IN VITRO MULTIPLICATION OF CASSAVA VARIETIES[1]

Renato Fernando Menegazzo[2]*
Max Emerson Rickli[3]
André Werlang Menegazzo[4]
Ana Daniela Lopes[5]
Candida Elisa Manfio[6]
Jana Koefender[7]

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ABSTRACT: The implantation of a model of sustainable development for agriculture can happen with the contribution of the mandiocultura. But for this, culture needs to be strengthened. *In vitro* propagation is an instrument for this purpose. Micropropagation can provide growers with large quantities of vigorous and healthy cassava seedlings in a short time. The objective of this study was to evaluate the *in vitro* establishment of four varieties of cassava cultivated in the municipality of Colorado do Oeste, State of Rondônia, popularly known as Arara, Caturra, Cacau Vermelha and Roxinha. For that, an experiment was carried out in the Laboratory of *In Vitro* Cultivation at the Pole of Technological Innovation of the University of Cruz Alta (UNICRUZ), with a completely randomized design in a 4 x 2 factorial scheme, with 6 replications. The treatments consisted of explants grown in Murashige and Skoog (MS) medium without the presence of growth regulator and MS medium supplemented with of 6-benzylaminopurine (BAP). The results indicate that the mean contamination percentage of the explants was 47.19%, differing among the varieties. The best growth response in culture media, in the multiple comparison of means (Scott-Knott's test, 5%), was obtained with MS medium without BAP addition, with significant difference between varieties. Under the conditions of this experiment, it was evidenced that micropropagation is a viable tool for obtaining varieties of interest, with desired phytosanitary qualities, with varietal and large-scale authenticity.
KEYWORDS: *Manihot esculenta*. MS medium. Micropropagation. Nodal explants. Popular varieties.

MULTIPLICAÇÃO IN VITRO DE VARIEDADES DE MANDIOCA

RESUMO: A implantação de um modelo de desenvolvimento sustentável para a agricultura pode acontecer com a contribuição da mandiocultura. Mas para isso, a cultura precisa ser fortalecida. A propagação *in vitro* é um instrumento para este fim. A micropropagação pode proporcionar aos produtores grande quantidade de mudas de mandioca vigorosas e sadias em um curto espaço de tempo. O objetivo deste trabalho foi avaliar o estabelecimento *in vitro* de quatro variedades de mandioca cultivadas no município de Colorado do Oeste, Rondônia, popularmente conhecidas como Arara, Caturra, Cacau Vermelha e Roxinha. Para isso, foi realizado um experimento no Laboratório de Cultivo *In Vitro* no Polo de Inovação Tecnológica da Universidade de Cruz Alta (UNICRUZ), com delineamento inteiramente casualizado em esquema fatorial 4 x 2, com 6 repetições. Os tratamentos consistiram em explantes cultivados em meio Murashige e Skoog (MS) sem a presença de regulador de crescimento e meio MS suplementado com 6-benzilaminopurina (BAP). Os resultados indicam que a porcentagem média de contaminação dos explantes foi de 47,19%, diferindo entre as variedades. A melhor resposta de crescimento em meios de cultura, na comparação múltipla de médias (teste de *Scott-Knott*, 5%), foi obtida com meio MS sem adição de BAP, com diferença significativa entre as variedades. Nas condições deste experimento, ficou evidenciado que a micropropagação é uma ferramenta viável para obtenção de variedades de interesse, com qualidades fitossanitárias desejadas, com autenticidade varietal e em larga escala.
PALAVRAS-CHAVE: *Manihot esculenta*. Meio MS. Micropropagação. Explantes nodais. Variedades populares.

MULTIPLICACIÓN *IN VITRO* DE VARIEDADES DE MANDIOCA

RESUMEN: La implantación de un modelo de desarrollo sostenible para la agricultura puede suceder con el aporte de la mandiocultura. Pero, para eso, la cultura necesita ser fortalecida. La propagación *in vitro* es un instrumento para ese fin. La micropropagación puede proporcionar a los cultivadores grandes cantidades de plántulas de mandioca vigorosas y saludables en poco tiempo. El objetivo de esta investigación ha sido evaluar el establecimiento *in vitro* de cuatro variedades de mandiocas cultivadas en el municipio de Colorado del Oeste, Rondônia, popularmente conocidas como Arara, Caturra,

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[1]Article partially extracted from a Master dissertation.
[2]Institute of Education of Rondônia (IFRO), Highway BR 435, Km 63, Colorado do Oeste, Rondônia, 76993-000, Brasil. *Corresponding author (renato.menegazzo@ifro.edu.br)
[3]State Maringá University (UEM), Avenue Ângelo Moreira da Fonseca, 1800, Umuarama, Paraná, 87506-370, Brasil.
[4]Campina Grande Federal University (UFCG), Street Aprígio Veloso, 882, Campina Grande, Paraíba, 58429-900, Brasil.
[5]Biotechnology Applied to Agriculture Program of Paranaense University (UNIPAR)
[6]Agricultural Research and Rural Extension Company of Santa Catarina (EPAGRI), Highway Admar Gonzaga, 1347, Florianopolis, Santa Catarina, 88034-901, Brasil.
[7]Professional Master's Degree in Rural Development at the Cruz Alta University (UNICRUZ)

Cacau Vermelha y Roxinha. Para eso, se realizó un experimento en el Laboratorio de cultivo *in vitro* en el Polo de Innovación Tecnológica de la Universidad de Cruz Alta (UNICRUZ), con delineamiento completamente casualizado en esquema factorial 4 x 2, con 6 repeticiones. Los tratamientos consistieron en explantes cultivados en medio Murashige y Skoog (MS) sin la presencia de regulador de crecimiento y medio MS suplementado con 6-bencilaminopurina (BAP). Los resultados indican que el porcentaje de contaminación promedio de los explantes fue de 47.19%, diferenciándose entre las variedades. La mejor respuesta de crecimiento en medios de cultivo, en la comparación múltiple de medias (prueba de Scott-Knott, 5%), se obtuvo con medio MS sin adición de BAP, con una diferencia significativa entre las variedades. Bajo las condiciones de este experimento, se evidenció que la micropropagación es una herramienta viable para obtener variedades de interés, con cualidades fitosanitarias deseadas, con autenticidad varietal y de gran escala.
PALABRAS CLAVE: *Manihot esculenta*. Medio MS. Micropropagación. Explantes nodales. Variedades populares.

Introduction

In order to be competitive, agribusiness must have a sustainable technological base which allows the generation of products at affordable prices for consumers; be linked to food safety; respect the environment; and conform to socially just standards (BRASIL, 2009). Science is a key element in this, as is the technological innovation. Together, can provide the conditions for agribusiness to achieve the aforementioned attributes and guide the sustainability path (TARAPANOFF, 2016).

At present, the world population gradually builds up an ecological awareness that evidences the need to implement a sustainable agricultural development, capable of considering as a whole the economic, social and ecological factors (BARROS; SILVA, 2010; BRANDENBURG, 2005; GIORDANO, 1995; MACHADO, 2009). Cassava crop is an activity which can contribute to the implementation of this sustainable model, provided is not adopted as intensive monoculture, which would generate risks of outbreaks of pests and diseases and depletion of soil nutrients (SILVA; MARTINS, 2010).

Perennial heliophilous plant, belonging to the family *Euphorbiaceae*, cassava (*Manihot esculenta* Crantz), among all crops, is supported by scientific studies such as higher calorie productivity, greater biological efficiency as a producer of energy and better adaptation to soil deficient in nutrients. Its root and by-products are consumed by more than 800 million people worldwide (NASSAR, 2006).

The genetic improvement of *M. esculenta* can contribute to raise the quality of the root production and, thus, increase the income of the producer. The combination of improved crop and the soil management, the high yield and drought and pest resistant varieties increases its productive potential to 23.2 tons, against the mean total yield of 12.8 tons per hectare in the last decade (FAO, 2013). The annual productivity per hectare is precisely the criterion used to evaluate the genetic improvement of the crop (NASSAR, 2006).

But it is not enough to have the best cultivar. It takes a lot of material and fast. For this, there are several methodologies, such as the in vitro culture. Micropropagation is also important for the process of multiplication of clone's uniform and with good phytosanitary status (OLIVEIRA, 2009). This importance is shown by the numbers. With micropropagation, the plant multiplication rate reaches 1:5 every 6 weeks. By the traditional method, each plant produces from 5 to 10 manivas, in a mean period of 12 months, at a propagation rate ranging from 1:5 to 1:10 (SANTOS et al., 2009).

The micropropagation of cassava is already a consolidated technique in Brazil. It has long been carried out both for the multiplication of varieties and for the production of material for in vitro conservation of germplasm (SOUZA et al., 2009). However, there are large variations between species and cultivars or varieties in terms of results. Therefore, the objective of this work was to evaluate the micropropagation potential of different cassava varieties planted in Colorado do Oeste, a municipality in the Southern Cone of Rondônia, in order to contribute to the planning of the production of matrices in the laboratory and strengthening of the cassava cultivation in the region.

Materials and Methods

The research was carried out in the Laboratory of *In Vitro* Cultivation of the Pole of Innovation and Technological of the Alto Jacuí, in the campus of the University of Cruz Alta (UNICRUZ), located at 28˚33˚45.3˚ of S latitude, 53˚37˚20.3˚ W longitude and 450m altitude in the municipality of Cruz Alta, Rio Grande do Sul, between March and August 2015. The experiment was carried out with four varieties of *M. esculenta* from Colorado do Oeste, Rondônia state. They are varieties of local origin, without registered selection processes, like many other varieties cultivated in the Country. Popularly known as Arara, Caturra, Cacau Vermelha and Roxinha, are varieties classified as sweet or "mesa" because they are normally used for human and animal fresh consumption (FUKUDA; OTSUBO, 2003).

The experiment was conducted in a completely randomized design in a 4 x 2 factorial design, with 6 replicates. Treatments consisted of explants grown on Murashige and Skoog (MS) medium without growth regulator and MS medium supplemented with of 6-benzylaminopurine (BAP).

The experimental activity was developed in two stages: 1. obtaining explants and evaluating survival; 2. *in vitro* multiplication compared to the use or not of BAP.

1. Obtaining explants

First, branches of cassava with two shoots were placed in plastic containers with 300 mL capacity, with about 300g of commercial substrate and a segment of manivas with 2 buds, planted horizontally, approximately 3cm deep. They were kept in a greenhouse (Laboratory of Plant Multiplication - UNICRUZ), with controlled environment - temperature of 26 °C and air humidity of 65% - until the emission of shoots, which served as the initial source of explants.

When the shoots reached a length of 10 cm, they were collected with a scalpel blade and placed in Becker glass with distilled water, immediately taken to the Laboratory of Vegetable Tissue Culture *In Vitro*. Disinfestation was

then carried out under constant stirring. At this moment, leaves were also removed from the shoots, but leaving 1cm of the petioles of the same.

For disinfestation, the shoots were washed in running water and immersed in carbendazim fungicide, for 10 min (1.5 mL 1000 mL^{-1}), alcohol 70° for 1 min and sodium hypochlorite 1.5% for 5 min in this sequence. Finally, they underwent triple washing with sterilized autoclaved distilled water in a laminar flow hood. The shoots were then cut into segments 1cm long for explanatory purposes.

2. In vitro multiplication

The segments of the cassava varieties were inoculated in 40 mL of MS medium (Murashige e Skoog 1962) plus 30g L^{-1} sucrose and 100 mg L^{-1} inositol. The pH was adjusted to 5.8 before the addition of 7g L^{-1} agar. These explants were inoculated into 300 mL glass vials, autoclaved at 121 °C and 1 atm pressure for 20 min prior to inoculation. After inoculation, they were kept in a growth room with a temperature of 25 ± 2 °C, photoperiod of 16h light and light intensity of approximately 40 µmol m^{-2} s^{-1}. From each of the four cassava varieties, 40 shoots were used to produce 80 explants each (320 in all). At 45 days, were evaluated survival, as well as deaths from fungal and bacterial contamination and oxidation. The results were analyzed by the T statistic, using the program Action for Excel (EQUIPE ESTATCAMP, 2014).

In order to verify the response of each of the varieties to the micropropagation and to the culture media, 48 explants cultivated *in vitro* in MS medium were used, 12 of each variety being divided into treatments with and without the use of BAP. At 21 days after the survival analysis, the number of shoots, internodes, leaves, bud length (cm), presence of callus, rooting and dry mass (g) of shoot and root were evaluated.

In the experiment with and without BAP, the normality of the data was evaluated by means of the *Komolgorov-Smirnov* test and the homogeneity of variances by the *Bartlett* test. For analysis of variance, the original data were transformed into √(Y+0.5). After that, a multivariate analysis of means of the significant variables was performed by the "F" test, using the *Scott-Knott* algorithm, with a 5% probability of error, with the aid of the Sisvar program 5.6 (FERREIRA, 2011).

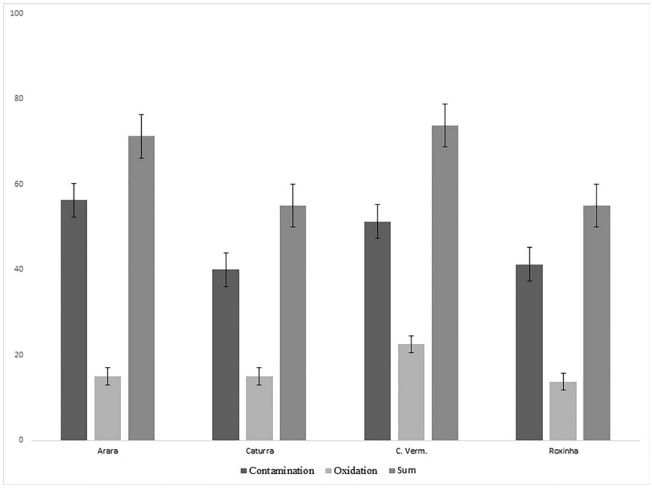
The transformed data, which did not present normal distribution of the non-homogeneous variances and errors, had the distribution of the variances evaluated by the *Kruskal-Wallis* non-parametric test, analogous to the Analysis of Variance (Anova), through the program Action for Excel (EQUIPE ESTATCAMP, 2014).

Results

The emergence of seedlings of varieties Arara, Caturra, Cacau Vermelha and Roxinha occurred in March. Of the 61 segments of branches of the Arara variety planted horizontally in plastic containers (300 mL) with commercial substrate, the formation of 64 shoots occurred. Of the 60 Caturra segments planted, 99 shoots were formed, 25 more than the total generated of 44 segments of Cacau Vermelha and 32 more than those produced by Roxinha.

The losses of explants by contamination and oxidation, respectively, were 45 (56.25%) and 12 (15%), adding 57 (71.25%) in the case of Arara; 32 (40%) and 12 (15%), adding 44 (55%) in Caturra; 41 (51.25%) and 18 (22.5%), adding 59 (73.75%) in Cacau Vermelha and 33 (41.25%) and 11 (13.75%), adding 44 (55%) in Roxinha (Figure 1). In terms of means percentages, the percentage of losses by contamination was 47.19% and by oxidation, of 16.56%. The total loss (contamination + oxidation) was 63.75%. In absolute numbers, the mean loss was 51 ± 8,124 explants. On the other hand, 23 explants of Arara (28.75%), 36 of Caturra (45%), 21 of Cacau Vermelha (26.25%) and 36 of Roxinha (45%) survived. The mean survival, therefore, was 29 ± 8,124 explants, or 36.25%.

Figure 1. Percentage of losses of explants of *Manihot esculenta* of Arara, Caturra, Cacau Vermelha and Roxinha varieties, originating in Colorado do Oeste-RO, by contamination and oxidation.



The losses of explants due to contamination and oxidation differed among varieties (P <0.05). The T-test statistic was 12.55533, with a p-value of 0.001089321 (Table 1). Thus, at a significance level of 5%, we accept the hypothesis which the mean losses of the tissues of cassava that have undergone explantation have been far from the nominal value, thus presenting a significant difference. This result is valid for comparison of Arara and Cacau Vermelha (mean loss of 72.5 ± 8.124) with Caturra and Roxinha (mean loss of 55 ± 8.124).

Table 1. Results of the analysis of the means loss of explants of cassava varieties Arara (71.25%), Cacau Vermelha (73.75%), Caturra (55%) and Roxinha (55%) due to contamination by fungi and bacteria and by oxidation by the Test T - Single Sample. Colorado do Oeste – RO, 2016.

Informations	Results
T-Statistic	12.55533
Degrees of Freedom	3
P-Value	0.00109
Sample mean	51
Standard deviation	8.124
Trust level	95%
Inferior limit	38.073

Superior limit	63.927

With and without BAP

The results of the analyzes of the studied factors (varieties and culture medium) are shown in Table 2. The responses of each of the varieties to the micropropagation are evidenced by the means followed by lower case letters, in the row of the table. The responses of the varieties to the culture media are corroborated by the means followed by capital letters in the column.

Table 2. Analysis of number of shoots, internodes and leaves, length of shoots, presence of callus and root and dry mass of M. esculenta varieties Arara, Caturra, Cacau Vermelha and Roxinha, cultivated in vitro, in MS medium, with and without BAP supplementation. Colorado do Oeste – RO, 2016.

Treatments	Varieties of *M. esculenta*			
	Arara	Cacau Vermelha	Caturra	Roxinha
Number of shoots				
With BAP	1.264 aA	1.375 aA	1.343 aA	1.268 aA
Without BAP	1.264 aA	1.343 aA	1.224 aA	1.282 aA
C.V. (%)	19.11	Mean:	1.295	± 0.240*
Number of internodes				
With BAP	3.182 aA	2.084 bA	2.398 bA	1.998 bA
Without BAP	1.629 aB	1.743 aA	1.751 aA	1.668 aA
C.V. (%)	33.86	Mean:	2.057	± 0.824
Number of leaves				
With BAP	3.148 aA	2.289 bA	2.318 bA	2.133 bA
Without BAP	0.88 aB	3.22 aA	2.33 aA	3.33 aA
C.V. (%)	34.22	Mean:	2.033	± 0.867
Length of shoots (cm)				
With BAP	3.748 aA	1.167 bA	1.283 bA	1.015 bA
Without BAP	0.840 aB	1.026 aA	0.934 aB	1.032 aA
C.V. (%)	17.55	Mean:	1.3812	± 0.245
Presence of callus				
With BAP	1.224 aA	1.167 aA	1.224 aA	1.224 aA
Without BAP	1.052 aB	1.052 aB	1.052 aB	0.707 bB
C.V. (%)	10.98	Mean:	1.002	± 0.110
Root presence				
With BAP	1.224 aA	1.224 aA	1.224 aA	0.994 bA
Without BAP	0.707 aB	0.707 aB	0.764 aB	0.707 aB
C.V. (%)	12.05	Mean:	0.944	± 0.138
Dry mass (g)				
With BAP	0.907 aA	0.761 bA	0.743 bA	0.726 bA
Without BAP	0.719 aB	0.751 aA	0.738 aA	0.722 aA
C.V. (%)	8.8	Mean:	0.759	± 0.067

Means not followed by the same letter, in the line (lower case) and in the column (upper case), do not differ significantly by the Scott-Knott test, at 5% probability of error. CV = Coefficient of variation. * = standard deviation.

Transformed data for √(Y+0,5)

Comparing the means, in the line, a significant interaction (P≤0.05) between the varieties was observed, that is, they responded differently to the micropropagation, specifically in relation to the number of internodes, number of leaves, length of shoots, presence of callus, presence of root and dry mass. Regarding the number of shoots, there was no significant interaction between the varieties (P≥0.05), that is, the segments of *M. esculenta* responded equally to the explanation. The best response to the *in vitro* establishment was the cultivar Arara, followed by Caturra, Cacau Vermelha and Roxinha, in this order.

The effect of the treatment on the varieties was also significant (P≤0.05) for the number of internodes and leaves, length of shoots and dry mass. The most favorable effect for the explanation of seed-seed segments was the MS medium without BAP. The non-significant interaction (P≥0.05) again occurred in relation to the variable number of shoots, indicating the need to carry out new work to understand the result.

Still in relation to the treatment effect on the varieties, the best response to treatment without BAP was also given by Arara, followed by Caturra, Cacau Vermelha and Roxinha. This was verified in relation to the number of internodes, number of leaves, shoot lengths and dry mass. Only in relation to this last variable, Red Cacao presented a better result than Caturra.

Regarding the presence of callus, the responses of the varieties to micropropagation were similar for all of them: small differences in means, but without statistical significance (P≥0.05) between Cacau Vermelha and Caturra. Roxinha was exception in medium without BAP. In it, the presence of callus was smaller, with statistical significance (P≤0.05) in comparison with the other varieties. Regarding the treatment effect on the varieties, the highest presence of callus was observed in the explants cultivated in medium supplemented with BAP, with significant difference (P≤0.05) among the plants tested.

Specifically on the root presence parameter, the responses of the varieties to micropropagation were similar for all of them: a slightly higher mean for Caturra, but with a non significant difference (P≥0.05) with Arara, Cacau Vermelha and Roxinha, in medium supplemented with BAP. In the medium without BAP, Roxinha was the one that generated the least roots, with statistical significance (P≤0.05) in comparison with the other varieties. Regarding the effect of the treatment on these plants, the greater presence of root was also observed in the explants cultivated in medium without BAP, with significant difference (P≤0.05) among the varieties.

Discussion

One of the basic principles for the tissue culture success depends in part on measures to control and prevent microbial contamination because it is a technique which provides a favorable environment for the growth of fungi and bacteria (PEREIRA et al., 2011). Contamination is caused by the entry of these biological contaminants into the culture medium, which comes from the explant (endogenous) or the environment (exogenous). The contaminating microorganisms compete with the explants for the nutrients of the culture medium, eliminating toxic metabolites in the medium, which can cause the seedling death (AMARAL, 2006).

When performing a clonal cleaning protocol test to obtain free cassava common mosaic virus (CsCMV) plants, Carnelossi (2010) reported losses from contamination (fungi and bacteria) ranging from 3.9% to 31% of the total number of cassava (CsCMV), 37%, depending on the variety studied. For oxidation, for example, the percentages were 32.5% of explant deaths of the cultivar Olho Junto and 39.57% of Pasquini. These percentages of contamination are lower than those obtained with Arara, Cacau Vermelha, Caturra and Roxinha, and those with higher oxidation.

The explanation for the difference in percentages of

contamination losses may be due to the fact that when the material comes directly from the field (in the case of varieties from Rondônia and Pasquini, cultivated in the Northwest region of Paraná), disinfestation is hampered by endogenous contamination, making the sodium hypochlorite has low effects due to its surface action (TRIGANO; GRAY, 2000), may have occurred with the present experiment, although sodium hypochlorite is considered an antiseptic.

Oxidation is another serious problem for the initial establishment of in vitro culture. This is due to the release of phenolic compounds by damaged cells during excision of the explants or due to the high levels of copper and iron contained in the culture medium (CAUDURO et al., 2014). Some enzymes oxidize the phenols forming quinones, responsible for the brown color of the cultures, in addition to causing inhibition of the growth and death of explants. It is a species--dependent process and no method is fully effective for all cultivated species (GIATTI; LIMA, 2007).

But the death of explants by oxidation can be reduced, if not avoided. In relation to the varieties originating in Colorado do Oeste -RO, this would mean a greater number of tissue segments for the matrices production in the laboratory. This is a fact which shows the results of research with PVP-40 and other antioxidants, according to which the addition of these compounds may be an alternative to contain the oxidation of explants and avoid their interference in regeneration (CAUDURO et al., 2014). The results on the survival of the explants of Arara, Caturra, Cacau Vermelha and Roxinha, could therefore have been better if the research had other developments, such as the addition of antioxidants to the culture medium, which can be done in the future.

Also with regard to work which can be performed to minimize the losses of explants by oxidation, future research has to consider that each genotype presents a specific regeneration potential. Thus, the study of tissue culture in vitro should be improved, considering a broad spectrum of external factors: culture medium, regulators concentration, light and temperature, favoring the development of morphogenetic potentials of each cultivar (OLIVEIRA et al., 2006).

The difference in the in vitro multiplication potential of varieties originating in Colorado do Oeste, evidenced by the responses of these varieties to micropropagation, is not an exclusive result. Aipim-Rosa, Cangaíba, Caravela, Cravela, and Pretinha, from the State of Sergipe, Oliveira et al. (2000) showed a pronounced effect of the genotype in the development in vitro of the seedlings. In this work, Cangaíba presented the highest multiplication, seedling height and root formation rates by subculture in comparison with the others, also showing a better response of one over the others.

To verify the treatment effect on the plants tested, the culture was compared in MS medium with and without BAP because the cytokinins are essential for the plants development. These plant hormones are responsible for the occurrence of cytokinesis, formation of chloroplasts, changes in metabolic rate, enzymatic activity, breakdown of apical dominance, mobilization of nutrients, retardation of senescence and formation of tissues and organs (KERBAUY, 2004).

As for the culture medium, the choice depends on the species in question and the purpose of the culture. The MS medium is used in tissue culture of most species and modifications and dilutions have been shown positive results for several of them (FARIA et al., 2007). With the addition of the cytokines BAP (6-benzylaminopurine) to the culture medium, it was sought, without success, to obtain the demonstrated efficacy in the multiplication of several species in vitro, especially in the favoring to the shoots formation (SOUTO, 2008). But the verified superiority of the MS medium on the MS + BAP treatment is not surprising, since the effect of the concentration of growth regulators varies according to the species (CORDEIRO et al., 2014).

Controversial results concerning the addition of BAP to MS medium were found in works with other *Euphorbiaceae*, such as *Croton antisyphiliticus* Mart. ex M. Arg. Oliveira et al. (2011), for example, found that supplementation gives better results in the number of shoots, but in vitro rooting is superior in culture medium without the addition of plant regulator. Specifically on cassava, better results without BAP supplementation were also verified by Silva, Ferreira and Gato (2015), for rooting parameters and multiplication rate, thus becoming the most suitable for seedling production.

Conclusions

The varieties of *Manihot esculenta* have different potentials of *in vitro* multiplication. The Arara variety shows higher seedling development in MS medium with absence of BAP growth regulators. The production of *M. esculenta* by micropropagation should be stimulated by plant breeding programs, but specific studies for each variety would maximize the *in vitro* multiplication process of varieties of interest, with desired phytosanitary qualities, on a large scale and with varietal authenticity. It is suggested, finally, studies about the addition of antioxidants to the culture medium to seek the losses of explants reduction by oxidation and acclimatization of seedlings of *M. esculenta* cvs. Arara, Cacau Vermelha, Caturra and Roxinha, obtained by explantation, to verify their physiological and molecular behavior when passing from a heterotrophic to an autotrophic state.

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No potential conflict of interest was reported by the authors.

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