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In vitro cloning of *Bambusa vulgaris* Schrad. ex J. C. Wendl.: Effect of culture systems, sucrose and activated charcoal supplementation

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ABSTRACT

Fast-growing forest species with multiple uses, like bamboo, have aroused interest for their silvicultural applications. Bamboo species are a valuable source of renewable raw material, and *Bambusa vulgaris* is an economically important species. However, there are limitations to large-scale cloning of adult-selected genotypes. This study aimed to evaluate the *in vitro* cloning of *Bambusa vulgaris* in different culture systems, sucrose and activated charcoal supplementation by the inter-simple-sequence repeat (ISSR) molecular markers. *In vitro* bud multiplication and shoot elongation were evaluated in three cultivation systems: semi-solid and liquid culture media, and temporary immersion bioreactor (TIB). The sucrose concentrations, 0 and 30 g L^{-1} were evaluated in the stages. Both the culture media were supplemented with 2.0 mg L^{-1} benzylaminopurine (BAP) and 0.5 mg L^{-1} α-naphthalene acetic acid (NAA). The absence and presence of activated charcoal (100 mg L^{-1}) were evaluated in the *in vitro* rooting. MS culture medium was supplemented with 2 mg L^{-1} indole-3-butyric acid (IBA), 1.0 mg L^{-1} NAA, and 0.5 mg L^{-1} BAP. Semi-solid culture medium supplemented with 30 g L^{-1} of sucrose presented superior emission of bud per explant. Liquid culture medium supplemented with 30 g L^{-1} of sucrose presented the most elongated shoots. Activated charcoal in the culture medium did not influence the adventitious rooting. Micropropagated plants showed genetic fidelity and were clones of the adult selected plant.

1. Introduction

The forestry sector is undergoing intense technological development. The main reason for this phenomenon is the high demand for forest products and by-products, due mainly to population growth and the increased use of renewable energy sources. Consequently, there is a need to expand the production of wood and its derivatives, thus intensifying the importance of increasing crop yield to supply the market (Moreira, 2011; Oliveira et al., 2019; Pereira et al., 2019). Fast-growing species with multiple uses are increasingly pursued to meet this demand. One such alternative to traditional world forest species is bamboo, which can serve as a wood substitute given that it contributes to the supply of forest resources and favors various ecological activities (Li et al., 2015).

There is a great diversity of bamboos in the world, and *Bambusa vulgaris* Schrad. ex J. C. Wendl. is considered an economically important species which can be used for various agronomic and ecological applications (INBAR, 2017). Bamboo is grown for many finalities, including reforestation, soil recovery, environmental protection, home construction, furniture making, the fabrication of agricultural implements, the use in handicrafts, and pulp production for papermaking (Cairo et al., 2018; Furlan et al., 2018; García-Ramírez et al., 2014; Ribeiro et al., 2020). However, bamboo propagation protocols have some shortcomings (Nogueira et al., 2019), and more research is

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needed to improve the production techniques of clonal plants for commercial applications. To overcome this problem, *in vitro* propagation can be adopted as a biotechnological tool for the production of clonal plants (Santos et al., 2019; Teixeira et al., 2021). The development of specific protocols for each species is essential: Studies on micropropagation show the use of different *in vitro* culture conditions. The bioreactors, and liquid media can optimize the process and reduce production costs, indicating the need for further research in this field of study (García-Ramírez et al., 2014; Moreira et al., 2013; Nogueira et al., 2017; Ribeiro et al., 2016; Teixeira et al., 2021; Mirzabe et al., 2022).

Genetic changes, defined as somaclonal variations, may occur in some growing conditions in the micropropagation (George et al., 2008). Somaclonal variations are considered disadvantageous when the main objective is to obtain clones (Konzen et al., 2021a). In this context, the micropropagation technique can be validated using molecular tools to evaluate plants' genetic fidelity *in vitro* (Konzen et al., 2017).

Our aim was to evaluate the cloning of *Bambusa vulgaris* in different *in vitro* culture systems, sucrose and activated charcoal supplementation.

2. Material and methods

2.1. General description and source of propagules

Shoots were collected from *Bambusa vulgaris* Schrad. ex J. C. Wendl. adult-selected plants (*i.e.*, three 12 year-old cloned plants from a single individual), in Lavras, Minas Gerais, Brazil (21°13′29.16″ S; 44°58′1.87″ W). The plants that served as a source of shoots were grown in 4-L pots containing washed sand and subsoil (1:1, v:v) as substrate and were placed in a greenhouse (Teixeira et al., 2021). Selected plants were fertigated weekly with a nutrient solution developed for growth and the emission of new shoots (Souza et al., 2022). Irrigation was performed daily, directly on the substrate, avoiding contact between water and the aerial part of the plants.

After shoot collection, the sheaths were removed, and their remnants were carefully scraped with a scalpel to expose the shoots (Teixeira et al., 2021). The material was then washed with deionized and autoclaved water and neutral detergent. Then, the shoots were cut down to 2-cm-long nodal explants and washed for 30 s in 70% hydroalcoholic solution, followed by a deionized and sterilized water wash. The explants were immersed in a sodium hypochlorite (NaOCl) solution (water:sodium hypochlorite, v:v, 1.00–1.25% active chlorine) for 10 min. At the end of the aseptic treatment, the explants were inoculated vertically in test tubes containing 10 mL of MS culture medium (Murashige and Skoog, 1962), which were sealed with polyvinyl chloride plastic film. The test tubes (20 × 100 mm) were placed in a growth room at 24 °C (± 1 °C) with 40 μmol m^{-2} s^{-1} irradiation and a 16-hour photoperiod.

2.2. In vitro multiplication and elongation

Nodal explants considered established were arranged into three different culture systems: (SSM) semi-solid medium in glass flasks (72 × 72 × 100 mm) with 50 mL of MS culture medium (Fig. 1A); (LM) liquid-medium in test tubes (25 × 150 mm) with 15 mL of stationary MS culture medium (Fig. 1B); and (TIB) temporary immersion bioreactor with 150 mL of MS culture medium, with an immersion sequence of 1 min every 3 h (Fig. 1C). Sucrose was added to all media in different concentrations (0 and 30 g L^{-1}).

Culture media were supplemented with 2.0 mg L^{-1} of benzylaminopurine (BAP) and 0.5 mg L^{-1} of α-naphthalene acetic acid (NAA) to induce bud multiplication and accelerate the production of viable shoots for *in vitro* rooting. The explants were placed in a growth room at 24 °C (± 1 °C), 40 μmol m^{-2} s^{-1} irradiation, and 16-hour photoperiod. The nutrient solution was renewed, and subcultures were performed every four weeks. Survival, number of buds, length of shoots, and vigour were evaluated at 60 days. Tissue vigour was scored on a scale from 1 to 4 (Fig. 2A).

The experiment was conducted in a completely randomized factorial design (3 × 2), testing three culture systems (SSM: semi-solid medium, LM: liquid medium, and TIB: temporary immersion bioreactor) and two sucrose concentrations (0 and 30 g L^{-1}). Each treatment consisted of 15 replicates (one explant per replicate).

2.3. In vitro rooting

Explants with 5 cm of length (*i.e.*, derived from the multiplication and elongation stages) were inoculated into glass flasks (72 × 72 × 100 mm) containing 50 mL of MS semi-solid culture medium supplemented with 2 mg L^{-1} of indole-3-butyric acid (IBA), 1 mg L^{-1} NAA, and 0.5 mg L^{-1} BAP and 30 g L^{-1} of sucrose. The treatments tested consisted of two concentrations of activated charcoal (0 and 100 mg L^{-1}). The flasks were placed in a growth room at 24 °C (± 1 °C) with 40 μmol m^{-2} s^{-1} irradiation and a 16-hour photoperiod. Rooting percentage and tissue oxidation were evaluated at 60 days. Oxidation was scored from 1 to 4 (Fig. 2B).

The experiment had a completely randomized design, testing two activated charcoal concentrations (0 and 100 mg L^{-1}). Each treatment consisted of 20 replicates (one explant per replicate).

2.4. Culture medium preparation

The culture medium was prepared with deionized water and 6 g L^{-1} agar (*i.e.*, for semi-solid culture medium, only). Sucrose was added according to treatment (*i.e.*, 0 or 30 g L^{-1}). The pH was adjusted to 5.8 with HCl (0.1 M) and NaOH (0.1 M) before adding the agar to the culture medium. Then the medium was autoclaved at 127 °C (1.5 kgf cm^{-2}) for 20 min. Plant growth regulators (PGR) were added to the culture medium before autoclaving.

2.5. Genetic fidelity

Young leaves were collected from *in vitro*–rooted plants and the selected plant to evaluate the genetic fidelity. DNA was extracted according to the protocol adapted from Ferreira and Grattapaglia (1998). For genetic fidelity analysis, 20 primers were used in PCR (Table 1).

ISSR reactions were prepared in microplates (PCR-96, Axygen Scientific). Three microlitres of DNA (standardized at 20 ng μL $^{-1}$ for all samples) and 10 μL of reaction mix [1.5 mM Phoneutria® PCR buffer, 1.5 mM dNTP, 1 U of Phoneutria® *Taq* polymerase (5 U μL $^{-1}$), *Taq* diluent (based on BSA and Tris HCl), 0.2 mM of each primer, and ultrapure water to complete the volume (4.2 μL)] were added to each well. The DNAs were amplified in a GeneAmp PCR System 9700 thermal cycler, with the following steps: 2 min at 94 °C for initial denaturation; 37 cycles of 30 s at 94 °C, 30 s at 42 °C, and 1 min at 72 °C; and a final extension for 7 min at 72 °C. The amplification products were separated in 1.5% agarose gel and stained with Gel Red™ (Uniscience). The amplicons were analysed visually.

2.6. Statistical analysis

Data were analysed with R software (version 4.2.1) (R Core Team, 2022) with the ExpDes package, version 1.1.2 (Ferreira et al., 2013). The data collected from the experiments were analysed for homoscedasticity and normality of residuals using the Hartley ($p > 0.05$) and Shapiro-Wilk tests ($p > 0.05$), respectively. According to the results of the Hartley and Shapiro-Wilk tests, the data were Box-Cox transformed. The data were then subjected to analysis of variance (ANOVA, $p < 0.05$), and the means were compared by Tukey's test ($p < 0.05$). Principal component analysis (PCA) was performed with the R software (R Core Team, 2022), using the "factoextra" R package (version 1.0.7) (Kassambara and Mundt, 2020).

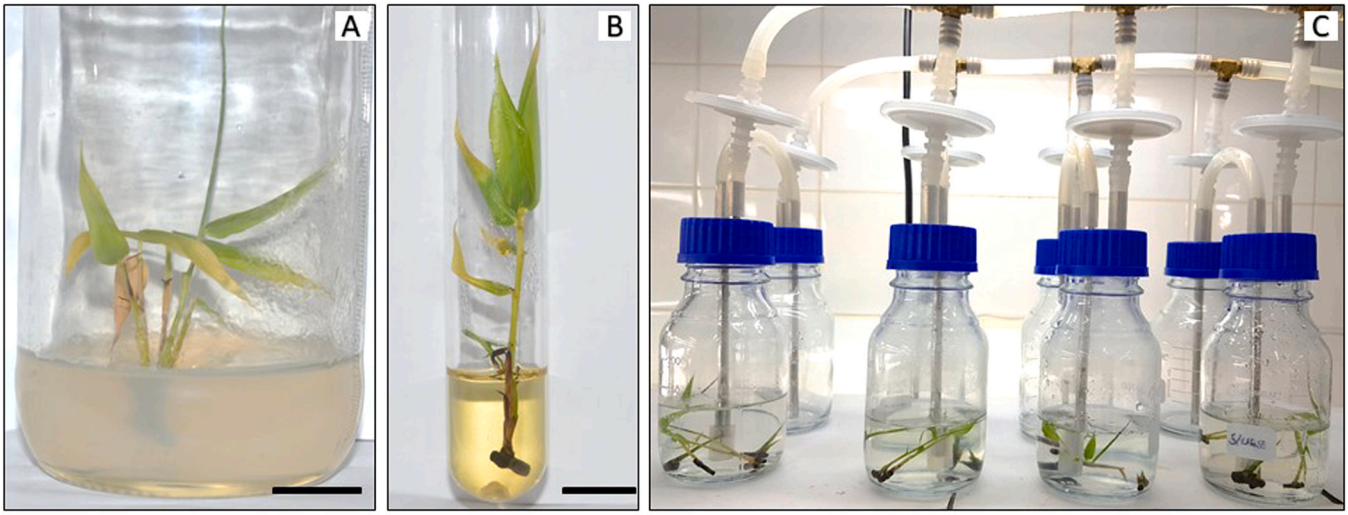

Fig. 1. Culture systems tested in the *in vitro* multiplication and elongation stages of *Bambusa vulgaris*. (A) SSM: semi-solid medium, (B) LM: liquid medium, and (C) TIB: temporary immersion bioreactor. Bar = 1.0 cm.

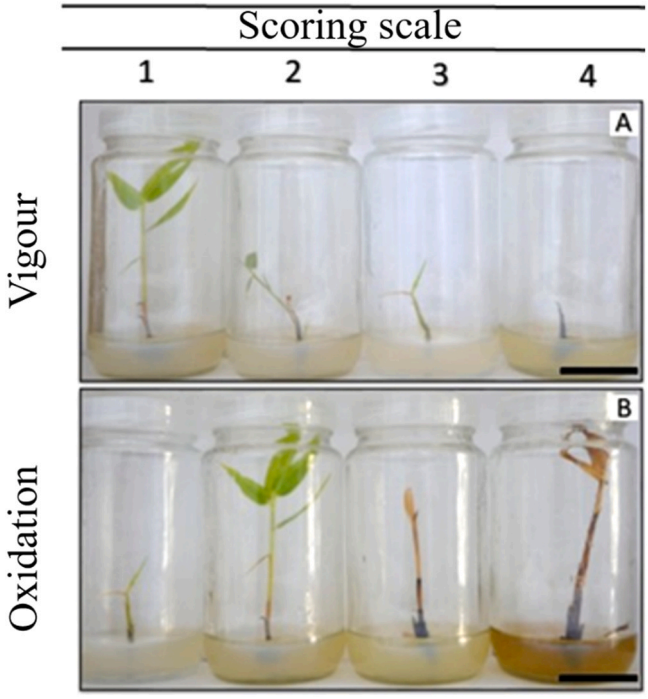

Fig. 2. Tissue vigour and oxidation evaluations of *Bambusa vulgaris* according to the scoring scale. (A) Vigour: 1 = Excellent: induction of bud and shoot with active growth, without apparent nutritional deficiency; 2 = Good: induction of bud and shoot but with reduced growth; 3 = Regular: small bud and shoot development and/or apparent nutritional deficiency; 4 = Poor: senescence and death of the explant; (B) Oxidation: 1 = Null: no oxidation; 2 = Low: reduced oxidation of the explant base; 3 = Intermediate: oxidation and color change in the culture medium; 4 = High: complete shoot oxidation (*i.e.*, darkened of the culture medium). Bars = 2.0 cm.

Table 1
Description of ISSR primers used in the genetic fidelity analysis of *Bambusa vulgaris in vitro*–rooted plants and selected plant.

Primer	Sequence
Becky	$(CA)_7$-YC
Chris	$(CA)_7$-YG
John	$(AG)_7$-YC
Manny	$(CAC)_4$-RC
Mao	$(CTC)_4$-RC
Omar	$(GAG)_4$-RC
UBC809	$(AG)_8$-G
UBC810	$(GA)_8$-T
UBC813	$(CT)_8$-T
UBC814	$(CT)_8$-TG
UBC825	$(AC)_8$-T
UBC827	$(AC)_8$-G
UBC834	$(AG)_8$-YT
UBC835	$(AG)_8$-YC
UBC840	$(GA)_8$-YT
UBC842	$(GA)_8$-YG
UBC848	$(CA)_8$-RG
UBC857	$(AC)_8$-YG
UBC898	$(CA)_6$-RY
UBC901	$(GT)_6$-YR

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

3. Results

3.1. In vitro multiplication and elongation

There was no interaction between culture systems and sucrose concentration (*i.e.*, factors) for survival (Fig. 3A-B). However, there was interaction for the factors when was considered the number of buds per explant (Fig. 3C) and shoot length (Fig. 3D).

In vitro multiplication and elongation of the *B. vulgaris* co-occurred. In this stage, the sucrose concentration presented a significant effect on survival, with the highest mean survival (86.6%) in the concentration of 30 g L^{-1}. In the absence of sucrose, there was only 20% of survival (Fig. 3A).

The culture system adopted did not affect the survival 60 days after the inoculation. The highest mean survival occurred under the semi-solid medium (65%), though it did not differ from that under the liquid medium (40%) or temporary immersion bioreactor (55%) (Fig. 3B). Thus, regardless of the culture system, *B. vulgaris* depends highly of the sucrose for its *in vitro* survival. Semi-solid culture medium presented 4.1 buds per explant at a concentration of 30 g L^{-1} sucrose (Fig. 3C) and was superior to the other treatments.

The length of elongated shoots differed according to the adopted cultivation system: The longest mean lengths were observed when the explants were subjected to the SSM and LM systems, at 7.9 cm and 10.8 cm, respectively, both at a concentration of 30 g L^{-1} sucrose (Fig. 3D). The most significant difference in shoot length occurred when the explants were supplemented with carbohydrate (30 g L^{-1}), the

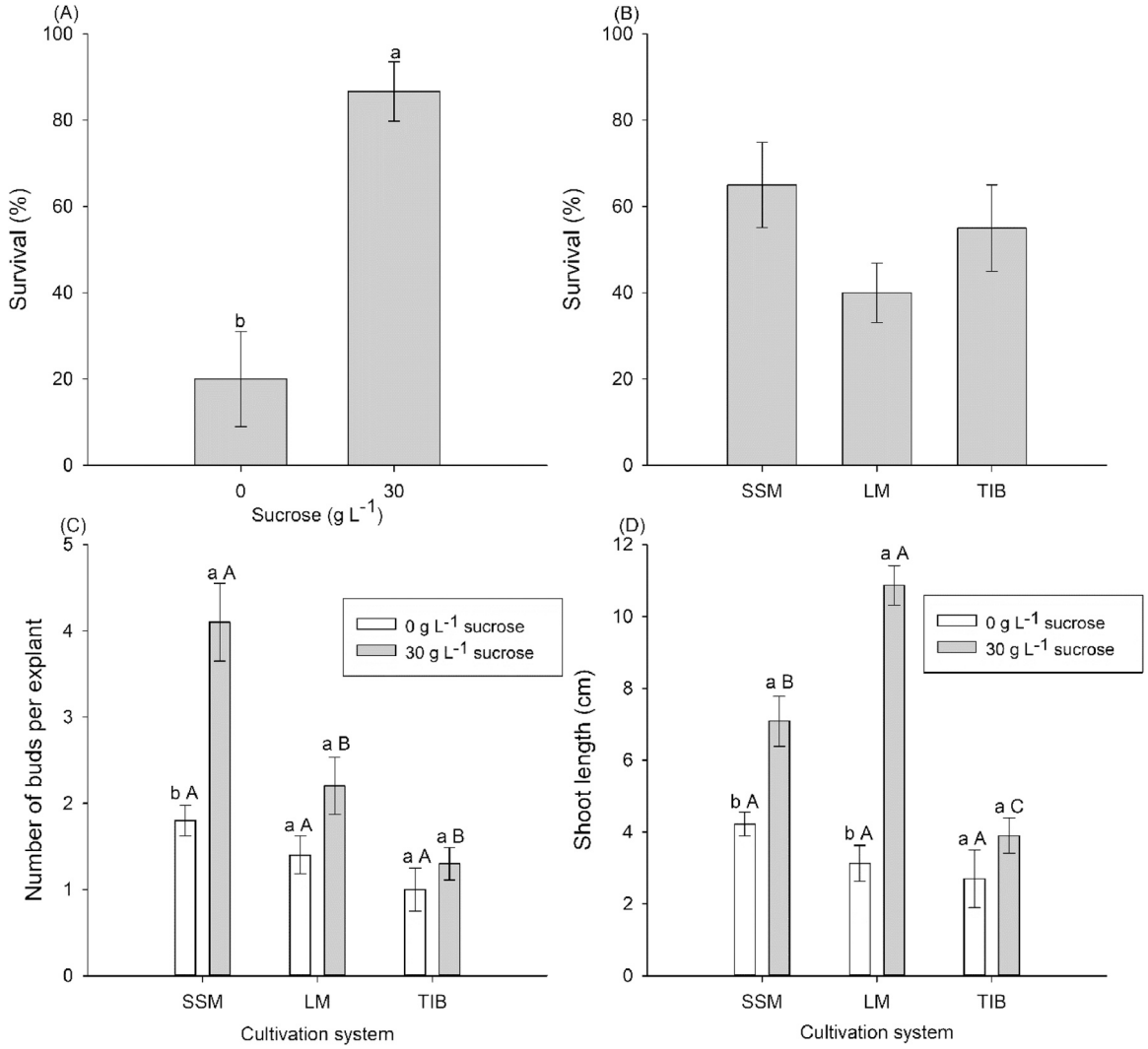

Fig. 3. Characteristics that were evaluated in the *in vitro* multiplication and elongation of *Bambusa vulgaris* in the different culture systems (SSM: semi-solid medium, LM: liquid medium, and TIB: temporary immersion bioreactor) and sucrose (0 and 30 g L^{-1}). (A) Survival according to sucrose concentration; (B) Survival according to culture system; (C) Number of buds per explant according to culture system and sucrose; (D) Shoot length according to culture system and sucrose. In (A), means followed by the same letter do not differ significantly by Tukey's test ($p < 0.05$). In (C) and (D), means followed by the same lowercase letter do not differ significantly between culture systems, and means followed by the same uppercase letter do not differ significantly between sucrose concentration by Tukey's test ($p < 0.05$). Data are mean ± standard error.

highest mean being observed in the LM (10.8 cm). These findings suggest that the LM system with sucrose supplementation is the most suitable for the shoot elongation of *B. vulgaris* under *in vitro* conditions, showing a strong dependence on the addition of carbohydrate to the culture medium.

Principal component analysis (PC1 and PC2) revealed that the SSM and TIB systems supplemented with 30 g L^{-1} of sucrose were the best treatments for vigour in the *in vitro* multiplication and elongation stages, given that the highest frequency of scores 1 and 2 (*i.e.*, accumulated value of 80%) (Fig. 4). LM supplemented with sucrose negatively correlated with vigour (Fig. 4). According to the scoring scale for vigour (Fig. 2A), the highest frequencies were observed for score 4 in all three systems without sucrose supplementation (SSM: 70%; LM: 80%; TIB: 80%) (Fig. 4).

3.2. In vitro rooting

There was no difference in adventitious rooting at 60 days after inoculation, according to the presence or absence of activated charcoal in the culture medium. The presence of adventitious roots was observed in the presence and absence of activated charcoal (Fig. 5A-C) in the culture medium, with 58.3% and 50.0% rooting, respectively. Therefore, using activated charcoal does not favour adventitious root induction or its emission in *B. vulgaris*.

According to PCA of the oxidation scoring scale (Fig. 2B), 80% of the explants cultivated in the culture medium with activated charcoal presented a score of 1; that is, they did not present tissue oxidation or a culture medium colour change (Fig. 6). In contrast, 60% of the explants subjected to the culture medium without activated charcoal had a score of 3, showing a high correlation (Fig. 6). Thus, to obtain *B. vulgaris* plants free of oxidation in the adventitious rooting stage, supplementation of 100 mg L^{-1} of activated charcoal to the culture medium is a viable procedure.

3.3. Genetic fidelity

In vitro–rooted plants were clones of the *B. vulgaris* selected plant. Evaluating genetic fidelity with 20 primers, 15 exhibited adequate characteristics (Fig. 7), with adequate amplification and discernible bands that were taken into consideration to determine genetic fidelity.

The 15 primers' results that showed amplification identified monomorphism in all bands for all individuals (Table 2). For identifying the

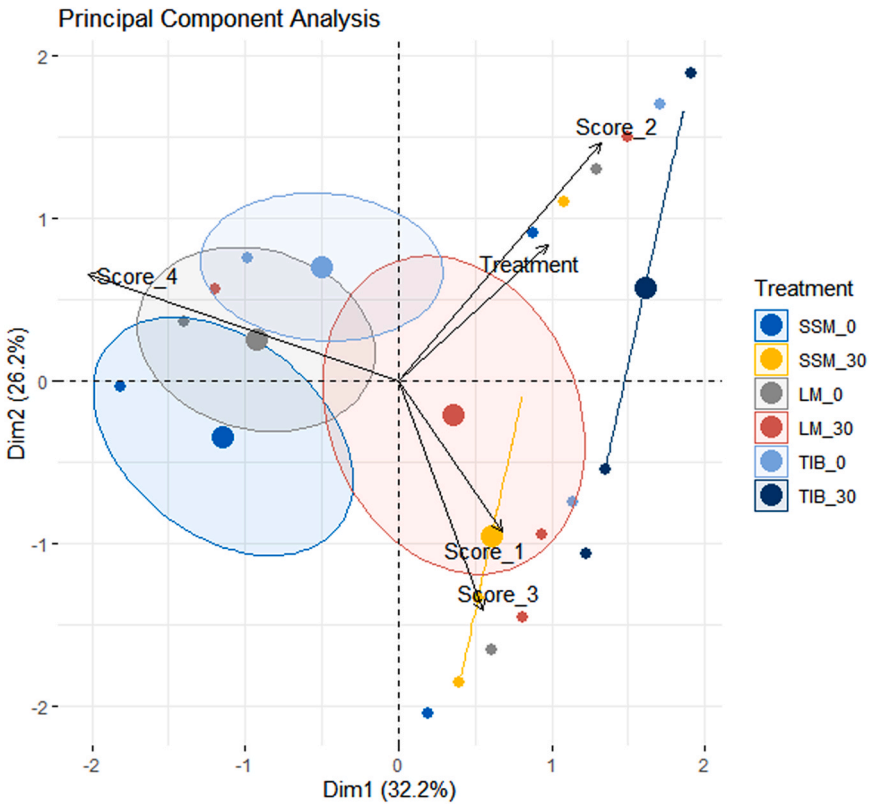

Fig. 4. Principal component analysis (PCA) of frequency of vigour based on the scoring scale of *Bambusa vulgaris* (Fig. 2A) in different culture systems and sucrose supplementation at 60 days. SSM_0 = semi-solid medium in the absence of sucrose, SSM_30 = semi-solid medium with supplementation of 30 g L^{-1} of sucrose, LM_0 = liquid medium in the absence of sucrose, LM_30 = liquid medium with supplementation of 30 g L^{-1} of sucrose, TIB_0 = temporary immersion bioreactor in the absence of sucrose, and TIB_30 = temporary immersion bioreactor with supplementation of 30 g L^{-1} of sucrose. Vigour: Score_1 = Excellent: induction of bud and shoot with active growth, without apparent nutritional deficiency; Score_2 = Good: induction of bud and shoot but with reduced growth; Score_3 = Regular: small bud and shoot development and/or apparent nutritional deficiency; Score_4 = Poor: senescence and death of the explant. Dim1 = Principal component 1 (PC1), Dim2 = Principal component 2 (PC2).

bands, strong intensity and clear separation from other bands were used as criteria. The absence of polymorphisms confirmed that there was no somaclonal variation.

4. Discussion

4.1. *In vitro* multiplication and elongation

Sucrose concentrations utilized in the culture medium for the *in vitro* multiplication and elongation of *B. vulgaris* affected the survival of the explants. There was no relationship between the culture system type and species survival.

Bambusa vulgaris demonstrated a strong dependence on carbohydrates for the maintenance of survival and success in multiplication. The absence of sucrose in the adopted culture can result in higher mortality of explants cultivated *in vitro* (Ribeiro et al., 2016). The plants do not have ideal CO_2 and O_2 concentrations for their development on *in vitro* culture, then, they may become partially heterotrophic. Thus, plants need an exogenous carbohydrate source for photosynthesis, sustain growth, and meet their metabolic needs. It means that the absence of an external energy source that benefits the biosynthesis of structural and functional components in the culture medium can cause explant mortality (George et al., 2008).

The mean buds per explant were higher with the semi-solid culture medium supplemented with 30 g L^{-1} sucrose. For the length of elongated shoots, the liquid medium with 30 g L^{-1} sucrose provided the best results. Using a liquid culture medium or temporary immersion bioreactor showed more satisfactory results for *in vitro* multiplication and elongation in *B. vulgaris* (Ribeiro et al., 2016), similarly found for shoot length.

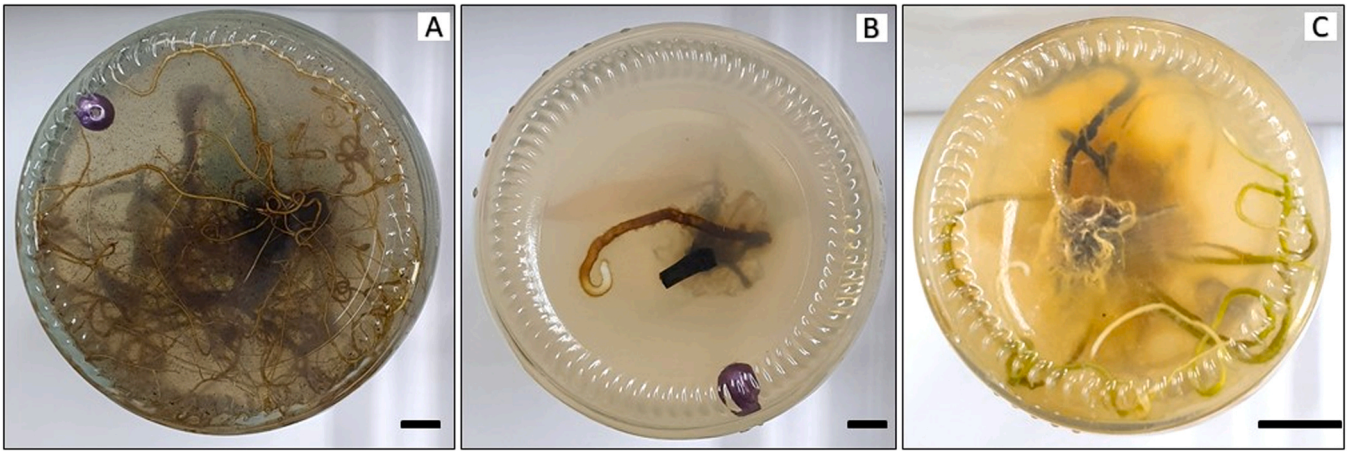

Fig. 5. *In vitro* emission of adventitious roots in *Bambusa vulgaris* explants. Legend: (A) rooting in culture medium with the addition of activated charcoal; (B) and (C) rooting in culture medium without the addition of activated charcoal. Bar = 1.0 cm.

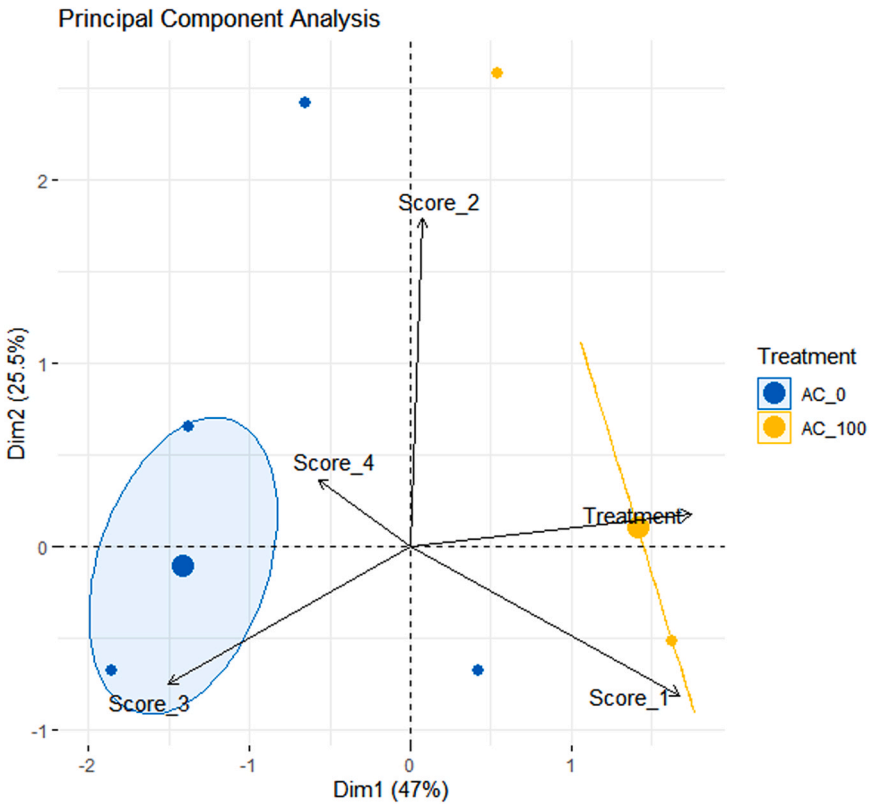

Fig. 6. Principal component analysis (PCA) of frequency of tissue oxidation based on the scoring scale of *Bambusa vulgaris* (Fig. 2A) in different activated charcoal at 60 days. AC_0 = absence of activated charcoal in culture medium, AC_100 = supplementation of 100 mg L^{-1} of activated charcoal in culture medium. Oxidation: Score_1 = Null: no oxidation; Score_2 = Low: reduced oxidation of the explant base; Score_3 = Intermediate: oxidation and color change in the culture medium; Score_4 = High: complete shoot oxidation (*i.e.*, darkened of the culture medium). Dim1 = Principal component 1 (PC1), Dim2 = Principal component 2 (PC2).

Highest mean elongated shoot length came about when the explants were grown in the culture system with a liquid medium. The more significant contact between the explant surface and the culture medium, the higher the growth is due to increased absorption of water and nutrients, given that the liquid medium offers uniform culture conditions (Nogueira et al., 2017).

During the *in vitro* culture, a critical issue is the physical state of the culture medium (*i.e.*, semi-solid or liquid). It may directly affect the *in vitro* development of explants due to differences in contact between the plants and the culture medium. Thus, it is necessary to define the composition and type of culture medium used since the nutritional requirements necessary for the growth and development of tissues depend on several factors, especially the species, genotype, and explants (Greenway et al., 2012; Mengarda et al., 2009; Mirzabe et al., 2022).

The adoption of different culture media and different systems have been described for the *in vitro* culture of several bamboo species, such as *Bambusa vulgaris* (García-Ramírez et al., 2014; García-Ramírez et al., 2019; Ribeiro et al., 2016), *Guadua angustifolia* (Gutiérrez et al., 2016), *Guadua longifimbriata*, and *Guadua angustifolia* (Costa et al., 2017), reinforcing the importance and the need for more in-depth studies to define micropropagation protocols for commercial applications of bamboo species.

Highest frequency of explants with score 1 of vigour response was observed in the SSM and TIB systems supplemented with 30 g L^{-1} sucrose (Fig. 4). The most frequent score was 4 in the semi-solid (70%), liquid (80%), and bioreactor (80%) cultivation systems without the

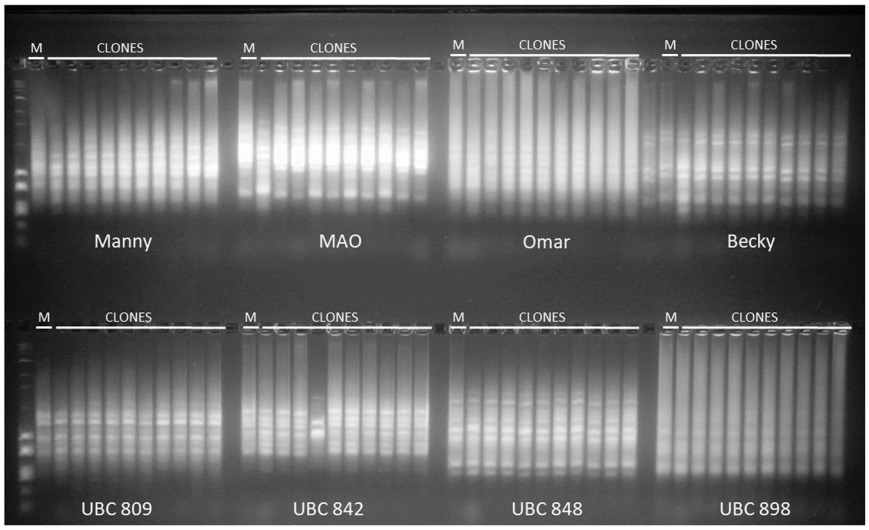

Fig. 7. Gel obtained using the ISSR primers Manny, MAO, Omar, Becky, UBC809, UBC842, UBC848, and UBC898 for *Bambusa vulgaris*. M: Selected plant, CLONES: *In vitro*–rooted plant.

Table 2
Results of primer amplification of *Bambusa vulgaris*.

Primer	Total amplicons	Monomorphic	Polymorphic
Becky	5	5	0
Chris	3	3	0
John	4	4	0
Manny	6	6	0
MAO	4	4	0
Omar	5	5	0
UBC809	4	4	0
UBC810	3	3	0
UBC813	0	0	0
UBC814	0	0	0
UBC825	5	5	0
UBC827	0	0	0
UBC834	0	0	0
UBC835	4	4	0
UBC840	5	5	0
UBC842	4	4	0
UBC848	4	4	0
UBC857	3	3	0
UBC898	3	3	0
UBC901	0	0	0

sucrose. Thus, the low vigour observed in *B. vulgaris* in the present study was probably caused by the lack of sucrose in the culture medium. The concentration and type of carbohydrates used in the culture medium are extremely important factors in the *in vitro* culture of plants as the source of energy to shoot and root morphogenesis (Carvalho et al., 2013; Silva et al., 2016). Thus, there is a need for carbohydrate supplementation in the culture medium to favour rapid *in vitro* growth and development of *B. vulgaris*.

4.2. In vitro rooting

The supplementation of active charcoal did not influence the *in vitro* rooting of *B. vulgaris*, and root formation was observed both in the absence and in the presence of charcoal in the culture medium. The rooting of bamboo shoots under *in vitro* culture is a key step for successful micropropagation (Sandhu et al., 2018; Konzen et al., 2021b), but bamboo species do not root easily when *in vitro* grown (Mudoi et al., 2013). This may occur because rhizogenesis is influenced by several factors, such as the concentrations of PGRs, luminosity, nutritional status, and genetic aspects of each individual (Geiss et al., 2009).

The finding that activated charcoal did not influence rooting may be related to the PGRs that were added at the same concentrations in all evaluated treatments, corroborating the observations of Ribeiro et al. (2020). According to Sandhu et al. (2018), bamboo rooting varies between species and is highly specific to each genotype. Therefore, this response may be attributed to endogenous hormone levels and to each explant's different exogenous PGR requirements. Activated charcoal has the physical ability to simulate dark conditions, under which roots typically develop better, and it has the chemical ability to retain some of the elements that make up the culture medium, acting as an adsorbent. However, the use of activated charcoal does not always have advantages, given that it can act both as a growth promoter and as an inhibitor; it can adsorb key nutrients for growth and release them gradually, and it can adsorb toxic compounds (Leitzke et al., 2009; Villa et al., 2014; Ribeiro et al., 2020).

There was a beneficial effect of adding activated charcoal to the culture medium on oxidation in the rooting (Fig. 5). The oxidation process is usually associated with tissue healing mechanisms, which cause the exudation of phenolic compounds and is determined by the darkening of the explants and culture medium (Mudoi et al., 2013; Van Winkle et al., 2003). Phenolic oxidation is often reported in *in vitro*–cultivated bamboo species, such as *Bambusa vulgaris* (Brondani et al., 2017; Furlan et al., 2018), *Dendrocalamus asper* (Brondani et al., 2017), *Guadua longifimbriata*, and *Guadua angustifolia* (Costa et al., 2017). Thus, the results obtained in the present study support the indication of activated charcoal to solve such problems in bamboo species. However, according to Furlan et al. (2018), further research is needed to effectively evaluate the adventitious rooting ability of *B. vulgaris* explants, given that protocols for this purpose are few.

4.3. Genetic fidelity

Plants showed no genetic variation from the propagule donor selected plant, even when subjected to PGRs, different cultivation systems, and eight subcultures (120 days). They are therefore considered authentic clones.

Commercial sectors focused on bamboo species have gained prominence in recent years, and everything indicates a promising future. In this context the establishment of technologies focused on the micropropagation of mature tissues free of somaclonal variation is a challenge (Singh et al., 2012; Singh et al., 2013; Konzen et al., 2021a). The possibility of genetic variation cannot be ruled out, so morphological, physiological, and molecular evaluations are fundamental to the commercial application of propagation technologies (Negi and Saxena, 2011). The occurrence of mutations cannot be predicted, and it seems that several factors can alter the frequency and nature of the somaclonal variation, such as the explant donor genotype, the culture conditions, the explant type, the culture medium, the use of PGRs, and the stress caused by *in vitro* culture (Venkatachalam et al., 2007). In general, somaclonal variation is a phenotypic variation that may be of genetic origin, that is, a chromosomal variation that becomes heritable in subsequent generations or of epigenetic origin, that is, a transient variation due to the physiological stress that the plant material is subjected to *in vitro* culture (Bairu et al., 2011; Wang and Wang, 2012). The genetic fidelity of micropropagated plants is commonly addressed in studies and is essential for understanding the factors involved in bamboo propagation (Konzen et al., 2017). In most cases, it is determined using DNA-based molecular markers.

ISSR markers applied for these purposes in bamboo species (*i.e.*, confirmation of genetic fidelity) were reported in *Bambusa balcooa* (Negi and Saxena, 2010), *Bambusa bambos* (Anand et al., 2013), *Bambusa vulgaris* (Teixeira et al., 2021), and *Guadua magna* and *Guadua angustifolia* (Nogueira et al., 2019). In these studies, no genetic variation in plants grown *in vitro* was observed in any of the culture systems evaluated, demonstrating the efficiency of clone production.

5. Conclusions

Semi-solid culture medium supplemented with 30 g L^{-1} of sucrose presented the best results for bud multiplication.

Liquid culture medium supplemented with 30 g L^{-1} of sucrose presented the best results for shoot elongation.

Semi-solid culture medium and TIB presented the best results for vigour.

Activated charcoal supplemented in the culture medium did not affect adventitious root formation, but reduced tissue oxidation.

Micropropagated plants showed genetic fidelity and were clones of the selected plant.

CRediT authorship contribution statement

DSG conducting the experiment, statistical analysis, writing, and discussion. DMSCS statistical analysis, writing and discussion. DC review, genetic stability analysis, and interpretation of results. GLT and LSO review and interpretation of results. GEB supervisor, was responsible for the obtention of funds and the administration of project, review and discussion. All authors revised the manuscript and approved the final version.

Data Availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no conflict of interest and this manuscript was not submitted in another journal.

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References

Anand, M., Brar, J., Sood, A., 2013. *In vitro* propagation of an edible bamboo *Bambusa bambos* and assessment of clonal fidelity through molecular markers. J. Med. Bioeng. 2, 257–261. https://doi.org/10.12720/jomb.2.4.257-261

Bairu, M.W., Aremu, A.O., Van Staden, J., 2011. Somaclonal variation in plants: causes and detection methods. Plant Growth Regul. 63, 147–173. https://doi.org/10.1007/s10725-010-9554-x

Brondani, G.E., Oliveira, L.S., Furlan, F.C., Ribeiro, A.S., 2017. Estabelecimento *in vitro* de *Bambusa vulgaris* Schrad. ex JC Wendl e *Dendrocalamus asper* (Schult. et Schult. F.) Backer ex K. Heyne. In: Drumond, P.M., Wiedman, G. (Eds.), Bambus no Brasil: da biologia à tecnologia. ICH, Rio de Janeiro, Brasil, pp. 86–102.

Cairo, P.C., Alonso, O.A., Yera, Y.Y., Urrutia, A.R., Mollineda, Á., Artiles, P.T., López, O.R., 2018. La biomasa de *Bambusa vulgaris* como alternativa para la recuperación de suelos degradados. Rev. Centro Agríc. 45, 51–58.

Carvalho, D.C., Silva, A.L.L., Schuck, M.R., Purcino, M., Tanno, G.N., Biasi, L.A., 2013. Fox grape cv. Bordô (*Vitis labrusca* L.) and grapevine cv. Chardonnay (*Vitis vinifera* L.) cultivated *in vitro* under different carbohydrates, amino acids and 6-benzylamino-purine levels. Braz. Arch. Biol. Technol. 56, 191–201. https://doi.org/10.1590/S1516-89132013000200004

Costa, F.A., Marques, A.A., Rondon, J.N., Cereda, M.P., 2017. Protocolo para micropropagação de duas espécies de *Guadua*. In: Drumond, P.M., Wiedman, G. (Eds.), ((Org.).). Bambus no Brasil: da biologia à tecnologia. ICH, Rio de Janeiro, Brasil, pp. 71–85.

Ferreira, E.B., Cavalcanti, P.P., Nogueira, D.A., 2013. ExpDes: Experimental Designs package. R package version 1.1.2.

Ferreira, M.E., Grattapaglia, D., 1998. Introdução ao uso de marcadores moleculares em análise genética. 2 ed. Brasília, Brasil: Embrapa-Cenargen.

Furlan, F.C., Gavilan, N.H., Zorz, A.Z., Oliveira, L.S., Konzen, E.R., Brondani, G.E., 2018. Active chlorine and charcoal affect the *in vitro* culture of *Bambusa vulgaris*. Bosque 39, 61–70. https://doi.org/10.4067/S0717-92002018000100061

García-Ramírez, Y., Barrera, G.P., Freire-Seijo, M., Barbón, R., Concepción-Hernández, M., Mendoza-Rodríguez, M.F., Torres-García, S., 2019. Effect of sucrose on physiological and biochemical changes of proliferated shoots of *Bambusa vulgaris* Schrad. ex Wendl in temporary immersion. Plant Cell Tiss. Organ Cult. 137, 239–247. https://doi.org/10.1007/s11240-019-01564-z

García-Ramírez, Y., Gonzáles, M.G., Mendoza, E.Q., Seijo, M.F., Cárdenas, M.L.O., Moreno-Bermúdez, L.J., Ribalta, O.H., 2014. Effect of BA treatments on morphology and physiology of proliferated shoots of *Bambusa vulgaris* Schrad. Ex Wendl in temporary immersion. Am. J. Plant Sci. 5, 205–211. https://doi.org/10.4236/ajps.2014.52027

Geiss, G., Gutierrez, L., Bellini, C., 2009. Adventitious root formation: new insights and perspectives. In: Beeckman, T. (Ed.), Annual Plant Reviews-root Development. Wiley-Blackwell, Hoboken, NJ, USA, pp. 127–156. https://doi.org/10.1002/9781119312994.apr0400

George, E.F., Hall, M.A., Klerk, G.D., 2008. Plant Propagation by Tissue Culture. Springer, Dordrecht, pp. 2008.

Greenway, M.B., Phillips, I.C., Lloyd, M.N., Hubstenberger, J.F., Phillips, G.C., 2012. A nutrient medium for diverse applications and tissue growth of plant species *in vitro*. In Vitro Cell. Dev. Biol. - Plant 48, 403–410. https://doi.org/10.1007/s11627-012-9452-1

Gutiérrez, L.G., López-Franco, R., Morales-Pinzón, T., 2016. Micropropagation of *Guadua angustifolia* Kunth (Poaceae) using a temporary immersion system RITA®. Afr. J. Biotechnol. 15, 1503–1510. https://doi.org/10.5897/AJB2016.15390

INBAR. INTERNATIONAL NETWORK FOR BAMBOO AND RATTAN., 2017. Brazil becomes the latest member of INBAR. Beijing. Disponível em: < 〈http://www.inbar.int/brazil_joins_inbar〉 > . Access: 10 May 2020.

Kassambara, A., Mundt, F., 2020. *Package* 'factoextra'. Version 1.0.7. 84 pp. < 〈https://cloud.r-project.org/web/packages/factoextra/index.html〉 > Access: 10 Mar. 2022.

Konzen, E.R., Perón, R., Ito, M.A., Brondani, G.E., Tsai, S.M., 2017. Molecular identification of bamboo general and species based on RAPD-RFLP markers. Silva Fenn. 51, 1691. https://doi.org/10.14214/sf.1691

Konzen, E.R., Pozzobon, L.C., Souza, D.M.S.C., Fernandes, S.B., Campos, W.F., Brondani, G.E., Carvalho, D., Tsai, S.M., 2021b. Molecular markers in bamboos: understanding reproductive biology, genetic structure, interspecies diversity, and clonal fidelity for conservation and breeding. In: Ahmad, Z., Ding, Y., Shahzad, A. (Eds.), Biotechnological Advances in Bamboo. Springer Singapore, Singapore, pp. 33–62. https://doi.org/10.1007/978-981-16-1310-4_2

Konzen, E.R., Souza, D.M.S.C., Fernandes, S.B., Brondani, G.E., Carvalho, D., Campos, W.F., 2021a. Management of bamboo genetic resources and clonal production systems. In: Ahmad, Z., Ding, Y., Shahzad, A. (Eds.), Biotechnological Advances in Bamboo. Springer Singapore, Singapore, pp. 207–228. https://doi.org/10.1007/978-981-16-1310-4_9

Leitzke, L.N., Damiani, C.R., Schuch, M.W., 2009. Multiplicação e enraizamento *in vitro* de amoreira-preta 'Xavante': efeito da concentração de sais, do tipo de explante e de carvão ativado no meio de cultura. Ciênc. Agrotec. 33, 1959–1966. https://doi.org/10.1590/S1413-70542009000700045

Li, P., Zhou, G., Du, H., Lu, D., Mo, L., Xu, X., Shi, Y., Zhou, Y., 2015. Current and potential carbon stocks in Moso bamboo forests in China. J. Environ. Manag. 156, 89–96. https://doi.org/10.1016/j.jenvman.2015.03.030

Mengarda, L.H.G., Povoas, L., Debiasi, C., Pescador, R., 2009. Estado físico do meio de cultura na propagação in vitro de Bromeliaceae. Sci. Agrar. Paranaensis 10 (6), 469–474.

Mirzabe, A.H., Hajiahmad, A., Fadavi, A., Rafiee, S., 2022. Temporary immersion systems (TISs): a comprehensive review. J. Biotechnol. 357, 56–83. https://doi.org/10.1016/j.jbiotec.2022.08.003

Moreira, A.L., Silva, A.B., Santos, A., Reis, C.O., Landgraf, P.R.C., 2013. *Cattleya walkeriana* growth in different micropropagation systems. Ciênc. Rural 43, 1804–1810. https://doi.org/10.1590/S0103-84782013001000012

Moreira, J.M.M.A.P., 2011. Potencial e participação das florestas na matriz energética. Pesq. Flor. Bras. 31, 363–372. https://doi.org/10.4336/2011.pfb.31.68.363

Mudoi, K.D., Saikia, S.P., Goswami, A., Gogoi, A., Bora, D., Borthakur, M., 2013. Micropropagation of important bamboos: a review. Afr. J. Biotechnol. 12, 2770–2785.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant 15, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Negi, D., Saxena, S., 2010. Ascertaining clonal fidelity of tissue culture raised plants of *Bambusa balcooa* Roxb. using inter simple sequence repeat markers. New For. 40, 1–8. https://doi.org/10.1007/s11056-009-9182-3

Negi, D., Saxena, S., 2011. *In vitro* propagation of *Bambusa nutans* Wall. ex Munro through axillary shoot proliferation. Plant Biotechnol. Rep. 5, 35–43. https://doi.org/10.1007/s11816-010-0154-z

Nogueira, J.S., Costa, F.H.S., Vale, P.A.A., Luis, Z.G., Scherwinski-Pereira, J.E., 2017. Micropropagação de bambu em larga-escala: princípios, estratégias e desafios. In: Drumond, P.M., Wiedeman, G. (Eds.), Bambus no Brasil: da biologia à tecnologia. ICH, Rio de Janeiro, Brasil, pp. 103–129.

Nogueira, J.S., Gomes, H.T., Scherwinski-Pereira, J.E., 2019. Micropropagation, plantlets production estimation and ISSR marker-based genetic fidelity analysis of *Guadua magna* and *G. angustifolia*. Pesqui. Agropecu. Trop. 49, e53743. https://doi.org/10.1590/1983-40632019v4953743

Oliveira, T.P.F., Barroso, D.G., Lamônica, K.R., Morais, T.C.B., Carvalho, G.C.M.W., 2019. Exigência nutricional e produtividade em minijardim clonal de *Toona ciliata* var. *australis*. Ciênc. Florest. 29, 1154–1167. https://doi.org/10.5902/1980509821276

Pereira, A.K.S., Longue Junior, D., Neto, C.S.M., Colodette, J.L., Gomes, F.J.B., 2019. Determinação da composição química e potencial de polpação da *madeira Pterogyne nitens* Tul. Ciênc. Florest. 29, 1490–1500. https://doi.org/10.5902/1980509831021.

R. Core Team, 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2022. < 〈https://www.r-project.org/〉 > Access: 11 Mar. 2022.

Ribeiro, A.S., Brondani, G.E., Tormen, G.C.R., Figueiredo, A.J.R., 2016. Cultivo *in vitro* de bambu em diferentes sistemas de propagação. Nativa 4, 15–18.

Ribeiro, A.S., Figueiredo, A.J.R., Tormen, G.C.R., Silva, A.L.L., Campos, W.F., Brondani, G.E., 2020. Clonal bamboo production based on *in vitro* culture. Biosci. J. 36, 1261–1273. https://doi.org/10.14393/BJ-v36n4a2020-48169

Sandhu, M., Wani, S.H., Jiménez, V.M., 2018. *In vitro* propagation of bamboo species through axillary shoot proliferation: a review. Plant Cell Tiss. Organ Cult. 132, 27–53. https://doi.org/10.1007/s11240-017-1325-1

Santos, D.W.R., Rocker, T.P., Ornellas, T.S., Guerra, M.P., 2019. Effects of a commercial biocide, kasugamycin and consistency of the culture medium on the *in vitro* establishment of bamboo. Pesqui. Agropecu. Trop. 49, 1–9. https://doi.org/10.1590/1983-40632019v4955435

Silva, I.M.C., Cuchiara, C.C., Winhelmann, M.C., Bianchi, V.J., Braga, E.J.B., Dutra, L.F., Peters, J.A., 2016. *In vitro* multiplication of pear tree cultivar Cascatense. Semin. Ciênc. Agrár 37, 581–594.

Singh, S.R., Dalal, S., Singh, R., Dhawan, A.K., Kalia, R.K., 2013. Evaluation of genetic fidelity of *in vitro* raised plants of *Dendrocalamus asper* (Schult. & Schult. F.) Backer ex K. Heyne using DNA-based markers. Acta Physiol. Plant. 35, 419–430. https://doi.org/10.1007/s11738-012-1084-x

Singh, S.R., Dalal, S., Singh, R., Dhawan, A.K., Kalia, R.K., 2012. Micropropagation of *Dendrocalamus asper* {Schult. & Schult. F.} Backer ex K. Heyne): an exotic edible bamboo. J. Plant Biochem. Biotechnol. 21, 220–228. https://doi.org/10.1007/s13562-011-0095-9

Souza, D.M.S.C., Martins, A.R., Fernandes, S.B., Avelar, M.L.M., Molinari, L.V., Gonçalves, D.S., Brondani, G.E., 2022. *In vitro* multiplication of *Eucalyptus pilularis* and *Eucalyptus grandis* × *E. urophylla* (urograndis eucalypt): effect of light quality in temporary immersion bioreactor. Mindanao J. Sci. Technol. 20, 72–86.

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., 2021. Clonal micro-garden formation of *Bambusa vulgaris*: effect of seasonality, culture environment, antibiotic and plant growth regulator on *in vitro* culture. Cerne 27, e-102979 https://www.doi.org/10.1590/01047760202127012979.

Van Winkle, S., Johnson, S., Pullman, G.S., 2003. The impact of gelrite and activated carbon on the elemental composition of plant tissue culture media. Plant Cell Rep. 21, 1175–1182. https://doi.org/10.1007/s00299-003-0637-2

Venkatachalam, L., Sreedhar, R.V., Bhagyalakshmi, N., 2007. Micropropagation in banana using high levels of cytokinins does not involve any genetic changes as revealed by RAPD and ISSR markers. Plant Growth Regul. 51, 193–205. https://doi.org/10.1007/s10725-006-9154-y

Villa, F., Pasqual, M., Silva, E.F., 2014. Micropropagação de híbridos de orquídea em meio knudson com adição de vitaminas do meio. MS, benzilaminopurina e carvão ativado. Semin. Ciênc. Agrár 35, 683–694.

Wang, Q.M., Wang, L., 2012. An evolutionary view of plant tissue culture: somaclonal variation and selection. Plant Cell Rep. 31, 1535–1547. https://doi.org/10.1007/s00299-012-1281-5