



Review Paper

In vitro culture of sempre-vivas species (*Comanthera*): a review

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Abstract

The term “sempre-viva” denotes plants whose structures retain their natural form and color after being cut and dried. For these reasons, they are commercially valuable for ornamental purposes. However, due to extractive overexploitation of their inflorescences, some of these species are considered endangered. The genus *Comanthera* includes the sempre-vivas species with greatest economic importance in Brazil. Previous studies have shown that tissue culture is a workable strategy for *in vitro* propagation and conservation of species of this genus. However, these studies are still incipient. Therefore, the objective of this review is to summarize the findings on the *in vitro* cultivation of species of the *Comanthera* genus, to serve as the basis for future research. The text is structured in two main topics: micropropagation and *in vitro* conservation.

Key words: *Comanthera*, *in vitro* culture, *in vitro* conservation, micropropagation, Sempre-viva.

Resumo

As sempre-vivas são plantas que mantêm o aspecto vivo de suas estruturas mesmo após serem coletadas, e por esta característica são comercializadas como ornamentais de alto valor econômico; contudo, devido à coleta extrativista de suas inflorescências algumas espécies se encontram em perigo de extinção. O gênero *Comanthera* compreende as sempre-vivas de maior importância econômica para o Brasil. Estudos apontam a cultura de tecidos como estratégia para a propagação e conservação *in vitro* de espécies deste gênero; entretanto as pesquisas publicadas ainda são incipientes. Diante disso, o objetivo desta revisão foi reunir publicações sobre o cultivo *in vitro* do gênero *Comanthera* com o intuito de fundamentar estudos futuros com suas espécies. Para tanto, esta revisão está estruturada em dois tópicos principais: micropropagação e conservação *in vitro*.

Palavras-chave: *Comanthera*, cultivo *in vitro*, conservação *in vitro*, micropropagação, Sempre-viva.

Introduction

Serra do Espinhaço is a mountain range that extends from the state of Minas Gerais to the state of Bahia, Brazil, and is noted for its wide biodiversity. According to ICMBio (2018), this region is considered by the United Nations Organization (ONU) to be the seventh leading biosphere reserve in Brazil, due to the great diversity of its natural resources and flora endemism.

Among the predominant species of Serra do Espinhaço are sempre-vivas, a designation given to plants whose structures maintain a living appearance for an extended period after being cut

and dried. These characteristics give these plants a high commercial value for ornamental purposes, such as decoration and production of bouquets and accessories. The sempre-vivas species most often found in commerce are those belonging to the families Xyridaceae, Cyperaceae, Gramineae and Eriocaulaceae (Giulietti *et al.* 1988).

Due to these potential uses, sempre-vivas plants have sometimes been overexploited as a source of subsistence or extra income by people living in or near the areas of their occurrence, causing reduction of these plants. Some species from the Eriocaulaceae family are classified as

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endangered, such as *Comanthera mucugensis* (Giul.) L.R. Parra & Giul. and *Comanthera elegans* (Bong.) L.R. Parra & Giul., or as vulnerable, such as *Comanthera curralensis* (Moldenke) L.R. Parra & Giul. (CNCFlora 2012). This problem has attracted the attention of researchers about the need to apply strategies for *ex situ* conservation of these genetic resources. For this purpose, one of the most successful biotechnological strategies is the use of tissue culture, encompassing a set of techniques such micropropagation and *in vitro* conservation.

Micropropagation allows the production of a large number of plants free from pathogens (good phytosanitary quality) in a brief time period and limited space (Junghans & Souza 2013; Coelho *et al.* 2016). As such, it is applied to produce seedlings for the ornamental market. The *in vitro* conservation technique consists of keeping cultured plants alive for as long as possible without the need for subculture. This can be achieved by alterations in the culture medium to reduce or suppress growth (Arrigoni-Blank *et al.* 2014), allowing maintenance of the genetic variability without the risks inherent to the field conditions.

The literature contains several studies of the micropropagation and *in vitro* conservation of different species of the genus *Comanthera*, but these are incipient, and no protocols have been established for commercial production of micropropagated plantlets, much less for the establishment of *in vitro* germplasm banks. In light of this context, the objective of this review is to summarize the publications on the *in vitro* cultivation of the genus *Comanthera*, to serve as a base for future studies of its species.

Micropropagation of sempre-vivas species

In vitro establishment

The pioneering studies focused on *Comanthera mucugensis* involved *in vitro* germination. Paixão-Santos *et al.* (2003) tested pre-germination treatments of the seeds before inoculation (chemical scarification with sulfuric acid, washing in running water, soaking and drying) and evaluated the effect of different concentrations of sucrose and gibberellic acid in culture medium containing water + 8 g.L⁻¹ of agar on *in vitro* establishment.

Their results indicated that the *C. mucugensis* seeds do not need to be submitted to chemical scarification and drying, and that soaking is favorable to germination. However, none of

the treatments led to germination higher than approximately 55%. The experiments also revealed that sulfuric acid is toxic to the seeds and that sucrose in the culture medium inhibits germination.

The low germination rates attained with all the treatments indicated the need to test new treatments for possible increase of the germination rate. In this respect, Silva *et al.* (2005a) obtained germination of 85.5% using seeds previously soaked in water sown in medium containing water + agar, at concentration of 7 g.L⁻¹, revealing that the seeds of the species do not have dormancy. The authors also assessed the influence of luminosity (presence or absence of light) and concluded that germination only occurs in the presence of light, indicating that the species is photoblastic positive.

Both Paixão-Santos *et al.* (2003) and Silva *et al.* (2005a) used a culture medium containing water and agar for germination, without nutrients and carbohydrates. This practice makes it necessary to transplant the recently germinated plants to a growth medium.

In this sense, Silva *et al.* (2005b) investigated the effect of five concentrations of sucrose (5, 10, 15, 20 and 30 g.L⁻¹) added to MS medium (Murashige & Skoog 1962) with half the normal saline concentration (MS ½) on the *in vitro* growth of *C. mucugensis* plants. The results indicated that the use of 15 g.L⁻¹ of sucrose is efficient for *in vitro* growth of the species. The authors suggested that new studies must be conducted to optimize the establishment step.

Paixão-Santos *et al.* (2006) tested different concentrations of MS salts (complete, ⅔, ½, ⅓ and ¼) on the initial growth of plants recently germinated *in vitro*. The results indicated that MS ½ medium tended to provide the best results.

The studies described above indicated that the germination and initial growth of *C. mucugensis in vitro* should be carried out in different culture media, which would increase the production costs and risks of contamination of the plant material during transfer. However, a later study with this species demonstrated that the *in vitro* establishment can be accomplished from seeds germinated in MS ½ medium supplemented with 15 g.L⁻¹ of sucrose, and the plants can be maintained in the same medium during *in vitro* growth (Lima-Brito *et al.* 2011a).

Besides *C. mucugensis*, studies on the *in vitro* establishment of other species of the genus *Comanthera* have also been reported. Pêgo *et al.* (2013) tested different concentrations (0, 25, 50,

75 and 100%) of woody plant medium - WPM (Lloyd & McCown 1980) supplemented with 15 g.L⁻¹ of sucrose for the germination of *Comanthera elegantula* (Ruhland) L.R. Parra & Giul. seeds. They concluded that the germination rate wasn't influenced by the saline concentrations, and attained rates higher than 80% in all the treatments.

The authors also evaluated the effect of different culture media (WPM and MS) and saline concentrations (50 and 100%), supplemented with 15 g.L⁻¹ of sucrose, on the *in vitro* establishment of plantlets. Then different concentrations of sucrose (0, 5, 10, 15, 20, 25 and 30 g.L⁻¹) were used to assess the plants' development. The best result for *in vitro* establishment and development was obtained with WPM 100% plus 17 g.L⁻¹ of sucrose.

The same methods for *in vitro* germination, establishment and development of *C. elegantula* were later applied by Pêgo *et al.* (2014) to *Comanthera elegans*. The best germination rate was near 70%, and WPM 100% plus 17 g.L⁻¹ of sucrose was the best treatment for *in vitro* establishment of the species.

A study for *in vitro* establishment of *Comanthera paepalophylla* (Silveira) L.R.Parra & Giul. subsp. *paepalophylla* was carried out by Pêgo *et al.* (2015), in which the authors evaluated the germination rate of seeds cultivated on medium containing only water and agar, or on WPM, and found rates of 70.9 and 60.5%, respectively, after growth for 30 days. In turn, to analyze the *in vitro* development, the authors used plantlets with height of 1 cm and tested the media MS and WPM with 50 and 100% salt concentrations, plus 15 g.L⁻¹ of sucrose and 8 g.L⁻¹ of agar, and concluded that the WPM 100% was better for plant development. That work is a pioneer regarding *in vitro* culture of *C. paepalophylla*, and its results serve as base for future studies of micropropagation and/or conservation of this sempre-viva plant.

WPM was also used by Moreira (2015) for *in vitro* establishment of *Comanthera bisulcata* (Körn.) L.R.Parra & Giul, supplemented with 15 g.L⁻¹ of sucrose. The author tested different immersion periods (1, 5, 10, 15 and 20 minutes) of the seeds in sodium hypochlorite (1%) on the *in vitro* germination and obtained the best result with 5 minutes (54% germination rate) after *in vitro* culture for 30 days. In that study, the author also evaluated the influence of different pH values (4.4, 5.0, 5.4 and 5.8), but did not find any significant differences in the *in vitro* germination rate, with average of 70%. Finally, Moreira (2015) analyzed

the influence of silver nitrate (AgNO₃) on the control of senescence and of polyvinylpyrrolidone (PVP) on oxidation control, attaining positive results in both experiments, where the best responses were obtained with 0.2 µM of AgNO₃ and 400 mg.L⁻¹ of PVP.

For *Comanthera curralensis*, Albuquerque *et al.* (2016) evaluated the effect of different salt concentrations in MS medium (50 and 100%) combined with 15 or 30 g.L⁻¹ of sucrose on *in vitro* establishment. The best germination rate was 62.75%, achieved with MS ½ medium and 15 g.L⁻¹, similar to the effects observed for *C. mucugensis*.

In vitro multiplication

The first published study of the *in vitro* multiplication of *Comanthera mucugensis* was carried out by Paixão-Santos *et al.* (2008), who tested different concentrations of 6-benzylaminopurine - BAP (0.0, 0.89, 1.78, 3.55, 7.14, 14.21 and 28.42 µM) and explant types (entire plant and nodal segments) obtained from plants established *in vitro* with 60 days of culture. As a morphogenic response, the authors only observed callogenesis, with percentages of 52.50 and 49.96% for nodal segments at the concentrations of 1.78 and 3.55 µM, respectively (Tab. 1). At these concentrations, the entire plant explants produced the best results for the same parameter, 71.25 and 68.75% (Tab. 1). Although the authors obtained a high rate of callus formation, they did not investigate this aspect regarding differentiation of shoots.

Later, Lima-Brito *et al.* (2011a) evaluated the *in vitro* morphogenesis of the same species with the use of the cytokinin BAP (0.00, 2.22 and 4.44 µM) in combination with the auxin naphthalene acetic acid - NAA (0.00, 1.34 and 2.68 µM), and different explant types (stem, leaf and root), obtained from *in vitro*-germinated plants with 180 days of culture. They found that the root explants were not responsive and the leaf explants were unable to regenerate shoots, but did form calli in small percentages (12.35 and 19.73%) when treated with 4.44 µM BAP and 2.68 µM NAA, respectively (Tab. 1).

However, unlike reported by Paixão-Santos *et al.* (2008), the authors observed regeneration of shoots in the stem explants by direct organogenesis on MS ½ medium without growth regulator, and by indirect organogenesis on medium containing BAP. In the direct case, the shoot regeneration rate was 58.75%, with 22 shoots per explant, while in the indirect case, the regeneration rates were 32.18

Table 1 – Studies on the *in vitro* morphogenesis of sempre-vivas species.

Reference	Type of morphogenesis	Explant	Growth regulator (μM)	RE%*	NSE**	Species
Paixão-Santos <i>et al.</i> (2008)	Callogenesis	Stem	1.78 BAP	52.50	0	<i>Comanthera mucugensis</i>
			3.55 BAP	49.96	0	
		Plant	1.78 BAP	71.25	0	<i>Comanthera mucugensis</i>
			3.55 BAP	68.75	0	
Lima-Brito <i>et al.</i> (2011a)	Callogenesis	Leaf	2.68 NAA	19.73	0	<i>Comanthera mucugensis</i>
			4.44 BAP	12.35	0	
	Direct organogenesis	Stem	No regulator	58.75	22	<i>Comanthera mucugensis</i>
			Indirect organogenesis	2.22 BAP	32.18	
		4.44 BAP	47.05	32		
Albuquerque (2013)	Indirect organogenesis	Leaf	0.88 BAP	Not evaluated	22.83	<i>Comanthera mucugensis</i>
Gurgel (2017)	Direct organogenesis	Leaf	No regulator	11	0.53	<i>Comanthera mucugensis</i>
Albuquerque (2013)	Callogenesis	Leaf	1.34 NAA	-	0	<i>Comanthera curralensis</i>
			Direct organogenesis	Stem	No regulator	
	Indirect organogenesis	Stem	0.22 BAP	-	7.5	<i>Comanthera curralensis</i>
Pêgo <i>et al.</i> (2013)	Callogenesis	Plant	2.68 NAA	More than 80	0	<i>Comanthera elegantula</i>
	Indirect organogenesis	Plant	4.54 TDZ	Not evaluated	More than 5	<i>Comanthera elegantula</i>
Pêgo <i>et al.</i> (2014)	Callogenesis	Plant	2.68 NAA	More than 80	0	<i>Comanthera elegans</i>
	Indirect organogenesis	Plant	2.68 NAA + 2.27 TDZ	Not evaluated	More than 25	<i>Comanthera elegans</i>
Moreira (2015)	Indirect organogenesis	Plant	2.68 NAA + 2.27 TDZ	50	Not evaluated	<i>Comanthera bisulcata</i>

* = Percentage of responsive explants; ** = Number of shoots/explant.

and 47.05% at concentrations of 2.22 and 4.44 μM , respectively, with averages of 28 and 32 shoots per explant (Tab. 1).

In both studies, calli appeared 15 days after inoculation. The results confirmed the advance achieved by Lima-Brito *et al.* (2011a) regarding

production of plants and showed that *in vitro* multiplication of the species in question is feasible.

With relation to the use of leaf explants of *C. mucugensis*, Albuquerque (2013) tested the effect of different BAP concentrations (0.00, 0.22, 0.44 and 0.88 μM) on the induction of indirect organogenesis

and found the concentration of 0.88 μM to be most efficient, inducing an average of 22.83 shoots per explant. However, the author suggested that higher concentrations should be tested.

Gurgel (2017) also assessed the morphogenic capacity of leaf explants, as well as stem explants of *C. mucugensis*. She tested the influence of different NAA concentrations (0, 4.9 and 9.8 μM) added to MS $\frac{1}{2}$ medium containing 2 g.L^{-1} of activated charcoal and 4.4 μM BAP. For the leaf explants, there was shoot regeneration only in the medium free of the growth regulator, with 11% responsive explants and 0.53 shoot per explant. On the contrary, for the stem explants, morphogenesis occurred at all concentrations, but there were no significant differences between them, indicating that the medium without NAA can be used for shoot multiplication via indirect organogenesis, with 60% responsive explants and 4.72 shoots per explant. Therefore, new tests can be conducted to investigate the morphogenic capacity of the leaf explants, which could enhance multiplication rates. Also, new treatments can be applied to increase shoot regeneration from stem explants. Finally, somatic embryogenesis can be tested to obtain microplants of this species.

In vitro multiplication of the sempre-viva *Comanthera curralensis* was carried out by Albuquerque (2013), evaluating the effect of different NAA concentrations (0.00, 1.34 and 2.68 μM) and BAP (0.00, 0.22, 0.44, 0.88, 1.76 and 3.52 μM) in leaf and stem explants. The author reported different morphogenic responses, such as callogenesis and direct and indirect organogenesis, and also observed high oxidation rates from both explant types (90.7% for stem and 79.2% for leaf explants). The best response for direct organogenesis was achieved with the stem explants in medium free of growth regulator, producing 42 shoots per explant, while for indirect organogenesis, the use of 0.22 μM BAP induced an average of 7.5 shoots per explants. Finally, regarding callogenesis, the best result was obtained using the leaf explants with 1.34 μM NAA.

Pêgo *et al.* (2013), in the study with *Comanthera eleganta*, also tested the effect of different combinations of the growth regulators thidiazuron - TDZ (0.0, 0.5, 1.0, 2.0 or 4.0 mg.L^{-1}) and NAA (0.0, 0.5 or 1.0 mg.L^{-1}) on the entire plantlet. The morphogenic response obtained was the formation of calli with 2.68 μM NAA, and regeneration of shoots by indirect organogenesis produced the best average results in

the combination of 4.54 μM TDZ with 2.68 μM NAA, or the use alone of 4.54 μM TDZ.

The same combinations and concentrations of growth regulators used by Pêgo *et al.* (2013) were again tested by Pêgo *et al.* (2014), with *Comanthera elegans* using the entire plant. The authors also observed the formation of calli and indirect shoots. Callogenesis was obtained in response to 2.68 or 5.36 μM NAA or in the combinations of 2.68 with 2.27, 5.36 with 4.54 and 5.36 with 18 NAA and TDZ, respectively. On the other hand, the formation of shoots occurred in combinations of 2.68 μM NAA with 2.27 TDZ, and 5.36 μM NAA with 4.54 TDZ.

In vitro multiplication using entire plantlets was described by Moreira (2015), evaluating the influence of different cytokinins (BAP, TDZ and kinetin - KIN) associated with 2.68 μM NAA. The author reported the occurrence of indirect organogenesis as a morphogenic response and concluded that the addition of 2.68 μM NAA + 2.27 μM TDZ was best for shoot induction, with a regeneration rate of 50%. This combination of growth regulators was then applied to compare two culture systems, liquid and semi-solid media. The best responses for relative growth rate and average plant weight were obtained in the liquid system. The author concluded that the liquid medium should be tested with other sempre-viva species, in order to enhance the responses of *in vitro* cultivation, besides reducing the cost with the gelling agent.

In light of the results obtained with the *Comanthera* species studied by Pêgo *et al.* (2013, 2014) and Moreira (2015), different explants should be tested in the multiplication step, so as to investigate all parts of *in vitro*-germinated plants, to broaden the possibilities for their use. In addition, the use of culture medium without growth regulator for shoot induction should be analyzed as a strategy to reduce costs, along with regeneration by direct organogenesis with the aim of preserving genetic fidelity.

In vitro rooting

The first study on the induction of *in vitro* rooting of *Comanthera mucugensis* was performed by Silva *et al.* (2005b), in which they tested different concentrations of sucrose (5, 10, 15, 20 and 30 g.L^{-1}) in MS $\frac{1}{2}$ medium for the formation of roots in plants germinated *in vitro* after 60 days of culture. They reported that after culture for 60 days, the concentration of 15 g.L^{-1} produced the

best rooting results, with average of 5.96 roots per plantlet (Tab. 2) and 2.59 mg of root dry matter. However, they did not evaluate the percentage of root formation or investigate the survival after the transfer of the plants to *ex vitro* condition. Furthermore, the induction of rooting was only tested in plantlets germinated *in vitro*, not in shoots from *in vitro* multiplication.

Lima-Brito *et al.* (2016) used MS $\frac{1}{2}$ medium containing 15 g.L⁻¹ of sucrose, supplemented with 4.9 μ M indolebutyric acid - IBA to induce roots from shoots of *Comanthera mucugensis* generated by direct organogenesis. The authors evaluated the effect of different levels of photosynthetically active radiation (PAR) (60, 120 and 300 μ mol m⁻² s⁻¹) and types of tube seals (PVC, cotton and plastic, enabling different aeration levels) on the *in vitro* rooting and observed a negative effect of increased aeration and light intensity on the growth of the microplants. The evaluations were also performed after 60 days of culture and the highest number of roots (14.55) was greater than that described by Silva *et al.* (2005b) (5.96). Furthermore, Lima-Brito *et al.* (2016) evaluated the length of the longest root and rooting percentage (between 94.39 and 98.00%) (Tab. 2). The microplants were later transferred to the *ex vitro* environment. Despite the progress achieved by Lima-Brito *et al.* (2016) in this micropropagation step, new studies should be conducted to try to find ways to reduce the time of *in vitro* growth and diminish the costs, given the high cost of the auxin used.

The application of auxin was also tested with *C. curralensis* by Albuquerque (2013), using different auxin types, AIB, NAA and indole acetic acid (IAA), at distinct concentrations (0.00, 0.22, 0.44, 0.88 and 1.76 μ M) to induce *in vitro* rooting.

The results indicated that the use of auxin was not effective in this step, with a rooting rate of 4.67% and averages of 3.65 and 0.48 (cm) for number and length of roots, respectively. Besides these low values, the author did not evaluate the rooting of shoots from *in vitro* multiplication.

Acclimatization

Lima-Brito *et al.* (2016) also acclimatized the *C. mucugensis* microplants by transferring them to pots containing substrate composed of soil + sand + vermiculite (1:1:1). The microplants were covered by the upper halves of PET bottles (uncapped after 15 days and removed after 30 days), maintained in a nursery under sombrite shading (70%) and watered daily. In this step, they tested the influence of different types of tube seal (PVC, plastic and cotton) and level of photosynthetically active radiation (PAR) (60 and 120 μ mol m⁻² s⁻¹) during the *in vitro* pre-acclimatization step on the plant *ex vitro* survival. This evaluation was carried out 90 days after transference to *ex vitro* conditions and the results indicated that 60 μ mol m⁻² s⁻¹ of PAR associated with PVC or plastic seal induced the best survival rates, 76.12 and 73.28%, respectively (Tab. 3). New studies should be conducted in order to increase plants' survival.

Another strategy was employed by Pereira *et al.* (2017) for acclimatization of microplants, analyzing the effect of inoculation of arbuscular mycorrhizal fungi (AMF) on the *ex vitro* plant survival. Native AMF, filtrate from the soil microbiota and AMF + soil filtrate were used, after 3 months 100% of the plants with AMF survived (Tab. 3), and after 11 months, the plants began to flower. At the end of the experiment, of the total of 8 plants/treatment, 5 survived with the

Table 2 – Studies on *in vitro* rooting of sempre-vivas species.

Reference	Treatment with best result	Percentage of responsive explants	Number of roots	Length of longest root (mm)	Species
Silva <i>et al.</i> (2005b)	15 g.L ⁻¹ of sucrose	Not evaluated	5.96	Not evaluated	<i>Comanthera mucugensis</i>
Lima-Brito <i>et al.</i> (2016)	4.9 μ M of IBA	97.80	14.55	15.6	<i>Comanthera mucugensis</i>
Albuquerque (2013)	No regulator	4,67	3,65	0,48	<i>Comanthera curralensis</i>

Table 3 – Studies on acclimatization of sempre-vivas species.

Authors	Survival (%)	Treatment with best result	Species
Lima-Brito <i>et al.</i> (2016)	76.12	Soil + sand + vermiculite + SRWL*	<i>Comanthera mucugensis</i>
Pereira <i>et al.</i> (2017)	100	AMF**	<i>Comanthera mucugensis</i>
Pêgo <i>et al.</i> (2013)	74	Sand + SRWL	<i>Comanthera elegantula</i>
Pêgo <i>et al.</i> (2014)	25.6	Sand, vermiculite and Plantmax™ + SRWL	<i>Comanthera elegans</i>

* = Strategy to reduce water loss; ** = arbuscular mycorrhizal fungi.

AMF treatment and 7 with the AMF + filtrate. The authors suggested further studies to assess the influence of the fungi alone.

Both studies demonstrated that it is possible to micropropagate *C. mucugensis*, but new acclimatization strategies still need to be studied in order to obtain a larger number of surviving plants and to establish the best fungus species for inoculation.

In the previously mentioned study with *Comanthera elegantula*, Pêgo *et al.* (2013) evaluated the influence of different substrate types (sand, vermiculite and Plantmax™) on the survival of acclimatized plants. After transfer to the substrate, the plants were covered by clear plastic bags, which were perforated weekly until their removal after 30 days. The authors concluded that sand produced the best results for the species, with an *ex vitro* survival rate of 74% (Tab. 3).

This same strategy was used by Pêgo *et al.* (2014) for acclimatization of *Comanthera elegans*, and there was no significant effect of the substrate types and pre-acclimatization on the survival rate of the plants, which was 25.6% (Tab. 3). Therefore, new studies should also be conducted with both *C. elegans* and *C. elegantula* seeking to develop more efficient acclimatization strategies to increase the survival of these species after transfer to the *ex vitro* environment.

In vitro conservation of sempre-viva species

The first study on the *in vitro* conservation of *Comanthera mucugensis* was carried out by Lima-Brito *et al.* (2011b), in which they tested the influence of different concentrations and combinations of osmotic agents (sucrose, mannitol and sorbitol) and temperature (18 and 25 °C) on the minimum growth induction of shoots obtained by direct organogenesis. Plants' survival rate was

evaluated after 180 days of culture and the authors concluded that the best treatment consisted of 15 g L⁻¹ of sucrose at 18° C, which induced survival of 97.5%, thus recommending it for the medium-term conservation of this species (Tab. 4).

A different strategy was tested by Lima-Brito *et al.* (2015) with *Comanthera mucugensis*, in which they applied different concentrations (0.0, 0.85, 1.70, 3.40 and 6.80 µM) of the growth regulators ancymidol or paclobutrazol to reduce the growth, also with shoots obtained by direct organogenesis. The authors evaluated plants' survival after the same period (180 days) as Lima-Brito *et al.* (2011b), and concluded that the culture medium without any growth regulator was more efficient, inducing a survival rate 81.25% (Tab. 4).

Both studies investigated *in vitro* conservation for only 180 days, and the authors used shoots formed by direct organogenesis. Therefore, further research is necessary to determine survival rates after longer periods of *in vitro* cultivation as well as using plants derived from *in vitro* germination, to allow the maintenance of the genetic variability of the species in *in vitro* collections.

The slow growth method was also applied by Albuquerque (2013) for *in vitro* conservation of the sempre-viva species *C. curralensis*. The author tested different osmotic agents, combining concentrations of sucrose (15, 30 or 45 g.L⁻¹) with mannitol or sorbitol (0 or 23 g.L⁻¹), and concluded that the use of 30 g.L⁻¹ sucrose induced the highest survival rate, 24.8%. Furthermore, the application of ancymidol and paclobutrazol was also evaluated, at different concentrations (0.00, 0.42, 0.85, 1.70 and 3.40 µM). The highest survival rate (63.25%) was obtained with 0.85 µM ancymidol. In this study, the authors used plants derived from *in vitro* germination and the conservation period was only 180 days, so further research is necessary with longer time frames.

Table 4 – Studies on *in vitro* conservation of sempre-vivas species through slow growth.

Authors	Treatment	Period	S%*	Explant	Species
Lima-Brito <i>et al.</i> (2011b)	MS ½ + 15 g.L ⁻¹ sucrose at 18 °C	180 days	97.5	Shoots	<i>Comanthera mucugensis</i>
Lima-Brito <i>et al.</i> (2015)	MS ½ free ancymidol or paclobutrazol	180 days	81.25	Shoots	<i>Comanthera mucugensis</i>
Albuquerque (2013)	30 g.L ⁻¹ sucrose	180 days	24.8	Plant	<i>Comanthera curralensis</i>
	0,85 µM ancymidol	180 days	63.25	Plant	<i>Comanthera curralensis</i>

* = Survival percentage.

Conclusion

Based on the results reported here, it is possible to infer that tissue culture is a viable alternative for propagation and conservation of the *Comanthera* genus. New studies should be carried out to optimize the protocols for production of seedlings/plantlets in commercial scale and for *in vitro* conservation in the medium range, as for *Comanthera mucugensis*.

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