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# Active metabolism during desiccation of the desiccation tolerant short-lived seeds of *Poincianella pluviosa* (DC.) L. P. Queiroz

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**ABSTRACT:** During embryogenesis and seed filling, developing seeds are metabolically highly active, while at the desiccation stage of tolerant seeds a decreased metabolism is expected. Seeds of *Poincianella pluviosa* present an extensive maturation cycle (11 months), longer than seed storability at room temperature (8 months). The metabolic profile of these seeds was characterized during maturation and drying, focusing in their role on seed behavior after shedding. Distinct responses of the embryonic axes and cotyledons to drying were observed depending on the developmental stage. Low proportions of tricarboxylic acids intermediaries until maturity indicated a low respiratory metabolism prior to the desiccation stage. Changes in shikimate, 4-coumarate, and quinate proportions suggest a metabolic shift towards the synthesis of chlorogenate isomers, found in high proportions in dispersed seeds. High proportions of lactate and glycerol were observed in immature seeds after artificial drying, but also in naturally dried axes of dispersed seeds. This suggests an active metabolism prior to drying and an osmotic stress under hypoxia, mechanisms that were never described before in desiccation-tolerant seeds. The results indicated that *P. pluviosa* seeds are dispersed with an incomplete metabolic *switch-off*, which can be related to their short lifespan.

Index terms: lactic acid, metabolism switch-off, primary metabolism.

**RESUMO:** Durante a embriogênese e deposição de reservas, sementes em desenvolvimento apresentam metabolismo fortemente ativo, enquanto na fase de dessecação de sementes tolerantes é esperado metabolismo reduzido. Sementes de *Poincianella pluviosa* apresentam um ciclo de maturação extenso (11 meses), maior que sua longevidade à temperatura ambiente (8 meses). O perfil metabólico dessas sementes foi caracterizado durante a maturação e secagem, enfocando seus papéis no comportamento das sementes após dispersão. Respostas distintas de eixos embrionários e cotilédones à secagem foram observadas dependendo do estádio de desenvolvimento. Baixas proporções de intermediários de ácidos tricarboxílicos até a maturidade indicaram um baixo metabolismo respiratório anterior à fase de dessecação. Mudanças nas proporções de chiquimato, 4-cumarato e quinato sugerem uma alteração metabólica em direção a síntese de isômeros clorogenatos, encontrados em altas proporções em sementes dispersas. Altas proporções de lactato e glicerol foram observadas em sementes imaturas após secagem artificial, mas também em eixos naturalmente secos de sementes dispersas. Isso sugere um metabolismo ativo anterior à dessecação e um estresse osmótico sob hipóxia, mecanismos nunca descritos antes em sementes tolerantes à dessecação. Nossos resultados indicam que sementes de *P. pluviosa* podem ser dispersas com um desligamento metabólico incompleto, que pode estar relacionado à sua baixa longevidade.

Termos de indexação: ácido lático, desligamento do metabolismo, metabolismo primário.

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## INTRODUCTION

Seeds of different plant species exhibit different metabolic behaviors at low water contents. In the case of desiccation tolerant seeds of some species, a *switch-off* in the primary metabolism seems to occur during the desiccation step, evidenced mainly by low respiratory activity (Bragante et al., 2018; Hell et al., 2019) and a decrease in the intermediaries of the tricarboxylic acid (TCA) cycle (Fait et al., 2006; Wang et al., 2016). This would avoid accumulation of oxidative processes in the mitochondria through reduced respiratory rates (Leprince et al., 1994). This mechanism was also suggested for the maintenance of desiccation tolerance (DT) in germinating seeds, in which oxidative processes generated during water loss depend greatly on the rate of respiratory metabolism prior to drying (Leprince et al., 1994). In contrast, the maturation of desiccation-sensitive (*i.e.,* recalcitrant) seeds lacks the desiccation step. Therefore, they maintain high metabolic activity during all maturation period (Bonjovani and Barbedo, 2019; 2020) and would switch directly to a germinative metabolism (Caccere et al., 2013). These seeds have a wide range of limits of tolerance to both desiccation and freezing after dispersal (Lamarca and Barbedo, 2015; Lamarca et al., 2016; Baruth et al., 2020), but frequently they need to be maintained at high levels of water content, resulting in high metabolic activity of both the seeds themselves and the microrganisms associated to them (Parisi et al., 2016; Barbedo, 2018; Parisi et al., 2019; Lamarca et al., 2020; Lamarca and Barbedo, 2021), which is a challenge for *ex situ* conservation (Berjak and Pammenter, 2014; Barbedo et al., 2013; Gasparin et al., 2020; Amorim et al., 2021; Srivastava et al., 2022).

Tropical forests are characterized by enormous genetic diversity and preservation of these resources is of major concern. The *ex situ* conservation of plant species through the storage of seeds in germplasm banks is an important strategy to accomplish this purpose (Liu et al., 2018; Walters and Pence, 2020; Barba-Espin and Acosta-Motos, 2022). *Poincianella pluviosa* (DC.) L. P. Queiroz (=*Cenostigma pluviosum* (DC.) Gagnon & G. P. Lewis) is a leguminous tree species widely distributed in almost all Brazilian phytogeographic domains, such as the Amazon, Caatinga, Cerrado, Atlantic forest and Pantanal (Lewis, 2015). This species has an extended period of seed maturation, being dispersed approximately 330 days after anthesis (DAA) (Silva et al., 2015). Seeds of *P. pluviosa* are tolerant to desiccation, keeping their viability up to 0.08 $g.g^{-1}$ of water (Silva et al., 2015), which would characterize them as orthodox seeds (Hong and Ellis, 1996). However, these seeds exhibit a short to medium storability at temperatures close to those found in nature, losing viability after 240 days at 20-25 °C at 0.09 $g.g^{-1}$ of water (Figliolia et al., 2001), and even at temperatures of 5-8 °C at dry state (0.11 $g.g^{-1}$ of water), storability can only be extended to 360 days (Pontes et al., 2006).

Improved storability of DT seeds is usually achieved by preservation at low temperatures in *ex situ* conservation banks, which indicate the urge of a better understading of the metabolic changes that occur during maturation and their consequences on seed longevity after shedding. For this purpose, this research aimed to characterize the physiological traits of *P. pluviosa* seeds during complete maturation and after artificial drying, by analyzing the metabolic profile, triacylglycerols, fatty acids membranes and soluble carbohydrates to correlate their role on seed behavior after shedding and to provide evidence of an active metabolism during the desiccation step.

## MATERIALS AND METHODS

The experiments were carried out with 35 trees of *Poincianella pluviosa* located in Rubião Jr, a *campus* of *Universidade Estadual Paulista* (UNESP), Botucatu, São Paulo state, Brazil (22°52’20’’S 48°26’37’’W). Inflorescences were tagged at the beginning of their anthesis, and pods were periodically harvested directly from the branches until natural shedding. Seven stages described in Silva et al. (2015) were selected, which represent the main phases of *P. pluviosa* seeds maturation. This also includes recently dispersed seeds (RDS), which were obtained directly from the ground and not exceeding 48 h after shedding.

Water content (g of water per g of dry weight, $g.g^{-1}$) were determined at each stage of seed maturation by using three replicates of five seeds, after oven drying (103 °C, 17 h) according to Brasil (2009). Additionally, the water potential

was measured with a Decagon WP4 potentiometer (Decagon Devices, Pullman, USA) based on the dew point of the air temperature in equilibrium with the sample (Silva et al., 2015). The germination analyzes that consisted in three replications of 15 seeds were carried out on rolls of Germitest paper previously moistened with tap water (using two sheets for the base and one for the covering - Brasil (2009), in germination chambers at 25 ± 1 °C. The percentage of germinated seeds (protrusion of at least 5 mm of the primary root) and of seedling with normal development (seedlings with at least 3 cm and no visual abnormal characteristics) were assessed every 2 days for 40 days. At the end of the analysis the speed of germination-aid was calculated according to Santana and Ranal (2004).

Developing seeds from the last five stages of maturation (S9 to S13) were dried directly in an oven with air circulation (40 °C) until they reach 0.21; 0.18; 0.14; 0.11 and 0.08 $g.g^{-1}$ (equivalent to 18, 15, 12, 10 and 7% on wet basis, respectively). Thereafter, they were subjected to the physical and physiological analyses as described. Those seeds that after rehydration exhibited the protrusion of the primary root of at least 5 mm were considered desiccation tolerant. To determine changes in metabolism, seeds from stages 10 and 12 were selected for biochemical analysis due to the higher contrast of DT behavior. Fresh seeds (initial) and seeds from both stages after drying to 0.08 $g.g^{-1}$ were dissected as described above.

For biochemical analyzes, embryonic axes and cotyledons from each stage were dissected, ground under liquid nitrogen with a mortar and stored at -80 °C until used.

The metabolic profile was analyzed using three to six replicates of samples (80-100 mg) which were extracted in a methanol:chloroform:water solution (12:5:1 - v/v) with adonitol (0.2 $mg.mL^{-1}$) added as internal standard for relative quantification. The mixture was stirred, incubated in a dry bath (60 °C, 30 min) and centrifuged (10 000 *g*, 2 min). The supernatant was transferred to another vial with the addition of the same volume of water and incubated at room temperature for 5 min. After incubation, centrifugation was repeated and 300 µL of the supernatant was separated and completely dried under vaccuum. The residue was re-dissolved in 150 µL of pyridine and derivatized with 50 µL of methoxyamine hydrochloride (20 $mg.mL^{-1}$) dissolved in pyridine and 50 µl of BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide), in a dry bath (80 °C, 60 min), according to Caccere et al. (2013). Finally, samples were injected in a gas chromatography-mass spectrometry (GC–MS) system (Agilent GC 6890 and MSD 5973N series, Agilent Technologies, USA). GC was performed using a HP-1701 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 analyzis was performed with the following program: 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 heating at 310 °C for 1 min. Mass spectra were recorded at 2 $scan.s^{-1}$ with a scan range of 50-600 m/z. Chromatogram and mass spectral data were analyzed using the Chemstation program (Agilent Technologies, USA). Peaks were identified and compared with authentic standards, and to databases of the National Institute of Standards and Technology Mass Spectral Library - NIST and the Golm Metabolome Database - GMD (Hummel et al., 2010). Metabolic data is showed here as relative metabolite content.

Lipid content and fatty acid composition were determined by triplicates. Samples (10-30 mg) from both tissues were freeze-dried and weighed, before extraction with hexane as a solvent in a soxhlet apparatus for 4 h. To determine the fatty acid composition of triacylglycerols and membrane lipids of axes and cotyledons, the neutral and polar lipid fractions of both tissues 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 twice with chloroform to elute the neutral lipid fraction and then washed twice with methanol in order to elute the polar lipids (Crane et al., 2003). After completely dried, fatty acids from each fraction were saponified and methyl-esterified, and the extract containing the fatty acid methyl esters was analyzed in a gas chromatography with flame ionization detector (GC-FID) system (HP 5809 series, Hewlett Packard, USA), according to Mayworm et al. (1998). Chromatogram data were evaluated with Chemstation (Hewlett Packard, USA). Fatty acids were identified and compared with authentic standards, and the percentage of fatty acid constituents were obtained by the display unit of the instrument.

Soluble carbohydrates contents were quantified by triplicates samples (14-60 mg) from both embryonic axes and cotyledons which were extracted in boiling ethanol (80%, 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 using the same procedure. The resulting ethanolic supernatants were combined and considered as the soluble sugar extracts. After complete drying under vaccuum, samples were re-suspended in deionized water, and analyzed by high-performance anion exchange chromatography coupled to pulsed amperometric detector (HPAEC/PAD), according to Mello et al. (2010).

The experiments were carried out following a completely randomized design. Seed physiological data were analyzed using ANOVA and means compared by Tukey´s test ($p < 0.05$) (Santana and Ranal, 2004). Principal component analyzis (PCA) was performed with data sets of metabolic profiles using PAST v. 3.01 (Hammer et al., 2001). Pearson´s correlation test was applied to identify compounds with strong correlations (> 0.9) between each identified compound and exclude from the PCA, to avoid missing other significant interactions in the data analyzes.

## RESULTS AND DISCUSSION

Drying immature seeds from S10 resulted in progressive decrease in germination from 72% at harvest to 30% at 0.08 $g.g^{-1}$ (Figure 1). In contrast, drying seeds from S12 did not exhibit a decrease in percentage of germination or normal seedling production after all drying levels (Figure 1). In addition, speed of germination of dried seeds presented threefold values at S12 compared to S10. Although the acquisition of DT has been progressive from S9 to S13, tolerance to low water content (0.08 $g.g^{-1}$ water) began to increase only after S10 (Figure 1). Therefore, for comparison, seeds at S10 were herein considered as more sensitive to desiccation as seeds from S12.

PCA (Figure 2) obtained from the metabolic profile during seed maturation (Figures 3 and 4) showed general metabolic changes in embryonic axes and cotyledons during maturation (Figures 2A and B). Both tissues presented similar developmental trajectories, characterized by a metabolic distinction along component 1 of immature stages (S3-S4), followed by S7, which correlates with the beggining of dry mass accumulation (Silva et al., 2015), from other stages (Figures 2A and B). In embryonic axes, intermediaries of the TCA cycle and shikimate (including chlorogenate isomers) contributed most to this separation, as well as sucrose and trehalose (Figure 2C). In cotyledons, similar changes were observed but less pronounced (Figure 2D). After S7, the metabolic events of both tissues were mostly explained by component 2, although scattered in opposite direction, from negative to positive in embryonic axes and from positive to negative in cotyledons (Figures 2A and B). During this period, the metabolism of axes was strongly affected by changes in ascorbate and xylose concentrations, opposed to raffinose, lactate and glycerol (Figure 2C), whereas in cotyledons, it was mainly represented by changes in shikimate and xylose opposed to chlorogenate isomers levels (Figure 2D).

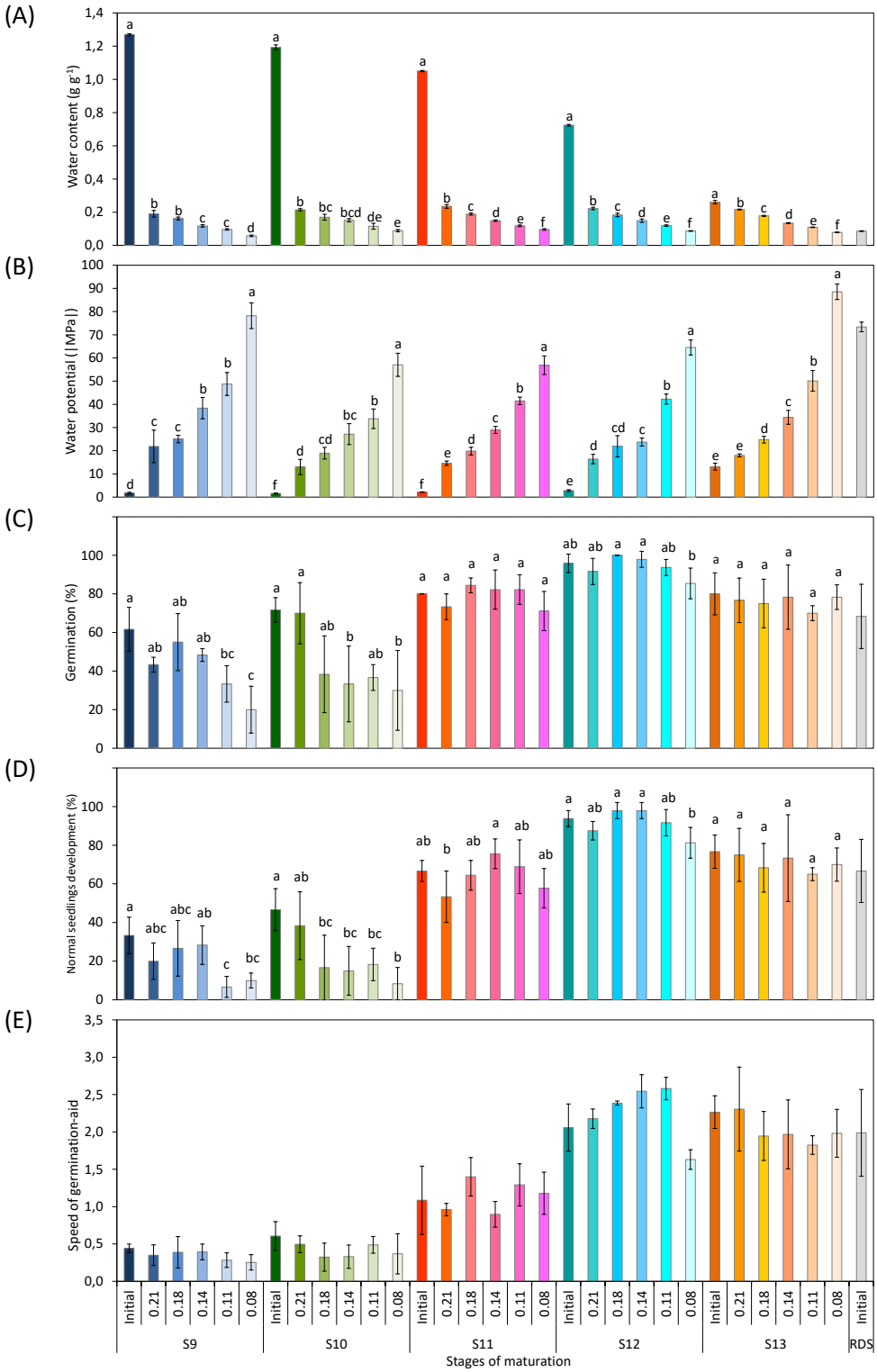


Figure 1. Seeds of *Poincianella pluviosa* during later maturation (between 286 to 325 DAA) classified into stages according Silva et al. (2015), before (Initial) and after artificial drying until 0.08 $g.g^{-1}$ water. (A) Water content ($g.g^{-1}$), (B) water potential (|MPa|), (C) Germination (%), (D) normal seedlings development (%) and (E) speed of germination-aid. 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.

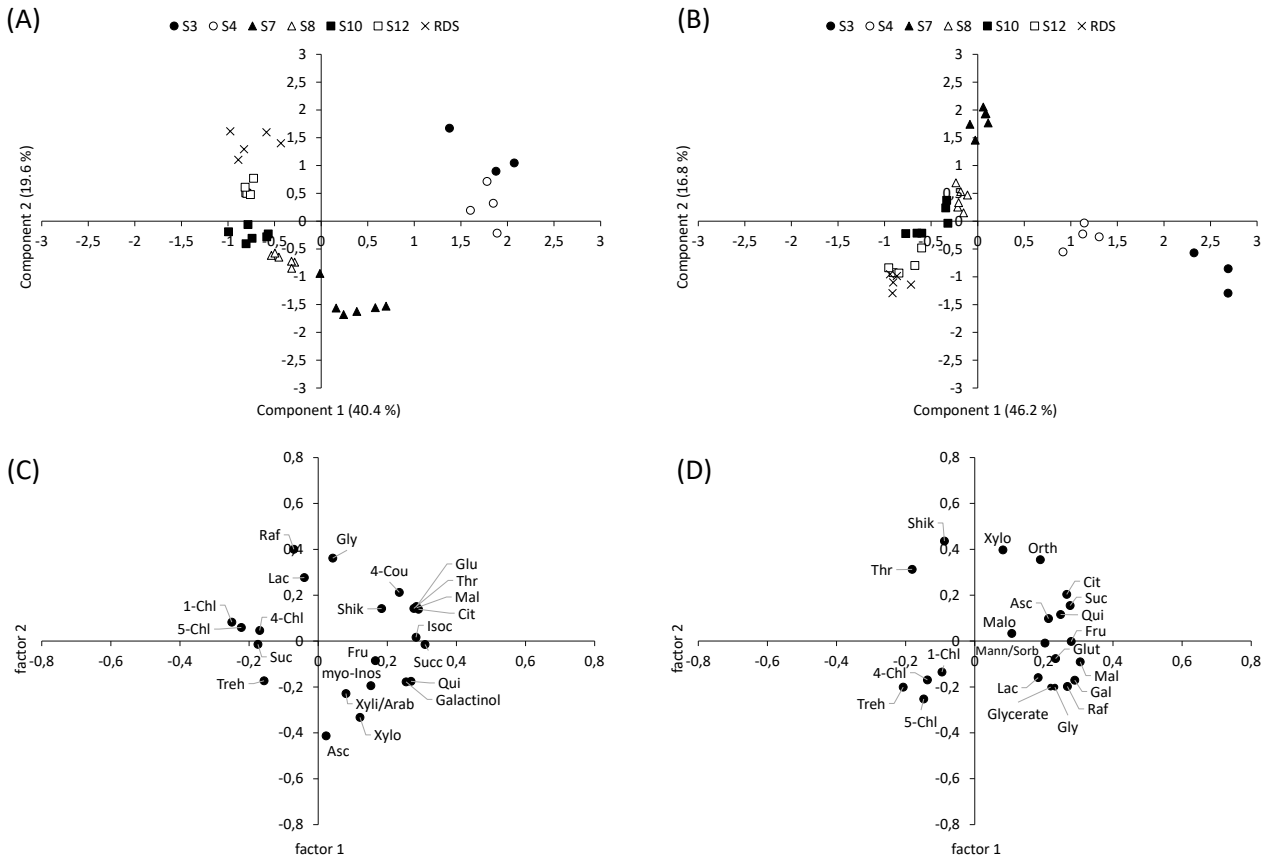


Figure 2. Principal component analysis (PCA) of metabolite profiles of embryonic axis (A) and cotyledons (B) and the respective loadings (C and D) during maturation of *Poincianella pluviosa* seeds (stages described in Silva et al., 2015). PCA is presented as the combinations of the first two dimensions. Each data point represents an independent biological sample.

PCA (Figure 5) obtained from the metabolic profile of desiccation sensitive (S10) and tolerant (S12) seeds (Figure 6), indicated that distinct metabolic changes occur if considered the stage of development and/or the tissue (Figures 5A and B). The metabolite distribution of embryonic axes along component 1 distinguished the maturation stage, mainly influenced by changes of galactinol, ononitol, xylose, 4-coumaric and intermediaries of the TCA, while dried axes from both stages were separated from wet counterparts along component 2, mostly by glycerol and lactate changes (Figures 5A and C). In cotyledons, the chlorogenate pathway (shikimate, 4-coumarate, 1- and 4-chlorogenate) along with galactinol, threitol, threonate, malonate and trehalose, contributed most to separation of maturation stages. Whereas dried cotyledons from both stages were clearly apart, mainly by changes of lactate, glycerol, and succinate (Figures 5B and D), similar as obtained from dried axes.

A wide range of fatty acids with C9 to C24 carbon chains, which comprised palmitic (C16:0), stearic (C18:0) and linoleic (C18:2) acids, characterized the neutral (*i.e.* triacylglycerol) and polar lipid fractions (*i.e.* membrane constituents) of *P. pluviosa* seeds (Table 1). However, in the case of embryonic axes of S10-dried seeds, linoleic acid was not detected as part of the neutral lipid fraction. Moreover, a very low unsaturated ratio was observed in the embryonic axes due to high levels of palmitic acid, while in the cotyledons no changes were observed (Table 1). In contrast, a small increase in the neutral lipid unsaturated ratio was observed in embryonic axes of S12-dried seeds, possibly due to the reduction in the proportion of palmitic, stearic, and oleic acids, together with an increase in linolenic acid (C18:3). Regarding the polar lipid fraction, a great decrease in the levels of linoleic acids simultaneous to an increase in palmitic acid in the embryonic axes of S10-dried seeds, resulted in a low unsaturated ratio, similar to that observed in the seeds at S12 (Table 1). On the other hand, no major changes were observed in the cotyledons of S10-dried seeds, as well in both tissues of S12-dried seeds.

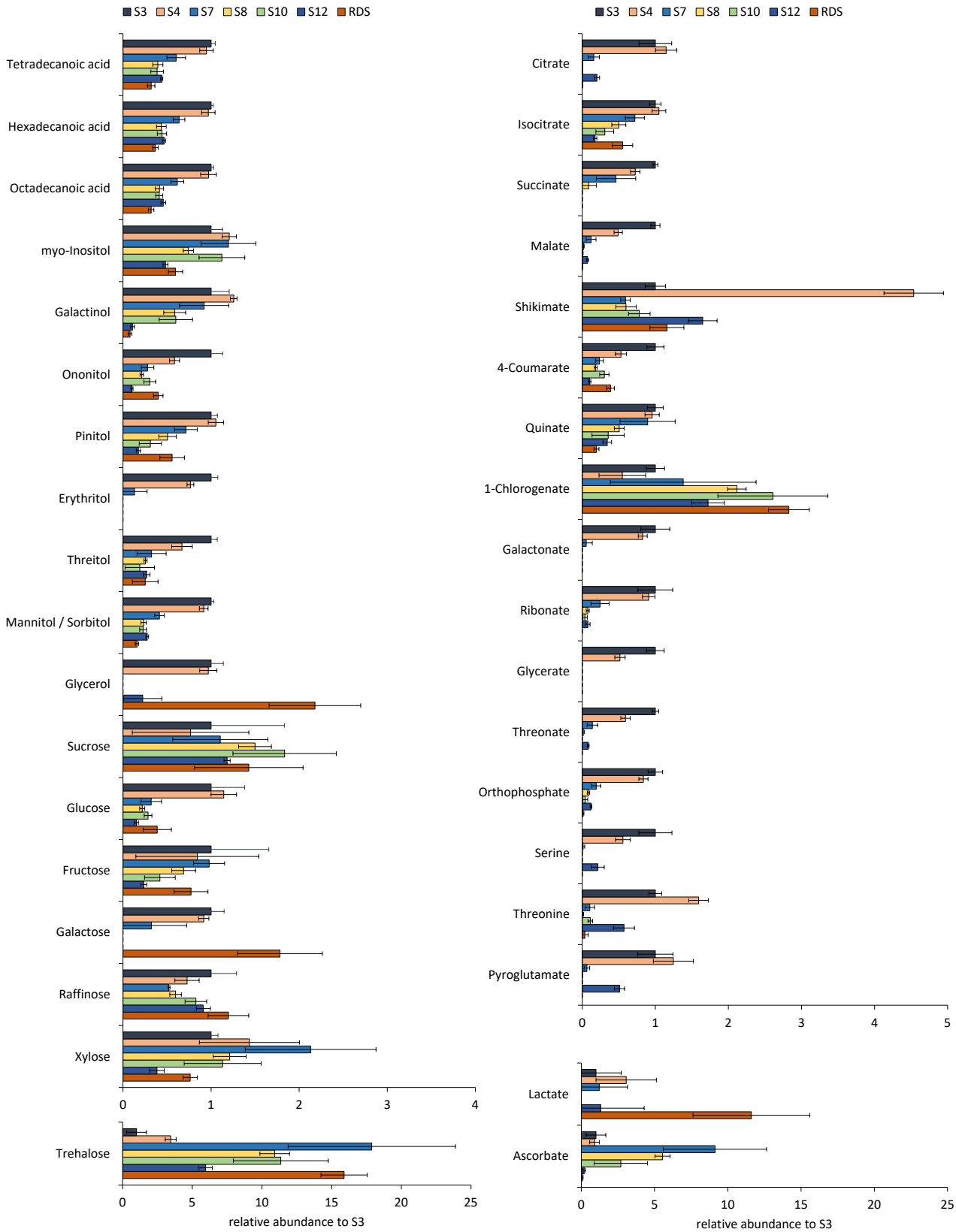


Figure 3. Metabolic profile of embryonic axis of *Poincianella pluviosa* seeds during maturation (stages described in Silva et al., 2015). Compounds were detected by GC/MS. All values were normalized from those found in S3. Values shown are means ± SD, with 3 or 6 replicates, see Material and Methods for details.

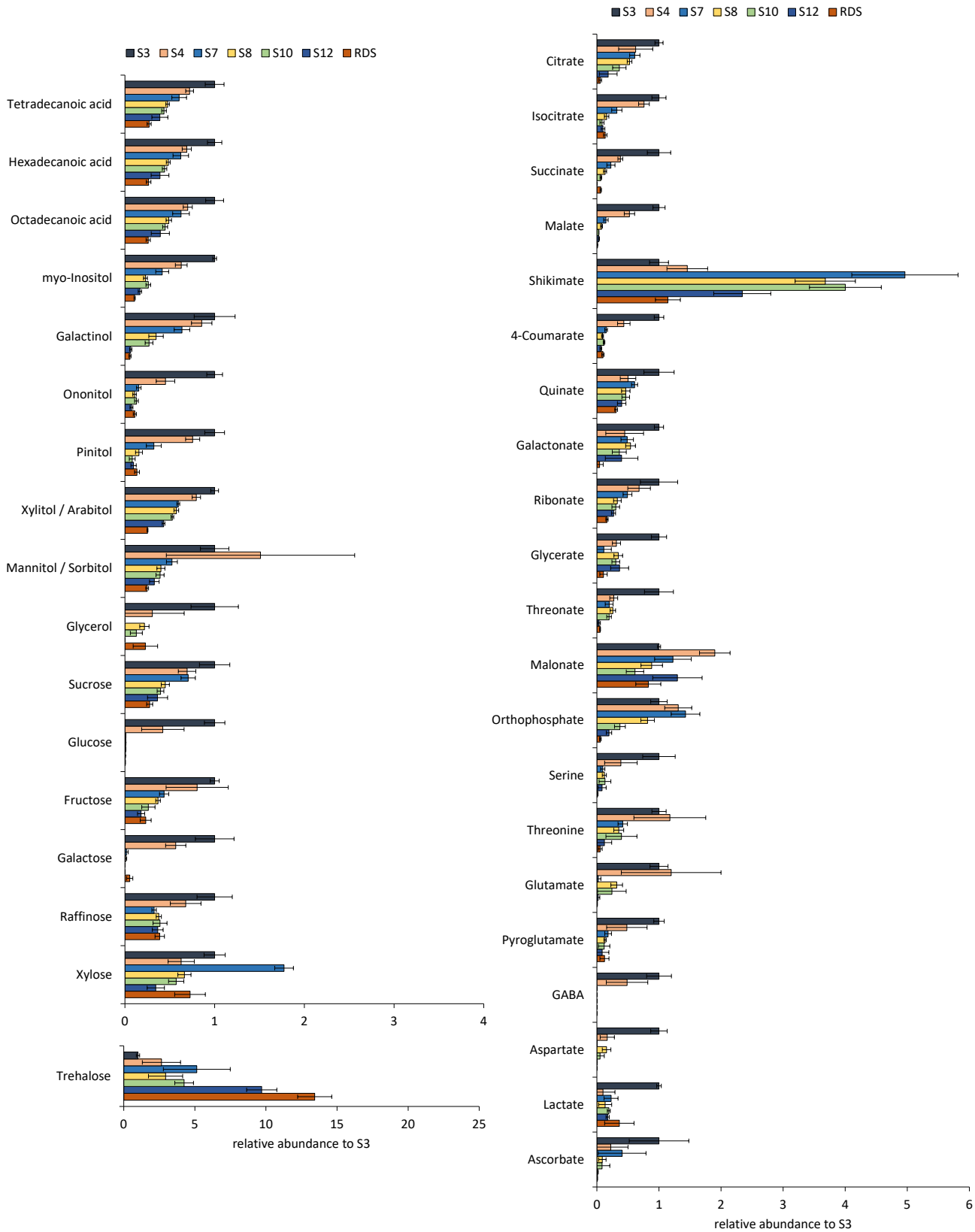


Figure 4. Metabolic profile of cotyledons of *Poincianella pluviosa* seeds during maturation (stages described in Silva et al., 2015). Compounds were detected by GC/MS. All values were normalized from those found in S3. Values shown are means ± SD, with 3 or 6 replicates, see Material and Methods for details.

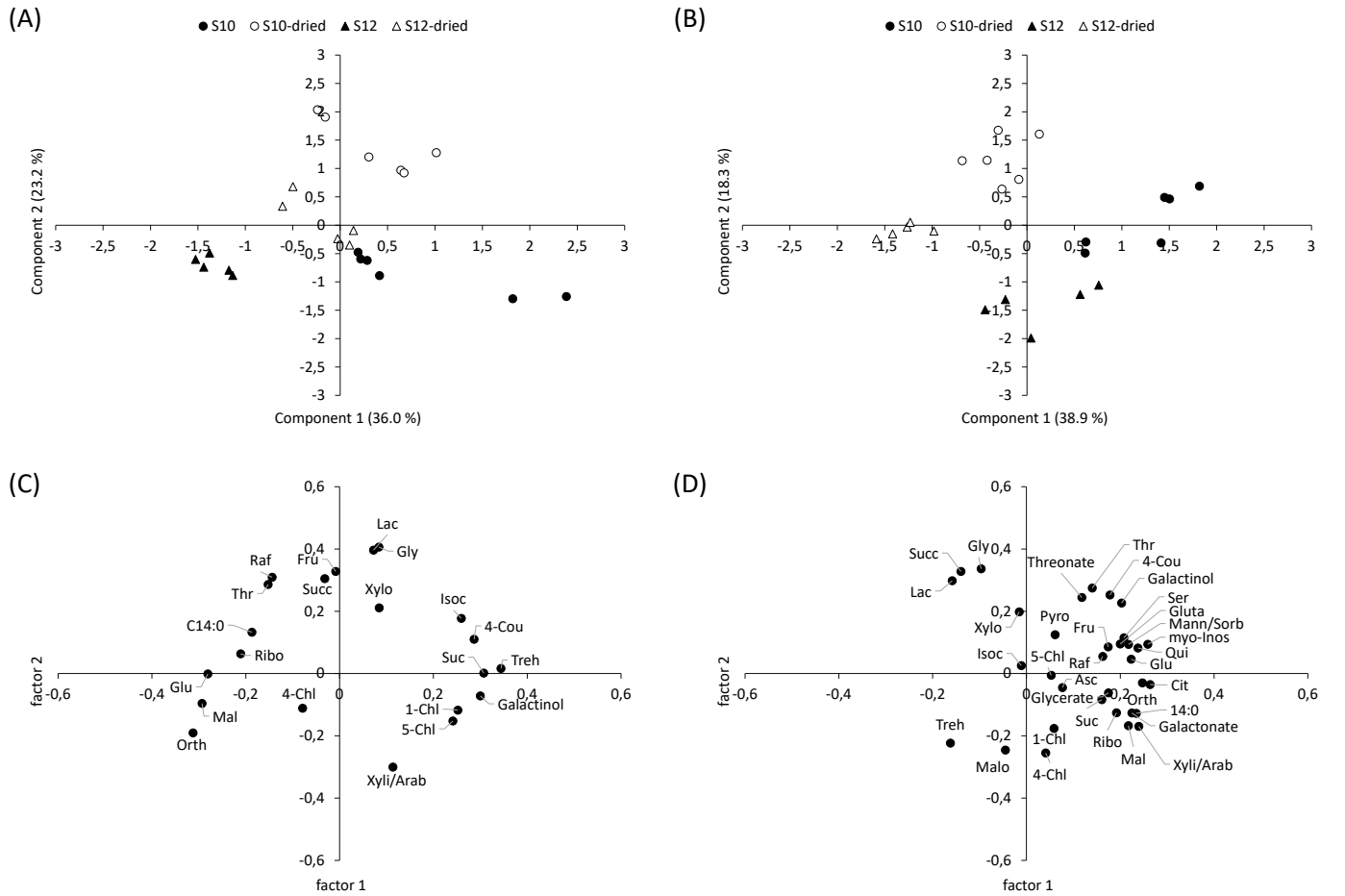


Figure 5. Principal component analysis (PCA) of metabolite profiles of embryonic axis (A) and cotyledons (B) and their respective loadings (C and D) of *Poincianella pluviosa* sensible (S10) and DT (S12) seeds and after drying until 0.08 $g.g^{-1}$ water. PCA is presented as the combinations of the first two dimensions. Each data point represents an independent biological sample.

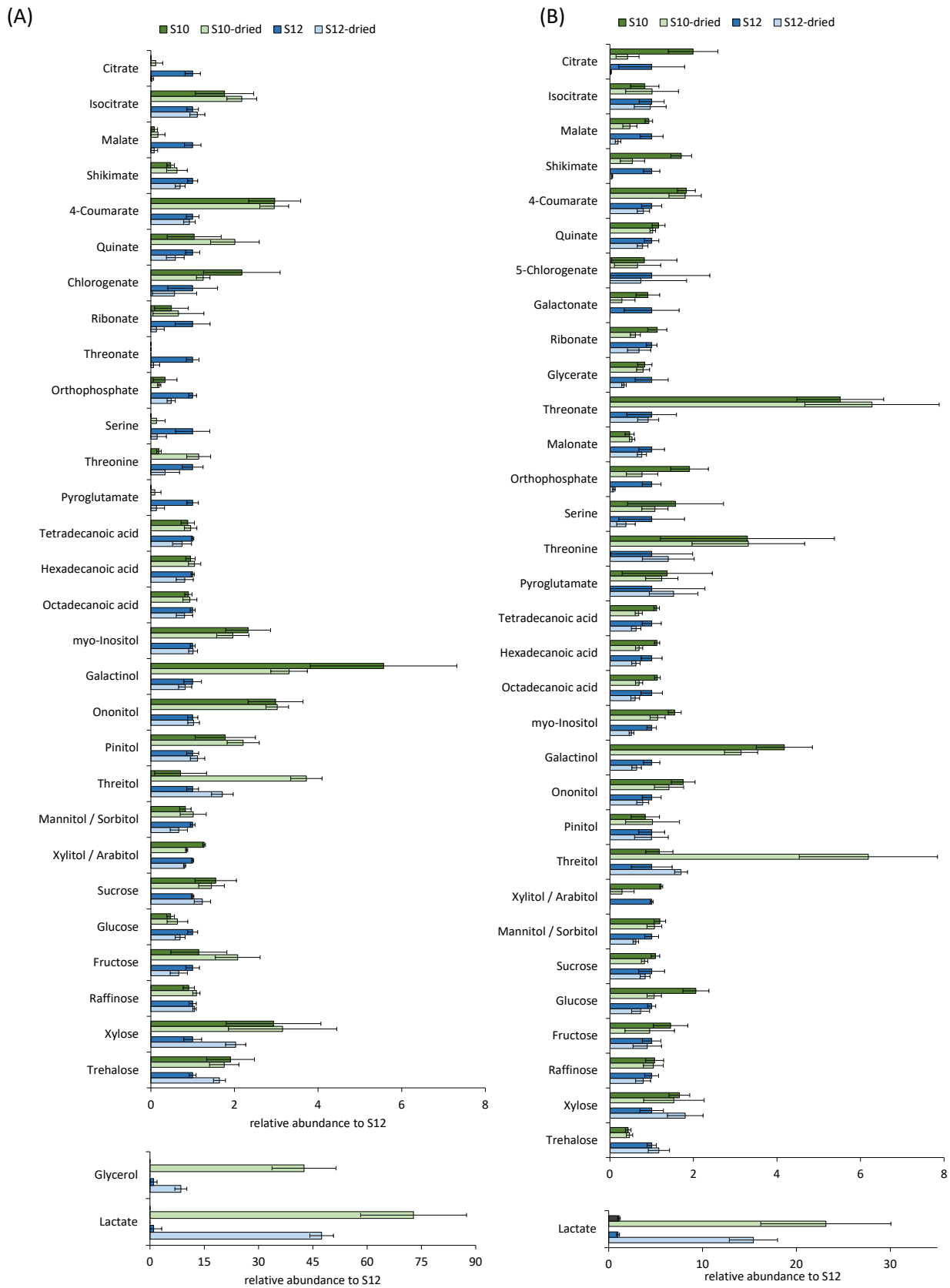


Figure 6. Metabolic profile of embryonic axis (A) and cotyledons (B) of *Poincianella pluviosa sensible* (S10) and DT tolerant (S12) seeds and after drying to 0.08 $g.g^{-1}$ water. Compounds were detected by GC/MS. All values were normalized from those found in S12.

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

| Stage | C9 | C14 | C16 | C18 | C18:1 | C18:2 | C18:3 | C20 | C22 | C24 | Total Unsat | Total Sat | U:S Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Embryonic axis* - neutral lipid fraction | | | | | | | | | | | | | |
| S10 | | | 33.1 ± 12.1 | 13.2 ± 5.5 | 9.6 ± 2.9 | 38.0 ± 16.1 | | 4.2 ± 1.6 | 3.4 ± 1.1 | | 47.6 | 53.9 | 0.88 |
| S10-dried | | 10.2 ± 4.1 | 51.0 ± 1.6 | 15.9 ± 0.9 | 7.3 ± 0.8 | nd | | 5.8 ± 0.1 | 4.5 ± 0.3 | | 7.3 | 87.4 | 0.08 |
| S12 | | 5.6 ± 1.2 | 38.7 ± 1.9 | 19.9 ± 3.8 | 13.8 ± 5.3 | 10.1 ± 0.7 | 3.4 ± 1.1 | 3.7 ± 0.5 | | | 27.3 | 67.9 | 0.40 |
| S12-dried | | 9.1 ± 2.4 | 33.0 ± 1.9 | 15.2 ± 1.5 | 8.3 ± 1.6 | 9.7 ± 1.3 | 20.6 ± 4.8 | | | | 38.6 | 57.3 | 0.67 |
| *Cotyledons* - neutral lipid fraction | | | | | | | | | | | | | |
| S10 | | | 15.5 ± 0.9 | 12.8 ± 1.2 | 8.2 ± 0.1 | 61.2 ± 0.7 | | 1.8 ± 0.1 | 0.6 ± 0.03 | | 69.4 | 30.7 | 2.26 |
| S10-dried | | | 15.8 ± 0.4 | 12.2 ± 0.9 | 7.9 ± 0.4 | 61.9 ± 1.6 | | 1.6 ± 0.1 | 0.6 ± 0.04 | | 69.8 | 30.2 | 2.31 |
| S12 | | | 21.6 ± 6.8 | 26.2 ± 5.6 | 11.8 ± 4.2 | 37.3 ± 11.7 | | 3.2 ± 1.4 | | | 49.1 | 51.0 | 0.96 |
| S12-dried | | | 26.3 ± 10.6 | 30.2 ± 6.6 | 12.2 ± 4.5 | 26.2 ± 15.4 | 1.7 ± 2.0 | 3.4 ± 1.5 | 0.9 ± 0.5 | | 40.1 | 60.8 | 0.66 |
| *Embryonic axis* - polar lipid fraction | | | | | | | | | | | | | |
| S10 | 4.4 ± 4.2 | | 39.7 ± 9.7 | 17.7 ± 1.5 | 9.6 ± 2.5 | 22.4 ± 9.2 | | 5.1 ± 1.2 | 4.3 ± 0.9 | 2.1 ± 0.4 | 32.0 | 73.3 | 0.44 |
| S10-dried | 6.8 ± 2.7 | | 53.1 ± 1.0 | 17.8 ± 2.1 | 5.4 ± 0.5 | 2.3 ± 1.5 | | 6.2 ± 0.7 | 5.3 ± 0.4 | 2.5 ± 0.1 | 7.7 | 91.7 | 0.08 |
| S12 | 8.4 ± 3.3 | 1.0 ± 0.1 | 46.0 ± 1.8 | 19.2 ± 2.7 | 8.6 ± 0.8 | 2.3 ± 0.8 | | 6.0 ± 1.0 | 4.5 ± 0.8 | 2.6 ± 0.5 | 10.9 | 87.7 | 0.12 |
| S12-dried | 8.4 ± 0.5 | 1.0 ± 0.04 | 43.8 ± 3.4 | 21.7 ± 4.0 | 7.5 ± 1.0 | 6.2 ± 4.8 | | 6.8 ± 1.2 | 5.1 ± 1.1 | 3.1 ± 0.6 | 13.7 | 89.9 | 0.15 |
| *Cotyledons* - polar lipid fraction | | | | | | | | | | | | | |
| S10 | nd | | 22.2 ± 1.2 | 18.1 ± 3.1 | 8.7 ± 1.2 | 47.6 ± 8.0 | | 2.2 ± 0.4 | | | 56.3 | 42.5 | 1.32 |
| S10-dried | nd | | 20.8 ± 1.8 | 16.5 ± 1.9 | 8.5 ± 1.6 | 52.9 ± 1.6 | | 1.9 ± 0.1 | | | 61.4 | 39.2 | 1.57 |
| S12 | 4.6 ± 2.9 | | 31.5 ± 2.1 | 35.9 ± 6.5 | 12.3 ± 2.5 | 11.7 ± 9.2 | | 4.0 ± 0.8 | | | 24.0 | 76.0 | 0.32 |
| S12-dried | 10.8 ± 3.7 | 1.0 ± 0.3 | 28.1 ± 8.8 | 31.1 ± 5.3 | 7.8 ± 0.8 | 19.3 ± 14.4 | | 3.4 ± 1.1 | 1.1 ± 0.1 | | 27.1 | 75.5 | 0.36 |

Figure 7 summarizes the results obtained in the metabolic profile of embryonic axes and cotyledons (Figures 3 and 4), in order to highlight the proposed metabolic pathways changes during *P. pluviosa* seed maturation and localized at the subcellular level. Also, two main moments of metabolic changes are indicated, a transition between S4 to S12 (Figures 7A and C) and other between S12 and RDS (Figures 7B and D).

Results showed a clear decrease in the proportions of TCA intermediaries in embryonic axes and cotyledons during maturation (Figures 3, 4), mainly after S4 (Figure 2). Moreover, changes of shikimate, 4-coumarate and quinate (Figures 3 and 4), as well as increased levels of chlorogenate isomers until the end of maturation (Figure 2), imply that the secondary metabolism was activated at the same period, suggesting that these isomers, which are derivatives of hydroxycinnamic acid, are accumulated through the shikimate pathway (Figures 7A and C). Together with the increased values of dry mass obtained from S7 seeds (Silva et al., 2015), the diversion from primary to secondary metabolism could denote a metabolic switch correlated with the start of seed filling. This phase also represents the onset of DT mechanisms, as observed in *P. pluviosa* (Figure 1) and a variety of DT seeds (Leprince et al., 2017, and references therein).

The ability to control the decrease of metabolism during drying is often described as an important characteristic of DT, which leads in seeds to a mature stage at quiescent state (Fait et al., 2006; Caccere et al., 2013; Leprince et al., 2017). A decrease in the proportions of TCA cycle intermediaries, associated with low respiratory rates, occurs at the desiccation phase of *Arabidopsis* seeds (Fait et al., 2006) or during seed filling of the sacred lotus *N. nucifera*, one of the longest-lived seeds (Wang et al., 2016). These results were also related to those obtained for *Paubrasilia echinata* seeds, in which a decrease in the respiratory rates measured by gas exchanges was observed during maturation (Araújo and Barbedo, 2017) which would, in turn, reduce seed susceptibility to oxidative processes during desiccation (Leprince et al., 1994). Interestingly, the chlorogenate isomers accumulated in both tissues during *P. pluviosa* seed maturation are well-known antioxidants (Amakura et al., 2013) and could minimize the effects of oxidative radicals in the dry state.

Mature seeds of *P. pluviosa* presented elevated DT during the late maturation stages, as evidenced by higher germination percentages in S11, S12 and S13 seeds after artificial drying than in seeds at S9 and S10 (Figure 1). Therefore, any potential drying damage during seed harvest seems not to be related to the short storability reported for these seeds (Figliolia et al., 2001). Moreover, a decrease in the unsaturated fatty acids ratio in both axes and cotyledons was observed between S10 and S12 (Table 1), which could be relevant since unsaturated fatty acids are primary targets of reactive oxygen species (ROS), commonly produced in several plant tissues during dehydration (Smirnoff, 1993).

However, immature seeds at S10 presented a marked decrease in the unsaturation rate of both triglycerides and membrane fractions of the embryonic axes (Table 1), together with high levels of glycerol (43 fold - Figure 6A) and succinate in both dried tissues (Figures 5C and D), which are potential products of fatty acid β-oxidation (Fait et al., 2006, Figure 8). Fatty acids degradation occurs naturally during the desiccation stage of *Brassica napus* embryos (approximately 15%), and in *Arabidopsis thaliana*, providing the energy needed for metabolic activity (Chia et al., 2005) or rapidly available amino acids to support metabolic recovery during imbibition (Fait et al., 2006). However, the metabolite data presented here do not support these observations, and only a small decrease of total lipids (~7%) was obtained in the cotyledons of S12-dried seeds, and even lower proportions were detected at S10.

Another component of the complex mechanism of DT in seeds involves sugars. The amount of sucrose decreased in S10-dried seeds (Figure 8A), while raffinose and stachyose were less than 0.6% (Figures 8C and D). The drying treatment of seeds at S12 neither led to significant changes in the content of starch and total soluble sugars nor of any other identified sugars (Figure 6). Instead of having a protective function, raffinose and stachyose might be considered ready sources of carbon for seedling establishment (Leduc et al., 2012). In fact, higher normal seedlings percentages are achieved between S10 and S12 (Silva et al., 2015), concomitantly with an increase in stachyose content (Figure 8C). The relatively high amounts of polyols found in *P. pluviosa* (Figure 7B) could play functions similar to those proposed for RFOs in the stabilization of macromolecules, as suggested earlier (Peterbauer and Richter, 2001; Leduc et al., 2012), and their synthesis can be induced when submitted to desiccation (Hell et al., 2021).

In contrast, an increase in the proportion of trehalose was observed in the axes of DT S12-dried seeds, reaching

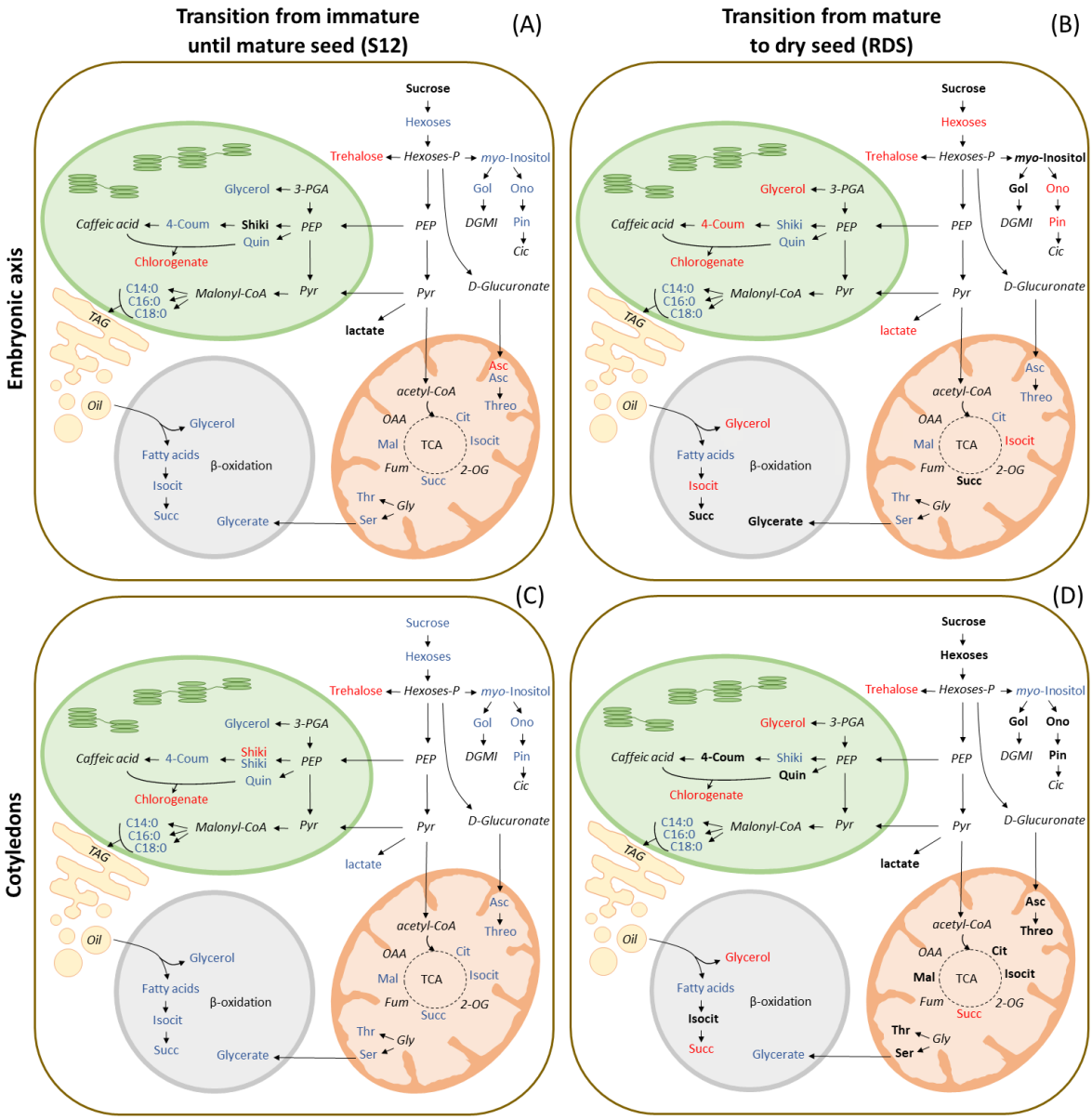


Figure 7. Schematic illustration of metabolites changes during seed maturation of *Poincianella pluviosa*. The metabolic pathways highlighted here were proposed according to metabolic changes observed and localized in the subcellular organelles. A and B represent metabolic changes in embryonic axes during seed maturation (data derived from Fig. 2). C and D represent metabolites changes in cotyledons during the same period (data derived from Fig. 3). A and C, indicate the transition from immature to mature seeds (from S3 to S12); B and D, indicate the transition from mature seeds to RDS (from S12 to RDS). Red and blue letters indicate metabolites whose levels either increased or decreased, respectively. Metabolites whose levels remained unchanged are indicated in bold. Metabolites whose levels were not detected are indicated in italics. Different organelles are highlighted in green (plastids), orange (mitochondria), grey (glyoxysomes), and gold (endoplasmic reticulum). Arrows represent one or multiple enzymatic steps. (3-PGA) 3-phosphoglyceric acid; (PEP) phosphoenolpyruvate; (Pyr) pyruvate; (Gol) galactinol; (Ono) ononitol; (DGMI) di-galactosyl myo-inositol; (Pin) pinitol; (Cic) ciceritol; (Asc) ascorbate; (Threo) threonate; (Gly) glycine; (Thr) threonine; (Ser) serine; (TCA) tricarboxylic acid; (Cit) citrate; (Isocit) isocitrate; (2-OG) 2-oxoglutarate; (Succ) succinate; (Fum) fumarate; (Mal) malate; (OAA) oxaloacetate; (Shiki) shikimate; (Quin) quinate; (4-Coum) 4-coumarate; (TAG) triacylglycerols.

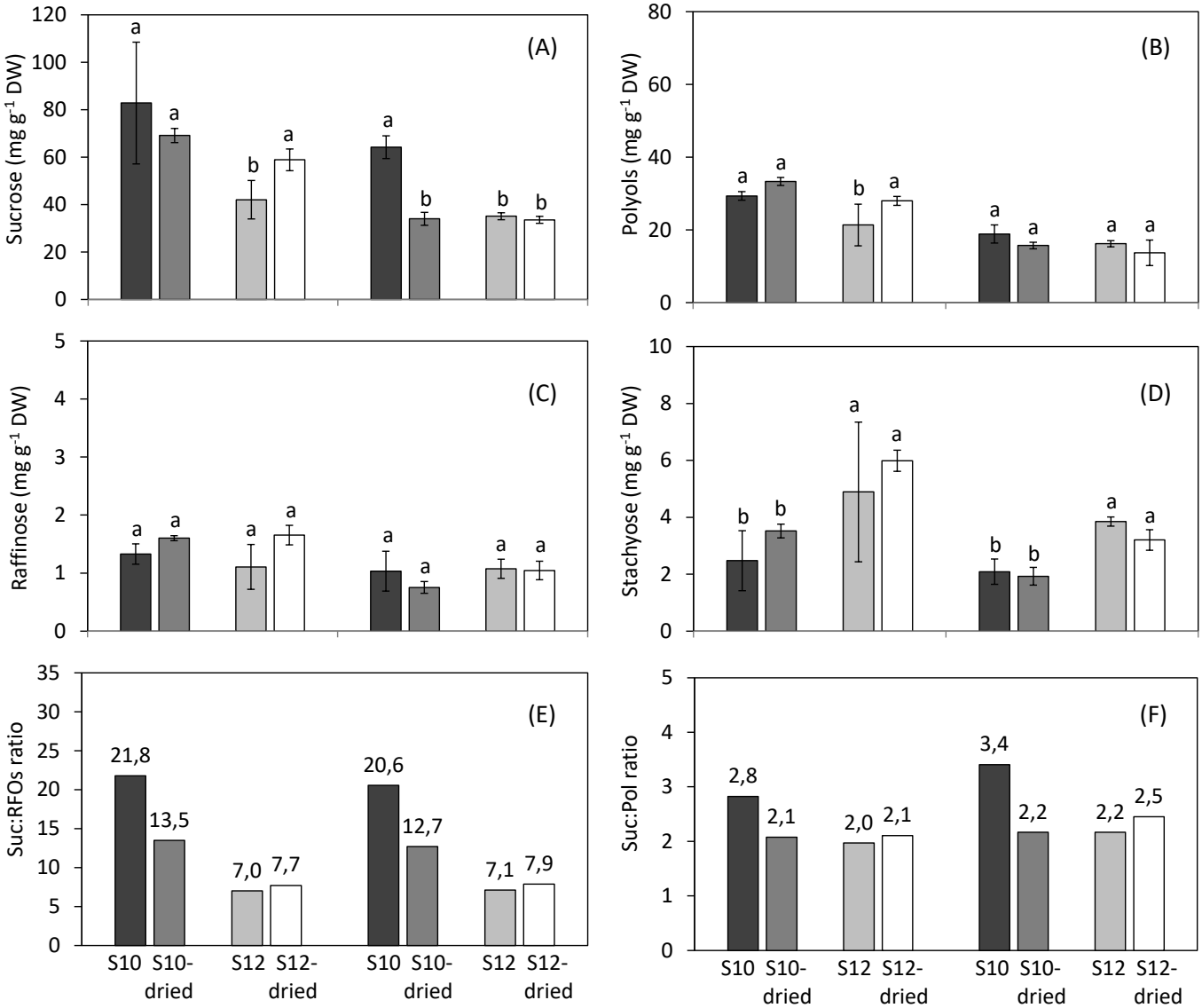


Figure 8. Soluble carbohydrate composition (mg.g$^{-1}$ DW) of embryonic axis and cotyledons of *Poincianella pluviosa* sensible (S10) and DT (S12) seeds and after drying until 0.08 g.g$^{-1}$ water. Sucrose (A); Polyols (B); Raffinose (C); Stachyose (D); Sucrose:Raffinose family oligosaccharides (RFOs) ratio (E); Sucrose:Polyols ratio (F). Within each compound and each tissue, means followed by the same letter do not differ significantly (Tukey´s test, $p < 0.05$, $n = 3$).

values close to those found in sensitive S10-dried seeds (Figure 6A). Although the proportion of trehalose in cotyledons was not affected by drying, it was higher in S12 than in S10 seeds (Figure 6B). Trehalose is a disaccharide accumulated in organisms that endure extreme environmental conditions, acting as a protein stabilizer (Eastmond and Graham, 2003; Kaushik and Bhat, 2003). However, traces of this compound are commonly found in a variety of plants, thus diminishing the putative protective role of this sugar.

Developing orthodox seeds are characterized by a hypoxic internal environment until the storage phase (Borisjuk and Rolletschek, 2008), which was also observed until shedding in the seeds of the recalcitrant *Inga vera* (Caccere et al., 2013). Evidence showed that the oxygen level in green embryos is regulated by the degree of maturity and relies on the lighting conditions of the environment, since embryo photosynthesis could minimize fermentative processes (Rolletschek et al., 2005; Borisjuk and Rolletschek, 2008; and references therein). An improved metabolic flux through

oxygen supply could reduce the formation of lactate at subsequent stages (Caccere et al., 2013). In this way, the high proportion of lactate in the cotyledons of *P. pluviosa* seeds at the beginning of the maturation (S3 - Figure 4) could be related to a putative initial low oxygen concentration inside the seed. During seed filling, the metabolic flux toward oxygen supply is correlated with a decreased lactate content (Figure 4) and the color of cotyledons, which remains green throughout entire *P. pluviosa* seed maturation (Silva et al., 2015). This condition results in an efficient energy supply and could be related to the large amounts of oil obtained in such seeds (~45 % - Silva et al., 2017), even with lactate still detected in cotyledons of mature seeds (Figure 4).

Considering that a decrease in the TCA rate is expected as far the maturation of DT seeds advances (Fait et al., 2006, Wang et al., 2016), the higher proportion of lactate and glycerol in S10-dried tissues rather than in S12-dried (Figure 6) could also indicate an elevated tissue metabolism prior to the drying treatment. Moreover, if considered that the progression of seed maturation will result in increasingly and orderly levels of germination, DT, vigor, and storability (Ellis, 2011; Barbedo, 2018), the possibly active metabolism in *P. pluviosa* axes might indicate an incomplete maturation of the embryo, which was also suggested for the different DT behaviors at shedding (Barbedo et al., 2013; Barbedo, 2018).

Similar as in *P. pluviosa*, seeds of *P. echinata* are also DT and present an even short-lifespan at room temperature (30 days - Mello et al., 2013; Santos and Barbedo, 2017), which is related to respiratory rates found during storage (Lamarca and Barbedo, 2012). In addition to the presence of lactate accumulation in the axes of *P. pluviosa* seeds at the RDS stage (Figures 3 and 7B), this result suggests that seeds are being dispersed without a complete metabolic shut-down that could be related to their short to medium lifespan at room temperature (240 days - Figliolia et al., 2001). Interestingly, when stored at lower temperatures, the viability of these seeds is maintained for longer periods (360 days - Pontes et al., 2006).

The present work showed that the major changes in the development of *P. pluviosa* seeds occur after early stages (S3 and S4), and at the latest stages of seed maturation (S12 and RDS), which are mainly characterized by a decrease in the primary energetic metabolism and the production of antioxidants via secondary metabolism both in embryonic axes and cotyledons. However, a low metabolic status of embryonic axes could still be present before shedding, evidenced by changes in lactate proportions that were correlated with drying and the physiological parameters analyzed. The main consequences of being dispersed without a complete metabolic shut-down could be the rapid viability loss of *P. pluviosa* seeds compared to other DT seeds, which would present an even lower respiratory metabolism and less damages. Altogether, these data emphasize the late maturation period as crucial for seed longevity and correlated with its metabolic status at shedding.

## CONCLUSIONS

*Poincianella pluviosa* seeds are dispersed with an incomplete metabolic *switch-off*, which can be related to their short lifespan.

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