In Vitro Techniques to the Conservation and Plant Regeneration of Malanga (*Colocasia esculenta* L. Schott)

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Abstract. Malanga (*Colocasia esculenta*) is a plant genetic resource that requires biotechnological strategies for conservation and propagation. One time-, labor-, and space-saving option is in vitro conservation and regeneration. The objective of this study was to develop a protocol for in vitro regeneration and conservation of germplasm of *C. esculenta* var. *criolla*. For conservation through minimal growth, we assessed several concentrations of Murashige and Skoog (MS) medium (one-third, one-half, and three-quarter strength), the growth retardant ancymidol (0, 1, 2, and 3 mg·L⁻¹), and the osmoregulator polyethylene glycol (PEG-8000 mw) at different concentrations (0, 10, 20, and 30 g·L⁻¹). For in vitro conservation, the percent survival, shoot number and length, and number of leaves and roots per explant were evaluated after 24 weeks. For in vitro regeneration, different concentrations of thidiazuron (TDZ: 0, 0.5, 1, 1.5, and 2 mg·L⁻¹) and 6-benzylaminopurine (BAP; 0, 1, 2, 3, and 4 mg·L⁻¹) were evaluated. After 4 weeks of cultivation, the percent response, shoot number, and number of leaves per explant were recorded. During in vitro conservation, it was noted that the treatment including 2 mg·L⁻¹ ancymidol resulted in a retarded development, without affecting the survival of the *C. esculenta* germplasm. With regard to shoot regeneration, 7.60 shoots per explant were obtained using 2 mg·L⁻¹ TDZ. Finally, 98% survival was achieved during the acclimatization process. This study will contribute to the establishment of genetic improvement programs through in vitro conservation and propagation of this valuable plant genetic resource.

Malanga (*Colocasia esculenta* L. Schott) is a tropical crop that is commercially valuable due to the high nutrient content in its

tubers, and for its uses in both medicine and the development of biofuels (Eleazu, 2016; Kaur et al., 2013; Ogali et al., 2016; Talukder et al., 2015). The leaves and corms of *C. esculenta* have a high content of starch, protein, vitamins, polysaccharides, and various trace elements that are used in the food industry (Jiang et al., 2012; Kaur et al., 2013). In addition, in this species, the presence of secondary metabolites with antitumor, antime-tastatic, antioxidant, and anti-inflammatory activity has been documented (Cambie and Ferguson, 2003; Chan et al., 2010; Eleazu, 2016; Kundu et al., 2012; Park et al., 2013; Yu et al., 2015). These nutritional characteristics and benefits make of malanga a commercially valuable plant genetic resource.

These resources are considered the basis of food security; for this reason, strategies aimed at its conservation and propagation should be implemented (FAO, 2018; Hidalgo and Cabrera, 2014). A strategy for the conservation of plant germplasm includes plant tissue culture techniques through the establishment of an in vitro germplasm bank (Gantait et al., 2018; Offord, 2017).

In vitro systems for midterm conservation offer the advantage of saving resources such as materials, space, and labor, in addition to preserving the plant material for an undetermined period of time through subculturing (da Silva et al., 2016; Pacheco et al., 2016). An option to reduce the in vitro development rate consists in reducing the concentrations of MS salts to minimize the source of nutrients (Mora et al., 2011; Pacheco et al., 2016), the use of growth retardants such as ancymidol (El-Dawayati et al., 2012; Sarkar et al., 2001), and osmoregulators as polyethylene glycol (PEG-800) (Seesangboon et al., 2018). In plant physiology, ancymidol produces a number of effects, including the inhibition of gibberellins (Guajardo et al., 2016). The osmoregulator agent PEG is an inert and nontoxic compound for in vitro cultures due to its high molecular weight, which prevents its entry into plant cells (de Araújo Silva et al., 2016; Rai et al., 2011).

In recent years, in vitro conservation has proved to be an efficient option to preserve germplasm from different plant species, such as cassava (*Manihot esculenta*) (Barrueto Cid and Carvalho, 2008), daylily (*Hemerocallis* spp.) (Chen et al., 2005), liquorice (*Glycyrrhiza glabra*) (Srivastava et al., 2013), vanilla (*Vanilla planifolia* Andrews) (Divakaran and Babu, 2009) (*Vanilla planifolia* jacks.) (Bello-Bello et al., 2015), and grape (*Vitis vinifera* L.) (Hassan et al., 2014).

For malanga, there are long-term in vitro conservation studies (Bessembinder et al., 1993; Sant et al., 2008). However, there is no a study of in vitro preservation in the medium term to ensure regeneration of preserved material. However, establishing the conservation strategy for a particular species requires the development of an in vitro regeneration system (Bonilla et al., 2015; da Silva et al., 2016).

In this context, the conventional method of proliferation of tubers from roots is labor-intensive and insufficient to meet the increasing population demand (Chavan et al., 2018). This study intended to develop an efficient protocol for in vitro conservation and regeneration of the germplasm of *C. esculenta*, as a contribution to implement strategies for the preservation of this valuable plant genetic resource.

Materials and Methods

Plant material. Plants used were obtained of the germplasm bank of Colegio de Postgraduados Campus Veracruz. For the disinfection of the plant material, tips of “criolla”-type malanga plants were cut and washed with soap and water. In the laboratory, these tips were immersed in a solution containing 1 g·L⁻¹ of fungicide (Cupravit, Bayer, Leverkusen, DE) and 1 g·L⁻¹ of bactericide

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(Agri-mycin, Pfizer, New York, EE) followed by rinsing with tap water. Corms measuring an average length of 5 cm were then removed in a laminar-flow hood. These were immersed in a 15% (v/v) NaCl solution containing two drops of Tween 20 per 100 mL of solution for 20 min, then in 96% ethanol for 1 min, and afterward were rinsed three times with sterile distilled water. Finally, a scalpel was used to extract the apical meristems (0.5 cm in length). All meristems were cultivated in MS medium (Murashige and Skoog, 1962) supplemented with 30 g·L⁻¹ sucrose and without plant growth regulators (PGR). Phytigel at 2.2 g·L⁻¹ was used as gelling agent. The medium pH was adjusted to 5.8 ± 0.2 and culture flasks were autoclaved at 1.5 kg·cm⁻² and 121 °C for 15 min. Cultures were incubated at 25 ± 2 °C under an illumination of 50 ± 5 μmol·m⁻²·s⁻¹ provided by fluorescent lamps.

In vitro conservation. For in vitro conservation by minimal growth, vitroplantlets measuring ≈1 cm in length were transferred to test tubes (22 × 220 mm) containing 15 mL of MS medium. Phytigel at 2.2 g·L⁻¹ was used as gelling agent. We evaluated different concentrations of MS salts (one-quarter, one-half, and three-quarter strength); a separate treatment was supplemented with the growth retardant ancymidol (1, 2, and 3 mg·L⁻¹) and the growth osmoregulator polyethylene glycol (PEG-8000) at different concentrations (10, 20, and 30 g·L⁻¹). The control treatment consisted of 100% (full-strength) MS medium. The pH, sterilization of the culture medium, and incubation were as described earlier. After 4 weeks of cultivation, the percent response and number of shoots, leaves, and roots per explant were recorded.

In vitro regeneration. Individual tips measuring 1 cm in length from the best minimum-growth treatment were transferred to 500-mL flasks containing 40 mL of MS medium. Different concentrations (0, 1, 2, 3, and 4 mg·L⁻¹) of BAP and (0, 0.5, 1, 1.5, and 2 mg·L⁻¹) of TDZ were evaluated. Phytigel at 2.2 g·L⁻¹ was used as gelling agent. The pH, sterilization of the culture medium, and incubation were as described earlier. All reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). After 4 weeks of culture, the percent response and the number of shoots and leaves per explant were recorded.

Acclimatization. Shoots measuring 5 to 7 cm in length and showing optimal root de-

velopment were rinsed with tap water. Subsequently, shoots were planted in sterile peatmoss + agrolite (1:1 v/v) using 72-well trays (5 × 5 × 8 cm). Plantlets were kept under greenhouse conditions, at 50% shade, 90 ± 5% relative humidity, and 30 ± 5 °C. After 8 weeks of cultivation, the percent survival was recorded.

Statistical analysis. All experiments included a fully random design and were run in triplicate. For in vitro conservation, 25 explants per treatment were used (one explant per test tube). For regeneration, 30 explants per treatment were used (three explants per culture flask). The data obtained were tested through an analysis of variance followed by a Tukey's test ($P \leq 0.05$) using the IBM SPSS Statistics software (Version 21 for Windows). The normality and homogeneity of the variance were tested using the Kolmogorov-Smirnov and Levene tests, respectively. Variables not meeting these statistical assumptions were natural log-transformed (LN). Percent data were arcsine-transformed before the statistical analysis.

Results

In vitro conservation. Significant differences were observed between the treatments evaluated for in vitro conservation (Table 1). The highest percent survival was observed in treatments with 1 and 2 mg·L⁻¹ ancymidol and in treatments with MS medium at one-half to three-quarter strength, with 95.83% and with 96.66% survival, respectively. The lowest percent survival was observed in medium supplemented with 30 g·L⁻¹ PEG, with 53.33%. The largest number of shoots per explant was observed in treatments with 10% and 20% PEG, with 3.0 and 3.2 shoots per explant, respectively (Table 1). In contrast, lower MS salts and 30% PEG produced a lower number of shoots per explant (Fig. 1). The longest shoots were noted in full three-quarter and one-half strength MS medium, with 10.1, 9.9, and 9.7 cm in length, respectively, and the shortest in medium supplemented with 2 and 3 mg·L⁻¹ ancymidol, with an average shoot length of 4.66 and 4.76 cm. As regards the number of leaves per shoot, significant differences between treatments were observed; however, values included 3 to 5 leaves per explant. In contrast, no significant differences were observed in the number of roots. For root length, all

ancymidol treatments resulted in smaller root size.

In vitro regeneration. Significant differences were observed between BAP and TDZ concentrations on malanga in vitro regeneration (Table 2). It was noted that the number of shoots per explant increased in medium supplemented with BAP and TDZ. However, the highest shoot regeneration was noted with the addition of 2 mg·L⁻¹ TDZ, with 7.60 shoots per explant, followed by 2, 3, and 4 mg·L⁻¹ BAP, with 6.10 to 6.8 shoots per explant. For reference, the control treatment only produced 3.4 shoots per explant (Table 2). The highest shoot length was observed in culture medium supplemented with 1, 1.5, and 2 mg·L⁻¹ TDZ, with an average length between 6.26 to 7.71 cm (Fig. 2). On the other hand, the largest number of leaves per explant was observed in the control treatment, with 3.80 leaves per explant, followed by 0.5 mg·L⁻¹ TDZ, with 3.57 leaves per shoot. For the variables fresh weight and dry weight, higher values were noted in the treatment containing 2 mg·L⁻¹ TDZ, followed by 4 mg·L⁻¹ BAP.

Acclimatization. A 98% survival was observed in *C. esculenta* plantlets subjected to the acclimatization process. These results ensured the efficiency of the in vitro regeneration protocol developed.

Discussion

Currently, biotechnological strategies involving plant tissue culture are a feasible option for mass propagation and conservation intended for the replenishment of natural populations for sustainable use in the future (Bapat et al., 2008). In this context, the establishment of in vitro germplasm banks through minimal growth has been used successfully in other species of interest in agriculture, including coconut (*Cocos nucifera* L.) (Lédo et al., 2014), red-tipped photinia (*Photinia ×fraseri* Dress) (Akdemir et al., 2010), pineapple (*Ananas comosus* L. Merr.) (Soneji et al., 2002), pumpkin (*Trichosanthes dioica*) (Singh et al., 2015), artichoke (*Cynara cardunculus* var. *scolymus*) (Tavazza et al., 2015), sugar cane (*Saccharum* spp.) (Bello-Bello et al., 2014; Nogueira et al., 2015), date palm (*Phoenix dactylifera*) (El-Bahr et al., 2016), passion flower (*Passiflora* spp.) (Pacheco et al., 2016), and vanilla (*Vanilla planifolia*) (Bello-Bello et al., 2015).

Table 1. Effect of the concentration of MS medium, growth retardants and polyethylene glycol (PEG) on the in vitro conservation of *C. esculenta*.

Treatment	Concn	Survival (%)	No. of shoots per explant	Shoot length (cm)	No. of leaves per shoot	No. of shoots per explant	Root length
Control	MS	91.66 ± 4.16 ab	2.20 ± 0.24 bc	10.10 ± 0.37 a	4.90 ± 0.23 a	14.50 ± 1.38 a	6.00 ± 0.26 a
MS	1/4	79.16 ± 8.33 bc	1.40 ± 0.16 c	6.20 ± 0.38 bc	3.41 ± 0.22 bc	16.25 ± 1.25 a	5.66 ± 0.22 a
	1/2	95.83 ± 4.16 a	1.63 ± 0.24 c	9.76 ± 0.42 a	4.90 ± 0.25 a	16.72 ± 1.14 a	6.08 ± 0.19 a
	3/4	95.83 ± 4.16 a	1.60 ± 0.26 c	9.90 ± 0.34 a	5.00 ± 0.23 a	16.18 ± 1.32 a	5.90 ± 0.27 a
Ancy (mg·L ⁻¹)	1	96.66 ± 3.33 a	2.00 ± 0.21 c	9.16 ± 0.24 a	4.83 ± 0.24 a	15.00 ± 1.18 a	4.00 ± 0.24 b
	2	96.66 ± 3.33 a	2.27 ± 0.14 c	4.76 ± 0.32 c	4.00 ± 0.26 abc	14.45 ± 0.94 a	3.69 ± 0.23 b
	3	79.16 ± 8.33 bc	2.30 ± 0.15 c	4.66 ± 0.30 c	3.22 ± 0.27 bc	13.72 ± 1.11 a	2.88 ± 0.26 b
PEG (g·L ⁻¹)	10	76.66 ± 1.66 bc	3.00 ± 0.21 a	6.58 ± 0.35 b	4.33 ± 0.35 ab	13.16 ± 0.99 a	6.26 ± 0.30 a
	20	66.66 ± 4.16 bc	3.26 ± 0.11 a	5.54 ± 0.36 bc	3.20 ± 0.20 bc	14.50 ± 0.79 a	6.07 ± 0.32 a
	30	53.33 ± 3.33 c	1.53 ± 0.19 c	5.60 ± 0.33 bc	3.00 ± 0.21 c	14.69 ± 0.73 a	6.50 ± 0.37 a

Figures represent the mean ± SE. Means with the same letter are not statistically different (Tukey, $P < 0.05$).

Fig. 1. Effect of the concentration of Murashige and Skoog (MS) medium, osmoregulator, and growth retardants on the in vitro conservation of *C. esculenta*. (A) MS medium (from left to right: 0%, 25%, 50%, and 75%, respectively). (B) Ancymidol (from left to right: 0, 1, 2, and 3 mg·L⁻¹, respectively). (C) Polyethylene glycol (from left to right: 0, 10, 20, and 30 g·L⁻¹, respectively). Bar = 1 cm.

Table 2. Effect of the growth regulators benzylaminopurine (BAP) and Thidiazuron (TDZ) on the in vitro multiplication of malanga.

Growth regulator	Dose (mg·L ⁻¹)	No. of shoots per explant	Shoot length (cm)	No. of leaves per shoot	Fresh wt (g)	Dry wt (g)
BAP	0	3.40 ± 0.43 c	5.30 ± 0.28 bc	3.80 ± 0.13 a	3.18 ± 0.38 c	0.28 ± 0.04 c
	1	5.90 ± 0.67 bc	3.10 ± 0.19 d	3.33 ± 0.18 bc	3.79 ± 0.32 bc	0.29 ± 0.01 c
	2	6.80 ± 0.82 ab	3.56 ± 0.24 cd	2.92 ± 0.14 bc	5.32 ± 0.17 ab	0.39 ± 0.01 b
	3	6.10 ± 0.59 ab	2.97 ± 0.15 d	3.00 ± 0.27 bc	5.33 ± 0.33 ab	0.41 ± 0.03 b
	4	6.50 ± 0.77 ab	3.58 ± 0.17 cd	3.00 ± 0.07 bc	4.24 ± 0.07 bc	0.36 ± 0.02 bc
TDZ	0.5	4.30 ± 0.67 bc	4.50 ± 0.25 bcd	3.47 ± 0.20 ab	3.21 ± 0.13 bc	0.39 ± 0.03 b
	1	4.66 ± 0.69 bc	7.71 ± 0.12 a	2.64 ± 0.11 c	4.50 ± 0.31 bc	0.35 ± 0.04 bc
	1.5	5.33 ± 0.63 bc	5.98 ± 0.34 bc	3.29 ± 0.11 bc	3.64 ± 0.18 bc	0.30 ± 0.02 c
	2	7.60 ± 0.77 a	6.26 ± 0.31 ab	2.84 ± 0.15 bc	5.14 ± 0.29 a	0.56 ± 0.03 a

Figures represent the mean ± SE. Means with the letter are not statistically different (Tukey, *P* < 0.05).

Fig. 2. Effect of the benzylaminopurine (BAP) and thidiazuron (TDZ) growth regulators on the in vitro regeneration of *C. esculenta* at 25 d of culture. (A) Control; (B–E) 1, 2, 3, and 4 mg·L⁻¹ BAP, respectively; (F–I) 0.5, 1, 1.5, and 2 mg·L⁻¹ TDZ, respectively.

However, none of these studies reports the use of ancymidol as an alternative in an in vitro conservation program.

There are reports of the in vitro conservation of malanga. Bessembinder et al. (1993) managed to conserve for 8 years vitroplantulas under minimum growth. However, they mentioned that prolonged addition of mannitol to the culture medium affected survival and regeneration, causing abnormalities and death of the explants. On the other hand, Sant et al. (2008) implemented the droplet vitrification technique in *Colocasia esculenta* var. *esculenta*. However, they did not cryopreserve plant material for a long time.

It has been extensively documented that ancymidol inhibits the biosynthesis of gibberellins, blocking the conversion of ent-kaurene to ent-kaurenoic acid, hence reducing internode length and leaf size (Hernández-Altamirano et al., 2018). In our study, we observed a relationship between ancymidol concentration and reduction in shoot length of *C. esculenta* cultured in vitro. This is consistent with reports for in vitro cultured plantlets of pumpkin (*Trichosanthes dioica*) (Singh et al., 2015), potato (*Solanum tuberosum*) (Sarkar et al., 2001), and fern (*Asparagus setaceus*) (Pindel, 2017).

On the other hand, the use of PEG in the in vitro conservation of *C. esculenta* reduced the percent survival with increasing PEG concentrations in the culture medium, due to the osmotic stress produced by this molecule. The change in the osmotic potential of the culture medium affects nutrient concentration and growth rate in vitro (Sahoo et al., 2018). In contrast to our results, PEG concentrations of 1 to 15 g·L⁻¹ have been successfully used in the in vitro conservation of various species, such as liquorice (*Glycyrrhiza glabra*) (Srivastava et al., 2013), jojoba (*Simmondsia chinensis*) (Bekheet et al., 2016), and vanilla (*Vanilla planifolia*) (Bello-Bello et al., 2015). MS salts at low concentrations have been used for in vitro conservation through minimum growth in cat's claw (*Uncaria tomentosa*) (Mora et al., 2011). This contrasts with our study because lower MS salts had no significant effect on the growth variables evaluated, relative to the control treatment. This was probably due to the low nutritional requirements of *C. esculenta*.

A successful in vitro conservation program requires the development of regeneration strategies such as micropropagation (Bonilla et al., 2015; da Silva et al., 2016). TDZ is a phenylurea used as growth regulator for a rapid and effective in vitro regeneration of plant species (Ali et al., 2018). Various studies have shown that the TDZ affects the endogenous production of cytokinins and auxins by regulating several genes that act on the transport of auxins and cytokinins (Dewir et al., 2018; Wannakraioj and Tefera, 2012). In addition, this PGR is commonly used for obtaining shoots or somatic embryos in a number of plant species (Dewir et al., 2018).

In our study, we obtained a larger number of shoots per explant of *C. succulent* using TDZ (2 mg·L⁻¹). These results contrast with

what is described by Du et al. (2006), who in *C. esculenta* var. *antiquorum* obtained the largest number of shoots per explant (4.7) in medium supplemented with 3.0 mg·L⁻¹ BAP + 0.1 mg·L⁻¹ TDZ. However, the regenerated plants were not part of an in vitro conservation program. It has been mentioned that TDS concentrations greater than 0.5 mg·L⁻¹ induce morphological malformations in a number of plant species cultured in vitro (Dewir et al., 2018). These malformations occur in the morphology of leaves and shoots, and also appear as swelling at the base of shoots (Dewir et al., 2006, 2018). However, these abnormalities depend on the high sensitivity of each individual species to TDZ. In our study, no malformations were apparent during the regeneration of shoots.

In conclusion, a protocol was established for the in vitro conservation of *C. esculenta* by inhibiting growth but with no effect on the percent survival. Also, an efficient protocol for in vitro regeneration of the preserved shoots was developed. In vitro conservation and regeneration systems contribute to germplasm preservation and reintroduction strategies of this valuable plant genetic resource.

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