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In-Vitro Micropropagation and Acclimatization of an Endangered Native Orchid Using Organic Supplements

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Abstract

In-vitro propagation is a technique that provides a vital solution for the conservation of endangered orchid species. The media used in tissue culture can be modified through the addition of inexpensive organic materials as an alternative to expensive synthetic additives. Some organic sources, such as coconut water and fruit juice, contain significant amounts of vitamins, amino acids, and organic compounds which can act as growth regulators, making these organic sources excellent additives for *in-vitro* cultivation. The aim of this study was to develop a protocol for *in-vitro* micropropagation and acclimatization of *Epidendrum nocturnum* using organic supplements in the growth media and various substrates at the acclimatization stage. Banana powder, coconut water, and potato dextrose were added to a basal seed sowing media and evaluated for seed germination percentage and plantlet growth. In addition, various substrates such as coconut coir, horticultural charcoal, sphagnum moss, and wood bark were evaluated for height, number of leaves, and number of shoots in the acclimatization portion of this study. The culture medium with coconut water showed a greater germination percentage (71.00% and 76.75%) compared with the control (37.50% and 45.50%) at 60 and 90 days after seed sowing, respectively. Media with organic supplements showed greater values of plant length and number of roots compared with the control. The combination of coconut coir and horticultural charcoal was shown to be more efficient than the combination of sphagnum moss, horticultural charcoal, and wood bark, as results showed greater values of plant height and number of leaves at 30, 90, and 120 days after transplantation in acclimatization of *E. nocturnum*.

Keywords

Tissue Culture, Native Orchid, Plant Growth Hormones, Climate Change, *Epidendrum nocturnum*

1. Introduction

Orchidaceae is one of the most important plant families in the ornamental horticulture industry. The beauty and variety of the shapes and colors of the flowers make them very appealing to growers and collectors alike. Increasing orchid popularity has led to unsustainable harvesting (often illegally), and this, along with habitat destruction, has been identified as major threat to their survival [1]. Florida is home to 106 native orchid species, of which 77 are listed as endangered or threatened [2]. Conservation efforts are challenging as orchids have complex reproductive strategies—their small seeds contain an undifferentiated embryo that lack an endosperm and have insignificant reserve material, as a result their natural germination process depends exclusively on associations with mycorrhizal fungi [3].

In-vitro propagation techniques serve as an important alternative for the conservation of endangered orchid species [4] [5] [6] [7]. The media used in tissue culture can be modified through the addition of inexpensive organic materials as an alternative to expensive synthetic materials [8]. Some widely available organic sources, such as coconut water and fruit juice, contain significant amounts of vitamins, amino acids, and organic compounds which can act as growth regulators, making these organic sources excellent additives for *in-vitro* culture and maintenance [9].

In tissue culture media, organic growth additives include potato dextrose, apple cider, maize extract, banana homogenate, coconut water, and other organic growth supplements that have the potential to boost the multiplication rate of regenerants and the production of orchid plantlets [10] [11] [12]. These organic growth supplements help to increase the number of shoots, roots, and leaves when added to the culture medium. In many orchid tissue cultures, the addition of organic based growth supplements has been shown to be essential to stimulate tissue growth, specifically the regeneration of shoots [13].

Epidendrum nocturnum, also known as the night-scented epidendrum, is native to Florida but has a wide distribution throughout the neotropics. This epiphytic orchid has white and yellow flowers ranging from 3 - 5 inches [14]. Traditionally, the orchid grows on a variety of trees throughout the swamps and hammocks of southern Florida, trees such as pond apple, pop ash, live oak, buttonwood, and cabbage palm [14]. Currently, *E. nocturnum* conservation status is endangered in south Florida (State of Florida Status) and ranked as imperiled by the Institute for Regional Conservation (IRC). There are similar studies on related orchid species; however there is not much literature on *in-vitro* micropro-

pagation and acclimatization of *E. nocturnum*. Therefore, the aim of this study was to develop a protocol for *in-vitro* micropropagation and acclimatization of *E. nocturnum,* using organic supplements in the culture media and to evaluate various substrates at the acclimatization stage. The findings of this study will be utilized to propagate and potentially increase the population of the endangered orchid in its natural habitat and urban environments in southern Florida.

2. Materials and Methods

2.1. Plant Material and Seed Surface Sterilization

The contents of a dehisced seed pod (collected by Fairchild Tropical Botanic Garden) were emptied onto a clean sheet of white paper, then transferred into a clean culture jar with a sealable lid. Added to the jar was 100 ml of 3% hydrogen peroxide (H_2O_2) and less than half a drop of liquid soap (acted as a surfactant). The jar was sealed, and solution was agitated with vortex for 60 seconds, allowed to incubate for 10 minutes, then agitated again for 60 seconds, and allowed to incubate once again until all soap bubbles dissipated. The jar and all materials were sterilized before being placed in a laminar flow hood. The sterilized seeds were then poured into another jar fitted with filter paper, adding more hydrogen peroxide (H_2O_2) as necessary to suspend and remove any remaining seeds from the jar. The filter paper with the seeds was then removed and allowed to dry before the seeds were sown onto the culture media.

Orchid seeds, due to their small size, can be difficult to quantify for germination studies. A method to subsample and estimate the number of seeds sown on each germination plate was established by drawing a line on the tip of a spatula with a sharpie and gathering seeds up to the line (**Figure 1(a)**), followed by counting the seeds under a light microscope at 15× magnification (**Figure 1(b)**). This procedure was repeated for a total of three counts and the average was calculated and used in this study (n = 60). The seeds were then sown directly onto the culture media by tapping the spatula over the germination plate. After the seeds were sown, containers were sealed with parafilm.

2.2. Culture Conditions

Five media treatments were prepared from a basal orchid seed sowing medium

Figure 1. *E. nocturnum* seeds: (A) Spatula used to quantify and sow seeds, (B) Light microscope at 15× magnification.

(PhytoTechnology P723) with charcoal and gelling agent. The control consisted of only the basal orchid seed sowing medium (BM) with 32.74 g/L. Treatment1 consisted of BM supplemented with 10 g/L banana powder from PhytoTechnology (B852, banana puree and maltodextrin). Treatment 2 consisted of BM supplemented with 10 ml/L coconut water from PhytoTechnology (C195, natural coconut water). Treatment 3 consisted of BM supplemented 5 g/L banana powder and 5 ml/L coconut water. Treatment 4 consisted of BM supplemented with 10 g/L potato dextrose from PhytoTechnology (P692, dehydrated infusion of potato). Approximately 30 mL of media was poured into 220 mL culture jars, with 10 replications for each treatment for a total of 50 containers. The media was autoclaved at 121˚C and 15 psi for 15 minutes. Once autoclaved, the media was transferred to a laminar flow hood and allowed to cool and solidify before being used for sowing the seeds. Data was collected by taking photos 30, 60, and 90 days after sowing (**Figures 2(a)-(d)**). After 90 days the pictures were uploaded to a computer and analyzed via the Microsoft photo app to determine final germination percentage. Germination was indicated by an enlarged embryo/protocorm-like body (PLB) [15] and percentage was determined using the following formula—(Germinated seeds)/(Total seeds sown) * 100 = Germination %. The length, number of roots, number of leaves, fresh weight, and dry weight of the seedlings were evaluated 120 days after sowing. Ten randomly selected plantlets were removed and evaluated from each replication across all treatments. The length was measured from the root tip to the leaf tip. The plants were sub-cultured into larger containers (**Figure 3**) using Phyto tech P668 orchid

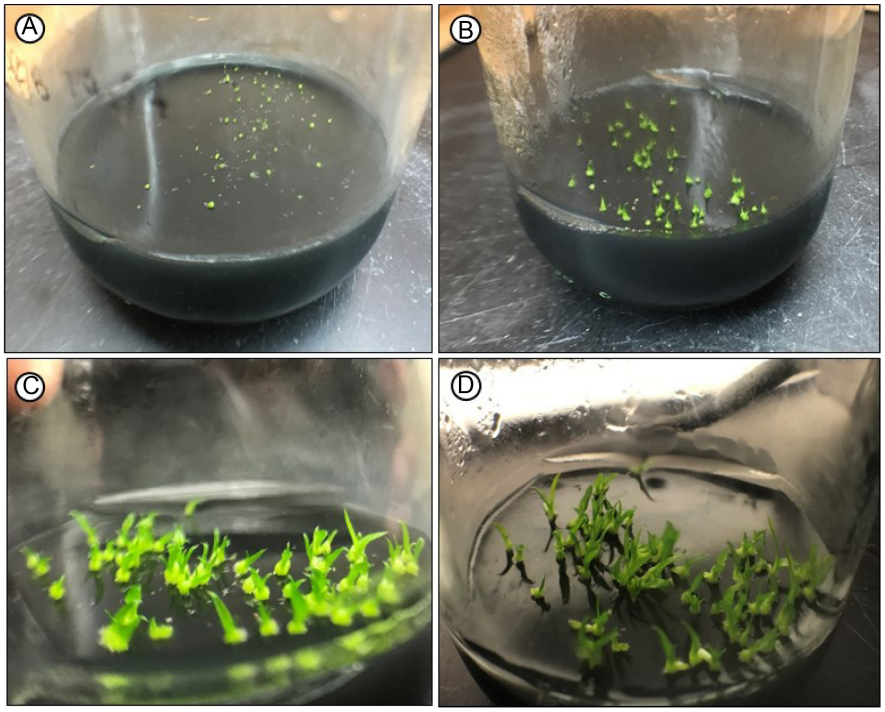

Figure 2. Seeds of *E. nocturnum* in culture media with Banana Powder (A) 30 days, (B) 60 days, (C) 90 days, and (D) 120 days after seed sowing.

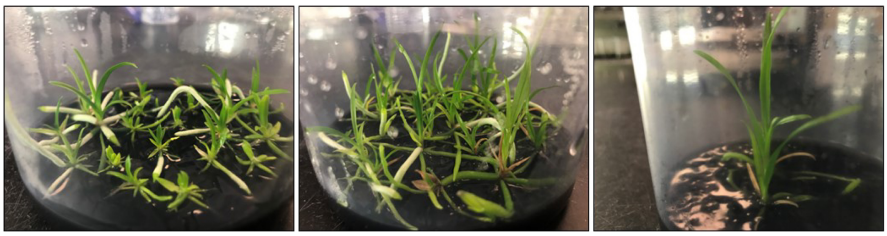

Figure 3. Sub-cultured *E. nocturnum*.

maintenance media amended with banana powder, and once large enough were used for the acclimatization portion of the study.

2.3. Acclimatization

Acclimatization began once the plants were "deflasked" in preparation for test substrates and greenhouse life (Figure 4(a)). After removing plants from their culture containers, excess media was rinsed off and they were placed on trays to drain. The plants were then treated with ProTeKt 0-0-3 fertilizer by misting with a spray bottle (1 mL/L). The tray was then covered with a clear plastic humidity dome for 24 hrs (hardening phase). After 24 hrs, the dome was removed, and the plants were transplanted into their respective substrates and transferred to the greenhouse (Figure 4(b)).

Four substrates were evaluated in this study and 50 plants were planted into each substrate (200 plants total, 50 cell tray for each substrate). The substrates evaluated were: 1) Coconut coir, 2) Coconut coir + Horticultural charcoal (2:1), 3) Sphagnum moss, and 4) Sphagnum moss + Horticultural charcoal + Wood bark (1:1:1)]. Each substrate was evaluated for growth characteristics (height: from base of media to "stem leaf", number of shoots, and number of leaves) at 30, 60, 90 and 120 days after transplanting (Figures 4(c)-(f)).

2.4. Statistical Analysis

In-vitro micropropagation experiment was established in a completely randomized design (CRD), with 5 treatments and 10 replications per treatment for a total of 50 containers. Acclimatization experiment was established in a CRD, with 4 treatments with a total of 50 pots per treatment. Data were subjected to analysis of variance (ANOVA), and the means were compared by Tukey's test at a 5% level of significance using the SISVAR statistical program [16].

3. Results

3.1. *In-Vitro* Micropropagation

No significant differences were found among treatments on germination percentage at 30 days after sowing (DAS) seeds (Figure 5(a)). However, significant differences ($P \leq 0.05$) were found among treatments at 60 and 90 DAS (Figure 5(b) and Figure 5(c)). Coconut water showed a greater germination percentage at 60 and 90 DAS (71.00% and 76.75%, respectively) compared with the control

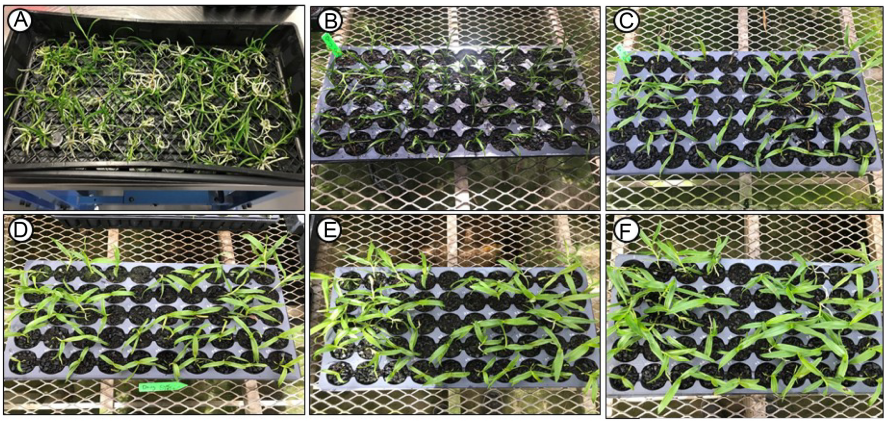

Figure 4. (A) Deflasked *E. nocturnum* plantlets; (B) Initial planting in Coconut coir + Horticultural charcoal substrate; (C) Plantlets in coconut coir + horticultural charcoal substrate 30 days after planting (DAP); (D) Plantlets in coconut coir + horticultural charcoal substrate 60 DAP; (E) Plantlets in coconut coir + horticultural charcoal substrate 90 DAP; (F) Plantlets in coconut coir + horticultural charcoal substrate 120 DAP.

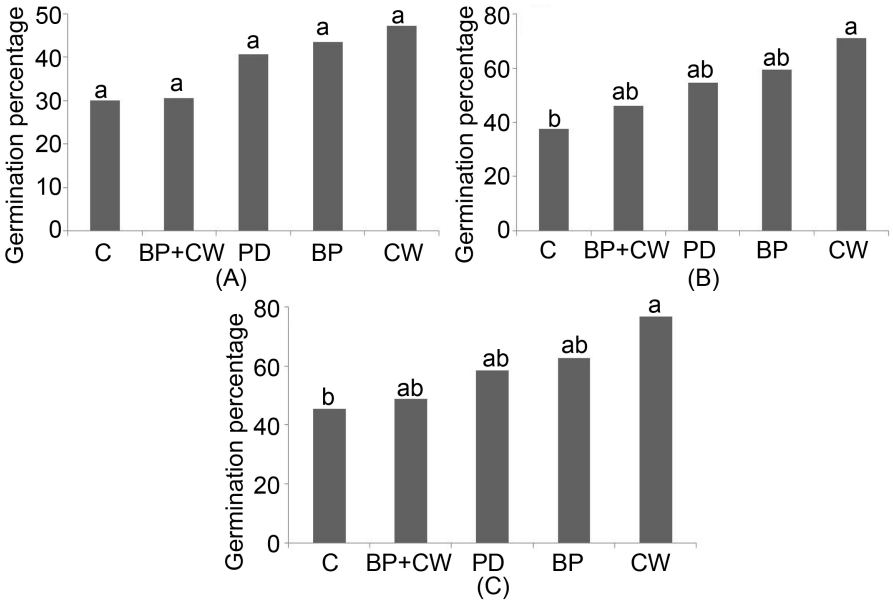

Figure 5. Germination percentage of *Epidendrum nocturnum* in each treatment at (A) 30, (B) 60, and (C) 90 days after sowing the seeds. Control, basal orchid seed sowing media (C), Banana Powder & Coconut Water (BP + CW), Potato Dextrose (PD), Banana Powder (BP), and Coconut Water (CW).

(37.50% and 45.50%, respectively) (**Figure 5(b)** and **Figure 5(c)**).

The various media showed a significant difference ($P \leq 0.05$) for length, number of roots, fresh weight, and dry weight in treatments with BP and BP + CW. However, there were no significant differences found among treatments for the number of leaves (**Table 1**). BP and BP + CW media showed an increase in length (20.10 mm and 19.80 mm, respectively) compared with other treatments. In addition, CW media showed an increase in length (11.90 mm) when compared with the control (8.20 mm). BP + CW media showed a greater number of

Table 1. Length, number of leaves, number of roots, fresh weight (FW), and dry weight (DW)of *Epidendrum nocturnum* plantlets on each media 120 days after sowing seeds. Banana Powder & Coconut Water (BP + CW), Potato Dextrose (PD), Banana Powder (BP), and Coconut Water (CW).

Medias	Length (mm)	Number of leaves	Number of roots	FW (g)	DW (g)
Control	8.20c	2.20a	0.30d	0.04c	0.0022c
BP	20.10a	2.00a	2.00ab	0.09a	0.0072a
BP + CW	19.80a	2.10a	2.10a	0.08a	0.0066a
PD	11.40bc	2.10a	1.50bc	0.06b	0.0048b
CW	11.90b	2.10a	1.30c	0.06b	0.0033bc

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$).

roots (2.10) compared with other treatments while the control showed the least number of roots (0.30) compared with the other treatments (Table 1).

All treatments showed greater fresh weight (0.09 g, 0.08 g, 0.06 g and 0.06 g, respectively) compared with the control (0.04 g). BP and BP + CW media showed greater fresh weight (0.09 g and 0.08 g, respectively) compared with PD (0.06 g), and CW media (0.06 g). BP and BP + CW showed greater dry weight (0.0072 g and 0.0066 g, respectively) compared with other treatments, including the control. PD media showed greater dry weight (0.0048 g) compared with the control (0.0022 g) (Table 1).

3.2. Acclimatization

The various substrates showed a significant difference for height and number of leaves 30 days after transplantation. However, no significant differences were found among treatments for the number of shoots (Table 2). The substrate Coconut coir + Horticultural charcoal showed greater height (28.52 mm) compared with Sphagnum moss + Horticultural charcoal + Wood bark (18.54 mm) 30 days after transplantation. In addition, Coconut coir + Horticultural charcoal showed a greater number of leaves (4.72) compared with Sphagnum moss (3.09), and Sphagnum moss + Horticultural charcoal + Wood bark (3.63). Lastly, Coconut coir showed a greater number of leaves (3.96) compared with Sphagnum moss (3.09) (Table 2).

The various substrates did not show a significant difference for height, number of shoots, and number of leaves of *E. nocturnum* plants 60 days after transplantation (Table 3).

The various substrates did not show a significant difference for the number of shoots, and number of leaves in *E. nocturnum* plants 30 days after transplantation. However, significant differences were shown among substrates for the height of the plants (Table 4). Coconut coir, and Coconut coir + Horticultural charcoal showed greater height (38.07 mm and 42.14 mm, respectively) compared

Table 2. Height, shoots, and number of leaves of *Epidendrum nocturnum* plants in various substrates 30 days after transplantation (DAT). Coconut coir (CC); Coconut coir + Horticultural charcoal (CC + HC); Sphagnum moss (SM); Sphagnum moss + Horticultural charcoal + Wood bark (SM + HC + WB).

Substrate	Height (mm)	Shoots	Number of leaves
	30 DAT		
CC	23.68ab	1.00a	3.96ab
CC + HC	28.52a	1.00a	4.72a
SM	21.58ab	1.00a	3.09c
SM + HC + WB	18.54b	1.06a	3.63bc

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$).

Table 3. Height, shoots, and number of leaves of *Epidendrum nocturnum* in various substrates 60 days after transplantation (DAT). Coconut coir (CC); Coconut coir + Horticultural charcoal (CC + HC); Sphagnum moss (SM); Sphagnum moss + Horticultural charcoal + Wood bark (SM + HC + WB).

Substrate	Height (mm)	Shoots	Number of leaves
	60 DAT		
CC	28.67a	1.06a	4.63a
CC + HC	28.70a	1.00a	4.71a
SM	26.28a	1.00a	4.12a
SM + HC + WB	22.90a	1.25a	4.59a

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$).

Table 4. Height, shoots, and number of leaves of *Epidendrum nocturnum* plants in various substrates 90 days after transplantation (DAT). Coconut coir (CC); Coconut coir + Horticultural charcoal (CC + HC); Sphagnum moss (SM); Sphagnum moss + Horticultural charcoal + Wood bark (SM + HC + WB).

Substrate	Height (mm)	Shoots	Number of leaves
	90 DAT		
CC	38.07a	1.04a	5.39a
CC + HC	42.14a	1.03a	5.78a
SM	30.60ab	1.00a	4.47a
SM + HC + WB	23.81b	1.22a	4.69a

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$).

with Sphagnum moss + Horticultural charcoal + Wood bark (23.81 mm) 90 days after transplantation (Table 4).

The various substrates showed a significant difference in height, and number

Table 5. Height, shoots, and number of leaves of *Epidendrum nocturnum* plants in various substrates 120 days after transplantation (DAT). Coconut coir (CC); Coconut coir + Horticultural charcoal (CC + HC); Sphagnum moss (SM); Sphagnum moss + Horticultural charcoal + Wood bark (SM + HC + WB).

Substrate	Height (mm)	Shoots	Number of leaves
	120 DAT		
CC	42.08a	1.09a	5.69ab
CC + HC	49.94a	1.03a	6.13a
SM	40.85ab	1.03a	5.06ab
SM + HC + WB	29.20b	1.25a	4.72b

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$).

of leaves in *E. nocturnum* plants 120 days after transplantation. However, no significant differences were shown among substrates for the number of shoots (Table 5).

Coconut coir, and Coconut coir + Horticultural charcoal showed greater height (42.08 mm and 49.94 mm, respectively) compared with Sphagnum moss + Horticultural charcoal + Wood bark (29.09 mm) 120 days after transplantation. In addition, Coconut coir + Horticultural charcoal showed a greater number of leaves (6.13) compared with Sphagnum moss + Horticultural charcoal + Wood bark (4.72) (Table 5).

4. Discussion

4.1. *In-Vitro* Micropropagation

The culture media with organic supplements showed an increase in germination, presumably because of differences in the balance and supply of organic and inorganic constituents [17] [18] [19] [20]. The greater germination percentage, when compared with the control, was found in culture media that contained coconut water (CW) alone. CW contains soluble sugars as a natural source of carbon, as well as amino acids and vitamins, such as thiamin, pyridoxine, ascorbic acid, and minerals [21] [22]. It also consists of various organic ions such as phosphorus, magnesium, potassium, calcium, iron, and manganese [23] [24], all of which have been shown to facilitate germination [11]. Another study showed that culture media supplemented with coconut water showed an increase in germination percentage in *Dendrobium antennatum* [25].

Organic supplements have been shown to enhance *in-vitro* orchid seed germination and seedling development of both commercial and threatened orchids [26]. Benefits of adding organic compounds to propagation media have been observed in orchid species such as *Cypripedium macranthos* [27], *Bulbophyllum dhaninivatii* [28], *Phalaenopsis* "Bahia Blanca" [29], and *Dendrobium antennatum* [25].

In this study, organic supplements showed greater values of length and num-

ber of roots of *Epidendrum nocturnum* plants. Organic supplements are a source of nutrients such as, vitamins, amino acids, fatty acids, carbohydrates, and minerals that facilitate growth [30].

Other researchers have shown results similar to those found in this study. Huh *et al*. [27] observed that adding organic supplements to the culture medium showed improved growth of orchid seedlings in *Cypripedium macranthos*. Kongbangkerd *et al*. [28] observed that adding organic supplements to the culture medium showed improved growth of *in-vitro* orchid (*Bulbophyllum dhaninivatii*) shoots compared with the control medium.

4.2. Acclimatization

Acclimatization is an important process to support successful plantlet transplantation from *in-vitro* to *ex-vitro* environments [31] [32] [33]. This process allows *in-vitro* plantlets to adapt to the natural environment which normally has higher light intensity and lower humidity compared to *in-vitro* conditions [31]. An important factor that influences orchid transplantation survival is the planting substrate [34] [35] [36].

The *Epidendrum nocturnum* plantlets in the Coconut coir + Horticultural charcoal substrate showed the greatest survival percentage with 90% (**Figure 4(f)**), followed by Coconut coir with 88%, Sphagnum moss with 80%, and Sphagnum moss + Horticultural charcoal + Wood bark with 68%, 120 days after transplantation.

In this study, Coconut coir + Horticultural charcoal substrate showed greater values of height and number of leaves compared with Sphagnum moss + Horticultural charcoal + Wood bark. In addition, Coconut coir showed greater values of height in *E. nocturnum* plants compared with Sphagnum moss + Horticultural charcoal + Wood bark at 90 and 120 days after transplantation (DAT).

Coconut fiber is a material resulting from the industrial processing of coconut husks (*Cocos nucifera*), which contain important nutrients for plant growth and development, such as potassium and phosphorus [37].

Charcoal provides a highly porous structure which, if pure or used as a substrate amendment, can increase porosity, water-holding capacity, and facilitate the proliferation of microorganisms beneficial to plant growth [38]. In addition, charcoal in acclimatization media can help prepare orchids for out-planting and attachment by facilitating the formation of velamen on the roots.

Santos *et al*. [39] observed that charcoal and coconut fiber, separately, are efficient for the acclimatization of *Epidendrum ibaguense*. Macedo *et al*. [40] concluded that the substrates sphagnum, charcoal, and coconut fiber are good options in the acclimatization of *Brassavola tuberculata*. These studies showed similar results to those found in this study, in which each substrate evaluated are good options for the acclimatization of *E. nocturnum*.

5. Conclusions

The culture media amended with coconut water showed a greater germination

percentage of *Epidendrum nocturnum* compared with the control. All organic supplements added to the culture media showed good seedling development of *Epidendrum nocturnum in-vitro*, most notably banana powder and coconut water.

All evaluated substrates were found to be good options in acclimatization of *Epidendrum nocturnum*. However, Coconut coir + Horticultural charcoal was shown to be the optimal option and more efficient than Sphagnum moss + Horticultural charcoal + Wood bark.

The methods explored in this study are critical to the conservation and sustainability of *Epidendrum nocturnum*, as well as other endangered orchid species. Many orchid species have coevolved with their pollinators and mycorrhizal fungi (many of which remain unidentified), and extinction may have an impact on a variety of organisms in ways that may not be recognized until it is too late. A complementary approach to the methods explored in this study is habitat conservation, which can help maintain current wild populations.

Possible future studies include the evaluation of *Epidendrum nocturnum* after out-planting on various native trees in its natural habitat and urban environments, trees such as live oak and buttonwood.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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