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Drought stress effects on germination and reserve degradation of *Aspidosperma polyneuron* seeds

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ABSTRACT: Seeds are exposed to diverse environmental factors when dispersed, including abiotic stresses, which will affect germination. Drought is the most important factor affecting germination, since water is essential for seed development. *Aspidosperma polyneuron* is a native endangered species which has been extensively exploited, mainly for timber extraction purposes. This study analyzed germination and degradation of soluble sugars and proteins of *A. polyneuron* seeds during germination under water stress. Seeds were placed on Germitest papers moistened with different water potential solutions, varying from 0.0 to -1.4 MPa. Degradation of soluble sugars and soluble proteins was assessed in five stages of germination at the different water potentials. *A. polyneuron* seeds lost germination capacity as the water potential decreased, being unable to germinate below -0.6 MPa. Glutelin was the most abundant storage protein in the seed, while prolamin was the least. Reserve degradation was altered in negative water potentials. Soluble proteins are degraded faster and soluble sugars are degraded slower as the water potential drops.

Key words: native species; seed imbibition; soluble proteins; soluble sugars; water potential

Efeito do estresse hídrico na germinação e degradação de reservas de sementes de Aspidosperma polyneuron

RESUMO: Quando dispersas, as sementes são expostas à diversos fatores ambientais, incluindo estresses abióticos, os quais irão afetar sua germinação. A seca é o fator mais limitante que afeta a germinação, já que a água é essencial para o desenvolvimento da semente. *Aspidosperma polyneuron* é uma espécie nativa em perigo de extinção, extensamente explorada para extração madeireira. Este estudo analisou a germinação e degradação de açúcares e proteínas solúveis de *A. polyneuron* durante a germinação sob estresse hídrico. Sementes foram dispostas em papéis Germitest umedecidos com soluções em diferentes potenciais hídricos, que variaram de 0,0 à -1,4 MPa. A degradação de açúcares e proteínas solúveis foi avaliada em cinco estágios de germinação nos diferentes potenciais hídricos. Sementes de *A. polyneuron* perderam capacidade germinativa conforme o potencial hídrico diminuiu, não germinando abaixo de -0,6 MPa. Glutelina foi a proteína de armazenamento mais abundante na semente, enquanto prolamina a menos presente. A degradação de reservas foi alterada em potenciais hídricos negativos, sendo que as proteínas solúveis foram degradadas mais rapidamente e os açúcares mais lentamente conforme o potencial hídrico diminuiu.

Palavras-chave: espécie nativa; embebição de sementes; proteínas solúveis; açúcares solúveis; potencial hídrico

Introduction

Aspidosperma polyneuron Müll. Arg. is a native species of the Atlantic Forest occurring naturally from South to Northeast Brazil in a variation of 850 to 2400 mm of annual rainfall (Ribas et al., 2005). *A. polyneuron* timber is of great quality and value, being used in civil and naval construction. The tree is also used in various ways for having astringent, antipyretic, anti-diarrheal, anti-malarial, and antimicrobial properties (Krentkowski & Duarte, 2012). Due to deforestation and overexploitation, *A. polyneuron* is listed in the IUCN red list of threatened species (IUCN, 1998). Its natural restoration is also hampered by low seasonal seed production, slow growth and irregular germination rates (Ribas et al., 2017).

When seeds are dispersed into the environment, they are subjected to different factors that affect germination. Seed germination is frequently the most critical phase during seedling establishment, dictating the existence or absence of a species at a particular site (Shi et al., 2014; Azerêdo et al., 2016). Drought is one of the most important environmental stresses which not only affects agriculture, but also in seedling establishment and reforestation (Fang et al., 2017). Water is the main factor for living organisms and one of the crucial environmental drivers regulating plant biogeography. A decrease in water availability leads to morphological, physiological, and metabolic modifications in plants, including seeds (Ferreira et al., 2015). The ability of seeds to tolerate water restraint has important conservation implications (Wang et al., 2009).

Water promotes and controls seed germination, favors tissue rehydration and oxygen entrance. Seeds begin to intensify metabolic activities from water uptake, generating nutrients and the energy necessary for embryonic axis growth (Loureiro et al., 2013). Furthermore, enzyme activation and use and translocation of stored substances demand water (Bello et al., 2008). The dynamic of seed reserves represents a crucial part of the germination process. The embryo starts the process with its own reserves, but growth maintenance will depend on a flow of soluble components from cotyledons to seed regions in full development (Pereira et al., 2015). Therefore, germination may be delayed or even prevented when water stress exceeds seed tolerance levels (Guedes et al., 2013; Parvin et al., 2014).

Physiological seed studies are essential to comprehend germination, and understanding the tolerance limit and capacity of seed adaptation to environmental stressors are determinant in this process (Dalberto & Braga, 2013). In this sense, high vigor seeds can be selected and used in restoring degraded areas, and therefore to propagate native species from seedling production in nurseries or through direct seed sowing (Ataíde et al., 2014).

Due to the great importance of seed reserves during germination and in order to understand the ecophysiological responses of *A. polyneuron* seeds to environmental factors, this study analyzed germination and the contents of soluble proteins and sugars during germination under water stress, while also associating reserve content and the capacity of seeds to germinate.

Material and Methods

A. polyneuron seeds were collected in 2014 in Terra Boa – PR (-23.794841, -52.450948). Seeds were received and stored in plastic bags in a refrigerator at 5°C at the Plant Physiology Lab of the Western Paraná State University, Cascavel, where the experiment took place from October 2016 to August 2017. The seeds had their wings removed before each experiment, then were sanitized using 1% sodium hypochlorite for 10 min and rinsed in distilled water to avoid fungi contamination (Brazil, 2013).

Seed initial water content and hydration curve

Initial water content (WC) was determined by the oven method at 105±2°C for 24 h using 4 replicated of 25 seeds (Brazil, 2013). WC was expressed in a percentage basis.

A hydration curve with eight replicates of 25 seeds placed on Germitest papers moistened with distilled water was conducted in order to select different stages for the biochemical analysis during germination. Replicates were weighted every hour on the first day, every two hours on the second, every four hours on the third and every 6 hours from the fourth day until half of the seeds were germinated.

Germination analysis

Four replicates of 15 seeds were set in Petri dishes containing three germitest papers. Papers were moistened with 7 ml of different polyethylene glycol (PEG 6000) solutions in order to simulate drought stress at different water potential levels (-0.2, -0.4, -0.6, -0.8, -1.0, -1.2, and -1.4 MPa). Distilled water was used as the control (0.0 MPa). The amounts of PEG used in the experiment were calculated following Villela et al. (1991). Samples were stored in germination chambers at 25°C, 12-hour photoperiod. Seeds were transferred to new Petri dishes every 3 days to maintain osmotic levels.

The variables analyzed to verify seed water stress tolerance were germination percentage, germination speed index (GSI) as described by Maguire (1962), and mean germination time (MGT) (Edmond & Drapala, 1958). The experiment was conducted for 25 days. Seeds were considered as germinated when radicle protrusion reached 2 mm (Hadas, 1976).

Reserve degradation

The test to verify the effects of water stress in the contents of soluble sugars and proteins during germination followed the same procedure as the germination analysis. Samples were taken from the germination chambers in periods based on the hydration curve. Samples were collected in each stage, frozen in liquid nitrogen to stop any metabolic activity and reserve degradation, and stored at -20ºC in a freezer. The collection stages were: control (S0, no hydration), middle of phase I (S1), beginning of phase II (S2), middle of phase II (S3) and beginning of phase III (S4).

Alterations in the of total soluble sugar (TSS), reducing sugar (RS), and glutelin (GLU), albumin (ALB), globulin (GLO) and prolamin (PRO) levels in the whole seed were determined using 100 mg of fresh mass of each sample. Readings were done in triplicate using a spectrophotometer.

Sugar extraction followed Garcia et al. (2006), in which three exhaustive extractions in the same sample were conducted using 80% ethanol at 80°C for 15 min. Samples were then centrifuged for 30 min. Next, supernatants were combined and utilized for quantifying TSS and RS. TSS contents were quantified through the anthrone method (Morris, 1948; Yemm & Willis, 1954), while RS were quantified through the dinitrosalicylic acid (DNS) method (Miller, 1959). The absorbances were measured in a spectrophotometer at 620 nm and 540 nm, respectively, and the results were expressed in mg g^{-1}.

Soluble protein extraction followed the seriated extraction method proposed by Suda & Giorgini (2000), in which each of the four proteins were extracted according to its solubility. First, distilled water extracted ALB, then GLO was extracted with 5% sodium chloride, PRO with 60% ethanol, and lastly 0.4% sodium hydroxide extracted GLU. The extracts were centrifuged and one protein was extracted every 24 h according to the solvent. We followed the methodology of Bradford (1976) for protein quantification using Coomassie Brilliant Blue G-250 dye and a casein protein calibration curve. Sample absorbances were measured in a spectrophotometer at 595 nm. The results were expressed in µg g^{-1}.

Statistical analysis

Data were subjected to polynomial regression to verify the data trends related to the different osmotic potentials, and the significances (p = 0.05) were tested using RStudio® software (RStudio Team, 2015). Selected models were based on the highest coefficient of determination and lowest standard error of the mean.

Results

WC and hydration curve

The initial WC was 3.81% (fig. 1). Seeds used in the hydration curve took approximately 78 hours to start germination, when WC was 48%. Germination was separated into three phases from the hydration curve, and the sample collection stages for the reserve degradation analysis were determined as the following: at 0 (S0), 18 (S1), 36 (S2), 56 (S3) and 78 (S4) hours.

Germination analysis

All measured indexes were significantly affected by changes in osmotic potential (Figure 2A-C). Germination percentage decreased as the osmotic potential decreased (R^2 = 0.9579, Figure 2A). Seeds under 0.0 MPa presented 82% germination, while only 38% germinated at -0.2 MPa, 17% at -0.4 MPa and 7% at -0.6 MPa. Seeds subjected to -0.8 MPa or less did not germinate.

Seeds subjected to low osmotic potentials delayed germination (R^2 = 0.9193, Figure 2B). GSI also decreased as

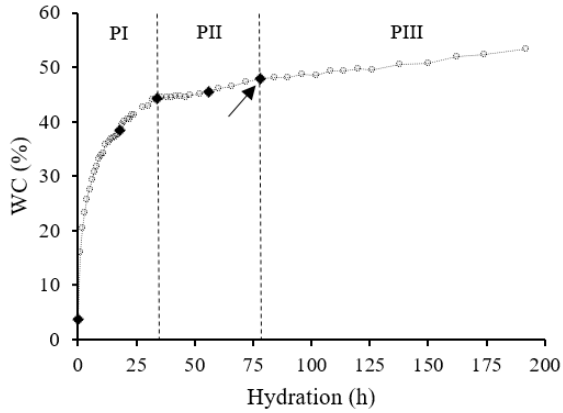

Figure 1. Hydration curve of *Aspidosperma polyneuron* seeds. PI: phase I. PII: phase II. PIII: phase III. Arrow indicates moment of radicle protrusion. Empty dots represent the sample weight moments. Black dots indicate the five sample collection stages – 0 (no hydration), 18 (middle of phase I), 36 (beginning of phase II), 56 (middle of phase II) and 78 (beginning of phase III) hours.

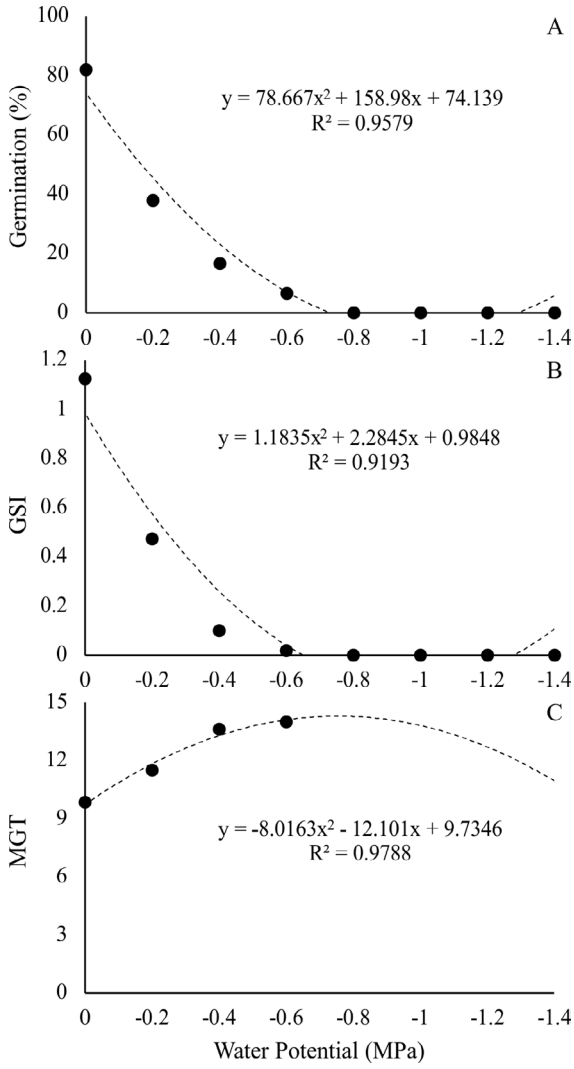

Figure 2. Regression analyses of *Aspidosperma polyneuron* seeds subjected to different osmotic potentials. A. Germination percentage (%). B. Germination Speed Index (GSI). C. Mean Germination Time (MGT).

water stress increased, showing 1.123 GSI at 0.0 MPa and reducing to less than half (0.473) at -0.2 MPa. GSI rates at -0.4 and -0.6 MPa were 0.098 and 0.017, respectively.

MGT increased as water potential decreased (R^2 = 0.9788). Seeds took about 10 days to germinate at 0.0 MPa. Under water stress conditions, seeds postponed germination to 11 days at -0.2 MPa and 14 days at -0.4 and -0.6 MPa (Figure 2C).

Reserve degradation

Seeds presented 532.44 mg g^{-1} of initial TSS (S0) and decreased throughout the experiment for all tested water potentials (Figure 3). Degradation at S1 was higher for 0.0, -0.2, -0.4 and -0.8 MPa, representing 50, 44, 47 and 44% decreases, respectively, while the lowest decrease (13%) occurred at -1.2 MPa in relation to S0. TSS levels remained

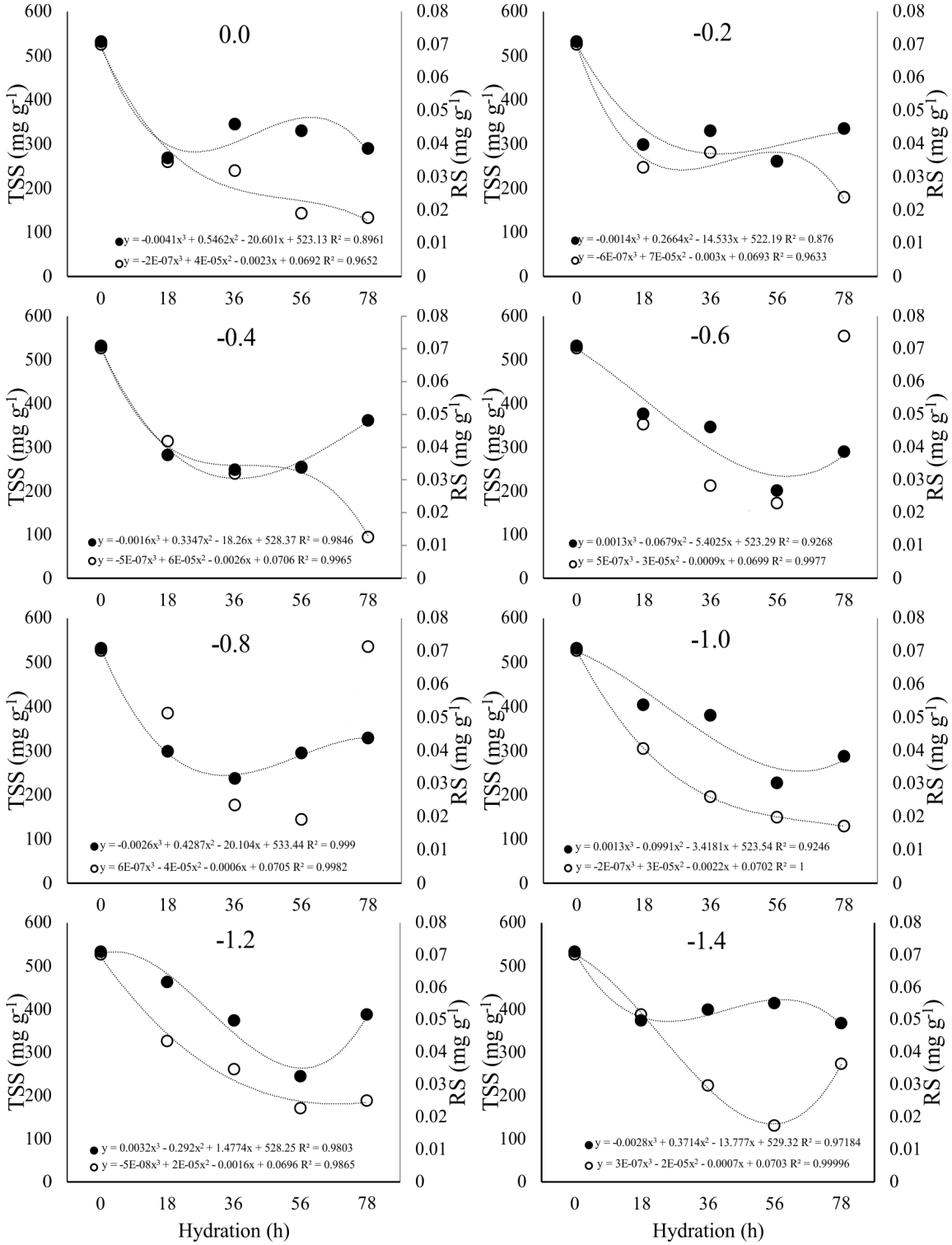

Figure 3. Degradation of total soluble sugars (TSS – ●) and reducing sugars (RS – ○) during germination (at 0 – S0, 18 – S1, 36 – S2, 56 – S3 and 78 hours – S4) of *Aspidosperma polyneuron* seeds subjected to water stress at different water potentials (0.0, -0.2, -0.4, -0.6, -0.8, -1.0, -1.2, -1.4 MPa).

constant at S2, declining once again at S3 for -0.6, -1.0 and -1.2 MPa. Treatments presented similar content by S4, varying from 287.4 to 387.73 mg g^{-1} and (excluding 0.0 and -1.4 MPa), the observed trend was to recover TSS (cv = 9.39%, p< 0.05).

RS contents at S0 was 0.07 mg g^{-1} (Figure 3), decreasing for all water potentials at S1. A higher decrease was observed at -0.8 MPa beginning in S2. In S3, the lowest content was presented by -1.4 MPa. RS contents at S4 for -0.4 and -1.0 MPa showed even higher decreases, with RS levels varying from 0.0127 to 0.0172 mg g^{-1} (82 and 76% decrease), respectively, when compared to S0. On the other hand, -0.6, -0.8 and -1.4 MPa exhibited an increase in RS levels, with -0.6 and -0.8 MPa reaching the same content presented by S0 (cv = 0.56%, p < 0.05).

The soluble protein that presented the highest levels in S0 was GLU, exhibiting 69.92 µg g^{-1} (Figure 4). A decrease of GLU started at S1, reaching 50% of initial content by S2. GLU contents were maintained constant for all water potentials at S3, while GLU increased at S4 for 0.0, -0.2 and -1.2 MPa (cv = 8.53%, p < 0.05).

Degradation of ALB started in S1 for all water potentials (Figure 4). ALB content at S0 was 60.14 µg g^{-1}, exhibiting the highest decrease at S1 for most water potentials, except 0.0

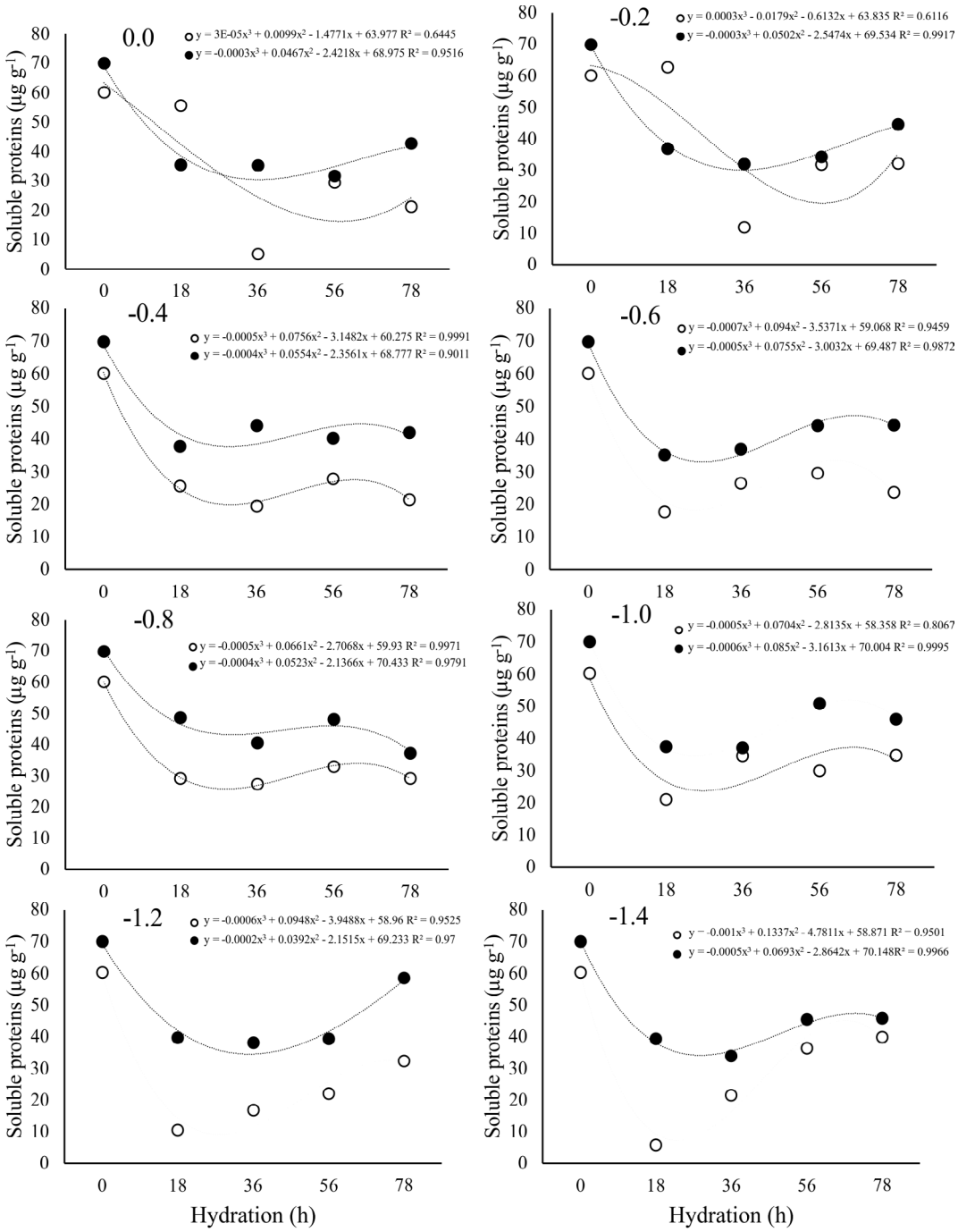

Figure 4. Degradation of glutelin (GLU – ●) and albumin (ALB – ○) during germination (at 0 – S0, 18 – S1, 36 – S2, 56 – S3 and 78 hours – S4) of *Aspidosperma polyneuron* seeds subjected to water stress at different water potentials (0.0, -0.2, -0.4, -0.6, -0.8, -1.0, -1.2, -1.4 MPa).

and -0.4 MPa, in which the lowest content appeared at S2. ALB content increased for 0.0, -0.2, -1.2 and -1.4 MPa starting from S3, while no significant changes occurred for the other water potentials. ALB levels remained similar to S3 at S4. Still, -1.4 MPa showed the highest content, while the lowest contents were observed for 0 and -0.4 MPa (cv 11.6%, $p < 0.05$).

Initial GLO content was 36.75 µg g^{-1} and degradation of GLO at S1 is visible at all water potentials (excluding -0.8 MPa) (Figure 5). GLO levels remained constant at S2 and S3. S4 is marked by a decrease at 0.0, -0.4 and -0.6 MPa and an increase at -0.2, -0.8, -1.2 and -1.4 MPa, while -1.0 remained constant. The lowest GLO contents were exhibited by 0.0 and -0.4 MPa, water potentials which presented 10.78 µg g^{-1} (cv = 13.37%, $p < 0.05$).

PRO presented the lowest content at S0 among the four soluble proteins (Figure 5). Initial content was 14 µg g^{-1}, decreasing at S1 for all water potentials except -0.2 and -1.4 MPa, which tended to remain continuous for all stages. The highest peak for the other water potentials appeared at S3. At S4, the PRO contents tended to decrease again, with the sharpest curves presented at -1.0 and -1.2 MPa (cv = 8.35%, $p < 0.05$).

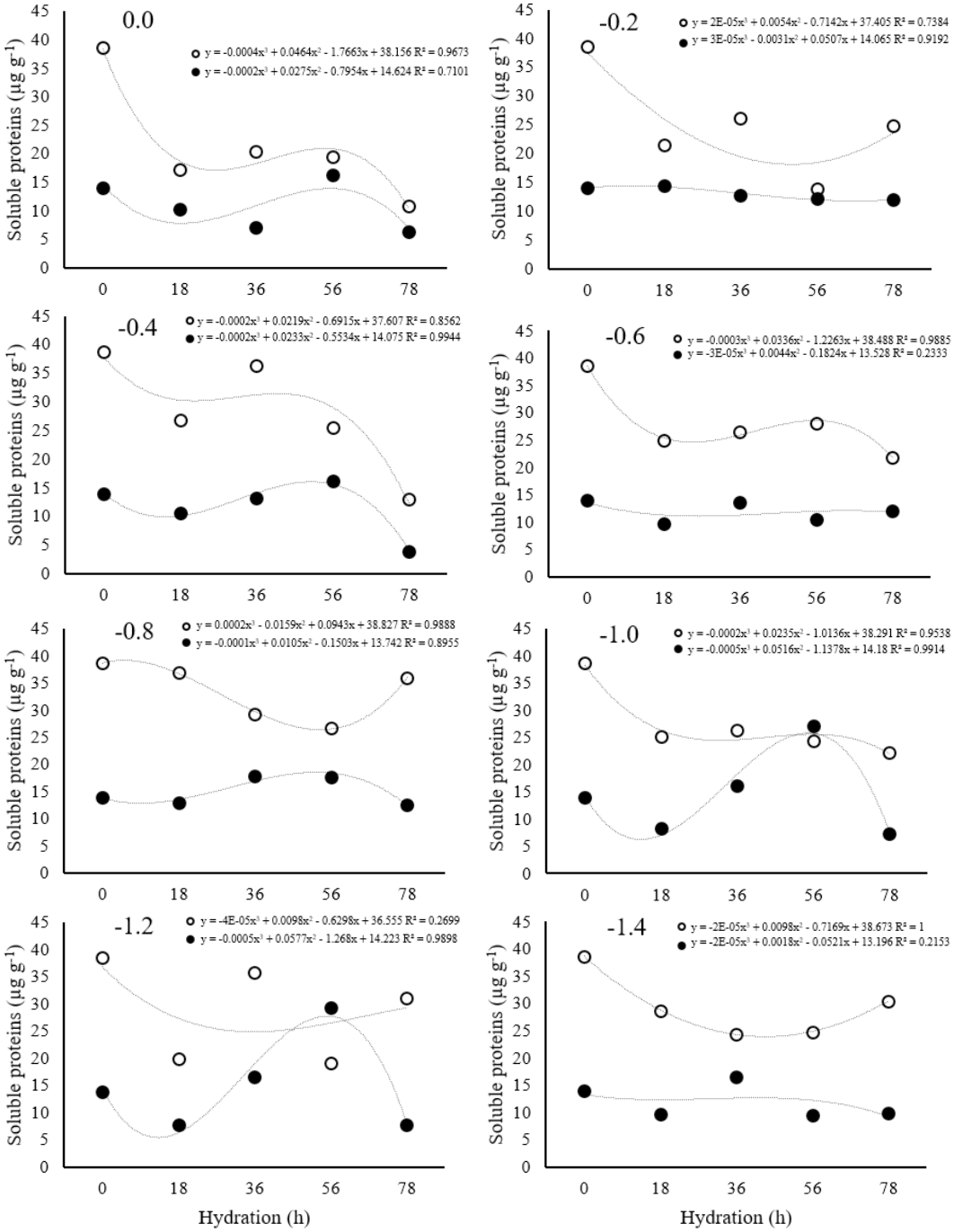

Figure 5. Degradation of globulin (GLO – ○) and prolamin (PRO – ●) during germination (at 0 – S0, 18 – S1, 36 – S2, 56 – S3 and 78 hours – S4) of *Aspidosperma polyneuron* seeds subjected to water stress at different water potentials (0.0, -0.2, -0.4, -0.6, -0.8, -1.0, -1.2, -1.4 MPa)

Discussion

Water stress significantly influenced *A*. *polyneuron* seed germination for all tested variables (Figure 2A-C). Negative water potentials can interrupt the sequence of germination events, especially at the beginning of seed hydration, delaying and decreasing the germination percentage and speed (Guedes et al., 2013). Water potentials below -0.6 MPa prevented seed germination (Figure 2A), indicating water restriction sensitivity presented by *A. polyneuron*. Germination is automatically blocked when osmotic potential reaches water stress critical limit (Kappes et al., 2010).

As water stress intensifies, the speed at which seeds acquire water from the environment decreases. Water potentials below -0.2 MPa already decreased GSI and increased MGT, representing a two, seven and 22-fold speed decrease at -0.2, -0.4 and -0.6 MPa, respectively, compared to the control (Figure 2B). The changes in GSI and MGT presented by *A. polyneuron* can be explained by the absence of minimum WC required for germination, which is around 48% WC in the case of *A. polyneuron*, as the necessary time for seeds to reach minimum water requirements for germination increases as the osmotic potential decreases (Bello et al., 2008).

Germination speed is vital for species survival and development, while mean germination time evaluates the velocity at which a species establishes in a niche or place (Silva & Medeiros Filho, 2006). The faster seeds germinate, the less seeds are exposed to adverse conditions (Secco et al., 2010). Germination percentage decline and delay suggest a low capacity of A*. polyneuron* seeds to adapt to areas affected by water stress.

Low germination rates of *A. polyneuron* under water stress may also be attributed to enzymatic activity reduction, since a water shortage restricts seed meristematic development (Teixeira et al., 2011). When turgor pressure decreases, water stress suppresses cellular growth and expansion, affecting the metabolism, growth and seedling establishment (Soares et al., 2015). Water availability is essential for germination, since water uptake is directly and indirectly involved in metabolic processes, such as tegument softening, oxygen entrance, embryo volume expansion and reserve mobilization (Guedes et al. 2013).

Reserve mobilization is completely altered in negative water potentials, as demonstrated in Figures 3, 4 and 5. Low water availability affects reserve assimilation and translocation of metabolized products from cotyledons to the embryo, consequently changing the way germination occurs (Teixeira et al., 2011). Therefore, the differences occurring in reserve degradation in each water potential may have caused alterations in embryo development at -0.2, -0.4 and -0.6 MPa, even preventing radicle protrusion at -0.8 MPa and below.

A. polyneuron seeds exhibited high TSS degradation levels in the first germination stage (S1), mainly in water potentials close to 0.0 MPa (0.0, -0.2, -0.4 and -0.8), decreasing more than 40% of initial content. TSS degradation occurs in the beginning of germination because soluble sugars are pre-produced carbohydrates in seeds which are the first substrate to be metabolized during pre-germination stage, functioning as metabolic resources and structural constituents of cells (Rosa et al., 2009; Erbas & Sanli, 2016).

Furthermore, TSS were slower degraded as water potential decreased in our experiments with *A. polyneuron* seeds. According to Rosa et al. (2009) and Reis et al. (2012), soluble sugars are required for growth and maintenance of the osmotic homeostasis of cells, adjusting the osmotic potential and protecting seeds from protein denaturation. In addition, germinating seeds and growing seedlings appear as the most vulnerable stages to soluble sugar fluctuations, particularly under environmental stresses such as drought, in which soluble sugar concentrations generally tend to increase (Rosa et al., 2009; Reis et al., 2012). *A. polyneuron* seeds exhibited a decrease until S3 and an increase in S4 for water potentials ranging from -0.2 to -1.2 MPa. The RS behavior was similar, tending to accumulate in negative water potentials, especially at -0.6, -0.8 and -1.4 MPa. Water stress may lead to intensify starch hydrolysis, which may generate increases in RS since they are a product derived from starch (Zaher-Ara et al., 2016). Additionally, RS serve various functions in seeds from energy storage to abiotic stress signaling and osmoregulation (Bhandari et al., 2016).

Overall, soluble proteins of *A*. *polyneuron* seeds exhibited earlier degradation under negative water potentials (Figures 4 and 5). The lower the water potential, the faster soluble proteins were decreased during germination for the *A. polyneuron* seeds tested in this study, which might explain the absence of seeds germinated below -0.6 MPa. Protein content is related to the physiological quality of the seed. Seeds exposed to environmental stresses may suffer deterioration and therefore present a decrease in protein levels (Henning et al., 2010).

Moreover, reduction in soluble protein concentrations may result from amplified activity of proteases or other catabolic enzymes which are activated under water stress. Since soluble proteins are hydrolyzed in response to changes in osmotic potential and increase in reactive oxygen species (ROS), lower protein levels are perceived as a characteristic symptom of oxidative stress presented by seeds exposed to water stress (Bano et al. 2013).

Storage proteins (GLU, ALB, GLO and PRO) are present in seeds but one type usually predominates in different plant families (Laudencia-Chingcuanco & Vensel, 2008). GLU was the most abundant soluble protein found by Bernardino-Nicanor et al. (2005) in *Psidium guajava* (Myrtaceae) seeds, while GLO was the most representative soluble protein in *Brachypodium distachyon* (Poaceae) seeds (Laudencia-Chingcuanco & Vensel, 2008). In this study, GLU was the predominant soluble protein in *A. polyneuron* seeds, while PRO was the protein present in reduced levels.

ALB is catabolized during germination phase I (S0 to S2), and an increase is observed during PII, which might be associated with seed rehydration and functional protein

biosynthesis (Dantas et al., 2008). The water potentials of 0.0, -0.4 and -0.6 MPa showed decreases in GLO. GLO proteins are synthesized in the endoplasmic reticulum, deposited in the storage vacuoles and are degraded easier than PRO (Laudencia-Chingcuanco & Vensel, 2008), which may explain the higher presence in *A. polyneuron* seeds at S0.

Irregular degradation of the storage proteins during germination of *A. polyneuron* seeds under water stress can reflect in slow (Figure 2B-C) and abnormal seedling growth, since storage proteins are important in germination and initial seedling development, generating new embryo tissues. Most amino acids used during early germination are derived from storage proteins and stored free amino acids (Rosental et al., 2014).

As reserve degradation is integrally related to germination, the speed at which sugars and proteins are consumed are reflected in the time seeds take to germinate (Silva et al., 2016). The longer MGT and slower GSI that *A. polyneuron* seeds presented under water stress conditions may be associated with the slower degradation of soluble sugars and faster degradation of soluble proteins as a way of protecting seeds from deterioration. Once seeds are unable to degrade and mobilize storage reserves and incorporate the derived products into the embryo axis under water stress conditions, seedlings originated by the seeds will present low growth rates (Medeiros et al., 2015), and consequently lower survivability chance in the environment.

Our results demonstrate the importance of evaluating water stress tolerance in seeds of different species. Reforestation needs to be based on knowledge and appropriate management in order to function properly. Adaptation and germinating capacity in adverse conditions confer ecological advantages to some species over others. Therefore, it is important to choose the right species for each different scenario. Seed germination and seedling survival depend on suitable reserve degradation. Finally, *A. polyneuron* seeds develop better in environments where water is regularly available or water shortages are minimum throughout development.

Conclusions

Germination of *A. polyneuron* seeds is disrupted in water potentials below -0.2 MPa, presenting lower germination rates and GSI and higher MGT. *A. polyneuron* seeds are incapable of germinating in water potentials below -0.6 MPa. Soluble sugars are consumed slower the more negative the water potential becomes, while soluble proteins are degraded faster in the same conditions.

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