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Paclobutrazol in the *in vitro* conservation of cassava genotypes

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ABSTRACT. *In vitro* germplasm conservation allows to extend the interval between subcultures without compromising the viability and genetic integrity of the plant, ensuring a backup of genotypes with high phytosanitary quality. Thus, this study aimed to verify the effect of four concentrations of Paclobutrazol® in inducing minimum growth in five *Manihot esculenta* accessions from the *in vitro* Active Germplasm Bank of Embrapa Cassava and Fruits. An experiment was installed using the Murashige and Skoog medium without addition and added with four concentrations of Paclobutrazol® (0.10, 0.20, 0.30, and 0.40 mg L^{-1}), in five *in vitro* accessions of *M. esculenta*: BRS Jari (BGM 2041), Cigana (BGM 0264), BRS Poti Branca (BGM 2017), TME 14, and BRS Novo Horizonte. The statistical design was completely randomized in a 5 x 5 factorial scheme, with 15 repetitions. After 120 days of cultivation, the following variables were evaluated: plant height (cm), number of green leaves, number of senescent leaves, number of mini-cuttings, number of shoots, and fresh and dry mass of shoots and roots (mg). Paclobutrazol® caused a reduction in plant height and gain in root mass for all accessions, in addition to preserving the number of green leaves and decreasing leaf senescence for most genotypes. There was a strong dependence of the genotype in relation to the concentration of Paclobutrazol®. The concentration of 0.20 mg L^{-1} showed potential in the *in vitro* conservation of *M. esculenta* genotypes.

Keywords: germplasm conservation; tissue culture; genetic improvement; biotechnology; *Manihot esculenta*.

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Introduction

The genus *Manihot*, belonging to the Euphorbiaceae family, has about 98 species originating from the American continent, comprising the Neotropical regions that range from the South of the United States to the North of Argentina (Rogers & Appan, 1973; Orlandini & Lima, 2014). Additional studies have expanded this list by describing endemic species, with some of them already classified as "endangered" following the criteria of the International Union for Conservation of Nature (Martins, Carvalho, Ledo, & Amorim, 2018; Martins & Ledo, 2015).

Manihot esculenta Crantz is the most relevant species within its genus due to the economic appeal of the cassava crop (Le et al., 2019). This is mainly attributable to the nutritional value of its tuberous roots and leaves in human and animal food, being a source of carbohydrates, vitamin C, calcium, and also other vitamins and minerals (USDA, 2016; Parmar, Sturm, & Hensel, 2017).

Because it is one of the main products that guarantee food security in various parts of the world and is a low-cost food, cassava has become a highly versatile crop, comprising the diet of approximately 800 million people (FAO, 2013; Parmar et al., 2017). The global production of cassava was a record in 2021, with a total of over 314 million tons, of which Brazil contributed with 18 million, occupying the 5th place among the most productive countries, behind Nigeria, Democratic Republic of Congo, Thailand and Ghana (FAO, 2021).

The cassava crop is mainly propagated vegetatively. That leaves it vulnerable to attack by pests, diseases (Sá et al., 2018) and bad weather, jeopardizing the preservation of genotypes with agronomic characteristics of interest for plant genetic improvement, which are usually maintained in the field by Research Institutions. Thus, it becomes necessary to seek effective strategies for the conservation of genetic resources as an alternative to germplasm banks kept in the field, which leave the material susceptible to environmental variations and attack by pests and pathogens.

In this context, tissue culture assumes great importance in the conservation of germplasm by enabling its maintenance *in vitro*, generating savings in physical space and labor (Ramírez-Mosqueda, Cruz-Cruz, Atlahua-Temoxtle, & Bello-Bello, 2019), and allowing to extend the interval between subcultures without compromising the viability and genetic integrity of the plant (Sá et al., 2021; Sá et al., 2022). Such collections serve as a backup of the genotypes, which, due to their *in vitro* condition, can be used in exchange between institutions (Carvalho et al., 2016) and development of new research.

In vitro conservation by slow growth can be achieved through some techniques, such as the use of low temperatures associated with different levels of photon flux density and photoperiod duration (George, Hall, & Klerk, 2007). However, the use of growth-inhibiting substances can also be used to delay plant cell metabolism and prolong subculture cycles for longer periods (Mendes et al., 2021).

In this circumstance, Paclobutrazol® (PBZ) has been used as an *in vitro* growth retardant for several species of woody and herbaceous plants, such as *Poincianella pyramidalis* (Silva, Nepomuceno, Soares, & Santana, 2019), *Laelia anceps* (Ramírez-Mosqueda et al., 2019), *Prunus armeniaca*, *Azarchta indica* (Padilla, Fernández-García, Olmos, Burgos, & Piqueras, 2015), *Citrus* (Mendes et al., 2021), and some wild species of the genus *Manihot* (Sá et al., 2021).

For *M. esculenta*, there are, so far, no studies on its application for minimal *in vitro* plant growth. Thus, considering the global socioeconomic relevance of this crop, and the scarcity of studies with PBZ for the conservation of germplasm in cassava varieties, this study aimed to verify the effect of four concentrations of Paclobutrazol® in inducing minimal growth *in vitro* in five accessions of *M. esculenta* from the Active Germplasm Bank of Embrapa Cassava and Fruits.

Material and methods

The experiment was carried out at the Tissue Culture Laboratory (LCT) of the Nucleus of Advanced Biology (NBA) of Embrapa Cassava and Fruits, located in Cruz das Almas, Bahia. Five cassava accessions were studied: BRS Jari (BGM 2041), Cigana (BGM 0264), BRS Poti Branca (BGM 2017), TME 14, and BRS Novo Horizonte.

As sources of explants, previously micropropagated plants were used, from which minicuttings with ± 1 cm in length and containing at least 1 lateral bud were extracted, excluding the apical and basal minicuttings because they had different physiological stages.

MS culture medium (Murashige & Skoog, 1962) was used with the addition of 20 g L^{-1} of sucrose, 0.01 mg L^{-1} of gibberellic acid (GA_3), naphthalene-acetic acid (NAA) and benzylaminopurine (BAP), in the absence and supplemented with four concentrations of PBZ: 0.10, 0.20, 0.30, and 0.40 mg L^{-1}. The culture medium, which was solidified with Phytagel® (2.4 g L^{-1}), had the pH adjusted to 5.8 and sterilized in an autoclave for 20 min. at 121°C. In a laminar flow chamber, the minicuttings of the different accessions were inoculated in test tubes containing 10 mL of the culture medium with their respective concentration of PBZ and cultivated under a temperature of 27 ± 1°C, a photoperiod of 16 hours and a flow density of photons of 30 µmol m^{-2} s^{-1}.

The experimental design used was completely randomized in a 5 x 5 factorial scheme, with five genotypes of *M. esculenta* and four concentrations of PBZ in addition to the control treatment without PBZ addition, totaling 25 treatments, with 15 replications consisting of a mini-cutting grown in a 25 x 125 mm test tube.

At 120 days of cultivation, the plants were evaluated for the following variables: explants responsive, plant height (PH, cm), number of green leaves (NGL), number of senescent leaves (NSL), number of mini-cuttings ±1 cm long (NMC), number of shoot (NS), 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). To evaluate the dry mass, the material was dehydrated in an oven with forced air circulation and a temperature of 70°C for 48 hours.

After the evaluation, the data obtained were submitted to the F test of the analysis of variance by the R software version 3.4 (R Development Core Team, 2018), using the 'ExpDes.pt' package. The polynomial regression models were adjusted for the average PBZ concentrations. Values were transformed into $\sqrt{x} + 0.5$ to meet the assumptions of the analysis of variance, except for the NGL, where the original data were used. For the averages obtained, non-transformed values were considered. For the calculation of the error in the Mean Square, the responsive explants for each treatment were considered, excluding the lost plots. In the regression equations, the minimum and maximum points that determined the optimal concentrations of PBZ were obtained considering the most effective concentration in the extension of the subculture, according to the behavior of the equations.

Results and discussion

Figure 1 shows the number of explants responsive to PBZ treatments, where an *in vitro* response was achieved for 69.07% of the explants. As there was an increase of PBZ to the treatments, it is observed that a greater number of explants did not respond to the treatment, evidencing the strong action of PBZ as a growth inhibitor.

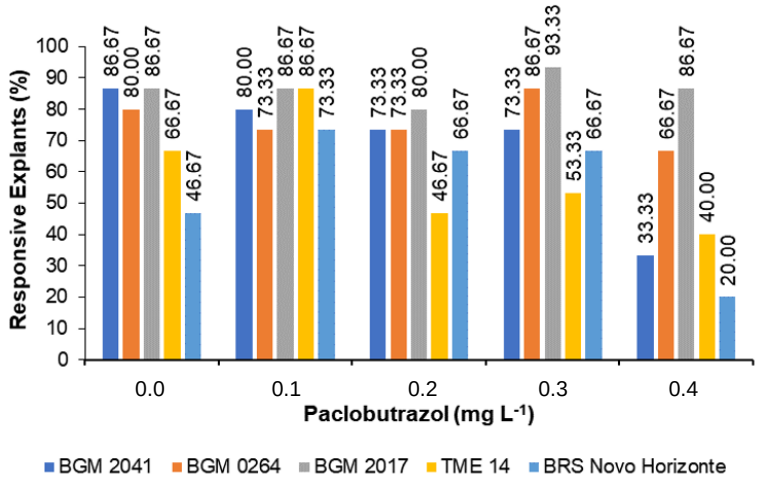

Figure 1. Responsive explants for accession BGM 2041, BGM 0267, BGM 2017, TME 14, and BRS Novo Horizonte, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation.

According to the analysis of variance (Table 1) there was a highly significant effect ($p < 0.01$) for all variables analyzed in the genotype x PBZ interaction. The high significance obtained is possibly a result of the wide genetic variability found in cassava, showing the great potential for exploring the crop in genetic improvement programs, which reinforces the need to preserve these genotypes in germplasm banks.

The coefficient of variation (CV) varied between 18.88% and 46.81% for the variables shoot dry mass and number of green leaves, respectively. Werner, Motta, Martins, Lima, and Schmidt (2012) consider that CVs, in experiments with tissue culture, usually have a large amplitude because it is a technique with hidden sources of variation and difficult to control. For the same authors, the genetic variability of the materials worked, the light intensity that each experimental plot receives and the physiological state of the plant that donor the explants exert a strong influence on the CV.

Table 1. Summary of analysis of variance for variables plant height (PH, cm), number of green leaves (NGL), number of senescent leaves (NSL), number of mini-cuttings ±1 cm long (NMC), number of shoot (NS), fresh weight of shoots (FWS, mg), fresh weight of roots (FWR, mg), dry weight of shoots (DWS, mg), and dry weight of roots (DWR, mg) for the genotypes BGM 2041, BGM 0264, BGM 2017, TME 14, and BRS Novo Horizonte, depending on the different concentrations of PBZ (mg L^{-1}), at 120 days of *in vitro* conservation.

		Middle Square								
SV	DF	PH	NGL	NSL	NMC	NS	FWS	FWR	DWR	DWR
Genotype	4	1.60 ^{ns}	20.22 *	1.17 ^{ns}	1.65 **	3.52 **	91.16 **	127.42 **	13.80 **	1.05 ^{ns}
PBZ	4	37.93 **	96.98 **	1.37 ^{ns}	4.91 **	4.61 **	856.06 **	89.98 **	33.66 **	25.10 **
Genotype x PBZ	16	2.67 **	25.91 **	2.64 **	1.17 **	1.10 **	114.32 **	84.38 **	9.76 **	8.97 **
Error	234	0.79	7.94	0.78	0.31	0.38	26.82	17.64	2.5	1.65
CV (%)		21.53	46.81	29.48	19.05	42.4	22.39	21.49	18.88	21.17
Mean		18.02	6.02	9.34	8.54	2.13	580.52	406.15	73.15	38.84

SV = Source of variation; DF = Degree of freedom; CV = Coefficient of variation; ns = not significant; ** and * = significant at 1% and 5%, respectively, based on the ANOVA F-test.

Other research with tissue culture also found similar CVs, such as Carvalho et al. (2016), which obtained CV from 13.52 to 42.78% in the conservation of *Citrus* genotypes, and that of Sá et al. (2018) that reached even higher amplitude, from 12.88 to 72.46% in *in vitro* multiplication of *Manihot* wild species.

Below are presented models of regression equations for the genotypes where a statistical difference was observed. For the variables where there were genotypes with non-significant responses (ns), the use of PBZ is not necessary for that evaluated parameter.

The regression models adjusted for the linear and quadratic equations, which had their determination coefficients (R^2) ranging from 51.72% to the number of green leaves in the TME 14 genotype to 98.97% for dry

weight of roots in genotype BGM 2017. These R^2 are consistent with those found by Mendes et al. (2021), which reached similar values, from 54.16 to 92.65% during *in vitro* conservation of *Citrus*, also using Paclobutrazol®, and Santos et al. (2021), which obtained percentages of 52.16 to 98.95% in *in vitro* multiplication of *Manihot* wild species.

Linear regression models were adjusted for the variable plant height in the genotype BGM 0264 and quadratic for the genotypes BGM 2017, TME 14 and BRS Novo Horizonte (Figure 2). For the accessions BGM 0264 and TME 14 the lowest estimated means (16.25 and 9.12 cm) were obtained at the optimal PBZ concentrations of 0.40 and 0.21 mg L^{-1}, respectively. For BGM 2017 and BRS Novo Horizonte, the highest estimated means (26.57 and 28.15 cm) were observed at the optimum concentrations of 0.18 and 0.13 mg L^{-1} of PBZ, respectively.

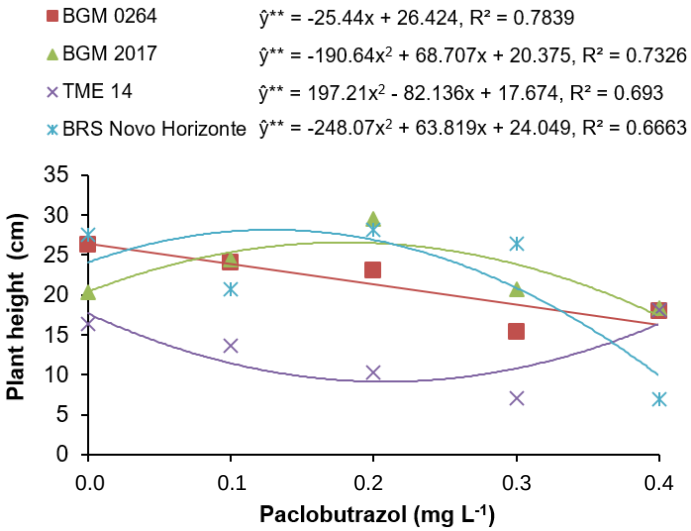

Figure 2. Plant height for accessions BGM 0267, BGM 2017, TME 14, and BRS Novo Horizonte, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

Therefore, with the increase in PBZ concentration, there was a reduction in the average height of the genotypes, generating smaller plants with shorter internodes. Except for TME 14 which, after the minimum point of the equation, tended to resume its growth at the highest concentration. Ramírez-Mosqueda et al. (2019), also reported reduced growth of *Laelia anceps*, due to the increment of PBZ in the culture medium. These results are in accordance with one of the objectives of *in vitro* germplasm conservation, which aims to induce shorter plant height, without compromising its development and regeneration capacity.

According to Seesangboon et al. (2018), PBZ is a triazole widely known as a gibberellin antagonist agent. Triazoles have a lone pair of electrons in the hybridized nitrogen-sp2 present in the heterocyclic ring, found at the periphery of the molecule, enabling a likely interaction with the iron protoheme of cytochrome P450, causing O_2 displacement (Rademacher, Fritsch, Graebe, Sauter, & Jung, 1987).

Thus, PBZ acts by specifically inhibiting the three oxidative steps of ent-caurene to ent-caurenoic acid (Hedden & Graebe, 1985) through inactivation of cytochrome P450-monoxygenase enzymes, located in the endoplasmic reticulum, hindering the biosynthesis of AG12-aldehyde (Kalra & Bhatla, 2018), a general precursor of all gibberellins studied so far, which act in cell elongation and internode extension in plants (Taiz, Zeiger, Møller, & Murphy, 2017). With this, there is a reduction in plant height, blocking the synthesis of gibberellins, without compromising the viability of the plant (Bisht, Rawat, Chakraborty, & Yadav, 2018).

The TME 14 and BRS Novo Horizonte genotypes showed different responses to the addition of PBZ in relation to the number of green leaves. It was possible to fit a linear regression model for TME 14 and a quadratic model for BRS Novo Horizonte (Figure 3). Accession TME 14 showed greater tolerance to PBZ, reaching the highest number of green leaves (6.25) at the highest concentration (0.40 mg L^{-1}), indicating tolerance of the genotype to the growth retardant. The BRS Novo Horizonte accession was more sensitive to higher concentrations of PBZ, showing a reduction in the number of green leaves from 0.13 mg L^{-1}. Note that in the BRS Novo Horizonte variety there is an increase in NGL up to the maximum point on the curve (0.13 mg L^{-1}), showing that at this concentration the growth retardant inhibited leaf senescence which is an important factor in the extension of *in vitro* plant subcultures.

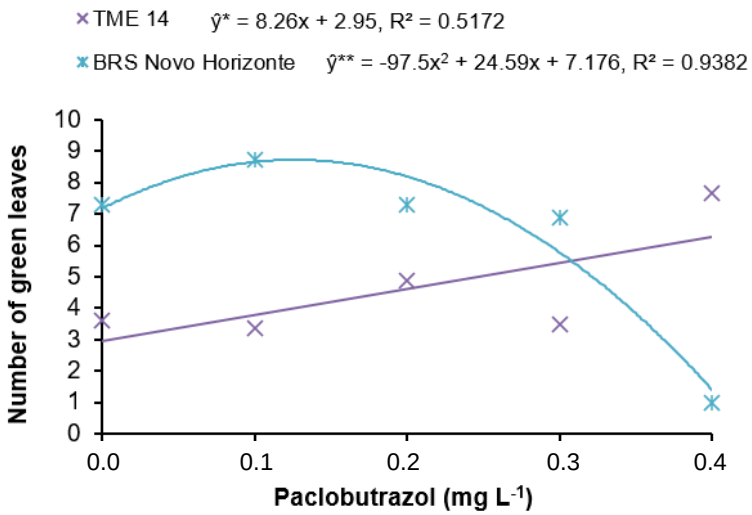

Figure 3. Number of green leaves for accessions TME 14 and BRS Novo Horizonte, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

Triazoles are associated with the reduction of endogenous ethylene levels in plants (Yahia et al., 2019), which in turn is responsible for regulating leaf senescence and abscission (Taiz et al., 2017). Thus, the likely inhibition of ethylene synthesis may have been the cause for maintaining the higher NGL in accession TME 14 when the highest concentration of PBZ was used.

For the number of senescent leaves, a linear regression model was adjusted for the genotype BGM 0264, which produced the lowest estimated value (5.25) at the concentration of 0.40 mg L^{-1} of PBZ, and a quadratic model for TME 14, with the lowest estimated value (7.18) at the concentration of 0.22 mg L^{-1} of the inhibitor (Figure 4), with an increase in senescence after the minimum point. Although TME 14 provided the highest NGL at the highest dose, the leaf senescence also increased progressively after the minimum point. This is a natural result, because at the highest dose of PBZ there was a greater production of the number of leaves (obtained by adding the NGL and NSL), which implies a greater leaf senescence.

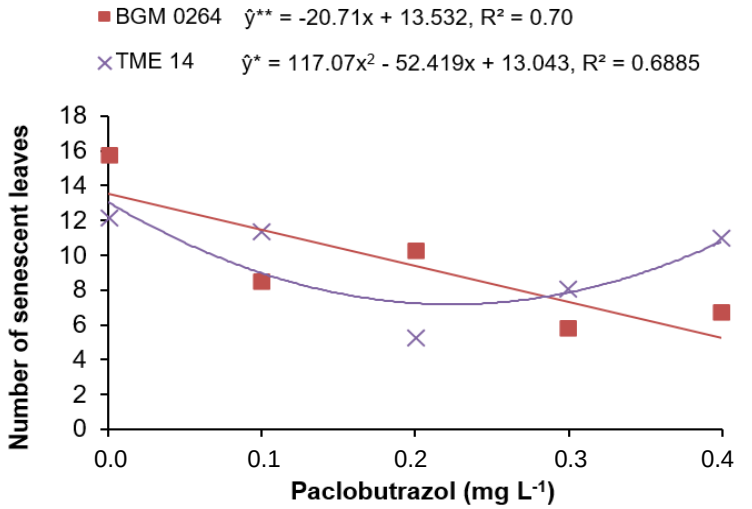

Figure 4. Number of senescent leaves for accessions BGM 0264 and TME 14, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

Research has shown that *M. esculenta* holds great genetic variability (Adjebeng-Danquah et al., 2020; Nunes, Uarrota, Moresco, & Maraschin, 2021). In the process of leaf abscission, the cells of the abscission zone need to develop competence to respond to ethylene (Taiz et al., 2017). Thus, the different morphological responses found for NGL and NSL can be attributed to the high genetic variation found in cassava, which can express different results for the same external stimulus in different accessions of the same species. It is possible that each of the genotypes presents different sensitivities to endogenous ethylene in the cells of the leaf abscission zone.

Regarding the number of mini-cuttings, quadratic regression models were adjusted for accessions BGM 0264 and TME 14, which produced the lowest estimated values at optimal concentrations of 0.30 mg L^{-1} (7.73) and 0.22 mg L^{-1} (5.57) of PBZ, respectively (Figure 5). The BRS Novo Horizonte accession also presented a quadratic regression model, where the highest estimated average (12.14) was obtained at the concentration of 0.10 mg L^{-1} of PBZ. After this concentration, there was a progressive decrease in the NMC. Even with the reduction in values, the plants still have a satisfactory number of mini-cuttings, which is fundamental for the subsequent restoration of the crop.

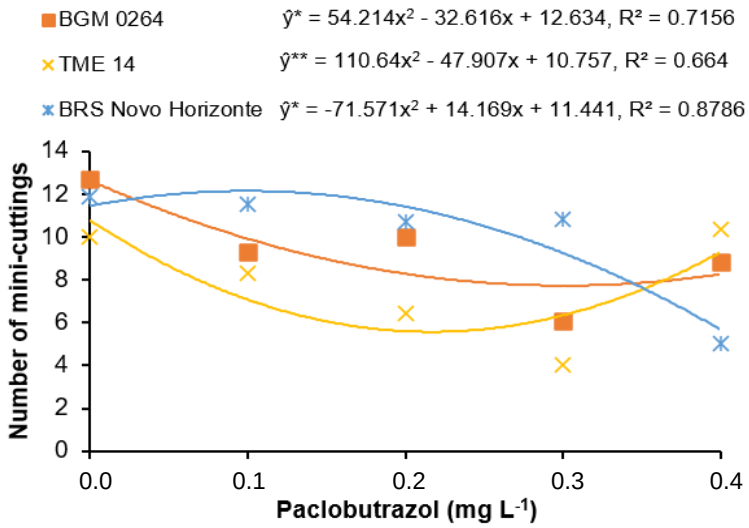

Figure 5. Number of mini-cuttings for accessions BGM 0264, TME 14, and BRS Novo Horizonte, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

With regard to the variable number of shoots, linear regression models were adjusted for accessions BGM 0264 and BRS Novo Horizonte, which generated the highest estimated values of 5.33 and 4.07, respectively, in the absence of PBZ (Figure 6), with an increasing reduction in the number of sprouts with the presence of the retardant in the culture medium. As the PBZ dose increased, the genotypes tended to reduce the number of mini-cuttings and the number of shoots, which was already expected, considering that PBZ also reduced plant height, due to the probable inhibition of gibberellin synthesis.

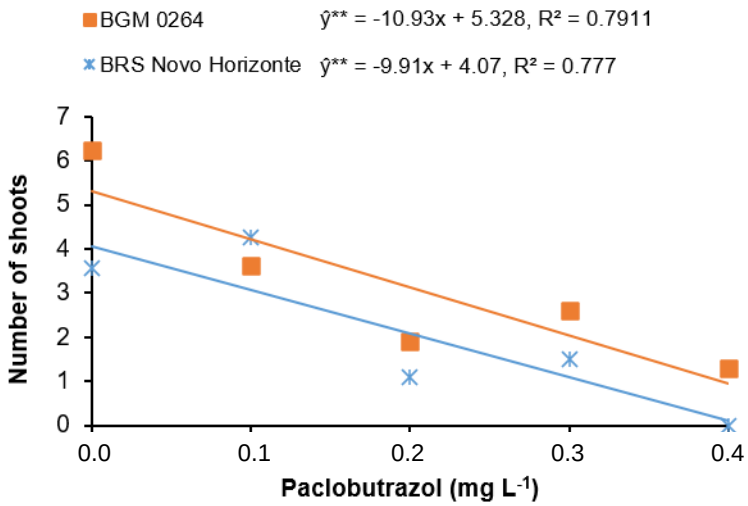

Figure 6. Number of shoots for accessions BGM 0264 and BRS Novo Horizonte, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

In the variable fresh weight of shoots, the lowest values were obtained from quadratic regression models for the accessions BGM 0264 (587.03 mg) and TME 14 (318.53 mg) at the respective optimal concentrations of 0.26 and 0.19 mg L^{-1} of PBZ, while for the BRS Novo Horizonte accession. The same quadratic model was adjusted where the highest estimated mean (881.2 mg) occurred at the concentration of 0.09 mg L^{-1} of PBZ (Figure 7).

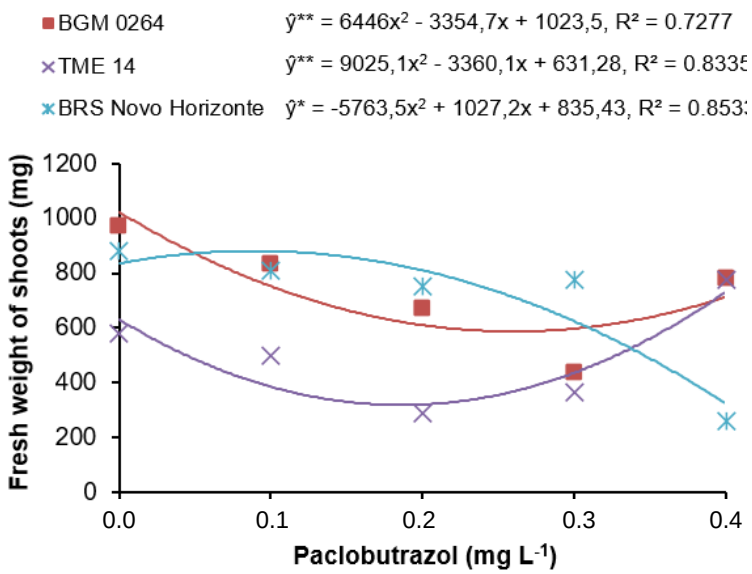

Figure 7. Fresh weight of shoots (mg) for accessions BGM 0264, TME 14, and BRS Novo Horizonte, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

For dry weight of shoots (Figure 8), a linear regression model was adjusted for the BRS Novo Horizonte genotype, which presented the highest estimated mean of 97.16 mg in the absence of PBZ. For accessions BGM 0264 and TME 14, quadratic models were adjusted, where the lowest estimated weight was obtained at concentrations of 0.28 and 0.20 mg L^{-1} of the growth retardant, with averages of 64.21 and 46.50 mg, respectively. The BRS Novo Horizonte accession reduced its fresh and dry weight of shoots as the PBZ doses increased. This is directly related to the reduction in plant height, which could be observed with increases in PBZ levels.

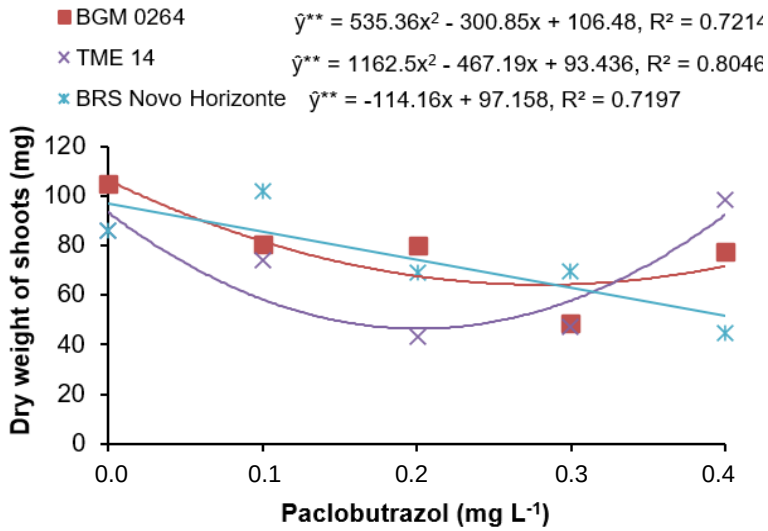

Figure 8. Dry weight of shoots (mg) for accessions BGM 0264, TME 14, and BRS Novo Horizonte, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

In the fresh weight of roots, a linear regression model was obtained for accession BGM 0264, which reached the highest weight (482.90 mg) at the highest concentration of PBZ (0.40 mg L^{-1}), and a quadratic model for the BGM 2017, which reached the highest value (579.27 mg) at the concentration of 0.23 mg L^{-1} of the regulator. The same model was also obtained for TME 14, which reached its lowest value (205.51 mg) at the concentration of 0.14 mg L^{-1} of PBZ (Figure 9).

For the dry weight of roots, a quadratic regression model was adjusted for accession BGM 2017, with the highest average obtained at the optimal concentration of 0.21 mg L^{-1} of PBZ (57.07 mg), and, from there, there was a tendency to reduce the weight. The same quadratic model was obtained for TME 14, which reached the lowest estimated value of 17.36 mg at the optimal dose of 0.16 mg L^{-1} of PBZ (Figure 10). Thus, the presence of PBZ favored the gain of fresh and dry weight in the roots of accession TME 14, which achieved a notable weight

gain in its roots at the highest concentration, when compared to the treatment without the retardant. The same occurred with BGM 0264, which had its fresh root weight increased in the highest concentration of PBZ.

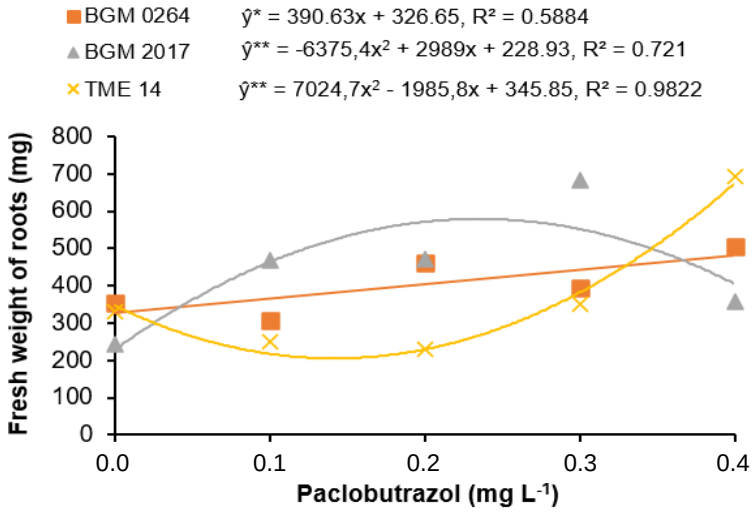

Figure 9. Fresh weight of roots (mg) for accessions BGM 0264, BGM 2017, and TME 14, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

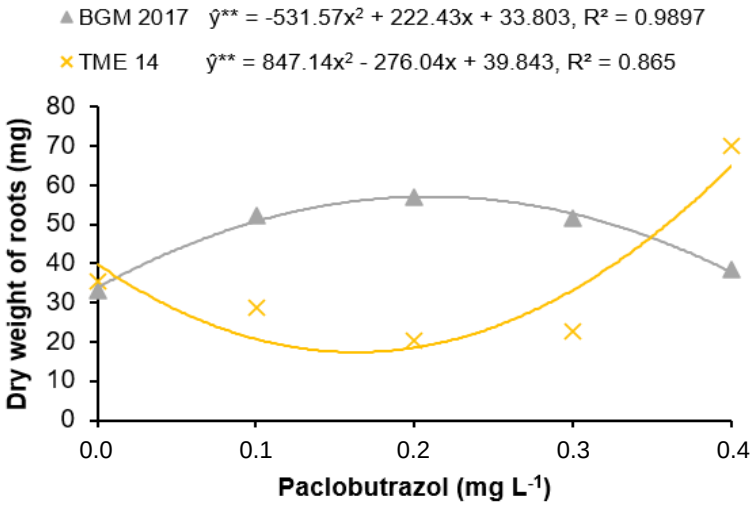

Figure 10. Dry weight of roots (mg) for accessions BGM 2017 and TME 14, with different concentrations of PBZ (mg L^{-1}), 120 days after *in vitro* conservation. ** significant at 1% and * significant at 5% probability by ANOVA F test.

Root growth and weight may be associated with increased partitioning of assimilates towards the roots, due to the lower demand for shoots caused by the action of PBZ (Wang, Byun, & Steffens, 1985). It is also reported an increase in dry weight in potato tubers (Tekalign & Hammes, 2005) and better development and starch accumulation in cassava roots (Panyapruek, Sinsiri, Sinsiri, Arimatsu, & Polthanee, 2016) due to the action of Paclobutrazol®.

Compared to the control treatment, Paclobutrazol® reduced height and favored the highest values of dry and fresh weight in the roots of all *M. esculenta* genotypes. Furthermore, even with the application of the retardant, the plants maintained the ability to preserve green leaves and reduce leaf senescence for most accessions.

Accession TME 14 proved to be resistant to PBZ, and the concentration of 0.40 mg L-1 favored its accumulation of fresh and dry weight of shoots and roots. Therefore, this genotype can tolerate higher doses of the growth retardant.

Silva et al. (2019) report that PBZ reduced shoot formation and favored thickening and root dry weight gain in *Poincianella pyramidalis* plants maintained *in vitro*. PBZ also helps to develop resistance in plants against biotic and abiotic stresses (Desta & Amare, 2021), a resistance that, associated with its known antifungal action, can facilitate the exchange of *in vitro* cassava germplasm to other institutions and companies, national or international markets, preventing the loss of material during transport.

The results found are promising for the conservation of cassava germplasm under *in vitro* culture conditions, due to the lack of studies for varieties of *M. esculenta*. It is suggested that complementary studies be carried out using a greater number of cassava genotypes, given the great genetic variability that the crop has, in order to validate a conservation protocol for the largest possible number of varieties. Other retardants and growth regulators can also be tested, combined with osmoregulatory agents, in order to prolong the *in vitro* conservation period of *M. esculenta* germplasm.

Conclusion

The effect of Paclobutrazol® is strongly dependent on the genotype. However, the concentration of 0.20 mg L^{-1} is most suitable for the *in vitro* conservation of cassava, in most of the studied accessions, as it allowed reducing plant height without compromising its viability.

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