

Gene Flow Between Cassava, *Manihot esculenta* Crantz and Wild Relatives

By

Nagib M. A. Nassar

Departamento de Genética e Morfologia
Universidade de Brasília, Brasília, Brazil

Abstract

Controlled and natural hybridization between cassava and wild relatives do occur. Barriers within the genus appear to be weak due to recent evolution of the group. All *Manihot* species examined cytogenetically have a chromosome number of $2n=36$. However, they behave meiotically as diploid. The weak interspecific barriers have led to an extremely heterozygous gene pool that may begin a sequence of hybridization followed by speciation. Introgression from cassava to a number of wild species has been detected by both morphological marker genes and molecular technique.

Cassava breeders are increasingly interested in wild relatives gene pools because it offers many opportunities of improving the crop by transferring alien genes. Gene flow or interfertility between these wild relatives and cassava is the limiting factor to turn these untapped genetic resources available and manipulated in breeding programs.

Gene flow from cassava to wild relatives may have played an important role in evolution of the wild population. The pollen parent in the initial cross may involve a cultivated clone or a recent escapee. The opposite pathway is true too. This may involve a wild *Manihot* plant that functions as the pollen parent in a cross with cultivated clone giving rise to a hybrid that backcrosses with wild plants. After several generations have passed the genes of cassava are swamped by the wild giving rise to what is known by introgression.

M. zenhneri Ule is an example of a wild species which may have evolved through this process. The strong morphological resemblance and affinity shown by electrophoresis (Grattapaglia et al., 1986) gives a clear evidence. Some factors may favour the hybridization in one or both of the two directions between wild *Manihot* species and cassava in nature. One of them is that both of the two species flower in the same time and for a long period. A second factor is the overlapping of the natural habitat of the wild species and area of cultivation of cassava enabling vectors to carry pollen between them.

Frequently the interspecific hybrid survives well and in many cases shows vigor and more fitness (Nassar, 1989). This vigor has been noted by this author in several cases of hybridization between cassava and wild *Manihot* species such as *M. neusana* and *M. pohlii* (see photo in the [gallery](#)). The meiotic division in several cases is regular and pollen fertility is reasonably viable (Nassar et al., 1996). This hybrid fertility would enhance introgression process and incorporation of species alleles from one to other. The electrophoresis study has revealed clearly the gene introgression from cassava to *M. zehneri*, *M. pilosa* and *M. glaziovii*.

The progressive introgression between cassava and a certain wild species may implicate in the extinction of the wild species and arising of a new one having genes of a parent species swamped by the other. The new species, being enriched by new genes will invade new areas and expand its geographic distribution. This is almost the case of *M. zenhneri* which was collected from states Bahia, Minas Gerais, S. Paulo and Mato Grosso do Sul. *M. reptans* and *M. cearulescens* are also cases of geographic expansion due to introgression (Nassar, 1979, 1982). *M. zenhneri* shows in electrophoresis analysis the highest affinity with cassava (Grattapaglia et al., 1986). Morphologically it is difficult to distinguish it from cassava. In controlled crosses it also exhibits high interfertility with cassava (Nassar, 1978). This case is similar to what reported by Harlan in *Helianthus* (1961), and by Dobzhansky in *Iris*, *Eucalyptus*, *Penstemon* and *Tragopogon* (1973).

All wild *Manihot* species examined cytogenetically have a chromosome number of $2n=36$ (Nassar 1978a) (Table I). Despite this high chromosome number, *Manihot* species behave meiotically as diploids. Therefore, they are believed to be allopolyploids and this polyploidization seems to have anticipated the emergence of the whole group and is responsible for their rapid speciation and their weak interspecific barriers, leading to interspecific hybridization. An extremely heterozygous gene pool is thus created, followed by differentiation; this begins a sequence of hybridization followed by speciation.

Nassar (1980a) reported frequent hybridization between *M. reptans* Pax and *M. alutacea* Rogers et Appan in sympatric natural habitats in which their population boundaries overlap.

Table1 - Chromosome Number in wild *Manihot* species

Species	Growth habitat	n	2n
<i>M. handroana</i>	Shrub	--	36
<i>M. jolyana</i>	Shrub	--	36
<i>M. tripartita</i>	Shrub	--	36
<i>M. reptans</i>	Shrub	18	--
<i>M. tweedieana</i>	Shrub	--	36
<i>M. humilis</i>	Subshrub	--	36
<i>M. pedicellaris</i>	Shrub	--	36
<i>M. gracilis</i>	Subshrub	18	36
<i>M. dichotoma</i>	Tree	--	36
<i>M. glaziovii</i>	Tree	18	--
<i>M. pseudoglaziovii</i>	Tree	--	36
<i>M. anomala</i>	Shrub	18	--
<i>M. zehntneri</i>	Shrub	18	--
<i>M. olighanta</i>	Subshrub	18	--
<i>M. nana</i>	Subshrub	18	--
<i>M. tomensosa</i>	Subshrub	18	--

Morphological marker gene leaf color and bract size were used to identify this interspecific hybridization ([see photos gallery](#)).

M. reptans is a typical example of natural hybridization between wild *Manihot* species. Its range has expanded during the past 100 years (Nassar, 1984) and this is attributed to the continuing gene introgression of *Manihot* species. Introgression of *Manihot reptans* with germplasm from other species allowed its ecotypes to penetrate and colonize areas where *Manihot reptans* (pure) had previously been unable to do so. This phenomenon was also noted in other species such as *Manihot cearulescens* (Nassar, 1980b). From a plant breeding viewpoint, the value of these hybrids lies in their ability to cross with the cultigen.

Marker genes lobe shape, the presence of stem nodes, flower disc color, fruit color, and fruit shape (see photos gallery) 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 by Nassar to identify hybridization. Interspecific hybrids of cassava with *M. glaziovii*, *M. neusana*, and *M. anomala* which were obtained by Nassar through controlled crosses, although their frequency was low. The meiotic behavior of several hybrids (cassava with *M. neusana* and cassava with *M. pseudoglaziovii*) was studied by Nassar (1992,1996), and results indicated low hybrid fertility between these species and cassava.

In the following presentation, a detailed description of the experiments carried by this author to study gene flow between cassava and wild relatives.

Production of Interspecific hybrids between cassava and *M. anomala*, and *M. neusana*

Nassar(1989) reported production of interspecific hybrids of two *Manihot* species namely *M. neusana* Nassar and *M. anomala* Pax with cassava through controlled crosses by vector insects.

Two wild *Manihot* species, *M. anomala* and *M. neusana* maintained in the living collection at the Experimental Biology Station, Universidade de Brasília, were used for this experiment.

In October 1982 the species were each planted in three rows alternated with cassava. In June 1983, 200 seeds were collected from each species and replanted in October 1984 for identification of possible natural hybridization. The following marker genes 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 habit, height, stem texture, and tuber formation were also recorded. In addition to the open pollination for the above mentioned species, 400 manual crosses with pollen of cassava cultivar Catelo were realized.

Of 200 seeds of *M. neusana*, only 43 seedlings emerged of which two hybrids were identified. Interspecific hybrids were identified by dominant markers from cassava; noded stem, setaceous bracteoles, ribbed fruit, and tuberculated root (Table2). Other characters provided indirect evidence of hybridization.

The 200 seeds collected from *M. anomala* gave rise to 112 seedlings. Of these, three seedlings showed characteristics of interspecific hybridization. Only one seedling

survived to maturity. This hybrid plant exhibited dominant phenotypes from cassava, namely ribbed fruit, red color of the flower disk, noded stem, and tuberous roots. (Table 2).

These results show that glabrous stem, setaceous-foliaceous bracteoles, red-creamy color of flower disks, variegated-green color of fruit, and ribbed-nonribbed fruit are simple marker genes that can be used to recognize interspecific barriers between *Manihot* species can be broken by the use of an abundant diversity of pollinator gametes transmitted by insect vectors. Interspecific crosses were difficult to fertilize manually in this work and previous crosses (Nassar et al. 1985). The 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 secondarily after geographic isolation. Nassar (1978b) postulated cassava itself is an interspecific hybrid that appeared by domestication some 200 years ago or less.

Breaking barriers between cassava and the wild relatives *M. pohlilii*

In 1996, Nassar reported results of hybridization of cassava with *M. pohlilii*. Hybrids of *M. pohlilii* and other species with cassava can be obtained, but at a very low frequency due to interspecific crossing barriers (Nassar et al. 1986). However, one technique for overcoming such barriers involves the use of pollen mixes which combine the pollen intended for syngamy with "mentor" pollen of the maternal species. This technique has been reported for a number of plant taxa including *Populus* (Stetler, 1986; Knox et al. 1972), *Malus* (Dayton, 1974), *Cosmos* (Howlett et al. 1975), and *Petunia* (Sastri and Shivanna, 1976). The role of the mentor pollen is to facilitate fertilization by foreign pollen. Apparently, the mentor pollen supplies proteinaceous substances which permit the foreign incompatible pollen grains to germinate (Knox et al., 1972). A treatment destroys the generative function of the pollen grain without affecting germination and growth of the pollen tube, and consequently does not affect stimulation. The stimulating effect of destroyed pollen is due to protein recognition substances. They are liberated by pollen grains while germinating, and have enzymatic and antigenic properties. These substances are localized in the internal layer of the pollen surface, and are correlated with pollen germination and growth of this tube on the stigma surface. They have been localized by cytochemical means in the cellulose intine (Knox et al., 1972). Many studies using this technique have been successful in overcoming an incompatibility barrier (Brewer and Henstra, 1974, Williams and Church, 1975). Subsequent work indicated the need to refine the preparation of the mentor pollen by using freeze thaw cycles or methanol treatment (Knox et al., 1972). The present communication reports the successful use of freeze-thawed mentor pollen in hybridizing cassava with *M. pohlilii*.

In addition to the mentor effect, other techniques seem to have potential for overcoming interspecific barriers. One of these may be the use of a bridge species. We were inspired by the capacity of a species, *M. neusana* Nassar, to cross easily with all *Manihot* species growing in the vicinity, and have used it as a bridge technique. Probably the only case is that which came from Dionne (1963) who used *Solanum aculeatum* Bitter as a bridge between *Solanum tuberosum* L. and *Solanum bulbocostatum* Dunal. We report here the use of *M. neusana* Nassar as a bridge between *M. esculenta* and *M. pohlilii*.

A natural hybrid between *M. pohlilii* and *M. neusana* Nassar was used as a bridge species. The hybrid was identified by fruit marker genes, which produce variegation of fruit color in *M. neusana*, and a straight white line in *M. pohlilii* fruit (see photos gallery). Crosses of this hybrid (named HNP) and cassava (clone EB05) were carried out from January 1994).

Flowers were taped shut for two days until they had been pollinated manually. Pollination of both the hybrid HNP and cassava was done with pollen mixes of cassava and *M. pohlilii*. *M. pohlilii* pollen used as mentor pollen was successively frozen for 5 min at -4°C and thawed for 30 min for a period of 105 min. The purpose of this treatment was to kill the mentor pollen and increase the chance of obtaining interspecific hybrid seed. To verify the presence of any autoincompatibility that would interfere in the controlled crosses in *M. pohlilii* and cassava, a controlled autopollination was undertaken. Cross between cassava and *M. pohlilii* (POH) were carried out using mentor pollen in one trial and untreated pollen in the second trial. Seeds were collected from both crosses and planted in the next growing season.

The pollination of *M. pohlilii* by untreated cassava pollen did not produce any fruit set (Table 2), while crosses with mixed pollen of cassava and mentor resulted in the production of 21 seeds, 4.9% of the possible maximum (assuming that every fruit has three ovules). Using cassava as a maternal parent, pollination by untreated pollen of *M. pohlilii* did not result in any seed. This clearly demonstrates the effect of mentor pollen in the crosses of cassava with *M. pohlilii*. It likewise means that the freeze-thawing treatment administered to *M. pohlilii* pollen, although killing it, did not affect its stimulatory function, so that all of the seed produced by the mentor effect had embryos and endosperm.

Table 2 - Crosses attempted between *Manihot pohlilii* and *M. esculenta* (cassava).

Treatment	Flowers Pollinated	Fruit N°	Seed N°
1 - without mentor			
2 - with mentor			
1° Cassava x <i>M. pohlilii</i>	145	0	0
2° <i>M. pohlilii</i> x Cassava	142	10	21

This result indicates that only the stigmatic barrier functions in preventing crossing in these species. Postfertilization mechanisms fail to prevent crossing. Only one plant germinated and could be raised from these seeds to severe dormancy. This plant bears fruits carrying the marker genes of both *M. pohlii* and cassava; a straight white line from *M. pohlii*, and winged fruit from cassava (see photos gallery). The mentor effect has also been successfully used in *Populus* and *Cosmos* in these genera, interspecific incompatibilities have been overcome by using compatible but dead pollen (Knox et al. 1972). These studies have suggested that this phenomenon is due to proteinaceous recognition factors released from the wall of the killed compatible pollen, masking the rejection reaction of the recipient stigma. Our report represents the first case of obtaining hybrid seed of *M. pohlii* and cassava and its further reproduction. In spite of the useful characters of *M. pohlii*, no successful breeding program has been carried out, due to a lock of hybrids between this species and cassava. Our study is the first to successfully cross these two species.

The use of *M. neusana* as a bridge species through the hybrid *M. neusana* - *M. pohlii* has improved seed set. When used as a maternal parent pollinated by cassava it gave seed in 3,5% of cases while the reciprocal crosses had greatly improved seed set, yielding 25,9% (Table 3). The combined treatment of both species (bridge and mentor) produced 3,5% seed production. The success of *M. neusana* as a bridge species between *M. pohlii* and cassava may occur because the two genomes of *M. neusana* carry different genetic mechanisms of cross incompatibility. This hypothesis is confirmed by our observations on crossing behavior of this species in the living collection of wild *Manihot* species. *M. neusana* has hybridized naturally with several *Manihot* species grown in the vicinity, e.g. *M. pseudoglaziovii* Pax et Hoffm., *M. caerulescens* Pohl, *M. salicifolia* Pohl, *M. pohlii* and *M. esculenta* itself (Nassar, 1989). Its hybrids with the above mentioned species were easily identified by the marker dominant gene of variegated fruit which came from *M. neusana* (see photos gallery). *M. neusana* is a newly emerging species, described and identified recently (Nassar, 1985).

Table 3 - Crosses attempted between cassava and *Manihot neusana* hybrids; fruit and seed produced following different treatments.

Treatment	Flowers Pollinated	Fruit Nº	Seed Nº
HNP x Cassava	161	14	17
Cassava x HNP	90	14	70
HNP x Cassava + mentor	208	19	22

Natural Hybridization between wild *Manihot* species

Hybridization between wild *Manihot* species do occur in nature, and it seems is responsible to rapid speciation in the genus. Natural hybrids between *M. reptans* and *M. alutacea* were observed in 1977 (Nassar 1978c) in two localities in the state of Goiás where *M. reptans* grows near *M. alutacea* and where these species have a sympatric distribution. Since interspecific hybrids have long been of special interest to breeders because of their heterogeneity, this case has been investigated in further detail.

Seeds of *M. reptans*, *M. alutacea* and their natural hybrid were collected from two localities in the state of Goiás 10 and 12 Km east of the city of Goiás Velho and were planted in a living collection of *Manihot* at the Universidade de Brasília. Observations of the following characters were recorded: growth habit, young stem, lamina texture and color, laminar veins, laminar lobes, bracteoles and fruit. Thirty bracteoles were measured on each of 10 plants of each species.

The morphology of *M. reptans*, *M. alutacea* and the hybrid are compared in Table 4. taxonomically, *M. reptans* and *M. alutacea* have been separated on the basis of growth habit, leaf shape and color, plant height, laminar texture and fruit shape and color. The presumed hybrid is similar to one or the other parent for characters except bracteole size, in which case the bracteoles are much larger than in either parent. Morphological measurements of the presumed hybrid provide indirect evidence of interspecific hybridization. From the scatter diagram (see photo gallery), a distinction between bracteole length, range of the two species and their hybrid is seen. Many workers expect the hybrid to occur in areas where distribution of the two parent species adjoin each other and where hybridization between the two is likely to be occurring continuously (Anderson 1953; Baker 1951; Harlan and de Wet 1963; Heiser 1961; Levin 1863)

The presumed hybrids which were collected from a site 10 Km east of Goiás Velho occurred in the absence of *M. reptans* stand being swamped by a large amount of pollen from the more abundant *M. alutacea*. Another possible hypothesis is that both species were present at this site at some early time and hybridized there;

Table 4. Comparison of morphological characters for *M. reptans*, *M. alutacea* and presumed hybrid

Character	<i>M. reptans</i>	<i>M. alutacea</i>	Presumed hybrid
Growth habit	Procumbent weak stemmed, ca 0.5 m, numerous stems arise from a woody base	Erect shrubs, 1- 1.5m	Erect shrubs, 2- 2.5m

Young stem texture and color	Glabrous, purplish tinged	Glabrous, deep red	Glabrous, purplish tinged
Lamina texture	Membraneous to slightly coraceous	Alutaceous	Membraneous
Laminar veins	Glabrous, bright green	Glabrous, bright red	Glabrous, bright red
Laminar lobes	Palmately 3-5 lobes, apex obtuse	Palmately 3-5 lobes, apex acute	Palmately 3-5 lobes, apex obtuse
Bracteoles	Setaceous 2-5 x 0,5-1,6 mm	Setaceous 7-9,5 x 1,6-3,4 mm	Semifoliaceous 10-20 x 4-10 mm
Fruits	Globose, without ribs, green, surface smooth	Globose, ribbed with bright red ribs, surface punctate	Globose, slightly ribbed, yellowish to redish ribs, surface smooth

Subsequently the environment changed and *M. reptans* disappeared since it was poorly adapted. Our observations show that *M. reptans* and *M. alutacea* will readily hybridize when grown sympatrically. Sympatric populations contain a large number of hybrids suggesting absence of barriers which may exclude the hybrids from the gene pool of the population. Nassar (1978c) referred to expansion of the *M. reptans* range in the last hundred years and attributed this to continuing introgression of genes of *Manihot* species. The hybrid of *M. reptans* with *M. alutacea* was identified by its bright red leaves, a characteristic of *M. alutacea* which served as a marker gene in the case.

A population of *M. reptans*, characterized fruits, two characteristics of cassava (*M. esculenta*) was found about 15 Km North of Corumbá de Goiás. Apparently, these two characteristics were acquired by *M. reptans* through introgressive hybridization with cassava. It seems that *M. reptans* became introgressed with germplasm from other species and was able to extend its ecotypic range and colonize new areas where the pure *M. reptans* had been previously unable to penetrate.

The hybrid type from Corumbá was collected from a disturbed habitat which may account for its weedy nature. It may be considered part of a weed-crop complex following the model described by Harlan and de Wet (1963) and others. Hawkes (1977) found these hybrids of great value in plant breeding programs because of their feasible crossability with the cultivated plant.

Cytogenetic Behaviour of Cassava hybrids with *M. neusana*

The wild *Manihot* species of *M. Neusana* Nassar was hybridized with the cassava clone Catelo through controlled hybridization with the help of pollinating insects (Nassar, 1989). An interespecific hybrid that combined marker genes of both parents was obtained. The marker genes were ribbed fruit, acquired from cassava, and variegated fruit color from *M. neusana*. This hybrid (HN) was backcrossed with cassava and used as a pollinator in the first trial and as a fruit carrier in the second trial. Seeds were obtained from both crosses, but only one plant could be raised from each; HO1 was the result of the interespecific hybrid (HN) as maternal plant (seed carrier), and HO4 resulted from crosses in which the interespecific hybrid (HN) was used as pollinator. The three hybrid plants (HN, H1 and H4) were studied cytogenetically for both meiotic and mitotic behavior. For the study of meiosis, inflorescences were fixed in a mixture of three parts absolute alcohol and one part glacial acetic acid and kept in a refrigerator for 24 h. The anthers were smeared with acetic carmine. Chromosome configurations in metaphase, chromosome distribution in anaphase I and tetrad formation were also studied. Pollen viability has been determined by using acetocarmine and iodine stain (Nassar, 1978a). For the mitotic study, root tips were left in 0,2% colchicine for 2 h and then fixed in acetic alcohol for 24 h. Before smearing with acetocarmine, they were treated with 1N HCl for 10 min.

One hundred pollen mother cells (PMCs) were studied in metaphase I of the interespecific hybrid *M. neusana* with cassava; 30 PMCs in metaphase II and 1000 tetrads of the same material were also investigated. The study of metaphase I showed different chromosome configurations, as shown in Table 5. The average bivalent frequency in all cells of metaphase I was 16,13 per cell. The high frequency of univalents was attributed to lack of synapses between chromosomes or failure of the two species to remain associated. Virtually the only other report on this subject is that of Magoon et al. (1970), in which chromosome pairing in the interespecific hybrid *M. glaziovii* (rubber tree) and cassava was studied and a regular synapses led the authors to conclude that there is a strong relationship between this species and cassava. Nassar et al. (1986) suggested that the material used by Magoon et al. was not a pure *M. glaziovii* but rather a natural interespecific hybrid between this species and cassava. If this were true, the supposed interespecific hybrid would be backcrossed progeny. The study of anaphase I showed that of 40 PMCs studied, 38 cells exhibited laggards, which were attributed to the occurrence of univalents resulting from no homologous chromosomes.

Anaphase II showed meiotic restitution. Of 33 PMCs studied in this phase, 5 cells exhibited a second meiotic restitution (SMR), forming 36 chromosomes on each pole. Apparently this phenomenon is a consequence of meiotic disturbance in the hybrid. An example of this disturbance was the breakdown of anaphases I. This was probably

due to disharmony between the two different genomes. Nassar (1991) documented this phenomenon in the interespecific hybrids of cassava with *M. pseudoglaziovii*. The presence of such restitution was confirmed in the following tetrad stage, in which the formation of both dyads and tetrads was observed.

In various crops, interespecific hybridization has led to the disturbance of meiotic division, with consequent meiotic restitution, e.g., in *Trifolium pratense* by Parrot and Smith (1984) and *Medicago* spp. by Vorsa and Bingham (1979). In manioc species, Hahn et al. (1990) reported 2n pollen formation in wild species in addition to certain clones of cassava. The detection of this phenomenon enabled these researchers to isolate triploid and tetraploid types from progeny that came from crosses of cassava with certain wild *Manihot* species, namely, *M. glaziovii* and *M. epruinosa*. These types proved much more productive than commercial clones used in Nigeria. Nassar (1991) manipulated the meiotic restitution occurring in the interespecific hybrids of *M. pseudoglaziovii* with cassava to produce triploid types that showed very good productivity under semiarid conditions. The discovery of the frequent occurrence of this phenomenon in interespecific hybrids of cassava offers an effective tool for the production of polyploid types by sexual means instead of the traditional method of colchicine applied to vegetative parts, which normally induces unstable, chimeral types (Abraham et al., 1964). An additional advantage is that production of triploid types may lead to production of trissomics among their progeny. If genes that control productivity in cassava are polygenes with additive model action, as is the case for many crops, certain trissomics of this crop may be more productive than their diploid ancestors. In general, the production of polyploidy via sexual means is advantageous from both genetic and evolution standpoints because it offers a vigorous heterotic effect and releases useful genetic variability adaptations.

Table 5 Frequency of Chromosome Configuration of Metaphase I in Interespecific Manihot Hybrids and Their Parents

	PCMs(N°)	Trivalents	Mean Bivalents	Univalents
<i>M. neusana</i>	20	-	18,00	-
Cassava	20	-	18,00	-
GN	100	-	17,00	1,58
HO1	30	1,86	16,13	0,13
HO4	100	1,63	12,41	8,84

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