Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Genetic, embryonic and anatomical study of an interspecific cassava hybrid

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Genet. Mol. Res. 9 (1): 532-538 (2010)
Received November 23, 2009
Accepted January 4, 2010
Published March 23, 2010

ABSTRACT. A molecular, anatomical and cytogenetic study of an interspecific hybrid between *Manihot esculenta* (cassava) and the wild species *M. oligantha* was carried out. Cytogenetics revealed relatively complete chromosome pairing and high viability of the pollen grains. Ovule structure examined by the clearing method showed polyembryony in 2.7% of the ovules. Doubling of the chromosome number resulted in an increase in polyembryony of up to 18% and a reduction in pollen viability. Multivalent formation was also observed. An anatomical study of stems of diploid and tetraploid hybrids showed a larger number of vascular bundles in the tetraploid type.

Key words: Genetics; Embryology; Anatomy; Cassava hybridization

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), also called yuca, mandioca or manioc, is the most important staple crop in the tropics and subtropics, where it serves as a food staple for more than 800 million people in the tropics and subtropics of South America, Africa and Asia (FAO, 2006). There are possibilities of its improvement for several purposes and directions (Nassar, 2006d; Nassar, 2007a,b,c; Nassar and Sousa, 2007). It is propagated vegetatively by stem cuttings, which perpetuate superior genetic combinations, but allow viral and bacterial diseases to accumulate. These reduce productivity and may eventually lead to the extinction of superior genotypes (Nassar, 1999). Systemic pathogen contamination could be avoided by seed propagation of the crop. However, this approach has not been possible, because the genetic superiority of individual clones breaks down due to genetic segregation in the progeny.

The heterozygosity responsible for vigor could be efficiently fixed by apomixis in cassava (Nassar and Collevatti, 2005). This phenomenon is defined as a process in which plants produce seeds without fertilization. This would bypass female meiosis and syngamy and produce embryos genetically identical to the maternal parent. Through apomictic reproduction, superior cassava genotypes could be maintained in successive generations. Apomixis in cassava was noted for the first time by Nassar (1980) while working on interspecific hybridization. In future studies, he and co-workers transferred its genes successfully to the cultivate (Nassar et al., 2000, 2008).

One of the obstacles that impede the cultivation of cassava in a large part of Brazil and Africa is the lack of sufficient tolerance to drought. Botanically, this character is determined for a large extent by the anatomic structure of both stem and root that enables the uptake of water and its storage in plant tissues. Therefore, we analyzed polyploidy effects in a productive interspecific hybrid and examined stem anatomy of the diploid and tetraploid forms.

MATERIAL AND METHODS

Cytogenetic and anatomic analyses were performed in diploid and tetraploid hybrids between *M. esculenta* and *M. oligantha* Pax. Tetraploid hybrids were obtained through polyploidization treatment, previously carried out by Nassar (2004).

For cytogenetic analysis, male buds were collected, fixed in Carnoy's solution, and stored in 70% ethanol under refrigeration. Anthers were smeared with carmine according to Swaminathan et al. (1954). Chromosome configurations in metaphase I and tetrad formation were studied. Pollen viability was determined using acetocarmine and iodine stain; thirty buds of each plant were examined.

The morphological development of embryo sacs was studied anatomically. Approximately 200 unpollinated pistillate buds collected from each hybrid, about 1 day before anthesis, were fixed in Farmer's fixative (l:3, glacial acetic acid:95% ethanol) in the field between 7:30 am and 12:00 pm. Fixed pistils were dissected under a dissection microscope. Dissected ovules were dehydrated in an ethanol series and cleared overnight in the benzyl benzoate-four-and-a-half fluid (BB-4 ½-lactic acid:chloral hydrate:phenol:clove oil:xylene:benzyl benzoate = 2:2:2:2:1:1, wt/wt), developed by Herr Jr. (1982), and treated in modified Herr's solution as previously reported by Nassar et al. (1997). Transparent ovules were then observed and photographed using Normarski differential interference contrast microscopy.

For the study of stem anatomy, samples were collected and fixed in 70% FAA and

stored in 70% ethanol. Free-hand sections were made and cleared in 50% sodium hypochlorite solution (Kraus and Arduin, 1997), stained with 1% safranin-alcian blue, dehydrated in an ethanol series and butyl acetate, and mounted in a synthetic resin (Paiva et al., 2006). Photomicrographs were taken using a Zeiss Axioskop microscope, and the images were captured with Motion Image Plus 2.0.

RESULTS AND DISCUSSION

Diploid hybrid showed a chromosome number of 2n = 36 and regular pairing (Figure 1A). The tetraploid type revealed 2n = 72, with most of the pairing consisting of bivalents (28 bivalents) and some representatives of quadrivalents (4 quadrivalents) (Figure 1B).

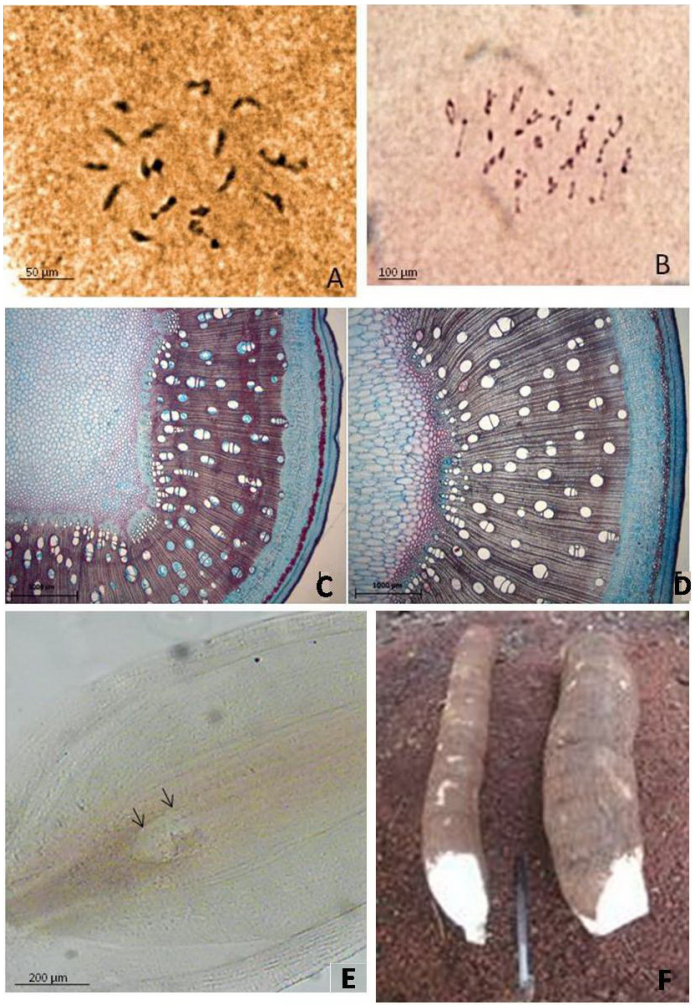

Figure 1. A. and **B.** Tetraploid and diploid metaphase I, respectively. **C.** and **D.** Stem section of the diploid and tetraploid types, respectively. **E.** Polyembryonic ovule with two embryos (arrows). **F.** Roots of the diploid and tetraploid types, respectively.

Tetrads were almost 100% normal in the 1176 spores of the diploid hybrid. Tetraploid ones, on the other hand, showed 81% abnormal tetrads of a total of 1065 counted spores. Pollen viability was higher in the diploid hybrid, reaching 83.5%, while in the tetraploid it was 64.8%.

The numbers of apomictic, non-apomictic and total ovules with their respective percentages are presented in Table 1.

Table 1. Apomixis data represented by number of ovules counted in diploid and tetraploid hybrids.

Hybrid	Non-apomictic	Apomictic	Total	Percent of apomixis
Diploid	213	6	219	2.7%
Tetraploid	164	36	200	18.0%

Both diploid and tetraploid hybrids showed apomictic structures represented by multiembryonic sacs (Figure 1C and D).

In cross-sections, the hybrid stems showed secondary growth characterized by the presence of phellogen and vascular cambium (Figure 1E and F).

In quantitative terms, the tetraploid had more developed secondary vascular tissues than did the diploid, in relation to secondary phloem as well as to secondary xylem; while growth rings were present in the diploid, they were absent in the tetraploid. Both hybrids showed articulated and branched laticifers in a vascular bundle of the bicollateral type. For more description of diploid and tetrapoid types, see Appendix.

Cruz (1968) and Magoon et al. (1969) determined a chromosome number of 2n = 38 for *Manihot glaziovii* and five other *Manihot* species. Nassar (1978) reported the same number for 8 wild *Manihot* species including *M. oligantha* Pax and cassava itself, both having a chromosome number of 2n = 36.

The polyploidized type of the hybrid between *M. esculenta* and *M. oligantha* showed multivalent formation ranging from three to four quadrivalents, while the diploid type formed regular bivalents in 17 of the 18 normal bivalents in cassava species. This is a striking feature of an interspecific hybrid where the formation of univalents is expected (Nassar et al., 1995). This is probably due to the introgressed nature of the parent *M. oligantha* used in hybridization. Nassar (1978) explained that his type used in crosses is not a pure type and that it is different from *M. oligantha* Pax in leaf type and tuber formation, characters that are assumed to have been acquired from cassava by natural hybridization followed by subsequent introgressive crosses in the direction of *M. oligantha*. From a plant breeding viewpoint, this is an advantageous aspect in our program, since bivalent configuration usually indicates the parental degree between species and the probability of gene transfer between them (Kumar et al., 1988; Nassar, 2003b,c; Panda et al., 2004).

This introgressed nature of the *M. oligantha* type, i.e., having cassava genes incorporated into it, explains the reasonable compatibility and regular pairing of its bivalents in meiotic metaphase, as confirmed by the formation of normal tetrads in almost all spores. The high pollen viability of the diploid hybrid also reflected this regular chromosome pairing and regular segregation in anaphase of the diploid hybrid.

The quadrivalent configuration of the tetraploid type produced unbalanced gametes and somewhat sterile pollen. Nassar (2004), and Husband et al. (2008) as well, have called attention to this serious limitation of certain polyploids in relation to the sterility of the plant.

The few multivalent associations occurring in metaphase I of the hybrid polyploid types resulted in abnormal tetrad formation. Parrott and Smith (1982) and Sala et al. (1989) attributed this meiotic abnormality to the presence of univalents. Apparently, multivalent formation led to irregular segregation and abnormal tetrad formation. It also resulted in a certain level of inviable pollen (Nassar, 2003a).

The nature of apomixis in *Manihots* seems to be different from that of others, as explained by Nassar (2001), where its facultative level is as low as 1-2%. Our results support this hypothesis where polyembryony was found at a frequency of 1% in the hybrid of cassava with *M. oligantha*.

A significant increase in the extent of polyembryony was observed in the polyploidized type of the hybrid cassava *M. oligantha*. This is in accordance with what was noted by Nassar (2006 a,b,c) in polyploid types of cassava hybrids with *M. anomala* Pohl and cassava hybrid with *M. glaziovii* Muller von Argau. He concluded that there is a strong correlation between ploidy and apomixis in this genus. Matzk et al. (2003) and Hojsgaard et al. (2008) also cited an association between apomixis and ploidy in several plants.

A difference between the diploid and tetraploid stems was noted in the cortex region. The tetraploid type has a greater diameter. However, the main structural differences were noted in the vascular tissues. The primary and secondary phloem cells were 3- to 4-fold larger in the tetraploid. The number of layers and starch content were also higher in the tetraploid plant than in the diploid.

The structures described above explain what had been reported earlier about sturdier, harder stems in tetraploid cassava (Graciano-Ribeiro et al., 2008). The greater diameter of vessels in the tetraploid type suggests its capacity of retaining a larger quantity of water than in diploid plants, which have a smaller diameter.

Increase in structure and organ size, number of layers, and cell size, and the observed increase in vascular tissues, induced by polyploidization, may confer drought resistance to our induced polyploidized type.

ACKNOWLEDGMENTS

Research supported by the Brazilian National Council for Scientific Research (CNPq) and the Brazilian Coordination Office for Qualifying Graduate Personnel (CAPES). The *Manihot* living collection at the Universidade de Brasília was established in the 1970's with support from the International Development Research Centre (IDRC), Canada, to whom we are grateful.

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Appendix. Description of the diploid and tetraploid types.

M. esculenta x *M. oligantha* diploid

Shrub, 2-2.5 m. Tuberous and cylindrical roots of approximately 30 cm in length and 12 cm in diameter; smooth surface, dark brown. Young stem purplish green, glabrous. Mature stem silver brown, prominent leaf scars, internodes spaced 5-10 cm, glabrous. Leaves alternate; stipules deciduous, 0.3-0.5 cm in length, lanceolate, entire, glabrous. Petioles greenish purple, about 18-23 cm long. Lamina nonpeltate, membranaceous, glabrous, palmately 3-7 lobed, median lobes oblong about 17-23 cm long, entire, apex acute to acuminate, venation camptodromous. Leaf dimorphism. Inflorescence monoecious, terminal panicle, about 3-5 cm long, and 5-7 branched, glabrous. Bracteoles and bractlets setaceous, margin entire, greenish yellow with traces of purple. Pistillate flowers restricted to the base of the inflorescence, 2-3 flowers follow each cluster of staminate flowers, pyramidal shape. Disc orange, glabrous, ovary subglobose, tricarpelar, slightly ribbed. Staminate flowers ovoid-ellipsoid, tepals about 0.5-1 cm long, stamens 10, in two whorls of five each of 0.3-0.9 cm, fillet and anthers white, pedicels purplish green, about 1.5 cm. Fruit spherical, smooth surface, prominently 6-ribbed, about 2.3 cm long, dehiscence septicidal. Seed oblong, about 1-1.4 cm long, caruncle moderately prominent.

M. esculenta x *M. oligantha* tetraploid

Tall shrub, 2 m approximately. Tuberous and cylindrical roots of approximately 50 cm in length and 9 cm in diameter; smooth surface, dark brown. Young stem purplish green, glabrous. Mature stem silver brown, prominent leaf scars, internodes spaced 4-8 cm, glabrous. Leaves alternate; stipules deciduous, 0.3-0.5 cm long, lanceolate, entire, glabrous. Petioles greenish purple, about 20-23 cm long. Lamina nonpeltate, membranaceous, glabrous, palmately 3-7 lobed, usually 3 big and 4 smaller, median lobes oblong about 7 cm wide, entire, apex acute to acuminate, venation camptodromous. Inflorescence monoecious, terminal panicle, about 3-5 cm long, and 5-7 branched, glabrous. Bracteoles and bractlets setaceous, margin entire, greenish yellow with traces of purple. Pistillate flowers restricted to the base of the inflorescence, 2-3 flowers follow each cluster of staminate flowers, pyramidal shape. Disc orange, glabrous, ovary subglobose, tricarpelar, ribbed. Staminate flowers ovoid-ellipsoid, tepals about 0.5-1 cm long, stamens 10, in two whorls of five each of 0.3-0.8 cm, fillet and anthers white, pedicels purplish green, about 1.5 cm. Fruit spherical, smooth surface, prominently 6-ribbed, about 2.5 cm long, dehiscence septicidal. Seed oblong, about 1-1.5 cm long, caruncle moderately prominent.

