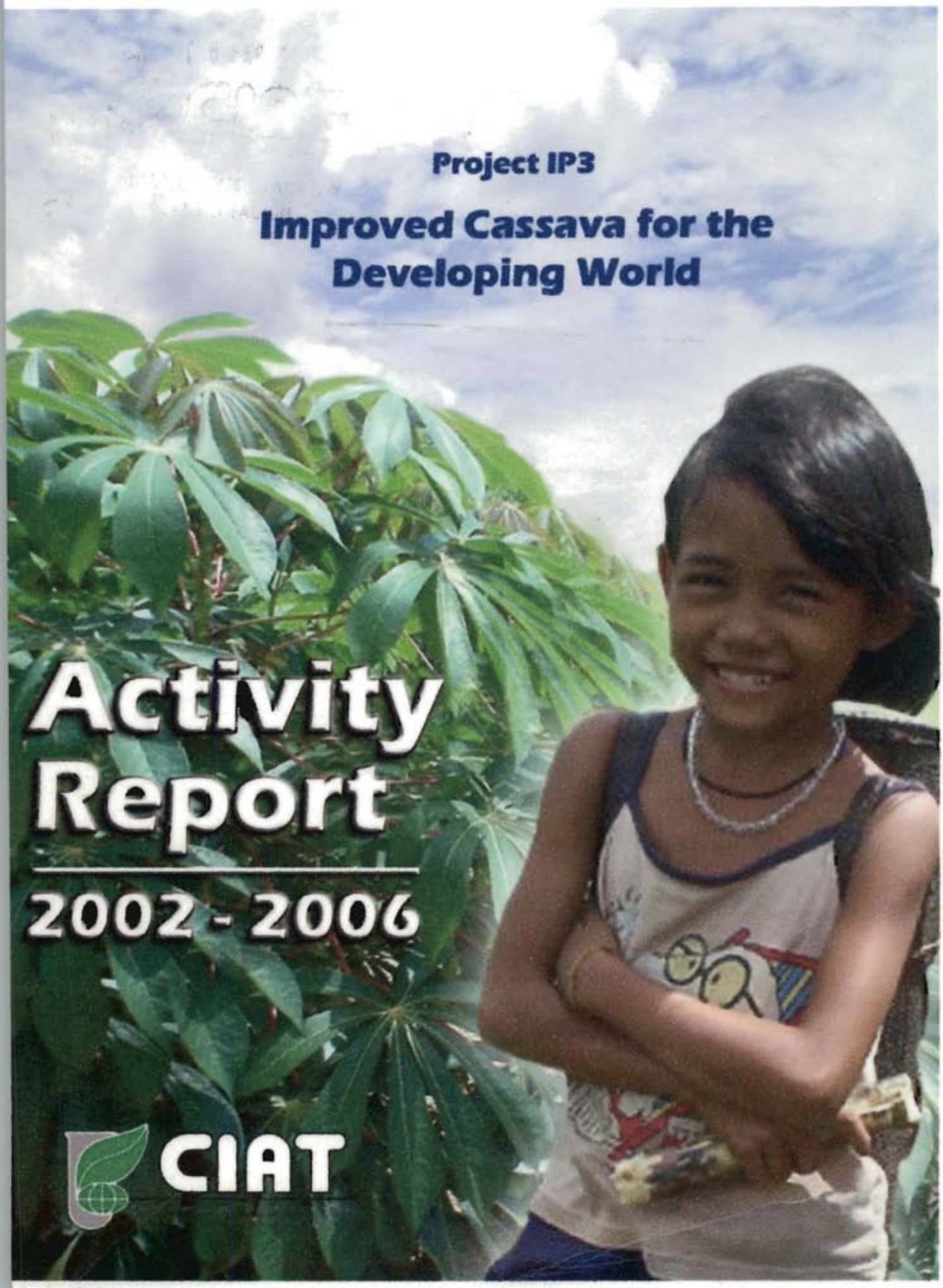
Project IP3

Improved Cassava for the Developing World



Activity Report

2002 - 2006

CIAT

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1. Description of cassava as a crop

1.1 Introduction of the crop

Cassava (*Manihot esculenta* Crantz), along with maize, sugarcane and rice, constitute the most important sources of energy in the diet of most tropical countries of the world. The species originated in South America (Allem, 2002), and was domesticated less than 10,000 years ago. Early European sailors soon recognized the advantages of the crop and carried it to Africa. From there, traders later introduced it to Asia. Until recently, cassava and its products were little known outside the tropical and subtropical regions where it grows. No major scientific efforts had been made to improve the crop. However, with the creation of the International Institute of Tropical Agriculture (IITA) in Nigeria and the International Center of Tropical Agriculture (CIAT) in Colombia in the early 1970's a new era began for cassava with the implementation of successful breeding projects, modernization of cultural practices and development of new processing methods (Cock, 1985; Jennings and Iglesias, 2002). National research centers in Brazil, Colombia, Cuba, India, Thailand and Vietnam among many other countries have conducted successful research on cassava as well.

Plant breeding has one of the highest rates of return among the investments in agricultural research. It has been reported that the remarkable increase in the productivity of many crops during the twentieth century was due to genetic gains achieved through crop breeding. Cassava has also benefited from technological inputs in the area of breeding (Kawano, 2003). New varieties in Africa, Asia, and Latin America and the Caribbean satisfy the needs of farmers, processors, and consumers, bringing millions of dollars in additional income to small farmers. New technologies in the area of tissue culture, genetic transformation and molecular biology have also made positive contributions (Calderón-Urrea, 1988; DeVries and Toenniessen, 2001; Fregene et al., 1997 and 2000; Puonti-Kaerlas et al., 1997).

Currently cassava is an important crop in regions at latitudes between 30° N and 30° S, and from sea level up to 1800 meters above sea level (Figure 1.1). Although its most common product is the starchy root, cassava foliage has an excellent nutritional quality for animal and human consumption and offers great potential. Cassava is the fourth most important basic food after rice, wheat and maize and is a fundamental component in the diet of millions of people. Scott et al. (2000) estimated that for the year 1993, annual production of cassava was about 172.4 million tons, with a value of approximately $US 9.31 billion. Between 1961-63 and 1995-97, cassava production increased at a rate of 2.35 % / year (Scott et al., 2000), a trend comparable to that found in other crops such as wheat (4.32%), potato (4.00%), maize (3.94%), yams (3.90%), rice (2.85%) and sweet potato (1.07%). Between 1994 and 2005 cassava productivity will increase by 1.1 % / year.

Cassava is a very rustic crop that grows well under marginal conditions where few other crops could survive. A large proportion of cassava varieties are drought tolerant, can produce in degraded soils, and are resistant to the most important diseases and pests. The crop is naturally tolerant to acidic soils, and offers the convenient flexibility that it can be harvested when the farmers need it.

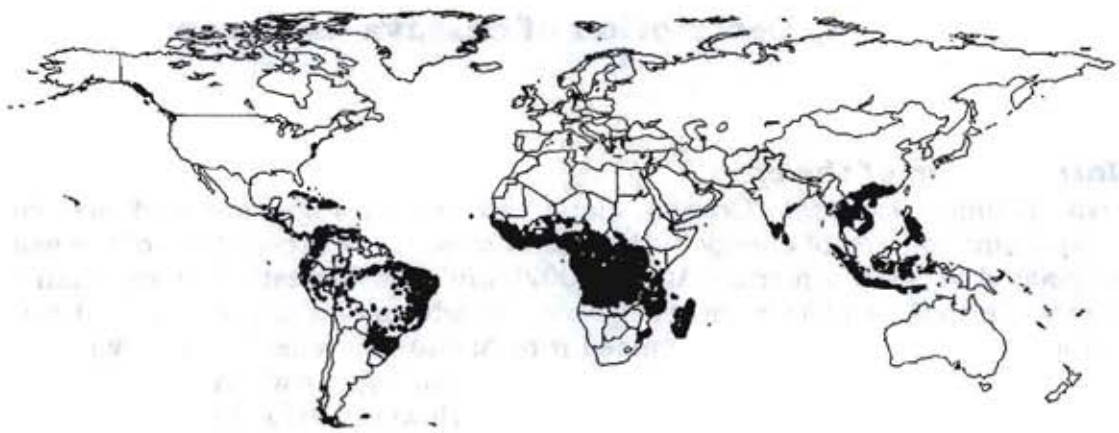

Figure 1.1 Major producing regions of cassava in the worls.

1.2 The plant and its uses.

Cassava is a perennial shrub. Basically every part of the plant can be utilized, but the starchy roots are by far the most commonly used product. Cyanogenic glucosides (CG) are found in every tissue except in the cassava seed. The most abundant CG is linamarin (about 85%), with lesser amounts of lotaustralin. The CG, synthesized in the leaves and transported to the roots, is broken down by the enzyme linamarase to produce HCN, a volatile poison (Andersen et al., 2000; Du et al., 1995; McMahon et al., 1995; Wheatley and Chuzel, 1995). Linamarin and linamarase accumulate in different parts of the cell, thus preventing the formation of free cyanide. However, most processing methods disrupts the tissues, allowing the enzyme to act on the substrate for a rapid release of cyanide. CG accumulation varies with genotypes, environment, agronomic practices, age of the plant and plant tissue, being highest in the leaves and peel of roots (Cock, 1985).

The starchy roots are a valuable source of energy and can be boiled or processed in different ways for human consumption. Roots can also be used for obtaining native or fermented starches and as dried chips, meal or pellets for animal feed. Cultivars with less than 100 mg CG kg^{-1} fresh weight in the roots are considered "sweet". Above this level cassava roots are considered "bitter". Depending on the processing methodologies, bitter or sweet clones may be preferred. In addition to the cyanogenic potential other relevant traits for the roots are dry matter content, percentage of amylose in the starch composition, protein and carotenoid contents. There is variation in starch quality in relation to its amylose percentage with a mean around 15 % (Wheatley et al. 1993). Cassava roots are low in protein content with an average of about 2-3% (dry weight basis). There have been, however, some preliminary results suggesting that protein content in the roots can be considerably higher (6-8%) in some landraces, particularly from Central America (CIAT, 2002). Yellow cassava roots have considerable amounts of carotenes (Chávez et al., 2000; Iglesias et al. 1997).

Cassava roots are not tubers and, therefore, cannot be used for reproductive purpose. A major consequence of this situation is their short shelf life (Beeching et al. 1998): within one or two days after harvest there is a rapid initiation of post-harvest physiological deterioration

(PPD). To date, little useful genetic variation to delay or reduce PPD has been found, and the solution to this problem remains one of the most important goals for cassava research.

Cassava stems are the most important source of planting material to propagate the crop. Cassava foliage is not widely exploited in spite of its high nutritive value, although consumption of leaves by human populations is relatively common in certain countries of Africa and Asia. Foliage is also used for animal feeding. Crude protein content in leaves typically ranges from 20 to 25% dry weight (Babu and Chatterjee, 1999; Buitrago, 1990; Gomez et al.,1983), but levels as high as 30% (dry basis) have been identified (Buitrago, 1990). Content of cyanogenic glucosides in the foliage is markedly higher (3800-5900 mg HCN kg^{-1} fresh weight), than in roots (4-113 mg HCN /kg fresh weight). Exploitation of foliage in cassava is expected to increase because of the recent developments and testing of mechanical harvesters and alternative cultural practices to exploit it (Cadavid Lopez and Gil Llanos, 2003).

1.3 Reproduction in cassava.

Cassava can be propagated either by stem cuttings or by sexual seed (Figure 1.2). However, the former is the most common practice used by farmers for multiplication and planting purposes. Propagation from true seed occurs occasionally in farmers' fields and, as such, is a starting point for the generation of useful genetic diversity (Alves, 2002). Most breeding programs generate seed through crossing, as a mean of creating new genetic variation. Occasionally botanical seed has also been used in commercial propagation schemes (Rajendran et al., 2000).

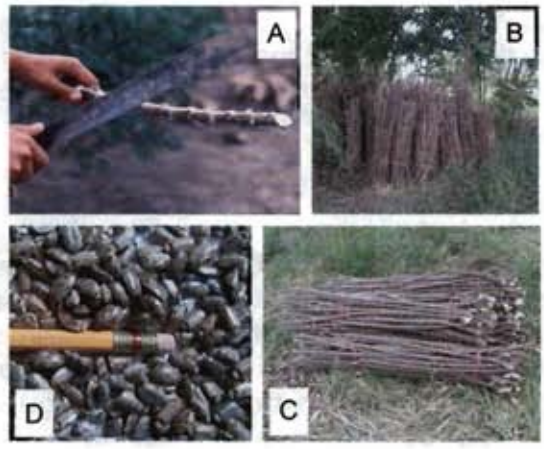

Figure 1.2. Vegetative and botanical seed of cassava. A: cutting the stems; B: storage of stems under a tree until planting time; c: preparing the stems for transport; D: botanical seed of cassava.

Cassava is monoecious, with female flowers opening 10-14 days before the male ones on the same branch (Figure 1.3). Self-pollination can occur because male and female flowers on different branches or on different plants of the same genotypes can open simultaneously (Jennings and Iglesias, 2002). Flowering depends on the genotype and the environmental conditions. Branching occurs when an inflorescence is formed. Because erect, non-branching

types, are frequently preferred by farmers, the crossing of elite clones in certain regions may become more difficult because of the scarcity of their flowers. Synchronization of flowering remains a difficult issue in cassava breeding. Some clones flower relatively early at 4 or 5 months after planting whereas others flower only at 8 to 10 months after planting. Because of this, and the time required for the seed to mature, it takes generally no less than a year to obtain seeds of a planned cross. On average, between one and two seeds (out of the three possibly formed in the trilocular fruit) per pollination are obtained. Several publications illustrate the procedures for controlled pollinations in cassava (Jennings and Iglesias, 2002; Kawano 1980). Seeds often have a dormancy period for a few months after maturity, and they require relatively high temperatures (30-35 °C) for optimum germination (Ellis et al, 1982).

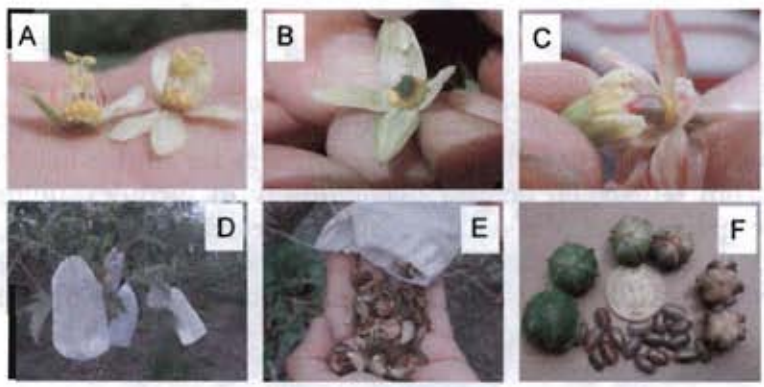

Figure 1.3. Flowering in cassava. A: Male flower; B: Female flower; C:Hand pollination for directed crosses; D: Cloth bags placed around the maturing fruit to capture the seeds; E: Harvest of seed; F: Fruits at different stages of maturity and botanical seed.

1.4 Breeding objectives

Breeding objectives depend on the ultimate use of the crop. Productivity plays a major role in industrial uses of cassava (i.e. starch production and dried roots for animal feed), whereas stability of production will be fundamental in the many regions where cassava is the main subsistence crop. Industrial uses of cassava require high dry matter content as the main quality trait for the roots, whereas human consumption will frequently emphasize cooking quality or starch characteristics over productivity, as a determining trait. Good cooking quality is usually associated with other morphological traits such as the color of the peel of the roots, the leaf petiole or the shoot. Farmers frequently reject any change in such morphological traits, although they may have little or no correlation with actual cooking quality. Because of those types of farmers and consumer preferences, participatory research and breeding approaches had to be developed for cassava breeding (DeVries and Toenniessen, 2001; Gonçalvez Fukuda et al., 2000; Gonçalvez Fukuda and Saad, 2001).

Other root quality traits relevant to different cassava breeding programs of the world are the cyanogenic potential in the roots (Dixon et al., 1994), early bulking capacity, higher protein content in the roots and reduced post-harvest physiological deterioration. Unfortunately, the

genetic variability for the latter two traits is limited in *M. esculenta* and, therefore, interspecific crosses with other *Manihot* species to introgress useful alleles have been attempted.

Stability of production is associated with resistance or tolerance to major biotic and abiotic stresses, the emphases vary with the target environments. In Africa Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD) are important constraints. A disease similar to CMD is also present in southern India. In certain regions of Latin America and the Caribbean (LAC), Frogskin Disease causes roots to become "corky" and commercially unusable. The causal agent has not yet been identified, although it has been suspected for many years that it may be a virus. Bacterial blight, induced by *Xanthomonas axonopodis* pv. *Manihotis* (also known as *X. campestris* pv. *manihotis*), is found in Asia, Africa and LAC, and can have devastating effects on yield and the availability of planting material, particularly in Africa and LAC (Hillocks and Wydra, 2002). Several fungal diseases also may affect cassava productivity. Super-elongation disease, induced by *Sphaceloma manihoticola* (Teleomorph: *Elsinoe brasiliensis*) is widespread in the Americas, from Mexico to Southern Brazil. In tropical lowlands with high rainfall *Cercospora, Cercosporidium, Phaeoramularia* or *Colletotrichum* species can affect cassava productivity (Jennings and Iglesias, 2002). *Phoma* species cause leaf and stem lesions in the tropical highlands. Several species of *Phytophthora* induce root rot but also different species of the genera *Sclerotium, Armillaea* and *Fusarium*. There are sources of genetic resistance to most of these diseases (CIAT, 2001; Hillocks and Wydra, 2002).

Several arthropod pests feed on cassava and can reduce productivity. The green mite (*Mononychellus tanajoa*) devastated cassava fields upon its introduction in Africa in the 1970s (Nyiira, 1975). Other mites important for cassava are *Tetranychus urticae, T. cinnabarinus, Mononychellus caribbeanae* and *Oligonychus peruvianus* (Bellotti et al., 2002). The mealybugs *Phenacoccus manihotis* and *P. herreri* feed on cassava fields of Africa and LAC, respectively. Thrips (particularly *Frankliniella williamsi* and *Scyrtotrips manihoti*) considerably reduce yields of susceptible genotypes. Clones with pubescent leaves in their early stages of development offer excellent levels of resistance to these insects (Belloti, 2002), and this trait has been broadly incorporated into improved varieties.

Whiteflies are among the most widespread pests in cassava. *Aleurotrachelus socialis* is the predominant species in northern South America, where it causes considerable crop damage through direct feeding. *Bemisia tabaci* is widely distributed in tropical Africa and several Asian countries. Until 1990 *B. tabaci* biotypes found in the Americas did not feed on cassava. The major effect of *B. tabaci* is as a vector of the devastating CMD disease in Africa. Several other species of whiteflies affect cassava in different regions. Genetic resistance to whiteflies in cassava has been found particularly for *A. socialis* in several germplasm accessions from the CIAT collection (Bellotti, 2002). Based on breeding work at CIAT, Colombia released the first whitefly-resistant variety of any crop, in 2002, targeted toward the Tolima Valley, where whiteflies typically devastate plantations.

There are several other arthropod pests affecting cassava roots, foliage and/or stems, particularly Lepidoptera, Diptera and Hemiptera. There is little or no genetic resistance to those pests and their management is commonly achieved through biological control measures. Recently, attempts to produce transgenic cassava have succeeded with the introduction of cry genes encoding insect-specific endotoxins (Bt toxins) from *Bacillus thuringiensis* (Fregene and Puonti-Kaerlas, 2002; Ladino et al. 2001).

There are a variety of abiotic factors limiting cassava productivity. The crop is frequently grown in drought-prone regions and/or on low fertility soils. It can also be found in alkaline or acidic soils, most frequently the latter. Some traits associated with adaptation to these conditions have been suggested (Jennings and Iglesias, 2002), such as: leaf longevity (CIAT 2001; Fregene and Puonti-Kaerlas, 2002), optimum Leaf Area Index, and ideal plant architecture (Hanh et al., 1979; Kawano et al. 1998; Kawano 2003). The capacity of the stems to withstand long storage periods (sometimes up to two months) from harvest to planting affects final density of established plants and is an important trait for areas with relatively long dry spells or erratic rainfall, because the storage period may extend to the point it compromises their viability. While there is known genetic variation for stem storability, it has not been a major breeding objective of any program so far.

1.5 Breeding scheme

As in most crop breeding activities, cassava genetic improvement starts with the assembly and evaluation of a broad germplasm base, followed by production of new recombinant genotypes derived from selected elite clones. Scientific cassava breeding began only a few decades ago and, therefore, the divergence between landraces and improved germplasm is not as wide as in crops with a more extensive breeding history. As a result, landrace accessions play a more relevant role in cassava than in other crops. Parental lines are selected based mainly on their *per se* performance and little progress has been made to use general combining ability (Hallauer and Miranda, 1988) as a criteria of parental selection. Crossing can be by controlled pollinations, done manually, to produce full sib families or else in polycross nurseries where open pollination results in half-sib families.

For open pollinations a field planting design developed by Wright (1965) is followed to maximize the frequency of crosses of all the parental lines incorporated in the nursery. Knowledge about flowering capacity is important in order to select a group of materials with synchronized flowering. When there are considerable differences in flowering habit a delayed planting and/or pruning of the earliest flowering genotypes may be required. At harvest the seed harvested from each clone are bulked to form a half-sib family. Seeds from full-sib families can be obtained in isolated open pollination plots were two clones are planted together and one of them, chosen to act as female progenitor is emasculated. Alternatively, several male-sterile clones have been identified, which can act as female parents.

The botanical seed obtained by the different crossing schemes (Kawano, 1980) may then be planted directly in the field (as done at IITA) to take advantage of the availability of irrigation and high temperatures. At CIAT, seeds are germinated in greenhouse conditions and the resulting seedlings transplanted to the field when they are about 20-25 cm tall (Jennings and Iglesias, 2002). Root systems in plants derived from botanical seed or vegetative cuttings may differ considerably. The tap roots from seedlings, tend to store fewer starches than roots from cuttings (Rajendran et al., 2000). Because of this, it is difficult, if not impossible, to correlate the root yield of clones at later stages in the evaluation/selection process with early results from the plants obtained from botanical seeds. However, when seeds are germinated in containers and later transplanted, the tap root often does not develop, and the seedling-derived plant may be more similar to subsequent stake-derived plants in terms of starchy root conformation.

The vegetative multiplication rate of cassava is low. From one plant, 5-10 cuttings typically can be obtained, although this figure varies widely by genotype. This situation implies a

lengthy process to arrive at the point where replicated evaluations across several locations can be conducted. It takes about 5-6 years from the time the botanical seed is germinated until the evaluation/selection cycle reaches the regional trial stage when several locations can be included. One further complication in a cassava program is the number of factors that can affect quality of planting material. For example, the original positioning of the vegetative cutting along the stem affects considerably the performance of the plant it originates. Cuttings from the mid-section of the stems usually produce better performing plants than those at the top or the bottom. This variation in the performance of the plant depending on the physiological status of the vegetative cutting, results in larger experimental errors and undesirable variation in the evaluation process.

Table 1.1 illustrates a typical selection cycle in cassava. It begins with the crossing of elite clones and ends when a few clones surviving the selection process reach the stage of regional trials across several locations. There is some variation among different cassava-breeding programs, regarding the numbers of genotypes and plants representing them through the different stages; however, the numbers presented in Table 1.1 are fairly common and illustrate the different stages required to complete a selection cycle and the kind of selection pressures generally applied.

Table 1.1. A typical selection cycle in cassava, beginning with the crossing of elite clones, through the different stages of the selection process (from Jennings and Iglesias, 2002).

Year	Activity	Number of genotypes	Plants per genotype
1	Crosses among elite clones	Up to 100,000	
2	**F1**: Evaluation of seedlings from botanical seeds. Strong selection for ACMV in Africa.	100000[a]; 50000[b]; 17500 [c]	1
3	Clonal Evaluation Trial **(CET)**	2000-3000 [a, b] 1800 [c]	6-12
4	Preliminary Yield Trial **(PYT)**	100 [a]; 300 [b]; 130 [c]	20-80
5	Advanced Yield Trial **(AYT)**	25 [a]; 100 [b]; 20-18 [c]	100-500
6-8	Regional Trials **(RT)**	5-30 [a, b, c]	500-5000

Figures for the cassava breeding at [a] IITA (Ibadan, Nigeria); [b] CIAT (Cali, Colombia) and [c] CIAT-Rayong Field Crops Research Center (Thailand). Averages from data in Kawano, 2003.

The first selection is conducted the second year on the nurseries with plants derived from botanical seed (**F1** in Table 1.1). Because of the low correlations between the performance at this early stage of selection and when the genotypes reach replicated trials, the early selec-

plots to increase within clone competition and reduce between clone competition. Figure 1.6 illustrates a typical PYT as currently conducted. Large genetic variability occurs among clones, even from the same family. Although poor performing clones are mostly eliminated at the **CET** stage, there is still a considerable variation in the **PYT** trials. This highlights the need for a gradual process of selection and the need to avoid strong selection pressures.

Figure 1.6. Preliminary yield trials (**PYT**). The photograph on the left shows two 2-rows plots with a total of 10 plants per plot. The photograph on the right shows the kind of contrast in root yields that can be observed in these PYTs.

With the initiation of replicated trial does the emphasis of selection shift from high-heritability traits to those of low heritability such as yield. Starting with **PYT** and increasingly during the Advanced Yield Trials (**AYT**) and the Regional Trials (**RT**) there will be a greater weight on yield and its stability across locations. Cooking quality, "poundability" (IITA), and "farinha" quality (Brazil) trials will also began at these stages, when the number of genotypes evaluated has been reduced to a manageable size. **AYT** are typically grown in 1-2 locations for two consecutive years. They have three replications per location and plots are four rows with five plants per row. Yield data is taken from the six central plant of the plot and the remaining 14 plants are used as source of planting material for the next season. **RTs** are conducted for at least 2 years in 4-10 locations each year. Plots have five rows with five plants per row. Yield data is taken from the nine central plants.

The clones that show outstanding performance in the **RTs** are released as new varieties and, often, incorporated as parents in the crossing nurseries. This completes a selection cycle and a new one begins. It should be pointed out that the selection scheme described above has the following characteristics:

- The process is indeed a mass phenotypic recurrent selection, because no family data are involved in the selection process.

- Few data are taken in early stages of selection, especially on genotypes that can be readily discarded by visual evaluation. Therefore, no data regarding general combining ability effects (≈ breeding value) are available for a better selection of parental materials.

- There is no proper separation between general (GCA ≈ additive) and specific (SCA ≈ heterotic) combining ability effects. The outstanding performance of selected materials is likely to depend substantially on positive heterotic effects, which cannot be transferred to the progenies sexually derived from them.

- Inbreeding has been intentionally omitted in the breeding scheme. Therefore large genetic loads are likely to remain hidden in cassava populations and useful recessive traits are difficult to detect..

- Two or more stages of selection may be based on unreplicated trials. A large proportion of genotypes is eliminated without the proper evaluation set up.

Because of the foregoing reasons there are some clear opportunities to further improve the efficiency and effectiveness of cassava breeding. Kawano et al. (1998) mention that during a 14-year period, about 372,000 genotypes, derived from 4130 crosses, were evaluated at CIAT-Rayong Field Crop Research Center. Only three genotypes emerged from the selection process to be released as official varieties. Nonetheless, it should be mentioned that these varieties have achieved remarkable success in Asia, with more that one million hectare planted. Similar experiences have been observed at IITA, CIAT- Colombia and Brazil. The resulting increases in productivity account for a higher income (about one billion $US annually) to the poor farmers who grow the improved germplasm (Kawano, 2003).

1.6 Adequate screening of genetic available variability.

Genetic variability available within *Manihot* has not been fully explored and screened. Therefore, this genetic wealth has not been fully exploited, and should offer interesting possibilities for the future. In part, the limited evaluation of cassava genetic variability is because the collection and maintenance of cassava germplasm is difficult, cumbersome and expensive. Compared with other crops, a relatively smaller number of accessions is maintained in the germplasm collections (5,728 and 32,445 for cassava and beans germplasm collections at CIAT, respectively). The remarkable genotype-environment interaction shown by cassava (Bueno, 1986), complicates the interpretation of evaluations across different environments. Furthermore, detection of some of the economically important traits in the roots is more difficult. For instance, the many different starch mutants in maize (popcorn, sweet, floury, waxy corn, etc.) are easily recognizable. No equivalent mutant has been reported for cassava.

Nutritional quality factors studied to date also show relatively low variation, with the exception of the high carotene levels found in yellow cassava roots (Iglesias 1997). Very little success has been obtained for increasing protein content in the roots. Few *M. esculenta* accessions from Meso America have been found to have higher levels of proteins in the roots (CIAT, 2002). An introgression of genes from related *Manihot* species into the *M. esculenta* gene pool may have occurred but these findings require further analysis and confirmation.

1.7 Perspectives and challenges

During the past 30-40 years, significant progress has been achieved in the initial phase of the scientific genetic improvement of cassava. In a way it could be said that the adaptation of the crop to more intensive cultivation systems has been completed. This process involved assembling major traits such as improved yield (mainly through a higher harvest index), low cyanogenic content (when desirable), improved plant architecture and resistance/tolerance to the major diseases and pests.

Future activities involve an increasing emphasis on complex traits such as higher yield and dry matter content in the roots, early bulking, etc., which are more difficult to improve. It is critical for cassava that efficient methods for the improvement of these complex traits are found to maintain the competitive edge that this crop currently has in tropical regions as an alternative to imported carbohydrate sources from temperate regions. Several approaches have been taken to address this situation in recent years. Modifications of the breeding scheme have been implemented for a more dynamic recurrent selection system and for obtaining valuable information on the breeding value of parental lines. Biotechnology tools have been adapted to cassava and are currently incorporated in different projects for its genetic improvement. A molecular map has been developed (Fregene et al., 1997; 2000; Mba et al. 2001) and marker assisted selection is currently used for key traits. Genetic transformation protocols are available and have been used successfully for the incorporation of different genes. Tissue culture techniques can also benefit cassava through the production of doubled-haploid lines.

One of the challenges for the crop is for a more extensive exploration to increase the germplasm collections and to develop approaches that will allow for an efficient evaluation of such germplasm. In this regard, tools for rapid identification of novel starch types are needed. The lack of genetic variability for overcoming the problem of post-harvest physiological deterioration remains a major bottleneck for cassava utilization and commercialization. For logistic reasons the unresolved problem of frog skin disease remain a frustrating bottleneck for research at CIAT Experimental Station in Palmira.

The inherent potential of cassava, its capacity to grow in marginal environments and the incorporation of new, powerful biotechnology tools as described in several articles from this special issue, offer a bright perspective for the crop and the people that depend on it.

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2. Production and identification of high-quality cassava

2.1 Introduction

Cassava (*Manihot esculenta* Crantz), along with maize, sugarcane and rice, constitute the most important sources of energy in the diet of most tropical countries of the world. In addition to its important role in subsistence farming and food security, cassava is acquiring an increasing role in rural development as raw material for many processing pathways. 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).

During the 1990s there was a drastic change in the economies of tropical and subtropical countries where cassava is grown. As a result of the globalization of the economies, it became obvious that tropical production of maize was not competitive compared with that from temperate regions. Several factors explain this situation (Pandey and Gardner, 1992): Differences in the photoperiod imply that a maize plant in Iowa (USA) or Pergamino (Argentina) producing for several more hours every day, the cool nigh temperature in temperate regions imply that the same plants will respire less every hour during the night, which is also shorter. The winter in temperate regions interrupts the cycle of pests, diseases and weeds a beneficial effect generally not present in the tropical world. Finally the farmers in temperate regions benefit from a considerably more refined technical support. For instance, the multinational hybrid seed system does not have a truly tropically-based hybrid maize research (perhaps as a result of the obvious limitations observed in tropical maize production of which they are in a better position to appreciate). Hybrids grown in the tropics are those originally developed for the subtropical environment. Because of these reasons there was an increased volume of temperate maize imported by tropical countries. Figure 2.1 shows the importation of maize in Colombia during the 1990s. This is not a result of unfair economic competition based on subsidies because Colombia imported maize from Argentina, which does not benefit from any subsidy.

As a result of the economic changes observed during the 1990 deep changes in the agriculture environment took place. For instance, a key agribusiness such as the poultry industry in Colombia saw for the first time ever, the strategic role of cassava in animal feed. For several years this sector financed research at CIAT for increased productivity of cassava with satisfactory results. The key objective of a production of 10 t/ha of dry matter (around 25-30 t/ha of fresh roots) was widely supported by results in commercial plantings. However, it became clear that in addition to high and stable productivity the cassava-breeding project had the opportunity of expanding and exploiting genetic variability that would generate clones in increased value for the different industrial processes where cassava can be an strategic raw material. Examples of key traits for the different industries are mentioned below:

<u>*Animal Feed*</u>: Cassava is an important commodity as source of energy in the diets. However, it has lower levels of protein and, therefore, its use imply the need to modify the composition of the diet with an additional source of protein (typically soybean derivatives). For this

reason, the rule of the thumb says that cassava cannot be more than 70% of the price of maize. Key qualitative traits for this industry would be finding cassava clones with higher levels of proteins in the roots. In addition the possibilities of other nutritional traits such as pro-vitamin A carotenoids would be beneficial.

Starch Industry: Cassava starch has properties of its own which make it particularly adapted (or not adapted) to certain uses. This sector has always requested novel cassava starch types to diversity its uses.

Ethanol and bioplastics: This is a relatively new demand for cassava products, which was accentuated with the recent increases in the price of oil. A "*sugary*" cassava would make the process of fermentation to produce ethanol of lactic acid (an alternative product in the pathway for the production of bioplastics) economically and environmentally less expensive.

Processed food: Acyanogenesis (roots without even traces of cyanogenic glucosides) has been a trait requested by this sector.

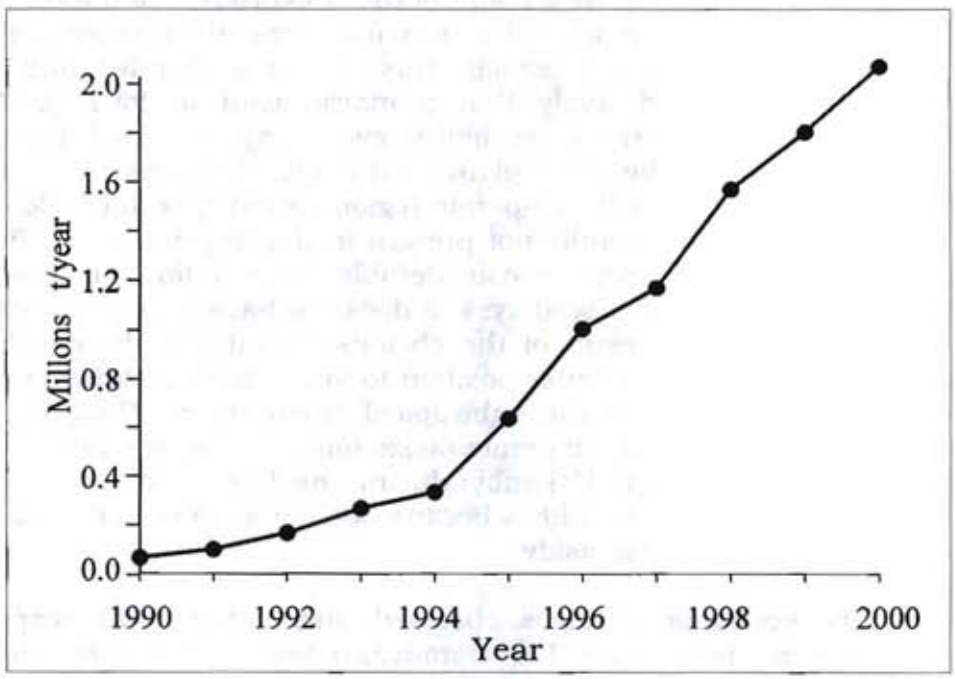

Figure 2.1. Annual importation of maize (million t/year) in Colombia during the decade of the 1990s.

Several factors have limited the actual impact of this crop in tropical agriculture. On the biological side, cassava breeding is cumbersome and relatively slow. Cultural biases have also affected cassava. For several decades, public and private sector investment favored investments for research and development in cereals such as maize, rice, wheat and sorghum

(Cock, 1985). Also, little genetic variability has been found for key economic traits perhaps as a result of the limited investments to conduct research on this crop in the previous decades.

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'*. The value of dried cassava roots, when used for the feed industry, is lower than that of maize because of their reduced levels of proteins (which is typically around 2 % dry weight basis, compared with about 8 % in maize kernels).

One 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 maize starch, 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). Figure 2.2 illustrates common cases of mutation in maize that are readily identifiable by visual inspection. To detect a mutation in the cassava root starch, the breeder will have to cut the roots and most likely will have to conduct a particular test (i.e. iodine test) or biochemical analysis to be able to pick potentially useful variants. It is possible, therefore, that clones with valuable traits have already been grown in breeding nurseries but could not be detected and, not showing an outstanding agronomic performance, they were unfortunately 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.

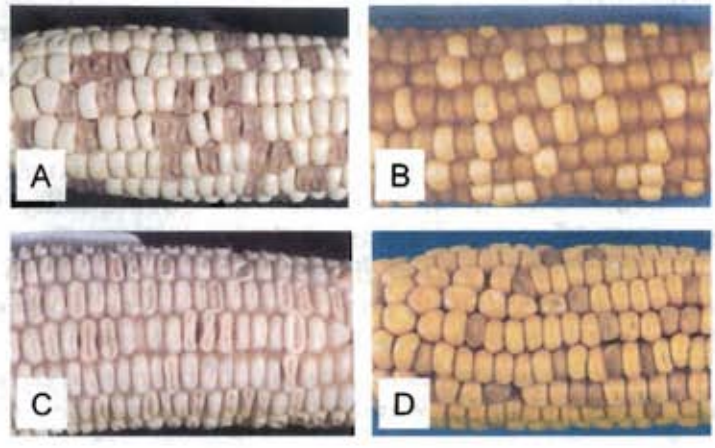

Figure 2.2. Examples of easy to identify mutations in the maize kernel. A: sweet corn induced by the *su* (sugary) mutation; B: high quality protein maize observed in the *op2* (opaque 2) mutation; C: sweet corn induced by the sh1 (shrunken 1) gene; and D: typical phenotype of the amylose expander 1 (*ae1*) gene.

The fact that roots are not reproductive or multiplicative organs may offer cassava (and other root crops) an advantage over the true-seed propagated crops. 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.

In spite of the problems mentioned above, the globalization of the economies and new technological breakthroughs are offering a unique opportunity for cassava never available to the crop before. Tropical production of maize is facing increasing problems to compete with maize from 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.

This chapter describes the approaches taken at CIAT for developing and identifying cassava clones with higher value in their roots for the different industrial uses of cassava. A fundamental set of principles justify this strategy in light of the mission of CIAT.

- Processing cassava needs to be done near the fields where it is produced because of the short shelf life of the roots. Therefore, promotion of cassava processing invariably results in **rural development**.

- For many years a bottleneck expressed by farmers in many different commissioned studies has been the lack of markets for cassava. Therefore, generating products that will encourage processing of cassava addresses the main problem of farmers. Turning cassava into a cash crop has been a major goal for this project with huge implications in **poverty alleviation**.

2.2 Materials and methods

Several strategies have been followed simultaneously to produce and/or identify cassava clones with high value for the industry. These strategies tried to overcome some of the limitations in cassava research described above.

Aggressive evaluation of landraces and improved germplasm: The first step given by the project has been to recognize the need to screen for root quality traits in the available germplasm. Obviously, CIAT has the unique advantage of hosting the worldwide cassava germplasm collection. However, only limited efforts have been made in the past to explore the variability present in this collection and its potential usefulness in cassava growth, utilization and/or processing. Through the recent years the cassava-breeding project has searched for resources to be able to screen the germplasm collection in search of commercially useful traits. In addition, routine evaluations are made in the germplasm developed in the crossing nurseries.

Inbreeding cassava to allow recessive mutations to express themselves: The introduction of inbreeding in the genetic improvement of cassava offers several advantages which have been described (Ceballos et al., 2004; Pérez et al., 2005a; 2005b). One of the advantages, which has a direct bearing with the theme of this chapter, is that it would allow for the

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

CIAT, with the support of the Rockefeller Foundation, and in collaboration with several cassava breeding programs in Africa, Asia and Latin America began in 2004 a research project to improve tolerance to inbreeding in elite cassava germplasm. Elite germplasm has been self-pollinated to generate a large number of botanical seed with varying degrees of inbreeding (Table 2.1). The project also involves the development of an 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 production of doubled haploids in cassava provides an appealing option for feasible introduction of inbreeding in cassava genetic improvement by drastically reducing the time required to produce homozygous parental lines.

Table 2.1 Number of self-pollinated botanical seed produced from a total of 74 elite germplasm in the cassava-breeding project at CIAT.

Parental clones	Number of partially inbred genotypes produced		
(#)	S_1	S_2	S_3
2	841	1272	218
16	7988	4397	0
56	9980	0	0
74 (Total)	**18809**	**5669**	**218**

Mutagenesis and the "TILLING" System: Breeders have used chemical products or irradiation such as gamma rays to induce mutations and generate genetic variability with relative success, particularly in the decades of the 1950s and 1960s (Maluszynski et al., 2001; Ahloowalia et al., 2004). Mutation breeding has a few drawbacks. Events are totally random, recessive in nature and usually appear as chimeras. Therefore, thousands of genotypes need to be evaluated before a useful mutation in the desired gene can be found. With the advent of molecular biology tools, an interesting system was developed to overcome some of the limitations of 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 plants ($\mathbf{M_1}$), they are self-pollinated. The resulting plants ($\mathbf{M_2}$) are then evaluated while DNA is extracted from them. For screening purposes, DNAs are pooled eightfold to maximize the efficiency of mutation detection. (description of the TILLING method adapted from Till et al., 2003).

CIAT is participating in a project led by Universidad Nacional de Colombia, which is supported by the IAEA (International Atomic Energy Agency). About 4,000 seeds from six different cassava clones were irradiated with gamma rays (using a Cobalt 60 source with a dosage level of 200 Gy) or with fast neutrons. Seeds were germinated and transplanted to the field early in 2004. Plants 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 $\mathbf{M_1}$ stage). As soon as plants started to produce viable flowers, they were self-pollinated. As many as 5,000 $\mathbf{M_2}$ seeds, from about 140 different $\mathbf{M_1}$ plants, have been obtained. Several genes related to starch biosynthesis are currently targeted for TILLING analysis.

Recurrent Selection to Modify Amylose Proportion in the Root Starch of Cassava:. Currently CIAT is finalizing the screening of the entire cassava collection (with starch samples from around 6,000 landraces and improved germplasm). This process has allowed toobtain a better idea of the actual range of variation in different traits including starch quality traits. Starch is made up of two main molecules, which vary in their degree of branching: amylose and amylopectin. The relative proportions of these two polymers determine many of the physical and chemical properties of the starch and, therefore, have important economic implications. For several years landraces and improved germplasm from the cassava improvement project have been evaluated for starch quality traits and allowed for the identification of a group of contrasting clones for amylose/amylopectin proportion.

Development of a high-capacity root 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 or their nutritional properties (Kawano et al., 1998; Jennings and Iglesias, 2002; CIAT, 2003; Kawano, 2003). It is possible, therefore, that along with the eliminated clones, valuable starch or nutritional 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 (Figure 2.2).

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 unknown mutations may also be available in cassava. A common need of many of the strategies described in this chapter is for the availability of a high capacity root quality analysis laboratory to screen large numbers of samples (>15,000) in search of those genotypes with novel pasting properties of starch or enhanced nutritional value.

Exploring the wild Manihot gene pool in search of novel traits: CIAT is actively searching for several valuable traits in *Manihot* species other than *M. esculenta*. These activities are described elsewhere in this report in more detail. The only source of dramatically delayed post-harvest physiological deterioration (PPD) in cassava roots has been identified in an inter-specific hybrid between cassava and *M. walkera*. The delayed PPD trait, originally from the wild *Manihot* parent, was successfully transferred to an F_1 inter-specific hybrid suggesting a dominant or additive gene action of gene(s) involved.

CIAT has also carried out a long-term project for higher protein content in the roots introgressed from accessions of wild relatives (*M. esculenta* subsp. *fabellifolia* and *M. tristis*) into cassava (CIAT, 2004). A total of 49 inter-specific crosses ranging from 6.39 to 10.46% in protein content have been selected and back-crossed into the elite *M. esculenta* clone 'MTai 8' ('Rayong 60' from Thailand). More than 6,000 back-crosses (BC) have been made. Further work is required to recover the root yield potential typical of *M. esculenta*, while maintaining (or even increasing) the current protein contents observed in the BC_1 populations. The issue of quality of proteins is also relevant and the profiling of amino acids is currently underway.

The only source of resistance to the cassava hornworm and a widely deployed source of resistance to cassava mosaic disease (CMD) were identified in 4th back-cross derivatives of *M. glaziovii* (Herrera et al., 2004). Moderate to high levels of resistance to white flies have been found in inter-specific hybrids of *M. esculenta* subsp. *flabellifolia* (CIAT, unpublished data). Again, resistance was recovered easily in F_1 inter-specific hybrids, suggesting a simple inheritance of the trait. Finally, accessions of *M. crassisepala* and *M. chlorosticta* are the only genotypes from the primary and secondary gene pool of the crop discovered to possess the waxy starch phenotype (CIAT, 2004). For several years now molecular marker tools and a modified advanced back-cross QTL scheme have been tested for cost-effective pyramiding of useful genes from cultivated and wild gene pools through the elimination of phenotypic evaluations in each breeding cycle.

Genetic Transformation: CIAT was the first institution to transform cassava. This event took place about 15 years ago. Since then a gradual improvement on the protocol for the genetic transformation of cassava has been achieved. Several genes have been introduced by different laboratories but the protocol need further improvement. Efficiency of transformation remains highly genotype-specific. The genetic engineering of industrial cassava varieties to produce waxy starch via anti-sense down-regulation of the GBSSI gene has been reported (Munyikwa et al., 1997; Salehuzzaman et al., 1993). GBSSI is the predominant starch synthase gene that catalyses the conversion of ADP-glucose into amylose. With funds from the Colombian Ministry of Agriculture and Rural Development, a similar project was initiated by CIAT. The isolation of a full-length GBSSI cDNA clone, construction of sense- and anti-sense transformation cassettes and their insertion into the genome have been described (CIAT, 2003). Two genetic constructions with the GBSSI gene in anti-sense and sense orientation in the vector pCAMBIA 1305.2 were made to achieve silencing of the gene. The constructs were initially introduced into friable embryogenic callus (FEC) of the model transformation genotype MNig11, from Nigeria, via *Agrobacterium tumefaciens*. Results of GUS transitory assay revealed a successful incorporation of the gene. Transformation of cassava accession MCol2215, from Colombia, was also attempted.

2.3 Results

A summary of the most interesting results from the activities described is presented in the following pages.

Aggressive evaluation of landraces and improved germplasm: This is a major undertaking of the project with no specific objective apart from identifying useful variants. Figure 2.3 provides result of amylograms that analyze the pasting properties of a typical cassava starch and that of a landrace (called NEP by local farmers) from the Central Vietnamese province of Hue. This graph suggest that starch from Nep is more stable and the gels it produces do not degrade as easily as those from normal cassava starch.

Table 2.2 summarizes the results of the most relevant information regarding the nutritional quality of cassava analyzed during the past few years. The main interest behind this information is the use of cassava as human food and animal feed. Of particular relevance is the variation observed for carotenoids and protein contents. Variation for Fe and Zn was originally found to be promising. However recent results suggest that Fe and Zn contents in cassava roots may be affected by the environment, particularly the edaphic conditions (not only Fe and Zn contents in the soil but also the pH of the soil) where cassava is grown. The

possibilities of increasing Fe and Zn contents in cassava roots are being further analyzed and will not be further described in this chapter. It should be mentioned that the work to increase carotenoids in cassava roots is part of the Harvest Plus initiative.

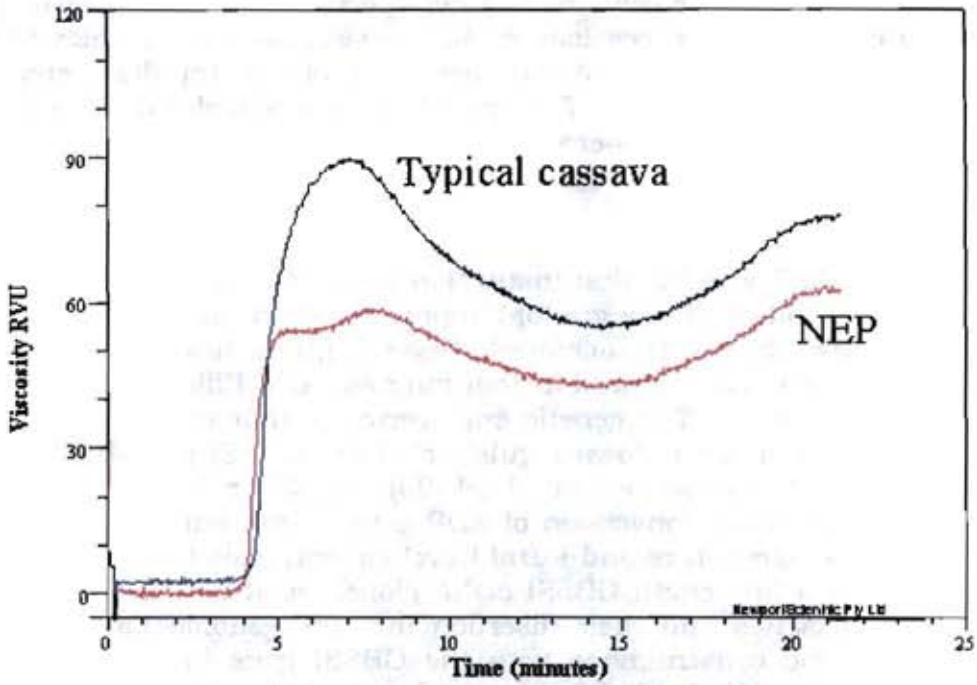

Figure 2.3. Comparison of pasting properties evaluated through the amylograms produced by rapid viscoanalyzer of starches from roots of a normal clone and from Nep of Central Vietnam. Farmers have long recognized special "sticky" properties of cooked roots from Nep. The clearly distinctive amylogram suggest that indeed the starch from this clone has different physico-chemical properties.

Considerable knowledge and products have been developed from the HarvestPlus initiative to increase carotenoids content in cassava roots (Chávez et al. 2005; Sánchez et al., 2005; Ceballos et al. 2005) not only in relation to the variation in concentration, but also its retention after different processing approaches. The maximum levels for total carotenoids content observed in directed crosses increased by about 30% the levels observed in the screenings, suggesting that the trait can be further improved by traditional breeding. As described elsewhere in this report molecular markers are being developed to understand the number of factors effectively contributing at the definition of carotenoids content in cassava roots. As a complement to the activities conducted by this project the genetic transformation approach is also being implemented by the Biotechnology Project at CIAT with the support of the HarvestPlus initiative.

One of the most interesting (and actually unexpected) result from the HarvestPlus initiative has been the discovery of very interesting variation in protein contents in cassava roots as illustrated by the data in Table 2.2. For the data presented in that table the N to protein conversion factor used was the standard 6.25. Hock-Hin and Van-Den reported in 1996 that, in the case of cassava roots, the conversion factor to estimate protein contents based on N

concentrations should probably range between 4.75 and 5.87. Preliminary results would suggest that the higher conversion factor (5.87) could better adjust to the measurements presented herein. However, the average between these two figures (5.31) was used instead as a conservative conversion factor between total nitrogen and protein content in the results provided below which have been accepted for publication (Ceballos et al., 2006).

Table 2.2. Simple descriptive statistics for the concentration of elements with potential nutritional relevance in cassava roots (dry weight basis)

	Sample size	Minimum	Maximum	Mean	Std. dev.
Elements measured in mg/kg					
Fe	600	6.0	230.0	17.1	15.2
Mn	600	0.45	5.0	1.4	0.6
B	580	1.14	9.91	2.0	0.6
Cu	599	0.79	40.31	5.8	5.4
Zn	600	2.63	37.52	7.5	3.6
Na	600	18.6	1230.0	129.2	147.3
Al	460	4.4	330	11.5	20.4
HCN*	2022	13.9	2561.7	263.7	324.2
Elements measured in %					
Ca	600	0.031	0.250	0.076	0.032
Mg	600	0.052	0.240	0.105	0.028
K	600	0.410	2.500	1.172	0.321
P	600	0.071	0.320	0.165	0.036
S	600	0.012	0.055	0.027	0.008
Protein	600	0.769	8.313	3.063	1.418
Total sugars	1755	0.2	15	2.876	2.028
Reducing sugars	1755	0.0	12.9	0.753	0.957
Root dry matter	2022	10.72	57.23	34.27	6.95
Total carotenoids (µg/g fresh tissue basis) measured by spectrophotometry					
Carotenoids	1789	1.02	10.40	2.457	1.351

*Cyanogenic potential

The preliminary results obtained by Chávez et al. (2005) suggested a good potential for genetic variability of protein content in cassava roots but it was based on single measurements. Therefore, an extensive data search was conducted to identify cassava germplasm whose roots had been analyzed for protein content more than once. A total of 149 clones from the germplasm collection at CIAT, as well as a few improved clones (Table 2.3), whose roots had been analyzed more than once were found. For each clone, the analysis was conducted in different years and on different roots from plants grown in CIAT experimental station in Palmira, Colombia. Therefore, differences in the environmental conditions where the plants were grown are mainly due to year-to-year climatic variation.

The different measurements over the years were conducted for different purposes and data had not been consolidated until now. Through the evaluations conducted these years roots

from the same clone were eventually analyzed more than once. One clone has been analyzed in four occasions. A group of eight clones had been analyzed three times and the remaining 140 clones, only twice. Although the data consolidated herein is mostly derived from evaluations made for other purposes, it provides replicated measurements and allows the estimation of errors for such measurements.

For these analyses, two plants per clone were harvested and the roots from these two plants combined together. From all the roots harvested from a given clone at a given time, four to five of them were randomly selected. Selected roots were peeled and washed. From the proximal, central and distal sections of each root a slice was taken. Samples from each root were mixed together and chopped into small pieces. Resulting chips were properly mixed to obtain a uniform sample of root tissue from the four to five original roots. A 100 g sample was then taken and dried in an oven with forced ventilation at 60 °C for 24 hours. Dried samples were then grinded in a mill with a stainless steel grinding tool. All sample processing was carried out to avoid, as much as possible, contamination from soil or any other source.

All solid samples were analyzed on an oven-dried basis. In the year 2000, root samples were sent to the Analytical Laboratory of University of Adelaide where the samples were analyzed using the total combustion gas chromatograph or Dumas method (Colombo and Giazzi, 1982). A Carlo Erba Instrument (model is NA 1500 series 2 Total Combustion Gas Chromatograph) was used in the quantification on 10-15 milligrams of samples. The limit of determination for the sample is calculated as 10 X the standard deviation of the blank.

All the remaining samples were analyzed at the plant tissue analytical laboratory at CIAT. Nitrogen determination was based on a modification of the Kjeldahl method (Skalar, 1995). The root samples were digested with a mixture of sulphuric acid, selenium and salicylic acid. The salicylic acid forms a compound with the nitrates present to prevent losses of nitrate nitrogen. The digestion of the samples started with hydrogen peroxide and, with this step, the larger part of the organic matter was oxidized. After decomposition of the excess of H_2O_2, the digestion was completed by concentrated sulphuric acid at elevated temperature (330 °C) with selenium as catalyst (Walinga et al. 1989; Novozamsky et al. 1983). Nitrogen was quantified colorimetrically on a segmented flow analyzer. In the coloring process, salicylate, nitroprusside (catalyst) ion. The absorption was measured at 660 nm (Krom, 1980; Searle, 1984). Statistical analysis was made using the square root transformation because the original data ranged from 1 to 7% (Gomez and Gomez, 1984).

The analysis of variance for the variation in protein content ($\sqrt{\ }$ %) indicated highly significant ($P \leq 0.01$) differences between the 149 clones evaluated. Results from Table 2.3 provide a strong evidence to support the hypothesis of a genetic origin in the variation in protein content in the cassava roots evaluated. In other words, to a considerable extent the variation ranging from 0.95 to 6.42% crude protein has a genetic origin. The relative magnitude of the error (variation from sample to sample from roots of the same clone) was small as well as the coefficient of variation, which was only of 6.09% (Gomez and Gomez, 1985; Steel and Torrie 1960). It should be emphasized that the disagreement between the ranges of protein presented in Tables 2.2 and 2.3 is due to the different N to protein conversion factors used (the standard 6.25 in Table 2.2 and the more conservative 5.31 in Table 2.3). These figures have not been corrected because data from Table 2.2 was published in Chávez et al. 2005 and those in Table 2.3 by Ceballos et al. 2006.

Table 2.3. Origin of the different accessions evaluated for protein content in root parenchyma over the years and for different purposes.

	# clones	Mean	St.Dev	Min	Max
Africa					
Nigeria	1	4.04	0	4.04	4.04
Asia					
Fiji	2	3.14	0.26	2.95	3.32
Indonesia	1	2.77	0.00	2.77	2.77
Malaysia	2	2.90	0.09	2.83	2.96
Philippines	1	2.47	0.00	2.47	2.47
Thailand	3	2.27	1.21	0.95	5.44
Central America, Caribbean and North America					
Costa Rica	3	2.66	0.75	1.81	3.21
Cuba	9	2.34	0.80	1.52	4.20
Dominican Rep.	2	3.05	0.57	2.64	3.45
Guatemala	10	4.19	1.14	2.71	5.83
Mexico	1	4.61	0.00	4.61	4.61
U.S.A.	1	3.12	0.00	3.12	3.12
Improved clones					
CIAT	8	3.61	1.61	1.57	5.44
South America					
Argentina	4	3.46	1.12	2.30	4.97
Brazil	18	3.50	1.18	1.66	5.71
Colombia	36	3.69	1.23	1.21	6.42
Ecuador	8	3.24	1.14	2.00	4.95
Paraguay	10	2.71	0.63	1.86	4.21
Peru	17	2.98	1.26	1.18	6.17
Venezuela	12	2.28	0.50	1.48	3.14
Across study	**149**	**3.24**	**1.19**	**0.95**	**6.42**

¶Average standard deviation for the different origins.

The total variation among the 149 clones evaluated is illustrated in Figure 2.4. It is clear that there is no clear pattern distinguishing the protein content of clones from specific regions or countries. As a group, however, accessions from Guatemala tended to show an above average performance, whereas cassava from Asia tended to have lower than average protein levels.

Results from this study are indeed very promising. It should be highlighted that results from only 149 clones have been analyzed and that the world cassava germplasm collection has more than 6000 accessions. Therefore, it is valid to assume that there are excellent possibilities of finding cassava clones with crude protein content in the roots with as much as 8% (or above). The possibilities of further increasing the natural range of variation for protein content through traditional recurrent selection methods are also very encouraging (Dudley, 1974). This activity will come to support on going research to increase protein content in cassava roots through inter-specific crosses (CIAT, 2003; Ceballos et al., 2005).

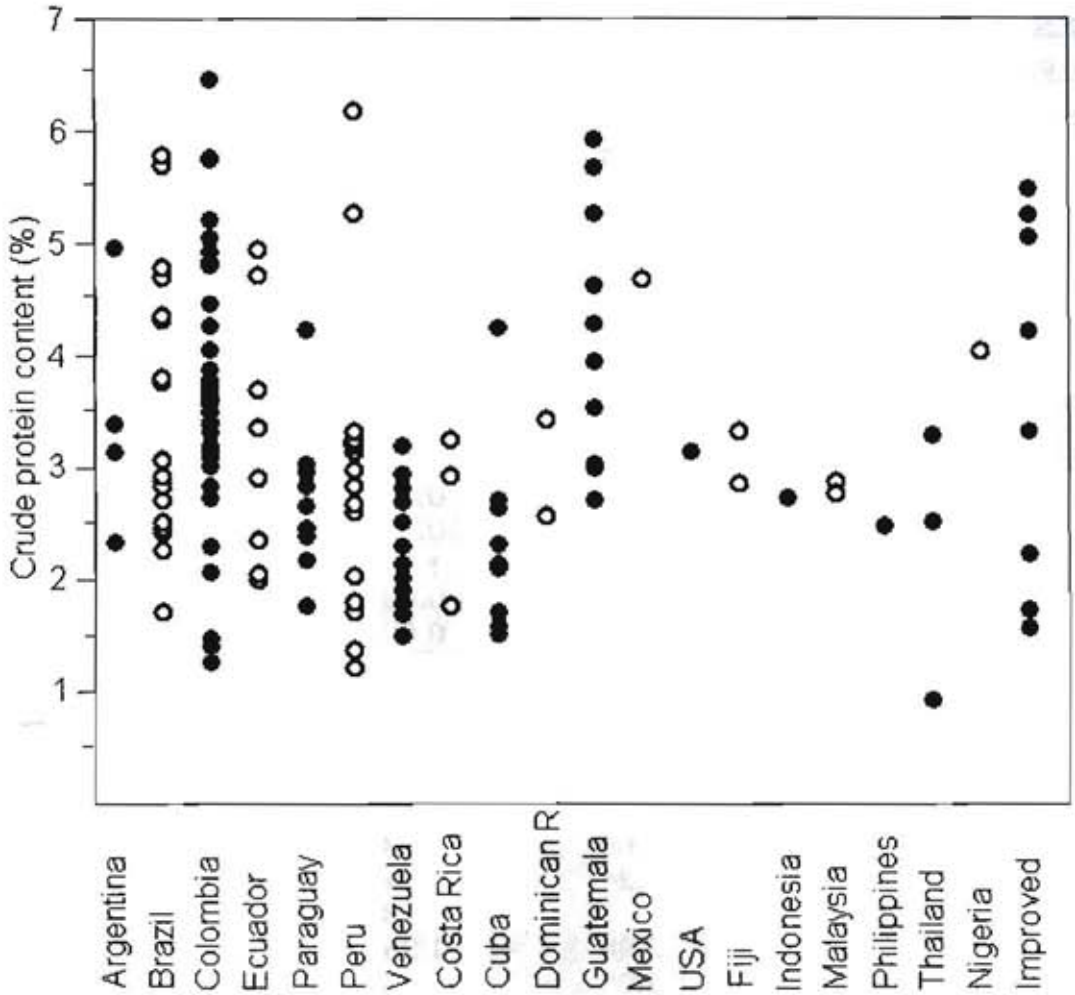

Figure 2.4. Variation in protein content in roots from 149 cassava accessions evaluated grouped by country. Each dot represents the average for a given clone.

These results and those published by Chavez et al (2005) provide valuable information justifying the need for a more aggressive screening of the genetic variability for nutritional traits in cassava and related *Manihot* species. The screening should not only focus on the roots but also consider the foliage, which has also been shown to be a valuable source of proteins, minerals and vitamins.

Based on the promising results reported in this article a group of "high-protein clones" have been planted and the protein content in the roots will be analyzed and the genotype by environment interaction assessed, to understand the relative importance of inheritance and environment in the expression of protein content in the roots. Amino acid profiling of the protein in these roots will also be conducted, but only after selecting a group of clones with contrasting levels of proteins in their roots.

It was surprising to observe the poor performance (for protein content) of some of the improved clones included in this study. Although this is not conclusive evidence, these results would suggest that improving cassava for higher productivity might result in a

gradual loss of protein content levels originally present in the landraces. Unless proper efforts are made to quantify and use protein content as criterion in the selection process continuous breeding for higher yields may unknowingly derive in a gradual reduction of protein content in the cassava roots. In fact the lowest protein content observed was in a clone from Thailand. Almost 100% of cassava grown in Thailand is improved germplasm. Probably no other country in Asia has made such an aggressive and successful campaign to release high-yielding varieties as Thailand (Kawano, 2003)

Perhaps the most important contribution of this research is to provide further evidence for the wide genetic variation in nutritional quality in staple crops such as cassava, which so far, has not been properly exploited. With the creation of the HarvestPlus Challenge Program consortium, several international agriculture research centers have made a significant and timely shift in their breeding objectives to include nutritional quality. That is a major departure from the way crop breeding had been envisioned during the last century and this article would further support the wisdom for such departure.

Inbreeding cassava to allow recessive mutations to express themselves: As mentioned above there are several reasons for introducing inbreeding in cassava genetic improvement. These reasons are further described in a different chapter of this report. One of these reasons, however, is the advantage that recessive traits can more easily express in partially or fully homozygous germplasm. In combination with the above strategy of an aggressive screening we have started to self-pollinate cassava germplasm in hopes of identifying useful mutants in cassava. Table 2.1 presented the number of self-pollinated seed produced in the last three years. There are several uses of this partially inbred lines and one of them is that of an increased possibility of expressing useful recessive traits. In March 1, 2006 a breakthrough for cassava research was made with the discovery of a mutation that drastically reduces the levels of amylose in the starch. This discovery started with a differential staining of the root after the application of an iodine solution. Figure 2.5 illustrates the results of such a technique in cassava roots and stems.

Figure 2.5. Differential staining or roots (photograph on the left) and stems (photograph on the right) with an iodine solution of a normal cassava clone (staining blue) and a waxy mutant (staining brown).

Further analysis in the quality laboratory demonstrated that the starch of the mutant discovered through the staining technique has, in fact, lower levels of amylose. The average percentage of

amylose was 2.93 (five different quantifications) rather than the usual 15-20%. Moreover, the starch from this clone provided a very distinctive amylogram when analyzed using the rapid viscoanalizer machine (RVA), as illustrated in Figure 2.6.

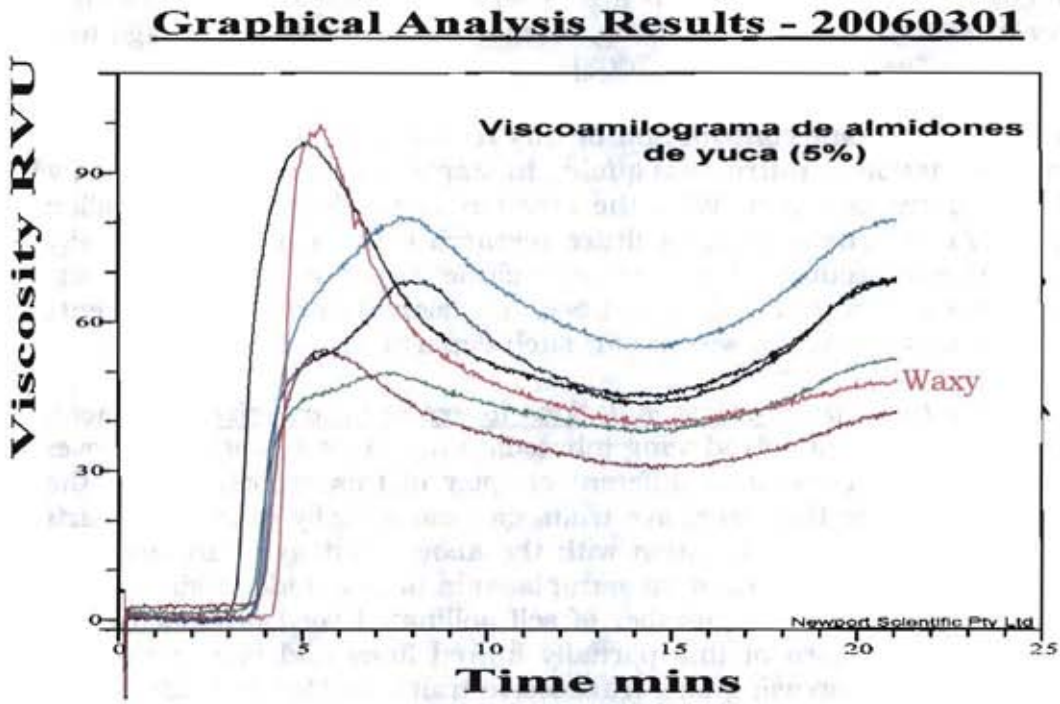

Figure 2.6. Amylograms of different cassava cultivars, including the waxy mutant ("ceroso") recently discovered. These amylograms also demonstrated the large variation in pasting properties observed in different clones.

Mutagenesis and the "TILLING" System. Few years ago a large population of botanical seed (**M_0** generation)from six different full sib families of cassava were irradiated with gamma rays or fast neutrons. The seed was germinated and the resulting **M_1** generation was grown during 2004 and self-pollinations made to produce the **M_2** generation that has overcome the problem of chimeras on one hand, and increases probabilities for recessive mutations to express phenotypically, on the other. Figure 2.7 present some of the abnormalities observed in these mutagenized plants that will be harvested during the last week of March, 2006.

As soon as M_1 plants started to produce viable flowers they were self-pollinated. The granule bound starch synthase GBSS I (Curtis Hannah, 2000) gene will be analyzed and key sequence(s) PCR-amplified for their use in the TILLING system. If any of the plants has 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. Unfortunately because of an administrative mistake at the Experimental Station where the M_0 plants were grown they were harvested before the process of self-pollination to obtain M_2 seed could be completed. Personnel quickly visited the field and recovered vegetative planting material from several hundreds of M_1 plants, which were then planted at CIAT Experimental Station in May 2005. Table 2.4 provides information of the number of M_1 plants that could be recovered and replanted. A total of 443 and 151 M_1 plants from seeds that had been treated with gamma

rays and fast neutrons could be recovered. As soon as the irradiated seed was germinated early in 2004 it was clear that seedlings and plants from seed treated with fast neutrons were considerably more affected with marked reduction in vigor. This effect has been carried out and is reflected by the lower number of M_1 plants (about 30%) that could be recovered compared with gamma rays.

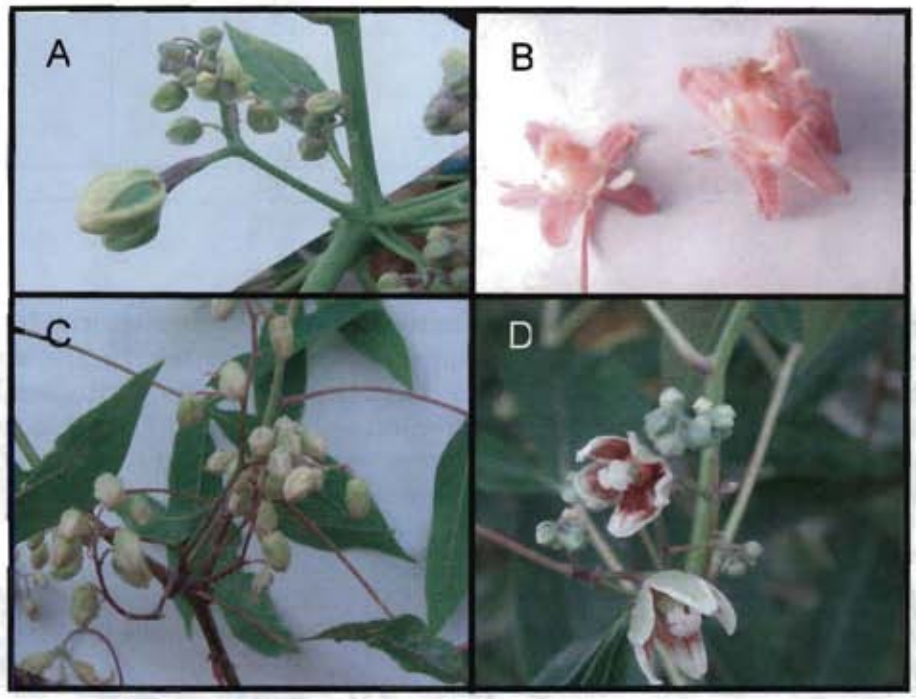

Figure 2.7. Anomalous flowers observed in $\mathbf{M_1}$ plants irradiated to induce mutations. **A**: A flower that does not open, yet produced fruits. **B**: Hermaphrodite flowers. **C**: Inflorescence that only contained male flowers. **D**: Inflorescence that only contained female flowers

In spite of the problems of an early harvest of the M_1 plants a considerable number of self-pollinations to produce M_2 seed could be made. The seed harvested earlier could be germinated and planted in May 2005 (Table 2.5). A second batch of seed that matured late was planted later in the season and will be soon transplanted to the field (Table 2.6). About 1500 M_2 plants are currently in the field, and another 500 are in the screenhouse.

Table 2.5 provides information regarding the number of M_1 plants from which the M_2 seed were harvested, separated by treatment. About 100 M_1 plants are currently represented in the M_2 generation (Table 2.5). The reduction in vigor in plants treated with fast neutrons reflects in the reduced number of M_2 seeds obtained from this treatment. However, it is clear that this effect was not transferred to the M_2 generation, at least based on the relative vigor of the plants in the field. It is important that the number of M_1 plants self-pollinated is maximized because each plant represent a unique mutation event (in fact several different events resulting in the chimeras mentioned above). It is for this reason that special efforts were made to recover as many M_1 plants as possible to continue the process of self-pollination (Table 2.6).

Table 2.4. Number of M_1 plants that could be recovered from the field that was harvested by mistake before all the plants could be self-pollinated. Planting material from these recovered plants was replanted in May 2005 to continue with the self-pollinations to produce M_2 seed.

Family	Number of M_0 seed originally treated with gamma rays or fast neutrons	M_1 plants recovered and replanted in May 2005	
		Gamma Rays	Fast Neutrons
CM 9331	300	51	20
SM 3015	300	35	19
SM 3045	300	69	6
GM 155	306	148	13
C-4	1574	122	90
C-127	25	18	3
Total	**2805**	**443**	**151**

DNA is currently being extracted from the M_2 plants for genetic characterization. In addition to thee TILLING system described above other approaches will be taken. The sequence of the GBSS-I gene was analyzed with the software CODDLE (Codons Optimized to Discover Deleterious Lesions) to define the most relevant regions of the gene. The **G**lycosil **T**ransferase Domain **(GT)** was found to be very sensitive. Primers were developed from the GT region and SSCP analysis (Single Strand Conformational Polymorphism) was conducted in a preliminary run.

Table 2.5. Number of M_2 plants transplanted to CIAT Experimental Station in May 2005. Information on the number of M_1 plants from which the M_2 seed was obtained and the vigor of the resulting M_2 plants is provided.

Family	M_1 plants	Vigor of M_2 plants			Total
		Poor	Intermediate	High	
	Plants derived from seed irradiated with gamma rays				
CM 9331	5	10	51	7	**68**
SM 3015	8	1	58	28	**87**
SM 3045	10	2	31	9	**42**
GM 155	13	18	111	19	**148**
C-4	37	135	612	30	**777**
C-127	2	0	13	0	**13**
Total	**75**	**166**	**876**	**93**	**1135**
(%)		14.6	77.2	8.2	
	Plants derived from seed irradiated with fasts neutrons				
CM 9331	1	0	4	1	**5**
SM 3015	4	0	14	0	**14**
SM 3045	1	0	1	1	**2**
GM 155	0	0	0	0	**0**
C-4	21	40	249	23	**312**
C-127	0	0	0	0	**0**
Total	**27**	**40**	**268**	**25**	**333**
(%)		12.0	80.5	7.5	

M_2 plants in the field have shown a large number of anomalies, which at this point in time can only refer to morphological traits such as different types of chlorosis, changes in the shape of the lobules of the leaves and/or the color of the leaves, and an interesting possibility of apomixis in female flowers that do not open but quickly produce fruits. It remains to be determined if these fruits contain seeds and if they are indeed viable seeds. The first batch of M_2 plants will be harvested during the last week of March, 2006 and carefully analyzed in search of useful variants.

Table 2.6. Seedlings from M_2 seed germinated and grown in the screenhouse and ready to be transplanted to the field. This batch of germplasm could not be transplanted earlier because they are the result of pollinations made late in the season and were not ready for planting in May 2005.

Family	Gamma rays		Fast neutrons	
	M_1 plants	M_2 seedlings	M_1 plants	M_2 seedlings
CM 9331	7	53	0	0
SM 3015	4	16	2	3
SM 3045	9	51	1	1
GM 155	11	53	2	10
C-4	16	247	19	124
C-127	1	6	3	8
Total	**48**	**426**	**27**	**146**

Recurrent Selection to Modify Amylose Proportion in the Root Starch of Cassava:. The extensive screening of cassava germplasm described above has led to the selection of two groups of cultivars with contrasting proportions of amylose in the starch of their roots (Table 2.7). 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, have important effects on dry matter content in the roots (Van Oirschot et al., 2000).

CIAT has initiated a divergent recurrent selection scheme for modifying starch quality traits through conventional breeding. Clones with low-amylose starch were crossed among themselves. The resulting progeny (single plants) will be grown and 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 the plants resulting from these crosses. The plants with lower proportion of amylose in their starch will be selected, replanted in a crossing nursery and crossed among themselves to start a second cycle of selection. A similar scheme will be used for high-amylose clones.

When there is limited genetic variability for a given trait of economic importance, breeders have looked to wild relatives of the crop, or else, have tried to generate it through mutation breeding. Recently the approach of genetic transformation has made huge progress for different traits in different crops. These three approaches are currently being implemented in cassava at CIAT. Below a brief description for each approach is provided.

It is acknowledged that the interest of this approach is now somewhat reduced after the discovery of the waxy cassava mutant described above. That mutation (likely to be a single

gene) is more valuable, from the breeding point of view, than the possibility of reducing amylose content based on the gradual modification of allelic frequencies of several genes. Nonetheless the recurrent selection approach leaves possibility of increasing the proportion of amylose in the starch (or, what is the same, a reduction of the other molecule in starch: the amylopectin). This is not a common mutation because the biosynthesis of amylopectin includes several genes and alternative pathways and, therefore, the possibilities of a single mutation are reduced.

Table 2.7. Description of cassava clones planted to make crosses aimed 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
Std. deviation	0.58	2.07
Minimum	9.66	21.82
Maximum	12.10	26.39
Number of clones	29	35

Development of a high-capacity root quality laboratory: CIAT has developed jointly with Universidad Nacional de Colombia a laboratory that will be able to generate thousands of amylograms per year using a battery of rapid viscoanalyzer (RVA), Brabender, differential scanning calorimeters (DSC), and other standard equipment and protocols. For crude protein content, the standardization of curves for the use of Near Infrared Analyzers will be pursued in 2005-2006.

Exploring the wild Manihot gene pool in search of novel traits: Results of these activities are described in more detail in a different section of this report. This strategy was nevertheless mentioned here to emphasize the integral approach taken with the implementation of several simultaneous approaches to maximize the chances of success of developing the different types of high-value cassava clones needed by different industries.

Genetic Transformation: Also results from this activity are mentioned elsewhere. A single transgenic event and five events for genotypes 60444 and MCol22, respectively, could be identified. After the six independent events obtained for the anti-sense constructs were identified, about 5 plants per event could be transferred to the screen house. The molecular characterization via PCR and Southern hybridization has been conducted and publication of results is underway. Southern analysis was performed using ten micrograms of total genomic DNA from the genetically modified plants and two non-transgenic cassava genotypes using the following restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *HaeIII*, and *DraI* . The RFLP probe was a full length GBSSI cDNA clone (clone-3) from a root cDNA library from TMS30572. Preparation and Southern hybridization of the filters were as described by Fregene et al. (1997).

Based on the positive results thus obtained, permission to transfer these plants to the field, following strict biosafety measures has also been requested. As soon as the plants grown in the field can be harvested the biochemical characterization of the starch from their roots will be conducted to confirm the waxy condition.

2.4 Conclusions

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 and the use of dried chips for animal feeding are clearly the most important examples of industrial uses in cassava, particularly in Asia (Chutharatkul, 2005). 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 products and increased emphasis on the search for value added traits/products.

Increased productivity has been achieved successfully through the development of high-yielding clones, improved fertilization and soil conservation approaches and/or better management of the planting materials (Chutharatkul, 2002; 2005; Hoang et al., 2005; Howeler, 2005; Howeler et al., 2005; Kawano, 2003). Mechanization is gradually being incorporated with the development of machinery specifically designed for cassava with reductions in production costs (Ospina, 2005). The exploitation of foliage for animal feeding opens a new avenue of uses of cassava. All these approaches have had a remarkable impact in the livelihood of millions of cassava farmers in different tropical and subtropical regions of the world.

The activities described in this Output illustrate a drastic change in the goals of the cassava-breeding project at CIAT toward the production and identification of high-value clones. Success in this regard will further contribute to poverty alleviation (a chronic and typical problem of many of the marginal agriculture land where cassava is one of the few crops that can be grown) and rural development (cassava roots need to be processed near the fields where it is harvested). Several approaches have been simultaneously implemented to produce and identify high-value clones: inter-specific crosses; systematic evaluation of the germplasm collection; creation of a high-capacity root quality laboratory; introduction of inbreeding in cassava genetic improvement; a divergent selection for high- or low-amylose starch; genetic transformation; and induction of mutations followed by the TILLING system and other molecular approaches are ongoing projects.

Two significant breakthroughs have been given: the discovery of cassava clones with crude protein levels 2-3 times higher than normal cassava and the identification of a cassava clone with waxy starch. However the most important step may be the fact that quality traits are now among the most important breeding objectives of our project. Implicit in this new strategy for cassava genetic improvement is the acknowledgement that these high-value cassava clones will target specific needs from the industry and, therefore, that a good-for-all variety will no longer be feasible. For instance, high-carotene, high-protein cassava clones are ideal for the feed industry (as well as for human consumption) but they will offer additional problems to the starch industry.

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3. Recent modifications in the breeding system of cassava

As stated in the introductory chapter of this report, cassava genetic improvement starts with the assembly and evaluation of a broad germplasm base, followed by production of new recombinant genotypes derived from selected elite clones. There are important limitations in cassava genetic improvement. The vegetative multiplication rate of cassava is low. From one plant, 5-10 cuttings typically can be obtained. This situation implies a lengthy process to arrive at the point where replicated evaluations across several locations can be conducted. It takes about 5-6 years from the time the botanical seed is germinated until the evaluation/selection cycle reaches the regional trial stage when several locations can be included. One further complication in a cassava program is the number of factors that can affect quality of planting material. For example, the original positioning of the vegetative cutting along the stem affects considerably the performance of the plant it originates. Cuttings from the mid-section of the stems usually produce better performing plants than those at the top or the bottom. This variation in the performance of the plant depending on the physiological status of the vegetative cutting, results in larger experimental errors and undesirable variation in the evaluation process.

The breeding scheme used before the turn of the century had been efficient to improve the traits that needed to be improved at that time. It can be said that between 1950 and 2000 the process of domestication of the crop was completed. Major achievements were made, regarding plant architecture, harvest index, resistance to pests and diseases and cooking quality. However, with the opportunities for industrial uses of cassava and the need to compete with "advanced" crops such as maize, changes in the breeding scheme were gradually introduced after the year 2000 and were already consolidated after the year 2003. The constraints in the breeding scheme used before 2000 and the approaches taken to overcome them will be summarized in this chapter.

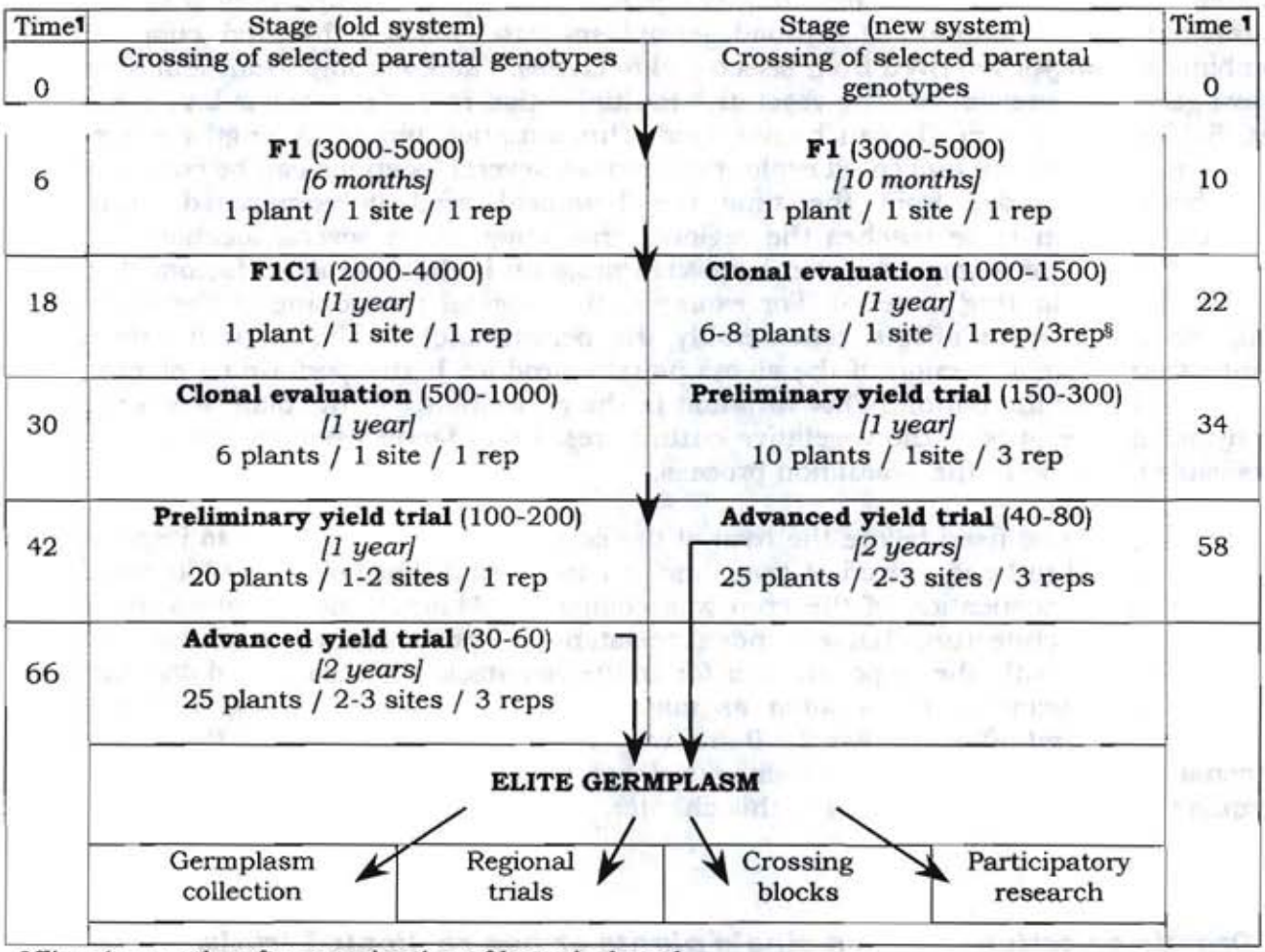
3.1 Drastic selection based in single plants or non-replicated trials.

Rationale

As illustrated in Table 1.1 of the introductory chapter of this report a typical recurrent selection cycle for cassava implies about six years. The first selection used to be based on single plant evaluations (F1 or F1C1 phase). The second year selected genotypes were planted in clonal evaluation trials (CETs), which were non-replicated evaluations of single-row plots with 6 plants. The third stage of selection was the preliminary yield trial (PYT), which also was non-replicated and based on 20-plant plots. Only at the advanced yield trials (AYT) more than one replication was introduced and at the regional trials (RT) several locations could finally be incorporated. It should be clear the implications of doing selection in non-replicated trials in the first three of the five stages of the recurrent selection cycle described in Table 1.1. Moreover, the genotype-by-environment interaction effect was only addressed in the last stage of selection. That scheme was probably very efficient for the traits improved in these early years of the scientific breeding of cassava, but is certainly not adequate for the needs of cassava genetic improvement of the third millennium.

Materials and methods

Few drastic changes were introduced after the year 2000 aiming at overcoming the problem mentioned above. Figure 3.1 illustrates the main differences between the breeding schemes used by the cassava-breeding project at CIAT before and after the year 2000.



¶ Time in months after germination of botanical seed.

§ One replication for clones within each "block" but three replications for families.

Figure 3.1. Basic cassava breeding schemes applied for each of the priority ecosystems. On the right is the new scheme currently under implementation have reached the Advanced Yield Trial phase in 2004.

The changes presented in Figure 3.1 can be summarized as follows. The F1 (or F1C1) selection stage was eliminated. The first evaluation in the target environment is now the CET, which was modified to include eight, rather than six plants/plot. The way the CETs were planted was modified as explained below in Section 3.2. The PYT now are based on three replications and ten-plant plots. AYT also have three replications and are conducted in two locations and for two years.

Results

The consequences of the changes in the breeding scheme are still to be measured since the first generation of materials developed entirely with the new system has not yet reached the regional trial phase. However, some conclusions can be readily made. It is obvious that eliminating the F1 phase implies a shortening of the time required to complete a selection cycle. On the negative side this means that there is no "filter" in the selection of the clones that reach the CET phase. Our experience, however, suggested that the F1C1 stage", being a single-plant evaluation was not an adequate "filter" anymore. Perhaps during the 1970s and 1980s the selection of plant traits such as plant architecture of harvest index could efficiently be made based on this single-plant evaluation. After so many years these traits are more or less fixed in our breeding populations and the traits that need to be improved (i.e. root productivity) cannot be selected properly through the old scheme.

It is obvious that increasing the size of the plots at the CET and the introduction of replicated PYTs should be beneficial and make the over all selection more precise. It is important to point out that the total number of plants used at the PYT is not much different. In the previous scheme we had only 20 plants in one replication, now we have 30 plants split in three replications. We have modified the planting system (as explained in the introductory chapter) to increase the within-plot competition and reduce the inter-plot competition.

To facilitate the process of selection we introduced the use of a selection index integrating the most relevant variables. To avoid the problems related to the magnitudes used to measure different variables, the index is constructed using standardized deviation units (Steel and Torrie, 1960). For example, a typical selection index for the Acid Soil Savannas environment of Colombia is:

$$SI = (FRY * 10) + (DMC*8) + (HI*5) - (PT\ 3) - (SED3)$$

where SI is the selection index; FRY = fresh root yield; DMC = dry matter content; HI = harvest index; PT = rating for plant type or architecture; and SED = rating for super elongation disease. Depending on the year we may choose to select for bacterial blight (CBB) or both diseases. The relative importance of each trait is weighted, as shown in the formula above, by a subjective assessment by the breeder. Negative signs are used for those variables where lower values represent most desirable phenotypes. Harvest index has been consistently favored as one relevant variable to be included in early stages of selection such as CET trials (Kawano et al., 1998). Plant architecture also plays an important role in early stages of selection (Hahn et al, 1979). Since the SI is estimated using the standardized values, a positive SI means a performance better than the average, while a negative one means a poor performance.

3.2 Large environmental effects in the Clonal Evaluation Trials.

Rationale.

A major problem with the CET was its large size (easily 2 ha in size) and the unavoidable environmental effect in the selection. This problem is particularly relevant in the case of cassava, because the relevant target environments for cassava are typically in "marginal" agriculture conditions and prone to large variation. Since CETs are the first stage of evaluation, only a few stakes (typically less than 10) are available for trials. So the introduction of replications that could help to overcome this problem is not practical.

Materials and methods

We have introduced the same simple principles suggested by Gardner in 1961. We first divide the plot were the CET is going to be planted in three "blocks" of about equal size. In the process we make the division to maximize differences among blocks and minimize variation within each block.

The replication of each clone is difficult to implement because of the lack of enough planting material available for CETs. On the other hand, clones are grouped in either full- or half-sib families. Since many clones are generally available from each family they are randomly allocated in one of these three blocks. In other words instead of planting all the clones from a given family together one after the other, they are split in three groups, which are planted in the three blocks the entire evaluation has been divided into (Figure 3.2). This approach allows for two interesting advantages:

- There is a replication effect for the families because all the clones from a given family are scattered in three "repetitions" in the field. The averages from all these clones are less affected by the environmental variation in such a large experiment.

- Selection is made within each block. This is similar to the stratified mass selection suggested by Gardner. This approach effectively overcomes the environmental variation that can be measured by comparing the means of each block.

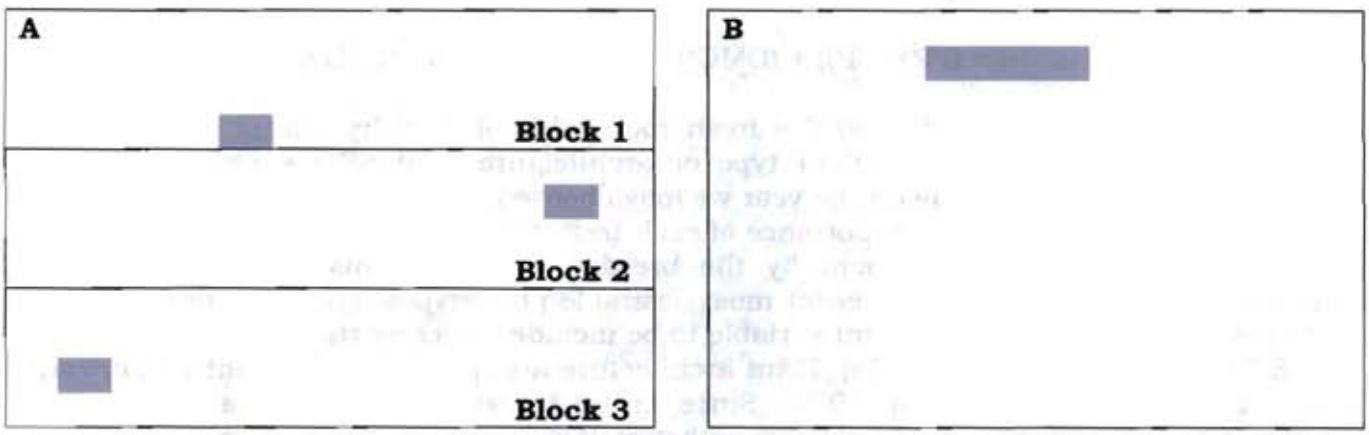


Figure 3.2. Advantage of splitting each family of clones in three groups that were randomly assigned to each of three blocks in the *CET*. (A= current procedure; B= previous situation).

Because all the clones from the *CET* were divided, the average performance of each family were more precisely estimated, since each family was scattered in three different parts of the field, whereas before it was concentrated in just one sector (Figure 3.2). As a consequence, the estimates of GCA (described in detail below in Section 3.3) for each family is much more precise.

A summary of the results from the *CET* harvested in 2003 for the three main target environments (Sub-Humid, Acid Soils and Mid Altitude Valleys) is presented in Table 3.1. The benefit of the introduction of stratified selection is directly proportional to the differences between the mean performances in each of the strata. In general variations in the order of 10-20% has been observed among average performances of the three blocks. This is, in other words, the gains in the precision attained by introducing the stratification of the CETs. Currently we are considering the possibility of increasing the number of blocks to four or five.

Table 3.1. Results of the *Clonal Evaluation Trials* for the three main target environments harvested in May 2003. Data present the variation between the three blocks in which each CET was divided.

Block	Yield (t/ha)		Harvest Index (0 to 1) ¶	Plant type (1 to 5) §	Dry matter content (%)	Selection Index
	Fresh roots	Dry matter				
Averages of the 412, 412 and 411 clones in Blocks 1, 2 and 3, respectively from the CET targeting the acid-soil savannas.						
Block 1	20.88	6.66	0.50	3.33	31.59	0.00†
Block 2	21.73	6.88	0.49	3.35	31.24	0.00†
Block 3	22.30	7.28	0.50	3.48	32.44	0.00†
Averages of the 749, 746 and 705 clones in Blocks 1, 2 and 3, respectively from the CET targeting the sub-humid conditions.						
Block 1	14.19	3.70	0.50	2.87	26.09	0.00†
Block 2	14.37	3.91	0.46	2.88	27.21	0.00†
Block 3	12.89	3.38	0.44	2.87	26.26	0.00†
Averages of the 605, 588 and 568 clones in Blocks 1, 2 and 3, respectively from the CET targeting the mid-altitude valleys.						
Block 1	24.05	8.86	0.63	2.68	36.61	0.00†
Block 2	28.08	10.21	0.57	2.63	36.02	0.00†
Block 3	27.51	9.76	0.54	2.97	35.09	0.00†

¶ The harvest index is obtained by dividing the production of commercial roots by total biomass (roots + aerial parts). Preferred harvest indexes are > 0.5.

§ Plant type integrates under one value, plant architecture, leaves health, and capacity to produce stakes on a scale where 1 = excellent and 5 = very poor is used.

† Average election index within blocks must be zero, because it is based on a combination of standardized variables.

3.3 Limited information from selections in the first two years of evaluation.

Rationale

One of the major decisions taken by any breeder is the selection of parents used to produce a new generation of segregating progenies. In cassava, this decision has been mainly based on the *per se* performance of each clone. Nonetheless, some empirical knowledge on the quality of progenies produced by different parents could be produced. This lack of organized information on the breeding values of parental lines used in the breeding projects was partially due to the fact that no data was taken and recorded during the first stages of selection (**CET** and **PYT** in Table 1.1) or else, it were incomplete. Moreover, before 2000 the

first stage of selection was based on single plants evaluations in the target environments. In that selection the only record kept was which plants were selected and which had been discarded. Little or no information for the basis of such decision was kept. Because large proportion of the selection was made at these early stages of selection (first 2-3 years of the 5-6 years required by each recurrent selection cycle) it was not possible to generate a balance set of data that would allow the breeder to have an idea of the relative performance of the progeny of each elite parental line. In other words we did not have a formal process to assess the breeding values the progenitors used in the breeding project.

Materials and methods

After the introduction of a new system to conduct the CETs it was decided not to conduct a visual selection but to spend the additional effort to quantify differences and record them. This implied spending five days rather than only two, harvesting these large trials. This additional effort was considered advantageous because it included measuring DMC which is a key trait that can be efficiently selected at this stage but was not; because quantifications are always less subjective that visual assessments, and because it allowed estimating general combining ability effects of the parental lines.

The current system therefore implies two or three additional days harvesting a CET. Data is recorded and selection indexes calculated. Selection is made within each stratum as explained in the previous section. Data from each family is then pooled across the three blocks in which it was planted. The stratification means that, in a way, there is a replication effect at the family level. Since a give progenitor may be used more than once, data from all the families in which a give progenitor participated are pooled together to obtain an idea of the general performance of all the progenies from a give parental clone.

Results

Results from the CET harvested in 2003 for the sub-humid environment have been chosen as an example of the kind of information that the current breeding system allows. These results are summarized in Table 3.2. A total of 39 parents participated in generating all the progenies evaluated in that CET. Some parents are used considerably more than others, to a large extent because of their flowering habit in Palmira where the crosses are made. MNGA 19 and SM 1433-4 were used as parent in 215 and 213 clones, respectively. On the other hand, SM 1657-14 and SM 1754-21 were the parents of only 21 and 28 clones respectively.

The interesting information from Table 3.2 comes from the proportion of clones selected from each half-sib family. For instance the four best parents, regarding the proportion of their progenies being selected, were Rayong 90, KU50 (Kasetsart University 50) , Rayong 60 and Rayong 5. All of these clones were developed in Thailand and show excellent adaptation to the sub-humid environment of Colombia. More than 40% of the progenies from each of these parents were selected (Table 3.2). On the other hand, none of the progenies from CM 6438-14, CM 7514-7 and SM 1431-2 were selected although they were not particularly small families (53, 56, and 33 clones respectively).

This system allows not only knowing the proportion of clones derived from a given parent that has been selected. Since there is recorded phenotypic data for each genotype in the CET, an average across all the progeny from a given progenitor for all the variables is available. It is possible, therefore, find out which progeny tends to have above average fresh root yield. It is

also possible, now, to conclude that the progeny from CM 6438-14 had an unacceptably low DMC (21.9%). This information is very valuable for defining which parents should stay in the crossing block, which should be removed and also to suggest crosses that may result in better progenies because the progenitors complement better their own advantages and defects.

Table 3.2. Number of progenies evaluated and selected from each progenitor. Data from the *Clonal Evaluation Trial* for the sub-humid environment (Santo Tomás, Atlántico Department, Colombia) harvested in 2003 .

	Progenitor	Family size	Progenies Selected (number)	Progenies Selected (%)		Progenitor	Family size	Progenies Selected (number)	Progenies Selected (%)
1	R 90	73	45	61.6	21	CM 4365-3	41	4	9.8
2	KU 50	64	30	46.9	22	SM 1657-14	21	2	9.5
3	MTAI 8 (R 60)	73	34	46.2	23	SM 1210-10	83	7	8.4
4	R 5	32	13	40.6	24	SM 1201-5	37	3	8.1
5	SM 1068-10	68	20	29.4	25	SM 1422-4	51	4	7.8
6	SM 2192-6	50	12	24.0	26	CM 7389-9	103	8	7.8
7	SM 1411-5	97	23	23.7	27	SM 1521-10	42	3	7.1
8	CM 7514-8	118	24	20.3	28	SM 1754-21	28	2	7.1
9	SM 1657-12	52	10	19.2	29	SM 1210-10	101	7	6.9
10	SM 643-17	32	6	18.8	30	SM 1619-3	29	2	6.9
11	MVEN 25	53	9	17.0	31	CM 8027-3	46	3	6.5
12	SM 1665-2	57	9	15.8	32	MNGA 19	215	12	5.6
13	CG 1141-1	33	5	15.2	33	CM 2772-3	28	1	3.6
14	SM 1511-6	87	13	14.9	34	SM 1600-4	61	2	3.3
15	SM 890-9	69	10	14.5	35	CM 7395-5	42	1	2.4
16	SM 1433-4	213	26	12.2	36	SM 805-15	73	1	1.4
17	SM 1565-17	108	13	12.0	37	CM 6438-14	53	0	0.0
18	CM 3372-4	52	6	11.5	38	CM 7514-7	56	0	0.0
19	CM 6754-8	49	5	10.2	39	SM 1431-2	33	0	0.0
20	SM 1438-2	109	11	10.1	**Average**				**13.6**

3.5 Absence of inbreeding.

Rationale

There are many advantages related to the introduction of inbreeding in cassava. As described in the chapter related to the production of high-value cassava, inbreeding would allow for the identification and exploitation of useful recessive traits. For the same reason it would also allow for the identification and elimination of undesirable alleles. Because no inbreeding is carried out, a sizeable genetic load (undesirable or deleterious genes) may prevent large and sustained genetic gains. The introgression of traits is cumbersome because the lack of inbred parents prevents the implementation of the back-cross scheme. For example, in the previous chapter we are reporting the discovery of a waxy starch mutant in cassava. The next step would be to introduce this trait in the highly successful clone KU50 grown in more that one million ha in SE Asia (Chutharatkul, 2005; Hoang et al., 2005; Howeler, 2005; Howeler et al., 2005; Kawano, 2003). It is also an outstanding

progenitor as suggested by data in Table 3.2. However since KU50 is heterozygous it is virtually impossible to recover it once it is crossed with the source of the waxy trait. Additional problems related to the lack of inbreeding in cassava relate to molecular markers, which are considerably more difficult when parental lines are not genetically fixed.

Because the crop is highly heterozygous, dominance and epistatic effects are likely to play a very important role in the performance of materials being selected (as suggested by data from the diallel studies described in this report). The current scheme can exploit non-additive effects because, once an elite clone is identified, it can be propagated vegetatively (therefore carrying along the gene combinations that define the dominance and epistatic effects). However, if the same elite clones are frequently selected as progenitors for the production of new segregating material, the current procedure has a bias because the breeding value of these clones are unlikely to be well correlated with their performance *per se*, precisely because of the distorting effects of dominance and epistasis. In other words, non-additive effects are exploited by the farmers when they vegetatively multiply an outstanding clone, but cannot be properly exploited by the breeder because the current scheme does not allow for a systematic, gradual and directed modification of allelic frequencies.

Materials and methods

In the year 2004 we initiated a project financed by the Rockefeller Foundation. The main objective of this project is to introduce inbreeding in the genetic improvement of cassava. However, because inbreeding would require about 9-10 years to attain acceptable levels of homozygosity, an efficient protocol for production of doubled-haploids (DH) through anther or microspore culture is critical. Inbreeding in cassava is desirable because:

- Reducing the genetic load (undesirable genes), which is expected to be large in cassava.
- Selection among DH would not be affected by dominance effects, which could be exploited through reciprocal recurrent selection.
- Additive effects among DH are twice as large as in the current array of evaluated genotypes.
- Homozygous lines are genetically fixed, and therefore, their genetic superiority (as progenitors) can be better exploited, than genetically unstable heterozygous parents.
- Germplasm exchange based on botanical seed is much easier than that of vegetative cuttings (Iglesias et al., 1994).
- Cleaning planting stocks from viral or other pathogens could be achieved without the need of meristem culture.
- Mutation breeding would be more easily implemented.
- The identification of useful recessive mutants would be greatly facilitated.
- The production of genetic stocks for basic and applied research would be feasible.

- For those projects exploiting polyploidy in cassava breeding (Sreekumari et al. 2000), the availability of haploids and DH is also desirable.
- The backcross breeding scheme could be implemented for the transfer of useful genes from one cassava inbred to another, or from an heterozygous clone to an inbred. In summary, DH would allow designing better performing hybrids compared to less efficient systems based on heterozygous parents.

Therefore, as explained above, the introduction of inbreeding in cassava offers several advantages. However, inbreeding is likely to induce a drastic reduction of vigor in the first few cycles of selection. This phenomenon is known as inbreeding depression. Maize was severely affected when it was first subjected to inbreeding. However, inbred maize lines yielding as much as 4 t/ha have now been developed (Duvick, 1999). Tolerance to inbreeding depression can be built in crops and fifth-generation inbreds have been developed at IITA (DeVries and Toenniessen, 2001). However, it is accepted that tolerance to inbreeding in cassava needs to be improved before full homozygosity can be attained through the production of DH lines. Developing families with tolerance to inbreeding is currently also an ongoing activity at CIAT, through recurrent selection schemes that involve inbred families (S_1 or S_2). Therefore, two main simultaneous activities have been planed and are currently underway:

- Development of a protocol for the production of doubled-haploid lines, tentatively from anther or microspore culture.
- Inbreeding elite germplasm to produce inbreeding-tolerant derivatives that, by definition, should be better progenitors because of their reduced genetic load.

To emphasize the integrality of our strategy it should be pointed out that the partially inbred lines developed from elite germplasm are also screened in search of new useful traits. It is in this process that we discovered the waxy mutant reported in the previous chapter.

A major concern about the implementation of this project was that inbreeding depression in cassava would be too high. In other words, that partially inbred plants would be too weak to survive. Since no study had been conducted to measure the magnitude of inbreeding depression in cassava the germplasm developed by this project has been used to answer this question. During the year 2004 a preliminary study was conducted and based on the experience gained from it a larger experiment was planted in May 2005. The first experiment consisted in three elite clones from which 25 S_1 plants had been obtained. These plants were cloned and planted in a randomized experiment with three replications (only one plant per replication). The experiment planted in 2005 is much larger. At least 100 S_1 plants from nine different elite lines were grown during 2004 and no less than 12 cuttings were obtained from each plant. The evaluation consists of three replications (three plants per replication) and the remaining cuttings will be used for multiplication purposes. The nine elite clones from which these S_1 clones were derived are: SM 1219-9, SM 1460-1, SM 1565-1, MTAI 8, SM 1511-6, SM 1665-2, SM 1669-5, SM 1669-7 and SM 1741-1. The trial will be harvested in February or March 2006.

It is expected that the first few cycles of self-pollinations will result in marked reduction of vigor (inbreeding depression associated with the genetic load of the parental lines). Therefore, selection for tolerance to inbreeding depression must be exerted. Basically at each successive generation of self-pollinated material the most vigorous plants are selected and self-pollinated again, or else, they are crossed among them to return to the "full vigor" status. Improving cassava (or any other crop for this matter) for tolerance to inbreeding has a common problem: selection is based on the vigor of the plant which may be because the plant is indeed tolerant to inbreeding and/or because the plant has lower levels of inbreeding than the average expected for that particular generation of inbreeding. In other words, this selection is biased by the differences in homozygosity levels of segregating partially inbred genotypes. This highlights the need for a method to measure the heterozygosity level in these partially inbred individuals to be used in a co-variance correction in the selection of phenotypically vigorous genotypes. Eventually molecular markers can also be used for determining regions in the genome that are particularly related to the expression of heterosis and for measuring genetic distances among inbred lines to direct crosses with higher probabilities of high heterosis.

Codominant simple sequence repeat (SSR) markers on a genome-wide basis are suitable for this purpose. The effect of self-pollination on vigor and heterozygosity will be analyzed in these 9 S_1 families. Hetererozygosity will be estimated in the S_1 families by 100 mapped SSR markers that cover over 80% of the cassava genome and plant vigor was estimated by dry root yield and plant biomass. Results, if they are considered useful, will assist in selecting the best performing and least heterozygous plants during inbreeding to identify superior partially inbred parental lines.

Results

We cannot fully report on the results of this activity. We have already mentioned the discovery of a useful mutant. However the introduction of inbreeding in cassava goes well beyond the possibilities of discovering useful recessive traits. There is a profound change in the way cassava genetic improvement proceeds. The main change is that rather than producing large number of segregating progenies in search of that one that will have an outstanding performance we can now "direct" genetic improvement in a more predictable way. Inbreeding (in parental clones) would facilitate the gradual and more consistent assembly of favorable gene combinations, which in the current system, occur just by chance.

As demonstrated elsewhere in this report epistatic effects have been show to be large for many important traits in cassava. To be able to efficiently exploit epistasis some kind of reciprocal recurrent selection would be convenient. But given the complexities of such systems it would be very difficult to implement if it was based on heterozygous segregating materials.

The main consequence of the introduction of inbreeding in cassava therefore, is that rather than just producing the segregating progenies that are screened in hopes of identifying a better commercial variety, we now concentrate in improving parents that will produce proportionally better progenies. Our strategy is to develop a stock of elite parental clones, which are partially of fully homozygous. The availability of this stock of elite parents offers many different advantages.

The back-cross breeding method can be implemented. A clone that has resistance to white flies can now be back-crossed to introgress into it resistance to ACMV (African Cassava Mosaic Disease). In few years it would be possible to have an improved version of this line combining the resistance to both biotic stresses but maintaining all the other characteristics unchanged.

The identification of heterotic patterns is now feasible. Then crosses to exploit non-additive effects (heterosis) can be directed, so the number of progenies evaluated is drastically reduced. Moreover, it is possible that once a good hybrid is found (as was the case of the now famous Mo17 x B59 cross in maize) we can improve each progenitor by making crosses of each parental clone with related materials (to introduce only limited amount of genetic variability). This would allow improving the way they complement each other. Since the two parental clones are fully or partially inbred it is feasible to make them to complement each other better in a gradual, consistent and systematic way. This is not possible when heterozygous parents are crossed because it results in an explosion of genetic variability that, ironically, results too large and makes the identification of useful segregating progenies very difficult.

One further advantage of working with stocks of elite germplasm is that exchange and storage of germplasm is greatly facilitated. Inbred materials can be maintained and reproduced through botanical seeds. Currently all the collection of elite germplasm needs to be constantly grown in the field or maintained in the in vitro collection. Both options are expensive and prone to eventual losses because of their inherent limitation that only few plants can be maintained. However, the facilitated exchange of germplasm is a much more relevant consequence. Currently exchange of germplasm has to be in vitro. This implies severe limitation in both the number of clones that can be shipped from one country to another and the number of plants representing each clone. Shipment of seed would be much easier.

Finally we could think of remaking a good hybrid by sexual crosses. This would allow cleaning the hybrids from the pathogens and non-pathogen organisms that eventually contaminate the planting material. This is much cheaper than the alternative approach of meristem culture.

3.6 Conclusions

Several approaches have been taken for a more efficient genetic improvement of cassava. Most of them are just improvement on the way the evaluation and selection was conducted. However, the introduction of inbreeding implies a drastic change that will hopefully change completely the way cassava is bred. The main ideas is to shift from making thousand of crosses, many of which are useless, to a system where a more directed approach is followed. The emphasis is now in developing a set of elite ***parents*** (not elite ***germplasm***), which are partially or fully inbred. Once these elite parents are identified they represent a solid base for further genetic gains. This is only possible if they possess certain degree of inbreeding. A significant event will take place during 2006 as part of the ongoing evolution of this activity. The Rockefeller Foundation has approved the organization of a brainstorming meeting at its Belagio Facilities in Italy. A group of maize breeders and perhaps breeder that have experience in other crops where heterosis is exploited (i.e. sunflower) will meet to discuss how heterosis can be better exploited in cassava when an efficient method for the

production of doubled haploids is developed. How can we identify heterotic groups in cassava? Should we look for heterotic patterns or just assume there is no pre-fixed pattern and that it is better to start "creating" heterotic groups? The answers to these questions are relevant not only to cassava, but to other crops as well (i.e. rice and tropical fruits).

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4. Development of technologies for the establishment of an IPM strategy for whiteflies on cassava

4.1 Introduction

Cassava is traditionally grown on small scale farming systems using few purchased inputs such as fertilizers or pesticides and where cassava is usually one of several crops grown. Distances between cassava fields may be considerable and this can contribute to the sporadic occurrence of some cassava pests. However, in Latin America there are indications of a shift towards larger-scale production units where cassava is grown as a plantation crop. In situations where cassava is utilized more as an industrial crop, it is advantageous for farmers to employ a multiple planting, multiple harvesting production system in order to meet the constant market demands of the processing industries.

In this type of production system, the cassava crop can be observed at several different growth stages in the same or surrounding farmer fields. Evidence now indicates that pest problems will be compounded in these overlapping production systems. Populations of certain pests, such as whiteflies and hornworms, and possibly mealybugs, tend to increase when a constant food supply, e.g. young cassava foliage, is available.

The continual food supply prevents or deters a break in the reproductive cycle of the insect, altering its population dynamics that could have been adversely affected by the lack of optimal foliage for feeding and reproduction. This situation is more apt to occur where environmental conditions such as an evenly dispersed rainfall pattern favors or provides for several planting dates throughout a one-year cycle.

In addition, crop management alternatives can be influenced where the availability of irrigation provides for more frequent plantings, especially in semi-arid or seasonally dry agroecosystems.

The aforementioned described conditions occur in certain cassava growing regions of Colombia, Venezuela and Brazil and other countries in Central and South America. In the coffee growing region of Colombia, rainfall is dispersed throughout the year and a prolonged dry period of three or more months is not uncommon. In Northeast Brazil (e.g. Bahia State) the availability of irrigation is resulting in large cassava plantations, some more than 3000 ha. In this seasonally dry region cassava was seldom planted more than twice during the year. The availability of irrigation had led to more frequent plantings (Osmar Lorenzi, personnel communication, 2005). A similar situation also occurs in the plains region of Venezuela. The Colombian coffee growing region (CCGR) and Northeast Brazil are reporting more frequent outbreaks of hornworm and increasing whitefly populations. Higher whitefly populations on cassava are recently reported from Central America, especially in Costa Rica and Guatemala as well as from Cuba.

4.2 Whiteflies on Cassava

Whiteflies are considered one of the world's major agricultural pest groups, attaching a wide range of crop hosts and causing considerable crop loss. There are nearly 1200 whitefly species with a host range that includes legumes, vegetables, fruit trees, ornamentals and root crops. As direct feeding pests and virus vectors, whiteflies cause major damage in agroecosystems based on cassava in the Americas, Africa and to a lesser extend in Asia.

The largest complex of whitefly pests on cassava is found in the neotropics where 11 species are reported. The most important species include *Aleurotrachelus socialis, Bemisia tuberculata, B. tabaci* and *Aleurothrixus aepim. A. socialis* and *A. aepim* cause considerable direct damage and yield losses in Northern South America and Brazil. *A. socialis* appears specific to cassava (no additional hosts have been identified) and predominates in Colombia, Venezuela, Ecuador and in certain regions of Central America.

A. aepim, which primarily attacks cassava but has other hosts, is found in high populations, causing yield losses in Northeast Brazil. *B. tabaci*, the vector of African cassava mosaic disease (ACMD) has a pan tropical distribution, feeding on cassava throughout Africa, several countries in Asia and more recently in the neotropics. ACMD is caused by several gemini viruses (Calvert and Tresh, 2002), and it has been speculated that the absence of ACMD in the Americas may be related to the inability of its vector, *B. tabaci*, to colonize cassava. Prior to the early 1990's, the *B. tabaci* biotyopes found in the Americas did not feed on cassava (Wool et al. 1994).

Whiteflies especially in the neotropics, cause direct damage to cassava by feeding on the phloem of the leaves. This causes symptoms such as chlorosis and leaf fall, which result in considerable reduction in root yield if prolonged feeding occurs. Yield losses resulting from *A. socialis* and *A. aepim* activity are common in Colombia and Brazil (Bellotti et. al. 1999; Farias, 1990). With *A. socialis* feeding, there is a correlation between duration of attack and yield loss. Infestations of 1, 6 and 11 months resulted in a 5%,, 42% and 79% yield reduction, respectively (Bellotti, 2002).

4.3 Integrated Management of Cassava Whiteflies

A successful integrated pest management program in cassava, especially for whitefly control will depend on having effective, environmentally sound, low cost pest management technologies available to cassava farmers. A successful IPM program requires farmer involvement in the development of technologies and in the decision making for appropriate implementation of control tactics. Equally important, a considerable amount of basic and applied research is required to understand the complexities of agricultural and biological systems involved.

This report will address several aspects of an integrated system in whitefly management in cassava. This includes the implementation of diagnostic cassava farmer surveys and an overview of recent research efforts and results in host plant resistance, biological control, cultural control and chemical pesticide use in cassava whitefly management. Most of this research was carried out in Colombia, where the major whitefly species is A.*socialis*. However results from this research are being utilized in other regions (e.g. Africa and Brazil) on additional whitefly species (e.g. *B. tabaci* and *A. aepim*).

Biological and agricultural systems are dynamic and change over time is certain to occur. This is especially true of whiteflies where a propensity to form new biotypes has been demonstrated. It should therefore be noted hat this is on-going research that will require a continued research effort for many years to come.

4.4 Casasava farmer surveys: Pest control practices

During the past five years cassava farmer surveys have been carried out in two important cassava growing regions of Colombia, the first was done in the Cauca/Valle del Cauca Departments from 2000 to 2002. The second was in the coffee growing region (CCGR), primarily in the Department of Quindio and this will be reported on in more detail.

A) The farmer survey in Cauca showed that cassava producers do not employ uniform criteria in cassava crop management. Numerous cassava varieties (approximately 20) were being grown, with the variety Chirosa being more frequently sown. Several types of fertilizers are applied with no consensus as to rate nor time of application. Several herbicides are used for weed control, including round-up, gramoxone and karmex, as well as hand weeding, and there is considerable variation in dosis and time of application.

Sixty eight percent of the 102 farmers surveyed indicated that they make their own decisions on crop management, with little or no outside technical advise and 83% of the farmers surveyed have received no assistance from any organization.

Surveys of pest problems resulted in identifying nine arthropod pest species. This is not surprising as the cassava crop is often accompanied by a diverse complex of arthropod pests, many of which are in low population and not of economic importance. However whiteflies were identified as the most important pest and probably causing yield losses, especially in the North Cauca region. Several chemical pesticides were being evaluated for whitefly control; drench applications with Confidor (Imidacloprid) was the only product to provide effective control of nymphs, and nymphal control is essential in reducing whitefly populations (Holguin and Bellotti, 2004).

B. Farmer surveys in Quindio Department were carried out in 2005 and are presently under way in Risaralda. The specific objectives of this survey include:

- Obtain information on current management practices being used by cassava producers.
- Provide cassava producers with information and training in integrated pest management and offer alternatives, techniques such as biological control and plant resistance, there-by reducing pesticide use.

Materials and Methods

Thirty cassava farmers were visited and interviewed in the region, especially in the Quindio Department. A survey questionnaire was designed for use during farm visits. These visits and interviews were designed with the purpose of obtaining information on the actual situation confronting cassava producers in the field, the severity of phytosanitary problems and farmer needs and priorities. Posterior to these surveys, random sampling was carried out in cassava fields to confirm the presence of the different arthropod pests and diseases and to try to determine pest populations or crop damage severity.

At the time of this survey meetings were held with farmer groups, students, technicians and agronomists in the region, providing them with information on pest biology and behavior and introducing some whitefly management practices.

Results:

Surveys were carried out with the assistance of entities such as the Federación Nacional de Cafeteros (The Colombian Coffee Federation) and directly with the Coffee Growers Committee's in each municipality, and ICA Regional Quindio. Cassava producers came from six municipalities in the Departments of Quindio (Armenia, Montenegro, Calarcá, Buena Vista, La Tebaida, Quimbaya and Circacia), where cassava is produced at altitudes varying from 1100 to 2900 masl.

Several pest species were detected feeding on cassava in this region. Whiteflies were the predominant pest found damaging cassava production. Whiteflies were the main pest on 52% of the farms surveyed followed by fruitflies (*Anastrepha sp.*) (20%) and mites (16%) (Figure 4.1).

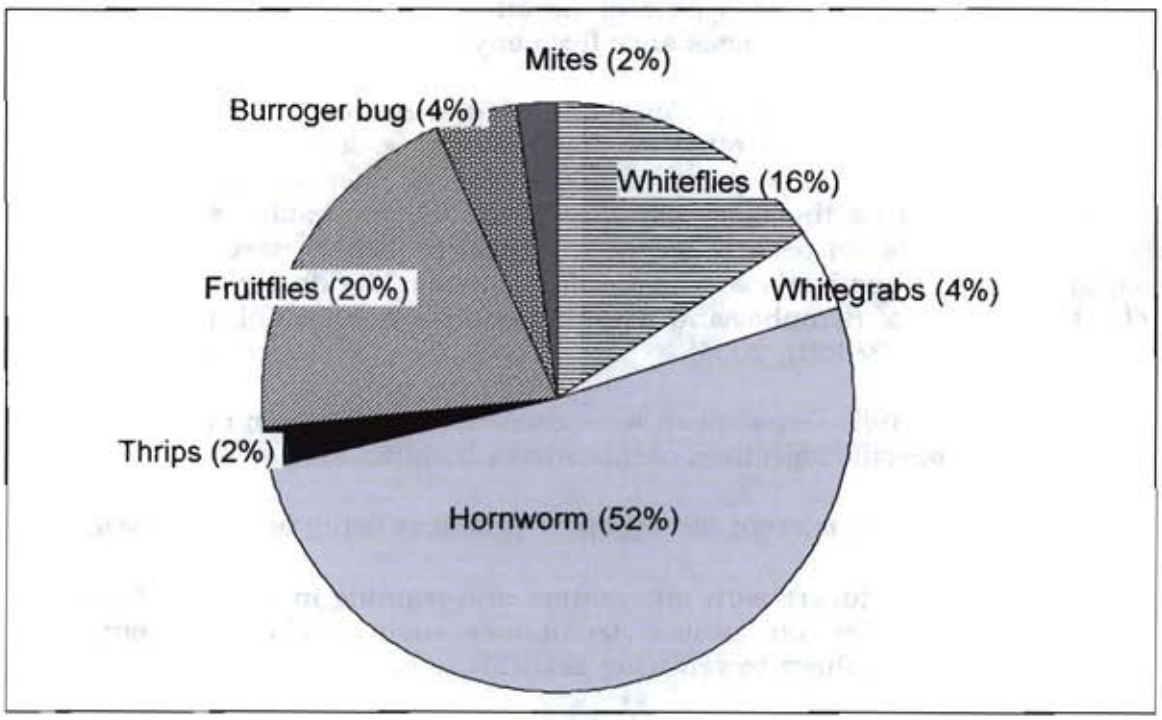

Figure 4.1 Arthropod pest populations on cassava farms in Quindío Department of Colombia. Figures indicate the percent of farms surveyed with each pest.

Additional arthropod pests detected in cassava fields included the cassava hornworm (*Erinnyis ello*), whitegrubs, thrips (*Frankliniella williamsi*), the burrower bug (*Cyrtomenus bergi*) and mites (*Mononychelus tanajoa*).

At the time of the survey, the municipality of Calarcá was most affected by whiteflies in cassava. This is reflected in the number of cassava farmers that was visited in this municipality (40% of the total).

Two species of whiteflies were collected feeding on cassava in the region, *Aleurotrachelus socialis* and *Trialeurodes variabilis* (Figure 4.2).

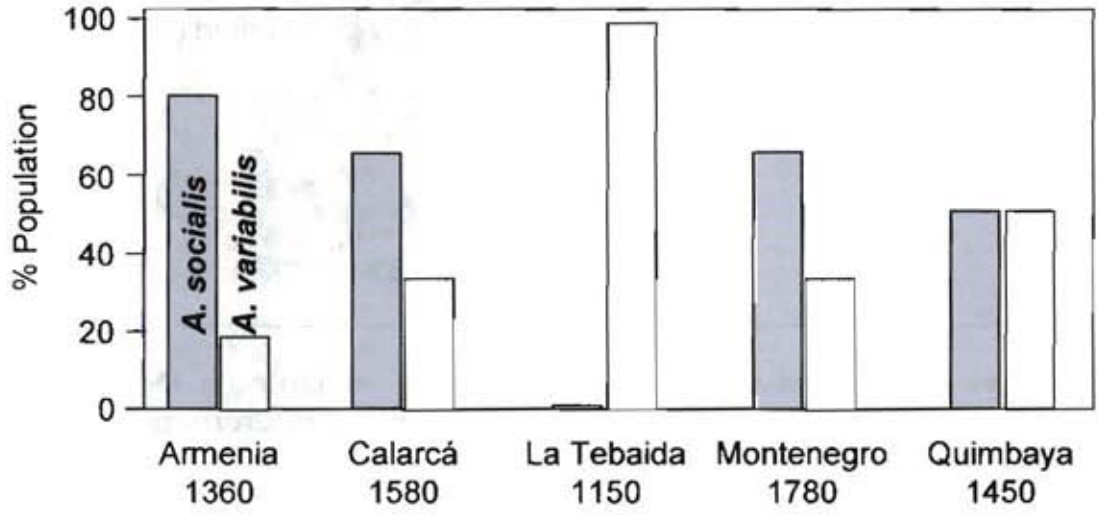

Figure 4.2 Percent whitefly species population found on cassava in five municipalities of Quindío Department in Colombia.

At the time of the survey, A. *socialis* was found in higher populations between 1360 and 1780 m.a.s.l., while *T. variabilis* was primarily found on cassava grown between 1100 and 1200 m.a.s.l. This is represented by the municipality of La Tebaida, where ambient temperatures are slightly higher than in the other municipalities in the survey.

The two most frequently grown cassava varieties in the region are Chirosa (MCol 2066) and ICA (HMC-1 and Catumare). Cassava is planted more frequently in monoculture (53.7%) than in association with other crop (46.3%) (Figure 4.3a). The crops most frequently grown in association with cassava are coffee (50.0%), beans (17,0%), citrus (17.0%), plantain (8.0%) (Figure 4.3b). It can also be noted that the whitefly species A. *socialis* predominated in the monoculture plantings of cassava while in the intercropping system the incidence of both species was nearly equal.

The damage or yield losses in cassava due to whitefly feeding in this region is being determined. Yield loses due to A. *socialis* feeding on cassava in other regions of Colombia have been recorded above 70% and averaging around 30 to 40% (Bellotti et.al.1999).The surveys also provided information on cassava farmer knowledge about pest biology, behavior, damage and ultimately about management of cassava pests. Results show that a great majority of producers, approximately 88% have little or no knowledge of whitefly biology, behavior and management.

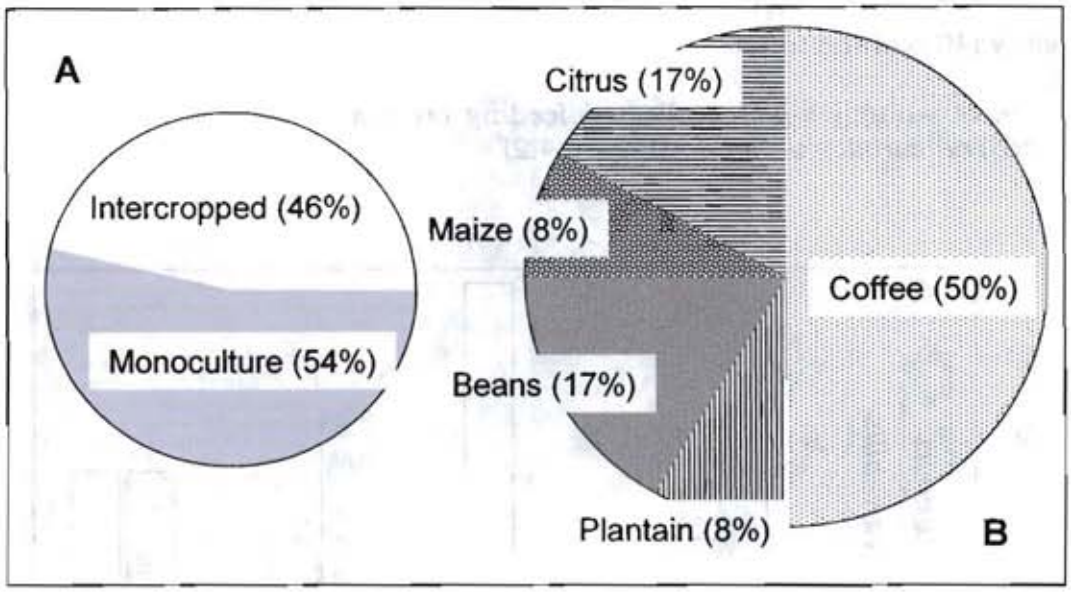

Figure 4.3 Cassava cropping systems being employed in Quindío Department of Colombia; a) monoculture vs. intercropping and b) percent of intercropped species.

Only 12% of the farmers surveyed claimed knowledge of whiteflies as a cassava pest. These results support the need for farmer training in recognizing cassava pest problems.Additional survey data shows that at present 50% of the cassava producers are applying chemical insecticides for whitefly control (Figure 4.4).

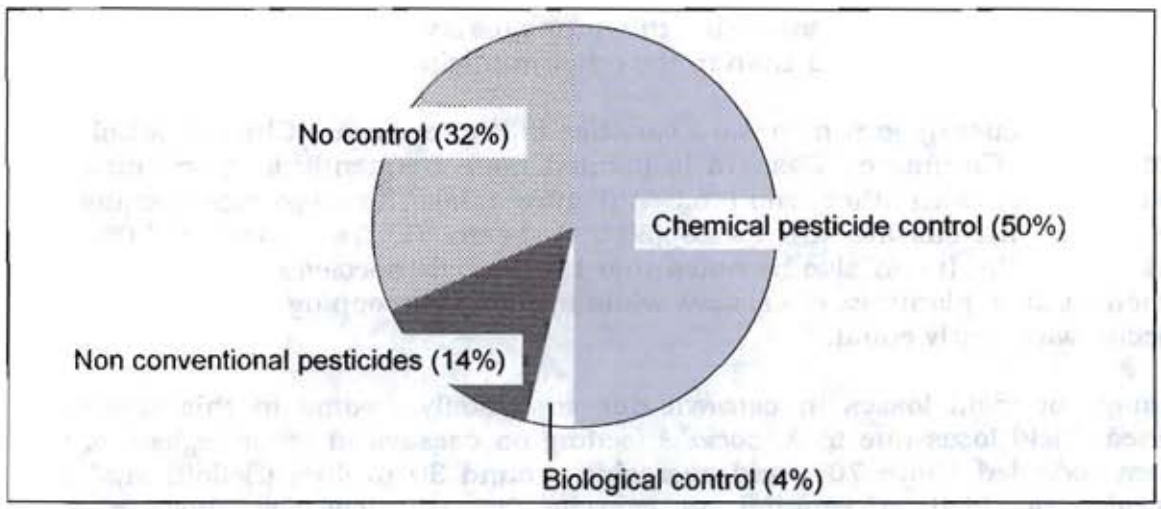

Figure 4.4 Whitefly control methods being used by cassava growers in Quindío Department of Colombia.

In most cases farmers receive no technical training or support. The continued high whitefly populations indicate that these pesticide applications are not effective in controlling the pest. Only 4% are presently using biological control, mostly entomopathogens such as *Beauveria bassiana* and *Lecanicillium lecanni* and predators like Crysopa. Approximately 14% of the farmers are using non-conventional pesticides; these are "home-remedy" types such as plant extracts and soap solutions. 32% of the farmers are not engaged in any whitefly control.

Cassava farmers are presently applying several different chemical pesticide products for whitefly control. The most popular is Sistemin (Dimethoate), being applied by nearly 48% of the farmers. Second is Actara (Tiametoxan) used by 19% of the growers, followed by Nodrin (9.5%) and Evisect (9.5%) (Table 4.1).

Products such Eviset (Tyocicam), Actara (Tiametoxan) and Trebon (Etoferprox) are reported as providing efficient control of whiteflies. However, farmers are not achieving adequate whitefly control, probably due to their lack of knowledge of whitefly biology and behavior. Pesticide applications are not timed to coincide with whitefly adult and 1st. instar nymphal populations when these species are most susceptible to chemical treatment.

Table 4.1 Percent usage by cassava growers of chemical pesticide products for cassava whitefly control in Quindío Department of Colombia.

PRODUCT/ACTIVITIES INGREDIENT	% UTILIZATION
Nodrin (Methomyl)	9.5
Sistemin (Dimetoato)	47.6
Evisect (Tyociclam)	9.5
Actara (Tiametoxam)	19.0
Thionil (Endosulfan)	4.8
Trebon (Etoferprox)	4.8

Discussion:
Similar surveys of cassava farmers in the Departments of Risaralda and Caldas have already been initiated as the project will be expanded into these two regions. Chemical pesticide and non-conventional pesticide products are being evaluated on farmer fields in the region to determine product efficiency and mode and timing of applications. Several biological control options will be evaluated. These include the use of entomopathogens and predator or parasite species. A whitefly resistant cassava variety is being compared to farmer varieties for acceptance as part of the varietal mixture.

The need to have farmers more knowledgeable about whitefly biology and behavior is obvious. Information sharing with growers has been initiated and courses in whitefly IPM have already been implemented. To date approximately 600 farmers and technicians have participated in these workshops and seminars.

4.5 Whitefly resistance in cassava

Host plant resistance (HPR) to whiteflies is rare in cultivated plants. The large-scale screening or evaluation of an extensive collection of cultivars, breeding materials, hybrids or selected wild or cultivated species for whitefly resistance has been limited (Bellotti and Arias, 2001). In many cases the range of germplasm evaluated is too limited to understand or obtain the diversity of whitefly resistance genes that may be available in a given crop species.

HPR studies initiated at CIAT more than 20 years ago have systematically been evaluating the nearly 6000 accessions in the CIAT cassava germplasm bank for resistance to whiteflies, especially *A. socialis*. This on-going research is carried out at primarily two sites in Colombia (CIAT HQ, Palmira and Nataima, Tolima, in cooperation with the Colombian Agricultural Research Corportation, CORPOICA) using natural *A. socialis* field populations. To date approximately 5500 clones (several evaluated more than once) have been evaluated in the field. Of these about 75% were considered susceptible with damage ratings above 3.5 (on a 1 to 6 scale). Emphasis is placed on those clones with damage ratings below 2.0 (about 8%). Most of these are probably escapes where the selection pressure is not high enough.

Sources of resistance to *A. socialis* have now been identified. Clone MEcu 72 has consistently expressed one of the highest levels of resistance. Additional cultivars expressing moderate-to-high levels of resistance include MEcu 64, M Per 334, M Per 415, M Per 273 and others. *A. socialis* feeding on resistant clones have less oviposition, longer development period, reduced size and higher mortality than those feeding on susceptible ones. *A. socialis* nymphal instars feeding on MEcu 72 and M Per 334 suffered a 72.5% and 77.5% mortality respectively, mostly in the early instars (Figure 4.5).

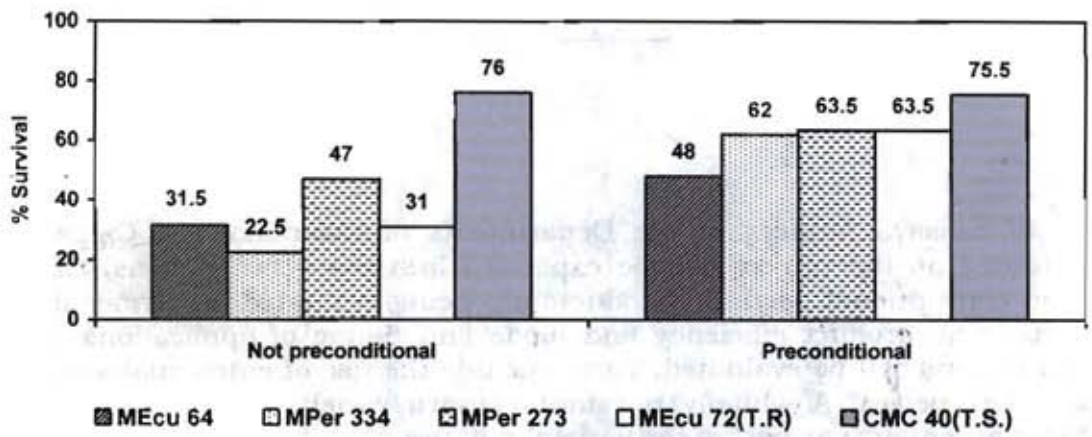

Figure 4.5 Percent survival of *Aleurotrachelus socialis* feeding on five cassava genotypes (resistant and susceptible) in the growth chamber (28±1°C, 60-70% RH, 12 hrs. light).

A cross between MEcu 72 (female parent, whitefly resistant) and M Bra 12 (male parent, high yielding, good plant type) resulted in 128 progeny and these were evaluated for whitefly

resistance, yield and cooking quality at CORPOICA, Nataima. Of these 128 progeny, 4 (CG 489-34, CG 489-31, CG 489-23 and CG 489-4) were selected for low whitefly populations and damage and favorable agronomic qualities. These four genotypes, along with susceptible controls and local farmer varieties were evaluated by CORPOICA at three sites in Tolima over a four year period.

Of the four, CG 489-31 was selected for high whitefly resistance, high yield and good cooking qualitie4s and officially released to farmers by the Colombian Ministry of Agricultural under the name Nataima -31, in 2003. Nataima -31 is now being commercially grown in several areas of Colombia, especially in Tolima and Cauca Departments, and is being evaluated in Quindio and Risaralda. Nataima 31 has attained yields as high as 33 T/ha, out yielding the regional variety Aroma (Figure 4.6), and requires little or no pesticide application.

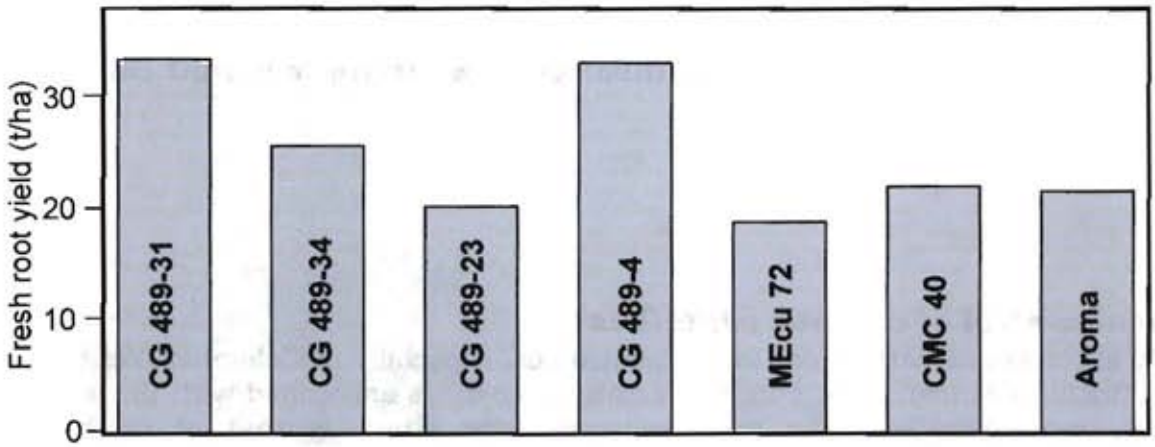

Figure 4.6. Agronomic Characteristics, Including Yield, Harvest Index and Dry Matter Content, of 10 Cassava Genotypes Evaluated under Whitefly (Aleurotrachelus socialis) Pressure at CORPOICA, Nataima (B), El Espinal, Tolima.

4.6 Host-plant resistance of South American Cassava genotypes to African and Indian whiteflies species

The whiteflies species, *Bemisia tabaci*, has a pan tropical distribution and is the vector of Cassava Mosaic Virus Disease (CMVD) in both Africa and India. This disease causes considerable yield loss and is a potential threat to cassava production in the Americas and Asia. In recent years high populations of *B. tabaci* have occurred on Cassava and is responsible for the rapid spread of the CMVD pandemic in East Africa. Losses of more than 50% have been reported (Legg et al. 2004).

Four Cassava genotypes; MCol 1468, MCol 2063, CG 489-34 and MEcu 72, with various degrees of resistance/susceptibility to *A. socialis* were sent by CIAT to NRI in the United Kingdom. NRI has established greenhouse colonies of Africa Cassava *B. tabaci* and *B. afer* and Indian Cassava *B. tabaci*.

Plantlets with 5 to 8 expanded leaves of the afore mentioned genotypes were placed individually in insect proof containers and exposed to 10 male and 10 female adult *B. tabaci*. Whitefly populations on each genotype were recorded at period of 18 and 38 days after infestation. Results indicate that the resistant genotype MEcu72 had the lowest rate of *B. tabaci* oviposition of the four genotypes evaluated. MEcu72 was introduced into Uganda during 2005 and will be included in a cassava-breeding program to develop whitefly resistant varieties.

4.7 The identification of genomic regions responsible for conferring resistance to whiteflies in cassava

(See Annex 1).

4.8 Determining the plant metabolites involved in whitefly resistant cassava varieties

(See Annex 2).

4.9 Biological control of cassava whiteflies

In recent field explorations carried out in the neotropics, especially in Colombia, Venezuela, Ecuador and Brazil, a considerable number of natural enemies associated with the whitefly complex have been identified. The most representative group is that of the micro-hymenopteran parasitoids. The richness of species in Colombia, Venezuela and Ecuador is primarily represented by the genera *Encarsia, Eretmocerus* and *Amitus*, frequently associated with *A. socialis* (Table 4.2) (Trujillo et at. 2004).

Gaps in knowledge about the natural enemy complex associated with the different whitefly species have limited the utilization and determination of their effectiveness in biological control programs. Consequently there is little knowledge on levels of parasitism, rates of parasitism by species, specification of the host and its effect on the regulation of whitefly population.

The parasitoid fauna of the whiteflies appeared to be more diverse in Colombia than in Ecuador. Eleven species of parasitoids representing 5 genera, 4 families and two super families, as well as 1 hyperparasitoid, were collected from the cassava growing regions of Colombia, while five species were collected from Ecuador and seven from Venezuela. There were notable differences among the different geographic regions. On the Caribbean Coast, *A. socialis* was parasitized by 8 species, with the genus *Erectmocerus* comprising 70% of the parasitoids. In the Andean region, Eretmocerus *sp.*, parasitized all whitefly species, but *E. pergandiella* was the predominant parasitoid of *T. variabilis*. In the Magdalena region, 73% of *A. socialis* were parasitized by *A. macgowni* followed by *Encarsia sp.* (26%).

Table 4.2. Whitefly parasitoids collected from cassava in diverse agroecosystems of Colombia, Venezuela and Ecuador.

	Colombia				Ecuador		Venezuela
Species	Caribe	Andean	Cauca Valley	Magdalena Valley	Coast	Highland	Plains
Amitus sp.					X		
A. macgowni				X			
Eretmocerus sp.	X	X	X	X	X	X	X
Encarsia sp.	X		X	X	X	X	
E. hispida	X	X	X				X
E. pergandiella	X	X					X
E. bellotti	X	X	X				
E. sofia	X		X				X
E. luteola	X		X				
E. cubensis							X
E. americana					X		
E. strenua	X						
Encarsia sp. prob. *variegata*	X						
Metaphycus sp.	X						X
Euderomphale sp.	X			X		X	X
Signiphora aleyrodis		X		X	X		X

In the Cauca region the number of parasitoid species on *A. socialis* was almost the same as that collected on the Caribbean Coast. However, the dominant genus was *Encarsia* (99%), represented by the species *E. hispida, E. Sophia, E. luteola* and A. *bellotti.*

Surveys have been initiated in the coffee growing region of Colombia to determine the parasitoid species complex associated with two whitefly species, *A. socialis* and *T. variabilis.* Additional studies are underway to determine the effect of parasitoids on whitefly densities.

More than 20 species of entomopathogens have been reported infecting whiteflies, including *Ascherosonia sp., Lecanicillium (Verticillium) lecanii, Beauveria bassiana and Paecilomyces fumosoroseus*; however a careful selection of the species is required, as well as the identification and evaluation of native isolates of entomopathogenic fungi. Greenhouse experiments at CIAT with isolates of L. lecanii resulted in 58-72% *A. socialis* nymphal mortality and 82% egg mortality (Aleán et al. 2004). At present *L. lecanii* is being formulated into a commercial product that should soon be available to cassava growers in Colombia.

Predators in general are less studied than parasitoids, and it is often difficult to accurately measure the impact that predators have on insect population dynamics in field situations. Predators that are most often observed feeding on cassava whiteflies (especially on *A. socialis*) are crysopids (Neuroptera: Crysopidae). Crysopids are generalist predators, feeding in the egg and immatures of numerous arthropod species. The crysopid species *Chrysoperla carnea* is frequently observed in cassava fields feeding on *A. socialis* immatures. A laboratory study to determine the efficiency of *C. carnea* on different instars of *A. socialis* was conducted.

Materials and Methods:
Studies were carried out in growth chambers and the greenhouse at CIAT (Temp 26°C and 67.5% RH). Whitefly adults and immatures were obtained from the *A. socialis* colony in the greenhouse (Var. CMC 40; Temp. 27±2°C, 60-70% RH). Adult *C. carnea* used in these studies were obtained from a commercial biocontrol company located in Palmira, Valle. The experimental design was completely randomized with five treatments and eight repetitions within each treatment. Each treatment corresponded to an *A. socialis* stage (egg, 3 nymphal instars, and pupae). Four male and four female *C. carnea* were released into each repetition/treatment. The experimental unit consisted of 500 cc plastic bottles with 2% nutrient agar. Cassava leaf discs containing 100 individuals of each developmental stage were placed on the agar in each plastic bottle. Adult *C. carnea* were released into each plastic bottle and consumption of the *A. socialis* development stages was recorded every four hours.

Results:
No significant differences were found in the consumption of different instars of *A. socialis* by *C. carnea* (Figure 4.7). No significant differences in consumption were observed between the nymphal and pupal stage. However, egg consumption was significantly different from that of nymphs and pupae (Figure 4.7). Egg and nymphal consumption was measured by recording the time required for 50% consumption of the prey stage being offered. *C. carnea* adult required 80 hours to consume 50% of these nymphal instars and pupae and 77 hours to consume 50% of the eggs offered.

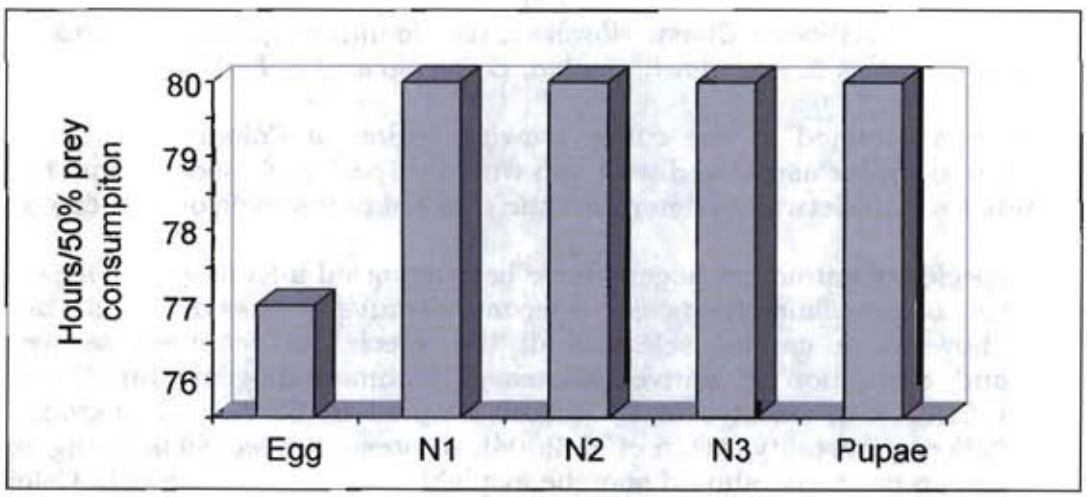

Figure 4.7. Consumption of *A. socialis* immatures by *C. carnea* (Neuroptera: Crysopidae) adults in laboratory studies (Duncan Multiple Comparison test at 0.05%).

Female *C. carnea* are slightly more voracious feeders of *A. socialis* immatures than are males. There was a significant difference in time required for females (78 hours) to consume 50% of the prey stage than males (80 hours).

The results for larval feeding of *C. carnea* were different from those of adult feeding. There resulted significant differences for *C. carnea* larval feeding on the different *A. socialis* prey instars. *C. carnea* preferred feeding on first and second instars. 50% consumption of first instar nymphs occurred in about 30 hours compared to about 70 hours for second instar nymphs, 78 hours for third instar and 80 hours for forth instar. 50% of egg consumption occurred at about 75 hours. It was also observed that most adult feeding was nocturnal, supporting evidence that the Crysopidae family is primarily nocturnal feeders. These results indicate that *C. carnea* could occupy an important role in reducing *A. socialis* populations planned to measure their impact on *A. socialis* populations.

4.10 Chemical Control

The shift from small cassava farms to larger plantations is often accompanied by an increase in phytosanitary problems, especially those associated with arthropod pests. The Cauca Department is still characterized by small, 1 to 3 ha. cassava plantings, while in the Cauca Valley larger cassava plantations are becoming more common. The predominant pest in both areas is the cassava whitefly, especially the species *Aleurotrachelus socialis*. The species *Trialeurodes variabilis* is frequently observed in moderate to high populations in Cauca as well as the coffee growing region (Quindio and Risaralda) where cassava is grown at higher altitudes (above 1200 m). Small farmersa are often resource limited and do not have access to agrochemicals, while larger farmers, with easy access to credit will often resort to the use of insecticides for pest control.

As part of an IPM program for cassava pest, especially whiteflies, for both small and large farmers, experiments were carried out to identify selective chemical pesticides (and biopesticides), determine doses and time of application for effective whitefly control with minimal effect on natural enemies. Farmer participation and training in IPM tactics are strategies to minimize pesticides application was an additional objective of this project.

Several products with new or novel active ingredients were evaluated for whitefly control on farmer fields in the region. Six different pesticides were applied, including Imidacloprid, Buprofezin, Carbosulfan, Tiametoxan, Diafentiuron and Piriproxifen. Foliar applications of these pesticides showed that Tiametoxan and Imidacloprid were most efficient in reducing whitefly populations. Tiametoxan treated plots had the highest root yield; however, this data was compromised due to the high incidence of Frogskin Disease (CFSD), that reduced root yield.

The nymphal stage best indicates pesticide efficiency since eggs and adult whitefly populations can vary due to migration from surrounding fields. All treatments reduced nymphal populations, but the lowest population was obtained using a concentrated suspension of imidacloroprid as a drench at planting at the high dosage of 0.8 and 0.6 lt/ha. This is considered the most efficient treatment.

A cost benefit analysis indicates that since larger farmers receive a higher price for their product, the C/B was above 1, and highest for the Tiametoxan treatment with a C/B value of 2.09 .This means that the producer receives 1.09 pesos for each peso invested. The C/B ratio for smaller farmers was below 1 for all treatments, except Tiametoxan where it was 1.34 to 1. These results indicate that chemical pesticide applications for whitefly control in cassava is generally uneconomical for small farmers (Holguín and Bellotti, 2004).

4.11 Cultural Control

(Agronomic factors in whitefly management). Whitefly populations, in particular those of *A. socialis* , can increase dramatically in certain cassava growing regions, The reasons for this rapid build-up in populations are not fully understood. *A. socialis* is more typically associated with lower altitudes and warmer temperatures as found in the Tolima Valley where the pest has been endemic for many years. However, in recent years *A. socialis* populations increased substantially in the Colombian Departments of Cauca, Valle del Cauca, and the coffee growing region of Quindio, Risaralda and Caldas, important cassava producing agrosystems.

This rapid population build-up could be caused by several factors that include:

- favorable environmental conditions.
- susceptible cassava varieties.
- lack of/or inefficiency of natural enemies.
- excessive and misuse of chemical pesticides.
- the introduction of a more virulent biotype.
- a high intrinsic rate of increase of the species.
- agronomic practices, including planting patterns and dates.

Undoubtedly, a combination of the above conditions has probably contributed to the whitefly population increases in the afore-mentioned regions.

Studies have been initiated to better understand the possible changes in *A. socialis* biology and population dynamics. This basic research will provide information on the potential of *A. socialis* to invade different cassava growing regions and will aid in developing effective pest management strategies. The immediate objective of this research is to determine the biology of *A. socialis* and estimate population parameters on two cassava genotypes. The genotype CMC-40 is a vigorous cultivar that is susceptible to whiteflies; Chirosa (MCol 2066) is a high yielding commercial cultivar being planted throughout the coffee growing region of Colombia.

Experiments are carried out on 30 to 40 day plants grown in plastic pots with sterile soil and maintained in the greenhouse at 30 $\pm$ 2°C and 50 to 60% RH. *A. socialis* adults are obtained from the CIAT colony that is maintained in the greenhouse (27 $\pm$ 2°C and 60 – 70% RH on cassava cultivar CMC-40).

Results show that *A. socialis* adults feeding on CMC-40 had a higher average longevity, greater oviposition, a longer development period and a higher net reproductive rate than those feeding on Chirosa (MCol 2066). However, adults feeding on MCol 2066 had an intrinsic rate of increase 40% higher than those feeding on CMC-40 (.3 4).

Table 4.3 Demographic parameters of *Aleurotrachelus socialis* feeding on CMC-40 and MCol 2066 (Chirosa).

	CMC 40	Chiroza (Mcol 2066)
Net Reproduction Rate (Ro)	33.74	6.2
Generation time (T)	44.23	36.71
Intrinsic rate of increase (r_m)	0.0296	0.0495
Days to duplicate population (DDP In $2r_m$	23.4	14

These results demonstrate the biotic potential of *A. socialis* to develop high populations on Mcol 2066 (Chirosa), inspite of the lower fecundity that occurs on this host. *A. socialis* will double its population in 14 days feeding on MCol 2066, while it requires 23 days on CMC-40 (Table 4.3).

It can be conclude from the results of this research that both CMC-40 and Chirosa are favorable host genotypes for rapid population increases of *A. socialis*. The shorter development time and the high rate of survival of *A.socialis* on Chirosa (MCol 2066) are indicators for high whitefly populations found on this genotype. These results help explain the high populations and damage to the cassava crop being experienced in the Colombian coffee growing region (Figure 4.8).

Figure 4.8. High survival of populations of *Aleurotrachelus socialis* feeding on Chirosa (MCol 2066) (a) and CMC-40 (b).

4.12 Whitefly (A. socialis) *Management*

The ability of whiteflies, especially *A. socialis*, to rapidly attain high populations indicates the need to introduce efficient management tactics early in the plant growth cycle (often during the first month). This could be particially achieved through the application of chemical pesticides. However, this will interfere with the effectiveness of the natural enemy

populations on often leads to repeated pesticide applications. A biopesticide might be advantageous in this situation and their effectiveness are being evaluated.

The employment of a resistant variety such as Nataime-31, has been shown to hinder or reduce this initial build-up of *A. socialis* populations and it is hypothesized that this provides the opportunity for move effective biological control. Field observations with Nataima-31 show that although there is an initial moderate adult population on young plants, the population does not increase sufficiently to cause economic damage.

4.13 The Successful Employment of a "Veda"

In cassava growing regions where there is a favorable rainfall pattern (e.g. no prolonged dry period) or the availability of irrigation exists, cassava is often grown "escalonada", or in overlapping cycles (e.g. multiple plantings) . This is ideal for the rapid build-up of whitefly populations as a constant food supply of young cassava leaves is available for whitefly adult feeding, high oviposition and nymphal development. Consequently it is difficult to adequately "break" the whitefly development cycle. Adults, upon emergence from the pupal stage, migrate to feed and oviposit on the recently germinated young plants in adjacent cassava fields.

This scenario occurred at the CIAT farm in Palmira several years ago and helps describe the present situation in the coffee growing region of Colombia (Quindio and Risaralda) and in regions of Central America and Brazil).

A successful tactic to counter this situation is through the implementation of a "veda". A "veda" is defined as an "interdiction" or "prohibition"; in practical terms it is a period of time when cassava is not present in the field. A 1 to 2 month "veda" has been employed at CIAT for the past four years and this has dramatically decreased whitefly *(A. socialis)* populations.

From about 1995 on ward, *A socialis* populations had constantly increased until it became impossible to continue cassava cultivation on the CIAT, Palmira station. Due to a favorable rainfall pattern (e.g. no prolonged dry period) and the availability of irrigation, cassava was being planted on almost a monthly basis and provided a constant food supply for high whitefly populations.

The success of this veda is enhanced by an important behavioral characteristic of *A. socialis;* it does not appear to have an efficient alternative host. *A. socialis* populations crash as adults cannot find an alternate host species. (For example, this same tactic would not be effective for the whitefly species *Bemisia tabaci* as it has numerous alternate hosts). The yearly implementation of the 1-2 month veda has become an efficient tactic to maintain lower whitefly populations.

The economic practicality of this tactic for cassava producers is questionable. In many regions, e.g. the coffee growing region, a constant supply of cassava roots is a desirable advantage for meeting the demands of the local fresh and processing markets. In this region farmers have traditionally employed multiple planting dates and in all probability continue to do so. This necessitates the need to seek alternative methods for whitefly management.

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Donor Institution: United States Department of Agriculture (USDA).

ANEX 1

4.14 . The identification of genomic regions responsible for conferring resistance to whiteflies in cassava

Due to the importance whitefly as pest, it is necessary to know about the nature of genes that confer resistance to whitefly in genotypes like MEcu-72. For this purpose we are using F1 segregation and the genetic expression of cross MEcu-72 (resistant genotype) x and very susceptible genotype (MCol-2246) and molecular markers. This would help to accelerate selection of resistant materials to whitefly and also to isolate resistant genes. We can hypothesize that these resistant genes may also be effective against other whitefly species, especially *Bemisia tabaci*, the species that is a vector of CMD, a virus that causes severe crop losses in Africa and Asia. Whitefly resistant genotypes (such as MEcu 72) from the neotropics are displaying resistance to *B. tabaci* in greenhouse trials being carried out by NRI in the UK (Progress report 2003).

An additional step toward a better understanding of attack response of whitefly to cassava, it is characterize genes that are involved in the downstream signaling cascades in plant defense responses. One tool that permits the unraveling of the complexities of gene expression is the establishment of a cDNA library, which has been developed with a highly effective method known as Substractive Hybridization. Using this approach, two mRNA populations, extracted from both resistant and susceptible genotypes, were examined to elucidate the differential gene expression between them.

Functional genomics tools such as the Microarray give a first comprehensive overview of the molecular basis of the cassava defense response to the whitefly attack and will help to understanding the defense mechanisms to other important pests and diseases, Microarray expression profiling will be used to identify putative early response regulatory and/or signaling genes. The application of molecular genetic analysis for cassava breeding has been limited compared to others crop. Recently progress has been made in the development of genomic and bioinformatics tools to increase our knowledge of cassava genome structure and cassava gene function. Lopez et al (2004) constructed a cassava Unigenes Microarray (5700 sequences), which is an unvalued resource to study global gene expression profiles.

The objective of this work in the contribution towards a better understanding of the plant-insect interaction and this information should help in the development of strategies for managing whitefly attack in cassava.

Materials and Methodos

For the present work we have used the F1 cross (family CM 8996, 276 individuals) between MEcu-72 (as the resistance parent) and MCol-2246 (as the susceptible parent) cassava cultivars from Ecuador and Colombia, respectively. The parents and their offspring were evaluated in the field at two sites: Nataima (Tolima) and Santander de Quilichao (Cauca). With this evaluation we intend to identify the gene segregation in the offspring and select the resistant and susceptible materials. Both parents were evaluated with 343 cassava SSRs (Simple Sequences Repeat) (Mba et al, 2001), we designed 105 pairs of SSRs primers from ESTs sequences (Bohorquez et al, 2004) of which 25 were evaluated. We are using AFLPs

(Vos, et al, 1995) and 15 random primers RAPDs to find markers associated to resistance for mapping and ultimately cloning the resistant genes.

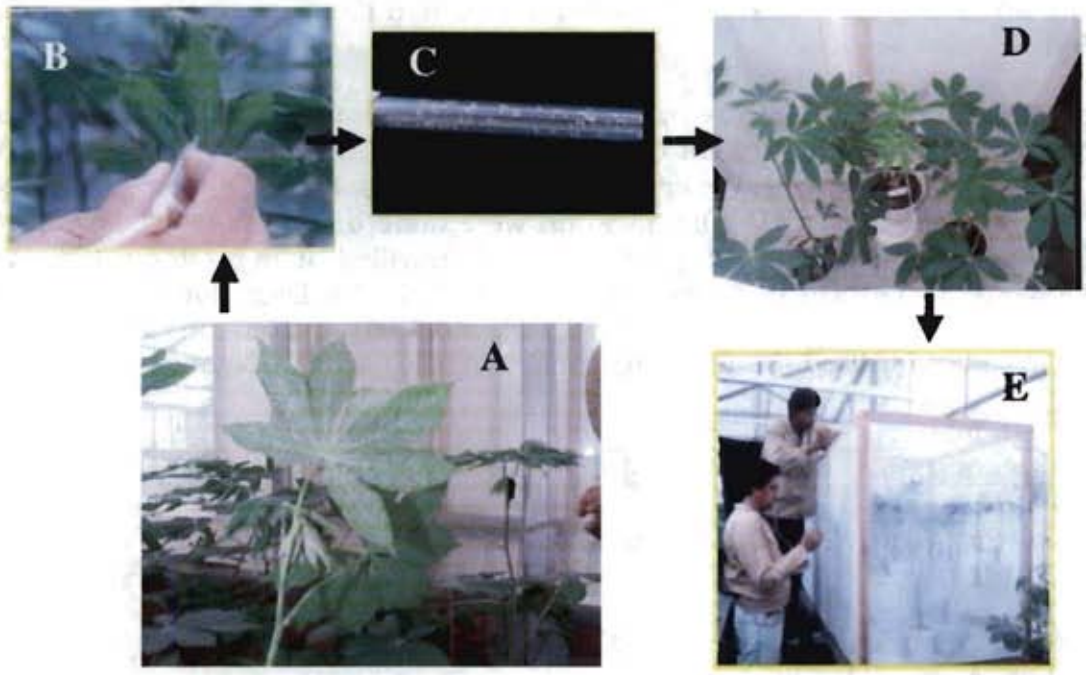


Figure 4. 9. Scheme of the greenhouse trials. A: CIAT colony of A. *socialis* developed on CMC-40. B: Two thousand adult male and female of A. *socialis* were removed from the CIAT colony with the aid of a bucal aspirator. C: tube containing the adults. D,E: These were placed in the cages, as previously described, and attached to the undersides of MEcu-72 and MCol-2246.

For the isolation of expressed sequences, we are using 28 plants of 40 days sown in pots; 7 of each genotype, (MEcu-72 resistance and MCol 2246 susceptible) are infested and 7 of each genotype are without infestation. These plants were placed in the greenhouse. They were infested with 300 whitefly adults per plant. A. *socialis* adults are obtained from the CIAT colony that is maintained in the greenhouse (27 $\pm$ °C temp. and 60 – 70% RH), on the cassava cultivar CMC-40. Each cage contained 2100 whiteflies (Figure 4.9). On the 2nd day the adults were removed and the eggs were allowed to develop. We collected leaves at six different times for the RNA extraction, in accordance with the whitefly life cycle. The first time was before the infestation, the 2nd time one day after the infestation, the 3rd time four days after, the 4th time six days, 5th time ten days and the 6th time 14 days after the infestation.

Differential Subtraction

For the isolation of expressed sequences we are using the following strategy: the genotype MEcu-72 infested is being used as a tester and the genotype MCol-2246 infested was used as a driver. The objective is to obtain constitutive resistant genes. The representational difference analysis of cDNA is divided into several phases, like the generation of a PCR

amplicon which is representative of the original mRNA from MEcu-72 and MCol-2246, then the subtractive hybridization of these amplicons MEcu-72 (Tester) and MCol-2246 (Driver), during which amplified portions of differentially expressed genes are enriched and common sequences are depleted, and ultimately the cloning and screening of the resulting products. At this moment we are using the Differential Subtraction Chain (DSC) technology according to Luo et al, (1999).

The RNA was isolated from young leaves colleted in the greenhouse. For the isolate total RNA we are using the *Rneasy Plant Mini Kit QIAGEN™.* Genomic DNA was removed prior to isolation of poly (A)$^+$ RNA with DNAse I. We are using *SV™. Total Isolation System of Promega.* First-strand cDNA synthesis and cDNA amplification were done using *SMART™. PCR cDNA Synthesis kit BD Biosciences.* The PCR products were purified using *QIAquick™. PCR Purification kit QIAGEN.* Once cDNAs amplified were purified, the Digestion-Ligation was carried out, in which the cDNA is digested with DpnII and then and adapters (BamI and BamII) are ligated. We standardized the following procedure of Digestion-Ligation process:

ATP (5 mM)	2,0
Adapters (*BamI, BamII*)* (10 µM)	6,0
T4 DNA Ligase (1U/µl)	4,0

**BamI* for MEcu-72 and *BamII* for MCol-2246

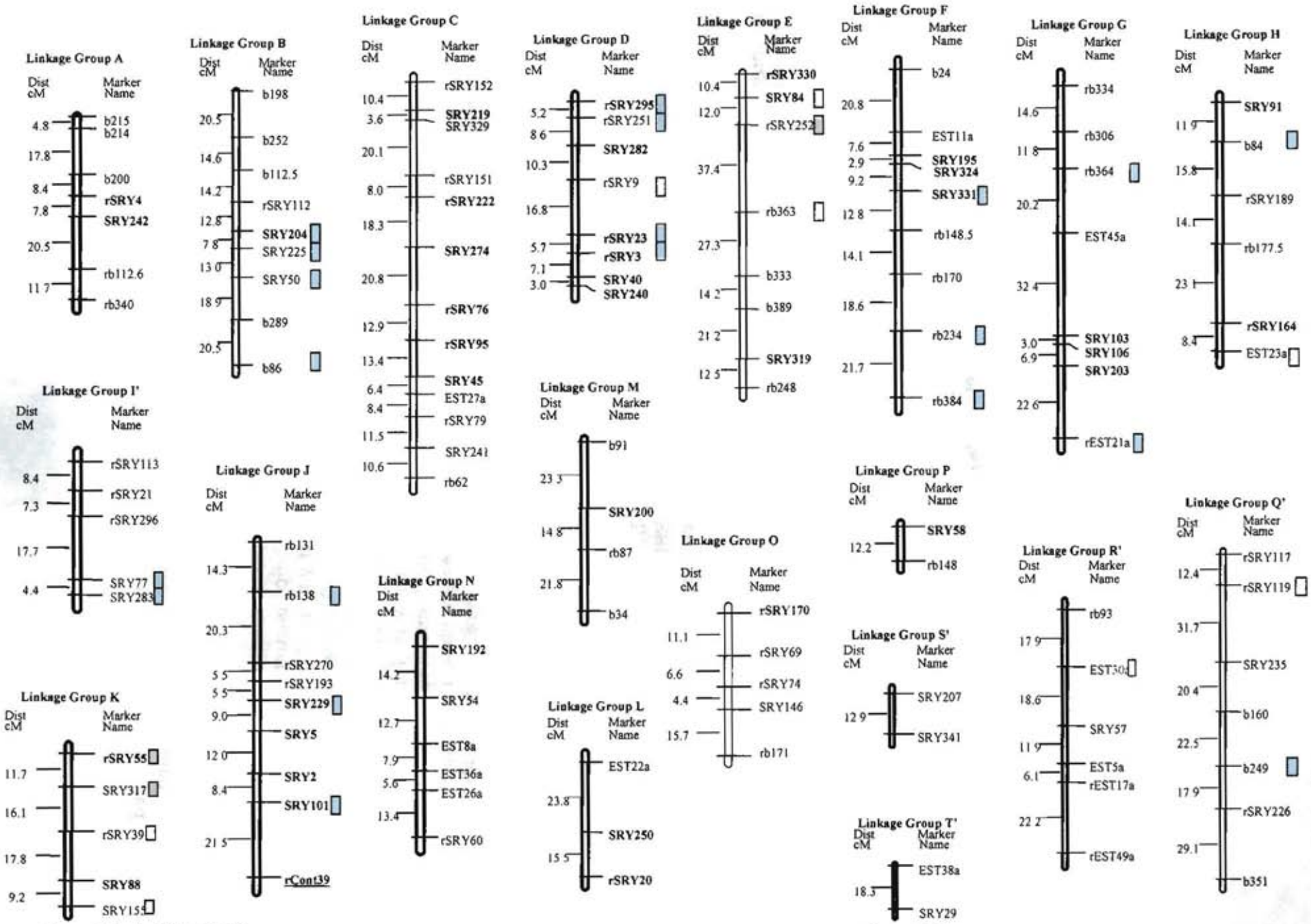
5-Incubate to 20°C for 2 hours.
6-Dilute with 10 µl of TE, pH: 8,0 (Dil. 1:1)

Then the amplicons are generated:

REAGENT	Volume (µl)
Distillated Water	37.3
Advantage Buffer 2™ 10X	5.0
dNTPs 20 mM (5mM cada uno)	0.5
Primer BamI, BamII 10 µM*	2.0
Advantage 2 Polymerase Mix™ (*BD Biosciences*)	0.2
Diluted Ligation Products	5.0

**BamI* for MEcu-72 and *BamII* for MCol-2246

Figure 4. 10 (Next page). Preliminary Cassava framework Map of MEcu-72 for Resistance to White Fly, consisting of SSRs, AFLPs and a RGA (Contig39) (Lod = 25 and theta = 25).


Linkage Group A
Dist cM
Marker Name
4.8 b215
b214
17.8
b200
8.4 rSRY4
7.8 SRY242
20.5
rb112.6
11.7 rb340
Linkage Group B
Dist cM
Marker Name
b198
20.5
b252
14.6
b112.5
14.2
rSRY112
12.8 SRY204
7.8 SRY225
13.0 SRY50
18.9
b289
20.5 b86
Linkage Group C
Dist cM
Marker Name
10.4 rSRY152
3.6 SRY219
SRY329
20.1
rSRY151
8.0 rSRY222
18.3
SRY274
20.8
rSRY76
12.9 rSRY95
13.4
SRY45
6.4 EST27a
8.4 rSRY79
11.5
SRY241
10.6 rb62
Linkage Group D
Dist cM
Marker Name
5.2 rSRY295
rSRY251
8.6
SRY282
10.3
rSRY9
16.8
rSRY23
5.7 rSRY3
7.1
SRY40
3.0 SRY240
Linkage Group E
Dist cM
Marker Name
10.4 rSRY330
12.0 SRY84
rSRY252
37.4
rb363
27.3
b333
14.2
b389
21.2
SRY319
12.5 rb248
Linkage Group F
Dist cM
Marker Name
b24
20.8
EST11a
7.6
2.9 SRY195
SRY324
9.2
SRY331
12.8
rb148.5
14.1
rb170
18.6
rb234
21.7 rb384
Linkage Group G
Dist cM
Marker Name
rb334
14.6
rb306
11.8
rb364
20.2
EST45a
32.4
3.0 SRY103
6.9 SRY106
SRY203
22.6
rEST21a
Linkage Group H
Dist cM
Marker Name
SRY91
11.9
b84
15.8
rSRY189
14.1
rb177.5
23.1
rSRY164
8.4 EST23a
Linkage Group I'
Dist cM
Marker Name
8.4 rSRY113
7.3 rSRY21
rSRY296
17.7
SRY77
4.4 SRY283
Linkage Group J
Dist cM
Marker Name
rb131
14.3
rb138
20.3
rSRY270
5.5 rSRY193
5.5 SRY229
9.0
SRY5
12.0
SRY2
8.4 SRY101
21.5
rCont39
Linkage Group K
Dist cM
Marker Name
rSRY55
11.7
SRY317
16.1
rSRY39
17.8
SRY88
9.2 SRY155
Linkage Group L
Dist cM
Marker Name
EST22a
23.8
SRY250
15.5 rSRY20
Linkage Group M
Dist cM
Marker Name
b91
23.3
SRY200
14.8
rb87
21.8 b34
Linkage Group N
Dist cM
Marker Name
SRY192
14.2
SRY54
12.7
EST8a
7.9
EST36a
5.6 EST26a
13.4
rSRY60
Linkage Group O
Dist cM
Marker Name
rSRY170
11.1
rSRY69
6.6
rSRY74
4.4 SRY146
15.7
rb171
Linkage Group P
Dist cM
Marker Name
SRY58
12.2 rb148
Linkage Group S'
Dist cM
Marker Name
SRY207
12.9
SRY341
Linkage Group T'
Dist cM
Marker Name
EST38a
18.3
SRY29
Linkage Group R'
Dist cM
Marker Name
rb93
17.9
EST30a
18.6
SRY57
11.9
EST5a
6.1
rEST17a
22.2
rEST49a
Linkage Group Q'
Dist cM
Marker Name
rSRY117
12.4
rSRY119
31.7
SRY235
20.4
b160
22.5
b249
17.9
rSRY226
29.1
b351

Results

We evaluated the OPERON™ set random primers in parents, of which 43 were selected for evaluation in the cross. An AFLP analysis was made of 128 combinations of primers with both parents (MEcu 72 and MCol 2246) and both bulks of 10 whitefly-resistant DNA and 10 susceptible DNA. We obtained 53 polymorphic combinations, in which there were 425 polymorphic bands between the resistant and the susceptible. All combinations were amplified in the F1. Approximately 155 of the SSRs evaluated were polymorphic in the parents and were evaluated in the F1 (286 individuals). For the construction of linkage map 103 SSRs, 1 RGA, 15 RAPDs and 57 AFLPs were analyzed of which 129 were anchored. A genetic linkage map of cassava was constructed with 129 markers segregating from the heterozygous female parent (MEcu-72) of an intraspecific cross. The map consists of 20 linkage groups, which represent approximately the haploid genome of cassava. These linkage groups span is 550,2 cM and the average marker density is 1 per 7,9 cM. The position of 129 markers, are shown in the Figure 4.10 on the framework (LOD = 25 and tetha (θ) = 25) molecular genetic map of cassava. Map distances are shown in Kosambi map units and analyzed by Mapmaker 2.0. So far, 41 SSRs markers were mapped (bold) on the cassava framework map (Fregene et al, 1997), the other 88 markers are new. The molecular data are being analyzed using QTL packages (QTL cartographer) to determine linkages between the SSR, RGA, RAPDs and AFLPs markers and the phenotypic characterization.

Association between Molecular Markers and Resistance

Preliminary analysis (X^2 and Simple Linear Regression at the 5% level) was done using SAS. Subroutine associations were found between 32 markers SSRs, RGA and AFLPs, shown by blue squares in Figure 4.10 and the field phenotypic characterization (score 1.0 to 2.0 of the levels of damage and populations). We observed that all markers anchored in the linkage group B, D, E, F. J and K are associated with the resistance. The molecular data are being analyzed using QTL packages (QTL cartographer) to determine linkage between the markers and the phenotypic characterization.

Functional Genomics: Differential Subtraction

High quality RNA of MEcu-72 and MCol-2246 (Figure 4.11) has been isolated.

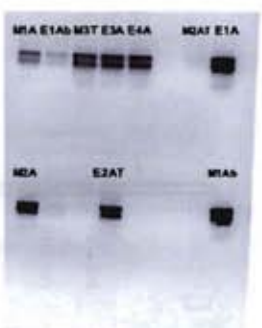


Figure 4.11. RNAs isolates with *Rnesy Plant Mini Kit QIAGEN* E=MECU-72 M= MCOL-2246 T=Witness plants.

Poly A+ mRNA isolation, cDNA amplification y purification.
The Poly A+ mRNA was isolated from total RNA and was used as a substrate for the generation of cDNA. The first-strand cDNA synthesis and the cDNA amplification were done (Figure 4.12). PCR products were then purified from the amplication of cDNA.

Figure 4.12. M: λ DNA digested with Pst I. cDNAs amplified with kit *SMART™*

Once cDNAs amplified were purified, the Digestion-Ligation and the amplicons generation were carried out.

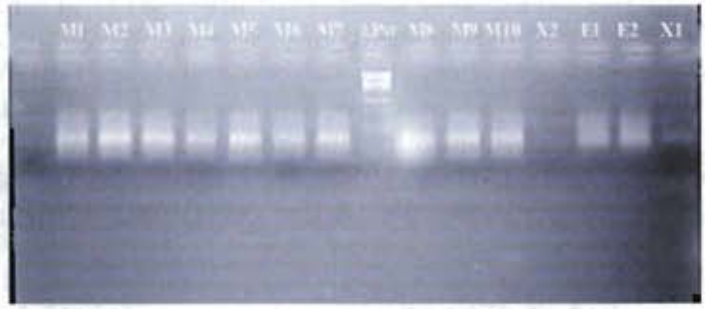


Figure 4.13 M: λDNA digested with Pst I. Amplicons of E=MECU-72 M= MCOL-2246 X=negative controls

Ongoing activities

- QTL analysis for whitefly resistance.
- Subtractive hybridization of the amplicon MEcu 72 (tester) and MCol 2246 (driver), the DSC technology according to Luo et al, (1999).
-

Strategy 2: the genotype MEcu-72 infested as tester and the same genotype without infesting as driver, to obtain induced defense genes.

- Cloning, sequencing and screening of the resulting products of expressed sequences during the defense response of MEcu 72 to whitefly attack.
- Microarray of clones in order to identify differentially expressed sequences.
- Hybridization with Cassava Unigenes Microarray.

ANEX 2

4.15. Determining the plant metabolites involved in whitefly resistant cassava varieties

The whitefly, *Aleurotrachelus socialis*, is a major pest of cassava, reducing root yield and the formation of cassava planting material (cuttings or stakes). Field evaluations during a 1, 6- and 11-month attack resulted in yield losses of 5, 42 and 79% respectively (Bellotti and Vargas, 1986). Whiteflies cause direct damage to cassava by feeding on the phloem of leaves, inducing leaf chlorosis and abscission, which results in reduction in root yield if feeding is prolonged (Bellotti, 2002). Additional yield reduction can be caused by the growth of a "sooty-mold" on whitefly exudates deposited on cassava leaves that deters photosynthesis (Bellotti and Vargas, 1986).

The CIAT cassava germplasm bank contains nearly 6000 accessions, of which 93% are landraces (locally selected cultivars), collected from tropical and subtropical regions of the world, but mainly from the Neotropics. This germplasm collection has been extensively screened in the field for whitefly (*A. socialis*) resistance and more than 5400 landrace cultivars have been evaluated. Sources of resistance to *A. socialis* have now been identified. The clone "MEcu 72" has consistently expressed high level of resistance. Several additional cultivars, including "MEcu 64; MPer 334, MPer 415, MPer 317, MPer216, MPer 221, MPer 266 and MPer 365, have expressed moderate to high levels of resistance. These results also indicate that *A. socialis* resistance may be concentrated in Peruvian and Ecuadorian germplasm. Greenhouse and field studies show that *A. socialis* feeding on resistant clones have less oviposition, longer development period reduced size and higher mortality than those feeding on susceptible ones (Arias, 1995). *A. socialis* nymphal instars feeding on MEcu 72 suffered a 72.5% mortality, mostly in the early instars (Arias, 1995, Bellotti and Arias, 2001).

Recent studies under controlled conditions in the growth chamber, *A. socialis* had a longer development cycle when feeding on MEcu 64, MEcu 72 and MPer 334 when compared to the susceptible control, CMC 40. Nymphal mortality was highest on MPer 334 (77.5%), followed by MEcu 64 and MEcu 72 with 68.5% and 68.0% respectively.

In addition, genomic sequences possibly involved in *A. socialis* resistance have been detected in MEcu 72 using AFLP and microsatelite markers (Bellotti, et al, 2003).

Plant strategies for resisting insect attack often involve biochemical factors or activities. Studies were therefore initiated to determine what plant metabolites might be involved in the development of *A. socialis* resistance found in the resistant genotypes. MEcu 64, MEcu 72 and MPer 334.

Materials and Methods

Whiteflies have piercing-sucking feeding habits; this has made it difficult to develop an artificial liquid diet that would allow testing the biological activity of protein extracts for each of the resistant and susceptible genotypes to determine the relationships between the protein and resistance to the whitefly.

The plan includes obtaining polyclonal antibodies from the immunization of rabbits against protein extracts for each of the materials, and later to determine by means of immunodetection, and the combination of Western Blot and 2D SDS-PAGE techniques, the differences between each of the protein extracts. The resistant genotypes evaluated were MEcu 72, MEcu 64 and MPer 334. The susceptible control was the genotype CMC40. This process will be carried out using healthy plants (non-infested), and plants infested with *A. socialis*, for each of the genotypes, to detect if a proteic response occurs in infested plants. In addition, *A. socialis* feeding on resistant plants will be examined for the presence of a plant protein.

Electrophoresis, employing polyacrylamide gels (PAGE), has proven to be a very useful technique for the analysis and characterization of complex protein mixtures. Nevertheless, since access into the interior of protein matrixes is limited, information generated about the individual components is usually restricted to molecular weight and isoelectric dots. The transfer of proteins by PAGE to an unfixed membrane permits the utilization of diverse tests for an improved characterization. One of the more precise applications for the transfer of proteins to membranes, is through immunodetection which consists of the identification and characterization of a fixed antigen by means of antibody tests (Timmons and Dunbar, 1990; Garfin, 1990; Anderson, 1988; Hames and Richwood, 1988; Dunbar, 1987).

Inmune-detection permits estimating by semiquantitative means, the mass or abundance of a specific protein in a determinate tissue. This technique is regularly employed in experimental studies in which the objective is to detect a specified protein or to observe its variation under diverse conditions.

Total Protein Extraction

To extract the total protein, cassava leaves (without petioles) were macerated in liquid nitrogen, obtaining a very fine powder that was subsequently homogenized for five hours at 4°C with the buffer Tris HCL, pH 8.0, and containing 1mM of EDTA (metalloprotease inhibitor), 5 mM of DTT (reduction agent), 1% PVP (antiphenolic), and 5 mM of PMSF (serine protease inhibitor) at a proportion of 1g macerated leaf to 3ml of buffer. The following step consisted of filtering this mixture and centrifuging it at 15000 rpm for 30 minutes at 4°C, to clarify the extract and eliminate vegetative tissue. The supernadant is dialyzed with a dialysis membrane of W.M. Co. 3.5 Kd and finally lyophilized to obtain an extract in powder form, in order to manipulate the concentration by weight units.

Immunization and Production of Polyclonal Antibodies against Cassava Proteins

Polyclonal antibodies were used as they contain different sub-classes of antibodies, including IgG, IGM, IGE, IgA and IgD. Each antibody represents the product of only one stimulated lymphocyte and its clonal progeny. An antigen complex such as a protein can contain several distinct or epitopes or determinant antigens, each of which is specifically recognized by antibodies from only one clonal lymphocyte (Dunbar and Schwoebel, 1990).

To produce polyclonal antibodies the following steps were developed:

- Two milligrams of each protein was dissolved in 1 ml of the buffer Tris-Glicina pH 6.8 and later emulsified with one ml of Freund's complete adjuvant.

- Four New Zealand breed rabbits were employed. Each of them was subcutaneously injected four times with 0.5 ml of each of the prepared proteins. The injections were applied to the animal's loin.
- After three weeks, the four applications were repeated on each rabbit, but at this time the proteins were emulsified with 1ml of Freund's incomplete adjuvant. Two of the injections were intermuscular.
- Ten days after the last injections, the animals were bled, obtaining 15-20 ml of blood from each.
- The collected blood was left at room temperature for 24 hours, than centrifuged and the serum was stored coagulated in aliquots for later analysis.

Test for Antibody Recognition using the Dot Blot Technique

A test for antibody recognition using the Dot Blot technique was carried out to verify that the antibodies produced were in good condition. The following steps were developed:

- One milligram of each of the proteins was dissolved with 200 µl of Tris Glycine (pH 6.8) buffer. On each nitrocellulose membrane 5 µl of the stock solution was applied to each of the proteins.
- Blockage of the nitrocellulose membrane with the sample in TBS containing 1% gelatin.
- Exposure of the membrane to 30 µl of the first antibody dissolved in 30 ml of blockage solution.
- Four washings of the membrane of 15-minutes each. The first three with TTBS (TBS containing 1% tween 20) and the last with TBS.
- Exposure of the membrane in 30 µl of the second antibody (Bound to PER) dissolved in 30 ml of the blockage solution.
- Four washings of the membrane of 15-minutes each. The first three with TTBS (TBS containing 1% tween 20) and the last with TBS.
- Addition of 5 ml of revealed solution (40 ml of TBS, 3 µl of hydrogen peroxide and 30 mg of 4 Chloro-1-Naphtol dissolved in 10 ml of methanol). This solution is preheated at 35°C.

SDS-PAGE

Using electrophoresis trials with polyacrilamide gels in disnatured conditions (SDS-PAGE) it was determined:

- Protein sample concentrations (mg/ml) carried on gel pools for a visualization of the bands. To do this, concentrations of 200 mg/ml, 100 mg/ml, 75 mg/ml, 50 mg/ml, 25mg/ml, 10 mg/ml and 2mg/ml were tested.
- Adequate concentrations of the resolving phase of the gel were achieved for a good view of the protein bands. To do this, concentrations of 10%, 14%, and 17% were tested. It should be noted that the phase stacking concentration was 4% at all times.
- Polymorphism by molecular weight for each of the proteins for each genotype evaluated. To do this a marker of the Prestained SDS-PAGE from Biorad Laboratories (with an arrange of 106 to 20.8 Kd) molecular weight was utilized.

These tests were carried out in a Biorad Mini Protean electrophoresis chamber and followed the protocol established by the manufacturer for both the electrophoresis as well as the staining of the gels.

First immunization tests were with healthy plants (no whitefly infestation) using the Western Blot Technique. This test seeks to determine the specificity of each antibody in the genotypes being evaluated. An SDS-PAGE electrophoresis of the proteic extracts was carried out for each of the genotypes, using the previously determined conditions. Subsequently a transfer of the bands obtained during electrophoresis was made to a nitrocellulose membrane using the Western Blot Technique. Antibody recognition was determined using the Dot Blot Technique, with the exception of the first two steps.

Results

Tests for antibody recognition using Dot Blot. By using the afore-described methodology a clear recognition of the antibodies for each of the genotype extracts was achieved and evaluated. In addition a good staining (concentration) of the polyclonal antibodies originating from each genotype was observed, owing to the high intensity of each marker (Figure 4.14).

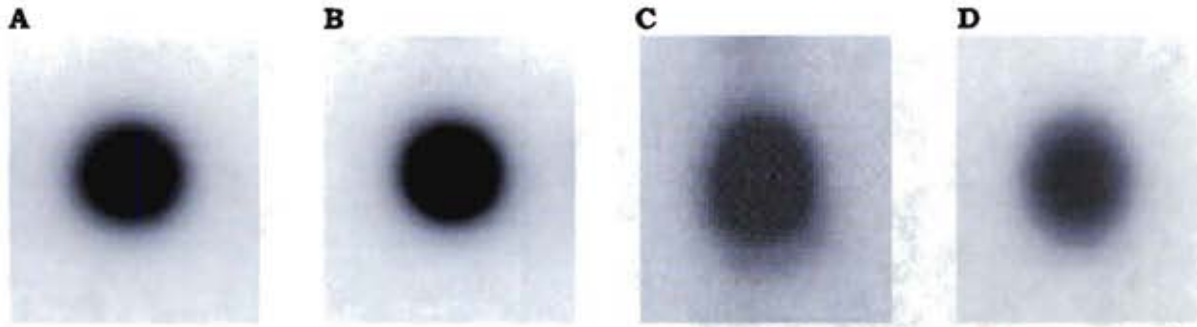


Figure 4.14. Test for antibody recognition using the Dot Blot technique. A: antibodies against MEcu 72, B: antibodies against MEcu 64, C: antibodies against MPer 334, D: antibodies against CMC 40.

These results indicate that the process for immunization and production of the antibodies using the described procedures was successful; therefore it is possible to continue with the cross-tests for immunodetection of proteins for both the varieties being evaluated, as well as for *A. socialis*.

SDS-PAGE Electrophoresis

It was determined that the protein sample concentration that best provides a good visualization of the bands is 2mg/ml. This concentration provided for well defined bands without vertical streaking of protein, as occurred with the other concentration evaluated (Figure 4.15).

The protein concentration that gave adequate results for the resolving phase by providing good visualization of the protein bands was 14% (Figure 4.15). With the other concentrations the distribution of the bands along the gel were not uniform and very congested on the lower part of the get at the 10% concentration, while they were congested at the top of the gel at the 17% concentration.

In Figure 4.15, polymorphic bands can be observed between the resistant and susceptible genotypes, with molecular weights between 47.5 and 35 Kd. A common polymorphic band is clearly noted in the resistant genotypes (black arrows), although it is less intense for MPer 334. The genotype MEcu 64 shows a high polymorphism as well as an additional band that is absent in the other genotypes (yellow arrow). The yellow circle on Figure 4.15, indicates the absence of these aforementioned protein bands on the susceptible genotype, CMC 40. These results are a good indication that these protein immunodetection tests should be continued on these genotypes; the differences shown between the resistant and susceptible genotypes are a good indication that a relationship may exist between these proteins and the presence of resistance to *A. socialis*.

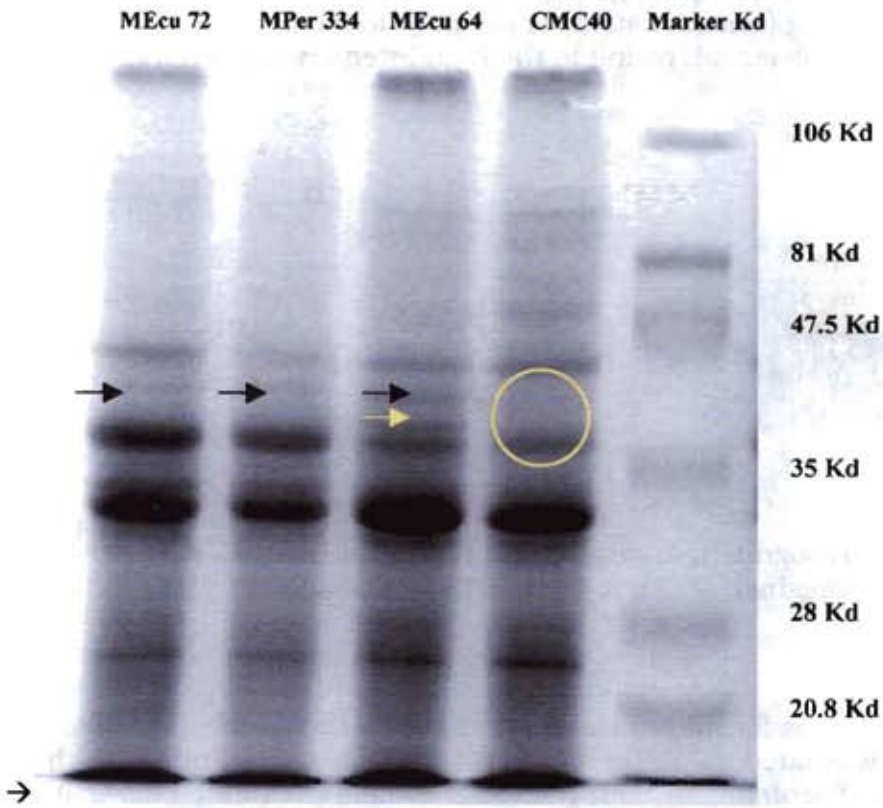


Figure 4.15. SDS-Page. Phase resolving concentration of 14%, Sample concentration of 2 mg/ml. The black arrow indicates the polymorphic band commonly present in the resistant genotypes and absent in the susceptible, CMC 40, indicated by the yellow circle. The yellow arrow shows an additional polymorphic band that is only evident in the resistant genotype MEcu 64.

First immunization test with healthy plants (no whitefly infestation) using the Western Blot Technique

Antibody specificity obtained from MEcu 64 with proteins originating from the same genotypes (B pool 3) can be observed in Figure 4.16. The four bands located in different positions, as indicated by the arrows, can only be observed in this genotype and are absent in the control (CMC 40) and the other resistant genotypes. These results support those obtained from SDS-PAGE electrophoresis, indicating that this genotype is markedly different than the other genotypes and this could be related to whitefly resistance.

In general, common bands, for all of the genotypes combined with all of the antibodies can be observed. This indicates common proteins, be they structural or functional, in the genotypes.

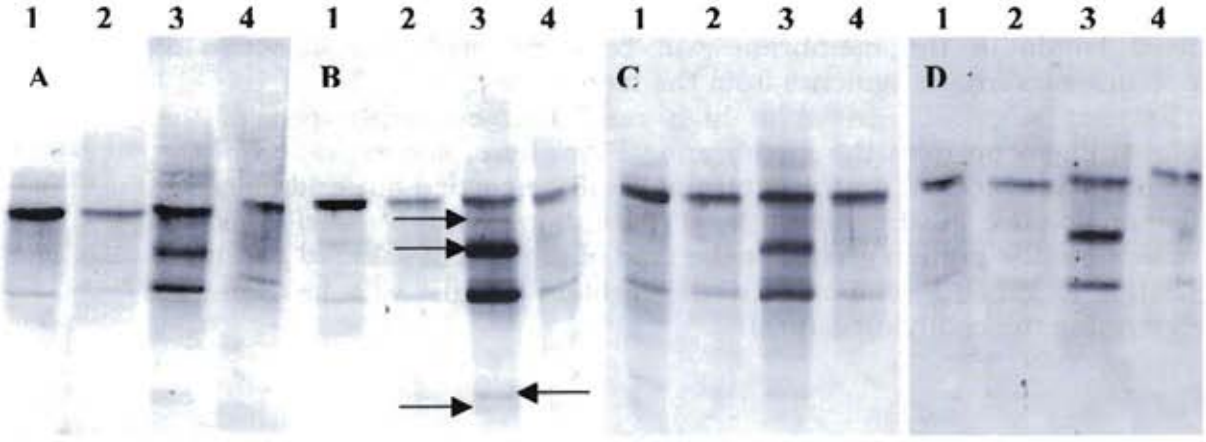


Figure 4.16. Immunodetection of healthy genotypes (non whitefly infested) using the Western Blot Technique. 1: MEcu 72, 2: MPer 334, 3: MEcu 64, 4: CMC 40. A: developed with antibodies of CMC 40; B: developed with antibodies from MEcu 64; C: developed with antibodies of MEcu 72; D: developed with antibodies from MPer 334. The black arrows signal the polymorphic bands of MEcu 64 that are absent in the other genotypes.

Projections

Present results indicate a distinct difference in the proteic behavior of at least one of the genotypes when free of whitefly (*A. socialis*) infestation. Therefore the following processes and activities are suggested:

- Development of a well defined proteic profile for each of the genotypes using more sensitive techniques, such as silver staining. This profile would be used as a reference for the protein behavior of each genotype in the absence of whitefly infestation and utilized for future comparisons.
- Obtain a proteic profile for whiteflies feeding on susceptible plants using more sensitive techniques. This profile would also be used as a protein behavior reference for whiteflies feeding on non-resistant genotypes.

- Determine the proteic profiles for resistant genotypes infested with feeding whiteflies, and compare these to the previously obtained profiles. Differences in the proteic activity would be established for the presence of the whitefly as well as the interaction with genotype resistance.
- Determine, through immunodetection, what proteins associated with each genotypes are found in the whiteflies feeding on them. This would provide a direct relationship with the proteic activity in whitefly resistant genotypes.
- Perform SDS-PAGE preparations of total proteins of the genotypes and whiteflies, especially where differences in the initial proteic profiles have been detected and corroborated through immunodetection; thereby extracting different bands, concentrating them in a gel and carry out an electro blot on the membrane.
- Once the electro blot is conducted on the membrane, digestion of the fixed bands in the membrane will be done, with the objective of obtaining internal fragments from the membrane.
- The next step will consist of high resolution electrophoresis of the eluted digestion from the membrane. From here, sequenciation of the amino acids blocked on the N terminal will be carried out and a search for analogues in the amino acid data bank will be performed to determine the protein group, or the type of protein, of selected bands.
- Lastly, a genetic sequence of the protein bands will be done to determine the codifying gene(s).

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5. Wild *Manihot* species source for resistance to artropod of cassava.

5.1 Introduction

Several cassava arthropod pests will significantly reduce root yield. Emphasis is given to two complementary systems, host plant resistance and biological control, for the effective environmentally sound and low cost methods of controlling cassava pests. Different levels of resistance to cassava pests have been identified within *M. esculenta*. For example resistance to mealybugs, lace bugs, stemborer and burrower bugs (within low HCN varieties) is very low; resistance levels to mites is low to moderate; resistance to thrips and whiteflies is moderate to high, while no resistance to hornworms and white grubs have been identified. Wild *Manihot* species are a wealth of useful genes for the cultivated species *Manihot esculenta* Crantz but their use in regular breeding programs is restricted by the long reproductive breeding cycle of cassava and linkage drag associated with the use of wild relatives in crop improvement.

5.1. Wild Manihot species as a source of resistance to cassava whiteflies, mites and mealybugs.

Wild relatives of cassava are important sources of genes for resistance to pest and diseases and longer shelf life. This "source" of resistance genes has been exploited for the control of Africa Cassava Mosaic Disease (ACMD) in Africa. ACMD resistance was obtained by intercrossing cassava varieties with *Manihot glaziouii* and other species of *Manihot*. Interspecies hybrids were backcrossed to cassava and this resulted in varieties highly resistant to ACMD. This research was initiated in the 1930's and 1940's, when modern biotechnology tools and information were not available.

The only source of dramatically delayed PPD has been identified in an inter-specific hybrid between cassava and *Manihot walkerae* (CIAT 2003), a unique source of resistance to the cassava hornworm was also identified in 4th backcross derivates of *M. glaziovii* (Chavarriaga et al 2004). This work reports preliminary results of a study directed to identify useful genes for pest and disease resistance in wild species of *Manihot* and to develop low cost marker tools for their rapid introgression into cassava.

In a first phase of the project, evaluations of two Brazilian wild species (*M. flabellifolia* y *M peruviana*) (Mueller) and commercial genotypes of *M. esculenta* were conducted in a screen house to identify levels of resistance to the mite, *Mononychellus tanajoa*, the mealybug, *Phenacoccus herreni* and, the whitefly *Aleurotrachelus socialis* Bondar (Figure 5.1). In addition transgenic cassava genotypes (Africa: TMS 60444) were tested for resistance to the cassava hornworm, *Erinnyis ello*.

Additional studies were conducted to develop a quick method to detect whitefly resistance, based on the number of eggs oviposited per female on a specific genotype. This will be a useful tool to evaluate resistance/susceptilbility in a great number of progenies from interspecific crosses between wild *Manihot* species and *M. esculenta* genotypes.

Figure 5.1. A: *Mononychellus tanajoa*, B: *Phenacoccus herreni* and C: *Aleurotrachelus socialis*

Materials and Methods

Screening for Natural Resistance

Plants of the genotypes CMC-40, MECU-72 from *M. esculenta*; MFLA 444-002 from *M. flabellifolia* y MPER 417-0003, MPER 417-005 from *M. peruviana*, were propagated. Four, 40 day old plants of each genotype, were placed individually in a fine nylon mesh screened cage. Infestations of *M. tanajoa*, *P. herreni* and *A. socialis*, obtained from greenhouse/ screenhouse colonies were introduced on individual plants in the following manner:

- Upper leaves of the five genotypes were infested with 200 *M. tanajoa* mites.
- *P. herreni* ovisacs were placed in the axil of upper plant leaves. First damage/population evaluation were made 10 days after infestation and continued every 10 days for eight weeks.
- The five genotypes were infested with 200 recently emerged (12h) *A. socialis* adults. Evaluations were initiated after five days and continued every ten days, for eight weeks.
- 4. Population and damage scales were employed for evaluating each of the pest species. These scales are based on a 1 to 6 rating where 1 indicate no pest population and no plant damage; while 6 indicate very high pest population (I.e. for whiteflies 6=> 4.000 nymphs pupae per leaf) and severe damage.

Rapid Selection Method for A. socialis

Ten plants of progenies from the interspecific cross of *M. esculenta* x *M. flabellifolia*, CW235-72, CW259-3, CW259-43, CW257-10, CW258-17, CW259-10 and commercial cassava variety, CMC-40 were sown in plastic pots. Five plants of each genotype, at 40 days after germination, were placed in nylon mesh cages (1mx1mx1m) for whitefly (*A. socialis*; obtained from CIAT reared colonies) infestation.

Ovipositional preferences was determined by introducing ten pair of recently emerged *A. socialis* adults (reared on CMC-40) into small leaf cages (2.5 cm diameter x 2.0 cm depth) attached to the underside of upper leaves of each genotype (Figure 5.2.A). After five days adultos were removed and the number of eggs oviposited recorded (Figure 5.2.B). Eggs were allowed to hatch and nymphs allowed to develop for ten days in order to estimate development to the third instars (Figure 5.2.C).

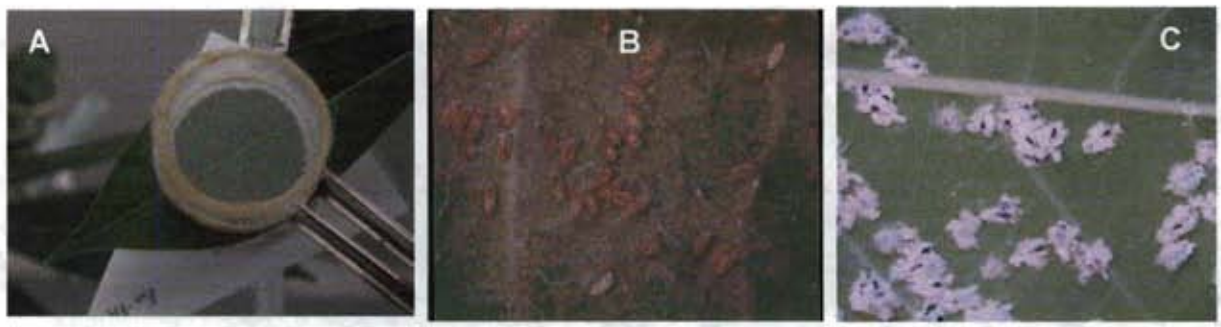

Figure 5.2. A: Small leaf cages B: Eggs and C: Nymphs

Results:
M. tanajoa: The mite infestation on the *M. flabellifolia* genotype (MFLA 444-002) was different from two *M. esculenta* genotypes (MECU-72 and CMC-40) but not from *M. peruviana* (MPER 417-005) (Tukey P<0.005). Leaf damage on MFLA444-002 was significatly different when compared with the *M. esculenta* genotypes, but similar to the two *M. peruviana* genotypes (Figure 5.3).

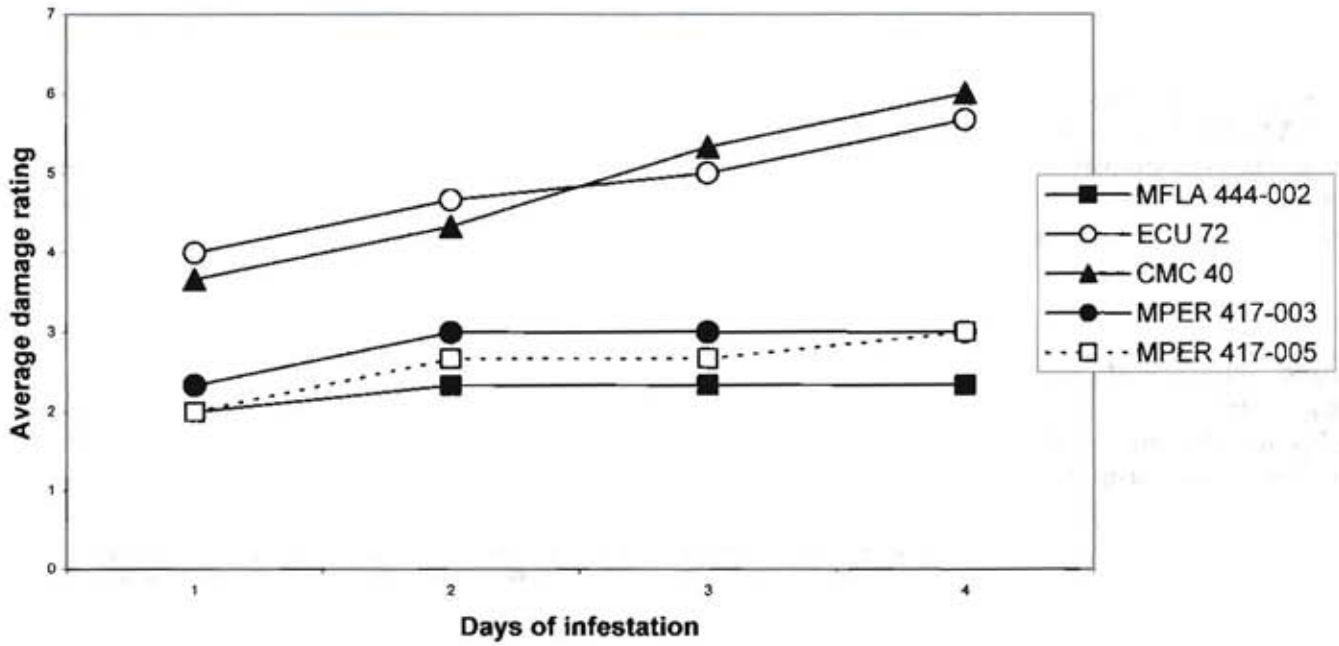

Figure 5.3. Mite (*Mononychellus tanajoa*) Damage rating on wild *Manihot* genotypes and commercial cultivars during a 25 day sampling period

P. herreni: MPER417-003 and MFLA 444-002 presented the lowest mealybug infestation levels (Figure 5.4). MPER417-003 had the lowest damage levels, suggesting possible resistance to this species.

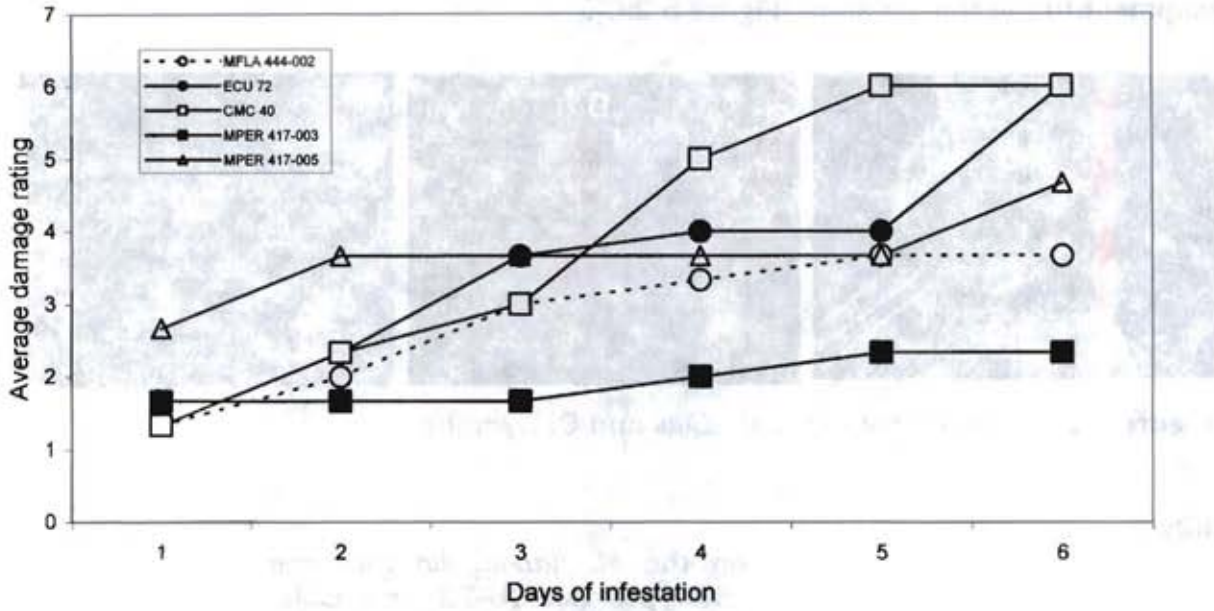

Figure 5.4. Mealybug (*Phenacoccus herreni*) damage ratings on wild *Manihot* genotypes and commercial cultivars (CMC-40, MEcu-72) during a 60 day infestation

A. socialis: Accessions from the wild *Manihot* species, MPER417-003, MPER417-005 and MFLA444-002all resulted in highly significant damage and population differences from the *M. esculenta* genotype CMC-40 (Tukey P<0.005). These results indicate a high degree of resistance in the wild genotypes. MECU-72 was previously selected as resistant to *A. socialis* and this resistance is confirmed in these results (Figure 5.5).

Rapid Selection Method for A. socialis

Development of *A. socialis* nymphs on cassava (interspecific progeny) genotypes displayed a behavior similar to the ovipositional rates on these genotypes. The genotype CW235-72 had the least development of nymphal stages, owing to very low ovipositional rates. CMC-40 and CW259-43 displayed the highest percentage differences (42% and 30% respectively) in nymphal development when compared to the initial ovipositional rates (Fisher's P<0.05). The genotypes CW235-72 and CW257-10 showed the least differences (0% and 17% respectively) between oviposition and nymphal development.

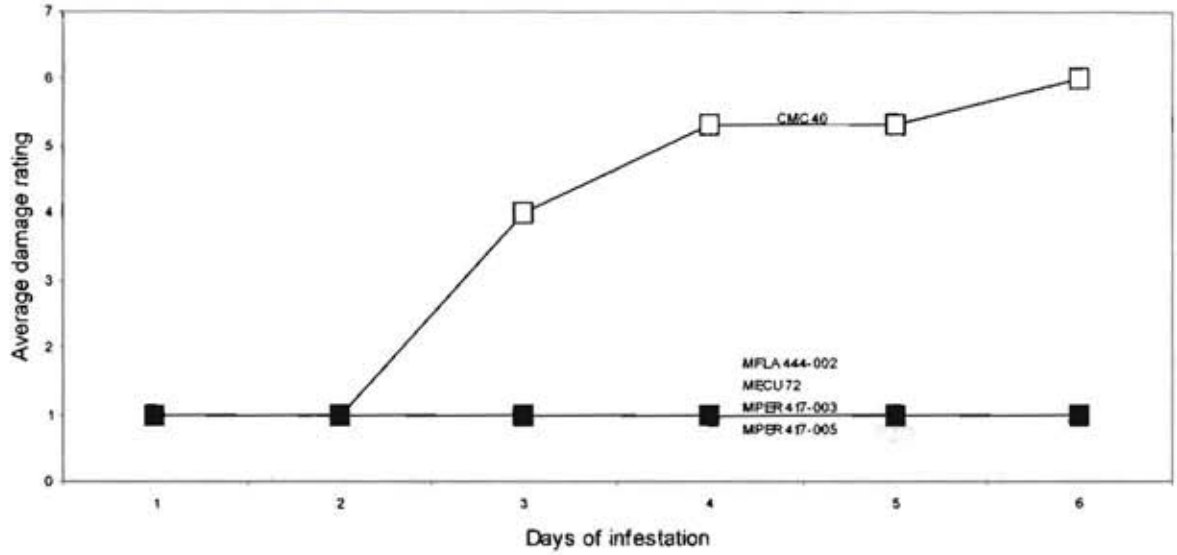

Figure 5.5. Whitefly (*Aleurotrachelus socialis*) damage ratings on the wild *Manihot* genotypes (MFLA 444-002, MPER 417-003) and *M. esculenta* cultivars CMC-40 and MEcu-72 during a 55 day infestation.

A regression analysis was performed in order to obtain a correlation between oviposition and nymphal development (3rd instar) of *A. socialis* on progeny of *M. esculenta* x *M. flabellifolia* (Figure 5.6). Results show a 87% correlation in development of the nymphal stages and number of eggs oviposited. It can therefore be concluded that number of eggs oviposited on a given genotype can be used as an indicator of the preference/resistance of *A. socialis* for that genotype.

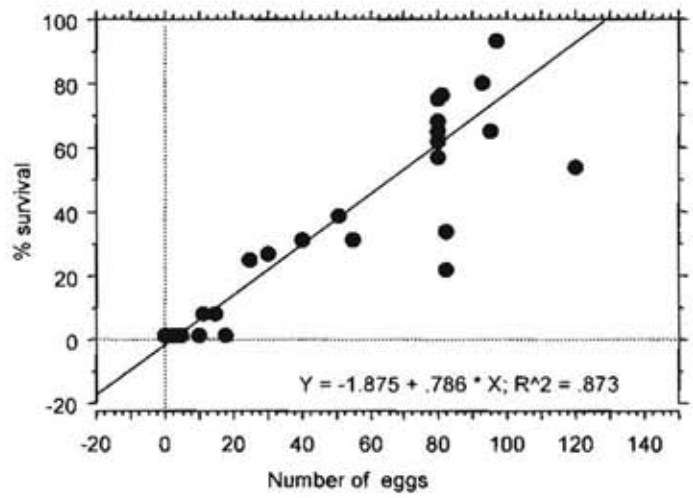

Figure 5.6. Correlation between oviposition and nymphal survival of the whitefly, *Aleurotrachelus socialis* on progeny of *M. esculenta* x *M. flabellifolia*

Conclusions and Ongoing Research

- The wild species genotypes MFLA444-002, MPER 417-003 and MPER417-005 displayed intermediate level of resistance to *M. tanajoa* and high levels of resistance to *A. socialis*.
- The genotypes MFLA 444-002 y MPER 417-003 showed moderate levels of resistance to *P. herreni*.
- The ovipositional rate (No. of eggs) of *A. socialis* on a given genotype is a good indication of the level of resistance of determined genotype.
- A project to develop low cost marker tool for accelerated marker-aided introgresion of useful genes into cassava gene pools in being funded under the GCP(Generation Challenge Programme) with participation of IARCs (CIAT, Cali, Colombia) and NARS in Brazil (CNPMF/ EMBRAPA Cruz das Almas, Bahia) and in Africa in Ghana (CRI, Kumasi), Nigeria (NRCRI, Umadike) and Uganda (NAARI, Namulonge).

Collaborators:
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5.2. Transgenic cassava (Africa genotype TMS 60444) as a source of resistance to the cassava hornworm, Erinnyis ello

Erinnyis ello, the cassava hornworm, is one of the most serious cassava pests in the neotropics (Bellotti et al, 1992). It has a broad geographic range, extending from the southern cone (Brazil, Argentina and Paraguay) of South America to the Caribbean Basin and southern USA. Hornworm larval feeding will defoliate cassava plants causing considerable yield reductions, especially if repeated attacks occur. Based on extensive research of this pest by CIAT and NAR's scientists an IPM program for hornworm control has been developed. The basis of this program is centered around biological control, especially the use of a baculovirus that has recently been developed as a commercial biopesticide (CIAT Annual Report, Project PE-1, 2002 and 2003).

The CIAT cassava germplasm bank consists of nearly 6,000 genotypes. Most accessions are traditional land race cultivars collected from farmers' fields. A high (60 to 70%) percentage of genotypes in this germplasm bank are consistently being grown in the field and subject to pest attack. Periodic evaluations of these genotypes when hornworm attacks have occurred have indicated that genetic resistance to *E. ello* is not available in cultivated cassava, *Manihot esculenta*.

Recently CIAT initiated research based on introducing insect resistant *Bacillus thuringiensis* (Bt) genes (Cry 1Ab) through *Agrobacterium*-mediated transformation into cassava embryonic tissue to develop lepidopteran resistant cultivars. Transgenic plants of the model variety of African origin, TMS 60444 (MNg 11) have been developed. This genotype is the

progeny of an interspecific cross of the wild species *Manihot glaziovii* and *M. esculenta*. *M. glaziovii* is also the source of resistance to ACMD (African Cassava Mosaic Disease), and in preliminary evaluations at CIAT has displayed resistance to other pests such us whiteflies. TMS 60444 was selected because of its high transformation capacity and relatively rapid regeneration (Bellotti et al, 2002).

The objective of this study was 1, to determine the leaf consumption rate of the cassava hornworm, *E. ello*, on different genetically modified lines the variety TMS 60444. and 2, to quantify the effect of the *Gen* Cry 1Ab in transgenic lines on the behavior and feeding of the cassava hornworm.

Materials and methods

Hornworm larvae were obtained from the laboratory/field colony maintained at CIAT. The cassava variety CMC-40, a susceptible genotype was grown out in farmers and CIAT fields. TMS 60444, non-modified genetically and resistant to the hornworm was grown at CIAT. The genetically modified lines L27, L80 and L92, originally from TMS 60444 were produced at CIAT.

The cassava hornworm, *E. ello*, colony is maintained by placing adults (male and females) in large field cages (2m x 2m x 2m) where females can readily oviposit on growing cassava plants. Eggs are removed to the laboratory where larval instars (5) develop in cages while feeding on cassava leaves. Recently emerged first instar larvae were used in all experiments; the first leaves fed-upon were those of each respective treatment.

The experiment had six treatments and twenty replications per treatment. The experimental arena was a plastic petri dish (15mm x 2.5mm) that contained excised cassava leaves. One first instar *E. ello* larvae was introduced into each petri dish and allowed to feed on the cassava leaf. All larvae were weighed on an analytical balance prior to being placed in the petri dish. It was therefore possible to record any weight gain or loss during the larval feeding period. Larvae were weighed every 24 hours and cassava leaves were replaced on a daily basis, until pupation or larval mortality occurred. Chi square analysis was used to evaluate mortality vs. variety (treatment).

Results

Hornworm (*E. ello*) mortality reached 100% on the transgenic lines L80, L92, and 85% mortality on L27 (Figure 5.7). On the latter 15% of the larvae reached the prepupal stage. Mortality on the non-modified control variety, CMC-40 was 25%. Mortality on the non-modified variety, TMS 60444, was 100%. The Chi square test showed that the mortality was independent of the genotype.

Peak mortality on the transgenic lines and non-modified TMS 60444 occurred during the first 3 to 5 days of larval development. Larval mortality on the susceptible control, CMC-40, first occurred at 6.6 days. There were no statistical differences between the transgenic genotypes and TMS 60444, but all four genotypes were statistically different from CMC 40.

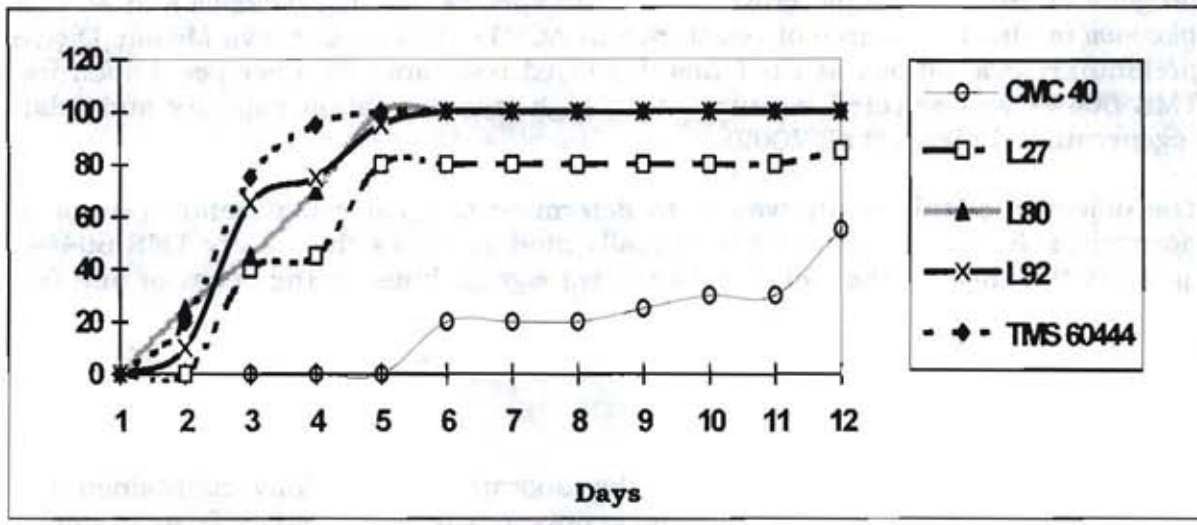

Figure 5.7: Percent mortality of cassava hornworm (*Erinnyis ello*) larvae consuming leaves from Bt transformed (L27, L80, L92) and non-transformed (CMC 40, TMS 60444) cassava genotypes (CIAT, 2004).

5.3. Studies on the biology and behavior of biotype "B" of Bemisia tabaci on wild Manihot sp, M. flabellifolia

The wild species within the genus *Manihot* are seen as potential source of genes for resistance in the control of major cassava pests. There is a precedence for this in that resistance to CMD resulted from an interspecific cross between *M. esculenta* and *M. glaziovii*. However, apart from this one successful case, wild Manihot species have not been exploited as a source of resistance to cassava pests and diseases. The objective of this present study is to evaluate biological, population and demographic aspects of Biotype "B" of *B. tabaci* found in Colombia, on *Manihot flabellifolia*.

Materials and methods

Plantlets of *M. flabellifolia* were obtained from the CIAT Biotechnology Unit (Agrobiodiversity and Biotechnology Project, SB-2) where they were propagated in-vitro. These were transplanted to plastic bags or pots. Eight 40-day old plants were selected and placed in nylon mesh wooden frame cages (1m x 1m x 1m).

The source of *B. tabaci* whiteflies was a CIAT established colony being reared on *Jatropha gossypiifolia* (Euphorbiacea). These had been reared for 15 generations on *J. gossypiifolia* in nylon meshed wooden cages (1m x 1m x 1m) in the growth chamber (25±2°C, 70±5% RH, 12:12 photoperiod). The species quality (uncontaminated) of the *B. tabaci* colony is periodically verified through RAPD-PCR testing of adults.

Longevity and fecundity were evaluated by placing 40 recently emerged adult pairs (40 males + 40 females) of *B. tabaci* from the *J. gossypiifolia* colony, in small clip cages (1.5 cm diameter + 2.0 cm depth) (one pair per cage), on the underside of *M. flabellifolia* leaves. Adults were removed every 48 hours to a different site on the leaf; this procedure was repeated until the natural death of the females. Fecundity was estimated by counting the number of eggs oviposited every 48 hours by each female, while longevity was estimated based on the number of days that females survived.

Development time, survival and female/male ratio was estimated by placing 50 two day old adults (males and females) removed from the *J. gossypiifolia* colony, in round clip cages (2.5 x 2.0 cm) on the underside of *M. flabellifolia* leaves. After six hours, adults were removed and 200 eggs were randomly selected.

Demographic parameters were calculated by combining data on development time and reproduction (l_x-m_x), generating life tables: 1) net reproduction rate (R_o), the average number of females that one female produces in one generation; 2) generational time (T), equal to that period between birth of the parents and of the progeny and 3) intrinsic rate of increase of the population (r_m), estimated using Carey's formula (1993),

$$\Sigma \exp(-r_m x)\, l_x m_x = 1$$

where x is the age of the female in days, l_x, the age of species survival, and m_x, the proportion of female progeny of one female at age x.

Results

Results show a range of *B. tabaci* female survival of 2 to 8 days when feeding on *M. flabellifolia*, with an average of 3.5 days (Table 5.1). An average of 3.3 eggs (range 1-16 eggs) were oviposited per female. Ninety percent of female initiated oviposition during the first 48 hours and by the 4th day, 87% of oviposition had occurred.

Table 5.1 Average longevity, average fecundity and rate of oviposition (eggs/female/2 days) of biotype "B" of *Bemisia tabaci* feeding on *Manihot flabellifolia* in the growth chamber.

Parameter	***M. flabellifolia***
Average longevity	3.5
Range	2-8
No. Insects	40
Average fecundity	3.3
Range	1-16
Average Oviposition rate	0.98
Range	0.25-4

Development time of *B. tabaci* (biotype B) individuals feeding on *M. flabellifolia* was 47.2 days (Table 5.2). The proportion of females was 50% and survival 8%.

The net reproduction rate (Ro) estimates that *B. tabaci* population will increase three fold during one generation (Table 5.3). *B. tabaci* will complete one generation in 48 days feeding on *M. flabellifolia*, resulting in seven generations in one year. In addition the r_m value indicates a 77% population decrease when compared to the reproductive rate of *B. tabaci* on its original host, *J. gossypiifolia*. Feeding on *M. flabellifolia*, *B. tabaci* requires 31 days to duplicate its population, compared to only 25 days on *J. gossypiifolia* (Carabalí, 2004).

Table 5.2 Development time, survival and proportion of females of Biotype "B" of *Bemisia tabaci* feeding on *M. Flabellifolia* (n=200) in the growth chamber.

Parameter	Values
Development time (days)	47.2
Rate of survival (%)	8
Proportion of females (%)	56

Table 5.3 Demographic parameters of individuals of biotype "B" of *Bemisia tabaci* feeding on *Manihot flabellifolia* (n=200) in the greenhouse.

Parameter	**Values**
Net reproduction rate (Ro) $\sum lxmx$	3.0
Generation time (T)	48.3
Intrinsic rate of increase (r_m)	0.0222
Days to duplicate population Ln2/rm	31.2

These results indicate that the wild *Manihot* species are a potential source of whitefly resistance genes and in particular a resistance source to biotype B of *B. tabaci*.

Contributors Arturo Carabalí, Adriano Muñoz, Anthony C. Bellotti.

5.4 References

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Carey, J.R. 1993. Applied demography for biologist. Nueva York. Oxford University Press. 206p.

6. Identification and Introgression of Useful Traits from Wild *Manihot* Relatives of Cassava

6.1 Introduction

Wild *Manihot* germplasm are a wealth of useful genes for the cultivated species *M. esculenta.* Several accessions of *M.esculenta* sub *spp flabellifolia, M.esculenta* sub spp *peruviana* and *M.tristis* collected in Brazil were found to have high protein content, between 10-18% (dry weight basis,) in the storage roots (CIAT 2003), dramatically delayed PPD has also been identified in an inter-specific hybrid between cassava and *Manihot walkerae* (2003). One of the major constraints of cassava production is post-harvest physiological deterioration (PPD), cassava roots generally start to deteriorate 24 – 48 hours after harvest, it is estimated that around 20% of annual total production in Africa (18 million tons of fresh roots) valued at US$ 480 million is lost by farmers, consumers and processors (FAO, 2002). In addition, a unique source of resistance to the cassava hornworm was identified in 4th backcross derivatives of *M. glaziovii* (Chavariagga et al. 2004) and moderate to high levels of resistance to white flies and green mites have been found in inter-specific hybrids of *M. esculenta* sub *spp flabellifolia* (CIAT 2004). An inter-specific cross between *Manihot tristis* and cassava produced several hybrids with very high dry mater content (DMC), over 50%.

But the use of wild relatives in regular breeding programs is complicated by the long reproductive breeding cycle of cassava, high genetic load that is released on backcrossing, and linkage drag associated with the use of wild relatives in crop improvement. A project was initiated at CIAT to accelerate the process of introgressing useful genes from wild relatives into cassava via a modified Advance Back Cross QTL (ABC-QTL) (Tanksley and Nelson 1996) breeding scheme. We describe here advances in the last 3 years in the introgression of high protein content, resistance to green mites, delayed post harvest physiological deterioration (PPD), and high dry matter content (DMC) from wild *Manihot* species into cassava.

6.2 Materials and Methodos

Proteins

An advanced back cross QTL (ABC-QTL) scheme to introgress high protein content from wild relatives into cassava is in its fifth year at CIAT. During the first year wild relatives of cassava collected in Brazil (CIAT 1995) were evaluated for high protein content using the Kjedahl method and in the second year genetic crosses were made between selected high protein genotypes and some improved elite parents, including some high beta-carotene varieties, were made. Wild by wild crosses were also carried out to investigate if combining favorable alleles from different populations or species of the wild accessions can further increase protein content. The inter-specific hybrids were evaluated for protein content in the third year, amino acid analysis of a few wild *Manihot* genotypes and inter-specific hybrids was also carried out. In the fourth year, selected inter-specific hybrids were backcrossed to elite cassava parents, the selection criteria used was high protein content in the F_1

individual and family. A total of 1,575 back crosses between the inter-specific hybrids CW 198 – 11, CW 205 – 2, CW 201 – 2 with MTAI 8 were made and 1,821 seeds obtained Of this number, 1,113 seeds were viable and germinated *in vitro* from embryo axes according to standard procedures in the cassava tissue culture laboratory at CIAT (CIAT 2003). The plantlets derived from embryo axes were multiplied, over 2-3 cycles, to produce 8-10 plants for screen house hardening. The plantlets were hardened in the screen house according to standard methods (CIAT 2003) and then transferred to a single row trial in the field at ICA-Corpoica. They are 5months old now and will be evaluated for protein content at 10 months after planting.

We also examined the use of SDS-PAGE gel analysis as a quick way to measure protein in the inter-specific hybrids. Root flour of 11 inter-specific hybrids (Table 6.1) having 6-8% of crude protein was evaluated using SDS-PAGE. The samples were evaluated using a saline buffer extraction protocol described by Jorgensen (2005, personal communication) with some modifications. Six different saline buffers were used (Table 6.2), a range of sample sizes between 40 mg and 500 mg; and two loading buffer; blue juice (CIAT 1996) and cracking buffer (Laemmli, 1970), were also tested to identify the most suitable. Samples were suspended in of 200 µl saline buffer, spun for 30 min at 15000 rpm in a table top centrifuge, and the supernatant carefully transferred to new tubes while avoiding the pellet as much as possible. The supernatant was spun for 10 min at 15000 rpm and the appropriate loading buffer (blue juice or cracking buffer), in different concentrations –1:1, 2:1, and 3:1 was added. Proteins were completely denatured by immersing the sample tubes in a boiling water bath for 5 minutes, the samples were briefly centrifuged at 14000 rpm for 5 min to pellet cellular debris and 12, 15 or 20 µl of supernatant loaded on the SDS-PAGE gel. SDS – PAGE denaturing electrophoresis was according to Laemmli (1970) as modified by CIAT (2003). Constant voltage of 180 V, 80 mA and 50 W, at 10 °C were applied until the tracking dye reached the bottom of the gel. A *Phaseolus vulgaris* seed protein sample was used as positive control in every gel assay. After electrophoresis, gels were stained over night at room temperature in a solution containing 50% Methanol, 10% Acetic acid and 0.25% Coomassie Brilliant Blue R. Gels were washed with water and de-stained by two changes of de-staining solution, first with 50% Methanol and 10% Acetic acid, and secondly with 5% Methanol and 7% Acetic acid.

Table 6.1 Inter-specific hybrids, and their percent protein content, selected for SDS-PAGE analysis

	Genotype	Maternal parent	Paternal parent	P.C. (%)		Genotype	Maternal parent	Paternal parent	P.C. (%)
1	CW 178-1	OW 132-2	CW 48-1	6.39	34	CW 177-1	OW 132-2	CM 1585-13	7.19
2	CW 178-2	OW 132-2	CW 48-1	6.44	35	CW 177-2	OW 132-2	CM 1585-13	6.55
3	CW 179-1	OW 132-2	MTAI 8	7.02	40	CW 177-7	OW 132-2	CM 1585-13	6.84
4	CW 179-2	OW 132-2	MTAI 8	7.86	42	CW 177-9	OW 132-2	CM 1585-13	6.81
8	CW 179-6	OW 132-2	MTAI 8	6.72	43	CW 177-10	OW 132-2	CM 1585-13	7.43
14	CW 179-12	OW 132-2	MTAI 8	7.8	61	CW 177-28	OW 132-2	CM 1585-13	6.7
23	CW 205-4	OW 231-3	MTAI 8	7.62	70	CW 177-37	OW 132-2	CM 1585-13	7.73
24	CW 205-5	OW 231-4	MTAI 9	7.25	84	CW 177-51	OW 132-2	CM 1585-13	7.05
28	WW 3-1	OW 132-2	OW 240-6	7.31	87	CW 177-54	OW 132-2	CM 1585-13	7.17
31	WW 3-4	OW 132-2	OW 240-6	6.33	89	CW 177-56	OW 132-3	CM 1585-14	6.77
33	WW 3-6	OW 132-2	OW 240-6	7.32					

Resistance to Green Mites

Following a very dry spell in January 2002 at CIAT Palmira and a subsequent heavy incidence of the green mites, 4 inter-specific hybrid families, CW68, CW65, CW67, and CW66, from a cross between cassava and *Manihot esculenta sub spp Fabellifolia* accession, were observed to show good resistance to cassava green mites (CGM). An attempt was made to introgress the resistance into cassava, by crossing selected inter-specific hybrids back to cassava concurrent to continued evaluation in the field. The green mite pest tends to attack in a localized manner and homogenous pest pressure in the field is uncommon therefore there is a need to evaluate plant materials over several years to avoid escapes due to low pest pressure. Figure 6.1 describes the scheme for the generation of inter-specific hybrids and backcross populations and the multi-stage evaluation of resistance to cassava green mites as well as perspectives for the development of molecular markers associated with resistance.

Table 6.2 Different saline buffers sample preparation for SDS-PAGE analysis of protein in root of inter-specific hybrids.

Saline buffers	**Reference**
NaCl 0.5 M pH 3.2, cracking 30 mg	Laemmli, 1970
Na_3PO_4 0.05M pH 7.0, PVP 2%	Jasso et al. 2002 (modificated)
KCl 0.1M, cysteine, pH 7.3	Bourdon, 1988
SDS 5%, glycerol 10%, Tris 80mM, DTT 25mM, pH 6.8	Carvalho. Et al, 2004
Na_3PO_4 0.05 M	Carvalho, 1992
Na_3PO_4 0.05M, PVP 2%, acetone wash, pH 7.0.	Rodriguez et al, 2002

The evaluation of green mite resistance is based on a severity of the damage scale with values between 1 - 6 where 1 is total absence of damage and 6 severe damage with complete leaf necrosis and apical defoliation. Evaluation was conducted by the cassava entomology group directed by Jose Maria Guerrero. In order to identify markers associated with resistance to CGM two sets of plant materials were employed: the BC_1 families, that have been evaluated over three cycles, and newly produced BC_2 (AR) families derived from crossing CGM resistant BC_1 individuals to CMD resistant parental lines, the resistant BC_1 individuals and AR families were planted in a single row or advanced yield trial (replicated) this year and will be evaluated January to March 2006. Genotypes with symptom damage of less than 3 will be bulked, resistant bulk, and those with scale 4-6 will be constituted into the susceptible bulks, bulks will be made on a family basis. The bulks will be evaluated with the 850 SSR markers available for cassava to select polymorphic markers, which will then be evaluated in the progeny to identify markers associated with CGM resistance.

Delayed Post Harvest Physiological Deterioration

The source of delayed PPD is an inter-specific hybrid CW429-1 obtained by crossing *Manihot walkerae* with cassava. This F_1 hybrid and 8 elite cassava genotypes namely: MCOL 1505, MPER 183, MTAI 8, CM523-7, HMC-1, MBRA 337, MCOL 2279 and CM 2772-3, having the widest variation for delayed PHD in the cultivated gene pool, were evaluated to compare the

novel source from the wild relative and known levels of PPD in the cultivated gene pool. Healthy commercial-sized roots of these plants were harvested 12 months after planting from the field with care to avoid physical damage. These roots were washed with tap water and disinfected with 1% hypochlorite and 50% ethanol for 10 minutes to prevent or reduce microbial growth. PPD quantification was done using the method of Wheatley et al. (1985) with a slight modification. Three evaluations were made on the 5th, 10th and 15th day after harvest (DAH) with 5 roots each. Roots were kept in a controlled environment chamber at 24-26°C and 60-80% relative humidity before PPD quantification. The proximal and distal root ends were cut off and a cling film was used cover the distal end. At each evaluation, seven 2-cm thick transversal cuts were made all along the root from the proximal end. A score, corresponding to the percentage of cut surface showing discoloration, of 1-10 (1=10%, 2=20%, etc.), was assigned to each slice. The mean PPD score for each root was calculated by averaging the score from 7 slices.

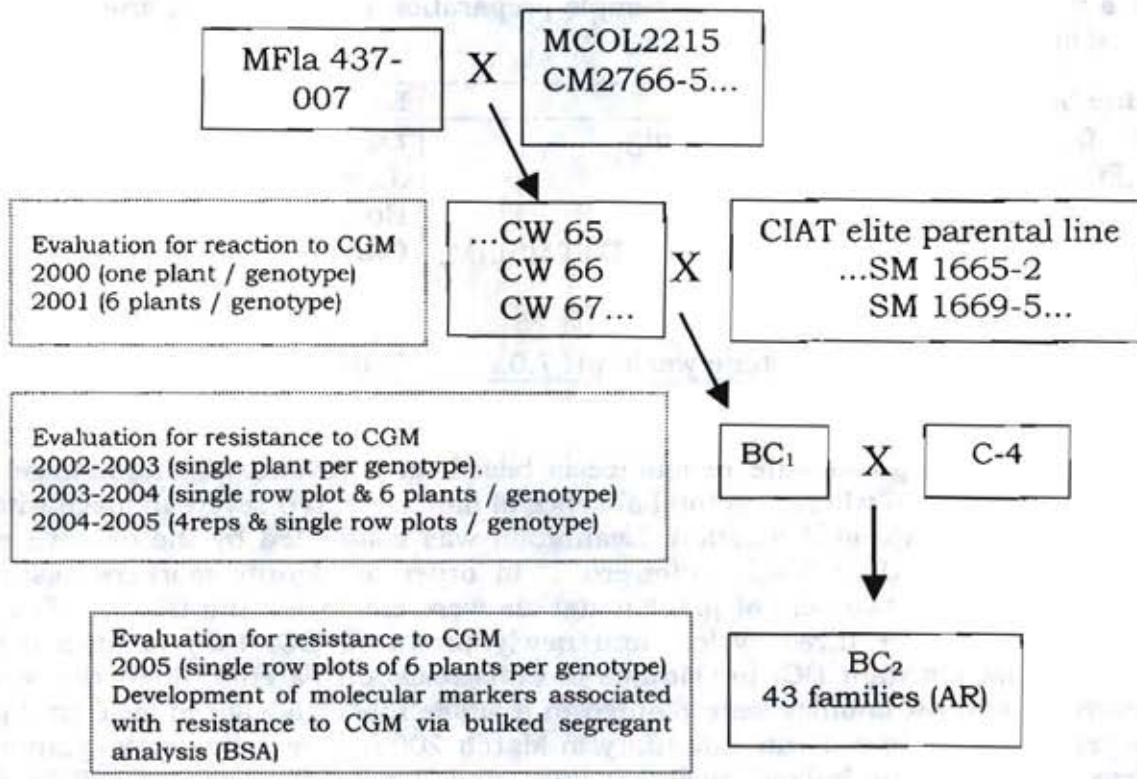

Figure 6.1 Pedigree of plant materials used for the introgression of CGM resistance from wild progenitors of cassava and evaluation of resistance

Back cross and self-pollinated mapping populations, for the identification of molecular markers for genes controlling delayed PPD, have also been developed from the delayed PPD line CW429-1. This line was crossed extensively to the elite cassava genotypes MTAI8, CM523-7, and SM909-25 to create 3 BC_1 families (BC_1 only in the sense of crosses to cassava) or self-

pollinated to generate an S_1 family. About 900 seeds were germinated *in vitro* from embryo axes according to standard procedures in the cassava tissue culture laboratory at CIAT (CIAT 2003). The plantlets derived from embryo axes were multiplied over 2-3 cycles to produce 8-10 plants for screen house hardening. The plantlets were hardened in the screen house according to standard methods (CIAT 2003) and transplanted to a single row trial in the field at ICA-Corpoica.

Dry matter Content

Crosses between elite varieties and accessions of the wild progenitor of cassava *Mannihot tristis* were made in 2000/2001. The F_1 inter-specific hybrids were backcrossed to the elite cassava lines to obtain first backcross (BC_1) generations. The BC_1 were evaluated for dry matter and a family, CW 208, showed very wide segregation for dry matter content, below 20 to over 50%, this is maybe the highest variation in a single family found to date at CIAT (CIAT, 2004). Bulk segregation analysis (BSA) of DMC in the family CW208 was carried out using SSR markers as described earlier (CIAT 2003). DNA samples from all genotypes were extracted from 1g of oven dried (48h at 50°C) leaves using a mini-prep version of the Dellaporta *et al.*, (1983) protocol. Between 500µg to 1000µg of high quality DNA was obtained from each extraction and DNA sample diluted to 10ng/µl for PCR amplification. SSR analysis was as described earlier by Mba et al. (2001). The available 850 SSR markers were used to screen the bulks and polymorphic ones used to screen individuals within the bulks, promising markers were used to screen the whole population.

6.3 Results

Proteins

A total of 4,271 sexual seeds organized into 58 families were obtained from inter-specific crosses between high protein accessions of *M. esculenta* sub spp *flabellifolia, M. tristis* and elite parents of the cassava gene pool. An evaluation of root protein content was made on a sub-set of 579 genotypes, selected according to root size, and a summary of the results are shown in Table 6.3. Results reveal that some wild genotypes such as OW 231-3, 280-2, OW132-2, and OW284-1 have good general combing ability for root protein content. It was also observed that crosses between 2 wild parents, both high in protein (WW), had more uniform high root protein progenies, compared to wild by cultivated crosses, suggesting that a number of genes for protein content might be recessive or additive. Amino acid profile revealed very high amounts of arginine, about half the total amount of amino acids, and low levels of methionine and lysine in the roots, but high levels in leaves (Table 6.4).

A summary of the first back cross generation generated is showed in Table 6.5. A total of 1113 sexual seeds from 3 families were germinated *in vitro*, of these 675 genotypes, 225 per family were multiplied and transferred to the screen house for hardening (Table 6.6). The rest was kept *in-vitro* against any failure of the selected genotypes during hardening or field transfer. Between 4 and 6 plants of the 675 genotypes were successfully hardened in the screen house and 4,932 plants were transplanted to the field at Corpoica

Table 6.3 Descriptive statistics of % Protein, based on dried root basis, in 31 inter-specific families . Genotypes OW230, 231, 180, 181, 189, are accessions of M. *esculenta* sub spp *flabellifolia*, while Genotype OW280, 284, 132, 131, 146 are accessions of *M. tristis*

Family	Mother	Father	Size	Maximum	Minimum	Average	Std. Deviation
CW 205	OW 231-3	MTAI 8	8	10.96	5.37	8.11	2.80
WW 14- 51	OW 181- 2	OW 280- 2	58	10.49	4.39	6.42	1.47
WW 40- 1	OW 284- 1	OW 280- 2	76	10.46	4.04	6.28	1.26
WW 22- 3	OW 231- 3	OW 240- 8	82	9.72	3.19	5.37	1.21
WW 41- 9	OW 284- 1	OW 146- 1	41	9.70	3.26	6.21	1.51
CW 177-1	OW 132-2	CM 1585- 13	63	8.52	3.75	5.71	1.04
CW 161	CW 56- 5	OW 189- 1	3	8.03	5.37	6.64	1.29
CW 160- 1	CW 56- 5	OW 181- 2	3	7.86	4.63	6.06	1.64
CW 179-1	OW 132-2	MTAI 8	17	7.86	3.80	5.65	1.17
WW 24- 2	OW 231- 3	OW 280- 2	35	7.80	3.42	5.63	1.09
CW 99- 26	CW 30- 29	OW 280- 1	30	7.66	3.85	5.27	0.87
CW 185	OW 180- 1	MTAI 8	6	7.58	3.10	5.13	1.66
WW 3-1	OW 132-2	OW 240-6	6	7.32	4.05	5.77	1.43
WW 39- 3	OW 280- 1	OW 280- 2	23	7.27	3.54	5.46	1.06
CW 256- 1	MCOL 1734	OW 280- 1	9	7.20	2.87	4.86	1.63
CW 200- 1	OW 230- 3	CW 47- 3	5	7.10	4.00	5.04	1.09
CW 201- 2	OW 230- 3	CW 56- 5	5	7.10	4.00	5.60	1.15
CW 73- 2	CM 1585- 13	OW 284- 1	12	6.84	4.27	4.97	0.86
CW 198	OW 230- 3	CW 30- 65	31	6.77	3.97	5.30	0.73
CW 212- 1	OW 284- 1	MCOL 1734	7	6.53	4.40	5.25	0.66
CW 251- 2	MCOL 1734	OW 189- 1	5	6.46	5.22	5.89	0.55
CW 203- 1	OW 230- 4	CW 48- 1	3	6.37	5.29	5.76	0.55
CW 184- 2	OW 180- 1	MCOL 1734	5	6.27	3.01	4.73	1.33
CW 204- 1	OW 231- 3	AM 244- 31	5	6.25	4.46	5.04	0.75
CW 183	OW 180- 1	CW 48- 1	2	6.05	4.66	5.36	0.98
WW 21- 10	OW 231- 3	OW 240- 6	14	5.86	3.11	4.74	1.00
WW 20- 2	OW 231- 3	OW 146- 1	8	5.74	3.30	4.98	0.90
CW 202- 2	OW 230- 4	CW 30- 73	4	5.66	4.02	4.61	0.72
WW 9- 1	OW 180- 1	OW 234- 2	2	5.58	5.39	5.48	0.13
CW 186- 1	OW 181- 2	CM 1585- 13	4	5.27	3.72	4.69	0.67
WW 19- 2	OW 230- 3	OW 240- 8	7	4.80	3.01	3.72	0.96
Total			579				

SDS-PAGE analysis of protein in root flour conducted using different samples sizes and different saline buffers revealed that 300 mg of sample and the saline extraction buffer Na_3PO_4 0.05M gave the best results of band resolution and intensity (Figure 6.2). The best loading buffer was blue juice and the best dilution was 2:1 and sample volume for the best resolution of bands in the gel was 50 µl.

Table 6.4 Total protein and Amino acid profile of root flour from 2 high protein inter-specific hybrids, 2 high protein *M. escuenta* sub spp *flabellifolia* accessions used as parent and a pooled leaf sample of 10 high leaf protein cassava varieties

Variety	**OW235-3 g/kg**	**CW66-18 g/kg**	**CW66-48 g/kg**	**OW132-2 g/kg**	**Leaf (pooled) g/kg**
N	15.7	13.8	14.9	18.7	35.5
CP=N*6.25	98	86	93	117	222
cys	0.3	0.6	0.3	0.4	2.9
met	0.6	0.2	0.5	0.5	3.5
asp	2.1	1.3	1.6	2.7	22.2
thr	1.1	0.5	0.8	1.1	9.4
ser	1	0.6	0.8	1.2	10.8
glu	7.7	4.7	8.6	9.7	31
pro	0.9	0.6	1	1.1	10.3
gly	0.9	0.7	0.8	1.1	10.7
ala	1.5	0.9	1.4	2.2	12.1
val	1.3	0.6	1	1.2	12.2
ile	0.8	0.5	0.7	0.8	9.5
leu	1	0.8	1	1.4	17.2
tyr	0.5	0.4	0.5	0.1	8.1
phe	0.7	0.5	0.7	0.9	11.4
gaba	0.9	0.7	0.8	1.5	2
his	1.1	0.9	1	1.5	4.7
ornt	1.3	0.6	3.8	1.8	0.1
lys	1.5	1.5	1.5	1.8	12.3
arg	27	28.5	23.8	27	11.1
trp	0.6	0.3	0.4	0.4	4.4
sum ex gaba, ornt	50.6	44.1	46.4	55.1	203.8
sumAA/CP	0.52	0.51	0.5	0.47	0.92
sumAA/N	3.23	3.2	3.12	2.94	5.74

The SDS-PAGE profiles of the root proteins in the crude extracts from the genotypes sampled are shown in Figure 6.2. The highest molecular weight protein group (2 bands) observed was between 103,000 and 77,000 Da and the lowest band 20,700 Da. There are reports of cassava proteins showing bands at 14,000-80,000 Da of molecular mass (Glaucia, 2001), using two-dimensional gel electrophoresis. Souza *et al*, 1998 reported the isolation of a major protein of MW of about 22,000, which was restricted to the parenchyma rather than the peel of the tuber. These results show that the patterns observed in our study are between the reported ranks.

Table 6.6 BC_1 Families established from embryo axes, hardened in the screen house and transferred to the field

No	BC1 Family	Donor parent	Female parent of donor	Male parent of donor	Recurrent Parent	% protein of donor parent	No of plants
1	B1P2	CW 198 - 11	OW 230 - 3	CW 30 - 65	MTAI 8	11.28	225
3	B1P5	CW 205 - 2	OW 231 - 3	MTAI 8	MTAI 8	10.54	225
4	B1P6	CW 201 - 2	OW 230 - 3	CW 56 - 5	MTAI 8	10.2	225

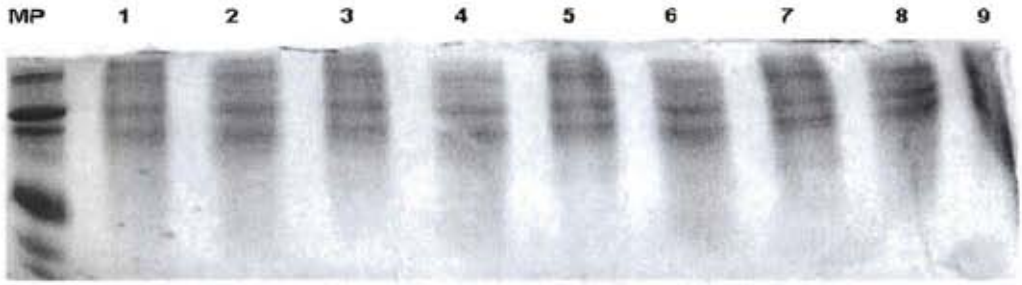

Figure 6.2 Visualization of bands in SDS-PAGE denaturing electrophoresis gel using several samples sizes prepared with Na_3PO_4 0.05M saline buffer of accessions number 31. 1) 200 mg of sample + Blue Juice; 2) 250 mg of sample + 2β- mercaptoethanol; 3) 250 mg of sample + Blue Juice; 4) 300 mg of sample + 2β- mercaptoethanol; 5) 300 mg of sample + Blue Juice; 6) 400 mg of sample + 2β- mercaptoethanol; 7) 400 mg of sample + Blue Juice; 8) 500 mg of sample + 2β- mercaptoethanol; 9) 500 mg + Blue Juice. MP is a protein molecular weight marker.

Green Mites

Individuals of the BC_1 families that appeared to possess resistance to CGM in focal points of great CGM damage in the seedling trial were crossed to CMD resistant parents to obtain recombinants that carry CMD and CGM resistance. Of the 429 BC_1 genotypes evaluated in the period 2002 (seedling trial), 2003 (clonal evaluation), and in 2004 (preliminary yield trial), 27 genotypes showed consistent high levels of resistance to the cassava green mites (Table 6.7). A major problem with these BC1 genotypes was the gradual loss of genotypes due to poor germination after storage of woody stems before planting as required by phyto-sanitary measures to control white flies of a one-month period of cassava-free fields. Of the 429 genotypes that were used to initiate evaluations in 2002 only 268 genotypes (62.8%) are left. Another trait of interest found in these backcross derivatives as very high dry matter content, a selection index based on resistance to green mites, dry matter, and yield was used to select 30 genotypes for establishment in the crossing block, a selection pressure was of 12%.

The BC_1 genotypes observed to be resistant to cassava green mites and BC_2 progenies (AR families) derived from some of the BC_1 individuals represent good plant materials to initiate discovery for markers associated with resistance to CGM. Resistant BC_1 individuals and AR

families were planted in a single row trial this year for evaluation during the dry period, January to March, in 2006. However, continuous rain during this period prevented mite attack therefore evaluation of the BC_2 lines, BSA is therefore going to be conducted on resistant and susceptible BC_1 lines.

Table 6.7 BC_1 progenies showing moderate to high resistance to CGM, the BCI individuals are being used for the development of markets.

Genotype	Female Parent	Male Parent	1st Objective	2nd Objective	Evaluation 2004	Evaluation 2004	Average 2003-2004
CW 215- 1	SM 909- 25	CW 66- 60	Z02	Green Mites	2	3	2.5
CW 220- 9	SM 1219- 9	CW 67- 123	Z02	Green Mites	1	3	2
CW 224- 6	SM 1460- 1	CW 66- 60	Z02	Green Mites	3	3	3
CW 225- 6	SM 1460- 1	CW 66- 62	Z02	Green Mites	1	3	2
CW 226- 10	SM 1460- 1	CW 66- 73	Z02	Green Mites	1	3	2
CW 226- 5	SM 1460- 1	CW 66- 73	Z02	Green Mites	2	3	2.5
CW 229- 7	SM 1511- 6	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 231- 19	SM 1565- 15	CW 66- 60	Z02	Green Mites	2	3	2.5
CW 235- 10	SM 1665- 2	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 235- 23	SM 1665- 2	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 235- 46	SM 1665- 2	CW 67- 87	Z01	Green Mites	2	3	2.5
CW 235- 82	SM 1665- 2	CW 67- 87	Z01	Green Mites	1	3	2
CW 236- 15	SM 1669- 5	CW 66- 19	Z01	Green Mites	1	3	2
CW 236- 16	SM 1669- 5	CW 66- 19	Z01	Green Mites	1	3	2
CW 236- 17	SM 1669- 5	CW 66- 19	Z01	Green Mites	1	3	2
CW 242- 2	SM 1669- 7	CW 67- 87	Z01	Green Mites	1	3	2
CW 246- 5	SM 1741- 1	CW 67- 91	Z02	Green Mites	4	3	3.5
CW 248- 2	SM 1778- 45	CW 67- 45	Z02	Green Mites	1	3	2
CW 258- 3	MTAI 8	CW 66- 60	Z01	Green Mites	2	2	2
CW 259- 21	MTAI 8	CW 66- 73	Z01	Green Mites	2	3	2.5
CW 259- 40	MTAI 8	CW 66- 73	Z01	Green Mites	2	3	2.5
CW 260- 5	MTAI 8	CW 66- 74	Z01	Green Mites	1	3	2
CW 76- 1	CM 3306- 4	CW 68- 3	Z01	Green Mites	2	3	2.5
CW 76- 5	CM 3306- 4	CW 68- 3	Z01	Green Mites	1	3	2
CW 76- 7	CM 3306- 4	CW 68- 3	Z01	Green Mites	1	3	2
CW 80- 1	CM 7951- 5	CW 67- 42	Z02	Green Mites	1	3	2
CW 81- 2	CM 7951- 5	CW 67- 98	Z02	Green Mites	1	2	1.5

Post harvest physiological deterioration (PPD)

Evaluation of inter-specific hybrid CW429-1 reveals that genes for delayed PPD have been transferred into cassava from a wild relative. Results of mean PPD values at 5 days after harvest (DAH) ranged from 0% in CW 429-1 and MBRA 337 to 44.85% in CM523-7 (Table 1). At 10 DAH, mean values ranged from 0% in CW429-1 to 58% in CM523-7, respectively.

The same trend was observed 15 DAH with CW 429-1 still displaying no visible sign of deterioration (Figure 2.3). Previous evaluation of delayed PPD shows nothing higher than 7 DAH has been found (Sanchez et al. 2005).

Table 6.8 Mean scores of post harvest physiological deterioration (PPD) in the inter-specific hybrid CW429-1 and 8 other elite varieties

Genotype	Previous PPD Status	Root Color	Dry matter (%)	PPD			
				5 DAH (%)	10 DAH (%)	15 DAH (%)	Average (%)
CW429-1	Tolerant	White	28.2	0.0	0.0	0.0	0.0
MTAI-8	Susceptible	Cream	41.5	33.7	42.3	22.9	33.0
CM 523-7	Susceptible	White	39.4	44.9	58.0	52.9	51.9
HMC-1	Susceptible	White	36.3	37.4	35.4	30.9	34.6
MCOL1505	Susceptible	White	35.1	26.6	29.7	27.1	27.8
MBRA337	Tolerant	Yellow	21.3	0.0	22.0	6.4	9.5
MPER183	Susceptible	White	32.1	39.7	19.6	45.7	35.0
CM 2772-3	Tolerant	Yellow	34.3	24.9	18.9	*	21.9
MCOL 2279	Tolerant	Cream	24.4	12.9	30.0	7.1	16.2

* High rot incidence did not allow for evaluation

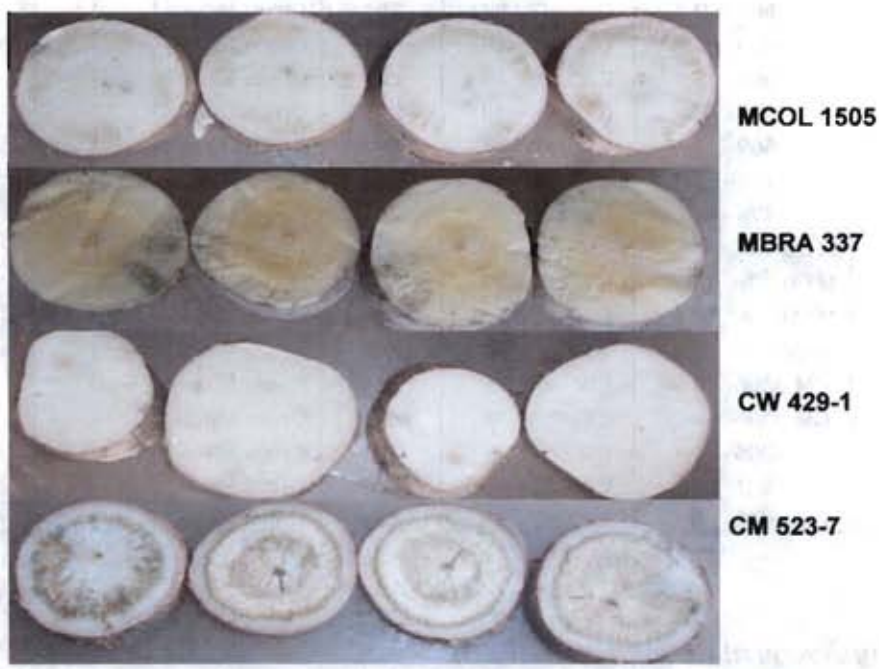

Figure 6.3 Visual representation of post harvest physiological deterioration (PPD) in the inter-specific hybrid CW429-1 and three elite cassava varieties

A total of 605 genotypes of back cross and self-pollinated populations have been obtained from CW429-1 and MNG11 (Table 6.10). Between 4 and 8 plants of each of the above genotypes have been successfully hardened in the screen house and transferred to the field. They will be evaluated at 10months after planting. The parental lines of these mapping populations are also to be evaluated with over 850 SSR markers to identify polymorphic markers for bulked segregant analysis and QTL mapping.

Table 6.9 BC_1 and S_1 mapping populations for QTL mapping of post-harvest deterioration (PHD).

Family	Female Parent	Male Parent	Seeds received (Number)	Non-viable seeds (Number)	Seeds planted (Number)	Genotypes obtained (Number)	% Recovery
B1PD280	CW 429-1		569	152	417	304	73%
B1PD284A	TAI 8	CW 429-1	108	28	80	21	26%
B1PD284B	CW 429-1	TAI 8	187	50	137	79	58%
B1PD289	CW 429-1	SM909-25	103	28	75	57	76%
TOTAL			**967**	**258**	**709**	**461**	**65%**

Dr matter Content

Segregation of dry matter content in the inter-specific family CW208 ranges from less than 20% to well over 40% (Figure 6.4). Distribution of the trait in this family reveals a non-normal distribution that suggests large QTLs genes might be involved (Figure 6.5).

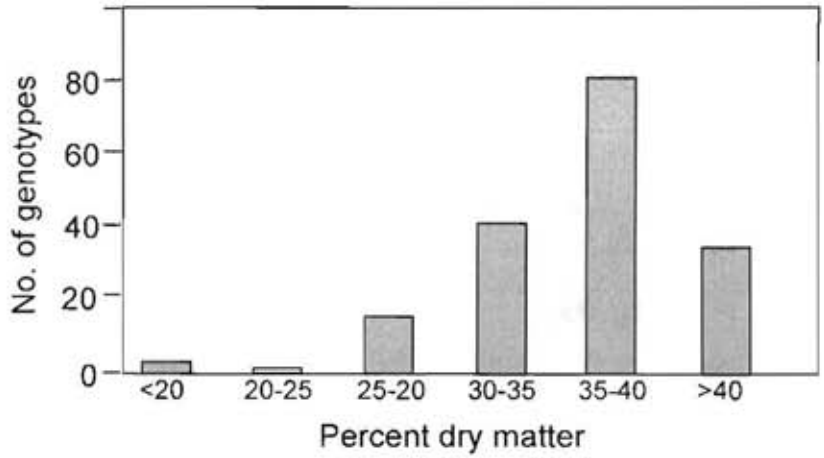

Figure 6.4 Frequency distribution of different classes of dry matter content in an inter-specific family CW208 obtained from a cross between MTAI8 and *M. tristis*

Of more than 600 SSR markers screened to date, 55 SSR markers were polymorphic between the DMC high and low bulks, of these 17 were polymorphic among the genotypes of the bulks (Figure 6.5). To determine promising markers for dry matter content, phenotypic data in individuals of the CW208 family was regressed on marker data classes of the 17 markers. However due to differences between the 2003 (seedling data) and the 2004 (clonal data), both data was used (Table 6.10), the bulks were made using 2004 data because it was from several plants as opposed to the 2003 which was from a single plant. The 2003 data identified SSRY 99, SSRY 141 and NS 169 with R^2 values of 22.68, 35.89 and 20.01 respectively while the 2004 data identified SSRY 11 (R^2=26.85). The phenotypic variance explained by these markers based on their regression coefficient, is enough to consider them as markers for marker-assisted selection (MAS). These are to be used to screen progenies derived from this family.

Table 6.10 Regression analysis results of markers polymorphic in the open bulks when used for screen the whole population.

Marker	R^2 values (2003 data)	R^2 value (2004)
SSRY 298	0.34	7.42
SSRY 306	2.7	7.74
C306R	0.6	7.64
SSRY 69	5.72	3.02
SSRY 99	**22.68**	0.07
SSRY 141	**35.89**	0.09
SSRY 11	1.19	**26.85**
SSRY 23	10.22	0
SSRY 27	7.1	16.6
SSRY 36	10.52	5.15
SSRY 47	1.91	9.97
SSRY 49	2.89	3.18
SSRY 54	6.9	15.37
SSRY 57	5.7	0.11
SSRY 60	0	6.66
SSRY 66	6.7	4.2
SSRY 75	1.5	7.9
NS 169	**20.01**	7.40

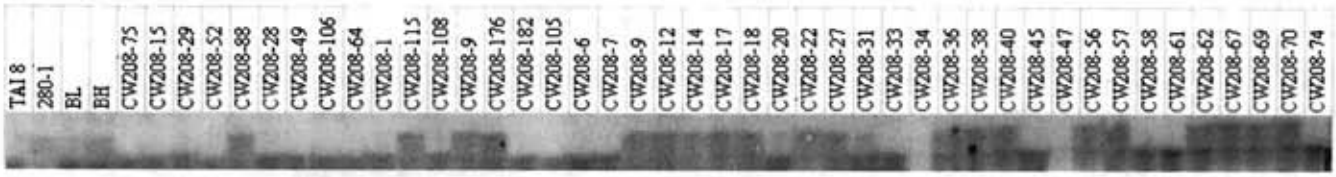

Figure 6.5 Acrylamide gel showing PCR amplification of marker SSRY 11 of parents bulks and individuals from the inter-specific family CW 208.

Perspectives

- QTL mapping of protein content in the BC_1 families and BSA of mite resistance, delayed PPD in the BC_1 families
- Completion of BSA of dry matter content in the CW208 family

6.4 References

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7. Detection and characterization of a phytoplasm and vectors associated with Cassava Frogskin Disease

7.1. Association between a phytoplasm and CFSD.

Cassava frogskin disease (CFSD) is an important disease affecting cassava roots, whose causal agent had remained unknown for many years despite its economic significance. CFSD attacks cassava roots, causing increasing numbers of deep lesions that eventually cause thin roots. Recently, CFSD has been reported with increasing frequency in Colombia, Brazil, and Venezuela. In Colombia, for example, incidence of up to 90% has been recorded in commercial fields in the production areas of Valle del Cauca, Cauca, Meta, and the North Coast. Disease symptoms consist of small, longitudinal fissures distributed throughout the root. As the roots increase in diameter, the fissures tend to heal, giving the injuries a lip form. Root cortex or epidermis presents a cork-like appearance that peels off easily. Depending on the severity of symptoms, the depth and number of lesions increase until the root becomes thin (Pineda *et al.*, 1983; Alvarez *et al.*, 2003). To develop appropriate management strategies for controlling the disease, the pathogen must be identified.

Recently, a phytoplasm –a microorganism that lack cell walls- was successfully detected and identified in CFSD-infected cassava roots, leaf, midribs, petioles, and peduncles in susceptible commercial varieties, using molecular tools such as nested PCR to amplify a fragment of the 16S rRNA gene, and techniques of microscopy (DAPI, Dienes' and electron). The phytoplasm was not detected in healthy plants from the same varieties harvested from fields free of disease. Various diseases have been reported as being caused by phytoplasm that induce symptoms in roots (Gatineau *et al.*, 2001; Pilkington *et al.*, 2004). Maust *et al.* (2003) observed necrosis in roots of coconut palms affected by the LY phytoplasm, causal agent of lethal yellowing, as well as reduced sugar concentrations in primary roots, suggesting that sugar transport from leaves to roots is affected by phytoplasm infection.

Phytoplasm are commonly transmitted between plants by homopterous insects. The microorganism first multiplies in the intestinal cells of their insect vectors and subsequently in the hemolymph after passing through the salivary glands. They infect internal organs such as the thoracic ganglion and fatty bodies (lipids) (Kawakita *et al.*, 2000). The transmission also can occur through grafting and the parasitic plant *Cuscuta*. Tetracycline antibiotics have been found to cause remission of symptoms in a number of diseases of suspected phytoplasm etiology, and thus were selected for attempted chemotherapy of CFSD.

Molecular tools and microscopy will be applied to detect phytoplasm in CFSD-infected roots, leaf midribs, petioles, and peduncles in different cassava varieties and genotypes found in various regions of Colombia. In addition transmission to *Cuscuta* (dodder plant), grafting and remission studies were conducted to elucidate the role played by the phytoplasm in this disease.

7.2 Materials and Methods

Phytoplasm sources: We took samples of leaf, stem, and root tissues from 39 genotypes of cassava (*Manihot esculenta* Crantz) grown in the field and greenhouse and naturally infected by CFSD (Table 7.1). The plants were collected from 2002 to 2004 in three areas of Colombia—North Coast, Valle del Cauca, and Cauca—where the incidence of CFSD is high.

We included as negative checks cassava plants obtained *in vitro* through meristem culture. Positive checks were samples of periwinkle (*Catharanthus roseus* (L.) G. Don) that clearly showed typical symptoms induced by phytoplasm—internode stunting or a clumping form of stunting in terminal buds.

Table 7.1. List of DNA fragments obtained from samples of tissues of 39 cassava varieties infected with Frogskin Disease. The samples were amplified by nested and direct PCR, using universal primers and primers specific for phytoplasm.

Code	Genotype	Site [a]	Tissue [b]	PCR [c]	Primers [d]	Code	Genotype	Site [a]	Tissue [b]	PCR [c]	Primers [d]
Y1	CM6740-7	VC	LmP/R[e]	+/+	A-C	**Y22**	CM9582-65	VC	LmP/R	+/+	A-B-C
Y2	CIAT Parrita	VC	LmP/S/R	+/+/+	B	**Y23**	CM9582-24	VC	LmP/R	+/+	A-B-C
Y3	CM523-7	VC	LmP/R[e]	+/+	B-C	**Y24**	MCR81	VC	LmP/R	+/+	A-B-C
Y4	Manzana	VC	LmP/R[e]	+/+	B-C	**Y25**	Venezolana	S	R	+	A-B-C
Y5	MBRA383	VC	LmP/R	+/+	B-C	**Y26**	MPER16	C	LmP/R	+/+	C
Y6	CM849-1	VC	LmP/R	+/+	B-C	**Y27**	MCOL634	C	LmP/R	+/+	C
Y7	CM5460-10	VC	LmP	+/+	C	**Y28**	MBRA829	C	LmP/R	+/+	C
Y8	CM2177-2	VC	LmP/R	+/+	B-C	**Y29**	SM1219-9	VC	LmP/R[e]	+/+	A-B-C
Y9	CM4919-1	VC	LmP/R	+/+	B-C	**Y30**	MCHN2	C	LmP/R	-/-	C
Y10	CM3306-9	VC	LmP[e]	+	B-C	**Y31**	HMC-1	C	LmP/R	+/+	C
Y11	CM3306-19	VC	LmP[e]	+	B-C	**Y32**	MARG2	C	LmP/R	-/-	C
Y12	MBRA856-54	VC	LmP[e]	+	B-C	**Y33**	MBRA325	C	LmP/R	+/+	C
Y13	MPER335	VC	R	+	C	**Y34**	MBRA839	C	LmP/R	+/+	C
Y14	MBRA856	C	LmP/R	+/+	C	**Y35**	MCOL1178	C	LmP/R	+/+	C
Y15	SM909-25	VC	LmP/R[e]	+/+	C	**Y36**	MCOL1468	C	LmP/R	+/+	C
Y16	CG6119-5	VC	LmP/R	+/+	C	**Y37**	MCUB74	C	LmP/R	-/+	C
Y17	MCOL2063	VC	LmP[e]	+	A-B-C	**Y38**	MBRA886	C	LmP/R	+/+	C
Y18	ICA Nataima	VC	LmP/R	-/+	C	**Y39**	MBRA882	C	LmP/R	+/+	C
Y19	SM1201-5	VC	LmP	-	C	**Y40**	MBRA383	C	LmP/R	+/+	B
Y20	GM228-14	VC	LmP	-	C	**Y41**	CM523-7	Q	LmP/R	-/-	B-C
Y21	CM9582-64	VC	LmP/R	+/+	A-B-C	**Y42**	Manzana	Q	LmP/R	-/-	B-C

[a] VC = Department of Valle del Cauca; C = Cauca; S = Sucre; Q = Quindío. [b] LmP = leaf midrib and petioles; R = roots; S = stems. [c] + = amplification positive for phytoplasm; - = amplification negative for phytoplasm. [e] Primers used for amplification were A = P1/P7–R16F2N/R16R2; B = R16mF2/R16mR1–R16F2N/R16R2; C = R16F2/R16R2–R16(III)F2/R16(III)R1. [d] Also showing foliar symptoms of chlorosis and deformed leaf blades.

Other checks included DNA from coffee crispiness phytoplasm (Ccp; GenBank Accession Number AY525125), facilitated by the National Coffee Research Center (CENICAFE, Colombia) and used as reference for the 16SrIII group (X-disease group); sugar beet phytoplasm (Rp; 16SrIII group) and pepper clover proliferation phytoplasm (Pcpp; 16SrVI

group), both facilitated by the National Institute for Agricultural and Food Research, Madrid, Spain; and lethal wilt oil palm phytoplasm (Lwop) (GenBank Accession Number AY739024) (Table 7.2) (Alvarez and Claroz 2003).

Table 7.2. Description of GenBank accession numbers of phytoplasm 16Sr RNA gene sequences used in this study

GenBank accesion no.	Strain
AJ430067	Apple proliferation (AP)
AF105315	Ash yellows strain AshY3 (AShY)
AY739024	Lethal wilt oil palm phytoplasm (Lwop)
L76865	Australian grapevine yellows phytoplasm (AGY)
AB052871	Bermudagrass white leaf (BGWL)
U15442	Candidatus *Phytoplasm aurantifolia* (CPA)
AB010425	Candidatus *Phytoplasm japonicum* (CPJ)
X76426	Candidatus *Phytoplasm mali* (CPM)
AY737646	Cassava frogskin disease strain (CFSDY17) ▪
AY737647	Cassava frogskin disease strain (CFSDY29)▪
AY787139	Cassava witches'-broom phytoplasm (CWB)
AF147706	Chayote witches'-broom (ChWBIII-Ch10) (ChWB)
AF373106	Cirsium white leaf phytoplasm (CWL)
L33761	Clover proliferation (CP)
AF189288	Clover yellow edge (CYE)
AF498307	Coconut lethal yellowing strain Jamaica (LYJ-C8) (CLY)
AF189214	Elm yellows (EY)
AF147708	Hibiscus witches'-broom (HWB)
L33764	Loofah witches'-broom (LWB)
AF248960	Mexican periwinkle virescence (MPV)
L33765	Peanut witches'- broom (PWB)
L33764	Pigeon pea witches'-broom (PPWB)
AB052874	Sugarcane white leaf phytoplasm (SWL)
AF060875	Virginia grapevine yellows VGYIII (VGY)
AF190227	Walnut witches'-broom (WWB)
L04682	Western X (WX)
AF294996	*Acholeplasma laidlawii*

▪ Sequence determined in this study.

Electron microscopy: Tissue exhibiting typical CFSD symptoms was chosen from four cassava genotypes. The root for each variety comprised different cuts directed mainly at the phloem. The tissue fragments were cut into 1 × 2 mm pieces to be prefixed in 2%–3% glutaraldehyde (0.1 M phosphate buffer, pH 7.3). The samples for electron microscopy were prepared by making ultra-thin (60–90 nm) sections with a Reichert Ultracut S ultramicrotome (North Central Instruments, Plymouth, MN). After post-fixation and precontrasting in uranyl acetate, they were dehydrated in an acetone series 50, 70, 90 (15 min each) and 100% (15 min, three times), and were embedded in Spurr's resin. A previous 18-h infiltration with acetone-Spurr (1:1) was done to facilitate the entry of resin into the tissues. The ultra-thin sections were mounted on copper grills, and images taken, using a

Megaview III digital camera system with SIS software (Soft Imaging System Corp., Lakewood, CO) on a JEOL 1200EX Woburn, MA scanning/transmission electron microscope (Japan Electron Optics Laboratory, Peabody, MA). Healthy plant samples were also processed to act as control.

DNA Extraction: DNA was extracted according to the protocol of Gilbertson and Dellaporta, 1983. Tissues from leaf veins, stems, and petioles, which had been conserved at -80°C, were macerated with liquid nitrogen, using a porcelain mortar. About 0.4 g of the pulverized tissue were mixed with 0.51 mL of extraction buffer (50 mM EDTA, pH 8.0; 500 mM NaCl; and 10 mM 2-mercaptoethanol) and agitated for 2 min at speed 8 in a blender (Vortex-Genie 2, model G560, Scientific Industries, Bohemia, NY). Then 90 µL of SDS at 10% were added and the whole agitated for 2 min before being incubated at 65°C for 10 min. Subsequently, 150 µL of potassium acetate at 5 M, pH 5.5, were added and the whole agitated for 2 min. The mixture was centrifuged at 14,000 rpm for 10 min and the supernatant (about 600 µL) collected. Then, 0.5 volumes of isopropanol at 100% (300 µL) were added and left to precipitate at -20°C for 30 min. The whole was then centrifuged at 14,000 rpm for 10 min, the supernatant eliminated, and the pellet washed with 500 µL of ethanol at 70%, centrifuging at 10,000 rpm for 5 min. Finally, the supernatant was eliminated and the pellet re-suspended in TE at 30 and 50 µL and 50°C. It was then incubated overnight with 2 µL of RNase A (10 mg/mL) at 4°C.

PCR amplification: Three pairs of universal primers—P1/P7 (Smart et al. 1996), R16mF2/R1 (Gundersen and Lee 1996; Schneider et al. 1993), and R16F2n/R2 (Gundersen and Lee 1996)—were used in nested PCR to amplify the region of the genes 16S rRNA and 23S rRNA. The amplified products of P1/P7 and R16mF2/R1 were diluted at 1:30 with sterilized and distilled water [deionized water (HPLC); J.T. Baker] for use as DNA mold in 1-µL quantities with primers R16F2n/R2.

Each reaction was put in 0.2-mL tubes carrying a volume of 25 µL, using final concentrations of 100 ng of DNA, 1X buffer, 3 mM $MgCl_2$, 1 U *Taq* polymerase (Promega, Madison, WI), 0.8 mM dNTPs (Invitrogen Life Technologies, Carlsbad, CA), and 0.1 µM of each primer (Operon Technologies, Inc., Alameda, CA).

For primers P1/P7, 35 cycles were carried out in a PTC-100 thermal cycler with a hot cover unit (MJ Research, Inc., Waltham, MA), following these conditions: 30 s (90 s for the first cycle) of denaturation at 94 °C, annealing for 50 s at 55 °C, and extension of the primer for 80 s (10 min in the final cycle) at 72 °C. Primers R16mF2/R16mR1 were amplified with 28 cycles, using the same conditions. The primer pair R16F2n/R16R2 was evaluated in a similar manner, but with an annealing temperature of 50 °C. The PCR products were visualized in a 1.5% agarose gel, stained with 0.75 µg/mL ethidium bromide, and analyzed in a Stratagene Eagle Eye® II video system (La Jolla, CA).

PCR amplification with specific primers: The DNA amplified with primers R16F2/R2 (Guo et al, 2000) was diluted at 1:50 and used as sample for re-amplification in nested PCR, using primers R16(III)F2/R16(III)R1 (Guo et al, 2000), designed specifically for the 16SrIII group of phytoplasm (X-disease group), using the same PCR conditions as mentioned above, carrying out 35 cycles of 1 min (2 min for the first cycle) of denaturation at 94 °C, annealing for 2 min at 50 °C, and extension of the primer for 3 min (10 min in the final cycle) at 72 °C. The analysis of the amplified products was carried out as previously described.

Restriction fragment length polymorphisms (RFLPs) analysis: To classify the phytoplasm in terms of the 15 groups so far reported, we amplified—using nested PCR, with the universal primers P1/P7 and R16F2N/R16R2, and the specific primers for the 16Sr III group R16F2/R16R2 and R16(III)F2/R16(III)R1—the sequence of gene 16Sr RNA (1.2 kb and 0.8 kb). The nested PCR product of universal and specific primers was then analyzed by digestion with restriction enzymes *Rsa*I, *Alu*I, *Mse*I and *Taq* I (Invitrogen Life Technologies, Carlsbad, CA). The enzyme *Taq* I was evaluated only with the PCR product obtained with specific primers. As controls, phytoplasm from the 16Sr I group (aster yellows, Lwop, Lethal wilt oil palm phytoplasm), 16Sr III group (X-disease, CCP, Coffee crispiness phytoplasm and RP, Sugar beet phytoplasm.), and 16Sr IX group (pigeonpea witches' broom, PP, as represented by phytoplasm from periwinkle), were used.

15 µL of the PCR product and added 2 µL of 10X buffer enzyme and 1 µL of the restriction enzyme (500 units/µL), were taken. This mixture was incubated for 16 h at 37°C (except for enzyme *Taq*I, which was incubated at 65°C). We then added 3 µL loading buffer (bromophenol blue at 0.25%, glycerol in water at 30%) and ran it in acrylamide gel at 5% for 1 h at 100 V, 24 mA, in TBE 1X buffer and stained with ethidium bromide at 10 mg/mL. The band patterns obtained with the RFLP technique were analyzed using the software package One-Dscan ™ Program (Stratagen, La Jolla, CA).

Cloning of PCR products and DNA sequencing: The PCR products were purified, using the QIAquick PCR Purification Kit (QIAGEN), ligated in pGEM-T Easy vector, which was introduced into the *Escherichia coli* strain DH5-α by electroporation at 2.4 kV/cm². Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media. Plasmids were extracted with a Plasmid Miniprep System Kit (Gibco-BRL). Positive inserts were observed by plasmid restriction with EcoRI and electrophoresis in 1.5% agarose gel. Different-sized (500-600 pb) fragments were selected for sequencing by automated dideoxy sequencing (ABI Prism 377-96 DNA Sequencer), using a DNA-sequencing kit from Applied Biosystems, with 3 µL water, 1 µL primer, 4 µL mix from kit, and 1 µL DNA. Other six PCR products were sequenced directly, using a DNA-sequencing kit from Applied Biosystems, with 3 µL water, 1 µL primer, 4 µL mix from kit, and 1 µL DNA. The selected clones assured a minimum of twice sequencing coverage for each base position by overlapping. The sequences were assembled using Sequencher 4.1 software and matched by nucleotide, using the Blastn tool in GenBank (www.ncbi.nlm.nih.gov). The nucleotide sequences determined in this study were registered in the GenBank data library (Table 7.2).

Phylogenetic Analysis: The 16S rRNA gene sequence similarities (1.2 Kb-R16F2n/R2) from CFSD phytoplasm, CFSDY17, CFSDY29 and 24 other phytoplasm strains representing fifteen phytoplasm groups and *Acholeplasma laidlawii* were aligned using DNAMAN version 4.13 (Lynnon BioSoft, 1999). Gaps and incomplete sequences were removed. The sequence alignment was then analyzed by means of Phylogenetic Analysis Using Parsimony (PAUP) by DNAman version 4.13 software. A phylogram was generated by performing bootstrap analysis of 1000 replicates for the estimation of stability and support for the inferred clades. *A. laidlawii* was selected as the outgroup to root the tree.

Chlortetracycline treatment of infected spouts: Cuttings of Secundina, M Bra 383 and CORPOICA Reina cassava genotypes, from affected plants were taken from plants grown in plastic pots. After removing the true leaves of the sprouts, they were rooted in deionized

water at different doses of chlortetracycline (0, 2.5, 5,10, 25, 50 ppm, injectable form, capsules can cause greater levels of intoxication of the plant, and are less effective against CFSD), with permanent oxygenation by a pump. The cuttings were incubated in a laboratory with a controlled temperature system (min. 20°C, max. 25°C), and 12-h alternate periods of light and darkness. The high humidity (66%-98%) was achieved through the use of closed boxes for the rooting of the cuttings.

Transmission The transmission of the pathogen was carried out on two cassava varieties infected with CFSD and evaluated with PCR as positive to Cfdp, using the ectoparasite *Cuscuta* sp. as a bridge transmitter and grafts (clefts and splices) into periwinkle (*Catharanthus roseus* (L.) G. Don) and healthy cassava.

The cassava variety used was SM 909-25, selected for severe symptoms in roots and the presence of symptoms—chlorosis and curling—in leaves under greenhouse conditions (23 °C and 80% RH). A clone (CW 94-21) from the CW family in CIAT's cassava genetic improvement program was also used. It had been found in the field with the characteristic symptoms of the disease in roots.

Transmission time was estimated from the establishment of *Cuscuta* in the plants corresponding to each treatment. The transmission period was about 2 months under greenhouse conditions (20–25 °C and 50%–90% RH). For transmission, the following treatments were carried out: (1) from infected cassava plants to healthy periwinkle plants; (2) infected periwinkle plants to healthy periwinkle plants; (3) infected cassava plants to healthy cassava plants; (4) healthy periwinkle plants to healthy periwinkle plants; and (5) healthy cassava plants to healthy periwinkle plants.

Transmissions by graft were carried out with cleft and splice grafts, using the leaf central nervure and terminal shoots of infected cassava plants. As stock, we used 2-month-old cassava plants and 5-to-6-week-old periwinkle plants. The treatments for this trial were (1) infected cassava plants to healthy periwinkle plants; (2) healthy periwinkle plants to healthy periwinkle plants; and (3) healthy cassava plants to healthy periwinkle plants. Each treatment was carried out with 6 replications. Once the *Cuscuta* plants were established and the grafts had developed well, monthly evaluations by PCR and by the characteristic symptoms reported in periwinkle were carried out.

7.3 Results

Electron microscopy: Diverse tissues (stem, leaf midrib, petioles, and roots) from numerous cassava plants were evaluated, but only some could be compared with the results obtained for nested PCR. In this study, guided by the results of the nested PCR, root tissues of four cassava genotypes susceptible to CFSD were first examined, which showed severe symptoms of the disease. Cells characteristic of phytoplasm were detected in root phloem. The phytoplasm structures observed were pleomorphic, comprising round, elongate, dumbbell, and ring-shaped elements, mostly 150 to 250 nm wide and 1000 nm long (Figure 7.1). The phytoplasm structures were limited only to phloem tubes and were never seen in large quantities.

Detection and classification of phytoplasm associated to CFSD: The affected plants of cassava and periwinkle exhibited symptoms typical of phytoplasm infections. Nested PCRs primed

by phytoplasm universal primer pair R16F2n/R2 resulted in the amplification of 1.2-kb DNA fragments of the 16S ribosomal DNA, indicating that the symptomatic cassava and periwinkle plants were infected by phytoplasm. The phytoplasm were detected in 35 of the 39 varieties of cassava tested exhibiting symptoms of CFSD, representing 89% of amplification (Table 7.1).

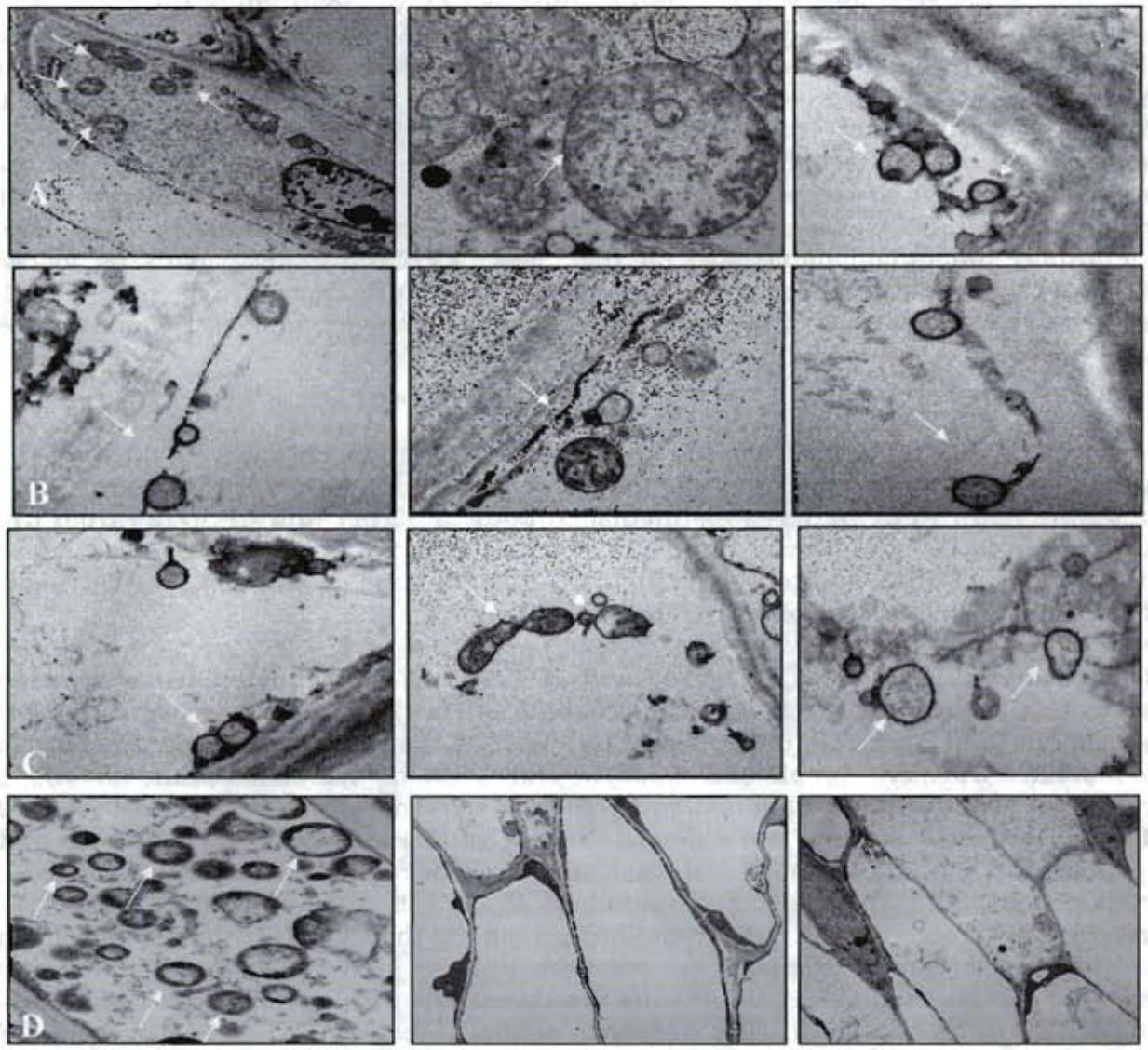

Figure 7.1. Micrographs, taken by cell transmission microscopy, of phytoplasm CFSD. (A) and (B) Infected cassava petiols. (C) Infected cassava roots and (D) Positive control (affected periwinkle) and healthy cassava petiols, left and right respectively.

RFLP analysis of the 16S rDNA PCR product indicated that the diseased cassava plants were infected by strains of a phytoplasm belonging to group 16SrIII (X-disease phytoplasm group). The presence of a group 16SrIII phytoplasm in all DNA samples was verified by

nested PCR assays primed by primer pair R16(III)F2/R1 yielded an amplified product of approximately 0.8 kb, while no amplified products were observed in Pcpp, Lwop and periwinkle used as controls.

The detected phytoplasm strains were classified on the basis of RFLP analysis of 16S rDNA amplified in PCR primed by primer pair R16F2n/R2, according to the classification scheme established by Lee et al, 1998. The collective RFLP patterns were compared with those published for other phytoplasm 16S rDNA (Gundersen et al. 1996). All of the cassava DNA samples tested yielded mutually indistinguishable collective RFLP patterns. The *Alu* I, *Mse* I, *Rsa* I and *Taq* I RFLP patterns observed for DNA from the 35 strains from cassava plants corresponded to RFLP patterns that have been found (Lee et al. 1998) only in phytoplasm strains belonging to group 16SrIII. Since the collective RFLP patterns indicated that the phytoplasm strains detected in the 35 diseased cassava plants were mutually indistinguishable, two of the phytoplasm strains Y17 and Y29, derived from two varieties growing in different fields, were selected as representative for further analysis. These strains were mutually indistinguishable also on the basis of collective RFLP patterns following analysis using two additional restriction enzymes (data not presented). These results lead us tentatively to conclude that these are strains of the same phytoplasm, which we term Cassava Frogskin Diseases phytoplasm (CFSD).

The collective RFLP patterns from use of the four restriction enzymes distinguished the CFSD phytoplasm strains from other phytoplasm classified in group 16SrIII (Figure 7.2). For example, the *Rsa* I RFLP patterns distinguished both CFSDY17 and CFSDY29 from LWOP phytoplasm, from the group 16Sr I and PP, from the group 16Sr IX (Figure 7.2A), and the *Alu* I and *Mse* I RFLP patterns distinguished both cassava strains from phytoplasm obtained from periwinkle and oil palm (Figure 7.2B and C).

Sequence analysis: Two strains, CFSDY17 and CFSDY29, of CFSD phytoplasm were selected for nucleotide sequencing of rDNA. PCR products of the 16S rRNA gene were sequenced using primers R16F2n and R16R2. As criteria, we took the number of correctly read bases of the amplified fragment, amplifications of the characteristic symptoms of the disease, and differences of genotype, and obtained two complete sequences, measuring 1260 and 1298 bp of 16Sr DNA gene region of two different cassava varieties, M Col 2063 (Y17) (leaf midrib and petiole) and SM 1219-9 (Y29) (external phloem from roots), which were classified and reported in GenBank with the accession numbers AY737646 (1260 bp) and AY737647 (1298 bp), respectively (Table 7.2). Sequence similarities among the CFSD-phytoplasm strains and group 16SrIII reference phytoplasm ranged from 98.8 to 99.6 % (Table 7.3). Since these values were greater than 97 %, the results are consistent with the placement of CFSDY17 and CFSDY29 in group 16SrIII. The sequence similarity between CFSDY17 and CFSDY29 phytoplasm was 99.9%, both showed the highest sequence homology with CYE phytoplasm (99.4% and 99.6%), and the lowest with WX phytoplasm (98.8% and 98.9%) (Table 7.3).

RFLP analysis of the 16SrIII group-specific rRNA primers pair, showed the same RFLP profiles for each of four enzyme digestions (Figure 7.3). Only two samples of CFSD are shown in each gel because digestion of the total amplified products with each of the four restriction enzymes gave an identical RFLP profile among all amplified CFSD phytoplasm samples.

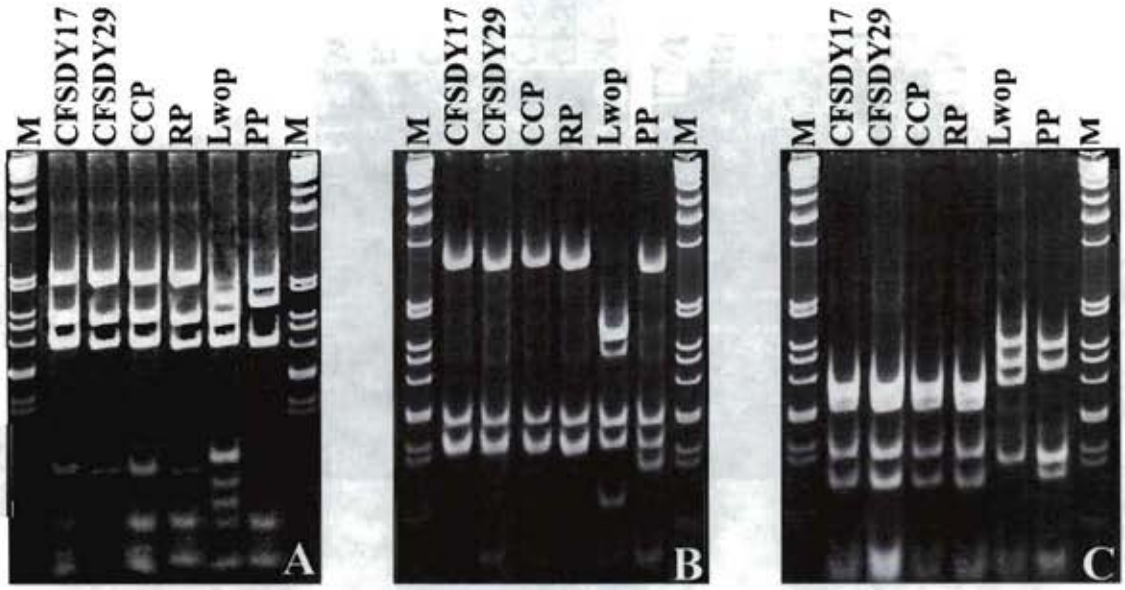

Figure 7.2. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA amplified in nested-PCR from two representative phytoplasm strains (Y17 and Y29) from cassava. First round of PCR was primed by P1/P7, followed by reamplification of target DNA in nested PCR primed by R16F2n/R2 on 5% polyacrylamide gels. DNA products from the nested PCR were digested with restriction endonucleases (A) *Rsa* I, (B) *Alu* I, (C) *Mse* I. M, 1kb DNA marker. CFSDY17, Cassava Frogskin diseases phytoplasm strain Y17. CFSDY29, Cassava Frogskin diseases phytoplasm strain Y29. CCP, Coffee crispiness phytoplasm. RP, *Sugar beet phytoplasm*. Lwop, Lethal wilt oil palm phytoplasm and PP, Periwinkle phytoplasm.

Table 7.3. Sequences similarities among 16S rDNAs from Y17 and Y29 phytoplamas and representative X-disease phytoplasm strains.[a]

	CFSDY29	VGY	WWB	WX	ChWB	CWL	CYE
CFSDY17	99.9	98.9	99.2	98.8	99.4	99.3	99.4
CFSDY29	***	99.0	99.3	98.9	99.5	99.4	99.6
VGY		***	99.5	99.1	98.9	99.0	99.5
WWB			***	99.4	99.2	99.2	99.6
WX				***	98.8	98.7	99.2
ChWB					***	99.3	99.5
CWL						***	99.3
CYE							***

[a] The fragment sequencing extend from the site of annealing of primer R16F2n to R16R2. Sequence similarities of 1.2 kb sequences of 16S rDNA were estimated using the alignment option of DNAMAN software. CFSDY17, Cassava frogskin disease strain Y17. CFSDY29, Cassava frogskin disease strain Y29. VGY, Virginia grapevine yellows VGYIII. WWB, Walnut

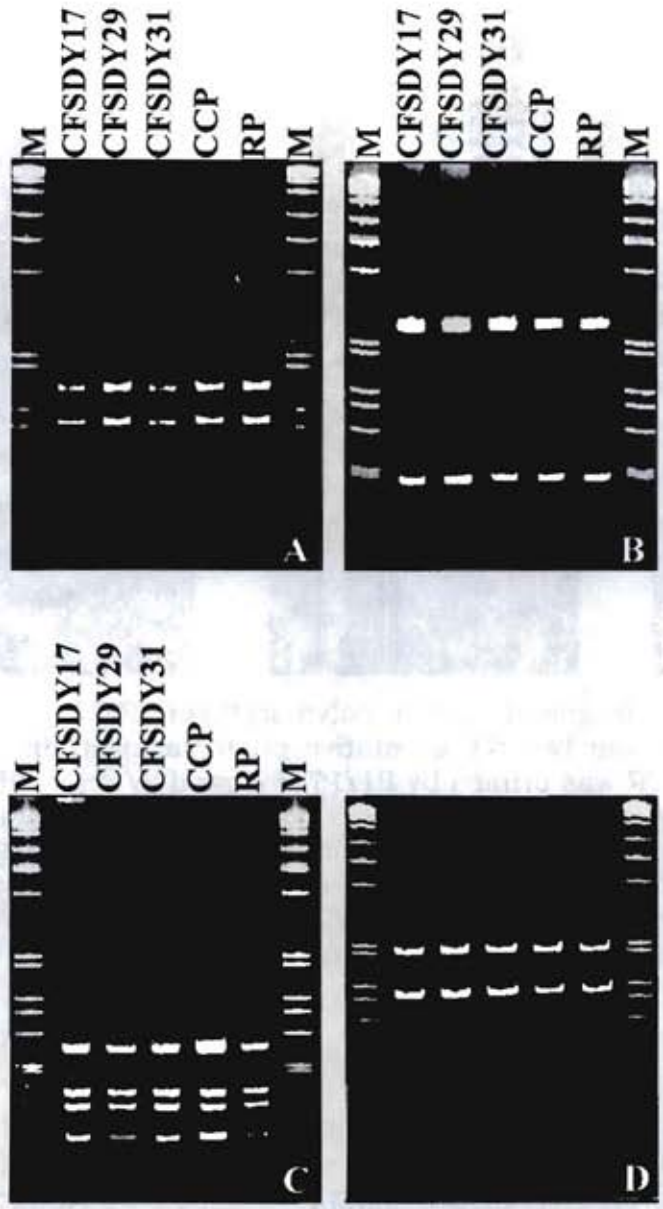

Figure 7.3. Restriction fragment length polymorphism (RFLP) profiles of amplified 16S rRNA products by primer pairs R16F2/R2 and nested X-disease phytoplasm (16SrIII) group-specific rRNA primers pair R16(III)F2/R1 (0.8 kb) on 5% polyacrylamide gels. (A) *Rsa* I, (B) *Alu* I, (C) *Mse* I and (D) *Taq* I. M, 1kb DNA marker. CFSDY17, Cassava Frogskin diseases phytoplasm strain Y17. CFSDY29, Cassava Frogskin diseases phytoplasm strain Y29. CCP, Coffee crispiness phytoplasm. RP, *Remolacha phytoplasm* witches'-broom. WX, Western X-disease. ChWB, Chayote witches'-broom (ChWBIII-Ch10). CWL, Cirsium white leaf phytoplasm. CYE, Clover yellow edge.

Phylogenetic analysis: A phylogenetic distance tree was constructed from a date set which included 16S rDNA sequences of the CFSD phytoplasm, 24 additional strains of phytoplasm from different crops, and *A. laidlawii* (Figure 7.4). Tree branching orders resolved by this

analysis were similar to and supported the same major phylogenetic groups identified in other studies (Montano et al, 2000; Galdeano et al, 2003). Also in this analysis, the CFSD phytoplasm clustered closely together with others known 16SrIII group strains, thereby supporting its assignment to this group. Strains from other phytoplasm belonging to 14 groups were included for comparison with group 16SrIII strains.

Inhibition of Chlortetracycline treatment of infected spouts: The inhibition of leaf symptoms (crispiness and clorosis) caused by CFSD was successful in two experiments using a dosage of 50 ppm chlortetracycline. The leaves of affected plants treated with 0 ppm of tetracycline showed presence of phytoplasm through nested PCR. The cuttings of the variety CORPOICA-Reina did not show CFSD foliar symptoms despite being infected and showing foliar symptoms in a glasshouse where cuttings were obtained to establish the experiment. Plants without the antibiotic formed roots and leaves within 3 weeks. Plants with the chemical treatments formed leaf shoots and rooting callus, but because of the phytotoxicity, no true leaves were formed.

Transmission: After 4 months of exposure, the pathogen's transmission was achieved through *Cuscuta* from infected cassava plants to healthy periwinkle plants and from infected to healthy periwinkle plants (treatments 1 and 2). Detection was achieved with nested PCR in 2 of the 6 replications for each treatment with variety SM 909-25. For the other three treatments and the other variety, positive amplifications were not obtained (Table 7.4).

Table 7.4. Results obtained with *Cuscuta* sp. and grafts as transmitters of phytoplasm from cassava infected with Cfdp to periwinkle.

Treatment		PCR (+)[a]	
		SM	CW
No.	Description	909-25	94-21
Cuscuta sp.			
1	Infected cassava to healthy periwinkle	2/6	0/6
2	Infected periwinkle to healthy periwinkle	2/6	0/6
3	Infected cassava to healthy cassava	0/6	0/6
4	Healthy periwinkle to healthy periwinkle	0/6	0/6
5	Healthy cassava to healthy periwinkle	0/6	0/6
Grafts (clefts and splices)			
1	Infected cassava to healthy periwinkle	5/6	3/6
2	Healthy periwinkle to healthy periwinkle	0/6	0/6
3	Healthy cassava to healthy periwinkle	0/6	0/6

a. Values refer to number of replications out of 6, where detection was successful.

Transmission by graft was generally high for treatment 1—of the 6 replications, 66% were detected as positive by nested PCR in the two infected varieties (SM 909-25 and CW 94-21). For the other two treatments, no positive samples were detected. Phytoplasm were detected, using grafts, 3 to 4 months after exposure (Table 7.4).

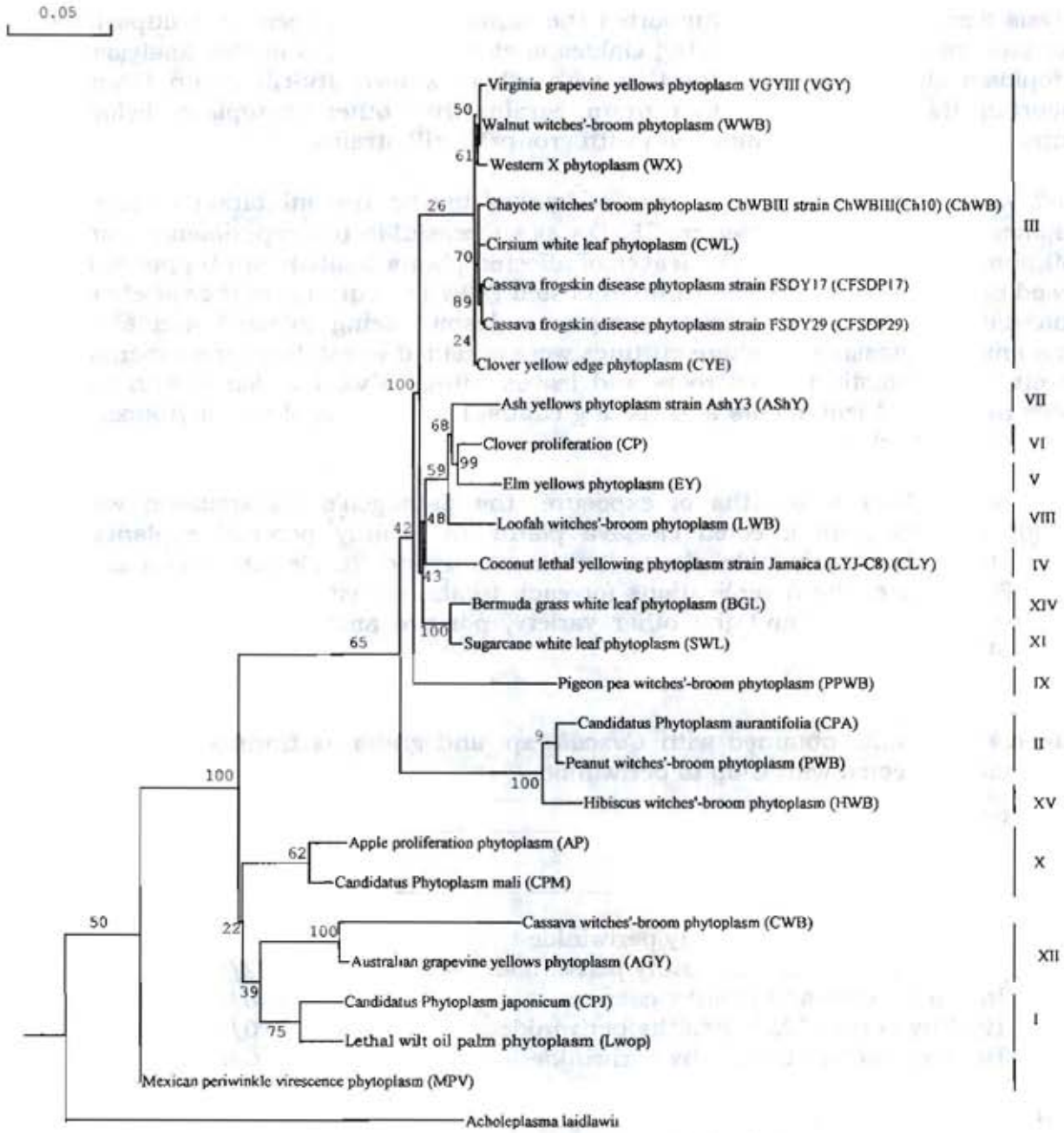

Figure 7.4. Phylogenetic tree of 16S rRNA gene sequences from 26 phytoplasm and *A. laidlawii* as outgroup, constructed by the Neighbor-Joining method. The branch lengths are proportional to the numbers of inferred character state transformations. Numbers on the branches are bootstrap (confidence) values. Roman numerals represent 16S rDNA RFLP groups according to ref. (Lee et al, 1998).

So far, we have not observed the characteristic symptoms caused by phytoplasm in periwinkle, even though we detected the presence of phytoplasm with the nested-PCR technique in infected cassava plants and later in periwinkle plants infected through *Cuscuta* or grafting. A major cause is the rather unfavorable environmental and greenhouse conditions for symptom expression.

7.4 Conclusions

Association of phytoplasm with CFSD, was confirmed, through DNA amplification with specific primers, designed recently, based on the sequence obtained in previous amplification by nested PCR. The phytoplasm was classified as X-Disease group. Cells characteristic of phytoplasm were detected in root phloem, by electron microscope. The phytoplasm structures observed were pleomorphic, comprising round, elongate, dumbbell, and ring-shaped elements, mostly 150 to 250 nm wide and 1000 nm long. The phytoplasm structures were limited only to phloem tubes and were never seen in large quantities. A phytoplasm was also detected in the insect *Scaphytopius marginelineatus* (Homoptera), trapped on cassava plants. Two DNA sequences of the Phytoplasm detected in cassava roots affected by CFSD, were reported to GenBank, which gave them accession numbers AY737646 and AY737647.

Characteristic foliar symptoms of CFSD were developed with the parasitic dodder plant (*Cuscuta* sp.) after infection by a pathogen transmitted from infected *in vitro* plants of cassava variety Secundina. Valencia et al. (1993), in studies on the transmission of the causal agent of witches' broom in cassava, caused by a phytoplasm, achieved 100% transmission between cassava and periwinkle, using *Cuscuta* sp. and grafts, after 3 months of exposure. This demonstrated that transmission of phytoplasm between these two different species is possible. These studies reported the expression of symptoms under growth chamber conditions with temperatures at 18–20 °C and RH at 44%–84%. The symptoms were not very severe, but vegetative depressions were observed in less than 6 months since transmission began.

The periods of incubation and optimal greenhouse conditions are fundamental for the expression of symptoms characteristic of phytoplasm in periwinkle plants. The plants evaluated as positive by PCR will be exposed to different periods and greenhouse conditions to seek the optimal for reproducing symptoms. This result provided evidence that CFSD is caused by a phytoplasm.

7.5.Identification possible vectors of cassava frogskin disease.

During 2003-2005 field surveys have been conducted in several regions of Colombia to collect possible vectors of CFSD. Emphasis is being given to homopteran species, especially those of the family Cicadellidae and Delphacidae, which are known vectors of phytoplasms and reoviruses (the later another candidate causal organism associated with CFSD). The objective of these studies was to identify the actual vector of CFSD (See CIAT PE1 Annual Reports, 2003, 2004 and 2005).

Specimens were collected from nine Departments in Colombia: Cauca, Quindío, Risaralda,

Tolima, Córdoba, Valle del Cauca, Meta, Atlántico and Sucre. Additional samples were collected from vegetation in fields adjacent to cassava fields (Table 7.5).

Observations during these collections indicate that the homopteran populations are very low in all of the sites surveyed. In some fields only 3 or 4 specimens were colleted. It was also observed that heavily weeded cassava fields contained a greater diversity of species, and this was especially noticeable as the diversity of weed species also increased. These observations may also indicate that many or most of the specimens we are collecting from cassava may not necessarily be feeding on cassava and are the present only because of the associated weed species.

Rearing Homopterans: It has been decided, that *Scaphytopius marginelineatus*, *Peregrinus maidis* and *Sogatella kolophon* are prime candidates as vectors of CFSD. This is based on the number of specimens we have collected and the frequency that we found these species in the different locations sampled. It is important to mention that in certain regions of Colombia CFSD seems not to spread from infected to healthy plants at least not with the rates of infections observed in Valle del Cauca Department. One explanation for these phenomena would be the absence in these regions of the insects suspected to be the vectors.

Methodologies developed for laboratory rearing of leafhoppers S. marginelineatus *(leafhopper) and plant hoppers:* Several field collections of this specie were made from cassava at the CIAT experimental stations in Santander de Quilichao (Cauca), during the months of August and September, when the populations of these species are high in these regions. This colony was maintained in a growth room under controlled conditions (26-27.5°C, 60-94% TH and 12:12 photoperiod). *S. marginelineatus* was able to feed and become established on the cassava varieties CM 6740-7, CMC 40 (MCOL 1468) and Secundina.

These results question the previously help observation that this species is only a sporadic visitor to the cassava crop. It was determined that approximate development period on cassava is 40.5 days under the aforementioned conditions.

The biology of S. marginelineatus: They are generally oviposited individually but 2 to 5 aligned eggs have been observed. As the incubation period advances, red ocular spots begin to appear and the characteristic of the embryo. Eggs hatch occurs 10 to 12 days after oviposition; 75.3% of the eggs hatched. There are five nymphal instars. Nymphs are mobile but can remain on the same leaf for parts of their cycle and occasionally nymphal instars exudates can be observed. The five nymphal stages last about 31.5 days. Egg to adult duration is about 40.5 days and adults can survive for 60 days. The population is polyvoltine. Adults (Figure 7.5) are very mobile and fly rapidly when the foliage is disturbed, peak activity is during the morning hours. *S. marginelineatus* is a piercing-sucking insect and damage is mechanical and physiological in that most cicadellidae are phloem feeders and may inject a toxin during the feeding; a gradual leaf yellowing occurs that increases in size until the whole leaf lobe is affected, after three to four days damage results in leaf fall (Figure 7.6).

Table 7.5. Homopteran species colleted from cassava fields at several locations in Colombia.

Dept.	Location		Species	Observations
Valle del Cauca	Palmira-CIAT	Cassava and weeds	*Scaphytopius* [1] *marginelineatus* *Empoasca bispinata* [1] *Peregrinus maidis* [2] *Sogatella kolophon*[2] *Perkinsiella* [2] *saccharicida* *Oliarus sp.* [3]	6 month cassava field plot, some cassava plants with CFSD
Cauca	Santander de Quilichao-Granja CIAT	Cassava and weeds	*E. bispinata* *Tylozygus fasciatus*[1] *Hortensia similis* [1] *Sogatella kolophon* Oliarus sp.	2 month cassava field plot, some cassava plants with CFSD
Quindio	La Tebaida	Cassava and weeds	*Planicephalus* [1] *flavicosta* *Agallia nielsoni* Freytag n. sp. [1]	5 month cassava field plot
	Armenia- La primavera	Cassava	*S. marginelineatus* *E. bispinata* *Stirellus bicolor* *Agallia nielsoni*	4 Month cassava field plot
	Marmato		*S. kolophon* *S. molina* [2]	
	Quimbaya-Vereda Querman	Cassava	S. *marginelineatus* *Hortensia similis* *Stirellus bicolor* *Planicephalus flavicosta*	4-5 months cassava field plot
Risaralda	Morelia-Santa Rita	Cassava	*Agallia n. sp.* *Stirellus bicolor* *Oliarus sp*	4 months cassava field plot
Tolima	Chicoral-Granja Nataima	Cassava	*Scaphytopius fuliginosus* *Empoasca bispinata*	Some cassava plants with CFSD.
	Gualanday	Cassava	*S. fuliginosus*	7 months cassava field plot
	Ambalema		*Empoasca bispinata*	6 months cassava field plot
	Espinal- San Francisco	Cassava	Empoasca bispinata	6-7 months cassava field plot
Meta	La Libertad		*Stirellus bicolor*	
Atlántico	Barahona-Palapa Caracoli	Cassava	*Empoasca bispinata* *S. marginelineatus* *Hortensia similis*	7-8 months cassava field plot
Córdoba	Cienaga de Oro	Cassava	*Empoasca bispinata*	7-8 months cassava field plot
Sucre	Corozal- Las penas	Cassava	*Empoasca bispinata* *S. marginelineatus*	7-8 months cassava field. Some cassava plants with CFSD.

1 F. Cicadellidae Identified by Dr. Paul Freytag (university of Kentucky)

2 F. Delphacidae, 3 F. Cixiidae : Identified by Dr. Stephen Wilson (University of Missouri)

Figure 7.5 Adult of S. *marginelineatus*.

Figure 7.6. Cassava leaves with typical damage.

Transmission of CFSD, evaluation of homopteran species as vectors:
Transmission studies were carried out in the entomology growth chamber (26.5°C, 65% RH and 12:12 photoperiod). Ten to fifteen adult *S. marginelineatus* were removed from the established colony and released into a nylon-meshed cage containing CFSD-infested cassava plants and allowed to feed for 7 days. "Secundina" is a CFSD indicator variety in that leaves readily express disease symptoms. After surviving *S. marginelineatus* were removed and places in cages with healthy Secundina plants and allowed to feed for 30 days. Plants were observed on a daily basis for CFSD leaf symptoms.

At the same time, *S. marginelineatus* adults were collected from a colony being reared on healthy Secundina plants, and were released into nylon meshed cages containing CFSD infected plants from variety MBRA 383. The adults remained on these plants until nymphs were obtained.

Adults that completed the 30 days feeding period on Secundina did not cause CFSD leaf symptoms on the healthy plants. It was therefore decided to do DNA extractions from the adults that feed on the CFSD infected Secundina as well as the adults and nymphs that feed on the infected MBRA 383. Adults from the healthy (CFSD free) colonies being reared on Secundina and beans were also evaluated. DNA extractions were carried out using the method described by Gilbertson and Dellaporta (1983) for PCR analysis.

Seventeen samples from the different developmental stages of *S. marginelineatus* were extracted and 1 to 2 individuals per sample were processed.

Nested PCR Analysis.
Of the 17 samples evaluated, a 50% amplification of the insects feeding on infected plants was obtained; the majority of these pertain to adults of *S. marginelineatus*. The presence of phytoplasms was detected by PCR (Figure 7.7).

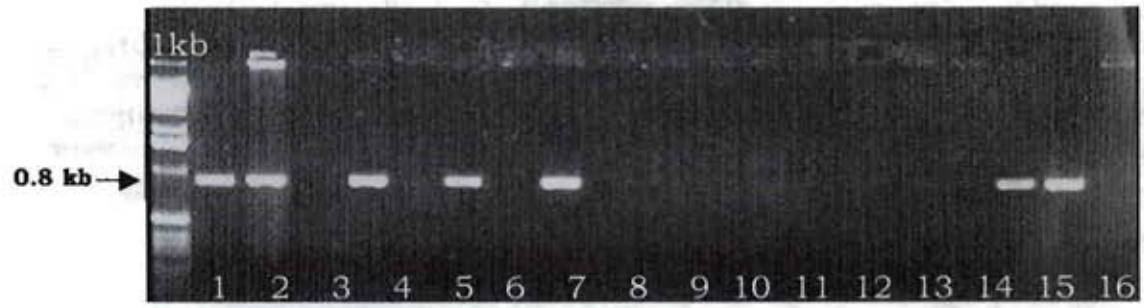

Figure 7.7. Presence of typical phytoplasms group 16SrIII for *S. marginelineatus* feeding on CFSD infected plants, lanes 1,2,4,6,8 and 17; lane 18 is the positive control and lane 19, the negative control; 1 kb: Molecular weight.

The sequences analyzed from the PCR products revealed that the fragments revealed that the phytoplasms from the insects was similar to *Cirsum* while leaf phytoplasms (Gen Bank acc. No. AF373106, 16SrIII X-disease group) with 100% homology in both fragments with a total of 800 pb sequenced. In addition a strong homology was found between the sequenced fragments from the insects and sequences reported in GenBank for phytoplasms associated with CFSD in the genotypes MCOL 2063 (AY737646) and SM 1219-9 (AY73647). This confirms that the amplified insects products are related to the phytoplasms associated with CFSD in cassava (CIAT, 2003). Based on these homologous results and on the Nested-PCR technique, new transmission studies are being evaluated, taking into consideration the previous evaluations done in plants considered healthy or diseased and later evaluating plants where the feeding homopterous insects were identified as possible vectors.

Peregrinus maidis *and* Sogatella kolophon *(planthoppers):*
P. maidis were collected from several experimental fields at CIAT where CFSD was present. It should be noted that several cassava fields were weedy, especially with *Rottboellia exalta* ("caminadora") and all nymphal instars of *P. maidis* were captured from this species. When weeds were removed, additional collections were made from these fields and high populations of *P. maidis* were again collected. This indicates that *P. maidis* can successfully feed on cassava in the absence of its preferred host.

S. kolophon was collected from several of the sites surveyed, while *S. molina* was only collected from fields in Armenia, Quindio. Cixiidae species are considered as possible vectors of phytoplasms, but few species have been studied.

Nymphs and adults of *P. maidis* and *S. kolophon* (Fam. Delphacidae) were collected from field plantings of cassava at CIAT, Palmira and Santander de Quilichao, Cauca, during the first trimester of 2005. *P. maidis* is placed on six plants of its natural host, *Rottboellia exalta* and *S. kolophon* on its host *Digitaria sp.* (Figures 7.8 and 7.9). At the same time *P. maidis* nymphs and adults were placed in a nylon-meshed cage containing both cassava and *R. exalta* (50% - 50%) in order to facilitate adaptation of *P. maidis* to cassava.

Greenhouse conditions were; Temp. 21 to 29°C; 63-100% RH and 12:12 hrs photoperiod. Experiments to adapt these homopteran species to cassava have been discouraging. Both species prefer to feed on their natural gramineous hosts rather than on cassava. Maximum survival on cassava is only four days once the grass hosts are removed. Although *P. maidis* is oligophagous, the adaptation to a "new" host appears to be difficult. However, these studies will continue.

Biological characteristics of P. maidis:
Hosts: this species is frequently found associated with maize, sorghum, millet, gramineous weeds and some shrubs and horticultural plants (Denno and Roderick, 1990). It is widely distributed, especially in the tropical and sub-tropical regions. Lifecycles studies: Under greenhouse conditions the egg to adult stages were 29-30 days. The egg stage is 10 to 12 days and nymphal development is 15 to 18 days. Females oviposit 17 $\pm$ 2.0 eggs per day and sex relation is 1.6 ♀; 1 ♂. There are five nymphal instars.

P. maidis is the recognized vector of five virus diseases, maize stripe, Iranian maize mosaic, maize mosaic, maize sterile stunt and finger millet mosaic, that affect maize and sorghum (S. Wilson, personal communication).

Figure 7.8. Adult of *Peregrinus maidis* feeding on *Rottboellia exaltata* leaves.

Biological characteristics of Sogatella kolophon:
Amply distributed, including the eastern Pacific, the Ethiopian region and in the new world it is reported from the USA, Mexico, Bermuda, Guyana and Argentina. This is the first established report from Colombia. The egg to adult stage under greenhouse conditions was 26 to 39 days. Egg eclosion is in 10 days and the average nymphal period is 15 to 17 days. Eggs are inserted in the central leaf vein and stem by using the ovipositor as a sword. Eggs are cream colored, turning more yellowish as they advance in development. Although eggs are similar to *P. maidis,* they are smaller.

Sexual dimorphism occurs in color and size; females are a clear yellow in color (Figure 7.9) while males are dark brown. *S. kolophon* is considered to be the vector of Brazilian wheat spike disease, Digitaria striate virus and maize sterile stunt virus.

Figure 7.9. Adult of *Sogatella kolophon* feeding on *Digitaria* sp. in the greenhouse

Evaluation of Peregrinus maidis *and* Sogatella kolophon *as possible vectors of CFSD:*

Virus acquisitions were conducted by first selecting 50 N3 and N4 nymphs from established colonies of P. *maidis* and *S. kolophon* in the greenhouse. These were allowed to feed on two-week cassava plants that showed marked symptoms of CFSD. These plants originated from stem cuttings of plants naturally infected with CFSD in the field that displayed typical root symptoms. This should guarantee the recovery of native strains of the CFSD virus.

The virology unit verified the presence of the CFSD pathogen in the plant, when the stem cuttings were selected. Samples were taken from the youngest leaves of the selected plants. The insects feed on cassava plants for 48 hours to acquire the virus. After this period the Virology Unit examined five individuals using PCR.

Individuals feeding on CFSD infected plants were separated into two groups: half were placed on *Digitaria sp.* plants (*S. kolophon*) and *Rottbollia (P. maidis)* for an initial period of 5 days. The second group was placed on healthy Secundina plants for 48 hours or until insect death. It is not known if these species can transmit the virus immediately after feeding on infested plants. To insure feeding the methodology used employed small cages that confined the insect to a specific site on the cassava plant. Nine cassava plants were used in the transmission trial and nine plants were employed as controls and held under the same conditions.

Inoculated plants were placed in the Virology Unit greenhouse under controlled conditions to wait expression of CFSD symptoms. Plants were observed daily during one-month period or until the appearance of symptoms.

Results.
Molecular studies with those individuals that have fed on plants with CFSD symptoms have provided conclusive results because phytoplasm was detected frequently in *S. marginelineatus* from affected cassava plants by CFSD. It is considered that the PCR methodology that was designed for insects is useful for phytoplasm detection in these insects.

At present CFSD transmission has not been achieved, as Secundina plants do not show leaf symptoms. During the course of these evaluations, changes in methodologies have occurred, but it is recognized that longer observational periods may be required as well as more detailed studies with these organisms.

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8. Disease Resistance in Cassava to Bacterial Blight and Phytophthora Root Rot

8.1. Resistance to Bacterial Blight

8.1.1. Introduction

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), is perhaps the disease that has caused more damage to this crop than any other disease over the last two decades. Losses to CBB have ranged from 12% to 90% (Lozano 1986). The disease has been reported in all regions where cassava is cultivated, in Asia, Africa, and Latin America.

A major strategy for managing CBB is to use varietal resistance, that is, to develop cassava varieties with lasting genetic resistance. This management alternative implies conducting genetic diversity studies of the pathogen to better understand the way it acts, and its co-evolution with its host. With such knowledge we can direct management strategies towards increasing crop productivity through disease resistance to targeted populations of the pathogen rather than fulfill a specific objective of overall resistance. Thus, we can prevent the economic imbalances that so often negatively affect the poorest farmers, who are the main producers of many important crops such as cassava.

Most of the breeding work at CIAT involves developing lines resistant to *Xam*. Many sources of resistance have been found, but about four cycles of evaluations in the field and evaluations with different isolates in the greenhouse are still needed to adequately differentiate escapes from truly resistant clones.

Although the genes controlling resistance are still unknown, molecular markers associated with the plant's phenotypic response to the disease can be detected through molecular biology techniques. Identified molecular markers comprise an important tool for breeders to select parents and identify resistant progenies. Previous studies on the genetics and number of disease loci involved in host-plant resistance to CBB revealed many QTLs as controlling disease resistance (Jorge et al. 2001). In this report, we describe our search for molecular markers. We evaluated 400 simple sequence repeats and identified resistance gene analogs, using four cassava varieties resistant to CBB and five non-specific primers.

Another important practice in managing CBB is to select and treat quality stakes. Immersion in hot water is a treatment that has been tested as effective in previous studies (CIAT 2001).

The objectives for this work were to analyze genetic variability of *Xam* isolates from different regions of Colombia, Venezuela, Brazil, and Cuba; to evaluate the resistance of promising cassava genotypes, elite clones and commercial varieties of cassava to *Xanthomonas axonopodis* pv. *manihotis*, causal agent of cassava bacterial blight (CBB); to develop RGAs and SSR markers associated with resistance to CBB; and to evaluate different genotypes and treatments for the management of CBB, and to train farmers in the zone.

8.1.2. Materials and methods

Characterizing Xanthomonas axonopodis *pv.* manihotis. Eighty five isolates of *Xam* were used, collected from different cassava varieties in Colombia, Brazil, Venezuela and Cuba. DNA from isolates was extracted, following the protocol by Boucher et al. (1985). A 1000-ng sample of DNA from five isolates was digested with two restriction enzymes (*Eco*RI and *Mse*I). The digested fragments were amplified with eight combinations of primers (Gibco-BRL, AFLP Analysis System for Microorganisms). The amplified products were denatured at 95°C and separated in polyacrylamide gels at 4% (w/v) in 0.5X TBE buffer for electrophoresis. To determine genetic variability among isolates, a phylogenetic tree was constructed, using the SAHN method and the tree option of NTSYS-pc 2.02 (FJ Rohlf, Exeter Software, New York).

Resistance to Xanthomonas axonopodis *pv.* manihotis

Evaluations in greenhouse

GM 315 family: This family was selected as the most segregant of four BC1 populations evaluated for reaction to CBB (*Xam*) in field conditions at Villavicencio in 2002 (CIAT, 2002). To confirm its response to CBB, 138 individuals of the progeny were evaluated under greenhouse conditions at CIAT, to several strains of *Xam*. Six-week-old plants were inoculated with a *Xam* suspension. The inoculated plants were incubated under greenhouse conditions for 5 days with 95% relative humidity (RH), and 28°C temperature. The pathogenicity evaluation was made 12, 19, and 26 days after inoculation, following a CBB severity scale where 1.0 corresponds to plants without symptoms, and 5.0 to plant death.

A figure was made of CBB frequency distribution in the 138 individuals of the GM 315 family evaluated, and another of the percentage of resistant, intermediate, and susceptible individuals. Individuals with values between 1.0 and 2.0 on the CBB severity scale were considered resistant, between 2.5 and 3.5, intermediate, and between 4.0 and 5.0, susceptible.

Promising cassava genotypes for Eastern Plains: During 2004 we evaluated, under greenhouse conditions, 65 promising cassava genotypes for agroecological zone 2 (Colombian Eastern Plains) for their resistance to *Xam*. We inoculated with four isolates obtained from Villavicencio (Meta). The plants were arranged in plots with four replications, with the isolate as the principal plot and the genotype as subplot. The susceptible controls were M Col 1505 and M Col 1522. The methodology for inoculation and evaluation was the same as described above.

Elite genotypes: In 2005, we selected 13 elite cassava genotypes for their agronomic characteristics and adaptation to agroecological zone 2 (Eastern Plains), together with 8 commercial varieties. We then evaluated these materials for resistance to two isolates (VM-12 and VM-14) of *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). The isolates came from cassava planted on the farm at CORPOICA "La Libertad" (Villavicencio) in June 2005. The methodology for inoculation and evaluation was the same as described above.

Field trials

Natural disease reaction of the genotypes, was evaluated with a disease severity scale from 1.0 to 5.0, where individuals with values in the CBB severity scale between 1.0 and 2.0 were considered resistant, between 2.5 and 3.5 intermediate, and between 4.0 and 5.0 susceptible.

Preliminary Yield Trial (EP) in Villavicencio: One hundred seventy five genotypes, planted in three different Preliminary Yield Trials (identified as experiments 1, 2, and 3) from selection in Clonal Evaluation Trial in 2002, were evaluated for reaction to CBB in Villavicencio, where these diseases are endemic. Four control varieties were evaluated for CBB resistance: Brasilera, Ica Catumare, CM 6438-14, and La Reina (CM 6740-7).

Advanced Yield Trial (ER) in Villavicencio: In a yield trial established in Villavicencio, 106 genotypes selected from Preliminary Yield Trials and from ER-2001, and varieties from a Regional Test (PR) were evaluated. The following cassava varieties were included as controls: Brasilera and La Reina (CM 6740-7) as susceptible, and Ica Catumare, Ica Cebucán, and CM 6438-14 as resistant.

Yield Trials in North Coast: We evaluated 96 cassava clones for their resistance to *Xam.* We planted 32 clones from an Advanced Yield Trial (ER) at La Unión (Sucre) during 2002 to 2004, and at Valencia, Chinú, and Ciénaga de Oro (Córdoba) in 2003 and 2004. We also evaluated 65 clones from a Preliminary Yield Trial (EP) at Corozal (Sucre). The experimental design was randomized complete blocks with 3 replications.

Back cross families: The progeny of four families (BC1) of cassava were characterized by reaction to CBB under natural disease pressure in Villavicencio 4 months after planting in a second evaluation cycle. The four families were:

Family	Cross	Individuals (no.)
GM 315	M Nga 19 × CM 9208-13	357
GM 316	M Nga 19 × CM 9208-26	399
GM 317	M Nga 19 × CM 9208-31	348
GM 318	M Nga 19 × CM 9208-73	238

The four families have a common male parent (MNga 19), resistant to various strains of *Xam.* Each individual was planted in plots of 6 plants. The highest value of disease expressed by plants within each plot was evaluated, and a figure drawn of the percentage of resistant, intermediate, and susceptible individuals of each BC1 family.

Diallel assay: A 10 × 10 diallel study, comprising 45 families with 30 plants each, was evaluated for reaction to CBB under natural disease pressure. The diallel was planted with three replicates at the Corporación Colombiana de Investigación Agropecuária (CORPOICA) "La Libertad" station. The 10 genotypes making up the diallel were:

CM 4574-7	CM 6740-7	CM 7033-3	SM 1219-9	SM 1565-15
SM 2058-2	SM 2219-11	HMC-1	M Per 183	M Tai 8

Molecular markers associated to CBB resistance

Resistant gene analog markers: A group of 32 primers NBS, Pto, XLRR and WipK, corresponding to conserved gene domains of plant disease resistance, were used to amplify similar sequences in the cassava genotypes selected as resistant to CBB.

The DNA of 4 resistant genotypes, together with that of M Nga 19 (parent resistant to CBB of the GM 315 family), and a group of individuals from the GM 315 family and acarid-resistant (AR) cassava family, were amplified by means of unspecific PCR in conditions of low annealing temperature, to obtain sequences associated with resistance to *Xam*, using degenerate primers.

The NBS primer is a sequence of the conserved domain of the nucleotide union site in the N gene of tobacco and RPS2 of *Arabidopsis* (Yu et al., 1996); XLRR is a sequence based on the region of Leucin-rich repeats of the RPS2 and Xa 21 rice genes (Chen et al., 1998); Pto is a kinase sequence involved in resistance in potato (Leister et al., 1996); WipK amplifies the conserved region of MAK kinase of coriander (Y12875), tobacco (D61377), and *Arabidopsis* (MPK3) (Ligterink et al., 1997); and KSU is a sequence that amplifies a region within the nucleotide binding site (NBS) domain.

The PCR product was submitted to electrophoresis in 2% agar gels in TBE 0.5X buffer, or in 6% acrylamide gels. From the DNA amplification of M Nga 19 and the resistant group of the GM 315 family visualized in acrylamide 6%, band elution was made and used as a template in a new PCR reaction with the degenerate primer that gave origin to the elution band, to augment the quantity of the sample to clone.

The PCR products and the elution bands were cloned to PGEM-T-easy vector, and then introduced into the *E. coli* DH5-α strain by electroporation. The transforming colonies were selected by color in LB/ampicilin/IPTG/X-gal medium. Clones with insertions greater than 300 pb were selected to be sequenced, and to seek homology with genes reported for disease resistance in various crops in the National Center for Biotechnology Information (NCBI) database, through application of the Basic Local Alignment Search Tool (BLASTx), where the sequence obtained with nucleotides sequences registered in the database was paired.

Simple sequence repeat markers: Towards the identification of SSR markers linked to CBB resistance, Bulk Segregant Analysis (BSA) was made in the family GM 315. Bulks were constituted from 11 resistant genotypes and 11 susceptible genotypes. BSA provides a method of focusing on regions of interest with molecular markers as against analyzing the entire genome (Michelmore, 1991). M Nga 19 (resistant parent) and contrasting bulks (11 individuals in each one) were evaluated, using 486 microsatellite primers, the SSR markers have been described elsewhere (Mba et al 2001; CIAT 2001).

Individuals from resistant and susceptible bulks were evaluated with candidate primers, which showed polymorphism between bulks. Candidate primers that showed polymorphisms between resistant and susceptible individuals were considered potential SRR marker associated with resistance. They were evaluated in the whole population to confirm association between SSR marker and CBB resistance in the field.

Association between SSR molecular marker and CBB resistance in the field was determined by a Chi-square Independence test (SAS Institute, 1999-2001). An association was considered significant if the probability of the null hypothesis (no association) was less than $P<0.05$ In order to evaluate marker reliability for resistance detection, its sensibility and specificity were measured, to know the probability for true positive and negative results, according to the marker.

Selection and treatment of cassava stakes

Three cassava varieties, La Reina, Vergara, and CM 4574-7 were planted on 0.5 ha at La Libertad farm (Villavicencio, Meta), and the following treatments were carried out:

- Healthy stakes were immersed for 10 min in a solution of Lonlife® at 2 cc/L.
- Healthy stakes were immersed for 10 min in a solution of copper oxychloride (at 3 g/L) and malathion (at 2 cc/L).
- Stakes infected with the bacterium *Xam* were placed in hot water (49 °C) for 49 min.
- Stakes infected with *Xam* and given no treatment.

8.1.3. Results

Characterizing Xanthomonas axonopodis *pv.* manihotis.

On analyzing the genetic variability of 85 isolates of *Xam* through the AFLP technique, three groups could be distinguished (Figure 8.1). The first group clustered at a similarity level of 0.6, and is formed of isolates from different localities in Colombia. The second group clustered at 0.7, and comprises 81% of the Venezuelan isolates included in this study, and 4 Brazilian isolates. The third group clustered at 0.4, and is formed by most of the Brazilian isolates, 3 isolates from Venezuela, 1 from Cuba, and 3 from Colombia. In this group, clustering below the 0.4 similarity level also occurred, indicating great genetic variability within the Brazilian locations, possibly related to the also high level of genetic diversity observed for the host plant (Roa et al. 1997; Sánchez et al. 1999). Cluster analysis led to the formation of three groups of isolates that could be separated by country (i.e., Brazil, Colombia, and Venezuela). Within these large groups, subgroups can be found, based on different areas within the countries.

Resistance to Xanthomonas axonopodis *pv.* manihotis

GM 315 family. The *Xam* strain isolated from the genotype GM 315-320 was selected as the most pathogenic on inoculating the susceptible check M Col 1505, and was used in evaluating the response of the GM 315 family under greenhouse conditions. The GM 315 family presented a segregation from resistance to susceptibility, and is normally distributed, confirming field evaluation results of 2002.A figure was made of the percentage of individuals resistant, intermediate, and susceptible to CBB (Figure 8.2). More than 60% of the evaluated individuals showed intermediate reaction to *Xam*, with values between 2.5 and 3.5 in the scale of disease severity, 23% were resistant, and 13.5% were susceptible.

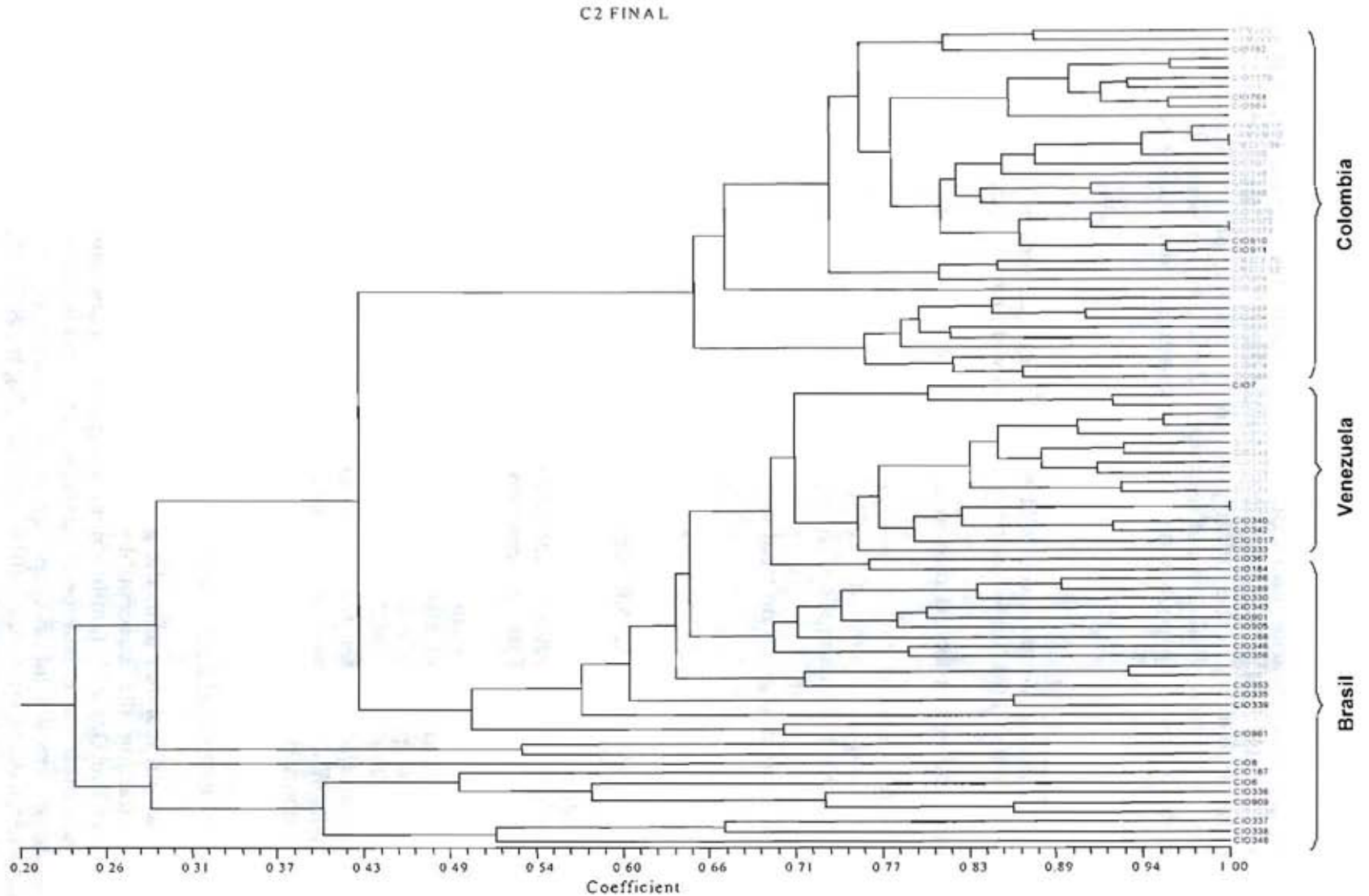

Figure 8.1. Similarity dendrogram of 85 isolates of *Xanthomonas axonopodis* pv. *manihotis*, based on AFLP analysis, using the unweighted pair-group method with arithmetic averaging (UPGMA) program of NTSYS-pc 2.02 (FJ Rohlf, Exeter Software, New York).

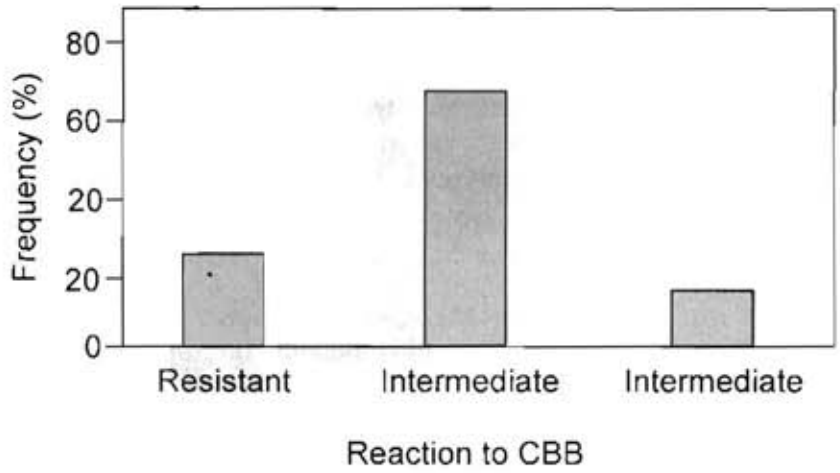

Figure 8.2. Reaction of family GM 315 progeny to cassava bacterial blight (CBB) under greenhouse conditions.

Promising cassava genotypes for Eastern Plains: Table 8.1. lists the highest reactions of the evaluated genotypes to four isolates of *Xam*. Genotypes CM 9459-13, CM 9460-41, and CM 9463-15 showed high resistance to CBB, with scores as high as 2.0. Commercial variety La Reina (CM 6740-7) showed an intermediate reaction to three isolates and was highly susceptible to another. The commercial variety Brasilera was susceptible to all four isolates. These results confirm field evaluations carried out in previous years (2002 and 2003 annual reports of Project IP-3). Elite genotype CM 4574-7 was susceptible to one isolate, and CM 6438-14 to two isolates.

Table 8.1. Reaction, under greenhouse conditions, of promising cassava genotypes for the Colombian Eastern Plains and susceptible check to three isolates of the bacterium *Xanthomonas axonopodis* pv. *manihotis*, obtained from Department of Meta.[a]

Genotype	Highest score[b]	Genotype	Highest score[b]
Brasilera	4.0		
CM 4574-7	4.0	CM 9464-33	2.5
CM 6438-14	4.0	CM 9464-36	2.5
CM 6740-7 (La Reina)	4.5	SM 2452-13	3.0
CM 9459-2	3.0	SM 2636-10	3.5
CM 9459-13	2.0	SM 2636-44	3.5
CM 9460-13	3.5	SM 2638-20	3.5
CM 9460-41	2.0	SM 2638-44	3.0
CM 9461-3	3.5	SM 2640-7	3.0
CM 9462-26	2.5	SM 2726-17	3.5
CM 9463-10	3.5	SM 2786-1	3.5
CM 9463-15	1.0	M Col 1505 (check)	4.0
CM 9464-19	3.0	M Col 1522 (check)	4.0

[a] Resistance is measured according to a scale of 1.0 to 5.0, where scores of 1.0–2.0 indicate resistant; 2.5–3.5, intermediately resistant; and 4.0–5.0, susceptible plants.
[b] Highest score for 4 isolates of *Xam*

Elite genotypes: Table 8.2 shows the highest severity value of the disease in the 4 replications. Isolate VM-12 was more aggressive than VM-14, demonstrating differences in pathogenicity. Clone CM 9460-3 was the most resistant to the two isolates inoculated, surpassing varieties such as ICA Catumare and ICA Cebucán, which had shown resistance to different isolates in research carried out in previous years.

Table 8.2. Resistance of elite clones and commercial varieties of cassava to two isolates of *Xanthomonas axonopodis* pv. *manihotis*, causal agent of cassava bacterial blight, from Villavicencio (Meta), Colombia.

Clone	Isolate[a]		Clone	Isolate[a]	
	VM-12	VM-14		VM-12	VM-14
Elite genotypes			Commercial varieties		
CM 9460-3	1.0	1.0	CM 4574-7	3.5	2.5
GM 220-59	3.0	3.0	Ginés	2.5	3.0
GM 223-15	2.0	2.5	ICA Catumare	3.0	2.5
GM 223-67	3.0	3.0	ICA Cebucán	3.0	2.0
GM 227-31	3.0	–	La Reina	3.5	2.5
GM 235-55	3.0	3.0	Vergara	3.0	1.0
GM 256-40	2.5	2.5	Verónica	4.0	1.5
SM 2726-17	2.5	–			

a. Values refer to scores on a scale, where resistant = 1.0–2.0; intermediately resistant = 2.5–3.5; susceptible = 4.0–5.0.

Field trials

Preliminary Yield Trial (EP) in Villavicencio: Eighty-nine genotypes (50.6%) were resistant to CBB, with scores up to 2.0 in the disease severity scale. The susceptible genotypes, Brasilera (score of 2.5) and Reina (score of 3.5), used as controls, showed slightly lower scores than in previous evaluations, indicating CBB pressure was not high in 2003. The most resistant genotypes were CM 9901-56, GM 219-31 and GM 240-31.

Advanced Yield Trial (ER) in Villavicencio: Genotypes SM 1697-1, SM 1143-18, SM 1565-15, SM 1807-1, and SM 1864-10, corresponding to 4.7% of genotypes, were highly resistant to CBB disease, with a score under 1.5 in the disease severity scale. Eight genotypes presented exudates on stem, and wilting, which correspond to a score of 3.0 in the disease scale. Five varieties and elite genotypes had intermediate reaction, scored up 2.5.

Yield Trials in North Coast: Ciénaga de Oro and Boca del Monte in Chinú were the sites with the highest disease pressure in 2003 and 2004, respectively. The ER clones with the highest resistance across all sites and years were CM 3306-4, M Tai 8, CM 4919-1, and SM 1438-2 (Table 8.3).

Table 8.3. Resistance of clones to cassava bacterial blight, corresponding to Advanced Yield Trials under field conditions in five sites in the Departments of Córdoba and Sucre, North Coast of Colombia.

Genotype	La Unión			Valencia		Ciénaga de Oro		Chinú 2004	
	2002	2003	2004	2003	2004	2003	2004	Heredia	Boca del Monte
CG 1141-1	2.0	1.0	2.0	1.0	1.0	2.0	2.0	1.0	3.0
CM 3306-4	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	–
CM 4919-1	–	1.0	1.0	1.0	1.0	2.0	1.0	1.0	2.0
CM 9560-1	2.0	1.0	1.0	2.0	1.0	2.0	1.0	1.0	2.0
M Tai 8	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	–
SM 1438-2	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0
SM 2545-22	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0
SM 2599-9	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	2.0
SM 2616-11	1.0	1.0	1.0	1.0	1.0	2.0	2.0	1.0	–
SM 2620-1	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	2.0
SM 2629-36	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	2.0
SM 2769-11	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0
SM 2773-21	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	2.0

a. Values refer to scores on a scale, where resistant = 1.0–2.0; intermediately resistant = 2.5–3.5; susceptible = 4.0–5.0.

Of the 65 clones evaluated in the EP, 40 showed resistance to CBB, 9 clones were intermediately resistant to CBB; and 14 were susceptible to CBB (Table 8.4).

Table 8.4. Resistance of clones to cassava bacterial blight (CBB) and superelongation disease (SED), corresponding to Preliminary Yield Trials under field conditions, Corozal (Sucre), Colombia.

Clone	CBB	Clone	CBB	Clone	CBB
CM 9907-41	2.0	CM 9966-57	2.0	GM 266-55	2.0
CM 9907-47	2.0	Ginés	1.0	GM 273-57	2.0
CM 9923-59	1.0	GM191-52	2.0	GM 274-1	2.0
CM 9926-35	2.0	GM 214-62	2.0	GM 274-10	2.0
CM 9926-49	2.0	GM 214-78	2.0	GM 274-14	2.0
CM 9952-37	2.0	GM 215-26	2.0	GM 290-14	2.0
CM 9957-33	2.0	GM 236-62	2.0	GM 290-20	2.0
CM 9957-35	3.0	GM 239-7	2.0	GM 290-39	1.0
CM 9957-75	2.0	GM 246-5	1.0	GM 290-42	1.0
CM 9958-32	2.0	GM 250-42	2.0	GM 290-65	1.0
CM 9958-44	2.0	GM 250-54	1.0	GM 302-48	2.0
CM 9958-76	2.0	GM 252-48	1.0	M Tai 8	2.0
CM 9958-80	2.0	GM 259-63	2.0	M Ven 25	2.0
CM 9966-45	2.0	GM 262-54	2.0		

a. Values refer to scores on a scale, where resistant = 1.0–2.0; intermediately resistant = 2.5–3.5; susceptible = 4.0–5.0.

*Back cross families:*At least 48% of individuals in each of the BC1 presented values less than 2.0 (Figure 8.3), which indicates that about half of the progeny of the four families showed high resistance to CBB in Villavicencio. Only in the GM 315 family did some individuals present a susceptible reaction to CBB, although the percentage was not representative (1%). There were no susceptible individuals in the other families (GM 316, GM 317, and GM 318). The CBB reactions registered in the four families may suggest that the natural pressure of the inoculum at the time of evaluation was very low compared to the pressure of the previous year, when the same families were evaluated and showed a good distribution of the disease in the progeny, the GM 315 family showing the largest segregation.

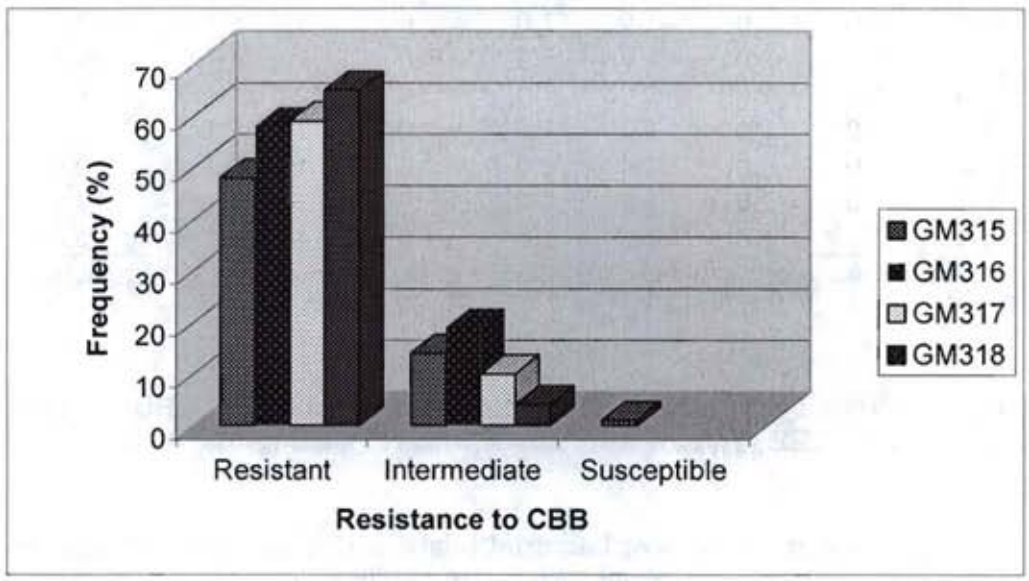

Figure. 8.3. Percentage of individuals resistant, intermediate, and susceptible to cassava bacterial blight (CBB) in the four BC1 families.

Diallel assay: Genotypes from crosses CM 4574-7 x SM 2058-2, and CM 4574-7 x HMC-1 showed higher specific combinatory ability (SCA) for resistance to CBB, with less disease severity. CM 4574-7 had higher general combinatory ability (GCA), showing the lowest average for severity of both diseases, taking into account the nine possible crosses with the other genotypes of the study. CM 6740-7 and HMC-1 showed the lowest GCA.

Resistant gene analog markers.

GM 315 family: The visualization of DNA of M Nga 19, and the resistant group of the GM 315 family, amplified with primers NBS, Pto, WipK, XLRR, and KSU, permitted the observation of a pattern of bands between 50 and 400 pb on agarose gel. Elution was made of a 500-pb band generated by the KSU primer in the four amplified genotypes, and of some bands between 200 and 400 pb observed in the acrylamide gel, for later cloning. The PCR product and eluted bands were cloned. Twelve NBS, 16 Pto, 15 Wipk and 32 XLRR clones were obtained with different primers, from DNA amplification of resistant genotypes.

The following clones showed homology with resistance genes reported in the NCBI database: X1, X5, X9, X15, X17, X18, and X19 from amplification with the XLRR primer, W5, W6, W9, and W10 from amplification with the WipK primer, P32, P41, and P36 from amplification with the Pto primer, and N31 from amplification with the NBS primer. For each of these clones, the presence of an open reading frame (ORF) was sought in the amino acid sequences deduced from the DNA.

The sequences that standardized with resistance genes reported in the NCBI, which also presented an ORF, were aligned among themselves, with sequences of known resistance genes, through the option of multiple alignments of sequences of the DNAman program version 4.13. A phylogenetic tree was constructed with them, through Parsimonia and bootstrap statistical analysis with 5000 replications (Figure 8.4), to determine the degree of similarity among the generated sequences and those reported.

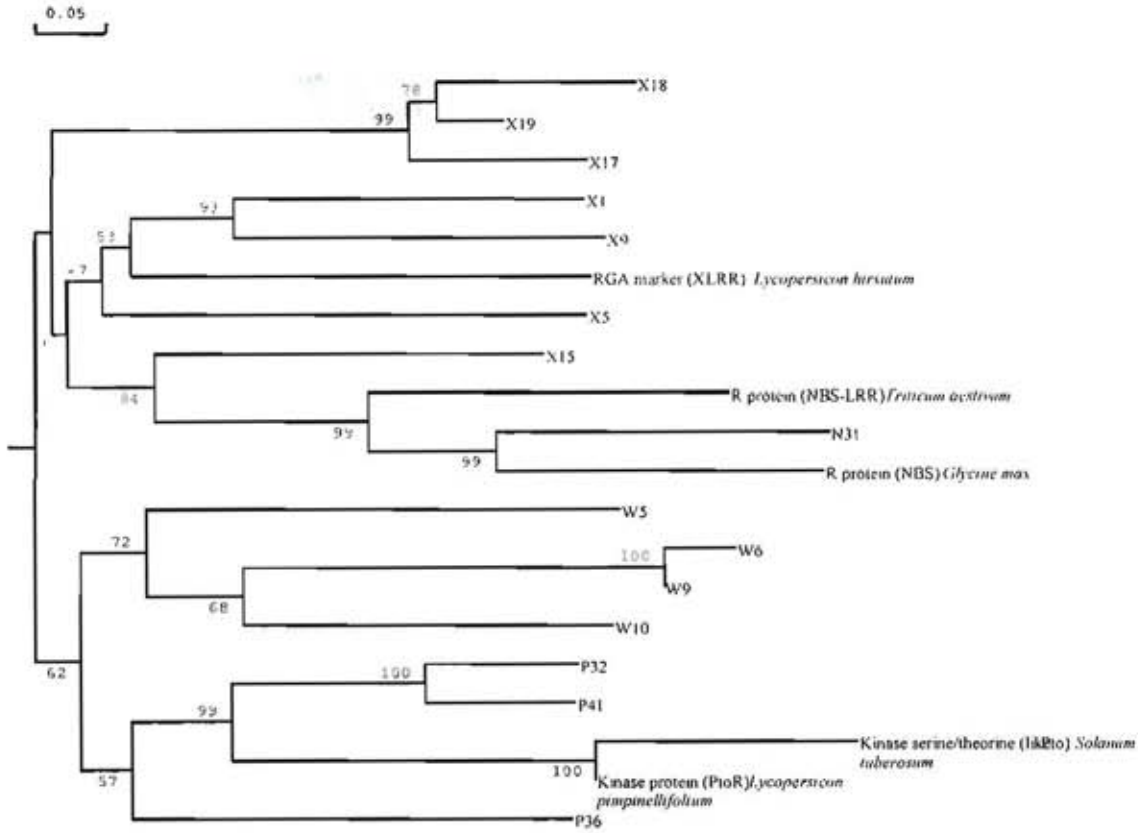

Figure 8.4. Phylogenetic tree of the sequenced clones that showed homology with resistance genes, carried out with Parsimonia and bootstrap analysis (5000 replications). X, clones XLRR; W, clones Wipk; P, clones Pto.

According to the phylogenetic tree, the sequences of clones from amplification with the XLRR primer (domain of Leucin-rich repeats) present similarity with the sequence of an RGA marker of the (XLRR) type from *Lycopersicon hirsutum*. Within the same group was found the sequence of a clone from amplification of the NBS primer (site of nucleotides union) that in turn shows high similarity with an R Protein type NBS-LRR from *Triticum aestivum* and

with an R Protein type NBS from *Glycine max*. The presence of XLRR and NBS clones in the same similarity group is explicable, taking into account that according to the type of resistance gene, the LRR and NBS domains are found together in the expressed protein, and are involved in pathogen recognition and signaling respectively, as in the case of the L6, N, RPP5, RPS2, and RPM1 genes, the first three mediating recognition of the virus and fungi, and the last two the recognition of bacteria, all at the level of cellular cytosol. Possibly, this group is indicating that a part of the XLRR sequences is encountering homology not only with the LRR region of resistance genes, but also with the NBS region of other resistance genes.

In addition, clones from amplification with the WipK primer grouped 62% of the bootstrap replicas with clones from amplification with the Pto primer; these last show similarity with a kinase serine/Threonine of Pto type, from *Solanum tuberosum* and a kinase (PtoR) protein from *Lycopersicon pimpinellifolium*.

Acarid-resistant (AR) cassava family: We chose the cassava crosses AR-1 and AR-9 for which high segregation was observed, to extract DNA from individuals belonging to these crosses to form bulk populations. One was of resistant individuals, and another was of susceptible individuals. We used 24 individuals from each cross to make the DNA mixtures. The crosses C 127 × CW 257-12 (AR-1) and C 243 × CW 257-12 (AR-9) showed significant segregation in studies on CBB, using bulked segregant analysis. The crosses permitted good phenotypic evaluation and are now being evaluated on a molecular level. To develop such a molecular evaluation, we selected the parents of the two contrasting groups, that is, C 127, CW 257-12 and C 243.

The resistant and susceptible bulks were formed, and 16 individuals were selected to represent each contrasting group, 8 resistant and 8 susceptible belonging to the AR-1 and AR-9 families, respectively. This group of 22 samples (4 parents, 2 bulks, and 16 individuals) was evaluated with 30 specific primers obtained from BAC libraries generated for studies seeking genes for resistance to *Xam* in cassava. Two primers (N-37 and N-38), designed by Llano (2004), were also used. They were then evaluated for their reaction to amplification and their level of polymorphism.

The primers BAC-27 and BAC-66 were selected as they each had a band showing polymorphism. These primers are possible candidates for amplifying one or more regions of the genome that may correlate with regions of resistance to the disease, expressed in a band that appears in resistant individuals but not in most individuals reported as susceptible. A clear example of such polymorphism found in the molecular evaluation of the contrasting groups was that obtained with primer BAC-66 (Figure 8.5).

The fragments generated with these specific primers are candidates for finding resistance gene analogs (RGAs). Once their bands are purified and their products sequenced, they will have the criteria needed for establishing whether they can be effective markers for regions of resistance to the disease produced by *Xam* and which are present in the genome of the AR cassava family.

On the basis of the positive predictive values, we suggest using the primers BAC-66 and BAC-27 in diagnostic tests for CBB within the AR family, as these can detect the presence of a band specific to most individuals resistant to the disease.

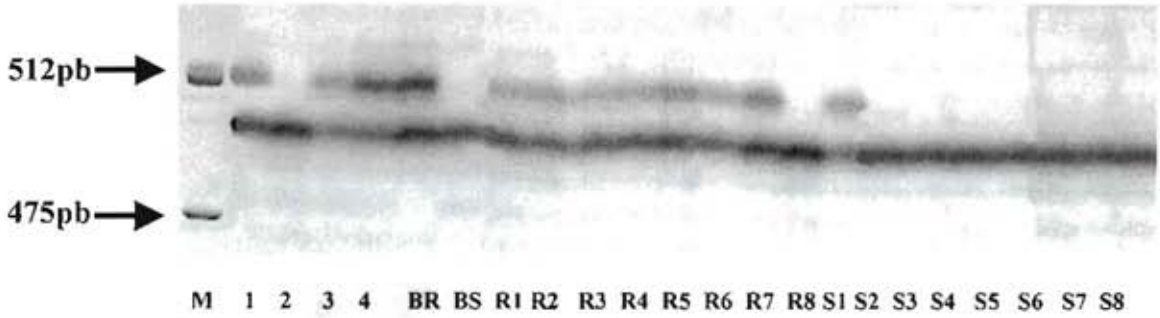

Figure 8.5.PCR amplification with primer BAC-66 of 22 samples of DNA from cassava genetic materials to lengths between 475 and 512 bp. Lane M = ladder of 30–330 bp; lanes 1–2 = parents of cassava family AR-1; lanes 3-4 = parents of cassava family AR-9; lane BR = bulk, resistant; lane BS = bulk, susceptible; lanes R1–R8 = resistant genotypes from the AR family; lanes S1–S8 = susceptible genotypes from the AR family.

Simple sequence repeat markers.

According to the frequency distribution for CBB resistance, the GM 315 family showed segregation for resistance/susceptibility and had high standard deviation (0.697) for individuals in each class: 46% of individuals of this family had a score between 2.1 and 2.5, thus showing intermediate resistance, whereas 6% scored less than 1.5 (i.e., resistant); and 2% scored more than 4.5 (i.e., susceptible).

Bulk segregant analysis of the family GM 315 revealed polymorphism between the parents and bulks with SSR markers. Primer SRRY65 showed differences between resistant and susceptible individuals of each bulk (Figure 8.6) and it was evaluated in the whole family (GM 315) (Figure 8.7)

RP RB SB **Resistant individuals** **Susceptible individuals**

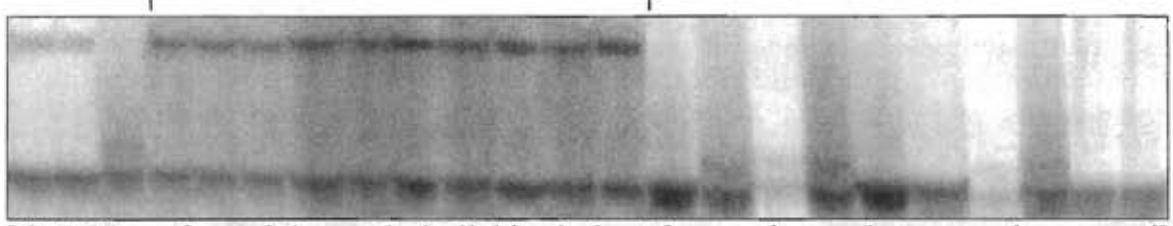

Figure 8.6. Primer SSRY65 evaluated in each individual that forms the resistant and susceptible bulks in family GM 315. RP: M Nga 19. RB: Resistant Bulk. SB: Susceptible Bulk

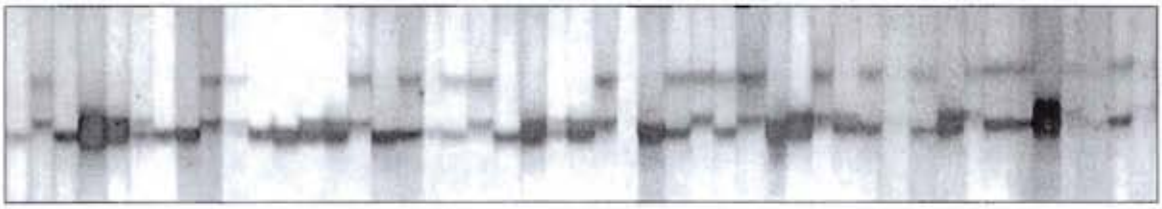

Figure 8.7. Some individuals from GM 315 family evaluated with SSRY65 marker

The presence of SSRY65 marker in resistant individuals of GM 315 family suggests association with CBB resistance from field evaluations. A probability of P=0.046 was obtained for association between marker SSRY 65 and field phenotypic data. Marker specificity was 66%, while its sensibility reached up to 55%, which was the percentage of individuals evaluated as resistant in the field, identifying the true positive results.

Selection and treatment of cassava stakes

The germination rate for all treatments was very low, with a maximum of 57.5%. Using healthy plant stakes, the variety with the lowest rate was Vergara (36.3%). Treatment with Lonlife® slightly improved germination rates for varieties La Reina and CM 4574-7 (Table 8.5).

For stakes from plants infected with CBB, the heat treatment reduced disease incidence to 3.1% in plants that developed from thin stakes, whereas incidence for thick stakes was 20.3% and the check (no treatment) 22.9%. Normally, germination is reduced when thin stakes are used. These results suggest that thick stakes should receive heat treatment over a longer period to guarantee cleaning of the material. However, farmers should be recommended to select more strictly for healthy plant stakes.

8.1.4. Discussion and conclusions

Previous studies have tested several molecular markers (repetitive fragment PCR, amplified fragment length polymorphism [AFLP], and ribotyping) for their usefulness in characterizing *Xanthomonas axonopodis* pv. *manihotis (Xam)* populations (Restrepo and Verdier 1997; Restrepo et al. 2004). The AFLP technique was the most informative and discriminatory for assessing genetic diversity in *Xam*, allowing us to conclude that *Xam* is a pathogen of considerable genetic diversity. Isolates from different ecological zones of Colombia fell into the same genetic group, indicating migration of the pathogen, probably through the movement of infected planting stakes. Even isolates from Brazil and Venezuela have been observed to share groups.

Taking into account that, for the farmer, varietal resistance is the most economic and efficient strategy for managing CBB, various sources of resistance were identified in both

elite genotypes and groups of materials being improved. Among the genotypes evaluated, CM 4574-7 stands out. This genotype also shows high general combinatory ability, making it a candidate for crossing, emphasizing resistance to CBB.

Table 8.5. Germination of stakes and dead plants from stakes infected with *Xanthomonas axonopodis* pv. *manihotis* receiving different stake treatments, Farm "La Libertad", Villavicencio, Department of Meta, Colombia.

Variety and treatment[a]	Germination (%)	Dead plants (%)[b]
La Reina		
Healthy stakes with Lonlife®	57.5	0.5
Healthy stakes with chemical treatment	54.3	0.2
Stakes with CBB, no treatment	35.4	22.9
Thick stakes with CBB, heat therapy	39.1	20.3
Thin stakes with CBB, heat therapy	23.1	3.1
CM 4574-7		
Healthy stakes with Lonlife®	54.6	0.0
Healthy stakes with chemical treatment	49.6	0.2
Vergara		
Healthy stakes with Lonlife®	29.6	0.6
Healthy stakes with chemical treatment	36.3	0.2

a. Healthy stakes were selected from plants with no symptoms of cassava bacterial blight (CBB).
b. From CBB-infected stakes.

To identify molecular markers associated with resistance to CBB, bulked segregant analysis is very useful (Michelmore 1991), allowing us to find an association between microsatellite marker SSRY 65 and resistance to CBB in the field and greenhouse. The specificity of the marker was greater in the greenhouse, as was the positive predictive value. This suggested that, under controlled conditions for evaluating the disease, we could be more certain of the marker's reliability, finding a higher probability of resistant individuals presenting band 1 generated by the marker and of susceptible individuals that would not present it.

We emphasize that although the greenhouse evaluations generated interesting results, in later trials, we had to increase the number of *Xam* strains evaluated to determine how the diversity of the pathogen affected the population, which, in the field, showed good segregation. Although we could not place marker SSRY 65 on the cassava map (the GM 315 family evaluated has no relationship with family K on which the only existing cassava map was generated), we could show that a clear association exists between the marker and resistance to CBB. The segregation presented by this family, as well as the contrasting nature of the parents, suggested its usefulness for later studies to evaluate against other pathogens and generate a new cassava map that would permit locating new markers that explain the resistance of cassava to different pathogens.

Different classes of RGAs were identified as associated with resistance to CBB. Results indicated that we could develop a strategy for seeking genomic regions associated with

resistance to CBB, designing specific primers based on sequences of RGAs detected in the cassava genome. The designed primers could be used to detect clear differences between amplified regions of individuals contrasting in their reaction to a determined pathogen. In contrast to SSR markers, the usefulness of this type of marker lies in its potential for use with different varieties of cassava and in the search for genomic regions associated with resistance to various pathogens, taking into account that the proteins for resistance have conserved domains. According to Bent (1996), an interesting characteristic of these genes for resistance is that they are related, gene by gene, with resistance to various diseases caused by fungi, viruses, bacteria, or nematodes.

For selecting and treating quality planting stakes, the hot-water treatment was identified as being more effective for thin stakes than for thick ones. The temperature reached was probably adequate to inhibit the bacteria in the planting material's deepest inner parts.

8.2. Resistance of Cassava to Phytophthora Root Rot

8.2.1 Introduction

A major problem of the cassava crop is root rot, caused by various species of *Phytophthora*. The disease is distributed throughout many production zones around the world, and losses of 70% to 80% to the disease have been reported, with average reductions of yield being estimated at 7.5 ton/ha. In Brazil, the species that most attacks cassava is *P. drechsleri* Tucker (Figueiredo and Albuquerque 1970), whereas in Colombia, it is *P. tropicalis* (CIAT, 2000), together with *P. drechsleri* (Oliveros et al. 1974) and *P. nicotianae* var. *nicotianae* (Soto et al. 1988; Lozano and Loke 1994). Other species reported as cassava pathogens in different countries are *P. erythroseptica* (Fassi 1957), *P. cryptogea* (CIAT 1991), *P. meadii*, and *P. arecae* (Barragán et al. 1998). The development of *Phytophthora* spp. is favored by inappropriate agronomic practices, ineffectual or unsuitable fungicides, transport of infected materials to zones free of the pathogen, and planting in compact or highly clayey soils (Takatsu and Fukuda 1990).

Currently at CIAT, selection for resistance to *Phytophthora* spp. is conducted under greenhouse conditions by inoculating cassava shoots and roots with isolates that had previously been characterized through molecular techniques, including digestion of the amplified ITS (i.e., the internal transcribed spacer region) of the 5.8s ribosomal gene with restriction enzymes, and appropriate pathogenicity tests. Deciphering the complexity of genetic resistance is a key element of plant breeding, particularly for diseases such as cassava root rot. We evaluated individuals of cassava family K for their reaction to root rots to better understand the genetics of resistance to three *Phytophthora* species. Genetic improvement for resistance to the disease can be more quickly and effectively achieved by using molecular markers.

A methodology was developed to evaluate resistance of cassava roots and leaves to *P. tropicalis* during its penetration and post-penetration phases. The methodology was then

validated in 22 cassava clones. Finally, the relationship between root resistance and leaf resistance was established in terms of the pathogen's two infection phases. To optimize the selection of cassava clones resistant to root rot caused by *P. tropicalis* biochemical and morphological markers were identified and validated. Other objectives were to identify disease resistance gene analogs, analyze homology in the cassava genome, and isolate disease resistance genes from monocotyledons through hybridization.

8.2.2. Materials and methods

QTL analysis:

In 2000 and 2001, we inoculated and evaluated the roots of 92 cassava clones belonging to family K (M Nga 2 × CM 2177-2) from Santander de Quilichao (Colombia). We used isolate 44 as inoculum. It was identified as *P. tropicalis* by sequencing the ITS region of its ribosomal DNA. We inoculated 10 to 12-month-old cassava roots, and extracted a cylinder of root, using a punch. A disk of mycelial growth was deposited in the orifice. Each inoculated root was placed in a plastic bag and on a plastic tray, and incubated for 7 days. Each inoculated root was cut transversely and the following measurements made: lengths and widths of lesion and cut, root length, and depth of inoculum in the root. The data were then processed through the Excel program. For analysis, the experimental unit was a root. The roots of 92 clones of cassava family K were harvested at CIAT. To analyze for QTLs, we used parental maps based on the segregation of alleles of the maternal and paternal clones according to different classes of markers (172 in maternal and 192 in paternal). These markers were restriction fragment length polymorphisms, random amplified polymorphic DNA, isoenzymes, microsatellites, expressed sequence tags, and known genes. The analysis and mapping were carried out with the QGene program, using simple regression or single-marker analysis. The dependent variable was the reaction to the pathogen and the independent variable was the number of alleles in the marker's locus, depending on the segregation of the individual. To minimize the detection of false positives, a significant association between the DNA marker and resistance to *Phytophthora* was determined if the probability of no QTL being present was less than 0 05.

Foliar and root resistance:

In Palmira (Colombia), roots were selected from 26 cassava clones. Roots were harvested, washed and disinfested with sodium hypochlorite, followed by ethanol, and then washed again. Leaves taken from the apical part of the stem of 22 clones, were harvested in Palmira and Jamundi (Colombia), disinfested in 10% ethanol, and washed twice in deionized water. For the experiments, we used isolate no. 44 from the collection held at CIAT and identified as *P. tropicalis*. Zoospores on disks of cassava leaves inoculated with isolate no. 44 were obtained as follows: disks of cassava leaves were extracted from 1-month-old plants. Eight disks were added to each of one petri dish per clon. The disks were then inoculated with fragments of mycelium in suspension, grown in nutritive broth. The petri dishes were incubated in the laboratory at temperatures between 25°C and 28°C and in alternating 12 h light and 12 h dark. The presence of sporangia was checked for daily.

Sporangia were harvested with a needle and then suspended in water containing Tween® 80. To inoculate the leaf lobes, a concentration of 1×10^4 zoospores/mL of inoculum was used. The roots were inoculated by placing a fragment of culture, 5 mm in diameter, in a perforation made with a punch (López and Lozano 1992). Re-isolation was obtained 5 days after inoculation by planting infected tissue in V8A medium modified with

antibiotics and fungicides (Sánchez 1998). Resistance to the penetration phase was determined according to the percentage of lesions, obtained at several inoculation sites in leaves and roots. The area covered by the lesion was used to indicate the degree of resistance during the post-penetration phase of infection in roots and leaves.

Cassava roots were inoculated without wounding by placing 3 to 9 disks of mycelial growth of *P. tropicalis* on each root's surface at several sites. The disks measured 5 mm in diameter and 1 mm thick. Each disk was then covered with masking tape. Each root was placed on a sterilized, moistened paper towel in a plastic bag. We evaluated four roots per cassava clone and incubated them at temperatures between 20°C and 25°C in the dark. As checks, one root per cassava clone was inoculated with a disk of medium culture with no mycelial growth. The 22 clones were organized according to a randomized complete block design.

At days 6, 9, and 12 after inoculation, transverse cuts were made to one or two roots per clone at the site where the inoculum was placed. The number of lesions was determined, and the infected area estimated. To determine the area infected by the pathogen, we took into account the area showing fluorescence under ultraviolet light at 365 nm (Spectroline®). To evaluate resistance to post-penetration, roots were inoculated by perforating the peel. Evaluation was carried out 6, 9, and 12 days later by making a transverse cut through each root section and determining the percentage of area infected, according to the symptoms described above. To determine resistance to penetration by the pathogen, 3 drops of each of the suspension of zoospores were deposited on the surface of the leaf lobe without wounding. Each drop was covered with a sterilized disk of filter paper, measuring 6 mm in diameter. To determine the resistance of leaf tissue, the midrib and blade of each leaf lobe was perforated with a punch with a 1-mm diameter. Each lobe was inoculated with a suspension of *P. tropicalis* at a concentration of about 1×10^4 zoospores/mL placed within the perforation, using a 200-µL micropipette. As negative checks, two leaf lobes of each clone were inoculated with sterilized distilled water.

All the lobes were incubated in the laboratory at temperatures between 20°C and 25°C. At 72, 96, 120, and 144 h of incubation, the lobes were evaluated in terms of the number of lesions formed and severity, using a semi-quantitative scale.

*Experimental design:*We evaluated 26 cassava clones inoculated with *P. tropicalis*, using four roots per clone and organized according to a randomized complete block design. Each root was inoculated between three and nine sites, which were each evaluated separately. The experimental unit was one root. Four to six roots per clone were used and the roots were organized according to the randomized complete block design. *Lobes.* Leaf lobes of the clones were organized according to the randomized complete block design. For each clone, four lobes were inoculated on their lower side at three inoculation sites, each of which was evaluated separately.

Statistical analysis: The roots were evaluated on 3 separate days. Observations made on the infected area of root parenchyma were standardized to generate an average per clone. To determine the significance of differences found between the reactions of clones in resisting the different phases of infection by *P. tropicalis* in roots and leaves, we carried out an analysis of variance and a LSD or Tukey's test, using the analytical package STATISTIX 8.0. The relationship between reaction at penetration and at post-penetration was proven by regression analysis.

Biochemical markers and agronomic traits associated with resistance: In Palmira (Department of Valle del Cauca, Colombia), roots of 10-month-old plants were selected from 26 cassava clones. The cassava roots were washed with running water to eliminate soil residues, peeled with a steel knife, and the peel then dried for 2 days at 40°C in an incubator. A mill was used to pulverize the peel. For each clone, iron and manganese contents were determined by atomic absorption spectrophotometry. Once harvested, six roots of each clone were washed with running water, then disinfected with 1% sodium hypochlorite for 1 min and 50% ethanol for 5 min, and given a final wash with sterilized deionized water. Each root received three 3-cm-long incisions to a depth of 1 cm. Three centimeters were cut off each end of the root and the new ends covered with cellophane. Each root was wrapped in moist, sterilized, paper toweling, placed in a plastic bag, and incubated at 20°C in the dark. To determine the area presenting fluorescence (scopoletin), the percentage of the fluorescent area was evaluated 10 days after harvest, based on seven transverse cuts made on each root on the day of evaluation. To measure the fluorescence in the roots, we used a dark booth with ultraviolet light at 365 nm (Spectroline®). Two types of resistance were evaluated: (1) resistance of peel to penetration, based on the frequency of lesions in the parenchyma with diameters greater than 1 mm, and obtained at several points of inoculation on the root; and (2) the size of lesion area was used to indicate resistance after the pathogen penetrated the parenchyma.

We carried out analyses of variance, using STATISTIX 8.0, to determine significant differences among the clones for area of fluorescence (scopoletin). Correlations were calculated between resistance and the following parameters: iron, manganese, iron-to-manganese ratio, and fluorescence (scopoletin). The correlations were then evaluated according to Pearson's coefficient and r^2.

Detection of RGAs: Root cylinders of cassava K (MNga 2 x CM 2177-2) and CM9582 (MBra 1045 x MCr 81) families were inoculated with mycelial discs from three species of *Phytophthora species* (*P. melonis*, *P. palmivora*, and *P. tropicalis*), to identify homologies of cassava genome regions to disease resistance genes from different crop species. The resistance of parents and progenies was also evaluated by measuring the volume of root showing rot symptoms 2, 4, and 6 days after inoculation. Two strategies were used to find resistance regions. The first consisted on hybridization with heterologous probes from maize and rice, using RFLP (restriction fragment length polymorphism), facilitated by Kansas State University (USA). The DNA of parents of the K family was digested with six enzymes selected based on previous work presenting polymorphic restriction in cassava. The probes were labeled with ^{32}P[dATP] and allowed to hybridize overnight, the film being revealed 15 days afterwards. The second strategy consisted of amplifying conserved regions of DNA, using PCR with degenerated NBS (nucleotide-binding sites) and Pto kinase primers. DNA was accordingly extracted from three cassava genotypes resistant to *Phytophthora* spp.— MBra 532, MBra 1045, and MCr 81—obtaining clones that were sequenced and homologated with known resistance genes, using the Blastx tool, in the NCBI (National Center for Biotechnology Information) database. Specific primers were designed with the sequences, allowing DNA regions of parental material and two bulks of 10 resistant individuals and 10 susceptible to be amplified. Bands were separated by denaturing polyacrylamide gel electrophoresis at 4% and 6%, and non-denaturing polyacrylamide gel or SSCP (single strand conformation polymorphism) at 10%. Sequencing was used to find single nucleotide polymorphisms (SNP).

8.2.3 Results

Resistance of family K to *P. tropicalis* was based on results obtained in 2003. Table 8.7 shows the results of the regression analysis for the simple marker as the percentage of infected area of roots inoculated in the laboratory. The markers identified eight QTLs located in the linkage groups C, H, J, N, Q, and V of the maternal map. The QTLs explained between 1.3% and 9% of phenotypic variance. NS911 was the most significant QTL, located in linkage group V. The markers identified six QTLs located in linkage groups A, D, I, M, and N of the paternal map. In this group, the most significant QTL was rGY32, which explained 11% of phenotypic variance and was found in linkage group A.

Table 8.7. QTLs that most explain phenotypic variance for resistance of cassava to root rot caused by *Phytophthora tropicalis*, as described by the percentage of infected area of the root.

Linkage group[a]	Markers[b]	F[c]	V[d] (%)	P[e]
Maternal map				
C (3)	rGY172	0 29	5.4	<0 500
H (8)	SSRY178	0.315	1.3	<0 500
J (10)	CDY76	0.163	4	<0 500
	K2a	0 40	8.6	<0 500
N (14)	SSRY13	0 78	4.2	<0 500
Q (17)	SSRY911	0 47	5.7	<0 500
V (22)	NS911	0 07	9	<0 070
	GY153	0 49	4.5	<0 500
Paternal map				
A (1)	rGY32	0 29	11	<0 100
D (4)	SSRY313	0.315	3.4	<0 500
I (9)	GY88	0.163	3.3	<0 500
	SSRY51	0 40	5.7	<0 500
M (13)	SSRY299	0 78	3.4	<0 500
N (14)	SSRY105	0 47	4.8	<0 500

a. Numbers in parentheses refer to linkage group number; b. Markers; c. F value; d. Phenotypic variance explained; e. Probability for no QTL associated.

Without wounding the roots, we obtained infection in all 26 clones inoculated with *P. tropicalis*. Differences were significant ($P < 0.05$) among the clones evaluated for root-peel resistance in the penetration phase, according to the percentage of observed lesions. By clone, lesions per root averaged between 18.8% (M Bra 383), and 97.3% (SM 1642-22). The percentage of lesions was relatively high in commercial varieties such as CM 523-7 (ICA Catumare, 66.2%), SM 2160-2 (59.8%), and M Per 183 (42.2%). Few lesions were recorded for M Col 2737 (26.8%), SM 1855-15 (31.9%), SM 1219-9 (23.8%), and CM 7951-5 (57.0%). These clones formed a cluster at the level of intermediate resistance (Tukey's test, $\alpha = 0.05$).

The percentage of lesions on leaf lobes, 3 days after inoculation, fluctuated between 36.8% and 100.0% (Table 8.8), with clones HMC-1 (36.8%) and SM 1779-7 (46.3%) being the most resistant, and SM 1871-33 (100.0%), CM 6660-21 (99.6%), and SM 1660-4 (92.4%) the most susceptible (LSD, $\alpha = 0.05$).

Table 8.8. Resistance to penetration and tissue resistance in roots and leaves of 26 cassava clones after inoculation with *Phytophthora tropicalis*, a causal agent of root rots. Averages are based on one experiment, otherwise values in parentheses indicate the number of experiments carried out.

Clone		Root resistance		Leaf lobe resistance	
		To penetration[a]	Of tissue[b]	To penetration[c]	Of tissue[d]
1	M Bra 383	18.8	54.4	–[e]	2.4 (2)
2	SM 1219-9	23.8	21.0	64.6	2.7 (2)
4	M Col 2737	26.8	6.4	–[e]	3.0 (2)
3	M Col 2760	28.8	40.8	66.0	3.1 (2)
5	SM 1855-15	31.9 (2)	19.4	81.3	3.6 (2)
6	HMC-1 (Manihoica P-13)[f]	38.2 (2)	–[e]	36.8 (2)	3.1 (3)
8	SM 2211-3	36.4	32.7	69.4 (2)	4.0 (3)
7	SM 2073-1	36.9	76.8	56.3	2.7 (2)
9	M Per 183 (Peruana)[g]	42.2 (2)	46.8	72.9	3.0 (2)
10	CM 8370-11	47.3	60.1	–[e]	3.2 (2)
11	SM 2085-7	47.5	70.9	72.9	2.4 (2)
13	CM 7951-5	57.0 (2)	25.6	84.1	2.5 (2)
12	SM 2198-4	55.6	56.4	72.9	2.9 (2)
15	SM 2160-2	59.8 (2)	64.3	72.9	2.7 (2)
16	SM 1871-33	59.8	54.0	100.0	3.2 (2)
17	SM 1520-16	59.8	41.8	–[e]	3.5 (2)
18	M Tai 8 (variety Tai)	59.8	41.3	86.8	3.2 (2)
14	SM 2058-2	61.7	43.1	64.6	3.5 (2)
19	SM 1965-1	64.0	46.0	64.6	3.3 (2)
21	CM 523-7 (ICA Catumare)	66.2 (2)	58.5	81.3 (2)	3.4 (3)
20	CM 8370-10	65.2	50.6	66.9	3.2 (2)
22	CM 6660-21	67.3 (2)	33.4	99.6	3.6 (2)
23	SM 1779-7	68.5	50.4	46.3	3.6 (2)
24	SM 1660-4	72.3	42.7	92.4 (2)	3.3 (3)
25	CM 7463-2	79.8	69.0	86.5 (2)	2.4 (3)
26	SM 1642-22	97.3	46.2	77.9 (2)	2.8 (3)
Minimum		18.8	6.4	36.8	2.4
Maximum		97.3	76.8	100.0	4.0
Average		52.8	46.1	73.5	3.1
SD[h]		18.9	16.8	15.6	0.4

a. No perforation of the inoculated tissue; resistance measured as percentage of lesions per inoculated root at day 10 after inoculation.
b. Inoculated tissue perforated; resistance measured as percentage of lesion area per inoculated root at day 10 after inoculation.
c. No perforation of the inoculated tissue; resistance measured as percentage of lesions per inoculated leaf lobe at day 3 after inoculation.
d. Inoculated tissue perforated; resistance is measured in terms of percentage of lesion area per inoculated lobe at day 5 after inoculation; scale of 1 to 5, where 1 = healthy tissue; 2 = mild symptoms of disease; 3 = intermediate symptoms; 4 = severe symptoms; 5 = whole leaf lobe is infected.
e. Not determined.
f. Check clone with a relatively high level of resistance to root rots, according to farmers in Colombia.
g. Check clone with a high level of resistance to postharvest physiological deterioration of roots (Teresa Sánchez, 2004, CIAT, unpublished data), but low levels of resistance to *P. tropicalis*.
h. Separation from the mean, according to Tukey's test at 5%.

Lesion size in roots varied significantly ($P < 0.05$) among the clones. SM 1660-4 was the most susceptible, with 64.3% of area infected (across two experiments; LSD, $\alpha = 0.05$). Detecting significant differences in percentage of lesions between clones, evaluating the lower side of leaf lobes was difficult. A combination ANOVA of experiments 1 and 2 did not help detect significant differences among the clones. However, HMC-1 (scale score of 3.1) and M Col 2760 (3.1) showed adequate levels of resistance in both experiments. We did not observe resistance in leaf tissue, as all the clones showed high degrees of susceptibility. However, we did see significant differences ($P < 0.05$) among the clones for leaf susceptibility to pathogen invasion, according to lesion size. Lesion sizes, averaging across three experiments, 5 days after inoculation (LSD, $P < 0.05$), for CM 7463-2 (scale score of 2.5), SM 1642-22 (2.8), and HMC-1 (3.0) were moderate (scoring 3.1 or less), whereas the relatively large lesions (scoring equal to or more than 3.0) were produced in SM 2211-3 (3.8), CM 523-7 (3.5), and SM 1660-4 (3.4).

Clone HMC-1 has a relatively high level of resistance to root rot in the field, according to evaluations carried out by cassava farmers in Colombia. Its reaction for leaf resistance (lesion size) to the pathogen is significantly smaller than that of the most susceptible clones. We need to include in trials validating the methodology, a larger number of clones with known reaction in the field to root rots. Clone M Per 183 has a high level of resistance to postharvest physiological deterioration of roots but with low levels of resistance to *P. tropicalis*. Data show that the root peel and parenchyma of clone CM 523-7 (ICA Catumare) is susceptible to *P. tropicalis*. However, clone SM 1855-15 (female parent is CM 523-7) has considerable levels of resistance in both root tissues (Tukey's test, $\alpha = 0.05$). A correlation of +0.31 (25 clones) was observed for root resistance during (in root peel) and after penetration (in root parenchyma), indicating that the two phases are moderately associated.

We did not obtain correlation (leaves of 22 clones, -0.01) between the reactions caused by the penetration and post-penetration phases of the pathogen in leaves. These two forms of resistance within each tissue (root or lobe) are apparently independent, suggesting that resistance to penetration cannot be predicted by tissue resistance. The analysis of Pearson's correlation carried out for the reactions during penetration phase of the pathogen in the leaves and roots of 22 clones showed a moderately positive relationship between the two organs ($r = +0.37$, $r^2 = 0.14$; Figure 8.8). The moderately positive correlation obtained among the leaves and roots suggests that leaves can be used to predict resistance of roots in cassava populations. To corroborate the validity of using leaf reaction as indicator of resistance to *P. tropicalis*, a representative population of cassava should be evaluated.

Analysis of the 22 clones indicates that most of the clones (15 of 22 clones, or 68%) have similar levels of resistance to penetration in the leaf and root peel. Four clones showed a level of leaf resistance that was higher than average, but their level of root-peel resistance to penetration showed they were susceptible. Assuming there is no interest in selecting susceptible clones, then the reactions of 44% of clones (i.e., 4 of 9 clones) selected for their leaf reaction were false because they did not have higher levels of resistance in the root peel.

For the 22 clones, the correlation between resistance in root parenchyma (size of infected area on day 10 after inoculation) and resistance in leaf tissue is -0.43, with $r^2 = 0.19$.

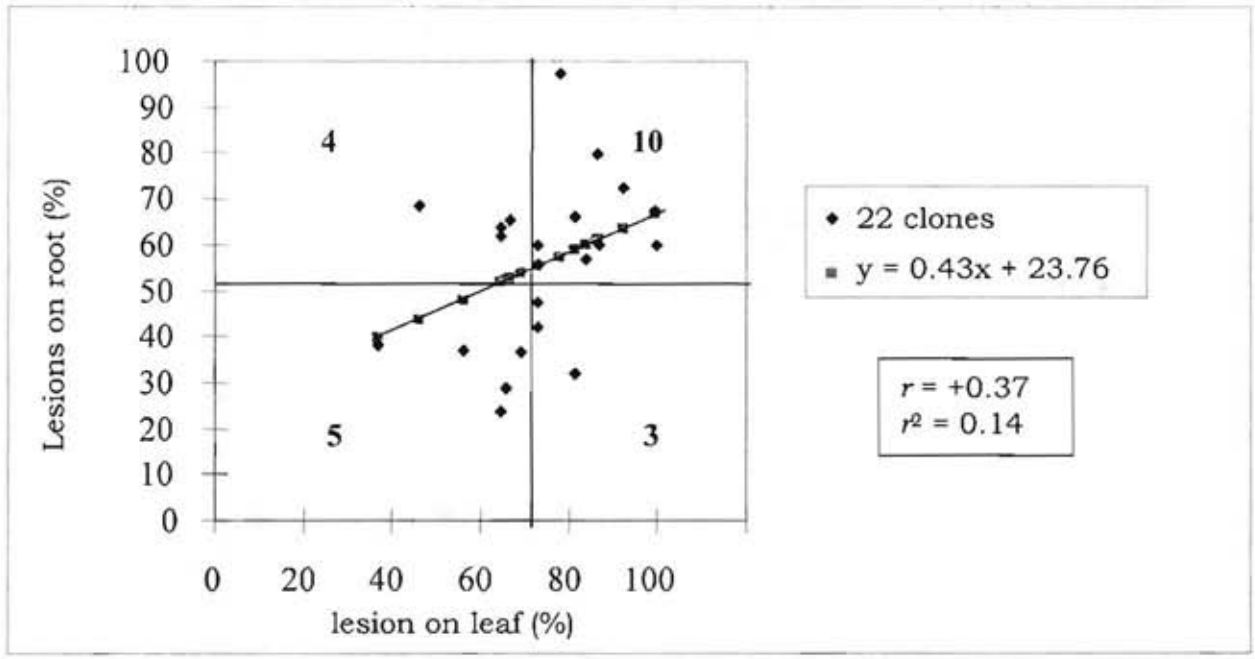

Figure 8.8. Relationship between percentage of lesions (at day 10 after inoculation) in roots and percentage of lesions (day 3) in leaf lobes of 22 clones inoculated with *Phytophthora tropicalis*, a causal agent of root rots. The number in each quadrant indicates the number of clones. The line represents a linear relationship between the two parameters.

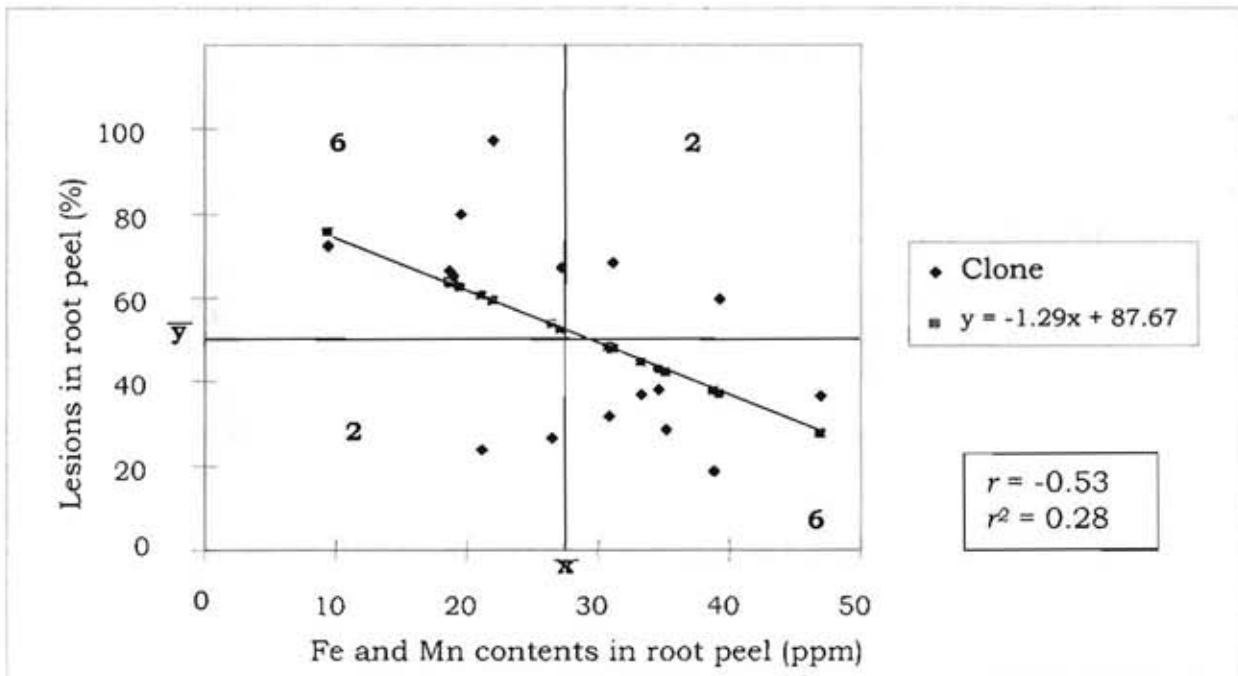

Figure 8.9. Relationship between resistance to *Phytophthora tropicalis* in roots (percentage of lesions, 10 days after inoculation) and the ratio of Fe to Mn contents in the root peel of 16 cassava clones. The bold number in each quadrant indicates the number of clones. The diagonal line represents a linear relationship between the two parameters.

Table 8.9. iochemical characteristics of the roots of 26 cassava clones and their resistance to *Phytophthora tropicalis*, causal agent of root rot.

Clone	Peel				Resistance	
	Fe (mg/kg)	Mn (mg/kg)	Fe/Mn (mg/kg)[a]	Fluoresc. in parench.[b]	Peel (% of lesions)	Parench. (% of infected area)
CM 523-7	119.1	6.4	18.7	47.8	47.8	58.5
CM 6660-21	255.6	9.4	27.2	17.6	17.6	33.4
CM 7463-2	184.7	9.4	19.6	60.8	60.8	69.0
CM 7951-5	–[c]	–	–	40.5	40.5	25.6
CM 8370-10	138.9	7.3	18.9	43.8	43.8	50.6
CM 8370-11	–	–	–	54.2	54.2	60.1
HMC 1	413.6	11.9	34.6	43.4	43.4	–
M Bra 383	278.9	7.2	38.8	33.2	33.2	54.4
M Col 2737	152.7	5.8	26.5	56.9	56.9	6.4
M Col 2760	239.4	6.8	35.3	34.3	34.3	40.8
M Per 183	–	–	–	41.7	41.7	46.8
M Tai 8	–	–	–	21.2	21.2	41.3
SM 1219-9	135.8	6.4	21.2	38.3	38.3	21.0
SM 1520-16	–	–	–	25.5	25.5	41.8
SM 1642-22	78.5	3.6	22.1	35.8	35.8	46.2
SM 1660-4	244.3	26.0	9.4	31.0	31.0	42.7
SM 1779-7	145.4	4.7	31.1	31.6	31.6	50.4
SM 1855-15	126.6	4.1	30.8	22.0	22.0	19.4
SM 1871-33	–	–	–	51.8	51.8	54.0
SM 1965-1	–	–	–	46.5	46.5	46.0
SM 2058-2	–	–	–	38.7	38.7	43.1
SM 2073-1	130.4	3.9	33.4	60.5	60.5	76.8
SM 2085-7	–	–	–	75.5	75.5	70.9
SM 2160-2	296.9	7.6	39.3	50.5	50.5	64.3
SM 2198-4	–	–	–	57.5	57.5	56.4
SM 2211-3	306.2	6.5	46.9	41.4	41.4	32.7
Minimum	78.5	3.6	9.4	17.6	17.6	6.4
Maximum	413.6	26.0	46.9	75.5	75.5	76.8
Average	202.9	7.9	28.4	42.4	42.4	46.1
SD	90.9	5.3	9.7	13.8	13.8	16.8

a. Ratio Fe/Mn in dry matter of root peel
b. Area (%) presenting fluorescence (scopoletin) evaluated 10 days after harvest
c. Not determined

Iron content in the root peel of the 16 clones is highly variable, fluctuating between 78.5 and 413.6 ppm. A coefficient of correlation was estimated as being -0.28 between Fe content and resistance (% of lesions in roots) to *P. tropicalis*. Although Mn is found in relatively low quantities—between 3.6 and 26.0 ppm—in the root peel and parenchyma of

the 16 clones, positive correlation was found with resistance to *P. tropicalis* (r = +0.21). This indicated a slight tendency for the percentage of lesions to increase with higher Mn content. A correlation of -0.53 (r^2 = 0.28) was found between resistance to penetration (% lesions on day 10) and Fe and Mn contents (in ppm) in the peel of 16 of the clones (Figure 8.9). The correlation between Fe and Mn contents is +0.42. Iron and manganese contents in the peel are not associated with resistance to *P. tropicalis* in the parenchyma.

Significant differences (P < 0.05) among the 26 clones were found with regard to area of fluorescence (scopoletin). Figure 8.10 presents the simple linear regression between fluorescence and the area of root parenchyma affected by *P. tropicalis*, based on averages of 25 of the clones. The coefficient of correlation for this association is +0.52, with r^2 = 0.28. Only clones M Bra 383 and SM 1779-7 showed low presence of fluorescence and high susceptibility to the pathogen (higher than the average for the other 25 clones). A correlation of -0.06 was found between the area of fluorescent parenchyma and resistance to *P. tropicalis* in the peel, thus indicating that no association exists between these evaluation parameters.

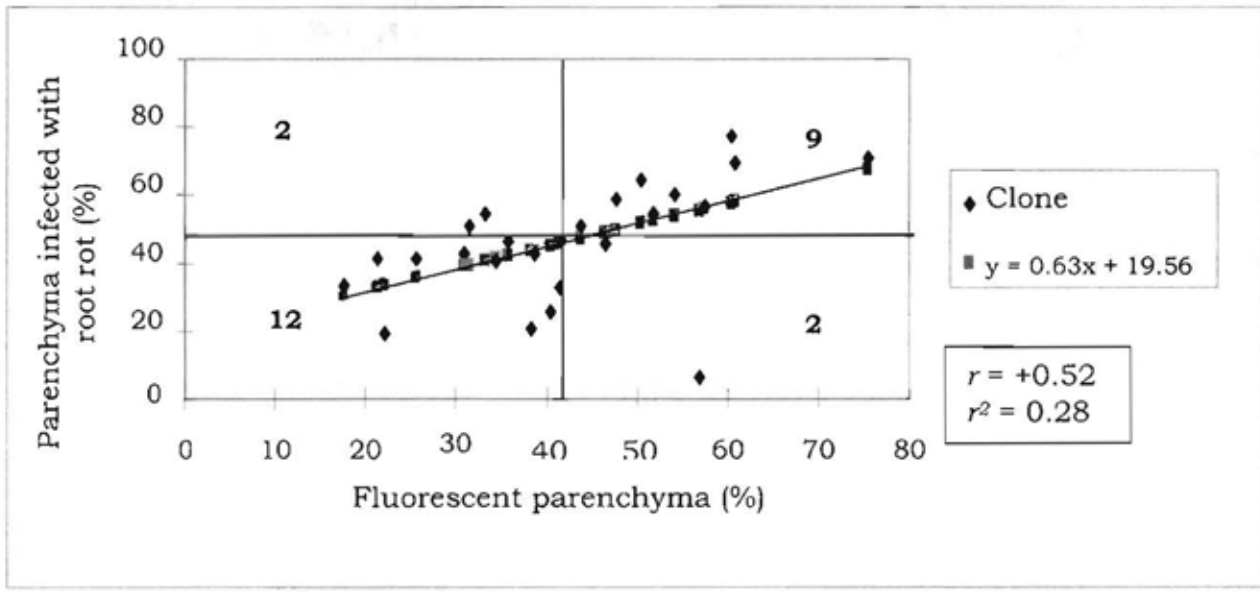

Figure 8.10. Relationship between the presence of scopoletin as observed by long-wave ultraviolet light and resistance of root tissue to *Phytophthora tropicalis* (causal agent of root rot) in 25 cassava clones. Both parameters are expressed as percentage of the area of a transverse cut of a root. The bold number in each quadrant indicates the number of clones. The diagonal line represents a linear relationship between the two parameters.

Five QTLs associated to *Phytophthora* spp resistance were identified in linkage groups E, G, H and O (Figure 8.11). By hybridization of the heterologous probe Pic 15, from maize, very faint bands were obtained when parental material was digested with *Eco*R V and *Hind* III. It was concluded that cassava has a very low homology with the genes from the monocotyledons tested. A total of 28 NBS and 2 Pto kinase clones were obtained using three

degenerate primers and subsequent cloning of the obtained fragments; of these, five showed homologous sequence with NBS-LRR RGAs (resistance gene analogs) reported by the NCBI (Table 8.10). Four of them showed open reading frames (ORF) with conserved motifs P-loop, kin-2, kin-3, and GLPL, of the NBS region, which means they were considered as RGAs. Three different RGAs were identified based on the phylogenic tree; of these, N-37 was similar to the Mi gene (non-TIR class); N-38 and K-1 were similar to the TIR genes L6 and RPP5; whereas N-33 was different from all the above. It was concluded that both TIR and non-TIR genes, subclasses of the NBS-LRR genes, are found in cassava. No polymorphism in the RGAs was found between the parents by PCR-RFLP, electrophoresis in polyacrylamide, SSCP, or SNPs, preventing the identification of association between the RGAs and resistance to *Phytophthora*.

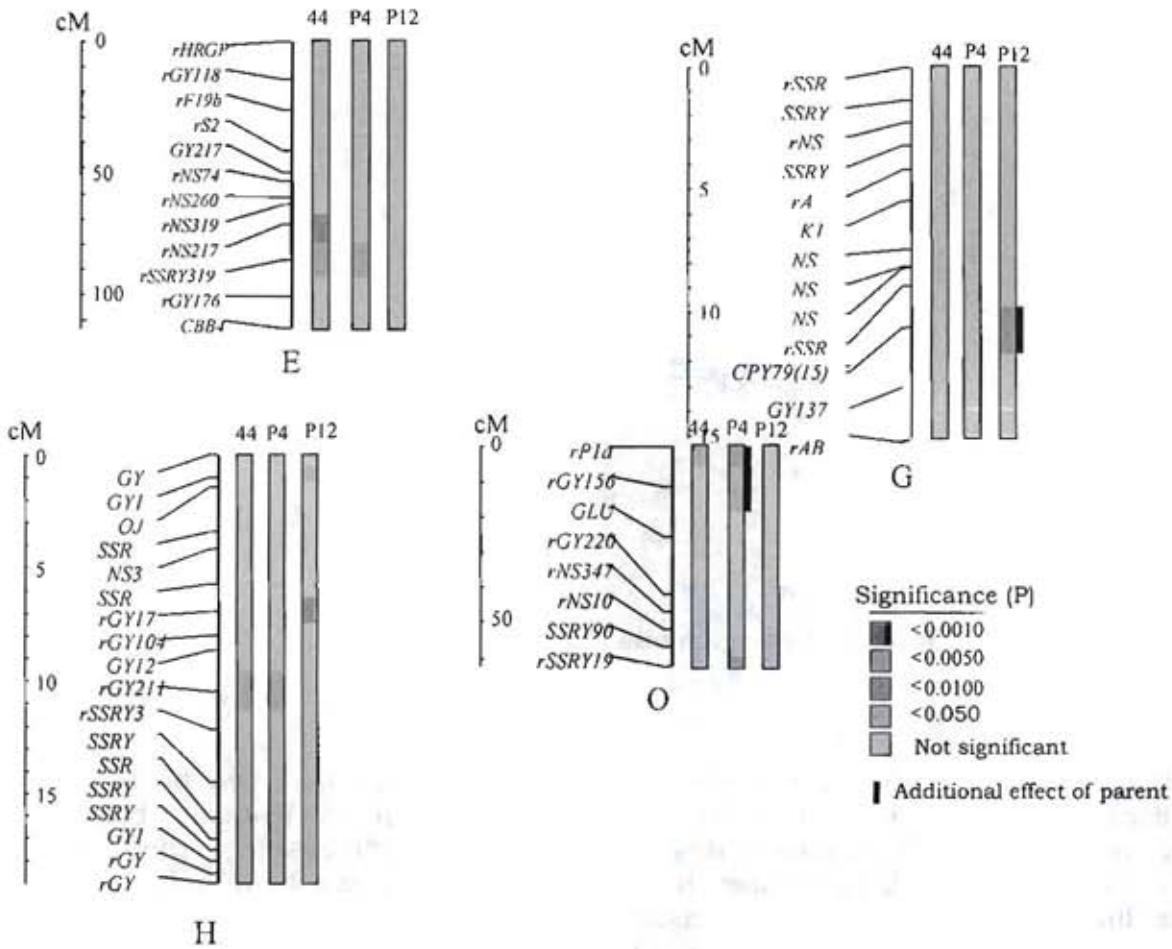

Figure 8.11. Map of identified QTLs, from the mother map (MNga 2), for resistance of the K family to *Phytophthora tropicalis* (44), *P. palmivora* (P4), and *P. melonis* (P12), in different linkage groups (E, G, H, and O). The color indicates QTL significance, according to the key at lower right of figure. The distance between molecular markers is shown in centimorgans (cM).

Table 8.10. Resistance genes analogs showing the highest homology with clones N-23, N-33, N-37, N-38, and K-1 isolated by PCR primers NBS and KSU from DNA of cassava genotypes M Bra 1045 and M Bra 532.

Protein codified by homologous sequences to each clone (GenBank) [a]	Species	Homology (bits) [b]	Probability of highest value	Identity (%) [c]	Positives (%) [d]
Clone N-23 (primer T7):					
Protein NBS-kinase Z2 (AF281282.1)	*Solanum tuberosum*	56	1e -07	35	47
Putative resistance gene analog, NBS-LRR (AF516642.1)	Malus prunifolia	54	5e –07	40	47
Clone N-33 (primer T7):					
Similar to resistance protein NBS/LRR (AF402735.1)	*Theobroma cacao*	103	1e -21	44	59
Candidate to resistance protein (AAC02202.1)	*Lactuca sativa*	97	1e -19	46	60
Clone N-37 (primer T7):					
Similar to resistance protein NBS/LRR (AF402735.1)	*Theobroma cacao*	92	1e -18	44	53
Homologous to disease resistance protein (AAD34880.1)	*Vigna unguiculata*	86.7	5e -17	44	53
Clone N-38 (primer T7):					
Putative protein similar to NBS-LRR (AF515627.1)	*Malus domestica*	126	8e -29	46	70
Resistance putative protein OB8 (AF363803-1)	*Phaseolus vulgaris*	125	2e -28	45	66
Resistance gene analog protein NBS-LRR-Toll (AF487946-1)	*Medicago sativa*	110	8e -24	42	67
Clone K-1 (primer T7):					
Putative resistance gene analog NBS-LRR (AF516642-1)	*Malus prunifolia*	146	1e -34	52	72
KNBS3, similar to resistance protein (AF325686.1)	*Glycine max*	142	2e -33	50	74
Resistance gene analog protein NBS-LRR-Toll (AF487946-1)	*Medicago sativa*	132	2e –30	50	72

[a]In parenthesis, National Center for Biotechnology Information (NCBI) code. [b]Homology (bits): Homology score according to number of homologous nucleotides and fragment length where there are homologous nucleotides. [c]Identity: Homologous genic products. [d]Positives: Homologous nucleotides.

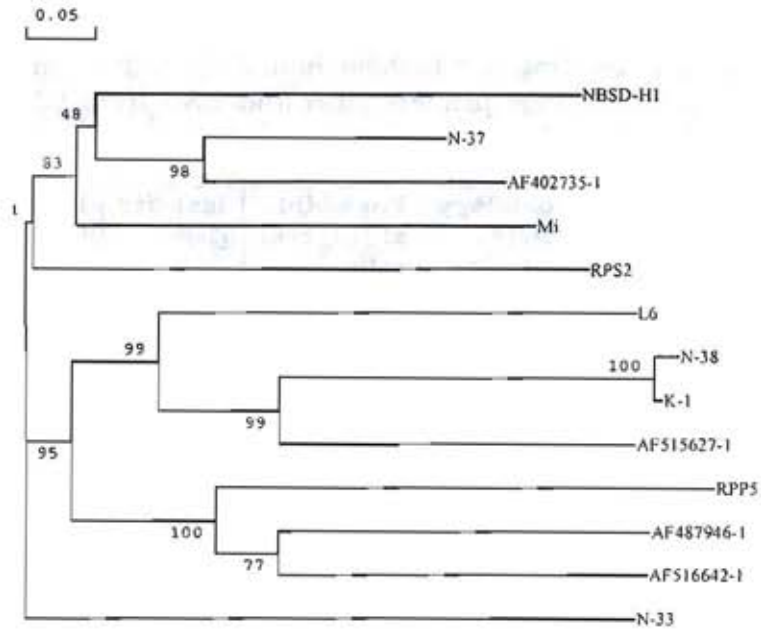

Figure 8.12. Phylogenetic tree of the amino acid sequences carried out with Parsimonia and bootstrap analyses (5000 replications) with the DNAman program, of the resistance gene analogues (RGAs) N-33, N-37, N-38, and K-1, compared with homologous sequences obtained from the National Center for Biotechnology Information (NCBI), two Toll protein and Interleukin-1 receptor (TIR) genes (L6 and RPP5), two non-TIR genes (RPS2 and Mi), the RGA of soya (NBSD-H1), and homologies found in the NCBI database (Table 8.10) AF402735-1, AF487946-1, AF515627-1, and AF516642-1.

8.2.4. Discussion and conclusions

The variability in the expression of resistance from year 2000 to 2003 indicates that cassava family K presents polygenes (Llano 2003). The QTLs associated with resistance to *P. tropicalis* in this study are different from the ones reported by Llano (2003). Nor were the linkage groups the same. Environment usually influences phenotypic expression, generating variation. Even so, certain clones of family K that had expressed intermediate resistance in 2000 continued expressing it in 2001.

Both parents of family K are susceptible to *P. tropicalis*. However, a group of clones in this family presented intermediate resistance. This indicates that the parents were heterozygotes and that both have resistance genes. Fregene et al. (1997) demonstrated that family K is heterozygous. This work is the first to report on an analysis of QTLs for resistance to root rot caused by *P. tropicalis* in a cassava population generating a map for each parent. The presence of individuals that are more resistant than the two parents and the detection of QTLs associated with the molecular markers of the map derived from the maternal parent of family K show that alleles for resistance that come from both parents contribute to resistance in the progenies. Such characteristics are well known in heterozygotic species and are useful for combining genetic factors of resistance in the same cultivar (Jorge et al. 2001). The markers rGY32, NS911, and K2a, which together explain 28.6% of phenotypic variation, will be evaluated in selection trials of cassava genotypes to

identify individuals resistant to root rot caused by *P. tropicalis*.

Agrios (1988) specified that resistance can be effective at the site of the pathogen's entry (i.e., penetration) or during its development within the host's tissue (i.e., post-penetration). In our study, the different cassava clones analyzed demonstrated that different levels of susceptibility exist for both phases of penetration and post-penetration during infection of leaves and roots by *P. tropicalis*. Such results occur according to inoculation method, suggesting that mechanisms of resistance operating in the penetration and post-penetration phases of the pathogen are different (Iwaro et al. 1997).

We observed high clonal variation in the resistance of root peel and parenchyma of cassava. No previous research has demonstrated that unlesioned cassava roots can serve as sites of infection for *Phytophthora*. This is the first report to determine resistance to *Phytophthora* in root peel. Although the responsible factors for *P. tropicalis* infection of root and leaf should be characterized completely, results suggest that two levels of resistance possibly exist in the roots. This implies that the selection of clones for resistance to root rot caused by *Phytophthora* should be carried out independently for penetration and for post-penetration, so that their combination may be used for breeding, thus increasing existing levels of resistance in cassava to *P. tropicalis*. The low correlation obtained between resistance of root peel to penetration and resistance of root parenchyma to post-penetration indicates that the mechanism conferring resistance is not systemic within the root. In several clones, we observed reductions in the size and number of root lesions, which may indicate quantitative resistance to *P. tropicalis*. Quantitative resistance is basically characterized by reduced growth rate, size, and number of lesions, latent period, and capacity to sporulate. These are controlled by several quantitative heredity genes (Parlevliet 2003). We found a negative correlation of -0.43 (r^2 = 0.19) between resistance of leaf tissue and resistance of root parenchyma. A biological reason may be that the translocation of photosynthetic products, which varies according to the physiological phases of the cassava plant, probably interferes with the level of resistance to *P. tropicalis* in leaves and roots.

This study indicates that resistance in root peel may be predicted by calculating the percentage of lesions found in leaf lobes inoculated in the laboratory or obtained from field plants. Thus, large populations of progenies can be evaluated during the plants' first phase in the field, hence, saving time and costs. The methodology developed for pre-selecting clones resistant to root rots by evaluating leaf lobes inoculated with *P. tropicalis* results in greater efficiency of cassava genetic-improvement programs. Adult roots, which may take a year to develop, are not required. Another advantage is the possibility of evaluating clones in the greenhouse or clones that are not adapted to the agroecological zones where germplasm banks are located. Obtaining leaves is easier than obtaining adult roots, especially in the greenhouse. The lack of a relationship between leaf reactions and root reactions in the post-penetration phase of the pathogen suggests that selection for this component of resistance is in the roots' enlargement phase.

We demonstrated that Fe and Mn contents in the root peel of 16 selected cassava clones and the ratio of these two microelements explain 28% of the phenotypic variation in resistance found in the clones. Garcia Mata and co-workers (2001) reported the effect of iron scarcity on *Phytophthora infestans*, showing that infection in cut potato leaves by *P. infestans* was drastically reduced when deferoxamine—an exogenous iron chelator—was applied. More analysis is needed to interpret these results. In tubers, messenger RNA of ferritin increased after treatment with an elicitor. These results suggest that iron has a

function in the interaction between potato and *P. infestans*. This is corroborated by findings that several soybean lines, resistant to different races of *Phytophthora sojae*, are tolerant of iron deficiency, which causes chlorosis of the plant (Helms et al. 2002; Orf and Denny 2000). However, Kaitany et al. (2000) report that plants of 12 soybean cultivars suffering high nutritional deficiency, particularly of iron, are more susceptible to *P. sojae*. A similar situation may occur in cassava.

In our study, a close relationship was found between the absence of scopoletin—a coumarin found in very low concentrations in fresh roots but which increases considerably after harvest (Rickard 1982)—and resistance to *P. tropicalis* in cassava roots. This discovery will make preselection of clones simpler and faster because inoculating root parenchyma with *P. tropicalis* will not be necessary. The use of this evaluation parameter in relation to resistance to *Phytophthora* is unknown for plants. However, the relationship between fluorescence of an area and deterioration has been reported by Rickard (1982). Agrios (1988) mentions scopoletin in relation to resistance.

We recommend integrating genetic improvement of cassava for biofortification, resistance to root rot caused by *P. tropicalis*, and post-harvest deterioration. The magnitude of the genotype-by-environment interaction for iron and manganese contents and area of root parenchyma with scopoletin is currently under study.

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9. **Molecular marker-assisted selection (for resistance to the cassava mosaic disease**

9.1 Introduction

Cassava mosaic disease (CMD) is the principal constraint of cassava production in sub-Saharan Africa and India. It is also a threat to South America where the disease is currently not reported but a biotype of the whitefly, *Bemisia tabaci*, vector of CMD viruses has recently become widespread (Polston and Anderson 1997) increasing the possibility that the CMD viruses (seven gemini viruses are known to cause CMD) or a native American gemini virus will cross over to cassava in the Neotropics. This is a frightening prospect for cassava production in Latin America considering that much of the Latin American cassava germplasm is highly susceptible to CMD (Okogbenin et al. 1998). The presence of CMD in Africa and India, and its absence in the Americas also limits the value of cassava germplasm from the crop's centre of diversity in Africa and India.

Host-plant resistance to CMD is the principal method of control and was first identified in third backcross derivatives of an inter-specific cross between cassava and its wild relative *M. glaziovii* (Storey and Nichols 1938; Jennings 1976). This source is the most widely deployed in Africa and it is thought to be polygenic with a recessive component (Jennings 1976; Hahn et al.1980). Recently, a second source of CMD resistance was identified in several local Nigerian cassava varieties and it is controlled by a single dominant gene *CMD2* (A. G. O Dixon 1989, unpublished data, Akano et al. 2002). The *CMD2* gene is currently being deployed in many parts of Africa. Molecular genetic mapping of *CMD2* identified eight molecular markers—five simple sequence repeats (SSR) and three sequence characterized amplified region (SCAR) markers—tightly associated with the dominant gene (Akano et al. 2002, CIAT 2004). The closest markers, RME1, a SCAR marker, and NS158, a SSR marker, located at 4 and 7 cM to *CMD2* respectively and explain about 70% of phenotypic variance (CIAT 2003; CIAT 2004). These markers open up the possibility of molecular marker-assisted selection (MAS) for breeding CMD resistance in neo-tropical cassava gene pools to protect them against an accidental introduction of the disease and also to permit the shipment of improved germplasm from CIAT to Africa and India. Molecular marker-assisted selection (MAS) for CMD resistance at CIAT is therefore both a pre-emptive measure, should in case the disease is accidentally introduced to Latin America, and a dynamic measure, to contribute CIAT's improved germplasm to India and Africa.

MAS for breeding CMD resistance have also been transferred to National Programs in Africa. Centralized breeding cassava programs of International Agricultural Research Centers (IARCs) is a multi-stage evaluation scheme lasting 8-10 years with farmers being introduced at the very end of the scheme. The low adoption of improved cassava genotypes coming from centralized breeding programs in many African countries led to the proposal of a decentralized and marker-assisted selection (MAS) breeding scheme in African NARS that uses MAS to reduce the sizes of breeding populations derived from crossing local varieties to improved introductions in a single cycle and introduce farmers by the 3rd year of the breeding program. A project to test this idea was initiated in Tanzania October 2003 with support from the Rockefeller Foundation. The proposed breeding project will take farmer preferred germplasm by agro-ecology and cross them to improved introductions that have resistance to Cassava Mosaic Disease (CMD), Cassava Green Mite (CGM), and Cassava Bacterial Blight (CBB). The progeny selected by MAS will be evaluated in a single season in

the corresponding agro-ecology and then evaluated over two cycles in collaboration with end-users (small farmers and cassava processors).

The Tanzanian MAS project seeks to improve local varieties for disease and pest resistance and provide a proof of concept for the MAS-PPB paradigm in cassava breeding but more importantly it is expected transfer useful variability from the crop's center of diversity of cassava to Africa. The concept has already been extended to three other NARs in Africa, namely Nigeria, Ghana, and Uganda, under the auspices of a Generation Challenge Program (GCP) competitive grant project 'Development of Low-cost Marker Technologies for Pyramiding Useful Genes from Wild Relatives of Cassava into Elite Progenitors'. It is expected to develop new varieties with resistance to cassava mosaic disease (CMD), delayed post-harvest physiological deterioration (PPD), and cassava green mite (CGM) through marker-assisted introgression of exotic genes from wild relatives from the center of origin into elite cassava progenitors. Cassava progenies with excellent resistance to CMD, CGM, and delayed PPD were shipped to NARs in Uganda, Ghana and Nigeria in the first year of the project for a MAS project similar to that ongoing in Tanzania. Simple molecular marker laboratories have also been established at the three participating NARs partners for the implementation of MAS. NARs partners include the National Root Crop Research Institute (NRCRI), Umudike, Nigeria, Crop Research Institute (CRI), Kumasi, Ghana, the National Animal and Agricultural Research Institute (NAARI), Namulonge, Uganda. A low cost method for MAS including, the use of agarose gels and PCR amplification of leaf squashes on FTA paper (Whatmann PLC, UK) are also being tested MAS in cassava.

9.2 Materials and Methods

MAS for CMD Breeding at CIAT (Neotropical cassava genepools)
Eighteen F_1 progenies of TME 3, a local Nigeria cassava variety having the *CMD2* gene, were established from embryo axes of sexual seeds at IITA and shipped to CIAT in 2000. These 18 genotypes are the source of CMD resistance for breeding at CIAT, they have been crossed extensively to elite parents of the four cassava gene pools as defined by agro-ecology, namely: the sub-humid lowland tropics, the acid-soil savannahs, mid-altitude valleys, and tropical highlands. The CMD-resistant progenies have also been crossed to beta- carotene and protein rich genotypes. Over ten thousand seeds have been obtained from crosses between CIAT lines and CMD resistant parent since the beginning of this program in 2002. A sub set of sexual seeds from the crosses were germinated in *vitro* from embryo axes according to standard protocols at CIAT (Fregene et al. 1998; CIAT 2002). After 3 or 4 weeks of growth, each embryo axes-derived plantlet was micro-propagated to obtain three to five plants. After another 4 weeks, leaves of all the plants were removed for molecular analysis and the plants micro-propagated again to obtain between 10 and 20 plantlets.

DNA was extracted from leaf tissue *of in vitro* plants using a rapid mini-prep method developed for rice (Nobuyuki et al. 2000). Two young leaves were placed in a 1.5ml eppendorf tube, containing 200 ul of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH = 8.0). The leaves were then crushed in the buffer, using a small pestle attached to a power drill, and incubated in a water bath at 100°C for 15 min. Next, 800 ul of TE buffer was added and the tube inverted gently several times to mix the contents, then centrifuged in a table-top centrifuge at 14,000 rpm for 10 min. The supernatant contained 10 to 20 ng/ul of total DNA, and 10 µl of this was used directly in the PCR. The supernatant was transferred to Eppendorf 96-well plates (Costar type), using an 8-tip multi-channel pipette for easy

dispensation into the 96-well PCR plates and for long-term storage. The DNA obtained was sufficient for 100 reactions and could be held in the Costar-type plates for 2 months at -20°C without DNA degradation.

All parental lines were evaluated with the 8 markers associated with *CMD2*, the progenies were evaluated with at least 2 polymorphic markers, preferably that flank the resistance gene. PCR amplification and PAGE or agarose gel analysis of the SSR or SCAR markers are as described by Mba et al (2003) and CIAT (2004). The gel image from the marker analysis was entered directly into an Excel sheet that contained information on the parents, tissue culture, and greenhouse records, and any phenotypic evaluation of the progenies. To reduce the cost of marker genotyping, PCR amplification of leaf squashes on FTA paper (Whatmann Inc., UK) was tested. Fresh leaves were harvested from 15 *in vitro* plantlets, 15 plants in the screen house, and 15 plants from the field, all plants were 2-3 months old. Leaf squashes were made using 0.5-2g of leaf tissue on FTA paper and a 1mm disc excised using a FTA paper punch supplied by the manufacturer (Whatmann Inc., UK). The FTA paper disc was either used directly in the PCR or washed as follows: the disc was transferred to a 96-well PCR plate and 200ul of 70% isopropanol was added and mixed using a pipette, the wash was repeated with IX TE (Tris 10mM, EDTA 1mM) PCR amplifications were conducted with both SCAR and SSR markers as described above.

Based upon the field performance of the CMD resistant hybrids generated in CIAT, particularly with respect to CMD resistance and yield, in Africa, new crosses were made between parents showing good specific or general combining ability, as demonstrated by progenies that showed superior agronomic performance in the field in Africa. Particular emphasis has been placed on crosses between CM523-7 and C4, parents of the CR14 family, progenies from this cross yielded progenies with excellent agronomic performance. Over 4000 sexual seeds were obtained from the new crosses made, the seeds were tested for viability and then established *in vitro* from embryo axes according to standard protocols at CIAT (CIAT 2003). DNA was isolated from leaf tissue harvested from one-month old tissue culture plants and analyzed with 2 polymorphic markers, NS158 and RME1 according to standard methods (CIAT 2003).

MAS for CMD resistance breeding in Tanzania (RF MAS and PPB Cassava project)

Following a decision made by CIAT management to permit direct transfer of cassava germplasm to African NARs without going through a third party, a committee was set up at CIAT to draw up guidelines for the safe transfer of cassava germplasm to Africa. The following recommendations were made by the committee:

- A request for cassava materials with information regarding the quarantine requirements of the receiving country.
- Only seeds from mother plants that are free of cassava frogskin disease (FSD) will be used. The mother plants will be inspected for root symptoms in the field. A significant sample of the mother plants will be tested using a diagnostic method appropriate for CFSD.
- A record of the results of testing will be kept, and one copy will be sent to ICA quarantine officer.
- Only plants that are placed *in vitro* through somatic embryo rescue will be exported.
- Permission to export plants must be obtained from ICA.

- All seed shipments from CIAT are accompanied by a Material Transfer Agreement (MTA).
- The receiving country will have a quarantine period before the release of these materials in the field. The quarantine facilities should be insect proof in order to be sure that no biological agents from the receiving country are introduced during the quarantine period.

Two different sets of germplasm coming out of the MAS for CMD resistance work at CIAT were shipped to Tanzania: the first group consists of 191 F_1 genotypes derived from crosses of elite CIAT lines to CMD resistant parents followed by MAS for CMD resistance (CR lines) and a second group of 335 BC_2 genotypes obtained from crossing CMD resistant lines to BC_1 derivatives of a wild progenitor of cassava having resistance to green mites(AR Lines). This second set of genotypes combines resistance to CMD and cassava green mites (CGM). Between 5 and 10 plants per genotype were shipped. The tissue culture plantlets were shipped in three batches, November 15 (CR plants), March 23 (AR first batch), and April 29 (AR second batch), to permit proper handling of plants in Tanzania. On arrival in Dar es Salaam the plants were received by plant quarantine officials from the Tropical Pesticide Research Institute (TPRI, Arusha) and transferred to the tissue culture growth room of Agricultural Research Institute (ARI) Mikocheni, Dar es Salaam. After 7 days in the growth room, to allow the plants recover from the stress of the journey, they were moved to the screen house at ARI-Kibaha for hardening according to standard methods laid down at CIAT (Roca et al. 1984). The plants were inspected after one month in the screen house by TPRI officials and at 2 months and molecular diagnostic for cassava frog skin disease (CFSD) performed just before transfer to the field. Molecular diagnostics for the presence of CFSD was carried out while the plants where in the green house to eliminate the possibility of an accidental introduction of the disease. A molecular diagnostic method for the detection of CFSD based on hybridization of an FSD cDNA clone CFSV-S5 was used. The method is a modification at CIAT of the to dsRNA extraction the Morris and Dodds method (1993).

After the second inspection and molecular diagnostics, the plants were transferred to the field at the Alawi estate, a 4000ha sisal plantation owned by the Mohammed Enterprises, interested in producing cassava for starch. Between 2 and 8 plants per genotype were established in the field in Kibaha in a single-row trial. The plants were evaluated for resistance to CMD 6 and 9 months after planting and at 11 months for yield and other traits of agronomic interest. The principal selection criteria in the introductions were resistance to CMD and CGM, little emphasis was therefore put on fresh root yield, number of roots and other traits that vary between tissue culture and clonally produced plants. Rather harvest index, a highly heritable trait was employed to select genotypes for the crossing block. The introductions were also evaluated for resistance to the cassava brown streak disease (CBSD), a major disease found in Tanzania in the expectation that a novel source of resistance to the disease could be found. For the local varieties the principal selection criteria was resistance to CBSD, a major production problem in the Eastern and Southern zones, and on farmer preferred characteristics, farmers were invited to participate in the culinary and agronomic evaluations of the local varieties

Concurrently, local cassava varieties were collected from the principal growing regions in Tanzania by NARS partners. Germplasm collected from the Eastern zone, around Tanga, Kibaha and the coastal areas of Dar es Salaam, and the Southern zone, Matwara, Lindi and Nachigwea, were established at the Alawi estate in Kibaha. Collections from the Lake region around Geita, Musoma, Tarime, Muleba and Kasulu districts were established at ARI-

Maruku. The experimental design of the trials was a random complete block design with 3 blocks and 10 plants per block and pest and disease resistance measured at 6 and 9 months after planting. Farmers from the different regions were invited at harvest (11 months after planting) to evaluate the collections after their own criteria and to select the best local land races for crosses.

A controlled and polycross crossing blocks were established at Chambezi experimental station situated about 60km North-west of Dar es Salaam for genetic crosses between the local introductions and local varieties. Three crossing blocks were established consisting of one controlled crossing block and two polycross blocks, a latin square design that maximizes the possibility of pollination between the local varieties and introduction under conditions of natural open pollination. The controlled crossing block and the two polycross blocks were planted in February 2005. The controlled crossing block consisted of 46 local cassava genotypes and 60 improved CIAT genotypes, the fifth block was planted with 25 CIAT genotypes as back up. All genotypes were planted at 1.8m x 2.0m spacing in 10-plant rows and a total of five blocks with each block having 30 genotypes. To increase chances of getting enough flowers, some of the commonly grown cassava genotypes for example Namikonga, Kalolo, Kitumbua, and Nanchinyaya, Kiroba Naliendele Kibaha were replicated four or three times. The two polycross blocks, one for the Eastern zone and one for the Southern contained 23 and 19 local cassava genotypes respectively along with 20 CIAT genotypes each. Chemical fertilizer NPK 20:10:10 was applied in split application, at one month after planting (MAP) and 2MAP respectively. To encourage flowering foliar fertilizer (COLJAP FLORESCENCIA) consisting of 10%N: 28%P:19% was applied on 12.7.05, 27.7.05, 31.8.05, and every two weeks after that. The crossing block was evaluated for CMD and CBSD resistance at 3 and 6 months after planting.

MAS for CMD resistance breeding in Nigeria, Ghana, and Uganda

CIAT has started using a modified version of the Advanced Backcross QTL scheme (Tanksley and Nelson 1996) to transfer useful genes from from wild relatives into cassava. Traits include resistance to CGM, hornworm, and white fly, and delayed post harvest physiological deterioration (PPD). CIAT has developed BC_1 and BC_2 populations with the above mentioned traits and have been multiplied *in vitro* for distribution to participating NARS and evaluation in the field at CIAT. A total shipment of 1,800 *in vitro* plants representing 100 CMD resistant (CR) genotypes and 200 CMD and mites resistant (ARs) were sent to the National Root Crop Research Institute (NRCRI), Umudike, Nigeria in two batches in November 2004 and April 2005. The materials were hardened in the screen house and inspected by officials of the Nigerian Plant Quarantine Services (PQS) and approval given for their transfer to the field. Between3-5 plants per genotype were successfully hardened and transplanted to the field for evaluation of disease and pest resistance at 6 and 9 months after planting. The materials were harvested at 11 months after planting and evaluated for resistance to CMD, CBB, and green mites. These materials will be shared with the Crop Research Institute (CRI), Ghana, located within the same West African region. Farmer-preferred varieties have also been collected in both Nigeria, twenty-three in number, and Ghana, thirty in number, and evaluated for pests and disease as well as farmer and end-user preference.

Similarly, 2246 plants comprising of CR and AR families, were introduced, as tissue culture plantlets from CIAT to Uganda in October 2004. Initially, the plantlets were placed in a growth room for 2 weeks, and thereafter potted in the screen house. In the screen house, the plantlets were potted in sterilized soil (3/4 top loam soil and ¼ sand soil), and placed in

the humidity chamber, where they were frequently supplied with both foliar and soil-applied fertilizers (Figure 1). The foliar-based fertilizers were intended to provide micro nutrients (Bo, Fe, Mn, Mg), while the soil based fertilizers provide macro nutrients (N, P, K). While in the screen house, observations on mortality and susceptibility to pests and diseases were recorded. The plantlets were transplanted to the field in March 2005, which coincided with the first rains of 2005. In the field, the entries were laid out in 10-plant rows, with a spacing of 1m between rows and 0.8m between rows. The number of plants per entry ranged from 1—10. To ensure accurate evaluations for CMD, the plantlets were established adjacent to older cassava fields with high inoculum for CMD. Although these progenies were primarily bred for CMD and CGM resistance, efforts were made to screen the materials for CBSD and cassava bacterial blight (CBB), whose inoculum was also high at NAARI. Evaluations for CMD, CGM and CBB were done using the standard 1-5 scale, were 1= resistant and 5= highly susceptible. CBSD was assessed basing on foliar symptom severity using the 1-5 scale, were 1= no symptoms and 5= defoliation, stem lesions and dieback. First and second assessments were respectively done in May and July 2005. A final assessment for CBSD will be done on the roots at harvest using a scale of 1-5, were 1= no necrosis and 5= >25% root necrotic. For entries with more than one plant, the means were computed from the phenotypic scores made.

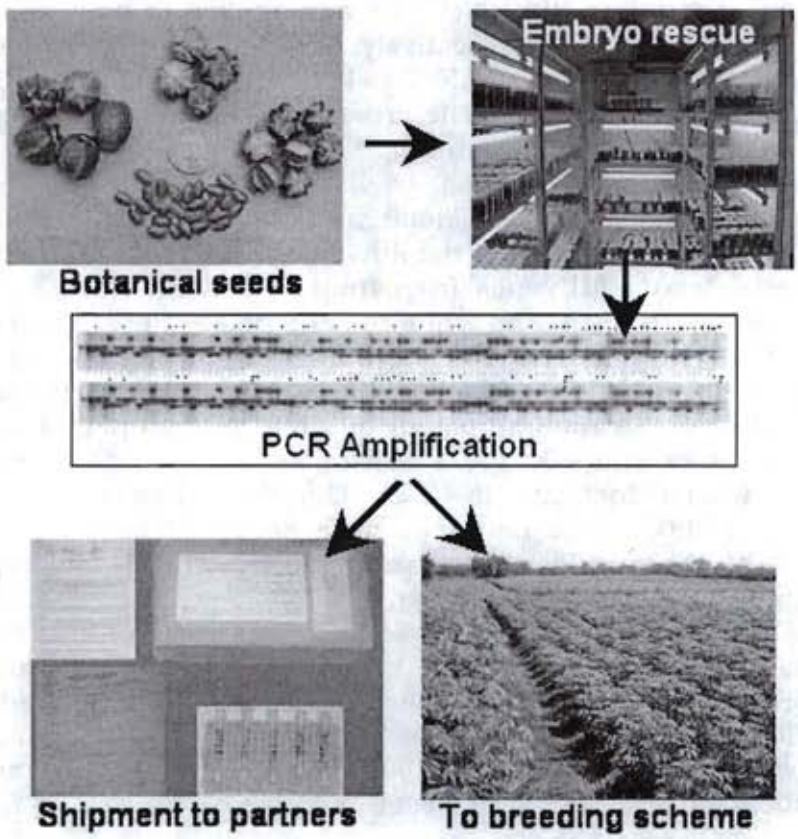

Figure 9.1 Schematic representation of steps employed in breeding for resistance to the cassava mosaic disease (CMD) in Latin America cassava gene pools. The entire process from sexual seeds to tissue plants for shipment or transfer to the screen house takes approximately 3 months

Additional *in vitro* materials having resistance to CMD, CGM, white flies and delayed PPD are being multiplied in CIAT and will be shipped by April to Nigeria, Ghana and Uganda. In addition to *in vitro* materials, CIAT has also shipped F_1 seeds to participating NARs to accelerate the introgression of desirable traits from wild relatives into the breeding scheme of participating NARs. The seeds were derived from three types of crosses: 1. crosses between *Manihot esculenta*; two. crosses between *Manihot esculenta* and wild *Manihot* species; and three. crosses between wild *Manihot* species). A total of 12,000 seeds were sent (i.e about 8,000 for NRCRI, 3,000 each for CRI and NAARI). At the moment about 750 seedlings have been transplanted to the field at the NRCRI, Nigeria after plant quarantine inspection.

9.3 Results

MAS for CMD resistance at CIAT

In 2003, 1,1000 genotypes were successfully established *in vitro* from embryo axes out of a total of sub-set of1, 550 seeds (70%) from 31 F_1 crosses between CIAT elite cassava parents and CMD resistant parents. They were micro-propagated and analyzed using molecular marker associated with CMD resistance. Figure 9.1 is a schematic representation of the process.

In 2004, 1291 genotypes out of a sub-set of1490 seeds (86%) were obtained from 43 BC_2 families as embryo axes culture and analyzed with markers associated with CMD resistance. In 2005 the number was 1,141 genotypes from a sub-set of1759 seeds (65%). In 2004, 191 genotypes of the F_1 and 335 genotypes of the BC_2 families were shipped to Tanzania (Tables 9.1 and 9.2). A total of 503 genotypes were successfully hardened in the screen house, transferred to the field, and evaluated for CMD resistance in the field at the Alavi estate where the CMD disease pressure was particularly high in 2004/2005.

Table 9.1. List of CR F_1 genotypes from with resistance to CGM and CMD resistance introduced to Tanzania this year

Code	Progenitor		Number of	Code	Progenitor		Number of
	Female	Male	Genotypes		Female	Male	Genotypes
CR11	MCOL 2206	C-127	15	CR44	C-39	CM3306-4	3
CR14	C-4	CM523-7	6	CR45	C-39	CM4574-7	9
CR15	CM523-7	C-33	2	CR46	C-39	SM1219-9	1
CR20	CM3306-4	C-33	5	CR49	C-243	CM4574-7	6
CR21	CM3306-4	C-243	3	CR51	C-243	OW280-1	5
CR24	CM7951-5	C-18	3	CR52	C-243	SM1219-9	15
CR25	CM7951-5	C-33	1	CR53	C-243	MCOL 2206	1
CR26	CM7951-5	C-39	1	CR54	C-243	MTAI 8	8
CR27	CM7951-5	C-243	8	CR55	MBRA 12	C-18	2
CR34	SM1741-1	C-18	5	CR57	MCOL 2206	C-18	4
CR35	SM1741-1	C-33	5	CR58	MMAL 66	C-18	2
CR36	SM1741-1	C-39	2	CR59	MTAI 2	C-18	6
CR37	C-4	CM4574-7	3	CR62	MTAI 8	C-39	3
CR41	C-18	MCOL 2056	3	CR8	C-4	MCOL 2206	13
CR42	C-18	MCOL 2206	5	CR9	C-4	MTAI 8	33
CR43	C-33	CM4574-7	13	**Total**			**191**

Table 9.2. List of AR genotypes from BC_2 families that combine resistance to CGM and CMD resistance introduced to Tanzania this year

Code	Progenitor		Number of	Code	Progenitor		Number of
	Female	Male	Genotypes		Female	Male	Genotypes
AR1-1	C-127	CW27-12	85	AR17-1	C-33	CW258-17	14
AR11-2	C-243	CW259-43	6	AR21-2	C-39	CW258-17	1
AR23-1	C-39	CW259-43	1	AR30-3	C-413	CW258-17	3
AR26-2	C-413	CW259-43	1	AR9-2	C-243	CW257-12	43
AR41-2	C-19	CW259-42	1	AR15-1	C-33	CW257-12	9
AR37-1	C-33	CW259-42	41	AR20-1	C-39	CW257-12	1
AR38-1	C-377	CW259-42	5	AR35-1	C-243	CW257-10	2
AR40-3	C-39	CW259-42	13	AR14-1	C-33	CW257-10	7
AR42-3	C-413	CW259-42	3	AR6-1	C-4	CW235-72	9
AR16-1	C-33	CW259-3	12	AR7-4	C-127	CW234-2	25
AR22-1	C-39	CW259-3	1	AR4-1	C-19	CW234-2	1
AR36-5	C-127	CW259-10	9	AR8-3	C-243	CW234-2	1
AR34-2	C-19	CW259-10	1	AR12-2	C-33	CW234-2	30
AR32-1	C-33	CW259-10	3	AR2-3	CW236-14	C-4	6
AR33-1	C-39	CW259-10	1	**Total**			335

Evaluations of all 503 genotypes at 6 and 9 months after planting revealed 224 genotypes with no visible foliar symptoms for CMD and176 genotypes that did not show any visible foliar symptoms for CMD and CGM (Table 9.3). The large number of susceptible varieties in the CR and AR genotypes that had been selected with markers for resistance to CMD conferred by the *CMD2* gene was unexpected. Further analysis of the results by family revealed that a number of large families had over 90% of genotypes susceptible, these families had the parent C127 in common (Fig 9.2). These results suggest that the C127 parent sent to CIAT from IITA along with 17 other F_1 progeny of TME3, the source of *CMD2*, as donor parents for CMD resistance is a susceptible genotype. When families having this parent were removed from the analysis, the percentage of resistant genotypes was 70%, which is the expected percentage, given that *CMD2* controls 70% of CMD resistance.

Molecular diagnostics, by PCR amplification using primers designed from the East African Cassava Mosaic Virus (EACMV), the predominant CMD virus in Eastern Tanzania, of genotypes with no visible symptoms presented PCR amplification of the virus in only the control and a single genotype, suggesting a low percentage of 'escapes' in the field evaluation (data no shown).

Table 9.3. CMD and CGM evaluations at 6 months after planting in Tanzania.

		#	CMD(-)/CGM(-)		CMD(-)/CGM(+)		CMD(+)/CGM(-)		CMD(+)/CGM(+)	
		Planted	#	%	#	%	#	%	#	%
AR	Genot.	325	127	39.1	1	0.3	191	58.8	6	1.8
Series	Plants	2282	837	36.7	9	0.4	1388	60.8	48	2.1
CR	Genot.	178	49	27.4	66	36.9	33	18.4	31	17.3
Series	Plants	750	210	28.0	231	30.8	152	20.3	157	20.9
Both	Genot.	503	176	34.9	67	13.3	224	44.4	37	7.3
Series	Plants	3032	1047	34.5	240	7.9	1540	50.8	205	6.8

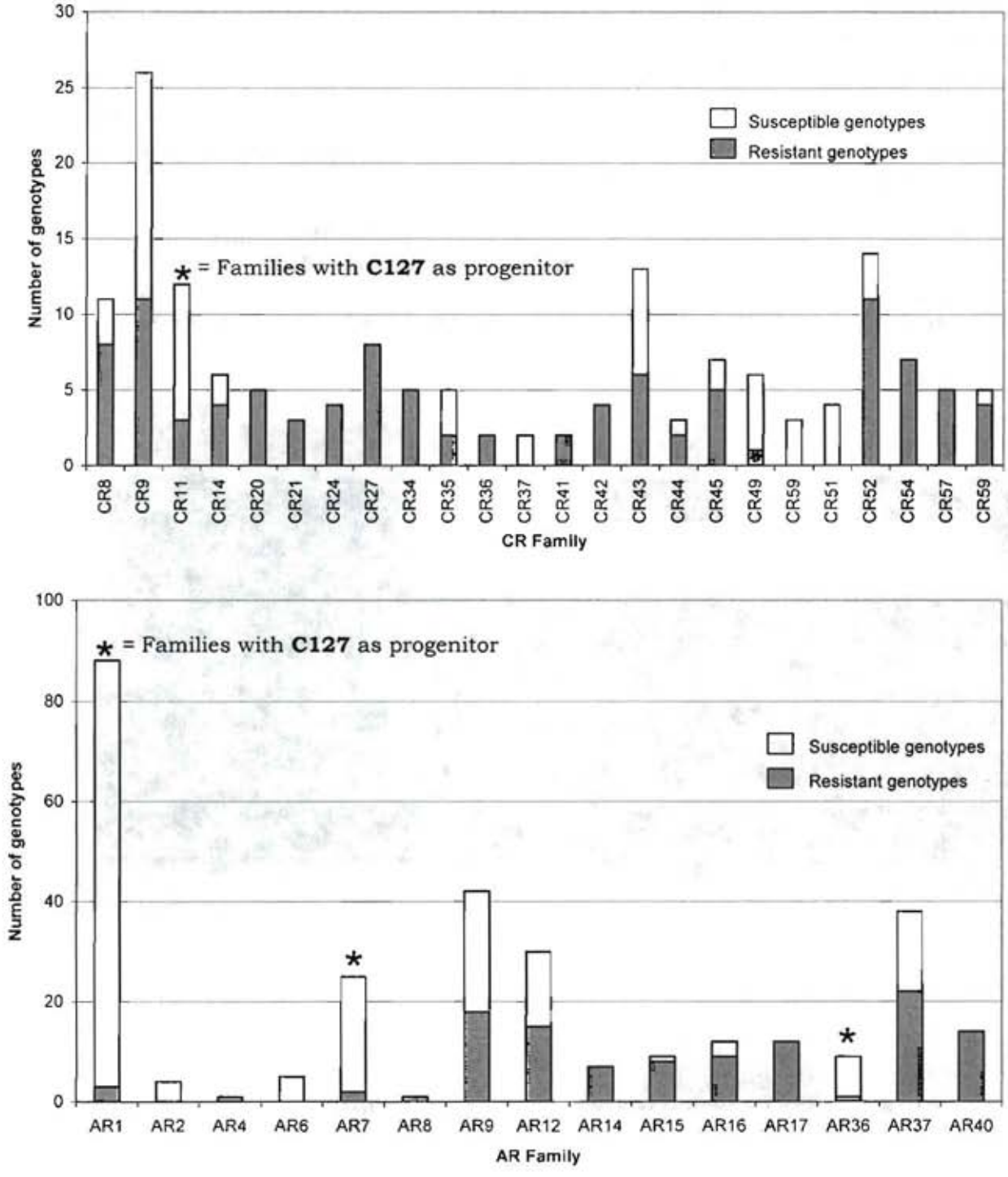

Figure 9.2. Response to CMD by family in the CR and AR genotypes.

The cassava molecular marker-assisted selection (MAS) lab currently has two persons working on MAS for CMD and together they can process 192 genotypes in 2 days or 480 genotypes per week or over 24,000 samples in a year. We are working on improving this by doing the grinding and DNA isolation in 96-well plates. Current costs of a single SSR marker data point analysis for cassava at CIAT is US$0.30, processing 24,000 samples in a year requires a budget of US$7,200. An attempt to lower the cost and labor of marker analysis via SCAR markers and PCR amplification of FTA paper discs with leaf squashes from *in vitro* plants was 100% successful with or without the washing step (Figure 9.3). Leaf squashes using leaves from screen house or field plants were also 100% successful but only after inclusion of the washing step. Elimination of the washing step led to a high number of failed PCR reactions suggesting that impurities from matured leaves were inhibiting the PCR reaction. This result suggests that FTA paper leaf squashes could replace the cumbersome DNA isolation step.

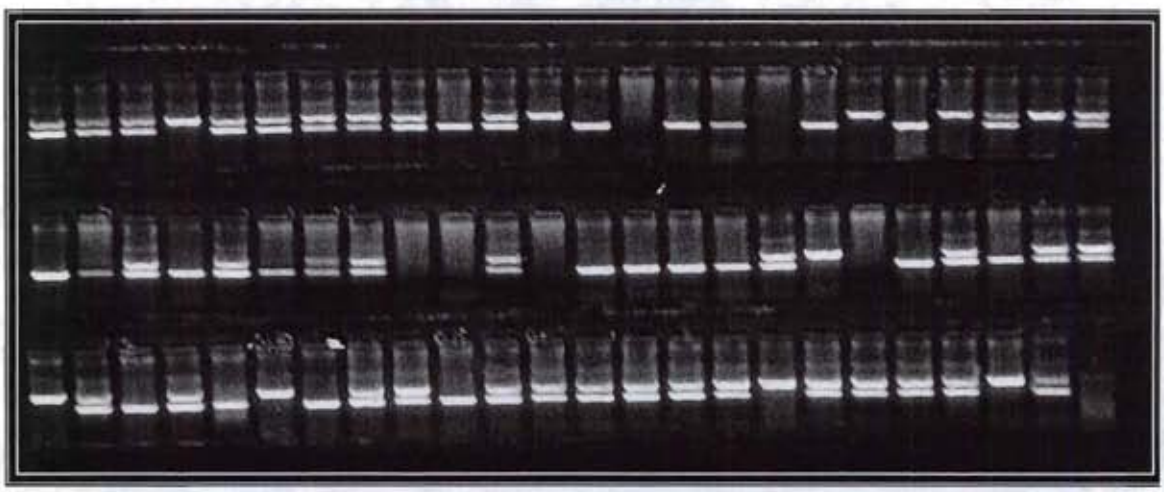

Figure 9.3. PCR amplification of BC_2 progenies using a SCAR marker developed from a RAPD marker RME1 located at less than 4cM from *CMD2*, and FTA paper leaf squashes. The larger weight allele is associated with resistance

MAS for CMD resistance breeding in Tanzania

A total of 191 CR and 335 AR genotypes were shipped to Tanzania, of this number 503 genotypes were successfully hardened and established in the field. The were harvested February 2005 and scored for high heritability traits such as dry matter content, harvest index, and plant architecture. A summary of agronomic evaluations of the CIAT introductions at harvest (11 months after planting) are shown in table 9.4. At harvest all roots from the genotypes harvested were visually inspected for root symptoms of cassava frog skin disease (CFSD), and no symptoms were found confirming earlier results of CFSD diagnostics that showed the introductions were free from CFSD. Eighty genotypes were selected for genetic crosses to local cassava varieties.

Table 9.4. Simple statistics of agronomic traits evaluated in the CIAT introductions harvested at 10 months after planting in the Alavi estate in Tanzania.

	Plants /clone (Number)	Roots /plant (Number)	Fresh root /plant (kg)	Fresh foliage /plant (kg)	Biomass /plant (kg)	Harvest Index (0-1)
Maximum	13.00	20.50	3.20	1.60	8.80	0.67
Minimum	1.00	1.50	0.39	0.18	1.18	0.19
Average	6.04	4.21	0.93	0.50	2.72	0.34
Std Dev.	2.34	2.23	0.49	0.28	1.14	0.10

Figure 9.4. Root necrosis class 3 symptom of the cassava brown streak disease (CBSD) (CBSD) in a root of the CIAT introduction CR27-9.

Results of cassava brown streak disease (CBSD), a major problem of cassava in Tanzania, evaluations of the CIAT introductions revealed that 9 genotypes had a symptom score of 3 (Figure 9.4) on a scale of 1 (no necrosis) to 5 (100% root necrosis), 72 genotypes a score of 2, and 32 genotypes a score of 1. Twenty-three other genotypes had a score of 1 but results were not conclusive due to the insufficient number of roots for these genotypes. A total of 60% of all genotypes showed some disease symptoms (score of 2 or 3). The high frequency of CBSD in these materials led to shelving the intended transfer of the CIAT introductions to Maruku for genetic crosses with materials from the Eastern Zone, CBSD is not known in the Lake zone and extra care needs to be taken that it is not introduced, crosses with genotypes from the lake zone were later done via transfer of pollen from Chambezi to Maruku. The high incidence of CBSD in these genotypes is expected as the disease is not known in South America and therefore there has not been any breeding for resistance to CBSD. But the genotypes (40%) that failed to show symptoms, need to be re-evaluated again, as these genotypes have introgression (for resistance to CGM) from a wild *Manihot* relative of cassava. The high incidence of CBSD in the CIAT materials also led to the decision to make crosses only to local varieties having resistance to CBSD.

A total of 290 varieties were collected from all over Tanzania: 80 varieties from the Eastern coastal region, 90 from the Southern region and 120 from the Lake region. The cultivars from the Eastern and Southern region were established at the Alawi estate while collections from the Lake region were established at ARI-Maruku. Both collections were harvested in the period February to March 2005 and evaluated for dry matter yield, harvest index, plant type, dry matter content, and culinary quality (Table 9.5). Farmer groups were invited for the harvest to take into considerations their criteria in choosing what varieties should be included in the crossing block.

Table 9.5. Mean HI, root yield in tons per hectare, number of roots per plant and number of harvested plants of cassava local varieties evaluated in the preliminary yield trial at Kibaha and Maruku

Zone	Harvest index	Root yield (tons/ha)	Number of roots per plant	No. of plants harvested
SZ	0.408	8.894	3.596	6.462
EZ	0.412	8.733	3.578	5.635
LZ	0.469	9.023	3.765	6.622

A total of 27 and 24 varieties were selected from varieties from the Southern and Eastern zones respectively, and 80 genotypes from the CIAT introductions (Table 9.6). The lower number of local varieties was due to resistance to CBSD as a selection criteria given the susceptibility of CBSD in the introductions. Flowering started on in July 2005, 5 months after planting in the following cultivars: Kalolo, Bwana Mrefu, Muzege, AR42-4, AR32-1, AR9-14, AR12-14 AND AR30-3. Pollinations started on 26.7.05 on the above mentioned cultivars with an emphasis on crosses between CBSD resistant locals and the CIAT introductions. This emphasis is based on combining resistance to CMD and CGM in the CIAT introductions with CBSD resistance in the local varieties to produce genotypes with good resistance to the principal abiotic constraints in the Eastern and Southern regions.

Over 60,000 pollinations were carried out and a total of 67,340 sexual seeds obtained. Given the limited resources to plant all these seeds, only 40% of the seeds were tested for viability (ability to sink in water) and germinated in seed trays. A total of 20,670 seeds were planted February 2006 in seed trays in the screen house in Kibaha (Fig 9.5) The seedlings will be transferred to the field late March 2006 and established in a seedling trial at the Chambezi sub-station of ARI-Mikocheni.

MAS for CMD resistance breeding in Nigeria, Ghana, and Uganda

A total of 100 CR and 200 AR genotypes were shipped to Nigeria, of this number 240 genotypes were successfully hardened and established in the field. They were harvested February 2005 and evaluated for dry matter content, harvest index, and plant architecture. Performance of some the introductions in Nigeria 11 months after planting are shown in Table 9.7.

Table 9.6. Local cassava varieties and CIAT introductions for genetic crosses.

Eastern Zone	Southern Zone	CIAT	CIAT	CIAT
1. Kitingisha 2	1. Kabinda	1. AR 21-2	28. AR 14-14	55. AR 16-16
2. Cheusi mwangia 1	2. Albert	2. CR 45-3	29. AR 16-1	56. AR 37-73
3. Kibangameno	3. Limbanga	3. CR 27-9	30. AR 38-6	57. AR 17-8
4. Kigoma mtoto	4. Sheria 1	4. CR44-6	31. CR 44-8	58. CR 21-10
5. Pamba	5. Mzungu	5. CR 45-10	32. CR20A-1	*Back ups*
6. Guzo	6. Kalolo (Mtwara)	6. AR 37-6	33. CR 45-9	1. AR 40-10
7. Kibwegere	7. Lipukalyene	7. CR 35A-9	34. CR 54B-44	2. AR 40-12
8. Muarusha	8. Kigoma red	8. AR 30-4	35. AR 35-1	3. AR 30-3
9. Mshelisheli	9. Saranga	9. CR 27-24	36. AR 16-3	4. CR 52A-19
10. Kalolo	10. Kiroba	10. CR 11A-20	37. AR 15-3	5. AR 17-5
11. Dide	11. Nanchinyaya	11. CR 45-1	38. AR 17-27	6. AR 16-5
12. Cheusi	12. Kalinda	12. CR 20B-2	39. AR 32-3	7. AR 38-3
13. Moshi wa taa	13. Mreteta	13. CR 43-13	40. AR 37-81	8. AR 43-12
14. Mahiza	14. Chimaji1	14. AR 17-18	41. AR 15-9	9. AR 17-23
15. Bwana mrefu	15. Namikonga	15. CR 11A-25	42. AR 9-44	10. AR 14-2
16. Kiroba	16. Kitumbua	16. AR 37-96	43. AR 40-11	11. AR 37-54
17. Mfaransa	17. Mreteta	17. AR 17-3	44. AR 12-14	12. AR 14-5
18. Mkiwa	18. Kalombe	18. AR 17-25	45. CR 52A-41	13. AR 37-38
19. Kibaha	19. NDL 90/34	19. AR 17-16	46. AR 42-3	14. AR 37-92
20. Kaniki	20. Toa pesa	20. AR 37-89	47. CR 20A-6	15. AR 9-54
21. Kasumoni	**Back ups**	21. AR 11-13	48. AR 15-10	16. AR 14-1
22. Kikombe	21. Tukuyu	22. CR 25-4	49. AR 9-18	17. AR 9-6
23. Bora kupata	22. Usilie chumbani	23. CR 52A-40	50. AR 12-11	18. AR 9-48
24. Muzege	23. Kichoko	24. AR 16-8	51. AR 41-2	19. AR 9-2
25. Jaribu tena	24. Bilali	25. AR 42-4	52. CR43-AR 9-22	20. AR 37-1
26. Kilokote		26. AR 9-25	53. AR 9-14	21. AR 30-5
27. Kifumulo		27. AR 11-12	54. AR 32-1	22. CR 8A-5
				23. AR 37-48
				24. AR 40-3
				25. AR 37-99

Figure 9.5. Seedling nursery of CIAT by local varieties crosses at ARI-Kibaha, Tanzania

Table 9.7. Performance of some CMD resistant lines from CIAT in Nigeria in the 2004/2005 planting season.

Genotype	Roots /plant (Number)	Fresh root /plant (kg)	Fresh roots Tons/ha	CBB symptom score	CMD symptom score	Stakes for Clonal trial (Number)
CR 14A-1	20	15	150	1	1	300
AR 38-3	10	12	120	2	1	300
CR 41-10	18	10	100	3	1	100
CR 36-2	15	8.5	85	2	1	100
CR 52A-41	14	7	70	4	2	100
AR 15-5	10	6	60	2	1	60
CR 26-1	10	5	50	2	1	56
CR 52A-25	8	4	40	3	1	140
CR 52A-22	12	3.5	35	4	2	17
CR 42-4	7	3	30	2	1	150
AR 37-108	6	3	30	2	1	200

In Uganda, 101 AR entries and 110 CR entries were evaluated for their reaction to CMD, CGM, CBSD, and CBB in the field (Figure 9.6, Table 9.8 and 9.9). Results are similar to that observed in Tanzania with families derived from C127 showing susceptibility to CMD in the field. Results further indicated that most of the entries were moderately susceptible to CBB.

Following the commissioning of the GCP project, CIAT appointed a visiting scientist, Dr. Emmanuel Okogbenin, a breeder with experience in molecular genetics to technically backstop project activities in the NARs. The visiting scientist is placed at the NRCRI, Nigeria but oversees project activities at the African NARs as well as serving as liaison officer between CIAT headquarters (PIs) and NARs partners (collaborating scientists). Activities so far supervised by the visiting scientists include the shipment of germplasm from CIAT to Africa and supervision of the post-flask management and transplant to the field.

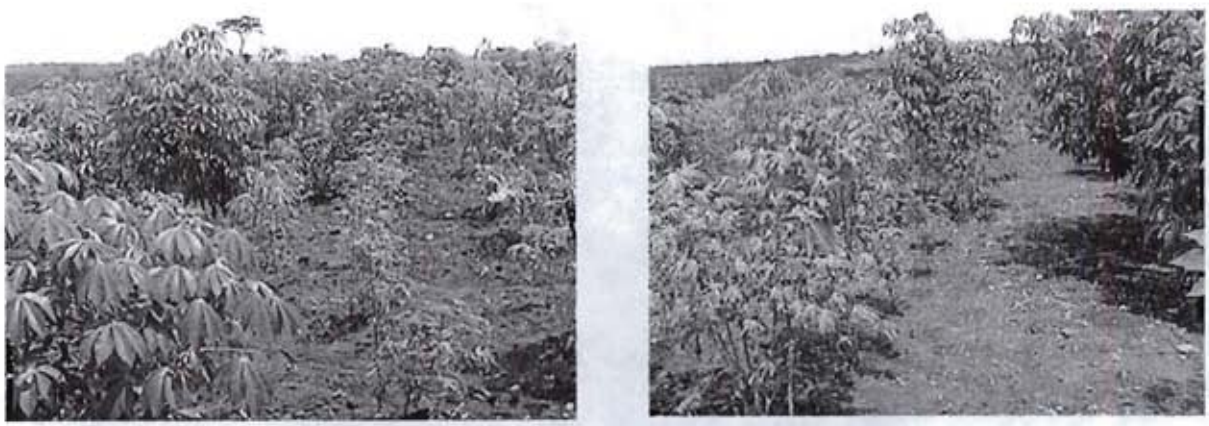

Figure 9.6. Field evaluations of CR (picture of the left) and AR (picture of the right) introductions in the field at NAARI, Namulonge, Uganda

The visiting Scientist has also been responsible in arranging for plant quarantine inspection and evaluation of the materials on the field together with collaborating scientists. The visiting scientist has successfully assisted the NRCRI, Nigeria to establish a MAS laboratory that is nearing completion in addition to backstopping the institute in its cassava breeding activities. In June, the visiting scientist in company of CIAT cassava geneticist visited CRI, Ghana to inspect the MAS laboratory and to deliver seeds from CIAT to the Institute. A similar visit was paid to NAARI in June 2005.

Table 9.8. Reaction of selected BC_2 populations (AR series) to pests and diseases at Namulonge, Uganda[1]

Entry	2 MAT			5 MAT			
	CMD	CGM	CBB	CMD	CGM	CBB	CBSD
AR1-11	1	1	1	4	2	2	1
AR1-129	1	1	1	3	1	1	1
AR1-140	1	1	2	5	1	4	1
AR1-158	1	2	1	5	3	2	1
AR1-167	1	1	1	4	2	2	1
AR1-28	1	1	2	4	2	2	1
AR1-33	1	1	1	4	2	2	1
AR1-37	1	1	1	4	1	4	1
AR1-41	1	1	1	4	1	2	1
AR7-14	1	1	1	4	1	2	1
AR7-35	1	1	2	3	2	2	1
AR9-1	1	1	2	1	1	2	1
AR9-11	1	1	1	3	1	1	1
AR9-12	1	1	1	1	1	2	1
AR9-14	1	1	1	1	1	2	1
AR9-15	1	1	1	1	1	2	1
AR9-18	1	1	1	1	1	2	1
AR9-2	1	1	1	1	1	2	1
AR9-23	1	1	1	1	1	2	1
AR9-24	1	1	1	1	1	2	1
AR9-27	1	1	1	1	1	2	1
AR9-28	1	1	1	5	1	4	1
AR9-4	1	1	1	4	2	2	1
AR9-44	1	1	1	1	1	2	1
AR9-95	1	1	1	1	1	2	1

[1] MAT= months after transplanting; CMD = cassava mosaic disease; CBB= cassava bacterial blight; CGM = cassava green mite; CBSD = cassava brown streak disease. Evaluations based on 1-5 scale; were 1= resistant and 5= highly susceptible

For efficient transfer of MAS technology to NARs, it is important that the necessary facilities are established and put in place at participating NARs institution for easy application and use of molecular marker technology in plant breeding. ARI-Mikocheni already has a molecular marker facility while the three African NARs (NAARI, CRI and NRCRI) in the projected have taken steps to set up a simple molecular markers laboratory with assistance from CIAT. A positive impact from this project is that NARs institutions have been challenged to invest considerably in molecular marker technology in plant breeding for the development of improved varieties. While GCP funds have been committed to the

procurement of laboratory equipment and accessories, NARs institutions have spent almost equal funds to support the establishment of MAS laboratory through the provision of laboratory space and renovation, provision of staff personnel, cabinet fittings and some lab equipment.

Table 9.9: Reaction of selected F1 populations (CR series) to pests and diseases at Namulonge, Uganda[1]

Entry	2 MAT			5 MAT			
	CMD	CGM	CBB	CMD	CGM	CBB	CBSD
CR11B-3	1	1	1	4	1	2	1
CR20A-1	1	1	1	2	1	2	1
CR 20A-2	1	1	1	2	1	2	2
CR20A-4	1	1	1	1	2	2	2
CR20A-6	1	1	1	1	1	2	3
CR21-10	1	1	1	4	1	2	1
CR21-6	1	1	1	1	1	2	3
CR24-3	1	1	1	1	1	2	1
CR24-7	1	1	1	1	1	2	1
CR25-4	1	1	1	1	1	2	1
CR34A-6	1	1	1	2	1	2	1
CR35-10	1	1	1	1	1	2	2
CR35-12	1	1	1	5	1	3	1
CR36-2	1	1	1	1	1	2	1
CR41-10	1	1	1	1	1	2	1
CR41-2	1	1	1	1	1	2	1
CR41-7	1	1	1	1	1	2	2
CR4-2	1	1	1	1	1	2	1
CR42-3	1	1	1	1	1	2	2
CR42-5	1	1	1	1	1	2	1
CR43-11	1	1	1	1	1	2	1
CR43-12	1	1	1	1	1	2	1
CR43-13	1	1	1	1	1	2	1
CR43-14	1	1	2	1	2	2	2
CR43-2	1	1	1	1	1	2	1
CR43-7	1	1	1	1	1	2	1
CR43-8	1	1	1	1	1	2	1
CR44-8	1	1	1	1	1	2	2
CR45-3	1	1	1	1	1	2	2
CR49-2	1	1	1	2	1	2	1
CR49-6	1	1	1	1	1	2	2
CR52A-1	1	1	1	1	1	2	1
CR52A-19	1	1	1	1	1	2	1
CR52A-25	1	1	1	2	1	3	1
CR53-3	1	1	1	1	1	2	1
CR54A-5	1	1	1	1	1	2	2
CR54A-7	1	1	1	2	1	2	3
CR93-7	1	1	1	1	1	2	1
CR9A-110	1	2	1	4	3	3	1
CR9A-121	1	1	1	4	4	3	1

[1] MAT= months after transplanting; CMD = cassava mosaic disease; CBB= cassava bacterial blight; CGM = cassava green mite; CBSD = cassava brown streak disease. Evaluations based on 1-5 scale; were 1= resistant and 5= highly susceptible

Important Outputs:

- Successful introgression of CMD resistance into Latin American cassava genepools via molecular marker-assisted selection (MAS).

- Introductions of CMD resistance germplasm from CIAT to Tanzania, Uganda, and Nigeria/Ghana for genetic crosses to local varieties to increase resistance to pest and diseases and broaden genetic diversity

Perspectives:

- Evaluation of the F1 progenies with markers associated with CMD in Tanzania
- Genetic crosses between CMD resistant introductions and local varieties in Nigeria, Ghana, and Uganda
- Continued MAS for introgression of CMD resistance to into Latin American cassava genepools.

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10. Genetic mapping of yellow color (carotenoids content) in cassava roots.

10.1 Background

The Harvest plus project in cassava seeks to improve, via conventional and genetic transformation methods, beta-carotene content in cassava and deploy pro-vitamin A dense cassava varieties in the fight against Vitamin A deficiency in the tropics. Naturally existing genetic variability for beta-carotene content in cassava is the basis for conventional improvement of beta-carotene content in cassava and knowledge of functional diversity provides for a more rational exploitation and faster progress in breeding. Furthermore, the cost-effectiveness of breeding for high beta-carotene content in cassava can be considerably enhanced if the mode of inheritance and the number of genes involved are known. The discovery of a wide segregation pattern of root color in two S_1 families from the Thai variety MTAI8 (AM320), provided an important resource for molecular genetic analysis of beta-carotene content in cassava. A study was initiated in 2003 to identify QTLs associated with yellow root color and also to associate genes in the beta-carotene biosynthetic pathway content with yellow root color, as a first step to analysis of functional diversity and development of markers for conventional breeding. To test if the hypothesis of multiple alleles at a few loci controlling yellow root color is true, markers associated with yellow root color were evaluated in 6 F_1 progenies generated from crosses of yellow fleshed cassava varieties crossed to either other yellow or white varieties. We describe here results of the identification of a single regions of the cassava genome associated with yellow root color.

10.2 Materials and Methods

Plant materials for the genetic analysis of beta-carotene color was 200 S_1 individuals obtained from selfing the Thai cassava variety MTAI (Rayong 60). MTAI8 is an improved variety developed in the CIAT-Thai cassava breeding program and the color of the root parenchyma is yellow. The S_1 progeny was established in a single row, of 4 plants, non-replicated trial in Summer 2003 at CIAT headquarters in Palmira, Colombia, and harvested after 10 months. Root parenchyma color is known to be highly correlated to beta-carotene content ($r=0.85$ at $P<0.05$), a color chart, 1 (white parenchyma) to 9 (pinkish parenchyma), was used in visual measurement of the root parenchyma color of the progeny. The experiment was repeated in the 2004/2005 this time in a replicated fashion of 8 replications and 1 plant per replication. Mean values and other calculations to estimate genetic and environmental effects of scores of root color in the S_1 progeny by year was performed for each trait using the SAS UNIVARIATE procedure (SAS institute 1996). Normality of distribution was tested ($P < 0.05$) with the W-statistic test described by Shapiro and Wilk (1965). All phenotypic analyses were performed on untransformed data.

Bulked Segregant, Analysis (BSA) of yellow root color

Bulked segregant analysis (BSA) (Michelmore et al. 1992) was employed to quickly identify molecular markers associated with regions of the genome that control beta-carotene content in cassava. Ten S_1 genotypes with white color of root parenchyma and 10 genotypes with pinkish colored parenchyma were selected based on results from the first year evaluation as individuals for bulks of high and low beta-carotene content. DNA was isolated from 1-2g of young leaves harvested from individual genotypes of each bulks using a modified Dellaporta (1983) mini-prep protocol. DNA of the individuals of each bulk was then combined and the

bulks genotyped with the 650 simple sequence repeat (SSR) markers available then for cassava according to methods described by Mba et al (2001). Markers polymorphic in the bulks were used to analyze individuals of the bulks and, where the polymorphism remained consistent in the individuals as with the bulks, the markers were analyzed in the entire family. Association with yellow or pinkish color was determined by a simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using the Microsoft Excel. The amount of phenotypic variance explained by each marker l was obtained from the R^2 value.

Quantitative trait loci (QTL) mapping

Molecular markers for QTL mapping of beta-carotene content came from a genetic map of the cassava genome drawn up using the S1 family AM320 and 130 SSR markers, map construction was as described earlier for cassava by Fregene et al. (1997). The genetic map of cassava provides DNA markers on a genome-wide basis to study the genetics of beta-carotene content in cassava. Genetic factors controlling these traits can be studied in terms of the size of their effects on the phenotype, gene action, the source of the favorable QTL alleles, and the relationship between QTLs underlying the different physiological processes. Phenotypic data of beta-carotene content was subjected to QTL analysis using untransformed data and marker genotypic data from the male- and female- derived maps of the F_1 mapping population. Cassava genome was scanned for the presence of QTL effect at 2.0 cM intervals using the computer package MAPMAKER/QTL 1.1 program and a free model QTL effect (Paterson et al. 1988; Lincoln et al. 1992a). A LOD score of 3.0 was used to estimate the most likely position of the QTL on the linkage map. For each LOD peak, we determined the LOD 1.0 support interval, that is the region in which the LOD score remains within 1.0 unit of the peak. The percent phenotypic variation explained by an individual QTL and effects were estimated at the most likely QTL position. Primary QTLs identified from the above step were fixed and the genome scanned again to search for other QTLs (secondary QTLs).

Genetic mapping of beta-carotene biosynthetic genes

The mapping population AM320 comprised of 200 S_1 plants obtained from self-pollinating MTAI8, an elite cassava cultivar developed by the CIAT-Thailand breeding program. This population is also being used for genetic mapping of cyanogenic glucosides and dry matter content, two traits that are high in MTAI8. Two cDNA clones each for phytoene synthase and 2 phytoene desaturase had earlier been obtained from a cDNA library of the cassava variety MNG2 (Andrea et al. unpublished data; CIAT 2002). Genetic mapping of the cDNA clones was as restriction fragment length polymorphism (RFLP). First, a parental survey of polymorphism was conducted using the restriction enzymes *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, and *Dra*I. Parental survey filters were made using 10ug of cassava genomic from the MTAI8 parent and 4 S_1 progenies DNA digested with the enzymes mentioned above and separated on a 0.9% agarose gels as described earlier (Fregene et al. 1997). Progeny filters containing restricted DNA from the 100 S_1 plants, including DNA from the parent in the first lane, were prepared using the restriction enzyme that revealed polymorphism in the parental survey. The raw RFLP data was read as codominant markers and joined with 100SSR markers already evaluated in the S1 population. Linkage analysis and genetic mapping was as described earlier (Fregene et al. 1997) using a LOD score of 4.0 and a recombination fraction of 0.3. Association between the markers and beta-carotene content, earlier evaluated in the S_1 cross was by single marker analysis using simple regression.

Genetic mapping of beta-carotene content from sources other than MTAI8

Plant materials for the genetic analysis of beta-carotene color were GM 708,GM 705, GM 734, CM 893, GM 734, CM 9816 F_1 families with progeny sizes ranging from 10 to 65 individuals. The F_1 progeny were established in a non-replicated trial at CIAT headquarters in Palmira, Colombia, and harvested after 10 months. Root parenchyma was visually measured using a scale of 1 (white parenchyma) to 9 (pinkish parenchyma) based on a color chart. Root parenchyma color is known to be highly correlated to beta-carotene content ($r=0.85$ at $P<0.05$). A total of 7 markers, namely SSRY313, NS980, SSRY92, SSRY251, SSRY9, and SSRY66, and NS717 found in the region of the cassava genome associated with root parenchyma color were used. The use of all 7 markers rather than just the 2, SSRY313 and SSRY251, tightly linked to beta carotene content is to increase the chances of finding polymorphism in the parents of all six crosses for genetic analysis of the trait. Parental lines of the 6 F_1 crosses were first evaluated for polymorphism in the 7 markers and polymorphic markers evaluated in the progenies. Association with color of root parenchyma was determined by a simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using the Microsoft Excel. The amount of phenotypic variance explained by each marker was obtained from the R^2 value.

10.3 Results

Phenotypic evaluation

Distribution of frequencies of different root color classes in the AM320 family was not normal as tested by was tested ($P < 0.05$) with the W-statistic test described by Shapiro and Wilk (1965). Correlation of data from 2003 and 2004/2005 was over 0.90, suggesting yellow root color is a stable trait with respect to the environment.

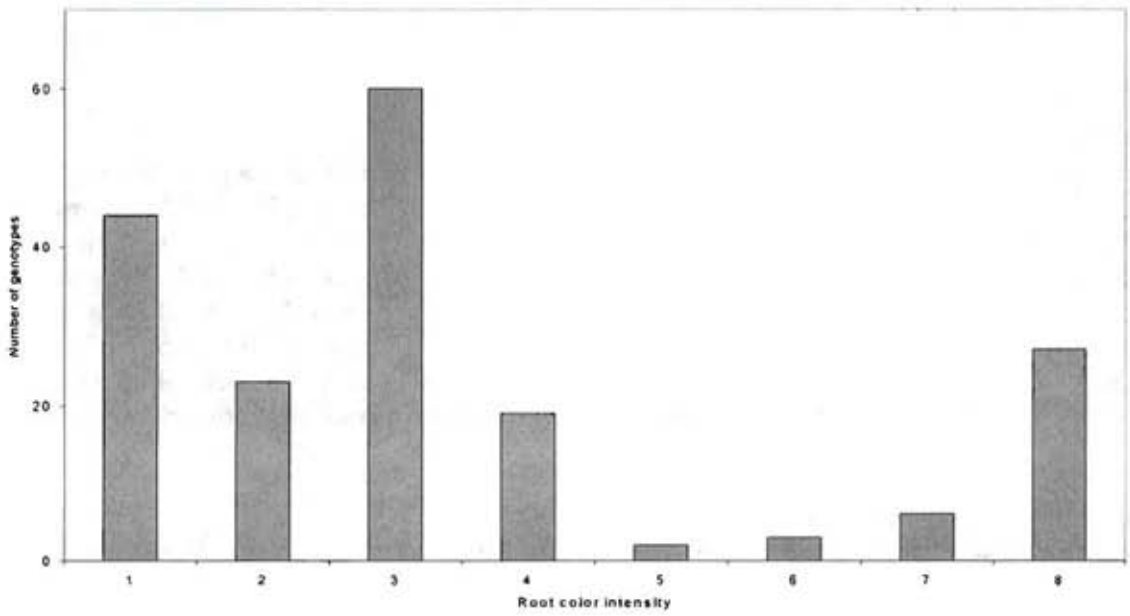

Figure 10.1. Distribution of frequencies of different root color classes in the AM320 family.

Bulked Segregant, Analysis (BSA)

A total of 8 markers, namely NS189, NS980, SSRY240, SSRY251, SSRY313, NS190, SSRY9, and SSRY63 were polymorphic in the bulks from the S_1 family AM320 derived from MTAI8. On analyzing individuals of the bulks, polymorphism was consistent in the individuals for markers SSRY251, NS980, SSRY313, NS190, SSRY240. All of the markers are located on linkage group D of the molecular genetic map of cassava. The 3 polymorphic markers were analyzed in all individuals of both S_1 and revealed to control between 30 and 40% of phenotypic variance for root color. Results reveal NS251 has the strongest association with beta-carotene content in the AM320 family (Figure10.2). This marker explains 40% of phenotypic variance for yellow root color.

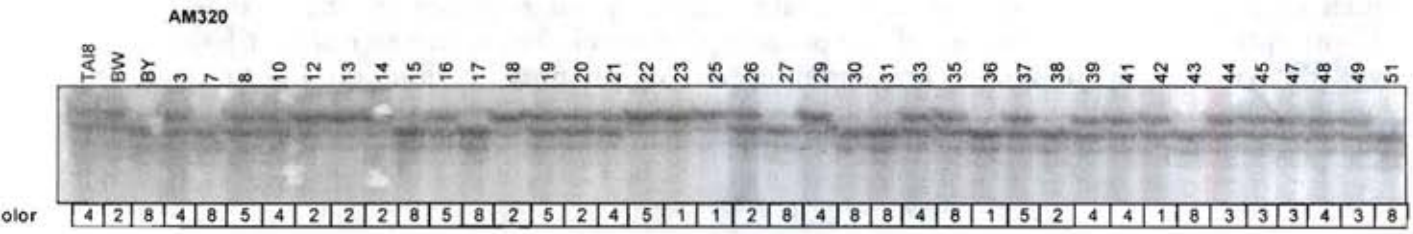

Figure 10.2. Silver stained polyacrylamide gel of PCR amplification of individuals from Family AM320 with SSR marker NS251. The color code is as follows: 8 = orange/pink colored roots, 1-2 = white colored roots, 4-5 = cream colored roots

It can be observed from Figure 10.2 that the most intense color, a score of 8, is associated with homozygosity of the smaller sized allele of NS251 in this family.

Quantitative trail loci (QTL) mapping

Quantitative trail loci (QTL) analysis revealed five intervals in the same genome region with a log likelihood of more than 3.0 and corresponds to two primary QTLs. The primary QTLs were fixed and the genome scanned for possible secondary QTLs. A very stringent condition (of at least 3-LOD) was used to declare secondary QTLs. A secondary QTL was detected in each of the two intervals harboring primary QTLs. The above results reveal that 2 QTLs, in the region between markers SSRY251 and NS109, SSRY313 and NS109, control yellow root color in cassava (Figure 10.3). The amount of phenotypic variance controlled is over 70%. The gene action of primary QTLs were consistent with dominance and/or additivity.

Genetic mapping of beta-carotene biosynthetic genes

Of the 2 genes used in the parental survey only the phytoene synthase gene revealed polymorphism in MTAI8 that segregated in the 4 S_1 progenies in the expected model with the restriction enzyme HindIII (Figure 10.4), phytoene desaturase was monomorphic. RFLP data from progeny hybridization permitted the mapping of the phytoene synthase gene in a linkage group different from that with SSRY251 a cDNA-SSR marker that was earlier found

to be associated with beta-carotene content in the same AM320 population. Incidentally SSRY251 shows very high homology to pyroxidine synthase, a gene known to be involved in the biosythesis of vitamin B6. Single point marker analysis by simple regression between the phytoene synthase gene, as independent variable, and beta-carotene content, as dependent variable revealed no association with the gene explaining 30% of phenotypic variance for the trait.

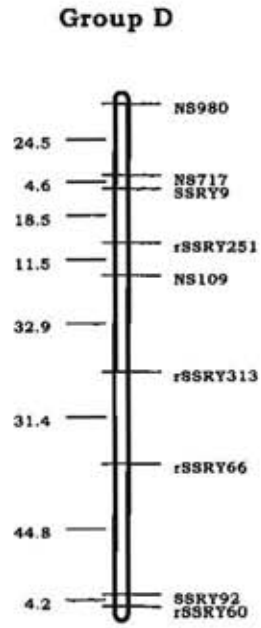

Figure 10.3 Linkage group G of the cassava genome showing the intervals where, between markers SSRY251 and NS109, SSRY313 and NS109, 2 primary QTLs for yellow root color are located.

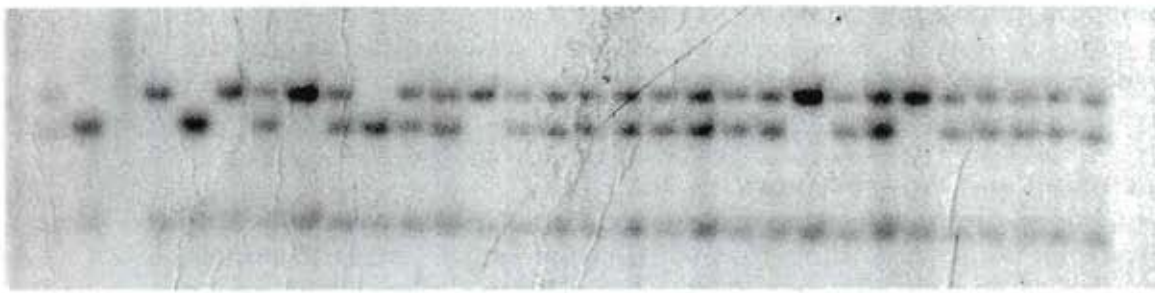

Figure 10.4. Southern hybridization of the phytoene synthase gene with *HindIII* digested DNA of progenies of AM320.

Genetic mapping of beta-carotene content from sources other than MTAI8

All 8 SSR markers found by BSA to be associated with yellow root color were polymorphic in the parents of the cross GM708 and segregated in the progenies, other crosses had between 2 and 5 polymorphic markers each. A simple regression of root parenchyma color in individuals of the GM708 cross on marker classes of the 7 markers revealed markers SSRY313 and SSRY92 each explained between 27 and 36% of phenotypic variance (Table 12.7). A similar result was found for crosses GM705 and GM734, where markers SSRY313 and SSRY9 explained between 25 and 30% of phenotypic variance. Analysis of marker association in the other crosses is yet to be completed. The differences in the variance explained for root parenchyma color in the different families suggests the presence of multiple alleles with different effects on the trait.

Table 10.1 Correlation (r-value) between SSR markers and root parenchyma color in 3 F_1 families obtained from crossing 2 yellow or a yellow and white variety

	SSRY-9	**SSRY66**	**SSRY92**	**SSRY251**	**SSRY313**	**NS717**	**NS980**
GM708	0.098282	0.109114	0.368828	0.12627	0.270877	0.117881	0.086813
GM705	0.212666	0.14199	0.089803	0.044901	0.237595	0.14199	0.08086
GM734	0.230383	0.505165	0	N/A	0.587795	N/A	0.016392

Important Outputs:

- Genetic mapping of for yellow root color, highly correlated to beta-carotene content in cassava, in an S1 progeny identified 2 QTLs located in adjacent intervals between markers SSRY251 and NS109, SSRY313 and NS109, that control over 70% of phenotypic variation.
- Markers earlier identified to be associated with beta carotene content in were also found to be associated with the trait in 3 F_1 progenies generated from crosses between 3 other yellow cassava varieties
- Genetic mapping of the phytoene synthase gene using an S_1 mapping population (AM320) from the yellow variety MTAI8, there was no association between beta-carotene content and the phytoene synthase gene.

Conclusion and Perspectives:

- Genetic mapping of beta-carotene content, as measured by high pressure liquid chromatography (HPLC) to refine genetic mapping studies of the trait, it has been shown that while yellow color is highly correlated with total carotenes, the correlation with beta-carotene is lower (0.4 to 0.6)
- Continuation of SSR markers analysis of test of allelism to estimate the effect of different alleles as a first step towards increasing beta-carotene content via combination of favorable alleles.

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Funding:
Harvest Plus

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11. Modification of Starch Amylose Content and Flowering in Cassava by Genetic Transformation

11.1 Introduction

Higher incomes from cassava in the developing world where the crop is generally found will require the development of novel industrial products from cassava. There are several novel starches that can be produced from cassava via genetic transformation, they include high amylopectin starch (waxy starch), via the down regulation of the granule-bound starch synthethase (GBSS) gene, and high amylose starch, from silencing the starch soluble synthase I, II, and III (SSS) genes. Industrial application of either high amylopectin or high amylose starches include the production of functional foods from high amylose starches or the use of high amylopectin starches in the food, paper, and adhesive industries and are markets with unlimited growth potential. With funds from the Colombian Ministry or Agriculture and Rural Development, a project was been initiated to genetically engineer industrial cassava varieties for the production of waxy starch using an anti-sense and sense construct of the GBSSI gene. GBSSI catalyses the conversion of ADP-glucose to amylose through the linkage of an ADP glucose to a preexisting glucan chain. Anti-sense disruption of the GBSSI gene has been employed to create potato transformants with 70-100% amylopectin via the down-regulation of the GBSSI gene (Salehuzzaman et al.,1993) and the disruption sense in sweet potato of the gene GBSS (Kimura et al., 2001).

Another transformation project being carried out is the control of the amount and timing of flowering through expression of flowering genes under the control of an ethanol-inducible promoter (AlcR gene expression system). The AlcR gene-expression system functions in many plants and works in controlled and field environments (Sweetman et al.2002). The principal objective of this project was to assess the effect of the expression of a set of floral developmental genes that are known to modify flowering time in Arabidopsis. The immediate benefits of this work include opening up to conventional breeding the many excellent cassava genotypes that are recalcitrant to flowering, and easing the difficulties of synchronizing flowering between cassava genotypes which currently flower at different times in the breeding cycle.

11.2 Materials and Methods

Isolation of a cassava GBSS cDNA clone.

More than 87,000 clones of a cassava root and leaf cDNA library cloned in the vector pCMV SPORT (GIBCO BRL Inc., USA) wer gridded onto high-density filters (Mba et al., 2000 unpublished data). The library was screened using a potato GBSS cDNA clones, a gift from Dr.Christine Gerhardt (Max Planck Institute, Cologne, Germany). The potato GBSS gene was labeled with [^{32}P] dATP by random primer labeling and hybridized overnight to the cDNA filters according to standard protocols for Southern hybridization used in cassava (Fregene et al., 1997). The filters were washed twice with 2X SSC +0.1% SDS at 60°C for 5 min, and autoradiography was al -80 °C using 2 intensifying screens. Positive clones were sequenced from the 5' and 3' ends to identify a full length GBSSI clone.

Cloning of GBSSI gene into transformation cassettes and genetic transformation of cassava
Primers were designed from published sequences of a full-length cassava cDNA of the GBSSI gene (Salehuzzaman et al., 1993). *BamHI* and *XbaI* restriction enzyme recognition sites were incorporated into the 5' end of the primers to enable sub-cloning of the cDNA clone in the sense and antisense orientation into the multiple cloning site (MSC) of the vector pRT101. The primers were used to amplify a full length cDNA clone obtained above, the PCR product was cleaned using the QIAGEN PCR Clean Up Kit (QIAGEN Inc., Los Angeles, CA) and digested with the appropriate enzymes. A 2.1Kb *BamHI* /*XbaI* fragment was subcloned in the sense and anti-sense orientation between the 35S promoter and the 35S polyadenylated terminator region of vector pRT101, a gift from Dr. Ryohei Terauchi, Iwate Biotechnology Research Center, Kitakami, Japan. The 35S promoter and GBSSI gene in pRT101 was liberated using the restriction enzyme *PstI*, separated on a agarose gel, eluted and cloned into the *PstI* site of the binary vector pCAMBIA 1305.2 having the GUSPlus^{R} and HPT reporter genes. The transformation cassettes were transferred into agrobacterium strain AGL1 or ABI

Friable embryogenic callus (FEC) of the cassava genotype TMS60444, Mcol.2215 y CM 3306-4 was transformed via *Agrobacterium tumefaciens* with the *GBSSI* gene cloned in anti-sense and sense orientation as described by Schopke et al (1996) but with some modifications. Briefly, FEC were massively produced by culturing nodal cuttings from *in vitro* plantlets in 4E media at a density of about 25 cuttings per Erlenmeyer glass flask. Explants produced were used to produce somatic embryos according to standard procedures (Schopke 1996). Axilliary buds of the explants were finely shredded and placed in MS4 media in a glass jar, 10 pieces per jar, for 3 to 4 weeks. Somatic embryos formed were excised from the rest of the tissue and placed in GD2-50Pi media solid for the induction of friable embryogenic callus (FECs; maximum of 9 clusters per dish). After 30 days, FECs that had developed in the clusters were sub-cultured in fresh GD2-50Pi media solid to increase amount of FECs. FECs obtained above were cultured again in GD2-50Pi media solid for one month.

To transform the FECs, acetosyringone [200μM] was added to each petri-dish and the FECs collected and re-distributed in clusters of 5mm width and 0.082g weight, about 20 clusters per dish. Following, 10μl of *agrobacterium* already transformed with the GBSSI construct in orientation sense or anti-sense was added to each cluster and left for 2-3 days at 21°C. The transformed FECs was collected with a sterile spatula and washed with GD2-50Pi liquid media supplemented with Cefotaxima or Claforan [0.5 mg/ml] for one week and an additional week under appropriate selection pressure (Higromicina 5mg/ml). Individual cell-lines of transformed FECs were allowed to proliferate in the GD2-50Pi solid media for 5 weeks after which they were transferred to MS2-1μMANA media for 2 to 3 weeks to allow the development of cotyledenous embryos and to continue with the process of regeneration plants.

Cloning of flowering inducing genes and genetic transformation of cassava
The gateway cloning technique was used to clone several flower meristem identity genes from *Arabidopsis thaliana*, generously donated by the Prof Coupland's lab of the Max Planck Institute of Plant Breeding Research (MPIZ), into two inducible vectors: an ethanol-inducible promoter and an estrogen receptor-inducible promoter. Three binary vectors: containing the ethanol inducible systems and the flowering genes Constans (ABI-pNew CO)

or Apetala (ABI-pNew API), and a vector with the steroid inducible gene and the Constans genes (AGL1-pER8 CO) were used for transformation. Agrobacterium cultures having the constructs ABI-pNew-CO, ABI-pNew-API and AGL1-pER8-CO were grown overnight with shaking (250rpm) in LB medium containing 19.6µl of acetosyringone (100mg/ml) with the selective antibiotics Kanamycin 50mg/ml, Chloramphenicol 30mg/ml and Carbenicillin 100mg/ml for ABI constructs and Carbenicillin 100mg/ml, Spectinomycin-Streptomycin 50mg/ml for AGL1 constructs at 28°C until the Optical Density, O.D 560 was between 0.5-1.0 Bacteria were pelleted and re-suspended in LB media, more acetosyringone was added and 10µl of the bacterial culture used to inoculate FECs from the cassava variety TMS 60444 that were then re-distributed in clusters of 5mm width and about 0.082g weight, about 20 clusters per dish of solid media GD2-50Pi + Acetosyringone.

The plates were placed in a vacuum apparatus to remove air trapped within the FEC to ensure direct and total contact of the agrobacterial culture to all the FEC units. The co-cultivation was carried out in darkness for 48hours at 21°C. Ten plates of TMS 60444 FEC were inoculated per construct. After co-cultivation, transformed FEC were collected with a sterile spatula and washed 4 times with GD2-50Pi liquid media supplemented with Cefotaxin (0.5mg/ml), the washing procedures were repeated each day for one week and for an additional week under appropriate selection pressure. Phosphinothricin -ppt (1mg/l) for pNew constructs and Hygromycin 5mg/ml for the pER8 constructs. Individual cell lines of transformed FECs were allowed to proliferate in the GD2-50Pi solid media for 4 weeks after which the medium was changed twice after 2weeks each under the appropriate selection pressure. The transgenic lines were selected on MS2-1µM NAA α-naphthalene acetic acid for more than 5weeks to allow the development of somatic embryos. The green somatic embryo from the transgenic FEC lines were transferred from the maturation medium to MS2-0.5% Activated Charcoal for cleaning, the procedure took over a month (some transgenic lines are still at this stage). Young matured green transgenic lines were transferred to elongation media MS2-0.2µM Gibberillin for elongation which takes about 3 weeks or more. After elongation, they were transferred to 17N for the development of roots for 4weeks after which they were hardened in the screen house in preparation for molecular and physiological analysis.

Molecular characterization of transgenic events for low amylose starch

Molecular characterization of the sense transgenic event was using PCR primers designed from the 3'and 5'ends of the GBSSI gene and primers for the *GUS* reporter gene, the PCR primers and conditions for PCR have also been described earlier (CIAT 2003). Southern analysis was using ten micrograms of total genomic DNA from transgenic plants from several events and 2 non-transgenic cassava genotypes as control using the following restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *HaeIII*, and *DraI* . The RFLP probe was a full length GBSSI cDNA clone (clone-3) from a root cDNA library from TMS30572. Preparation and Southern hybridization of the filters were as described by Fregene et al. (1997).

11.3 Results

Three GBSS cDNA clones were obtained form screening the cassava library and sequenced, and one was found to be a complete cDNA clone. The cDNA clone has the ATG start codon 81 bp down stream from the beginning of the cDNA sequence and a stop codon about 100 bp from the poly-A tail. PCR amplification with the designed primers yielded a fragment about 2.1 kb in size that corresponds to the full-length GBSSI cDNA clone(Figure 11.1).

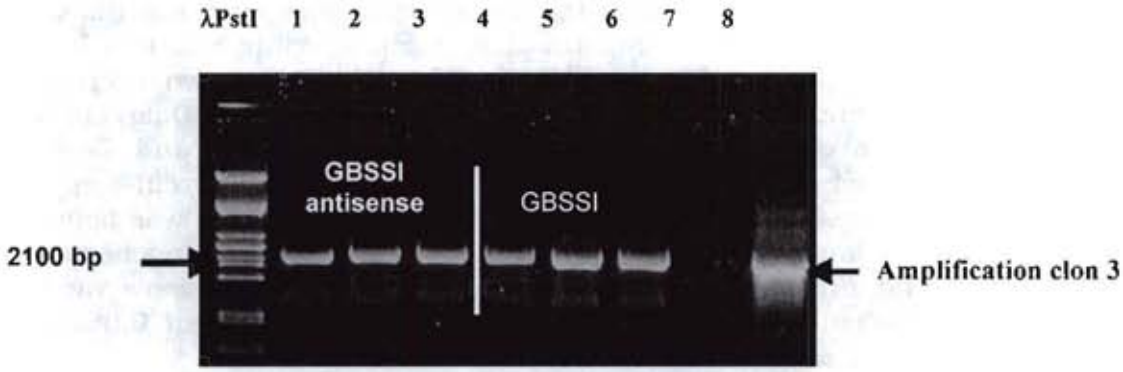

Figure 11.1. PCR amplification of the GBSSI cDNA clone using primers to introduce restriction enzyme sites at the ends of the gene. The first lane on the right is molecular weight marker Lambda DNA digested with *PstI*, the next three lanes are PCR of the gene GBSSI in anti-sense and sense (lane 4-6) orientation respectively.

The resulting PCR fragment, in the sense of anti-sense orientation, digested with *BamHI* and *XbaI* restriction enzyme, was cloned into the MCS of pRT101, following which, the GBSSI gene, promoter and terminator sequences was excised with *PstI* and the resulting fragment cloned into the PstI site of pCAMBIA (Figure 11.2).

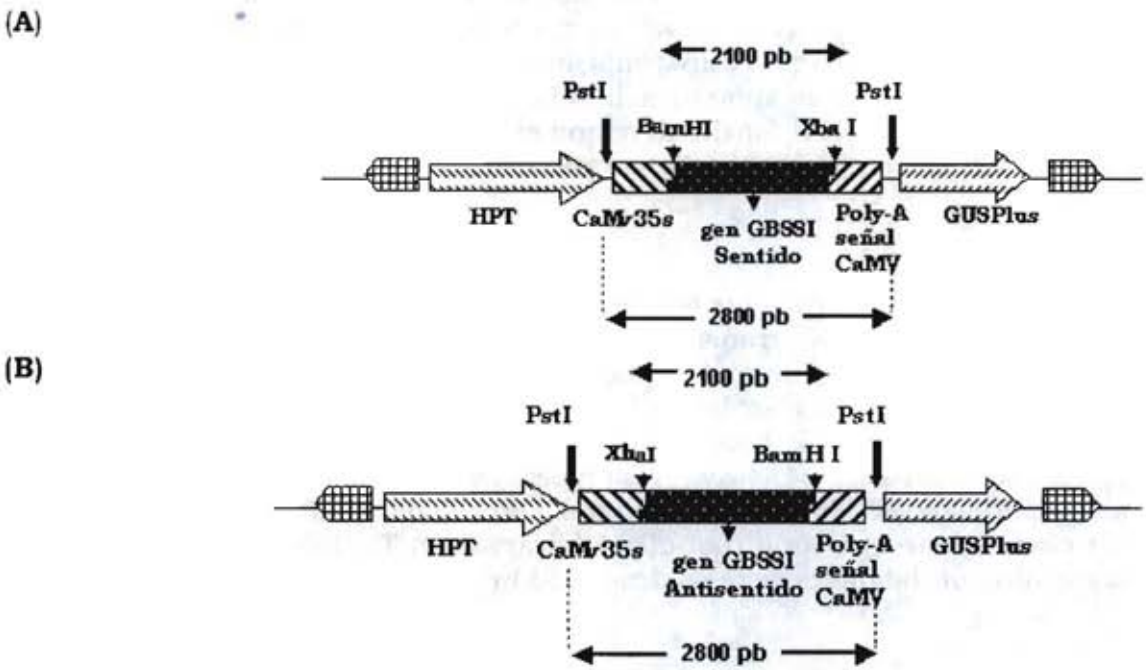

Figure 11.2. Gene constructs of GBSS in sense (A) and anti-sense (B) in the binary vector pCAMBIA 1305.2

Results of GUS transitory assay revealed a successful incorporation of the gene (Figure 11.3).

Figure 11.3. Positive test of GUS in Cotyledonary embryos of variety TMS 60444 with the GBSSI sense gene construct in the vector pCAMBIA 1305.2

A total of 123 independent transgenic lines with the GBSS in sense orientation and 86 lines with the GBSS anti-sense were obtained using the model transformation genotype 60444 as demonstrated by *gus* assay (Figure 11.4). Sixty one independent transgenic events in sense orientation sense and 43 in anti-sense were produced and hardened in the screen house (Figure 11.5).

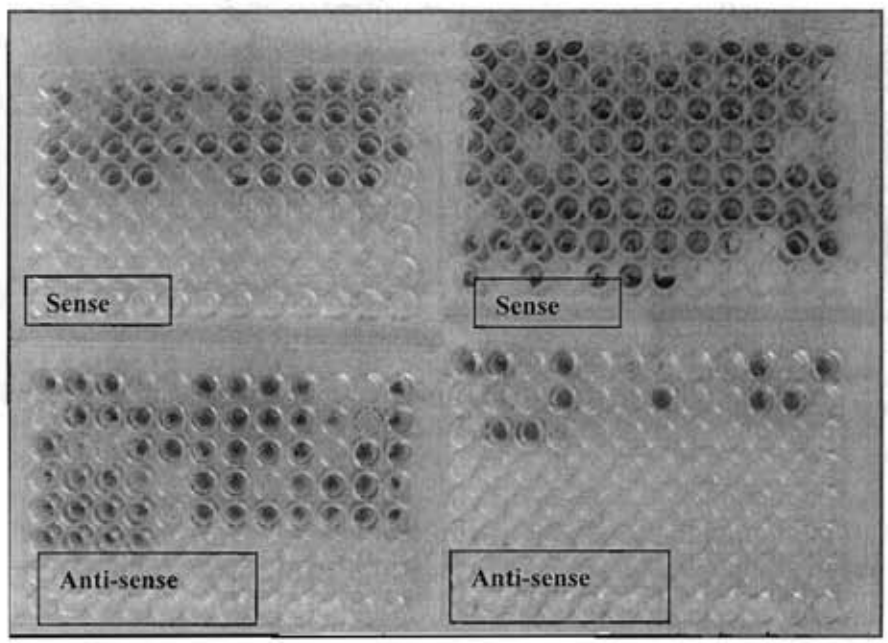

Figure 11.4 Positive *gus* assays of FEC variety 60444 using two different transformation constructs, each one of these samples are independent lines.

Molecular analysis to confirm the successful integration of the GBSSI construct was by PCR amplification and Southern hybridization. PCR amplification with the GBSSI and *GUS* gene primers yielded the expected size of a 2.1 kb and 700 bp fragment respectively in the transgenic plants of (Figure 11.6). Southern hybridization of the GBSSI to genomic DNA digested with *PstI* also gave the expected size of 2.1kb in the transgenic plants but not in the control non-transgenic plants suggesting stable integration in the genome (Figure 11.7). Large molecular weight bands in the non-transgenic plants are indigenous GBSSI genes and introns usually found in cassava.

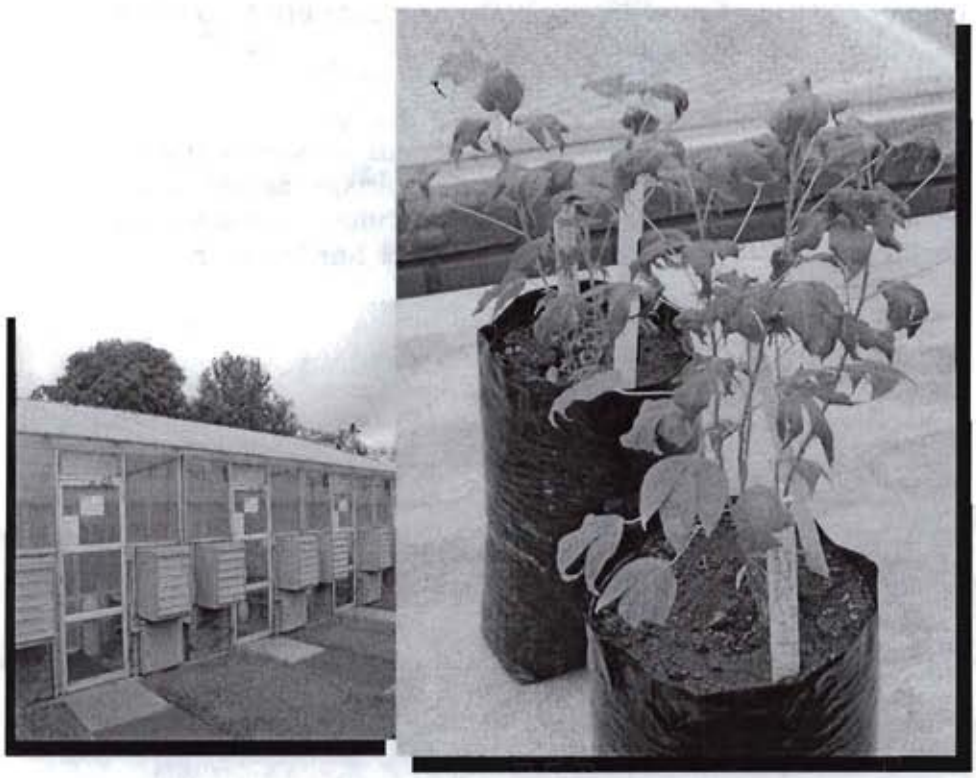

Figure 11.5. GBSSI sense transgenic plant in biosafety green house.

Figure 11.6. (A) PCR amplification of the *gus* using primers specific for the gene. The first and last lanes are molecular weight marker Lambda DNA, digested with *PstI*, the next four lanes from the left (section GUS) are PCR amplification of the *GUS* gene in regenerated transgenic plants transformed with the GBSSI sense construct, the next two lanes are control PCR amplification of the construct in the plasmid pCAMBIA 1305. (B) PCR amplification using primers specific for the GBSSI gene. The first four lanes in the section on the right (labeled GBSSI) are PCR amplification of the gene in regenerated transgenic plants transformed with the GBSSI sense and the last two lanes are control PCR staring of a cDNA GBSS clone (clone 3).

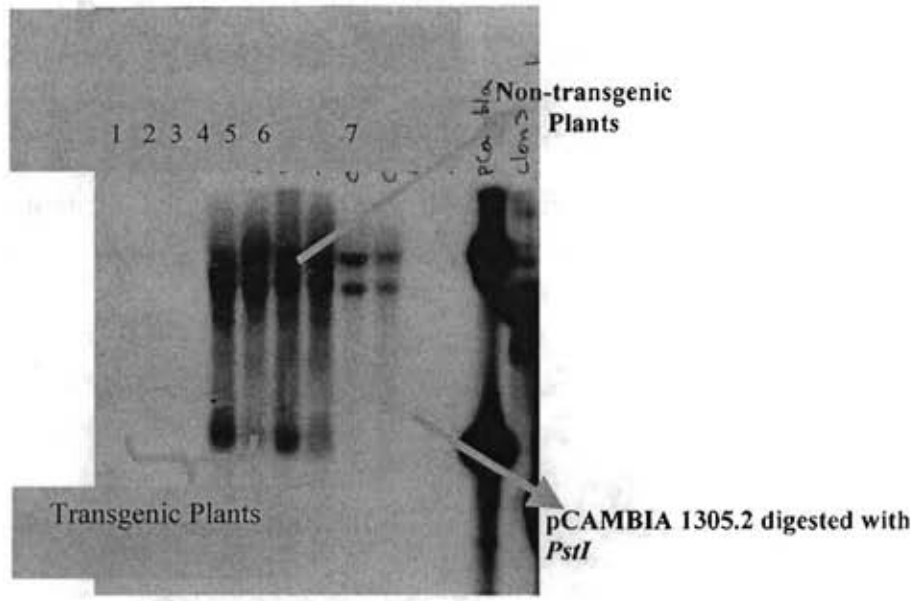

Figure 11.7. Southern hybridization of a GBSS full-length clone hybridized to genomic DNA digested with *Pst I* of transgenic, non-trangenic and the transformation construct. Lane 1-4 are transgenic plants transformed with a sense GBSSI construct, lanes 5-6 are non-transgenic control DNA, lane 7 is the construct in pCAMBIA1305.2. Large molecular weight bands in the non-transgenic plants are indigenous GBSSI genes in cassava containing introns.

Following the confirmation of successful and stable incorporation of the sense GBSSI construct into the genome of the cassava genotype 60444 as revealed by molecular characterization of transgenic plants, the stage is set for biochemical evaluation of roots in field experiments. An application for permission to transfer the transgenic plants to the field was made to the Colombian authorities through the CIAT bio-safety committee and approval for field evaluation has recently been received, the transgenic plants are being planted in the field now and biochemical evaluation of the waxy phenotype will be conducted 10 months after planting

Transformation of cassava with flowering genes

Agrobacterium mediated transformation of the 60444 variety using 2 floral genes is at different stages (Fig 11.8, Fig 11.9, and Table 11.1). Several independent transgenic lines have been obtained for the ABI-pNew-CO construct and are presently being elongated (Figure 11.9) after which they which they will be transferred to 17N rooting media. There are several 70 API lines and 60 CO lines in the proliferation phase in activated charcoal (Figure 11.9).

Figure 11.8. Independent transgenic ethanol inducible constans (CO) lines at the elongation stage.

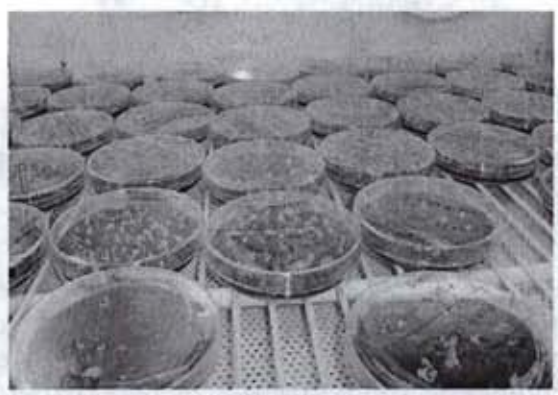

Figure 12.8 Independent transgenic cassava lines of ethanol inducible Constans and Apetala 1 lines at the proliferation stage in activated charcoal.

Table 11.1; Summary of the process of Agrobacterium mediated transformation of the model cassava variety 60444 with 4 different flowering gene constructs.

Start date	23rd May	23rd May	23rd May	15th July
Number of plates	30	30	30	30
1st stage of growth	3rd June	3rd June	3rd June	28th July
1st isolation of transformed clusters	5th –8th July	5th –8th July	11th July	No transformed cluster
Number of plates	34	55	8	15th July
Selection media	GD2-50Pi + Cefotaxin + 1mg/Lppt	GD2-50Pi + Cefotaxin + 1mg/Lppt	GD2-50Pi + Cefotaxin + 5mg/LHygromycin	
2nd isolation	1st –10th August	1st –10th August	1st –10th August	
Number of plates	51	89	Very low transformation efficiency	
Selection media	GD2-50Pi + 1mg/L ppt	GD2-50Pi + 1mg/L ppt		
Approx. total number of clusters	~ 650	~ 900		
Maturation Media MS2- BAP + 1mg/L ppt	29th August 15plates, min.of 9 clusters per plate	29th August 16plates, min.of 9 clusters per plate	22nd September 3 clusters	
MS2-1uM ANA	30th Aug –6 Sept	30th Aug-12Sept		
Number of plates	64plates, min.of 9 clusters per plate	99 plates, min.of 9 clusters per plate		
MS3-0.5%CA Somatic Embryos	6th October 10lines 7th Nov, 60 independent lines	6th October 1 line 7th Nov, 70 independent lines		
MS2-0.2GA3	4 transgenic lines			

Important Outputs:

- Production of 137 sense and 86 anti-sense (GBSSI gene) independent transgenic lines from of the variety 60444
- Several independent transgenic lines of the variety 60444 transformed with ethanol-inducible or steroid- inducible constructs containing the flowering genes Constans and Apetala

Perspectives:

- Biochemical evaluation of GBSSI sense and anti-sense transgenic events on roots from 10 months old field plants.
- Molecular and physiological analysis, including ethanol induction under screen house conditions, of several independent transgenic events for the Constans and Apetala gene constructs.

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12. Quantitative genetic studies to assess the relative importance of additive, dominance and epistatic effects in cassava

12.1 Introduction

This chapter reports the results of interesting studies on the inheritance of relevant traits for cassava in three different agro-ecosystems. Several articles were published as a result of these studies (Cach et al. 2005a; 2005b; Calle et al., 2005; Jaramillo et al. 2005; Pérez et al. 2005a; 2005b).

Several studies have been conducted on the genetic variability in cassava landraces from Africa (Asante and Offei, 2003); Central America (Zaldivar et al., 2004) and South America (Carvalho and Schaal, 2001; Elias et al, 2001a; Peroni and Hanazaki, 2002). For a crop that is vegetatively propagated, remarkable intravarietal variation has been found as a result of volunteer seedlings originating from unmanaged sexual reproduction in farmers' gardens (Elias et al, 2001b; Pujol et al., 2002; Sambatti et al., 2001). Outcrossing has been found to be prevalent, although self-pollination can also occur (Meireles da Silva et al. 2003), and is followed by selection for heterozygosity in the field (Pujol et al., 2002).

An interesting dynamic process takes place in farmers' fields. In spite of the vegetative propagation of the crop and the strong differences originating in the physiological (Eke-Okoro et al., 2001) and sanitary (Lozano and Nolt, 1989) conditions of the planting material, a quasi-natural breeding process takes place. Shamans (Salik et al., 1997) or efficient farmers (Sambatti et al., 2001) have been found to be key players in this informal genetic improvement process.

Little progress has been achieved in understanding the inheritance of agronomically relevant traits. Few articles on the inheritance of quantitative traits have been published (Easwari et al., 1995; Easwari and Sheela, 1993; 1995; 1998). In this regard cassava is an interesting case. Although a molecular map has already been developed (Fregene et al., 1997; Mba et al., 2001) and many studies of genetic variability among and within landraces have been conducted (Asante and Offei, 2003; Elias et al., 2001a, b; Elias et al., 2004; Peroni and Hanazaki, 2002; Sambatti et al., 2001; Zaldivar et al., 2003), little knowledge on quantitative genetics has so far been generated. Losada presented a Ph.D. thesis on quantitative genetics of cassava in 1990, using a diallel scheme. This work, however, was not published in any scientific journal in spite of its value. The heterozygous nature of cassava complicates work to improve the existing molecular map and implement marker-assisted selection. Different authors have suggested that cassava is a segmental allotetraploid (Umanah and Hartman, 1973; Magoon et al., 1969), which would further increase the complexities of gene interactions within and between loci and between homolog and homeolog genomic components.

Cassava is an interesting crop because its vegetative propagation allows the estimation of within-family genetic variation and, indirectly, the relative importance of epistatic effects. Genetic studies analyzing the importance of epistatic effects are not very common, particularly in annual crops. Accurate measurement of epistatic effects for complex traits, such as yield, is difficult and expensive. Reports in the literature on the relevance of epistasis are not as frequent as those estimating additive and dominance variances or

effects and generally take advantage of the vegetative multiplication that some species offer (Comstock et al. 1958; Foster and Shaw 1988; Isik et al. 2003; Rönnberg-Wästljung, and Gullberg 1999; Rönnberg-Wästljung et al. 1994; Stonecypher and McCullough 1986). In many cases these reports are on forest trees. Because of the complexities of these analyses and the costs involved, reports in the literature related to epistatic effects are frequently based on a limited number of genotypes.

Holland (2001) published a comprehensive review on epistasis and plant breeding. Several cases of significant epistasis have been reported in self- (Brim and Cockerham, 1961; Busch et al. 1974; Gravois, 1994; Hanson and Weber, 1961; Pixley and Frey, 1991; Orf et al., 1999) and cross-pollinated (Ceballos et al.,1998; Eta-Ndu and Openshaw, 1999; Lamkey et al., 1995; Melchinger et al., 1986; Wolf and Hallauer, 1997) crops. According to Holland (2001) finding significant epistasis seems to be easier in self- than in cross-pollinated species and in designs based in the contrasts of means rather than the analysis of variances.

12.2 Materials and methods

Three different studies were conducted using three different sets of parents targeting specific environments: sub-humid, acid soil and mid-altitude valleys environments. Table 12.1 provides information about the parents used for each environment. Of particular interest is the parent for mid-altitude valleys MECU 72, (a landrace from Ecuador) was one of the parents and has host plant resistance to the white fly *Aleurotrachelus socialis* (Bellotti et al., 1999, Bellotti and Arias, 2001). Controlled pollinations were performed following the standard procedures described by Kawano (1980). Each year, about 30 elite clones are planted and crossed in the breeding nurseries at CIAT's experimental station in Palmira (Valle del Cauca Department, Colombia), to produce segregating progenies adapted to this sub-humid environment. The parents used in this study where those that produced enough seeds for each of the required F_1 crosses in a complete, balanced diallel set. That was the main selection criterion in the choice of parental materials. Inbreeding has not been extensively used in cassava breeding. The elite parental clones, therefore, are considered to have the reduced levels of inbreeding typical of panmictic populations. Reciprocal crosses were pooled: progenitors served as father or mother of the clones evaluated.

Table 12.1. Parents used in the diallel studies conducted in three target environments for cassava.

Sub-humid conditions	Acid soil savannas	Mid-altitude valleys
MTAI 8	CM 4574-7	CM 6740-7
CM 6754-8	CM 6740-7	SM 1219-9
CM 8027-3	CM 7033-3	SM 1278-2
SM 805-15	SM 1219-9	SM 1636-24
SM 1565-17	SM 1565-15	SM 1673-10
SM 1411-5	SM 2058-2	SM 1741-1
SM 1219-9	SM 2219-11	HMC 1
SM 1657-12	HMC 1	MECU 72
SM 1665-2	MPER 183	MPER 183
	MTAI 8	

Seeds produced from the crosses were germinated and grown in a screen house and transplanted to the field after 2 months at CIAT's station in Palmira. A total of seed ranging from 8639 to 12,022 was produced for the studies (Table 12.2) from the nine or ten parental lines involved in this study. The average number of seeds (as well as the range) produced for each cross is also provided in Table 12.2. Cassava seeds are frequently weak, and therefore not all the seed germinates and produces vigorous seedlings that can be transplanted to the field. In addition, not all transplanted seedlings will develop vigorous enough "mature" plants from which to extract the necessary planting material. For this study at least six good quality stakes were necessary. Table 12.2 describes the number of seed germinated, seedlings transplanted and the number of plants that could produce six stakes. For each cross at least 30 genotypes could be produced and that was the number of genotypes representing each F_1. At harvest time six vegetative cuttings for each of the 30 genotypes of each F_1 family were obtained. Unavoidably there was selection based on the capacity of the plants to produce six good-quality vegetative cuttings at this stage. Indeed, this was the only selection criterion used to define the 30 genotypes to represent each F_1 family. Table 12.2 also list the number of crosses for which we could find fewer than 30 genotypes to represent the family.

Table 12.2. Number of seed produced and germinated, seedlings transplanted and number of vigorous F1 plants that produced enough stakes for the diallel study to be conducted in each of three target environments.

Relevant information	Sub-humid conditions	Acid soil savannas	Mid-altitude valleys
Seed produced	11548	12,022	8639
Average number of seed/cross	321	267	240
Range of seed produced / cross	37 - 1525	73-791	26 - 791
Germinated seed	3585	4697	3355
Transplanted seedlings	3213	4251	3037
Plants that produced enough stakes	2817	3871	2610
Crosses with less that 30 clones	1	1	3

Trials were planted in two representative locations for each of the three target environments. A randomized complete block design was used. Each replication contained 36 plots for the nine-parents diallels (sub-humid environment and mid-altitude valleys) and 45 plots for the 10-parent diallel (acid soil savannas). Each plot was planted with all the genotypes representing each F_1 cross. Experimental plots contained eight rows with seven plants per row. The first and last rows and the first and last plant within each row were filled with border plants. The rest of the plot (6 x 5 = 30 plants) was used to plant the experimental material. The 30 clones constituting each F_1 cross were planted together in the respective plots of each replication. Fertilization and general agronomic practices were followed according to the recommendations for each type of environment (Cach et al. 2005a; Jaramillo et al. 2005; Pérez et al. 2005a). Trials were 10 months after planting (the usual age for harvesting cassava in this environment).

Plants were hand-harvested individually and results averaged across the 30 clones of each F_1 cross. All the roots produced by each plant were weighed as well as the above-ground biomass (stems and foliage). Harvest index was measured as the ratio of root weight to total biomass. Root dry matter content was estimated using the specific gravity methodology (Kawano et al., 1987). Reaction to insects, plant-type architecture and general root appearance were scored using a scale of 1 to 5 where 1 is resistant or excellent and 5 is susceptible or very poor (CIAT 2003). Plant-type score integrated important characteristics such as plant vigor, erect architecture with few branches and reduced branching angle, which together ensure an adequate capacity to produce vegetative cuttings.

Statistical model

The analysis of variance was conducted following the expectations for each mean square described in Table 12.3. The analysis takes advantage of the full- (FS) and half-sib (HS) families that the diallel mating design creates. As is commonly the case, a few plants died or failed to develop normally to be harvested. Therefore in a few F_1 crosses fewer that 30 clones were actually evaluated in the field in each of the three replications at the two locations. To take into consideration this lack of uniformity, the harmonic (not the arithmetic) mean was used as ***k*** in the expected mean squares formulas (Vencovsky and Barriga 1992). The total genetic variance was partitioned into between-family variation (σ^2_{F1}) and the within-family variation ($\sigma^2_{c/F1}$). The between-family variation, in turn, was partitioned into the well-known variances related to general (σ^2_{GCA}) and specific (σ^2_{SCA}) combining ability, which in turn allow the estimation of σ^2_A and σ^2_D (Griffing 1956; Hallauer and Miranda 1988):

$$\sigma^2_{GCA} = (\text{Cov.HS}) = 1/4\sigma^2_A + 1/16\ \sigma^2_{AA} + 1/64\ \sigma^2_{AAA} + \ldots \text{ etc.} \quad [1a]$$

$$\sigma^2_{SCA} = (\text{Cov.FS} - 2\ \text{Cov.HS}) = 1/4\ \sigma^2_D + 1/8\ \sigma^2_{AA} + 1/8\ \sigma^2_{AD} + 1/16\ \sigma^2_{DD} \ldots \text{ etc.} \quad [1b]$$

Genetic parameters were estimated using the following mean squares from Table 1:

$$\sigma^2_{GCA} = [\ MS_{31} - MS_{32} - MS_{41} + MS_{42}\ ] / rak\ (p-2) \quad [2a]$$

$$\sigma^2_{SCA} = [\ MS_{32} - MS_{42}\ ] / rak \quad [2b]$$

Variance for these estimates were calculated as follows (Becker, 1985; Vega 1987):

$$\text{Var}\ (\sigma^2_{GCA}) = \{2/[rak(p-2)]^2\}\ [(MS^2_{31}/df_{31}+2)+(MS^2_{32}/df_{32}+2)+(MS^2_{41}/df_{41}+2)+(MS^2_{42}/df_{42}+2)] \quad [3a]$$

$$\text{Var}\ (\sigma^2_{SCA}) = [2/(rak)^2]\ \ [(MS^2_{32}\ /\ df_{32}+2) + (MS^2_{42}\ /\ df_{42}+2)] \quad [3b]$$

In this evaluation, in addition to the usual between-family variation, the vegetative propagation of cassava allowed the analysis of within-family variation. By cloning individual genotypes, they could be planted in two locations with three replications in each location. Therefore it was possible to partition the within-family variation into its genetic ($\sigma^2_{c/F1}$), genotype by environment ($\sigma^2_{c/F1*E}$) and environmental (σ^2_e) components, as illustrated in Table 12.3.

The within-family analysis allows estimation of the relative importance of epistatic effects. In the absence of epistasis the following equation holds true (Hallauer and Miranda 1988):

$$\sigma^2_{c/F1} - 3\ \text{Cov FS} + 4\ \text{Cov HS} \approx 0 \qquad [4]$$

Therefore, a test statistics for the significance of epistatic variance can be constructed by using estimates of the parameters on the left side of the equation. The variance for this test statistic is expected to be large (Hallauer and Miranda, 1988) because of the complexity of this linear function. The variance was estimated following the principles established in Lynch and Walsh (1998) and Isk et al. (2003), as follows:

$$\begin{aligned}\text{Var (Test)} &= \text{Var}\,[\sigma^2_{c/F1} - 3\,(\sigma^2_{SCA} + 2\,\sigma^2_{GCA}) + 4\,\sigma^2_{GCA}] \\ &= \text{Var}\,[\sigma^2_{c/F1} - 3\,\sigma^2_{SCA} - 6\,\sigma^2_{GCA} + 4\,\sigma^2_{GCA}] \\ &= \text{Var}\,[\sigma^2_{c/F1} - 3\,\sigma^2_{SCA} - 2\,\sigma^2_{GCA}] \\ &= \text{Var}\,(\sigma^2_{c/F1}) + \text{Var}\,(3\,\sigma^2_{SCA}) + \text{Var}\,(2\,\sigma^2_{GCA}) - 6\,\text{Cov}\,(\sigma^2_{c/F1}, \sigma^2_{SCA}) - \\ &\quad 4\,\text{Cov}\,(\sigma^2_{c/F1}, \sigma^2_{GCA}) + 12\,\text{Cov.}\,(\sigma^2_{SCA}, \sigma^2_{GCA}) \end{aligned} \qquad [5]$$

However, since Cov $(\sigma^2_{c/F1}, \sigma^2_{SCA}) = 0$ and 4 Cov $(\sigma^2_{c/F1}, \sigma^2_{GCA}) = 0$, the formula can be simplified:

$$\text{Var (Test)} = \text{Var}\,(\sigma^2_{c/F1}) + 9\,\text{Var}\,(\sigma^2_{SCA}) + 4\,\text{Var}\,(\sigma^2_{GCA}) + 12\,\text{Cov}\,(\sigma^2_{SCA}, \sigma^2_{GCA}) \qquad [6]$$

The last term in the equation can be estimated as:

$$\begin{aligned}\text{Cov}\,(\sigma^2_{SCA}, \sigma^2_{GCA}) = [(1/rak) * (1/rak(p-2)] * [&\text{Cov}\,(MS_{32}, MS_{31}) - \text{Cov}\,(MS_{32}, MS_{32}) - \text{Cov} \\ &(MS_{32}, MS_{41}) + \text{Cov}\,(MS_{32}, MS_{42}) - \text{Cov}\,(MS_{42}, MS_{31}) + \text{Cov}\,(MS_{42}, MS_{32}) + \text{Cov} \\ &(MS_{42}, MS_{41}) - \text{Cov}\,(MS_{42}, MS_{42})]\end{aligned}$$

in the above equation:

$$\text{Cov}\,(MS_{32}, MS_{31}) = \text{Cov}\,(MS_{32}, MS_{41}) = \text{Cov}\,(MS_{42}, MS_{31}) = \text{Cov}\,(MS_{42}, MS_{41}) = 0$$
$$\text{Cov}\,(MS_{32}, MS_{32}) = \text{Var}\,(MS_{32})$$
$$\text{Cov}\,(MS_{42}, MS_{42}) = \text{Var}\,(MS_{42})$$

Therefore,

$$\begin{aligned}&\text{Cov}\,(\sigma^2_{SCA}, \sigma^2_{GCA}) = \\ &= [(1/rak) * (1/rak(p-2)] * [-\text{Var}\,(MS_{32}) - \text{Var}\,(MS_{42}) + 2\,\text{Cov}\,(MS_{32}, MS_{42})] = \\ &= -[2/(r^2a^2k^2(p-2)] * [\,(MS_{32})^2/(df+2) + MS_{42})^2/(df+2)\,]\end{aligned}$$

Equation 6 can now be written as follows:

$$\begin{aligned}&\text{Var (Test)} = \\ &\text{Var}\,(\sigma^2_{c/F1}) + 9\,\text{Var}\,(\sigma^2_{SCA}) + 4\,\text{Var}\,(\sigma^2_{GCA}) - 12\,[2/(r^2a^2k^2(p-2)]*[\,(MS_{32})^2/(df+2) + MS_{42})^2/(df+2)]\end{aligned}$$

The estimates of σ^2_{GCA} and σ^2_{SCA} additive and dominance variances but these estimates are biased upward because they contain portions of epistatic variances (Equations 1a and 1b).

Table 12.3. Analysis of variance and expected mean squares for a 9-parents diallel design in which the 30 cassava genotypes representing each F_1 cross were clonally propagated.

Source of variation	Degrees freedom [1]	MS	Expected mean squares
Environment (E)	a-1	MS_1	
Rep/E	a(r-1)	MS_2	
F_1	[p(p-1)/2]-1	MS_3	$\sigma^2_e + k\,\sigma^2_\varepsilon + rk\,\sigma^2_{F1*E} + rka\,\sigma^2_{F1}$
GCA	p-1	MS_{31}	$\sigma^2_e + k\,\sigma^2_\varepsilon + rk\,\sigma^2_{SCA*E} + rk(p-2)\,\sigma^2_{GCA*E} + rka + \sigma^2_{SCA} + \ldots$ $\ldots + rka(p-2)\,\sigma^2_{GCA}$
SCA	p(p-3)/2	MS_{32}	$\sigma^2_e + k\,\sigma^2_\varepsilon + rk\,\sigma^2_{SCA*E} + rka\,\sigma^2_{SCA}$
F_1*E	(a-1)([p(p-1)/2]-1)	MS_4	$\sigma^2_e + k\,\sigma^2_\varepsilon + rk\,\sigma^2_{F1*E}$
GCA*E	(a-1)(p-1)	MS_{41}	$\sigma^2_e + k\,\sigma^2_\varepsilon + rk\,\sigma^2_{SCA*E} + rk(p-2)\,\sigma^2_{GCA*E}$
SCA*E	(a-1)(p(p-3)/2)	MS_{42}	$\sigma^2_e + k\,\sigma^2_\varepsilon + rk\,\sigma^2_{SCA*E}$
Error (a)	a([p(p-1)/2]-1)(r-1)	MS_5	$\sigma^2_e + k\,\sigma^2_\varepsilon$
Clones/F_1	(p(p-1)/2)(k-1)	MS_6	$\sigma^2_e + r\,\sigma^2_{c/F1*E} + ra\,\sigma^2_{c/F1}$
Clones/F_1*E	(p(p-1)/2)(k-1)(a-1)	MS_7	$\sigma^2_e + r\,\sigma^2_{c/F1*E}$
Error (b)	a(p(p-1)/2)(k-1)(r-1)	MS_8	σ^2_e

[1] a= number of environments evaluated (2); r= number of replications within each environment (3); p= number of parents involved in the diallel crosses (9); k= number of cloned genotypes representing each F_1 cross (30).

The analyses of between-family variation were published elsewhere (Cach et al. 2005a, Calle et al. 2005; Jaramillo et al. 2005). In those articles genetic effects, rather than genetic variances, were of interest and they were considered fixed effects. In this chapter, however, the analysis of within-family variance and the relative importance of epistatic effect are of prime interest. All effects, therefore, were considered random and normally distributed. The 30 genotypes representing each F_1 cross are clearly a random sample of all possible genotypes that could possibly be derived from the respective parents. The only criterion defining which genotype would be used was the capacity to produce six stakes in an environment different from the target environment where the evaluation was conducted. The parents involved in this study were among a group of about 30 clones characterized by their adaptation to the three different target environments used in these diallels (sub-humid, acid soil and mid-altitude valleys environments). The respective parents for each diallel are considered to be part of a reference population of clones adapted to the target environment.

The actual parents eventually included were those that allowed for a balanced set of progenies for the study. Therefore, the main criterion for the selection of the parental lines was based on their capacity to flower and produce adequate samples of botanical seed from many different crosses. It is difficult to assess the impact (if any) of this selection because crossings are made in the mid-altitude valleys environment where CIAT headquarters are located, but the evaluation was conducted in a different environment in the case of the diallels for sub-humid environments and the acid soil savannas. This is important because the flowering habit, which profoundly affects plant architecture vary drastically from one environment to the other. A non-branching, erect type in the sub-humid environment may be bushy and flower profusely at Palmira. Because of this situation it can be assumed that

the effect of selection of parents at Palmira had a neutral impact on the general performance of the progenies selected and evaluated for this study.

The analysis of variance for the between-family variation follows the method 4 proposed by Griffing (1956). The usual assumptions for Method 4 analysis are: regular diploid behavior during meiosis; absence of cytoplasmic effects; linkage equilibrium, relatives are random members of a specified population and, because of the vegetative propagation of cassava, negligible C-effects (Libby and Jund, 1962). In the case of cassava, C-effects would result from differences in the physiological/sanitary status between F_1 mother plants and/or among the six stakes used to clone each genotype and these differences would be confounded with the environmental and/or genotype x environment interactions components of variation. Since the F_1 plants from which the six stakes were taken had been grown in Palmira under excellent management practices, differences (if any) in the physiological/sanitary status of these vegetative cuttings are reasonably expected to be small and negligible.

12.3 Results

Results for each of the three different studies will be presented separately and concentrating on the within-family variation, which provides a general idea of the relative magnitude of the different components of the genetic variation.

Sub-humid environment

The analysis of variance and other details of this study have been published (Cach et al., 2005a; 2005b). Based on the magnitude of the estimates for between- and within-family genetic variances, a large proportion of the genetic variability (79-93%) remained as within family variation (Table 12.4). These results agree with observations during the selection in evaluation trials where large numbers of crosses among elite parental lines are represented by several clones. As expected, the lowest within-family variation (79% of total genetic variance) was measured for a relatively simply inherited trait such as the reaction to thrips (Bellotti 2002), which showed the only statistically significant additive variance. The tolerance/resistance in outstanding parents transmitted to the progeny tended to accentuate differences among families and reduce the variability among sister clones. A similar situation was observed in for the mid-altitude valleys environment for other pests. However, it is clear that a considerable within-family variation still remained even for the reaction to thrips. On the other hand, complex traits such as root and foliage yields showed a larger partitioning of the total genetic variance (> 90%) into the within-family variation, suggesting that there were, comparatively, smaller differences in the breeding values of the progenitors.

The within-family variation suggested not only important genetic effects, but also significant genotype-by-environment variation for all variables analyzed. This interaction implies that reliable selection can only be made when enough planting material for replicated trials at more than one location, has been produced. In practice, this means the third or fourth stage in the selection process (Ceballos et al., 2004). One alternative for overcoming this problem would be to modify the clonal evaluation trials (first stage in the selection process), which currently is conducted as a non-replicated trial at a single location, with seven plants per genotype (Ceballos et al., 2004). The total number of plants per genotype can be raised to eight so that two trials, at two different locations, and with four plants per genotype at each

location can be planted. Although the costs related to this change are large, and the logistic complications considerable, the data provided by this experiment (and other similar studies) suggest that they may be justifiable.

Table 12.4. Variances and test for epistasis from the evaluation of a diallel set combining data from two locations (Pitalito and Sto. Tomás) in Atlántico Department, Colombia. Within parenthesis the standard error for each estimate.

Genetic parameter	Thrips (1-5)	Fresh root yield	Fresh foliage yield	Harvest Index	Dry matter content	Dry matter yield
σ^2_G (Between F_1)	0.225	13.09	11.53	0.0010	0.772	0.694
σ^2_G (Within F_1)	0.641	127.21	131.86	0.0037	5.556	9.977
σ^2_G (Total)	0.867	140.30	143.39	0.0048	6.328	10.671
σ^2_A	0.419 (0.211)	17.82 (13.75)	11.93 (12.59)	0.0009 (0.0010)	1.452 (0.985)	0.741 (0.933)
σ^2_D	0.231 (0.068)	23.87 (11.15)	27.02 (10.00)	0.0027 (0.0011)	0.765 (0.497)	1.589 (0.919)
Epistasis Test¶	0.259 (0.119)	100.40 (12.74)	105.64 (11.84)	0.0013 (0.0009)	4.257 (0.673)	8.414 (0.990)

¶ Test for epistasis = $\sigma^2_{c/F1}$ – 3 Cov. FS + 4 Cov. HS

Dominance effects were very important for thrips, harvest index and root and foliage yields, with variance estimates significantly different from zero (estimates two times or more the size of the respective standard error). Only the score for thrips and dry matter content showed larger estimates for the additive compared with the dominance variance (Table 12.4). This highlights the importance of heterosis in cassava breeding for many relevant traits, which in turn justifies the implementation of a reciprocal recurrent selection scheme for cassava genetic improvement.

Epistatic effects were significant for all variables, except harvest index, based on the test for epistasis (Table 12.4). It was surprising to see the size and generalized significance of epistatic effects. To a large extent this may be the result of the large size of this experiment, which resulted in large degrees of freedom for the overall analysis, including the number of clones within family and the number of replications and environments employed. However, the large and frequent epistasis found in this study may also be the result of the evolutionary history of this species that can multiply both sexually or clonally. It is feasible that cassava has evolved to take advantage of favorable gene combinations resulting from dominance and epistatic relationships by fixing them through the vegetative mode of reproduction.

Acid-soil savannas

Results of this diallel have been published (Calle et al., 2005; Pérez et al., 2005b). The comparison of the relative magnitude of between- and within-family variation suggested that a large proportion of the genetic variability in cassava was detected as within-family variation for fresh root and foliage yields (Table 12.5). These results reaffirm the observations made during the selection in evaluation trials where large number of crosses among elite parental lines, are represented by several clones. The within-family genetic variances for harvest index, dry matter content and plant type score were larger than for between-family variation, but the difference was not as large as for the root and foliage yields. On the other hand, the score for super-elongation disease (SED) induced by the fungus *Sphaceloma manihoticola,* showed larger variation in the between- compared with the within-family component. Larger between-family variation was observed for reactions to thrips, white flies and mites in the diallels for the sub-humid and mid-altitude valleys environments and with a different set of parental lines (Cach et al. 2005b; Pérez et al., 2005a). The relatively simple inheritance for resistance to diseases or pests (with a strong dominance component) generate large variation between the averages of progenies involving one or two resistant parents compared with those from susceptible ones, with relatively little or no variation among the individual genotypes or clones within each family.

Table 12.5. Variances and test for epistasis from the evaluation of a diallel set from ten parents combining data from two different edaphic environments at CORPOICA – La Libertad (Villavicencio) in Meta Department, Colombia. Within parenthesis the standard error for each parameter.

Genetic parameter	Fresh root yield	Fresh foliage yield	Harvest Index	Dry matter content	Plant type score	SED† score
σ^2_G (Between F_1)	1.649 (2.954)	1.325 (3.094)	0.0010 (0.0006)	1.600 (0.664)	0.089 (0.039)	0.237 (0.055)
σ^2_G (Within F_1)	21.082 (2.297)	38.557 (3.242)	0.0030 (0.0003)	3.216 (0.169)	0.121 (0.012)	0.088 (0.066)
σ^2_A	-1.485 (6.321)	1.172 (8.035)	0.0015 (0.0016)	3.379 (2.399)	0.160 (0.144)	0.523 (0.234)
σ^2_D	9.028 (7.930)	3.384 (6.594)	0.0011 (0.0013)	0.873 (0.666)	0.096 (0.033)	0.092 (0.050)
Epistasis Test¶	15.054 (6.740)	35.433 (6.858)	0.0014 (0.0012)	0.872 (1.294)	-0.031 (0.077)	-0.242 (0.139)

¶ Test for epistasis = $\sigma^2_{c/F1}$ – 3 Cov. FS + 4 Cov. HS

†SED = super elongation disease induced by the fungus *Sphaceloma manihoticola*

The cassava-breeding project at CIAT has recently started to generate data from the earlier phases of the selection process called Clonal Evaluation Trials (CET) that allow an estimation of the breeding value of parents used in generating these trials (Ceballos et al., 2004). These modifications have been described in this report as well (Chapter 3). In general, these estimations of breeding values based on the CET will be effective in traits were the genetic variation is concentrated in the between-family component or shows strong additive effects. Selection of outstanding parents for a given trait such as SED score, will

tend to generate uniform progenies also outstanding for that trait. This, in turn, could allow the implementation of the Backward GCA Selection described by Mullin and Park in 1992. For characteristics such as fresh root yield, with strong non-additive effects and large within-family variation, the selection of outstanding parents would not be enough and individual clone analysis, within a given family, would be required.

In this diallel, epistatic effects were important for fresh root and foliage yields. These results agree with those observed in similar studies conducted for the other environments.

Mid altitude valleys

Results from this diallel have been published (Jaramillo et al., 2005; Pérez et al., 2005a). Genetic variation has been partitioned into the additive and nonadditive genetic effects. Dominance is a measure of nonadditivity of allelic effects within loci and epistasis describes the nonadditivity of effects between loci. Simple statistical arguments suggest that epistatic interactions are likely to be important in the expression of many quantitative traits. In this diallel, as in the previous ones, a large proportion of the genetic variability was found to be within-family variation (Table 12.6). It was surprising to find such a large variation for the within-family component, for reaction to the two pests was mostly attributable to additive variation.

Table 12.6. Variances and test for epistasis from the evaluation of a diallel set from nine parents combining data from two different mid-altitude valleys environments at CIAT–Palmira and Jamundi in Valle del Cauca Department, Colombia. Within parenthesis the standard error for each parameter.

Genetic parameter	Fresh root yield	Harvest Index	Dry matter content	Reaction to mites	Reaction to whiteflies
σ^2_G (Between F_1)	42.8 (13.3)	0.0016 (0.0004)	1.19 (0.43)	0.271 (0.067)	0.345 (0.115)
σ^2_G (Within F_1)	288.9 (19.2)	0.0029 (0.0002)	2.25 (0.21)	0.188 (0.107)	0.119 (0.120)
σ^2_A	11.9 (24.7)	0.0029 (0.0015)	1.43 (1.33)	0.571 (0.271)	0.994 (0.467)
σ^2_D	152.1 (49.1)	0.0018 (0.0008)	2.47 (0.89)	0.170 (0.065)	-0.210 (0.132)
Epistasis Test¶	168.9 (40.2)	0.0001 (0.0010)	-0.32 (0.92)	-0.225 (0.179)	-0.221 (0.279)

¶ Test for epistasis = $\sigma^2_{c/F1}$ – 3 Cov. FS + 4 Cov. HS

The magnitude and generalized significance of σ^2_D highlights the importance of nonadditive genetic effects (heterosis) in this allogamous species. Only the reactions to pests showed significant estimates for σ^2_A, not only in this diallel but in the other two, as well. In this

study, fresh-root yield was the only trait showing significant conditioning by epistatic effects, following the same trend observed in the previous studies.

1.4 Conclusions

The consideration of random genetic effects in the analysis of this diallel study can be questioned. The number of parents (nine) and clones representing each F_1 cross (≈30) may not be enough to properly represent the genetic variability in the reference population (cassava adapted to midaltitude valleys). However, the number of parents and clones within each F_1 cross in this study required the analysis of more than 1000 genotypes in a total of six replications. Even small increases in the number of parents and/or clones within each F_1 cross would result in an unmanageably large experiment. The number of F_1 families and clones per cross is representative of those typically used in the clonal evaluation trials in the cassava-breeding project at CIAT (Ceballos et al., 2004). Any bias that may affect the conclusions in this study (including those from linkage disequilibrium), therefore, would be similar to that affecting the breeding efforts this study aims to improve. Selection of parents in this kind of study is somewhat unavoidable, as acknowledged in Costa e Silva et al. (2004), and yet the genetic effects still can be considered random.

Few studies have found as significant epistatic effects in annual crops as grain yield for maize (Ceballos et al., 1998; Gamble, 1962; Lamkey et al., 1995; Moreno-González and Dudley, 1981; Narro et al., 2000) and other traits (McConnell and Gardner, 1979; Olatinwo et al., 1999), as well as in perennial crops (Foster and Shaw, 1988; Rönnberg-Wästljung et al., 1994). In many other cases, however, epistatic effects may have been large but failed to reach statistical significance, in part, because of the size of the standard errors typical for complex linear functions (Hallauer and Miranda 1988; Holland, 2001). In this study, however, this was not the case. Results from these studies further demonstrate the importance of epistasis in complex traits such as fresh-root yield and to expose the limitation of most quantitative genetic studies based on the assumption of negligible epistasis. These results would also help explain the difficulties in finding QTL that satisfactorily explain the phenotypic variation observed in complex traits such as yield (Kao and Zeng, 2002).

CIAT has recently introduced modifications that allow for the estimation of GCA effects in early stages of the selection process (Ceballos et al., 2004). This, in turn, allows the implementation of the Backward GCA Selection described by Mullin and Park in 1992. Results from these studies suggest that this approach would be ideal for traits such as the reaction to thrips (Table 12.4), SED (Table 12.5), and whiteflies or mites (Table 12.6) given the importance of GCA effects and the comparatively low relevance of dominance and epistatic effects. For complex traits such as fresh-root yield, however, the prevalence of non-additive effects suggested by this study, would require a different approach.

The phenotypic clonal selection used for cassava breeding takes advantage of the vegetative reproduction of the crop. In selecting outstanding clones all genetic effects (additive, dominance and epistatic) are exploited (Ceballos et al., 2004; Mullin and Park, 1992). However, the current recurrent selection system lacks the capacity to direct genetic improvement in such a way that the frequency of favorable (within or between loci) genetic combinations is maximized. To achieve this, special efforts to design parental clones that produce better crosses are required.

The mass phenotypic recurrent selection used for cassava genetic improvement exploits additive, dominance and epistatic genetic effects (Ceballos et al., 2004; Jennings and Iglesias, 2002; Hershey, 1984). However, the current breeding scheme cannot direct genetic improvement in such a way that the frequency of favorable genetic combinations (within or between loci) is efficiently maximized. To achieve this, special efforts to design parental clones (preferably with some degree of inbreeding) that produce better crosses and/or a reciprocal recurrent selection (Hallauer and Miranda, 1988), are required. The development of clones specifically designed for their utilization as parents in breeding nurseries would be one alternative that offers interesting advantages. Introduction of inbreeding in these parental clones would facilitate the gradual and consistent assembly of favorable gene combinations, which in the current system occur just by chance. Inbreeding would also facilitate the reduction of the genetic load of this crop, which is expected to be relatively large at this point in time.

Moreover, inbred parents offer additional advantages: a) Promote the discovery of useful recessive traits (e.g. waxy starch); b) Genetic load, which is expected to be high based on published results (Kawano et al. in 1978), could be reduced; c) Inbred parents would allow for the implementation of the back-cross scheme (Allard, 1960). This in turn will enhance the usefulness of valuable traits (e.g. genes for resistance to whiteflies, novel starch properties, etc.), which can then be transferred from one parental clone to another; d) Molecular markers and traditional genetic studies would be greatly facilitated; e) Germplasm exchange based on botanical seed is much easier than that of vegetative cuttings (Iglesias et al., 1994). This would promote south-to-south collaboration among the few cassava breeding projects of the world; and f) Cleaning planting stocks from viral or other pathogens could be achieved without the need of meristem culture.

One major constraint for the introduction of inbreeding in cassava is the time required for it. The production of doubled haploids through anther or microspore culture is an interesting approach that would reduce the time required to obtain homozygous genotypes. This, in turn, will maximize the exploitation of dominance and epistatic genetic variation, which have been found to be significant in this study. CIAT is currently executing a project financed by the Rockefeller Foundation to develop the protocol for the production of doubled-haploids in cassava.

The methodology and results of this study can be useful for other semi-perennial crops that can be vegetatively propagated. The diallel design employed, although cumbersome, it not very different from the traditional CET used in cassava (Ceballos et al., 2004). The main differences were the restriction of a balanced set of crosses and the fact that each genotype (or clone) was planted in six, single-plant plots, rather than in a single six-plant plot. A balanced set of crosses allows for proper estimation of genetic parameters. The planting scheme allows a more precise estimation of the genetic value of each clone, because of the reduced impact of genotype x environment interaction. As a matter of fact, the data produced from this diallel study was used in the same way as that from CETs and the best clones were selected to continue in the following stages of selection (preliminary yield trials, advanced yield trials and regional trials).

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