

## STOMATAL AND NONSTOMATAL COMPONENTS TO INHIBITION OF PHOTOSYNTHESIS IN LEAVES OF SUGAR BEET PLANTS UNDER SALT STRESS

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### ABSTRACT

Sugar beet (*Beta vulgaris* L.) plants grown in sand culture were gradually exposed to different levels of salinity (0, 50, 150, 250, and 350 mM, NaCl + CaCl<sub>2</sub> in 5:1 molar ratio) and photosynthetic rates of individual attached leaves were measured during salinisation period at external CO<sub>2</sub> concentrations ranging from approximately 70 to 1500  $\mu\text{mol CO}_2 \text{ mol}^{-1}$  air. Salinity dramatically decreased net photosynthesis ( $A_{\text{CO}_2}$ ) and stomatal conductance ( $g_s$ ). Net photosynthesis was plotted against computed leaf internal CO<sub>2</sub> concentration ( $C_i$ ), and the initial slope of this  $A_{\text{CO}_2}$ - $C_i$  curve was used as a measure of photosynthetic ability. Leaves from plants exposed to 50 mM salinity showed little change in photosynthesis, whereas those treated to high levels of salinity had up to 91.5% inhibition, with increase in CO<sub>2</sub> compensation point. Leaves appeared healthy and leaf chlorophyll content increased with increasing salinity. Although partial stomatal closure occurred with salinisation but reductions in photosynthesis were partly non-stomatal at high levels of salt treatment. Photosynthetic ability was inversely related to the concentration of either Na<sup>+</sup> and Cl<sup>-</sup> in the leaf laminae sampled at the end of experimental period.

**Key words:** Photosynthetic ability, Salt stress, Stomatal conductance, Sugar beet.

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## تحقیقات کشاورزی ایران

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### ممانعت اجزای روزنه ای و غیر روزنه ای از فتوسنتز برگهای گیاهان چغندر قند تحت تنش شوری

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#### چکیده

گیاهان چغندر قند (*Beta vulgaris*) در محیط شنی کاشته شدند و به تدریج تحت سطوح مختلف شوری ۰، ۵۰، ۱۵۰، ۲۵۰ و ۳۵۰ میلی مول مخلوط نمکهای کلرور سدیم و کلرور کلسیم به نسبت مولی ۵:۱ قرار گرفتند و میزان فتوسنتز برگها در طول دوره تنش شوری در غلظت های مختلف گاز کربنیک (۷۰ تا ۱۵۰۰ میکرومول CO<sub>2</sub> در مول هوا) تحت بررسی قرار گرفت. تنش شوری میزان فتوسنتز خالص (A<sub>CO2</sub>) و هدایت روزنه ای (g<sub>s</sub>) را بشدت کاهش داد. منحنی میزان فتوسنتز خالص و غلظت گازکربنیک داخل برگ (C<sub>i</sub>) رسم شد و شیب اولیه این منحنی (منحنی A<sub>CO2</sub>- C<sub>i</sub>) تعیین و بعنوان قابلیت فتوسنتزی مورد استفاده قرار گرفت. فتوسنتز برگ گیاهانی که تحت تنش شوری ۵۰ میلی مول قرار داشتند در مقایسه با تیمار شاهد تغییر کمی نشان داد در حالیکه فتوسنتز برگ گیاهانی که تحت سطوح بالای شوری بودند تا ۹۱/۵٪ کاهش نشان داد و هم چنین نقطه جبرانی آنها افزایش یافت. برگهای گیاهان تحت تنش شوری سالم بنظر می رسیدند و مقدار کلرفیل برگ با افزایش شوری افزایش یافت. اگرچه با افزایش

غلظت شوری روزنه ها بسته تر شدند و در نتیجه فتوسنتز کاهش یافت؛ اما بخشی از کاهش مقدار فتوسنتز بویژه در سطوح بالای شوری (۲۵۰ و ۳۵۰ میلی مول) به فاکتور های غیر روزنه ای مربوط می شود. قابلیت فتوسنتزی با غلظت یونهای  $\text{Na}^+$  و  $\text{Cl}^-$  موجود در برگ ارتباط معکوسی نشان داد.

## INTRODUCTION

Most plants exposed to salinity show less  $\text{CO}_2$  uptake by their leaves than the same plants not exposed to salinity (17, 19). The photosynthetic capacity of plants grown under saline conditions is depressed depending on type of salinity, duration of treatment, species and plant age (1, 10, 19). Many studies have concluded that the reduction in photosynthesis in response to salinity is to some extent the result of reduced stomatal conductance (2, 5, 11, 21, 27, 28) and consequently restriction of the availability of  $\text{CO}_2$  for carboxylation (8, 27). The reduction in stomatal conductance ( $g_s$ ) under salt stress is estimated to be substantial, with the intercellular  $\text{CO}_2$  concentration reduced by up to 30% (13). Although reduction of photosynthesis by salinity is partly due to a reduced stomatal conductance, non-stomatal inhibition of photosynthesis, caused by direct effect of NaCl on the photosynthetic apparatus have been reported for some species (19, 26, 29). These non-stomatal factors consist of a reduced efficiency of ribulose 1,5-bisphosphate carboxylase (RuBPCase), reduction of RuBP regeneration, the sensitivity of photosystem II to NaCl and a reduction in chlorophyll pigments to absorb light (3, 4, 7, 25).

Carbon assimilation is central to plant growth and productivity, and a better understanding of the factors that contribute to its inhibition by excess salinity may provide future strategies for plant improvement. Although several studies have shown that photosynthetic rates of some plants are reduced by salinity, information on associated physiological changes occurring during salinisation is lacking. Therefore, the aims of present study were to assess photosynthetic ability, chlorophyll content and ionic relations that occur in individual attached leaves of sugar beet when plants are exposed to salt stress.

## MATERIALS AND METHODS

### Plant culture and salinisation

Seeds of sugar beet plants (*Beta vulgaris* cv Madison) were sown 5 mm deep in plastic containers (40 × 20 × 10 cm) filled with vermiculite. After emergence, seedlings were

transplanted to 15 cm diameter plastic pots containing washed sand and with a saucer under them to prevent leaching after irrigation and transferred to a controlled environment chamber in the Agriculture Building of The University of Newcastle upon Tyne. The growth conditions were  $26 \pm 1$  °C/  $16 \pm 1$  °C (day/night), relative humidity was between 45-55%. Photon flux density (PFD) was about  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$  at canopy height with 16-h photoperiod. Five levels of salinity (0, 50, 150, 250, 350 mM NaCl + CaCl<sub>2</sub> in 5:1 molar ratio) were imposed through irrigation from the time most plants were at the 4-leaf stage and continued for 8 weeks. Salts were added to the modified Hoagland nutrient solution (23). The pots were flushed out with saline water contain nutrient every week to ensure homogeneity of salinity and nutrient supply in the growth medium. To prevent shock to plants, irrigation started with 50 mM saline water and was increased by 50 mM every other day until reaching each salinity level.

### **Gas-exchange measurement**

Gas exchange patterns in sugar beet leaves were studied in the presence of salinity under controlled environmental condition. Net photosynthesis ( $A_{\text{CO}_2}$ ) of the attached youngest fully expanded leaf was measured by enclosing the middle part of leaf in the cuvette of a Combined Infra Red Gas Analysis System (CIRAS-1 Portable photosynthesis system) at week 8<sup>th</sup>. The area of cuvette that caught full illumination was  $2.5 \text{ cm}^2$ . Measurements and results were displayed on the analyzer display panel and also recorded on the data storage system. In order to study photosynthetic ability, photosynthetic rates of individual attached leaves were measured at different levels of external CO<sub>2</sub> concentrations ranging from approximately 70 to 1500  $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$  and saturated light intensity ( $1500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ).

### **Leaf Chlorophyll Content**

Extraction of chlorophyll pigments from the finely ground leaf samples was carried out using 80% acetone. The absorbance of optically clear filtrates were measured at 664 and 647 nm using a Beckman spectrophotometer. The chlorophyll *a*, *b* and total chlorophyll ( $\mu\text{g/cm}^2$ ) were computed using the equations given by Porra *et al.* (24).

### **Leaf Chlorophyll Fluorescence**

Leaf chlorophyll fluorescence measurements were done on youngest fully expanded leaves at week 8<sup>th</sup> by using the Plant Efficiency Analyser (PEA) (Hansatech, Norfolk England). Measurement of maximum and minimum chlorophyll fluorescence ( $F_m$  and  $F_0$ , respectively) was

used to assess photosynthetic tolerance to environmental constraints. The  $F_v$  (variable fluorescence) /  $F_m$  ratio ( $F_v = F_m - F_0$ ) gives an indication of the status of the photosynthetic apparatus, since fluorescence emission is inversely proportional to the amount of light utilized photosynthetically. Determination of  $F_v/F_m$  ratio was made on dark- adapted leaves of all treatments.

### **Leaf ion analysis**

For  $Na^+$  analysis, the leaves blade that were used for gas-exchange measurement were removed from the plants and were ashed in furnace for 24h at 500 °C. The ashes were dissolved in 50% Hydrochloric acid, diluted in distilled water and filtered through a sheet of Whatman paper.  $Na^+$  contents were determined by flame photometer. Chloride ( $Cl^-$ ) determinations were made on a distilled water extract of the dried sample, shaken for 24 h and then filtered through a 90 mm Whatman No.2 filter paper. The amount of  $Cl^-$  was measured by ion-exchange chromatography using DIONEX Model DX 500, fitted with a CD20 conductivity Detector, IP 25 pump and AS 14 Ion Exchange Column.

Specific leaf area (SLA) is defined as the unit of leaf area per weight of leaf materials. It could be calculated as follow:

$$SLA = L_A/L_W$$

Where  $L_A$  is the leaf area ( $cm^2$ ) and  $L_W$  is weight of leaf (g).

### **Data analysis**

This experiment was carried out base on randomized complete block design. The data for all characters were analysed using the analysis of variance procedure of Statistical Analysis System (SAS) software, version 6.12. Means were compared by Duncan's multiple range tests at the 0.05 probability level for all comparisons.

## **RESULTS AND DISCUSSION**

Leaf photosynthesis ( $A_{CO_2}$ ) significantly ( $P < 0.0001$ ) decreased with increasing salt concentration. Leaf photosynthesis of plants grown at low (50 mM) and high (350 mM) levels of salinity declined 19.3% and 91.6% respectively compared with controls (Table 1). The adverse effect of salinity on  $A_{CO_2}$  was associated with a significant decrease in stomatal conductance. Stomatal conductance of plants irrigated with low level of salinity (50 mM) was decreased 24%

compared with controls. At high level of salinity stomatal conductance was very low (89.5% decrease) (Table 1).

Although, stomatal conductance ( $g_s$ ) and levels of salinity were highly correlated ( $r = -0.94$ ) (Fig. 1),  $g_s$  might not be the only reason for photosynthesis reduction at high levels of salt concentrations. The ratio of intercellular  $CO_2$  ( $C_i$ ) to ambient  $CO_2$  ( $C_a$ ) concentration decreased up to 150 mM salinity compared to control (Fig. 2). In spite of decreasing  $A_{CO_2}$  and  $g_s$  with increasing levels of salinity,  $C_i/C_a$  ratio not only showed no significant reduction at 250 mM compared to control plants but on the contrary increased at 350 mM salt concentration (Fig. 2). In other words,  $C_i$  concentration increased at high levels of salinity. This showed that non-stomatal factors could be involved in reduction of photosynthesis at high levels of salinity.

Leaf chlorophyll *a*, chlorophyll *b* and total chlorophyll content generally increased with increasing salinity (Fig. 3). The amount of leaf total chlorophyll and photosynthesis showed a negative correlation in the presence of salinity (Fig. 4). This could be due to the negative effect of salinity on photosynthesis, and its positive effect on chlorophyll contents.

The presence of salts (NaCl and  $CaCl_2$ ) in the root medium induced an important increase in  $Na^+$  and  $Cl^-$  concentrations in leaves (Fig. 5). Leaf sodium concentration increased up to 7 times control value at the highest salt treatment while  $Cl^-$  content in leaves increased up to 21 times control value at the same salinity.

Table 1. Photosynthesis ( $A_{CO_2}$ ) and stomatal conductance ( $g_s$ ) (means $\pm$ S.E) of sugar beet plants at ambient  $CO_2$  concentration ( $370 \mu mol CO_2 mol^{-1}$  air) and  $1500 \mu mol photon m^{-2} s^{-1}$  light intensity. Each number is the mean of four measurements. The numbers in pranthesis are percent of reduction compared to control.

Salinity	0 Control	50mM	150mM	250mM	350mM
$A_{CO_2}$	16.33 $\pm$ 0.53 (0)	13.18 $\pm$ 0.37 (19.30)	9.90 $\pm$ 0.52 (39.36)	3.53 $\pm$ 0.35 (78.3)	1.38 $\pm$ 0.19 (91.58)
$g_s$	317.5 $\pm$ 13.77 (0)	241.3 $\pm$ 8.28 (24.02)	126.3 $\pm$ 8.60 (60.24)	60.0 $\pm$ 5.40 (81.10)	33.3 $\pm$ 3.50 (89.53)

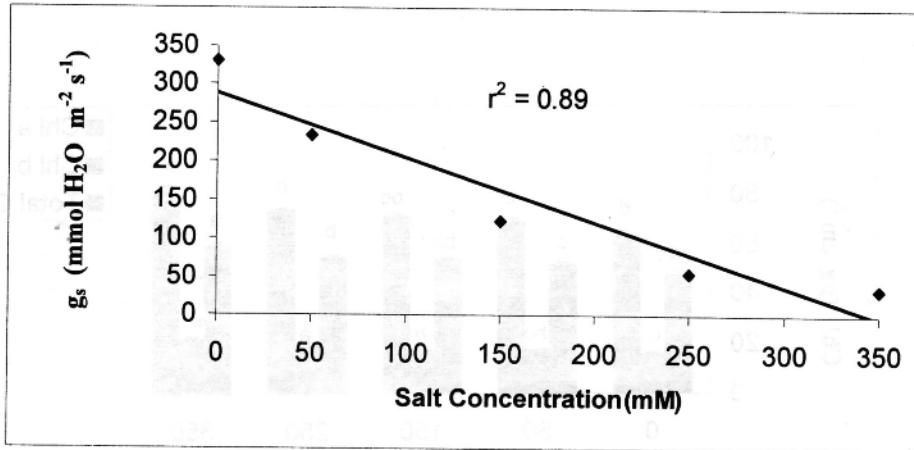


Fig. 1. Stomatal conductance ( $g_s$ ) of sugar beet plants at five levels of salinity. Values represent the mean rate of gas exchange for measurements made on youngest expanded leaf (youngest leaf over 50% full size). Each point is the average of four measurements.

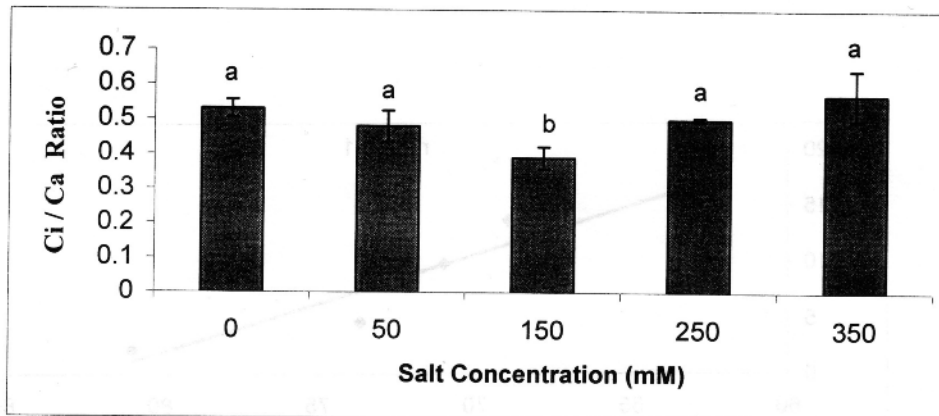


Fig. 2. Ratio of intercellular to ambient  $\text{CO}_2$  concentration ( $C_i/C_a$ ) of youngest fully expanded leaves of sugar beet at different levels of salinity. Each point is the average of four measurements. Vertical lines are standard error of the means and different letters indicate significant differences within salt concentrations according to the Duncan test ( $P < 0.05$ ).

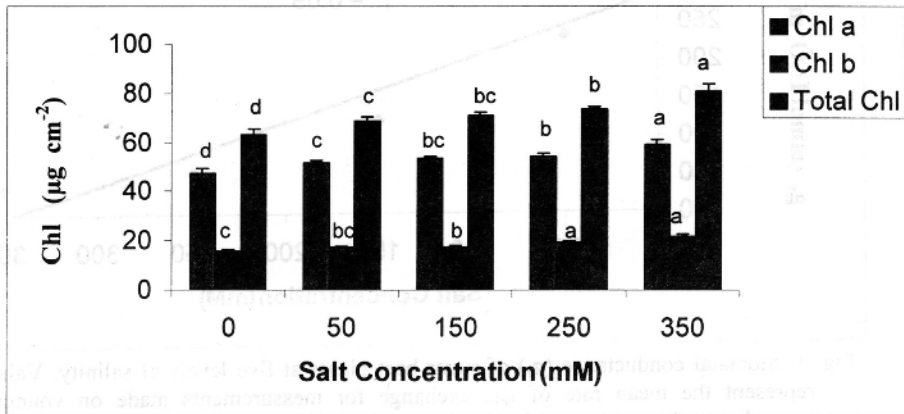


Fig 3. Amount of chl *a*, chl *b*, and total chlorophyll of youngest sugar beet leaves of different levels of salinity. Each histogram is the mean of four measurements. Vertical lines are standard error of the means and different letters indicate significant differences within salt concentrations according to the Duncan test ( $P < 0.05$ ).

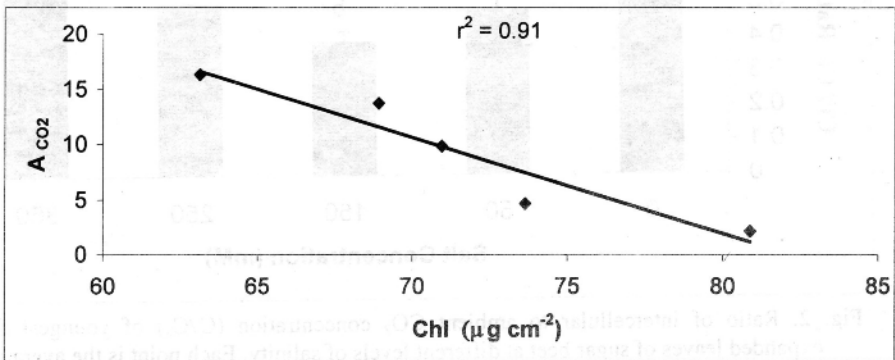


Fig. 4. Relationship between photosynthesis ( $A_{\text{CO}_2}$ ) and total leaf chlorophyll content at different levels of salinity. Each point is the mean of four measurements.



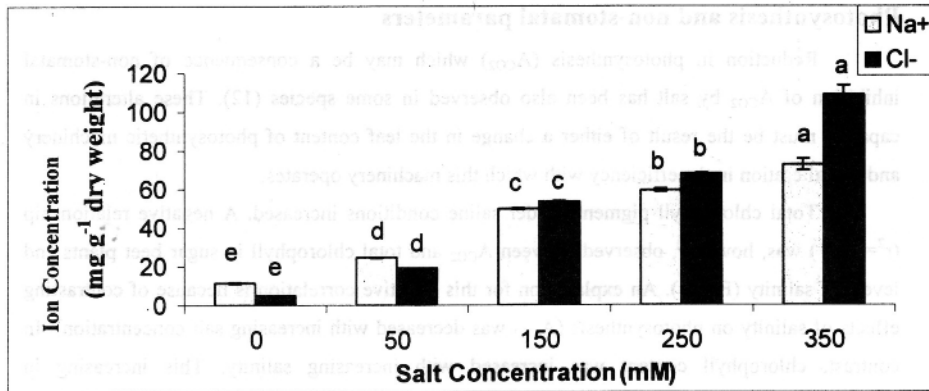


Fig. 5. Leaf sodium (Na<sup>+</sup>) and Chloride (Cl<sup>-</sup>) concentration after long term exposure to different salt concentrations. Each bar is the average of four replications. Vertical lines are standard error of the mean and different letters indicate significant differences within salt concentrations according to the Duncan test ( $P < 0.05$ ).

### Photosynthesis and stomatal parameters

This study indicate substantially lower net photosynthesis rate with increasing salinity. Similar results were obtained with other plants by Everard *et al.* (14), Flanagan and Jefferies (15) and Guanghai *et al.* (17). In the present study, increased salinity treatments often had significantly smaller stomatal conductance. This has been found in most other studies on halophyte species (9, 31). Everard *et al.* (14) reported that photosynthesis at intermediate salinity (100 mM) in celery (*Apium graveolens* L.) is limited by a decrease in stomatal conductance, and at the highest level (300 mM), carboxylation capacity (by measuring Ci) and electron transport (fluorescence) were the apparent prevailing limitations to photosynthesis. Lower rates of net photosynthesis at higher levels of salt concentrations were associated with decreased stomatal conductance (Fig. 1). Decreased stomatal conductance however had relatively little effect on intercellular CO<sub>2</sub> (C<sub>i</sub>) concentration, which responded in a complex fashion. If stomatal closure was the main reason for decreased rates of net photosynthesis at high levels of salinity (250 mM and 350 mM), it would be expected that there would be lower intercellular CO<sub>2</sub> (C<sub>i</sub>) concentrations. The absence of such an observation of salinities above 150 mM suggests that a non-stomatal effect limited photosynthesis at higher salinities.

### Photosynthesis and non-stomatal parameters

Reduction in photosynthesis ( $A_{CO_2}$ ) which may be a consequence of non-stomatal inhibition of  $A_{CO_2}$  by salt has been also observed in some species (12). These alterations in capacity must be the result of either a change in the leaf content of photosynthetic machinery and / or alteration in the efficiency with which this machinery operates.

Total chlorophyll pigments under saline conditions increased. A negative relationship ( $r^2= 0.91$ ) was, however, observed between  $A_{CO_2}$  and total chlorophyll in sugar beet plants and levels of salinity (Fig. 4). An explanation for this negative correlation is because of contrasting effects of salinity on photosynthesis ( $A_{CO_2}$  was decreased with increasing salt concentration). In contrast, chlorophyll content was increased with increasing salinity. This increasing in chlorophyll can be due to negative effect of salinity on specific leaf area (SLA) (Fig. 6). As SLA is considered as a measure of leaf density or thickness (10), leaves of stressed plants become thicker than leaves of non-stressed plants, and thicker leaves contain more cells per leaf area unit. Therefore, as both  $A_{CO_2}$  and chlorophyll concentration are expressed per leaf area, chlorophyll content could not be a limiting factor on photosynthesis in the presence of salinity.

The ratio of variable to maximum fluorescence ( $F_v/F_m$ ) was significantly reduced amongst salinity levels but not between control and 50 mM (Fig. 7). Maximum level of fluorescence ( $F_m$ ) was lower in salt treated plants than in control, which suggests that there might have been some damage to photosystem II reaction centers, or in transfer of excitation energy from the antenna to the reaction centers due to salinity. Some researchers have found that salt stress is accompanied by oxidation damage in plants (16, 18). Lower  $F_v/F_m$  in salt stressed compared to the control plants indicates that RuBP regeneration, which needs adequate electron translocation from PSII to electron acceptors, might be disturbed by salinity. However, in this study although there were significant differences in  $F_v/F_m$  in different salt treatments, values of  $F_v/F_m$  were relatively high for both control and salt stress treatments (0.91 – 0.76) (Fig. 7). Similar values of  $F_v/F_m$  were found by Belkoudja *et al.* (6) and Kafi (20) for barley and wheat respectively, grown in the presence of salinity.

Net photosynthesis was plotted against computed leaf internal  $CO_2$  concentration, and the initial slope of  $A_{CO_2}$ - $C_i$  curves were calculated (Fig. 8). The initial slope of the  $A_{CO_2}/C_i$  curve is a convenient measure of efficiency often defined as the carboxylation efficiency (CE), and greater decreases in CE were observed with increase salinity level (Fig. 9). Changes in carboxylation efficiency with salinisation have been reported previously (7, 13) and may indicate a lower activity of RuBisCO because the initial slope of  $A_{CO_2}/C_i$  curve is thought to be

controlled mainly by ability of the enzyme to fix CO<sub>2</sub> (30). Plants treated to 50 mM salinity had little or no decline in photosynthetic ability after 8 weeks of salinisation, indicating that sugar beet plants are able to tolerate moderate levels of salinity for long period of time. For those plants salinized to higher levels (150, 250 and 350 mM), photosynthetic ability was reduced. Surprisingly, leaves of plants at higher salinity remained healthy looking and continued to photosynthesize, although at the reduced rate, when as much as 18.5% of their dry weight was composed of harmful ions (Na<sup>+</sup> and Cl<sup>-</sup>). This attests to either the plants ability to compartmentalize these ions, or to the durability of photosynthetic process. Although partial stomatal closure also occurred (Fig. 1) but it does not seem to be the main factor of reductions in photosynthesis at high levels of salt treatment. The reasons are similar to those discussed by Lu and Zhang (22) for wheat leaves exposed to salt stress. As can be seen in Fig 2. C<sub>i</sub>/C<sub>a</sub> ratio decreased up to 150 mM salinity, however, at 250 and 350 mM despite reduction in A<sub>CO2</sub> and g<sub>s</sub>, the C<sub>i</sub>/C<sub>a</sub> ratio actually increased. It is likely that the increased intercellular CO<sub>2</sub> concentration (C<sub>i</sub>) was mainly associated with the decreased CO<sub>2</sub> fixation of mesophyll cells due to the decreased CO<sub>2</sub> assimilation capacity (22). Correlation between leaf elemental composition and photosynthetic ability revealed that levels of both Na<sup>+</sup> and Cl<sup>-</sup> in the leaf tissue are proportional to the degree of photosynthetic reduction. No threshold concentration for damage is seen for either of these two ions. A similar result for Na<sup>+</sup> in rice (32) and Na<sup>+</sup> and Cl<sup>-</sup> in *Capsicum annuum* (7) has been found. It can be said that either or both effects of Na<sup>+</sup> and Cl<sup>-</sup> concentration inhibited photosynthesis. However, sodium has been implicated as being more harmful than Cl<sup>-</sup> because in our experiment it showed a closer correlation (r=-0.98) with inhibition of photosynthesis rather than Cl<sup>-</sup> (r=-0.95) (but not significantly).



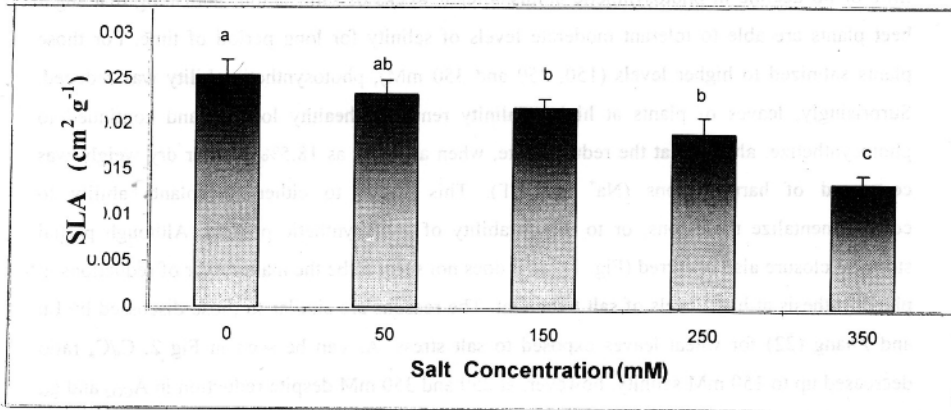


Fig. 6. Effect of different levels of salinity on specific leaf area (SLA) of sugar beet plants after 16 weeks of salinisation. Each bar is the average of four replications. Vertical lines indicate standard error of the means and different letters indicate significant differences within salt concentrations according to the Duncan test ( $P < 0.05$ ).

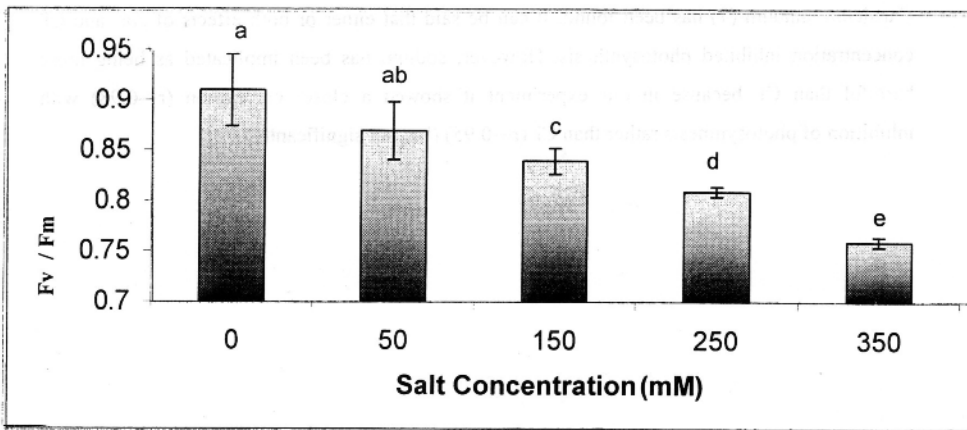


Fig. 7. Chlorophyll fluorescence:  $F_v/F_m$  ratio of youngest expanded leaves of sugar beet plants at different levels of salinity. Each histogram is the mean of four measurements. Vertical bars are standard error of the mean and different letters indicate significant differences within salt concentrations according to the Duncan test ( $P < 0.05$ ).

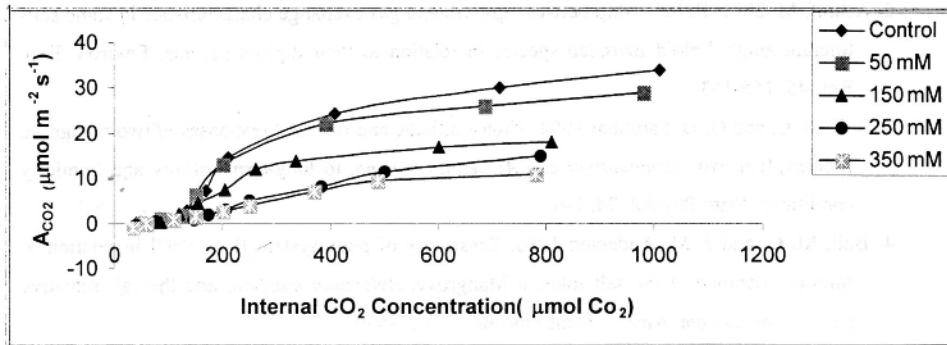


Fig. 8. Relation between net assimilation rate and substomatal CO<sub>2</sub> concentration for individual attached leaves of sugar beet under different salt concentrations. Each point is the mean of four replications.

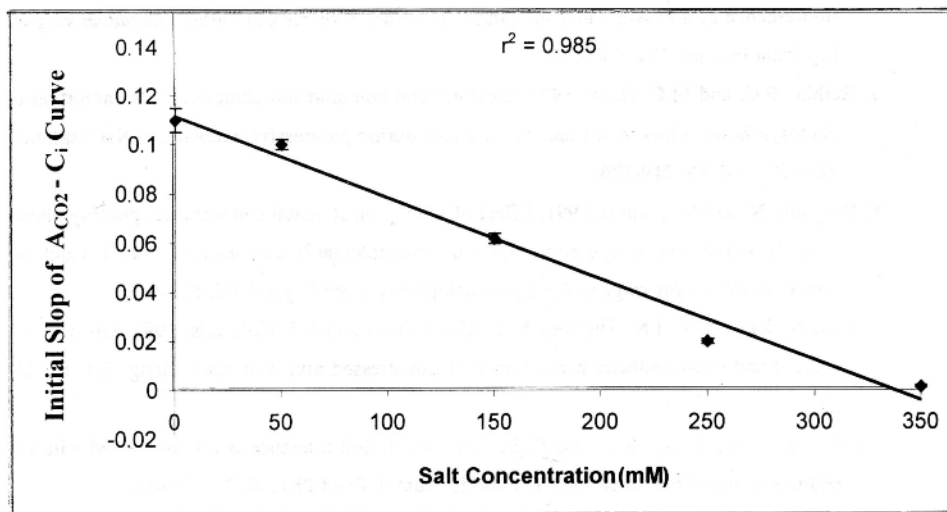


Fig. 9. Correlation between the initial slope of the ACO<sub>2</sub>/C<sub>i</sub> curve and different levels of salt treatments. Each point is the mean of four measurements.

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