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Proline and trehalose in maize seeds germinating under low osmotic potentials

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Key words:
Zea mays
germination
water restriction
osmoregulation
compatible solutes

ABSTRACT

Although it is relatively well known that adult plants tend to accumulate proline and trehalose in their tissues as a physiological mechanism in response to drought, there is scarce information about the development of this physiological response in seeds. Thus, the objective of this research was to verify if maize seeds are able to develop mechanism of osmoprotection, when are germinating under low osmotic potential, and the possibility to use the levels of trehalose and proline in a defined seed part, aiming to differentiate genotypes regarding drought tolerance. The experiment was performed as a factorial arrangement of 2 x 5 (2 hybrids x 5 osmotic potential) within a completely randomized design, with four replicates. It was found that the proline content in the embryo axis of maize seeds germinating under water limitation is directly proportional to the intensity of this stress. Distinct hybrids show different proline levels accumulated in the embryo axis, when seeds are germinating under the same conditions of water limitation. The trehalose content tends to decrease in the embryo axis and in the endosperm of maize seeds germinating under increasing water limitation, but the reduction is not directly proportional to osmotic potential.

Palavras-chave:
Zea mays
germinação
restrição hídrica
osmoregulação
solutos compatíveis

Prolina e trealose em sementes de milho germinando em baixos potenciais osmóticos

RESUMO

Embora seja relativamente bem conhecido que as plantas adultas tendem a acumular prolina e trealose como mecanismo fisiológico em resposta à seca, há pouca informação sobre o desenvolvimento desta resposta em sementes. Assim, esta pesquisa objetivou avaliar se sementes de milho são capazes de desenvolver mecanismos de osmoproteção quando postas para germinar em condições de baixos potenciais osmóticos, bem como a possibilidade de usar os níveis de trealose e prolina como indicadores bioquímicos para diferenciar genótipos quanto à tolerância à seca. O experimento constou de um esquema fatorial de 2 x 5 (2 híbridos x 5 potenciais osmóticos) conduzido em um delineamento inteiramente casualizado com quatro repetições. Observou-se que o teor de prolina no eixo do embrião de sementes de milho postas para germinar sob limitação hídrica, é diretamente proporcional à intensidade do estresse. Híbridos distintos mostram diferentes níveis de prolina no eixo embrionário quando as sementes germinam nas mesmas condições de limitação hídrica. O teor de trealose diminui, tanto no eixo embrionário como no endosperma de sementes de milho postas para germinar sob crescente limitação hídrica mas a redução não é diretamente proporcional à intensidade do estresse.

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INTRODUCTION

The understanding on drought tolerance in crop plants is a matter of great relevance, once water limitation is one of the greatest constraints for agriculture (Rodriguez-Salazar et al., 2009; Xoconostle-Cázarez et al., 2010). Maize is one of the most important commodities produced worldwide (FAO, 2014) and drought has a strong limiting effect on seed germination, on plant development and on the yield of this crop. Thus, knowledge on mechanisms that enable maize cope with drought has a fundamental importance, especially considering that this crop provides significant part of human food across the world, which is under risk in a scenery of growing global warming. Although there are many studies regarding the strategies of plant drought tolerance, most part have focused mainly on parameters measured in the green tissues of plant shoots (Abo-El-Kheir & Mekki**,** 2007; Lopes et al., 2011). While there is no doubt that seed performance reflects on later plant development (Mondo et al., 2013), reports on the development of protective mechanism in seeds are still scarce in the literature (Thakur & Sharma, 2005; Mohammadkhani & Heidari, 2008; Špoljarević et al., 2011).

Based on studies with adult or young plants, it was stated that generally the response to water limitation is the development of an osmoprotection mechanism. This mechanism is initiated by an osmotic adjustment to keep plant metabolism running, at least at a minimum rate, and for a relatively short period of time under drought (Mohammadkhani & Heidari, 2008). This phenomenon comprises the synthesis of certain compatible solutes that may have a prime role on the protection of key proteins, on stabilizing cell membranes, and on the control of instable chemicals species (Mohammadkhani & Heidari, 2008, Xoconostle-Cázarez et al., 2010). In this case, those compatible solutes are often designated as osmoprotectors. Proline and trehalose are some of the main compatible solutes largely studied in the evaluation of plants to detect tolerance to drought, and often used as plant breeding tool to segregate sensible and tolerant genotypes (Trovato et al., 2008; Anami et al., 2009). Although trehalose has been considered as osmoprotector, lately some authors have reported that it should not be regarded only as a protective sugar, and that further investigation is required to elucidate its exact role in the stress tolerance in plants (Paul et al., 2008; Fernandez et al., 2010). It is also advocated that trehalose and proline are used as energy sources, and that proline may be metabolized as nitrogen source readily available for plant re-establishment just after the period of water limitation (Kavi Kishor et al., 2005; Trovato et al., 2008).

Although the seed is a plant part, it is different from other green plant organs, frequently studied in research about the development of osmoptotection mechanism. During their formation, the seeds are typically importer of photoassimilates, while green plant tissues are characteristically producers and exporters of those compounds (Ludewig & Flügge, 2013). However, during the germination process, the seeds, at the same time have some tissues working as a non-photossynthetic source (e.g. endosperm) and others as sink (e.g. embryo axis) of photoassimilates. Thus, our hypothesis is that when seeds are germinating they may have a distinct behavior compared to that of largely studied green tissues, regarding the capacity to develop osmoprotection mechanism. Moreover, it is possible that distinct seed tissues have different behaviors on this physiological response. This hypothesis is supported by the knowledge that the response and tolerance of crop plants to abiotic stress are quantitative traits controlled by many different genes (Lopes et al., 2011). This fact reinforces the idea that different organs, or even different tissues of a same plant organ, may have distinct behavior regarding the degree of response to water stress (Abo-El-Kheir & Mekki, 2007; Mohammadkhani & Heidari, 2008; Anami et al., 2009, Tardieu, 2012). Therefore, specific study to better understand this phenomenon in the seed are matter of great interest.

Under natural conditions, several factors affect seed germination simultaneously. Thus, it is very difficult to insulate the effect of a single factor (e.g. water deficit) to be studied under these conditions. As a way to circumvent this problem, water-stress conditions may be simulated in laboratory by using solutions with different osmotic potentials, which are usually obtained by adding distinct concentrations of polyethylene glycol (PEG) in the germination medium. PEG is often used for this purpose because it is chemically inert, non-toxic to the seeds, and works as an strong osmotic agent (Villela et al., 1991; Queiroz et al., 2000; Meneses et al., 2011).

Considering the aforementioned, this research was idealized with the objective to evaluate if maize seeds are able to accumulate proline and trehalose as mechanism of osmoprotection, when germinating under low osmotic potential. Also, to check the possibility to use the levels of trehalose and/or proline in a defined seed part, as a biochemical indicator to differentiate genotypes regarding drought tolerance.

MATERIAL AND METHODS

Treatments included seeds of 2 maize genotypes subjected to 5 levels of osmotic potential, composing 10 treatments, with 4 replicates. The assays were carried out with maize seeds of similar size, belonging to the single-cross hybrids: DKB-390 (Dekalb), and DAS-2B710 (Dow AgroScience). Before the assays, the seeds were treated with nistatine solution (1%) in order to guarantee their sanity during the tests. Each experimental unit was constituted of two plastic boxes (gerbox®), of size 11.0 x 11.0 x 3.5 cm, containing 50 seeds (25 seeds each box) deposited on a sterile germitest® paper sheet. The distinct osmotic potential treatments were imposed by soaking the paper sheet with: Sterile distilled water (control, corresponding to potential 0.0 MPa) or solutions with different PEG 6000 concentrations to obtain the osmotic potentials corresponding to: -0.3; -0.6; -0.9; and -1.2 MPa, following indications of Villela et al. (1991). All experimental units were randomly distributed in a germination chamber adjusted to temperature of 30 ± 2 °C, atmosphere with 60 ± 2% relative humidity, and photoperiod of 12 h, as indicated by Brasil (2009). Every 03 days, the seeds were transferred to new boxes containing the respective fresh treatments in order to mantain the desired osmotic potential constant. Germination evaluations were performed daily until 10 days after imposition of treatments, considering as germinated seeds those with primary root protrusion at least 2 mm long. These data were used to determine the germination index (GI) and the germination speed index (GSI), as proposed by Maguire (1962). The average germination time (ATG) was obtained according to that described by Edmond & Drapala (1958). Dry mass allocation (DM) among seed tissues was determined after 10 days of imposition of treatments. For this determination, the embryo axis and the endosperm were excised. The aerial part (when present) and the roots were cut off quite close to the surface of the seed coat. All these tissues were dried (by lyophylization) constant weight, and the dry mass was measured using an analytical scale. After that, the samples were ground in the presence of liquid nitrogen, before biochemical analysis. The proline content in the endosperm (EN) and embryo axis (EA) was determined according to the method proposed by Bates et al. (1973). The trehalose content was determined with the enzymatic method described by Neves et al. (1994), except that the trehalase used in this assay was obtained from the fungus *Rhizopus microsporus*, following indications of Queiroz et al. (2008). The statistical analysis of the data was performed in a factorial arrangement of 2 x 5 (2 hybrids x 5 osmotic potentials) within a completely randomized design, with four replicates. Data significance was assessed by analysis of variance (F test). Polynomial regression analysis was performed to better evaluate the quantitative effect of osmotic potential, and to study the interaction hybrid x osmotic potential (when significant ($p < 0.05$)). Figures were made by choosing the polynomial equation with the highest coefficient of determination (R^2), and highest F test significance (Barbosa & Maldonado Júnior, 2010). Also, Pearson's correlation was performed among studied variables.

Results and Discussion

In studies on the effects induced by a given stress, it is necessary initially to ensure that the stress was actually imposed at desired levels. With this concern, physiological measurements were carried out, and data showed that the imposed osmotic potentials were effective ($p < 0.01$) to induce changes on the measured variables, except for embryo axis dry matter (Table 1).

Except for GSI, ATG and proline in the endosperm, the osmotic potential had effect independent from the genotypes ($p > 0.05$). In the case of embryo axis dry matter, the interaction between hybrid and osmotic potential showed a low significant effect ($p < 0.05$), although no significant effect ($p > 0.05$) was observed for hybrids or osmotic potentials, independently (Table 1).

The restriction on water availability induced a reduction of about 47% for DKB-390 hybrid (DKB) and 52% for DAS-2B710 (DAS) in the germination index (GI) of seeds, considering data obtained at the lowest osmotic potential (-1.2 MPa, Figure 1A).

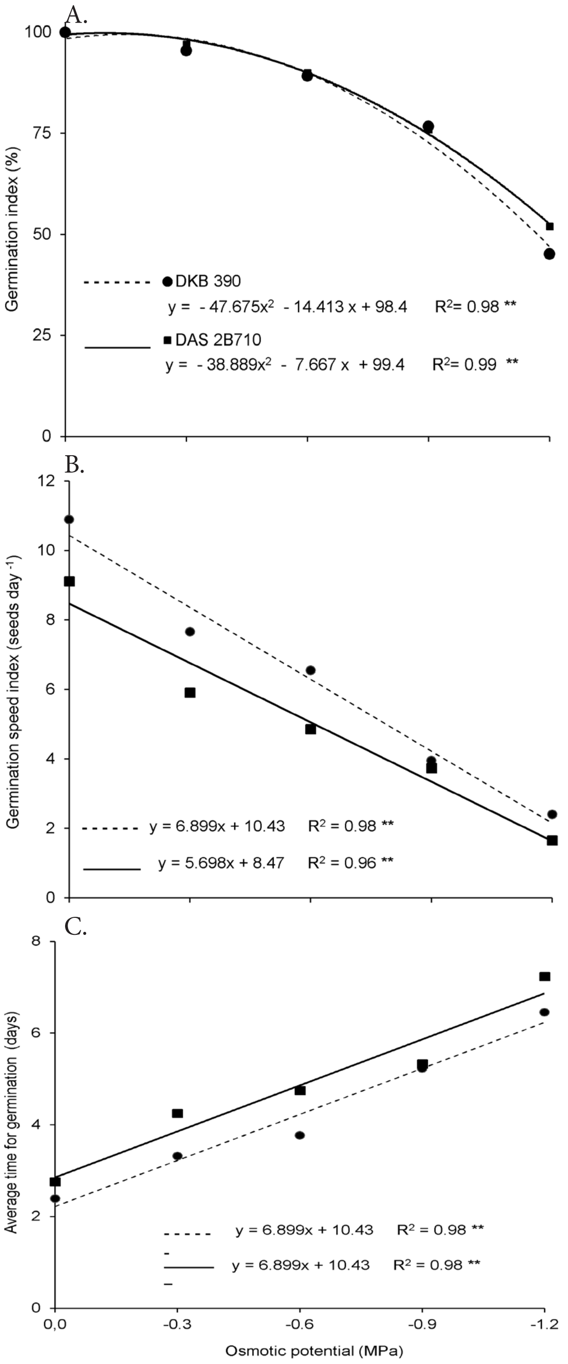

Figure 1. Germination Index (A), germination speed index (B), and average time for germination (C), for maize seeds of the genotypes DKB-390 and DAS-2B710, after germinating for 10 days under varying osmotic potential

Table 1. Analysis of variance (F test) for germination index (GI), germination speed index (GSI), average time for germination (ATG), and dry matter of shoot, root, endosperm and embryo axis

Variables	GI	GSI	ATG	Shoot	Root	Endos-perm	Embryo axis	Proline		Trehalose	
								Endosperm	Endosperm	Endosperm	Embryo axis
Hybrids (H)	15.75**	64.93**	52.57**	12.16**	0.19^{ns}	0.08^{ns}	0.13^{ns}	19.49**	1.01^{ns}	7.74**	0.32^{ns}
Osmotic potential (P)	79.40**	310.90**	271.94**	11.36**	18.00**	8.70**	2.20^{ns}	510.38**	123.03**	9.09**	4.01**
Interaction (H x P)	0.21^{ns}	4.36**	3.80**	1.78^{ns}	1.57^{ns}	2.60^{ns}	3.03*	12.31**	2.28^{ns}	2.54^{ns}	1.45^{ns}
CV (%)	10.31	12.09	8.59	31,27	32.46	13.60	20.15	10.41	13.42	30.73	16.24

** and * - Significant at $p < 0.01$ and $p < 0.05$ level of probability, respectively; ^{ns} - Not significant; CV - Coefficient of variation

The germination speed index (GSI) was reduced proportionally to water limitation (Figure 1B), while the mean germination time (AGT) showed an inverse behavior for both genotypes (Figure 1C). These results indicate that water stress harmed germination, so seeds demanded more time to germinate. The GSI of the DKB hybrid, at the highest and the lowest studied osmotic potentials, were 10.43 and 2.15 germinated seeds per day, respectively (Figure 1B). These values were higher than 8.47 and 1.63 germinated seeds per day of the DAS hybrid, at the respective same potentials.

The seeds of DAS hybrid tended to show higher ATG and lower GSI compared to DKB (Figures 1B and 1C). These data showed that GI, GSI and ATG are suitable to describe the process of germination under adverse conditions. However, due to the significant effects of the interaction between genotypes and the osmotic potential (Table 1, and Figures 1B and 1C), the use of GSI and ATG as tools to distinguish the response of the hybrids to water limitations seems to be unsure. Besides reducing seed germination (Figure 1B), low water availability was detrimental to the seedlings growth (Table 1, Figure 2C and 2D).

The detrimental effect of water limitation on seedlings growth has been also observed by Mohammadkhani & Heidari (2008), and it was expected, shoot and root as because depend on the endosperm as source of energy, besides metabolites, until the plantlet achieves autonomy for its own support (Aoki et al., 2006).

With the decrease in water availability (from 0 to -1.2 MPa) the seeds tended to show more dry mass kept in the endosperm (Figure 2A), and the shoot and the roots tended to decrease growth (Figures 2C and 2D). The hybrid DKB kept more endosperm dry mass, and less shoot growth in comparison to DAS (Figs. 2A and 2C). For the DKB, the embryo axis dry mass remained constant with increasing stress, while this variable tended to increase in DAS (Figure 2B).

The accumulation of proline in seed tissues was proportionally increased by increasing the water limitation (Figure 3A). Nevertheless, most part of the total proline of the seed was found in the embryo axis. This is an indication that this tissue is much more sensitive than endosperm to the accumulation of proline, and this trend was observed in both studied hybrids (Figure 3A).

The accumulation of proline in the tissues by reducing osmotic potential is an evidence that maize seeds are also able to develop osmoprotection mechanism, as frequently occurs in adult plants. The higher proline accumulation in the embryo axis, compared to that of endosperm, seems to be related to the greater metabolic activity and substantial presence of soluble carbohydrates in the embryo axis. This statement is based on the observations of Mohammadkhani & Heidari (2008), in which the accumulation of proline is relatively dependent on the levels of soluble carbohydrates in the tissues, since sucrose has a positive effect on the accumulation of this osmoprotectant. Although the endosperm of maize seeds is a storage organ, rich in carbohydrates, it is composed mainly of starch and other insoluble carbohydrates. Also, the endosperm is a tissue highly dependent on the embryo to promote the mobilization of its storage compounds, during

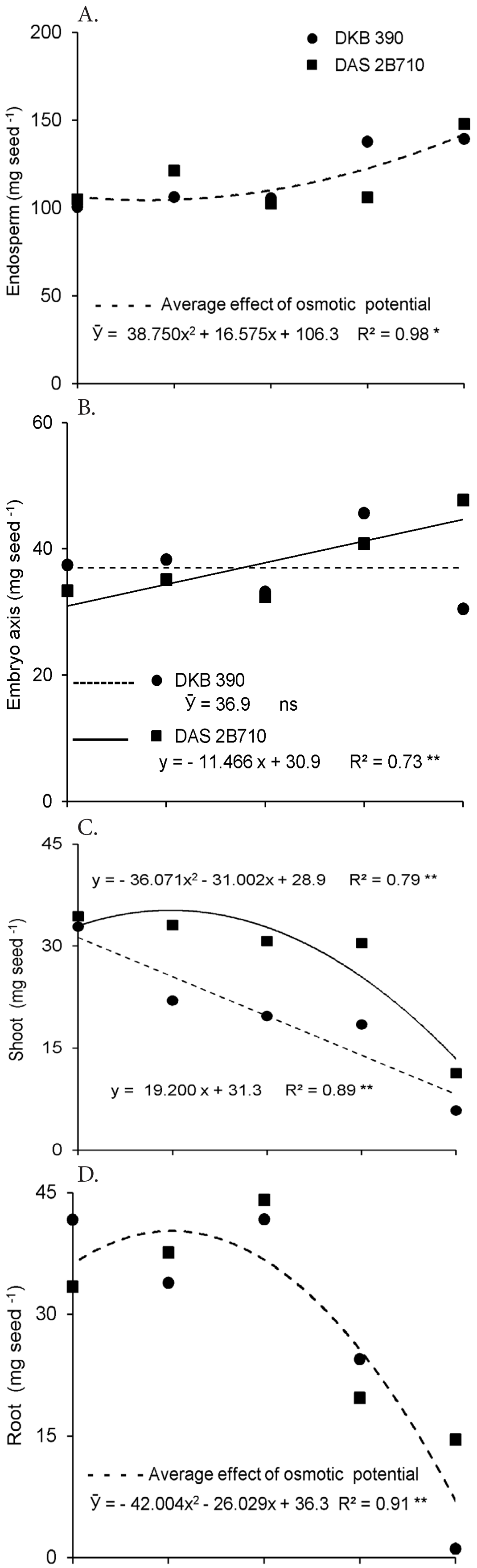

Figure 2. Dry mass kept in the endosperm (A), and dry mass of the embryo axis (B), shoot (C) and root (D), from seeds of two maize hybrids (DKB-390 and DAS-2B710) after 10 days germinating under varying osmotic potential

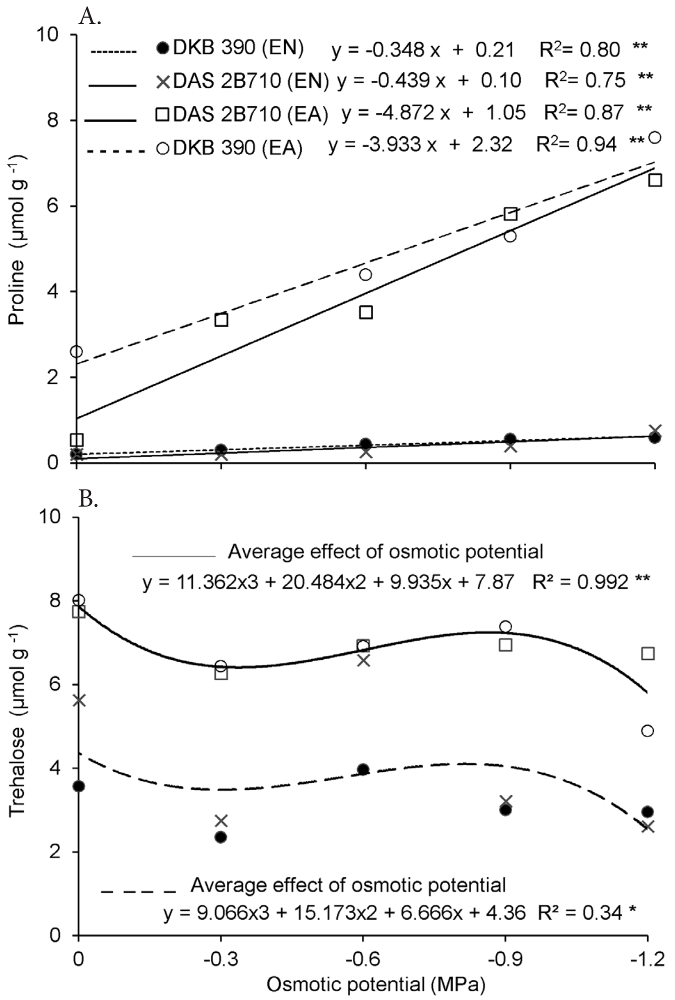

Figure 3. Levels of proline (A), and trehalose (B), in the dry matter of endosperm (EN) and embryo axis (EA) of seeds from the maize hybrids DKB-390 and DAS-2B710, after 10 days germinating under varying osmotic potential

germination. Because of that, the endosperm normally has a lower metabolism activity compared to that of embryo axis, and it seems that its metabolic activity is even more reduced under drought (Sánchez-Linares et al., 2012). These facts are, probably, what may justify the low proline synthesis and accumulation in the endosperm tissue, in seeds germinating under water limitations. Considering that proline accumulated much more in the embryo axis than in the endosperm, also that this accumulation was directly proportional to the level of the imposed stress, and that the values were different ($p < 0.01$) for genotypes (Table 1, Figure 3A), this compound may be considered as a biochemical indicator of tolerance to drought in these hybrids, when quantified in the embryo axis. Besides being an osmoprotector compound, it is speculated that the proline can also act as an inducer of secondary dormancy of embryo axis under drought, as observed for sorghum (Thakur & Sharma, 2005). Thus, this amino acid may take part in the system that protects key vital molecules of seed tissues and also, in some way, associated or not to the abcisic acid (Thakur & Sharma, 2005), may be involved in the induction of the embryo axis dormancy under low water availability, which agrees with the results of this research.

Trehalose was found in the endosperm and embryo axis of the germinating seeds, and its content varied with osmotic potential ($p > 0.05$) independently of the hybrid (Table 1, Figure 3B). The trehalose content in the embryo axis of seeds germinating with no water limitation (0.0 MPa) was clearly ($R^2 = 0.99$, $p < 0.01$) higher than that in the stressed ones (Figure 3B). Also, under intense stress (-1.2 MPa), the trehalose level in the embryo axis tended to reduce even more. In the endosperm tissue, the trehalose concentration was lower than that for embryo axis. Although data of trehalose in the endosperm have presented a less clear adjustment to the average effect equation ($R^2 = 0.34$, $p < 0.05$), the behavior of this equation was similar to that observed for embryo axis data. These results indicate that with increase of stress, the seeds tended to consume trehalose, rather than accumulate it. This behavior is opposite to the trend observed for the proline contents (Figure 3B).

The fact that higher concentrations of trehalose were detected in seeds after 10 days germinating under no water limitation suggests that this compound must be produced by the endosperm as a metabolite for the germination process. Also, the lower concentrations found in seeds germinating under stress conditions may be an indication that its production, from the reserves stored in the endosperm, decreased more than its consumption by seed tissues. Then, these results suggest that trehalose in maize seeds germinating under water stress is more used as energy source than as osmoprotector compound. This explanation is reinforced by the fact that the increase in the proline accumulation under more limiting conditions certainly required extra carbon, and energy source, and trehalose must have been used to support proline synthesis. Therefore, in seeds germinating under low osmotic potential, the trehalose may have a similar role to its isomer sucrose, as suggested before by Grennan (2007). Moreover, trehalose presented similar tendencies of the GI and GSI, which are variables related to growth (Tables 1 and 2). So, this is another evidence that this carbohydrate has more importance as storage compound than as osmoregulator, in maize seeds germinating under stress conditions.

The analysis of interactions among the studied variables (Table 2) indicated that variables that quantify the germination process showed high correlation among themselves as well as with some metabolic variables. Thus, the seeds with faster germination (high GSI, and low ATG) also presented more dry mass of shoot and root as well as higher trehalose content in the embryo axis, compared to those having low GSI and high ATG. The opposite has occurred with the behavior of proline content, either in the endosperm or embryo axis.

The negative correlation among the proline content in the endosperm with the dry matter of shoot and root (Table 2) indicates that, under stress conditions, the metabolism in the proline synthesis pathway had priority in detriment of the germination process. It is interesting to mention that the embryo axis dry matter does not show significant correlation with any other studied variable (Table 2). This is an indication that the seed tends to keep the embryo axis alive and protected from the effects of the water limitation, mainly by accumulating proline in this tissue. Also, the negative correlation between the levels of proline in the embryo axis and trehalose in the endosperm (Table 2) reinforces the idea that, under water

Table 2. Correlations (Pearson test) among the studied variables, measured in maize seedlings after 10 days germinating under decreasing osmotic potential

	GS	ATG	ProEN	ProEA	TreEN	TreEA	SDM	ENDM	EADM	RDM
GI	0.98**	-0.88**	-0.89**	-0.68*	0.47^{ns}	0.65*	0.75*	-0.55^{ns}	-0.19^{ns}	0.68*
GS		-0.96**	0.92**	0.64*	-0.46^{ns}	0.67*	-0.83**	0.57^{ns}	0.17^{ns}	-0.79**
ATG			-0.89**	-0.47^{ns}	0.36^{ns}	0.63^{ns}	0.83**	-0.49^{ns}	-0.14^{ns}	0.85**
ProEN				0.49^{ns}	-0.41^{ns}	-0.43^{ns}	-0.85*	0.57^{ns}	0.32^{ns}	-0.73*
ProEA					-0.64*	-0.56^{ns}	-0.56^{ns}	0.82**	0.27^{ns}	-0.47^{ns}
TreEN						0.35^{ns}	0.38^{ns}	-0.65*	-0.42^{ns}	0.57^{ns}
TreEA							0.63^{ns}	-0.44^{ns}	0.18^{ns}	0.48^{ns}
SDM								-0.69*	0.01^{ns}	0.68*
ENDM									0.27^{ns}	-0.55^{ns}
EADM										-0.21^{ns}

GI - Germination index; GSI - Germination speed index; ATG - Average time for germination; ProEN - Proline content in the endosperm; ProEA - Proline content in the embryo axis; TreEN - Trehalose content in the endosperm; TreEA - Trehalose content in the embryo axis; SDM - Shoot dry mass; ENDM - Endosperm dry mass; EADM - Embryo axis dry mass; RDM - Root dry mass; * Significant correlation at $p < 0.05$; ** Significant correlation at $p < 0.01$; ^{ns} not significant ($p > 0.05$)

stress, the trehalose is carried to the embryo to be used as energy and carbon source to the synthesis of proline.

Conclusions

1. Maize seeds are able to develop mechanism of osmoprotection when germinating under water limitation.

2. The proline contents in the embryo axis of maize seeds germinating under water limitation are directly proportional to the intensity of this stress.

3. Distinct hybrids show different proline levels accumulated in the embryo axis, when seeds are germinating under the same conditions of water limitation.

4. The content of trehalose decreases in the tissues of maize seeds germinating under water limitation in trend not directly proportional to the intensity of this stress.

5. Trehalose in maize seeds germinating under low osmotic potential is more used as energy source for germination and proline synthesis in the embryo axis, than as osmoprotector compound.

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