

## Alleviation of Salinity-Induced Dormancy by Growth Regulators in Wheat Seeds

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**Abstract:** The effect of 2 growth regulators, betaine and thiourea, was examined to learn the extent to which they can alleviate salinity-induced dormancy of wheat seeds. Germination velocity (as measured by Timson index) progressively decreased with increasing salinity, while thiourea and betaine, alone or in combination, elevated the germination velocity of wheat seeds. Timson index showed significant improvement in germination velocity when thiourea was used at 225 mM NaCl. Likewise, the adverse effect of salinity on growth parameters, like root and shoot lengths and fresh and dry weights, was also alleviated considerably when growth regulators were added. The application of growth regulators in combination with NaCl resulted in increased level of reducing sugars in the embryo and a decrease in the endosperm coupled with enhanced amylase activity, suggesting significant recovery in the mobilization rate of soluble sugars from endosperm to embryo. Thiourea was more effective in the mobilization process compared to betaine. Significance of growth regulators in alleviating the salt stress in crop plants is discussed.

**Key Words:** Salinity, thiourea, betaine, germination velocity, reducing sugars

### Introduction

Growth promoters such as GA and kinetin, regulators, i.e., nitrate and thiourea, and compatible osmotica, like proline and betaine, are well-known to alleviate salinity-induced dormancy in wild plants (Plyler & Proseus, 1996; Yoshiba et al., 1997; Khan et al., 1999; Khan & Ungar, 2001). Growth regulators like nitrate, nitrite, and thiourea often stimulate germination (Esashi et al., 1979; Aldosaro et al., 1981). Treatment with thiourea has been shown to be highly effective in promoting germination when dormancy is related to salt stress (Esashi et al., 1979; Khan & Ungar, 2001). Effectiveness of thiourea and related growth regulators depend on the plant species, climatic zone, and strength of soil salt solution. Germination of the species of temperate salt marshes are reported to respond positively to the application of growth regulators, but there is little information available regarding their effects on tropical plants (Ungar, 1991; Plyler & Proseus, 1996) Khan & Ungar (1996) reported that at low salinities, both thiourea and nitrate significantly improved germination of *Atriplex patula* L.

Brown seeds of *Arthrocnemum indicum* (Willd.) Moq. germinated more successfully at salinity concentration as high as 1000 mM by the use growth regulators compared to black seeds, which did not respond to 1000 mM NaCl (Khan et al., 1998). Alleviation of NaCl-induced dormancy by growth regulators like GA and kinetin has also been reported by several other researchers (Okusanya & Ungar 1983; Ungar, 1984; Tirmizi, 1988; Ismail, 1990; Plyler & Proseus, 1996). Gorham (1995) hypothesized that compatible osmotica, like betaine and proline, increase the salt tolerance of plants in the growth stage of development, but their effect in relationship to seed germination has been reported for only a few species. Sufficient information has accumulated on the adverse effects of salinity on seed germination and its alleviation by the addition of growth regulators; but few studies attempt to investigate the biochemical mechanisms involved in the inhibition process and its alleviation (Bolarin et al., 1995; Prado et al., 2000).

Physiologically, thiourea offsets the effect of ABA and decreases the level of cytokine in plant tissues subjected

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to water stress due to drought, salinity, or supra optimal temperatures (Kabar & Baltepe, 1989). Compatible osmotica, such as proline and betaine, have also been hypothesized to increase the salt tolerance of plants in the growth stage of development (Gorham, 1995); but their effects on seed germination of crop plants are poorly understood because very few species have been studied in this respect (Poljakoff-Myber et al. 1994).

The objectives of this study were to investigate the extent to which thiourea and betaine can alleviate salinity-induced dormancy on wheat seeds, and to examine the biochemical changes (carbohydrate status) during seed germination under saline media alone, and in combination with growth regulators, because carbohydrate is the major seed reserve in wheat.

## Material and Methods

### Germination Test

Seeds of *Triticum aestivum* L. var. Pasban-90 were obtained from The Pakistan Agriculture Research Centre (PARC), University Campus, Karachi. They were sterilized with 0.50% NaOCl (sodium hypochlorite) solution for 1 min and thoroughly washed in sterilized water before use in the experiments. The germination tests were performed in 20 mm diameter test tubes. Seeds were placed on Whatman No. 3 filter paper channels (3.5 × 16 cm strips of filter paper folded to form channels) and were germinated under controlled conditions (25 ± 2 °C during a 13-h light period of about 25 μmole m<sup>-2</sup> s<sup>-1</sup> white florescent light and 19.5 ± 2 °C during 11 h of darkness). The experiment was designed as a factorial experiment with Factor I as salinity, which included 4 levels (0, 75, 150 and 225 mM NaCl), while Factor II constituted amendments, which included 3 levels - 0, 10 mM thiourea, and 50 μM betaine. In each test tube, 5 ml of test solution or distilled water was added. Small amounts of the respective solutions were added when the test tubes were beginning to dry out. Treatments and controls were replicated 4 times with 20 seeds in each test tube. On the 15<sup>th</sup> day, fresh weight, and root and shoot length of germinated seedlings were measured. Subsequently, the seedlings were placed in an oven at 80 °C for 24 h and dry weights determined. Each value was expressed as an average of 10 randomly chosen seedlings per replicate. Relative water uptake was measured after 60 min of imbibition and it was expressed as net gain in

weight of the seeds. Seeds were considered germinated with the emergence of the radical. Percent germination was recorded every alternate day of the 15-day period. The rate of germination was estimated by using a modified Timson index of germination velocity = G/t, where G is the percentage of seed germination at 2-d intervals, and t is the total germination period. The maximum possible value of using this index with our data was 50 (i.e., 1000/20). The higher values represented faster rates of germination.

### Sugars Determination

Sugar content was recorded after 4, 6, 10, and 14 h of imbibition in combinations of thiourea and betaine, each with 225 mM salinity because of effectiveness of this concentration to induce stress. The different tissues (0.5g fresh weight of each embryo and endosperm) were crushed and homogenized with a pestle and mortar in 2 ml of 80% ethanol solution. After heating the homogenate in a water bath at 75 °C for 10 min, the insoluble residue was removed by centrifuging at 5000 g for 10 min. The precipitate was re-extracted with 2 ml of 80% ethanol at 75 °C and then re-centrifuged. The ethanol extracts were pooled separately and concentrated with a rotary evaporator at 70 °C under vacuum. From the extract obtained above, total sugars were estimated by the method of Dubois et al., (1956). For reducing sugars, 1 ml of extract and 1 ml of mixed copper reagent were heated in a boiling water bath (unstirred thermostatic-controlled water bath with sensitivity ± 0.3 °C) for 20 min. The contents of the setup were cooled in cold water at 4 °C and 1 ml of arsenomolybdate reagent was added to each test tube with constant shaking in an electric shaker (Shaker, Vibrax VXR) until the froth disappeared. After 10 min, the volume was made to 5 ml with double distilled water. OD was recorded at 500 nm in a Shimadzu UV 1260 mini spectrophotometer and amounts were expressed in mg g<sup>-1</sup> using a standard graph. Glucose was used as the standard for reducing sugars.

### Extraction and estimation of amylase activity

Amylase activity was recorded after 4, 6, 10, and 14 h of imbibition in combinations of thiourea and betaine, each with 225 mM salinity because of the effectiveness of this concentration to induce stress, which effects the

mobilization of reducing sugars, i.e., glucose, fructose, and sucrose, from endosperm to the embryo. Seeds were crushed and extracted (4 ml of buffer per 1 g tissue) in chilled 1% NaCl in 0.2 M phosphate buffers (pH 6.5) at intervals of 4, 6, 10, and 14 h of imbibition. The extract was centrifuged at 12,000 g for 20 min at 4 °C and the clear supernatant thus obtained was used as crude amylase. Amylase activity was determined using soluble starch as the substrate. One enzyme unit (EU) liberates 1  $\mu$ mole of reducing sugars (calculated as glucose) per min at 25 °C and pH 6.5 under the specified condition from soluble starch (Decker, 1977).

### Statistical Analysis

The data sets were subjected to factorial analysis of variance followed by Bonferroni test to determine the significance ( $P < 0.05$ ) of differences between individual treatments using SPSS (Nie et al., 1980).

## Results

### Effects of salinity, thiourea, and betaine on germination

Increased salinity inhibited the germination of wheat seeds; but a few seeds did germinate at 225 mM NaCl (Figure 1a and b). Thiourea alone or in combination with

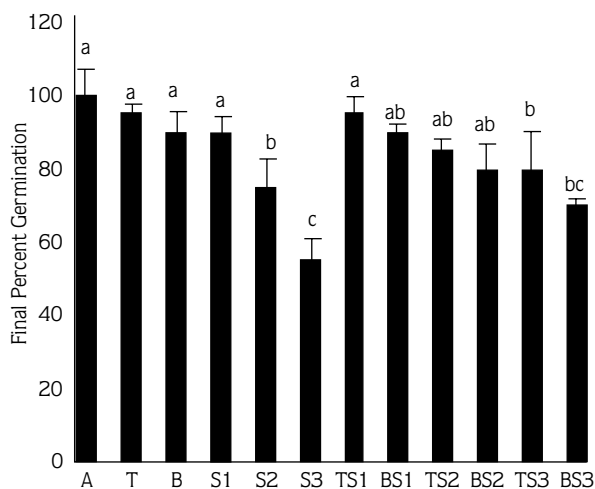


Figure 1. Effect of growth regulators on germination lengths and weights of *Triticum aestivum* L. Symbols on X-axis: A = Distilled water; S1 = 75 mM NaCl; S2 = 150 mM NaCl; S3 = 225 mM NaCl; B = Betaine; T = Thiourea. Note: Values at each concentration having same letters are not significantly different ( $P > 0.05$ )

NaCl markedly enhanced germination velocities and showed significant alleviation of salinity-induced dormancy. Use of thiourea along with NaCl considerably minimized inhibitory effects of NaCl compared to betaine. The application of thiourea in combination with a high concentration (225 mM) of NaCl significantly improved the germination velocity; however, increases in germination velocities were found in this order:

#### For betaine:

Distilled Water > 50  $\mu$ M betaine = 75 mM NaCl > betaine-75 mM NaCl > betaine-150 mM NaCl > 150 mM NaCl > betaine+225 mM NaCl > 225 mM NaCl;

#### For thiourea:

Distilled Water > 10 mM thiourea > thiourea-75 mM NaCl > 75 mM NaCl > thiourea-150 mM NaCl > 150 mM NaCl > thiourea-225 mM NaCl > 225 mM NaCl.

### Relative Water Content

Relative water uptake was slowed down by salinity (Figure 1c and d). Application of growth regulators enhanced the rate of water uptake compared to salinity treatment. Use of thiourea showed greater effects than betaine in considerably increasing water uptake; however, betaine did not improved the rate of water uptake significantly under saline medium, and the difference was non significant. While results indicated that thiourea significantly enhanced ( $P < 0.05$ ) water uptake more so than the control and betaine at 225 mM NaCl, at lesser concentration, relative water contents in the thiourea-salinity treatment were more compared to betaine-salinity treatment.

### Seedling Growth

Root and shoot lengths were also significantly ( $P < 0.05$ ) affected by salinity (Figure 1e & f). Again, the application of thiourea resuscitated total seedling length (Figure 2b), especially the shoot length. Use of NaCl induced inhibition in seedling growth and, consequently, a greater decrease in root and shoot lengths compared to the control treatment, while use of thiourea, as well as betaine, in combination with various NaCl concentrations showed improved root and shoot growth over the salinity treatments. Figure 2b shows that thiourea in combination with saline treatment of 225 mM proved to be most

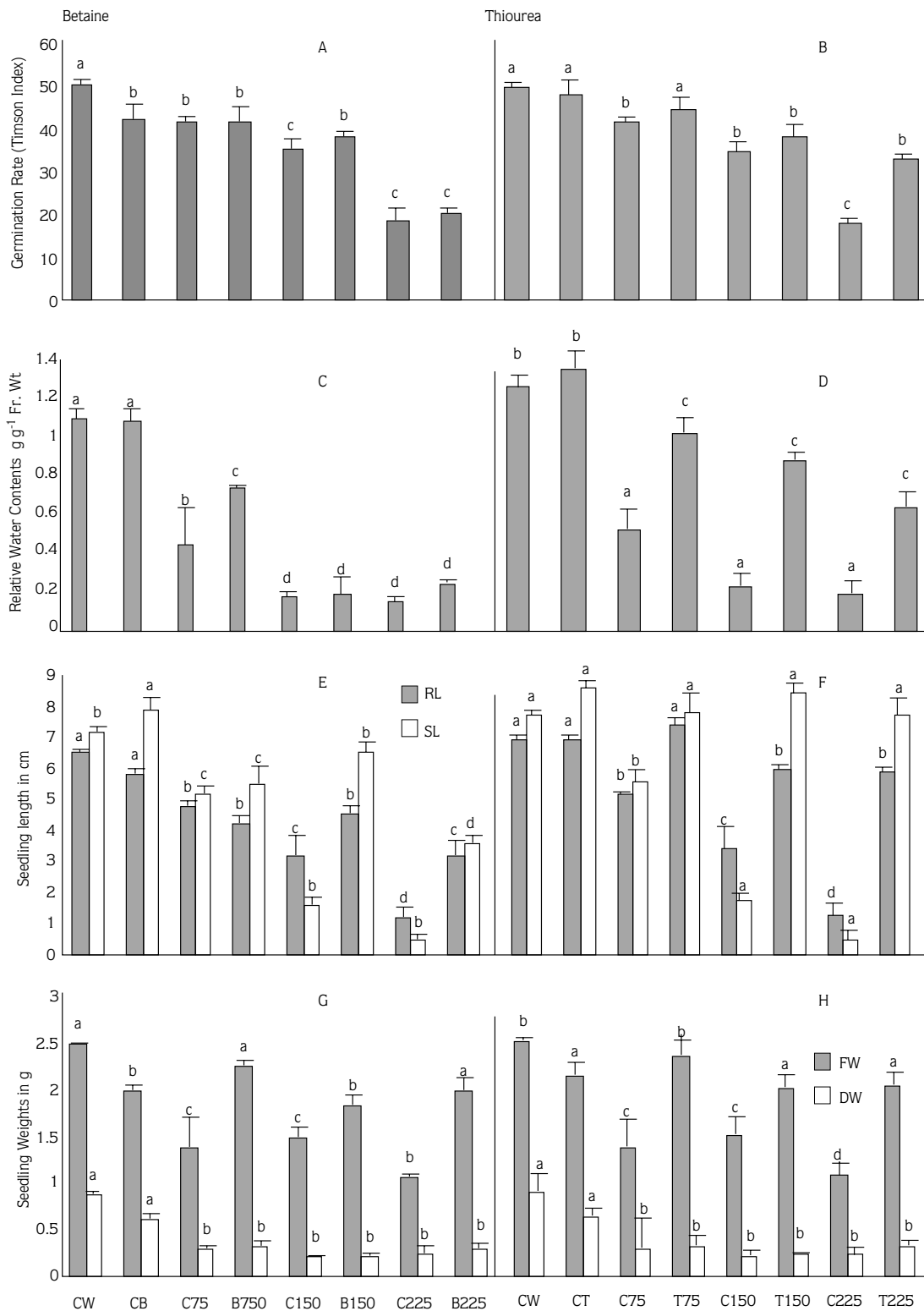


Figure 2. Effect of growth regulators on germination velocity, relative water contents, seedling lengths and weights of *Triticum aestivum* L. Symbols on X-axis: CW = Distilled water; S1 = 75 mM NaCl; S2 = 150 mM NaCl; S3 = 225 mM NaCl; CB = Betaine, CT = Thiourea. Note: Values at each concentration having same letters are not significantly different ( $P > 0.05$ ).

effective, compared to betaine, in considerably increasing total seedling length, while salinity negatively affected seedling growth (Figure 3). The greatest variation in root /shoot ratio was observed at 150 and 225 mM salinities (Figure 2a), indicating the ability of roots to withstand higher saline concentrations. At higher salinity values, shoots were more severely affected; thus, showing the sensitivity of shoots to salinity. The application of thiourea and betaine greatly promoted seedling lengths by improving shoot lengths as compared to saline treatments, and also stabilized the root/shoot ratio, suggesting the potential of growth regulators.

### Seedling Biomass

Growth measured in terms of dry and fresh weights responded negatively to NaCl application (Figure 1g & h). The 2 growth regulators enhanced fresh weight more than dry weight ( $P < 0.05$ ). Thiourea was found to be more potent in alleviating salinity-induced inhibition in growth compared to betaine. Thiourea along with 225 mM NaCl caused significant improvement in fresh weights compared to dry weights ( $P < 0.05$ ). Likewise, betaine in combination with saline solution tended to increase the fresh weight, with almost no effect on dry weight. The betaine-NaCl combination was less effective than the

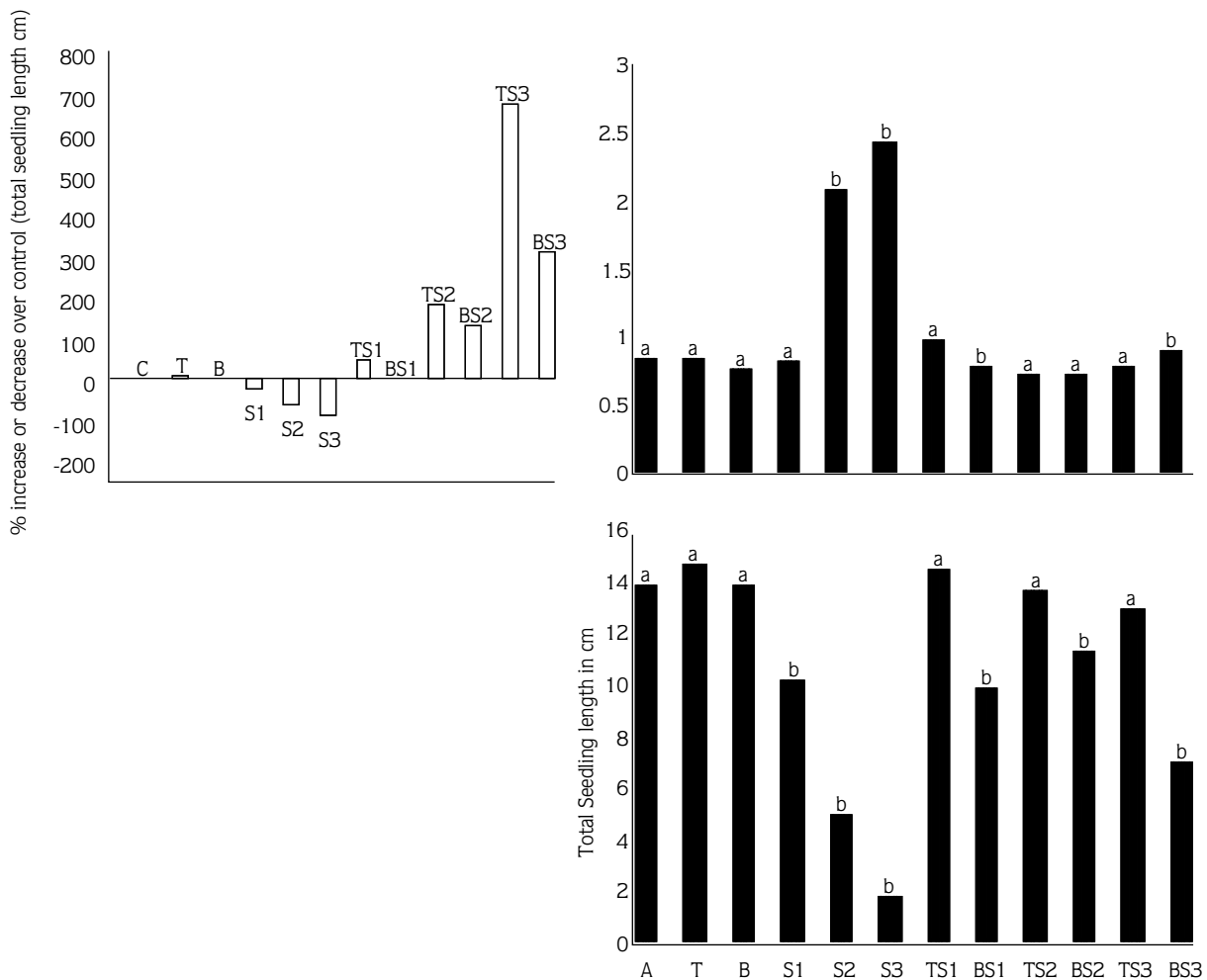


Figure 3. Percentage increase or decrease over control, total seedling length and root shoot ratio by the application of growth regulators in saline media. Symbols on X-axis: C = Distilled water; S1 = 75Mm NaCl; S2 = 150mM NaCl, S3 = 225 mM NaCl; B = Betaine; T = Thiourea  
 Note: Values at each concentration having same letters are not significantly different ( $P > 0.05$ ).

thiourea-NaCl combination in improving seedling biomass.

### Sugar Contents

Changes in the total soluble sugar content during germination (Table 1) were observed. It was found that total and reducing sugars increase with time and a comparatively higher buildup of sugars was noted in the embryos than the endosperms that were subjected to salinity stress. Reducing sugars in embryos increased with time with thiourea-NaCl treatment, showing considerable mobilization of sugar from endosperm to embryo. Thiourea caused a greater increase in reducing sugars compared to betaine and control treatments throughout the experiment. Salinity stress retarded the concentration of reducing sugars in both endosperm and embryo.

### Amylase activity

Application of growth regulators in saline medium had considerable effect on amylase activity (Figure 4). At 225 mM NaCl, amylase activity was retarded compared to the control treatment, with a slight peak at 10 h (Figure 4).

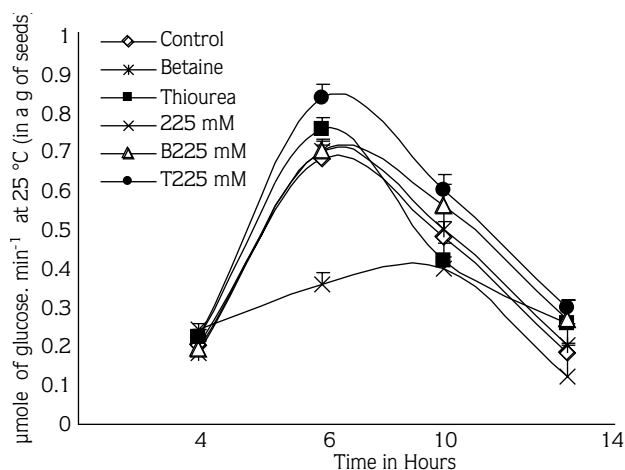


Figure 4. Changes in amylase activity of wheat seeds during germination due to the use of growth regulators, with or without saline medium (225 mM NaCl).

Throughout the experiment, the use of growth regulators significantly ( $P < 0.05$ ) increased amylase activity by alleviating salinity-induced suppression of the germination process. Compared to betaine, thiourea markedly enhanced amylase activity, which peaked 6 h after imbibition. Treatments other than salinity showed maximum increase after 6 h of imbibition.

Table 1. Changes in sugar contents in embryonic axis and endosperm during germination at various time points

Time (h)	Sugar Content (mg.g <sup>-1</sup> FW)							
	Total				Reducing			
	Control	225mM	B+225mM	T+225mM	Control	225mM	B+225mM	T+225mM
Embryo								
0	20.2 ± 0.55	20.2 ± 0.55	20.2 ± 0.55	20.2 ± 0.55	15.2 ± 0.05	15.2 ± 0.05	15.2 ± 0.05	15.2 ± 0.05
4	23.5 ± 0.88	22.2 ± 1.06	22.5 ± 1.04	23.9 ± 1.2	16.5 ± 0.02	10.2 ± 0.88	10.0 ± 0.22	12.5 ± 0.66
6	25.7 ± 0.56	22.5 ± 0.55	22.3 ± 1.2	25.08 ± 0.88	17.25 ± 0.048	10.5 ± 1.02	12.82 ± 1.02	15.9 ± 1.08
10	26.1 ± 1.25	23.4 ± 0.88	25.4 ± 0.88	27.6 ± 1.06	21.3 ± 0.033	10.8 ± 0.66	15.5 ± 0.33	20.0 ± 0.66
14	28.0 ± 0.25	24.3 ± 1.02	27.3 ± 1.08	29.5 ± 0.88	19.25 ± 0.045	10.88 ± 0.33	12.8 ± 1.03	15.20 ± 0.33
Endosperm								
0	14.2 ± 0.55	14.2 ± 0.55	14.2 ± 0.55	14.2 ± 0.55	14.2 ± 1.02	14.2 ± 1.02	14.2 ± 1.02	14.2 ± 1.02
4	13.2 ± 0.05	17.3 ± 1.02	13.6 ± 0.55	12.2 ± 0.88	10.3 ± 0.25	9.2 ± 1.02	9.50 ± 2.3	10.0 ± 0.66
6	12.5 ± 0.02	17.6 ± 0.88	12.5 ± 1.02	13.0 ± 1.02	8.05 ± 0.88	8.8 ± 0.33	9.0 ± 0.66	7.5 ± 0.33
10	11.25 ± 0.048	17.9 ± 0.76	10.6 ± 1.02	11.5 ± 1.02	6.7 ± 0.56	8.6 ± 0.88	8.8 ± 0.45	6.05 ± 0.88
14	10.3 ± 0.033	19.3 ± 1.02	11.5 ± 1.04	10.8 ± 0.45	5.5 ± 1.25	8.2 ± 0.26	8.8 ± 1.02	5.25 ± 0.44

Symbols: B+225 = Betaine + 225 mM NaCl; T+225 = Thiourea + 225 mM NaCl

## Discussion

While salinity greatly contributed to the low germination rate of *T. aestivum* seeds, addition of thiourea and, to a lesser extent, betaine to the salt solution tended to improve germination, amylase activity, mobilization of reducing sugars from endosperm to embryo, as well as seed water uptake, seedling length, and fresh and dry weights. The alleviating effect of thiourea on *T. aestivum* clearly suggests that it is a more compatible osmoregulator compared to betaine. This is consistent with previous studies that have demonstrated the effectiveness of thiourea in alleviating salinity-induced inhibition of germination (Esashi et al., 1979; Yoshiyama et al., 1996). The principal reasons for the ameliorative effect of thiourea is due to its counteractive effect on ABA production (Kabar and Baltepe, 1989) and that it also controls the adverse hormonal changes resulting from water stress induced by drought, salinity, or high temperature (Kabar and Baltepe, 1989; Sultana et al., 2000; Bano and Aziz, 2003). In addition to ABA, thiourea is also known to break dormancy and overcome the negative effect of temperature on seed germination (Esashi et al., 1979; Aldosaro et al. 1981). Nitrogenous compounds, such as nitrate and thiourea, are also reported to promote germination (Esashi et al., 1979; Aldosaro et al., 1981; Bewley and Black, 1994; Yoshiyama et al., 1996). Poljakoff-Mayber et al. (1994) monitored proline and betaine levels in *Kosteletzkya virginica* seeds during the process of germination and found low concentrations of proline; however, when seeds were germinated in a saline medium, the concentration of proline increased substantially, while the level of betaine declined. They suggested that proline could provide a compatible osmotica to the saline medium for germinating seeds compared to betaine. Interestingly, they did not find any effect of the external application of either proline or betaine on germination under both control and saline medium. On the other hand, Khan and Ungar (1997) reported that both proline and betaine alleviated the innate dormancy of *Zygophyllum simplex* L. seeds, but failed to improve salinity-enforced dormancy. Proline and betaine, which often increase the tolerance of plants to high levels of salinity by acting as osmoregulators (Schobert, 1977; Gorham, 1995), may be of significance as compatible solutes in seeds, but their in situ biochemical mechanism during seed germination under saline medium requires further investigation.

Schobert (1977) opined that betaine and thiourea alleviate salinity-induced dormancy by virtue of their ability to act as osmoregulating substances in cell cytoplasm. Known for enhancing the drought tolerance of plants in arid or semi-arid environments through osmotic adjustments, osmoregulation is considered an eco-physiological mechanism to counter the stresses posed by the environment.

Alteration in the physiological and biochemical behavior of seeds during germination and early seedling growth under saline medium is not uncommon. A number of reports suggest that a hyper-saline environment causes delay in germination (Prado et al., 1995, 2000), inhibits seedling growth (Dash & Panda, 2001), reduces enzyme activities, retards the mobilization rate of soluble sugars (Prado et al., 2000; Ashraf et al., 2002), and affects other metabolic and molecular responses (Hanson & Hitz, 1982; Ingram & Bartlets, 1996). When the 2 growth regulators were used in conjunction with saline medium, significant improvement resulted in seedling length, fresh weight, sugar mobilization, and amylase activities. The observed increase in fresh weight can be attributed to cell enlargement by water absorption, cell vacuolation, and turgor-driven wall expansion (Dale, 1988; Prado et al., 2000). During germination and early seedling growth, cell division and enlargement require proper transportation of respiratory substrates in the form of soluble sugars from seed storage organs to the site of embryo growth (Bewley & Black, 1994). Accumulation of sugars not only acts as a source of energy, but also increases fresh weight and provides the carbon skeleton to synthesize specific osmolytes such as proline and betaine that are used for adaptive and/or defensive responses against stresses, including salinity (Prado et al., 2000). In addition, sugars, such as raffinose and sucrose, are suggested to have important roles in protecting cells from water stress; these solutes are available for osmoregulation or function as protectants of macromolecules and membrane systems (Leopold, 1990; Bray, 1997). It has been suggested that high molecular weight carbohydrates converted to soluble sugars, i.e., glucose, maltose, sucrose, and fructose, are readily transportable to the sites where they are required for growth (Mayer & Poljakoff-Mayber, 1975; Bewley & Black, 1994). Hence, it is conjectured that the use of growth regulators along with salinity treatments nullified the inhibitory effect of salinity on growth by restoring the

mobilization rate of soluble sugars and their conversion to low molecular weight sugars. Accumulation of reducing sugars in the embryo and reduction in total sugars in endosperm, and increased amylase activity also supports this hypothesis. Similarly, improvement in seedling length and weights can also be ascribed to the higher mobilization of reducing sugars and their accumulation in the embryo in response to the application of thiourea and betaine under saline media. Previous reports suggest environmental stresses, such as salt-stress-induced enhancement in sugar levels at the site of growth, serves as an adaptive mechanism to minimize or overcome stress to which plants are exposed. Soluble sugars, principally glucose and maltose, are known to accumulate in plants in response to a variety of environmental stresses (Macleod & Orquodale, 1958; Gorham et al., 1981; Wang et al. 1996; Prado et al., 2000; Gill et al., 2002). Such an increase in reducing sugars during germination may help the seeds to overcome the inhibition enforced by high levels of salinity. Such an increase helps to maintain the water content and thus ensures continuous growth and cellular functions. Accumulation of soluble sugars in response to thiourea and betaine in saline media is expected to be beneficial to plants for coping with salinity stress. Although it is generally agreed that salinity and water stress induce soluble sugar accumulation (Wang and Stutte, 1992; Kameli & Losel 1995; Prado et al., 2000), there is some disagreement with the suggestion that metabolically labile primary metabolites, including reducing sugars, are "compatible" cytosolutes, since many of them have effects on cytoplasmic enzymes and could become incompatible in high concentrations (Rozema et al., 1978). Given the importance of embryonic growth, it is essential to hydrolyze the stored sugars into reducing sugars absorbed in the scutellum and transported via phloem to provide an immediate source of respirable substrate for early seedling growth (Bewley & Black, 1994). Inhibition of amylase activity at 225 mM NaCl solution slows down the mobilization rate of soluble sugars, which in turn,

delays germination. The combination of growth regulators, thiourea and betaine, with NaCl significantly alleviated the inhibitory effects on amylase activity and mobilization of reducing sugars, which led to the successful completion of germination, particularly with thiourea. It has been shown that exogenously supplied sucrose induces the expression of the amylase gene in leaf-petiole of sweet potato (Nakamura et al., 1991). It is possible that a similar mechanism is present in cucumber cotyledons; the accumulated sucrose might be responsible for the induction of  $\beta$ -amylase. It has been reported that water stress enhances the expression of  $\alpha$ -amylase in barley (Jacobsen et al., 1986). Although they did not determine the contents of sugars,  $\alpha$ -amylase induced by water stress may play a role in increasing the contents of sugars, though there is no direct evidence for this working hypothesis at present. Though the present study does not deal with  $\alpha$ -amylase and  $\beta$ -amylase separately, the available evidence suggests that mobilization and accumulation from endosperm to embryo is possible through enhanced amylase activity. This enhancement was possible by the application of growth regulators under saline medium.

Gill & Singh (1985) have suggested that exposure of plants to environmental stresses such as salinity and water stress can hinder germination, growth, respiration, and other related processes. Gill et al., (2002) have also hinted at the possibility of enhancement in the levels of reducing sugars to overcome stress and, thus, pave the way for germination and further growth. Given the importance of growth regulators, it is concluded that growth regulators such as thiourea and betaine considerably alleviated salinity-induced dormancy by inducing significant enhancement in amylase activity, which resulted in a subsequent increase in the mobilization of reducing sugars from endosperm to embryo. Consequently, these biochemical changes led to improvement in germination velocities and facilitated early seedling growth.

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