UNESP – UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
INSTITUTO DE BIOCIÊNCIAS

MATURAÇÃO DE SEMENTES DE *POINCIANELLA PLUVIOSA* (DC.) L.P.QUEIROZ (SIBIPIRUNA): SEMENTES TOLERANTES À DESSECAÇÃO E DE BAIXA VIABILIDADE NO ARMAZENAMENTO

JOÃO PAULO NALDI SILVA

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Sumário

RESUMO: Sementes apresentam diversos comportamentos de tolerância à dessecação e longevidade após a dispersão, quando um metabolismo reduzido ou desligado proporcionaria maior sucesso no armazenamento. Esses comportamentos podem ser agrupados em categorias que facilitam o uso de técnicas para conservação em bancos de germoplasma, ou ainda, considerados como um gradiente de respostas entre sementes de diferentes espécies. Uma recente abordagem é de que esses diferentes comportamentos residiriam no estádio de maturidade no qual as sementes se desprendem da planta mãe, sendo sementes sensíveis à dessecação as dispersas em uma fase muito imatura. Entretanto, as variações do metabolismo durante o processo de maturação e suas consequências na viabilidade das sementes após a dispersão são ainda pouco abordadas. O objetivo desta tese foi avaliar as alterações do perfil metabólico primário, de carboidratos e ácidos graxos, durante a maturação e contextualizá-las na contribuição para a longevidade, utilizando sementes de *Poincianella pluviosa* como modelo de estudo. O período de maturação dessas sementes é extenso e maior do que sua viabilidade no ambiente. Sementes imaturas são capazes de produzir plântulas e resistir à secagem bem cedo na maturação. A maturação dessas sementes é caracterizada pela diminuição do metabolismo energético primário concomitante à produção de antioxidantes via metabolismo secundário e acúmulo de compostos osmoprotetores, e os últimos meses da maturação são essenciais para grande aumento de vigor. Esses resultados foram comparados às sementes de *Caesalpinia echinata*, demostrando que baixas temperaturas de armazenamento elevam a longevidade dessas sementes, indicando a presença de um metabolismo reduzido após a dispersão. Ambas apresentam mecanismos antioxidantes, embora não pareçam suficientes para controlar processos oxidativos durante o armazenamento. Diferenças na extensão da maturação entre as duas espécies explicariam a longevidade dessas sementes após a dispersão, quando um possível avanço na maturação proporcionaria uma maior redução do metabolismo energético primário.

Termos para indexação: ácidos graxo, carboidratos, longevidade, metabolismo, tolerância à dessecação

ABSTRACT: Seeds present different behaviors with respect to desiccation tolerance and storability after shedding, when a lower or switch-off metabolism provide greater success in storage. These behaviors can consist in categories that facilitate the use of techniques for conservation in germplasm banks, or even considered as a gradient among seeds of different species. A recent approach is that these different behaviors reside in the mature stage in which seeds are shed from the mother plant, being sensitive to desiccation seeds dispersed in a very immature stage. However, variations of the metabolism during maturation process and its consequences on seed viability after dispersion are still not well understood. The aim of this thesis was to evaluate the changes in the profile of the primary metabolism, carbohydrates and fatty acids during maturation and contextualize them to contribute to storability, using seeds of *Poincianella pluviosa* as model. The maturation period of these seeds is extensive and larger than its viability under environmental conditions. This process is characterized by a decrease of the primary energetic metabolism concomitant with the production of antioxidants via secondary metabolism and an accumulation of osmoprotectant compounds, wherein the last months of maturation are essential for large increase of seed vigor. These results were compared to seeds of *Caesalpinia echinata*, showing that low temperatures of storage increase these seeds storability, suggesting the presence of a reduced metabolism after dispersion. Both present antioxidant mechanisms, although not seem enough to control oxidation processes during storage. Differences on the extension of maturation between the two species can explain the storability of these seeds after shedding, when an advance in maturation would provide a greater reduction of primary energy metabolism.

Keywords: fatty acids, carbohydrates, storability, metabolism, desiccation tolerance

1. Introdução geral

O conhecimento sobre a longevidade das sementes no ambiente foi inicialmente baseado na capacidade destas em resistir à secagem na época da dispersão, já que a redução do metabolismo, por meio da redução do teor de água e da temperatura, é a principal técnica para conservação de sementes.

Sementes que resistem à secagem até umidade abaixo de 10% e ao congelamento são classificadas como ortodoxas, e podem ser armazenadas por períodos prolongados. Já as recalcitrantes, dispersas ainda úmidas e intolerantes à secagem abaixo de 20%, não suportam longos períodos de armazenamento (Roberts, 1973). Mais tarde, uma terceira categoria foi observada. Sementes intermediárias são dispersas com elevado teor de água, como nas recalcitrantes, mas resistem à secagem até 12% de umidade, com maior sucesso no armazenamento (Hong & Ellis, 1996).

Apesar da categorização em três grupos distintos ser útil (Ellis et al., 2007), são observados diversos comportamentos das sementes após a dispersão, e compreenderiam um *continuum* formado pelo máximo de recalcitrância em um extremo e o máximo de ortodoxia no outro (Berjak & Pammenter, 2007; Daws et al., 2008). Estes diferentes comportamentos poderiam, ainda, ser o resultado do quanto a maturação de cada semente se estendeu, evidenciado pela redução da umidade da semente durante este processo, e aliado às condições ambientais e às características de cada espécie, acumuladas no seu processo evolutivo (Barbedo et al., 2013).

Embora a redução do metabolismo seja um objetivo prático das técnicas para a conservação de sementes, há poucas informações na literatura que retratam as consequências das alterações do metabolismo ao longo da maturação com a longevidade das sementes após a dispersão. Para tal, o presente trabalho teve como objetivos:

- descrever a maturação de sementes de *Poincianella pluviosa* como modelo de estudo;
- analisar as alterações do perfil metabólico e de compostos protetores como carboidratos e ácidos graxos durante a maturação e a tolerância a dessecação dessas sementes;
- comparar com sementes de *Caesalpinia echinata*, que produz sementes de baixa longevidade e possui maior quantidade de informações acerca dos processos de maturação e longevidade.

2. Revisão de literatura

2.a Maturação de sementes

O período de desenvolvimento de uma semente pode ser dividido convencionalmente em três fases. A primeira fase é a de histodiferenciação, caracterizada por expressiva divisão celular acompanhada de um aumento rápido no peso fresco e no conteúdo de água da semente. Após este evento, segue-se a fase de expansão celular e de deposição de reservas nas células. Finalmente, o desenvolvimento da maioria das sementes termina com uma fase pré-programada da secagem nas sementes tolerantes, com redução considerável do teor de água (entre 90 a 95%) e do metabolismo da semente e do embrião, passando a um estado quiescente (Black & Pritchard, 2002); nas sementes não tolerantes, o elevado teor de água é mantido, sendo metabolicamente ativas quando dispersas, podendo a semente germinar quando ainda está ligada à planta mãe (Barbedo & Marcos Filho, 1998). Entretanto, o tipo e a intensidade do metabolismo podem diferir, dependendo da fase de desenvolvimento e o conteúdo de água no momento da dispersão.

O estudo da maturação é uma importante forma de se conhecer o comportamento de reprodução das espécies e possibilita prever o estabelecimento e a época adequada de colheita. Além disso, pode-se obter material genético de boa qualidade fisiológica, que é a base para os programas de conservação genética e recuperação de áreas degradadas (Figliolia & Kageyama, 1994). As variáveis mais analisadas em estudos de maturação são as dimensões de sementes, as características externas (tais como cor, textura, brilho e flexibilidade do tegumento), o conteúdo de água e de massa seca, germinação, capacidade de produzir plântulas normais e vigor (Marcos Filho, 2005; Carvalho & Nakagawa, 2012). Vários trabalhos também incluem as características dos frutos, principalmente ao estabelecer um diagnóstico visual do momento adequado para a colheita ou para identificar as características do fruto que correspondem à melhor qualidade fisiológica das sementes (Borges et al., 2005)

Embora o comportamento geral do processo de maturação possa ser previsível, apenas as características da semente ou do fruto não são confiáveis para definir o real estádio de maturidade, especialmente considerando as diferentes espécies ou, dentro da mesma espécie, de diferentes regiões ou épocas. No entanto, o teor de água é mais confiável, homogêneo e, aparentemente, mais independente das condições em que as sementes são formadas (Barbedo et al., 2013).

2.b Comportamentos das sementes após dispersão

A tolerância à dessecação de sementes é um comportamento fisiológico que pode ser considerado como resultado do processo de seleção natural em ambientes com expressivas

sazonalidades térmica ou hídrica. Assim, as espécies que produzem sementes tolerantes à dessecação supostamente necessitaram de sistemas capazes de resistir a condições impróprias à germinação de suas sementes logo após a dispersão. Espécies cujas sementes são dispersas com elevados teores de água e são intolerantes à dessecação, por sua vez, podem ter sido selecionadas em ambientes com distribuição regular de suficiente quantidade de água e temperaturas adequadas para a germinação das sementes e para o estabelecimento de plântulas durante o ano todo (Barbedo & Marcos Filho, 1998).

Apesar desse comportamento diferenciado entre as sementes ter sido detectado desde o início do século passado (Kidd, 1914; Castro & Krug, 1951; Zink & Rochele, 1964), apenas na década de 70 foi proposta uma classificação dessas sementes, incluindo-se também a capacidade e previsibilidade de seu armazenamento. Dessa forma, foram denominadas sementes ortodoxas as que resistem à secagem até umidade abaixo de 10%, característica que permite armazená-las em temperaturas negativas, mantendo a viabilidade por períodos prolongados; em outro extremo foram designadas as recalcitrantes, que são dispersas ainda úmidas, não toleram secagem até cerca de 20% e consequentemente não suportam muito tempo de armazenamento (Roberts, 1973).

Inicialmente, o estudo do comportamento de sementes que apresentavam comportamento não ortodoxo foi baseado na umidade das sementes na época de dispersão, analisando a viabilidade dessas sementes após remoção de água dos tecidos de forma gradativa (Hong & Ellis, 1990). Com o avanço no conhecimento sobre as categorias inicialmente criadas, notou-se um terceiro grupo de sementes, caracterizadas pela dispersão com elevado teor de água, como nas recalcitrantes, mas que suportam serem desidratadas até 10-13% de umidade, ou seja, não tão intensamente até valores suportados pelas ortodoxas mas bem inferiores aos valores suportados pelas recalcitrantes; dessa forma, apresentam maior chance de sucesso no armazenamento. Essas sementes foram denominadas intermediárias (Hong & Ellis, 1996).

Apesar da categorização das sementes em três grupos distintos ser útil (Ellis et al., 2007), hoje se considera que esse comportamento engloba um *continuum*, formado pelo máximo de ortodoxia em um extremo e o máximo de recalcitrância no outro (Berjak & Pammenter, 2007; Daws et al., 2008). O conceito de *continuum* engloba a grande variabilidade que ocorre não apenas entre, mas também dentro da mesma espécie. Trabalhos recentes indicam que o local de origem dessas sementes possui efeito significativo no desenvolvimento da semente e no grau de tolerância à dessecação que pode ser adquirida pelas espécies (Daws et al., 2004; 2006).

Uma visão mais recente é a de que o teor de água assume um papel decisivo na maturação até atingir valores próximos de 50%. Depois deste ponto, diferentes resultados podem ser

observados, dependendo de quanto foi estendido o período de maturação, em conjunto com as condições ambientais e as características apresentadas de cada espécie acumuladas durante o processo evolutivo da espécie (Barbedo et al., 2013).

2.c Metabolismo, carboidratos e ácidos graxos

Sementes em desenvolvimento são metabolicamente muito ativas, principalmente durante a embriogênese e a etapa de acúmulo de reservas (Borisjuk & Rolletschek, 2008). A alta atividade respiratória é correlacionada com a necessidade do embrião em fornecer energia para o crescimento e a síntese de compostos de reserva (Vigeolas et al., 2003; Borisjuk et al., 2004). Na etapa de dessecação de sementes tolerantes ocorre uma grande perda de água, considerado como uma preparação para o período de repouso no estado seco (Angelovici et al., 2010). Existem poucos trabalhos que abordem especificamente o metabolismo respiratório durante toda a maturação das sementes. Entretanto, o desligamento do metabolismo respiratório parece ocorrer na fase de dessecação de sementes de *Arabidopsis*, principalmente, pela redução dos componentes do ciclo do ácido tricarboxílico (Fait et al., 2006), o que diminuiria as taxas respiratórias e evitaria processos oxidativos na mitocôndria, levando ao aumento da tolerância à dessecação (Pammenter & Berjak, 1999). De forma oposta, algumas sementes não passam por uma fase de dessecação e possuem um elevado teor de água e da atividade respiratória quando são dispersas, como observado em sementes de *Inga vera* (Caccere et al., 2013), sementes altamente sensíveis à desidratação e de difícil armazenamento (Berjak & Pammenter, 2007; Bonjovani & Barbedo, 2008).

Processos e mecanismos de proteção vêm sendo identificados sobre diversos aspectos, e juntos promovem a tolerância à dessecação das sementes, embora o modo como operam e sua interação ainda não estejam muito bem compreendidos (Berjak et al., 2007). Entre outros mecanismos que podem estar envolvidos no processo de tolerância à dessecação, o acúmulo de carboidratos não estruturais no período de maturação foi bastante investigado e parece invariável que a sacarose e certos oligossacarídeos da série da rafinose (OSR), como a rafinose e estaquiose são acumulados durante a maturação, na fase de secagem, de sementes tolerantes a dessecação (Steadman et al., 1996; Obendorf, 1997; Black et al., 1999; Hoekstra et al., 2001; Buitink et al., 2003; Leduc et al., 2012). Além disso, a alta concentração de sacarose é também comum nos tecidos secos das plantas revivescentes (Berjak et al., 2007). Outros açúcares como os ciclitóis livres e galactosil ciclitóis também se acumulam em algumas sementes e parecem contribuir na tolerância à dessecação de maneira semelhante aos OSR (Obendorf, 1997; Peterbauer & Richter, 2001), quando estes não estão evidenciados (Horbowicz et al., 1998; Steadman et al., 2000; Borges et al., 2006).

O papel da sacarose no estado seco em tecidos da semente já foi bastante discutido (Berjak et al., 2007), esse dissacarídeo teria um papel dinâmico de impedir a aproximação das proteínas de membranas umas às outras, substituindo fisicamente a água quando esta é removida na secagem e, dessa forma, prevenindo sua proximidade lateral (Hoekstra et al., 2001). Esta proximidade promoveria mudanças nos fosfolípideos e em alguns componentes da membrana que são acompanhadas pela exclusão de proteínas integrais (Bryant et al., 2001; Koster & Bryant, 2005; Halperin & Koster, 2006). Assim como a sacarose, trealose é um dissacarídeo não redutor acumulados por organismos que suportam vários estresses ambientais como seca, calor ou temperaturas muito baixas (Wingler, 2002; Eastmond & Graham, 2003), e é encontrado em grandes quantidades durante a dessecação das plantas revivescentes *Selaginella lepidophylla* (Adams et al., 1990). Trealose atuaria como um estabilizador de proteínas, protegendo a conformação da proteína na desidratação (Kaushik & Bhat, 2003).

Outra visão sobre o papel dos carboidratos seria a da ação benéfica associada a um sistema antioxidante bastante eficiente (Walters et al., 2002). Carboidratos solúveis e o sistema antioxidante, tais como o ciclo ascorbato-glutationa e compostos fenólicos poderiam ser parte de um sistema redox integrado contra o estresse oxidativo (Van den Ende & Valluru, 2009). Açúcares tais como sacarose, OSR, frutanos e ciclitóis atuariam não apenas como osmoprotetores e estabilizantes de membranas celulares, mas também como agentes contra espécies reativas, função semelhante dado para os compostos fenólicos (Nishizawa et al., 2008; Van den Ende & Valluru, 2009; Peshev et al., 2013). Estes açúcares poderiam também dispor de NADH necessário para a ação das enzimas do ciclo ascorbato-glutationa, tais como monodehidroascorbato redutase e glutationa redutase, e também estão correlacionados com o aumento dos níveis dos antioxidantes ácido ascórbico e glutationa durante o estresse oxidativo (Nishizawa et al., 2008).

Os ácidos graxos também atuam na defesa das células das sementes em condições de estresse nas alterações da saturação de ácidos graxos durante a perda de água, modificando a fluidez da membrana (Liu et al., 2006; Mello et al., 2010). Menores quantidades de ácidos graxos saturados foram detectados em sementes tolerantes à dessecação, em comparação com as intolerantes, em triacilgliceróis e ácidos graxos constituintes da membrana (Liu et al., 2006; Mello et al., 2010). Estas modificações também impediriam a ação dos radicais oxidativos em ácidos graxos insaturados, que poderiam resultar na morte celular (Halliwell & Gutteridge, 1999).

Embora um aumento da insaturação de ácidos graxos poderia diminuir a probabilidade de transição de fase cristalina para gel em teores de água muito baixos, melhorando a tolerância

à dessecação de sementes (Liu et al., 2006), uma baixa quantidade de insaturação poderia diminuir a fluidez da membrana, tornando a bicamada rígida, e reduzindo perda de solutos (Quartacci et al., 2002). Em *Ramonda serbica*, a redução de insaturação dos ácidos graxos, em conjunto com mudanças nos componentes de membranas, tais como fosfolipídeos, fosfatidiletanolamina, cerebrosídeos e esteróis livres limitam a tendência da membrana em formar uma configuração não-lamelar durante a secagem, dessa forma mantendo a integridade da membrana (Quartacci et al., 2002). A presença do ácido azeláico nas membranas também podem ser um indicativo de dano oxidativo da membrana, uma vez que é considerado como um marcador da fragmentação de lipídeos induzida por radicais livres (Zoeller et al., 2012.), um processo caracterizado pela fragmentação do ácido linoleico (C18:2) catalisada por espécies oxidativas (Schneider et al., 2008). Também foi proposto que a peroxidação de lipídeos não enzimática protege as células contra o estresse oxidativo pela eliminação de espécies reativas (Mene-Saffrané et al., 2009).

2.d Espécies modelos de estudo

Poincianella pluviosa (DC.) L.P.Queiroz (= *Caesalpinia pluviosa* DC.) está incluída em Leguminosae, uma das mais importantes e numerosas famílias tropicais. Sua situação taxonômica foi revista (Lewis, 1998; Queiroz, 2009) mas ainda está em discussão (Souza et al., 2013). Esta espécie apresenta uma ampla distribuição no Brasil, abrangendo quase todos os domínios fitogeográficos (Amazônia, Caatinga, Cerrado, Mata Atlântica e Pantanal - Lewis, 2013; Queiroz, 2009). Informações limitadas sobre sementes dessa espécie são disponíveis. A semente madura de sibipiruna apresenta cerca de 50% de lipídios, 32% de carboidratos solúveis, 7,7% de amido e 6,8% de proteínas solúveis (Corte et al., 2006), podem ser armazenadas por até 360 dias em câmara fria, porém em ambiente natural, perdem sua capacidade germinativa em 240 dias de armazenamento (Figliolia et al., 2001). São visualmente parecidas com as sementes de pau-brasil, com o mesmo tom amarronzado nas sementes maduras, porém maiores em comprimento e largura. As sementes imaturas apresentam tegumento fino e transparente, com cotilédones verdes e eixo embrionário bem definido (Silva, 2010).

Caesalpinia echinata Lam., o conhecido pau-brasil que deu nome ao nosso País, ou ibirapitanga (madeira vermelha) na linguagem tupi-guarani, é pertencente à família Leguminosae, subfamília Caesalpinioideae, e apresenta distribuição natural restrita à floresta pluvial tropical atlântica e está ameaçada de extinção (Rocha, 2004). Apresenta sementes planas e irregularmente orbiculares, com 1 a 1,5 cm de diâmetro (Cunha & Lima, 1992), presentes entre 1 a 2 sementes por fruto (Lewis, 1998), com coloração verde clara no início do desenvolvimento até uma coloração parda, quando maduras (Borges et al., 2005).

Essas sementes toleram secagem até atingirem níveis abaixo de 8% de água (Barbedo et al., 2002) e vêm se mostrando de grande interesse para estudos de tolerância à dessecação, acumulando amido como principal polissacarídeo de reserva, seguido de alguns ciclitóis como o pinitol e ciceritol (Borges et al., 2006; Garcia et al., 2006), com apenas traços de rafinose e estaquiose (Borges et al., 2006; Garcia et al., 2006). Perdem a viabilidade rapidamente, mesmo quando secas, se armazenadas em temperaturas acima de 20°C (Barbedo et al., 2002), mas mantêm a viabilidade por mais de 5 anos se armazenadas em temperaturas negativas (Mello et al., 2013). E foi possível induzir essa tolerância em sementes imaturas ao aplicar estresse hídricos (Leduc et al., 2012).

3. Chapter 1

The maturation period of *Poincianella pluviosa* (Caesalpinioideae) seeds is longer than their storability

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The maturation period of *Poincianella pluviosa* (Caesalpinioideae) seeds is longer than their storability

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Abstract

The persistence of viable seeds in the soil is an important way to assure plant propagation. However, some species produce seeds with very short lifespan, requiring specific strategies to guarantee their propagation. Seeds of *Poincianella pluviosa* have medium to short storability at room temperature and no information is available on the development of such seeds. Early harvest may provide immature seeds with low vigor and harvesting seeds after maturity can accelerate their deterioration. In the present work we characterize morphological and physiological changes that occur in two consecutive developmental cycles of *P. pluviosa* seeds. These analyses allowed defining 13 stages in the annual cycle. The maturation period (315-330 days after anthesis - DAA) was higher than seed viability at natural environment. Maximum values of dry mass, germination and vigor were obtained in the last month of maturation, before natural desiccation phase. Higher temperatures during maturation increased vigor of immature seeds, producing normal seedlings before the physiological maturation point. Results show that seeds in different stages are dispersed together at the end of maturation period, despite the physiological maturity was identified 315-320 DAA. Possible strategies to increase seed viability in environmental conditions are discussed.

Keywords: *ex situ* conservation, physiological maturity, seed dispersal, seed maturation, sibipiruna.

1. Introduction

Seed development starts with the fertilization of the ovule followed by a period of extensive cell division and differentiation. After this phase, dry matter is transferred from the maternal plant to the seed which has its water content progressively reduced (Bewley et al., 2013) until abscission indicating, for most species, the end of reserve accumulation and the achievement of mass maturity (Ellis and Pieta Filho, 1992).

Some seeds can maintain viability for thousands of years, representing an important feature for plant survival. However, the longevity of seeds can be regulated by how far is advanced the maturation process. An incomplete process can result in both poor seed germination and low persistence of a seed bank (Barbedo et al., 2002; Sallon et al., 2008). Therefore, characterization of the maturation cycle is important for maintaining seed viability during storage, since it depends on the initial quality of seeds (Bonjovani and Barbedo, 2008), and on the wide spectrum of desiccation tolerance behavior evidenced at the end of seed maturation (Berjak and Pammenter, 2008). Precocious harvesting, for example, can produce seed lots with low vigor (Kermode, 1990; Probert and Hay, 2000). On the other hand, late harvesting can accelerate seed deterioration because the environmental conditions are frequently unsuitable for seed storage (Butler et al., 2009). Therefore, the understanding of the maturation processes is crucial to determine the ideal time of harvesting seeds with high quality (Borges et al., 2005).

Poincianella pluviosa (DC.) L.P.Queiroz (= *Caesalpinia pluviosa* DC.) is included in the Leguminosae, one of the most numerous and important neo-tropical families. Its taxonomic classification was reviewed (Lewis, 1998; Queiroz, 2009) but is still under discussion (Souza et al., 2013). This species shows a wide distribution in Brazil, covering almost all phytogeographic domains (Amazon, Caatinga, Cerrado, Atlantic forest and Pantanal - Lewis, 2013; Queiroz, 2009). Excepting a recent ontogenetic study (Souza et al., 2013), limited information about seeds of this species is available. They are not photosensitive (Ferraz-Grande and Takaki, 2006), accumulate predominantly lipids (about 50% of total weight - Corte et al., 2006) and their storability at room temperature is about 240 days (Figliolia et al., 2001; Pontes et al., 2006).

In the present work we characterize the physiological maturity of seeds of *P. pluviosa* and describe the morphological and physiological changes occurring during the whole

maturation period aiming at an understanding of this process and to contribute to conservation strategies of underexploited neo-tropical species. Information about different strategies of tree species to maintain seed viability in the natural environment is discussed.

2. Material and methods

2.1. Plant material

Trees (*ca*. 35) planted in Rubião Jr, a *campus* of the Universidade Estadual Paulista (UNESP) in Botucatu, SP, Brazil (22º52'20''S 48º26'37''W) had inflorescences tagged at the beginning of their anthesis. Pods were harvested directly from the branches at 80, 161, 203, 217, 245, 259, 285, 301 and 315 days after anthesis (DAA), in 1P (September/2009 to August/2010), and after 97, 174, 202, 216, 230, 245, 258, 286, 301, 317 and 323 DAA, in 2P (September/2010 to July/2011). These were considered as the age of fruits/seeds. Additionally, seeds were obtained directly from the ground, not exceeding 48 h after shedding, which were named recently-dispersed seeds (RDS).

2.2. Meteorological data

Values of minimum and maximum temperatures and rainfall, from flowering to dispersion of each period of maturation were obtained from an automatic meteorological station located in the Rubião Jr *campus* (Botucatu/São Paulo).

2.3. Fruit and seed analyses

Fruits and seeds (three replicates of five fruits and fifteen seeds) of each stage were biometrically characterized with respect to color, texture and size (length, width and thickness, in mm). The seeds were removed from the pods manually and were also evaluated for water and dry mass contents, germination and seedling development.

The water content (g of water per g of dry weight, g g^{-1}) and dry mass (g $seed^{-1}$) were determined gravimetrically in an oven at 103 ºC ± 3 ºC for 17 h, according to ISTA (2004) with four replicates of five seeds. The water potential was measured with a Decagon WP4 potentiometer (Pullman, USA) based on the dew point. Germination tests were carried out in four replications of 15 rolls (two sheets for the base and one for the covering) of Germitest paper previously moistened with tap water (ISTA, 2004). The rolls were maintained in a germination chamber (25 ± 1 ºC) and were evaluated every 2 days until 40 days, registering the percentage of germinated seeds (protrusion of at least 5 mm of primary root), normal seedling development (seedlings with at least 3 cm and no visual malformations) and, at the end of the analysis was calculated the speed of germination-aid (Maguire, 1962).

Data are means ± standard deviation of at least four replicates of 20 seeds per treatment that comprises the physical and physiological analyses; the points in the graphics were connected when they indicate continuous development.

3. Results

3.1. Morphological features of seeds and fruits

The maturation process of *Poincianella pluviosa* seeds is extremely long (*ca.* 11 months) and was characterized in 13 distinct stages distributed in a complete cycle of 330 DAA (Fig. 1). During this period, several morphological changes were observed in the dehiscent fruits, including the greenish color which turns to brown at the end of maturation, at the same time they become rigid. After seed dispersion, the fruits usually are twisted, dry and detached from the mother plant.

The seed coat of *P. pluviosa* is green until 300 DAA, when turned completely to brown (Fig. 1), however, it was possible to find some dispersed seeds with green color. The size of the seeds showed different pattern of development when compared to the fruits (Fig. 2). The seed length, width and thickness reached maximum values at stage 12 (Figs. 2b, d, f). On the other hand, there was no significant variation in fruits size during almost all the maturation process (Figs. 2a, c), except for the thickness, which had a slight increase until 300 DAA (Fig. 2e), and could be related to the accumulation of dry matter (Fig. 3b). In addition, the decrease in thickness in the last two stages could be related to the loss of water (Fig. 3a), corresponding to natural desiccation phase at the end of the seed maturation process.

3.2. Water content, water potential and seed dry mass

The water content of the seeds decreased gradually from the beginning (*ca.* 3.5 g g^{-1}) to the end (*ca.* 0.6 g g^{-1}) of maturation, with a greater decrease in the last stages (Fig. 3a). It is interesting to note that, despite the water content was reduced from the first to the last stage, the water potential remained unchanged during most part of the maturation process (Fig. 3a). In recently dispersed seeds (RDS), the water content was 0.09 g g^{-1} and very low water potential (-73.4 MPa). Dry matter accumulation, that was also similar in 1P and 2P, gradually increased until the last month of maturation. However, seeds of 2P showed dry matter higher than 1P at the last two stages, mainly at stage 12 (0.376 and 0.261 g $seed^{-1}$, respectively, Fig. 3b).

3.3. Seed vigor

Higher temperature ranges were registered in the second period of analysis (2P), especially after 200 DAA (Fig. 4), and could be related to the highest rate of seed germination in 2P (Fig. 3c). However, seeds with 300 DAA in both periods (1P and 2P) presented the same

germination percentage (around 70%) until they reached the maximal values (100%) at stages 12 and 13 (Figs. 3c, d). The main difference between 1P and 2P was the capability of the seed lot to produce normal seedlings, which was higher in 2P (Fig. 3e). It is interesting to note that RDS of *P. pluviosa*, usually utilized as source of seeds in reforestation programs, showed low rates of germination and normal seedling development (Fig. 3c, e).

4. Discussion

4.1. The extensive seed maturation process

The maturation process of seeds of *P. pluviosa* could be included into the most extensive ones so far reported in Angiospermae, being distributed along 330 DAA. Long periods of seed maturation were also described for seeds of *Cedrela fissilis* (8-9 months), *Coffea sp.* (10-11 months), and for some palms with seed maturation ranging from 180 to 400 days (Chapin, 1999; Corvello et al., 1999; Dussert et al., 2000; Pérez et al., 2012).

Despite the long time needed to complete seed development and maturation, the major changes were observed just at the end of the maturation process when the fruits become brown and could be an indicator of the last stage prior to dehiscence (Fig. 1). However, the seeds undergo morphological changes during maturation, with maximum values of length, width and thickness at stage 12 (Figs. 2b, d, f), indicating the point of maximum dry matter accumulation is close to seed dispersal.

4.2. Influence of temperature range on seed vigor

Although similar pattern was observed throughout most of the maturation process in 1P and 2P, the higher seed dry matter observed in the last two stages in 2P (Fig. 3b) could be a result of differences in meteorological conditions, as also observed for other species (Daws et al., 2004; Martins et al., 2009). In seeds of *Eugenia pyriformis*, for example, dry matter accumulation was influenced by variation of temperature between two consecutive years (Lamarca et al., 2013). It is possible to observe, in Figure 4, that the highest temperature range was registered in 2P, especially after 200 DAA, which could allow higher accumulation of dry matter.

The high percentage of seeds capable to germinate could also be related to the higher temperature range during the first stages of development (Fig. 3c). In fact, the increase of air temperature during seed development increases germination of various species (Fenner, 1991; Martins et al., 2009). The lower temperature registered in 1P (Fig. 4a) and the lower dry matter accumulated by these seeds (Fig. 3b) could be related to their lower capacity to produce normal

seedlings (Fig. 3e). Similar behavior was described earlier to seeds of several species (Kermode, 1990).

4.3. Dispersed seeds are not the most vigorous ones

Seeds of *P. pluviosa* are dispersed in the driest period of the year (July to August, Fig. 4) in the southeast region of Brazil which could be related, in addition to the long maturation period, with seed tolerance to water stress, as suggested by Daws et al. (2004), Dussert et al. (2000) and Pritchard et al. (2004) for other species. In agreement, seeds of *P. pluviosa* could be viable under natural environment until the next rainy period (December to January, Fig. 4), despite their short storability at room temperature (Figliolia et al., 2001). However, differently from the usual procedures, RDS are not as the most suitable seeds for storage or seedling production as those ones at stage 13, since RDS showed lower rates of germination and normal seedling development (Fig. 3c and e). In addition, green seeds were also found in the RDS lot (Fig. 1), suggesting that some immature seeds can be dispersed with the mature ones, thus resulting in a heterogeneous lot of seeds. Seeds of *Galanthus nivalis* and *Narcissus pseudonarcissus* (Amaryllidaceae), from moist temperate woodlands where shed when they are immature and considerable embryo development occurred after shedding (Newton et al., 2013). Implication of dispersal of immature seeds in neo-tropical forests was also recently discussed. The degree of immaturity in which seeds are detached from the mother plant was considered crucial for desiccation tolerance and seed longevity (Barbedo et al., 2013).

Seeds of *P. pluviosa* contained the highest dry mass (*i.e.*, physiological maturity, Bewley et al., 2013) between 315-323 DAA (stage 12), immediately prior to the natural desiccation. Some authors consider that increase in seed quality, including seed longevity, can occur between periods of maximum accumulation of dry matter and seed dispersion (Hay and Probert, 1995; Probert et al., 2007). According to this concept, seeds of *P. pluviosa* should be harvested at stage 13, when natural desiccation already occurred and their water content was close to 0.26 g g^{-1} (Fig. 3a).

4.4. Long maturation period as a strategy to maintain seed viability

While tolerant to desiccation, seeds of *P. pluviosa* do not exhibit characteristics typically found in classic models of tolerant seeds. *P. pluviosa* seeds do not accumulate raffinose family oligosaccharides (RFO), such as raffinose and stachyose, but cyclitols (Silva, J.P.N. unpublished data), which are related to membrane stabilization during the dry state (Obendorf, 1997), and have low viability when stored at room temperature (Figliolia et al., 2001). Similar characteristics were observed in seeds of brazilwood, which also accumulate cyclitols and only traces of RFO (Borges et al., 2006; Mello et al., 2010). Brazilwood seeds can be stored at

temperatures below 0 °C and remain viable under this condition for as long as five years (Mello et al., 2013). When stored at room temperature these seeds lose their viability even quicker (after 30 days - Barbedo et al., 2002) then *P. pluviosa,* just after the short maturation period (50-60 DAA), evidencing an extremely narrow window of production and viability in natural conditions (Borges et al., 2005). In contrast, the maturation process of *P. pluviosa* is so extensive that exceeds the viability of these seeds during storage in natural conditions. This suggests that the long maturation period could be a strategy to maintain seeds able to develop new plantlets even during the maturation process.

As a matter of fact, the extensive maturation cycle of *P. pluviosa* corroborates the concept that seed banks are not the only way to guarantee plant propagation by seeds. The monoembryonic seeds of *Eugenia* species (Salomão and Allem, 2001) are sensitive to desiccation (Delgado and Barbedo, 2007, 2012) and do not support storage for periods longer than one year (Barbedo et al., 1998; Kohama et al., 2006; Maluf et al., 2003). Conversely, they are able to germinate successively up to 6-8 times if the previous one fail to occur (Teixeira and Barbedo, 2012), thus maintaining the seed alive in the environment for a period that exceeds the actual period of seed viability under storage, if considered that the germination of these seeds takes 1-2 months to occur (Delgado and Barbedo, 2007).

Another interesting strategy is found in seeds of Arecaceae species in which the germination period varies from 100 days to 13 months under natural conditions (Koebernik, 1971). These seeds exhibit morpho-physiological dormancy (Baskin and Baskin, 2001), generally caused by the presence of a rudimentary embryo with a hard but slightly permeable coat (Pérez et al., 2008), which act as a barrier to the water stream causing a slow imbibition as well as protecting the seed against dehydration (Pérez et al., 2008; 2012; Pritchard et al., 2004). Similarly as seen in *Eugenia* seeds, the period in which seeds of Arecaceae germinate could also be higher than the seed viability period under storage, between 2-12 months (Broschat and Donselman, 1987; Ellis et al., 1991; Martins et al., 2009). Taken together, these examples represent alternative ways to both the desiccation tolerance and/or the dormancy to guarantee the perpetuation of the species. These strategies could allow the establishment of seedlings in different periods of the year and climatic conditions.

The maturation study of *P. pluviosa* seeds highlights a new strategy of a neo-tropical tree species to overcome adverse environmental conditions expected to be present in the variety of phytogeographic domains where the species can be found (Lewis, 2013; Queiroz, 2009). In addition, the information provided here contributes to a better understanding of seed behavior

allowing the possibility to obtain seeds with high quality and consequently to increase the availability of *P. pluviosa* for reforestation and conservation purposes.

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The authors declare that they have no conflict of interest.

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Fig. 1

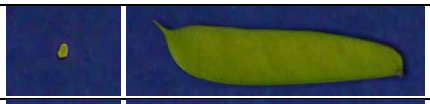
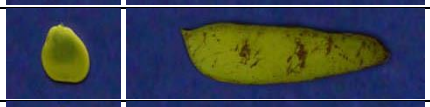
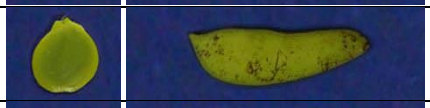
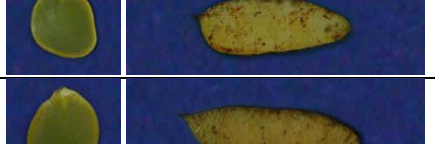
Stage	DAA	Visual aspects		Characteristics	
		Seed	Fruit	Seed	Fruit
S1	80-97			bright green, thin and fragile coat	light green and very flexible
S2	145 to 175			bright green, cotyledons smaller than the coat	light green, with brown spots, very flexible
S3				bright green, cotyledons of size close to the coat	
S4	200			green, coat thin and fragile, very flexible	
S5	215				
S6	230			green, rough coat and fragile, very flexible	green, with brown spots, flexible
S7	245				
S8	260			green, rough coat and fragile, flexible	
S9	285				
S10	300				
S11	307 to 330			green with brown spots, rough coat, flexible	green, with brown spots, slightly flexible
S12				light brown and opaque, rigid rough coat, slightly flexible	
S13				brown and opaque, and rigid rough coat	pale brown or brown, rigid
Dispersed				green to brown, opaque, and rigid rough coat	brown or grey, rigid, often twisted.

Fig. 1. Morphological characteristics of *P. pluviosa* fruits and seeds throughout 13 stages of maturation. The pods were harvested directly from the branches of marked flowers and the estimated time to reach each stage was calculated after two periods of seed maturation: between Sep/09 and Aug/10 (1P) and between Sep/10 and Jul/11 (2P). Bars: seeds= 1 cm, fruits= 2 cm.

Fig. 2

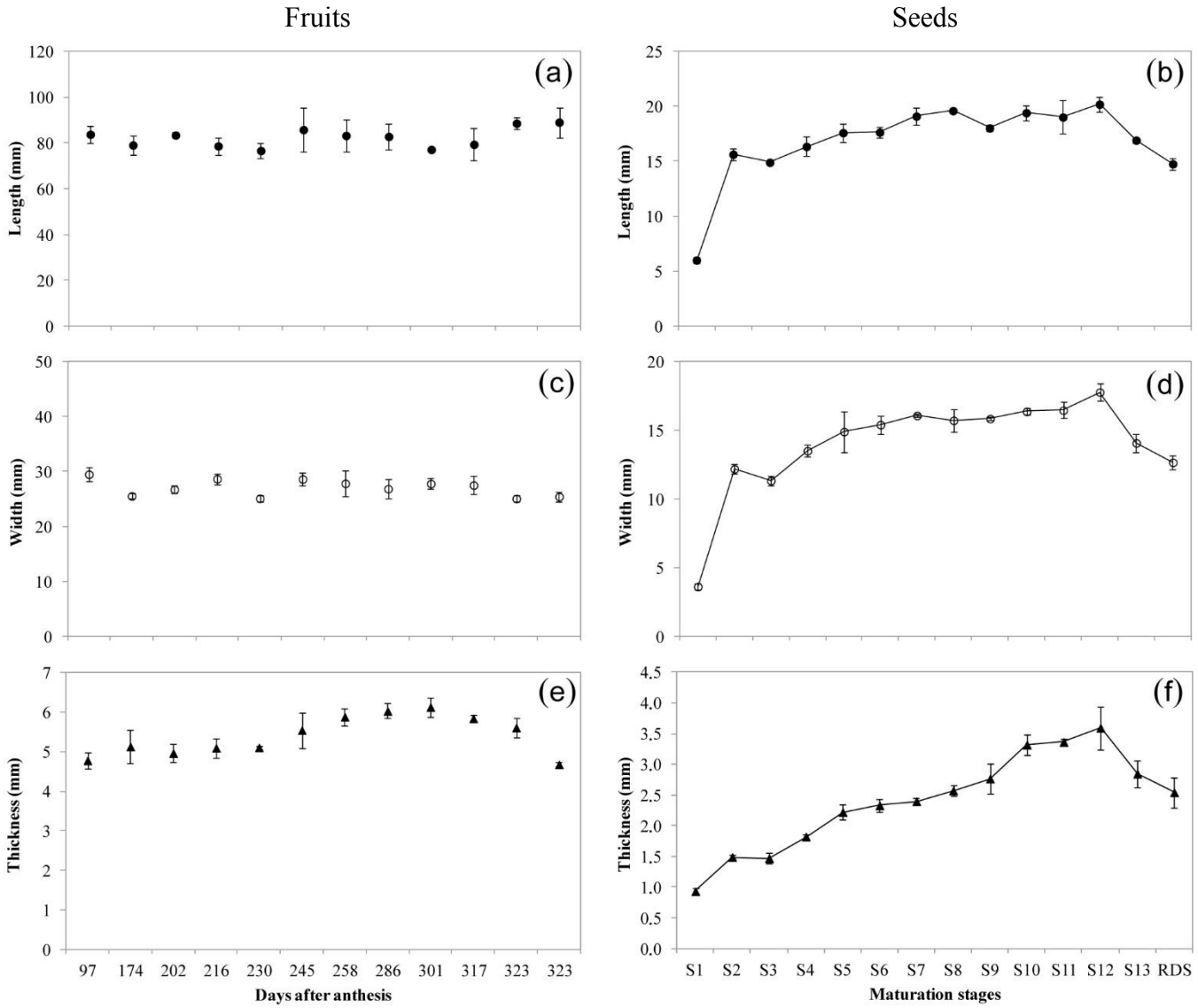


Fig. 2. Changes in dimensions of *P. pluviosa* fruits (a, c and e) collected during Sep/10 to Jul/11 (2P), and dimensions of seeds (b, d and f) classified into 13 stages. RDS, seeds recently dispersed. Data are average ±SD of three replicates of five fruits or fifteen seeds. The points were connected when they indicate continuous development.

Fig. 3

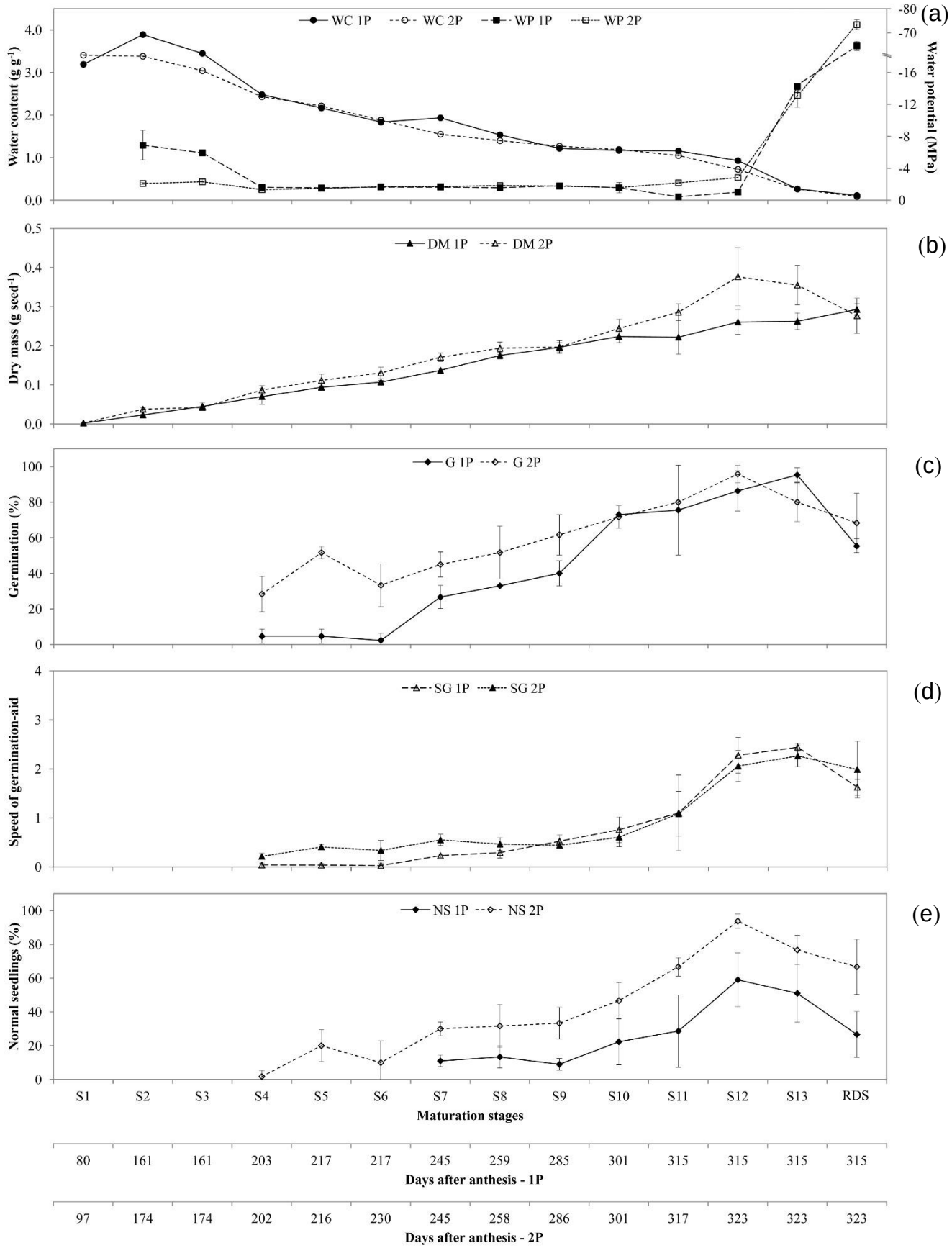


Fig. 3. Physical and physiological characterization (average ±SD) of *P. pluviosa* seeds defined each stage of maturation throughout two consecutive periods. Sep/09 to Aug/10 (1P) and Sep/10 to Jul/11 (2P). Water content (WC - g g^{-1}) and water potential (WP - MPa) - (a); Dry matter (DM - g $seed^{-1}$) - (b); Germination (G - %) - (c); Speed of germination-aid (SG) - (d); Normal seedlings (NS - %) - (e).

Fig. 4

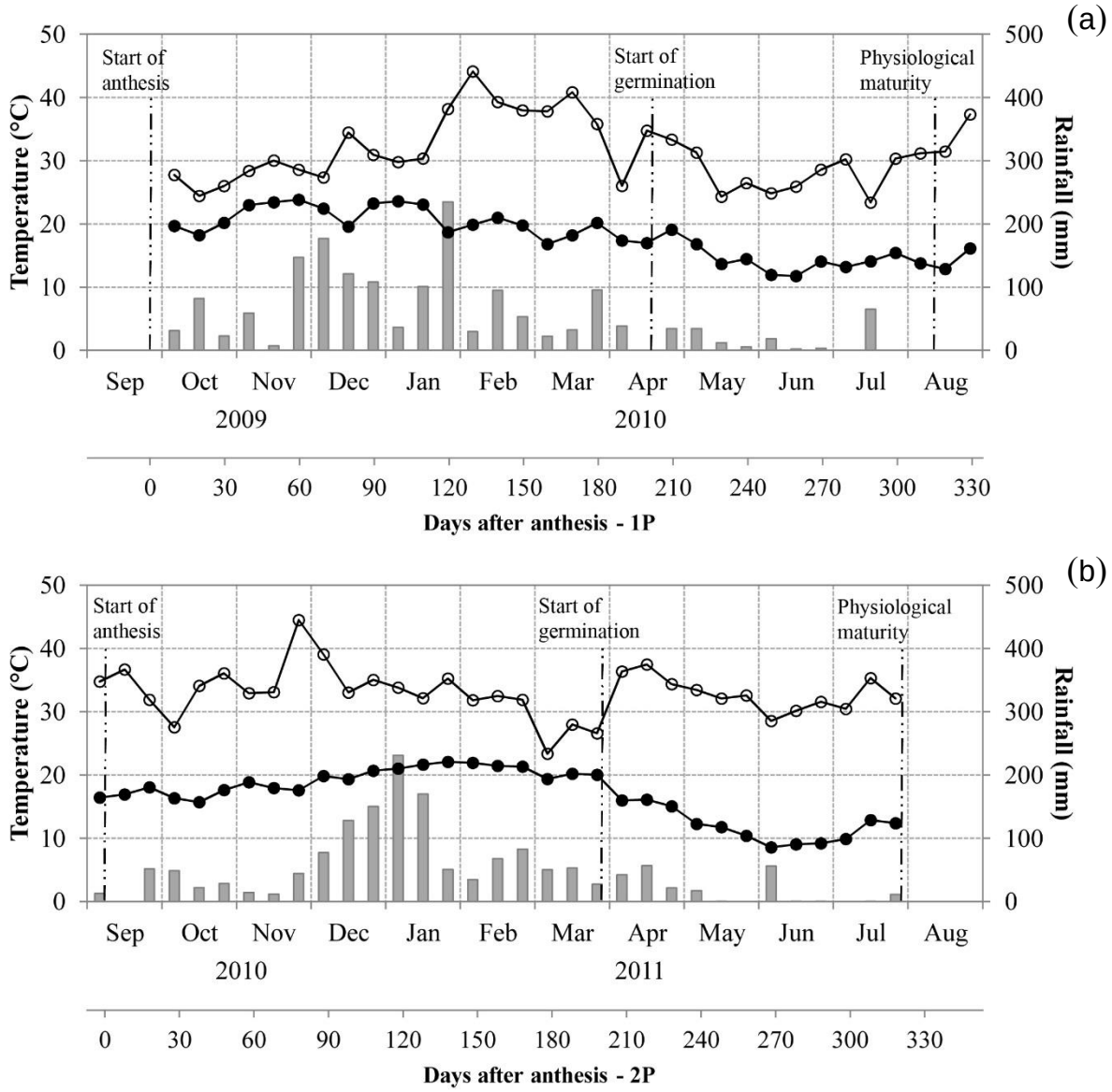


Fig. 4. Meteorological data registered in Botucatu, SP, Brazil, throughout two periods of complete seed maturation. Sep/09 to Aug/10 (1P) - (a); Sep/10 to Jul/11 (2P) - (b). Maximum (○) and minimum (●) temperatures and rainfall (columns). The beginning of seed maturation (start of anthesis), germination (radicle protrusion) and physiological maturity point are indicated to each analyzed period.

4. Chapter 2

A switch of primary to secondary metabolism prior the desiccation phase characterizes the maturation of *Poincianella pluviosa* (Caesalpinioideae) seeds

Abstract

Developing seeds are metabolically highly active during embryogenesis and seed filling, while a decreased metabolism is expected at the desiccation step of tolerant seeds maturation. Seeds of *Poincianella pluviosa* present an extensive maturation cycle (11 months) being higher than seed storability at room temperature (8 months) requiring specific strategies to guarantee its propagation. The objective of this work was characterize the metabolic profile during seed maturation and changes of soluble carbohydrates, triacylglycerols and fatty acids membranes constituents to better understand their role on seed behavior after shedding. Decreased proportions of citrate, isocitrate, succinate and malate until maturity (S12) indicates low respiratory metabolism prior the desiccation step. During maturation, changes of shikimate, 4-coumarate and quinate proportions suggest the production of chlorogenate isomers, known antioxidants, found in elevated proportions in dispersed seeds, along with lactate and glycerol. Seeds of *P. pluviosa* accumulates large quantities of oil (~45%), mainly composed by linoleic acid, compared to starch and soluble carbohydrates (both ~9%), composed by sucrose, cyclitols and traces of raffinose and stachyose. Decreased level of unsaturation in membrane fatty acids and increased proportion of trehalose in dried seeds suggest elevated protection of membrane integrity against oxidative stress and water loss. These results provide evidence that seeds of *P. pluviosa* prepare for the dried period, indicated by the metabolic shift between respiratory to secondary metabolism to produce antioxidants, and present different mechanisms to avoid damages during desiccation.

Keywords: carbohydrates, chlorogenic acid, fatty acids, metabolic profile, trehalose.

2.1 Introduction

Poincianella pluviosa (DC.) L.P.Queiroz (= *Caesalpinia pluviosa* DC.) is a leguminous tree species widely distributed in Brazil and covers almost all phytogeographic domains, such as Amazon, Caatinga, Cerrado, Atlantic forest and Pantanal (Lewis, 2013; Queiroz, 2009). Seeds of *P. pluviosa* exhibit a short to medium storability when stored at natural tropical environmental conditions (20-25 °C), losing their viability in 240 days (Figliolia et al., 2001), while at low temperatures (5-8 °C), the seed germination is maintained for 360 days (Pontes et al., 2006). Intriguing, these seeds present an extensive maturation period, being dispersed about 330 days after anthesis (DAA - Silva et al., 2014, Chapter 1), with the physiological maturity achieved only at 307 DAA, before the desiccation step. However, it is possible to obtain seedlings from seeds in earlier stages of development. In fact, it is suggested that the long maturation period could be a strategy to maintain seed viability even during the maturation process (Silva et al., 2014, Chapter 1).

Mature seeds of *P. pluviosa* contain 50% of lipids, 32% of soluble carbohydrates and 7-8% of starch and soluble proteins (Corte et al., 2006) but no information is found about the profile or how these reserves are accumulated during maturation. These seeds are not photosensitive to germinate (Ferraz-Grande and Takaki, 2006), and are tolerant to desiccation, keeping their viability up to 0.08 g g^{-1} DW (Silva et al., 2014, Chapter 3). Seeds of *Caesalpinia echinata* share similar characteristics, a short storability at natural environment (less than 90 days - Barbedo et al., 2002) while viability is maintained for 5 years under sub-zero temperatures (Mello et al., 2013). The low viability of *C. echinata* seeds are related to respiration rates, possibly leading to oxidative processes (Lamarca and Barbedo, 2012), indicating that a respiratory metabolism could still be present in this seed at maturity.

Developing seeds are metabolically highly active, mainly during embryogenesis and seed filling (Borisjuk and Rolletschek, 2008). The high respiratory activity is correlated with the necessity to provide energy for growth and synthesis of storage compounds (Vigeolas et al., 2003; Borisjuk et al., 2004). At the last period of maturation, a great loss of water occurs in desiccation tolerant seeds, preparing for a quiescent period (Angelovici et al., 2010). Indeed, a switch-off of the primary respiratory metabolism seems to occurs in the desiccation phase of *Arabidopsis* seeds, mainly by the decrease of the tricarboxylic acid cycle components (Fait et al., 2006), which would decrease the respiratory rates and avoid oxidative processes in the mitochondria, leading to increased desiccation tolerance (Pammenter and Berjak, 1999). In contrast, some seeds lacks the desiccation phase and possess an elevated water content and respiratory rate at shedding, as observed in seeds of *Inga vera* (Caccere et al., 2013), being very

sensitive to desiccation and difficult to storage (Berjak and Pammenter, 2007; Bonjovani and Barbedo 2008). The maintenance of high respiratory rates, among others factors, could also indicate a non-completed maturation, influenced by environmental conditions and the species characteristics (Barbedo et al., 2013).

The acquisition of desiccation tolerance takes place in the later stages of seed maturation and has been correlated with increased amounts of protective compounds, including sugars and antioxidants (Black et al., 1999; Berjak and Pammenter, 2007; Vicré et al., 2004; Tommasi et al., 1999), among others. Sucrose, trehalose, raffinose family oligosaccharides (RFOs) and cyclitols appear to buffering the water loss and stabilizing polymers during desiccation (Hoekstra et al., 2001; Peterbauer and Richter, 2001; Kaushik and Bhat, 2003). Fatty acids also function in defense of seed cells against stressing conditions, by changes in fatty acid saturation during water loss, modifying the membrane fluidity (Liu et al., 2006; Mello et al., 2010). Lower quantities of saturated fatty acids was also detected in desiccation tolerant seeds, compared to intolerant ones, in both triacylglycerols or membrane constituents (Liu et al., 2006; Mello et al., 2010). These changes could also prevent the action of oxidative radicals on unsaturated fatty acids that ultimately results in cell death (Halliwell and Gutteridge, 1999).

In the present work we characterized changes in the metabolic profile throughout *P. pluviosa* seed maturation and analized profiles of triacylglycerols, constituents of fatty acids membranes and soluble carbohydrates in embryonic axis and cotyledons to better understand their role on seed behavior after shedding.

2.2 Material and methods

2.2.1 Plant material

The experiments were carried out with 35 trees located in Rubião Jr, a *campus* of the Universidade Estadual Paulista (UNESP) in Botucatu, SP, Brazil (22º52'20''S 48º26'37''W), during September/2010 to July/2011. Inflorescences were tagged at the beginning of their anthesis and pods were harvested directly from the branches at 174, 202, 245, 258, 301 and 323 days after anthesis (DAA), comprising the main phases of seed maturation classified into different stages (Silva et al., 2014, Chapter 1). In addition, seeds were obtained directly from the ground, not exceeding 48 h after shedding, which were named recently dispersed seeds (RDS).

For biochemical analyses, embryonic axes and cotyledons of each stage (20-30 seeds each) were dissected and immediately frozen in liquid nitrogen. Both were ground in mortar and pestle with liquid nitrogen and stored at -80 ºC until use.

2.2.2 Extraction and analyses of soluble carbohydrates and starch

The soluble carbohydrates of each stage (14-60 mg, in triplicate for both axis and cotyledons) were extracted with boiling 80% ethanol (v/v) for 15 min. The supernatants were recovered after centrifugation (1000 *g*, 15 min) and the residues were manually homogenized and re-extracted twice in boiling 80% ethanol for 15 min. The resulting ethanolic supernatants were combined and considered as the soluble sugar extracts. The amounts of total carbohydrates were determined colorimetrically by the phenol-sulfuric acid method (Dubois et al., 1956), using glucose as standard and results were expressed as mg per g of dry weight (mg g^{-1} DW). The residues were freeze-dried and used for quantification of starch as described by Amaral et al. (2007). The extracts were deionized through anion exchange columns and analyzed by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC/PAD). The HPAEC/PAD system (Dionex ICS-3000, USA) is composed of a CarboPac PA-1 column (2 x 250 mm - Dionex, USA) and a gradient of 150 mM sodium hydroxide (eluent B) and water (eluent A) with the following programme: 0-25 min, 66.7% eluent B; 25-30 min, 100% eluent B; 30-35 min, 66.7% eluent B, with flow rate of 0.25 mL min^{-1}. Sugars were identified by co-chromatography with authentic standards (Sigma-Aldrich Co., USA).

2.2.3 Extraction and analyses of fatty acids

Samples were freeze-dried and weighed (10-30 mg, in triplicate for both axis and cotyledons) before extraction with hexane as solvent in a soxhlet apparatus during four hours. The resulting extract was completely evaporated and the amount of total lipid of each stage was expressed as mg per g of dry weight (mg g^{-1} DW). To determine the possible changes in the fatty acid composition of triacylglycerols and membrane lipids during seed maturation, neutral and polar lipid fractions of both axis and cotyledons were separated. This was performed by loading the total lipid fraction, re-dissolved with 200 µl of chloroform, onto a solid-phase extraction cartridge (SepPak Silica, Waters, USA) previously flushed and primed with chloroform. The cartridge was washed with chloroform twice to elute the neutral lipid fraction and then washed with methanol twice to elute the polar lipids (Crane et al., 2003). After completely dried, fatty acids from each fraction were saponified and methyl-esterified according to Mayworm et al. (1998) with modifications. Briefly, sample was solubilized in 200 µl of toluene before incubation with 4 mL of sulfuric acid 5% methanolic solution and 2 mL of toluene in a dry bath (80 ºC, 60 min). The mixture was transferred to a centrifuge tube with 4 mL of sodium chloride 0.5 M and 1 mL of dichloromethane and slightly stirred before centrifugation (3000 *g*, 5 min). The organic phase was separated in another tube, whereas 1 mL

of dichloromethane was added in the aqueous phase and the procedure repeated. The organic phase was separated again and combined with the former, and 4 ml of sodium chloride 0.5 M was added and the procedure repeated. After the recovery of the organic phase, sodium sulfate was added, mixed well and the centrifugation repeated. Finally, the extract containing the fatty acid methyl esters was separated and completely evaporated before injection.

The residue was solubilized in hexane and analyzed in a gas chromatography with flame ionization detector (GC-FID) system (HP 5809 series). GC was performed utilizing a HP-INNOWAX column (30 m x 0.32 mm x 0.5 µm - Agilent, USA). The injection temperature was set at 220 °C and the detector at 270 °C. Helium was used as the carrier gas at a flow rate of 2.1 mL min^{-1}. The analysis was performed with the following programme: 1 min of isothermal heating at 150 °C, followed by a 15 °C min^{-1} oven temperature ramp rate to 225 °C, followed by a 5 °C min^{-1} increase to 260 °C, maintaining for 7 min. Chromatograms data were evaluated by using the Chemstation program (Hewlett Packard, USA). Fatty acids were identified and compared by co-chromatography with authentic standards (Sigma-Aldrich Co., USA), and the percentage of fatty acid constituents were obtained by the areas under the pick (display unit of the instrument).

2.2.4 Extraction and analysis of the metabolite profile

Samples of embryonic axes and cotyledons of each stage (80-100 mg, in three to six replication) were extracted in methanol:chloroform:water solution (12:5:1 - v/v) with adonitol (0.2 mg/mL), added as internal standard for quantification. The mixture was stirred and incubated in a dry bath (60 °C, 30 min), centrifuged (10000 *g*, 2 min), with the supernatant transferred to another vial, when the same volume of water was added. Centrifugation was repeated after incubation at room temperature for 5 min, with 300 µl of the supernatant separated and completely dried. The residue was re-dissolved in 150 µl of pyridine and derivatization with 50 µl of methoxyamine hydrochloride (20 mg/mL, dissolved in pyridine) and 50 µl of BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) in a dry bath (80 °C, 60 min), according to Roessner et al., (2001). Finally, samples were injected in a gas chromatography-mass spectrometry (GC–MS) system (Agilent GC 6890 and MSD 5973N series). GC was performed using a HP-5 MS column (30 m x 0.25 m x 0.23 µm - Supelco, USA). The injection temperature was set at 230 °C, the interface at 250 °C, and the ion source adjusted to 150 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The analysis was performed with the following programme: 5 min of isothermal heating at 70 °C, followed by a 5 °C min^{-1} oven temperature ramp rate to 310 °C, and a final 1 min of heating at 310 °C. Mass spectra were recorded at 2 scan s^{-1} with a scan range of 50-600 m/z. Chromatogram and mass spectral

data were evaluated by using the Chemstation program (Agilent Technologies, USA). The peaks were identified and compared to authentic standards, the National Institute of Standards and Technology Mass Spectral Library - NIST, and the Golm Metabolome Database - GMD (Hummel et al., 2010). The metabolic data were reported here as relative metabolite content as it is commonly used in metabolic profiling studies to ease interpretation of large data sets.

2.2.5 Analyses of seed quality

Water content (g of water per g of dry weight, g g^{-1}) and dry mass (g $seed^{-1}$) were determined for each seed maturation stage by using four replicates of five seeds, after oven drying (103 ºC, 17 h) according to ISTA (2004). Additionally, water potential of seeds was measured with a Decagon WP4 potentiometer (Decagon Devices, USA) based on the dew point, according to Bonjovani and Barbedo (2008). The germination analyses (four replications of 15 seeds) were carried out on rolls of Germitest paper previously moistened with tap water (two sheets for the base and one for the covering - ISTA, 2004), in germination chambers at 25 ± 1 ºC and were evaluated every 2 days until 40 days (Borghetti, 2005). The percentage of germinated seeds (protrusion of at least 5 mm of primary root) and normal seedling development (seedlings with at least 3 cm and no visual abnormal characteristics) were scored.

2.2.6 Statistical analyses

The experiments were carried out with a completely randomized design and data were statistically analyzed by using ANOVA and Tukey test at 5% of probability for comparison of means (Santana and Ranal, 2004). Principal component analysis (PCA) was performed for sugars, sugar alcohols, amino acids, fatty and organic acids, using the software Statistica 12 (StatSoft Inc., USA).

2.3 Results

The patterns of *P. pluviosa* seeds maturation under field conditions have been already described (Silva et al., 2014, Chapter 1), with 13 distinct stages distributed in a complete cycle of 330 DAA (Table 1). The maximum seed dry mass was reached nearly at the end of maturation (0.376 g $seed^{-1}$ in S12), but the water content decreased gradually throughout the growth phase (*ca.* 3.04 to 0.72 g g^{-1}). Although the seed water potential remained unchanged during most part of the maturation process, a sharp decrease of water content was observed in the desiccation period (after S12), and in dispersed seeds with very low water content (0.09 g g^{-1}) and water potential (-73.4 MPa). Germinability of these seeds began at S4, reaching 50% of germination at S8 and the maximum at S12. Immature seeds were capable to produce low amounts of normal seedlings (30% at S7 and S8), reaching 50% at S10, and the highest values

at S12 (Table 1). RDS showed lower rates of germination and normal seedling development, probably due to immature seeds dispersed along with the mature ones (Silva et al., 2014, Chapter 1).

The contents of starch and total soluble sugars are low compared to the amount of total lipids of *P. pluviosa* seeds during maturation (Figs. 1a, b). Starch contents remained stable until the end of maturation, with the highest concentration in cotyledons at S10 and S12 (98.1 and 71.3 mg g^{-1}, respectively - Fig. 1b). In the embryonic axis, starch decreased from S4 to S7 (54.1 mg g^{-1} and 18.9 mg g^{-1}, respectively), while the content of total sugars remained stable during seed maturation (Fig. 1a). However, in cotyledons a large decline in total soluble sugars occurs from S3 (188.2 mg g^{-1}) to S8 (69.6 mg g^{-1}), remaining unchanged until seed dispersal (Fig. 1b). The content of total lipids in cotyledons is already high at the beginning of the maturation (151 mg g^{-1} in S3), increasing until S12, when reaches 475.9 mg g^{-1} (Fig. 1b), stage with the highest dry matter obtained (Table 1). While lipids have not been detected in embryonic axes at early stages (Fig. 1a), in S7 it was greater (174 mg g^{-1}) than the contents of starch and total sugars (103.6 mg g^{-1} together) and remained high until the end of maturation (208.1 mg g^{-1} at S12).

Analysis of HPAEC/PAD showed that sucrose and cyclitols are the major soluble carbohydrates present in *P. pluviosa* seeds (Fig. 2). During seed maturation, while high concentration of sucrose (more than 60 mg g^{-1} DW - Fig. 2c) was obtained during most part of maturation cycle, the proportions of glucose and fructose decrease slowly in the axis until S12 (Figs. 2a, b). Similar variation was observed in cyclitols, though in lesser extent (Fig. 2d). Except at the beginning of maturation, in cotyledons similar changes occurs throughout seed maturation, when glucose and fructose decrease rapidly until S7, remaining stable from S8 on (Figs. 2a, b). Minor quantities of the raffinose family oligosaccharides (RFOs), raffinose and stachyose, were found in axis and cotyledons during maturation (Figs. 2e, f), although an increase of stachyose occurs in both until the end of maturation (Fig. 2f). Hence, the sucrose:RFOs ratio decreases during maturation (Fig. 2g), mostly due to reduction in sucrose concentration (Fig. 2c), reaching the lowest values at the end of maturation (S12 and RDS). Since seeds of *P. pluviosa* presents low amount of RFOs the sucrose:cyclitols ratio was also calculated during maturation, however no trends were observed during the analyzed period (Fig. 2h).

The analysis of fatty acids composition showed that hexadecanoic (C16:0, palmitic acid), octadecenoic (C18:0, stearic acid), 9-octadecenoic (C18:1, oleic acid) and 9,12-octadienoic (C18:2, linoleic acid) acids were the major fatty acids of the neutral lipid fraction (*i.e.* triacylglycerols) of *P. pluviosa* seeds (Table 2). In embryonic axis, in addition to 9,12,15-

octatrienoic acid (C18:3, linolenic acid) it was detected fatty acids with shorter (C12-C14) and longer (C22) carbon chains length. In cotyledons, it was observed a slow decrease of the unsaturated ratio after S4 until seed dispersion. In embryonic axis these changes are clear after S8 when the proportions of the major unsaturated fatty acids decreases to lowest values until RDS (Table 2).

A wide range of fatty acids characterized the polar lipid fraction (*i.e.* membrane constituents), with carbon chains length between C9 to C24 (Table 3), although the major components are similar to those of the neutral fraction. Except by the presence of the nonanedioic acid (C9:0, azelaic acid) in both axis and cotyledons, most notably in RDS (Table 3). As observed in triacylglycerols, the proportions of membrane unsaturated fatty acids decreases during maturation, mainly by the very low values obtained for linoleic acid after S10 (Table 3). At the same time, the increased proportions of saturated fatty acids, such as palmitic and arachidic (C20:0) acids (and also stearic acid in cotyledons), result at the last stages of seed maturation (S12 and RDS) in the lowest unsaturated ratio (Table 3).

Marked decreases were observed in the metabolite proportions during maturation of *P. pluviosa* seeds (Figs. 3, 4), mainly associated with the primary metabolism, such as organic acids of the tricarboxylic acid cycle (TCA), amino acids, fatty acids and sugars (including sugar alcohols). The decrease of organic acids of TCA cycle is clear after S7 in both axis and cotyledons, as citrate, isocitrate, succinate and malate reaches minimum levels before greater losses of water at the end of maturation, with exception of isocitrate, which increases in embryonic axis at RDS (Fig. 3). At this stage, significant increases in lactate (more than tenfold) and glycerol were observed in the embryonic axis (Fig. 3), when seeds presented very low water content (Table 1). In contrast to TCA acids cycle, great changes were observed in shikimate levels, increasing in the cotyledons at the middle of the maturation period with further reduction in the last stages (Fig. 4). A decrease in 4-coumarate and quinate during maturation were also observed (Figs. 3, 4), both acids related with shikimate pathway, as well an accumulation of three chlorogenate isomers in both axis and cotyledons. The abundance of some chlorogenate isomers at S3 was zero, thus not allowing to normalize as with other compounds (for this see Figs. 5c, d).

Fatty acids that could be detected in this analysis, such as tetradecenoic, hexadecenoic and octadecenoic acids, decreased during maturation. Moreover, a decrease of glycerol and relatively stable proportion of malonate also occurred in cotyledons (Fig. 4), both related to fatty acids biosynthesis. At the same period, a general decline of sugars were observed in both axis and cotyledons (Figs. 3, 4) as described early for the HPLC analysis (Fig. 2). However, in

this analysis it was also detected trehalose, which have a great increase in the cotyledons (Fig. 4), mainly in the last two stages (ten to fifteen fold, S12 and RDS, respectively). In embryonic axis the trehalose changes were also great, starting early (at S7), followed by a decrease at S12 (still fivefold than S3), before increasing again at RDS (Fig. 3). Another sugar detected was xylose, which increased in axis and cotyledons up to S7 before start to decrease until the last stages of maturation.

Seeds of *P. pluviosa* present different sugar alcohols such as ononitol, pinitol, erythritol, threitol, mannitol / sorbitol and xylitol / arabitol. In general, the proportions of these compounds decrease during seed maturation, except by galactinol and *myo*-inositol in embryonic axis, remaining constant during a large extent period until the last two stages of maturation (Fig. 4). Different from other compounds, amino acids were poorly detected in this analysis. However, reductions of serine, threonine, glutamate, pyroglutamate, GABA and aspartate proportions were observed during maturation in both axis and cotyledons (Figs. 3, 4).

The principal component analysis (PCA) of the metabolic profile showed that metabolic changes occurred in embryonic axis between each stage of seed maturation (Fig. 5a), except at the beginning (S3 and S4) or at the end of maturation (S12 and RDS), suggesting that a more pronounced metabolic switch occurred on such stages. A great metabolic change also occurred in cotyledons after S4, and is possible to observe that S10 samples appear scattered between an immature group (S7 and S8) to a mature one (S12 and RDS, Fig. 5b). Components 1 and 2 together explain 64% of the variation in embryonic axis (Fig. 5a), which was strongly affected by changes in the organic acids and some sugars, such as chlorogenate isomers, trehalose and sucrose (Fig. 5c). In the cotyledons, components 1 and 2 together explain less than 56% of the variation (Fig. 5b), while the compounds that considerable contribute to the sample discrimination were practically the same as those observed in embryonic axis, the chlorogenate isomers and the sugar trehalose (Fig. 5d).

2.4 Discussion

Seeds of *P. pluviosa* preferentially accumulate lipids as carbon reserve, as observed in the large amounts of total lipids obtained in the cotyledons (475.9 mg g^{-1} DW - Fig. 1b) and in the axis (210.2 mg g^{-1} DW - Fig. 1a). In addition, the elevated proportions of malonate in the cotyledons (Fig. 4) could serve as constant source of malonil-CoA maintaining a higher synthesis of fatty acids until S12 (Fig. 1b), as implied in the decrease of glycerol and free fatty acids proportions (Fig. 4). The seed triglycerides (neutral lipid fraction) that is mainly composed of palmitic (16:0), stearic (18:0) and oleic (18:1) acids, with elevated proportions of

linoleic acid (18:2, Table 2), are commonly found in seeds of other leguminous species (Mayworm et al., 1998; Mello et al., 2010), and are also major constituents of membranes (polar lipid fraction - Table 3). Some authors correlate the presence of unsaturated fatty acids with seed desiccation tolerance behavior, since desiccation sensitive seeds typically present higher proportions of saturated fatty acids than the tolerant ones (Mello et al., 2010), and this was also found for the membranes of such seeds (Liu et al., 2006).

Interestingly, a reduction of the proportion of unsaturated fatty acids occurred during maturation of *P. pluviosa* seeds as shown by the unsaturated ratio (Table 2), mainly in the polar lipid fraction and in the axis (Table 3). Similar results were observed in the resurrection plant *Ramonda serbica*, when a general decrease of the unsaturation proportion of phospholipids and total lipids, was caused by dehydration (Quartacci et al., 2002). While an increased rate of fatty acid unsaturation could diminishes the probability of crystal to gel phase transitions in lower water contents, improving seed desiccation tolerance (Liu et al., 2006), a lower unsaturation rate could decrease membrane fluidity, rendering the bilayer tighter and rigid, with reduced solute leakage (Quartacci et al., 2002). In *R. serbica*, the decrease of fatty acid unsaturation, allied to changes in membrane constituents, such as phospholipids, phosphatidylethanolamine, cerebrosides and free sterols limited the tendency to form the non-lamellar configuration during drying, thus stabilizing membrane integrity (Quartacci et al., 2002).

In *P. pluviosa* seeds, the decrease of fatty acids unsaturation occurred especially at S12, stage prior to desiccation step (Table 1). Although only the general profile of membrane fatty acids was analyzed (Table 3), *P. pluviosa* seeds go through to a desiccation step (Silva et al., 2014, Chapter 1) and were capable to tolerate drying until very low water content (Chapter 3), indicating similar behavior as observed in *R. serbica*. Another benefit to lower fatty acid unsaturation would be to avoid the generation of reactive oxygen species that commonly occurs during dehydration in various plant tissues (Smirnoff, 1993). These changes can prevent lipid peroxidation, mainly of unsaturated fatty acids of the extensive oil bodies that ultimately result in cell death (Halliwell and Gutteridge, 1999). In fact, the presence of azelaic acid (Table 3) could indicate an oxidative membrane damage, since it is regarded as a marker for free radical-induced lipid fragmentation (Zoeller et al., 2012), a process characterized by the fragmentation of esterified linoleic acid (18:2) catalyzed by oxidative species (Schneider et al., 2008). It has also been proposed that non-enzymatic lipid peroxidation protects cells from oxidative stress by scavenging reactive species (Mène-Saffrané et al., 2009). In this way, the high proportion of azelaic acid in the polar lipid fractions in RDS (Table 3) indicates an increased oxidized membrane with low risk of uncontrolled lipid peroxidation after seed desiccation step.

As expect by seeds that accumulate large quantities of oil, the amount of starch and soluble sugars obtained during maturation were low in both axis and cotyledons (Fig. 1). Sugars are thought to act as compatible solutes during the initial phase of water loss, stabilizing macromolecules, maintaining the conformation and functionality of cell membranes and contributing to the glassy state (Hoekstra et al., 2001). Sucrose and RFOs have been considered the most important sugars to fulfill this function (Blackman et al., 1992), mainly in legumes (Horbowicz and Obendorf 1994). However, raffinose and stachyose were detected in low levels during maturation of *P. pluviosa* seeds (less than 0.6%), in which mature seeds were capable to tolerate desiccation until very low water contents (Table 1, Chapter 3). Horbowicz and Obendorf (1994) proposed a correlation between seed storability and ratio of sucrose to RFOs in seed tissues. Seeds with sucrose:RFOs ratio lower than 1.0 could maintain viability under storage to periods higher than 10 years, whereas those with ratio higher than 1.0 would maintain viability to periods lower than 10 years. The sucrose:RFOs ratio obtained for both axis and cotyledons of *P. pluviosa* seeds were close to 10.0 (Fig. 2g), which is consistent with the short seed lifespan at room temperature (240 days - Figliolia et al., 2001). In *P. pluviosa* seeds, rather than protective function, the increase of stachyose content during maturation could be a source of carbon during germination, as also observed for *C. echinata* seeds (Leduc et al., 2012). Alternatively, it could be related with the polyols metabolism.

Similar or even higher amounts of cyclitols instead of RFOs are frequently found in other legume seeds, and they could play the same function in stabilizing macromolecules (Peterbauer and Richter 2001; Borges et al., 2006; Leduc et al., 2012). However, there was no visible trend in sucrose:cyclitols ratio (Fig. 2h) as observed in *C. echinata* seeds (Leduc et al., 2012), despite their similar short-term storability (Figliolia et al., 2001; Barbedo et al., 2002). The GC-MS analysis show that most of the identified cyclitols decreases during seed maturation (Figs. 3, 4), except galactinol and *myo*-inositol in the axis (Fig. 3). It should be mentioned that two other polyols were detected in *P. pluviosa* seeds, both compounds increasing during maturation and presumably count with higher degree of polymerization. However, different from these, ciceritol, a di-galactoside cyclitol synthetized through pinitol pathway by the addition of galactosyl residue in galactopinitol (Peterbauer and Richter, 2001) and found in highly amounts in *C. echinata* seeds (Borges et al., 2006), was not detect in our samples. Comparative chromatography suggests these two unknown cyclitols as galactopinitol A and B, being similar to what was found in *C. echinata* seeds (Borges et al., 2006). On the other hand, could also indicate a different pathway, as *myo*-inositol pathway, which results in both di- and tri-galactosyl *myo*-inositol through galactinol, pathways currently little understood (Obendorf

et al., 2013). Duo to the great structural similarity between such compounds and the absence of commercial standards their identification requires further and specific analyses.

The proportion of trehalose increased markedly in both axis and cotyledons in dispersed seeds (RDS - Figs. 3, 4). Trehalose is a non-reducing disaccharide accumulating in organisms that withstand several environmental stresses such as drought, heat or freezing temperatures (Wingler, 2002; Eastmond and Graham, 2003) and is found in high amounts during desiccation of the resurrection plant *Selaginella lepidophylla* (Adams et al., 1990). This sugar act as a protein stabilizer, protecting protein conformation from dehydration (Kaushik and Bhat, 2003). However, trehalose is commonly found in traces in a variety of plants, diminishing the protective role of this sugar. In this case, trehalose, mainly the trehalose precursor, trehalose-6-P, act as a regulatory molecule, especially in sugar influx and ABA signaling during vegetative development (Eastmond and Graham, 2003; Avonce et al., 2004). The technique employed here does not allow to estimate the actual amount of trehalose present in axis and cotyledons. However, the elevated proportions of trehalose found during maturation, mainly in embryonic axis (Fig. 3), in addition of the increased proportion in RDS axis and cotyledons (Figs. 3, 4), suggest that trehalose could act as a metabolic regulator of seed maturation, changing to a protective function during the desiccation step.

As other part of the complex mechanism of desiccation tolerance presented by seeds, the cell wall composition plays a central role during the desiccation step in tolerant seeds due to the essential maintenance between the association of cell wall and plasma membrane (Vicré et al., 2004). High proportions of arabinans (arabinose-containing polysaccharides) could increase cell wall flexibility (Moore et al., 2008) in contrast, high proportions of galactans (galactose-containing polysaccharides) implied a great mechanical resistance of the cell wall (McCartney et al., 2000). In fact, some desiccation tolerant seeds present cell wall with high proportions of arabinose (Gomez et al., 2009), while *Inga vera*, one of the most sensitive seeds, presents cell wall rich in galactose (Caccere et al., 2013). In *P. pluviosa* seeds, the changes observed in xylose and xylitol/arabitol (Figs. 3, 4), precursors of the hemicellulose xylan and arabinan, might be related to modifications of the cell wall composition during maturation and desiccation step.

It is often described as an important characteristic of seed desiccation tolerance the capability to switch-off the metabolism, leading to a mature seed in a quiescent state. In fact, a decrease of the tricarboxylic acid cycle (TCA) intermediates, related to low respiratory rates, occurs in the desiccation phase of *Arabidopsis* seeds (Fait et al., 2006). In contrast, embryos of *I. vera*, present high metabolic rate throughout seed maturation and without a desiccation step

(Caccere et al., 2013). In *P. pluviosa* seeds, the decrease of TCA intermediates in axis and cotyledons during maturation was clear, suggesting a decreased primary metabolism, before the desiccation phase (Figs. 3, 4). These results indicate a decrease in respiration rates contributing to avoid oxidative processes (Pammenter and Berjak, 1999; Fait et al., 2006). In addition, changes in the proportions of shikimate, 4-coumarate and quinate (Figs. 3, 4), as well the increased proportions of chlorogenate isomers until the last stage of maturation (Fig. 3), imply that the secondary metabolism was still active. This result also suggest that the chlorogenate isomers, hydroxycinnamic acid derivatives, are accumulated by the shikimate pathway. Interestingly, the chlorogenate isomers, mainly 5-chlorogenate (or 5-caffeoylquinic acid), are well known antioxidants (Giraldo et al., 2007; Amakura et al., 2013), and allied to the decreased activity of the TCA cycle, could be an important defense against oxidative radicals in the dry state.

The high proportion of lactate in cotyledons at the beginning of seed maturation (Fig. 4) might be associated with a putative low oxygen concentration inside the seed. This was also observed in the first stages of development of orthodox seeds (Borisjuk and Rolletschek, 2008) as well in seeds of *I. vera* (Caccere et al., 2013). Current modifications in the embryo greening during maturation might favor photosynthesis (Borisjuk et al., 2004) that could contribute significantly to improve metabolic fluxes through the oxygen supply, resulting in a lower formation of lactate in the subsequent stages (Caccere et al., 2013), as well favoring oil synthesis (Vigeolas et al., 2003). The color of *P. pluviosa* cotyledons remains green throughout seed maturation (Silva et al., 2014, Chapter 1) which could result in an efficient energy supply and could be related to the large amounts of oil obtained (Fig. 1b).

In contrast, the embryonic axis became white at S10, possibly resulting in a restriction of oxygen supply (Borisjuk and Rolletschek, 2008). However, a great increase of lactate, as well glycerol, was observed in embryonic axis just in RDS (Fig. 3), when seeds are already dried (Table 1), indicating that the oxygen availability probably decreased due to the removal of water, although not occurs in cotyledons (Fig. 4). Nevertheless, other compounds such as sugars (raffinose and trehalose) and fumarate and succinate could accumulate specifically during seed desiccation (Angelovici et al., 2010). Therefore, the later accumulation of lactate and glycerol, as well isocitrate (Fig. 3) might serve as substrates to reactivate the TCA cycle or the glyoxilate cycle at the beginning of germination, when the oxygen supply will rise with the entrance of water.

Taken together our findings evidenced that major changes occur in early development (S3 and S4), and at the last stages of seed maturation (S12 and RDS), characterized by a

decrease of the energetic primary metabolism and the production of antioxidants via secondary metabolism in both, embryonic axis and cotyledons. Mature seeds of *P. pluviosa* present very low level of unsaturation in membrane fatty acids, which could limit phase transition, as well elevated proportion of trehalose, which could together protect membrane integrity against oxidative stress and water loss. Changes in trehalose metabolism could also be a signal of seed maturity, changing from the regulatory function to a protective role.

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Table 1.

Table 1. Water content, water potential, germination, normal seedling development and dry mass of *Poincianella pluviosa* seeds during maturation from 174 to 323 days after anthesis (DAA). Stages classified according Silva et al. (2014), chapter 1. RDS, recently dispersed seeds. Within each column, means followed by the same letter do not differ significantly (Tukey´s test, $P < 0.05$, $n = 4$).

Stage	DAA	Water content ($g\ g^{-1}$)	Water potential (MPa)	Germination (%)	Normal seedlings (%)	Dry mass ($mg\ seed^{-1}$)
S3	174	3.04 a	-2.3 b	0 e	0 d	0.042 e
S4	202	2.43 b	-1.3 b	28 d	2 d	0.087 de
S7	245	1.55 c	-1.7 b	45 cd	30 c	0.171 cd
S8	258	1.40 c	-1.8 b	52 bc	32 c	0.194 bc
S10	301	1.19 d	-1.6 b	72 b	47 bc	0.244 bc
S12	323	0.72 e	-2.8 b	96 a	94 a	0.376 a
RDS	323	0.09 f	-73.4 a	68 b	67 b	0.277 b

Fig. 1

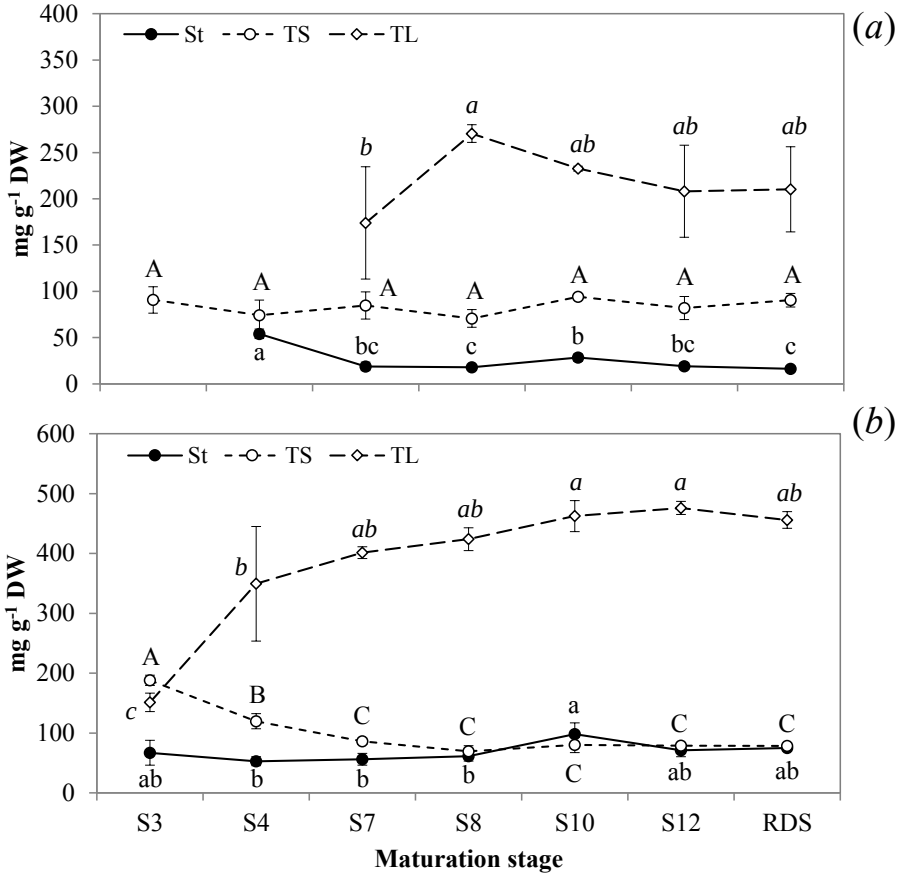

Fig 1. Contents (mg g^{-1} DW) of starch (St), total soluble sugars (TS) and total lipids (TL) of *Poincianella pluviosa* embryonic axis (*a*) and cotyledons (*b*) during maturation (stages as described in Table 1). Means sharing the same letter are not significantly different among stages of maturation (Tukey's test, $P < 0.05$, $n = 3$). Small letters compare starch; capital letters compare total soluble sugars and italic letters compare total lipids.

Fig. 2

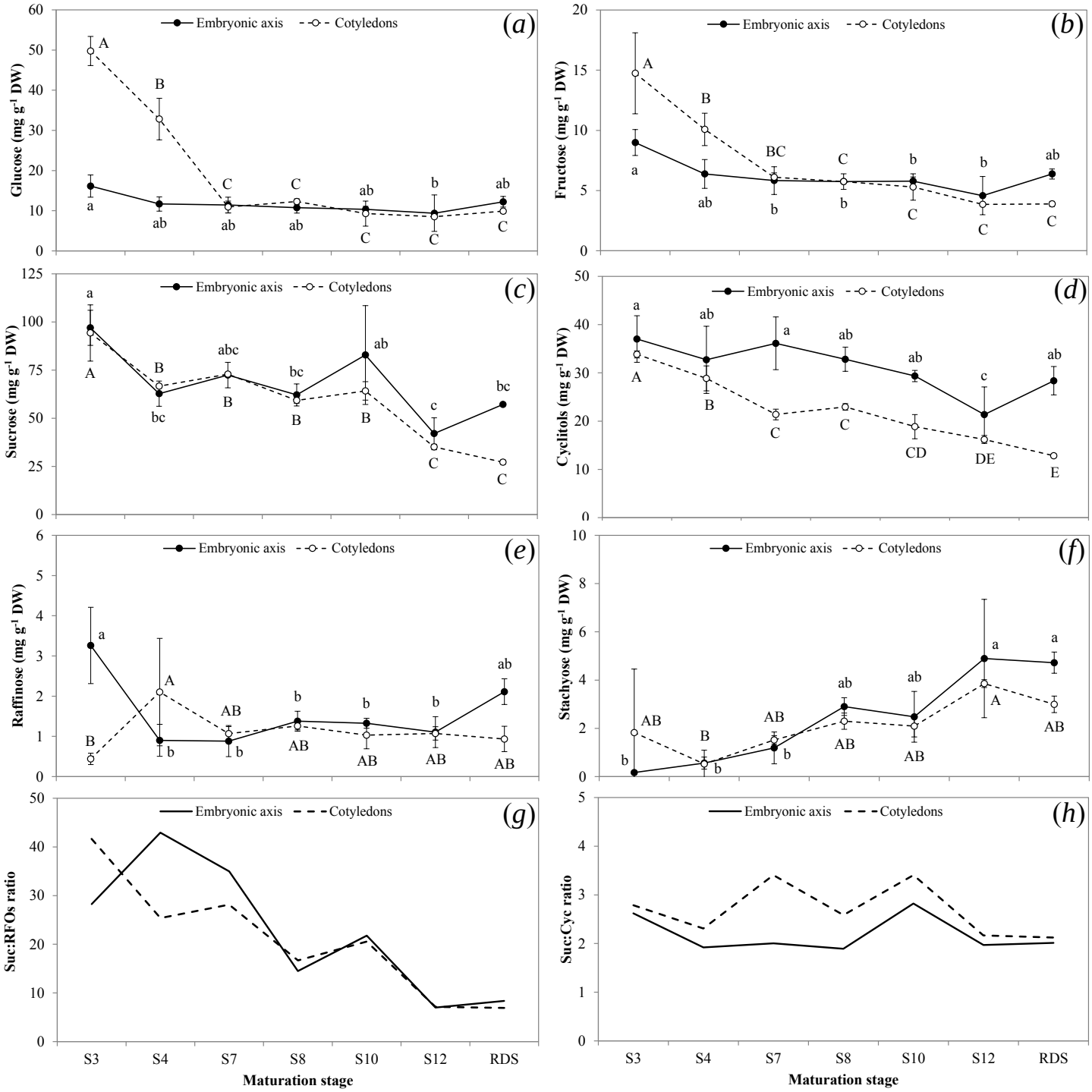

Fig 2. Soluble carbohydrate composition (mg g^{-1} DW) of embryonic axis (●, fill line) and cotyledons (○, dashed line) of *Poincianella pluviosa* seeds during maturation (stages as described in Table 1). Glucose (*a*); Fructose (*b*); Sucrose (*c*); Cyclitols (*d*); Raffinose (*e*); Stachyose (*f*); Sucrose:Raffinose family oligosaccharides (RFOs) ratio (*g*); Sucrose:Cyclitol ratio (*h*). Means sharing the same letter inside each compound are not significantly different among stages of maturation (Tukey's test, $P < 0.05$, $n = 3$). Small letters compare embryonic axis; capital letters compare cotyledons.

Table 2

Table 2. Total fatty acid composition (%. *n* = 3. neutral lipid fraction) of *Poincianella pluviosa* embryonic axis and cotyledons during maturation (stages as described in Table 1). The sum of saturated and unsaturated fatty acids are indicated as well the unsaturated:saturated ratio.

Stage	C9	C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:2	C18:3	C20	C20:1	C22	C22:1	C24	Total Unsaturated	Total Saturated	Unsat/Sat Ratio
Embryonic axis - neutral lipid fraction																		
S3		12.5	12.0	4.5	15.3		7.3	8.9	39.4							52.8	47.2	1.12
S4		1.8	2.2		17.3		4.5	8.8	60.1	2.1	1.7		1.5			71.0	29.0	2.45
S7					19.3		6.3	7.7	61.4	1.3	2.1		1.8			70.4	29.6	2.38
S8		0.8	0.8		18.6		5.9	7.4	61.7	1.3	2.2		1.8			70.3	30.1	2.33
S10					33.1		13.2	9.6	38.0		4.2		3.4			47.6	53.9	0.88
S12		4.9	5.6		38.7		19.9	13.8	10.1	3.4	3.7					27.2	72.8	0.37
RDS		7.2	6.5		38.0		14.3	10.3	5.0	15.9	4.4					31.1	70.3	0.44
Cotyledons - neutral lipid fraction																		
S3					17.6		6.0	16.3	58.7		1.5					74.9	25.1	2.99
S4					16.1		8.1	12.5	63.3							75.8	24.2	3.13
S7					15.5		7.9	9.4	65.0		1.6		0.7			74.5	25.8	2.89
S8					15.6		10.3	9.1	63.2		1.8					72.3	27.7	2.61
S10					15.5		12.8	8.2	61.2		1.8		0.6			69.4	30.6	2.26
S12					21.6		26.2	11.8	37.3		3.2					49.1	50.9	0.96
RDS					17.5		18.1	8.6	53.4		2.2					61.9	37.8	1.64

Table 3

Table 3. Total fatty acid composition (%. *n* = 3. polar lipid fraction) of *Poincianella pluviosa* embryonic axis and cotyledons during maturation (stages as described in Table 1). The sum of saturated and unsaturated fatty acids are indicated as well the unsaturated:saturated ratio.

Stage	C9	C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:2	C18:3	C20	C20:1	C22	C22:1	C24	Total Unsaturated	Total Saturated	Unsat/Sat Ratio
Embryonic axis - polar lipid fraction																		
S3			4.8		21.5		19.0	18.4	36.3							54.7	45.3	1.21
S4					38.0		20.1		42.0							42.0	58.0	0.72
S7	4.1	8.6	6.2		34.1		11.9	10.3	35.4		5.8					45.7	70.7	0.65
S8		2.9	3.2		36.8		19.2	9.1	28.1		5.8		5.1			37.2	73.1	0.51
S10	4.4				39.7		17.7	9.6	22.4		5.1		4.3		2.1	34.1	73.3	0.47
S12	8.4		1.0	2.7	46.0	2.0	19.2	8.6	2.3		6.0		4.5		2.6	18.1	87.6	0.21
RDS	14.6				47.5		17.9	4.7	1.5		6.1	1.1	4.5		2.5	9.7	93.1	0.10
Cotyledons - polar lipid fraction																		
S3					26.4		11.7	25.4	36.5							61.9	38.1	1.62
S4		6.5			20.0		11.7	10.4	51.4							61.8	38.2	1.62
S7					18.3		10.7	8.5	62.5							71.0	29.0	2.45
S8		1.7	2.2	7.8	19.6	3.9	13.0	7.4	53.6		1.8					72.7	38.3	1.90
S10					22.2		18.1	8.7	47.6		2.2			2.9		59.2	42.5	1.40
S12	4.6				31.5		35.9	12.3	11.7		4.0					24.0	76.0	0.32
RDS	10.8		1.3	1.7	30.7		30.6	8.2	17.8		3.5					27.7	76.9	0.36

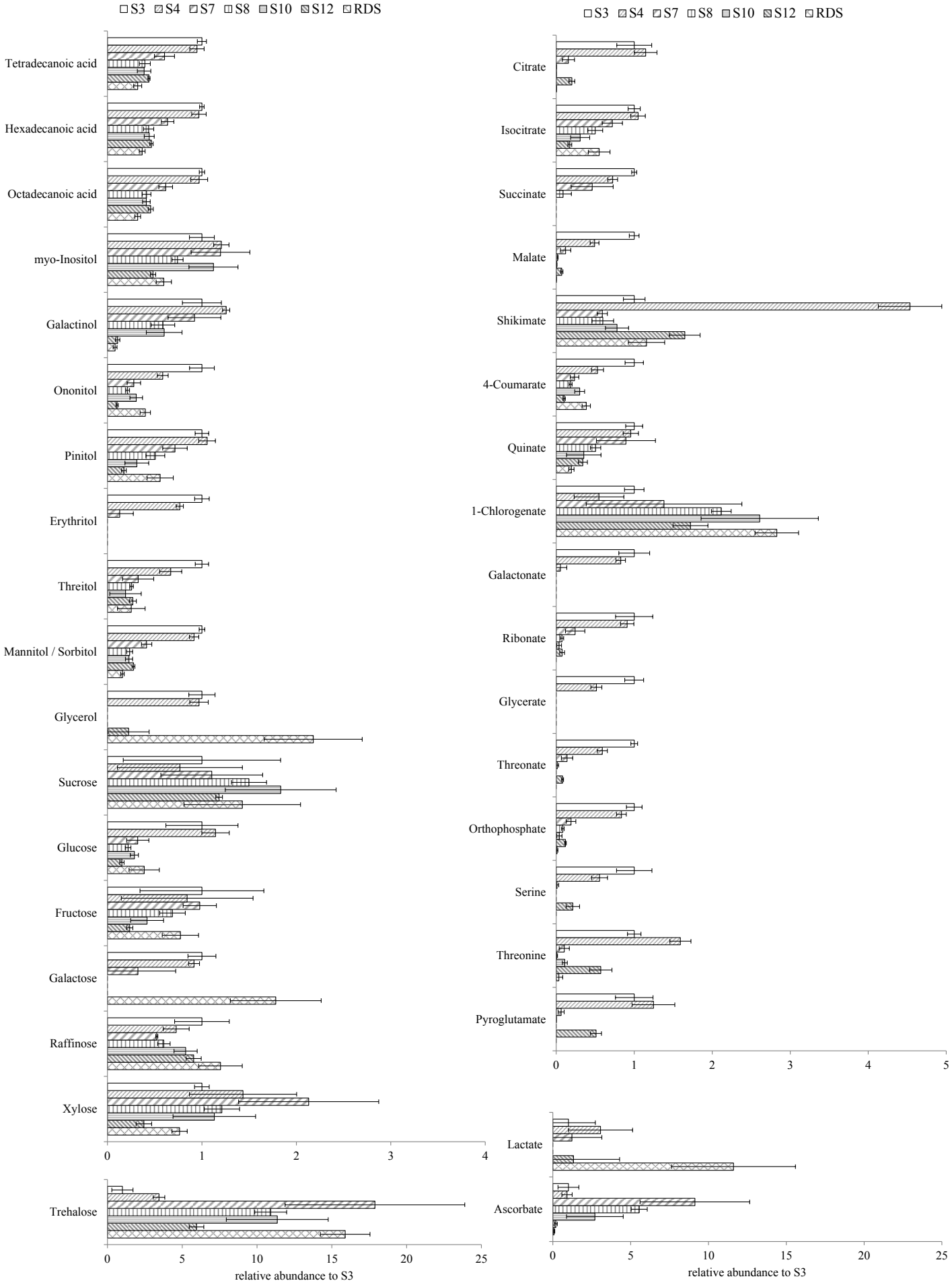

Fig 3. Metabolic profile of embryonic axis of *Poincianella pluviosa* seeds during maturation (stages described in Table 1). Compounds were detected by GC/MS. All values were normalized from those found in S3.

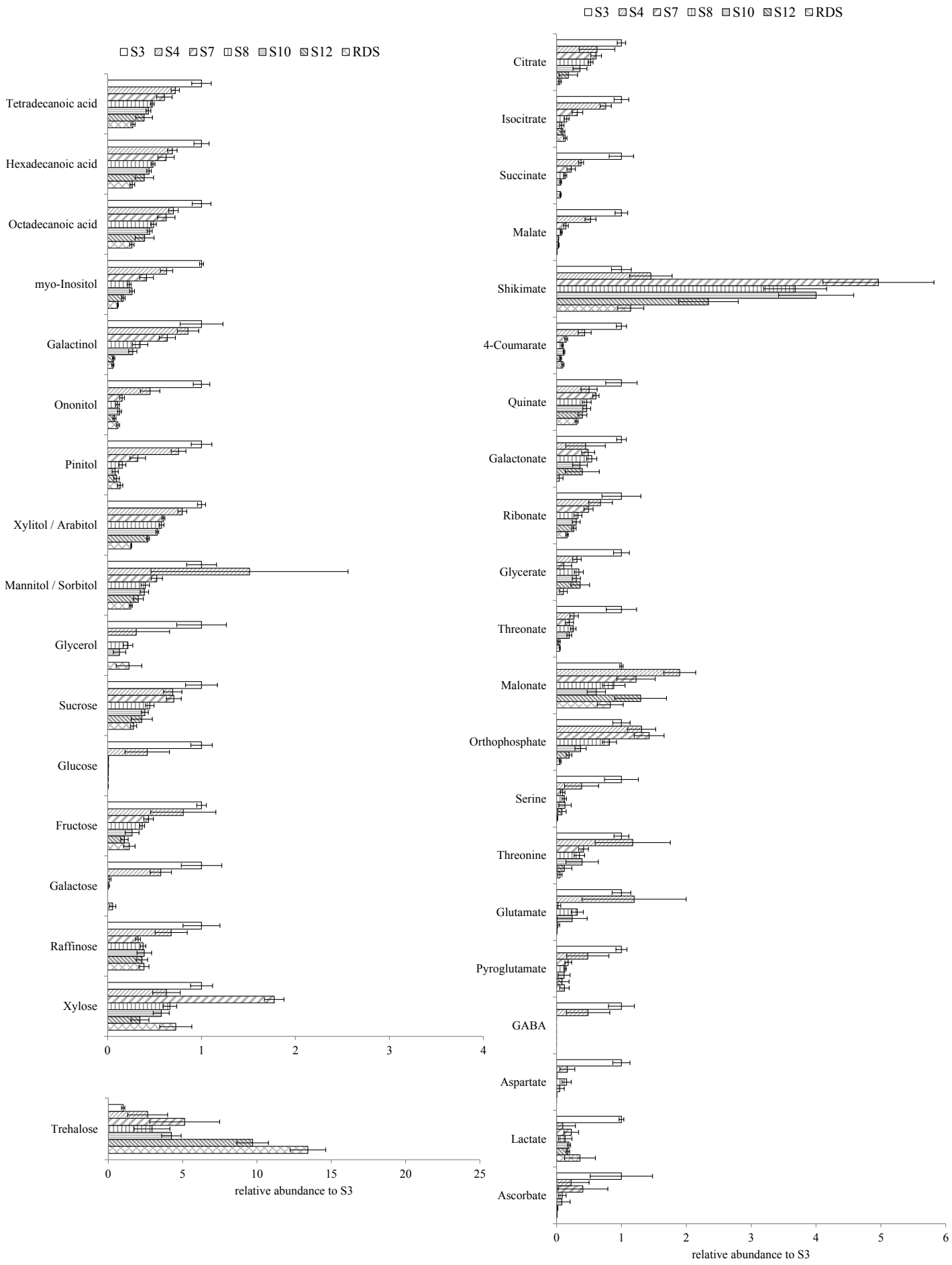

Fig 4. Metabolic profile of cotyledons of *Poincianella pluviosa* seeds during maturation (stages described in Table 1). Compounds were detected by GC/MS. All values were normalized from those found in S3.

Fig 5.

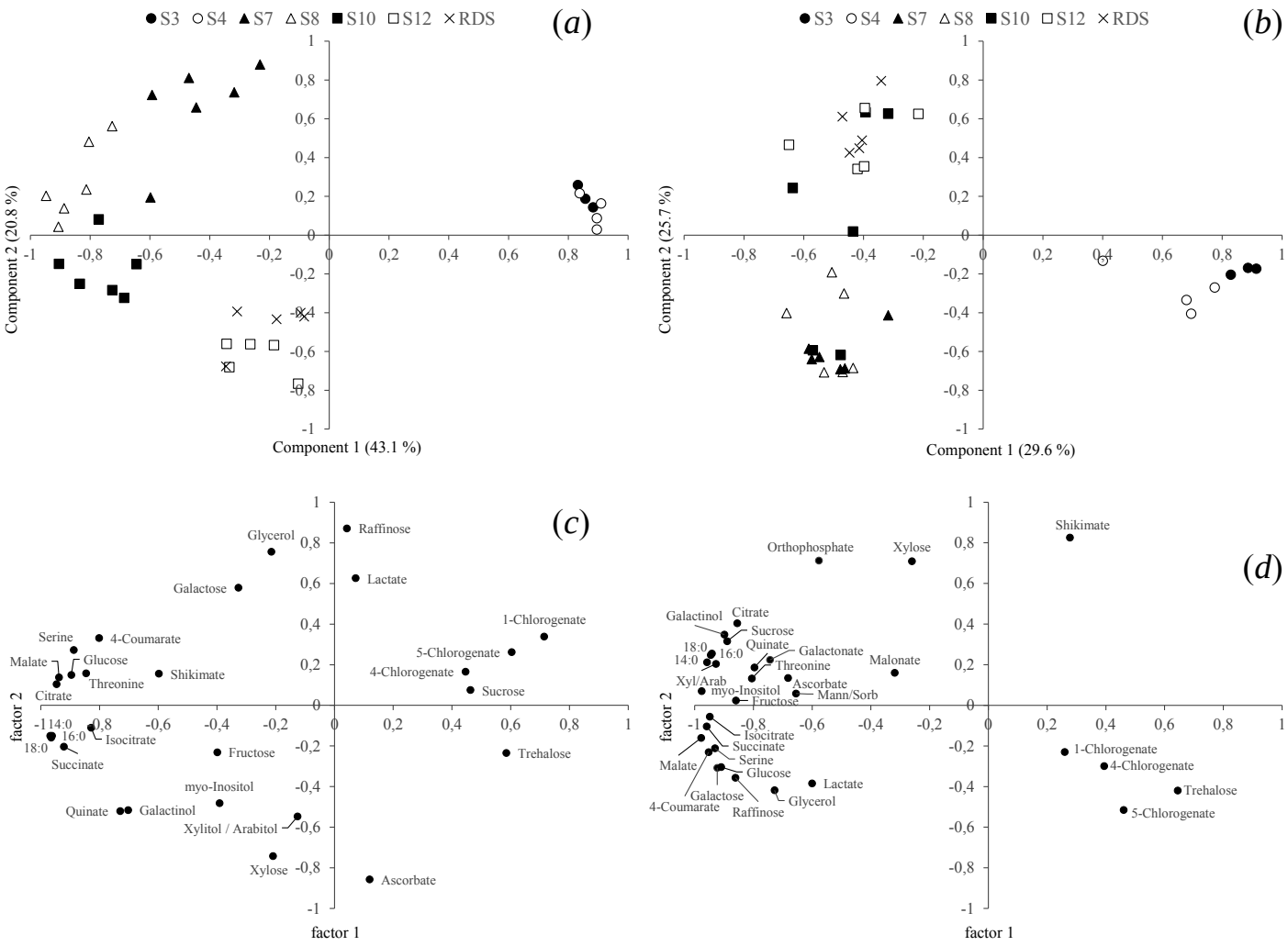

Fig 5. Principal component analysis (PCA) of metabolite profiles of embryonic axis (*a*) and cotyledons (*b*) and the respective loadings (*c* and *d*) during maturation of *Poincianella pluviosa* seeds (stages described in Table 1). PCA is presented as the combinations of the first two dimensions. Each data point represents an independent biological sample.

5. Chapter 3

The later maturation of *Poincianella pluviosa* (Caesalpinioideae) seeds is characterized by an early desiccation tolerance and a fermentative metabolism after drying

Abstract

The last period of desiccation tolerant (DT) seeds maturation is characterized by great loss of water, when cytoplasm condenses and water deficit could damage cell membranes if protective mechanism are not active. Seeds of *Poincianella pluviosa* present an extensive maturation cycle characterized by decreased respiratory metabolism until physiological maturity, but have short lifespan at natural environment. We characterized the last stages of *P. pluviosa* seed maturation and their tolerance to drying, analyzing changes of carbohydrates, triacylglycerols, fatty acids membranes and the metabolic profile of immature and mature seeds to correlate to its DT behavior. At S9, 20% of germination was obtained after drying until 0.08 g g^{-1}, while is ~100% at S12. Distinct responses of embryonic axis and cotyledons to drying was obtained depending the maturity stage. No changes in unsaturated ratio of fatty acids was observed in cotyledons of S10-dried seeds, while linoleic acid was not detected in axis triglycerides. Trehalose proportions are not affect by drying. Increased proportions of glycerol and lactate occur after drying, mainly in S10-dried seeds. The inability of immature seeds to modify unsaturation level of fatty acids is correlated to their desiccation sensitivity. The higher proportion of lactate and glycerol in dried immature seeds suggest an active metabolism prior to drying and an osmotic stress under hypoxia. The DT in immature seeds indicate that the long maturation of *P. pluviosa* is a strategy to maintain seeds able to develop plantlets during maturation.

Keywords: fatty acids, glycerol, hypoxia, lactic acid, metabolic profile.

3.1 Introduction

During maturation, the water content assumes a decisive role in seeds and decreases slowly until reaching values near 1.0 g of water g^{-1} DW (or 50% in w.b.). After this point on, different results can be observed depending on how extended was the maturation period, allied to the environmental conditions and the characteristics of each species accumulated during evolutionary process (Barbedo et al., 2013). Great loss of water at the last period of maturation characterizes the desiccation tolerant seeds, resulting in dried seeds prepared for a quiescent period and thereafter for germination (Angelovici et al., 2010). In contrast, desiccation-sensitive seeds lack this desiccation step, switching to a germination metabolism and exhibiting limits of desiccation tolerance after dispersal (Berjak and Pammenter, 2007; Caccere et al., 2013; Lamarca et al., 2013).

Throughout desiccation, the cytoplasm condenses and the intracellular components become more crowded. These conditions lead to various undesirable interactions, such as protein denaturation and cell-organelle membrane fusion (Hoekstra et al., 2001). Water deficit could damage cell membranes by inducing changes in composition and function of their constituents, influencing the physical state of membranes and their activities (Quartacci et al., 1995). Differences in fatty acids unsaturation of triacylglycerols and membrane constituents were observed between desiccation tolerant (DT) and sensitive seeds, being more elevated in DT seeds (Liu et al., 2006; Mello et al., 2010). In *P. pluviosa* seeds, some differences in fatty acids composition were observed (Chapter 2) and was thought to be related to membrane fluidity, restriction of solute leakage and maintenance of the membrane integrity (Quartacci et al., 2002; Liu et al., 2006; Mello et al., 2010).

In general, seeds accumulate large amounts of storage compounds during maturation, such as carbohydrates, proteins and oils, the main sources of energy and nutrients for germination. Some of these reserves can act as protective compounds against dehydration during desiccation. They include disaccharides, such sucrose and trehalose (Black et al., 1999; Kaushik and Bhat, 2003), and raffinose family oligosaccharides (RFOs) or cyclitols, when RFOs are not present (Horbowicz and Obendorf, 1994; Peterbauer and Richter, 2001). The function of such sugars appears to buffering the water loss and stabilizing polymers during desiccation (Hoekstra et al., 2001; Kaushik and Bhat, 2003). The presence of antioxidative defenses (Tommasi et al., 1999; Bailly, 2004) and changes in the physical structure of the cell wall (Vicré et al., 2004; Caccere et al., 2013) also contribute to DT in seeds, among other mechanisms. However, a controlled switch-off of the metabolism is considered essential to

prevent the production of reactive species in the dry state, leading to increased desiccation tolerance at the dispersal (Hoekstra et al., 2001; Pammenter and Berjak, 1999).

Developing seeds are metabolically highly active, mainly during embryogenesis and seed filling (Borisjuk and Rolletschek, 2008). Although a reduced metabolism is expected in the desiccation phase, indicated by a decrease in the tricarboxylic acid cycle intermediates (Fait et al., 2006; Chapter 2), in highly desiccation sensitive seeds of *Inga vera*, an elevation of the respiratory rate was reported at seed dispersal (Caccere et al., 2013). The high respiratory activity is necessary to provide energy to the young embryo for cell division and synthesis of storage products (Vigeolas et al., 2003; Borisjuk et al., 2004). However, low internal levels of O_2 was observed due to a low permeability on maternal seed tissues, which could limit respiration and potentially induce fermentation (Borisjuk et al., 2004). Metabolic responses upon hypoxia is typically observed in the first stages of seed development (Borisjuk and Rolletschek, 2008; Caccere et al., 2013) and disappear during the storage phase (Borisjuk et al., 2004).

Seeds of *Poincianella pluviosa* (DC.) L.P.Queiroz (= *Caesalpinia pluviosa* DC.) present short to medium storability (240 days - Figliolia et al., 2001) while the physiological maturity is achieved around 300 days after anthesis (DAA), comprising an extensive maturation period of 330 DAA (Silva et al., 2014, Chapter 1). This period is characterized by a decrease of tricarboxylic acid cycle intermediates, in contrast to increased levels of the shikimate pathway components, followed by a major drop in unsaturation levels of the fatty acids membrane constituents until physiological maturity (Chapter 2). *P. pluviosa* seeds accumulate large quantity of lipids, mainly linoleic and palmitic acids, resulting 45-50% of oil in mature seeds (Corte et al., 2006; Chapter 2), with low amounts of starch and soluble carbohydrates, mainly composed by sucrose and cyclitols, and traces of RFOs. Furthermore, elevated levels of trehalose in axis and cotyledons of dried seeds was related to the observed DT in recently dispersed seeds (RDS - Chapter 2).

P. pluviosa is a tree species that occurs in almost all phytogeographic domains in Brazil, including Amazon, Caatinga, Cerrado, Atlantic forest and Pantanal (Lewis, 2013; Queiroz, 2009). Neotropical forests constitute a reservoir of genetic diversity due to the enormous ecosystems and species distributed in a variety of biogeographical divisions, comprising a crucial role in the environmental stability. However, it is necessary to understand seed behavior with respect to DT in order to improve seed viability during storage, providing tools for *ex situ* conservation of plant species.

In the present work, we characterize the physiological traits of *P. pluviosa* seeds during the last stages of maturation and after imposed drying. We also analyzed the changes of triacylglycerols, fatty acids membranes, soluble carbohydrates and metabolic profiles of immature and mature seed axis and cotyledons to better understand its DT behavior.

3.2 Material and methods

3.2.1 Plant material

The experiments were carried out with 35 trees located in Rubião Jr, a *campus* of the Universidade Estadual Paulista (UNESP) in Botucatu, SP, Brazil (22º52'20''S 48º26'37''W), between June to July in 2011. Inflorescences were tagged at the beginning of their anthesis, pods were harvested directly from the branches at 286, 301, 317 and 323 DAA (Table 1), comprising the last five stages of seed maturation according to Silva et al. (2014 - Chapter 1). In addition, seeds were obtained directly from the ground, not exceeding 48 h after shedding, which were named recently dispersed seeds (RDS).

3.2.2 Drying treatments

Seeds from each stage of maturation were dried directly in an oven with air circulation (40 °C) until they reached five levels of water content: 0.21; 0.18; 0.14; 0.11 and 0.08 g g^{-1} water (equivalent to 18, 15, 12, 10 and 7% water in wet basis, respectively). Then, seeds from all treatments were subjected to determination of the water content (g of water per g of dry weight, g g^{-1}) and dry mass (mg $seed^{-1}$) by using four replicates of five seeds, after oven drying (103 ºC, 17 h - ISTA, 2004). Additionally, water potential of seeds was measured with a Decagon WP4 potentiometer (Decagon Devices, USA) based on the dew point, according to Bonjovani and Barbedo (2008). Seeds that exhibited in germination tests (four replications of 15 seeds) protrusion of the primary root after rehydration (at least 5mm) were considered desiccation tolerant.

Fresh seeds (initial) from stages 10 and 12, and from both stages after drying until 0.08 g g^{-1} water, were collected for biochemical analysis. Embryonic axes and cotyledons were dissected and immediately frozen in liquid nitrogen (30 seeds per replicate). Both were ground in mortar and pestle with liquid nitrogen and stored at -80 ºC until use.

3.2.3 Extraction and analyses of soluble carbohydrates and starch

Soluble carbohydrates of each treatment (14-60 mg, in triplicates for both embryonic axis and cotyledons) were extracted with boiling 80% ethanol (v/v) for 15 min. The supernatants were recovered after centrifugation (1000 *g*, 15 min) and the residues were manually homogenized and re-extracted twice in boiling 80% ethanol for 15 min. The resulting

ethanolic supernatants were combined and considered as the soluble sugar extracts. The amounts of total carbohydrates were determined colorimetrically by the phenol-sulfuric acid method (Dubois et al., 1956), utilizing glucose as standard and results were expressed as mg per g of dry weight (mg g^{-1} DW). The residues were freeze-dried and used for quantification of starch as described by Amaral et al. (2007). The extracts were analyzed by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC/PAD) as described in Chapter 2. Sugars were identified by co-chromatography with authentic standards (Sigma-Aldrich Co., USA).

3.2.4 Extraction and analyses of fatty acids

Samples were freeze-dried and weighed (10-30 mg, in triplicates for both axis and cotyledons) before extraction with hexane as solvent in a soxhlet apparatus during four hours. The resulting extract was evaporated to dryness and the amount of total lipid of each sample was expressed as mg per g of dry weight (mg g^{-1} DW). To determine changes in fatty acid composition of triacylglycerols and membrane lipids during drying, neutral and polar lipid fractions of both axis and cotyledons were separated by loading the total lipid fraction onto a solid-phase extraction cartridge (SepPak Silica, Waters, USA) as described by Crane et al. (2003). The extracts of fatty acids from each fraction were analyzed by gas chromatography with flame ionization detector (GC-FID), as described in Chapter 2. Data were evaluated by using the Chemstation program (Hewlett Packard, USA). Fatty acids were identified by co-chromatography with authentic standards (Sigma-Aldrich Co., USA), and the percentage of fatty acid constituents was obtained by the areas under the picks (display unit of the instrument).

3.2.5 Extraction and analysis of the metabolite profile

Samples of embryonic axis and cotyledons of each treatment (80-100 mg, in five or six replicates) were extracted in methanol:chloroform:water solution (12:5:1 - v/v) and quantified using adonitol (0.2 mg/mL), as internal standard. The mixture was stirred and incubated in a dry bath (60 °C, 30 min), centrifuged (10000 *g*, 2 min), with the supernatant transferred for another vial, when the same volume of water was added. Centrifugation was repeated after incubation at room temperature for 5 min, with 300 µl of the supernatant separated and completely dried in speed vac. The residue was re-dissolved in 150 µl of pyridine and derivatization with 50 µl of methoxyamine hydrochloride (20 mg/mL, dissolved in pyridine) and 50 µl of BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) in a dry bath (80 °C, 60 min), according to Roessner et al. (2001). Finally, samples were injected in a gas chromatography-mass spectrometry (GC–MS) system (Agilent GC 6890 and MSD 5973N series), as described in Chapter 2. Chromatogram and mass spectral data were evaluated by using the Chemstation

program (Agilent Technologies, USA). The peaks were identified and compared to authentic standards, the National Institute of Standards and Technology Mass Spectral Library - NIST, and the Golm Metabolome Database - GMD (Hummel et al., 2010). The metabolic data were reported here as relative metabolite content as it is commonly used in metabolic profiling studies to ease interpretation of large data sets.

3.2.6 Statistical analysis

The experiments were carried out with a completely randomized design and data were statistically analyzed by using ANOVA and Tukey test at 5% of probability for comparison of means (Santana and Ranal, 2004). Principal component analysis (PCA) was performed for sugars, sugar alcohols, amino acids, fatty and organic acids, using the software Statistica 12 (StatSoft Inc., USA).

3.3 Results

During the last two months of *P. pluviosa* seed maturation the water content and the water potential change from a high hydrated state (1.27 g g^{-1} and -1.8 MPa, at S9) to a very dried and low one (0.09 g g^{-1} and -73.4 MPa, in RDS), while the seed dry mass markedly increased (44% - Table 1). At this period, seed vigor also increased substantially, with elevated rates of germination, normal seedlings and speed of germination-aid in the last stages of maturation (maximum at S12) compared to S9 and S10 (60-70% germination and 30-50% of normal seedlings - Table 1). RDS showed lower rates of germination, normal seedling development and dry mass than the previous stages, indicating that immature seeds were dispersed at the same time with mature ones (Silva et al., 2014, Chapter 1).

Dried immature seeds are sensitive to desiccation below 0.11 g g^{-1} water, as seen by the decrease of germination rates at S9 and S10 (Fig. 1), although the acquisition of DT has been apparently progressive from S9 to S13. At S12, highest rates of germination, normal seedlings and speed of germination were obtained after drying treatments (Fig. 1b). Low values of seed germination and vigor were obtained at S13 compared with the previous one and no significant change was observed in seed viability at this stage after drying treatments (Fig. 1b). Therefore, seeds at S10 undergo to a decrease in viability when subjected to drying, suggesting they are still immature. Conversely, seeds at S12 were practically not affected by the drying treatments and nearly all of them produced normal seedlings.

Lipids are the main carbon storage compounds of *P. pluviosa* seeds corresponding to ~20% in embryonic axis and ~45% in cotyledons of seed dry weight (Fig. 2). Lipid content remained stable in both stages after treatments, although in embryonic axis the drying treatment

led to great variations in both stages (Fig. 2a). Total soluble sugars also remained stable in both axis and cotyledons and treatments, while starch content decreased from S10 to S12, mainly in embryonic axis. The drying treatment led to a decrease of starch in embryonic axis of seeds from S10, but not in S12-dried seeds (Fig. 2a).

Sucrose was the major neutral sugar in embryonic axis and cotyledons of *P. pluviosa* seeds and decreases in both axis and cotyledons from S10 to S12 (Fig. 3c). The drying treatment led to a decrease of sucrose content in seeds from S10, mainly in the cotyledons. In contrast, in the cotyledons of S12-dried seeds the sucrose content remained stable, while is observed a tendency to increase in embryonic axis of the same treatment (Fig. 3c). The highest values of stachyose were observed in seeds from S12 (Fig. 3f), while the raffinose content was practically the same in both stages (Fig. 3e). However, their levels were very low when compared with sucrose and cyclitols (Figs. 3c, d). Except for the embryonic axis, the drying treatment did not affect the amount of raffinose and stachyose in seeds from both stages (Figs. 3e, f). Hence, the sucrose:RFOs ratio decreased in both axis and cotyledons in S10-dried seeds (Fig. 3g), mostly by the reduction of sucrose concentration (Fig. 3c), reaching values close to that obtained in seeds from S12. Similar results were obtained in sucrose:cyclitols ratio, reaching in seeds from S10, in both axis and cotyledons, equal values compared to mature seeds (Fig. 3h), while these parameters remained practically stable in S12-dried seeds.

Linoleic (C18:2) and palmitic (C16:0) acids were the major fatty acids present in embryonic axis and cotyledons of *P. pluviosa* seeds, followed by low proportions of stearic (C18:0) and oleic (C18:1) acids (Table 2). In neutral lipid fraction, the proportions of linoleic acid in both axis and cotyledons decrease from S10 to S12, with a small increase in levels of palmitic, stearic and oleic acids. The ratio of unsaturated:saturated fatty acids decreases with maturity.

Drying treatments in seeds from S10 led to non-detected levels of linoleic acid in the embryonic axis, and high proportions of palmitic acid, resulting in a very low unsaturated ratio, while in cotyledons no changes were observed (Table 2). In contrast, in embryonic axis of S12-dried seeds a small increase of unsaturated ratio was observed, possibly caused by reduction in the proportion of palmitic, stearic and oleic acids, together with an increase of linolenic acid (C18:3). In cotyledons of S12-dried seeds, a reduction in unsaturated ratio was mainly caused by a decrease in the proportion of linoleic acid. Increased proportion of medium-chain fatty acids in embryonic axis from both stages after drying was also observed, such as in lauric (C12:0) and myristic (C14:0) acids compared with the respective fresh seeds (Table 2).

Similar as observed in neutral lipid fraction, the unsaturated ratio of polar lipids decreased in seeds from S10 to S12 in both axis and cotyledons, mainly by the low values obtained for linoleic acid (Table 2). The drying treatment in seeds from S10 led to a great decrease in linoleic acid proportion in embryonic axis, simultaneous to an increase in palmitic acid, which results in a low unsaturated ratio, even lower than that obtained in seeds from S12. In contrast, no major changes was observed in cotyledons of S10-dried seeds, as well in both axis and cotyledons at S12-dried seeds. Except by a small increase of linoleic acid in cotyledons after drying, it was clearly observed similar unsaturated ratios. A small proportion of azelaic acid (C9:0) was also detected in both axis and cotyledons of *P. pluviosa* seeds. However, azelaic acid was not detected in cotyledons of seeds from S10, while an increase was observed in S12-dried seeds (Table 2).

Changes in the metabolic profile in dried-seeds from both stages corresponded mainly to compounds related to primary respiratory metabolism, such as organic acids of the tricarboxylic acid cycle (TCA), amino acids and fatty acids (Fig. 4). The drying treatment led to a decrease of citrate and malate proportions in both axis and cotyledons in mature seeds, while in the embryonic axis of S10-dried seeds these acids were already at low levels (Fig. 4a). Different from others metabolites, isocitrate proportions were similar in axis and cotyledons and after drying, whereas a marked increase of lactate occurred mainly in immature seeds (Fig. 4). Similar results were observed in glycerol and succinate proportions, the last in a lesser extent (Fig. 5), although in cotyledons the abundance of glycerol in S12 was zero, as well succinate in both axis and cotyledons, thus not allowing to normalize as with other compounds. The increased proportion of glycerol obtained in S10-dried seeds was also higher than S12-dried seeds (Fig. 4a).

Fatty acids also detected in this analysis, such as myristic, palmitic and oleic acids, remained unchanged in embryonic axis from both stages, while a decrease was observed in cotyledons of S12-dried seeds (Fig. 4b). High proportions of cyclitols, such as *myo*-inositol, galactinol, ononitol and pinitol were detected in axis of immature seeds compared to mature ones (Fig. 3a). In addition, a great proportion of threitol was observed in S10-dried seeds in both embryonic axis and cotyledons, higher than in seeds from S12. Sugars commonly related to dehydration protection, such as sucrose and raffinose did not exhibit great changes between both stages and after drying. In contrast, an increase of trehalose proportion was observed in axis of S12-dried seeds, reaching values similar to that found in S10-dried seeds (Fig. 4a). In cotyledons, although trehalose was not affected by drying, it was higher in mature seeds than in immature ones (Fig. 4b).

The principal component analysis (PCA) of the metabolic profiles evidenced that the metabolism of embryonic axes differed between stages of maturity, although become similar after drying (Fig. 5a). In contrast, the metabolism of cotyledons of fresh seeds from both stages was similar, while after drying marked differences were observed (Fig. 5b). Components 1 and 2 together explain more than 70% of the variation in embryonic axes (Fig. 5a), while in the cotyledons, components 1 and 2 together explain more than 52% of the variation.

3.4 Discussion

Results highlighted that the last months of maturation of *P. pluviosa* seeds are crucial to improve seed vigor and dry matter accumulation (Table 1, Fig. 1), despite the extensive maturation cycle of 330 DAA (Silva et al., 2014). Increased DT was observed during this period, as evidenced by the elevated rate of germination after drying comparing seeds from S9 and S10 with seeds from S11, S12 and S13 (Fig. 1b). The ability to germinate, survive desiccation and the potential longevity are commonly installed and increased in this order during maturation of DT seeds (Pieta-Filho and Ellis, 1991; Sanhewe and Ellis, 1996; Butler et al., 2009), and are related to mass maturity at the end of the seed-filling (Ellis and Pieta Filho, 1992). Although *P. pluviosa* immature seeds showed high sensitivity to desiccation, it was possible to notice that about 20% of seed from S9 germinate after drying until 0.08 g g^{-1} (Fig. 1). This indicates that the start of DT processes appears early during maturation, close to the beginning of seed capability to produce normal seedlings (S7, 245 DAA - Silva et al., 2014, Chapter 1), and becomes more evident as the protection mechanisms are consolidated with the progress of maturation. This was observed when dried seeds from S12 maintained high rates of germination, normal seedlings and speed of germination after any drying level (Fig. 1b).

Sucrose and RFOs have been considered the most important sugars in the acquisition of DT (Blackman et al., 1992), mainly in legume seeds (Horbowicz and Obendorf 1994). However, these sugars were detected in very low levels in mature seeds of *P. pluviosa* (Chapter 2), as seen in seeds from S12 (Fig. 3). The decrease in starch occurred after drying in desiccation sensitive seeds from S10 did no led to an increase of total soluble sugars (Fig. 2a). In fact, the quantities of sucrose decreased in cotyledons of S10-dried seeds (Fig. 3c) while raffinose and stachyose remained unchanged (Figs. 3e, f). In DT seeds from S12, the drying treatment did not led to changes in starch content, total soluble sugars nor in any other identified sugar (Fig. 3). This was confirmed by the similar ratios of sucrose:RFOs and sucrose:cyclitols (Figs. 3g, h). Raffinose and stachyose could be a ready carbon source for seedling establishment, more than act as a protective function, as observed for *C. echinata* seeds (Leduc et al., 2012).

The lack of RFOs and the relative higher amounts of cyclitols found in *P. pluviosa* are frequently found in other legume seeds, as *C. echinata* (Borges et al., 2006). In such cases, cyclitols are considered to play the same function as RFOs in stabilizing macromolecules (Peterbauer and Richter 2001; Leduc et al., 2012). However, the sucrose:cyclitols ratio decreased in S10-dried axis and cotyledons and were similar as found in seeds from S12 (Fig. 3h), with no improvement in seed viability (Fig. 1b). In addition, as observed by GC-MS analysis, most of the polyols identified were present in seeds from S10 in higher proportions compared to mature ones (Fig. 4), and some of them decreases after drying, as *myo*-inositol and galactinol, with exception of threitol.

Threitol is a sugar alcohol that act as cryoprotectant, by stabilizing protein structure at low temperature, and found in high concentrations among other polyols in freeze-tolerant Alaskan beetles that are capable to endure temperature of -60 °C (Walter et al., 2009). The threitol biosynthetic pathway has not been fully characterized in any organism. In humans, threitol is the major end product of xylose catabolism (Pitkänen, 1977), while in Alaskan beetles the synthesis of threitol was by the pentose phosphate pathway (Walter et al., 2009). In *P. pluviosa* seeds, an increase in threitol proportion was observed in both stages and both axis and cotyledons after drying (Fig. 4), and were greater in S10-dried than S12-dried seeds, pronounced in the cotyledons (Fig. 4b). The technique employed here does not allow estimate the actual amount of threitol present in the axis and cotyledons, and S10-dried seeds presented high sensibility to desiccation (Fig. 1). However, as an early seed vigor was observed (Chapter 1), processes related to DT and freeze-tolerance might be in progress in seeds from S10.

In contrast, an increase in trehalose proportion was observed in axis of DT S12-dried seeds, reaching values close to those found in sensitive S10-dried seeds (Fig. 4a). Although the proportion of trehalose was not affected by drying in cotyledons, it was higher in seeds from S12 compared to S10 (Fig. 4b). Trehalose is a disaccharide that accumulates in organisms capable to endure extreme environmental conditions, acting as a protein stabilizer (Wingler, 2002; Eastmond and Graham, 2003; Kaushik and Bhat, 2003). While the proportion of trehalose in seeds of *P. pluviosa* increased during maturation (Chapter 2), the results shown here indicate that trehalose metabolism to drying could be specific to axis or cotyledons and to the degree of seed maturity.

The decrease in fatty acid unsaturation in both embryonic axis and cotyledons of *P. pluviosa* obtained between seeds from S10 and S12 was related to maturation (Table 2), and were described previously (Chapter 2). Although not common compared to some orthodox seeds (Liu et al., 2006), a low unsaturation could be one of the factors that allows decreases in

membrane fluidity, rendering the bilayer tighter and rigid, and decreasing possible solute leakage, as observed in resurrection plant *Ramonda serbica* (Quartacci et al., 2002). However, the drying treatment in immature seeds led to a large decrease of unsaturation rate in embryonic axis of both triglycerides and membrane fractions and could indicate fatty acid degradation (Table 2), as well related to the higher levels of glycerol (43 fold - Fig. 4a) or succinate in dried axis and cotyledons (Figs. 5c, d).

Degradation of fatty acids occurs during desiccation step of *B. napus* embryos (around 15 %), and it is also detected in *A. thaliana*, and was described to provide energy needed for metabolic activity in this period (Chia et al., 2005) or amino acids rapidly available to support metabolic recovery during imbibition (Fait et al., 2006). However, only a small reduction of total lipids was obtained (~7%) in cotyledons of S12-dried seeds, and compared to RDS during maturation, the decrease of total lipids in cotyledons at S12 was even lower (~4%, Chapter 2). The proportions of free fatty acids remained stable in axis of both stages (Fig. 4a) while in cotyledons even a decrease in S12-dried seeds was observed (Fig. 4b). However, the presence of succinate in dried seeds (Figs. 5c, d) might also indicate fatty acid degradation, since was a potential product of β-oxidation, as fumarate (Fait et al., 2006) that was not detect here. In axis of S12-dried seeds minor changes occurred, mainly an increase of linolenic acid (Table 2), as also observed after the desiccation at the end of maturation (Chapter 2).

In contrast, no changes were observed in cotyledons of S10-dried seeds, even the reduction of linoleic acid that normally occurs during maturation (Table 2, Chapter 2). Moreover, the absence of azelaic acid, a fatty acid signed as marker of free radical-induced lipid fragmentation, as well related to protection from lipid peroxidation (Mène-Saffrané et al., 2009; Zoeller et al., 2012) in cotyledons of S10-dried seeds, could indicate that immature cotyledons are not fully prepared to support drying until low levels of water content.

The decrease of TCA cycle components after drying was similar to what was observed during maturation of *P. pluviosa* seeds (Chapter 2). Citrate and malate decreased in both axis and cotyledons, although, in cotyledons, the drop of these two acids were greater in mature seeds after drying (Fig. 4b). This response could represent a lower primary respiratory metabolism, which would decrease the respiration rates and contribute to avoid oxidative processes in the quiescent state (Pammenter and Berjak, 1999; Fait et al., 2006). Differently from that observed during maturation (Chapter 2), the compounds related to shikimate pathway do not present a clear trend. In addition, the proportions of the chlorogenate isomers, well known antioxidants (Giraldo et al., 2007; Amakura et al., 2013), seemed to decrease after drying (Fig. 4), possibly related to an increase generation of reactive radicals during drying.

Developing seeds are characterized by a hypoxic internal environment until the storage phase (Borisjuk and Rolletschek, 2008), including some oilseeds such as rape (Vigeolas et al., 2003). Recent evidences show that the oxygen level in green embryos is regulated by the maturity degree and relies on the lighting conditions of the environment, when embryo photosynthesis could minimize fermentative processes (Rolletschek et al., 2005; Borisjuk and Rolletschek, 2008, and references therein). Interestingly, an accumulation of lactate and glycerol were observed in the embryonic axis of *P. pluviosa* RDS (Chapter 2). The low oxygen concentration probably due to water removal during seed dehydration, diverted the pyruvate produced in glycolysis from mitochondria, converting to lactate in the cytosol. In fact, the accumulation of lactate and glycerol could serve as substrates to reactivate the TCA or the glyoxilate cycles at the beginning of seed germination, as observed for a wide range of compounds during desiccation step (Angelovici et al., 2010).

Similarly, an increase of glycerol could be due to the degradation of fatty acids (Chia et al., 2005) or an increased synthesis. In yeast *Saccharomyces cerevisiae*, an elevated synthesis of glycerol via glycolysis occurs after osmotic stress under hypoxia, acting as an important osmoregulator (Nevoigt and Stahl, 1997). Despite some evidences on fatty acids degradation in drying seeds as discussed earlier, the increase of glycerol could be related to osmotic stress responses. Moreover, the higher proportion of lactate and glycerol in dried immature axis and cotyledons rather than in mature ones might also be related to an elevated metabolism prior to the drying treatment.

The indication of DT in immature seeds gives support to the suggestion that the long maturation period of *P. pluviosa* could be a strategy to maintain seeds able to develop new plantlets during the maturation process (Chapter 1), since some immature seeds are capable to survive after drying till 0.08 g g^{-1} DW (Fig. 1). However, the distinct responses of embryonic axis and cotyledons to drying could be related to different progress of DT mechanism in each part. The highly sensitivity to desiccation of immature seeds (S10) was possibly related to the inability to modify unsaturation level of fatty acids from triglycerides and membranes in the cotyledons, or prevent degradation of triglycerides in the embryonic axis. In mature seeds (S12) the presence of trehalose, rather than RFOs, in addition to changes in fatty acid profile, could together act in the preservation of the membrane integrity during drying. Specific analysis on energetic metabolism, composition of membrane lipids and fatty acid catabolism are necessary in future to infer how the metabolism act to improve DT at the final stages of *P. pluviosa* seed maturation.

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Table 1.

Table 1. Water content, water potential, germination, normal seedling development and dry mass of *Poincianella pluviosa* seeds during later maturation (between 286 to 323 days after anthesis - DAA). Stages classified according Silva et al. (2014), chapter 1. RDS, recently dispersed seeds. Within each column, means followed by the same letter do not differ significantly (Tukey´s test, $P < 0.05$, $n = 4$).

Maturation Stage	DAA	Water content ($g\ g^{-1}$)	Water potential (MPa)	Germination (%)	Normal seedlings (%)	Dry mass ($mg\ seed^{-1}$)
S9	286	1.27 a	-1.8 c	62 b	33 d	0.197 b
S10	301	1.19 b	-1.6 c	72 b	47 cd	0.244 b
S11	317	1.05 c	-2.2 c	80 ab	67 bc	0.286 ab
S12	323	0.72 d	-2.8 c	96 a	94 a	0.376 a
S13	323	0.26 e	-13.1 b	80 ab	77 ab	0.355 a
RDS	323	0.09 f	-73.4 a	68 b	67 bc	0.277 ab

Fig 1.

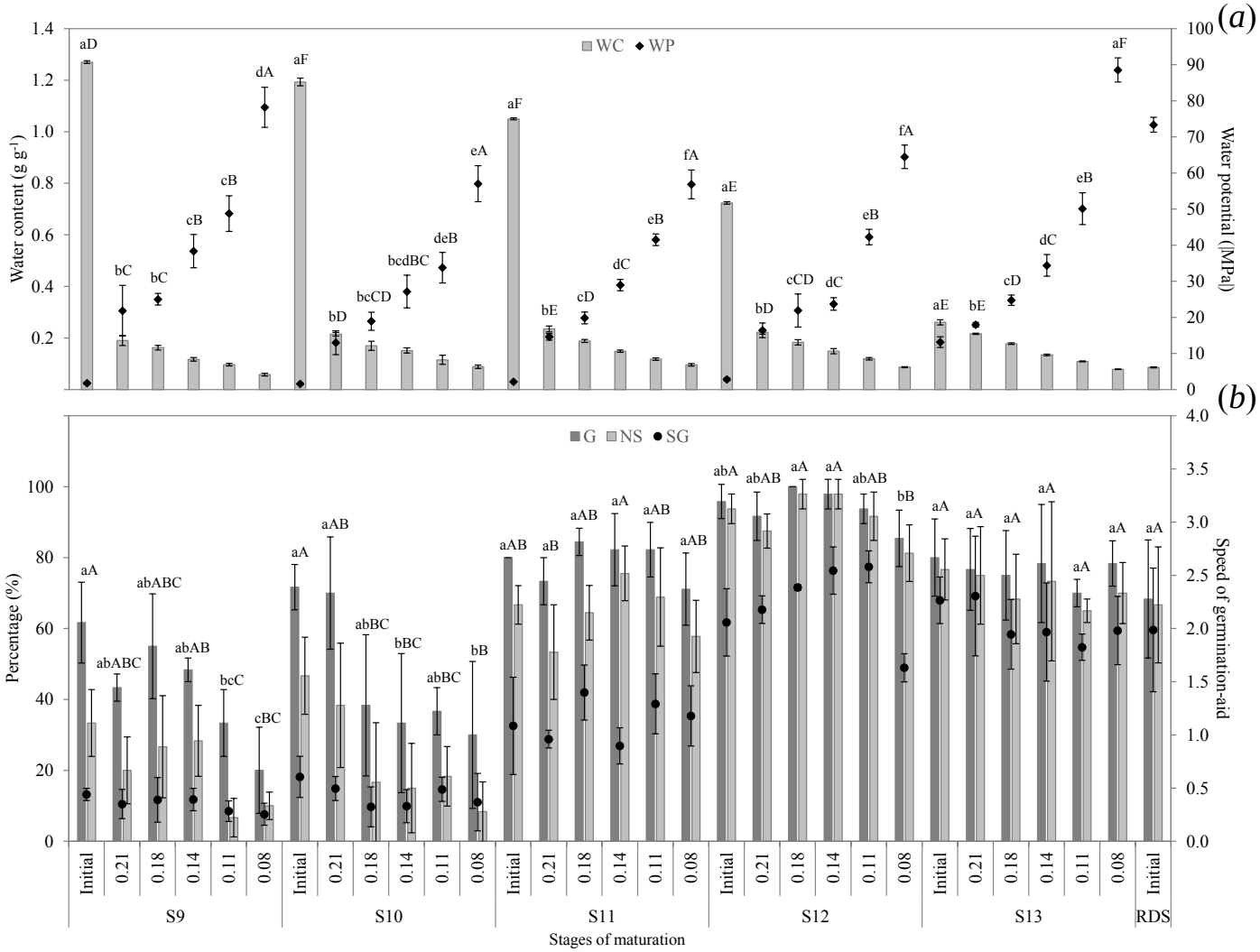

Fig 1. Seeds of *Poincianella pluviosa* during later maturation (between 286 to 325 DAA) classified into stages according Silva et al. (2014), chapter 1, before (Initial) and after drying until 0.08 g g^{-1} water. (*a*) Water content (WC - g g^{-1}) and water potential (WP - |MPa|). (*b*) Germination (G - %), normal seedlings development (NS - %) and speed of germination-aid (SG). RDS, recently dispersed seeds. Means sharing the same letter are not significantly different (Tukey´s test, $P < 0.05$, $n = 3$). Letters compare drying treatments inside each stage of maturation. Small letters compare water content (*a*) and germination (*b*). Capital letters compare water potential (*a*) and normal seedling development (*b*).

Fig. 2

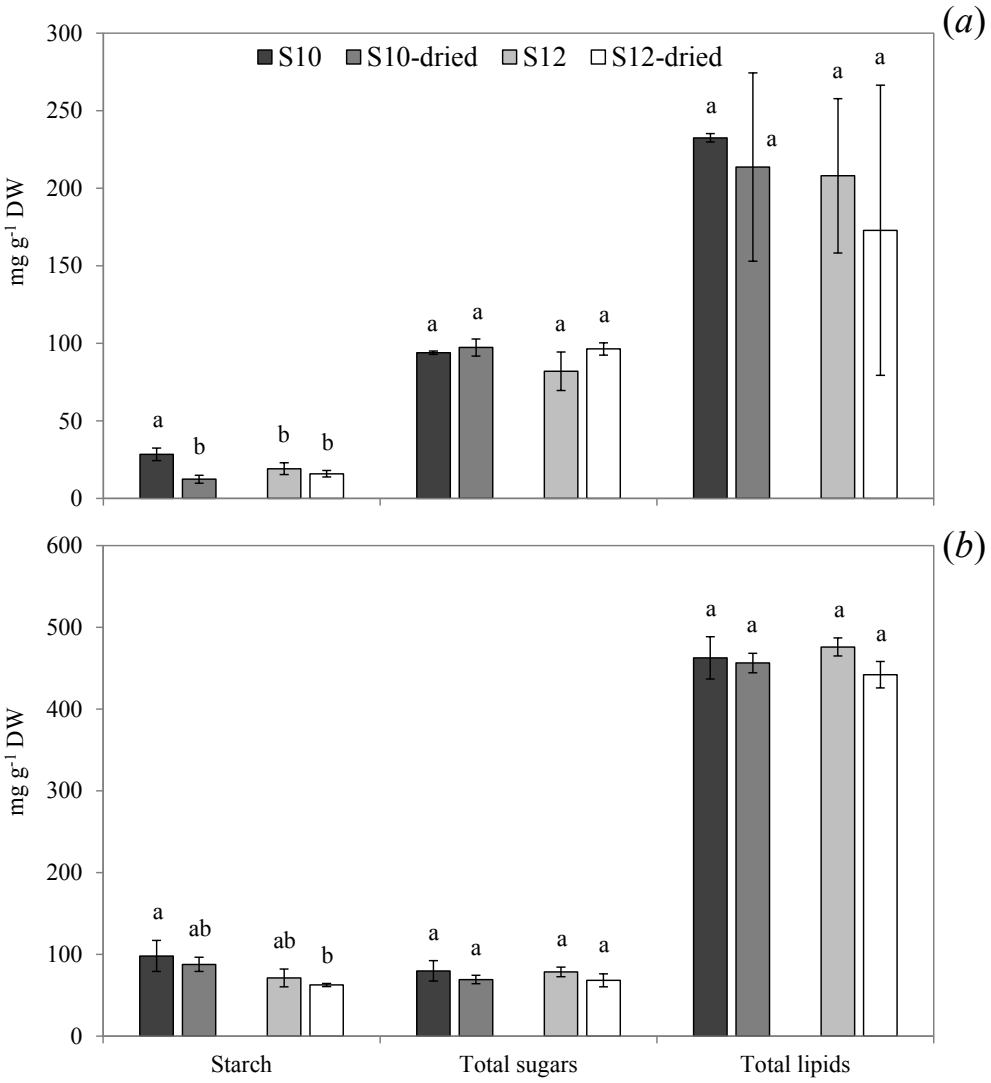

Fig 2. Contents (mg g^{-1} DW) of starch, total soluble sugars and total lipids of embryonic axis (*a*) and cotyledons (*b*) of *Poincianella pluviosa* immature (S10) and mature (S12) seeds and after drying until 0.08 g g^{-1} water. Within axis and cotyledons and each compound, means followed by the same letter do not differ significantly (Tukey´s test, $P < 0.05$, $n = 3$).

Fig. 3

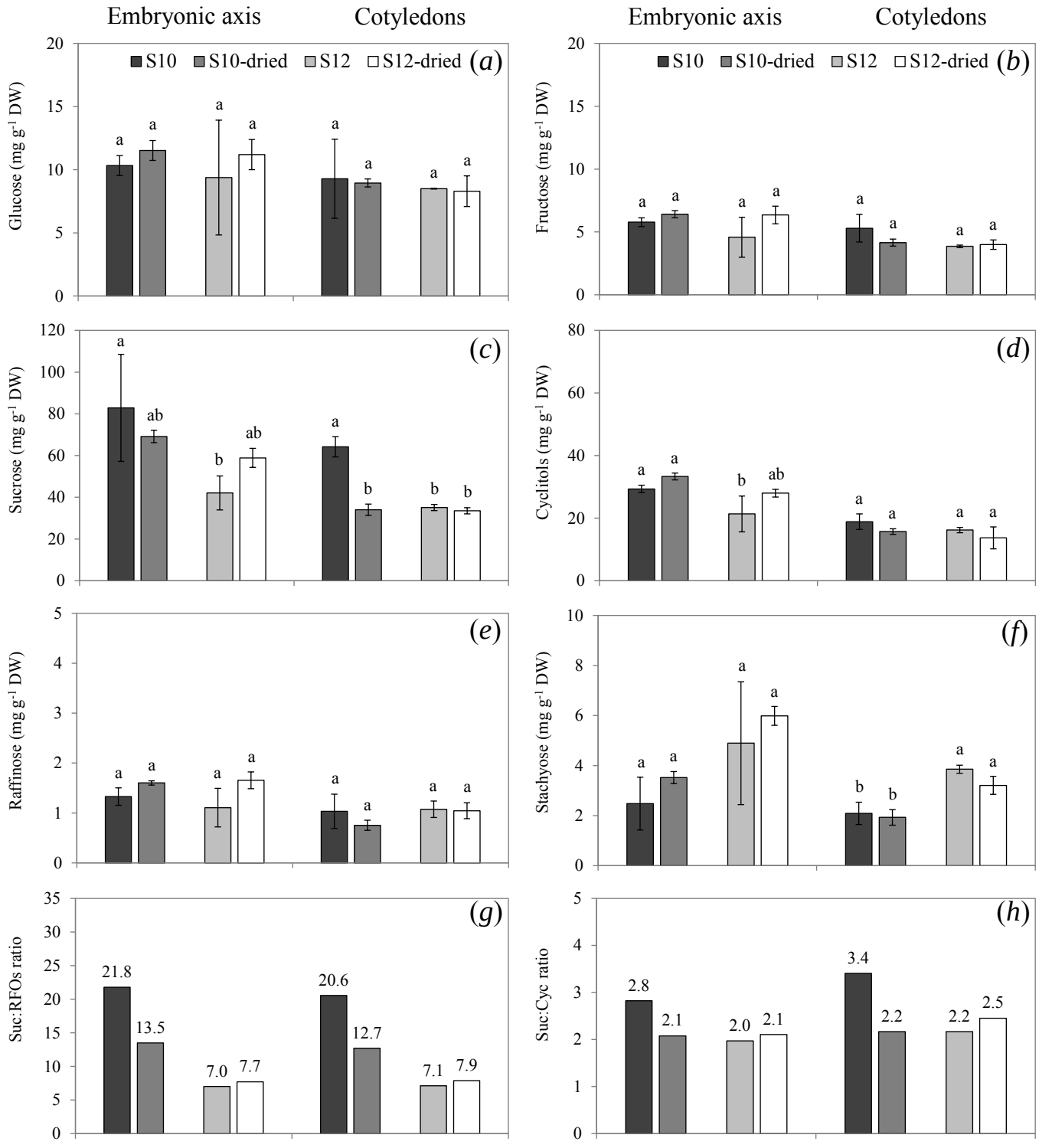

Fig 3. Soluble carbohydrate composition (mg g^{-1} DW) of embryonic axis and cotyledons of *Poincianella pluviosa* immature (S10) and mature (S12) seeds and after drying until 0.08 g g^{-1} water. Glucose (*a*); Fructose (*b*); Sucrose (*c*); Cyclitols (*d*); Raffinose (*e*); Stachyose (*f*); Sucrose:Raffinose family oligosaccharides (RFOs) ratio (*g*); Sucrose:Cyclitol ratio (*h*). Within each compound and axis and cotyledons, means followed by the same letter do not differ significantly (Tukey´s test, $P < 0.05$, $n = 3$).

Table 2

Table 2. Total fatty acid composition (%, neutral and polar lipid fraction. $n = 3$) of *Poincianella pluviosa* embryonic axis and cotyledons of immature (S10) and mature seeds (S12) and after drying until 0.08 g g^{-1} water. The sum of saturated and unsaturated fatty acids are indicated as well the unsaturated:saturated ratio. nd = non-detected

Stage	C9	C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:2	C18:3	C20	C20:1	C22	C22:1	C24	Total Unsaturated	Total Saturated	Unsat/Sat Ratio
Embryonic axis - neutral lipid fraction																		
S10					33.1		13.2	9.6	38.0		4.2		3.4			47.6	53.9	0.88
S10-dried		8.8	10.2		51.0		15.9	7.3	nd		5.8		4.5			7.3	96.1	0.08
S12		4.9	5.6		38.7		19.9	13.8	10.1	3.4	3.7					27.2	72.8	0.37
S12-dried		7.4	9.1		33.0		15.2	8.3	9.7	20.6						38.6	64.6	0.60
Cotyledons - neutral lipid fraction																		
S10					15.5		12.8	8.2	61.2		1.8		0.6			69.4	30.6	2.26
S10-dried					15.8		12.2	7.9	61.9		1.6		0.6			69.8	30.2	2.31
S12					21.6		26.2	11.8	37.3		3.2					49.1	50.9	0.96
S12-dried					26.3		30.2	12.2	26.2	1.7	3.4		0.9			40.1	60.8	0.66
Embryonic axis - polar lipid fraction																		
S10	4.4				39.7		17.7	9.6	22.4		5.1		4.3		2.1	34.1	73.3	0.47
S10-dried	6.8				53.1		17.8	5.4	2.3		6.2	2.1	5.3		2.5	12.3	91.7	0.13
S12	8.4		1.0	2.7	46.0	2.0	19.2	8.6	2.3		6.0		4.5		2.6	18.1	87.6	0.21
S12-dried	8.4		1.0	0.9	43.8		21.7	7.5	6.2		6.8		5.1		3.1	17.7	89.9	0.20
Cotyledons - polar lipid fraction																		
S10					22.2		18.1	8.7	47.6		2.2			2.9		59.2	42.5	1.40
S10-dried					20.8		16.5	8.5	52.9		1.9					61.5	39.2	1.57
S12	4.6				31.5		35.9	12.3	11.7		4.0					24.0	76.0	0.32
S12-dried	10.8		1.0		28.1	0.9	31.1	7.8	19.3		3.4	1.5	1.1			29.6	75.6	0.39

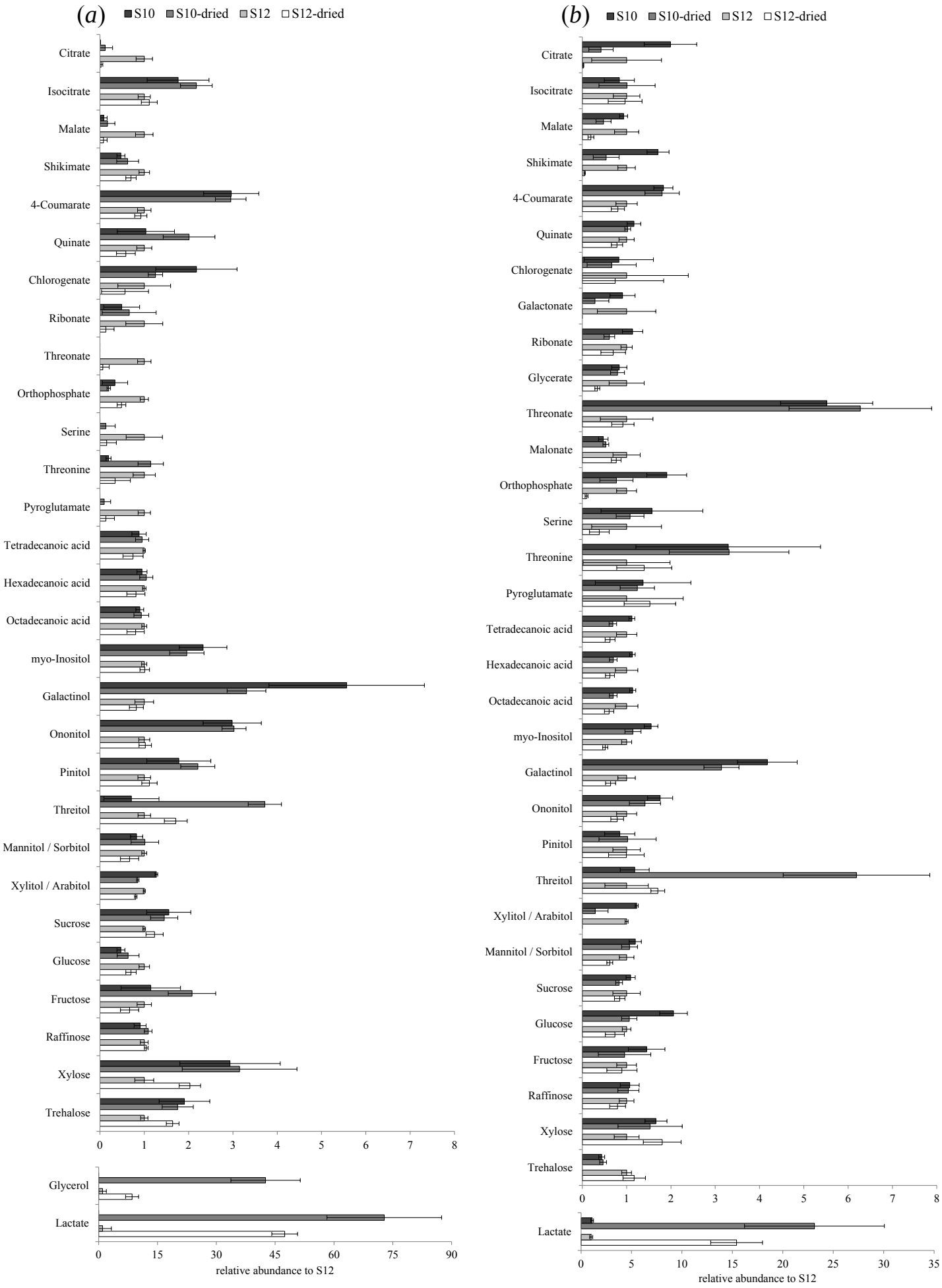

Fig 4. Metabolic profile of embryonic axis (*a*) and cotyledons (*b*) of *Poincianella pluviosa* immature (S10) and mature (S12) seeds and after drying until 0.08 g g^{-1} water. Compounds were detected by GC/MS. All values were normalized from those found in S12.

Fig 5.

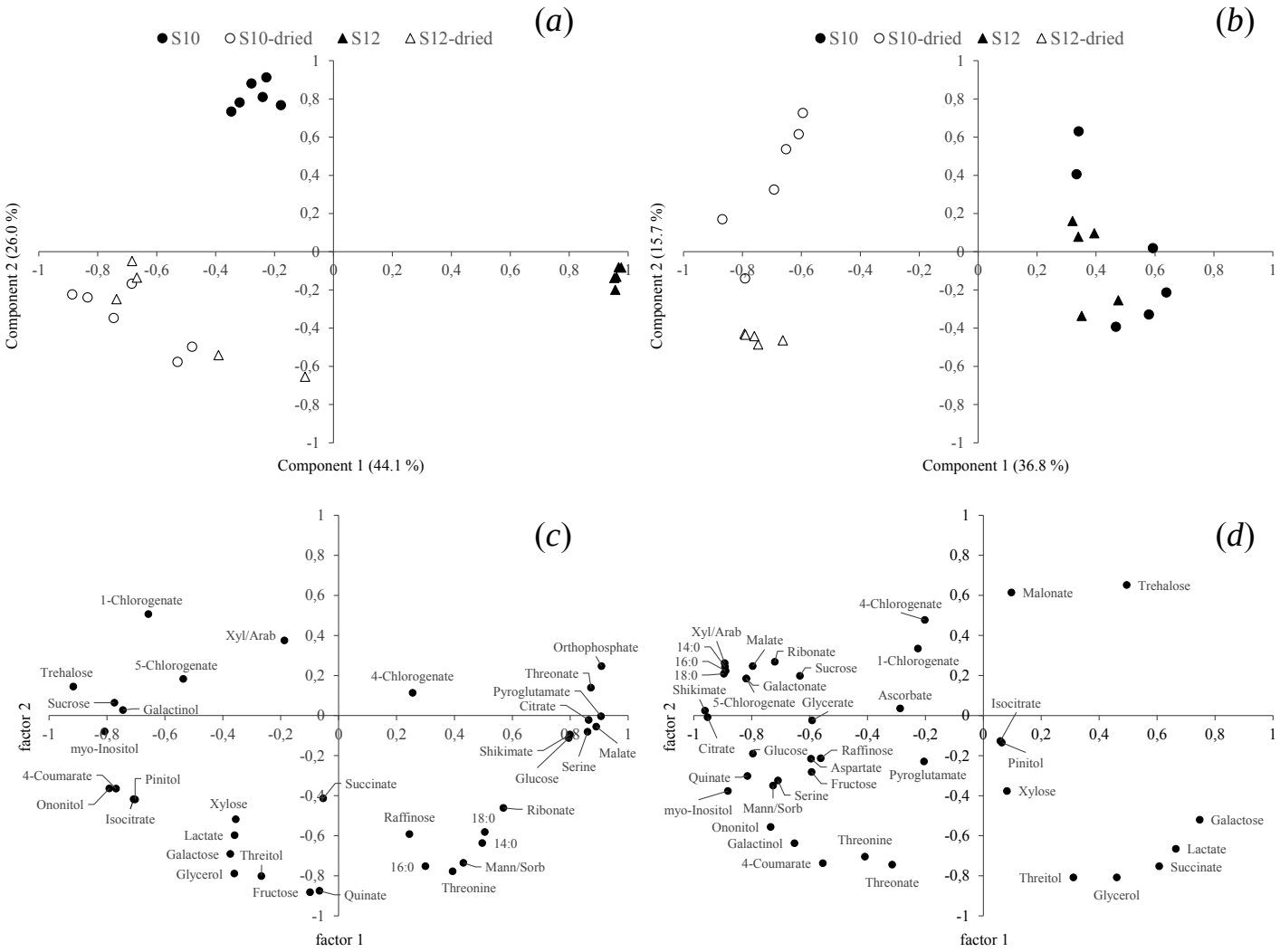

Fig 5. Principal component analysis (PCA) of metabolite profiles of embryonic axis (*a*) and cotyledons (*b*) and their respective loadings (*c* and *d*) of *Poincianella pluviosa* immature (S10) and mature (S12) seeds and after drying until 0.08 g g^{-1} water. PCA is presented as the combinations of the first two dimensions. Each data point represents an independent biological sample.

6. Chapter 4

A comparative study of *Poincianella pluviosa* and *Caesalpinia echinata* (Caesalpinioideae) seeds indicate that a reduced metabolism after shedding is controlled by low temperatures.

Abstract

Seeds of *Poincianella pluviosa* and *Caesalpinia echinata* are desiccation tolerant but present short to medium lifespan at natural environment. Decreased respiratory metabolism characterize the maturation of *P. pluviosa* seeds, while in *C. echinata*, respiratory rates at maturity are related to seed low storability. *C. echinata* seeds maintain viability for 5 years if stored at subzero conditions, but no information about freezing tolerance of *P. pluviosa* seeds are known. The reduction of metabolism by low temperatures, allied to an integrated redox system could limit oxidative process during storage. We analyze comparatively seeds of both species concerning morphophysiological characteristics of maturation, main reserve and antioxidant compounds, and stored *P. pluviosa* seeds under low and freezing temperatures to better understand these seeds post shedding behavior. *P. pluviosa* seeds are shed during winter while *C. echinata* during summer, but both can resist until the next rainy period. *P. pluviosa* seeds present several oil and proteins bodies while *C. echinata* accumulates large quantities of starch and present coat with several stomata. Mature seeds of both species presented high antioxidant capability in hydrophilic phase and high amounts of glutathione. Cotyledons of *P. pluviosa* have high antioxidant capability in lipophilic phase and high quantity of polyphenols. Cotyledons of both species present high levels of lipid peroxidation, but it is higher in *C. echinata* seeds. *P. pluviosa* seeds maintain viability for 2 years at low and subzero conditions. These results indicate that both seeds present antioxidant mechanisms, but not enough to control oxidation at high temperatures. Differences of the storability period under low temperatures between these seeds suggest a reduced metabolism after shedding, higher in *C. echinata*.

Keywords: freezing, seed storage, seed maturation, seed conservation, oxidation.

4.1 Introduction

Tropical forests are characterized by enormous genetic diversity and attention has concerned on how to maintain these resources available (Joly et al., 2010). *Ex situ* conservation of plant species through storage of seeds in germplasm bank is an important tool to accomplish this purpose. However, seeds present distinct responses to desiccation and freezing and more information is necessary to improve seed viability during storage (Barbedo and Marcos Filho, 1998).

For example, seeds of *Poincianella pluviosa*, *Caesalpinia echinata* and some species of *Salix* sp. tolerate high levels of desiccation but present short storability in the natural environment (Maroder et al., 2000; Figliolia et al., 2001; Barbedo et al., 2002). The low viability of these seeds under storage is believed to be due to oxidative processes, caused by photooxidation in an incomplete dedifferentiation of the chloroplast in *Salix* sp. (Maroder et al., 2003; Roqueiro et al., 2010) or by respiratory activity in *C. echinata* (Lamarca and Barbedo, 2012). No information is available on the possible mechanisms involved in the short storability of *P. pluviosa* seeds.

According to Figliolia et al. (2001), seeds of *P. pluviosa* lose viability within eight months when stored at room temperature (20-25 °C) but it is possible to extend their storability for one year when stored at low temperatures (Pontes et al., 2006). In comparison, *C. echinata* seeds exhibit a short lifespan (90 days) when stored at room temperature (Barbedo et al., 2002) and at low temperatures (7 °C) the viability is maintained for 18 months (Hellmann et al., 2006), increasing for 5 years if they are stored at freezing temperatures (-20 °C - Mello et al., 2013).

Poincianella pluviosa (DC.) L.P.Queiroz (= *Caesalpinia pluviosa* DC.) and *C. echinata* Lam. are included in the Leguminosae, one of the most numerous and important neo-tropical families (Queiroz, 2009; Lewis, 2013), both presenting seeds with interesting characteristics. *P. pluviosa* is widely distributed and covers almost all phytogeographic domains in Brazil, such as Amazon, Caatinga, Cerrado, Atlantic forest and Pantanal (Queiroz, 2009; Lewis, 2013), and present an extensive maturation cycle of 11 months (Silva et al., 2014 - Chapter 1). In contrast, *C. echinata* is scarcely found in its original habitat, the Atlantic forest (Rocha et al., 2007) being endangered to be extinct (Pilatti et al., 2011), and present a short maturation cycle of 60 days (Borges et al., 2005). Mature seeds of *P. pluviosa* contain large quantity of lipids (45-50%), mainly linoleic and palmitic acids, and low amount of starch (Corte et al., 2006; Chapter 2). On the other hand, mature seeds of *C. echinata* contain more than 40% starch and 17% lipids (Garcia et al., 2006; Mello et al., 2010). Both seeds also accumulate soluble sugars, mainly

sucrose and cyclitols, and traces of raffinose and stachyose (Borges et al., 2006; Garcia et al., 2006; Chapter 2).

It is suggested that soluble carbohydrates and the antioxidative system such as the ascorbate-glutathione cycle and phenolic compounds could be part of an integrated redox system of plant stress physiology (Van den Ende and Valluru, 2009 and references therein). Sugars such as sucrose, raffinose family oligosaccharides, fructans and cyclitols would act not only as osmoprotectants and as stabilizers of cellular membranes but also as scavengers of reactive species, similar function given for phenolic compounds, such as caffeic and chlorogenic acids (Nishizawa et al., 2008; Van den Ende and Valluru, 2009; Peshev et al., 2013). These sugars could also replenish NADPH, necessary for enzymes action of ascorbate-glutathione cycle, such as monodehydroascorbate-reductase and glutathione reductase, and are correlated with increased levels of the antioxidants ascorbate and glutathione during oxidative stress (Nishikawa et al., 2008).

Information concerning seed viability during storage and the involvement of the antioxidant system in seeds can help understanding their storage behavior. In the present work, we analyze morphological characteristics of seeds of *P. pluviosa* and *C. echinata* during seed maturation, their main reserve compounds and some components of the antioxidant system. Information on the storage behavior of *P. pluviosa* seeds under different conditions for two years, including sub-zero temperature was also provided aiming to increasing knowledge on *ex situ* seed conservation.

4.2 Material and methods

4.2.1 Plant material

Fruits and seeds of *P. pluviosa* were harvest at 210 DAA (immature) and 330 DAA (mature) from plantations located in Botucatu, SP, Brazil (22º52’ S 48º26’ W). For *C. echinata*, seeds were harvested at 45 DAA (immature) and 60 DAA (mature) from plantations located in Jaú, SP, Brazil (22º19’ S 48°50’ W). After harvest, fruits were transferred to the laboratory and seeds were processed for histochemical analysis as described below. Additionally, seeds from both species were obtained directly from the ground, not exceeding 48 h after shedding, which were named recently dispersed seeds (RDS). RDS of *P. pluviosa* were utilized in the storage treatments as described below. RDS seeds from both species were utilized to antioxidants and lipid peroxidation analyzes.

4.2.2 Morphological analysis of the seed coat and reserve compounds

Immature and mature seeds of *P. pluviosa* and *C. echinata* were fixed in Karnovsky's glutaraldehyde solution (modified by Kraus and Arduin, 1997) for 24h. For scanning electron microscopy (SEM), seeds were further dehydrated with 100% ethanol and rinsed in a hexamethyldisilazane (HMDS) series (33.3, 50.0, and 66.6% v/v in 100% ethanol) and then three times in 100% HMDS for 1 min each (Jeger et al., 2009) to dry the material. Samples were mounted on stubs, coated with gold palladium in a Hummer 6.2 sputtering system (Anatech, Union City, CA, USA) and viewed with a JSM-541OLV SEM (JEOL, Tokyo, Japan) at 10 kV. Digital images were edited using Adobe Photoshop version 7.0. For light microscopy observations, material was embedded using standard methods for Leica historesin® (Heraeus Kulzer, Hanau, Germany) and serially sectioned at 5 μm thickness. Sections were stained with toluidine blue O (Sakai, 1973) and mounted in water.

The main classes of metabolites that compose both structures (seed coat and reserve parenchyma) were *in situ* detected using the following histochemical tests: Sudan black B and Sudan IV to identify the lipophilic compounds (Pearse, 1985), Osmium tetroxide to unsaturated lipids (Ganter and Jolles, 1969/1970), Nile blue to acid and neutral lipids (Cain 1947) and Copper acetate/rubeanic acid for total fatty acids (Ganter and Jollès, 1969/1970), periodic acid-Schiff's reaction (PAS) to detect 1,2-glycol groups present in total polysaccharides (McManus, 1948), Lugol reagent to identify starch grains (Johansen, 1940), Xilidine Ponceau (Vidal, 1970) and Coomassie blue (Fisher, 1968) reagents for total proteins. Simultaneous standard control procedures (McManus, 1948; High, 1984) were performed. Observations and digital images were acquired using an Olympus BX53 compound microscope equipped with an Olympus Q-Color 5 digital camera and Image Pro Express 6.3 software.

4.2.3 Analyses of seed quality during storage

Poincianella pluviosa seeds (RDS) were stored in paper bags at 20 ± 7 ºC, 5 ± 2 °C, and -20 ± 5 ºC. Analysis of seed viability during storage at different temperatures was evaluated after 4, 8, 12, 18 and 24 months through germination tests. Before germination tests seeds were stored for 24 h at room temperature for acclimation. Germination (three replications of 15 seeds each) was carried out on rolls of Germitest paper previously moistened with tap water (ISTA, 2004), at 25 ± 1 ºC and evaluated every 2 days until 40 days (Borghetti, 2005). The percentage of germinated seeds (protrusion of at least 5 mm of primary root), normal seedling development (seedlings with at least 3 cm and no visual abnormal characteristics) were scored, and at the end of the analysis was calculated the speed of germination-aid (Maguire, 1962). Seed water content (g g^{-1} DW) and dry mass (g $seed^{-1}$) were determined by using three replicates of five seeds, after oven drying (103 ºC, 17 h) according to ISTA (2004). Data were statistically

analyzed by the F test at the 5% level of probability and were compared by Tukey test ($P < 0.05$, $n = 3$). It was used a completely randomized design in a factorial scheme 3 storage temperatures x 6 storage periods (Santana and Ranal, 2005).

4.2.4 Analysis of glutathione content

Embryonic axis and cotyledons (0.08 g and 0.1 g in triplicate, respectively) of both species were homogenized with eight volumes of cold 5% meta-phosphoric acid at 4°C in a porcelain mortar. The homogenate was centrifuged (20.000 *g*, 15 min, 4 °C), and the supernatant was collected for analysis of glutathione (GSH) contents as described in De Pinto *et al.* (1999) and calculated on a fresh weight basis.

Briefly, the glutathione pool was assayed using 0.2 ml aliquots of the supernatant neutralized with 0.8 ml of 0.5 M phosphate buffer (pH 7.5). For glutathione disulfide (GSSG) assay, the GSH was masked by adding 20 µl of 2-vinylpyridine to the neutralized supernatant, whereas 20 µl of H_20 was added in aliquots utilized for total glutathione pool (GSH plus GSSG) assay. Glutathione content was measured in 1 ml of reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM 5,5'- dithiobis (2-nitrobenzoic acid), and 0.1 ml of sample obtained as described above. The reaction was started by adding 3 units of glutathione reductase and was monitored by measuring the change in absorbance at 412 nm during 1 min. GSH was estimated as the difference between the amount of total glutathione and that of GSSG. A standard curve for GSH in the range of 0-30 µM/ml was utilized for quantification.

4.2.5 Analysis of total antioxidant capability (TAC)

Embryonic axis and cotyledons (0.03 g and 0.1 g in triplicate, respectively) of both species were homogenized with sixty volumes of 50 mM sodium phosphate buffer pH 7.5 in a cold porcelain mortar. The homogenate was centrifuged (15000 *g*, 30 min, 4 °C) and the supernatant, named as hydrophilic phase, was separated and kept on ice until use. To the residue, thirty volumes of acetone was added and thoroughly shaken before centrifugation (15000 *g*, 30 min, 4 °C). The supernatant was separated and named as lipophilic phase. Total antioxidant capability of both fractions was determined using Trolox equivalent capacity assay based on the ability of antioxidant molecules to quench the 2,2´-azino-bis-3-ethylbenzothiazo-line-6-sulfonic acid (ABTS•) radical into a colorless product (Re et al., 1999). Trolox (6-hydrolxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as an antioxidant standard. The capability of the lipophilic and the hydrophilic phases to scavenge the ABTS• was expressed as Trolox equivalent using a standard dose-response curve. ABTS was dissolved in 0.1 M phosphate buffer pH 7.4 to a 5 mM concentration and the radical (ABTS•) was produced

by adding 1.76 mM potassium persulfate. The mixture was kept in the dark at room temperature for 16h before use. The ABTS• working solution was freshly made by diluting the original solution with 0.1 M phosphate buffer or ethanol to an absorbance of 0.7 (± 0.02) at 734 nm. To measure the antioxidant activity, the delta absorbance in 1 min was calculated. This was performed at least three times in triplicate.

4.2.6 Measurement of total polyphenols

Extraction of total polyphenols was performed according to Arranz and Saura-Calixto (2010). Embryonic axis and cotyledons (0.02 g and 0.1 g in triplicate, respectively) of both species were homogenized with forty volumes of acidic methanol (HCl)/water (50:50, v/v; pH 2) in a porcelain mortar. The homogenate was transferred to a centrifuge tube and thoroughly shaken in a planar centrifuge (1h, 25 °C). After this period, the tubes were centrifuged (5000 *g*, 10 min, 25 °C) and the supernatant separated in another tube. To the residue, forty volumes of acetone/water solution (70:30, v/v) was added, followed by vigorous shaking and centrifugation in a planar centrifuge was repeated (1h, 25 °C). Methanolic and acetonic extracts were combined and used to quantify the polyphenols in samples and calculated on a fresh weight basis.

The total phenolic content was determined by Folin-Ciocalteu assay (Singleton et al., 1999) with minor modifications. Briefly, 200 µl of extract was added to 425 µl of water and 125 µl of Folin-Ciocalteu reagent. The solution was mixed, and after 8 min of incubation (25 °C, in the dark), 1.25 mL of 7% Na_2CO_3 and 1 ml of H_2O were added. Finally, the mixture was incubated for 90 min (25 °C, in the dark), and the adsorption at 760 nm was subsequently measured using water as blank. To calculate the total phenolic contents, a standard curve with gallic acid was used.

4.2.7 Analysis of lipid peroxidation

The level of lipid peroxidation in axis and cotyledons of both species was measured in terms of malondialdehyde (a product of lipid peroxidation) content determined by the thiobarbituric acid reaction according to Zhang and Kirkham (1996). Embryonic axis and cotyledons (0.03 g and 0.5 g in triplicate, respectively) were homogenized with ten volumes of 0.1% trichloroacetic acid in a porcelain mortar. The homogenate was centrifuged (10000 *g*, 10 min), and aliquots of the supernatant (100 µl for axis and 1 ml for cotyledons) was diluted (1:5) with 20% trichloroacetic acid containing 0.5% thiobarbituric acid. The mixture was shaken, heated at 95 °C for 30 min and then quickly cooled in an ice bath. After cooling, the tube was centrifuged (10000 *g*, 10 min) and the extract adsorption at 532 nm was subsequently measured. The value for the nonspecific absorption at 600 nm was subtracted from the 532 nm reading.

The concentration of malondialdehyde was calculated using malondialdehyde extinction coefficient of 155 mM cm^{-1} and the results are expressed as μmol g FW^{-1}.

4.3 Results

4.3.1 Morphological characteristics of fruits and seeds

Similarities are observed between fruits and seeds of *P. pluviosa* and *C. echinata* (Fig. 1). Both present green fruits and seeds, which acquire a brownish color when mature. Fruits of *C. echinata* present several aculeus, absent in *P. pluviosa*, with generally larger fruits (Figs. 1a, c–e). Immature seeds (245 DAA) of *P. pluviosa* present a thin and translucent coat, allowing the visualization of the green cotyledons (Fig. 1b), that become rigid and more opaque when mature (Fig. 1d). In *C. echinata* seeds, the coat is dense with brown spots not allowing to visualize changes in the color of embryonic axis and cotyledons, both becoming white with maturity.

Micromorphological examination of seed coats from both species allowed recognition of different patterns (Fig. 2). The immature seed coat of *P. pluviosa* presented entire papillate aspect (Fig. 2a), becoming plasmolysed and presenting irregular fissures when mature and dried (Fig. 2c). In *C. echinata*, the immature seed coat presented several stomata (Fig. 2b), that are maintained when mature, although not easily visualized due to high plasmolysis when dried (Fig. 2d). In addition, many amyloplasts occupying the entire lumen of parenchymatic cells were visualized in sections of the cotyledons (Fig. 2d, *inset*).

4.3.2 Reserve compounds

Structurally, the cotyledons cells of *P. pluviosa* differ from those of *C. echinata* (Fig. 3) the protoplast is denser and present a number of lipid droplets and few amyloplasts occupying a parietal position in the parenchyma cells (Fig. 3a). PAS reagent and Lugol's test confirmed the presence of starch grains inside the amyloplasts (Figs. 3f, g). Lipophilic compounds are the major reserves, being visualized as numerous droplets inside the cotyledons cells (Figs. 3a-e). The nature of these lipophilic compounds is mainly of fatty acids (Fig. 3e), probably unsaturated and neutral (as indicated by Fig. 3c-d). Moreover, protein bodies are also found co-occurring with lipophilic droplets (Fig. 3h), indicating that both constituents predominate in the seed reserves.

Unlike *P. pluviosa,* starch is the main reserve compound in *C. echinata* seeds, as shown in Figure 4. The membrane of the amyloplasts can be visualized after staining with Toluidine Blue (Fig. 4a), and the starch grains evidenced by PAS reaction (Fig. 4f) and Lugol's reagent (Fig. 4g). Fewer and smaller lipophilic droplets close to the cell wall are shown in Fig. 4b, and

could be of unsaturated and neutral nature as determined by tests with OsO_4 and Nile blue, respectively (Figs. 4c, d). They represent minor constituents of *C. echinata* seeds. Nonetheless, mostly the lipids (as fatty acids) and proteins identified by histochemical tests represent structural constituents of the seed, i.e., the protoplast.

4.3.3 Viability of P. pluviosa *seeds during storage*

Recently dispersed seeds (RDS) present low water content (0.09 g g^{-1}), 47% germination and 30% normal seedling development. The seed water content did not change significantly during storage at freezing temperatures, while decreased to ~0.06 g g^{-1} after 4 months of storage at 5 °C, and increased to 0.14 g g^{-1} after 12 months of storage at 20 °C (Fig. 5a). However, only seeds stored at 5 °C and -20 °C maintained the germination rates and normal seedling development after 24 months of storage (Figs. 5b, d), while at 20 °C, minimal values of both parameters were obtained after 12 months (Fig. 5b).

4.3.4 Analysis of antioxidants, polyphenols and lipid peroxidation

Seeds of *P. pluviosa* and *C. echinata* presented high antioxidant capability in the hydrophilic fraction (more than 15 nmol Trolox equiv g^{-1}), higher in embryonic axis than in cotyledons. In contrast, the lipophilic phase presented a very low antioxidant capability in both species (less than 3 nmol Trolox equiv g^{-1}), except the cotyledons of *P. pluviosa* seeds (13.6 nmol Trolox equiv g^{-1} - Fig. 6a). Very high concentration of total glutathione, with elevated concentrations of reduced glutathione (GSH) was also found in both axis and cotyledons of such seeds (Fig. 6b). Similar to what was observed for lipophilic phases in TAC analysis, the quantity of polyphenols is higher in cotyledons of *P. pluviosa* (34.6 µmol gallic acid equiv. g^{-1}) compared to embryonic axis (13.8 µmol gallic acid equiv. g^{-1}), and with *C. echinata* seeds (12-16 µmol gallic acid equiv. g^{-1} - Fig. 6c). Cotyledons of both species presented elevated level of lipid peroxidation compared to embryonic axis, being higher in *C. echinata* (Fig. 6d).

4.4 Discussion

4.4.1 Shedding period and seed storability in natural environment

Although fruits and seeds of *P. pluviosa* and *C. echinata* present great similarities (Fig. 1), the maturation of both species present marked physiological differences. The maturation of *P. pluviosa* seeds commonly starts from August to October when a new anthesis phase occurs, followed by an extensive period of 11 months, ending in the driest period (July to August) that characterizes winter in southeast region of Brazil (Silva et al., 2014, Chapter 1). Conversely, the anthesis of *C. echinata* plants growing in the same region starts close (September to November - Rocha, 2004), but the complete seed maturation finish in 60-65 days, shedding in

periods of highest rainfalls (November to January) at the beginning of summer (Borges et al., 2005). Although seeds of both species present short lifespan in field conditions, 3 months for *C. echinata* (Barbedo et al., 2002) and 8 months for *P. pluviosa* (Figliolia et al., 2001), both can maintain viability until the start of rainy period.

4.4.2 Strategies to optimize internal O_2 level and accumulate reserves

Despite similar morphological changes in the maturation of seeds from both species were observed (Fig. 1), the immature seed coat of *C. echinata* present several stomata while they were not observed in *P. pluviosa* seeds (Fig. 2a). The presence of stomata in immature seeds of *C. echinata* contrasts to the seed coat of another related legume *Caesalpinia ferrea*, characterized by the presence of osteosclereids layers, as well as fibers and macrosclereids in mature seeds (Teixeira et al., 2004), possibly related to the impermeability to water shown by these seeds. Interestingly, a hypoxic internal environment characterizes developing seeds during most part of the maturation period (Borisjuk and Rolletschek, 2008). In this way, the presence of stomata in immature seed coat might facilitate gas exchange to the embryo, maintaining elevated internal levels of oxygen, stimulating the accumulation of reserve compounds (Vigeolas et al., 2003; Rolletschek et al., 2005). Although stomata were not observed in *P. pluviosa* seed coat at any stage of maturation (Figs. 2a, c), the maintenance of green cotyledons until maturity could act as internal O_2 adjuster (Borisjuk and Rolletschek, 2008 and references therein). These characteristics allowed embryo photosynthesis, providing control of oxygen level and favoring accumulation of reserve compounds (Rolletschek et al., 2005; Borisjuk and Rolletschek, 2008). In mature seeds (Figs. 2b, d), the stomata could facilitate water entrance and might be related to the fast imbibition rate observed during germination of *C. echinata* seeds, when seed water content triples after 10 min of imbibition (Lamarca et al., 2009). In comparison, in *P. pluviosa* seeds this only occur after 4 hours of imbibition (Silva, 2010), probably affected by the great amount of oil (Figs 3b-e), despite the fissures observed in mature seed coat (Fig. 2c), possibly caused by constrictions during seed desiccation.

4.4.3 Oil bodies, starch and desiccation tolerance

Seeds of *P. pluviosa* preferentially accumulate lipids, as observed in morphological analysis by the presence of several oil bodies (Figs. 3b-e), followed by relatively high amount of proteins (Fig. 3h), while starch is the main carbon reserve in *C. echinata* seeds (Fig. 4g). Our results are consistent with previous works (Corte et al., 2006; Borges et al., 2006; Mello et al., 2010; Chapter 2) although specific analyses of proteins in these seeds are necessary. Even with higher quantity of lipids, as observed in cotyledons of *P. pluviosa* seeds (~45% - Chapter 2)

compared to cotyledons of *C. echinata* seeds (18% - Mello et al., 2010), both have a similar fatty acids profile, high proportions of linoleic acid (46-54%) followed by stearic (18-26%) and palmitic (13-18%) acids (Mello et al., 2010; Chapter 2). In addition, mature seeds of *C. echinata* present great quantities of sucrose and cyclitols, that is less pronounced in *P. pluviosa* seeds and both species have just traces of oligosaccharides such raffinose and stachyose (Borges et al., 2006; Mello et al., 2010; Chapter 2).

Lipids and sugars in seeds of both species could have important role in water movement, maintaining the conformation and functionality of cell membranes and protecting embryo against injuries during seed dehydration (Hoekstra et al., 2001; Mello et al., 2010). Sugars such sucrose, raffinose and cyclitols favor desiccation tolerance when buffering the water loss, stabilizing polymers during desiccation (Hoekstra et al., 2001; Peterbauer and Richter, 2001). While the presence of unsaturated fatty acids, such as linoleic acid, could influence fluidity of oil bodies and membranes and is correlated to seed desiccation tolerance (Liu et al., 2006; Mello et al., 2010), changes in classes of lipid membranes (*i.e.* phospholipids, phosphatidylethanolamine) could be another form to alter membrane physical characteristics (Quartacci et al., 2002). Interestingly, a decrease in the unsaturation ratio of membrane fatty acids in embryonic axis was observed during maturation of *P. pluviosa* seeds (Chapter 2), although no information about membrane fatty acid constituents was found for *C. echinata* seeds. However, seeds of *P. pluviosa* and *C. echinata* are desiccation tolerant maintaining high germinability even after drying to 0.08 g g^{-1} (Barbedo et al., 2002; Chapter 3).

4.4.4 Low and freezing temperatures improve seed viability of both species

The low rates of germination and normal seedling development initially observed in RDS of *P. pluviosa* are probably related to the presence of immature seeds in the same lot, as suggested by Silva et al. (2014 - see chapter 1). However, the viability of such seeds was maintained during storage at cold and freezing temperatures for at least two years (Fig. 5b), which did not occur at 20 °C after eight months. Similar results were obtained by Figliolia et al. (2001) and Pontes et al. (2006), although, these authors did not evaluate longer periods of storage or utilized freezing temperature as in the present study. Seed storage at low temperatures is beneficial to many species, though it may be detrimental to others (Maroder et al., 2000; Crane et al., 2003; Bonjovani and Barbedo, 2008). *P. pluviosa* seeds tolerate storage at low and freezing temperatures, which is known to reduce the metabolism of many seeds and growth of microorganisms that occur during storage, contributing to maintain seed viability for long periods. Although this might not be a rule for all seeds, as indicated by the elevated respiratory

rates of desiccation sensitive seeds of *Inga vera* stored at decreasing levels of temperature (Bonjovani, 2011).

Seeds of *C. echinata* and some species of *Salix* are also desiccation tolerant and present low viability when stored in environments without temperature or light control (Roqueiro et al., 2010; Mello et al., 2013). The low viability of *C. echinata* seeds is related to respiration rates observed in mature seeds even when they have low water content (0.11 to 0.16 g g^{-1}), indicating that oxidative processes should occurs during storage (Lamarca and Barbedo, 2012). In *Salix nigra* seeds, chloroplasts do not complete dedifferentiation during maturation drying, retaining chlorophyll and maintaining their endomembrane system intact in the dried state. In consequence, they are very susceptible to photooxidation during storage, when light intensity increases the production of free radicals (Maroder et al., 2003; Roqueiro et al., 2010). Interestingly, the cotyledons of *P. pluviosa* remain green throughout maturation (Silva et al., 2014, Chapter 1), although no information about organelle dedifferentiation is known. Moreover, the decreased viability of *P. pluviosa* seeds stored at 20 °C was correlated to the increased water content obtained after one year of storage (Figs. 4a, b). Therefore, a better control of seed water content and light conditions under storage could be efficient to improve viability of such seeds.

Seeds of *P. pluviosa* kept at 5 °C presented great reduction in water content, but remained viable for two years and showed no significant differences in germination percentage compared to seeds kept frozen and do not loose water during storage (Figs. 5a, b). In contrast, when *C. echinata* seeds are stored at low but positive temperatures (2 °C or 8 °C), low rates of germination and normal seedling development were obtained after two years, while at sub-zero temperature the viability of these seeds was maintained for 5 years (Hellmann et al., 2006; Mello et al., 2013). Similar results were reported for *Salix alba* seeds, an higher viability was achieved when stored at freezing temperature (5 months) than at 5 °C (2 months - Moroder et al., 2000). Taking into account the higher viability of *P. pluviosa* seeds in natural environment (240 days) compared to *C. echinata* (90 days) or *S. alba* (15 days), it is reasonable to argue that even seeds stored at 5 °C would present decreased vigor with longer periods then two years of storage. In this way, negative temperatures are the better option to maintain viability of these seeds for longer periods.

4.4.5 Protective mechanisms against reactive oxygen species

Due to evidences that oxidative processes were taking place in mature seeds of *C. echinata* and this could be related with low viability of such seeds in tropical natural

environment, RDS of both species were compared in terms of antioxidant characteristics and lipid peroxidation of embryonic axis and cotyledons (Fig. 6).

It is evident that seeds of both species have higher antioxidant capability in hydrophilic than in lipophilic phase (Fig. 6a), possibly related to large amounts of glutathione found in their axis and cotyledons (Fig. 6b), or by chlorogenic acid, suggested to be accumulated during maturation of *P. pluviosa* seeds (Chapter 2). Glutathione is a major cellular hydrophilic antioxidant and redox buffer, and together with ascorbate, these two redox pairs comprise the plant protection against reactive oxygen species (Foyer and Noctor, 2011). The ascorbate system is turned off at the final stages of maturation in desiccation tolerant seeds, and only moderate amount of the oxidized form of ascorbate is found (Arrigoni et al., 1992; Tomassi et al., 1999). In contrast, the glutathione pool is present in dry seeds both in the reduced (GSH) and oxidized (GSSG) forms (Tommasi et al., 2001). GSSG is reduced immediately back to GSH by the action of glutathione reductase in metabolically active tissues. However, because enzymes presumably cannot be active in the dry state, desiccated seeds accumulate GSSG depending on the extent of oxidative stress (Kranner and Grill, 1993; Bailly, 2004; Kranner et al., 2006). In fact, the redox potential of the glutathione pool, indicated by the ratio between GSSG and GSH, could be utilized as an indicator of seed viability (Kranner et al., 2006). In this concern, RDS of *P. pluviosa* and *C. echinata* presented relatively low amounts of GSSG and high level of total glutathione (Fig. 6b), that could provide protection against reactive oxygen and nitrogen species. However, since glutathione could be present in different cell compartments, it is necessary to localize and correlate the GSH pool in seeds from both species presenting different levels of storability.

The high quantity of total polyphenols obtained in cotyledons of *P. pluviosa* seeds (Fig. 6c) is probably related to the high antioxidant capability found in the lipophilic phase (Fig. 6a), suggesting that compounds such tocopherol is present. Tocopherol is commonly found in green parts of higher plants and oil seeds, with ability to directly quench reactive oxygen species or indirectly protect tissues against lipid peroxidation (Kamal-Eldin and Appelqvist, 1996). Furthermore, the large amounts of oil bodies present in cotyledons of *P. pluviosa* (Figs. 3b-e) would have a higher protection against reactive species compared to embryonic axis or to *C. echinata* seeds (Figs. 6a, c).

The insufficient antioxidant control during the dried state allows the accumulation of oxidative damage to macromolecules, contributing to seed deterioration, leading to viability loss with severe lipid peroxidation, particularly of the poly-unsaturated fatty acids (Pukacka et al., 2007). However, the highest levels of lipid peroxidation obtained in RDS were found mainly

in cotyledons of both species, followed by increased levels in the axis of *C. echinata* compared to *P. pluviosa* (Fig. 6c). Although axis and cotyledons from both species present high antioxidant capability (Fig. 6a) and high amounts of glutathione (Fig. 6b), the production of reactive species in these seeds would be higher than the protective capacity in a short period during storage.

Sensitive desiccation tolerant seeds possess antioxidant mechanisms (Pammenter and Berjak, 1999 and references therein), but during dehydration they likely are insufficient because these seeds cannot down-regulate metabolism as in desiccation tolerant seeds (Fait et al., 2006; Caccere et al., 2013). Although a clear decrease of the respiratory metabolism occur throughout *P. pluviosa* seeds during seed maturation (Chapter 2), the production of reactive species in dried seeds of *C. echinata* is related to respiratory rates found during storage (Lamarca and Barbedo, 2012). Taking into account the similar low viability in natural environment and the improved storability obtained by decreasing temperatures (Mello et al., 2013; Fig. 5) providing lower metabolic activity, a low, but active respiratory process could be happening in seeds of both species, being pronounced in *C. echinata*.

Even producing seeds with short to medium lifespan, seeds of *P. pluviosa* and *C. echinata* are capable to survive in the natural environment, either by shedding in the rainy period and germinating rapidly or by resisting the dry state for longer periods. Taken together, results show that seeds of both species have specific strategies to regulate the internal oxygen concentration during maturation providing large amounts of reserves at maturity. Moreover, both seeds present antioxidant protection mechanisms, although could be not enough to control oxidation for longer periods during storage at high temperatures. The difference of the storability period observed between these seeds in natural environment could be related to a specific and reduced seed metabolism after shedding.

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Fig. 1

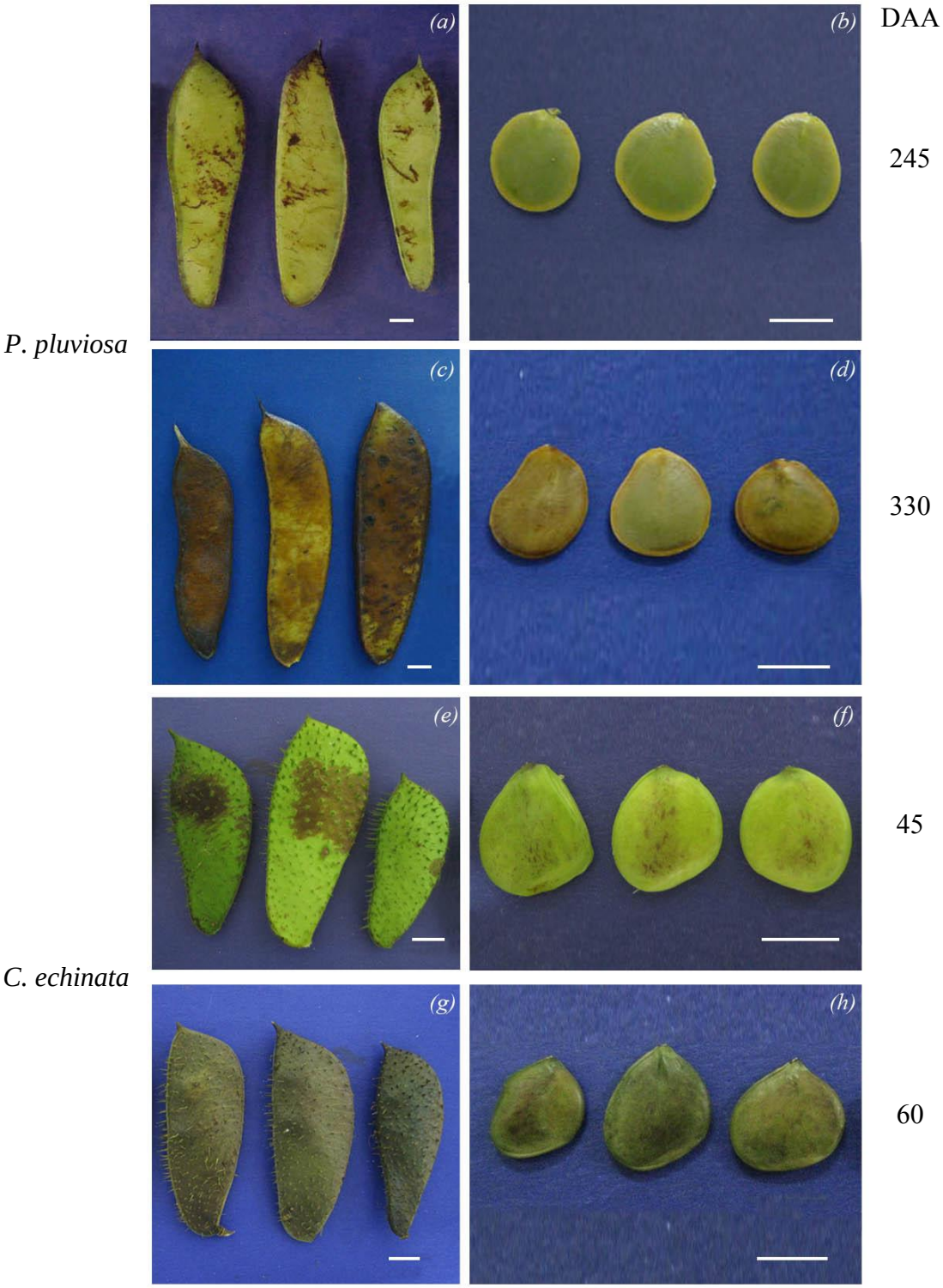

Fig 1. Fruits and seeds of *Poincianella pluviosa* (*a*, *b*, *c* and *d*) and *Caesalpinia echinata* (*e*, *f*, *g* and *h*), collected at 245 DAA (*a* and *b*); 330 DAA (*c and d*), 45 DAA (*e* and *f*); 60 DAA (*g* and *h*). DAA, days after anthesis. Bars means 1cm.

Fig. 2

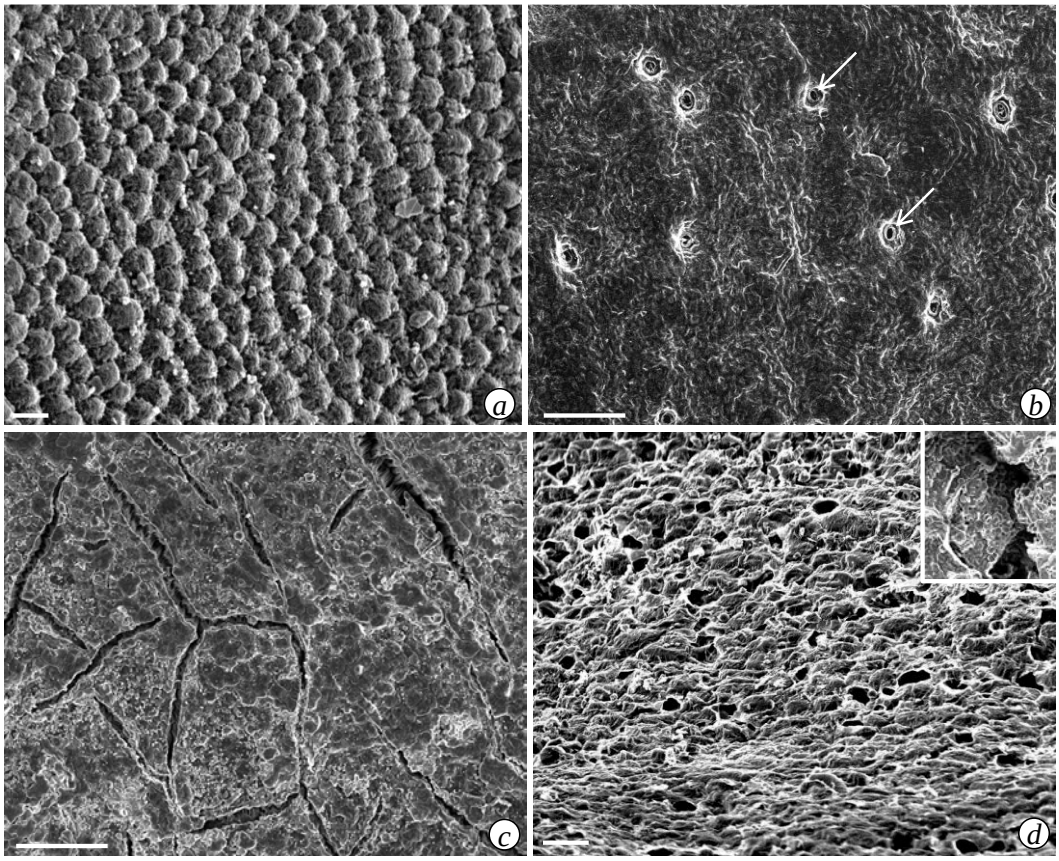

Fig 2. Immature (*a*-*b*) and mature (*c*-*d*) seed coats of *P. pluviosa* (*a*, *c*) and *C. echinata* (*b*, *d*). Note the plasmolysed aspect of the seed coat of mature seeds (*c*-*d*). Note the papillate aspect of the immature seed coat (*a*) and the occurrence of fissures when mature (*b*). Note the occurrence of stomata (arrows) in immature seed coat hardly viewed on mature one. Note the starch grains inside cotyledons cells (*inset*). Scale bars: 10µm (*a*, *d*), 100µm (*b*, *c*).

Fig. 3

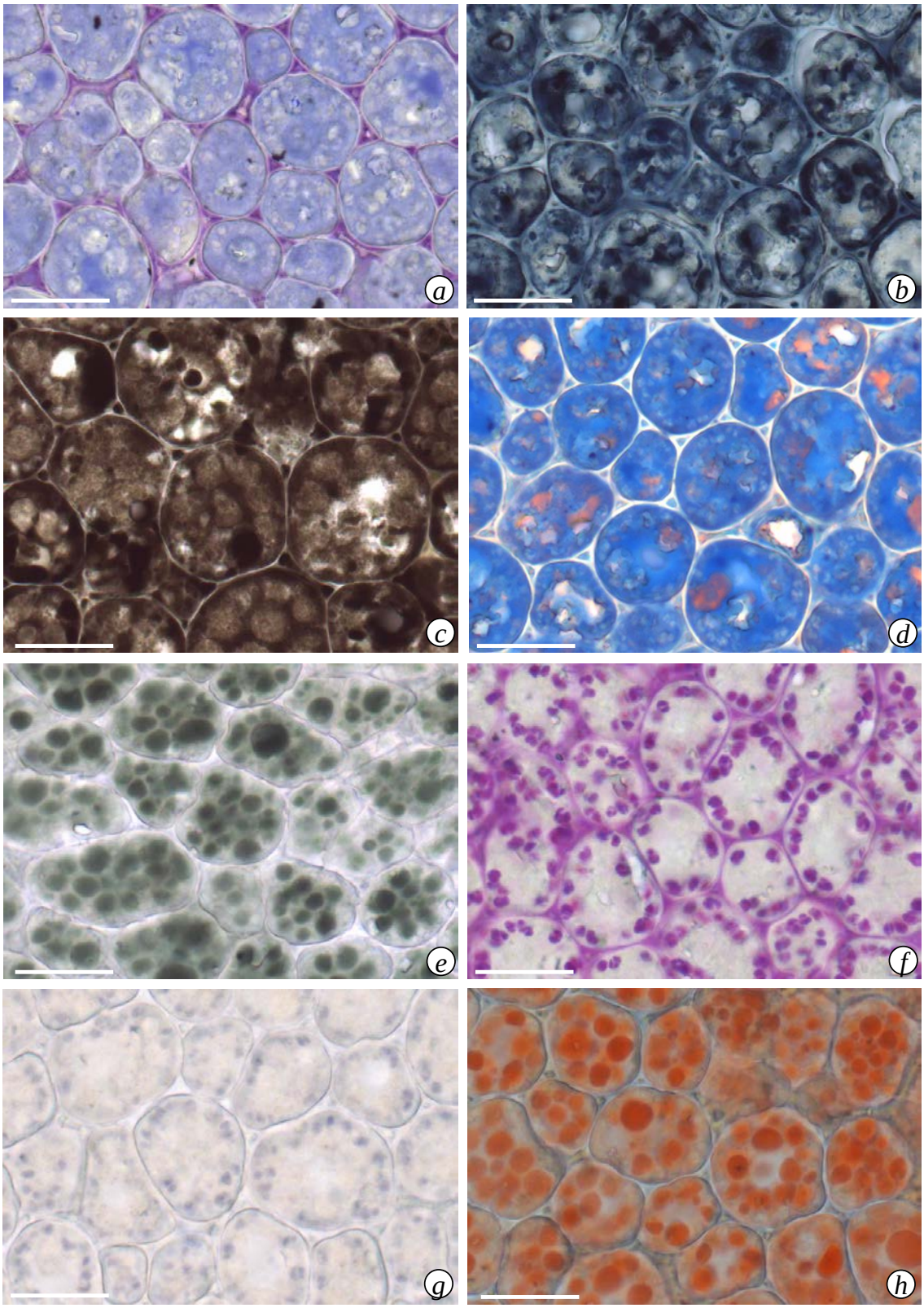

Fig 3. Structure and histochemistry of *P. pluviosa* cotyledons cells. (*a*) Toluidine blue O. Structure (*b*) Sudan black B. Lipophilic compounds. (*c*) OsO_4. Unsaturated lipids (*d*) Nile blue. Acid (blue) and neutral (pink) lipids (*e*) Cupper acetate/rubeanic acid (fatty acids). (*f*) PAS reaction. Total polysaccharides. (*g*) Lugol reagent. Starch grains. (*h*) Xilidine Ponceau. Proteins. Scale bars: 75µm.

Fig. 4

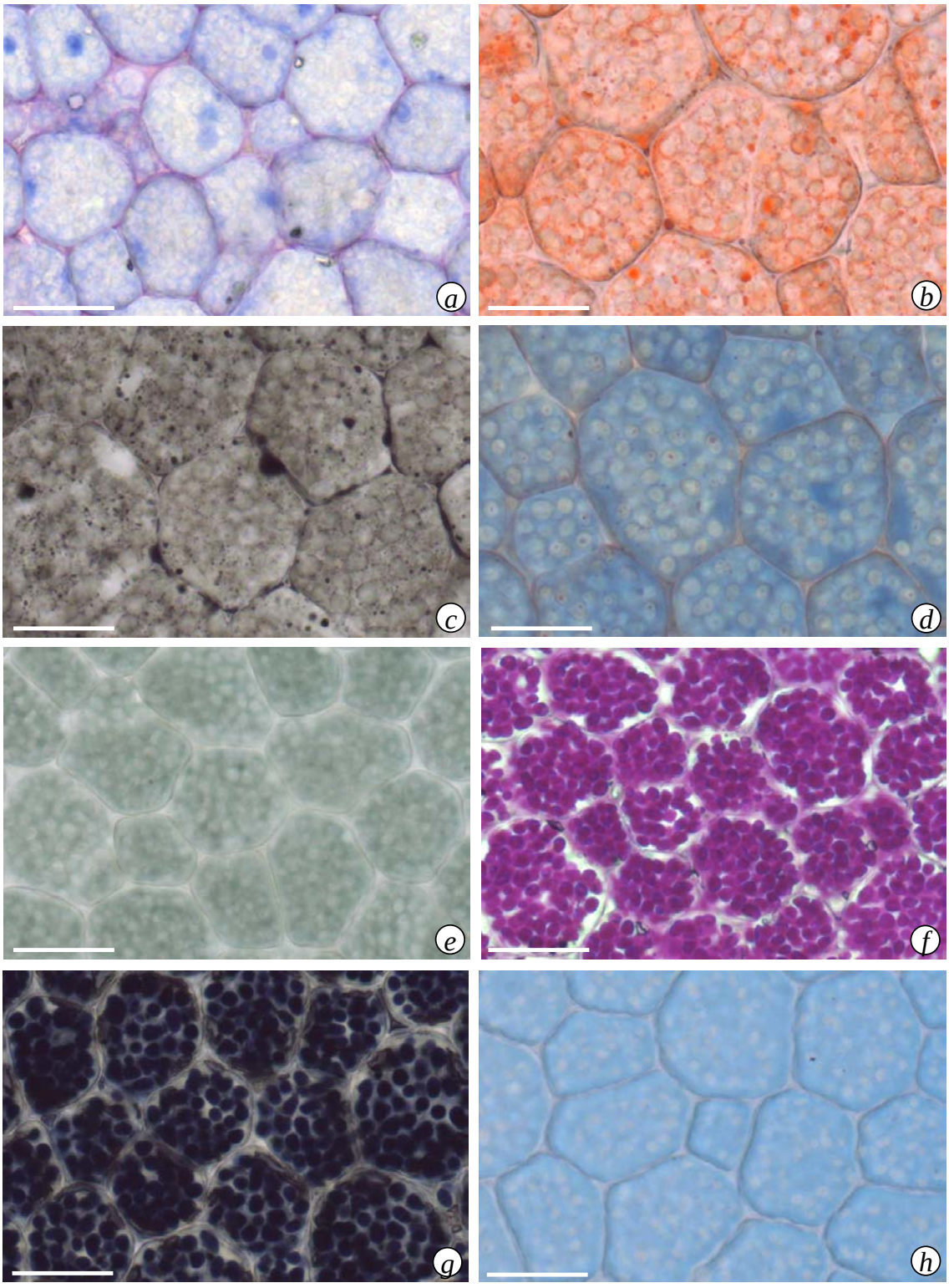

Fig 4. Structure and histochemistry of *C. echinata* cotyledons cells. (*a*) Toluidine blue O. Structure (*b*) Sudan IV. Lipophilic compounds. (*c*) OsO_4. Unsaturated lipids (*d*) Nile blue. Acid (blue) and neutral (pink) lipids (*e*) Cupper acetate/rubeanic acid (fatty acids). (*f*) PAS reaction. Total polysaccharides. (*g*) Lugol reagent. Starch grains. (*h*) Coomassie blue. Proteins. Scale bars: 75µm.

Fig. 5

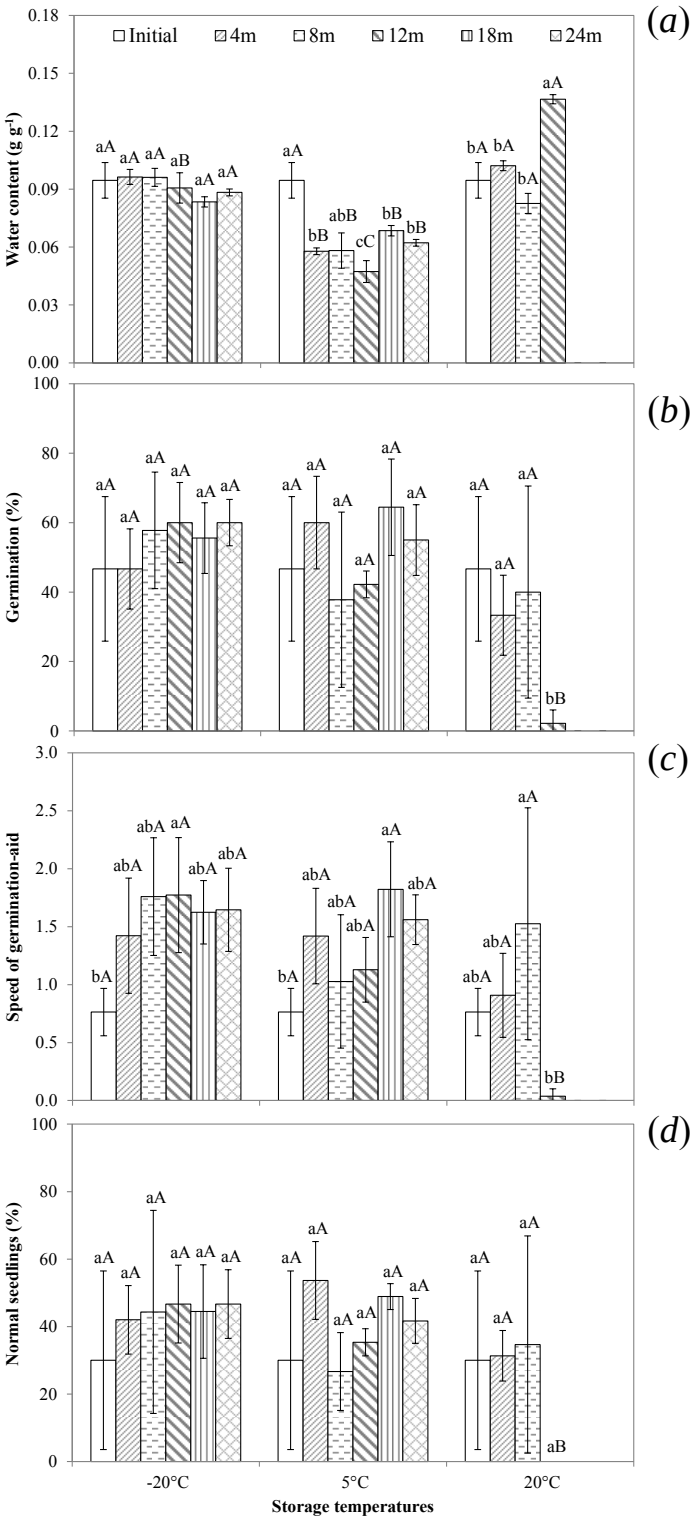

Fig 5. Water content (*a* - g g^{-1} DW), germination (*b* - %), speed of germination-aid (*c*) and normal seedlings development (*d* - %) of *Poincianella pluviosa* seeds during 0, 4, 8, 12, 18 and 24 months of storage at -20 °C, 5 °C and 20 °C. Means sharing the same letter are not significantly different (Tukey's test, $P < 0.05$, $n = 3$). Small letters compare storage time, capital letters compare storage temperature.

Fig. 6

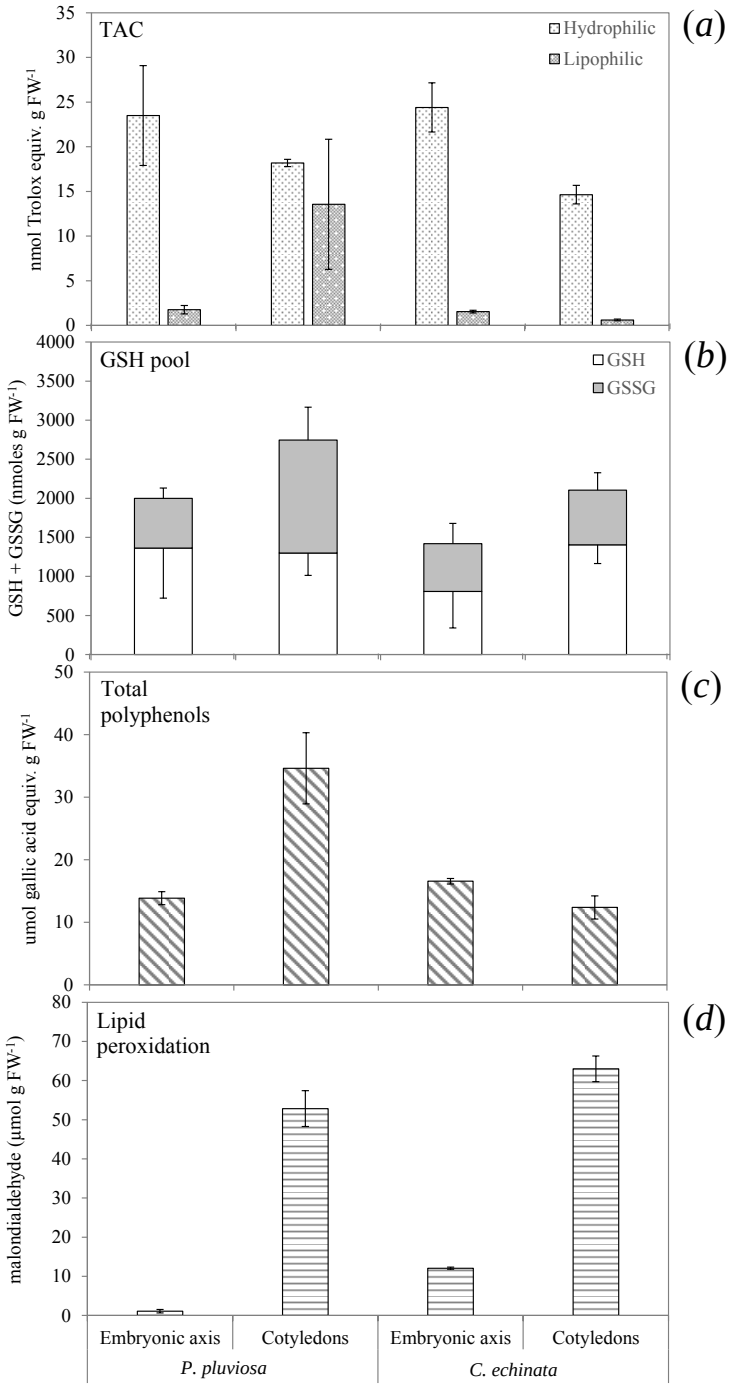

Fig 6. Total antioxidant capability (TAC) of hydrophilic and lipophilic extracts (*a*); total glutathione pool (GSH, glutathione reduced and GSSG, glutathione oxidized - *b*); total soluble polyphenols (*c*), and lipids peroxidation in terms of malondialdehyde contents (*d*) in embryonic axis and cotyledons of RDS of *P. pluviosa* and *C. echinata*.

7. Considerações finais

No presente trabalho ficou evidenciado que a capacidade de sementes de *Poincianella pluviosa* manterem a viabilidade no estado seco está relacionada com a redução do metabolismo energético primário e com o acúmulo de compostos osmoprotetores e antioxidantes, que ocorre ao longo da maturação. De fato, a capacidade da semente em desligar o metabolismo é um dos principais contrapontos considerados entre sementes tolerantes (ortodoxas) e intolerantes à dessecação (recalcitrantes).

Durante a maturação das sementes ortodoxas os processos de germinação, tolerância à dessecação e potencial de armazenamento prolongado são instalados nessa ordem (Pieta-Filho & Ellis, 1991; Sanhewe & Ellis, 1996). É evidente que os últimos meses de maturação das sementes de *P. pluviosa* são essenciais para um grande aumento de vigor, observado nas elevadas porcentagens de germinação e de tolerância à dessecação obtidas até o estádio da semente madura. Entretanto, a viabilidade dessas no ambiente é muito reduzida se comparada às sementes ortodoxas clássicas. Recentemente, foi sugerido que os diferentes comportamentos observados ao final da maturação das sementes seriam o resultado do quanto este processo se estendeu, influenciado pelas condições ambientais em que a planta-mãe foi submetida e pelas características de cada espécie acumuladas ao longo de seu processo evolutivo (Barbedo et al., 2013). Nesse sentido, sementes de *P. pluviosa* poderiam ter sido dispersas antes de adquirirem uma maior capacidade de armazenamento, ou seja, ainda pouco tempo antes do máximo período de maturação possível.

Essa suposição ganha força quando se compara o processo de maturação das sementes de *P. pluviosa* e de *Caesalpinia echinata*. Mesmo formando sementes tolerantes à dessecação, ambas possuem processos de maturação diferenciados (Figura 1). Apesar da instalação da germinação e do desenvolvimento de plântulas aparecerem proporcionalmente em estádios bastante imaturos em *P. pluviosa*, fica evidente que sementes de *C. echinata* apresentam ambos os processos ainda mais cedo, em uma curta maturação. E, ainda, indicam um investimento desta espécie em uma rápida germinação, mais do que para o armazenamento, logo após a dispersão (Figura 1). Esse resultado sugere que a menor longevidade das sementes de *C. echinata* é resultado de uma maturação ainda mais incompleta do que em sementes de *P. pluviosa*.

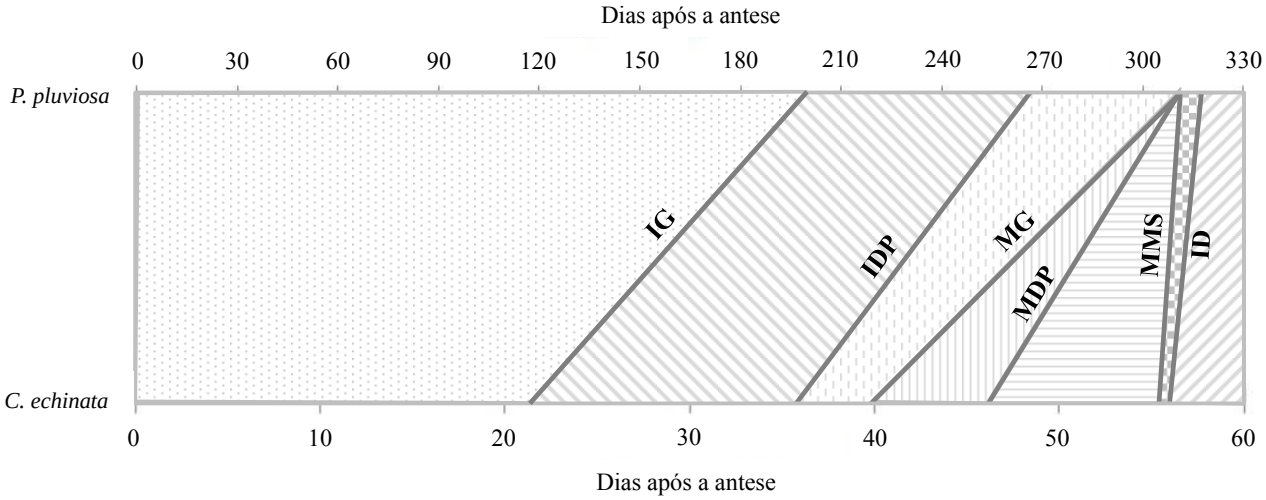

Figura 1. Esquema comparativo da maturação de sementes de *Poincianella pluviosa* e *Caesalpinia echinata* indicando os principais processos fisiológicos que ocorrem em ambas as sementes neste período a partir de informações extraídas de Borges et al. (2005) e do Capítulo 1. IG, início da germinação. IDP, início do desenvolvimento de plântulas. MG, máximo de germinação. MDP, máximo de desenvolvimento de plântulas. MMS, máximo de matéria seca. ID, início da dessecação.

A extensão da diferença de maturação entre as duas espécies poderia explicar a longevidade dessas sementes após a dispersão. Embora temperaturas de armazenamento baixas ou negativas elevem o período que ambas as sementes se mantêm viáveis, ele é sempre menor em *C. echinata*. A baixa viabilidade de *C. echinata* está relacionada a taxas respiratórias observadas nas sementes maduras, que levariam a processos oxidativos durante o armazenamento. O mesmo processo deve ocorrer nas sementes de *P. pluviosa*, mas em menor grau. Dessa maneira, uma maior redução do metabolismo energético proporcionaria maior longevidade da semente e deve estar ligada ao maior avanço do processo de maturação. O fato de que os processos fisiológicos são adquiridos ao longo da maturação, como germinação e tolerância a dessecação, em conjunto com a observação obtida através da análise do perfil metabólico de que eixos embrionários e cotilédones respondem de modo diferenciado à secagem, indicam que o processo de maturação pode ocorrer de maneira diferente nos dois tecidos. Esse resultado ajudaria a explicar os diversos comportamentos da tolerância à dessecação de sementes observados após a dispersão.

Portanto, a maior dificuldade em conservar a viabilidade de sementes se dá pela falta da compreensão do processo de maturação e do seu papel nas diferentes espécies. Em vista disso, são necessárias análises específicas sobre o metabolismo energético nos tecidos das sementes durante o processo de maturação para inferir como ele atua no processo de amadurecimento e longevidade de sementes.

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