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Hydrolytic enzyme activity (phytase and α-amylase) is decisive in the germination metabolism of maize seeds under salt stress

A atividade de enzimas hidrolíticas (fitase e alfa amilase) é decisiva no metabolismo germinativo de sementes de milho sob estresse salino

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ABSTRACT

The mobilization of reserves is essential for seedling formation and can be influenced by seed vigor. This work evaluated how seed vigor and reserve mobilization influence maize seedling formation under salt stress. The high-vigor seeds comprised the seeds directly from the lot, while the low-vigor seeds were subjected to an artificial aging process to reduce the vigor. Seeds were germinated in a substrate moistened with water (control) or saline solution (100 mmol L^{-1} NaCl) and were submitted to germination and vigor tests (first germination count, cold test, and seedling performance). Biochemical profiling (phytic acid and inorganic phosphorus, total and soluble protein, starch and soluble sugars, α-amylase and phytase activity) was determined at 0 (quiescent seeds), 2, 4, 6, and 8 days after sowing. High-vigor seeds had better physiological performance and higher reserve hydrolysis rates under salt stress than low-vigor seeds. High-vigor seeds also showed earlier enzymatic activity than low-vigor, demonstrating a temporal difference in reserve hydrolysis during the stress. The highest differences in reserve hydrolysis occurred 2 days after sowing and differentiated the germinative metabolism of high and low-vigor seeds under salt stress.

Index terms: *Zea mays* L.; phosphorus; physiological performance.

RESUMO

A mobilização de reservas é fundamental para a formação de plântulas e pode ser influenciada pelo vigor das sementes. O objetivo do trabalho foi avaliar como o vigor de sementes e a mobilização de reservas influência a formação de plântulas de milho em estresse salino. Foram utilizados dois lotes de sementes de milho, as sementes de alto vigor (HV) foram as sementes provenientes diretamente do lote, e as sementes de baixo vigor (LV) foram obtidas através da redução artificial do vigor. As sementes foram germinadas em substrato umedecido com água (controle) e com solução salina (100 mmol L^{-1} NaCl). As sementes foram submetidas ao teste de germinação e de vigor (primeira contagem de germinação, teste de frio e desempenho de plântulas). O perfil bioquímico (ácido fítico e fósforo inorgânico, proteína total e solúvel, amido, açúcar solúvel, atividade da α-amilase e fitase), foi determinada temporalmente em 0 (sementes quiescentes), 2, 4, 6 e 8 dias após a semeadura. As sementes de alto vigor tiveram melhor desempenho fisiológico e as maiores taxas de hidrólise de reservas sob stress salino quando comparadas com as sementes de baixo vigor. As sementes de alto vigor apresentaram atividade enzimática antecipada em relação as de baixo vigor, demonstrado diferença temporal na hidrólise de reservas. As maiores diferenças na hidrólise dos compostos de reserva ocorrem dois dias após a semeadura e diferenciam o metabolismo germinativo das sementes de alto e baixo vigor sob condições de estresse.

Termos para indexação: *Zea mays* L.; fósforo; desempenho fisiológico.

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Introduction

Maize is one of the most important cereal crops worldwide and an important source of food, feed, and biofuels (Yin et al., 2020). Maize has expressive productive potential. However, its productive stability is limited by factors such as low prolificacy and high interspecific competition. Thus, proper seedling establishment is important to ensure crop success. Germination and plant formation are important to plant establishment and sensitive to biotic and abiotic stresses (Tarnawa et al., 2023).

Besides abiotic stresses, climate change poses a challenge to researchers and breeding programs to develop crops tolerant to heat stress, drought, waterlogging, salinity, and other climatic factors (Lopes, 2023). Salinity stress can significantly affect crop performance (Ahmad et al., 2023) and has a negative effect on maize seed germination and seedling formation (i.e., reduced length of the shoot and root system) (Beyaz & Dai, 2022). In this way, the early stages of maize crop development are the most sensitive to saline stress (Sabagh et al., 2021). The ability to overcome saline stress is dependent on several factors, including morphological, biochemical, physiological, and molecular characteristics of plants.

Seed vigor has a great influence on responses to abiotic stresses, such as saline stress, and can be determinant during seed germination and seedling formation (Padilha, Coelho, & Sommer, 2022). Seed vigor is a complex physiological trait, including seed germination, seedling emergence and growth, as well as seed storability and stress tolerance (Zhao et al., 2021). Seed vigor is the sum of those properties that determine the germination activity, performance of seed lots, and proper seedling formation in a wide range of environments (Bagateli et al., 2022).

During germination, storage compounds are hydrolyzed to support a rapid root and shoot growth until the seedling reaches photoautotrophy. Endosperm stores the main fraction of the reserve components of maize seeds, such as starch and proteins, whereas the scutellum contains mainly lipids and phytate (Feenstra et al., 2017; Nadeem et al., 2014).

Hydrolytic enzymes are possibly associated with rapid and uniform germination and vigorous seedling development (Ali et al., 2018). Vigorous seeds with higher enzymatic activity release soluble compounds with lower molecular weight that fuel metabolism during germination. Thus, we suppose that low molecular weight compounds favor the development of vigorous seedlings during saline stress.

Associations of biochemical components and seed vigor are reported in maize (Andrade, Coelho, & Uarrota, 2020; Prazeres, Coelho, & Souza, 2021). However, how vigor acts on germination and seedling formation during saline stress is unclear. This study aimed to verify if seed vigor is a determinant for seedling formation and how reserve mobilization is associated with seedling performance in salt stress.

Material and Methods

Experimental design

The experiment was carried out at the Seed Analysis Laboratory of the Agroveterinary Center, Santa Catarina State University (27°47’34.3” S; 50°18’16.3” W). Seeds (1000 g) of the commercial hybrid 30F53VYH produced in the 2019/2020 harvest were homogenized using a sample divider (Brasil, 2009) to obtain four replicates of 250 g (Coelho et al., 2010). Replicates were stored in a cold chamber with a relative humidity of 50% and 12 °C temperature until the 2020/2021 harvest, when the analyses were performed.

The experimental design was entirely randomized in a 2 x 2 factorial scheme with two vigor levels (high-vigor and low-vigor) and two germination conditions [control (CTR, no salt) and 100 mM NaCl (SSC)]. High-vigor (HV) seeds comprised seeds stored as described above and directly used for the experiment, while the low-vigor (LV) seeds were subjected to an artificial aging process to reduce the vigor. For that, seeds were placed in gerbox boxes containing 40 mL distilled water and maintained at 45 °C and 95% ± 2% relative humidity for 72 h. Afterward, low-vigor seeds were dried until the moisture content was 13% ±1%. . The effects of the seed vigor throughout salt and control germination conditions were compared.

Determination of germination and seed vigor

Seeds (four replications of 50 seeds) were arranged on sheets of germitest paper previously moistened with distilled water or 100 mM NaCl solution in a volume equivalent to 2.5 times the weight of the dry paper. Seeds were scattered on the paper and placed in a Magensdorf-type germination chamber at 25 °C. The first and the final germination counts were performed after 4 and 7 days respectively (Brasil, 2009).

For the cold tolerance test (CT), paper rolls were kept in a cold chamber at 5 ± 1 ºC. After 7 days, we evaluated the germination percentage.

Vigor index was determined with four replicates of 20 seeds distributed in the upper third of papers moistened as described above. Seedling performance was evaluated at 2, 4, 6, and 8 days after sowing (DAS). Root length (RL), shoot length (SL), and total seedling length (TSL) were measured with a digital caliper and expressed in millimeters (mm^{-1}). For obtaining root dry mass (RDM), shoot dry mass (SDM), and total seedling dry mass (TSDM), seedlings were dried at 80 ºC for 24 h and the results were expressed in milligrams (mg^{-1}) (Krzyzanowski et al., 2020).

Biochemical profile

The biochemical profile was carried out with three replications of 50 seeds at 0 (quiescent seeds), 2, 4, 6, and 8 DAS in water or 100 mM NaCl solution. The embryo and embryonic axis were excised from the quiescent seeds (T0), whereas. the embryonic axis and endosperm + scutellum were separated to evaluate samples at 2, 4, 6, and 8 DAS.

For determining the soluble sugar (SS) content, seeds were ground, sugars were extracted with 80% ethanol and quantified using the anthrone reagent and a standard curve according to Clegg (1956). The results were expressed in mg g^{-1} soluble sugar per seed dry weight. After soluble sugar extraction, pellets were dried, resuspended in perchloric acid, and the supernatant reacted with the anthrone reagent to obtain the starch content (ST) compared with a standard glucose curve (McCready et al., 1950). The results were expressed in mg g^{-1} starch per seed dry weight.

Phytic acid (PA) content was determined as described by Latta and Eskin (1980), using 3 mL of the extract and 2 mL of Wade’s reagent. The results were expressed in mg g^{-1} phytic acid per seed dry weight. Inorganic phosphorus (Pi) was extracted with 12.5% trichloroacetic acid (w/v) containing 0.025 M $MgCl_2$ as described by Wilcox et al. (2000) and quantified using the method of Chen, Toribara and Warner (1956) based on a standard curve with different concentrations of KH_2PO_4. The results were presented in mg g^{-1} of seed dry weight.

Total protein (TP) content was determined by an official method (Deutsch, 1986) multiplying the nitrogen content obtained by the factor 6.25. The results were expressed in mg.g^{-1} of total protein per seed dry weight. Soluble protein (SP) was extracted using sodium acetate buffer (240 mmol L^{-1} pH 5.5) containing 0.005% Triton X-100 (v/v) and 5 mmol L^{-1} of $CaCl_2$ and protein quantification was according to Bradford (1976). The results were expressed in mg.g^{-1} per seed dry weight.

The soluble protein extract was used to determine the activity of α-amylase (EC 3.2.1.1) and phytase (EC 3.1.3.8). α-amylase was assayed using the 3,5-dinitrosalicylic acid (DNS) method described by Miller (1959), following the procedure described by Sun and Henson (1991). Phytase was assayed following the method described by Engelen et al. (2001) modified by Ou et al. (2011). The results were expressed in units of enzyme per milligram of protein (U mg^{-1}). An enzyme activity unit is defined as the amount of enzyme required to produce 1 µmol of maltose (α-amylase) or phosphate (phytase) per minute under trial conditions.

Data analysis

The germination test, first germination count, and cold test data were transformed using arcsen $\sqrt{x}$ / 100 to meet the theoretical assumptions of the F-test (i.e., normality and homogeneity). The data were submitted to variance analysis and means were compared by the Tukey's test ($P \leq 0.05$). When a significant effect was detected, polynomial regression models using the statistical software Sisvar (Ferreira, 2011) were generated based on the statistical significance (F test) of the coefficient of determination (R2) as an indicator of model fit. Principal components analysis (PCA) was applied to identify the effect of vigor on physiological and biochemical responses using the R Statistical Software (R Core Team, 2023). Results were visualized using the 'factoextra' R package (Kassambara & Mundt, 2020).

Results and Discussion

High-vigor seeds (HV) showed better performance in the first germination count (FGC), germination (G), and CT compared to the low-vigor seeds (LV) in both CTR and SSC treatments (Table 1). Salt stress did not reduce the germination rate, however, it reduced the speed of germination, as observed in the FGC results. This delay in germination time may be generally attributed to osmotic stress in phase I and ionic stress in phase II (Uçarli, 2020). Salinity stress impairs seed germination either ionically through the formation of Na^+ and Cl^- ions or osmotically through reduced water absorption resulting in an imbalance in nutrient uptake and toxicity (Sharif et al., 2019; Beyaz & Dai, 2022).

Table 1: Germination (G) and vigor (First Germination Count - FGC and Cold Tolerance - CT) of maize seeds with high (HV) and low vigor (LV) subjected to salt stress.

Vigor level	G (%)		FGC (%)		CT (%)
	CTR	SSC	CTR	SSC	
HV	98 aA	98 aA	95 aA	79 aB	96 a
LV	85 bA	85 bA	69 bA	32 bB	58 b
CV (%)	1.61		3.03		7.85

Significant differences (Student's t-test P<0.05) in columns and rows are indicated by lowercase and uppercase letters, respectively. SSC: salt stress condition. CTR: control. CV: Coefficient of variation.

The high-vigor seeds showed the highest seedling performance compared to low-vigor in the tested conditions (CTR and SSC), regardless of the germination time (Figure 1). Here, salinity stress decreased total seedling length by 33% (Figure 1a, b) and root length by 61% (Figure 1c, d), independent of the vigor level.

At 2 DAS, seedlings from high-vigor seeds showed higher shoot dry mass (SDM), root dry mass (RDM), and total seedling dry mass (TSDM) in both evaluated conditions (Figure 2). However, at 8 DAS, salt stress decreased SDM by 84%, RMD by 42%, and TSDM by 62% in seedlings from high vigor seeds compared to control. In low-vigor seedlings, this decrease was 68% in SDM, 32% in RDM, and 50% in TSDM (Figure 2). Salt stress reduced the conversion rate of seed storage reserves and increased the root/shoot ratio (Khalid et al., 2023; Li et al.; 2022).

Seedling emergence constitutes the most critical stage in the plant life cycle. In this study, high-vigor seeds showed better seedling performance compared to low-vigor seeds under salt stress. This abiotic stress reduces physiological growth parameters (i.e., seedling length and dry mass) (Shanko et al., 2016).

Seed reserves are usually hydrolyzed and mobilized faster in high-vigor seeds, providing low-molecular weight compounds acting as building blocks and energy supply for seedling formation. High-vigor seeds showed higher total protein hydrolysis and better seedling performance compared to low vigor in both growth conditions (CTR and SS) (Figure 3). Protein hydrolysis releases amino acids that will form new proteins (with enzymatic or structural roles), are sources of carbon skeletons, and supply energy for metabolic activites (Feenstra et al., 2017). The higher total protein hydrolysis increased the availability of soluble proteins in the endosperm after 4 DAS (Figure 3c, d) for high-vigor seeds in the control and stress conditions. This may contribute to high-vigor seeds overcoming salt stress faster.

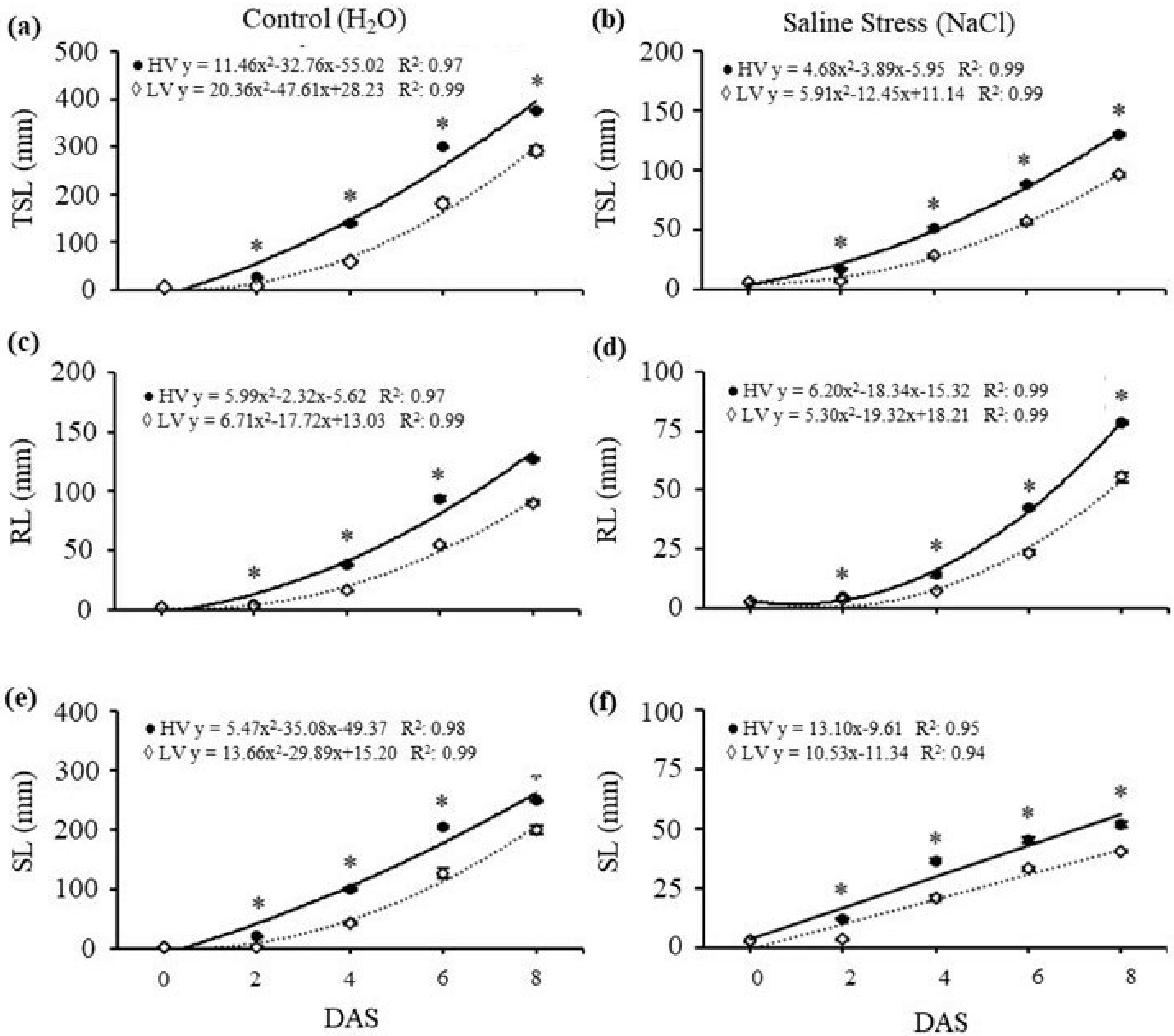

Figure 1: Seedling performance (total seedling length – TSL, root length – RL, and shoot length - SL) of maize seeds with high vigor (HV) and low vigor (LV) subjected to salt stress. At each time point, significant differences between HV and LV are indicated by asterisks (Student’s t-test P<0.05).

We observed a decrease in soluble protein content in the endosperm of low-vigor seeds subjected to salt stress after 4 DAS (Figure 3d). Soluble proteins include enzymes and transport proteins. The faster decrease in SP shown by the high-vigor seeds may be associated with *de novo* synthesis and organic components (Figure 3e).

High-vigor seeds also showed higher phytase activity at 2 DAS in control and salt stress conditions (Figure 4a, b). A positive association exists between phytase activity and phytate content (Bouajila et al., 2019), also observed in our study (Figure 4a, b, c, d). The earlier phytase activity favored Pi availability in the endosperm for metabolic reactions during germination (Figure 4e) with mobilization to the embryonic axis (Figure 4g). The main difference in inorganic phosphorus content in the embryonic axis between the experimental conditions and the seed vigor levels was observed at 2 DAS (Figure 4c, d). The higher phytase activity favored minerals and phosphorus availability, resulting in better seedling performance (Dong et al., 2020).

Low-vigor seeds had a higher available phosphorus content from 4 DAS onwards. This result may indicate lower use efficiency and greater demand for supporting the metabolism. This inefficient phosphorus use can be reflected in seedling performance, as shown by the lower dry mass and length in both conditions (Figure 1, Figure 2).

We observed a reduction in seed starch (ST) content during germination regardless of vigor and tested condition (Fig. 5a, b). Starch is a source of soluble sugars for growth points in the embryonic axis. α-amylase is the most important enzyme in starch hydrolysis (Feenstra et al., 2017) and is crucial during the initial phase of germination. High-vigor seeds showed higher α-amylase activity at 2 DAS (Figure 5c, d), whereas in low-vigor seeds the higher activity was observed at 6 DAS.

This increase in starch hydrolysis resulted in higher soluble sugar content in the endosperm (Figure 5e) and the embryonic axis (Figure 5g). Starch hydrolysis was similar between high-vigor and low-vigor seeds under salt stress. However, high-vigor seeds showed greater availability of soluble sugars in the endosperm and scutellum from the 4 DAS onwards (Figure 5f) and a higher ability to mobilize soluble sugars to the embryonic axis (Figure 5g). The availability of sugars is essential to germination and seedling formation and this greater accumulation may explain the better physiological performance and vigorous seedling formation in high-vigor maize seed under salt stress (Figure 1 and Figure 2). Sugars such as sucrose, fructose, and trehalose function as osmoprotectants and osmotic regulators, protecting cell membranes and eliminating toxic reactive oxygen species (ROS) (Sami et al., 2016). Environmental stresses such as those caused by salinity damage the activity of the hydrolytic enzymes, and increase the levels of lipid peroxidation and ROS (Catão et al., 2020).

The PCA indicated differences in metabolic responses to salt tolerance in low and high-vigor seeds during germination (Figure 6). The two components (PC1 – 73.2% and PC2 – 13.7%) explained together 86.9% of the data variance.

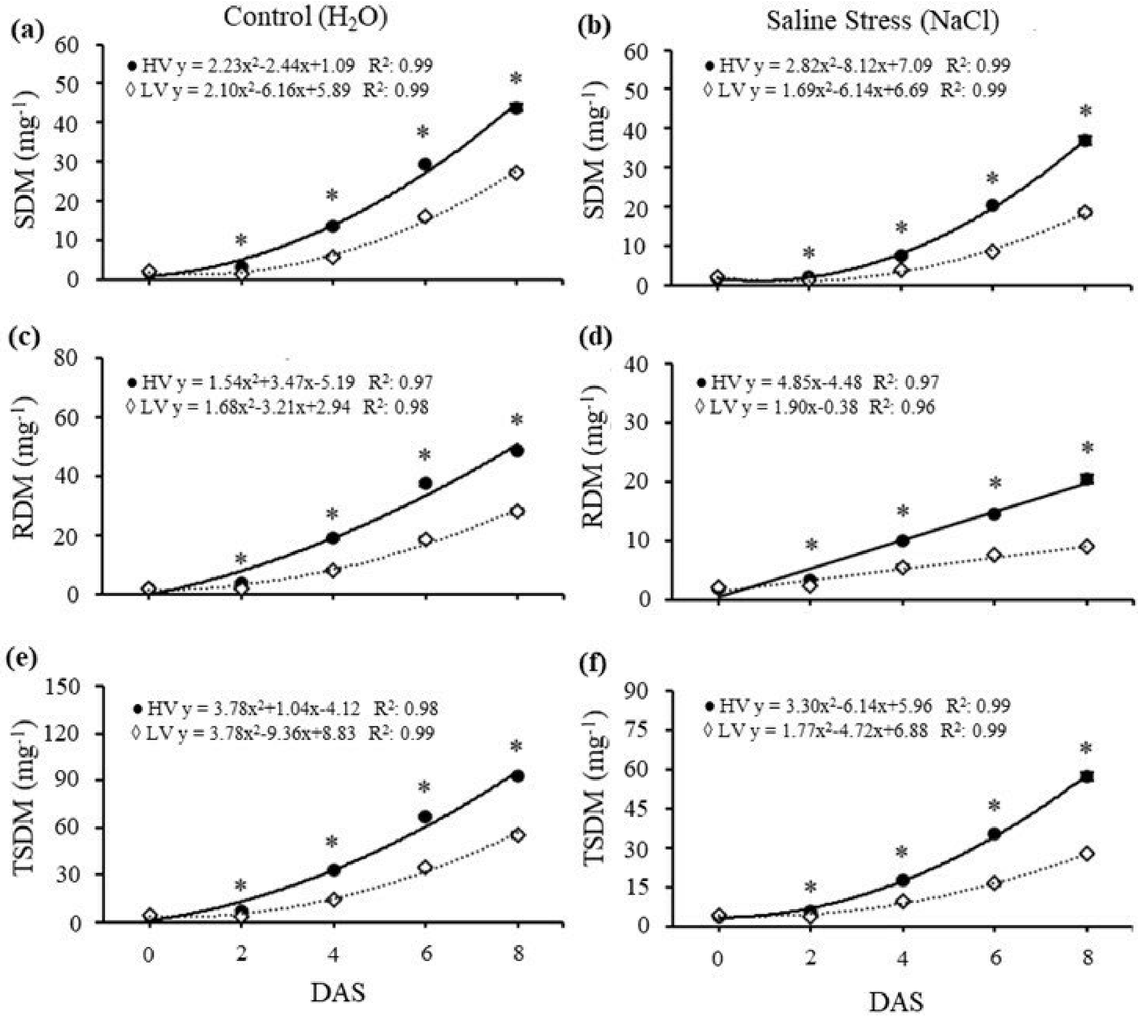

Figure 2: Shoot dry mass (SDM), root dry mass (RDM), and total seedling dry mass (TSDM) of high vigor (HV) and low vigor (LV) maize seeds subjected to saline stress. At each time point, significant differences between HV and LV are indicated by asterisks (Student's t-test P<0.05).

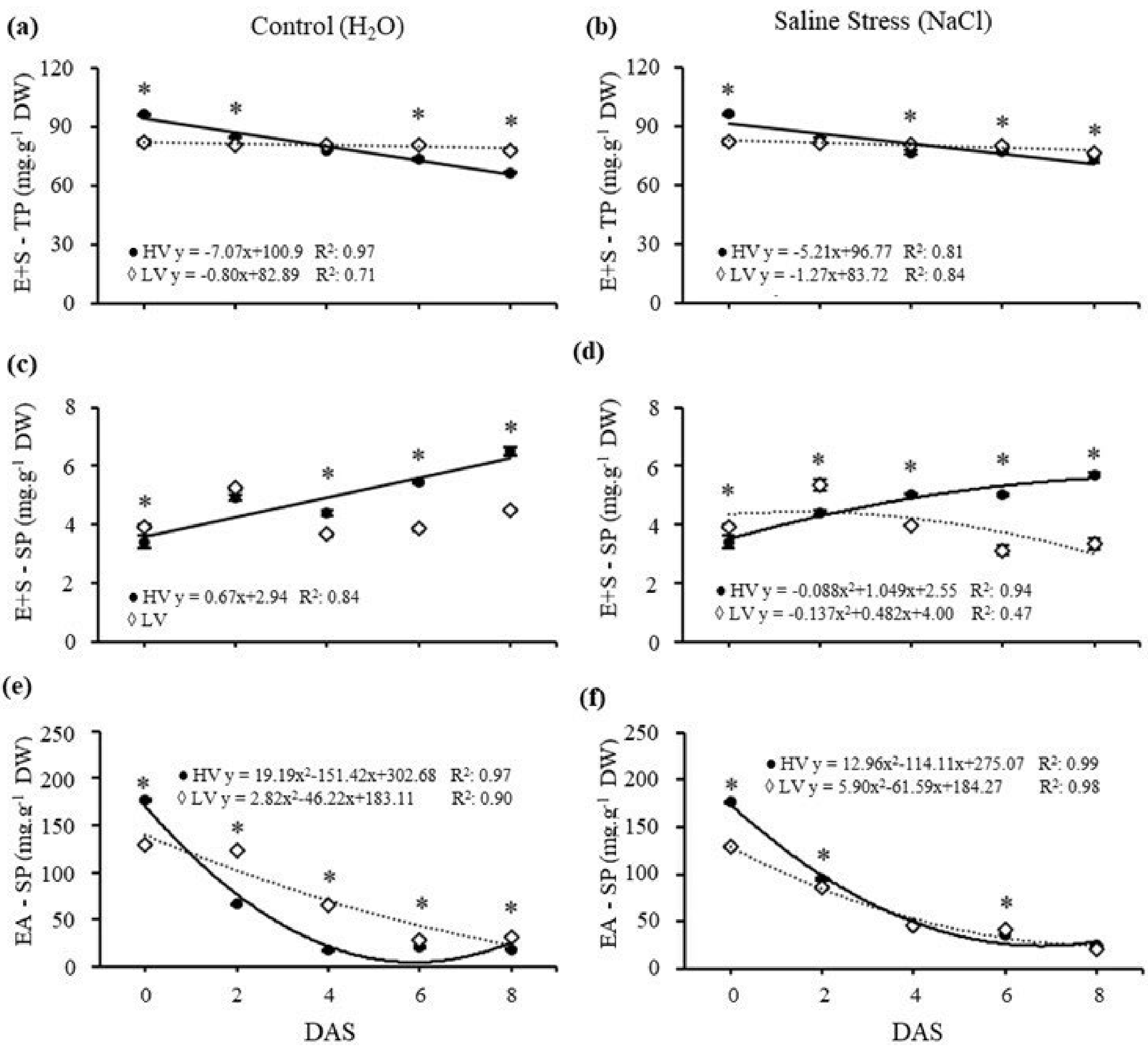

Figure 3: Total protein (TP) and soluble protein (SP) content in endosperm + scutellum (E+S) and embryonic axis (EA) of high vigor (HV) and low vigor (LV) maize seeds germinated under salt stress and control conditions. At each time point, significant differences between HV and LV are indicated by asterisks (Student's t-test P<0.05).

Growth parameters (RL, TSL, SL, DRM, TSDM, and SDM), SS, and Pi) positively influenced the separation of samples along PC1. Pi has a role in regulating and directing reserves (SS) in metabolism. In this way, the higher Pi and SS content are related to better seedling performance. We also observed that TP, ST, and FT negatively influenced the separation of samples in this axis.

SP, α-amylase, and phytase contributed to separate samples along PC2. TP, ST, and FT were related to initial germination (2 and 4 DAS). These results suggested that the reserve hydrolysis was lower in initial time points.

Total protein content was associated with low vigor at 4 DAS in seeds subjected to salt stress. Soluble protein (SP) content was decisive for the improved seedling performance in high-vigor seeds. Storage proteins are the main source of amino acids during the early stages of germination. High-vigor seeds have quickly hydrolyzed proteins into amino acids, mobilizing them into growth. metabolism. Our results confirm that reserves are more efficiently mobilized to form seedlings in seedlings with greater vigor (Nerling, Coelho, & Brümmer, 2022).

From 4 DAS onwards, the metabolic differences between the lots were accentuated. Low-vigor seeds in the control condition showed higher phytase and α-amylase activity at 6 DAS. In the salt stress condition, low-vigor seeds showed higher activity of hydrolytic enzymes at 8 DAS, indicating a delay in seed metabolism and physiological performance.

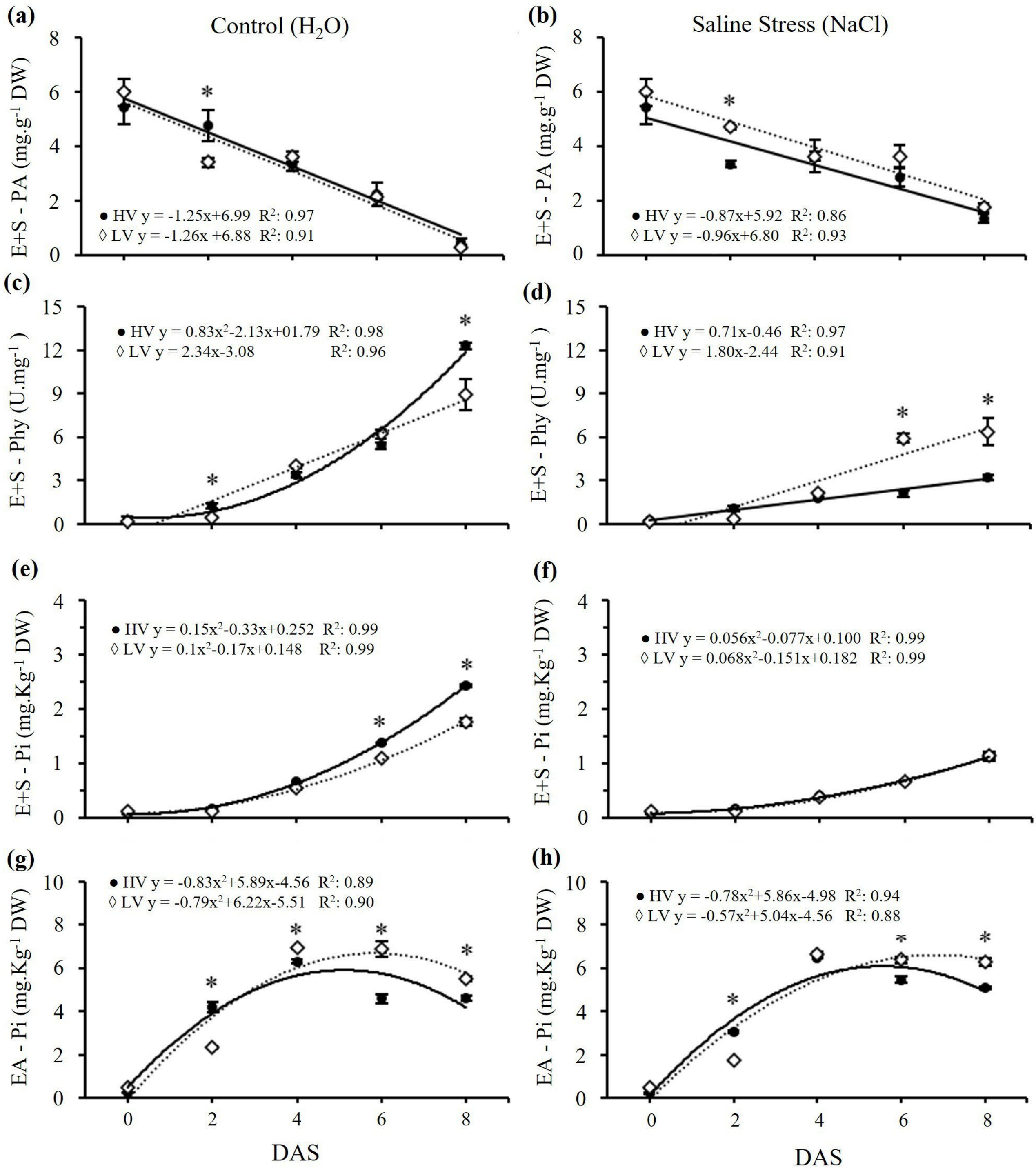

Figure 4: Phytic acid (PA), inorganic phosphorus (Pi) content, and phytase activity (Phy) in endosperm + scutellum (E+S) and embryonic axis (EA) of high vigor (HV) and low vigor (LV) maize seeds germinated under salt stress and control conditions. At each time point, significant differences between HV and LV are indicated by asterisks (Student's t-test P<0.05).

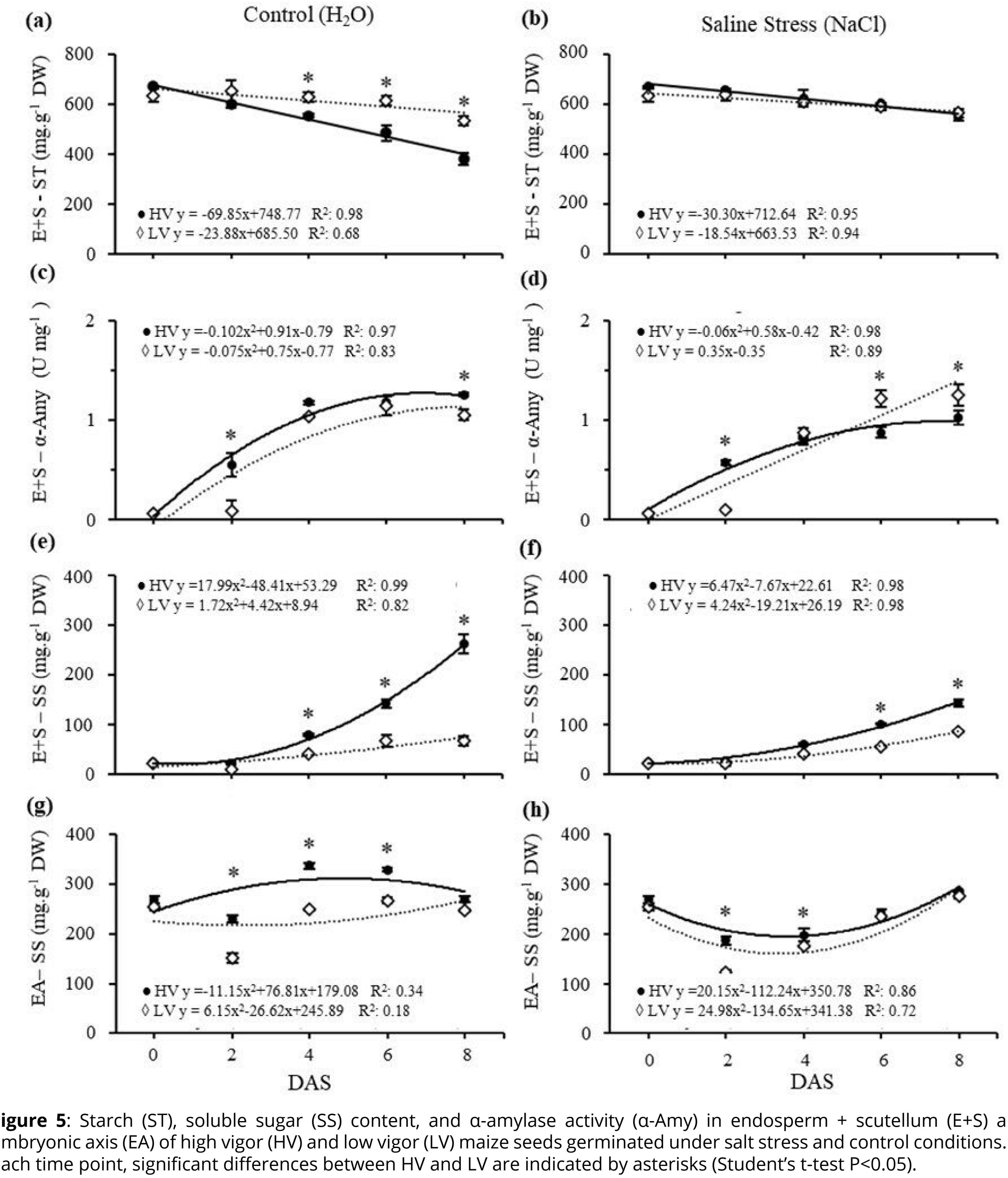

Figure 5: Starch (ST), soluble sugar (SS) content, and α-amylase activity (α-Amy) in endosperm + scutellum (E+S) and embryonic axis (EA) of high vigor (HV) and low vigor (LV) maize seeds germinated under salt stress and control conditions. At each time point, significant differences between HV and LV are indicated by asterisks (Student's t-test $P<0.05$).

One of the main goals of this study was to identify how reserve mobilization is associated with seedling performance in salt stress. NaCl concentrations affected the germination and maize seedling development. The reserve hydrolysis rate and the availability of soluble compounds were anticipated in high-vigor seeds compared to low vigor (Figure 7). Hydrolysis rates changed throughout germination, and the metabolism of high and low-vigor seeds was different over the period evaluated. We observed that the mobilization rate of soluble protein and Pi remained high for a longer time in high-vigor seeds (up to 4 DAS and from 6 DAS onwards). In contrast, the highest mobilization of SP and Pi was observed late in low-vigor seeds. These differences indicate that the efficiency in the reserve hydrolysis is associated with seed vigor. The longer seed germination time can affect seed performance in the field, compromising the speed, emergence uniformity, and establishment of seedlings.

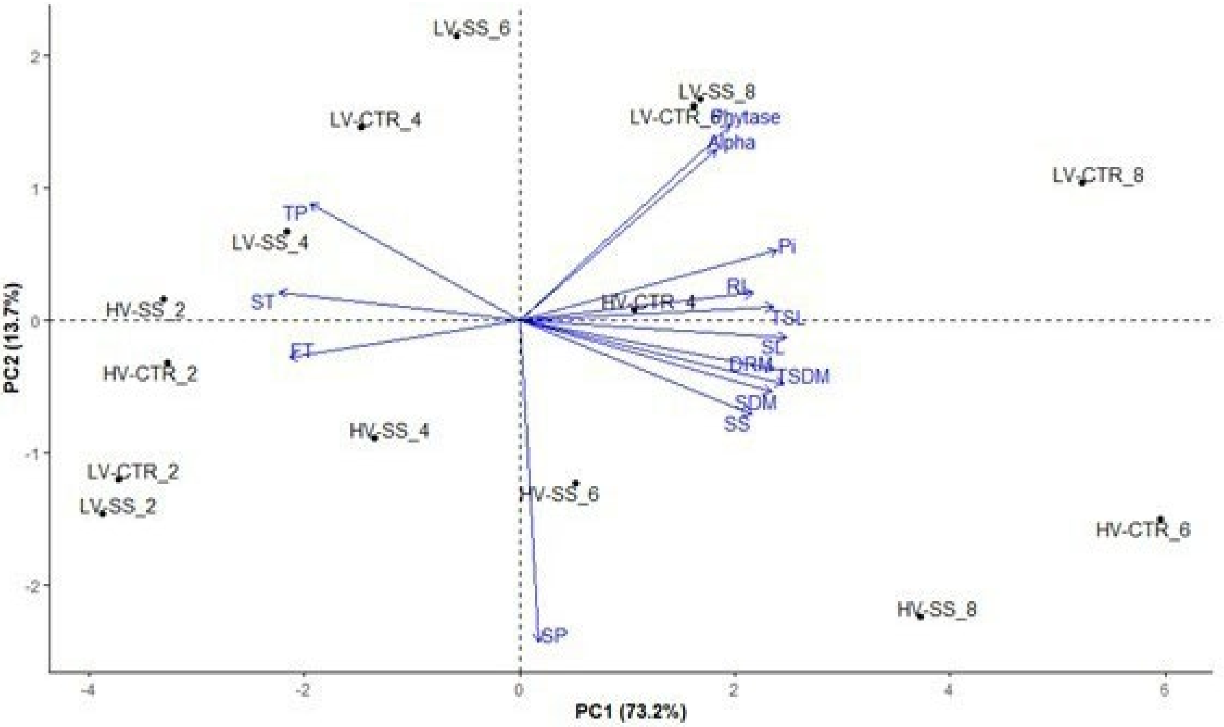

Figure 6: Principal component analysis (PCA) of growth parameters and metabolites of high vigor (HV) and low vigor (LV) seeds during germination in water (CTR) and saline solution (SS). The time points evaluated were at 2, 4, 6, and 8 DAS. PC1 = first principal component; PC2 = second principal component.

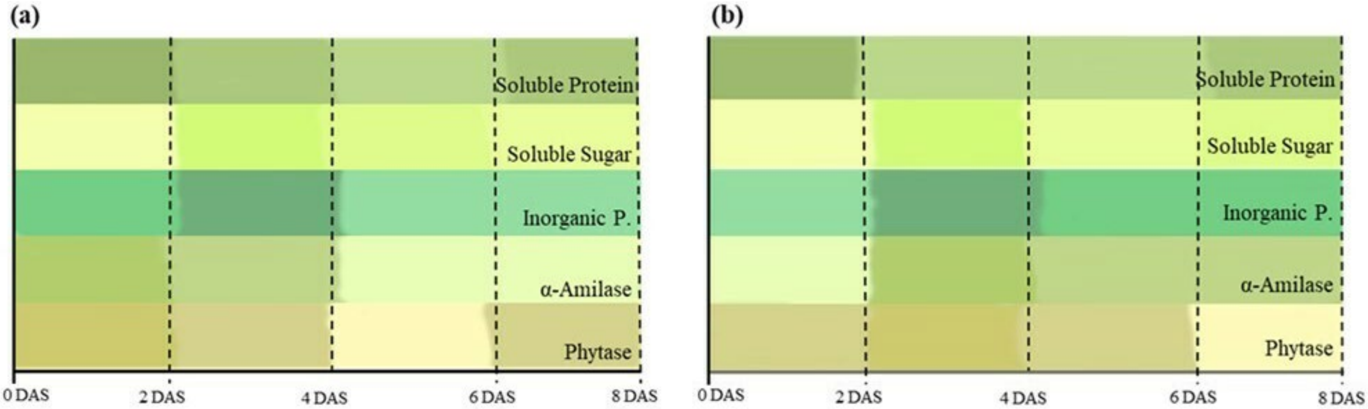

Figure 7: Time course of germination and changes in the rate of reserve hydrolysis in high vigor (a) and low vigor (b) seeds. The darker color indicates a higher hydrolysis rate .

Conclusions

The high-vigor seeds grew better and the resulting seedlings had stronger performance and faster hydrolysis capacity, which led to rapid reserve mobilization and use under salt stress. This response is influenced by higher activity of α-amylase and phytase.

Author Contributions

Conceptual idea: Coelho, C.M.; Sommer, A.S., Methodology design: Sommer, A.S.; Padilha, M.S., Data collection: Sommer, A.S.; Padilha, M.S., Data analysis and interpretation: Sommer, A.S.; Padilha, M.S.; Nerling, D., Writing and editing: Sommer, A.S.; Nerling, D. Nerling, D.

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