UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO CIÊNCIAS AGRÁRIAS
PROGRAMA DE PÓS-GRADUAÇÃO RECURSOS GENÉTICOS VEGETAIS

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Caraterização morfológica do androceu e do grão de pólen em diferentes estádios de desenvolvimento floral de *Dendrocalamus asper* (Schult. & Schult.) Backer ex K. Heyneke e *Bambusa tuldoides* Munro

Florianópolis
2020

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Tese submetida ao Programa de Programa de Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do título de doutor em Ciências
Orientador: Prof.ª Dr.ª Rosete Pescador
Coorientadora: Prof.ª Dr.ª Cristina M. Ribas dos Santos

Florianópolis
2020

Ficha de identificação da obra elaborada pelo autor,
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Souza, Priscila Fernandes de
Caraterização morfológica do androceu e do grão de pólen em diferentes estádios de desenvolvimento floral de Dendrocalamus asper (Schult. & Schult.) Backer ex K. Heyneke e Bambusa tuldoides Munro / Priscila Fernandes de Souza ; orientador, Rosete Pescador, coorientador, Cristina Magalhães Ribas dos Santos, 2020.
81 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro de Ciências Agrárias, Programa de Pós Graduação em Recursos Genéticos Vegetais, Florianópolis, 2020.

Inclui referências.

1. Recursos Genéticos Vegetais. 2. Bambu. 3. Reprodução Sexuada. 4. megaesporogênese. 5. megagametogênese. I. Pescador, Rosete. II. Santos, Cristina Magalhães Ribas dos. III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Recursos Genéticos Vegetais. IV. Título.

Priscila Fernandes de Souza

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em Ciências.

____

Coordenação do Programa de Pós-Graduação

____

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Orientador(a)

Florianópolis, 2020

Este trabalho é dedicado à minha mãe, Ivone, à minha avó Anita e ao meu marido Fylipe

AGRADECIMENTOS

Agradeço primeiramente a Deus, por me acolher e me guiar nos momentos mais difíceis e me proporcionar viver esse momento.

À Universidade Federal de Santa Catarina e ao Programa de Pós-Graduação em Recursos Genéticos Vegetais, docentes, discentes e colaboradores, pela formação e confiança a mim concedidas.

À minha orientadora Rosete Pescador, por acreditar no meu potencial, pelos ensinamentos, conselhos e amizade.

À minha coorientadora Cristina M. Ribas dos Santos, pela disponibilidade e ensinamentos.

À banca examinadora, por aceitar o convite, pelas críticas e sugestões.

Aos meus amigos do Núcleo de Pesquisas em Biotecnologia e Desenvolvimento Vegetal (NPBV), Elizandra Pradella, Nadhine Nostrani, Rafaela Rizzolo, Mariana Schvamba, Mariana Schlickmann, Bruna Andriolli, Sebastian Montoya e Giuliano Rigo, por estarem sempre dispostos a ajudar e pelas conversas de motivação e força.

Ao Joe Ree, pela amizade, ajuda na execução dos experimentos e na tradução dos artigos.

Aos amigos Willian Goldini Costa e Julia Zappelini, pela ajuda nas coletas.

À minha família, em especial a minha mãe Ivone e minha avó Anita, pelo apoio e por acreditarem no meu potencial.

Ao meu marido e melhor amigo Fylipe Aguilar, por me apoiar nessa jornada e por compreender os momentos de ausência.

A minha amiga/irmã Lidiane Guedes, pelo apoio e amizade incondicional.

Ao Laboratório Central de Microscopia Eletrônica (LCME), pelos ensinamentos e por ter me auxiliado na realização das imagens de microscopia eletrônica.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), projeto “Tecnologias para o desenvolvimento sustentável da cadeia produtiva do bambu no sul do Brasil” (Edital 66/2013), pelo apoio financeiro.

E a todos aqueles que direta ou indiretamente contribuíram para a realização deste trabalho.

OBRIGADA!

RESUMO

As informações sobre a reprodução de *Bambusa tuldoides* e *Dendrocalamus asper* são incipientes pois, para a maioria dos bambus, a floração é imprevisível, variando de anual a floração após 120 anos de desenvolvimento vegetativo. Os objetivos deste trabalho foram caracterizar morfologicamente o androceu e gineceu em diferentes estádios de desenvolvimento das flores de *B. tuldoides* e *D. asper* e elucidar aspectos relacionados à reprodução e à não produção de cariopses. Foram realizadas a contagem do número de grãos de pólen e óvulos.flor^{-1}, a razão pólen/óvulo, testes de geminação in vitro e viabilidade dos grãos de pólen in vivo, identificação dos diferentes estádios de desenvolvimento floral e a caracterização morfológica e do desenvolvimento do androceu e do gineceu. Em *D. asper* verificou-se que as flores apresentam sete estádios de desenvolvimento (quatro iniciais, pré-antese e dois de antese) e protoginia como estratégia para evitar a endogamia. As características florais e do grão de pólen indicam que a espécie é anemófila. Foram registrados os estádios de micrósporo livre (estádio dois de desenvolvimento floral), micrósporo vacuolado (estádio cinco) e grãos de pólen maduros (antese). Até a maturação final, os grãos de pólen apresentaram incremento na quantidade e tamanho dos amiloplastos e de organelas como mitocôndrias, sendo dispersos com presença de amido e no estádio de desenvolvimento tricelular. Na antese, o grão de pólen é monoporado com a abertura do poro do tipo opérculo, padrão de superfície da exina rugular e a estrutura do teto espinhosa. O gineceu de *D. asper* consiste em um ovário ovado, um estilete longo e um estigma plumoso. O óvulo maduro é anátropo e bitegumentado. A formação do saco embrionário inicia-se no estádio dois de desenvolvimento floral e finaliza seu desenvolvimento na pré-antese. Em *D. asper* a depressão por endogamia de ação precoce parece ser o motivo para a não produção de cariopses. Em *B. tuldoides*, nos três estádios florais analisados, verificou-se o desenvolvimento normal das anteras. Porém, em relação aos micrósporos, observou-se que no estádio um, inicia-se a retração do citoplasma, o rompimento da membrana nuclear e das mitocôndrias. Nos estádios de pré-antese e antese continua a deposição de amido, a esporoderme apresenta-se incompleta e o desenvolvimento do pólen se dá de forma anormal, pois termina com a degradação total do citoplasma. A inviabilidade desses grãos de pólen anormais impede o desenvolvimento de cariopses em *B. tuldoides*. O gineceu de *B. tuldoides* consiste em um ovário obovado, estilete curto e dois estigmas plumosos. O óvulo é ortotrópo e bitegumentado. A formação do saco embrionário inicia-se na pré-antese e finaliza o desenvolvimento na antese. Esses resultados sugerem que a não produção de cariopses em *B. tuldoides* se deve à inviabilidade do grão de pólen e em *D. asper* supõe-se que seja devido a depressão por endogamia de ação precoce.

Palavras-chave: Bambu. Megaesporogênese. Megagametogênese. Microesporogênese. Microgametogênese. Reprodução sexuada.

RESUMO EXPANDIDO

Introdução

Os bambus têm obtido reconhecimento como espécies florestais de grande valor, por apresentar características físicas e mecânicas, que os tornam alternativas para o desenvolvimento de produtos, substituindo a madeira nativa ou reflorestada. Desempenham ainda, papel importante na preservação da biodiversidade e dos solos, bem como na conservação da água.

Dentre as espécies mais utilizadas de bambus destaca-se o *Dendrocalamus asper* (Schult. & Schult.) Backer ex K. Heynek, nativa da China valorizada pelos seus brotos comestíveis que são vendidos como enlatados em todo o mundo e a espécie *Bambusa tuldoides* Munro, nativa da China e que se propagou por todo o Sudeste Asiático sendo amplamente cultivada em regiões tropicais e subtropicais da América. No Brasil é uma das mais difundidas, tendo sido introduzida pelos portugueses no período colonial.

Apesar da grande importância econômica dos bambus pouco se sabe sobre a sua reprodução. Isso se deve principalmente à floração imprevisível, variando de anual à floração após 120 anos de crescimento vegetativo.

O conhecimento dos aspectos relacionados ao desenvolvimento das anteras, do grão de pólen, óvulo e ovário são de grande importância para a propagação e conservação tanto para *D. asper* quanto para *B. tuldoides*.

Objetivos

Os objetivos deste trabalho foram caracterizar morfologicamente o androceu e gineceu em diferentes estádios de desenvolvimento das flores de *B. tuldoides* e *D. asper* e elucidar aspectos relacionados à reprodução e à não produção de cariopse.

Metodologia

As inflorescências de *Bambusa tuldoides* foram coletadas de touceiras matrizes, localizadas no Parque Cidade das Abelhas-UFSC (27°32’20,04” S, 48°30’10,87” O), na cidade de Florianópolis, Santa Catarina, em janeiro/2017. As inflorescências de *Dendrocalamus asper* foram coletadas na Fazenda dos Bambus, pertencente ao Instituto Jatobás, na cidade de Pardinho, São Paulo (23°06’37,33” S, 48°22’05,60” O), em junho/2017.

Foram coletadas amostras da antera e ovário em ambas as espécies em três estádios de desenvolvimento da inflorescência. Em *B. tuldoides* os estádios de desenvolvimento da inflorescência foram: estádio um, estádio de pré-antese e antese e em *D. asper* foram: estádio dois de desenvolvimento, na pré-antese e na antese (estádio seis). Nestes estádios de desenvovimento floral foram feitas análises sob microscopia de luz (óptica) e histoquímica utilizando azul de toluidina e lugol, microscopia eletrônica de transmissão e varredura. No estádio de pré-antese em *D. asper,* foi realizado com os grãos de pólen o teste de germinação *in vitro*, viabilidade *in vivo*, contagem de grãos de pólen e razão pólen/óvulo. No estádio de pré-antese em *B. tuldoides,* foi realizado com os grãos de pólen o teste de germinação *in vitro* e a viabilidade *in vivo*.

Resultados e Discussão

As flores de *Dendrocalamus asper* apresentam sete estádios de desenvolvimento (quatro estádios iniciais, pré-antese e dois estádios de antese). *D. asper* possui como estratégia para evitar a endogamia a protoginia favorecendo a polinização cruzada. As características florais e do grão de pólen indicam que a espécie é anemófila. O número médio de grãos de pólen.flor^{-1} de *D. asper* foi de 7525, estando dentro do valor encontrado para espécies anemófilas dentro da família Poaceae em outros trabalhos. A razão P/O de D. asper (7525) é menor do que a de

plantas tipicamente polinizadas pelo vento. Em um estudo com *Phyllostachys nidularia* a razão P/O encontrada também está abaixo das plantas anemófilas, porém esta espécie é polinizada pelo vento**.** Os pólens de *D. asper* apresentaram viabilidade de 62,5%, valor considerado baixo quando comparado com outras espécies. Porém alguns trabalhos relatam que a viabilidade do pólen pode cair horas após a sua antese o que pode ter ocorrido com *D. asper*, uma vez que a viabilidade foi avaliada 36 horas após a coleta das inflorescências.

Foram registrados os estádios de micrósporo livre (estádio dois de desenvolvimento floral), micrósporo vacuolado (estádio cinco) e grãos de pólen maduro (antese). Até a maturação final, os grãos de pólen apresentaram incremento nas quantidades e tamanhos dos amiloplastos e de organelas como as mitocôndrias, sendo dispersos com presença de amido e no estádio de desenvolvimento tricelular. Na antese o grão de pólen é monoporado com a abertura do poro do tipo opérculo, padrão de superfície da exina rugular e a estrutura do teto espinhosa.

O gineceu de *D. asper* consiste em um ovário ovado, um estilo longo e um estigma, sendo todas as estruturas plumosas. No estádio dois do desenvolvimento floral o óvulo maduro é anátropo e bitegumentado. O tegumento externo e interno possui duas camadas. A formação do saco embrionário inicia-se no estádio dois de desenvolvimento floral e finaliza o desenvolvimento na pré-antese. Na antese observam-se os resquícios das células do tegumento externo e o embrião colapsado envolto por uma camada de células irregulares do endotélio e do endosperma. Em algumas amostras observou-se o início do desenvolvimento da cariopse com o acúmulo de amido, porém essa estrutura também colapsou. A depressão por endogamia de ação precoce parece ser o motivo para a não produção de cariopses.

Em *Bambusa tuldoides*, nos três estádios florais analisados, pode-se perceber o desenvolvimento normal das anteras como observado em outras espécies. Porém, apesar das anteras de *B. tuldoides* apresentarem desenvolvimento e deiscência normais, os grãos de pólen não são funcionais no momento da sua liberação. Em relação à microgametogênese, após a degeneração das células do tapete da antera espera-se que o micrósporo passe por processos como a mitose e acúmulo de amido, culminando com a liberação de um pólen maduro e viável. No entanto, o que se observa no estádio um de desenvolvimento floral de *B. tuldoides* é uma sequência de eventos degenerativos que acarretam na inviabilidade dos micrósporos livres e a presença de micrósporos vazios e deformados. Observou-se que no estádio um, inicia a retração do citoplasma, o rompimento da membrana nuclear e das mitocôndrias. Nos estádios dois e quatro continua a deposição de amido, a esporoderme apresenta-se incompleta e o desenvolvimento do pólen se dá de forma anormal, pois termina com a degradação total do citoplasma. A esporoderme do micrósporo é incompleta, pois não apresenta a intina. Os resultados obtidos sugerem que a inviabilidade dos grãos de pólen impede a formação de cariopses.

O gineceu de *B. tuldoides* consiste em um ovário obovado, estilete curto e dois estigmas plumosos. O ovário possui uma pilosidade em sua extremidade superior que se estende até o estilete. No estádio um do desenvolvimento floral, o óvulo de *B. tuldoides* maduro é ortotrópo e bitegumentado. A micrópila é formada por ambos os tegumentos externo e interno. A formação do saco embrionário inicia-se na pré-antese e finaliza o desenvolvimento na antese.

Considerações Finais
Este trabalho permitiu a caracterização morfológica e do desenvolvimento do androceu e gineceu em *B. tuldoides* e *D. asper*. Durante esse processo de caracterização foi possível concluir que a não produção de cariopses em B. tuldoides se deve a inviabilidade do grão de pólen e em D. asper supõe-se que seja devido a depressão por endogamia de ação precoce. Sendo este trabalho o primeiro realizado com essas espécies.

Palavras-chave: Bambu. Reprodução sexuada. Micrósporos anormais. Endogamia de ação precoce.

ABSTRACT

Little is known about the reproduction of *Dendrocalamus asper* and *Bambusa tuldoides* because, for most bamboos, flowering is unpredictable, ranging from annual to flowering after 120 years of vegetative development. The objectives of this work were to morphologically characterize the androecium and gynoecium at different stages of development of the flowers of *D. asper* and *B. tuldoides* and clarify aspects related to reproduction and no production of caryopses. Number of pollen grains and ovules per flower, pollen/ovule ratio, in vitro twinning and pollen grain viability in vivo were evaluated and the different stages of floral development identified. Further, we performed a morphological analysis of androecium and gynoecium. In *D. asper* Seven distinct stages of flower development were identified; four initial stages, a pre- anthesis stage, and two stages of anthesis. *D. asper* pseudospikelets avoid inbreeding by means of protogyny. The floral and pollen characteristics suggest that the species is anemophilous. The ultrastructural characteristics of free microspores (stage two of floral development), vacuolated microspores (stage five) and mature pollen (anthesis) were analysed. During maturation, pollen grains accumulate larger and more numerous amyloplasts and organelles such as mitochondria. Pollen disperse in the tricellular development stage. Pollen is monoporate with an operculum-like pore, with a rugulate structure and a spinose tectum. *D. asper*'s gynoecium consists of an oval ovary, a long style and a single stigma. The mature ovule is anatropous and bitegmic. The formation of the embryo sac starts at stage two of floral development and ends its development in the pre-anthesis. In *D. asper*, early-acting inbreeding depression seems to be the reason for not producing caryopses. In *B. tuldoides*, in the three floral stages analysed, the anther dehiscence appeared to be normal. In contrast, microspores began to develop abnormally starting as early as the first flower development stage: retraction of the cytoplasm; rupture of the nuclear and mitochondria membrane. As the interior machinery of the microspores degenerated, starch accumulated within numerous amyloplasts during stages two to four of flower development. The sporoderms of these microspores were similarly incomplete: though they possessed an exine, they lacked an intine. The results here obtained suggest that the non-viability of these abnormal pollen grains prevents the development of *B. tuldoides* caryopses. B. *tuldoides*'s gynecium consists of an obovated ovary, a short style and two plumose stigmas. The ovule is orthotropous and bitegmic. The formation of the embryo sac starts in the pre-anthesis and finishes the development in the anthesis. These results suggest that the non-production of caryopses in *B. tuldoides* is due to the non-viability of the pollen grain and in *D. asper* it is assumed that it is due to early-acting inbreeding depression.

Keywords: Bamboo. Megasporogenesis. Megagametogenesis. Microsporogenesis. Microgametogenesis. Sexual reproduction.

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5 CARATERIZAÇÃO MORFOLÓGICA DO GINECEU EM *BAMBUSA TULDOIDES* MUNRO E *DENDROCALAMUS ASPER* (SCHULT. & SCHULT.) BACKER *EX* K. HEYNEK 56

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1 INTRODUÇÃO GERAL

Os bambus são membros do grupo taxonômico das grandes gramíneas lenhosas (Família Poaceae, Subfamília Bambusoideae, Tribo Andropogoneae), compreendendo 1250 espécies dentro de 75 gêneros (SCURLOCK *et al.*, 2000) distribuídos principalmente nos trópicos, mas ocorrem naturalmente em zonas subtropicais e temperadas de todos os continentes em latitudes de 46° N a 47° S e do nível do mar a 4000 m de altitude, com exceção da Europa (WILLIAMS *et al.*, 1994).

A maior riqueza de espécies é encontrada na Ásia (China: 626, Índia: 102, Japão: 84, Mianmar: 75, Malásia: 50), cobrindo uma área de mais de 180.000 km² (BYSTRIAKOVA *et al.,* 2003). Na China chega a 33.000 km² ou 3% da área florestal total do país (QIU et al., 1999) e na Índia 96.000 km² ou cerca de 13% da área florestal total (SHANMUGHAVEL *et al*., 1996). Na América, o Brasil possui o maior número de espécies, representando 89% dos gêneros e 65% das espécies que são relatadas no Novo Mundo (FILGUEIRAS; GONCALVES, 2004), com destaque para os bambus herbáceos, com aproximadamente 110 espécies, que estão concentradas na região Neotropical do Brasil, Paraguai, México e Argentina (JUDZIEWICZ *et al*., 1999). As maiores florestas de bambu natural, conhecidas como 'tabocais' no Brasil e 'pacales' no Peru, cobrem cerca de 6.000 km^2 no Brasil, Peru e Bolívia (FILGUEIRAS; GONCALVES, 2004).

No Brasil foram encontradas 256 espécies, das quais 176 são endêmicas, sendo as regiões de maior ocorrência o Sudeste com 57,4% e o Centro Oeste com 9,0% (GRECO *et al*., 2015).

Os bambus apresentam reconhecimento como uma espécie florestal de grande valor por apresentar características físicas e mecânicas, que os tornam uma opção alternativa para o desenvolvimento de produtos, substituindo madeira nativa ou reflorestada (BONILLA *et al.*, 2010). São utilizados como fonte de material para móveis, construção, celulose, bioenergia e alimento (SCURLOCK *et al*., 2000). Desempenham também papel importante na preservação da biodiversidade e dos solos e na conservação da água (EMBAYE, 2000).

A grande variabilidade de usos dos bambus os torna um fator de desenvolvimento econômico amplamente explorado em vários países do continente asiático (COSTA *et al.*, 2015). No Brasil, foi instituída a Política Nacional de Incentivo ao Manejo Sustentado e ao Cultivo do Bambu (PNMCB Lei n.º 12.484/2011), cujo objetivo é fomentar o

desenvolvimento da cultura do bambu no Brasil por ações governamentais e empreendimentos privados (BRASIL, 2011).

Dentre as espécies mais utilizadas de bambus destacam-se *Dendrocalamus asper* (Schult. & Schult.) Backer *ex* K. Heynek. e *Bambusa tuldoides* Munro.

Dendrocalamus asper é nativa da China e é valorizada pelos seus brotos comestíveis, que são vendidos como enlatados em todo o mundo. Além disso, seus colmos maduros são utilizados na fabricação de celulose e papel (Figura 1) (AYRA *et al*., 2001).

A produção de sementes de *D. asper* é irregular, uma vez que a floração ocorre aos 100-120 anos de idade. Como outras espécies de bambu, também é monocárpico, ou seja, cada população individual apresenta um único evento com floração e frutificação maciça e sincrônica (GUILHERME *et al.*, 2017; NADGIR *et al*., 1984).

Bambusa tuldoides é nativa da China e espalhou-se por todo o sudeste asiático, sendo amplamente cultivada em regiões tropicais e subtropicais da América (GUERREIRO; LIZARAZU, 2010). No Brasil é uma das espécies mais difundidas, tendo sido introduzida pelos portugueses na época da colonização (AZZINI *et al*., 1988). É principalmente utilizado na contrução civil, em cercas e tutoramento de culturas (Figura1) (GRECO *et al*., 2011).

A principal característica fisiológica de *B. tuldoides* é o florescimento esporádico que ocorre em alguns colmos das touceiras, porém com reduzida ou nenhuma produção de cariopses (AZZINI *et al.* 1988). Segundo o estudo de Guerreiro e Lizarazu (2010), o intervalo de sua floração na América do Sul é cerca de 23 anos de desenvolvimento vegetativo e o seu comportamento é monocárpico e pode ou não senescer após a floração.

A floração dos bambus, de forma geral, é irregular, na maioria das espécies, florescendo em períodos de 20 a 60 anos, podendo chegar a 120 anos de permanência em estádio vegetativo. Estes eventos de floração podem ocorrer de duas formas, floração esporádica ou gregária. A floração esporádica pode ocorrer individualmente ou em populações de algumas espécies. Por sua vez, na floração gregária, indivíduos da mesma espécie florescem simultaneamente e produzem grande quantidade de sementes (cariopses), porém esses aglomerados podem morrer após a floração (PILCHER; 2004; WARRIER *et al*., 2004). Além disso, as cariopses de bambus germinam dentro de 3-7 dias, apresentando uma alta taxa de germinação (60-90%), porém perdem a viabilidade após 1-2 meses em temperatura ambiente (SURENDRAN *et al*., 2003; WARRIER *et al*., 2004). Embora as cariopses possuam uma alta taxa de germinação, existem relatos de ausência de sua formação

em algumas espécies (DAS *et al*., 2017; JIJEESH *et al*., 2014; KOSHY; JEE, 2001; KOSHY; PUSHPAGADAN, 1997; WANG *et al*., 2015).

Apesar da grande importância econômica dos bambus, poucos estudos foram realizados sobre a sua biologia reprodutiva. Estudos sobre o correto padrão de desenvolvimento das anteras, do pólen e do gineceu, são essenciais para a obtenção de embriões e sementes bem formados na maioria das espécies de plantas (REISER; FISCHER, 1993; SILVA *et al*., 2017; ZHANG *et al*., 2014). Além disso, as características dos grãos de pólen são importantes nas questões ligadas à taxonomia vegetal, uma vez que o conhecimento da sua morfologia e taxonomia podem ser utilizados como instrumentos de múltiplas pesquisas científicas em botânica sistemática, paleobotânica, paleoecologia, análise polínica, dentre outras (LORSCHEITTER, 2006; MAZARI *et al*., 2017; STEPHEN, 2014).

Desse modo, o conhecimento sobre o desenvolvimento do androceu e do gineceu são de grande importância para a propagação e conservação destas espécies. Os objetivos deste trabalho foram caracterizar morfologicamente o androceu, os grãos de pólen e o gineceu em diferentes estádios de desenvolvimento das flores de *D. asper* e *B. tuldoides* visando elucidar aspectos relacionados à reprodução e à não produção de cariopses.

Figura 1. Touceiras de bambu. **a.** Touceira de *Dendrocalamus asper*, Fazenda dos bambus, Pardinho – São Paulo. **b.** Touceira de *Bambusa tuldoides*, Cidade das Abelhas – Universidade de Santa Catarina, Florianópolis – Santa Catarina.

Fonte: Imagens do autor (2020)

2 OBJETIVOS

2.1 OBJETIVO GERAL

Estudar aspectos da biologia reprodutiva, estrutura do androceu e do gineceu e o desenvolvimento e a morfologia do grão de pólen,em três estádios de desenvolvimento floral de *Dendrocalamus asper* e *Bambusa tuldoides*.

2.2 OBJETIVOS ESPECÍFICOS

- Caracterizar os estádios de desenvolvimento floral de *D. asper* e *B. tuldoides*;
- Caracterizar o desenvolvimento das anteras de *D. asper* e *B. tuldoides*;
- Caracterizar o desenvolvimento e morfologia dos grãos de pólen de *D. asper* e *B. tuldoides*;
- Determinar o número de grãos de pólen e óvulos.flor^{-1} e a razão pólen/óvulo em *D. asper*;
- Caracterizar o gineceu e o gametófito feminino em três estádios de desenvolvimento das flores em *D. asper* e *B. tuldoides*.

3 FLOWERING AND MORPHOLOGICAL CHARACTERIZATION OF *DENDROCALAMUS ASPER* ANDROECIUM AND POLLEN GRAINS

3.1 ABSTRACT

Little is known about the reproduction of *Dendrocalamus asper* because it flowers only every 100 to 120 years. In the present work we describe some reproductive features of this bamboo and characterise flowers and pollen at various developmental stages. Number of pollen grains and ovules per flower, pollen/ovule ratio, *in vitro* twinning and pollen grain viability *in vivo* were evaluated and the different stages of floral development identified. Further, we performed a morphological analysis of androecium and pollen development. Seven distinct stages of flower development were identified; four initial stages, a pre-anthetic stage, and two stages of anthesis. *D. asper* pseudospikelets avoid inbreeding by means of protogyny. The floral and pollen characteristics suggest that the species is anemophilous. The ultrastructural characteristics of free microspores (stage two of floral development), vacuolated microspores (stage five) and mature pollen (anthetic) were analysed. During maturation, pollen grains accumulate larger and more numerous amyloplasts and organelles such as mitochondria. Pollen disperse in the tricellular development stage. Pollen is monoporate with an operculum-like pore, with a rugulate structure and a spinose tectum.

Keywords: Bamboo, wind-pollination, microgametogenesis, reproductive biology

Disponibilidade: https://doi.org/10.1080/00173134.2020.1736148

3.2 INTRODUCTION

Bamboos are large woody grasses of the Poaceae family (subfamily Bambusoideae), which is comprised of about 1,250 species within 75 genera distributed mainly in the tropics (Rao & Rao 1998). They occur naturally in the subtropical and temperate zones of all continents except Europe and Antarctica in latitudes from 46 ° N to 47 ° S and from sea level to up to 4000 m above sea level (JUDZIEWICZ *et al*., 1999; SUNGKAEW *et al*., 2009).

Bamboos are valuable for industry because of the physical and mechanical characteristics of their fibres (BONILLA *et al*., 2010). Bamboos also play an important role in the preservation of soil quality and water conservation, which, in turn, preserves biodiversity by providing an ecosystem for a wide variety of organisms (EMBAYE, 2000). *Dendrocalamus asper* (Schult. & Schult.) Backer *ex* K. Heynek, a bamboo native to China, is used in industry for several main reasons: its shoots are edible and may be eaten fresh or sold as canned goods; its culms may be used for boards, furniture, musical instruments, containers, household items, handicrafts, etc; and it may also be used as biofuel (ARYA *et al*., 2001; SINGH *et al*., 2012).

Despite the economic importance of bamboos, little is known about their reproduction in general. This is mainly due to unpredictable flowering periods, which may range from annually to only after 120 years of vegetative growth (JANZEN, 1976). In addition, most species die after flowering (JANZEN, 1976). *Dendrocalamus asper* flowers after 100 to 120 years of vegetative growth (NADGIR *et al*., 1984), and this rarely-studied event is of great importance for sexual reproduction and regeneration of the population (HUANG *et al*., 2002).

The characterisation of anther and pollen development, as well as pollen release mechanisms, are essential for understanding embryo and seed formation in most species of angiosperms (ZHANG *et al*., 2014). In addition, before bamboo breeding programs may begin, more-detailed knowledge of the floral biology and breeding behaviour of bamboos is needed (NADGAUDA *et al*., 1993). Furthermore, pollen morphology plays an integral role in taxonomy, which is then essential for scientific research in systematic botany and evolutionary aspects in various groups of plants palaeobotany, palaeoecology, and pollen analysis, among other fields (DÓREA *et al*., 2017; MAZARI *et al*., 2017; STEPHEN, 2014).

The present work analyses *D. asper* flower and pollen development in order to better understand the reproductive biology and consequently to provide tools for its propagation and genetic improvement.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

Dendrocalamus asper inflorescences were collected from 30 mother plants located at the Bamboo Farm of the Jatobás Institute in the city of Pardinho / SP / Brazil (23 ° 06'37.33 "S, 48 ° 22'05.60 " W). The collection was carried out in June 2017. At the moment of collection, the inflorescences were packed in plastic bags, placed among ice packs in an insulated container and transported to the Laboratory of Developmental Physiology and Plant Genetics (LFDGV) and the Central Laboratory of Electronic Microscopy (LCME) of the Federal University of Santa Catarina (UFSC), Florianópolis / SC / Brazil campus.

3.3.2 Flower developmental stage identification and microgametogenesis

One hundred inflorescences were collected to identify flower development stages. These were analysed under stereomicroscope (Olympus brand, model SZH10). The inflorescences were described according to WATSON *et al.* (1992). The developmental stages of microgametogenesis were determined according to Sharma et al. (2015).

3.3.3 Pollen germination *in vitro*

Pollen germination was determined according to a modified protocol adapted from ALMEIDA *et al*. (2011). Four different culture media containing 100 ml of distilled water, 1 g of agar and different concentrations of sucrose (%)/boric acid (mg L-1) were evaluated using the following proportions: M1 - 0/5; M2 = 0/20; M3 = 10/0; M4 = 10/10. The components were added to a vessel and heated until dissolved without boiling. After reaching a temperature of 27°C, pollen grains collected in the pre-anthetic stage were sprinkled evenly across the medium and kept at this temperature for 24 and 48 hours for germination. Two hundred pollen grains were observed and the percentage of germinating pollen determined. A completely randomised design with four replicates was used.

3.3.4 *In vivo* pollen viability

Ten flowers were collected at the pre-anthetic stage to determine the viability of pollen grain *in vivo*. Six anthers were removed from each flower, macerated with one drop (2 µl) of acetic carmine dye on a labelled glass slide and covered with a coverslip. After five minutes, the pollen grains were counted under an optical microscope (Olympus, model BX40) at 10x magnification. Pollen grains that stained red were considered viable, and uncoloured pollen were considered non-viable. The results were expressed as a percentage of viable pollen grains (JIJEESH *et al*., 2012). A completely randomised design with four replicates was used, each replicate being represented by a slide.

3.3.5 Pollen count

Pollen was collected from thirty flowers at the pre-anthetic stage. At the time of collection, the number of anthers per flower was counted following CRUDEN (1977). The anthers were stored in Eppendorf tubes containing 500 µl of an 85% lactic acid solution. Subsequently, the tubes were agitated and two 1.5 µl samples of the solution with released pollen grains were placed on a slide and analysed under 10x magnification with an optical microscope (Olympus, model BX40).

The number of pollen per anther (N) was determined by multiplying the average number of pollen of each sample (X) with the volume of lactic acid in the solution (500 µl), and dividing this value by the product between the volume of lactic acid in the sample (1.5 µl) and the number of anthers in each tube (4). To calculate the number of pollen grains produced per flower, the average pollen grain estimate per anther was multiplied by the number of anthers per flower (KEARNS; INOUYE, 1993).

3.3.6 Pollen/Ovule ratio (P/O)

Thirty flowers were collected in the pre-anthetic stage to count the number of ovules per flower. The ovary of each flower was removed, sectioned longitudinally on a glass slide and the number of ovules quantified with the aid of a stereomicroscope (Olympus brand, model SZH10) (CRUDEN, 1977). The pollen/ovule ratio (P/O) was obtained by dividing the number of pollen grains per flower by the number of ovules per flower (CRUDEN, 1977).

3.3.7 Scanning electron microscopy

Samples from six anthers from three flower developmental stages (stage two, pre-anthetic and anthetic - stage six) were fixed in a 2.5% glutaraldehyde solution with 0.1 M sodium cacodylate buffer (pH 7.2) and 0.2 M sucrose for 24 hours. Subsequently, the samples were dehydrated with a gradual ethanol series (30%, 50%, 70% and 90% and 100%) for 30 minutes at each concentration, except for 100% ethyl alcohol, which had two changes of 30 minutes each followed by critical point drying with CO_2 in an EM-CPD-030 (Leica, Heidelberg, Germany) (adapted from SCHMIDT *et al.*, 2012). The dried samples were covered with 20 nm of gold with a metalliser (Baltec, CED 030) (SCHMIDT *et al.*, 2012). The samples were then observed with a Jeol scanning electron microscope (model JSM-6390LV).

The pollen grains were classified according to the terminology of PUNT *et al*. (2007) and HESSE *et al*., (2009).

3.3.8 Light microscopy analysis

Samples from six anthers from three flower developmental stages (stage two, pre-anthetic stage and anthetic - stage six) were fixed in a 2.5% paraformaldehyde solution with a 0.2 M phosphate buffer (pH 7.3, 1:1 ratio) for 24 h at 4°C. After fixation, the samples were washed three times with phosphate buffer for thirty minutes (BOUZON, 2006) and dehydrated in a gradual ethanol series (SANDERS *et al*., 1999). Subsequently, the samples were pre-infiltrated in Leica ™ historesin with ethanol PA at a ratio of 1:1 per 72 hours and infiltrated into Leica ™ historesin for 72 hours, according to manufacturer's instructions. A rotating microtome (model CUT 4055) was used to produce 5 µm-thick transverse and longitudinal sections (NAKAMURA *et al*., 2010). Sections were stained with toluidine blue (O'BRIEN *et al*., 1965) to help distinguish cell and tissue types and Lugol solution to identify starch reserves. Sections were placed in the reagent for ten minutes and washed in distilled water (JOHANSEN, 1940). Observations and recordings were performed using a fluorescence microscope (Leica, model DM5500 B). Afterward, 30 pollen collected at anthetic (stage six) were randomly analysed and polar axis (P) and equatorial diameter (E) were measured and P/E ratio calculated. Measurements were made with the Leica LAS AF Lite 4.0.11706 program.

3.3.9 Transmission electron microscopy analysis

Samples from six anthers from three flower developmental stages (stage two, pre-anthetic stage and anthetics - stage six) were fixed in a 2.5% glutaraldehyde solution buffered with 0.1 M cacodylate (pH 7.2) for 24 hours. Afterward, the samples were washed four times in the same buffer. They were then post-fixed in 1% OsO_4 in 0.1M cacodylate buffer, pH 7.2 (1: 1) for four hours at room temperature (PUESCHEL, 1979). The material was washed twice in 0.1M cacodylate buffer pH 7.2 at 30 minutes each, dehydrated in a series of acetone solutions (30%, 50%, 70%, 90% and 100%), for 30 minutes at each concentration with a final wash in 100% acetone for an additional 30 minutes (adapted from COIMBRA *et al*., 2004). After dehydration, the material was infiltrated with Spurr resin and polymerised in horizontal oven molds at 70 ° C for 24 h (Spurr 1969). The material was sectioned with an ultramicrotome (Leica, model EM UC 7), allocated on copper grids and contrasted with 5% uranyl acetate and lead citrate (REYNOLDS, 1963). The samples were observed and recorded in a transmission electron microscope (Jeol, JEM 1011).

3.4 RESULTS

3.4.1 Identification of stages of flower development

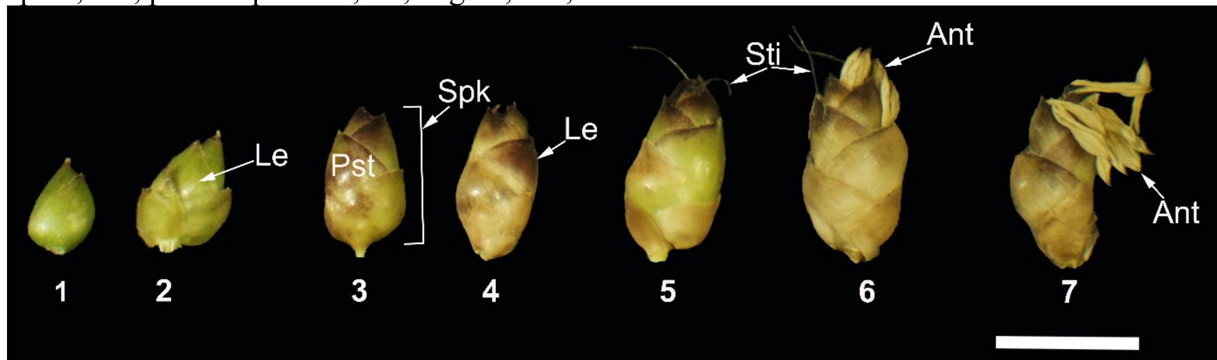
The inflorescences of *Dendrocalamus asper* are indeterminate with pseudospikelets consisting of spikes with spikelet tufts containing a bisexual flower each. From stages one to four, the gynoecium and the androecium are protected by the closed lemma. At stage five (pre-anthesis) the lemma opens, initiating the exposure of the style-stigma in the flowers of the upper part of the inflorescence. In stage six (anthetic) anthers and style-stigma are exposed, and anthers release the pollen. Stage seven (anthetic) is characterised by senescence of anthers and finalisation of pollen dispersal (Table I).

The exposure of the stigma in stage five at pre-anthesis and, afterwards, the exposure of the anthers in stage six (anthesis) suggest that *D. asper* pseudospikelets display protogyny. Flowers do not have nectaries or perceptible odours, and the inflorescences have many flowers with large anthers (Figure 1).

Table I. Identification of stages of development of inflorescences of *Dendrocalamus asper*.

Development stage	Characteristics
Initial stages 1-4	Gynoecium and androecium are protected by the lemma
Stage 5 (pre-anthetic)	Opening of the lemma and exposure of stigma
Stage 6 (anthetic)	Exposure of the anthers and stigma
Stage 7 (anthetic)	Anther senescence and pollen liberation

Figure 1. Stages of *Dendrocalamus asper* inflorescence development. 1 to 4. Initial stages of development with the pseudospikelets (florets) closed and protected by the lemma; 5. Pre-anthesis: exteriorization of the stigma at the top of the inflorescence; 6. Anthesis: exteriorization of stigma and anthers in upper florets. 7. Senescent anthers. *Le*, lemma; *Spk*, spike; *Pst*, pseudospikelets; *Sti*, stigma; *Ant*, anther. Scale bar - 5 mm.



Fonte: Souza *et al*. (2020a)

3.4.2 Pollen count, Pollen/Ovule ratio (P/O), *in vitro* pollen germination and *in vivo* pollen viability

At stage six of flower development, a single ovule per flower is present in each gynoecium. The androecium consisted of six anthers per flower. The number of pollen per anther is estimated to about 1,254 and 7,525 totally per flower resulting in a pollen/ovule ratio of 7,525.

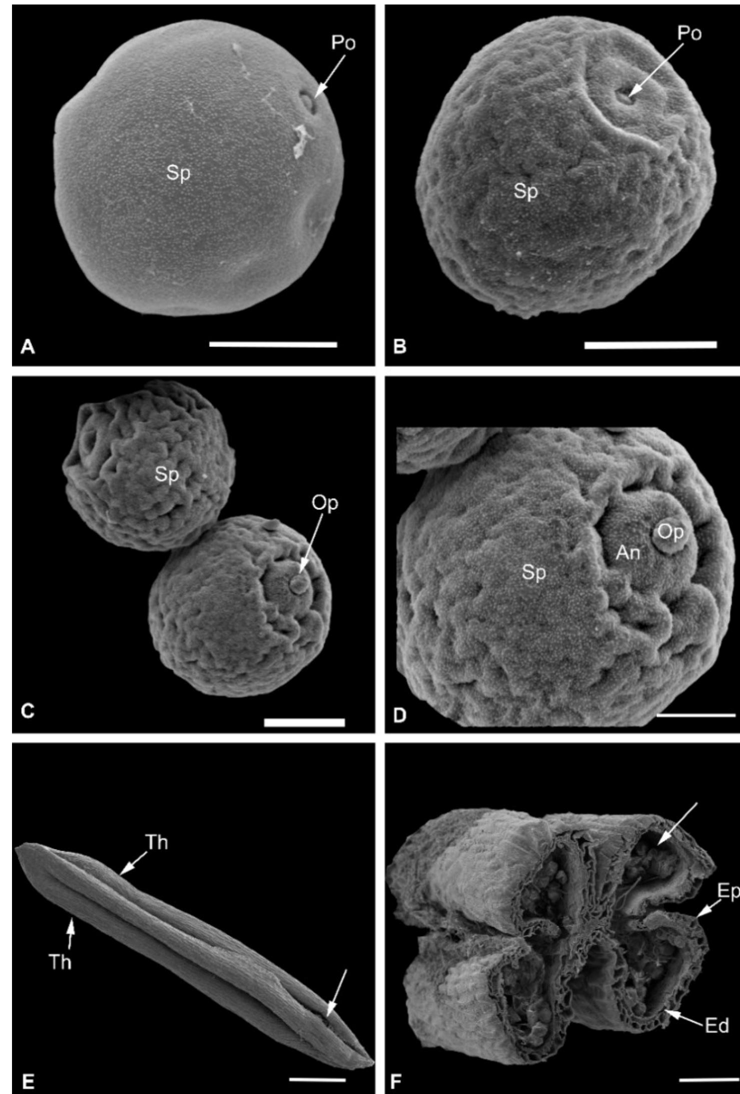
The *in vitro* viability test showed that the pollen grains were twinned and staining with acetic carmine (*in vivo* viability) showed that 62.5% of pollen were viable.

3.4.3 Analysis of anthers and microgametogenesis

At flowering stage two, microspores are detached and characterized by a developed pore, a developing sporoderm and a lack of an annulus (Figure 2A). In stage five, the microspores are vacuolated. They show a distinct pore and a structural annulus, with sporoderm beginning to develop structural characteristics (Figure 2B). The mature pollen grain at stage six of flower development (anthetic) is monoporate with an operculum-like aperture (Figures 2C-D and 5G-H). At this stage the microspores are tectate-spinose with a rugulate ornamentation (Figures 2C-D and 5I) with polar axis (P) of 37.88-48.44 µm, equatorial diameter (E) 38.41-45.47 µm and P/E ratio 1.03, suggesting that the pollen has a prolate-spheroidal shape. At anthesis anthers are bithecate, tetrasporangiate and with rupture of the stomium allowing release of the pollen (Figure 2E-F).

At stage two of flower development the tetrasporangiate anther has a uni-stratified epidermis with juxtaposed cells and an endothecium with cells smaller than those of the epidermis. The tapetum is secretory with a layer of cells intact around the locus of the anther and uni-nucleate and, occasionally, binucleate cells. The cell walls of the epidermis, endothecium, middle layer and tapetum stains blue with toluidine blue, indicating cellulosic compounds. Well-developed cells with evident connections to the blue-stained central vascular bundles are present in the parenchymatic tissue between the locules. Free microspores, also blue-stained, in the locule show non-polarised nuclei and an undeveloped sporoderm (Figures 3A-B and 4A). Further, these grains show no signs of starch accumulation because no sign of Lugol stain appeared. In stage five, cellulosic reinforcement of the epidermis cell walls leads to a more intense blue stain. The tapetum cells and the middle layer, in contrast, begin to degenerate. Vacuolated microspores show polarised nuclei located opposite of the pore. Bicellular pollen grains—the vegetative and generative cells—appear with an increase in sporoderm thickness and a greater deposition of cellulosic compounds (Figures 3C-D). At this stage, starch accumulates in the cytoplasm of microspores and bicellular pollen (Figure 4B). During anthetic (stage six), mature pollen grains have three nuclei, and the sporoderm is fully developed with an intense blue stain (Figure 3E). At this stage, ostium cells begin to degenerate (Figure 3F) and starch accumulates (Figure 4D).

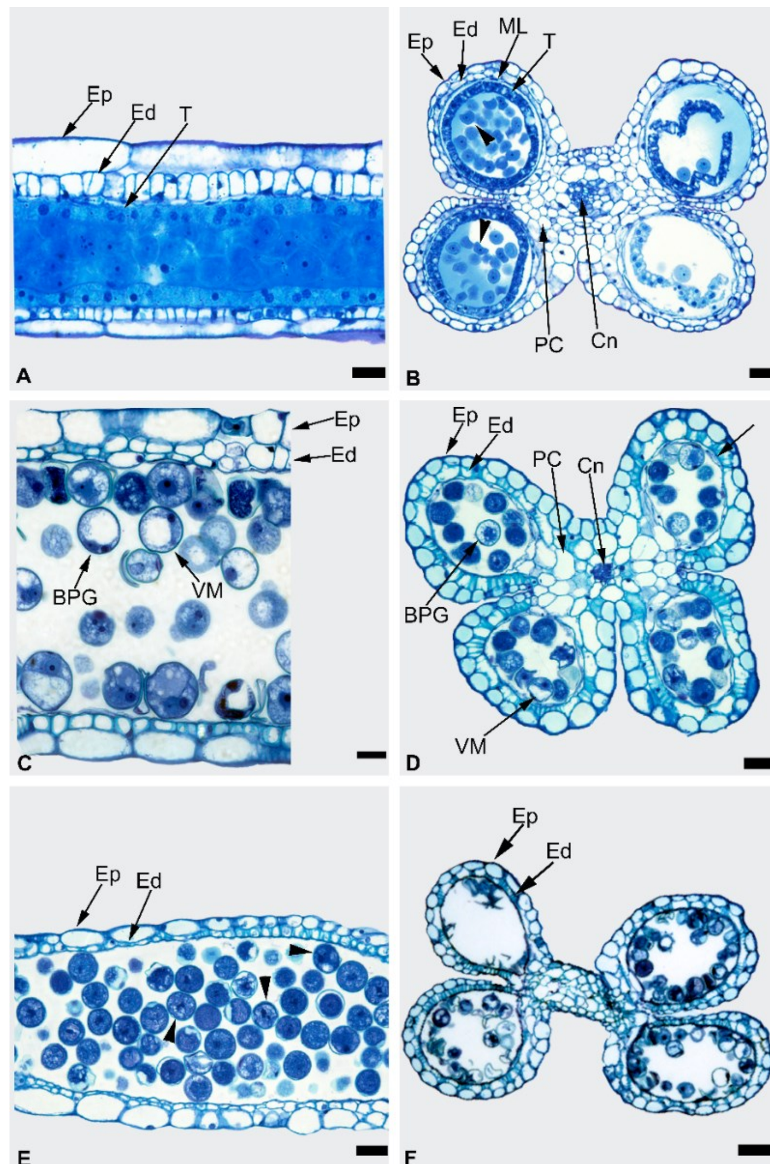
Figure 2. Scanning electron microscopy of *Dendrocalamus asper* pollen grains and anthers: **A.** Stage 2: Free microspore with formed pore and forming sporoderm. **B.** Stage 5: vacuolated microspore with pore and sporoderm in early ornamentation. **C.** Anthetic: mature pollen with well-formed sporoderm and pore. **D.** Anthetic: Oblique polar view of mature pollen grain showing sporoderm, pore with developed operculum and annulus. **E.** Anthesis: Overview of the anther showing theca and the stomium (arrow). **F.** Anthetic: Cross section of the anther showing the sporangia with the mature pollen grains inside (arrow) and the epidermis and endothecium forming cells. *Th*, theca; *Sp*, sporoderm; *Po*, pore; *Op*, operculum; *An*, annulus; *Ep*, epidermis; *Ed*, endothecium. Scale bar - 10 µm (A-C); 5 µm (D); 500 µm (E); 100 µm (F).



Fonte: Souza *et al*. (2020a)

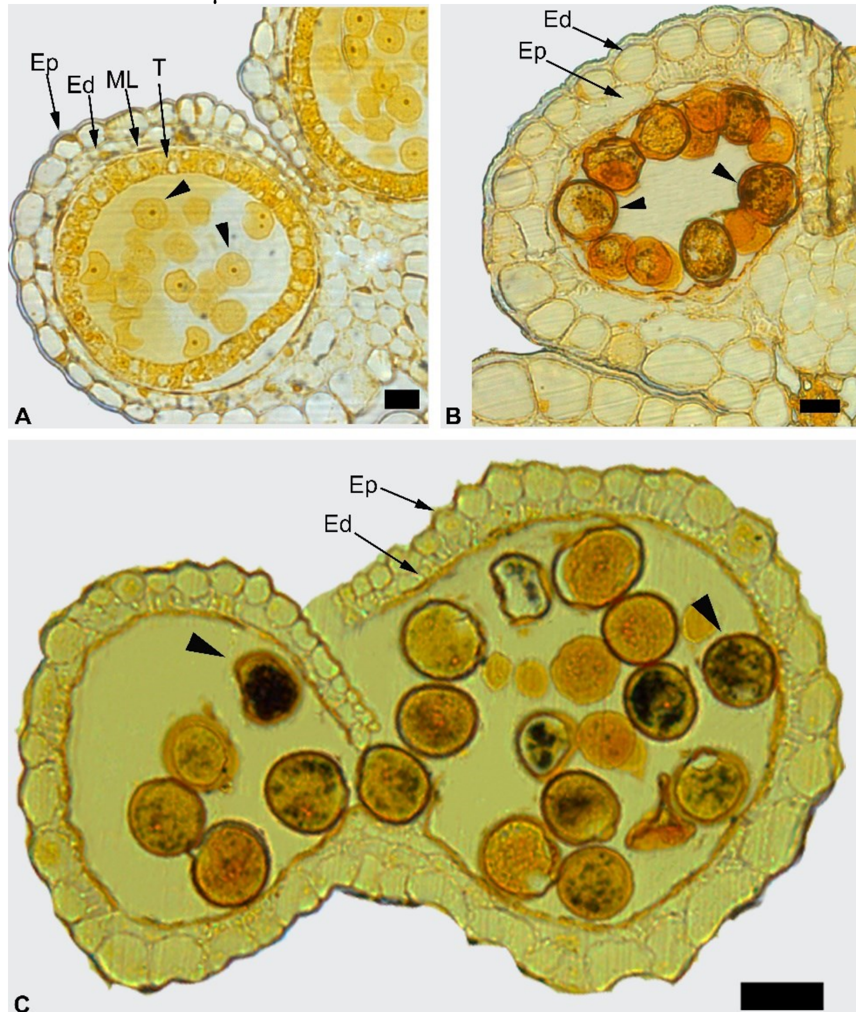
Figure 3. Light microscopy analysis of the development of *Dendrocalamus asper* anthers, microspores and pollen grains. A, C and E. Longitudinal sections. B, D and F. Cross sections. **A.** Stage 2: the epidermis, endothecium and tapetum of an anther. **B.** Stage 2: Tetrasporangial anther showing the epidermis, endothecium, middle layer and intact tapetum cells. The

stomium, parenchymal cells and the connective tissue in the central anther region were observable. The arrowheads indicate the free microspores. **C.** Stage 4: Anther showing the epidermis and endothecium. Vacuolated microspores and the onset of bicellular pollen formation were observed. **D.** Stage 5: Anther showing epidermis, endothecium, remaining tapetum cells (arrow), vacuolated microspore, and bicellular pollen. The parenchyma-forming cells between the locules and the stomium were observed. **E.** Anthetic: Anther showing the epidermis, endothecium and mature pollen grains (arrowheads). **F.** Anthetic: anther showing persistence of epidermis and endothecium and rupture of the stomium region. *Ep*, epidermis; *Ed*, endothecium; *T*, tapetum; *ML*, middle layer; *St*, stomium; *PC*, parenchyma cells; *Cn*, connective tissue; *VM*, vacuolated microspore; *BPG*, bicellular pollen grains; *MPG*, mature pollen grains. Toluidine blue dye. Scale bar - 50 µm.



Fonte: Souza *et al*. (2020a)

Figure 4. Light microscopy of starch accumulation during the development of *Dendrocalamus asper* microspores and pollen grains. A, B and C. Cross sections of anther. **A.** Stage 2: Anther showing the epidermis, endothecium and, more internally, the median layer and the intact tapetum. Arrowheads indicate microspores without starch deposition. **B.** Stage 5: Anther showing epidermis, endothecium, and tapetum cell degeneration. Arrowhead indicates the beginning of starch deposition in vacuolated microspores. **C.** Anthetic: anther showing the epidermis and endothecium. Increased starch deposition in mature pollen grains (arrowhead) was observed. *Ep*, epidermis; *Ed*, endothecium; *T*, tapetum; *ML*, middle layer. Lugol reagent. Scale bar - 50 µm.

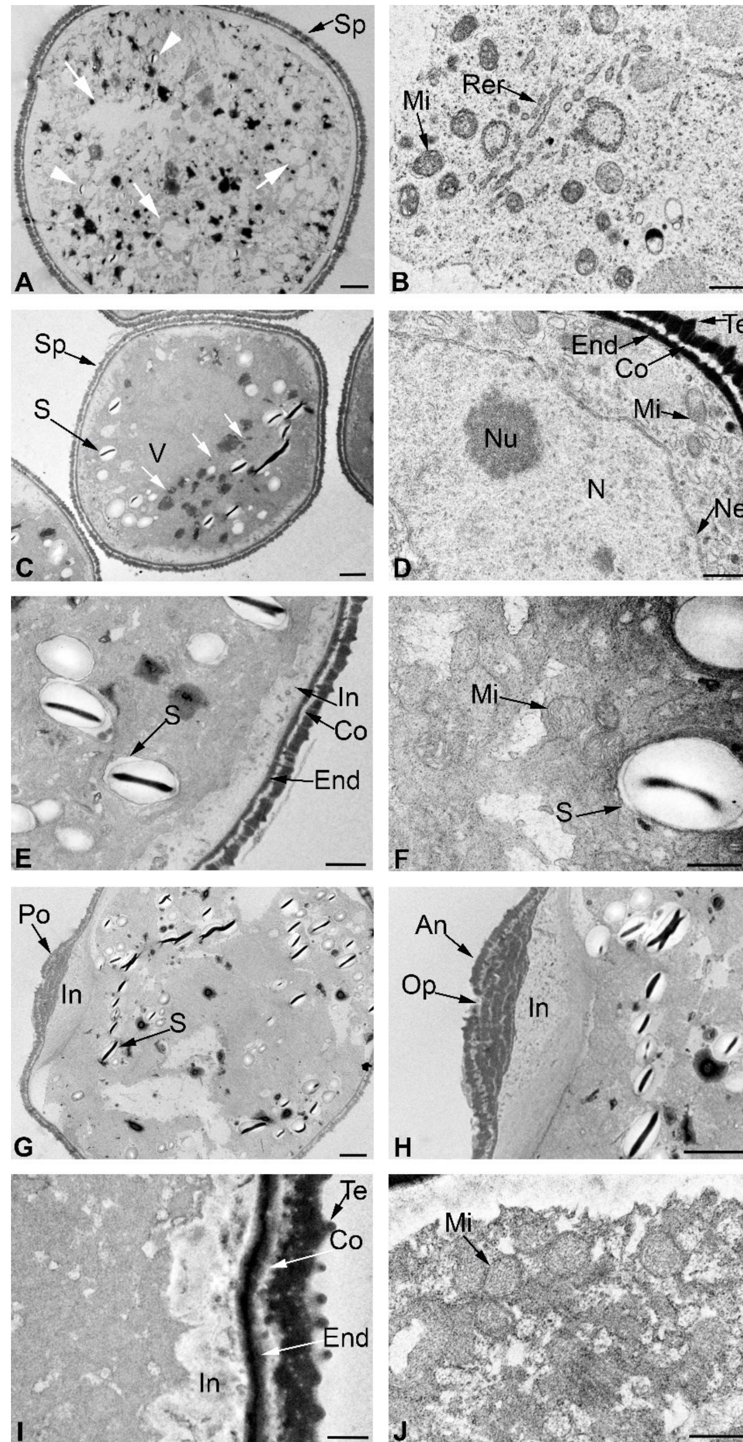


Fonte: Souza *et al*. (2020a)

The ultrastructural characteristics of microspores and mature pollen grains in three stages of floral development of *D. asper* is shown in Figure 5. During stage two, free microspores show small amyloplasts and the presence of numerous vacuoles within their cytoplasm. Mitochondria and rough endoplasmic reticulum are distributed regularly. The microspore sporoderm is incomplete and composed only of columella, endoexine and the beginning of intine formation (Figures 5A-B). At stage five, the vacuolated microspores show

incomplete sporoderms composed of endoexine, columella and intine (Figures 5C-D). The number of mitochondria and the number and size of amyloplasts increase by this stage. The presence of a large vacuole polarises the mitochondria, the amylopast and the nucleus toward the microspore periphery (Figures 5C-5F). At anthetic (stage six), amyloplasts accumulate and the mature pollen grains develop complete sporoderms composed of the exine (tectum, columella and endoexine), the intine and a developed operculum formed by the thickening of the exine and intine (Figures 5G-5I). In the cytoplasm, numerous mitochondria are present (Figure 5J). A summary of *D. asper* microgametogenesis is summarised in Table II.

Figure 5. Transmission electron microscopy of microspores and mature *Dendrocalamus asper* pollen. A and B show stage two of floral development; C, D, E and F show stage five of floral development; and G, H, I and J show anthesis. **A.** Free microspore overview showing sporoderm in formation with few starch grains (arrowhead) and small vacuoles (arrow). **B.** Free microspore showing mitochondria and rough endoplasmic reticulum dispersed in the cytoplasm. **C.** Overview of the vacuolated microspore showing the sporoderm, starch grains and a large vacuole. Cytoplasm-forming structures were concentrated in the microspore periphery (arrows). **D.** Detail of the microspore showing the nucleolus, part of the nucleus, envelope, mitochondria, and exine-forming layers. **E.** Detail of the vacuolated microspore showing the exine layers in formation (tectum, columella and endoexine) and the intine. Starch grains were present. **F.** Vacuolated microspore showing the cytoplasm in which several mitochondria and starch grains were present. **G.** Overview of mature pollen showing increased amount and size of amyloplasts. The well-formed sporoderm, the pore and the intine were observable. **H.** Detail of mature pollen with pore composed of annulus and operculum. More internally, the intine was beginning to thicken. **I.** Detail of mature pollen showing the layers of the complete sporoderm formed by the exine (tectum, columella and endoexine) and the intine. **J.** Detail of mature pollen showing the cytoplasm in which numerous mitochondria were present. *Sp*, sporoderm; *Mi*, mitochondria; *Rer*, rough endoplasmic reticulum; *S*, starch granule; *Nu*, nucleolus; *N*, nucleus; *NE*, nuclear envelope; *Te*, tectum; *Co*, columellae; *End*, endexine; *In*, intine; *Po*, pore; *Op*, operculum; *An*, annulus; *V*, vacuole. Scale bar - 2 µm (A, C, G, H); 1 µm (E); 0.5 µm (B, D, F, I, J).



Fonte: Souza *et al*. (2020a)

Table II. Developmental stages during *Dendrocalamus asper* microgametogenesis

Developmental stage	Characteristics
Free microspore	Free spores with a non-polarised nucleus. Presence of a pore without ornamentation.
Vacuolated microspore	Gametophytes with polarised nuclei before first mitosis. Presence of the pore and annulus.
Bicellular pollen	Gametophytes with two cells—vegetative and generative. Beginning of starch reserve accumulation.
Mature pollen	Trinucleated pollen containing a vegetative nucleus and two spermatic ones.

Fonte: Souza *et al*. (2020a)

3.5 DISCUSSION

3.5.1 Identification of flower development stages

Identifying the stages of flower development for bamboos, which have an extensive flowering interval, such as *Dendrocalamus asper*, is of great interest because it is a rare occurrence. A detailed analysis of pollen development and viability during different stages of flower development is essential to fully understanding the reproductive cycle of the plant (STANLEY; LINSKENS, 1974) and is useful to breeding programs where it is essential to collect pollen at an appropriate stage of maturity. In this study, we observed that pollen of *D. asper* reaches maturity at the time of anthesis when the anther is fully exposed.

The exposure of the stigma at stage five (pre-anthetic) and later of the anthers at stage six (anthetic), suggest that *D. asper* uses the protogyny strategy to avoid inbreeding (Figure 1). According to JIJEESH *et al*. (2012), dichogamy is observed in most bamboo species and protogyny is more common than protandry in many genera: e.g. *Ochlandra travancorica* (Beddome) Bentham*, O. wightii* (Munro) C.E.C.*, O. scriptoria* C. E. C. Fischer, *Phyllostachys nidularia* Munro*, P. heteroclada* Oliver*, P. nuda* McClure and *Bambusa striata* Lodd. *ex* Lindl. (GOPAKUMAR; MOTWANI, 2013; HUANG *et al*., 2002; JIJEESH *et al*.; 2014; KOSHY; HARIKUMAR, 2001). Protogyny is especially common in many members of *Dendrocalamus*: e.g. *D. membranaceus* Munro*, D. sinicus* L. C. Chia & J. L.

Sun, *D. sikkimensis* Gamble, *D. longispathus* Kurz. (Rawnal) and *D. strictus* Nees. (CHEN *et al*., 2017; JIJEESH *et al*., 2012; LALNUNMAWIA; SAILO, 2017; NADGAUDA *et al*., 1993).

Many species of bamboos are only little dependent on sexual reproduction; instead, they mostly rely on multiplying clonally through the growth of complex systems of underground rhizomes from which roots and stalks originate and can thus grow across extended areas over time (GUILHERME *et al*., 2017; POHL, 1991). However, sexual reproduction is essential in the long term to ensure the sustainability of species populations as it provides an independent dispersal phase, greater genetic diversity and potential for adaptation to new environments (RAMAWAT *et al*., 2014; WILCOCK; NEILAND, 2002).

Adaptive morphological characteristics of inflorescences, such as the growth of many large anthers as well as lack of nectaries, colour and perceptible odours, are characteristic of anemophily (FAEGRI; VAN DER PIJL, 1979; SODERSTROM; CALDERÓN, 1971). In addition, anemophily is accompanied by the production of large amounts of pollen to increase the likelihood of successful pollination. A small number of pollen produced per anther could be offset by a large number of flowers, and the reduction in flower numbers is usually balanced by high anther/pollen production (ABOULAICH *et al*., 2009).

3.5.2 Pollen count, Pollen/Ovule ratio (P/O) and pollen viability *in vivo* at anthesis

Little is known about pollination and other aspects of the reproductive biology of bamboos, however, anemophily is dominant (RUIZ-SANCHEZ *et al*., 2017). The average number of pollen per flower in *Dendrocalamus asper* is 7,525. A study of 38 species of Poaceae showed very high variability in number of pollen per flower from 61.11 to 36,135.54 depending on the species (PRIETO-BAENA *et al*., 2003). Three were wind-pollinated and showed pollen counts similar to that of *D. asper*: *Avena sterilis* L. (7,354.44), *A. barbata* L. (7,323.33) and *Cynodon dactylon* L. (7,495.53). The average number of pollen per flower in *Dendrocalamus asper* is also within the range for other anemophilous species of Poaceae reported from other studies. REDDI and REDDI (1986) in a study of 54 species of Poaceae showed a range from 165 to 17,775 and ABOULAICH *et al*. (2009) in a study of 38 species showed a range from 22.33 to 15,923.34 pollen per flower. Much higher values were found by RADAESKI and BAUERMANN (2016) who reported 213,000 grains per flower for the anemophilous species *Bromus catharticus* Vahl.

The pollen/ovule (P/O) ratios of anemophilous plants generally range from 10^4 to 10^6 (FAEGRI; VAN DER PIJL, 1979). According to the present study, the P/O ratio for *Dendrocalamus* of 7,525 is smaller than that of typically wind-pollinated plants. In a study of *Phyllostachys nidularia* the P/O ratio was also smaller than that for anemophilous plants in general, but this species, nonetheless, displayed many of the characteristics common for wind-pollinated plants (HUANG *et al*., 2002). These authors, however, suggested that visiting insects may play an active role in pollination of *P. nidularia* through dislodging a cloud of pollen from the anthers. Bees would, therefore, accelerate and synchronise pollen release over a short period of time, which would take longer to be released in their absence.

The role of insects in the pollination of *Dendrocalamus asper* is unknown, but during the collection of inflorescences honeybees (*Apis mellifera*) were frequently observed on the inflorescences. Therefore, it is possible that these bees could be accelerating the release of pollen grains just as in *P. nidularia*. This same mechanism has also been reported for *Dendrocalamus membranaceus* and *D. sinicus* (CHEN *et al*., 2017). *D. strictus* and *Ochlandra travancorica*, although typically anemophilous, were visited by *Apis mellifera* and *O. scriptoria* C. E. C. Fischer by *A. cerana*, (KOSHY; HARIKUMAR, 2001; NADGAUDA *et al*., 1993; VENKATESH, 1984). Many wind-pollinated species have few ovules per flower, so only one or a few pollen grains are needed to fertilise a given flower (WHITEHEAD, 1969), which may explain the existence of only one ovule per flower in *Dendrocalamus asper*.

Pollen viability is of interest because it is indicative of how well one plant may pollinate another (SHIVANNA *et al*., 1991). The 62.5% viability of *Dendrocalamus asper* pollen may be considered low when compared with other species (GROMBONE-GUARATINI *et al*., 2011; JIJEESH *et al*., 2009; JIJEESH *et al*., 2012; RAMANAYAKE; WEERAWARDENE, 2003;). Pollen viabilities for the bamboos *Aulonemia aristulata* (Döll) McClure, *Pseudoxytenanthera monadelpha* (Thw.) Soderstrom and Ellis, *Dendrocalamus sikkimensis* Gamble and *Melocanna baccifera* (Roxburgh) Kurtz ex Skeels were 90, 92-95, 90-92 and 85.8%, respectively (GROMBONE-GUARATINI *et al*., 2011; JIJEESH *et al*., 2009; JIJEESH *et al*., 2012; RAMANAYAKE; WEERAWARDENE, 2003). However, some studies reported that pollen viability may decrease hours after anthetic. *Ochlandra travancorica* (Beddome) Bentham and *O. wightii* (Munro) C.E.C. pollen at anthetic were 80-100% viable, but this decreased to 59% after another hour (GOPAKUMAR; MOTWANI, 2013). Similarly, *Ochlandra scriptoria* C. E. C. Fischer pollen viability after anthesis was

91.38%, but viability fell to 50% after 7-9 hours (KOSHY; HARIKUMAR, 2001). The relatively low viability of *Dendrocalamus asper* may therefore be explained, at least in part, due to the 36 hours between inflorescence collection and evaluation of pollen viability.

3.5.3 Anther developmental analysis

The anther contains the reproductive and the non-reproductive tissues that are responsible for the production and release of pollen grains (GOLDBERG *et al*., 1993). During *Dendrocalamus asper* floral development, the tetrasporangiate and anther develop an epidermal outer wall and a well-structured endothecium with a middle layer (Figures 3A-B). Each of these tissues and cells performs specialised tasks (ESAU, 1977): the endothecium and middle layer provide structural support and act in dehiscence and the epidermis provides structural support, dehiscent function, water loss prevention and gas exchange (ESAU, 1977; KOLTUNOW *et al*., 1990; WEBERLING, 1989).

FURNESS and RUDALL (2001) classified the anther tapetum into two main types: secretory, in which a layer of tapetum cells remains intact around the anther locule; and plasmodial, in which a multinucleated plasmodium is formed by degeneration of cell walls and fusion of its protoplasts within the locus of the anther. *Dendrocalamus asper* have the secretory type, which is considered a general feature in Poaceae anthers (Figures 3A-B) (NAKAMURA *et al*., 2010). Monocots, however, may display either type: the secretory type is predominant in Poales, but the plasmodial type has been reported in the Sparganiaceae and Typhaceae families (FURNESS; RUDALL, 1998). The tapetum plays a critical role in development because it provides a source of nutrients for developing pollen, a source of exine and lipid precursors for pollen coating and sporophytic recognition proteins (FURNESS; RUDALL, 2001; SHI *et al*., 2015). Starting from stage five of flower development, the tapetum begins to degenerate (Figures 3C-D). According to SANDERS *et al*. (1999), the exine wall forms and the microspore becomes vacuolated as the tapetum is steadily degenerated until the pollen mitotic divisions are completed. Tapetum cell death also results in the deposition of tryphine on the surface of maturing pollen (BEDINGER, 1992). These substances are lipoid and protect pollen grain from dehydration, as well as facilitate communication and adhesion between pollen and stigma (SHI *et al*., 2015).

After stage five of flower development, the anther dehiscence process begins, in which a sequence of cell destruction culminates in the rupture of the stomium during stage six

(anthetic), releasing mature pollen. The degeneration of the septum generates a bilocular anther, which is followed by rupture of the stomium cells (Figures 3D-F and 4C). SANDERS *et al*. (1999) described the main events that occur during dehiscence in *Arabidopsis* similar to those observed in *Dendrocalamus asper.* First, the middle layer and tapetum degenerate while the endotecial layer expands. The thickening of the endotecial layer leads to anther dehiscence and pollen release. Endothecium development is coordinated with pollen maturation and tapetum and middle layer degeneration. Deposition of lignocellulosic compounds thickens and expands cell walls, which is essential for providing the mechanical strength needed for anther dehiscence (WILSON *et al*., 2011).

3.5.4 Microgametogenesis analysis at different stages of floral development

During microgametogenesis, several internal modifications occurr as free microspores develop into mature pollen. While they are in the free microspore stage, the juvenile pollen contains only several small vacuoles that fuse into one large vacuole, giving rise to the vacuolated microspore (Figures 3C-D, 5A and 5C). This cytological reorganization of vacuoles prior to mitosis results in nuclear migration to a specific site near the cell periphery, a process also found to have occurred during *Zea mays* L. and *Brachypodium distachyon* (L.) Beauv. pollen development (BEDINGER, 1992; SHARMA *et al*., 2015).

While the nucleus is polarised opposite the pore, the first asymmetric mitotic division occur, producing bicellular pollen grains (Figures 3C-D). The first division of mitotic microspores give rise to two cells: a vegetative cell and a generating cell. Subsequently, the generating cell undergoes mitosis to produce two sperm nuclei. This pattern resembles same patterns observed in *Olyra humilis* Nees, *Sucrea monophylla* Soderstr, *Axonopus aureus* P. Beauv, *Paspalum polyphyllum* Nees *ex* Trin., *Cloris elata* Nees and *Eragrostis solida* Desv. (NAKAMURA *et al*., 2010).

During microspore mitosis, mitochondria accumulate in the cytoplasm (Figures 5D, 5F and 5J). Mitochondria are one of the major synthesis sites of ATP, indicating an active metabolism related to mitotic divisions (LOGAN, 2006). This event was also observed during mitosis in *Arabidopsis thaliana* (REGAN; MOFFATT, 1990).

However, according to BLACKMORE *et al*. (2007), before pollen grains are released from anthers, three important processes must occur: reserve accumulation in the form of starch or lipids; the addition of tryphine to the exine after tapetum degeneration; and

cytoplasm dehydration. Starch accumulation in *Dendrocalamus asper* begins in the free microspore stage. Amyloplasts became larger and more numerous throughout all subsequent stages of pollen development.

According to BAKER and BAKER (1979), two classes of pollen grains can be found among angiosperms: ones which contain little-to-no starch at time of dispersal and ones that contain considerable starch reserves. *Dendrocalamus asper* pollen is classified as the latter because, at the time of dispersal, these pollen grains contain large amounts of starch (Figures 4C and 5G-H). Starch accumulation during the final stages of pollen maturation is critical, not only because starch is a reserve energy source for pollen germination, but also because it serves as a checkpoint for pollen maturity (DATTA *et al*., 2002). Three species of monocots within the Bromeliaceae family—*Aechmea recurvata, Dyckia racinae and Tillandsia aeranthos*—similarly accumulated starch during maturation starting from the free microspore phase and, at the moment of dispersion, the starch was completely hydrolysed (OLIVEIRA *et al*., 2015). These products, including sucrose and other oligosaccharides, protect membranes during pollen desiccation, leading to greater pollen viability (FRANCHI *et al*., 1996).

3.5.5 Analysis of sporoderm formation during microgametogenesis

During *Dendrocalamus asper* floral development, several stages of microgametogenesis were characterized, which begins shortly after the end of sporogenesis with the dissolution of callose around the microspores (BEDINGER, 1992). In stage two of floral development, the sporoderm of free microspores is still developing with the gradual deposition of the different exine and intine forming layers in the vacuolated microspore until the sporoderm is complete (Figures 2A and 5A). Pollen have an exine coating that protects them during the dispersion process, and the unique characteristics of the exine ornamentation are useful for plant identification and taxonomic studies (STEPHEN, 2014). Digital processing of exine ornamentation images from scanning electron microscopy allowed the differentiation of six Poaceae species from south eastern Amazonia in Brazil (GUIMARÃES *et al*., 2017). *Dendrocalamus asper* pollen have a rugulate ornamentation pattern, which is different than the granular pattern of *Olyra humilis* and *Sucrea monophylla* pollen; the spinule pattern of *Axonopus aureus* P. Beauv., *Paspalum polyphyllum* Nees *ex* Trin., *Eragrostis solida* Nees and *Chloris elata* Desv pollen; and the microechinate pattern observed in a study of 11 species of Poaceae (NAKAMURA *et al*., 2010; RADAESKI *et al*., 2018).

The sporoderm is essential for plant reproduction because its resistant physical properties promote survival in adverse environments (SHI *et al*., 2015). Pollen grain wall formation begins shortly after microspore formation (BLACKMORE *et al*., 2007).

The intine of *Dendrocalamus asper* pollen forms during the free microspore stage (Figures 5E and 5G-I), which provides the pollen with structural integrity, as well as playing a role in germination and growth of the pollen grain pollen tube (SHI *et al*., 2015). Tryphine is later added to the sporoderm after the degeneration of the tapetum (BLACKMORE *et al*., 2007), where it serves multiple purposes: it fills the gaps in the exine, protects male gametophytes from dehydration, and facilitates the recognition of stigma-compatible pollen (SHI *et al*., 2015).

Though *Dendrocalamus asper* free microspores have a pore with a distinguishable operculum, it underwent further development as the annulus developed and the exine and intine thickened during pollen maturation (Figures 2A-D, 5A, 5D-E and 5G-I). The presence of the operculum and intine thickening are of great importance because it protects the cytoplasm from dehydration, especially in wind-pollinated plants (FURNESS; RUDALL, 2003). In addition to its physiological importance, pore description is useful for determining taxonomy (SHARMA *et al*., 2015).

Mature pollen grain of *Dendrocalamus asper* is classified monoporated with an operculum-like pore. Elongated apertures are a predominant characteristic in the pollen of Poales, however, pore-like openings are also found in Thyphaceae of the graminoid-restiid (LINDER; RUDALL, 2005). Of pollen samples collected from 49 species of Gramineae in the Venezuelan Andes, most species have monoporated pollen grains, except for *Agrostis sp.*, *Bromus sp.*, *Calamagrostis sp.*, *Cliirsqirea sp*. and *Melinis minutiflora* P. Beauv, which are diporated (SALGADO-LABOURIAU; M. RINALDI, 1990). RADAESKI *et al*. (2018) studied the pollen morphology of different native genera of Poaceae native to Southern Brazil and found that all species were monoporated except for the species *Pharus lappulaceus* Aubl., which had either a monoporate or diporate aperture. Finally, RADAESKI *et al*. (2016) studied 68 species of the Poaceae native to the Brazilian state of Rio Grande do Sul and found, as in other studies, that all species were monoporate except for the species *Paspalum pauciciliatum* (Parodi) Herter, which had either a monoporate or diporate aperture.

The mature pollen grains of *D. asper* are larger to medium-sized when compared to other studies the Poaceae family (RADAESKI *et al*., 2016; RADAESKI *et al*., 2018; SALGADO-LABOURIAU; M. RINALDI, 1990). RADAESKI *et al*. (2018) found pollen

grains with 22-77 µm, SALGADO-LABOURIAU and M. RINALDI (1990) found 17.5-60.6 µm and RADAESKI *et al*. (2016) found 22-46 µm.

3.6 CONCLUSIONS

In the present work, seven stages of floral development for *Dendrocalamus asper* were characterised. Pollen grains undergo successive modifications (free microspore, vacuolated microspore and mature pollen grains) and disperse at the tricellular stage containing considerable starch reserves. At anthesis, pollen is monoporate with an operculum-like pore and a rugulate and spinose exine surface pattern. *Dendrocalamus asper* has as strategy to avoid inbreeding using a protogyny pattern of flower development. The floral characteristics and presence of the operculum in the pollen grain indicate that *Dendrocalamus asper* is wind pollinated. To the best of our knowledge this is the first report describing in full details the reproductive biology of the bamboo *D. asper*.

3.7 ACKNOWLEDGMENTS

This study was supported by the National Council for Scientific and Technological Development (CNPq, Proc. 457726/2013-0), and Rosete Pescador (302105/2017-4) an Miguel Pedro Guerra (302798/2018-8) productivity scholarships. The authors also thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the doctoral level scholarship to the first author and the Jatobás Institute for the donation of plant material.

4 MALE STERILITY IN *BAMBUSA TULDOIDES* MUNRO

4.1 ABSTRACT

Despite their great economic importance, relatively little is known about bamboo sexual reproduction because they usually spread through rhizomes and have long intervals between flowering periods. *Bambusa tuldoides* is no exception; the intervals between flowering periods are about 23 years, and often does not result in successful caryopsis production. The aim of the present work was to characterise *Bambusa tuldoides* sexual reproduction at three stages of flower development and investigate possible male sterility. Pollen was cultured onto several types of culture medium in order to encourage germination, but not a single of the thousands of observed pollen germinated under any condition. Anthers and microspores were analysed by scanning electron microscopy, transmission electron microscopy and optical microscopy techniques. Anther dehiscence appeared to be normal when compared to other species. In contrast, microspores began to develop abnormally starting as early as the first flower development stage: retraction of the cytoplasm; rupture of the nuclear and mitochondria membrane. As the interior machinery of the microspores degenerated, starch accumulated within numerous amyloplasts during stages two to four of flower development. The sporoderms of these microspores were similarly incomplete: though they possessed an exine, they lacked an intine. The results here obtained suggest that the non-viability of these abnormal pollen grains prevents the development of *Bambusa tuldoides* caryopses.

Keywords: Bamboo, genetic diversity, scanning electron microscopy, abnormal microspores.

Recebido: 27 agosto de 2019
Aceito: 26 dezembro de 2019

Disponibilidade: https://link.springer.com/article/10.1007/s00709-019-01479-8
de Souza, P.F., dos Santos, C.M.R., Ree, J. et al. Protoplasma (2020).
https://doi.org/10.1007/s00709-019-01479-8

4.2 INTRODUCTION

Natural bamboo populations (family Poaceae, subfamily Bambusoideae) are present on almost every continent of the globe except Europe and Antarctica (SCURLOCK *et al*., 2000; WILLIAMS *et al*., 1994). Among the countries of the Americas, Brazil has the highest diversity, with 33 genera and about 250 species, of which about 160 are endemic, in addition to the presence of more than 20 exotic species. (FILGUEIRAS; GONÇALVES, 2004; FILGUEIRAS *et al*., 2013).

Bamboo is particularly valuable for industry, because the quality and tensile strength of its wood may be used to manufacture high quality goods, such as construction material, furniture, bulk cellulose, bioenergy and also the meristematic region of young shoots is used as food (BONILLA *et al*., 2010; SCURLOCK *et al*., 2000). Fast-growing bamboo cultivation may indirectly reduce deforestation and soil erosion while preserving plant biodiversity (EMBAYE, 2000). Despite the great economic importance of bamboos their propagation is mostly based on vegetative methods (MUDOI *et al*., 2013).

Bambusa tuldoides Munro, a woody bamboo native to China, is widely cultivated in the tropical and subtropical America (GUERREIRO; LIZARAZU, 2010). First introduced by the Portuguese during the colonial period, *B. tuldoides* has since become one of the most widespread species through Brazil (AZZINI *et al*., 1988). *Bambusa tuldoides* flowering periods are sporadic with an interval of about 23 years of vegetative development, during which bamboo spread asexually through growing new stems from clumps (AZZINI *et al*., 1988; GUERREIRO; LIZARAZU, 2010). A survey on sporadic flowering of six woody bamboo species introduced in Brazil showed that only four flowering episodes of *B. tuldoides* were observed in 1954, 1984, 2005 and, most recently, in 2017 in three different locations. However, caryopsis formation occurred only in a single flowering event in a population in Santa Teresinha, Goias, Brazil, and in Argentina (FILGUEIRAS; SILVA, 2007; GUERREIRO; LIZARAZU, 2010).

The absence of caryopsis formation reported in other bamboo species may be related to male sterility, though many studies only analysed pollen grains with light microscopy (DAS *et al*., 2017; JIJEESH *et al*., 2014; KOSHY; PUSHPAGADAN, 1997; KOSHY; JEE, 2001; WANG *et al*., 2015). There are comparatively fewer studies on ultrastructural processes resulting in male sterility, and many of those reports studied cultivated or mutant plants (SANDERS *et al*., 1999; KU *et al*., 2003; ZHOU *et al*., 2011) or the formation of unisexual

female flowers (COIMBRA, 2004; HADDAD *et al*., 2018). However in other bamboo species the absence of caryopsis does not occur, such as in *Arundinaria alpina*, *Oxytenanthera abyssinica, Dendrocalamus membranaceus, D. hamiltonii, D. brandisii, Bambusa arundinacea* (BAHRU *et al*., 2015; LAKSHMI *et al*., 2014; RAWAT; THAPLIYAL, 2003; SINGH; RICHA, 2016; THAPLIYAL *et al*., 1991; WARRIER *et al*., 2004; XIE *et al*., 2016).

Knowledge of the aspects related to pollen grain development is of great importance for the propagation and conservation of *B. tuldoides* because it is intrinsically linked to successful embryo and seed formation (ZHANG *et al*., 2014). Thus, this work aimed at to characterise the anthers and pollen grain of *B. tuldoides* in three stages of flower development through histochemical and ultrastructural techniques looking at to elucidate aspects related to male sterility.

4.3 MATERIAL AND METHODS

4.3.1 Vegetable material

Bambusa tuldoides inflorescences were collected from mother plants located in Parque Cidade das Abelhas-UFSC (27 ° 32'20.04” S, 48°30'10.87” W), in Florianópolis, Santa Catarina, Brazil, in January 2017. At the time of collection, the inflorescences were packaged in plastic bags and placed in ice-containing Styrofoam boxes to be transported to the Plant Physiology and Developmental Genetics Laboratory (LFDGV) and to the Central Electron Microscopy Laboratory (LCME) of the Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil. Voucher specimens were deposited in the Herbarium FLOR – UFSC (FLOR 63000).

4.3.2 Identification of flower developmental stages

One hundred inflorescences were analysed under a stereomicroscope (Olympus, model SZH10), to determine flower development stages (MCCLURE, 1966).

Bambusa tuldoides inflorescences, also called spikes, are composed of structural units called spikelets and a single bisexual flower. In stages one and two of flower development, the gynoecium and androecium are protected by the lemma. Anthesis begins at stage three with the opening of the palea and lemma and the exteriorization of the anthers. In stage four

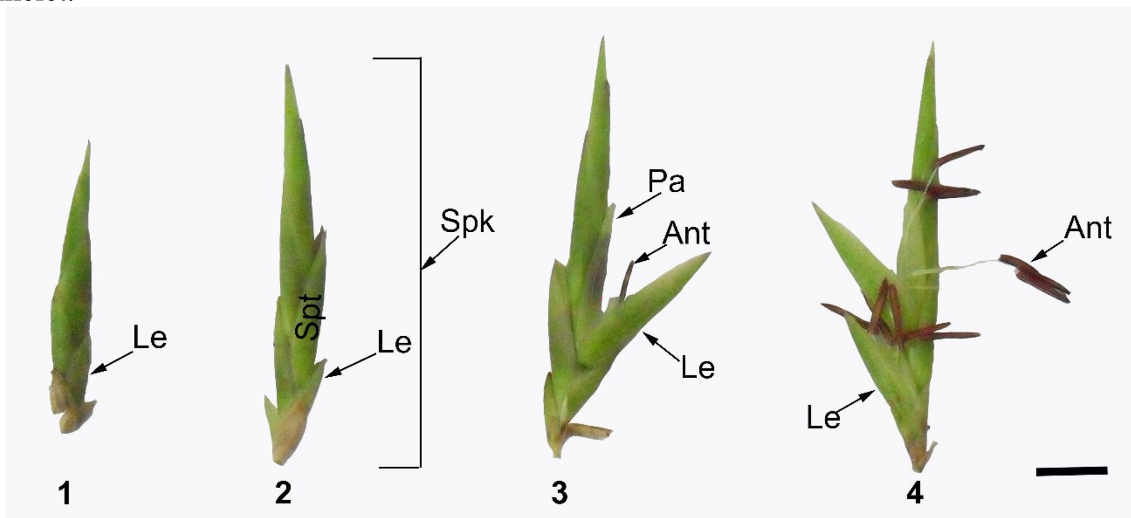
(anthesis), the anthers are externalised with subsequent dehiscence and pollen release (Table 1). Spikelets lacked nectaries and noticeable odors (Figure 1).

Table I - Developmental stages of *Bambusa tuldoides* inflorescences

Stages of flower development	Characteristics
Initial stage 1	Gynoecium and androecium are protected by the lemma
Stage 2 (pre-anthesis)	Lemma begins to open
Stage 3 (anthesis)	Lemma and palea are fully open and anthers begin to exteriorize
Stage 4 (anthesis)	Anthers are fully exteriorized and have undergone dehiscence

Fonte: Souza *et al*. (2020b)

Figure 1. Developmental stages of *Bambusa tuldoides* spikelets: 1. Early stage of development: gynoecium and androecium are protected by the lemma; 2. Pre-anthesis: lemma begins to open; 3. Beginning of the anthesis: lemma and palea are fully open and anthers begin to exteriorize; 4. Anthesis: anthers are fully exteriorized and have undergone dehiscence. Scale bar: 5 mm. References: *Le*, lemma; *Pa*, palea; *Ant*, anther; *Spk*, spike; *Spt*, spikelet.



Fonte: Souza *et al*. (2020b)

4.3.3 *In vivo* pollen viability

Ten flowers were collected at the pre-anthesis stage to determine the viability of pollen grain *in vivo*. Six anthers were removed from each flower, macerated with one drop (2 µL) of acetic

carmine dye on a labeled glass slide and covered by a coverslip. After five minutes, the pollen grains were counted under an optical microscope (Olympus, model BX40) under 10x magnification. Pollen grains that stained red were considered viable, and uncolored pollen grains were considered non-viable. The results were expressed as a percentage of viable pollen grains (JIJEESH *et al*., 2012). A completely randomised design with four replicates was used, each replicate being represented by a slide.

4.3.4 *In vitro* pollen grain germination

Pollen grain germination viability was determined based on an adapted methodology of ALMEIDA *et al*. (2011). Three separate experiments, consisting of four replicates for all treatments applied, each conducted to investigate pollen germination on culture medium containing 100 mL of distilled water, 1g of agar, and the additives listed below:

- Experiment 1: sucrose (10, 15, 30, 45%) and boric acid (0, 80, 160 mg / L);
- Experiment 2: sucrose (10, 15, 30, 45%); boric acid (0, 40, 80 mg / L) and 400 mg / L calcium chloride.
- Experiment 3: calcium chloride (mg / L), sucrose (%) and boric acid (mg / L) - Treatment 1: 1500/10/0; Treatment 2: 500/10/0; Treatment 3: 1500/0/0; Treatment 4: 500/0/0; Treatment 5: 1500/10/300; Treatment 6: 500/10/300.

The components were added to a container and heated until dissolved without boiling. After the medium cooled to about 27 ° C, pollen collected from flowers in development stage two (pre-anthesis) were added and kept at this temperature for 24 and 48 hours for germination. Two-hundred pollen grains were counted per replicate for presence of a clearly-visible pollen tube. A completely randomized design was used, with the experiments 1 and 2 in factorial scheme.

4.3.5 Light microscopy analysis

Samples from six anthers at three flower developmental stages (stage one, pre-anthesis and anthesis), were fixed in paraformaldehyde solution (2.5%) in phosphate buffer (0.2M) pH 7.3 at a ratio of 1: 1 for 24 h at 4°C. After fixation, the material was washed three times in phosphate buffer for 30 minutes (Bouzon 2006) and dehydrated in a gradual ethanol series (SANDERS *et al*., 1999). Subsequently, the samples were pre-infiltrated in LeicaTM

historesin with absolute ethyl alcohol at a ratio of 1: 1 for 72h and infiltrated into LeicaTM historesin for 72h, according to the manufacturer's instructions with minor alterations. The samples were sectioned on a rotary microtome (microTec, model CUT 4055) to produce 5 µm thick transverse and longitudinal sections (NAKAMURA *et al*., 2010). Sections were stained with toluidine blue (O'BRIEN *et al*., 1965) and Lugol histochemical test for starch identification. Sections were placed in either reagent for 10 minutes and washed in distilled water (JOHANSEN, 1940). Observations and records were performed using a light microscope (Leica, model DM5500 B).

4.3.6 Transmission electron microscopy analysis

Samples from six anthers at three stages of flower development (stage one, pre-anthesis stage and anthesis) were fixed in 2.5% glutaraldehyde solution buffered with 0.1 M cacodylate (pH 7.2) for 24h. The samples were then washed four times in the same buffer. They were then post-fixed in 1% OsO_4 in 0.1M cacodylate buffer, pH 7.2 (1: 1) for 4 h at room temperature (PUESCHEL, 1979). Subsequently, the material was washed twice in 0.1 M cacodylate buffer, pH 7.2 (30 minutes each), dehydrated in an acetone series (30%, 50%, 70%, 90% and 100%) for 30 minutes at each concentration with the 100% acetone stage repeated twice more (adapted from COIMBRA *et al*., 2004). After dehydration, the material was slowly infiltrated with Spurr resin and polymerised in horizontal oven molds at 70 ° C for 24 h (SPURR, 1969). The material was sectioned with a ultramicrotome (Leica) allocated on copper grids and stained with 5% uranyl acetate and lead citrate (REYNOLDS, 1963). The samples were observed and recorded with a transmission electron microscope (Joel, model JEM 1011).

4.3.7 Scanning electron microscopy analysis

Samples from six anthers at three flower developmental stages (stage one, pre-anthesis stage and anthesis) were fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.2) with 2 M sucrose for 24 hours. Subsequently, the samples were dehydrated with an ethanol series (30%, 50%, 70% and 90% and 100%) for 30 minutes at each concentration and then washed twice more with 100% ethanol (adapted from SCHMIDT *et al*., 2012) and critical point dried with CO2 (Leica, model EM-CPD-030). The dried samples were covered with 20

nm gold in a metalliser (Baltec, CED 030) (Schmidt et al. 2012). The samples were observed and documented in a Scanning Electron Microscope (SEM) (Jeol, model JSM-6390LV).

4.4 RESULTS

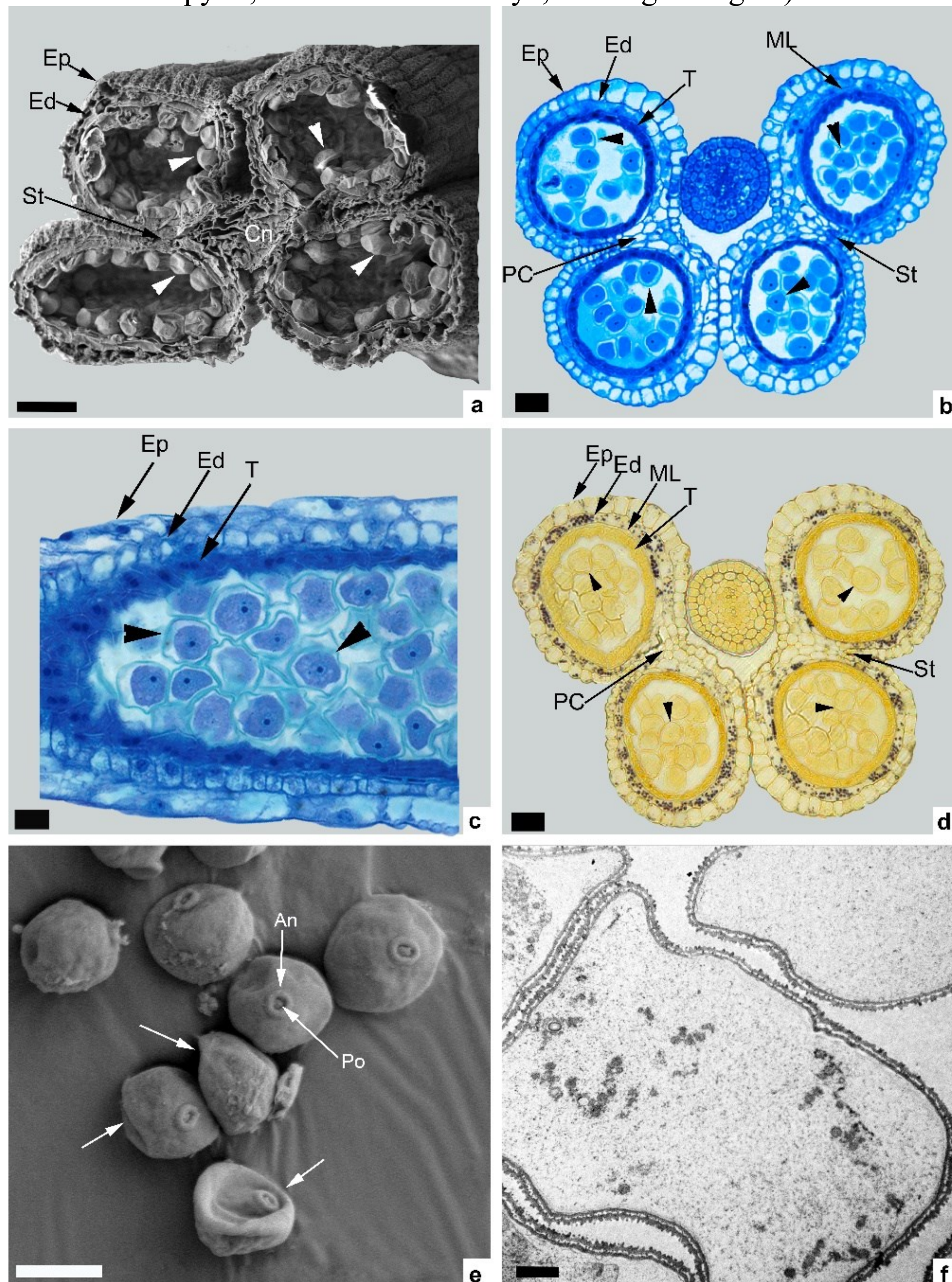
4.4.1 *In vitro* pollen grain germination, in vivo pollen viability, anthers and microspores analysis

By staining with acetic carmine (*in vivo* viability), we were able to observe that 68.12% of pollen were viable. However, despite many different treatments pollen germination was not observed. Because of these unexpected results, we used histological analysis to investigate possible reasons for non-germination of pollen grains.

In the stage one, the tetrasporangiated anther had a wall with an unistratified epidermis with juxtaposed cells, followed by a well-structured endothecium, middle layer, and irregularly-shaped uni and binucleated tapetum cells (Figures 2a-d). In the endothecium starch dispersed in the cytoplasm of cells was evident (Figure 2d). The tapetum was of a secretory type, as opposed to a plasmodial type, because a layer of cells remained intact around the anther's locus with uninucleated and occasionally binucleated cells (Figure 2b-c). The cell walls of the epidermis, endothecium, middle layer and tapetum were stained blue (toluidine blue) indicating cellulosic compounds. In the parenchymatic tissue between the anther locules, well-developed cells formed a clear connection to the central vascular bundle (stained blue) (Figures 2b-c). Free microspores had non-polarised nuclei, blue-stained sporoderms, and signs of cytoplasm retraction (Figures 2b-c). At this stage of inflorescence development, deformed microspores showing cytoplasm poor in organelles (Figures 2e-f). The free microspores showed signs of a retraction of the cytoplasm (Figures 3a-c and 3e), with a rupture of the cytoplasmic membrane (Figures 3b-c and 3e). Most mitochondria had membrane disruption (Figures 3b and 3d-e). The microspore sporoderm at this stage was incomplete: the exine (tectum, columella and endoexine) was formed, but the intine was absent (Figure 3f). Scanning electron microscopy showed that the pore and annulus formation in microspores (Figure 2e).

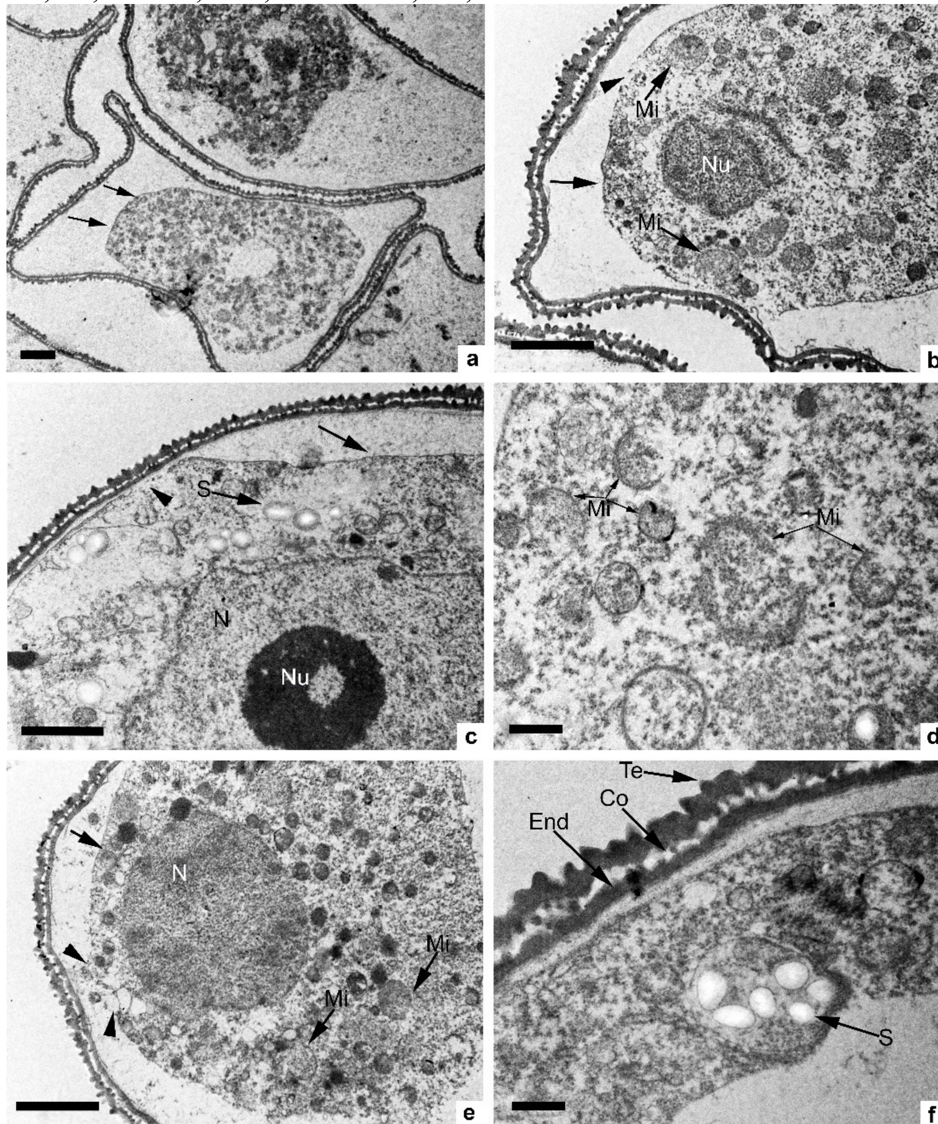
Figure 2. Scanning and transmission electron micrographs and light microscopy of the *Bambusa tuldoides* anthers and microspores at stage one of flower development. a, b, d - Cross sections. c - Longitudinal sections **a.** Epidermis, endothecium, connective tissue and

stomium in the anther. Arrowheads indicate microspores. **b.** Epidermis, endothecium, middle layer and irregularly-shaped tapetum cells. Remaining the parenchyma cells and stomium. In the locule, free microspores with retraction of the cytoplasm (arrowheads) were present. **c.** Epidermis, endothecium, irregularly-shaped tapetum cells and free microspores with retraction of the cytoplasm (arrowhead). **d.** Starch deposition in epidermis, endothecium, middle layer. Microspores without starch deposition were present (arrowheads). **e.** Free microspores with irregular shapes (arrows). Pore and annulus formation in microspores. **f.** Free microspores with irregular shapes and showing cytoplasm poor. Scale bar: a, b, c, d – 50 µm; e – 20 µm; f – 2 µm. References: *Ep*, epidermis; *Ed*, endothecium; *T*, tapetum; *ML*, middle layer; *St*, stomium; *PC*, parenchyma cells; *Cn*, connective tissue; *An*, annulus; *Po*, pore. (Optical microscopy: b, c – toluidine blue dye; d – Lugol reagent)



Fonte: Souza *et al*. (2020b)

Figure 3. Electron transmission micrograph of *Bambusa tuldoides* microspores at stage one of flower development. **a.** Irregularly-shaped free microspores with retraction of the cytoplasm (arrows). **b.** Microspore with ruptured plasma membrane (arrowheads), cytoplasm retraction (arrow) and membrane rupture in mitochondria. **c.** Microspore with rupture (arrowhead) and retraction of the plasma membrane (arrow). Cytoplasmic content with dispersed amyloplasts. **d.** Microspore with some disruption of mitochondrial membranes. **e.** Microspore with plasma membrane rupture (arrowheads) and cytoplasm retraction (arrow). Some disruption of mitochondrial membranes close to the nucleus. **f.** Microspore with sporoderm formed by exine (tectum, endoexine and columella) and starch deposited amyloplast inside. Scale bar: a, b, c, e – 2 µm; d, f - 0.5 µm. References: *Nu*, nucleolus; *N*, nucleus; *S*, starch granule; *Mi*, mitochondria; *Te*, tectum; *End*, endoexine; *Co*, columellae.



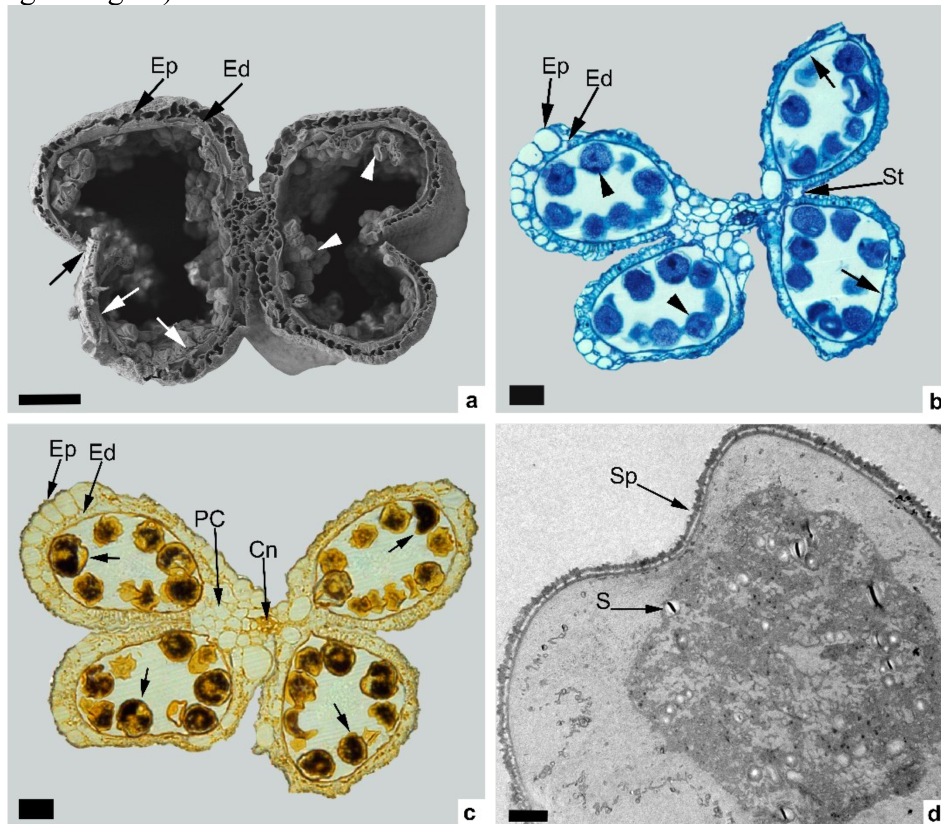
Fonte: Souza *et al*. (2020b)

In the stage two (pre-anthesis), the epidermis and endothecium cells remained with the same level of blue-stain without indication of thickening of the walls of these cells (Figure 4b). The tapetum cells and the middle layer began to degenerate, and, elsewhere, the

connective regions between the anther locules began to break down (Figures 4a-b). The microspores sporoderm thickness remained unchanged, nuclei became hard to visualise (Figure 4b). At this stage, positive Lugol stains identified the beginning of starch reserve accumulation (Figure 4c-d).

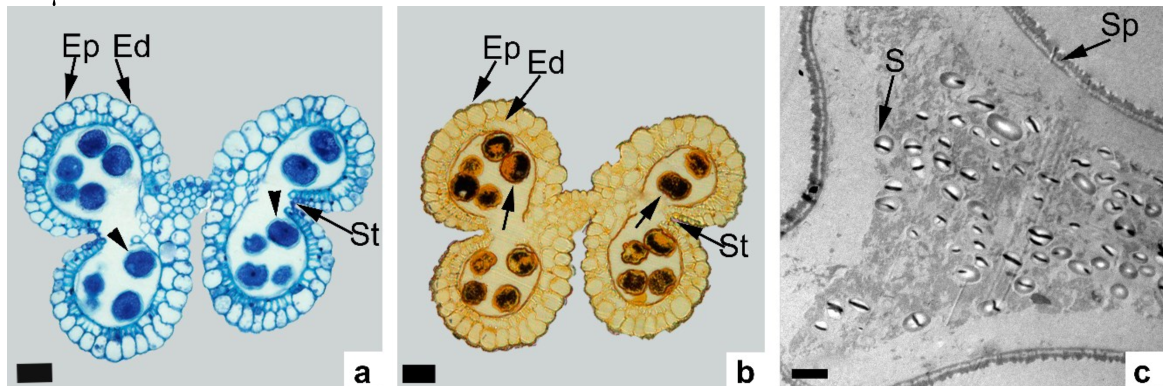
At the stage four (anthesis), the total destruction of the walls between the anther locules and the dissolution of the tapetum cells was observed (Figure 5a-b). The stomium had ruptured, and few pollen grains were present inside the anther. Microspores were stained in blue, which indicated the deposition of cellulosic compounds on the sporoderm. Starch continued to accumulate according to the intensity of Lugol staining inside the microspores (Figures 5b-c). The sporoderm was maintained and the evident retraction of the cytoplasm was clearly visible at this stage with the total destruction of the plasma membrane, organelles and cytoplasmic content, and neither the nucleus nor the nucleolus could be visualised (Figures 5c).

Figure 4. Scanning and transmission electron micrographs and light microscopy of the *Bambusa tuldoides* anthers and microspores at stage two of flower development: a, b, c - Cross sections. **b.** Epidermis, endothecium, remaining tapetum cells (arrows) and stomium. Irregularly-shaped micropores (arrowheads). **c.** Epidermis, endothecium and anther tapetum cell degeneration. The parenchyma cells can be seen between the locules, the connective tissue and the region of the stomium. Microspores (arrows) with starch deposition in the cytoplasm. **d.** Microspores with starch deposition. Scale bar: a – 100 µm; b, c – 50 µm; d – 2 µm. References: *Ep*, epidermis; *Ed*, endothecium; *St*, stomium; *PC*, parenchyma cells; *Cn*, connective tissue; *S*, starch granule; *Sp*, sporoderm. (Optical microscopy: b – toluidine blue dye; c – Lugol reagent)



Fonte: Souza *et al*. (2020b)

Figure 5. Electron transmission micrograph and light microscopy of the *Bambusa tuldoides* anthers and pollen grains at stage four of flower development: a, b. Cross sections. **a.** Epidermis, endothecium, tissue rupture in the anther stomium region and the free microspores (arrowheads) with retracted cytoplasm and irregular shapes. **b.** epidermis, endothecium and tissue rupture in the anther stomium region. Microspores with higher starch deposition in the cytoplasm (arrows). **c.** Microspores with increased starch deposition and numerous amyloplasts. Clear retraction of the cytoplasm with destruction of the plasma membrane, organelles and cytoplasmic content. The sporoderm remained intact. Scale bar: a, b – 50 µm; c – 2 µm.



Fonte: Souza *et al*. (2020b)

4.5 DISCUSSION

4.5.1 Analysis of anther in different stages of floral development

The anther contains the reproductive and non-reproductive tissues that are responsible for the production and release of pollen grains. During the stage one of *B. tuldoides* flower development, the tetrasporangiate anther, with the epidermal outer wall, a subepidermal inner layer called the endothecium and the well-structured middle layer, could be easily identified. More internally, we found the secretory tapetum with irregularly shaped cells (Figures 2a-c). At the stage two, both the tapetum and the middle layer had degenerated further (Figures 4a-c), thus starting the anther dehiscence process. SANDERS *et al*. (1999) described the main events that occur during pollen grain release in *Arabidopsis*: the middle layer and tapetum degenerate; the endothelial layer expands; the septum degenerates to form the bilocular anther; and the stomium cells rupture. Similarly, we observed that the destruction of the locules wall led to the generation of the bilocular anther (Figure 4a). During stage four (anthesis), the stomium ruptured (Figures 5a) and released pollen grains into the environment. According to WILSON *et al*. (2011), anther development, coupled with functional pollen

release, is fundamental for plant reproduction. However, although *B. tuldoides* anthers had normal development and dehiscence, pollen grains were not functional at the time of release.

Genetic defects in anther development often cause male sterility through the production of nonfunctional pollen grains (KU *et al*., 2003). One cause may be interference with the process of tapetum development or degeneration, which are both tightly regulated (PARISH and LI, 2010). Premature or late programmed cell death (PCD) in these cells promotes disruption of pollen grain development (HADDAD *et al*., 2018). The tapetum acts as a source of nutrients for developing pollen grains; as well as a source of exine and lipid precursors for their coating and sporophytic recognition proteins (FURNESS; RUDALL, 2001; SHI *et al*., 2015). Removal of this vital source of many compounds could lead to drastic defects in pollen. For example, *Sorghum bi-color* (L.) Moench strains that had changes in tapetum development (anomalous nuclei and increased vacuolization) produced sterile pollen grains because the flow of nutrition and other compounds was disrupted (TSVETOVA; ELKONIN, 2013). The abnormal expansion of *Bambusa sinospinosa* tapetum cells caused trinucleated microspores to shrink and develop a deformed sporoderm (WANG *et al*., 2015).

According to PARISH and LI (2010), tapetum development is sensitive to abiotic stress. Indeed, the tapetum cells of the pollen of a thermal-sensitive *Oryza sativa* L. strain subjected to high temperatures died prematurely (KU *et al*., 2003). NGUYEN *et al*. (2009) also observed sterility of *Oryza sativa* L. *japonica* cv R31 pollen when subjected to water stress for three days. But in *Bambusa tuldoides* tapetum degradation begins at stage two and the *in vivo* viability showed that 68.12% of pollen were viable (Figure 2b-c). So this is not the cause of the unviability of pollen grains.

4.5.2 Analysis of pollen grain at different stages of floral development

After anther tapetum cell degeneration, the microspore is expected to undergo mitosis and starch accumulation, culminating in the release of mature and viable pollen (BEDINGER, 1992; DATTA *et al*., 2002). However, in *B. tuldoides* pollen grains are unviable.

In *B. tuldoides* the first indication of microspore degeneration is the retraction and disruption of the plasma membrane (Figures 3a-c and 3e). Similarly, the plasma membrane of *Actinidia deliciosa* microspores showed signs of retractions, but all other cellular organelles, including mitochondria, had well-preserved structures (COIMBRA, 2004). In contrast, *B. tuldoides* microspores showed ruptured mitochondria membranes (Figures 3b and 3d-e). The

presence of functional mitochondria is important in the development of microspores, as these provide the vast majority of ATP needed to support an active cellular metabolism (LOGAN, 2006; REGAN and MOFFATT, 1990).

In the following stages of pre-anthesis and anthesis, these organelles were no longer visible, leaving only starch grains and the incomplete sporoderm presenting the exine without the formation of the intine (Figures 3f, 4d and 5c). In the free microspore stage, intin formation begins and ends during pollen grain maturation (BLACKMORE *et al*., 2007). The main components of intin are pectin, cellulose, hemicellulose, hydrolytic enzymes and hydrophobic proteins. These are necessary for maintaining structural integrity, as well as playing a role in germination and growth of the pollen grain pollen tube (SHI *et al*., 2015).

Starch deposition in microspores is one of the main processes that must occur before pollen grains are released from anthers (BLACKMORE *et al*., 2007). Starch biosynthesis during the late stages of pollen maturation is critical for the pollen of many types of plants, not only because starch is a source of energy and reserves for pollen germination, but also serves as a checkpoint for pollen maturity. Therefore, pollen infeasibility may be associated with starch deficiency (DATTA *et al*., 2002). In the case of *B. tuldoides* microspores, starch was continually deposited even with cytoplasm and nucleus degeneration until anthesis. The first signs of starch deposition occurred at stage one (Figures 3c and 3f); and starch continued to accumulate from pre-anthesis to anthesis until microspores were filled with amyloplasts (Figures 4c-d and 5b-c). In *Lilium hybrida* cv. Citronella a similar process was observed, in which the cytoplasm progressively degenerated after microspore mitosis, but the cytoplasm of the pollen grains contained a large amount of starch (VARNIER *et al*., 2005).

Carbohydrates produced by the sporophyte and dispersed in the locular fluid are absorbed for microspore development (CLEMENT; AUDRAN, 1995) and can have different destinations: consumed immediately; transformed into other molecules; polymerised to form the intine; or stored as starch grains (PACINI; FRANCHI, 1983). This event could explain the continuity of starch deposition in the *B. tuldoides* microspore cytoplasm even with the degeneration of cytoplasmic structures.

4.5.3 Pollen grain unviability and genetic diversity

Plants may reproduce sexually or asexually, and each method is controlled by distinct mechanisms with contrasting genetic consequences. The most common form of asexual

reproduction is clonal growth, in which vegetative modules (ramets) are produced by the parental genotype (genet) (VALLEJO-MARIN *et al*., 2010). In bamboos, due to the long period of vegetative growth, many species rely little on sexual reproduction. These reproduce clonally through a complex system of underground rhizomes from which the roots and stalks emerge, allowing them to spread over large areas (GUILHERME *et al*., 2017; POHL, 1991). However, as asexual propagation can only produce genetically identical offspring, it is unable to introduce new genotypes into the population. Thus, dependence on vegetative regeneration can lead to a genetically homogeneous population (KITAMURA; KAWAHARA, 2011).

Sexual reproduction is essential in the long term to ensure the sustainability of species populations as it provides an independent dispersal phase, greater genetic diversity and potential for adaptation to new environments (RAMAWAT *et al*., 2014; WILCOCK; NEILAND, 2002). Sexual reproduction in bamboos, therefore, is necessary for population regeneration (HUANG *et al*., 2002). A study of *Dendrocalamus giganteus* populations located in China showed that there is low genetic variation within the populations of this species. The authors attributed the low genetic variation to its long vegetative phase and widely separated flowering / seed episodes during the species life cycle (TIAN *et al*., 2012). These are typical features of various woody bamboo species (JANZEN, 1976). *Bambusa tuldoides*, likewise, have long vegetative periods between flowering events, but, beyond that, the scarce-to-absent production of caryopses may be related to pollen unviability, leading to a potential loss of genetic diversity due to inefficient sexual reproduction.

The unviability of pollen grain and the consequent non-production of caryopses has also been reported in *B. striata, B. vulgaris, B. sinospinosa, D. giganteus* and *B. balcooa* (DAS *et al*., 2017; JIJEESH *et al*., 2014; KOSHY; PUSHPAGADAN, 1997; KOSHY; JEE, 2001; WANG *et al*., 2015). In B. *striata* the non-production of caryopses and flowering after a long growing season was the focus of several studies leading researchers to speculate about the possible risk of extinction of the species (JOHN; NADGAUDA, 1997; KOSHY; PUSHPAGADAN, 1997; KOSHY; JEE, 2001). The absence of *B. balcooa* caryopses led DAS *et al*. (2017) to state that effective conservation strategies would require careful planning. Despite the knowledge about the lack of caryopsis production in several bamboo species, these works do not generally address how this fact can influence the genetic variability and conservation of these species.

Loss of genetic variation may reduce population viability due to inbreeding depression, reduced adaptability to new environments or, in the case of plants, genetic self-

incompatibility systems and a subsequent reduced availability of compatible partners (ELLSTRAND; ELAM, 1993).

4.6 CONCLUSION

During development, pollen grains showed accumulation of amyloplasts, a seeming loss of organelles, signs of cytoplasm retraction, rupture of the cytoplasmic membrane and an incomplete sporoderm. These pollen grains were released into the atmosphere, but were unviable, may be responsible for the non-production of caryopses. Considering that caryopses have not been observed in this study (and in others also), *B. tuldoides* may maintain their populations mainly, or, perhaps, solely by means vegetative propagation.

4.7 ACKNOWLEDGMENTS

This study was supported by the National Council for Scientific and Technological Development (CNPq, Proc. 457726/2013-0), and RP (302105/2017-4) and MPG (302798/2018-8) productivity scholarships. The authors also thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the doctoral level scholarship to the first author.

5 CARATERIZAÇÃO MORFOLÓGICA DO GINECEU EM *BAMBUSA TULDOIDES* MUNRO E *DENDROCALAMUS ASPER* (SCHULT. & SCHULT.) BACKER *EX* K. HEYNEK

5.1 RESUMO

Os estudos sobre biologia reprodutiva dos bambus tem como ênfase à viabilidade, morfologia e desenvolvimento do grão de pólen. Porém, estudos sobre o gametófito feminino são escassos na literatura. O objetivo deste estudo foi caracterizar o gineceu e o gametófito feminino em *Dendrocalamus asper* e *Bambusa tuldoides*, visando elucidar aspectos relacionados a não produção de cariopses. O gineceu em três estádios de desenvolvimento floral foi analisado por meio de técnicas de microscopia eletrônica de varredura e microscopia de luz. O gineceu de *B. tuldoides* consiste de um ovário obovado, estilete curto e dois estigmas plumosos. O óvulo é ortotrópo e bitegumentado. A formação do saco embrionário inicia-se na pré-antese e finaliza o desenvolvimento na antese. O gineceu de *Dendrocalamus asper* consiste em um ovário ovado, um estilete longo e um estigma plumoso. O óvulo maduro é anátropo e bitegumentado. A formação do saco embrionário inicia-se no estádio dois de desenvolvimento floral e finaliza o desenvolvimento na pré-antese. A não produção de cariopses em *B. tuldoides* não pode ser explicada pelo desenvolvimento do saco embrionário. Em *D. asper* a depressão por endogamia de ação precoce parece ser o motivo para a não produção de cariopses.

Palavras chave: Bambu, colapso do embrião, gametófito feminino, megagametogênese.

5.2 INTRODUÇÃO

Os bambus são membros do grupo taxonômico das grandes gramíneas lenhosas (subfamília Bambusoideae, família Poaceae/Andropogoneae), compreendendo 1250 espécies dentro de 75 gêneros de ocorrência natural em quase todos os continentes do globo, exceto Europa e Antártida (SCURLOCK *et al*., 2000; WILLIAMS *et al*., 1994).

Os bambus possuem um amplo espectro de usos, que vão desde a fabricação de artesanatos, construção civil à alimentação (BONILLA *et al*., 2010; SCURLOCK *et al.*, 2000), tornando-os um fator de desenvolvimento econômico amplamente explorado em vários países do continente asiático (COSTA *et al*., 2015). No continente americano, na Colômbia e no Equador são usados como material de construção popular não somente para construção civil, mas também na criação de agroindústrias, o que fomenta o desenvolvimento rural (GUILHERME *et al*., 2017). Já no Brasil foi instituída a Política Nacional de Incentivo ao Manejo Sustentado e ao Cultivo do Bambu (PNMCB Lei n.º 12.484/2011), cujo objetivo é fomentar o desenvolvimento da cultura do bambu por ações governamentais e empreendimentos privados (BRASIL, 2011).

Dentre as espécies de bambu mais utilizadas destacam-se o *Dendrocalamus asper* (Schult. & Schult.) Backer *ex* K. Heynek, espécie nativa da China, valorizada pelos seus brotos comestíveis que são vendidos como enlatados em todo o mundo, e *Bambusa tuldoides* Munro, também nativa da China e amplamente cultivada em regiões tropicais e subtropicais da América (ARYA *et al*., 2001; GUERREIRO e LIZARAZU, 2010).

Apesar da grande importância econômica dos bambus, poucos estudos foram realizados sobre a sua biologia reprodutiva devido a imprevisibilidade de sua floração, que varia de 10-120 anos de crescimento vegetativo. Em *B. tuldoides* o florescimento é esporádico, com intervalo de tempo de 23 anos de desenvolvimento vegetativo. Já *D. asper* floresce após 100 a 120 anos de vida das plantas (GUERREIRO e LIZARAZU, 2010; NADGIR *et al*., 1984; PILCHER, 2004; RAMANAYAKE e WEERAWARDENE, 2003;).

A maioria dos estudos sobre biologia reprodutiva dão ênfase a viabilidade, morfologia e desenvolvimento do grão de pólen (GUIMARÃES *et al*., 2017; HORN e CLARK, 1992; NAKAMURA *et al*., 2010; SOUZA *et al*., 2020b). Porém, estudos sobre o gametófito feminino são escassos na literatura, limitando-se a algumas espécies tais como, *Bambusa tulda*, *B. multiplex, B. sinospinosa, Thyrsostachys siamensis*, *Arundinaria simonii* f. *heterophylla*, *Dendrocalamus giganteus*, *Neosinocalamus affinis* (BHANWRA *et al*., 2001;

LIN *et al.*, 2018; LIN e DING, 2013; WANG *et al.*, 2015). Embora o desenvolvimento do gineceu em angiospermas compartilhe características comuns, as diferentes espécies mostram uma variedade de características de desenvolvimento (SILVA *et al.*, 2017). Tal conhecimento pode fornecer uma base para futuras pesquisas sobre conservação de sementes e desenvolvimento das espécies.

O objetivo deste estudo foi caracterizar o gineceu e o gametófito feminino em três estádios de desenvolvimento das flores nas espécies *Dendrocalamus asper* e *Bambusa tuldoides*, visando elucidar aspectos relacionados a não produção de cariopses, sendo este o primeiro trabalho que estuda aspectos do desenvolvimento reprodutivo feminino nestas espécies.

5.3 MATERIAL E MÉTODOS

5.3.1 Material vegetal

As inflorescências de *Bambusa tuldoides* foram coletadas de touceiras matrizes, localizadas no Parque Cidade das Abelhas-UFSC (27°32’20,04” S, 48°30’10,87” O), na cidade de Florianópolis, Santa Catarina, em janeiro/2017.

As inflorescências de *Dendrocalamus asper* foram coletadas na Fazenda dos Bambus, pertencente ao Instituto Jatobás, na cidade de Pardinho, São Paulo (23°06’37,33” S, 48°22’05,60” O), em junho/2017.

A inflorescências recém coletadas foram acondicionadas em sacos plásticos e colocadas em caixas de isopor com gelo durante o transporte até o Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal (LFDGV) do Departamento de Fitotecnia e Laboratório Central de Microscopia Eletrônica (LCME) da Universidade Federal de Santa Catarina (UFSC), campus Florianópolis, Santa Catarina.

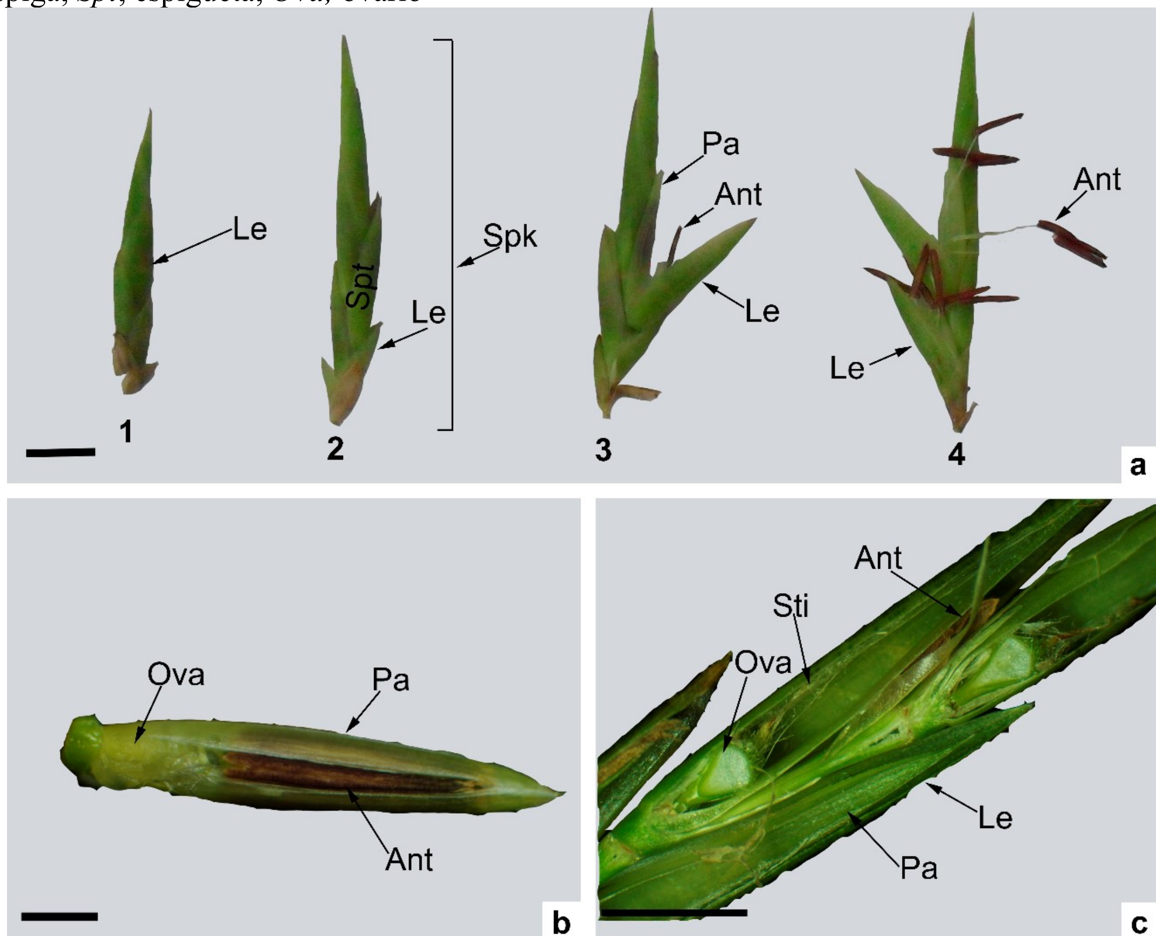
Exsicatas de *B. tuldoides* e *D. asper* foram depositadas no Herbário FLOR – UFSC, sob os códigos FLOR 63000 e FLOR 64899, respectivamente.

5.3.2 Análise sob microscopia de luz

Foram coletadas dez amostras do gineceu em ambas as espécies em três estádios de desenvolvimento da inflorescência. Em *Bambusa tuldoides* os estádios de desenvolvimento da

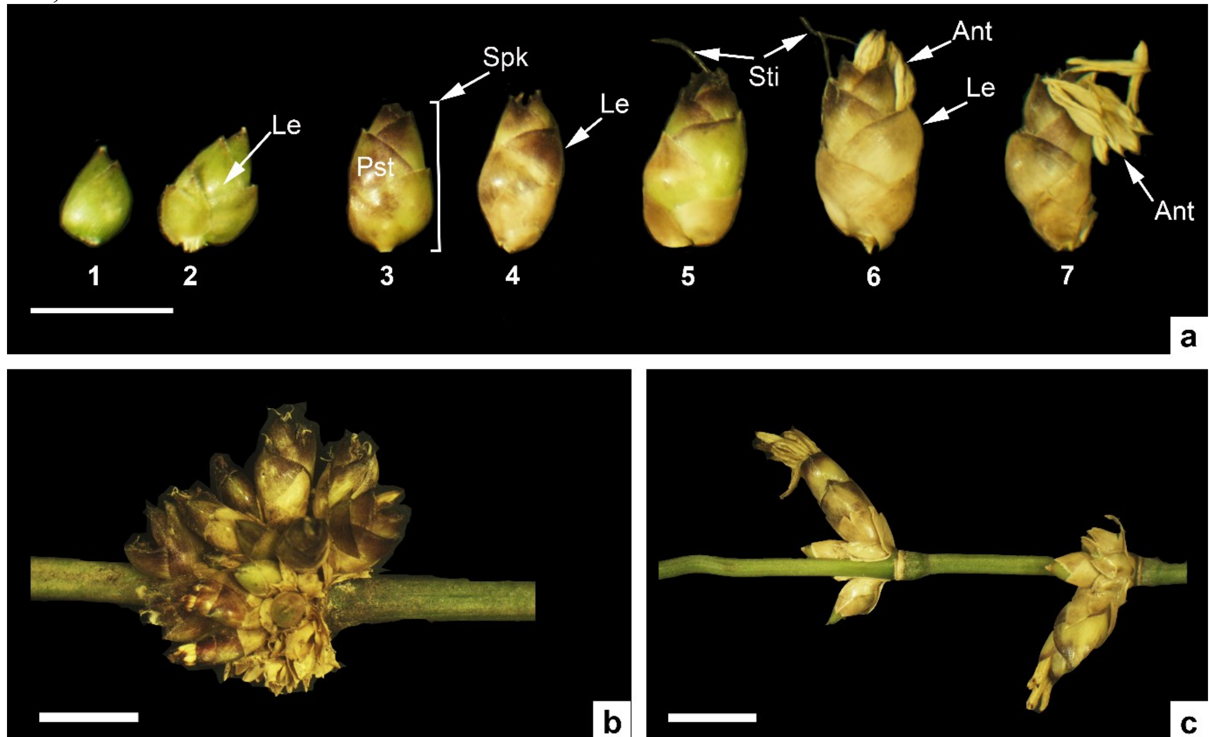
inflorescência foram: estádio um, estádio de pré-antese e antese (Figura 1) e em *Dendrocalamus asper* foram: estádio dois de desenvolvimento, na pré-antese e na antese (estádio seis) (Figura 2).

Figura 1. Estádios de desenvolvimento das espigas de *Bambusa tuldoides*. **a.** 1. Estádio inicial de desenvolvimento: gineceu e androceu protegidos pela lema; 2. Pré-antese: a lema começa a abrir; 3. Início da antese: abertura da pálea e lema e a exteriorização das anteras; 4. Antese: exteriorização das anteras e sua deiscência. **b.** Pré-antese: anteras e ovário protegidos pela pálea. **c.** Antese: ovário e estigma protegidos pela lema e pálea e antera senescente. Barra de escala = a, c - 0,5 cm; b – 1 cm . Legenda: *Le*, lema; *Pa*, palea; *Ant*, antera; *Spk*, espiga; *Spt*, espigueta; *Ova,* ovário



Fonte: adaptado SOUZA *et al.* (2020b)

Figura 2. Estádios de desenvolvimento das inflorescências de *Dendrocalamus asper*. **a.** 1 a 4. Estádios iniciais de desenvolvimento com as pseudoespiguetas (floret) fechadas e protegidas pela lema; 5. Pré-antese: exteriorização do estigma na parte superior da inflorescência; 6. Antese: exteriorização dos estigmas e das anteras nas psedoespiguetas superiores. 7. Anteras senescentes. **b – c**. Ramos com inflorescências em diferentes estádios de desenvolvimento. Barra de escala – 5 mm. Legenda: *Le*, lema; *Spk*, espiga; *Pst*, pseudoespigueta; *Sti*, estigma; *Ant*, antera.



Fonte: adaptado SOUZA *et al.* (2020a)

As amostras foram fixadas em solução de paraformaldeído (2,5%) em tampão fosfato (0,2M) pH 7,3, na proporção de 1:1, durante 24 h, à temperatura de 4ºC. Após a fixação, o material foi lavado três vezes em tampão fosfato, por 30 minutos (BOUZON, 2006), e desidratado em série etílica gradual (SANDERS *et al*., 1999). Posteriormente, as amostras foram pré-infiltradas em historesina LeicaTM com álcool etílico PA na proporção de 1:1 por 72h e infiltradas em historesina LeicaTM por 72h, de acordo com instruções adaptadas do fabricante. As amostras foram seccionadas em micrótomo rotativo (marca microTec, modelo CUT 4055) produzindo secções transversais e longitudinais de 5 µm de espessura. As secções foram coradas com azul de toluidina (O'BRIEN *et al*., 1965) e foi realizado teste histoquímico com Lugol para identificação de amido. As secções foram colocadas no reagente por 10 minutos e lavadas em água destilada (JOHANSEN, 1940). As observações e

registros foram realizados com auxílio de microscópio de florescência (marca Leica, modelo DM5500 B).

5.3.3 Análise sob microscopia eletrônica de varredura

Foram coletadas dez amostras do gineceu em ambas as espécies em três estádios de desenvolvimento da inflorescência. Em *Bambusa tuldoides* os estádios de desenvolvimento da inflorescência foram: estádio um, estádio de pré-antese e antese e em *Dendrocalamus asper* foram: estádio dois de desenvolvimento, na pré-antese e na antese (estádio seis).

As amostras foram fixadas em glutaraldeído a 2,5% e em tampão cacodilato de sódio 0,1 M (pH 7,2) adicionado 0,2 M de sacarose, por 24 horas. Posteriormente as amostras foram desidratadas com série etílica gradual (30%, 50%, 70% e 90% e 100%) 30 minutos em cada concentração, exceto a o álcool etílico 100%, com duas trocas de 30 minutos cada (adaptado de SCHMIDT *et al.*, 2012) e secas com ponto crítico de CO_2 EM-CPD-030 (Leica, Heidelberg, Alemanha). As amostras secas foram cobertas com 20 nm de ouro, em metalizador (Baltec, CED 030) (Schmidt et al. 2012). As amostras foram observadas e documentadas em Microscópio Eletrônico de Varredura (MEV) (marca Jeol, modelo JSM-6390LV).

5.4 RESULTADOS

O gineceu de *Bambusa tuldoides* consiste em um ovário obovado, estilete curto e dois estigmas plumosos. O ovário possui uma pilosidade em sua extremidade superior que se estende até o estilete (Figura 3a).

No estádio um do desenvolvimento floral, o óvulo de *B. tuldoides* maduro é ortotrópo e bitegumentado (Figura 3b). A micrópila é formada por ambos os tegumentos externo e interno. O tegumento externo possui duas ou três camadas, exceto na micrópila onde há mais de três camadas. O tegumento interno possui duas camadas (Figura 3b). Ainda no estádio um de desenvolvimento floral é possível observar o megasporócito (célula mãe do megásporo), distinguível pelo seu tamanho, citoplasma denso e núcleo grande, localizado próximo a região calazal (Figura 3c).

No estádio pré-antese o nucelo se degenera e o tegumento interno se transforma no endotélio (Figuras 3d-e). As células do endotélio possuem núcleos proeminentes (Figura 3e).

Observa-se neste estádio o início da formação do saco embrionário, com a presença de um grande vacúolo (Figura 3e).

Na antese é possível observar o saco embrionário contendo a célula central com os dois núcleos polares e um grande vacúolo (Figura 3f).

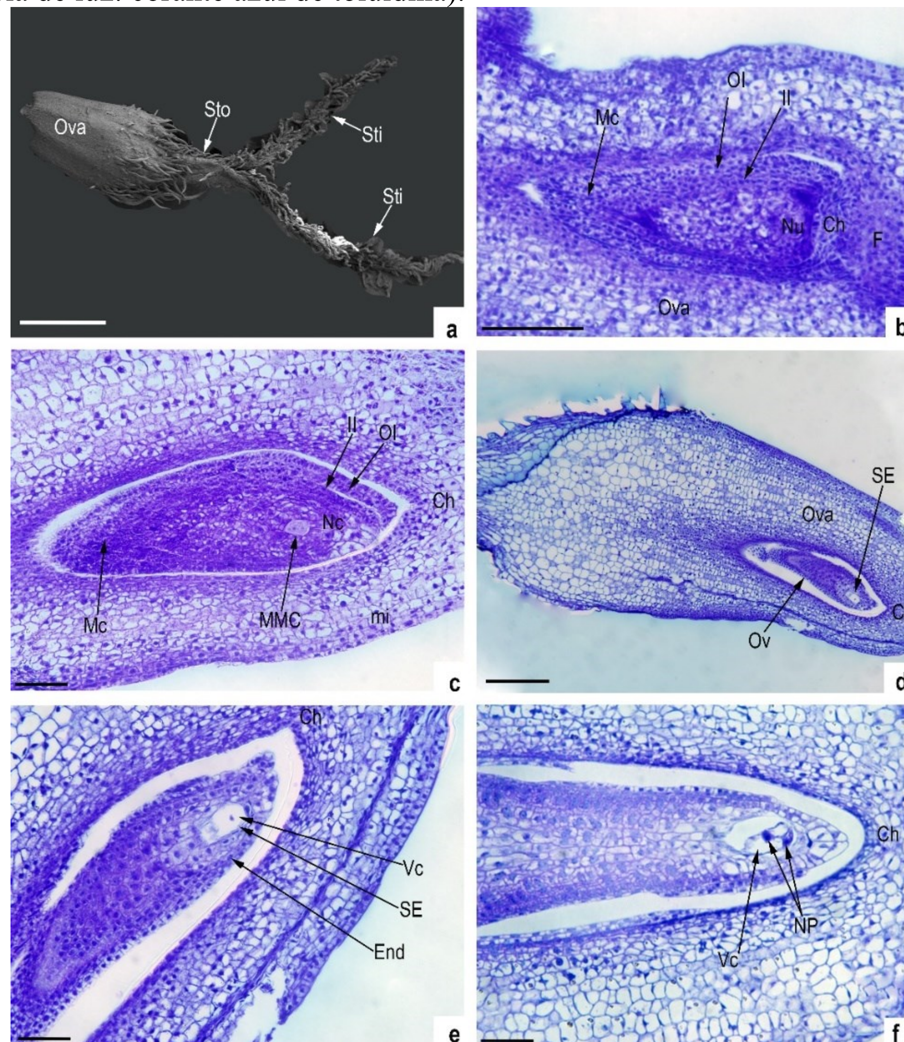
O gineceu de *Dendrocalamus asper* consiste em um ovário ovado, um estilo longo e um estigma, sendo todas as estruturas plumosas (Figura 4a).

No estádio dois do desenvolvimento floral o óvulo de *D. asper* maduro é anátropo e bitegumentado (Figura 4b). O tegumento externo e interno possui duas camadas (Figura 4c). Ainda no estádio dois é possível observar o início da formação do saco embrionário que apresenta um dos núcleos formados após a primeira mitose do megásporo (Figura 4c).

No estádio de pré-antese é possível observar a micrópila formada por ambos os tegumentos externos e internos e o saco embrionário maior do que no estádio dois (Figura 5a).

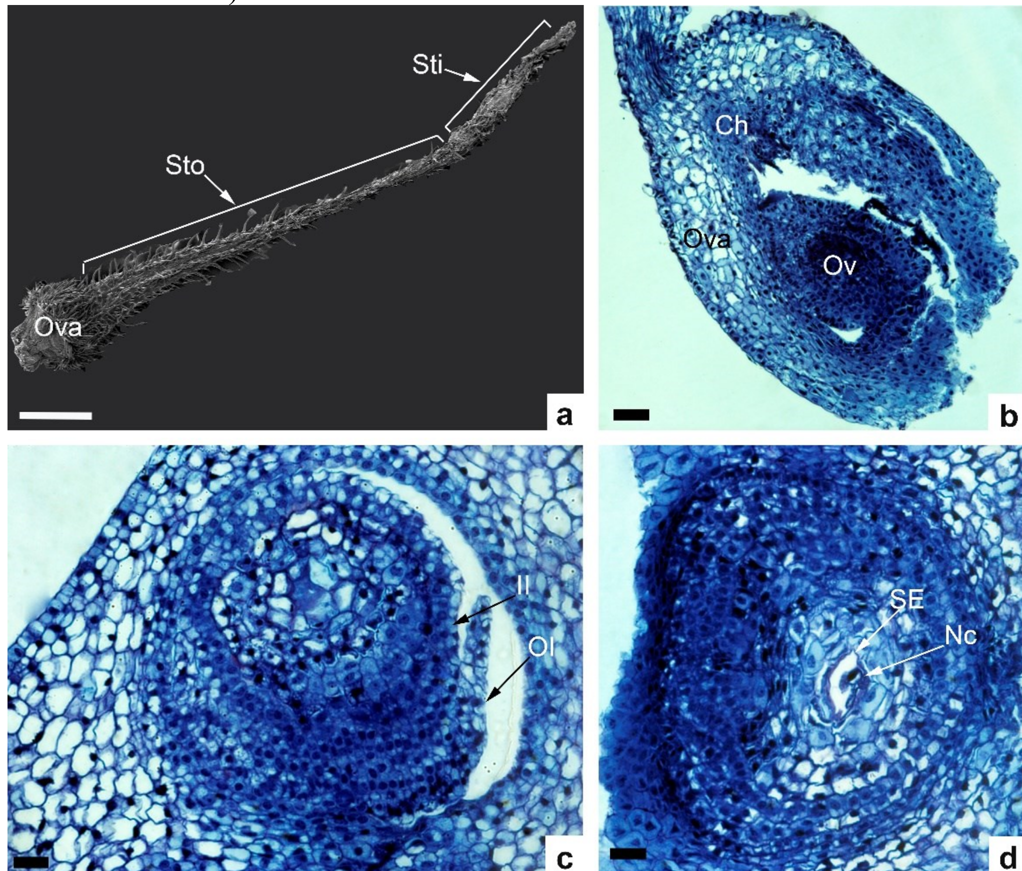
Na antese observam-se os resquícios das células do tegumento externo e o embrião colapsado envolto por uma camada de células irregulares do endotélio e do endosperma (Figura 5b). O acúmulo de amido não foi observado em nenhuma dessas estruturas (Figura 5c). Em algumas amostras observou-se o início do desenvolvimento da cariopse com o acúmulo de amido, porém essa estrutura também colapsou (Figura 5d).

Figura 3. Eletromicrografias de varredura e microscopia de luz do gineceu *de Bambusa tuldoides* em três estádios de desenvolvimento das flores. **a.** Antese: O gineceu composto por um ovário obovado, um estilete curto e dois estigmas plumosos. **b.** Estádio 1: Ovário mostrando as regiões do funículo e calaza e o óvulo com os tegumentos interno e externo, nucelo e região da micrópila. **c.** Estádio 1: Ovário mostrando a região da calaza, tegumentos interno e externo, nucelo, região da micrópila e a célula mãe do megásporo. **d.** Pré-antese: Visão geral do ovário mostrando a região da calaza, óvulo e o início da formação do saco embrionário. **e.** Pré-antese: Óvulo mostrando a região da calaza e o início da formação do saco embrionário, com a presença de um grande vacúolo e do endotélio. **f.** Antese: Óvulo mostrando a região da calaza e o saco embrionário contendo a célula central com dois núcleos polares e um grande vacúolo. Barra de escala: **a** - 500 µm; **b** - 100 µm; **c, e, f** - 50 µm; **d** - 200 µm. Legenda: *Ova*, ovário; *Ov*, óvulo; *Sto*, estilete; *Sti*, estigma; *F*, funículo; *Ch*, calaza; *Nu*, nucelo; *Mc*, micrópila; *II*, tegumento interno; *OI*, tegumento externo; *MMC*, célula mãe do megásporo; *SE*, saco embrionário; *End*, endotélio; *Vc*, vacúolo; *NP*, núcleo polar. (Microscopia de luz: corante azul de toluidina).



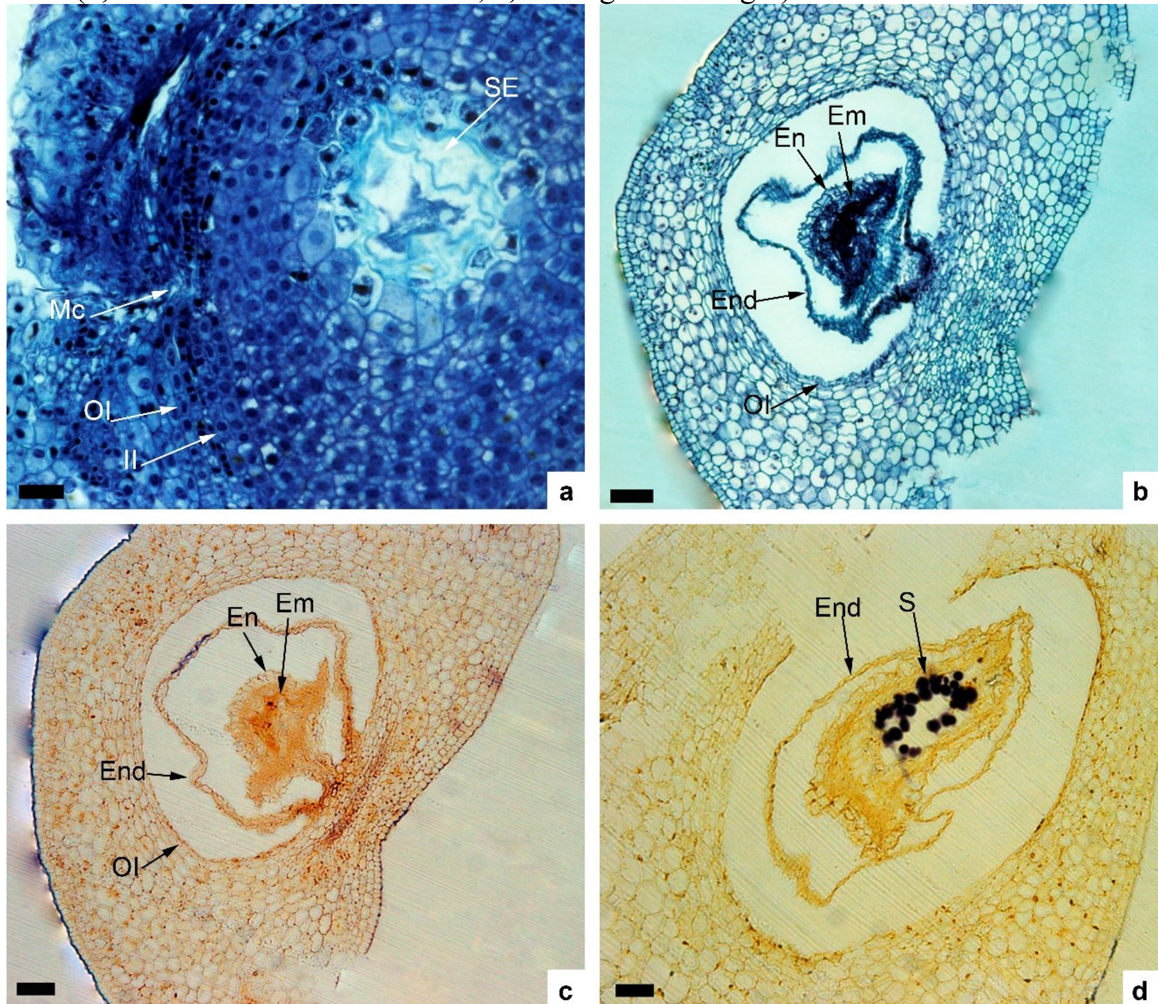
Fonte: Imagens do autor (2020)

Figura 4. Eletromicrografia de varredura e microscopia de luz do gineceu de *Dendrocalamus asper* em diferentes estádios de desenvolvimento das flores. **b.** Secção longitudinal. **c, d.** Secção transversal. **a.** Antese: O gineceu composto por um ovário ovado, um estilo longo e um estigma, ambos plumosos. **b.** Estádio 2: Ovário mostrando a região calaza e o óvulo. **c.** Estádio 2: Óvulo mostrando os tegumentos interno e externo. **d.** Estádio 2: Óvulo mostrando o início da formação do saco embrionário e um dos núcleos formados após a primeira mitose do megásporo. Barra de escala: **a** - 500 µm; **b, c, d** - 50 µm. Legenda: *Ova*, ovário; *Ov*, óvulo; *Sto*, estilete; *Sti*, estigma; *Ch*, calaza; *II*, tegumento interno; *OI*, tegumento externo; *SE*, saco embrionário; *Nc*, núcleo formado após a primeira mitose do megásporo. (Microscopia de luz: corante azul de toluidina).



Fonte: Imagens do autor (2020)

Figura 5. Microscopia de luz do gineceu de *Dendrocalamus asper* em diferentes estádios de desenvolvimento das flores. **a.** Pré-antese: Óvulo mostrado os tegumentos internos e externos, a micrópila e o saco embrionário. **b.** Antese: Ovário mostrando o embrião colapsado, os resquícios de células do tegumento externo, o endotélio e o endosperma. **c.** Ovário mostrando o embrião colapsado, os resquícios de células do tegumento externo, o endotélio, o endosperma e a ausência de amido em nestas estruturas. **d.** Cariopse colapsada mostrando as células do endotélio e o acúmulo de amido. Barra de escala: 50 µm. *SE*, saco embrionário; *II*, tegumento interno; *OI*, tegumento externo; *End*, endotélio; *En*, endosperma; *Em*, embrião; *S*, amido. (**a, b** - corante azul de toluidina; **c, d**- reagente de lugol).



Fonte: Imagens do autor (2020)

5.5 DISCUSSÃO

5.5.1 Caracterização do óvulo e ovário

A anatomia e morfologia do gineceu em diferentes espécies de plantas são divergentes, quanto ao número de estigmas, curvatura óvulo, número de tegumentos, nucelo, tipo de saco embrionário, dentre outros (SILVA *et al*., 2017).

Nos bambus o estigma é variável entre gêneros ou entre espécies dentro de um gênero. Na maioria das espécies de bambus estudadas é plumoso e varia em número de um, três ou quatro, diferindo, portanto, do encontrado para *B. tuldoides* que possui dois estigmas (Figura 3a) (BHANWRA *et al*., 2001; LIN *et al*., 2018; LIN e DING, 2013; RAMANAYAKE e WEERAWARDENE, 2003). Por outro lado, *Neosinocalamus affinis* e *Thyrsostachys siamensis* possuem um estigma, assim como *D. asper* (Figura 4a) (BHANWRA *et al*., 2001; WANG *et al*., 2015).

O formato do ovário nas flores de bambus varia de obovado, ovado, alongado a elíptico corroborando ao encontrado neste estudo (Figuras 3a e 4a) (BHANWRA *et al*., 2001; BHATTACHARYA *et al*., 2006; LIN e DING, 2013; RAMANAYAKE e WEERAWARDENE, 2003).

A curvatura do óvulo varia de ortotrópico a anátropo. *Thyrsostachys siamensis* possui óvulo ortotrópico assim como *B. tuldoides* e *Bambusa multiplex* e *Arundinaria simonii f. heterophylla* possui óvulo anátropo assim como *D. asper* (Figura 3b) (BHANWRA *et al*., 2001; LIN *et al*., 2018; LIN e DING, 2013).

Com relação ao número de tegumentos, os óvulos geralmente têm um (unitegumentado) ou dois (bitegumentado) tegumentos (LATTAR *et al*., 2016). Estudos com *Bambusa tulda*, *B. multiplex*, *Thyrsostachys siamensis*, *Dendrocalamus hamiltonii*, *Arundinaria simonii f. heterophylla*, *Neosinocalamus affinis* mostraram que essas espécies são bitegumentadas assim com *B. tuldoides* e *D. asper* (Figuras 3b e 4c) (BHANWRA *et al*., 2001; GOPAL e RAM, 1987; LIN *et al*., 2018; LIN e DING, 2013; WANG et al., 2015).

5.5.2 Saco embrionário

Os diferentes estádios de desenvolvimento floral de *Bambusa tuldoides* e *Dendrocalamus asper* permitiu o registro de algumas etapas da formação do saco embrionário (megagametogênese).

A reprodução sexual é um processo importante no ciclo de vida das plantas, pois é essencial para a produção de sementes e gerar uma nova população de descendentes (ZHANG *et al.*, 2015). Portanto, o desenvolvimento reprodutivo feminino bem-sucedido deve passar por vários eventos, começando com a especificação do megasporócito, que subsequentemente produz um megásporo funcional (megasporogênese) (REISER e FISCHER, 1993). Em *B. tuldoides* a célula mãe do megásporo está presente no estádio um de desenvolvimento floral (Figura 3c).

Posteriormente inicia-se a formação do saco embrionário (megagametogênese) (REISER e FISCHER, 1993). Em *B. tuldoides* esta etapa inicia-se no estádio de pré-antese e em *D. asper* já no estádio dois de desenvolvimento floral com a primeira mitose do megásporo (Figuras 3d-e e 4d).

O padrão de desenvolvimento do saco embrionário pode ser classificado em polygonum (monospórico), bispórico e tetraspórico. No tipo polygonum, após a primeira mitose do megásporo, os dois núcleos migram para polos opostos e cada um dos dois núcleos se divide mais duas vezes, resultando em oito células (REISER e FISCHER, 1993). Em *B. tuldoides* parte desse processo de celularização é visualizado no estádio de pré-antese com a formação dos dois núcleos polares (Figura 3f).

O saco embrionário do tipo polygonum tem sido relatado em espécies do mesmo gênero de *B. tuldoides* e *D. asper* (GOPAL e RAM, 1987). Porém, neste estudo não foi possível determinar para as duas espécies o padrão de desenvolvimento do saco embrionário.

O desenvolvimento completo do saco embrionário em *B. tuldoides* se dá na antese, pois é quando se observa a formação dos núcleos polares (Figura 3f). Já em *D. asper* ocorre na pré-antese, pois na antese observou-se a formação do embrião e o início do desenvolvimento da cariopse (Figuras 4b-d).

5.5.3 Formação da cariopse

Não foi observada a produção de cariopses em *Bambusa tuldoides* e *Dendrocalamus asper* neste estudo.

A baixa produção ou mesmo a não produção de sementes/cariopses tem sido relatada em diversos estudos e atribuída principalmente à inviabilidade do grão de pólen, à autoincompatibilidade fisiológica (SI) e a depressão por endogamia de ação precoce (ARAUJO *et al.*, 2007; SAGE *et al.*, 1999; SOUZA *et al.*, 2020b).

Em estudo realizado por Souza et al. (2020b) sobre o desenvolvimento grão de pólen nessa população e floração, mostrou que a não produção de cariopses em *B. tuldoides* se deve à inviabilidade dos grãos de pólen; já em *D. asper* o fato não está relacionado à viabilidade do grão de pólen, já que esses apresentam um desenvolvimento normal e são viáveis quando dispersos, corforme estudo também realizado nessa mesma floração.

Na autoincompatibilidade fisiológica, um sistema de auto reconhecimento baseado na genética reduz a frequência de autofecundação bem-sucedida e aumenta a heterose. Esse mecanismo é reconhecido principalmente como uma barreira pré-zigótica que pode envolver uma falha do pólen ao germinar no estigma e/ou prejudicar o crescimento posterior do tubo polínico (DE NETTANCOURT, 1997). Porém, esse não é o caso de *D. asper*, pois o saco embrionário é fecundando dando origem a um embrião colapsado (Figuras 5b-c).

Já a depressão por endogamia de ação precoce provoca aborto de descendência homozigótica durante o desenvolvimento do embrião devido à presença de alelos recessivos deletérios (ARAUJO *et al.*, 2007; HUSBAND e SCHENSKE, 1996). A depressão por endogamia de ação precoce tem sido relatada como motivo da pouca produção de sementes em *Brachiaria brizantha* (ARAUJO *et al.*, 2007). Esse também parece ser o motivo da não produção de cariopses em *D. asper*, uma vez que essa espécie teve uma floração intensa em todas as touceiras em 2016, mas em 2017, quando o material foi coletado, a floração foi insipiente e nem todas as touceiras floresceram. Como *D. asper* apresenta um longo período de crescimento vegetativo, essa população pode ter se multiplicado clonalmente por meio de um complexo sistema de rizomas subterrâneos e estes indivíduos aparentados podem ter trocado material genético ente si (GUILHERME *et al.*, 2017; POHL, 1991).

5.6 CONCLUSÕES

O gineceu de *B. tuldoides* consiste em um ovário obovado, estilete curto e dois estigmas plumosos. O óvulo é ortotrópo e bitegumentado. A formação do saco embrionário inicia-se na pré-antese e finaliza o desenvolvimento na antese.

O gineceu de *Dendrocalamus asper* consiste em um ovário ovado, um estilete longo e um estigma plumoso. O óvulo maduro é anátropo e bitegumentado. A formação do saco embrionário inicia-se no estádio dois de desenvolvimento floral e finaliza o desenvolvimento na pré-antese.

O desenvolvimento do saco embrionário em *B. tuldoides* não pode ser explicada pelo não explica a não produção de cariopses. Em *D. asper*, a depressão por endogamia de ação precoce parece ser o motivo para a não produção de cariopses.

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7 CONCLUSÃO FINAL

Este trabalho, o primeiro realizado com *D. asper* e *B. tuldoides,* permitiu a caracterização morfológica e o desenvolvimento do androceu, grãos de pólen e gineceu nessas espécies.

D. asper e *B. tuldoides*, florescem após um longo período de crescimento vegetativo, o que torna dificícil estudos sobre a sua biologia reprodutiva. Os estudos sobre a biologia reprotutiva realizados neste trabalho serão importantes para futuros trabalhos sobre melhoramento genético e propagação dessas espécies.

Com os resultados obtidos, foi possível concluir que não ocorre a formação de cariopses em *B. tuldoides* devido à inviabilidade do grão de pólen. E para *D. asper* supõe-se que a causa seja a depressão por endogamia de ação precoce. Portanto as espécies tem como rota principal de reprodução a propagação vegetativa, criando uma população geneticamente homogênea.

Sugere-se o estudo sobre a diversidade genética dessas espécies na população estudada ou em outras populações. Esses estudos seriam importantes para ajudar a determinar as causas da inviabilidade do grão de pólen em *B. tuldoides* e se endogamia de ação precoce é realmente a causa da não produção de cariopses em *D. asper*. Ademais, corfirmaria a importância da variabilidade genética na implantação de plantios dessas espécies quando o objetivo for a produção de cariopses e consequentemente a manutenção da diversidade genética.