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Salt Stress and Phytohormone (ABA)-Induced Changes in Germination, Sugars and Enzymes of Carbohydrate Metabolism in *Sorghum bicolor* (L.) Moench Seeds

MEENU THAKUR AND ARUN DEV SHARMA[1]
Department of Biotechnology, Lyallpur Khalsa College, G T Road, Jallandhar-144001, Punjab, India
[1]Corresponding author's e-mail: goldi77700@yahoo.com

ABSTRACT

The effect of NaCl and ABA on germination, sugars and enzymes of carbohydrate metabolism in sorghum embryos and endosperm was investigated. Both phytohormone and salt stress influenced different aspects at physiological and biochemical level. Germination decreased markedly under NaCl and ABA treatments. Subsequently, an influence of salt stress and ABA on total and reducing sugars along with amylase activities and invertase activities was also observed, reflecting the impact of exogenous abiotic treatments on interconversion between carbohydrates and correlating enzymes. There was a considerable increase in the total and reducing sugar content in embryos and endosperm following both treatments. Both α- and β-amylase activities were enhanced by salt and ABA treatments, with former (α-) enhanced more than latter, and were significantly correlated with the concentrations of soluble sugars in the embryos and endosperm. In addition, both ABA and NaCl decreased the mobilization of starch, resulting in high starch levels in control embryos and endosperm compared with treated tissues. The reduction in starch content was more under NaCl treatment than ABA, in embryos and endosperm. The other two enzymes, acid-invertase and alkaline-invertase, showed significant enhancement upon both treatments in embryos. On the contrary, in endosperm, only acid-invertase activity was significantly increased, whereas, alkaline-invertase activity was least affected. Based upon all results, a possible physiological role of these sugars along with enzymes of carbohydrate metabolism in germinated sorghum seeds under salt stress and phytohormone ABA is discussed.

Key Words: Amylase activity; ABA; Growth; Invertase activity; Sorghum

INTRODUCTION

Plants adapt to stresses by different mechanisms, including changes physiological and biochemical processes (Bohnert *et al.*, 1995). Adaptation to all these stresses is associated with metabolic adjustments that lead to the accumulation of organic solutes such as sugars, polyols, betaines and proline (Flowers *et al.*, 1977; Gorham *et al.*, 1981). Among these accumulating solutes, starches and lipids give rise to sugars (Amuti & Pollard, 1977; Koster & Leopold, 1988) during seed germination and these are transported to sites where they are required for growth (Mayer & Poljakoff-Mayber, 1975). Soluble sugars also seem to play an important role in osmotic regulation of cells during germination (Gorham *et al.*, 1981). In addition to this role, sugars also regulate the expression of some genes involved in germination of the seeds (Yu *et al.*, 1996). Accumulation of sugars, a characteristic of mature seeds appears to be central to the development of desiccation tolerance (Hoekstra *et al.*, 2001, for review). Sugars also facilitate vitrification (a phenomenon in which intracellular water hardens like glass with no ice crystal formation during freezing or chilling stress) thus avoiding the damage caused by crystallisation as water is withdrawn (Williams & Leopold, 1989). Earlier studies report on carbohydrate accumulation with correlating carbohydrate-metabolizing enzymes during various abiotic stresses in temperate grasses and cereals where long-term carbohydrate storage occurs during reproductive development (Meier & Reid, 1982). However, there are few studies on the modulation of osmoprotectants and correlating enzymes in the germinated seeds, because, metabolism of these compounds can be affected by a number of environmental factors such as irradiance, temperature, salinity and type of ion present (Bohnert *et al.*, 1995). Thus, the variation that occurs in carbohydrate levels and enzymes of carbohydrate metabolism, during early germination is poorly understood and information on physiological events involved in this process is scarce. Therefore, in the continuation of our previous study (Gill *et al.*, 2003), in this study, we present details on germination and status of soluble carbohydrates in germinating embryos and endosperm in correlation with carbohydrate metabolizing enzymes during early germination in sorghum seeds under salt stress and to the application of ABA. Sorghum is a C_4 grass that is well adapted to semiarid and arid tropics (Quinby, 1974) where salinity is the major problem. Moreover this grain crop is the fifth most important cereal grown worldwide, due in large parts to its unusual tolerance to adverse environmental conditions (Doggett, 1988).

MATERIALS AND METHODS

Seed germination and growth conditions. Grains of *Sorghum bicolor* (L.) Moench were surface sterilized with 1% mercuric chloride followed by 70% ethanol. Seeds were thoroughly rinsed with deionized water and imbibed for 6 h. After imbibation, seeds were placed in petriplates containing sterile filter sheets, moistened with 2 mL of distilled water (in case of control), ABA (100 µm) and NaCl (0.41 M, -1.86 MPa) (Gill *et al.*, 2003, Soderman *et al*., 1996). Germination percentages and biochemical analysis were estimated after 14h using radicle protrusion (appearance of radicle 2 mm in length) as a criterion (Gill *et al.*, 2003). Each treatment was repeated three times independently of each other and each replicate included 100 seeds (i.e. 300 seeds per treatment). In order to determine the influence of treatments on germination, mean of the three replicates were taken and seed germination per 100 seeds was calculated. For biochemical analysis, tissues from each replicate independently of other replicate were combined and used for further studies. Embryos and endosperm were separated and stored immediately in liquid nitrogen until further analysis. Parts of these tissues were weighed to obtain the fresh weight (FW). The dry weight (DW) was obtained after drying the different tissues at 75°C till constant weight. Tissue water content was obtained from the (FW-DW/FW) ratio.

Extraction and estimation of sugars. Sugars were extracted twice with 80% ethanol at 90°C followed by 4 times extraction with 70% ethanol as described in Gill *et al.* (2003). From this extract as obtained above, the reducing sugars were quantitatively estimated by Nelson's method (Nelson, 1944), and total sugars were estimated according to Dubois *et al.* (1956).

Extraction and estimation of starch. Extraction and estimation of starch was carried out as described in Sawhney and Singh (2000). The sugar free pellet was resuspended in 52% (w/v) perchloric acid and incubated in closed test tubes at 96°C for 3 min. After cooling, the extract was centrifuged for 5 min at 10,000 rpm and the resulting pellet was washed again with cold 52% perchloric acid. The starch containing supernatants were combined and analyzed by the anthrone method (Sawhney & Singh, 2000)

Extraction and assay of acid and alkaline invertases. Both acid and alkaline invertases were extracted from embryos and endosperm essentially following the method of Sawhney and Singh (2000), by grinding the tissues with mortar and pestle at 0-4°C using 50 mM sodium acetate (pH 5.0) for acid invertase and 50 mM glycine NaOH buffer (pH 10.5) for alkaline invertase. The homogenate was centrifuged at 12,000 rpm for 15 min, and the supernatant collected. Invertase activities were assayed by measuring the reducing sugars released as described in Sharma *et al.* (2002). One unit (U) of invertase (acid and alkaline) is equivalent to the amount of enzyme liberating 1 µmole of product per min under assay conditions.

Extraction of amylases and their determination. The extraction of α-amylase and β-amylase from embryos and endosperm were carried out by strictly following Gupta *et al.* (1993) using 50 mM calcium acetate (pH 6.0) and 50 mM sodium acetate (3.6) extracting buffers, respectively. Amylases were extracted by crushing 100-150 mg of tissue in a pestle and mortar with 50 mM calcium acetate (pH 6.0). The mixture was aged for 1 h at room temperature and centrifuged at 10,000 rpm for 10 min. The supernatant was heated at 70°C for 20 min to inactivate the β-amylase and then cool to 0°C. The precipitates were removed by centrifugation at 10,000 rpm for 10 min and supernatant was used for measuring the α-amylase activity. For extracting β-amylase, the tissues were homogenized in 50 mM sodium acetate (pH 3.6) containing 0.1 mM EDTA. The suspension was left for 1 h at 0°C, centrifuged and the clear supernatant assayed for β-amylase. The amylase activities were assayed by measuring the reducing sugars released as described in Sawhney and Singh (2000). One unit (U) of amylase (α and β) is equivalent to the amount of enzyme liberating 1 µmole of product per min under assay conditions.

Statistical analysis. A statview ANOVA program as used for statistical analysis of the data. Values for treatments with in each tissue were compared using one-way analysis of variance and student's *t*-test for differences between pairs of data if the ANOVA ($LSD_{0.05}$) revealed significance.

RESULTS

Seed germination under different treatments. Seed germination in distilled water reached the maximum (97%) in 14h whereas germination rate was adversely affected by both NaCl and ABA treatments (Table I). However, NaCl caused a considerably greater decrease in germination (34%) as compared with ABA (63%) treatment. Subsequently, the germination percentages increased to more than 90% after 72h under NaCl and ABA treatments (data not shown).

NaCl- and ABA-induced changes in physiological and biochemical parameters in the 14 h germinated embryos. A significant reduction in embryo FW and DW was observed for both treatments. However, the maximum decrease in FW accompanied by DW, was caused by salt treatment (Table II). The tissue water content (FW-DW/FW) ratio, a measure of expansion growth of embryo in distilled water showed a substantial increase, whereas water content of embryos was adversely affected after NaCl treatment.

As compared with control, imposition of NaCl and ABA treatments resulted in a significant increase in total sugar content (Table III). Reducing sugar content was also significantly higher after both treatments, however, the rate of increase was more under NaCl treatment. In both the treatments, the starch content was considerably lower than control embryos (Table III), however the decrease was more

pronounced under salt treatment.

The activity of α- and β-amylases were significantly higher under both treatments, with former (α-) enhanced more than latter (Table IV). The enhancement was more under salt treatment than ABA. As compared with control, β-amylase activity also showed significant increase under both treatments. In general, acid- and alkaline-invertase activities were considerably high after both treatments than water-irrigated control, however, the contribution of acid-invertase was higher.

NaCl- and ABA-induced changes in physiological and biochemical parameters in endosperm after 14 h of seed germination. As compared with control, a significant decrease in FW was observed only after NaCl treatment (Table II). DW was least affected under both treatments. Further, a significant decrease in TWC was observed only after NaCl treatment. Like embryos, as compared with control, a significant increase in total and reducing sugar content was observed under both treatments, however, the increase was more under NaCl than ABA treatment (Table III). As compared with that of control, starch content was significantly less after both treatments, especially in salt-treated endosperm (Table III).

Similar to embryos, as compared with control, a significant increase in α- and β-amylase activities was observed after both treatments (Table IV). However, the enhancement was more in α-amylase activity. Acid-invertase activity was considerably higher after both treatments, however, increase was more under salt treatment. Contrary to acid invertase, the alkaline-invertase activity was least affected under salt and ABA treatments.

Table I. Effect of NaCl and ABA on germination of sorghum seeds. Data represents the mean of three replicates ±SE

Treatment	Germination (%)
Control	97±1
NaCl	^{d}34±3
ABA	^{d}63±2

d, represent significant difference vs. control ($P \leq 0.05$).

Table II. Effect of NaCl and ABA on fresh weight (FW), dry weight (DW) and tissue water content of germinated seeds of sorghum. Data represents the mean of three replicates ±SE

Treatment	FW (mg tissue^{-1})	DW (mg tissue^{-1})	Tissue water content
Embryo Control	7.00±0.88	3.00±0.45	0.57±0.11
NaCl	^{d}1.60±0.54	^{d}0.92±0.11	^{d}0.43±0.12
ABA	^{d}0.52±0.21	^{d}0.21±0.10	^{ns}0.58±0.12
Endosperm Control	47.01±2.50	41.11±5.60	0.12±0.05
NaCl	^{d}43.20±2.0	^{ns}41.53±6.60	^{d}0.04±0.01
ABA	^{ns}45.03±4.4	^{ns}34.52±9.70	^{ns}0.23±0.21

d, represent significant difference vs. control ($P \leq 0.05$), ns, not significant

DISCUSSION

In order to gain insight into the physiological and biochemical changes occurred during salt stress and phytohormone (ABA) treatments, we studied the sugar content changes in correlation with amylases and invertases in sorghum embryos and endosperm. In earlier research, Gill and Singh (1985) has reported that the germination, growth, respiration and other related processes could be affected in seeds that are subjected to environmental stresses. Changes in any one of these processes can affect other metabolic activities, particularly the carbohydrate metabolism that plays an important role in germination and seed development. Imposition of NaCl as well as ABA treatments resulted in a significant decrease in germination. Subsequently, the germination percentages increased to more than 90% after 72 h under NaCl and ABA (data not shown). The decrease in germination particularly under salt stress may be due to the fact that seeds seemingly develop an osmotically enforced "*dormancy*" under water stress conditions. However, the decrease in germination rate observed under ABA treatment may be attributed to metabolic alterations. The dormancy-inducing hormone ABA, which is also known as stress hormone, may also be involved in inhibiting the seed germination by restricting the availability of energy and metabolites (Garciarrubio *et al.*, 2003).

It has been reported that water stresses cause an active conversion of starch to sugars in bean leaves (Stewart, 1971). If this is also case with sorghum seeds, the phenomenon that the stress causes a decrease in starch content and an increase in sugar content is explainable. The accumulated starch is probably an energy reserve for the high-energy process of organogenesis and provides for osmotic agents in the form of free sugars (Thorpe *et al.*, 1986). This assertion is further supported by studies with a variety of plants that demonstrate a water stress-induced conversion of hexoses and other carbohydrates, such as starch, into sugar alcohols (polyols) and proline (Perez-Alfocea & Larher, 1995; Wang *et al.*, 1996). In such a case, amylase activities induced by water stress like stimuli is possibly involved in starch degradation. This assertion was substantiated by the observation that, the increase in sugar levels accompanied by decrease in starch content in embryos and endosperm was directly linked to the activity of α-and β-amylases in the embryos and endosperm, which is in agreement with the existing reports (Monerri *et al.*, 1986; Gupta *et al.*, 1993). This increase might be considered to play an important role in osmotic adjustment, which is widely regarded as an adaptive response to water deficit conditions (Kameli & Losel, 1995). Furthermore, the results showed that enhanced reduction of starch in embryos and endosperm under salt and ABA treatments was closely associated with an increase of the α-amylase activity. The increase was more under NaCl treatment than ABA in both

Table III. Effect of NaCl and ABA on sugar and starch contents in the germinated seeds of sorghum. Data represents the mean of three replicates ±SE

Treatment	Sugar content (μg mg^{-1}DW)		Starch content (μg mg^{-1}DW)
	Total	Reducing	
Embryo			
Control	31.00±5.6	11.0±1.6	10.12±2.0
NaCl	^{d}68.21±6.6	^{d}39.15±3.3	^{d}2.71±0.5
ABA	^{d}54.12±4.8	^{d}20.92±4.2	^{d}5.01±0.6
Endosperm			
Control	11.02±1.1	7.6±1.0	32.01±2.3
NaCl	^{d}26.23±2.1	^{d}15.21±2.8	^{d}8.02±1.1
ABA	^{d}20.11±2.8	^{d}10.22±2.0	^{d}15.20±2.1

d, represent significant difference vs. control (P≤ 0.05), ns, not significant

Table IV. Effect of NaCl and ABA on amylase and invertase activities in the germinated seeds of sorghum. Data represents the mean of three replicates ±SE

Treatment	Amylase activity (Units.10^{-5} mg^{-1} protein)		Invertase activity (Units.10^{-5} mg^{-1} protein)	
	α	β	Acid	Alkaline
Embryo				
Control	40.21±5.6	20.12±5.6	10.21±1.0	5.20±1.3
NaCl	^{d}160.1±5.4	^{d}48.11±6.6	^{d}25.31±2.5	^{d}12.10±2.4
ABA	^{d}80.12±6.5	^{d}30.22±5.4	^{d}20.23±2.8	^{d}8.23±2.8
Endosperm				
Control	17.11±2.1	10.22±1.8	5.22±1.4	1.71±0.50
NaCl	^{d}40.25±3.3	^{d}20.10±2.5	^{d}10.22±2.1	^{ns}1.58±0.61
ABA	^{d}36.28±4.2	^{d}15.20±1.0	^{d}8.6±1.8	^{ns}1.59±0.60

d, represent significant difference vs. control (P≤ 0.05), ns, not significant

the tissues. Though, β-amylase activity was also associated with both soluble sugars and starch remobilization, it was less enhanced by the both treatments, when compared to α-amylase, which is in accordance with the earlier studies (Yang *et al.*, 2001). In this study, a high correlation α-amylase activity with soluble sugars and reduction of starch suggests that the fast hydrolysis of starch under water deficit is mainly attributed to the enhanced α-amylase activity in embryos and endosperm.

Moreover, a strong association between reducing sugar content and both acid- and alkaline-invertase activities was observed in the embryos under both treatments. Whereas in endosperm only acid-invertase activity was found to be closely related with sugar contents under both treatments. However, alkaline activity was least affected, indicating the differential regulation of invertases by ABA and NaCl in a tissue dependent manner. The regulation of invertase by phytohormone ABA indicates an essential link between the molecular mechanism of ABA action and primary metabolism. Changes in invertase activity have also been reported to correlate with reducing sugar content in maize (Trouverie *et al.*, 2003) and in chickpea (Kaur *et al.*, 2003).The induction by invertase by abiotic stimuli supports the suggestion that invertase is an important component of stress response.

In conclusion, the present study indicates that changes observed under abiotic conditions are associated with acclimation of sorghum seeds to water deficit conditions that lead to an increase in synthetic activity and associated changes. Additionally, in this study we found a strong negative correlation between germination and sugars or correlating enzymes under salt stress or plant growth regulator. Although, ABA, NaCl decreased germination, however, in all the treatments the levels of soluble sugars were increased. In future, we could find out more with context to germination and correlating enzymes. But, from the above data it became apparent that under less germination higher levels of sugars along with correlating enzymes might be involved in acclimation (keeping developing embryonic axis in resting state) of germinating seeds by modulating internal osmoregulatory solutes. Further, investigations are needed to enhance the understanding on the effect of different abiotic stresses and growth hormones during early seed development.

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