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Evaluation of cassava plants generated by somatic embryogenesis in different stages of development using molecular markers

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Abstract

Background: Cassava (*Manihot esculenta* Crantz) is a crop that is high in carbohydrates in the roots and in protein in the leaves, important for both human consumption and animal feed, and also has a significant industrial use for its starches. In this study we evaluated the genetic variability with molecular markers in different stages in micropropagated plants from somatic embryos of Venezuelan native clone 56. **Results:** Three markers were used: ISTR, AFLP and SSR, finding that ISTR showed the highest polymorphism among individuals tested. With AFLP a high similarity between the evaluated individuals was observed and with SSR total monomorphism was seen. Using cluster analysis it was found that individuals from an embryo labeled as fasciated at the beginning of the somatic embryogenesis process were grouped as independent of the other plants when analyzed at the acclimatization stage. The differences found with the different markers used are discussed. In field trials, micropropagated plants had a yield between 4 and 5 times the average yield of cassava in Venezuela. **Conclusion:** Despite variability in terms of DNA markers, somatic embryogenesis is suitable for mass propagation of highly performing cassava clones.

Keywords: AFLP, genetic stability, ISTR, SSR, UPGMA

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a tropical plant species, very efficient in terms of carbohydrate production, drought resistant and able to produce in poor soils. Its high productivity makes it an important source of calories in food and animal feed, as well as a raw material for industry. However, the productivity of this crop may be low because it is normally propagated vegetatively by cuttings and replanting plant material that has been used previously, which is old and often infested with viruses and bacteria. This creates the need to obtain whole plants by an alternate route to the traditional one, making it important to have *in vitro* plant propagation systems. There are two modes of *in vitro* propagation, microcutting cultures using apical or axillary meristems and somatic embryogenesis (SE) in which plant tissue is subjected to a dedifferentiation process by producing a callus, then inducing production of embryos and allowing the development of plants from them. The SE process has the additional advantage that it can be potentially automated allowing production of large numbers of plants at a reasonable cost.

Several protocols have been developed that allow the induction of somatic embryos from cultivation of immature leaves and the subsequent production of cassava plants (Taylor et al. 2005), a process that is highly dependent on the genotype of the plant that donor the explant. Although these regeneration systems provide efficient propagation procedures, they should also ensure the genetic stability of the

individuals obtained with respect to source material. Both aspects should be taken into account in the use of modern biotechnology in improving cassava cultivars.

It has been assumed that species that are vegetatively propagated and generated by asexual reproduction are clones genetically identical to the plant that gave rise to them, an assertion that has never been proven (Raven et al. 2005). But now it is known that regardless of the type of propagation, sexual or asexual, there is always variation and that asexual reproduction also introduces variability, both *in vivo* in agaves (Infante et al. 2003; Infante et al. 2006) and during *in vitro* cultivation (González et al. 2003; Sanchez-Teyer et al. 2003), due to the high rate of variation in somatic tissues, which is transmitted to the offspring during asexual reproduction. However, this variability may lie in noncoding or highly repeated regions of the genome, therefore they may not have a visible phenotypic effect.

To study the genetic differences in cassava propagated through somatic embryogenesis, we used three different molecular markers: i) ISTR (Inverse Sequence-Tagged Repeat), a molecular marker technology based on PCR which uses the universal presence of reverse transcriptase sequences of copia-like elements in genomes. Specific primer pairs are used to produce PCR fragments of a particular length that serve as markers (Demey et al. 2004); ii) AFLP (Amplified Fragments Length Polymorphism) markers based on selective amplification of genomic restriction fragments by PCR (Vos et al. 1995); iii) SSR, microsatellites specific to cassava (Mba et al. 2001).

In this work we evaluated plants obtained through somatic embryogenesis using molecular markers in the *in vitro* development stage and in nurseries during their acclimation, as well as their performance after one cycle of field planting.

MATERIALS AND METHODS

Plant Material

Clone 56 was used, a native of Venezuela, belonging to the Germplasm Bank of the Instituto de Estudios Avanzados. As a control, we used a mature plant planted in Dividive (Aristides Bastidas municipality, Yaracuy state, Venezuela). Samples were taken from plants *in vitro* and acclimated plants in greenhouse phase. They were identified as follows: 56c: field plant; 56V: plants *in vitro* propagated by microcutting; EN1-6: plants obtained from a normal somatic embryo which has been multiplied *in vitro* by microcuttings; EF1-6: plants obtained from a fasciated somatic embryo which has been multiplied *in vitro* by microcuttings; S21-6: acclimated plants issued from the normal embryo; F21-6: acclimated plants issued from the fasciated embryo; and S01-4: acclimated plants propagated by microcuttings. All individuals evaluated were the same but analyzed at different stage of development.

Somatic embryogenesis

Somatic embryogenesis was induced according to the protocol proposed by Taylor et al. (2005). Immature unilobed leaves were sectioned with a scalpel and placed on semi-solid MS medium enriched with 50 mM Picloram for 20 days. The calli produced were transferred to GD medium (Gresshoff and Doy, 1974) with 50 mM Picloram and received 3 subcultures of 4 weeks each. In the second change to GD medium, friable embryogenic calluses (FEC) were selected and transferred to MS medium without hormones until the appearance of SE. Later these embryos were separated and placed in 4E medium for growth. Microcutting propagated plants (SO), and those issued from a normal embryo (EN) or from a fasciated embryo (EF) were acclimatized in the greenhouse.

DNA extraction

Young leaves were used from all the samples described above, following the protocol of Keb-Llanes et al. (2002). DNA was stored at -20ºC until analysis. Total genomic DNA was quantified in a PerkinElmer Lambda ® 35 UV / Vis spectrophotometer. The DNA quality was verified by electrophoresis in 1% agarose gel with 0.5 X TBE buffer.

ISTR

A modification of the procedure described by Demey et al. (2004) was used for development of ISTRs. The amplifications were carried out using 25 ng DNA, 0.3 pM of each primer (ISTRF x ISTRB), 1x PCR buffer, 3 mM $MgCl_2$, 1.25 U Taq polymerase and 0.25 mM dNTPs in a 20 µl final volume. The amplification program consisted of an initial denaturation step of 95ºC for 3 min, followed by 40 cycles of 95ºC for 30 sec, 45ºC for 1 min, 72ºC for 2 min, then 72ºC for 10 min and a final cycle of 4ºC. This procedure was used with 4 combinations of primers: F1 x B1, F10 x B6, F10 x B1 and F9 x B6 (Table 1).

Table 1. Primers used: ISTR F, Primers forward, ISTR B, primers backward. SSR Cassava Microsatellites.

ISTR F1	5'-GGACTCCACCAAGAATACC-3'	
ISTR F9	5'-TTACCTCCTCCATCTCGTAG-3'	
ISTR F10	5'-TAAGCAAGCATCTCGGAG-3'	
ISTR B1	5'-ATCAGGAAGGTCTGTAAAGC-3'	
ISTR B6	5'-GGTTCCACTTGGTCCTTAG-3'	
SSR	**FOWARD**	**REVERSE**
SSRY12	5'-AACTGTCAAACCATTCTACTT GC-3'	5'-GCCAGCAAGGTTTGCTACAT-3'
SSRY21	5'-CCTGCCACAATATTGAAATGG-3'	5'-CAACAATTGGACTAAGCAGCA-3'
SSRY47	5'-GGAGCACCTTTTGCTGAGTT-3'	5'-TTGGAACAAAGCAGCATCAC-3'
SSRY51	5'-AGGTTGGATGCTTGAAGGAA-3'	5'-GGATGCAGGAGTGCTCAACT-3'
SSRY64	5'-CGACAAGTCGTATATGTAGTATTCACG-3'	5'-GCAGAGGTGGCTAACGAGAC-3'
SSRY69	5'-CGATCTCAGTCGATACCCAAG-3'	5'-CACTCCGTTGCAGGCATTA-3'
SSRY82	5'-TGTGACAATTTTCAGATAGCTTCA-3'	5'-CACCATCGGCATTAAACTTTG-3'
SSRY116	5'-CGTTTTCCTGTTAAATCTTGCAT-3'	5'-TAGAGCAGCTGCAAAGCAAA-3'
SSRY129	5'-CTTTTTGCCAGTCTTCCTGC-3'	5'-AATGGATCATGTTCAATGTCTTC-3'
SSRY130	5'-GGTCCCTGATAGTTGATAATGGAT-3'	5'-CTTTTTGCCAGTCTTCCTGC-3'
SSRY133	5'-AGCATGTCATTGCACCAAAC-3'	5'-CGACTGCATCAGAACAATGC-3'
SSRY142	5'-CTTTTTGCCAGTCTTCCTGC-3'	5'-AATGGATCATGTTCAATGTCTTC-3'
SSRY189	5'-TGGGCTGTTCGTGATCCTTA-3'	5'-CATGAGTTTAAAAATTATCACATCCG-3'

AFLP

This technique was developed following the protocol of Infante et al. (2003) with modifications. From the extracted DNA, 100 ng were taken for some samples and 250 ng for others, digestion was carried out with 5U of ECO RI and 5U of MSE I and 1U of T4 DNA ligase was used for ligation and it was left at room temperature overnight. Thereafter the protocol was followed strictly. The primer combinations were: EAAC+MCAA, EAAC+MCAC, EAAC+MCAG, EAAC+MCTA, EACG+MCAA, EACG+MCAC, EACG+MCAG, EACG+MCTA.

SSR

The primers used were: SSR 12, 21, 47, 51, 64, 69, 82, 116, 129, 130, 133, 142 and 189 (Table 1). The thermal cycles consisted of an initial denaturation step of 5 min at 94ºC, followed by 30 cycles of 94ºC x 30 sec, 55ºC x 1 min and 72ºC x 2 min and a final extension of 5 min at 72ºC, following the protocol of Mba et al. (2001). The amplification products in all cases were separated by 4% polyacrylamide gel electrophoresis (1X TBE), where the samples were loaded concentrated either by vacuum aspiration in a SpeedVac ® system for 20 min or by evaporation by continuous heating for 10 min at 96ºC until reaching a volume of 4 µl, and then it was added an equal volume of loading buffer (98% formamide, 10 mM EDTA pH 8, 0.5% bromophenol blue and 0.5% xylene cyanol). The gel was stained with silver salts, using a kit from Promega Corporation (Madison, WI, USA), following the manufacturer's instructions.

Statistical analysis

To estimate the similarity between plants from different types of propagation, we used a matrix of 1/0 (presence/absence) obtained from polyacrylamide gels. The Dice coefficient was calculated. Then a cluster analysis was performed by the UPGMA method (Unweight Pair-Group Method with Arithmetic Mean) using the NTSYSpc program. The results were plotted in a dendrogram. The tree topology was assessed by bootstraping 200 times with 25% resampling using the programs Freetree and Treview X.

Field trial

To test performance in the field, 210 plants, 140 from each type issued from SE and 70 from microcutting propagation, were planted in a commercial field in San Carlos (Cojedes state, Venezuela). After one year in the field, plants were harvested. To measure yield, roots from 20 plants from each type, taken at random, were used. Results were extrapolated in Kg fresh roots/h assuming a density of plantation of 10,000 plants/ha.

RESULTS

The SE of cassava clone 56 was obtained from unilobed leaves of an *in vitro* microcuttings placed on semi-solid MS medium enriched with 50 mM Picloram. After 15 days, the leaf tissue was transformed into a light green-yellow and translucent cell mass. After transferring them to GD medium, they formed friable embryogenic structures (FEC) within 8 weeks, which have a creamy yellowish colour. On the average, 8 structures developed from each leaf. Embryos were obtained from FEC when they were transferred to hormone-free MS medium. In clone 56, seven embryos were obtained from one FEC of which five were separated. Germinated embryos were classified: one of them as normal by having a slender stem and well-formed leaf lobes and a second one as fasciated, having a thicker than normal stem and fused leaves (Figure 1b and Figure 1c). Plants obtained from two types of embryos were *in vitro* propagated by the microcutting method then acclimatized to the greenhouse. Morphologically, plants deriving from the two types of embryos were similar in the greenhouse. No abnormalities in either the aerial part or the roots were observed in the plants obtained from the embryo considered as fasciated at the beginning of the process (Figure 1d and Figure 1e).

ISTR

The four tested combinations generated 248 bands, of which 232 were polymorphic, representing 93.5% of polymorphic loci. The minimum (0.45) and maximum (0.85) values of the coefficient of genetic similarity show the existence of genetic variability among cassava individuals from the two types of *in vitro* propagation (microcutting and SE). In the dendrogram presented in Figure 2, the formation of two groups and two unique individuals (S23, S26) was observed. Group I was made up of individuals 56c, 56v, S01, S02, S21, S22, EN1, EN2, EN3, EN5, EN4, EN6, S03, S04, EF1, EF3, EF2, EF4, EF6, EF5, F21, S24 , S25 and F22, and group II consisted of F23, F24, F26 and F25. The first group included plants issued from the two methods of propagation analysed *in vitro* as well as in the greenhouse. The second group included only plants analysed in the greenhouse and deriving from the fasciated embryos. The 2 individuals which were not pooled (S23 and S26) were field plants originated from the normal embryo.

AFLP

The eight primer combinations produced a total of 191 bands with 48 polymorphic bands, accounting for 25.1%. The number of bands per individual and per combination of primers ranged between 19 (E + AAC x CTA) and 61 (E + AAC x M + CAA). The minimum similarity coefficient found was 0.89 between individuals 56C and F22 and the maximum was 0.99 among individuals EN2 and S03. The cluster analysis shows the formation of two homogeneous groups, according to the dendrogram in Figure 3. The first group includes plants from the two methods of *in vitro* propagation, both in greenhouse and *in vitro* conditions, composed of individuals 56V, EN1 to EN6, S21 to S26, S01 to S04 and EF1 to EF6. Group 2 includes only plants from fasciated embryos analysed in acclimatization stage, composed of individuals F21 to F26.

SSR

None of the 13 primers that were evaluated (SSRY 12, 21, 47.51, 64, 69, 82, 116, 129, 130, 133, 142 and 189) showed polymorphism, *i.e.* that for the region of the genome studied by the above mentioned primers (Figure 4).

Field trial

Once acclimated, plants were planted in the field in farm conditions. The yield was 43,000 Kg fresh roots/ha for the plants issued from microcuttings (56C), 40,700 Kg/ha for the ones issued from the normal embryo (56N) and 57,600 Kg/ha for the ones from the fasciated somatic embryo (56F) (Figure 5). This performance contrasts with the average yield of cassava in Venezuela which is 12,000 Kg/ha (Marín, 2002), and in Latin American (13,196 Kg/ha) (http://www.infoagro.com/hortalizas/yuca.htm).

DISCUSSION

In this study, the production of somatic embryos in cassava was induced using Picloram, since in earlier work by Capote et al. (2000), it was demonstrated in *Allium cepa* cytology studies that 2,4-D induced triploidy while with Picloram the plant cells retained their diploid state. It was carried out, due to the interest in mass production of plants for this crop, to guarantee the quality of the propagated material distributed to the farmers. However, the cassava embryos showed two different morphologies in the early stages of development, normal and fasciated. To analyze these discrepancies and to compare it with the method of clonal propagation by microcuttings and with the mother plant of this cultivar, three types of markers were used: ISTR, AFLP and SSR. The results showed that the levels of variability are different according the type of marker used, being higher with the ISTR than with AFLP and monomorphic with SSR. This is explained by the nature of each marker. ISTR are based on Copia-like elements (retrotransposons) found in the genomes of plants. With advances in DNA sequencing on a large scale, it has been shown that retrotransposons are not a rare component of the genome but constitute an important fraction of the genetic material of many eukaryotes. For example, they occupy 45% of the genome of Cacao Criollo *(Theobroma cacao)* (Argout et al. 2011). The AFLP are based on restriction enzymes. The differences between the ISTR and AFLP are explained by the fact that each type of marker studies a different region of the genome, as demonstrated in agaves (Infante et al. 2006). While there is no evidence about the preferences of insertion of retrotransposons in plants, they mostly are located in heterochromatic repetitive regions of the genome (Dasilva et al. 2002; Argout et al. 2011), while AFLP are located in euchromatic regions. However, despite the different levels of variability, both markers were able to group the individuals studied in a similar way, as seen in the dendrograms generated by each of them. Particularly, they show that plants issued from the fasciated embryo were separated from other individuals when analysed at the acclimatization stage. Tissue cells during SE go through a callus phase which increases the likelihood of changes and decreases genetic stability (Smykal et al. 2007). Among other reasons, that can be due to the activation of mobile elements, which causes insertions, deletions and rearrangements in the genome, leading to changes in it. The activity of transposons is associated with the patterns of methylation/demethylation of DNA. For their part, SSR are markers that have high levels of inter and intraspecific variability. Therefore, they are useful for genotyping, individual identification, pedigree and paternity analysis. The results obtained with different markers show that although there is variability found with AFLP and ISTR, the individuals are the same during the process, because SSR patterns are conserved.

The evaluation of patterns of markers in different stages of plant development shows that the profiles are changing during development and that genetic variability is introduced in all stages of the process. These observations indicate that somatic embryogenesis is a process that generates changes in the DNA *per se*, as it is also the case for microcutting and stem cutting methods. These changes have been related to alterations in the concentrations of auxins, cytokinins, the extent of the *in vitro* culture, as well as the stress itself that this system causes, given the conditions to which plants are subjected. When these alterations in the genome cause changes in the phenotype it is called somaclonal variation. However, all these changes in morphology *in vitro* can be reversed when the plants are carried to the field (Sanchez-Teyer et al. 2003; Sharma et al. 2007; Venkatachalam et al. 2007). In this work, a phenotype called fasciated, found in the early stages of the SE process, reverted to normal phenotype in the greenhouse stage, giving rise to normal plants.

The plants obtained showed a better performance in the field in the first planting season, reaching a productivity per plant between 4 and 5 times higher than the average yield of cassava in Venezuela (Figure 5), the most productive plants being the ones from the fasciated embryo (56F), with a yield 4.7 times higher than the average cassava yield in Venezuela. Even they showed 41% higher yield per hectare than the plants obtained from the normal somatic embryos (56N).

What is then the mechanism that preserves the morphology of cells and ultimately the plant's shape in a process that generates so much variability? The relationship between genotype and phenotype is a complex problem (Dowell et al. 2010). Classically, development has been seen as a pre-programmed and deterministic process. However, studying the biology of systems of initiation of flower development in *A. thaliana*, Espinosa-Soto et al. (2004) find that it exists a limited number of possibilities for flower forms, which is determined by the regulatory dynamics of the genetic network, finding differences in network architecture in *Arabidopsis* and *Petunia hybrida*. In other study, Traas and Moneger (2010) find that minor changes in the rules of interaction do not alter significantly the simulation results in various angiosperms, thus the regulatory network is very robust. The consequence is that all flowers in general have the same organs and a certain geometric similarity. These results indicate that gene expression patterns have some possible states. Then, not everything is possible. Even if we assume a random expression of the genes involved in cell morphology or body shape, the disturbances present in a highly nonlinear system make it to converge towards 10 predefined fixed attractors, each with an array of gene expression that characterizes the inflorescence cells, sepals, petals, estaminoids and flower carpel primordial cells (Alvarez-Buylla et al. 2008). It is then possible that such a self-regulation system corrects the morphological differences initially observed in the fasciated phenotype in our study. The variability detected with our markers did not have an effect on the phenotype in the greenhouse and in the field. Similarly, Venkatachalam et al. (2007) reported field reversal of malformations found *in vitro*. This allows us to assert that SE did not generate important changes. Therefore, this propagation method is a process suitable for mass propagation of high performance cassava clones.

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REFERENCES

ÁLVAREZ-BUYLLA, E.R.; CHAOS, A.; ALDANA, M.; BENÍTEZ, M.; CORTES-POZA, Y.; ESPINOSA-SOTO, C.; HARTASÁNCHEZ, D.A.; LOTTO, R.B.; MALKIN, D.; ESCALERA SANTOS, G.J. and PADILLA-LONGORIA, P. (2008). Floral morphogenesis: Stochastic explorations of a gene network epigenetic landscape. *PLoS ONE,* vol. 3*,* no. 11, p. e3626. **[CrossRef]**

ARGOUT, X.; SALSE, J.; AURY, J.M.; GUILTINAN, M.J.; DROC, G.; GOUZY, J.; ALLEGRE, M.; CHAPARRO, C.; LEGAVRE, T.; MAXIMOVA, S.N.; ABROUK, M.; MURAT, F.; FOUET, O.; POULAIN, J.; RUIZ, M.; ROGUET, Y.; RODIER-GOUD, M.; BARBOSA-NETO, J.F.; SABOT, F.; KUDRNA, D.; AMMIRAJU, J.S.; SCHUSTER, S.C.; CARLSON, J.E.; SALLET, E.; SCHIEX, T.; DIEVART, A.; KRAMER, M.; GELLEY, L.; SHI, Z.; BERARD, A.; VIOT, C.; BOCCARA, M.; RISTERUCCI, A.M.; GUIGNON, V.; SABAU, X.; AXTELL, M.J.; MA, Z.; ZHANG, Y.; BROWN, S.; BOURGE, M.; GOLSER, W.; SONG, X.; CLEMENT, D.; RIVALLAN, R.; TAHI, M.; AKAZA, J.M.; PITOLLAT, B.; GRAMACHO, K.; D'HONT, A.; BRUNEL, D.; INFANTE, D.; KEBE, I.; COSTET, P.; WING, R.; MCCOMBIE, W.R.; GUIDERDONI, E.; QUETIER, F.; PANAUD, O.; WINCKER, P.; BOCS, S. and LANAUD, C. (2011). The genome of *Theobroma cacao*. *Nature Genetics,* vol. 43, no. 2, p. 101-108. **[CrossRef]**

CAPOTE RODRÍGUEZ, A.; FUNDORA MAYOR, Z. and PÉREZ DÍAZ, O. (2000). Estudio de la variabilidad inducida en células y plántulas de cebolla (*Allium cepa, L*.) cv. Caribe-71 regeneradas *in vitro*. *Biotecnología Aplicada,* vol. 17, no. 4, p. 241-246.

DASILVA, C.; HADJI, H.; OZOUF-COSTAZ, C.; NICAUD, S.; JAILLON, O.; WEISSENBACH, J. and CROLLIUS, H.R. (2002). Remarkable compartmentalization of transposable elements and pseudogenes in the heterochromatin of the *Tetraodon nigroviridis* genome. *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, no. 21, p. 13636-13641. **[CrossRef]**

DEMEY, J.R.; GAMEZ, E.; MOLINA, S. and INFANTE, D. (2004). Comparative study of the discriminating capacity of AFLP and ISTR markers for genetic analysis of *Agave fourcroydes*. *Plant Molecular Biology Reporter,* vol. 22*,* no. 21, p. 29-35. **[CrossRef]**

DOWELL, R.D.; RYAN, O.; JANSEN, A.; CHEUNG, D.; AGARWALA, S.; DANFORD, T.; BERNSTEIN, D.A.; ROLFE, P.A.; HEISLER, L.E.; CHIN, B.; NISLOW, C.; GIAEVER, G.; PHILLIPS, P.C.; FINK, G.R.; GIFFORD, D.K. and BOONE, C. (2010). Genotype to phenotype: A complex problem. *Science,* vol. 328, no. 5977, p. 469. **[CrossRef]**

ESPINOSA-SOTO, C.; PADILLA-LONGORIA, P. and ÁLVAREZ-BUYLLA, E.R. (2004). A gene regulatory network model for cell-fate determination during *Arabidopsis thaliana* flower development that Is robust and recovers experimental gene expression profiles. *The Plant Cell,* vol. 16, no. 11, p. 2923-2939. **[CrossRef]**

GONZÁLEZ, G.; ALEMÁN, S. and INFANTE, D. (2003). Asexual genetic variability in *Agave fourcroydes* II: Selection among individuals in a clonally propagated population. *Plant Science,* vol. 165, no. 3, p. 595-601. **[CrossRef]**

GRESSHOFF, P. and DOY, C. (1974). Derivation of a haploid cell line from *Vitis vinifera* and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. *Zeitung Pftlanzenphysiologie,* vol. 73*,* p. 132-141.

INFANTE, D.; GONZÁLEZ, G.; PERAZA-ECHEVERRÍA, L. and KEB-LLANES, M. (2003). Asexual genetic variability in *Agave fourcroydes*. *Plant Science,* vol. 164, no. 2, p. 223-230. **[CrossRef]**

INFANTE, D.; MOLINA, S.; DEMEY, J.R. and GAMEZ, E. (2006). Asexual genetic variability in *Agavaceae* determined with inverse sequence-tagged repeats and amplification fragment length polymorphism analysis. *Plant Molecular Biology Reporter,* vol. 24, no. 2, p. 205-217. **[CrossRef]**

KEB-LLANES, M.; GONZÁLEZ, G.; CHI-MANZANERO, B. and INFANTE, D. (2002). A rapid and simple method for small scale DNA extraction in *Agavaceae* and other tropical plants. *Plant Molecular Biology Reporter*, vol. 20, no. 3, p. 299a-299e. **[CrossRef]**

MARÍN, D.C. (2002). Rendimiento y producción agrícola vegetal: Un análisis del entorno mundial (1997-1999) y de Venezuela (1988-2001). *Agroalimentaria*, vol. 8, no. 15*,* p. 49-73.

MBA, R.E.C.; STEPHENSON, P.; EDWARDS, K.; MELZER, S.; NKUMBIRA, J.; GULLBERG, U.; APEL, K.; GALE, M.; TOHME, J. and FREGENE, M. (2001). Simple sequence repeat (SSR) markers survey of the cassava (*Manihot esculenta* Crantz) genome: Towards an SSR-based molecular genetic map of cassava. *TAG Theoretical and Applied Genetics,* vol. 102, no. 1, p. 21-31. **[CrossRef]**

RAVEN, P.; EVERT, R. and EICHHORN, S. (2005). *Biology of Plants*. W.H. Freeman and Company, N.Y. USA, 944 p. ISBN 9780716710073.

SANCHEZ-TEYER, L.F.; QUIROZ-FIGUEROA, F.; LOYOLA-VARGAS, V. and INFANTE, D. (2003). Culture-induced variation in plants of *Coffea arabica* cv. caturra rojo, regenerated by direct and indirect somatic embryogenesis. *Molecular Biotechnology,* vol. 23, no. 2, p. 107-115. **[CrossRef]**

SHARMA, S.; BRYAN, G.; WINFIELD, M. and MILLAM, S. (2007). Stability of potato (*Solanum tuberosum* L.) plants regenerated via somatic embryos, axillary bud proliferated shoots, microtubers and true potato seeds: A comparative phenotypic, cytogenetic and molecular assessment. *Planta,* vol. 226, no. 6, p. 1449-1458. **[CrossRef]**

SMYKAL, P.; VALLEDOR, L.; RODRIGUEZ, R. and GRIGA, M. (2007). Assessment of genetic and epigenetic stability in long-term *in vitro* shoot culture of pea (*Pisum sativum* L.). *Plant Cell Reports,* vol. 26, no. 11, p. 1985-1998. **[CrossRef]**

TAYLOR, N.; CHAVARRIAGA, P.; RAEMAKERS, K.; SIRITUNGA, D. and ZHANG, P. (2005). Development and application of transgenic technologies in cassava. *Plant Molecular Biology,* vol. 56, no. 4, p. 671-688. **[CrossRef]**

TRAAS, J. and MONEGER, F. (2010). Systems biology of organ initiation at the shoot apex. *Plant Physiology,* vol. 152, no. 2, p. 420-427. **[CrossRef]**

VENKATACHALAM, L.; SREEDHAR, R. and BHAGYALAKSHMI, N. (2007). Genetic analyses of micropropagated and regenerated plantlets of banana as assessed by RAPD and ISSR markers. *In Vitro Cellular and Developmental Biology-Plant,* vol. 43, no. 3, p. 267-274. **[CrossRef]**

VOS, P.; HOGERS, R.; BLEEKER, M.; REIJANS, M.; VAN DE LEE, T.; HORNES, M.; FRITERS, A.; POT, J.; PALEMAN, J.; KUIPER, M. and ZABEAU, M. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research,* vol. 23, no. 21, p. 4407-4414. **[CrossRef]**

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Figures

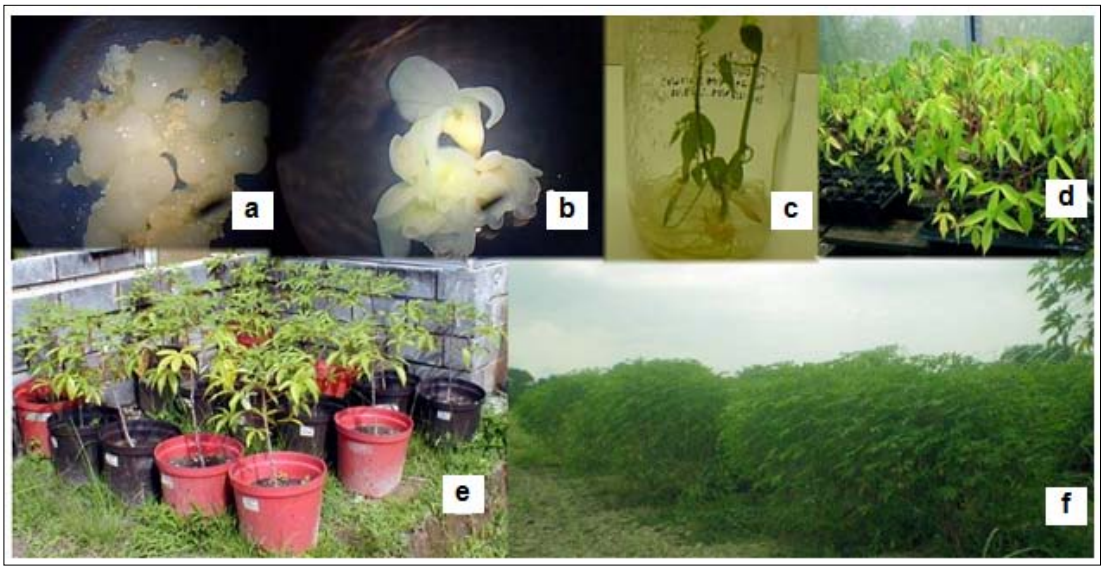

Fig. 1 Different stages in the development of cassava plants (Clone 56) through somatic embryogenesis. (a) Embryogenic friable calli on GD medium, (b) Somatic embryos on MS medium without hormones, (c) Normal (left) and Fasciated (right) germinated embryos, (d) Acclimation in Canadian peat, (e) Seven month old plants in pots, (f) Eleven month old plants in the field.

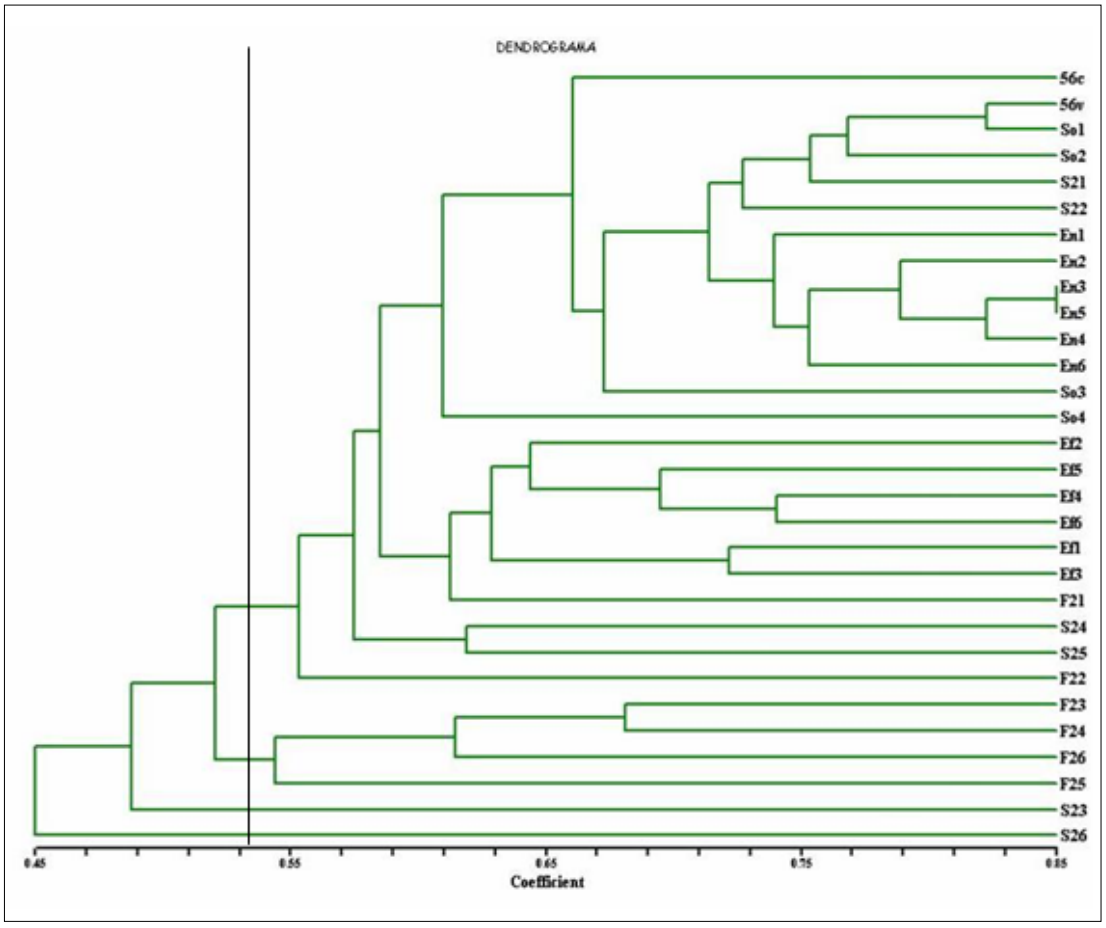

Fig. 2 Dendrogram generated using ISTR. Mother plant from the field (56c*)*, *in vitro*propagated plant by microcutting method (56v), stem cuttings with 30 days in a greenhouse (S01 to S04), *in vitro* plants obtained from a normal somatic embryo (En1 to En6), greenhouse plants obtained from a normal somatic embryo (30 days in greenhouse) (S21 to S6) *in vitro* plants obtained from a fasciated somatic embryo (Ef1 to 6), greenhouse plants obtained from a fasciated somatic embryo after 30 days in a greenhouse (F21 to F6).

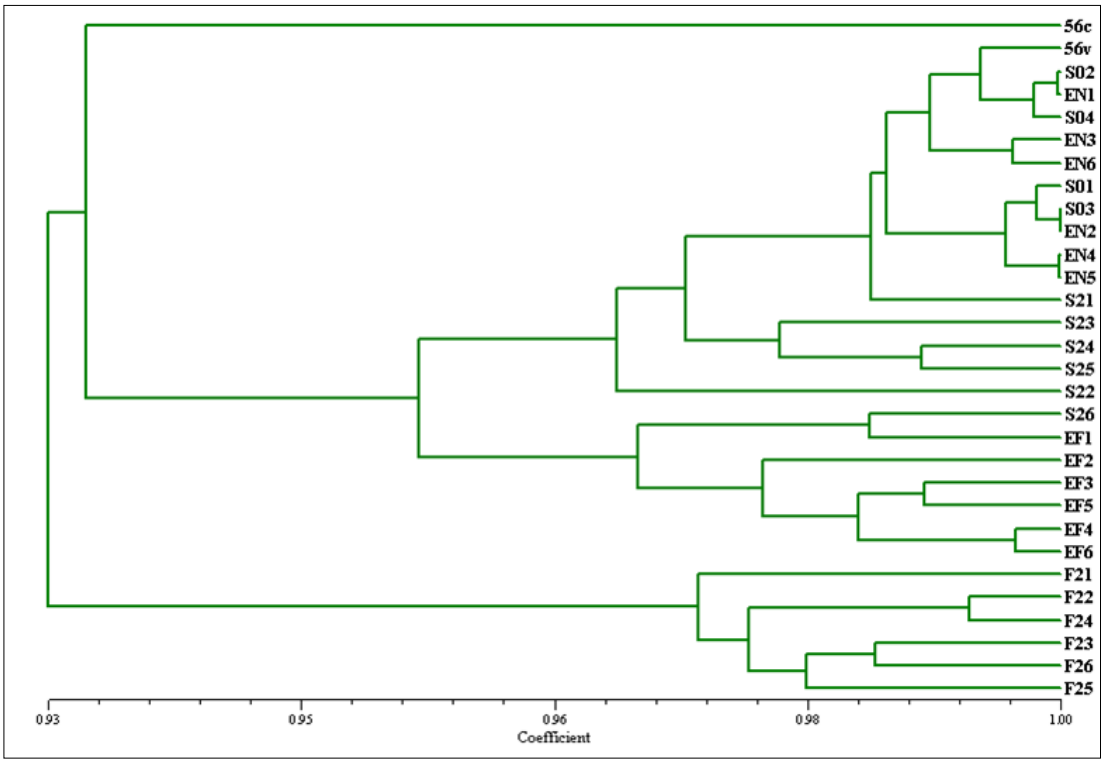

Fig. 3 Dendrogram generated using AFLP. Mother plant from the field (56c), *in vitro* propagated plant by microcutting method (56v), stem cuttings with 30 days in a greenhouse (S01 to S04), *in vitro* plants obtained from a normal somatic embryo (En1 to En6), greenhouse plants obtained from a normal somatic embryo (30 days in greenhouse) (S21 to S6) *in vitro* plants obtained from a fasciated somatic embryo (Ef1 to 6), greenhouse plants obtained from a fasciated somatic embryo after 30 days in a greenhouse (F21 to F6).

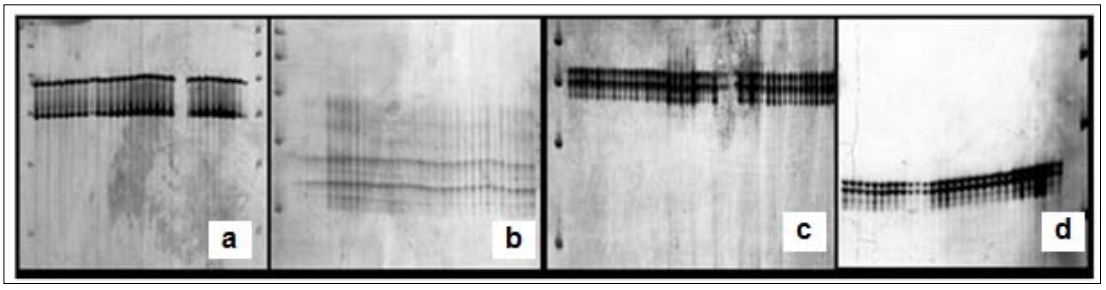

Fig. 4 SSR gels in 6% polyacrylamide. (a) -SSRY 133; (b) -SSRY 82; (c) -SSRY 21; (d) -SSRY 142.

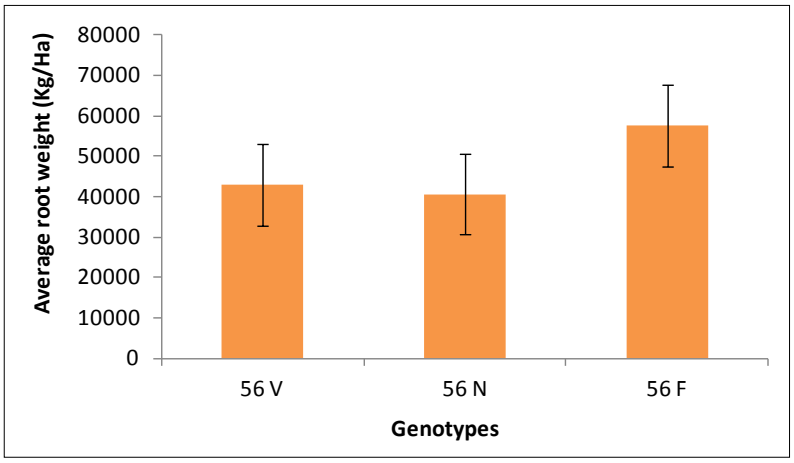

Fig. 5 Yield in Kg/ha after the first harvest for plants issued from a normal somatic embryo (56 N), for plants issued from an abnormal somatic embryo (56 F), for plants propagated by microcuttings (56 V).