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Research Article

Effects of Salinity: Calcium Interaction on Growth and Nucleic Acid Metabolism in Five Species of *Chenopodiaceae*

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Abstract: Seed germination, seedling growth, and some enzyme activity of nucleic acid metabolism were studied in 5 members of *Chenopodiaceae* [*Beta vulgaris* L., *Chenopodium quinoa* Willd., *Spinacea oleracea* L., *Allenrolfia occidentalis* (S.Watson) Kuntze, *Atriplex hortensis* L.] under NaCl salinity alone or combined with 0.5 mM $CaSO_4$. High salinity delayed radical emergence and decreased germination percentage in all plants. Combined $CaSO_4$ reduced inhibition of seed germination in *B. vulgaris*, *S. oleracea*, and *A. hortensis*, but increased it in *C. quinoa* and *A. occidentalis*. $CaSO_4$ reduced plant growth to a greater degree than high salinity did. High salinity decreased total nucleic acid content in all plants, except *C. quinoa*. Combined $CaSO_4$ enhanced this effect in *B. vulgaris*, *S. oleracea*, and *A. hortensis*, but alleviated it in *C. quinoa* and *A. occidentalis*. Salinity progressively activated deoxyribonuclease (DNase I) and $CaSO_4$ enhanced its activation in all plants, except *B. vulgaris*. Salinity increased endonuclease activity, except in *S. oleracea* and *A. occidentalis*. Addition of $CaSO_4$ increased endonuclease activity in *C. quinoa*, *S. oleracea*, and *A. occidentalis*. Salinity inhibited ribonuclease A (RNase A) activity, but increased it in *C. quinoa* and *S. oleracea*, whereas $CaSO_4$ alleviated such inhibition in *A. occidentalis* and *A. hortensis*. Salinity increased ribonuclease T (RNase T) activity in all plants, especially in *C. quinoa*, *S. oleracea*, and *A. occidentalis*.

Key Words: *Chenopodiaceae*, growth, nucleic acid, DNase I, endonuclease, RNase A and RNase T

Introduction

Osmotic stress is one of the effects of salinity on plants. It is induced by a decrease in soil water potential, which is reflected morphologically as a decrease in leaf expansion. In saline environments, plant adaptation to salinity during germination and early stages of growth is crucial for the establishment of species (Ungar, 1991, 1995). The seedling stage is the most vulnerable stage of the life cycle of plants, whereas germination determines where and how seedling growth begins (Gutterman, 1993; Kigel, 1995). An important stage in the life cycle of plants that is vulnerable to salinity stress is seed germination (Mayer & Poljakoff-Mayber, 1982; Biss et al., 1986; Corchete & Guerra, 1986; Kurth et al., 1986). Seed germination involves imbibition of water, activation and formation of enzyme systems, mobilisation of storage reserves, and growth and establishment of the seedling. All these processes may be adversely affected by NaCl (Levitt, 1980). Salinity has inhibitory effects on seed germination by limiting water uptake and arresting radical emergence, although the ion toxic influence of salt cannot be excluded (Alwan et al., 1989; Sharma & Yamdagni, 1989).

Plant growth is affected by the interaction of Na^+ or Cl^-, as well as by mineral nutrients, causing imbalance in nutrient availability, uptake, or distribution within plants (Grattan & Grieve, 1992). Thus, a high concentration of Na^+ in the external solution causes a decrease in both K^+ and Ca^{2+} concentrations in plant tissues. This decrease could be due to the antagonism of Na^+ and K^+, or Ca^{2+} at sites of uptake in roots (Gronwald et al., 1990; Lynch & Lauchli, 1998).

There are reports indicating the importance of adequate levels of Ca^{2+} in alleviating the deleterious effects of salinity on plant growth (Epstein, 1972; Rains, 1972; Gong & Yang, 1994). This is due to the role of

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Ca^{2+} in maintaining the selectivity and integrity of the membrane, and hence decreasing the passive accumulation of Na^{+} in plant tissues (Hu et al., 1997). Two processes are of considerable importance in the establishment of seedlings in a saline environment: cell elongation and maintenance of a balanced nutrient ion uptake, both of which require Ca^{2+} (Epstein, 1972; Mengel & Kirkby, 1982).

Because the *Chenopodiaceae* species used in this study are cultivated under various conditions throughout the world, comparisons between varietals are questionable; nonetheless, identification of varietal differences in response to salinity between some *Chenopodiaceae* species was the aim of the present study. The differences in nucleic acid content and nucleolytic enzyme activity were determined in order to detect if they had similar responses to salinity stress during their growth. It was also determined if the changes in nucleolytic enzyme activity, especially ribonuclease, could support the hypothesis that they could be used as a marker for salt stress.

Materials and Methods

Seeds of 5 *Chenopodiaceae* species, Ruby Red Chard, Swiss Chard (*Beta vulgaris* L. var. *flavescens* Lam.), quinoa (*Chenopodium quinoa* Willd. cv. Andean Hybrid), spinach (*Spinacea oleracea* L.), *Allenrolfia occidentalis* (S.Watson) Kuntze, and garden orach (*Atriplex hortensis* L.) were obtained from Thompson and Morgan (Jackson, NJ, USA), and were sterilised and germinated in a dark incubator at 25 °C. All experiments consisted of 8 treatments. The non-saline treatment was modified Hogland's nutrient solution (Epstein, 1972; Table 3-1). The salt treatments were NaCl at –3, –9.5 and –14.2 bar in addition to Hogland's solution. The other 4 treatments contained an additional 0.5 mM $CaSO_4$ with Hogland's nutrient solution or in combination with salt treatment solutions.

Six replicates of 100 seeds of each species were placed in 9-cm plastic petri dishes containing a standard blue germination blotter moistened with 10 ml of each solution. The solutions were replaced every 2-3 days.

Radical protrusion was taken as the criterion for germination. Plants were harvested at the end of the experiment (15 days after seed germination). Shoot and root lengths were recorded in 10 germinated plants for each treatment. Fresh samples were immediately used for the determination of nucleic acid content and enzyme activity. A known weight of the fresh seedling was placed in a mortar and homogenised in 20 ml of distilled water. The filtrate was separated from the residue and used in the study.

Deoxyribonuclease (DNase 1) assay

Estimation of DNase I was carried out by the method of Kunitz (1950), in which 0.5 ml of sample was mixed with 2.5 ml of buffer substrate (pH 5.0), and E/30 s for 5 or 10 min at 260 nm was measured against a blank. The volume activity was equal to $(3.0 \times 1000)/0.5 \times \Delta E$ (units/ml sample).

Endonuclease assay

Endonuclease activity was measured by the method of Linn & Lehman (1965), in which 0.5 ml of the sample was mixed with 1.0 ml DNA buffer (pH 8.0) and 0.55 ml of distilled water. An aliquot was taken at zero time and another after 30 min for the blank. The incubation solution (0.3 ml) was mixed with 0.2 ml of distilled water and 0.5 ml of 1 M perchloric acid. The mixture was centrifuged at 3000 rpm for 5 min, and 0.1 ml of the supernatant was taken, mixed with 2.9 ml of distilled water, and measured at 260 nm. $\Delta E = E_{sample} - E_{blank}$. Volume activity was equal to $(1.6 \times 1.0 \times 3.0)/(0.3 \times 0.1 \times 0.05) \times \Delta E$ (units/ml sample).

Ribonuclease A (RNase A)

Estimation of RNase A was carried out according to Kunitz (1946). In this method, 0.5 ml of the sample was mixed with 1.5 ml of ribonuclease solution (0.055%) and 1.45 ml of distilled water. The optical density was measured every 0.5 min for 10 min, and then after 1, 2, and 3 h at 300 nm. The volume activity was equal to $(3.0 \times 10)/\varepsilon \times 1.0 \times (0.05) \times (E_o - E_t)/t \times 1/E_o - E_f)$ (Kunitz units/ml sample). The extinction coefficient, ε, and the optical density, E_o, were extrapolated to the time when the reaction started. E_t was the optical density after t time (2-4, according to the position of the line), and E_f was the final optical density after completion of the reaction (approximately 3 h).

Ribonuclease T (RNase T)

RNase T was measured according to Egami et al. (1964). The sample (0.1 ml) was mixed with 0.25 ml of Tris buffer (pH 7.5), 0.1 ml of 0.02 M EDTA, and 0.3 ml of distilled water. The mixture was placed in a water bath

at 37 °C for 5 min, then 0.25 ml of 0.012% ribonuclease solution was added and the mixture kept at 37 °C for 15 min. The reaction was stopped by adding 0.25 ml perchloric acid/uranyl acetate solution (22 ml 70% perchloric acid + 78 ml distilled water + 750 ml uranyl acetate), followed by 5-min centrifugation at 3000 rpm. To 0.2 ml of the clear supernatant was added 4.8 ml of distilled water. The optical densities of the sample and blank were measured at 260 nm. The volume activity was equal to (31.2 × 10,000)/0.01× ΔE (Egami units/ml sample).

Estimation of nucleic acid content

Nucleic acid content was estimated spectrophotometrically by the method of Chaykin (1970).

Statistical analyses

Significant variation in the estimated variable in relation to the different treatments was determined using one-way analysis of variance (ANOVA) and the complementary test least significant difference (LSD), using SPSS version 11 (2001, SPSS Inc., Chicago).

Results

The germination of *B. vulgaris*, *S. oleracea*, and *A. occidentalis* was delayed in comparison to that of *C. quinoa* and *A. hortensis* (Table 1). The last 2 species expressed the greatest germination percentage, whereas the first 3 species showed the lowest germination percentage under control and different salinity treatments. In general, germination percentage decreased significantly in response to increased NaCl concentration alone, or in the presence of $CaSO_4$, especially in *S. oleracea* (Table 1). ANOVA indicated that seed germination percentage decreased significantly in *C. quinoa* ($P < 0.001$), *A. hortensis* ($P < 0.001$), *A. occidentalis* ($P < 0.001$), *S. oleracea* ($P < 0.001$), and *B. vulgaris* ($P < 0.001$). Addition of 0.5 mM $CaSO_4$ slightly reduced the effect of NaCl on seed germination percentage with all NaCl concentrations in *B. vulgaris, S. oleracea*, and *A. hortensis*, but it increased the effect of NaCl in *C. quinoa* and *A. occidentalis*, particularly at –3.0 and –9.5 bar, respectively (Table 1).

Table 1. Germination percentage of the 5 species of *Chenopodiaceae* after 15 days of exposure to different concentrations of NaCl, with or without the addition of 0.5 mM $CaSO_4$. The results are the mean of 4 replications.

Treatments	*B. vulgaris*	*C. quinoa*	*S. oleracea*	*A. occidentalis*	*A. hortensis*
NaCl alone					
0.0	38.50	98.00	39.00	48.25	96.25
–3.0	32.75	98.50	28.75	47.50	94.50
–9.5	25.25	92.75	23.75	34.50	89.00
–14.2	15.25	86.25	14.25	19.00	92.75
NaCl combined with $CaSO_4$					
0.0	30.00	98.00	37.00	41.25	90.50
–3.0	38.75	95.75	32.00	41.75	95.75
–9.5	28.25	91.25	25.00	27.50	91.00
–14.5	17.00	86.75	15.75	18.75	93.50
F	19.985	101.848	64.192	89.569	6.027
P <	0.001	0.001	0.001	0.001	0.001
LSD	2.787	0.718	1.575	1.431	1.751

Root and shoot length in *C. quinoa, S. oleracea*, *A. occidentalis*, and *A. hortensis* were reduced with increasing NaCl concentration (Figure 1C). In *B. vulgaris*, seedling length was significantly reduced ($P < 0.001$), as shown in Figure 1A. On the other hand, the addition of $CaSO_4$ to NaCl caused a greater reduction in root and shoot length in *C. quinoa, S. oleracea*, and *A. occidentalis*, and a greater reduction in shoot length in *A. hortensis* (Figure 1D). The response of root length varied among the 5 species; however, the reduction in root length was significant and more than 75% in *A. occidentalis* ($P < 0.001$) and *S. oleracea* ($P < 0.006$) when treated with –14.2 bar NaCl mixed with $CaSO_4$. Root length in *C. quinoa* declined significantly (55%) ($P < 0.001$) in response to –9.5 bar NaCl, and in *A. hortensis* it declined significantly (ca. 30%) ($P < 0.001$) when treated with –14.2 bar NaCl.

The highest nucleic acid content of the control plants was recorded in *A. hortensis*, while the lowest content was recorded in *C. quinoa* (Figure 2A). Application of NaCl treatments slightly reduced nucleic acid content, but addition of $CaSO_4$ to saline solutions resulted in a significant reduction in NaCl's effect in *C. quinoa* ($P < 0.001$) and *A. occidentalis* ($P < 0.001$), while it caused a significantly greater reduction in nucleic acid content in *B. vulgaris* ($P < 0.001$), *S. oleracea* ($P < 0.001$), and *A. hortensis* ($P < 0.001$).

The activity of DNase I increased along with increased salinity stress in all of the studied plants, except *A. hortensis*, where DNase I activity significantly decreased ($P < 0.001$) (Figure 2B). At the highest concentration NaCl treatment, enzyme activity was significantly enhanced (4 times that of the control) in *B. vulgaris* ($P < 0.001$). The combination of $CaSO_4$ with NaCl treatments significantly reduced the effect of NaCl on enzyme activity in *C. quinoa* ($P < 0.001$), *S. oleracea* ($P < 0.001$), and *A. occidentalis* ($P < 0.001$). Similar results were obtained in *A. hortensis*, except that of the control. Additionally, the combination of $CaSO_4$ and NaCl significantly decreased the effect of NaCl on enzyme activity in *B. vulgaris*.

Endonuclease activity was affected by NaCl treatments and the effect varied among the studied plants (Figure 3A). Salinity significantly inhibited the enzyme activity in *S. oleracea* ($P < 0.001$) and *A. occidentalis* ($P < 0.001$), but generally increased the activity in *B. vulgaris* ($P < 0.001$), *C. quinoa* ($P < 0.001$), and *A. hortensis* ($P < 0.001$). *A. hortensis* expressed a remarkably higher endonuclease activity under all NaCl treatments compared to the control values. Addition of $CaSO_4$ to the NaCl solution significantly increased the activity of the enzyme in *C. quinoa*, *S. oleracea*, and *A. occidentalis*, under all treatments. Conversely, addition of $CaSO_4$ reduced the enzyme activity in *B. vulgaris* and *A. hortensis* under NaCl treatments.

RNase A activity under the control condition was similar in all examined plants, as indicated by insignificant variation (Figure 3B). NaCl treatments inhibited the enzyme activity in *B. vulgaris, A. occidentalis*, and *A. hortensis*, but induced the activity in *C. quinoa* and *S. oleracea*. Combining $CaSO_4$ with NaCl solution slightly reduced the inhibitory effect of NaCl on endonuclease activity in *A. occidentalis* and *A. hortensis*, while it decreased its activity in *B. vulgaris, C. quinoa*, and *S. oleracea*.

RNase T activity varied in response to salinity among the studied plants. Its activity significantly increased with increasing salinity in *B. vulgaris* ($P < 0.001$) and *C. quinoa* ($P < 0.001$), and in *A. hortensis* it decreased at –9.5 bar ($P < 0.001$). A slight reduction effect was observed in RNase T activity in *A. occidentalis* ($P < 0.001$). $CaSO_4$ in combination with NaCl reduced RNase T activity in *B. vulgaris* and *A. hortensis*, while it reduced the inhibitory effect of NaCl in *C. quinoa, S. oleracea*, and *A. occidentalis*.

Discussion

Germination percentage of the studied species was highest in control or low salinity conditions, but higher salinity levels caused a gradual suppression of germination. It has been reported that inhibition of germination in *A. occidentalis* occurs at high concentrations of NaCl (Gul & Weber, 1999; Tattini & Gucci, 1999). The rate of suppression due to NaCl stresses varied among the studied species. In addition, salinity delayed the emergence of radicals in some of the studied species (*S. oleracea, B. vulgaris*, and *A. occidentalis*), and the delay was longer in *S. oleracea* than in the other species. Coincidentally, a decline in seed germination percentage with increasing salinity was reported in *Atriplex* spp. (Khan & Ungar, 1984; Katembe et al., 1998; Ungar, 1996), in *A. occidentalis* (Khan & Ungar, 1997), and in *B. vulgaris* (Ghoulam & Fares, 2001). Conversely, Jacobsen et al. (1998) found that

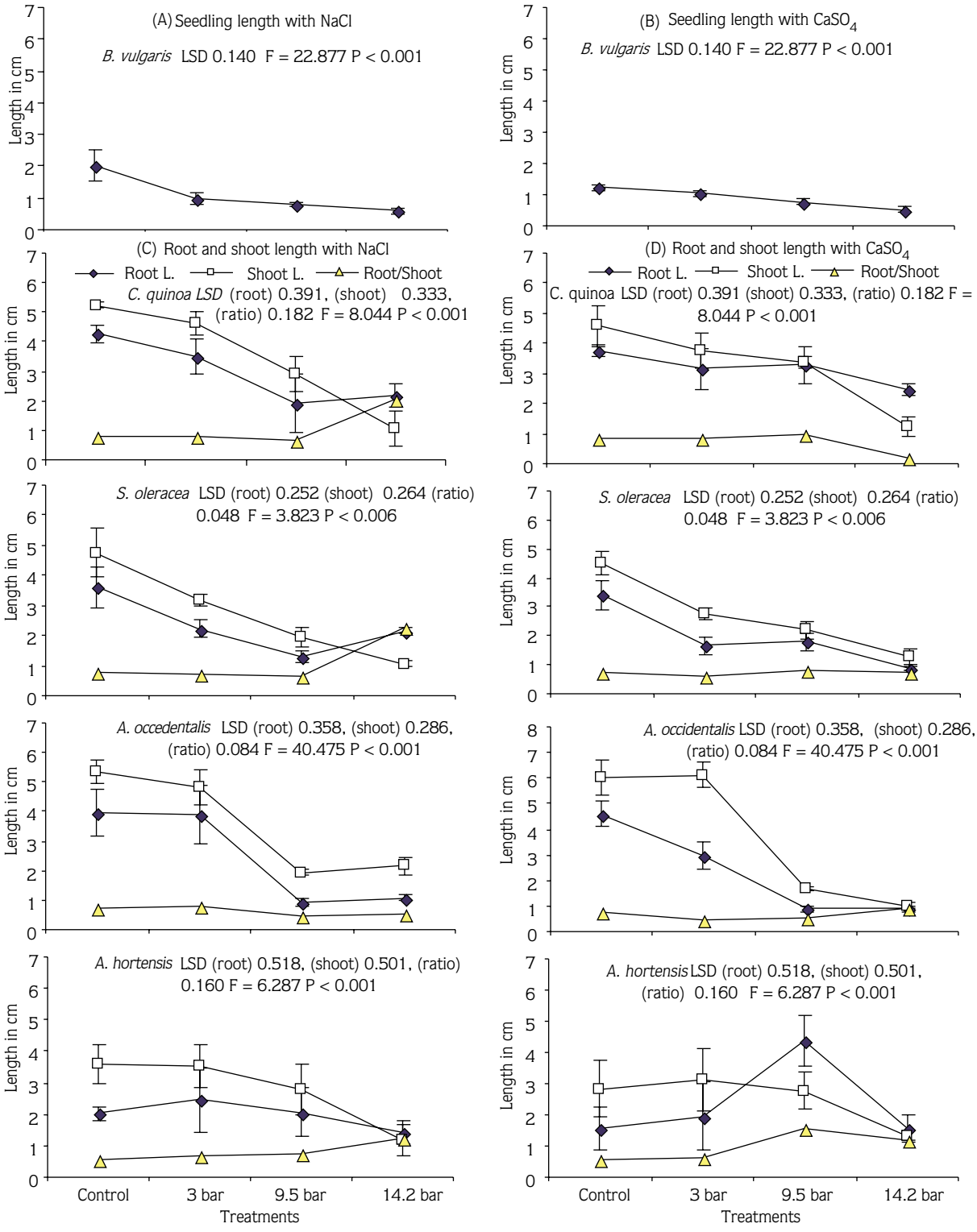

Figure 1. The seedling length of *B. vulgaris* (A) and (B), root, shoot length and root/shoot ratio (C) and (D) of *C. quinoa*, *S. oleracea*, A. occidentalis and *A. hortensis* under different concentration of NaCl singly or in combinations with 0.5 mM $CaSO_4$. The results are means of 4 replicates ± standard deviation.

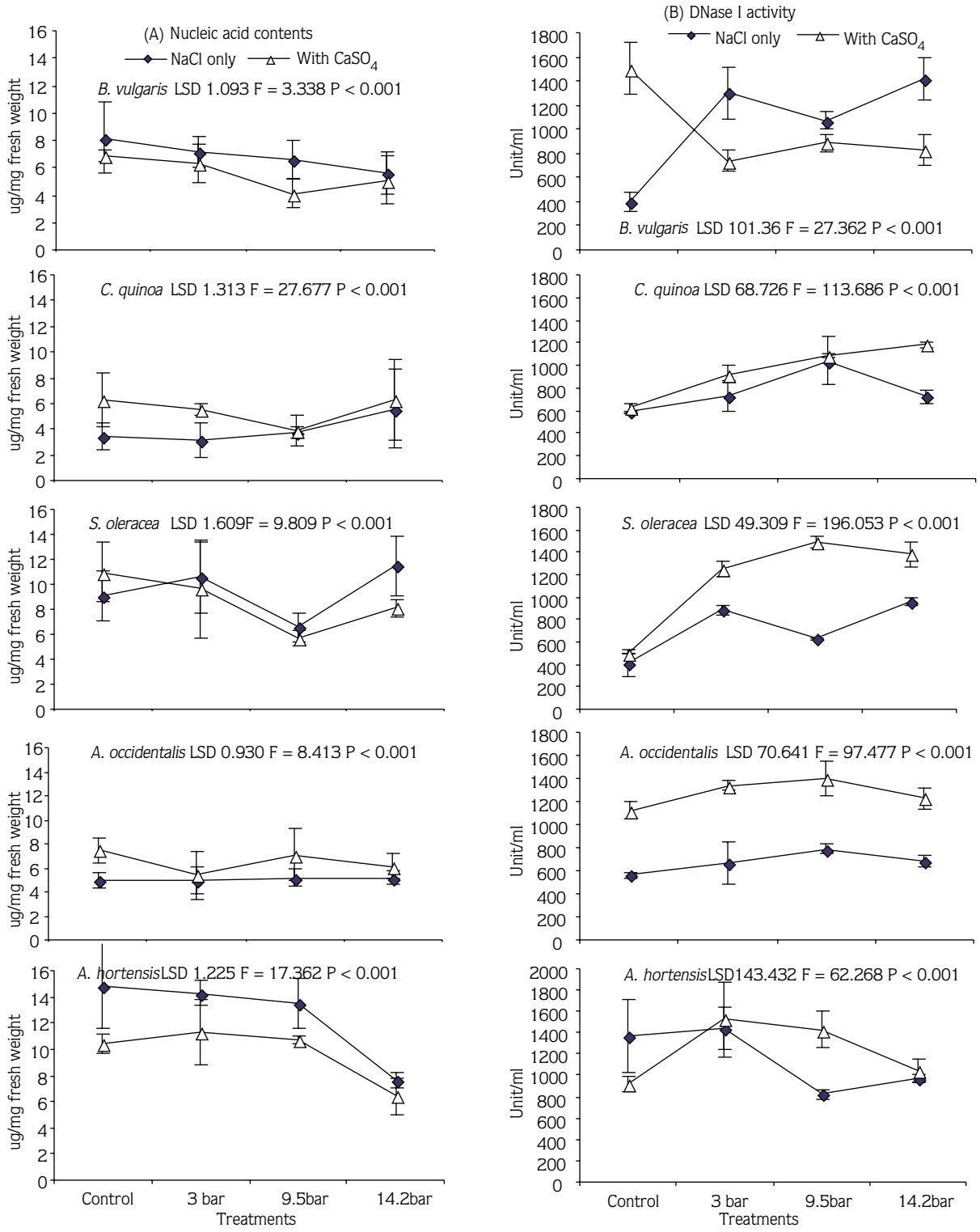

Figure 2. The total nucleic acids (A) and DNase I activity (B) of *B. vulgaris*, *C. quinoa*, *S. oleracea*, A. occidentalis and *A. hortensis* under different concentrations of NaCl singly or in combination with 0.5 mM $CaSO_4$. The results are means of 4 replicates ± standard deviation.

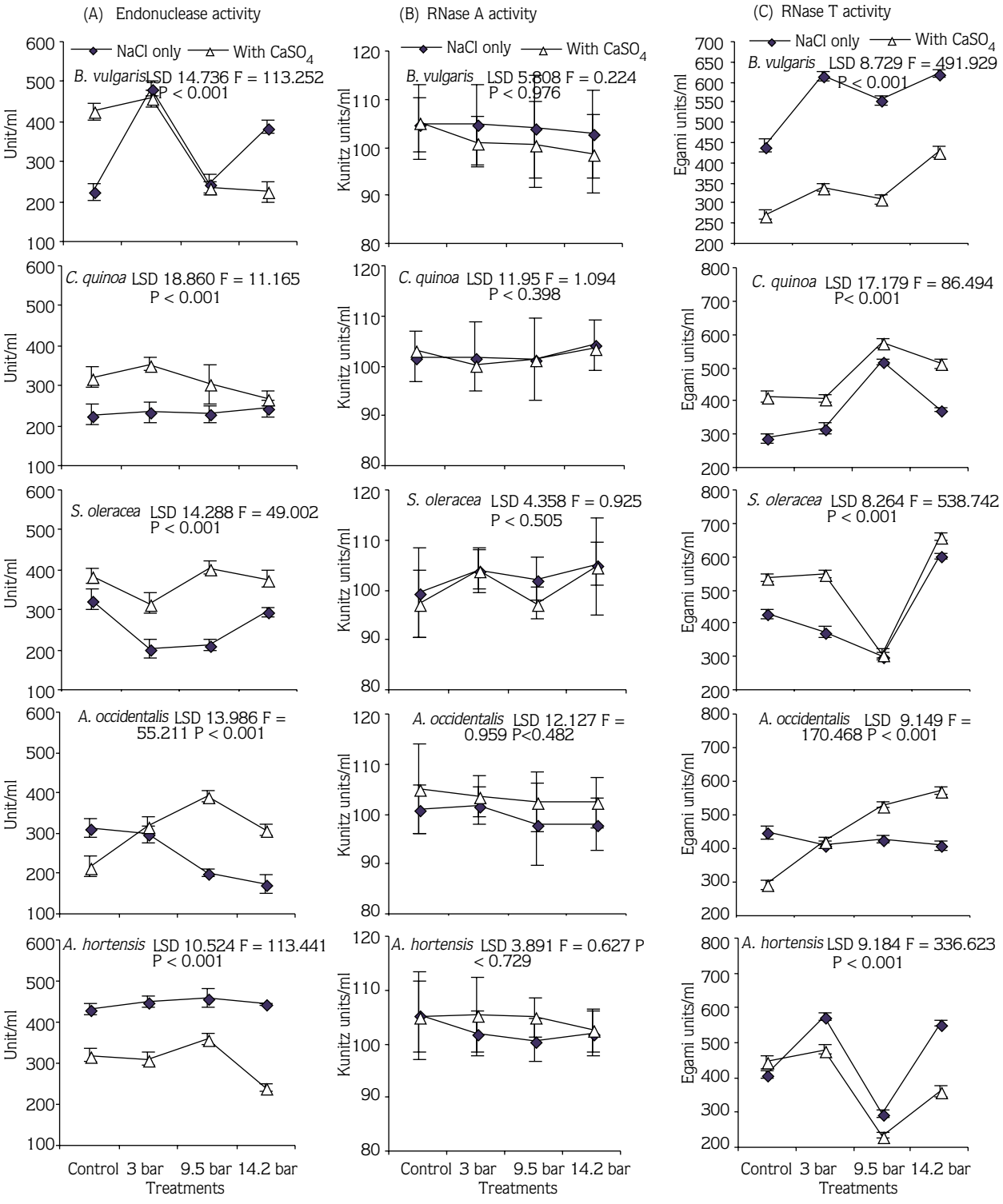

Figure 3. Endonuclease (A), ribonuclease A (B) and ribonuclease T (C) activities of *B. vulgaris*, *C. quinoa*, *S. oleracea*, *A. occidentalis* and *A. hortensis* under different concentrations of NaCl singly or with 0.5 mM $CaSO_4$. The results are means of 4 replicates ± standard deviation.

350 mM NaCl concentration stimulates *C. quinoa* germination percentage and 700 mM stimulates it to a greater degree. Addition of $CaSO_4$ alleviated the NaCl inhibitory effect on seed germination in *B. vulgaris, S. oleracea*, and *A. hortensis*, but led to greater inhibition in *C. quinoa* and *A. occidentalis*. It is also remarkable that *A. hortensis* had the highest NaCl stress tolerance in comparison to the other studied plant species. *A. hortensis* showed the highest germination percentage under the highest NaCl concentration, whereas *S. oleracea* could be considered the most sensitive plant to salinity as it expressed the lowest percentage of germination.

The present results support previously reported results of germination suppression caused by increased salinity (McCarty & Dudeck, 1993; Mahmood et al., 1996; Shen & Wang, 1999; Khan et al., 2000). It was reported that addition of mannitol solution to NaCl concentrations in sugar beet indicates that the inhibitory influence of NaCl on seed germination was principally a specific ionic effect and only a slight inhibition could be attributed to an osmotic effect (Al-Karaki, 2000). In the present study, the combination of $CaSO_4$ and NaCl affected germination percentage negatively, which is in agreement with the results reported by Debez & Chaibi (2001). Reduction of salinity's inhibitory effect on germination by Ca^{2+} was also reported by Hamed (2000). In this study, very similar or higher germination percentages than in the control were perhaps due to the high salinity tolerance of most *Chenopodiaceae* (Ungar, 1995).

Salinity did not affect only germination of the studied species, but also reduced shoot and root growth. The reduction was more apparent in shoot growth and this increased the root:shoot ratio; thus, directing growth toward roots is a strategy of adaptation for plants in their search for water. The root:shoot ratio increased as a result of both salt and water stress, and it was due to a greater reduction of shoot growth than root growth (Gomes Filho et al., 1996). Based on the results obtained by Gomes Filho et al. (1996) and on the fact that a high root:shoot ratio has been used as an index of water stress tolerance (Kramer, 1983), it seems that *C. quinoa* and *S. oleracea* showed a high salt tolerance compared to the other studied species. Addition of $CaSO_4$ slightly increased the growth of shoots and roots, and its effect was obvious at the highest stress level. The increase was more in shoot growth than root growth, which was reflected in the decrease in the root:shoot ratio.

The nucleic acid content differed according to the type of species. The maximum content was recorded in *A. hortensis*. Sodium chloride led to a reduction in nucleic acid level when it was applied alone and this could be attributed to the general reduction that occurred due to salinity stress, as reported by Hamed (2004). Greater reduction was apparent when NaCl was applied in combination with $CaSO_4$, except in *C. quinoa* and *A. occidentalis*.

All of the studied plants, except *A. hortensis,* showed a progressive increase in DNase I activity with increased salinity. This showed that for plant cells protecting the DNA when under salt stress was a priority, which is confirmed by the data reported by Hasegawa & Bressan (2000), who observed a salinity stress-induced reduction in cell elongation, but no reduction in cell division. Addition of $CaSO_4$ to NaCl increased DNase I activity in most of the examined plants, whereas NaCl stress reduced DNase- and RNase-specific activity in alfalfa and lentil (Yupsanis et al., 2001).

The endonuclease activity in *B. vulgaris, C. quinoa,* and *A. hortensis* increased with different salinity treatments, while it decreased in *S. oleracea* and *A. occidentalis*. Adding $CaSO_4$ activated endonuclease activity in stressed *C. quinoa, S. oleracea,* and *A. occidentalis.* This indicated that it reduced the inhibitory effect of salinity on the enzyme activity in the 3 plants. *A. occidentalis* showed greater activity under the combination of NaCl and $CaSO_4$. This could have been due to the effect of Ca on Na uptake (Hu et al., 1997). Calcium sulphate decreased endonuclease activity in *B. vulgaris* and *A. hortensis* when combined with NaCl. This decrease may indicate that the effect of 0.5 mM $CaSO_4$ was not due to its increase in osmotic stress, but the sensitivity of the 2 plants to Ca ions.

The activity of RNase A decreased with increased salinity stress levels. Increased RNase A activity was observed in *S. oleracea* and at the highest NaCl stress level in *C. quinoa.* Conversely, RNase T had an increasing trend under all NaCl stress levels. This indicated that the 2 enzymes responded differently to NaCl stress in the studied species. These results are in agreement with other previous reports (Vieira da Silva, 1970; Yi & Todd, 1979; Rouxel et al., 1989). They observed significant

increases in leaf RNase activity under salt or water stress. These results support the hypothesis that salt and water stress always induces increased RNase activity (Vieira da Silva, 1970; Lauriere, 1983; Rouxel et al., 1989). It was suggested that there was a correlation between salt stress tolerance and increased RNase activity (Rouxel et al., 1989). In contrast, the differences in enzyme behaviour in response to salt stress vary depending upon the time of seedling development and in the space according to plant organ (Rouxel et al., 1989).

The 2 enzymes, RNase A and RNase T, responded differently to the combination of NaCl stress with $CaSO_4$ in the studied plants. RNase A increased and RNase T decreased in *B. vulgaris*; however, in the other studied plants the activity of both enzymes decreased. This may indicate little importance of $CaSO_4$ addition on the activity of RNase A and RNase T under NaCl stress conditions.

In general, the obtained reduction in nucleic acids by salinity in both *B. vulgaris* and *A. hortensis* was accompanied by a reduction in the activities of the enzymes (DNase I, endonuclease, RNase A and RNase T). This indicated a general inhibitory effect of salinity due to the great sensitivity of the 2 species to salinity. In the other species, the increase in nucleic acid content at low salinity levels was accompanied by an increase in the activity of the studied nucleolytic enzymes. This may indicate different degrees of salinity tolerance in the studied species. The response to the combination of $CaSO_4$ at different NaCl levels showed that it was dependent on the plant species.

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