol*.

Physicochemical characteristics

Like fresh cassava, *growol* is a source of carbohydrate. Other constituents (proteins, fats, minerals) are limited. Table 1 shows the chemical composition of cassava and *growol*. Barret (1984) mentioned that in general, traditional food processing caused a decrease in proteins and vitamins. But the loss of nutrient content caused by the process is quite low compared with its advantages, such as the decrease in cyanide or other antinutritional content (Steinkraus, 1977; Rascana, 1986).

There are some physicochemical changes during *growol* fermentation: hardness, a reduction of sugars, total acids and degrees of acidity (pH).

Hardness

Fermentation times and temperature affect *growol's* hardness. It is determined by using a penetrometer and changes from 1.483 mm/second to 15.61 mm/sc (Figure 2). Change is slow during the first 12 h, then increases

gradually during the next 12 (Gustati, 1987). The process of lessening cassava hardness correlates with enzyme activity (cellulase, pectinase and amylase). The metabolism and growth chemical reaction increases in correlation with increasing temperature, up to the temperature limit that causes enzyme destruction preventing further chemical reaction (Pederson, 1971).

Reducing sugars

During fermentation, reducing sugars decrease from 1.0%-1.45% (fresh cassava) to 0.45%-0.60% (*growol*). This is caused by reducing sugars decomposing to pyruvic acids, and microorganisms break down the metabolism to organic acids. The decreasing rate of reducing sugars is relatively low at the lower temperature (30 °C) compared to those of the higher (35, 40 °C) (Figure 3).

Total acids

Microbial metabolism during *growol* fermentation results in the formation of organic acids. Prior to this, monosaccharides and disaccharides decompose, becoming pyruvic acid. The total acid changes from 1.1-1.5 ml NaOH/100 g to 2.1-2.8 ml/100 g cassava. Figure 4 shows that total acids increase gradually in the first 12 h of fermentation, then quickly decrease in the following 36 h at a fermentation temperature of 30 °C, or slowly at 34 °C.

Decree of acidity (pH)

The forming of organic acids during fermentation results in decreasing pH—from approximately 6.6 to 4.4. Figure 5 shows that fermentation temperatures (30, 35 and 40 °C) follow similar patterns. During the first 12 h of fermentation, pH decreases quickly, slowing in the next 36 h. Besides the role of microorganism, it is predicted that there are some constituents of cassava that can control pH and act as a buffer system. Lindsay (1976) mentioned that organic acids, protein and phosphate function as pH buffers.

Sensory evaluation

The sensory evaluation of *growol*, in terms of texture, stickiness, aroma and taste, was also conducted using the scoring difference test method. Results indicated that fermentation temperature affects the texture and stickiness of *growol* (Figure 6).

Texture of *growol* ranges from slightly hard to hard. Lowering the fermentation temperature results in harder *growol*. Similarly, with hardness, the lower the fermentation temperature the less sticky the *growol*. But *growol*'s aroma and taste are not significantly affected by the fermentation temperature.

MICROBIAL EVALUATION IN *GROWOL* FERMENTATION

Isolation and microbial counting

Five types of medium were used to isolate different types of microorganisms in *growol* fermentation: aerob mesophyl bacteria, aerob bacteria (bacteria total), lactic acid bacteria, mould, and yeast. The samples of *growol* fermentation liquid were diluted (by 10-fold dilution series), then planted in a suitable medium using the spread plate method. A Quebec counter was used to count the colony and the standard plate count to determine the total life cell or colony forming units (cfu).

Total bacteria had changed over the 120 h of *growol* fermentation. At first, total bacteria were around log 6.03-log 8.50 cfus/ml of fermentation medium. This increased gradually during the first 48 h and reached log 8.31-log 10.8 at the end of 48 h. Fermentation results in a relatively constant level (or number) of total bacteria. This differs from the change in total yeasts during fermentation of *growol*. At the start of fermentation, total yeasts are around log 2.6-log 3.7 cfu/ml and increase gradually over 72 h (log 3.7-log 4.0), then decrease at the end of fermentation.

Isolate characterization and identification

Isolates were first observed for their morphology (cell shape) and their reaction to gram staining, using the Hucker modification (Salle, 1961). *Moraxella* sp. gave positive oxidase, while *Acinetobacter* sp. had negative oxidase (Jay, 1978). Glucose fermentation was used to differentiate *Micrococcus* sp. and *Staphylococcus* sp.

Table 2 shows the 91 isolates collected over 120 h of fermentation. There are 84 bacteria isolates, 6 yeast isolates and 1 unidentified. The isolates which have been purified are originally from seven families of bacteria: *Enterobacteriaceae, Neisseriaceae, Micrococcaceae, Streptococcaceae, Bacillaceae, Lactobacillaceae* and the *Coryneform* group. Table 3 shows the types of isolated microbia. There is more *Lactobacillus* found during isolation, followed by *Coryneform*, yeast, *Enterobacteriaceae* and *Streptococcus*.

Microbial succession during *growol* fermentation

Many types of microbia grow in *growol* fermentation, especially from well water and cassava. Some of them (*Coryneform, Streptococcus, Bacillus* and *Acinetobacter*), grow during initial fermentation. The first two play a role in acid formation during glucose fermentation. It is believed that *Bacillus* affects hydrolization of starch to glucose (Skerman, 1967).

After 24 h fermentation, *Streptococcus* and *Acinetobacter* decreased, while the amount of *Coryneform* still increased. Acids changing, pH, and potential reduction-oxidation cause a decrease in the amount of bacteria. Dissolving ascorbic acid in the fermentation medium from the raw material results in a greater inhibition of oxygen diluted in the medium. At this stage, yeast begins to develop, following the growth of *Moraxella* sp., *Lactobacillus* sp. and *Enterobacteriaceae*.

At the start of hour 48 of fermentation, *Lactobacillus* sp., *Moraxella* sp. and *Acinetobacter* sp. grow faster. *Coryneform* sp. and *Streptococcus* sp. are relatively constant. The ph of fermentation medium slightly decreases at the beginning of hour 72 of fermentation but it remains relatively constant in the continuing fermentation. At the end of fermentation, all bacteria and yeast tend to decrease, except *Moraxella* sp.

Microbial growth during fermentation of *growol* is affected by substrate changes such as pH, soluble oxygen and total acids. Microorganisms found were, in order: (1) *Streptococcus* sp., (2) *Coryneform* group, (3) Yeast, *Enterobacteriaceae, Bacillus* sp. and *Acinetobacter* sp., (4) *Lactobacillus* sp., and (5) *Moraxella* sp.

From this, it is clear that traditional *growol* fermentation, usually taking from 2 to 5 days, is not dominated by certain microorganisms. Thus *growol* fermentation can perhaps be classified as heterofermentation (Haseltine, 1979).

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Table 1. Chemical composition of cassava and *growol*.

Constituents		Cassava	*Growol*
Protein	(%, db)	0.98	0.32
Crude fat	(%, db)	0.13	0.08
Ash	(%, db)	2.13	0.20
Starch	(%, db)	83.95	80.89
Other carbohydrates	(%, db)	12.81	18.51

Table 2. Amount of isolate in traditional *growol* fermentation.

Isolate	Fermentation times (h)					
	0	24	48	72	96	120
Lactobacillus	4	2	2	1	2	1
Streptococcus	1	1	1	0	2	3
Staphylococcus	1	0	1	0	0	0
Planococcus	1	1	0	0	1	0
Micrococcus	1	1	0	0	0	0
Acinetobacter	2	0	1	1	1	1
Moraxella	2	1	0	1	1	0
Coryneform group	2	2	4	4	1	4
Enterobacteriaceae	1	1	6	4	0	1
Bacillus	0	0	1	2	1	0
Yeast	1	1	1	1	1	1
Unidentified	1	0	0	0	0	0

SOURCE: Rascana (1986).

Table 3. Types of microbial isolates in traditional *growol* fermentation.

Isolate	Fermentation times (h)					
	0	24	48	72	96	120
Lactobacillus	+	+	+	+	+	+
Streptococcus	+	+	+	-	+	+
Staphylococcus	+	-	+	-	-	-
Planococcus	+	+	-	-	+	-
Micrococcus	+	+	-	-	-	-
Acinetobacter	+	-	+	+	+	+
Moraxella	+	-	-	+	-	-
Coryneform group	+	+	+	+	+	+
Enterobacteriaceae	+	+	+	+	-	+
Bacillus	-	-	+	+	+	-
Yeast	+	+	+	+	+	+
Unidentified	+	-	-	-	-	-

SOURCE: Rascana (1988).

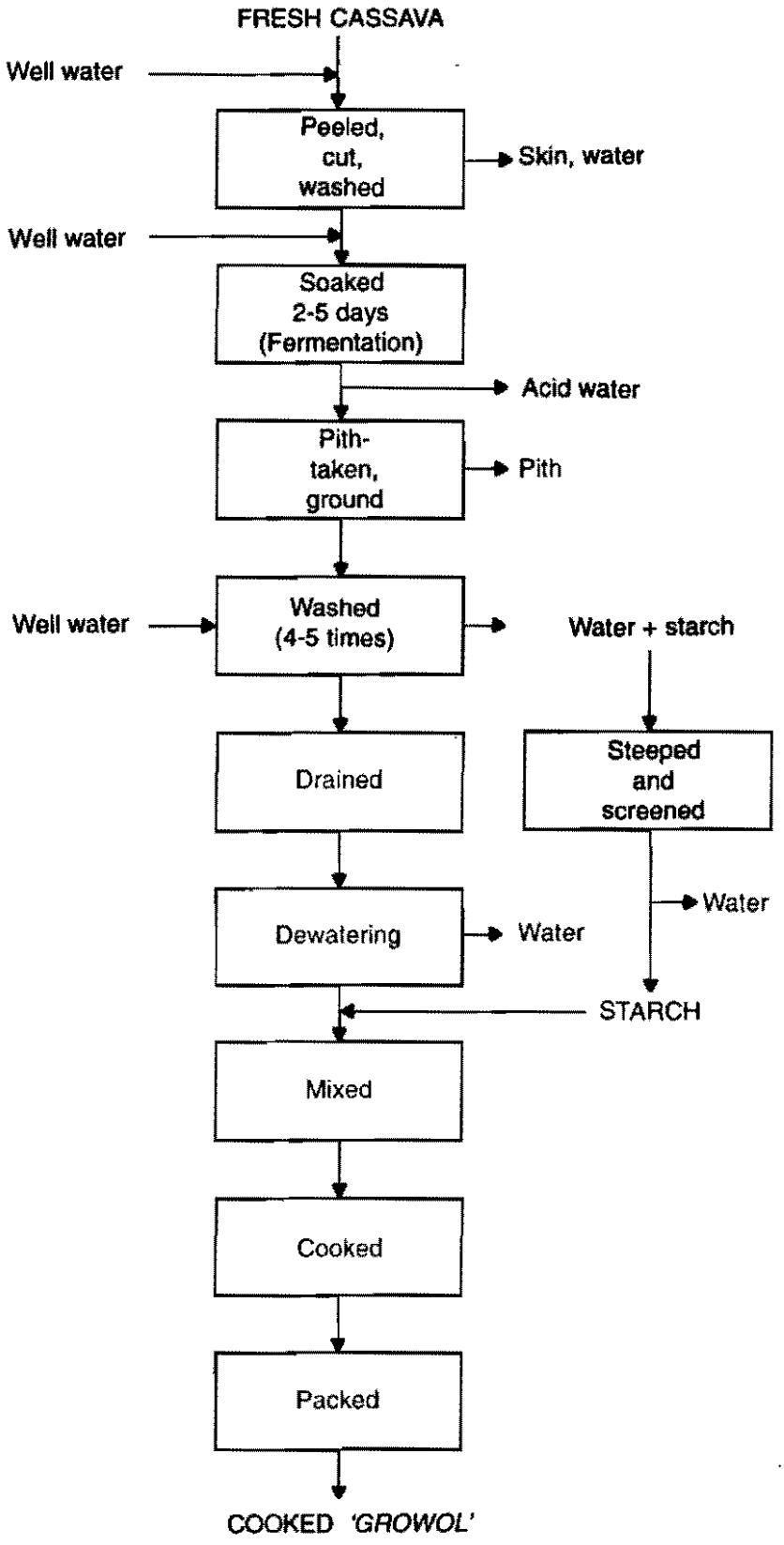

Figure 1. Flowsheet of *'growol'* procedure.

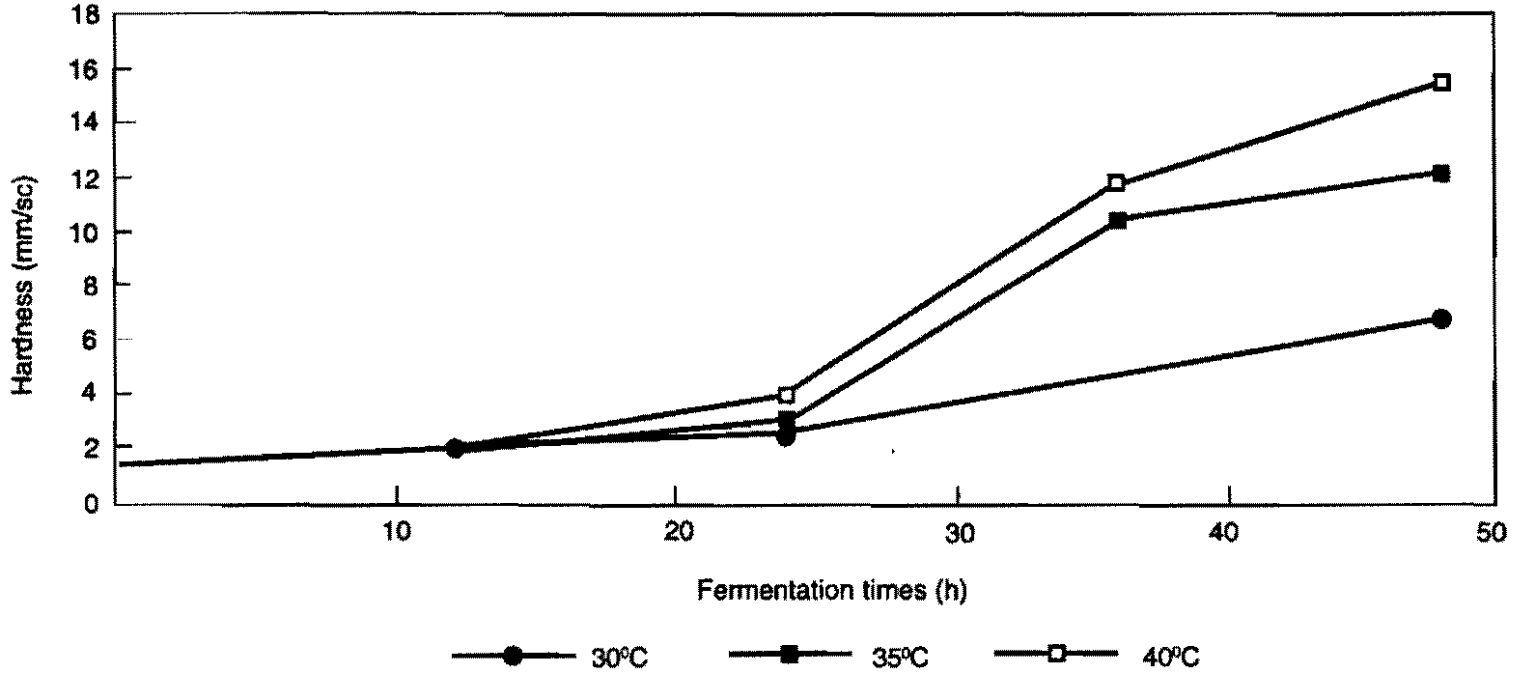

Figure 2. Changing of hardness in cassava Aldira I during fermentation of *growol*.

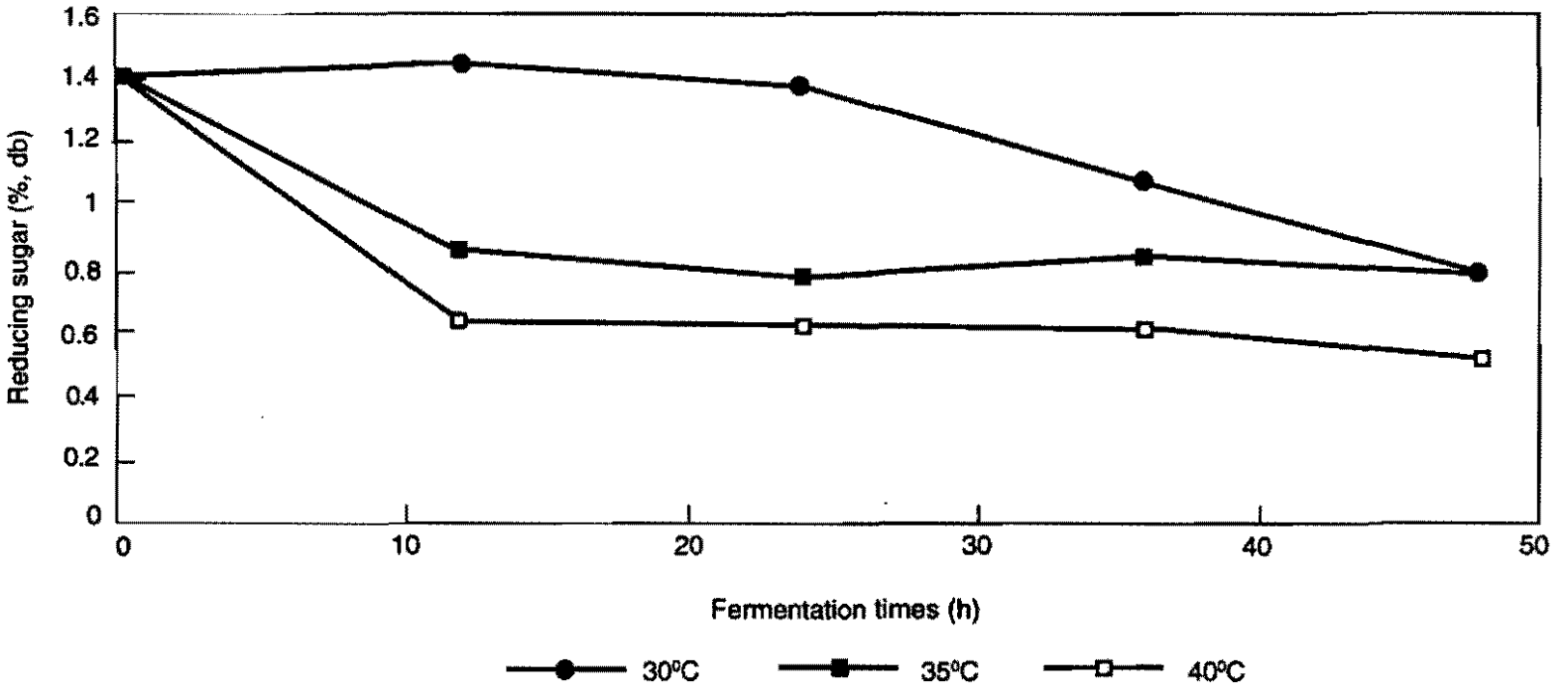

Figure 3. Changing of reducing sugars in cassava Aldira I during fermentation of *growol*.

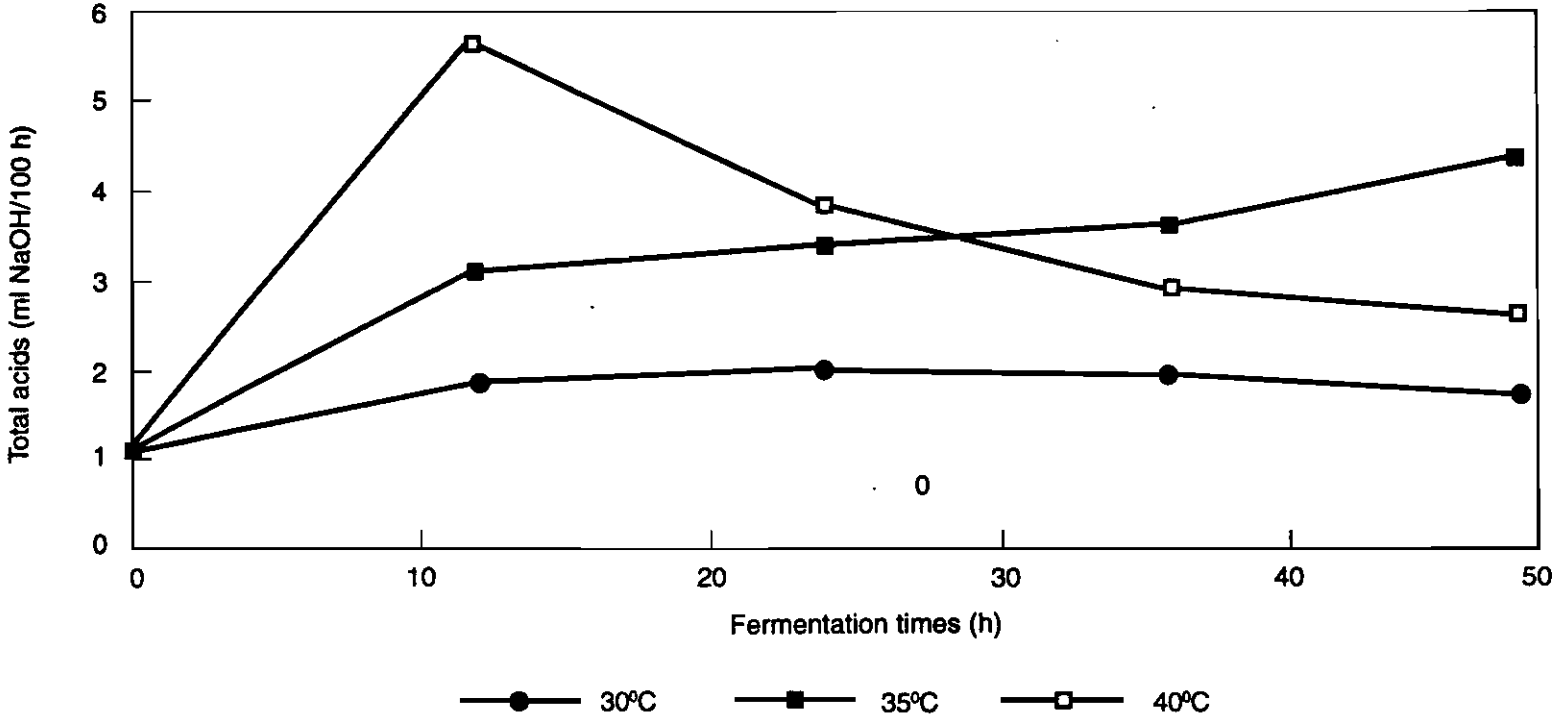

Figure 4. Changing of total acids in cassava Aldira I during fermentation of *growol*.

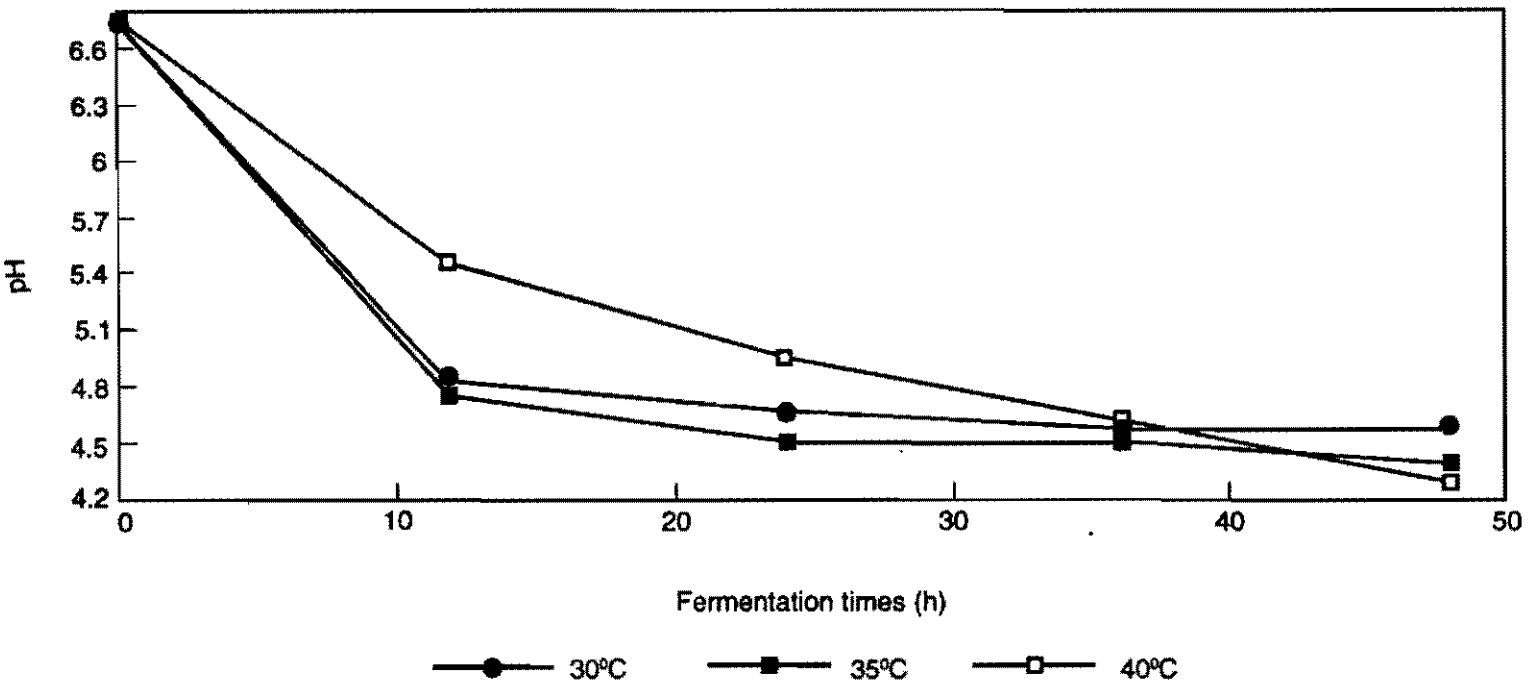

Figure 5. Changing of pH in cassava Aldira I during fermentation of *growol*.

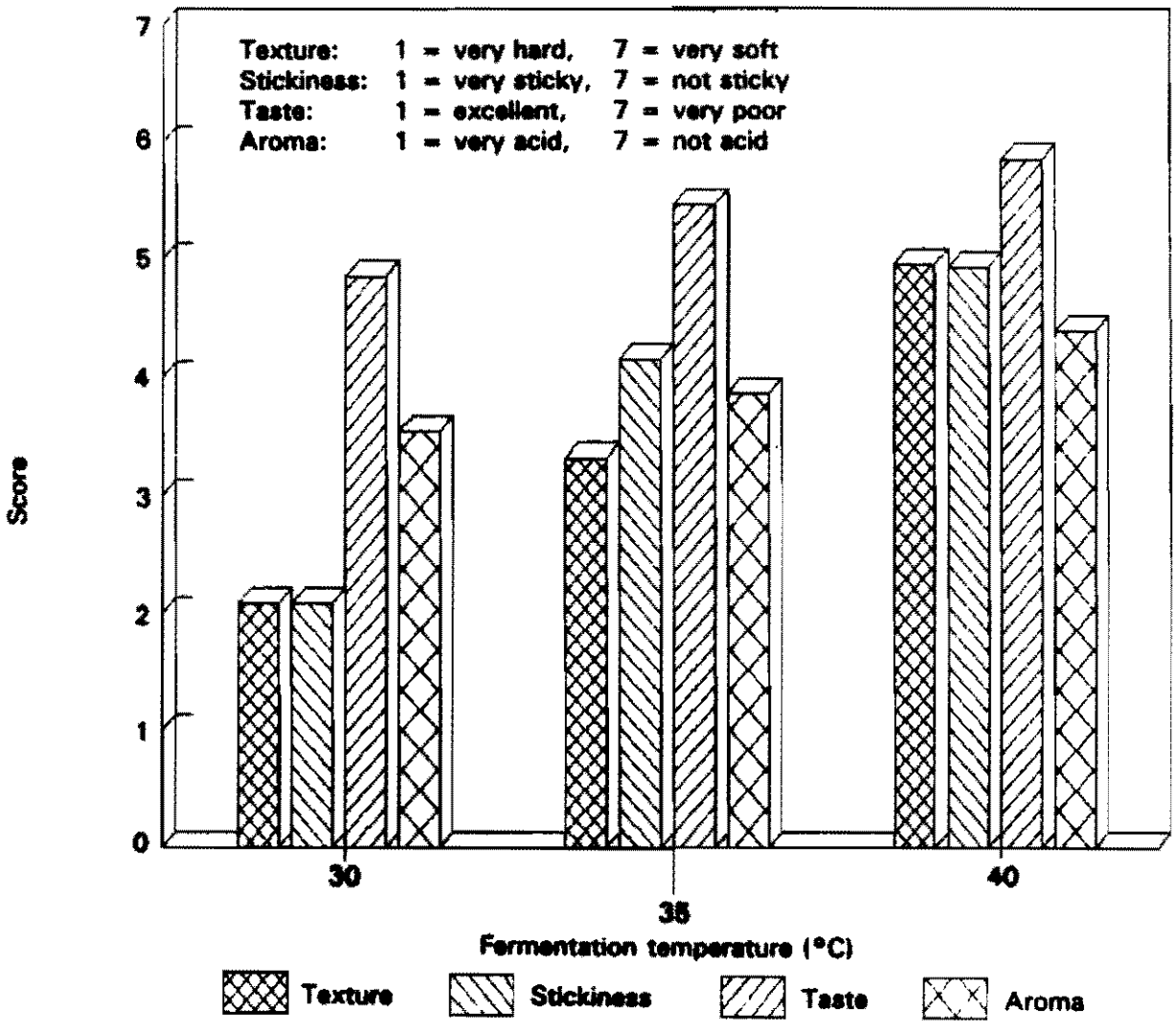

Figure 6. Sensory evaluation of '*growol*'.

CHARACTERIZATION OF CASSAVA ROOT PROTEINS

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Although cassava roots are a good source of dietary carbohydrates, they are uniquely deficient in protein, which comprises about from 1% to 2% of their dry weight. The purpose of this study is to characterize the proteins present in the roots of significant cassava clones. Background work involved determining the nitrogen balance in the roots and in whole plants of cassava grown under conditions of varying nitrogen supply and deficient or reduced sulphur. Detailed examination of the root proteins from cassava cultivars CMC 40 and M Col 1684 revealed that at least these cultivars contain no major storage proteins; however, a significant protein, with Mr about 25,000, was found in M Col 1684 and was purified by preparative SDS-polyacrylamide gel electrophoresis. Attempts at amino acid sequencing indicated that this protein was N-terminal blocked. A polyclonal antibody was, however, raised to this protein and used to screen cDNA libraries to mRNA from M Col 22 roots constructed in lambda gt11 and lambda Moselox. Several clones were isolated from the latter library and are currently being sequenced.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the fourth most important food crop in the tropics. It is grown predominantly for its secondary thickened roots, which comprise from 20% to 40% dry matter, of which 85% is starch (Cock 1985a; Kay, 1973). Cassava leaves are eaten as a vegetable by humans and used as animal feed. Cassava flour can be used as a partial substitute for wheat flour in breadmaking; however, if the substitution is greater than 30%, bread quality is very poor (Almazan, 1990).

Cassava storage roots only contain from 1% to 2% protein (dry weight). The overall quality of the storage root protein is limited by sulphur-containing amino acids (Yeoh and Chew, 1977), which can cause protein deficiency in people consuming cassava as a staple, with no protein supplement (Cock, 1985b). Cassava leaves contain up to 10 times the protein in the storage roots, but are still limited by the low levels of the essential amino acids cysteine and methionine (Nandakumaran et al, 1978). Root peel is discarded in food preparation, but has higher levels of protein than the edible parenchyma. The peels of low-cyanide varieties are often used as a nourishing animal feed (Gómez and Noma, 1986).

There are two reasons for manipulating the protein content and nutritional status of cassava roots. In the first place, if the protein content is improved,

protein supplements would not be needed to avert deficiency in people consuming cassava as a staple. But improved protein content is not enough, protein quality must also be enhanced since increased protein would not solve the problem of protein deficiency if the amino acids are not balanced. In the second place, the manipulation of cassava protein would make cassava root flour more useful for breadmaking and thus alleviate the dependance on expensive wheat flour.

The cassava plant is propagated vegetatively, using pieces of primary stem from 20 to 30 cm long from plants at least 10 months old (Kay, 1973). The initial growth rate of the cutting depends on its storage reserves. The leaves do not start to photosynthesize fully for up to 3 weeks after planting, so the larger and thicker the cutting, the more likely it is to survive. Roots are formed within 5 days, and the number of roots that will undergo secondary thickening to form storage roots is determined early during plant growth, usually by leaf carbohydrate supply (Hunt et al., 1977).

The nutritional requirements of cassava have long been a subject of debate. Despite the fact that in long-term fertility trials cassava has been shown to extract much less nitrogen (N) and phosphorus (P) per unit dry matter than other crops (Howeler, 1991), many growers consider that cassava depletes the soil of minerals, and therefore should only be grown on almost completely exhausted soils. Although it might be supposed that cassava would respond to N fertilization to produce more storage roots, as occurs with other crops, several experiments have shown that excess N causes accumulation of cyanogenic glucosides, early leaf abscission, and partial decomposition of the roots (Tankou et al., 1990). It also can cause excessive top growth at the expense of root formation and growth (Cenpukdee and Fukai, 1991; Okeke et al., 1982). Howeler and Cadavid (1983) found that even after three consecutive crops of cassava there was still no significant response to N fertilization. Root starch content in fertilized plants has also been found to decrease with excess N (Malavolta et al., 1954).

Storage proteins, just like any storage product, are elaborated specifically at times of plenty or excess, to make a reserve for when conditions are harsher. Storage proteins are abundant in storage organs such as seeds and roots. They may be restricted to specific types of cells or tissues and are usually located in storage vacuoles. Storage proteins also tend to have a high amide content and usually follow a temporal pattern of storage followed by utilization. For example, in some temperate trees, storage occurs in the bark in the winter months, and mobilization follows in spring (Greenwood et al., 1986). Seeds store proteins which are mobilized at germination when the embryo starts to expand.

Cassava storage roots do not appear to contain a storage protein, and although leaf proteins have only been partially characterized, no single protein has so far been identified as fulfilling a storage function (Shewry et al., 1992; Yeoh and Chew, 1977). The starch present in cassava roots acts as a storage reserve to

enable the plant to survive periods of drought and other adverse conditions, and seasonal depletion has been observed to occur (O'Hair, 1989). In other plants, the storage organs may act as propagules, such as the seeds and tubers of yam and sweet potato. In the case of cassava, although the root may be expected to store protein, this is not essential as N would be available and could possibly be stored in other plant organs such as the leaves. In fact, it has been reported that cassava plants rarely drop their leaves, even under stress, but so far they have not been shown to store N, either as protein or in any other form (Baker et al., 1989; Yao et al., 1988). The effect of nutritional stress on the cassava plant was studied specifically regarding the N balance within the roots. An cDNA clone, with an abundant protein of Mr 25,000, was also isolated and characterized to facilitate the eventual improvement of cassava root quality.

MATERIALS AND METHODS

Plant material

Stock material was propagated in the form of 10-month-old cuttings of two cassava cultivars: M Col 1684, from South America, and CMC 40, from Africa. Stakes, 24 cm long, were planted in a mixture of 25:75 sand:soil in pots 10 cm in diameter, and grown for 3 months in a heated greenhouse with a constant temperature of 25 °C day and 15 °C night, with a 16-h daylength. The plants were grown for another 6 to 8 months, after which roots were harvested as necessary. Storage roots were used for protein fractionation and N determination.

Thirty-two 24-cm-long stakes of both varieties were divided into two lots planted in pots 24 cm in diameter. One lot was planted in vermiculite and the other in perlite. The plants were then divided into four treatments or growth media (4 plants/treatment): low N + S, low N - S, high N + S, and high N - S. Plants were grown for 3 months and watered 3 times a week with 1 liter of nutrient solution (Table 1). Any necessary watering between applications was done with distilled water. After 3 months, the plants were cut back to two nodes above the original stake to encourage the manifestation of deficiency symptoms. The plants were left to grow for another 4 weeks.

Protein extraction and N determination

Tubers from the stock material were harvested at 10 months after planting (MAP), separated into peel and parenchyma, and milled to pass through a 2.5-mm sieve, the flours were sequentially extracted with four solvents to give six samples: raw flour, water extract, 1 M sodium chloride (NaCl) extract, 1% sodium dodecyl sulphate (SDS) extract, 1% SDS + 2% 2-β-mercaptoethanol (2ME) extract, and residue. The extracts and the residue were all dialyzed against distilled water and

then lyophilized. The resulting 12 extracts for each cultivar were analyzed for N content.

Water extracts obtained by the above method were fractionated using the Biorad Prep Cell. A 5-cm 10% resolving gel was poured into the tube (3 cm in diameter), and topped with a 3 cm stacking gel. The sample was loaded onto the gel, and run for 3 to 4 h at 40 mA, until the blue dye reached the bottom of the gel. At this point, 2.5-ml fractions of elute were collected every 2.5 minutes for another 4 h. After washing with ice-cold ethanol, SDS-PAGE gels of the dried fractions were run.

Polyclonal antibody synthesis

A polyclonal antibody to a protein of Mr about 25,000 was raised in a rabbit, and the crude serum used for immunodetection.

Construction and screening of cDNA library of root mRNA of M Col 22 in lambda Moselox

Total RNA was extracted from fresh roots of cassava cultivar M Col 22 using the method of Salehuzzaman et al. (1992) and was purified through Qiagen resin. An oligo(dT)cellulose column was used to isolate mRNA, which was synthesized into double-stranded cDNA using the Amersham cDNA synthesis kit; 50 ng of the resulting cDNA was modified with EcoR1 adaptors and ligated into lambda Moselox arms and packaged into the protein coats following the procedures outlined in the Amersham rapid cloning kit. The library so constructed had a titre of 6.7 x 10^5 pfu/ml. A mixture of 0.1 ml of 10^3 pfu/ml phage to 0.1 ml of BL21(DE3)pLysE *E. coli* cells were plated onto a 9-cm 2 x YT agar plate and grown for 6 h at 37 °C. An IPTG impregnated nitrocellulose filter was then laid on the plate and incubated for another 4 h at 37 °C. The filters were marked asymmetrically and removed, washed in TBS, and then placed in 1% BSA solution for 0.5 h, to block non-protein sites. Filters were then immersed in 1:1000 primary antibody solution for 4 h, followed by 1 h in 1:2000 horseradish peroxidase-conjugated secondary antibody solution. After washing, the filters were developed with hydrogen peroxide and diaminobenzidine to show positive signals.

RESULTS AND DISCUSSION

The N distribution in the storage roots of cassava cultivars CMC 40 and M Col 1684, grown as described above, was examined. The raw flours of cultivar M Col 1684 give N values of 5.7 mg/g dry weight for the peel, and 2.1 mg/g dry weight for the parenchyma (Figures 1 and 2). The same difference of 2-fold or

more in N value can also be seen in cultivar CMC 40, with values of 0.47 mg/g and 0.18 mg/g, respectively (Figures 3 and 4). Although the largest amount of this N was extracted in the water fraction, this was also where differences occurred between the dialyzed and nondialyzed fractions. Large differences also occurred with 1 M NaCl extracts of both cultivars, almost certainly due to soluble cyanogenic glucosides, free animo acids, and short peptides small enough to pass through the dialysis membrane. Losses during dialysis of the other extracts may have been due to the proteins remaining on the dialysis tubing during extract transfer prior to lyophilization.

In each case, approximately 25% of the original N was left in the insoluble residue. The differences between the N content of the raw flour and the overall N recovered may be due to the action of linamarase. When water is added to cassava flour, the linamarase acting on the cyanogenic glucosides would be intact, giving a higher N content than can be accounted for by the protein content.

Storage roots from cultivar CMC 40 have approximately 15% less protein in total per g dry weight compared with those of M Col 1684. In both cases, however, the protein contents are less than 1% of the dry weight, so the difference is probably not nutritionally significant. The N balance in the cassava root, with its bias towards the peel, merits further study in view of its implications for any genetic manipulation envisaged. If the plant naturally sequesters more N into the peel, which is discarded during food preparation, then any attempt to increase the protein levels in the currently consumed tissues through genetic engineering would have to overcome this tendency. If the peels could be made culturally or palatably acceptable for human and animal consumption, then this tendency would not be a problem.

Regarding the effects of N and S deficiency and enrichment on the growth of M Col 1684 and CMC 40, the greatest differences among treatments were observed on vermiculite for cultivar M Col 1684 (Figure 5), and on perlite for cultivar CMC 40 (Figure 6). In both cases, the plants grown with S were taller, with longer internodes and also longer petioles. The plants grown with low S had longer leaves of a yellower color, and thinner and spiky leaflets. The youngest leaves started to form with only three leaflets, instead of the usual five or seven. In addition, the lower leaves of the plants grown with low S senesced and died more quickly than those on the plants grown with high S.

The differences between the plants grown with high and low N were less obvious. The plants with high N were greener and had more leaves, which agrees with the results of Cenpukdee and Fukai (1991) and Okeke et al. (1982). Leaves were also larger and with less noticeable veins.

A total of 40,000 clones of the lambda Moselox library were screened with the 25kD polyclonal antibody, of which nine were found to be positive after a

secondary screen. These were further studied by subcloning and restriction mapping. One clone is currently being sequenced.

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Table 1. Nutrient solutions.

Reagent	Type of nutrient solution			
	High N + S	High N - S	Low N + S	Low N - S
KNO_3	0.708 g	0.708 g	—	—
KH_2PO_4	0.136 g	0.136 g	0.136 g	0.136 g
$Ca(NO_3)_2.4H_2O$	1.180 g	1.180 g	0.472 g	0.472 g
NH_4CL	0.107 g	0.107 g	—	—
$Mg(NO_3)_2.6H_2O$	0.439 g	0.439 g	0.4099 g	0.4099 g
NaCl	0.0059 g	0.0059 g	0.0059 g	0.0059 g
EDTAFeNa	0.0184 g	0.0184 g	0.0184 g	0.0148 g
$CaCl_2.6H_2O$	—	—	0.438 g	0.438 g
$MgCl_2$	—	0.0962 g	—	0.0962 g
KCl	—	—	0.522 g	0.5 ml
Trace elements	0.5 ml	0.5 ml	0.5 ml	—
$MgSO_4.7H_2O$	0.277 g	—	0.277 g	—
Water	1 l	1 l	1 l	1 l

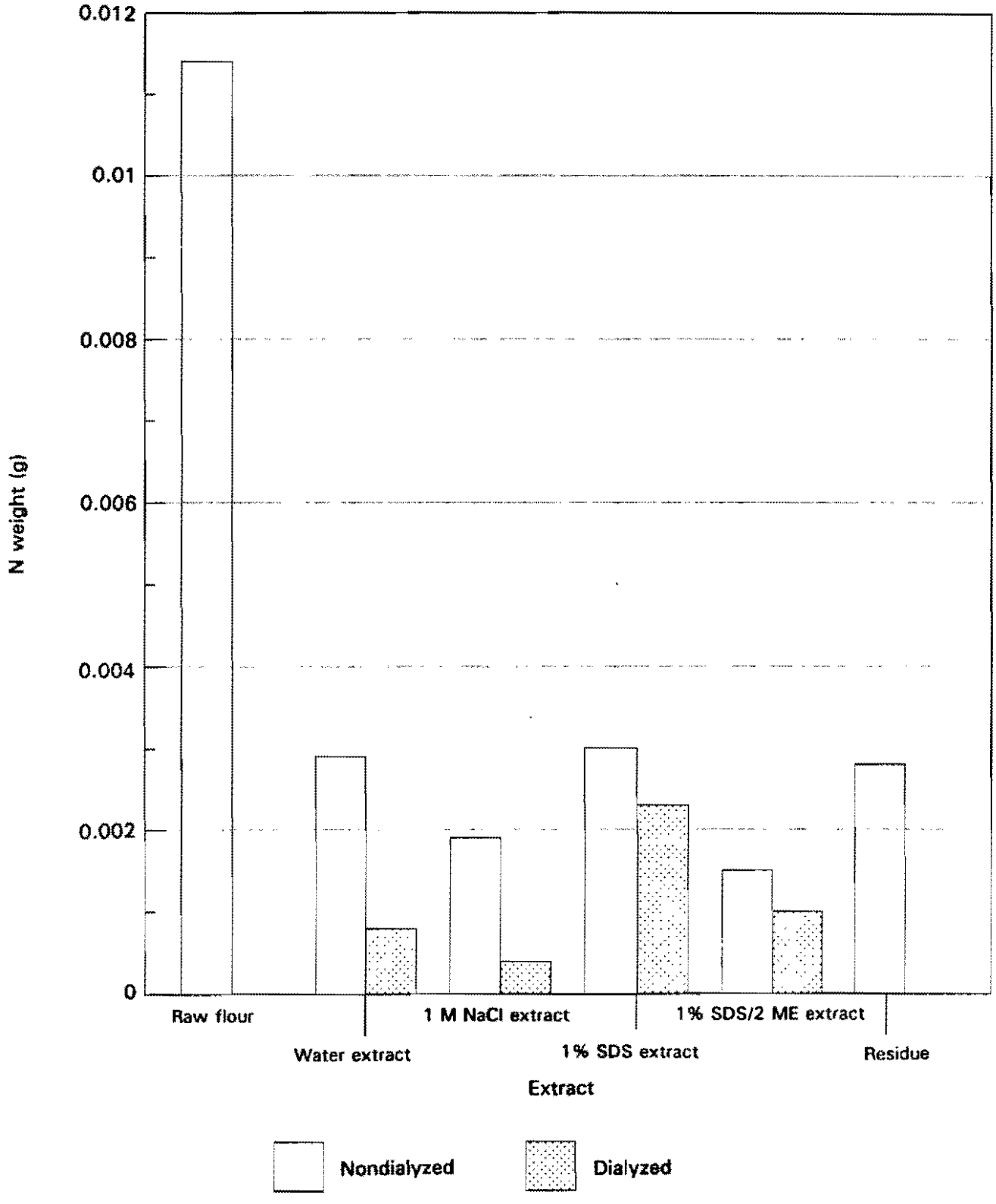

Figure 1. Nitrogen distribution in six extracts (dialyzed and nondialyzed) of the peel of cassava cultivar M Col 1684.

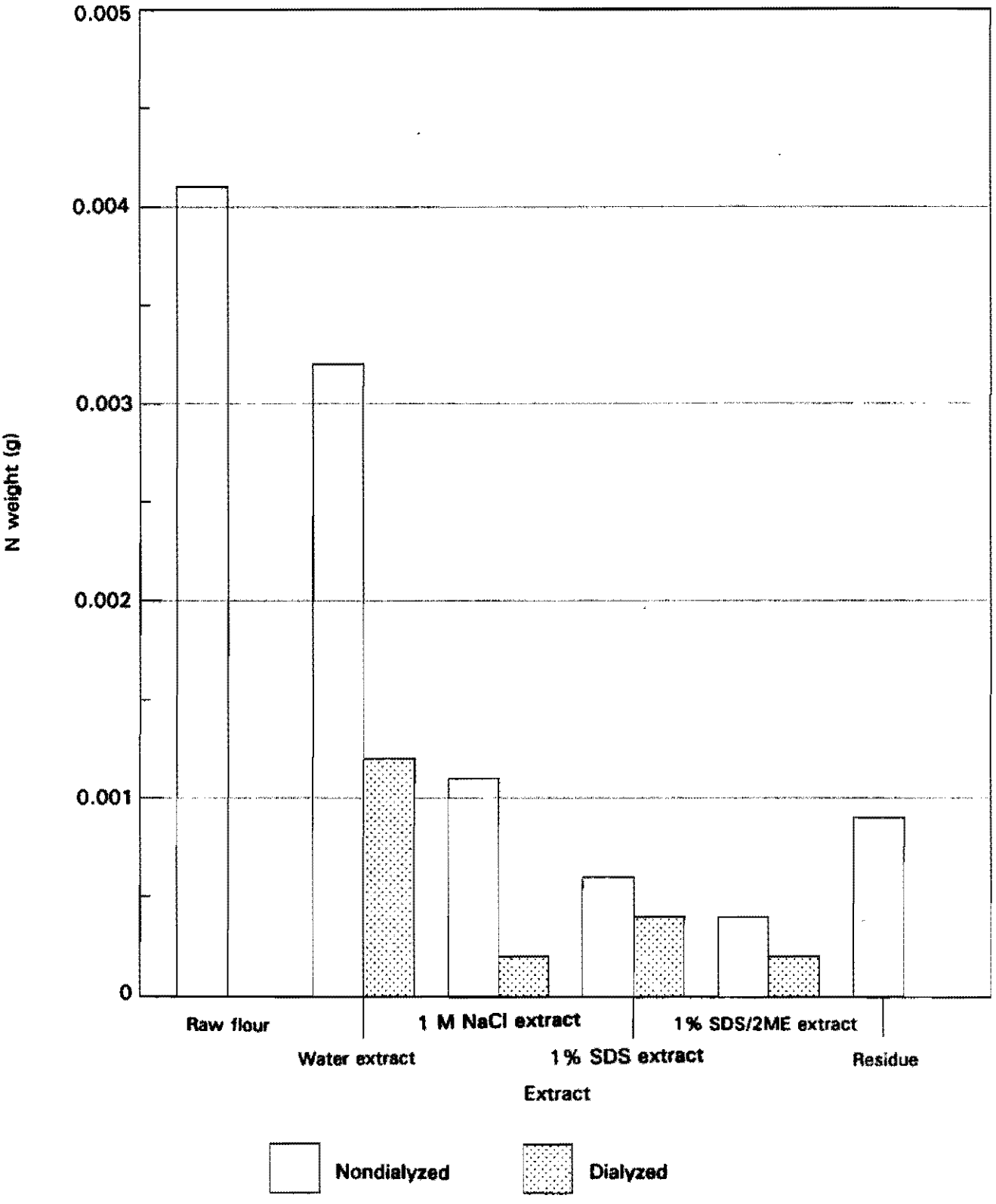

Figure 2. Nitrogen distribution in six extracts (dialyzed and nondialyzed) of the parenchyma of cassava cultivar M Col 1684.

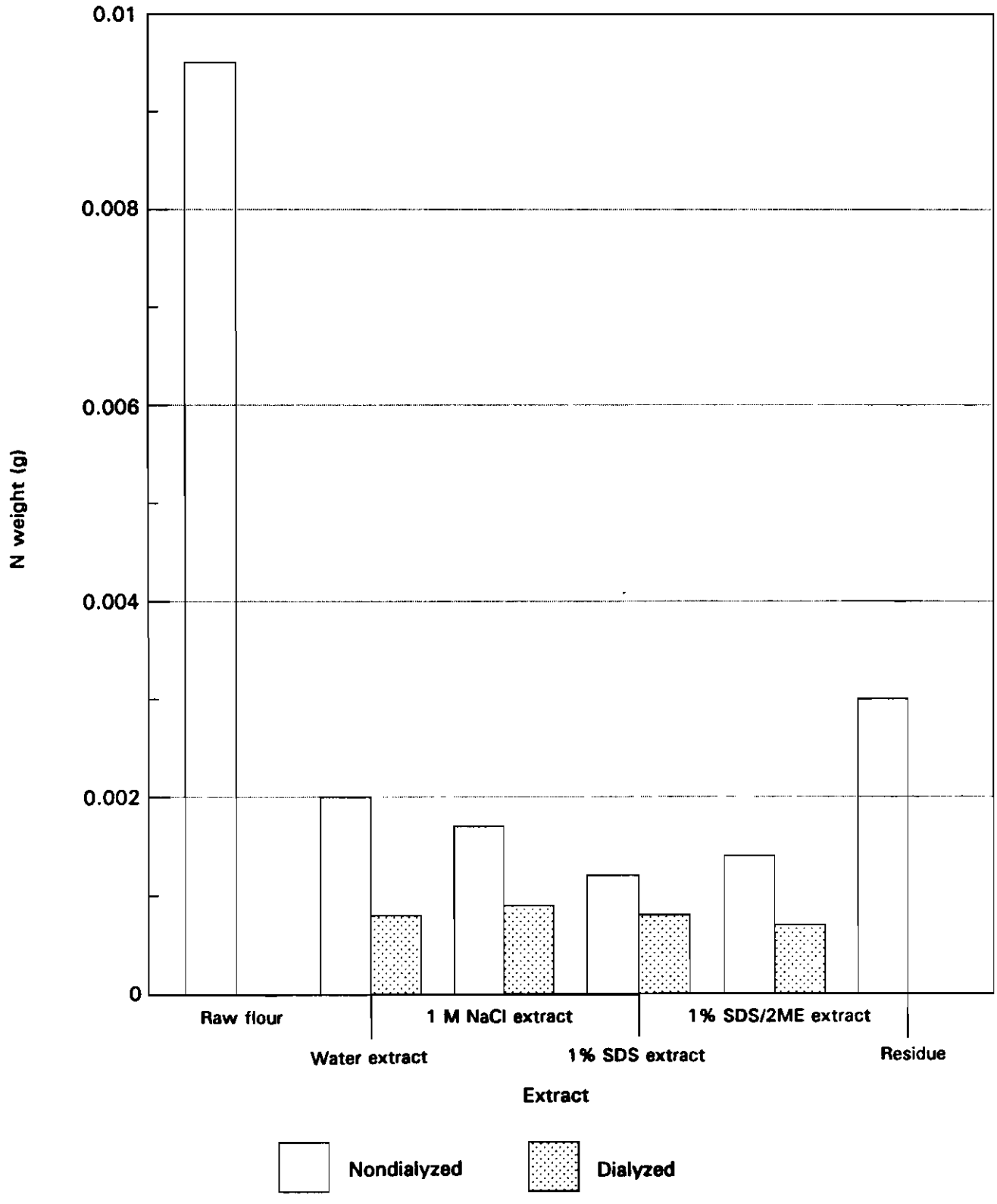

Figure 3. Nitrogen distribution in six extracts (dialyzed and nondialyzed) of the peel of cassava cultivar CMC 40.

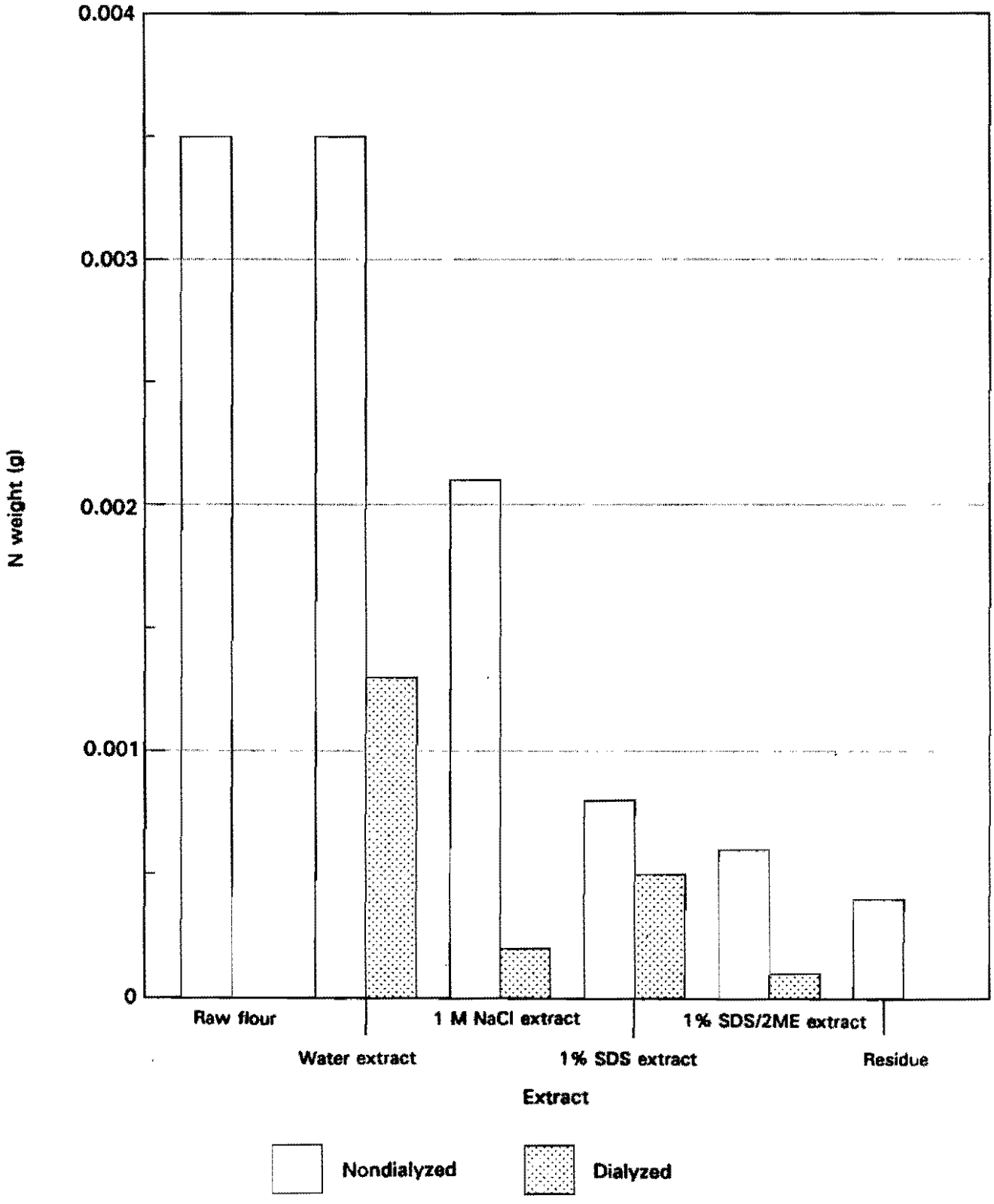

Figure 4. Nitrogen distribution in six extracts (dialyzed and nondialyzed) of the parenchyma of cassava cultivar CMC 40.

Figure 5. Deficiency symptoms expressed by cassava cultivar M Col 1684 grown under four nutrient conditions.

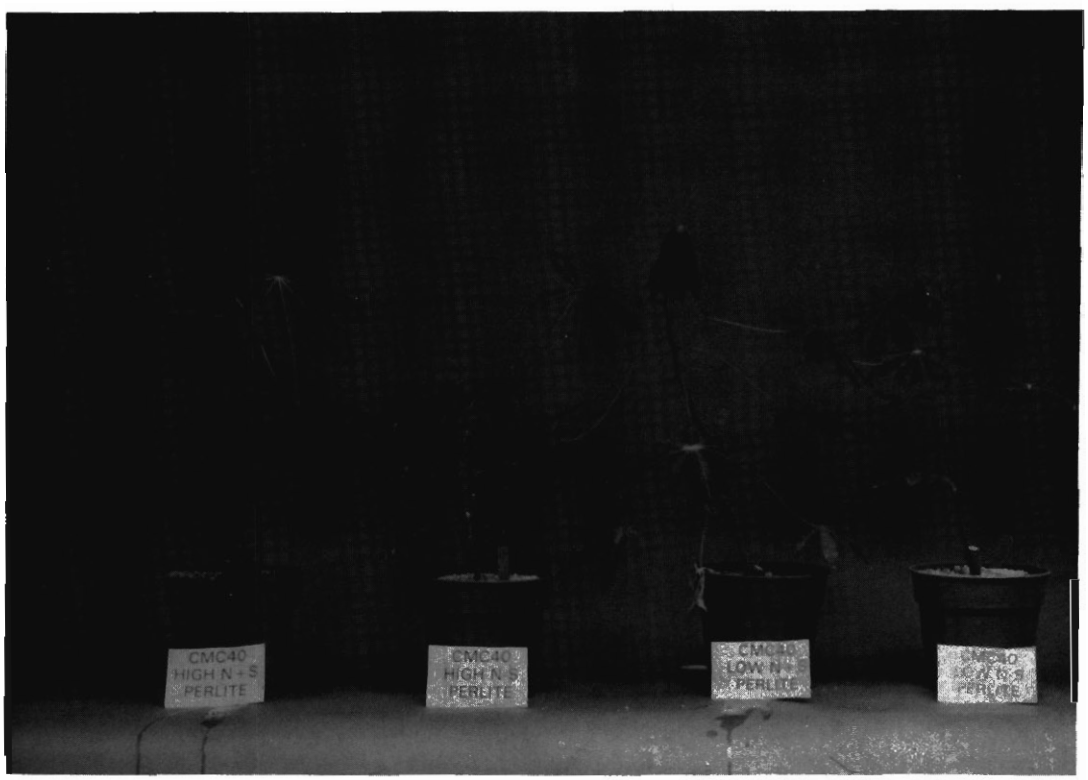

Figure 6. Deficiency symptoms expressed by cassava cultivar CMC 40 grown under four nutrient conditions.

PHYSIOLOGICAL DETERIORATION IN CASSAVA: AN INCOMPLETE WOUND RESPONSE?

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The rapid postharvest physiological deterioration (PD) of cassava is a serious problem for the grower, and necessitates prompt consumption or processing of the roots upon harvesting. Physiologically and biochemically PD shares many features with wound responses studied in more fully characterized plant systems, such as changes in amounts and composition of membrane lipids, increased activity of peroxidases and phenol oxidases, increases in activity of enzymes of phenolic biosynthesis, and the accumulation of phenolic compounds. While some of these processes involve the activation of pre-existing enzymes, others involve the *de novo* synthesis of the new proteins; for example we have shown the appearance of protein bands on SDS-PAGE following the *in vivo* labelling of polipeptides. In most plants with a normal wound response it is the healing of wound repair which reduces and finally cuts off the source of signals which trigger the cascade of reactions due to wounding. In cassava this negative feedback loop seems to be inadequate so that the wound response is sustained and spreads throughout the whole root rather than remaining localized. Our results and observations are discussed in terms of this perspective and of suggested targets for the modulation of PD.

INTRODUCTION

One of the major problems with cassava is the rapid postharvest deterioration which renders it unpalatable as food. Initially deterioration is due to physiological processes which occur within 2 to 3 days of harvest and is subsequently followed by microbial deterioration within 5 to 7 days. These events necessitate the prompt consumption or processing of cassava soon after harvest. The physiological deterioration (PD) is observed as discoloration of the vascular tissues and storage parenchyma (vascular streaking) and is accompanied by biochemical changes typical of wound responses in other plant systems. This included: changes in the amounts and composition of membranes lipids, increases in activity of phenylalanine ammonia lyase (PAL); phenolic biosynthesis; the accumulation of phenolic compounds such as catechins, proanthocyanadins and scopoletin; increased activity of pheroxidases and phenol oxidases; and ethylene production. In harvested cassava, roots held under conditions favorable for wound healing/curing (80%-90% relative humidity, 25-30 °C), the above wound responses remain localized near areas of mechanical damage and continue to develop rapidly throughout the roots resulting in discoloration of the storage parenchyma.

While some of the events occurring during PD involve the activating of pre-existing components, many require the *de novo* synthesis of enzymes and compounds (Hirose, 1986; Plumbley and Rickard, 1991). Here we present some data on the *novo* biosynthesis of proteins during PD, discuss the phenomenon in terms of wound responses in more fully studied plants systems, and examine potential strategies for the modulation of PD.

MATERIALS AND METHODS

Cassava cultivars M NGA 1 and CMC 40 were grown in pots in the glasshouse with 25 °C days and 15 °C nights and with a daylength of at least 16 h. Small storage roots were produced after 6 to 9 months of growth. A plug was taken from the middle of the root using a size 12 cork-borer and cut into 2 mm thick slices. The slices were incubated in a chamber placed in a 25 °C incubator. Air which had been bubbled through water was passed through the chamber. Four slices were removed at intervals and placed on watch glasses. Onto each slice was dropped 100 μl of an aqueous solution containing radio-labelled amino acids (EXPRE $^{35}S^{35}s$, NEN, du Pont: >70L-[^{35}S] methionine, >18%L-[^{35}S]cysteine) with an activity of 15 μCi/100 μl. The watch glasses were then covered with a glass plate and incubated at room temperature for 2 h. To each slice were added 100 μl of protein extraction buffer (62.5 mM tris Cl (pH 6.8), 10% glycerol, 2% SDS, 0.001% bromophenol blue, 50mM dithiothreitol), which was rapidly ground. The cellular debris was pelleted in a microfuge and the supernatant rapidly frozen in liquid nitrogen and stored at 80 °C. The incorporation of radioactive amino acids into proteins was determined by scintillation counting using standard methods. Radioactive proteins were separated by SDS- polyacrylamide gel electrophoresis (SDS-PAGE), fixed in acetic acid, soaked in Amplify (Amersham), dried and exposed to Hyperfilm-β-max(Amersham) at -70 °C for 7-14 days.

RESULTS

The incorporation of radio-labelled amino acids into *de novo* synthesized proteins was determined over a 48-h period in root discs kept in humid aerated conditions (Figure 1). The percentage incorporation increases with time indicating a major increase in translational activity. The root, when harvested, was essentially translationally dormant and continued so for the first 6 to 8 h. But by 24 h after harvesting/wounding there has been a dramatic increase in translational activity, which persists for at least up to 48 h after harvesting. The two cultivars, M NGA 1 and CMC 40, exhibit comparable results. These results show that upon harvesting/wounding there is a major synthesis of new protein. But they do not indicate whether this was due to increased synthesis of proteins already present in the root or whether novel proteins were induced which were not present in the unwounded root.

SDS-PAGE of the above protein extracts show a changing profile of protein synthesis with time (Figure 2, Table 1). Three bands are common to extractions at 0, 2, 4 and 6 h postwounding, with estimated molecular masses (M_r)0f 86,000, 43,000 and 32,000. A protein band, estimated M_r180,000, is detected in all lanes except lane one (proteins labelled at time zero). The 43,000 M_r band is not detectable in samples extracted at 24 and 48 h but two new proteins, M_r70,000 and 55,000, are present at both these times. A new protein band of M_r92,000 is present at 24 h and of M_r130,000 at 48 h.

DISCUSSION

Experimental data

The experimental data show that wounding of the cassava root initiates a dramatic increase in protein synthesis. While some of the new protein synthesis may be of proteins which pre-existed in the unwounded root, others are novel proteins. Certainly, the profile of proteins synthesis changes during the 48 h post wounding. So far we have not characterized any of these *de novo* synthesized proteins but evidence from other studies of cassava postharvest PD and from other plant wounding systems would suggest that these proteins include enzymes involved in defence and biosynthesis of defence related compounds. This experimental work needs to be extended through the increased precision of characterization of the *de novo* synthesized proteins, for example by two dimensional gel electrophoresis, and ultimately by the molecular cloning of their corresponding cDNAs.

Plant wound responses and physiological deterioration

The immediate effect of wounding plant tissue is the physical disruption of the protective barriers. Tissues close to the wound site are likely to experience dehydration, lowered carbon dioxide levels, enhanced levels of oxygen and increased exposure to microorganisms. These effects compromise the survival of the tissue, both close to the wound and some distance away from it. In most plants, many of the changes resulting from wounding can be considered as part of an orchestrated cascade of responses (Bowles, 1990). These responses result in the defence of the wounded plant and subsequently in the sealing of the exposed tissues by the regeneration of a barrier (periderm). Common wound responses directly involved in defence include lytic enzymes (glucanase and chitinase), protease inhibitor (PI) proteins and hydroxyproline-rich glycoproteins (HRPGPs) production. Other enzymic proteins such as PAL and chalcone synthase (CHS) lead to the biosynthesis of phenolics which may act directly as defence compounds (quinones, phytoalexins), or can form polymers such as lignin which render cell walls more resistant to water loss and microbial enzymes.

The overall wound response thus results in the restoration of the integrity of the damaged plant. The processes involved include: the formation and movement of signals from the damaged tissues, the perception by undamaged cells of these signals (resulting in the activation of pre-existing enzymes), and the expression of genes for a wide range of enzymic and other proteins concerned with defence, containment and repair. Wounding also induces the formation of further signal substances that serve to amplify and sustain the primary effects, and leads to a coordinated response of whole tissues and organs. Some responses to wounding take place close to the wound, others take place at a distance. Some are initiated within minutes, others take place hours or even days after the damage has been inflicted. As the normal internal environment of the plant is reinstated, wound signal formation is suppressed and the wound-response processes are down-regulated.

Cassava physiological deterioration appears to share many of the common characteristics of plant wound responses. But the sealing and healing aspects necessary to the successful completion of the wound response seem to be poorly expressed and less localized in harvested cassava roots (Booth, 1976; Rickard and Coursey, 1981). The negative feedback loop from wound healing to the suppression of wound-induced signals and responses is inadequate so that the responses are sustained and extend through the whole root. Unlocalised responses in cassava may be due to a failure of early suberization/lignification at the wound surface, or slow development of periderm, caused by unresponsiveness of the root tissue to signals which normally initiate the sealing and healing processes (for more detailed discussion of these points see Beeching et al., 1994).

The control of PD in cassava by genetic means is an important and realistic objective that could be approached by several means. The technology of genetic modification could be used to generate mutants in which a key enzyme is inactivated thereby suppressing all the subsequent cascade reactions of PD. The modification of the ripening process in tomato by the insertion of antisense genes for polygalacturonase and ACC synthase (Smith et al., 1990; Hamilton et al., 1990) shows the potential of this approach. A key enzyme in the phenylpropanoid pathway, such as PAL, could be an appropriate target. But this pathway plays multiple and important roles in plant development. Therefore, the antisense construct would require being under the control of a strictly PD-specific promoter. A major difficulty with this strategy is that cassava genotypes with impaired wound responses may show increased susceptibility to pathogens, as has been observed in other plants (Lamb et al., 1989).

The alternative approach would be to complete and enhance the feedback loop which appears to be inadequate in the cassava root wound response. A rapid and localized wound healing response could reduce deterioration resulting from dehydration and the entry of oxygen and microorganisms into the root, whilst probably allowing the initial wound response to be down regulated as in other plant systems. Early studies reported a limited variation in susceptibility to PD (Booth,

1976), but other work demonstrated that the conditions under which the cassava has grown may have a significant effect on the level of susceptibility expressed (Wheatley, 1980). Differences in wound healing properties do not appear to have been investigated in cassava. While it is possible that an adequate wound healing response has been lost due to selection in *M. esculenta,* this may not be the case in the other *Manihot* species with which cassava can form hybrids. These species should be examined to identify potential parents with the genetic complement required for an efficient wound healing process.

The problem of physiological deterioration in cassava needs to be addressed by either the above or other approaches. The impression we gain is that, while there is a collection of circumstantial and descriptive data in cassava PD, there is a lack of rigorous and detailed background information. Such biochemical and molecular data on all aspects on the cassava root's response to harvesting are urgently required.

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Table 1. Estimated molecular mass ($x10^{-3}$) of cassava root proteins extracted at different postwounding times (hours), and separated by SDS-PAGE. Values in bold represent the prominent bands.

0 h	2 h	4 h	6 h	24 h	48 h
				230	230
	180	180	180	180	180
			170	170	170
			145		145
					130
				92	
86	86	86	86	86	86
				70	70
				58	58
				55	55
43	43	43	43		
				42	42
					39
					36.5
				36	
35				35	35
33					
32	32	32	32		32
				29.5	29.5
					25
				21	21
				14.2	14.2
					12

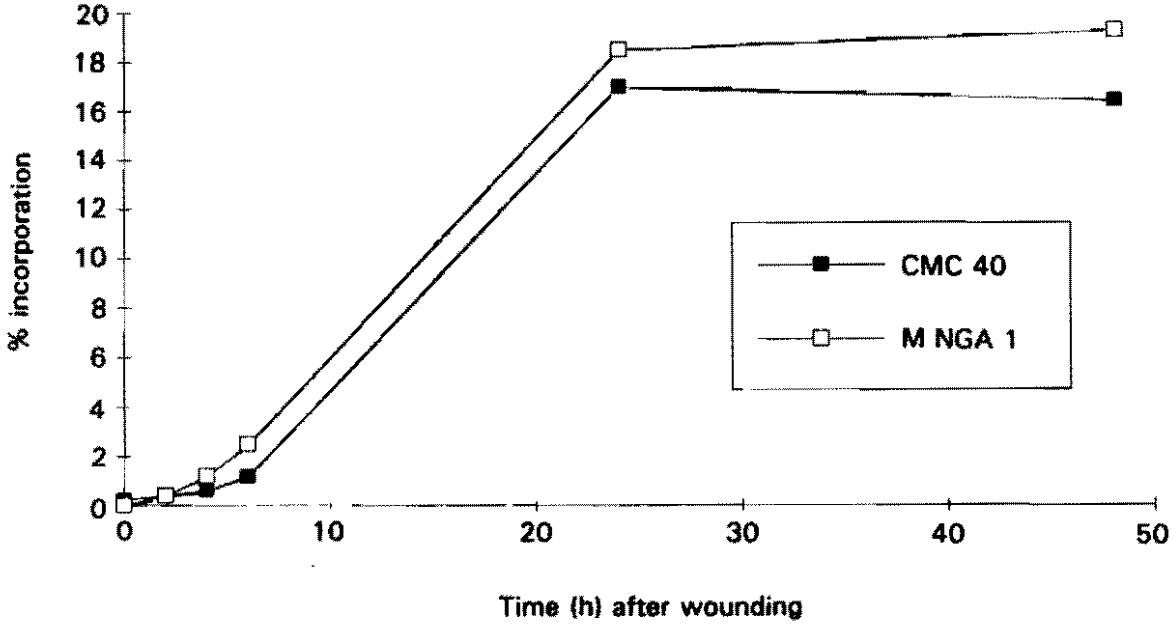

Figure 1. Incorporation of radioactive amino acids into protein in root discs of cassava varieties CMC 40 and M NGA 1 after wounding.

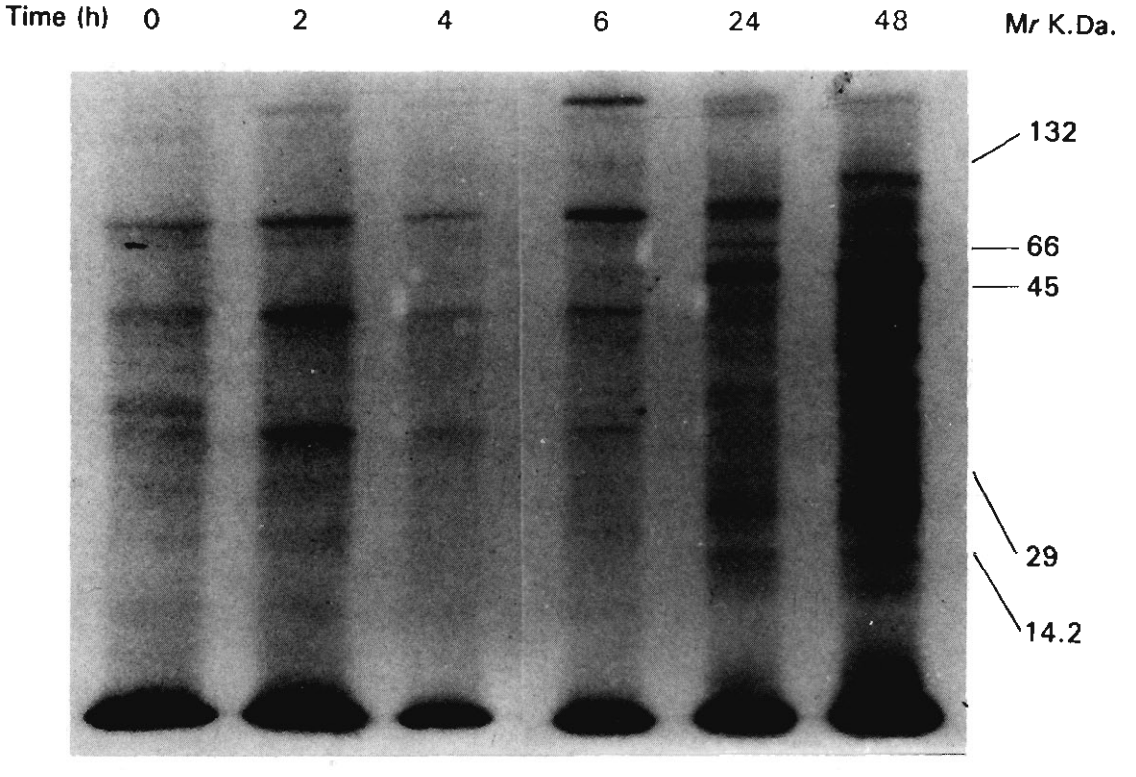

Figure 2. SDS-PAGE of radiolabelled polypeptides from cassava variety CMC 40 extracted at different times (hours) post wounding.

USING *ASPERGILLUS NIGER* TO PROCESS RAW CASSAVA FOR ANIMAL HUSBANDRY

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Cassava and its by-products are found to be limited as food. Bioconverting these materials into protein by microbial fermentation of selected strains of fungi is a promising way of improving the quality of animal food. We found that *Aspergillus niger* TH3 92 and its by-products from batch-submerged and batch solid state fermentation, improved SCP production and protein enrichment from cassava. We obtained the final biomass of 22 g (dry base containing 30% crude protein) from 50 g of raw cassava by batch-submerged fermentation. The medium for batch solid state fermentation has many components: 1000 g raw starch substrate; 13 g urea; 25 g ammonium sulphate; 50 g super phosphate; 9 g potassium chloride; and 1350 g tap water. The total protein in various fermented media of raw cassava-spent grains was increased by 80%, of cassava-maize by 100%, of raw cassava fiber-maize by 120%, and of raw cassava chips by 400%. At present the batch solid-state fermentation is being applied effectively in Vietnam.

INTRODUCTION

Like many other Southeast Asian countries, Vietnam makes full use of many traditional and non sterile fermentation techniques to produce wine, spirits, vinegar, sauces, and soy-based foods. We are reporting on the modification of traditional techniques for myco-biomass production from available raw, starchy materials such as cassava, cassava fiber, maize, spent grains (in breweries) and the minerals N, P, and K. Selected strains of filamentous fungi grow on raw starchy materials in nonsterile conditions (surface, solid and submerged fermentation).

MATERIALS AND METHOD

Materials

Cassava starch, cassava chips and maize can be purchased in the markets.

Cassava fiber can be taken from the Cassava Processing Factory, Son Tay Province, and spent grains from the Hanoi Brewery.

Chemicals are technical grade.

Microorganisms

The CAM collection has 10 strains fungi found to be good protein producers with the addition of starch. These strains were cultured on Czapek-Dox medium and maintained at refrigerated temperature.

Submerged state fermentation medium

The medium has the following components: 50% raw cassava flour; 0.5% urea; 1% super phosphate; 0.15% potassium chloride; and 4% pH.

Solid-state fermentation medium

The nutritional solution used for solid-state fermentation medium contains the following components per each 1000 g of raw substrate (dry basis): 6,5 g urea; 12,5 g ammonium sulfate; 25 g super phosphate; 0.15 g potassium chloride; and 675 g water.

Analytical methods

We determined starch content of substrate using the HCl hydrolysed method of keeping 100 mg dry substrate and 5 ml HCl in a boiling water-bath for 3 hours. We used the Micro-Bertrand method (Klein, 1932) to determine the total reducing sugar. We calculated the percentage of starch as follows:

$$\% \text{ starch} = \frac{\text{Reducing sugar} \times 0.9}{100}$$

We obtained biomass from submerged culture and dried it at 70 °C until weight was constant to determine dry biomass. We used the Kjeldahl method (AOAC, 1980) to determine total protein content of fermented media. We calculated the protein content by multiplying the quantity of nitrogen obtained by 6.25.

RESULTS AND DISCUSSION

Submerged fermentation

Figure 1 shows that *Aspergillus niger* TH3 93 is the best biomass producer from both raw soluble starch and raw cassava media. A previous report on the higher

activity of raw starch digestion glucoamylase of this strain in comparison with others (Dung et al., 1993a) supported this result. Dung et al. obtained the final biomass of 22 g/1 (dry basis, containing 30% crude protein) at hour 44 hour of fermentation (Figure 2). At that time, the amount of reducing sugar left was about 2-5 g/1 but no starch was detected.

Protein enrichment of various kinds of raw starch by batch solid-state fermentation with *A. niger* TH3 92 seemed promising. When we added the nutrition solution obtained from our previous study (Dung et al., 1993b), the total protein contents of fermented media of cassava-spent grains increased by 80%, that of cassava-maize by 100%, that of cassava fiber-maize by 120%, and that of cassava chips by 400% compared with the initial contents of total protein (Figures 3, 4, 5, and 6).

Compared to the submerged fermentation method, the quality of these fermented products is not so high in terms of protein content but this method is much more applicable and easily accepted by farmer households. Using prepared inoculum of *A. niger* TH3 92 with detailed instructions for making the nutritional solution, they can easily prepare their animal feed from available raw starch materials without cooking. Feeding pigs with fermented cassava-maize increased the yield of meat by 20% and reduced the amount of food used for each unit of meat production by 15%. On the other hand, inoculating the fermented products with "yeast-cake" significantly improved the nutritional value (vitamins and proteins) and flavor of the final products. Further studies are being carried out to confirm results.

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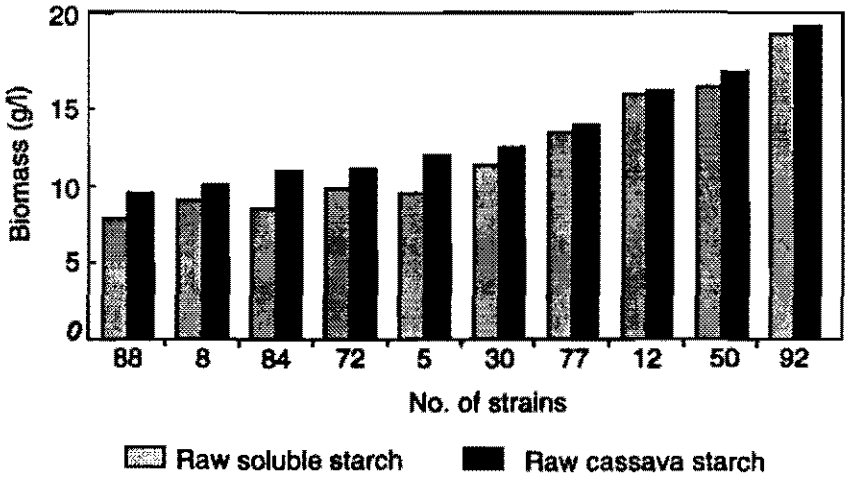


No. 88 = *Rhizopus nigricans*
No. 8 = *Aspergillus candidus*
No. 84 = *Humicola grisea*
No. 72 = *Penicillium corylophylum*
No. 5 = *Mucor mucedo*
No. 30 = *Mucor hiemalis*
No. 77 = *Cylindrocapon* sp.
No. 12 = *Cunninghamella eligaus*
No. 50 = *Cunninghamella echinulata*
No. 92 = *Aspergillus niger* (TH3 92)

Figure 1. Biomass of various strains on two raw starch media.

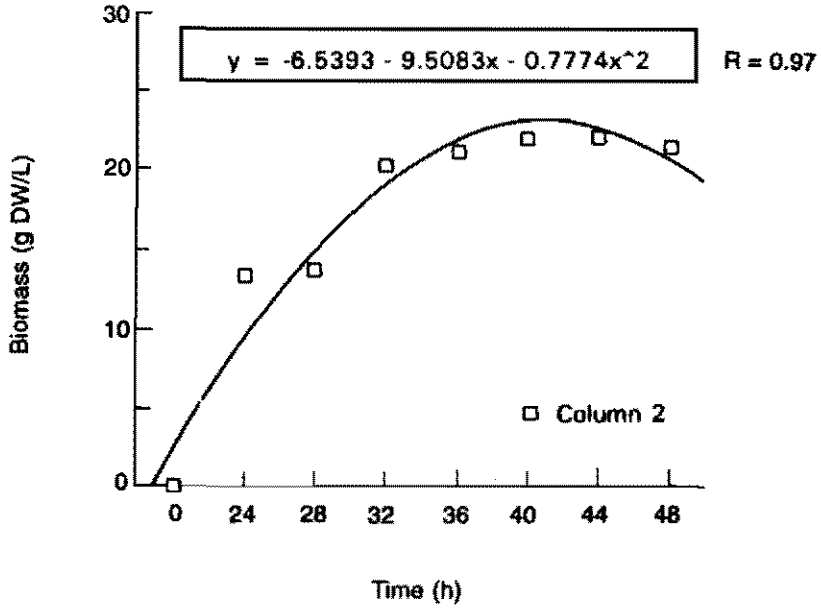


Figure 2. Kinetics of biomass accumulation of *Aspergillus niger* TH3 93.

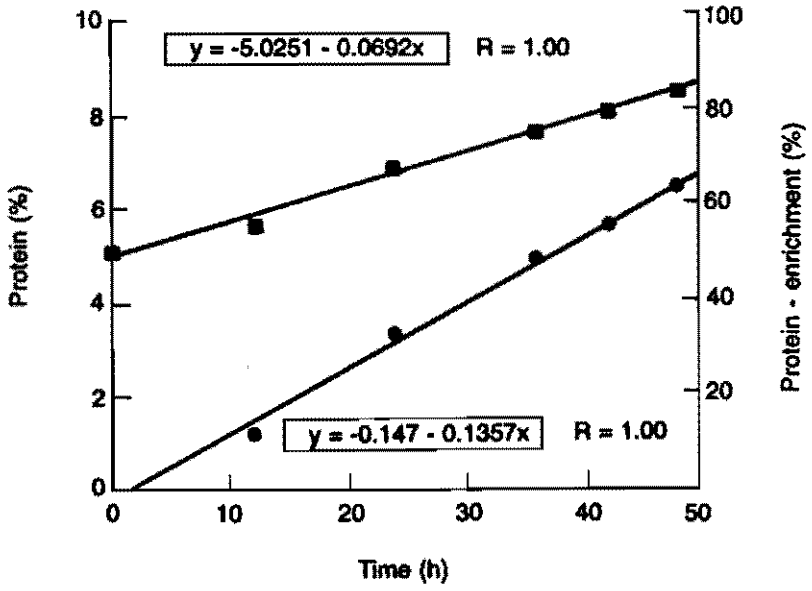
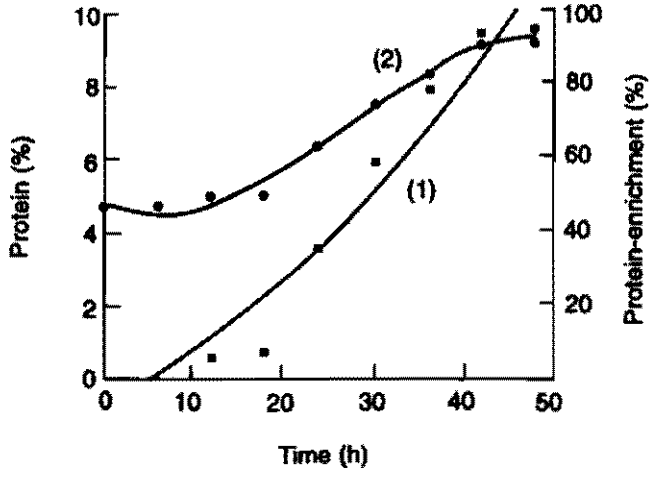
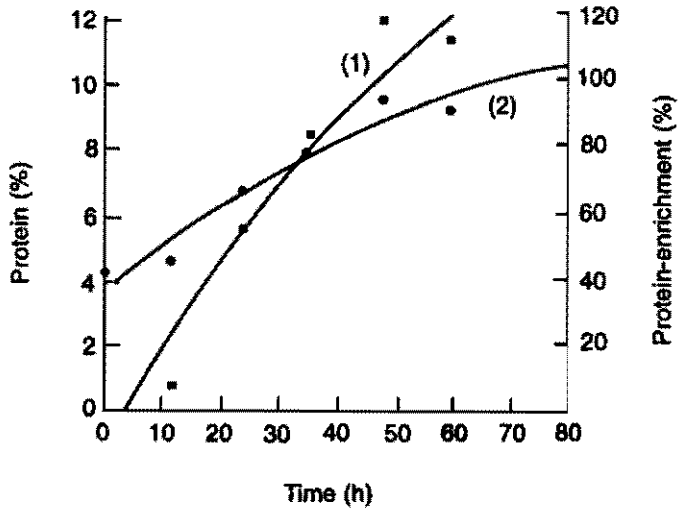


Figure 3. Protein enrichment of raw cassava-spent grains by solid-state fermentation of *Aspergillus niger* TH3 93.



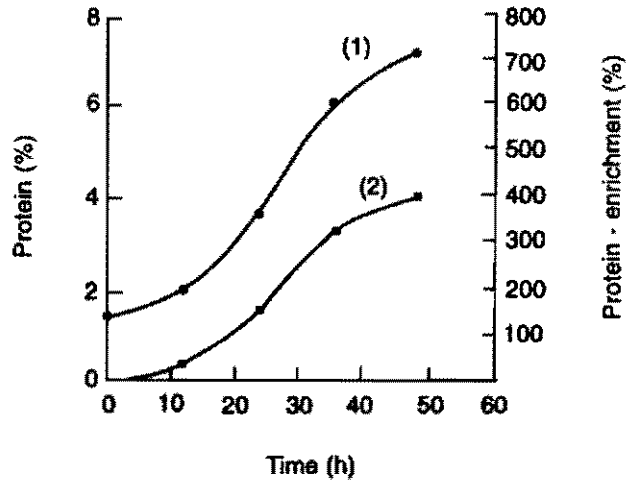
y(1) = -0.748 + 0.117X + 0.002X2 R = 0.97
y(2) = 4.845 - 0.123X + 0.011X2 - 1.37e-4X3 R = 0.99

Figure 4. Protein enrichment of raw cassava-maize by solid-state fermentation of *Aspergillus niger* TH3 92.



$y(1) = -1.031 + 0.305X - 0.001X^2$ R = 0.97
$y(2) = 3.863 + 0.134X - 6.560e\text{-}4X^2$ R = 0.97

Figure 5. Protein enrichment of raw cassava fiber-maize by solid-state fermentation of *Aspergillus niger* TH3 92.



$y(1) = 1.456 - 0.056X + 0.009X^2 - 1.13e\text{-}4X^3$ R = 1.00
$y(2) = 0.02 - 0.042X + 0.007X2 - 8.24e\text{-}5X3$ R = 1.00

Figure 6. Protein enrichment of raw cassava chips by solid-state fermentation of *Aspergillus niger* TH3 92.

CASSAVA ROOT PROTEINS: ISOLATION AND CHARACTERIZATION OF THE MAJOR ALBUMINS FROM THE PEEL AND CELLULOSIC PARENCHYMA

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We have analyzed by SDS-PAGE the protein profile of the peel and cellulosic parenchyma of cassava (*Manihot esculenta*) roots and found striking qualitative and quantitative differences. Through the use of a combination of chromatographic techniques (gel filtration, ion exchange and reverse phase HPLC) we isolated the major albumin of each of these tissues. The protein from cellulosic parenchyma showed a molecular weight of 22,000. Antibodies were raised in rabbits against the 22K protein and used to show (by a tissue imprinting technique and by western blot),that this protein is present only in the cellulosic parenchyma. We used these antibodies to study the deposition pattern of this protein as tuberization proceeds.

INTRODUCTION

Cassava (*Manihot esculenta*) is widely cultivated in South America, South East Asia and Africa, where its tuberized roots are a very important source of carbohydrates (Cock, 1985). Lack of basic knowledge about the biochemistry of the cassava plant is hampering the application of new biotechnological tools to improve certain agronomic traits of the crop, such as nutritional quality, disease resistance, starch quality. For example, it is widely accepted that one of the priorities in cassava biotechnology should be improving the qualitative protein in the roots, yet no major proteins from any of the root tissues have been isolated and characterized in any detail (Shewry et al., 1993). Here we present data on two major proteins isolated from the peel and cellulosic parenchyma.

MATERIAL AND METHODS

Plant material

We used cassava (cv. Tapicinea) roots from plants grown at the experimental station of the Centro Nacional de Pesquisa de Agroindustria Tropical (CNPAT/ EMBRAPA) at Pacajus, Ceará.

Protein extraction and characterization

We removed the periderm, separated the peel and cortex, dipped them into liquid nitrogen and freeze-dried them. We then prepared a very fine powder. We extracted proteins with NaCl 1% (1:5,w/v) for two hours at 4 °C. We added polyvinylpyrrolidone (2%,w/v) to remove phenolics. We centrifuged (10,000 g, 4 °C) the supernatant then extensively dialyzed it against distilled water and collected, freeze-dried and kept the albumin fraction at -20 °C until used. We purified the protein using fractionation of freeze-dried albumin fractions by ion exchange chromatography and reverse phase HPLC (Miranda et al., 1993).

Protein deposition during root "tuber" development

We collected and separated young roots according to their diameter: up to 0.3 cm (stage 1); 0.3 to 0.6 (stage 2); 0.6 to 1.0 (stage 3); 1.0 to 1.5 (stage 4); 1.5 to 2.0 (stage 5); 2.0 to 2.5 (stage 6); 2.5 to 3.0 (stage 7). We prepared the albumin fractions from each of these root groups as described above. After SDS-PAGE electrophoresis (Laemmli, 1970) of b-mercaptoethanol treated samples, we transferred proteins to a nitro-cellulose membrane as described in (Towbin et al., 1979). We probed against polyclonal antibodies raised in rabbits against the 22K protein purified from the cellulosic parenchyma.

We used a tissue imprinting technique (Cassab, 1993) to visualize the distribution of the 22K protein within the peel and cellulosic parenchyma. We transversely cut fully mature tuberized roots with a sharp blade and gently pressed them against a nitro-cellulose membrane. We then probed the proteins bound to the membrane against polyclonal antibodies raised against the 22K protein purified. We dipped the membranes for two minutes in a solution of ponceau reagent for protein staining.

RESULTS AND DISCUSSION

The peel and cellulosic parenchyma from cassava roots have protein patterns which are quantitative and qualitatively different (Figures 1 and 2). Figure 1 shows that among the peel albumins there is a major protein of MW 66K and in the cellulosic parenchyma one of the major proteins has a MW of 22K. We have isolated both of these proteins. We purified the 22K protein from the cellulosic parenchyma (Figure 3) by a combination of ion-exchange chromatography and reverse phase HPLC. We raised antibodies against this protein in rabbits. We used these antibodies to show that this protein is present predominantly in the cellulosic parenchyma (Figures 4 and 5).

Figure 6 shows the SDS-PAGE protein profile of the cellulosic parenchyma of tuberized roots at different stages of development. It does not reveal any major change in the quantitative or qualitative pattern of protein composition in the stages of development analyzed. We could see by anatomical analysis that even in roots at stage I of development, the process of root "tuber" formation was well under way (data not shown). Figure 7 shows that the concentration of the 22K protein remains fairly constant in all of the developmental stages analyzed. The 22K protein could not be detected in extracts of very young roots obtained from stem cutting 8 to 20 days after planting.

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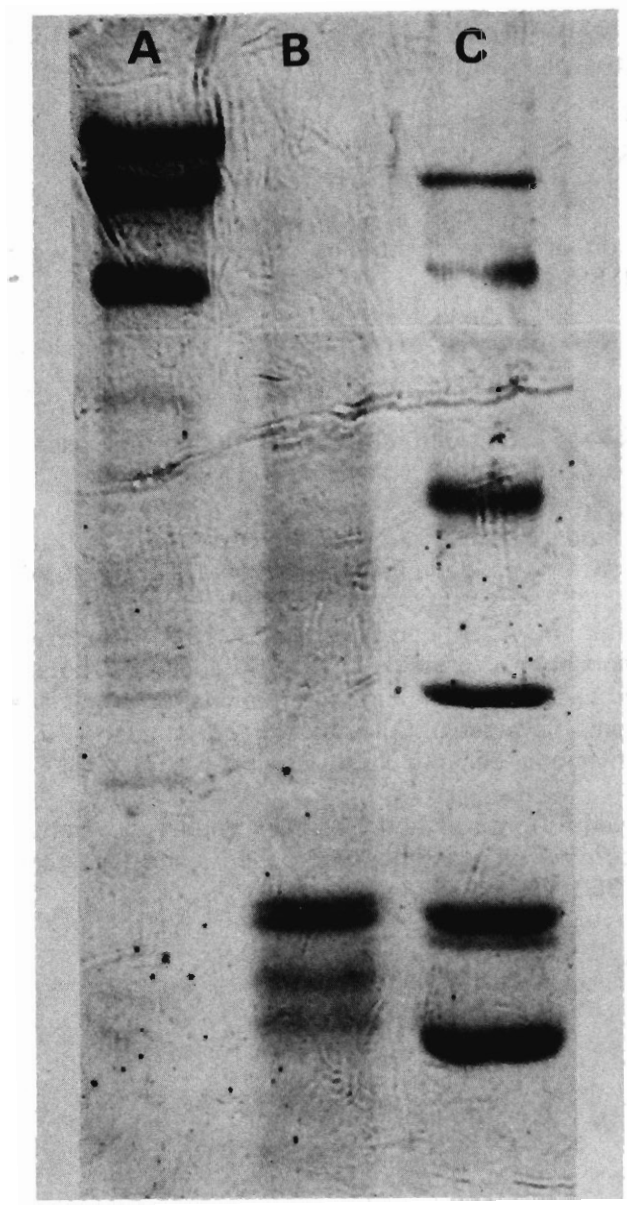

Figure 1. SDS-PAGE of total water-soluble proteins from the peel (lane A) and cellulosic parenchyma (lane B) of cassava roots. Lane C, molecular weight standards.

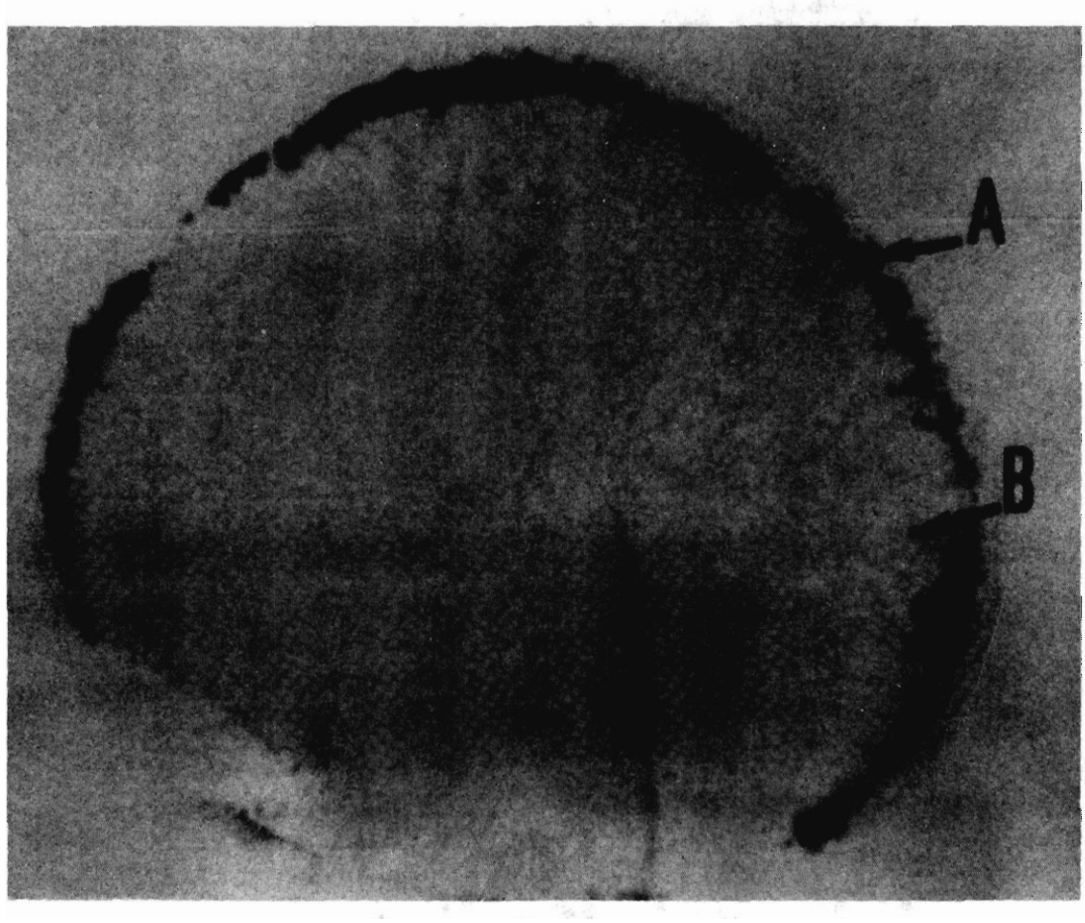

Figure 2. Tissue-print on nitro-cellulose membrane of a cross section of a tuberized root of cassava. The print was stained with Ponceau reagent. (A) peel; (B), cellulosic parenchyma.

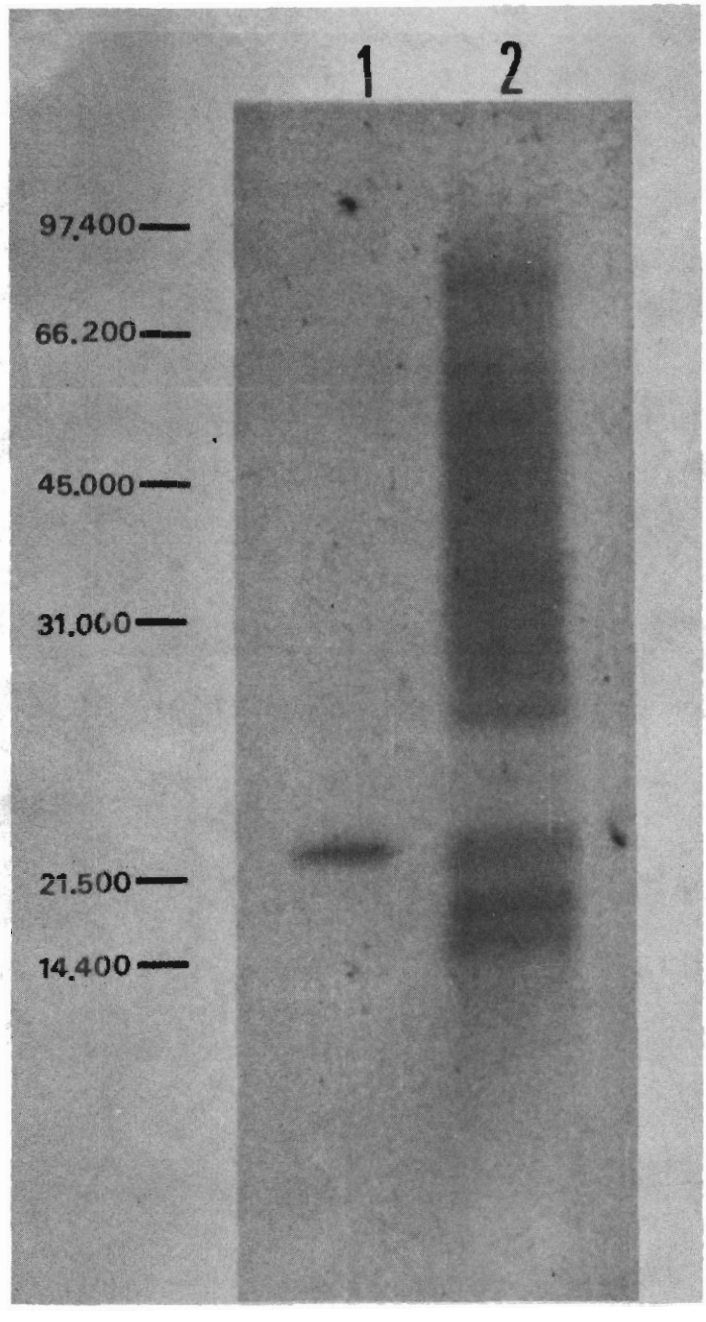

Figure 3. SDS-PAGE of purified albumin from cassava roots. Lane 1, purified albumin; lane 2, total water-soluble proteins. The molecular weight of the protein standards is indicated on the left.

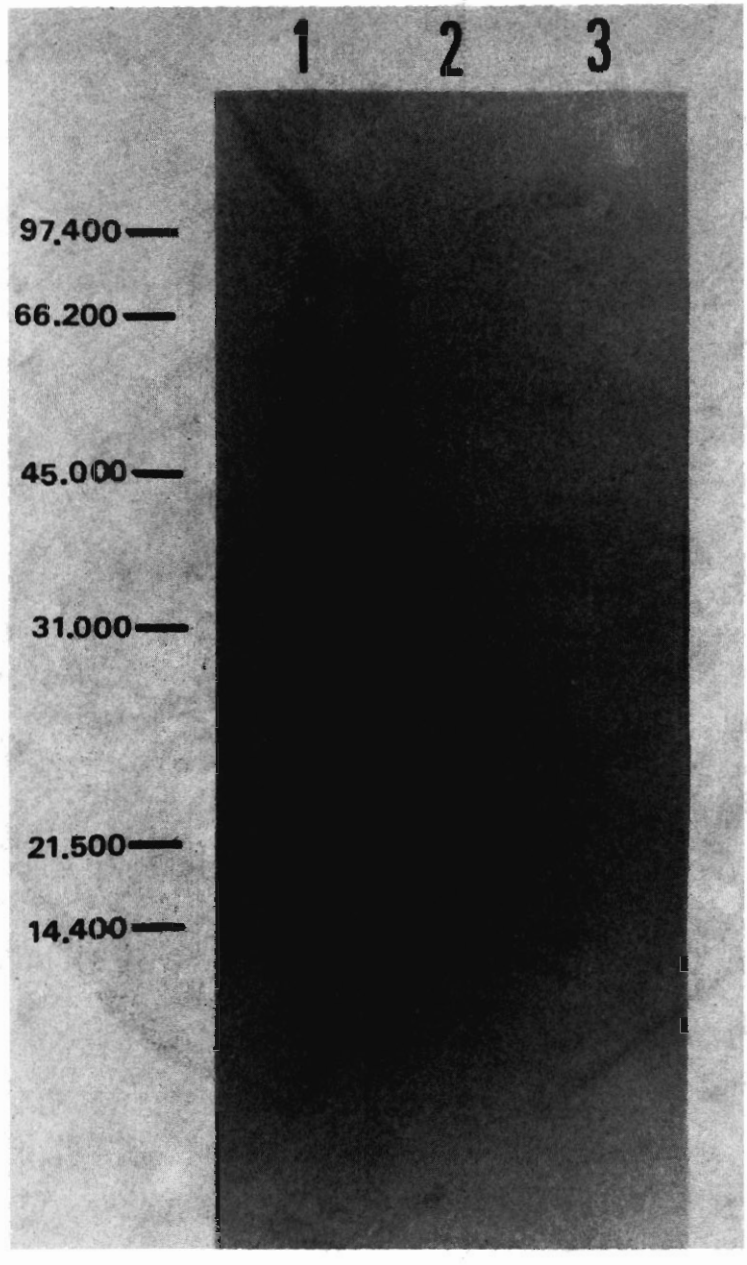

Figure 4. Western blot analysis of total water-soluble proteins from the cellulosic parenchyma (lane 1), purified 22K protein (lane 2) and total water-soluble protein from the peel (line 3). The print was reacted with polyclonal antibodies (diluted 1:1000) against the 22K protein and detected with alkaline phosphatase-conjugated anti-goat immunoglobulins antibodies. The molecular weight of the protein standards is indicated on the left.

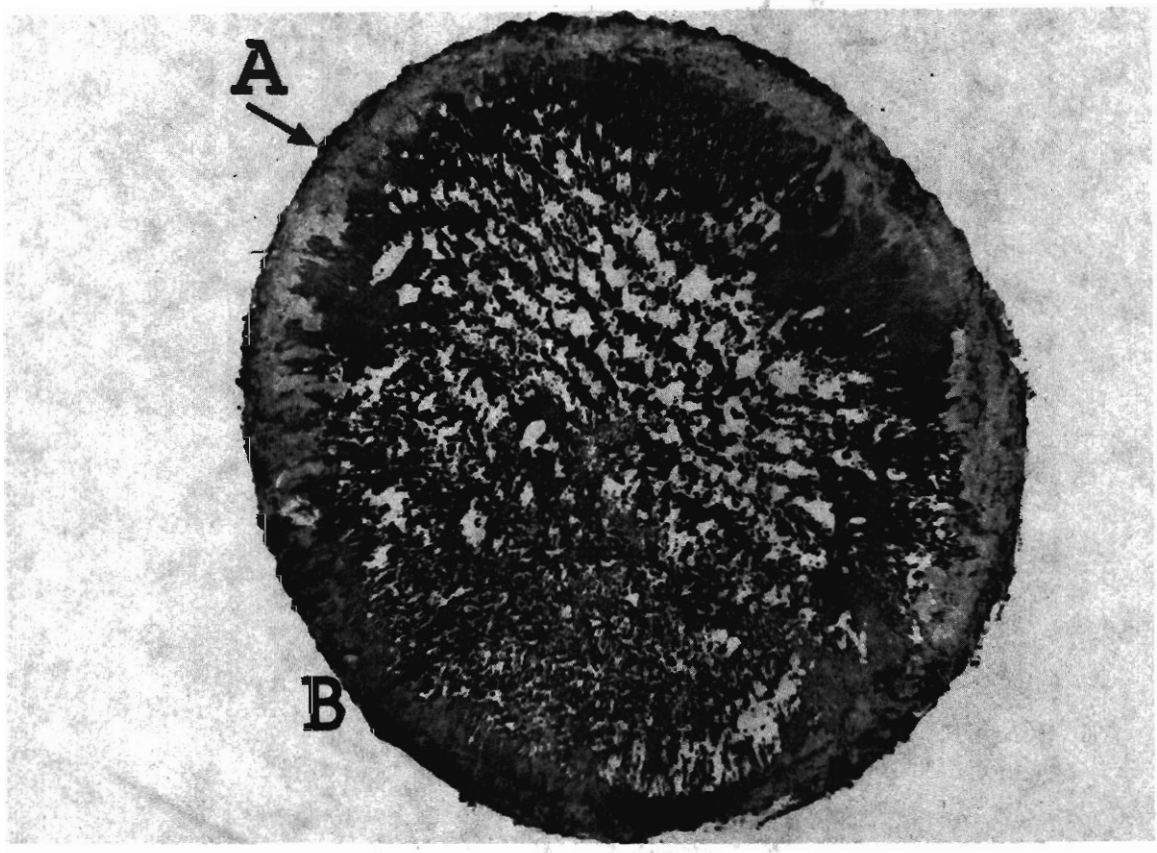

Figure 5. Tissue-print immunoblot of a cross-section of mature cassava tuberized root. The print was reacted with polyclonal antibodies (diluted 1:1000) against the 22K protein and detected with alkaline phosphatase-conjugated anti-goat immunoglobulins antibodies. (A) peel; (B) cellulosic parenchyma.

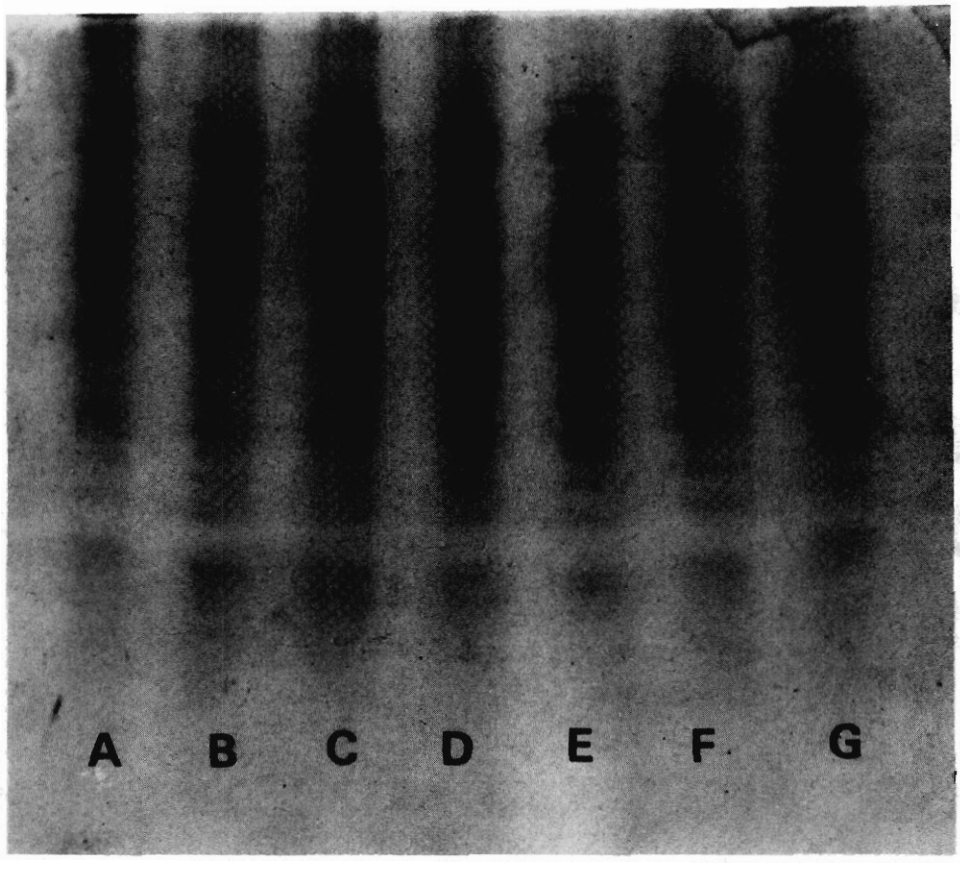

Figure 6. SDS-PAGE analysis of total proteins from cassava roots at different stages of development. Lane A, stage I; Lane B, stage II; Lane C, stage III; Lane D, stage IV; Lane E, stage V; lane F, stage VI; and lane G, stage VII.

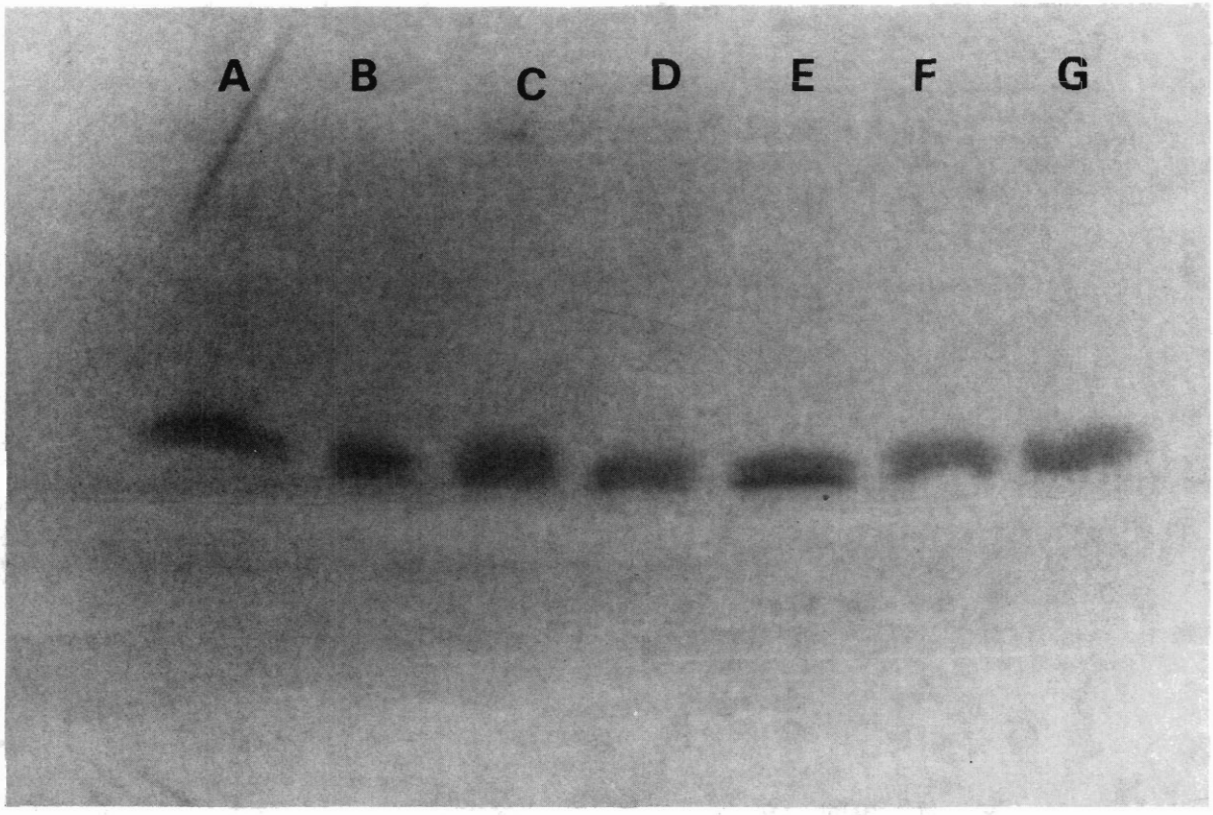

Figure 7. Western blot analysis of accumulation of the 22K cassava protein during root "tuber" development. The print was reacted with polyclonal antibodies (diluted 1:1000) against the 22K protein and detected with alkaline phosphatase-conjugated anti-goat immunoglobulins antibodies. Lane A, stage I; lane B, stage II; lane C, stage III; lane D, stage IV; lane E, stage V; lane F, stage VI; and lane G, stage VII.

THE EFFECT OF PROCESSING PARAMETERS ON GARI QUALITY

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Gari production is a form of solid-state fermentation and various factors such as moisture content, particle size, pH and type of fermenter affect the fermentation process. The effect of these factors on the reduction of cyanide content, the use of starter culture, and various methods in the production of low cyanide, good quality, gari have been studied. The optimum fermentation period for cyanide detoxification is 72 h while the optimum period of fermentation for high quality gari is 48 h. The optimum mash moisture content for cyanide detoxification was 80%. The optimum particle size of mash was obtained when graters, perforated with nails of size 2.12 mm, 2.72 mm and 3.24 mm, were used for grating. Keeping the pH of the fermenting mash in a non porous container above 5 reduced the bound cyanide by 93% after 72 h. *Bacillus subtilis* (B) and *Candida krusei* (C) strains increased the hydrolysis of cyanogenic glucoside while a mixed culture of both strains or a combination with *Lactobacillus plantarum* strains produced the most acceptable gari.

INTRODUCTION

Over 500 million people in the world consume cassava (*Manihot esculenta* Crantz). Over 200 million people in sub-Saharan Africa depend on cassava as a source of calories (Idowu, 1990). About 70% of Nigeria's daily calorie intake is cassava based (IITA, 1985). Nigeria is the world's foremost cassava producer and consumes about 90% of its own production (Longe, 1979). Up to 60% of this is consumed in the form of gari. Yearly, 67 kg of gari is consumed per head in Nigeria, mainly in the form of eba porridge (Onyekwere et al., 1989). Gari quality varies as processing conditions vary. The inconsistency in gari quality on the market is due to increasing industrialization, urbanization and the consequent growing demand for this product. A recent survey of gari samples from urban and rural markets revealed a wide variation (4-50 ppm) in the cyanide content (Bolade, 1990). The presence of cyanide in gari has been implicated as the cause of certain neuropathological diseases in Nigeria (Oshuntokun, 1981). Controlling the processing parameters can improve the quality.

Gari production is a form of solid-state fermentation. Various factors such as moisture content, particle size pH and type of fermenter affect this fermentation process. Our Department focuses research on two aspects of cassava fermentation: how to reduce cyanide content; and the use of starter cultures towards this end. We here present results of work carried out to date.

MATERIALS AND METHODS

Type of cassava

We used cassava roots of a varietal clone, TMS 30572, obtained from IITA and the University of Agriculture farm, Abeokuta, Ogun state, Nigeria. The roots were from 18- and 12-month old plants.

Type of cultures

We used the starter cultures *Lactobacillus plantarum* (L), *Bacillus subtilis* (B), *Candida krusei* (C), obtained from the Food Microbiology Laboratory of the Department of Food Science and Technology, University of Agriculture, Abeokuta, Ogun State.

Culture cultivation

We made pure culture inocula from *L. plantarum* suspension in glycerol, and suspensions of *B. subtilis* on Nutrient Agar (Oxoid) slants and of *C. krusei* on Malt Extract Agar (Oxoid) slants. We added five ml of sterile peptone water to cultures on slants and shook it to make a suspension. We prepared inocula for the mixed cultures by inoculating 50 ml of MRS broth (Mann, Rogosa, Sharpe, 1960) with *L. plantarum*, of nutrient broth (NB) with *B. subtilis*, and Yeast Extract broth (YEB) with *C. krusei*. We incubated the cultures at 28°C for 48 h agitating them on a reciprocating orbit shaker at 100-150 rpm.

Preparation of cassava mashes

We manually peeled, washed and grated the cassava roots. We made five different graters by perforating a galvanized iron sheet 0.32 mm thick using iron nails 4.75 mm, 3.84 mm, 3.24 mm, 2.72 mm and 2.12 mm in diameter. The rasping surfaces of these graters varied in size of protruding rims of nail openings. We used them to grate the roots manually. We designated the cassava mash samples prepared using these graters as G475, G384, G324, G272, and G212. We made a sixth sample, designated BLC, by blending G212 in a waring blender for 3 min.

We sterilized the cassava mash for 30 min in a U.V. sterilizer (Millipore) chamber using four germicidal lamps. We used a 3-ml aliquot of each culture suspension as inocula for every 150 g of sterilized cassava mash.

Gari processing methods

We studied six different methods of processing cassava into gari:

Method 1 - TMF (traditional method). Peel, wash and grate the roots into mash; ferment in jute bags and simultaneously dewater mash using a screw press. Sift the resulting cake and garifying to a moisture content of 8%-10%.

Method 2 - NPC. As for method 1 (TMF) but fermentation is carried out in a nonporous container, necessitating a separate dewatering step.

Method 3 - SCM. As for method 2 (SCM) but sterilize the mash and inoculate with starter cultures.

Method 4 - MCM. As for method 2 (NPC) but increase the moisture content of the mash to 80% before fermenting.

Method 5 - NAM. As for method 2 but add a local neutralizing agent (Trona; a mixture of potassium carbonate and potassium bicarbonate) to maintain the pH of the mash at about 6.0.

Method 6 - SDM. As for method 4 (MCM) but incubate the mash for 24 h followed by sun drying for 48 h then dewater.

Chemical analysis

We determined the pH of the fermenting cassava mash and gari product by adding 50 ml sterile water to 5 g of sample, mixing and decanting the supernatant and reading the pH with a Kent pH meter model 7020. We determined the titratable acidity (expressed as % lactic acid) by titrating 25 ml of the supernatant used for the pH determination with 0.1N, NaOH to pH 8.3 following the method of Mbugua (1981). We used the phenol sulfuric acid method of Dubois et al. (1956) to determine the reducing sugar, while using the methods of Cooke et al. (1978) and Ikediobi et al. (1981) to determine the cyanide content in the cassava mash, cake and gari. We profiled the colour of gari samples on a Munsel (1975) color chart while determining the particle size distribution of gari samples by Akingbala's (1982) method.

We carried out sensory evaluation of gari in batches. We used a 7-member panel of judges consisting of University of Ibadan staff and students familiar with gari. The panelists used the nine hedonic scale preference analysis (Larmond, 1977) to express their degree of like or dislike of gari. The quality factors assessed for gari were colour, taste, aroma, particle size and general acceptability. We

analyzed the result of the sensory evaluation using Duncan's multiple range test (Steele et al., 1980).

RESULTS AND DISCUSSION

Effect of duration of fermentation on cyanide content and gari quality

The total, bound and free cyanide contents of the samples decreased with an increase in fermentation time until 72 h, after which there was no effective reduction in cyanide concentration. Free cyanide decreased from 55.3 mg/kg dry matter to 11.2 mg/kg matter at 72 h, while the bound cyanide decreased from 57.9 mg/kg at time 0 to a constant value of 14.3 mg/kg beyond 72 h (Figure 1).

The residual cyanide content in gari ranged from 1.9 to 11.6 mg/kg DM. Gari made from fresh unfermented cassava mash had a residual cyanide content of 11.6 mg/kg DM. Gari of cassava mash fermented for 24 h, had a cyanide concentrations of 8.6 mg/kg DM, for 48 h of 5.1 mg/kg DM, for 72 h of 2.3 mg/kg DM and for 96 h of 1.9 mg/kg DM. Calculated as a percentage of the initial cyanide content in the mash, the residual cyanide in gari ranged from 10.3% to 1.7%, with 7.6% for mash fermented for 24 h, 4.5% for 48 h and 2% for 72 h.

Gari colour varied with period of fermentation. The nonfermented gari was white, the colour changing progressively to yellow with longer fermentation (Table 1). Browning occurs during garification due to Maillard reaction involving amino acids and sugars present in the cassava mash (Ketiku and Oyenuga, 1970). But the colour changes shown in Table 1 may be due more to HCN reducing the sulphur-containing amino acids in the mash and making the yellow colour of sulphur prominent with increase in fermentation time (Nartey, 1973).

The judging panel preferred fermented gari to nonfermented gari in all quality factors assessed. Gari from mash fermented for 24, 48 and 72 h scored the same with respect to all sensory parameters except general acceptability where gari fermented for 24 h was rated less acceptable than the others (Table 2).

Effect of moisture content of mash on the residual cyanide content and quality of gari

We reduced the moisture content of freshly grated mash from 65% to 60%, and 55% by pressing and increased to 75% and 80% by adding water and kept it in a nonporous container for 72 h before dewatering, sifting and garification. Adding water to the freshly grated cassava spontaneously increased the measurable total cyanide content probably through hydrolysis or leaching of soluble cyanoglucoside, thereby also reducing the residual total cyanide in the cake. The increased

hydrolysis depleted the cyanide by 91.7% in the cake from 80% moisture mash, and 73% in the cake from 55% moisture mash. It also made similar reductions in gari: 1.9% residual total cyanide in gari from 80% moisture mash, and 12.2% in gari from 55% moisture mash (Figure 2). Over 98% total cyanide was removed when moisture content of mash was 80%, compared to 95% for the traditional method with mash of 65% moisture content. The residual cyanide content in the gari made from mash with 55% moisture was 30.7 mg HCN/kg DM, with 60% it was 29.8, with 75% it was 22.9, with 80% it was 11.6 and with the traditional method 19.2 mg HCN/kg DM.

Effect of pH on the cyanide content and gari quality

We added 0.3% (w/w) of trona (a mixture of potassium carbonate and bicarbonate) to study the effect of pH on the cyanide content and gari quality. The pH of the mash varied between 5 and 7 at an initial moisture content of 80%. The total cyanide content in the mash was reduced from 113.2 to 14.9 ppm compared to 24.9 ppm by the traditional method after 72 h. The bound cyanide was reduced to 3.6 ppm compared to 15.2 ppm in the mash obtained by the traditional method. Maintenance of this pH effectively reduced the cyanide content. But the gari quality was poor in terms of colour, taste, aroma, and general acceptability compared to that obtained by the traditional method.

Effect of particle size on cyanide content and gari quality

We studied the effect of particle size by grating cassava with graters of different pore sizes and subjecting the mash to fermentation for 72 h. The pore sizes of the graters were 212 mm, 272 mm, 324 mm, 384 mm and 475 mm. We prepared a fine mash by blending the 212 mm sample in a blender for three mins and named it BLC. We named the samples G212, G272, G324, G384, and G475 in order of increasing coarseness. Samples G212, G272, and G324 had similar particle size distribution had the highest rate of reduction in cyanide content compared to samples G475 and BLC (Figure 3). The optimum grater sizes for cyanide reduction were G212, G272 and G324. Gari from the above samples was highly rated in terms of color, taste, aroma, mouldability, stickiness and general acceptability (Table 3).

Effect of different methods of gari production on the cyanide content and quality of gari

Sun drying the cake made from mash fermented for 72 h (SDM) reduced the cyanide content by 99.5% of its initial total, leaving less than 3 ppm cyanide in the

gari. The residual cyanide content in the gari made by TMF is 3.0 mg HCN/kg DM, by NPC it is 2.5, by MCM it is 2.2, by NAM it is 1.9 and by SDM it is 2.7 mg HCN/kg DM. These represent 95.0%, 97.8%, 98.1%, 98.3% and 99.5% reductions in total cyanide content of the fresh cassava mash from which the samples were made (Figure 4). All the methods used for gari preparation were effective in reducing cyanide to levels less than the FAO/WHO recommended value of 10 ppm. However gari produced by the MCM method was rated highest with respect to colour, taste, aroma, particle size and general acceptability (Table 4).

Effect of starter cultures on cyanide reduction and gari quality

When we used starter culture of *Lactobacillus plantarum* (L) as inoculum, the reduction in bound cyanide content was 56.5%, using *Bacillus subtilis* (B) it was 87%, using *Candida krusei* (C)it was 92.2% and in the control with chance inoculation it was 75.2%. Similarly when the starter cultures were used in the combination BC the reduction in bound cyanide was 87.1%, with LB it was 92%, with LC it was 88.2%, and with LBC it was 61.8% (Figure 5). These results indicate that the *Bacillus subtilis* (B) and *Candia krusei* (C) strains increased the hydrolysis of cyanogenic glucoside.

The gari samples from mash inoculated with single culture were preferred to the control sample with natural inoculation. The gari from *Lactobacillus* inoculated mash scored highest with respect to colour, taste, and general acceptability. The gari from *Bacillus* inoculated mash scored highest with respect to particle size while the gari from *Candida* inoculated mash scored highest with respect to aroma (Table 5).

In terms of taste and general acceptability, the gari samples from mash inoculated with mixed cultures were significantly better ($P<0.05$) than that from the control with chance inoculum. The gari from BC inoculated mash scored highest with respect to colour, taste and aroma while the gari sample from LB inoculated mash scored highest with respect to particle size. The gari samples from BC, LC and LBC had the same score with respect to general acceptability (Table 6).

CONCLUSION

The optimum fermentation period for cyanide detoxification is 72 h while the optimum period of fermentation for sensory quality of gari is 48 h. The residual cyanide content in gari decreased from 9.5 to 30 ppm as the moisture content in the mash decreased from 80%-55%. Adding water resulted in high quality gari. Adjusting pH of the fermenting cassava mash above 6 resulted in reduction of the bound cyanide content of the mash but the gari was of poor quality. The size distribution of the mash had an influence on cyanide detoxification and the quality

of gari. Cassava mash produced from graters perforated with iron nails of diameter 2.12 mm, 2.72 mm and 3.24 mm had rapid cyanide detoxification and low cyanide content after 96 h of fermentation while cassava mash from a grater perforated with iron nails of diameter 4.75 mm and that produced by blending the mash had a lower rate of detoxification. Fine mash resulted in fine gari while coarse mash resulted in coarse gari. The general acceptability of gari increased with the fineness. The reduction in bound cyanide content when starter cultures of *Bacillus subtilis* and *Candida krusei* were used singly or as mixtures were higher compared to the control with chance inoculum. In addition, fermentation using single or mixed culture inocula produced gari that was better than the control. Gari of mixed culture containing *Bacillus subtilis* and *Candida krusei* was the most acceptable.

This study has shown that the final quality properties of gari, like colour, taste and aroma, are direct manifestations of various biochemical reactions that occur during fermentation. Some of the biochemical reactions such as cyanide hydrolysis, reducing sugar, and acid production, depend on favorable conditions for enzyme activity and stability such as pH, initial moisture condition, and substrate concentration, which are also important in solid state fermentation. A study of the enzymes involved in the different biochemical process is therefore necessary. The only enzyme that has not been adequately investigated is hydroxynitrile lyase which we are studying in our laboratory.

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Table 1. Changes in color of samples of gari with different fermentation periods.[a]

Method of color determination	Color in gari sample				
	NF	F24	F48	F72	F96
Munsel chart	White	Pale yellow	Pale yellow	Very pale yellow	yellow
Sensory panel	White	cream	cream	yellow	yellow

a. NF = not fermented; F24 = 24 h; F48 = 48 h; F72 = 72 h; F 96 = 96 h.

Table 2. Mean* sensory scores for quality of samples of gari with different fermentation periods**.

Sensory quality	Mean scores of gari samples				
	NF	F24	F48	F72	F96
Color	5.14b	7.14a	7.14a	7.29a	5.00b
Taste	5.14b	6.00a	6.71a	6.57a	5.57b
Aroma	4.29b	6.00a	6.57a	6.57a	6.00a
Particle size	6.14a	6.14a	6.29a	6.29a	6.14a
General acceptability	5.00c	6.43b	7.14a	6.86a	5.43c

* Means in the same row followed by the same letters are not significantly different ($P<0.05$).
** NF = not fermented; F24 = 24 h; F48 = 48 h; F72 = 72 h; F96 = 96 h.

Table 3. Mean* scores for gari of different particle sizes**.

Sensory quality	Mean scores of gari samples					
	BLC	G212	G272	G324	G384	G475
Colour	7.43a	6.86a	6.86a	6.86a	6.71a	5.86b
Taste	6.57a	6.57a	6.57a	6.43a	5.71a	5.86a
Aroma	6.29a	6.14a	6.14a	6.14a	6.57a	5.14c
Particle size	7.00a	6.71ab	6.71ab	6.57b	6.57b	5.14c
General acceptability	6.57a	6.43a	6.14a	6.14a	5.86ab	5.00b

* Means in the same row followed by the same letters are not significantly different ($P<0.05$).
** Gari samples made from grated cassava mash using graters perforated with iron nails of different diameters as follows: G212 = 2.12 mm; G272 = 2.72 mm; G324 = 3.24 mm; G3.84 = 3.84 mm; G475 = 4.75 mm; BCL = blend G212.

Table 4. Mean* sensory scores for gari quality produced by different methods**.

Sensory quality	Mean scores of gari samples				
	TMF	NPC	MCM	NAM	SDM
Color	5.43b	6.00ab	6.00ab	2.00c	6.44a
Taste	5.00b	5.1b	6.43a	4.43b	5.00b
Aroma	5.14a	6.14a	6.60a	4.86b	5.33a
Particle size	5.57a	6.00a	6.00a	5.57a	6.00a
General acceptability	5.71c	6.29a	6.43a	4.43b	6.11a

* Means in the same row followed by the same letters are not significantly different ($P<0.05$).
** Gari samples prepared by different methods: TMF = Traditional method of fermentation; NPC = Fermentation in nonporous container; MCM = Fermentation with 80% moisture content; NAM = Fermentation with neutralizing agent; SDM = Fermentation for 24 h with sun drying of mash for 48 h.

Table 5. Mean* scores for gari made from mash inoculated with different starter cultures**.

Sensory quality	Mean scores of gari samples			
	NPC	L	B	C
Color	5.14b	7.57a	7.00a	7.29a
Taste	5.29a	7.00a	6.57a	6.86a
Aroma	5.00b	5.71ab	7.29a	7.14a
Particle size	6.43a	7.00a	7.29a	7.14a
General acceptability	5.43b	7.00a	6.86a	6.86a

* Means in the same row followed by the same letters are not significantly different ($P<0.05$).
** Gari samples made using *L. plantarum* (L), *Bacillus subtilis* (B) and *Candida krusei* (C) as single starter culture in fermenting cassava mash. NPC was made from cassava mash by chance inoculum in a non porous container.

Table 6. Mean* scores for gari from cassava mash inoculated with mixed cultures**.

Sensor quality	Mean scores of gari samples				
	NPC	BC	LB	LC	LBC
Color	6.00b	7.57a	7.00a	7.43a	7.29a
Taste	5.86a	6.86a	6.29a	6.71a	6.14a
Aroma	5.71a	6.00a	5.71a	5.86a	5.86a
Particle size	7.14a	7.14a	7.71a	7.57a	7.43
General acceptability	5.86b	7.14a	7.00	7.14a	7.14a

* Means in the same row followed by the same letters are not significantly different ($P<0.05$).
** Mixed cultures used in fermenting cassava mash in non porous container: BC = *B. subtilis* and *C. krusei*; LB = *L. plantarum* and *B. subtilis*; LC = *L. plantarum* and *C. krusei*; LBC = *L. plantarum*, *B. subtilis* and *C. krusei*; NPC = chance inoculum.

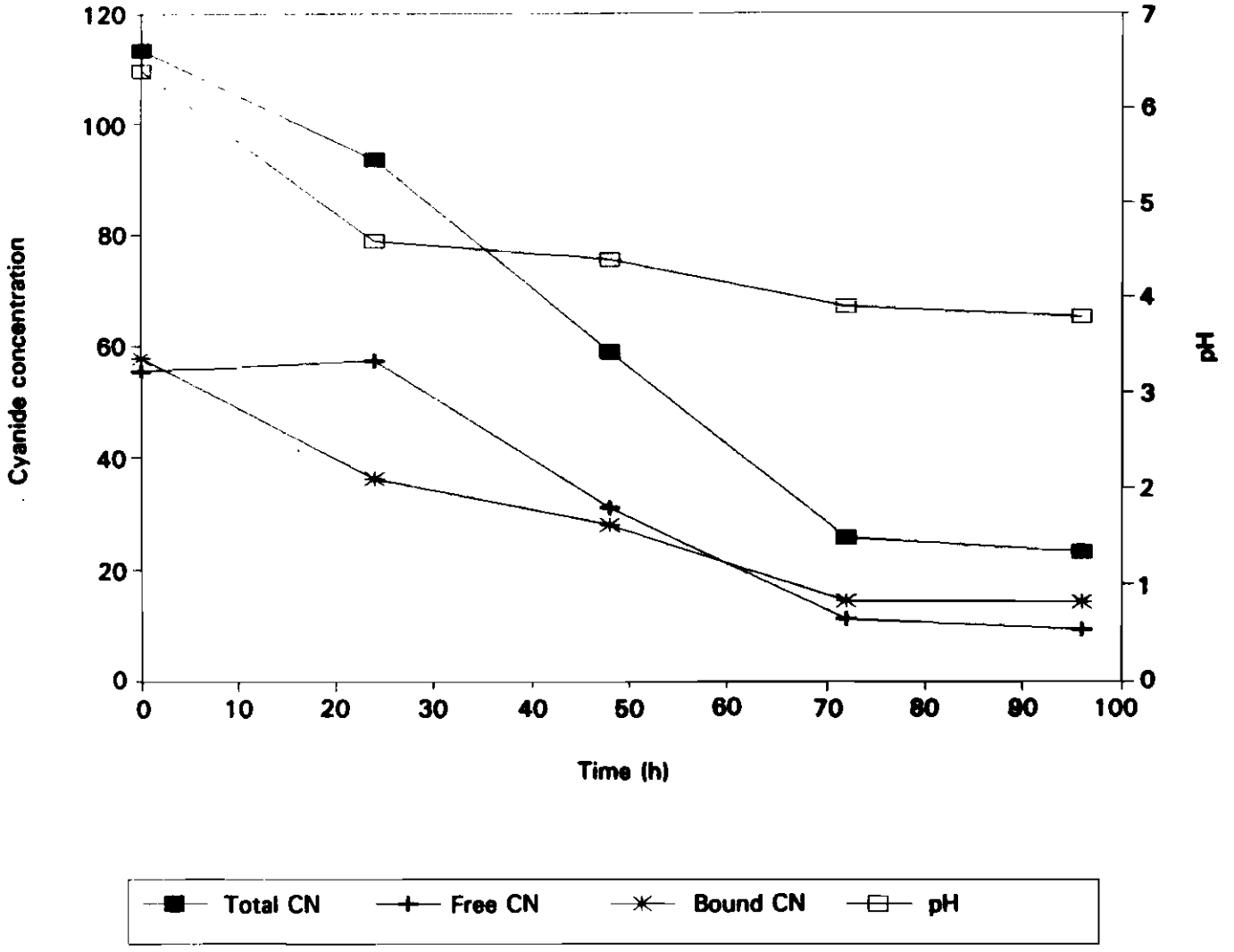

Figure 1. Cyanide and pH profile in cassava mash during fermentation in nonporous container.

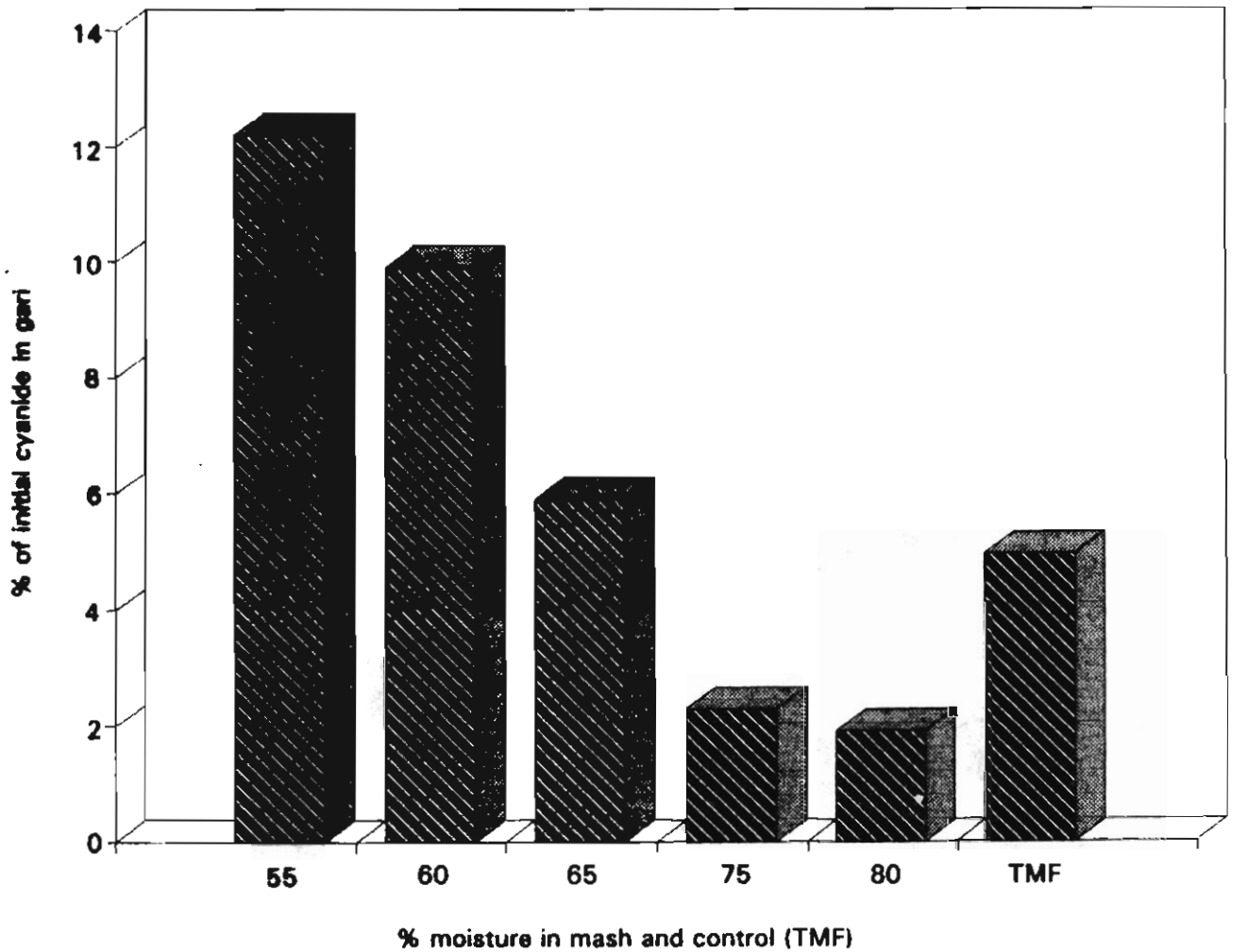

Figure 2. Effect of moisture content on residual cyanide in gari. TMF is gari produced by traditional method from mash with 65% moisture content.

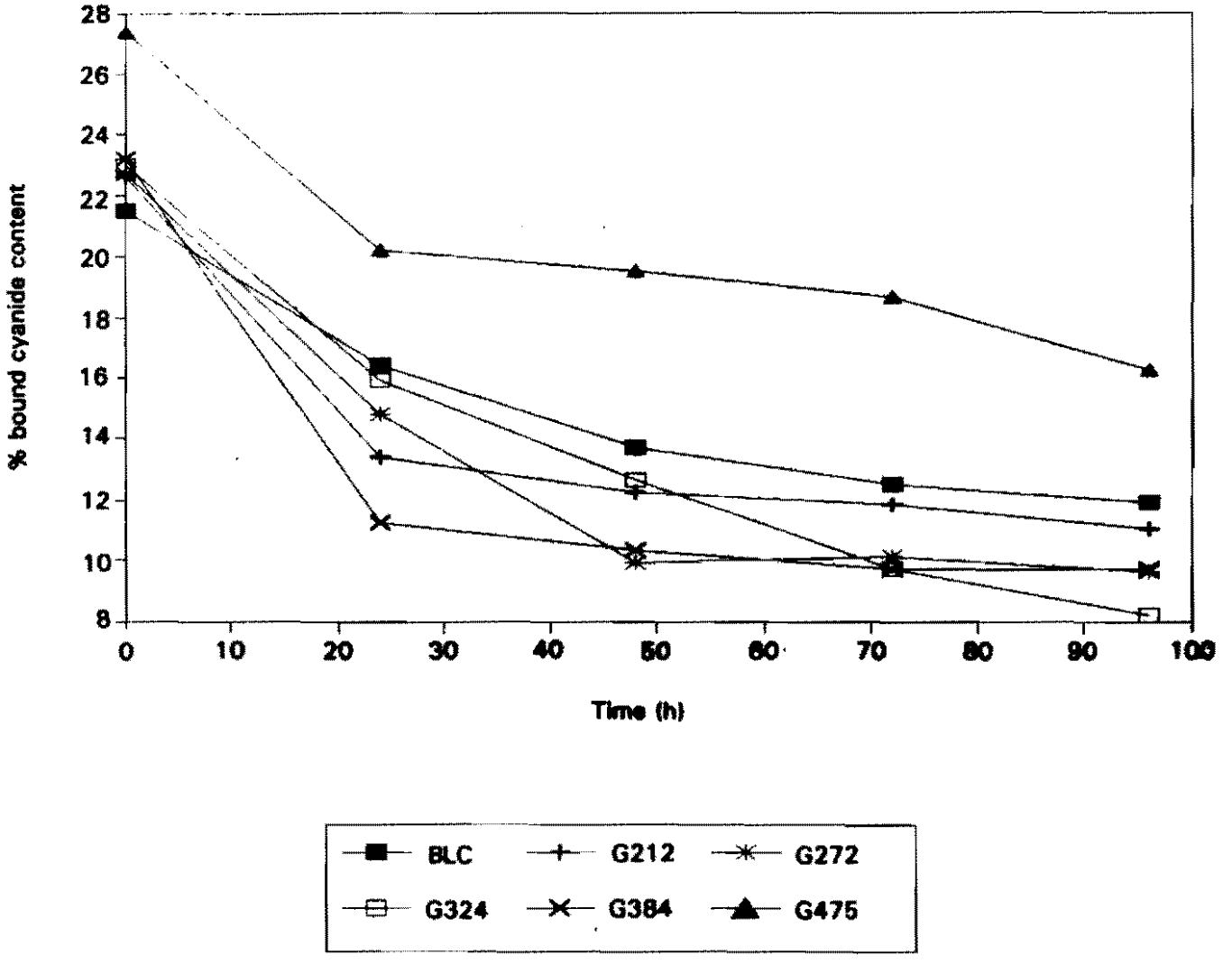

Figure 3. Bound cyanide profile in cassava mash produced with graters of different sizes. Coarseness increases in the order BCL < G212 < G272 < G324 < G384 < G475.

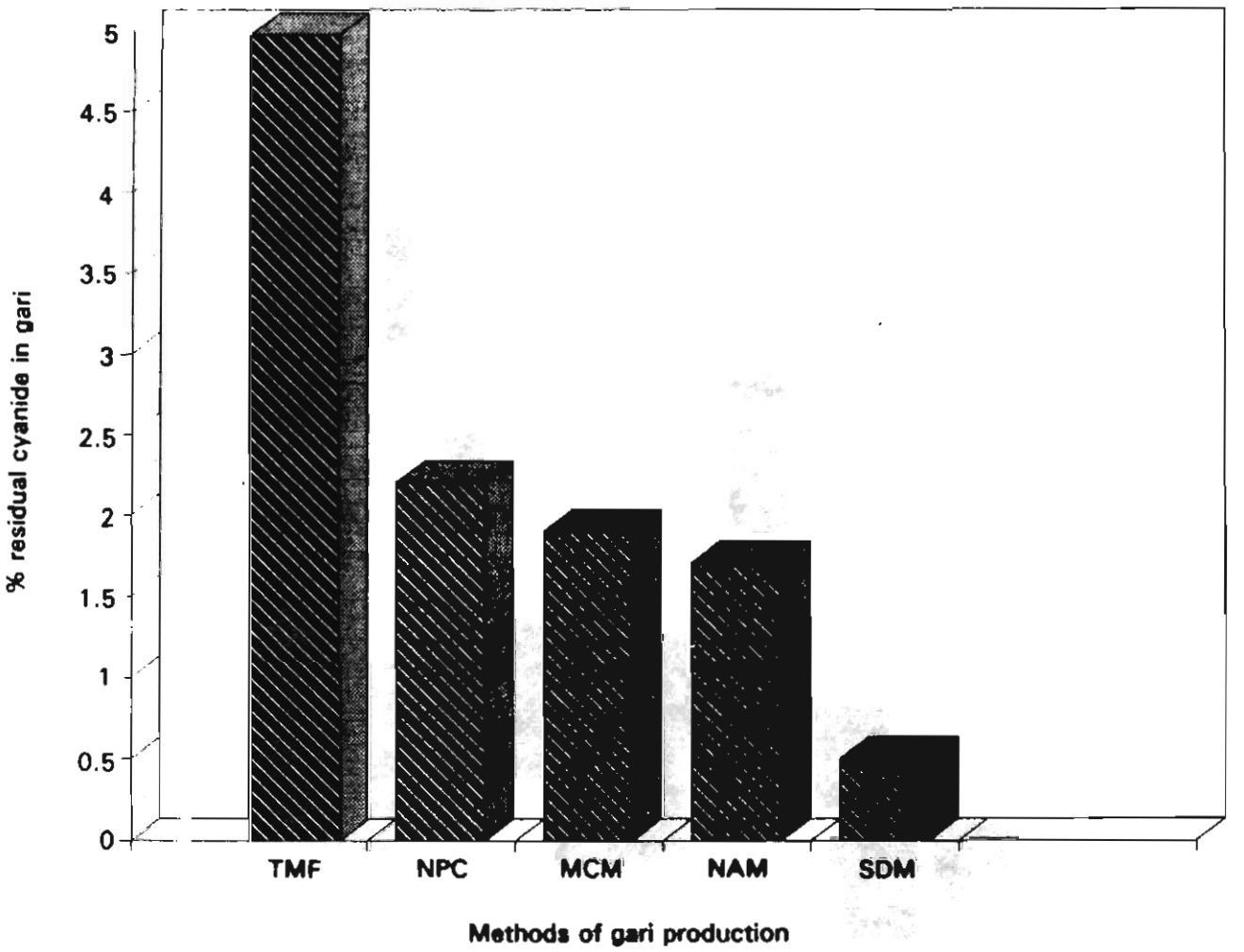

Figure 4. Effect of different methods of gari production on residual cyanide content: TMF gari by traditional method from mash in jute bag with 65% moisture content; NPC gari produced by TMF but in nonporous container; MCM gari produced by MCM from mash with 80% moisture; NAM gari produced by NPC with trona added to mash; SDM gari produced by MCM with sun drying.

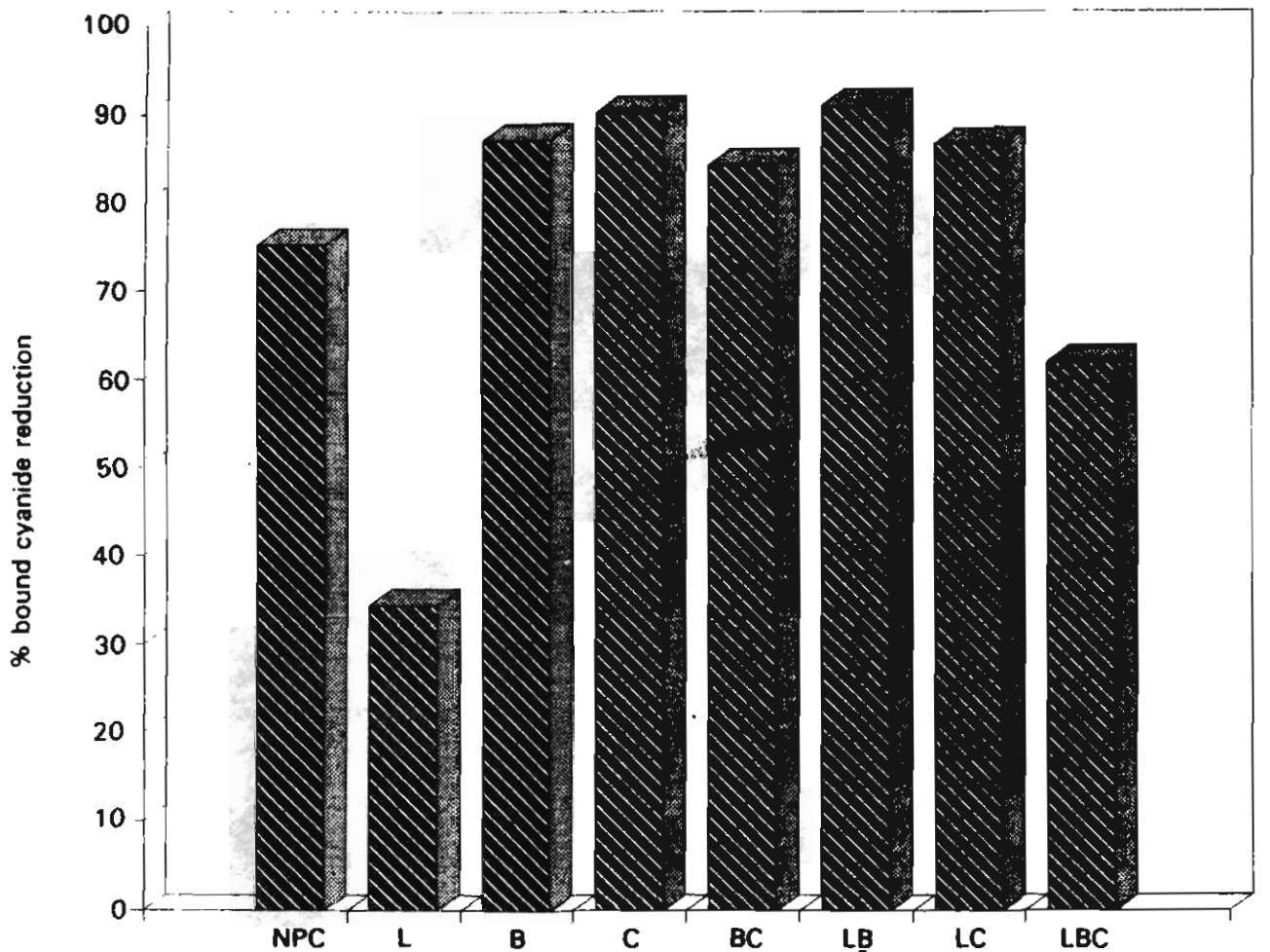

Figure 5. Effect of single and mixed starter cultures on bound cyanide reduction in cassava mash after 72 h of fermentation. *L. plantarum* (L), *B. subtilis* (B), *C. krusei* (C), NPC control with chance inoculum.

AN IMPROVED FERMENTATION PACKAGE FOR OPTIMAL DETOXIFICATION OF HIGH CYANIDE CASSAVA ROOTS AND PEEL

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The fermentation process previously devised using a mixed culture resulted in slightly higher retention of cyanide in fermented roots and peel from cassava cultivars with high cyanide content. The system was therefore improved upon by introducing steps like change of steep water during fermentation, prolonging fermentation for peel and sun drying the fermented products. The efficacy of fermentation with change of steep water at every 24 h (T2) and 48 h (T3) in eliminating cyanide from roots and peel of three high cyanide cultivars (initial total cyanide 332-432 mg/kg DM as HCN equivalent in roots and 1416-1537 mg/kg in peel) was compared with non-fermented samples (T1). Total cyanide (roots) was reduced to 27-41 (T2) and 32-42 mg/kg (T3) through fermentation (72 h) while retention in nonfermented roots was 226-295 mg/kg. Bound cyanide was hydrolyzed extensively during fermentation. Fermentation (120 h) reduced the total cyanide in peel to 190-198 (T2) and 171-218 mg/kg (T3) as against 395-596 mg/kg in nonfermented peel (T1). Fermentation followed by sun drying eliminated 90%-94% total cyanide from roots, of which 88%-92% was removed during fermentation itself. From 70% to 74% total cyanide was eliminated from non-fermented roots, of which 30%-36% only was removed during incubation and the rest during sun drying. Fermentation and sun drying removed 96%-97% total cyanide from peel of which 84%-88% was contributed by fermentation. In non-fermented peel, 89%-94% total cyanide was eliminated of which 60%-74% was through incubation and the rest through sun drying. A significant difference in cyanide removal was not obtained between T2 and T3, indicating that change of steep water at 48 h only (roots) and 48 h intervals (peel) followed by sun drying could effectively detoxify high cyanide cassava roots and peel and render them safe for food of feed use. The role of microorganisms in elaborating linamarase to help in the hydrolysis of linamarin during fermentation is discussed.

INTRODUCTION

Toxicity of cassava arises from the hydrolytic release of cyanide from the bound cyanogenic glucosides, linamarin and lotaustralin. Varietal variations exist in the content of the glucosides as well as the hydrolytic enzyme, cyanogenic beta-glucosidase (linamarase). Traditional processing techniques reduce the cyanogen levels in cassava but residual cyanide is usually present in many processed forms (Cooke and Maduagwu, 1978; Ketiku et al., 1978). Toxicity from processed cassava depends on the residual content of cyanohydrins and bound glucosides (Rosling, 1988).

Fermentation has been reported as one of the best ways of eliminating cyanogens from cassava (Ayernor, 1985; Mahungu et al., 1987; Bokanga et al., 1988; Padmaja et al., 1993). It is reported to improve the textural qualities of the fluor and impart a good favor to the products (Oyenuga, 1968; Oyewole and Odunfa, 1989). Several workers have reported the use of linamarase producing microorganism in detoxification of cassava roots (Ikediobi and Onyike, 1982a, 1982b; Ikediobi et al., 1985; Okafor and Ejiofor, 1986; Legras et al., 1990; Padmaja et al., 1993).

Cassava peel contains very high amounts of cyanogens as compared to the roots (Padmaja et al., 1993). Effective exploitation of peel as an animal feed depends on the efficiency of cyanogen removal. Fermentation has been suggested as a means of detoxifying the peel (Akinrele, 1964; Pido et al., 1979; Padmaja et al., 1993).

A problem often encountered during natural fermentation of cassava roots is the foul smell emanating from the fermentation vats. Studies conducted earlier at this laboratory showed that this difficulty could be overcome through the use of a mixed culture inoculum (George et al., 1991). The pattern of cyanogen breakdown during fermentation with the mixed culture inoculum had been studied using a low cyanide cultivar (M4) and a high cyanide cultivar (H 165) and the results reported (Padmaja et al., 1993). Although the process was effective in eliminating almost all the cyanogens from the low cyanide cultivar, there was a higher residual cyanide in the fermented roots of the high cyanide cultivar (H 165). The system was therefore improved by introducing steps like change of steep water during fermentation, prolonging fermentation for peel and sun drying the fermented products. We report the results of this modification study in this paper.

MATERIALS AND METHODS

Organisms, culture conditions and fermentation procedures

Preparation of the inoculum, enumeration of microflora and fermentation procedures were previously reported (George et al., 1991). The mixed culture inoculum source used in the study contained 7.5×10^5 ml^{-1} *Lactobacilli*, 3.5×10^5 ml^{-1} *Streptococci* and a bacterial count in nutrient agar comprising 6.5×10^5 ml^{-1} *Corynebacteria* and yeast cells 0.7×10^5 ml^{-1}.

Three high cyanide cassava cultivars, H 165, H 97 and T-300, were selected for the study and roots were obtained from plants of uniform maturity (10 months) grown under the same conditions at the Institute Farm. Cassava roots were peeled and cut into prismoid pieces. A total of 150 g of root was put into 500 ml beakers and then covered with 300 ml water. Two replications were maintained for each of three treatments. T1 served as the control in which fermentation was totally

arrested through the use of an antimicrobial combination (1.5 ml each of 1% thiomersal, penicillin and streptomycin sulfate). In the case of T2 and T3, 3.0 ml of mother liquor containing the mixed inoculum was added to each beaker and the beakers covered with muslin cloth and incubated at 30 ± 1 °C for 72 h (steep water was changed at 24 h and 48 h and replaced with fresh water in the case of T2, while steep water was changed only at 48 h in the case of T3).

Cassava peel (skin and rind) was chopped to Ca. 1-1.5 cm long pieces and 50 g samples were put in 150 ml water. The peel samples (T2 and T3) were inoculated with 1.0 ml mother liquor and allowed to ferment for 120 h. Steep water was changed and replaced with fresh water every 24 h for T2 and every 48 h for T3. T1 served as the control nonfermented peel, where fermentation was arrested as described earlier.

Analytical procedures

After incubation/fermentation for the respective periods, the steep liquor was decanted and the root and peel pieces were blotted. One part was used for the determination of the cyanogen content and dry matter content (initial and incubated) and another part was sun dried. Cyanogen content was also determined in the sun-dried and powdered roots and peel from the experiments. Duplicate samples were taken from each replication, and cyanogens (total cyanide, acetone cyanohydrin and free cyanide) were quantified by combining the methods of O'Brien et al. (1991) and Nambisan and Sundaresan (1984). In order to assess the contribution of fermentation in comparison to direct sun drying in eliminating cyanogens, a parallel set of chopped roots (all three cultivars) and peel was directly sun-dried, powdered and cyanogens quantified as above.

Steep liquor cyanide fractions were also quantified for the various treatments from root and peel experiments by the above method. Initial pH of the steep liquor (O h) as well as the final pH after incubation/fermentation were determined using an Elico pH meter with combined electrode.

RESULTS AND DISCUSSION

Table 1 shows the effect of change of steep water at 24 h and 48 h (for T2) and 48 h only (for T3) during 72 h fermentation time, on the residual content of cyanide in the fermented roots. The initial total cyanogen content in fresh cassava roots of the three high cyanide cultivars ranged from 331-432 mg HCN kg^{-1}DM, with a glucosidic cyanide content of 302-391 and a non-glucosidic cyanide content of 29-41 mg HCN kg^{-1} DM. After incubation for 72 h, the total cyanide was reduced to 226-294 mg kg^{-1}DM in the nonfermented set of roots (Table 1). In the case of the fermented roots, there was a highly significant reduction in the total and glucosidic cyanide for all three cultivars. There was no significant difference

between T2 and T3, indicating that change of steep water once at 48 h only during fermentation was sufficient to bring down the cyanide content.

The nonfermentation and fermented roots were sun dried after 72 h and Table 2 gives the residual cyanide in the dried flour. In the nonfermented roots, the total and glucosidic cyanide were reduced during the subsequent sun-drying stage (Tables 1 and 2). It was also found that there was no pH change during the 72 h incubation (Figure 1) indicating that microbial growth and fermentation could be totally arrested in the nonfermented control roots (T1). Because of this, it is likely that the linamarase in the nonfermented roots is active after 72 h incubation and during the sun drying, it helps in eliminating more cyanogens from the roots. In the case of fermented roots, the subsequent sun drying did not result in additional loss of cyanogens (Table 2). There was a substantial decreases in the pH (3.6-4.1) of the steep liquor from the fermented roots. It is known that linamarase exhibits optimal activity at a pH of 5.5-6.0 (Ikediobi and Onyike, 1982a) and thus at the acidic pH prevailing in the fermented roots, linamarase may be either suboptimally active or inactive with the result that during sun drying, additional loss of cyanogens is unlikely. It was reported from an earlier study that cyanogenic-glucosidase activity increase rapidly within 12 to 18 h of fermentation with the mixed culture inoculum (Padmaja et al., 1993) whereas in the nonfermented roots, increase in activity was observed only towards the latter stages of incubation.

The relative contribution of incubation/fermentation and sun drying towards total cyanide removal from cassava roots indicated that in the case of nonfermented roots, out of 70%-74% cyanogen removal, 30%-36% was contributed by incubation while 34%-42% removal occurred at the sun drying stage (Padmaja et al., 1994). But in the case of fermented roots (T2 and T3), fermentation was almost totally responsible for cyanogen removal and the contribution of sun drying was minimal. As compared to the plain sun-dried flour of all the three cultivars, the incubated and fermented roots retained much less cyanide (Padmaja et al., 1994). The beneficial role of fermentation on cyanogen removal was brought out from the studies of several workers (Bokanga et al., 1988; Ikediobi et al., 1985; Mahungu et al., 1987; Okafor and Ejiofor, 1986, 1990; Arihantana and Buckle, 1987).

Cassava peel contains much higher amounts of cyanide than the edible roots (Padmaja et al., 1993; Dufour, 1988; Akinrele, 1964). The three high cyanide cultivars used in the study had total cyanide in the range of 1416-1538 and glucosidic cyanide 1011-1273 mg HCN kg^{-1}DM (Table 3). Because of the high linamarase activity in the peel (Padmaja et al., 1993) considerable decrease in the total and glucosidic cyanide occurred in the incubated as well as fermented peel within 120 h. In comparison to the nonfermented peel, fermented peel evidently had much lower cyanide values (Table 3). Subsequent sun drying further helped in the loss of greater quantities of cyanogens form nonfermented as well as fermented peel (Table 4). Even after 120 h fermentation, the peel linamarase appears to be active at the low pH prevailing, which may have helped in additional loss of cyanide

during drying. It is also likely that due to the very high linamarase activity in fresh peel, some activity may be remaining even after 120 h. The initial pH of steep liquor from peel (Figure 1) was only 4.7-5.1 which was less than that from the roots. It is also possible that the peel linamarase has a pH optimum different from that of root linamarase. But this needs further confirmation through more studies.

The relative contribution of incubation/fermentation and sun drying towards cyanide removal from peel also makes it clear that, in contrast to the roots, sun drying of fermented peel has helped in an additional loss of 8%-12% cyanogens (Padmaja et al., 1994). It was found that fermentation with the mixed culture inoculum could remove as high as 97%-98% cyanogens from peel. Change of steep water at 48 h intervals (two changes of water during 120 h) was only necessary to detoxify the peel to safe levels.

Quantification of cyanide fractions in the steep liquor from root and peel experiments indicated that fermentation had helped in creasing the acetone cyanohydrin and free cyanide levels of steep liquor (Figures 2 and 3). By replacing the steep water at 48 h (for roots) and at 48 h intervals (for peel), we could shunt away from the steep liquor the acetone cyanohydrin and free cyanide that were likely to exert a feedback inhibition on linamarase, thus pulling the equilibrium in favor of linamarin hydrolysis. Replacing the acidic steep liquor with fresh water also helped to bring back the pH to around 5.5-6.0 which is conducive to the further autolytic breakdown of acetone cyanohydrins.

The study showed that replacing the steep water at 48 h (for roots) and 48 h intervals (for peel) during fermentation was necessary to optimally detoxify the high cyanide roots and peel. Figure 4 shows a schematic of the improved fermentation process for high cyanide roots. The scheme is similar for high cyanide peel except that a peel:steep liquor ratio of 1:3 has to be maintained and steep liquor should be replaced with fresh water at 48 h and 96 h of fermentation.

CONCLUSION

The improved fermentation package suggested in the study was found to be highly effective in detoxifying high cyanide cassava roots and peel, thus making them safe for human consumption or animal feed.

It was also found that there was no significant difference in cyanogen elimination when steep water was replaced at 24 h or 48 h intervals indicating that only water change at 48 h (for roots) and 48 h intervals (for peel) was needed. This could also help in economizing in the process operation.

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Table 1. Effect of change of steep water during fermentation on the residual cyanide content of fermented cassava roots.

Cassava cultivars/ Cyanide fractions		Initial cyanide (0 h)	Residual cyanide content[a] (mg HCN kg^{-1} DM)		
			Non-fermented control (T_1)[b]	T_2[b]	T_3[b]
H165	Total	432.89	294.74	32.08	32.10
		(24.23)	(59.80)	(7.45)	(9.50)
	Glucosidic	391.17	224.20	18.06	20.39
		(29.42)	(72.32)	(4.13)	(6.26)
	Non-glucosidic	41.72	70.54	14.02	11.71
		(3.43)	(8.67)	(4.53)	(5.07)
H97	Total	331.93	230.48	27.28	33.77
		(64.07)	(45.45)	(8.06)	(1.26)
	Glucosidic	302.50	178.97	16.28	12.86
		(81.15)	(60.63)	(8.81)	(6.11)
	Non-glucosidic	29.42	51.51	11.00	20.91
		(6.69)	(2.57)	(1.83)	(5.59)
T300	Total	358.73	226.91	41.83	41.90
		(68.91)	(58.23)	(9.40)	(8.10)
	Glucosidic	321.30	154.64	16.30	5.64
		(89.50)	(69.69)	(7.89)	(0.40)
	Non-glucosidic	37.42	72.27	25.53	36.26
		(5.62)	(8.95)	(3.82)	(8.38)

a. Residual cyanide in the roots immediately after incubation/fermentation.

b. T_1 = Incubated (72 h) with anti-microbials; T_2 = Fermented (72 h) with water change at 24 h and 48 h; T_3 = Fermented (72 h) with water change at 48 h only.

Figures in parentheses indicate standard deviation (observations on four samples).

Table 2. Effect of subsequent sun drying of fermented cassava roots on the residual cyanide content.

Cassava cultivars	Cyanide fractions	Residual cyanide content[a] (mg HCN kg^{-1} DM)		
		T_1[b]	T_2[b]	T_3[b]
H 165	Total	111.68	27.36	30.80
		(9.50)	(8.15)	(4.07)
	Glucosidic	32.00	9.12	14.70
		(8.95)	(2.04)	(5.56)
	Non-glucosidic	79.68	18.24	16.10
		(3.17)	(3.17)	(0.14)
H 97	Total	97.92	27.20	32.04
		(1.81)	(0.45)	(2.72)
	Glucosidic	65.28	12.48	15.40
		(1.29)	(0.65)	(2.56)
	Non-glucosidic	32.64	14.72	16.64
		(2.72)	(0.91)	(0.91)
T 300	Total	102.72	35.52	37.12
		(6.79)	(0.45)	(4.53)
	Glucosidic	49.60	20.16	22.40
		(1.92)	(1.92)	(3.85)
	Non-glucosidic	53.12	15.36	14.72
		(5.43)	(1.81)	(1.81)

a. Residual cyanide in the roots immediately after incubation/fermentation.

b. T_1 = Incubated (72 h) with anti-microbials; T_2 = Fermented (72 h) with water change at 24 h and 48 h; T_3 = Fermented (72 h) with water change at 48 h only.

Figures in parentheses indicate standard deviation (observations on four samples).

Table 3. Effect of change of steep water during fermentation on the residual cyanide content of fermented cassava peel.

Cassava cultivars/ Cyanide fractions		Initial cyanide (0 h)	Residual cyanide content[a] (mg HCN kg^{-1} DM)		
			Non-fermented control (T_1)[b]	T_2[b]	T_3[b]
H165	Total	1416.40	498.08	198.62	218.13
		(61.10)	(96.93)	(19.25)	(10.73)
	Glucosidic	1065.65	65.18	56.27	90.78
		(16.67)	(40.64)	(17.52)	(6.60)
	Non-glucosidic	350.75	432.90	140.35	127.35
		(72.89)	(68.19)	(6.86)	(15.40)
H97	Total	1537.98	395.21	194.91	203.63
		(211.51)	(94.08)	(20.50)	(20.72)
	Glucosidic	1272.91	41.06	85.93	84.18
		(229.67)	(5.02)	(18.92)	(1.70)
	Non-glucosidic	265.07	354.15	108.98	119.45
		(49.15)	(90.53)	(7.16)	(19.52)
T300	Total	1506.47	596.62	190.31	171.15
		(157.84)	(111.10)	(6.31)	(21.06)
	Glucosidic	1011.73	90.71	87.49	50.67
		(137.83)	(45.75)	(9.33)	(24.88)
	Non-glucosidic	494.74	505.91	102.82	120.48
		(60.38)	(78.75)	(12.97)	(3.47)

a. Residual cyanide in peel immediately after incubation/fermentation.

b. T_1 = Incubated (120 h) with anti-microbials; T_2 = Fermented (120 h) with water change at every 24 h; T_3 = Fermented (120 h) with water change at every 48 h.

Figures in parentheses indicate standard deviation (observations on four samples).

Table 4. Effect of subsequent sun drying of fermented cassava peel on the residual cyanide content.

Cassava cultivars	Cyanide fractions	Residual cyanide content[a] (mg HCN kg^{-1} DM)		
		T_1[b]	T_2[b]	T_3[b]
H 165	Total	141.84	35.64	48.60
		(23.60)	(0.51)	(2.55)
	Glucosidic	62.28	24.81	33.12
		(18.26)	(0.66)	(1.44)
	Non-glucosidic	79.56	10.83	15.48
		(10.69)	(0.04)	(1.53)
H 97	Total	97.92	52.20	42.84
		(10.55)	(3.56)	(1.53)
	Glucosidic	46.44	38.16	28.80
		(8.44)	(4.31)	(1.44)
	Non-glucosidic	51.48	14.04	14.04
		(4.58)	(0.51)	(0.51)
T 300	Total	84.60	43.56	38.52
		(2.55)	(1.53)	(4.48)
	Glucosidic	30.96	27.36	26.28
		(1.44)	(1.44)	(3.59)
	Non-glucosidic	53.64	16.20	12.24
		(1.53)	(2.55)	(2.04)

a. Residual cyanide in peel immediately after incubation/fermentation.

b. T_1 = Incubated (120 h) with anti-microbials; T_2 = Fermented (120 h) with water change at every 24 h; T_3 = Fermented (120 h) with water change at every 48 h.

Figures in parentheses indicate standard deviation (observations on four samples).

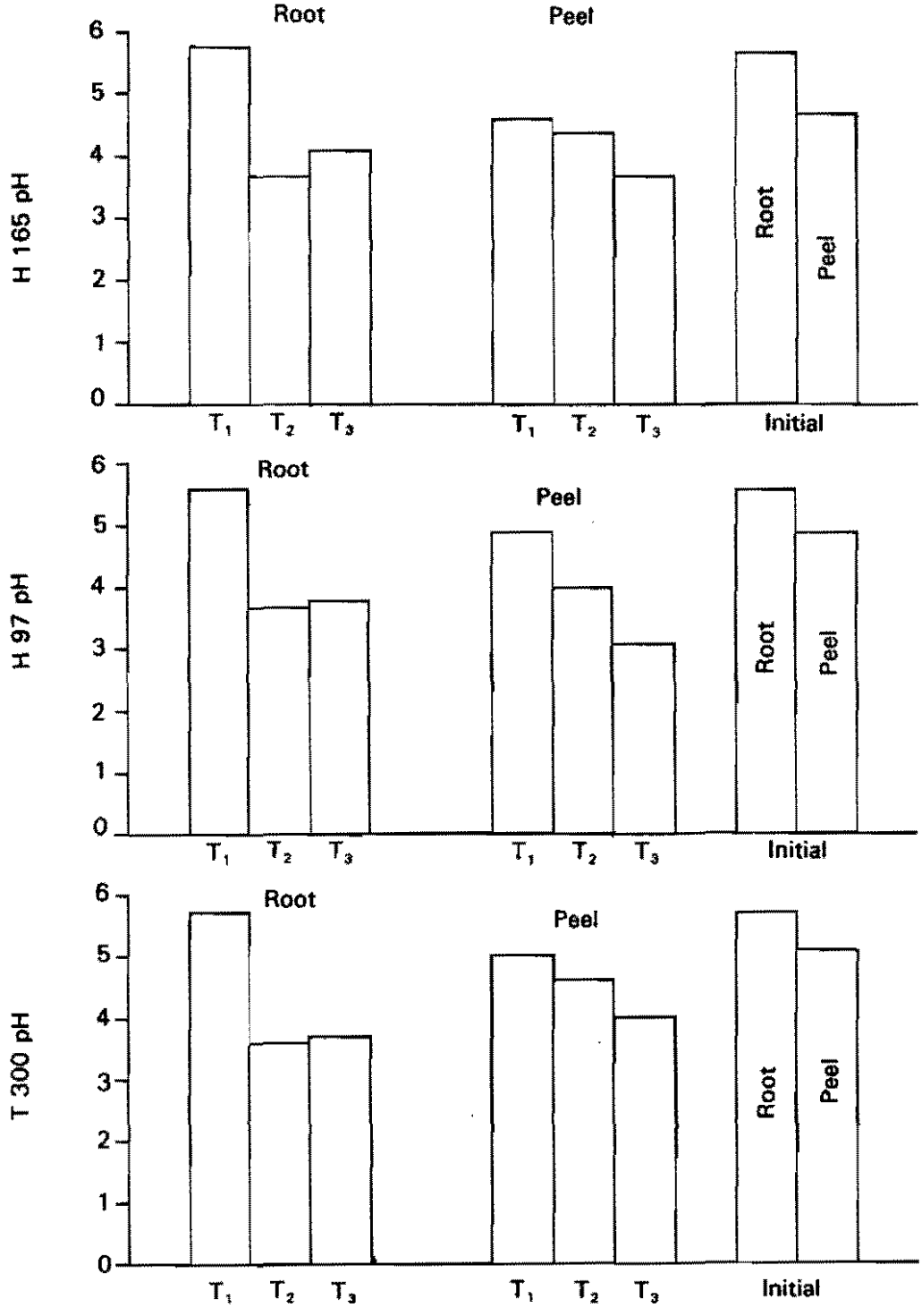

Figure 1. pH of steep liquor from root (after 72 h) and peel (after 120 h) experiments.

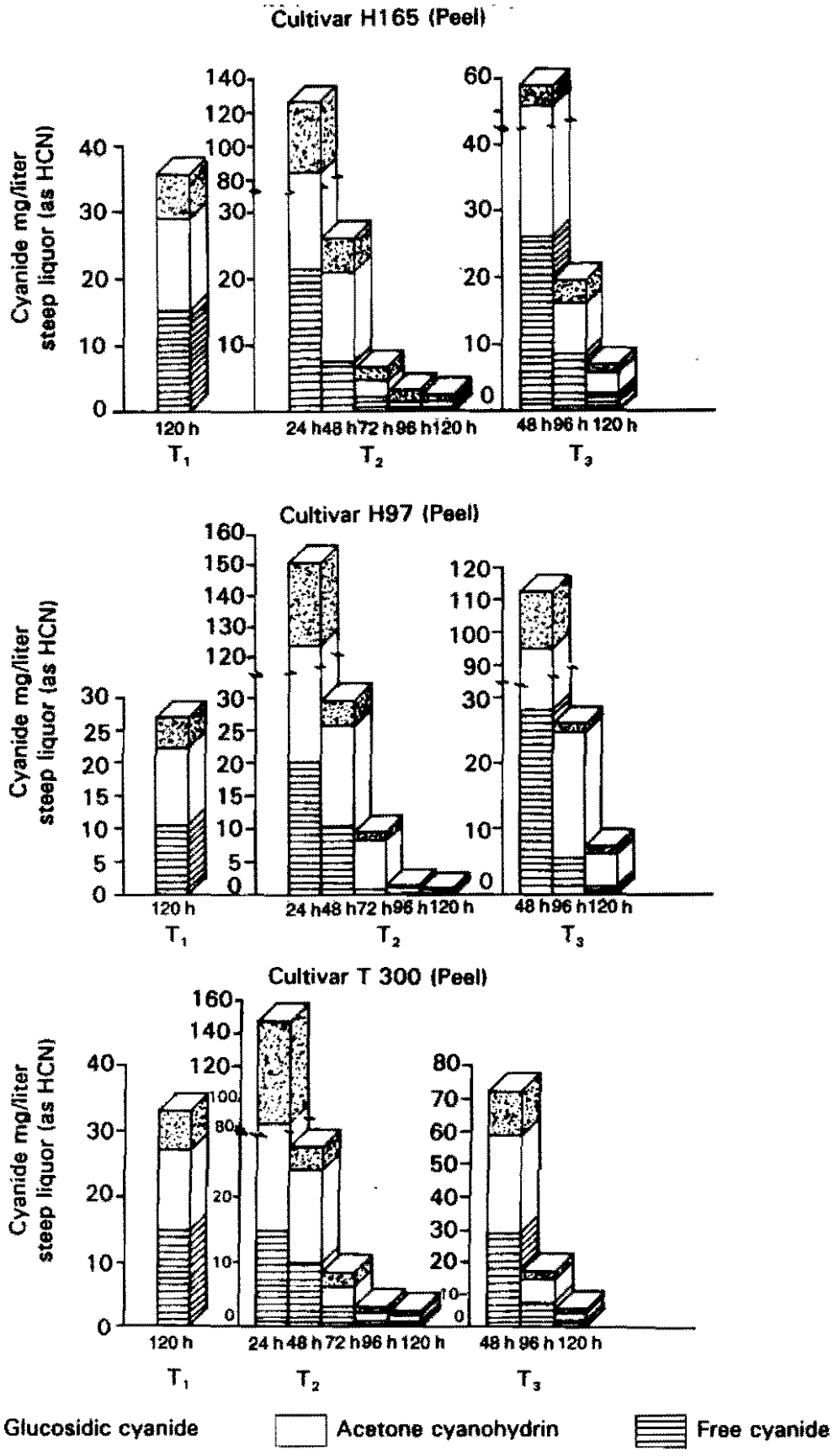

Figure 2. Steep liquor cyanide fractions from fermented and non-fermented cassava roots: T_1 = fermented; T2 = fermented with 24 h change of water; T3 = fermented with water change at 48 h only.

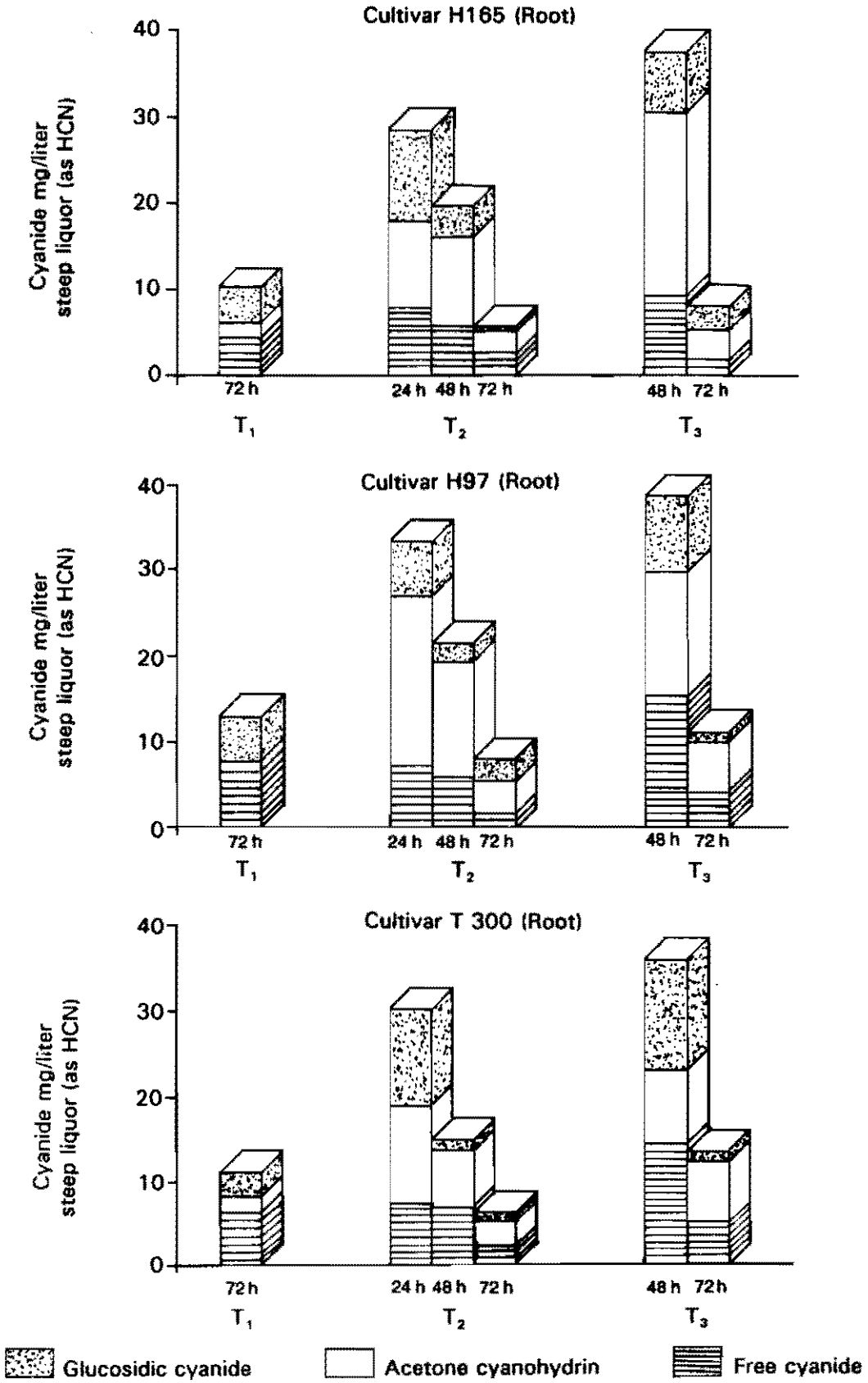

Figure 3. Steep liquor cyanide fractions from fermented and non fermented cassava roots: T_1 = non-fermented; T2 = fermented with 24 h change of water; T3 = fermented with water change at 48 h only.

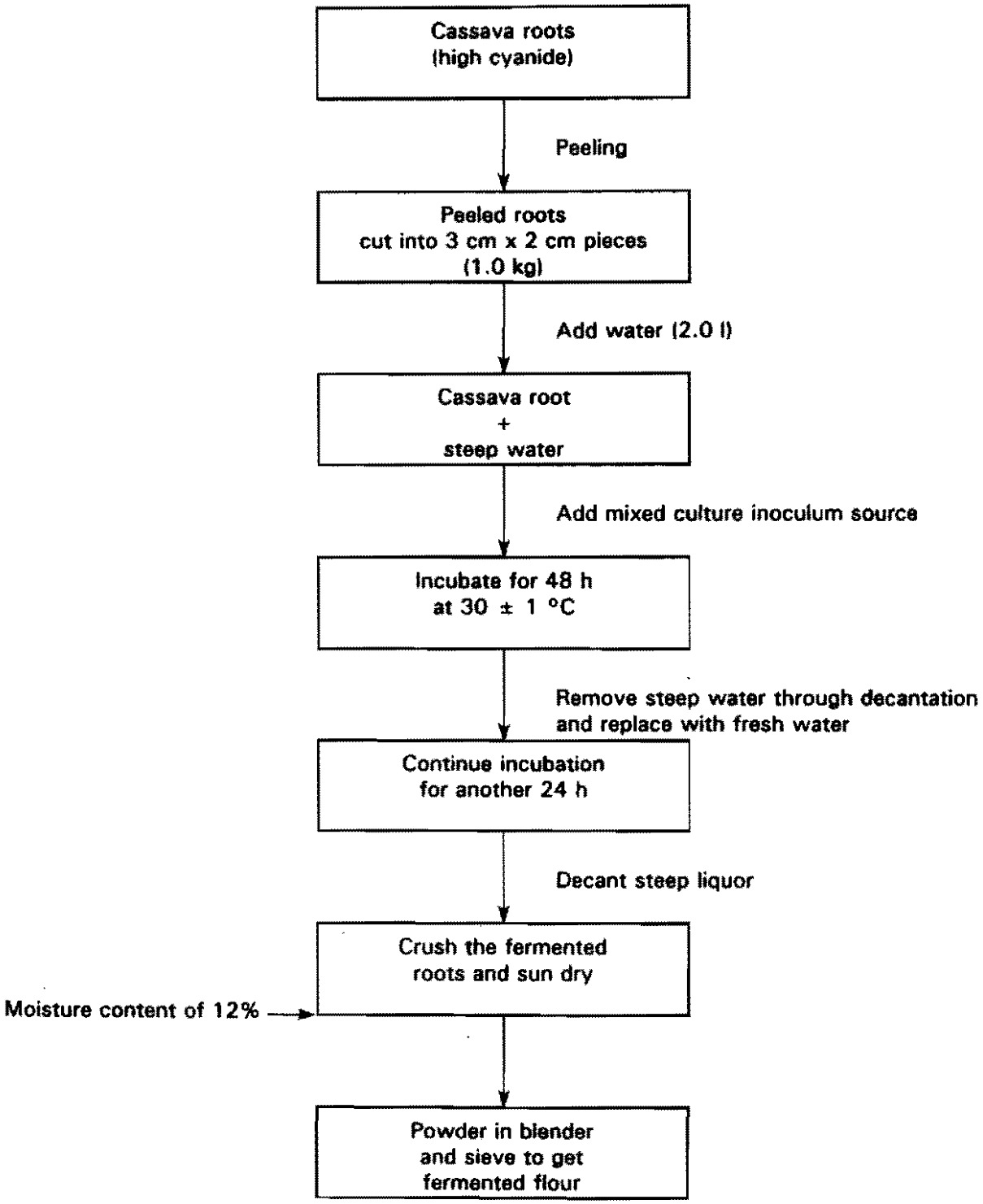

Figure 4. Schematic of improved fermentation process for high cyanide cassava roots.

ENSILING AS AN INNOVATIVE BIOTECHNOLOGICAL APPROACH FOR CONSERVATION OF HIGH CYANIDE CASSAVA TUBERS FOR FEED USE

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A major disadvantage in ensiling cassava roots is the production of large quantities of effluent which ultimately reduces the nutrient quality and shelf life of silage. A technique was perfected to obtain stable quality silages from whole cassava roots through the use of effluent absorbents like rice straw and cassava starch factory waste (CSFW). The most feasible combinations to obtain silages with good shelf life were cassava roots:rice straw (90:10) and cassava roots:CSFW (80:20). There was a rapid fall in pH within 2 days of ensiling itself resulting from a high lactic acid production. After 72 days of ensiling, the pH values ranged from 3.6-4.0 units and the lactic acid levels were 4.8-11.4 g 100 g^{-1} DM. Amino acid profiles of the modified silages indicated that there was good conservation of amino acids during ensiling. The mode of cyanoglucoside hydrolysis during ensiling was studied using a high cyanide cultivar, H 165. Although the total cyanide reduced from 450 to 368 mg HCN kg^{-1} DM only during a siling period of 33 days, there was almost 50% reduction in the bound glucoside. The acid pH prevailing in the silages retarded the hydrolysis of acetone cyanohydrin to free cyanide. Initial cyanide load entering the silo could be reduced by exposing the roots at ambient temperature or to sunlight for 4 h. The retention of bound cyanide in such treated silages was lesser than in the untreated silages. Further detoxification could be done through either boiling the silage for 10 min in water or sun-drying the silage.

INTRODUCTION

Ensiling has been known since very early times as a means of preserving high moisture fodder crops (Woolford, 1985). But the microbiological and chemical changes taking place in silages were understood only about 40 years ago (Kempton and San Clemente, 1959; Langston et al., 1958). The natural ensiling process goes through several stages, the first being the plant enzymes' use of the trapped oxygen inside the silo. The creation of anaerobic atmosphere facilitates multiplication of lactic acid flora using the available sugars. This leads to the production of lactic acid and a consequent drop in pH of the silage.

As compared to the vast amount of literature on ensiling of green fodder crops, reports on silage fermentation of cassava are few (Covarrubias et al., 1989; Limon, 1991; Padmaja and Balagopalan, 1991; Saucedo et al., 1990a, 1990b). Extensive studies have been conducted on the use of cassava as animal feed, yet the rapid perishable nature of the roots is limiting. On-farm use at the production

site itself is an alternate possibility but depends on year-round availability of roots. With a view that ensiling may provide a useful means of preserving the nutritive value of cassava roots and allow a constant supply of feeding material, work was begun on the use of cassava roots for ensiling. Earlier studies conducted in this laboratory indicated that the large quantity of effluent produced during ensiling of cassava roots alone, led to a watery and soft silage with poor shelf life (Padmaja and Balagopalan, 1991). The present study was undertaken with three aims: to study the biochemical changes taking place in modified whole root cassava silages; to study the mode of cyanogen breakdown during ensiling; and to evaluate techniques to reduce cyanide retention in cassava silages.

MATERIALS AND METHODS

Experiment 1: Biochemical changes in modified whole root cassava silages

Preparation of silage

Freshly harvested roots of cultivar H1687 from the Institute farm were washed free of dirt and chopped to 3- to 4-cm length along with rind in a Malaysian chipping machine. Dehydrated rice straw (RS) was cut to the same length and mixed in varying proportions of root:RS (70:30, 80:20 and 90:10) and filled immediately into polypropylene bottle silos. In another set of silos, cassava starch factory waste (CSFW) was mixed, in the same proportions as above with roots. Two replications were maintained for each treatment and for the sampling frequencies of 2, 7, 15, 21, 33, 49 and 72 days. Sufficient consolidation and external sealing were ensured in all the silos. Initial samples were collected at the time of filling the silos and subsequent samples were collected at each interval by taking out the silage, mixing well by hand and collecting two representative samples from each replicate.

From the appearance as well as the keeping quality of the silages studied over a period of six months, it was concluded that 90:10 (cassava roots:RS) and 80:20 (cassava roots:CSFW) were the most feasible combinations for obtaining silages with good shelf life. So only the data for these treatments are reported under this experiment.

Biochemical analysis

To determine pH, a 10 g sample from each treatment was put in 100 ml distilled water, stirred occasionally for 10 min, and the pH taken in an Elico pH meter using a combination electrode.

Lactic acid content of the silage was determined by the method of Barker and Summerson (1941). Amino acid profile of the silages (initial vs 72 days' ensiled) was studied at the Natural Resource Institute, Chatham, UK, using a Biotronik LC 5000 amino acid analyzer (Spackman et al., 1958; Spindler et al., 1984).

Experiment 2: Mode of cyanogen breakdown during ensiling

The pattern of hydrolysis of cyanogens during ensiling was studied using a high cyanide cultivar, H165, for the most stable silage combinations—cassava roots:RS (90:10) and cassava roots:CSFW (80:20). The silos were prepared as in Experiment 1, using chopped whole roots of H165. Since the silage stabilizes and conspicuous biochemical changes occur during the first stages of ensiling, studies on cyanogen changes during ensiling were restricted to 33 days of ensiling. The total and non-glucosidic cyanide contents of fresh and of ensiled cassava were estimated by combining the methods of O'Brien et al. (1991) and Nambisan and Sundaresan (1984). Dry matter content of the silages was determined by the oven-drying method.

Experiment 3: Evaluation of techniques to reduce cyanide retention in silages

The effect of certain physical pretreatments like exposing the silage mixes to ambient temperature (30 ± 1 °C) for 2 and 4 h or to sunlight similarly, then using it for filling the silos, was studied to find out the cyanide retention in silages. Control silos were made with fresh silage mixes. After ensiling for 33 days, the silos were opened and the silage mixed thoroughly by hand. One lot of the silage mix, i.e., cassava (cultivar H1687):rice straw, was directly sun-dried while a second lot was boiled in water for 10 min and then cyanide determined in the boiled silage. Total cyanide and non-glucosidic cyanide were determined in the initial silage mix as well as the 33 days' ensiled cassava, dried cassava mix and boiled cassava mix by the methods of O'Brien et al. (1991) and Nambisan and Sundaresan (1984).

RESULTS AND DISCUSSION

Experiment 1: Biochemical changes in modified whole root cassava silages

pH changes

A decrease in pH was observed within 2 days of ensiling cassava:RS (90:10) and cassava:CSFW (80:20) silages. This was followed by a gradual decrease in pH up to 21 days, after which the decrease was not so pronounced (Figure 1). A similar decrease in pH was also observed when cassava roots were ensiled with molasses and/or urea (Padmaja and Balagopalan, 1991). Saucedo et al. (1990b)

reported a decrease in pH to 4.0 in silages made with dry cassava chips inoculated with a *Lactobacillus* sp. Covarrubia et al. (1989) reported a reduction in pH from 6.32 to 4.15 units after 42 days of ensiling cassava roots with swine waste. The rapid decrease in pH obtained in the study tended to stabilize the silage and prevent multiplication of putrefying organisms.

Lactic acid changes

The initial lactic acid content of 0.25-0.35 g 100^{-1} DM in the silages increased to 2.2-3.1 g 100^{-1} within 2 days of ensiling (Figure 2). There was a continuous build-up of lactic acid in the cassava:CSFW (80:20) silages during the 72 days ensiling period (Figure 2). In the case of cassava:RS silages, a slight fall in the lactic acid concentration occurred after 15 days which remained almost at the same level till 49 days and then increased further in the 72 day sample. Unlike in the silages made with dry cassava chips (Saucedo et al., 1990b), the lactic acid production was quite high in the wet silages in our study. This may be due to the effective diffusion of lactic acid in the wet, fermenting mass preventing its local accumulation. Whittenbury et al. (1976) reported that growth of homolactic flora during ensiling resulted in very high levels of lactic acid in silages. Covarrubias et al. (1989) also reported higher lactic acid levels in cassava root meal ensiled with swine waste.

Amino acid profile in modified silages

Amino acid profiles studied for the initial and 72 days' ensiled samples of the two silage combinations indicate that there was good conservation of most of the amino acids during ensiling (Table 1). In the case of cassava:RS silages, only Arginine was considerably reduced as a result of ensiling (3.48 in the initial vs 1.95 in the ensiled). This may be due to microflora utilizing arginine more. Literature on the amino acid profiles of ensiled cassava is lacking. Decrease in some of the amino acids like valine, isoleucine, leucine and arginine was noticed in the ensiled cassava:CSFW silages.

Experiment 2: Mode of cyanogen break down during ensiling

The pattern of change in total cyanide reduction during ensiling studied using a high cyanide cultivar H165, indicated that about 18%-19% decrease in total cyanide occurred within 33 days of ensiling (Table 2). Retention of acetone cyanohydrin and free cyanide was found to be greater in the silages. Under the acid pH prevailing in the silages, the autolytic breakdown of acetone cyanohydrin is prevented (Fomunyam et al., 1985). It is also likely that linamarase is only suboptimally active under the acid pH. After 33 days of ensiling, about 24% of bound glucosides was retained in the cassava:RS silage and 37% in cassava:CSFW. There are few reports on the cyanogen changes taking place during ensiling.

Gómez and Valdivieso (1988) observed that during ensiling whole root chips, cyanide content was reduced to 25%-36% of the value after an ensiling period of 26 weeks. Tewe (1991) reported losses of up to 98% in free cyanide during ensiling of cassava roots with poultry litter for 8 weeks. Our study showed that free cyanide was retained to a greater extent, when cassava roots were ensiled with silage effluent absorbents like RS or CSFW. It is possible that the highly soluble acetone cyanohydrins and free cyanide can leach out into the effluents during ensiling. In an earlier study (Padmaja and Balagopalan, 1991), we reported that total cyanide was not detected in molasses-treated, whole root cassava silages. However the effluent production was high in that silage and the finished silage had soft and watery consistency.

Experiment 3: Evaluation of techniques to reduce cyanide retention in silages

Table 3 presents the effect of exposure of mixed silages to ambient temperature and sunlight on the cyanide retention in silages. It was found that both total and glucosidic cyanide were considerably reduced in the silage mixes on exposure to ambient temperature (30 ± 1 °C) or sunlight for 4 h. When such exposed mixes were used for making silages (33 days ensiling), the retention of bound and non-glucosidic cyanide fractions was much less than in the unexposed silages.

It was found that the residual cyanide in silage could be further reduced by boiling the silages or by sun-drying them (Table 3). Both glucosidic cyanide and acetone cyanohydrins were substantially low in the boiled or sun-dried silages.

CONCLUSIONS

The study showed that stable quality silages can be made from whole root cassava chips using silage effluent absorbents like RS and CSFW. The initial cyanide load entering the silo could be reduced by exposing the roots to ambient temperature or sunlight for 4 h. Further reduction in cyanide in composite silages was possible by boiling the silages or sun-drying them.

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Table 1. Amino acid[a] profile in modified silages (cassava cultivar H 1687).

Amino acids	Cassava:rice straw silage		Cassava:CSFW silage	
	Initial (0 h)	Ensiled (72 days)	Initial (0 h)	Ensiled (72 days)
Aspartic acid	3.45	3.51	4.69	4.06
Threonine	1.74	1.77	2.31	2.00
Serine	1.77	1.55	2.46	2.03
Glutamic acid	9.63	10.21	13.97	12.47
Glycine	2.40	2.65	2.99	2.56
Alanine	3.04	3.48	3.93	3.77
Valine	2.47	2.72	3.42	2.57
Isoleucine	1.64	1.58	2.31	1.81
Leusine	2.79	2.57	3.78	2.47
Tyrosine	1.59	1.76	2.25	1.50
Phenyl alanine	1.83	1.50	2.29	1.83
Histidine	4.72	4.80	6.42	5.06
Arginine	3.48	1.95	5.49	2.35
Proline	1.60	1.60	1.60	1.60
Total lysine	1.55	1.76	2.98	2.25
Cystine	1.23	1.41	1.53	1.14
Methionine	0.73	0.70	0.98	0.66

a. Expressed as g 16 g^{-1} Nitrogen.

Table 2. Cyanogen removal during ensiling of whole root cassava silage mixes (cassava cultivar H 165).

Silage combination	Days of ensiling	Cyanide fractions		
		Total cyanide	Glucosidic cyanide	Non-glucosidic cyanide
	0	450.73	238.83	211.90
	2	437.98	108.13	329.85
	7	375.15	42.13	333.02
Cassava:RS (90:10)	15	348.87	52.99	295.88
	21	373.85	51.25	322.60
	33	367.71	47.87	319.84
	0	356.79	205.56	151.23
	2	309.97	76.94	233.03
Cassava:CSFW (80:20)	7	303.65	87.74	215.91
	15	310.28	90.98	219.43
	21	255.77	85.58	170.19
	33	286.47	66.04	220.43

Table 3. Effect of exposure to ambient temperature/sunlight and boiling/sun-drying on the residual cyanide in modified silage[a] (cassava cultivar H 1687).

Cyanide fractions[b]	Treatments[c]			
	Initial	Ensiled (33 days)	Boiled (after ensiling)	Sun-dried (after ensiling)
		T^1		
Total	234.10	220.56	56.00	65.46
Glucosidic	144.92	65.57	10.09	21.10
Non-glucosidic	89.18	155.00	45.91	44.36
		T^2		
Total	228.00	210.60	62.40	72.50
Glucosidic	155.20	53.29	9.56	17.60
Non-glucosidic	72.80	157.31	52.80	55.00
		T^3		
Total	144.00	132.60	43.40	48.40
Glucosidic	105.00	24.50	8.10	15.90
Non-glucosidic	38.90	108.10	35.40	32.00
		T^4		
Total	215.80	206.10	62.30	73.20
Glucosidic	159.20	39.70	19.50	30.40
Non-glucosidic	56.70	166.40	42.80	42.80
		T^5		
Total	161.30	156.00	75.90	85.50
Glucosidic	102.20	50.90	13.60	23.50
Non-glucosidic	59.10	105.10	62.30	62.00

a. Cassava root:rice straw (90:10).

b. mg HCN kg^{-1} DM.

c. T_1 = immediately filled to silos; T_2 and T_3 = exposed to ambient for 2 and 4 h, respectively. T_4 and T_5 = exposed to sun light for 2 and 4 h, respectively.

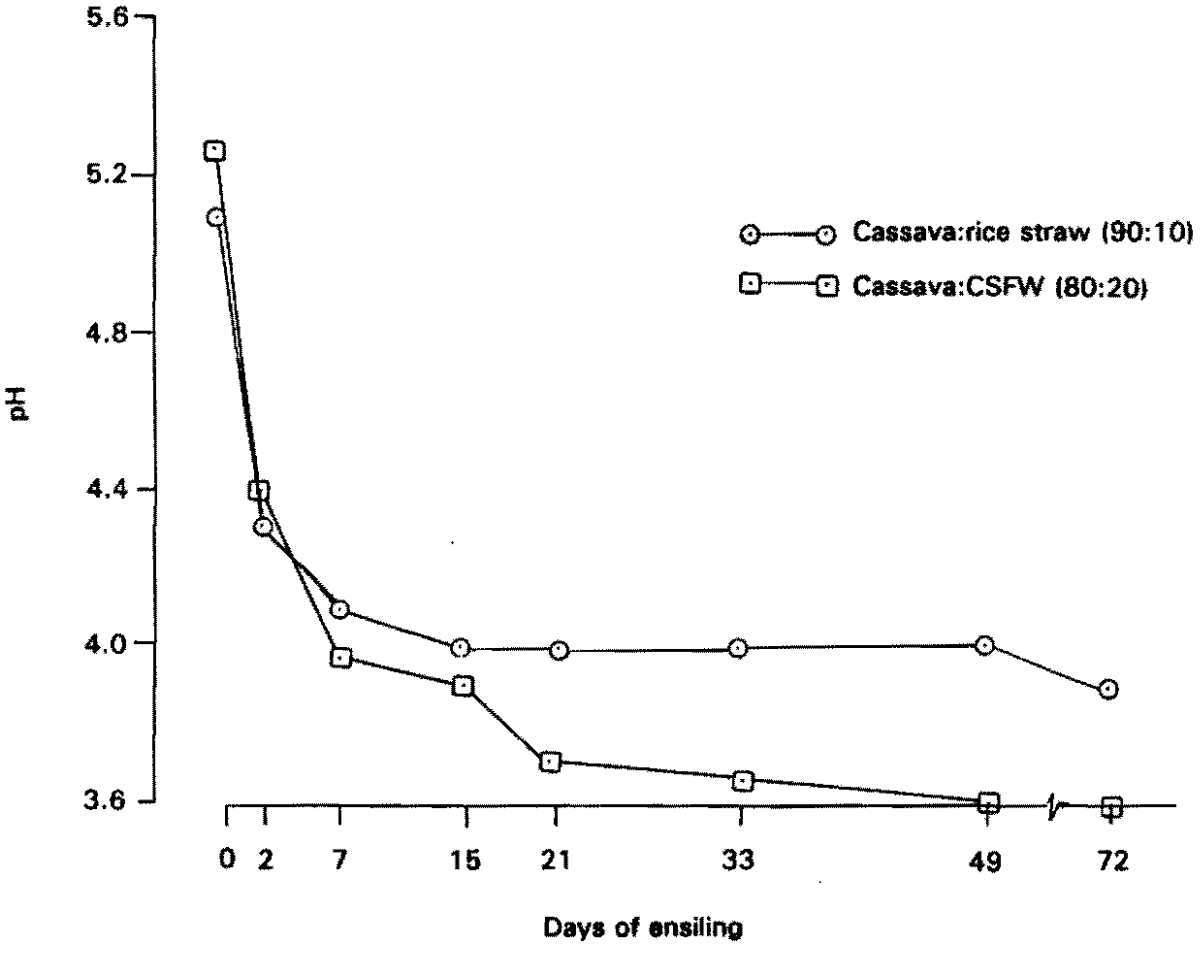

Figure 1. pH changes during ensiling of modified cassava silages.

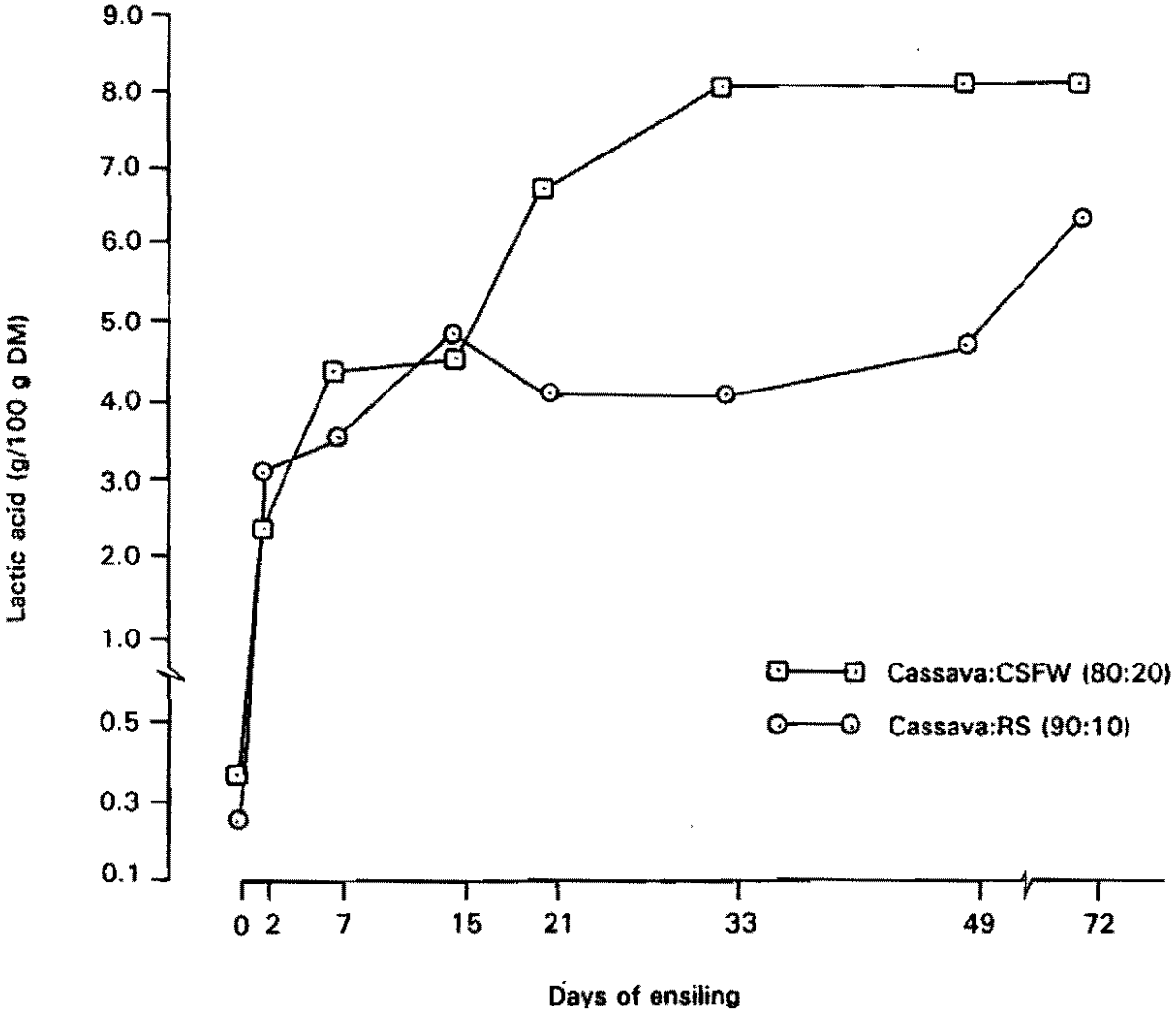

Figure 2. Lactic acid changes during ensiling of modified cassava silages.

STUDY ON THE INDUCTION OF STORAGE ROOT FORMATION IN CASSAVA GROWN UNDER *IN VITRO* CULTURE AND NUTRITION SOLUTION

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Our long-term goal to study a root-specific promoter in cassava requires presently nonexistent information about storage root development; hence our interest in developing an *in vitro* system for storage root development under controlled conditions, to isolate genes expressed specifically in the root and associated with root thickening. Attempts to induce root thickening formation under *in vitro* conditions have failed. Here we report our study with an *in vitro*-induced root thickening system, its reproduction in greenhouse-grown plants and its comparison with field-grown plants. Several *in vitro* cultured experiments were carried out to study the induction of cassava thickened root formation by combination of benzyl aminoporine (BAP) and naphthalene acetic acid (NAA), different carbon sources and concentrations, and different stage of root development. Root morphological and anatomical patterns were observed after induction *in vitro* and greenhouse and natural occurrence in field-grown plants. Two-dimensional protein gel electrophoreses were performed at different stages of storage root development in field-grown plants. Results indicate that the formation of storage roots *in vitro* is reproducible in nutrient solution in greenhouse-grown plants. Uniform storage root formation was obtained either with the combination of 0.10 mg/l benzyl aminopurine (BAP) and 0.3 mg/l ANA, applied once or applied at a weekly interval. The system works either with cassava plants grown *in vitro* or in nutrient solution. Root morphological change is observed 48 h after hormone application; but anatomical changes up to 15 days after induction under *in vitro* conditions indicated no secondary growth such as that observed in the natural field-grown plant. Results from a two-dimensional protein gel electrophoresis indicated a complex protein pattern as the root thickens. Different proteins observed during early stages of root thickening may be candidates for gene isolation. Experiments are being designed to reproduce the natural system under controlled conditions and to isolate genes during the early events of root thickening.

INTRODUCTION

Processes of plant development, such as cassava storage root initiation and development, involve differential expression of genes to control changes in organ growth pattern, differentiation of tissue and specialization of cells that become a starch deposit. Several endogenous and environmental factors appear to influence these processes during root thickening (Nair and Sinha, 1968; Mogilner et al., 1967; Carvalho and Ezeta, 1983; Lowe et al., 1976; Indira and Ramanujan, 1979; Indira and Maini, 1973). Although there is little information on cassava, it indicates the

possibility for hormonal roles in the regulation of root thickening (Melis and van Staden, 1985). Indeed preliminary observations by Cabral et al. (1993) and preliminary results obtained by Carvalho et al. (1993) indicate the possibility of using hormone induction of root thickening in *in vitro*-cultured cassava plants. Thus hormone induction of root thickening may offer a route to follow in order to study its control in *in vitro* culture and its validation in relation to the natural field-grown plant. A controlled, regulated developmental system in cassava roots is important in the goal of our work, to obtain a root-specific promoter that is developmentally regulated.

Our current results include preliminary observations on root thickening in cassava in *in vitro* culture, the validation of this system in relation to field-grown plants, description of comparative morphology and anatomy of the system under study and finally the protein profile of three different stages of root development in field-grown plants.

METHODS

Plant material

Field-grown plants

Cassava plants (cv. Pioneira) were harvested 60 days after planting, and roots at different stages of development were stored at -20 °C until used. Root development stages were defined as A = root of < 0.5 cm in diameter, B = roots with a diameter of 0.5 to 2.0 cm, and C = roots of > 2.0 cm in diameter (Figure 1A), used for protein extraction.

In vitro-cultured plants

Standard MS (Murashige and Skoog, 1962) medium was used to grow a one-node stem of cassava with one bud. After 2 weeks' growth or 2.5 cm-long roots, hormone treatments were applied in the tubes. Visual results were taken for 10 replicated plants evaluated 2 weeks after cultivation of the explants on the hormone-containing medium (Table 1). Table 2 shows the response of storage root formation to different sources of carbon and mode of applying hormone treatments to the growth medium in *in vitro* cultured cassava plants. All treatment solutions were applied for 2 weeks after cultivation of the explants on MS medium without hormone. The standard treatment refers to the most effective hormone concentration treatment combination—0.1 mg/l of BAP + 0.3 mg/l of naphthalene acetic acid (NAA)—in MS medium with 0.08 M sucrose in promoting root swelling. Observations were made on the appearance of storage roots, root morphology and anatomy.

Greenhouse nutrient-solution experiments

A rapid propagation system was used to produce young sprouts from field stem cutting-propagated plants. The new branches were transferred to water and/or nutrient solution until the root system was established; then a hormone treatment was applied in concentrations based on previous *in vitro* culture experiments. Observations were made on the appearance of storage roots, root morphology and anatomy.

Observations and analysis

Morphological and anatomical observations

Visual root swelling and photographic records of root thickening were made in the three plant culture systems. Anatomical observations were made by traversal and longitudinal sectioning of the swollen root. A starch stain of roots sectioned by hand was done at different stages of root development, using the standard iodine procedure. Sections of roots were made by hand and observed in optical microscopes with natural light.

Protein extraction

About 5.0 g of fresh roots at three stages of root thickening (A, B and C) were ground in a polytron at medium speed for three minutes with saline phosphate buffer. Samples were exhaustively dialyzed against phosphate buffer, concentrated in a freeze dryer and kept frozen at -20 °C.

Protein gel separation

Two-dimensional gel electrophoresis methods were used to separate proteins from different root development stages. A native-PAGE direction gel with an ampholyte pH range of 4.0 to 8.0 was processed in a tube apparatus system. For the second dimension, a 10% SDS-PAGE was used.

RESULTS

Tables 1 and 2 show our root observations in the *in vitro* cultured cassava plant's response to hormone treatments, to source of carbon and to mode-of-treatment application. The results indicate that root swelling could be induced by the treatment that included the application of 0.1 mg/l BAP and 0.3 mg/l NAA, either at the beginning of the *in vitro* culture of the explant (Table 1) or 2 weeks into the growing period before hormone treatment (Table 2). However, the latter method produced roots morphologically more like roots from natural field-grown plants

(Figure 1) than the former method. Based on these results and observations, this hormone treatment combination was selected as the standard induced hormone treatment. As root swelling morphology showed response to the application of BAP alone, it was decided to test this effect in combination with the later application of NAA. The response of root swelling to the application of BAP early and NAA later seems to be tissue dependent. Observations indicated that the slow response of root swelling to applying BAP early is consistent with the overall effect of cytokinin activity (cell division) in the cortical meristem. While the rapid response of applying NAA early is consistent with the rapid growth effect on cell enlargement by auxin and therefore the epidermal cells of the root pop up because there is no effect on cell division. Together these results indicate that cytokinin is the most important regulating factor in studying cassava swollen root formation. Our current focus on these preliminary results includes the testing of several different forms of cytokinin alone or in combination with auxins, in order to establish an *in vitro* storage root development system for cassava.

Figure 1 shows the morphological observations of thickened root and the anatomical patterns of the roots in the three plant culture systems. In natural field-grown plants (Figure 1A), it is observed that any root in the plant can become a storage roots regardless of the root origin. But the number of thickened roots is higher in the wounded, cut part of the old stem than in the basal section of the bud where the shoot originates. In cv. Pioneira, a necked type of thickened root is formed and root enlargement follows apical growth. In cassava, primary apical root growth is accompanied by secondary cambial growth of the root. This makes cassava storage root formation a unique system, distinct from potato tuber formation, which is a differentiated stem tissue. Root enlargement, with a morphological resemblance to tubers, is observed after applying the combination of 0.1 mg/l BAP and 0.3 mg/l NAA, either in nutrient solution-grown plants (Figure 1B) or *in vitro* cultured plants (Figure 1C). Anatomical observations were made to establish whether root enlargement in nutrient solution-grown and *in vitro*- cultured plants had the same root growth and tissue differentiation pattern as in the natural field-grown plant. Figure 1A indicates a typical secondary growth pattern in thickened roots of natural field-grown plants which is not in the hormone-treated plants in nutrient solution (Figure 1B) or *in vitro* culture (Figure 1C).

These results indicate that although the morphological swelling of the roots in hormone-treated plants resembles the naturally thickened root, it may not fully induce thickening by means of secondary growth. Hormone-induced growth was observed only in the cortical meristem of the root, not in the cambial meristem. These results are consistent with hormone concentration as determined in different sections of thickened roots of cassava by Melis and van Staden (1985), who showed high cytokinin activity in cambium as well as in the cortical meristem. Although the hormone balance in both meristems is different, cytokinin in both root sections may play the major role in root thickening. This is also consistent with our results (Tables 1 and 2). In our results, the lack of full hormone-treatment effect

on true storage root formation be due to rapid response to high cytokinin concentrations used on the outermost cell layers of the root and reaching the cortical meristem first, so as to penetrate the root tissue. In our system, the primary root tissue enlargement is in the cortex due to the effect of the hormone in the cortical meristem, not in the innermost meristematic cambium region. As our results are not fully conclusive, further research is currently under way to define a hormone source, concentration combination and time of application to fully induce secondary growth under controlled conditions.

In our search for differential gene expression during storage root development in cassava roots, we defined three stages of root development (Figure 2). Starch staining revealed the presence of starch grains in all the stages studied (Figures 2A, 2C and 2E). But protein content and composition (Figures 2B, 2D and 2F) varied with the stage of development of the storage root in the natural field-grown plant. Protein content in cv. Pioneira was 1.56 mg/g fresh wt. root in stage A; 2.26 in stage B and 2.47 in stage C. Electrophoreses separation of the proteins indicated different patterns in all stages of root development studied (Figures 2B, 2D and 2F). Preliminary observations indicate that the optimum separation pH gradient in the first protein profile dimension of the gel may not be the same for all stages of development. Stage A of the storage root (Figure 2B) has few proteins and the simplest protein profile of all. Further research is underway to improve the separation resolution of proteins to be purified and characterized during root development, not initiation, as our initiation system is still being developed.

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Table 1. Response of storage root development in *in vitro* cultured cassava plants at different concentrations of benzylaminopurine (BAP) and naphthalene acetic acid (NAA).[a]

NAA (mg/l)	BAP (mg/l)				
	0	0.1	0.3	1.0	3.0
0	-	-	+ +	-	-
0.01	-	+ +	-	-	-
0.03	-	+ + + +	+	-	-
0.1	-	+ + +	-	-	-
0.3	-	+ + + +	+ +	-	-

a. - = no response.
+ to + + + + = increased level of appearance of swollen roots in response to hormone treatment.

Table 2. Response of storage root formation to different sources of carbon and mode of application of the hormone treatments to the growth medium in *in vitro* cultured cassava plants.

Treatment	Concentration	Formation of swollen roots[a]	Observation
Standard[b]		+ + + +	
Control		-	
Glucose	0.2 M	+ + + +	
	4.0 M	+ + + +	
Sucrose	0.4 M	+ + + +	
BAP + NAA[c]		+ + + +	Slow swelling
NAA + BAP[d]		-	Rapid swelling with epidermal cell enlargement; sponge-like root formation

a. (+) = formation of swelling roots, (-) = none formed.

b. Standard treatment = most effective hormone concentration treatment combination (0.1 mg/l of benzylaminopurine [BAP] + 0.3 mg/l of naphthalene acetic acid [NAA]) in Murashige and Skoog medium with 0.08 M sucrose in promoting root swelling.

c. NAA applied 7 days after BAP.

d. BAP applied 7 days after NAA.

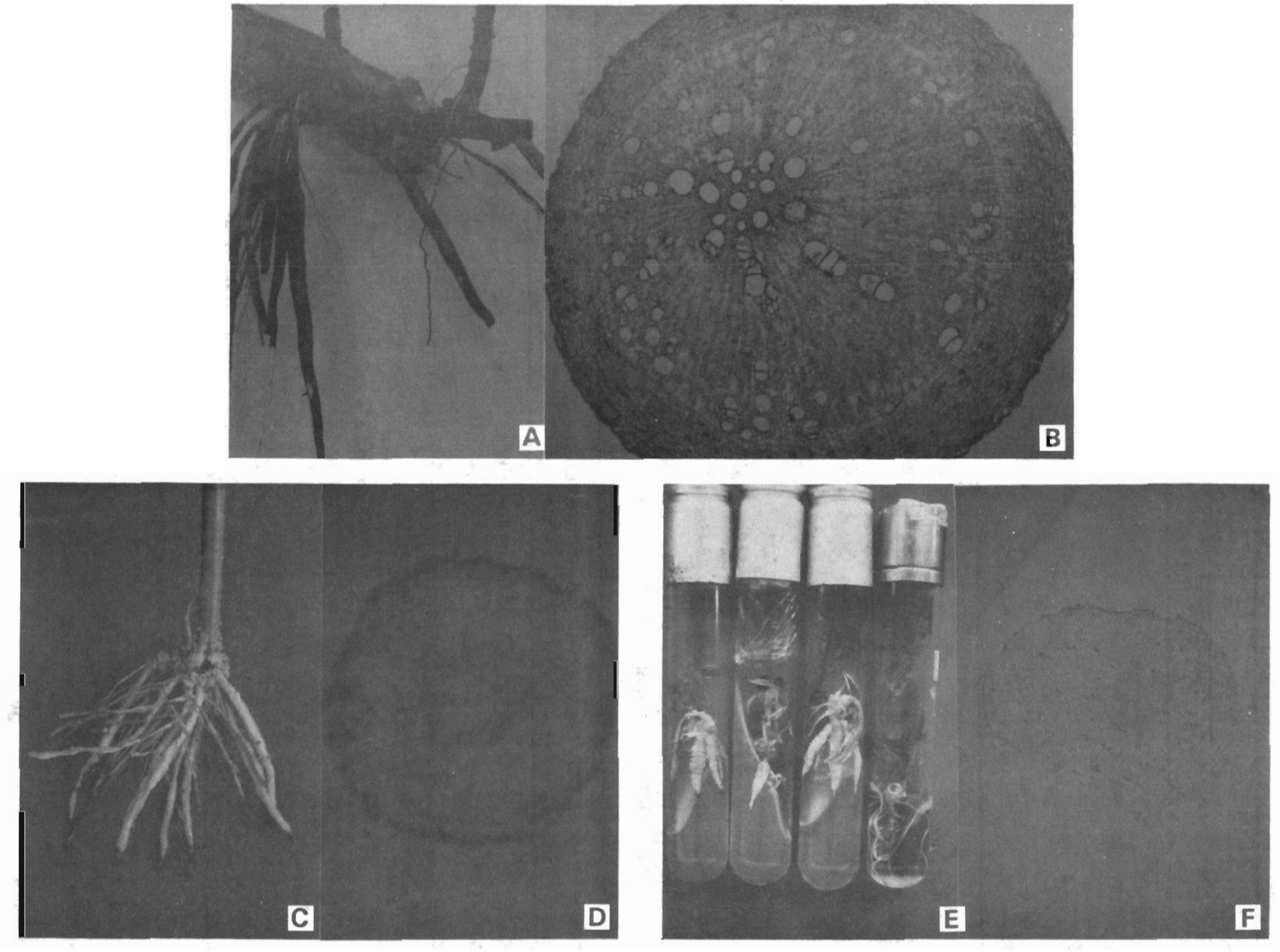

Figure 1. Morphological patterns of cassava storage roots growing in different culture systems: (A) field-grown plant of cv. Pioneira harvested 60 days after planting; (B) its corresponding anatomical pattern; (C) hormone-treated plant growing in nutrient solution (D) its corresponding anatomical pattern; (E) hormone-treated plant growing *in vitro*; (F) its corresponding anatomical pattern.

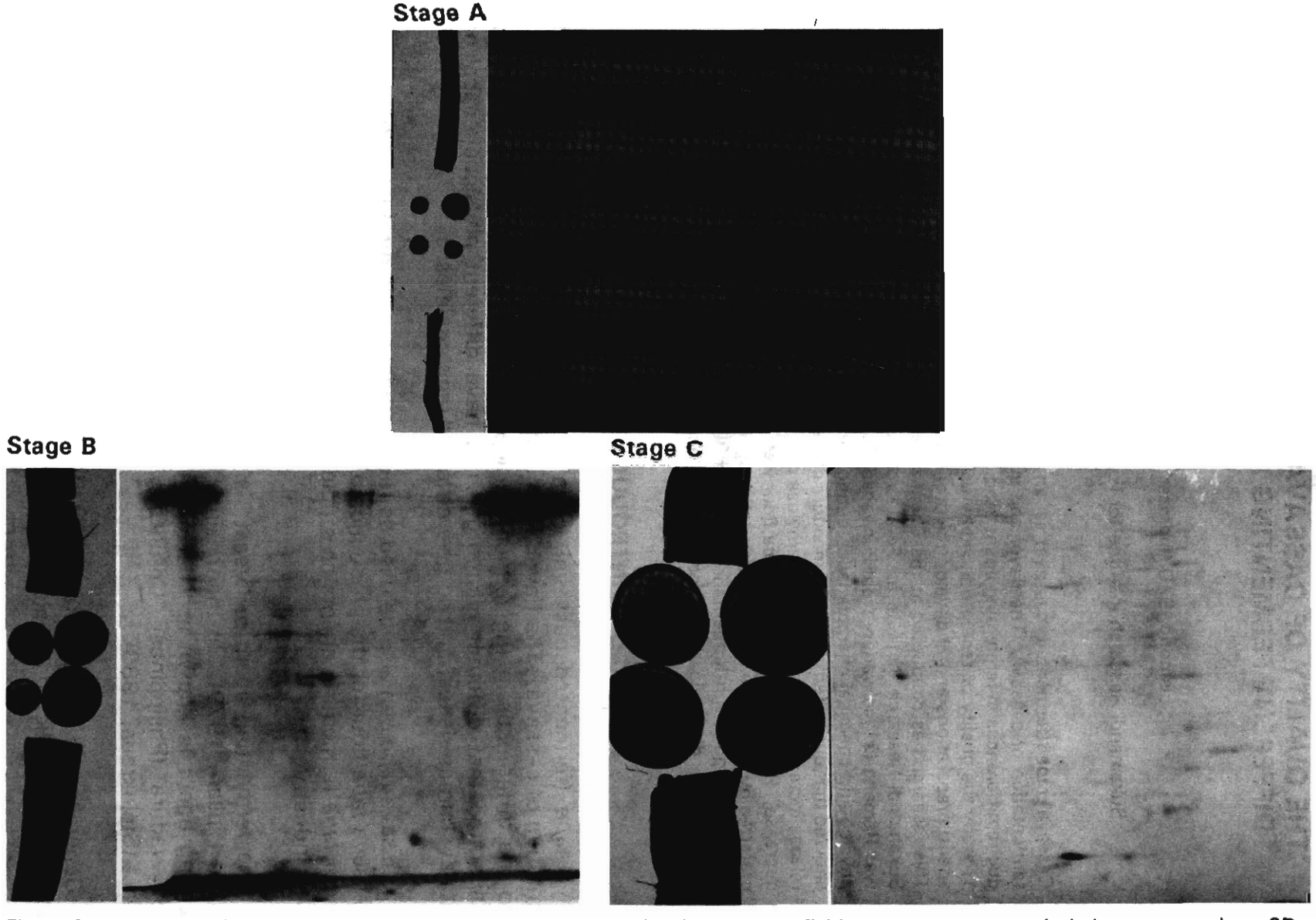

Figure 2. Definition of three different stages of storage root development in field-grown cassava and their correspondent 2D gel electrophoreses profile. Hand-sectioned roots were stained for starch with the iodine method. Two-dimensional gel were performed with pH range of 4.0 to 8.0 in a 10% native-PAGE and 10% SDS-PAGE in the second direction.

THE QUALITY OF CASSAVA *TAPE*, USING SEVERAL DIFFERENT FERMENTING AGENT PREPARATIONS

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Cassava *tape* (fermented cassava) is an Indonesian traditional food made by fermentation of cassava. The flavor and quality of *tape* in the market are variable due to differences in fermenting agent used. The objective of this research were to: 1) study the quality of *tape* produced by using different ragi (traditional fermenting agent) preparations and 2) develop the proper technology for *tape* flour production. This research has conducted at the Chemical and Technological Laboratory at the Sukamandi Research Institute for Food Crops (SURIF). The cassava variety used was Remang (a local variety from Purwadadi-Subang). Observations were made of physico and chemical characteristics during the fermentation process. The results showed that during fermentation, moisture content, total sugar content and CO_2 gas release increase and of starch and amylose contents, O_2 gas content and pH of *tape* decrease. Recovery of *tape* and *tape* flour were 67% and 30%, respectively. *tape* preferred by the panelists were those using ragi from a supermarket and a traditional market in Bogor, with fermentation period of 30 h. *Ragi* and *tape* flour have good prospects for diversification in food and food industries.

INTRODUCTION

Cassava *tape* (fermented cassava) is an Indonesian traditional food made by fermentation of cassava. The flavor and quality of *tape* in the market are variable due to the differences in the fermenting agent used. Dwidjoseputro and Wolf (1970) stated that different microorganisms have different physicochemical or functional characteristics, and so give different *tape* qualities.

Cassava *tape* can also be used as raw material for the production of alcohol and cassava *tape* flour for the production of bread. The method of production and quality of *tape* fermenting agents be studied because *tape* has good prop pects in the food industry.

The fermenting agent or "yeast" is a solid inoculum which is traditionally produced (Tuilan, 1979). In Indonesia is known as "*ragi*". In other countries, fermenting agent is known as "Jin paing" (Malaysia), "Loog pang" (Thailand), "Bubot levadura" (Philippines), "Ch'u (China) and in India is known by the name of "Bakhar" (Steinkraus, 1983).

Generally, the raw material of *ragi* production are rice, flour and a mixture of spices such as garlic, galingale, pepper and chili. Different methods of *ragi*

production will results in different kinds and numbers of active microorganisms during the fermentation process (Jonsen, 1984).

The spices in the *ragi* production produce aroma, flavor and stimulate the activities of the desired microorganism. Soedarsono (1972) reported that the additional garlic in the *ragi* production will inhibit the growth of amylolitic microorganisms (fungi) and the use of 6% chili can stimulate the growth of all microorganisms present in the *ragi* (Saono, 1981).

There are various kinds of microorganisms: fungi, "khamir" and bacteria, in the *ragi* (Kozaki, 1979). The fungal and khamir species present in the *ragi* different, depending on the origin and method of production the *ragi*. Saono (1981) reported that the microorganisms present in the *ragi* which play important roles in fermentation are: species of genus *Mucor, Rhizopus* and *Amylomyces*; species of genus *Endocopsis, Saccharomyces, Hanzenula* and *Candida*; and species of the *Pediococcus* and *Bacillus*.

This research was designed to: 1) study the quality of *tape* produced using different *ragi* preparations and 2) develop the proper technology for *tape* flour production.

MATERIALS AND METHODS

This research was conducted at the Chemical and Technological Laboratory at Sukamandi Research Institute for Food Crops (SURIF), from December 1993 to February 1994. The cassava variety used was Remang (local variety from Purwadadi-Subang). The *ragi* used was obtained from a supermarket in Bogor and traditionally produced *ragi* from traditional markets in Bogor and Sukamandi.

Ragi production

Generally *tape ragi* is traditionally produced from rice flour and various spices to promote the growth of microorganism in the fermentation process (Table 1 and Figure 1).

Methods of cassava *tape* and *tape* flour production

The cassava was peeled and washed, and then partially steamed, cooled, and finally inoculated with 0.5% *ragi* uniformally. It was then fermented in a bamboo mat container and covered with banana leaves. Each experimental unit used 1.5 kg of cassava (Figure 2).

The process of *tape* flour production was done by grinding the dried *tape*. *Tape* was dried in a dryer using temperature of 70-75 °C for 16 h. A drying temperature above 80 °C can promote browning. Grinding was done by using a grinding machine. Manual grinding is difficult because the *tape* has high contents of moisture and sugar, so that it tends to coagulate and is difficult to sieve (Figure 3). The chemical composition of *tape* flour can be seen in the Table 3 (BBIHP, 1989).

The completely randomized block designed was used with 3 replications with treatments as follows:

1. *Ragi* preparations:

   SMB = *ragi* from supermarket in Bogor
   TMB = *ragi* from traditional market in Bogor
   TMS = *ragi* from traditional market in Sukamandi

2. Fermentation duration:

   D3 = 3 h
   D25 = 25 h
   D47 = 47 h

Observations are made for: 1) moisture content (Oven method); 2) starch content (Anthrone method); 3) amylose content (Yun and Matheson, 1990); 4) sugar content (Refractometer); 5) pH (pH meter); 6) CO_2 and O_2 gas releases (Warburg apparatus); 7) *tape* recovery; 8) temperature of package; 9) ambient temperature and humidity, and organoleptic test of *tape* and *tape* flour (Hedonic method).

RESULTS AND DISCUSSION

Physico chemical changes that occur during the cassava fermentation process can be assessed on the based of the moisture content, starch content, sugar content, amylose content, pH, CO_2 gas release, O_2 gas content, *tape* recovery and organoleptic test of *tape* and *tape* flour. In addition, environmental factors such as temperature in the package, ambient temperature and humidity can affect the rate of fermentation.

Moisture content

During fermentation, the moisture content of the *tape* increases from 60% to 68% (Figure 4). In the *tape* produced using *ragi* from the Sukamandi traditional market,

the moisture content increases up to 20 h of fermentation, and then is followed by constant moisture content. In the *tape* produced by using *ragi* from Bogor Supermarket, the sharp increase of the moisture content occurs after 25 h of fermentation, and in the *tape* produced by using *ragi* from Bogor traditional market the increase continues up to 47 h of fermentation. The increase of moisture content is due to the glycolysis and saccharification processes occurring during fermentation, marked by the present of a mucus substance.

Starch content

The starch content of the *tape* decreases during the fermentation because starch is consumed by the fermenting microorganisms. The rate of the decrease of starch content of the *tape* using *ragi* from Bogor supermarket is greater than those in the *tape* using *ragi* from Bogor and Sukamandi traditional market.

The starch content of fresh cassava is 34%, while that of *tape* after 3 h of fermentation is 4%-5% this means that the rate of starch utilization during the growth of microorganisms is high (Figure 5).

Amylose content

The amylose content of the *tape* also decreases during fermentation. After 25 h of fermentation the amylose content of *tape* is less than 26% this means that *tape* is becoming softer, especially *tape* using *ragi* from supermarket and traditional market in Bogor (Figure 6).

Sugar content

During the fermentation the sugar content of the *tape* increases up to 21%, reaches a maximum from 15-30 h of fermentation, and then decrease slowly. The pattern of this changes agree with that of starch discomposition to reducing sugar during fermentation (Figure 7). According to Rahayu (1980) the maximum of sugar content during fermentation is 16%-18%.

pH content

The observation show that during the fermentation process the pH of *tape* decrease from the beginning to 20 h of fermentation, using all the three kinds of *ragi* studied (Figure 8). The desirable pH and temperature of *tape* during fermentation are 4.5-7.0 and 40 °C, respectively (Rahayu, 1980; Suparto, 1982). The pH decrease is due to the process of starch discomposition to simple sugar and organic acids.

The longer the fermentation, the more acids are produced. The decline of pH of *tape* using *ragi* from Sukamandi traditional market is greater than those of *tape* using *ragi* from the supermarket and traditional market in Bogor. This is due to the differences in the method of *ragi* production and the kinds of microorganisms present (Saono et al., 1974; Apandi et al., 1979).

CO_2 gas release

The maximum CO_2 gas released by the three kinds of *ragi* reaches a maximum after 15-35 h of fermentation. The CO_2 gas release is caused by the fermentation of alcohol by khamir, in which piruvic acid produced by the glycolysis process is discomposed by carboxylase enzyme, with Mg^{++} as catalyst, to acetaldehyde and CO_2 (Braverman, 1963). The maximum CO_2 gas release by *ragi* from the Sukamandi traditional market (22.4 ml/2 g) is greater than those from *ragi* from the Bogor supermarket (14.45 ml/2 g) and the Bogor traditional market (13.86 ml/2 g) (Figure 9). This difference is due to the greater rate of fermentation activity of *ragi* from Sukamandi traditional market.

O_2 gas content

The O_2 gas utilization follows the pattern of fermentation and differs between the three kinds of *ragi*. The O_2 gas utilized by *ragi* from Sukamandi traditional market is lower because its O_2 gas utilization is earlier than *ragi* from the supermarket and traditional market in Bogor. Maximum O_2 gas utilization during the fermentation was attained after 20-45 h of fermentation (Figure 10).

Tape recovery

Average *tape* recovery is 67.6%, calculated from fresh cassava by the weight, as shown in Table 2. Wilda (1981) stated that *tape* flour recovery is about 30% calculated from *tape* (by the weight).

Tape acceptability test

Based on the physico chemical characteristic the best *tape* quality is obtained between 15 to 35 h of fermentation. The results of *tape* organoleptic test can be seen on Figure 9.

The *tape* produced by the 3 kinds of *ragi* are all preferred by the panelists (in terms of color, aroma, flavor, texture and acceptability). The *tape* using *ragi* from supermarket and traditional market in Bogor are apparently preferred over that using

ragi from the Sukamandi traditional market. This reflects the changes in of sugar, starch, amylose, pH, CO_2 and O_2 and package temperature after 25-30 h of fermentation at 27-31 °C ad relative humidity of 78%-80% (Figure 11). *Ragi* from the supermarket and traditional market in Bogor gives a higher temperature than that from Sukamandi traditional market; this may be a useful indicator that the fermentation product using *ragi* from the supermarket and traditional market in Bogor is preferred to that from using the Sukamandi traditional market (Figure 12).

Tape flour

Physical characteristics of *tape* flour were: 67.47% whiteness using a whiteness tester, and flour particle size of 60 mesh.

CONCLUSIONS

1. During fermentation there are increases in moisture content, total sugar content and CO_2 gas release and decreases of starch and amylose contents, O_2 gas content and pH of *tape*.

2. The recoveries of *tape* and *tape* flour are 67% and 30%, respectively.

3. *Tape* preferred by the panelist are those using *ragi* from the supermarket and traditional market in Bogor, with a fermentation period of 30 h.

4. *Ragi* cannot be concluded on the basis of this research.

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Table 1. The composition of *tape ragi* raw materials.

Raw materials		Percent to rice flour (%)
1.	Rice flour	100
2.	Spices:	
	garlic	0.50 - 18.70
	white pepper	0.05 - 6.20
	galingale	2.50 - 50.00
	red chili	0.25 - 6.20
	cinamon	0.05 - 3.50
	black pepper	0.30 - 2.50
	dill/fennel	2.50 - 3.00
	sugar cane	1.00 - 12.50
	citroen	2.50
	coconut water	50.00

SOURCE: Saono (1981).

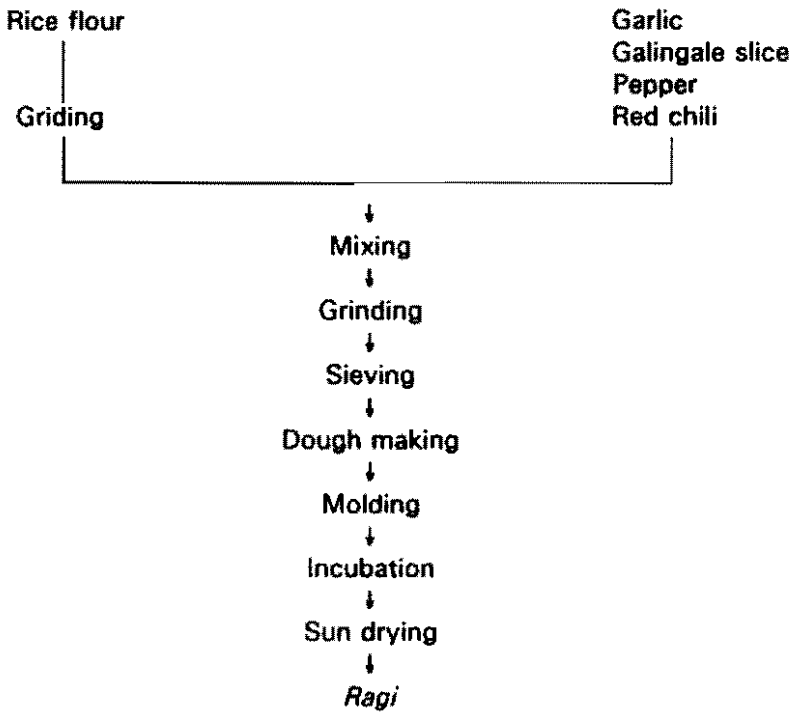


Figure 1. *Ragi* production.

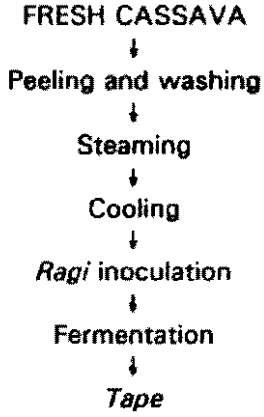


Figure 2. The flow chart of cassava *tape* production.

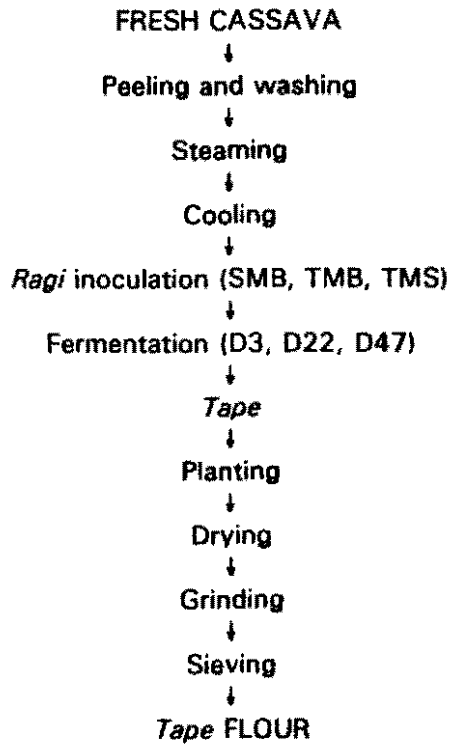


Figure 3. The flow chart of cassava *tape* flour production.

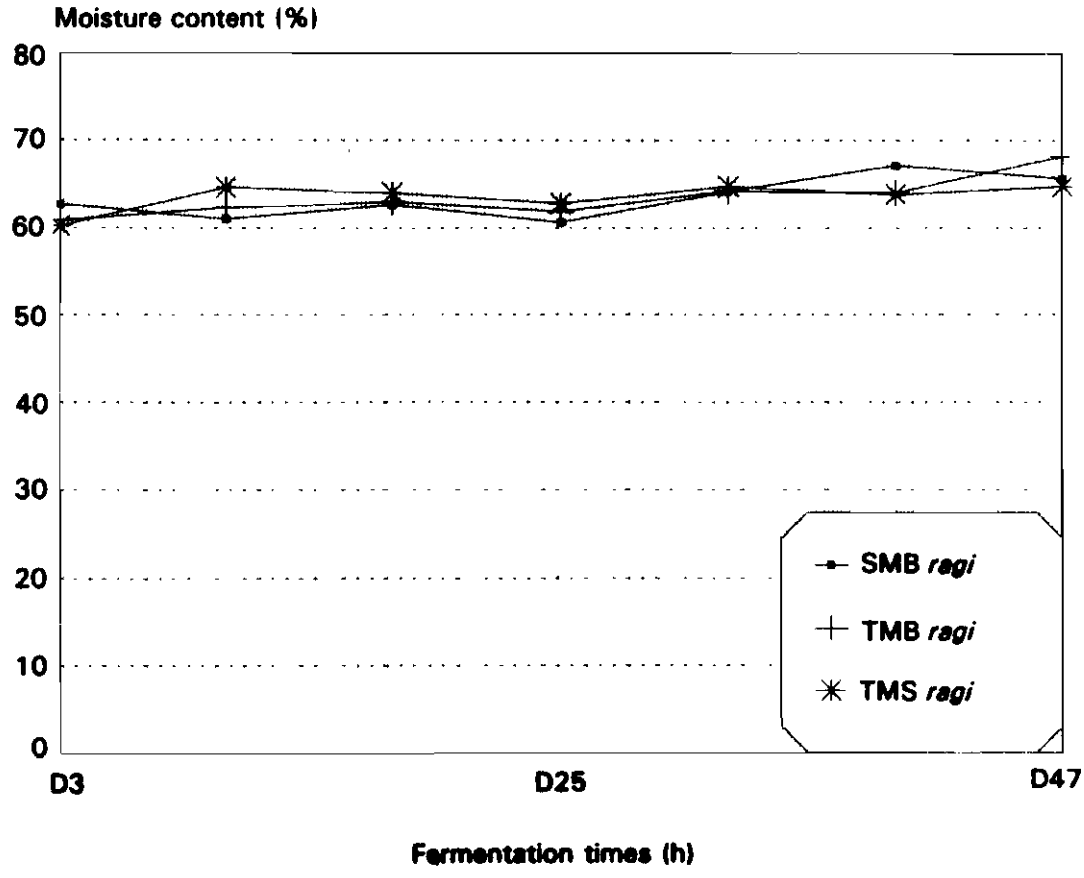


Figure 4. The effects of fermentation time and kinds of *ragi* on *tape* moisture content.

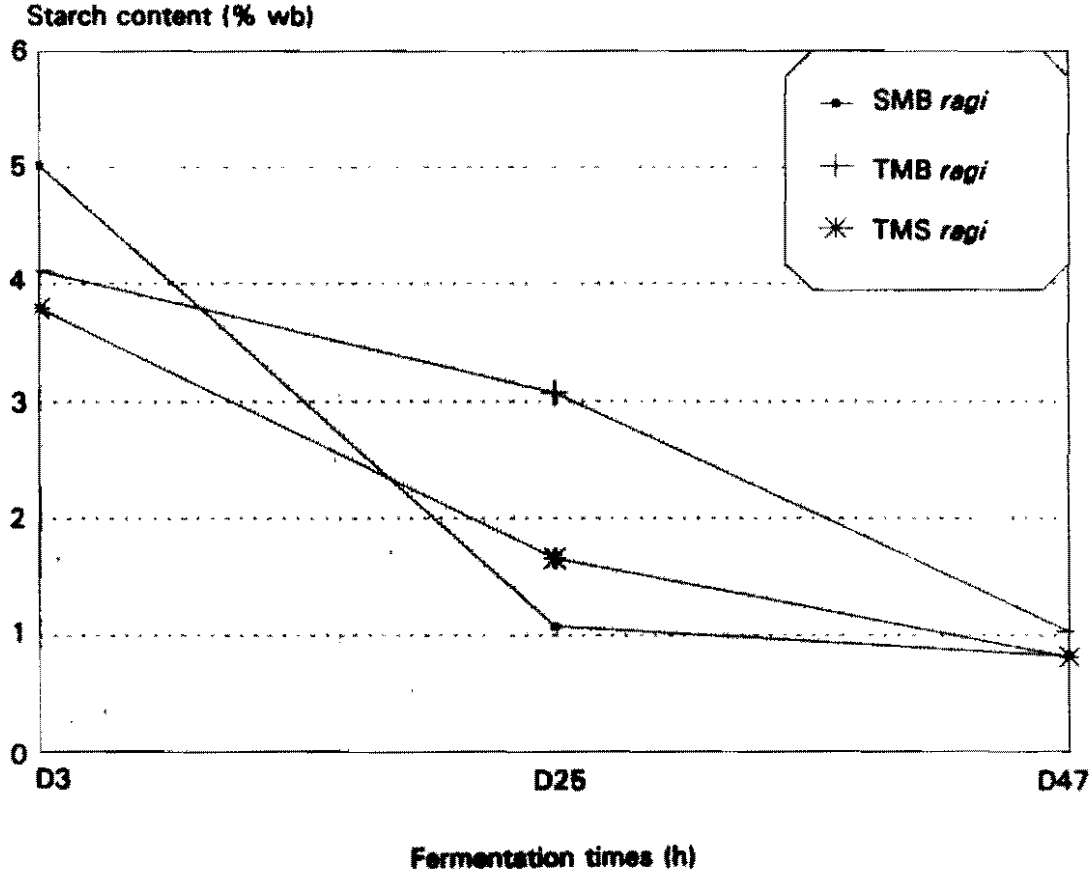


Figure 5. The effects of fermentation time and kinds of *ragi* on *tape* starch content.

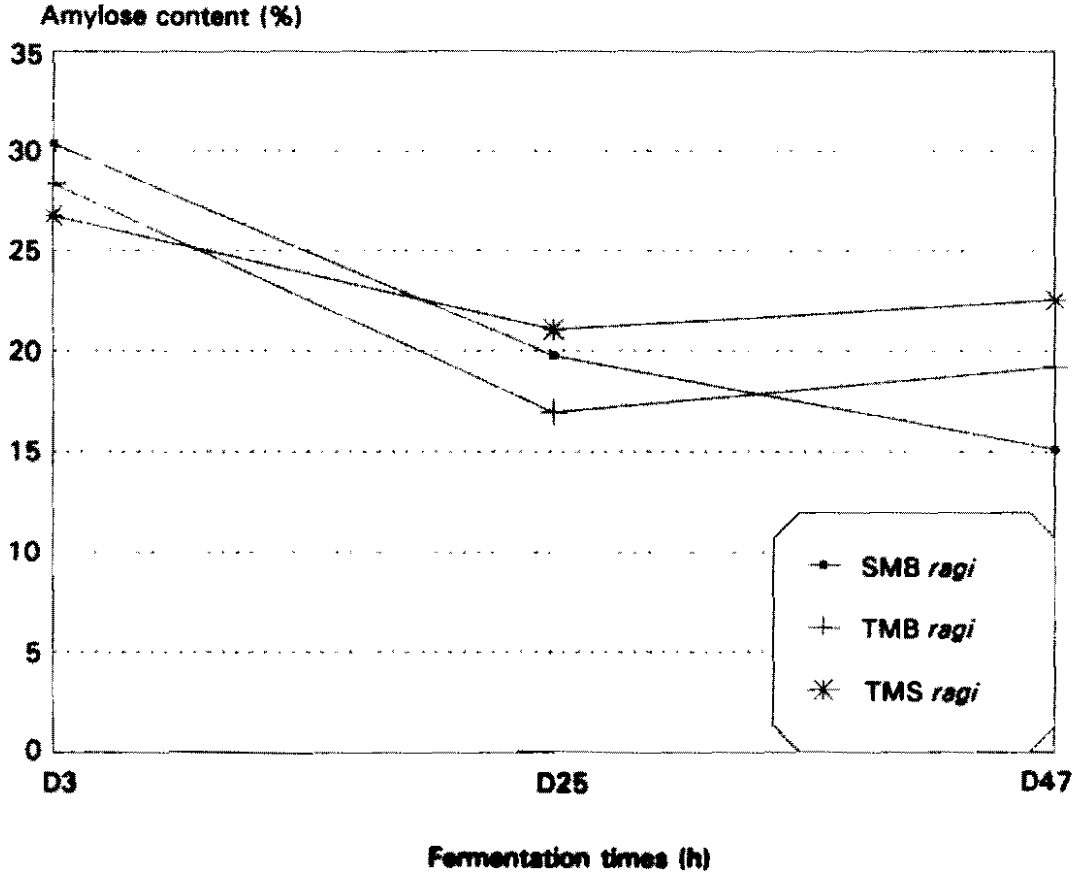


Figure 6. The effects of fermentation time and kinds of *ragi* on *tape* amylose content.

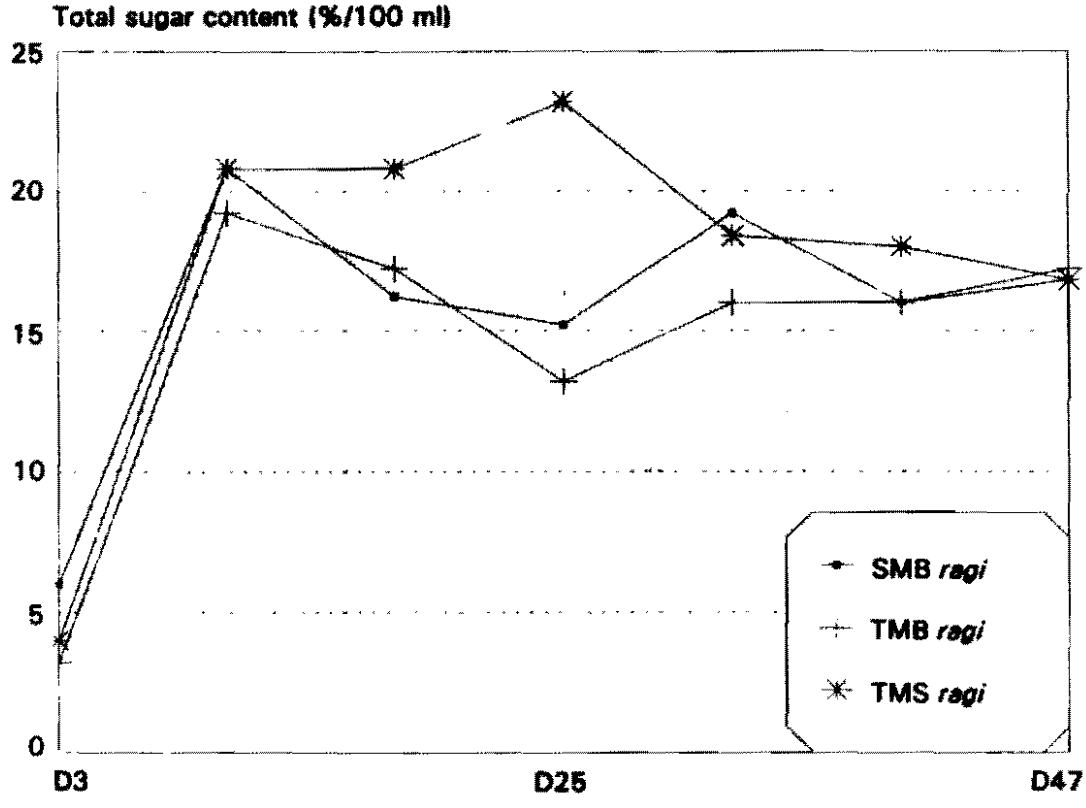


Figure 7. The effects of fermentation time and kinds of *ragi* on *tape* sugar content.

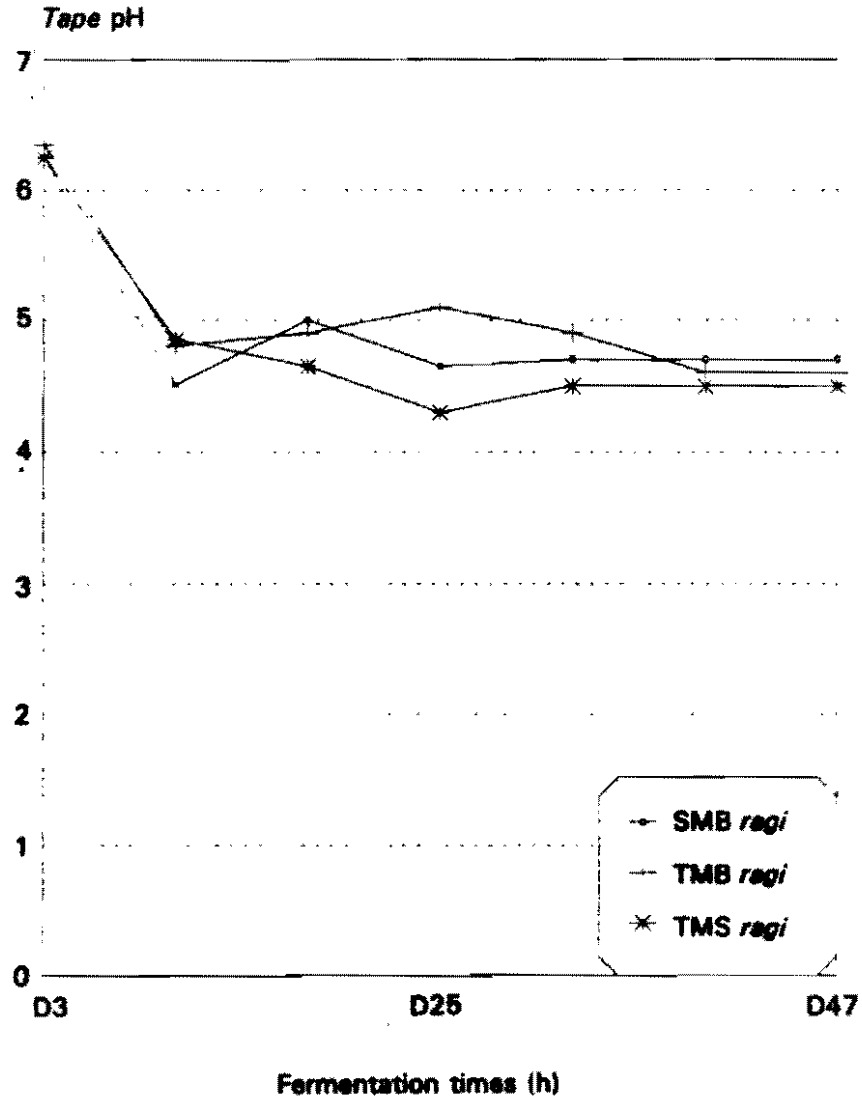


Figure 8. The effects of fermentation time and kinds of *ragi* on the *tape* pH.

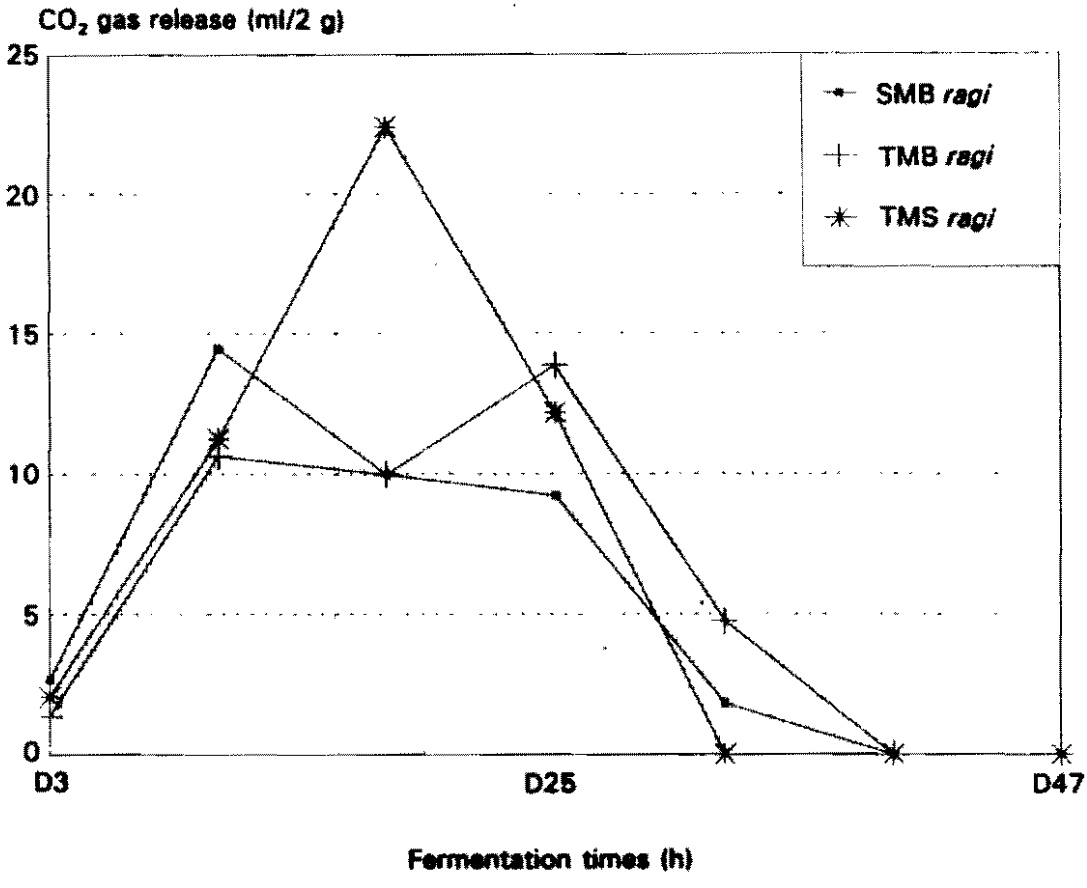


Figure 9. The effects of fermentation time and kinds of *ragi* on the release of CO_2 from *tape*.

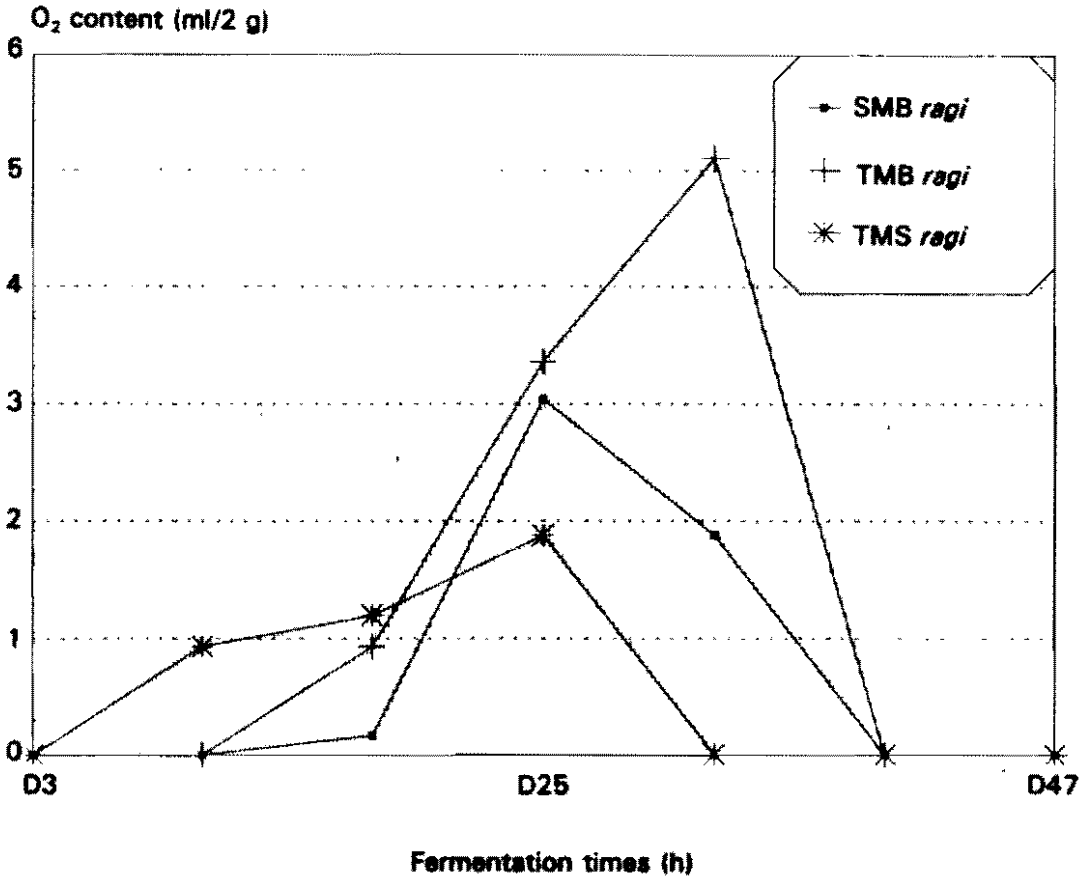


Figure 10. The effects of fermentation time and kinds of *ragi* on *tape* O_2 gas content.

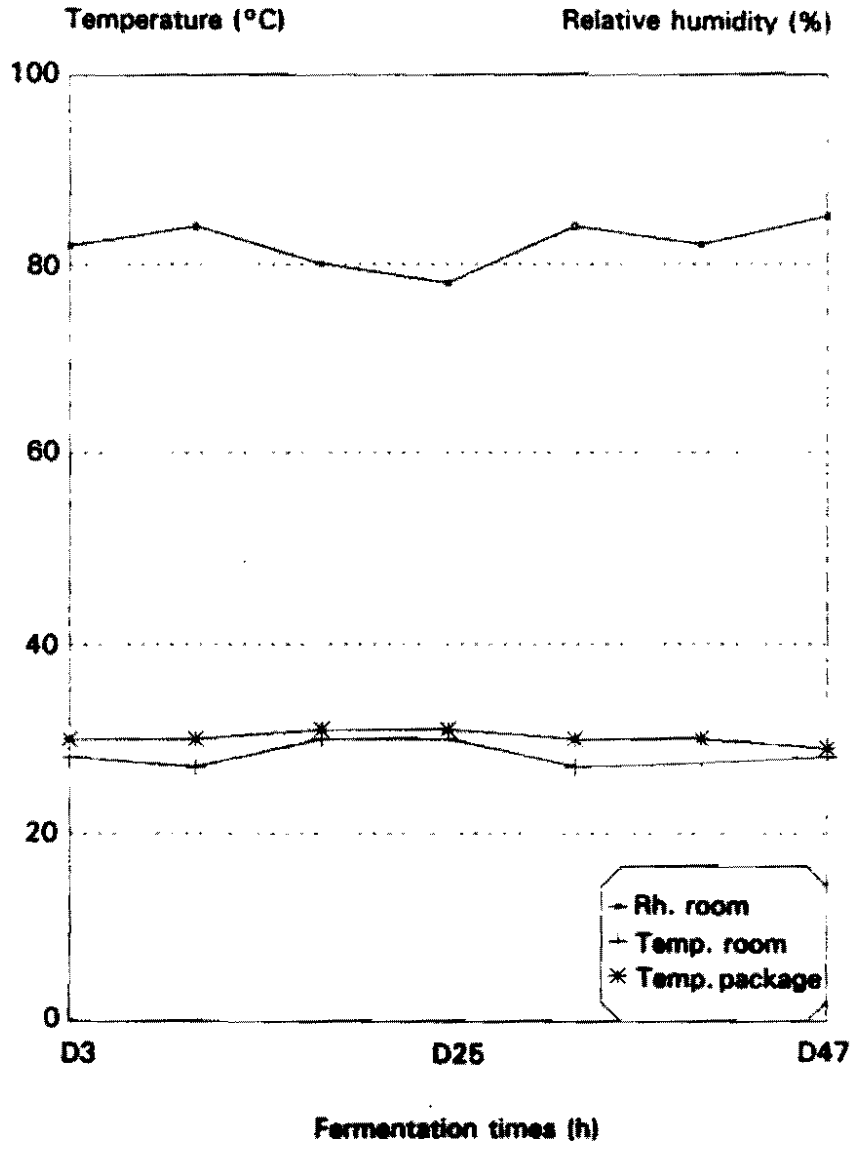
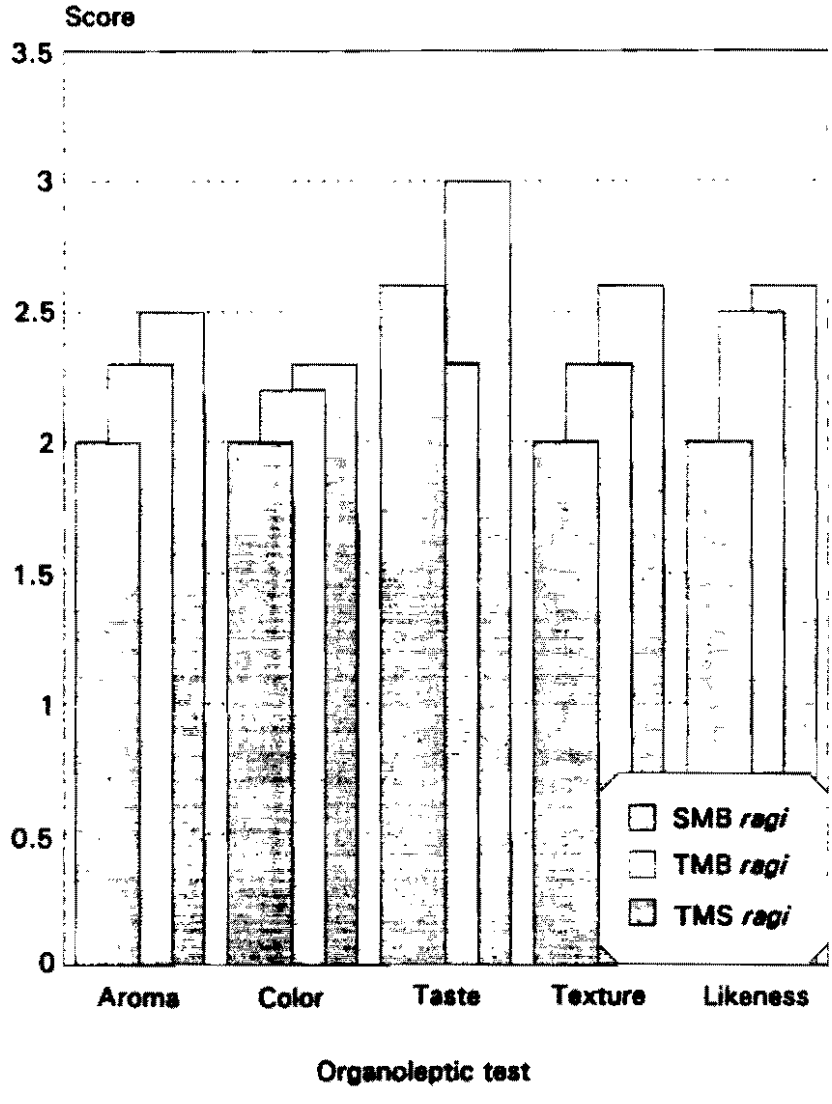


Figure 11. The temperature and relative humidity of the fermentation process.



(Score: 1 = very preferred, 2 = preferred, 3 = medium, 4 = unpreferred)

Figure 12. The effects of fermentation time and kinds of *ragi* on *tape* preference.

SCREENING OF CIAT CASSAVA GERMPLASM DIVERSITY: THE RELATIONSHIP BETWEEN CYANOGENIC POTENTIAL AND CASSAVA STARCH FUNCTIONAL PROPERTIES

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[2]Centro Internacional de la Papa (CIP), stationed at Central Research Institute for Food Crops (CRIFC), Bogor, Indonesia;
[3]Natural Resources Institute (NRI), Kent, United Kingdom;
[4]Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia

In 1991, a core cassava germplasm collection was established at the Centro Internacional de Agricultura Tropical (CIAT) comprising 633 accessions. It attempts to reflect the genetic diversity of the entire cassava germplasm collection, held at CIAT, of over 5000 clones. The establishment of this core collection provided an opportunity to start the characterization of cassava root biodiversity. In 1992, 565 available clones of the core collection were analyzed. At CIAT, plants were harvested from 10-12 months of age, and the following evaluations undertaken:

Total cyanogenic potential (CNP) of root peel and parenchyma.

Dry matter content of root peel and parenchyma.

Amylose content of the extracted starch from root parenchyma.

Starch and sugar contents of root parenchyma.

Proximal analysis (protein, fibre, lipids, etc.).

Figure 1 shows the results of dry matter, Figure 2 those of cyanogen content, and Figure 3 of amylose content distribution. Results show great variability among cassava clones, reflecting the biodiversity of the international core collection held at CIAT. Dry matter content ranged from 13% to 48% (Figure 1); total parenchymal CNP was 17 to 4126 mg/kg (dry matter basis) (Figure 2); amylose content ranged from 13 to 28% (Figure 3).

The 565 analyzed clones were statistically distributed into 10 clusters (Figure 4). For the purposes of the statistical analysis, variables of relevance in cassava processing were selected, such as dry matter content, percentage of amylose and CNP.

The general analyses of the cassava root peel and parenchyma were carried out as part of the CIAT Cassava Program's germplasm evaluation work.

Since 1993, the characterization of starch samples was undertaken as part of a research program on cassava starch biodiversity organized by the Centre de Coopération International en Recherche Agronomique pour le Développement, Département des systèmes agroalimentaires et ruraux (CIRAD-SAR) and the European Community. It was jointly financed in partnership with CIAT, with the involvement of several institutional partners in Latin America and Europe.

From the 10 clusters of the core collection, 33 clones were chosen and grown at CIAT headquarters. Roots were harvested at 9 months maturity. The pasting properties were evaluated by viscoamylographic studies using a 6% starch suspension (Figure 5). Physicochemical composition and starch functional properties were widely distributed. Gelatinization temperature varied between 60 and 67 °C; maximum viscosity ranged between 400 and 8850 Brabender Units; rheological behavior was extremely variable. Large differences were observed even among clones within the same cluster, indicating the probable influence of edaphoclimatic parameters.

The 15 clones with the highest and lowest CNP from the core collection were selected for detailed starch functionality studies. A correlation between cassava CNP and pasting properties of starch is shown in this study. The high cyanogen clones had lower maximum starch viscosity than low cyanogen clones (Table 1).

Amazonian population groups' preference for high cyanogen varieties may be related to the starch functional properties of these varieties. These populations have developed sophisticated traditional methods to reduce the total CNP of processed cassava products to innocuous levels and to obtain the desired functional properties of cassava flour and starch. Further studies are presently being conducted to investigate the effect of the natural ecosystem (soil, rainfall, temperature and solar intensity) upon functional properties of cassava starch. A better knowledge of the functional properties of cassava starch will enable recommendations to be made regarding agricultural practices and cassava cultivars suitable for specific industrial applications. This should lead to improvements in the use of cassava germplasm resources.

Table 1. Mean value of starch functional properties for 15 clones of extreme low and high total parenchyma cyanogen content.

	Low cyanogen group		High cyanogen group		Significant* difference (P=)	
	Core collection analyzed in 1991	Core collection analyzed in 1993	Core collection analyzed in 1991	Core collection analyzed in 1993	Core collection analyzed in 1991	Core collection analyzed in 1993
Total cyanogen (mg/kg fresh basis)	13	22	461	692.2	0.0001	0.0001
Amylose (%)	22.9		21.6		n.s.	
Gelatinization temperature (°C)	64.1	62.8	64.7	62.7	n.s.	n.s.
Maximum viscosity (BU)[a]	509.5	478	359.5	406	0.0001	0.06
Viscosity at 90 °C (BU)	391.5	313	230.2	240	0.0001	0.0007
Viscosity after 20 min at 90 °C (BU)	232.6	204	133.5	145	0.0001	0.0003
Viscosity at 50 °C after cooling (BU)	391.3	355	198.7	226	0.0001	0.0009
Ease of cooking[b] (min)	10.2	8.2	6.3	6.7	0.0001	0.06
Gel instability[c] (BU)	276.9	269	222.1	261	0.014	n.s.
Gelification index[d] (BU)	158.7	154	65.3	81	0.0001	0.002

* Student's comparison t-Test.
a. Brabender Units.
b. Time to Vmax - Time to gelatinization.
c. Vmax - V90/20.
d. V50 - V90/20.

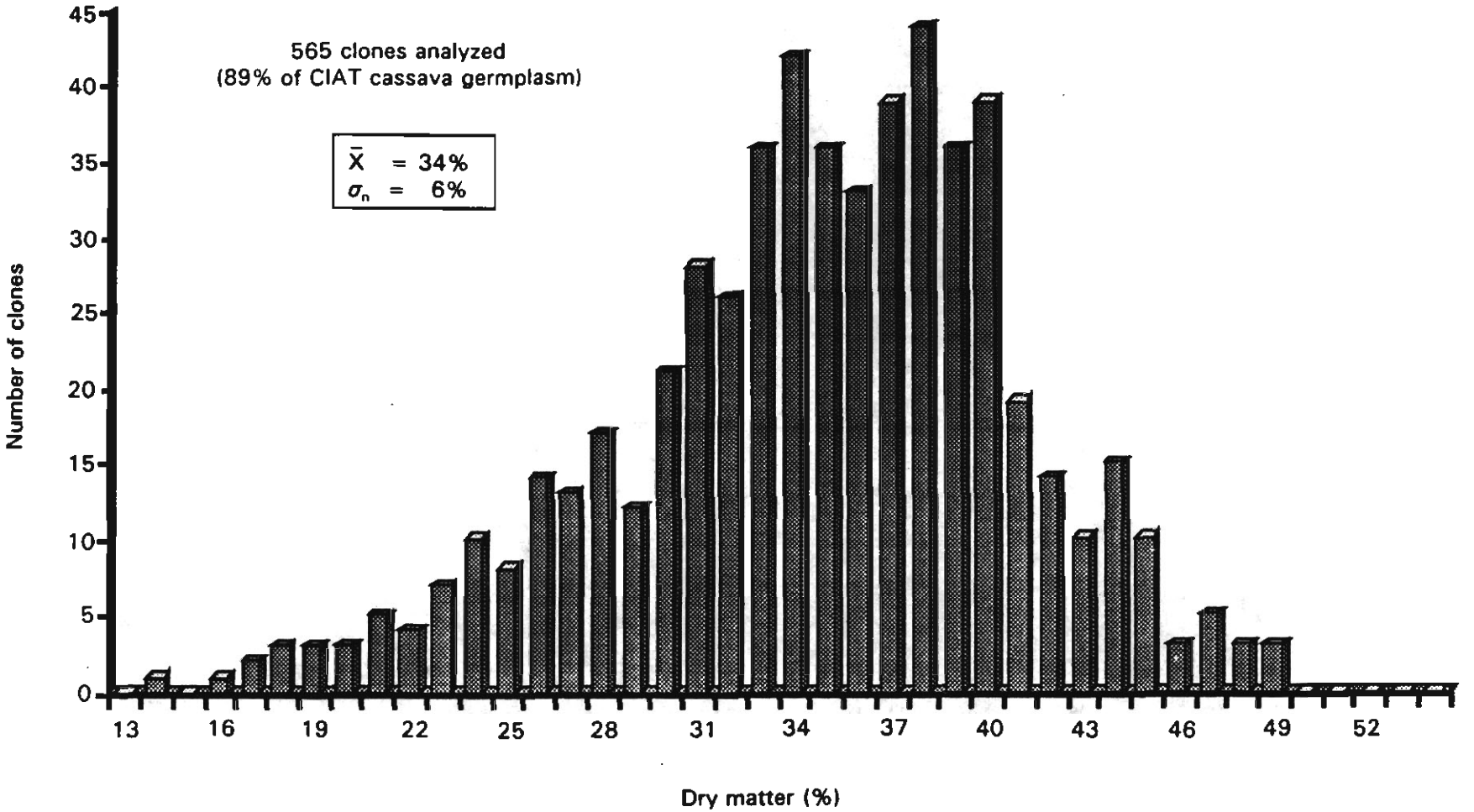

Figure 1. Distribution of dry matter content in cassava germplasm held at CIAT.

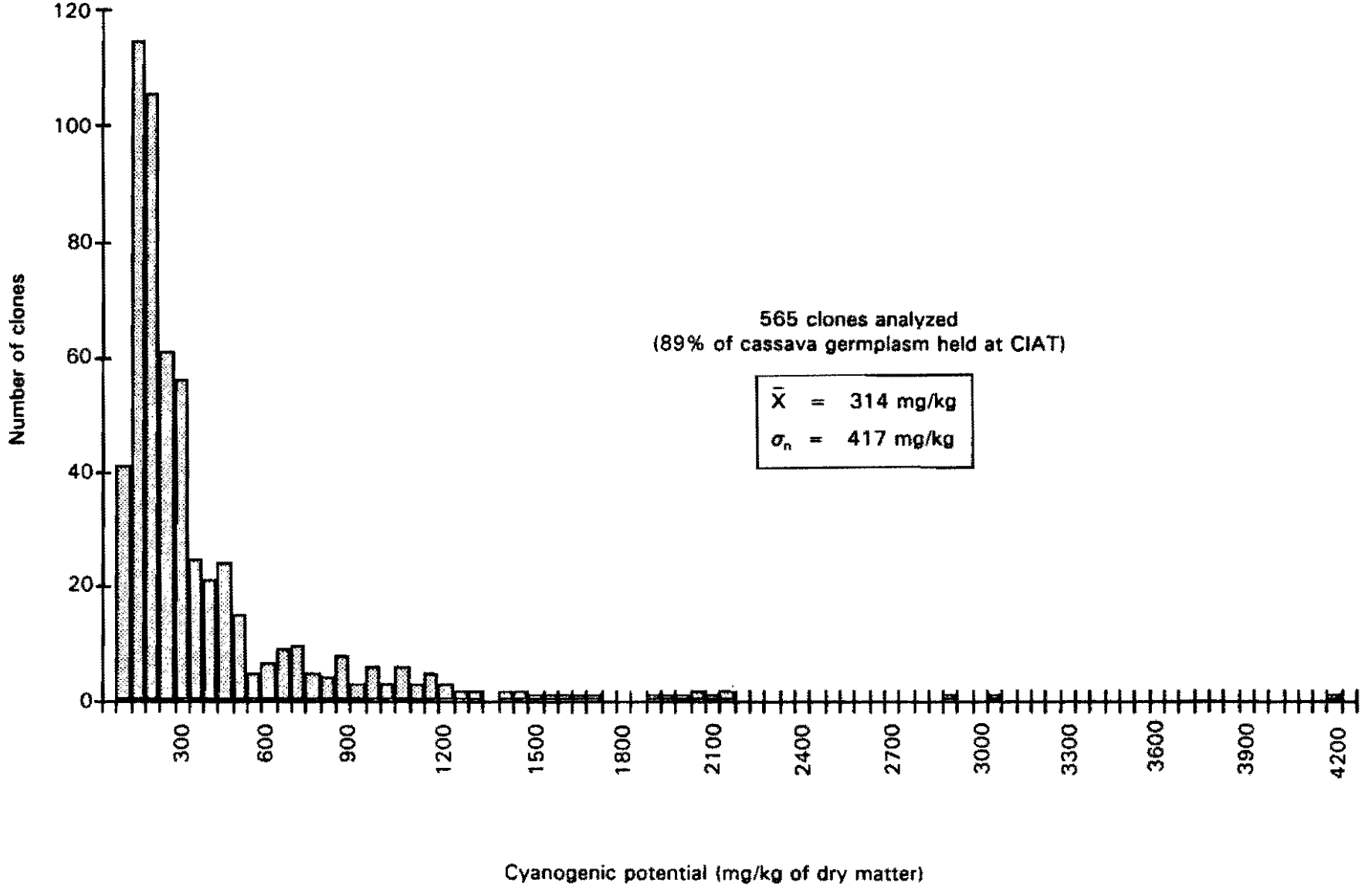

Figure 2. Distribution of parenchyma total cyanogenic potential in cassava germplasm held at CIAT.

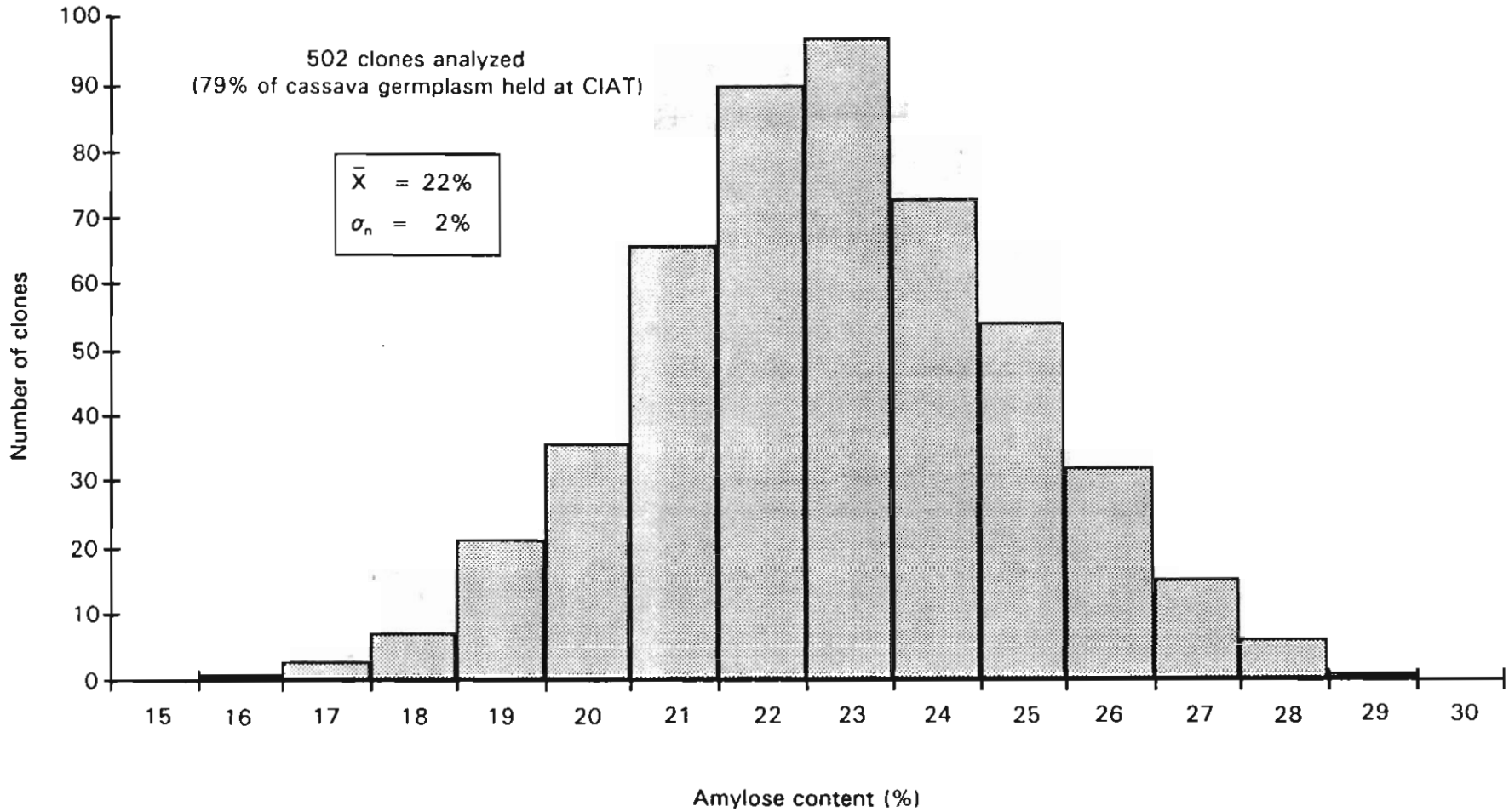

Figure 3. Distribution of amylose content in starch extracted from cassava germplasm held at CIAT.

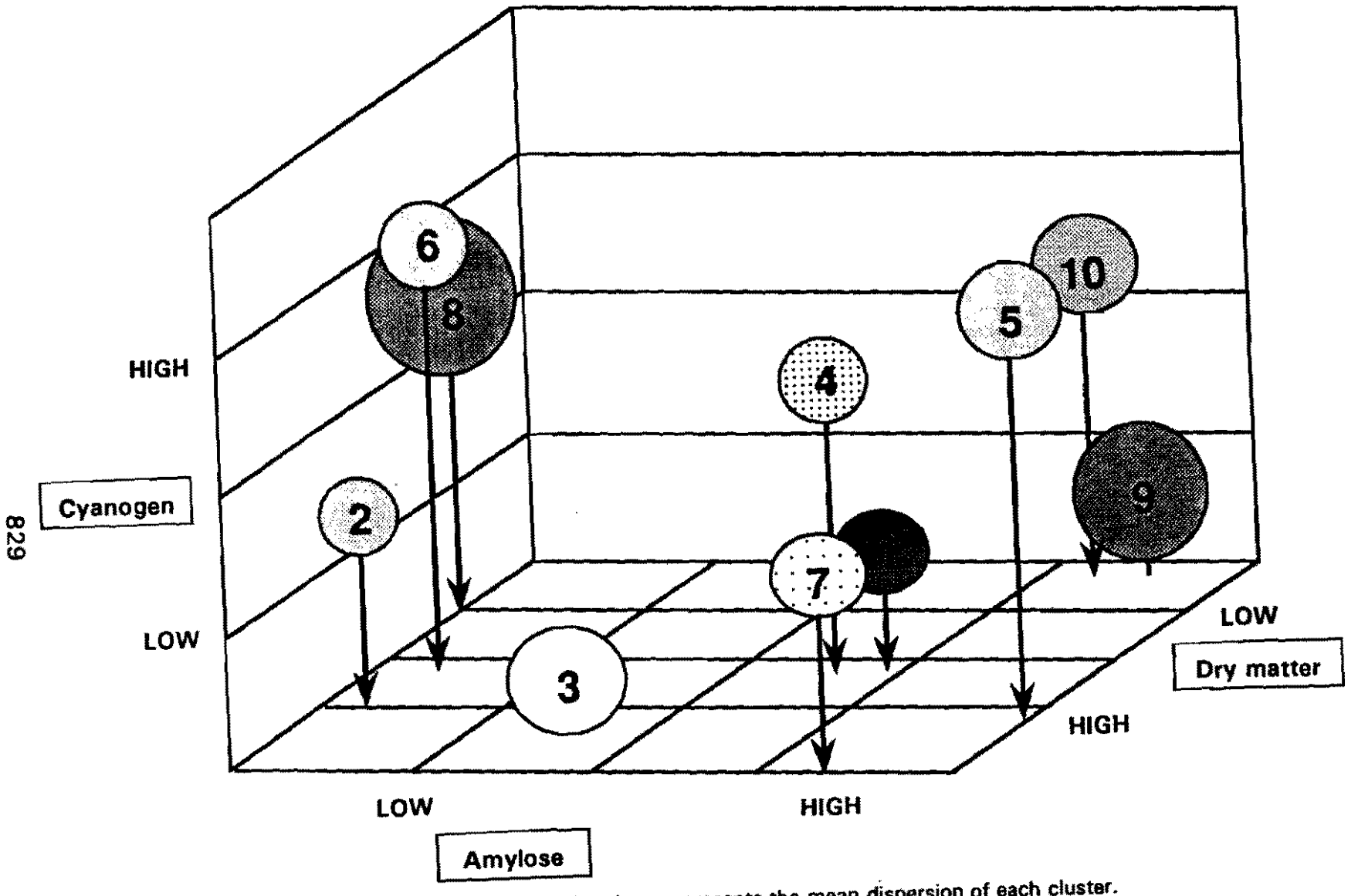

Figure 4. Representation of 10 clusters of clones.

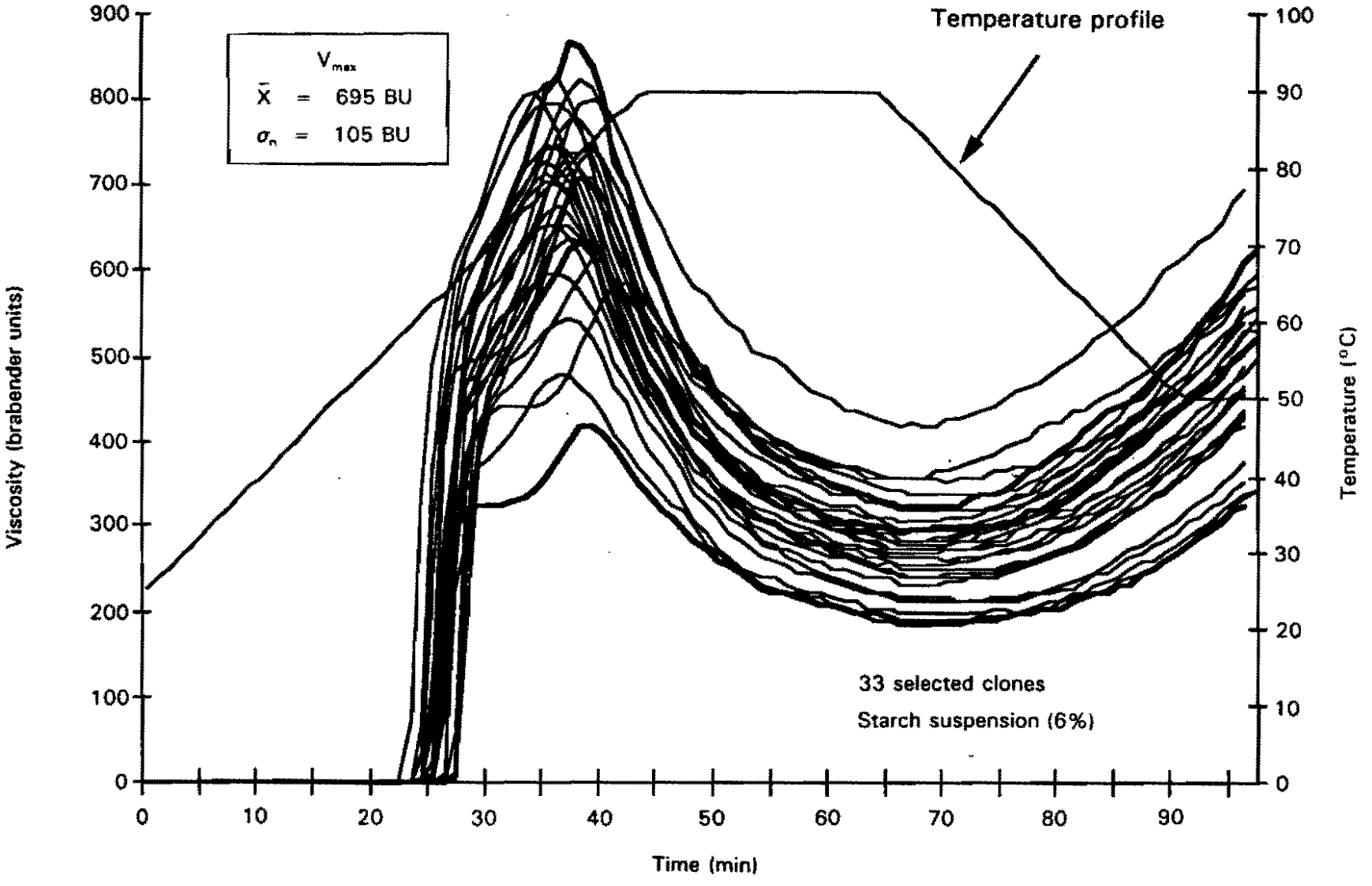

Figure 5. Variation in cassava pasting properties.

WORKING GROUP REPORTS

REPORT OF THE WORKING GROUP ON NEEDS ASSESSMENT AND PRIORITY SETTING FOR CASSAVA BIOTECHNOLOGY

Conveners: G. Henry and J. K. Lynam

Rapporteur: J. K. Lynam

Socio-economics research has focused on integrating needs assessment into a priority setting framework for use by CBN and other cassava researchers. During the meeting, additional areas for socio-economic research to support biotechnological research and applications were identified. In general, the limited number of socio-economists working on cassava have not yet been well integrated into CBN; socio-economic research directly supporting or linked to biotechnological research is as yet non-existent.

Needs assessment

Integrating a user perspective into CBN priority setting will be based on needs assessments of cassava end-users, including farmers, processors and consumers. The CBN commissioned a first needs assessment in northern Tanzania. Several methodological issues arose out of the study that require resolutions in future needs assessments. These include the following:

1. Should needs assessment ascertain farmer priorities or only evaluate farmer problems? If the former, how are priorities aggregated across farmers with different priories or across farmers, processors and consumers?

2. A minimum data set for all needs assessments is required to assist in developing a more systematic understanding across the range of cassava systems and to develop a replicable methodology.

3. The delineations of farmers' field problems do not easily translate into single trait characteristics, which are required for setting of priorities in biotechnological applications.

4. Future case studies employing needs assessments might usefully be linked to possible applications of biotechnology, such as micro-propagation or integrated pest management.

5. A mechanism is needed for better linking of socio-economic researchers in cassava. This will be especially important in developing and applying the minimum data set and in assembling the results for easy access by other researchers.

Priority setting

The development of a priority setting framework is one of the three objectives of the CBN. The scope of the exercise is global. It should assist in establishing priorities in both conventional cassava research as well as biotechnology. The intent of developing such a priority setting capacity is not to rigidly fix priorities by which financial resources would be allocated but rather to provide the most accurate information in a form that can be used by decision-makers. Given the relatively low level of funding provided to cassava research, a priority setting capacity would provide the needed justification in increasing donor funding. Because the potential of cassava is still not widely accepted, the rigor of the analysis will have to be better than for such crops as rice or wheat.

The central element of the priority setting system is a database integrating data from cassava farmers, processors, and consumers. There are several key design features of the planned database. Because the scope of the system is global, the database structure will have two levels. The first, or macro-level will consist of geo-referenced databases for the distribution of cassava production, the agro-climatic conditions under which it is grown, and the different markets in which cassava is utilized. This will produce a production/utilization stratification, weighted by the distribution of cassava. Within this stratification will be embedded the second, or micro-level of the database. This level will integrate the needs assessment, farmer and processor surveys, and crop assessment data. This database will be used to evaluate the potential impact of alternative biotechnological applications. This assessment will then be integrated with an evaluation of the cost and feasibility of developing such technologies.

Case studies

Technology assessment was identified as another area requiring socio-economic research. The priority area in this regard was assessment of present and potential cassava "seed" production and distribution systems. The research would focus on evaluation of the potential farmer demand for cassava artificial "seed" (encapsulated micropropagules) and true seed in comparison to farmers current use of stake cuttings and the use of micro-propagation techniques within varietal distribution systems. Other areas of potential application of biotechnology where similar studies could be designed include integrated pest management, impact of acyanogenetic varieties, and evaluation of environmental regulations on cassava processing.

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

REPORT OF THE WORKING GROUP ON *MANIHOT* GENOME AND GENETIC IMPROVEMENT

Convenor and Rapporteur: G. Kochert

Three main topics were discussed in the meeting:

- Molecular markers: types and advantages/disadvantages of each
- Map construction
- Utilization of the map and molecular markers

The meeting opened with a discussion led by Dr. Mike Gale (John Innes Centre) on the first topic above. RFLP markers were judged to be valuable because of reproducibility and transferability between different populations, however, RFLP analysis is time consuming and expensive. Dr. Martin Fregene (CIAT) related his experience at CIAT using nonradioactive methods for RFLP analysis. These techniques are particularly valuable in developing countries, where it is difficult to obtain isotopes. RAPD markers were judged to be useful for genetic distance estimates and for supplementary mapping use, such as filling in gaps in an RFLP map. The chief advantage of RAPDs is that one can generate a great deal of data very quickly without cloning and sequencing and without use of isotopes. Major limitations of RAPDs are poor reproducibility and difficulty in transferral of mapped markers to new populations. Sequence tagged sites (STS) markers would probably exhibit such a low frequency of polymorphism in cassava that they would have little use in mapping. AFLPs would seem to be useful, primarily for population studies or germplasm screening, and AFLPs do not have obvious utility in mapping. Microsatellites have several advantages for mapping, including a high level of polymorphism and reproducibility of the results. The main disadvantage is the high cost to produce and test each marker.

The pattern of segregation observed for molecular markers in cassava was discussed next. The traditional view has been that cassava is an allotetraploid with a diploid pattern of chromosome segregation (few multivalents). Duplication of molecular markers would thus be expected, however, most RFLPs thus far appear to be single copy and to segregate as a single locus. The mapping population being used at CIAT and the University of Georgia is an F1 of about 90 individuals. A pseudo test cross mapping strategy is being used. In this way recombination in either the female or male parent can be monitored, and two maps are, in effect, produced. No problems were reported with the mapping population, and map construction is proceeding satisfactorily. However, the full population of 150 plants should be utilized to maximize resolution.

Map utilization goals were outlined by Dr. Merideth Bonierbale (CIAT). At CIAT crosses are being made to produce populations segregating for important traits such as cyanogenesis, insect resistance (whitefly), photosynthetic rate and virus

resistance. It is not possible to screen progeny for African cassava mosaic virus resistance in South America, and collaboration is being arranged with a suitable test site.

The main limitation to map utilization in cassava was judged to be the time-consuming nature of cassava population development. As virtually all agronomic traits of cassava are quantitative, large populations will be needed for adequate scoring. The ability to propagate plants clonally will, however, be an important advantage for replication of tests.

Databases such as those being developed for most major US crops were also discussed. One question was when a database for cassava should be started.

Germplasm screening using molecular markers is also an area of great promise for managing cassava collections. Work of this sort is already underway at CIAT, the University of Bath, ORSTOM, and other places.

Recommendations from the Working Group

1. Continue to add markers to the map. Concentrate on RFLPs (genomic and cDNAs) and microsatellites. Also cloned genes of known function should be added to the map in an effort to tie the map to other maps being developed.

2. Implement a database for cassava that would include information on maps and markers, germplasm, genetic stocks, research literature and cassava researchers.

3. Continue to develop segregating populations for tagging agronomic traits. Try to produce populations of at least 150-200 individuals for analysis of QTLs.

4. Initiate some sort of formal training program for those who wish to use molecular markers in their research.

5. A formal system for distribution of clones and molecular marker information needs to be established with funding to support this actively.

6. A "standard" cassava variety is proposed for genome work. This will provide a reference genome for probe characterization and RFLP and RAPD patterns. This variety should be ICA Cebucan (CM-2177-2).

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

REPORT OF THE WORKING GROUP ON *MANIHOT* GERMPLASM CONSERVATION, EXCHANGE AND MICROPROPAGATION

Convenors: G. G. Henshaw and W. M. Roca

Rapporteur: G. G. Henshaw

1. Micropropagation

- As the technology is already available and the need has been established in certain regions, immediate consideration should be given to the implementation of a pilot project in which micro- propagation techniques are used for the delivery of pathogen-tested planting materials to farmers. This would probably involve a blend of micropropagation and CIAT 'rapid propagation' techniques appropriate to local needs and circumstances.

- The first stage should involve a multi-disciplinary feasibility study and cost-benefit analysis for a selected region. This should take into account the implications for other clonally-propagated crops which might benefit from the availability of a common facility.

- Although cassava micropropagation procedures are already well established, there should be some research into the development of a more 'appropriate' technology with reduced infra-structure dependency ("low-tech biotech"). In particular, any reduction in the need for a constant electricity supply would be advantageous.

2. Germplasm storage and transfer

- The further development of ambient-temperature storage procedures such as oil overlay techniques would be useful and they could be investigated in national programs.

- Cryoconservation techniques will be important for the international germplasm collections as a back-up to the in vitro storage procedures currently employed. The techniques could be further improved and recent developments involving encapsulation and/or vitrification should be investigated.

- The monitoring of genome stability during storage will be important but this will only become feasible as high-density gene maps become available.

- There should be some publicity concerning the availability of third-country quarantine procedures for cassava.

3. **True seed and artificial seed technology**

- The investigations of the feasibility of developing a true-seed for cassava, which were given a high priority at a previous meeting of the network, should be brought to a conclusion.

- These investigations should now take into account the possibility of there being an alternative approach involving an artificial seed technology based on the recently developed embryogenic suspension cultures.

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

REPORT OF THE WORKING GROUP ON CASSAVA REGENERATION AND TRANSFORMATION

Convener: C. Fauquet

Rapporteurs: N. J. Taylor and C. Schöpke

The first point noted is that there are now 9 groups working on this topic, which represents a 50% increase from CBN I.

The second point is that, in comparison with CBN I, most of the recommendations have been partially or totally fulfilled:

- efficient of recovery of plants
- new routes of regeneration
- obtention of putative transgenic cassava plants by *Agrobacterium*-mediated transformation
- obtention of fully transgenic cassava embryoids by particle-gun-mediated transformation.

Report on regeneration

1. Somatic embryogenesis
   - positive results achieved in South America, Africa, India and Indonesia, but there is still a strong genotypic dependence
   - major improvement in culture systems and recovery of plants
2. Histological study has established that secondary embryogenesis is a multicellular event initiated in deep layers. This result explains the difficulty of obtaining fully transgenic embryos by particle gun or by *Agrobacterium*.

   Similar studies are encouraged with other regeneration systems.
3. Organogenesis: CBN I recommended the development of alternative regeneration systems, and CBN II heard a report of the production of organogenesis from somatic cotyledons. This represents, in theory, an excellent target tissue for *Agrobacterium* transformation.

4. Embryogenic suspension cultures offer a very significant potential as target tissue for producing transgenic plants, especially by particle gun delivery. Similar systems have been successfully used with cereals. There is some minor concern about the reaction of somaclonal variation, but there is a major advantage for selection and massive production. In addition, the embryogenic suspension constitutes an excellent source for producing protoplasts.

5. New approaches: The production of organogenic shoots from proliferating ancillary buds has been shown. This could constitute a new target for *Agrobacterium*-mediated transformation and, above all, for microtargeting-mediated transformation.

6. Genotypic variation: Local institutes have a role to play in screening locally important cassava genotypes for their morphogenic capabilities. A range of growth regulators and micronutrients, especially copper, should be tested.

7. Somaclonal variation is a slight concern, but the molecular tools needed to evaluate this variability are not available yet.

Report on transformation

1. The recommendation of CBN I has been followed, and the methods of transformation by *Agrobacterium*, particle gun and electroporation have been tested for cassava.

2. *Agrobacterium*-mediated transformation: There is a general agreement that *Agrobacterium*-mediated transformation is highly desirable. Considerable efforts went into developing supervirulent strains of *Agrobacterium*. One natural strain of cassava used by CIAT has led apparently to the production of possible fully transgenic plants. These preliminary results will have to be confirmed and repeated. There is a need for disarmed vectors which should be made available to everyone.

   Two target tissues have been used: somatic embryogenic tissue and organogenic tissue. With the former, there are problems of bacterial attachment to the transformable cells, but this could eventually be overcome by microtargeting associated by *Agrobacterium*. The latter might be more effective for *Agrobacterium* transformation.

3. Particle gun-mediated transformation: Two gun types have been tested: the particle gun (Finner's gun) and the BioRad gun, giving comparable results.

The particle Finner's gun is cheaper ($250) to purchase or to build than the BioRad gun ($10,000) and shooting is cost-free (see R. Sayre, Ohio State Univ., for details).

This system of transformation has been used to achieve a high frequency of transient and stable transformation of cassava

Two types of target tissue have been used: somatic embryogenic tissue, that always leads to the production of chimeric embryos, and embryogenic suspension, produces fully transgenic embryoids.

4. Electroporation: CBN II heard the first report of transient expression on cassava using electroporation-mediated transformation, a technique that still requires tailoring. Embryogenic suspensions could constitute an excellent target tissue.

5. New approaches: A genotypic independent transformation system is needed; microtargeting will be used very soon. The silicon carbide whiskers system could be used on embryogenic suspension, but is not a top priority.

6. Promoters

   - The 35S promoter works in cassava tissues, except in root tissues, and is suitable for developing transformation systems.

   - To use genes of interest, host- and tissue-specific promoters are needed. This type of research should start as soon as possible.

7. Marker genes/selection: All the selectable markers currently used are not very selective. Therefore new markers are needed, for example, luciferase. Non-lethal GUS-type markers are needed for cassava.

   - Some research groups may attempt to use GUS as a selectable marker, as it is used for soybean or sunflower.

Recommendations for cassava regeneration

1. Further develop embryogenic suspensions, particularly for increased regeneration frequency; test the method with a range of cultivars.

2. Repeat the work of regeneration by somatic embryogenesis with numerous cassava cultivars.

3. Develop and compare systems to produce direct organogenesis from somatic cotyledons, especially for *Agrobacterium*-mediated transformation.

4. Develop alternative target tissue for microtargeting, such as regeneration from ancillary buds.

5. Investigate microspore culture and ovule/embryo culture.

6. Recommend national institutes to screen genotypes of local agronomic importance for morphogenic potential.

Recommendations for cassava transformation

1. Further develop transformation using the three systems already tested, particularly to repeat the apparently successful results obtained by CIAT.

2. Test new approaches.

3. Develop combined systems: define the appropriate target tissue for each transformation system.

4. Develop alternative promoters.

5. Test and implement new selection systems.

6. Identify, as soon as possible, supervirulent natural strains of *Agrobacterium* and made them available to researchers.

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

REPORT OF THE WORKING GROUP ON CYANOGENESIS

Rapporteur: M. A. Hughes

This topic was discussed by the Working Group on Biotic Stresses and the Working Group on Processing. In addition, an informal working group consisting of Prof Hughes, Dr. Sayre, Dr. Bokanga, Dr. Okeke and Dr. Thro discussed Cloning of Cyanogenic genes for Cassava Transformation.

Considerable progress has been made since CBN I with 4 new cyanogenesis genes reported in the literature, many of the molecular tools produced and significant advances in our understanding of the biochemistry of cyanogenesis. The "state of the art" in this field is excellent with the research gap being relatively small compared with many of the other fields discussed this week. As a result the technical feasibility of the objectives is high.

The working group on cyanogenesis and gene cloning reaffirms the recommendations of the Cassava Safety Workshop with respect to cyanide and food safety,

These recommendations include:

1. The development of transgenic plants using agronomically important cultivars which over-express linamarase and alpha-hydroxynitrilase to facilitate cyanogen detoxification.

2. The development of transgenic plants which synthesize linamarin in only shoots or roots or which are completely acyanogenic. These plants will prove useful for determining the physiological significance of cyanogen content.

3. Field testing of such transgenic plants to determine yield parameters including sensitivity to herbivore damage and quality.

4. Final phase testing of plants, selected from field trials, with small farmers.

In addition, the working group recommends further investigations on the potential role of cyanogens in crop protection using transgenic and non-transgenic plants with varying cyanogen content and catabolic enzyme (linamarase and alpha-hydroxynitrilase) activity.

The time scale for objectives of recommendations 1 and 2 is estimated as 2-4 years, after the development of a cassava transformation system. The time scale for objectives 3 and 4 is more difficult to assess but plans have been made to implement these in due course.

The major constraints of their work are:

1. a reliable cassava transformation system.

2. the identification of tissue specific cassava promoters.

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

REPORT OF THE WORKING GROUP ON BIOTIC STRESS ON CASSAVA PRODUCTION

Convener: B. D. Harrison

Rapporteur: R. M. Cooper

Highlights

Virus diseases of cassava

1. Transgenic resistance. Viral coat protein - mediated resistance to common cassava mosaic virus (CCMV) works in *Nicotiana benthamiana* but is less promising for African cassava mosaic virus (ACMV). However, the first evidence of stronger transgenic resistance to ACMV has been obtained —conferred by a disfunctional ACMV replicate gene.

2. Cassava geminiviruses. Three viruses have been distinguished by comparison of their DNA sequences and epitope profiles: ACMV, East African cassava mosaic virus (EACMV) and Indian cassava mosaic virus (ICMV). The three viruses can be detected and distinguished by their reactions with selected monoclonal antibodies (MAbs). The viruses can also be detected and distinguished by PCR-based tests —even in single whiteflies.

Other pathogens and pests of cassava

- Progress but no great highlights.

Report of Working Group on Biotic Stress

Several scientists around the world are studying the occurrence, properties, epidemiology and control of cassava pathogens and pests but few are using the tools of biotechnology in this work. This summary is restricted to the use of biotechnological approaches:

a. to study the nature and properties of pathogens and pests and

b. to solve problems caused by pathogens and pests that are not being solved by conventional approaches.

Members of the Working Group considered that the principle agents meeting those criteria are as follows:

a. Cassava mosaic gemini viruses - the top priority.

b. Cassava bacterial blight (CBB) - second priority.

c. Cassava root rots (fungal complex?).

Root rots have been suggested to be an important problem in several countries but further work is needed on their etiology and significance before the relevance of biotechnological approaches can be assessed.

Agents causing leaf lesions such as *Cercospora* spp. (eg in Malaysia) and *Colletotrichum* (in Nigeria) were considered to merit further research but are not currently amenable to biotechnological approaches.

Major arthropod pests such as mealybug and cassava greenmite were considered to be amenable to control by plant breeding and/or the use of predators and parasites.

Biotechnological methods (molecular markers) are being used to develop and monitor IPM systems to control arthropods.

Technologies available

a. Diagnostics and pathogen variation

From work described in the plenary session it is clear that cassava can be infected by three distinct whitefly —transmitted geminiviruses (African cassava mosaic (ACMV), East African cassava mosaic, Indian cassava mosaic viruses). It cannot be assumed that results obtained with one of these viruses will apply to the other two, eg, genetic resistance, epidemiology, vector transmissibility.

The three viruses can be detected and distinguished by ELISA with selected monoclonal antibodies. Also the viruses can be detected and distinguished by PCR for special purposes, such as samples from single virus-carrying whiteflies. In the Working Group the need to develop a robust and wholly monoclonal antibody-based test for the geminiviruses, and suitable for transfer to all cassava growing countries, was emphasized.

In addition at least twelve other viruses have now been recorded in cassava and serological methods are available for detecting several of them. Test plant methods must be used for the others.

The need to study genetic variation in pathogens such as CBB and ACMV was underlined.

b. Gene mapping

Work has been initiated to map genes for resistance to ACMV and CBB and the working group considered that this should be encouraged and extended to resistance genes for other major pathogens and pests.

c. Transgenic plants

Work described in the plenary session provided the first evidence that plants transformed with a gene based on a dys functional version of ACMV replicate show considerable resistance to the virus. This resistance is expressed as resistance to infection coupled with a decrease in severity of symptoms in those plants that become infected. However, this resistance has so far been tested only in an artificial system (*Nicotiana benthamiana*). The urgent need to continue this work and to explore allied resistance strategies was agreed. It is important that any transgenic resistance should be tested against all three cassava mosaic geminivirus and its durability assessed. The progress in transferring this type of resistance to cassava is dependent upon the development of satisfactory transformation procedures.

As regards bacterial and fungal pathogens the Working Group felt that an alertness should be maintained to the development of applicable transgenic strategies based on candidate genes such as lysozyme and cecropins. The outcome of tests with other plant species will be a valuable guide to the usefulness of such approaches with cassava.

Points of discussion

- the importance of damage caused by red mites in some environments was emphasized.
- if transgenic resistance is successful for ACMV it would not require a great additional effort to apply it to EACMV and ICMV.
- the dynamic nature of interactions between pathogens, pests, virus vectors and their hosts should be borne in mind and a watching brief maintained on the emergence of major problems associated with changes in genetic constitution of the pests and pathogens or their introduction into new areas.
- a need was expressed for diagnostic reagents for the cassava geminiviruses for use in cassava-growing countries.

- further studies are needed on other newly discovered or poorly studied viruses in cassava with a view to developing diagnostic tests for them. Care should be taken to ensure that none of these viruses should be disseminated in clonal material sent from one country to another or used in schemes for propagating virus-tested stocks.

- there is an almost total lack of information on mechanisms of bacterial and fungal pathogenesis and host resistance in cassava. Identification of key events in resistance could facilitate the identification of genes suitable for use in transgenic resistance.

- there are indications that high cyanide levels may confer some resistance to pests which have affected cassava only recently or for which cassava is not the main host.

Recommendations

1. Cassava mosaic geminiviruses (highest priority)

    - transgenic resistance (transgenic cassava plants 2-4 years after availability of transformation protocol); field tested lines for field scale planting an additional 5 years).

    - wholly monoclonal antibody-based ELISA (1 year if funded).

    - transfer of monoclonal antibody-based ELISA to cassava growing countries (from 2 years if funded).

    - characterization of variant forms (2 years if funded).

2. Other viruses (intermediate priority)

    - characterization and development of diagnostic tests (from 2 years if funded).

3. Cassava bacterial blight (intermediate priority)

    - molecular variation among isolates within and between geographical areas (2-3 years).

    - key biochemical components of disease resistance (2-3 years).

    - search for somaclonal variation in resistance by exposing embryogenic suspension cultured cells to bacterial materials (2-3 years).

4. Fungal pathogens

- maintain watching brief for emergence of applicable biotechnological approaches.

5. Arthropod pests

- no urgent need for biotechnological approaches for direct control of major pests was identified but the situation should be kept under review.

Gene mapping (high priority)

- mapping genes for resistance to ACMV, CBB and other major pathogens and pests.

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

REPORT OF THE WORKING GROUP ON ABIOTIC STRESS ON CASSAVA PRODUCTION

Convener and Rapporteur: R. Howeler

A limited amount of research has been conducted using biotechnological approaches to try to improve the uptake or utilization of plant nutrients as well as to prevent the excessive uptake of toxic elements such as AL or NA. The latter is easier to achieve than the former. Little or no research has been conducted using cassava due to a lack of basic knowledge about its physiological processes and which genes determine them. This will be the main obstacle in genetic engineering for improved plant nutrition and in alleviating other abiotic constraints.

The main objective of the working group was to assess the feasibility of using biotechnology—especially gene transfer—as a means of speeding up genetic improvement with respect to nutrient absorption and use or the alleviation of other abiotic constraints, vis-a-vis the use of conventional breeding or agronomic practices.

Concerning abiotic constraint affecting cassava production, the following observations can be made:

1. Drought

   Cassava has several mechanisms to tolerate drought and as a species is extremely drought tolerant. Still, varietal differences in this character have been identified, and large numbers of accessions and breeding lines are being evaluated under severe drought conditions.

2. Flooding

   Not so common, but in some locations, very important. Cassava is very susceptible to flooding, and most plants die if flooded for more than 4-5 days. Limited germplasm screening shows some genetic differences.

3. Low temperatures

   Important at high altitude and in subtropics.

4. High temperature with dry winds

   Only important in some isolated areas.

5. Shade

Important when cassava is intercropped under coconut or rubber. Some varietal differences observed, but in general cassava yields are seriously reduced.

6. Acid soils

Cassava is extremely acid-soil tolerant (up to 85% Al sat.); Al toxicity and extreme acidity will reduce yields only in isolated areas such as the Eastern Plains of Colombia and peat soils in Malaysia.

7. Low N

Cassava requires 200-300 kg N/ha for a normal crop, but about 2/3 of this is recycled as fallen leaves and crop residues. N is the main limiting nutrient in most soils in Asia, as well as in sandy soils in Latin America.

8. Low P

Cassava is extremely mycorrhizal dependent, but with an efficient association of vesicular-arbuscular mycorrhiza (VAM), it tolerates very low levels of available soil P (± 4 ppm P). Relatively little P is removed in the root harvest.

9. Low K

Cassava requires 150-200 K_2O/ha for normal growth, and about 2/3 of this is removed in the root harvest, leading to soil-K depletion if the crop is grown continuously without adequate K input.

10. Low Ca, Mg, S

Only important in some isolated areas.

11. Low micronutrients

Mainly Zn deficiency; others in isolated areas only.

12. High salt

Cassava is very susceptible to high salt or Na, but it is seldom cultivated in saline/alkaline soils.

Table 1 shows the abiotic constraints considered in decreasing order of importance and ranked as to whether the problem can be solved best through genetic manipulation (conventional breeding or biotech) or by cultural practices.

Conclusions

1. In the short-to-medium term, most problems are best solved through cultural practices, except drought stress, in which breeding, possibly with biotechnological assistance, can make a major contribution. After identifying genotypes which are contrasting in terms of drought tolerance and studying the inheritance of this tolerance, the mapping of genes might be considered. Once principal genes are identified, drought tolerance might be enhanced through molecular-marker assisted selection or gene transfer.

2. In the medium-to-long term, nutrient use efficiency might be enhanced through breeding and selection under low-fertility conditions. In Asia, however, most farmers will try to increase yields through fertilizer application.

3. In some isolated areas, genetic improvement for flood, shade, or wind tolerance will be very useful.

Recommendations

1. It is recommended that CBN stimulate more basic research on the various physiological processes that make cassava tolerant to drought, to study the major genes controlling these processes, and possibly to map out these genes so as to target better any possible gene transfer with the objective of further improving the crop's drought tolerance.

2. The feasibility of transferring the genes responsible for cassavas acid-soil tolerance into other plant species could be assessed.

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

Table 1. Abiotic constraints to cassava production and relative value of genetic manipulation and agronomic practices.

Constraint	Genetic manipulation	Agronomic practice
Drought	+ + + (drought tolerance)	+ (irrigation, time of planting)
K-deficiency	+ (increased K efficiency)	+ + + (K fertilizer, ash)
N-deficiency	+ (increased N efficiency)	+ + + N application, reduce N losses)
P-deficiency	+ (increased P efficiency)	+ + + (optimize VAM association; P fertilizer)
Flooding	+ + (short duration varieties)	+ + (site selection, ridging)
Cold temperature	+ + (cold tolerance)	+ + (date of planting)
High wind	+ + + (short stature varieties)	+ (method of planting; date of planting)
Soil acidity	+ + (Al tolerance)	** (liming)
Shade	+ + + (shade tolerance)	+ (plant population, fertilizer application)
Salinity	+ + + (salt tolerance)	+ (S or gypsum application)

REPORT OF THE WORKING GROUP ON CASSAVA PROCESSING, QUALITY AND NEW PRODUCTS

Convenor and Rapporteur: N. Poulter

1. This Working Group was newly created (not having been in existence at CBN I in Colombia in 1992) because of the importance of conventional biotechnology options in the post-harvest sector and prospects for more immediate impact with beneficiaries.

2. The group identified seven important theme areas for discussion, each of which were discussed in turn in greater detail. Discussion was focused to provide an update on the state of our current knowledge in these areas, the research gap and feasibility of producing usable research outputs and their time frame and the problems and opportunities existing in each of them. The group then attempted to integrate this knowledge to provide an indication of relative priorities.

3. The seven theme areas were:

   Post-harvest deterioration

   Thickened storage (tuberous) root conformation

   Storage proteins

   Cyanogens

   Starches

   Micro-nutrients, and

   Environmental pollution control and value added processing

4. Post-harvest Deterioration (PHD): This was considered to be a key constraint to the trading and consumption of fresh cassava, and also significantly influences the efficiency and quality of starch and flour extraction industries. PHD is a major causal reason for post-harvest losses which can be as high as 20% globally. A significant impact can be achieved if the expression of PHD was minimized for 3-4 weeks. Current knowledge on the biochemistry and physiology of PHD is substantial, but gene control of these pathways is very poorly understood or researched. Prospects, with concerted action, to elucidate these controls were good with a time frame of 3-5 years before the

basic information were to become available. Comprehensive screening of germplasm, requiring the further development of rapid methods, for assessment of natural variability of this trait were highly recommended and could be undertaken in parallel with more fundamental molecular studies.

5. Thickened storage (tuberous) root conformation: The ease of harvesting of the plant has a significant impact on labor needs and costs. More compact roots may be easier to harvest, with less damage to the roots and could thereby reduce the costs of labor associated with cassava production, a fifth of which can be attributed to harvesting. Knowledge in this theme area is almost entirely lacking and the prospects for biotechnological interventions are therefore unknown, but likely to be limited over a long term time frame. However, tuberous root conformation is a highly heritable trait which is screened in conventional breeding programs, where any continued focus of attention on this trait should be directed.

6. Storage proteins: The issue of quantity and quality of proteins in cassava roots was animatedly discussed. Key points included the need to source proteins in the diet from the roots when the cassava leaves contain significant quantities of apparently good quality protein (this might also apply to micro-nutrients see below), the possible negative implications of the presence of proteins in starch extraction processing, and the implications of sustainable production of higher protein roots if there were to be an increased requirement for nitrogen from already impoverished and low-fertility soils (soil/sink relationships). The current understand of protein synthesis and manipulation of genetic material in cassava is poor and it is in its formative stages. Any biotechnological interventions at the genome level must be considered in a long time frame and there is currently no guarantee ofsuccess. However, an improved understanding is considered valuable in broadening our knowledge of the biology of cassava. More comprehensive screening of germplasm for high protein types might be given emphasis. Some are already available at IITA.

7. Cyanogens: It was considered that conventional post-harvest processing technologies under normal circumstances effect the reduction of cyanogens to acceptable levels and that our knowledge of the food science and biochemistry of this subject was well advanced. This is in agreement with the conclusions of the International Workshop on Cassava Safety held recently at IITA. However, under conditions of food insecurity short cuts in processing can occur and problems may then arise from exposure to residual cyanogens in the foods consumed. The incidence of these problems may increase in frequency and significance as the causative effects, eg drought, war etc become more prevalent. Tools exist to raise the linamarase and hydroxynitrile lyase activity in transformed bacteria responsible for natural fermentations and these interventions might specifically assist in these

circumstances. This would indicate that the further development of improved starter cultures for cassava fermentation may have significant impact. The importance of possible relationships between cyanogen levels (Cyanogen potential - CNP) and other traits, including diseases/pest tolerance, and quality characteristics, particularly starch qualities was stressed.

8. Starches: The issues of quantity and quality of starches differ depending on the end uses, particularly industrial users versus consumers. Such issues include the efficiency of starch extraction and implications for reducing pollution effects of waste streams, starch digestibility both in the modified starch industry and from the human nutrition perspective and consumer acceptability including organoleptic and rheological qualities. The opportunities for improving the competitiveness of cassava starch with other traditional starch sources is high. The cloning and characterization of starch biosynthetic genes of cassava is fairly well advanced and the key constraint to impact is effective transformation and regeneration. However, it was noted that more comprehensive screening of germplasm to assess more closely the natural variability in the content and qualities of this major component of cassava, requiring the further development of appropriate rapid methodologies of quality assessment, is required. It was concluded that prospects for significant impact in this theme area were within a short to medium time frame and that our knowledge of the science was well advanced. High priority was therefore accorded to future research on starch.

9. Micro-nutrients: The issues of micro-nutrient availability to consumers, including particularly Vitamins A and B and iron and the nutritional policies being developed by IFPRI stimulated the inclusion of this theme area in the groups discussions. The state of knowledge regarding micro-nutrient production/accumulation and prospects for biotechnological interventions was limited and weak. Prospects for alternative solutions to problems that may exist in those populations most at risk from micro-nutrient deficiencies may be most viable, including the use of cassava leaves. Further evaluation of this theme area is required.

10. Environmental pollution control and value added processing. These can often include conventional biotechnological interventions, including improvements in the efficacy and use of microorganisms. The use of microorganisms to enrich animal feeds, to derive products from starches, including ethanol and colorants and to reduce the polluting effects of effluent streams through reducing chemical and biological oxygen demand (COD and BOD) have high potential for significant impact in an expanding commercial sector. The state of knowledge in the theme area is generally well advanced and prospects for impact in the short term is high.

11. It was stressed repeatedly during the working group's deliberations that for all theme areas, robust studies are required to evaluate more thoroughly the need and likely impact of any interventions, the socio-economic constraints and opportunities which might guide priority setting within the working group and the need to consider institutional strengthening, training and dissemination at all levels of the subject.

Note: For more information or the names of participants in this working group, contact the working group convener or the CBN Coordinator.

STEERING COMMITTEE RESEARCH HIGHLIGHTS REPORT

Report prepared by C. M. Fauquet, G. G. Henshaw and N. Poulter

Review presentations on cassava research areas other than biotechnology reflected the fact that many cassava problems still need basic research to better appreciate the needs and the potential solutions. Other presentations brought the latest developments in plant biotechnology, regardless of species, and this aspect of the program that may be further developed at CBN III. The number of biotechnology teams working on cassava has increased and should be further increased. CBN should play a role in attracting increased inputs to cassava biotechnology research. Socio-economics research has focused on integrating needs assessment into a priority setting framework for use by CBN and other cassava researchers. During the meeting, other areas for socio-economic research to support biotechnological research and applications were also identified. In general, the limited number of socio-economists working on cassava have not yet been integrated into CBN; socio-economic research directly supporting or linked to biotechnological research is as yet non-existent.

The development of a priority setting framework is one of the three objectives of the CBN. The scope of the exercise is global. It should assist in establishing priorities in both conventional cassava research as well as biotechnology. The intent of developing such a priority setting capacity is not to rigidly fix priorities by which financial resources would be allocated but rather to provide the most accurate information in a form that can be used by decision-makers. Given the relatively low level of funding provided to cassava research, a priority setting capacity would provide the needed justification in increasing donor funding.

Technology assessment was identified as another area requiring socio-economic research, once constraints and priorities had been identified. For example the priority area in this regard would be assessment of present and potential cassava "seed" production and distribution systems. The research should initially focus on the evaluation of well-established micropropagation technology for the production and distribution of micropropagules, either to intermediate multiplication nurseries producing traditional stake cuttings or directly to the farmer, and could be extended to consider the implications of true or artificial seed. Other areas of potential application of biotechnology where similar studies could be designed include integrated pest management, impact of acyanogenic varieties, and evaluation of environmental regulations on cassava processing.

During this meeting the first molecular map of cassava was presented. The work is progressing rapidly: 150 markers have already been inserted. By the end of 1995, there should be enough markers to produce a map that could be used for

practical purposes. The coordination of the efforts between CIAT and the University of Georgia is excellent and common decisions have been made.

Map utilization goals were outlined and at CIAT crosses are being made to produce populations segregating for important traits such as cyanogenesis, insect resistance (whitefly), photosynthetic rate and virus resistance. The main limitation to map utilization in cassava was judged to be the time-consuming nature of cassava population development. As virtually all agronomic traits of cassava are quantitative, large populations will be needed for adequate scoring.

Studies on *Manihot* wild species initiated several years ago, are progressing fairly well and they demonstrate that the center of origin for *Manihot esculenta* is probably Brazil. A better knowledge of the relationships between these species should allow scientists in the long term to use some of these species as a source of natural genes for cassava improvement.

Implementation of a database for cassava that would include information on maps and markers, germplasm, genetic stocks, research literature and cassava researchers, has been recognized as a need and it has been proposed that CBN could initiate this database as a network project.

Micropropagation of cassava cultivars has been recognized as a biotechnological tool that is ready for implementation and it has been stated that CBN should be more proactive in this area. Several examples of utilization of this technique (China, Congo, Uganda...), were presented. These techniques, developed on a large scale, in association with virus cleaning technologies, could be extremely beneficial for cassava farmers. Funding should be sought to set-up such projects in Africa.

Progress has been made in cryo-preservation techniques that demonstrated the potential usage of such technologies for germplasm conservation purposes. With the development of embryogenic suspensions and the development of embryo-encapsulation protocols, the production of artificial seeds is now a technical possibility. This raised the question of the benefit to the farmers of true seeds and/or artificial seeds. Some socio-economic studies have been initiated by CIAT and it was questioned if the CBN should on its own initiate a study in this respect. It has been stated that investigation is needed to assess effects of existing well-established micropropagation technology. For the longer term, investigation is needed to consider to consider implications of completely new technologies based either on "artificial seed" (encapsulated micropropagules) or on true seed.

Since CBN I, tremendous progress has been made in cassava regeneration. Plant recovery efficiency of somatic embryogenesis has been boosted to high levels and it has been demonstrated that this method of regeneration can be extended to a number of cultivars in different countries. The range of regenerable genotypes

has still to be expanded and the CBN can play an active and important role in this domain.

New regeneration pathways have been investigated and, in particular, a highly regenerable embryogenic suspension has been obtained with at least two cassava cultivars. This is considered to be a breakthrough for cassava transformation purposes, and the system has already been used by one group to produce fully transgenic embryoids. Other ways to regenerate cassava need more investigations and should be pursued in order to offer alternatives for transformation.

Cassava transformation is a bottleneck for many scientists that already have genes ready or being developed in the near future. A lot of progress has been made in this field and CIAT presented the first putative transgenic cassava plants. The final proofs will be provided in the next months and if confirmed, it would be a door opened for other groups to repeat these results. There are now nine teams in the world working on cassava transformation. These teams will use all the techniques of transformation available. In conjunction with the improved regeneration protocols, it is hoped that this high priority goal will be achieved in a few months.

If cassava transformation is really close, new needs will appear, including root specific promoters and transformation/regeneration protocols for particular cassava genotypes. It has been requested that the network should identify the most important cassava cultivars to be used in the future for genetic engineering.

Three major biotic stresses have been identified: cassava geminiviruses (ACMV...), cassava bacterial blight (CBB) and cassava root rots. Serological and molecular diagnostic tests are available for several viruses. It was recommended that these tests should be developed for technology transfer to national programs on a large scale. Work has been initiated to map genes for resistance to ACMV and CBB and it was considered that this should be encouraged and extended to resistance genes for other major pathogens and pests. Coat protein-mediated resistance to cassava common mosaic virus works in tobacco, but is less promising for ACMV. However, the first evidence of stronger transgenic resistance to ACMV, conferred by a dysfunctional ACMV replicase gene, has been obtained. Transformation of cassava is considered as the major constraint to the development of resistant plants by genetic engineering. As regards bacterial and fungal pathogens an alertness should be maintained to the development of applicable transgenic strategies based on candidate genes such as lysozyme and cecropins. The outcome of tests with other plant species will be a valuable guide to the usefulness of such approaches with cassava.

A limited amount of research has been conducted using biotechnological approaches to try to improve the uptake or utilization of plant nutrients as well as to prevent the excessive uptake of toxic elements like Al or Na. Little or no

research has been conducted using cassava due to a lack of basic knowledge about physiological processes and about which genes determine them. For cassava, most problems are best solved through cultural practices, except drought stress, in which either conventional breeding or biotechnology can make a major contribution. After identifying genotypes which are contrasting in terms of drought tolerance and studying the inheritance of this tolerance, the mapping of genes might be considered. Once principal genes are identified, drought tolerance might be enhanced through gene transfer.

Considerable progress has been made since CBN I with four new cyanogenesis genes reported in the literature, many of the molecular tools produced and significant advances in our understanding of the biochemistry of cyanogenesis. The "state of the art" in this field is excellent with the research gap being relatively small compared with many of the other fields discussed during the week. An excellent report was given about the Cassava Safety Workshop held at IITA, Nigeria in 1994, relative to the cyanogens in cassava and although their role is still unclear, it has been recommended to engineer several enzymes in cassava in order to better understand the effects on quality and production, of under or over expressing catabolic enzymes in the cyanogenesis pathway.

The cassava processing and quality working group identified seven important theme areas: post-harvest deterioration, root conformation, storage proteins, cyanogens, starches, micro-nutrients, and microbial biotechnologies for environmental pollution control and value-added processing. Discussion was focused to provide an update on the state of our current knowledge in these areas, the research gap and feasibility of producing usable research outputs. For all these areas, robust studies are required to evaluate more thoroughly the socio-economic constraints and opportunities and likely impact which might guide priority setting within the working group and the need to consider institutional strengthening, training and dissemination at all levels of the subject.

There is a general consensus that the CBN is maturing and increasingly well organized. Exchange of information within the network is increasing. It is also a common feeling that CBN should be more pro-active in many respects including seeking funds, initiating actions and developing studies either on CBN funds or external funds whenever possible.

CONCLUDING REMARKS

R. Best

Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia

I would like to thank the organizers for giving me the opportunity of saying a few words at this final session. I do so not so much in my capacity as leader of CIAT's Cassava Program but rather an interested and close observer of the evolution and development of the CBN and as a participant in CBN II.

Not having an official function within the organizational structure of the CBN—since Willy Roca very ably represents CIAT on the Steering Committee—gives me, I hope, the opportunity to be objective as to the progress being made with respect to the orientation and development of the Network.

I do not want to dwell on the scientific progress of CBN. This has been very adequately summarized by the respective working groups and one cannot help but be infected by the enthusiasm of people such as Claude Fauquet as they communicate to us the excitement of the progress in their work. There are two issues that I should like to deal with: one on the relative formality or informality of the CBN, the other on a number of institutional aspects that I believe the CBN may need to address in the future.

Formality versus informality. Ann Marie (CBN Coordinator) refers to the CBN as an "informal forum for biotechnologists, their collaborators, and cassava research users". I presume that she uses the term informal in the sense that CBN membership is open to all interested parties as a means of facilitating the incorporation of a wide range of individuals and institutions with diverse interests and disciplines, and of also enhancing the free flow of information between members.

Despite this "informality" in terms of membership, the CBN has in reality a very formal structure. The Network is implemented within the framework of a specific project that has a goal and specific outputs to deliver. Its operation is guided by a Steering Committee, supported by a Scientific Advisory Committee, it holds scientific meetings, publishes a newsletter and has a small grant facility. And, as we have appreciated this week, it provides a forum for trying to match technology supply to the concrete needs—or demand—of the end users of biotechnology applications.

The issue that arises is the extent to which CBN should try to influence or manage the prioritization of research. John Lynam in his summing up suggested

that CBN does not have a role in controlling this process and it is my opinion that it would be very dangerous for the CBN to act in this capacity. Any attempt to stifle scientific creativity in the search for new tools or the generation of new knowledge on the crop would undoubtedly be detrimental to overall progress towards realizing the full potential of cassava. CBN does have, however, a clear responsibility and mandate to provide clear and concise quantitative information on the principal constraints to and opportunities for realizing the crop's potential. And, leading from that, an attempt needs to be made to estimate the costs and benefits of differential technological interventions to resolve those constraints or opportunities. This is not a passive process and requires the intellectual input and honesty of all those associated with the CBN.

This type of analysis is not only of interest to the CBN but to all those of us that are involved in cassava R and D. As such, this activity will receive the unconditional support of CIAT's Cassava Program. To my knowledge, the rigorousness of the priority setting process actually being undertaken is far ahead of similar exercises for other commodities, a fact that should stand us in good stead for capturing additional resources for our work.

Turning to institutional issues. Not being party to the details of the discussions leading up to the organization of the workshop, I may be speaking out of turn. However, I was surprised that the issues of biosafety and intellectual property rights hardly got a mention during the week. While I do not believe that, at a scientific meeting, these issues should take up a lot of time, CBN needs to maintain its members informed of developments in these areas and draw attention to possible institutional obstacles that could influence the availability or use of biotechnological applications in the future.

A further institutional issue that I consider to be of importance to the CBN is the relative capacity of different cassava producing countries to harness the advances in biotechnology. This capacity obviously varies significantly from country to country. Any strategy that the CBN might wish to pursue in terms of human resource development should not be biased towards those relatively more advanced countries that presently have the infrastructure and human capital to benefit from biotechnological applications. In two years time, CBN III will be held in Africa and I believe by that time the Network should have made progress towards addressing this issue.

Putting on my Cassava Leader's hat and now representing CIAT, the "home" of the CBN, I would like to acknowledge the support received from the Netherlands Foreign Ministry and, in particular, the dedication of Hans Wessels. It is seldom that a donor representative takes such an active and interested part in the running of a project. The open and frank dialogue that this engenders is obviously beneficial for both parties. Thanks are also due to the other sponsors of this meeting: the Rockefeller Foundation, the Thai Tapioca Development Institute, the Technical

Centre for Agricultural and Rural Cooperation and the United States Agency for International Development. We would also like to recognize all those institutions and individuals that have either financed representatives or used their own resources to participate in the meeting.

Speaking now on behalf of the participants, I would like to express our appreciation to the members of the Steering Committee and to the Coordinator for the time and effort they put into planning the meeting. A very special vote of thanks goes to CRIFC and the team of very dedicated organizers led by Dr. Ahmed Dimyati. I should like you to join with me in extending to them our gratitude for their warm hospitality and untiring efforts to make our stay in Indonesia unforgettable. Thank you.

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