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*Full Length Research Paper*

# Micropropagation and acclimatization of *Bauhinia cheilantha* (an important medicinal plant)

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**The objective of this study is to establish a micropropagation protocol for *Bauhinia cheilantha*. This was undertaken through an evaluation of 6-benzylaminopurine (BAP) effects, alone or in combination with a-napthaleneacetic acid (NAA), on the morphogenesis of different explants, taken from 20 day old seedlings grown *in vitro*. In the rooting, different auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and NAA were tested under the influence of activated charcoal. The nodal segments showed an organogenic capacity which is 2.4 times greater than the cotyledonary segment. The highest number of shoots (2.84) was obtained at 2.0 $mgl^{-1}$ BAP. The use of 0.5 $mgl^{-1}$ IAA, 1.0 $mgl^{-1}$ NAA or 0.25 $mgl^{-1}$ IBA gave the highest percentage of rooting (about 65.0%). Concerning the activated charcoal (AC), there was a beneficial effect in most of the analyzed characteristics at a concentration of 0.5 $gl^{-1}$. The culture tube, with forced ventilation during rooting, increased the percentage of seedling survival during the acclimatization in greenhouse. This finding reveals that it is possible to obtain *in vitro* culture of *B. cheilantha* using the segment nodal as a source of explants in woody plant medium (WPM) supplemented with BAP.**

**Key words:** *Bauhinia cheilantha*, woody, *in vitro* culture, organogenesis, seedling node, activated charcoal.

## INTRODUCTION

*Bauhinia cheilantha* (Bong.) Steud., commonly known as mororó or pata-de-vaca, is a common leguminous plant in the *Caatinga*, which is the principal ecosystem in Brazil's semi-arid regions. The specie has a socioeconomic importance due to its fodder and medicinal value. Traditional medicine attributes anti-inflammatory, antidiabetic, sedative, antiparisitic, digestive and expectorant properties to the aerial parts of the plant (Lorenzi and Matos, 2008), and as such, its antinociceptive activity has been proven scientifically (Silva et al., 2005). Moreover, its hypoglycaemic action has been observed in experiments conducted on several species of the genus (Silva and Cechinel-Filho, 2002; Negri, 2005).

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**Abbreviations: AC,** Activated charcoal; **BAP,** 6-benzylaminopurine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **NAA,** a-naphthaleneacetic acid; **PGR,** plant growth regulators; **WPM,** woody plant medium; **PET,** polyethylene terephthalate.

Since it is known that in 2010, diabetes growth will increase to 6.4% of the world's population (285 million adults); various countries have joined forces to investigate medicinal plants with powerful hypoglycaemic actions (Shaw et al., 2010). As such, *B. cheilantha* is a semi-arid plant with high medicinal value that could be used to treat this disease in other parts of the world.

In this context, plant tissue culture through micropropagation makes the rapid multiplication of selected genotypes possible, allowing the useful metabolites to be collected in greater quantities (George, 2008), as well as providing an alternative means of propagation, given that in the field, the presence of seed tegument dormancy hinders the swift production of uniform plants (Seiffert, 2006).

The micropropagation of woody species has been proven to be a viable method for the production of explants, which is of great value for the conservation of native species and the recovery of degraded areas (Merkle and Nairn, 2005). On the other hand, there are several difficulties observed during the *in vitro* cultivation such as: Infection by endophytic bacteria not removed during

surface disinfestation (Harry and Thorpe, 1994), phenolic browning (Dobránszki et al., 2010), low shoot multiplication rates (Nhut et al., 2008), excessive leaf drop and internode elongation (Nepomuceno et al., 2009) and making rooting of the aerial parts more difficult (Souza and Pereira, 2007). In turn, it affects the survival of explants during the acclimatization phase.

Due to the fact that there are no existing studies concerning the tissue culture of this species, the objective of this paper is to establish a protocol for the *in vitro* regeneration and acclimitization of *B. cheilantha*.

## MATERIALS AND METHODS

### Culture media and culture conditions

The woody plant medium (WPM) (Lloyd and McCown, 1980) was solidified with 0.6% (w/v) agar (Himedia®) and supplemented with 3% sucrose (w/v). The pH was adjusted to 5.7 ± 0.01 with 0.1 N NaOH or 0.01 N HCl before autoclaving at 121°C for 20 min. All cultures were incubated in a culture room maintained at 60% relative humidity, 25 ± 2°C under a 16 h photoperiod with a photon flux density of 60 $\mu mol.m^{-2}.s^{-1}$ provided by cool white fluorescent tubes.

### Plant material

First, the *B. cheilanta* seeds underwent mechanical scarification, marking the tegument on the opposite side to the hilum. Next, the scarified seeds were disinfested, in laminar flow chamber, by immersing them in 70% ethanol for one minute, followed by a solution of 2.5% active chlorine containing two drops of neutral detergent for 15 min and rinsed successive times in sterile water. Ten seeds were then inoculated in each Petri dish containing previously sterilized germtest paper, humidified with sterile water and closed with PVC film. At about 72 h after sowing, when the shoots have emitted their first roots (1.0 cm long), the seedlings were transferred to test tubes. Explants obtained as a result of the *in vitro* germination were taken from the seedlings after 15 to 20 days and used in subsequent experiments.

### Shoot development

Six types of explants were used for shoot induction (hypocotyls, cotyledonary segments, cotyledon, epicotyl, nodal and internodal segment) and the explants were inoculated in different concentrations of 6-benzylaminopurine (BAP) (0.0-2.0 $mgl^{-1}$) and α-napthaleneacetic acid (NAA) (0.0-0.5 $mgl^{-1}$).

The experimental design used was completely randomized in a 6 x 5 x 3 factorial scheme, giving a total of 90 treatments. Each treatment consisted of 5 repetitions, each of which is made up of 5 test tubes containing an explant. However, 30 days after inoculation, the explants were evaluated based on the following variables: Percentage of responsive explants and number of shoots per explant.

### Rooting

The shoots obtained in the WPM culture medium that was supplemented with 2.0 mg $L^{-1}$ BAP, measuring between 1.5 and 5.0 cm in length, were transferred to a culture medium supplemented with different auxins: Indole-3-butyric acid (IBA), NAA and indole-3-acetic acid (IAA) in different concentrations (0.0-1.0 $mgl^{-1}$) and AC (0.0-1.0 $gl^{-1}$).

The experimental design was completely randomized in a 3 x 4 x 3 factorial scheme, giving a total of 36 treatments. Each treatment consisted of six repetitions, each of which is composed of 5 test tubes containing a shoot. However, 45 days after inoculation, the following variables were observed: Rooting percentage and the number of adventitious roots per shoot.

### Acclimatization

The plants rooted *in vitro* were concomitantly maintained in different culture vessels with forced ventilation (polyvinylchloride (PVC) film; plastic lid without PVC film and cotton plug), after which they were transferred to poly bags containing organic matter and, lastly, placed in a greenhouse. The plants were covered with polyethylene terephthalate (PET) bottles with lids in order to maintain the relative humidity in the microenvironment and kept in a greenhouse at 70% luminosity during acclimatization. During this period, the lid of the PET bottle was unscrewed on the 7th day so as to reduce the relative humidity and was taken off on the 16th day, while the bottle itself was removed on the 30th day. After 30 days, the survival rates of the seedlings in the three treatments were evaluated. Each treatment consisted of 10 repetitions, each of which was composed of six bags containing one seedling.

### Statistical analysis

The data obtained were subjected to variance analysis (ANOVA) and the statistical averages were compared by the Tukey test, with the level of significance set at 5% for the qualitative factors and adjustments using polynomial regression equations for the quantitative factors. As such, the analysis was performed with the software SISVAR (Ferreira, 2008).

## RESULTS AND DISCUSSION

### Shoot development

The adventitious bud producing capacity was greatly influenced by the type of explants (Table 1). The morphogenetic capacity of *B. cheilantha* nodal explants (96.4%) was superior to that of the cotyledonary explants (39.9%) (data not shown). The values observed were superior to those reported by Borchetia et al. (2009) for *Camellia sinensis,* given that the maximum shoot regeneration from nodal segments was 32.8% in a WPM culture medium supplemented with BAP 3.0 $mgl^{-1}$. They were also superior to the results obtained by Tripathi and Kumari (2010) who achieved a response rate of 41.6% in *Spondias mangifera* explants in BAP 2.0 $mgl^{-1}$. The values also differ from the results found for *Parapiptadenia rigida*, in that the percentages for cotyledonary segments (98.8%) were superior to the rates obtained with nodal segment explants (80.0%) (Kielse et al., 2009). It was not possible to observe shoot formation in the remaining explants, as such, the ANOVA test was only conducted with responsive explants. It is probable that the combinations and/or types of growth regulators used in the *in vitro* culture of this species were not sufficient to show this specific cellular level response.

**Table 1.** Factorial ANOVA of responsive explants (RE), shoot numbers per explant (SN), percentage of rooting shoots (PR) and number of root primordial per explants (NR) of *B. cheilantha*.

| Source of variation | Shoot development | | | Source of variation | Rooting | | |
|---|---|---|---|---|---|---|---|
| | DF | Mean square | | | DF | Mean square | |
| | | RE [X] | SN [Z] | | | PR [Y] | NR [X] |
| Explant type (E) | 1 | 23.175** | 45.342** | Auxin type (A) | 2 | 0.185** | 0.069** |
| BAP concentration (B) | 4 | 0.0578 [NS] | 5.390** | Auxin concentration (C) | 3 | 0.161** | 0.155** |
| NAA concentration (N) | 2 | 0.143* | 3.000** | AC concentration (AC) | 2 | 0.0672 * | 0.166** |
| E X B | 4 | 0.070[NS] | 2.616** | A X C | 6 | 0.243** | 0.078** |
| E X N | 2 | 0.118 [NS] | 0.193 [NS] | A X AC | 4 | 0.282** | 0.208** |
| B X N | 8 | 0.086 [NS] | 1.469** | C X AC | 6 | 0.371** | 0.169** |
| E X B X N | 8 | 0.0424 [NS] | 1.076** | A X C X AC | 12 | 0.045** | 0.036** |
| Error | 120 | 0.044 | 0.343 | Error | 180 | 0.018 | 0.012 |
| CV (%) | | 19.69 | 40.89 | CV (%) | | 20.81 | 8.63 |

**Significant at P < 0.01; *Significant at P < 0.05; [NS] Non-significant; [X] Data transformed in arc sin $\sqrt{\%}$; [Y]; [X] Data transformed in $(x + 1)^{0.5}$; [Z] Data not transformed; CV coefficient of variance; DF, degree of freedom.

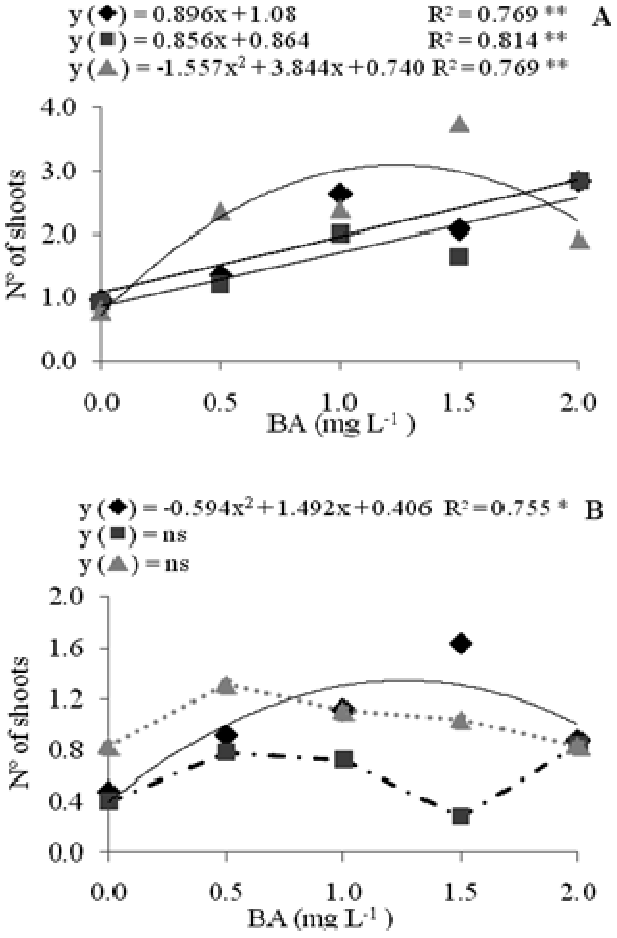


**Figure 1.** Number of shoots obtained from nodal segment (A) and cotyledonary segment (B) of *B. cheilantha*, after 30 days in WPM containing different concentrations of NAA and BAP (◆ 0.0 $mgl^{-1}$, ■ 0.25 $mgl^{-1}$ and ▲ 0.5 $mgl^{-1}$ NAA) (** Significant at P < 0.01; * Significant at P < 0.05; [NS] Non-significant).

For the number of shoots per explant variable, a highly significant triple interaction effect was observed (explant type, BAP concentration and NAA concentration) (Table 1). The response curve for NAA (0.5 $mgl^{-1}$) indicates that the use of BAP in an estimated concentration of 1.23 $mgl^{-1}$ achieves the highest estimated value (3.11 shoots/ explant) for the number of shoots from nodal segments. Any increase of BAP beyond this estimated concentration tends to prejudice shoot formation, probably due to a decrease in the natural endogenous substance levels that promote cell division. On the other hand, in lower concentrations of NAA (0.25 $mgl^{-1}$), or in its absence, shoot induction was increased as a result of the increase in BAP concentrations. A higher shoot value (2.84) was observed, in both conditions, in the presence of BAP (2.0 $mgl^{-1}$) (Figure 1A). Since there was a linear increase for this variable, the concentrations above BAP (2.0 mg $L^{-1}$) could still be tested. Tamta et al. (2008) in the *in vitro* cultivation of *Quercus semecarpifolia* and *Searsia dentata* (Prakash and Staden, 2008), achieved an average of 5.5 shoots in the culture media containing BAP 4.50 e 2.25 $mgl^{-1}$, respectively.

The results achieved for *B. cheilantha* are superior to those reported for other woody species. In the guava species, *Alibertia edulis*, a maximum average of 2.0 shoots was obtained from nodal segments inoculated in a ¼ MS culture medium supplemented with BAP (0.5 mg $L^{-1}$) (Silva et al., 2008). Bopana and Saxena (2008) reported a maximum average of 1.45 shoots from nodal segments of *Asparagus racemosus*, using a MC medium supplemented with BAP (0.5 $mgl^{-1}$). Gomes et al. (2010), studying the *in vitro* multiplication of *Arbutus unedo*, observed the formation of 1.75 shoots from the same explant inocu-lated in a culture medium containing BAP 2.0 $mgl^{-1}$, and it was concluded that the interaction of BAP and NAA does not favor the multiplication rate of these species. Mean-while, for *Populus trichocarpa,* Kang

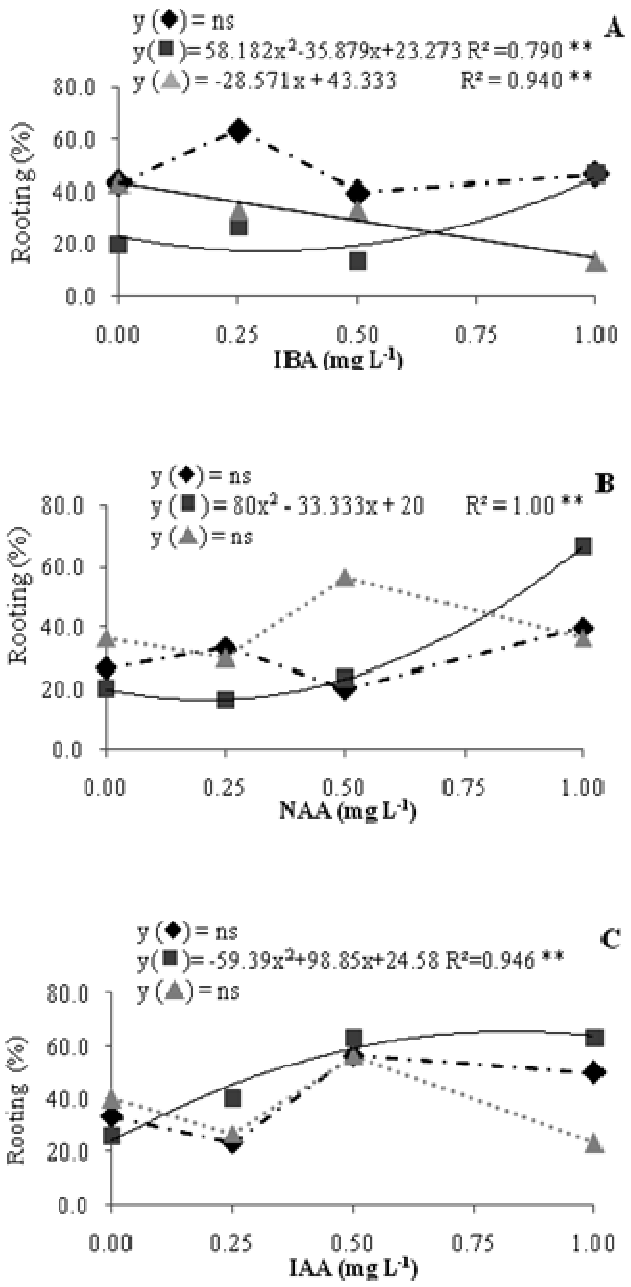


**Figure 2.** Percentage of rooting shoots of *B. cheilantha*, after 45 days in WPM with different concentrations (0.0, 0.25, 0.5 and 1.0 mg $L^{-1}$), auxin types (A, IBA; B, NAA and C, IAA) and in addition, with different concentrations of AC (◆ 0.0, ■ 0.5 and ▲ 1.0 $gl^{-1}$) (** Significant at $P < 0.01$; * Significant at $P < 0.05$; $^{NS}$ Non-significant).

et al. (2009) did not report a significant difference in shoot numbers (2.4 shoots/nodal segment) formed in a medium supple-mented with, or without, BAP. In another species from this family, *Bauhinia vahlii*, only one shoot per nodal segment was observed in the presence of BAP (0.56 $mgl^{-1}$) (Dhar and Upreti, 1999), while Mathur and Mukunthakumar (1992) achieved an average of 3.2 shoots in a MS medium culture supplemented with BAP (3.2 $mgl^{-1}$), using the nodal segments of *Bauhinia variegata*.

A lesser quantity of shoots is formed using cotyledon segments, in comparison to nodal segments, independent of the interaction of BAP and NAA concentrations tested. In the absence of NAA, the response curve achieved its maximum value of estimated shoots (1.34) when the culture medium was supplemented with BAP (1.26 $mgl^{-1}$) (Figure 1B). According to Staden et al. (2008), the interaction of two or more growth regulators can have a synergetic or antagonistic effect depending on various circumstances, given that the presence of a determined regulator can affect the biosynthesis or metabolism of the other regulator, thus altering endogenous substance levels. Furthermore, the environmental factors inherent in the process can modify the response of the regulators, or even neutralize them.

## Rooting

According to the variance analysis, the triple interaction (explant type, auxin concentrations and activated carbon) significantly influenced rooting rate and number of adventitious roots per shoot (Table 1). In the presence of IBA, the maximum rooting rate observed was 63.33% in the absence of activated carbon (Figure 2A). Whilst in NAA and IAA, the highest rooting rates achieved in the presence of 0.5 mg $L^{-1}$ activated carbon were 66.67 and 65.7%, respectively (Figures 2B and C). Analyzing these results, it can be inferred that the rooting rate for *B. cheilantha* was not significantly influenced by differences in plant growth regulator (PGR). In contrast, in the culture of *Vitex agnus-castus*, the rooting rate for shoots maintained in the culture medium with IBA was 2.25 times the rate achieved with NAA or IAA, independent of their concentrations (Balaraju et al., 2008). Similar results were observed for the cultivation of *Pinus massoniana*, in that NAA favored greater root development (82%) in shoots in comparison to IBA (32%) (Zhu et al., 2010).

Results, similar to those of *B. cheilantha*, were reported by Peternel et al. (2009), in the cultivation of *Populus tremula*, where a rate of 60% was obtained in ½ Murashige and Skoog (MS) culture medium supplemented with 1.0 mg $L^{-1}$ IAA without activated charcoal (AC). Similar rates (65.5%) were found in *Prunus salicina* cv. Bruce cultivated with 3.0 mg $L^{-1}$ IBA (Canli and Tian, 2009). Higher rooting rates (90.0%) were obtained in *Liquidambar styraciflua* shoots cultivated in ½ WPM with 0.5 mg $L^{-1}$ NAA (Durkovic and Lux, 2010); whereas, in *Cornus canadendis* (Feng et al., 2009) and *Persea americana* (Nhut et al., 2008), independent of NAA concentrations, it was not possible to induce the formation of adventitious roots. In the cultivation of *B. vahlii*, rates of 38.8 and 30.4% were found in 1.0 mg $L^{-1}$ NAA and IBA, respectively (Dhar and Upreti, 1999). However, this was in contrast to a rate of 96% in *B. variegata* shoots

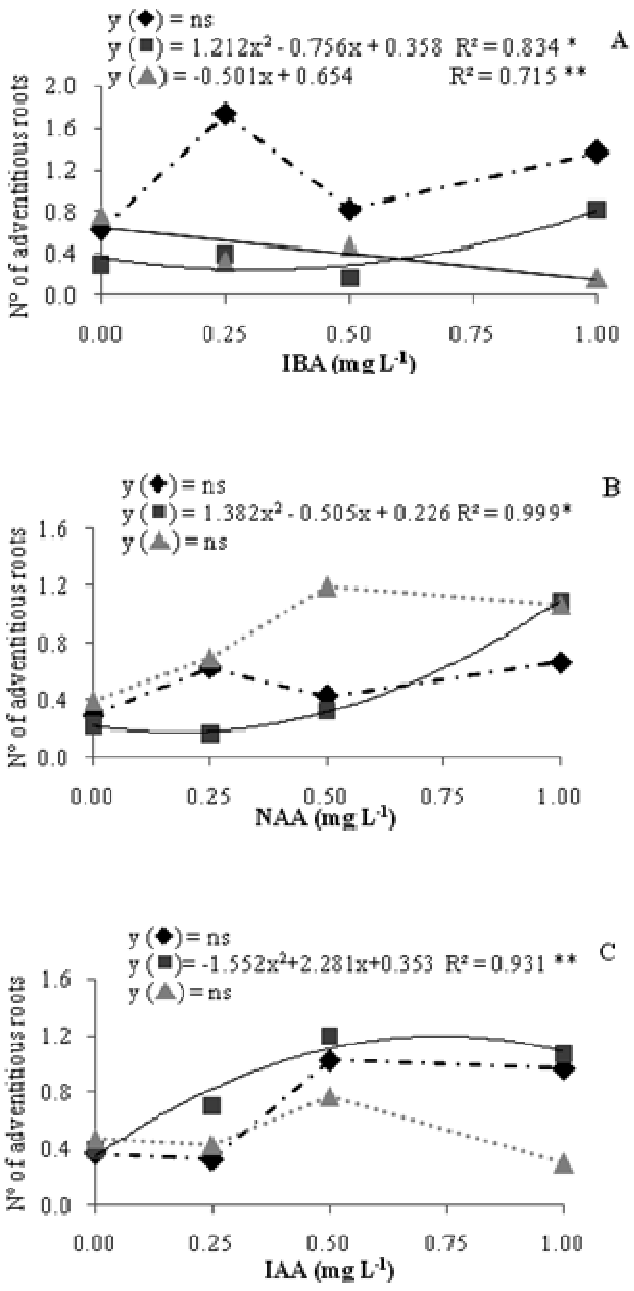


**Figure 3.** Number of adventitious roots of *B. cheilantha,* 45 days after inoculation in WPM culture medium with different types (A, IBA; B, NAA and C, IAA) and concentrations (0.0, 0.25, 0.5 and 1.0 $mgl^{-1}$) of auxins and supplemented with different concentrations of AC (◆ 0.0, ■ 0.5 and ▲ 1.0 $gl^{-1}$) (** Significant at $P < 0.01$; * Significant at $P < 0.05$; $^{NS}$ Non-significant).

(Mathur and Mukunthakumar, 1992).

The rhizogenesis of *B. cheilantha* did not induce callus formation at the base of the shoots. This behavior may have facilitated root formation in the shoots even in the absence of exogenous auxins, something which has not been reported in other woody species, such as *Pistacia vera* (Tilkat et al., 2009). However, the levels of exogenous auxins present in the *B. cheilantha* shoots were not sufficient to promote rooting at levels necessary for satisfactory acclimatization. For the number of adventitious roots variable, the highest observed value (1.47) occured with IBA and without AC (Figure 3A), whereas, for the concentrations of NAA and IAA tested, the highest values (1.10 and 1.19) were achieved in the presence of 0.5 mg $L^{-1}$ AC (Figures 3B and C). In the absence or presence of the major concentration activated charcoal tested, a significant mathematical model adjustment was not possible for this variable, with the exception of IBA auxin supplemented with 1.0 g $L^{-1}$ AC. In this case, a descending linear tendency ($p < 0.01$) for the variable was observed (Figure 3A). The beneficial effect of carbon comes, in part, from its absorption capacity, in that it modifies the composition and the pH of the culture medium and, as such, is able to improve or regulate the *in vitro* growth (Thomas, 2008). The highest AC concentration tested may have altered more intensely the pH of the medium. Also, it may have absorbed more nutrients and PGR from the culture medium, as such, negatively influencing the rooting stage of the shoots.

## Acclimatization

The highest survival rates (86.5 and 78.33%) in acclimatization occured when the test tubes were closed with plastic lids or cotton during the *in vitro* rooting stage, with no significant difference between these treatments. However, they both differed significantly for the lowest rate (61.4%) observed in the test tubes closed with PVC film (data not shown). The increased aeration allowed by the plastic lids and cotton may have stimulated an improvement in stomatal function, cuticular wax formation and/or a more accentuated development of the leaf mesophyll (Ziv and Chen, 2008), which, in turn, may have prevented a more intense dehydration of the plants when they were transferred to normal environmental conditions, thus, improving the quality of the transplants and the *ex vitro* survival rate.

In conclusion, the present study describes an effective method for the induction of adventitious shoots from nodal stem segments of *B. cheilantha* cultured on a WPM medium supplemented with BAP. Additionally, it sets out a combined rooting and hardening protocol developed for producing shoots capable of high survival rates in field, upon transplantation. Furthermore, this work provides important information for the large-scale regeneration and germplasm conservation of *B. cheilantha*.

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