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The hydrolysis dynamic of storage reserves in maize seed germination helps to explain differences in inbred lines and hybrid seed vigor

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ABSTRACT: Storage reserves composition is directly related to the manifestation of seed vigor. However, the physiological potential expression in inbred lines, as well as hybrids from the storage reserves hydrolysis, is not clear. Thus, the aim of this study was focused on verifying if there are differences in the hydrolysis of seed storage reserve of maize inbred lines or hybrids during germination, and also checking if the changes in hydrolysis are associated with seed vigor. The seeds of inbred lines and hybrids were submitted to germination test, vigor by accelerated aging and cold tolerance. Biochemical profiling (total protein content, soluble proteins, phytate, inorganic phosphorus, starch, and soluble sugars), was temporally determined at 0 h (quiescent seeds), 12 h, 24 h, 30 h, and 48 h after imbibition. The seeds of inbred lines showed a longer time for root protrusion and less vigor when compared to hybrids. The highest hydrolysis rates in inbred lines were observed in phase I and at the end of phase II of germination. In hybrids, the rates remained, on average, constant throughout germination. The greatest differences in the hydrolysis of reserve compounds occur in phase II of germination and differentiate the germination metabolism of hybrids and inbred lines.

Index terms: metabolism, physiological potential, root protrusion, *Zea mays* L.

RESUMO: A composição das reservas está diretamente relacionada à manifestação do vigor da semente. No entanto, a manifestação do potencial fisiológico de linhagens e híbridos a partir da hidrólise de reservas não está elucidada. O objetivo neste trabalho foi verificar se há diferenças na hidrólise de compostos de reserva de sementes de linhagens ou híbridos de milho durante a germinação e verificar se eventuais alterações estão associadas ao vigor de sementes. A qualidade fisiológica foi avaliada pelo teste de germinação e de vigor por envelhecimento acelerado e teste de frio. A dinâmica de hidrólise foi determinada durante a embebição e germinação temporalmente em 0 h (sementes quiescentes), 12 h, 24 h, 30 h e 48 h por meio da determinação do conteúdo de ácido fítico e fósforo inorgânico, proteína total e solúvel, amido e açúcar solúvel. As linhagens apresentaram maior tempo para protrusão radicular e menor vigor em relação aos híbridos. As maiores taxas de hidrólise em linhagens foram observadas da fase I e ao final da fase II da germinação. Nos híbridos, as taxas se mantiveram, em média, constantes ao longo da germinação. As maiores diferenças na hidrólise dos compostos de reserva ocorre na fase II da germinação e diferenciam o metabolismo germinativo de híbridos e linhagens.

Termos para indexação: metabolismo, potencial fisiológico, protusão radicular, *Zea mays* L.

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INTRODUCTION

Seed germination is the beginning stage of the crop life cycle. It's strongly related to seedling survival rate and grain yield (Han et al., 2017). Germination *stricto sensu is* a complex process from water uptake by quiescent seeds (imbibition) to root protrusion (Rajjou et al., 2012). Water uptake actives storage reserves hydrolysis and compound mobilization to seedling growth. The water uptake pattern is divided into three phases and can provide information about the physiological and metabolic processes that occur during germination. Phase I is a rapid water uptake phase (Lopes et al., 2013), DNA damage repairing, resuming seed respiratory metabolism (Han et al., 2013). Phase II is a plateau phase, mitochondria synthesis and translation of storage mRNA occurred. Phase II is considered an active phase of metabolism which reserves mobilization is initiated. Phase III is the post-germination stage characterized by root protrusion. Mobilization of reserves is one of the most critical events in germination, they provide precursors and energy for the biosynthetic processes (Han et al., 2013).

Although mobilization of seed reserves is considered a post-germination process, some studies indicate that hydrolysis and mobilization of reserves occur during germination (Ehrhardt-Brocardo and Coelho, 2022). Storage reserves hydrolysis is induced by gibberellin secretes into the endosperm. These hormones induce the development of hydrolytic enzymes such as α-amylase and β-amylase in the aleurone layer (Rosental et al., 2014, Galland and Rajjou, 2015).

Starch, present in greater amounts in cereals, has greater hydrolysis among the reserves used during germination in *Sorghum bicolor* (Elmaki et al., 1999) and *Avena sativa* (Chen et al., 2016). During seed germination, starch is hydrolyzed into glucose by the action of the α-amylase enzyme. Hydrolyzed products are transported to the vascularized region of the scutellum, which connects to the phloem for embryonic axis growth (Han et al., 2017). Storage proteins are hydrolyzed into amino acids by proteolytic enzymes. Amino acids may remain in storage tissues; however, most are translocated to developing embryonic axis tissues, being used for the synthesis of enzymes and structural proteins (Rosental et al., 2014). Phytic acid is the major storage form of phosphorus in maize seeds. Phytic acid is hydrolyzed by the action of phytase, producing inositol and phosphates that are remobilized for the growing embryo (Nadeem et al., 2014).

Germination is influenced by genetic, environmental and seed endogenous factors (Joosen et al., 2013). The genotype influences the composition, hydrolysis and mobilization of storage reserves during germination, besides seed vigor. Seed vigor depends on the correct synthesis and accumulation of reserves, such as mRNAs and proteins, which enable efficient reactivation of cellular metabolism after seed hydration. This efficient reactivation active mechanisms that combat deterioration, with antioxidant and DNA repair responses, converging to obtain vigorous seedlings (Rajjou et al., 2012; Han et al., 2013).

The relationship between seed reserves and germination was studied in *Triticum aestivum* (Han et al., 2017), *Oryza sativa* (Cheng et al., 2015; Sun et al., 2015; Hu et al., 2016; Cheng et al., 2018), *Sorghum bicolor* (Yang et al., 2016), *Glycine max* (Pereira et al., 2015; Bellaloui et al., 2017). Also, in model species like *Medicago truncatula* (Vandecasteele et al., 2011), *Arabidopsis thaliana* (Shrestha et al., 2016) and in six wild grassland species (Zhao et al., 2018). In maize some authors studied the relationship of seed reserve with germination and vigor. Nerling et al. (2018) verified that seeds with higher content of inorganic phosphorus and soluble sugars have superior physiological quality. Prazeres and Coelho (2020), observed that vigorous hybrids was more efficient in mobilizing proteins and soluble sugars during germination under water deficit. Andrade et al. (2019) verified that hybrids genotypes with higher seed reserve utilization efficiency have higher vigor.

There are important advances in understanding the mechanisms of storage reserves hydrolysis and its relationship with germination. However, there may be differences in the reserve hydrolysis in hybrids and maize lines, and this difference can be associated with seed vigor. Thus, the aim of this study was focused on verifying if there are differences in the hydrolysis of seed storage reserve in inbred lines and hybrids during germination, and also checking if the changes in hydrolysis are associated with seed vigor.

MATERIALS AND METHODS

The study was carried out with five maize lines and five simple hybrids, obtained in a breeding program of a cooperative located in São Miguel do Oeste – SC, Brazil in the 2014/2015 harvest. The maize ears were collected and threshed manually, seeds dried in the shade until they reach 13% of moisture. A representative sample of seeds of each genotype was individually homogenized to obtain a working sample (900 g) (Brasil, 2009). Work samples were stored in a cold chamber with a relative humidity of 50% and 12 °C temperature until 2016 when the analysis was performed.

Determination of the standard curve of hydration was performed by observing the change in seed moisture content during germination until at least 50% of seeds (T50) in each replication presented radicle protrusion (2 mm). The moisture content was determined in quiescent seeds (0 h) and at predetermined hydration times: 12 h, 24 h, 30 h, and 48 h. At each time point, seed water content was measured by taking as a reference the standard oven method at 105 ± 3 °C for 24 h (Brasil, 2009).

The physiological quality of seeds was evaluated by germination test, cold tolerance, and accelerated aging tests. The germination test consisted of four subsamples of 50 seeds and was conducted in the vertical position at 25 ± 1 ^{o}C. Assessments of normal seedlings were carried out five and eight days after the test, as indicated in the Rules for Seed Testing (Brasil, 2009). The accelerated aging test was conducted, using four replications of 50 seeds each, which were distributed on aluminum screens fixed on the inside of plastic boxes and 40 mL of water added. The boxes were closed and kept in an aging chamber for 72 h at 45 °C, according to Marcos-Filho (2015b). After this period, the seeds were germinated at 25 °C and, on the fourth day, normal seedlings counting was carried out. The cold tolerance test was conducted using four replications of 50 seeds each. The seeds were placed between sheets of germitest paper, moistened with distilled water, and kept in a cold chamber at 5 ^{o}C for seven days, after this period, seeds were germinated and the normal seedlings were evaluated similarly to the germination test.

The hydrolysis of storage reserve of each line and hybrid was carried out according to a standard curve of hydration, at hydration times: 0, 12, 24, 30, and 48 h. Phytic acid (PA) content at each time was determined as described by Latta and Eskin (1980), using 3 mL of the extract and 2 mL of Wade's reagent. Readings were taken in a spectrophotometer at 500 nm. The results were expressed in $mg.g^{-1}$ of phytic acid per dry weight seed. The inorganic phosphorus (PI) content was determined using 0.100 g of dried ground seeds. The sample was extracted twice, for two minutes, with 4 mL of 12.5 % trichloroacetic acid (w/v) in 0.025 M $MgCl_2$. Each extract was centrifuged at 10,000 g_n for 10 min and filtered through Whatman number 1 filter paper (Raboy and Dickinson, 1984). The filtered extracts were combined, diluted to 12.5 mL and inorganic phosphorus was determined colorimetrically, according to Chen et al. (1956). Results were expressed in $\mu g.g^{-1}$ of inorganic phosphorus per dry weight seed. Total protein (TP) content was determined by an official method (AOAC, 1995), and results were expressed in $mg.g\text{-}^{1}$ of total protein per dry weight seed. Soluble protein (SP) extraction was performed as described by Azevedo et al. (1998) and Garcia et al. (2006). Soluble protein determination was care out according to the Bradford method (1976). The results were expressed in $mg.g\text{-}^{1}$ of soluble protein per seed fresh weight. For soluble sugar (SS) content determination, seeds were ground and extracted with 80% ethanol, using anthrone reagent according to Clegg (1956). The solution was then allowed to cool and its absorbance was measured at 620 nm. The results were expressed in $mg.g\text{-}^{1}$ of soluble sugar per dry weight seed. For starch (ST) determination, after sugar extraction, each of the sample pellets was dried, resuspended in 20 mL H_2SO_4 0.2N, and boiled for 2 h. The supernatant was reacted with anthrone reagent according to Clegg (1956) and read in a spectrophotometer at 620 nm. The results were multiplied by 0.9 (glucose conversion factor to starch) and were expressed in $mg.g\text{-}^{1}$ of starch per dry weight seed.

The changes in hydrolysis rates of storage reserves were calculated as (C2–C1) x 100/C1, C1 represents the content at quiescent seeds (0 h) and C2 represents the content at the four subsequent sampling times (12 h, 24 h, 30 h, and 48 h) during germination and early seedling growth. Analysis of variance (ANOVA), Scott Knott's test at 5% probability, and principal component analysis (PCA) were applied to identify the effect of lines and hybrid combinations on physiological and biochemical responses. Statistical analyzes were implemented in R software (R Core Team, 2016).

RESULTS AND DISCUSSION

The water content of the seeds was evaluated over 48 h and allowed to identify the standard curve of hydration of seed, was observed that inbred lines and hybrids of maize have a triphasic model of hydration (Figure 1A). After sowing, the seeds quickly absorbed water, characterizing phase I of germination, completed after 12 h of hydration. Root protrusion (T50) occurred at the end of phase II, after 30 h of hydration in hybrids and 36 h in inbred lines. After 30 h of hydration, the inbred lines showed a statistically higher moisture content (45.1%) compared to hybrids seed (42.0%) (Figure 1A). Phase III was marked by an increase in water absorption from the seeds.

The difference in hydration behavior among inbred lines and hybrids may be related to the physiological quality of the seeds. The speed of root protrusion can influence the speed of seedling formation. Seed with low vigor has slow germination, characterized by an increase in the period of water absorption until root protrusion (Marcos-Filho, 2015a). The inbred lines showed a longer time for root protrusion compared to hybrids seed. This difference in the pattern may be related to the lower physiological quality of the inbred lines seed.

There are no differences in inbred lines and hybrids germination percentages (Figure 1B). However, differences in seed vigor were verified. The hybrids showed low vigor, an average of 80% by AA test and 85% by cold test, compared to inbred lines, an average of 41% by AA and 77% by cold test (Figures 1C and 1D). These results indicate a positive correlation between rapid root protrusion and high seed vigor. Prazeres and Coelho (2017) also observed a rapid root protrusion in hybrids with high germination and vigor percentage. From the phytotechnical point of view, early

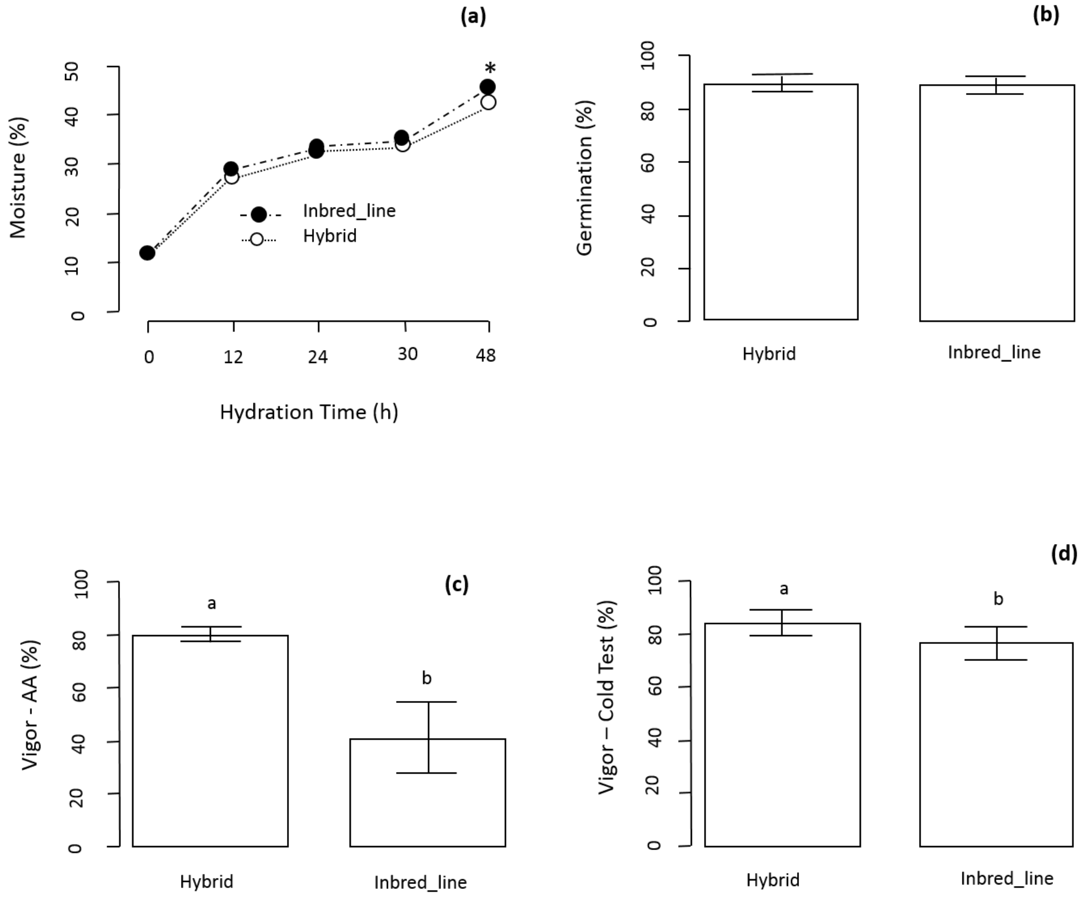

Figure 1. Hydration pattern (a), germination (b) and vigor by accelerated aging test (c) and cold test (d) in maize inbred lines and hybrids seeds.

germination allows for the rapid establishment of the stand. Besides enabling the overcoming of adverse conditions to which the seeds are subject to the field during the initial establishment phase.

Seed metabolism is activated to supply nutrients for the resumption of embryo growth after hydration (Marcos-Filho, 2015a). The rate of reserve hydrolysis during seed germination of inbred lines and hybrids are shown in Figure 2. The rate of hydrolysis of phytic acid (PA), total protein (TP), and starch (ST) in inbred lines and hybrids maize decreased with the germination stage. The PA hydrolysis rate was higher in inbred lines compared to hybrids (Figure 2A). After 48 h from the beginning seed hydration, the inbred lines and hybrids hydrolyzed 70% and 40% of the PA respectively, in relation to the initial content (quiescent seeds). We observed a higher PA hydrolysis rate in the first 12 h of hydration in

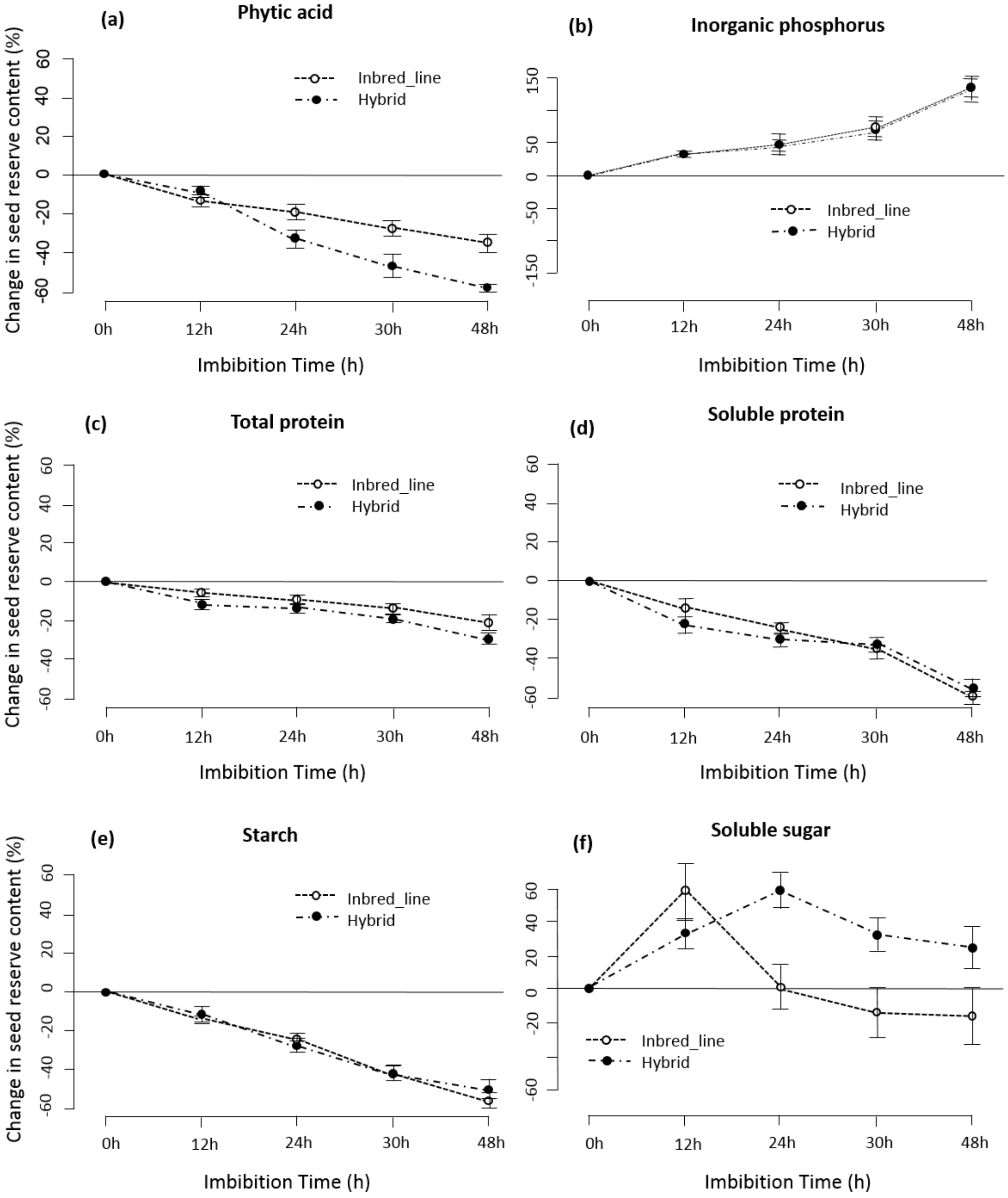

Figure 2. Change (%) in seed (a) phytic acid content, (b) inorganic phosphorus, (c) total protein, (d) soluble protein, (e) starch and (f) soluble sugar content during imbibition time.

hybrids. In inbred lines, the hydrolysis rate was higher in 24 h and remained higher until the end of the period evaluated (48 h). The time of 24 h, comprises the phase II of germination, which was lower for hybrids and larger for inbred lines. A larger phase II is associated with the longer period for the action of metabolic repair mechanism or indicating an incomplete repair (Bewley et al., 2013; Marcos-Filho, 2015a).

The decrease in PA can be attributed to the increase in phytase activity, which results in the formation of myo-inositol and inorganic phosphorus (PI) during germination. Despite the higher rate of PA hydrolysis observed in the inbred lines, the rate of PI availability was similar between hybrids and inbred lines, showing an increase in the germination process (Figure 2B). Hydrolyzed forms of phosphorus were temporarily stored in the seeds before being translocated to the developing embryo. This temporary phosphorus stored explains the increase of these compounds in seeds, both in inbred lines and hybrids. PA is an important antioxidant; thus, we speculate the prolongation of phase II and the higher rate of PA hydrolysis observed in inbred lines are indicate a higher period for the metabolic repair compared to hybrids, resulting in a higher time for root protrusion.

The total protein (TP) hydrolysis rate was higher in the inbred lines. After 48 h of hydration, inbred lines hydrolyzed approximately 30% of TP reserves while hybrid hydrolysis was 22% (Figure 2C). There were no differences between inbred lines and hybrids throughout germination for soluble protein (SP) (Figure 2D). Changes in protein content in germination indicate the existence of a dynamic regulatory process (Han et al., 2017). In this way, the higher TP hydrolysis inbred lines may be indicative of higher energy expenditure of these genotypes during the germination process, especially of compounds associated with *de novo* synthesis, DNA repair, and antioxident response. In this sense, the hybrids were more efficient in the hydrolysis and mobilization of proteins to the growing points than the inbred lines.

Starch (ST) hydrolysis rate was similar for strains and hybrids throughout germination (Figure 2E). From quiescent seeds (0 h) until 48 h of hydration, the ST content decreased 56% for the hybrids and 51% for the inbred lines. The ST hydrolysis occurs by the action of α-amylase, β-amylase, debranching enzyme, and α-glucosidase, forming simple sugars faster consumed by the growing embryo. Sugars and hormones play an important role in regulating germination (Ma et al., 2017). Genetic control associated with ST and sugar content in maize crosses was studied by Santos et al. (2017). These authors found that starch amounts between reciprocal crosses were not correlated with the vigor of the seed lot.

Inbred lines and hybrids had increased 28% and 43%, respectively, in the content of soluble sugar (SS) until 12 h of hydration (Figure 2F). The maximum content of SS was observed in 24 h of hydration in inbred lines, followed by a gradual reduction of the compost in the seeds. There was the maintenance of high levels of soluble sugars after 24 h in the inbred line seed. There was a demand for the compound, evidencing a higher time for cell organization and combat reactive oxygen species (ROS) in inbred lines. Sugars such as sucrose, fructose, and trehalose function as osmoprotectants and osmotic regulators, protecting the cell membrane and eliminating toxic ROS (Keunen et al., 2013; Sami et al., 2016). The maximum rate of hydrolysis of SS was observed in 12 h of hydration in hybrids, followed by a gradual reduction in subsequent periods. Thus, the period of cell organization and repair was faster in hybrids compared to inbred lines, indicated by anticipated root protrusion and greater seed vigor.

PCA was applied to identify similarities between genotypes and variables. Figure 3 shows the PCA of the biochemical profile of inbred lines and hybrids during germination. The genotypes were separated into two groups: inbred lines and hybrids, confirming differences in reserve metabolism between them throughout germination (Figure 3). The total variance explained by the two main components (PC) was 88.8%. PCA1 represented 65.2% of the total variance, with PI, ST, and SP having a high contribution to this component. PCA2 represented 23.6% of the total variance, with SS, PA, and TP with high contributions to this component.

We verified an association of ST, SS, PA, and PI with the inbred lines. These compounds are involved with energy and antioxidant metabolism. On the other hand, TP and SP are associated with hybrids (Figure 3). We hypothesize hybrids may have hydrolyzed proteins into amino acids, mobilizing them to grow points faster than inbred lines. This efficiency was reflected in the early root protrusion in the hybrids (Figure 1A). Genotypes with greater vigor are more efficient in using reserves to form normal seedlings (Ehrhardt-Brocardo and Coelho, 2016; Andrade et al., 2019). Maize plants from high seed vigor demonstrated a greater capacity to combat oxidative stress with the accumulation of proline and increased activity of antioxidant enzymes (Prazeres et al., 2021). According to Nerling et al. (2018), genetic divergence related to physiological quality and biochemical composition indicated that genetic diversity between inbred lines also leads to differences in metabolism associated with maize seed germination.

The biochemical profile analysis of the hybrids indicated different responses along the hydration times (Figure 3). It was possible to verify the formation of two groups in maize hybrids (Figure 3). The first group comprised the quiescent seeds, 12 h and 24 h of hydration, and the second group corresponds to 30 h and 48 h of hydration time. Quantitative hybrids traits TP and SP were the most important in the first group formation. Protein hydrolysis by the action of proteases synthesized in vacuoles produces amino acids that can be used in the synthesis of new amino acids used in germination (Bewley et al., 2013). Germination requires the coordinated induction of several non-uniform processes involving starch, lipids, and proteins distributed between the tissues and organs of the seeds (Feenstra et al., 2017), making the process complex.

The PCA of the inbred line seed metabolism throughout germination indicated different responses from that observed for the hybrids (Figure 3). The 12 h and 24 h of hydration proved to be similar and were influenced by carbohydrates and PA. It is possible to observe a higher association of ST, SS, and PA with 12 h time of hydration. We observed the highest rate of hydrolysis for PA, TP, and SP at 12 h in the inbred lines (Figures 2A, C, and D).

In our study, the SS showed a higher association with the phase II of germination. After 24 h there was a gradual increase in the SS rate. This increase is also consistent with the hydrolysis of starch in the inbred lines (Figure 2E). In maize metabolomics analysis of quiescent seeds and at four points during inbred lines germination, Feenstra et al. (2017) detected 162 analytes, of which 63 were chemically identified. The authors verified differences in the metabolome of the two inbred lines and alterations in this profile during the germination process. Our study found similar results.

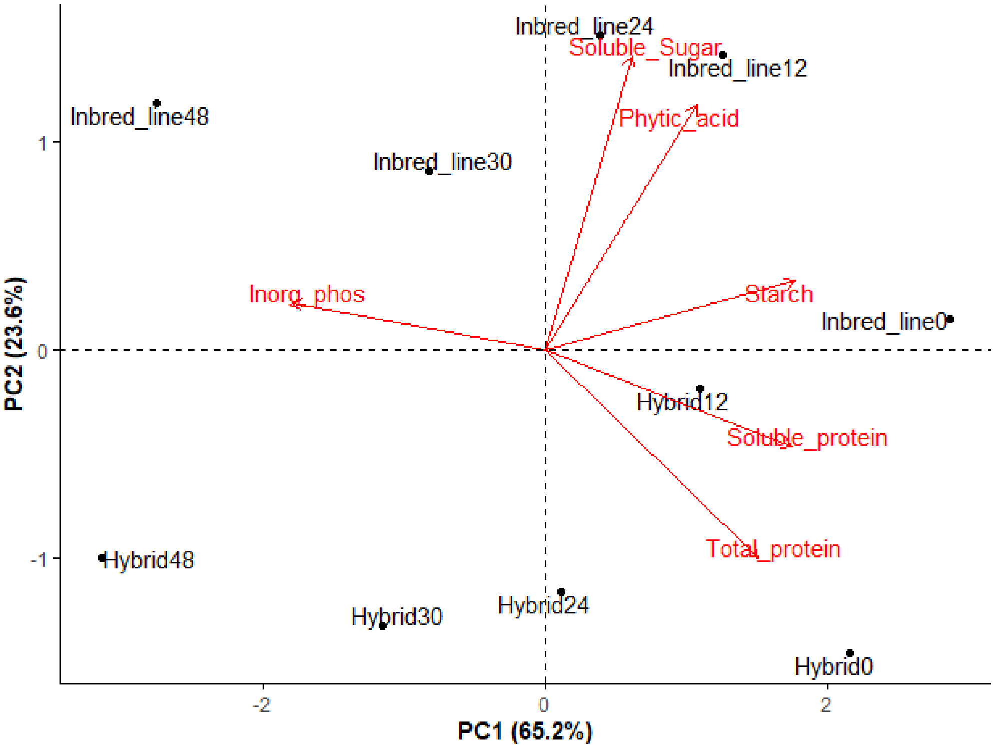

Figure 3. Principal component analysis (PCA) of metabolites of inbred lines and hybrids metabolite profiles during seed germination. Time periods include quiescent seed, 12, 24, 30 and 48 h. PC1 = first principal component; PC2 = second principal component.

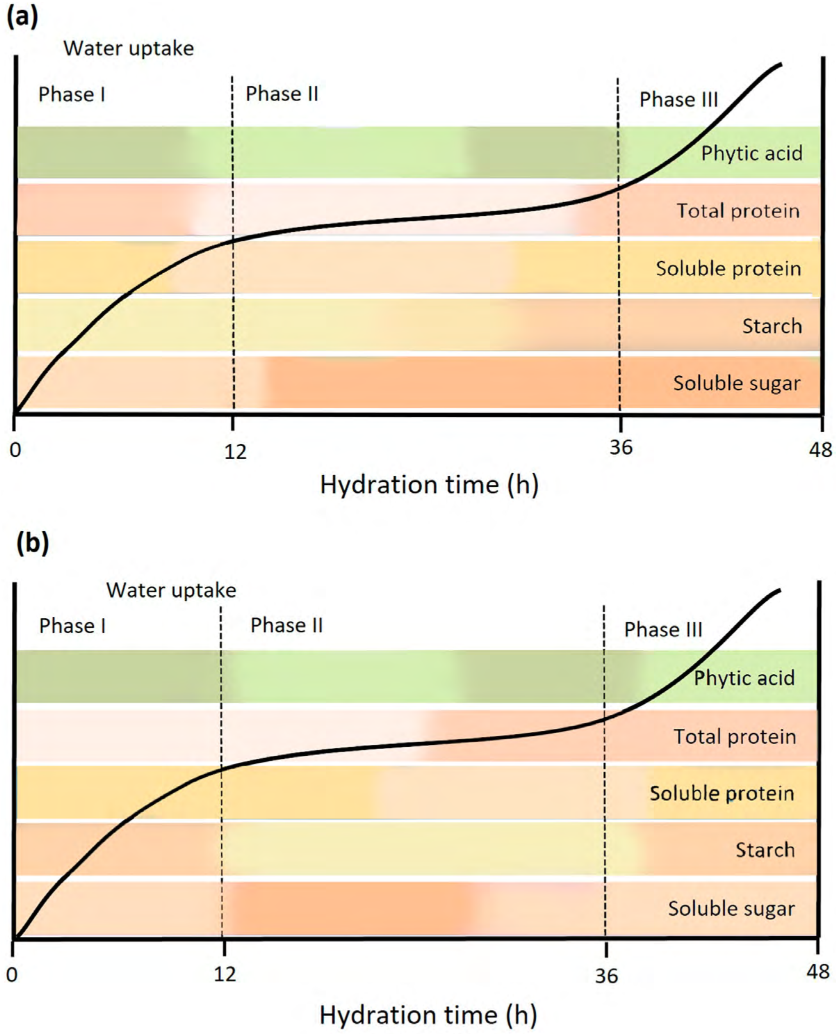

Figure 4. Time course of water uptake and changes in the rate of hydrolysis in reserve compounds associated with germination in inbred line (a) and hybrids (b) seeds. The darker color indicates where there was a higher rate of hydrolysis of compounds in the course of germination.

The main metabolic changes between lines and hybrids occurred in phase II of germination (Figure 4). In the inbred lines, the highest hydrolysis rates were observed in phase I and at the end of phase II of germination, near to root protrusion. In the hybrids, the hydrolysis rate remained distributed throughout the germination process. These differences indicate that the efficiency in the hydrolysis of accumulated reserves is associated with seeds vigor. Seed vigor is a complex property that determines its potential for the emergence and rapid and uniform establishment under a wide range of environmental conditions (Rajjou et al., 2012). The longer time for radicle protrusion can affect seed performance in the field, compromising the speed and uniformity of emergence and establishment of seedlings.

CONCLUSIONS

Maize hybrids show earlier root protrusion to the inbred lines. The higher differences in the hydrolysis of reserve compounds occur in phase II and differentiate the germination metabolism of hybrids and inbred lines.

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