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Biochemical Responses of Dimorphic Seeds of *Arthrocnemum indicum* Willd. during Germination, Inhibition, and Alleviation under Saline and Non-Saline Conditions

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Abstract: Dimorphic seeds of *Arthrocnemum indicum* (a halophytic shrub) exhibit diverse germination responses following salinity treatment and subsequent exposure to growth regulators by adjusting their physiological and biochemical processes. The present study examined the effects of 24-h and 72-h NaCl treatment on germination, germination velocity, and the associated biochemical changes in protein and RNA contents of 2 seed morphs, and compared these effects after transfer into distilled water or 10 mM thiourea solution. Treatment for 24 and 72 h in NaCl solution induced a significant reduction in the rate of germination and final percent germination of both seed morphs. No germination took place in brown winter seeds in 300 and 400 mM NaCl treatment. In a separate experiment, seeds previously treated with NaCl for 24 and 72 h were transferred to distilled water or thiourea, and final germination was recorded. Germination velocities were higher following transfer into thiourea than into distilled water for black summer seeds, but not for brown winter seeds. The damage caused by salinity to total protein and RNA contents were not completely alleviated by the use of thiourea or distilled water, but showed only a limited tendency to recover from salinity stress. The seeds imbibed in different salt concentrations for different time periods exhibited different capacities to recover after their transfer into distilled water or thiourea. The inhibitory effects of NaCl on germination were more pronounced on brown seeds than on black seeds.

Key Words: Distilled water, DNA, germination, protein, RNA, salt, thiourea

Introduction

Arthrocnemum indicum Willd. is a perennial halophytic shrub of the family Chenopodiaceae, which is found in tropical marshes that are frequently inundated with seawater. *A. indicum* is characterized by a bushy appearance and mostly occurs as pure patches, or rarely, with other species (1). *A. indicum* produces dimorphic seeds, which are characterized as black or brown. Dimorphic seeds of halophytes show different germination responses to salinity, temperature, moisture, and growth regulating chemicals. Growth regulators such as thiourea, GA_3, and kinetin have exhibited different effects on germination (2). The effects of GA_3, kinetin, thiourea, nitrate, nitrite, proline, and temperature on seeds grown in saline medium have been extensively studied (2,3-9), but biochemical changes during germination in halophytes have not been reported. Therefore, the present study investigated the changes in protein and RNA synthesis during germination under salinity stress, as well as the roles played by distilled water and thiourea solution in alleviating the stress. The main objectives of the present study were to determine the effect of salinity stress on germination and its relation to protein and RNA synthesis, and to assess the biochemical changes that take place during stress alleviation with distilled water and thiourea.

Materials and Methods

Experiment 1

Seeds of *Arthrocnemum indicum* were collected in May and June 2004 and from December 2004 to January 2005 from sand dunes and flats on sandpits around the Karachi coast and Gadani. Seeds were

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separated into the 2 dimorphic forms and surface sterilized using benlate as a fungicide. Germination was carried out in 50 tight-fitting plastic petri dishes 9 cm in diameter, which contained 5 ml of test solution. Each dish was placed in a 10 cm diameter plastic petri dish as an added precaution against loss of water by evaporation. Seeds were germinated in a Hotpack programmed, refrigerated growth chamber with an alternating temperature regime of 20 and 30 °C, where the higher temperature coincided with the 12-h light period (Sylvania Cool White florescent lamp; 25 µmol m^{-1} s^{-1}; 400-700 nm) and lower temperature coincided with the 12-h dark period. Concentrations of 0, 100, 200, 300, and 400 mM NaCl were used (0 served as control). Four replicates of 25 seeds for each seed morph were used for each treatment. Seeds were considered to have germinated upon the emergence of the radicle. Percent germination was recorded on alternate days for 15 days. The rate of germination was estimated using a modified Timson index; germination velocity = Σ G/t, where G is the percentage of seed germination at one day intervals and t is the total germination period. The maximum possible value using this index with our data was 50 (i.e. 1000/20). The higher the value, the more rapid the rate of germination. Seeds were imbibed in salt concentration for 24 and 72 h and then transferred into distilled water or thiourea (10 mM) solution. The seeds were placed in a germinator and percent germination and rate of germination were recorded on alternate days for 15 days.

Experiment 2

A preliminary test was carried out to examine the timing of radicle emergence under control and saline conditions in order to plan a schedule for testing various biochemical processes during germination. The experiment was divided into 2 parts. Firstly, 200 healthy seeds were placed in 50 × 9 cm tight fitting glass petri dishes with 10 ml of test solution, i.e. 0, 100, 200, 300, and 400 mM NaCl (0 served as control for salinity). The biochemical tests for protein, RNA, and DNA contents were determined after 24 and 72 h imbibitions. In the second part of the experiment, 150 seeds were imbibed in each of the different saline solutions (0, 100, 200, 300, and 400 mM NaCl), and 75 seeds from each petri dish were transferred into distilled water or 10 mM thiourea solution. The changes in protein, RNA, and DNA contents were recorded 24 and 72 h after transfer into distilled water or thiourea. Each dish was covered with a 12 cm diameter glass petri dish as an added precaution against loss of water by evaporation. The whole setup was placed in a growth chamber (Hotpack programmed, refrigerated incubator). The photoperiod, light intensity, and relative humidity were 13 h, 300 µmol m^{-1} s^{-1}, and 70%, respectively at an alternating temperature regime of 20 and 30 °C. Each treatment and control was replicated 4 times.

Extraction and estimation of protein and RNA content

Twenty-five seeds were collected randomly from all treatments and controls and crushed with a mortar and pestle in 10 ml of 5% TCA. The crushed material was centrifuged at 400 rpm for 20 min. Then, 5 ml of 0.5 N NaOH was added and the mixture was incubated at 37 °C for 16 h. The optical density (OD) of the clear solution was measured at 260 nm and 280 nm using a Shimadzu UV mini spectrophotometer against a reagent blank. The protein was estimated by the method outlined by Boyer (10) and expressed in mg g^{-1} 25 $seeds^{-1}$. Prior to the estimation of total RNA contents, 25 seeds were extracted in ice-cold 60% perchloric acid. Lipid was removed by repeated washing in a mixture of ethanol-ether-chloroform in a ratio of 2:2:1. The residue was then hydrolyzed in 5 ml of 5% TCA. Total RNA was calculated by the formula described (10) and expressed in µg/g/25 seeds.

Statistical analysis

ANOVA analysis was used to determine significant differences among means. A Bonferroni test was carried out to determine if significant ($P < 0.05$) differences occurred in individual treatments.

Results

The study showed that salinity gradually reduced the germination velocity (Timson index) and final percent germination of both seed morphs. No germination was recorded at 300 and 400 mM NaCl treatment in brown winter seeds, while black summer seeds germinated at 300 mM (Figure 1). Likewise, the germination velocity of black seeds was slightly higher than that of brown seeds.

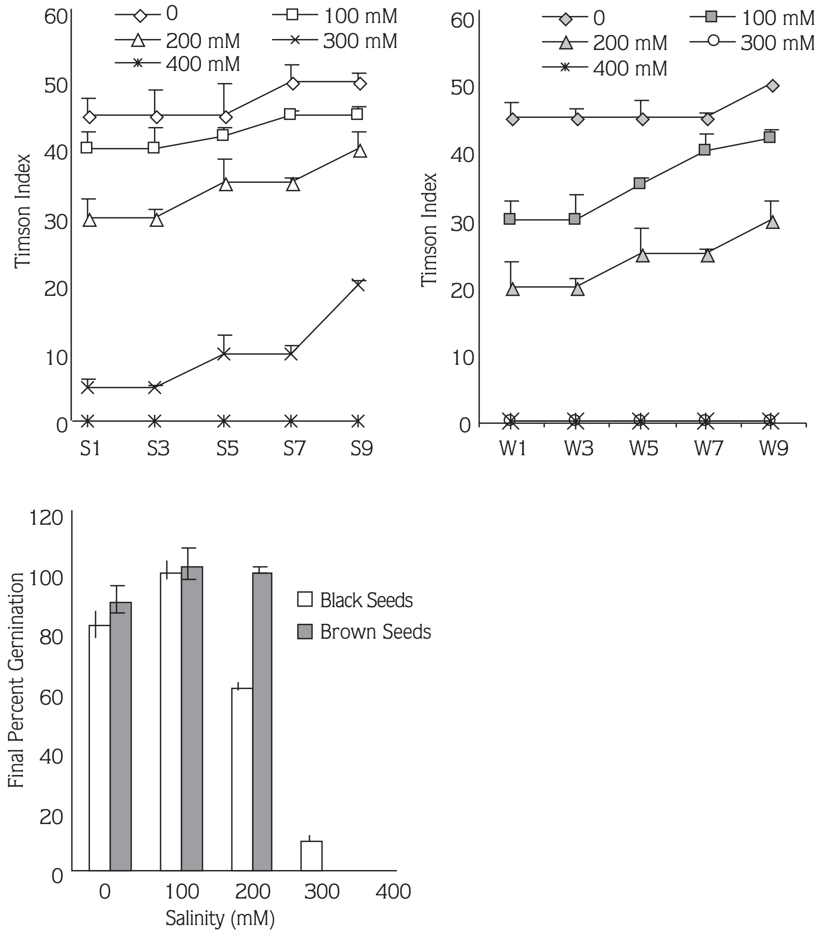

Figure 1. The effect of salinty on germination and germination velocity of dimorphic seeds of *A. indicum*.
X axis symbols: W = winter seeds; S = summer seeds; 1, 3, 5, 7, and 9 = days

The seeds that were imbibed in different concentrations of NaCl solution for 24 and 72 h, and subsequently transferred into distilled water, showed different patterns of recovery with significant increases in percent germination and Timson index value (Figures 2, 3, 4A, and 4C). Seeds exposed to 300 and 400 mM NaCl showed delayed recovery in germination velocity; however, summer seeds showed a linear increase in germination velocity (Timson index) compared to winter seeds in high concentration and prolonged exposure to NaCl. Germination velocity of winter seeds slowed as the concentration and exposure to NaCl increased. Final percent germination was higher in summer seeds than in winter seeds for all treatments (Figures 4a and 4C). Both seed morphs showed significant recovery in final percent germination after transfer into distilled water.

When the seeds were transferred from NaCl to thiourea solution, the recovery of the seeds with respect to germination velocity was better than in distilled water (Figures 4B and 4D); however, 400 mM-treated seeds showed 75% final percent germination in black summer seeds and 50% in brown winter seeds. Seeds exposed to 400 mM salinity showed increased germination velocity in thiourea compared to summer seeds transferred to distilled water. Data relating to final percent germination and velocity revealed significant recoveries in distilled water and thiourea solution after prolonged exposure to NaCl concentrations.

Seeds exposed to 24- and 72-h salinity before transfer into distilled water or thiourea solution showed significant decreases in protein contents compared with the controls. Black seeds, however, showed less protein

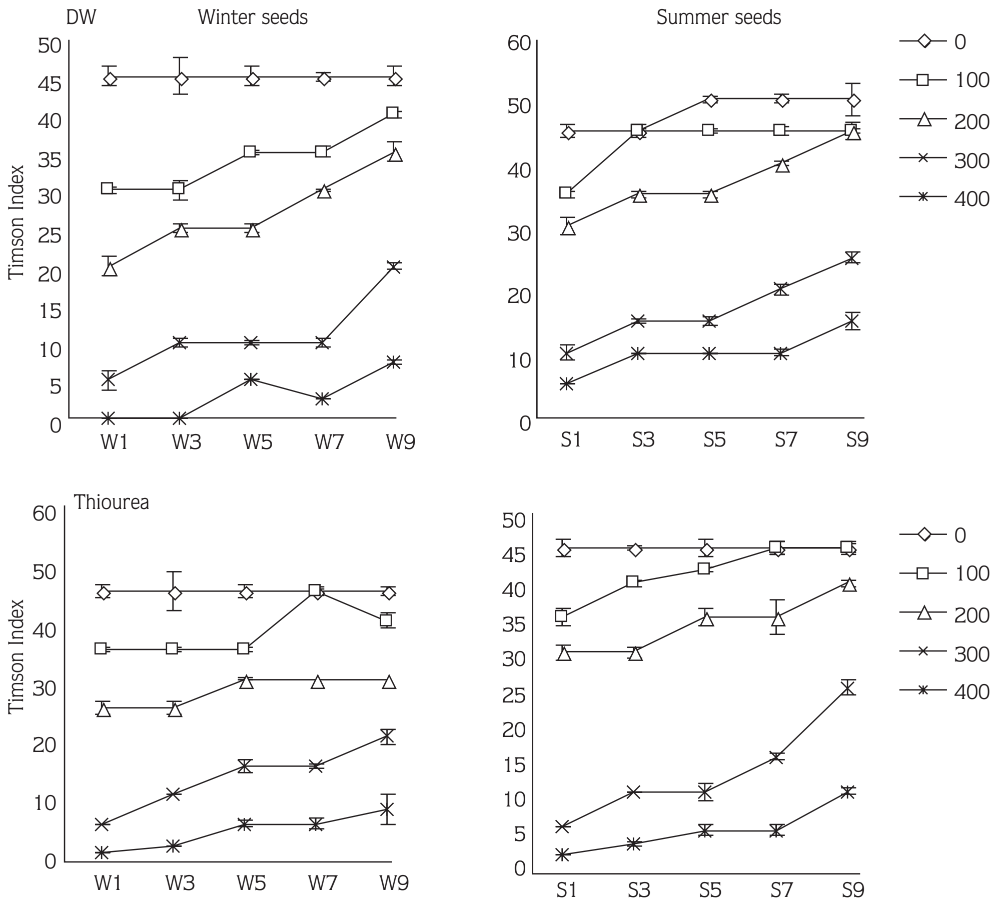

Figure 2. Germination velocity of *A. indicum* transferred to distilled water or thiourea following 24 h in NaCl solution.
X axis symbols: W = winter seeds; S = summer seeds; 1, 3, 5, 7, and 9 = days

content in thiourea solution than in distilled water. In contrast, brown winter seeds exposed to the same level of treatment showed a greater decrease in protein content (Table 1). Increase in protein was recorded when black seeds were transferred to thiourea solution after 24-h NaCl treatment. It was revealed in the present study that thiourea solution was better in alleviating the inhibitory effect of NaCl on protein synthesis compared to distilled water. Similarly, it was observed that the duration of NaCl treatment had pronounced effects on the timing of resumption of protein synthesis. Black seeds showed a slight decrease compared to the controls, indicating more rapid recovery than brown seeds.

The values of RNA did not differ significantly between black and brown seeds transferred to distilled water after 24-h and 72-h NaCl treatment; hence, only slight variations were noted (Table 2). However, higher reductions were recorded for 24-h NaCl treatment 24 and 72 h after transfer into distilled water. Seeds transferred to thiourea solution showed a smaller decrease compared to seeds transferred to distilled water; however, brown seeds showed a greater decrease compared to black seeds. Similarly, the RNA content of both seed morphs showed significant decreases (except black seeds imbibed in 100 mM NaCl for 72 h) when transferred to distilled water. This decrease in RNA content was, nevertheless, alleviated more by the thiourea solution than distilled water for 24-h NaCl treated seeds. Brown seeds showed greater reductions compared to black seeds.

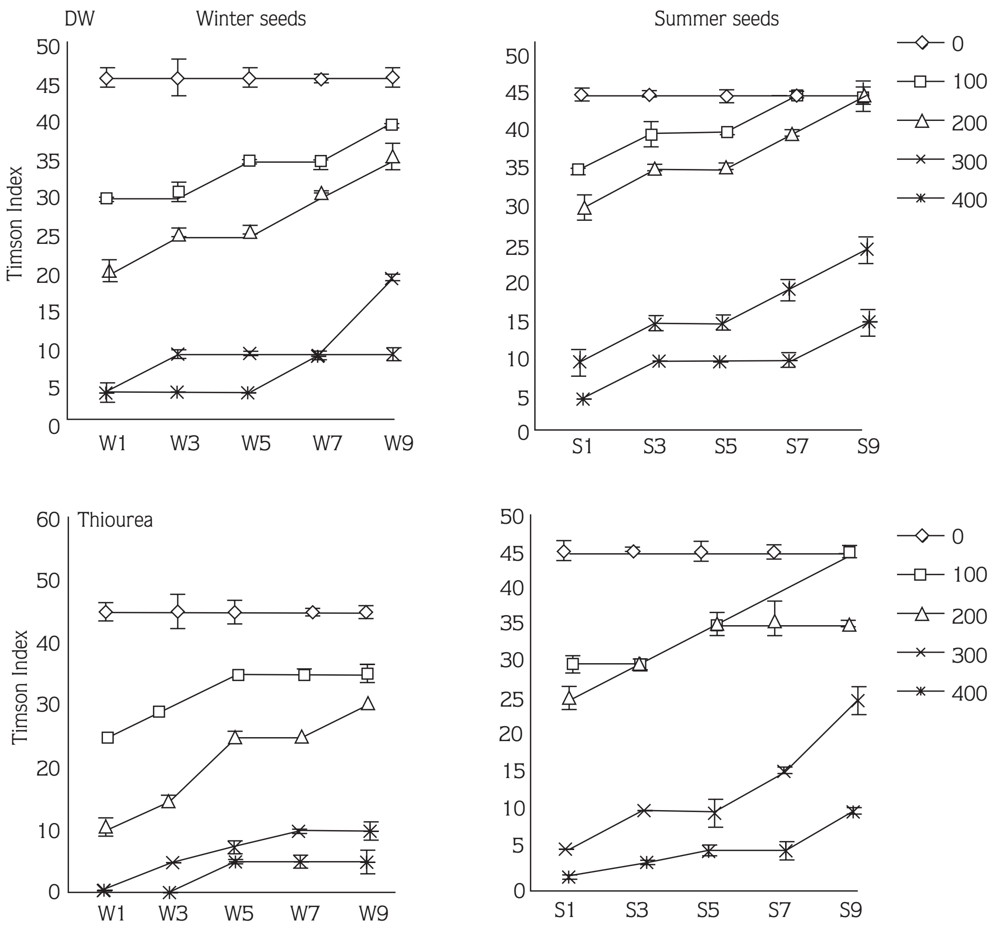

Figure 3. Germination velocity of *A. indicum* after transferring to distilled water or thiourea after 72 h in NaCl solution.
X axis symbols: W = winter seeds; S = summer seeds; 1, 3, 5, 7, and 9 = days

Discussion

Diverse germination responses of both seed morphs of *A. indicum* were observed in saline medium and differential recovery patterns were noted after transferring to distilled water or thiourea. Seeds exposed to 300 and 400 mM NaCl showed delayed recovery in germination velocity. On recovery in distilled water, black summer seeds showed a linear increase in germination velocity (Timson index), with 75% final percent germination, compared to brown winter seeds (50%). Contrary to the recovery in distilled water, 400 mM NaCl-treated black seeds showed a 65% final percent germination and brown seeds showed a 40% final percent germination after the seeds were transferred into thiourea.

Observation revealed that thiourea solution led to better alleviation of the inhibitory effects of NaCl on protein synthesis compared to distilled water. Likewise, it was observed that the duration of NaCl treatment had pronounced effects on the time taken for the resumption of protein synthesis. Black seeds exhibited a reduction in protein content to a lesser extent than controls, thus indicating more rapid recovery than brown seeds. It was obvious from the experiments that the decrease in RNA content was alleviated more by the thiourea solution than by distilled water, especially after 24-h NaCl treatment. Brown seeds showed a greater decrease in RNA content compared to black seeds. The reduction in protein under NaCl stress conditions recorded in this investigation is in accordance with the results of other investigations, in which a high concentration of NaCl affected protein

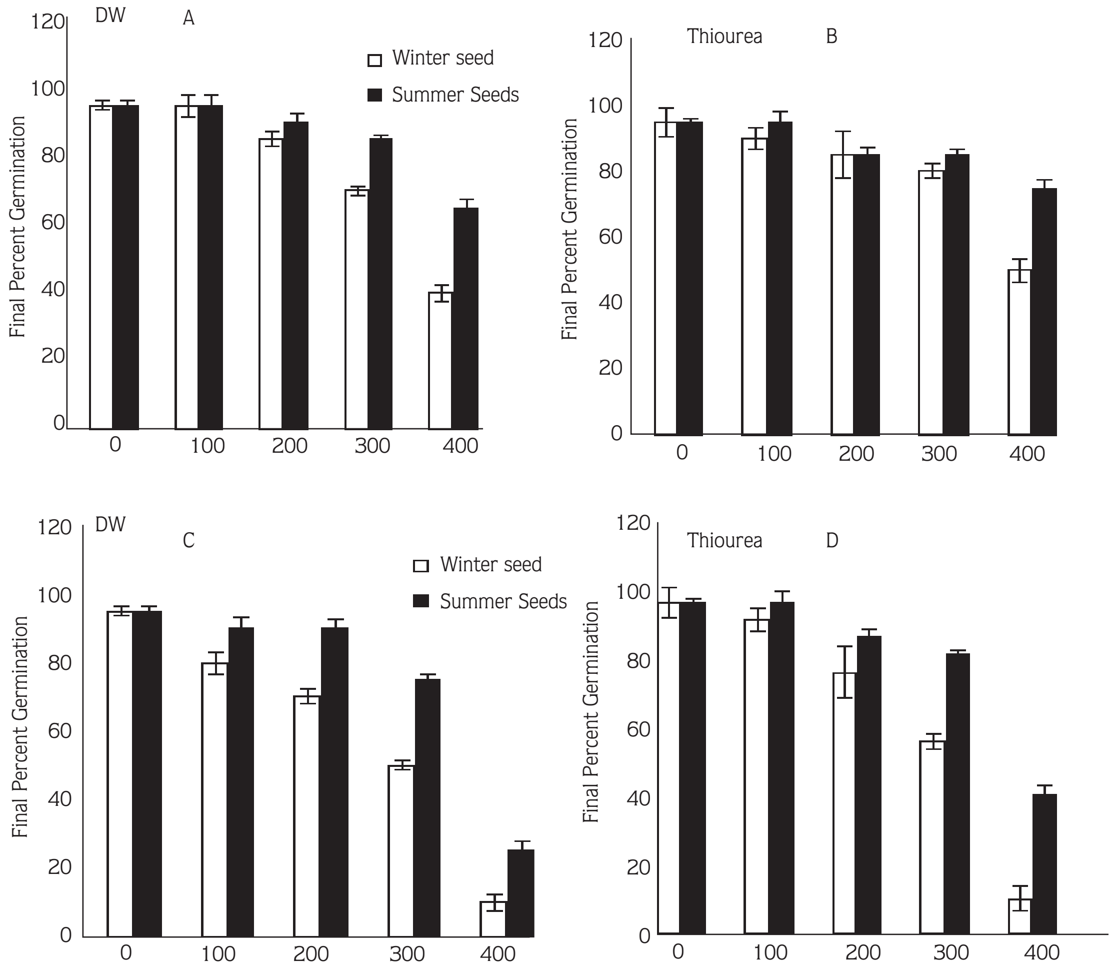

Figure 4. Final percent germination of *A. indicum* after transferring in distilled water or thiourea. Graph symbols: DW = distilled water; A, B = Transfer after 24-h NaCl exposure; C,D = transfer after 72-h NaCl exposure

synthesis through the inhibition of some enzymes that regulate the process (11-16). Recovery of seed germination in thiourea and distilled water reflected the enhancement of germination and Timson index (germination velocity), which could have been due to resumption of metabolic activities, as evinced by increased protein synthesis, once the seeds were removed from NaCl solutions and transferred into distilled water or thiourea solution. Metabolic activities, like protein, nucleic acid, and RNA synthesis, are retarded by salinity (17,18), which results in the inhibition of germination and a reduction in germination velocity.

In addition, alteration in the biochemical processes of dimorphic seeds of halophytes, such as *A. indicum*, during germination and early seedling growth in saline medium is common. A number of reports suggest that hyper-saline environments cause delayed germination (13) by reducing hydrolytic enzyme activities and retarding the mobilization rate of metabolites (16). During germination and early seedling growth, cell division and enlargement require transportation of respiratory substrates, in the form of soluble sugars, and low molecular weight protein from seed storage organs to the site of growth (19). RNA synthesis is initiated almost immediately upon imbibition, but its rate is slow during germination or seedling growth, when RNA is actively synthesized (18-20). Since the processes of DNA and RNA synthesis are related to protein synthesis, reductions in RNA synthesis ultimately

Table 1. Changes in total protein content of 2 seed morphs of *Arthrocnemum indicum* Willd. after transfer to distilled water or 10 mM thiourea solution.

NaCl Solution (mM)	Increase or decrease in total protein contents over control mg g^{-1}							
	Distilled Water							
	*Treatment 1				#Treatment 2			
	Brown Seeds		Black Seeds		Brown Seeds		Black Seeds	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
100	–25	–16	–20	–26	–55	–65	–25	–44
200	–14	–79	–18	–74	–45	–88	–45	–48
300	–94	–86	–13	–81	–37	–104	–78	–95
400	–54	–104	–18	–74	–85	–124	–75	–114

NaCl Solution (mM)	Thiourea Solution (10 mM)							
	*Treatment 1				#Treatment 2			
	Brown Seeds		Black Seeds		Brown Seeds		Black Seeds	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
100	–80	–50	10	3	–79	–69	–40	–27
200	–100	–85	8	15	–115	–98	–42	–55
300	–79	–90	–12	–13	–126	–99	–57	–64
400	–95	–85	–11	–12	–81	–94	–69	–64

*Treatment 1 = 24-h NaCl treated seeds

#Treatment 2 = 72-h NaCl treated seeds

( ) increase over control, (–) decrease over control

reduce the enzymatic protein content. Hence, it could be inferred that protein and nucleic acid synthesis were restored once seeds were removed from the salt solution and imbibed in thiourea or distilled water. The promotion of seed germination by nitrogenous substances, including thiourea, nitrate, and nitrite, has been previously reported (19,21). The stimulatory effect of inorganic nitrates and nitrites are frequently reported as exerting a marked stimulation effect on the seed germination of a wide range of species. This effect has been previously recorded for some dicotyledonous seeds and grains of the family Poaceae (22). Furthermore, nitrate is known to interact with light in affecting seed germination (23).

Moreover, seeds of halophytes and non-halophytes germinate better in distilled water, but differ in their ability to germinate at higher salinities. Halophytes remain viable for an extended period when immersed in saline water (24). It has been proposed that thiourea counteracts the effects of increased ABA and reduces the level of cytokinin in plant tissue exposed to drought induced by water stress, salinity, or high temperature (25). According to Gul and Weber (26), treatment with thiourea is highly effective in alleviating the inhibition of germination by salinity or high temperature, through a physiological process.

The present study strongly revealed that germination-associated biochemical processes, such as RNA and

Table 2. Changes in total RNA contents of two seed morph of *Arthrocnemum indicum* Willd. after transfer in DW and 10 mM thiourea solution.

	IIncrease or decrease in total RNA content over control (µg mg^{-1})							
	Distilled Water							
NaCl Solution (mM)	*Treatment 1				#Treatment 2			
	Brown Seeds		Black Seeds		Brown Seeds		Black Seeds	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
100	–13	–32	–12	–32	–24	–1	–28	1
200	–14	–51	–24	–47	–38	–29	–35	–29
300	–36	–55	–36	–54	–40	–48	–16	–47
400	–54	–74	–55	–71	–53	–52	–50	–50
	Thiourea Solution (10 mM)							
NaCl Solution (mM)	*Treatment 1				#Treatment 2			
	Brown Seeds		Black Seeds		Brown Seeds		Black Seeds	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
100	–23	–43	–2	–2	–27	–31	–29	–55
200	–53	–64	–4	–11	–41	–52	–30	–69
300	–79	–78	–32	–13	–65	–70	–44	–77
400	–83	–19	–39	–42	–93	–98	–48	–90

*Treatment 1 = 24-h NaCl treated seeds
#Treatment 2 = 72-h NaCl treated seeds
( ) increase over control, (–) decrease over control

protein synthesis, are affected by high NaCl concentrations. Distilled water and thiourea alleviated the inhibitory effect of high NaCl concentration on protein and RNA synthesis. In addition, prolonged exposure of seeds to high NaCl concentration has an irrecoverable effect on germination, velocity, protein, and RNA synthesis. Consequently prolonged exposure of seeds to high NaCl concentrations and their subsequent transfer to distilled water or thiourea solution showed decreases in protein and RNA contents, resulting in lower germination velocity and final percent germination compared to the control seeds.

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