ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 9, Issue, 01, pp.45295-45301, January, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

CLONAL MICROPROPAGATION OF NEWS CASSAVA VARIETIES (*MANIHOT ESCULENTA* CRANTZ) ASSISTED BY INTER-RETROTRANSPOSON AMPLIFIED POLYMORPHISM AND RETROTRANSPOSON-MICROSATELLITE AMPLIFIED POLYMORPHISM MARKERS

[1]Betty Cristiane Kuhn, [2]Jaqueline da Silva Manzatti, [3]Afonso Pepinelli, [2]Patricia Rozin Carnelossi, [2]Eliezer Rodrigues de Souto, [3]Claudete Aparecida Mangolin and [3,*]Maria de Fátima Pires da Silva Machado

[1]Department of Bioprocess Engineering and Biotechnology Federal University of Technology (UTFPR) Dois Vizinhos-PRBrazil, Estr. Boa Esperança, 85660-000, Dois Vizinhos PR Brazil
[2]Department of Agronomy, State University of Maringá, Av. Colombo, 5790, 87020-900 Maringá PR Brazil
[3]Department of Cell Biology and Genetics, State University of Maringá, Av. Colombo, 5790, 87020-900 Maringá PR Brazil

ARTICLE INFO

Article History:
Received 10th October, 2016
Received in revised form
28th November, 2016
Accepted 15th December, 2016
Published online 31st January, 2017

Key words:

Manihot esculenta,
Grown regulator,
Meristem-tip,
Virus,
Genetic diversity.

ABSTRACT

Current assay has established protocols for micropropagation of three cassava varieties (*Manihot esculenta*) of commercial and industrial interest, and index the plants for CsCMV virus. The molecular markers based on retrotransposons LTRs (IRAP and REMAP markers) were employed to test the hypothesis that varieties with greater genetic similarity can develop in the same culture medium while varieties with smaller genetic similarity should require different culture media. Meristem-tips of the three varieties were inoculated in four concentrations of naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) in the MS culture medium. After the establishment of the meristem-tip, the plantlets were transferred to media supplemented with higher cytokinin (6BA) and auxins (NAA and IBA) concentrations containing gibberellic acid for faster development. The Fécula Branca variety showed 100% of regenerated plants in the medium containing IBA, while the IPR-União variety showed 100% of regenerated plants in the medium containing NAA. Use of the IRAP and REMAP markers showed that plants with greater genetic similarity can develop in the same culture medium while varieties showing the lowest similarity coefficient generated plantlets in culture medium containing different auxin types. So that the use of IRAP and REMAP markers may be indicated as a preliminary strategy to program the in vitro cultivation of the genotypes with greater genetic similarity in media supplemented with the same combination of growth regulators.

Citation: Betty Cristiane Kuhn, Jaqueline da Silva Manzatti, Afonso Pepinelli *et al*. 2017. "Clonal Micropropagation of news cassava varieties (*Manihot esculenta* crantz) assisted by Inter-retrotransposon amplified polymorphism and Retrotransposon-microsatellite amplified polymorphism markers", *International Journal of Current Research*, 9, (01), 45295-45301.

INTRODUCTION

Cassava (*Manihot esculenta*, Crantz), an important food crop for millions of people worldwide (Raemakers 2001), is the fourth greatest source of calories after rice (*Oryza sativa*), sugarcane (*Saccharum officinarum*) and maize (*Zea mays*) (Medina *et al*., 2007). The low protein content of cassava roots makes it a source of energy with low allergenic potential (Ceballos *et al*., 2006). Cassava is a perennial shrub of the family Euphorbiaceae (Wongtiem *et al*., 2011) native to South America (Oliveira *et al*., 2000), but grows in all tropical and subtropical regions (Fan *et al*., 2011). Cassava features easy propagation systems, satisfactory performance even in dry and low fertility soils, and low demands for sophisticated cultural requirements (Medina *et al*., 2007). Moreover, it provides a high return per unit of energy used in its cultivation (Mejía-Agüero *et al*., 2012). Cassava is multiplied mainly by stem cutting which is actually a slow process when compared with grain crops (Santana *et al*., 2009). The crop cycle depends on whether the early or late varieties are grown: the first type varieties are harvested eight months after planting, whereas the second type varieties are collected 18 months post planting (Santana *et al*., 2009). The vegetative propagation of cassava may also be affected by several diseases, especially systemic, which are transmitted through successive generations (Oliveira *et al*., 2000). Since cassava has predominantly vegetative propagation, the loss of crop is heightened due to the planting

Corresponding author: Maria de Fátima Pires da Silva Machado
Department of Agronomy, State University of Maringá, Av. Colombo, 5790, 87020-900 Maringá PR Brazil

of suboptimal material, or by spreading diseases (Bull *et al*., 2011). The most frequent disease in Brazil is the CsCMV (Cassava Common Mosaic Virus) (Soares *et al*., 2010) which causes significantly impact on crop yields, root quality, economic costs, marketability, consumer availability and commercial processes (Bull *et al*., 2011). In fact, in the northwest region of the state of Paraná (Brazil), CsCMV infects more than 90% of plants from all cassava cultivars (Silva *et al*., 2011). As a strategy in order to minimize the proliferation of infected stems, the in vitro tissue culture may be an alternative for the production of healthy cassava seedlings. The meristem-tip culture is an important tool which enables the production of a great deal of seedlings in a short time and in a reduced space, coupled to plants free of fungi, bacteria, viruses and pests (Roca *et al*., l989). After the establishment of tissue culture, the generated clones should be indexed with virus detection methods. Variations of ELISA have been used to detect viruses and more recently a new method has been established to improve virus detection sensitivity. An immunocapture-RT-PCR(IC-RT-PCR) protocol was established by Silva *et al*. (2011) and has been reported to be more sensitive and a faster protocol than ELISA alone. Although the meristem-tip culture is a promising method to produce virus-free cassava plants, its application to new cultivars requires improved protocols since different genotypes may have different developments in the culture media (Oliveira *et al*. 2000; Feitosa *et al*. 2007; Fan *et al*. 2011). According to this premise, an expectation is that varieties with greater genetic similarity can develop in the same culture medium while varieties with smaller genetic similarity should require different culture media. This hypothesis was tested beside the present study in order to establish a protocol for the in vitro generation of three new cassava cultivars (Fécula Branca, Olho Junto, and IPR União) indexed for CsCMV virus. The Fécula Branca, Olho Junto, and IPR União are high-yielding cassava cultivars of commercial and industrial interest.

The molecular marker based on retrotransposons LTRs (Kalendar *et al*., 1999) was employed to investigate the genetic relationship among the three cassava cultivars. The Inter-retrotransposon amplified polymorphism (IRAP) and the Retrotransposon-micro-satellite amplified polymorphism (REMAP) markers are abundant in the cassava genome and produce a high number of reproducible DNA segments (Kuhn *et al*., 2016). It may be possible that the meristem-tip culture of cassava cultivars showing high genetic similarity may be established in culture medium with the same supplements and that the IRAP and REMAP markers may be useful for preliminary planning of the assays for in vitro propagation of new varieties of cassava.

MATERIALS AND METHODS

Selection of cassava varieties

In current study, the cassava varieties Fécula Branca, IPR-União, and Olho Junto were obtained from the cassava germplasm bank maintained at the IAPAR (Agronomic Institute of Paraná, Brazil). Fresh stem cuttings measuring 20-30 cm, with 5-8 nodes, were cultivated in plastic trays and kept under greenhouse conditions at the Universidade Estadual de Maringá, Maringá PR Brazil. Cassava leaves were harvested from plants in the greenhouse (Figure 1) and submitted to PTA-ELISA analysis prior to in vitro propagation. The CsCMV virus is widespread in the cassava and was detected in all plants tested. Consequently all parental plants were infected by the virus and clonal cleaning was required. The shoots harvested from the plants with the lowest virus index tested by PTA-ELISA were used for in vitro propagation (Figure 1B and C). Two plants of each Fécula Branca (FB1 and FB2) Olho Junto (OJ1 and OJ2) variety and one plant of IPR-União with the lowest virus index tested by PTA-ELISA were used for analysis of the IRAP and REMAP markers and to in vitro propagation.

IRAP and REMAP amplification

Genomic DNA obtained of leaves from the parental varieties used for clone regeneration in vitro was isolated with CTAB (cetyltrimethylammonium bromide) protocol (Knapp and Chandlee 1996), adapted by Carvalho (Cenargen – Embrapa). The genomic DNA was dissolved with TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and quantified with picodrop (Pico200 spectrophotometer). PCR reactions were performed in 25 µL volume: 30 ng of DNA, 1xPCR buffer (75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 4 pmol of each primer, 100 µL dNTP, and 1 U of PrimeSTAR DNA polymerase (Takara Bio, Tokyo, Japan). Amplification was performed in a MJ Research PTC-200 PCR Peltier Thermal Cycler (Bio-Rad, Hercules, USA). After 10 min of initial denaturation at 95 °C, amplifications were carried out in 36 cycles at 94 °C for 30 sec; at 55 °C for 30 sec and at 72 °C for 3 min, with a final extension of 10 min at 72 °C. The PCR products were identify by electrophoresis with 3% agarose (ECOGEN, AG-0600, Madrid, Spain) in 1xTAE buffer followed by EtBr staining and UV visualization. PCRs were repeated three times with similar results. The primer combinations (AYF8, AYF9, AYF2 x AYF3, AYF3 x AYF8, AYF5 x AYF9, AYF4 x SSR5) designed and tested by Kuhn *et al*. (2016), were used for DNA amplification. The amplified DNA segments were analyzed by comparing IRAP and REMAP profiles of each plant in terms of presence or absence of each DNA segment. Plants' similarity was calculated by Jaccard's coefficient, while UPGMA cluster analysis was performed with NTSYS-pc software (Rohlf 1989).

Culture establishment

Shoot tips (2.0 cm) were collected from the cassava plants and washed in distilled water with two drops of Tween 20 for 10 minutes. Sterilization was subsequently carried out in a laminar airflow cabinet under aseptic conditions, or rather, the shoots were sterilized by immersion in alcohol 50% for 1 minute, followed by 0.5% calcium hypochlorite for 5 minutes, and cleaning in sterilized water for three times during 2 minutes. Meristem-tips with no leaf primordial (<0.4 mm) were aseptically dissected and transferred to test tubes (15 x 2.5 cm) containing 10 ml of solidified MS medium, supplemented with different concentrations of grown regulator (Table 1); the pH of all media was adjusted to 5.8 before adding agar and autoclaved for 15 min at 121 °C and 1.2 kPa. The tubes with explants were incubated at 26 ± 2 ºC within a 16-hour photoperiod provided by white fluorescent lamps (25.3 µmol m^{-2} s^{-1}). Each experiment consisted of 10 explants (meristem tip) and the data were recorded three weeks after the beginning of the experiments. Data were analyzed according to the development and mortality rate of seedlings. Growth was analyzed 60 days after inoculation. The meristem tip provided plantlets in vitro which were divided into 2 at 4 pieces (with

side shoots) for fast multiplication. One mother plant generated between 5 and 15 cloned plants in each subculture, and each meristem tip was sub-cultured 5 times. After the five subcultures, the plantlets generated were transplanted to plastic cups with substrate Plant Max covered by cups to maintain humidity for 1 week. They were then taken to the green house and the cup was gradually removed, following Souza *et al*. (2008). Ten regenerated plantlets were randomly selected for each cultivar and indexed for CsCMV virus.

PTA-ELISA virus detection

Young cassava leaves were harvested from plantlets grown from meristem-tip culture after 5 sub-culture cycles (plus an additional 60 days in greenhouse acclimatization) and 10 random plantlets for each line cultivated in vitro were tested. Conditions for PTA-ELISA analysis followed Mowat and Dawson (1987). Each sample was tested in triplicate wells of a polystyrene microtiter plate. Extracts from healthy cassava cv. (IAC 12) plants were used as negative controls, whereas extracts from an infected cassava cv. Baianinha plant was used as positive control. Tests were considered positive when the absorbance (A405) rate of each sample was at least two times greater than that of the respective healthy control plant.

IC-RT-PCR detection

After the PTA-ELISA, the virus-free plants were also tested by IC-RT-PCR, a more sensitive method than ELISA. Immunocapture of CsCMV virions was performed according to protocol by Silva *et al*. (2011) with 50 µL of virus-specific IgG, incubated for 180 min at 37 ^{o}C and subsequently washed three times with PBS-Tween. Tubes were filled with 50 µL of extract and incubated at 6 ^{o}C overnight, followed by two washes with PBS-Tween and one wash with DEPC-treated water prior to RT-PCR. Complementary DNA (cDNA) was prepared in a final volume of 20 µL. Tubes were filled with 1 µL of random primers (500 ng), 2 µL of dNTPmix (10 mM) and 3 µL of DEPC-treated water, heated at 65 °C for 5 min and immediately chilled on ice. A mixture of 4 µL of 5× first strand buffer, 2 µL of DTT (0.1M), 1 µL of RNAse OUT (40 U) and 200 U of M-MLV were added to the tubes. They were heated at 25 °C for 10 min and then at 42 °C for 50 min. For the PCR, 5 µL of the cDNA was placed in a new tube, followed by 5 µL of 10× PCR Buffer (200 mM) Tris-HCl (pH 8.4), 500 mM KCl], 3 µL of $MgCl_2$ (50 mM), 2 µL of dNTP mix (10 mM), 2 µL of each primer (10 µM), 1 µL of *Taq* DNA polymerase (5 U) and 30 µL of DEPC treated water. The primer set used in this assay is universally used for members of the genus *Potexvirus* (Gibbs *et al*. 1998).

RESULTS

Genetic relationship among the parental plants of the three cassava cultivars propagated in vitro

The primers combinations (AYF8, AYF9, AYF2 x AYF3, AYF3 x AYF8, AYF5 x AYF9, AYF4 x SSR5) amplified a total of 72 DNA segments of which 43 (59.72%) were polymorphic in parental plants of the three cassava cultivars Fécula Branca (FB1, FB2), IPR-União (IPRU), and Olho Junto (OJ1 and OJ2) propagated in vitro (Table 2). The size of amplified products ranged between 115 pb in the AYF5 x AYF9 primer and 2.5 kb in the AYF2 x AYF3 primer. The primer AYF2 x AYF3 amplified the highest number (16) of DNA segments with 37% of polymorphism. The highest polymorphism level (76.92%) was shown by the primer AYF9. The dendrogram by Jaccard coefficient (Figure 2) showed that the molecular marker IRAP and REMAP analysis divided the parental cassava plants into two groups. The first group was formed by the Fécula Branca 1 (FB1), Fécula Branca 2 (FB2) and IPR-União (IPRU) plants, while the second group was formed by the Olho Junto 1 (OJ1) and Olho Junto 2 (OJ2) plants. The highest similarity coefficient (0.8769) was observed between FB1 and FB2 plants while the lowest similarity coefficient (0.6176) was observed between OJ2 and FB1 plants. A low similarity coefficient (0.6799) was also observed between OJ1 and OJ2 plants. The similarity coefficient (0.7166) between IPRU and OJ2 plants was higher than the similarity between OJ1 and OJ2 (0.6779) and the similarity coefficient in OJ1 and FB1 and FB2 was also low (0.6567).

In vitro cassava propagation

After 60 days the meristems cultivated in the culture media C1 (0.4 mg · L^{-1} of 6BA, 0.2 mg · L^{-1} of NAA and 3% of sucrose) and C2 (0.4 mg · L^{-1} of 6BA, 0.2 mg · L^{-1} of IBA and 3% of sucrose) generated callus with no regeneration of plantlets. In the culture media C3 (0.2 mg · L^{-1} of 6BA, 0.1 mg · L^{-1} of NAA and 3% of sucrose), C4 (0.2 mg · L^{-1} of 6BA, 0.1 mg · L^{-1} of IBA and 3% of sucrose), C5 (0.04 mg · L^{-1} of 6BA, 0.02 mg · L^{-1} of NAA and 3% of sucrose), and C6 (0.04 mg · L^{-1} of 6BA, 0.02 mg·L^{-1} of IBA and 3% of sucrose) most regeneration plantlets die, whilst those that did not die had their growth impaired and presented a yellow color. In the culture media C7 (0.04 mg · L^{-1} of 6BA, 0.02 mg · L^{-1} of NAA, 3% of sucrose, and 0.05 mg · L^{-1} of GA_3) and C8 (0.04 mg · L^{-1} of 6BA, 0.02 mg · L^{-1} of IBA, 3% of sucrose, and 0.05 mg · L^{-1} of GA_3), death rate was low (<30% and <20%, respectively). The plantlets of the Fécula Branca varieties showed satisfactory growth in the C8 medium, whereas variety IPR União grew in the C7 medium and variety Olho Junto showed low growth in C7 and C8 culture media (Table 3). The plantlets grown in the culture media C7 and C8 were then transferred to culture medium with a higher concentration of cytokinin and auxins: 0.2 mg · L^{-1} of 6BA and 0.1mg · L^{-1} of NAA in the culture medium $C9^7$, and 0.2 mg · L^{-1} of 6BA and 0.1 mg · L^{-1} IBA in the culture medium $C10^8$, where the seedlings were kept growing for a further 30 days.

Although the culture media C7 and C8 were efficient to develop cassava meristems, higher concentrations of cytokinin and auxins in $C9^7$ and $C10^8$ culture media were needed to complete the in vitro development of seedling from the Fécula Branca, IPR-União and Olho Junto varieties. GA_3 had to be added in C7 and C8 culture media for the efficient development of the meristems. It was also efficient to the growth of the Fécula Branca, IPR-União and Olho Junto seedlings in $C9^7$ and $C10^8$ culture media since the concentration of GA_3 (0.05 mg · L^{-1}) was maintained in the two culture media $C9^7$ and $C10^8$ (Figure 3). The culture media $C9^7$ and $C10^8$ with their higher concentration of auxin and cytokinin than the culture media C7 and C8 provided a fast development, shoot elongation and root formation (Figure 4). Rapid root induction and elongation is one of the most important steps toward fast and successful production of *in vitro* regenerated shoots. The presence of roots in the cassava seedlings, proportional to the development of the aerial parts, is beneficial to multiplication; they provide the absorption of

nutrient and the production of shoots which will be used as explants in the sub-cultures (Oliveira *et al*., 2000). After the establishment of the cassava development protocol, two plantlets of Fécula Branca (FB1 and FB2) obtained from $C10^8$ culture medium, one plantlet of IPR União from $C9^7$, one plantlet of Olho Junto (OJ1) from $C9^7$ and another plantlet of Olho Junto (OJ2) from $C10^8$ were chosen and sub-cultivated (cut perpendicular to the midrib) into three sections: lower, middle and upper, with a shoot in each explant. The side shoots were also cut and each section was considered an explant. The plantlets were sub-cultured five times at every 40 days in their respective $C9^7$ and $C10^8$ culture media. After the five subcultures, 15 cloned plants were obtained from each FB1, FB2, IPRU, OJ1, and OJ2 plantlets.

Generated cloned plants were transplanted to plastic cups with substrate, covered by cups to maintain humidity for 1 week; they were then taken to the greenhouse and the cup was gradually removed (Figure 5). The regeneration capacity to survive under field conditions is important as it determines the success of in vitro propagation. The regenerated plants were successfully acclimated with a high survival rate (Table 3).
Rooted plants were acclimatized successfully in the greenhouse, with a high survival rate and no observable morphological aberrations. The development of each meristem of the Fécula Branca variety in $C8/C10^8$ culture media generated 14-15 acclimated cassava plants after five subcultures in $C10^8$ culture medium. Further, the development of each meristem of the IPR-União variety in $C7/C9^7$ culture media generated 13 acclimated cassava plants after five subcultures in $C9^7$ culture medium. Perspectives for the Fécula Branca variety propagated in the $C8/C10^8$—culture media established in current study comprised the generation of 140-150 clones from one plant with 10 apical meristems, while the perspective for the IPR-União variety propagated in $C7/C9^7$culture media is the generation of approximately 130 clones. Similar reasoning and perspectives may be applied to the Olho Junto variety. Thus, it is possible to establish the clonal propagation of the Fecula Branca, IPR-União and Olho Junto varieties at a less than 12-month period and at a more than 10-fold proportion by employing the culture media developed in current study.

Virus indexing by PTA-ELISA and IC-RT-PCR

Leaves of 10 randomly selected plants were used for the PTA-ELISA assay after successful acclimatization. The cassava meristem-tips were inoculated with <0.5mm and PTA-ELISA index showed a low index virus-free regenerated plants (Figure 6). Only one of the plantlets was virus free in the Fécula Branca 1 samples. The Fécula Branca 2 did not have any virus-free plant. Whereas the Olho Junto 1 samples showed four virus-free plants by the PTA-ELISA assay, the Olho Junto 2 had only one virus-free plantlet. The IPR–União samples had the highest rate of clonal cleaning, with nine virus-free plants by PTA-ELISA indexing. Since the PTA-ELISA was not efficient to detect low virus levels, the samples were also analyzed according to IC-RT-PCR protocol developed by Silva *et al*. (2011) to increase the sensitiveness in the CsCMV virus detection (Table 4). IC-RT-PCR revealed that some plants identified as virus-free by ELISA were not clean. Although four plants in the Olho Junto 1 cultivar were virus-free by PTA-ELISA, two were indexed as infected by IC-RT-PCR. Two plants in the IPR-União cultivar were also false negative and demonstrated that IC-RT-PCR indexing was more sensitive than PTA-ELISA. Results showed that most plants generated by *in vitro* culture have CMV virus infection. The technique was efficient for viral cleaning only in the case of the cassava cultivar IPR-União. In the IPR-União cultivar, 70% of the meristem-tip cultures were successful cloned and generated virus-free plants indexed by PTA-ELISA and IC-RT-PCR methods.

The viral cleaning in meristem-tips of cassava also seems to be related with the level of viral infection in the parental plants used in obtaining the explants. This is due to the fact that a lower level of viral infection was detected in the parental IPR-União than that of levels of viral infection in parental plants of the Fécula Branca and Olho Junto varieties prior to *in vitro* propagation (Figure 1).

Figure 1. A: Cassava plants in the greenhouse. B: Cassava stem. C: Shoot tips

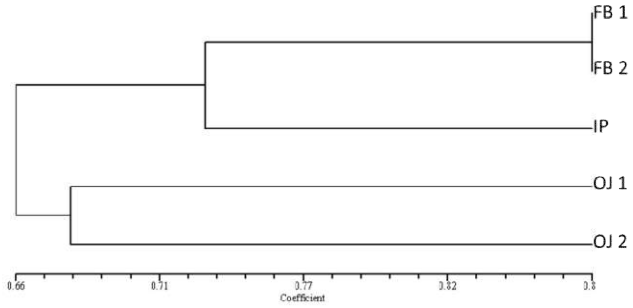

Figure 2. Dendrogram from the parental plants, using molecular marker based on retrotransposon (IRAP and REMAP). FB 1 (Fécula Branca 1), FB 2 (Fécula Branca 2), IP (IPR-União), OJ 1 (Olho Junto 1), OJ 2 (Olho Junto 2)

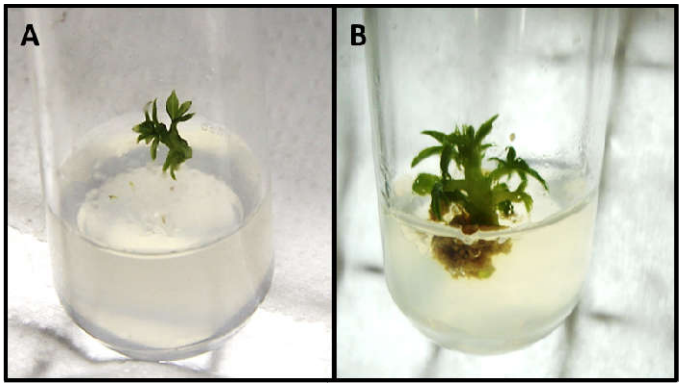

Figure 3. Meristem tip development of the cassava cultivar IPR-União. A: plantlet generated in C7 culture medium after 60 days. B: Plantlet transferred to $C9^7$ culture medium after 30 days growth

Figure 4. Growth of cassava plantlets in culture media C9[7] and C10[8] provided A: shoot elongation and B: root formation

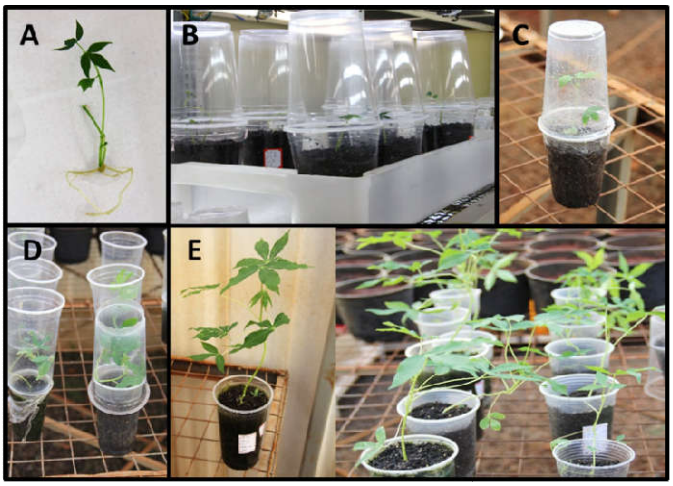

Figure 5. A: Clean plantlet ready for acclimatization. B: Plantlets covered by plastic cup. C: Plantlets in the greenhouse. D: Plastic cup was removed gradually. E: successfully acclimated plant in the greenhouse

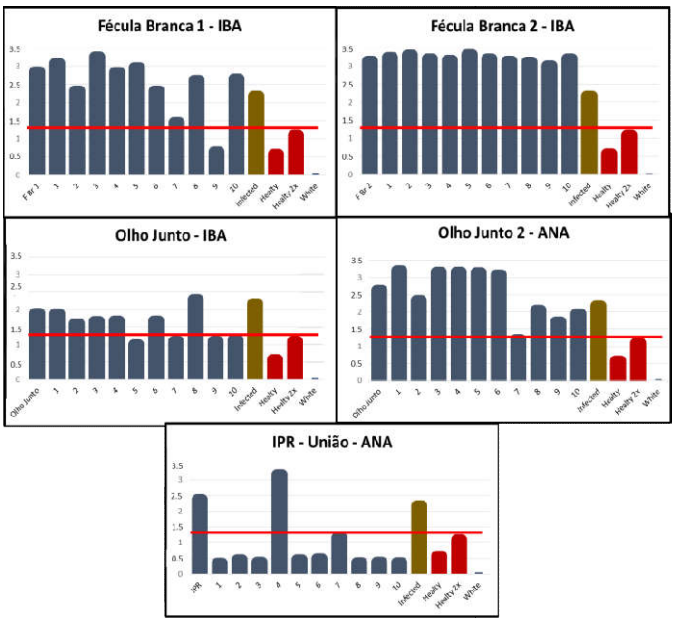

Figure 6. PTA-Elisa assay of the acclimatized cassava plants. Each graph represents the mother plant and 10 cloned plants (1-10). The red line represents the minimum value to be considered virus free

Table 1. MS medium (Murashige and Skoog, 1962), supplemented with different concentrations (mg · L^{-1}) of 6-Benzylaminopurine (6-BA), Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), Gibberellic acid (GA_3) and sucrose (%) for the establishment of cassava tissue culture

Medium	6BA	NAA	IBA	GA_3	Sucrose
C1	0.4	0.2	-	-	3%
C2	0.4	-	0.2	-	3%
C3	0.2	0.1	-	-	3%
C4	0.2	-	0.1	-	3%
C5	0.04	0.02	-	-	3%
C6	0.04	-	0.02	-	3%
C7	0.04	0.02	-	0.05	3%
C8	0.04	-	0.02	0.05	3%
C9[7]	0.2	0.1	-	0.05	2%
C10[8]	0.2	-	0.1	0.05	2%

C9[7]: seedlings formed on C7 [which have a lower concentration of 6BA (0.04 mg · L^{-1}) and NAA (0.02 mg · L^{-1}) to avoid callus formation] after 60 days were transferred to C9[7]; **C10[8]:** seedlings formed on C8 [which have a lower concentration of 6BA (0.04 mg · L^{-1}) and IBA (0.02 mg · L^{-1}) to avoid callus formation], after 60 days were transferred to C10[8].

Table 2. Polymorphism of markers IRAP and REMAP by the analysis of the cassava cultivars Fécula Branca 1, Fécula Branca 2, IPR-União, Olho Junto1 and Olho Junto2

Primer	TNS	PS	%PS
AYF8	11	8	72.72%
AYF2 x AYF3	16	11	68.75%
AYF5 x AYF9	11	4	36.36%
AYF3 x AYF8	12	6	50.00%
AYF4 x SSR5	9	4	44.44%
AYF9	13	10	76.92%
Total	72	43	59.72%

TNS: Total number of amplified segments; PS: polymorphic DNA segments

Table 3. Growth of plantlets from the different cassava cultivars in different culture media. R: Regenerated plantlets; C: Callus formation

	Cultivars		
Culture medium	Fécula Branca	IPR União	Olho Junto
C1	100% C	80% C	100% C
C2	100% C	90% C	100% C
C3	70% R / 0% C	80% R / 0% C	90% R / 40% C
C4	80% R	80% R	80% R
C5	60% R	80% R	30% R
C6	50% R	60% R	20% R
C7	70% R	100% R	90% R
C8	100% R	80% R	100% R
C9[7]	--	100% R	100% R
C10[8]	100 % R	--	100% R

Table 4. Acclimatization of cassava plants generated from *in vitro* culture

	Acclimatized plants	Survived plants	Survival rate
Fécula Branca 1*	15	15	100%
Fécula Branca 2*	15	14	93.3%
Olho Junto 1*	15	13	86.6%
Olho Junto 2**	15	14	93.3%
IPR – União**	15	13	86.6%

*Plantlets cultivated in culture medium with IBA.
**Plantlets cultivated on culture medium with NAA.

Table 5. Virus-free plants of the cassava cultivars

Cultivar*	PTA-ELISA	IC-RT-PCR
Fécula Branca 1 (IBA)	10%	10%
Fécula Branca 2 (IBA)	0%	0%
Olho Junto 1 (IBA)	40%	20%
Olho Junto 2 (IBA)	10%	10%
IPR União (NAA)	90%	70%

*Exclusive analysis of plants generated by in vitro tissue culture.

DISCUSSION

The meristem-tip culture of cassava cultivars showing the highest genetic similarity revealed by the IRAP and REMAP markers was established in culture medium with the same supplements according our preliminary hypothesis. The FB1 and FB2 plants showing the highest genetic similarity (0.8769) generated cassava plantlets in culture medium supplemented with IBA, while the OJ2 and FB1 plants showing the lowest similarity coefficient (0.6176) generated plantlets in culture medium containing different auxin types: NAA (OJ2) and IBA (FB1). Use of the IRAP and REMAP markers was also important to justify that plants from the same variety (OJ1 and OJ2) showing low genetic similarity (0.6799) are generate in medium supplemented with different auxin types: IBA (OJ1) and NAA (OJ2). This way, the genetic variability within a variety may determine their development in the culture medium with one or more growth regulators. The varieties OJ1 and FB2 with low genetic similarity were generated in culture medium containing the same growth regulator (NAA) indicating that additional factors beyond the genotype may be related with the in vitro response of explants. The premise is that different genotypes of cassava may have different developments in the culture media (Oliveira *et al*., 2000; Feitosa *et al*., 2007; Fan *et al*., 2011), but in fact when an explant is inoculated into a culture medium, the responses to exogenous growth regulators depend to a great extent on the physiological status (level of endogenous hormones) of the donor plant (Tang *et al*., 2008). Our study is the first to show that plants with greater genetic similarity reveled by molecular markers (IRAP and REMAP) can develop in the same culture medium. It is important now to extend this assessment and investigating a larger number of varieties, considering also the genetic diversity within each variety in order to strengthen our preliminary evidences. The IRAP and REMAP may be indicated to assessed the genetic diversity within and inter cassava varieties. The IRAP and REMAP markers system have been used for studying the genetic diversity and structure genetics of populations in helianthus (Vukich *et al*., 2009), flax (Smýkal *et al*., 2011) and maize (Kuhn *et al*., 2014), while a high genetic diversity was also observed in the cassava varieties analyzed in current study. Use of different composition and concentration of plant growth regulators in the culture medium showed that minor changes after the inoculation are important factors to determine growth and development of the cassava meristem tip from the Fécula Branca, IPR-União and Olho Junto varieties. The transference of the explants after inoculation to the culture media with higher concentration of auxin and cytokinin provided a fast development, shoot elongation, and root formation. An elaborate intermediary strategy was need. GA_3 besides the different concentrations of auxin had an effective role in the organogenesis of the cassava meristem tip development. Gibberellins´ main effect is the stimulation of the growth of organs already formed. Oliveira *et al*. (2000) and Souza *et al*. (2008) also used 0.05 mg $\cdot$ L^{-1} of GA_3 to obtain a good development of roots and aerial part of cassava plantlets.

Despite the low rate of virus-free plants obtained after the establishment of clones of the three cassava varieties, the experiments in current study were important to show that the immunocapture-RT-PCR protocol is a more sensitive protocol than ELISA alone. Only 10% (FB1 and OJ2) - 70% (IPR-União) of the meristem-tip cultures were successful cloned and generated virus-free plants indexed by PTA-ELISA and IC-RT-PCR methods. In clones of OJ2 20% of the micropropagated plants were virus-free plants indexed by IC-RT-PCR methods. An increase in rate of microprogated plants free of virus in the culture media here established will be achieved by inoculation of smaller meristem-tips. As expected, the micropropagation of new varieties of cassava required culture media with different combinations and concentrations of growth regulators and also required specific and elaborate steps. So that the use of IRAP and REMAP markers to estimate the genetic similarity coefficient between varieties may be indicated as a preliminary strategy to program the in vitro cultivation of the genotypes with greater genetic similarity in media supplemented with the same combination of growth regulators. The possibility of making a plan for the cloning of different varieties reducing the number of experimental tests in vitro is a strategy that saves time and capital.

Acknowledgments

The authors would like to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasília DF Brazil) for its financial support.

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