Original Article

MULTIPLICATION, ROOTING *IN VITRO,* AND ACCLIMATIZATION OF *Brassavola tuberculata* Hook. (ORCHIDACEAE), AN ORCHID ENDEMIC TO THE BRAZILIAN ATLANTIC RAINFOREST

MULTIPLICAÇÃO, ENRAIZAMENTO in vitro E ACLIMATIZAÇÃO DE Brassavola tuberculata *Hook (ORCHIDACEAE), UMA ORQUÍDEA ENDÊMICA DA MATA ATLÂNTICA BRASILEIRA*

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ABSTRACT: The objective of this study was to promote the establishment of an *in vitro* culture of *Brassavola tuberculata*, testing different concentrations of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) on multiplication and rooting, evaluating different substrates during acclimatization, as well as the effect of *in vitro* treatments. After germination, the seedlings of *B. tuberculata* were subjected to culture on MS medium supplemented with different concentrations of NAA and BAP, and multiplication and rooting were assessed. During acclimatization, different substrates were tested: S1, Plantmax® and vermiculite (1: 1); S2, Plantmax® and grit (1: 1); and S3, dust fern. Also the effect of the *in vitro* culture treatments was evaluated: T1, control; T5, (2.5 µM NAA +5 µM BAP); and T7, (5 µM NAA + 0 µM BAP). The favorable balance of cytokinins promoted by treatment T5 yielded the largest number of shoots and leaves in *B. tuberculata*. The greatest length of leaves and roots, and highest root number were observed in the treatment T7, favored by the presence of auxin. This treatment had a positive effect with respect to plant acclimatization: T7 associated with substrate S1 provided the most suitable conditions for acclimatization of seedlings of *B. tuberculata*, providing greater number and length of leaves, and high survival rate.

KEYWORDS: 6-benzylaminopurine. *In vitro* establishment. Naphthalene acetic acid. Substrates. Tissue culture.

INTRODUCTION

Orchids are among the most appreciated and most commercially valuable ornamental plants. Nevertheless, many species are at risk of extinction owing to the destruction of their habitat and predatory extractivism (COLOMBO et al., 2004; ROBERTS and DIXON, 2008). *Brassavola tuberculata* Hook is an epiphytic species with very wide occurrence, whose limits as a species are still considered indeterminate due to the specie's variability (HERMANN et al., 2011). As seen in most species of Orchidaceae, *B. tuberculata* Hook has tiny seeds which, under natural conditions, germinate through association with mycorrhizal fungi that provide the required nutrients. Even so, the natural germination is very difficult, with rates as low as 5% (RASMUSSEN, 2002).

One of the main advantages of *in vitro* germination in orchids is the achievement of higher germination rates compared with natural conditions. Furthermore, it serves as an *in vitro* conservation method, which contributes to reducing the risk of extinction (UNEMOTO et al., 2007; FERREIRA and SUZUKI, 2008).

The formulation of the culture medium is essential for the development of the explant, whereby the presence of necessary constituents (minerals, vitamins and growth regulators) stimulates proliferation, rooting and growth (FARIA et al., 2002). According to Shimura and Koda (2004), growth regulators such as auxins and cytokinins influence the development of orchid explants. For *Cymbidium giganteum* and four varieties of *Phalaenopsis* cultivated *in vitro*, the use of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) provided significant responses in the development of shoots (NAGARAJU and PARTHASARATHY, 1999; PARK et al., 2002; HOSSAIN, 2008; HOSSAIN et al., 2009).

Another crucial step in the *in vitro* cultivation of orchids is acclimatization. During this phase, the loss of plants can be very high, constituting a limiting factor in the multiplication process. In particular, for orchid seedlings germinated *in vitro*, in an asymbiotic manner, it becomes necessary to find suitable substrates that permit the vegetative establishment of these seedlings (COLOMBO et al., 2005).

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However, due to the restrictions in natural propagation exhibited by orchids, studies on their propagation process are important, not only to improve their production, but also to collaborate with their preservation. Nevertheless, no published reports are available on *in* and *ex vitro* establishment of *B. tuberculata* Hook. Therefore, the objective of the present research was to promote the establishment of cultivation in this species, testing different concentrations of NAA and BAP on multiplication and rooting *in vitro*, and evaluating different substrates, as well as the effects of *in vitro* cultivation treatments, on acclimatization.

MATERIAL AND METHODS

A capsule containing seeds of *B. tuberculata* Hook (Orchidaceae) was collected from a private plant nursery in the city of Alegre – ES, Brazil. The capsule was disinfected by immersion in 70% ethanol (Merck®) for 15 min, followed by 2.5% sodium hypochlorite solution for 20 min, and rinsing three times with autoclaved dH_2O.

After the disinfection step, performed under laminar flow hood, seeding was carried out. Approximately 0.01 g of seeds were placed onto Petri dishes (90 x 15 mm) containing 30 mL of Murashige and Skoog (MS) medium (MURASHIGE; SKOOG, 1962) supplemented with 30 g L^{-1} sucrose, and 7.0 g L^{-1} agar (Vetec®); the pH was adjusted to 5.7. This culture was maintained at 25 ± 1ºC under 16/8 h light/dark regime, with 36 µmol $m^{-2}s^{-1}$ light radiation.

The germination was characterized by the formation of a protocorm, a tuberiform structure characteristic of the germination of orchid seeds (KRAUS et al., 2006).

After 90 days, the seedlings of *B. tuberculata* Hook germinated *in vitro*, showing approximately 1 cm in length and three leaves, were transferred to glass jars containing 30 mL of MS medium supplemented with different concentrations of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) (Table 1).

Table 1. MS culture medium supplemented with different concentrations of NAA and BAP.

TREATMENTS	ANA (µM)	BAP (µM)
T1	0.0	0.0
T2	0.0	5.0
T3	0.0	10.0
T4	2.5	0.0
T5	2.5	5.0
T6	2.5	10.0
T7	5.0	0.0
T8	5.0	5.0
T9	5.0	10.0

The seedlings were maintained under the *in vitro* germination conditions described above. After 30, 60 and 90 days, the average number of shoots, leaves and roots and the length of the longest leaf (cm) and longest root (cm) were assessed.

For the multiplication and rooting *in vitro,* a completely randomized design was adopted, with nine treatments and ten replicates per treatment. Each replication consisted of a glass jar containing ten seedlings. The results were subjected to analysis of variance, and means were compared by the Scott-Knott test at 5% significance level (THE R FOUNDATION FOR STATISTICAL COMPUTING PLATFORM 2014).

Two experiments were conducted during acclimatization, in order to: (1) determine the most suitable substrate; and (2) verify the influence of treatments T1, T5 and T7 on acclimatization, as described below:

1 - Substrates for acclimatization: T1 seedlings were removed from the culture medium and transferred into pots containing different substrates: S1, Plantmax® and vermiculite (1: 1); S2, Plantmax® and sand (1: 1); and S3, dust fern. The plants were maintained for 15 days in a growth chamber, covered with plastic bags, at 25 ± 1ºC and 16/8 h light/dark regime, with 36 µmol $m^{-2}s^{-1}$ light radiation. After this period, the seedlings were transferred to greenhouse. After 90 days, the

survival rate, number of leaves and length of the largest leaf (cm) were assessed.'

2 - Effect of treatments on acclimatization: Seedlings from the treatments T1, T5 and T7 were acclimatized using substrate S1 (Plantmax® and vermiculite) in the ratio 1:1. The seedlings went through the same process described above. At 60 days, the survival rate was evaluated, as well as the number of leaves and length of the largest leaf (cm).

For the acclimatization stage a completely randomized experimental design was adopted with three treatments and ten replicates per treatment. Each replication consisted of one seedling into a plastic cup containing 50 mL of substrate. The results were subjected to analysis of variance, and the means were compared by Tukey test at 5% significance level (THE R FOUNDATION FOR STATISTICAL COMPUTING PLATFORM 2014).

RESULTS AND DISCUSSION

After 30, 60 and 90 days of *in vitro* culture under different treatments, it was observed that the addition of plant growth regulators to the culture medium had a differential effect on each variable analyzed (Figure 1 and 2). At the 90-day assessment, the highest averages for shoot number were observed in treatments T3 (0 µM NAA + 10 µM BAP) and T5 (2.5 µM NA + 5 µM BAP) (Figure 1A). Treatment T5 also stood out at the 30- and 60-day evaluations.

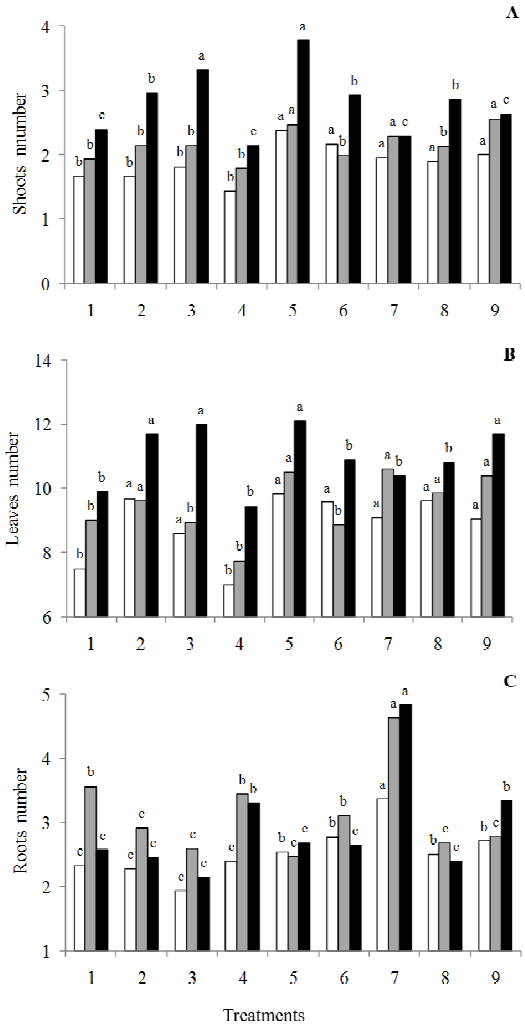

Figure 1. Effect of NAA and BAP combinations in the multiplication of *B. tuberculata*: A – number of shoots; B – number of leaves; C – number of roots. Assessment at 30 (white bars), 60 (gray bars) and 90 days (black bars). Mean values with the same letter between treatments (for each evaluation period) are grouped by the Scott-Knott test at 5% probability (n = 10).

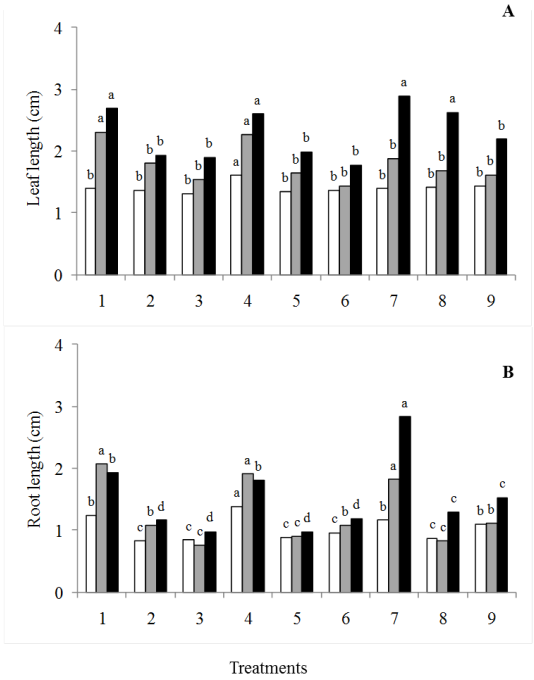

Figure 2. Effect of NAA and BAP combinations on the growth of *B. tuberculata*: A – leaf length; B- root length. Assessment at 30 (white bars), 60 (gray bars) and 90 days (black bars). Mean values with the same letter between treatments (for each evaluation period) are grouped by the Scott-Knott test at 5% probability (n = 10).

The different combinations of auxin/cytokinin showed that increased formation of buds occurred in the absence or at low concentrations of NAA in combination with BAP. The same combination was show to increase the number of formed leaves, whereby the use of high concentrations of BAP in treatments T2, T3, T5 and T9 showed significantly higher means (Figure 1B).

BAP, as well as most cytokinins derived from adenine (aminopurine), has a key role in differentiation and plant regeneration in most species (CASTRO et al., 2002). For the hybrid Blc. Owen Holmes Ponkan x *Brassavola digbiana* no. 2 (GIATTI; LIMA, 2007), the combination favorable to cytokinin (↓auxin/↑cytokinin) proved suitable for multiplication of shoots and leaf formation, just as attested by the results obtained for *B. tuberculata* (Figure 1A and B). The same combination has also been suggested for the species *Cymbidium giganteum* (HOSSAIN et al., 2010), *Cymbidium* (PATHAK et al., 2001), and *Cattleya aurantiaca* (PIERIK; STEEGMANS, 1972).

Analysis of the average number of roots (Figure 1C) and longest root length (Figure 2B) showed a higher mean value for the treatment T7 (5 µM NAA + BAP 0 µM). Similar results were observed for the genera *Dendrobium* and *Oncidium*, in which the presence of NAA provided the highest number of roots (TALUKDER et al., 2003; SORACE et al., 2007).

Auxins and cytokinins are the main hormones involved in regulating plant growth and development, especially in the processes that determine the architecture of the roots and favor rooting. Auxins in particular have a positive effect on the early occurrence of root differentiation from cells (LASKOWSKI et al., 2008; BIELACH et al., 2012). As shown in this work for the species *B. tuberculata*, rooting was observed in the presence of 5 µM NAA auxin in the culture medium.

In the analysis of leaf length, treatments T1 (control), T4, T7 and T8 were statistically similar at day 90 (Figure 2A). Considering the response from the control treatment, it is evident that, despite presenting a small seed, with structure devoid of nutritional reserve, when germinated under ideal conditions, *B. tuberculata* can develop physiological mechanisms that synthesize hormones responsible for the regulation of plant growth as regards leaf length.

Treatment T7, besides showing higher average for root number and length, also stood out among the top averages for leaf length (Figure 2). The presence of NAA promotes, thus, a better response to the elongation of the leaf.

However, the treatments with BAP concentrations higher than NAA concentrations

presented smaller leaf length. In some cases, the increase in the concentration of cytokinin in the culture medium may cause slower development, and decrease the length of shoot leaves (GRATTAPAGLIA; MACHADO, 1998; ERIG et al., 2002; NICIOLI et al., 2008).

Thus, regarding the 90 days of culture *in vitro*, it was concluded that the balance favoring cytokinins offered by treatment T5 (2.5 µM NAA + 5 µM BAP) promoted greater number of shoots and leaves in *B. tuberculata*. The greatest length of leaves and roots as well as increased root number were seen in treatment T7 (5 µM NAA + 0 µM BAP), favorable to auxin (Figure 1, 2 and 3C).

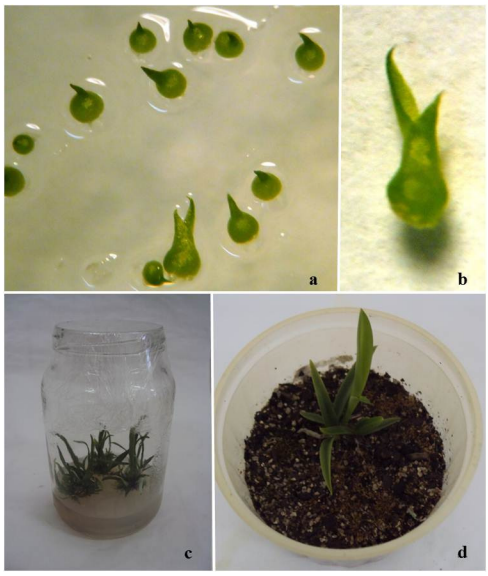

Figure 3. Establishment and multiplication *in vitro*, and acclimatization of *B. tuberculata*: A – seed germination and establishment *in vitro* on MS medium for 60 days; B – protocorm with a pair of leaves in detail; C – multiplication and rooting of seedlings at 90 days on MS medium supplemented with 5 µM NAA + BAP 0 µM (treatment 7); D – seedlings from treatment 7 after 60 days of acclimatization in substrate Plantmax® and vermiculite (1:1).

In the acclimatization phase, the mean S1, S2 and S3 treatments for the number of leaves (NL), length of the longest leaf (LL), and survival rate (S) were statistically equal by Tukey test at 5% probability. However, S1 had 100% survival (Table 2).

Table 2. Effect of different substrates on the growth of *B. tuberculata*: NL– number of leaves; LL– average length of the longest leaf; S – survival percentage assessed at 90 days.

Substrates	NL	LL (cm)	S (%)
S1	4.35 a	3.24 a	100.00 a
S2	4.20 a	2.32 a	95.00 a
S3	5.35 a	2.48 a	95.00 a
CV%	20.02	27.31	9.44

* Means with the same letters in columns are not statistically different by Tukey test at 5% probability (n = 10). S1 = Plantmax and vermiculite (1: 1); S2 = Plantmax and sand (1: 1); S3 = dust fern. CV% = coefficient of variation of average.

Regarding the evaluation of treatment effects, the treatment T7 stood out with the highest averages for leaf number and length (NL and LL) and survival (S), though not statistically different from T1 (Table 3).

Table 3. Effect of treatments on acclimatization of *B. tuberculata*: NL – number of leaves; LL – average length of the longest leaf; S – survival percentage assessed at 60 days.

Treatments	NL	LL (cm)	S (%)
T1 (S1)	2.70 ab	0.72 b	70 ab
T5 (S1)	1.12 b	1.22 b	50 b
T7 (S1)	3.35 a	2.38 a	95 a
CV%	42.35	27.83	27.76

* T1 (control); T5 = 2.5 µM of NAA + BAP 0 µM; T7 = 5 µM of NAA + BAP 0 µM; S1 = Plantmax and vermiculite (1:1). CV% = Coefficient of variation of average. Means with the same letters in columns are not statistically different by Tukey test at 5% probability (n = 10).

B. tuberculata is an epiphytic orchid that depends primarily on the development and aeration of its roots. Thus, *in vitro* rooting is a prerequisite for adaptation of the seedlings to the *ex vitro* environment (GANTAIT et al., 2009). In previous evaluations, T7 was the treatment with the highest number and length of roots (Figure 3 C and D). According to Moraes et al. (2002), in study regarding root development *in vitro*, the combination of vermiculite and Plantmax® contributed to a good root aeration, and thus to the seedling development.

For other species of the orchid family, the use of tree fern fiber has been reported for the acclimatization of seedlings of *Dendrobium nobile* (MORAES et al., 2002), and sand coarse + Plantmax® for *Epidendrum ibaguense* Lindl. (MENEGUCE et al., 2004) propagated *in vitro.* Although many studies indicate the use of tree fern fiber as substrate, due to providing better physiological conditions for seedling acclimatization, its extraction is forbidden in nature. For this reason, despite its good qualities, an alternative must be found for this substrate (DEMATTÊ and DEMATTÊ, 1996).

In the present work, the previous conditions of *in vitro* culture which promoted greater number and length of roots, i.e., MS medium supplemented with 5 µM NAA + BAP 0 µM (T7), associated with the substrate Plantmax® and vermiculite (1:1) (S1), were best for acclimatization of seedlings of *B. tuberculata* propagated *in vitro* (Figure 1D). Moreover, they provided increased length of leaves and high survival rate.

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RESUMO: Objetivou-se, com este trabalho, promover o estabelecimento do cultivo *in vitro* de *Brassavola tuberculata*, testando diferentes concentrações de ANA e BAP na multiplicação e no enraizamento, e avaliar diferentes substratos e o efeito dos tratamentos de cultivo *in vitro* na aclimatização. Após a germinação das sementes, as plântulas de *B. tuberculata* foram submetidas ao cultivo em meio MS suplementado com diferentes concentrações de ácido naftaleno acético (ANA) e 6-benzilaminopurina (BAP), sendo avaliados a multiplicação e o enraizamento. Foram testados diferentes substratos: S1 (Plantmax e vermiculita (1:1)); S2 (Plantmax e areia (1:1)) e S3 (pó de xaxim) na aclimatização e, posteriormente, o efeito dos tratamentos do cultivo *in vitro:* T1 (controle), T5 (2,5 ANA + 5 BAP) e T7 (5 ANA + 0 BAP), na aclimatização. O balanço favorável às citocininas promovido pelo tratamento T5 (2,5 µM ANA + 5 µM BAP) promoveu maior número de brotos e de folhas em *B. tuberculata*. O maior comprimento das folhas, das raízes e maior número de raízes foi observado no tratamento T7 (5 µM ANA e 0 µM BAP), favorável a auxina. Este tratamento apresentou efeito positivo com relação a aclimatização das plantas: T7 associado ao substrato S1, Plantmax e vermiculita (1:1) proporcionou melhores condições para a aclimatização das plântulas de *B. tuberculata*, propiciando maior número e comprimento das folhas, e elevada taxa de sobrevivência.

PALAVRAS-CHAVE: Acido naftaleno acético. 6-benzilaminopurina. Cultura de tecidos. Estabelecimento *in vitro*. Substratos.

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