

Review

Cassava genetic resources and their utilization for breeding of the crop

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ABSTRACT. Wild cassava relatives are perennials and vary in growth pattern from nearly acaulescent subshrubs to small trees. They have been used as a source of useful characters such as high protein content, apomixis, resistance to mealybug and mosaic disease, and tolerance to drought. Indigenous clones are a potential source of β -carotene and lycopene. Apomixis genes have been transferred to the crop successfully through interspecific hybridization, and apomictic clones arising from these hybrids are now being grown at the Universidade de Brasília. Interspecific hybrids produced earlier were polyploidized and had their fertility restored. Different useful types of chimera were also produced.

Key words: Apomixis, Drought, Protein, Amino acids

WILD *MANIHOT* SPECIES - A BOTANICAL REVIEW

Wild cassava relatives are perennials and vary in growth pattern from nearly acaulescent subshrubs to small trees. Procumbent, semiherbaceous subshrubs, shrubs, and trees are found in the genus (Figure 1A-G). The branching pattern is typically dichotomous or trichotomous, having at the branching point a terminal inflorescence. Bark of the woody species is generally smooth. Many of the species are laticiferous, and some species, particularly *Manihot glaziovii* (Ceará rubber), are cultivated in Brazil and in some countries (Nigeria) for rubber production (Rogers, 1965; Rogers and Appan, 1973). This species was used by Storey and Nichols in the 1930s in Tanzania (formerly Tanganyka) to transfer resistance to mosaic disease. Many species such as the tripartita group have their stems adapted to dry periods; they die back to a root crown regularly and shed their leaves during the dry season. The majority of *Manihot* species are found on limestone-derived and well-drained soils.

All *Manihot* species are monoecious and a few are dioecious, which make them obligate out-crossers. In many species, they are protogynous, i.e., pistillate flowers open before staminate flowers of the same inflorescence. Pollination is done by insects to whose bodies the sticky pollen adheres. This cross-pollination phenomenon leads to the formation of extremely heterozygous gene pools. Being allopolyploid species, partially apomictic, and having weak barriers in addition to their allogamous nature, have led to the rapid speciation of this group and formation of the large number of species (Nassar, 1999, 2000, 2004).

All species of the genus *Manihot* are native to South America (particularly Brazil). The only species found in other tropical regions of the world are those that have been introduced since Columbus' voyages to the American continent. The species of *Manihot* are all rather sporadic in their distribution and rarely become dominant among the local vegetation. The majority of these species are found in relatively dry regions, and only a few are found in rainforest regions. Their typical habitats are clearings in the forest, as in the case of *M. anomala*. So they are typically heliophiles that grow only in the absence of shading. Many of these species such as *M. pohlii*, *M. zehntneri* and *M. grahamii* are weedy types capable of invading newly disturbed areas, and are frequently found on limestone-derived and well-drained soils. All of the species are damaged by frost with exception of a few, such as *M. grahamii* and *M. neusana*, whose native distribution includes regions with occasional frost.

According to Rogers and Appan (1973), 98 *Manihot* species have been recognized. Only one species, *Manihotoides pauciflora*, is known in the closest related genus *Manihotoides*. Several of its attributes are not found in any *Manihot* species, including single-flower inflorescences, which is a primitive characteristic compared with the multi-flowered inflorescence in *Manihot*, and leaves borne at the apex of short, condensed stems arising from branchlets. Such evidence suggests that this species is the probable origin of all the *Manihot* group. Unfortunately, this species is on the verge of extinction, and may be eventually extinct (Nassar, 1999, 2002c).

Rogers and Appan (1973) classified *Manihot* species into 19 sections, varying from trees in the section Glazioviannae to subshrubs, nearly acaulescent, in the section Stipularis. The species in this latter section are also characterized by being more dioecious than monoecious, which is the opposite in all other *Manihot* species. Other sections, such as Tripartitae and Graciles, are perennial subshrubs with large woody roots; their stems frequently die back to the root crown in response to dry periods or fires.

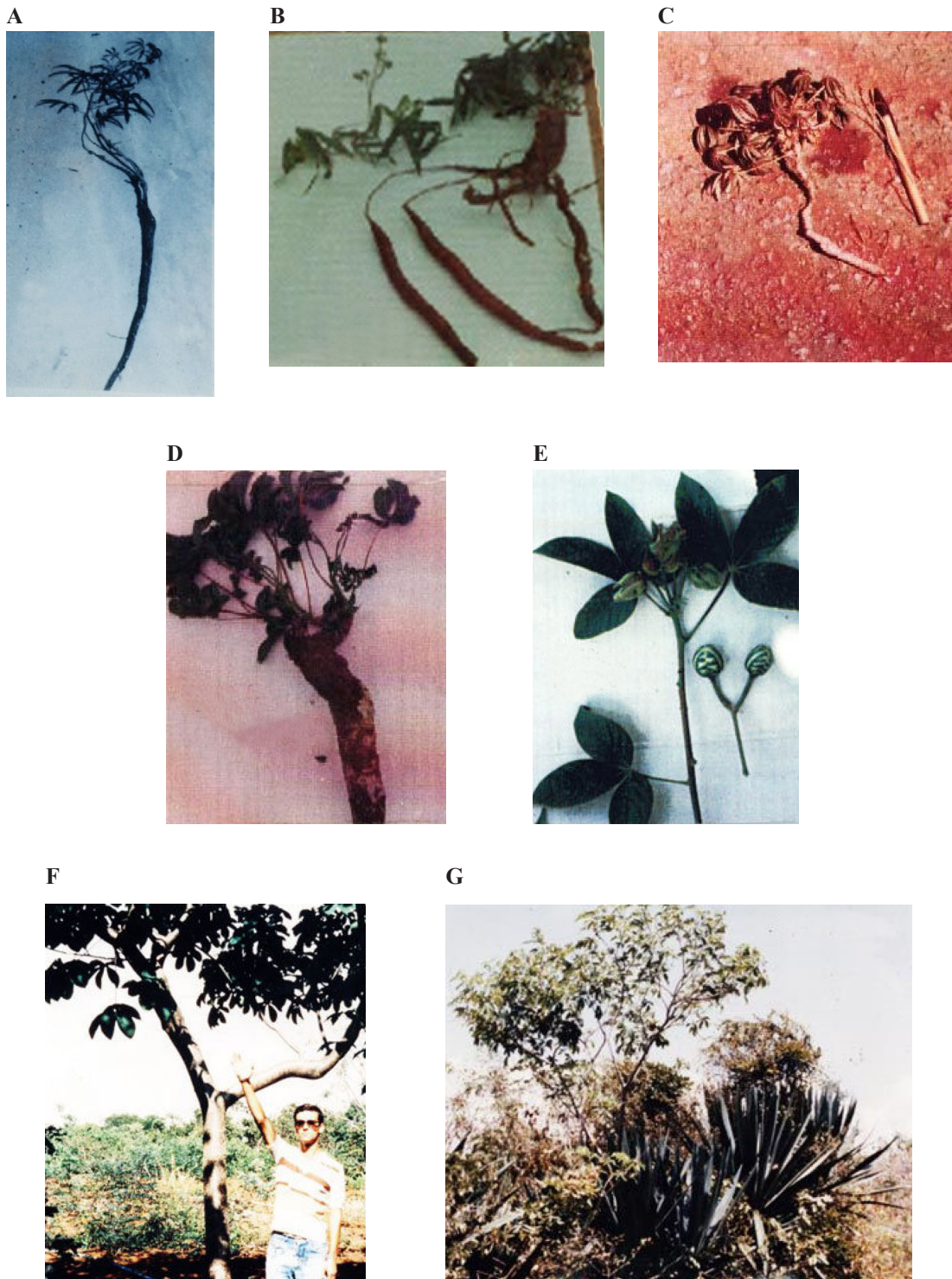


Figure 1. Wild cassava relatives varying in growth pattern from nearly acaulescent subshrubs to small trees. **A.** *Manihot falcata*. **B.** *M. oligantha*. **C.** *M. stipularis* Pax. **D.** *M. nana* Muell. **E.** *M. neusana*. **F.** *M. glaziovii*. **G.** *M. pseudoglaziovii*.

All *Manihot* species are native to tropical regions of the New World, particularly Brazil and Mexico. Nassar (1978d) defined four centers of diversity for these species: Mexico and northeast, central, and southwest Brazil. Microcenters of diversity for these species exist within central Brazil, where large numbers of species are concentrated in small areas (<50 km in diameter) (Nassar, 1978a-f, 1979a,b, 1980a,b, 1982, 1984, 1985, 1986, 2003a,b). These microcenters arose due to the frequent hybridization between species and the heterogeneous topography of their habitats, which helps isolate fragmented gene pools that lead to speciation. Tree-like species, such as *M. glaziovii* and *M. pseudoglaziovii*, are found in northeastern Brazil, whereas short species and subshrubs are found in central Brazil.

WILD *MANIHOT* SPECIES AND THEIR INTERSPECIFIC HYBRIDS

Wild *Manihot* species have been used by this author through hybridizing them with cassava. Probably the most impressive case is the interspecific hybrid of *M. oligantha* with cassava. This hybrid has high protein content, which reaches 4% of peeled roots, i.e., double the protein content in common cassava, combined with low HCN content of 90 mg per kg (Nassar and Dorea, 1982). Recently, genes for apomixis from the wild *M. neusana* species were transferred successfully (Nassar, 2000, 2003f,g, Nassar et al., 2000). Probably the most important utilization of wild *Manihot* species is the discovery of resistance to mealybug in *M. glaziovii*, and its transfer to the cassava gene pool through interspecific hybridization (Nassar, 1996, 2006b). This interspecific hybrid could be polyploidized and its fertility restored (Nassar, 2003e).

Natural hybridization occurs among wild *Manihot* species and between them and cassava (Nassar, 1984, 1989). Barriers within the genus appear to be weak due to recent evolution of the group. All wild *Manihot* species that have been examined cytogenetically have a chromosome number of $2n = 36$ (Nassar, 1978a,b). Despite this high chromosome number, *Manihot* species behave meiotically as diploids. Therefore, they are believed to be allopolyploids, and this seems to have anticipated the emergence of the whole *Manihot* group.

Morphological markers for lobe shape, the presence of stem nodes, flower disc color, fruit color, and fruit shape (Figures 2, 3, 4) were discovered in controlled crosses between cassava and wild *Manihot* species, as well as in natural hybrids between cassava and different species. These genes were used to identify hybridization. Interspecific hybrids of cassava with *M. glaziovii*, *M. pseudoglaziovii*, *M. aesculifolia*, *M. pilosa*, *M. dichotoma*, *M. pohlii*, *M. neusana*, and *M. anomala* were obtained through controlled crosses, although their frequency was low. The meiotic behavior of several hybrids (cassava with *M. neusana* and *M. pseudoglaziovii*) was studied by Nassar (1994, 2002a) and Nassar et al. (1996a), and the results indicated low hybrid fertility between these species and cassava.



Figure 2. Natural hybrid (right) of *Manihot alutecea* (left) with *M. reptans* (medium).



Figure 3. Fruit shape and color as markers in the hybrid.



Figure 4. Morphological markers winged fruit (left) and variegated fruit (right).

PRODUCTION OF CASSAVA CULTIVARS BY INTERSPECIFIC HYBRIDIZATION

Cassava cultivars are deficient in many economic characteristics such as resistance to insects, diseases, and drought and have low protein content (Nassar, 2000; Nassar and Dorea, 1982; Nassar and Grattapaglia, 1986). This can be attributed to the mode of evolution in the species and modifications of the allogamy system of the plant (Nassar and O'Hair, 1985). Lost genes can be restored to the gene pool of the cultigen by interspecific hybridization with wild relatives that possess these genes (Nassar and Grattapaglia, 1986). Wild species of cultivated crops have frequently been used as an important source of genetic diversity and have been employed effectively in a variety of breeding programs (Stalker, 1980). There are interspecific barriers to hybridization, but these are weak and can be overcome in different ways (Nassar et al., 1996b).

Nassar (1978f, 1980a, 1984, 1989, 1992, 1994, 2006a) reported the production of interspecific hybrids of several *Manihot* species with cassava through controlled crosses by insect vectors (Figures 5 and 6). The following morphological markers were used to identify interspecific hybrids: variegated color of fruit dominant to smooth, red color of flower disk dominant to yellow, setaceous bracteole dominant to foliaceous, and noded stem dominant to smooth. Observations of growth pattern, height, stem texture, and tuber formation were also recorded. Other characters provided indirect evidence of hybridization. The hybrid plants ex-

hibited dominant phenotypes from cassava, namely, ribbed fruit, red color of the flower disk, noded stem, and tuberous root (Table 1). These results show that glabrous stem, setaceous-foliaceous bracteoles, red-creamy color of flower disks, variegated-green color of fruit, and ribbed-non-ribbed fruit are simple morphological markers that can be used to recognize interspecific hybridization. It is evident that interspecific barriers between *Manihot* species can be overcome by the use of an abundant diversity of pollinator gametes transmitted by insect vectors. Interspecific crosses were difficult to fertilize manually in the present and in previous crosses (Nassar, 1980a). This evidence suggests that barriers between cassava and other *Manihot* species are weak and recently evolved. It seems they have arisen not as a primary isolating event but rather secondarily after geographic isolation. Nassar (1978b) postulated that cassava is an interspecific hybrid that appeared through domestication some 3000 years ago.



Figure 5. Hybrid of *Manihot pseudoglaziovii*.



Figure 6. Clone UnB-120 selected from the interspecific hybrid *Manihot cearulescens* x cassava progeny.

Table 1. Comparison of morphological characteristics of *Manihot neusana*, cassava and their hybrid.

Characteristic	<i>M. neusana</i>	Cassava	Hybrid
Growth pattern	Procumbent shrub (1.5-2 m)	Erect shrub (1.5-2 m)	Erect shrub (1.5-2 m)
Young stem texture	Hairy	Glabrous	Hairy
Bracteoles	Foliaceous	Setaceous	Setaceous
Fruits	Globose, without ribs, variegated	Ovoid, ribbed, green	Ovoid, ribbed, variegated
Tuber formation	None	Forms tubers	Forms tubers
Growth pattern	Erect shrub (2-2.5 m)	Erect shrub (1.5-2 m)	Erect shrub (1.5-2 m)
Young stem texture	Hairy	Glabrous	Hairy
Bracteoles	Semi-foliaceous	Setaceous	Setaceous
Flower disk color	Anomala	Lobed; 5 lobes	Anomala
Fruit	Globose, without ribs	Ovoid, ribbed	Ovoid, ribbed
Tuber formation	Scarcely forms tuberous roots	Forms tuberous roots	Forms tuberous roots

Interspecific hybrids of cassava with *M. glaziovii* and *M. pseudoglaziovii* were produced (Nassar, 1996), and propagated by cuttings and planted alternately with clone Sonora. This clone was chosen because of its high resistance to bacterial wilt. Seeds were collected, planted, and whole plants were grown. In March 1994, these plants were reproduced by cuttings; six of each were planted for assessing yield and survival during the dry season from June to October (5 months). In November 1994, surviving plants were evaluated for root formation. Ten clones were selected and given to the Semi-Arid Centre at Pernambuco for propagation and cultivation under semi-arid conditions of northeastern Brazil. The selected clones were characterized morphologically according to Rogers and Appan (1973) and Nassar and Grattapaglia (1986). This characterization was aimed at detecting the association of different morphological characters with tolerance to drought.

Morphological characterization showed that certain characters were associated with tolerance to drought. All selected clones have a notable brown, thick, and rough superficial epidermis. It seems that the brown-colored thick epidermis is associated with tolerance to drought because of its isolating nature, which impedes evaporation. All the wild species investigated by this author have fibrous roots with brown external color and their epidermal layer is thick. This character may be therefore inherited from the wild. Graner (1942) reported that this character is dominant to white. Anatomically, the distinct portion of the enlarged root is composed of three sections. The first is a layer referred to as the phelloderm which is generally composed of the above-mentioned epidermis, a subepidermis, and a thicker inner layer. The phelloderm is thick and easily separated from the next inner layer. Second is a layer of parenchymatous cells that constitutes the bulk of the root and is the carbohydrate storage region. The third layer is the portion called the cortex or flesh at the center of the root which is a well-defined central vascular core. As noted previously, the outer epidermis is so thin that it is difficult to measure, but it is possible to evaluate its thickness using the naked eye. It is about 0.5 mm in the thickest types.

The second interesting case in selected clones is the prominence of leaf scars on stems. All selected clones have a prominent enlarged leaf scar. This character which seems to be well associated with enlarged root formation in the hybrid progeny apparently was inherited from cassava. All wild species studied by this author have a smooth stem without any leaf scar. All selected clones have deep fibrous roots in addition to enlarged ones. It seems that this character is inherited from the wild. This appears to be a mechanism of cassavas to tolerate drought since they are capable of capturing water from long distance. Both wild species and their interspecific hybrids produced long, deep roots from the fourth month onward, reaching 4 or 5 m when the plants were one year old.

Another mechanism of tolerance to drought is the thick epidermal layer, probably because of its structure, it impedes evaporation. The dieback of the vegetative parts to the

crown in the dry season was the third common character shown by all the selected clones. Presumably, this behavior helps plants to reduce respiration and consumption of carbohydrate deposits. From this study, it is obvious that breeders can make use of morphological characterization as a criterion to detect the association between morphological characters and drought tolerance and consequently select genotypes complying with this objective.

THE TRANSFER OF APOMIXIS GENES FROM *MANIHOT* SPECIES TO CASSAVA

Apomixis means seed formation without fertilization. In cassava, it is an alternative to reproduction through cuttings which normally is practiced by farmers. The latter type of propagation leads to accumulation of viral and bacterial diseases that reduce productivity and may cause extinction of superior genotypes. Thus, by the use of apomictic plants in propagation, systemic pathogens could be avoided, and the genetic segregation in the progeny is prevented. Plant-produced stems through apomixis from a contaminated clone will be free from viral and bacterial germs and can begin a new cycle of clone life in place of its extinction. If apomixis was found or had been introduced into the excellent Brazilian clones such as Guaxupé and Vassourinha, they would not have become extinct, and could have been preserved for a longer time. The use of apomixis in preserving superior genotypes and filtering the bacterial contamination provides benefits to international cassava programs that export their germplasm routinely. It is sufficient in this case, for the destination country to produce only one plant and further reproduce it vegetatively to maintain the original superior genotype.

Facultative apomixis was discovered in the wild cassavas *M. dichotoma* and *M. glaziovii* (Nassar, 1994, 2003c; Nassar et al., 1998). It was noted earlier in *M. neusana* - a species characterized by extreme resistance to bacterial wilt and stem borers (Nassar, 1985). Interspecific hybridization was carried out to transfer these useful genes to the cultigen (Nassar, 1989, 1995, 2002b, 2006a; Nassar and Collevatti, 2005; Nassar et al., 2006). The clearing method was used to detect apomixis (Nassar et al., 1998). The anatomical studies of ovules showed that the embryo was formed by apospory from a somatic cell in the nucellus. The megasporogenesis in ovules with aposporous development proceeds normally up to a certain moment when nucellar cells enlarge and the nuclei divide to form aposporous embryo sacs (Figure 7A,B). These aposporous embryo sacs appear to develop faster than sexual embryo sacs, probably because they are not delayed by meiotic division (Asker, 1979; Nogler, 1984). In some cases, development of apospory embryo sacs from cells within the sexual one was noted. Both the aposporous and sexual embryo grew in parallel and finally coexisted.

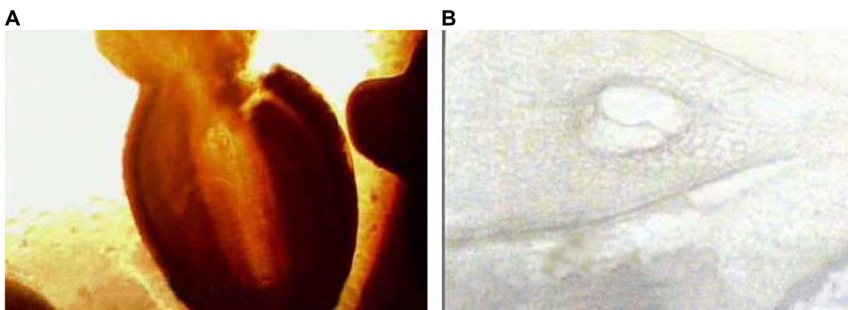


Figure 7. Different forms (A, B) of multiembryos in an aposporic ovule.

This observation confirms results from a previous study (Nassar, 1995), where two seedlings grew side by side; one of which was apomictic and one of sexual origin. Nogler (1984) reported that aposporous and sexual processes coexisted in one individual ovule producing several embryos of *Potentilla*.

This study documents the survival of two aposporous embryo sacs beside a sexual one, all of them at a developed stage in the ovule (Figure 7A,B).

The cytogenetic study showed that of the 25 individuals examined, 13 plants were sterile, and the percentage of pollen viability ranged from 4 to 15% (Table 2). Two plants had $2n + 1$ while the rest were $2n$. The other 12 plants were highly fertile with pollen viability ranging from 92 to 97%. Their chromosome number was $2n$.

Table 2. Chromosome constitution in relation to pollen viability and apospory in apomictic progeny.

Clone	Pollen viability (%)	Apospory (%)	Chromosome number
1	9.2	1.2	$2n$
2	6.8	1.5	$2n + 1$
3	4.9	1.7	$2n$
4	2.0	1.6	$2n + 1$
5	4.7	1.1	$2n$
6	6.1	1.2	$2n$
7	15.6	1.3	$2n$
8	4.1	1.4	$2n$
9	8.3	1.7	$2n$
10	5.6	1.3	$2n$
11	4.1	1.8	$2n$
12	4.6	1.4	$2n$
13	4.7	1.3	$2n$

The embryonic study revealed that all of the sterile plants were partially apomictic while the fertile plants were sexual. Sterility apparently leads to apomixis. Sterility is caused by consistent defects of meioses due to lack of pairing. All of these sterile plants showed asynapsis in meiotic metaphase. The formation of univalents ranged from 4 to 6 per cell. The irregular chromosome-segregation in these sporocytes must lead to genetically unbalanced and aborted gametes. It seems that this sterility triggers certain genes of apomixis to act. Apomixis will function and be established in such genotypes since it is favored by natural selection as it offers an escape from lethality, providing a perpetuation of the current genotype.

In summary, the nature of apomixis in cassava is different from other types found in other crops since it is present at very low levels (1-2%). It depends on meiotic irregularity that often causes sterility in plants. This sterility triggers a certain gene in cassava that activates a number of somatic cells in the nucellus or in the sexual embryo sac to form aposporic embryo sacs.

POLYPLOIDIZATION OF THE INTERSPECIFIC HYBRIDS

Four interspecific hybrids between cassava and wild *Manihot* species, obtained earlier by this author, were used for polyploidization. These hybrids are *M. neusana* x *M. esculenta*; *M. glaziovii* x *M. esculenta*; *M. aesculifolia* x *M. esculenta*, and *M. pohlii* x *M. esculenta*. Twenty vegetative buds of stem cuttings of each hybrid were soaked in 0.2% colchicine aqueous solution for 24 h. Sprouting shoots were examined for leaf shape. Pollen viability was estimated and pollen mother cells were studied to determine chromosome number.

To study pollen mother cells at meiotic division (Figure 8), the buds were fixed in absolute alcohol-glacial acetic acid, smeared and stained with acetocarmine. Pollen viability was estimated using acetocarmine-iodine mixture. Five hundred pollen grains were examined in each cross. To distinguish different types of chimeras and tetraploid tissue, stomata and guard cells on leaf surface and leaf shape were examined on both sides of the emerging stem after colchicine treatment.



Figure 8. Meioses of tetraploid interspecific hybrid cassava x *Manihot glaziovii*.

The colchicine treatment resulted in the production of both complete and chimeral tetraploid stems with tissues having different ploidy levels growing side by side on the same stem. This result is due to the stratified arrangement of cells in the meristem treated with colchicine, and derivation of mature tissue from these layers. The derivative cells of the outermost layer of the tunica form epidermis. The second layer (LII) forms the subepidermal tissues. The third layer (LIII) forms the pith and vascular tissue. The chimeras were distinguished as sectorial or periclinal. The frequency of polyploids obtained from 20 buds treated is given in Table 3.

Table 3. Frequency of polyploids obtained in four interspecific hybrids between cassava and wild *Manihot*.

Hybrid total	Chimera total	Polyploids	Tetraploids	Frequency of polyploids (%)
<i>M. esculenta</i> x <i>M. neusana</i>	1	3	4	20
<i>M. esculenta</i> x <i>M. glaziovii</i>	5	-	5	25
<i>M. esculenta</i> x <i>M. pohlii</i>	2	2	4	20
<i>M. esculenta</i> x <i>M. aesculifolia</i>	-	3	-	15

IDENTIFICATION OF CHIMERAS

It was possible to identify the chimera tissues on the basis of pollen grain viability, leaf shape and stem anatomy. The polyploid section of the stem in sectorial chimeras had short leaves while the diploid side developed narrow and longer diploid form. In case of periclinal chimera, pollen viability, leaf shape and stomata size were used as a selection criteria. Pollen is formed from LII while leaves are differentiated and form the first layer (LI). In periclinal chimeras, the leaf is different and stomata enlarged, and pollen viability is much higher than in diploid plants. All the chimeras in the interespecific hybrid *M. esculenta* x *M. neusana*, and *M. esculenta* x *M. glaziovii* were sectorial while two sectorial, one periclinal chimeras were obtained in the cross cassava x *M. aesculifolia*. In sectorial chimeras, pollen grain viability on one side was low as in a diploid while on the other side it was as high as observed in the tetraploids. The size of pollen grains was notably larger in the latter part. The pollen grain size in the periclinal chimeras reflects the ploidy level of this layer (Table 4).

Table 4. Pollen viability in diploid and tetraploid sectors.

Interspecific hybrid Diploid tissue	Pollen viability (%)		Tissue
	Diploid	Tetraploid	
Cassava x <i>M. neusana</i>	18		92
Cassava x <i>M. glaziovii</i>	11		90
Cassava x <i>M. pohlii</i>	13		93
Cassava x <i>M. aesculifolia</i>	15		91

INSTABILITY OF CHIMERAS

Little information is available about the production of chimera in root crops and much less in cassava, but the use of polyploidy in cassava breeding is frequently reported by Indian breeders. Probably, the most interesting reports came from the Indian team at Thiruvanthpuram (Sreekumari et al., 1999). Their article reports the production of total tetraploids in cassava, but it does not mention chimera induction. Since the appearance of chimera is a frequent phenomenon after colchicine treatment, it is possible that the resulting chimeras were overlooked or simply ignored in the above research. Due to stratification of the shoot apex, cytochimeras with different ploidy levels appear in each layer of tissue and their derivatives when the buds are treated by colchicine (Figure 9). A competition between tetraploid and diploid tissues in chimeras was reported earlier (Stewart et al., 1974). This competition leads to the loss of desired traits. Only the chimeras in LII (periclinal) have a chance of transmitting desirable characteristics to their progeny. In the chimeral sectors observed by us, the stem exhibited diploid phenotype after about six months of growth, restoring the normal leaf shape which became narrow, and pollen viability was that of a diploid. It seems that the growth rate of tetraploid tissue is slower than that of the diploid, and tetraploid tissue is often overgrown by diploid tissue. However, it is possible to propagate tetraploid tissue through somatic selection. This was done by cutting the apical buds of the chimeral stem, followed by removal of the lateral shoots growing from the diploid sector and allowing only the tetraploid side branches to grow.

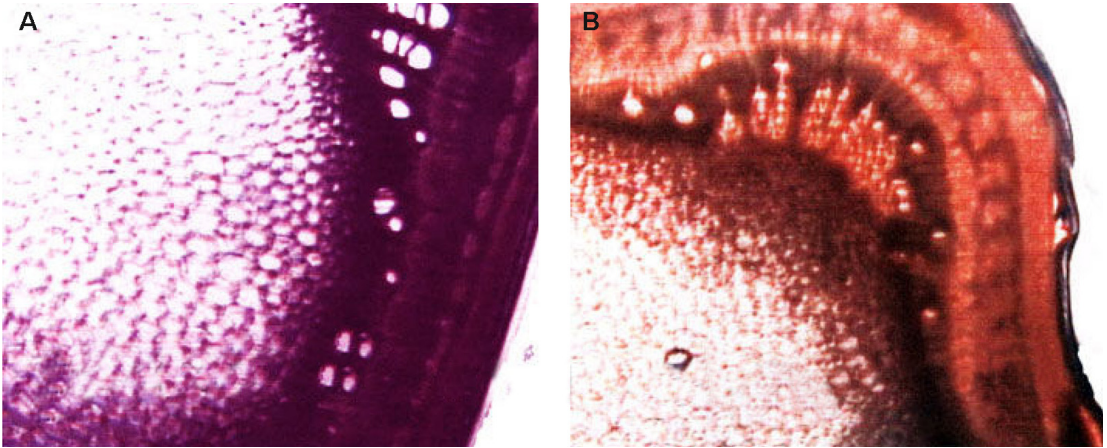


Figure 9. Diploid section (A) and tetraploid section in a sectorial chimera (B).

One of the striking features of polyploidization is the restoration of the fertility of the interspecific hybrids. Yet, a very small portion of unviable pollen formed in the polyploids due to the formation of 3% multivalents in the polyploidized tissue. Quadrivalent formation occurs in cassava itself. Fertility restoration in the interspecific hybrids through polyploidization improves chances of using the wild species for crop improvement. This means the creation of new tetraploid species with high fertility and capable of self-reproduction maintaining their unique characteristics with a new closed gene pool in every interspecific hybrid (Sreekumari and Abraham, 1997; Nassar, 2002a). This technique allows the incorporation of desirable genes in further crosses. The strategy involves backcrossing the polyploidized interspecific hybrids with cassava followed by selection for the desirable traits in the progeny. Preferential autosyndetic pairing between chromosomes of cassava may result in the elimination of the majority of chromosomes of the wild species during meiotic segregation. Even selfing of a fertile hybrid may produce useful recombination between wild *Manihot* species and cassava. One interesting approach in utilizing the induced polyploid types could be to cross them with the facultative apomictic clones, which may lead to the production of apomictic triploid clones that combine both heterosis and polyploidy.

AMINO ACIDS IN CASSAVA AND THE INTERSPECIFIC HYBRID ICB-300

Cassava roots are a poor source of protein in spite of its quality and the proportion of amino acids therein. Methionine and lysine are, however, limiting amino acids in the root. If cultivars can be bred with a higher quantity of these amino acids, it would enhance the value of cassava as a food or feed (Nassar and Marques, 2005). Only about 60% of total nitrogen in cassava is derived from amino acids and about 1% of it is in the form of nitrates and hydrocyanic acid. The remaining 38 to 40% of total nitrogen remains unidentified (Diasolua et al., 2002, 2003).

Cassava proteins are comparable to rice protein in digestibility. The biological value (Block and Michell equivalent) of the total protein is 48%. The crude protein content

of roots appears to be relatively stable and constant with maturity of the plant. According to Close et al. (1953), the protein of processed cassava includes the highest percentage of glutamic acid and the lowest of methionine (1%). Osuntokun et al. (1968) reported that both cystine and cysteine are involved in cyanide detoxification. Cyanide is produced when the glycoside linamarine is hydrolyzed by linase.

Powder sample from a cassava cultivar (UnB-01), an interspecific hybrid between cassava and *M. oligantha* (ICB-300 Diploid), and its offspring (ICB-300 progeny 4, ICB-300 progeny 9, ICB-300 progeny 3, and ICB-300 progeny 10) were analyzed. For this analysis, 500-mg samples of cassava root powder were extracted with 1 mL 10 mM HCl for 4 h, at 25°C, under agitation at 1200 rpm in a Thermemixer (Eppendorf, Hamburg, Germany). The suspensions were then centrifuged for 4 min at 6000 rpm in a bench centrifuge. The supernatant (800 µL), called acid extract, and the remaining powder were dried down in a SpeedVac vacuum centrifuge (Savant, NY, USA). The dried powder was extracted in the same way with 1 mL 10 M NH₄OH producing an alkaline extract. The vial with acid extract was resuspended with 10 mM HCl, washed with the same dilute acid. The total extract was exhaustively dialyzed against MilliQ water and vacuum-dried in a SpeedVac centrifuge. Aliquots of 150 µg of each extract were dissolved in 75 µL 100 mM HCl. Acid hydrolysis of the samples was performed in 6 M HCl under vacuum for 24 h at 109°C. After acid hydrolysis, the hydrolyzed samples were solubilized of 100 mM HCl, and 50 µL was injected into an amino acid analyzer, Hitachi I:8500 (Tokyo, Japan). The analyses for determination of amino acid compositions were performed in triplicate. The total protein content of the samples was calculated by summing up the amounts of the amino acids.

Amino acid compositions from *Manihot* proteins were determined by analyzing sample extracts which were dialyzed against water to remove free amino acids, salts, monosaccharide, and other small molecules (Nassar and Souza, 2007). Tryptophan could not be analyzed since it is degraded upon acid hydrolysis. By summing the amounts of the amino acids found, it was possible to determine the protein content for each sample.

Among the six samples analyzed in this study (Table 5), interspecific hybrid ICB-300 offspring 3 Root showed the highest amount of protein (1654 g/100 g sample powder), followed by ICB-300 Diploid (1454 g/100 g), and ICB-300 progeny 9 (0.922 g/100 g). The other samples (ICB-300 progeny 10 Root, ICB-300 progeny 4 and UnB-01 Root) were poorer in protein content (0.350 g/100 g). Essential amino acids were more concentrated also in progeny 3 Root (His, Leu, Lys, Met, Phe, and Val) and ICB-300 Diploid (Ile and Thr), with low or undetectable amounts in the other cultivars. Thus, progeny 3 Root and ICB-300 Diploid would be more interesting for human consumption based on such nutritional characteristics.

Progenies 3 and 9 showed an equal amount of protein, i.e., double that of common cassava, indicating high heritability of this character and the possibility of selecting for high protein cassava. Essential amino acids in cassava are arginine, histidine, isoleucine, leucine, phenylalanine, threonine, tryptophan, and valine, whereas methionine and tryptophan are lacking. The essential amino acid profile of cassava seems to be deficient in sulfur-containing amino acids (methionine, cystine and cysteine) (Bailey, 1961). Osuntokun et al. (1968) pointed out that both cysteine and cystine are involved in cyanide detoxication (cyanide is produced

Table 5. Amino acid (AA) profile in peeled roots of cassava cultivar UnB, its interspecific hybrid with *Manihot oligantha* - namely ICB-300 Diploid, and its offspring (progenies 3, 10, 4, and 9).

AA	UnB-01	Progeny 3	Progeny 10	Progeny 4	Progeny 9	ICB-300 Diploid
Ala	0.020	0.093	0.017	0.019	0.040	0.098
Arg	0.037	0.261	0.061	0.082	0.320	0.108
Asp	0.016	0.146	0.023	0.033	0.052	0.137
Cys	0.027	0.029	0.026	0.025	0.026	0.025
Glu	0.039	0.222	0.044	0.065	0.151	0.221
Gly	0.012	0.078	0.012	0.015	0.037	0.075
His	0.000	0.038	0.010	0.010	0.027	0.036
Ile	0.008	0.068	0.010	0.010	0.018	0.069
Leu	0.016	0.131	0.013	0.000	0.041	0.127
Lys	0.010	0.098	0.020	0.019	0.034	0.079
Met	0.014	0.041	0.004	0.000	0.019	0.037
Phe	0.016	0.129	0.058	0.000	0.065	0.120
Pro	0.000	0.054	0.000	0.000	0.000	0.066
Ser	0.012	0.088	0.013	0.018	0.033	0.078
Thr	0.008	0.061	0.007	0.013	0.022	0.066
Tyr	0.000	0.000	0.000	0.000	0.000	0.000
Val	0.019	0.115	0.027	0.025	0.039	0.112
Total	0.254	1.654	0.344	0.336	0.922	1.454

Data are reported as sample mass (g per 100 g).

when cyanogenic glucoside-linamarine present in cassava is hydrolyzed by linamarinase or by acid). Cysteine is mainly detoxified by conversion to thiocyanate, in the process of which it reacts with cysteine and cystine. Excessive detoxication may be responsible for the low concentration of sulfur-containing amino acids.

Action was taken before to increase protein content of cassava roots by interspecific hybridization with wild species - namely *M. saxicola* and *M. mefanobasis*. Over a period of 10 years beginning in 1932 and ending with the Japanese occupation of Java in 1942, Bolhuis (1953) carried out a program of cassava breeding for increased protein content in roots. Crosses with *M. saxicola* yielded a few seedlings with as much as 2% protein in fresh roots. In the clones, he propagated from these seedlings and protein content fell back to typical levels. Jennings (1957) reported that the roots of the F₁ progeny of *M. esculenta* x *M. melanobasis* possessed approximately twice as much protein as their cassava parent. The offspring were lost and not cultivated anywhere, probably because of poor root yield.

Barros and Bressani (1967) and researchers at Centro Internacional de Agricultura Tropical (CIAT) reported cultivars with high protein nitrogen content (7%). It is, however, doubtful if the nitrogen referred to in such cultivars was protein or the breakdown of cyanogenic glucosides. It is, therefore, not unlikely that the reported cultivars of high nitrogen content turn out to be nothing but bitter cultivars with high glucoside content. Another interfering factor for assessing protein as total nitrogen is the humidity while drying the material. Excessive drying of the root powder may increase drastically the percentage of nitrogen by 3-fold. Thus, it is important to determine protein content as amino acids jointly with the evaluation of nitrogenous matter.

INDIGENOUS CASSAVA CULTIVARS AS A SOURCE OF CAROTINOIDS

Vitamin A deficiency results in progressive eye damage. It is a serious problem in northern and northeast Brazil (Simmons, 1976; Flores and de Araújo, 1984; Dricot-d'Ans et al., 1988) and many other areas of the country (Desai et al., 1980; Wilson and Da Silva

Nery, 1983; Favaro et al., 1986; Gonçalves-Carvalho et al., 1995). Pro-vitamin A carotenoids are an inexpensive source since they are found to be abundant in plants. Since cassava is one of the most important sources of food in northeast Brazil, selecting high carotene content clones may contribute significantly to reduce the incidence of vitamin A deficiency in developing countries (FAO, 2003).

The average requirement of β -carotene recommended by WHO for adults ranges between 2.4 to 3.5 mg. The range of carotene content in cassava was assessed to select clones rich in carotene and good palatability. To evaluate carotenoids in cassava cultivars and hybrids, 10 g mature roots and 5 g leaves were extracted three times with acetone (5 mL/g). The filtered acetone extract was added to a separation funnel containing petroleum ether, distilled water and ethyl ether (100:100:0.3, v:v:v). The aqueous fraction was discarded, and the organic fraction was submitted to saponification. Saponification was preferred since it removes accompanying lipids and chlorophylls (Nassar, 2003d; Nassar et al., 2005).

The vitamin A values were calculated according to the conversion factor given by NAS-NRC (1989), where 6 μg trans- β -carotene correspond to 1 μg retinol equivalent, and the activities are related as follows: 100% for trans- β -carotene, 50% for trans- α -carotene and cis- β -carotene (Britton, 1995). All the cassava clones showed the same major carotenoids in different concentrations. Lutein, trans- α -carotene and trans- β -carotene were separated and identified according to Davies (1976) and Britton (1995).

The colorimetric method for the characterization of cassava clones in terms of parenchyma color proved to be useful and is pragmatically used to detect if variation exists. Among the cassava clones studied, the most impressive one was UnB-400 with 236 $\mu\text{g/g}$ lutein *vis-à-vis* zero in other cultivars. This antioxidant material is extremely important for health conditions of poor people. The same clone has a reasonable quantity (2.2 $\mu\text{g/g}$), which is considered by WHO sufficient for daily requirements of adults, considering that a person consumes normally 500 g of cassava daily. The presence of lutein at such high levels adds to the valuable importance of this cultivar. In palatability tests, this clone was one of the most outstanding. It was easily cooked within 5 to 10 min maximum, turning to a very soft mass like a cream. The clones showed very low HCN content, as judged by the taste.

The most striking result is the content of both trans- β -carotene and lutein in leaves of clones UnB-400 and ICB-300 (Figure 10). The trans- β -carotene reached 27.40 $\mu\text{g/g}$ in the former, making this clone one of the richest sources of this precursor of vitamin A available for poor people. ICB-300 had almost 20 $\mu\text{g/g}$ of this type of carotene, making it also a very rich source. This clone (ICB-300) is a hybrid ensuing from crossing cassava with the wild relative *M. oligantha*. It has 5% protein compared to 1.5 to 2% in common cassava (Nassar and Dorea, 1982). The amazing result was the amount of lutein in UnB-400 and ICB-300: 3081 and 9108 $\mu\text{g/g}$, respectively. Such quantities mean that they contain 4 to 12 times more carotenoid than do normal clones (Nassar et al., 2005, 2007).

Apparently, the hybrid ICB-300 having 4% protein in the roots, 20 $\mu\text{g/g}$ trans- β -carotene, 9108 $\mu\text{g/g}$ lutein in the leaves is an excellent source of these important components. UnB-400 is a very good source of precursors of vitamin A, considering its excellent palatability. ICB-300 clone is a very good ingredient to be added to wheat flour for making bread considering its high protein and carotene content. The Brazilian government is now examining the possibility of mixing cassava flour with wheat to reduce importations of the latter. An obstacle though is

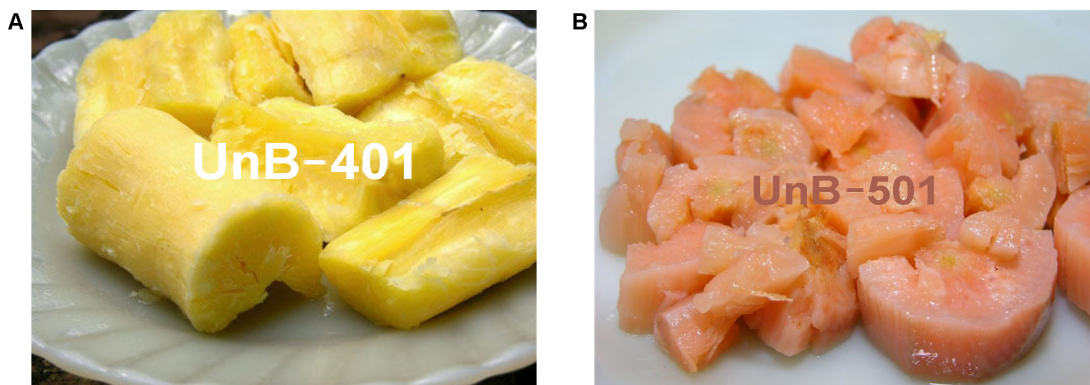


Figure 10. Selected indigenous clone for high carotenoids (β carotene (A) and lycopene (B)).

the low level of protein in common cassava compared to 7% in wheat. Using the flour of the hybrid ICB-300 may resolve this problem. Being rich in vitamin A precursor in its leaves adds an advantage to its future use. These results show the need for assessing cassava interspecific hybrids for carotene content because previous research showed that a cassava hybrid ensuing from crosses with *M. dichotoma* had double the carotene content (22 mg/kg) *vis-à-vis* that (13 mg/kg) of cassava (Nassar et al., 2004).

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