CLONAL BAMBOO PRODUCTION BASED ON *in vitro* CULTURE

PRODUÇÃO DE PLANTAS CLONAIS DE BAMBU A PARTIR DO CULTIVO in vitro

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ABSTRACT: Bamboo species are an alternative for the composition of forest plantations. However, their potential has not been explored due to the hard time in producing large-scale clonal plants. Thus, the aim this work was to evaluate the *in vitro* establishment, bud multiplication and *ex vitro* rooting of *Bambusa vulgaris*. The first experiment tested different systemic and contact fungicide solutions, based on exposure time, during the establishment phase. Established explants were subjected to evaluation of residual fungicide effect on subcultures during the multiplication and elongation phases. The second experiment evaluated the influence of activated carbon on *ex vitro* survival and on adventitious rooting. Explant immersion in liquid culture medium added with 1.0 mL of fungicide for 120 hours has favored the *in vitro* establishment and reduced fungal contamination. On the other hand, it favored the shoot emission of shoots per explant during the multiplication phase. Both rooting induction culture medium and mini-incubator system use were effective in enabling adventitious root formation. The presence of activated carbon in the rooting induction culture medium resulted in a higher clonal plant survival rate.

KEYWORDS: *Bambusa vulgaris*. Cloning. *In vitro* establishment. Micropropagation. Multiplication. Adventitious rooting.

INTRODUCTION

Bamboo species are a perennial, renewable and fast-growing resource that presents high yield per field, low cost and diverse use, besides being considered carbon accumulators (CALEGARI et al., 2007; GUIMARÃES JÚNIOR; NOVACK; BOTARO, 2010). Given their high regrowth capacity, they do not require constant replanting in explored fields and can be grown in eroded soils (DA MOTA et al., 2017). Such features turn bamboo plants into an alternative to meet the growing global demand for forest products.

Bambusa vulgaris Schrad ex Wendl is the most common bamboo species distributed in the Brazilian territory (DO VALE; MOREIRA; MARTINS, 2017). This species has been used for ecological applications such as recovering of degraded soils; as well as in industrial applications such as alcohol, cellulose and paper production; and in the manufacture of handicrafts, furniture, fences and scaffolding (GUIMARÃES JÚNIOR; NOVACK; BOTARO, 2010; LIMA NETO et al., 2010). Bamboo species have potential for carbon sequestration and stand out as one of the main non-timber products (MOGNON et al., 2017). Such features translate into great economic potential and income source for farming families (AFONSO; SILVA, 2017). However, according to Mendes et al. (2010), in homogeneous bamboo plantations present high plant density with low rotation and no replanting, a fact that affects the nutritional aspects of crop fertilization, with emphasis on potassium, which is the most exported nutrient.

B. vulgaris is a monocarpic species, whose flowering and fruiting take long periods of time; besides, it presents low seed germination rate (most of them are sterile), a fact that makes it unfeasible producing seedlings by sexual reproduction (SINGH et al., 2012a; SANDHU; WANI; JIMÉNEZ, 2018). Therefore, despite its potential, species *B. vulgaris* has not been extensively grown in Brazil due to the hard time producing large-scale clonal plants.

Micropropagation technique is an alternative to enable mass plant production, since it reduces time and physical space, and enables increased control over propagated material health and genotype reproduction (ERIG; SCHUCH, 2005). Thus, this technique can be used to get clonal plants from matrices, such as *B. vulgaris* species, that face propagation difficulties through other methods. However, microorganism contamination and adventitious root formation in *Bambusa vulgaris* are the main limiting factors of *in vitro*

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establishment and rooting stages, respectively (GARCÍA-RAMÍREZ et al., 2010; MUDOI; SAIKIA; BORTHAKUR, 2014; TORRES; HOULLOU; DE SOUZA, 2016a; TORRES; LEMOS, 2017). Thus, different strategies such as *in vitro* establishment in different seasons (GARCÍA-RAMÍREZ et al., 2010; SANDHU; WANI; JIMÉNEZ, 2018), use of chemicals to reduce fungal and bacterial contamination under *in vitro* conditions (GARCÍA-RAMÍREZ et al., 2007; TORRES; HOULLOU; DE SOUZA, 2016a; BRONDANI et al., 2017; TORRES; LEMOS, 2017; FURLAN et al., 2018), search for adequate concentrations of auxins, cytokinins and other plant growth regulators capable of increasing shoot multiplication and adventitious rooting (NDIAYE et al., 2006; RAMÍREZ et al., 2012; MALINI; ANANDAKUMAR, 2013), as well as the use of bioreactors (RIBEIRO et al., 2016) have been adopted to develop a viable protocol for *in vitro* plant production of *B. vulgaris*. Thus, the aim of the current study was to develop a micropropagation protocol to enable clonal production of *B. vulgaris* plants.

MATERIAL AND METHODS

Experiment 1 - *In vitro* establishment and multiplication

Material collection and preparation

Shoots were collected from six-year-old *Bambusa vulgaris* Schrad. ex J. C. Wendl plants in Cuiabá County, Mato Grosso State, Brazil. Shoots presenting dormant buds were washed in distilled and autoclaved water added with liquid detergent. Next, shoots were sectioned in the region next to the buds in order to get explants, which consisted of 1.0-to-1.5-cm-long nodal segments, whose leaves were fully removed. The leaf sheath insertion was previously scraped to reduce microorganism contamination (FURLAN et al., 2018; BRONDANI et al., 2017).

In vitro establishment

Uniform nodal segments (explants) were immersed in hydroalcoholic solution (70%) for one minute; next, they were immersed in sodium hypochlorite solution (NaOCl, 2.0-2.5% active chlorine) under constant stirring for 10 minutes. After asepsis, explants were rinsed three times with distilled and autoclaved water and inoculated into glass tubes (2 × 10 cm) filed with 2 mL of MS culture medium (MURASHIGE; SKOOG, 1962). They were grown in incubation room under the following conditions: temperature of 25°C (±2°C), photoperiod of 16 hours and brightness of 32 μmol m^{-2} s^{-1}.

Two 5,6-dihydro-2-methyl-1,4-oxathie-3-carboxanilide (systemic action mode) and tetramethylthiuram disulphide (contact action mode) fungicide concentrations (0.1 and 0.2 mg L^{-1}) were evaluated in comparison to *in vitro* establishment, by taking into consideration different explant immersion times (72 and 120 hours); as well as to the control treatment (0 hour), which was characterized by the lack of contact between explant and fungicide (Table 1). Fungicides were added to the culture medium prior to autoclaving.

Table 1. Immersion time and fungicide solutions tested during *in vitro* establishment of *Bambusa vulgaris* nodal segments.

Treatment	Time[1] (hours)	Active principle (mg L^{-1})	
		Systemic fungicide	Contact fungicide
T1[2]	0	0.1	0.1
T2[2]	0	0.2	0.2
T3	72	0.1	0.1
T4	72	0.2	0.2
T5	120	0.1	0.1
T6	120	0.2	0.2

[1]Time of explant immersion in liquid culture medium added with fungicide. [2]Treatments that did not allow contact between explant and fungicide (i.e., control).

Explants were transferred to glass tubes filed with 2 mL of liquid MS culture medium after the immersion times set for each treatment were over (Table 1). At 14 days, all explants were transferred to glass tubes filed with 5 mL of semi-solid MS culture medium added with agar (7 g L^{-1}) and sucrose (30 g L^{-1}) (Figure 1).

The experiment has followed a completely randomized design with factorial arrangement (3 × 2); three times of explant immersion in culture medium and two fungicide concentrations were

tested (Table 1). Each treatment comprised 40 explants. Contamination (i.e., fungal and bacterial) and oxidation rates were evaluated at the end of the 30th day. Non-contaminated and non-oxidized explants were classified as established.

***In vitro* multiplication and shoot emission**

Established explants were transferred to MS culture medium supplemented with agar (7 g L^{-1}), sucrose (30 g L^{-1}), benzylaminopurine (3 mg L^{-1}) and naphthaleneacetic acid (0.50 mg L^{-1}). Subcultures were carried out for new culture medium every 30 days by keeping the same medium composition. Shoots larger than 1.5 cm were sectioned next to the axillary bud and transferred to new glass tubes to enable getting new explants. The number of explants emitting shoots, the number of shoots per explant and the mean shoot length per explant were evaluated in each subculture.

The experiment has followed a completely randomized design with factorial arrangement (3 × 2 × 4), three times of explant immersion in culture medium, two fungicide concentrations and four subculture times (0, 30, 60 and 90 days). After the previous stage of the experiment was over, the number of replications was set for each treatment; it ranged from 8 to 28 replications. This variation was taken into consideration in the statistical analysis, based on the application of a mathematical model to different replications.

Experiment 2 - *Ex vitro* rooting and acclimatization

Plant material preparation and *in vitro* establishment

Shoots were collected from six-year-old *B. vulgaris* plants in Cuiabá County, Mato Grosso State, Brazil. Shoots presenting dormant buds were washed in distilled and autoclaved water added with liquid detergent. Next, shoots were sectioned in the region next to the buds to enable getting the explants, which consisted of 1.0-to-1.5-cm-long nodal segments, whose leaves were fully removed. The leaf sheath insertion was previously scraped (FURLAN et al., 2018; BRONDANI et al., 2017) and the material was immersed in solution comprising distilled water and fungicides such as 5,6-dihydro-2-methyl-1,4-oxati-3-carboxanilide (0.2 mg L^{-1}) and tetramethylthiuram disulphide (0.2 mg L^{-1}) for 24 hours. After the exposure time was over, shoots were rinsed with distilled and autoclaved water, and then sectioned in the region close to the buds.

Explants consisted of 1.0-to-1.5-cm-long nodal segments, whose leaves were fully removed. Explants were immersed in hydroalcoholic solution (70%) for one minute; next, they were immersed in sodium hypochlorite solution (NaOCl, 2.0-2.5% active chlorine) for 10 minutes. After asepsis, explants were rinsed three times with distilled and autoclaved water, and inoculated into glass tubes (2 × 10 cm) filed with 5 mL of MS liquid culture medium. They were grown in incubation room at 25°C (±2°C), under 16-h photoperiod and brightness equal to 32 μmol m^{-2} s^{-1}.

***In vitro* multiplication and shoot emission**

Twenty-one-day-old established explants were transferred to MS culture medium supplemented with agar (7 g L^{-1}), sucrose (30 g L^{-1}), benzylaminopurine (3 mg L^{-1}) and naphthaleneacetic acid (0.50 mg L^{-1}) in order to enable shoot multiplication and elongation. After 30 days, explants showing shoots larger than 2 cm were transferred to rooting induction medium.

***Ex vitro* rooting and acclimatization**

After multiplication phase and shoot emission, explants presenting shoots larger than 2 cm were transferred to rooting induction medium, where they remained for seven days. The culture medium comprised autoclaved distilled water, agar (7 g L^{-1}), sucrose (30 g L^{-1}), indole-3-butyric acid (1 mg L^{-1}) and activated charcoal (1 g L^{-1}). The control treatment comprised culture medium without activated charcoal. Next, explants were transferred to a mini-incubator system (BRONDANI et al., 2018) (Figure 2), which consisted of a styrofoam tray divided into 9.93-mL cells filled with a mixture of commercial organic substrate based on pinus bark and vermiculite (1:1, v/v). The styrofoam tray was covered with plastic bag and placed in a plastic box filed with 10 mm of water to keep humidity inside it. Explants were grown in incubation room at 25°C (±2°C), under 16-h photoperiod and brightness equal to 32 μmol m^{-2} s^{-1}. Root survival rate, rooting, as well as root number and length were evaluated after 45 incubation days in mini-incubator. The experiment has followed a completely randomized design with factorial arrangement. Two activated coal concentrations (0 and 1 g L^{-1}) were tested in the *ex vitro* rooting of *B. vulgaris* explants. Each treatment comprised 36 explants.

The acclimatization of micropropagated plants was divided in two stages. In the first stage, plants that have survived after 45 days in a mini-incubator system were transplanted into plastic cups (250 mL), where they remained for 30 days in

environment under controlled temperature, humidity and luminosity conditions. Shoots were covered with transparent plastic cups to avoid mortality caused by too low relative humidity. The coverage was gradually removed (every seven days) over time. In the second stage, plants were transferred to larger plastic cups (500 mL) and stored in shade house for 30 days (50% shade).

Statistical analysis

Data measured in all the experiments were subjected to the Shapiro-Wilk's test ($p < 0.05$) and Bartlett's test ($p < 0.05$) in order to verify the normality and homogeneity of the variances, respectively; they were transformed through Box-Cox whenever necessary. After analysis of variance (ANOVA, $p < 0.05$) the means were compared through Duncan's test ($p < 0.05$), according to their significance.

RESULTS

Experiment 1 - *In vitro* establishment and multiplication

Microorganisms were observed in all treatments, a fact that evidenced a hard time controlling contamination (Figure 1). However, it was possible obtaining *B. vulgaris* explants in all fungicide treatments (Figure 2).

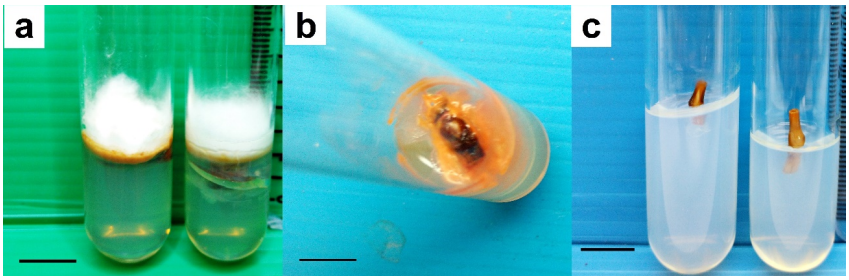

Figure 1. Fungal contamination (a), bacterial contamination (b) and mortality (c) observed in *Bambusa vulgaris* explants during *in vitro* establishment (Bar = 1 cm).

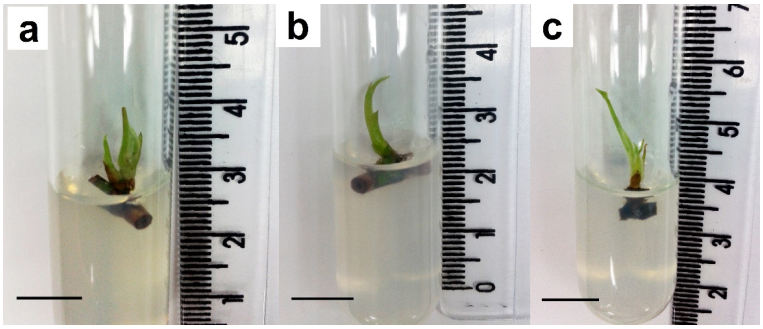

Figure 2. Established *Bambusa vulgaris* explants.
(a) Control. b) Explants immersed in fungicide for 72 hours. (c) Explants immersed in fungicide in 120 hours (Bars = 1 cm).

The fungal contamination of explants immersed in medium added with 0.2 mg L^{-1} of fungicide for 120 hours was lower than that of the fungicide-free control (0 hours). There were no differences in fungal contamination between explants treated with 0.1 mg L^{-1} of fungicide for 72 or 120 hours (Table 2). Fungal contamination did not show significant interaction between fungicide concentration and immersion time. The fungicide treatment did not inhibit bacterial growth (Table 2). The *in vitro* establishment of *B. vulgaris* explants was greater in plants treated with 0.2 mg L^{-1} of fungicide for 120 hours (Table 2). Thus, based on the analyzed data, *B. vulgaris* explant immersion in medium added with 0.2 mg L^{-1} of fungicide for 120 hours decreased fungal contamination and improved *in vitro* establishment.

There was not difference in the number of explants emitting shoots and mean number of shoots per explant in plants immersed in medium added with 0.1 mg L^{-1} of fungicide for 72 or 120 hours. However, the number of explants emitting shoots was larger in plants treated with 0.2 mg L^{-1} of fungicide for 72 hours (Table 3). The mean number of shoots per explant was also larger in explants treated with 0.2 mg L^{-1} of fungicide for 72 or 120 hours (Table 3). Shoot length in explants treated with 0.1 or 0.2 mg L^{-1} of fungicide for 72 or 120 hours was longer than that of the fungicide-free control (0 hours) (Table 3).

Table 2. Percentage of fungal contamination, bacterial contamination, and explants establishment of *Bambusa vulgaris* regarding treatments at 30th days.

Variable	Immersion time (hours)[1]	Fungicide (mg L^{-1})		Mean
		0.1	0.2	
Fungal contamination (%)	0	35.00 (± 7.63)	50.00 (± 8.00)	42.50^{b}
	72	50.00 (± 8.00)	70.00 (± 7.33)	60.00^{a}
	120	37.50 (± 7.75)	25.00 (± 6.93)	31.25^{b}
	Mean	40.83	48.33	44.58
Bacterial contamination (%)	0	40.00^{aA} (± 7.84)	10.00^{aB} (± 4.80)	25.00
	72	22.50^{aA} (± 6.68)	22.50^{aA} (± 6.68)	22.50
	120	25.00^{aA} (± 6.93)	30.00^{aA} (± 7.33)	27.50
	Mean	29.16	20.83	25.00
In vitro establishment (%)	0	65.00^{aA} (± 7.63)	50.00^{aA} (± 8.00)	57.50
	72	50.00^{aA} (± 8.00)	22.50^{bB} (± 6.68)	36.25
	120	47.50^{aA} (± 7.99)	70.00^{aA} (± 7.33)	58.75
	Mean	54.16	47.50	50.83

[1] Time of explant immersion in liquid culture medium added with two fungicide concentrations. Data were expressed as mean (± SE). Means followed by equal uppercase letters on the line did not statistically differ from each other, whereas means followed by lowercase letters in the same column did not statistically differ from each other, Duncan's test ($p < 0.05$).

Table 3. Percentage of explants emitting shoots, mean number of shoots per explant and mean shoot length of *Bambusa vulgaris* immersed in culture medium added with two fungicide concentrations for 0, 72 and 120 hours.

Variable	Immersion time (hours)[1]	Fungicide (mg L^{-1})		Mean
		0.1	0.2	
Explants emitting shoots (%)	0	31.08^{aA} (± 10.95)	13.51^{cB} (± 05.52)	22.29
	72	37.03^{aA} (± 12.69)	42.50^{aA} (± 11.37)	39.76
	120	27.45^{aA} (± 12.87)	24.39^{bA} (± 10.19)	25.92
	Mean	31.85	26.80	29.32
Mean number of shoots per explant (shoot explant^{-1})	0	1.84^{aA} (± 0.13)	0.26^{bB} (± 0.08)	1.05
	72	1.91^{aA} (± 0.23)	1.71^{aA} (± 0.14)	1.81
	120	2.07^{aA} (± 0.27)	1.95^{aA} (± 0.21)	2.01
	Mean	1.94	1.31	1.62
Mean shoot length per explant (cm explant^{-1})	0	0.79^{bA} (± 0.08)	0.11^{cB} (± 0.04)	0.45
	72	0.97^{abB} (± 0.14)	1.37^{aA} (± 0.11)	1.17
	120	0.99^{aA} (± 0.16)	0.72^{bB} (± 0.10)	0.86
	Mean	0.92	0.74	0.83

[1] Time of explant immersion in liquid culture medium added with two fungicide concentrations. Data were expressed as mean (± SE). Means followed by equal uppercase letters on the line did not statistically differ from each other, whereas means followed by lowercase letters in the same column did not statistically differ from each other, Duncan's test ($p < 0.05$).

Explants immersed in culture medium added with fungicide for 120 hours recorded larger number of shoots per explant throughout the subcultures (Table 4). On the other hand, shoot length in explants treated with fungicide for 72 hours was longer than that of the control (0 hour) or 120 hours along the subcultures (Table 4). Explants immersed in medium added with 0.1 or 0.2 mg L^{-1} of fungicide presented similar shoot length throughout the subcultures (Table 5).

Experiment 2 - *Ex vitro* rooting and acclimatization

After multiplication phase and shoot emission, explants presenting shoots larger than 2 cm were transferred to rooting induction medium added with 0 or 1 g L^{-1} of activated charcoal. Next, they were transferred to a mini-incubator system (BRONDANI et al., 2018). Explants immersed in 1 g L^{-1} of activated charcoal recorded higher survival rate than the non-treated ones (Table 6).

Table 4. Mean number of shoots per explant and mean length of shoots of *Bambusa vulgaris* based on immersion time in culture medium added with fungicide, and on different subcultures.

Variable	Subculture (days)[1]	0 hours	72 hours	120 hours	Mean
Mean number of shoots per explant (shoot explant^{-1})	0	0.17^{bC} (± 0.10)	1.18^{bB} (± 0.12)	1.60^{bcA} (± 0.40)	0.98
	30	0.37^{bB} (± 0.16)	1.31^{bA} (± 0.12)	1.18^{cA} (± 0.13)	0.96
	60	1.64^{aB} (± 0.18)	1.99^{aA} (± 0.21)	2.01^{bA} (± 0.27)	1.88
	90	1.74^{aC} (± 0.20)	2.37^{aB} (± 0.29)	2.78^{aA} (±0.29)	2.30
	Mean	0.98	1.71	1.89	1.53
Mean shoot length per explant (cm explant^{-1})	0	0.07^{bC} (± 0.04)	1.05^{bcA} (± 0.17)	0.78^{bB} (± 0.17)	0.63
	30	0.21^{bC} (± 0.09)	1.82^{aA} (± 0.28)	1.20^{aB} (± 0.33)	1.08
	60	0.61^{aB} (± 0.13)	1.18^{bA} (± 0.13)	0.72^{bB} (± 0.12)	0.84
	90	0.74^{aB} (± 0.07)	0.94^{cA} (± 0.08)	0.73^{bB} (± 0.07)	0.80
	Mean	0.41	1.25	0.85	0.83

[1] Subculture and time of explant immersion in liquid culture medium. Data were expressed as mean (± SE). Means followed by equal uppercase letters on the line did not statistically differ from each other, whereas means followed by lowercase letters in the same column did not statistically differ from each other, Duncan's test ($p < 0.05$).

Table 5. Average length of shoots of *Bambusa vulgaris* in relation to two concentrations of fungicide along different subcultures.

Subculture (days)[1]	Fungicide (mg L^{-1})		Mean
	0.1	0.2	
0	0.78^{bA} (± 0.10)	0.35^{cB} (± 0.10)	0.57
30	1.25^{aA} (± 0.28)	0.65^{bB} (± 0.17)	0.95
60	0.85^{bA} (± 0.11)	0.93^{aA} (± 0.13)	0.89
90	0.81^{bA} (± 0.04)	0.81^{abA} (± 0.08)	0.81
Mean	0.92	0.69	0.80

[1] Subculture and two fungicide concentrations. Data were expressed as mean (± SE). Means followed by equal uppercase letters on the line did not statistically differ from each other, whereas means followed by lowercase letters in the same column did not statistically differ from each other, Duncan's test ($p < 0.05$).

Table 6. Mean values recorded for *Bambusa vulgaris* explants that survived after 7-days of exposure to the root induction medium added with two activated charcoal concentrations; and to 45 days in mini-incubator system.

Activated charcoal (g.L^{-1})[1]	Survival (%)
0	63.88^{b} (± 7.02)
1	88.88^{a} (± 7.96)
Mean	76.38

[1] Data were expressed as mean (± SE). Means followed by same letters did not statistically differ from each other, Duncan's test ($p < 0.05$).

The percentage of rooted explants (mean number = 27.77%), the number of roots per explant (mean number = 1.50 roots) and root length per explant (mean length = 2.5 cm) did not show significant difference. The survival rate and *ex vitro* rooting of *B. vulgaris* explants grown in rooting induction medium was higher in plants subjected to mini-incubator than in the ones subjected to acclimatization stages 1 and 2 (Table 7). Only the rooted explants have survived (Figure 3).

Table 7. Survival percentage and *ex vitro* rooting of *Bambusa vulgaris* explants exposed to rooting induction medium; based on mini-incubator and acclimatization methods.

Stage	Survival (%)	Rooting (%)
Mini-incubator	76.38	27.77
Acclimatization (Stage 1)	27.77	27.77
Acclimatization (Stage 2)	27.77	27.77

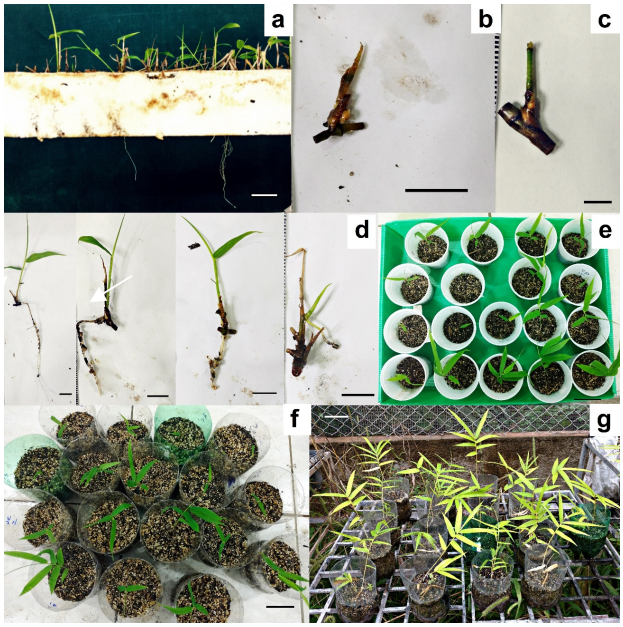

Figure 3. Detail of *Bambusa vulgaris ex vitro* rooting steps in mini-incubator and acclimatization stages.
(a) Explants after 45 days in mini-incubator (roots under the styrofoam container are easily observed). (b) Dead explant after 45 days in mini-incubator. (c) Surviving explant without root formation after 45 days in mini-incubator. (d) Rooted explants after 45 days in mini-incubator. (e) Surviving plants after the first acclimatization stage. (f) Plants transferred to plastic containers (500 mL). (g) Surviving plants after the second acclimatization stage (Bar a, e-g= 5.0 cm; b-d= 1.0 cm).

DISCUSSION

The silvicultural and technological potential of bamboo species has not been properly exploited in Brazil due to the hard time producing clonal plants at commercial scale, among other factors. Given the seed propagation complexity often observed in monocarpic species and in traditional vegetative methods (vegetative propagation), several scholars have succeeded in propagating bamboo species based on *in vitro* cultivation (SINGH et al., 2012a; ANAND; BRAR; SOOD, 2013; NURHAYANI; MEGIA; PURNAMANINGSIH, 2018; SANDHU; WANI; JIMÉNEZ, 2018).

Obtaining viable material during *in vitro* culture stages depends on several factors such as genotype, physiological state of parent plants, harvest and explant type, asepsis, culture medium (nutrients, vitamins, plant growth regulator concentrations and types), incubation conditions (photoperiod, irradiance and temperature) and operators' ability. For example, the main setback during *in vitro* establishment lies on fungal and bacterial contamination. Some reports have succeeded in adding fungicides and antibiotics to the culture medium (RIBEIRO et al., 2016; BRONDANI et al., 2017; FURLAN et al., 2018; SANDHU; WANI; JIMÉNEZ, 2018). This strategy was adopted in the present study and it enabled significant microorganism reduction.

Fungi and bacteria are undesirable during *in vitro* propagation because they can impair explants multiplication and elongation due to competition for nutrients in the culture medium (OLIVEIRA et al., 2015). Thus, it is essential emphasizing several studies aimed at reducing and inhibiting microbial contamination during the micropropagation of several bamboo species such as *B. vulgaris* (RAMANAYAKE; MEEMADUMA; WEERAWARDENE, 2006; TORRES; HOULLOU; DE SOUZA, 2016a; TORRES et al., 2016b; TORRES; LEMOS, 2017), *B. bambos* (ANAND; BRAR; SOOD, 2013), *B. nutans* (NEGI; SAXENA, 2011), *Guadua angustifolia* (JIMÉNEZ et al., 2006; CORREA; MORENO; GONZÁLEZ, 2014), *Dendrocalamus asper* (SINGH et al., 2012b), *D. hamiltonii* (SINGH et al., 2012a) and *D. strictus* (PANDEY; SINGH, 2012). On the other hand, there are groups of endophytic microorganisms that have beneficial relationship with plants. Microorganisms with characteristics capable of helping plant development - either in growth promotion, phytohormone production or in the defense against pathogens - were observed during *B. vulgaris in vitro* culture (COLETTA et al. 2010; COLETTA et al., 2011).

Fungi incidence (Figure 1A) in the current study may have been caused by several factors such material source (matrices planted in the field, where there is no control over phytosanitary conditions, tend to present higher microorganism contamination

rate), fungicide type and concentration, as well as endogenous microorganisms (ANDRADE; BAGATIM; JASPER, 2014). The highest fungal contamination rate was observed in explants immersed in fungicide for 72 hours; however, it decreased after 120 hours of exposure (Table 2).

Torres, Houllou and De Souza (2016a) and Torres et al. (2016b) observed lack of bacterial contamination (Figure 1B and Table 1) in culture medium added with antibiotics. Despite the satisfactory results achieved in the aforementioned studies, it is important exercising caution at the time to use such a strategy due to the high cost of the products and the likelihood of phytotoxicity. In addition, antibiotics should be only used for specific contaminants (each antibiotic has an action spectrum). Contaminated material disposal and autoclaving are the most practical solution (TAMBARUSSI et al., 2017).

The reduced survival percentage (Figure 1C and Table 1) resulting from longer exposure to the fungicide can be associated with the action of the product, since, although fungicide addition to the culture medium in *in vitro* establishment helps controlling contamination, it can have toxic effects on the explant such as growth inhibition and even tissue death (BRONDANI et al., 2013). Although the active principle 5,6-dihydro-2-methyl-1,4-oxathi-ine-3-carboxanilide added with tetramethylthiuram disulfide accounted for low mortality rate in the present study, it did not cause phytotoxicity during *Butia capitata* (SOUZA et al., 2013) and *Passiflora edulis* (VILLALOBOS; VARGAS; GARCÍA, 2009). This principle results from the combination between systemic and contact fungicides. Systemic fungicides have a selectivity type capable of eradicating and suppressing infections caused by pathogens without damaging host cells. The non-specific mode of action of the contact fungicide, which is highly toxic to plant cells, is what may have led to the death of *B. vulgaris* explants, although at low rate (GARCIA, 1999).

The *in vitro* establishment phase (Figure 2 and Table 1) can be influenced by several factors such as genetic material, explant type, asepsis method and phytotoxicity, and physiological conditions of mother plants (BRONDANI et al., 2013). Explants from field-planted matrices often present lower establishment percentage than the ones from matrices located in protected sites subjected to nutritional and phytopathological control (BRONDANI et al., 2011). Seasonality is another important factor in micropropagation; and for *B. vulgaris*, *D. giganteus* (RAMANAYAKE; YAKANDAWALA, 1997), *D. asper* (SINGH et al., 2012b) and *D. hamiltonii* (SINGH et al., 2012a) has indicated that collection time can influence explant establishment. During the rainy season - when the present study was carried out - shoots collected in fields presented higher microorganism incidence when they were *in vitro* cultivated (SINGH et al., 2012b).

The increased number of new explants deriving from the shoot section (Table 3) may have suggested hormonal regulator action by the fungicide. The fungicide stimulated shoot multiplication and elongation; consequently, it stimulated new explant formation. Fungicides' action as cell division stimulant was observed in other studies (ANDRADE; BAGATIM; JASPER, 2014). According to Skene (1972), this phenomenon can take place due to changes in the active principle, which result from culture medium autoclaving. Such changes cause the active principle to act as plant growth regulator, however, new experiments must be carried out in order to confirm such action.

Pandey and Singh (2012) have also reported reduced percentage of explants emitting shoots due to longer exposure to germicidal products. These researchers have observed a larger number of shoots after treatment with calcium hypochlorite and mercury chloride, during *D. strictus in vitro* multiplication, although the number of explants emitting shoots reduced when the time of exposure to the products got longer. The highest percentage of explants emitting shoots was recorded in the present study (42.50% of explants emitting shoots); this outcome corroborates results of experiments conducted with *B. vulgaris* (approximately 40%) (TORRES et al., 2016b), *B. nutans* (45%) (MUDOI; SAIKIA; BORTHAKUR, 2014) and *Guadua angustifolia* (40%) (JIMÉNEZ et al., 2006). High percentage of bamboo explants emitting shoots (up to 97%) had already been observed by other researchers (GARCÍA-RAMÍREZ et al., 2007; SINGH et al., 2012b; PANDEY; SINGH, 2012; SINGH et al., 2012a; TORRES; HOULLOU; DE SOUZA, 2016a). In these cases, the better results may be associated with inherent characteristics of the matrix plant (SANDHU; WANI; JIMÉNEZ, 2018; ARAÚJO et al., 2019).

The mean number of shoots per explant in the current study could have been higher if it had followed the recommendations by Negi and Saxena (2011) and Pandey and Singh (2012), who found that the use of high cytokinin concentration (5 mg L^{-1} of 6-benzylaminopurine - BAP) in the multiplication phase resulted in increased number of *B. nutans* and *D. strictus* shoots, respectively.

However, it is essential highlighting that, to a certain point, increased cytokinin concentrations may be harmful to plants (SINGH et al., 2012a). Devi, Bengyella and Sharma (2012) and Jiménez et al. (2006) observed that high BAP concentrations reduced multiplication in bamboo species (i.e., the number and length of shoots).

Subcultures are another factor positively affecting the number of shoots per explant. In addition to increase shoot rate during multiplication, the promotion of successive subcultures can also restore the juvenile characteristics of adult tissues and favor rooting (SANDHU; WANI; JIMÉNEZ, 2018). Thus, the number of subcultures required during multiplication phase depends on micropropagation goals (BRONDANI et al., 2017).

Mean shoot length was higher in explants treated with fungicide (Table 5), due to its phyto-regulatory action (SKENE, 1972; ANDRADE; BAGATIM; JASPER, 2014). The reduction observed in this variable after 30 subculture days may be associated with the increased number of shoots.

The highest survival rate observed in the presence of activated charcoal (Table 6) may be associated with its ability to retain toxic substances found in the culture medium (such as 5-hydroxymethyl-furfural, which results from sucrose dehydration during autoclaving) or with inhibitory substances found in the agar, which mitigate the action of toxic metabolites such as phenolic substances, ethylene and abscisic acid, which, in their turn, are eliminated by the explant (SANDHU; WANI; JIMÉNEZ, 2018). Despite the benefits of activated charcoal addition to the culture medium during adventitious rooting (adsorption of toxic or inhibitory substances and reduction of light incidence in the root formation region to stimulate adventitious system formation) (LEITZKE; DAMIANI; SCHUCH, 2009), the presence of this substance was not significant for variables such as rooting, and number and length of roots. Similar results were observed during *D. hamiltonii* rooting (SOOD et al., 2002; SINGH et al., 2012a).

The mean rooting rate observed in the current study was 27.77%. This outcome corroborates the studies by Ramanayake, Meemaduma and Weerawardene (2006) and Ndiaye et al. (2006), who used the same indole-3-butyric acid (IBA) concentrations during *B. vulgaris* rooting. Overall, bamboo species have a hard time *in vitro* rooting, which makes it difficult to get a large number of explants with roots (MUDOI; SAIKIA; BORTHAKUR, 2014). Auxin addition to the culture medium is of paramount importance to enable rhizogenesis in bamboo plants (LIMA NETO et al., 2010). The absence of this plant growth regulator resulted in lack of rooting in *B. vulgaris* explants (RAMANAYAKE; MEEMADUMA; WEERAWARDENE, 2006). Increased auxin concentrations led to better results during *B. vulgaris* (NDIAYE et al., 2006; RAMANAYAKE; MEEMADUMA; WEERAWARDENE, 2006; MALINI; ANANDAKUMAR, 2013), *B. nutans* (NEGI; SAXENA, 2011) and *D. hamiltonii* (SINGH et al., 2012a) rooting.

The mortality of explants that did not show root formation was already expected. The nutrient media used during *in vitro* culture are responsible for supplying essential substances in order to enable explant growth. However, the formation of functional roots is fundamental for the survival of micropropagated plants subjected to conditions *ex vitro*. Lack of plant mortality during the acclimatization period suggested functional adventitious root formation. The advantages of *ex vitro* rooting (directly on the substrate) comprise quality root system formation (which reduces mortality during acclimatization) and reduced micropropagation production costs in comparison to *in vitro* rooting (SANDHU; WANI; JIMÉNEZ, 2018).

The transfer of explants *in vitro* grown from humidity and brightness control conditions to greenhouse conditions often implies plant death (BRONDANI et al., 2011). It is important gradually conducting this transfer to assure the survival of plants at acclimatization. The adoption of two acclimatization stages - the first one in a laboratory under controlled temperature, humidity and luminosity conditions, and the second one in a shade house - was efficient in enabling *B. vulgaris* adaptation to *ex vitro* conditions, which resulted in the survival of all plants (Figure 3 and Table 7).

Briefly, results in the current study have shown that fungicide addition to the culture medium during the establishment reduced fungal contamination, which is a recurrent issue during the establishment of bamboo species, as well as accounted for better results during the multiplication phase. On the other hand, the adoption of culture medium to induce adventitious root formation in the rooting stage, in association with the methodology applied during the acclimatization, provided interesting results. The methodology proposed in the present study enabled generating a protocol aimed at forming clonal samplings of *B. vulgaris* suitable to be used in several forest chain sectors.

B. vulgaris explant immersion in liquid culture media added with 1 mL L^{-1} of fungicide for

120 hours resulted in the lowest fungal contamination and in the highest percentage of established explants. Explants immersed in liquid culture medium added with 1 mL L^{-1} of fungicide for 120 hours in the *in vitro* multiplication presented the best results for shoot emission rate and mean number of shoots per explant. Furthermore, the presence of activated charcoal in the culture medium, during *ex vitro* rooting, resulted in increased plant survival. Finally, the use of two different acclimatization stages - the first one under controlled temperature, luminosity and humidity conditions and the second one in the shade-house - was efficient in enabling the adaptation and survival of clonal plants under *ex vitro* conditions.

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RESUMO: As espécies de bambus são uma alternativa para a composição de plantios florestais. Entretanto, esse potencial não tem sido explorado devido à dificuldade de produção de mudas clonais em larga escala. Assim, objetivo deste trabalho foi avaliar o estabelecimento *in vitro*, a multiplicação e o enraizamento *ex vitro* de *Bambusa vulgaris*. No primeiro experimento foram testadas diferentes soluções de fungicida sistêmico e de contato em relação ao tempo de exposição durante a fase de estabelecimento. Os explantes estabelecidos foram avaliados quanto ao efeito residual do fungicida durante subcultivos nas fases de multiplicação e alongamento. No segundo experimento, foi avaliada a influência do carvão ativado sobre a sobrevivência e enraizamento *ex vitro*. Durante o estabelecimento *in vitro*, a imersão de explantes em meio de cultura líquido contendo alíquota de 1,0 mL de fungicida durante 120 horas favoreceu o estabelecimento e reduziu a contaminação fúngica, enquanto na fase de multiplicação, houve o favorecimento da emissão de brotos por explante. O meio de cultura de indução ao enraizamento e uso de sistema de mini-estufim foram efetivos para a formação de raízes adventícias e a presença de carvão ativado resultou em uma maior sobrevivência das mudas clonais.

PALAVRAS-CHAVE: *Bambusa vulgaris*. Clonagem. Estabelecimento *in vitro*. Micropropagação. Multiplicação. Enraizamento adventício.

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