PHYSIOLOGIA PLANTARUM 102: 487–492. 1998
Printed in Ireland—all rights reserved

ISSN 0031-9317

NaCl-induced accumulation of glycinebetaine in four subtropical halophytes from Pakistan

M. Ajmal Khan, Irwin A. Ungar, Allan M. Showalter and Howard D. Dewald

Khan, M. A., Ungar, I. A., Showalter, A. M. and Dewald, H. D. 1998. NaCl-induced accumulation of glycinebetaine in four subtropical halophytes from Pakistan. – Physiol. Plant. 102: 487–492.

Variation in both the total content and the concentration of glycinebetaine in response to increasing soil salinity was studied in the salt-secreting *Atriplex griffithii* Moq., the leaf succulent *Suaeda fruticosa* (L.) Forssk., the stem succulent *Haloxylon recurvum* Bunge ex Boiss. and the osmotically adjusting desert grass *Halopyrum mucronatum* (L.) Stapf. collected from a subtropical maritime desert in Pakistan. Glycinebetaine content (mmol kg^{-1} dry weight) increased with increasing NaCl in *Atriplex griffithii*, *Haloxylon recurvum* and *Halopyrum mucronatum*, but peaked at 600 m*M* NaCl for *Suaeda fruticosa* and declined thereafter. Glycinebetaine concentration (mmol l^{-1} tissue water) increased with increasing salinity in all four halophyte species and was sufficiently high to serve as an osmoticum in all cases.

Key words – *Atriplex griffithii*, compatible osmotica, glycinebetaine, halophyte, *Halopyrum mucronatum*, *Haloxylon recurvum*, NaCl, salt stress, *Suaeda fruticosa*.

M. A. Khan, Dept of Botany and Range Science, Brigham Young Univ., Provo, UT 84602, USA; I. A. Ungar (corresponding author, e-mail ungar@ohiou.edu) and A. M. Showalter, Molecular and Cellular Biology Program and Dept of Environmental and Plant Biology, Ohio Univ., Athens, OH 45701-2979, USA; H. D. Dewald, Dept of Chemistry, Ohio Univ., Athens, OH 45701-2979, USA.

Introduction

A variety of mechanisms contribute to the salt tolerance of halophytes (Gorham 1995). Most of the highly salt-tolerant halophytes are salt "includers" and are able to withstand high tissue salt concentrations (Flowers et al. 1986). It is suggested that compartmentation of ions in vacuoles and accumulation of compatible solutes in the cytoplasm, as well as presence of genes for salt tolerance, confer salt resistance (Gorham 1995). Compatible osmotica, such as glycinebetaine, are hypothesized to function in osmoregulation of the cytoplasmic compartment of cells or as an osmoprotectant for proteins (Schobert 1977, Flowers et al. 1986, Gorham 1995, Bohnert and Jensen 1996). Increases in salinity of the growth media for halophytes result in an increase in the concentration of compatible osmolytes that comprise a relatively small number of low-molecular-mass organic compounds, including proline (Stewart and Lee 1974, Treichel 1975, Tal et al. 1979, Weimberg et al. 1982), glycinebetaine (Storey and Wyn Jones 1975, Hall et al. 1978, Wyn Jones et al. 1984), sugars (Gorham et al. 1985), and polyols (Muralithran et al. 1992). Compatible solutes provide an environment compatible with macromolecular structure and function (Yancey et al. 1982). It is hypothesized that inorganic ions are preferentially excluded from the surface proteins and their hydration sphere in the presence of glycinebetaine or other compatible osmotica (Crow et al. 1988, Jacoby 1994, Bohnert and Jensen 1996).

Species in the Chenopodiaceae produce glycinebetaine in response to water and salt stress (Marcum and Murdoch 1992). Circumstantial evidence indicates that stress-induced glycinebetaine accumulation is adaptive, since it may function as a non-toxic cytoplasmic osmoticum or an osmoprotectant.

Received 30 July, 1997; revised 17 December, 1997

Indeed, glycinebetaine is considered to be a compatible solute in salt-tolerant plants (Wyn Jones 1984, Gorham et al. 1985) and it occurs primarily in the cytoplasm (Gorham and Wyn Jones 1983, Wyn Jones 1984). The possible contribution of glycinebetaine to cytoplasmic osmotic adjustment in *Sporobolus virginicus* was estimated, assuming it is located in the cytoplasm, which comprises 10% of the total cell volume. If this percentage is accurate and the majority of glycinebetaine is sequestered in the cytoplasm, its concentration (200–300 m*M*) would be sufficient for total osmotic adjustment of the cytoplasm (above a basal cytoplasmic osmotic potential of 200–300 mOsm kg^{-1}) at high salinities (Marcum and Murdoch 1992). A positive correlation between salt concentration and accumulation of glycinebetaine was reported in *Atriplex semibaccata* and *A. halimus* (Koheil et al. 1992), *Spartina alterniflora* (Cavalieri 1983), *Sporobolus virginicus* (Marcum and Murdoch 1992), *Limonium* sp. (Hanson et al. 1991), *Suaeda monoica* (Storey and Wyn Jones 1979), and in a number of grass species (Marcum 1995).

The purpose of the present paper is to determine the effect of NaCl salinity on the glycinebetaine accumulation of salt-secreting *Atriplex griffithii* var. *stocksii* Moq., the osmotically adjusting grass *Halopyrum mucronatum* (L.) Stapf., the stem succulent *Haloxylon recurvum* Bunge ex Boiss., and the leaf succulent *Suaeda fruticosa* (L.) Forssk., all halophytes native to a subtropical maritime desert in Pakistan.

Materials and methods

Seeds of *Atriplex griffithii* var. *stocksii*, *Suaeda fruticosa*, *Haloxylon recurvum* and *Halopyrum mucronatum* were collected from populations at Sands Pit beach, Karachi, Pakistan, and stored at a cool temperature (5°C). Seeds were germinated in 36-cm-diameter plastic pots filled with coarse sand. Plants were grown for 2 weeks in a growth chamber (Conviron, Pembina, ND, USA) at a thermoperiod of 15 h–30°C day (500 µmol photons m^{-2} s^{-1}, 400–700 nm) and 9 h–25°C night and irrigated with distilled water. In order to avoid shock stress, the salinity of half-strength Hoagland's No. 2 solution (Moore 1960) was increased gradually at 2-day intervals until required concentrations were reached. Plants were subirrigated with three NaCl concentrations ranging from 0 to 1 000 m*M* depending on species used. Distilled water was added at 24-h intervals to compensate for losses due to evapotranspiration. The solutions were changed weekly. Plants were grown for 17 weeks and harvested prior to dispersal of seeds. Fresh and dry weights of plants were determined.

For glycinebetaine measurements, 0.5 g of plant material was boiled in 10 ml of water for 2 h at 100°C in a dry heat bath. Samples were diluted with a 50 m*M* potassium dihydrogen phosphate buffer adjusted to pH 4.6. This was the carrier buffer that was also used in the HPLC system. The sample was cooled and filtered using a 0.45-µm membrane filter (Gelman, Ann Arbor, MI, USA), and then used directly to measure glycinebetaine with a Hewlett Packard HP 1050 modular 3D HPLC (Boise, ID, USA) with quaternary pump, on-line degasser, autosampler, and diode array detector with a stainless steel flow cell (6 mm path length, 8 µl volume). Separations were performed on a 250 × 4 mm i.d. stainless steel column packed with 10 µm Nucleoside 100-10SA (Phenomenex, St. Torrance, CA, USA). Flow rate was at 1.2 ml min^{-1}. Glycinebetaine and choline standards were run at 1, 10 and 100 mmol l^{-1}, while trigonelline standards were run at 0.1, 1 and 10 mmol l^{-1}.

An ANOVA analysis was used to determine if significant differences were present among means. A Bonferroni test was carried out to determine if significant ($P < 0.05$) differences occurred between individual treatments (SPSS 1994).

Results

Glycinebetaine content in *Suaeda fruticosa* increased significantly ($P < 0.05$) from 0 to 200 m*M* and remained unchanged ($P < 0.05$) in saline treatments up to 800 m*M* NaCl. However, glycinebetaine content decreased significantly ($P < 0.05$) at 1 000 m*M* NaCl to a level similar to that of the non-saline control (Fig. 1). In contrast, glycinebetaine concentration (mmol l^{-1} tissue water) showed small variation at lower salinities, but at 600 m*M* NaCl its concentration increased significantly ($P < 0.05$) to 200 m*M* and remained unchanged ($P > 0.05$) with further increases in salinity to 1 000 m*M* NaCl (Fig. 1). Therefore, although glycinebetaine content decreased with an increase in salinity beyond 800 m*M* NaCl, glycinebetaine still achieved high concentrations under such saline conditions.

Glycinebetaine content in *Atriplex griffithii* increased significantly ($P < 0.05$) with increasing NaCl concentration from about 1 200 mmol kg^{-1} in the control to 1 700 mmol kg^{-1} in the 360 m*M* NaCl treatment (Fig. 2). The glycinebetaine concentration in *A. griffithii* increased significantly ($P < 0.05$), about threefold in the highest salinity treatment (360 m*M* NaCl) in comparison to the control (Fig. 2).

Glycinebetaine content (mmol kg^{-1} dry weight) of *Haloxylon recurvum* did not change at low salinities but increased significantly ($P < 0.05$) in the highest salinity treatment (360 m*M*) (Fig. 3). Here, glycinebetaine concentration (mmol l^{-1} tissue water) increased significantly ($P < 0.05$) about threefold in the highest salinity treatment in comparison to the control (Fig. 3).

Halopyrum mucronatum displayed significant ($P < 0.05$) increases in both glycinebetaine content and con-

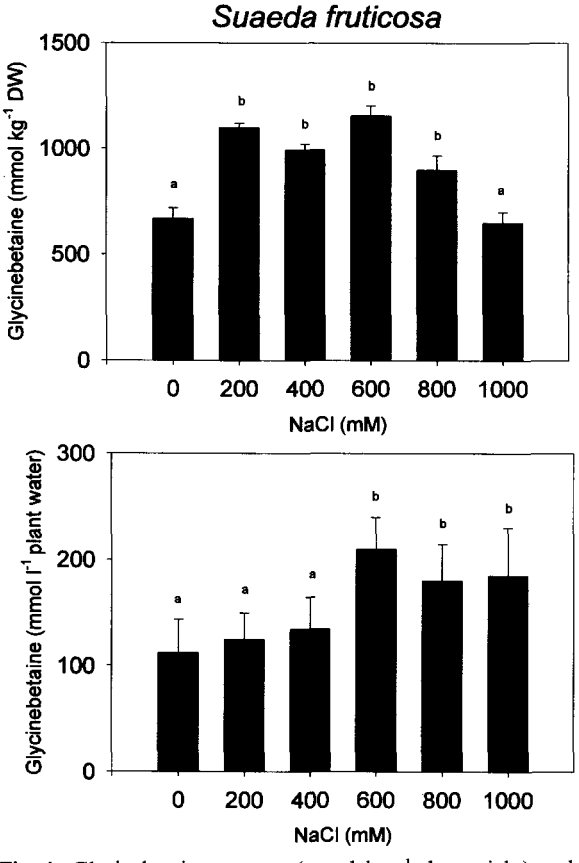

Fig. 1. Glycinebetaine content (mmol kg^{-1} dry weight) and concentration on a tissue water basis (mmol l^{-1}) of *Suaeda fruticosa* shoots exposed to NaCl from 0 to 1 000 m*M*. Means between different salinity concentrations with different letters are significantly ($P < 0.05$) different from each other according to a Bonferroni multiple comparison procedure.

centration with increasing salinity (Fig. 4). Glycinebetaine concentration increased significantly ($P < 0.05$), approximately 20-fold in the highest salinity treatment (180 m*M* NaCl) in comparison to the control.

Discussion

Many halophytes regulate turgor by accumulating NaCl to reach concentrations that are higher than that in the saline medium (Jacoby 1994). Compartmentation of salts in plant cells was suggested as a mechanism of adaptation to salt accumulation (Storey and Wyn Jones 1977). In plants that accumulate large amounts of salts in their cells, these salts are stored in the vacuole where they can function in osmotic adjustment. Organic solutes that are compatible with enzyme functions may thus play an important role in osmotic adjustment in the cytoplasmic compartment of plant cells.

It is hypothesized that organic solutes, which cause a minimum amount of perturbation of macromolecular stability and therefore function, accumulate in the cytoplasm of eukaryotic cells in order to adjust to low osmotic potentials (Stewart and Lee 1974, Storey et al. 1977). This adaptation may occur at 350–400 mOsm kg^{-1} (−1.4 to −1.6 MPa) (Wyn Jones 1981). Glycinebetaine accumulation is a well-documented metabolic feature exhibited by many halophytes, particularly from certain families such as the Chenopodiaceae (Storey et al. 1977) and in perennial succulent halophytes collected from arid environments (M. A. Khan and M. Popp, unpublished data). The present paper presents data on the endogenous levels of glycinebetaine present in four subtropical maritime desert perennial halophytes in response to increased medium salinity. Shoot biomass production of *S. fruticosa*, *A. griffithii*, and *H. mucronatum* was inhibited 51% at 800 m*M* NaCl, 20% at 360 m*M* NaCl and 74% at 180 m*M* NaCl, respectively (M. A. Khan, unpublished data). Growth of *H. recurvum* was not inhibited in up to 360 m*M* NaCl. Considering the increase in glycinebetaine content on a dry weight basis, halophytes such as the salt-secreting

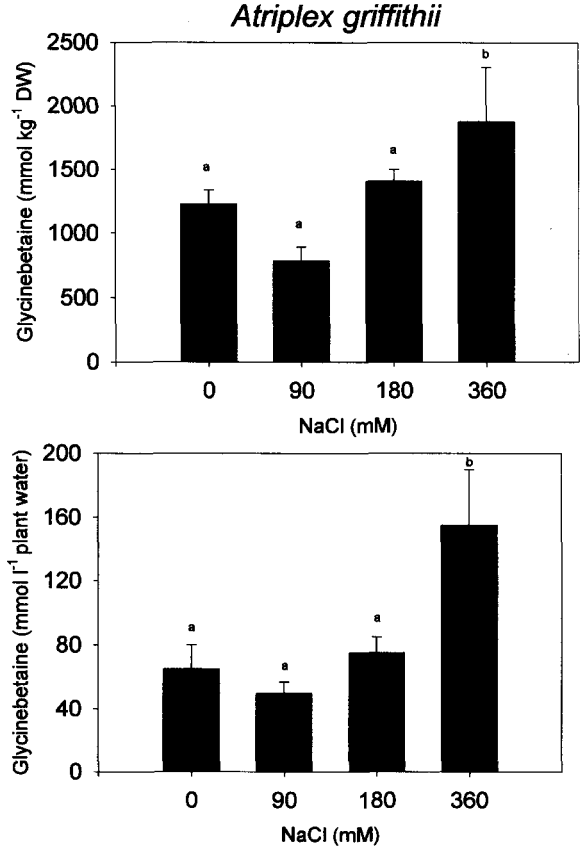

Fig. 2. Glycinebetaine content (mmol kg^{-1} dry weight) and concentration on a tissue water basis (mmol l^{-1}) of *Atriplex griffithii* shoots exposed to NaCl from 0 to 360 m*M*. Means between different salinity concentrations with different letters are significantly ($P < 0.05$) different from each other according to a Bonferroni multiple comparison procedure.

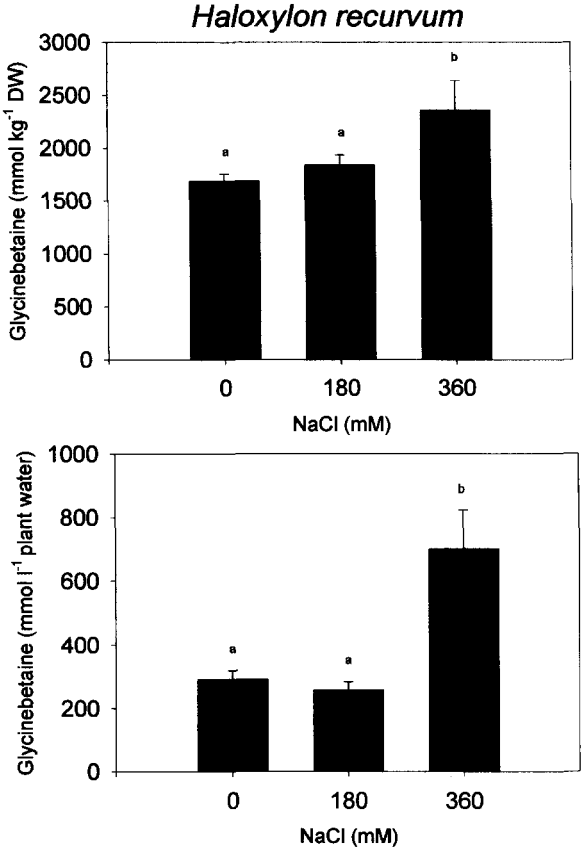

Fig. 3. Glycinebetaine content (mmol kg^{-1} dry weight) and concentration on a tissue water basis (mmol l^{-1}) of *Haloxylon recurvum* shoots exposed to NaCl from 0 to 360 m*M*. Means between different salinity concentrations with different letters are significantly ($P < 0.05$) different from each other according to a Bonferroni multiple comparison procedure.

Atriplex griffithii, stem succulent *Haloxylon recurvum* and the osmotically adjusting grass *Halopyrum mucronatum* showed a progressive increase, while in the leaf succulent halophyte *Suaeda fruticosa* glycinebetaine content increased at 200 m*M* NaCl and declined at 1 000 m*M* NaCl. However, the concentration of glycinebetaine on a plant water basis increased at the higher salinities in all four halophytes studied.

Marcum and Murdoch (1992) showed that glycinebetaine accumulated in the shoot tissues of *Sporobolus virginicus* with increasing salinity and proposed that glycinebetaine may act as a compatible solute. Levels of glycinebetaine were sufficiently high to induce osmotic adjustment of the cytoplasm at high salinities. Cavalieri (1983) determined that total glycinebetaine content was approximately 10 times higher than proline content in *Spartina alterniflora*. 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 to 2 days (Cavalieri 1983). After removal of salinity stress, proline content fell rapidly but glycinebetaine content remained unchanged. A positive relationship between salt concentration and accumulation of glycinebetaine was also reported for a number of halophytes (Storey and Wyn Jones 1975, Hanson et al. 1991, Koheil et al. 1992, Marcum and Murdoch 1992, Marcum 1995). In contrast, Guy et al. (1984) reported that glycinebetaine content of *Salicornia europaea* on a dry weight basis actually declined with exposure to progressively higher levels of NaCl. However, Guy et al. (1984) determined that this could be due to the high salt content in plant tissues, and when they reanalyzed data on an organic matter basis, they found that glycinebetaine increased with increasing salinity, a pattern similar to that of the four halophytes studied in our investigation.

Indeed, the concentration of glycinebetaine was high enough in all four species examined to serve as an osmoticum. Glycinebetaine concentration was lowest in

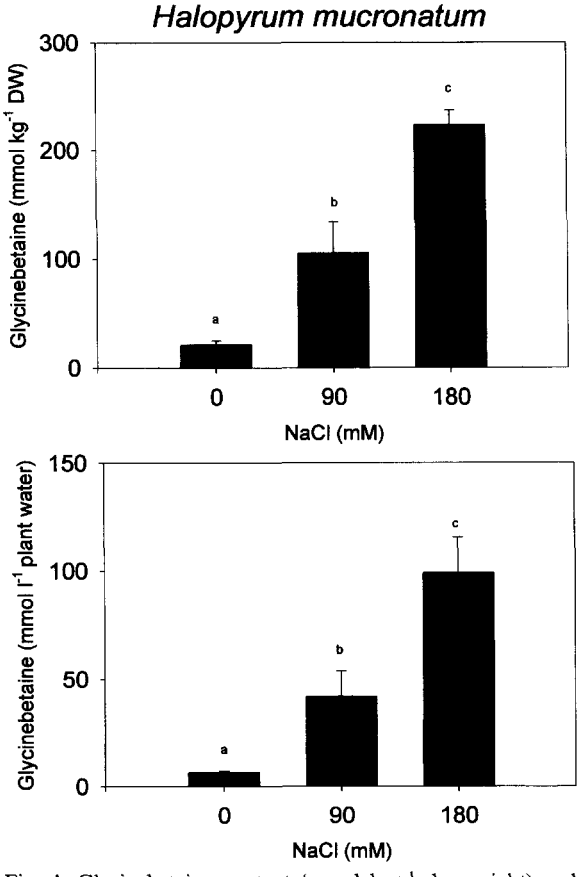

Fig. 4. Glycinebetaine content (mmol kg^{-1} dry weight) and concentration on a tissue water basis (mmol l^{-1}) of *Halopyrum mucronatum* shoots exposed to NaCl from 0 to 180 m*M*. Means between different salinity concentrations with different letters are significantly ($P < 0.05$) different from each other according to a Bonferroni multiple comparison procedure.

Halopyrum mucronatum (100 mmol l^{-1}), as compared to 200 mmol l^{-1} in *Suaeda fruticosa*, 600 mmol l^{-1} in *Haloxylon recurvum* and 160 mmol l^{-1} in *Atriplex griffithii*. Grasses usually have relatively low concentration of glycinebetaine (96–126 mmol l^{-1} tissue water) at high salinities (400 m*M* NaCl) (Marcum and Murdoch 1992). Briens and Larher (1982) showed that monocotyledons (*Puccinellia maritima* and *Spartina townsendii*) used both carbohydrate and nitrogenous compounds as osmotica; this may explain the lower concentration of glycinebetaine in *Halopyrum mucronatum*.

Glycinebetaine concentration in *Atriplex barclayana* leaves increased slightly when culture salinity was raised to 100 m*M* NaCl and then remained constant up to a level of 400 m*M* NaCl (Nerd and Pasternak 1992). However, our data with *Atriplex griffithii* showed an increase in glycinebetaine (mmol l^{-1} plant water) with increasing salinity, which is in agreement with results for *Atriplex spongiosa* (Wyn Jones et al. 1977) and *Atriplex halimus* (Wyn Jones et al. 1981).

Various species of *Suaeda* showed a progressive increase in glycinebetaine levels with increasing salinity (Storey and Wyn Jones 1975, Storey et al. 1977, Wyn Jones 1981, Brien and Larher 1982), but none reported a decline in glycinebetaine content on a dry weight basis at higher salinities after an initial increase as the case of *Suaeda fruticosa*. Since our calculation of the concentration of glycinebetaine on a tissue water basis increased with increasing salinity, we conclude that the increase in glycinebetaine concentration was the result of a decrease in tissue water content. This would account for the different patterns of glycinebetaine accumulation observed in the 1 000 m*M* NaCl treatment when calculations were made on a dry weight vs tissue water basis.

Overall, our results are in agreement with previous reports that Chenopodiaceous halophytes accumulate glycinebetaine as osmoticum and that its level increases with increasing salinity, except for *Suaeda fruticosa* for which we observed highest concentrations at 600 m*M* NaCl. The present work will serve as an important basis for future investigations designed to determine the mechanisms of salt tolerance in mangrove and salt desert species in Pakistan.

Acknowledgments – We would like to thank the Council for International Exchange of Scholars for providing a Fulbright Scholar Research Grant and Ohio University for a Postdoctoral Fellowship to M. Ajmal Khan.

References

Bohnert, H. J. & Jensen, R. J. 1996. Strategies for engineering water-stress tolerance in plants. – TIBTECH 14: 89–97.

Briens, M. & Larher, F. 1982. Osmoregulation in halophytic higher plants: A comparative study of soluble carbohydrates, polyols, betaines and free proline. – Plant Cell Environ. 5: 287–292.

Cavalieri, A. J. 1983. Proline and glycinebetaine accumulation by *Spartina alterniflora* Loisel. in response to NaCl and nitrogen in a controlled environment. – Oecologia 57: 20–24.

Crow, J. H., Crow, L. M., Carpenter, J. F., Rudolph, A. S., Wistrom, C. A., Spargo, B. J. & Anchordoguy, T. J. 1988. Interactions of sugars with membranes. – Biochim. Biophys. Acta 974: 367–372.

Flowers, T. J., Hajibagheri, M. A. & Clipson, N. J. W. 1986. Halophytes. – Q. Rev. Biol. 61: 313–337.

Gorham, J. 1995. Mechanisms of salt tolerance of halophytes. – *In* Halophytes and Biosaline Agriculture (R. Choukr-Allah, C. V. Malcolm and A. Hamdy, eds), pp. 207–223. Marcel Dekker, Inc., New York, NY. ISBN 0-8247-9664-0.

– & Wyn Jones, R. G. 1983. Solute distribution in *Suaeda maritima*. – Planta 157: 344–351.

– , Wyn Jones, R. G. & McDonnell, E. 1985. Some mechanisms of salt tolerance in crop plants. – Plant Soil 89: 15–40.

Guy, R. D., Warne, P. G. & Reid, D. M. 1984. Glycinebetaine content of halophytes: Improved analysis by liquid chromatography and interpretation of results. – Physiol. Plant. 61: 195–202.

Hall, J. L., Harvey, D. M. R., Flowers, T. J. & Kent, B. 1978. Evidence for the cytoplasmic localization of betaine in the leaf cells of *Suaeda maritima*. – Planta 140: 59–62.

Hanson, A. D., Rathinasabapathi, B., Chamberlin, B. & Gage, D. A. 1991. Comparative physiological evidence that β-alanine betaine and choline-O-sulfate act as compatible osmolytes in halophytic *Limonium* species. – Plant Physiol. 97: 1199–1205.

Jacoby, B. 1994. Mechanisms involved in salt tolerance by plants. – *In* Handbook of Plant and Crop Stress (M. Pessarakli, ed.), pp. 97–124. Marcel Dekker, Inc., New York, NY. ISBN 0-8247-8987-3.

Koheil, M. A. H., Hilal, S. H., El-Alfy, T. S. & Leistner, E. 1992. Quaternary ammonium compounds in intact plants and cell suspension cultures of *Atriplex semibaccata* and *A. halimus* during osmotic stress. – Phytochemistry 31: 2003–2008.

Marcum, K. B. 1995. Salt tolerance in the grass (Poaceae) subfamily chloridoideae. – *In* Biology of Salt Tolerant Plants (M. A. Khan and I. A. Ungar, eds), pp. 231–237. Department of Botany, University of Karachi, Pakistan. ISBN 0-9648260-1-1.

– & Murdoch, C. L. 1992. Salt tolerance of the coastal salt marsh grass, *Sporobolus virginicus* (L.) Kunth. – New Phytol. 120: 281–288.

Moore, R. H. 1960. Laboratory guide for elementary plant physiology. – Burgess Publishing Company, Minneapolis, MN.

Muralithran, M. S., Chandler, S. & van Steveninck, R. F. M. 1992. Effects of NaCl and Na_2SO_4 on growth and solute composition of highbush blueberry (*Vaccinium corymbosum*). – Aust. J. Plant Physiol. 19: 155–160.

Nerd, A. & Pasternak, D. 1992. Growth, ion accumulation, and nitrogen fractioning in *Atriplex barclayana* grown at various salinities. – J. Range Manage. 45: 164–166.

Schobert, B. 1977. Is there an osmotic regulatory mechanism in algae and higher plants? – J. Theor. Biol. 68: 17–26.

SPSS. 1994. SPSS 6.1 for Windows Update. – SPSS Inc. Chicago, IL. ISBN 0-13-182338-8.

Stewart, G. R. & Lee, J. A. 1974. The role of proline accumulation in halophytes. – Planta 120: 279–289.

Storey, R. & Wyn Jones, R. G. 1975. Betaine and choline levels in plants and their relationship to NaCl stress. – Plant Sci. Lett. 4: 161–168.

– & Wyn Jones, R. G. 1977. Quaternary ammonium compounds in plants in relation to salt resistance. – Phytochemistry 16: 447–453.

– & Wyn Jones, R. G. 1979. Response of *Atriplex spongiosa* and *Suaeda monoica* to salinity. – Plant Physiol. 63: 156–162.

– , Ahmed, N. & Wyn Jones, R. G. 1977. Taxonomic and ecological aspects of the distribution of glycinebetaine and related compounds in plants. – Oecologia 27: 319–332.

Tal, M., Rosental, I., Abramovitz, R. & Forti, M. 1979. Salt tolerance in *Simmondsia chinensis*: Water balance and accumulation of chloride, sodium, and proline under low and high salinity. – Ann. Bot. 43: 701–708.
Triechel, S. 1975. The effect of NaCl on the concentration of proline in different halophytes. – Z. Pflanzenphysiol. 76: 56–68.
Weimberg, R., Lerner, H. R. & Poljakoff-Mayber, A. 1982. A relationship between potassium and proline accumulation in salt-stressed *Sorghum bicolor*. – Physiol. Plant. 55: 5–10.
Wyn Jones, R. J. 1981. Salt tolerance. – *In* Physiological Processes Limiting Plant Productivity (C. B. Johnson, ed.), pp. 271–292. Butterworths, London. ISBN 0-408-10649-2.
– 1984. Photochemical aspects of osmotic adaptation. – *In* Recent Advances in Phytochemistry, 3: Photochemical Adaptations to Stress (B. N. Timmerman, C. Steelink and F. A. Loewus, eds), pp. 55–78. Plenum Press, New York, NY. ISBN 0-306-41720-0.
– & Storey, R. 1981. Betaine. – *In* Physiology and Biochemistry of Drought Resistance in Plants (L. G. Paleg and D. Aspinall, eds), pp. 171–204. Academic Press, New York, NY. ISBN 0-12-544380-3.
– , Gorham, J. & McDonnell, E. 1984. Organic and inorganic solute content as selection criteria for salt tolerance in Triticeae. – *In* Salinity Tolerance in Plants (R. C. Staples and G. H. Toenniessen, eds), pp. 189–204. John Wiley & Sons, New York, NY. ISBN 0-471-89674-8.
Yancey, P. H., Clarke, M. E., Hand, S. C., Bowlus, R. D. & Somero, G. N. 1982. Living with water stress: evolution of osmolyte systems. – Science 217: 1214–1222.

Edited by T. C. Vogelmann