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In vitro conservation of mango (*Mangifera indica* L.) Ubá and Carlota cvs. through culturing immature embryos

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Abstract: The *in vitro* rescue of immature embryos can be employed both for preservation of mango germplasm and rescue of hybrids produced from crosses to obtain traits of interest. The objective of this work was to establish a protocol for *in vitro* rescue of immature embryos aiming to preserve mango germplasm. Immature embryos of two mango varieties, 'Carlota' and 'Ubá', were inoculated in MS/2 medium supplemented with 100 mg L^{-1} of cysteine, 0.5 mg L^{-1} of gibberellic acid (AG_3) and 30 g L^{-1} of sucrose. The experimental design was completely randomized with 30 repetitions, each composed of two embryos/flask. After *in vitro* growth for 84 days, some of the plantlets were transferred for acclimatization. The parameters evaluated were plant height, number of leaves and leaf, stem and root dry weight. The germination began on the sixth day after seeding, and the immature embryos presented a high oxidation rate, with 60% oxidized after 72 hours. The plantlets from immature embryos showed better development than those from aborted embryos. The results showed the viability of rescuing mango embryos in the immature phase and of their *in vitro* conservation for a period of 12 months.

Key words: *Mangifera indica* L., plantlet, polyembryony, tissue culture, zygotic embryos.

INTRODUCTION

The mango (*Mangifera indica* L.) is one of the most popular tropical fruits worldwide (FAOSTAT 2020), and global output in 2018 reached roughly 55,384 metric tons, with the leading countries being India, with 40.7%, China (10.4%), Thailand (8%), Mexico (4.8%) and Indonesia (3.9%) (Altendorf 2017).

For the purpose of supporting genetic improvement programs and efficient germplasm conservation, various studies have been carried out to investigate the genetic diversity of mango, including the development of a genetic map (Samal et al. 2012, Alves et al. 2016, Kuhn et al. 2017). Due to the importance of the crop and the large number of improvement programs, germplasm banks under field conditions are maintained by various institutions, in countries such as India, Brazil and Australia (Souza et al. 2011, Dillon e al. 2013, Ribeiro et al. 2013, De et al. 2014). India maintains the world's largest and most representative mango germplasm collection, at the Indian Institute of Horticultural Research (IIHR) in Bangalore, with around 728 accessions maintained in the field (ICAR-IIHR 2018). These plants are kept in the field in clonal form, on rootstocks that vary depending on the collection. Although the maintenance of these varieties in the field is important, it is also costly and laborious, and the plants are vulnerable due to biotic and abiotic

problems. On the other hand, since mango seeds are recalcitrant, with very short longevity, *in vitro* conservation or cryopreservation can be an efficient way to preserve germplasm as a security backup.

However, various studies have revealed difficulties of mango tissue culture, such as exudation of polyphenols, endogenous bacterial contamination and tissue recalcitrance, among other drawbacks (Thomas & Ravindra 1999, Litz et al. 2000, Krishna & Singh 2007, Krishna et al. 2008, Al-Busaidi et al. 2016).

Very little information has been published about the *in vitro* culture of mango embryos, which can be carried out to obtain explants for induction of somatic embryogenesis for micropropagation of elite varieties (Xiao et al. 2004) or for rescue of zygotic embryos, reducing the time necessary for genetic improvement by allowing rapid characterization and selection (Pérez-Hernández & Grajal-Martín 2011). However, there are virtually no reports of this technique for preserving mango germplasm as an alternative to establish duplicates for security backup purposes. This is an application that has not been widely investigated but can be another alternative to complement the conservation of germplasm of this important species.

Litz et al. (2000) reported that the culture of zygotic embryos normally suffers from high contamination rates and high concentrations of polyphenols exuded in the culture medium, causing necrosis of the tissues and reducing the rescue success. The initial disinfestation of the explants and the reduction of oxidation of these cultured tissues are among the main challenges to the success of *in vitro* culture techniques of mango varieties, as reported initially by Erig & Schuch (2003).

However, Pérez-Hernández & Grajal-Martín (2011) investigated the culture of embryos to rescue cultivars and reported a success rate of 83% for the cultivars 'Lippens' and 'Keitt' after growing immature embryos in liquid medium during the maturation period, and mentioned the importance of the interaction between the culture system and medium.

Despite reports of culturing zygotic embryos for rescue of hybrids, to the best our knowledge there are no reports of culturing nuclear embryos for *in vitro* conservation of mango germplasm, a strategy that can be used with polyembryonic varieties.

Therefore, the aim of this study was to develop a protocol for culturing nuclear embryos of the polyembryonic mango cultivars 'Ubá' and 'Carlota' for the preservation of germplasm in the medium term, as well as to establish an efficient acclimatization procedure for rescue of the preserved plants for the purpose of demonstrating the viability of the proposed technique.

MATERIALS AND METHODS

In vitro germination

Immature fruits were collected of the polyembryonic varieties 'Ubá' and 'Carlota', with average diameter of 5.0 cm (approximately 13 to 20 days after pollination). The fruits were washed in tap water and detergent and then immersed in a 50% sodium hypochlorite solution (2% active chlorine) for 40 minutes under agitation. Next, the disinfested fruits were transferred to a laminar flow cabinet where the embryos were retrieved and inoculated in culture medium with half the normal concentration of MS salts (Murashige & Skoog 1962), supplemented with 100 mg L^{-1} of cysteine, 0.5 mg L^{-1} of gibberellic acid and 30 g L^{-1} of sucrose. The medium was solidified with 2.2 g L^{-1} of Phytagel® and the pH was adjusted to 5.8. The embryos were maintained in a growth room at a temperature

of 27 ± 1 °C, photon flux density of 30 µmol m^{-2} s^{-1} and photoperiod of 16 hours.

Daily evaluations were conducted for 30 days after establishment to observe the germination percentage, bacterial and/or fungal contamination, and percentage of oxidized embryos (presence of polyphenols). After that, the evaluations were carried out weekly until 84 days after seeding, when the surviving germinated embryos were counted.

In vitro conservation

The viable plantlets obtained after 84 days were incubated in a conservation room at a temperature of 22 ± 1 °C, photon flux density of 22 µmol m^{-2} s^{-1} and photoperiod of 12 hours, the most propitious conditions for slow growth by reduction of the cell metabolism. This is most appropriate for germplasm conservation. At the end of 365 days of growth, the following variables were appraised: plant height (cm); number of green leaves, number of senescent leaves and number of roots. During this 365-day interval, no subculture was performed.

Acclimatization of the plants

After 365 days the plants were removed from the flasks, washed with abundant water to remove the residual culture medium and taken to a greenhouse (temperature of 27 °C and 50% of luminosity) where they were planted in acrylic cups containing the commercial substrate Vivato®. Irrigation was performed every three days. Then the substrate was saturated with a solution of indole-3-butyric acid (IBA) at concentration of 0.01, 0.1 or 1.0 mg L^{-1}, plus a control treatment without addition of IBA. The plants were covered with other cups to prevent dehydration by excessive transpiration. The following variables were evaluated: survival percentage; plant height (cm); and number of leaves, on the 15th, 30th and 60th days after transfer to the substrate.

After 60 days, the plants were removed from the substrate and the leaves, roots and stems were separated and dried in a forced-air oven at 60 °C until reaching constant weight, to measure the dry weight (g) of each part, using a precision scale.

Statistical analysis

For the *in vitro* conservation trials of the experimental design was completely randomized, with 30 repetitions per variety, where each repetition consisted of two embryos per flask. The resulting data were submitted to analysis of variance and the means were compared by the Tukey test at 1% probability. For the acclimatization test the experimental design was completely randomized, with four treatments and 16 repetitions, where each repetition was composed of one cup holding one plant. The data were submitted to analysis of variance with the SAS Institute program (2010) and the means and standard deviations were calculated.

RESULTS AND DISCUSSION

In vitro germination of the embryos

The germination began seven days after placement of the embryos in the culture medium, and the germination rates were 33% and 16% for the 'Ubá' and 'Carlota' varieties, respectively (Figure 1). The germination was observed during 84 days, and stability was reached as of 50 days after inoculation, with the highest values for the 'Ubá' variety, indicating the difference in behaviors between the two varieties (Figures 1 and 2a, b). The embryos, besides being immature, had different sizes, which interfered with uniform germination and partly explains the long germination time.

The germination began on the sixth day after seeding, and the immature embryos presented a high oxidation rate, with 60% oxidized after 72 hours (Table I). Bacterial and fungal contamination was detected in the embryos of both varieties, with 58% in 'Ubá' and 36% in 'Carlota' (Table I). The contamination by bacteria was more pronounced than by fungi, and again the 'Ubá' was more afflicted, with approximately 40% of the embryos being contaminated, versus 18% for the 'Carlota' variety.

The bacterial contamination might have come from endophytes, while the origin of the fungal contamination might have been more closely related with inadequate handling or insufficiency of the disinfestation system. In some plant species, endophytic bacteria on the seeds are beneficial, participating in the germination and establishment of the plants by acting as growth promoters and biological controls (Holland & Polacco 1994, Truyens et al. 2015), but they can be a hindrance to tissue culture. In this study, we did not use any type of antibiotic for bacterial control, which can partly explain the high bacterial contamination rates.

The differences in the germination rates between the varieties were marked, with 80% of the embryos germinated of the 'Ubá' variety and 50% of 'Carlota' (Figure 1). The embryos were considered to have germinated when emitting a radicle, irrespective of whether it later developed into a root. In fact, some of the embryos developed the aerial part without a substantial root system. This result was observed for both varieties, i.e., plantlets with well-developed aerial part but poorly developed root system. This lack of roots, however, did not impair the *in vitro* development of the plantlets or their subsequent rescue, since even with precarious root systems the plants could be successfully acclimated.

Not all the embryos germinated, and among those that did, not all survived. On average, three embryos germinated per fruit, but only two survived. The loss of these embryos was mainly due to malformations that prevented the normal development, which might have been related to their physiological immaturity.

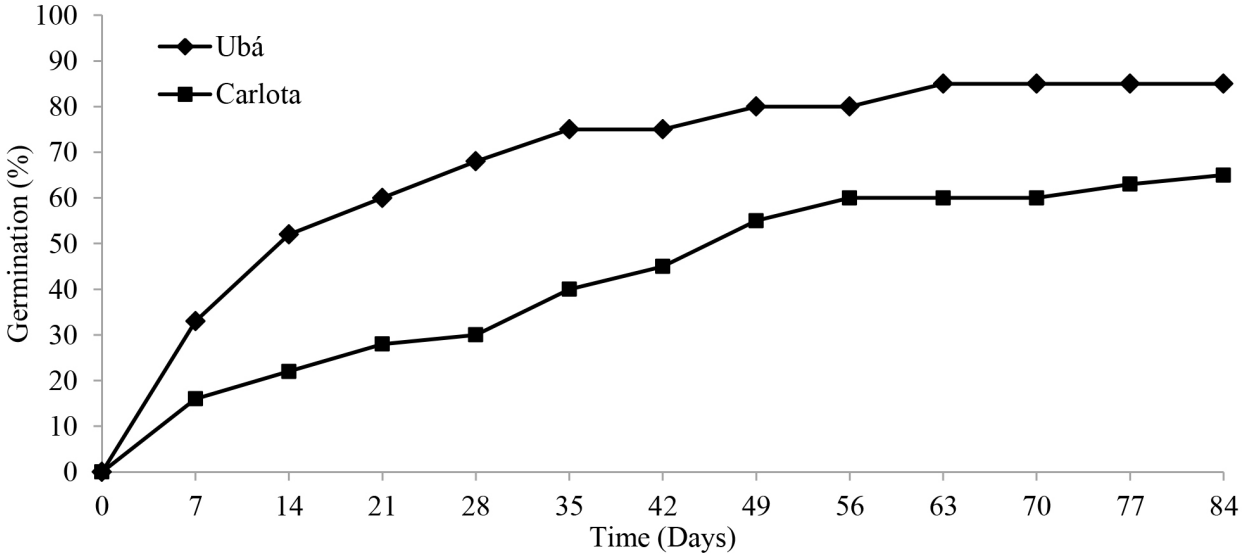

Figure 1. Germination percentages of embryos of the mango varieties 'Ubá' and 'Carlota' during 84 days.

Table I. Percentage of contamination (fungal and bacterial) and oxidation of embryos inoculated *in vitro* in the mango varieties 'Ubá' and 'Carlota'.

Embryos (%)	'Ubá'	'Carlota'
Contaminated by fungi	10	16
Contaminated by bacteria	26	42
Oxidized	58	62

In vitro conservation

The plantlets that developed after germination were incubated in a conservation room for 365 days, after which they were evaluated (Figure 2c). The parameters selected for this type of evaluation are important for conservation studies, since they reflect the plant's development and its state of conservation during the period. The criterion used was the presence of a developed aerial part, with expanded leaves. The presence of roots was not a determinant for selection of plants for conservation.

In studies involving *in vitro* conservation, it is common to try to reduce the plants' metabolism to reduce their growth and thus the number of subcultures, to facilitate management of the collection and avoid genetic variations (Souza & Pereira 2007, Santos et al. 2012, Silva et al. 2016). However, in this study the material conserved consisted of plants from *in vitro* germination of immature nuclear embryos, whose nutritional demands are much more complex, besides the existence of differences between the embryos themselves. Therefore, we placed priority on obtaining healthy plantlets able to develop and remain viable for a prolonged period in the conservation conditions.

The variables number of senescent leaves, number of germinated embryos and number of surviving embryos differed significantly between the two varieties, with all three being greater for the 'Ubá' variety (Table II). These results indicate that the 'Ubá' variety develops better than the 'Carlota', suggesting a slightly faster metabolism, since the former variety presented approximately 70% of the leaves in senescence (average of 4.27) in the evaluation period, indicating the need for subculture in fresh medium (Table II). The results obtained with the 'Carlota' variety were very interesting in terms of conservation, since the plants maintained their physiological integrity for a longer period, although having lower germination and survival percentages, aspects that can be improved by alterations in the disinfestation step. All the embryos of both varieties that survived developed normally, without any morphological abnormalities, even though the root development was not always abundant.

In mango, the number of nuclear embryos in polyembryonic varieties can vary from 1 to 13, with records of averages of 6.4 and 4.3 embryos for the 'Ubá' and 'Espada' varieties, respectively (Borges et al. 1999). From comparing these numbers with the *in vitro* germination rates observed in this study, it is possible to note the importance of physiological maturity as well as the *in vitro* conditions for germination of these embryos, although the germination results were very satisfactory, since the aim was conservation rather than multiplication.

In the case of polyembryonic varieties of citrus, the number of embryos per seed also varies between and within varieties, as a result

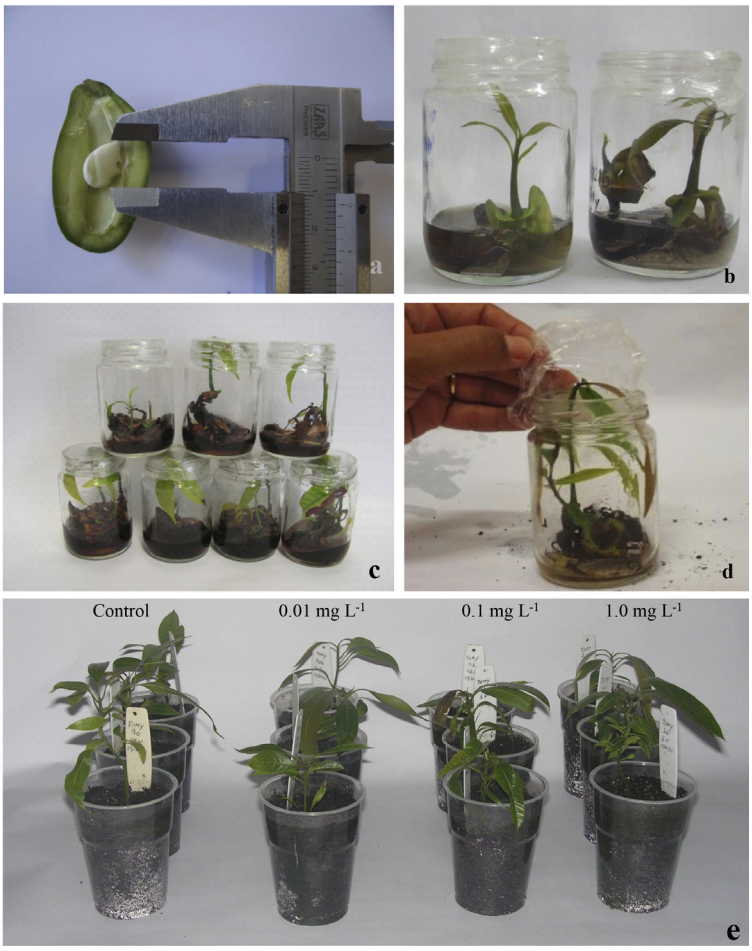

Figure 2. a) Immature mango embryos used for *in vitro* establishment. b) Germinated embryos; c) Plants from nuclear embryos of the mango variety 'Ubá' conserved *in vitro* after 365 days. d) Acclimatization procedure. e) Acclimated mango plants from different IBA treatments.

of environmental factors and the genotypes involved in the hybridizations. In hybrid seeds of *Citrus sinensis* (L). cv. Natal crossed with *P. trifoliata*, Ribeiro et al. (1999) found up to 20 embryos in seeds of immature fruits. However, other citrus varieties, such as 'Cravo' lime, produced much lower averages, with only 1.5 embryos per seed (Soares Filho et al. 2000).

Despite the differences observed between the two varieties and the heterogeneous germination of the embryos, many of the germinated plantlets of both varieties were able to develop and survive for a period of 12 months, making this type of conservation workable for polyembryonic mango varieties. However, there are no guarantees that the embryos cultivated are all nuclear, so there is a need for molecular testing to confirm that the conserved plants are clones of the parent plant. Still, although the more precise identification of the nuclear embryos is a relevant step to consider regarding this *in vitro* conservation technique of mango plants, the results of this study are promising.

The culture of apical meristems or axillary buds, the techniques most often applied for *in vitro* conservation of many species, is very difficult for mango, mainly due to the high contamination rates and exudation of polyphenols in the culture medium, rapidly killing the inoculated explants. Therefore, the alternative of using nuclear embryos should be considered for further research, to develop improvements and establish a protocol.

Table II. Plant height (cm), numbers of green leaves, senescent leaves, roots, germinated and surviving embryos from cultures of the mango varieties 'Ubá' and 'Carlota' after 12 months of *in vitro* conservation.

Variables	'Ubá'	'Carlota'
Plant height (cm)	5.85 a	5.52 a
Number of green leaves	6.10 a	5.82 a
Number of senescent leaves	4.27 a	0.75 b
Number of roots	2.21 a	1.98 a
Number of germinated embryos	2.87 a	2.00 b
Number of surviving embryos	2.24 a	1.66 b

Different letters in the row indicate significant differences by the Tukey test (p<0.01).

Acclimatization and effect of IBA on the plants' development

The rescue of plants preserved *in vitro* is a crucial step in a conservation protocol, and in the case of this work, more specifically due to the low root formation of both varieties. Therefore, we tested the use of IBA for the purpose of promoting the establishment of the plants in autotrophic conditions.

No significant differences were observed between the cultivars or the IBA doses with respect to plant height and number of leaves and roots. Therefore, we present the results referring to these variables considering the two cultivars together. The survival rate was recorded for all the treatments and cultivars. Low mortality rates were noted in all treatments, with values ranging from 17.65% for the control group and the plants given an IBA dose of 1 mg L^{-1} to 23.53% for the doses of 0.01 and 0.1 mg L^{-1}. However, there were no significant differences for the plant height variable 15, 30 and 60 days after acclimatization with the different IBA doses in relation to the control (Figure 2e). After 15 and 30 days, there were no differences in plant height, in contrast with the measurements on the 60th day, when all the plants had nearly double the size (Figure 3a). In relation to the number of leaves and dry weight of the plant parts, the addition of IBA influenced positively these variables in all three evaluations (15, 30 and 60 days after acclimatization (Figure 3b, c).

The use of auxins during the *in vitro* rooting phase, mainly IBA, has been reported in the literature as promoting better survival rates of many plant species (Fermino Júnior et al. 2011, Rathore et al. 2015), but in this work the application of IBA in the acclimatization step did not result in any improvement in the survival rate of the plants, which formed roots in the commercial substrate without addition of any growth regulator.

These results show that sufficient numbers of plants developed and grew, which can be considered positive for consolidation of a protocol, since many species have problems during this step due to death of plants.

The acclimatization step of plants produced by *in vitro* culture can be a limiting factor for the success of the process, so great care is needed to avoid loss of plants by excess transpiration. Before starting the acclimatization, the number of expanded leaves was evaluated so as to have a benchmark to measure the growth of the plants during the maturation period. The number of expanded leaves is an important parameter for the success of acclimatization, and requires special precautions.

The absence of waxes, such as cutins and serines, among other leaf surface components,

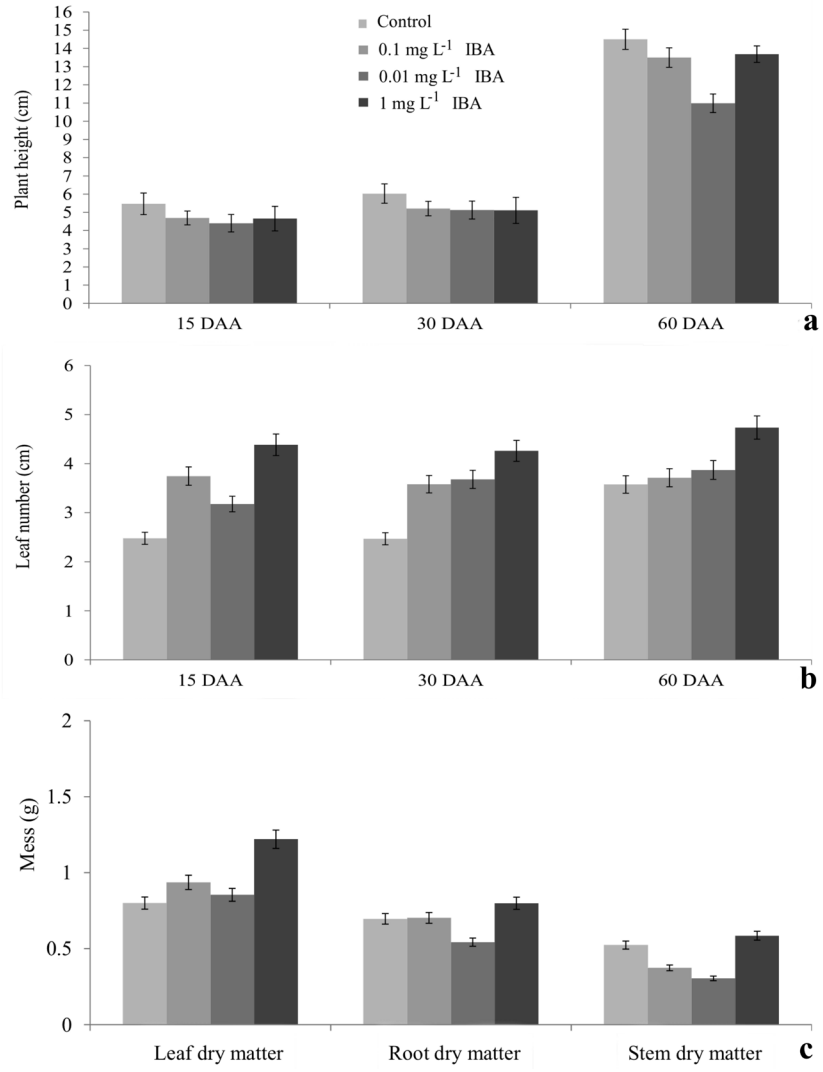

Figure 3. Plant height (cm) (a) and number of expanded leaves (b) of mango ('Ubá' and 'Carlota') plants treated with different concentrations of IBA 15, 30 and 60 days after acclimatization and leaf, root and stem dry weight (c) 60 days after acclimatization.

along with the limited functionality of the stomatal and photosynthetic structures, can impair the survival of the plantlets or plants generated *in vitro*, and consequently hamper the entire process (Rogalski et al. 2003, Souza & Pereira 2007). Called the hardening phase, the purpose of acclimatization is to induce the development of these structures while the plants are protected (in an environment with high moisture) against water loss due to excessive transpiration, and to allow the photosynthetic process to start functioning.

All the plants not treated with IBA survived in the first 30 days after acclimatization. Similar results were obtained by Martins (1998), studying lychee, who observed no positive influence of IBA on the survival of cuttings. Roncatto et al. (1999) and Roberto et al. (2001), investigating the effect of IBA on the rooting of "Valencia' orange cuttings, also found no influence of this growth regulator on the cutting survival percentage.

CONCLUSIONS

The results showed the viability of rescuing mango embryos in the immature phase and of their *in vitro* conservation for a period of 12 months.

The 'Carlota' variety maintained its physiological integrity longer than the 'Ubá' variety.

The growth regulator IBA did not affect the acclimatization of the mango plants.

The two cultivars presented similar behavior in the acclimatization step.

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Author contributions

Dra. Fernanda Vidigal Duarte Souza: responsible for the obtention of funds and the administration of project, wrote the manuscript, reviewed and corrected the English version and discussed the results.
M.Sc. Celma C. Rodrigues: conducted field work, collected the material and wrote the manuscript;
Dr. Everton H. Souza: statystical analyses, analyzed the data, prepared figures and/or tables, wrote the manuscript and discussion of the results.
Dr. Clovis P. Peixoto: collaboration and review and the interpretation of results.
All authors critically revised the manuscript and approved the nal version.