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In vitro regeneration of shoot segments of *Manihot esculenta* varieties cultivated in northern Brazil

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ABSTRACT

The objective of this work was to evaluate the *in vitro* establishment of BRS Kiriris, BRS Poti, Água Morna, Paraná and Jurará of *Manihot esculenta* varieties. For this purpose, the shoot apices were inoculated in test tubes containing five mL of MS medium, supplemented with 0.02 mg/L naphthaleneacetic acid (NAA), 0.04 mg/L 6- benzylaminopurine (BAP) and 0.05 mg/L gibberellic acid (GA_3). The experimental design was completely randomized (CRD), with five varieties and six replicates, where each replicate consisted of five tubes containing one explant, totaling 30 explants per treatment. The number of explants contaminated by fungus and/or bacteria and necrosis were evaluated every seven days. After 30 days of *in vitro* culture, the following variables were evaluated: survival, mortality, seedling height, number of green leaves, number of roots and length of the largest root. The data concerning the analyzed variables were transformed to √(x + 0.5) and then subjected to Analysis of Variance. The averages were compared using the Tukey test at 5% probability using the statistical program Sisvar 5.6. According to the results obtained, the BRS Poti variety obtained lower contamination rate and greater *in vitro* survival in relation to the other varieties. The Jurará variety showed the best results in the development of aerial part and root system *in vitro*. On the other hand, the Paraná variety did not obtain satisfactory results for the *in vitro* establishment.

Index terms: Cassava; growth regulators; i*n vitro* culture.

INTRODUCTION

Cassava (*Manihot esculenta*) is a species that presents several advantages over other agricultural crops, such as the use of leaves, stems and roots, as well as being the second world raw material for starch (Buhari, 2017; Vilpoux, 2008). In Brazil, the northern region accounts for 43% of cassava production, with Pará state being the largest national producer, with over 6 million tons of the roots in 2016 and grow in 90% of rural properties in the state of Pará (Instituto Brasileiro de Geografia e Estatística - IBGE, 2018; Modesto Júnior; Alves, 2016).

However, its productivity is declining over the years, due to the insufficient availability of material for planting, caused by the physiological aging, caused by constant multiplication and climatic factors, such as drought. Associated with this is the lack of agronomic practices in pest and disease control (Mattos et al., 2000; Silva; Cereda; Fiorini, 2002). In the western region of Pará, root rot caused by the fungus *Fusarium solani* has been registered since 2002, affecting most of the cassava crops and, consequently, affecting crop productivity (Poltroniere et al., 2002).

The method most often used for the vegetative propagation of Manihot esculenta consists of cuttings or staves of the mother plant, but this system often results in the transmission of pests and diseases to the following generations, a fact that affects the crop yield (Cerqueira; Faria; Santos, 2016). Thus, the culture of plant tissues, being a set of methods of *in vitro* cultivation of cells, tissues and organs, controlled nutritional and environmental conditions (Hussain et al., 2012), through *in vitro* micropropagation presents several desirable advantages such as, high rates of multiplication in a short period of time, production of disease-free material and the homogeneity of its growth (Bhalang et al., 2018).

However, the great genetic variability of cassava presents different morphogenetic responses *in vitro*, both for nutritional demands and for different cultivation conditions. Therefore, this work proposes to evaluate the *in vitro* establishment of BRS Kiriris, BRS Poti, Água Morna, Paraná and Jurará of *M. esculenta* varieties.

MATERIAL AND METHODS

For the present study, staves were selected from vigorous cuttings and apparently without diseases, through morphological observation, aged 10 months.

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These were cut with 10 to 15 cm from the soil surface and then taken to the Laboratory of Micropropagation of Plants *In Vitro*, from the Federal University of Western Pará (UFOPA) in partnership with the Federal Institute of Education, Science and Technology do Pará (IFPA) in the first half of 2018.

The wells were segmented with a hand saw on cuttings from 15 to 20 cm in length. Then, these were treated in solution with sodium hypochlorite, at a concentration of 1.25% (active chlorine content between 2.0 to 2.5% w / w in 1L) in 3L of water, with immersion for 5 minutes. After treated, the cuttings were planted in plastic trays, containing coconut fiber (SoCoco) and commercial substrate (Tropstrato®), in the proportion of 1: 1. The trays were stored in a nursery under a 70% screen for a period of 20 days, being irrigated daily.

After this period, the shoot apices presented suitable for *in vitro* cultivation, which were removed with the aid of a scalpel blade, at a size of approximately 1.5 to 2.0 cm. The apexes collected were taken to the laboratory and, in a laminar flow chamber, the explants were cleaned. For this, the apexes were placed into lints and submerged in 70% alcohol for one minute. Soon after, they were immersed in autoclaved distilled water for 15 seconds and then in commercial sodium hypochlorite solution (Qboa®) at 0.25% (v/v) for three minutes. They were then washed in autoclaved distilled water for three consecutive times, for approximately 15 seconds (Souza et al., 2013).

After asepsis, a gradual elimination of the leaves and stipules was carried out with the aid of a stereoscopic microscope, until it was possible to visualize the shoot apices, a bright structure of 0.3 to 0.5 cm in size, with 1-3 leaf primordial (Souza et al., 2013). The explants were then inoculated into test tubes (30x150 mm) containing 5 mL of MS culture medium (Murashige; Skoog, 1962 - Sigma-aldrich), previously autoclaved at 121°C for 20 minutes. The medium was supplemented with 20 g/L sucrose, 0.02 mg/L naphthaleneacetic acid (NAA), 0.04 mg/L 6-benzylaminopurine acid (BAP) and 0.05 mg/L gibberellic acid (GA_3), solidified with 2 g/L Phytagel® and pH adjusted to 5.7 ± 0.1 (Souza et al., 2013).

The flasks were kept in a growth room with illumination by cold white fluorescent lamps, with photon flux density of 30 µmol/m^2/s, photoperiod of 16 hours and temperature of 27 ± 2 °C, where they remained for 30 days. The evaluations were carried out every seven days after inoculation *in vitro*, when the variables number of explants contaminated by fungus and/or bacteria and necrosis were observed. Regarding the necrosis variable, we considered those explants that presented total oxidation.

After 30 days of *in vitro* cultivation, the final evaluation of the experiment was performed, when variables such as survival, mortality, seedling height, number of green leaves (NGL), number of roots (NR) and length of the largest root (LR – cm) were analyzed. Seedlings height and root length were measured with the aid of a pachymeter, from the sprout base to the insertion of the youngest leaf and the sprout base to the end of the highest root, respectively.

The experimental design used was completely randomized (DIC) for the variables seedling height, number of green leaves, number of roots and length of the largest root, with 5 varieties and 6 repetitions, where each repetition consisted of five tubes containing an explant, totaling 30 explants per treatment. Contamination, survival and mortality rates were assessed by percentage. The data were subjected to the normality test with subsequent transformation of the data to $\sqrt{(x + 0.5)}$ for the variables number of green leaves, number of roots and root lengths, and then they were subjected to Analysis of Variance (ANOVA) and the averages were compared using the Tukey test at 5% probability, using the statistical program SisVar 5.6 (Ferreira, 2011).

RESULTS AND DISCUSSION

According to the analysis of variance (Table 1), it was observed that there was a significant difference between the treatments for most of the variables in the *in vitro* establishment of *Manihot esculenta*.

In the evaluation of the percentage of contamination (Table 2), the lowest percentage was observed for the BRS Poti variety, which presented 13.13% of losses, in which 3.33% was caused by bacteria, 6.67% by fungi and 3.13% by death of the explants. BRS Poti also had the highest survival rate of the explants, 86.87%. On the other hand, the Paraná variety had the lowest survival rate, only 30%, due to contamination and death losses of the explants, which together added up to 70%. This fact

may be related not only to asepsis, but also to the great genetic variability among *in vitro* cultures, where varieties of the same species may have different requirements (Santos et al., 2015).

Table 1 – Analysis of variance, indicating degrees of freedom (DF), the coefficient of variation (CV) and the average squares (AS) of the variables height of the seedlings (HS- cm), number of green leaves (NGL), number of roots (NR) and length of the largest root (LR - cm), in the *in vitro* establishment of different varieties of *Manihot esculenta*.

Source of variation	DF	AS			
	HS (cm)	NGL	NR	LR (cm)	
Treatments	4	0.00*	0.00*	0.00*	0.00*
Error	25	0.24	0.09	0.07	0.09
Total	29				
Overall Average		1.88	1.85	1.36	1.35

* significant by the Tukey test at 5% probability.

ns not significant.

Table 2 – Percentage of fungal and bacterial contamination, survival and mortality of explants of different varieties of *Manihot esculenta* after 30 days of *in vitro* establishment.

	Contamination			
	Fungi	Bacteria	Survival	Mortality
Cultivars	(%)	(%)	(%)	(%)
Paraná	6.67	43.33	30.00	20.00
Jurará	3.33	16.67	66.67	13.33
BRS Kiriris	6.67	10.00	73.33	10.00
Água Morna	6.67	6.67	70.00	16.66
BRS Poti	6.67	3.33	86.87	3.13

Indexes of up to 15% of contamination are considered normal for the *in vitro* establishment phase (Oliveira et al., 1997), so data obtained from the Jurará, Água Morna and BRS Poti varieties are within this normality. The contamination index depends on the age and type of the explant, the concentration of the sterilant solution and the exposure time of the explant to the solution, making it necessary to adapt the protocol to each culture (Ferreira; Santos; Bragado, 2009). In addition, explants extracted from matrices originating in the field tend to present higher levels of contamination (Santos; Chagas; Guimarães, 2015). For the varieties analyzed in this work, besides having origin in the field, it should be considered that a pre-treatment was not performed to aid in asepsis.

Plant height is an important variable because it is directly related to the number of nodes that will be recovered in new germinations (Moreira et al., 2003), allowing efficiency in the subsequent multiplication phase, due to a good response to the culture medium and the conditions set in the establishment. For this, the Jurará, BRS Poti and BRS Kiriris varieties obtained the best means, but did not differ significantly from each other or the Água Morna variety when compared to the Paraná variety. Jurará (2.30 cm), BRS Poti (2.21 cm) and BRS Kiriris (2.11 cm) varieties showed better adaptation to the culture medium and to the growing conditions, which provided the greatest development of the aerial part. The same did not happen with the Paraná variety, presenting a inferior result compared to the other (Table 3).

Table 3 – Average values for height (HS – cm), number of green leaves (NGL), number of roots (NR) and length of roots (LR – cm) of different varieties of *Manihot esculenta*, after 30 days of *in vitro* establishment.

Varieties	HS	NGL	NR	LR
Paraná	1.15 b	1.51 b	0.22 c	0.35 c
Jurará	2.30 a	4.29 a	3.02 a	2.55 a
BRS Kiriris	2.11 a	3.55 a	1.40 b	1.27 abc
Água Morna	1.61 ab	2.66 ab	1.03 bc	0.99 bc
BRS Poti	2.21 a	3.44 a	2.02 ab	2.39 ab

Means followed by the same lowercase letters in the column do not differ by the Tukey test at 5% probability.

Sesay et al. (2018), in their work with three varieties of cassava (Slicass 6, Slicass 11 and Cocoa), used as one of their treatments the interaction of BAP, NAA and GA_3 at concentrations of 0.01 mg/L, 1.5 mg/L and 1.0 mg/L, respectively, obtaining an average of 2.13 cm for height of the seedlings, superior result when compared to the treatment that contained only 0.05 mg/L BAP (1.11 cm). Different results were found by Mapayi et al. (2013), where MS medium in the absence of growth regulators showed better growth for three cassava genotypes evaluated

for micropropagation. In this way, it is observed that there is a great variation in the *in vitro* response and in the requirement of growth regulators among cassava varieties.

For the variable number of green leaves, the varieties that stood out the most were Jurará (4.29), BRS Kiriris (3.55) and BRS Poti (3.44), which did not differ among themselves nor with the Água Morna variety (2.66). However, they were superior to the Paraná variety, with 1.51 leaves.

Wasswa, Alicai and Mukasa (2010), in the *in vitro* establishment of cassava, analyzing different concentrations of BAP and dichlorophenoxyacetic acid (2.4-D), found that the absence of plant growth regulators could provide the highest number of leaves, with 4.82. Larkin and Scowcroft (1981) reported that sufficient amounts of the growth regulator can be produced naturally by the explants, leading to the dispensability of supplementation. The number of leaves formed is an essential parameter for the seedling, being important for the acclimatization phase as well as for the best development in the previous stages of *in vitro* propagation, as the leaves are the main source of photosynthesis, being of great importance in strengthening of *ex vitro* plants (Bell; Bryan, 1993). Thus, there was a good *in vitro* development of the varieties studied in this work.

Regarding the number of roots, the Jurará variety stood out in comparison to other varieties, with an average of 3.02 roots per seedlings, except for the BRS Poti variety (2.02) that did not presented a significant difference. On the other hand, the Paraná variety had the lowest mean for number of roots. In the cassava crop, a rooting of the seedlings is important because stronger and longer roots promote greater absorption of nutrients in the medium, stimulating the survival of the plant for longer periods (Shiji et al., 2014). Yandia et al. (2018), when analyzing the effect of the culture medium on the *in vitro* rooting of four cassava cultivars for *in vitro* propagation, observed that the response of each cultivar varied according to the culture medium used (Six-mois, M61/061, Yalipe and Rendre). This demonstrates the effect of the genotype on *in vitro* culture, also verified in this work.

For the root length variable, the varieties that presented the best means were Jurará (2.55 cm), BRS Poti (2.39 cm) and BRS Kiriris (1.27 cm). The Água Morna (0.99 cm) and Paraná (0.35 cm) varieties, presented the lowest results. In micropropagation of cassava, the presence of roots in the plants in balanced quantity with the development of the aerial part is beneficial to promote greater absorption of nutrients and, consequently, greater production of nodal segments, which will serve as explants for the next subcultures (Oliveira; Silva, 1997).

In view of the above, the concentrations of growth regulators of 0.02 mg/L NAA, 0.04 mg/L BAP and 0.05 mg/L GA_3, in the culture medium established by the protocol of Embrapa for the micropropagation of cassava (Souza et al., 2013), promoted a adaptation of the varieties, regenerating green seedlings, with a number of leaves and a satisfactory rooting.

Therefore, the varied behavior in the *in vitro* development of cassava seedlings demonstrated the pronounced effect of the genotype of the varieties studied. The regeneration potential does not only depend on the composition of the culture medium and the different growth regulators, but also on the genotypic differences, which will directly influence the *in vitro* morphogenetic response of this culture.

CONCLUSIONS

The BRS Kiriris, BRS Poti, Paraná, Água Morna and Jurará de Manihot esculenta varieties were established *in vitro*, according to the methodology presented, but the BRS Poti variety had the lowest contamination rate and the highest survival rate. The Jurará and BRS Poti varieties showed the best development *in vitro*, both for the aerial part and for the root system. The Paraná variety did not obtain satisfactory results for the analyzed variables.

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