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Micropropagação de *Dendrocalamus asper*, *Bambusa multiplex* e *Bambusa oldhamii*

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Thiago Sanches Ornellas

Micropropagação de *Dendrocalamus asper*, *Bambusa multiplex* e *Bambusa oldhamii*

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Micropropagação de *Dendrocalamus asper*, *Bambusa multiplex* e *Bambusa oldhamii*

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Este trabalho é dedicado aos meus pais,

Eliana e Eduardo

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RESUMO

Bambus são gramíneas da subfamília Bambusoideae. O interesse mundial e demanda por diversas espécies de bambus vêm crescendo nos últimos anos. A diversidade de espécies e os múltiplos usos de várias destas são os principais motivos de destaque atual deste grupo de plantas. O aumento na procura por espécies prioritárias de bambus associado às dificuldades da produção de mudas pode resultar em uma escassez de material para o estabelecimento de plantios comerciais. Embora os bambus possam ser reproduzidos por sementes, para diversas espécies a disponibilidade deste tipo de propágulo é baixa. O florescimento de algumas espécies ocorre em intervalos de décadas e muitas vezes não são previsíveis. Além disto, as sementes quando disponíveis, possuem curta viabilidade. Os métodos vegetativos são os mais empregados. Porém, a taxa de sucesso destes pode variar em função de época do ano, tipo de propágulo e métodos propagativos. Ademais, muitas vezes as mudas produzidas são volumosas e com logística de transporte custosa. Neste cenário as biotecnologias associadas a propagação de plantas são adequadas para o desenvolvimento de métodos de propagação mais eficientes e independentes de condições ambientais. A micropropagação de bambus vem sendo desenvolvida desde o início dos estudos de cultura de tecidos vegetais, e grande variação de protocolos e resultados são observados na literatura sobre o tema. O presente trabalho teve como objetivo estabelecer culturas *in vitro* de bambus de interesse comercial, assim como determinar metodologias aplicadas à micropropagação destas espécies. Após uma breve introdução e justificativa, três capítulos são apresentados: (1) A micropropagação do bambu *Dendrocalamus asper* por meio da embriogênese somática a partir de inflorescências jovens; (2) A micropropagação de *Bambusa multiplex* por meio de organogênese de ramos axilares; e (3) O estabelecimento de culturas *in vitro* de *Bambusa oldhamii* como base para o desenvolvimento de um protocolo de micropropagação por meio de organogênese de ramos axilares. Para as três espécies alvos deste estudo foram estabelecidas culturas *in vitro.* Embriões somáticos de *D. asper* foram regenerados e convertidos em plantas a partir de inflorescências jovens (estádio pré-antese). Um protocolo foi estabelecido para *B. multiplex* a partir de segmentos nodais. Três experimentos foram realizados para determinar a dose ideal de Benzilaminopurina e qual a posição mais adequada para coleta de segmentos nodais em plantas de *B. oldhamii.* O estabelecimento de culturas *in vitro* a partir de material vegetativo de plantas matrizes em condições de campo é um dos principais entraves para a micropropagação de bambus lignificados. O presente estudo resultou em culturas de três espécies de potencial ornamental, além de outros usos de importância econômica. A partir destas culturas diversos novos estudos deverão ser realizados a fim de elucidar questionamentos de cunho básico e aplicado. O aperfeiçoamento destes protocolos permitirá a produção em larga escala de plantas com qualidade fisiológica e fitossanitária, além de permitir a seleção e conservação de genótipos superiores.

Palavras-chave: Propagação *in vitro*. Bambuseae. Embriogênese somática. Organogênese.

ABSTRACT

Bamboos are grasses of the subfamily Bambusoideae. The worldwide interest and demand for several bamboo species have grown in recent years. The diversity of species and the multiple uses of several of them are the main reasons for the current prominence of this group of plants. This increased demand for priority bamboo species associated with difficulties in plantlets production can result in a shortage of material for commercial plantations. Although bamboos can be reproduced by seeds, the availability of this type of propagule is low for several species. The flowering of some species occurs at intervals of decades and is often not predictable. Furthermore, when seeds are available, they have short viability. Vegetative propagation means are the most applicable methods. However, their success rate may vary depending on the species, the year's season, type of propagule, and propagation methodology employed. In addition, the seedlings produced are often bulky and have costly transport logistics. In this scenario, biotechnologies associated with plant propagation are suitable for developing more efficient methods independent of environmental conditions. Bamboo micropropagation has been developed since the beginning of plant tissue culture studies, and a wide range of protocols and results are observed in the literature about the theme. The present work aimed to establish *in vitro* cultures of bamboos with a commercial interest and determine methodologies feasible for the micropropagation of these species. After a brief introduction and justification, three chapters are presented: (1) *Dendrocalamus asper* bamboo micropropagation through somatic embryogenesis from young inflorescences; (2) *Bambusa multiplex* organogenesis micropropagation through axillary branches; and (3) *In vitro* cultures establishment of *Bambusa oldhamii* as a basis for the development of a micropropagation protocol through axillary branch organogenesis. For the three target species of this study, *in vitro* cultures were established. Somatic embryos of *D. asper* were regenerated and converted into plants from young inflorescences (pre-anthesis). An initial protocol was established for *B. multiplex* from nodal segments, resulting in acclimatized plants. Three experiments were conducted to determine the ideal dose of Benzilaminopurine, and which is the most suitable position for collecting nodal segments in *B. oldhamii* plants. The establishment of *in vitro* cultures from field-condition-mother plants is one of the main constraints for the lignified bamboos micropropagation. The present study resulted in cultures of three multiple-purpose bamboo species with established worldwide economic and environmental importance. Several new studies should be carried out from these cultures to elucidate questions of a basic and applied scientific nature. The improvement of these protocols can allow the large-scale production of plants with physiological and phytosanitary quality and allow the selection and conservation of superior genotypes.

Keywords: *In vitro* propagation. Bambuseae. Somatic embryogenesis. Organogenesis.

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LISTA DE ABREVIATURAS E SIGLAS

BAP – Benzilaminopurina/ Benzilaminopurine

mT – meta-Topolina/ meta-Topolin

TDZ - Thiadiazuron

NAA – Ácido Naftaleno acético/ Naphtaleneacetic acid

IBA – Ácido Indol Butírico/ Indolebutyric acid

2,4-D – Ácido diclorofenoxiacético/ Dichlorophenoxyacetic acid

2-iP - Isopentenyladenine

Kin – Cinetina/ Kinetin

INBAR – Rede Internacional do Bambu e Ratam/ International Network for Bamboo and Rattan

BambuSC – Associação Catarinense do Bambu

GL – Gay-Lussac

PGR – Regulador de crescimento vegetal/ Plant Growth Regulator

PCR – Reação em Cadeia de Polimerase/Polymerase Chain Reaction

PVP – Polivinilpirolidona/ Polyvinylpyrrolidone

EDTA – Ácido etilenodiamino tetra-acético/ Ethylenediamine tetraacetic acid

LTR - Long terminal repeats/ Repetições terminais longas

TE – Transposable elements/ Elementos transponíveis

TBE – Tampão Tris-Borato-EDTA/ Tris-Borate-EDTA Buffer

LISTA DE SÍMBOLOS

º grau /degree

± mais ou menos/ more or less

® marca registrada

^{TM} Trademark

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INTRODUÇÃO

"Bambu" é um termo genérico utilizado para as gramíneas (família botânica Poaceae [syn. Gramineae]) pertencentes à subfamília Bambusoideae. Esta é considerada a única linhagem da família Poaceae que se diversificou em ambiente florestal (Judziewicz et al., 1999; Bamboo Phylogeny Group [BPG], 2012).

A subfamília Bambusoideae pode ainda ser classificada em três tribos botânicas: Arundinarieae, Bambuseae e Olyreae. Em termos práticos estas tribos agrupam, respectivamente, os bambus lignificados de clima temperado ou de altitude, bambus lignificados de clima tropical, e bambus herbáceos (Clark et al., 2015). Devido ao seu potencial colonizador de gramínea e sua evolução em ambientes florestais, os bambus são nativos da Ásia, Oceania, África e Américas, além de terem sidos introduzidos na Europa. As mais diversas condições ambientais favoreceram especificidades durante a especiação de bambus, que ocorrem desde nível do mar até 3500 m de altitude, sendo o regime de chuvas e as temperaturas mínimas os fatores determinantes de ocorrência das espécies (Hoogendoor et al. 2013).

Atualmente são reconhecidos 127 gêneros botânicos de bambus, somando mais de 1680 espécies. Com os avanços das discussões taxonômicas e o avanço das tecnologias moleculares, o número de espécies de bambus tem sido dinâmico e crescente nas últimas décadas, devido a reclassificação de alguns táxons e a descrição de novas espécies (Clark e Oliveira, 2018).

Na prática, esta diversidade pode ser exemplificada por meio da comparação de dimensões entre o "menor bambu do mundo", *Raddiella vanessiae* Judziewicz & Sepsenwol (Judziewicz e Sepsenwol, 2007) e o "maior bambu do mundo", *Dendrocalamus sinicus* L.C. Chia & J.L. Sun. O primeiro alcança uma altura de planta de alguns centímetros e cabe na palma da mão, enquanto o segundo pode chegar a mais de 30 metros de altura e um diâmetro de colmo de até 30 cm (Li et al. 2021).

Entre as três tribos de bambus, a tribo Bambuseae agrupa em 74 gêneros cerca de 970 espécies. Esta diversidade de características mofológicas, anatômicas e fisiológicas é resultado da evolução em diversos ambientes e da coevolução com uma diversidade de organismos. Essas espécies possuem importância ambiental, social, e econômica ao redor do mundo. Algumas destas possuem importância cultural, fazendo parte de tradições e religiões

de várias nações. No Brasil, ocorrem de forma nativa apenas bambus das tribos Bambuseae e Olyreae, sendo conhecidas 258 espécies nativas classificadas em 35 gêneros, dos quais 12 são considerados endêmicos (Filgueiras e Viana, 2017).

Como a maioria das plantas monocotiledôneas, os bambus não apresentam meristemas secundários e, portanto, não apresentam crescimento secundário. Ou seja, o diâmetro que o colmo jovem (broto) possui ao emergir do solo é praticamente o diâmetro que este terá até sua senescência. Apesar do uso da terminologia "lenhoso" ser comumente difundida para delimitar os bambus não herbáceos (i.e., tribos Bambuseae e Arundinareae), é preferível que estes bambus sejam descritos como "lignificados". O uso do termo lenhoso provavelmente ocorre devido ao termo anglicano "woody" utilizado para descrever os bambus de grande porte.

Nas últimas décadas, os bambus vêm sendo utilizados de forma crescente como substitutos da madeira em diversos processos industriais. Como um recurso renovável perene, quando cultivados os bambus contribuem de forma importante para a preservação de florestas nativas. No entanto, a demanda industrial por matéria prima de bambus põe em risco reservas naturais de algumas espécies com potencial de uso, impondo a necessidade urgente de implantação de áreas cultivadas. Vários gêneros da tribo Bambuseae, incluindo os prioritários, apresentam espécies com longos tempo de juvenilidade, apresentando longos intervalos entre florescimentos, impossibilitando a produção regular de mudas por meio de sementes. Estas sementes, quando disponíveis, apresentam curta viabilidade e são importante recurso para fauna, o que torna ainda mais difícil sua utilização para produção de mudas (Rao et al. 1998).

Os métodos convencionais de propagação são por meio de divisão de touceira, secção de colmo com rizoma, segmento nodal de colmo, mergulhia e alporquia de colmo. Estes métodos apresentam grande variação na taxa de sucesso. Ainda a sazonalidade na obtenção de propágulos e o elevado custo de produção tornam-se gargalos para a base da cadeia produtiva dos bambus (Ray e Ali, 2017).

Frente a estas dificuldades na produção de mudas de bambus, a micropropagação vem sendo desenvolvida visando à propagação massal e conservação ex situ de genótipos elite. Além destas aplicações, o florescimento e fertilização *in vitro* de bambus também são ferramentas alternativas ao melhoramento genético dificultado pelos longos ciclos.

O primeiro relato do cultivo *in vitro* de bambus ocorreu em 1968 por Alexander e colaboradores (1968), os quais relataram a germinação de embriões zigóticos em meio de

cultura. Para bambus lignificados, diversos protocolos regenerativos via organogênese ou embriogênese somática foram estabelecidos nas últimas décadas (Mudoi et al., 2013). Porém, devido à alta diversidade de Bambuseae e a genótipo dependência nas repostas morfogenéticas *in vitro*, são necessárias adaptações e aperfeiçoamentos nos protocolos para espécies de bambus (Sandhu et al., 2018).

Durante o cultivo *in vitro* os tecidos podem sofrer variações somaclonais devido ao ambiente e método de cultivo empregados, e principalmente ao uso contínuo de reguladores de crescimento exógenos (Oprins et al., 2004). Para o estabelecimento de um protocolo *in vitro* de propagação massal comercial é importante a garantia de fidelidade clonal das plantas obtidas. Variações somaclonais podem não se manifestarem por características morfológicas ou expressar fenotipicamente somente na maturidade. Atualmente métodos de detecção de variantes somaclonais por marcadores moleculares são importante ferramenta para validar protocolos da micropropagação.

Várias espécies de Bambuseae apresentam ciclos de florescimento longos (até 120 anos), florescimento gregário e monocárpico (Janzen, 1976). Os mecanismos genéticos e fisiológicos destes fenômenos ainda não foram completamente elucidados. Devido a isso, diversos bambus são classificados baseando-se em características estéreis, o que dificulta a sua correta identificação botânica. Ao longo das últimas décadas, pelo desenvolvimento dos estudos genéticos e aumento dos florescimentos registrados, tem-se identificado novas espécies e corrigido classificações dentro do grupo (Clark e Oliveira, 2018).

Os bambus são considerados recursos florestais não madeireiros de ocorrência mundial e muito utilizados como substitutos da madeira (Lobovikov et al., 2007). Um estudo preliminar da *Global Forest Resources Assessment* estimou baseado em apenas em 33 países, uma área de 31,5 milhões de hectares de bambus no mundo.

O cultivo de bambus permite um manejo sustentável devido ao crescimento rápido, colheitas constantes, altos rendimentos e qualidade de fibras (Zehui, 2007). Além disso, prestam serviços ambientais. Assim, de acordo com Zengyao (2001) um hectare de floresta de bambu pode reter 1000 toneladas de água, aumentando a umidade relativa em 5-10% e reduzindo a temperatura abaixo do dossel em 3-5 °C. Além disto, o mesmo autor relata um incremento na interceptação e na taxa de permeabilidade do solo quando comparada a pastagens.

Os bambus ainda apresentam uma ampla gama de usos e potenciais tais como, combustível (biomassa, etanol, carvão, pellets, entre outros), construção civil, produtos manufaturados (laminados, *lumbers*, *oriented-strand board* [OSB] e compósitos), produtos têxteis, movelaria, paisagismo e decoração, entre outros, incluindo diversos usos tradicionais (Akinlabi et al., 2017)

Cadeia produtiva no Brasil

O Brasil possui potencial para cultivos de bambus tanto de espécies nativas quanto exóticas. Das nativas destacam-se espécies do gênero Guadua, que apresentam colmos de grande diâmetro e paredes espessas, conferindo assim resistência e durabilidade. Espécies dos gêneros Merostachys e Chusquea são utilizadas tradicionalmente para artesanatos e cestarias, além de apresentarem potencial de uso ornamental e para recuperação de áreas degradadas. Dentre as exóticas, as de maior potencial pertencem aos gêneros Bambusa, Dendrocalamus e Phyllostachys.

O maior plantio comercial de bambus do Brasil encontra-se no Nordeste, onde o Grupo Industrial João Santos utilizava o bambu *Bambusa vulgaris* como matéria prima celulósica na produção de cartão duplex e papel Kraft para embalagens. Para isso, o grupo contava com o cultivo em Coelho Neto – MA, com uma área de 30.000 ha da espécie *Bambusa vulgaris* (Lobovikov et al., 2007; PNF, 2005). Outra recente iniciativa, do Grupo Suzano – Papel e Celulose e o Instituto Jatobás, é a Fazenda dos Bambus localizada em Pardinho-SP, onde são cultivadas 62 espécies de bambus e são realizadas pesquisas sobre a cultura (www.fazendadosbambus.com.br). Ainda no estado de São Paulo, o Projeto LUPA disponibiliza mapas com a distribuição de algumas áreas cultivadas de bambus. Podem-se observar concentrações de plantios na região de Mogi das Cruzes e Registro, apresentando talhões de até 120 ha. Em Santa Catarina os cultivos mais expressivos são cerca de 40 ha no município de Frei Rogério, 60 ha em Rio Negrinho, e 40 ha em São Cristovão do Sul.

Quanto ao cenário de políticas públicas, a cadeia produtiva do bambu no Brasil tem avançado nos últimos anos. Publicado pelo Ministério do Meio Ambiente, por meio do Programa Nacional de Florestas, o documento que apresenta reflexões sobre o uso sustentável do bambu no Brasil cita a importância da necessidade de conservação das espécies nativas por meio da implantação de bancos regionais de germoplasma (PNF, 2005).

Em abril de 2011 foi firmado entre o Ministério da Ciência, Tecnologia e Inovação do Brasil e o Ministério da Ciência e Tecnologia da China um memorando de entendimento para

promoção da cooperação bilateral em Ciência e Tecnologia na área de desenvolvimento do bambu. Em setembro do mesmo ano foi decretada a lei n° 12.484/2011 que institui a Política Nacional de Incentivo ao Manejo Sustentado e ao Cultivo do Bambu (PNMCB) e que previa apoio ao desenvolvimento tecnológico da cadeia produtiva do bambu. O Ministério da Ciência, Tecnologia e Inovação (MCTI) em conjunto com Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) lançaram uma chamada pública com o objetivo de estruturar a Rede Nacional de Pesquisa e Desenvolvimento do bambu, visando atender às demandas tecnológicas dessa nova política nacional (MCTI/Ação Transversal/CNPq n.º 66/2013). Este edital visou a formação de cinco centros de referência de estudos com bambus no Brasil. Como resultado, a Universidade Federal de Santa Catarina, por meio do Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal foi contemplada para o desenvolvimento na região Sul do Brasil.

Em 2002 o Brasil foi convidado pela International Network for Bamboo and Rattan (INBAR) para ser país membro, mas o convite foi declinado com a justificativa de excesso de gastos com as contribuições anuais. Após 14 anos, em 25 de agosto de 2016 entrou em vigor o decreto legislativo aprovando o texto do Acordo com o INBAR, o que torna o país membro da rede (BRASIL, 2016). Em janeiro de 2022 foi aprovada a Lei Ordinária Estadual N° 18.341 que instituiu a Política Estadual de Incentivo ao Desenvolvimento da Cadeia Produtiva do Bambu em Santa Catarina (SANTA CATARINA, 2022).

Reprodução e propagação de bambus

Novas plantas de bambus podem ser obtidas por método sexuado (sementes) ou pelo método assexuado (partes da planta). Embora o uso de sementes para a produção de mudas possa apresentar vantagens, sua relativa escassez impede seu uso comercial ou em larga escala. Por isso, a maioria dos bambus prioritários é geralmente propagada por métodos vegetativos (Figura 1).

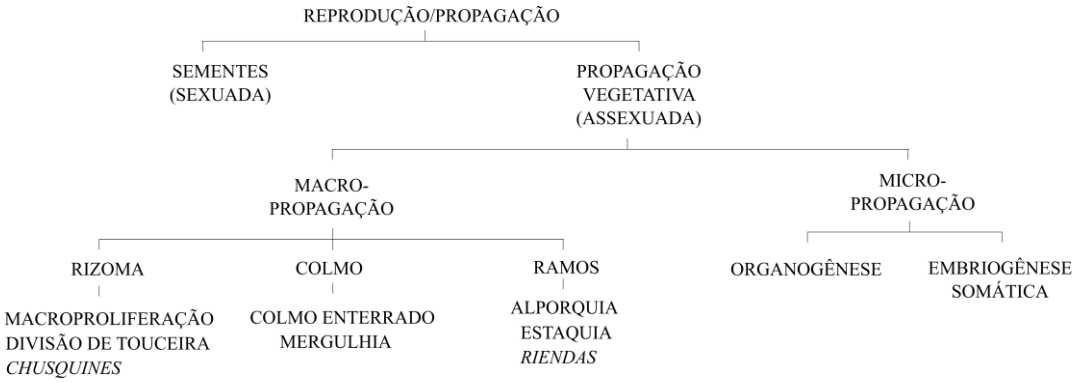

Figura 1. Esquema gráfico dos métodos de reprodução utilizados para bambus (Poaceae:Bambusoideae)

Apesar do potencial que os bambus possuem para usos industriais, a seleção de genótipos com características superiores de acordo com os objetivos (brotos, colmo, biomassa, serviços ambientais) e sua posterior propagação, ainda necessitam atenção. A diversidade de características e os usos mais convenientes de cada espécie demandam o desenvolvimento de processos específicos (Hoogendoorn et al. 2013)

O uso como substituto de madeira coloca atualmente o bambu como matéria prima à prova em processos industriais já consolidados. Isto, associado à falta de conhecimentos agronômicos e à lenta implantação de novos cultivos comerciais, impõe pressão sobre populações naturais (Gielis e Oprins, 2002). O aumento da área plantada, aperfeiçoamento do cultivo e seleção de germoplasma elite devem ser pesquisados visando maior eficiência produtiva e melhores condições de cultivo (John et al. 1994). Neste contexto, as biotecnologias para a propagação, melhoramento e conservação *ex situ* podem colaborar para o estabelecimento e desenvolvimento sustentável da cadeia produtiva.

Propagação convencional

Devido à escassez, imprevisibilidade e curta viabilidade das sementes dos bambus lignificados, a grande maioria das espécies é propagada pelos métodos assexuados. É importante ressaltar que há uma variação importante de respostas aos diversos métodos em função de vários fatores que serão elencados abaixo, mas principalmente devido às características intínsecas às espécies. Essas diferenças de eficiência dos métodos de propagação nos diferentes bambus são provavelmente devido à processos evolutivos em diferentes ambientes e à domesticação de algumas espécies. Embora o potencial propagativo não tenha sido diretamente um dos critérios para o estabelecimento de espécies prioritárias de

bambus (Priority Species of Bamboo and Rattan - Rao e Ramanatha Rao, 1998), outros critérios como, por exemplo, importância relativa em termos de uso atual, conhecimento sobre o grau de domesticação e comercialização, e germoplasma disponível ou rapidamente disponível, resultaram em umas lista com 20 bambus prioritários, na qual apenas 4 foram considerados não domesticados.

Biotecnologias associadas à propagação

A atual demanda pelo uso industrial do bambu como matéria prima excedeu a oferta, e as abordagens multidisciplinares das biotecnologias são importantes na superação deste desequilíbrio (Thapa et al., 2018).

As dificuldades de realizar o melhoramento convencional de bambus são atribuídas ao longo ciclo entre florescimentos, e assim, a seleção e fixação de genótipos com características superiores na propagação vegetativa por meio da micropropagação é uma alternativa eficiente para incremento de produtividade dos cultivos (John et al., 1994). A micropropagação é um método eficaz para a produção de mudas em escala maior que 500.000 plantas por ano (Gielis e Oprins, 2002)

A organogênese direta por brotações axilares é o método mais utilizado para propagação massal de bambus devido a sua eficiência propagativa e sua segurança quanto à baixa ocorrência de variação somaclonal (Gielis e Oprins, 2002; Negi e Saxena, 2010). Para diversas espécies foram estabelecidos protocolos para a propagação em larga escala de mudas de qualidade fisiológica e fitossanitária, e com fidelidade genotípica (Sandhu et al., 2018).

Diversos trabalhos relatam a ocorrência de florescimento em culturas de bambus *in vitro*. O primeiro relato foi realizado por Nadgauda e colaboradores (1990) para *B. arundinarea* e *D. brandisi* em culturas estabelecidas a partir de sementes. Estudos acerca do florescimento *in vitro* de bambus são fundamentais na elucidação do complexo padrão de florescimento dos bambus, e para fins de melhoramento e hibridização de espécies (Yuan et al. 2011; Yuan et al., 2017).

Os trabalhos mais atuais relatando protocolos de regeneração *in vitro* continuam sendo focados no aumento da eficiência da fase de estabelecimento de culturas, sobretudo pela redução das taxas de contaminações e escurecimento enzimático (Ray e Ali, 2017). Neste sentido, estudos relatam a identificação de microrganismos e seus potenciais benefícios, inclusive para o co-cultivo *in vitro*.

A fase de multiplicação também tem sido estudada visando o aumento de rendimento das culturas, por meio do incremento das taxas de multiplicação e reduções de custos e mão de obra. Neste sentido muitos estudos mostram efeitos positivos do uso de meio de cultura líquido para culturas *in vitro* de bambus (Saini et al., 2016). Embora isso reduza significativamente os custos, o contato contínuo das plantas com o meio favorece a ocorrência de hipoxia e desordens fisiológicas, como por exemplo, hiperhidricidade (Hazarika, 2006).

Para a rota regenerativa de embriogênese somática, os trabalhos mais recentes visam desenvolver protocolos a partir de tecidos vegetativos, sem a dependência de material reprodutivo, como as escassas sementes (Ye et al., 2017). Alguns trabalhos relatam o uso de sistemas de embriogênese somática para a transformação genética de plantas para variedades tolerantes ao frio (Qiao et al., 2014).

Antecedentes

Desde 1985 vêm sendo realizadas pesquisas no campo da cultura de tecidos, e amplo conhecimento sobre a embriogênese somática, sistemas de imersão temporária, e conservação *in vitro* foi gerado por meio da estrutura do LFDGV, sob a coordenação do prof. Miguel Pedro Guerra. Desde o ano de 2012, o grupo de pesquisas em cultura de tecidos do LFDGV vem desenvolvendo trabalhos de ciência básica e aplicada à propagação de bambus. O projeto "Tecnologias para o desenvolvimento sustentável da cadeia produtiva do bambu no Sul do Brasil" iniciou em 2013 e foi formado por 10 subprojeto nas mais diversas áreas do conhecimento acerca do tema.

Com relação ao tema da micropropagação de bambus, até o presente momento dois protocolos completos para a organogênese direta foram estabelecidos para as espécies *D. asper* e *G. chacoensis* (Ornellas et al., 2017, 2019). A partir destas culturas estabelecidas, diversos outros estudos foram realizados pelo grupo (Marchetti, 2019; Polesi et al., 2019; Rigo, 2020; Belincanta et al., 2021), além de trabalhos que seguem em andamento. Atualmente as pesquisas do grupo estão focadas no estabelecimento *in vitro* de novas espécies, aperfeiçoamento de protocolos previamente estabelecidos, estabelecimento de protocolos de embriogênese somática, conservação *ex situ* de germoplasma e caracterização e uso de microrganismos endofíticos de bambus.

Estruturação da Tese

O presente trabalho foi estruturado em três capítulos. O primeiro capítulo apresenta um protocolo de indução da calogênese em inflorescências jovens e regeneração de plantas por meio da embriogênese somática bambu gigante, *Dendrocalamus asper*. O segundo capítulo trata sobre os efeitos de diferentes citocininas na multiplicação e enraizamento de culturas organogênicas de *Bambusa multiplex*, um bambu ornamental. No terceiro capítulo avaliou-se os efeitos da Benzilaminopurina e da posição dos explantes na planta matriz durante a introdução e estabelecimento de culturas *in vitro* de *Bambusa oldhamii*, um bambu de múltiplos usos e resistente às baixas temperaturas.

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CAPÍTULO 1 - Somatic embryogenesis from young inflorescences of the giant bamboo *Dendrocalamus asper* (Schult f.) Backer ex Heyne

RESUMO

Os bambus são um importante produto florestal não-madeireiro em todo o mundo com crescente interesse devido às suas aplicações ecologicamente corretas. Além de seu uso como brotos comestíveis e colmos para uso estrutural, o bambu gigante, *Dendrocalamus asper*, é um bambu ornamental imponente para horticultura. O presente trabalho teve como objetivo estabelecer culturas *in vitro* de calos e a regeneração de plantas por meio da embriogênese somática a partir de inflorescências jovens de *D. asper*. As inflorescências pré-antese foram coletadas, desinfestadas e submetidas à indução de calos em meio basal MS suplementado com ácido 2,4-diclorofenoxiacético (2,4-D) a 0, 9, 18, 27 e 36 µM em combinação com 9 µM de Isopenteniladenina (2-iP) ou 9 µM Cinetina (Kin). Os diferentes tipos de calos que se desenvolveram foram caracterizados e subcultivados em 0, 4,5, 9 e 18 µM de 2,4-D em combinação com 9 µM de 2-iP ou Kin para multiplicação e diferenciação. Adicionalmente, o efeito do corte do explante ao meio e sua orientação no meio de cultura foi testado a fim de melhorar a indução de calos. A redução subsequente de 2,4-D para 4,5 µM resultou na maturação dos embriões somáticos. Estes foram transferidos para um meio sem fitorreguladores para a conversão em plantas. O presente trabalho mostrou a viabilidade do uso de inflorescências como explantes e a eficiência do uso de 2-iP em combinação com 2,4-D para indução de calos e regeneração de plantas *in vitro* por meio de embriogênese somática.

Palavras-chave: Espigueta de bamboo; Indução de calos; Micropropagação; Embriogênese somática secundária

ABSTRACT

Bamboos are an important worldwide non-timber forest product with rising interest due to their environmentally friendly applications. Besides its use as edible sweet shoots and culms for structural uses, the giant bamboo, *Dendrocalamus asper* is an imposing ornamental bamboo for horticulture. The present work aimed to establish *in vitro* callus culture and plant regeneration through somatic embryogenesis starting from young inflorescences of *D. asper*. Pre-anthesis inflorescences were collected, disinfested, and subjected to callus induction on MS basal medium supplemented by 2,4-Dichlorophenoxyacetic acid at 0, 9, 18, 27, and 36 µM in combination with 9 µM of Isopentenyladenine (2-iP) or 9 µM Kinetin (Kin). The different calli types that developed were characterized and subcultured in 0, 4.5 , 9 , and 18 µM of 2,4-D in combination with 9 µM of 2-iP or Kin for multiplication and differentiation. Additionally, the effect of explant cutting in half and its orientation on culture media were evaluated to improve callus induction. The 2,4-D was essential for callus induction and, combined with the two cytokinins, resulted in embryogenic callus induction and somatic embryos regeneration. The subsequent reduction of this auxin to 4.5 µM resulted in somatic embryo maturation. Somatic embryos transferred to a plant growth regulator-free medium resulted in plantlet germination. The present work showed the feasibility of using inflorescences as explants and the efficiency of using 2-iP in combination with 2,4-D to callus induction and *in vitro* plant regeneration through somatic embryogenesis.

Keywords: Bamboo spikelet; callus induction; micropropagation; secondary somatic embryogenesis

INTRODUCTION

Despite the "bamboo" generic term, the Bambusoideae subfamily (Poaceae family) comprises a wide diversity with more than 1680 species currently identified (Soreng et al. 2017; Clark and Oliveira, 2018), displaying a broad range of applications. The increasing worldwide bamboo interest is due to their economic and environmental sustainability, as they are a noteworthy ecosystem service provider (cultural, supporting, provisioning, and regulating) (Paudyal et al. 2019). Although practically every part of the bamboo is useful for food, medicine, handicrafts, construction, biomass, and matter for industry (Liese et al. 2015; Akinlabi et al. 2017), the most elegant application of bamboos is as the whole plant in the landscape ornamentation. Due to the spiritual or cultural connotation, or only because of the unique giant grass characteristics, the ornamental use of bamboos on gardens promotes benefits on both the physiological and psychological levels for the people (Wang et al. 2021; Zhang et al. 2021).

Dendrocalamus asper is a tropical non-invasive bamboo with majestic culms that can reach up to 30 m of height and more than 15 cm of diameter. Every year, the shoots sprouting is an eye-catching phenomenon, not just because of their huge dimension, but also because they provide a colorful-dynamic effect on the gardens. When those shoots emerge from the soil, they show dark-purple leaves. They are protected by bronzed sheaths, which fall after the rapid shoot elongation (+50 cm/day), revealing a whitish-brown velvety hair in basal nodes and conspicuous white nodes on the superior portion of the culm. Along with the maturation, the natural green color of the culms becomes more evident and contrasts with the new sprouting shoots and the young culms of the following season. Those peculiar features turn *D. asper* as desired bamboo with a high ornamental value for horticulture (Li and Kobayashi 2004; Banik 2016).

D. asper can be propagated by seeds, culm cuttings, air-layering, offsets, macroproliferation, and micropropagation (Singh et al. 2012; Zang 2019). Although seeds can be an easy way to obtain seedlings, the flowering cycle of *D. asper* happens in intervals of around 30-100 years, and until now is an unpredictable phenomenon. When available, seeds have short viability and an unknown genetic background (Arya et al. 1999; Banik, 2016). Because of that, the vegetative propagation methods are the most practiced. However, they are infeasible on a large-scale plantlet demand due to the size of some propagules type and high labor and transport costs (Arya et al. 1999). Besides this, a fluctuation in rooting efficiency occurs over the seasons and between the genotypes (Ray and Ali 2017a).

For those reasons, micropropagation has consolidated as a process for large-scale quality plantlets production, with additional advantages for breeding, *ex situ* conservation, and basic research purposes. The bamboo *in vitro* culture establishment can be reached from different explants, such as isolated zygotic embryos, whole seeds, shoot apex, nodal segments, leaves, roots, and inflorescences (Ojha et al., 2009). Inflorescences can be the last somatic resource to *in vitro* regeneration of selected genotypes in the case of monocarpic flowering (Arya et al., 2008). Due to the scarcity of that kind of explant, few papers reported the flowering tissue employment for bamboos micropropagation. In Table 1 is presented a summary of relevant reports of inflorescence tissue culture approaches for Bambusa and Dendrocalamus species over the last decades. Due to the rare availability of inflorescences, the present work aimed to establish a callus induction and plant regeneration through somatic embryogenesis from young inflorescences of *Dendrocalamus asper.*

Table 1 Reports of micropropagation from flower structures of some important Bambusa and Dendrocalamus species.

Species	Pathway	Explant	PGRs		Reference
			Callus Induction	Plant Regeneration	
B. oldhamii	SE	Young inflorescences	2,4- D (13.4 µM) + Kin (9.3 µM)	2,4-D (13.4 µM) + Kin (9.3 µM)	Yeh and Chang (1986 a)
B. beecheyana var. beecheyana	SE	Young inflorescences	2,4 – D (13.4 µM) + Kin (9.3 µM)	NAA + IBA	Yeh and Chang (1986 b)
B. oldhamii (= *Sinocalamus latiflora*)	SE	Anthers	2,4 – D (4.5 µM) + BAP (4,4 µM)	-	Tsay et al. (1990); Tsay et al. (1996)
B. oldhamii, *D. latiflorus*, and *D. asper*	SE	Immature inflorescences	2,4-D (13,5 - 27 µM)	NAA (1,4 – 5,4 µM) + BAP (2,2 – 8,8 µM)	Prutpongse and Gavinlertvatana (1992)
D. giganteus	Indirect Org.	*In vitro*-regenerated spikelets	2,4-D (33.9 µM) + NAA (16.1 µM)	-	Ramanayake and Wanniarachchi (2003)
B. edulis	Direct Org; SE	Inflorescences	2,4-D (45.3µM)	-	Lin et al. (2005)
B. balcooa	SE	Pseudospikelets	2,4-D (4.5 µM)	BAP (22.2 µM)	Gillis et al. (2007)
D. latiflorus	SE	Inflorescences	2,4 – D (13.4 µM) + Kin (9.3 µM)	TDZ (0.45 µM)	Lin et al. (2007)
D. asper	Direct Org.	Young and mature inflorescences	-	BAP (31.1 µM)	Arya et al. (2008)
B. multiplex	SE	Spikelets	2,4-D (18.1 µM)	Kin (13.9 µM) + BAP (13.3 µM)	Yuan et al. (2009)
D. latiflorus	SE	Anthers	BAP (1,33 µM) + NAA (5.37 µM) + PAA (110.17 µM)	BAP (8.89 µM) + Kin (2.32 µM) + NAA (1,08 µM) + PAA (110.17 µM)	Qiao et al. (2013)

PGR – Plant growth regulator; SE – Somatic embryogenesis; Org. – Organogenesis; 2,4-D – Dichlorophenoxyacetic acid; Kin – Kinetin; NAA – Naphthaleneacetic acid; IBA -Indoleacetic acid; TDZ – Thiadiazuron; BAP – Benzylaminopurine; PAA – Phenylacetic acid.

MATERIAL E METHODS

Plant material

Young inflorescence (pre-anthesis stages) of *Dendrocalamus asper* were collected at Fazenda dos Bambus (-23.110470 °; -48.368193 °), of the Jatobás Institute in the municipality of Pardinho, São Paulo, Brazil. Branches with young synflorescences (Fig. 1a) were collected along with the sporadic flowering of about 20 clumps in a plantation of 600 plants.

Disinfestation and isolation of inflorescences

The explant disinfestation method was carried out in three steps: (1) Branches containing developing synflorescences were selected. Prophylls and senescent glumes were removed before the branches were immersed in 70 °GL ethanol for two minutes and transferred to sodium hypochlorite solution (2% active chlorine) with Tween^{TM} 20 (1 drop / 100 ml) and kept under constant stirring for 20 minutes. The branches were then rinsed twice with sterile deionized water and exposed for 10 minutes to the chamber's airflow on sterile filter paper for drying; (2) Synflorescences were excised from the branches and subjected to immersion in 70 °GL ethanol for 30 seconds, followed by immersion in sodium hypochlorite solution (2% active chlorine) supplemented with Tween^{TM} 20 for 5 minutes, and then rinsed with sterile deionized water; (3) The central inflorescences of each synflorescence were selected and reduced by the removal of the lowermost glumes. Then, they were subjected to sodium hypochlorite solution (2% active chlorine) with Tween^{TM} 20 under agitation for 15 minutes, followed by a triple wash in sterile deionized water. Before inoculation, the inflorescences were subjected to a further size reduction, by removing 1 to 2 basal anthecia and the excision of the basal tissue damaged by chlorine.

Callogenesis induction

The basal culture media used for all the experiments consisted of the MS saline formulation (Murashige and Skoog, 1962), supplemented with 30 $g.L^{-1}$ of sucrose, 250 $mg.L^{-1}$ of polyvinylpyrrolidone, 2 $ml.L^{-1}$ of Morel's vitamins (Morel and Wetmore, 1951), 1 $g.L^{-1}$ of glutamine, 1 $g.L^{-1}$ of Myo-inositol and 0.5 $g.L^{-1}$ of hydrolyzed casein. The PGRs were added before the pH of the medium was adjusted to 5.8. The media were sterilized by autoclaving for 15 minutes at 121 °C. Two subsequent induction experiments were carried out to determine: (1) the ideal 2,4-D concentration in combination with the cytokinins 2-

Isopentenyladenine (2-iP) or Kinetin (Kin), and (2) the effect of explant sectioning and its position onto the culture media. All the cultures were kept in the dark at 25 ± 1 °C.

Exp. 1: Effects of 2,4-D combined with 2-iP or Kin

After the disinfestation procedure, inflorescences were inoculated in culture media containing 0 µM, 9 µM, 18 µM, 27 µM, and 36 µM 2,4-D in combination with 9 µM 2-iP or 9 µM Kin. That cytokinins concentration was settled based on the induction medium proposed by Yeh and Chang (1986a). The treatments consisted of 8 repetitions of a Petri dish (60 mm X 15 mm), each containing 5 explants inoculated on 15 ml of culture medium. At 30 days after *in vitro* inoculation, the contamination rate, the callus induction rate, the oxidation rate, and the swelling of the explants were evaluated.

Exp. 2: Effect of the explant sectioning and its position onto the medium

After determining the ideal callus induction concentration, disinfested inflorescences were inoculated longitudinally sectioned or non-sectioned (entire). The sectioned explants were inoculated onto the medium in two ways: with the area exposed by the cut (1) upwards or (2) downwards, in contact with the culture medium. The treatments consisted of 20 repetitions of a Petri dish containing 4 explants inoculated on 15 ml of culture medium. At 30 days after inoculation, the callus induction and oxidation rates were evaluated, and the obtained calli were characterized according to their morphology.

Multiplication and differentiation

Calli cultures maintained by two subcultures in the induction medium were transferred to multiplication media with 0 µM; 4.5 µM; 9 µM; 18 µM 2,4-D. Those concentrations were proportional reductions of 100%, 75%, 50%, and 0% of the ideal dose of 2,4-D used for callus induction, as established in experiment 1. At 30 days of transfer to the new treatments, the rate of oxidation, differentiation, and the frequency of each type of callus multiplied were evaluated.

Statistical analysis

Percentage data were obtained by averaging the binomial values of occurrence in each Petri dish. The analysis of variance assumptions was verified by analyzing the residues distribution of the model and the application of Bartlett's homoscedasticity test. When necessary, the percentage values were submitted to sine arc transformation. Statistical analyzes were

performed in the R Studio environment (R 3.6.2). Variance analysis was performed using the "base", "stats" (R Core Team, 2019), and "agricolae" (Mendiburu, 2019) packages.

Somatic embryos conversion and acclimatization

Isolated and fused somatic embryos were transferred to the basal culture medium without PGR for maturation and plantlet conversion. The cultures were maintained for 15 days in the dark and subsequently exposed to the 16 h photoperiod for another 30 days. Finally, the obtained plantlets were subcultured to the basal medium in test tubes for 30 days for axillary branching, elongation, and root development before the acclimatization step. Then, the plantlets were transferred to 1 L pots containing a commercial substrate (Tropstrato FT - Vida Verde). Those plants were acclimatized in a shading greenhouse (65 % shading net and 20° C – 30°C) under intermittent nebulization (80 % - 100 % relative humidity).

Histochemical analysis

Samples of induced inflorescences, histo-differentiated callus section, and isolated somatic embryos were fixed in glutaraldehyde for 48 hours. The material was dehydrated in an increasing ethanol series up to anhydrous conditions (Ruzin, 1999). Therefore, the samples were infiltrated and included in hydroxyethyl methacrylate (Leica Historesin, Germany). Cross-sectional and longitudinal sections of 5 µm thickness were obtained in a rotating microtome RM 2125 (Leica, Germany) and stretched into slides. The sections were stained with toluidine blue in pH 6.8 phosphate buffer according to O'Brien et al. (1964) for the observation and identification of anatomical structures. The images were captured in a DP71 camera attached to the BX-40 microscope (Olympus, Japan).

Potential applicability of the protocol in other species

After defining the most suitable concentration of 2,4-D combined with 9 µM 2iP, we tested the proposed protocol in other bamboo species. We used inflorescences of *Bambusa oldhamii* Munro and *B. tuldoides* Munro, both as a sporadical flowering event. The *B. oldhamii* plant was a young plant, shade-stressed under a running bamboo (*Phyllostachys bambusoides*) canopy, at Sítio Vagalume (-27.660420 °; -49.151541 °), in Rancho Queimado, Santa Catarina, Brasil. Flowers of *B. tuldoides* are more common to find in isolated flowering culms in some clumps, and they were obtained at Fazenda Experimental da Ressacada (-27.682318 °; -48.537550 °) in Florianópolis, Santa Catarina, Brazil. Flowers of both species were subjected to the tree-step assepsy process described above and introduced *in vitro* in the most

suitable 2,4-D and 2iP concentrations defined for *D. asper* to validate the methodology and observe their effects on other species of the Bambuseae tribe.

RESULTS AND DISCUSSION

Disinfestation and isolation of inflorescences

The three-step disinfestation methodology and the closed-flowering compactness of the explants resulted in a high rate of viable cultures. In the first experiment, a contamination rate of only 4.2 % was observed after 30 days of the *in vitro* introduction. In experiment 2, only 3.7 % of the explants presented microorganisms manifestation. In both *in vitro* introductions, of these few contaminated explants, most of them manifested a late and slow-growing microorganism. Few cases of mycelial growth were observed.

Bamboos *in vitro* cultures are commonly recognized as hard-to-establish due to their microorganisms association and morphological characteristics, which usually can make disinfestation difficult (Tsay et al. 1990; Ray and Ali 2017b). Endogenous contamination is an important constraint during establishing bamboos *in vitro* cultures, mainly in explants obtained from mature and field-growth plants (Oprins et al. 2004). Lin et al. (2007), using *D. latiflorus* inflorescences for callus induction, reported the loss of 70 % of explants due to contamination. Arya et al. (2008) reported the loss of 30 % - 40 % of the explants due to contamination in the induction of shoots from *D. asper* inflorescences. Other studies that used inflorescences or isolated floral pieces from bamboo as explants did not report the contamination rates after disinfestation (Yeh and Chang, 1986a, 1986b; Lin et al., 2003; Lin et al., 2005; Qiao et al., 2013).

Callus induction and oxidation

In the first days after *in vitro* introduction, the swelling of the explants was observed, most notably in the treatments containing only cytokinin (2-iP or Kin). Rates of 52% and 25% of swollen explants were observed, respectively, in response to treatments containing only 9 µM 2-iP or 9 µM Kin. Yeh and Chang (1986 a, b) reported similar results of swelled explants during callus induction from two *Bambusa* species inflorescences. Cytokinins are plant growth regulators involved in many complex physiological mechanisms, playing a key role in cell enlargement and proliferation. During the anthesis, most of the spikelet subtending organs are in full-growing for anthers and stigma exsert, and susceptible to exogenous PGRs increases effects. Most of the explants in 2,4-D-free treatments showed tissue oxidation from

the second subculture. The indeterminate flowering bamboos present inflorescences with a basal bud, which can regenerate new inflorescences, known as pseudospikelets (McClure, 1966). It is possible under *in vitro* conditions to revert the flowering process by shoots regeneration through exogenous hormonal stimulus, mainly by cytokinins PGRs (Ramanayake and Yakandawala, 1998; Arya et al. 2008). Another possible approach is the transfer of the flowering to the *in vitro* condition through the inflorescences' *in vitro* introduction and its further multiplication (Lin et al. 2003 b). Despite the *D. asper* having that kind of buds, its removal during the disinfestation procedure (glumes successive remotion and basal cutting before inoculation) impaired any direct inflorescence multiplication or shoot regeneration in the present study, even in the cytokinin alone treatment. The following flowering opportunities should consider pre-formed buds for *in vitro* inflorescence establishment, aiming for a continuous explant *in vitro* source.

Callus induction occurred exclusively in treatments containing 2,4-D, and most of them originated from the basal sectioned region of the inflorescences (Fig. 1b).

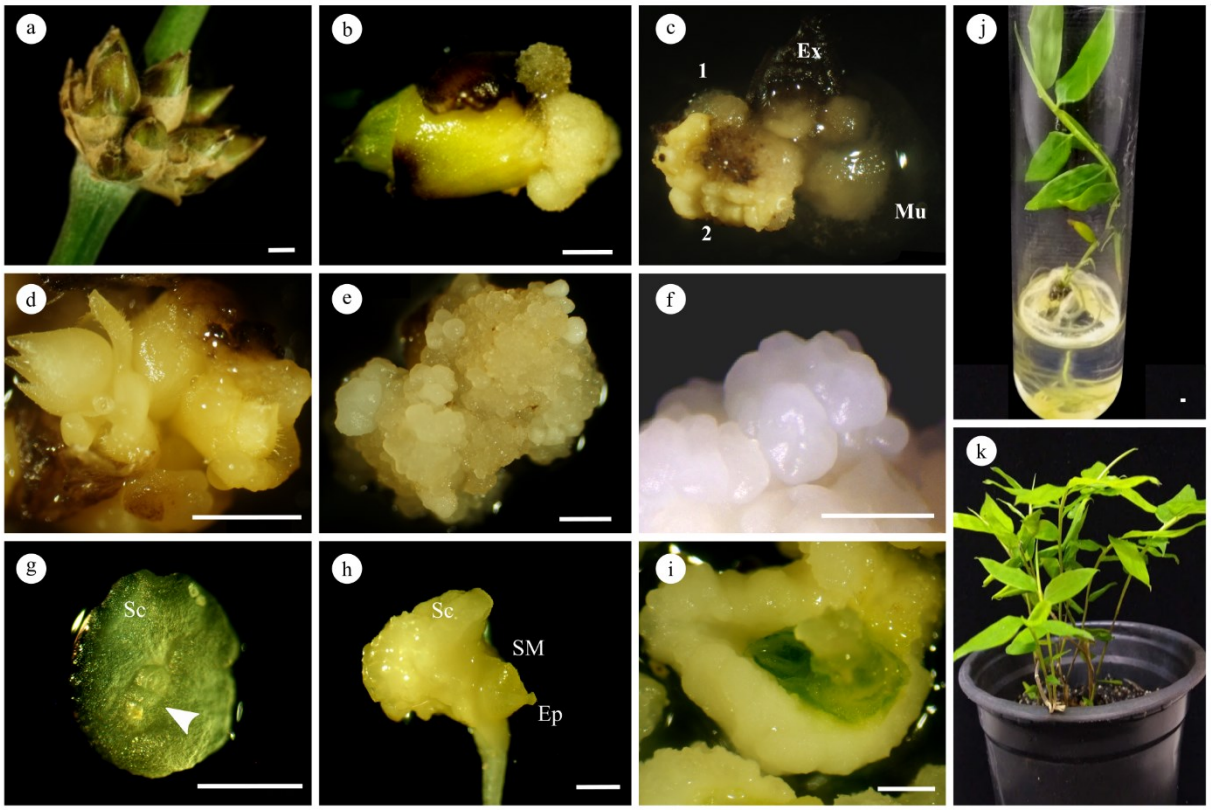

Fig. 1 Somatic embryogenesis from young inflorescence of *Dendrocalamus asper*. (a) Synflorescence; (b) Callus induction on the basal region of inflorescence after 30 days of the *in vitro* introduction. (c) The different callus morphology obtained from inflorescence at the 60th day of induction, 1- Type 1 callus, 2- Type 2 callus, Mu- Mucilaginous callus, Ex- Oxidated explant; (d) Type 2 callus regenerating inflorescence-like structure. (e) Type 2 callus regenerating somatic embryos; (f) Somatic embryos; (g) Somatic embryo with foregrounded embryonic axis (arrowhead); (h) Initial plantlets conversion of a somatic embryo during the dark-phase, Sc- Scutellum, SM- Shoot meristem, Ep- Epiblast; (i) Somatic embryos with green shoot apex during the light-phase of plantlet

conversion, showing a rough scutellum starting secondary embryogenesis; (j) Somatic embryo-converted plantlet; (k) Acclimatized plantlet. Bars = 1 mm

An increase in the 2,4-D doses raised the callus induction rate, regardless of the combined cytokinin (Table 2). That effect was stronger in response to 2,4-D and 2iP, reaching the higher callus induction rate (77.5 %).

Table 2 Callus induction rate and oxidation rate of explants on the 30th day of *Dendrocalamus asper* inflorescences *in vitro* culture.

CK (9 µM)	**2,4-D (µM)**	**Induction (%)**	**Oxidation(%)**
2-iP	0	00.0±00.0 c	87.5±05.3 a
	9	40.0±10.7 b	65.0±07.3 ab
	18	72.5±11.9 a	32.5±11.9 cd
	27	65.0±08.2 a	67.5±07.5 ab
	36	77.5±08.0 a	20.0±08.4 d
Kin	0	02.5±02.5 c	52.5±10.0 bc
	9	30.0±11.9 b	70.0±11.9 ab
	18	37.5±04.5 b	72.5±05.2 ab
	27	45.0±10.5 b	60.0±10.7 ab
	36	40.0±08.4 b	57.5±08.0 bc

CK - Citokynin; 2,4-D – Dichlorophenoxyacetic acid; 2-iP- 2-isopentenyl adenine; Kin – Kinetin;
Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test (p <0.05). Means obtained from 8 repetitions (n=40).

Even though callogenesis was also induced in the 2,4-D combined with Kin, the auxin increment did not significantly increase the calli induction. The combination 18 µM 2,4-D + 9 µM 2iP was fixed as the ideal for callus induction due to the high induction effect associated with a low oxidation rate in the inflorescence explants. The 2,4-D has been reported for callogenesis and somatic embryogenesis in several bamboo species (Obsuwan et al., 2019). In young shoots of *D. hamiltonii* (Godbole et al., 2002) and zygotic embryos of *P. edulis* (Yuan et al., 2013), callus induction occurred only in a culture medium containing 2,4-D, and the induction frequency was affected by its concentration. For leaves, roots, and nodal segments of *D. asper* calli induction, it was necessary 30 µM 2,4-D (Ojah et al. 2009). For inflorescences of *B. oldhamii*, *D. latiflorus*, and *D. asper* calli were obtained using 13.5-27 µM of 2,4-D on the culture medium (Prutpongse e Gavinlertvatana, 1992).

Histological sections of explants revealed a cell proliferation on floret subtending tissues (i.e., glume, palea, and lemma) after 7 days of induction (Figure 3a). Those structures were membranous and vascularized, which in association with injury stress during the early stage can be suitable tissues for callus induction. Considering that the palea and lemma are two bract-like structures, i.e., a modified leaf (Lombardo and Yoshida 2015), we could very likely assume that the medium composition of our protocol might be successfully applied to other young vegetative explants as well.

Calli morphology

Most of the obtained cultures showed more than one type of calli, already in the first 30 days of induction (Fig. 1c). In general, three distinct types of calli were obtained: A less common but generally initial was a mucilaginous translucid callus with whitish cell clusters dissociated (Fig. 2a).

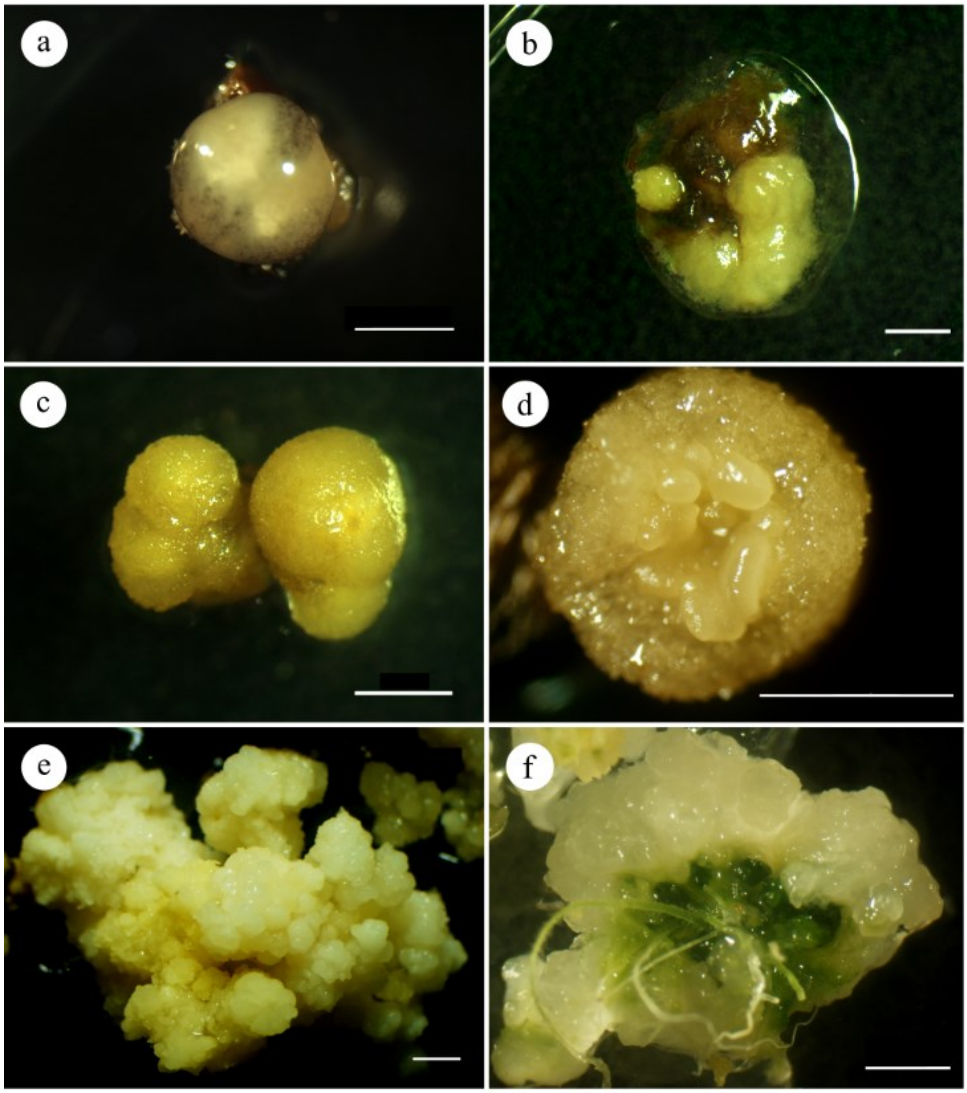

Fig. 2 *Dendrocalamus asper* inflorescence-derived calli types. (a) Mucilaginous callus; (b) Type 1 callus regenerating from a mucilaginous callus; (c) Type-1 callus; (d) Type-2 callus regenerating from type-1 callus; (e) Type-2 callus regenerating somatic embryo; (f) Secondary somatic embryogenesis on a scutellum tissue of a fused converting somatic embryos cluster. Bars= 2 mm

The most common type 1 callus was translucid to yellowish with friable texture and small-nodular growth (Fig. 2c). The type 2 callus was yellowish to white, with a compact appearance and initial protoderm development, showing a smoother surface from where somatic embryos developed (Fig. 2e). A gradual and successive regeneration occurred among these calli. Type 1 callus could be obtained from the mucilaginous, and the type 2 embryogenic calli were usually derived from that primary one (Fig. 2b,d). Histological analysis of type 2-derived from type 1 callus revealed a protoderm organization around a meristematic center (Figure 3b). Qiao et al. (2013) reported the development of embryogenic calli derived from primary calli generated from *D. latiflorus* anthers. The simultaneous different calli morphology occurrence was also reported for *B. glaucescens* (Jullien and Van,

1994). In general, type 1 callus was fast-growing and more prone to oxidation as compared to type 2.

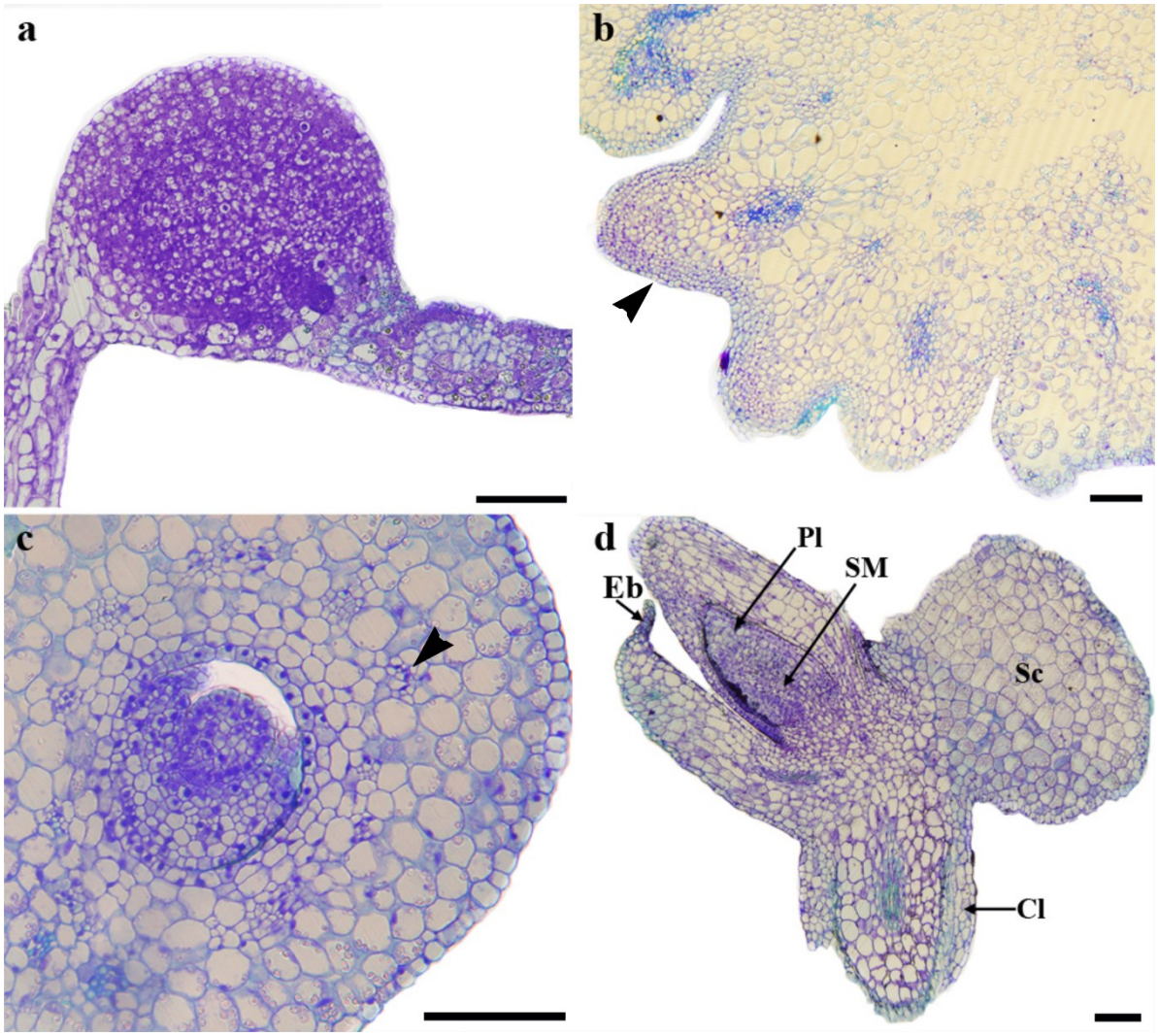

Fig. 3 Histological analysis in *Dendrocalamus asper* calli and somatic embryo obtained from young inflorescences. (a) Longitudinal section of inflorescence lemma tissue at 7th day of *in vitro* induction showing an initial callogenesis; (b) Longitudinal section of callus with an organized protoderm developing (arrow) from a type 1/type 2 callus; (c) Cross-section of the scutellar somatic embryo showing meristematic centers around the embryo axis (arrowhead); (d) Longitudinal section of a somatic embryo, Sc- Scutellum, Pl- Plumule, SM- Shoot Meristem, Eb- Epiblast, Cl- Coleorhiza. Bars = 100 µm

Few type-1 calli had regenerated root clusters, probably originating from somatic embryos that were not classified as so during the selective subculture or even the direct root regeneration from type-1 callus. As soon as the roots touched the culture medium surface, a mucilaginous loose callus induction was observed, similar to the reported by Yeh and Chang (1986 b) and Chang and Lan (1995). That observation again highlights the potential of bamboo roots as a vegetative starting material for *in vitro* callus induction.

Calli multiplication and histodifferentiation

The combination of 2,4-D with the different cytokinins resulted in differential responses of the explants to oxidation occurrence and calli type produced (Table 3).

Table 3 Occurrence rates of type 1 and type 2 calli, morphogenetic differentiation, and oxidation of *Dendrocalamus asper* calli cultures derived from young inflorescence, during the somatic embryogenesis multiplication step.

CK (9 µM)	2.4-D (µM)	Callus occurrence		Differentiation (%)	Oxidation (%)
		Type 1 (%)	Type 2 (%)		
2-iP	0.0	44.4 ± 06.1 bc	22.2 ± 06.1 ab	16.7 ± 09.9 ab	55.6 ± 06.1 a
	4.5	72.2 ± 13.8 ab	44.4 ± 12.2 ab	22.2 ± 09.6 ab	16.7 ± 09.9 ab
	9.0	94.4 ± 4.81 a	27.8 ± 08.9 ab	22.2 ± 06.1 ab	5.56 ± 04.8 b
	18.0	100.0 ± 00.0 a	16.7 ± 11.8 b	16.7 ± 11.8 ab	00.0 ± 00.0 b
Kin	0.0	25.9 ± 08.8 c	58.3 ± 11.8 a	43.7 ± 08.3 a	27.1 ± 12.6 ab
	4.5	90.5 ± 08.9 a	33.3 ± 09.6 ab	00.0 ± 00.0 b	23.8 ± 13.1 ab
	9.0	71.4 ± 10.6 ab	09.5 ± 08.9 b	09.5 ± 05.7 b	33.3 ± 13.6 ab
	18.0	25.0 ± 17.7 c	25.0 ± 11.3 ab	00.0 ± 00.0 b	58.3 ± 17.7 a

CK - Citokynin; 2,4-D – Dichlorophenoxyacetic acid; 2-iP- 2-isopentenyl adenine; Kin – Kinetin.
Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test ($p < 0.05$). Means obtained from 8 repetitions (n=40).

When in combination with 2,4-D, 2-iP effectively promoted the induction of type 1 callus compared to Kin. In contrast, Kin reached the higher rate of type 2 callus in the 2,4-D free medium. Regardless of the cytokinin, the 2,4-D reduction to 4,5 µM or its total removal of the culture media promoted type 2 calli and the regeneration of somatic embryos. The type of cytokinin affected the oxidation rate of the cultures. In 2-iP alone-medium or in combination with reduced levels of 2,4-D (4.5 µM 2,4-D + 9 µM de 2-iP), there was an increase in calli oxidation rates, especially on the type 1 calli. In the Kin-containing media, 2,4-D did not influence oxidation rates. The oxidation incidence is usually observed during the multiplication step of bamboo calli cultures. Qiao et al. (2013) reported the common occurrence of *D. latiflorus* anther-derived calli during the multiplication step.

From the second subculture on the induction medium, type 2 callus, inflorescence-like structures (Fig. 1d), and somatic embryos regenerated (Fig. 1e-f). In induced callus from *D. latiflorus* anthers, shoots were obtained during the induction step (Qiao et al. 2013). Yeh e Chang (1986 b) reported the somatic embryos' regeneration during the 16 months long culture

on 2,4-D and Kin-containing medium. In the present work, an increase in the cytokinin:auxin ratio promoted differentiation of structures on type 2 callus and subsequent differentiation of somatic embryos. Similar observations occurred for *D. hamiltonii* with the gradual reduction of 2,4-D and NAA concomitant with BAP increases in the culture medium (Godbole et al., 2002). As for many plant calli cultures, a higher cytokinin concentration than auxin in bamboos can enhance the shoots and embryos regeneration. In bamboos calli cultures, a concentration-ratio of 2:1 (cytokinin: auxin) was feasible to regenerate shoots (Prutpongse and Gavinlertvatan 1992).

Explant sectioning

The longitudinal sectioning of inflorescences further increased the callus induction rates than non-sectioned inflorescences (Table 4).

Table 1

Table 4 Callus induction and explant contamination rates of *Dendrocalamus asper* young inflorescences in the function of longitudinal section and the explant orientation on the culture medium.

Longitudinal sectioning	Explant orientation inoculation	Induction (%)	Contamination (%)
-	Non-sectioned	80,4 ± 04,5 b	5,0 ± 2,3 a
+	Downward cutting	63,3 ± 06,7 c	3,7 ± 2,0 a
+	Upward cutting	98,7 ± 01,2 a	2,5 ± 1,7 a

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test (p <0.05). Means obtained from 20 repetitions (n=80).

The orientation of these half-inflorescences onto the culture medium also influenced the callus induction rates. The cut-surface inoculation in contact with the culture medium (downward cutting) reduced the callus induction rate. The inoculation of half-inflorescence in an upward cutting position resulted in a significant increment in the callus induction rate, resulting in a callus induction in almost 100 % of the explants. The explant sectioning and its inoculate position onto the medium did not influence the contamination rates observed. Regardless of the treatment, most calli started on the sectioned basal region of the inflorescence. Similar observations were reported (Godbole et al. 2002), in which initial swelling and cellular proliferation began from the cut ends segmented shoots of *D. hamiltonii*. Explants inoculated with cut sections upward oriented developed calli from the borders. It was also observed callus initiation from the anthers. After 30 days, a subsequent subculture on the same induction medium of those anther-derived calli resulted in oxidation, and embryonic

callus (type 2) was not observed. Although anther-derived callus could result in haploid plants, interesting for breeding purposes (Tsay et al. 1990), the low induction rate compared to the enclosing tissues of inflorescences showed the need for improvement on the induction procedure in anthers. The inflorescence longitudinal section enhanced the induction rate by relieving the physical blocking of the enclosing tissues of spikelets. Additionally, mechanical wounding is recognized and commonly adopted for cellular differentiation induction by overexpression of stress-related genes (Fehér et al. 2003; Wójcik et al. 2020). Furthermore, the explant size-reduction increases the surface-area-to-volume ratio, promoting the stress triggered by the *in vitro* environment. Large explants or intact organs can show, even that explanted, a well-organized symplastic pathway and stable cellular metabolism, which can lead to recalcitrance to *in vitro* responses. The wounding of tissues often results in symplastic domain rupture and can stimulate the establishment of new domains and plasmodesmata cell-to-cell communication, promoting differentiation and expression of pluripotentiality (Bonga, 2016). Even though the small size of the inflorescences used as explants for those experiments, the sectioning showed a feasible and straightforward technique to increase the explant number, callus induction area, and the callus induction rate. This result suggests that using the thin cell layer technique may be beneficial to increase callus induction from bamboo spikelets.

Secondary somatic embryogenesis

A scutellum deformation was observed when somatic embryos were kept in a multiplication medium containing the higher 2,4-D levels (9 µM to 18 µM). That occurred mainly on somatic embryos in late developmental stages (Fig. 1h). The scutellar tissue of zygotic and somatic embryos was 2,4-D-responsive for other Dendrocalamus species (Sumathi et al. 2003; Zhang et al., 2010). In our work, during the selective multiplication, easily detached and rounded-shaped somatic embryos were subcultured isolated on 18 µM 2,4-D + 9 µM 2-iP (induction medium). Just a minor growth on the root region of the embryo axis was observed. At the same time, a distinguished cellular proliferation occurred around the somatic embryo axis, specifically on the scutellum tissue (Fig. 1i), where it was possible to observe meristematic centers (Figure 3c). That observation led to an understanding of the origin of scutellum-fused embryos clusters and evidenced the 2,4-D inducing effect of secondary embryogenesis in the scutellum tissues. The maintenance of embryogenic calli in 2,4-D containing media leads to secondary embryogenesis from the regenerated embryos. Plant regeneration from these fused embryo clusters (Fig. 2f) was inefficient because of their non-

synchronic maturation. Long-term cultivation on a 2,4-D-containing medium led to clusters of reduced-size fused somatic embryos, which were unable to maturation and plant regeneration. Therefore, a further study focusing on the control of secondary somatic embryogenesis is suggested to improve normal embryo regenerating rates and control the repetitive multiplication step.

Plantlet's regeneration

Somatic embryos regenerated in all the tested media during the multiplication step. Normal somatic embryos had translucent to white color showing a regular rounded to spherical shape with the embryonal axis detached on their central region. Most of them were easily detachable from the callus surface (Fig. 1g). The conversion to plantlets occurred faster from the round somatic embryos with smooth epidermis than from those with the rough epidermis (Fig. 1f-i) or with fused-scutellum (Fig. 2f). Greening of the embryonic axis was observed after 7-10 days of 16 h photoperiod exposition, and shoots and roots developed after 10-14 days. Histochemical analysis showed a considerable starch accumulation in the scutellum tissue (Figure 3d), which probably provoked the yellowish to the white color of the somatic embryos. Similar features were observed in *Phyllostachys edulis* somatic embryos (Yuan et al. 2013). That increased starch content on scutellum tissue was probably due to the sucrose enriched medium and the lack of endosperm and aleurone layer on somatic embryos. Both structures are primarily responsible for the starch accumulation and starch related-enzymes in seeds. In somatic embryos of D. hamiltonii, the scutellum substituted those structures in the amylase accumulation and starch deposition (Godbole et al. 2004). In the present work, the successfully converted plantlets were transferred to test tubes with the basal medium, where synchronous rooting and shoot elongation was observed. The typical broad-leaves development of embryonic seedlings was also observed during the plantlet elongation (Fig. 1j). After 45 days, converted plants started tillering and were acclimatized (Fig. 1k).

Potential applicability of the protocol in other species

Inflorescences of *B. oldhamii* and *B. tuldoides* showed related results when exposed to the 2,4-D and 2iP combination for callus induction. For both cases, the collection of early-stage inflorescences allowed the establishment of cultures and callus induction. Despite the few inflorescences accessed from sporadical flowering, types 1 and 2 calli regenerated from inflorescences and led to the formation of primary and secondary somatic embryos.

Differently of Dendrocalamus spp., the species which belongs to the Bambusa genus show the open-flowering type (Zhang 2002). Due to that, the collection of young-stage material is even more necessary to keep the inflorescence compactness and resistance to the disinfestation procedure and the less microbial load. For *B. oldhamii*, as in *D. asper* cultures, were observed inflorescences-like structures regenerating from type-2 callus (Figure 4).

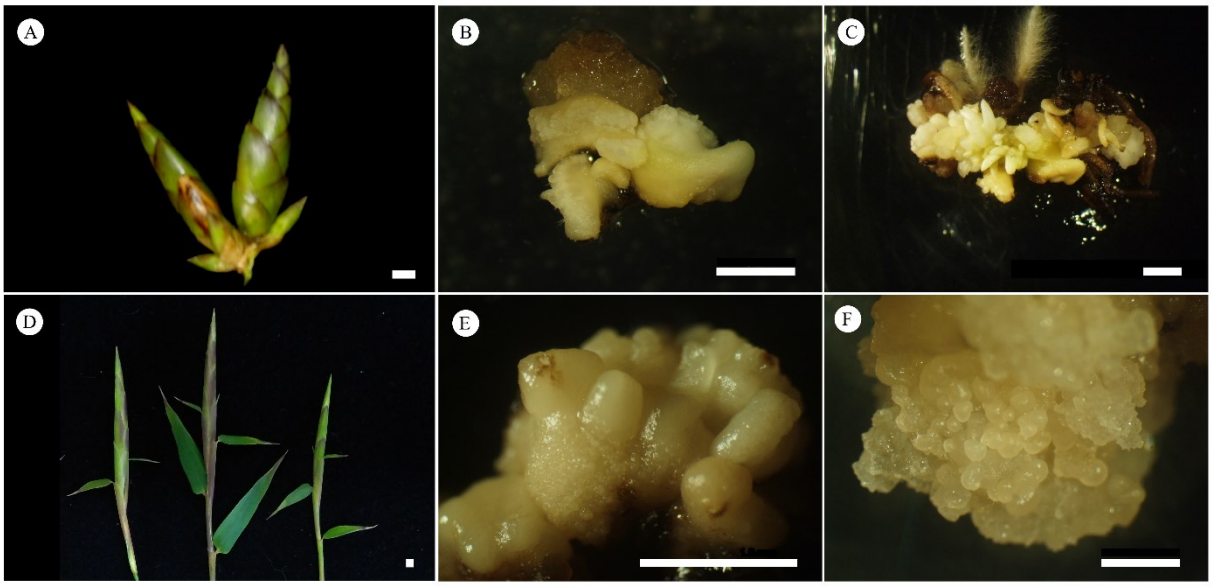

Fig. 4 Preliminary observation in callus induction and somatic embryo regeneration in *Bambusa oldhamii* and *B. tuldoides* using MS medium supplemented with 2,4-D and 2-iP. (A) *Bambusa oldhamii* inflorescences; (B) *B. oldhamii* morphogenetic differentiation on type-2 callus derived from type-1 callus; (C) *Bambusa oldhamii* somatic embryos regeneration with leaf-like scutellum; (D) *Bambusa tuldoides* inflorescences; (E) Type 2 callus regenerated from type 1 callus in *Bambusa tuldoides* (F) Regenerated globular somatic embryos from a *Bambusa tuldoides* type 2 callus. Bars = 2 mm

The young inflorescences used as explants in the present study are morphologically similar to young vegetative shoots emitted from culm buds during the sprouting season. That similarity suggests the need to evaluate if the procedure was also suitable for young shoots of *in vitro* organogenesis regenerants plants, which can be longitudinally or transversally sectioned. A similar approach was already proposed for *Dendrocalamus hamiltonii* (Godbole et al. 2002) and effectively applied for *Thyrsostachys siamensis* (Obsuwan et al. 2019). Its application with the present induction medium 2,4-D and 2iP combination could be more practical from the point of view of plant material availability than floral explants. Further investigation should be performed in that way, mainly because nodal segments are most applicable. The *in vitro* culture establishment can provide a more significant number of explants for callus induction and plant regeneration.

CONCLUSION

The present work described a somatic embryogenesis protocol from young inflorescences of the giant bamboo, *Dendrocalamus asper* (Figure 5).

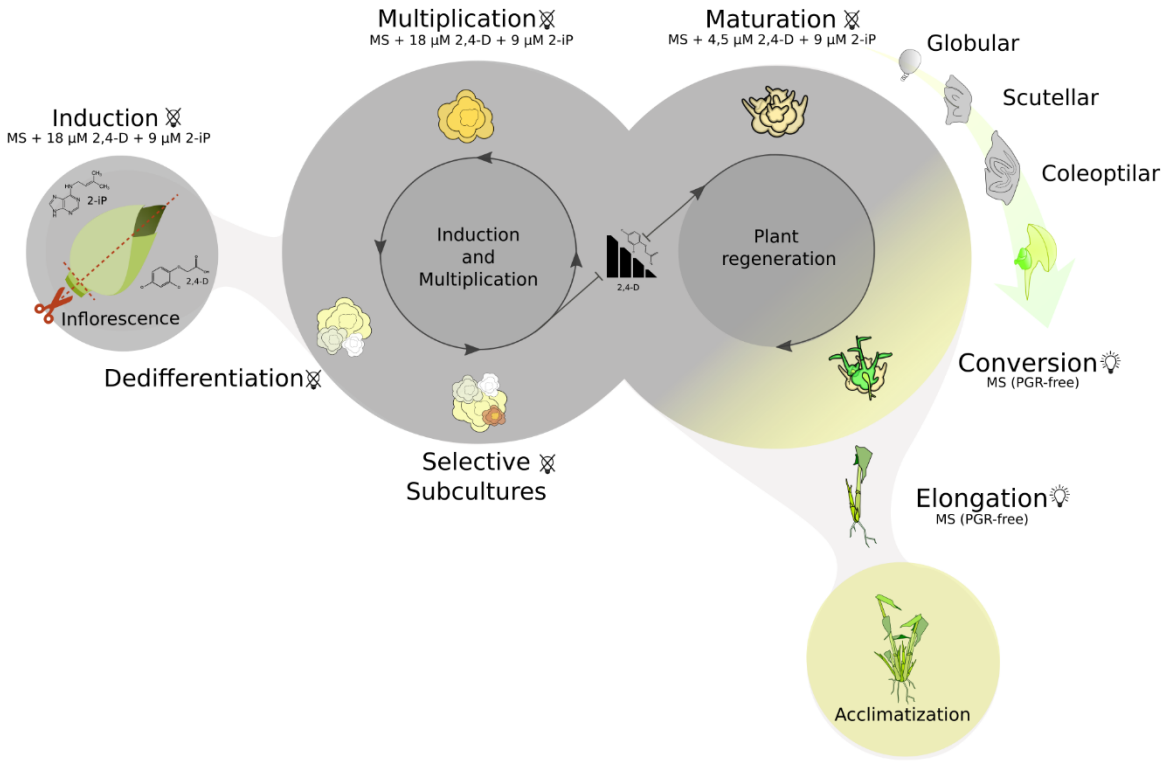

Fig. 5 Graphical summary of *in vitro* somatic embryogenesis protocol for the giant bamboo *Dendrocalamus asper*.

A 3-step disinfestation procedure using ethanol and sodium chlorine in inflorescence explants allowed more than 95% of explants to be successfully *in vitro* introduced. That procedure, combined with the embryogenic competence of the young enclosing tissues of inflorescence, resulted in an efficient *in vitro* callus culture establishment. 2,4-D was essential for callus induction, and the cytokinins tested influenced the type of callus obtained from inflorescences, which was correlated with their embryogenic capacity. The longitudinal sectioning of inflorescences before inoculation further increased the callus induction rate and the number of initial explants, improving the efficiency of *in vitro* introduction. When combined with 2-iP or Kin, the 2,4-D concentration reduction resulted in a rapid somatic embryos regeneration from the obtained calli. Those results corroborated the already known benefits of Kin utilization for bamboo micropropagation and introduced the 2-iP as a feasible

cytokinin alternative for bamboo somatic embryogenesis from inflorescence explants. The subculture of the embryos to the induction medium led to secondary embryogenesis from scutellum tissues, resulting in fused and asynchronous embryos maturation. The somatic embryos obtained were converted into plantlets in a PGR-free medium. Embryogenic cultures induced from inflorescences allow the large-scale propagation of selected genotypes. These cultures are also a feasible method for the rescue and *ex situ* conservation of elite genotypes in the case of a monocarpic flowering event. The results presented here are important to the scale-up multiplication of this multipurpose bamboo, emphasizing the ornamental industry.

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CAPÍTULO 2 - Clonal *in vitro* propagation of *Bambusa multiplex* (Lour.) Raeusch. ex Schult. & Schult. f. through axillary proliferation.

RESUMO

Bambus são importantes produtos florestais não madeireiro. Diversas espécies de bambus são interessantes para uso ornamental devido às suas variações fenotípicas, como o *Bambusa multiplex* e suas variedades botânicas. Esses grupos de plantas são propagados convencionalmente por divisão em touceira, que é trabalhosa e resulta em mudas volumosas para transporte. No Brasil, é considerada uma espécie introduzida e empregada para cercas vivas e bordas de jardins, porém ainda em pequena escala devido à escassez de plantas. O ciclo de floração é documentado como esporádico, o que torna imprevisível a disponibilidade das sementes. Como alternativa ao método de propagação convencional, as técnicas de cultura de tecidos são uma ferramenta importante para se atingir uma propagação em larga escala de plantas com qualidade fisiológica e genética. Além disso, o estabelecimento de culturas *in vitro* permite a fixação de genótipos selecionados, melhorando a qualidade dos plantios na direção de características de interesse do mercado. Porém, o estabelecimento *in vitro* de culturas de bambus lignificados ainda apresenta entraves, como por exemplo, microrganismos associados às plantas matrizes e a sazonalidade de explantes em condições adequadas para introdução *in vitro*. O presente trabalho teve como objetivo estabelecer um protocolo para o cultivo *in vitro* de *Bambusa multiplex*, a fim de tornar esta espécie e suas variedades botânicas mais disponíveis para a cadeia produtiva brasileira do bambu Além disto, avaliar os efeitos de diferentes fitorreguladores na micropropagação deste bambu ornamental. Segmentos nodais obtidos de uma touceira jovem foram introduzidos *in vitro*. As culturas estabelecidas foram multiplicadas em meio de cultura MS suplementado com 5 µM de diferentes citocininas: Benzilaminopurina (BAP), meta-Topolina (mT), e Thidiazuron (TDZ). Adicionalmente, aplicou-se o tratamento 5 µM BAP + 2,5 µM Ácido Naftaleno acético. Após a fase de multiplicação, as plantas foram transferidas para o meio de enraizamento (MS sem fitorreguladores). As plantas enraizadas foram aclimatizadas e monitoradas quanto ao rendimento quantico máximo do fotossístema II (Fv/Fm) por 90 dias. A fidelidade genotípica das plantas regeneradas foi verificada por meio da técnica *Sequence-specific amplified polymorphism* (S-SAP). A introdução *in vitro* resultou em 67% dos segmentos nodais brotados e 25% destes apresentando a manifestação de microrganismos (contaminação). Todos os tratamentos testados apresentaram maiores taxas de multiplicação em comparação ao tratamento controle (sem fitorreguladores). A citocinina utilizada na multiplicação influenciou n fase de enraizamento, sendo mT a citocinina que menos inibiu o enraizamento após a multiplicação. O tratamento adicional de BAP+ANA resultou em enraizamento abundante nas culturas. Porém, plantas albinas foram geradas a partir deste tratamento. Sob condições *ex vitro*, 85 % das plantas sobreviveram. Estas alcaçaram valores de Fv/Fm médio de 0.761 aos 90 dias de aclimatização. As 12 combinaçoes de primers de S-SAP resultaram em um total de 47 bandas em gel de agarose e 100% de monorfismo entre as plantas. O presente trabalho estabeleceu um protocolo de micropropagação para o bambu ornamental *Bambusa multiplex*.

Palavras-chave: Micropropagação. Bambu de cerca. Fluorescência da clorofila a.

ABSTRACT

Bamboos are relevant non-timber forest products. Several bamboo species are interesting for ornamental purposes, such as *Bambusa multiplex* and its botanical varieties. These plants are propagated conventionally by clump division, which is labor-intensive and results in large plantlets for transport. In Brazil, it is considered an introduced species used for hedges and garden edges, but still on a small scale due to the scarcity of plants. The flowering cycle is documented as sporadic, making seed availability unpredictable. As an alternative to the conventional propagation method, tissue culture techniques are essential for achieving large-scale plants with physiological and genetic quality. In addition, the establishment of *in vitro* cultures allows the establishment of selected genotypes, improving the quality of plantations in the direction of market interest. However, the *in vitro* establishment of lignified bamboo cultures still presents obstacles, such as microorganisms associated with field mother plants and the seasonality of explants suitable for *in vitro* introduction. The present work aimed to establish a protocol for the *in vitro* propagation of *Bambusa multiplex* to make this species and its botanical varieties more available for the Brazilian bamboo production chain. In addition, to evaluate the effects of different phytoregulators on the micropropagation of this ornamental bamboo. Nodal segments obtained from a young clump were introduced *in vitro*. Established cultures were grown in MS culture medium supplemented with 5 µM of different cytokinins: Benzylaminopurine (BAP), meta-Topoline (mT), and Thidiazuron (TDZ). Additionally, the treatment of 5 µM BAP + 2.5 µM Naphthalene Acetic Acid was applied. After the multiplication phase, the plants were transferred to the rooting medium (MS without phytoregulators). The rooted plants were acclimatized and monitored for the maximum quantum yield of photosystem II (Fv/Fm) for 90 days. The clonal fidelity evaluation of the regenerated plants was verified by the Sequence-specific amplified polymorphism (S-SAP) technique. The *in vitro* introduction resulted in 67% of the sprouted nodal segments and 25% being discarded because of microorganisms manifestation (contamination). All cytokinins-containing treatments evaluated showed higher multiplication rates than the control treatment (without phytoregulators). The cytokinin used in the multiplication influenced the rooting phase, with mT being the cytokinin that least inhibited the rooting after multiplication. The BAP+ANA treatment resulted in great rooting in the cultures. However, albino plants were generated from this treatment. Under *ex vitro* conditions, 85% of the plants survived. These reached average Fv/Fm values of 0.761 at 90 days of acclimatization. The 12 combinations assessed of S-SAP primers resulted in 47 bands on agarose gel and 100% monomorphism between plants. The present work established a micropropagation protocol for the ornamental bamboo *Bambusa multiplex*.

Key words: Micropropagation. Hedge bamboo. Chlorophyll *a* fluorescence

INTRODUCTION

Bambusa multiplex is sympodial bamboo (Bambusoideae:Poaceae) of the Bambuseae tribe. *B. multiplex* has a beautiful growth habit of small stature and densely dispersed leaves on their top-arched branches. Due to its known ornamental purposes, *B. multiplex* is spread worldwide because it is suitable for hedges, live fences, natural barriers, and landscape design (Dransfield and Widjaja 1995). Because of that traditional employment in gardens contours and borders, this species is known as "hedge bamboo". Regardless of the common name, botanical varieties are used as isolated or potted plants in ornamental gardens, drawing attention because of their differentiated characteristics of grown habits and variable size and colors variations on leaves and culms. Besides the ornamental purpose, *B. multiplex* is also widely distributed because of their hardy cold tolerance among the clumping bamboos (Young 1946). There are at least 7 recognized varieties of *B. multiplex*, with variations as culm and leaves striped variegation (Alphonso-karri, Stripstem, Silverstripe, Yellowstrip, Viridi-striata), reduction on the stature of plants and size of the leaves (Fernleaf, Riviereorum), exotic shaping (Willowy), and coloration of the culms (Alphonso-karri) (Young 1946; Shi et al. 2014). The origin of *B. multiplex* species is controversial. Some authors report that as cultigen (i.e., it is found only under cultivation) (Dransfiel and Widjaja 1995; Sharma and Nirmala 2015) and others as originated from southeast Asia (Li et al. 2008). McClure (*apud* Young 1946) reported wild *B. multiplex* in Kwangtung Province – China. Regardless of the origin, nowadays, the hedge bamboo is widely cultivated around the world and is considered the most widely distributed clumping bamboo in China (Shi et al. 2020).

Due to the long flowering cycle of many bamboos, the taxonomic identification practice occurs most of the time based on their vegetative traits. For *B. multiplex*, that is even more difficult due to its significant intraspecific variability and the transience of some phenotypes. Among many nomenclatures adopted, the most common synonyms are *Bambusa glaucescens* and *Bambusa nana* (Holttum 1956). It can be cultivated from sea level up to 2000 m altitude (Stapleton 1994) and grows on general soil conditions, in fields, mountains, low hills, and riversides (Li et al. 2008).

Young (1946) reported the possible effect of soil fertility on the stability and reversion of some varieties of *B. multiplex*. As one of the hardiest species among the clumping bamboo, the hedge bamboo tolerates negative temperatures for some periods. That feature of cold-

resistant is an important quality for bamboo genetic breeding and hybridization (Yuan 2011), allowing this bamboo to be cultivated in a wide latitude range.

There are many reports of sporadic flowering for *Bambusa multiplex* (Zheng et al. 2020). In Brazil, Filgueiras and de Silva (2007) registered a sporadic flowering of the species with no caryopsis formation and no death of plants after the flowering episode.

Among the other utilization, *B. multiplex* is very suitable for handcrafts, weaving, and basketry due to its features of small but strong and flexible culms with long internodes (Yuan et al. 2009; Li and Kobayashi 2004).

In the environmental necessities and potentials, *B. multiplex* is a suitable alternative to leptomorphs bamboos for environmental purposes because of their non-running rhizomes. Their rapid growth, a small port, and a broad root system are essential characteristics for hill soil recovery or landscape regeneration (Barbosa and Diniz 2010). The *B. multiplex* adventitious root system turns it into an attractive bamboo for slope conservation by increasing slope soil resistance (Kobayashi et al. 2006). . The species' potential for salt tolerance is another trait interesting for seawater flooding areas (Inoue et al. 2014).

Industrial paper pulp production is another potential for *B. multiplex* uses. These uses are possible because of their long fibers, which can reach more than 2.5 mm in length (Seethalakshmi et al. 1998; Li and Kobayashi 2004). Also, there are many reports on the traditional use of the leaves of *B. multiplex* for medicinal purposes, for example, against cooling down and nose bleeding (Shi et al. 2021). Young (1946) reported that the young shoots, which had not yet emerged from the topsoil, are edible. Despite the small size of the *B. multiplex* shoots, they are edible and commercially exploited in some countries of Asia (Chongtham et al. 2011; Bhaskaran et al. 2015).

Despite the wide versatility of the *B. multiplex*, their most common use is for ornamental purposes, either by the growth habit and size or by the stem and leaves variegation. Any variability in the trivial sense of tall and green bamboo is a desirable character in bamboo for landscaping (Zheng et al. 2021). Although the high value of the ornamental purposes of their varieties, the significant number of plants used for hedges-barrier is actual market demand. Moreover, mainly propagation method is the offset method, whereas culm cuttings are not an efficient method for propagation (Stapleton 1994). In addition, the plantlets obtained by clump division are usually voluminous and costly to produce and transport. Also, an initial bulk of propagules are not always available for large-scale propagation. Seed usage is not a feasible alternative. Based on XIX century flowering registers of Germany, Bangladesh, Sri Lanka, India, and Singapore, a cycle of 28-31 years

was established as the flowering period of *B. multiplex* (Seethalakshmi et al. 1998). Furthermore, flowering can frequently occur sporadically, but it usually results in a low seed setting (Zheng et al. 2020).

Faced with that scenario, the biotechnologies associated with plant propagation can be valuable tools for large-scale plant cloning, with insurance for producing quality and true-to-type plantlets. The present work had the objective to compare the effect of PGR on multiplication and rooting of *Bambusa multiplex in vitro* cultures as the first approach to a micropropagation protocol establishment for plantlets production.

MATERIAL AND METHODS

Plant material

In vitro culture was established from a 2-years old *Bambusa multiplex* plant accessed from a particular garden in Florianópolis – Santa Catarina. A just elongated culm was selected with 15-20 not sprouted nodes. The culm sheath color changing and the basipetal sprouting of lateral buds was followed to select the collecting timing. The trimmed culms were transported in a plastic bag to prevent dehydration and were immediately disinfested for *in vitro* culture establishment.

In vitro introduction

The entire culms were scrubbed with 70° GL ethanol-soaked cotton, removing all the sheath leaves. Nodal segments with around 20 mm with a dormant bud protected by sheath were trimmed. The sheaths were manually removed, and the nodal segments were immersed in a 3 ml L^{-1} Kasugamicin solution for 20 min in agitation. After that, those explants were transferred to a 70° GL ethanol solution and agitated for 1 min. Then, the explants were transferred to a 1.5 % (active chlorine) sodium hypochlorite solution with a drop of Tween 20® and kept in agitation for 15 min. The disinfested nodal segments were transferred for sterile deionized water and agitated for 3 minutes in a flow chamber cabinet. This step was repeated thrice. After that, the explants were arranged on sterile absorbent paper. Each nodal segment was trimmed (2-3 mm in each extremity) before the inoculation onto the culture medium.

Culture media and growth conditions

The basal culture medium used for all the experiments was MS (Murashige and Skoog 1962) supplemented with Morel's vitamin (Morel and Whitmore 1951) and 250 mg L^{-1} Polyvinylpyrrolidone. For *in vitro* introduction, this basal medium was supplemented with 10 µM of Benzilaminopurine (BAP) and 2 $ml.L^{-1}$ of Plant Preservative Mixture - PPM® (Plant Cell Technology, USA). The phytorregulator supplementation was with 5 µM of three different cytokinins for multiplication. For the rooting process, the ionic force of macronutrients (except for Calcium) was half-reduced. All the experiments were conducted in a grow room with a controlled temperature (25° C ± 2°C) and maintained under a 16 h photoperiod provided by tubular fluorescent lamps with 40 µmol photons. cm^{-2}. s^{-1} intensity.

In vitro multiplication

The established cultures were transferred to media containing 5 µM meta-Topolin (mT), 5 µM Benzylaminopurine (BAP), or 5 µM Thidiazuron (TDZ), to evaluate the effects of different cytokinins for the *in vitro* multiplication of *B. multiplex*. For auxin effects observation, a supplementary treatment of 5 µM BAP combined with 2.5 µM Naphthalneacetic acid (NAA) was conducted. The response rate was obtained by the occurrence rate of axillary shoots regeneration. The multiplication rate was calculated by the ratio among the final and the initial number of shoots. The mean height of shoots, rooting rate, number of roots, and root length per clump was obtained during the multiplication. Phenotypic features were visually evaluated for the occurrence of yellowing leaves, shoot browning, shoot oxidation, and hyperhydricity. The plants were multiplicated at least for 2 cycles of 45 days in each treatment before the data collection.

In vitro rooting

Due to the spontaneous rooting observed in some plants in all different cytokinins evaluated during the multiplication stage, no additional auxin was used in the rooting medium. Instead, it reduced the macronutrients' ionic strength in the MS basal medium. This approach was applied to verify a differential residual effect on rhizogenesis inhibition between the cytokinins evaluated for multiplication. Plants with 3-4 shoots without induced roots obtained from the multiplication step were transferred to the rooting medium. After 45 days, the occurrence rate of roots and their number and length per plant were evaluated.

Ex vitro acclimatization

For acclimatization, rooted and elongated plants were selected. Those plants were dark-acclimated for at least 60 minutes. The chlorophyll fluorescence parameters were obtained using a MINIPAM fluorometer (Walz, Germany). The initial fluorescence (Fo) and the maximum fluorescence (Fm) were measured by a saturating light pulse always in dark-acclimated leaves.

The Fv/Fm value was calculated by the variable fluorescence (Fv) and de Fm ratio, where Fv is the difference between Fm and Fo values. The plants were washed in deionized water for the residual remotion of the culture medium. Then, they were transferred to a 290 cm^3 tube-pot containing vermiculite-based commercial substrate, burnt rice husk, and organic compost (1:0.5:0.5; v/v/v). The plants were cultivated for 90 days in a greenhouse (50% shading) with intermittent irrigation by nebulization during the light period. The Fv/Fm monitoring was conducted at day 0 (*in vitro* condition), 7, 15, 30, 45, and 90 of the *ex vitro* acclimatization.

Clonal fidelity assessment

Clonal fidelity verification was conducted at the 12^{th} *in vitro* cultivation cycle. The leaves of three randomly selected plants of each multiplying treatment (NAA + BAP, BAP, mT, and TDZ) were collected for DNA extraction by CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987). Aims to assess the S-SAP method's sensibility, DNA from *Bambusa multiplex* var. fernleaf was used as a positive control. That is a dwarf variety of the same species and shows smaller leaves densely distributed on the branches. Additionally, DNA of *in vitro* mutant phenotypes (*i.e.,* albino and variegated lineage samples) was isolated as potential contrasts to further comparisons. The obtained DNA integrity was verified in a 1% (w/v) agarose gel electrophoresis. Then, the DNA quality verification and quantity estimative were measured in a spectrophotometer Nanodrop 1000 (Thermo Scientific, U.S.A.). The clonal fidelity was assessed using the Sequence-specific amplified polymorphism (S-SAP) method (Waugh et al. 1997). About 0.5 µg DNA sample was subjected to the simultaneous enzymatic restriction by 5 U of EcoRI and 5 U of MseI endonucleases (Thermo Fisher Scientific Inc.) in 2x TANGO Buffer (Thermo Scientific, U.S.A.) for 1 h at 37 °C. The EcoRI (5 ρmol. μl^{-1}) and MseI (50 ρmol. μl^{-1}) adapters (Table 1) were ligated to the restricted DNA to form the fragments templates during 3 hours at 37° C in Restriction-Ligation buffer, T4 DNA Ligase (1 U . $reaction^{-1}$), ATP (10 mM), and ultrapure

water. After the restriction-ligation procedure, the product was diluted at 1:10 in TrisEDTA (low EDTA).

Table 1 Adapters and selected primers for clonal fidelity assessment in *Bambusa multiplex in vitro* cultures.

Adapters	**Sequence (5'-3')**
MseI adapter 1	GACGATGAGTCCTGAG
MseI adapter 2	TACTCAGGACTCAT
Primers	**Sequence (5'-3')**
MseI + CGT	GATGAGTCCTGAGTA CGT
MseI + CAT	GATGAGTCCTGAGTA CAT
MseI + CCG	GATGAGTCCTGAGTA CCG
3'CRM	TCGGGCAATCACCCAACGGGTGCACATCAG
5'Del	ATTTTTTATATAAAATGTCGGGTCGTGATA
3'Galadriel	GTGCGAGAAAAATAAATCGACCCTCTTTCA
3'Reina	AACCCCAGTAGATCTACTACGTAGCTAACA

The pre-amplification reaction was carried out with 5 µl of the diluted DNA template in 45 µL of a pre-amplification mix (EcoRI + A primer [50 ng.µL^{-1}], MseI + C primer [50 ng.µL^{-1}], Taq DNA polymerase [5 U.µL^{-1}], PCR Buffer [1X], MgCl2 [25 mM], dNTP mix [5 mM], ultrapure water). The thermocycler was set to 25 cycles with the following cycle profile: a 30 s denaturation at 94 °C, a 60 s annealing at 56 °C, and a 60 s extending at 72 °C. Then, the pre-amplification product was diluted at 1:10 in TrisEDTA (low EDTA). Subsequently, 5 µL of diluted pre-amplification product and 15 µL of amplification mix (MseI + CNN primer [10 ng.µL^{-1}], RLT retrotransposon primers (3'CRM, 5'Del, 3'Galadriel, and 3'Reina) [10 ng.µL^{-1}], Taq DNA polymerase [5 U.µL^{-1}], PCR Buffer [1X], MgCl2 [25 mM], dNTP mix[5 mM], ultrapure water). The thermocycler was set to 35 cycles for amplification reaction with the following cycle profile: a 30 s denaturation at 94 °C, a 30 s annealing at 65 °C, and a 60 s extension at 72 °C. During the first 12 cycles, the annealing temperature was reduced by 0.7 °C per cycle. From the 13° to 35° cycle, the annealing temperature was 56 °C. (Vos et al. 1995). The obtained fragments of the selective amplification were separated and evaluated in a 2 % (w/v) low endosmosis agarose gel in TBE buffer electrophoresis (100 V) during around 2 h of running. The gels were photographed in the MultiDoc-It^{TM} (UVP, Cambridge, UK) and evaluated by count bands number per primer combination and monomorphism/polymorphism rate between the samples. The monomorphism rate was obtained by evaluating the presence/absence of bands among the lanes samples. Differing bands could indicate differences in the DNA fragments patterns among samples and, thus, the possible somaclonal variation identification.

RESULTS

In vitro introduction

After 45 days of *in vitro* introduction, 67 % of the explants (Figure 1a) sprouted. Most of them showed multiple shoots sprouting (Figure 1b). Of the sprouted nodal segments, 25 % manifested microorganisms' growth and were discarded as contaminated. The simple disinfestation procedure used for bamboos *in vitro* establishment in our laboratory resulted in an effective culture establishment for *B. multiplex*.

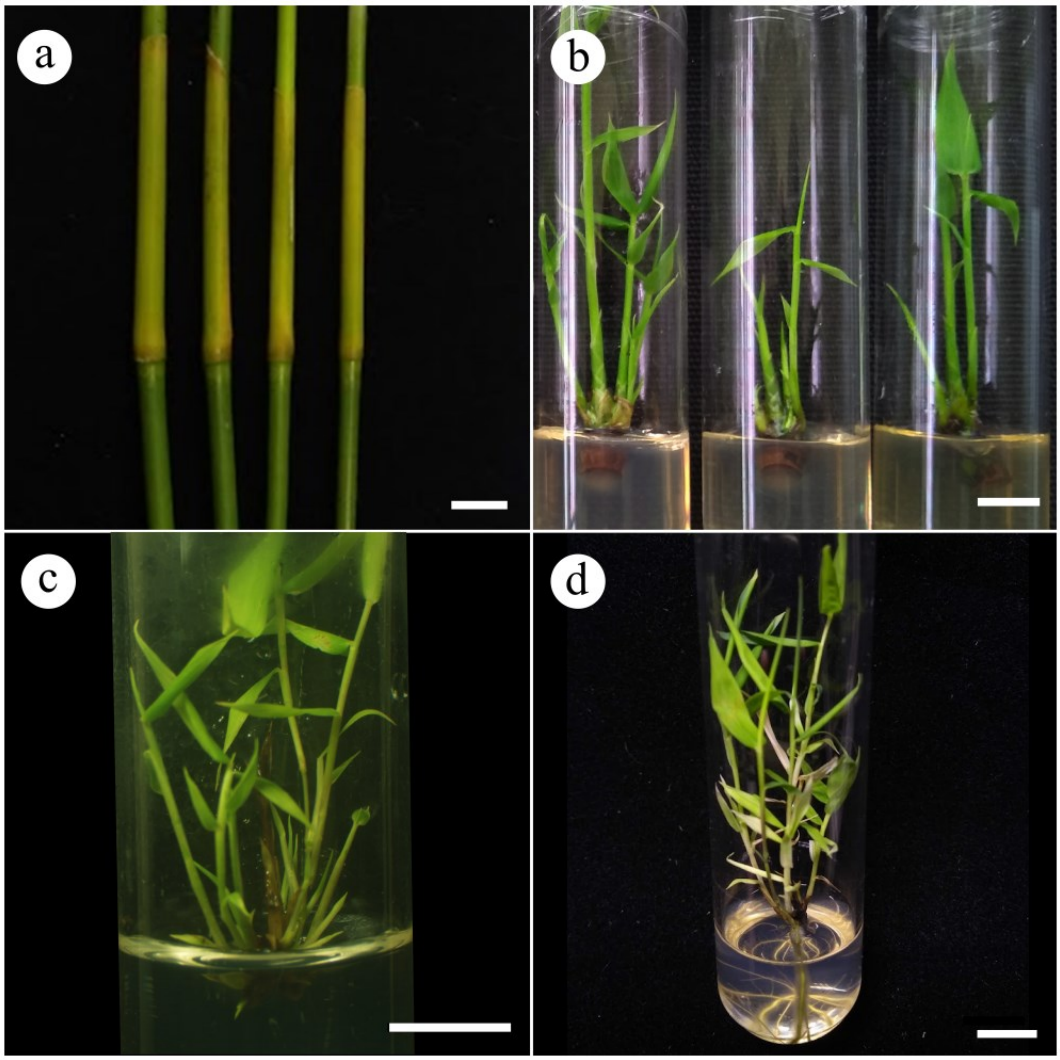

Figure 1 *Bambusa multiplex* micropropagation. (a) Nodal segments explants; (b) Sprouted nodal segments after 45 days of *in vitro* introduction; (c) *In vitro* plant under multiplication; (d) Rooted plant after 45 days in a half-strength MS plant growth regulator-free medium. Bars = 1 cm

In vitro multiplication

The cytokinins-containing treatments enhanced new axillary buds development and reduced the shoot height of the culture over the multiplication step (Table 1). Despite the NAA presence in combination, that response was the most effective in the BAP-containing treatments. Regardless of the cytokinin used, all the treatments with PGR multiplied more

than the control treatment (PGR-free), reaching up to 84 % of multiplication response. The highest multiplication rate was observed in both treatments containing BAP. However, no significant differences were observed among the cytokinins treatments. A differential response was observed in the shoot height between the tested cytokinins, which reduced that parameter compared to the PGR-free control treatment over the multiplication phase.

Although no statistical differences were observed, the combination of NAA with BAP showed a reduced effect in shoot height reduction compared to the BAP-only treatment. That BAP-only treatment resulted in the most reduction in the shoots' height, while TDZ resulted in the lowest reduction in this parameter among the cytokinins. The PGR-free medium contrasted with the cytokinin-containing treatments, reaching less shoot height reduction.

As expected, the only-cytokinin treatments showed reduced rooting and small roots during the multiplication (Table 2). The 2.5 µM NAA supplementation to BAP enhanced the rooting occurrence and the number of the roots but did not differentiate from the control medium.

Table 2 Effects of different growth regulators on the axillary development response, multiplication rate, and shoot height in the *Bambusa multiplex in vitro* cultures.

Auxin (2.5 µM)	**Ck (5 µM)**	**Response (%)**	**Multiplication rate**	**Shoots height (cm)**
-	**-**	57.5 ± 2.5 b	1.25 ± 0.06 b	2.20 ± 0.15 a
NAA	**BAP**	81.4 ± 5.9 a	1.66 ± 0.09 a	1.51 ± 0.07 bc
-	**BAP**	84.3 ± 2.0 a	1.77 ± 0.10 a	1.35 ± 0.05 c
-	**mT**	72.1 ± 5.6 ab	1.61 ± 0.13 a	1.40 ± 0.05 bc
-	**TDZ**	68.1 ± 9.2 ab	1.59 ± 0.11 a	1.58 ± 0.05 b

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test (p <0.05). Ck – Cytokinin; NAA -Naphtaleneacetic Acid; BAP – Benzilaminopurine; mT – meta-Topolin; TDZ – Thiadiazuron.

The rooting occurrence was observed in plants of all treatments over the multiplication phase (Table 3).

Table 3 Effects of different growth regulators in the rooting rate, roots number, and roots length on *Bambusa multiplex in vitro* cultures during the multiplication phase.

Auxin (2.5 µM)	Ck (5 µM)	Rooting (%)		Roots number		Roots length (cm)	
-	-	41.1 ± 5.0	a	2.59 ± 0.44	a	6.79 ± 1.04	a
NAA	BAP	47.3 ± 7.7	a	2.58 ± 0.24	a	1.18 ± 0.16	b
	BAP	9.35 ± 4.0	b	1.57 ± 0.30	ab	1.64 ± 0.32	b
	mT	15.9 ± 5.7	b	1.67 ± 0.33	ab	1.72 ± 0.37	b
	TDZ	16.7 ± 4.3	b	1.20 ± 0.13	b	1.75 ± 0.42	b

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test (p <0.05). Ck – Cytokinin; NAA -Naphtaleneacetic Acid; BAP – Benzilaminopurine; mT – meta-Topolin; TDZ – Thiadiazuron.

During the multiplication step, physiological disorders were observed at different rates in response to the PGRs treatments (Table 4).

Table 4 Effects of different growth regulators in the yellowing, oxidation, and browning occurrence rates on *Bambusa multiplex in vitro* cultures during the multiplication phase.

Auxin (2.5 µM)	Ck (5 µM)	Yellowing (%)		Oxidation (%)		Browning (%)		Leaf variegation (%)	
-	-	22.0 ± 2.7	a	5.0 ± 2.9	ab	7.0 ± 4.4	a	2.5 ± 2.5	a
NAA	BAP	21.3 ± 3.3	a	0.0 ± 0.0	b	13.0 ± 4.2	a	7.2 ± 2.5	a
	BAP	16.1 ± 3.5	a	4.0 ± 1.9	ab	12.3 ± 2.0	a	4.0 ± 1.9	a
	mT	6.6 ± 2.2	b	2.1 ± 2.1	ab	4.2 ± 4.2	a	0.0 ± 0.0	a
	TDZ	19.7 ± 1.5	a	10.6 ± 5.5	a	13.6 ± 3.9	a	1.5 ± 1.5	a

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test (p <0.05). Ck – Cytokinin; NAA -Naphtaleneacetic Acid; BAP – Benzilaminopurine; mT – meta-Topolin; TDZ – Thiadiazuron.

The occurrence of yellowing was the most common, affecting all the treatments. That yellowing was usually followed by browning or oxidation of that organs. In most affected plants, that symptoms occurred only in the tallest or already elongated culms, and new green shoots remained in development. The affected organs were removed during the medium refreshing. Hyperhidricity symptoms were observed in only a few plants in BAP and TDZ treatments, only when shoots grew into the medium.

Another abnormality observed during multiplication was the occurrence of variegated leaves (Figure 2).

Figure 2 Variegation range in *Bambusa multiplex* leaves during *in vitro* multiplication. (a) Normal green-phenotype; (b) Variegated-phenotype; (c) Albino-phenotype. Leaves bars = 0,2 cm; Culms bars = 0,5 cm.

The variegation color varied from white to a creamy color. Most of the variegation occurred longitudinally on the leaves' borders (Figure 2b) but also in the median region of the leaves. No variegation was observed in sheaths or culms tissues. Two independent albino cultures (Figure 2c, 3d) were initiated in NAA+BAP treatment from a cluster of microshoots developed in the base of clumps (Figure 3). The albino lineage was established by subculturing and kept a continuous multiplication. The elongated shoots still albinos and developed a purple color on the internodes.

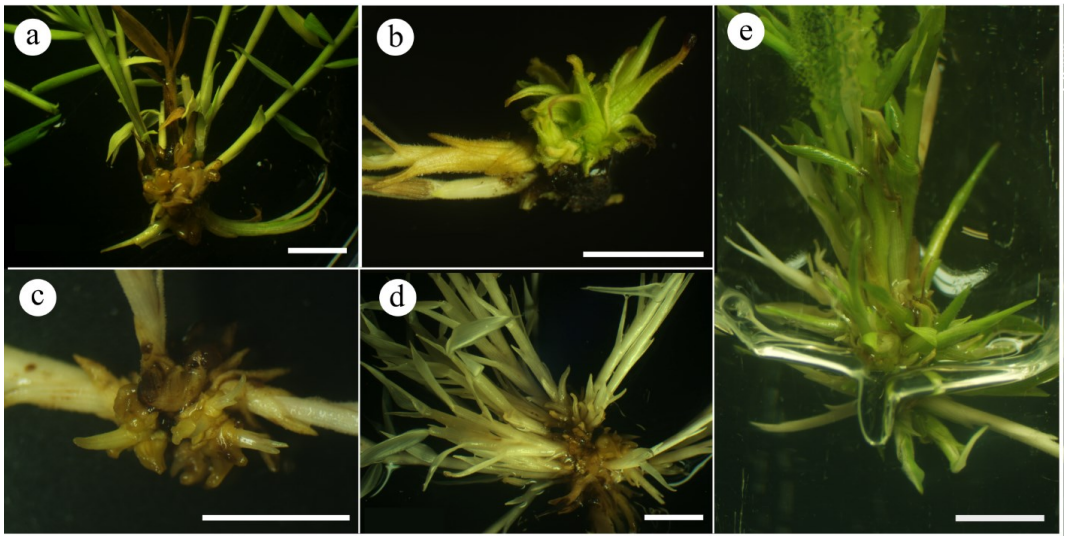

Figure 3 Effects of 2.5 µM NAA + 5 µM BAP on *Bambusa multiplex in vitro* cultures. (a) Regeneration of microshoots from the basal region of *in vitro* clump; (b) Green shoots regenerating from the microshoots clusters; (c) Albino shoots regenerating from the microshoots cluster; (d) Albino shoots regenerated and continuos microshoots proliferation; (e) Green, variegated and albino shoots regenerated in a fastgrowing culture. Bars = 0,5 cm

During the multiplication of cultures, a clump was kept for 2 subcultures (3 months) in 5 µM mT and had flowered (Figure 4a), which was a unique occurrence of *in vitro* flowering of *B. multiplex* cultures.

In vitro rooting

Figure 4 *Bambusa multiplex in vitro* flowering and rooting. (a) Rooted and flowering clump from meta-Topolin multiplication; (b) Microshoot cluster-derived clump rooting after transferring for half strength PGR-free MS medium; (c) Rhizome induction in a rooting shoot of NAA+BAP treatment; (d) Rooted and elongated plant at ready for soil transference in acclimatization procedure; (e-f) Plantlets at 90th day of acclimatization; Typical leaves distribution of the species. Note the variegated leaves on the basal position of an *ex vitro* regenerated culm. Bars = 0.5 cm

After multiplication, plants from the different cytokinins treatments rooted when transferred to a half-strength MS PGR-free medium. However, the cytokinin employed to multiplication influenced the rooting quality and occurrence rate, resulting in differential rhizogenesis response (Table 5).

Table 5 Effect of different growth regulators remotion after multiplication step, in the rooting rate, roots number, and roots length of *Bambusa multiplex in vitro* cultures after 45 days on half-strength and PGR-free MS medium

Previous treatment		Rooting			
Auxin (2.5 µM)	**Ck (5 µM)**	**PGR-free**	**Rooting (%)**	**Roots number**	**Roots length (cm)**
NAA	**BAP**	**MS/2**	72.8 ± 2.4 a	2.2 ± 0.7 b	2.2 ± 0.5 a
-	**BAP**	**MS/2**	40.0 ± 5.8 b	1.0 ± 0.0 a	6.1 ± 1.5 a
-	**mT**	**MS/2**	71.7 ± 5.8 a	1.2 ± 0.2 b	1.4 ± 0.4 a
-	**TDZ**	**MS/2**	41.1 ± 4.8 b	1.7 ± 0.3 ab	4.4 ± 2.0 a

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test (p <0.05). PGR – Plant Growth Regulator; MS- Murashige and Skoog medium; Ck – Cytokinin; NAA -Naphtaleneacetic Acid; BAP – Benzilaminopurine; mT – meta-Topolin; TDZ – Thiadiazuron.

Plants multiplicated in meta-topolin medium reached up to 70 % of rooting after 60 days. Although no exogenous auxins were imperative for rooting, its use in NAA + BAP treatment during multiplication improved the rooting rate. It increased the number of the roots compared to the BAP alone treatment on the rooting step.

The plants subcultured in NAA + BAP treatment showed developed culms with prominent nodes and rhizome formation in the new shoots (Figure 4c) when transferred to the rooting medium. That trait was not observed in PGR-free control treatment nor any other multiplication treatments with cytokinins. In general, most plants from the NAA + BAP treatment showed enhanced root visual quality and a high amount of radicular hair (Figure 4d).

Ex vitro Acclimatization

After 90 days in *ex vitro* conditions, around 85 % of plants survived (Figure 4e). Those plants reached a stable Fv/Fm value of 0.761± 0.007. The Fv/Fm dynamic is presented in Figure 5.

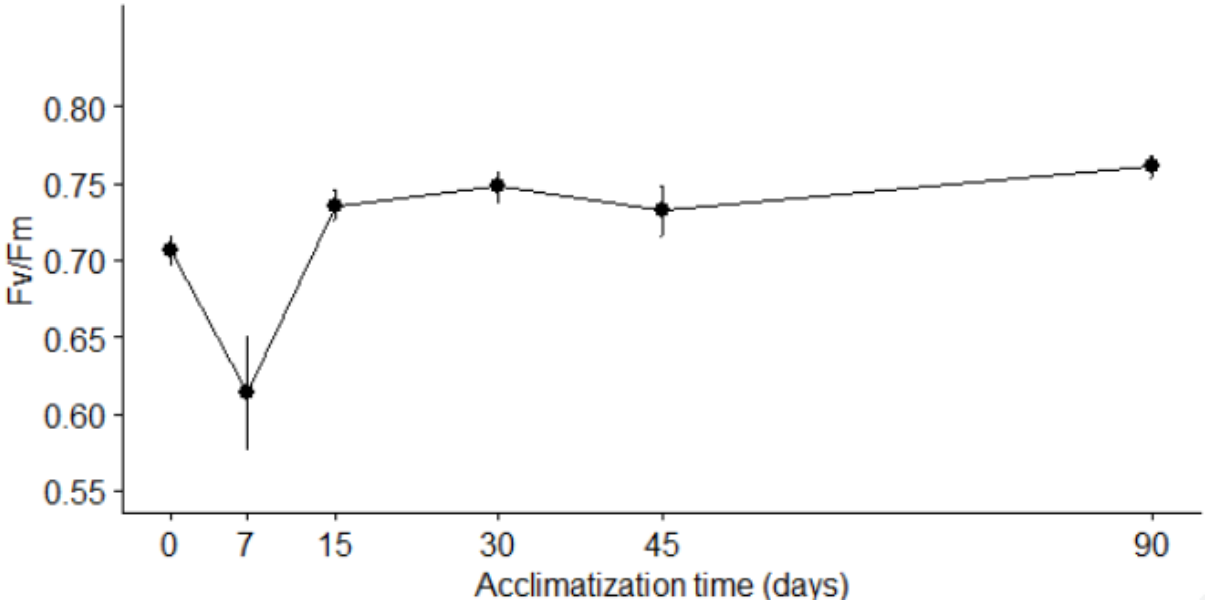

Figure 5 Graphical tracking of maximum quantum yield of photosystem II (Fv/Fm) over the 90 days of *Bambusa multiplex ex vitro* acclimatization. Error bars represent the standard error of the mean.

After *in vitro* rooting and elongation, the plants transferred to *ex vitro* conditions showed a rapid decline in Fv/Fm chlorophyll-a parameter, due to a decline in Fm values. In contrast, Fo values were constants over those 90 days (Figure 6).

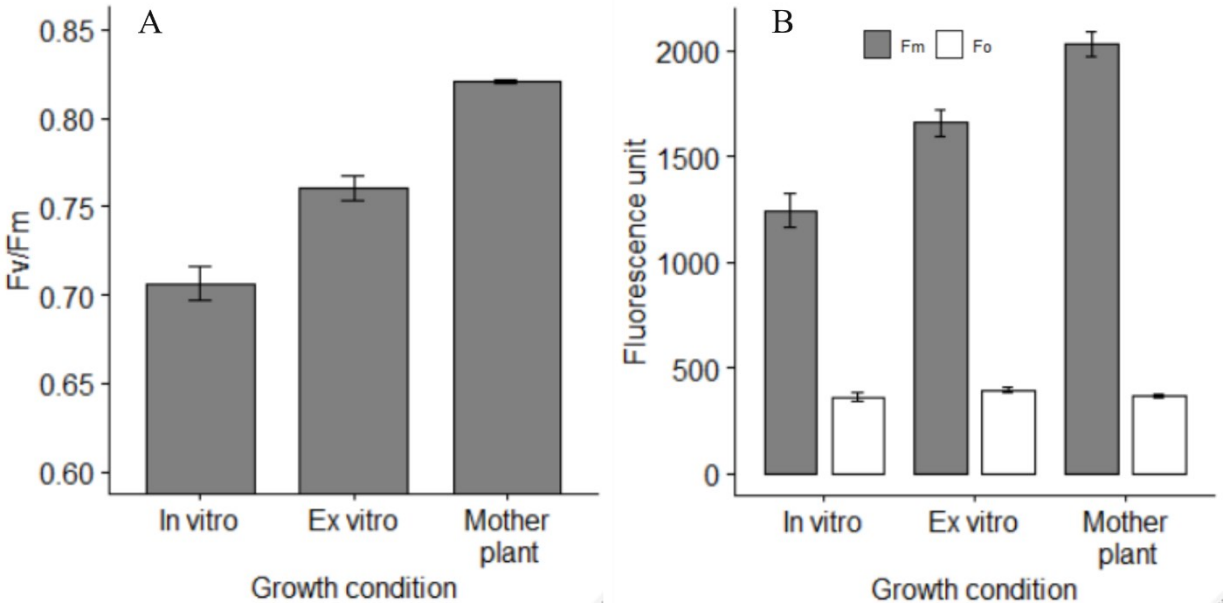

Figure 6 Chlorophyll fluorescence parameters on the field-mother plant, *in vitro* cultures, and *ex vitro* acclimatized plantlets (90th day of *ex vitro* condition). Fv/Fm – Maximum quantum yield of photosystem II; Fm – Maximum fluorescence yield; Fo – minimum fluorescence yield.

The new culms shoots were observed from the 15th day on most plants. Variegated leaves were observed in *ex vitro* developed culms (Figure 4f), like the *in vitro* cultures.

Clonal fidelity assessment

Despite the contrasting phenotypes and possible positive control, no polymorphism was observed among the twelve primers combination assessed. The number of scorable gel bands varied from 2 to 10 bands produced per primer combination, resulting in a mean of 3,9 bands per primer combination (Table 6).

Table 6 Number of scorable bands and monomorphism occurrence rate in 2% agarose gel of different primers combinations on *Bambusa multiplex* clonal fidelity assessment after 12 subcultures of *in vitro* cultures

No.	Primer Combination	Number of scorable bands	Monomorphism (%)
1	MseI + CGT/3'CRM	4	100
2	MseI + CGT/3'Del	3	100
3	MseI + CGT/3´Galadriel	2	100
4	MseI + CGT/ 5'Reina	5	100
5	MseI + CAT/3'CRM	3	100
6	MseI + CAT/3'Del	3	100
7	MseI + CAT/3´Galadriel	3	100
8	MseI + CAT/ 5'Reina	5	100
9	MseI + CCG/3'CRM	4	100
10	MseI + CCG/3'Del	10	100
11	MseI + CCG/3´Galadriel	3	100
12	MseI + CCG/ 5'Reina	2	100
	Average	**3.9**	**100**
	Total	**47**	**100**

DISCUSSION

As for conventional propagation means, the propagules availability can be a limitation for starting *in vitro* culture. The mother plants' conditions and growing season are crucial to establishing bamboos *in vitro* cultures (Mudoi et al. 2014). For *in vitro* introduction of bamboos nodal segments, the explant collection should be done during the active growth period, usually associated with the rainy and warm seasons, usually when endogenous hormones balance is favorable for bud sprout (Ramanayake and Yakandawala 1997). Besides the quality and the timing of nodal segments explants collection, the phytosanitary and physiological conditions of the mother plants also play a crucial role in the establishment success of *in vitro* cultures (Ornellas et al. 2019). Despite the few materials accessed in the present work, those above cited elementary points led to the efficient establishment of *in vitro* cultures.

During the initial phase of those cultures, many constraints could affect the *in vitro* rate of success, such as late microorganism manifestation, browning, oxidation, and

hyperhydricity. Most of those limitations could occur or be overcome by *in vitro* conditions adjustment (Hazarika 2006). Cytokinins type, concentration, and the plant exposition duration are essential for an efficient micropropagation process. Those factors should continuously be improved to reach quality plants with efficiency in clonal propagation. Although no differences were observed in multiplication rate, plants qualities and morpho-physiological responses were differentially expressed among the treatments. For bamboos, the most used cytokinin is BAP, although TDZ and mT showed positive results in a few bamboo micropropagation reports (Lin et al. 2007; Ornellas et al. 2015). In our previous experience with other Bambuseae *in vitro* cultures, the common rooting in the cytokinin-containing medium during the multiplication may suggest a low concentration of this PGR-type. Another paper about *B. multiplex* organogenesis also reported the spontaneous rooting during the multiplication (Bordolodoi et al. 2013). However, new experiments should be performed to tune cytokinin concentrations for multiplication rates improvement.

In vitro flowering

Flowering is a crucial phase, and its occurrence timing can vary widely among the plants. Most vegetables recombine genes and keep their variability for evolution adaptations. Bamboos show a unique flowering behavior, especially those that present monocarpic flowering (i.e., the plants die after the flowering and seed production) (Janzen 1976). The juvenile phase of many priorities woody bamboos can last for decades, even a century. The flowering mechanisms in those bamboos remain a mystery that needs to be puzzle-solved with the advances in scientific knowledge, especially molecular-based-biotechnologies. Although natural and artificial hybrids bamboos are known (see Zhang 2002), the major bottleneck for hybridization and full-domestication is a lack of synchronization in the flowering events among species or populations (Gielis and Debergh 1998). In this context, *in vitro* flowering is pertinent, allowing pollen production/storage or even *in vitro* fertilization and seed production (Murthy et al. 2012). The *in vitro* flowering was already reported for at least 13 bamboo species, being the first described by Nadgauda et al. (1990). Despite a long time since that, the *in vitro* flowering phenomena and its control remain considered not understood (Yuan et al. 2017). In the 60's decades, Young and Haun (1961) reported that almost all botanical forms of *B. multiplex* had flowered sporadically in field conditions.

The *B. multiplex in vitro* flowering was reported in a study in which the flowering has occurred for seven other bamboo species. Among those eight flowered bamboos *in vitro* cultures, only *B. multiplex* plants survived after the flowering (Prutpongse and

Gavinlertvatana 1992). In the present work, the flowered clump episode was in an mT-containing medium, after a long-term (3 months) subculture.

In in-field conditions, the *B. multiplex* flowering is considered sporadic (i.e., not with a long flowering period, good stigma receptivity, and high pollen viability. Those reproductive features associated with a high cold resistance turn the hedge bamboo into a desired bamboo hybridization resource (Yuan et al. 2009). Besides that, *in vitro* flowering is still a valuable material for research of flowering phenomena (Yuan et al. 2017). The author also observed flowering plants of that species at "Paraiso del Bambú y la Guadua" - Colômbia (2018). The bamboo taxonomist and the collection owner, Dra. Ximena Londoño confirmed the survival and continuous flowering after two years (Londoño, 2021, personal communication).

In vitro rooting

After the multiplication, about 56 % of the plants were rooted when they were subcultured in a PGR-free medium. The cultivation at cytokinin-enriched medium is the most used method for plant multiplication in micropropagation protocols. For Bambuseae species, the most employed cytokinin is BAP (Mudoi et al. 2013). A high-dosage or long-term cultivation in the cytokinin-containing medium can lead to rooting recalcitrance. The rooting difficulty is one major constraint of axillary branches bamboos micropropagation (Yasodha et al. 2008). A factor for Bambusa species is related to an intrinsic feature of rooting potential of species/genotypes. In micropropagation protocols, the plants are usually transferred for a rooting/elongation medium after multiplication, occurring concomitant or in separate steps. Usually, to induce rhizogenesis, exogenous auxins are applied (Sandhu et al. 2018). Despite the rhizogenesis observation due to cytokinin removal from the culture medium, poor rooting can difficult plantlets elongation and leaves development, leading to less *ex vitro* survival. The auxin supplementation to the culture medium is essential to quality rooting achievement. Like Bordoloi et al. (2013), spontaneous rooting was observed in the *B. multiplex* cultures of the present work, mainly in those multiplicated in a cytokinin-auxin-containing medium. A follow subculture in PGR-free medium enhanced rooting and elongation of *B. multiplex in vitro* plants (Bordoloi et al. 2013). Although spontaneous rooting *in vitro* bamboos cultures are not common, few species can show that (Jimézes et al. 2006; Ornellas et al. 2019). For *B. glaucescens* (syn. *B. multiplex*), various auxins evaluated enhanced rooting compared to the PGR-free medium (Shirin and Rana 2007). Supplementary experiments should be carried out to evaluate the rooting quality of plants in auxins-enriched media and the ideal concentration of these. That improvement can enhance the *ex vitro* acclimatization process regarding

survival rates and acclimatization time. Consequently, that improvement can increase the quality of plants and reduce the costs of the produced plantlets.

Ex vitro acclimatization

A high survival rate was achieved during the acclimatization procedure. Bamboo survival is usually high in acclimatization, varying from 70 % to 100 % (Sandhu et al. 2018). For *B. multiplex*, the *ex vitro* acclimatization could result in survival rates of 70 % (Yuan et al. 2009) to 100 % (Bordoloi et al. 2013). Considering the efficiency lost in the micropropagation process when plants die within the final phase, new attempts should ensure maximal plant survival.

At the *ex vitro* acclimatization step, the micropropagated plants are exposed to a drastic environmental change. In comparison to the *in vitro* conditions, two critical points in the *ex vitro* acclimatization are the increase of light intensity and the decrease of relative humidity (Hazarika 2003). Both conditions are considered when the plants are transferred to *ex vitro* conditions in a shaded greenhouse with intermittent nebulization. Most of the micropropagation protocols only report the survival rates during that critical process. The chlorophyll fluorescence analysis is a rapid and non-destructive analysis of the photosystem II (PSII) apparatus. The Fv/Fm parameter is an easy-to-apply index adopted for monitoring the stress condition on PSII (Maxwell and Johnson 2000). A substantial decrease in the Fv/Fm parameter during the first 7 days of *ex vitro* acclimatization suggests photoinhibition. The analyzed marked leaves kept the green color during all acclimatization periods. Only a few plants that showed yellowing and subsequent senescence of marked leaf did not survive the acclimatization. The further increase in Fv/Fm values since the 7^{th} day indicated a recovery of PSII and subsequent acclimatization of the plants. After 90 days of *ex vitro* condition, the plantlets showed a lower Fv/Fm value than their mother plant, which was in the non-stress Fv/Fm values levels (Björkman and Demmig 1987). That difference evidence possible photoinhibition in the plantlets, despite the shading provided to mitigate the environmental changes over the acclimatization.

Leaves Variegation

Although variegations are not the standard in plant phenotypes, their occurrence is commonly reported in bamboos (Oprins et al. 2004). Many bamboo varieties were selected due to their stable striped variegations or some degree of albinism in the plant's organs (Shi et al. 2021). Under *in vitro* conditions, other grasses usually regenerate leaves with chlorophyll

gaps (Kumari et al. 2009). Those phenotypic variations are not always stable. It can be a transient effect in plants caused by environmental stresses and ceasing after plants reach homeostasis (Chen et al. 2018). Both variegated and albino phenotypes can be valuable sources for basic science knowledge about biochemical and physiological chlorophyll pathways (Liu et al. 2007, Wu et al. 2009; Duarte-Aké et al. 2016) and practical uses (Chang and Lan 1995). Many ornamental Bambusoideae species show different variegation degrees, varying from a few temporary leaves stripes to whole albino leaves. The variety (or cultivated form) "Silverstripe" of *B. multiplex* is characterized by the white-striped or even albino basal leaves of branches, which improve the ornamental potential compared to the typical all-green form. That pigments variation was attributed to an impairment in chloroplastidial biosynthesis (Chen et al. 2017).

As a type of variegation, albino leaves are frequently observed in bamboos (Lin et al. 2007; Zang et al. 2019). The albinism is considered a recessive monogenic trait and frequently occurs in bamboo seedlings (Alexander and Kanddaswami 1966; Saxena 1990; Kader et al. 2001). Under *in vitro* conditions, its occurrence was attributed to long-term subcultures in *D. asper* (Kumar et al. 2018). For *B. edulis in vitro*-cultivated over 8 years, two albino occurrences were observed (Liu et al. 2007). Albino plants are reported in somatic embryogenesis cultures of *B. oldhamii* (Ho and Chang 1998), *B. edulis* (Lin et al. 2004), and *B. multiplex* (Liang 1996 *apud* Lin et al. 2004). By organogenesis means, albino regenerants were also observed in *B. oldhamii* (Lin et al. 2007) and *B. edulis* (Lin and Chang 1998). A subsequent evaluation of *B. edulis* albino cultures revealed different chloroplast gene deletion in distinct albino lineages of the same *in vitro* culture (Liu et al. 2007), while another albino-cause mechanism was reported to *B. oldhamii* (Lin et al. 2007). The albinism could be reverted in *B. tulda* when the sucrose concentration in the culture medium was reduced to 2 % (Saxena 1990). Although albinism can be considered a beautiful feature in plants, its occurrence can impair *ex vitro* acclimatization of cultures (Lin et al. 2007).

Clonal fidelity

Somaclones are *in vitro* regenerated plants that show distinct phenotypes of the mother plant from which the explants were obtained (Larkin and Scowcroft 1981). Somaclonal variants are a significant constraint in tissue culture means focused on genetic resources conservation or elite genotype cloning. Nevertheless, its incidence can benefit breeding since somaclonal variants can be sources of different features or a new variety with improved

characteristics. Also, in ornamental plants, differential appearance features usually can conquest new market niches. That somaclonal variance may occur due to many factors related to *in vitro* environmental conditions (e.g., culture media composition, low light, and high humidity conditions) (Krishna et al. 2016). Many phenotypic features can easily be detected by simple plant observation (e.g., dwarfing, leaf, and other organs variegation). However, mutants can often only be recognized in the field after years, bringing economic losses during the culture's productive period.

In bamboos micropropagation and the other plants, the somaclonal variants manifestation can also occur. In general, because of the callus phase in some *in vitro* pathways and its more unstable cell proliferation, the axillary shoots multiplication is usually the most cautious method to avoid *in vitro* bamboo variants (Gielis and Oprins 2002).

The ubiquitous nature of transposons and their potential for genetic diversity development are notable features that enable their use as molecular markers (Schulman et al. 2012). Molecular markers are helpful biotechnology tools for bamboo breeding, genomics studies, and taxonomy issues (systematics and identification).

Besides the bamboo's particularities about the long flowering cycles and difficulty to species conventional identification, the specific and intraspecific levels (e.g., forma and varieties) can be characterized by molecular approaches (Gielis et al. 2004). Transposable elements (TE) can create spontaneous variation in DNA (Bourque et al. 2018).

With the improvement of molecular biotechnologies, genome sequencing of bamboos started to be accessed (Wu et al. 2009; Zhang et al. 2011; Zhao et al. 2018; Guo et al. 2019; Li et al. 2021). It is expected to increase molecular information about the plants of the Bambusoideae subfamily. TE are the most repetitive sequences in eukaryotic genomes. The Galadriel, Reina, Del (or Tekay), and CRM are Long Terminal Repeat Retrotransposons (LTR-RT) lineages grouped in the Chromovirus (Orozco-Aria et al. 2019). For the annotated genome of *Phyllostachys heterocycla*, 59 % of that consisted of TE. Of those, 37.3 % were LTR-RT, in which Gypsy-type and Copia-type were the most common with 24.6 % and 12.3 % of the repetitive sequences (Peng et al. 2013). In the draft genome of the pachymorph bamboo *Phyllostachys edulis,* Zhao, and coworkers (2018) reported transposable elements (TE) insertions in 51,62% of all annotated genes. The draft genome of *Raddia distichophylla* and *R. guianensis*, both herbaceous bamboos (Bambusoideae, Olyra), revealed 49.08 % of TE, in which LTR retrotransposons were the most abundant type (Li et al. 2021). The retrotransposons can be activated by stress exposure. The *in vitro* environment is undoubtedly a stressful condition for any life cultivation, due to the *ex situ* circumstances and various

stimuli provided during the process. Retrotransposons can influence the occurrence of somaclonal variations in *in vitro* cell or tissue culture (Grandbastien 1998).

Among the typical phenotype plants propagated for 12 subcultures in different PGRs, the monomorphism observed was 100 %. Regardless of the cytokinin used for multiplication, the sampled plants showed identical fragments pattern in the random fragment analysis by using an MseI + LTR-based combination of primers. Despite the occurrences of albino plants in *B. multiplex* cultures, the bamboo micropropagation through axillary buds' proliferation is considered the safest method to avoid somaclonal variation (Oprins et al. 2004). The 12 primers combinations adopted for genetic fidelity generated 47 strong electrophoresis bands *for B. multiplex* micropropagated plants. A mean of 3,9 bands per primer combination. No polymorphism could be detected among normal-phenotype plants and mutants-phenotype plants (albinos and variegated). Due to the probable monogenic nature of variegation, the use of random molecular markers could not easily identify polymorphisms. Another possibility is that the mutation occurred in the plastome and could not be identified by nuclear retrotransposons-based primers. The tested primers combinations could not even access polymorphism among the *B. multiplex* and its botanical variety (*B. multiplex* var. fernleaf) used as a positive control.

The albino lineage observed during the multiplication phase in the micropropagation of *B. multiplex* was unexpected. In our previous experiences with other sympodial bamboos (e.g., *Dendrocalamus asper*, *Guadua chacoensis*, and *Bambusa oldhamii*), transient variegation of leaves and albino plants was only observed in plants obtained from seeds of *D. asper*. Under natural conditions, various Bambusa species show variegations on culms, culm sheets, and leaves (*e.g., Bambusa vulgaris* f. vittata, *Bambusa ventricosa* f. kimmei, *Bambusa eutuldoides* varieties). Specifically for *B. multiplex*, among the known varieties, 5 of them show some variegation type. The origin of that infraspecific taxa is hard-to-identify, but their stability could be proved by the widespread and the long-time cultivation.

CONCLUSIONS

A protocol for the ornamental bamboo *Bambusa multiplex* micropropagation was established. All the different cytokinins evaluated (BAP, mT, and TDZ) enhanced the multiplication of the cultures, and differential responses in morphological and physiological features were observed. A residual effect of cytokinins was also observed in the rooting step. Over the multiplication, albino and variegated leaves occurred, and lineages were established. Due to

the essential ornamental purpose of the *Bambusa multiplex* species, the somaclonal plants with variegated leaves can be considered a benefit. Around 85 % of the acclimatized plants survived under *ex vitro* conditions. The acclimatized plants reached an Fv/Fm value of 0.761± 0.007 after 90 days of the acclimatization procedure. The acclimatized plants should be observed for the stability of the variegated phenotype. Despite the monomorphism observed among the MseI + LTR combination of primers, the PCR-based technique and electrophoresis on agarose reveal an easy and cheap way to monitor *in vitro* cultures. Further studies should be conducted for clonal fidelity assessing for varieties discrimination and true-to-type plantlets certification.

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CAPÍTULO 3 - Efeitos da Benzilaminopurina e seleção de explantes no estabelecimento de culturas *in vitro* de *Bambusa oldhamii* Munro

RESUMO

Bambusa oldhamii é um bambu de grande porte originário da Ásia e com características promissoras ao cultivo na região Sul do Brasil. A espécie é mundialmente difundida devido a importância do uso de seus brotos no setor alimentício. Apesar das potencialidades na iminente cadeia produtiva brasileira do bambu, não há disponibilidade de matrizes, mudas, e tecnologias para a propagação em escala comercial da espécie. O presente estudo teve o objetivo de gerar informações básicas para o desenvolvimento de um protocolo de micropropagação de *B. oldhamii*. Foram realizados três experimentos: (1) Efeitos da Benzilaminopurina (BAP) na introdução e estabelecimento de culturas *in vitro*; (2) Introdução *in vitro* de segmentos nodais obtidos de ramos laterais em diferentes posições no colmo; (3) Efeito da posição do segmento nodal em ramos jovens e maduros para o estabelecimento de culturas *in vitro*. O uso de BAP afetou a velocidade de brotação em segmento nodais e as taxas de oxidação dos brotos obtidos. O uso de 10 µM de BAP + 2,5 µM de ANA foi considerado como adequado para os experimentos seguintes. A posição no colmo dos ramos doadores de segmentos nodais não influenciou o estabelecimento de culturas quanto aos parâmetros avaliados. Os segmentos nodais da região mediana de ramos jovens resultaram em menores taxas de contaminação por microrganismos e subsequente incremento nas taxas de estabelecimento de culturas *in vitro.* Os resultados obtidos fornecem as bases para o estabelecimento de um protocolo para a micropropagação em larga escala de *Bambusa oldhamii.*

Palavras-chave: Segmento nodal; Introdução *in vitro*; Bambuseae

ABSTRACT

Bambusa oldhamii is a large bamboo originally from Asia and with promising characteristics for cultivation in southern Brazil. The species is spread worldwide due to the importance of using its shoots in the food sector. Despite the potential in the Brazilian bamboo production chain, mother plants, seedlings, and technologies are unavailable for propagating the species on a commercial scale. The present study aimed to generate basic information for the development of a micropropagation protocol for *B. oldhamii*. Three experiments were conducted: (1) Effects of Benzylaminopurine (BAP) on the introduction and establishment of *in vitro* cultures; (2) *In vitro* introduction of nodal segments obtained from lateral branches at different positions in the culm; (3) Effect of the position of the nodal segment in young and mature branches for the establishment of *in vitro* cultures. The use of BAP affected the budding speed in nodal segments and the oxidation rates of the shoots obtained. The use of 10 µM of BAP + 2.5 µM of ANA was considered adequate for the following experiments. The position in the culm of the donor branches of nodal segments did not influence the establishment of cultures regarding the evaluated parameters. The nodal segments of the median region of young branches resulted in lower rates of contamination and a subsequent increase in the rates of the establishment of *in vitro* cultures. The results obtained provide the basis for establishing a protocol for large-scale micropropagation of *Bambusa oldhamii.*

Keywords: Nodal segment**;** *In vitro* introduction; Bambuseae

INTRODUÇÃO

Bambusa oldhamii Munro (Bambusoideae:Poaceae) é um bambu nativo do sudoeste Asiático. Considerado um bambu de grande porte, seus colmos podem atingir 12-15 m de altura e diâmetro de mais de 12 cm (Hsu, 2000). A espécie possui potencial econômico devido aos seus colmos tradicionalmente utilizados para a produção de mobiliário e estruturas leves, embora não haja limitações para o uso em construções civis. Além disso, devido sua arquitetura de planta ereta, as touceiras apresentam potencial de uso ornamental (Schiva, 1999) e como cortina vegetal em propriedades rurais. Na Ásia, a espécie é amplamente difundida devido a facilidade de cultivo e a longevidade do período de safra de brotos comestíveis, que pode estender-se sobre períodos de entressafras de outras espécies (Lan et al., 2019). O extrato de suas folhas também possui características antioxidantes e efeitos inibidor da peroxidação lipídica comprovados para o uso alimentício e farmacêutico (Lv et al., 2012).

No Brasil, a espécie ainda é pouco difundida e incipientemente cultivada. Contudo, devido aos seus múltiplos propósitos e sua tolerância à baixas temperaturas, ela se mostra promissora para o cultivo no país, especialmente na região Sul do Brasil. Embora a importância desta espécie para a região seja reconhecida informalmente por entidades locais, não existem documentos sobre a recomendação desta ou de outras espécies para plantios comerciais de bambus.

Assim como as outras espécies de bambus consideradas prioritárias (Rao et al., 1998), *B. oldhamii* possui um longo ciclo de florescimento sem um intervalo regular registrado. A espécie apresenta florescimento esporádico de poucos colmos ou touceiras, sem necessariamente ocorrer a senescência da touceira após o evento. Estes fatos implicam na escassez e imprevisibilidade da disponibilidade de sementes para a propagação sexuada da espécie. Embora, comparativamente à outras espécies de Bambuseae, apresente taxas razoáveis de sucesso dos métodos convencionais de propagação, a sazonalidade, a baixa disponibilidade de matrizes e a lacuna no conhecimento sobre a ocorrência de pragas e doenças em propágulos, ainda são entraves para os plantios comerciais da espécie no Brasil.

Neste cenário, biotecnologias associadas à cultura de tecidos e análises moleculares são ferramentas úteis para fomentar a produção de plantas em escala comercial. Além disso, ambas metodologias são utilizadas para o diagnóstico e eliminação de vírus do mosaico (BaMV) que acomete a espécie na Ásia e pode reduzir em 50 % a produtividade de brotos (Hsu et al., 2000; Lin et al., 2007). Embora a ocorrência desta virose não seja relatada no

Brasil, a lacuna no conhecimento sobre a espécie é uma vulnerabilidade quanto a potencial disseminação da doença no país.

O estabelecimento de um protocolo regenerativo *in vitro* para a espécie pode fortalecer o fomento à produção em larga escala de plantas de genótipos selecionados com qualidade fitossanitária e fisiológica para o estabelecimento de plantios comerciais. Trabalhos anteriores estabeleceram metodologia para propagação *in vitro* via organogênese (Lin et al., 2007; Thiruvengadam et al., 2011) e via embriogênese somática (Yeh e Chang, 1986; Prutpongse e Gavinlertvatana, 1992) para essa espécie. Contudo, a validação e adaptação de protocolos são necessárias em bambus devido à ocorrência de respostas genótipo-dependentes, bem como às variações nas condições ambientais das matrizes doadoras de explantes (Hirimburegama e Gamage, 1995).

Assim, o presente estudo objetivou avaliar a concentração ideal de BAP no meio de cultura e a posição ideal do segmento nodal nas touceiras para a introdução e estabelecimento de culturas *in vitro* de *Bambusa oldhamii* a partir de matrizes cultivadas nas condições climáticas de Florianópolis, SC, Brasil.

MATERIAL E MÉTODOS

Segmentos nodais de plantas matrizes jovens (2-4 anos) da espécie *Bambusa oldhamii* foram utilizados como explantes para o estabelecimento de culturas *in vitro*. Ramos jovens com as gemas ainda protegidas pela bainha foram obtidos de plantas matrizes cultivadas no Centro de Ciências Agrárias da Universidade Federal de Santa Catarina, em Santa Catarina, Brasil.

Para os experimentos de introdução *in vitro* os segmentos nodais foram desinfestados de acordo com as seguintes etapas: (1) Os ramos foram esfregados com algodão embebido em álcool 70 °GL até a retirada de tricomas e folhas das bainhas; (2) Segmentos nodais foram excisados com cerca de 20 mm de comprimento; e (3) tiveram suas bainhas manualmente retiradas: (4) Os segmentos foram mantidos por 15 minutos em solução à 3 $ml.L^{-1}$ de Kazumin® (Casugamicina) por 20 minutos sob agitação. (5) Em seguida, em câmara de fluxo laminar (CFL), estes segmentos foram transferidos para frascos esterilizados e submetidos a imersão em álcool 70 °GL por 1 minutos sob agitação; e posteriormente (6) transferidos para novo frasco esterilizado e imersos em solução de hipoclorito de sódio a 1,5% de cloro ativo suplementado com Tween 20® (1 gota / 100ml de solução) por 15 minutos sob agitação; Então, (7) os segmentos nodais foram submetidos a tríplice lavagem em água deionizada

estéril e (8) colocados sobre papel absorvente estéril para secagem do filme de água antes da inoculação no meio de cultura.

O meio de cultura basal utilizado foi composto da solução salina de MS (Murashige e Skoog, 1962), suplementado com 2 $ml.L^{-1}$ de vitaminas de Morel (Morel e Wetmore, 1951). Os fitorreguladores e outros suplementos, quando utilizados, foram adicionados ao meio de cultura antes da aferição do pH para 5,8. Os meios foram solidificados com 1,5 $g.L^{-1}$ de Phytagel® e esterilizados em autoclave por 15 minutos a 121 °C. Os experimentos de introdução *in vitro* foram realizados utilizando-se tubos de ensaio (25 mm X 150 mm) contendo 10 ml de meio de cultura.

Com o objetivo de gerar informações básicas para o aperfeiçoamento da metodologia de introdução *in vitro* e estabelecimento de culturas por meio de segmentos nodais de *Bambusa oldhamii*, foram realizados os seguintes experimentos.

Exp. 1 – Efeitos do BAP na introdução in vitro

Avaliou-se o efeito das concentrações de Benzilaminopurina (BAP) para a indução de brotação em segmentos nodais e estabelecimento de culturas *in vitro* de *B. oldhamii*. Após o procedimento de desinfestação, os segmentos foram inoculados em tubos de ensaio contendo o meio de cultura basal contendo *Plant Preservative Mixture*® (PPM®) à 0,2% (v/v) e suplementado com 2,5 µM de ANA como controle, e com 10 µM, 20 µM, 30 µM e 40 µM de BAP também suplementados com 2,5 µM de Ácido Indolacético (ANA). Foram inoculados 16 segmentos nodais por tratamento. Os subcultivos foram feitos a cada 15 dias e aos 45 dias avaliou-se as taxas de contaminação por microrganismos (fungos e bactérias – avaliação visual), de brotação, de oxidação dos brotos gerados, e de estabelecimento de culturas. Este experimento foi repetido por 3 vezes, totalizando 240 segmentos nodais introduzidos.

Exp. 2 – Efeito da posição do ramo no colmo

Segmentos nodais de ramos principais de um colmo de uma touceira jovem (2 anos) foram introduzidos *in vitro*. Os ramos foram classificados de acordo com a sua posição no colmo (sentido acropetal) em: basais (6°, 7° e 8° nós), medianos inferiores (9°, 10° e 11° nós), medianos superiores (12°,13° e 14° nós) e apicais (15°, 16° e 17° nós). Os segmentos foram desinfestados e inoculados em tubos de ensaio contendo o meio de cultura basal suplementado com 2,5 µM de ANA e 10 µM de BAP, e PPM® à 0,2% (v/v). Aos 30 dias da introdução *in vitro* foram avaliadas as taxas de contaminação por microrganismos (fungos e bactérias –

avaliação visual), de brotação, de oxidação, de indução de calos na região do meristema intercalar, e o número médio e altura média de brotos obtidos por segmento nodal. Os dados de porcentagem foram obtidos pela média de três tubos contendo um segmento nodal cada. Foram introduzidos 12 segmentos nodais por tratamento, totalizando 48 segmentos nodais.

Exp. 3 – Efeito da posição do segmento nodal em ramos jovens e maduros

*S*egmentos nodais de ramos principais de colmos de 1 a 2 anos obtidos de touceiras maduras, com cerca de 10 anos de idade, foram introduzidos *in vitro*. Foram selecionados ramos com a maioria das gemas ainda protegidas pelas bainhas. Devido a heterogeneidade no desenvolvimento destes em função da posição do colmo na touceira e da variação de idade dos colmos, os ramos foram separados em duas classes: ramos jovens (42,4 ± 16,9 cm de comprimento) e ramos maduros (108,6 ± 20,4 cm de comprimento). Os segmentos foram desinfestados e inoculados em tubos de ensaio contendo meio de cultura basal suplementado com PPM® à 0,2% (v/v) e contendo 2,5 µM de ANA e 10 µM de BAP. Aos 30 dias da introdução foram avaliadas as taxas de contaminação por microrganismos (fungos e bactérias – avaliação visual), de brotação, de oxidação, e de indução de calos na região do meristema intercalar. Os dados de porcentagem foram obtidos da média de 3 tubos contendo um segmento nodal cada. Foram utilizados de 10-20 segmentos por tratamento, totalizando 176 segmentos nodais introduzidos.

Análises estatísticas

Para todos os experimentos realizou-se a prévia verificação dos pressupostos da análise de variância. A normalidade dos dados foi verificada por meio de representação gráfica Quantil-Quantil dos resíduos, e a homocedasticidade foi verificada pelo teste de Bartlett. Quando houve descumprimento de pressupostos, aplicou-se a transformação dos dados por arco seno [$\sqrt{(x/100)}$]. Em caso de não satisfação dos pressupostos após a transformação, realizou-se o teste não paramétrico de Kruskal-Wallis. Em caso de conformidade dos pressupostos, os dados foram submetidos à análise de variância (ANOVA) à 5%. Quanto esta foi significativa, aplicou-se o teste de separação de médias de Duncan à 5%. Para o experimento de doses de BAP no estabelecimento de culturas a partir de segmentos nodais realizou-se a análise de regressão logística binomial por modelos generalizados mistos (GLM). Todas as análises foram realizadas em plataforma R Studio (R

Core Team, 2019) e os pacotes utilizados foram: agricolae (Mendiburu, 2019), stats (R Core Team, 2019), e ggplot2 (Wickham et al., 2016).

RESULTADOS

Exp. 1 –BAP estimula a brotação em segmentos nodais de B. oldhamii

As taxas de contaminação por microrganismos observadas alcançaram um valor de 28,9 % aos 45 dias após a introdução *in vitro* (Figura 1). A maior parte das contaminações ocorreu devido à manifestação de fungos, observados pelo desenvolvimento de estruturas miceliais associadas aos explantes.

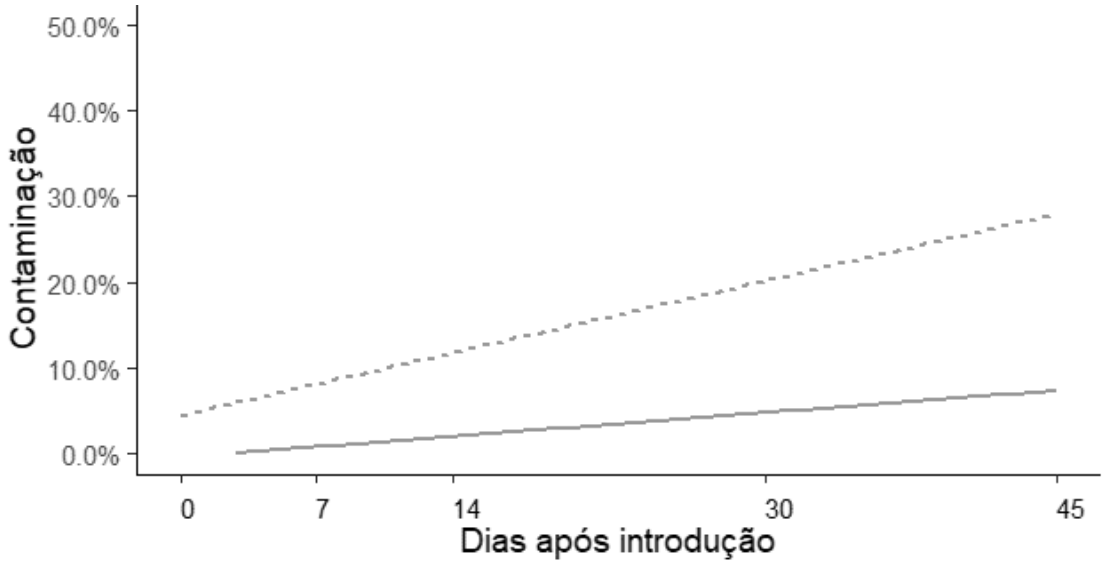

Figura 1 Contaminação fúngica (linha contínua) e bacteriana (linha tracejada) ao longo do estabelecimento *in vitro* de culturas de *Bambusa oldhamii*.

Foram observadas contaminações por microrganismos tardias, ocorridas após os 45 dias de cultivo, o que sugere a presença de microrganismos de natureza endofítica nos segmentos nodais utilizados com explante. Esse tipo de contaminação ocorrida após o segundo subcultivo também pode indicar um efeito biostástico, porém não biocida do supressor microbiológico PPM^{TM} suplementado ao meio de cultura.

O incremento de BAP no meio de cultura aumentou as taxas de brotação dos segmentos nodais até os 30 dias após a introdução *in vitro* (Figura 3).

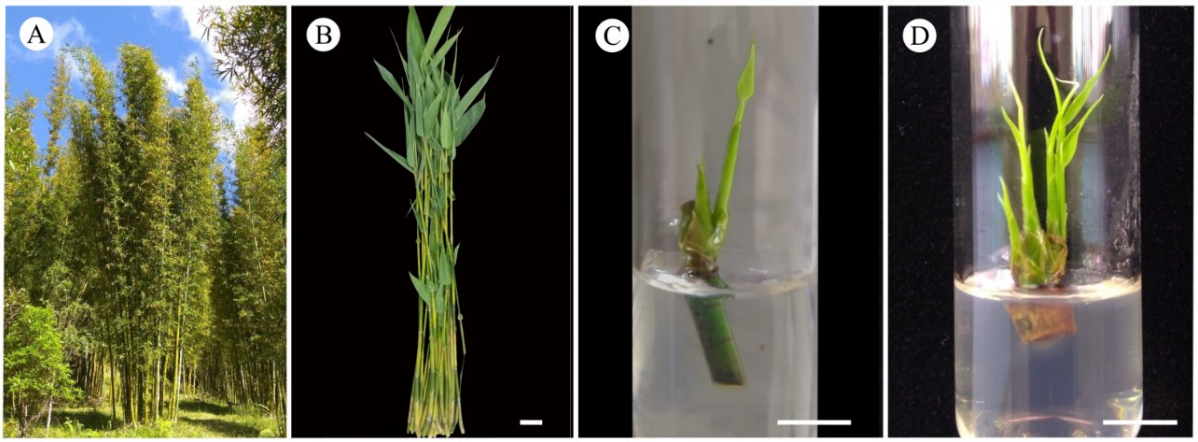

Figura 2 Introdução *in vitro* de *Bambusa oldhamii*. (A) Touceira madura; (B) Ramos primários; (C-D) Segmentos nodais *in vitro* apresentando múltiplas brotações simultâneas. Barras = 5 mm.

Observou-se um aumento na ocorrência das brotações até os 30 dias da introdução *in vitro* para todas as doses de BAP, sem aumento expressivo nos 15 dias consecutivos (45 dias). Porém ocorreram novas brotações tardias ao longo do experimento sobretudo no tratamento sem adição de BAP ao meio de cultura. A interação entre os fatores tempo e doses de BAP foi significativa (p=0,0089), evidenciando um efeito promotor de BAP na velocidade de indução de brotação nos segmentos nodais (Figura 3).

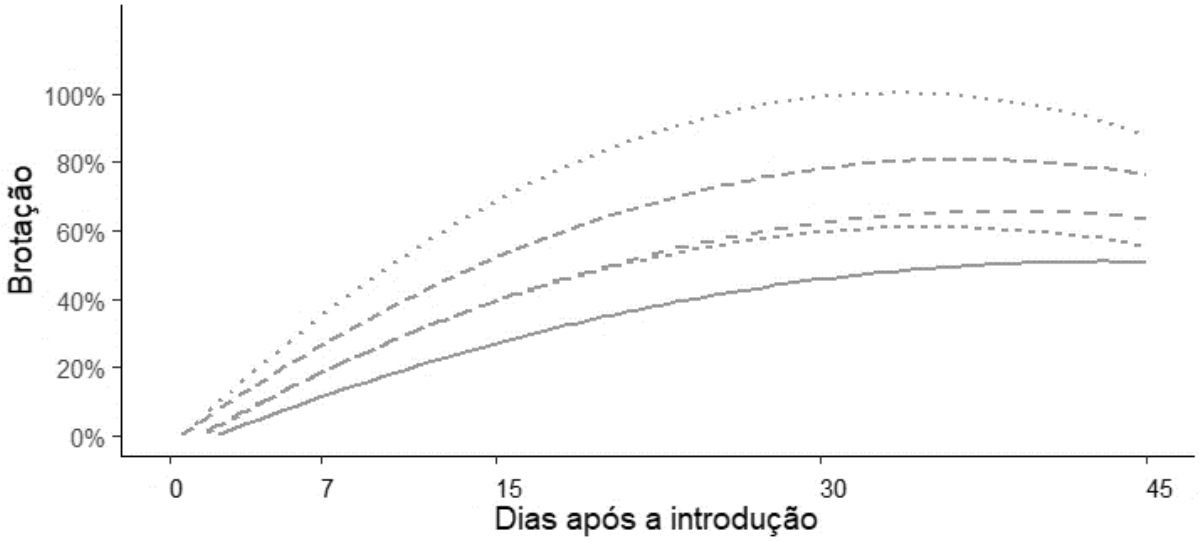

Figura 3 Brotação *in vitro* de segmentos nodais de *Bambusa oldhamii* em resposta à distintas doses de BAP (—) 0 µM; (···) 10 µM; (– –) 20 µM; (– ·) 30 µM; (···) 40 µM

O estabelecimento de culturas foi avaliado pela manutenção de brotações saudáveis ao longo dos 45 dias de cultura *in vitro* (*i.e.,* Segmentos brotados, não contaminados e não afetados por desordens fisiológicas). Embora a taxa de brotação tenha sido incrementada pelo aumento de BAP no meio de cultura, este incremento também resultou em maior ocorrência

de oxidação e/ou escurecimento de brotações nos tratamentos com as maiores doses testadas, 30 µM e 40 µM (Figura 4).

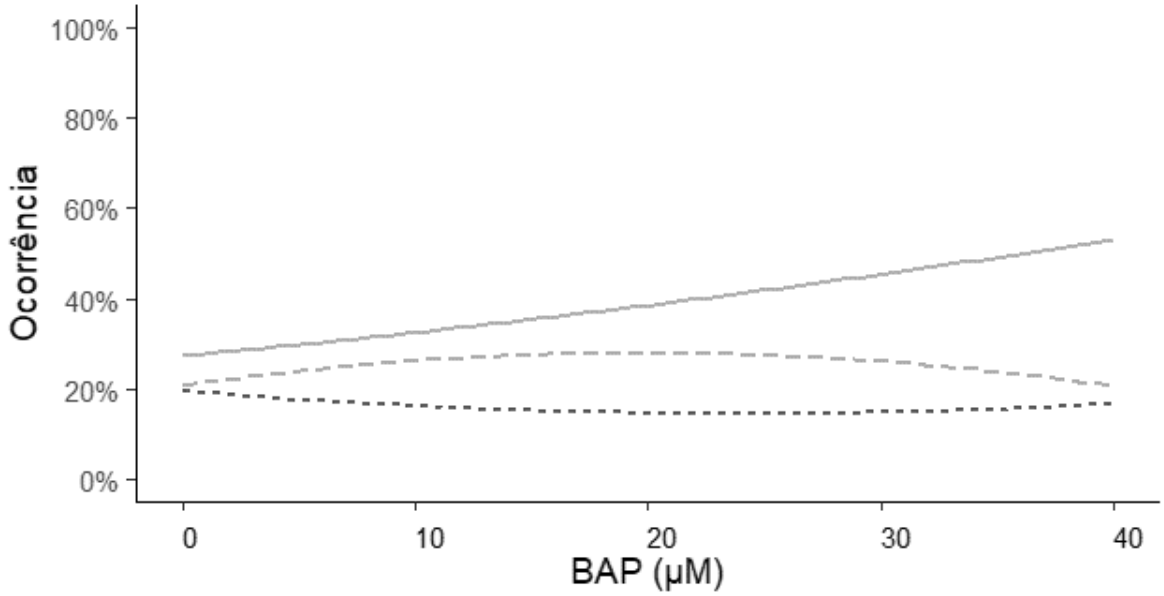

Figura 4 Estabelecimento *in vitro* de culturas de *Bambusa oldhamii* por meio de segmentos nodais. (—) Brotação; (- - -) Contaminação; (– –) Estabelecimento.

Exp. 2 - Efeito da posição do ramo no colmo

Aos 30 dias em cultivo *in vitro* somente foram observadas contaminações por bactérias. Não foi observada ocorrência diferencial da manifestação de bactérias nos explantes das diferentes posições no colmo (p=0,7364). A taxa de brotação geral foi de 49,1 ± 8,0 %. Cerca de 75 % dos segmentos nodais dos ramos da região mediana superior brotaram. Dos segmentos obtidos de ramos da região basal do colmo apenas 33% apresentaram brotação. Não foi detectada diferença estatística no efeito da posição dos ramos no colmo (p=0.6018). Consequentemente, a taxa de estabelecimento não foi diferente entre os segmentos obtidos de regiões distintas do colmo (p=0.7802), embora esta taxa tenha variado de 16,67 % a 62,50 %, respectivamente nas posições apical e mediana superior do colmo. O intumescimento das gemas ocorreu na maioria dos segmentos inoculados. Porém, não foi observada diferença significativa deste parâmetro nas diferentes posições dos ramos no colmo (p= 0.5484). Já quanto a formação de calo na região do meristema intercalar do segmento nodal (calo intercalar), também não se observou diferença significativa (p= 0.1428) (Figura 6 e 7).

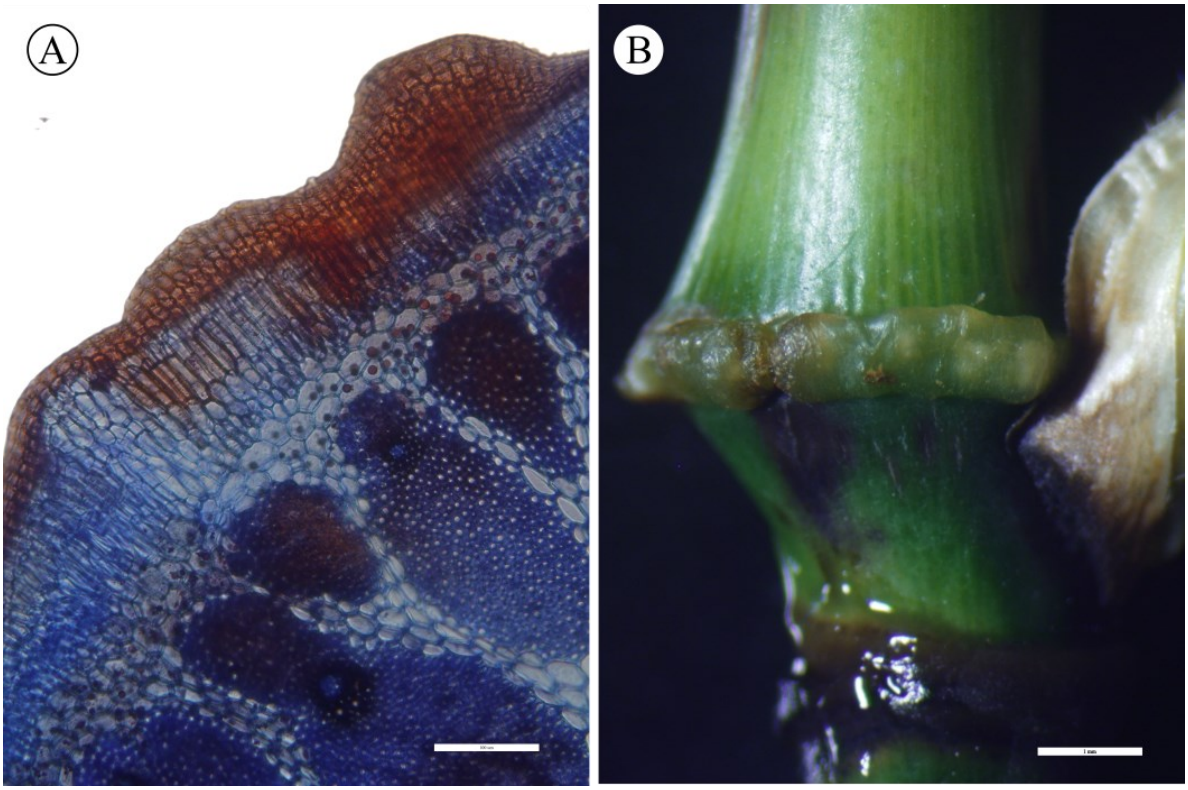

Figura 5 Formação de calos na região da crista nodal em segmentos nodais de *Bambusa oldhamii* introduzidos *in vitro*. (A) Secção transversal de calo formado na região da crista nodal corada com azul de Astra e Safranina; (lâmina produzida por. Marco Antônio Neto) Barra= 100 µm; (B) Calo na crista nodal; Barra = 1 mm.

Os brotos obtidos aos 30 dias de introdução *in vitro* não apresentaram diferenças significativas quanto a altura média (p=0.9517) e o número de brotos gerados por segmento nodal (p=0,6154) (Figura 8).

De maneira geral não foram observadas diferenças significativas quanto aos parâmetros avaliados em segmentos nodais obtidos de diferentes regiões de um colmo jovem para a introdução *in vitro* de *Bambusa oldhamii.*

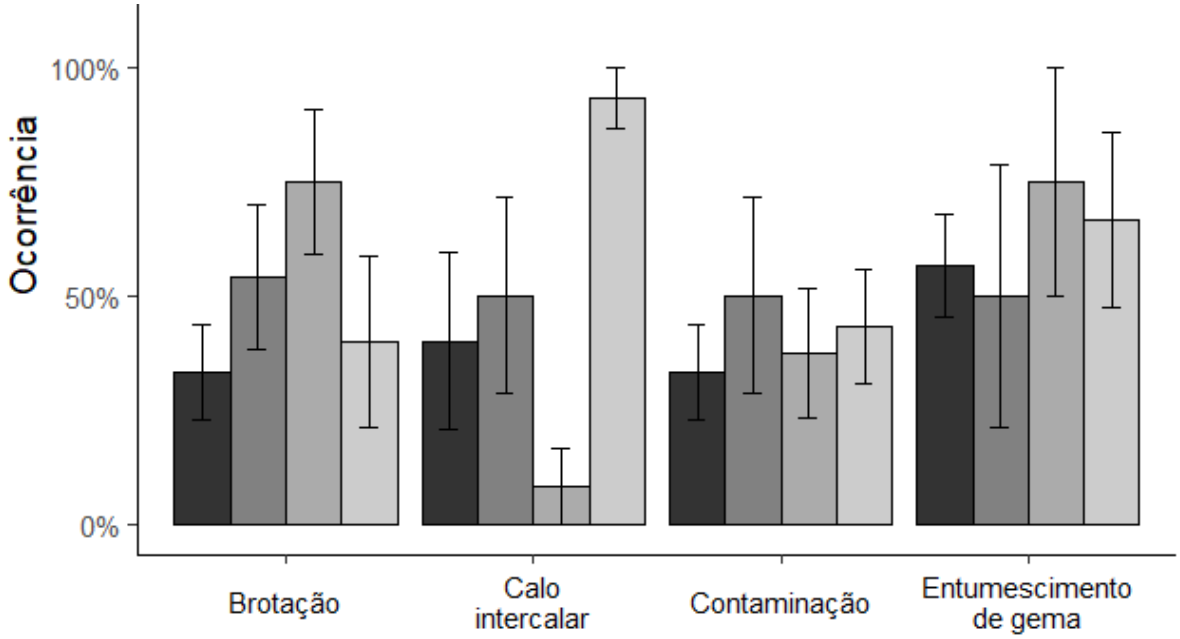

Figura 6 Taxas de ocorrência de brotações, formação de calos na região do meristema intercalar, contaminação e intumescimento de gemas em segmentos nodais de *Bambusa oldhamii* durante a introdução *in vitro* em função da posição do ramo no colmo. Regiões do colmo: (■) basal, (■) mediana inferior, (■) mediana superior, e (■) apical.

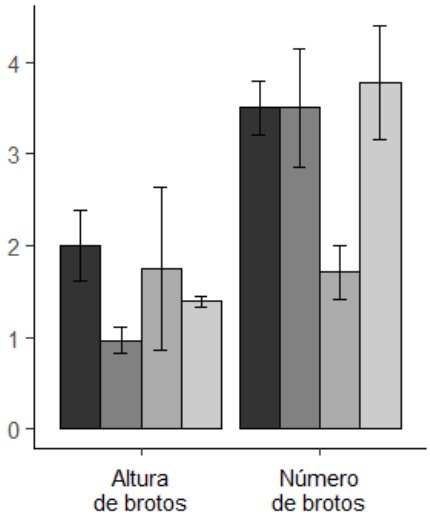

Figura 7 Altura média e número de brotos em segmentos nodais brotados de *Bambusa oldhamii* aos 30 dias da introdução *in vitro* em função da posição do ramo no colmo. Regiões do colmo: (■) basal, (■) mediana inferior, (■) mediana superior, e (■) apical.

Exp. 3 – Efeitos da posição do segmento nodal em ramos jovens e maduros

A maturidade do ramo e a posição do segmento nodal neste foram fatores importantes quanto a ocorrência de contaminação por microrganismos na introdução *in vitro* de *B. oldhamii.* Ramos jovens apresentaram menor ocorrência de contaminação comparativamente aos ramos em estádio de desenvolvimento mais avançado ($p<0,01$). Os segmentos nodais obtidos a partir de ramos jovens apresentaram 30,9 % de contaminação, enquanto segmentos

obtidos de ramos maduros resultaram em 59,1 % de ocorrência de manifestação de microrganismos. A posição do segmento no ramo também apresentou diferenças significativas quanto a ocorrência de contaminações por microrganismos ($p<0,01$) (Figura 9). Segmentos de ambas as extremidades apresentaram mais contaminação comparativamente a região central do ramo, sendo que a posição 3 e 4 apresentaram as menores taxas, 21,8 % e 9,1 % respectivamente. No caso apenas dos ramos jovens, 8,3 % dos segmentos da posição 3 contaminaram e nenhum dos segmentos da posição 4 contaminou.

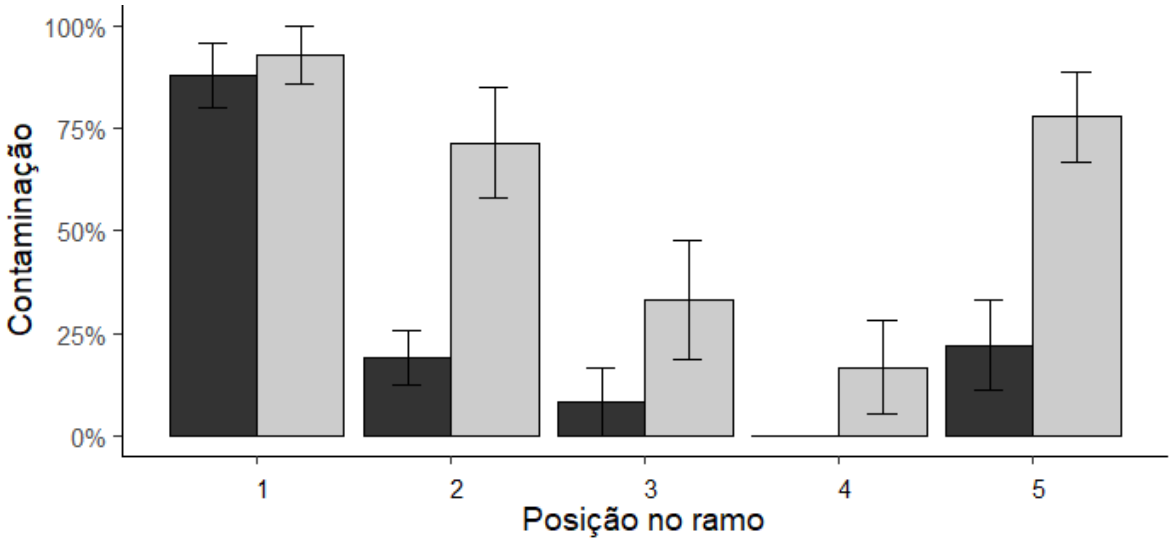

Figura 8 Ocorrência de contaminação na introdução *in vitro* de *Bambusa oldhamii* em função da posição do segmento nodal nos ramos (■) jovens e (■) maduros.

Quanto aos tipos de contaminação visualmente constatadas, a maior parte delas ocorreu por manifestação de bactérias (32,24 %) e apenas 15,02 % ocorreram por manifestação de fungos (Figura 9). Se forem considerados os efeitos do tipo de ramo, tanto para ramos jovens quanto para ramos mais desenvolvidos, não foi observada normalidade dos resíduos dos modelos. O teste de Kruskal-Wallis resultou em diferença significativa apenas para a ocorrência de fungos, a qual foi maior em ramos maduros, comparativamente à ramos jovens. Para a ocorrência de bactérias não houve distinção entre os tratamentos deste fator.

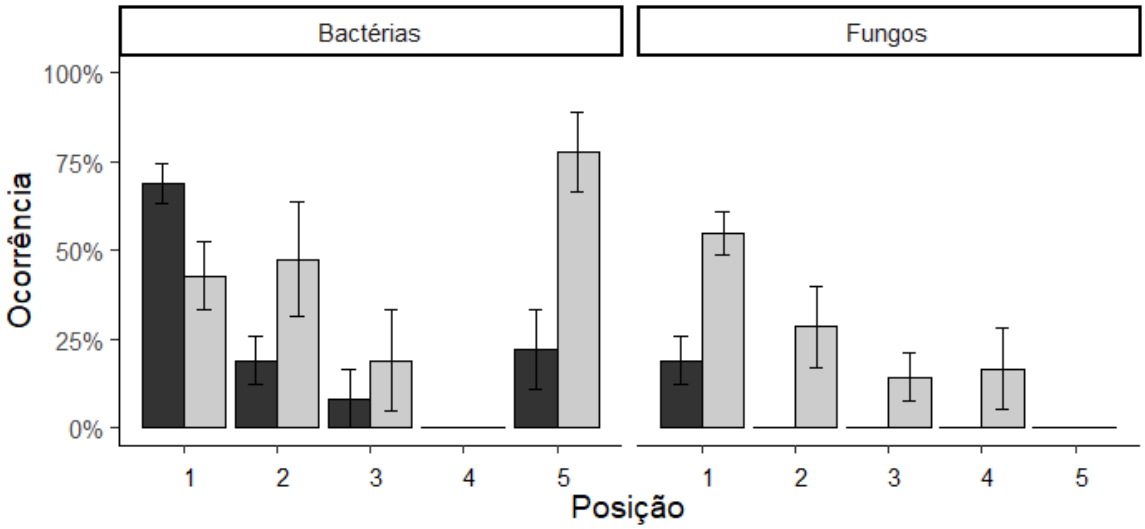

Figura 9 Ocorrência de bactérias e fungos na introdução *in vitro* de *Bambusa oldhamii* em função da posição do segmento nodal nos ramos (■) jovens e (■) maduros

A ocorrência de brotação dos segmentos nodais não foi afetada pela maturidade do ramo. Porém, a posição do segmento nodal no ramo foi um fator significativo para a obtenção de gemas brotadas (Figura 10). Para ambos os fatores não foi observada a normalidade dos resíduos do modelo. Segundo o teste de Kruskal-Wallis, as ocorrências de brotações dos segmentos nodais foram semelhantes para ambos os tipos de ramos utilizados. Porém os segmentos nodais na região central do ramo apresentaram maiores porcentagens de brotação comparativamente aos segmentos nodais das extremidades dos ramos.

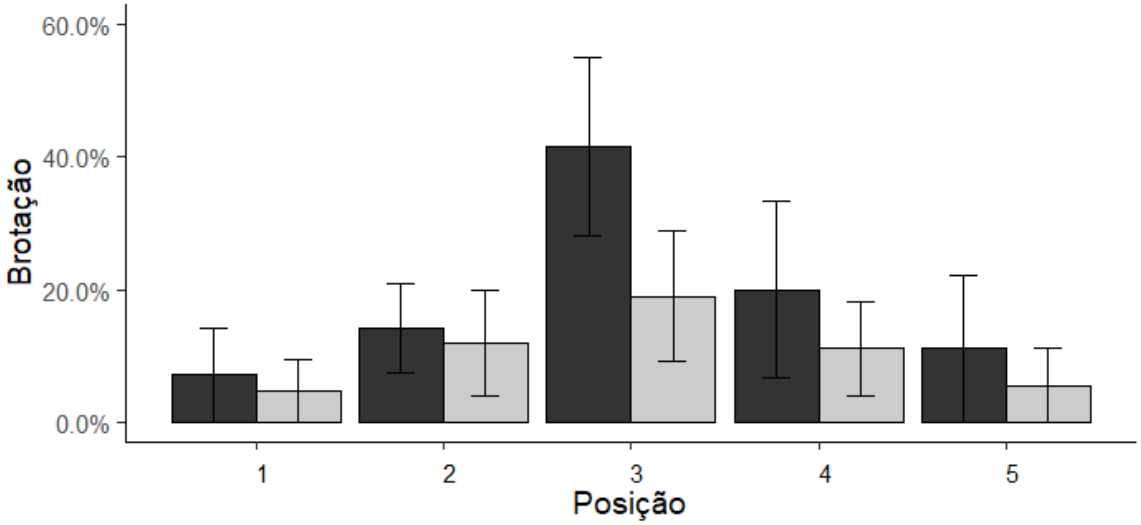

Figura 10 Ocorrência de brotação na introdução *in vitro* de *Bambusa oldhamii* em função da posição do segmento nodal nos ramos (■) jovens e (■) maduros.

Para o parâmetro número de brotos não houve diferenças significativas para os fatores testados. A análise de variância não revelou diferenças estatísticas no número de brotos por segmento nodal quanto ao tipo de ramo (p=0,9687) ou quanto a posição do segmento nodal no ramo (p=0,3071).

O estabelecimento de culturas apresentou valores semelhantes aos de brotação e não foi influenciado pelos fatores avaliados (Figura 11). Os dados não apresentaram homocedasticidade, tampouco normalidade dos resíduos do modelo. O teste de Kruskal-Wallis demonstrou diferenças apenas quanto a posição do segmento nodal no ramo, independentemente da maturidade deste. Os segmentos da região central do ramo apresentaram maiores taxas de estabelecimento de culturas comparativamente aos segmentos das extremidades. A maior porcentagem de estabelecimento ocorreu com segmentos nodais obtidos da posição 3 com uma média de 25,64 % de estabelecimento, sendo que nos ramos jovens essa ocorrência foi de 33,33 % e nos ramos maduros de 19,05 %.

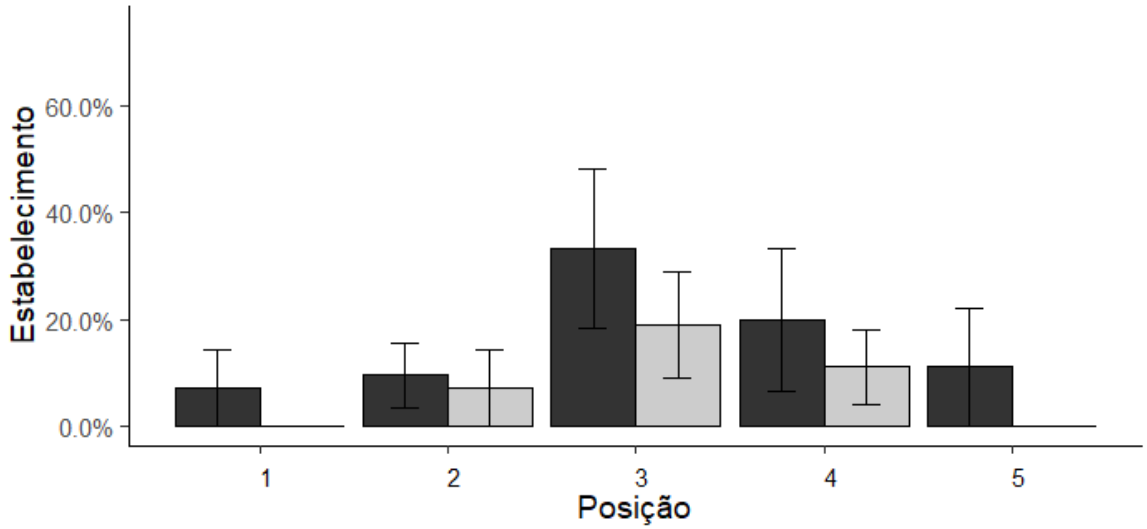

Figura 11 Estabelecimento de culturas *in vitro* de *Bambusa oldhamii* em função da posição do segmento nodal em ramos jovens (■) e ramos maduros (■).

O intumescimento das gemas após a inoculação é um indicador visual de resposta fisiológica às novas condições *in vitro* (Figura 12). Este parâmetro mostrou-se significativamente diferente quanto ao tipo de ramo utilizado (p=0,0006). Os ramos mais jovens apresentaram maior ocorrência de intumescimento de gemas que os ramos maduros e houve um aumento de ocorrência desta resposta no sentido acropetal.

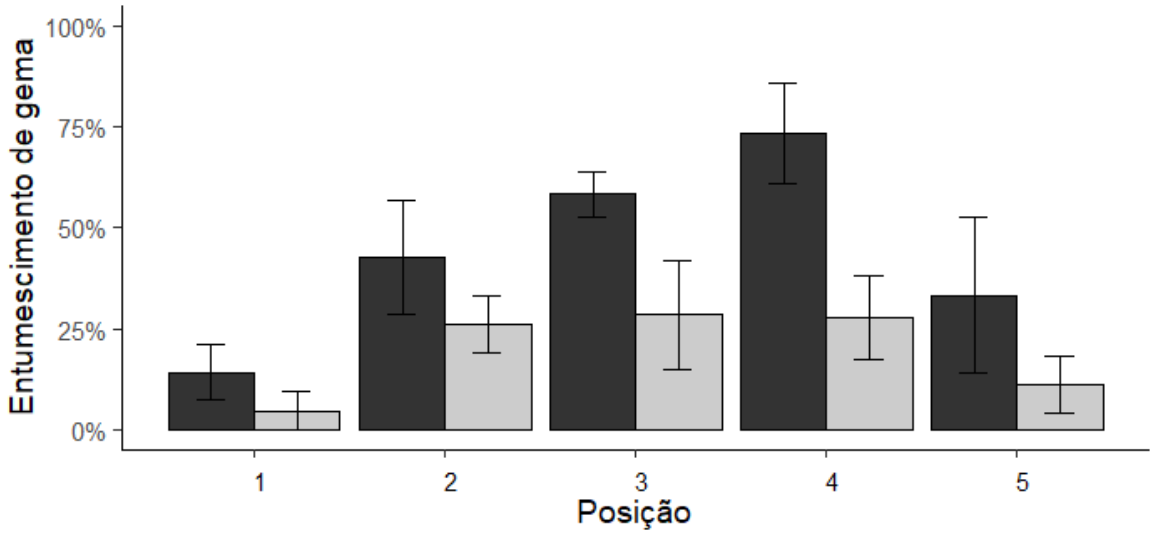

Figura 12 Taxa de intumescimento de gemas durante a introdução *in vitro* de *Bambusa oldhamii* em função da posição do segmento nodal em ramos jovens (■)e ramos maduros (■).

DISCUSSÃO

Bambusa oldhamii é um bambu lignificado de grande porte. Nos últimos anos, a procura por esta espécie é crescente no Sul do Brasil, devido aos seus múltiplos usos e sua tolerância à baixas temperaturas. Para a aplicação de *B. oldhamii* na formação de cortinas vegetais, produção de varas e seus derivados, e brotos comestíveis, há uma necessidade de produção de mudas em grandes quantidades. Em experimentos anteriores com a espécies, a falta de explantes homogêneos em quantidade e qualidade devido à escassez de plantas matrizes foi um fator limitante para o estabelecimento. Portanto, três touceiras foram plantadas no Centro de Ciências Agrárias da Universidade Federal de Santa Catarina. As quais possibilitaram os experimentos deste capítulo. Considerando ainda os resultados anteriores, nos quais *B. oldhamii,* a terceira espécie de Bambuseae acessada pelo grupo do LFDGV para estabelecimento de culturas *in vitro*, apresentou a ocorrência de desordens fisiológicas ainda não observadas até então. O principal problema foi o escurecimento enzimático e necrose (oxidação) de brotos, que na maior parte dos casos impossibilitou o estabelecimento de culturas *in vitro*. Outro problema relevante foi a heterogeneidade das repostas observadas quanto as condições do material utilizado para introdução *in vitro.* Por estes dois motivos, decidiu-se realizar de maneira sistematizada experimentos para rastrear possíveis variações nas condições dos segmentos nodais, e determinar a melhor posição na touceira para obtenção de explantes, visando aumentar a eficiência do processo de introdução *in vitro* de *B. oldhamii.*

O estímulo da brotação em segmentos nodais de bambus é influenciado por diversos fatores e a presença de citocinina no meio de cultura é um fator importante no estabelecimento de culturas *in vitro*. O primeiro experimento teve como objetivo determinar a concentração mais adequada de BAP no meio de cultura para a indução de brotações em segmentos nodais de ramos laterais. Em um estudo utilizando meristemas isolados de segmentos nodais, a concentração de 0,45 μM de TDZ foi utilizado para o estabelecimento de culturas (Lin et al. 2007; Silveira et al. 2020). As doses de 4,4 μM a 6,6 μM de BAP foram eficientes para regenerar os maiores números de brotos por segmento nodal ao longo dos ciclos iniciais (63 dias) de cultivo *in vitro* de *B. oldhamii.* Observou-se que este efeito de BAP foi superior que o de KIN nos mesmos tipos de explantes testados (Thiruvengadam et al. 2011). Mais recentemente, em estudo focado nos tratamentos para redução da contaminação por microrganismo durante a introdução *in vitro,* nenhum fitorregulador foi adotado para a quebra de dormência das gemas (Pasqualini et al. 2019). No presente trabalho, enquanto um aumento nas concentrações de BAP resultaram em aumento no número de segmentos nodais brotados, e na velocidade da indução da brotação. Porém, os segmentos cultivadores nestas altas concentrações foram mais susceptíveis à ocorrência de escurecimento enzimático e necrose de folhas e brotos alongados.

Quanto a manifestação de microrganismos, o estabelecimento de culturas *in vitro* por meio de segmentos nodais é reconhecido como um dos principais gargalos da micropropagação de bambus (Ray e Ali, 2017). Segmentos nodais são explantes complexos, que apresentam diversos tipos de tecidos distintos em um gradiente de diferenciação. Entre estes, é importante destacar que os tecidos vasculares já estão desenvolvidos em segmentos nodais adequados para estabelecimento de culturas. Esta vascularização é uma importante fonte de microrganismos endofíticos. Além disto, o procedimento adequado de desinfestação deve levar em consideração a redução da carga microbiana ao longo das etapas e os devidos cuidados para evitar microrganismos epifíticos. Os trabalhos mais antigos com *B. oldhamii* não relatam sobre a ocorrência de contaminações durante o estabelecimento *in vitro* (Lin et al. 2007; Thiruvengadam et al. 2011). Enquanto em trabalhos mais recentes com a espécie, é notável a adoção do agente biocida PPM®, composto a base de duas isothiazolonas. Pasqualini e colaboradores (2019) relatam que a imersão dos explantes em meio de cultura líquido contendo 4 ml. L^{-1} foi o melhor tratamento para evitar a manifestação de microrganismos. Altas taxas de contaminações foram relatadas por Silveira e colaboradores (2020), com valores de 50 % a 100 % de ocorrência de manifestação de microrganismos em

função da época do ano em que os explantes foram coletados. No presente estudos, entre os três experimentos realizados, as taxas de contaminações variaram de 29 % a 59 %.

A posição no colmo do ramo de onde foram obtidos os segmentos nodais não influenciou o processo de introdução *in vitro* de *B. oldhamii.* Durante o rápido alongamento do colmo após a época de brotação, as gemas são recobertas pelas bainhas. A brotação dos ramos só inicia após o alongamento total do colmo. Para *B. vulgaris* a brotação dos ramos laterais ocorre no sentido basípeto (Banik, 1980). Em observações das plantas matrizes, o mesmo comportamento foi observado. Embora haja um gradiente no desenvolvimento dos ramos laterais, esta condição não interferiu significativamente no processo de introdução *in vitro* e no estabelecimento de culturas nas condições do presente estudo. É importante destacar que este experimento foi realizado utilizando-se plantas jovens como doadoras de explantes. A condição de cultivo e sobretudo a idade da planta matriz também podem influenciar neste efeito da posição sobre a brotação e até sobre a contaminação de segmentos nodais (Chowdhury et al., 2004).

A posição do segmento nodal no ramo principal foi um fator importante para o processo de introdução *in vitro*. Ramos jovens respondem mais efetivamente à quebra de dormência de gemas em comparação a ramos maduros contendo gemas brotadas (Saxena e Bhojwani, 1993). A região mediana de ramos jovens foi considerada a mais responsiva para a introdução *in vitro* e estabelecimento de culturas para *D. longispathus* (Saxena e Bhojwani, 1993), *B. vulgaris* (Hirimburegama e Gamage, 1995), e para *B. nutans* (Mudoi et al., 2014). Assim como nos referidos trabalhos, para *B. oldhamii* observou-se uma melhor resposta de brotação na região mediana dos ramos jovens. O entumescimento da gema em resposta à inoculação no meio de cultura é um importante marcador da primeira mudança observável, quando os prófilos abrem-se e o ápice do broto torna-se visível (Hirimburegama e Gamage, 1995).

CONCLUSÕES

O presente estudo demonstrou dois fatores fundamentais para o estabelecimento de culturas *in vitro* do bambu *Bambusa oldhamii,* a concentração ideal de BAP para a indução da brotação em segmentos nodais e a posição na planta em que estes segmentos devem ser obtidos. Para a indução de brotações dos segmentos nodais, o incremento de BAP aumentou a taxa de brotações, além de reduzir o tempo de ocorrência destas. Embora concentrações elevadas tenham sido eficientes para a iniciação de culturas por meio de segmentos nodais,

efeitos adversos podem ser observados em subcultivos subsequentes. Considerou-se o uso de 10 µM BAP + 2,5 µm ANA como adequados para o estabelecimento de culturas a partir de segmentos nodais. Para a coleta destes segmentos, observou-se que os explantes obtidos de ramos principais da região mediana superior e região apical de colmos jovens apresentaram altas taxas de indução de brotações, embora os explantes obtidos de outras regiões do colmo também foram viáveis para o estabelecimento de culturas *in vitro.* Quanto a posição do segmento nodal no ramo, os explantes obtidos da região mediana das ramificações principais foram os que brotaram mais e apresentaram as menores taxas de contaminação por microrganismos. Os resultados obtidos mostram o potencial da micropropagação de *Bambusa oldhamii*, delimitando fatores importante deste processo.

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CONSIDERAÇÕES FINAIS

Os bambus são recursos genéticos vegetais de importância global. Atualmente seus usos e potenciais vêm sendo mundialmente reconhecidos e divulgados devido a sua importância socioeconômica e ambiental. O presente trabalho é o relato de parte significativa de um esforço aplicado ao estabelecimento de culturas *in vitro* e ao avanço acadêmico no conhecimento frente à pontos limitantes no processo de micropropagação de bambus lignificados. Esse projeto iniciou-se a partir da interação do LFDGV e a BambuSC para a submissão e consequente aprovação do projeto "Tecnologias para o Desenvolvimento Sustentável da Cadeira Produtiva do Bambu no Sul do Brasil". O subprojeto "Macro e Micropropagação e conservação *in vitro* de germoplasmas de bambu" resultou em diversos avanços na micropropagação de bambus. Porém, o fator limitante sempre foi o estabelecimento de culturas *in vitro.* A escassez de plantas matrizes das espécies alvo, e a manifestação de microrganismos durante a introdução *in vitro* foram as principais dificuldades encontradas ao longo do subprojeto. Vencida esta etapa, diversos trabalhos de graduandos e pós-graduando puderam ser executados com as culturas de bambus estabelecidas *in vitro.* O presente estudo teve como meta principal o estabelecimento de culturas *in vitro*, de forma que metodologias e plantas fossem geradas, visando o fortalecimento da crescente cadeia produtiva do bambu no país. Bambus de reconhecida importância econômica foram micropropagados com sucesso por meio das rotas da embriogênese somática e organogênese direta. O estabelecimento de um protocolo de micropropagação envolve diversos procedimentos e seu desenvolvimento resulta em novas perguntas e hipóteses a serem respondidas e testadas. As culturas estabelecidas permitirão os avanços no conhecimento científico e aplicado acerca dos bambus lignificados, não apenas no campo da propagação, mas também no conhecimento sobre a fisiologia, genética e conservação dos bambus.