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Effect of pre-treatment with ethanolamine on the response of *Helianthus annuus* L. to salt stress

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Abstract

The accumulation of compatible solutes is one of the strategies that plants have developed to tolerate salt stress. Proline and betaine are the main metabolites that accumulate in various species of higher plants in response to salt stress. In *Helianthus annuus* L., pre-treatment of seeds with ethanolamine led to enhanced seedling tolerance to conditions of saline stress during germination, as evidenced by the greater growth of pretreated seedlings (EAS group) versus untreated seedlings (S group), evaluated through such parameters as length, water and chlorophyll content. During the germination period, a considerable increase was observed in proline levels (up to 300%) in seedlings subjected to saline stress, whereas in the EAS group, the proline increment was much smaller (20%). Starting from the fourth day of germination, betaine levels in seedlings pretreated with ethanolamine and then with water (EAW group) and in EAS showed a significant increase versus C (control) and S seedlings, possibly because such a precursor promotes betaine biosynthesis. This could be responsible for the enhanced growth observed in EAS versus S seedlings, as well as for preventing the decrease in chlorophyll content in the EAS group. The accumulation of betaine seems to correlate with the greater tolerance of these seedlings against stress induced by sodium chloride.

1. Introduction

Germination and development stages of seedlings are crucial to determine potential crop yield. Should the seed be subjected to conditions of saline stress during these periods, detrimental effects on plant growth may occur, affecting production in the long term [6]. One of the mechanisms developed by organisms to acquire tolerance to saline stress consists of accumulating so-called compatible solutes, since these fail to interfere with normal biochemical reactions [3, 7]. Other mechanisms that contribute to tolerance include ion uptake, and synthesis of membrane lipids and diverse proteins [3]. Among the main compatible osmolytes are proline, betaine and some sugars. The amino acid proline, as well as betaine and other quaternary ammonium compounds, accumulate in various species of higher plants [5, 25]. On the whole, it is accepted that the accumulation of compatible osmolytes in the cell can prevent the exit of water or increase its entry, providing the required turgidity for cell expansion [12]. In addition, other functions may be attributed to these osmolytes. Recently, it has been suggested that proline accumulation during stress could serve as a reserve supply of carbon and nitrogen for later recovery of the plant following stress [11, 15], and that proline accumulation is manifested only when there is injury to plant tissues [10]. It has been advanced that substrates participating in the biosynthesis of proline, rather than proline itself, may act as compatible osmolytes to compensate for stress-induced imbalances [10, 17]. Betaine is a protective protein-stabilizing osmolyte reported to stabilize photosystem II protein complexes in higher plants, thus avoiding

the dissociation of extrinsic regulatory proteins [32, 33].

The study of biochemical and molecular processes through which plants tolerate environmental stress offers useful tools, employed in genetic engineering, to improve the yields of a crop [4, 9]. Therefore, it is of interest to study the biochemical responses elicited by saline stress in plants like sunflower which make up a large resource for the production of oils.

Though capable of accumulating betaine [30], *Helianthus annuus* is a species moderately sensitive to saline stress [24], a property that still remains poorly understood, indicating that, so far, the role played by protective osmolytes is unclear.

Besides, it is known that pre-treatment with natural amino alcohols such as ethanolamine or choline, exerts a protective effect against saline stress in corn and barley plants [20, 23] and it has also been suggested that a change in the membrane phospholipid profile is responsible for tolerance of salinity in buffalograss [22].

In view of the above information, the goal of the present work was to determine the influence of ethanolamine pre-treatment on *Helianthus annuus* L. seeds subjected to saline stress during germination. Results demonstrate that one of the compatible osmolytes, betaine, plays a leading role in protection against saline stress during this stage of the ontogenetic cycle.

2. Materials and methods

2.1 *Seed culture and treatments*

Surface sterilized sunflower seeds (*Helianthus annuus* L.) were placed in Petri dishes with distilled water in a controlled environmental chamber, with a 16-h photoperiod (175-μmol m^{-2} s^{-1} photon flux density) and a 26/20 °C day/night regime for 18 h. Seeds were then distributed in plastic trays (12×12 cm) with three layers of filter paper moistened with the solutions indicated for each treatment, as follows: control in water (C), in water for 6 h and then treated with 100 mM NaCl (S), treated with 100 mM ethanolamine for 6 h and then in water (EAW), and treated with 100 mM ethanolamine for 6 h and then subjected to 100 mM NaCl (EAS). Each treatment consisted of 10 plastic trays each containing 20 seeds. Samples were collected randomly at 1, 2, 3, 4, 5, 6, and 7 days and extracted for chlorophyll, betaine, proline, and phospholipid determination.

Water content was also measured and expressed as a percentage according to the following equation:

$$\text{Water content (WC) (\%)} = (\text{FW} - \text{DW})/\text{FW} \times 100,$$

where FW is fresh weight and DW is dry weight.

2.2 *Recovery of seedlings*

At the end of the experiment described above (day 7), seedlings were transferred to 20 cm plastic pots containing vermiculite, maintained in the same environmental conditions and watered daily with a nutrient solution [14] for 7 days.

2.3 *Chlorophyll content*

Chlorophyll content was determined 5, 6, and 7 days after germination and in recovered plants. It was extracted by homogenizing and boiling 0.5 g fresh weight of green tissues (leaf plus stem) of the seedlings in 15 ml 95% ethanol. After centrifugation for 10 min at 5000 rpm, the chlorophyll content was analyzed spectrophotometrically on the ethanolic supernatant at 654 nm, as described by Wintermans and De Mots [31].

2.4 *Proline determination*

Seedlings (0.5 g FW) were homogenized in 5 ml of 3% sulphosalicylic acid. After centrifugation for 10 min at 5000 rpm, proline was estimated spectrophotometrically at 520 nm using the ninhydrin method [1]. Purified proline was used for standardization.

2.5 *Betaine assay*

Seedlings (0.5 g FW) were homogenized with 5 ml of methanol, and methanol extracts were phase-separated with chloroform and water as described previously [26, 27]. After evaporating the aqueous phase to dryness in an air stream, 4 ml of distilled water and 0.3 ml of a slurry of Dowex-50 ion exchange resin in the H^+ form [18,21] were added. Betaine was determined according to the periodide method [29].

2.6 *Phospholipid content*

Seedlings (0.5 g FW) were homogenized with 6 ml of chloroform:methanol (1:2 v/v) and extracted according to Blight and Dyer [2]. Phospholipids were separated by thin-layer chromatography [28] and the phosphate content of the different spots was determined according to Kahovcova and Odavic [16].

Table 1. Time course effect of different treatments on sunflower water content. Water content (%) was measured as described in Materials and Methods. C (control); S (6 h in water and then treated with 100 mM NaCl); EAW (pretreated with 100 mM EA for 6 h and then in water); EAS (pretreated with 100 mM EA for 6 h and then subjected to 100 mM NaCl). Data are the means ± SEM of three independent experiments with two replicated measurements. Different letters within the same day indicate significant differences ($P < 0.05$) according to Tukey's multiple range test

Group	Time (days)				
	3	4	5	6	7
C	42 ± 3^a	51 ± 3^a	81 ± 2^a	91 ± 3^a	91 ± 4^a
S	43 ± 3^a	41 ± 3^b	60 ± 2^b	70 ± 2^b	72 ± 3^b
EAW	42 ± 4^a	52 ± 3^a	80 ± 2^a	92 ± 2^a	90 ± 3^a
EAS	41 ± 3^a	53 ± 3^a	72 ± 2^c	91 ± 3^a	91 ± 4^a

2.7 *Statistical analysis*

Figures in the text and tables indicate mean values ± SEM. Differences between control and treated seeds were analyzed by one-way ANOVA, taking $P < 0.05$ as significant, according to Tukey's multiple range test.

3. Results

3.1 *Effect of pre-treatment with ethanolamine on growth, water content and chlorophyll levels in sunflower seeds subjected to saline stress*

To determine the growth of *Helianthus annuus* L. seedlings, starting from the third day of the experiment, their length and water content (WC) were measured (Figure 1, Table 1). The length of S seedlings was significantly lower than that of the C group over the whole experiment, reaching a length 5-fold less at the seventh day of treatment (Figure 1). Stress induced root necrosis but secondary roots failed to appear, and therefore root length was more affected than shoot length under salt stress conditions (data not shown).

WC was significantly lower (20%) in S seedlings starting from the fourth day as compared with the C group and this decrease persisted up to the seventh day (Table 1).

The protective effect of ethanolamine pre-treatment against saline stress was shown by the greater length and WC of EAS versus S seedlings (Figure 1, Table 1). On the seventh day, mean EAS length was twice as much as S and was accompanied by the appearance of secondary roots in the former seedlings. Ethanolamine pre-treatment (EAW) failed to exert any effect on seedling length or WC; values were similar to group C (Figure 1, Table 1).

Total chlorophyll was determined in green tissue from five days post-germination. Chlorophyll levels in S seedlings were lower than those of C seedlings (Figure 2). On the seventh day it was observed that the former possessed roughly 35% less chlorophyll than the latter. Seedling pre-treatment with ethanolamine (EAS) prevented the decrease in chlorophyll caused by saline stress (Figure 2).

3.2 *Levels of compatible osmolytes*

In the S group, proline levels were significantly increased (200% versus controls) starting from the fifth day and peaking at the seventh day of the experiment, with an increase of roughly 300% above that of the controls; while in EAS seedlings, significantly smaller increments were observed (Figure 3).

When betaine content was determined, it was observed that control seedlings reached the maximal betaine level (4.3 μmoles g^{-1} FW) on the fourth day after germination (Figure 4) and, thereafter, levels diminished. In seedlings subjected to saline stress, no increase was observed in betaine content, which had a similar profile to that of the C group. In contrast, seed pre-treatment with ethanolamine led to a significant betaine increase, both in the EAW and in the EAS group, as compared to the control seedlings. The peak betaine level in both groups (roughly 8 μmoles g^{-1} FW) was reached on day four (Figure 4), and remained high, with respect to the C group, up to seven days after germination.

3.3 *Phospholipid determination*

When phospholipids phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine and phosphatidylinositol were studied, no variations were recorded, either in total phospholipid levels or in their profiles according to the various treatments. All groups displayed values similar to those of control seedlings (data not shown).

3.4 *Plant recovery*

On the third day of recovery, both EAS and control plants had two pairs of leaves, while only on the fifth day did the second pair appear in the S seedlings

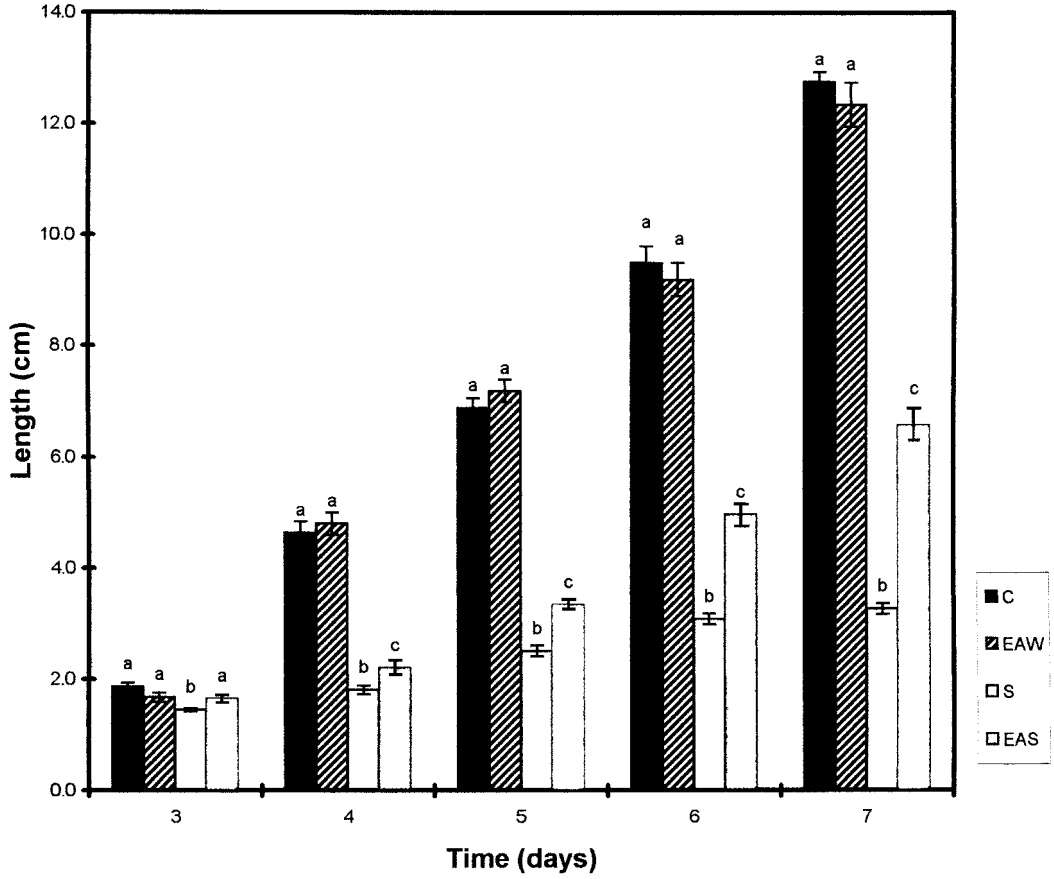

Figure 1. Effect of the various treatments on length (root plus shoot) during seedling development in sunflower. Values are means of three experiments with two replicated measurements, and bars indicate SEM. Different letters corresponding to the same day represent significant differences ($P < 0.05$) according to Tukey's multiple range test.

(data not shown). On the seventh day of recovery, it was observed that root length of EAS plants was 40% greater than that of S (Table 2).

Seedlings pre-treated with ethanolamine and subjected to saline stress (EAS) had a greater recovery in chlorophyll levels (85% of control plant value) than those of the S group, which reached 70% of control value (Table 2).

No differences were observed in shoot length among the groups studied, following the recovery period (Table 2).

Table 2. Effect of recovery on root and shoot development and on chlorophyll content in sunflower plants. Samples were taken after 7 days of recovery as described in Materials and Methods. C (control); S (6 h in water and then treated with 100 mM NaCl); EAW (pretreated with 100 mM EA for 6 h and then in water); EAS (pretreated with 100 mM EA for 6 h and then subjected to 100 mM NaCl). Replicates and statistical analysis, as in Table 1

Group	Root length (cm)	Shoot length (cm)	Chlorophyll content (μg g^{-1} FW)
C	13.0 ± 0.6^{a}	6.9 ± 0.4^{a}	980 ± 30^{a}
S	6.6 ± 0.3^{b}	6.8 ± 0.5^{a}	693 ± 18^{b}
EAW	12.9 ± 0.4^{a}	6.8 ± 0.5^{a}	950 ± 20^{a}
EAS	9.1 ± 0.5^{c}	6.9 ± 0.4^{a}	810 ± 10^{c}

4. Discussion

Despite the large amount of literature on the effect of salinity on diverse plant tissues and cells, the function of compatible osmolytes in salt injury still remains controversial. To our knowledge, there are no previous reports on ethanolamine pre-treatments during germination in sunflower plants subjected to salt stress.

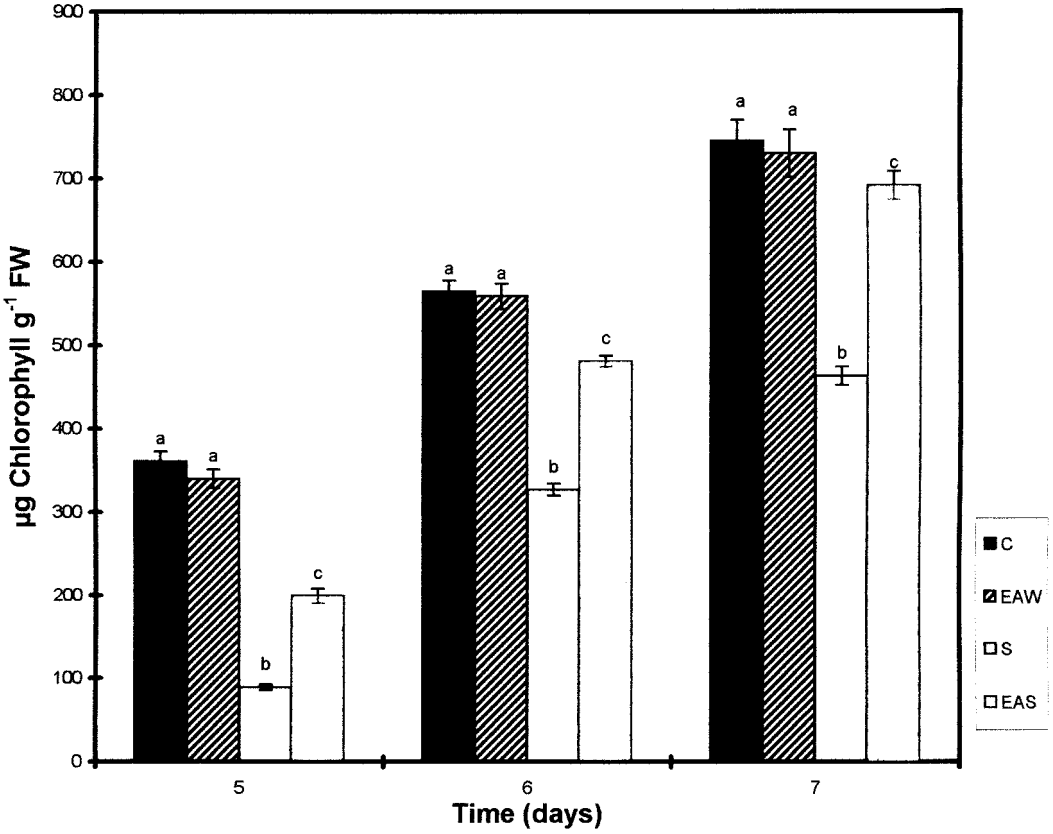

Figure 2. Effect of the various treatments on chlorophyll content during seedling development in sunflower. Replicates and statistical analysis, as in Figure 1.

Helianthus annuus L. is a plant moderately sensitive to stress [24]. Pre-treatment of sunflower seeds with ethanolamine led to enhanced seedling tolerance to conditions of saline stress during germination, as evidenced by the greater growth of EAS versus S seedlings evaluated through such parameters as length, WC and chlorophyll content (Figures 1 and 2, Table 1).

During the germination period, a considerable increase was observed in proline levels (up to 300%) in seedlings subjected to sodium chloride treatment. However, such an increase in a protective osmolyte was insufficient to protect seedlings against damage caused by salinity conditions. In seedlings pre-treated with EA and subjected to saline stress (EAS group), the proline increment was much smaller (20%) (Figure 3). Hare et al. [12] have contended that proline content increases when there is an injury to plant tissue. Possibly in seedlings pre-treated with ethanolamine and subjected to saline stress, where growth is greater and plant status better, damage is less and, therefore, proline levels are hardly increased with respect to the control seedlings. On the other hand, the inhibitory effect of betaine on proline accumulation would provide an alternative explanation for this finding, in agreement with the results obtained by Gibbon et al. [8] and Larher et al. [19] with rape leaf discs.

Previous reports have shown that *Helianthus annuus* L., a member of the *Compositae* family, is a plant that contains considerable amounts of betaine and is able to accumulate a greater quantity under conditions of saline stress [30]. However, in the present work we found that the levels of betaine in control seedlings seven days after germination barely reached a value of 1 μmol g^{-1} FW and that saline treatment failed to produce a significant increase (Figure 4).

Starting from the fourth day of germination, pre-treatment with ethanolamine led to a significant increase in betaine levels in EAW and in EAS versus C and S seedlings. Such an increase may be attributed

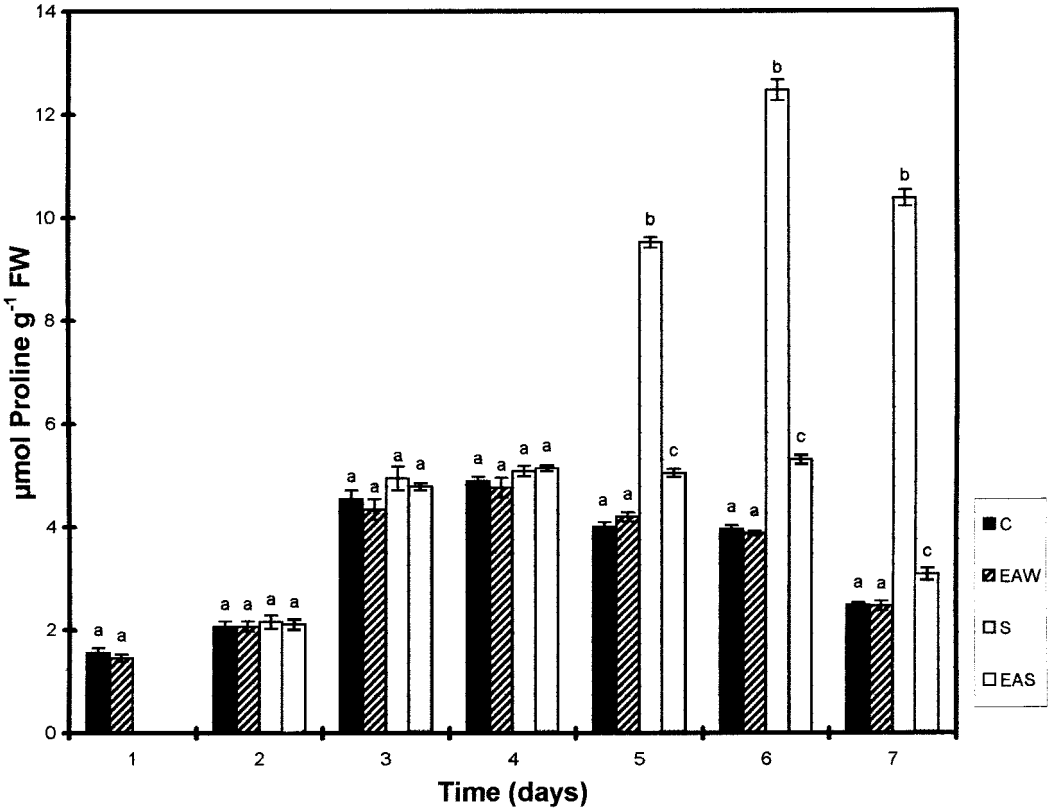

Figure 3. Effect of the various treatments on proline levels during seedling development in sunflower. Replicates and statistical analysis, as in Figure 1.

to the fact that the addition of this precursor (ethanolamine) promotes betaine formation by stimulating its biosynthesis [13].

The protective role of betaine against saline stress, both in higher plants and in bacteria and animals, is widely recognized [25]. The significant increase of this osmolyte in plant tissue from seeds pre-treated with ethanolamine would help to explain the increase in tolerance to salinity. The accumulation recorded in seedlings starting from the fourth day could be responsible for the enhanced growth observed in EAS versus S seedlings, as well as for preventing the decrease in chlorophyll content in the EAS group.

It is known that altered composition of membrane phospholipids plays a major role in tolerance to saline stress [22]. During the germination period of *Helianthus annuus* seeds pre-treated with ethanolamine and subjected to conditions of saline stress, neither qualitative nor quantitative changes were observed in the levels of the five assayed phospholipids, with respect to the control seedlings. Based on these findings, the protective effect of ethanolamine cannot be ascribed to variations in phospholipid quantity and/or profile.

The mechanisms by which plants defend themselves against saline stress are indeed manyfold; many are still unclear and they may vary according to the ontogenetic stage. Although *Helianthus annuus* L. is a betaine-accumulating plant, it is moderately sensitive to stress induced by sodium chloride. Weretilnyk et al. [30] reported that sunflower possesses a low activity of one of the enzymes responsible for its biosynthesis, namely betaine aldehyde dehydrogenase (BADH). The results obtained in this work strongly suggest that the moderate sensitivity presented by this species is attributable to its limited capacity to accumulate betaine during germination (groups C and S, Figure 4).

Pre-treatment with ethanolamine in quantities nontoxic to the plant showed that an increase in betaine most likely takes place during germination, because its biosynthetic precursor, ethanolamine, could activate BADH, thus enhancing the accumulation of betaine. The accumulation of this osmolyte seems to correlate

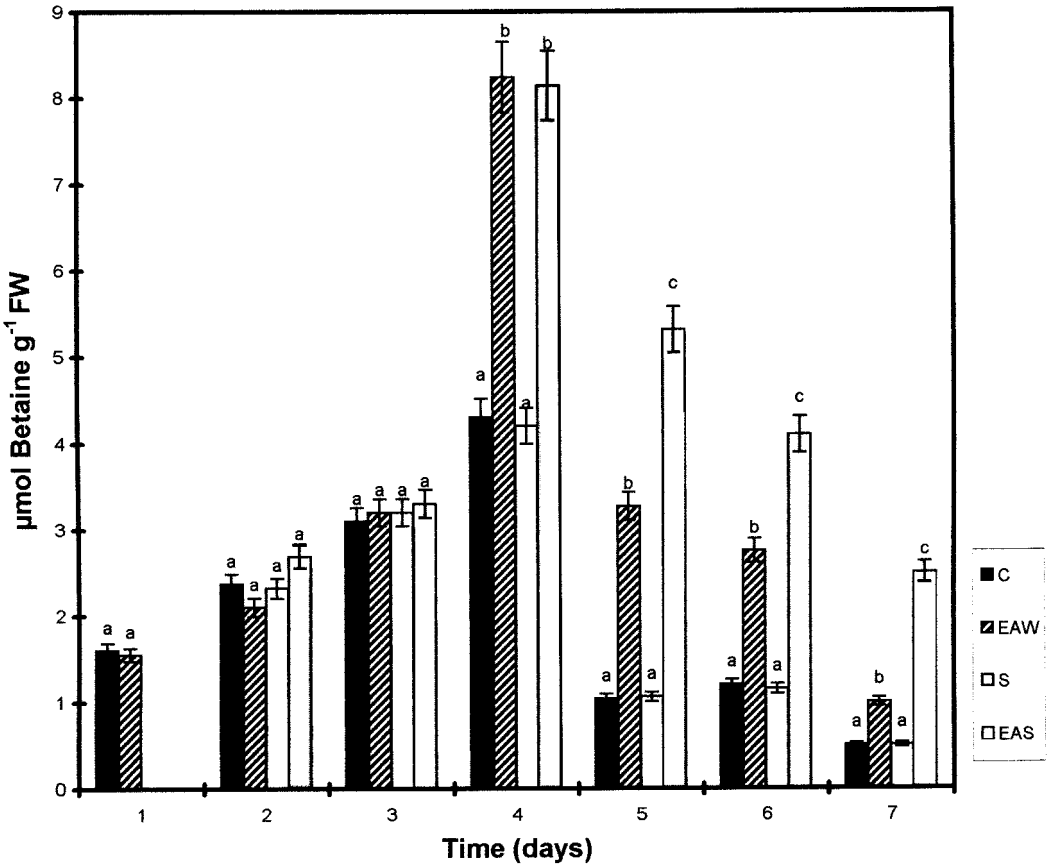

Figure 4. Effect of the various treatments on betaine levels during seedling development in sunflower. Replicates and statistical analysis, as in Figure 1.

with greater tolerance of these seedlings against stress induced by sodium chloride. In a more general context, it could be said that the formation of a compatible osmolyte such as betaine, capable of stabilizing membranes and proteins, is responsible for the increase in tolerance against saline stress.

To sum up, the present work demonstrates clearly that betaine, rather than an increase in proline and/or phospholipid content and composition, is responsible for the increase in tolerance to saline stress when sunflower seeds are pre-incubated with ethanolamine.

Acknowledgements

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