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RESEARCH PAPER

High concentrations of Na^+ and Cl^- ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress

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Abstract

Despite the fact that most plants accumulate both sodium (Na^+) and chloride (Cl^-) ions to high concentration in their shoot tissues when grown in saline soils, most research on salt tolerance in annual plants has focused on the toxic effects of Na^+ accumulation. There have also been some recent concerns about the ability of hydroponic systems to predict the responses of plants to salinity in soil. To address these two issues, an experiment was conducted to compare the responses to Na^+ and to Cl^- separately in comparison with the response to NaCl in a soil-based system using two varieties of faba bean (*Vicia faba*), that differed in salinity tolerance. The variety Nura is a salt-sensitive variety that accumulates Na^+ and Cl^- to high concentrations while the line 1487/7 is salt tolerant which accumulates lower concentrations of Na^+ and Cl^-. Soils were prepared which were treated with Na^+ or Cl^- by using a combination of different Na^+ salts and Cl^- salts, respectively, or with NaCl. While this method produced Na^+-dominant and Cl^--dominant soils, it unavoidably led to changes in the availability of other anions and cations, but tissue analysis of the plants did not indicate any nutritional deficiencies or toxicities other than those targeted by the salt treatments. The growth, water use, ionic composition, photosynthesis, and chlorophyll fluorescence were measured. Both high Na^+ and high Cl^- reduced growth of faba bean but plants were more sensitive to Cl^- than to Na^+. The reductions in growth and photosynthesis were greater under NaCl stress and the effect was mainly additive. An important difference to previous hydroponic studies was that increasing the concentrations of NaCl in the soil increased the concentration of Cl^- more than the concentration of Na^+. The data showed that salinity caused by high concentrations of NaCl can reduce growth by the accumulation of high concentrations of both Na^+ and Cl^- simultaneously, but the effects of the two ions may differ. High Cl^- concentration reduces the photosynthetic capacity and quantum yield due to chlorophyll degradation which may result from a structural impact of high Cl^- concentration on PSII. High Na^+ interferes with K^+ and Ca^{2+} nutrition and disturbs efficient stomatal regulation which results in a depression of photosynthesis and growth. These results suggest that the importance of Cl^- toxicity as a cause of reductions in growth and yield under salinity stress may have been underestimated.

Key words: Chloride, faba bean, salt tolerance, sodium.

Introduction

Salinity stress is a limitation to the productivity of agricultural crops worldwide. It has been estimated that almost 80 million hectares of arable lands worldwide is currently affected by salinity (FAO, 2008). While improvements in land and water management can alleviate the problem, improving the tolerance of crop species to salinity will also contribute to increases in productivity. The soil solution of saline soils is composed of a range of dissolved salts, such as NaCl, Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl, and Na_2CO_3, each of which contribute to salinity stress, but NaCl is the most prevalent salt and has been the focus of much of the work on salinity to date (Rengasamy, 2002;

Munns and Tester, 2008). The reductions in growth from high salinity are the consequences of both osmotic stress inducing a water deficit and the effects of excess Na^+ and Cl^- ions on critical biochemical processes (Munns and Tester, 2008).

Understanding the mechanisms of tolerance of crop plants to high concentrations of NaCl in soils may ultimately help to improve yield on saline lands. The comprehensive study on the changing levels of several major phytohormones during salt-induced leaf senescence of tomato showed the sequence of physiological events involved in this process that directly reduces crop productivity (Albacete *et al.*, 2008; Ghanem *et al.*, 2008). Early events during the osmotic phase of salt stress can delay leaf appearance, retard leaf expansion, and promote leaf senescence prior to the large accumulation of toxic ions (Rajendran *et al.*, 2009; Tavakkoli *et al.*, 2010). However, this may be a transient effect and prolonged exposure to high salinity can exacerbate damage once Na^+ and Cl^- ions accumulate to high concentrations. Consequently, homeostasis of Na^+ and Cl^- is an important mechanism to reduce NaCl stress in higher plants. Excess Na^+ is frequently assumed to be largely responsible for the reductions in growth and yield under salinity (Kingsbury and Epstein, 1986; Chi Lin and Huei Kao, 2001; Tsai *et al.*, 2004; Hong *et al.*, 2009) and therefore the mechanisms by which Na^+ ions enter the cells have been studied intensively and Na^+ extrusion mechanisms, which aid salt tolerance, are known in molecular detail (Amtmann and Sanders, 1998; Tester and Davenport, 2003; Apse and Blumwald, 2007). Many studies have treated tissue Na^+ concentration in NaCl-stressed plants as a measure of tolerance, despite many examples of the lack of correlation between salt sensitivity and leaf ionic concentrations (Greenway and Munns, 1980; Genc *et al.*, 2007). However high concentrations of Cl^- are often found in tissues of plants growing under salt stress (Gorham 1990; Kingsbury and Epstein, 1986) and less attention has been given to the possible toxicity of excess levels of Cl^-, to salt stress.

The importance of high Cl^- concentrations to salt tolerance and the mechanism of Cl^- tolerance are less well understood than that of Na^+ transport (Britto *et al.*, 2004; Teakle and Tyerman, 2010), despite its being the predominant anion in most saline soils. Chloride is an essential micronutrient that regulates enzyme activities in the cytoplasm, is a co-factor in photosynthesis, acts as a counter anion to stabilize membrane potential, and is involved in turgor and pH regulation (Xu *et al.*, 2000;White and Broadley, 2001). However, Cl^- can be toxic to plants at high concentrations, with critical concentrations for toxicity estimated to be 4–7 mg g^{-1} for Cl^- sensitive species and 15–50 mg g^{-1} for Cl^--tolerant species (Xu *et al.*, 2000; White and Broadley, 2001). Control of Cl^- transport and Cl^- exclusion from shoots is correlated with salt tolerance in many species, particularly legumes, such as *Trifolium* (Winter, 1982; Rogers *et al.*, 1997), *Medicago* (Sibole *et al.*, 2003), *Glycine* (Luo *et al.*, 2005), and *Lotus* (Teakle *et al.*, 2007), and woody perennials, for example, *Citrus* and *Vitis* (Romero-Aranda *et al.*, 1998; Moya *et al.*, 2003). Severe leaf chlorosis and depression of photosynthesis were found for red kidney bean (*Phaseolus vulgaris*) (Hajrasuliha, 1980), and high concentrations of Cl^- led to a decrease in the growth rate. Previous investigations on soybean (*Glycine max* L.) clearly indicated a sensitivity of this species to high concentrations of Cl^- (Lauchli and Wieneke, 1979; Parker *et al.*, 1983). High tissue Cl^- concentrations found in salt-treated beans has been considered to be the principal cause of salt-induced growth reduction (Marschner, 1995), although investigations by Montero *et al.* (1998) and Sibole *et al.* (1998) strongly suggest that bean is also extremely sensitive to Na^+. Slabu *et al.* (2009) concluded that for faba bean (*Vicia faba*) grown at high NaCl concentration, Na^+ is the primary toxic ion, because it interferes with K^+ uptake and disrupts efficient stomatal regulation resulting in unproductive water loss and necrosis whereas Cl^- induces chlorotic toxicity symptoms due to chlorophyll degradation. However, in that study no measurements of plant growth were presented to assess the relative importance of Na^+ and Cl^-.

Salinity is a major constraint to crop production in Australia (Rengasamy, 2002) but there has been some recent debate about the importance of soil Cl, and by implication plant Cl^- uptake, as the principal cause of damage and yield loss at field level (Dang *et al.*, 2010). Based on analysis of a number of field trials of wheat and chickpea crops, Dang *et al.* (2008) concluded that the Cl^- concentration in the soil was more important in reducing growth and yield than Na^+. They estimated that the critical subsoil Cl^- concentration (defined as the concentration that reduces the growth or yield by 10%) was 490 mg Cl^- kg^{-1} soil, which was exceeded in many cases in the soils in their region of study. They found that Cl^- concentration in the youngest mature leaf of bread wheat, durum wheat, and chickpea varied much more with increasing levels of subsoil salinity than with Na^+ concentration (Dang *et al.*, 2006), suggesting that Cl^- toxicity was relatively more important to growth than Na^+ toxicity. However, subsequent crop simulation modelling of the effects of salinity suggested that soil osmotic effects was the overriding factor influencing yield rather ion toxicity (Hochman *et al.*, 2007).

Studies in the past to determine whether Na^+ is more or less toxic than Cl^- have tended to be based on solution culture (Kingsbury and Epstein, 1986; Martin and Koebner, 1995; Luo *et al.*, 2005; Slabu *et al.*, 2009). Given the debate about the possible differences in growth in solution culture and in soil (Gregory *et al.*, 2009; Tavakkoli *et al.*, 2010), it is surprising there have been no studies to compare the responses to Na^+ and Cl^- toxicity in soil-based studies. Therefore, the aim of the present study was to assess the relative importance of toxicity of Na^+ versus Cl^- using two genotypes of faba bean differing in their ion exclusion mechanism and salt tolerance in a soil-based experiment.

Materials and methods

The experiment was conducted in a field soil using two varieties of faba bean, Nura and line 1487/7. In a previous hydroponic screening experiment, line 1487/7 showed a superior ability to exclude Na^+ (1253 versus 1653 mmol kg^{-1} DW) and particularly Cl^- (730 versus 1888 mmol kg^{-1} DW) than Nura when grown at 75 mM NaCl, which was associated with greater salt tolerance (93% versus 60% relative growth). These differences in the concentrations of Na^+ and Cl^- between 1487/7 and Nura were confirmed in measurements from field trials at sites with moderately high levels of salinity and the differences in ion exclusion were also related to grain yield at the sites (E Tavakkoli *et al.*, unpublished data).

The experiment compared the effects of 100 mmol kg^{-1} Na^+ (applied as a range of sulphate, nitrate, and phosphate salts), 100 mmol kg^{-1} Cl^- (applied as a range of calcium, magnesium, and potassium salts) and 100 mmol kg^{-1} NaCl at a similar electrical conductivity (EC) in a soil-based system. The soil used in the experiment was from the A horizon (topsoil) of a sandy loam red Chromosol (Isbell, 1996) collected from Roseworthy (34.51° S, 138.68° E), South Australia. Following collection, the soil was air-dried and ground to pass through a 5 mm sieve. A soil-water characteristic curve was determined using the pressure plate method (Klute, 1986) and the soil moisture content at field capacity (–10 kPa, equivalent to 37% w/w) was estimated. Four treatments were compared which consisted of: control (no amendment), Na^+-treated, Cl^--treated, and NaCl-treated salinity. The Cl^--treated, Na^+-treated, and NaCl-treated saline soils were prepared by dissolving a mixture of Cl^- salts (15 mM $CaCl_2$, 15 mM $MgCl_2$, and 40 mM KCl), a mixture of Na^+ salts (15 mM Na_2SO_4, 15 mM Na_2HPO_4, and 40 mM $NaNO_3$), and NaCl salt (100 mM NaCl) in milliQ H_2O and spraying each solution on a 2 cm layer of soil to reach field capacity moisture content. The Cl^-, Na^+, and NaCl-dominated saline soils so obtained were thoroughly mixed and covered with plastic to reduce evaporation and left for 5 d at 25 °C to reach equilibrium. To measure the ionic concentrations of the four synthesized soils, soil samples were moistened to field capacity (water potential at –10 kPa) and centrifuged at 4000 *g* for 30 min to extract the soil solution which was then filtered through 0.25 µm filter paper. Electrical conductivity and osmotic potential (π) of the solutions were measured and these represent the EC (EC_{FC}) and osmotic potential (π_{FC}) of the soil solution at field capacity. Measurements were made at field capacity because, in order to assess the effects of salinity on plant growth, a measure of the concentration of soluble salts in the soil solution at a reference water content is required. A desirable reference water content for experimental studies is the field capacity water content rather than the saturation water content, which is the conventional measure of EC (EC_e), and which is 2–3 times the field capacity water content. All three treatments had similar values for EC_{FC} and π_{FC} (Table 1). The extracted soil solutions were analysed for ionic concentration by using inductively coupled plasma–optical emission spectrometry (ICP-OES). The characteristics of the soils used in the different treatments are shown in Table 1. The method for producing the Na^+-enhanced and Cl^--enhanced soils resulted in changes in the nutrients associated with the balancing cations (K^+, Ca^{2+}, and Mg^{2+}) and anions (S, P, and NO_3) (Table 1). This was an unavoidable effect of the treatments, which potentially could interact with the salt treatments. However, tissue analysis using ICP-OES (see below) showed that there were no deficiencies or toxicities which may have adversely affected growth, apart from those of the nutrient targeted by the treatments.

Table 1. Osmotic potential (π; MPa), pH, electrical conductivity (dS m^{-1}), and ionic concentration (mM) of the treatment soil solutions extracted at field capacity

Values are means of two replicates.

Treatment	π_{FC}	pH	EC_{FC}	Ca^{2+}	Mg^+	Na^+	K^+	P	S	Cl^-
Control	–0.01	7.8	1.2	5.8	2.7	2.2	1.9	0.012	2.2	1.9
Na	–0.48	7.6	8.8	6.5	3.2	80.9	2.7	0.387	17.2	1.3
Cl	–0.50	7.7	8.4	27.6	14.6	2.4	33.2	0.011	2.0	103.0
NaCl	–0.49	7.9	9.0	6.4	3.9	83.1	2.5	0.010	2.1	111.4

Pots, 6 cm in diameter and 25 cm deep, were lined with plastic bags and filled with 1200 g of air-dry soil to a bulk density of 1.35 Mg m^{-3}. Prior to transplanting, basal fertilizer was applied to each pot, the composition of which (in mg pot^{-1}) was: NH_4NO_3 (380), KH_2PO_4 (229), $CaCl_2$ (131), $MgCl_2$ (332), $CuCl_2$ (10.7), $ZnCl_2$ (11), Na_2MoO_4 (6.84), and H_3BO_3 (15) and thoroughly mixed through the soil. All soils with basal fertilizer were wet to field capacity with deionized water and allowed to equilibrate for 4 d at 25 °C. Uniformly sized seeds of each genotype were surface-sterilized in 70% ethanol for 1 min, followed by soaking in 3% sodium hypochlorite for 5 min, then rinsed three times with deionized water. Seeds were germinated and grown in University of California (UC) 1:1 peat:sand mix for 10 d and then one faba bean seedling was transplanted to each pot. The experiment was conducted in a temperature-controlled growth chamber with day/night temperatures of approximately 23/19 °C. The intensity of photosynthetically active radiation was measured using a Li-Cor quantum sensor meter (Model LI-1000, Li-Cor, Lincoln, NE, USA) and varied from 380 to 410 mmol m^{-2} s^{-1}. The pots were weighed and watered to field capacity every two days.

Measurements

An infrared, open gas exchange system LI-6400 (Li-Cor, Inc., Lincoln, NE, USA) coupled with an integrated fluorescence chamber head (Li-6400-40 leaf chamber fluorometer; Li-Cor Inc., Nebraska, USA) was used to measure instantaneous gas exchange and chlorophyll fluorescence. All measurements were taken on the fourth and fifth leaflets of the last fully emerged leaf blade 4–5 h into a 9 h photoperiod on the day after the plants were rewatered to field capacity. The settings were chosen to match growth chamber conditions. Leaf temperature was maintained at 25 °C, light intensity was set at 800 µmol photons m^{-2} s^{-1} with a red/blue light source, and the CO_2 concentration was set at 400 µmol mol^{-1}. Leaf to air VPD was maintained at 1.1 kPa. The photosynthetic rate (A), stomatal conductance (g_s), and substomatal CO_2 concentration (C_i) were recorded. Fluorescence parameters were estimated from the measurements of chlorophyll fluorescence on light-adapted leaves (Genty *et al.*, 1989). The efficiency of energy harvesting by open reaction centres of photosystem II for light-adapted leaves was calculated as

$$\frac{F_v'}{F_m'}=\frac{F_m'-F_m'}{F_m'}$$

where F_v' is the variable fluorescence, F_o' is the minimal fluorescence of a momentarily darkened leaf, and F_m' is the maximal fluorescence during a saturating flash of light >7 mmol m^{-2} s^{-1}. Photochemical quenching (qP) and non-photochemical quenching (NPQ) were also calculated:

$$qP=\frac{F_m'-F_s}{F_m'-F_o'}$$

$$NPQ=\frac{F_m-F_m'}{F_m'}$$

where F_s is the steady-state fluorescence and F_m is the maximal fluorescence yield which was determined after exposure to a 0.8 s saturating flash of light. The actual photochemical efficiency of

photosystem II (Φ_{PSII}) was determined by measuring steady-state fluorescence (F_s) and maximum fluorescence during a light-saturating pulse of 8000 μmol m^{-2} s^{-1} (F'_m)

$$\Phi_{PSII} = F'_m - \frac{F_s}{F'_m}$$

Estimates of the amount of chlorophyll in the leaves were made using a hand-held SPAD 502 meter (Minolta, Osaka, Japan). This provides a unitless index ranging from 0 to 100 that is proportional to the chlorophyll content of the leaves (Markwell *et al.*, 1995; Hoel and Solhaug, 1998). Average SPAD readings were calculated from four measurements from the leaflet tip to the leaflet base of the fourth and the fifth leaflets. To measure osmotic potential of leaf sap a disc of Whatman GF/B glass micro-fibre paper was placed in the barrel of a 2 ml plastic syringe so that it covered the outlet hole. A fresh leaf was then put in the barrel, the plunger was re-inserted, and the tip of the syringe was sealed with Blu-Tack®. The syringe was frozen in liquid nitrogen and, still sealed, was thawed to ambient temperature. When temperature equilibration was complete, the plunger and Blu-Tack were removed and the barrel of the syringe was placed in a 15 ml centrifuge tube, with its tip resting inside a 1.5 ml Eppendorf tube. After centrifugation at 2500 *g* for 10 min at 4 °C, the osmolality of a 10 μl sample collected in the Eppendorf tube was measured by a calibrated vapour pressure osmometer (Model 5520; Wescor, Inc., Utah, USA).

Plants were harvested 49 d after sowing and 1 d after watering to field capacity. Fresh weight was measured and plants dried at 70 °C for 72 h and dry weights were recorded. Shoot moisture content was estimated from the difference between the fresh weight and the dry weight. The nutrient concentration of the whole shoot was measured after digestion in the nitric acid/hydrogen peroxide in a closed vessel (50 ml polypropylene centrifuge tube) using a hot block digestion system. Elemental analyses of plant samples were performed using ICP-OES (Zarcinas *et al.*, 1987). The fresh and dry weights were recorded, and the whole shoot moisture content was calculated. To measure shoot Cl^- concentration, the shoots were digested in 40 ml of 1% HNO_3 at 95 °C for 6 h in a 54-well HotBlock (Environmental Express, Mt Pleasant, South Carolina, USA). Chloride concentrations of the digested extracts were determined using a Cl^- analyser (Model 926, Sherwood Scientific, Cambridge, UK). The concentration of ions are presented on a molar basis because reduced tissue water content would itself increase the concentration of ions in the cellular fluids and this is what presumably determines toxicity (Flowers *et al.*, 2010; Tavakkoli *et al.*, 2010).

Statistical design and analysis

The experimental design was a factorial completely randomized design comprised of four salinity treatments×two faba bean genotypes (Nura and 1487/7) with three replicates. Statistical analyses were performed using R 2.7.1 (R Development Core Team, 2006). Data for growth, ion content, and moisture content were analysed using ANOVA to determine if significant differences were present among means. Differences among the mean values were assessed by Least Significant Differences (LSD). Relationships between individual variables were examined using simple linear correlations and regressions which were performed using SigmaPlot (version 10).

Results

Soil properties

The different salt treatments had similar values for *EC*, π_{FC}, and pH (Table 1). The Na^+-treated and NaCl-treated soils had comparable levels of Na^+ in the soil solution and the Cl^--treated and NaCl-treated had similar concentrations of Cl^-. The use of different salt solutions in the four treatments resulted in some variation in the concentrations of Ca^{2+}, Mg^{2+}, K^+, P, and S. However, in all cases, the variation in plant nutrient concentrations was much lower than those in the soil solution and all were within the physiologically normal range for faba bean (see below). While the effects of this variation in the other cations and anions can not be completely discounted, the much greater range in and the final concentrations of Na^+ and Cl^- in the plants means that the responses to salt that were observed in faba bean were largely due to the effects of Na^+, Cl^- and NaCl.

The effects of excess Na^+ and Cl^- on growth and water use of faba bean

Increases in EC_{FC} caused by the Na^+, Cl^-, or NaCl treatments significantly (P <0.05) reduced dry weight, plant height and leaf SPAD values in both genotypes but the responses of Nura and line 1487/7 differed (Table 2). High concentrations of soil Cl^- decreased the growth of faba bean more than high concentrations of Na^+, but the two varieties differed in their sensitivity. The dry weight of line 1487/7 was not significantly reduced by the Na^+-treated soil but was significantly reduced by the Cl^--treated (24% reduction) and NaCl-treated soil (36% reduction). By contrast, the shoot dry matter of Nura was decreased by 23% by high Na^+, by 40% by the Cl^--treated soils and decreased further by 55% by the NaCl-treated soil. In both genotypes, the reduction in plant height followed a similar pattern as dry weight. The SPAD values were significantly increased by the Na^+ treatment whereas they were reduced by 27% in 1487/7 and by 40% in Nura when grown in Cl^--treated or NaCl-treated soils, respectively (Table 2).

Daily water use increased consistently throughout the experiment in the control treatment in both varieties. Salinity reduced daily water use from 10–20 d after transplanting (Fig. 1). The reductions tended to be greater in the Cl^--treated and NaCl-treated soils, especially in Nura.

Table 2. The shoot dry matter, plant height, and leaf chlorophyll of two genotypes of faba bean (line 1487/7 and Nura) grown on soils treated with Na^+, Cl^-, and NaCl salts for 49 d

Values are means (n=3). $LSD_{0.05}$ for the Variety×Salt treatment is shown as the interaction was significant.

	Control	Na^+-soil	Cl^--soil	NaCl-soil
	Dry weight (g)			
1487/7	1.498	1.417	1.173	0.980
Nura	1.608	1.230	0.963	0.798
$LSD_{0.05}$=0.115				
	Height (cm)			
1487/7	29.2	23.5	19.4	17.1
Nura	28.8	20.5	15.2	15.8
$LSD_{0.05}$=2.611				
	Leaf chlorophyll (SPAD unit)			
1487/7	43.1	46.5	31.5	30.2
Nura	45.0	48.5	27.2	28.5
$LSD_{0.05}$=2.43				

Twelve days after transplanting there was a large drop in daily water use in the Cl^- and especially the NaCl treatments in Nura, followed by a slow recovery. This did not occur with line 1487/7. At about 35 d after transplanting, daily water use declined sharply in all salt treatments in Nura and in Cl^--treated and NaCl-treated soils in 1487/7.

Differences in cumulative water use developed after about 15 d. The greater sensitivity of Nura to Na^+, Cl^-, and NaCl is clearly evident. In Nura, differences in total water use between the Na^+ and Cl^- treatments appeared after 15 d from the start of the experiment, whereas differences occurred almost 20 d later in 1487/7. The reduction due to the presence of NaCl was greater than that due to Na^+ and Cl^- individually, but it was less than the additive effect of the two. In line 1487/7 the NaCl treatment reduced total crop water use by 59%, the Cl^- treatment reduced water use by 50% whereas the Na^+ treatment reduced water use by 30%. In Nura, plant water uptake was reduced by 74% in the NaCl –treated soil, by 60% by Cl^--treated soil and by 40% by Na^+-treated soils (Fig. 1).

Whole shoot ion concentration and leaf osmotic potential

For plants in non-saline soil, tissue Na^+ concentrations were approximately 3–4 mM and no difference was apparent between the two genotypes. As soil Na^+ concentration increased, so too did the tissue Na^+ concentration in both genotypes (P <0.001) (Table 3), but the concentrations in the shoots of Nura were higher in both Na^+ and NaCl systems compared to those of line 1487/7. The concentration of Cl^- also increased in both the Cl^- and NaCl systems and the Cl^- concentrations were consistently greater than the tissue Na^+ concentrations (up to 2-fold). The increase in the Cl^- concentration in Nura was significantly greater than that in 1487/7 for both Na^+ and Cl^- (Genotype×Treatment interaction, P <0.001) (Table 3). Potassium and Ca^{2+} concentrations of both genotypes decreased significantly in the tissues of plants exposed to Na^+ or NaCl salinity. Line 1487/7 maintained higher concentrations of K^+ and Ca^{2+} than Nura (P <0.05) and this was associated with the lower Na^+ concentrations in the shoots. The use of different salt solutions in the four treatments also resulted in significant variation in Mg^{2+}, P, and S in the soil solution (Table 1), but this was not reflected in the plant tissue nutrient concentrations (Table 3). In all cases, the variation in tissue concentrations were small and the values were within the physiologically normal range (Reuter and Robinson, 1997).

Leaf osmotic potential also decreased in saline treatments compared with the control (Table 3). Although both Na^+ and Cl^- reduced the leaf osmotic potential the greatest reduction occurred when plants were grown in NaCl. Nura

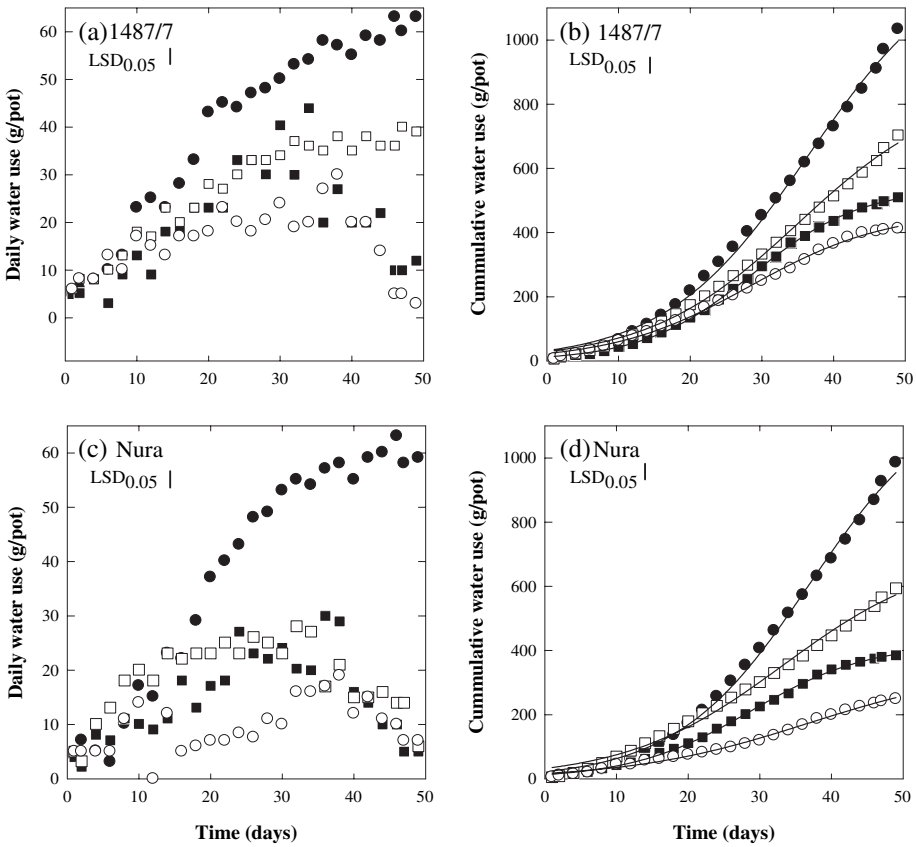

Fig. 1. The daily changes of water use and cumulative water use of line 1487/7 (a, b) and Nura (c, d) grown on soils treated with Na^+ (open squares), Cl^- (filled squares) or NaCl (open circles) salts compared with control (closed circles) treatments. Values are averages (n=3). Vertical bars represent $LSD_{0.05}$ for the Variety×Salt treatment.

Table 3. The leaf osmotic potential (MPa) and whole plant shoot concentration of Ca^{2+}, Mg^{2+}, Na^+, K^+, P, S, and Cl^- (mM basis) of two genotypes of faba bean (line 1487/7 and Nura) grown on soils treated with Na^+, Cl^-, and NaCl salts for 49 d

Values are means (n=3). $LSD_{0.05}$ for the Variety×Salt treatment is shown as the interaction was significant.

	OP	Ca^{2+}	Mg^{2+}	Na^+	K^+	P	S	Cl^-
1487/7								
Control	–0.49	66	22	3	134	11	10	7
Na-soil	–0.68	47	21	41	107	15	19	10
Cl-soil	–0.69	69	21	4	139	10	9	88
NaCl-soil	–0.71	45	20	38	84	10	12	85
Nura								
Control	–0.55	75	29	4	140	11	12	11
Na-soil	–0.75	39	27	58	54	17	20	15
Cl-soil	–0.79	77	25	3	144	12	14	155
NaCl-soil	–0.82	36	24	61	58	10	13	159
$LSD_{0.05}$	0.04	3.3	2.2	3.4	4.9	1.6	1.1	4.5

had significantly lower leaf osmotic potential compared to line 1487/7.

Gas exchange and chlorophyll fluorescence parameters

The gas exchange parameters of both genotypes decreased with increasing salt concentration of the soil solution. Photosynthetic rates (A) of both varieties were the similar in the control treatment. However, in Na^+-treated, Cl^--treated, or NaCl-treated soils, Nura had a significantly lower rate of photosynthesis than 1487/7 (Table 4). The rate of photosynthesis of plants which were grown in Na^+-soil was decreased by 11% (line 1487/7) and by 24% (Nura) while Cl^--treated soil reduced it further by 15% in both varieties. However, the largest reduction occurred in NaCl treatments.

Stomatal conductance (g_s) of both genotypes was more sensitive to salinity treatments than A but followed a similar pattern of reduction (Table 4). Nura had consistently lower g_s than 1478/7 under salt stress. The relative intercellular CO_2 concentration (C_i/C_a) was reduced by just 8% in Na^+-treated soil with little difference between the two genotypes, whereas there was a larger effect of the Cl^- treatment on C_i/C_a. The low values in the NaCl treatment largely reflected those of the Cl^- treatment. Nura had a consistently lower C_i/C_a than 1487/7 in the Cl^- and NaCl system. Transpiration efficiency (TE) remained unchanged in Na^+-treated soil and Cl^--treated soil, but increased by 9% (1487/7) and 42% (Nura) at NaCl treatments (Table 4).

Salinity induced by Na^+, Cl^-, and NaCl salts had also a marked effect on four key fluorescence parameters, but the effect of the Na^+ treatment was much less than that of the Cl^- and NaCl treatments. Growing plants in a Na^+-treated soil reduced F_v'/F_m' by 9% in 1487/7 and by 14% in Nura compared with 24% (1487/7) and 38% (Nura) in the Cl^--treated soil. When the soil was treated with NaCl, F_v'/F_m' was reduced further: by 31% (1487/7) and 48% (Nura) (Table 5). A similar pattern was observed in Φ_{PSII} and qP. A significant positive relationship between the sensitivity of F_v'/F_m' (r=0.81) and Φ_{PSII} (r=0.84) with g_s was indicated among both genotypes, indicating that these parameters may not be independent or are co-regulated (Tables 4, 5). As the proportion of PSII reaction centres that remained open (qP) was reduced for both genotypes, the portion of fluorescence quenching associated with thermal energy dissipation (NPQ) increased significantly (Table 5). There were significant negative correlations between g_s and NPQ (r= –0.93) and between A and NPQ (r= –0.93).

Discussion

The relative contributions of Cl^- and Na^+ toxicity to yield reductions of broadacre crops is not well understood as much of the work on salinity has focused on the effects of Na^+ with little consideration of the importance of Cl^- (Dang *et al.*, 2008; Tavakkoli *et al.*, 2010; Teakle and Tyerman, 2010). In many studies on salt tolerance it has not been possible to determine whether the detrimental effects on growth associated with NaCl stress are due to Cl^-, Na^+ or to a contribution from the two since Na^+ and Cl^- are combined with their respective counteranions/countercations. Responses to salinity in hydroponics and soil may also be fundamentally different (Tavakkoli *et al.*, 2010) and so to evaluate the relative effects of Na^+ and Cl^-, a soil-based system is needed to simulate the responses in field-grown plants.

The method employed here used a combination of different salts to generate soils enhanced with Na^+, Cl^-, and NaCl, but all at the same EC and π. The maintenance of the same π over the treatments is critical as changes in soil salt composition also affects soil π which can also affect plant growth. The use of soils with similar EC and osmotic potential but different concentrations and combinations of Na^+ and Cl^- allowed a comparison of the effects of Na^+ and Cl^- on growth. However, the method inevitably led to differences in the concentrations of the other balancing ions (Ca^{2+}, Mg^{2+}, K^+, S, and P) in the soil solution (Table 1). Since the availability of ions such as Ca^{2+} and K^+ can alter salinity responses (Genc *et al.*, 2010), the different concentrations may also affect the responses. However, the variation in concentrations of these ions in the plant tissue was considerably less that that measured in the soil solution (Table 3). This may have been due in part to the buffering capacity of the soil affecting the activity of the ions in solution (Khasawneh and Copeland, 1973). Moreover, the values, while showing some variation among the salinity treatments, are more typical of the concentrations found in fertile soil (Epstein and Bloom, 2005) than commonly used in hydroponic studies.

Much of the recent work on salinity has been centred on the toxic effects of Na^+ while the role of Cl^- has scarcely been investigated. Thus, it is not surprising that the literature on Cl^- uptake and its involvement in salt toxicity is small and the results equivocal. For example, Kingsbury and Epstein (1986) attempted to separate the toxic effects of

Table 4. Changes in photosynthetic parameters, *A* (μmol CO_2 m^{-2} s^{-1}), g_s (mol m^{-2} s^{-1}), C_i:C_a, *T* (mmol m^{-2} s^{-1}), and *TE* of intact leaf of two varieties of faba bean (line 1487/7 and Nura) grown in control condition or soils treated with Na^+, Cl^-, and NaCl for 49 d

Values are means (n=3). $LSD_{0.05}$ for the Variety×Salt treatment is shown as the interaction was significant.

	Control	Na^+-soil	Cl^--soil	NaCl-soil
	CO_2 assimilation rate (*A*)			
1487/7	20.8	18.4	15.6	13.1
Nura	21.9	16.6	13.1	11.5
$LSD_{0.05}$=0.524				
	Stomatal conductance (g_s)			
1487/7	0.77	0.66	0.50	0.43
Nura	0.80	0.55	0.39	0.27
$LSD_{0.05}$=0.030				
	Partial pressures of CO_2 in inside of leaf and in the air (C_i:C_a)			
1487/7	0.82	0.76	0.62	0.54
Nura	0.80	0.73	0.41	0.40
$LSD_{0.05}$=0.022				
	Transpiration (*T*)			
1487/7	4.12	3.70	2.98	2.39
Nura	4.22	3.10	2.52	1.58
$LSD_{0.05}$=0.106				
	Transpiration efficiency (*TE*)			
1487/7	5.04	5.03	5.29	5.49
Nura	5.19	5.31	5.19	7.37
$LSD_{0.05}$=0.292				

Table 5. Changes in fluorescence parameters: efficiency of light harvesting (F_v'/F_m'), actual quantum yield of PSII electron transport (Φ_{PSII}), photochemical quenching (q*P*), and non-photochemical quenching (*NPQ*) of intact leaf of two varieties of faba bean (line 1487/7 and Nura) grown in control condition or soils treated with of Na^+, Cl^-, and NaCl for 49 d

Values are means (n=3). $LSD_{0.05}$ for the Variety×Salt treatment is shown as the interaction was significant.

	Control	Na^+-Soil	Cl^--soil	NaCl-soil
	F_v'/F_m'			
1487/7	0.58	0.53	0.44	0.40
Nura	0.59	0.51	0.36	0.30
$LSD_{0.05}$=0.048				
	Φ_{PSII}			
1487/7	0.42	0.39	0.22	0.19
Nura	0.41	0.38	0.12	0.10
$LSD_{0.05}$=0.019				
	q*P*			
1487/7	0.72	0.70	0.45	0.40
Nura	0.71	0.68	0.30	0.27
$LSD_{0.05}$=0.042				
	NPQ			
1487/7	0.77	0.84	1.35	1.76
Nura	0.76	0.92	2.01	2.28
$LSD_{0.05}$=0.065				

Na^+ and Cl^- by comparing the growth rates of a salt-sensitive and a salt-tolerant wheat in a series of isosmotic solutions. They concluded that the toxicity effects of high NaCl on the growth of the sensitive wheat was a function of the Na^+ rather than the $C1^-$ ion. However, the sole counter-anion in the Na^+ treatment was nitrate (120 mM in the culture solution), which can be phytotoxic (Clement *et al.*, 1978). Other studies with rice seedlings (Chi Lin and Huei Kao, 2001; Tsai *et al.*, 2004) and wheat seedlings (Kinraide, 1999) also concluded that the Cl^- toxicity hypothesis is false. A weakness of these experiments is that were short-term, with rice and wheat seedlings being grown in solution culture for only 5 d and 2 d, respectively. For crop plants growing under field conditions, this is unrealistic. On the basis of the two-phase model of salt injury (Munns *et al.*, 1995) it is more likely that specific ionic toxicity is building up over a longer period, as occurs with plants cultivated under a field situation. Thus, a relationship which may arise under short-term studies may not necessarily reflect that observed in the longer term.

Salinity reduced biomass production and water uptake of faba bean plants (Fig. 1; Table 2) and from the results it was clear that plants were more sensitive to Cl^- than to Na^+. However, the data also show that when leaf Cl^- concentrations are high, the presence of the Na^+ ions as dominant cation exacerbates the severity of the alterations (Fig. 1; Tables 2, 3). The measurements of ion composition and plant biomass were made at the end of the experiment, when there had been a considerable increase in Na^+ and Cl^- in the plants. However, significant reductions in growth can occur before Na^+ (and presumably Cl^-) reach phytotoxic levels (Albacete *et al.*, 2008; Munns and Tester, 2008). The trends in water use (Fig. 1) provide a useful surrogate for plant growth and these suggested that Nura suffered a significant reduction in growth in the NaCl and Cl^- treatments at about 12 d after transplanting followed by a slow recovery. This may reflect transient slowing in leaf

expansion in Nura as the seedlings were increasingly exposed to salt stress similar to that reported for tomato (Albacete *et al.*, 2008). The effect was not seen with the Na^+ treatment even though the soil has a similar π_{FC}. It was also not observed in line 1487/7. The slowing followed by the large reduction in water use later in the experiment may have been associated with the development of ion toxicity. Therefore, while the differences in growth at the end of the experiment were strongly associated with differences in ion accumulation, short-term effects of the treatments early in the experiment may also have contributed to the treatment effects.

A significant reduction in plant K^+ and Ca^{2+} was indicated with increasing Na^+ concentration under Na^+ and NaCl treatments (Table 3). Previous studies have demonstrated the overriding importance of Na^+ for many plant species as a reason for ion-specific damage during salt stress. Toxicity of Na^+ in metabolic processes results from its ability to compete with K^+ for binding sites and to inactivate enzymes and essential cellular functions and, consequently, crops growing in saline soils may suffer the dual injury of Na^+ toxicity and low K^+ concentrations (Amtmann and Sanders, 1998; Munns and Tester, 2008; Tester and Davenport, 2003). In the present study, line 1487/7 exhibited a higher leaf K^+:Na^+ ratio (~2.5) compared with Nura (~0.9) as a result of significant Na^+ exclusion and better maintenance of leaf K^+ concentrations under Na^+ and NaCl stress which can be associated with higher salt tolerance (Gorham *et al.*, 1990; Dvořak *et al.*, 1994). Na^+–Ca^{2+} interactions under salt stress also have important effects on plant membrane properties and ion transport and content and lead to changes in cytoplasmic Ca^{2+} activity and therefore physiological properties such as plant growth, photosynthesis, nutrition, water and ion transport (Cramer *et al.*, 1985, 1989; Cramer, 2002). A higher concentration of Ca^{2+} in leaves of line 1487/7 can enhance the replacement of displaced Ca^{2+}, thus restoring cell wall stability and plasma membrane integrity, facilitating higher K^+/Na^+ selectivity, increasing Na^+ exclusion and so improving plant salt tolerance (Zhang *et al.*, 2010).

A significant decline in leaf chlorophyll with increasing leaf Cl^- concentration was observed. However, plants grown under high Na^+ by itself showed a significant increase in leaf chlorophyll (Table 2). Therefore, Na^+ toxicity was not the primary reason for the degradation of chlorophyll in plants grown under high NaCl and the decreased chlorophyll may be induced by increased Cl^- concentration. Heber and Heldt (1981) also provided evidence that chloroplasts exhibit a high permeability for Cl^-, and a treatment with NaCl resulted in the accumulation of Cl^- and decline in SPAD values. The reduction in leaf chlorophyll with salt stress may thus be related to the reduction in photosynthetic capacity at a given internal CO_2 concentration (Seemann and Critchley, 1985). Under high Na^+ concentration A was mostly limited by g_s which suggests that the reduction in assimilation of CO_2 was due to stomatal factors. However, plants under Cl^- and NaCl treatments showed a reduction of C_i:C_a in parallel with a reduction in g_s indicating specific damaging effects of Cl^- on the functioning of the chloroplasts in addition to stomatal limitations. Similarly, other studies showed that both stomatal and non-stomatal factors affect photosynthesis at moderate and high levels of salinity (James *et al.*, 2002; Tavakkoli *et al.*, 2010). Correlations between a reduction in photosynthesis and toxic concentrations of Na^+ and Cl^- have been observed in a number of species including bean, cotton, citrus, grapevine, and rice. Evidence in support of this comes from strong negative correlations between ions and photosynthetic activity, where Na^+ has been implicated primarily in crop species such as rice and wheat and Cl^- in woody perennials such as citrus and grapevines. Results from the present experiment show negative relationships between both Na^+ and Cl^- accumulation and A, however, the greater effect of Cl^- on growth and photosynthesis suggest that under NaCl stress, Cl^- accumulation may determine the response to salinity in faba bean plants (Table 4). High concentrations of Cl^- can be detrimental to the integrity of the cell and affect photosynthetic processes directly through membrane damage or enzyme inhibition, if the vacuole can no longer sequester incoming ions. Seemann and Critchley (1985) found that high Cl^- concentrations (250–300 mM) in the chloroplast of *Phaseolus* correlated negatively with the efficiency of Rubisco. In that study, similar Cl^- concentrations were found in both the cytoplasm/chloroplasts and in the vacuole, indicating a break-down in vacuolar compartmentation.

Analysis of chlorophyll fluorescence parameters provided insights into the physiological responses of two faba bean genotypes to toxic concentrations of Na^+ and Cl^-. If high internal salt levels were to be toxic and directly affect the photosynthetic machinery, then one might expect this to be reflected in reduced chlorophyll fluorescence. There was a significant reduction in both the efficiency of light harvesting of PSII ($\frac{F'_v}{F'_m}$) and actual quantum efficiency of PSII (Φ_{PSII}) in both genotypes, but line 1487/7 maintained a higher capacity of the PSII system compared with Nura. Fluorescence data also indicate that the decline in A in saline soils is likely to be a consequence of toxic Cl^- concentrations. While Na^+ and Cl^- accumulated to high concentration (Table 3), Φ_{PSII} and $\frac{F'_v}{F'_m}$ declined markedly in Cl^- and NaCl treated soils (Table 5). The proportion of PSII reaction centres that remained open (qP) was also reduced, indicating that this decrease in Φ_{PSII} was due to feedback regulation caused by processes such as photoinhibition as well as salt-induced photodamage. To our knowledge, this is the only report in which the effects of Na^+ and Cl^- on photochemical efficiency has been examined independently of each other and only the third report of Φ_{PSII} being correlated to g_s, following the results of a study of grape leaves subjected to progressive drought (Flexas *et al.*, 2002) and barley plants under saline treatments (Jiang *et al.*, 2006). A reduced quantum yield, as found in the present experiment (Table 5) may result from a structural impact of high Cl^- concentration on PSII. Salinity has been concluded to affect reaction centres of PSII either directly (Masojidek and Hall, 1992) or via an

accelerated senescence (Kurra-Hotta *et al.*, 1987). A structural change of PSII, its immediate surrounding or both is suggested by the increase of *NPQ* in plants grown at high salt concentration (Table 5). The rise in *NPQ* may also reflect the fact that reduced CO_2 assimilation decreases demand for products of electron transport, and thus increases thermal dissipation of light energy (James *et al.*, 2002). Greater oxidative damage can result and the increase in *NPQ* is considered to be a way of minimizing damage. The significant negative correlations between *NPQ* and *A* and *NPQ* and g_s are consistent with this.

A possible effect of Cl^- on plant cell function has been suggested by the sensitivity of leaf RuBP carboxylase levels, and therefore rate of assimilation of CO_2, to leaf Cl^- level in *Prunus* sp. and photosynthesis of the plant. If these effects were combined with those generated by the loss of K^+ and Ca^{2+} homoeostasis, a drastic loss of plant viability could readily ensue. It is also important to emphasize that exclusion of Na^+ and Cl^- and the K^+/Na^+ discrimination character described here are not seen as the only mechanisms controlling salt tolerance in faba bean. Other mechanisms, such as osmotic tolerance, which operate in totally different ways can also contribute to growth reduction under saline conditions (Munns *et al.*, 2002) and its relative importance compared to specific ion toxicity increases at higher levels of salinity stress (Tavakkoli *et al.*, 2010). Thus the exclusion trait is only one mechanism which must be integrated with other characters in the pyramiding approach to designing and breeding crop plants with greater salt tolerance.

In conclusion, the results of this study indicated that exposure to high concentrations of Cl^- is a major cause of losses in yield due to soil salinity in faba bean. The greater reductions in growth under NaCl treatment compared with Na^+ and Cl^- separately, suggests that high concentrations of Na^+ and Cl^- are limiting growth through different mechanisms but simultaneously. High concentrations of Na^+ interferes with K^+ and Ca^{2+} nutrition while high Cl^- concentration reduces the photosynthetic capacity due to chlorophyll degradation. These results have been interpreted to mean that the toxicity of NaCl is not merely the result of uptake of excess Na^+, a belief that lies behind many attempts to select for salt tolerance on the basis of tissue Na^+ levels. The results of this work provide support for further studies on the toxicity of the Cl^- ion, but these still need to be tied into a general response of the whole plant to NaCl stress. Further experiments with genotypes differing in Na^+ and Cl^- accumulation are needed to resolve fully the contribution of these ions to the salt-induced decline in growth.

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