Influence of salinity on cotton nitrogen status

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ABSTRACT

High salinity affects nitrogen status of cotton in a number of ways. In our experiments, it did not significantly reduce the total nitrogen content of dry matter, but did alter the proportions of different nitrogen-containing components. Glycinebetaine concentrations increased, while leaf, petiole and stem nitrate-nitrogen decreased. Both greenhouse and field experiments under nitrogen-deficient conditions revealed that glycinebetaine concentrations were maintained even when growth was limited by nitrogen supply.

Introduction

Nitrogen, a substrate required for cell growth, is severely affected under salt stress (Luque and Bingham, 1981). The interaction between nitrogen and salinity has been studied using several plant species, including peanut, wheat and maize (Lewis *et al.,* 1989; Silberbush and Lips, 1988). NH_4^+ was a suitable nitrogen source under non-saline conditions for some species, whereas for other species (e.g. cotton), NH_4^+ produced much less growth than did NO_3^-. Furthermore, detrimental effects of salinity were more pronounced in NH_4^+ fed plants than in NO_3^- fed plants (Lewis *et al*., 1989). Plants grown under saline conditions absorb excessive amounts of inorganic cations and anions, with Cl^- and Na^+ being the predominant ions absorbed (Martinez and Cerdá, 1989). The presence of Cl^- inhibits the uptake of NO_3^-, while increasing NO_3^- reduces Cl^- concentrations in the leaves (Deane-Drummond, 1986). Under such conditions, the competing effect between Cl^- and NO_3^- uptake has implications for crop production (Kafkafi *et al*., 1982). Leaf nitrogen concentrations were unaffected or increased by salinity (Bhivare and Nimbolkar, 1984; Seemann and Critchley, 1985). Contradictory results in response to salinity have also been found in cotton. Leaf nitrogen content decreased in tomato (Brougnoli and Björkman, 1992). Total N contents have been reported to increase (Pessarakli and Tucker, 1988), to remain the same (Hernando *et al*., 1967) or to decrease (Papadopoules *et al*., 1985). Thus, the effects of salt stress on nitrogen contents in plants are inconsistent and unpredictable.

Glycinebetaine appears to be a critical determinant of stress tolerance in some plants. It is an efficient compatible solute (Le Rudulier *et al*., 1984) and its presence is strongly associated with the growth of plants in dry and/or saline environments (Rhodes and Hanson, 1993). The accumulation of glycinebetaine is induced under stress conditions (Gorham, 1995), and the level of glycinebetaine is correlated with the degree of enhanced tolerance to stress (Saneoka *et al*., 1995a, b). Exogenous application of glycinebetaine improves the growth and survival of a wide variety of plants under various stress conditions (Allard *et al*., 1998; Gorham *et al*., 1998; Hayashi *et al*., 1998).

Glycinebetaine and structurally related compounds have stabilizing effects on proteins and membranes (Pollard and Wyn Jones, 1979; Incharoensakdi *et al*., 1986; Naidu *et al*., 1987; Papageorgiou and Murata, 1995; Gorham, 1995). Water deficit and salinity can lead to denaturation of proteins and disruption of membrane structures. Glycinebetaine prevented inactivation of aphanothece halophytica ribulose-1,5-bisphosphate carboxylase activity due to salt, heat or cold stress (Incharoensakdi *et al*., 1986), and decreased the salt-induced inhibition of phosphoenolpyruvate kinase activity by salt in both monocot and dicot species (Manetas *et al*., 1986).

Maintaining integrity of a plant's photosynthetic machinery is important for sustaining growth under environmental stress. The protective effects of glycinebetaine on macromolecules, such as complex proteins and membranes, which have been well demonstrated *in vitro*, are also recognizable *in vivo*. Under various stress conditions, glycinebetaine protected the PSII complex from photo-induced inactivation in transgenic cells of *Synechococcus* and in higher plants. The protection by glycinebetaine of the photosynthetic machinery against photo-induced damage can be attributed to acceleration of the recovery of the PSII complex from such damage (Deshnium *et al.*, 1997; Alia *et al*., 1999; Holmström *et al*., 2000). Glycinebetaine also protects the photosystem II (PSII) complex by stabilizing the association of the extrinsic PSII complex proteins in the presence of salt (Murata *et al*., 1992). PSII complexes that lack their extrinsic proteins are protected from inactivation by glycinebetaine under extremes of temperature or pH (Mohanty *et al*., 1993), but not from the inhibitory effects of Na^+ ions on oxygen evolution (Papageorgiou *et al*., 1991).

Glycinebetaine has been obtained from Synechococcus cells that have been transformed with the codA gene (Deshnium *et al*., 1997). These strains were found to have an enhanced tolerance to low temperature stress. However, glycinebetaine did not affect the unsaturation of fatty acids in *Synechococcus* (Deshnium *et al*., 1997) or *Arabidopsis* (Alia *et al*., 1999) transgenic strains, so this suggests that the enhancement of low-temperature tolerance by glycinebetaine is due to some other in vivo action of glycinebetaine. There is some evidence for the involvement of glycinebetaine in the protection of transcription and translation processes under stress conditions. Rajendrakumar *et al*. (1997) reported that glycinebetaine decreases the melting temperature in vitro of double-stranded DNA. Such a destabilizing effect on DNA might facilitate replication and transcription in vivo in a high-salt environment. Allard *et al*. (1998) observed that exogenous application of glycinebetaine to wheat seedlings induced the expression of cold-inducible genes, suggesting that

glycinebetaine has an ability to enhance the transcription *in vivo* of genes that are involved in stress tolerance.

Processes involved in gene expression are highly susceptible to various kinds of stress such as high concentrations of salt and extreme temperatures. Bourot *et al*. (2000) have recently demonstrated that glycinebetaine behaves *in vivo* like chaperonin, i.e. glycinebetaine may stabilize the transcriptional and translational machinery for the efficient expression of genes under stress conditions.

Glycinebetaine may not be beneficial under all conditions, however, as its accumulation has been associated with increased incidence of some insect pests such as aphids (Araya *et al*., 1991) and fungal diseases such as *Fusarium* (Pearce *et al*., 1976). Whereas mannitol may function to shield susceptible thiol-regulated enzymes (such as phosphoribulokinase) from inactivation by hydroxyl radicals in plants, glycinebetaine was not found to be effective as a hydroxyl radical scavenger (Shen *et al*., 1997). At the whole plant level, accumulation of glycinebetaine has been correlated with growth under stress in some species (Colmer *et al*., 1995), with freezing tolerance in barley (Kishitani *et al*., 1994), and with maintained nitrogen fixation under osmotic stress (Riou and Le Rudulier, 1990).

This paper addresses the question of whether salinity influences plant growth through effects on nitrogen nutrition.

Experimental procedure

Experiment 1

The first experiment was conducted in a heated greenhouse at Pen-y-Ffridd field station, University of Wales, Bangor from October to November 1995. The minimum temperatures were 25/20 °C day/night with a photoperiod of 16 h d^{-1} (Natural daylight supplemented with 400 W Son-T high pressure sodium lamps; Osram, UK).

Three seeds of cotton (*Gossypium hirsutum* L. cv. Acala SJ2) were sown in a mixture of equal parts of loam-based John Innes No. 1 compost, fine grade horticultural vermiculite and Seramis (sintered clay granules) in 2-liter pots. The experiment was conducted in a flood bench with 200 liter nutrient reservoirs. Macronutrients concentrations were, 1 mol m^{-3} KNO_3, 1 mol m^{-3} KH_2PO_4, 2 mol m^{-3} $MgSO_4$, and 4 mol m^{-3} K_2SO_4 (Hoagland No. 1) and micronutrients were 0.5 ml per liter (Hoagland and Arnon, 1950). $CaCl_2$ was added at 10 mol m^{-3} to all the treatments. The experiment comprised 10 treatments with 16 replicates in a factorial design. There were two levels of NaCl (0 and 200 mol m^{-3}). The salt was dissolved in the nutrient reservoirs at the time of sowing.

For ion analysis, the second youngest fully-expanded leaf, its petiole and the stem below the petiole were collected and frozen in micro-centrifuge tubes for chemical analysis. For sap extraction the samples were thawed and mechanically disrupted with a tapered stainless steel rod. Sap was centrifuged into a second microcentrifuge tube after piercing the original tube with a pin. For Na^+ and K^+ analyses, the sap samples were diluted with deionised water and measured with a flame photometer. For anions, the saps of four replicated plants were pooled together to make one sample. The samples were analyzed by ion exchange HPLC (Dionex 2000i, Dionex (UK) Ltd).

Chlorophyll estimations were made with a Minolta (SPAD) chlorophyll meter. Overall, the SPAD value is quite well correlated with chlorophyll concentration (Hoel and Solhaug, 1998). SPAD is an acronym for Soil Plant Analysis Development.

For nitrogen determination, the dry leaves of four replicated plants were pooled together to make one sample. Total nitrogen contents of leaf samples were analyzed by the Kjeldahl method.

Betaine was measured by the Reineckate method. The Reineckate Assay for Quaternary Ammonium Compounds (QACs) is based on the same basic principles as the Periodide assay (Wall *et al.*, 1960). Reinecke salt $NH_4[Cr(NH_3)_2(SCN)_4]$ F.W. 336 (Fluka AG, CH-9470 Buchs) is a chromium compound which complexes with the quaternary ammonium group thereby precipitating it out of solution. The glycinebetaine-reineckate precipitate is formed under acid conditions, and is collected and redissolved in NH_4OH (1 kmol m^{-3}) for subsequent spectrophotometric quantification. The method is described below:

Twenty mm^3 of leaf sap was added to 200 mm^3 H_2SO_4 (1 kmol m^{-3}) saturated with Reinecke salt, in each well of a 96 well (8 x 12) filter plate (Unifilter™ 800 mm^3, Whatman Polyfiltronics®). The base of the filter plate contains a hydrophobic cellulose filter. This prevents leakage of solution at times when it is not being filtered under vacuum. On the underside of each well is a protruding drip director that directs the flow of filtrate to the appropriate well of the 96 well collecting plate beneath.

Twenty mm^3 of glycinebetaine standards (0-100 mol m^3) were put into two columns of wells in the filter tray. The mixture was refrigerated for 10 min to allow precipitate to form.

Tray contents were filtered with the aid of a vacuum chamber (Univac™, Whatman Polyfiltronics®) and the filtrate discarded. The accumulated precipitate of glycinebetaine-reineckate in each well on the filter was dissolved by adding 300 mm^3 NH_4OH (1 kmol m^{-3}), and the dissolved filtrate was collected in a 96 well, 900 mm^3 collecting tray (Whatman Polyfiltronics®),

using a slight vacuum. This step was repeated if all precipitate in the filter tray was not dissolved. One hundred mm^3 of dissolved precipitate was then put into each well of a clear 96 well, 200 mm^3 reading plate (Uniplate™, Whatman Polyfiltronics®). The concentration of dissolved glycinebetaine-reineckate was ascertained by measuring solution density at 340 nm using a 96 well plate spectrophotometer (EL 340 microplate, Bio-Kinetics Reader). The QAC-Reineckate measurement may be used as a measure of glycinebetaine concentration, given that glycinebetaine is the major (over 90%) QAC in cotton (Gorham, 1996).

Experiment 2

Thirty-two seeds of Acala SJ2 were sown in sand in plant pots. For the first 28 days all plants were watered twice per week with excess 'Phostrogen' plant food. From day 29 to day 42 half of the plants received full-strength Hoagland's solution, while the other half received nitrate-free Hoagland's solution ($Ca(NO_3)_2$ and KNO_3 were replaced with $CaCl_2$ and K_2HPO_4). Between days 43 and 56 half of the plants in each nitrogen treatment received salt (200 mol m^{-3} NaCl + 10 mol m^{-3} initially in increments of 50 mol m^{-3} per day). There were thus four treatments:

1. Normal nitrogen, no salt (N+S-)
2. Normal nitrogen, 200 mol m^{-3} NaCl (N+S+)
3. No nitrogen, no salt (N-S-)
4. No nitrogen, 200 mol m^{-3} NaCl (N-S+)

Plants were harvested on day 56, and sap extracted from young and old leaves as described above. Glycinebetaine concentrations in sap were measured by the Reineckate method (see above).

Nitrogen content of dried leaves was measured with a LECO® CHN-2000 Carbon, Hydrogen and Nitrogen Elemental Analyser. One hundred mg DW of leaf sample was accurately weighed and enclosed in a tin foil holder. The samples were placed in a circular sample holder from where they fell into a combustion chamber. O_2 gas was pumped through the furnace and the sample combusted to CO_2, H_2O, N_2 and NO_2. These gasses are then passed through infrared detectors to determine the C and H contents, and through thermal conductivity detectors to determine N content.

Results

Experiment 1

In the absence of salinity, concentrations of nitrate and chloride were similar in leaves, petioles and stems of cotton (Table 1). Leaf concentrations were about one half of the concentrations in petioles and stems. Increasing salinity from 0 to 200 mol m^{-3} NaCl produced an increase in chloride concentrations in leaves, stems and petioles. At the same time the nitrate concentrations in all three tissues decreased by about one half. The osmotic effect of the decreased nitrogen concentrations was more than offset by the increase in chloride.

Leaf chlorophyll concentrations (per section of leaf, measured as SPAD readings), did not decrease with increasing salinity, but were higher in the treatments with high nitrogen nutrition (Figure 1a). This confirmed visual observations that the salt-treated plants had darker green leaves. This could only partly be explained by increases in leaf thickness induced by salinity. Similarly, total nitrogen concentrations (mmol kg^{-1} dry weight) did not decline with increasing salt in the high nitrogen treatment, and were even increased at 200 mol m^{-3} salt in the low nitrogen treatment (Figure 1b).

Experiment 2

In the second experiment, elemental analyses (Table 2) showed that carbon and hydrogen (as % of dry weight) were hardly affected by the different treatments, which were high or low nitrogen nutrition in combination with high or low salinity. Nitrogen concentrations in young and old leaves were reduced by about 50% in the low nitrogen treatments, but were not significantly different between high and low salinity treatments at either level of nitrogen nutrition.

Concentrations of the major nitrogen-containing compatible solute, glycinebetaine, were higher in all the high salinity treatments compared with the relevant low salinity treatments (Figure 2). Concentrations were higher, and increased more, in the young leaves than the old leaves. In low nitrogen treatments, the concentrations of glycinebetaine were not significantly different, or were slightly reduced, compared with the equivalent high nitrogen treatments.

Discussion

In both experiments, salinity did not reduce total nitrogen or chlorophyll concentrations, and increased the concentrations of glycinebetaine. There was a reduction in nitrate-nitrogen, but the osmotic effect of this was small compared with the increase in chloride. Thus the decrease in nitrate could either be the result of reduced root uptake of nitrate (Deane-Drummond, 1986), and/or its replacement as a vacuolar osmoticum by chloride. Inhibitory effects of chloride on nitrate uptake are seen mainly under conditions of low supply of both ions. The inhibition of nitrate uptake by the roots might not be apparent in the shoots since shoot growth is inhibited by salinity to a greater extent than root growth. The result is little change in most nitrogen fractions in the shoot. The idea that shoot growth in salt-treated plants is inhibited by nitrogen supply is thus difficult to sustain. It is more likely that changes in cell wall rheology are the primary causes of reduced growth in saline conditions.

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Table 1. *Effect of salinity on tissue nitrate and chloride concentrations (Experiment 1).*

NaCl (mol m^{-3})	Tissue	Nitrate (mol m^{-3})	Chloride (mol m^{-3})
0	Leaf	42 ± 2	62 ± 7
	Petiole	133 ± 5	104 ± 20
	Stem	109 ± 5	124 ± 15
200	Leaf	23 ± 3	246 ± 16
	Petiole	58 ± 8	304 ± 33
	Stem	52± 4	337 ± 8

Table 2. *Percentage C, H and N in young and old leaves of cotton plants under various nitrogen and salinity treatments at day 56. Mean ± standard error, n=3 (Experiment 2).*

Treatment	Young Leaves			Old Leaves		
	% C	% H	% N	% C	% H	% N
N+ S-	46.7 ± 0.4	6.5 ± 0.1	3.9 ± 0.3	43.4 ± 2.0	6.2 ± 0.3	2.8 ± 0.1
N+ S+	46.6 ± 0.8	6.5 ± 0.1	4.2 ± 0.1	41.4 ± 0.3	5.9 ± 0.1	3.0 ± 0.4
N- S-	45.6 ± 0.5	6.4 ± 0.2	2.0 ± 0.1	40.7 ± 0.6	6.1 ± 0.1	1.3 ± 0.1
N- S+	46.2 ± 0.3	6.3 ± 0.1	2.3 ± 0.2	45.7 ± 1.7	6.8 ± 0.5	1.5 ± 0.3

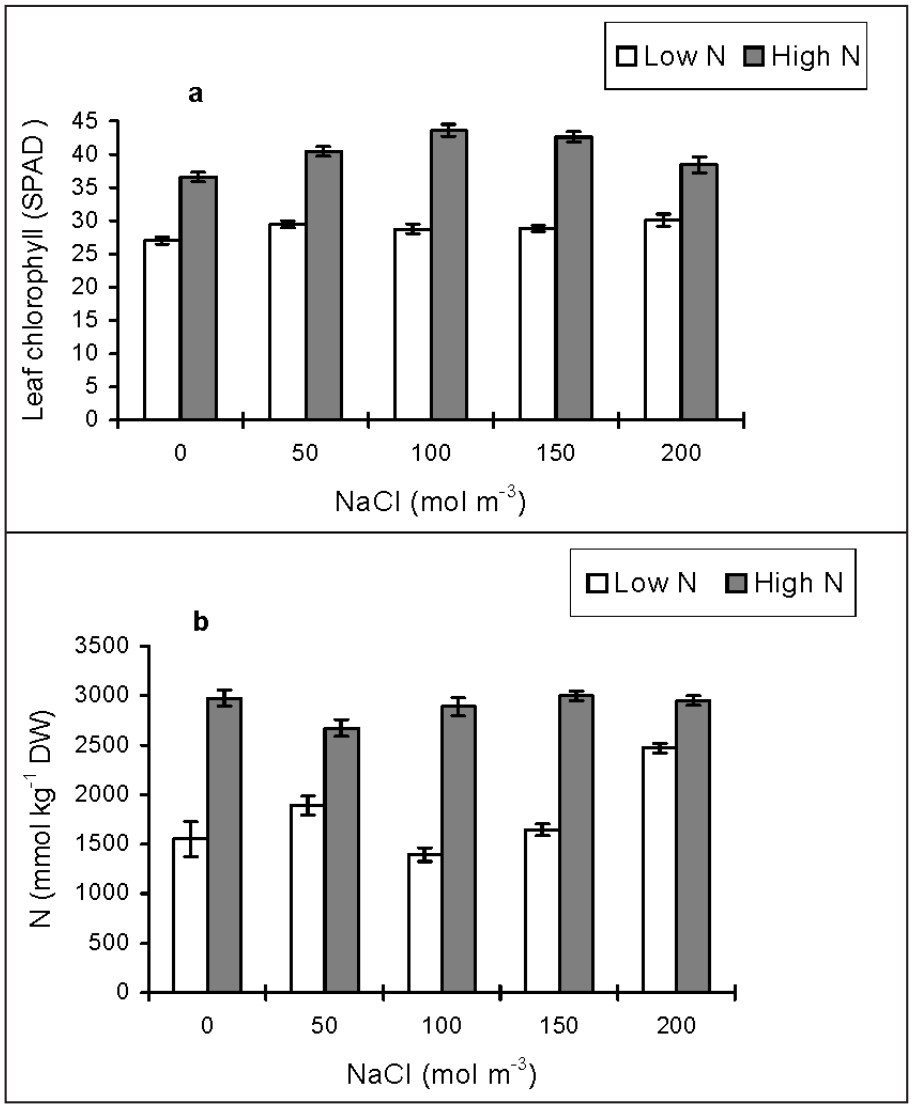

Figure 1.
Leaf chlorophyll measurements (SPAD readings) and nitrogen concentrations in dry cotton leaves in plants subjected to a range of salt concentrations under conditions of low (empty bars) or high (hatched bars) nitrogen nutrition (Experiment 1).

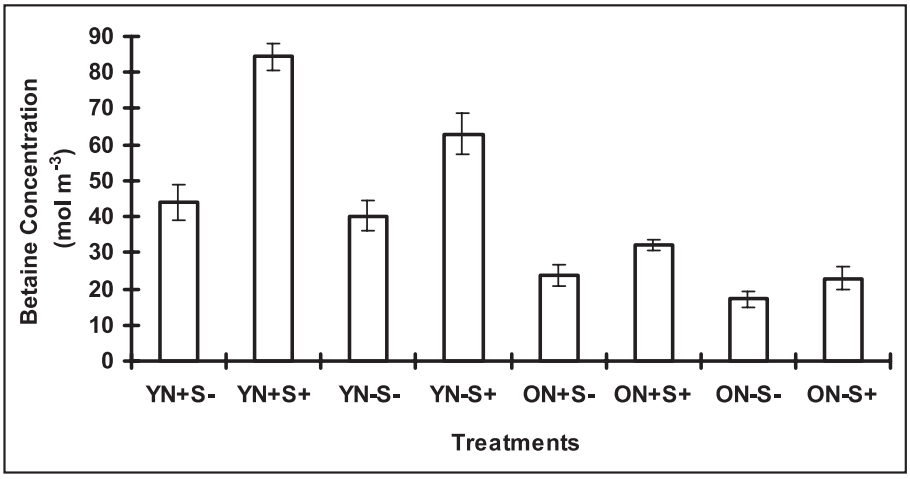

Figure 2.
Glycinebetaine concentrations in young (Y) or old (O) leaves of cotton subjected to high (N+) or low (N-) nitrogen nutrition in combination with low (S-) or high (S+) salinity (Experiment 2).