Efficacy of silver nitrate for slow-growth conservation of cassava (*Manihot esculenta Crantz*). Determination of viability and genetic stability.

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Introduction

Over the past few years, efforts have been made to develop more efficient methods for *in vitro* conservation of cassava. Research has been focused to develop a conservation system that permits to reduce growth using an ethylene inhibitor, silver nitrate, in order to open ways to safe-duplicate the collection and increase time between regenerations. Ethylene (C_2H_4) is produced by plants and is known to have various effects on growth in plant tissue culture (De Proft et al, 1985; Huxter et al. 1979). Silver nitrate not only had a beneficial effect on six varieties of cassava survival after prolonged maintenance *in vitro*, but also was highly effective in limiting the microshoot height and presenting a lower rate of defoliation (Mafla G. et al 2000).

Maintenance of viability and genetic stability is of great importance in any *in vitro* conservation approach, and no *in vitro* culture system will be acceptable if it induces loss in viability and introduces a high risk of genetic instability (Withers 1980).

In our study we examined the genetic stability using morphological, biochemical and molecular analyses of the six varieties grown for a period of 20 months in presence of silver nitrate; we also analyzed the impact of silver nitrate on growth rate *in vitro* for a wide range of varieties.

Materials and Methods

This research comprises two steps: the first step analyses the genetic stability using morphological, biochemical and molecular analyses; the second step has been undertaken to know whether or not silver nitrate is effective to reduce growth and increase the conservation lag on the rest of the collection.

Plant material: Six cassava varieties were evaluated: ARG 2, BRA 337, COL 2056, NGA 16, VEN 329A and CM 2177- 2. They were maintained as *in vitro* plants on medium containing 58.8 $_u$M (Ag3) and 70.6 $_u$M (Ag4) of silver nitrate and *in vitro* storage medium 8S as control (8S) (CIAT 1984). The materials were regenerated and propagated *in vitro*, acclimatized in greenhouse and eventually planted in the field. Four plants from each treatment for each of the six varieties were selected for morphological analysis. Later of these plants, stakes were harvested in field and taken to the greenhouse for later biochemical and molecular analyses (the two different treatments and a control for each variety).

***Morphological analysis*:** Twenty-two morphological descriptors, which are used for the characterization of cassava, were used in order to determine the degree of variation (CIAT- CNPMF 1995). Four plants from each treatment for each of the six varieties were studied.

***Biochemical analysis*:** A total of fifteen enzyme systems were analyzed by using PAGE and SGE electrophoresis. The methodology for isozyme extraction and electrophoresis was the one reported by Ramirez et al. (1987). The gels were stained, fixed, and stored according to the methodology compiled in CIAT (1988).

Molecular analysis: The DNA fingerprinting technique selected was the AFLP technique because of the magnitude of genome coverage. In order to guarantee reproducibility of results (as AFLPs generate a great amount of analyzable fragments), two DNA extractions were done on different plants for the same material (1 and 2, see Fig. 1). The AFLP protocol followed was the one reported by Chavarriaga et al. (1999) and following the method of AFLP Analysis System I.

Determining effectiveness: A total of 4,827 varieties have been subcultured in presence of silver nitrate (58.8 M, 2 tubes per variety). A sample of 2,870 cassava varieties of 24 regions of origin has been included in this group. After 2 months of conservation, observations were recorded on stem length; each treatment included two replicates and data of stem length were averaged. Evaluations should be pursued to determine for how long silver nitrate can extend the time of conservation for the wide range of varieties.

Results and discussion

Morphological analysis: The results showed that reactions of materials for most of the evaluated descriptors remained constant in the different treatments. We observed significant variation only in the length of the central lobe and petiole length for the variety BRA 337, wich presented an average significantly higher in the treatment Ag4 (Table 1). The environment factors (light, soil, and plant vigor) have considerable effect on the expression of these morphological characters (CIAT-CNPMF 1995). In general, using morphological descriptors, we have shown that cassava plants remain true to type after *in vitro* conservation under slow-growth conditions.

	Width of lobe[a] (cm)			Length of lobe[a] (cm)			Length of petiole[a] (cm)		
Varieties	8S	Ag3	Ag4	8S	Ag3	Ag4	8S	Ag3	Ag4
ARG 2	31.2[a*]	35.0[a]	28.7[a]	16.0[a]	17.5[a]	16.5[a]	28.5[a]	32.5[a]	23.5[a]
BRA 337	25.2[b]	24.2[a]	31.0[a]	17.5[b]	18.2[b]	22.0[a]	24.2[b]	26.0[ab]	30.0[a]
COL 2056	45.7[a]	46.5[a]	48.2[a]	19.0[a]	18.7[a]	18.7[a]	31.5[a]	29.5[a]	32.7[a]
NGA 16	58.7[a]	55.7[a]	51.5[a]	20.5[a]	18.7[a]	17.2[a]	30.5[a]	28.7[a]	27.5[a]
VEN 329A	41.0[a]	31.0[a]	45.7[a]	18.2[a]	13.2[a]	19.5[a]	32.2[a]	22.0[a]	34.2[a]
CM 2177- 2	27.2[a]	24.2[a]	24.7[a]	23.7[a]	22.2[a]	23.0[a]	35.7[a]	34.2[a]	33.5[a]

Table 1. Evaluation of traits of cassava *in vitro* plants regenerated after culturing with silver nitrate.

Biochemical analysis: The fifteen isozyme systems selected to monitor the genetic stability, presented a good activity and repeatabilty in cassava. In addition, they also show polimorphism between varieties (Table 2). Twenty genes are involved in these systems, wich represent a tiny coverage of the genome, but an easy and cheap comparison to start with (Lefévre & Charrier 1993; Sarria et al.1993). The electrophoretic patterns obtained for these fifteen isozymes, are the same for control and the two different treatments for each variety. These results suggest genetic stability for the genes involved in the control of these isozymes.

Isozymes	IUBNC[1/]	Gels[2/]	Tissues	Polymorphism Between varieties
Esterease	E.C.3.1.1.-	PAGE	Root	Excelent
Glutamate Oxaloacetate Transaminase	E.C.2.6.1.1.	PAGE	Root	High
Acid phosphatase	E.C. 3.1.3.2	PAGE	Root	High
Diaforase	E.C.1.6.-.-	PAGE	Root	High
Peroxidase	E.C. 1.11.1.7	PAGE	Root	Absent
Shikimic acid Dehydrogenase	E.C. 1.1.1.25	SGE	Leaf	Medium
Malate Dehydrogenase	E.C. 1.1.1.37	SGE	Root	Medium
Isocitrate Dehydrogenase	E.C.1.1.1.42	SGE	Root	Medium
Phosphoglucomate Isomerase	E.C.5.1.3.9	SGE	Root	Medium
Phosphoglucomate Mutase	E.C.5.4.2.2	SGE	Root	Low
Malic enzyme	E.C.1.1.1.40	SGE	Root	Medium
glucose-6-phosphate dehydrogenase	E. C. 1.1.1.49	SGE	Leaf	Medium
glutamate dehydrogenase	E.C. 1.4.1.2	SGE	Leaf	Low
formate dehydrogenase	E. C. 1.2.1.2	SGE	Leaf	Low
fumarase	E. C. 4.2.1.2	SGE	Leaf	Low

[1/]UBNC: Nomenclature committee of the Biochemistry International Union.
[2/]PAGE: Polyacrylamide gel for electrophoresis.
[2/] SGE: Starch gel for electrophoresis.

Table 2. Isoenzymatic systems used as genetic markers to monitor the genetic stability.

Molecular Analysis: The most useful primer combinations were considered those having the highest polymorphism, reproducibility and scorability of AFLP patterns, and also those generating a reasonable number of clearly detectable total fragments. As a result, three primer combinations were selected for the subsequent analysis: (1) E-AAC/M-CTA (Figure 1), (2) E-AAG/M-CTG and (3) E-ACA/M-CTG. These three primer combinations have generated an average of 63 defined, monomorphic and polymorphic bands between varieties. The AFLP fingerprints obtained are the same for control and the two different treatments for each variety. These results indicate no variation for the evaluated regions of the genome for the six cultivated cassava accessions.

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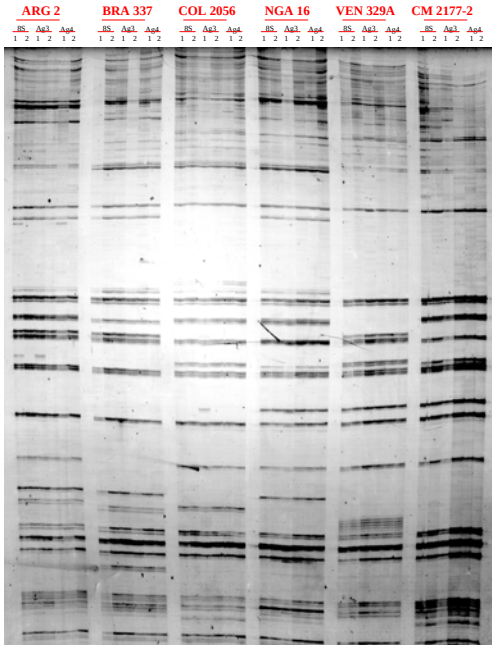

Figure 1. DNA stability monitored with AFLP (E-AAC/M-CTA, the primer combination used).

Determining effectiveness: We have found that silver nitrate with concentration 58.8 $_u$M (Ag3) reduced the growth three times as compared to the standard 8S (Control). The general average for the 2,870 cassava varieties evaluated in silver nitrate medium was 2.7 cm and 8.7 cm in the standard medium. Figure 2 shows the principal effect of silver nitrate on slow growth in the wide range of varieties (coming from 23 different countries and a group of breeding lines). After 7 months of conservation a total of 167 varieties that were cultivated without silver nitrate began to deteriorate, showed defoliation, thus requiring to carry out the subculture. The plants that were in silver nitrate media even continued in conservation under good conditions.

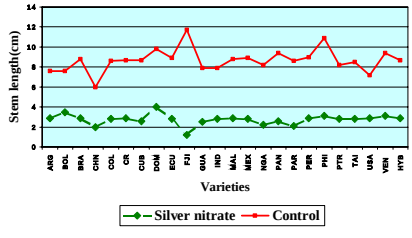

Figure 2. Effect of silver nitrate on *in vitro* growth of cassava varieties.

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