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Special Supplement: Bamboo

Effects of a commercial biocide, kasugamycin and consistency of the culture medium on the *in vitro* establishment of bamboo[1]

Daniela Werner Ribeiro dos Santos[2], Théo Piucco Rocker[2], Thiago Sanches Ornellas[2], Miguel Pedro Guerra[2,3]

ABSTRACT

The contamination by microorganisms and oxidation of explants in the *in vitro* establishment of bamboo are recurrent problems for its micropropagation. In the present study, effects of the biocide Plant Preservative Mixture (PPM™), the antibiotic kasugamycin and the consistency of the culture medium were evaluated in the *in vitro* establishment of *Bambusa vulgaris*, *Phyllostachys bambusoides* and *Dendrocalamus asper*. The presence of PPM™ in the culture medium had a significant effect using 2 mL L^{-1} or 4 mL L^{-1} concentrations, as well as in the liquid culture medium, increasing the plants established in the autumn. Kasugamycin promoted variable responses for all the three species, depending on the season. There was interaction among the factors, demonstrating that higher rates of viable plants can be obtained by combining different strategies to reduce the oxidation and contamination. For the *in vitro* establishment of *B. vulgaris*, *P. bambusoides* and *D. asper*, it is recommended to add 2 mL L^{-1} or 4 mL L^{-1} of PPM™ to the liquid culture medium.

KEYWORDS: *Bambusoideae*, bamboo contamination, bamboo micropropagation.

RESUMO

Efeitos de um biocida comercial, casugamicina e consistência do meio de cultura no estabelecimento *in vitro* de bambu

A contaminação por micro-organismos e oxidação dos explantes no estabelecimento *in vitro* de bambu são problemas recorrentes na sua micropropagação. Avaliaram-se os efeitos do biocida Plant Preservative Mixture (PPM®), do antibiótico casugamicina e da consistência do meio de cultura no estabelecimento *in vitro* de *Bambusa vulgaris*, *Phyllostachys bambusoides* e *Dendrocalamus asper*. A presença de PPM® no meio de cultura apresentou efeito significativo nas concentrações de 2 mL L^{-1} ou 4 mL L^{-1}, assim como o meio de cultura líquido no aumento de plantas estabelecidas no outono. A casugamicina promoveu respostas variáveis para as três espécies, dependendo da estação do ano. Houve interação entre os fatores, demonstrando que maiores índices de plantas viáveis podem ser obtidos aliando-se diferentes estratégias para diminuir a oxidação e a contaminação. Para o estabelecimento *in vitro* de *B. vulgaris*, *P. bambusoides* e *D. asper*, é recomendada a adição de 2 mL L^{-1} ou 4 mL L^{-1} de PPM® ao meio de cultura líquido.

PALAVRAS-CHAVE: *Bambusoideae*, contaminação em bambu, micropropagação de bambu.

INTRODUCTION

The economic importance of bamboos in Brazil is no longer related to craftwork and furniture production. Today, these plants and their products are used in the construction of houses, kiosks, glue-laminated products and for food consumption in the form of bamboo shoots (Azzini & Beraldo 2001, Greco & Cromberg 2011).

The *Bambusa vulgaris* Schrad ex Wendl, *Phyllostachys bambusoides* Sieb. & Zucc. and *Dendrocalamus asper* (Schult. & Schult. f.) Baker ex K. Heyne species, all from Asia, have a potential for planting in Brazil (Greco & Cromberg 2011). They are used as raw material for paper production, construction of temporary structures and fences in rural areas, manufacturing of furniture and laminates, ornamental plants and for the production of edible shoots (Pereira & Beraldo 2008, Singh et al. 2012a).

Traditionally, bamboos have been propagated by seeds or vegetatively, but both techniques have limitations, such as low seed viability, long flowering periods, limited availability of propagules, seasonal dependence and low survival rates. Thus,

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the lack of bamboo seedlings with genetic and phytosanitary quality may become a bottleneck in the production chain, since conventional propagation methods cannot meet the demand (Oprins et al. 2004, Mudoi et al. 2013, Singh et al. 2013a). The *in vitro* propagation of these plants is an appropriate biotechnological tool for large scale propagation and regenerative routes based on organogenesis that has been successfully employed. Since there is a high diversity of bamboo species, it is difficult to obtain a single micropropagation protocol that works for all of them. Therefore, there is a need to test and refine *in vitro* culture conditions for each species/genus (Jiménez & Guevara 2007).

Several factors influence the *in vitro* establishment of bamboo, such as contamination by microorganisms, oxidation of explants, season of the year, juvenility of explants and consistency of the culture medium (Mudoi et al. 2013, Singh et al. 2013b).

Some authors report difficulty in establishing explants of different bamboo species *in vitro* due to high contamination rates (Das & Pal 2005, Jiménez et al. 2006, Jiménez & Guevara 2007, Yasodha et al. 2008, Singh et al. 2013a, Mudoi et al. 2014). For bamboo, the antifungal agents benomyl, carbendazim and mancozeb (Ramanayake & Yakandawala 1997, Jiménez & Guevara 2007, Singh et al. 2013b, Mudoi et al. 2014) and the antibiotics agrimycin, kanamycin, streptomycin and gentamycin (Jiménez & Guevara 2007, Nadha et al. 2012, Mudoi et al. 2014) have been widely used for the disinfection of explants prior to placing *in vitro*.

A chemical agent that has been used to eliminate or control microorganisms *in vitro* is the Plant Preservative Mixture (PPM™ - Plant Cell Technology, Washington DC, USA). PPM™ penetrates the cell wall of microorganisms and inhibits several key enzymes in the citric acid cycle and the electron transport chain, acting as a biocide of broad spectrum. Studies on various species of bamboo indicate that the use of PPM™ in the cultivation medium at concentrations of 0.1-20 % during the initial stages of *in vitro* cultivation resultes in lower contamination rates (Jiménez et al. 2006, Jiménez & Guevara 2007, Ogita et al. 2008, Mudoi et al. 2013).

Oxidation is a common occurrence in some bamboo explants and is considered to be a major problem for the *in vitro* establishment of some bamboo species (Mudoi et al. 2013). The exudation of phenolic compounds causes the darkening and necrosis of tissues that have suffered some kind of injury, such as the cut edge of the explant, limiting the uptake of nutrients from the culture medium and the development of the plant (George et al. 2008, Mudoi et al. 2014). For bamboo, different strategies that aim to reduce oxidation are reported, such as the addition of anti-oxidants to the culture medium (Sanjaya et al. 2005) like polyvinylpyrrolidone (PVP) (Jullien & Van 1994). In addition, the collection time of the explants has also been studied as a way of reducing oxidation (Mudoi et al. 2014). The success of each strategy is dependent on the species. During the establishment stage, a gel culture medium is generally used, but, for bamboo, some authors have reported a greater success with the use of liquid culture media (Sood et al. 2002, Das & Pal 2005, Arya et al. 2006, Ogita et al. 2008).

This study aimed to establish *in vitro* cultures of *Bambusa vulgaris*, *Phyllostachys bambusoides* and *Dendrocalamaus asper* during two seasons, with the use of the antibiotic kasugamycin and the biocide PPM™, as well as to evaluate the consistency of the culture medium.

MATERIAL AND METHODS

The study was developed at the Universidade Federal de Santa Catarina (Florianópolis, Santa Catarian state, Brazil). The used donor plants were: *Bambusa vulgaris* - adult clump (27º34'56"S; 48º30'17"W); *Phyllostachys bambusoides* - two-year-old seedlings kept in a pot under greenhouse conditions (27º34'50"S; 48º30'15"W); *Dendrocalamaus asper* - adult clump (27º36'07"S; 48º31'29"W).

The experiments were carried out during two seasons: spring (October and November 2016) and autumn (April and May 2017). The experimental design was a completely randomized factorial scheme (3 x 2 x 2, being three concentrations of PPM™ x absence/presence of kasugamycin x liquid or solid consistency), with five replicates per treatment. Each replicate consisted of the mean of two and three test tubes for *B. vulgaris* and a mean of three test tubes for *P. bambusoides* and *D. asper*. Each treatment consisted of 14 test tubes for *B. vulgaris* and 15 test tubes for *P. bambusoides* and *D. Asper*, containing one explant per tube. The treatments comprised concentrations of Plant Preservative Mixture™ - PPM™ solution (Plant Cell Technology,

Washington DC, USA) of 0 mL L^{-1}, 2 mL L^{-1} and 4 mL L^{-1} added to the culture medium; the addition or not of the antibiotic kasugamycin (Kasumin™ - Arysta Lifescience) at the concentration of 3 mL L^{-1} for *B.vulgaris* and *P. bambusoides* and 1 mL L^{-1} for *D. asper*; and two consistencies of culture medium, liquid or solid, the latter being added with an agar gelling agent (5 g L^{-1}).

Primary and secondary branches were collected and taken to the laboratory, where the sheaths were removed and cleaned with cotton soaked in 70 % alcohol. These branches were sectioned into nodal segments containing a dormant bud (1.5 ± 0.5 cm) and kept at 4 ºC, for 6 h. Next, the nodal segments were immersed in a Tween 20 solution (1 drop/100 mL of sterile deionized water - SDW) that was stirred for 20 min. Then, they were immersed in a solution containing the bactericide Agrimycin (3 g L^{-1} in SDW) for 15 min, followed by a SDW wash. After the pre-asepsis, the explants were transferred to a laminar flow chamber, where they were immersed in 70 % alcohol for 2 min, followed by immersion in 2 % NaOCl (active chlorine) for 15 min. Three washes were performed with SDW. The explants were then immersed in 5 % of PPM™ solution for 16 h. Prior to the inoculation into the culture medium, the oxidized tissue was removed from the ends of the segments.

The culture medium was composed of MS salts (Murashige & Skoog 1962) supplemented with 3 % of sucrose and 2 mL L^{-1} of Morel's vitamins (Morel & Wetmore 1951), 15 µM of 6-benzylaminopurine (BAP), 250 mg L^{-1} of polyvinylpyrrolidone (PVP), 40 mg L^{-1} of adenine sulphate and PPM™ (0 mL L^{-1}, 2 mL L^{-1} or 4 mL L^{-1}). In the treatments containing antibiotic, kasugamycin was added at the aforementioned concentrations. The pH of the culture media was adjusted to 5.8 before the addition of 5 g L^{-1} of agar (for the solid culture medium) and its autoclaving for 15 min at 121 ºC and 1.1 atm pressure.

All explants from the three evaluated bamboo species were maintained at 25 ± 2 ºC, under a 16-h photoperiod, illuminated by white tubular fluorescent lamps at 40-50 µM m^{-2} s^{-1}. Analyses were performed after 25 days of cultivation, based on the presence/absence of microorganisms (fungi and bacteria), oxidation (darkening of the explant) and establishment (sprouting of the bud with absence of contamination and oxidation).

All statistical analyses were carried out in the R software (R Core Team 2014), using the "Agricolae" package (Mendiburu 2015). Analyses of variance were performed and the effects of factors and interactions were considered significant when $p < 0.05$. The means were compared using the Duncan test.

RESULTS AND DISCUSSION

At 25 days after the inoculation, the experiments were concluded and the collected data used for all the analyses and interpretations. Figure 1 shows the steps of the *in vitro* establishment process for *Bambusa vulgaris*.

In general, cultures from the *B. vulgaris* explants collected in the spring (Table 1) were not influenced by the presence or absence of PPM™ in

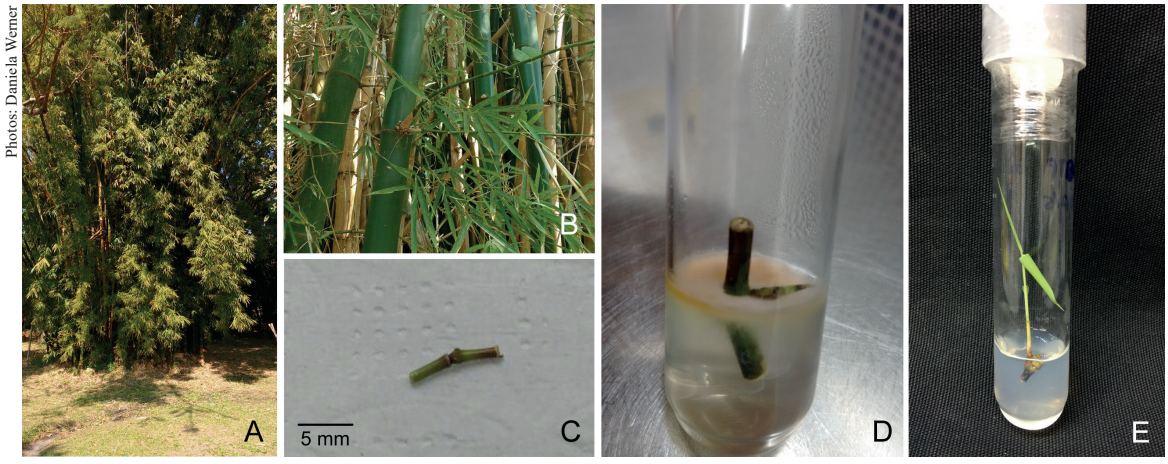

Figure 1. Stages of the *in vitro* establishment for *Bambusa vulgaris*: A) mother plant where the explants were obtained; B) axillary branches; C) nodal segment; D) explant contaminated with fungus; E) established plant.

Table 1. Statistics for the oxidation, contamination and *in vitro* establishment of nodal segments of *Bambusa vulgaris* collected in the spring and autumn.

Factor	Spring			Autumn		
	Oxidation (%)	Contamination (%)	Establishment (%)	Oxidation (%)	Contamination (%)	Establishment (%)
PPM™ (mL L^{-1}) (P): means[1]						
0	51.7 ± 7.4 a	57.5 ± 5.7 a	10.8 ± 4.6 a	1.7 ± 1.7 a	75.0 ± 6.7 a	22.5 ± 6.6 b
2	66.7 ± 5.4 a	34.2 ± 6.3 a	10.8 ± 4.6 a	6.7 ± 3.1 a	40.0 ± 6.9 b	53.3 ± 7.3 a
4	61.7 ± 7.4 a	40.8 ± 6.1 a	12.5 ± 5.2 a	6.7 ± 3.9 a	29.2 ± 6.5 b	50.0 ± 7.2 a
Kasugamycin (K): means[1]						
Absent	52.2 ± 4.9 b	46.7 ± 5.6 a	12.8 ± 3.9 a	4.4 ± 2.1 a	58.9 ± 6.4 a	33.9 ± 6.6 b
Present	67.8 ± 5.6 a	41.7 ± 4.8 a	10.0 ± 3.9 a	5.5 ± 2.8 a	37.2 ± 6.0 b	50.0 ± 5.6 a
Consistency of the culture medium (M): means[1]						
Liquid	63.3 ± 5.6 a	50.5 ± 5.4 a	5.5 ± 2.8 b	7.8 ± 3.1 a	34.4 ± 7.0 b	55.5 ± 6.9 a
Solid	56.7 ± 5.3 a	37.8 ± 4.8 a	17.2 ± 4.5 a	2.2 ± 1.5 a	61.7 ± 4.8 a	28.3 ± 4.2 b
Interactions: F-test						
P x K	2.16^{ns}	1.82^{ns}	1.56^{ns}	0.15^{ns}	0.56^{ns}	4.96*
P x M	0.13^{ns}	0.59^{ns}	0.06^{ns}	1.34^{ns}	0.92^{ns}	1.13^{ns}
K x M	1.44^{ns}	1.05^{ns}	0.84^{ns}	0.10^{ns}	3.34^{ns}	3.31^{ns}
P x K x M	2.16^{ns}	0.93^{ns}	0.06^{ns}	0.15^{ns}	0.92^{ns}	0.76^{ns}

[1] Means followed by the same letter in the same column and factor do not differ statistically according to the Duncan test at 5 % of probability. PPM™: Plant Preservative Mixture.

the culture medium for the evaluated parameters. There were significant differences ($p < 0.05$) for the oxidation between the presence and absence of kasugamycin, with the absence of the antibiotic promoting less oxidation of the explants (52.2 %) than its presence (67.8 %). During this season, a high percentage of oxidation (above 50 %) was observed for the explants. The *in vitro* establishment was influenced by the consistency of the culture medium, where the solid consistency had a higher percentage of developed explants, but did not show any apparent microbial contamination ($p < 0.05$), when compared to the liquid medium. The analysis of the interaction between factors did not highlight significant differences for the *B. vulgaris* collected in the spring.

In the autumn, the *B. vulgaris* explants were influenced by all the evaluated factors, regarding the *in vitro* contamination and establishment; however, for the oxidation, there was no significant difference between the factors ($p < 0.05$) (Table 1). There were also no significant differences between the concentrations of PPM™ (2 mL L^{-1} and 4 mL L^{-1}) for both the contamination and *in vitro* establishment. A double interaction (PPM™ x kasugamycin) significantly influenced ($F = 4.07$; $p < 0.05$) the *in vitro* establishment, where the media containing 2 mL L^{-1} of PPM™ and the presence of kasugamycin (58.3 %) had the highest percentage of established explants. Regardless of the results generated by the statistical analysis, the culture media with 4 mL L^{-1} of PPM™ (29.2 %), presence of kasugamycin (37.2 %) and liquid consistency (34.4 %) had the lowest contamination percentages. The culture media with 2 mL L^{-1} of PPM™ (53.3 %), presence of kasugamycin (50 %) and liquid consistency (55.5 %) had the highest percentages of established explants.

The *in vitro* responses of the *Phyllostachys bambusoides* explants collected in the spring were significantly affected by all factors (Table 2). Both kasugamycin and the consistency of the culture medium presented significant differences ($p < 0.05$) in the oxidation of the explants. The lowest oxidation percentages were observed in the kasugamycin-free medium (20 %) and solid culture medium (15.5 %). There was a significant difference ($p < 0.05$) for microbial contamination between the concentrations of PPM™ and the absence/presence of kasugamycin. The culture medium containing 4 mL L^{-1} of PPM™ (10 %) and the medium with the addition of kasugamycin (14.4 %) had the lowest percentages of contamination within their respective factor groups. There were also significant differences between the concentrations of PPM™ and the consistency of the culture media for the *in vitro* establishment, where higher percentages of established explants were observed in the solid medium (63.3 %) *versus* liquid medium. There was no significant difference in the interaction of the

Table 2. Statistics for the oxidation, contamination and *in vitro* establishment of nodal segments in *Phyllostachys bambusoides* collected in the spring and autumn.

Factor	Spring			Autumn		
	Oxidation (%)	Contamination (%)	Establishment (%)	Oxidation (%)	Contamination (%)	Establishment (%)
PPM™ (mL L^{-1}) (P): means[1]						
0	28.3 ± 6.6 a	26.7 ± 6.2 a	41.7 ± 7.9 b	0.0 ± 0.0 a	63.3 ± 5.3 a	35.0 ± 5.7 b
2	30.0 ± 6.8 a	26.7 ± 6.2 a	46.7 ± 7.4 ab	5.0 ± 3.6 a	45.0 ± 5.0 b	43.3 ± 4.3 b
4	25.0 ± 6.3 a	10.0 ± 4.3 b	63.3 ± 5.9 a	1.7 ± 1.7 a	31.7 ± 8.2 b	60.0 ± 8.6 a
Kasugamycin (K): means[1]						
Absent	20.0 ± 4.7 b	27.8 ± 5.3 a	47.8 ± 5.7 a	1.1 ± 1.1 a	50.0 ± 5.2 a	48.9 ± 5.2 a
Present	35.5 ± 5.5 a	14.4 ± 5.5 b	53.3 ± 6.3 a	3.3 ± 2.4 a	43.3 ± 6.0 a	43.3 ± 5.8 a
Consistency of the culture medium (M): means[1]						
Liquid	40.0 ± 5.1 a	22.2 ± 4.3 a	37.8 ± 4.9 b	1.1 ± 1.1 a	38.9 ± 6.0 b	56.7 ± 6.0 a
Solid	15.5 ± 4.4 b	20.0 ± 5.2 a	63.3 ± 6.0 a	3.3 ± 2.4 a	54.4 ± 4.9 a	35.5 ± 4.2 b
Interactions: F-test						
P x K	1.55^{ns}	0.72^{ns}	0.03^{ns}	0.25^{ns}	1.47^{ns}	0.07^{ns}
P x M	1.55^{ns}	0.72^{ns}	0.27^{ns}	0.25^{ns}	31.02***	45.56***
K x M	0.11^{ns}	0.48^{ns}	0.50^{ns}	2.65^{ns}	0.62^{ns}	5.88*
P x K x M	1.55^{ns}	1.61^{ns}	0.03^{ns}	0.25^{ns}	2.88^{ns}	1.82^{ns}

[1] Means followed by the same letter in the same column and factor do not differ statistically according to the Duncan test at 5 % of probability. PPM™: Plant Preservative Mixture.

factors for the *P. bambusoides* collected in the spring. Regardless of the results generated by the statistical analysis, the medium containing 4 mL L^{-1} of PPM™ had the highest percentage of established explants (63.3 %) among the concentrations.

In the autumn, the microbial contamination and *in vitro* establishment responses of the *P. bambusoides* explants were influenced by the presence of PPM™ and the consistency of the culture medium (Table 2). There was a significant difference ($p < 0.05$) between the absence and presence of PPM™ and between the consistencies of the culture media, where the media with 2 mL L^{-1} of PPM™ (45.0 %) and 4 mL L^{-1} of PPM™ (31.7 %) and the medium with a liquid consistency (38.9 %) had the lowest percentages of contaminated explants. Thus, the highest percentages of established explants were observed in the media containing 4 mL L^{-1} of PPM™ (60 %) and a liquid consistency (56.7 %). There was a double interaction (PPM™ x medium consistency) that significantly influenced the apparent microbial contamination ($F = 31.0241$; $p < 0.05$) and the *in vitro* establishment ($F = 45.6$; $p < 0.05$). The lowest percentage of contaminated explants (3.3 %) and the highest percentage of established explants (93.3 %) were observed in the culture media containing 4 mL L^{-1} of PPM™ and liquid consistency. There was also a double interaction (kasugamycin x medium consistency) that significantly influenced the *in vitro* establishment ($F = 5.9$; $p < 0.05$), where a higher percentage of established explants was observed in an enhanced kasugamycin culture medium and liquid consistency (60 %).

The oxidation and contamination responses, but not the establishment, were significantly affected by the factors in the *D. asper* explants collected in the spring (Table 3). Significant differences ($p < 0.05$) were observed in the oxidation and contamination among the PPM™ concentrations. Regardless of the results generated by the statistical analysis, 0 mL L^{-1} of PPM™ provided the lowest oxidation (20 %) among the PPM™ concentrations and 4 mL L^{-1} of PPM™ promoted a lower microbial contamination (33.3 %) than 0 mL L^{-1} and 2 mL L^{-1} of PPM™. There were also significant differences ($p < 0.05$) between the consistencies of the culture medium, where the liquid medium had a lower percentage of oxidized explants (23.8 %) than the solid medium. In addition to the PPM™, the contamination was also influenced by the presence of kasugamycin in the culture medium ($p < 0.05$), where lower percentages of contaminated explants were observed in the medium with added antibiotic (29.8 %), when compared to those without the antibiotic (50 %). There was a triple interaction (PPM ™ x kasugamycin x culture medium consistency) that influenced the microbial contamination ($F = 11.5$; $p < 0.05$), where the lowest percentages of contamination were observed in

Table 3. Statistics for the oxidation, contamination and *in vitro* establishment of nodal segments in *Dendrocalamus asper* collected in the spring and autumn.

Factor	Spring			Autumn		
	Oxidation (%)	Contamination (%)	Establishment (%)	Oxidation (%)	Contamination (%)	Establishment (%)
PPM™ (mL L^{-1}) (P): means[1]						
0	20.0 ± 7.0 b	56.7 ± 4.8 a	25.0 ± 5.6 a	13.3 ± 5.1 a	61.7 ± 7.7 a	16.7 ± 5.1 b
2	37.1 ± 8.9 ab	29.7 ± 5.9 b	13.1 ± 5.5 a	8.3 ± 3.3 a	41.7 ± 7.2 b	28.3 ± 6.1 ab
4	50.0 ± 8.0 a	33.3 ± 7.7 b	20.0 ± 5.3 a	16.7 ± 5.1 a	38.3 ± 7.7 b	31.7 ± 5.1 a
Kasugamycin (K): means[1]						
Absent	30.0 ± 6.3 a	50.0 ± 5.5 a	20.0 ± 4.7 a	13.3 ± 4.1 a	40.0 ± 6.7 b	32.2 ± 4.9 a
Present	41.7 ± 6.8 a	29.8 ± 4.9 b	19.1 ± 4.3 a	12.2 ± 3.4 a	54.4 ± 5.9 a	18.9 ± 3.8 b
Consistency of the culture medium (M): means[1]						
Liquid	23.8 ± 5.9 b	44.1 ± 5.9 a	23.9 ± 4.6 a	16.7 ± 3.8 a	31.1 ± 5.3 b	35.5 ± 4.5 a
Solid	46.7 ± 6.5 a	36.7 ± 5.0 a	15.5 ± 4.3 a	8.9 ± 3.5 a	63.3 ± 5.0 a	15.5 ± 3.8 b
Interactions: F-test						
P x K	3.07^{ns}	1.67^{ns}	0.41^{ns}	1.01^{ns}	9.81**	3.12^{ns}
P x M	1.11^{ns}	0.74^{ns}	0.05^{ns}	0.00^{ns}	3.83^{ns}	3.12^{ns}
K x M	0.82^{ns}	0.00^{ns}	2.30^{ns}	0.38^{ns}	0.02^{ns}	0.00^{ns}
P x K x M	3.07^{ns}	11.86**	0.41^{ns}	0.25^{ns}	0.00^{ns}	0.06^{ns}

[1] Means followed by the same letter in the same column and factor do not differ statistically according to the Duncan test at 5 % of probability. PPM™: Plant Preservative Mixture.

the culture media containing 2 mL L^{-1} of PPM™, kasugamycin and a liquid consistency (0.17 %).

In the fall, responses regarding the apparent microbial contamination and *in vitro* establishment of *D. asper* explants were influenced by all factors (Table 3). There were significant differences ($p < 0.05$) among the concentrations of PPM™, where the media containing 2 mL L^{-1} of PPM™ (41.7 %) and 4 mL L^{-1} PPM™ (38.3 %) had lower percentages of contamination than 0 mL L^{-1} PPM™ (61.7 %). Regardless of the results generated by the statistical analysis, the medium containing 4 mL L^{-1} of PPM™ (31.7 %) had the highest percentage of established explants within its factor. The kasugamycin factor also had a significant effect ($p < 0.05$), where its absence provided lower percentages of contamination (40 %) and higher percentages of explants established *in vitro* (32.2 %) than its presence. There was also a significant difference between the culture media consistencies ($p < 0.05$). The liquid medium had the lowest percentage of contamination (31.1 %) and a greater establishment (35.5 %). There was a double interaction (PPM™ x kasugamycin) that influenced the microbial contamination ($F = 9.8$; $p < 0.05$), where the lowest percentage of contaminated explants (10 %) was observed in the culture media with 4 mL L^{-1} of PPM™ and no kasugamycin.

From the data obtained for the three bamboo species in this study, PPM™, kasugamycin and the consistency of the culture medium influenced the oxidation, contamination and establishment rates of the *in vitro* culture. However, it was observed that the responses may be variable among the species and seasons when the explants were collected and introduced *in vitro*.

Season is an important factor to be considered in the collection and establishment of *in vitro* cultures (Tisserat 1985). Some studies with bamboo species show the importance of the time of the year to reduce the contamination and increase the rates of bud dormancy breaking, that is, the beginning of the sprouting and development of the bud. Ornellas et al. (2017) obtained a high percentage of *in vitro* contamination in *D. asper* collected in the summer (hot and rainy season), in Brazil. Mudoi et al. (2014) obtained the highest establishment rates of *Bambusa nutans* explants in the autumn, in India. Explants collected during higher growth seasons tend to reach higher rates of *in vitro* bud development (Das & Pal 2005). Despite the fact that seasons were not compared, it was observed that *B. vulgaris* and *D. asper* had a higher number of established explants in the autumn, a season marked by a lower growth for these species. This result is different from other studies that evaluated the *in vitro* establishment at different seasons (Singh et al. 2012a, Singh et al. 2012b, Banerjee et al. 2011). However, there are reports of other

plant species that had higher *in vitro* establishment rates when the explants were collected during the lower growing season, such as *Sapium sebiferum*, Euphorbiaceae (Siril & Dhar 1997), and *Myrica esculenta*, Myricaceae (Bhatt & Dhar 2004).

In the spring, the factors significantly affected the oxidation of the explants of the three species, the microbial contamination of *P. bambusoides* and *D. asper* and the *in vitro* establishment of *B. vulgaris* and *P. bambusoides*. In the autumn, the oxidation was not influenced by the factors, whereas the contamination and *in vitro* establishment were influenced by the factors in the three bamboo species. Despite the addition of the adsorptive agent PVP to the culture medium, the explants collected in the spring presented high oxidation rates, when compared to those collected in the autumn. A probable explanation for this result may be the aggressiveness of the asepsis process in explants with more juvenile and less lignified tissues that are produced in the spring in tropical species. *B. vulgaris* and *D. asper* showed higher numbers of oxidized explants in the spring and fall, respectively (data not shown). In general, the exudation of phenolic compounds has been reported to be lower when temperatures are lower (Andersone & Ievinsh 2002).

In the present study, PPM™ influenced the microbial contamination and *in vitro* establishment in the three species when collected in the autumn and influenced the microbial contamination of *P. bambusoides* and *D. asper* when collected in the spring. Ogita et al. (2008) were successful in the aseptic germination of *Phyllostachys meyeri* using 0.1 % of PPM™ in sterile distilled water. Jiménez et al. (2004, 2006) reported the efficiency of 2 mL L^{-1} of PPM™ in the *in vitro* establishment of nodal segments of *Guadua angustifolia* grown in a greenhouse, but observed that this concentration was not efficient for explants collected from plants in the field.

The presence of kasugamycin in the culture medium reduced the microbial contamination in explants of *B. vulgaris* collected in the fall and in explants of *P. bambusoides* and *D. asper* collected in the spring. It was also verified that the presence of kasugamycin promoted an increase in the oxidation of explants in *B. vulgaris* and *P. bambusoides* collected in the spring.

Kasugamycin (Kasumin™) is a systemic antibiotic, presented in a liquid form, which allows its dilution in the culture medium. Nadha et al. (2012) added kanamycin and streptomycin to the culture medium to decrease the growth of bacteria of the *Pantoea* genus.

The consistency of the culture medium also influenced the contamination and *in vitro* establishment of the three bamboo species when the explants were collected in the autumn. Lower contamination rates were obtained in the liquid culture medium, probably because the explants were totally immersed and surrounded by PPM™ and kasugamycin, which effectively acted in the control of microorganisms. In the liquid medium, higher rates of established explants were also obtained. In the spring, the solid culture medium was more effective in the *in vitro* establishment of *B. vulgaris* and *P. bambusoides*. In the *in vitro* establishment stage, gelled culture media are generally used, because they allow a better visualization of the microbial contamination and keep the explant free from hypoxia.

Singh et al. (2012b) did not obtain good results when using a liquid culture medium in the *in vitro* establishment of *D. asper*. However, several studies report the preference for the liquid medium in order to start the crops of *Bambusa balcooa*, *B. nutans*, *B. salarkhanii* and *B. vulgaris* (Islam & Rahman 2005). According to Marulanda et al. (2005), the liquid medium has a greater distribution and availability of nutrients, what contributes to the growth of the explants. Also, higher rates of *in vitro* multiplication and growth are reported when using a liquid culture medium, such as for *Bambusa balcooa* (Das & Pal 2005), *Dendrocalamus giganteus* (Arya et al. 2006), *Bambusa glaucescens* (Shirin & Rana 2007) and *Phyllostachys meyeri* (Ogita et al. 2008). The low multiplication rate and slow growth in solid media, when compared to liquid media, may be attributed to the fact that the gelling agent releases water, nutrients and phytoregulators more slowly, not keeping up with the metabolism of some species (Singh et al. 2013b). The majority of studies that report the *in vitro* cultivation of bamboo species used solid media (Marulanda et al. 2005, Jiménez et al. 2006). However, we suggest the use of a liquid medium, not only based on the results of this study, but also because of its easy preparation and lower cost.

The interactions between the factors showed that lower percentages of microbial contamination and higher percentages of explants established *in vitro* can be obtained by combining different asepsis agents in a liquid culture medium.

CONCLUSIONS

1. The presence of PPM™ and kasugamycin in the culture medium and the consistency of the medium influence the oxidation, apparent microbial contamination and *in vitro* establishment of *Bambusa vulgaris*, *Phyllostachys bambusoides* and *Dendrocalamus asper*;
2. The addition of the antibiotic kasugamycin to the culture medium presents variable responses for the three bamboo species, both in the spring and autumn. Its use is recommended only for the *in vitro* establishment of *B. vulgaris*;
3. It is recommended that the *in vitro* establishment of *B. vulgaris*, *P. bambusoides* and *D. asper* be carried out in the autumn, with the addition of 2 mL L^{-1} or 4 mL L^{-1} of PPM™ to a liquid culture medium.

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