Research Article
doi: 10.1590/01047760202127012979
vol(27), 2021

CERNE

Clonal micro-garden formation of *Bambusa vulgaris*: effect of seasonality, culture environment, antibiotic and plant growth regulator on *in vitro* culture

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SILVICULTURE

ABSTRACT

Background: We have developed a micropropagation technique methodology to clonal micro-garden formation of *Bambusa vulgaris* selected adult plant. Collection site (i.e., stock plant cultivated in different environments) and seasonality of shoot collection (i.e., months of the year) effects on *in vitro* culture were evaluated. Explants that showed normal development were *ex vitro* rooted in mini-incubator system, and three culture media (WPM, MS and deionized water + agar) supplemented with plant growth regulators (IBA, NAA and BAP) and antibiotic (streptomycin sulphate and culture medium free - control) were evaluated. Micropropagated plants were acclimatized in a shadow house and transferred to a semi-hydroponic system for establishment of a clonal micro-garden. The efficacy of the cloning protocol was determined with genetic fidelity analysis by ISSR molecular markers.

Results: Considering all inoculations, 21.9% of nodal segments were *in vitro* established in nine shoot collections. The rooting percentage was 36.6%, and no interactions were observed between the use of culture medium and antibiotic. Culture medium free antibiotic resulted in 80.0% of survival and 50.0% of adventitious rooting. Micropropagated plants presented adequate growth and adaptation to *ex vitro* conditions in the clonal micro-garden. Molecular analyses by ISSR markers indicated the absence of genetic variations, and histological analyses revealed normal adventitious root formation originating from meristematic cells.

Conclusion: The formation of a clonal micro-garden was demonstrated, proving the feasibility of the tested technique. Our results contribute to the development of a clonal propagation protocol for *B. vulgaris* selected adult plants.

Keywords: Bamboo, Clonal multiplication, *In vitro* culture, Genetic fidelity, Adventitious rooting

HIGHLIGHTS

Seasonality and stock plant culture environment influences the *in vitro* establishment of nodal segment.
Fungal contamination is the main factor responsible for explant mortality on *in vitro* establishment phase.
Multiplication and elongation phases occur simultaneously.
Ex vitro adventitious rooting is not influenced by the culture medium and antibiotic.
ISSR molecular markers were efficient for clonal identification.
Clonal micro-garden of bamboo is possible to be established.

TEIXEIRA, G. C.; GONÇALVES, D. S.; MODESTO, A. C. B.; SOUZA, D.M.S.C.; CARVALHO, D.; MAGALHÃES, T. A.; OLIVEIRA, L. S.; TEIXEIRA, G. L.; BRONDANI. G. E.. Clonal micro-garden formation of *Bambusa vulgaris*: effect of seasonality, culture environment, antibiotic and plant growth regulator on in vitro culture. CERNE, v. 27, e-102979, doi: 10.1590/01047760202127012979

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Received: 13/07/2021 Accepted: 18/10/2021

Open Access
CERNE
ISSN 0104-7760

INTRODUCTION

Bamboo plants are from the Poaceae family and the subfamily Bambusoideae, they are of great commercial value for their versatility (INBAR, 2015). There are traditional, ecological, and industrial applications to bamboo plants, such as food, carbon sequestration and cellulose production (Ramakrishnan et al., 2020; Mustafa et al., 2021). Because of high plasticity regarding climate conditions, bamboos are highly adaptable, what makes some of these species cosmopolitan - they can be found practically anywhere on the planet (80% of bamboo forest lands and species are distributed across the Asian Pacific), including Brazil (Akinlabi et al., 2017; Bhadrawale et al., 2018; Kumar et al., 2021; Mustafa et al., 2021; Sawarkar et al., 2021).

In Brazil, bamboo cultivation is not expressive, and to encourage the culture the Law Number 12,484 was created (PNMCB, 2011), and it institutes a National Policy for Encouragement, Sustainable Management, and Bamboo Cultivation and aims the development of the culture through government actions and private corporations.

The production of clonal bamboo plants needs technological advances to effectively contribute to the encouragement of their cultivation aimed at the production of sustainable biomass. The bamboo species presents variations in seed production (Ramanayake et al., 2001; Vengala et al., 2008), a fact that limits the production of plants by seeds, and thus, vegetative propagation is a significant alternative for plant multiplication (Furlan et al., 2018; Ribeiro et al., 2016; Ribeiro et al., 2020; Konzen et al., 2021a). Although frequent, bamboo vegetative propagation by traditional methods, such as the breaking up of clumps, still cannot meet the growing demands for seedlings and the expectations generated with the increase in product diversification (Akinlabi et al., 2017; Rajput et al. 2020). Therefore, further studies related to clonal plants production techniques at industrial scale are needed.

In this context, micropropagation is often employed since it permits large-scale production and smaller genetic variations, besides promoting rejuvenation/reinvigoration of plant tissue. The micropropagation of bamboo, however, is a process that still presents some challenges. The most frequent ones are endophytic organism contamination, hyperhydricity, oxidation of the culture medium due to the release of phenolic substances, unstable multiplication rate and rooting difficulty (Ray and Ali, 2017; Sandhu et al., 2018). For minimising such challenges, researchers have conducted studies that test new asepsis techniques, new culture medium, exogenous applications of plant growth regulators and factors that promote events such as shoot induction and adventitious rooting (Furlan et al., 2018; Sandhu et al., 2018; Lin et al., 2019; Ribeiro et al., 2020; Silveira et al., 2020; Konzen et al., 2021a).

Micropropagation is expected to produce healthy individuals that are genetically identical to the selected plant (Nogueira et al., 2019; Ornellas et al., 2019). Some clonal variations, through the production of calluses (e.g., indirect organogenesis), can occur due to prolonged *in vitro* culture time (Lin et al., 2019). For checking for the occurrence of these events, genetic fidelity tests and anatomical tissue analyses can be performed after micropropagated plants acclimatization (Konzen et al., 2021b).

Our study aimed to develop a micropropagation protocol for *Bambusa vulgaris* and to form a clonal micro-garden in a semi-hydroponic system. For this purpose, the following effects were evaluated: (*i*) the effect of seasonality and tissue origin (stock plant culture environment) in the *in vitro* establishment, (*ii*) the effect of antibiotic application as a disinfecting agent and different culture media in adventitious rooting, (*iii*) *ex vitro* rooting in micro-incubator and acclimatization. The efficacy of the cloning protocol was assessed by genetic fidelity analyses by using Inter-simple Sequence Repeat (ISSR) molecular markers and by histological analyses aiming to describe the adventitious root formation.

MATERIAL AND METHODS

Explant source and *in vitro* establishment

Shoots were collected (situated at 1 meter high) from a selected adult plant of *Bambusa vulgaris* Schrad. ex J. C. Wendl., in Lavras, Minas Gerais, Brazil. Collection site - stock plant cultivated in different environments (A1 – adult plant cultivated in the field – selected plant - tissue donor; A2 – clonal plants grown in vessel suspended in a greenhouse, with irrigation performed directly on the substrate; and A3 – clonal plants grown in vessel suspended in a shadow house with 50% of shade and with a micro-sprinkler system) were evaluated. Clonal plants of A2 and A3 environments were produced by rooting of culm cuttings collected from a selected plant (approximately, 12-years-old) of the A1 environment.

Nine *in vitro* inoculations of nodal segment (i.e., explant) were performed in different times - seasonality on shoot collection (i.e., SC1, SC2, SC3, SC4, SC5, SC6, SC7, SC8, and SC9) from 2017 to 2018 (Table 1). Mean temperature (°C) in the 30 days prior to shoot collection, minimum temperature (°C) in the 30 days prior to shoot collection, maximum temperature (°C) in the 30 days prior to shoot collection, and mean precipitation (mm) in the 30 days prior to collection were measured. The weather data were collected from the Brazilian National Institute of Meteorology (INMET) website and corresponded to the OMM:83687 weather station, Lavras, MG, Brazil (Table 1).

Fungal solution (1 mg L^{-1} Captan, Orthocide 500®) was applied 48 h before shoot collection. Leaf sheaths of the shoot were removed and the remaining lignified tissues were scraped (Furlan et al., 2018; Ribeiro et al., 2020). Shoots were washed with sterile deionized water and neutral-pH detergent, and reduced to the nodal segment (i.e., explant) measuring 2.0 cm of length with one axillary bud, which was immersed in a solution with sodium hypochlorite (NaClO) (deionized water:sodium hypochlorite, v/v, 1.00-1.25% of active chlorine) for 10 minutes. The explants were then transferred to a laminar flow cabinet where they were washed four times with sterile deionized water and inoculated in test tubes (20 x 150 mm) with 10 mL of MS culture medium (Murashige and Skoog, 1962).

Tab. 1 Mean values of minimum (Tmin), mean (Tmea) and maximum (Tmax) temperatures and the mean precipitation (Prec) according to *Bambusa vulgaris* shoot collection (SC) months/years, Lavras, Minas Gerais, Brazil.

SC	Month / Year	Tmin (°C)	Tmea (°C)	Tmax (°C)	Prec (mm)
SC1	April / 2017	19.6	21.5	24.0	152.1
SC2	May / 2017	16.7	19.1	21.7	88.0
SC3	July / 2017	12.1	16.1	18.5	0.0
SC4	September / 2017	15.2	18.7	23.1	1.5
SC5	October / 2017	17.5	22.1	26.4	101.4
SC6	November / 2017	18.9	21.9	24.2	77.4
SC7	January / 2018	19.3	22.6	25.3	272.8
SC8	February / 2018	17.9	23.1	25.3	81.8
SC9	March / 2018	20.8	23.4	25.3	93.3
	Mean(1)	17.6(±2.5)	20.9(±2.3)	23.8(±2.3)	96.5(±78.3)

[1] Date presented as: average (± standard deviation).

MS culture medium was prepared with deionized water, 6.0 g L^{-1} of agar and 30.0 g L^{-1} of sucrose. The pH value of culture medium was adjusted to 5.8 with HCl (0.1 M) and/or NaOH (0.1 M), prior to the addition of agar. Culture medium was autoclaved at 127°C (~ 1.5 kgf cm^{-2}) for 20 minutes. The nodal segments were inoculated vertically, sealed with polyvinyl chloride (PVC) plastic film and kept in a growth room at 24°C (± 1°C), under irradiation of 40 µmol m^{-2} s^{-1} and photoperiod of 16 hours.

The experiment was conducted in a random design in factorial arrangement (3 × 9), considering three stock plant culture environment (A1, A2, and A3) and nine shoot collections (SC1, ..., SC9) with 10 replications of one explant. Percentage of *in vitro* establishment, fungal contamination and loss of explant caused by tissue oxidation and/or bacterial contamination (considered as other events) were evaluated at 30 days.

Data were analysed using the Bartlett's test ($p > 0.05$), Shapiro-Wilk test ($p > 0.05$) and analysis of variance (ANOVA, $p < 0.05$). For all response variables, the data were transformed with the equation (X+1)^0.5. According to the significance of the ANOVA, the mean values were compared using the Scott-Knott's test ($p < 0.05$). In addition, a Pearson correlation test ($p < 0.05$) was performed with data on maximum temperature, mean temperature, minimum temperature and mean precipitation.

In vitro multiplication and elongation

Established nodal segments (i.e., 30 days after inoculation) were transferred to glass flasks (72 × 72 × 100 mm) with 40 mL of semi-solid MS culture medium supplemented with 2.0 mg L^{-1} of 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} of α-naphthaleneacetic acid (NAA). The culture medium was prepared with deionized water, 6.0 g L^{-1} of agar and 30.0 g L^{-1} of sucrose. After supplementation with plant growth regulators, the pH value of culture medium was adjusted to 5.8 with HCl (0.1 M) and/or NaOH (0.1 M). Agar was added to the culture medium, and the solution was distributed in the glass flasks. The glass flasks were autoclaved at 127ºC (~ 1.5 kgf cm^{-2}) for 20 minutes. The explants were subcultured at 21 days.

After the fifth subculture, half of the explants showed bacterial contamination, and these were transferred to test tubes (20 x 150 mm) containing 10 mL of liquid MS culture medium supplemented with 200.0 mg L^{-1} of streptomycin sulphate-based antibiotic, where they were kept for one week. The antibiotic solution was filtered (Millipore Filter®, 0.22 µm) and applied in culture medium. After this period, all explants (with and without bacterial contamination) were properly identified and subcultured into test tubes (20 x 150 mm) with the same treatment as used in the initial elongation stage, but with liquid medium. After 15 days, the explants were subcultured in semi-solid MS culture medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Sixteen subcultures were subcultures were performed for maintenance and shoots elongation.

Survival and *ex vitro* rooting

Explants from the elongation phase (i.e., standard: two shoots > 2.5 cm, and absence of senescence) were treated in the absence (culture medium free-antibiotic) and presence of antibiotic. Three semi-solid culture media were tested to stimulate adventitious rooting: M1 – Wood Plant Medium, WPM (Lloyd and McCown, 1980); M2 – MS culture medium; and M3 – deionized water + agar. All media were supplemented with 1.0 g L^{-1} of activated charcoal, 2.0 mg L^{-1} of indole-3-butyric acid (IBA), 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. The culture media were prepared with deionized water, 6.0 g L^{-1} of agar and 30.0 g L^{-1} of sucrose. After the culture supplementation media with plant growth regulators, the pH value was adjusted to 5.8 with HCl (0.1 M) and/or NaOH (0.1 M). Agar was added to the culture medium, and the solution was distributed in 40 mL glass flasks (72 × 72 × 100 mm). The flasks were autoclaved at 127ºC (~ 1.5 kgf cm^{-2}) for 20 minutes. The explants remained in rooting medium for 7 days, and were then subjected to *ex vitro* rooting in a mini-incubator system (Brondani et al., 2018).

The explants were transferred to cell trays (12.5 mL per cell) with a substrate of a mixture of medium vermiculite and decomposed pine bark and coir organic matter (1:1:1, v/v). For the maintenance of high relative humidity, the tubes were covered with polyethylene film, forming a mini-incubator (Brondani et al., 2012; Brondani et al., 2017), which was kept in a growth room at 24°C (± 1°C), under the irradiation of 40 µmol m^{-2} s^{-1} and photoperiod of 16 hours.

The experiment was conducted in a random design in factorial arrangement (3 × 2), considering three culture

media (M1 – WPM, M2 – MS, and M3 – deionized water + agar) and antibiotic application (culture medium free-antibiotic and presence of antibiotic), with five replications of one explant. Percentages of survival, adventitious rooting, number of leaves and number of roots per explant were evaluated at 45 days.

Data were analysed with the Bartlett's test ($p > 0.05$), Shapiro-Wilk test ($p > 0.05$) and analysis of variance (ANOVA, $p < 0.05$). The data were transformed with the equation (X+0.5)^0.5 for the survival percentage and (X+1)^0.5 for adventitious rooting percentage. The mean values were compared using the Scott-Knott's test ($p < 0.05$).

Clonal micro-garden formation

In vitro rooted plants were transferred to a shadow house, with a shadow plastic mesh used to reduce the natural light by 50.0% for acclimatization in 20 days. After the acclimatization phase, micropropagated plants were transferred to a semi-hydroponic system, with a suspended structure with periodic irrigation and drip irrigation. Sand substrate filled the structure and supported the micro-stumps.

Histological analyses

Samples of shoot, root-shoot transition and root regions were fixed in FAA70 solution (Johansen, 1940) for 3 days and then transferred to 70% alcohol for 24 hours. Dehydration was performed in an ethanol series at 80, 90 and 100% at 30-minute intervals and left in a 1:1 (v/v) resin solution (resin:alcohol) for 24 hours. The material was left for 2 hours in pure resin and then embedded in historesin. The sections, measuring 8 µm, were cut using a benchtop microtome and then stained with toluidine blue – pH 4.7. The semi-permanent slides were prepared with Acrilex® glass varnish, analysed and photographed under an optical microscope, with the images being captured on a micrometric scale. Descriptive analysis was performed for each sample for identification of the tissues arrangement near adventitious root emergence and evidence of meristematic zones.

Genetic fidelity

DNA extractions were performed according to the protocol proposed by Ferreira and Grattapaglia (1998). Thirteen universal primers, whose specifications are shown in Table 2, were used to evaluate the diversity of the genetic materials. ISSR reactions were prepared in microplates (PCR-96, Axygen Scientific), with 3 µL of DNA (standardized at 20 ng μL^{-1} for all samples) and 10 µL of reaction mix [1.5 mM of Phoneutria® PCR buffer, 1.5 mM of dNTP, 1 U of Phoneutria® Taq polymerase (5 U μL^{-1}), Taq diluent (with BSA and Tris HCl) and 0.2 mM of each primer, brought to the final volume with ultrapure water (4.2 µL)].

RESULTS

In vitro establishment

The lowest percentage of fungal contamination was observed in SC6 (November / 2017) with adult plant cultivated in the field – A1 (1.9%), differing for the other treatments (Figure 1).

Tab. 2 Specifications of ISSR primers used in genetic fidelity analyses of *Bambusa vulgaris* micropropagated plants

Primer	Sequence
John	$(AG)_7$-YC
Manny	$(CAC)_4$-RC
Mao	$(CTC)_4$-RC
Omar	$(GAG)_4$-RC
Chris	$(CA)_7$-YG
Becky	$(CA)_7$-YC
UBC809	$(AG)_8$-G
UBC827	$(AC)_8$-G
UBC835	$(AG)_8$-YC
UBC840	$(GA)_8$-YT
UBC842	$(GA)_8$-YG
UBC848	$(CA)_8$-RG
UBC898	$(CA)_6$-RY

Note: R = purine (A or G) and Y = pyrimidine (C or T).

In general, the lowest fungal contamination was observed in explants from greenhouse (A2) and in the months of collection with low rainfall indexes (Table 1) SC2 (May / 2017), SC3 (July / 2017), SC4 (September / 2017), SC5 (October / 2017) and SC8 (February / 2018) (Figure 1).

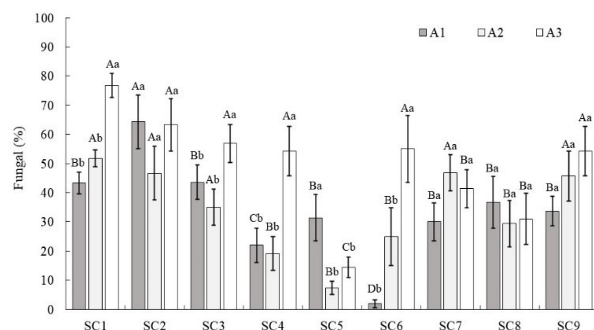

Fig. 1 Percentage of *in vitro* fungal contamination of *Bambusa vulgaris* explants according to stock plant culture environment and shoot collection. Mean values followed by equal capital letter considering the same stock plant culture environment, and equal lowercase letter considering the same shoot collection, do not differ statistically by the Scott-Knott's test. Legend: SC – shoot collection; A1 – adult plant cultivated in the field – selected plant; A2 – clonal plants grown in vessel suspended in a greenhouse, with irrigation performed directly on the substrate; and A3 – clonal plants grown in vessel suspended in a shadow house with 50% shadow and a micro-sprinkler system. Data are presented as the mean ± standard error.

The percentage of contamination by other events (i.e., tissue oxidation and/or bacterial contamination) as well as fungal contamination showed interaction between factors ($p < 0.05$). The lowest average was observed in the first month of collection SC1 (April / 2017) considering A2 (23.2%) and A3 (19.4%), with statistical difference for A1 (31.7%) (Figure 2).

The factors acted independently for percentage of *in vitro* establishment ($p < 0.05$). Different months of shoot collection used for the introduction phase, influenced the *in vitro* establishment of *B. vulgaris*, at 30 days after inoculation. The highest percentage average of *in vitro*

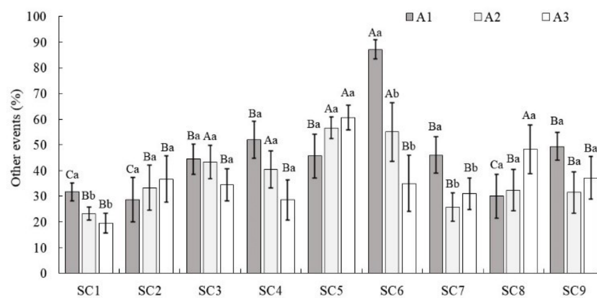

Fig. 2 Percentage of other events (i.e., bacterial contamination and/or tissue oxidation) of *Bambusa vulgaris* explants according with stock plant culture environment and shoot collection. Mean values followed by equal capital letter considering the same stock plant culture environment, and equal lowercase letter considering the same shoot collection do not differ statistically by the Scott-Knott's test. Legend: SC – shoot collection; A1 – adult plant cultivated in the field – selected plant; A2 – clonal plants grown in vessel suspended in a greenhouse, with irrigation performed directly on the substrate; and A3 – clonal plants grown in vessel suspended in a shadow house with 50% shadow and a micro-sprinkler system. Data are presented as the mean ± standard error.

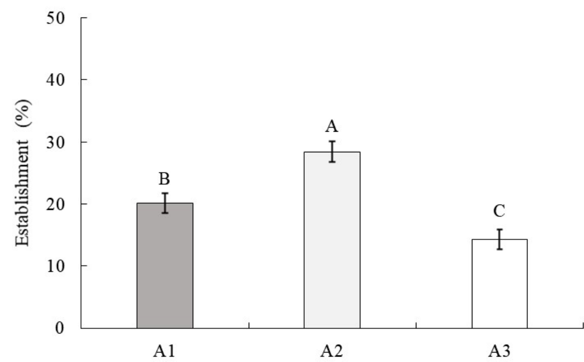

Fig. 4 Percentage of *in vitro* establishment of *Bambusa vulgaris* explants according with stock plant culture environment. Mean values followed by equal capital letter do not differ statistically by the Scott-Knott's test. A1 – adult plant cultivated in the field – selected plant; A2 – clonal plants grown in vessel suspended in a greenhouse, with irrigation performed directly on the substrate; and A3 – clonal plants grown in vessel suspended in a shadow house with 50% shadow and a micro-sprinkler system. Data are presented as the mean ± standard error.

establishment occurred during SC1 (21.4%), SC4 (28.8%), SC5 (30.2%), SC7 (26.4%) and SC8 (31.2%), with a significant difference for the other treatments (Figure 3).

A2 environment provided the best results (28.4%) regarding the *in vitro* establishment in view of the different origins of the vegetative propagule, differing from the A1 (20.1%) and A3 (14.3%) (Figure 4).

Survival, *ex vitro* rooting and clonal micro-garden

In vitro multiplication and elongation phases occurred simultaneously, in which the explants were sub-cultured in liquid MS medium and semi-solid MS medium. When they reached the desired standard (i.e., two shoots > 2.5 cm, and absence of senescence), they were placed in rooting medium and then transferred to the mini-incubator on *ex vitro* conditions.

The results were not significant for either survival or rooting percentages (Figure 5). After 45 days, the survival percentage was 50.0% (Figures 5A and C), and adventitious rooting was 36.6% (Figures 5B and D). Considering the culture media, the highest survival percentage was observed in M2 (80.0%) and the lowest in M1 (30.0%) (Figure 5A). Regarding the use of antibiotic, the highest survival percentage was found in the absence of this chemical agent (53.3%) (Figure 5C). For the rooting, considering the culture medium, the best result was also observed in M2 (50.0%) (Figure 5B); regarding the use of the antibiotic, the best result was observed in the culture medium free-antibiotic (46.7%) (Figure 5D).

Among the explants acclimatized in the greenhouse with subsequent formation of the clonal micro-garden (Figure 6), 71.0% had roots and adequate growth and formation of shoots, which were retained after being transferred to the gutter system in a sand bed.

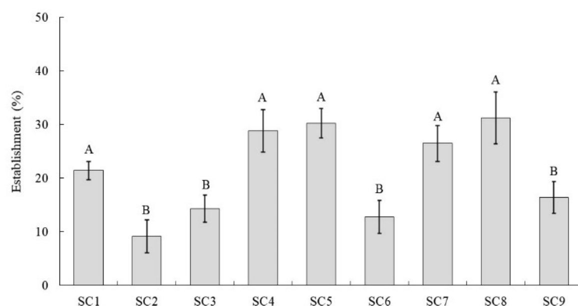

Fig. 3 Percentage of *in vitro* establishment of *Bambusa vulgaris* explants according with shoot collection. Mean values followed by equal capital letter do not differ statistically by the Scott-Knott's test. Data are presented as the mean ± standard error.

Histological analyses

Histological sections of the nodal segment of *B. vulgaris* (Figures 7A-D) showed that tissue redifferentiation occurred in the formation of adventitious roots (Figures 7B-D). A direct connection was observed between the redifferentiation region and the new adventitious roots, without the occurrence of indirect organogenesis (callus) (Figures 7C-D). The adventitious roots were formed in the node's region, which may be due to the greater number of meristematic cells. Identification of the pith, pericycle, aerenchyma and the presence of root hairs (Figures 7B) was possible from observing the adventitious roots. Root hairs are common in adventitious bamboo roots and facilitate the absorption of solutes by the plant (Furlan et al., 2018; Ribeiro et al., 2020). In the stem, in the portion above the node and the root-stem transition region (Figures 7C-D), there was a reduction in the cells and the emergence of a central void, which characterise the hollow region of the bamboo.

Genetic fidelity

The micropropagated explants were faithful copies of the *B. vulgaris* selected plant. Only ten primers tested presented adequate amplification and discernible bands, which were considered for the evaluation of genetic fidelity.

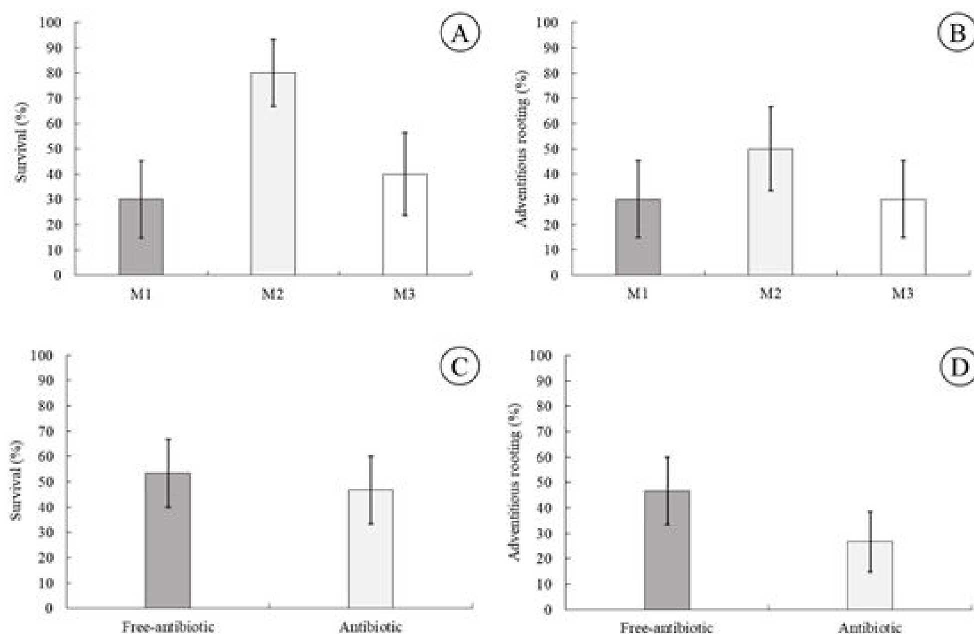

Fig. 5 Percentage of survival and *ex vitro* adventitious rooting of *Bambusa vulgaris* explants at 45 days. (A) Survival according to the culture medium. (B) Adventitious rooting according to the culture medium. (C) Survival according to the use of antibiotic. (D) Adventitious rooting according to the use of antibiotic. * There was no significant difference between treatments by Scott-Knott's test. M1 – WPM, M2 – MS, and M3 – deionized water + agar. Data are presented as the mean ± standard error.

Fig. 6 Clonal micro-garden of *Bambusa vulgaris*. (A) One the day of transfer to semi-hydroponic system, and (B) after 45 days. Legend: yellow and red bars = 5 cm.

For the identification of the bands, the strong intensity and clear separation from the other bands were used as criteria. Thus, a total of 39 bands were observed, representing an average of approximately four bands per primer. All bands were considered monomorphic, i.e., presented the same profile for all individuals (Table 3, Supplementary document). The absence of polymorphism confirms that no somaclonal variation occurred in the subcultures.

DISCUSSION

In vitro establishment

Tissue contamination is an undesirable aspect of the *in vitro* introduction process. Fungal contamination is reported in several bamboo species, as well as *Bambusa tulda* (Bhadrawale et al., 2018), *B. vulgaris* (Furlan et al., 2018; Ribeiro et al., 2020), *Drepanostachyum luodianense* (Lin et al., 2019), *Guadua angustifolia* (Nogueira et al., 2019), *Guadua chacoensis* (Ornellas et al., 2019), *Guadua magna* (Nogueira et al., 2019) and *Guadua latifolia* (Leão et al., 2020). However, the results showed differences between stock plant cultivated in different environments (A1, A2, and A3) and shoot collection (SC1, …, SC9) factors at 30 days of *in vitro* cultivation. Thus, it is possible to determine the better donor source of *B. vulgaris* explants and the shoot collection for *in vitro* establishment. Probably, the initial precipitations of the rainy season contributed to the washing of the tissues, eliminating particles of dust that contained fungal spores. In addition, the environmental conditions favor the emission of new shoots with greater vegetative vigour and low fungal contamination. However, this effect should be further investigated, in view of the precipitation intensity and frequency effects, which can influence the *in vitro* establishment of explants.

High fungal contamination according to the increase in average precipitation was reported by other authors in studies with bamboo (Sandhu et al., 2018; Vale et al., 2019; Leão et al., 2020), and may be related to ideal conditions for the fungal proliferation. High temperatures may limit the growth and development of fungi, what correlates with the decrease in the occurrence of fungi in SC5 (Figure 1), the month with the highest maximum temperature. It is important to emphasize the maintenance of the stock plant in a controlled environment, such as the greenhouse, it contributes to the reduction of fungal contamination of shoots, due to less exposure to environment conditions.

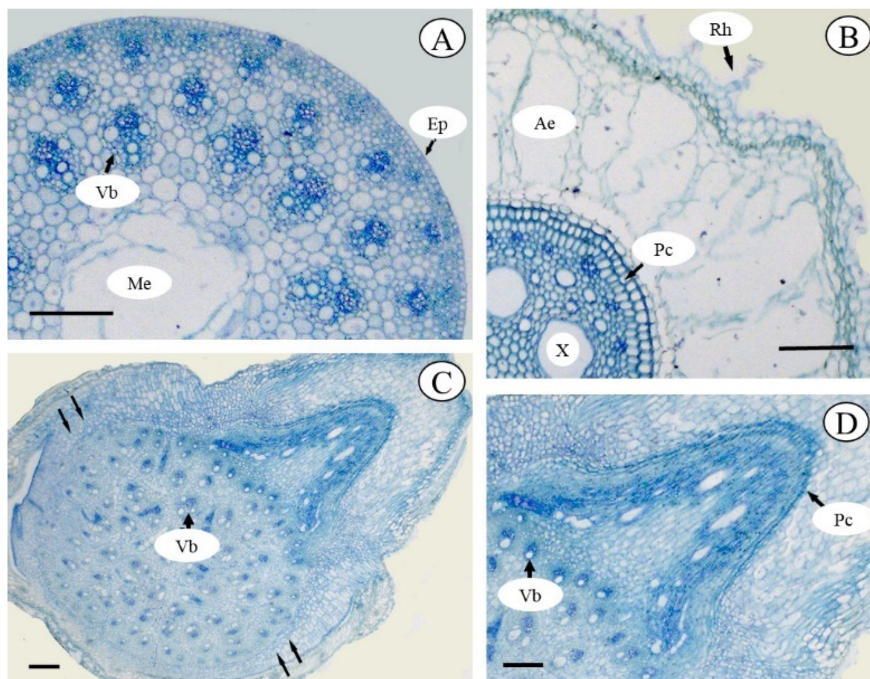

Fig. 7 Cross-sections of micropropagated *Bambusa vulgaris* tissues. (A) Internodal region of the culm. (B) Adventitious root. (C) Root-stem transition region where the arrows indicate the region of onset of cell redifferentiation. (D) Details of the formation of the adventitious root. Legends: Ae = aerenchyma; Ep = epidermis; Vb = vascular bundles; Me = medullary region (pith); Pc = pericycle; Rh = root hairs; and X = xylem. Bar = 100 μm.

Contamination has been a problem associated with the micropropagation of bamboo, which can influence the development of explants (Furlan et al., 2018; Ribeiro et al., 2020). These results may be related to the cultivation environment, high humidity and temperature, which increases fungal proliferation (Silveira et al., 2020). Therefore, methodologies aimed at overcoming or reducing contamination on the surface of the tissues are important in the propagation system. In general, the low percentages of fungal contamination and other events (bacterial contamination and/or tissue oxidation) from the observed months, denote the potential of using a greenhouse to obtain propagules free from contaminants. Therefore, it is possible to proceed with the *in vitro* multiplication phase, denoting the importance of the high percentage of proliferation of shoots, and consequently *in vitro* establishment of explants.

Results of *in vitro* establishment of *Bambusa vulgaris* (Figure 3) are similar to those found in *B. tulda* (Bhadrawale et al., 2018), *G. chacoensis* (Ornellas et al., 2019) and *B. oldhami* (Silveira et al., 2020). Thus, different responses of *B. vulgaris* explants were observed, varying according to the months of collection and origin. The maintenance of the stock plants in a protected environment contributes to less exposure to environmental variations and consequently the obtention of more vigorous shoots with lower levels of contamination. In addition, because the distribution of stock plants in the greenhouse there was a reduction in fungal contamination with greater distance between plant root systems, due to less mobility of microorganisms in tissues (Sandhu et al., 2018; Torres et al., 2019; Konzen et al., 2021a). It is important to highlight that the greatest source of fungal contamination through endophytic fungal occurs in the root tissues in bamboos (Torres et al., 2019).

The success in establishment of *in vitro* of bamboo shoots from adult stock plants is difficult, mainly due to contamination. As an alternative to reduce microbial contamination in *B. vulgaris,* Furlan et al. (2018) observed a significant reduction in fungi when using active chlorine added to culture medium. However, they also found low percentages of *in vitro* establishment (16.0%). Ribeiro et al. (2016) tested different culture systems on *in vitro* introduction of *B. vulgaris* nodal segments and observed 100.0% of established explants, when compared to the conventional system. However, not only disinfection procedures should be investigated, but also the months of collection and origin of the explant. These factors are significant on *in vitro* culture and there are still few studies involving its effect on bamboo species. The knowledge of the relationship between the collection times and the origin of the vegetative propagules in *B. vulgaris*, allowed greater efficiency in the *in vitro* establishment, providing a difference in the responses to micropropagation.

Survival, *ex vitro* rooting and clonal micro-garden

Culture medium did not significantly influence the *ex vitro* rooting and acclimatization. This result may be associated with the residual effect of plant growth regulators on endogenous tissues, indicating that this may be the determining factor for adventitious rooting stimulation. All media were supplemented with 1.0 g L^{-1} of activated charcoal, 2.0 mg L^{-1} IBA, 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. The residual effects of plant regulators used in the multiplication and elongation phases, mainly BAP, may have contributed to the inhibition of the rooting process under *ex vitro* conditions, as with *Garcinia mangostana* (Goh et al., 1990), *Alcantarea imperialis* (Martins et al., 2020). Additionally, the period of time that the explants remained in the rooting induction media, which, despite having generated the necessary stimulus for the rhizogenesis in the explants (36.6%), may have been insufficient for others. Among other authors, Bhadrawale et al. (2018) and Rajput et al. (2020)

observed that the use of higher auxin concentrations may cause greater rooting.

Pandey and Singh (2012) found difficulties with the *in vitro* rooting of *Dendrocalamus strictus*. The authors estimated IBA, indole-3-acetic acid (IAA) and NAA in MS medium supplementation, and the only treatment that led to rooting was the one containing 5.0 mg L^{-1} IBA. For *Bambusa balcooa*. Rajput et al. (2020) observed that, in the absence of IBA, no root induction occurred and that, with the increase of IBA concentrations the rooting percentage also increased. However, the rooting obtained with concentrations above 3.0 mg L^{-1} IBA did not show significant differences, suggesting that this could be the ideal concentration.

Additionally, the different responses of the explants may be associated with the ontogenetic age of the explants, which affects the ability of cells to respond to stimuli and to re-differentiate themselves in new morphogenic routes, as in the formation of adventitious roots. Even with the absence of differences between treatments, the use of MS culture medium without the addition of antibiotic generated 80.0% survival and 50.0% adventitious rooting of the explants and was therefore recommended for *ex vitro* rhizogenesis of *Bambusa vulgaris*. The result obtained in this treatment is considered satisfactory because the study here involved the cloning of adult plants. This result is promising for breeding programmes of species that aim to establish plantations.

The formation of clonal micro-garden in a semi-hydroponic system is widely used for several forest species (Brondani et al., 2012; Brondani et al., 2018; Araújo et al., 2019; Konzen et al., 2021a) and allows greater micropropagation efficiency. However, for bamboo, it is not so widespread. Through this study, with the establishment of the clonal micro-garden of *B. vulgaris* (Figure 6A-B), investigations of the effects of this cultivation system on the species are now possible.

The appropriate performance under external environmental conditions may be related to the use of *ex vitro* rooting method in mini-incubators, corroborating the findings of Nogueira et al. (2019), Ornellas et al. (2019), Bag et al. (2019) and Rajput et al. (2020), the method reduced the stress caused during acclimatization through a gradual transition of the humidity, temperature and nutritional conditions, thus preventing the death of any rooted individual. An adequate *ex vitro* performance makes the possibility of implementing a clonal micro-garden workable since it allows the frequent production of complete plants, besides facilitating the acclimatization process.

Histological analyses

Direct connection in the formation of adventitious roots, the anatomical analysis suggests a satisfactory result of the methodology employed. This is because the use of antibiotics may stimulate the occurrence of calluses (Tambarussi et al., 2015), whose presence in a vascular connection region is undesirable because the connection region is fragile, and calluses may compromise the functionality of the root (Furlan et al., 2018; Ribeiro et al., 2020). The large extent of re-differentiation events also suggests the formation of well-structured roots with likely good performance in nutrient absorption.

Many aerenchyma cells were also observed in the roots of other monocotyledons by Furlan et al. (2018), being an indication of its functionality. The formation of aerenchyma in the root helps in the gas exchange between the root system and the shoots, which allows greater diffusion of gases such as oxygen and ethylene, causing the root to be aerated even when the external environment is hypoxic, such as may occur on *in vitro* conditions (Yin et al., 2010).

Genetic fidelity

RAPD-RFLP markers were efficient for molecular identification of bamboo genera and species, providing a cost-effective and accurate method for conservation, management and plant breeding (Konzen et al., 2017; Konzen et al., 2021b). The lack of genetic variations in the micropropagation process suggests that the protocol was efficient, with no interference from the use of antibiotics in the material's decontamination or the number of subcultures. Antibiotics can stimulate the production of calluses, cell structures that dedifferentiate and/or re-differentiate, providing greater susceptibility to the occurrence of somaclonal variation (Nogueira et al., 2019).

No genetic variation was observed, even after 16 subcultures for approximately 10 months. Sandhu et al. (2018) reported the existence of a correlation between *in vitro* culture time and increased genetic instability. The *in vitro* culture conditions and plant growth regulators used in the *B. vulgaris* micropropagation are not very effective in promoting genetic alterations. Genetic fidelity was also observed with molecular markers for *G. magna* (Nogueira et al., 2019), *G. angustifolia* (Nogueira et al., 2019), *Bambusa bambos* (Anand et al., 2013), *Dendrocalamus asper* (Singh et al., 2013) and *D. strictus* (Goyal et al., 2015). The use of ISSR molecular mardekers in other cultures also allowed the evaluation of genetic fidelity in micropropagated individuals. The method was considered a fast and reproducible alternative, capable of confirming the genetic integrity of the crops, as *Eleusine coracana* (Babu et al., 2018), *Tecomella undulata* (Chhajer and Kalia, 2016), and *Tetrastigma hemsleyanum* (Peng et al., 2015).

CONCLUSIONS

Low percentage of fungal contamination and other events (i.e., bacterial contamination and/or tissue oxidation) for the shoot collection denote the potential of using A2 environment (i.e., clonal plants grown in vessel suspended in a greenhouse, with irrigation performed directly on the substrate) for the *in vitro* establishment of *B. vulgaris* nodal segments. Seasonality influences the *in vitro* establishment of nodal segments. *Ex vitro* rooting percentage was 36.6%, and no interactions were observed between the use of culture media and antibiotic. MS culture medium antibiotic free resulted in the better survival percentage (80.0%) and adventitious rooting (50.0%). Micropropagated plants have genetic fidelity in relation to the selected adult plant.

Histological analyses revealed normal adventitious root formation originating from meristematic cells. The formation of a clonal micro-garden of *B. vulgaris* was established and can be used for the propagation of selected adult plants.

ACKNOWLEDGMENTS

The authors thank the National Counsel of Technological and Scientific Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, 471370/2013-4, 305938/2016-9, 405025/2018-1, 304058/2019-0), Coordination for Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Finance Code 001), and Foundation for Research of the State of Minas Gerais (Fundação de Amparo a Pesquisa do Estado de Minas Gerais – FAPEMIG, APQ-00273-16). They also thank Dra. Ana Luiza de Oliveira Timbó for the laboratory support.

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SUPPLEMENT TABLE

Tab. 3 Results of amplifications per primer for *Bambusa vulgaris*.

Primer	Total of bands	Monomorphic	Polymorphic
John	4	4	0
Manny	3	3	0
Mao	5	5	0
Omar	3	3	0
Chris	4	4	0
Becky	0	0	0
UBC809	3	3	0
UBC827	4	4	0
UBC835	0	0	0
UBC840	3	3	0
UBC842	4	4	0
UBC848	6	6	0
UBC898	0	0	0