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Proline and glycinebetaine accumulation by *Spartina alterniflora* Loisel. in response to NaCl and nitrogen in a controlled environment

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Summary. The interaction of salinity and nitrogen availability on the growth and accumulation of proline and glycinebetaine by *Spartina alterniflora* Loisel. grown in hydroponic solution in controlled environements was investigated. Growth of shoots was reduced by increasing salinity and enhanced at higher nitrogen levels. Proline was accumulated when a threshold salinity was reached. At higher nitrogen levels, the threshold salinity to induce proline accumulation was lowered and the amount of proline accumulated was enhanced. Glycinebetaine content was higher in plants grown at higher nitrogen levels, but was less affected by salinity and nitrogen levels than proline. Glycinebetaine contents were approximately 10 times higher than proline contents. When plants that were non-stressed for 1 month were exposed to salinity stress, proline and glycinebetaine increased immediately and reached a plateau in 1–2 days. After the removal of salinity stress, proline disappeared rapidly but glycinebetaine content remained unchanged. Unlike NaCl, neither proline nor glycinebetaine had a significant effect on the in vitro activity of malate dehydrogenase activity from leaves.

Introduction

Various species of halophytes accumulate high concentrations of organic solutes in response to salinity (Stewart and Lee 1974; Flowers et al. 1977; Storey et al. 1977; Wyn Jones et al. 1977; Jefferies et al. 1979; Gorham et al. 1980; Cavalieri and Huang 1981). These compounds are thought to function as compatible osmotica in the cytoplasm (Flowers et al. 1977; Gorham et al. 1980; Wyn Jones et al. 1977). *Spartina alterniflora* Loisel. is the dominant halophytic species along the eastern coast of the United States. The plants growing in the marsh accumulate the imino acid proline and the quaternary ammonium compound glycinebetaine in response to an increase in soil salinity. This accumulation also varies with nitrogen availability (Cavalieri and Huang 1981). The molecules of proline and glycinebetaine are composed of 12% nitrogen and a significant drain on the plant nitrogen balance may result from their accumulation (Storey et al. 1977; Jeffries et al. 1979; Cavalieri and Huang 1981).

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In this paper, I report experimental results on the growth of roots and shoots and the accumulation of proline and glycinebetaine in *S. alterniflora*. Experiments were conducted in controlled environment growth chambers and hydroponic solutions so that the effects of nitrogen and salinity could be accurately evaluated. The kinetics of accumulation and disappearance, as well as the cytoplasmic compatibility of the organic osmotica on enzyme activity, was also investigated.

Materials and methods

Spartina alterniflora Loisel. plants were collected from the marsh at the Baruch Institute Field Laboratory, Georgetown, South Carolina, and maintained in sand with nutrient solution in the growth chambers. When plantlets formed at the bases of the plant were 5–10 cm tall, they were used for hydroponic experiments. The hydroponic solution was modified from that of Haines and Dunn (1976). Iron as Sequestrene (CIBA-Geigy Corp. Ardely, New York) was included at a final concentration of 10 mg Fe/l. Ammonium chloride and NaCl were added as described in the individual experiments. Plants were supported in foam rubber and grown in 1 qt. mason jars wrapped in aluminum foil. The growth chambers (Model # GCOP-101-73, Environmental Growth Chambers, Integrated Development and Manufacturing Co., Chagrin Falls, OH) were adjusted to a photoperiod of 14 h light (26° C) and 10 h dark (18° C) period. The illumination level was 143 cal. $cm^{-2} \cdot d^{-1}$.

Leaf and root samples for proline and glycinebetaine determinations were collected and frozen in liquid nitrogen, then stored in a −20° C freezer until the assays were performed. Free proline was extracted from 0.2 g samples of the frozen tissue and assayed by the method of Singh, Paleg and Aspinall (1973). Glycinebetaine was extracted from 0.5 g samples and assayed by the method of Pearce et al. (1976). All data points represent averages of at least 4 replicates.

An aliquot of plant extract obtained by the method of Pearce et al. (1976) was spotted onto a thin layer chromatography plate coated with 250 μm of Silica Gel G (Applied Science Labs). The plate was developed in 90:10:4 (v/v/v) methanol:acetone:HCl (11 M). Glycinebetaine was detected with I_2 fumes. Anhydrous glycinebetaine was used as a standard (Sigma Chemical Co). Two spots were visible from the plant extracts and were scrapped off and assayed by the periodic method of Pierce et al. (1976). Only the

spot co-migrating with the glycinebetaine standard reacted in the colorimetric assay, and the color developed was proportional to the amount of glycinebetaine in the spot.

Malate dehydrogenase (EC 1.1.1.37) was extracted and assayed as described earlier (Cavalieri and Huang 1977). Five g of tissue were ground with a mortar and pestle in 10 ml of grinding medium (0.1 M Tris-citrate buffer pH 7.5, 2 mM dithiothreitol). The crude extract was squeezed through eight layers of cheesecloth and centrifuged at $1,000 \times g$ for 10 min. The supernatant was desalted by passing it through a column of Sephadex G-25 equilibrated with the grinding medium. All steps were conducted at 4 C. The enzyme activity was assayed spectro-photometrically in 0.1 M Tris-citrate buffer, pH 7.5, 0.14 mM NADH, 1 mM oxaloacetate, and NaCl, proline or glycinebetaine at concentrations as described in the text.

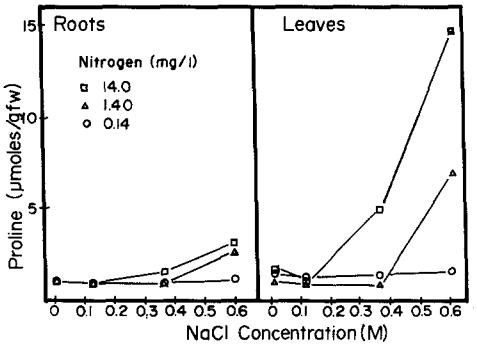

Fig. 1. Proline accumulation by roots and leaves of *Spartina alterniflora*. Plants were grown hydroponically in NH_4Cl and NaCl levels indicated for two months and were sampled. Each point is the average of 4 measurements

Results

Effects of nitrogen and salinity on growth. The effects of nitrogen and salinity levels on the growth *S. alterniflora* were examined in plants grown hydroponically in a growth chamber for 2 months. The results are shown in Table 1. As a general trend, the plants produced lower live shoot dry weight with higher NaCl levels. This trend was the case for each of the three nitrogen levels. At each NaCl level, plant dry weight was higher in the higher nitrogen level than in the lower nitrogen level. Live plus dead shoot weight was used as an indication of total above ground production throughout the course of the experiment. Shoot height was increased at 0.5% NaCl, but decreased at higher NaCl levels. Root weight showed no clear trend with the levels of salinity or nitrogen; however, root length (the length of the longest root) decreased with increasing salinity for each of the three nitrogen levels. Generally speaking, fewer new shoots were produced in plants at the higher salinities regardless of the nitrogen levels. The shoot/root ratio was higher at higher nitrogen levels. Plants in the lowest nitrogen level (0.14 mg N/l) had shoot/root ratios that were below 2 whereas in the highest nitrogen level (14 mg/l), the ratios were above 2. In the two higher nitrogen levels, increased NaCl levels resulted in a trend toward lower shoot/root ratios.

Effect of nitrogen and salinity on proline and glycinebetaine content. Proline content in the leaves of plants grown hydroponically for 2 months increased with elevated salinity of the nutrient solution (Fig. 1). This increase was also affected by the nitrogen levels in the nutrient solution. Increased nitrogen levels lowered the salinity threshold for proline

Table 1. Growth and biomass production of *Spartina alterniflora* plants grown hydroponically in a growth chamber. The plants were grown from plantlets for two months, harvested and the measurements made. Values represent averages of six plants from two separate experiments. ±1 standard deviation

NaCl (%)	Shoots			Roots		# new shoot per plant	Shoot/root (wt/wt)
	Height (cm)	Dry Weight (g)		Length	Dry Weight (g)		
		live	dead				
Low N 0.14 mg/l							
0	16.5± 2.7	0.99±0.58	0.40±0.12	34.7± 2.1	0.87±0.67	1.5	1.6
0.5	20.4± 5.7	0.84±0.53	0.36±0.32	41.4±20.3	0.78±0.76	1.2	1.5
1.5	19.7± 4.9	0.98±0.42	0.32±0.13	39.9±11.2	0.75±0.25	2.0	1.7
2.5	21.1± 2.2	1.13±0.38	0.29±0.12	26.5± 5.2	1.05±0.56	0.7	1.4
3.5	17.7± 2.5	0.75±0.21	0.35±0.07	13.5± 9.9	0.62±0.14	0	1.7
Medium N 1.4 mg/l							
0	23.5± 4.9	1.79±0.19	0.24±0.04	64.0±24.0	1.02±0.49	2.0	2.0
0.5	27.5±13.8	1.58±1.64	0.50±0.33	38.4± 9.2	1.17±0.93	1.0	1.8
1.5	26.8± 9.1	1.62±1.05	0.48±0.31	40.7±11.7	1.43±0.92	1.3	1.5
2.5	23.8± 1.1	1.38±0.27	0.46±0.17	27.2± 4.6	1.05±0.52	1.0	1.4
3.5	18.7±10.9	1.05±1.55	0.31±0.20	11.8±10.2	0.98±0.54	0.5	1.7
High N 14 mg/l							
0	34.4±13.0	2.51±1.19	0.31±0.16	24.7± 7.8	0.52±0.22	2.0	5.4
0.5	39.0±11.9	3.05±1.67	0.27±0.11	33.6± 2.4	1.02±0.70	1.0	3.2
1.5	27.1± 3.5	1.24±0.42	0.25±0.06	30.1±14.2	0.57±0.27	1.4	2.6
2.5	22.3± 3.5	1.63±0.40	0.33±0.13	21.4±14.6	0.73±0.38	1.2	2.7
3.5	22.8± 7.7	1.23±0.77	0.38±0.06	17.0± 4.9	0.75±0.31	1.0	2.1

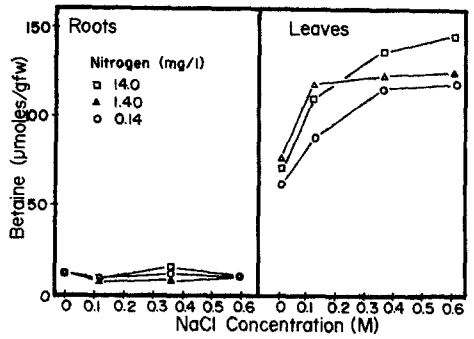

Fig. 2. Glycinebetaine accumulation by roots and leaves of *Spartina alterniflora*. Plants were grown hydroponically in NH_4Cl and NaCl regimes indicated for two months and were sampled. Each point is the average of at least 4 measurements

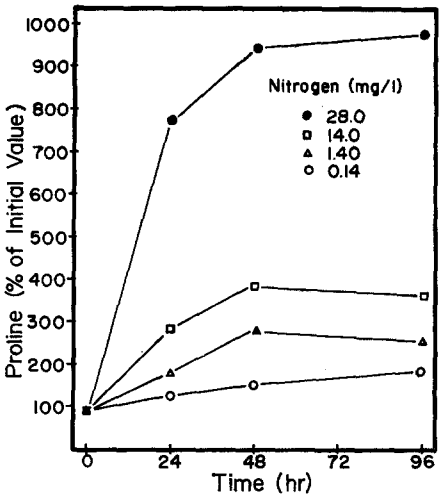

Fig. 3. Time course of proline accumulation in leaves of *Spartina alterniflora* grown in a growth chamber. Plants were grown hydroponically in nutrient solutions with NH_4Cl levels as indicated for one month. Plants were then placed in nutrient solutions containing NH_4Cl as indicated plus 0.5 M NaCl (−25 bars) at time 0. Proline content was monitored by harvesting plants at the time indicated. Initial values of the three lower nitrogen treatments are shown in Fig. 1, and the initial value of the treatment with 28.0 mg/l was 0.5 μmoles per g fresh weight

accumulation from greater than 0.5 M to 0.1 M NaCl and at the same time increased the proline content at the higher salinity levels. Roots showed a similar response (Fig. 1), although the proline content in the roots was quite low.

Glycinebetaine content in leaves of plants grown hydroponically was influenced by salinity and nitrogen to a much lesser extent than the proline content (Fig. 2). Plants grown in the absence of added NaCl contained about 60–70 μmoles/g fresh weight of glycinebetaine. The glycinebetaine content approximately doubled at the highest salinity level (0.6 M NaCl). Increased nitrogen content of the nutrient solution only slightly enhanced the glycinebetaine content at the higher salinities. Roots contained a relatively small amount of glycinebetaine, 10 μmoles/g fresh weight, and showed essentially no change with increasing salinity or nitrogen levels (Fig. 2).

Time course of proline and glycinebetaine accumulation. The plants were grown in nutrient solution without NaCl for

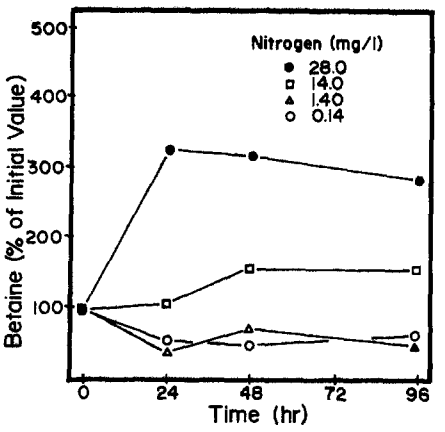

Fig. 4. Time course of glycinebetaine accumulation in leaves of *Spartina alterniflora* grown in a growth chamber. Plants were grown hydroponically in nutrient solutions with NH_4Cl levels as indicated plus 0.5 M NaCl (−25 bars) at time 0. Glycinebetaine content was monitored by harvesting plants at the time indicated. Initial values of the three lower nitrogen treatments are shown in Fig. 3, and the initial value of the treatment with 28.0 mg/l was 65 μmoles per g fresh weight

1 month and then transferred to nutrient solution containing 0.5 M NaCl (−25 bars). Proline started to accumulate immediately after the transfer, and the amount accumulated was proportional to the nitrogen level in the nutrient solution (Fig. 3). At all nitrogen levels, the accumulation leveled off after 48 h.

Increases in glycinebetaine accumulation also occurred in plants growing at 28 and 14 mg N/l after the plants were transferred to solutions containing 0.5 M NaCl (−25 bar) (Fig. 4). The increase was much lower than that of proline and the amount leveled off somewhat faster after the plants had been transferred to the higher salinity. There was a slight decrease in glycinebetaine content in plants growing in the lower nitrogen regimes in the first 96 h (Fig. 4); however, after two months the level increased (Fig. 2).

Time course of proline and glycinebetaine disappearance. Plants grown in nutrient solution with 28 mg N/l and 0.5 M NaCl (−25 bars) for 1 month were transferred to a similar nutrient solution but without NaCl. Proline content began to decrease immediately, and continued to decline to 50% of the initial level after 96 hr (Fig. 5). In contrast, the glycinebetaine content was much less dynamic. After 96 h, negligible reduction in the content had occurred (Fig. 5).

Effect of proline and glycinebetaine on the in vitro activity of malate dehydrogenase. Neither proline nor glycinebetaine at concentrations up to 0.5 M affected the in vitro activity of malate dehydrogenase extracted from the leaves of *S. alterniflora* (Fig. 6). The malate dehydrogenase activity measured was that in the crude leaf extract which contained all the isozymes. No differential salt tolerance of malate dehydrogenase isozymes was found in *S. alterniflora* (Cavalieri and Huang 1977). The decrease in the enzyme activity was 15% in 1 M proline and 35% in 1 M glycinebetaine. Nevertheless, the in vitro activity of malate dehydrogenase was much less sensitive to these compounds than to NaCl at equal concentrations.

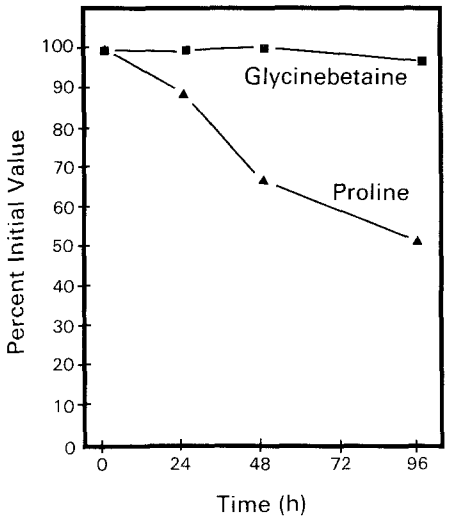

Fig. 5. Time course of decrease in proline and glycinebetaine contents in *Spartina alterniflora* leaves after removal of salinity stress. Plants were grown hydroponically in nutrient solutions containing 28 mg/l of nitrogen and 0.5 M NaCl for 1 month and then transferred to a similar nutrient solution but without NaCl at time 0

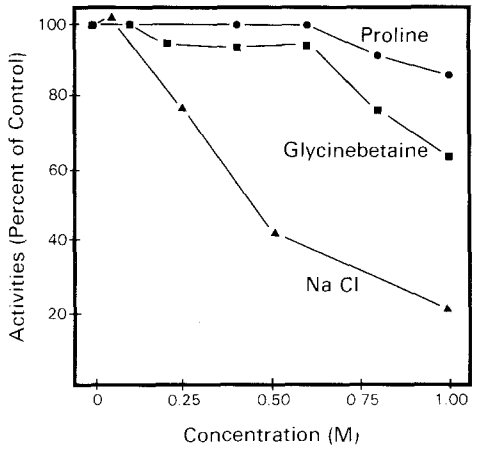

Fig. 6. Effect of proline, glycinebetaine or NaCl on the in vitro activity of malate dehydrogenase from *Spartina alterniflora* leaves

Discussion

S. alterniflora plants growing in the marsh show wide variation in productivity and morphology. This variation was shown to be in part a response of the plants to soil salinity (Nestler 1974; Valiela et al. 1978) and nitrogen availability (Gallagher 1974; Valiela et al. 1978; Mendelssohn 1979; Cavalieri and Huang 1981). The adverse effects of high salinity and inadequate nitrogen supply on the plants have also been shown in growth chamber experiments by Haines and Dunn (1976) and Parrondo et al. (1978). The growth parameters measured in the present experiments generally support these studies. The current findings of increased shoot/root ratios in plants growing in high nitrogen and low salinities differ from those of Haines and Dunn (1976), but support the observations of Parrondo et al. (1978). Increased shoot/root ratios indicate that the above ground standing crop in the marsh should not be used as a direct measurement of the total productivity, especially when comparing different marshes, different areas of a marsh, or different times of a year within an area of a marsh. Longstreth and Strain (1977) have shown that salinity as high as 3% inhibits photosynthesis in *S. alterniflora* under low but not high irradiance. Therefore, in our growth chamber studies where the plants were grown at a relatively low illumination level (143 $cal \cdot cm^2 \cdot d^{-1}$), the reduction in growth due to salinity would be greater than that in the field.

Accumulation of free proline has been shown to occur in response to salinity stress, and has been suggested to function as a cytoplasmic osmoticum in a number of halophytic species (Stewart and Lee 1974; Flowers et al. 1977; Wyn Jones et al. 1977; Rozema et al. 1978; Cavalieri and Huang 1979; Stewart et al. 1979). In *S. alterniflora*, the amount of proline accumulated would only make a small contribution to osmoregulation when plants were grown at high soil salinities. The accumulation of proline in *S. alterniflora* was also strongly affected by the availability of nitrogen. Since maintenance of a favorable osmotic balance for growth should be of primary importance for the plant, proline would not constitute a suitable cytoplasmic osmoticum in this species. Since changes in salinity were made in the hydroponic system, the plants responded to stress immediately and no lag phase in accumulation occurred, as was reported on *Limonium* grown in sand culture (Cavalieri and Huang 1979).

The quaternary ammonium compound glycinebetaine has been proposed to be of significance in osmoregulation in a number of species, particularly members of the Graminaceae and Chenopodiaceae (Storey et al. 1977; Stewart et al. 1979; Gorham et al. 1980). Glycinebetaine content in *S. alterniflora* was high enough to make a significant contribution to the leaf solute potential. From the current data in which the glycinebetaine content was 50–100 μmoles/g fresh weight, concentrations of 68–130 mM exist if glycinebetaine is distributed throughout the cells. Wyn Jones et al. (1977) and Hall et al. (1978) have shown glycinebetaine to be localized in the cytoplasm and not in the vacuoles in *Sueada maritima* and red beet. If this is the case in *S. alterniflora,* the concentration of glycinebetaine in the cytoplasm would be 270–545 mM assuming 75% of the cell content to be vacuolar, or 680–1,360 mM assuming 90% of the cell content to be vacuolar. This would represent (−8 to −16) or (−21 to −40) bars, respectively, in the cytoplasm. The glycinebetaine content was high enough to account for the low leaf solute potentials that would occur in turgid plants in the field having leaf water potentials reported (Cavalieri and Huang 1981). Although glycinebetaine content was altered due to environmental changes, it was less affected by salinity or the availability of nitrogen than was proline. Glycinebetaine accumulated rapidly after the plants were exposed to high salinity, but decreased very slowly if at all once the salinity was lowered. Glycinebetaine content was reduced only 2% after 96 h, a reduction of 3.0 μmoles/gfw. This small reduction in glycinebetaine may have resulted from dilution by growth during this time. Studies with barley also indicate that glycinebetaine degradation is very slow (Hanson and Nelson 1978). This response seems appropriate for a plant like *S. alterniflora* which grows in habitats where soil salinities are often high.

Proline and glycinebetaine appear to be compatible with functioning of the cellular machinary since malate dehydrogenase activity was uneffected by concentrations of up to 0.5 M. Malate dehydrogenase from *S. alterniflora* was

somewhat more sensitive to glycinebetaine than to proline. Enzymes from glycophytes such as barley as well as those from several halophytes are also uneffected by proline or glycinebetaine at high concentrations (Pollard and Wyn Jones 1979; Flowers et al. 1979). Glycinebetaine is likely to be the primary organic compound functioning as a cytoplasmic osmoticum in *S. alterniflora*, with proline making a small contribution at certain times of the year and locations in the marsh. Since both proline and glycinebetaine contain substantial amounts of nitrogen in their molecules (12%), the accumulation of these compounds results in a drain on the plant nitrogen, a drain that may be large enough to adversely affect plant growth, particularly those plants growing in the high marsh regions with high soil salinities and low nitrogen availability (Jefferies et al. 1979; Cavalieri and Huang 1981).

It has recently been established that while halophytes may have optimal growth at very low salinities, these species are often restricted to high salinity areas because they cannot compete with glycophytes in non-saline areas (Szwarcbaum and Waisel 1973; Barbour 1978). Glycinebetaine and proline contents in *S. alterniflora* grown in nutrient solution without salinity stress account for at least 11% of the total leaf nitrogen. This allocation of nitrogen for cytoplasmic osmoregulation, while essential when growing in saline areas, represents a drain on the plant resources and may contribute to the reduced competitive ability of the halophytes when growing in non-saline situations.

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