

IN VITRO CONSERVATION AND ACCLIMATIZATION OF EPIDENDROIDEAE (Orchidaceae) FROM SERGIPE, BRAZIL

CONSERVAÇÃO IN VITRO E ACLIMATIZAÇÃO DE EPIDENDROIDEAE (Orchidaceae) DE SERGIPE, BRASIL

Thays Saynara Alves MENEZES-SÁ¹, Maria de Fátima ARRIGONI-BLANK²,
Andrea Santos da COSTA³, Rosana Barroso FEITOSA-ALCANTARA⁴,
Arie Fitzgerald BLANK², José Magno Queiroz LUZ⁵

1. Doutoranda em Agricultura e Biodiversidade, UFS, São Cristóvão, SE, Brasil. thayssaynara@yahoo.com.br; 2. Professor do Departamento de Engenharia Agrônômica, Universidade Federal de Sergipe, Av. Marechal Rondon s/n, São Cristóvão, SE, Brasil; 3. Pós-doutoranda em Agricultura e Biodiversidade, UFS, São Cristóvão, SE, Brasil; 4. Doutora em Biotecnologia. 5. Instituto de Ciências Agrárias, Universidade Federal de Uberlândia, Av. Amazonas s/n, Uberlândia-MG, Brasil.

ABSTRACT: Orchids are known for the beauty, exuberance, and color of their flowers and therefore represent one of the most coveted ornamental plants. Due to their high commercial value, several illegal practices of removal and commercialization have led to the verge of extinction. This study aimed to develop protocols for *in vitro* conservation under slow growth and acclimatization of species of the subfamily Epidendroideae (Orchidaceae), from the state of Sergipe, Brazil. Two experiments were conducted: the first one tested four concentrations of MS (MURASHIGE and SKOOG, 1962) (25%, 50%, 75%, 100%), three orchid species (*Catasetum macrocarpum*, *Oeceoclades maculata*, and *Polystachya estrellensis*), and two temperatures (18 and 25°C); the second one tested three combinations of carbon sources and osmotic regulators (20 g L⁻¹ sucrose; 10 g L⁻¹ sucrose + 5 g L⁻¹ de manitol; 10 g L⁻¹ sucrose + 5 g L⁻¹ sorbitol) and the same three species and two temperatures. For the *in vitro* conservation of *C. macrocarpum*, *O. maculata*, and *P. estrellensis* over 450 days, the use of 25% of MS salts or 20 g.L⁻¹ sucrose at 25°C is recommended. *C. macrocarpum* seedlings were acclimatized using sand + shredded pine bark + worm castings at a ratio of 2:2:1 (w:w:w). The use of sand alone is recommended for the acclimatization of *O. maculata*.

KEYWORDS: *Catasetum macrocarpum*; *Oeceoclades maculate*; *Polystachya estrellensis*; Slow growth; Substrate.

INTRODUCTION

The Orchidaceae family is the most diverse of all angiosperm families. It has approximately 35,000 species distributed over 1,800 genera and more than 100,000 hybrids. ROBERTS and DIXON (2008) grouped this family in five subfamilies, which are spread worldwide (MOREIRA et al., 2007), and the greatest diversity is found in the tropics (RECH et al., 2011). Orchids are ornamental plants of economic and botanical interest (CHUGH et al., 2009); they are also important for the medical, cosmetic, and food industries (GALDIANO-JUNIOR et al., 2012).

The several tropical species and hybrids exhibit varied shapes, colors, and flowers, which are commercially exploited worldwide. The rapid economic development has increased the anthropic exploitation of natural resources in an exaggerated fashion. Some orchid species are endangered owing to forest devastation or the intense extractive activity by collectors and traders (COLOMBO et al., 2004), which has caused the loss of genetic material

and variability in native populations. Conservation practices have increasingly relied on biotechnological applications, such as *in vitro* conservation.

The *in vitro* conservation has two procedures: slow growth, which involves reducing plant metabolism; and cryopreservation, with the complete growth suppression by storage at ultra-low temperatures. The *in vitro* conservation of plants allows maintaining a large number of specimens over a small footprint, without experiencing adverse weather conditions or risks associated with fieldwork (FARIA et al., 2006). This technique aims at the highest survival rate and genetic stability with the lowest frequency of subcultures.

The *in vitro* slow growth procedure maximizes the interval between subcultures or prolongs it indefinitely (MARCO-MEDINA and CASAS, 2012). It has been used for the conservation of several plant species, such as *Vanilla planifolia* (DIVAKARAN et al., 2006), *Epidendrum chlorocorymbos* (LOPEZ-PUC, 2013), and *Paphiopedilum* (NG and SALEH, 2011). This

technique allows the storage of healthy germplasm with prolonged subculture intervals and thus reduce maintenance time and costs (SANTOS et al., 2012; PEIXOTO et al. 2017).

The acclimatization is characterized as a gradual adaptation of micropropagated plants in a greenhouse before transferring them to the field, aiming at minimizing the effects of a sudden change of environments, as they had been kept in a totally controlled and aseptic place (POSPÍLOVÁ et al., 2007). This acclimatization consists of two stages: rooting (*in vitro* and *ex vitro*) and transfer to non-sterile conditions with controlled temperature and humidity (SILVA et al., 2003).

This process is not an easy task since microplants need to change their metabolism from heterotrophic (*in vitro*) to autotrophic (SILVA et al., 2003). Therefore, the choice of substrate is fundamental to the success of acclimatization. The substrate must have good physico-chemical characteristics that allow the maintenance of the root system and the gas exchange between the roots and the environment to facilitate the plant's development (FREITAS et al., 2011).

This study aimed to develop protocols for *in vitro* conservation under slow growth and acclimatization of species of the subfamily Epidendroideae (Orchidaceae), from the state of Sergipe, Brazil.

MATERIAL AND METHODS

The collected individuals were maintained in a greenhouse at the Department of Agronomic Engineering of the Federal University of Sergipe, Brazil. Seeds were collected in 2012 from specimens with ripe fruits and were used to establish the *in vitro* cultures. *Catasetum macrocarpum* Rich. ex Kunth., *Oeceoclades maculata* Lindl., and *Polystachya estrellensis* Rchb. f. were used in the *in vitro* conservation experiments, and *C. macrocarpum* and *O. maculata* were used in the acclimatization of micropropagated seedlings.

The species were established using the collected fruits. Seed capsules were washed with water and neutral detergent, disinfected in a laminar flow hood in 70% alcohol (v/v) for 1 minute and a 5% sodium hypochlorite solution for 20 minutes, and then triple-washed with autoclaved distilled water. After disinfection, capsules were opened, and seeds were isolated in Murashige and Skoog (MS) salt medium, with half the concentration of salts, plus 30 g L⁻¹ sucrose, 7 g L⁻¹ agar, and 1 g L⁻¹ activated charcoal. The tubes were maintained in a biochemical oxygen demand (BOD) chamber, at a

controlled temperature of 25 ± 2°C and a 12-hour photoperiod (luminous intensity of 60 μmol m⁻² s⁻¹). After reaching 1-2 cm in height, seedlings were used in the *in vitro* conservation and acclimatization experiments.

The *in vitro* conservation experiments were conducted in the Laboratory of Plant Tissue Culture and Breeding, and the acclimatization experiment was performed in a greenhouse, located in the Department of Agronomic Engineering of the Federal University of Sergipe.

All the *in vitro* conservation experiments used MS as the culture medium, which had the pH adjusted to 5.8 ± 0.1 before autoclaving (121±1°C and 1.05 atm) for 15 minutes. Plants were inoculated in test tubes and maintained in a growth room, at a controlled temperature of 25± 2° C, with a 12-hour photoperiod (luminous intensity of 60 μmol m⁻² s⁻¹), in a BOD chamber (18°C).

Reduction of MS salt medium and culture temperature

A completely randomized experimental design was used, in a 4x3x2 factorial scheme, with five replications, four MS salt medium concentrations (25%, 50%, 75%, and 100%), three species (*C. macrocarpum*, *O. maculata*, and *P. estrellensis*), and two temperatures (18 and 25°C). Each replication consisted of six tubes containing one shoot each.

Osmotic regulators and culture temperature

A completely randomized experimental design was used, in a 3x3x2 factorial scheme, with five replications, to test three combinations of carbon sources and osmotic regulators (20 g L⁻¹ sucrose; 10 g L⁻¹ sucrose + 5 g L⁻¹ mannitol; and 10 g L⁻¹ sucrose + 5 g L⁻¹ sorbitol), three species (*C. macrocarpum*, *O. maculata*, and *P. estrellensis*), and two temperatures (18 and 25°C). Each replication consisted of six tubes containing one shoot each.

Experiments evaluations

For the *in vitro* conservation experiments, evaluations were performed after 450 days. Survival rate (%), presence of roots (%), and leaf color were evaluated, following a scoring scale, in which 5 corresponded to 100% of green leaves and shoots; 4 corresponded to the start of leaf drying; 3 corresponded to 30-50% of dead leaves; 2 corresponded to over 50% of dead leaves; and 1 corresponded to 100% of dead leaves and shoots. Plant height was evaluated according to a scoring scale (LEMOIS et al., 2002), in which 4

corresponded to a height equal to the initial height; 3 corresponded to up to the double of the initial height; 2 corresponded to heights between the double and triple of the initial height; and 1 corresponded to over the triple of the initial height.

Seedling acclimatization

The seedlings acclimatization experiment used the species *C. macrocarpum* and *O. maculata*. The acclimatization of *P. estrellensis* was not possible due to the lack of available seedlings.

Seedlings established *in vitro* after approximately 120 days in MS medium were removed from the vials, washed in running water to eliminate any culture medium adhered to the roots, and transferred to 72 polystyrene trays containing the different treatments.

A completely randomized design was used, with five replications. The following eight substrates were tested (w/w/w): sand + worm castings (1:1); sand + coconut coir + worm castings (2:2:1); sand + shredded pine bark + worm castings (2:2:1); sand + shredded pine bark + coconut coir + worm castings (2:1:1:1); sand; sand + coconut coir (1:1); sand + shredded pine bark (1:1); and sand + shredded pine bark + coconut coir (2:1:1). Each experimental unit was composed of six seedlings. The experiment was conducted in a greenhouse with an intermittent irrigation system and covered with a 50% shade screen. Seedlings were maintained under the described treatments for 180 days. The following variables were analyzed: survival rate (%), number of leaves, height (cm), and shoots and roots dry weight (mg). Shoots and roots were dried in a forced-air circulation oven, at 60°C, until constant weight, to determine shoots and roots dry weight.

Statistical analysis

Results were subject to analysis of variance (ANOVA), and when necessary, the means were compared by the Tukey's test, at the 5% probability level. For the MS medium concentration, a polynomial regression was applied, using the SISVAR software (FERREIRA, 2011). The percentage variables were transformed into the arcsine of the square root of (X/100). In the acclimatization experiments, the means were compared by the Scott-Knott's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

MS salt medium reduction and culture temperature

At 450 days of *in vitro* conservation, the survival of the plants in response to the salt concentration and temperature were evaluated. *O. maculata* and *P. estrellensis* had the highest survival rate when maintained at 18°C, showing values between 90 and 100% (*O. maculata*) and 88 and 100% (*P. estrellensis*). At this temperature, *O. maculata* responded positively to the increase of salt concentrations in the MS medium. Conversely, *P. estrellensis* had a negative response to the increase in the salt concentration, showing a reduction in the survival rate. *C. macrocarpum* had the minimum survival rate in response to 25% MS salts. (Table 1).

The evaluation of seedling survival at 25°C revealed that the salt concentration was significant for all the species. However, this temperature was beneficial for *C. macrocarpum* (97% of survival) when compared with cultivation at 18°C (survival below 35%) (Table 1). The low survival rate observed for *C. macrocarpum* at 18°C is probably because these plants are found in a coastal forest environment (*restinga*), where temperatures range from 25 to 35°C, and thus, 18°C is below the range of tolerance for this species (MONTEIRO et al., 2012). In *Cochlospermum regium* (CAMILLO et al., 2009), temperatures below 20°C also reduced the survival rate of individuals. Temperatures below the ideal range for the cultivation of a species is one of the most commonly used strategies to induce slow growth. However, the physiological changes caused by cold temperatures may be irreversible and prevent future growth. The range of tolerance to lower or higher temperatures varies among species and even among genotypes or cultivars of the same species.

Regarding the leaf color at 450 days of *in vitro* conservation, the temperature of 18°C was favorable for *O. maculata* and *P. estrellensis* since the leaves remained completely green (score 5). For these species, the use of low temperatures possibly increased the viability of the cultures by slowing the tissue degradation process (Table 1). Low temperature has been proven efficient for reducing the growth of several species and retaining greener leaves for a longer time, as observed in *Aechmea blanchetiana* (SANTA-ROSA et al., 2013) and potato (ARRIGONI-BLANK et al., 2014). However, low temperatures can also be limiting for other species, as observed in this study for *C. macrocarpum*, which had scores higher than 2, indicating over 50% of dead leaves (25°C). Therefore, this species was not tolerant to lower temperatures (18°C) (Table 1).

Table 1. Seedling survival (%), leaf color (scores 1-5), and seedling height (scores 1-4) of the three species of the subfamily Epidendroideae, at 450 days of conservation, according to the species, MS salt concentration, and temperature.

MS Salts (%)	Survival (%)		Leaf coloration (scores 1 to 5)		Seedling height (scores 1 to 4*)	
	Temperature		Temperature		Temperature	
	18°C	25°C	18°C	25°C	18°C	25°C
	<i>C. macrocarpum</i>		<i>C. macrocarpum</i>		<i>C. macrocarpum</i>	
25	23.75 B	96.25 A	1.66 B	3.84 A	3.76 A	1.75 B
50	27.50 B	96.25 A	1.03 B	3.66 A	3.71 A	1.66 B
75	42.50 B	96.25 A	1.09 B	3.65 A	3.57 A	1.66 B
100	48.75 B	100.00 A	1.02 B	2.65 A	3.51 A	1.67 B
Equation (Y)=	13.1250+0.3600x	94.3750+0.060x	2.368-0.0354x+0.0002x ²	4.345-0.0143x	3.8610-0.0035x	1.8700-0.005880x+0.00004x ²
	R ² =94.95%	R ² =80.00%	R ² =88.71%	R ² =73.17%	R ² =96.37%	R ² =93.89%
CV(%)	8,74		18,32		2,63	
	<i>O. maculata</i>		<i>O. maculata</i>		<i>O. maculata</i>	
25	90.00 B	100.00 A	4.96 A	4.00 B	3.20 A	3.10 A
50	93.33 B	100.00 A	4.93 A	4.00 B	3.20 A	3.10 A
75	100.00 A	100.00 A	4.90 A	4.00 B	3.13 A	2.80 B
100	100.00 A	100.00 B	4.80 A	4.00 B	3.20 A	2.73 B
Equation (Y)=	ns	ns	5.0333-0.0021x	ns	3.2833-0.0036x+0.00002x ²	3.2833-0.0056x
			R ² =91.43%		R ² =40.00%	R ² =86.47%
CV(%)	5,44		1,36		4,35	
	<i>P. estrellensis</i>		<i>P. estrellensis</i>		<i>P. estrellensis</i>	
25	100.00 A	100.00 A	4.46 A	3.60 B	3.06 A	2.13 B
50	100.00 A	100.00 A	4.46 A	3.76 B	3.10 A	2.10 B
75	96.66 A	96.66 A	4.63 A	3.83 B	3.03 A	2.10 B
100	88.00 A	96.66 A	4.60 A	3.80 B	3.00 A	2.10 B
Equation (Y)=	ns	ns	ns	ns	ns	ns
CV(%)	8,01		6,96		4,16	

Means followed by the same uppercase letters between temperatures do not statistically differ by the Tukey's test at the 5% probability level. CV - coefficient of variation. Color scores: 5 = completely green leaves and shoots; 4 = beginning of leaf drying; 3 = 30-50% dead leaves; 2 = over 50% dead leaves; and 1 = completely dead leaves and shoots. Height scores: 4 = height similar to initial height (1-2 cm); 3 = up to the double of the initial height; 2 = between the double and triple of the initial height; and 1 = over the triple of the initial height.

O. maculata and *P. estrellensis* plants maintained at 25°C had scores of approximately 4, indicating the beginning of leaf drying. This temperature was only better than 18°C for *C. macrocarpum*, following the same survival trend (Table 1). Plants with high color scores (greener) should be chosen because senescence is not acceptable for slow growth conservation. Moreover, plants must remain viable for future growth (SANTOS et al., 2012).

The analysis of seedling height at 450 days of cultivation revealed the minimal growth for *C. macrocarpum* at 18°C. This species showed the lowest scores when compared with the other species at all concentrations of MS salts. However, under this temperature, only 35% of *C. macrocarpum* plants survived (Table 1). When maintained at 25°C, this species showed greater growth than the others

(Table 1). In general, 25°C resulted in greater seedling growth, except for *O. maculata* seedlings, which exhibited lower growth using 25 to 100% salts at 18°C (Table 1). Similar behavior was observed in *A. blanchetiana*, which maintained the lowest growth indices at 360 days at lower temperatures (SANTA-ROSA et al., 2013).

When performing *in vitro* conservation, plant height is an important variable to indicate the subculturing time because when the medium is exhausted, the plant growth is affected by the lack of nutrients. At this point, the number of senescent leaves increases, indicating a weak physiological state and the need to change the medium.

Osmotic regulators and culture temperature

Seedling survival rates throughout 450 days of conservation under slow growth were between 80 and 100% for all species, regardless of the culture

temperature, except for *C. macrocarpum* at the temperature of 18°C, whose survival rate of microcultures reduced. A mean survival rate of 25% (Table 2) indicates that the temperature of 18°C should not be used in slow growth conservation for that species. No significant differences were observed between osmotic regulators in the analysis of the survival rate at 25 °C, and the values ranged

from 88 to 100% (Table 2). A higher survival rate was detected for *C. macrocarpum* seedlings at this temperature when compared with the survival rate at 18°C (Table 2). Similar results were found in *Agave nizandensis*, which was *in vitro*-conserved for 300 days, with sucrose:mannitol:sorbitol (30:50:50 g L⁻¹), at 25°C (BALCH et al., 2012).

Table 2. Seedling survival (%), leaf color (scores 1-5), and seedling height (scores 1-4) of three species of the subfamily Epidendroideae, at 450 days of conservation, in function of species, osmotic regulators, and temperature.

Osmotic Regulators (g L ⁻¹)	Species		
	<i>C. macrocarpum</i>	<i>O. maculata</i>	<i>P. estrellensis</i>
Survival			
-----Temperature 18°C -----			
Sucrose (20)	23.75 aB	100.00 aA	92.00 aA
Sucrose + Mannitol (10:5)	21.25 aB	84.00 bB	92.00 aA
Sucrose + Sorbitol (10:5)	28.75 aB	84.00 bB	88.00 aA
-----Temperature 25°C -----			
Sucrose (20)	98.75 aA	100.00 aA	96.00 aA
Sucrose + Mannitol (10:5)	96.25 aA	100.00 aA	92.00 aA
Sucrose + Sorbitol (10:5)	93.75 aA	100.00 aA	88.00 aA
CV(%)	11.33		
Leaf color			
-----Temperature 18°C -----			
Sucrose (20)	1.89 aB	4.36 aA	4.00 aA
Sucrose + Mannitol (10:5)	1.80 aB	4.63 aA	3.80 aA
Sucrose + Sorbitol (10:5)	1.84 aB	4.36 aA	3.70 aA
-----Temperature 25°C -----			
Sucrose (20)	3.92 aA	3.96 aA	3.43 aA
Sucrose + Mannitol (10:5)	3.98 aA	3.90 aA	3.30 aA
Sucrose + Sorbitol (10:5)	3.82 aA	3.90 aA	3.10 aA
CV(%)	9.13		
Seedling height			
-----Temperature 18°C -----			
Sucrose (20)	3.24 aA	2.86 aA	2.90 aA
Sucrose + Mannitol (10:5)	3.63 aA	3.00 aA	2.93 aA
Sucrose + Sorbitol (10:5)	3.65 aA	3.00 aA	3.00 aA
-----Temperature 25°C -----			
Sucrose (20)	2.98 aA	2.40 aA	2.03 aB
Sucrose + Mannitol (10:5)	3.04 aB	2.86 aA	2.10 aB
Sucrose + Sorbitol (10:5)	3.03 aB	2.80 aA	2.20 aB
CV(%)	9.94		

Means followed by the same lowercase letters in the same column and Greek letters between temperatures do not differ statistically by the Tukey's test at 5% probability. Color scores: 5 = completely green leaves and shoots; 4 = beginning of leaf drying; 3 = 30-50% dead leaves; 2 = over 50% dead leaves; and 1 = completely dead leaves and shoots. Height scores: 4 = height similar to the initial height (1-2 cm); 3 = up to the double of the initial height; 2 = between the double and triple of the initial height; and 1 = over the triple of the initial height. CV – coefficient of variation.

Regarding the color, *P. estrellensis* and *O. maculata* had the highest scores at the temperature of 18°C. The latter species showed the highest mean (4.45), indicating the beginning of leaf drying, regardless of the osmotic regulator. This same temperature was not favorable for color maintenance in *C. macrocarpum* because this species had approximately completely dead leaves

and shoots (score 1) at 450 days of conservation (Table 2). Leaf color at 25°C had a greater uniformity of scores (3.10-3.98), with scores closer to 3, which indicates between 30 and 50% of dead leaves (Table 2). When comparing the two temperatures, 18 °C enabled seedlings to maintain greener leaves, possibly due to the slower metabolism, although the temperature of 25 °C was

the most favorable for *C. macrocarpum*. *A. fasciata* also had greener leaves when conserved at 25°C for 360 days (COSTA et al., 2012).

The analysis of seedling height at 450 days of cultivation at the temperature of 18°C revealed that the combination of sucrose with mannitol and sorbitol led to the high scores for *C. macrocarpum* (3.6, up to the double of the initial height) and *P. estrellensis* and *O. maculata* (between the double and triple of the initial height) (Table 2). The analysis of temperatures indicated that 25°C was favorable to greater seedling heights, regardless of the osmotic regulator (Table 2). After 450 days of *in vitro* conservation of *C. macrocarpum*, *O. maculata*, and *P. estrellensis*, the explants originated from all the treatments reestablished their growth when placed in normal *in vitro* cultivation.

For *in vitro* conservation, slow plant growth is important for avoiding successive subcultures and preserving the plant's capacity to recover and multiply. A viability analysis is required to certify the successful development of a conservation protocol. Therefore, the three species of orchid microplants were placed in the MS medium for the evaluation of their development. The viability analysis showed that the concentrations of MS salts and osmotic regulators used in the conservation protocol did not hinder the responsive capacity of the culture. Thus, the plants maintained high vigor and development, even after months without changing the culture medium.

Seedling acclimatization

The ANOVA test showed significant differences for substrate mixtures after 180 days of acclimatization. For *C. macrocarpum*, the comparison between substrates revealed mean survival rates ranging from 50% to 100% (Table 3). The best results were obtained in mixtures that contained worm castings (90-100% survival) (Table 3). Worm castings might have contributed to the development of seedlings since it is an organic material rich in nutrients (nitrogen, phosphorous, potassium, magnesium, calcium, and several micronutrients). Therefore, this substrate favors the seedlings survival over the cultivation (KNAPIK and ANGELO 2007).

The different mixtures of substrates did not influence the survival rate of *O. maculata* during the acclimatization experiment since the values ranged from 83 to 100% (Table 3). These values were probably the result of the ability of this species to adapt to different environments, from sandy to rainforests soils. Owing to its adaptation and environmental colonization, this species is

considered as a bioindicator orchid for anthropic forest fragments (DUBBERN et al., 2013).

The analysis of plant height for *C. macrocarpum* showed that the substrate mixture sand + shredded pine bark + coconut coir + worm castings (2:1:1:1) resulted in the tallest plants (5.8 cm) (Table 3). Similar results were obtained in the acclimatization of *Cattleya guttata* x *Laelia tenebrosa*, using mixtures containing coconut coir and fiber (COLOMBO et al., 2005). For *O. maculata*, the use of sand and worm castings (1:1) led to the greatest plant growth (6.51 cm) (Table 3).

C. macrocarpum reached the largest number of leaves per plant when using the mixtures containing sand + worm castings (1:1) and sand + shredded pine bark + worm castings (2:2:1). Conversely, *O. maculata* reached the smallest number of leaves per plant when using sand + worm castings (1:1) and sand + coconut coir + worm castings (2:2:1) (Table 3). The combination of worm castings with coconut coir also led to an increase in the number of *O. maculata* leaves during acclimatization (ROCHA et al., 2009). Seedlings with a larger number of leaves have higher survival rates in the field since leaves are responsible for capturing solar energy to produce organic material through photosynthesis (ASSIS et al., 2009).

The analysis of the shoot dry weight of *C. macrocarpum* revealed that the lowest values were obtained in the mixtures of substrates that did not contain worm castings [sand; sand + coconut coir (1:1); sand + shredded pine bark (1:1); sand + shredded pine bark + coconut coir (2:1:1)], with values between 4.5 and 11.3 mg. However, the highest values of shoot dry weight (43.4 mg) was provided by the mixture of sand + shredded pine bark + worm castings (2:2:1). This fact might have been the result of the improved nutritional aspects of the substrates containing worm castings and the presence of sand and shredded pine bark, which offer better physical properties and adequate water supply (Table 3).

Table 3. Survival rate (%), plant height, number of leaves, and shoots and roots dry weight (mg) of *Catasetum macrocarpum* and *Oeceoclades maculata*, in function of different substrate mixtures, at 180 days of cultivation.

Substrates	<i>C. macrocarpum</i>	<i>O. maculata</i>
	----- Survival (%) -----	
S:WC (1:1)	90.0 a	83.3 a
S:CC:WC (2:2:1)	100.0 a	96.7 a
S:SPB:WC (2:2:1)	100.0 a	96.7 a
S:SPB:CC:WC (2:1:1:1)	90.0 a	100.0 a
S	76.7 b	100.0 a
S:CC (1:1)	80.0 b	90.0 a
S:SPB (1:1)	53.3 c	100.0 a
S:SPB:CC (2:1:1)	50.0 c	100.0 a
CV (%)	14.54	
	----- Plant height (cm) -----	
S:WC (1:1)	4.0 c	6.5 a
S:CC:WC (2:2:1)	3.9 c	5.9 b
S:SPB:WC (2:2:1)	5.2 b	5.2 c
S:SPB:CC:WC (2:1:1:1)	5.8 a	5.0 c
S	3.6 d	4.7 c
S:CC (1:1)	3.3 d	4.2 d
S:SPB (1:1)	3.3 d	2.8 e
S:SPB:CC (2:1:1)	3.2 d	2.6 e
CV (%)	8.88	
	----- Number of leaves -----	
S:WC (1:1)	6.8 a	2.8 b
S:CC:WC (2:2:1)	5.7 b	3.3 b
S:SPB:WC (2:2:1)	6.3 a	4.3 a
S:SPB:CC:WC (2:1:1:1)	5.5 b	4.3 a
S	4.2 c	4.4 a
S:CC (1:1)	4.1 c	4.2 a
S:SPB (1:1)	4.4 c	3.7 a
S:SPB:CC (2:1:1)	3.5 c	3.9 a
CV (%)	16.78	
	----- Shoots dry weight (mg) -----	
S:WC (1:1)	28.5 b	16.6 b
S:CC:WC (2:2:1)	20.8 b	23.5 b
S:SPB:WC (2:2:1)	43.4 a	33.5 a
S:SPB:CC:WC (2:1:1:1)	24.2 b	34.7 a
S	4.5 c	33.8 a
S:CC (1:1)	11.3 c	38.6 a
S:SPB (1:1)	10.5 c	14.4 b
S:SPB:CC (2:1:1)	9.2 c	18.4 b
CV (%)	34.38	
	----- Roots dry weight (mg) -----	
S:WC (1:1)	34.7 a	11.2 c
S:CC:WC (2:2:1)	18.9 b	16.6 c
S:SPB:WC (2:2:1)	39.7 a	24.0 b
S:SPB:CC:WC (2:1:1:1)	32.2 a	27.4 b
S	9.0 b	45.4 a
S:CC (1:1)	14.4 b	24.0 b
S:SPB (1:1)	27.6 a	13.1 c
S:SPB:CC (2:1:1)	19.1 b	14.9 c
CV (%)	44.38	

Means followed by the same lowercase letters in the columns do not differ by the Scott-Knott's test ($p < 0.05$). S:WC – sand + worm castings (1:1); S:CC:WC – sand + coconut coir + worm castings (2:2:1); S:SPB:WC – sand + shredded pine bark + worm castings (2:2:1); S:SPB:CC:WC – sand + shredded pine bark + coconut coir + worm castings (2:1:1:1); S – sand; S:CC – sand + coconut coir (1:1); S:SPB – sand + shredded pine bark (1:1); S:SPB:CC – sand + shredded pine bark + coconut coir (2:1:1). CV – coefficient of variation.

In general, higher values were observed for *O. maculata*, and the substrates sand + shredded pine bark + worm castings (2:2:1), sand + shredded pine bark + coconut coir + worm castings (2:1:1:1), sand, and sand + coconut coir (1:1) provided the highest values (Table 3). Similar results were reported for the acclimatization of *Phalaenopsis* ssp. using mixtures containing pine bark, which enabled greater dry weight accumulation and might have promoted greater moisture retention (AMARAL et al., 2010).

Considering the variable root dry weight in *C. macrocarpum*, a higher value was provided by the substrates sand + worm castings (1:1), sand + shredded pine bark + worm castings (2:2:1), sand + shredded pine bark + coconut coir + worm castings (2:1:1:1), and sand + shredded pine bark (1:1), with a mean of 33.58 mg of biomass. For *O. maculata*, however, the highest biomass was obtained with the substrate sand (45.46 mg) (Table 3). Generally, the mixtures of coconut coir, sand, and pine bark favored moisture retention and aeration, improved the physical conditions of the plants, and enabled the root development and growth (AMARAL et al., 2010), which corroborated the results obtained for *C. macrocarpum*. Nevertheless, the highest root dry weight for *O. maculata* was obtained when using sand. This result probably occurred because *O. maculata* is a terrestrial orchid commonly found in sandy environments. *O. maculata* is indicated as an

invasive species in disturbed environments, which assists in the diagnosis of interference in forest fragments (DUBBERN et al., 2013) and (UENO, 2013).

CONCLUSIONS

For the *in vitro* conservation of *C. macrocarpum*, *O. maculata*, and *P. estrellensis* throughout 450 days, 25% MS salts at a temperature of 25°C, or 20 g L⁻¹ sucrose at 25°C should be used.

For the acclimatization of *C. macrocarpum* seedlings for 180 days, the substrate composed of sand + shredded pine bark + worm castings (2:2:1) is recommended. Conversely, for the acclimatization of *O. maculata* seedlings at 180 days, the exclusive use of sand is enough.

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RESUMO: As orquídeas são conhecidas pela beleza, exuberância e cores de suas flores e por isso representam uma das plantas ornamentais mais cobiçadas. Devido ao alto valor comercial, muitas são as práticas ilegais de retirada e comercialização de orquídeas, o que leva as espécies à beira de extinção. O objetivo deste estudo foi desenvolver protocolos para a conservação *in vitro* sob crescimento lento e aclimação de espécies da subfamília Epidendroideae (Orchidaceae) do Estado de Sergipe, Brasil. Dois experimentos foram realizados: o primeiro ensaio testou quatro concentrações dos sais MS (MURASHIGE e SKOOG, 1962) (25, 50, 75 e 100%), três espécies (*Catasetum macrocarpum*, *Oeceoclades maculata* e *Polystachya estrellensis*) e duas temperaturas (18 e 25°C); o segundo ensaio testou três combinações de fontes de carbono e reguladores osmóticos (20 g L⁻¹ de sacarose; 10 g L⁻¹ de sacarose + 5 g L⁻¹ manitol; 10 g L⁻¹ de sacarose + 5 g L⁻¹ sorbitol), três espécies (*C. macrocarpum*, *O. maculata* e *P. estrellensis*) e duas temperaturas (18 e 25°C). Para executar a aclimação, oito substratos foram testados para as espécies *C. macrocarpum* e *O. maculata*. Para realizar a conservação *in vitro* de *C. macrocarpum*, *O. maculata* e *P. estrellensis* ao longo de 450 dias, recomenda-se a utilização 25% dos sais de MS e de 20 g L⁻¹ de sacarose a 25°C. Mudanças de *C. macrocarpum* foram aclimatizadas utilizando areia + casca de pinus + húmus de minhoca (2:2:1). Recomenda-se a utilização de areia para aclimação de *O. maculata*.

PALAVRA-CHAVE: *Catasetum macrocarpum*. *Oeceoclades maculata*. *Polystachya estrellensis*. Crescimento lento. Substrato

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