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# Somatic Embryogenesis and Ploidy Stability in Cassava (*Manihot esculenta* Crantz) Cultivars Regenerated by *in Vitro* Culture of Young Leaves

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**Summary** The regeneration and cytogenetic analysis of 7 cassava (*Manihot esculenta* Crantz) cultivars obtained by organogenesis and somatic embryogenesis were investigated. In the first phase, callus formation was induced in young leaves using MS medium supplemented with 1.0, 5.0, 10.0, 15.0 and 20.0 mg/l of 2,4-D or without a growth regulator (control). In the second phase (development), the calli obtained in the above treatments were transferred to MS medium supplemented with factorial combinations of $GA_3$ and BAP at 0, 0.5, 1.0, 1.5 and 2.0 mg/l. Results of the first phase showed that 2,4-D induced the formation of both friable and compact calli. The compact calli displayed root and shoot formation, whilst somatic embryos were obtained only from friable calli in the medium with 1.0 mg/l $GA_3$. Plants obtained from somatic embryos were regenerated in MS medium supplemented with 0.04 mg/l BAP, 0.02 mg/l NAA and 0.05 mg/l $GA_3$. Cytogenetic analysis in 167 regenerated plants from somatic embryogenesis and 176 from organogenesis showed $2n=36$ as a normal diploid chromosome number.

Cassava is the most important cultivated species in the tropics and subtropics, with thousands of accessions and cultivars. Cassava roots are rich in carbohydrates and have been used mainly in the production of flour for human consumption in developing countries, where calorific deficiencies and malnutrition are widespread (Fukuda *et al.* 1996).

In recent years, micropropagation techniques have been used for the multiplication of pathogen-free and more productive materials. Among these techniques, shoot regeneration and organogenic callus culture are the most significant for inducing plant formation from petioles, roots and nodal segments (Eskes *et al.* 1974, Parke 1978, Tilquin 1979, Roca 1984, Smith *et al.* 1986, Oliveira *et al.* 2000). Somatic embryos of cassava have also been induced from immature leaves of plants propagated *in vitro* and from shoot tips (Szabados *et al.* 1987, Matsumoto *et al.* 1991). In cassava, this technique is a process that can offer important possibilities, not only for genotype propagation but as a tool for genetic improvement associated with cellular and molecular genetic methods (Vasil 1996).

On the other hand, plant cell and tissue cultures undergo genetic erosions and show changes of various types, especially in chromosome numbers and ploidy level (Bajaj 1990, Choi *et al.* 2000). *Manihot esculenta* is considered as a species of allotetraploid origin with $2n=36$ and base chromosome number $x=9$ (Perry 1943). The occurrence of both spontaneous and induced polyploids has been reported in some plants (Carvalho *et al.* 1999, Nassar 2000). However, polyploidy in cassava does not appear to be very frequent. Hahn *et al.* (1992), for instance, reported only 1 tetraploid specimen with $2n=72$ among 11,480 plants.

In the present work, the effect of certain growth regulators on plant regeneration obtained from young leaves of 7 cassava cultivars was studied. The cytological study of regenerated plants was carried out using conventional techniques, in order to evaluate their chromosome number.

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## Material and methods

The cassava (*M. esculenta* Crantz) MBRA222, MBRA235, MBRA254, Trouxinha, Cachoeirinha, CM955 and Mcol-1468 cultivars were supplied by the *in vitro* Germplasm Bank of the Agricultural and Livestock Research Institute (IPA) of Pernambuco State, Brazil.

Axillary multiplication of the material was carried out using nodal segments. The explants were placed on MS (Murashige and Skoog 1962) medium containing 20 g/l sucrose, 0.5 mg/l thiamine, 0.04 mg/l BAP (6-benzylaminopurine), 0.02 mg/l NAA ($\alpha$-naphthaleneacetic acid), 0.05 mg/l $GA_3$ (gibberellic acid) and 7.5 g/l agar.

Callus induction was carried out using young leaves with lengths varying from 3 to 8 mm. The leaves were inoculated in MS medium containing 2,4-D (2,4-dichlorophenoxyacetic acid) at 1.0, 5.0, 10.0, 15.0 and 20.0 mg/l. As a control, MS medium was used without growth regulators. The calli were transferred to MS medium, with a factorial combination of $GA_3$ and BAP at 0, 0.5, 1.0, 1.5 and 2.0 (mg/l) comprising 25 treatments.

The somatic embryos were germinated in a multiplication medium containing growth regulators in the concentrations described above. The regenerated plants were acclimatized in a substrate composed by vermiculite, sand and organic matter in the ratio 1 : 1 : 1.

For mitotic analysis, young root tips in active growth were pre-treated with 8-hydroxyquinoline (0.002 M) at 8°C for approximately 20 h, fixed in ethanol–acetic acid (3 : 1, v/v) for 2–24 h and stored in a freezer. Root tips were hydrolyzed in 5 N HCl for 20 min at room temperature, squashed in 45% acetic acid, frozen in liquid nitrogen to remove the coverslip, dried in air, stained in a 2% Giemsa solution and mounted in Entellan (Merck), according to Guerra (1983). The cells were photographed with Imagelink Kodak HQ ASA 25 film for cytological analysis, and Kodak ASA 400 film for callus culture.

## Results and discussion

All cassava cultivars formed calli in most of the 2,4-D levels in the first culture stage (induction). A concentration of 1.0 mg/l 2,4-D induced only a limited expansion of the explants and formation of globular structures near the central leaf vein, without callus formation. The other levels of 2,4-D induced great expansion and formation of globular structures. During the second week of *in vitro* culture, all explants showed callus formation of 2 types: a friable green callus with uniform arrangement (type CV) and a compact yellow callus with an irregular arrangement (type CA) (Fig. 1a).

Table 1 shows the morphogenetic responses of the cassava cultivars to a combination of $GA_3$ and BAP levels used. Only Mcol-1468 and MBRA235 cultivars formed large numbers of somatic embryos in the culture medium containing 1.0 mg/l $GA_3$. At 0, 0.5, 1.5 and 2.0 mg/l $GA_3$, there was no formation of somatic embryos. In this case, the embryos were formed from CV-type calli (Fig. 1c), whereas the CA-type calli showed no embryo formation, even though they displayed significant root regeneration. Multiple shoots were regenerated from MBRA222, MBRA254 Trouxinha, Cachoeirinha and CM955 cultivars, in the medium containing BAP and combinations BAP and $GA_3$ (Fig. 1b).

The formation of different callus types was reported by Jimenez and Bangerth (2001) during the embryogenic process in carrot (*Daucus carota*). In this species, translucid calli did not produce somatic embryos, while calli composed of preglobular and globular embryos were competent to regenerate somatic embryos. In the case of cassava, there was an embryogenic response only for CV calli. This result might suggest the occurrence of cells with different susceptibilities in the route from callus formation to induction and development of somatic embryos. On the other hand, the use of $GA_3$ incorporated into the somatic embryogenesis process has been reported in several species.

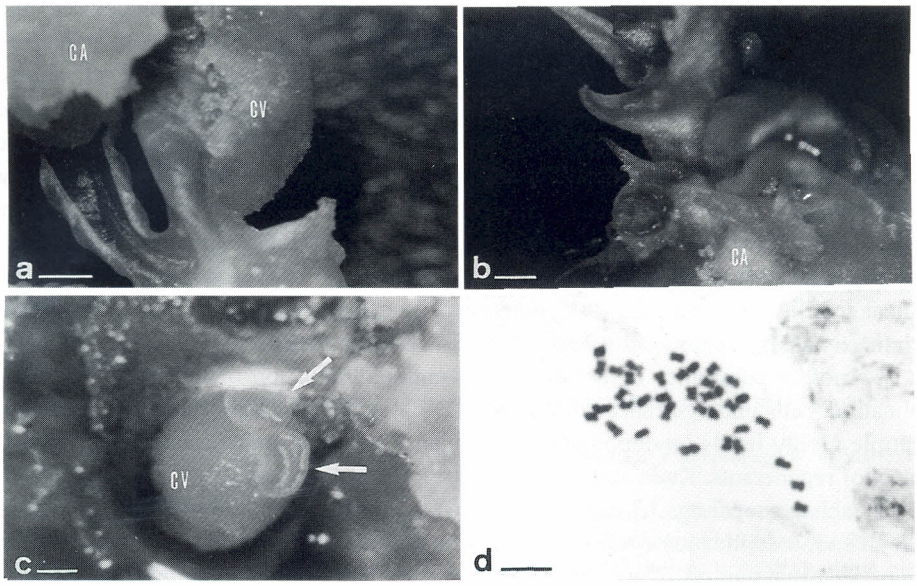


Fig. 1. Process of somatic embryo formation of cassava from callus cultures. a) Leaf explant showing the formation of callus CV type (green) and CA type (yellow). b) Shoot regeneration from callus CA. c) Somatic embryos (arrows) obtained from callus CV. d) Metaphase displaying $2n=36$ metacentric to submetacentric chromosomes. Bars represent 1 mm in a, b, c, and 5 $\mu$m in d.

Table 1. Morphogenetic responses of the cassava (*Manihot esculenta* Crantz) cultivars analysed according to a factorial combination in mg/l, of $GA_3$ (gibberellic acid) and BAP (6-benzylaminopurine)

| Callus type | $GA_3$ | BAP | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.5 | 1.0 | 1.5 | 2.0 |
| CV | | | | | | |
| | 0 | NR | NR | C | C | C |
| | 0.5 | NR | NR | NR | C | C |
| | 1.0 | SE | C | C | C | C |
| | 1.5 | NR | C | C | C | C |
| | 2.0 | NR | C | C | C | C |
| CA | | | | | | |
| | 0 | NR | NR | S | S | S |
| | 0.5 | NR | NR | S | S | S |
| | 1.0 | R | NR | S | S | S |
| | 1.5 | R | R | S | S | S |
| | 2.0 | R | R | R | S | S |

NR=not response, SE=somatic embryogenesis, C=callus growth, S=shoot formation, R=root formation.

Table 2. List of the cassava (*Manihot esculenta* Crantz) cultivars analysed with respective morphogenetic response, number of regenerated plants and chromosome numbers

| Cultivar | Morphogenetic response | Number of regenerated plants | $2n$ |
|---|---|---|---|
| Mcol-1468 | SE | 89 | 36 |
| MBRA235 | SE | 78 | 36 |
| MBRA222 | O | 34 | 36 |
| MBRA254 | O | 31 | 36 |
| Trouxinha | O | 23 | 36 |
| Cachoeirinha | O | 54 | 36 |
| CM955 | O | 34 | 36 |

SE=somatic embryogenesis, O=organogenesis.

In cassava, for example, Matsumoto *et al.* (1991) demonstrated the importance of $GA_3$ for somatic embryogenesis, pointing out 1.0 mg/l as a level for obtaining good induction.

Under cytogenetic analysis, all 167 regenerated plants from somatic embryos and 176 from organogenesis showed $2n=36$ chromosomes (Table 2) with metacentric to submetacentric morphology (Fig. 1d). In some cases it was not possible to visualize the centromere and secondary constric-

tion positions due to the small size and degree of chromosome condensation. In general, plant cells growing in an artificial culture environment have numerous genetic changes, such as increased frequencies of single-gene mutations, chromosome breakages, transposable element activations, quantitative trait variations, and modifications of normal DNA methylation patterns (Banerjee-Chattopadhyay *et al.* 1985, Kaeppler and Phillips 1993, Do *et al.* 1999). In asparagus, for instance, Kunitake *et al.* (1998) reported a positive relationship between chromosome variation in embryogenic calli-derived plants and duration of subculture. In the present work, however, no ploidy variations were observed in the regenerated plants. The karyological constitution appears to play a role in determining the frequency of variation due to the *in vitro* culture condition and cassava probably has karyological stability. Angel *et al.* (1996) demonstrated genetic stability by RFLP, RAPD and DNA fingerprinting in cassava plants cultivated *in vitro* for 10 years, reinforcing the karyological stability hypothesis. Another possibility is the absence of endoreduplication of nuclear DNA. During endoreduplication, cells undergo repeated rounds of DNA synthesis without mitosis, resulting in cells with multiple ploidy levels (endopolyploidy). Explants of a polysomatic nature may influence the ploidy level of regenerants. Kudo and Kimura (2001), for instance, reported that most of the somatic tissue of cabbage seedlings (*Brassica oleracea* L.) contains multiple ploidy cells. In this case, cells with an endoreduplication cycle could generate polyploid plants if their competence for regeneration has been maintained. Thus, the high percentage (33%) of tetraploid transformed cabbage plants reported by Metz *et al.* (1995) may be due to the hypocotyl explants used for *in vitro* culture (Kudo and Kimura 2001). Future directions are necessary for research into the genetic stability of plants cultivated *in vitro.* This may be useful in decreasing the occurrence of somaclonal variation and polyploidization in *in vitro* cultures.

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## References

Angel, F., Barney, V. E., Tohme, J. and Roca, W. M. 1996. Stability of cassava plants at the DNA level after retrieval from 10 years of *in vitro* storage. Euphytica **90**: 307–313.

Bajaj, Y. P. S. 1990. Biotechnology in Agriculture and Forestry 11-Somaclonal Variation in Crop Improvement I. Berlin: Springer-Verlag.

Banerjee-Chattopadhyay, A. Y. S., Schwemmin, A. M. and Schwemmin, D. J. 1985. A study of karyotypes and their alterations in cultured and *Agrobacterium* transformed roots of *Lycopersicon peruvianum* Mill. Theor. Appl. Genet. **71**: 258–262.

Carvalho, R., Guerra, M. and Carvalho, P. C. L. 1999. Occurrence of spontaneous triploidy in *Manihot esculenta* Crantz. Cytologia **64**: 137–140.

Choi, H., Lemaux, P. G. and Cho, M. 2000. High frequency of cytogenetic aberration in transgenic oat (*Avena sativa* L.) plants. Plant Sci. **156**: 85–94.

Do, G., Seo, B., Ko, J., Lee, S., Pak, J., Kim, I. and Song, S. 1999. Analysis of somaclonal variation through tissue culture and chromosomal localization of rDNA sites by fluorescent *in situ* hybridization in wild *Allium tuberosum* and a regenerated variant. Plant Cell Tiss. Org. Cult. **57**: 113–119.

Eskes, A. B., Varga, A., Staritsky, G. and Bruinsma, J. 1974. Callus growth and rooting of cassava (*Manihot esculenta* Crantz) stem segments cultured *in vitro.* Acta Bot. Neerlandica **23**: 315–320.

Fukuda, W. M. G., Costa, I. R. S., Vilarinhos, A. D. and Oliveira, R. P. 1996. Diversidade Genética e Manejo de Germoplasma de Mandioca. In: Banco de Germoplasma de Mandioca: Manejo, conservação e caracterização. Série Documentos **86**: 10–32.

Guerra, M. 1983. O uso do Giemsa na citogenética vegetal: comparaçáo entre a coloração simples e o bandeamento. Ci. Cult. **35**: 190–193.

Hahn, S. K., Bai, V. K. and Asiedu, R. 1992. Spontaneous somatic tetraploids in cassava. Jap. J. Breed. **42**: 303–308.

Jimenez, V. M. and Bangerth, F. B. 2001. Endogenous hormone levels in explants and in embryogenic and non-embryogenic cultures of carrot. Physiol. Plant. **111**: 389–395.

Kaeppler, S. M. and Phillips, R. S. 1993. DNA methylation and tissue culture-induced variation in plants. *In vitro* Cell. and Develop. Biology-Plant **29**: 125–130.

Kudo, N. and Kimura, Y. 2001. Patterns of endopolyploidy during seedling development in cabbage (*Brassica oleracea* L.). Ann. Bot. **87**: 275–281.

Kunitake, H., Nakashima, T., Mori, K. and Tanaka, M. 1998. Somaclonal and chromosomal effects of genotype, ploidy and culture duration in *Asparagus officinalis* L. Euphytica **102**: 309–316.

Matsumoto, K., Cabral, G. B., Teixeira, J. B. and Rech, E. L. 1991. Embriogênese somática a partir de folhas imaturas de mandioca *in vitro*. Rev. Bras. Fisiol. Veg. **3**: 107–110.

Metz, T. D., Dixit, R. and Earle, E. D. 1995. *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var. *italica*) and cabbage (*B. oleracea* var. *capitata*). Plant Cell Rep. **15**: 287–292.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tabacco tissue cultures. Physiol. Plant. **15**: 473–497.

Nassar, N. M. A. 2000. Cassava, *Manihot esculenta* Crantz, genetics resources: their collection, evolution and manipulation. Adv. Agron. **69**: 179–230.

Oliveira, R. P., Gomes, T. S. and Vilarinhos, A. D. 2000. Avaliação de um sistema de micropropagação massal de variedades de mandioca. Pesq. Agrop. Bras. **35**: 2329–2334.

Parke, D. 1978. Tissue culture of cassava on chemically defined media. Physiol. Plant. **42**: 195–201.

Perry, A. B. 1943. Chromosome number and phylogenetic relationships in the Euphorbiaceae. Amm. Bot. **26**: 352–360.

Roca, W. M. 1984. Cassava. In: Sharp, W. R., Evans, D. A., Ammirato, P. V. and Yamada, Y. (eds). Handbook of Plant Cell Culture: Crop Species. Macmillan, New York. pp. 269–301.

Smith, M. K., Biggs, B. J. and Scott, K. J. 1986. *In vitro* propagation of cassava (*Manihot esculenta* Crantz). Plant Cell Tiss. Org. Cult. **6**: 221–228.

Szabados, L., Hoyos, R. and Roca, W. 1987. *In vitro* somatic embryogenesis and plant regeneration of cassava. Plant Cell Rep. **6**: 248–251.

Tilquin, J. P. 1979. Plant regeneration from stem callus of cassava. Can. J. Bot. **57**: 1761–1763.

Vasil, I. K. 1996. Milestones in crop biotechnology-Transgenic cassava and *Agrobacterium*-mediated transformation of maize. Nature Biotech. **14**: 702–703.