Reprint from Canadian Journal of Plant Science, 98:349-352 (1998)

Molecular and embryonic evidence of apoximis in cassava interspecific hybrids (*Manihot* spp.)

By
Nagib M. A. Nassar[1], Marco André Vieira[1], Clibas Vieira[2] and Dario Grattapaglia[3]

[1]Departamento de Agronomia, Universidade de Brasília, C.P. 04477
Brasília, DF, 70919-000
[2]Departamento de Fitotecnia, Universidade Federal de Viçosa
Viçosa, MG, 36571-000
[3]Laboratório de Genética de Plantas, CENARGEN-EMBRAPA, C.P. 02372
Brasília, DF, 70879-970

Received 14 February 1997 - Accepted 11 November 1997

Nassar, N. M. A., Vieira, M. A., Vieira, C. and Grattapaglia, D. l998. **Molecular and embryonic evidence of apomixis in cassava interspecific hybrids** (*Manihot* **spp.**). Can. J. Plant Sci. **78**: 349-352. In cassava, apomixis could fix hetereozygosity and prevent the transmission of systemic pathogens which complicate vegetative propagation of the crop. Evidence from maternal inheritance of RAPD markers and the structure of the embryonic sac in large progeny sets of two distinct genotypes have further confirmed that apomixis occurs in cassava. Here, we have built on an earlier report of apomixis in cassava in four ways (l) we estimated the rate of facultative apomixis in the range of 2% (2) we detected the occurrence of apomixis in a second genotype, derived form a different interspecific cross; (3) apomictic behavior was demonstrated in an Fl individual and (4) embryonic evidence showed that the mode of apomixis is aposporic. Since apomixis was detected in an Fl interspecific hybrid it is possible that genes for apomixis could be transferred directly to cultivated cassava from a wild relative.

Key words: cassava, interspecific hybrid.

Nassar, N. M. A., Vieira, M. A., Vieira, C. et Grattapaglia, D. l998. **Preuve moléculaire et embryonnaire d'apomixie chez les hybrides interspécifiques de manioc** (*Manihot* **spp.**). Can. J. Plant Sci. **78**: 349-352. Chez le manioc, l'apomixie pourrait être un moyen de fixer l'hétérozygotie tout en évitant la transmission des pathogènes systémiques qui rendent compliquée la propagation végétative de la culture. Les indications obtenues à partir de la transmission maternelle de marqueurs RAPD et à partir de la structure du sac embryonnaire dans de grandes populations issues de 2 génotypes distincts, permettent de confirmer que l'apomixie existe dans le manioc. Dans la présente communication, qui s'appuie sur une communication antérieure, nous avons 1º estimé aux alentours de 2 % le taux d'apomixie facultative, 2º décelé la présence d'apomixie dans un second génotype dérivé d'un croisement interspécifique distinct, 3º mis en évidence un comportement apomictique dans une plante Fl et 4º mis en évidence une preuve embryonnaire que le mode d'apomixie est aposporique. vu que l'apomixie a été décelée dans la Fl d'un hybride interspécifique, les genes commandant l'apomixie pourraient être transférés au manioc cultivé directement à partir d'une espece sauvage apparentée.

Mots clés: Manioc, hybride interspécific.

Cassava (*Manihot esculenta*) also named yuca, mandioca or manioc, is the most important staple crop in the tropics and subtropics, being a food staple for more than 600 million people (Food and Agriculture Organization, l994). Cassava is propagated vegetatively by stem cuttings which perpetuate superior genetic combinations, but allow viral and bacterial diseases to be accumu1ated. These reduce productivity and may eventually lead to the extinction of superior genotypes. 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. 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 apomitic 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 interespecific hybridization. Later its occurence was observed genetically (Nassar 1994) and confirmed by RAPD marker analysis in a single clone (Grattapaglia et al. 1996).

Our working hypothesis is that a truly apomictic seedling would display an identical RAPD pattern of bands to the maternal parent for all marker polymorphisms. Non-maternal bands in the offspring would reject the hypothesis of apomixis and indicate their zygotic nature. The word extends our earlier report of molecular detection of apomixis in cassava by analyzing more progeny individuals and shows further evidence

from embryological studies for facultative apomictic behavior in two distinct cassava genotypes.

MATERIAL AND METHODS

Two putative faculative apomitic cassava clones were used in this study clone 031 was selected based on vigor in an F2 population resulting from a cross between an interspecific hybrid (*M. dichotoma* x *M. esculenta*) and a cultivated clone, Branca Santa Catarina. Clone 200 is an F1 individual from hybridization between cultivated cassava and *M. glaziovii*. These clones were described by Nassar and Grattapaglia (l986).

Embryo Sac Analysis

The morphological development of embryo sacs was studied histologically. Unpollinated pistillate buds collected, about 1 d before anthesis, and pollinated pistils, collected 2 d after anthesis, were fixed in Farmer's fixative (l:3 = glacial acetic acid: 95% ethanol) in the field between 07:30 h and 12:00 h. Fixed pistils were dissected under a dissection microscope (magnification x 40, transmitted light). Dissected nucellus and ovules were dehydrated in ethanol series and cleared overnight in the benzyl-benzoate-four-and-a-p half (BB-4 1/2) fluid (lactic acid: chloral hydrate: phenol: clove oil: xylene: benzyl benzoate = 2:2:2:2:1:1, wt/wt devised by Herr (1992), and treated in a modified Herr's fluid as previously reported by Ogburia and Adachi (1994). Transparent ovules were then observed and photographed microscopically at 400x magnification using Normarski's differential interference contrast.

DNA Extraction and RAPD Assay

The two sets of matemal parents and 67 offspring individuals, 37 individuals of clone 31 and 30 individuals of clone 200 were genotyped with 24 selected arbitrary ten base primers selected earlier for high band content and discrimination power in cassava (Grattapaglia et al. 1996). Total genomic DNA was isolated from 200 mg of fresh leaf tissue ground in liquid nitrogen using the CTAB protocol of Doyle and Doyle (1978), modified by the addition of 1 % PVP and 1 % 2-mercaptoethanol. DNA concentration was estimated by gel electrophoresis comparing the fluorescence intensities of the ethidium bromide stained samples to those of lambda DNA standards. For the RAPD assay, working stocks of genomic DNA were diluted in water at a concentration of 2.5 ng µL-1.

Arbitrary primer (kits OP-A through OP-Z) were obtained from Operon Tecnologies Inc. (Alameda, CA). Amplification reactions (13 mL) were carried out according to Williams et al. (1990) with the following modification: 0.4 mM ten-base primer, 10 mg µL-1 non-acetylated bovine serum albumin (New England Biolabs), 5 to 10 ng of genomic DNA and 1 unit of Taq DNA polymerase. Amplications were performed in 96-well microwell plates using an MJ Research PT -100 thermal controller. RAPD products were analyzed by electrophoresis in l.5 or 2.0% agarose gels containing 0.2 mg µL-1 ethidium bromide. Gel images were captured and digitalized with an Eagle- Eye II system (Stratagene, CA). Gel scoring was performed directly from the gel images on a computer screen and images were stored electronically on a laser CD. A set of 24 arbitrary primers selected earlier for high band content and discrimination power in cassava (Grattapaglia et al. l996) was used. The presence or absence of RAPD fragments was scored by visual inspection of the gel images. Informative RAPD markers were identified as described previously (Grattapaglia and Sederoff. l994). Two replicate RAPD analysis experiments including DNA extractions, RAPD assays and marker scoring were carried out with the set of selected primers on the putatively apomictic individuals to confirm the patterns of bands. Although it would seem un likely that the maternal parent and a zygotic progeny individual could have an identical combination of more than l00 RAPD fragments, it could be argued that it is possible. As described in our previous report (Grattapaglia et al. l996), to exclude this possibility we used the statistical procedure described by Novy et al. (l994) to show that the complete similarity between the two samples is not an artifact resulting from a limited number of RAPD markers surveyed, but rather has a biological basis.

RESULTS AND DISCUSSION

Each selected primer amplified an average of 8.25 clearly interpretable RAPD fragments with a range of 5 to 14 fragments. A total of 198 clearly interpretable and reproducible RAPD markers were surveyed in this study.

This number of markers was considered to provide a representative genome coverage for the objective of this study. Eighty-one percent of the scored RAPD bands were monomorphic in the two families. The remaining bands were polymorphic and thus useful for testing the hypothesis proposed herein.

Progenies of clones 031 and 200 displayed a high uniformity of DNA fingerprints. However, except for one individual in each progeny it was possible to find markers that readily showed that individuals were not derived from apomixis. In the progeny of clone 031 individual 4 (see photo gallery , Fig.54) showed a pattern of RAPD bands identical to the maternal one for all primers examined as did individual 5 in the progeny of clone 200. Given the number of markers surveyed, the probability that complete uniformity in RAPD markers between the maternal parents and their respective progeny individuals happened due to chance alone was equal or less than 103 in both clones. Therefore, it is likely that apomixis was detected in progenies of both clones, 031 and 200, at a rate of 3.13 and 2.70%, respectively. These results clearly indicate that the type of apomixis detected in this study is facultative and occurs at very low frequency.

A total of 261 ovules were analyzed histologically. In both clones, we observed aposporic sacs inside the sexual embryo sacs (see photo gallery Fig. 46). This abnormality was found in 2.36 and 1.49% of the ovu1es of clones 031 and 200, respectively. Similar results using the same histological clarification technique were reported in *Cenchrus ciliaris* (Young et al. 1979). These results strongly suggest that the mechanism responsible for apomixis in cassava is apospory (development of aposporic embryo sacs). Apospory in the angiosperms is the most common mechanism responsible for apomixis. In this type of apomixis one or more somatic cells from the ovule enlarges considerably and becomes vacuolated, producing a new embryo sac (Asker 1980).

This type of apomixis would explain why more than one zygote per seed was found in clone 031 by Nassar (1994). The presence of apomixis in both clones 031 and 200 shows the genes for apomixis in cassava are probably found more frequent than expected. sources of genes controlling apomixis in wild species that are relatives of com, sugar beet, wheat, and several forage grass have already been reported (Asker 1979).

In conclusion this report documents more evidence that apomixis occurs in cassava, by presenting a combination of molecular and embryonic evidence in a larger set of progeny. We have added to our previous report (Grattapaglia et al. 1996) of apomixis in cassava in four fundamental ways: (1) we were able to estimate the percentage of facultative apomixis is about 2%; (2) we detected apomixis in a second genotype, clone 200, derived from a different interspecific cross; (3) we demonstrated apomictic behavior in an F1 individual; and (4) we were able to show embryonically that apomoxis in the two genotypes investigated was aposporic. Furthermore, because clone 200 is an F1 interspecific hybrid, it seems possible to directly transfer genes for apomixis from a wild relative to cultivated cassava.

Abbreviations: RAPD, random amplified polymorphic DNA

ACKNOWLEDGMENTS

This work was partially supported by the National council for scientific and Technological Development (CNPq) to N.M.A.N., and a research grant to D.G. from PADCT/SBIO – FINEP. M.A.R.V had a Msc. Fellowship from CNPq. Special thanks go to the Intemational Development Research) Centre (IDRC) Ottawa, for the support in establishing the living *Manihot* collection at the Universidade de Brasília.

REFERENCES

Asker, S. 1979. Progress in apomixis research. Hereditas 91: 231-240.

Asker, S. 1980. Gametophytic apomixis: elements and genetic regulation. Hereditas 93: 277-293.

Doyle, J. J. and Doyle, J. L. 1978. Isolations of plant DNA from fresh tissues. Focus 12: 13-15. Food and Agrjculture oOrganjzation 1994. FAO yearbook. FAO, Rome, Italy.

Grattapaglja, D. and Sederoff, R. R. 1994. Genetic linkage maps of *Eucalyptus grandis* and *E. urophylla* using a pseudo-test cross strategy and RAPD markers. Genetics 137: 1121-1137.

Grattapaglia, D. Costa e Sjlva, C. and Nassar, N. M. A. 1996. Strict maternal inheritance of RAPD fingerprints confirms apomixis in cassava (*Manihot esculenta* Crantz). Can. J. Plant Sci. 76:379-382.

Herr , H. M. Jr .1982. An analysis of methods for permanently mounting ovules cleared in four-and-a-half type clearing fluids. Stain Technol. 57: 161-169.

Nassar. N. M. A. 1980. Attempts to hybridize wild *Manihot* species with cassava. Econ. Bot. 34: 13-15.

Nassar, N. M. A. 1994. Development and selection for apomixis in cassava, *Manihot esculenta* Crants. Can. J. plant. Sci: 74: 857-858.

Nassar, N. M. A. and Grattapaglia, D. 1986. Variabilidade de clones de mandioca em relação a fertilidade e aspectos morfológicos. Turrialba 36: 555-559.

Novy, R. G. Kobak, C., Gofreda, J. and Vorsa, N. 1994. RAPDs identify verietal misclassification and regional divergence in cranberry [*Vaccinium macrocapon* (Ait.) Pursh]. Theor. Appl. Genet. 88: 1004-1010.

Ogburia, M. N. and Adachi, T. 1994. An improved cleared-pistil tecnique for rapid in toto observation of embryo sac malformation in cassava (*Manihot esculenta* Crantz). proceedings of the second Intemational Scientific Meeting cassava Biotecnology Network. Bogor, Indonesia. pp. 117-127.

Williams, J. G. K., Kublik, A. R., Livak, K. J., Rafalski, J. A. and Tingy, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Young, B. A., Sherwood, R. T. and Bashaw, E. C. 1979. Cleared-pistil and thick-

sectioning techniques for detecting aposporous apomixis in grasses. Can. J. Bot. 57: 1668-1672.