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Germination and initial development *in vitro*, and acclimatization of *Oeceoclades maculata* (Orchidaceae), aiming at its reintroduction in natural environments

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ABSTRACT. *Oeceoclades maculata* is a terrestrial orchid species that has potential for commercial purposes. Taking that into consideration, the present investigation aimed at studying its germination and initial development *in vitro* as well as its acclimatization. The influence of Murashige and Skoog (MS), Knudson C (KC), and Vacin and Went (VW) media in the presence and absence of 0.3% activated charcoal on *in vitro* germination and protocorm development were investigated. The effects of different concentrations of BA in combination with 0.5 mg L^{-1} NAA on seedling multiplication and growth were evaluated. The possibility of using dark-grown stem segments for micropropagation and acclimatization under laboratory and field conditions was also assessed. The results indicated that the most adequate media for germination were full-strength MS enriched with activated charcoal or KC supplemented with 1.5 mg L^{-1} BA in combination with 0.5 mg L^{-1} NAA. In terms of protocorm development, KC supplemented with 1.5 mg L^{-1} BA alone or in combination with 0.5 mg L^{-1} NAA provided the best results. The addition of 1.5 mg L^{-1} BA in combination with 0.5 mg L^{-1} NAA to KC medium favored the best results for seedling multiplication and development. The use of dark-grown stem segments is a viable alternative for the micropropagation of *O. maculata*. Regarding acclimatization, 100% survival of plants was observed during the initial phases and under field conditions average survival was 53.33%.

Keywords: activated charcoal; culture media; etiolation; orchid protocorm development.

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Introduction

Orchidaceae is one of the largest families in the plant kingdom, encompassing approximately 7% of all angiosperms; it is widely distributed in the world although more abundant and diversified in the tropics, especially in Asia and the Americas (Chase et al., 2015; Mezzalira & Kuhn, 2019). According to Flora do Brasil (2020), 252 genera of orchids occur in Brazil, out of which 26 are endemic. This diversity is reflected in the different shapes and colors of their flowers and leaves. Orchid species are predominantly herbaceous and can exhibit epiphytic, terrestrial, climbing, rupicolous and even saprophytic growth forms (Suzuki, Moreira, Nakabashi, & Ferreira, 2009).

In spite of their great diversity, many orchid species are under threat, primarily as a consequence of the decrease in their natural habitats, over-collection from the wild and indiscriminate trade (Moreira, Tomba, & Zonetti, 2007). For these reasons, the propagation of these species in nurseries and laboratories may thereby decrease the chances of their extinction. Therefore, *in vitro* techniques have been extensively used for their multiplication to meet conservation programs and commercial demands (Sopalun, Thammasiri, & Ishikawa, 2010; Zhang, Lee, Deng, & Zhao, 2013).

The most adequate conditions for orchid seed germination and plant development *in vitro* can be specific to the genus and species (Suzuki et al., 2009; Ferreira, Oliveira, Suzuki, Silva, & Soares Junior, 2018), which makes studies focusing on the use of different culture media relevant for the comprehension of physiological processes involved in the propagation and growth of these species. Culture media incorporate essential components for the development of a plant such as macro- and micronutrients, carbohydrates and vitamins, among others, which must be at appropriate concentrations to support the growth and development of the

plant material (Lassaga, Bretón, Gieco, Milisich, & Dittrich, 2010). A supplementary constituent that has been used in culture media of several species and has proved to be effective for the *in vitro* propagation of orchids (Moraes, Faria, & Cuquel, 2005; Prizão, Gonçalves, Gutierre, Mangolin, & Machado, 2012) is activated charcoal. Its use is a result of the fact that it has a structure composed of fine pores with great internal surface area which results in the adsorption of inhibitory or toxic substances released by the explants or present in the culture medium in harmful amounts and it is also added to the culture medium as an antioxidant (Chagas et al., 2005; Thomas, 2008). In addition to the nutritional components of culture media, growth regulators play a strong role on the development of plants *in vitro*. Among the growth regulators commonly added to the culture media of orchids are auxins and cytokinins. They are responsible for the regulation of meristematic growth, cell division, root formation and other physiological processes, and must be combined by type and concentration for the stimulation of development *in vitro*.

Some studies have reported that *in vitro* multiplication of orchids can be achieved by using etiolated stem segments (Ferreira, Suzuki, Pescador, Figueiredo-Ribeiro, & Kerbauy, 2011; Rodrigues, Santos, Takane, & Carvalho, 2017; Juras, Puragatto, Ferreira, & Suzuki, 2020). This is possible because plants that grow in the absence of light undergo a special kind of development known as skotomorphogenesis (etiolation) which result in the formation of elongated internodes and scale-like leaves that do not accumulate chlorophyll (Rodrigues et al., 2017). Ferreira et al. (2011) affirmed that these characteristics are especially interesting in the case of orchids because they typically have very short internodes and etiolation facilitates the division of the stems into small segments holding an axillary bud which can be used as explants for micropropagation.

One of the challenges when plants originate from *in vitro* propagation is the need for a period of hardening and acclimatization before the newly formed plants can be transferred to the natural environment. The transfer of plants to natural conditions can be harmful to them if the process is not cautiously conducted because it represents a stress to most plants. Very few studies in Brazil have addressed aspects related to the reintroduction of orchids into natural environments. Schmidt and Zotz (2002) state that the monitoring of survival and development of orchid species produced *in vitro* and introduced in their natural habitats tends to be a complex process and requires patience coupled with precision and logistical support since, under field conditions, these species exhibit slow growth and take a long time to reach maturity.

Taking the above into consideration, the *in vitro* propagation of orchids and their acclimatization and reintroduction into natural environments are essential for conservation purposes. Moreover, the use of these techniques allows for the study of the physiology of these plants and helps in the understanding of factors related to their development and reproduction. Thus, the present investigation aimed at studying the germination and initial development *in vitro* as well as the acclimatization (under laboratory and field conditions) of *Oeceoclades maculata*, a terrestrial orchid species that has potential for commercial purposes. The possibility of using dark-grown stem segments for its micropropagation was also evaluated.

Material and methods

Plant species and material

Oeceoclades maculata (Lindl.) Lindl. is a terrestrial, sympodial orchid that occurs extensively throughout most Brazilian states except for Acre and Amapá and is considered a naturalized species (Flora do Brasil, 2020). It is commonly found in humid and dry areas and has short roots with low levels of tuberization. Potted individuals of *O. maculata* in blossom are very charming, do not require a great deal of caring and thus have commercial appeal.

Fifteen mature fruits of *O. maculata* prior to dehiscence and collected from several different plants growing in the wild and also in a shade-house at the Environmental Studies Center (Neamb), *Universidade Federal do Tocantins* (UFT), Brazil, were the source of seeds for the present study. These fruits were carefully washed with detergent and water prior to seed removal. After being removed from the fruits, seeds were thoroughly mixed in a sterile beaker. A portion of about 25 mg of these seeds was initially immersed in 80 mL of deionized water for 30 min. Subsequently the water was discarded, and seeds were surface sterilized with 60 mL of a 15% (v/v) commercial bleach (2.5% active chlorine) in deionized/autoclaved water solution for 10 min. followed by three 15 min. rinses in deionized/autoclaved water. After the last rinse, 10 mL of the water were removed, and a 50 mL aqueous suspension of seeds was readied for inoculation onto the culture media.

Germination and initial development

The influence of three culture media on *in vitro* germination were investigated: Murashige and Skoog (1962) used at two different concentrations of its macronutrients (full- and half-strength) [MS; ½MS], Knudson (1946) [KC], and Vacin and Went (1949) [VW], in the presence or absence of 0.3% activated charcoal (AC). They were all supplemented with 0.4 mg L^{-1} thiamine, 100 mg L^{-1} myo-inositol and 2% sucrose. The pH of the media was adjusted to 5.85 ± 0.01, and 0.2% Phytagel (Sigma Co., USA) was added to the media before autoclaving at 121°C and 105 kPa for 15 min. The effects of the addition of 1.5 mg L^{-1} benzyladenine (BA) alone or in combination with 0.5 mg L^{-1} naphthaleneacetic acid (NAA) to the culture media mentioned above were also evaluated. For each treatment there were ten replicates which consisted of a 100 mL glass jar (covered with plastic transparent lids) containing 40 mL of culture medium onto which 250 mL of the aqueous suspension of seeds mentioned above (containing ca. 200 seeds) were inoculated. The cultures were kept in a growth room at 27 ± 1°C under a 16 hours photoperiod provided by cool-white fluorescent lamps (Empalux, Brazil) at 30-35 μmol m^{-2} s^{-1}. The germination analysis was carried out according to Suzuki et al. (2009). Material from five replications for each treatment were placed on microscope slides (five slides per replication) and analyzed with the use of a dissecting microscope. The counts encompassed all protocorms on each slide, which were gridded to aid in counting. These procedures were carried out under a laminar flow hood (Veco, Brazil, model FUH).

Ninety days after the start of germination, the individuals contained in all replications were evaluated in relation to the stages of protocorm development (morphological characteristics). Four different developmental stages were considered according to Suzuki et al. (2009). Five microscope slides per replication containing protocorms/seedlings were prepared and analyzed by means of a dissecting microscope. The percentages of individuals obtained for each developmental stage and for each replication were multiplied by 1, 2, 3, and 4 (weights) according to their respective stages, so that the growth index could be calculated according to Spoerl (1948), as modified by Ferreira, Vasconcelos, Silva, Oliveira, and Suzuki (2017). The growth index of each replication was the sum of all stages of their individuals.

Effects of growth regulators on multiplication and development

Taking into consideration that KC medium and the addition of BA and NAA favored seedling formation (based on the results obtained for the growth index), the effects of this culture medium supplemented with different concentrations of BA (1, 1.5, and 2.0 mg L^{-1}) in combination with 0.5 mg L^{-1} NAA on seedling multiplication and growth were also evaluated. The control treatment consisted of growth regulator-free culture medium. Seedlings aged 6 months and 1.5 ± 0.5 cm in length (which had all roots removed) grown *in vitro* were used as explants in the experiments. There were four replicates for each treatment. Each replicate consisted of 250 mL glass jars filled with 50 mL of culture medium (covered with plastic transparent lids) containing five explants (n = 20). The experimental conditions were the same as described for the germination experiment. The results were analyzed after 120 days of culture according to the following variables: number of shoots and roots produced per inoculated explant, length of the longest shoot (measured from the base of the plant to the apex of the longest leaf) and root as well as dry matter of shoots and roots.

Micropropagation from dark-grown stem segments

In order to verify the possibility of *in vitro* multiplication of *O. maculata* by using etiolated stem segments, plants originated from *in vitro* germination were grown on KC medium supplemented with 0.4 mg L^{-1} thiamine, 100 mg L^{-1} myo-inositol and 4% sucrose according to Ferreira et al. (2011). Plants were cultivated in 250 mL glass flasks (three plants per flask) containing 50 mL of culture medium (covered with black plastic caps) which were kept under dark conditions for 360 days at 25 ± 1°C. After that period, etiolated stems were divided into 1 cm segments containing at least one axillary bud. These segments were then transferred to the same culture medium except for the fact that sucrose concentration was reduced to 2%. Twenty 90 mL glass flasks containing 50 mL of culture medium covered with plastic transparent caps were used. Ten etiolated stem segments were inoculated per flask. Flasks were kept in a growth room under the same environmental conditions described for the previous experiments. The results were evaluated 30 days after transferring the etiolated stem segments to the presence of light considering the percentage of segments that originated shoots.

Acclimatization

The process of acclimatization consisted of taking 100 plants (bearing roots and leaves and approximately 4 cm in length) from the culture vessels and thoroughly washing them in running water for removal of growth medium. They were transferred to ten community pots (transparent plastic containers with covers - 16 cm height × 12 cm basal width) each containing 10 plants. Different proportions (v/v) of Bioplant (Bioplant Agrícola Ltda., Minas Gerais State, Brazil) and Ouro Negro (Aparecida de Goiânia, Goiás State, Brazil) were tested: 0:100; 100:0; 25:75; 50:50; 75:25. Pots were kept for 8 weeks in a growth room under the same experimental conditions described for the germination experiment. Four weeks after transferring plants to the community pots their covers were removed. They were watered three times a week until the substrate reached the saturation point and plants were sprinkled with 50 mL of deionized water once a day. After eight weeks, an evaluation was carried out based on the percentage survival of individuals. Following that, they were transferred to individual plastic pots (7 cm height × 6 cm basal diameter, perforated at the base) containing a substrate composed of Bioplant and Ouro Negro at 50:50 (v/v) and were kept in the same growth room (first acclimatization phase). Eight weeks later they were relocated to a shade-house with 75% retention of solar radiation flux and were watered every other day for three months. Another evaluation was carried out based on percentage survival of the individuals (second acclimatization phase).

For the process of acclimatization under field conditions (third acclimatization phase), plants measuring approximately 5 cm in length and an average of 1.5 leaves per individual were planted in two sub-areas at the Neamb/UFT Woods 20 meters apart from one another. Fifteen individuals were planted in each sub-area (n = 15) at least 2 meters apart. They were 2 years old, originated from *in vitro* propagation and were initially acclimatized as described in the previous paragraph. Evaluation was based on survival percentage of the individuals and newly formed shoots, and the development of the individuals was assessed by plant height (measured from the base of the plant to the apex of the longest leaf) and the number of leaves and shoots produced 15 months after the beginning of this experimental study. The results were associated with the physical properties of the soil and the relative humidity and light intensity of the two sub-areas, measured by a digital thermo-hygrometer (Lufft 512010) and a digital light meter (General Tools DLM2), respectively.

Experimental design and statistical analysis

The experiments were set up in a completely randomized experimental design. The results were tested for normality by the Shapiro-Wilk test. Those with normal distribution were evaluated by the Analysis of Variance (ANOVA) and the means were compared by the Tukey test at the 5% probability level. The results that did not present a normal distribution were analyzed by the Kruskal-Wallis test and the means were compared by the Dunn test at the 5% probability level. Percentage results were arcsine transformed to normalize variation. The experiments were repeated twice, except for the field experiment. The 5.0 BioEstat software package was used for the statistical analysis.

Results and discussion

Germination and initial development

The onset of germination of *O. maculata* seeds was visible 35 days after sowing. The results obtained in the different culture media assessed are shown in Table 1. No significant differences ($p < 0.05$) were detected among the germination percentages of the different culture media. However, the best outcome was verified on MS medium enriched with activated charcoal and on KC supplemented with BA+NAA (66.06 and 65.3%, respectively). In general, it was observed that media enriched with activated charcoal showed the best germination percentages when compared to the AC-free ones. This component has a network of fine pores and great internal surface area to which many substances, including toxic materials and phenolic compounds, can be adsorbed. These substances negatively interfere with the growth of the cultivated plant material. According to Thomas (2008), activated charcoal plays an important role in the germination of certain orchid species, especially terrestrial ones.

Table 1. Germination percentages and growth indices of *Oeceoclades maculata* on Murashige and Skoog (MS and ½ MS), Knudson (KC), and Vacin and Went (VW) media supplemented with 1.5 mg L^{-1} benzyladenine (BA) alone or in combination with 0.5 mg L^{-1} naphthaleneacetic acid (NAA), in the presence or absence of 0.3% activated charcoal (AC). Values followed by the same letter (in rows) are not significantly different according to the Tukey's test at 5% probability.

Culture medium	Germination (%)	Growth index
MS AC	66.06 a	100.00 b
KC BA+ANA	65.33 a	295.63 a
½ MS BA+ANA	59.99 a	139.38 ab
VW AC	59.46 a	100.00 b
½ MS AC	58.67 a	100.00 b
VW BA	58.00 a	192.41 ab
KC AC	57.00 a	100.00 b
MS BA+ANA	56.23 a	201.83 ab
KC	54.99 a	100.00 b
VW BA+ANA	54.48 a	196.49 ab
MS BA	53.63 a	220.11 ab
½ MS	50.94 a	104.86 ab
VW	50.01 a	100.00 b
½ MS BA	49.60 a	182.43 ab
KC BA	49.30 a	311.34 a
MS	45.07 a	116.69 ab

Soares, Rosa, Macedo, Sorgato, and Rosa (2012) stated that the responses of orchid seeds when sown on media supplemented with activated charcoal vary among species. They observed that for *Brassavola tuberculata* AC is vital for seed germination. In the case of *O. maculata* its presence favored seed germination but was not critical for this process. Hossain (2008) verified that AC improved germination of *Epidendrum ibaguense* seeds as well as protocorm development and root formation. This author affirms that the property of AC to adsorb inhibitory compounds or toxic substances was most likely responsible for the results obtained. Hossain, Sharma, Silva, and Pathak (2010), on the other hand, did not detect significant differences in the germination of *Cymbidium giganteum* seeds on MS and KC media enriched or not with activated charcoal.

Auxins are important hormones in the process of plant morphogenesis and some authors state that their interaction with cytokinins on the *in vitro* germination of orchid seeds stimulates positive effects (Krapiec, Milaneze, & Machado, 2003; Puchooa, 2004). The results obtained in the present study for *O. maculata* showed a positive interaction of BA (1.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) for seed germination, since most of the culture media supplemented with these growth regulators revealed better results than the BA-free and NAA-free media or those enriched with BA only. However, it is necessary to emphasize that the presence of growth regulators is not crucial for seed germination of this orchid species.

Godo, Komori, Nakaoki, and Miyoshi (2010) reported that the addition of BA to the culture medium favored the germination of *Calanthe tricarinata* seeds, a terrestrial orchid species. Although the presence of NAA in the culture medium was positive for germination of this species, the results obtained with 0.2 mg L^{-1} BA alone were significantly greater than those verified when 0.2 mg L^{-1} NAA or higher concentrations were added to the medium. Deb and Imchen (2006) observed that the presence of BA or BA+NAA was essential for the germination of the terrestrial orchid *Malaxis khasiana*. Unlike these two studies, the presence of BA alone caused a decrease in *O. maculata* germination. These results show that the use of growth regulators and their ideal concentrations for the germination of orchid seeds differ among species.

Regarding protocorm development, the media supplemented with growth regulators showed the best responses, although significant differences ($p < 0.05$) were not detected among most treatments (Table 1). The four initial developmental stages of *O. maculata* are shown in Figure 1. The highest growth index was achieved on KC supplemented with BA (331.34). Abraham, Augustine, and Thomas (2012) reported that protocorms of *Coelogyne nervosa* developed better (formation of a greater number of leaves and roots) when seeds germinated on MS enriched with 3 mg L^{-1} BA and 0.5 mg L^{-1} NAA. Protocorm development of *O. maculata* (leaf and root formation) on growth regulator-free media was not observed, except for AC-free MS and ½ MS on which the growth indices were markedly reduced. In this case MS

provided better protocorm development (higher growth index), probably due to the fact that is it rich in nutrients. As *O. maculata* is a terrestrial species, its nutritional requirements are possibly more elevated than epiphytic species (Ferreira et al., 2017).

It was interesting to observe that based on the growth index, culture media supplemented with activated charcoal did not promote leaf or root formation. This shows that although activated charcoal favored seed germination, that is, protocorm formation, protocorm development was inhibited in the presence of this component. Thomas (2008) stated that even though activated charcoal promotes growth and development of several species, cases of inhibition are also found in the scientific literature. Activated charcoal can also act as an adsorbent of vitamins, inorganic ions and growth regulators, thus having positive or negative effects on explant development, depending on the species (Schneiders, Pescador, Booz, & Suzuki, 2012).

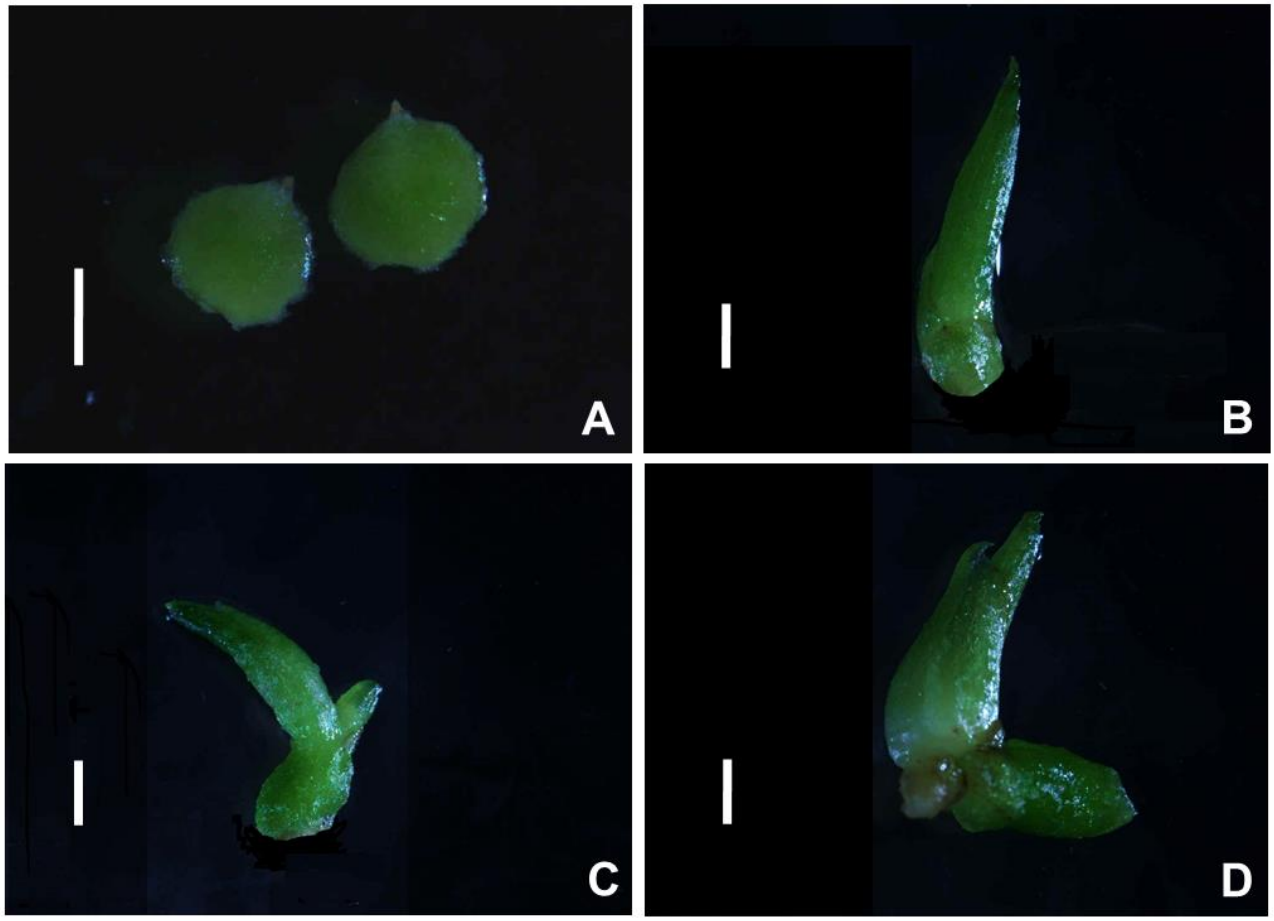

Figure 1. Developmental stages in *Oeceoclades maculata* protocorms. A. Stage 1 - swollen green embryos (protocorm phase). B. Stage 2 - protocorm bearing one leaf. C. Stage 3 - protocorm bearing two leaves. D. Stage 4 - seedling with leaves and one root. Scale bars = 1 mm.

Effects of growth regulators on seedling multiplication and development

The results concerning the effects of different BA concentrations in the presence of 0.5 mg L^{-1} NAA on multiplication and development of *O maculata* seedlings are displayed in Table 2. The addition of BA at 2 mg L^{-1} provided the best results for shoot formation, even though significant differences were not detected among treatments. Different results were obtained by Mahendran, Muniappan, Ashwini, Muthukumar, and Bai (2013) who reported that increasing BA concentration from 1 to 2 mg L^{-1} caused a decrease in shoot formation of *Cymbidium bicolor*. In different orchid species it has been demonstrated that organogenesis and growth are highly favored by the interaction of auxin and cytokinin (Picolotto et al., 2017; Adhikari & Pant, 2019).

In terms of root formation, the best response was observed at 1.5 mg L^{-1} BA, although significant differences were not detected among treatments. An increase in BA concentration to 2 mg L^{-1} caused a

decrease in root formation. Moreover, it is necessary to point out that the number of roots formed was considered very low. Cid (2000) affirmed that the long exposure of the explants to BA may hinder root formation. This fact might have caused the poor root formation observed since the explants were cultivated for 120 days in the medium containing BA. Ferreira et al. (2018) also verified that the addition of BA to the culture medium inhibited root formation in *Alatiglossum fuscopetalum* and *Catasetum macrocarpum*, respectively. Although cytokinins are not commonly associated with the formation of roots, a few studies have shown that for some orchid species this process is favored by the presence of cytokinins in the culture medium (Roy, Patel, Patel, Sajeev, & Deka, 2011; Soares et al., 2012).

Table 2. Effects of benzyladenine (BA) and naphthaleneacetic acid (NAA) on the multiplication and development of *Oeceoclades maculata* after 120 days of *in vitro* culture. NS = number of shoots; LSL = longest shoot length; NR = number of roots; LRL = longest root length; SDM = shoot dry matter; RDM = root dry matter. Values represent averages per inoculated explant and those followed by the same letter (in rows) are not significantly different according to the Tukey's test at 5% probability.

BA/NAA (mg L^{-1})	NS	NR	LSL (cm)	LRL (cm)	SDM (g)	RDM (g)
0.0/0.0	0.07 a	0.75 a	1.65 b	0.41 b	0.0474 a	0.0025 a
0.5/0.5	0.50 a	1.06 a	1.31 b	0.36 b	0.0805 a	0.0027 a
1.0/0.5	0.65 a	1.48 a	1.56 b	0.41 b	0.0531 a	0.0065 a
1.5/0.5	0.81 a	1.93 a	4.60 a	1.62 a	0.0479 a	0.0078 a
2.0/0.5	0.87 a	1.00 a	1.43 b	0.30 b	0.0331 a	0.0063 a

Regarding shoot and root length, the best result was detected at 1.5 mg L^{-1} BA, which was significantly higher than the other treatments. The increase in BA concentration to 2 mg L^{-1} caused a decrease in length of shoots and roots. In *A. fuscopetalum* and *C. macrocarpum* the presence of BA significantly inhibited the growth in length of shoots and roots, respectively (Ferreira et al., 2017, Ferreira et al., 2018). Lane (1979) stated that the reduction in development when cytokinin concentrations are elevated results from the toxic effects of high amounts of this growth regulator. Mahendran et al. (2013), on the other hand, reported an increase in the shoot length of *C. bicolo*r when BA concentration was raised from 1 to 2 mg L^{-1}.

No significant differences ($p < 0.05$) among treatments were detected in dry-matter values for the shoot and root. However, discrete decreases were observed at concentrations above 0.5 mg L^{-1} BA for shoot dry matter. Contrasting results were observed by Asghar, Ahmad, Hafiz, and Yaseen (2011), who verified that BA concentrations up to 2.0 mg L^{-1} BA promoted significant increases in shoot dry matter of *Dendrobium nobile* var. Emma White. In *C. macrocarpum*, Ferreira et al. (2018) reported that the addition of BA to the culture medium in the presence of 0.5 mg L^{-1} NAA caused a decrease in dry-matter values for the shoot and root. In the present study, on the other hand, the addition of BA not only favored root dry matter accumulation, but the best result was verified at 1.5 mg L^{-1} BA.

Micropropagation from dark-grown stem segments

After 360 days of growth in the dark, several white-colored, etiolated shoots lacking chlorophyll of *O. maculata* bearing reduced scale-like leaves were observed (Figure 2A). In fact, etiolation started after ca. 4 months of dark incubation, but the number of etiolated shoots was very low and they were not long enough to provide sufficient material for micropropagation, which only occurred after 360 days. Similar observations were made by Soares, Pasqual, Rodrigues, and Araujo (2010) in *Laelia crispata* but in a shorter period of time, that is, after 150 days of dark incubation. The longer period of time required for etiolation of *O. maculata* was possibly due to its slow growth when compared to other orchid species. Nevertheless, the dark-grown shoots could be easily divided into ca. 1 cm-long segments containing at least one axillary bud (Figure 2A, B, and C).

Thirty days after transfer to the presence of light 45% of the etiolated stem segments regenerated shoots (Figure 2E) and gave rise to new plants ca. eight weeks later. Ferreira et al. (2011) reported that 70% of the dark-grown stem segments of *Dendrobium* Second Love regenerated shoots five weeks after transfer to light conditions. These authors also demonstrated that the addition of growth regulators such as cytokinins did not significantly increase shoot regeneration after transfer of etiolated stem segments to the presence of light. This indicates that the endogenous levels of this hormone (which is involved with axillary bud development) in etiolated shoots are sufficiently adequate for the resumption of bud growth and the formation of a new shoot.

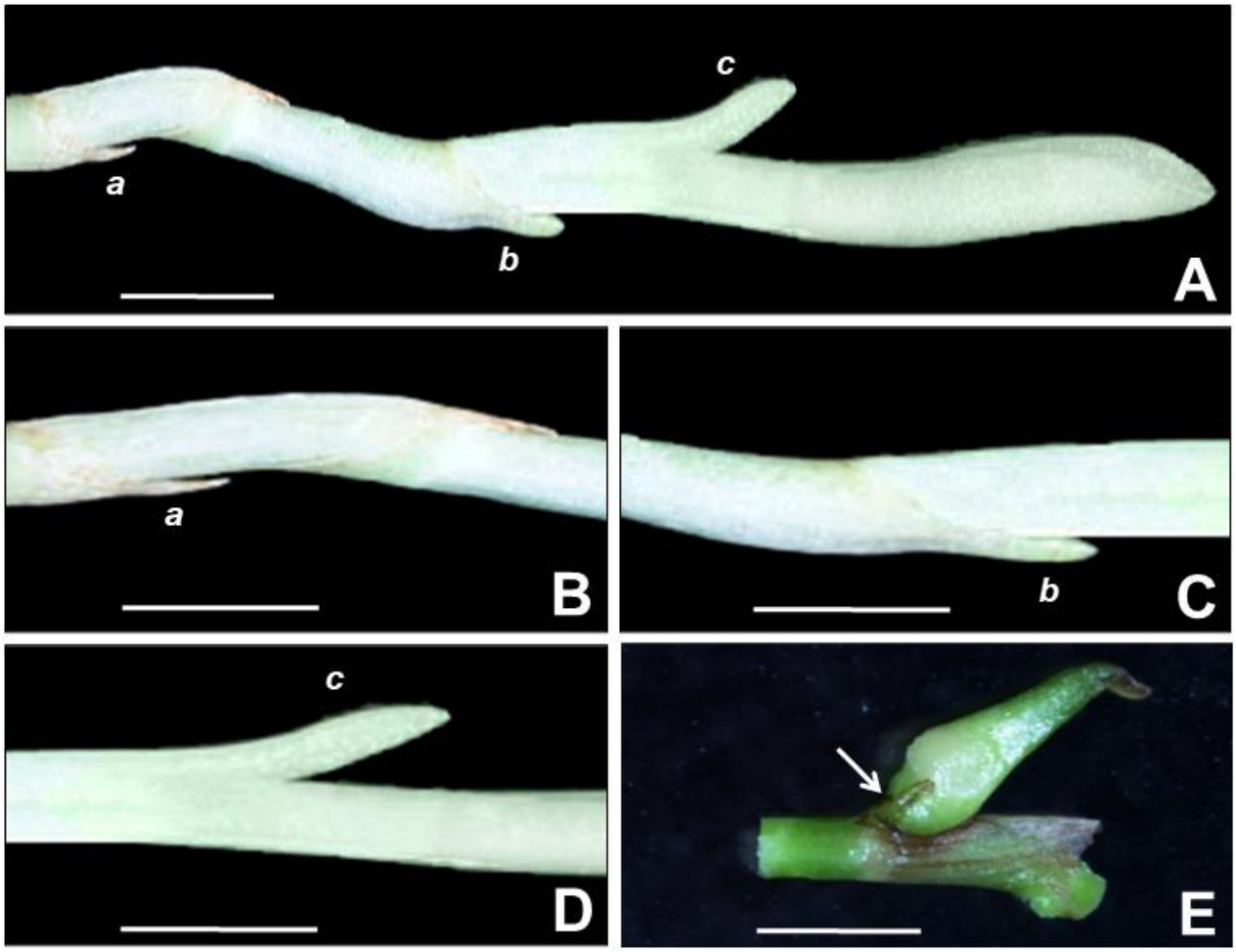

Figure 2. Diagram illustrating the micropropagation of *Oeceocades maculata* by using dark-grown stem segments. A. Etiolated stem after 360 days of incubation in the dark showing scale- like leaves (*a*, *b* and *c*). B-D. Sequence of an etiolated stem which was divided into ≈1 cm segments. The apical segment in A is discarded and the other three (B-D) are transferred to the presence of light. E. Green shoot formed 30 days after transfer of a dark-grown stem segment to the presence of light. This shoot is formed from an axillary bud which is protected by the scale-like leaf (arrow). Scale bars = 0.5 cm

Acclimatization

When plants are propagated under *in vitro* conditions, a gradual acclimatization process is necessary, which usually consists of three phases: a) the removal of the new plants from the culture medium and their cultivation in a solid substrate under laboratory conditions; b) the transfer of these plants to a greenhouse or a shade-house and c) their transfer to the natural environment. Regarding the acclimatization of *O. maculata*, all three phases were accomplished. After the first two phases (Figure 3A and B) the survival percentage was maximum (100%), which means that all substrates and proportions tested as well as the growth conditions were highly satisfactory. As their leaves are coriaceous, they are less susceptible to water loss which certainly aids survival, especially under controlled environmental and growth conditions such as the ones to which *O. maculata* plants were subjected in the present study. Upon studying the effects of different substrates, Menezes et al. (2019) verified that survival of *O. maculata* plants varied from 83 to 100% after 6 months of growth under shade-house conditions with 50% retention of solar radiation flux and an intermittent spray system (second acclimatization phase). The fact that shading percentage was higher (75%) might have contributed to the maximum survival of *O maculata* in the present study. Ferreira et al. (2018) reported that survival of *C. macrocarpum* was 96.6 and 75% at the end of the first and second acclimatization phases, respectively, under the same growth conditions of our study. As the leaves of this species are much thinner and thus more prone to water loss than those of *O. maculata*, the observed survival percentage at the end of the second phase was much lower. These results obtained for *O. maculata* might also have resulted from the fact that this orchid species is terrestrial and adapted to different substrates varying from sandy to clayey ones (Menezes et al., 2019).

Regarding its acclimatization under field conditions (third acclimatization phase; Figure 3C, D), the results showed that survival was much higher in sub-area 1 (73.33%) than in sub-area 2 (33.33%). Probably the

edaphic conditions related to greater water retention due to the more elevated clay content as well as the greater solar radiation in sub-area 1 (Table 3) may have contributed to the higher survival percentage observed. On the other hand, survival of newly formed shoots did not differ markedly between sub-areas 1 and 2 (84.7 and 76.7% respectively). The presence of these new shoots is essential for the survival and development of the individuals, since they are meant to grow until blooming, thus ensuring reproduction. They are also important when dividing a sympodial orchid, which is the case of *O. maculata*, for propagation purposes; it is recommended that three to five shoots be left together when division is carried out. Upon analyzing the distribution of *O. maculata* in a Caribbean tropical forest, Cohen and Ackerman (2009) verified that it occurs predominantly in clay soils and that as this species has a relatively shallow root system it develops better in flat areas which provides protection against the chances of getting swept away by heavy rains or landslides. Populations of different orchid species native to Australia decreased in number due to erosion in relatively steep terrains (Scade, Brundrett, Batty, Dixon, & Sivasithamparam, 2006). Souza-Leal and Pedroso-de-Moraes (2014) also reported the presence of many well-established individuals of *O. maculata* in a flat Cerrado area with clay soil in Mogi Guaçu, São Paulo State, Brazil. In the present study the areas where the individuals were planted are also flat, which might have, beside other factors, contributed to their survival.

In contrast, Bogarín and Pupulin (2007) reported that *O. maculata* individuals can also be found growing in less clayey and drier soils, like the one in sub-area 2, keeping their leaves all year round and, sometimes, forming plant clusters. These results show that this species seems to be able to adapt to different environmental conditions. Souza-Leal and Pedroso-de-Moraes (2014) draw attention to the fact that *O. maculata* exhibits considerable adaptive plasticity, which corroborates the statement of Cohen and Ackerman (2009), who affirm that this species develops well in various edaphic and climatic conditions and, thus, can even be considered an invasive species. Indeed, according to Souza-Leal and Pedroso-de-Moraes (2014), *O. maculata* is not native to Brazil, but a naturalized species.

Figure 3. Acclimatization of *Oeceoclades maculata*. A. Individuals in round community pots (16 cm height × 12 cm basal width) eight weeks after transfer from the *in vitro* culture medium (first acclimatization phase). B. A *O. maculata* plant at the second acclimatization phase in an individual plastic pot (7 cm height × 6 cm basal diameter). In C and D individuals of *O. maculata* growing in sub-areas 1 and 2 (respectively) at the Neamb/UFT Woods. Scale bars: 10 cm (C and D).

In terms of development no significant differences ($p < 0.05$) between the areas were detected in terms of the average number of leaves and shoots, although the best results were observed in sub-area 1 (Table 4). In relation to the increase in height, the individuals in this area exhibited significantly better results than those in sub-area 2. It is possible that edaphic conditions, particularly the greater clay content, may have contributed to greater water retention in this sub-area, making it more readily available for the plants which favored their growth in height besides leaf and shoot formation. In addition, the greater light intensity verified in this sub-area (Table 3) might have promoted more elevated photosynthetic rates and, thus, a superior performance of the individuals. It is worth mentioning that in sub-area 1 we observed one blooming individual, which indicates the existence of adequate conditions for this important developmental phase.

Table 3. Average temperature, relative humidity and light intensity (from January/2019 through March/2020), and soil chemical and physical characteristics of sub-areas 1 and 2 at the Neamb/UFT Woods in Porto Nacional, Tocantins State, Brazil, where the individuals of *Oeceoclades maculata* were grown. SA = Sub-area; CEC = Cation Exchange Capacity.

Variable	SA 1	SA 2	Variable	SA 1	SA 2
Calcium (cmol dm^{-3})	1.40	1.81	CEC	6.5	7.6
Magnesium (cmol dm^{-3})	0.82	1.01	Sand (%)	35	42
Aluminum (cmol dm^{-3})	0.1	0.1	Silt (%)	23	24
Potassium (mg dm^{-3})	34.0	50.0	Clay (%)	42	34
Phosphorus (mg dm^{-3})	0.7	0.7	Texture	clay	Medium
Organic matter (%)	0.4	0.8	pH	4.55	4.62
Relative humidity (%)	59.3	57.8	Temperature (°C)	29.8	29.7
Light intensity (μmol m^{-2} s^{-1})	242.29	133.38			

Table 4. Average increase in height and average number of leaves and shoots of *Oeceoclades maculata* individuals cultivated in two sub-areas at the Neamb/UFT Woods in Porto Nacional, Tocantins, after 15 months of growth. Values followed by the same letter (in lines) are not significantly different according to the Dunn's test at 5% probability.

Variable	Sub-area 1	Sub-area 2
Average number of leaves	2.02 a	1.48 a
Average number of shoots	2.91 a	2.20 a
Average increase in height (cm)	1.31 a	0.10 b

Oeceoclades maculata easily adapts to different edaphic and climatic conditions (Cardoso, 2014). Its establishment in various regions seems to be closely related to light availability and stomatal density since, although it is a species well adapted to disturbed or undisturbed soils, it exhibits a combination and convergence of anatomical characteristics of its different organs that allow for its adaptation to environments with high light intensities as well as to those that are humid and shady (Riverón-Giró et al., 2017). Such adaptability has been demonstrated by Rahal, Souza-Leal, and Pedroso-de-Moraes (2015), who reported the occurrence of *O. maculata* in areas of high light intensities in Araras, São Paulo State, Brazil, whereas, on the other hand, Krahl, Cogo, and Valsko (2014) found a well-established population of this species in a shady area of a semi-deciduous forest in the State of Espírito Santo, Brazil, blooming from January through March. In the present study flowering was also observed in this period.

Conclusion

These results presented in this study indicated that the protocol for seed germination, protocorm and seedling multiplication and growth were effective for the *in vitro* propagation and development of *O. maculata*. The use of dark-grown stem segments proved to be a viable alternative for the micropropagation of this species. The acclimatization procedures developed were successful for plant survival and growth of plants both under lab and field conditions. Thus, the *in vitro* propagation of *O. maculata* and its acclimatization as described herein are efficacious for its multiplication and can be used for the re-establishment of this species in Brazilian Savanna areas.

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