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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (BIOLOGIA VEGETAL)

Respostas Fisiológicas e Morfológicas de Sementes de Palmito Juçara (*Euterpe edulis* Mart.) à Luz, Temperatura, Desidratação e Aplicação de Giberelinas.

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Dissertação apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Biologia Vegetal).

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Dedico à minha família, grande representante desta que é a maior das Instituições.

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RESUMO

Euterpe edulis (Mart.), ou palmiteiro juçara, é uma espécie-chave da mata Atlântica ameaçada de extinção devido à alta taxa de corte não seletivo. Seu desenvolvimento a partir da germinação é muito lento, resposta típica das espécies da família Arecaceae. Visando melhorar sua reposição no meio natural, objetivou-se entender e otimizar a germinação de sementes de *E. edulis*. No primeiro estudo objetivou-se determinar: condições ótimas de temperatura e luz (claro X escuro); o início da fase de retomada do metabolismo da curva de embebição, para a aplicação de tratamentos promotores de germinação; e a resposta à aplicação de hormônios durante a germinação. Foi observado que a aplicação de 50 mg L^{-1} de ácido giberélico (GA_3), aos 12 dias após a semeadura (DAS), a 25ºC no escuro, aumentou o desempenho germinativo (porcentagem, velocidade e sincronização da germinação) de sementes de *E. edulis*. Não foram observados indícios anatômicos de divisão e/ou alongamento celular em sementes 15 dias após o tratamento com GA_3. De posse dessas informações, no segundo estudo foram testadas as hipóteses: (i) sementes de *E. edulis* apresentam maior desempenho germinativo quanto mais bem hidratado for o tecido; (ii) por serem recalcitrantes, sementes de *E. edulis* têm desenvolvimento contínuo; logo, é indiferente o momento (0 ou 12 DAS) da germinação em que é aplicado GA_3; (iii) alterações anatômicas indicativas de crescimento embrionário só podem ser observadas após 15 dias da aplicação de GA_3; (iv) de que o maior desempenho germinativo de sementes de *E. edulis* tratadas com GA_3 é resultado da ação hormonal indutiva da mobilização de reservas. Através de testes de germinação, de quantificação de reservas e anatomia das sementes respostas fisiológicas a perda de água e a aplicação de GA_3, foram confirmadas as hipóteses (i), (ii) e (iii). A hipótese (iv) não pôde ser confirmada. Conclui-se que, apesar de a característica de lento desenvolvimento do embrião de *E. edulis* ser mantida, a aplicação exógena de GA_3 em qualquer fase da curva de embebição acelera a germinação de sementes de *E. edulis*, podendo ser um alento à degradação da espécie.

ABSTRACT

Euterpe edulis (Mart.), or jussara palm, is a key species from the Atlantic forest threatened to extinction due to the high rate of non-selective cutting. His development from germination is very slow, a typical response of species from the Arecaceae family. In order to improve its natural replacement on the environment, this study aimed to understand and optimize the germination of *E. edulis* seeds. In the first study was determined: the optimal conditions of temperature and light (light X shade), the start of metabolism resumption of imbibition curve, when seeds could be treated for germination enhancement and the germination response to hormone application during germination. It was observed that the application of gibberellic acid (GA_3) 50 mg L^{-1}, 12 days after sowing (DAS), 25°C in the dark, increased germination performance (percentage, speed and synchronization of germination) of *E. edulis* seeds. There was no anatomical evidence of division and / or cell elongation in seeds 15 days after treatment with GA_3. With this observations, the second study tested the hypotheses: (*i*) seeds of *E. edulis* have higher germination performance as much better hydrated is the tissue, (*ii*) being recalcitrant, seeds of *E. edulis* have continuous development, therefore it is irrelevant the moment (0 or 12 DAS) of germination in with GA_3 is applied, (*iii*) anatomical abnormalities indicative of embryonic growth can only be observed after 15 days from the application of GA_3, (*iv*) that the greatest performance of germination of *E. edulis* with GA_3 is the result of inductive hormonal mobilization of reserves. Physiological responses to water loss and application of GA_3 were obtained through germination tests, quantification of reserves and seed anatomy. The hypotheses (*i*), (*ii*) and (*iii*) were confirmed. The hypothesis (*iv*) could not be confirmed. We conclude that although the maintenance of the characteristic of slow development of embryo, the exogenous application of GA_3 at any stage of imbibition curve accelerates the germination of *E. edulis* seeds, being a boost to the degradation of the species

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1. INTRODUÇÃO

Euterpe edulis (Mart.), ou palmiteiro juçara, é uma espécie ecologicamente chave da mata Atlântica. Encontra-se na lista de espécies ameaçada de extinção devido à alta taxa de corte não seletivo. Seu desenvolvimento a partir da germinação é muito lento, resposta típica das espécies da família Arecaceae.

Dadas as características morfofisiológicas de suas sementes, sua dificuldade de propagação natural, além de sua depredação não seletiva, esta espécie chave da mata Atlântica encontra-se ameaçada de extinção, carecendo de medidas que possam auxiliar na propagação mais eficiente da espécie.

Existem poucos trabalhos publicados sobre o balanço hormonal da semente, germinação e estabelecimento de plântulas de *E. edulis*. Isso justifica estudos sobre esses assuntos para esta espécie, visto que a aceleração na propagação dos palmiteiros poderia ser um alento à sua degradação. Assim, poder-se-ia melhorar sua reposição no meio, através de cultivo artificial como uma ferramenta de manejo sustentável, preservando a espécie e a flora e fauna a ela associadas.

Em um primeiro estudo, objetivou-se (i) testar condições de luminosidade (claro *versus* escuro) e de temperatura em que ocorre maior desempenho germinativo de sementes (frutos despolpados) de *E. edulis*. Nessas condições, determinou-se ainda (ii) a curva de embebição de sementes da espécie, com o intuito de se (iii) testar diferentes concentrações de ácido giberélico (GA_3) aplicado no momento da retomada da hidratação de sementes de *E. edulis* (início da fase estacionária – fase II – da curva de embebição), para que os efeitos do GA_3 fossem os mais eficientes na obtenção de maior desempenho germinativo.

Diante das informações obtidas no primeiro estudo, que indicavam que a embebição inicial das sementes (fase I da curva de embebição) poderia ser uma reidratação de correção de frutos dispersos, em um estudo subsequente, foi testada a hipótese de que (i) sementes de *E. edulis* apresentam maior desempenho germinativo quanto mais bem hidratado for o tecido. Além disso, nesse segundo estudo, hipotetizou-se que (ii) por serem recalcitrantes, sementes de *E. edulis* têm desenvolvimento contínuo, sendo indiferente a fase da germinação em que GA_3 é aplicado. Também, como no primeiro estudo não houve indícios anatômicos de divisão e/ou alongamento celular embrionário após a aplicação de GA_3 na semeadura ou aos 12 dias após a semeadura, no segundo estudo verificou-se se (iii) alterações anatômicas indicativas de crescimento embrionário só podem ser observadas após 15 dias da aplicação de GA_3.

Investigou-se ainda se (iv) o maior desempenho germinativo de sementes de *E. edulis* tratadas com GA_3 é resultado da ação hormonal indutiva da mobilização de reservas (lipídeos e carboidratos) das sementes.

As hipóteses foram testadas medindo-se o desempenho germinativo (porcentagem de sementes germinadas, tempo e velocidade de germinação e índice de sincronização da germinação) de sementes com diferentes umidades iniciais e embebidas em GA_3 (50 mg L^{-1} – dosagem definida pelos resultados do primeiro estudo) em diferentes momentos do processo de germinação. Avaliações anatômicas do endosperma e do embrião e quantificação de reservas também foram realizadas em vários momentos do processo de embebição e germinação de sementes tratadas e não tratadas com 50 mg L^{-1} de GA_3.

2. REVISÃO BIBLIOGRÁFICA

A Floresta Atlântica e o palmiteiro juçara como espécie chave

E. edulis, de reprodução sexuada, necessita de seis a nove anos para a produção de sementes e sua exploração atual reduz drasticamente a regeneração natural, provocando seu desaparecimento da floresta (NODARI & GUERRA, 1986). A espécie é muito explorada porque seu único palmito (meristema apical comestível) é apreciado como iguaria. Para consegui-lo é preciso cortar a planta, que é incapaz de perfilar. Seu corte é ilegal, mas muitas quadrilhas vendem o produto para restaurantes (GALETTI & FERNANDEZ, 1998). Isso é feito de forma não-seletiva, atingindo indivíduos adultos produtores de sementes, mas também plantas jovens com mais de dois metros (ANDRADE & PEREIRA, 1997).

E. edulis é considerada uma espécie-chave, porque associa a sobrevivência de espécies da fauna e da flora, devido a sua frutificação abundante e seu pericarpo possuir alta concentração de carboidratos, fornecendo alimento para cerca de 30 espécies de aves e 13 de mamíferos (DRANSFIELD et al., 1988). Contudo, as sementes de *E. edulis* mostram características morfofisiológicas que dificultam sua sobrevivência e propagação. A redução do grau de umidade da semente a valores inferiores a 35% diminui a porcentagem de germinação (ANDRADE et al., 1996). Tais resultados classificam suas sementes como recalcitrantes, o que é confirmado por Panza et al. (2004). Ao serem desidratadas abaixo de 12 a 30% de umidade, perdem a viabilidade e mesmo armazenadas em condições úmidas, mostram longevidade que varia de algumas semanas a poucos meses (ANDRADE & PEREIRA, 1997).

Em sementes recalcitrantes, a dessecação desencadeia alguns processos como a desnaturação de proteínas, alterações na atividade das enzimas peroxidases e danos no sistema de membranas, resultando na completa perda de sua viabilidade (BERJAK & PAMMENTER, 1997; NAUTIYAL & PUROHIT, 1985). Em geral, sementes de palmeiras são consideradas de curta longevidade e frequentemente perdem sua viabilidade após períodos de duas semanas a três meses (DE LEON, 1958).

A Propagação e a vulnerabilidade de *E. edulis*

A curta longevidade restringe o prazo de utilização das sementes de *E. edulis*, exigindo-se que a semeadura artificial seja feita logo após sua extração dos frutos (STUBSGAARD, 1990). A ocorrência de adversidades ambientais entre a frutificação e a dispersão dos frutos, tais como geadas e secas, pode diminuir a oferta de plântulas na mata,

em virtude das dificuldades de manutenção do banco de sementes (FONSECA & FREIRE, 2003). Os frutos maduros caem abaixo da palmeira num raio não maior do que 2 m do estipe. Logo, em condições favoráveis, inúmeras plantas aparecem ao pé da planta. Poucas, porém, sobrevivem e tornam-se adultas (BOVI et al., 1992), devido à competição por água, nutrientes e luz.

A proporção de sementes germinadas em condições de mata é, em média, de 50% (BOVI et al., 1992). Essa baixa germinação, se comparada a resultados de laboratório e viveiro, deve-se, principalmente, a estresses hídricos (dispersão de frutos entre março e agosto), manutenção da polpa envolta à semente e à ação de microorganismos, insetos e pequenos animais, especialmente os roedores.

A polpa mantida ao redor da semente pode conter inibidores da germinação, como compostos fenólicos, diminuindo a velocidade de germinação. A polpa afeta ainda a porcentagem final de emergência por ser um substrato rico para o desenvolvimento de microorganismos. Em condições naturais, a germinação leva de três a seis meses para se completar. No entanto, quando é feito o despolpamento do fruto, o processo germinativo se completa de 60 a 90 dias (BOVI et al., 1992) . Em seu hábitat, o despolpamento ocorre com o auxílio de pássaros que comem a sua polpa e regurgitam a semente completamente limpa (PIZO & SIMÃO, 2001).

Além do dessecamento, sementes de *E. edulis* mantidas em ambientes com temperaturas iguais ou inferiores a 15°C têm baixa viabilidade (OLIVEIRA et al., 2002) e o desempenho germinativo é diminuído significativamente ROBERTO & HABERMANN, 2010). Temperaturas de 30-20ºC (dia-noite) e 25ºC (constante) promovem alta porcentagem de germinação e velocidade de emergência de plântulas normais (ANDRADE et al., 1999). Sementes de *E. edulis* também mostram o maior desempenho germinativo quando germinadas a 25ºC (constante), independentemente de germinarem no claro ou no escuro (ROBERTO & HABERMANN, 2010).

Desenvolvimento do embrião e balanço hormonal

Embriões da família Arecaceae parecem ter estrutura primitiva por ocasião da dispersão de sementes, não apresentando tecido discernível e mostrando-se como cilindros de tecido não-diferenciado (TOMLINSON, 1960). Embora o desempenho germinativo natural seja baixo, acredita-se que a semente madura de *E. edulis* se apresente completamente formada e apta para a germinação (BOVI & CARDOSO, 1978). A análise anatômica de embriões de *E. edulis* revelou que estes possuem tecidos diferenciados por ocasião da

dispersão e possui inúmeras mitocôndrias, retículo endoplasmático rugoso e complexos de Golgi, que indicam que o mesmo encontra-se em estado metabolicamente ativo (PANZA et al., 2004). Segundo estes mesmos autores, tais características estão associadas a estratégias de contínuo desenvolvimento, sem a interposição, na maturidade, de uma fase de menor umidade do tecido e baixo metabolismo, como nas espécies de sementes ortodoxas.

Por tratar-se de semente recalcitrante, os entraves da germinação estão possivelmente ligados à perda de umidade e viabilidade da semente, além da característica de desenvolvimento contínuo do embrião. De fato, o endosperma de *E. edulis* aparece em estado quiescente, com lipídios, proteínas, minerais e mananos armazenados como reservas insolúveis, revelando a falta de remobilização de reservas (PANZA et al., 2004).

Especialmente nos cereais, a ação de hormônios vegetais no processo de transferência energética e protéica para o embrião em desenvolvimento foi muito estudado (WOODGER et al., 2004). O endosperma dessas espécies é circundado pela camada celular de aleurona, que contém numerosos corpos protéicos, bem como vesículas armazenadoras de lipídios, os oleossomos. A camada de aleurona é um tecido cuja função limita-se à síntese e à secreção de enzimas hidrolíticas. Estas atuam na quebra dos compostos de reserva da semente durante a germinação, originando açúcares, aminoácidos e outros produtos mais simples transportados ao embrião. As enzimas alfa- e beta-amilase atuam sobre a degradação do amido. A primeira produz oligossacarídios, que são degradados pela segunda, resultando em maltose, que é convertida à glicose, pela maltase (GUERRA, 2004). Estudos com isótopos radioativos evidenciaram que as giberelinas podem aumentar a síntese de alfa-amilase (JACOBSEN et al., 1995).

Além da atuação das giberelinas na ativação de enzimas hidrolíticas, elas também podem promover a divisão e o alongamento celular do embrião. As citocininas também são consideradas promotoras da germinação, uma vez que atuam no ciclo celular, promovendo a divisão mitótica, além de agirem sobre a diferenciação dos tecidos. Já o ácido abscísico (ABA) e compostos fenólicos são conhecidos como inibidores da germinação (BEWLEY & BLACK, 1994; CARDOSO, 2004). Contudo, a germinação pode ocorrer na presença de inibidores desde que a concentração destes seja menor que a de promotores. Essa dormência fisiológica depende do balanço entre promotores e inibidores, podendo ser superada com promotores ou ampliada pela aplicação de inibidores (WEAVER, 1987).

No caso da germinação de *E. edulis*, o uso de 50 mg L^{-1} de ácido giberélico (GA_3) aumentou o desempenho germinativo de sementes de *E. edulis* à 25 ºC no escuro, promovendo maior porcentagem e velocidade de germinação; o uso de benzil aminopurina

(BAP), um tipo de citocinina, não exerceu efeito do desempenho germinativo (ROBERTO & HABERMANN, 2010).

O uso de GA_3 no intervalo de concentrações de 40 a 60 mgL^{-1} confirmou-se como indutor do aumento de desempenho germinativo em vários lotes de sementes de *E. edulis* (ROBERTO & HABERMANN, 2010). Apesar de esses autores terem identificado que o início da fase estacionária da curva de embebição de sementes de *E. edulis* ocorre em aproximadamente 12 dias após a semeadura (DAS), os autores não encontraram indícios anatômicos de divisão e/ou alongamento celular pelo efeito do GA_3 aplicado no momento da semeadura (após dispersão) ou aos 12 DAS, mesmo após 15 dias da aplicação de GA_3. Segundo estes autores, a reidratação ocorrida até os 12 DAS foi apenas corretiva, uma vez que os lotes testados não atingiram umidade de dessecação crítica para as sementes da espécie, estabelecidas por Andrade et al. (1996) e Panza et al. (2004). De fato, sementes de *E. edulis* são recalcitrantes e sementes recalcitrantes não apresentam retomada de atividades metabólicas por ocasião do início da fase estacionária da curva de embebição (PAMMENTER & BERJAK, 1999). Especificamente, o embrião de *E. edulis* possui característica de contínuo e lento desenvolvimento (TOMLINSON, 1960; PANZA et al., 2004; ROBERTO & HABERMANN, 2010).

Os resultados de ROBERTO & HABERMANN (2010) também não indicaram alterações anatômicas na parede e/ou no conteúdo de reserva das células do endosperma após 15 dias da aplicação de GA_3. Isso sugere que o efeito confirmado do GA_3 no aumento do desempenho germinativo possa ser explicado por diferenças anatômicas evidenciáveis somente após 15 dias da aplicação de GA_3; ou ainda, que as diferenças na mobilização de reservas também só possam ser notadas após 15 dias da aplicação de GA_3.

Reservas Nutricionais e a germinação de sementes

Durante a germinação das sementes, são mobilizadas as reservas do endosperma para o desenvolvimento do embrião. Em sementes de Arecacea, o embrião é uma massa indiferenciada de células que se desenvolve a medida que as reservas são mobilizadas lenta e localmente, num processo denominado pós-maturação (PANZA et al., 2004; ROBERTO & HABERMANN, 2010). Nesses casos, o desenvolvimento embrionário não é completado durante a maturação da semente e a principal reserva é o manano de parede celular, que confere dureza e proteção ao embrião em formação. Por apresentarem baixa longevidade e não suportarem dessecação, sementes recalcitrantes necessitam de rápido desenvolvimento. Na maioria das sementes recalcitrantes, o embrião encontra-se em estágio de maturação

proporcionalmente mais desenvolvido, possuindo baixa concentração de reservas de carbono, que se constituem de proteínas e fitinas (reservas de transferência de matéria) (BUCKERIDGE et al, 2004).

O sincronismo entre a mobilização de reservas e o crescimento e desenvolvimento da planta é feito por meio de um sistema de comunicação entre os tecidos que permite aumentar a eficiência do processo como um todo. Durante a germinação há sinalização e transporte hormonal e a mobilização de reservas inicia-se a partir de um sinal que vem do embrião. A degradação das reservas do endosperma inicia-se pela ativação da transcrição dos genes que codificam as hidrolases e a consequente síntese e transporte das enzimas até o local da ação enzimática (BEWLEY & BLACK, 1994).

E. edulis é uma espécie recalcitrante, da família Arecacea. Panza et al (2004) observaram que suas sementes apresentam embrião desenvolvido e apto para iniciar o processo de germinação, porém têm baixas reservas de carboidratos disponíveis (solúveis), além de altos teores de reservas estruturais (mananos).

Desta forma, estudos bioquímicos e anatômicos da composição e mobilização de reservas, associados a sementes tratadas e não tratadas com giberelinas em diferentes momentos do processo de germinação podem auxiliar no entendimento do processo germinativo de sementes de *E. edulis*.

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3. MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSES OF THE RECALCITRANT *Euterpe edulis* MART. SEEDS TO LIGHT, TEMPERATURE AND GIBBERELLINS[1]

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Abstract

Euterpe edulis is a key species of the Atlantic forest, but its seeds exhibit slow germination, which is a limiting factor for the successful establishment of the plant. This study assessed the capacity, time, rate, and synchrony of germination as a function of light *vs.* dark at different temperatures. At 25ºC in the dark, which gave the optimum response, it was possible to note a time representing the beginning of the stationary phase (phase II) of the imbibition curve. We hypothesised that gibberellin (GA_3) and/or cytokinin (benzylaminopurine, BAP) applied at that imbibition time could improve germination performance, accelerating the germination of *E. edulis* seeds, enhancing propagation techniques used in palm tree nurseries, which provide palm plants for the recomposition of degraded fragments in the Atlantic forest. It was confirmed that GA_3 at 50 mg. L^{-1} improved these responses. However, phase II could not be demonstrated as a better time to improve germination performance compared to the time immediately after sowing, since there were no embryo and/or endosperm anatomical differences between these times. This evidence supports published explanations for low germination performance of many palm fruits: primitive structure of the seed embryo and continuous development after fruit dispersion in the shade of the understorey of the Brazilian Atlantic rainforest.

Keywords: Atlantic forest; imbibition curve; jussara palm; seed germination physiology

Introduction

Euterpe edulis or the Jussara palm is considered a key species in the Brazilian Atlantic rainforest because of its abundant fructification; it also produces fruits with high sugar content, valuable as a food resource for birds. *E. edulis* plants are desirable because of the edible apical meristem, but obtaining the meristem requires total plant extraction, rendering *E. edulis* a vulnerable species (DRANSFIELD et al, 1988). The species reproduces sexually and requires six to nine years to fruit. Current usage lowers its natural rate of regeneration (NODARI & GUERRA, 1986).

The successful establishment of a key species in a specific environment depends, primarily, on the ability of its seeds to germinate quickly (GOMES et al, 2006). Under natural conditions, *E. edulis* fruits are dispersed by birds, which regurgitate depulped fruits (PIZO & SIMÃO, 2001; GENINI et al, 2009); but as far as we know, there is no information on germination of depulped *E. edulis* fruits under natural conditions. Under controlled conditions, however, *E. edulis* fruits take three to six months to germinate, and seeds (depulped fruits) complete germination within 60 to 90 days (BOVI et al, 1992). The mean moisture content of *E. edulis* seeds is around 48% (PANZA et al, 2004). Reducing water content of seeds below 35% decreases the germination responses (ANDRADE et al, 1996), classifying the seeds as recalcitrant (ANDRADE & PEREIRA, 1997).

E. edulis seeds generally germinate in the dark, and seem to reproduce best in forest surroundings (ORLANDE et al, 1996), but previous studies have not tested germination in light *vs.* dark. Some authors use constant light for *E. edulis* germination tests (MARTINS et al, 2004), but none have tested the effects of light *vs.* dark on germination responses.

The slow germination of *E. edulis* seeds can be attributed to two factors: the thick and hard mesocarp and endocarp of many palm fruits and the primitive structure of the palm-seed embryo (MULLET et al, 1981). Numerous organelles related to carbon and protein metabolism were observed in the *E. edulis* embryo (PANZA et al, 2004), indicating continuous development, almost without the interposition, at maturity, of a dry state.

Many methods have previously been attempted to accelerate the germination of palm seeds (MULLET et al, 1981). The use of plant growth regulators, such as gibberellins (GA) and cytokinins (Cyt), would contribute largely to accelerate the germination of palm seeds and ameliorate the propagation techniques used in palm tree nurseries, which provide palm plants for the recomposition of degraded fragments in the Atlantic forest.

Some gibberellins are involved in storage mobilisation to the embryo, especially in cereals (WOODGER et al, 2004), but GA are also involved in cell elongation. In addition, Cyt promote embryo growth, controlling the cell cycle and mitosis (FINKELSTEIN, 2004).

For orthodox seeds, any treatment to accelerate germination should be applied after the beginning of the stationary phase (phase II) of the imbibition curve, when seed metabolic activities are reinitiated (FINKELSTEIN, 2004). However, this time is species-dependent and is also influenced by environmental conditions. There may be no second initiation of metabolic activity in the recalcitrant *E. edulis* seeds (PANZA et al, 2004).

We found the optimum light conditions and temperature for *E. edulis* seed germination, and we confirmed that there is a time representing the beginning of phase II of the imbibition curve. It was hypothesised that gibberellic acid (GA_3) or Cyt (benzylaminopurine) applied at the beginning of phase II would improve germination performance, since *E. edulis* seeds might exhibit slow germination due to low GAs and/or Cyt contents. We nonetheless examined the anatomy of seed embryos treated with GA_3.

Materials and Methods

Plant material

Mature fruits of *E. edulis* Mart. were harvested from 10 palm trees growing in fragments of a swamp forest in the municipality of Ajapi (24°00’ S, 75°30’ W), São Paulo (SP) state, Brazil in July 2006. The fruits were transported in closed plastic bags to the laboratory (IB – UNESP), where the assays were conducted.

Fruits were depulped through friction against a steel sieve under water flow. Seeds from the 10 trees were mixed together as one seed lot. Inert materials and fruits that were damaged or smaller than 10 mm in diameter were discarded. For storage, the seed lot was packed in a 20-µm thick, securely closed plastic bag at 8ºC. Seed quality tests were performed before experiments were set up.

Light and temperature experiments

To prevent fungal infection during the germination period, seeds were immersed in a solution of sodium hypochlorite (5%, v/v) for 15 min. The seed water content (gravimetrically determined; 103 ± 2ºC until constant mass) and the embryo viability (tetrazolium test) percentage were determined on the day that germination tests were set up. Both measurements were performed using three replicates of twenty seeds, following the standard procedures of the International Seed Testing Association (1993).

Germination responses to artificial light (78 µmol. m^{-2} s^{-1}, provided by fluorescent lamps) versus dark were assessed at different constant temperatures (15, 20, 25, 30, and 35ºC). Seeds were sown inside hand-folded channels of several layers of filter paper wetted with distilled water, which were inside opaque (black) plastic boxes (gerbox) (replicates). These channels allowed high seed-paper/water contact, since seeds were 12 ± 2 mm in diameter. The test was performed with 30 seeds per replicate. The number of germinated seeds was monitored daily until no seed had germinated for at least 14 days. We calculated the percentage of germinated seeds or germinability (%G); the mean germination time [$T = (\Sigma(n_i.T_i)/\Sigma n_i)$, where n_i is the number of germinated seeds in the time interval from T_i-1 to T_i]; the mean germination rate [V = (1/T), where T is the mean germination time]; and the synchronisation index ($U = -\Sigma RF.Log_2 RF$, where RF is the relative frequency of germination), according to Labouriau & Agudo (1987) and Ranal & Santana (2006). This study was conducted as a randomised experimental design with two light conditions (light *vs.* dark) and five temperature conditions (15, 20, 25, 30, and 35 °C), with six replicates. Data were subjected to two-way ANOVA, and mean results were compared by Tukey's test ($P < 0.05$) after transforming %G into arc sin of $\sqrt{(G\%/100)}$.

Imbibition curve

The imbibition curve was determined using the light and temperature conditions which resulted in the highest germination performance in the previous test. The same seed lot and experimental procedures described above were adopted. The seed moisture content was initially determined, and subsequent replicate (30 seeds) water mass gain was registered daily for the first 15 days. When the replicate water gain stabilized (beginning of the stationary phase of the imbibition curve), observations were performed every other day. Germinated seeds (showing the protrusion of the germinative button) were eliminated from replicates, so the curve was plotted using fresh mass per seed against time, with subsequent regression analysis.

Gibberellin and cytokinin tests

With the results of the first and second studies, the germination responses (%G, T, V and U) to gibberellin (GA_3) and Cyt (benzylaminopurine, BAP) were tested using a different seed lot, as seeds from the first lot could have lost viability and vigour during storage, because of their recalcitrant behaviour (PANZA et al, 2004). The seed lot was obtained from six adult ten-year-old *E. edulis* plants from the Experimental Garden of the Rio Claro Campus of the

São Paulo State University (IB-UNESP, Rio Claro) (22°25' S, 47°33' W), Brazil. These seeds were obtained from depulped mature fruits harvested during the 2007 dispersion period.

Seeds were sown under the same conditions previously described [inside hand-folded channels of wetted filter paper, inside black gerboxes (replicates), containing 30 seeds; six replicates; using light and temperature conditions which resulted in the highest germination performance in the first study]. At the beginning of phase II of the imbibition curve, as estimated in the second study, seeds were separately taken from the replicates and immersed for 24 h (VALIO, 1976) in aerated solutions containing GA_3 (Gibco BRL, Grand Island, NY, USA) at 50, 100, 150, and 200 $mg.L^{-1}$ or BAP (Acros Organics, New Jersey , USA) at 30, 60, and 90 $mg.L^{-1}$. Distilled water (control solution) was also used. After immersion, every seed replicate was returned to its respective gerbox for daily observation of germination, as per Labouriau & Agudo (1987). Data were submitted to one-way ANOVA, and mean results were compared by Tukey's test ($P < 0.05$).

Light microscopy study

Using the same seed lot used to measure plant growth regulator influence on germination performance, seeds were sown under the same conditions as above. The plant growth regulator at the dosage that returned the highest germination performance in the previous test was applied at different times, both immediately after sowing and at the beginning of the stationary phase of the imbibition curve. Embryo development was examined anatomically in treated and untreated (distilled water) seeds, which were randomly sampled from the replicates on the third and 15^{th} day after treatment.

Part of the mesocarp and endocarp were cut from the seed samples (without cutting the seed embryo) to facilitate the absorption of the FAA 70 [Formalin-Aceto-Alcohol; 90% (v/v) of ethyl alcohol at 70% (v/v), 5% (v/v) of glacial acetic acid and 5% (v/v) of formalin], solution in which they were fixed (JOHANSEN, 1940) for 15 days, to avoid extensive oxidation of the plant tissue. Next, seed samples were preserved in 70% ethanol (v/v). The samples were embedded in glycolmethacrylate historesin (GERRITS, 1991) and sectioned with a rotary microtome. The embryo longitudinal sections (3-5 μm) were stained with a 0.05% solution of toluidine blue (O'BRIEN et al, 1964), mounted in permount and observed under a light microscope. Images were captured with the IM50 (Leica, Germany) software.

Results

The seed lot tested to determine the influence of light and temperature on germination responses had a water content of 39.7 ± 2% and initial embryo viability of 72%.

Seeds started germinating at 15°C, reaching the optimum germination responses at 25°C and 30°C, regardless of the light condition. Seeds were still able to germinate at 35°C, but the percentage of germinated seeds (%G) was around 60% for seeds incubated in the dark and below 50% for seeds incubated in the light. At 25°C and 30°C, %G was similar ($P < 0.05$) for both the light and dark conditions (Fig. 1). This same response pattern was observed for the mean germination time (T), mean germination rate (V), and synchronisation index (U). The only significant difference between light *vs.* dark occurred for T and U measured at 15°C; seeds incubated in the light or in the dark showed the same germination performance at 25°C and 30°C (Table 1).

Table 1. Germination time (T), germination rate (V), and synchronization index (U) of *E. edulis* seeds at different temperatures and light conditions, after 60 days of incubation.

	Mean germination time (day)		**Mean germination rate (day^{-1})**		**Synchronization index (bits)**	
T (ºC)	Light	Dark	Light	Dark	Light	Dark
15	38.9 ± 1.3 **aA**	29.1 ± 2.7 **bB**	0.026 ± 0.001 **bA**	0.035 ± 0.003 **cA**	1.75 ± 0.27 **cA**	1.30 ± 0.27 **cB**
20	30.3 ± 1.1 **bA**	27.5 ± 0.6 **bA**	0.033 ± 0.001 **bA**	0.036 ± 0.001 **bA**	3.79 ± 0.59 **aA**	3.63 ± 0.32 **aA**
25	19.4 ± 0.4 **cA**	20.6 ± 0.5 **cA**	0.052 ± 0.001 **aA**	0.049 ± 0.001 **aA**	3.72 ± 0,18 **aA**	3.67 ± 0.25 **aA**
30	17.3 ± 0.4 **cA**	21.3 ± 0.9 **cA**	0.058 ± 0.001 **aA**	0.047 ± 0.002 **aB**	3.27 ± 0.22 **abA**	3.63 ± 0.33 **aA**
35	33.1 ± 2.4 **bA**	37.5 ± 1.0 **aA**	0.030 ± 0.002 **bA**	0.027 ± 0.001 **cA**	2.81 ± 0.28 **bA**	2.78 ± 0.26 **bA**

The same lower case letters in columns (among temperatures), and the same upper case letters in rows (light *vs.* dark) are not significantly different by Tukey's test ($P < 0.05$); Mean values ± standard deviation *(n = 6)*.

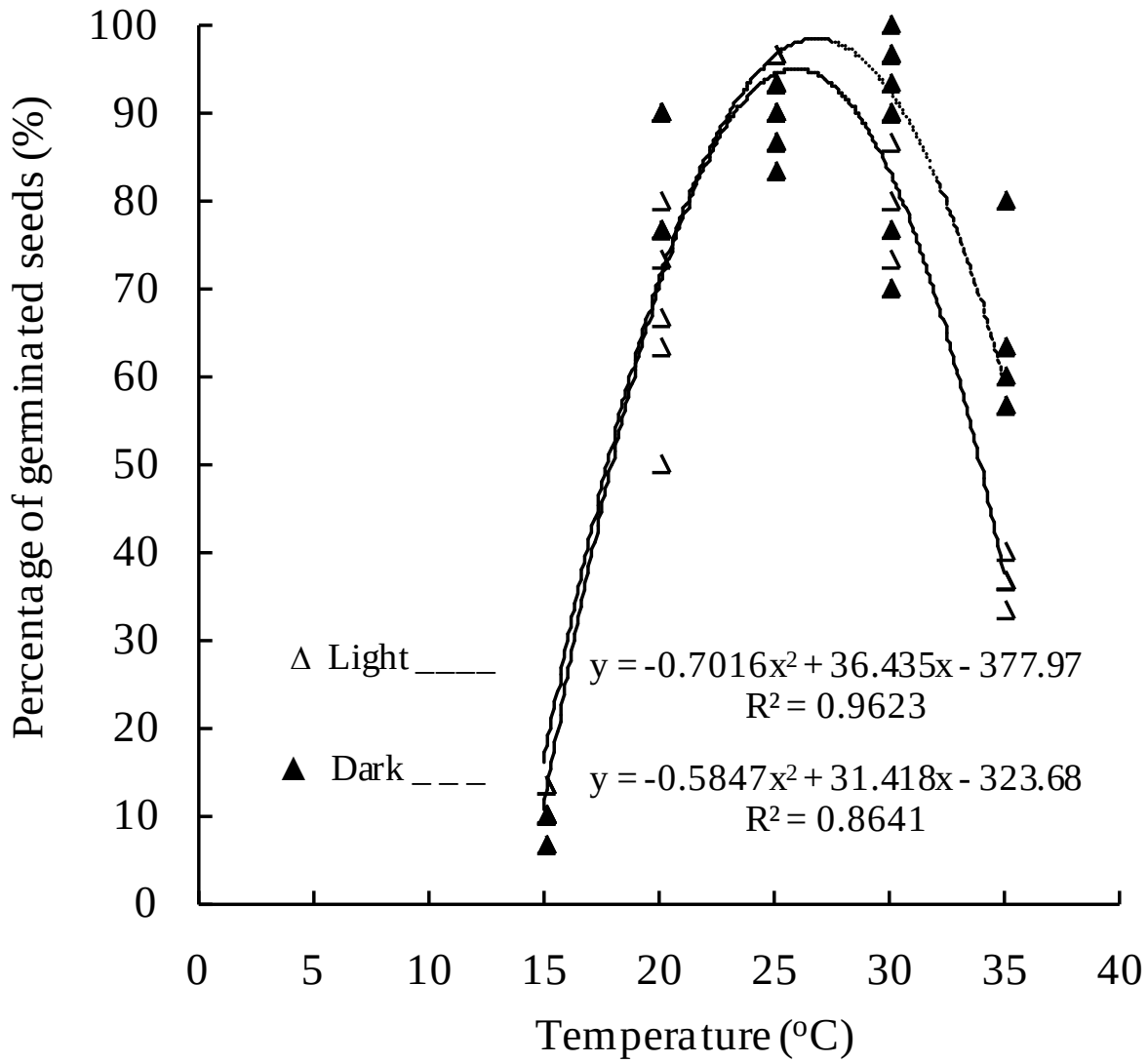

Fig. 1 Individual readings (replicates; *n = 6*) of the percentage of germinated *E. edulis* seeds at different incubation temperatures, in the light (Δ) and in the dark (▲).

The imbibition curve of seeds incubated at 25°C in the dark showed an initial water imbibition from the first to the tenth day, but the seed fresh mass began to stabilise around 12 days after sowing (Fig. 2).

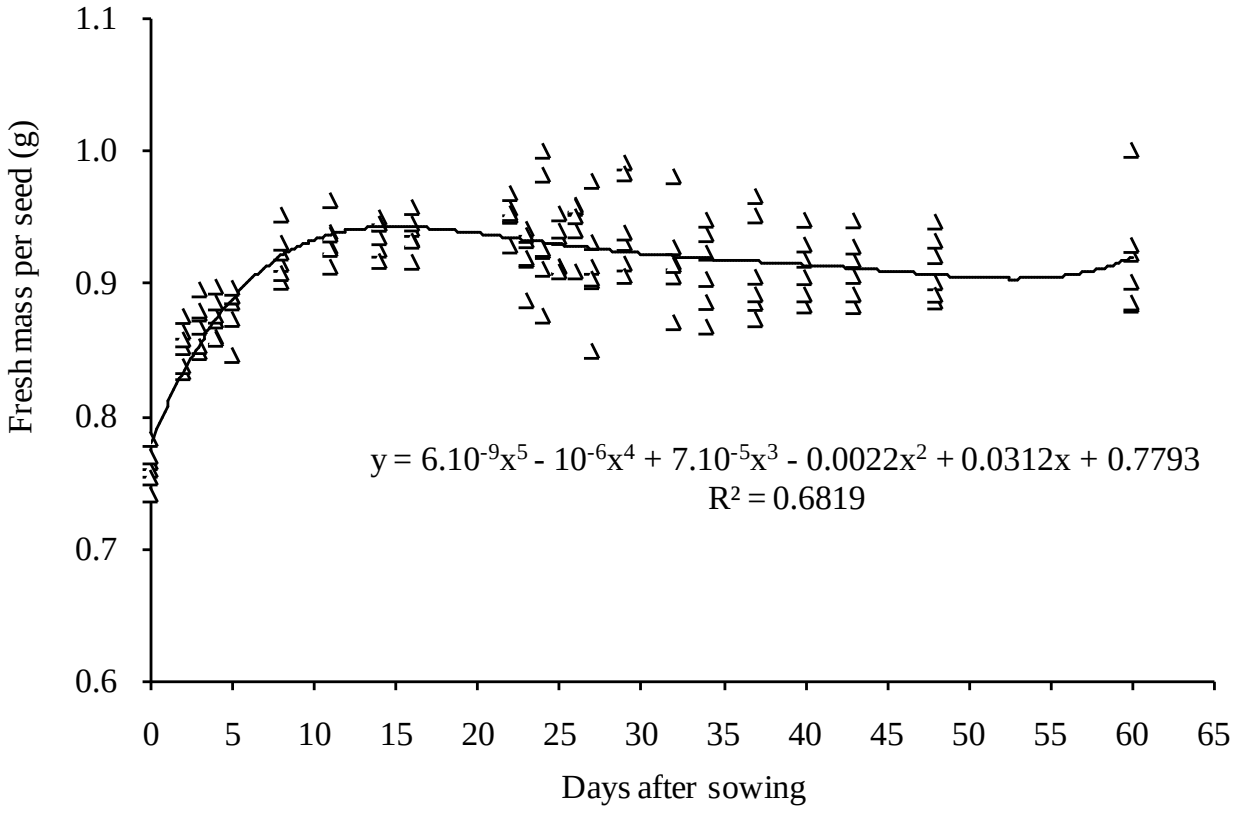

Fig. 2 Individual readings (replicates; *n = 6*) of the fresh mass per *E. edulis* seed, incubated at 25°C in the dark for 60 days.

When a different seed lot was used to test plant hormones applied on the 12th day after sowing (at 25°C in the dark), the initial seed water content was 40.5 ± 2% and the embryo viability was about 70%. Gibberellic acid (GA_3) at 50 $mg.L^{-1}$ increased the germinability (%G). In contrast, 100 and 150 $mg.L^{-1}$ GA_3 returned, statistically, the same results as the control treatment (no plant hormones). A dosage of 200 $mg.L^{-1}$ GA_3 decreased germinability to even lower than that observed for the control treatment and similar to the effects of BAP (Fig. 3). Compared to the other treatments, 50 $mg.L^{-1}$ of GA_3 also promoted the highest mean germination time and rate. The highest synchronisation of germination (lowest synchronisation index, U), however, occurred when 200 $mg.L^{-1}$ of GA_3 was applied (Table 2).

Table 2. Germination time (T), germination rate (V), and synchronization index (U) of *E. edulis* seeds incubated for 60 days at 25°C in the dark, either treated with gibberellic acid (GA_3) or cytokinin (benzylaminopurine) on the 12th day after sowing.

Treatments	Mean germination time (day)	Mean germination rate (day^{-1})	Synchronization index (bits)
Control	29.0 ± 2.3 **b**	0.035 ± 0.003 **bc**	3.76 ± 0.31 **ab**
GA_3 50mg.L^{-1}	22.2 ± 1.2 **c**	0.045 ± 0.002 **a**	4.43 ± 0.31 **a**
GA_3 100mg.L^{-1}	41.6 ± 2.4 **a**	0.024 ± 0.001 **c**	3.86 ± 0.24 **ab**
GA_3 150mg.L^{-1}	32.9 ± 1.4 **a**	0.030 ± 0.001 **e**	4.03 ± 0.74 **ab**
GA_3 200mg.L^{-1}	38.6 ± 1.4 **a**	0.026 ± 0.001 **c**	3.27 ± 0.74 **b**
BAP 30 mg.L^{-1}	35.0 ± 2.9 **a**	0.029 ± 0.003 **c**	3.53 ± 0.92 **ab**
BAP 60 mg.L^{-1}	26.2 ± 4.1 **b**	0.039 ± 0.005 **bc**	4.30 ± 0.74 **ab**
BAP 90 mg.L^{-1}	37.6 ± 1.7 **a**	0.027 ± 0.001 **b**	3.78 ± 0.59 **ab**

The same letters in columns are not significantly different by Tukey's test ($P < 0.05$); Mean values ± standard

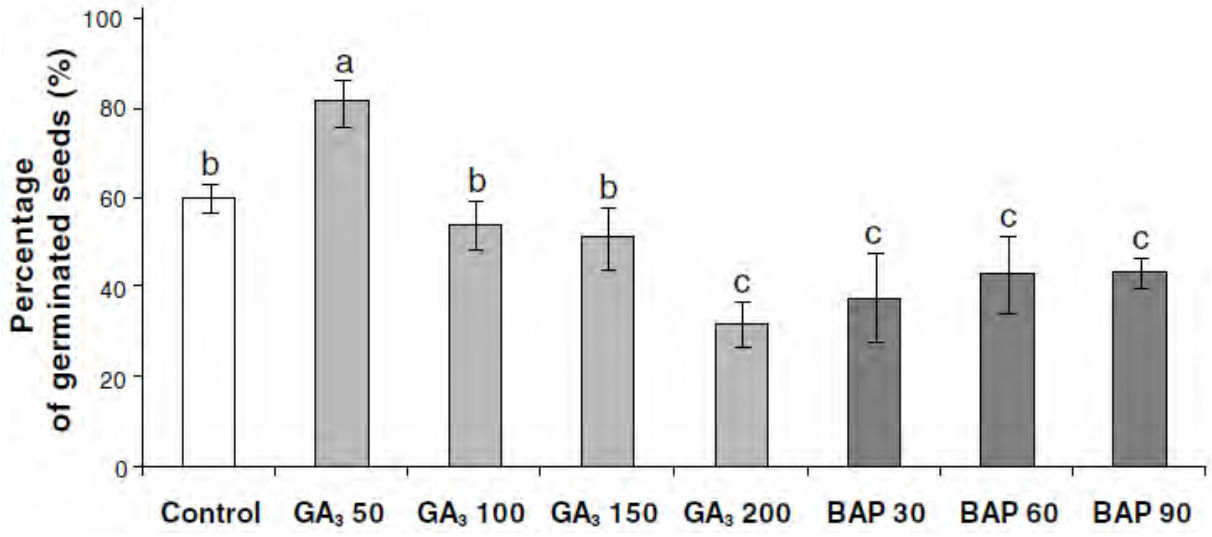

Fig. 3 Percentage of germinated *E. edulis* seeds, incubated at 25°C in the dark, after 24 h immersion in solutions of different dosages of gibberellic acid (GA_3) and benzylaminopurine (BAP), applied 12 days after sowing. Columns represent mean values ($n = 6$) and vertical bars are standard deviation. Same letters indicate lack of statistical significance by Tukey's test ($P < 0.05$).

E. edulis seeds present a massive and hard endosperm (Fig. 4A). The proximal region of the embryo consists of the embryo axis and the apical cotyledon zone, and the distal region of the embryo comprises the bottom part of the cotyledon (Fig. 4B). The embryos of untreated seeds (Fig. 4C, D) and the embryos of seeds treated with 50 $mg.L^{-1}$ of GA_3, either immediately after sowing (Fig. 4E, F) or at the beginning of phase II of the imbibition curve (Fig. 4G, H), showed round, isodiametric and uniform cells 15 days after treatment. The endosperm of untreated seeds (Fig. 4I, J) and the endosperm of seeds treated with 50 $mg.L^{-1}$ of GA_3 immediately after sowing (Fig. 4K, L) displayed unchanged cell contents and intact cell walls three days (Fig. 4I, K) and 15 days (Fig. 4J, L) after treatment. There were no structural differences between endosperm cells of the GA_3-treated seeds (Fig. 4K, L) and endosperm cells of untreated seeds (Fig. 4I, J).

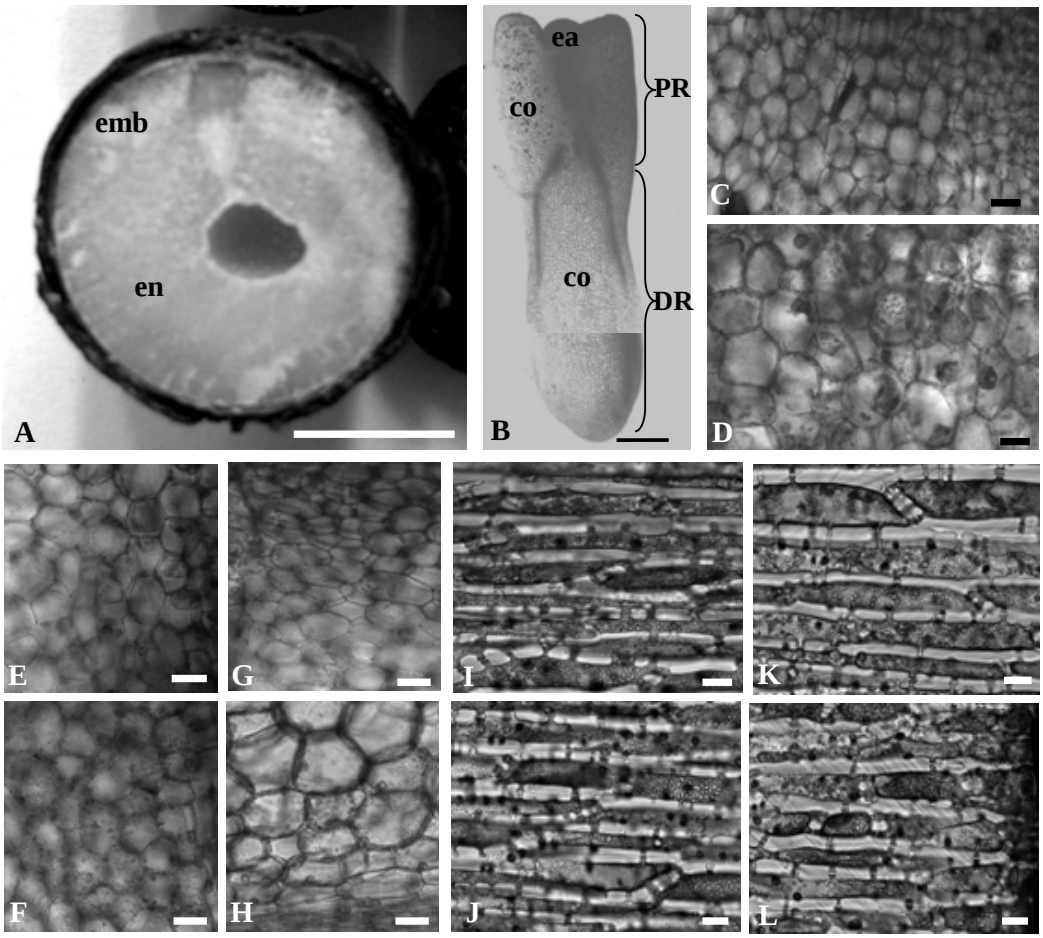

Fig. 4 *Euterpe edulis* seeds in longitudinal sections. **A**. Micrograph of a longitudinal section of an *E. edulis* seed showing the embryo (**emb**), the endosperm (**en**), and the seed coat (**arrow**). **B**. Embryo of a seed treated with GA_3 12 days after sowing: longitudinal section obtained 15 days after the GA_3 application, showing the embryo axis (**ea**) and the apical cotyledon (**co**) zone in the proximal region (**PR**), as well as the bottom part of the cotyledon (**co**) in the distal region (**DR**). **C-D**. Details of an untreated seed embryo: longitudinal sections obtained 15 days after sowing (**C**. Proximal region; **D**. Distal region). **E-F**. Details of an embryo of a seed treated with GA_3 immediately after sowing: longitudinal sections obtained 15 days after the GA_3 application (**E**. Proximal region; **F**. Distal region). **G-H**. Details of an embryo of a seed treated with GA_3 12 days after sowing: longitudinal sections obtained 15 days after GA_3 application (**G**. Proximal region; **H**. Distal region). **I-J**. Details of the endosperm of an untreated seed: longitudinal sections obtained three days after sowing (**I**) and 15 days after sowing (**J**). **K-L**. Details of the endosperm of a seed treated with GA_3 immediately after sowing: longitudinal sections obtained three days (**K**) and 15 days (**L**) after the GA_3 treatment. Scale bars: A = 1 cm; B = 1 mm; C-L = 5 µm.

Discussion

Seed water content of the seed lot used to measure the influence of light and temperature on germination responses was above 35%, which is considered critical to the seed viability and germination performance of this species (ANDRADE et al, 1996).

The seed responses to temperature are in agreement with the results of Andrade et al (1999), who observed that 30-20ºC (day-night) and 25ºC (constant) promoted the highest percentage of germinated seeds (%G) and mean germination rate (V) of normal *E. edulis* seedlings. The range of 20-30ºC, constant or alternating, has regularly been used for *E. edulis* seed germination tests (ANDRADE et al, 1996; ANDRADE et al,1999; MARTINS et al, 2004).

Constant or alternating (photoperiod of 8 h) fluorescent light (20 to 80 $\mu mol.m^{-2}.s^{-1}$) has also been employed in *E. edulis* germination tests performed in lab incubators, but without any cited reference. Under natural conditions, *E. edulis* seeds generally germinate in the shade of the Atlantic rainforest (ORLANDE et al, 1996), forming a dense bank of seedlings. These plants can show high growth rates as irradiance reaches up to 20-30% of full sunlight, often near a forest clearing (NAKAZONO et al, 2001). However, 80 $\mu mol.m^{-2}.s^{-1}$, regularly used inside lab incubators, could be considered shade, since the natural understorey irradiance of the Atlantic forest and swamp forests (where *E. edulis* also occurs) varies between 20 and 80 $\mu mol.m^{-2}.s^{-1}$ during the day (data not shown). Therefore, dark *vs.* high irradiance (higher than 1000 $\mu mol.m^{-2}.s^{-1}$) has not yet been tested for *E. edulis* seeds. Moreover, the high irradiance response (HIR) of phytochrome A (phyA), which is known to control the germination of non-photoblastic seeds, does not display photo-reversibility. It also does not obey the law of reciprocity (TAKAKI, 2001; TAIZ, 2006a), in which the magnitude of the response depends on the product of the fluence rate and the irradiation time (TAIZ, 2006a). This could justify the intermittent low irradiance used inside lab incubators for long periods of time, as in the case of *E. edulis* seeds, which take more than 60 days to complete germination. *E. edulis* seeds are considered non-photoblastic. For other species under specific conditions (water stress), the phyA presence can be demonstrated in typical “non-photoblastic seeds”, inhibiting germination at high irradiances (TAKAKI, 2001) and causing these seeds to be re-classified as non-photoblastic seeds. Notwithstanding, our results showed that seeds incubated under constant fluorescent light (78 $\mu mol.m^{-2}.s^{-1}$) or dark had the same germination performance, especially under temperatures which resulted in the highest %G and V, and also at the lowest T and U (25°C and 30°C). Thus, light absence and 25°C seem to be effective and are recommended for *E. edulis* germination tests.

Our results showed an initial water imbibition, with subsequent fresh mass stabilisation, approximately 12 days after sowing. For most orthodox seeds, this fresh mass stabilisation indicates the beginning of the stationary phase of the imbibition curve (phase II), when seed metabolic activities are expected to begin again (FINKELSTEIN, 2004).

The beginning of the stationary phase could represent the best time to treat seeds in order to accelerate their germination. In fact, 50 $mg.L^{-1}$ of GA_3 applied 12 days after sowing, promoted the highest germination performance, having induced higher %G and V, and lower T, compared to the other treatments. Meanwhile, BAP (Cyt) resulted in lower germination performance, even when compared to the control (Fig. 3; Table 2). GA_3 might have induced or accelerated some storage mobilisation to the *E. edulis* embryo, a phenomenon which has been especially well described in cereals (WOODGER et al, 2004). Gibberellins may also induce seed embryo cell elongation and division responses (FINKELSTEIN, 2004; TAIZ, 2006b). Cytokinins, on the other hand, may promote embryo growth, controlling cell cycling and mitosis (FINKELSTEIN, 2004; TAIZ, 2006c). However, 15 days after treatment, there were no indications or signs of recent cell division or elongation in embryos of untreated or GA_3-treated seeds. Additionally, the endosperm of untreated and GA_3-treated seeds did not show any changes with regard to cell content or intact cell walls, neither three nor 15 days after treatment. This may indicate a lack of storage reserve mobilisation for at least 15 days after treatment. Accordingly, the *E. edulis* endosperm appeared to be in an inactive state, showing no evidence of storage mobilisation. Meanwhile, its seed embryo cells presented numerous mitochondria, as well as rough endoplasmic reticulum and Golgi apparatus cisternae, indicating that they were in an active state, but developing slowly (PANZA et al, 2004). In fact, the *E. edulis* seed embryo may be seen as very primitive, with differentiation into recognisable plant structures occurring at a late stage in the germination process rather than during seed development (TOMLINSON, 1960). Actually, the protodermis, parenchyma, and procambium are recognisable at the time of *E. edulis* fruit dispersion, but cells from most tissues lack insoluble storage reserves and are highly vacuolated (justifying the recalcitrant behaviour) (PANZA et al, 2004).

Therefore, it was not possible to specifically determine the physiological cause(s) of improvement of germination performance with GA_3 application (Fig. 3; Table 2). Even though we did not histochemically test the longitudinal sections, it was possible to corroborate the results of Panza et al (2004), who described the *E. edulis* embryo as a very slow-developing seedling. The GA_3 treatment, which provoked increased seed germination

performance after 60 days of incubation, seemed to be insufficient to cause structural and/or physiological effects at three or 15 days after its application.

When we tested the effects of light and temperature on the germination responses, untreated seeds germinated at 25ºC in the dark exhibited 88.9 %G (Fig. 1). When testing the influence of plant growth regulators on the germination response (Fig. 3), seeds germinated under the same light and temperature conditions and also not treated with GA_3 (control) displayed 60 %G. This discrepancy in %G occurred because the seed lots used in each of these studies were different. It would be difficult to concomitantly perform the three studies (light and temperature responses; imbibition curve; GA_3 and Cyt responses), because each study generated results that were useful to simplify and reduce the number of treatments in the following study. Notwithstanding, GA_3 did improve the germination performance of *E. edulis* seeds (Fig. 3; Table 2). Moreover, similar results obtained for the synchronization index between the first (Table 1) and the third (Table 2) studies confirmed the quality of the seed lots used, as well as their seed water content and viability percentage.

The beginning of the stationary phase of the imbibition curve could not be demonstrated to be a better time for treatments intended to improve the germination performance, in comparison to the time immediately after sowing. Anatomically, there were no structural differences between these times (Fig. 4C-H), although GA_3 (50 $mg.L^{-1}$) did promote the highest germination performance of these seeds after 60 days of incubation (data not shown). Indeed, the range of 40-60 $mg.L^{-1}$ of GA_3 later resulted in increased germination performance (high %G and V, and low T) of a different *E. edulis* seed lot (data not shown). The fact that there was an initial water imbibition, with a fresh seed mass stabilization 12 days after the beginning of the imbibition, does not necessarily imply that embryo metabolism was reactivated. *E. edulis* seeds have recalcitrant behaviour (ANDRADE & PEREIRA, 1997; PANZA et al, 2004), and recalcitrant seeds have no metabolism reactivation because their seed metabolic activities are not switched off after fruit dispersion, as is the case in orthodox seeds (PAMMENTER & BERJAK, 1999). Furthermore, the immediate cause of the inability of the recalcitrant seed tissues to switch off metabolism is suggested to be desiccation sensitivity (PAMMENTER AND BERJAK, 1999). In this way, the 12-day imbibition represented a rehydration of 20% of the maximum seed water content: the seed fresh mass went from ±0.75 g after sowing to ±0.95 g at the stationary phase (Fig. 2). Considering the initial 39.7% of seed water content, this means that seeds started imbibing at around 0.65 $g.g^{-1}$ dry mass basis and reached more than 1.0 $g.g^{-1}$ dry mass basis at the stationary phase. The highly recalcitrant tropical species *Avicennia marina* starts suffering lethal damage at the

meristematic root primordial of the seed embryo at water contents below 0.5 $g.g^{-1}$ dry mass basis (PAMMENTER & BERJAK, 1999). Therefore, this 12-day initial imbibition may represent only some sort of *corrective* seed rehydration, considering the enormous desiccation sensitivity of this species (ANDRADE & PEREIRA, 1997; PANZA et al, 2004). Specifically for *E. edulis* seeds, there are strong indications that germination starts around shedding and that the embryo exhibits continuous and very slow development (PANZA et al, 2004). Our anatomical results corroborate these findings. Thus, this corrective *E. edulis* seed rehydration does not seem to reactivate seed metabolism activities, and the immediate physiological cause(s) of the GA_3 improvement of *E. edulis* germination performance remain to be determined.

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4. GERMINATION OF RECALCITRANT *Euterpe edulis* MART. SEEDS RELATED TO WATER CONTENT AND TREATMENT WITH GIBBERELLIC ACID.

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Abstract

To accelerate the germination of *Euterpe edulis* (Mart.) seeds, a key species of Atlantic forest, and allow its replacement on enviroment, it was found that exogenous application of gibberellin (GA_3) 12 Days After Sowing (DAS) increases the speed of species seeds germination. However it was not observed anatomical evidence of division and / or cell elongation in the embryo 15 days after treatment with GA_3. In this study the objective was to review the timing of application of GA_3, related to anatomical observations and nutritional reserves assessment for a longer period and to determine the effect of seeds hydration on germination performance of *E. edulis* seeds. Germination tests with seeds at different initial moisture contents (51 ± 1%, 47 ± 1%, 43 ± 1% and 39 ± 1%) confirmed the hypothesis that seeds of *E. edulis* have higher germination performance as most well-hydrated is tissue, confirming the species as recalcitrant. In germination tests with seeds treated with GA_3 at 0 and 12 DAS and untreated, both treatments showed higher germination performance compared to untreated seeds, without differences among themselves, confirming the hypothesis that, due to their continued development of the embryo, it is irrelevant the moment (0 or 12 DAS) in seed germination in wich is applied GA_3. Anatomical changes indicative of embryo growth in GA_3-treated seeds were observed 45 and 60 days after the application. There was weak mobilization of reserves (free carbohydrates and fat) during germination and the hypothesis that the increased performance of germination of *E. edulis* with GA_3 is the result of hormonal inductive mobilization of reserves could not be confirmed.

Keywords: Atlantic forest, jussara-palm, gibberellins, seed germination physiology.

Introduction

Euterpe edulis, or jussara palm, is considered to be a key species in the Atlantic forest. It associates the fauna (birds and mammals), as its fruits dispersers (GENINI et al., 2009), and the flora, since it grows under the shade of pioneer species (NAKAZONO et al., 2001). *E. edulis* takes six to nine years to fruit, but illegal and precocious plant extraction, to sell its desirable and edible palm heart (the apical meristem), led to the almost disappearance of the species from many areas (DRANSFIELD et al., 1988).

The germination ability of the seed may be considered to be extremely important for the establishment of any species in a certain environment (GOMES et al., 2006). On the other hand, the time of germination of *E. edulis* de-pulped fruits (seeds) ranges between 60 and 90 days (BOVI et al, 1992). This slow germination response may be associated with the thick and hard mesocarp and endocarp of *E. edulis* seeds (MULLET et al., 1981), seed hormonal imbalance, a lack of reserve mobilization, intrinsic slow embryo cell development, and morphological seed dormancy, which is frequent in seeds of palm species (PANZA et al., 2004, 2007). The slow plant development is also typical of palm species (NAKAZONO et al., 2001). But temperature and environmental moisture, influencing the seed water content may be also involved in the slow germination of *E. edulis* seeds.

E. edulis seeds are considered to be recalcitrant, losing viability when their water content reaches values below 30% (ANDRADE, 2001). It seems that there is a general relationship between the recalcitrant behavior of seeds and the continuous development of the embryo, which is observed in *E. edulis* seeds (PANZA et al., 2004). The endosperm of *E. edulis* seeds appears in a quiescent state, with lipids, proteins, minerals and mannans stored as insoluble reserves, revealing a lack of reserve mobilization to the embryo (PANZA et al., 2004).

The germination performance (percentage of germinated seeds, time of germination and germination rate) of *E. edulis* seeds is high at 25°C, regardless of the light conditions, but it significantly decreases under lower temperatures (MARTINS et al., 2004; ROBERTO & HABERMANN, 2010).

A previous study of the imbibition curve of *E. edulis* seeds indicated that the beginning of the stationary phase of the curve occurs at approximately 12 days after sowing (DAS), and applying any enhancer of germination performance on this date would return maximum results (ROBERTO & HABERMANN, 2010). In this same study, gibberellic acid (GA_3) used at 50 mg L^{-1} and applied at 12 DAS induced high germination performance; however, there were no signs of anatomical changes in *E. edulis* embryos or endosperm of

GA_3-treated seeds, neither three nor 15 days after treatment. On the other hand, recalcitrant seeds do not show metabolism reactivation during early stationary phase of the imbibition curve (PAMMENTER & BERJAK, 1999).

Thus, we could predict that the best germination performance for the successful establishment of this key species may be achieved only after a corrective re-hydration of *E. edulis* de-pulped fruits, which occurs until approximately 12 days after the imbibition has started on the soil surface of the forest. Keeping in mind that GA_3 induces a high germination performance of *E. edulis* seeds, whose embryo shows a continuous and slow development (PANZA et al. 2004), it would be irrelevant the moment of the imbibition curve that GA_3 is applied. We also raised the hypothesis that signs of anatomical changes caused by GA_3 on the embryo or endosperm can be observed after more than 15 days GA_3 is applied. In addition, we checked whether there was any evidence that GA_3 induces seed reserve mobilization. The hypotheses were tested by measuring the germination performance of *E. edulis* seeds with distinct water content. In addition, seeds were also treated with GA_3 at different moments of the seed imbibition curve, and had their germination performance evaluated. Anatomical embryo and endosperm analyses were performed as a framework for the functional studies in GA_3-treated and non-treated seeds at different moments of the seed imbibition curve.

Material and Methods

Plant material

Mature fruits were obtained from six adult ten-year-old *E. edulis* plants from the Experimental Garden at the Rio Claro Campus of the Univ Estadual Paulista (IB-UNESP, Rio Claro) (22°25' S, 47°33' W), Brazil. The fruits were harvested in March/April, 2009, and were immediately immersed in water for 48 hours, so that the epicarp could be easily removed through friction against a nine-mm^2 steel sieve under tap water.

Germination tests

To study *E. edulis* seeds with distinct water content, and GA_3-treated and non-treated seeds that were evaluated at different moments of the imbibition curve, germination tests were performed as follows.

The seeds were immersed in a solution of sodium hypochlorite (5%, v/v) for 15 min, in order to prevent fungal infection during the germination period. The seed water content was gravimetrically determined (105 ± 2°C until constant mass) and the embryo viability

(tetrazolium test) percentage were both measured on the same day that germination tests were set (LIN, 1988). Such measurements were performed using three replicates of twenty seeds, according to standard procedures of the International Seed Testing Association (2005).

Seeds were sown inside hand-folded channels of several layers of filter paper wetted with distilled water, which were disposed inside black (opaque) plastic boxes, or gerbox (replicates) that were maintained under dark at 25°C, inside B.O.D. chambers. These channels allowed high seed-paper/water contact, since seeds were 12 ± 2 mm in diameter. The tests were performed using 30 seeds per replicate. The number of germinated seeds was monitored daily until no seed had germinated for at least 14 days. The percentage of germinated seeds or germinability (%G); the relative germination frequency ($RF = n_i:Nt$, where n_i is the number of germinated seeds between times $t_i–1$ and t_i); the mean germination time [$T = (\Sigma(n_i.T_i)/\Sigma n_i)$, where n_i is the number of germinated seeds in the time interval from T_i-1 to T_i]; the mean germination rate [$V = (1/T)$, where T is the mean germination time]; and the germination synchronization index ($U = -\Sigma RF.Log_2RF$, where RF is the relative frequency of germination) were calculated according to Labouriau & Agudo (1987) and Ranal & Santana (2006).

Germination test of seeds with distinct initial water content

Germination variables (%G, RF, T, V, and U) of *E. edulis* seeds were tested in response to the following different seed lot water content: 51%, 47%, 43%, and 39%.

After determining the initial seed lot water content (51.6 ± 1%), six replicates made of 30 seeds each were disposed inside gerboxes, as previously described, which were kept inside the B.O.D. chambers. The remaining seed lot was divided into three sub-lots that were subjected to oven drying at constant 45°C , for 24, 48 and 72 hours. After such times, water contents of the three sub-lots were measured again and returned values of 47 ± 1%, 43 ± 1%, and 39 ± 1%, respectively. Such sub-lots with these seed water contents (treatments) were submitted to the above-mentioned germination tests. Data were subjected to a one-way analysis of variance (ANOVA), and mean results were compared by the Tukey's test ($P < 0.05$), after transforming %G into arcsin of $\sqrt{(G\%/100)}$.

Germination tests of GA_3-treated and untreated seeds

After the conclusion of the study that tested seeds with distinct water content, the same original seed lot that had exhibited 51.6 ± 1% of water content was used to evaluate the germination performance under the influence of GA_3. The germination variables (%G, RF, T,

V, and U) were measured in GA_3-treated and non-treated *E. edulis* seeds. The GA_3 (gibberellic acid, Gibco BRL, Grand Island, NY, USA) was applied using a 50 mg L^{-1} GA_3 solution, in which seeds were immersed for 24 hours. The GA_3 was applied before sowing, or 0 days after sowing (DAS), and at the beginning of the stationary phase of the imbibition curve, or 12 DAS, according to Roberto & Habermann (2010). Data were subjected to a one-way analysis of variance (ANOVA), being the presence of GA_3 applied at 0 DAS or at 12 DAS the cause of variation. Mean results were compared by the Tukey's test ($P < 0.05$), after transforming %G into arcsin of $\sqrt{(G\%/100)}$.

Quantification of seed reserves

Another experiment to test *E. edulis* seeds in response to GA_3 was set, and it was identical and concomitant with the germination test immediately described above. The seeds were randomly and periodically sampled from the six replicates (gerbox) to quantify seed reserves (carbohydrates and lipids), as well as to anatomically analyze their seed embryo and endosperm. Sampling was performed at the 15^{th}, 30^{th}, 45^{th}, and 60^{th} day after sowing.

For the reserve analysis, seed samples were oven dried at 45°C for 8 hours, and were ground in a ball mill for 10 minutes. The lipid quantification was done according to Bligh & Dyer (1959). One g of the oven-dried ground seeds was submitted to an organic extraction using 10 mL of chloroform and 10 mL of methanol. The solid extract was removed through a filter paper, and it was added 5 mL of chloroform and 10 mL of deionized water to the aqueous filtrate. After centrifugation (5400 rpm for 15 minutes), the lower phase (chloroform) was collected and dried in beakers of known mass. The quantification of lipids was gravimetrically calculated, and results, which were obtained in triplicate, were expressed in mg of total lipids per g of dry seed. For each evaluation date (15^{th}, 30^{th}, 45^{th}, and 60^{th} DAS), data from the three different treatments (untreated seeds, and GA_3-treated seeds at 0 DAS and 12 DAS) were submitted to a one-way ANOVA, and mean results were compared by the Tukey's test ($P < 0.05$).

Extraction of total free carbohydrates (D-glucose, D-fructose, and D-mannose) from seeds was performed according to Takaki & Dietrich (1980). One g of the oven-dried ground seeds was submitted to an organic extraction using 15 mL of 70% ethanol (v/v) for 60 minutes (reflux extraction). Subsequently, the extract was centrifuged (5400 rpm for 30 minutes) and the supernatant was separated for quantification of free carbohydrate. Quantification was done by the phenol-sulfuric method (DUBOIS et al., 1956), using glucose as standard. Results were obtained in triplicate and were expressed in mg per g of dry seed.

For each evaluation date (15th, 30th, 45th, and 60th DAS), data from the three different treatments were submitted to a one-way ANOVA, and mean results compared by the Tukey's test ($P < 0.05$).

Light microscopy study

Seeds that were sampled (at the 15th, 30th, 45th, and 60th DAS) from replicates of the above-mentioned experiment had part of the mesocarp and endocarp cut in order to facilitate the absorption of the FAA 70 [Formalin-Aceto-Alcohol; 90% (v/v) of ethyl alcohol at 70% (v/v), 5% (v/v) of glacial acetic acid and 5% (v/v) of formalin]. The seeds were fixed in this FAA solution (JOHANSEN, 1940) for 15 days, to avoid tissue oxidation. Next, seed samples were preserved in 70% ethanol (v/v). Seeds from each of the three treatments had their embryo and an adjacent piece of endosperm excised, which were subsequently dehydrated in a series of normal-butyl alcohol (NBA) (FEDER & O'BRIEN, 1968). The embryos and the endosperm samples were embedded in 2-hystoresin hydroxiethylmetacrilate (Leica Microsystems, Wetzlar, Germany) and sectioned with a Reichert-Jung microtome (2040, Leica Microsystems, Wetzlar, Germany). The embryo longitudinal sections (3-5 μm) were stained with a 0.05% solution of toluidine blue (FEDER & O'BRIEN, 1968), mounted in synthetic resin (Entellan, Merck Chemicals, Darmstadt, Germany) and observed under a light microscope (DMLB, Leica Microsystems, Wetzlar, Germany). Images were captured with a digital camera (DFC-290, Leica Microsystems, Wetzlar, Germany), which is functionally connected to the DMLB light microscope.

Results

The seed lot that had water content of 51.6 ± 1%, concomitantly had an embryo viability of 80 ± 1.5%.

The distinct seed water contents did not cause different percentage of germinated seeds, but definitely influenced time (T) and rate (V) of *E. edulis* seed germination. The higher the seed water content, the higher the V, and lower the T (Table 1). Seeds that showed water content of 39% demonstrated a relative frequency of germination peak 20 DAS, whereas the seeds with higher water contents exhibited such peaks 10 DAS, and showed more elevated values (Fig. 1).

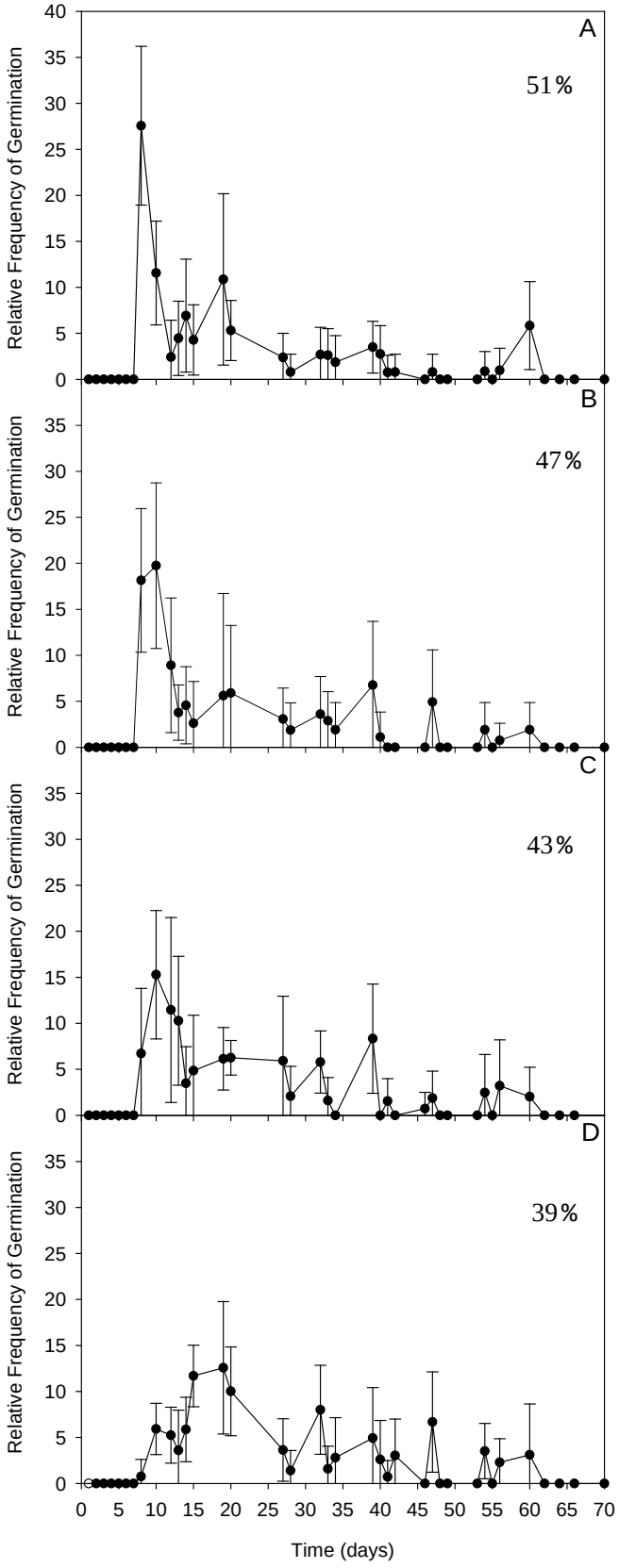
A
51%
B
47%
C
43%
D
39%
Relative Frequency of Germination
Time (days)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
0 5 10 15 20 25 30 35 40

Fig 1. Relative frequency of germinated *Euterpe edulis* seeds, incubated at 25ºC in the dark, in response to seed water contents of 51% (**A**), 47% (**B**), 43% (**C**) and 39% (**D**). Dots represent mean values (*n = 6*) and vertical bars are standard deviation.

Table 1. Germination time (T), germination rate (V), synchronization index (U), and the percentage of germination (%G) of *E. edulis* seeds at different seed water contents, after 60 days of incubation.

Seed water content	T	V	U	%G
51%	20.8 ± 3.5 **b**	0.06 ± 0.006 **a**	3.0 ± 0.3 **a**	57.2 ± 7.7 **a**
47%	21.3 ± 3.2 a**b**	0.05 ± 0.012 **ab**	2.8 ±0.2 **a**	53.9 ± 8.5 **a**
43%	23.1 ± 3.3 **ab**	0.05 ± 0.008 **ab**	3.0 ± 0.24 **a**	56.1 ± 9.3 **a**
39%	26.0 ± 2.3 **a**	0.04 ± 0.006 **b**	3.1 ± 0.2 **a**	58.9 ± 7.8 **a**

For each variable, same letters in columns (among seed water contents) are not significantly different by the Tukey's test ($P < 0.05$); Mean values ± standard deviation *(n = 6)*.

GA_3-treated seeds showed significantly higher V, and lower T, compared to untreated seeds. However, T and V were the same, regardless of the moment (0 or 12 DAS) GA_3 was applied. GA_3 induced a higher %G, compared to untreated seeds, only when it was applied at 0 DAS (Table 2). GA_3-treated seeds reached a higher cumulative germination, in comparison to untreated seeds (Fig. 2). GA_3-treated seeds at 0 DAS showed higher relative frequency of germination in relation to untreated seeds, at approximately 45 DAS. However, the values of such parameter were higher for untreated rather than GA_3-treated seeds at the 55^{th} DAS (Fig. 3).

Table 2. Germination time (T), germination rate (V), synchronization index (U), and the percentage of germination (%G) of untreated and GA_3-treated *E. edulis* seeds. GA_3 treatments (50 mg L^{-1}) were applied immediately after sowing (0 days after sowing, 0 DAS) and at 12 DAS.

Treatments	T	V	U	%G
Untreated seeds	51.5 ± 3.1 **b**	0.019 ± 0.0012 **b**	2.9 ± 0.2 **a**	65.0 ± 5.5 **b**
GA_3-treated (0 DAS)	48.5 ± 1.4 **a**	0.020 ± 0.0006 **a**	3.2 ± 0.2 **b**	76.1 ± 2.5 **a**
GA_3-treated (12 DAS)	49.1 ± 2.2 **a**	0.020 ± 0.0009 **a**	3.6 ± 0.1 **c**	72.2 ± 2.7 **a**

For each variable, the same letters in columns (among treatments) are not significantly different by the Tukey's test ($P < 0.05$); Mean values ± standard deviation *(n = 6)*.

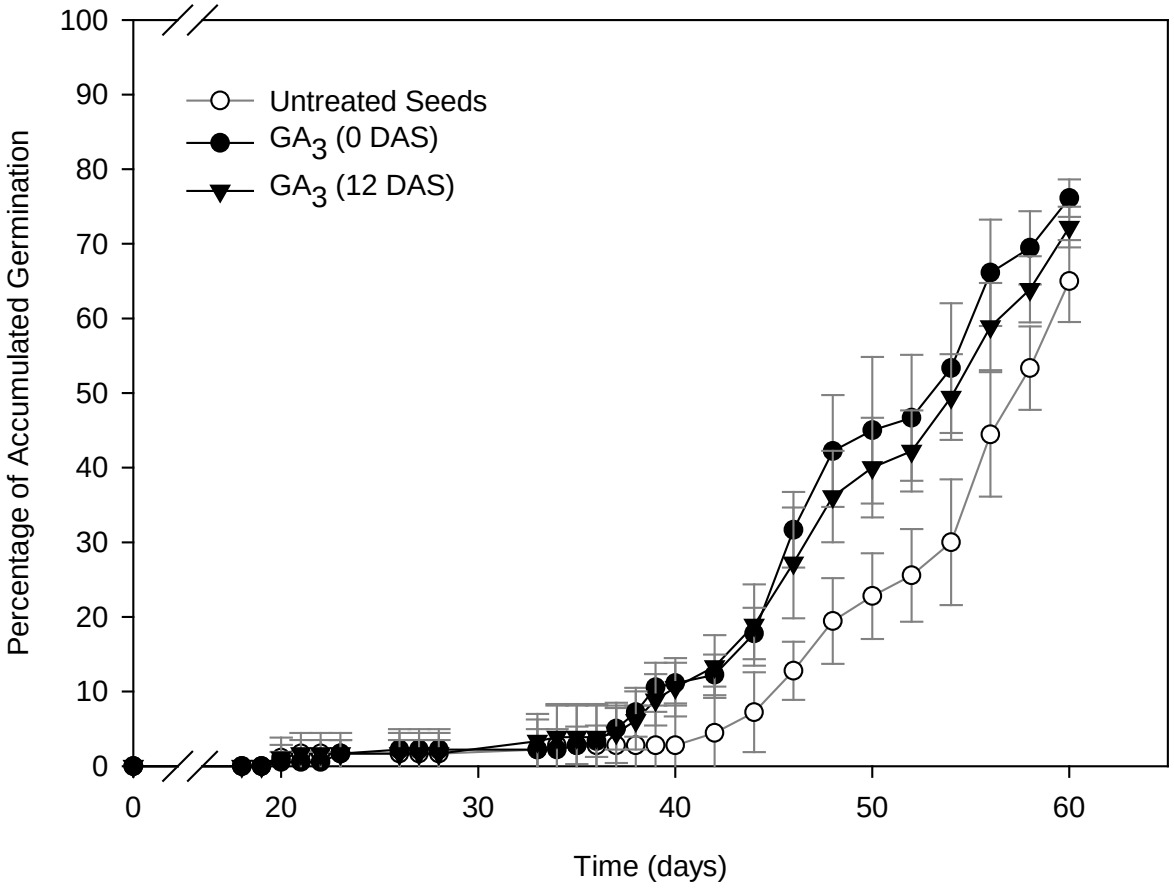

Fig 2. Percentage of accumulated germination of untreated and GA_3-treated *Euterpe edulis* seeds, incubated at 25ºC in dark. GA_3 treatments (50 mg L^{-1}) were applied immediately after sowing (0 days after sowing, 0 DAS) and at 12 DAS. Dots represent mean values (*n = 6*) and vertical bars are standard deviation.

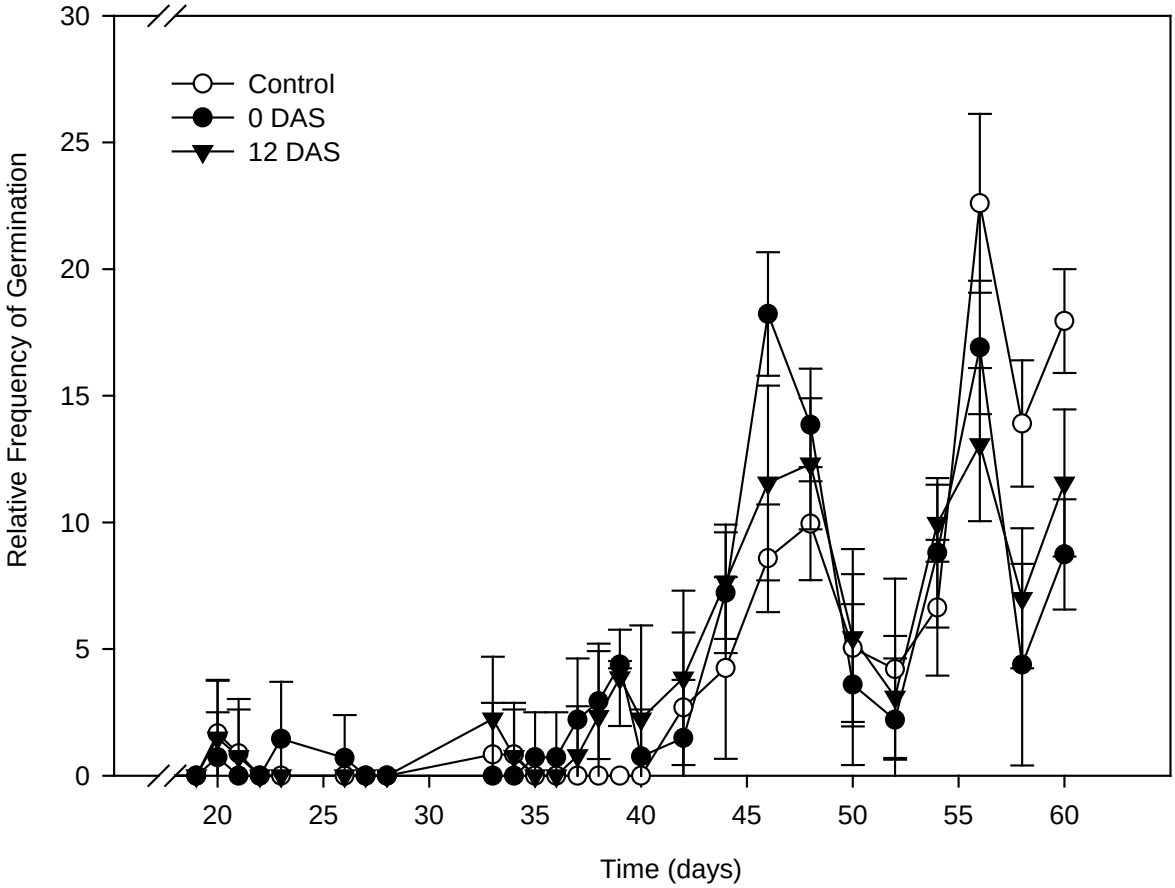

Fig 3. Relative frequency of germinated *Euterpe edulis* untreated seeds and GA_3-treated (50 mg L^{-1}) seeds, immediately after sowing (0 days after sowing, or 0 DAS) and at 12 DAS. Dots represent mean values (*n = 6*) and vertical bars are standard deviation.

GA_3-treated seeds exhibited larger cells in the embryo (Fig. 4K) and in the endosperm (Fig. 4O and 4V), in comparison to the embryo (Fig. 4D) and endosperm (Fig. 4H) cells of untreated seeds at 60 DAS. Such anatomical contrasts between GA_3-treated and untreated seeds for the embryo (Fig. 4A and 4P) and for the endosperm (Fig. 4E, 4L, and 4S) cells could not be observed at 15 DAS. Some small but inconclusive anatomical differences possibly caused by the GA_3 could be noted at 30 DAS (Fig. 4B, 4F, 4I, 4M, 4Q, and 4T) and at 45 DAS (Fig. 4D, 4H, 4K, 4O, and 4V).

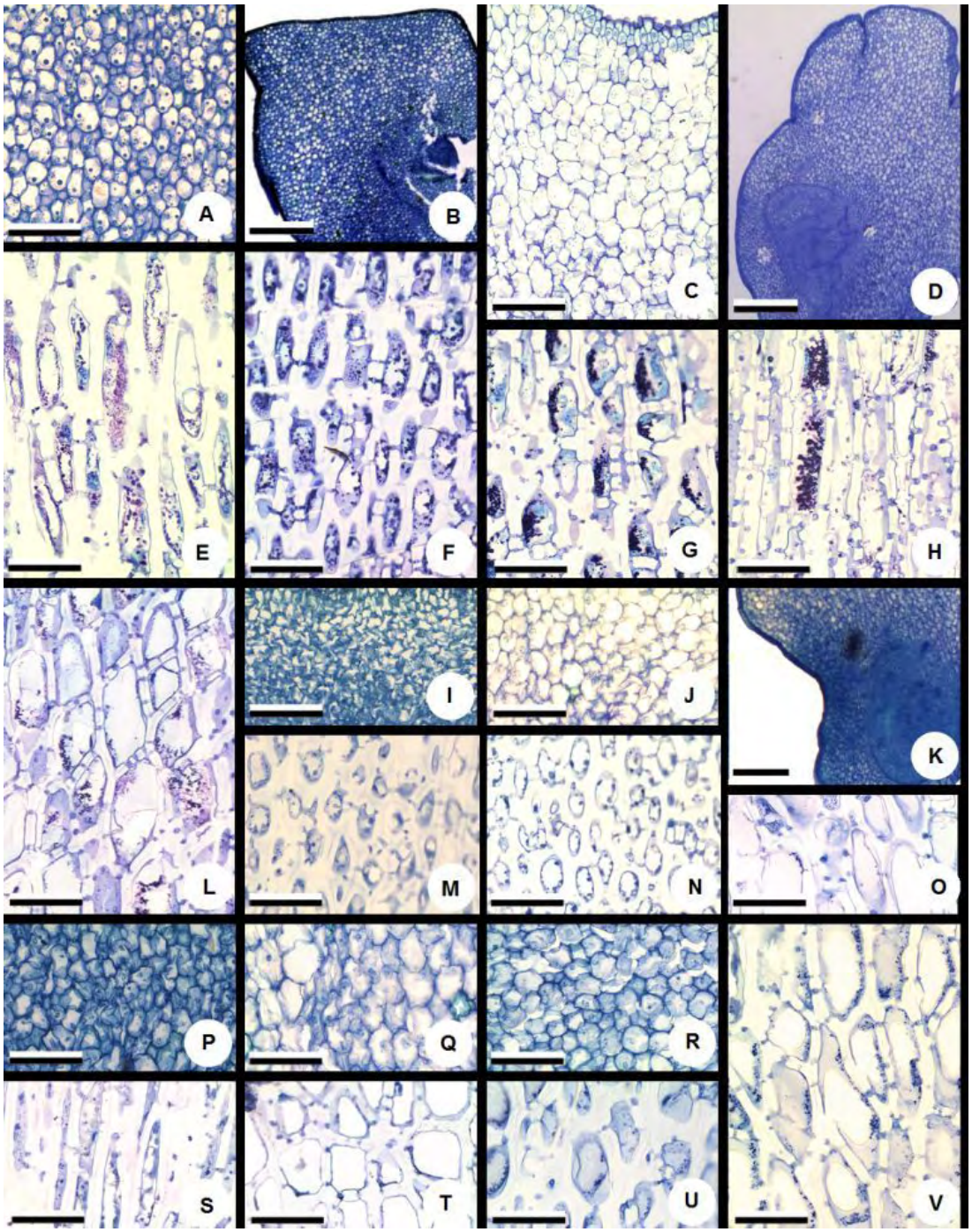

Fig 4. Photomicrographs of longitudinal sections of the apical zone of the embryo (A, B, C, D, I, J, K, P, Q e R) and the endosperm (E, F, G, H, L, M, N, O, S, T, U e V) of *Euterpe edulis* untreated seeds (A-H), and GA_3-treated (50 mg L^{-1}) seeds, immediately after sowing (0 days after sowing, or 0 DAS) (I-O) and at 12 DAS (P-V). Anatomical analysis were performed at 15 (A, E, L, P e S), 30 (B, F, I, M, Q e T), 45 (C, G, J, N, R e U), and at 60 (D, H, K, O e V) DAS. Scale bars: A, C-J, L-V = 10μm; B and K = 20 μm.

Free carbohydrates contents significantly differed among GA_3-treated and untreated *E. edulis* seeds at 15 and 45 DAS. GA_3-treated seeds showed higher carbohydrates contents in comparison to untreated seeds at 45 DAS, but seeds treated at 0 DAS and 12 DAS presented the same values (Fig. 5A). The total lipid contents were different among GA_3-treated and untreated seeds at 15, 30 and 60 DAS. There were great variations among treatments at 30 DAS, and minor differences at 15 and 60 DAS. Therefore, except for the 30th DAS, seed lipid contents ranged from 5 to 7 mg per g of dry seed (Fig. 5B).

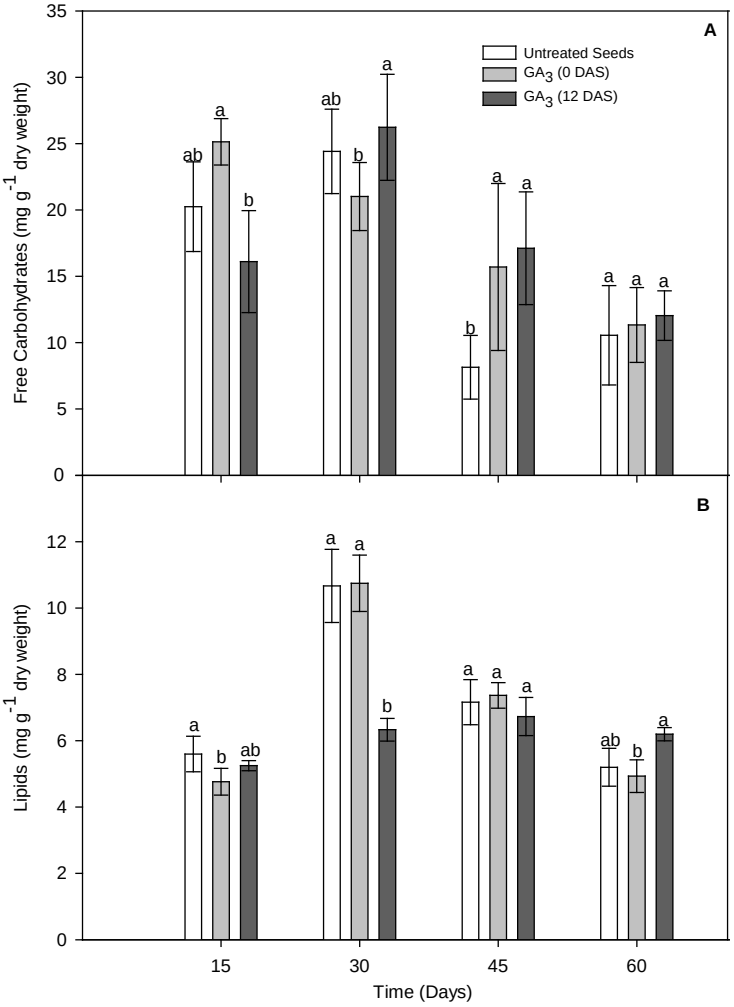

Fig 5. Total free carbohydrate (**A**) and total lipid (**B**) contents in *Euterpe edulis* untreated seeds and GA_3-treated (50 mg L^{-1}) seeds, immediately after sowing (0 days after sowing, or 0 DAS) and at 12 DAS. The reserves quantification were performed at 15, 30, 45, and e 60 DAS For each evaluation date, the same letters indicate a lack of significant difference among treatments, by the Tukey's test ($P < 0.05$). Columns represent mean values (*n =3*) and vertical bars are standard deviation.

Discussion

E. edulis seeds exhibiting 45% of water content maintains the same percentage of germinated seeds (%G) until reaching water contents of 30-35%, values below which the seed viability is lost (ANDRADE, 2001). Palm seeds harvested in riparian forests from warm regions show critical water content of 19% (SALOMÃO et al, 2009). We also found the same %G in seeds that had water contents between 51% and 39%. However, the rate (V) and time (T) of germination were higher as seed water content increased (Table 1). *E. edulis* seeds that had 39% of water content exhibited its relative frequency of germination peak ten days later, in comparison to the seed lot that had 51% of water content. In addition, such peak of the 39%-water-content seeds was lower in relation to the other seed lots tested (Fig. 1). Therefore, our results confirm our prediction that *E. edulis* seeds increase the germination performance as seed hydration is augmented. In fact, decreases in water content of recalcitrant seeds can slow their metabolism, as well as maintaining a low embryo development during the pre-germination phases (BERJAK et al, 1992; FINCH-SAVAGE, 1996). Then, considering the natural dispersion of *E. edulis* de-pulped fruits by dispersers, theoretically, the highest germination performance and seed metabolism events for its successful establishment may start only after a corrective re-hydration of these seeds, which occurs during the first 12 days of seed imbibition (ROBERTO & HABERMANN, 2010) on the soil surface of the forest.

For the above reasons, we used the fully-hydrated seed lot (51 ± 1% of water content) when testing the gibberellic acid (GA_3) on *E. edulis* seeds, despite the fact that this study was performed after the experiment testing different seed water content was concluded. Seeds of this species treated with GA_3 in the range of 40 to 60 mg L^{-1} show improved germination performance (ROBERTO & HABERMANN, 2010), although the precise mechanism for improving it has not been clearly specified. GA_3-treated seeds demonstrated a higher V and T, in comparison to untreated *E. edulis* seeds. However the moment of the imbibition curve that GA_3 was applied (0 or 12 DAS) did not cause any difference in V, T, or %G (Table 2). In our previous work (ROBERTO & HABERMANN, 2010) there was increased germination performance of *E. edulis* seeds treated with GA_3 at 12 DAS, and although strongly suggestive, we could not prove that the stationary phase of the imbibition curve (when the fresh seed mass stabilizes) does not necessarily imply that embryo metabolism was reactivated and that it would not necessarily represent the best moment for the GA_3 application. In the present study, it was clear that the moment of the imbibition curve that GA_3 was applied is irrelevant, corroborating our hypothesis, and emphasizing the recalcitrant seed behavior of *E. edulis* (ANDRADE, 2001). In addition, considering that germination of

GA_3-treated and untreated seeds were evaluated after the conclusion of the study that tested seeds with distinct water content, it is valuable to comment that the time of germination found in the first study was half of the T found in the second study, reinforcing even more its recalcitrant seed behavior.

In the orthodox cereal seeds, it is well established that GA_3 stimulates cells to secrete hydrolytic enzymes, such as α-amylase, which degrades starch reserves in the endosperm (WOODGER et al., 2004). On the other hand, *E. edulis* seed reserves are mostly composed of lipids, proteins, and mannans (PANZA et al., 2004). In *E. edulis* seeds, total lipids may represent 0.45% of the endosperm dry mass (PANZA et al., 2009), which is similar to our results of 0.006 g of total lipids per g of dry seed, throughout the germinating period (Fig. 5B). Unsaturated fatty acids, however, represent approximately 60% of total endosperm fatty acids (PANZA et al., 2009).

Expression of hydrolytic enzymes in germinating cereal seeds, as well as its endosperm cellular changes seems to occur within few days after GA_3 treatments (MATTHEWS et al., 2002). However, endosperm (and embryo) anatomical changes from GA_3-treated to untreated *E. edulis* seeds were not observed immediately after the GA_3 application (Fig. 4E, 4L, and 4S), but it could be noticed only at 60 DAS (Fig. 4H, 4O, and 4V). In fact, the endosperm of *E. edulis* seeds is observed to be in an inactive state, showing no seed reserve mobilization (PANZA et al., 2004).

Our results of slow anatomical responses of the embryo cells when under the influence of GA_3 reinforces the observation that *E. edulis* embryo possesses a conserved characteristic of continuous and very slow development (PANZA et al., 2004). In seeds treated at 0 or 12 DAS, the GA_3 promoted higher contents of total free carbohydrates at 45 DAS, in comparison to untreated seeds (Fig. 5A). Interestingly, the relative frequency of germination was also significantly higher in GA_3-treated seeds around 45 DAS, in comparison to untreated seeds (Fig. 3). On the other hand, the relative frequency of germination of untreated-seeds were higher than GA_3-treated seeds around 55 DAS (Fig. 3), a period in which the same contents of total free carbohydrates were found for GA_3-treated and untreated seeds (Fig. 5A). Although elevated contents of total free carbohydrates were found for GA_3-treated seeds around 45 DAS, the GA_3 was applied 45 and 33 days before this observation, respectively for when the GA_3 was applied at 0 DAS, and at 12 DAS. Moreover, the unlikely lipid degradation role of the GA_3 (as starch degradation stimulated by GA_3 in cereal seeds) was not sustained by our anatomical evidence, which indicated that at 45 DAS the endosperm and embryo cells were displaying relatively unchanged cell contents and intact cell walls (Fig. 4C,

4G, 4J, 4N, 4R, and 4U). Thus, the high germination performance of *E. edulis* seeds caused by GA_3 (ROBERTO & HABERMANN, 2010), which was corroborated by our results (Table 2), is not related to seed reserve mobilization.

GA_3-treated seeds showed larger cells in the embryo (Fig. 4K) and in the endosperm (Fig. 4O and 4V), in comparison to the embryo (Fig. 4D) and endosperm (Fig. 4H) cells of untreated seeds only at 60 DAS. This observation endorses our prediction, based on our previous work (ROBERTO & HABERMANN, 2010), that signs of anatomical changes caused by GA_3 on the embryo or endosperm of *E. edulis* seeds can be observed after more than 15 days GA_3 is applied. Therefore, it seems more reasonable to propose that GA_3 improves the germination performance of *E. edulis* seeds because this plant regulator induces cell expansion in the embryo (and in the endosperm). In fact, there is strong evidence that gibberellins stimulate the expansion of seed embryos (BEWLEY & BLACK, 1994). Nevertheless, in the case of *E. edulis* seeds, GA_3 effects seem to adhere to the conserved characteristic of this species of a very slow embryo development (PANZA et al., 2004).

In nature, the highest germination performance of *E. edulis* de-pulped fruits may be achieved only after imbibition “corrects” seed water content to maximum values. However, even with maximum seed water content, germination performance of this species will tend to decline as seeds age, substantiating the recalcitrant seed behavior of this species. GA_3 accelerates the *E. edulis* germination not because of any seed reserve mobilization, but as a result of seed cell expansion, including embryo cell expansion, which is naturally slow (PANZA et al., 2004).

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