DAYANNE MEDRADE SILVA

FLORAL CHARACTERIZATION, POLLEN GRAIN DESCRIPTION, VIABILITY, GERMINATION AND PRODUCTIVE PERFORMANCE OF *Castanea crenata* SIEBOLD & ZUCC (FAGACEAE) IN TROPICAL REGIONS

LAVRAS-MG

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Botânica Aplicada, área de concentração em Botânica Aplicada, para a obtenção do título de Doutor.

Prof. Dr. Rafael Pio DAG/UFLA

Orientador

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2020

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Silva, Dayanne Medrade.
Floralcharacterization, pollen grain description, viability, germination andproductive performance of *Castanea crenata*Siebold & Zucc (Fagaceae) in tropical regions / Dayanne Medrade Silva. - 2020.
79 p.

Orientador(a): Rafael Pio.
Coorientador(a): Vânia Helena Techio.
Tese (doutorado) - Universidade Federal de Lavras, 2020.
Bibliografia.

1. Viabilidade Polínica. 2. Receptividade Estigmática. 3. Produção. I. Pio, Rafael. II. Techio, Vânia Helena. III. Título.

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APROVADA em 28 de maio de 2020.

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Orientador

LAVRAS - MG
2020

Aos meus pais Glória e Devenir, por todo o amor. Aos meus pais adotivos Maria e Rufino, por toda a dedicação ao longo desses 28 anos. À minha madrinha Luciana, por sempre acreditar em mim e me incentivar. À minha avó Maria Francisca (in memoriam), por todo amor, carinho e os ensinamentos.

Dedico

AGRADECIMENTOS

Em primeiro lugar agradeço à Deus, por me capacitar e fortalecer para chegar até aqui.

À Universidade Federal de Lavras, especialmente ao Programa de Botânica Aplicada, pela oportunidade. Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa de doutorado. Às agências de fomento FAPEMIG e CAPES pelo auxílio no desenvolvimento do projeto.

Aos coordenadores do curso de Botânica Aplicada, Dr. Evaristo e Dra. Marinês, por procurá-los diversas vezes e sempre ser atendida.

À Dra. Silvana e ao CDRS-SP pela disponibilidade em coletar o material.

Ao Professor Dr. Rafael Pio e a Professora Dra. Vânia por me orientarem e por muitas vezes me aconselharem nesse doutorado, que não foi fácil.

À todos os colegas de trabalho do Pomar 1000 Grau, em especial Dani, Tatu, Paula, que muito me ajudaram. Ao pessoal do Laboratório de Citogenética, onde passei muito tempo.

Aos técnicos dos laboratórios onde desenvolvi minha pesquisa, Felipe Fogaroli, Evaldo Tadeu, Aline Norberto e Elisa de Melo.

À equipe da Docência Voluntária do GBI 175, Professor Dr. Welison Pereira, Professora Dra. Giovana Torres, a Técnica Iara Reinaldo, foi essencial em minha vida acadêmica essa experiência e aos meus queridos alunos que me fizeram dar o melhor de mim.

À Carol Zambon e ao seu esposo Luiz Fernando, que muito me auxiliaram no início da pesquisa, não só me ensinando técnicas, como também me hospedando para que a pesquisa acontecesse.

Às minhas amigas de Goiás Gleice, Gisely, Julie e Stefânia, por sempre estarem me incentivando e mandando boas vibrações.

À minha amiga Naty, por sempre se lembrar de mim e tirar um tempinho para ver como estou.

Aos companheiros de casa ao longo desses anos, Jeff, Lud e Palomita, conviver com vocês foi muiiiiito bom.

Ao meu amigo mexicano Alejandro, gratidão por todo carinho, apoio e tudo o mais que veio com sua amizade.

À Gra, Angel, Tatá e Ingrid por todo carinho, apoio e rolês na represa.

Aos meus queridos amigos Ray, Bea, Camila e Debs, as risadas no Porão ficarão sempre em minha memória.

Ao clubinho dos rolês e conversas produtivas, Alex, Caio, Átila, Othon, Ana Luísa, Lari, Ana Gabriela, Nati da Cunha, gratidão pelos momentos de descontração.

Aos amigos Will e Iago, pelas noites de "Langanha", vocês estarão sempre em meu coração.

Ao Kevinho, por sempre me chamar para tomar aquele açaízinho e me proporcionar momentos de distração.

Às amigas Gabis e Mara, por todo o apoio frente as minhas diversas crises de ansiedade e depressão, sem vocês eu não teria conseguido.

Ao Pablo, você é um amigo incrível e sempre me incentivou nesses dez anos de amizade. Amo você baby.

À minha amiga Raquel, muito obrigada por tudo, você se tornou uma irmã nesses anos, sem você provavelmente eu não estaria terminando tudo isso, pois além de sempre corrigir meus trabalhos, ainda tinha tempo para me acompanhar em uns copinhos.

Aos amigos que ganhei na Bélgica Ricardo e Germana, conhece-los foi um enorme presente.

À minha psicóloga Renata Rodarte, que me salvou na pior crise que tive na reta final do curso.

Aos colegas da Botânica, por todos os momentos de distração, em especial Flávio Zagotta, Elias Roma, Kaline Fernandes, Lucas Muñoz, por todos os nossos rolês, fossem para comer ou tomar algo.

À Lindinha, pelos bons momentos que passamos juntas e por termos recuperado nossa amizade. Ahhh e por traduzir o abstract.

À Juara e ao Alex, por sempre me receberem em SJDR, ao Francisco e a Carla, por me receberem em STL, esses passeios salvaram minha vida.

Ao Avenida Beer e Postinho, pelos momentos de alegria e também aqueles choppinhos.

Aos meus pais e irmã de Lavras, Sr. Israel, Dona do Carmo e Isamara, obrigada por me adotarem e sempre me fazerem sentir em casa.

À minha família de Goiânia, em especial meus pais adotivos Maria e Rufino, minha mãe Glória, minha madrinha Luciana, gratidão pela paciência e também por sempre acreditarem em mim.

Aos meus primos Lilian, Juliana, Gabriel e Filé e aos meus irmãos Johnathan, Diogo, Aline e Julie, por me apoiarem. Ao meu sobrinho Nicolas por me mostrar o maior sentimento do mundo, titia te ama muito.

Ao meu pai Dedé, minha madrasta Mônica e meu irmãozinho Rafael, pela viagem inesquecível, que ajudou muito a manter a saúde mental ao final do doutorado.

Ao Elvis, meu gatinho, que sempre me apoiou durante as noites sem dormir.

À cidade de Lavras, por me acolher ao longo desses cinco anos.

À todos que contribuíram de alguma forma para que eu chegasse aqui.

GRATIDÃO!!!

“A ciência nunca resolve um problema sem criar pelo menos outros dez.”

George Bernard Sha

RESUMO

O gênero *Castanea* Mill., compreende dez espécies, apresenta árvores monoicas com amentilhos estaminados e andróginos e de portes variados. Com origem na China, as castanheiras estão distribuídas no mundo, sobretudo nas regiões da Ásia e Europa. A castanheira possui múltiplas finalidades, que vão desde a utilização na gastronomia a fabricação de utensílios, além do fruto não apresentar glúten e ter proteína de boa qualidade. A domesticação do gênero ainda está em processo, uma vez que a produção mundial, ainda conta com castanheiros naturais e a produção em larga escala, requer plantações homogêneas. Estudos como biologia floral, fertilidade do grão de pólen e de produtividade, podem oferecer subsídio para outros programas e assim contribuir para que se obtenha essas plantas homogêneas, pois com demanda por alimentos é necessário aumentar a produção e ainda investir em meios mais sustentáveis. Os estudos do gênero, são em sua maioria, voltados para a espécie *Castanea sativa* Mill., por ser mais conhecida e produzida, deixando a desejar os estudos para as outras espécies. Com isso, o objetivo desse trabalho foi apresentar a caracterização floral da espécie *Castanea crenata* Siebold e Zucc., desenvolver protocolo de germinação para as cultivares do gênero, verificar a viabilidade polínica através de testes colorimétricos, classificar o grão de pólen e apontar as cultivares mais indicadas para produção em regiões tropicais. Para a caracterização floral, desenvolvimento do protocolo de germinação, avaliação da viabilidade polínica e classificação do grão de pólen, foram utilizadas nove cultivares de *C. crenata* e dois híbridos (*C. crenata* x *Castanea sp*). O material foi coletado na Coordenadoria de Desenvolvimento Rural Sustentável -CDRS em São Bento do Sapucaí – São Paulo e o experimento foi realizado no Laboratório de Citogenética da Universidade Federal de Lavras-MG. As flores foram caracterizadas através da observação das partes florais e auxílio da literatura e a germinação foi obtida através de testes de meios de cultura. A viabilidade do grão de pólen foi avaliada utilizando-se dois corantes, o Carmim Propiônico e Corante Alexander. Os grãos de pólen foram classificados em viáveis e inviáveis. Para descrição do grão de pólen, foi aplicada a técnica de acetólise e os grãos de pólen classificados conforme Erdtman (1943). Para avaliar a produtividade, foram utilizadas quatro cultivares de *C. crenata* e conduzido na Unidade Experimental do Pomar da Universidade Federal de Lavras- MG, as coletas foram realizadas ao longo de quatro safras. As inflorescências da espécie *C. crenata*, são do tipo amento estaminados ou andróginos, onde as flores femininas se encontram localizadas na base das masculinas. O melhor meio de cultura em que se proporcionou a germinação do tubo polínico, foi contendo 6 g L^{-1} de ágar, 46,5 g L^{-1} de sacarose e 460,23 mg L^{-1} de ácido bórico na ausência de nitrato de cálcio, pH 5,25 onde a germinação chegou a 30% e as cultivares indicadas para serem doadoras de grãos de pólen foram ‘Morioase” e ‘Tamatsukuri’. Os grãos de pólen, foram classificados como prolato, isopolar, tricolpado, com colpo constritivo longo, heteroaperturado, zonocolpato e com exina lisa e a viabilidade polínica novamente indicou a cultivar ‘Morioase” como tendo a maior taxa de grãos viáveis. Quanto a produtividade, os estudos indicaram as cultivares ‘Taishowase’ e ‘Okuni’, como as mais promissoras para cultivo em regiões tropicais.

Palavras-chave: Viabilidade polínica. Receptividade estigmática. Produção.

ABSTRACT

The genus *Castanea* Mill., is comprised of ten species; monoecious trees with staminate and androgynous catkins of varying sizes. Originating from China, chestnuts are distributed widely around Asia, Europe and all around the world. The chestnut tree has multiple purposes, ranging from use in gastronomy to the manufacture of utensils since the fruit does not have gluten and has good quality protein. The domestication of the genus is still in process since world production still counts on natural chestnut trees and large-scale production requires homogeneous plantations. Studies such as floral biology, pollen grain fertility and productivity can provide support for other programs and thus contribute to obtaining these homogeneous plants. With the demand in the food market, it is necessary to increase production and invest in more sustainable means. Most studies of the genus are focused on the species *Castanea sativa* Mill., as it is more commonly known and produced, leaving studies on other species mostly disregarded. The objective of this work was to present the floral characterization of the species *Castanea crenata* Siebold and Zucc., develop a germination protocol for the cultivars of the genus, verify the pollen viability through colorimetric tests, classify the pollen grain and point out the most suitable cultivars for production in tropical regions. For floral characterization, development of the germination protocol, evaluation of pollen viability and pollen grain classification, nine cultivars of *C. crenata* and two hybrids (*C. crenata* x *Castanea* sp) were used. The material was collected at Chestnuts from the Sustainable Rural Development Coordination - CDRS, in São Bento do Sapucaí-SP and the fixed material was prepared at the Cytogenetics Laboratory of the Federal University of Lavras-MG. The flowers were characterized by observation of the floral parts. The use of literature and germination was obtained through tests of culture media. Pollen viability was assessed using two dyes, Carmine Propionic and Alexander Dye. Pollen grains were classified as viable and non-viable. To describe the pollen grain, the acetolysis technique was applied and the grains were classified according to Erdtman (1943). In the productivity experiment, four cultivars of *C. crenata* were used and conducted at the Experimental Unit of the Orchard of the Federal University of Lavras-MG, the collections were carried out over four harvests. The inflorescences of the species *C. crenata*, are of the type staminate or androgynous, where the female flowers are located at the base of the male flowers. The best culture medium in which pollen tube germination was provided was 6 g L^{-1} of agar, 46.5 g L^{-1} of sucrose and 460.23 mg L^{-1} -1 of boric acid in the absence of nitrate. Calcium, pH 5.25 where germination reached 30% and the cultivars indicated to be donors of pollen grains were ‘Morioase’ and ‘Tamatsukuri’. The pollen grains were classified as prolate, isopolar, tricolporate, with long constricting colostrum, heteroapertured, zonocolporate and with exine smooth. Pollen viability indicated that the cultivar ‘Morioase” has the highest rate of viable grains. As for productivity, the studies indicated that the cultivars ‘Taishowase’ and ‘Okuni’ are the most promising for cultivation in tropical regions.

Keywords: Pollen viability. Stigmatic receptivity. Production.

SUMÁRIO

PRIMEIRA PARTE

1INTRODUÇÃO

O gênero *Castanea* Miller pertence à família Fagaceae, e estudos apontam o Hemisfério Norte como centro de origem, as espécies do gênero são distribuídas principalmente na Ásia e Europa (BUENO; PIO, 2014; BRAGA; RODRIGUES; OLIVEIRA, 2015).

Informações quanto ao número de espécies, apresentam divergências, o "*The Plant List*", descreve nove espécies estabelecidas, já os autores Bueno e Pio (2014), citam a ocorrência de sete espécies, a controvérsia, pode ser devido as sinonímias presentes no gênero.

Apenas quatro espécies apresentam destaque na economia: a *Castanea crenata* Siebold & Zucc., conhecida como castanha-japonesa, a *Castanea dentata* (Marsh) Borkh ou castanha-americana, a *Castanea molíssima* Blume, também chamada de castanha-chinesa e a *Castanea sativa* Mill., que é mais conhecida e cultivada, popularmente nomeada de castanha-portuguesa (BUENO; PIO, 2014; PIO et al., 2014) .

A castanheira é muito versátil, utilizada na fabricação de móveis e alimentação, humana e animal. O consumo de castanhas, principalmente em épocas natalinas é uma tradição (BUENO; PIO, 2014). O fruto apresenta baixo teor de gordura, além de ser rico em proteínas, vitaminas e livre de glúten. Esse último, é um dos fatores para o aumento de seu consumo, principalmente por parte dos portadores de doença celíaca (BUENO; PIO, 2014; PIO et al., 2014; BRAGA; RODRIGUES; OLIVEIRA, 2015).

Em 2018, a produção mundial foi de 2.353.825 toneladas de castanhas (FAOSTAT, 2018). A Ásia produziu 2.112.252 toneladas de castanhas, a Europa foi responsável por 154.612 toneladas e as Américas produziram 86.719 toneladas de castanhas (FAOSTAT, 2018). Diversos países, como a China, Portugal, Turquia, Itália e Espanha, produzem castanhas e investem no melhoramento genético dessas espécies, sendo a produção exportada para as demais partes do mundo.

A castanha exibe enorme potencial para produção em diferentes condições climáticas, desde climas temperados a climas mais quentes, uma vez que possui boa adaptação, dependendo da origem genética (BUENO; PIO, 2014). Silva et al. (2019), constataram ao longo de quatro safras o potencial da castanheira em clima tropical em Minas Gerais, e as estimativas de produtividade estiveram entre 689,4 a 2.585,7 kg ha^{-1}.

Além dos estudos em campo, como os de produção e produtividade, existem diversos estudos e técnicas utilizando grãos de pólen, como os testes de viabilidade por coloração e germinação e outras técnicas mais refinadas como a microscopia eletrônica de varredura e acetólise que permitem a classificação da morfologia do grão de pólen. Esses estudos auxiliam na escolha de plantas candidatas ao melhoramento genético (TECHIO et al., 2006).

No caso da *Castanea crenata* Siebold & Zucc., não há relatos recentes e esses tipos de avaliações, além de contribuírem com a propagação e preservação da espécie, oferecem subsídio para estudos futuros de melhoramento genético e taxonomia do grupo.

Como a castanheira é amplamente cultivada em regiões de clima mais frio e no Brasil a exploração econômica é recente, torna-se necessário quantificar a adaptação agronômica e o potencial produtivo das cultivares, principalmente nas condições climáticas subtropicais e tropicais, devido ao crescente interesse do cultivo nos estados de São Paulo e Minas Gerais.

Em um plano superior, é necessário estabelecer as bases de um programa de melhoramento genético visando a seleção de cultivares que possam proporcionar incrementos produtivos em regiões de inverno ameno e tropicais.

Com isso, o objetivo deste trabalho, foi caracterizar as flores da *Castanea crenata*, desenvolver protocolo de germinação, caracterizar o grão de pólen, em função da morfologia e viabilidade polínica, além de apontar as cultivares mais promissoras da espécie, para o cultivo em regiões tropicais.

2 REFERENCIAL TEÓRICO

2.1 Aspectos botânicos do gênero *Castanea*

A castanheira, como é popularmente conhecido o gênero *Castanea* Mill., são frutíferas, com espécies diploides (2n = 2x = 24) e árvores monoicas com flores estaminadas e andróginas no mesmo indivíduo (VALDIVIESSO, 1999; ABREU, 2007).

As flores estaminadas são agrupadas em amentilhos unissexuais e as flores femininas são localizadas na base das flores masculinas (VALDIVIESSO, 1999). As árvores são de portes variados, apresentam diferenças ecológicas e morfológicas entre si, que permite a diferenciação das espécies, a partir das características das folhas e número de frutos (GOMES-LARANJO et al., 2007; BOUNOS, 2014).

O fruto da castanheira é chamado de castanha e é do tipo aquênio, com formato ovóide a subgloboso, pericarpo seco e fica contido em cúpula ou ouriço, proveniente das brácteas, que apresentam espinhos, as dimensões dos ouriços são variáveis, sendo os de menor porte os oriundos de castanheiras florestais e de maiores dimensões os de castanheiras comerciais (GOMES-LARANJO et al., 2007; BELTRAME, 2013).

A *Castanea crenata* pode atingir de 15 a 20 metros de altura, apresenta ramos finos e arredondados, de cor castanho avermelhado e brilhantes. As folhas são elípticas medindo até 16 cm de comprimento, com base arredondada e limbo com a margem denteada. As inflorescências são do tipo amento e medem entre 7 e 20 cm de comprimento, as flores femininas ficam na base e apresentam-se de 1 a 3. Os ouriços são revestidos por brácteas e armazenam de 1 a 3 castanhas (GOMES-LARANJO et al., 2007).

A *Castanea dentata* chega a alcançar 30 a 40 metros de altura, ramos glabros, levemente esbranquiçados. As folhas são oblongo-lanceoladas com aproximadamente 25 cm de comprimento, margem denteada, os amentilhos medem até 20 cm e as flores femininas se encontram isoladas ou em cimeiras. Os ouriços apresentam de 2 a 3 castanhas, a principal diferença da castanha-americana, é em relação a forma de dispersão, pois é polinizado por insetos (MEIRELES et al., 2005; GOMES-LARANJO et al., 2007).

A *Castanea molíssima* pode medir 20 metros de altura, os ramos glabros esbranquiçados, as folhas são oblongo-lanceoladas e elíptico-lanceolada, com cerca de 25 cm, margens serradas, amentilhos com até 15 cm e ouriços com 1 a 3 castanhas (GOMES-LARANJO et al., 2007).

A *Castanea sativa* chega a atingir 40 metros de altura, seus ramos são glabros cinzentos, as folhas oblongo-lanceoladas medindo de 10 a 25 cm, margem denteada, no entanto com dentes aristados, amentilhos masculinos com até 20 cm e flores femininas em cimeiras de 3. Os ouriços apresentam de 2 a 3 castanhas (GOMES-LARANJO et al., 2007; BELTRAME, 2013).

Por apresentar flores masculinas e andróginas, há uma controvérsia quanto a funcionalidade do grão de pólen do gênero Castanea. Beyhan e Serdar (2008 e 2009), afirmam que o grão de pólen de origem andrógina é menos funcional do que os de origem masculina. No entanto, Valdiviesso (1999), observou que as taxas de germinação dos grãos de pólen de flores andróginas, em algumas cultivares de *C. sativa*, foram superiores aos de flores masculinas, cerca de 58,3 e 12,7%, respectivamente, e em um estudo recente,

realizado por Silva et al. (2020), as taxas de germinação dos grãos de pólen em cultivares de *C. crenata*, atingiram 29,99% utilizando flores mistas, neste mesmo estudo as avaliações da viabilidade polínica a partir de testes colorimétricos foram superiores a 80%.

O período de floração da castanheira se divide em cultivares precoces, semi precoces e semi tardias, sendo que o estigma se encontra receptivo nesses períodos. A fecundação é cruzada, com isso, existe a necessidade de introdução de diferentes cultivares para polinização em caso de pomar comercial, e para que isso aconteça o período de floração entre elas precisa ser simultâneo (VALDIVIESSO, 1999).

De acordo com Giovanetti e Aronne (2011), a castanheira dispõe de dupla forma de dispersão, sendo por via anemófila e entomófila, em um estudo realizado por Sabugosa-Madeira et al (2008), foi observado que a quantidade de grãos de pólen, são menores em dias chuvosos, o que demonstra que em dias mais ensolarados, a polinização é favorecida.

A espécie *Castanea crenata* apresenta quantidade de grãos de pólen em suas anteras variando entre 8,91 e 58,09, logo um amentilho pode conter de 19,29 a 2.083 grãos de pólen, e isso pode ser explicado como uma adaptação da espécie e/ou cultivar (ZAMBON et al., 2014; SILVA et al., 2020).

2.2 Origem, importância econômica e aspectos produtivos do gênero *Castanea*

Indícios fósseis apontaram para a existência das castanhas desde o período Terciário no Hemisfério Norte, em depósitos de âmbar, da região do Báltico, foram encontradas partes florais bem conservadas de castanheiras, com datas da época do Eoceno Superior (STEWART; ROTHWELL, 1993; DANE et al., 2003).

Huang, Dane e Norton (1994) apontou a China como centro de origem do castanheiro e diversidade do gênero. A *Castanea molíssima* teria divergido por duas vias de migração, uma em direção à América do Norte, dando origem a *Castanea dentata*, e a segunda via, em direção a Europa, originando a *Castanea sativa* (JAYNES, 1975). Os autores Huang, Dane e Norton (1994) e Dane et al. (2003), sugerem que a *C. molíssima*, de fato seja a provável progenitora das demais espécies.

Na colonização da América, a cultura da castanheira apresentou relevância na economia, pois supria as necessidades alimentícias (BUENO; PIO, 2014), e o fornecimento de madeira de qualidade (VALDIVIESSO; COSTA, 2006). Segundo

Gomes-Laranjo et al. (2007), a castanheira também assumiu papel de suma importância para os habitantes de diversas regiões montanhosas no Continente Europeu.

Além disso, com o advento da tecnologia, a castanha passou a ser utilizada de formas mais elaboradas, como na produção de suflês, compotas, bolos e recentemente na produção de biocombustível e substrato para o cultivo de cogumelos, (GOMES-LARANJO et al., 2007; BRAGA; RODRIGUES; OLIVEIRA, 2015). A flor da castanha apresenta propriedades antimicrobianas e antitumorais, o que poderá ser explorado pela indústria farmacêutica (CAROCHO et al., 2014).

Na América do Sul, as castanhas são importadas basicamente de Portugal (98,5%) e uma pequena porcentagem da Espanha (1.5%) (BUENO; PIO, 2014). As castanheiras requerem clima ameno e umidade adequada para o crescimento e boa produtividade (MUZAFFAR et al., 2016). De acordo com Gomes-Laranjo et al. (2006), as castanheiras quando expostas as temperaturas elevadas reduzem em 60% a atividade fotossintética durante o dia, o que demonstra o potencial do cultivo em regiões tropicais. Em regiões com temperaturas mais elevadas, há redução da atividade antioxidante nas castanhas (DINIS et al., 2012), mas não há perdas em outras propriedades químicas, como açúcares e proteínas (PIO et al., 2014).

A produção de frutíferas de clima temperado em regiões tropicais é um grande desafio, devido às baixas condições de refrigeração deste clima, mas existem cultivares que possuem menor exigência térmica por isso se deve selecionar cultivares para cada macrorregião (PIO et al., 2019).

2.3 Estudos do Grão de Pólen

O grão de pólen é o microgametófito das plantas e são armazenados nas anteras, sua morfologia pode variar tanto de um gênero para outro, quanto entre espécies do mesmo gênero (JUDD, 2009).

A polinização é o evento em que ocorre a transferência de grãos de pólen, entre flores masculinas e femininas, sendo responsável pela formação de frutos e sementes, a dispersão do grão de pólen, pode ser através de insetos polinizadores, vento ou água (WOLOWSKI et al., 2019).

Quando se trata de produção de alimentos, a polinização é um processo fundamental para garantir colheitas significativas para o abastecimento de alimentos à população. Em escala global, estima-se que mais de 75% das espécies vegetais utilizadas

na agricultura sejam dependentes de animais para polinização e em regiões tropicais, 94% das plantas também são polinizadas por animais (ALVES et al., 2010; OLLERTON; WINFREE; TARRANT.; 2011).

Além da necessidade de uma polinização entomófila, a formação do fruto pode ser influenciada por vários outros fatores, estruturais, morfológicos e ambientais, como horário de liberação do pólen e viabilidade polínica, receptividade do estigma, fragilidade e sobrevivência do óvulo, compatibilidade genética, conteúdo de açúcar total de seu néctar, além de temperatura, umidade e luminosidade (ALVES et al., 2010).

Basicamente, quanto maior for à quantidade de pólens viáveis e compatíveis no estigma, maior a probabilidade de êxito na fertilização. Contudo, dependendo do tipo de pólen envolvido, o processo de fertilização pode ser afetado por fatores genéticos e fenotípicos, ocasionando má formação da semente e do fruto (LEITE; SOUZA, 2003).

Quando o processo de polinização e/ou a qualidade do pólen são deficientes ocorre problemas na fecundação do óvulo, acarretando em aborto ou má formação das sementes. Com isso, haverá um maior número de lóculos vazios dentro do pericarpo, consequentemente a má conformação e inviabilizando o fruto para a comercialização (SIMÃO, 1998).

Estudos do grão de pólen são de extrema importância, tanto do ponto de vista botânico, que inclui a conservação, discriminação e descrição das espécies, quanto do ponto de vista do melhoramento genético, uma vez que, oferece subsídio para o programa de espécies melhoradas (SILVA, 2017). Além de expressarem parte do genoma, os grãos de pólen carregam características, que permitem inferir sobre evolução, além de auxiliar nos estudos taxonômicos, palinológico e melhoramento genético (FERGUSON, 1985).

A viabilidade do pólen, está intimamente relacionada às condições climáticas, fatores como temperatura, sincronização no período de floração, nutrição da planta, entre outros, esses estudos podem ser *in vitro* e *in vivo* (SHIVANNA; RANGASWAMY, 1992).

Os estudos *in vivo*, envolve procedimentos como a polinização controlada e requer estudos prévios, a fim de verificar a receptividade dos estigmas e abertura das anteras para liberação dos grãos de pólen. Por esse motivo, uma alternativa relativamente mais rápida e menos onerosa são os ensaios *in vitro* (SHIVANNA; RANGASWAMY,1992; NOGUEIRA et al., 2015).

De acordo com Shivanna e Rangaswamy (1992), os estudos *in vitro*, como de germinação, permitem simular um ambiente natural, que seja favorável ao

desenvolvimento do tubo polínico, porém as condições também podem influenciar na germinação, sendo interferências geradas pela quantidade de água, pH e nutrientes fornecidos (FERREIRA et al., 2007, RAMOS et al., 2008).

O teste de germinação de grãos de pólen in vitro é imprescindível para programas de melhoramento genético de frutíferas, pois, através dessas análises preliminares torna-se possível verificar sua viabilidade, assim como realizar as primeiras inferências sobre problemas de esterilidade intrínsecos das cultivares em estudo (PIO et al., 2004). Os resultados da germinação do grão de pólen, através de meios de cultura, podem ser inferidos, como viável, quando o tamanho do tubo polínico é maior ou igual ao diâmetro do grão de pólen, e inviável, quando o tubo é menor ou inexistente (SHIVANNA; RANGASWAMY,1992).

A germinação *in vitro* emprega a utilização de um meio de cultura para determinar a aptidão da germinação dos grãos de pólen. Sendo que para uma alta taxa de germinação é necessário a compreensão de uma série de fatores como temperatura, pH, ajustes da composição do meio para a espécie trabalhada e período de emissão do tubo polínico.

A temperatura em que o pólen é exposto durante sua fase germinativa é diretamente relacionada com o desenvolvimento do tubo polínico. Chagas et al. (2010) trabalhando com desenvolvimento de tubos polínicos de porta enxerto de Pereiras observaram que até 28 °C houve favorecimento da germinação e que temperaturas mais elevadas acarretaram em diminuição dessa porcentagem. Silva et al. (1999) trabalhando com germinação de grão de pólen de maracujá, observaram que em temperaturas entre 22 e 24 °C houve diminuição da taxa de germinação do pólen e temperaturas entre 27 e 28 °C favoreceram seu aumento.

Outro fator importante para o meio de cultura é o pH, sendo que seu ajuste varia de acordo com a espécie em estudo, influenciando diretamente na disponibilidade de fitorreguladores e nutrientes, além de intervir diretamente na solidificação do meio.

Chagas et al. (2009), em seu estudo com diferentes cultivares de *Prunus persica* (L.), obtiveram como melhor resultado na germinação dos grãos de pólen em meio com pH 5,5. Os mesmos autores estudando germinação de grãos de pólen de *Pyrus betulaefolia* e *Pyrus calleryana*, concluíram que o ideal para a germinação do pólen dessas espécies é o pH variando entre 5,2 e 5,8, respectivamente (CHAGAS et al., 2010).

Com relação ao ajuste dos componentes básicos do meio sabe-se que vários compostos orgânicos e inorgânicos interferem na germinação in vitro, dos quais o ágar, a sacarose, o cálcio e o boro são os mais importantes (BOLAT; PIRLAK, 1999).

O ágar tem como função a solidificação do meio de cultura, possui como atributos a facilidade da incorporação dos nutrientes, além de proporcionar umidade relativa constante, fornecendo um ambiente onde os grãos de pólen possam se desenvolver. A concentração de ágar varia dependendo da espécie. Chagas et al. (2009), em seu estudo com *Prunus persica* (L.) obteve o melhor resultado de germinação (55,42%), com 10 g L^{-1} ágar. Os mesmos autores estudando germinação de grãos de pólen de porta enxertos de pereira, conseguiram as maiores taxas de germinação à medida que aumentaram a concentração de ágar no meio de cultura (CHAGAS et al., 2010).

A sacarose tem como finalidade o fornecimento de energia nos processos biossintéticos envolvidos no crescimento, diferenciação e morfogênese celular. Xie et al. (2004), estudando germinação de grãos de pólen de pêras asiáticas e Chagas et al. (2009), estudando *Prunus persica* (L.), constataram que as maiores porcentagens de germinação foram alcançadas com as maiores concentrações de sacarose adicionadas ao meio de cultura, enquanto Barbosa et al. (1991) em seu trabalho realizado com pessegueiros e nectarineiras subtropicais obtiveram os melhores resultados de germinação em meios com baixa concentração açúcar.

O nitrato de cálcio utilizado no meio de cultura tem por finalidade diminuir a sensibilidade dos grãos de pólen e do tubo polínico as alterações no meio básico, sua principal função é contribuir para a resistência e crescimento linear do tubo polínico com diminuição de sua permeabilidade (BHOJWANI; BHATNAGAR, 1974).

O boro é um micronutriente que vai desempenhar um importante papel na germinação e crescimento do tubo polínico. Possui a propriedade de interagir com as moléculas de sacarose presentes no meio de cultura, aumentando a eficiência de absorção desse composto pelo grão de pólen, favorecendo o crescimento do tubo polínico (DANTAS et al., 2005).

Brewbaker e Kwack (1963) trabalhando com germinação de pólen in vitro de 86 espécies distribuídas em 39 famílias de angiospermas constataram que o cálcio e o boro têm um papel fundamental no início da germinação do tubo polínico.

Chagas et al. (2009), em seu estudo com *Prunus persica* (L.) e Barbosa et al. (1991), em seu trabalho com pessegueiros e nectarineiras, constataram que o requerimento desses micronutrientes na germinação de grãos in vitro variam de acordo com a espécie e da cultivar. Além disso, existe a necessidade do estabelecimento correto desses compostos dentro do meio de cultura, pois, seu excesso ou deficiência podem

promover a não germinação ou rompimento dos grãos de pólen, levando a um falso resultado (RAMOS et al., 2008).

Os testes colorimétricos são utilizados para testes de viabilidade polínica, em que corantes com diferentes afinidades são empregados para discriminar os grãos viáveis e inviáveis. Esse tipo de teste pode ser realizado tanto com o grão de pólen *in vivo*, quanto com material fixado.

Existem diferentes tipos de corantes para a determinação da viabilidade polínica, usualmente os corantes de Alexander e Carmim acético e propiônico, são mais empregados nessa técnica. O corante de Alexander permite corar os grãos viáveis e inviáveis de colorações diferentes, devido a sua composição, que é a base de fucsina ácida e verde malaquita e o Carmim Propiônico, cora de vermelho os grãos viáveis e permanece incolor os não viáveis (ALEXANDER, 1980).

O DAPI ou 4',6'-diamino-2-fenil-indolé um fluorocromo, que possui afinidade pelo material genético e vem sendo utilizado tanto para investigação da viabilidade polínica, quanto estudos para marcação de tubo polínico (BRAMMER; TONIAZZO; POERSCH 2015). Munaretto et al. (2019), utilizando o DAPI para investigação da viabilidade polínica em cevada, foi indicado como sendo eficiente para a discriminação de grãos abortados e não abortados.

No entanto, a coloração do grão de pólen, poderá ser influenciada pelas características do próprio grão, isso porque, se a exina for muito espessa dificulta a penetração do corante, sendo necessárias outras técnicas para auxiliar nos estudos morfopolínicos (SILVA, 2017).

Um outro tipo de estudo que costuma ser aliado a compreensão do grão de pólen é a acetólise, que consiste em um método químico, que remove os conteúdos internos e externos do grão, mantendo apenas a exina, facilitando visualização ao microscópio de luz e permitindo a classificação dos mesmos (ERDTMAN, 1960).

A microscopia eletrônica de varredura é outra técnica bastante utilizada na caracterização da morfologia polínica, através da técnica, é possível avaliar os aspectos como a simetria do grão de pólen e presença de ornamentação na exina (HALBRITTER et al., 2018).

Mert e Soylu (2007), descreveram os grãos de pólen da espécie *Castanea sativa*, como sendo prolatos e perprolatos, ocorrendo essa variação, em função do tipo de planta (macho fértil e estéril). O experimento de Evrenosoğlu e Misirli (2009) também

corroboram com essa classificação e puderam ser confirmadas através de microscopia eletrônica de varredura.

O uso do grão de pólen na discriminação e agrupamento de espécies e gêneros, vem crescendo exponencialmente. Wrońska-Pilarek et al. (2016) agruparam 67 espécies de carvalhos do gênero *Quercus* L. de acordo com sua região de origem, isso foi possível, através das análises das características dos grãos de pólen, como tamanho, forma e número de abertura.

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SEGUNDA PARTE - ARTIGOS

ARTICLE 1 - Floral characterization and pollen germination protocol for *Castanea crenata* Siebold & Zucc

(Published, 2020 at South African Journal of Botany)

ARTICLE 1 -Floral characterization and pollen germination protocol for Castanea crenata Siebold & Zucc

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ABSTRACT

Chestnuts have male and hermaphrodite flowers in the same individual. The male flowers are grouped into unisexual catkins, while the female flowers are located at the base of male inflorescences. There is divergence in the functionality of pollen grains among chestnut species. As this species requires cross-pollination, germination tests are essential in breeding programs. The objective of this study was to comparatively evaluate pollen viability as a function of the type of flower using colorimetric assays to develop a culture medium protocol for *in vitro* pollen germination and characterize the floral morphology of the Japanese chestnut (*C. crenata*). Eleven Japanese chestnut cultivars were evaluated for characterization and description of the floral morphology, pollen viability by staining, and *in vitro* germination with determination of the percentage of viability regarding the type of inflorescence (staminate x hermaphrodite). In addition, a culture medium was established to induce germination of chestnut pollen and determination of the percentage of pollen germination *in vitro*, as well as quantification of the number of anthers per flower and pollen grains per anther and per flower for all cultivars. There was no difference in pollen viability between androgynous and staminate catkins. The culture medium containing 6 g L^{-1} of agar, 46.5 g L^{-1} of sucrose, and 460.23 mg L^{-1} of boric acid in the absence of calcium nitrate, pH 5.25, promoted the best conditions for *in vitro* pollen germination. Cultivars with the highest percentage of viability recorded the lowest amounts of pollen per anther and per flower. This characteristic may influence the number of flowers necessary for extraction of anthers in crosses and the success of hybridization in the field.

Keywords: Hybridization; cross-pollination; pollen viability; chestnut; androgynous catkin

1. Introduction

The *Castanea* genus comprises seven species, including *C. sativa* Miller (sweet chestnut – originated in Portugal), *C. crenata* Siebold & Zucc. (Japanese chestnut - originated in Japan), *C. mollissima* Blume (Chinese chestnut – originated in China) and *C. dentata* (Marsh.) Borkh (American chestnut – originated in North America) (Bueno and Pio, 2014). This plant has been gaining prominence in the food industry and has been incorporated into haute cuisine because the fruit, in addition to being low in fat, is a high-quality protein source (Pio et al., 2014). Given its importance as one of the most important fruit species since ancient times, the tradition of consuming chestnuts during the Christmas holiday persists, especially in countries of the Iberian Peninsula (Bueno and Pio, 2014).

The chestnut is a monoecious tree, with male and hermaphrodite flowers in the same individual, wherein the male flowers are grouped into unisexual catkins, while the female flowers are located at the base of some male inflorescences (Valdiviesso, 1999; Beyhan and Serdar, 2008). Beyhan and Serdar (2008 and 2009) reported that pollen grains derived from androgynous/hermaphrodite inflorescences are less functional than male inflorescences. However, in a study conducted with *Castanea sativa* Mill., Valdiviesso (1999) described a higher germination rate of androgynous inflorescences than male inflorescences in some cultivars. In the case of *C. crenata* Siebold & Zucc., no reports in the literature have addressed the functionality of pollen grains.

The domestication of chestnut is still underway, and several natural populations of chestnuts are also responsible for the worldwide production of the nut (Bueno and Pio, 2014). The domestication process is long and involves techniques such as controlled hybridization, which is constantly used in the field, aiming to generate new hybrids to increase variability.

To support breeding programs, knowledge of the floral characteristics in the available germplasm, such as pollen viability and germination ability, is of great importance for the selection of genitors for use in hybridization. Analysis of the pollen fertility of genitors collected in the field is an indispensable preliminary condition for crosses (Chagas et al., 2010). To perform crosses for hybrid formation, it is essential to understand the intervarietal and species-specific compatibility, but the available information on the floral biology of the chestnut plant is limited. In this scenario, more detailed studies on the floral differentiation, morphology, and compatibility between

pollen and stigma are essential, especially for *C. crenata* Siebold & Zucc (Valdiviesso, 1999).

The flowering period of the chestnut is divided into early, semi-early, and semi-late. Considering that fertilization is achieved through cross-pollination and that the stigma is receptive during this period, the presence of a pollinating tree with a flowering period that occurs simultaneously with the other plants in the orchard is recommended (Valdiviesso, 1999). Thus, hybridization success is also related to the pollen germination capacity. According to Nogueira et al. (2015), to assess *in vivo* pollen germination, it is necessary to perform field crosses and, subsequently, monitor the fruiting. However, this process is time-consuming and expensive, so methods such as the evaluation of pollen viability through staining or *in vitro* germination may be interesting alternatives in attempts to reduce costs and time, as well as assist in decision-making.

Several factors may influence pollen germination *in vitro*, including the pH, temperature, and composition of the culture medium. The pH is one of the most critical variables during the germination process among various components of the culture medium and cultivation conditions, and when properly adjusted, this variable can favor germination, improving the utilization of available nutrients (Ramos et al., 2008). To date, no efficient protocol for the induction of *in vitro* germination of chestnut pollen has been described, and studies on floral biology and palynology are limited.

Thus, the objective of this study was to comparatively evaluate pollen viability as a function of the flower type, using colorimetric assays, with the goal of developing a culture medium protocol for *in vitro* pollen germination and characterizing the floral morphology of the Japanese chestnut (*C. crenata*) to provide support for further studies on reproductive biology and breeding**.**

2. Materials and methods

2.1 Plant material

The experiments were conducted in August and September 2017, and 11 Japanese chestnut cultivars were evaluated: ‘Ibuki’, ‘Isumo’, ‘Kinshu’, ‘Km1’, ‘Km2’, ‘Morioase’, ‘Okuni’, ‘Senri’, ‘Taishowase’, ‘Tamatsukuri’, and ‘Tiodowase’. The plants were 15 years old and located in the fruit tree cultivar collection of the *Coordenadoria de Desenvolvimento Rural Sustentável* (Coordination Office for Sustainable Rural Development, CDRS) in the São Bento do Sapucaí municipality, state of São Paulo, Brazil.

During the study, the following experiments were implemented and evaluated:

- Characterization and description of the floral morphology of *Castanea crenata*;
- Pollen viability assay by staining to determine the difference in percentage of viability according to the type of inflorescence (staminate x hermaphrodites);
- Establishment of the optimum culture medium, with the definition of the pH and the concentrations of agar, sucrose, calcium nitrate, and boric acid to induce pollen germination in the chestnut;
- Determination of the percentage of pollen germination *in vitro*, quantification of the number of anthers per flower and of the pollen grains per anther and per flower for all cultivars.

The cultivars choice for testing and description, was based on availability at the time of colleted.

2.2 Characterization and description of the floral morphology of *Castanea crenata*

For the characterization and floral description of the species, three plants of the 'Tamatsukuri' cultivar in the reproductive stage were selected. For each individual, androgynous and staminate inflorescences were collected at different stages of development. The qualitative morphological evaluations involved the analysis of verticils (calyx, corolla, gynoecium, and androecium) and the results were recorded and illustrated in photographs with appropriate scale and optical conditions.

2.3 Pollen viability assay by staining

For these analyses, mature hermaphrodite and staminate inflorescences of the 'Taishowase' cultivar were used. Fully formed anthers were collected in the morning and excised under a stereoscopic microscope. The slides were prepared by the squash technique (Guerra and Souza, 2002) and stained separately with Alexander's stain (Alexander, 1980) and 2% propionic carmine.

For Alexander's staining, the pollen grains were considered viable when they exhibited a purple color without deformations and were considered inviable when stained green. For staining with 2% propionic carmine, the pollen grains were considered viable when stained red and inviable when colorless.

This experiment was conducted using a completely randomized design, with five slides per stain and 200 grains of pollen analyzed per slide. The percentage of viable pollen was calculated as a function of the total number of pollen grains evaluated. All

slides were observed under a light microscope (Carl Zeiss, AxioLabA1) equipped with a micro camera (AxioCam ICc1) to capture the images.

2.4 Establishment of the culture medium for *in vitro* pollen germination

In this experiment, the composition of the culture medium specific for chestnut pollen germination was determined to obtain the maximum germination rate possible. The components used to assemble this experiment were based on studies on viability and *in vitro* germination of other fruit trees and recommendations for the composition of the culture medium used for *Castanea sativa* (Beyhan and Serdar, 2009; Nogueira et al., 2015).

The cultivar used to establish the culture medium was 'Tamatsukuri' due to its early flowering compared with the other cultivars. Inflorescences with flowers in the pre-anthesis stage were collected, and the anthers were subsequently removed and slightly macerated to release the pollen grains, which were then placed in Petri dishes containing culture medium.

To determine the culture medium and ideal conditions for chestnut pollen germination, tests were performed in a germination chamber (BOD) with a controlled temperature at 27°C, absence of light, and considering the following treatments:

A) Agar concentration (4.6.8 and 10 g L^{-1});

B) Sucrose concentration (0, 30, 60, and 90 g L^{-1});

C) pH (3.5, 4.5, 5.5, and 6.5);

D) Boric acid concentration (0, 400, 800, and 1200 mg L^{-1});

E) Calcium nitrate concentration (0, 200, 400, and 800 mg L^{-1}).

Twenty-four hours after inoculation, the pollen grains were evaluated under a light microscope (Carl Zeiss, AxioLabA1, 10x objective). The experimental design was completely randomized using four replicates, in which each replicate consisted of one Petri dish with four fields of view. The pollen viability was determined as a function of the percentage of germinated pollen, and pollen grains with pollen tube lengths equal to or greater than their diameters were considered viable, as recommended by Shivanna and Rangaswamy (1992).

2.5 Determination of the percentage of *in vitro* pollen germination and quantification of the number of anthers per flower and of pollen grains per anther and per flower for all cultivars

After defining the culture medium suitable for pollen germination of the Japanese chestnut, the germination percentage was determined for the 11 cultivars. For this

purpose, the inflorescences of each cultivar were collected separately, and the pollen preparation and evaluation were conducted according to the above-described procedures (item 4). The experimental design was completely randomized (CRD) using 11 cultivars and four replicates, in which each replicate consisted of one Petri dish with five fields of view.

The number of anthers per flower/cultivar was determined by analyzing three inflorescences, from which 10 pre-anthesis flowers were randomly removed and the number of stamens per flower quantified. At this stage, the experimental design was completely randomized (CRD) using 11 cultivars and 10 replicates, in which each replicate consisted of a flower bud.

To quantify the number of pollen grains/anther/flower, five inflorescences with flowers in the pre-anthesis stage were initially collected from each of the 11 cultivars. Then, 100 anthers/inflorescence were randomly selected and stored in uncapped microtubes at a controlled temperature (27°C) for 24 hours in the absence of light to determine the occurrence of anther dehiscence and release of pollen grains.

After 24 hours, 1000 μL of lactic acid was added to the microtubes, which were incubated for an additional 24 hours. Then, a 10-μL sample of the solution (lactic acid + pollen grains) from each microtube was placed in a Neubauer chamber to count the number of pollen grains under a light microscope (Carl Zeiss, AxioLabA1, 10x).

The number of pollen grains/anther was calculated by multiplying the mean number of pollen grains of each sample by the total volume of lactic acid in the solution (1000 μL) and dividing this value by the product between the volume of lactic acid in the sample (10 μL) and the number of anthers in each tube (one hundred). The number of pollen grains per flower was calculated by multiplying the estimated mean number of pollen grain per anther by the mean number of anthers per flower.

The experimental design was completely randomized using five replicates, in which each replicate consisted of a microtube with four counts in the Neubauer chamber.

2.6 Analysis of the results

To characterize the floral morphology, a descriptive analysis of the observed floral structures was performed and compared with data available in the literature. All results were recorded using photographs.

Regarding the other experiments, the collected data were analyzed by analysis of variance, the quantitative means were fitted to a linear or quadratic regression at 5%

probability, and the qualitative means were analyzed by the Scott & Knott test. The analyses were performed using SISVAR software (Ferreira, 2011).

3. Results and Discussion

The chestnut has inflorescences in the form of catkins, which can be classified as a hermaphrodite, mixed or androgynous catkins, which carry one or more female inflorescences at the base of the staminate inflorescences, and unisexual or staminate catkins, in which the inflorescence is composed only of staminate flowers (Beyhan and Serdar, 2008). These characteristics were well evidenced in the ‘Tamatsukuri’ cultivar evaluated in this study, as shown in Figures 1 and 2. With respect to the female inflorescence, the 'Tamatsukuri' cultivar exhibited inflorescences consisting of three flowers arranged in a row, one terminal and two lateral, with multiple glabrous stigmas (Figure 1A, B and D). These flowers were surrounded by a cupule covered with green and acuminate bracts (Figure 1C) that, as the inflorescence differentiated into fruit, originated the burr (Figure 1E).

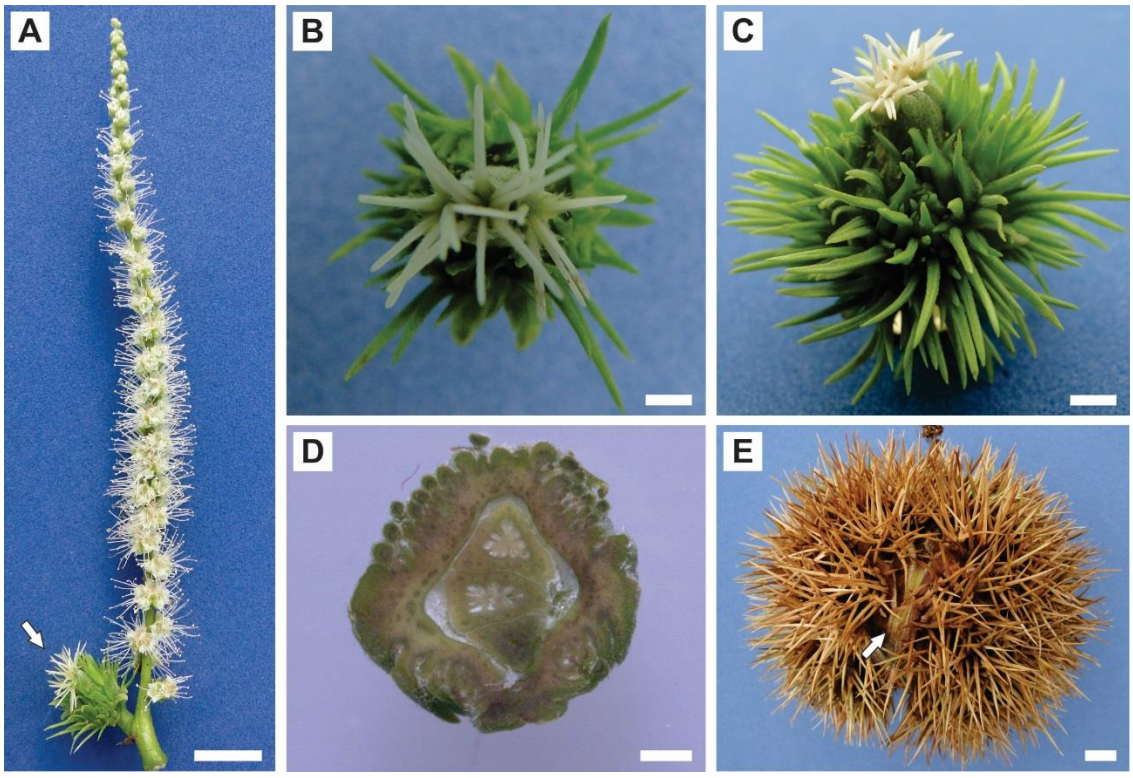

Figure 1. The androgynous catkin, pistillate inflorescence, and spiny capsule of the Japanese chestnut ‘Tamatsukuri’ cultivar (*Castanea crenata* Siebold & Zucc). A - Androgynous catkin with details of the pistillate inflorescence at the base of the staminate inflorescence (white arrow). B- Above view of the pistillate inflorescence, showing the

three flowers arranged in a row (white arrows). C. Frontal view of the pistillate inflorescence, showing the cupule, covered by acuminate green bracts. D. Cross-section of a pistillate inflorescence showing the three flowers inside that make up the inflorescence. E. Spiny capsule or burr with details of the dehiscence line (white arrow). Scale bar: A and E = 10 mm, B = 3 mm, C = 5 mm, and D = 1 mm. Photos: A, B, C, and E= Carolina Ruiz Zambon; D = Dayanne Medrade Silva.

These observations corroborated the results published by Valdiviesso (1999), who also reported the occurrence of groups of three to seven female flowers in the cupule of an inflorescence. Bueno and Pio (2014) stated that with a greater the number of flowers in the cupule and greater fertilization of these flowers and differentiation into fruits, the smaller is the size of each achene contained in the burr. The same authors also reported that the number of pistillate flowers in one inflorescence varies among different chestnut species and cultivars.

Regarding staminate flowers, they were arranged in groups of five to seven flowers in a pentagonal conformation called the glomerule (Figure 2A, B, E, and F). The glomerules were individualized and arranged in a spiral along the axis of the catkin (Figure 2B and E).

The reproductive study and characterization of different chestnut cultivars by Valdiviesso (1999) showed that the flowers that make up the glomerule are subsessile and extremely compact. The author also reported that the flower positioned towards the apical region of the inflorescence is larger and usually the first to bloom. In the floral evaluations performed using the ‘Tamatsukuri’ cultivar in the present study, the same pattern was observed regarding the structure of the glomerules and floral anthesis. However, the flowers directed towards the apical apex of the catkin were often not different in size compared with the other flowers of the respective glomerulus; however, they were in the beginning of anthesis (Figure 2C and F).

Valdiviesso (1999) also noted that the chestnut may have inflorescences containing astaminate flowers, which had silky filaments with a white color as long as the androecium of the staminate flowers. In the ‘Tamatsukuri’ cultivar, astaminate flowers were not observed at the beginning of anthesis, but the evaluated staminate flowers were in the intermediate and late stages of anthesis (Figure 2D). In these flowers, it was possible to separate the anther/filament structures with greater ease, and

inflorescences containing flowers in the post-anthesis stage were also observed to present a large number of filaments without anthers.

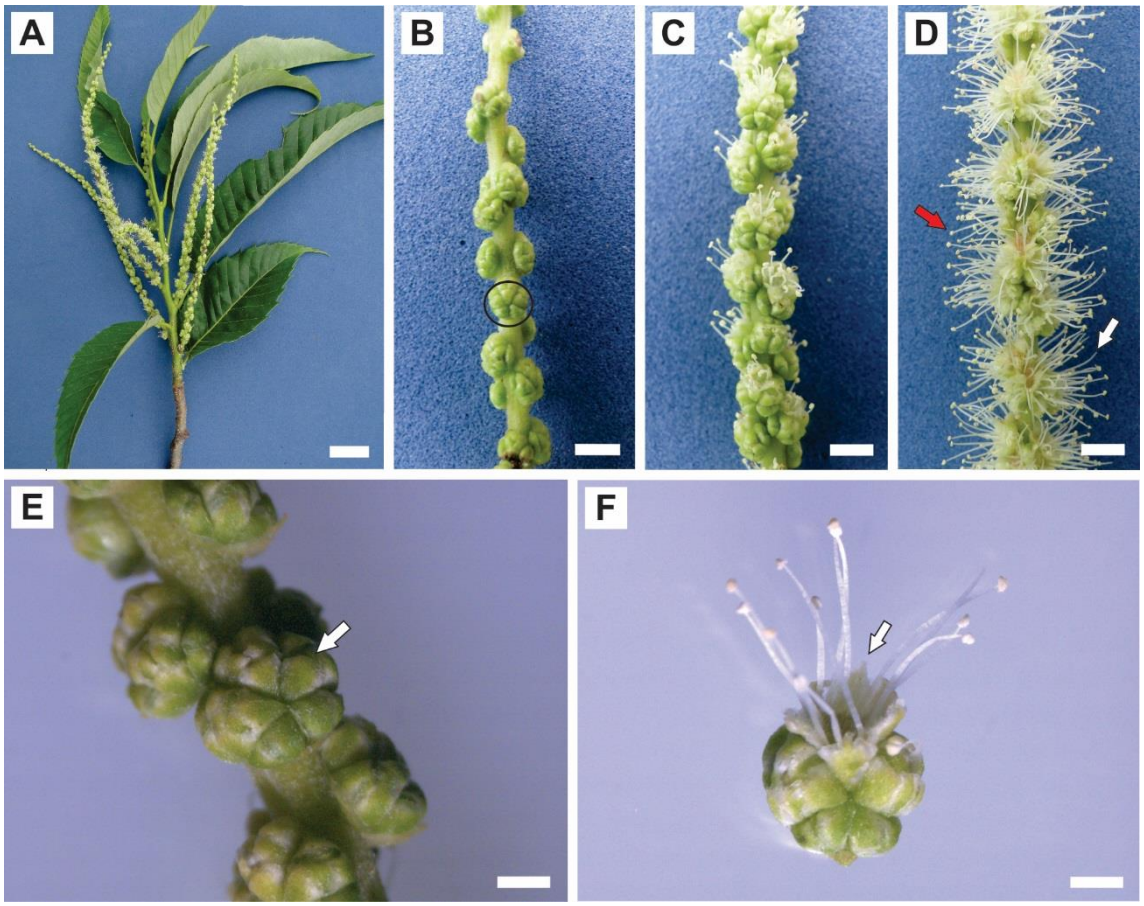

Figure 2. Staminate catkins, glomerules and staminate flowers of the Japanese chestnut (*Castanea crenata* Siebold & Zucc), 'Tamatsukuri' cultivar. A- Set of staminate catkins. B- Staminate catkin in pre-anthesis - details of the set of flowers forming a glomerule (Circle). C- Staminate catkin with the flower of the apical region of each glomerule in anthesis. D- Staminate catkin in anthesis - details of the anther and filament (red arrow) and the filament without the anther (white arrow). E- Staminate catkin in pre-anthesis - details of a flower composing the glomerule. F- Glomerule with a flower from the apical region in anthesis. Scale bar: A = 20 mm, B, C and D = 3 mm, E and F = 1 mm. Photos: A, B, C, and D = Carolina Ruiz Zambon; E and F = Dayanne Medrade Silva.

Regarding the evaluation of pollen grains by staining, the 'Taishowase' cultivar showed a high pollen viability rate for both stains, above 80%, without significant differences between them or between the different inflorescence types evaluated (Table 1).

Table 1 – Pollen viability (%) of the *Castanea crenata* 'Taishowase' cultivar evaluated in hermaphrodite and androgynous flowers and comparatively between the two stains.

Flower Type	**Pollen viability**
Hermaphrodite	81.84 a
Androgynous	81.50 a
CV (%) =8.58	
Type of stain	**Pollen viability**
2% propionic carmine	82.94 a
Alexander's stain	80.40 a
CV (%)= 38.23	

Equal lowercase letters in the column for the flower type and for the type of stain do not differ statistically ($p>0.05$) by the Scott Knott test.

In previous studies with different chestnut genotypes (*Castanea sativa* L.), Beyhan and Serdar (2008 and 2009) reported that androgynous catkins produce nonfunctional pollen grains and thus exhibit a lower viability compared with staminate inflorescences. Although the same phenomenon was not observed in the evaluated cultivars herein (Table 1), it cannot be completely ruled out in *C. crenata* considering that the inflorescences were randomly sampled.

In the experiment performed to determine the most suitable culture medium for the germination of Japanese chestnut pollen grains, there was a significant interaction between the agar and sucrose concentrations. The quadratic regression revealed that the agar concentration of 6 g L^{-1} induced the highest percentage of pollen germination (Figure 3A). Breakdown of the equation showed that the sucrose concentration of 46.5 g L^{-1} with 6 g L^{-1} of agar promoted 14.9% germination.

The role of agar, according to Silva et al. (2016), is to promote solidification of the culture medium, ensure a constant relative humidity, promote osmotic equilibrium, and facilitate the incorporation of nutrients, aiding formation of the pollen tube. A possible explanation for the higher percentage of *in vitro* germination in the medium containing 6 g L^{-1} agar was that this concentration favored the best absorption of water and nutrients necessary for formation and growth of the pollen tube.

Sucrose, in turn, has the function of providing energy for the biosynthetic processes involved in cell growth and differentiation (Silva et al., 2016). Under the

present experimental conditions, the addition of increasing sucrose concentrations to the culture medium up to 46.5 g L^{-1} resulted in an increase in the percentage of germinated pollen grains. However, when higher concentrations were used, a decrease in the germination rate was observed, which might have been related to the increase in the solute concentration of the culture medium leading to disruption and damage to the cellular structures of the pollen grains (Dantas et al., 2005).

Regarding pH, pollen germination started at pH values above 3.5, and the maximum germination percentage was 15.34% at pH 5.25. The pH is a very important factor for establishing a culture medium because it directly influences the physiological processes of pollen grains (Zambon et al., 2014). Similar results have been found in the establishment of culture media for *in vitro* germination of pollen grains of other fruit species, such as peach (pH 5.5) (Chagas et al., 2009) and pear (pH 5.2 and 5.8) (Chagas et al., 2010). According to Pierik (1987), the optimal pH for pollen germination of most plant species is between 5 and 6.5, and values outside this range result in suspension of *in vitro* growth and development.

Another important observation related to the composition of the culture medium regarded the effect of boric acid, which increased the pollen germination percentage of Japanese chestnut. The highest germination percentage (24.43%) was obtained with a concentration of 460.23 mg L^{-1} of boric acid. Studies have shown that boric acid plays an important role in the formation and development of the pollen tube, and the concentrations used were varied according to the species studied. In the culture medium, boron interacts with sucrose, forming a sugar-borate ionizable complex that reacts with the plasma membrane, enabling greater growth of the pollen tube (Zambon et al., 2014). The addition of boric acid to the culture medium likely favored the formation of this complex, increasing the percentage of pollen germination.

Regarding calcium nitrate (Figure 3C), the best results were obtained in its absence, with approximately 29% germination. Similar observations have been reported by Nogueira et al. (2015) and Zambon et al. (2014), who found a decrease in the germination percentage with increasing calcium nitrate concentrations in the culture medium.

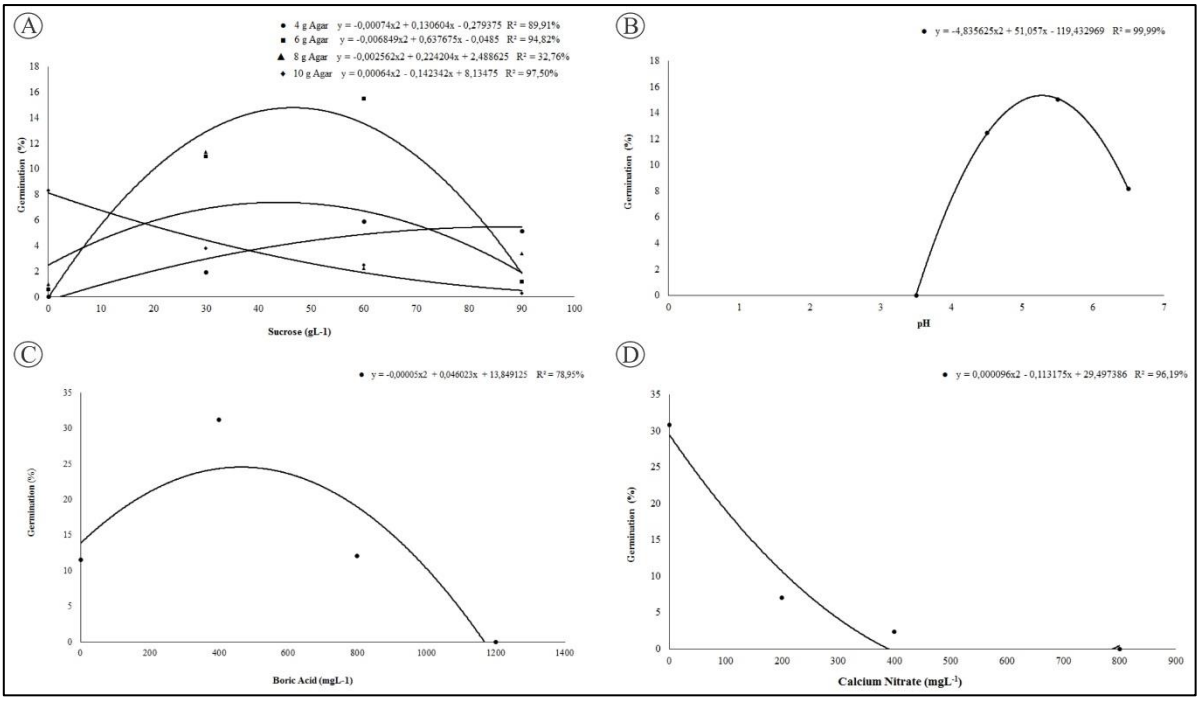

Figure. 3. In vitro pollen germination percentage of the Japanese chestnut 'Tamatsukuri' cultivar. (A) Different concentrations of agar and sucrose. (B) Different pH levels. (C) Different concentrations of boric acid. (D) Different concentrations of calcium nitrate.

Based on the above considerations, the recommended culture medium to obtain the best results for *in vitro* pollen germination of Japanese chestnut should have the following composition and conditions: agar - 6 g L^{-1}, sucrose - 46.5 g L^{-1}, boric acid - 460.23 mg L^{-1}, pH 5.25 for culture in a germination chamber (BOD), a controlled temperature of 27°C, and absence of light.

The above data for *in vitro* pollen germination in the culture medium revealed significant differences among the cultivars (Table 2). The mean grouping test resulted in six groups considering the percentage of germination of the different cultivars. The best results were observed for cultivars 'Morioase' (34.04%) and 'Tamatsukuri' (29.99%), followed by 'Senri' (26.88%) and 'KM2' (20.59%). The remaining cultivars had germination percentages ranging from 4% to 16% (Table 2). This difference in pollen germination probably resulted from the intrinsic characteristics of each cultivar. Similar results have been acquired in other studies of pollen viability in fruit tree species (Chagas et al., 2010; Zambon et al., 2014; Nogueira et al., 2015).

Another aspect that must be considered is that pollen viability can vary considerably between individuals of a species and between samples of the same

individual. According to Shivanna and Rangaswamy (1992), the flowering period, environmental changes, and genotypic differences may contribute to such variability. Pollen viability and longevity also depend on the cellular composition. Shivanna and Rangaswamy (1992) indicated that the greatest limitation of *in vitro* germination tests is the difficulty related to obtaining satisfactory germination in many species, especially those presenting tricellular pollen grains, as this type of pollen grain germinates faster and has reduced longevity than bicellular species (Hanna and Towill, 1995).

Regarding the quantification of the number of anthers per flower, variation was observed among the different cultivars studied, with cultivar ‘KM1’ exhibiting the highest mean number of anthers per flower (39.17), followed by ‘Isumo’ (31.50), ‘Senri’ (27.33), ‘Tamatsukuri’ (26.50), and ‘Tiodoase’ (24.67) (Table 2). Other studies on fruit tree species have shown that the number of stamens per flower may vary among different cultivars. Albuquerque Junior et al. (2010) observed differences in the number of stamens per flower among the different apple tree cultivars studied. Barbosa et al. (1991) evaluated 25 peach and nectarine cultivars and observed differences in cultivars evaluated for this trait. It is important to note that a greater number of stamens per flower does not necessarily indicate a greater amount of pollen grains. In addition, the number of anthers per flower of a cultivar may vary annually due to the climatic, nutritional, and environmental conditions in which the plants develop (Albuquerque Junior et al., 2010).

In evaluations of the number of pollen grains per anther and the number of pollen grains per flower, significant differences were detected with the formation of different groups by the mean grouping test. Cultivars 'KM1' (58.09), ‘Isumo’ (45.72), 'Senri' (39.29), and ‘KM2’ (33.31) had the highest values for the number of pollen grains per anther (Table 2). For the trait number of pollen grains per flower, cultivar 'KM1' had the highest mean (2083.23), followed by cultivars ‘Isumo’ (1553.23) and ‘Senri’ (1174.09) (Table 2).

A possible explanation for the higher pollen percentages in these cultivars may be related to Zambon et al. (2014). In addition, genotypes with greater pollen viability and numbers of pollen grains per anther and per flower have an adaptive advantage at the time of pollination, which may favor an increase in fruit formation and, consequently, in production during harvest (Figueiredo et al., 2013).

Table 2 – Mean *in vitro* pollen germination, number of anthers per flower, number of pollen grains per anther, and number of pollen grains per flower of different Japanese chestnut cultivars.

Cultivars	*In vitro* germination (%)	No. of Anthers per flower	No. Pollen per anther	No. Pollen per flower
IBUKI	3.69 f	-	8.91 e	-
ISUMO	13.51 d	31.50 b	45.72 b	1553.23 b
KINSHU	9.17 e	16.67 d	22.35 d	410.71 e
KM1	8.30 e	39.17 a	58.09 a	2083.23 a
KM2	20.59 c	16.42 d	33.31 c	571.44 d
MORIOASE	32.04 a	22.25 c	20.72 d	385.90 e
OKUNI	15.13 d	22.17 c	24.32 d	493.98 d
SENRI	26.88 b	27.33 b	39.29 c	1174.09 c
TAISHOWASE	16.34 d	12.83 d	12.97 e	166.40 e
TAMATSUKURI	29.99 a	26.50 b	11.50 e	331.35 e
TIODOASE	6.38 f	24.67 b	21.09 d	777.95 d
CV%	13.65	17.63	17.76	19.49
Mean	16.55	23.87	27.75	794.83

Means followed by the same letter in the columns do not differ by the Scott-Knott test ($p>0.05$).

4. Conclusion

There was no difference in pollen viability between androgynous and staminate catkins. The culture medium containing 6 g L^{-1} of agar, 46.5 g L^{-1} of sucrose, and 460.23 mg L^{-1} of boric acid in the absence of calcium nitrate, pH 5.25, provided the best conditions for *in vitro* pollen germination, reaching approximately 30% viability.

Regarding the germination of pollen grains, cultivars ‘Morioase’ and ‘Tamatsukuri’ showed the highest percentage of viability, but lower amounts of pollen per anther and per flower, which may influence the number of flowers required for anther extraction in crosses and the success of field hybridizations.

Acknowledgments

The authors would like to thank the Brazilian National Council for Scientific and Technological Development - CNPq, Foundation for Research Support of the State of Minas Gerais - FAPEMIG, and Coordination for the Improvement of Higher Education Personnel - CAPES for financial support for this research.

Conflict of interest

The authors declare that they have no conflicts of interest.

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ARTICLE 2 - Pollen grain description and viability in *Castanea crenata* Siebold & Zucc (Fagaceae)

(Prepared in accordance with PAB)

ARTICLE 2- Pollen grain description and viability in *Castanea crenata* Siebold & Zucc (Fagaceae)

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Abstract

The chestnut tree (*Castanea crenata*) is a monoecious plant, with androgynous and staminate flowers. Most studies regarding the description of pollen grains and pollen viability have concentrated on *Castanea sativa*. However, due to several attributes and mainly the recent agroeconomic importance of *C. crenata*, it is fundamental to characterize the morphology and viability of the *C. crenat*a pollen grain, contributing to breeding and genetic conservation of the species. The acetolysis technique and ultrastructural analysis by scanning electron microscopy was used for the characterization of pollen grain morphology. Pollen viability was determined by colorimetric tests using Alexander's dye and propionic carmine. The analysis of pollen grains via acetolysis showed that there is no morphological variation related to flower type, except for the aperture length, which was higher in androgynous flowers. The grains were characterized as prolate, isopolar, tricolpate, with long colpi constricts, heteroapertured, zonocolpate, and with psilate (smooth) exine. Both dyes were efficient to determine pollen viability and indicated that the *C. crenata* cultivars and hybrids have high viability rates, with emphasis on the cultivar 'Morioase'.

Index terms: Acetolysis; Chestnut**;** Pollen fertility; Pollen morphology; genetic resources.

1. Introduction

Castanea Miller is a genus belonging to the Fagaceae family (Corredoira et al. 2017; Mellano et al. 2018), popularly known as chestnut tree, which comprises nine species (Govaerts, 2020). *Castanea sativa* Miller, *Castanea crenata* Siebold & Zucc., *Castanea molissima* Blume, and *Castanea dentata* (Marsh.) Borkh are considered the most important species of the genus (Bueno and Pio, 2014; Pio et al. 2014).

Although the genus *Castanea* presents species native to temperate zones in Asia, Europe and the eastern United States (Corredoira et al. 2017; Mellano et al. 2018), China is considered the most significant site of genus diversity, being pointed out as the probable center of geographical dispersion (Gomes-Laranjo et al. 2007).The species of this genus attend multiple purposes, which gives them great agroeconomic importance, both for the production of nuts, as for wood for furniture, utensils, and even as a substrate in the cultivation of edible mushrooms (Corredoira et al. 2017). Chestnuts contain high-quality protein and little fat (3% of total weight) and are gluten-free. They also contain vitamin C, potassium, low sodium, and approximately 45% starch, in addition to fatty acids, which can vary among species and cultivars (Pio et al. 2014).

In this scenario, some challenges still need to be overcome, as the chestnut tree still undergoes the domestication process and part of the production comes from natural populations made up of pure plants or hybrids naturally originated, mainly in the Iberian Peninsula, where one of the largest exploration areas is located.

Among the various strategies, access to genetic diversity, analysis of the domestication process, and studies of gene flow among different species and gene pool are essential. These studies may involve the analysis of floral biology, adaptation, genetic recombination, cytogenetic characters, and aspects related to palynology.

Research in these areas is even more important if we consider that although there is high genetic diversity, there is currently a risk of ecotype and variety loss because of the species of the genus, such as *Castanea sativa* Mill. are susceptible to certain diseases, such as the ink, caused by the pathogen *Phytophthora* sp. (Cepêda, 2014; Mellano et al. 2018). Therefore, initiatives that aim to collect and evaluate a higher number of genotypes and/or wild species available are shown to be determining factors for better use of genetic diversity.

Hybridizations allow expanding the genetic base, adding desirable genes (Granato, 2010) related to the increase in the quality and production of fruits and wood, as well as increasing disease resistance. Success in hybridizations depends on different factors, such as genomic homologies, the potential for gene introgression, compatibility in crosses, and simultaneity in flowering, among others. In addition to these, aspects related to fertility, including pollen viability studies, also need to be considered. Dafni and Firmage (2000) emphasize that pollen viability studies are essential for pollination biology, genetic interaction, incompatibility, and fertility, in addition to dispersion and gene flow assessment.

Genetic divergence through multivariate techniques has been used in breeding programs to assist in the predisposition of genetic variability (Ivoglo et al. 2008). For *Castanea*, studies on this topic are limited. The available studies, for the most part, are related to *C. sativa*. Furthermore, the pollen grain has characteristics, which can be used to discriminate species and even cultivars (Evrenosoğlu and Misirli, 2009).

Thus, the objective of this study was to characterize the morphology and viability of the pollen grain of *C. crenata*, contributing to breeding programs, genetic conservation and characterization of the species.

2. Material and methods

2.1 *Plant material*

The evaluations were made in eight cultivars of *Castanea crenata* ('Ibuki', 'Isumo', 'Kinshu', 'Morioase', 'Okuni', 'Senri', 'Tamatsukuri' and 'Tiodowase') and two hybrids between *Castanea crenata* x *Castanea sp*. ('Km1' and 'Km2'). The inflorescences were collected in the morning, in the collection of Chestnuts from the Sustainable Rural Development Coordination - CDRS, located in the municipality of São Bento do Sapucaí, São Paulo, Brazil.

2.2 *Pollen grain evaluation using the acetolysis technique*

The technique employed followed the recommendations of Erdtman (1960), modified by Melhem et al. (2003). The flower buds were macerated with the aid of a needle in a microtube containing acetic acid 45% and centrifuged for 10 min. at 2500 rpm. The supernatant was then removed and replaced with distilled water and again centrifuged for 10 min at the same speed and discarded at the end. The 1mL acetolytic solution (1 sulfuric acid: 9 acetic anhydride) was added and the preparation was kept in a water bath at 85ºC for four minutes.

The material was again centrifuged twice. In the last exchange, in addition to the distilled water, four drops of ethyl alcohol were added, and the supernatant removed after each centrifugation. After the last centrifugation, the supernatant was replaced with glycerin water 50%.

The material was stored in microtube at 10°C for 24 hours until the preparation of slides. The glycerinated water was discarded and the material was placed on slides with portions of glycerinated gelatin (100 mL of distilled water, 100 mL of glycerin, 17 g of

colorless gelatin, and 1 g of phenol) heated in a lamp, covered with a coverslip and sealed with paraffin.

The slides were evaluated in a light field microscope (Carl Zeiss, AxioLabA1), equipped with a micro camera (AxioCam ICc1), to capture the images. For each cultivar, five slides were evaluated. The polar axis (P) and equatorial axis (E) measurements were obtained from the evaluation of ten pollen grains per slide. The exine thickness (ET), length (CL), width (CW), and margin (MC) of the colpi, length (AL), width (AW) and margin (AM) of the aperture were obtained from the evaluation of five pollen grains in equatorial view. Measurements were made using ImageJ software version 1.44 (Research Services Branch, U.S. National Institutes of Health, Bethesda, MD, USA.).

2.3 *Ultrastructure analysis of pollen grain using scanning electron microscopy*

The anthers of the cultivar ‘Taishowase’ were fixed in Karnovsky solution. The samples were washed in 0.05M sodium cacodylate buffer (3 times - 10 minutes each). Subsequently, they were dehydrated in a progressive acetone series (25%, 50%, 75%, 90% and 100% - 3 times for 10 minutes), and subjected to critical point CO_2 desiccation in BAL-TEC equipment, CPD-030, fixed in metallic support with silver glue and covered with metallic gold (10 nm) in a BAL-TEC equipment, SCD-050. The analyzes were performed using a scanning electron microscope LEO-EVO 40, XVP.

2.4 *Assessment of pollen viability*

The anthers were excised of androgynous and staminate inflorescences, under a stereomicroscope, to release the pollen grains. The slides were prepared using the squash technique (Guerra and Sousa, 2002) and stained separately with Alexander's dye (Alexander, 1980) and 2% propionic carmine.

By Alexander's dye, pollen grains were considered viable and non-viable according to the purple or green color, respectively. For staining with 2% propionic carmine, pollen grains were considered viable when presenting red and unviable, if colorless.

The evaluations were made in five slides and 200 pollen grains/slide/dye. The percentage of viable pollen was obtained as a function of the total number of pollen grains evaluated. All slides were observed under a light field microscope (Carl Zeiss, AxioLabA1), equipped with a micro camera (AxioCam ICc1), to capture the images.

2.5 *Data analysis*

The data were submitted to the Scott -Knott test ($p < 0.05$) (Scott-Knott, 1974) using the SISVAR software (Ferreira, 2003). Pollen grains were classified according to Erdtman's recommendations (1943).

Dissimilarity based on the Gower algorithm (1971) was used to quantify the divergence between accessions. Cluster analysis was performed by the hierarchical method *Unweighted Pair-Group Method Using an Arithmetic Average* (UPGMA). The co-phenetic correlation coefficient (CCC) was estimated according to the methodology described by Sokal and Rohlf (1962) to test the efficiency of the hierarchical clustering method. The cut-off point of the dendrogram formed by the UPGMA method was defined as proposed by Mojena (1977). Cluster analyzes were performed using the Genes Software (Cruz, 2013).

3. Result and discussion

The analysis of pollen grains via acetolysis, in androgynous and staminate flowers of the cultivar 'Taishowase,' showed that there is no morphological variation related to

the type of flower, except for the aperture length, as the androgynous flowers have larger dimensions (Figure 1A and Table 1). Considering that these results confirmed that there was no difference for 92% of the parameters evaluated between the two types of flowers, the acetolysis technique was applied to the other cultivars and hybrids but evaluating random/mixed flower buds.

The pollen grains of the evaluated genotypes presented a ratio between the polar and equatorial axes (P/E) ranging from 1.33 to 1.54 (Table 2) and, therefore, were classified as prolates, considering the criteria proposed by Erdtman (1943). This classification is similar to that described by Grygorieva et al. (2015) for *Castanea sativa* and by Mert and Soylu (2007) for cultivars of *Castanea sativa* (fertile male and sterile male). However, these latter authors observed variable morphologies, because, in addition to the prolate, they identified grains of perprolate pollen in the fertile male cultivars, while the sterile male cultivars presented the morphology classified as subprolate.

The lowest value for the ratio between the polar and equatorial axes was observed in cultivar 'Ibuki' (1.33) and the highest (1.54) in cultivars 'Tamatsukuri' and 'Tiodowase' (Table 2), Evrenosoğlu and Misirli, (2009), cited approximate averages (1.75-2.02 µm) in the cultivars Osmanğlu and Seyrekdiken from *Castanea sativa*.

The polar diameter of the pollen grains varied between 11.55 and 15.14 µm, and they were classified as small, according to Halbritter et al. (2018). In *C. crenata*, the dimensions of the pollen grains are similar to those of *C. sativa*: 13.33-21.30 µm; 11.3-16.7 µm and 2.58-24.39 µm described, respectively, by Mert and Soylu (2007); Evrenosoğlu and Misirli (2009) and Grygorieva et al. (2015).

Exine thickness varied between 0.59 and 0.98 µm, and exhibits a smooth (psilated) surface, without ornamentation, confirmed through the images of MEV (Figure 1D-E). This observation coincides with the description by Grygorieva et al. (2015) in *C. sativa*.

Halbritter et al. (2018) reported that the thin exine pattern acts as a probable facilitator for germination and contributes to the dispersion of the pollen grain.

Several species pollinated by the action of the wind, according to Judd et al. (2009), exhibit smooth exine. Sabugosa-Madeira et al. (2008a) also describe that, in anemophilous plants, the pollen coat is generally poorly developed or almost absent. According to Giovanetti and Aronne (2011), the chestnut tree has a double form of dispersion (anemophilous and entomophilic). However, anemophilia is considered the main form of transport of pollen grains of the species. In an experiment with *Castanea sativa*, Sabugosa-Madeira et al. (2008b) observed that, on rainy days, the concentration of pollen in the air is lower. Therefore, the morphological characteristics of the pollen grains of *Castanea crenata*, may indicate an adaptation for efficiency in pollen dispersion of the species.

The pollen grains of *C. crenata*, are dispersed in monads, that is, isolated, isopolar, tricolpate (Figure 1A), long constricted colpi heteroapertured and zonocolpates. They present subcircular scope and radial symmetry. Evrenosoğlu and Misirli (2009) also classified the pollen grain of *Castanea* as being tricolpated, with the presence of ectoapertures and dispersed in monads. According to Shah et al. (2005), the tricolpated pollen grain is a shared character in the Fagaceae family.

According to Beyhan and Serdar (2008 and 2009), pollen grains of *Castanea sativa* androgynous inflorescences are less functional than those of staminate. However, in analyzes carried out by Silva et al. (2020), it was found there was no difference in the pollen viability of this species (androgynous 81.84%, staminate 81.50% viable pollen grain) regardless of the type of inflorescence, which is why, in the present study, random/mixed buds were used mixed to estimate the viability.

Table 1 - Average values (µm) of the pollen grain characteristics of *Castanea crenata*, cultivar 'Taishowase,' in the function of the type of inflorescence (androgynous x staminate), being P - polar diameter; E - equatorial diameter; P/E - equatorial/polar diameter ratio; ED - equatorial diameter; AS[1] - acolpi side; ET – exine thickness; CL - colpi length; CW – colpi width; CM - colpi margin; AL – aperture lenght; AW – aperture width; AM - aperture margin.

Flower	P*	E	P/E	ED	AS[1]	ET	CL	CW	CM	AL	AW	AM
Androgynous	13.35 a	9.54 a	1.40 a	9.39 a	6.98 a	0.77a	8.26a	0.57a	0.52[a]	1.46a	0.97 a	0.46 a
Staminate	13.24 a	9.17 a	1.45 a	9.19 a	7.28 a	0.81a	8.22a	0.57a	0.42a	1.14b	0.66 a	0.50 a
CV(%)	4.60	6.24	3.07	5.10	8.01	10.99	6.21	13.25	25.13	7.40	10.84	26.91

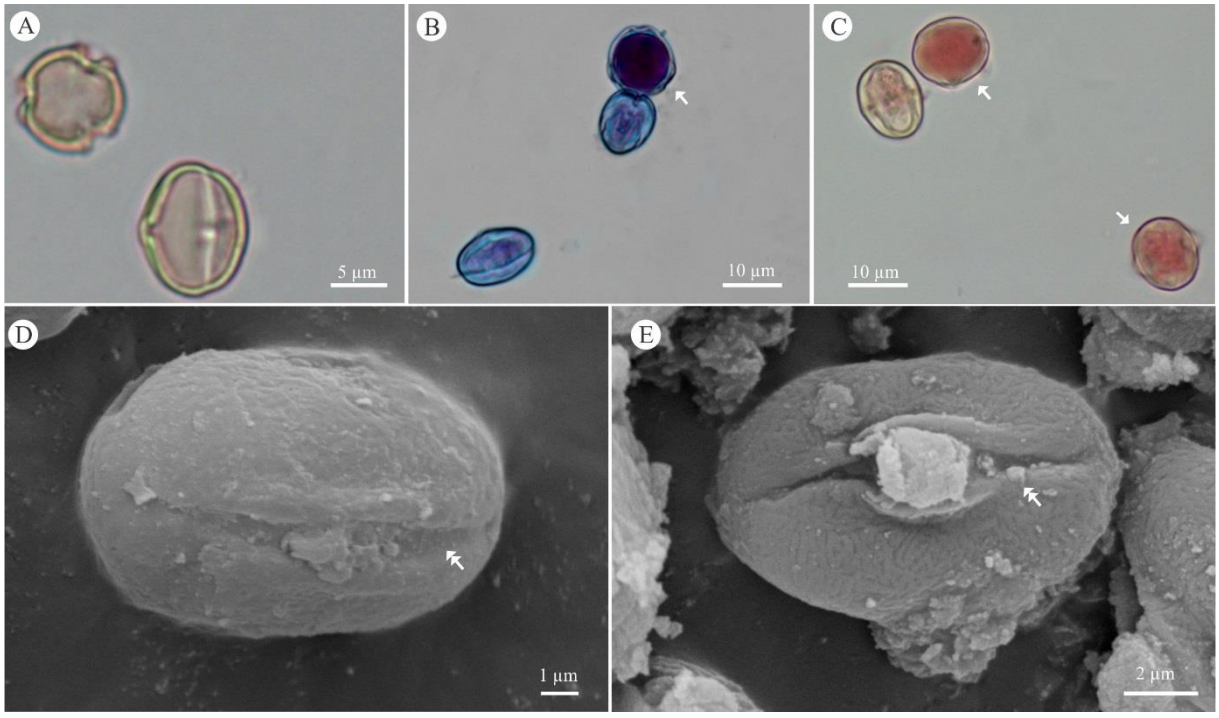
Means followed by the same letter in the columns do not differ by the Scott-Knott test (p>0.05).

Table 2 - Mean values (µm) of the pollen grain characteristics of *Castanea crenata*. Being P - polar diameter; E - equatorial diameter; P/E - equatorial/polar diameter ratio; ED - equatorial diameter; AS[1] - acolpi side; ET – exine thickness; CL - colpi length; CW – colpi width; CM - colpi margin; AL – aperture lenght; AW – aperture width; AM - aperture margin.

Cultivar/hybrids	P*	E	P/E	ED	AS[1]	ET	CL	CW	CM	AL	AW	AM
Ibuki	11.55e	8.65 c	1.33d	8.73 c	6.80 c	0.59b	8.33 b	0.55 b	0.38 a	0.74 b	0.81 a	0.44 b
Isumo	14.16c	9.34 b	1.52a	9.77 b	7.23 b	0.84a	9.93 a	0.53 b	0.36 a	0.89 a	0.73 a	0.54 a
Kinshu	12.02d	8.50 c	1.42c	8.93 c	7.08 b	0.68b	8.12 b	0.51 b	0.33 a	0.57 c	0.56 a	0.45 b
Km1 (hybrids)	14.59b	9.73 a	1.50a	10.25 a	7.64 a	0.89a	10.91 a	0.71 a	0.38 a	0.85 a	0.73 a	0.51 a
Km2 (hybrids)	15.14 a	10.02 a	1.51 a	10.02 a	7.55 a	0.98a	10.41 a	0.64 a	0.39 a	0.85 a	0.81 a	0.54 a
Morioase	14.66 b	10.06 a	1.46 b	10.26 a	7.76 a	0.91a	10.86 a	0.61 a	0.47 a	0.86 a	0.63 a	0.55 a
Okuni	14.16 c	9.50 b	1.47 b	9.82 b	7.43 a	0.63b	10.18 a	0.60 a	0.47 a	0.75 b	0.67 a	0.54 a
Senri	12.29 d	8.45 c	1.46 b	8.50 c	6.49 c	0.61b	8.24 b	0.47 b	0.40 a	0.67 b	0.63 a	0.44 b
Tamatsukuri	14.69 b	9.56 b	1.54 a	10.29 a	8.05 a	0.69b	9.74 a	0.52 b	0.41 a	0.88 a	0.75 a	0.61 a
Tiodoase	14.41 c	9.39 b	1.54 a	9.70 b	6.98 b	0.92a	8.91 b	0.56 b	0.34 a	0.77 b	0.71 a	0.58 a
CV(%)	2.49	2.68	2.27	3.18	4.79	16.09	6.99	15.40	19.33	10.70	21.41	15.26

Means followed by the same letter in the columns do not differ by the Scott-Knott test (p>0.05)

Figure 1 - Pollen grains of *Castanea crenata*, cultivar ‘Taishowase.' (A) Pollen grains obtained by the acetolysis technique, polar (left) and equatorial (right) view; (B) Viable pollen grains (arrow) and non-viable stained by Alexander' dye; (C) Viable pollen grains (arrow); and non-viable stained with the 2% propionic Carmine dye; (D-E) Pollen grains morphology obtained by Scanning Electron Microscopy, aperture (two headed arrow).



In general, the cultivars showed high viability rates (Table 3), with a higher percentage (90.88%) for the cultivar 'Morioase', the lowest (51.42%) for the hybrid 'Km2'. According to Techio et al. (2006), pollen viability can vary between species and even in samples from the same individual, supporting the information as mentioned above.

The values verified in the analyzes were close to the rates described by Beyhan and Serdar (2008), (61-93%) and Cêpeda (2014) (24.1-55.3%), both performed with *Castanea sativa*. Viability may vary, due to factors such as climatic conditions and genotype (Shivanna

and Rangaswamy, 1992) Furthermore, the loss of viability is continuous, and germination will depend on conditions, if favorable for development (Dafni and Firmage, 2000).

Table 3 - Percentage of the pollen viability of *Castanea crenata* cultivars.

Cultivar/hybrids	GV (%)*	
	Alexander	Carmim
Ibuki	67.47 Ab	94.51 Aa
Isumo	75.91 Aa	67.45 Aa
Kinshu	63.53 Ab	67.16 Aa
Km1 (hybrids)	79.33 Aa	74.17 Aa
Km2 (hybrids)	51.42 Ab	22.47 Bb
Morioase	90.88 Aa	97.13 Aa
Okuni	80.58 Aa	80.24 Aa
Senri	84.98 Aa	68.65 Aa
Tamatsukuri	78.36 Aa	89.56 Aa
Tiodoase	88.01 Aa	78.97 Aa
CV(%)	13.97	

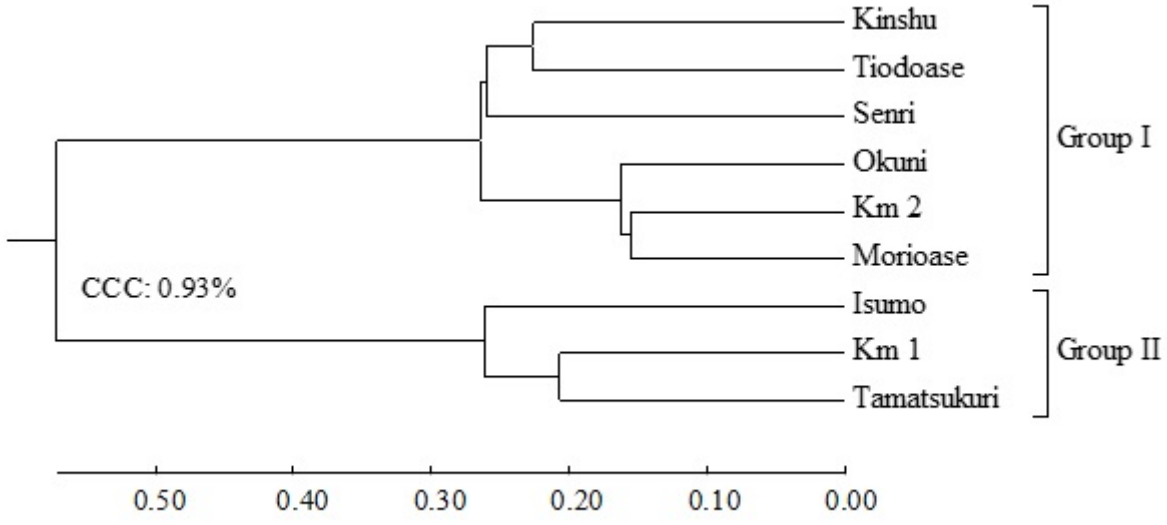
*Capital letters compare data among cultivars (column) and lower letters, between dyes (lines). Means followed by the same letter, do not differ statistically from each other by the Scott-Knott Test ($p < 0.05$).

The dyes were useful in distinguishing viable and non-viable pollen grains (Figure 1B-C), emphasizing that the efficiency depends on the penetration of the dye in the pollen grain, since very thick walls or those with the presence of ornamentation, can prevent dye penetration,

compromising the staining (Techio et al. 2006). In general, Alexander dye has different constituents with basic and acidic chemical properties that differentially stain the cell wall and protoplasm, discriminating viable and non-viable pollen grains with more excellent reliability (Alexander, 1980). However, there was no difference in the viability data obtained with both dyes, except for the cultivars ‘Ibuki’ and ‘Kinshu’.

The cluster analysis based on the Gower index and grouping by UPGMA showed a cophenetic correlation coefficient of 0.93 with the formation of two groups (Figure 2) considering the cut-off line suggested by Mojena (1977). The average dissimilarity was 0.391, with the cultivars ‘Morioase’ and ‘Km2’ being the least dissimilar (0.155). On the other hand, the cultivars ‘Morioase’ and ‘Kinshu’ were the most dissimilar (0.696).

Figure 2 - Dendrogram of genetic dissimilarity, cultivars of *Castanea crenata*, established by the UPGMA method, using the Gower distance based on 12 morphological characters of the pollen grain.



The diversity results allow inferring about the genetic variability that exists between the groups formed since it exists within more similar cultivar groups and between the different cultivar groups. This information may contribute to and guide future research strategies. The exploration of this variability can happen through the selection of cultivars from groups 1 and 2 with higher averages for the main morphological characteristics of the pollen grain.

4. Conclusion

1. There is no morphological difference between pollen grains of androgynous and staminate origin.

2. The pollen grains of *Castanea crenata*, are prolate, isopolar, tricolpate, with long constricting colpi, heteroapertured, zonocolpate, and with psilate (smooth) exine.

3. The cultivars and hybrids of *C. crenata* have high rates of pollen viability, with emphasis on the cultivar 'Morioase'.

Acknowledgments

The authors would like to thank the Brazilian National Council for Scientific and Technological Development- CNPq (140247/2018-1), Foundation for Research Support of the State of Minas Gerais – FAPEMIG, and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

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ARTICLE 3 - Productive performance of chestnut trees for cultivation in tropical regions

(Published, 2019 at Journal of Agricultural Science)

ARTICLE 3 -Productive performance of chestnut trees for cultivation in tropical regions

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Abstract

Chestnut trees are economically exploited in temperate regions of Asian countries, Oceania and the European Iberian Peninsula. Chestnuts are sources of protein and carbohydrates and are eaten in many ways, mainly roasted during cold temperatures. Chestnuts may be suitable for cultivation in tropical regions, but to do this, the potential cultivars' productive performances should be quantified. Thus, this study determined the performances of chestnut cultivars in a tropical region using the Taishowase, Isumo, Tamatsukuri and Okuni cultivars. The cultivars' phenological development, including the beginning and end of flowering and harvesting, as well as each cultivar's nut production were evaluated over four production cycles. The Taishowase and Okuni cultivars were shown to be the most promising for tropical regions because they showed greater adaptability and production stability.

Keywords: *Castanea* spp., productivity, genotype × environment interaction

1. Introduction

Chestnut trees belong to the Fagaceae family and the *Castanea* genus, which comprises seven species, including *C. sativa* Miller, *C. crenata* Siebold & Zucc., *C. molissima* Blume and *C. dentata* (Marsh.) Borkh. (Bueno & Pio, 2014). These species were named for their place of origin and are known as the Portuguese chestnut (Portugal), Japanese chestnut (Japan and South Korea), Chinese chestnut (China) and American chestnut (North America), respectively (Yamanishi et al., 2010).

Chestnuts are widely enjoyed for their nutritional characteristics, especially in countries with temperate climates (Kan et al., 2017; Benedetti et al., 2018). Chestnuts contain high-quality protein and little fat (3% of the total weight) and are gluten-free. They also contain vitamin C, potassium, low sodium and approximately 45% starch, in addition to fatty acids, which can vary among species and cultivars (Pio et al., 2014).

In South America, chestnuts are mainly imported from Portugal (98.5%), with a small

percentage from Spain (1.5%) (Bueno & Pio, 2014). Chestnut trees require mild climate and adequate moisture for growth and good productivity (Muzaffar et al., 2016). When exposed to high temperatures, they reduce their photosynthetic activity by 60% during the day (Gomes-Laranjo et al., 2006), demonstrating their potential for cultivation in tropical regions. In regions with higher temperatures, the chestnuts' antioxidant activity is decreased (Dinis et al., 2012), but no other losses in chemical properties, such as sugars or proteins, are observed (Pio et al., 2014).

Production of temperate fruit trees in tropical regions is challenging due to the low cooling conditions, but some cultivars have lower thermal requirements; thus, cultivars must be selected for each macroregion (Pio et al., 2019). Some commercial orchards in the highlands in Brazil demonstrate potential for cultivating *Castanea crenata* × *Castanea* sp. hybrids (Pio et al., 2017), but no data exist in the literature on chestnut production in tropical regions.

To select more stable individuals with higher productive performances, studies on the genotype × environment (GE) interaction play important roles in determining a genotype's adaptation and suitability to the environment and thus may aid in selecting chestnut cultivars for tropical regions. Therefore, this study determined the performance of chestnut cultivars in a tropical region.

2. Method and Methods

The study was conducted in the municipality of Lavras, Minas Gerais state, Brazil, located at 21°14' south latitude and 45°00' west longitude, at an average altitude of 918 metres, from September 2011 to February 2018. Based on the Köppen classification, the regional climate is type Cwb (mesothermal or tropical highland climate), with dry winters and rainy summers.

Seedlings aged 15 months from the Taishowase, Isumo, Tamatsukuri and Okuni cultivars and grafted on Taishowase rootstock were acquired from a commercial nursery and planted in the field in September 2011, spaced 4 m between rows and 5 m between trees (density of 500 trees per hectare). The experiment was conducted in randomized blocks, containing four treatments (cultivars), with five blocks and two useful trees per experimental unit.

Throughout the experiment, weeds were controlled in the experimental area, and compost (3 L per tree) was applied twice, once in June and once in November of each cultivation year. The compost was a 3:1 mixture of decomposed plant material to cattle manure. Soil was analysed

during the four cultivation years, revealing the following parameter values: 5.7 pH, 46.3 g.dm^{-3} organic matter, 136.9 mg.dm^{-3} phosphorus, 10.1 mmolc.dm^{-3} calcium, 3.1 mmolc.dm^{-3} magnesium, 14.2 sum of bases and 15.5 cation exchange capacity. For plant maintenance, 300 g of ammonium sulphate was applied, divided into two applications (one in October and one in January), plus 200 g of simple superphosphate and 200 g of potassium chloride applied in September and 150 g of lime per plant applied in May.

In the 2014/15, 2015/16, 2016/17 and 2017/18 production cycles, the chestnut cultivars' phenology was described, including the beginning and end of flowering and harvesting. The flowering and harvesting durations were calculated in days. To quantify the productive performance during the four production cycles, the cupules were harvested every four days per plot, and the nuts were removed, counted and weighed. The total number of nuts, mean nut weight, production (kg tree) and estimated yield (kg ha^{-1}, considering a density of 500 trees per ha) were calculated at the end.

The interaction between years and cultivars was viewed using the genotype and genotype × environment (GGE) biplot method (genotype main effect and genotype by environment interaction) as described by Yan et al. (2000) and analysed per Oliveira et al. (2010) using the R program, considering the simplified model of two principal components (Equation 1):

$$\bar{Y}_{ij} - \mu_j = \lambda_1 \gamma_{i1} \alpha_{j1} + \lambda_2 \gamma_{i2} \alpha_{j2} + \rho_{ij} + \bar{\varepsilon}_{ij},$$

where $\lambda_1 \gamma_{i1} \alpha_{j1}$ is the first principal component (PCA1) of the effect of the genotypes (G) + the interaction (G×E); $\lambda_2 \gamma_{i2} \alpha_{j2}$ is the second principal component (PCA2) of the effect of the genotypes (G) + the interaction (G×E); λ_1 and λ_2 are the eigenvalues associated with PCA1 and PCA2, respectively, and γ_{i1} and γ_{i2} are the scores of PCA1 and PCA2, respectively, for the genotypes; α_{j1} and α_{j2} are the scores of PCA1 and PCA2, concomitantly for the environments; ρ_{ij} is the residue of the genotype × environment interaction, corresponding to the principal components not retained in the model; and $\bar{\varepsilon}_{ij}$ is the residue of the model with normal distribution, zero mean, and variance σ^2/r (where σ^2 is the variance of the error between plots for each environment, and r is the number of replications). Statistical analyses were conducted as split plots in time, with time (production cycles) constituting the main plots and the cultivars constituting the split plots.

The data were submitted to analysis of variance, and the averages were compared via Tukey's test ($p \leq 0.05$) using the Genes Program Software for PCs (Cruz, 2013). Adaptability and stability were analysed using GGE biplot methodology (Yan & Rajcan, 2002), and the graphs were generated using R Program software for PCs.

3. Results and Discussion

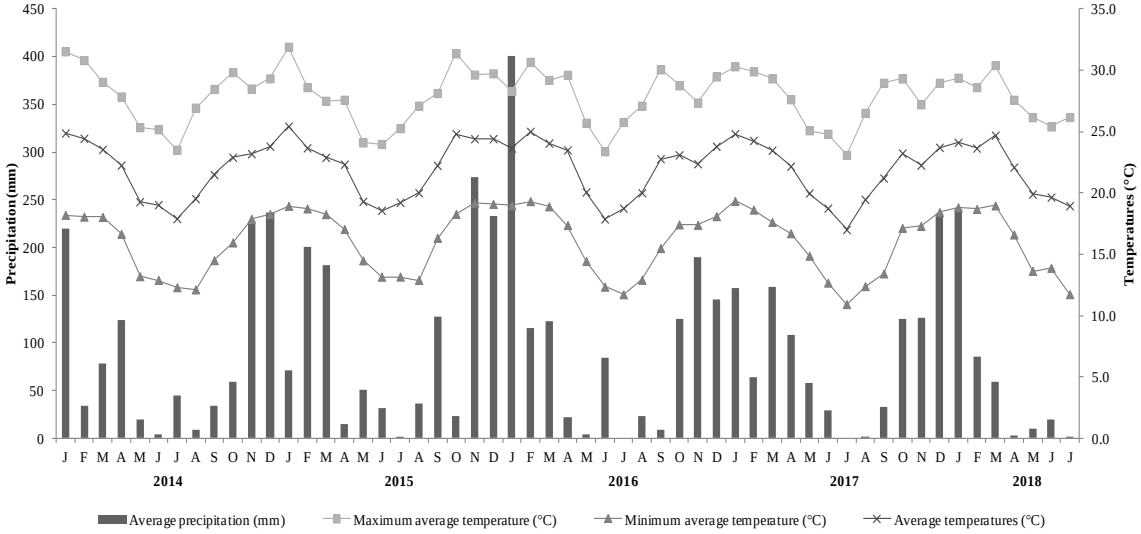
In the first production cycle (2014/15), the flowering began in the second half of July and ended in early October, and harvesting began in the first half of November until the end of January of the following year (Table 1). The flowering period duration was very short in the Isumo cultivar, which flowered between the beginning of August and the second half of September, leading to a short harvest period (45 days). This finding evidences this cultivar's lack of adaptation to mild winter climatic conditions.

Table 1. Phenological description: beginning the flowering (BF), end the flowering (EF), duration of flowering (DF), beginning harvest (BH), end the harvest (EH), duration of harvest (DH) of four chestnut cultivars (*Castanea crenata* × *Castanea* sp.) in four production cycles.

Cultivars	BF	EF	DF (days)*	HB	EH	DH (days)*
			Productive year 2014/15			
Taishowase	20/07	08/10	80 Ca	28/11	27/01	60 Ba
Isumo	01/08	20/09	50 Bb	21/11	05/01	45 Aa
Tamatsukuri	23/07	25/09	64 Bab	20/11	22/01	63 ABa
Okoni	03/08	16/09	75 Ba	07/11	12/01	66 ABa
			Productive year 2015/16			
Taishowase	20/07	11/11	114 Ba	26/11	27/01	63 Ba
Isumo	05/08	19/10	75 Bbc	20/11	22/12	52 Aa
Tamatsukuri	23/07	22/09	61 Bc	12/11	23/01	72 ABa
Okoni	30/07	01/11	95 Aab	18/11	26/01	69 ABa
			Productive year 2016/17			
Taishowase	15/08	23/11	100 BCa	22/11	30/03	130 Aa
Isumo	23/08	24/10	63 Bb	22/11	13/01	53 Ab
Tamatsukuri	19/08	19/10	61 Bb	05/11	23/01	80 Bb
Okoni	23/08	08/11	78 Bb	05/11	02/02	90 Bb
			Productive year 2017/18			
Taishowase	20/07	13/02	182 Aa	30/11	09/04	130 Aa
Isumo	13/07	10/11	120 Ab	17/11	26/01	72 Ab
Tamatsukuri	13/07	30/10	109 Ab	20/08	23/11	95 Ab
Okoni	13/07	21/10	101 Ab	17/11	18/02	93 Ab
CV (%) Subplots			13.2			28.6
CV (%) Plots			13.3			28.1

* Uppercase letters compare the production cycles, and lowercase letters compare the cultivars; means followed by the same uppercase or lowercase letters do not differ significantly by Tukey's means comparison test ($P \leq 0.05$).

In the second production cycle (2015/16), flowering began early and was concentrated between the end of July and beginning of August (Table 1). The early flowering may have been due to the trees' maturity or the climate because during the production cycle, the mean maximum temperatures were higher for July and August (Figure 1). Consequently, the harvest period was extended and continued until the end of January in the Taishowase and Okuni cultivars. Per



Medina Mora et al. (2018), chestnut cultivar flowering periods naturally vary between years.

Figure 1. Mean minimum and maximum temperatures and cumulative rainfall for the production cycles.

In the third production cycle (2016/17), all cultivars began flowering late in the second half of August (Table 1). However, the Taishowase and Okuni cultivars showed a greater flowering range. Harvesting these cultivars began in November and persisted until the end of the last half of March. A prolonged flowering period may not favour cultivar development because of the greater exposure to climatic adversities such as temperature drops and water deficit (Souza et al., 2013).

In the fourth production cycle (2017/18), flowering began early (Table 1). The end of the flowering period was more prolonged, which helped increase the flowering duration, which persisted between 101 days (Okuni) and 182 days (Taishowase). The beginning of the harvest period varied by cultivar, with the earliest cultivar harvest (Tamatsukuri) beginning in the second half of August and the latest cultivar harvest (Taishowase) at the end of November. Pio et al. (2014) studied the dehiscence and beginning and end of the harvest for different chestnut cultivars in highland regions and found that the Tamatsukuri cultivar was considered an early

cultivar whose harvest began in the second half of October. Notably, the late cultivar Taishowase showed the same behaviour in all evaluated production cycles, with harvesting always beginning in the second half of November. This finding evidences the Taishowase cultivar's adaptation to the edaphoclimatic conditions of tropical regions. This longer harvesting period is important because hot summers and climatic events, such as excessive rains, concentrated at the end of the year can damage fruits and impair harvests, as occurred in the production cycles (Figure 1).

During the chestnut's annual cycle, flowering is the most critical phase. Chestnuts are monoecious, with staminate (male) and pistillate (female) flowers on the same tree (Vossen, 2000). Mixed catkins sprout from the one-year-old lignified branches. Staminate catkins grow from the basal shoot, while bisexual catkins grow more towards the shoot tip. These bisexual catkins will have one to three pistillate flowers at their bases, with the remaining flowers being staminate. Each pistillate flower differentiates into three pistils. If pollinated and fertilized, the ovaries of all three will developed flattened nuts; if only one is pollinated, a larger, rounder nut will develop (Pio, 2018).

Under the tropical conditions in Brazil, chestnut trees begin flowering in late winter and the beginning of spring based on the species and cultivar (Pio, 2018). Some cultivars may release pollen before the pistillate flowers are receptive, requiring cross-pollination (Medina Mora et al., 2018). Thus, pollinator trees must be cultivated and appropriately arranged in the orchard with a fully or partially synchronized flowering period to obtain satisfactory productivity.

Thus, given the overlapping flowering period, the Okuni cultivar can be used as a pollinator and Taishowase as a commercial cultivar in commercial chestnut orchards. Of note, flowering periods may vary depending on the year, place, farming practices and, especially, edaphoclimatic conditions.

Regarding the productive performance variation in the evaluated production cycles (Table 2), the Taishowase cultivar differed significantly in the mean number of nuts in all cycles, followed by the Tamatsukuri cultivar, which reached its best potential in the 2017/18 cycle. The Okuni cultivar had the highest mean nut weight in all production cycles, followed by the Isumo cultivar in the 2016/17 and 2017/18 cycles. Mean nut weight is an important criterion in cultivar selection (Zenginbal et al., 2018). In this sense, the Okuni, Tamatsukuri and Isumo cultivars presented higher mean nut weights over the production cycles, although all cultivars exceeded

a mean of 5 to 10 g, as discussed by Zenginbal et al. (2018).

For the variables production per tree and estimated yield, the Taishowase cultivar obtained the best productive performance, production of 5.2 kg of nuts per tree and an estimated yield of 2,585.7 kg ha^{-1} for all study cycles, followed by the Tamatsukuri and Okuni cultivars in only the 2017/18 cycle.

Vossen (2000) indicated that pollen patterns can influence pollinated nuts, especially regarding fruit weight, due to the xenia phenomenon. That is, pollen from a tree with well-developed nuts can alter its receptor's genetic characteristics, leading to qualitative and quantitative changes in nut size. Thus, the finding that the Okuni cultivar's mean nut weight excelled across all the production cycles reinforces the indication for using this cultivar as a pollinator in commercial orchards.

The GGE biplot was analysed for the estimated yield data of the different chestnut cultivars, using principal components and the modified centroid method (Figure 2). Figure 2A illustrates the best genotype performance over the years (i.e., 'which-won-when'). Yan and Kang (2003) proposed the polygon view of a biplot to visualize the genotype and environment interaction. The genotype in the vertex of the polygon is the winner in the years falling in this sector.

The lines perpendicular to each side of the polygon are lines of equality between adjacent genotypes (Yan; Tinker 2006). The equality line is useful for comparing genotype performance in a year. For example, the equality line between Tamatsukuri and Taishowase (Figure 2A) indicates that Taishowase was better in all years except 2017/18. Among the three vertex cultivars, Taishowase, Tamatsukuri and Isumo, most production cycles were in the Taishowase cultivar sector, reinforcing this cultivar's performance in most of the years evaluated.

Subsequently, the cultivars' adaptability and stability regarding estimated yield was evaluated based on their distances from the "ideal genotype" (Figure 2B). The centre of the concentric circles represents the ideal genotype position (Yan; Tinker 2006). The Taishowase and Okuni cultivars are closer to the concentric circle; therefore, they are considered the most desirable in terms of average performance and yield stability. Conversely, Isumo was farthest from the ideal genotype position, thus being the most unstable.

Table 2. Total number of nuts, mean nut weight, production (kg/tree) and estimated yield (kg/ha) of four chestnut cultivars (*Castanea crenata* × *Castanea* sp.) in four production cycles.

Cultivars	2014/15	2015/16	2016/17	2017/18	General average**
			Total number of nuts*		
Taishowase	189.6 Da	290.8 Ca	508.4 Ba	596.2 Aa	396.3 a
Isumo	37.4 Bb	27.4 Bc	19.2 Bc	207.2 Ac	72.8 d
Tamatsukuri	71.4 Bb	84.2 Bb	57.0 Bbc	563.2 Aa	194.0 b
Okuni	46.8 Bb	52.6 Bbc	100.4 Bb	377.2 Ab	144.3 c
CV (%)Subplots		13.3			
CV (%) Plots		14.8			16.5
			Mean nut weight (g)		
Taishowase	10.6 Ac	11.8 Ac	13.2 Ab	14.2 Ab	12.5 a
Isumo	17.2 Aab	18.0 Aab	19.0 Aa	19.4 Aa	18.4 a
Tamatsukuri	14.8 Ab	15.2 Ab	15.6 Ab	16.0 Ab	15.4 a
Okuni	20.0 Aa	20.6 Aa	20.8 Aa	21.8 Aa	20.8 a
CV (%)Subplots		10.3			
CV (%) Plots		17.5			2.9
			Production (kg tree)		
Taishowase	2.0 Ba	3.5 Ba	6.6 Aa	8.6 Aa	5.2 a
Isumo	0.6 Bb	0.5 Bb	0.4 Bc	4.0 Ab	1.4 b
Tamatsukuri	1.1 Bab	1.3 Bb	0.9 Bbc	9.0 Aa	3.1 ab
Okuni	0.9 Bab	1.1 Bb	2.1 Bb	8.3 Aa	3.1 ab
CV (%)Subplots		23.0			
CV (%) Plots		26.1			26.9
			Estimated yield (kg ha^{-1})		
Taishowase	1006.9 Ba	1752.6 Ba	3300.4 Aa	4282.9 Aa	2585.7 a
Isumo	316.9 Bb	247.7 Bb	182.6 Bc	2010.5 Ab	689.4 c
Tamatsukuri	530.2 Bab	640.9 Bb	444.8 Bc	4496.0 Aa	1528.0 b
Okuni	466.6 Bab	547.8 Bb	1046.1 Bb	4142.6 Aa	1550.8 b
CV (%)Subplots		23.0			
CV (%) Plots		26.1			26.9

* Means followed by the same uppercase letter in a row and lowercase letter in a column do not differ significantly by Tukey's means comparison test ($P \leq 0.05$).

** Means followed by the same letter do not differ significantly by Tukey's means comparison test ($P \leq 0.05$).

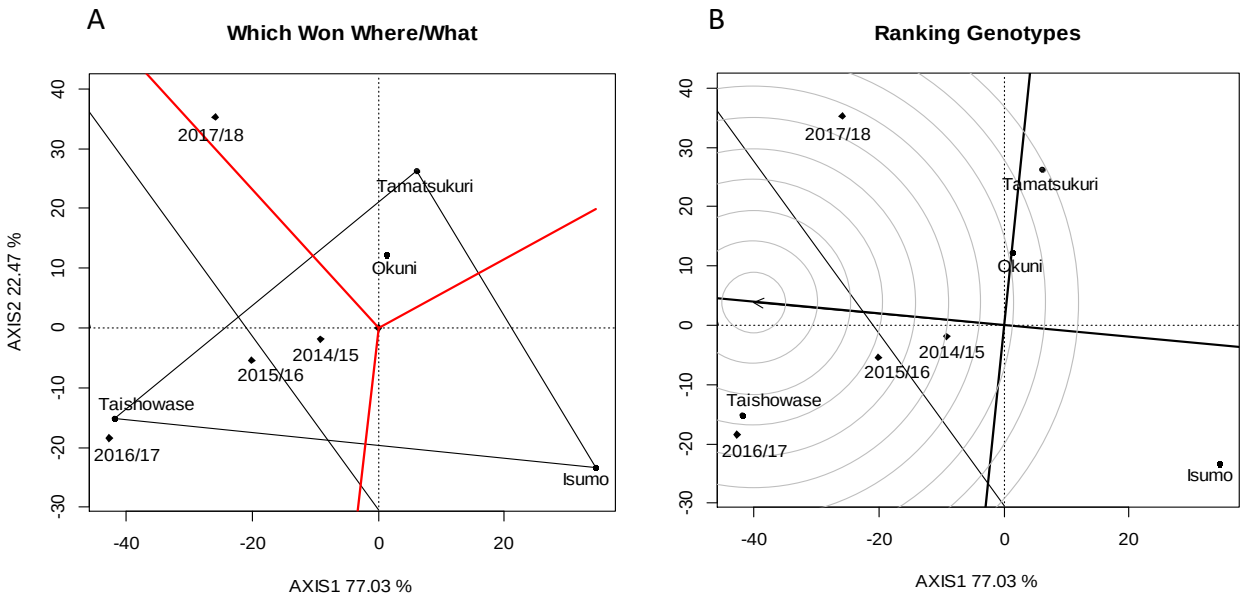


Figure 2. GGE biplot showing the winning genotypes relative to production years (A) and comparison of genotypes with the ideal genotype (B).

Under mild winter conditions, cultivars from temperate regions can vary greatly from one cycle to another in their flowering period and productive performance, which can be attributed to the instability of biotic and edaphoclimatic factors during the cycle, characteristic of tropical regions (Petri et al., 2008; Bettiol Neto et al., 2011). Dinis et al. (2010) suggest that pollination and embryonic growth are strongly influenced by climatic conditions that vary from site to site at different altitudes and over years, often being the main cause of production variability in terms of chestnut tree quantity and quality.

4. Conclusions

Chestnut harvesting is concentrated between the first half of November and the second half of April, with Tamatsukuri being the earliest cultivar and Taishowase being the latest.

The Taishowase and Okuni cultivars are the most promising for tropical regions because they showed greater adaptability and productive performance stability.

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