



***In vitro* germination, initial development and acclimatization of *Cattleya nobilior* Rchb. f. (Orchidaceae): an approach to curb the eventual endangerment of this exuberant, near-threatened Cerrado species**

Germinação *in vitro*, desenvolvimento inicial e aclimatização de *Cattleya nobilior* Rchb. f. (Orchidaceae): uma abordagem para evitar a eventual extinção dessa exuberante e quase ameaçada espécie de Cerrado

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RESUMO: O objetivo deste estudo foi avaliar a influência de meios de cultura na germinação *in vitro* e no desenvolvimento inicial de *Cattleya nobilior* e aclimatizar essa espécie utilizando palhada de soja (*Glycine max*) como componente do substrato, com a finalidade de produzir plantas em larga escala para reintroduzi-la em ambientes naturais. Características anatômicas associadas ao desenvolvimento durante a aclimatização também são descritas. A influência dos meios de Murashige e Skoog, Knudson, e Vacin e Went na germinação e desenvolvimento dos protocormos *in vitro* foi investigada. A aclimatização foi realizada utilizando-se diferentes proporções de palhada de soja (PS) e Bioplant (BP) como substratos. Os estudos anatômicos foram conduzidos com folhas das plantas durante a aclimatização. KC foi o meio de cultura mais adequado tanto para a germinação quanto para o desenvolvimento inicial. Para a aclimatização dessa espécie recomenda-se inicialmente a utilização de um substrato composto por 60% PS e 40% BP. Ao transferir as plantas para viveiro, deve-se utilizar uma mistura de 40% PS e 60% BP. As características anatômicas foliares observadas são típicas de orquídeas epifíticas adaptadas a ambientes pobres em recursos hídricos, o que indica que a OS não afetou negativamente o desenvolvimento da espécie. A propagação *in vitro* de *C. nobilior* aqui descrita é eficiente para sua multiplicação com fins comerciais e de conservação, e a PS pode ser utilizada como componente alternativo do substrato para sua aclimatização.

PALAVRAS-CHAVE: Anatomia foliar, Meio de cultura, Palhada de Soja

ABSTRACT: The objective of the present study was to evaluate the effects of different culture media on the *in vitro* germination and initial development of *Cattleya nobilior*, and to acclimatize this species using soybean straw as a substrate component, aiming the production of plants at large scale to reintroduce it in natural environments. Anatomical characteristics associated with development during acclimatization are also depicted. The influence of Murashige and Skoog, Knudson, and Vacin and Went culture media on the *in vitro* germination and protocorm development were assessed. Acclimatization was accomplished using different proportions of soybean straw (SS) and Bioplant (BP) as substrates. Anatomical studies were conducted on the leaves of plants during acclimatization. KC was the most suitable culture medium for both germination and initial development of *C. nobilior*. Regarding acclimatization, it is recommended that a substrate composed of 60% SR and 40% BP be initially used. Upon transfer to shade-house conditions, plants should be grown in a mix of 40% SR: and 60% BP. The leaf anatomical characteristics observed are typical of epiphytic orchids adapted to water-poor environments, which indicates that SR did not negatively affect the development of the species. The *in vitro* propagation of *C. nobilior* as described herein is efficient for its multiplication for commercial and conservation purposes and SS can be used as an alternative component of the substrate for its acclimatization.

KEYWORDS: Foliar anatomy, culture media, soybean straw.

INTRODUCTION

In the Cerrado domain, the Orchidaceae family is considered the third most important in terms of representativeness (BARROS et al., 2015). In Brazil, 2,449 species distributed in 237 genera (KRAHL et al., 2014) have been described, which places the country as one of the largest holders of orchid diversity in the Americas and the world, with 1,620 endemic species (BARROS et al., 2015). The genus *Cattleya* is composed of 45 species distributed in the tropics from Mexico to Paraguay. Among them the species *Cattleya nobilior* stands out for having broad capacity for genetic recombination, structure, beauty, and durability of its flowers. These characteristics contribute to its intense exploitation, which, together with habitat degradation, can promote the vulnerability of the species (BIANCHETTI, 2007; SILVA et al., 2009).

The conservation of orchid species can be subsidized by *in vitro* cultivation techniques (WOTAVOVÁ-NOVOTNÁ et al., 2007; PONERT et al., 2013; YEUNG, 2017), which have been widely used for scientific and conservation purposes as they result in high germination percentages when compared to natural conditions (STANCATO et al., 2001; ARAÚJO et al., 2006; FARIA et al., 2012). The germination and development responses of orchids are peculiar to each species and depend on the specific nutritional needs at the different stages of development, making critical the choice of the culture media that best meet these specificities to promote the success of asymbiotic culture (STEWART; KANE, 2006; PAUL et al., 2012).

Therefore, specific studies related to the mineral composition of culture media for species of Orchidaceae are important to understand their role in the biochemical processes involved in the germination and initial development of this group of plants. According to the germination responses and respective basic nutritional needs of each species in the *in vitro* process, Stewart (1989) divides orchids into two large groups, the first group consisting of the species in which the seeds germinate in nutritionally simpler culture media such as KC and VW and a second group of the species that need nutritionally richer media, that is, with higher macronutrient content, such as MS.

Before being taken into the natural environment, plants produced *in vitro* require a gradual period of acclimatization so that they can survive *ex vitro* conditions. During

this period the plant depends on, among other factors, the type of substrate used in the transplant substrate, which should have good capacity for water retention and, at the same time, favor gas exchange, availability of nutrients and adequate pH for roots (SOUZA 2003; SANTOS; TEIXEIRA, 2010; FIGUEIREDO; KOLB, 2013). Different alternative substrates have been used for the acclimatization of orchids, including coconut fiber, pine bark, charred rice husk, piaçava fiber, sugarcane bagasse, barbatimão bark, coffee husk, almond husk, corn cob and charcoal (LONE et al., 2008; MEURER et al., 2008; YAMAMOTO et al., 2009; SANTOS; TEIXEIRA, 2010; ASSIS et al., 2011; SASAMORI; ENDRES JUNIOR, 2014). Another substrate option that could be used in the acclimatization process of these species is soybean straw (*Glycine max*), which has potential for this purpose.

Another aspect that deserves attention and that contributes to the understanding of factors involved with plant development is anatomy. Anatomical studies are important for understanding plant adaptations to certain environments and understanding physiological responses during early stages, considered the most critical in the life cycle of many species, and which determine the survival and establishment of these plants in the environment (FERREIRA et al., 2015). The relevance of studies related, particularly, to leaf anatomy in orchids is substantiated by providing clarification on taxonomic and ecological aspects of this family (SILVA et al., 2014; CARNEIRO et al., 2017), and, therefore, can help in understanding its behavior during the acclimatization phase to the natural environment.

In view of the above, the objective of this work was to evaluate the influence of different culture media on the *in vitro* germination and initial development of *Cattleya nobilior* (a near-threatened species) and develop a protocol to acclimatize this orchid species using soybean straw (*Glycine max*) as a substrate component. Morphological and anatomical characteristics associated with seedling development during acclimatization are also depicted.

MATERIALS AND METHODS

Plant species and material

Cattleya nobilior Rchb. f. is an epiphytic or rupicolous orchid found in the Cerrado domain distributed in the states of Goiás, Mato Grosso, Mato Grosso do Sul, Pará, Rondônia, Tocantins, Maranhão, and the Federal District. Its occurrence has also been recorded in Paraguay and Bolivia (BIANCHETTI, 2007; BARROS et al., 2015). Flowering occurs in August and September and, due to the beauty of its flowers (Fig. 1), the species experiences collection pressure by collectors and/or merchants (BIANCHETTI, 2007; RODRIGUEZ et al., 2009; BARROS et al., 2015), and may possibly be included in lists of endangered species if no action is taken to prevent it. Even though it is not considered threatened with extinction, concern for the conservation status of this species is necessary, thereby making it impossible to frame it accurately and safely as threatened (BRASIL, 2008). At present, according to CNCFlora (2012), *C. nobilior* is a near-threatened (NT) species.



Figure 1. *Cattleya nobilior* in the Orchidarium of the Center for Environmental Studies (Neamb), Campus of Porto Nacional of the Federal University of Tocantins: (a) Detail of a flower; (b) Detail of a fruit. Bars = 2 cm.

For the present study, seeds of *C. nobilior* from five fruits collected from individuals cultivated in the Orchidarium of the Center for Environmental Studies (Neamb), Campus of Porto Nacional of the Federal University of Tocantins were used. Before inoculation of the seeds in the culture media, they were soaked in autoclaved and deionized water for 30 minutes and then submitted to a sterilization process which consisted of immersion in 15% (v/v) commercial sodium hypochlorite solution with two drops of household detergent for 10 min. Subsequently, they underwent a double 15-minute wash with 50 mL of autoclaved and deionized water. After the second washing, 30 mL of water were removed and the aqueous suspension of seeds (20 mL) was used for sowing in the culture media. This process was carried out in a Veco model FUH 12 laminar flow chamber (São Paulo, Brazil).

Influence of culture media on *in vitro* germination and initial development

The following three culture media were tested: Knudson medium (1946) [KC], Vacin and Went (1949) [VW] and Murashige and Skoog (1962) [MS], at concentrations of 100 and 50% of their macronutrients (MS and ½MS, respectively). All media were supplemented with 0.4 mg.L⁻¹ thiamine, 100 mg.L⁻¹ of myo-inositol and 2% sucrose. The pH of each culture medium was adjusted to 5.8 ± 0.1 before the addition of 0.2% Phytigel. The media were sterilized in an autoclave at 121° C and 105 kPa for 20 min. Soon after sterilization, the seeds were inoculated on the media with the aid of a micropipette whose dosage was 250 µL of the aqueous suspension mentioned above, containing approximately 550 seeds. For each culture medium, 10 replicates were performed, consisting of glass bottles with a capacity of 90 mL, containing 40 mL of culture medium, closed with plastic lids. The cultures were kept in a growth room with a temperature of 26 ± 1° C, a photoperiod of 16 hours, and light intensity of 35-40 µmol.m⁻².s⁻¹. Thirty days after visualization of the first indications of germination, indicated by the presence of chlorophyllous material in the culture medium, germinated seeds were counted, i.e., seeds with a swollen embryo called the protocorm phase, using four replicates per treatment. For this evaluation, the material contained in each

repetition was placed on a gridded slide to facilitate the counting of germinated seeds, which was performed under a Mikros stereoscopic microscope (São Paulo, Brazil).

Ninety days after the beginning of germination, the individuals contained in four replications, per treatment, were evaluated according to the number of organs formed. Four stages of development were considered: stage 1 - protocorm (swollen green embryos); stage 2 - protocorm with one leaf; stage 3 - protocorm with two leaves and stage 4 - seedling (leaves and roots). Substantiated by the present evaluation, the growth index was calculated based on Spoerl (1948), that is, the percentage of individuals obtained at each stage of development, by repetition, was multiplied by the weights 1, 2, 3 and 4 according to the respective stages. The mean sum of all stages of development present was used to calculate the growth index of each repetition.

Acclimatization

The acclimatization process was carried out in two phases. In the first, 180 plants (with roots and leaves already formed) of approximately 2 to 3 cm in height, were used from the *in vitro* cultivation. These were washed in running water for the removal of the culture medium and transferred to 36 plastic containers (round pots measuring 150 mm diam. × 94 mm high, with transparent lids and a capacity of 1000 mL), each containing 5 individuals. Six replicates (pots) were performed for each treatment. The treatments were composed of soybean straw and the commercial substrate Bioplant (Minas Gerais, Brazil), in the following proportions (%): 100:0; 80:20; 60:40; 40:60; 20:80 and 0:100. Soybean straw, the alternative substrate tested, was acquired from a harvest at Fazenda Boa Sorte in the municipality of Porto Nacional, Tocantins, during the harvest period of the region (March/2017). After becoming totally dry, all the aerial part of the plants (except fruit and seed), was harvested manually and then ground with the aid of a mechanical grinder. The pots containing the plants remained closed and were kept in a grow room for 60 days with an irrigation of 250 mL of water every 15 days, performed with the aid of a spray (first phase). After this period, the surviving plants were analyzed according to the following variables: plant height (from the base to

the extreme tip of the largest leaf), length of the largest root, number of leaves and roots, as well as the percentage of survival.

In the second phase, the plants (from the first phase) were transferred to individual plastic pots (7 cm high × 6 cm basal diameter) containing the same treatments described in the first phase except those containing only soybean straw. These remained in the grow room for 10 days and were irrigated daily. Subsequently, they were transferred to a shade-house with 75% shading and received daily irrigation to the point of water saturation of the substrate. After 90 days, the same evaluations described in the first phase were performed.

Anatomical analyses

Anatomical analyses were performed on plant leaves at the end of the second acclimatization phase. These analyses were conducted at the Anatomy Laboratory in the Center of Environmental Studies (Neamb) of the Federal University of Tocantins (UFT). Three plants from each treatment were used. Cross-sections of the largest leaf were made, dividing it into three parts: base, middle, and apex. For light microscopy, leaf portions were immersed in 50% FAA (50% formaldehyde, acetic acid and ethyl alcohol) in individual transparent vials, with a screw cap, properly identified for each treatment. Subsequently, the vials were placed in a desiccator coupled to a vacuum pump, where they remained for 24 hours to remove air from the tissues and facilitate the penetration of the fixative into the anatomical part. After this period, the 50% FAA was replaced with 60% ethyl alcohol P.A. for one hour and then 70% ethyl alcohol P.A. After 24 hours, each piece was embedded using paraffin + 8% beeswax (JOHANSEN, 1940).

A Leica RM2245 rotary microtome (Hessen, Germany) was used to perform the cuts (12 µm thick). The cuts were stained with 1% Alcian Blue and 1% Safranin (Luque et al., 1996). Subsequently, they were adhered to slides with Haupt adhesive, which were assembled using Canada balsam. For the observation of the structures, a Leica DM 500 optical microscope (Hessen, Germany) with a coupled Leica ICC50 HD digital camera (Hessen, Germany) were used to obtain photographic documentation.

Statistical analyses

The experimental design used was completely randomized. The data were submitted to analysis of variance and the means compared by the Tukey test at 5% significance. To obtain parametric assumptions, homogeneity was tested by the Levene's test and normality by the Shapiro-Wilk test, being transformed according to Box-Cox when necessary. All analyses were performed using the R statistical software (version 3.4.2).

RESULTS AND DISCUSSION

Influence of culture media on *in vitro* germination and initial development

Twenty days after inoculation of *C. nobilior* seeds in different culture media, germination was observed. Different results have been observed for other species of the genus *Cattleya*: as reported by Suzuki et al. (2010) for *C. bicolor*, which formed protocorms at 15 days after inoculation; and by Schneiders et al. (2012) for the species *C. forbesii*, whose protocorms were observed 30 days after seed inoculation in KC, MS, and VW media. For the species *Alatiglossum fuscopetalum* (FERREIRA et al., 2017) and *Catasetum macrocarpum* (FERREIRA et al., 2018) the first indications of germination were observed at 10 and 15 days, respectively, after symbiotic inoculation of seeds, periods also different from that observed in the present study. It is noted that germinative responses of Orchidaceae species may present variations among different genera and even among species of the same genus, and therefore species-level studies are relevant for determining the time factor needed to meet the specific needs of each species (SUZUKI et al., 2010).

The results obtained for the germination of *C. nobilior* are presented in Table 1. It was observed that there were no significant differences among the MS, ½MS and KC media, although the highest percentage of germination was verified in KC medium (46.71%). Such results were similar to those found by Suzuki et al. (2009) for the species

Hadrolaelia tenebrosa, where KC medium provided the highest seed germination rate. Probably, KC medium favored the best result for *C. nobilior* due to the fact that it is a balanced medium, that is, it is not as rich in nutrients as MS medium and does not have low concentrations as in VW. Thus, the KC medium meets the nutritional needs for seed germination of this species, which develops naturally in epiphytic environments. Epiphytic species when grown *in vitro* generally do not present great demands in relation to the concentrations of mineral salts in the medium, because they inhabit environments with nutrient scarcity (MORAES et al., 2009; CORDEIRO et al., 2011; CUNHA et al., 2011). Thus, the results obtained in the present study indicate that *C. nobilior* does not require high levels of mineral nutrients for embryo development and consequent germination.

The lowest percentage of germination of *C. nobilior* seeds was observed in VW medium. On the other hand, the species *Cyrtopodium punctatum* and *C. bicolor* showed a higher percentage of germination in VW medium (DUTRA et al., 2009; SUZUKI et al., 2010, respectively). These authors relate these results to the presence of ammonia in the culture medium, which is considered more efficient than nitrate for seed germination of several orchid species, since VW medium has the highest ammonia/nitrate ratio when compared to KC and MS media. However, the results obtained for *C. nobilior* show that the presence of nitrate in the culture medium was probably beneficial for seed germination of this species. Raghavan and Torrey (1964) had already reported that the mature seeds of *C. mollie* and *C. trianaei* use both NH_4^+ and NO_3^- for their development and subsequent seedling growth, as will be discussed later.

Table 1. Germination percentage and growth index (90 days after germination) of *Cattleya nobilior* in MS and $\frac{1}{2}$ MS (Murashige and Skoog), KC (Knudson) and VW (Vacin and Went) media.

Culture medium	Germination (%)	Growth index
MS	44.41 a	234.65 ab
$\frac{1}{2}$ MS	42.16 a	271.02 ab
KC	46.71 a	308.04 a
VW	21.60 b	193.11 b

Values followed by different letters (columns) show significant variation among treatments by Tukey's test at the 5% probability level.

The germination percentage of orchids in asymbiotic cultures presents a percentage variation between 50 and 95%, and can reach up to 100%, as described by Ferreira et al. (2011). However, some species may present germination percentages below the values observed in *C. nobilior* in the present study. Dutra et al. (2009) also verified low germination percentages for the species *Cyrtopodium punctatum* (26.1% in VW medium) and this value was higher than those found in ½MS and KC media.

The results obtained for the initial development evaluated through the growth index are shown in Table 1. Figure 2 shows the four stages used to evaluate the development of *C. nobilior* after germination. It was observed that KC medium promoted more effectively the development of *C. nobilior* protocorms, because it presented a higher percentage of plants in the more advanced stages of development (stage 4), although it did not present significant differences when compared to MS and ½MS media. The initial development analysis performed after 180 days of *in vitro* sowing by Suzuki et al. (2010) of *C. bicolor* also showed that KC medium was the most promising, as it presented protocorms in all stages of development with the highest proportion found in stage 4, as verified in the present study. These data indicate, therefore, that these species present good development in a nutrient medium more similar to the oligotrophic environment they inhabit naturally. In addition, it is possible that the presence of nitrate in the culture medium contributed to the observed result. Dijk and Eck (1995) also reported that the protocorms of *Dactylorhiza incarnata* developed best in the presence of a higher concentration of NO₃⁻ in the culture medium.

The KC medium was also the most effective for the initial development of *Cyrtopodium saintlegerianum* (SILVA et al., 2017). Schneiders et al. (2012) also recommended KC medium for propagation of *Cattleya*, *Laelia*, *Laeliocattleya*, and *Brassocattleya*. Thus, seed germination and post-germination development of *C. nobilior* in the culture media tested suggest that this species found the most appropriate nutritional conditions in KC medium. On the other hand, Koene et al. (2019) reported that for *Acianthera prolifera* ½MS provided better protocorm development than MS and KC after 12 weeks of culture. These results show that medium requirements for protocorm development vary among orchid species.

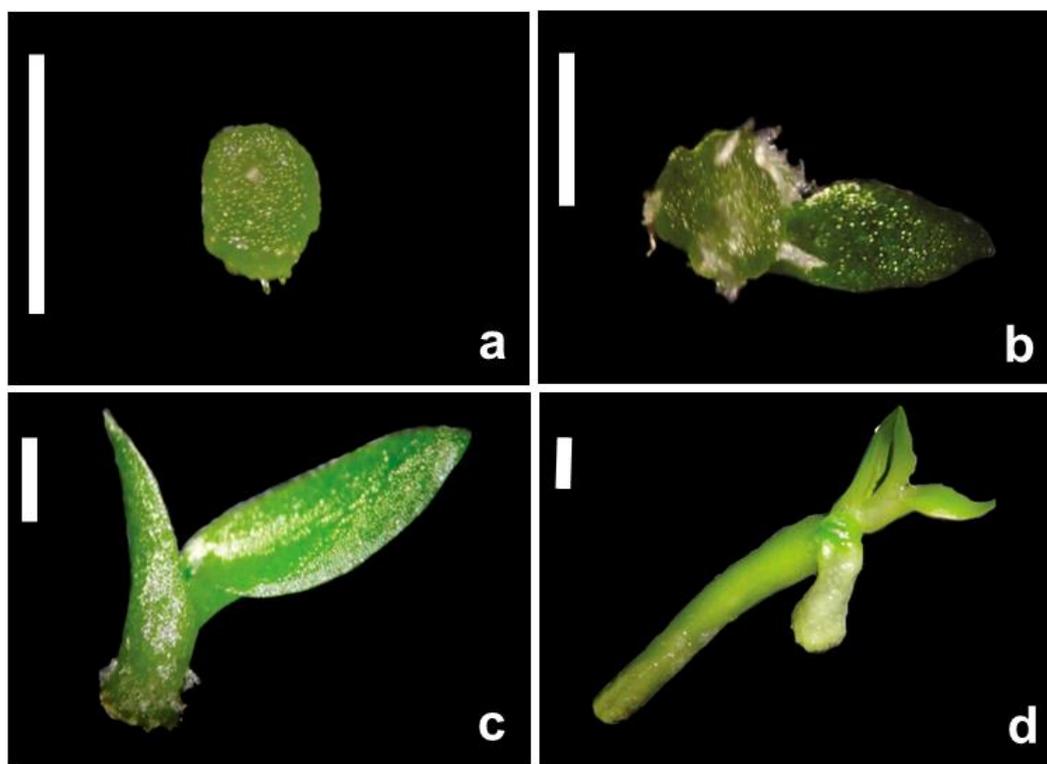


Figure 2. Stages of development of *C. nobilior* 90 days after the first indications of germination *in vitro*: (a) chlorophyllous protocorm; (b) protocorm with one leaf; (c) protocorm with two or more leaves; (d) seedling with leaves and one or more roots. Bars = 1 mm.

Acclimatization

The survival percentage of *C. nobilior* plants from *in vitro* germination and cultivated in substrate composed of soybean straw and Bioplant in the two acclimatization phases is described in Table 2. The plants transferred to the substrate exclusively containing straw did not survive. Therefore, this treatment is not recommended for acclimatization of *C. nobilior*. It is possible to infer that this fact occurred due to factors such as metabolic activities of straw decomposers, which cause increased temperature and release of compounds (SILVA et al., 2009), which may impair root development. Assis et al. (2011), when testing coffee husk-based substrate in

Cattleya cultivation also reported that it is not recommended to use it as a single substrate.

The presence of straw in proportions between 20 and 80% added to Bioplant showed positive results for the species in the first acclimatization phase, presenting a survival percentage equal to or greater than 70%. This result is higher than that described by Dorneles and Trevelin (2011) in studies with a species of the same genus (*C. intermedia*) whose average survival percentage was 53% in *Sphagnum* substrate and 27% survival of plants acclimatized in substrate composed solely of pine bark. Similar results were observed by Macedo et al. (2014) and Mengarda et al. (2017) with the species *Brassavola tuberculata*, whose average survival percentage was also 70% in different substrates (*Sphagnum*, charcoal, coconut fiber, vermiculite, sand and tree-fern powder).

Table 2. Survival percentage of *C. nobilior* in the first and second acclimatization phases after 60 and 90 days of *ex vitro* cultivation, respectively, using substrates composed of different proportions of soybean straw (SS) and Bioplant (BP).

Treatments SS (%):Bioplant (%)	Phase 1 (%)	Phase 2 (%)
100:0	0.00	NT*
80:20	73.3	40.0
60:40	80.0	60.0
40:60	70.0	66.7
20:80	76.7	43.5
0:100	83.3	36.0

* NT = not tested.

The treatment containing only Bioplant substrate showed a higher survival rate although the addition of soybean straw to Bioplant also provided satisfactory survival rates (between 70 and 80%). Therefore, the use of soybean straw at proportions between 20 and 80% added to Bioplant as substrate in the initial phase of acclimatization was adequate for the survival of *C. nobilior* plants in this first phase of transfer to *ex vitro*

conditions, which provides a reduction and mitigation of costs with commercial substrate, since soybean straw can be acquired at very low values or even at no cost, depending on the reuse or not of soybean bagasse by soybean producers.

In the second phase of acclimatization, there was a reduction in the percentage of survival of individuals in all treatments. Of these, the lowest rate observed was in the treatment that contained exclusively Bioplant probably because this substrate quickly loses moisture when exposed to the shade-house environment. Dorneles and Trevelin (2011) reported that during the period of acclimatization of orchids obtained *in vitro* the loss of individuals may exceed 50% in a greenhouse during the first six months, a period that is more susceptible to losses and also a consequence of major changes in cultivation conditions (KOZAI et al., 1992). It is possible that during the second acclimatization phase, when the plants were cultivated under shade-house conditions (Fig. 3) the decrease in atmospheric humidity contributed to an increase in the transpiration rate and, thus, caused a decrease in the survival percentage of individuals. The transfer of plants to the shade-house occurred in the month of July, a period with high temperatures and high evaporative rates.

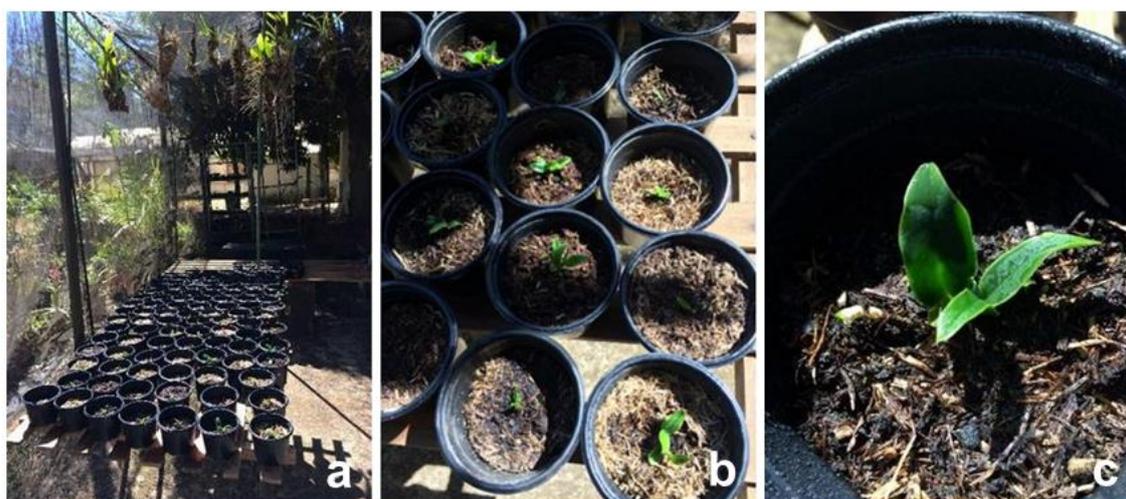


Figure 3. *Cattleya nobilior* at the second acclimatization phase under shade-house conditions with 75% retention of solar radiation flux: (a) General view of plants in plastic pots 7 cm high \times 6 cm basal diameter; (b) Detail of some plants in pots. (c) Close-up view of an acclimatized plant in a pot.

Table 3 contains the results of the growth variables evaluated in both phases. There were no significant differences among treatments for most of the variables analyzed in both phases. In relation to plant height, there were no significant differences among treatments in the first phase. In the second phase, a significant difference was observed ($P < 0.05$) among the treatments that contained 0 and 20% straw, showing that the plants that were under the influence only of the Bioplant substrate presented lower height while those cultivated in the presence of 20% soybean straw exhibited better results for this variable. Bioplant used alone also caused a decrease in plant height when compared to other treatments. Lone et al. (2008), when testing new substrate alternatives for acclimatization of *C. intermedia*, verified that substrates containing coconut fiber may be substitutes for tree-fern powder and sphagnum, since the results for the variables analyzed, including shoot length, did not differ statistically among these substrates or were superior to the sphagnum. Thus, it is verified that plant bagasse such as soybean straw can be alternative components of substrates for the acclimatization of orchids.

Table 3. Average height (PH), numbers of leaves (NL) and roots (NR), and length of the longest root (LLR) of *Cattleya nobilior* at the end of the first and second acclimatization phase using substrates composed of different proportions of soybean straw (SS) and Bioplant (BP).

SS : BP	Phase 1				Phase 2			
	PH (cm)	NL	NR	LLR (cm)	PH (cm)	NL	NR	LLR (cm)
0:100	2.63 a	3.2 a	3.20 ab	1.81 ab	2.17 a	3.0 a	2.44 b	1.16 a
20:80	2.91 a	3.6 a	4.22 ab	2.20 a	3.26 b	2.9 a	5.60 a	2.06 b
40:60	2.84 a	4.1 a	4.00 ab	1.78 ab	2.68 ab	2.3 a	3.00 a	1.62 ab
60:40	2.93 a	3.7 a	4.61 a	2.38 a	2.84 ab	2.6 a	3.47 a	1.81 ab
80 :20	2.73 a	2.9 a	2.47 b	1.43 b	2.61 ab	2.2 a	3.00 a	1.35 ab

Values followed by different letters (columns) show significant variation among treatments by Tukey's test at the 5% probability level.

Regarding the number of leaves, no significant differences were observed among treatments or losses of this organ during the two phases of acclimatization, that is, all plants remained with leaves of intense green color and apparently healthy, as can be observed in Figure 3c, corresponding to the second phase. Dorneles and Trevelin (2011) described that leaf permanence is probably associated with the specificities of each species and/or the physical and chemical conditions of the environment during the acclimatization process. As the atmospheric environment was quite uniform in the first phase of the present study (grow room with constant temperature and light intensity), the differences found are possibly a response to the substrate. In this sense, it is possible that in this phase the presence of soybean straw contributed to an average increase of 1 leaf at a proportion of 40%.

Concerning the variable of the number of roots, it was observed that in the first phase there were no significant differences among the treatments included in the interval between 0 and 60% soybean straw. However, the treatment containing 80% soybean straw caused a decrease in this variable when compared to the other treatments. In the second phase, the results showed no significant differences among treatments containing 20 to 80% soybean straw content. However, the treatment containing Bioplant as a single substrate resulted in a significant decrease in this variable. Regarding the length of the longest root, no significant differences were observed among most treatments in the first phase of acclimatization although in 80% soybean straw there was a decrease in this variable in relation to the other treatments. In the second phase, only one difference was observed between the treatments containing 0 and 20% soybean straw, with a significant decrease in root length when Bioplant was used alone. However, the best results were observed in the interval between 20 and 60% soybean straw. Together, these results demonstrate that, in general, the different treatments did not strongly influence the formation and growth in extension of the roots.

Assis et al. (2011) also reported that there were no differences among treatments in the evaluation of coffee husk-based substrates for root development of (*Cattleya forbesii* × *C. labiata*) × *C. labiata*. Dorneles and Trevelin (2011), during the acclimatization of *Cattleya intermedia*, reported that there was a decrease in the length of

the root of plants growing in Bioplant substrate, as observed in the present study especially in the second acclimatization phase. For Lone et al. (2008) epiphytic orchids, such as *Cattleya* for example, when grown in pots have better development in substrates of relatively thick texture and good drainage favoring free access to air and light for root development. On the contrary, substrates that do not present these characteristics tend to limit root development. Colombo et al. (2005) also stated that good drainage of the substrate, a characteristic also observed in soybean straw, is fundamental for the healthy development of epiphytic orchid roots.

In view of the results obtained, it was observed that *C. nobilior* was tolerant to the different proportions of Bioplant and soybean straw used. Therefore, it is possible to infer that soybean straw associated with Bioplant is a viable alternative for acclimatization of *C. nobilior*. In addition to providing survival rates that are compatible with those found in the literature and contributing to plant development, the use of soybean straw favors the protection of the environment since it is a natural plant material, thus avoiding the extraction of native species such as tree-fern (*Dicksonia sellowiana*) and *Sphagnum* spp. that have been used as substrate. The physical characteristics of this substrate component might have possibly contributed to aeration and moisture retention (ABATI et al., 2017), in addition to the availability of nutrients (LIU et al., 2015), factors necessary for the development of both the shoot and root systems of *C. nobilior* during acclimatization.

Since this process is considered one of the most critical and delicate stages of micropropagation, *C. nobilior* presented adequate performance during the acclimatization process performed in the present study, although an 14.6% average decrease in plant survival was observed in the second phase, which corroborates the findings of other studies with orchids (KEITHLY et al., 1991; DORNELES; TREVELIN, 2011).

Anatomical analyses

In cross-section (Fig.4), the leaves of *C. nobilior* commonly present a uniseriate epidermis and are hypostomatic. This characteristic was found in the leaf anatomy of other species of the same genus such as *C. walkeriana*, *C. araguaiensis*, and *C. bicolor* (Zanega-Godoy and Costa, 2003), *C. violacea* (SAONCELLA et al., 2017), *C. jenmaniè* and *C. lawrenceana* (CARNEIRO et al., 2017), as well as for different genera such as *Oeceoclades maculata* (Riverón-Giró et al., 2017). According to Saoncella et al. (2017), the presence of stomata only on the abaxial face is a common characteristic in species of the family Orchidaceae and has already been observed for several species of the tribe Epidendroideae and subtribe Laeliinaea in which the species *C. nobilior* is inserted. Around the atrium outside the ostiole, a pronounced cutinized thickening was observed that constitutes a stomatal crest which contributes to the formation of a suprastomatic chamber (Fig. 4d), similar to those observed by Zanega-Godoy and Costa (2003) when describing the leaf anatomy of *Cattleya* species of the Brazilian central plateau and Silva and Milaneze-Gutierre (2004) when describing *C. walkeriana*. According to these latter authors, this chamber is a common feature in epiphytic orchids which contributes to the reduction of leaf transpiration, seeing that they face high temperatures and low water availability. The plants analyzed in the present study were growing in a shade-house environment during the dry season (July-September), a period in which high temperatures occur associated with low relative humidity, as mentioned earlier.

It is noted that on both sides of the leaf blade in cross-section, the external periclinal walls of epidermal cells are thick, almost always presenting convexity, especially along the margin. Also, the cuticle (Fig. 4a and c) is thick along the margin and across the entire adaxial and abaxial surfaces homogeneously, however, in general the wall of the cells present in the adaxial surface is always thicker. Adjacent to the epidermis, a hypodermis was observed, formed by a layer of cells with different shapes from the rest of the parenchyma of the mesophyll. This layer was observed in the leaves of the plants of all acclimatization treatments. This result suggests that the proportional use of straw did not cause changes in *C. nobilior*. Vascular bundles (Fig. 4a) were observed in all leaves analyzed. This structure was also observed by Zanega-

Godoy and Costa (2003) and Diniz et al. (2011) when studying the same species of the present study and in other species of the same genus studied by Silva and Milaneze-Gutierrez (2004) and Lando et al. (2016). According to Silva and Milaneze-Gutierrez (2004), in addition to sap conduction, vascular bundles perform the function of supporting the leaf blade. The study conducted by Lando et al. (2016) was also with acclimatized plants and, according to these authors, the hypodermic strata are adaptive structures formed in response to acclimatization.

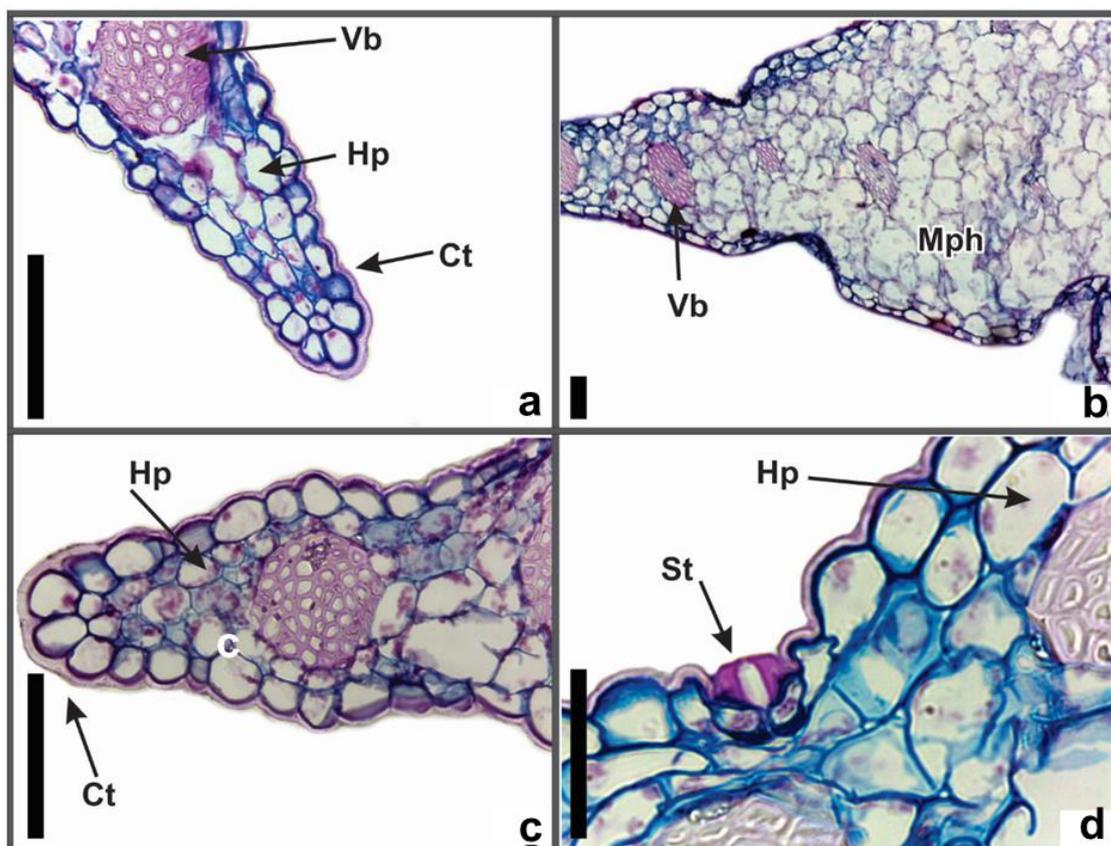


Figure. 4. Anatomical aspects of cross-sections of the basal region of leaves of *Cattleya nobilior* (Orchidaceae) after 150 days of acclimatization: (a) cross-section of leaf of plant grown in 100% Bioplant; (b, c) cross-section of leaf of plant grown in 60% soybean straw; (d) cross-section of leaf of plant grown in 20% soybean straw, showing detail of a stomate. Bars = 100 μ m. St = stomate; Hp = hypodermis; Vb = vascular bundle; Mph = mesophyll; Ct = cuticle.

The mesophyll (Fig. 4b) was homogeneous, compact, composed of chlorenchyma (spongy), a common characteristic in this family as described by Silva and Milaneze-Gutierrez (2004). A similar result was reported for *Cattleya xanthina* in which the cells in the mesophyll in the *in vitro* condition were homogeneous with chlorenchyma, but heterogeneous under *ex vitro* conditions during the acclimatization process (LANDO et al., 2016). One of the common characteristics in the mesophyll of orchid species is the presence of idioblasts with raphide crystals, which are frequently found in *C. nobilior* as noted by Zanega-Godoy and Costa (2003) and which were also found in the present study. These structures were also observed in *C. walkeriana* by Silva and Milaneze-Gutierrez (2004). Ferreira et al. (2015) suggest that these crystals play a defensive role against herbivory by reducing leaf digestibility or, depending on the type/shape, may lead to the death of the herbivore. This characteristic becomes important when it is desired to reintroduce species produced *in vitro* into the natural environment.

The anatomical studies carried out at the end of the second acclimatization phase showed that there were no differences among the leaves of the plants used in the different treatments. Leaf anatomical characteristics of epiphytic species of the family Orchidaceae as observed in the present study, including a thick cuticle that acts as a hydrophobic barrier protecting the plant against high intensities of solar radiation, against excessive transpiration, and the action of pathogens, (JAVELLE et al., 2010), the formation of stomatal crests, the positioning of stomata on the abaxial surface and the presence of a hypodermis, are considered highly efficient structural and physiological adaptations in plants adapted to environments with little water availability, high light intensity and high transpiration rates (ZANENGA-GODOY; COSTA, 2003; DINIZ et al., 2011).

CONCLUSIONS

In summary, Knudson C medium was the most suitable for the seed germination and initial development of *C. nobilior*. With regard to the acclimatization process, it is recommended that the initial substrate be composed of 60% soybean straw and 40%

Bioplant, since these proportions favored a high survival rate as well as better development of the root system. When transferring the plants to shade-house conditions, a substrate composed of 40% soybean straw and 60% Bioplant favored the highest survival rate. Considering that soybean straw is available in large quantities and presents physicochemical characteristics that, when associated with Bioplant, do not negatively interfere in the development of *C. nobilior* as observed in the present study, its use as an alternative substrate in place of tree-fern powder and *Sphagnum* is recommended. The leaves of this species in plants undergoing acclimatization showed leaf anatomy characteristic of the genus *Cattleya*, including anatomical structures related to epiphytic plants adapted to environments with little water availability. This reveals that soybean straw did not lead to structural changes that might affect the development of the species.

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