



A clue to the role of apomixis in *Manihot* speciation

By

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Abstract

Apomixis is development of embryos without fertilization. Apomictic genes occur in wild cassava. Inter-specific hybridization was successfully performed between cassava and the wild species *Manihot glaziovii*. A polyploid genotype from this inter-specific hybrid was produced with the use of colchicine. The induced polyploid type restored fertility, exhibiting apomixis. Embryonic analysis and molecular examination have confirmed the apomictic nature of its progeny. The hybrid as well as its interspecific hybrid exhibit resistance to mealy bug. The apomixis induced by polyploidy indicates the role of this phenomenon in *Manihot* speciation. Given this material's resistance to mealy bug, its seeds have been made available to breeders in West and East Africa.

Key words: Apomixis, Interspecific hybridization, Embryonic analysis, Molecular analysis, Polyploidy

Cassava, *Manihot esculenta* Crantz, is the main staple for more than 800 million people living in the tropics (FAO, 2007). In some locations around the world, such as Northeast Brazil, the lowlands of Ghana and Nigeria, and some islands in Indonesia and the Pacific Ocean, it provides more than 70% of the calories consumed daily by the local population (FAO, 1975). Among other crops, it is credited with having high calorie productivity, high biological efficiency as an energy producer, year-round availability and adaptation to suboptimal soils (Nassar, 2004). The cultivars of

cassava are vegetatively propagated by stem cuttings that maintain superior genotypes. However, this asexual propagation system favours the accumulation of viruses and bacteria, lowering crop yield, and leading to degeneration of many excellent cultivars (Hahn et al., 1980). Should seeds be used to propagate the crop, systemic pathogen contamination may be avoided. However, the breakdown of selected heterozygous genotypes due to genetic segregation in the progeny has always precluded this approach. If apomictic seeds were made available, that could resolve this problem while maintaining heterozygosity.

Throughout West and East Africa, cassava cultivation is highly constrained by several strains of cassava mosaic disease and mealy bug (Nassar and Ortiz, 2006). Cassava mosaic disease was contained in the 1980s and 1990s by resistant cultivars raised from inter-specific hybrids (Hahn et al., 1980). However, cultivars resistant to cassava mosaic disease proved to be extremely susceptible to mealy bug owing to the high susceptibility of the parent clone (Branca Santa Catarina cassava cultivar).

Inter-specific hybrids ensuing from hybridization of distant species generally exhibit high sterility due to lack of chromosome pairing (Nassar, 2003). For this reason, polyploidizing such interspecific hybrids is needed to restore the fertility of the inter-specific hybrid. Chromosome duplication of inter-specific hybrids may rarely occur in nature, as is the case of the classic work by Karpishinco on *Raphanus sativus* x *Brassica oleracea*. Artificially induced polyploidy in inter-specific hybrids is less frequent in the literature, probably due to the difficulty of conducting such experiments.

Manihot glaziovii Muell. is a possible source of resistance to mealy bug and is traditionally used as a source of resistance to cassava mosaic disease (Storrey and Nichols, 1938, Nassar, 1978, Hahn et al., 1980). Nevertheless, it is very difficult to obtain hybridization of this species and cassava. In this research work polyploidy was induced in an inter-specific hybrid between cassava and *M. glaziovii*, for combining potential host plant resistance to both cassava mosaic disease and mealy bug. Furthermore, such bred-*Manihot* germplasm can be transferred as true seed to cassava researchers in Africa.

Material and Methods

An interspecific hybrid between cassava and *Manihot glaziovii* Muell. was obtained in 1992 (Nassar, 1994). This hybrid was propagated vegetatively, and further screening revealed its resistance to mealy bug. The hybrid was polyploidized artificially using colchicine. Colchicine treatments were carried out by applying a 0.2% aqueous solution to lateral buds three times, over a period of 36 hours, using moist cotton fixed around the bud. The emerging shoots were screened for the formation of chimera or total tetraploidy (Nassar, 2002, Nassar, 2003). In order to identify sectorial chimera, leaf shape and form on both sides of the shoots were compared, and stomata morphology was observed. To identify periclinal chimera, buds were examined for meiotic chromosome counting, leaf shape and form of developed shoot also being noted. For meiotic chromosome counting, buds were fixed in absolute alcohol-glacial acetic acid, smeared and stained with acetocarmine. Pollen viability was estimated by an acetocarmine-iodine mixture. Tetraploid shoots were selected and propagated by cuttings, raising a tetraploid population. From these tetraploid plants, embryo sacs of 200 ovules were observed using the clearing method to verify apomixis (Nassar, 1989). Additionally, 40 seeds from an open-pollination experiment were collected and planted to ascertain mating system. The progeny genotype was examined and compared to the mother plant using six microsatellite loci (GA-12, GA-13, GA-16, GA-21, GA-126, GA-

131) developed for *M. esculenta* (Chavarriga, 1998). These loci had previously been used and characterized for the study of the mating system on *M. glaziovii* and on the UnB 307 clone, a hybrid between *M. glaziovii* and cassava (Nassar and Collevatti, 2005, Nassar et al., 2006). Micro-satellite amplification and genotyping were performed as described elsewhere (Nassar and Collevatti, 2005).

Results

The six loci used in this research presented one or two alleles (Table 1). The two individuals of *M. esculenta* (positive control) presented clear amplification for all loci (Table 1). Both individuals of *M. esculenta* presented the same genotype for all loci.

From the progeny of the GxM hybrid, all sibs were identical to the mother plant, indicating that sibs were sired by apomixis (Table 1).

Table 1. Genotype of the mother plant GxM/M and its progeny, based on six microsatellite loci (allele size in base pair) transferred from *M. esculenta*. Control, genotype of control individual of *M. esculenta*; GxM/M – hybrid *M. glaziovii* X *M. esculenta* – mother plant; GxM/01 to GxM/42 – GxM/M progeny.

Individual	GA12	GA13	GA16	GA21	GA126	GA131
Control	140/140	140/140	104/104	114/114	180/180	98/116
GxM/M	140/160	140/140	104/116	114/114	184/214	100/100
GxM/01	140/160	140/140	104/116	114/114	184/214	100/100
GxM/02	140/160	140/140	104/116	114/114	184/214	100/100
GxM/04	140/160	140/140	104/116	114/114	184/214	100/100
GxM/05	140/160	140/140	104/116	114/114	184/214	100/100
GxM/06	140/160	140/140	104/116	114/114	184/214	100/100
GxM/07	140/160	140/140	104/116	114/114	184/214	100/100
GxM/08	140/160	140/140	104/116	114/114	184/214	100/100
GxM/09	140/160	140/140	104/116	114/114	184/214	100/100
GxM/10	140/160	140/140	104/116	114/114	184/214	100/100
GxM/11	140/160	140/140	104/116	114/114	184/214	100/100
GxM/12	140/160	140/140	104/116	114/114	184/214	100/100
GxM/13	140/160	140/140	104/116	114/114	184/214	100/100
GxM/14	140/160	140/140	104/116	114/114	184/214	100/100
GxM/15	140/160	140/140	104/116	114/114	184/214	100/100
GxM/16	140/160	140/140	104/116	114/114	184/214	100/100
GxM/17	140/160	140/140	104/116	114/114	184/214	100/100
GxM/18	140/160	140/140	104/116	114/114	184/214	100/100
GxM/19	140/160	140/140	104/116	114/114	184/214	100/100
GxM/20	140/160	140/140	104/116	114/114	184/214	100/100

GxM/21	140/160	140/140	104/116	114/114	184/214	100/100
GxM/22	140/160	140/140	104/116	114/114	184/214	100/100
GxM/23	140/160	140/140	104/116	114/114	184/214	100/100
GxM/24	140/160	140/140	104/116	114/114	184/214	100/100
GxM/25	140/160	140/140	104/116	114/114	184/214	100/100
GxM/27	140/160	140/140	104/116	114/114	184/214	100/100
GxM/28	140/160	140/140	104/116	114/114	184/214	100/100
GxM/29	140/160	140/140	104/116	114/114	184/214	100/100
GxM/30	140/160	140/140	104/116	114/114	184/214	100/100
GxM/31	140/160	140/140	104/116	114/114	184/214	100/100
GxM/32	140/160	140/140	104/116	114/114	184/184	100/100
GxM/33	140/160	140/140	104/116	114/114	184/184	100/100
GxM/34	140/160	140/140	104/116	114/114	184/184	100/100
GxM/35	140/160	140/140	104/116	114/114	184/184	100/100
GxM/36	140/160	140/140	104/116	114/114	184/184	100/100
GxM/37	140/160	140/140	104/116	114/114	184/184	100/100
GxM/38	140/160	140/140	104/116	114/114	184/184	100/100
GxM/39	140/160	140/140	104/116	114/114	184/184	100/100
GxM/40	140/160	140/140	104/116	114/114	184/184	100/100
GxM/41	140/160	140/140	104/116	114/114	184/184	100/100
GxM/42	140/160	140/140	104/116	114/114	184/184	100/100

Colchicine treatment applied to lateral buds induced both totally tetraploid and chimeral shoots. These totally tetraploid shoots were obtained by somatic selection followed by vegetative propagation. This made it possible to establish a population of tetraploid plants. Chromosome counts undertaken at metaphase I in selected individuals exhibited regular pairing of 34 bivalents with the exception of one quadrivalent.

We examined 200 ovules of both the diploid and tetraploid polyploid type. While no ovule of the diploid type displayed a multi-embryonic sac, it was possible to classify 56 ovules of the tetraploid type as multi-embryonic. This may be regarded as clear evidence of facultative apomixis (Fig. 1).

Discussion

Colchicine treatment applied to lateral buds was effective in obtaining polyploidy. It was followed by selection of somatic buds, and propagating of selected shoots by vegetative means. Chromosome counts in metaphase 1 revealed regular pairing of 34 bivalents with the exception of one quadrivalent.

We examined 200 ovules of both the diploid and tetraploid polyploid types. While no ovule of the diploid type displayed a multi-embryonic sac, it was possible to detect 56 multi-embryonic ovules of the tetraploid type (Fig.1).

Polyploidy was successfully achieved by the use of colchicines followed by selection of somatic buds, and propagating

of selected shoots by vegetative means. Chromosome counts were undertaken at metaphase 1 in selected individuals. This metaphase exhibited regular pairing of 34 bivalents with the exception of one quadrivalent. Some authors have attributed this multivalent to the amphiploid nature of cassava (Nassar et al.1995, Nassar, 2004). Pollen viability reached 93% compared to 9% for the diploid inter-specific hybrid. This result is similar to those obtained for other cassava interspecific hybrids (Nassar, 1994, Nassar, 2002). Regular bivalent formation of the tetraploid type indicates distinct speciation of *M. glaziovii* and may explain the difficulty of obtaining interspecific hybridization between this species and cassava (Nassar, 2007).

Storrey and Nichols⁵ reported the first and only successful trial of hybridizing cassava with *M. glaziovii*. They also successfully bred cultivars with resistance to cassava mosaic disease from this hybrid. However, the nature of their initial material (a pure *M. glaziovii* or an introgressed type of *M. glaziovii* with cassava) is uncertain (Nassar, 1978a, Nassar, 1978b, Nassar, 2003).

We examined 200 ovules of both of the diploid and tetraploid polyploid types. While no ovule of the diploid type displayed a multi-embryonic sac, it was possible to detect 56 ovules of the tetraploid type as multi-embryonic. This may be regarded as clear evidence of facultative apomixis (Fig. 1). The formation of multi-embryonic sacs in cassava ovules has been reported and described as proof of apomixis in cassava (Nassar, 1994, Nassar, 2002). These materials were derived from inter-specific hybrids in which the wild species was the donor of apomixis genes (Nassar and Collevatti, 2005, Nassar, 2006). We likewise confirmed apomixis using microsatellite markers, previously developed for cassava (Charriaga, 1998) and already used for study of the mating system in *M. glaziovii* and in interspecific hybrids of *Manihot* (Nassar and Collevatti, 2005, Nassar et al.,2006). When 40 sibs from an open-pollinated progeny of a polyploid type were genotyped using six microsatellite loci, all sibs presented the same genotype as the mother plant, demonstrating that the sibs were sired by apomixis.

Host plant resistance to mealy bug was also achieved. None of the 40 sibs was infected by mealy bug. Nevertheless, neighbour plants of a clone called UnB 400, planted in alternate rows, were densely infected. Since the polyploidized hybrid is fertile and facultatively apomictic, cassava breeders may benefit from it to develop cultivars resistant to mealy bug. The grown seed of this polyploid hybrid will maintain resistance to mealy bug without segregation in their new field. Mealy bugs are major pests in some parts of sub-Saharan Africa (Nassar and Ortiz, 2007, Nassar and Ortiz, 2008). We can thus conclude that an experimentally produced interspecific hybrid has been polyploidized. Polyploidization restored fertility and had the important effect of introducing apomixis and so giving rise to a new species.

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Figure Captions

Fig.1. Multi-embryonic ovule in polyploidized interspecific hybrid



Fig.2. Metaphase 1 of polyploidized type

