Ciência e Agrotecnologia, 42(6):598-607, Nov/Dec. 2018
http://dx.doi.org/10.1590/1413-70542018426024718

Culture media for the multiplication of wild *Manihot* species

Meios de cultura para a multiplicação de espécies silvestres de *Manihot*

Jucieny Ferreira de Sá[1*], Emília dos Santos Sampaio[1], Maria Inês de Souza Mendes[2], Karen Cristina Fialho dos Santos[3], Antônio da Silva Sousa[3], Carlos Alberto da Silva Ledo[3]

[1]Universidade Federal do Recôncavo da Bahia/UFRB, Cruz das Almas, BA, Brasil
[2]Universidade Estadual de Santa Cruz, Ilhéus, BA, Brasil
[3]Empresa Brasileira de Pesquisa Agropecuária/Embrapa, Embrapa Mandioca e Fruticultura, Cruz das Almas, BA, Brasil
*Corresponding author: jucienyferreira@hotmail.com
Received in October 10, 2018 and approved in November 21, 2018

ABSTRACT

The cassava propagation system is slow and favors disease transmission through successive generations. Micropropagation is an alternative to overcome the aforementioned limitations, besides allowing the generation of a larger number of pest- and pathogen-free plants. Therefore, the aim of the present study is to investigate the effect of culture media on the multiplication *in vitro* of five wild *Manihot* species. The experiment followed a completely randomized design, at factorial arrangement 5 (wild *Manihot* species) x 6 (culture media), with 11 repetitions. Explants consisted in nodal segments (91 cm long and one lateral bud) of species *Manihot flabellifolia*, *M. tristis*, *M. caerulescens*, *M. chlorosticta* and *M. jacobinensis*, which were extracted *in vitro* from the collection of wild cassava species. One segment was placed in each test tube added with 10 mL of MS media 0.01, 17N, $12A_3$, 4E, 8S and WPM, and kept for 90 days in growth room under 30 µmol m^{-2} s^{-1}irradiance, temperature 27 ± 1 °C and 16h photoperiod. Variables plant height (cm), number of green leaves, number of senescent leaves, number of shoots, number of microcuttings, fresh and dry shoot mass, fresh and dry root mass (mg) and callus mass (mg) were analyzed. Our results showed that the culture medium $12A_3$ was not responsive to any of the species; however, if one takes into consideration variables plant height and number of microcuttings, this medium can possibly be used in the micropropagation of other wild species belonging to genus *Manihot.*

Index terms: Cassava; wild parental; nutritional media; tissue culture; micropropagation.

RESUMO

O sistema de propagação da mandioca é vagaroso e favorece a transmissão de doenças para sucessivas gerações. A micropropagação é uma alternativa para disponibilizar uma maior quantidade de plantas isentas de pragas e patógenos. Portanto, o objetivo desse estudo foi verificar o efeito de meios de cultura na multiplicação *in vitro* de cinco espécies silvestres de *Manihot*. O experimento foi realizado no delineamento experimental inteiramente ao acaso, em esquema fatorial 5 (espécies silvestres de *Manihot*) x 6 (meios de cultura), com 11 repetições. Os explantes consistiram de segmentos nodais das espécies *Manihot flabellifolia*, *M. tristis*, *M. caerulescens*, *M. chlorosticta* e *M. jacobinensis*, com 1 cm de tamanho e uma gema lateral. Colocou-se um segmento por tubo de ensaio, contendo 10 mL dos meios de cultura MS 0,01, 17N, $12A_3$, 4E, 8S e WPM, mantendo-os durante 90 dias em sala de crescimento com irradiância de 30 µmol m^{-2} s^{-1}, temperatura de 27± 1 °C e fotoperíodo de 16 horas. Foram analisadas as variáveis altura de planta (cm), número de folhas verdes, número de folhas senescentes, número de brotos, número de microestacas, massas fresca e seca de parte aérea (mg), massas fresca e seca de raízes (mg) e massa de calo (mg). O meio de cultura $12A_3$ não foi responsivo para nenhuma das espécies, no entanto, considerando-se às variáveis altura de planta e número de microestacas os demais meios podem ser utilizados na multiplicação *in vitro* das espécies estudadas, e possivelmente, podem ser utilizados na micropropagação de outras espécies silvestres do gênero *Manihot*.

Termos para indexação: Mandioca; parentais silvestres; meios nutritivos; cultura de tecidos; micropropagação.

INTRODUCTION

Choosing the most appropriate culture medium is an essential factor during multiplication, given the importance of its components in the regeneration process *in vitro*. Physical support for the explant and the supply of all nutrients participating in the growth and development of vegetal material stand out among functions performed by the medium. There are many culture medium formulations; however, the one formulated by Murashige and Skoog (1962) is mostly widespread. It is universally known as the MS medium and has been broadly applied in studies about tissue cultures, such as the nodal segment multiplication, somatic embryogenesis induction, and embryo rescue and cultivation.

According to Villa et al. (2009), the culture medium is essentially composed of water (distilled, deionized, reverse osmosis), inorganic macronutrients (N; K; Ca; Mg; P; S; Si), inorganic micronutrients (Cl; Fe; B; Mn; Na; Zn;

Cu; Ni; Mo), vitamins (nicotinic acid, pyridoxine, thiamine), amino acids (tyrosine, L-arginine, L-serine), sources of organic nitrogen (glycine; inositol), carbohydrates (sucrose), solidifying agents and/or gelatin (agar, Phytagel®) - when the medium is used in its solid form, and synthetic phytoregulators (auxins, cytokinins, gibberellins).

Overall, tissue culture strategies are adopted when conventional sexual and vegetative propagation systems are not satisfactory. Propagation in cassava culture can happen through sexual reproduction. However, according to Cerqueira et al. (2016), the most often used propagation method lies on stakes or manivas from the mother plant. Nevertheless, this system contributes to pest and disease transmission, with emphasis on the systemic one, to the following generations, fact that can affect culture yield.

Thus, micropropagation becomes a promising alternative in comparison to the conventional cassava vegetative propagation method, since it allows the production of phytosanitary-quality plants, among other advantages. Culture medium 4E (ROCA et al., 1991) is applied to *Manihot esculenta* Crantz in the explant establishment stage *in vitro*; media 17N (CIAT, 1982) and MS 0.01 (Souza et al., 2008) are adopted during multiplication, and medium $12A_3$ (Mafla et al., 2010) is recommended by CIAT for the micropropagation of wild species belonging to genus *Manihot*, whereas 8S is used to conserve germplasm *in vitro* (CIAT, 1984). Many studies have been performed in this field and they have shown the efficiency of this technique in cassava cultures (Demeke et al., 2014; Shiji et al., 2014; Kabir et al., 2015; Mongomake et al., 2015; Anjum; Shazia, 2015).

Accordingly, cultivation *in vitro* also emerges as a feasible strategy to the propagation of wild cassava species of great importance for cassava breeding programs. However, studies focused on multiplication methods applied to these species have not been enough. The quite heterogeneous behavior of each of the presented species, whenever they are micropropagated, has been one of the main barriers. Therefore, studies about the adjustment of methodologies that allow efficient multiplications have become essential. Thus, the aim of the present research was to investigate the effect of culture media on the multiplication *in vitro* of five wild species belonging to genus *Manihot*.

MATERIAL AND METHODS

The experiment was conducted between November 2016 and February 2017 in the Tissue Culture Laboratory (LCT) of the Advanced Biology Center (NBA) at Embrapa Cassava and Fruit Culture (CNPMF), in Cruz das Almas County, Bahia State, Brazil. Accessions characterized as *Manihot flabellifolia* Pohl; *M. tristis* Müll.Arg; *M. caerulescens* Pohl; *M. chlorosticta* Standl. and *M. jacobinensis* Müll.Arg were used in the present study. These accessions were provided by the collection *in vitro* of Embrapa Cassava and Fruit Culture. Plants were sectioned in flow chamber in order to get the explants, which were cut into micropiles (at approximately 1cm length, with at least one gem). Subsequently, the micropiles were inoculated in test tubes (2.5cm x 15cm) with 10 mL of the assessed culture media. Next, the test tubes containing the explants were kept for 90 days in growth room under irradiance 30 $\mu mol.m^{-2}\ s^{-1}$, temperature 27± 1 °C and 16h photoperiod. Culture media MS 0.01 (Souza et al., 2008), 17N (CIAT, 1982), $12A_3$ (Mafla et al., 2010), 4E (Roca et al., 1991), 8S (CIAT, 1984) and WPM (Lloyd; Mc Cown, 1980)], which were autoclaved for 20 minutes at 120 °C, are presented in Table 1.

After this period, plants were subjected to the evaluation of the following traits: plant height (PH; cm), number of green leaves (NGL), number of senescent leaves (NSL), number of shoots (NS), number of micropiles 1cm (NMP), shoot fresh mass (SFM; mg), root fresh mass (RFM; mg) and callus mass (CM; mg). All plant material was identified and placed in forced air circulation oven at 70 °C for 48 hours. Subsequently, the shoot dry mass (SDM; mg) and the root dry mass (RDM; mg) were determined. The study followed a completely randomized design at factorial arrangement 5x6 - 5 wild species belonging to genus *Manihot* and 6 culture media, with 11 repetitions. Each repetition was composed of one explant (micropile) placed in a test tube. Data recorded after the evaluation were subjected to Scott-Knott test, at 5% probability level. The numbers of green leaves, senescent leaves, shoots and micropiles were assessed by transforming them into $\sqrt{x+0.5}$, in order to fulfill assumptions based on analysis of variance. Statistical analyses were conducted in the '*ExpDes.pt*' package implemented in the R software, version 3.4.2 (R Development Core Team, 2017).

RESULTS AND DISCUSSION

It is necessary taking into account the interaction among some factors to achieve efficient development *in vitro* such as the case of plant physiological status, cultivation and genotype/species conditions, since they will contribute to crop yield. Genus *Manihot* presents wide genetic variability (Nassar; Grattapaglia, 1986); therefore, it is possible observing quite different behaviors in the species assessed in the current study.

Table 1: Composition of culture media 4E, 17N, 8S, MS 0.01, $12A_3$ and WPM, which were used in the multiplication of five wild species belonging to genus *Manihot*.

Components (mg.L)	Culture Media					
Macronutrients	4E	17N	8S	MS 0.01	$12A_3$	WPM
NH_4NO_3	1,650.0	577.5	1,650.0	1,650.0	1,650.0	400.0
KNO_3	1,900.0	665.0	1,900.0	1,900.0	1,900.0	556.0
$CaCl_2.2H_2O$	450.0	154.0	450.0	450.0	450.0	96.0
$MgSO_4.7H_2O$	370.0	129.5	370.0	370.0	370.0	370.0
KH_2PO_4	170.0	59.5	170.0	170.0	170.0	170.0
$FeSO_4.7H_2O$	27,8.0	9.73.0	27.8	27.8	27.8	27.8
K_2SO_4	-	-	-	-	-	990.0
$Na_2EDTA.2H_2O$	37.3	13.1	37.3	37.3	37.3	37.3
Micronutrients						
KI	0.830	0.291	0.830	0.830	0.830	-
H_3BO_3	6.200	2.170	6.200	6.200	6.200	6.200
$MnSO_4.4H_2O$	22.300	7.805	22.300	22.300	22.300	22.300
$ZnSO_4.7H_2O$	8.600	3.010	8.600	8.600	8.600	8.600
$Na_2MoO_4.2H_2O$	0.250	0.090	0.250	0.250	0.250	0.250
$CuSO_4.5H_2O$	0.025	0.009	0.025	0.025	0.025	0.250
$CoCl_2.6H_2O$	0.025	0.009	0.025	0.025	0.025	-
Vitamin + Hexitol						
Thiamine-HCl	1.0	1.0	1.0	0.1	1.0	1.0
Pyridoxine-HCl	-	-	-	0.5	-	0.5
Nicotinic acid	-	-	-	0.5		0.5
Glycine	-	-	-	2.0		2.0
Inositol	100.0	100.0	100.0	100.0	100.0	100.0
Growth regulators						
ANA	0.02	0.01	0.01	0.01	-	-
BAP	0.04	-	0.02	0.01	-	-
AG_3	0.05	0.01	0.10	0.01	-	-
Kinetin	-	-	-	-	0.20	-
Other supplements						
Activated charcoal	-	-	-	-	1.000.0	-
$CuSO_4.5H_2O$	-	-	-	-	500.0	-
Sucrose	20,000.0	20,000.0	20,000.0	20,000.0	20,000.0	20,000.0
Phytagel®	2,400.0	2,400.0	2,400.0	2,400.0	2,400.0	2,400.0
pH	5.8	5.8	5.8	5.8	5.8	5.8

According to the analysis of variance presented in Table 2, species and culture media, as isolated factors, had significantly influenced ($p < 0.01$) most of the assessed variables, except for variable 'number of shoots', which only had significant effect ($p < 0.01$) on factor 'species'.

On the other hand, it is essential pinpointing the significant interaction between species and means in almost all variables, although there was no interaction in variable 'number of shoots', fact that did not influence the recorded results.

The cultivation *in vitro* of different wild plant species led to quite heterogeneous growth, as demonstrated by the respective coefficients of variation (CV), which ranged from 12.88% to 72.46% (Table 2) in variables 'number of shoots' and 'root fresh mass', in the correct order. These results are similar to results found in some other studies. Cardoso et al. (2018) recorded CV values ranging from 6.52% and 54.43% in the multiplication of *Manihot esculenta* Crantz varieties *in vitro*, whereas Miranda et al. (2016) found CVs between 12.25% and 99.53% in the multiplication of *Eremanthus incanus* Less *in vitro*.

According to Werner et al. (2013), there are few studies related to adequate CV values of variables assessed in experiments conducted in the plant-tissue culture field. Authors of these studies state that the accuracy of experiments conducted in tissue culture can be low due to the influence of some factors such as genetic material variability and plant physiological stage, which gave birth to explant type; and the chemical and physical characteristics of culture media, luminosity intensity and cultivation temperature.

Table 3 presents the results of interaction in variable plant height, as well as allows verifying how culture media MS 0.01, WPM and 8S helped finding higher values observed for the cultivation *in vitro* of the assessed species. The highest PH value (15.13cm) was recorded for species *M. chlorosticta* when it was cultivated in culture medium MS 0.01. This value did not statistically differ from the value observed in medium WPM (14.49).

Table 2: Summary of analysis of variance applied to variables plant height (PH; cm), number of living leaves (NLL), number of dead leaves (NDL), number of shoots (NS), number of micropiles (NMP), shoot fresh mass (SFM; mg), shoot dry mass (SDM; mg), root fresh mass (RFM; mg), root dry mass (RDM; mg) and callus mass (CM) of species belonging to genus *Manihot* (*M. flabellifolia*; *M. tristis*; *M. caerulescens*; *M. chlorosticta and M. jacobinensis*) in six culture media, for 90 days cultivation *in vitro*.

SV	DF	MS									
		PH	NLL	NDL	NS	NMP	SFM	SDM	RFM	RDM	CM
Species	4	153.63**	20.87**	4.12**	0.49**	1.01**	89.01**	1.09**	77.14**	0.60**	9.56**
Media	5	211.27**	7.17**	2.37**	0.06^{NS}	4.67**	133.81**	1.55**	25.28**	0.43**	6.04**
Species * Media	20 (18^{1}. 15^{2})	43.53**	1.04**	0.59*	0.01^{NS}	0.53**	29.49**	0.42**	29.00**	0.15**	1.97**
Error	201 (135^{1}.140^{2})	13.51	0.37	0.29	0.03	0.22	6.98	0.11	6.67	0.04	0.79
CV (%)		49.20	27.45	36.12	12.88	25.37	51.99	47.84	72.46	68.83	54.78
Mean		7.47	5.36	2.23	1.28	3.25	160.65	21.52	112.74	9.47	51.36

SV = Source of variation; DF^{1} = Degree of freedom due to loss of treatments for RFM and RDM variables; DF^{2} = Degree of freedom due to loss of treatments for CM variable; MS = middle squares; ns = non-significant; **and* = significant at 1% and 5% probability level, respectively, in the F test by ANAVA.

Table 3: Mean plant height values (PH; cm) recorded for five species belonging to genus *Manihot* in six nutritional media at the 90^{th} cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	5.16bB	3.54aB	6.85bA	7.56aA	8.28bA	9.65bA
M. tristis	9.41aA	1.75aB	6.96bA	7.33aA	7.30bA	8.97bA
M. caerulescens	3.24bC	1.05aC	10.31bB	6.48aC	13.75aA	7.81bB
M. chlorosticta	7.92aB	4.40aC	15.31aA	8.77aB	9.71bB	14.49aA
M. jacobinensis	2.80bB	1.45aB	7.09bA	6.29aA	5.89bA	4.92cA

Means followed by the same lowercase letters in the column and by the same uppercase letter on the line belong to the same group in the Scott-Knott test at 5% significance level.

According to Dezan et al. (2012), plant development is positively influenced since the MS medium is quite concentrated in macronutrients, micronutrients, vitamins and amino acids. Some studies about cassava (*M. esculenta* Crantz) (Mapayi et al., 2013), yam (*Dioscorea rotundata* Poir.) (Simões et al., 2017), tamarillo (*Solanum betaceum* Cav.) (Copatti et al., 2016) and peach [*Prunus persica* (L.) Batsch] (Reis et al., 2012) micropropagation based on MS evidenced the effectiveness of culture medium in the development *in vitro* of different species.

It is worth highlighting that the composition of media MS 0.01 and 8S has lower total nitrogen concentration than the MS medium, and it can cause late growth in explants. Thakur and Kanwar (2008) using MS and WPM medium in the *in vitro* multiplication of *Pyrus pyrifolia* (Burm F.) Nakai, did not observe statistical differences in shoot length. A similar result was found by Lencina et al. (2014) who, when using the WMP, MS and ½ MS medium, also did not observe statistical differences in shoot length. However, this behavior was not observed in the present study, fact that likely indicates a normal growth relation to cassava adaptation to less fertile soil in *vitro*.

Values depicted in Table 4 refer to results of interactions between factors 'species' and 'culture media' in variable 'number of living leaves'. Culture media MS 0.01, 4E, 8S and WPM were statistically higher than media 17N and $12A_3$ in species *M. flabellifolia* and *M. jacobinensis*.

Overall, the species *M. jacobinensis* was statistically higher than the other assessed species, and this outcome evidences higher NLL values (15.22, 15.20, 14.37 and 13.40), which were proportional through media MS 0.01, 8S, WPM and 4E, respectively (Table 4). According to Ribeiro et al. (2014), the largest number of leaves allows the formation of auxiliary gems and of internodes in *Zantedeschia aethiopica* (L.) Spreng., and this process reflects the increased number of nodal segments and, consequently, it increases the multiplication rate throughout the sub-cultivation procedure.

Based on Taiz and Zeiger (2008), leaf senescence is one of the physiological explanations enabling plant survival. Culture medium WPM led to lower values in variable 'number of dead leaves', with emphasis to the species *M. flabellifolia* and *M. tristis,* which did not show dead leaves (Table 5).

Table 4: Mean number of living leaves (NLA) of five species belonging to genus *Manihot* in six nutritional media at the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	3.25bB	1.86aB	4.50bA	5.20bA	4.89bA	5.00bA
M. tristis	6.00aA	0.00aB	3.00bA	5.16bA	5.33bA	6.00bA
M. caerulescens	1.54bA	0.00aB	2.25bA	1.43cA	3.62bA	2.82bA
M. chlorosticta	3.50bA	2.33aA	3.14bA	3.75bA	3.25bA	4.50bA
M. jacobinensis	6.00aB	0.75aC	15.22aA	13.40aA	15.20aA	14.37aA

Means followed by the same lowercase letter in the columns and by the same uppercase letter on the lines belong to the same group in the Scott-Knott at 5% significance level.

Table 5: Mean number of dead leaves (NDL) of five plant species belonging to genus *Manihot* in six nutritional media in the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	1.50aA	0.71aA	0.25bA	1.50aA	1.44cA	0.00bA
M. tristis	1.25aA	1.50aA	1.80bA	2.16aA	1.16cA	0.00bA
M. caerulescens	2.63aA	1.00aA	2.62aA	2.86aA	2.62cA	1.63aA
M. chlorosticta	1.90aB	2.55aB	5.00aA	2.75aB	3.50bA	2.30aB
M. jacobinensis	1.66aC	1.25aC	3.33aB	4.10aB	6.60aA	0.75bC

Means followed by the same lowercase letter in the columns and by the same uppercase letter on the lines belong to the same group in the Scott-Knott test at 5% significance level.

Therefore, based on this result, the medium WPM was more responsive to leaf senescence reduction. According to Flores et al. (2015), data about senescence are used to determine that largest interval possible between sub-cultivations, which would end up in the production of the largest number of seedlings, however without harming the physiological quality of the plant. This larger interval between sub-cultivations is also relevant for the conservation *in vitro* of the germplasm when it is applied to explore the maximum vigor and feasibility.

The production of nodal segments is a relevant factor for micropropagation, since it reflects the generation of new plants at each sub-cultivation (Flores et al., 2009). All the assessed species presented similar behavior when they were cultivated in media $12A_3$, 4E, 8S and WPM, (Table 6).

However, the behavior of the species *M. jacobinensis* and *M. chlorosticta* was statistically higher than the other species in medium MS 0.01. Thus, they showed the highest NMP values, 6.11 and 5.14 respectively (Table 6). This result is similar to that found by Freitas et al. (2016), who developed a micropropagation protocol for *Justicia pectoralis* and found that MS was the most responsive medium among the assessed media in the multipropagation of nodal segments.

It is worth highlighting that, by associating these results with those presented in Table 4, the same culture media responsible for the highest NLL values were the ones allowing the highest NMP means, namely: MS 0.01, 4E, 8S and WPM. This relation between these two variables reinforced the previously mentioned statement by Ribeiro et al., (2014), who says that the larger number of leaves implies a higher multiplication rate due to the increased formation of nodal segments.

Table 7 shows the results related to the outcomes of interaction in variable 'fresh shoot mass', which evidenced that only species *M. tristis* did not statistically differ in the assessed culture media.

The opposite result was observed in species *M. caerulescens,* which recorded higher SFM value (400.00 mg) in medium 8S. This species was followed by *M. chlorosticta* and *M. jacobinensis* that recorded values 312.86 mg and 297.78 mg in medium MS 0.01, respectively (Table 7). The composition of these media are based on MS, which also stimulates greater shoot fresh mass production in mulberry multiplication *in vitro* (Villa et al., 2005), since these plants absorbed more water due to the increased osmotic potential of the medium.

It is important highlighting that the increased shoot fresh mass reflects the vigor of micropropagated plants and, consequently, the production of new better-quality explants. It is also known that more vigorous plants produced *in vitro* imply higher survival rates in the acclimatization stage.

Based on values of variable 'shoot dry mass' shown in Table 8, only species *M. flabelifolia* and *M. tristis* did not statistically differ in the culture media factor. On the other hand, the highest SDM values (52.28 mg and 34.61 mg) were observed in species *M. caerulescens* and *M. jacobinensis* in medium 8S.

The statistical differences in shoot dry mass between treatments indicate the possibility of different accumulation of photoassimilates due to differentiated absorption of minerals by part of the genotypes (Pereira et al., 2001). Overall, culture medium 8S led to the highest SDM, NLA, NDL and SFM means.

According to outcomes of the interaction between factors 'species' and 'culture media' for variables root fresh mass (Table 9), the species *M. chlorosticta* recorded the highest RFM value (285.11; mg) in medium MS 0.01 and it formed the statistically highest group in comparison to the other species in this medium. However, this value did not statistically differ from the result found when this species was cultivated in WPM medium (258.22 mg).

Table 6: Mean number of micropiles (NMP) from five plant species belonging to genus *Manihot* in six nutritional media at the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	2.50aA	1.43aB	2.50bA	3.60aA	3.89aA	3.11aA
M. tristis	4.12aA	0.00aB	2.40bA	2.50aA	3.33aA	3.83aA
M. caerulescens	1.00bB	0.00aB	3.62bA	2.86aA	4.62aA	2.54aA
M. chlorosticta	2.90aB	1.00aC	5.14aA	3.62aB	3.50aB	4.90aA
M. jacobinensis	1.66bB	1.00aB	6.11aA	4.70aA	5.10aA	4.12aA

Means followed by the same lowercase letter in the columns and by the same uppercase letter on the lines belong to the same group in the Scott-Knott at 5% significance level.

According to Miyata et al. (2014), an efficient root growth process is related to high nitrogen, manganese and zinc concentrations in the composition of medium MS. The greater root formation presented by heterotrophic plants grown *in vitro* consisted in a desirable characteristic to improve acclimatization process efficacy, despite the low efficiency of the root system (Sousa et al., 2015).

Values shown in Table 10 for the variable 'root dry mass' evidenced that the highest value (24.64 mg) was recorded for the species *M. chlorosticta* in medium WPM. However, this number did not present statistical difference from the response recorded for *M. jacobinensis* in the same medium, but it did differ from the numbers shown by the other species. It is worth highlighting that

Table 7: Mean shoot fresh mass (SFM; mg) of five plant species belonging to genus *Manihot* in six nutritional media at the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	80.00aB	64.29aB	147.50bA	157.00bA	182.22cA	140.00bA
M. tristis	107.50aA	37.50aA	80.00bA	100.00bA	90.00dA	106.67bA
M. caerulescens	82.72aC	10.00aC	170.00bB	122.86bB	400.00aA	141.82bB
M. chlorosticta	117.00aB	113.33aB	312.86aA	137.50bB	170.00cB	250.00aA
M. jacobinensis	100.00aB	112.50aB	297.78aA	255.00aA	275.00bA	148.75bB

Means followed by the same lowercase letter in the columns and by the same uppercase letter on the lines belonged to the same group in the Scott-Knott test at 5% significance level.

Table 8: Mean shoot dry mass (SDM; mg) of five plant species belonging to genus *Manihot* in six nutritional media at the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	13.52aA	10.97aA	19.15bA	19.15bA	24.76cA	19.30bA
M. tristis	16.16aA	6.45aA	13.24bA	16.88bA	12.46dA	14.21bA
M. caerulescens	14.49aB	1.55aC	23.77bB	16.01bB	52.28aA	24.71bB
M. chlorosticta	14.75aB	15.62aB	31.07aA	16.53bB	22.06cB	34.32aA
M. jacobinensis	18.83aB	16.97aB	33.11aA	30.72aA	34.61bA	17.23bB

Means followed by the same lowercase letters in the columns and by the same uppercase letters on the lines belong to the same group in the Scott-Knott test at 5% significance level.

Table 9: Means of root fresh mass (RFM; mg) of five plant species belonging to genus *Manihot* in six nutritional media at the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	67.61aB	181.6aA	77.23bB	125.22aB	197.70aA	93.00bB
M. tristis	77.82aA	26.23bA	53.75bA	68.80aA	66.46bA	51.61bA
M. caerulescens	8.00aA	-	42.25bA	32.60aA	43.40bA	82.90bA
M. chlorosticta	105.17aB	146.55aB	285.11aA	75.58aB	130.68aB	258.22aA
M. jacobinensis	84.05aB	-	13.76bB	45.50aB	60.18aB	270.20aA

Means followed by the same lowercase latter in the columns and by the same uppercase letter on the lines belong to the same group in the Scott-Knott test at %% significance level.

there was root formation in culture medium $12A_3$ (Tables 9 and 10), which had activated coal in its composition. This result does not comply with the statement by Chapla et al. (2009), who said that this substance simulates darkness, and it can favor root system development *in vitro*.

However, Erig et al., (2004) investigated the root growth of *Pyrus communis* L. *in vitro* and found that coal addition to the culture medium can be a beneficial strategy. Moreover, Oliveira-Cauduro et al., (2014) suggest that the presence of this substance can help the absorption of other compounds found in the culture medium, among than one can find phytoregulators, fact that can compromise root system formation.

According with the results recorded for the outcomes of callus mass interaction (Table 11) shows that the culture media 4E, 8S and MS 0.01 recorded the highest values for this variable, with emphasis to species *M. chlorosticta* (84.76 mg), *M. flabellifolia* (83.40 mg) and *M. jacobinensis* (82.93 mg), in the media mentioned above, respectively. Contrasting result was found for the regeneration of *Eucalyptus camaldulensis in vitro* (DIBAX et al., 2010), in which the culture medium WPM indicated superior callus induction.

Overall, the assessed species presented quite different behaviors, and such outcome is likely related to the variability of species belonging to genus *Manihot* and to differences in the production of photoassimilates. Culture medium plays a key role in cell and tissue growth responses. Moreover, its composition can be changed based on the need of each explant type and on the species under study (Torres et al., 2001). Changes in the culture medium can be made either in the basic composition (minerals and vitamins) or in the types and concentrations of growth regulators. Accordingly, Faye et al. (2015) found that the responses from the assessed variables changed based on the type and concentration of growth regulators by using different phytoregulators in organogenesis conducted *in vitro*.

Variables 'plant height' and 'number of micropiles' were highly discriminating for culture medium determination in each species during the *in vitro* culture process. According to the recorded results, and by taking into account the aforementioned variables, it is possible saying that species *M. chlorosticta* and *M. jacobinensis* presented the best performance when they were cultivated in culture medium MS 0.01, whereas the species *M. caerulescens* and *M. tristis* were more responsive in media 8S and 17N, respectively. WPM and 8S were the most effective media for species *M. flabellifolia* in variables 'plant height' and 'number of micropiles'.

Accordingly, differences between these data and other experiments of the same nature can also result from the specification of culture media for each species, since different carbohydrate, minerals, vitamins and growth regulator combinations can stimulate, or not, the growth of organs, tissues or cells, and plant development (George et al., 2008).

These contrasting results reinforce the hypothesis that it is not possible generalizing a single culture medium for the *in vitro* cultivation of wild species belonging to genus *Manihot.* Therefore, the importance of adjusting a nutritional medium for each one, or at least to one group of species, is evident.

Table 10: Means of root dry mass (RDM; mg) of five plant species belonging to genus *Manihot* in six nutritional culture media at the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	4.91aB	21.10aA	5.76bB	9.44aB	10.62aB	8.73bB
M. tristis	5.32aA	2.0bA	4.55bA	4.03aA	2.53bA	51bA
M. caerulescens	0.70aA	-	9.40bA	2.56aA	3.83bA	7.50bA
M. chlorosticta	6.81aC	17.99aB	18.28aB	5.93aC	10.62aC	24.64aA
M. jacobinensis	11.80aA	-	1.10bA	3.45aB	4.42bB	20.06aA

Means followed by the same lowercase letter in the columns and by the same uppercase letter on the lines belong to the same group on the Scott-Knott test at 5% significance level.

Table 11: Means of callus mass (CM; mg) in five *Manihot spp*. species in six nutritional media at the 90th cultivation days *in vitro*.

Species	Culture Media					
	17N	$12A_3$	MS 0.01	4E	8S	WPM
M. flabellifolia	64.05aA	-	30.00bB	74.69aA	83.40aA	4.80aB
M. tristis	34.02aA	-	26.75bA	39.58bA	13.70bA	11.86aA
M. caerulescens	24.80aA	-	-	24.50bA	32.45bA	61.75aA
M. chlorosticta	50.54aA	22.50aB	72.72aA	84.76aA	67.91aA	11.75aB
M. jacobinensis	30.73aB	-	82.93aA	69.98aA	53.79aA	30.62aB

Means followed by the same lowercase letter in the columns and by the same uppercase letter on the lines belonged to the same group in the Scott-Knott test at 5% significance level.

CONCLUSIONS

The assessed wild species belonging to genus *Manihot* grown in culture medium $12A_3$ were not responsive. However, the other media can be used for the *in vitro* culture of the assessed wild *Manihot* species, if one takes into account variables 'plant height' and 'number of micropiles' as the most important ones in a micropropagation protocol.

REFERENCES

ANJUM, P.; SHAZIA, M. Rapid propagation of a biodiesel plant cassava (*Manihot esculenta* Crantz) through tissue culture. **International Journal of Biology and Biotechnology**, 12(3):369-372, 2015.

CARDOSO, M. N. et al. Influência de luz e sacarose no crescimento *in vitro* de mandioca. **Nucleus**, 15(1):85-93, 2018.

CERQUEIRA, F. B.; FARIA A. J. G. de; SANTOS, P. F. dos. Desenvolvimento inicial da mandioca 'Cacau' sob diferentes posições da maniva. **Tecnologia & Ciência Agropecuária**, 10:16-21, 2016.

CHAPLA, P. I. et al. PH, carvão ativado e agentes geleificantes do meio de cultura no crescimento *in vitro* de *Miltonia flavescens* Lindl. **Plant Cell Culture & Micropropagation**, 5(2):87-93, 2009.

CIAT. **El cultivo de meristemas para la conservación de germoplasma de yuca *in vitro***; unidad audiotutorial. (CIAT. Guia de Estudio. Serie 045C-05.03). Cali, 1984. 44p.

CIAT. **El cultivo de meristemas para el saneamiento de clones de yuca**; unidad audiotutorial. (CIAT. Guia de Estudio. Serie 045C-02.05). Cali, 1982. 45p.

COPATTI, A. et al. Reguladores vegetais na multiplicação *in vitro* de Tamarilo (*Solanum betaceum)*. **Revista da Jornada da Pós-Graduação e Pesquisa Congrega Urcamp**, 13:89, 2016.

DEMEKE, Y. et al. Effects of plant growth regulators on *in vitro* cultured nodal explants of cassava (*Manihot esculenta* Crantz) clones. **African Journal of Biotechnology**, 13(28):2830-2839, 2014.

DEZAN, L. F. et al. Crescimento *in vitro* de *Schomburgkia gloriosa* Lindl. em meio de cultivo simplificados. **Idesia**, 30(2):53-58, 2012.

DIBAX, R. et al. Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis* Dehn and histological study of organogenesis *in vitro*. **Brazilian Archives of Biology and Technology**, 53(2):311-318, 2010.

ERIG, A. C.; SCHUCH, M. W.; BRAGA, E. J. B. Enraizamento *in vitro* de pereira (*Pyrus communis* L.) cv. Carrick. **Ciência Rural**, 34:275-277, 2004.

FAYE, A. et al. Effects of different hormones on organogenesis *in vitro* of some varieties of cassava (*Manihot esculenta* Crantz) grown in Senegal. **African Journal of Plant Science**, 9(8):305-312, 2015.

FLORES, R. et al. Benzilaminopurina (BAP) e thidiazuron (TDZ) na propagação *in vitro* de *Pfaffia glomerata* (Spreng.) Pedersen. **Revista Brasileira de Plantas Medicinais**, 11(3):292-299, 2009.

FLORES, R. et al. Otimização da produção de plantas *in vitro* de cultivares de *Ipomoea batatas*. **Revista de Ciências Agrárias**, 38(3):429-437, 2015.

FREITAS, R. M. O.; NOGUEIRA, N. W.; PRAXEDES, S. C. Multiplicação de anador (*Justicia pectoralis*) *in vitro*. **Revista Verde de Agroecologia e Desenvolvimento Sustentável**, 11(3):159-163, 2016.

GEORGE, E. F.; HALL, M. A.; KLERK, G. J. de. **Plant propagation by tissue culture**. 3. ed. Dordrecht: Springer, 2008. 501p.

KABIR, M. H. et al. *In vitro* propagation of cassava (*Manihot esculenta* Crantz). **Nuclear Science and Applications**, 24(1-2):23-28, 2015.

LENCINA, K. H. et al. *In vitro* establishment and growth of apuleia seedlings. **Ciência Rural**, 44(6):1025-1030, 2014.

LLOYD, G.; Mc COWN, B. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. **International Plant Propagators Society Proceedings**, (30):421-427, 1980.

MAFLA, G. et al. **Manual de procedimientos para la conservación *in vitro* del germoplasma del género *Manihot*.** Cali: CIAT, 2010. 54p.

MAPAYI, E. F. et al. Optimization of *in-vitro* propagation of cassava (*Manihot esculenta* Crantz) genotypes. **Journal of Agricultural Science**, 5(3):261, 2013.

MIRANDA, N. A. et al. Meio de cultura, reguladores de crescimento e formas de vedação de tubos de ensaio na multiplicação *in vitro* de candeia (*Eremanthus incanus* Less.). **Scientia Forestalis**, 44(112):1009-1018, 2016.

MIYATA, L. Y.; VILLA, F.; PASQUAL, M. Culture media used in the micropropagation of orchids hybrids. **Semina: Ciências Agrárias**, 35(4):1731-1738, 2014.

MONGOMAKE, K. et al. Somatic embryogenesis and plant regeneration of cassava (*Manihot esculenta* Crantz) landraces from Cameroon. **Springer Plus**, 4(1):477, 2015.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, 15(3):473-497, 1962.

NASSAR, N. M. A.; GRATTAPAGLIA, D. Variabilidade de clones de mandioca em relação a fertilidade e aspectos morfológicos. **Turrialba**, 36:555-559, 1986.

OLIVEIRA-CAUDURO, Y. D. et al. Organogênese indireta a partir de explantes foliares e multiplicação *in vitro* de brotações de *Eucalyptus benthamii* x *Eucalyptus dunnii*. **Ciência Florestal**, 24(2):347-355, 2014.

PEREIRA, J. E. S.; FORTES, G. R. de L.; SILVA, J. B. da. Crescimento de plantas micropropagadas de macieira em casa de vegetação com aplicações de ácido giberélico. **Pesquisa Agropecuária Brasileira**, 36(6):881-886, 2001.

R DEVELOPMENT CORE TEAM. 2017. **R:** A language and environment for statistical computing, reference index version 3.4.2. R foundation for statistical computing, Vienna, Austria. Available in: <http:// www.R-project.org>, Access in: 19 may, 2017.

REIS, L. et al. Estratificação *in vitro* de embriões zigóticos de pessegueiro em diferentes meios de cultura e concentrações de sacarose. **Revista Brasileira de Fruticultura**, 34(3):653-660, 2012.

RIBEIRO, M. D. N. O. et al. *In vitro* propagation of *Calla lily*: Adenine sulphate and 6-benzilaminopurine. **Ornamental Horticulture**, 20(1):21-26, 2014.

ROCA, W. M. et al. Eliminación de virus y propagación de clones en la yuca (*Manihot esculenta* Crantz). In: ROCA, W. M.; MROGINSKI, L. A. (Ed.). **Cultivo de tejidos en la agricultura:** Fundamentos y aplicaciones. Cali: CIAT, 1991. p.403-420.

SHIJI, R. et al. Effect of NAA and IBA on *in vitro* regeneration and hardening in cassava (*Manihot esculenta* Crantz.). **Journal of Root Crops**, 40(2):12-20, 2014.

SIMÕES, K. da S. et al. Micropropagação de Inhame da Costa sob concentrações de BAP em distintos meios de cultura. **Magistra**, 28(3-4):342-351, 2017.

SOUSA, G. G. de et al. Aclimatização de *Brassavola tuberculata* Hook embebida com ANA em diferentes substratos. **Horticultura Brasileira**, 33(2):208-2015, 2015.

SOUZA, A. da S. et al. **Micropropagação da mandioca mediante ápices caulinares e segmentos nodais**. (Embrapa Mandioca e Fruticultura Tropical. Circular Técnica, 88). Cruz das Almas, BA: Embrapa Mandioca e Fruticultura Tropical, 2008. 12p.

TAIZ, L.; ZEIGER, E. **Fisiologia vegetal**. Porto Alegre: Artmed, 2008. 819p.

THAKUR, A.; KANWAR, J. S. Micropropagation of 'Wild Pear *'Pyrus pyrifolia* (Burm F.) Nakai. I. Explant Establishment and Shoot Multiplication. **Notulae Botanicae Horti Agrobotanici Cluj-Napoca**, 36(1):103-108, 2008.

TORRES, A. C. et al. **Meio e condições de incubação para a cultura de tecidos de plantas**: Formulações de meios de cultura de tecidos de plantas. Embrapa Hortaliças. Circular Técnica, 24. Brasília: Embrapa Hortaliças, 2001. 19p.

VILLA, F. et al. Multiplicação *in vitro* da amoreira-preta 'Ébano' em diferentes concentrações de meio MS e BAP. **Ciência e Agrotecnologia**, 29(3):582-589, 2005.

VILLA, F. et al. Sacarose e um aditivo orgânico complexo na micropropagação de amoreira-preta (*Rubus sp*.). **Plant Cell Culture & Micropropagation**, 5(1):1-8, 2009.

WERNER, E. T. et al. Coeficiente de variação como medida da precisão em experimentos de cultura de tecidos de plantas. **Plant Cell Culture & Micropropagation**, 8(1-2):27-36, 2013.