CASSAVA

1. Cryopreservation of shoot-tips

Cryopreservation provides a means for the long-term storage of cassava clones, seed and pollen. With the collaboration of the IBPGR, that provided support for a post-doctoral fellow (Ma. Luisa Marin) and research assistance (R. Escobar) research was conducted on cassava cryopreservation between 1988-90.

Due to the lack of reproducibility of previous cryopreservation reports, we decided to use seeds as specimens for freezing experiments. In 1989, 90-100% seed germination was obtained after rapid immersion of whole seeds into liquid nitrogen. Thawing had to be slow to prevent seed shattering. In 1988 very few shoot tips survived cryopreservation, and in 1989 shoot tips consistently survived freezing to -25 to -35°C. Only in 1990 we were able to obtain on average 70% shoot tip survival and 20% plant formation from cryopreserved shoot tips in liquid nitrogen (-196°C).

The strategy applied consisted first in developing a protocol with var. MCol22 and then test the system with other cassava genotypes. We studied every single step of the process, paying special attention to conditions which could prevent or minimize growth of ice crystals within the tissue, such as composition of the culture medium and the kind and amount of cryoprotectant used prior to freezing. Use of sorbitol, sucrose and DMSO in the pre-culture medium, and the elimination of water from the surface of the explants, contributed to increase tissue survival to 90% and shoot formation to 50%.

The use of small shoot tips and purified cryoprotector increased shoot formation. Interestingly, direct immersion into liquid nitrogen gave higher recovery rate as well as higher shoot formation than slow freezing (Table 1).

We tested the cryopreservation response of 14 cassava genotypes, with MCol 22 as a check. Viability and shoot formation obtained varied from 4-80% and 0-50%, respectively. Our work in 1991 focused on improving the pre-culture, cryoprotection and after- freezing culture conditions. We have significantly increased the response of low responding genotypes and obtained shoot formation from non-responding ones. It seems that genotypic differences in response are not due to the actual freezing step, but to the culture conditions prior and after freezing. Recently, we have also succeeded in the cryopreservation of cassava pollen. This concerted effort should lead to the development of a cassava base gene bank.

Table 1. Effect of shoot tip size, cryoprotection and freezing rate on viability and shoot formation after freezing in liquid nitrogen (cv M Col 22).

Treatment	Average values (%)	
	Viability	Shoots
Shoot tip size		
small (0.5-1 mm)	71A	57A
large (2-3 mm)	14B	0B
Cryoprotection		
sterile DMSO (ampules)*	90A	44A
standard DMSO*	83AB	33A
without cryoprotection*	60B	11B
cryoseeds	67AB	0B
Freezing rate**		
fast	86A	57A
slow	68B	29B

* Shoot tips were dried prior to freezing.
** Fast = direct immersion in liquid nitrogen
Slow = 0.5°C/min.
Averages with the same letter are not significantly different at 0.05 level.

2. **Towards a haploid method: culture of immature pollen**

Haploids and double haploids will be of significant value to cassava as a research tool and for various applications in genetics, evolutionary studies for the expression of important recessive genes, and for developing a breeding system for true cassava seed.

Initially we carried out background research on the reproductive behavior of cassava, including *in situ* and *in vitro* pollen germination, pollen tube growth and isolation and culture of young zygotic embryos. The microsporogenesis of cassava was also characterized and macroscopic parameters have been defined which correlate with critical microspore developmental stages.

The *in vitro* culture of whole anthers has only lead to the formation of callus from anther cells. Therefore in the last year we have focused our attention to devicing a technique for culturing isolated immature pollen. The technique utilizes some 50 flowers, i.e. 50,000 microspores, per petri dish in hanging droplets.

Immature pollen at the mitosis stage did not divide in culture as it would be expected. The pollen rapidly formed exine components which rendered the pollen impermeable to the medium.

Therefore we have shifted our work to the culture of microspores at the tetrad stage. Optimal culture density was 10^5 microspores per ml medium. Tetrad-stage microspores cultured under high osmotic conditions, and then transferred to a medium with lower osmolarity, were able to divide mitotically and gradually pushed their way out of the tetrad's callose. Outside, the tetrad division continued, but at a slower rate. Microcalli would then become visible. Microspores isolated from highly fertile varieties responded better than those from highly sterile ones.

In summary we have been able to change the gametophytic fate of cassava microspores to initiate a sporophytic development. This is the first necessary step towards androgenetic development. We will focus our future activities on enhancing callus induction from tetrad microspores and monitor this development cytologically.

3. **Genetic transformation**

Development of genetic transformation requires the availability of:

- A genetic construct containing appropriate selectable and/or reporter markers, a constitutive or tissue specific promoter;
- A DNA transfer methodology;
- An efficient plant regeneration protocol;
- Technology to monitor the transcriptional and post-translational products of gene expression and the inheritance of the introduced gene.

We have demonstrated earlier the regeneration of cassava plants by somatic embryogenesis on immature leaves or apical meristems (Fig. 1).

Agrobacterium-mediated genetic transformation

- Because the efficiency of *Agrobacterium* infection is genotype dependant, we have first screened four cassava varieties (M Col 22, M Col 1505, M Mex 55 and M Cub 74) with 25 *A. tumefaciens* strains. M Col 1505, which also regenerates well through somatic embryogenesis, was highly susceptible to the strains 1182, 1183, C58C1, B6S3 and EHA 101.

- We used the plasmid construct pGV1040 provided by PGS, Belgium. This construct contains two selectable markers (the bar gene and the nptII gene) and one reporter gene (uid A) which are driven by strong promoters (NOS and CaMV 35S).

- Cassava somatic embryos produced endogenous GUS activity, which did not occur in leaves and stems. Using Kosugi's technique, which includes methanol (Kosugi et al., 1990), somatic embryos were still GUS positive at higher substrate (X-Gluc) concentrations with respect to the original GUS assay (Jefferson et al,. 1986, 1987), but not detectable at lower X-Gluc concentrations.

- Leaves and stems were much more susceptible (8 mg/l) than somatic embryos (32 mg/l) to the herbicide BASTA.

- The antibiotic Kanamycin was highly detrimental to somatic embryogenesis.

We will now define the conditions for efficient *A. tumefaciens* infection of MCol 1505 in order to initiate the work on transformation and regeneration. We intend to use both a cointegrate vector approach as well as the binary vector system.

Genetic transformation by particle bombardment

The particle gun available at the BRU accelerates DNA-coated metallic microprojectiles to high speed, in such a way that they can penetrate cell walls, thus being a vehicle for the introduction of foreign DNA into plant cells. This can result in stable integration of the DNA into the plant genome with low frequency.

- Transient GUS activity was demonstrated in cassava tissues 3 days after bombardment.

- Vacuum and distance for the particle bombardment have been optimized.

- Recently we have obtained GUS expression on the tips of somatic embryos 3 days after bombardment of embryogenic callus at the globular stage (Fig. 1).

Progress has been made with respect to the frequency and intensity of GUS expression on somatic embryos. Stable genetic transformation of somatic embryos will be sought for by selecting on herbicide containing media.

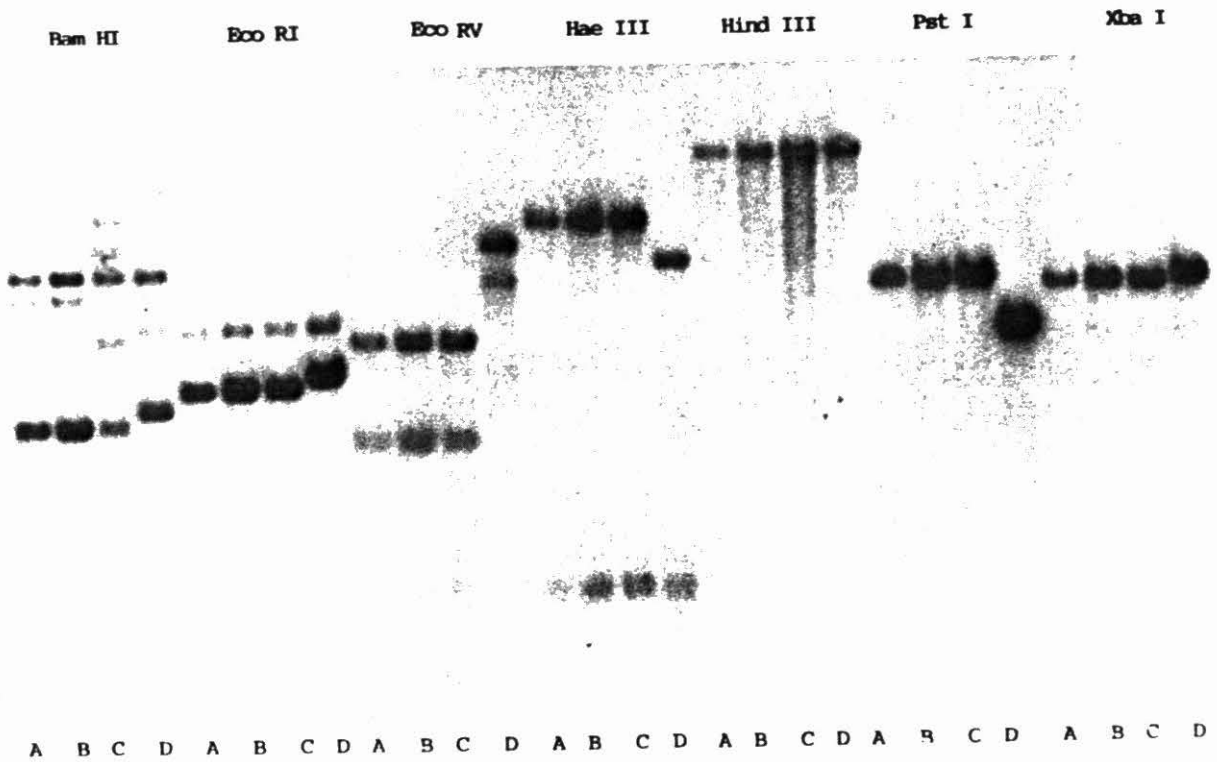


Figure 1. Restriction fragment length polymorphism (RFLP) with 7 restriction enzymes between A= M Thai 8, B= M Col 1505, C= M Nig 5 and D= *A. aesculifolia*. Note polymorphisms only with *M. aesculifolia*.

References

Jefferson RA, Burgess SM and Hirsh D (1986) ß-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc Natl Acad Sci USA 83, 8447-8451.

Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6, 3901-3907.

Kosugi S, Ohashi Y, Nakajima K and Arai Y (1990) An improved assay for ß-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous ß-glucuronidase activity. Plant Sci 70, 133-140.

4. DNA fingerprinting

In contrast to other methods, DNA fingerprinting covers the genome extensively, detects variation in coding and non-coding regions of the genome and is insensitive to developmental and environmental variations. In a collaborative project with the IBPGR we have been developing DNA-based techniques for the analysis of genetic diversity of *Manihot*. The construction of a genomic library has been achieved using the var M Col 22. DNA was digested with each one of the restriction enzymes: Pst I, Eco RI, Bam HI, Xba I and Hind III. Digested DNA was ligated into the plasmid pUC 19 and the constructs transformed into *E. coli* DH5α; insert sizes ranged from 0.2 to 7 Kb.

DNA isolated from varieties M Col 22, M Col 1505 and CM 507-35 was digested with the enzymes Apa I, Bam HI, Dra I, Eco RI, Eco RV, Hae III, Hind III, Hinf I, Hpa I, MspI, Pst I, Taq I and Xba I. We found that methylation sensitive-enzymes like Apa I and Pst I cut cassava DNA very ineffectively. Polymorphisms were detected with several clones; one Hind III clone was able to discriminate between the three varieties.

We have also used other probes for detecting polymorphism in cassava. The human minisatellite probe (Jeffrey's probe) was ineffective in detecting polymorphism; however, the phage M13 probe (Vassart et al., 1987) and several randomly amplified polymorphic DNA markers (RAPD) (Williams et al., 1990) did yield sufficiently variable band patterns as to differentiate between the varieties tested (Fig. 2B and C). We are testing those probes to analyze genetic stability of morphologically and isoenzymatically similar varieties and cassava clones which have been under *in vitro* storage for over 10 years.

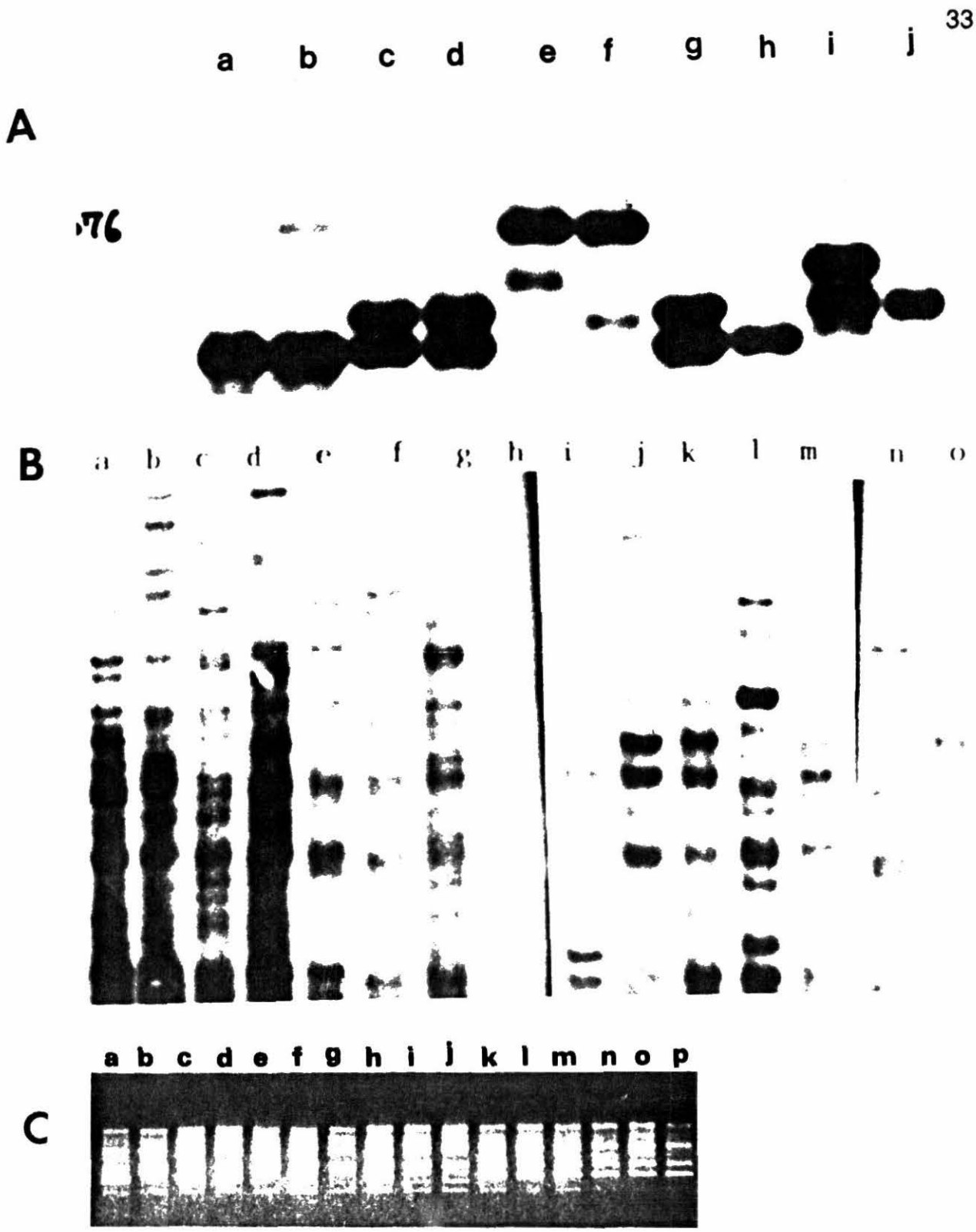


Figure 2. Molecular fingerprinting of cassava varieties; (A) EST isozyme fingerprints of 10 varieties (a-j); (B) M13 probe DNA fingerprints of 15 varieties (a-o); and (C) RAPD fingerprints of 16 varieties (a-p).

Genotyping of cassava by isozyme and DNA fingerprinting techniques will be useful to assess genetic variability, define gene pools and contribute to the construction of the cassava molecular map.

References

Vassart G, Georges M, Monsiuer R, Brogas H, Lequarre AS and Christphe D (1987) A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. Science 235, 683-684.

5. **Molecular mapping**

With the support of the Rockefeller Foundation we have initiated a research project to construct the molecular and physical maps of cassava using random genomic, cDNA and YAC libraries. The project involves cooperation between the University of Georgia, Washington University, IITA and CIAT.

The maps will be useful to analyze the genomic structure of cassava and its wild relatives, and to tag agronomically important traits, simply and quantitatively inherited. Eventually the map will be useful to isolate and clone cassava genes.

The project builds up on the experience gained at CIAT in the last year. Thus, protocols for DNA extraction, digestion and hybridization have been implemented. A random Pst I genomic library was generated using the var M Col 22. Some 200 single copy clones of a total of 500 have been characterized, insert sizes ranging from 0.2 to 3 Kb.

Recent developments in this project include:

- Cassava DNA digested with Eco RI, Eco RV, Xba I and Hind III libraries provide probes with highest polymorphism rates.

- Polymorphism between cv M Col 22 and its wild relative *M. aesculifolia* was dramatically higher than the polymorphism found between two cultivated genotypes, as expected (Table 2, Fig. 1). Four-cutter restriction enzymes displayed less frequency of polymorphism than six-cutter enzymes for cultivated genotypes for fragments larger than 2 kb.

- Polymorphism displayed by Dra I was extremely low, indicating that regions rich in adenine and thymine may not be hot spots for mutation in cassava.

Table 2. Degree of polymorphism detected with at least one restriction enzyme* using five genomic libraries as source of probes [%].

Library	M Col 22 vs. M Col 1505	*M. aesculifolia* vs. M Col 1505
Pst I	60	85
Xba I	60	85
Hind III	55	95
Eco RI	40	60
Bam HI	30	45

* Best restriction enzymes in order of polymorphism are: Eco RV, Xba I, Eco RI, Hind III.

6. **Biochemistry of Cassava Starch Fermentation**

Sour cassava starch is a regionally very important small industry product, the departments of Valle and Cauca producing almost 80% of the national total. The product is consumed mainly locally as pandeyuca, besitos, rosquillas, and other local classics. The fermentation process still takes place under quite rudimentary conditions in the rallanderias. Inspite of some improvements in the machinery and sedimentation tanks over the last few years, the main problem encountered remains the reproducibility of the process, the lack of inocula, and well defined marketing criteria.

Gerard Chuzel, from CIRAD/CEEMAT-Cassava Utilization Section CIAT, has been pursuing a holistic approach to this problem, and putting together the Cassava Utilization Section, the BRU, and UniValle, microbiological, physicochemical, and biochemical studies have been performed (see also BRU Annual Report 1990; and Abstracts of the Workshop "Avances sobre almidón de yuca" Jun 17-20, 1991, CIAT). A very thorough microbiological analysis is well documented in the thesis work of Carlos Figueroa (1991). The fermentation process, which is mainly anaerobic, is carried out to a great extent by lactic amylolytic bacteria of the genera Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, and Lactococcus.

Last year we reported the electrophoretical separation of amylolytic activities and their transfer to starch containing gels (SCGs) for their characterization. This year we set for the detailed characterization of amylases from the principal bacteria

involved in the process. It is the partial amylolytic degradation of the starch granules, as shown by electron microphotography, that confers to the starch its rheological properties for panification.

Excreted amylases were extracted from the fermentation mass in acetate buffer, as to preserve the optimal acidic conditions. Proteins were then precipitated from the supernatant by ammonium sulfate, and after dialysis of the resuspended sample, chromatographed on DEAE anion exchanger resin. The enriched amylases were then quantified and characterized on SCGs by iodine staining and by enzymatic assays (colorimetric assays either with iodine after hydrolysis of starch or after the liberation of p-nitrophenol from PNP-heptaglucoside).

Strains with strong amylolytic activities have been selected using the methods described, after analysis of supernatants from pure liquid cultures (see Fig. 3). Characteristic patterns of supernatant proteins have also been obtained by silver staining of polyacrylamide gels. In the next step the amylolytic enzymes of these strains will be characterized as to their pH and temperature optima, substrate preferences, cofactors, inhibitors, and kinetic properties, and also the elaboration of an amylolytic profile throughout the fermentation process will be pursued.

References

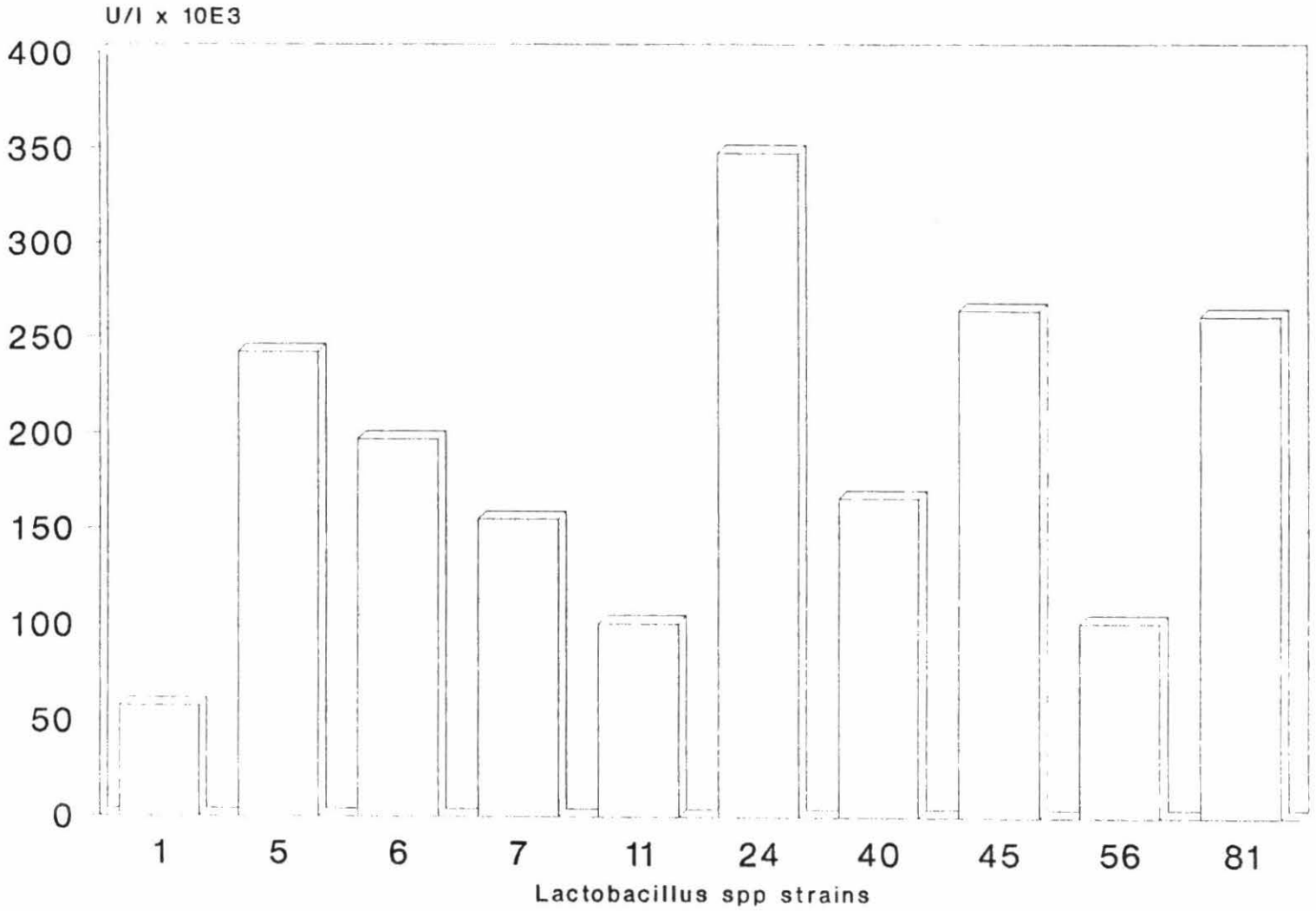
Figueroa C (1991) Fermentación del almidón de yuca. Tesis, UniValle.

7. **Molecular Mechanisms of CO_2 assimilation**

Certain cassava photosynthetic parameters seem to lie between C3 (bean) and C4 (maize) plants (El-Sharkawy and Cock, 1987). This suggests that cassava might have developed mechanisms for efficient photosynthesis, especially under drought and high temperature conditions (Schuster and Monson, 1990). Earlier work at CIAT has demosntrated that there is a wide range of genotypic variability concerning this physiological behavior. Now, it remains to be established which are the actual mechanisms underlying these characteristics. The final objective is to use this knowledge for developing screening methodologies which can be incorporated into breeding programs searching for improved biomass production, water and nitrogen use efficiency.

One approach we are initiating is to develop *in situ* hybridization techniques on leaf tissue using photosynthetic gene probes to assess their compartmentalization patterns. For this purpose, homologous genes from cassava have to be used; therefore we must first isolate these genes from cassava gene libraries using heterologous probes from maize (provided by T. Nelson, Yale University). We are

Amylolytic Activity of Lactic Bacteria



1U = 1 µeq reducing ends/min at 37 C

in the process of constructing a genomic library from cassava. We have observed high homology between the maize genes and cassava by hybridization of total DNA with the maize probes, which indicates that these genes are highly conserved, hence, facilitating the task of isolating the cassava genes. The genes we are working on are: RUBISCO (rbcs), malate dehydrogenase (mdh), malic enzyme (me) and PEP-carboxylase (ppc). Another target would be the glycine decarboxylase, an enzyme that is involved in CO_2 recycling, a mechanism that maybe related to cassava's C3-C4 intermediate characteristics (Hylton et al., 1988).

References

El-Sharkawy MA and Cock JH (1987) C3-C4 intermediate photosynthetic characteristics of cassava (*Manihot esculenta* Crantz). Photosynth Res 12, 219-235.

Hylton CM, Rawsthorne S, Smith AM, Jones DA and Woolhouse HW (1988) Glycine decarboxylase is confined to the bundle-sheath cells of leaves of C3-C4 intermediate species. Planta 175, 452-459.

Schuster WS and Monson RK (1990) An examination of the advantages of C3-C4 intermediate photosynthesis in warm environments. Plant, Cell and Environment 13, 903-912.