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Abscisic acid and *in vitro* conservation of *Manihot* wild species

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ABSTRACT. Abscisic acid (ABA) is associated with bud dormancy, leaf abscission, and germplasm growth inhibition in *in vitro* conservation. We evaluated the effects of ABA in four wild *Manihot* accessions and one cassava accession (*M. esculenta* Crantz) to refine *in vitro* conservation methods for these species. The experiment was performed at the Laboratory for Tissue Culture from Embrapa, Cruz das Almas, Bahia State, Brazil. The statistical design was completely random in a 5 × 5 factorial scheme [(5 ABA dosages (0, 0.25, 0.50, 0.75, and 1 mg L^{-1}) and 5 *Manihot* species (*M. pseudoglaziovii*, *M. tristis*, *M. flabellifolia, M. chlorosticta,* and *M. esculenta*)], with 15 replicates. Mini-cuttings of 1 cm were used, each inoculated in 10 mL of modified Murashige and Skoog medium, solidified with Phytagel® (2.4 g L^{-1}) containing the respective ABA dosages. Tubes containing these mini-cuttings were placed in a germplasm conservation room with an irradiance of 30 µmol m^{-2} s^{-1}, temperature of 22 ± 1°C, and photoperiod of 12 hours. Plant height (cm), the number of living and senescent leaves, shoots, and mini-cuttings (1 cm), and fresh and dry weights of shoots and roots (mg) were evaluated after 150 days. Growth reduction was prominent in *M. pseudoglaziovii*, *M. tristis*, and *M. flabellifolia* during the *in vitro* conservation period. In the present study, the addition of ABA did not promote the expected reduction in plant growth.

Keywords: growth inhibitor; plant genetic improvement; *in vitro* germplasm.

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Introduction

The *Manihot* genus has economic importance, particularly *M. esculenta Crantz* (cassava), which has a high commercial value. Cassava roots are cultivated worldwide and serve as a staple food for millions of people, primarily in tropical and subtropical regions of Africa, Asia, and Latin America (Kuria et al., 2017). According to FAO data (2019), for that year, the world production of cassava was over 303 million tons, with Nigeria, Democratic Republic of Congo, Thailand, Ghana, Brazil, and Indonesia being the main producing countries. Brazil is the fifth largest producer of cassava globally, with a production of more than 17 million tons of roots.

The wild *Manihot* species have the potential for use in genetic improvement, as according to Fukuda and Iglesias (2006), they have resistance and tolerance genes against biotic factors, which through introgressive hybridization can enable the development of more productive varieties that are less susceptible to the negative factors affecting cassava (*M. esculenta* Crantz) crops. Using wild species for breeding increases the genetic diversity in plant breeding programs of cultivated species (Fukuda & Iglesias, 2006).

In vitro conservation of germplasm using tissue culture techniques has emerged as an alternative to germplasm banks in the field, which are exposed to pests and pathogens, climatic conditions, vandalism, human failures, and errors in access identification, among others. *In vitro* conservation is convenient, as it is economically feasible and practical (Withers & Williams, 1998; Sanghamitra, Samantaray, Bagchi, & Mandal, 2019), which allows for the conservation of a large quantity of plants in a reduced space, enables the preservation of security copies of field accessions, and simplifies germplasm exchange and trade between institutions, regions, and countries.

In vitro conservation may be achieved under minimum growth conditions, where changes in culture medium are performed in order to slow down or suppress part of the cell growth of tissues and organs, with the main objective of increasing the interval between sub-cultures at a maximum or extending them over a

long period (Roca, Nolt, Mafla, Roa, & Reyes, 1991; Withers & Williams, 1998). This would reduce the labor force and the required area for preservation and provide immediate access to the entire germplasm while promoting genetic stability of the plants, preserving their characteristics after storage periods, and reducing the number of lost individuals (Kovalchuk, Lyudvikova, Volgina, & Reed, 2009; Santos, Lédo, Lédo, Souza, & Silva Junior, 2011).

One strategy for minimal growth consists of the addition of growth retardants to the culture medium, since they are synthetic compounds that reduce stem elongation without harming plant viability and could intensify leaf coloration (Roca, Arias, & Chavéz, 1991; Withers & Williams, 1998). Additionally, these compounds inhibit cell division in the subapical meristem of buds and reduce leaf production and root growth (Gianfagna, 1987).

Abscisic acid (ABA) operates in numerous ways in plants, influencing the regulation of various physiological processes and allowing plants to remain protected under stress conditions, such as excess salinity, water deficit, and thermal stress (Rai et al., 2011). It is also considered a growth inhibitor and is associated with the dormancy of buds, seeds, and underground organs (Moosikapala & Te-chato, 2010; Taiz & Zeiger, 2017). Furthermore, it induces stomatal closure, which provides protection against water deficit by reducing water loss from transpiration that could limit carbon assimilation and consequently biomass production (Taiz & Zeiger, 2017). ABA also has direct effects on foliar, flower, and fruit abscission.

In vitro conservation represents one of the most used alternatives to preserve and provide cassava germplasm, primarily because it is vegetatively propagated. This strategy is relevant for the conservation of wild species that are difficult to preserve in the field or *in vitro*. Despite various studies concerning the evaluation of the effects of ABA on the *in vitro* conservation of germplasm (Watt, Thokoane, Mycock, & Blakeway, 2000; Lemos, Ferreira, Alencar, Ramalho Neto, & Albuquerque, 2002; Gopal, Chamail, & Sarkar, 2004; Rai et al., 2011; Sá, Ledo, & Ledo, 2011; Santos, Arrigoni-Blank, & Blank, 2012), there is little data on its impacts on cassava (Sato et al., 2001; Barrueto Cid & Carvalho, 2008), and no literature has been found regarding the effects on wild species of *Manihot*.

The lack of available information to improve *in vitro* conservation methods for *Manihot* wild species justifies further studies to develop efficient methodologies. This study was a pioneer in tissue culture using ABA in the *in vitro* conservation of species of the *Manihot* genus that aimed to evaluate the effect of different concentrations of ABA in four wild accessions of *Manihot* and one of cassava (*M. esculenta*) to enhance *in vitro* protocols for conservation of wild species through minimizing plant growth.

Materials and methods

The experiment was performed from September 2019 to February 2020 at the Tissue Culture Laboratory, Advanced Biology Nucleus from Embrapa Cassava and Fruits, Cruz das Almas, Bahia State, Brazil. Five wild species were used in the experiment: *Manihot pseudoglaziovii* Pax & Hoffman, *M. tristis* Müll.Arg, *M. flabellifolia* Pohl, *M. chlorosticta* Standl, and access BGM 0868 (Thin Bark) of *M. esculenta* Crantz, from the Embrapa Cassava and Fruits *in vitro* collection.

Previously micropropagated plants were used to obtain the explants. These plants were selected in a laminar flow cabinet to obtain mini-cuttings of approximately 1 cm in length and containing one bud. The mini-cuttings were then inoculated in test tubes (2.5 × 15 cm) containing 10 mL of Murashige and Skoog (1962) culture medium following Souza, Souza, Santos-Serejo, Junghans, and Silva Neto (2008), supplemented with ABA at concentrations of 0, 0.25, 0.50, 0.75, and 1 mg L^{-1}, hardened with Phytagel® (2.4 g L^{-1}), adjusted to a pH of 5.8, and autoclaved for 20 min. at 120°C.

A completely random design with a factorial scheme of 5 × 5, consisting of five ABA concentrations and five *Manihot* accessions with 15 replicates was used in the experiment, where each parcel constituted one explant (mini-cutting) cultivated in one test tube.

Test tubes containing the explants were kept for 150 days in a germplasm conservation room with an irradiance of 30 µmol m^{-2} s^{-1}, temperature of 22 ± 1°C, and photoperiod of 12 hours.

Plants were then evaluated for plant height (PH, cm), number of living leaves (NLL), number of senescent leaves (NSL), number of shoots (NS), number of mini-cuttings with a size of 1 cm (NMC), fresh weight of shoots (FWS; mg), and fresh weight of roots (FWR; mg). The material was then identified and set in a forced air oven at 70°C, and after 48 hours, when the weight of the sample remained constant, the dry weight of shoots (DWS; mg) and dry weight of roots (DWR) were determined.

Registered data were subjected to variance analysis and the F-test. The means of each accession were compared using the Tukey test at 5% probability, and mean values of the ABA concentrations were adjusted to polynomial regression models. The number of living leaves, senescent leaves, shoots, and mini-cuttings were transformed to $\sqrt{x + 0.5}$, to fulfill variance analysis presuppositions. Statistical analyses were performed using the package "*ExpDes.pt"* (Ferreira, Cavalcanti, & Nogueira, 2018) implemented in the R software version 3.4 (R Development Core Team, 2018).

Results and discussion

The results of the analysis of variance are shown in Table 1 and reveal that the accessions used in the present study had a highly significant influence on all the variables analyzed, possibly because of the wide genetic variability of the *Manihot* genus (Nassar & Grattapaglia, 1986; Venturini et al., 2016), a factor that affirms its potential for exploration in plant breeding programs.

Table 1. Summary of the analysis of variance for plant height (PH; cm), number of living leaves (NLL), number of senescent leaves (NSL), number of shoots (NS), number of mini-cuttings (NMC), fresh weight of shoots (FWS; mg), dry weight of shoots (DWS; mg), fresh weight of roots (FWR; mg), and dry weight of roots (DWR; mg), from accessions of *Manihot pseudoglaziovii, M. tristis*, *M. flabellifolia*, *M. chlorosticta,* and BGM 0868 (Thin Bark) with varied concentrations of abscisic acid (ABA; mg L^{-1}), after 150 days of *in vitro* conservation.

SV	DF	PH	NLL	NSL	NS	NMC	FWS	DWS	FWR	DWR
ABA	4	9.82^{ns}	1.14*	1.05^{ns}	0.96^{ns}	0.28^{ns}	20605.00^{ns}	656.90^{ns}	34453.00^{ns}	1150.20**
Accessions	4	1541.89**	3.50**	26.49**	14.37**	1.85**	1203792.00**	20515.00**	675764.00**	10792.00**
ABA * Accessions	16	77.44^{ns}	0.99**	1.05*	0.86^{ns}	0.18^{ns}	96572.00^{ns}	1167.40^{ns}	24894.00^{ns}	975.9**
Error	185	46.90	0.45	0.54	0.56	0.19	51296.00	814.90	16117.00	326.70
Mean		13.16	4.11	8.28	7.26	0.76	398.67	57.43	287.31	32.88
CV (%)		52.02	33.47	26.44	28.40	42.34	56.81	63.51	44.19	54.97

SV = Source of variation; DF = Degree of freedom, ns = non-significant, **, and * = significant at 1% and 5% probability, respectively, by the F-test.

There was a highly significant effect ($p < 0.01$) on the interaction of ABA × Accessions for NLL and DWR, and a significant effect ($p < 0.05$) was observed in NSL, whereas most variables had no significant effect on the isolated ABA factor (Table 1).

In vitro conservation of the accessions showed different growth results when considering the coefficients of variation (CV), which varied from 26.44% to 63.51% (Table 1) for NSL and DWS, respectively. According to Werner, Motta, Martins, Lima, and Schmildt (2013), these values may be associated with the inconsistent plant growth observed in *in vitro* studies, which is common in tissue culture works, thereby safeguarding experimental precision. During *in vitro* conservation of *Hancornia speciosa*, Sá, Ledo, and Ledo (2011) observed a coefficient of variation oscillating from 35.65% to 112.44%, while Rezende et al. (2018) obtained a CV from 18.9% to 82.10% when studying *in vitro* conservation of *Physalis peruviana* L. under minimal growth.

The mean values of the variables that separately showed significance for factor accessions are presented in Table 2.

Table 2. Mean values for plant height (PH; cm), number of shoots (NS), number of mini-cuttings (NMC), fresh weight of shoots (FWS; mg), dry weight of shoots (DWS; mg), and fresh weight of roots (FWR; mg) from accessions BGM 0868 (Thin Bark), *Manihot chlorosticta, M. flabellifolia, M. pseudoglaziovii, and M. tristis*, after 150 days of *in vitro* conservation.

Accessions	PH	NS	NMC	FWS	DWS	FWR
BGM 0868	15.87 b	9.39 a	1.00 ab	491.83 ab	80.96 a	440.85 a
M. chlorosticta	22.65 a	11.20 a	1.67 a	591.55 a	77.14 a	385.79 a
M. flabellifolia	11.45 c	6.41 b	0.43 bc	384.90 b	53.15 b	155.97 c
M. pseudoglaziovii	7.71 c	5.05 bc	0.68 bc	236.15 c	49.11 b	282.51 b
M. tristis	8.21 c	4.28 c	0.14 c	209.99 c	27.13 c	184.42 c

Means followed by different letters within columns are statistically different according to Tukey's test at 5% probability.

To attain effective *in vitro* conservation, it is important to consider that plant metabolism reduction must be associated with the preservation of plant viability (Withers & Williams, 1998; Arrigoni-Blank et al., 2014). It is desirable that PH and number of senescent leaves have inferior mean values, while the other variables present higher means, as this allows the verification of the feasibility of the growth reduction and later, the recovery and multiplication of plants kept under *in vitro* conservation.

Manihot pseudoglaziovii, M. tristis, and *M. flabellifolia* showed the lowest mean values for PH of 7.71, 8.21, and 11.45 cm, respectively, with no statistical differences between them. Vieira, Silva, Zaffari, and Feltrim

(2014) revealed that the addition of 0.10 mg L^{-1} ABA to the culture medium promoted the lowest PH, proving the inhibition effect of this plant growth regulator in *Allium sativum* L. In addition, abscisic acid can modify the synthesis or activity of cytokinins, which are used in the culture medium to break the apical dominance of shoots and increase the multiplication rate (Lemos, 2000).

BGM 0868 (9.39) and *M. chlorosticta* (11.20) presented higher mean values for the number of shoots compared to the other accessions. This result is in contrast with observations of Barrueto Cid and Carvalho (2008), who stated that a total inhibitory effect occurred in *M. esculenta* at concentrations higher than 2.6 mg L^{-1} of ABA during *in vitro* conservation. However, those authors kept cassava plants at the concentrations mentioned for only 90 days, while the present study used an extended period of 150 days with ABA concentrations lower or equal than 1 mg L^{-1}. Lemos et al. (2002) demonstrated that adding 1 mg L^{-1} ABA to the culture medium MS allowed the preservation of sugarcane micro-plants for 12 months.

The superior behavior of the accessions previously mentioned was also observed with NMC, FWS, and FWR. Therefore, these accessions showed sustained viability, and could support higher dosages of the inhibitor. In addition, the strong performance of these variables may guarantee efficient multiplication and adequate maintenance of plants after the *in vitro* preservation period.

Although the interaction between ABA concentrations and *Manihot* accessions was significant for the number of living leaves, the linear decreasing equation model only fit *M. tristis* with biological meaning (Figure 1).

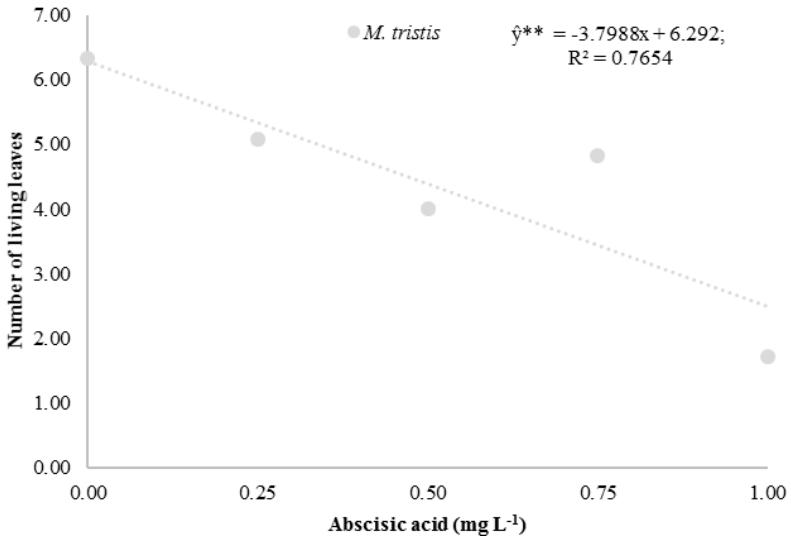

Figure 1. Number of living leaves produced by *Manihot tristis,* with different concentrations of ABA (mg L^{-1}), 150 days after *in vitro* conservation.

As observed in Figure 1, the estimated value (6.29) for *M. tristis* was obtained in the absence of ABA; however, the number of living leaves decreased as the concentration increased. Despite this result, living leaves were observed at the maximum ABA dosage (1 mg L^{-1}), showing that the plants conserved viability and could tolerate higher dosages of the inhibitor.

This result corroborates that of Arrigoni-Blank et al. (2014), who used 2 mg L^{-1} of ABA in the conservation of sweet potato genotypes IPB-052 and IPB-007 and registered reduced plant growth and preservation of green leaves, allowing later regeneration and multiplication of these genotypes. Bello-Bello, García-García, and Iglesias-Andreu (2015) established an *in vitro* conservation protocol for *Vanilla planifolia* Jacks. at a dose of 3 mg L^{-1} of ABA.

Concerning the variable NSL, the fitting of a linear increasing equation model with biological meaning was possible only for *M. pseudoglaziovii* (Figure 2). A lower estimated value (5.52) was obtained in the absence of ABA, with the number of senescent leaves increasing as the inhibitor dosage increased, proving that ABA promotes senescence rather than abscission itself (Taiz & Zeiger, 2017).

The significance of the interaction between the factors studied and DWR is shown in Figure 3, where a linear decreasing equation model with biological meaning was obtained for BGM 0868 (Thin Bark), with the highest estimated value of 69.70 mg obtained in the absence of ABA. Despite the DWR reduction with the increase of ABA concentration there was observed an estimated value of 39.36 mg at the maximum dosage

(1 mg L^{-1}), possibly related to the inhibitory effect of ABA on root growth in an *in vitro* cultivation system (Pilet & Barlow, 1987). Kaminska, Skrzypek, Wilmowicz, Tretyn, and Trejgell (2016) showed that the addition of ABA to the culture medium significantly reduced the rooting of *Taraxacum pieninicum* Pawl.

The opposite was observed in *M. pseudoglaziovii*, with a linear increasing equation model and higher estimated value of 53.24 mg at a dosage of 1 mg L^{-1}, suggesting that DWR was incorporated with the increment of ABA concentrations.

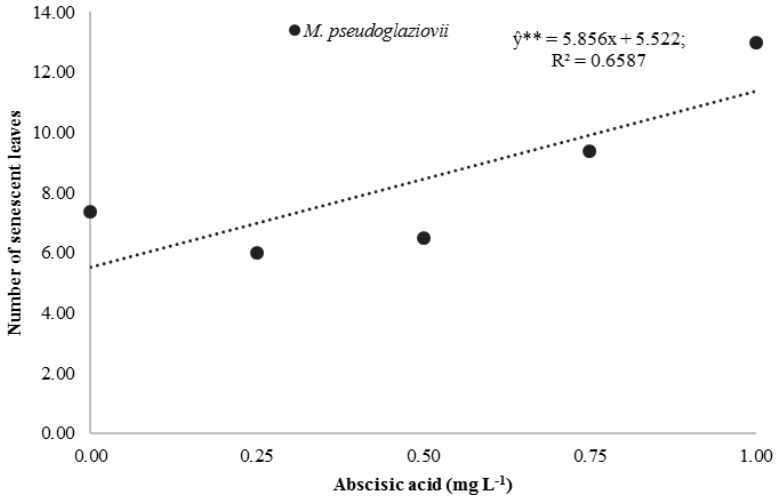

Figure 2. Number of senescent leaves in *Manihot pseudoglaziovii* with to different concentrations of ABA (mg L^{-1}), 150 days after *in vitro* conservation.

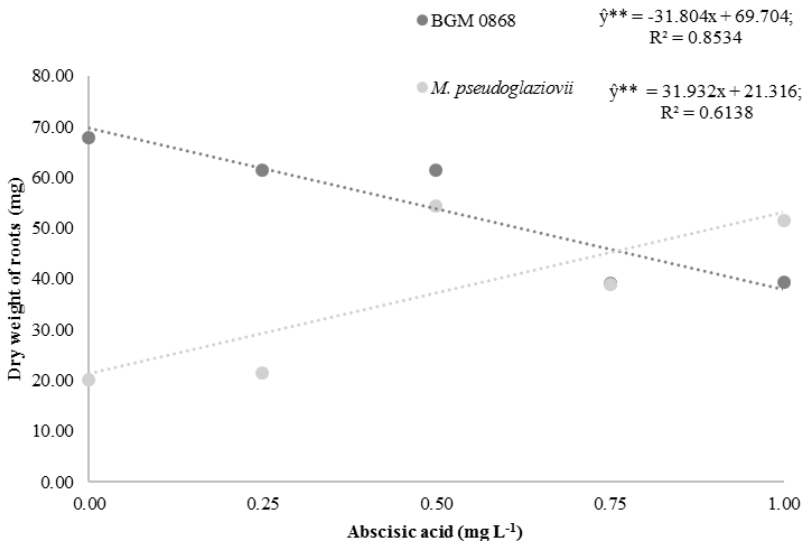

Figure 3. Dry weight of roots produced by BGM 0868 (Thin Bark) and *Manihot pseudoglaziovii* with different concentrations of ABA (mg L^{-1}), 150 days after *in vitro* conservation.

When considering the behavior of the accessions within each ABA concentration regarding living leaves (Table 3), we verified that in the absence of ABA at 0.50 mg L^{-1} there was no significant difference between the accessions. In general, independent of ABA concentrations, *M. flabellifolia* and *M. pseudoglaziovii* were responsible for the highest and lowest means of the number of living leaves, respectively. In contrast, Cabrera et al. (2019) stated that during the *in vitro* conservation of *Ipomoea batatas* (L.) no leaf formation was observed in plants grown in media containing ABA.

As displayed in Table 3, *M. tristis* had the lowest means for NSL with a value of 1.71, at a concentration of 1 mg L^{-1} ABA, which was statistically different from all other accessions. In contrast, *M. chlorosticta* attained the highest mean of 13.89 at a dosage of 0.25 mg L^{-1} ABA. According to Taiz and Zeiger (2017), leaf senescence is an adverse process that triggers degradation of cellular contents and promotes destruction of organelles, harming the energy potential of plants.

Table 3. Mean values for number of living leaves, number of senescent leaves, and dry weight of roots (mg) in plants from accessions BGM 0868 (Thin Bark), *Manihot chlorosticta, M. flabellifolia, M. Pseudoglaziovii, and M. tristis* according to concentrations of ABA (mg L^{-1}), 150 days after *in vitro* conservation.

Accessions	Abscisic acid (mg L^{-1})				
	0	0.25	0.50	0.75	1
	Number of living leaves				
BGM 0868	3.33 a	3.42 ab	4.00 a	6.80 a	5.82 a
M. chlorosticta	3.80 a	2.78 ab	1.13 a	5.10 ab	2.71 ab
M. flabellifolia	6.88 a	4.92 a	4.44 a	4.45 ab	6.50 a
M. pseudoglaziovii	4.38 a	1.71 b	3.00 a	1.87 b	1.60 b
M. tristis	6.33 a	5.09 a	4.00 a	4.83 ab	1.71 b
	Number of senescent leaves				
BGM 0868	12.44 b	10.58 bc	14.00 c	8.40 b	11.91 bc
M. chlorosticta	11.40 b	13.89 c	11.62 bc	12.90 b	13.29 c
M. flabellifolia	8.25 ab	7.92 bc	5.33 ab	3.36 a	6.20 b
M. pseudoglaziovii	7.37 ab	6.00 b	6.50 abc	9.38 b	13.00 c
M. tristis	4.55 a	2.00 a	2.55 a	2.33 a	1.71 a
	Dry weight of roots (mg)				
BGM 0868	67.94 a	61.37 a	61.32 a	39.02 a	39.36 ab
M. chlorosticta	28.52 b	29.11 b	67.14 a	26.4 ab	37.9 ab
M. flabellifolia	27.28 b	24.72 b	18.82 c	9.11 b	14.82 bc
M. pseudoglaziovii	20.16 b	21.54 b	54.45 ab	38.83 a	51.43 a
M. tristis	13.78 b	16.64 b	26.52 c	17.03 ab	12.16 c

Means followed by different letters within the column differ according to Tukey's test at 5% probability.

The highest mean values for DWR were observed in BGM 0868 (Thin Bark), at concentrations of 0, 0.25, and 0.75 mg L^{-1} ABA, as showed in Table 3. *M. chlorosticta* and *M. Pseudoglaziovii* had higher means at concentrations of 0.50 and 1 mg L^{-1}, respectively, and the lowest means were observed for *M. tristis* at concentrations of 0, 0.25, and 1 mg L^{-1} ABA. Sato et al. (2001) observed that the use of ABA in the micropropagation of some species aids in the formation of reserve organs, while Yamaguchi and Street (1977) stated that this inhibitor favors the development of roots, especially at low concentrations. However, in the present study, the addition of ABA did not substantially increase the dry weight of roots.

According to Kerbauy (2008), abscisic acid blocks the action of auxins and gibberellins, making it useful for the *in vitro* conservation of plant species. The responses of *Manihot* genotypes to ABA addition in the culture medium were diverse, possibly related to the genetic characteristics of the genotypes used in the present study. A similar conclusion was attained by Arrigoni-Blank et al. (2014), who determined that the addition of ABA in the culture medium of sweet potato was efficient for some genotypes, while others showed detrimental effects. Sanghamitra et al. (2019) obtained satisfactory growth reduction results in *Dioscorea* using higher dosages (10 mg L^{-1}) of ABA.

Given that ABA interferes with various aspects of plant development, antagonizing the action of growth promoters (auxins, cytokinins, and gibberellins), and considering that cytokinins stimulate cell division favoring the development of the plant, ABA in this study effectively blocked the action of cytokinins, thereby, inhibiting growth.

Sato et al. (2001) demonstrated that the inhibitory effect of abscisic acid on growth and development can be used for *in vitro* conservation of cassava germplasm; however, prior to implementation, further studies are required, considering that ABA may increase some stimulating processes at the morphological, physiological, cellular, and molecular levels (Parthier et al., 1992).

Considering the importance of safeguarding genetic resources and the particularities of wild species, it is essential to continue research studies testing the same dosages used in the present work in different accessions and higher dosages in those previously studied to devise clearly defined protocols for each species.

Conclusion

The growth reduction of *M. pseudoglaziovii, M. tristis,* and *M. flabellifolia* was greater than that of the other species tested in this study, considering the variables analyzed during the *in vitro* period.

The addition of abscisic acid did not have the expected effect on plant growth reduction; therefore, it is not possible to recommend a specific dosage for *in vitro* conservation of the species studied.

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