OUTPUT 2
Genetic base of cassava and related *Manihot* species evaluated and available for cassava improvement: higher commercial value.

The overall objective of this output is to generate genetic stocks and knowledge about genetic variability for starch quality traits. The main activities focus in developing and identifying high-value cassava germplasm. Traits involving higher commercial value turn particularly around novel starch types. Related issues are the need for a better understanding of the biochemical and genetic basis of these high-value traits. Because of the scarcity of institutions conducting research on cassava new methods for assessing, identifying and exploiting genetic variability are required and need to be developed by CIAT.

Rationale

Cassava roots are valued for their starchy properties. They are used for fresh consumption, fermentation and drying, rasping and drying, chipping, pelleting, starch extraction and alcohol production. More recently high value-added products, such as precooked, frozen croquettes and fried chips, have been developed and have an increasing presence in urban markets. High starch content is an important component of root quality for nearly all uses of cassava (Jennings and Hershey, 1985). A limiting characteristic for the human or animal consumption of cassava roots is their content of cyanogenic glucosides (Kakes, 1990). Cyanide is largely removed by the traditional processing methods of grating, fermenting and/or drying. Cultivars with less than 100 mg HCN per kg of fresh root are considered to be *'sweet'*.

Several factors have limited the actual impact of this crop in tropical agriculture. On the biological side, cassava breeding is cumbersome and slow and little genetic variability has been found for key economic traits particularly for those determining starch quality. Cultural biases have also affected cassava. For several decades, public and private sector investment was biased favoring investments for research and development in cereals such as maize, rice, wheat and sorghum (Cock, 1985).

In spite of the problems mentioned above, the globalization of the economies and new technological breakthroughs are offering a new opportunity for cassava, which was never available to the crop during the past. Tropical production of maize is facing increasing problems to compete with maize produced in temperate regions. This situation has prompted government and private sector of many tropical countries to turn to cassava as a competitive alternative to imported maize. In addition, advances in molecular biology, genetic engineering, plant-tissue culture protocols and starch technologies provide important tools that will allow bridging the main gaps between cassava and the cereals. Two major constraints are to be solved in relation to the objectives of this output.

Genetic improvement of cassava

Genetic improvement of cassava has not been as consistent and efficient as in other crops and has some constraints. As in every crop improvement project, elite cassava clones are crossed to produce new segregating families. Each individual produced, as well as the parental clones used, is highly heterozygous. Once a superior genotype is identified (a process that requires no less than six years), it is vegetatively multiplied to take advantage of the reproductive habits of this crop (Kawano et al., 1998; Jennings and Iglesias, 2002). This

system (except for the vegetative multiplication) is similar to the ones used for autogamous crops (beans, wheat, rice, etc.) as well as for the hybrid maize industry. However, there is a major difference because cassava is seldom self-pollinated to produce partially or completely inbred (homozygous) lines from the segregating progenies. The system also bears some similarities with recurrent selection used in many crops, but there is a significant difference because in cassava there is not an actual population whose allelic frequencies are modified through evaluation and selection, as in true recurrent selection schemes.

From the practical point of view, implementing a traditional recurrent selection method in cassava offers some problems. Pollinations (Kawano, 1980) are slow and inefficient. It takes about 16 to 18 months since a given cross is planned until the recombinant seed is finally obtained (usually field operations have to adjust to the occurrence of the rainy season, and if that is the case, then planting can only be done 24 months after planning the cross). The third year would be used to grow the plant from the botanical seed. During the fourth year, a clonal evaluation could finally be carried out. Therefore a typical recurrent selection method would require no less than four years. In this case, however, no self-pollinating for reducing genetic load would have been included, and few additional years will be required for the release of a variety.

The time required for each selection cycle in cassava has limited the genetic gains for higher productivity in the crop. It should be emphasized that because the highly heterozygous condition of cassava in every stage of the breeding process, consolidation of genetic gains is difficult, due to the inherent genetic instability of the heterozygous status. Moreover, the absence of inbreeding allows for large genetic loads (deleterious or undesirable alleles carried by an individual) in the *M. esculenta* gene pool, further limiting genetic progress.

Genetic variability for starch quality traits.

*O*ne additional disadvantage for cassava to play a more important role in tropical agriculture is the relatively low genetic variability for root starch traits. Compared with the many economically advantageous mutations found and exploited, for example, in the maize kernel (sweet corn, pop corn, waxy corn, opaque 2, etc.), very little variability has been reported for cassava. It is valid to assume that such variability exists in the crop, and at least two main reasons could explain why it has not been readily found and reported: **a)** Starch mutations in the roots are more difficult to detect than in grain kernels (where they can be easily identified by visual inspection without the need of any sophisticated tests). To detect a mutation in the cassava root starch, the breeder will have to break the roots and most likely will have to conduct a particular test (such as the iodine test) to be able to pick potentially useful variants. It is possible, therefore, that waxy cassava clones have already been grown in breeding nurseries but could not be detected and, not showing an outstanding agronomic performance, they were discarded; **b)** The known starch mutants are usually recessive. The fact that cassava seldom undergoes inbreeding drastically reduces the chance of (expectedly) low-frequency recessive alleles, to express in the phenotype.

The fact that roots are not reproductive or multiplicative organs may offer cassava (and other root crops) an advantage over the grains. It is valid to assume that cassava roots could withstand mutations that would otherwise be lethal for reproductive organs such as the kernels of cereals. Figures 2.1 through 2.4 provide a general illustration of the kind of variations found for key root starch traits in different studies conducted at CIAT. Currently CIAT is finalizing the analysis of the entire cassava collection (starch samples from around

6000 landraces and improved germplasm). However, further research is needed to confirm the extent of environmental influence on the observed variation. For example, it has been documented that age, cultural practices and environment, as well as genetic differences, play an important effect in dry matter content in the roots (van Oirschot at al., 2000).

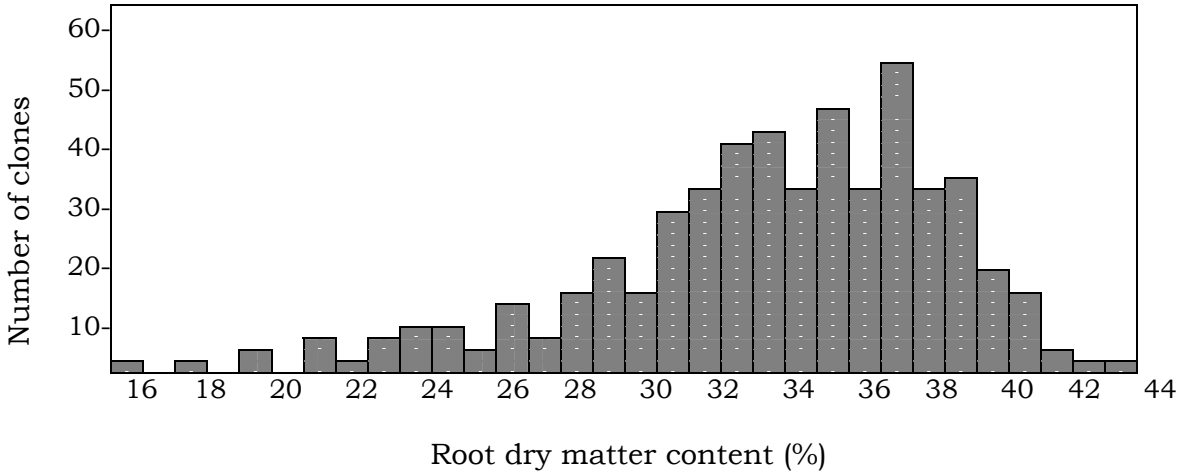

Figure 2.1. Typical distribution of dry matter content in roots from a large sample of cassava clones.

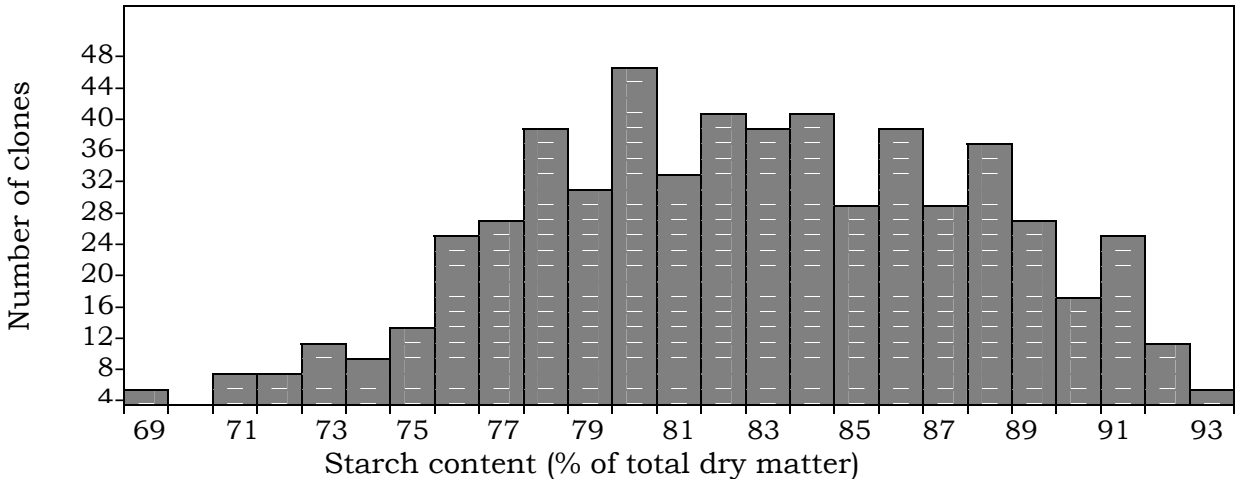

Figure 2.2. Variation in starch content (as percentage of the total dry matter) in roots from a large sample of different cassava clones.

The strategies that CIAT and many other cassava research institutions are following can be summarized in three key objectives: a) continue the research for increased yields and reduced costs of production; b) widen the uses of cassava, including the exploitation of foliage; and c) increase the emphasis on the search for value added traits/products. The ultimate and common objective of all these strategies is to increase the income of cassava

farmers; open new alternatives to processors that would in turn stimulate rural development and increase the demand for cassava products. The present article focuses on the activities currently underway at CIAT in its search for value-added traits, such as those modifying starch physicochemical properties.

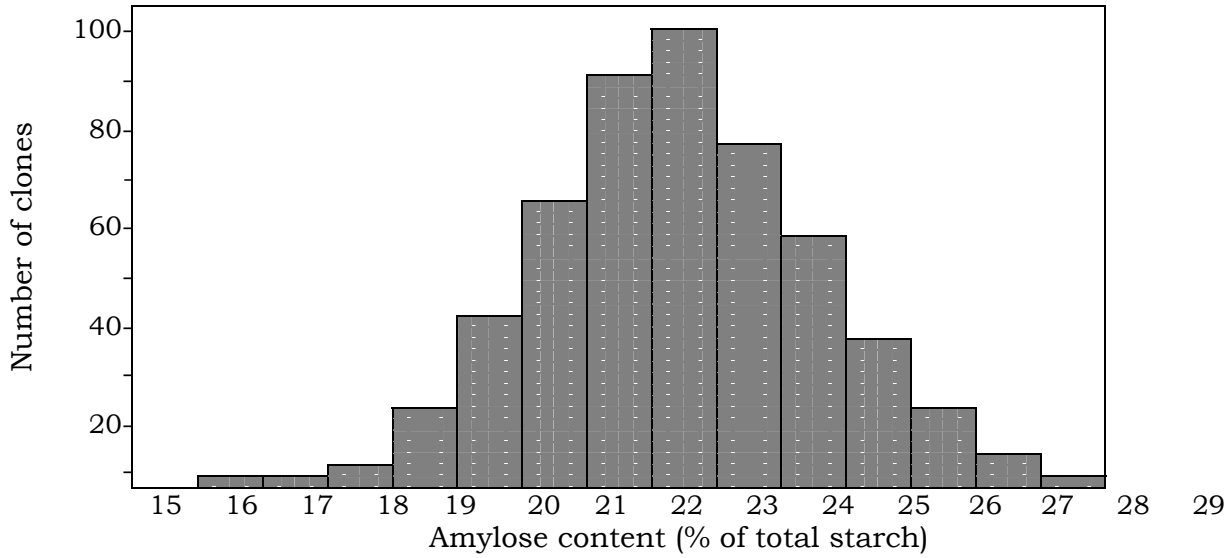

Figure 2.3. Variation in amylose content (as percentage of the total starch) in roots from a large sample of different cassava clones.

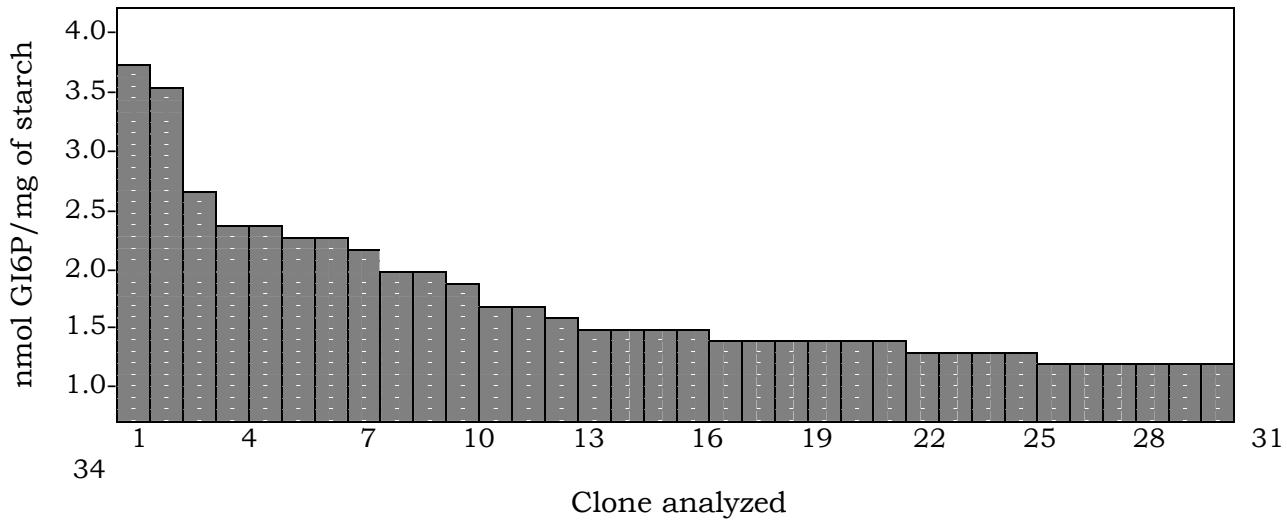

Figure 2.4. Variation in GI6P associated with the starch in roots from a sample of 34 cassava clones

CIAT has currently implemented several approaches at to modify the physical and chemical properties of cassava root starch. These approaches are briefly described below.

The activities related to this Output are long term and reveal an important strategy that the cassava-breeding project at CIAT has gradually defined. It is difficult at this point in time to predict when or what kind of novel starch will be created and identified. Below there is a description of the status of each of the approaches followed with the goal of producing novel starch types in cassava.

Activity 2.1. A cassava-breeding scheme based on the production of doubled-haploids

When an elite clone is self-pollinated two important events occur: **a)** the unique, specific combination of alleles present in the original genotype is broken, therefore loosing the agronomic superiority that the clone had; **b)** self-pollination forces that half of the loci on average to become homozygous. This facilitates the elimination of undesirable, deleterious alleles that, although present in the original clone, remain hidden because of the predominant heterozygosity. In other words, self-pollinated progenies allow for a reduction of the genetic load originally present in the clone, therefore becoming better progenitors themselves. In a way, self pollination allows to "*concentrate*" the desirable genes originally present in the elite clone.

If inbreeding was pursued until near or complete homozygosity, then the transfer of desirable traits through the back-cross scheme becomes feasible. Also homozygosity "*captures*" genetic superiority because of its inherent stability. Therefore each cassava improvement cycle would be a consolidating step that could help further progress in a more consistent and predictable way. On the other hand, each time a hybrid is used as parent, the process goes back to the initial stage because of the unavoidable genetic instability of a heterozygous material. In this case, progress cannot be easily consolidated or sustained through time, but only in a rather inefficient way.

In summary, inbred lines are better progenitors because, by definition, they carry lower levels of genetic load. The breeding value of a given genotype becomes more apparent when dealing with homozygous lines. Also if a breeding process is based on the use of inbred lines the transfer of valuable traits is greatly facilitated because the back-cross scheme becomes feasible. The availability of inbred lines in cassava would also benefit other areas in addition to breeding. Genetic and molecular marker analysis would be greatly facilitated if homozygous lines were available. The only way to maintain germplasm in cassava is either by growing the plants in the field or by maintaining them under tissue culture systems. Inbred lines, on the other hand, could be maintained and shipped in the form of botanical seed. Phytosanitary problems could be reduced significantly if maintenance and multiplication of genetic stocks were based on the use of botanical seed. Therefore, germplasm exchange among the few cassava breeding projects in the world would be greatly facilitated bypassing the need to use vitro-plants. Finally, elite clones could be reproduced by crossing the same parental lines from which they were originally derived.

One final advantage of inbreeding in cassava is that in addition of exposing recessive deleterious alleles so that they can be eliminated from the gene pool, it also allows the

identification of useful recessive traits (such as the starch quality mutants found in different crops), which would lead to the development of value-added genetic stocks.

There are, however, two major obstacles for the incorporation of inbreeding in cassava genetic improvement. Because of the large genetic load expected to be present in most cassava gene pools, inbreeding may lead to a rapid loss of vigor and, eventually, to non-viable plants. This is a common process known as inbreeding depression. Maize showed strong inbreeding depression and tolerance to it had to be gradually developed in maize adapted to temperate (early 1900s), and tropical (during the 1960s) regions. A similar approach will be used in cassava. Elite germplasm will be self-pollinated successively until vigor becomes too low. Then sister lines derived from the same elite clone will be crossed, generating several populations. Each population (or lineage) will be composed by the progenies derived from the same elite clone. By default, they will carry reduced genetic load and increased tolerance to inbreeding.

A second problem still remains even if inbreeding depression was negligible. It would require about ten years to develop highly homozygous inbred lines through successive self-pollinations. This period is too long and would not allow cassava to sustain the genetic progress rate observed in other crops with which cassava competes. Therefore, an alternative and faster method for the production of homozygous lines needs to be developed. Tissue culture protocols for the production of doubled-haploid lines from anthers (occasionally from ovules) have been developed for many different crops and are routinely used for their genetic improvement. The production of doubled haploids in cassava provides an appealing option for a feasible introduction of inbreeding in cassava genetic improvement. This technology does not involve the use of transgenesis.

CIAT, with the valuable support of UNDP and Rockefeller Foundation, and in collaboration with several cassava breeding programs (including Thailand) began in 2004 a research project to simultaneously improve tolerance to inbreeding in elite cassava germplasm. Jointly with Wageningen University from The Netherlands, CIAT is also coordinating a project to develop the anther culture protocol for the production of doubled haploids. As soon as partially inbred lines are produced the search of novel starch types will immediately began.

The elite germplasm that is included in the crossing nurseries every year has been self-pollinated as well as used for the production of new hybrids. During the past year a total of 2793 botanical seed (615 S_1s and 2178 S_2s) were germinated. Eventually only 1995 seedlings derived from these botanical seeds were vigorous enough to be transplanted in the field (348 S_1s and 1647 S_2s) where they will be used for yet another round of self-pollinations to increase even further the level of inbreeding and also as source of stakes. Each partially inbred produced will be clonally kept for further phenotypic, genetic and/or molecular studies.

During the past year, therefore, a heavy emphasis was invested in the production partially inbred cassava germplasm. A total of 8436 self-pollinations were made to produce S_1 botanical seed (50% average homozygosity); 3645 self-pollinations to produce S_2 seed (75% average homozygosity) and 391 self-pollinations to produce S_3 seed (87.3% average homozygosity). As soon as the starch quality laboratory reaches its capacity to process the thousands of genotypes developed, the starch derived from them will be carefully screened in search of useful mutants.

Activity 2.2 Mutagenesis and the "TILLING" system.

There are few alternative approaches when no immediate genetic variability is available for the breeders to develop a desired product. Obviously the first approach is to identify a genetic source for the desired trait within the same gene pool (for instance *M. esculenta*). If this approach is unsuccessful, breeders have frequently sought for the desired trait in the gene pool of related wild relatives. For instance some of the sources of resistance to the African Cassava Mosaic Disease seem to have originated in crosses between *M. esculenta* and *M. glaziovii*. This approach has the disadvantage that along with the desired trait, many undesirable ones are also introduced from the wild relative species. Many years of additional work are usually required to eliminate undesirable genes while maintaining the useful trait.

Another traditional approach used by many breeders (particularly in the decades of the 1950s and 1960s) was the induction of genetic variability through the use of mutagenic agents (chemical products such as EMS or irradiation such as gamma rays). Mutation breeding has few drawbacks. Events are totally random, so thousand of genotypes need to be evaluated until a useful mutation in the desired gene can be found. Furthermore, since mutations typically take place at individual cells, a large frequency of chimeras is very common. Chimeras will lead to drastic changes in the following generations, until the desired mutant can be stabilized. Finally, the common recessive nature of the mutations made it difficult to identify them through the phenotypes.

With the advent of molecular biology tools, an interesting system was developed to renew the interest in mutation breeding. DNA TILLING (for *Targeted Induced Local Lesions in Genome*) has been successfully used in different plant species (McCallum et al. 2000; Perry et al. 2003; Till et al. 2003). Sexual seeds are mutagenized and, to avoid ambiguities caused by chimeras in the first generation (**M_1**), plants are self-pollinated and the resulting **M_2** plants evaluated. DNA is extracted from each M_2 plant. For screening, DNAs are pooled eightfold to maximize the efficiency of mutation detection. PCR is performed using 5'- end labeled gene-specific primers to target the desired locus, and heteroduplexes are formed by heating and cooling the PCR products. **CEL I** nuclease is used to cleave at base mismatches, and products representing induced mutations are visualized with denaturing polyacrylamide gel electrophoresis (description of the TILLING method adapted from Till et al., 2003).

CIAT is participating in a project lead by Universidad Nacional de Colombia, which is supported by the IAEA (International Atomic Energy Agency). About 4000 seeds from six different cassava clones were irradiated at IAEA facilities at Seibersdorf, Austria. Half the seeds were irradiated with gamma rays (using a Cobalt 60 source with a dosage level of 200 Gy) and the remaining half, with fast neutrons. Seeds were germinated and transplanted to the field early in 2004 (Table 2.1). By years end the plants will have been carefully evaluated in search of promising mutant forms (although it is recognized that the occurrence of chimeras and the lack of expression of recessive mutations will certainly reduce the probabilities of finding such mutants at the M_1 stage). Also, as soon as plants start to produce viable flowers they will be self-pollinated as indicated in Table 2.1. In the meantime the granule bound starch synthase GBSS I (Curtis Hannah, 2000) will be analyzed and key sequence(s) PCR-amplified for their use in the TILLING system.

Table 2.1. Tentative number of self-pollinations that will be sought from **M1** cassava plants currently grown in the field at Palmira, Colombia. More than 4000 self-pollinated seed will be generated.

Family	Number of **M1** plants	Treatment	Plants to be self-pollinated	Number of self-pollinations	Total of self-pollinated seed
CM 9331	150	γ - irradiation	20	15	300
	150	Fast neutrons	20	15	300
SM 3015	150	γ - irradiation	20	15	300
	150	Fast neutrons	20	15	300
SM 3045	150	γ - irradiation	20	15	300
	150	Fast neutrons	20	15	300
GM 155	153	γ - irradiation	20	15	300
	153	Fast neutrons	20	15	300
C-4	787	γ - irradiation	50	15	750
	787	Fast neutrons	50	15	750
C-27	25	γ - irradiation	10	20	200
	25	Fast neutrons	10	20	200

If any of the 3000 plants have a mutation in the GBSS I gene, the TILLING system will be able to detect it, even if it is in the heterozygous condition and does not show in the phenotype. Figures 2.5 and 2.6 shows the plants from irradiated seed in the greenhouse and then after transplantation to the field.

For this work two students from National University of Colombia – Sede Palmira (one undergraduate and the second enrolled in the Ph.D program), have been invited to participate. It is interesting to mention that a very similar approach has been successfully implemented for the production and identification of a potato clone which had one of the four alleles of the GBSSI (potato is a tetraploid species) mutated. The resulting material will be capable of producing amylose-free starch.

Figure 2.5. **A**: Seedlings from botanical seeds that were irradiated with gamma rays (left) or fast neutrons (right). **B**. A seedling showing obvious symptoms of abnormal development.

Figure 2.6. **A.** Seedlings from botanical seeds that were with gamma rays or **B.** fast neutrons.

Activity 2.3 Recurrent selection to increase and reduce amylose proportion in the root starch of cassava.

Figures 2.1 through 2.4 depicted the kind of variation observed for key cassava root traits. A third approach that CIAT has just begun for modifying starch quality traits is through conventional breeding (Figure 2.7). Using the kind of information provided in Figure 2.3, CIAT will begin crossing clones with roots whose starch have low-amylose proportion. Ten to twenty such clones will be crossed among themselves (shaded areas in each distribution curve). The resulting progeny (single plants) will be harvested and the amylose content of the starch in their respective roots will be measured. It is expected that there will be considerable variation among these plants resulting in the L1 curve depicted in Figure 2.7. The plants with roots whose amylose proportion is lower than those of the parental clones will be selected. The remaining progeny will be discarded. Selected plants will be crossed among themselves to start a second cycle of selection, which results in the L2 distribution curve. A similar scheme will be used for clones with high-amylose clones, resulting in the H1 and H2 successive distribution curves.

The scheme illustrated in Figure 2.7 is a theoretical response to recurrent selection. This response is based on the assumption that amylose proportion in the cassava root starch can be modified through gradual changes in the allelic frequencies of genes that have a quantitative inheritance.

As described in Figures 2.1to 2.4 there is some variation in quality traits of cassava root starch. The information generated in the last few years has been used for identifying clones with high- and low-amylose. Table 2.2 describes the main characteristic of the two groups of clones. In-vitro plants from the germplasm bank were recovered from the selected clones, hardened and transplanted to the field in March 2004. As the materials begin to flower crosses within each group will be made in order to generate the first cycle of selection for the high- and low-amylose populations. The segregating genotypes will be analyzed the those plants with the lowest (in the low-amylose population) or highest (in the high-amylose population) amylose levels will be crosses to generate the second cycle of selection and so

forth. Eventually, some crosses between high- and low-amylose genotypes will be made to conduct genetic studies on the inheritance of this trait.

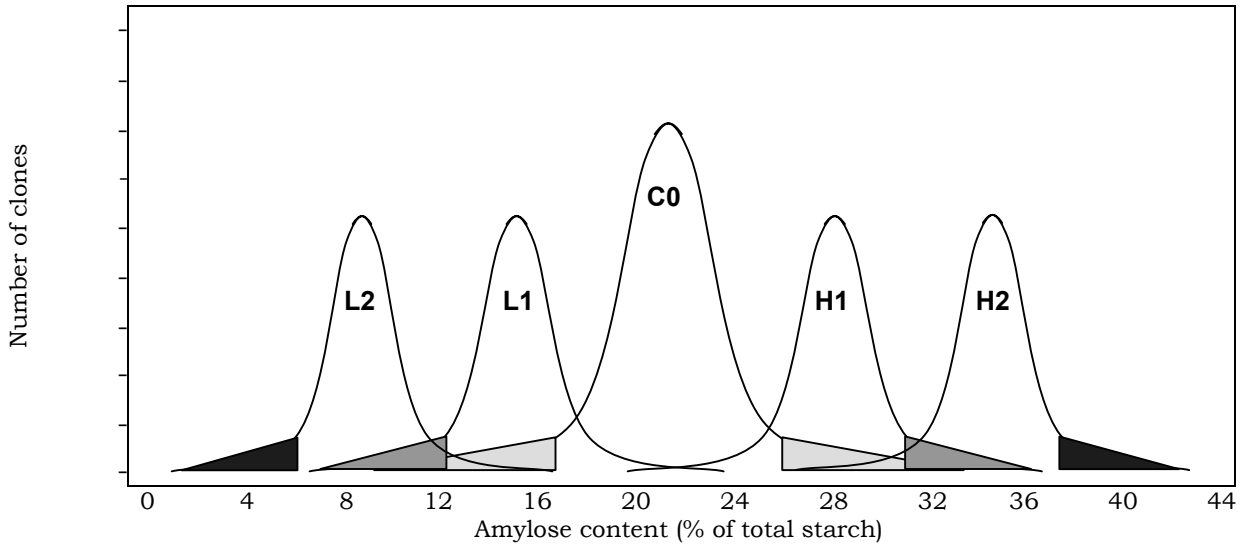

Figure 2.7. Variation in amylose content (as percentage of the total starch) in roots as a result of a divergent recurrent selection scheme. **C0**: Original cycle; **L1** and **L2**: two consecutive cycles for lower amylose content; **H1** and **H2**: two consecutive cycles for higher amylose content.

In addition to the crosses and selection process described above stability and genetic studies will be conducted to determine how much of the phenotypic observations that originated the selection of clones described in Table 2.2 is genetic and how much is environmental.

Table 2.2. Description of cassava clones planted to make crosses aiming at a divergent recurrent selection for high- and low-amylose content in the root starch

Parameter	**Low-amylose**	**High-amylose**
Average	11.17	22.74
St.Deviation	0.58	2.07
Minimum	9.66	21.82
Maximum	12.10	26.39
Number of clones	29	35

Activity 2.4. High-capacity starch quality laboratory

Each year, cassava-breeding projects around the world produce thousands of new genotypes. Early stages of selection eliminate a large proportion of these new genotypes without analyzing the quality of their starches (CIAT, 2003; Jennings and Iglesias, 2002; Kawano et al., 1998; Kawano, 2003). It is feasible, therefore, that along the eliminated clones valuable starch quality traits have also been discarded. One of the problems, as explained above, is that starch mutants in cassava roots are not as readily identifiable as those in the cereal kernel.

Some of the approaches described above (TILLING system for mutation breeding or the iodine test) are specifically targeting the identification of known mutations (i.e. waxy starch). However, it is valid to assume that known (as well as unknown and yet to be discovered) mutations may be available in cassava. A common need for many of the strategies described in this output is the availability of a high capacity starch analysis laboratory to screen large number of samples in search of those with novel pasting properties.

CIAT and other institutions (CLAYUCA, Chemical Engineering Department from Engineering School, Universidad Nacional de Colombia-Bogotá Campus; Agriculture Department from Agronomy School, Universidad Nacional de Colombia-Palmira Campus; CENICAFE and INYUCAL) have developed a collaborative project for the creation of a high-capacity starch quality laboratory. This consortium has successfully submitted a proposal to COLCIENCIAS (Colombian granting agency) for setting up a laboratory that will be able to generate thousands of amilograms per year using available equipments (i.e. rapid viscoanalyzers, Brabender, Differential Scanning Calorimeter) and purchasing two new RVSs. Resources have also been assigned for the extraction of the starch samples.

Figure 2.8 provide illustrations of few amylograms obtained using the Brebender equipment. As can be seen in the horizontal axis, the Brabender requires 90 minutes to complete and amylogram. The main advantage of the RVAs is that it only needs 20 minutes to provide information similar to that provided by the more standard Brabender. The problem of the RVA is the cost, because each apparatus costs about 35,000 US$.

In Figure 2.8 photographs of Brabender, RVA and DSC equipments are included for illustration. It is expected that the high-capacity starch quality laboratory (HCSL) will be located within the Agronatura Scientific Park and evolve towards a semi-autonomous operation coordinated by National University of Colombia – Palmira Campus and CIAT. The administrations of National University of Colombia – Palmira Campus and CIAT are already considering the requirements for the physical expansion of the current facilities to accommodate the new equipment and considering the legal implications and status that this semi-autonomous HCSL would have.

An interesting feature of the proposal successfully submitted to COLCIENCIAS is the participation of the private processing sector. In this case a cassava-starch processing factory is part of the consortium contributing with fresh and in-kind resources and know-how capacities. This company operates in the northern coast of Colombia.

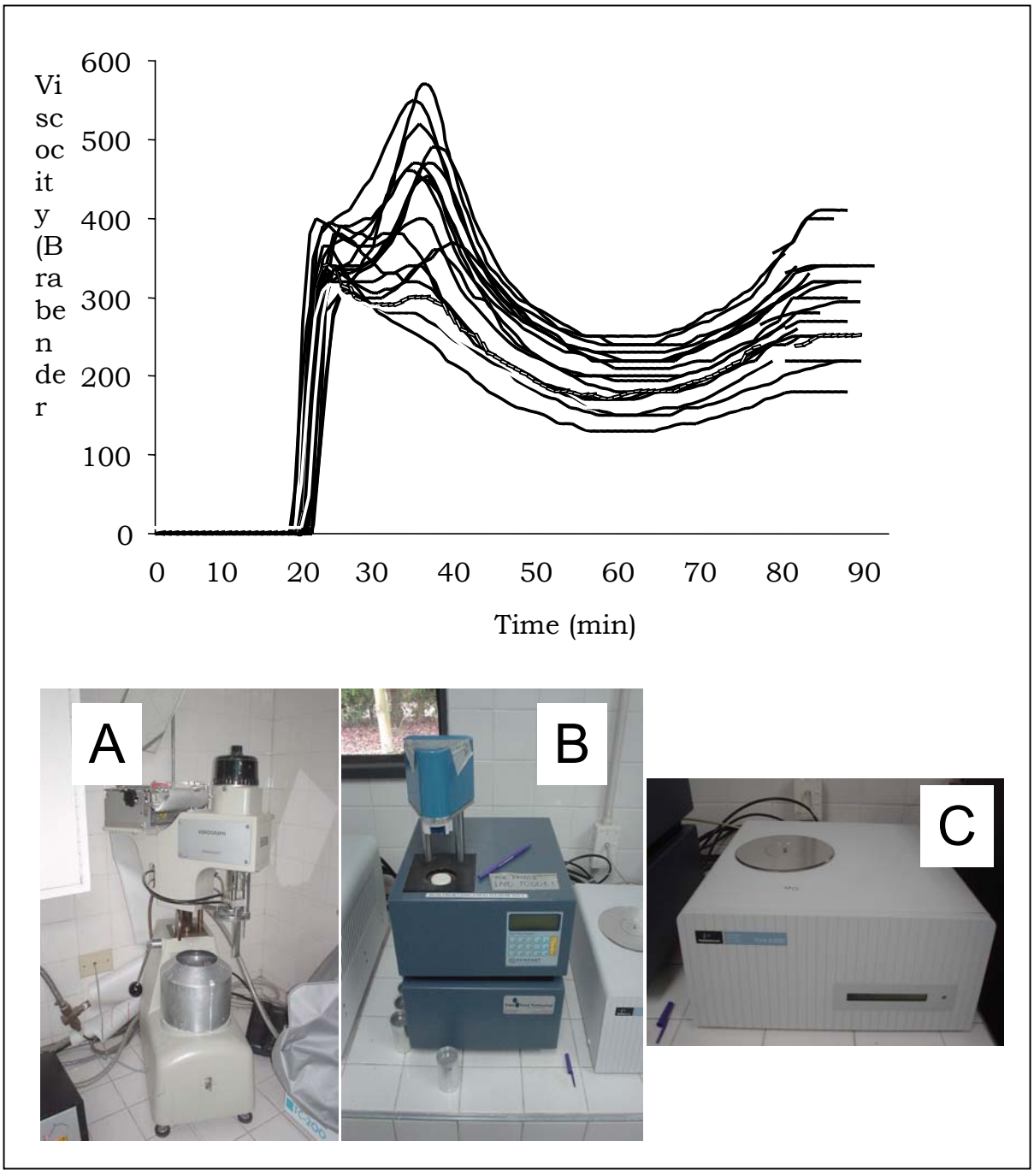

Figure 2.8. Illustration of different amilograms and equipment for the identification of novel starch types: A= Brabender; B= RVA; and C= DSC.

Conslusions

Cassava is an important crop for the agriculture of many tropical and subtropical countries. It remains one of the most relevant commodities for subsistence farming as food security, and it is acquiring an increasing role in rural development as raw material for many processing pathways. Starch production from cassava roots is clearly one of the most important examples of industrial uses in cassava, particularly in Asia (Chutharatkul, 2002). To maintain this trend and make cassava even more competitive several approaches have to be taken simultaneously: increased yields and reduced costs of production; widened uses of cassava (i.e. exploitation of foliage); and increased emphasis on the search for value added traits/products.

The justification for this strategy can be found in the maize productive chain. Maize is a commodity directly competing with cassava in many areas, including starch. Corn, for example offers the case of "waxy maize", a mutation that inhibits the synthesis of amylose. Therefore waxy starch is only made up of amylopectin. Farmers growing waxy maize receive a 30% additional return for their efforts. This is a very appealing situation because the modification to degrade amylose or separate it from amylopectin is not made in a factory by chemical and/or physical means but in the plant using the metabolism of a useful genetic mutation. The environment, therefore, benefits because there is a reduced impact in the modification of native starches on one hand, and the farmer benefit from a considerably higher return. Similar benefits are expected to happen from the novel starch types that this activity aims at creating and identifying.

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