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Accumulation of Proline and Glycinebetaine in *Spartina alterniflora* Loisel. in Response to NaCl and Nitrogen in the Marsh

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Summary. The possible interaction of high soil salinity and low soil nitrogen content in affecting the growth of *Spartina alterniflora* Loisel in the high and low marshes of the Eastern U.S. was explored. Throughout the whole growing season, the short plants growing in the high marsh, where there was a higher soil salinity and lower available soil nitrogen, contained more proline and glycinebetaine and showed a lower leaf water potential than the tall plants growing in the low marsh. In both short and tall plants, the proline content varied more than 10 fold throughout the growing season, with the highest content occurring in spring and fall. In contrast, the glycinebetaine content in both short and tall plants remained fairly constant throughout the growing season, and was consistently at least 10 fold higher than the proline content. It is estimated that 19–30% of the total leaf nitrogen was in the form of proline and glycinebetaine in the short plants, and 14–27% in the tall plants. Ammonium nitrate fertilization in the field resulted in increased growth, higher proline and glycinebetaine contents, and lower water potentials in the short plants, but had little effect on these parameters in the tall plants. We suggest that in the low marsh, the plants can obtain sufficient nitrogen for osmoregulation and other metabolism. In the high marsh with higher soil salinity and lower nitrogen content, the plants have to allocate a even greater proportion of the already limited nitrogen supply for osmoregulation. Thus, nitrogen available for osmoregulation and other nitrogen-requiring metabolism is insufficient, resulting in reduced growth.

Introduction

Salt marshes of the Atlantic Coast of the U.S. are dominated by *Spartina alterniflora* Loisel. This grass grows in essentially pure stands in the lower part of the marsh, and is responsible for the high productivity of the salt marshes (Keefe 1972; Turner 1976). The growth of *S. alterniflora* is not uniform throughout the marsh since the plants vary from 2 m to 10–40 cm in height. The tall plants are found along creek banks and the short plants occur in the high marsh, and a gradient of plant height between the two regions exists. This situation is found along the entire Atlantic Coast of North America, as well as the gulf coast of the U.S. (Miller and Egler 1950; Kerwin and Pedigo 1971; Mooring et al. 1971; Gallagher 1974; Squires and Good 1974; Sullivan and Daiber 1974; Mendelssohn and Marcellus 1976; Valiela et al. 1978).

The height forms of *S. alterniflora* have been shown conclusively to be genetically uniform (Mooring et al. 1971; Shea et al. 1975; Valiela et al. 1978) and their occurrence therefore reflects a response of the plants to the environment. It now appears that two major environmental factors are responsible for the variation. One of them is the availability of nitrogen. In the marsh, *S. alterniflora* growth is correlated with the availability of nitrogen in the soil. Soil closer to the tidal creeks (with tall plants) contains more available nitrogen than that more distant from the creeks (Van Raalte et al. 1974). This situation is due in part to the decreased aeration in the soil in the high marsh. When the high marsh (with short plants) is fertilized with nitrogen, plant height and productivity increase. Fertilization of the low marsh (with tall plants) has little effect (Gallagher 1974; Valieda et al. 1978). In fertilization experiments lasting four years, short plants attain a height approaching that of the tall plants (Veliela et al. 1978). In controlled environmental chambers, short plants also show better growth in higher nitrogen treatment (Haines and Dunn 1977). The other major factor affecting the growth of *S. alterniflora* is soil salinity (Mooring et al. 1971; Nixon and Oviatt 1973; Haines and Dunn 1976; Nestler 1978; Parrondo et al. 1978; Valiela et al. 1978). Salinities in the high marsh are higher due to less frequent tidal flooding, and as a consequence, the continuous evaporation concentrates the salts in the soil.

S. alterniflora, like other halophytes, can osmoregulate when growing in saline environments. The high amount of NaCl found in the tissue is thought to be largely sequestered in the cell vacuoles, since it would otherwise inhibit or damage the cytoplasmic metabolic machinery (Flowers et al. 1977; Wyn Jones et al. 1977; Cavalieri and Huang 1979, Pan et al. 1981). To balance the low osmotic potential in the vacuole, the free amino acid proline and quaternary ammonium compound glycinebetaine accumulate in the cytoplasm (Stewart and Lee 1974; Wyn Jones et al. 1977; Cavalieri and Huang 1979; Stewart et al. 1979, Pan et al. 1981). Since the two compounds, each having 12% of nitrogen in the molecule, may accumulate to levels as high as 165 μmoles/gfw, their accumulation is a severe drain on the nitrogen resources. The situation is especially critical in the high marsh having higher soil salinity, where the plants can least afford the nitrogen drain due to the already insufficient supply of nitrogen in the soil.

In this paper, we examine the accumulation of proline and glycinebetaine in *S. alterniflora* as an osmoregulatory response to salinity. We suggest that this drain of nitrogen for osmoregulation is a major factor in the occurrence of the growth forms.

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Materials and Methods

Fertilization Experiments

Six permanent plots (3 m × 3 m) were laid out in the high and low marsh region containing short and tall *S. alterniflora*, respectively, at the Belle W. Baruch Institute field laboratory, Georgetown, South Carolina. Both the short plants and the tall plants were growing in essentially pure stands. The plots of tall plants were set 1–2 m away from a creekbank and therefore did not contain the tallest plants in the marsh. In each area of the marsh, three plots were fertilized with ammonium nitrate (TePee Industries, Box 546 Pamplico, S.C.) at a rate of 15 g nitrogen/m^2/month, while the other three plots were maintained as controls. The fertilizer was added each month to holes made at regular intervals throughout the plots. Leaf samples for the analysis of proline and glycinebetaine content, as well as soil water samples, were taken monthly. Samples were also taken from random positions in the marsh and were found to be comparable to those of the control plots.

Standing Crop Biomass

In order to determine the standing crop biomass, a single harvest was made at the end of the growing season on Nov. 3, 1978. Plots were sampled by cutting all above ground plant material in four 0.25 m^2 quadrats/plot. The plant material was bagged, washed, dryed in an oven at 100° C, and weighed. Dry weight (live and dead), stem height, and density were determined for all the plants in each quadrat.

Assays

Leaf samples for the determinations of proline and glycinebetaine were collected in the field, immediately frozen in liquid nitrogen, and stored in a −20° C freezer until assay. The second leaf from the growing tip was sampled. Glycinebetaine was extracted from 0.5 g tissue and assayed by the method of Pearce et al. (1976). Specificity of the assay for glycinebetaine in extracts from *S. alterniflora* was established by thin layer chromatography using Dragendoff reagents as the stain (Pearce et al. 1976). Free proline was extracted from 0.2 g tissue and assayed by the method of Singh, Paleg and Aspinall (1973). Each data point represents the average of at least 4 replicates.

Water Potential Measurements

Leaf water potential measurements were made with a Wescor HR 33T Dew Point Hygrometer with a Model C-52 Sample chamber (Logan, Utah). Leaves were washed with distilled H_2O, blotted dry, and cut into a size appropriate for the sample chamber with care to minimize cut edges. An equilibration period of 30 min. and a cooling time of 10 s were used.

Field Interstitial Water Measurements

Soil water samples were taken at the base of the plants using a thin, perforated plastic tube and a manual vacuum pump as described by Gardner (1975). The soil water was first centrifuged to remove soil particles. The salinity was determined using a Bausch and Lomb Refractometer, and ammonia was determined with Technician Autoanalyzer immediately after sampling.

Results

Growth Parameters

The characteristics of plants growing in the marsh at the end of the growing season of 1978 are shown in Table 1. In the high marsh, plants had an average stem height of 22.9 cm and a high density of 712 plant stems/m^2. Each plot had an average standing crop biomass of 302 g/m^2. In the low marsh, plants had a higher average stem height (103 cm), at a lower density (144 stems/m^2), and had about 4 times more standing crop biomass than the plots of short plants. Although the dead matter in the various plots shows a trend similar to that of the living plants, the influx and efflux of dry matter during tidings and storms throughout the whole growing season make it impossible to evaluate these data. Differences between the control plots containing tall plants and short plants are significant at the 95% confidence level for all the parameters listed in Table 1.

The short plants responded to the fertilization with increased stem height, stem density, and biomass (Table 1). All the differences were significant at the 95% confidence level. The tall plants in the fertilized plots showed only slight variation in these parameters from the tall plants in the control plots; none of the variation was significant at the 95% confidence level (Table 1). In our single measurement, the control plots containing short plants had less soil ammonia than the control plots containing tall plants, and increased soil ammonia contents were found in both fertilized plots of short and tall plants.

Proline Content

The proline content in the plants was analyzed monthly in the growing season of 1978 (Fig. 1). It was always higher in the short plants than in the tall plants, and higher content of proline in the short plants correlates well with the higher salinities found in these plots (Fig. 1). The proline content in the short plants was increased substantially by fertilization of the plots, whereas the proline content in the tall plants was not affected by fertilization. A significant difference between the proline content in the fertilized tall plants and that in the control tall plants was found only in September. The proline content was higher in Spring and Fall, but varied considerably from month to month.

Table 1. Characteristics of *Spartina alterniflora* and soil ammonia in plots with and without nitrogen fertilization. Harvests were made in November, 1978

	High Marsh (Short plants)		Low Marsh (Tall plants)	
	Control	Fertilized	Control	Fertilized
Average stem height (cm)	22.8 ± 1.3	25.9 ± 2.2	102.9 ± 14.2	106.1 ± 5.1
Stems #/m^2	712.0 ± 145.6	1,259.4 ± 99.2	144.1 ± 6.4	102.2 ± 0.2
Standing crop biomass (g/m^2)	302.4 ± 70.4	689.6 ± 111.8	1,211.2 ± 249.6	1,596.8 ± 662.4
Dead Matter dry weight (g/m^2)	284.8 ± 30.4	476.8 ± 145.6	352.0 ± 98.6	324.8 ± 121.6
Soil ammonia * (μmole/L)	9.0 ± 8.9	53.9 ± 21.7	28.6 ± 13.8	95.5 ± 23.8

* measured in January, 1979

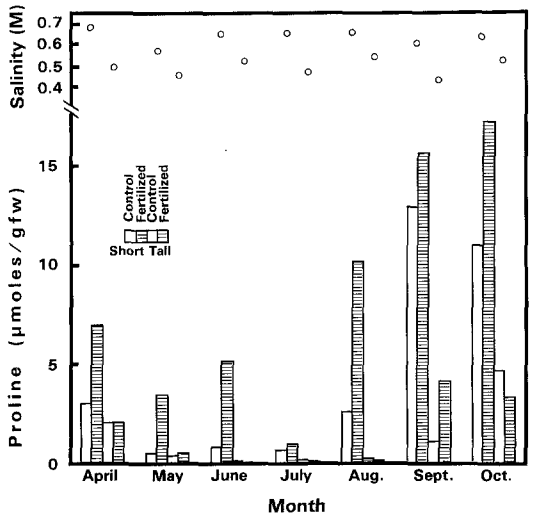

Fig. 1. Proline content in the second fully expanded leaves of short and tall *Spartina alterniflora* in control and fertilized plots in the growing season of 1978. Circles indicate the soil salinity at the time of sampling

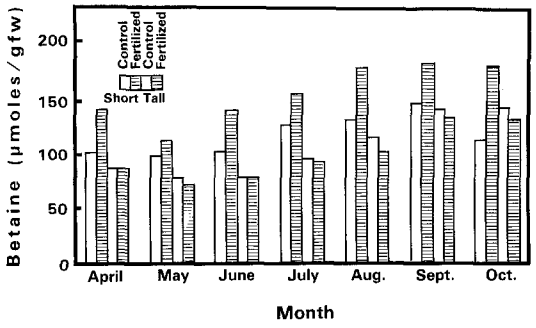

Fig. 2. Glycinebetaine content in the second fully expanded leaves of short and tall *Spartina alterniflora* in control and fertilized plots in the growing season of 1978. Sampling of leaves for proline (Fig. 1) and glycinebetaine (Fig. 2) contents were carried out at the same time each month. For soil salinities, see Fig. 1

Glycinebetaine Content

In field-grown plants, the glycinebetaine content was approximately 10-fold higher than the proline content (Fig. 2). Again, the glycinebetaine content was higher in the short plants than in the tall plants, but the difference is not as drastic as that for proline (Fig. 1). The higher content of glycinebetaine correlates well with the higher salinity in the high marsh (Fig. 1). Fertilization increased the glycinebetaine content in the short plants but had little effect on that of the tall plants. Unlike the proline content, little variation was found to occur in the glycinebetaine content from month to month, in either tall or short plants. However, there was a slight trend toward increased glycinebetaine content at the end of the growing season.

Leaf Water Potentials, Osmotica, and Fertilization

Measurements of soil salinity, leaf water potential, proline content and glycinebetaine content were made together on October 1, 1978 (Table 2). As expected (Fig. 1), soil salinity was higher in the high marsh (with short plants) than in the low marsh (with tall plants). The proline and glycinebetaine content was higher in the short plants than in the tall plants, and fertilization increased the contents only in the short plants but not in the tall plants. Leaf water potentials were more negative in the short plants than in the tall plants, and fertilization further reduced the leaf water potential of the short plants, but had little effect on that of the tall plants (significant at 95% confidence level). The reduction in water potential in the short plants due to fertilization correlates well with the increase in proline and glycinebetaine content. There was no effect of fertilization on water potential or glycinebetaine and proline content in tall plants.

Discussion

Soil salinity and nitrogen have been shown to be the most important factors limiting the distribution of growth forms of *S. alterniflora* (see Introduction). Our results agree with these reports. Nitrogen fertilization increases the growth of the short plants but not the tall plants. While our measurement of soil ammonia indicates lower nitrogen availability in the high marsh, we have made only one set of analysis and substantial temporal variation of soil ammonia content in the marsh has been recorded (Mendelssohn 1979; Chalmers 1979). Nevertheless, our findings agree that nitrogen availability to the short plants is reduced.

In the high marsh, *S. alterniflora* growing in higher salinity allocates more of the available nitrogen, organic carbon, and energy to the production of proline and glycinebetaine for osmoregulation. The allocation of extra nitrogen is especially critical, since the available soil nitrogen in the high marsh is already low. Although the plants allocate more of the limited nitrogen resource to produce osmotica, the amount of osmotica is still not enough to prevent a reduction in growth. In fact, a nitrogen dificiency may arise as a consequence of the diversion of nitrogen to osmoregulation, resulting in a reduction in growth of the short plants. Our fertilization experiments support such a notion. The plants in the high marsh after nitrogen fertilization accumulate more proline and glycinebetaine, resulting in a reduction in water potential and an increase in growth. The plants in

Table 2. Soil salinity, leaf proline and glycinebetaine content, and leaf water potentials of *Spartinia alterniflora* in plots with and without nitrogen fertilization

	High Marsh (Short Plants)		Low Marsh (Tall Plants)	
	Control	Fertilized	Control	Fertilized
Salinity (M)	0.7	0.7	0.48	0.48
Proline (μmoles/gfw)	10.5±3.7	15.5±4.8	4.3±0.4	3.2±0.4
Glycinebetaine (μmoles/gfw)	130.6±10.6	160.6±9.9	110.8±6.6	110.7±4.6
Leaf Water Potential (bars)	−44.6±1.5	−49.5±3.6	−40.8±6.0	−38.4±2.0

the low marsh with relatively lower soil salinity and higher nitrogen content are evidently able to synthesize enough proline and glycinebetaine for osmoregulation without limiting the amount of nitrogen available for growth. Application of nitrogen fertilizer to these tall plants has little effect. The amount of proline and glycinebetaine accumulated in control and fertilized plants is adequate to account for the reduction in cytoplasmic water potential, based on the estimation of Flower et al. (1977) and Wyn Jones (1979).

The magnitude of the drain of cellular nitrogen into proline and glycinebetaine can be estimated. Both compounds contain 12% nitrogen in their molecules. Estimates of leaf nitrogen content of *S. alterniflora* vary from 0.54% to 2.5% of the dry weight (Taschdjian, 1954; Mendelssohn and Marcellus 1976; Woodhouse et al. 1976; Chalmers 1979). In order to make the most conservative estimate, the highest nitrogen content is assumed. From our data, field-grown tall plants with or without fertilization contained between 14.3 and 27.4% of the total leaf nitrogen in proline and glycinebetaine, depending on the month. For field-grown short plants with and without fertilization, the figures are 19.3% to 30.4%, and 21.1% to 27.8%, respectively. These figures are similar to those on other halophytes (Storey et al. 1977; Jefferies et al. 1979), and probably represent minimal estimates.

In either the short or the tall *S. alterniflora,* the proline content fluctuates more than 10 fold during the growing season, and it further depends on the soil salinity and nitrogen availability (Fig. 1). On the contrary, the glycinebetaine content remains fairly constant throughout the growing season, with variation of less than 1 fold depending also on the soil salinity and nitrogen availability (Fig. 2). The recognition of these factors affecting the content of proline and glycinebetaine is important. Sampling plant tissues for proline and glycinebetaine content in one particular region in the field at a certain time of the year may give illusive results of whether or not the plant species does or can accumulate proline and/or glycinebetaine. Furthermore, the availability of nitrogen in the soil at the location of sampling is critical, as our data have illustrated.

We should emphasize that although osmoregulatory requirements results in decreased growth of plants in the high marsh, other factors also contribute. These other factors include the energy allocation for extra salt gland function and ion compartmentation (Haines and Dunn 1976), the interference of excess ions with nutrient uptake (Epstein 1969), the effect of high salinity and low soil water potential on the morphology of the leaves (Longstreth and Strain 1977) and photosynthesis (Giurgevich and Dunn 1979), and the decreased light penetration of denser short plants (Valiela et al. 1978). The effect of these other factors may contribute to the failure of short plants to completely match the growth of the tall ones even after long term fertilization (Valiela et al. 1978). However, our findings indicate that osmoregulation is a significant sink for the nitrogen resources in plants throughout the marsh, particularly in areas where the salinity is high and the availability of nitrogen is low.

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