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Additive effects of Na⁺ and Cl⁻ ions on barley growth under salinity stress

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Abstract

Soil salinity affects large areas of the world's cultivated land, causing significant reductions in crop yield. Despite the fact that most plants accumulate both sodium (Na⁺) and chloride (Cl⁻) ions in high concentrations 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. It has previously been suggested that Cl⁻ toxicity may also be an important cause of growth reduction in barley plants. Here, the extent to which specific ion toxicities of Na⁺ and Cl⁻ reduce the growth of barley grown in saline soils is shown under varying salinity treatments using four barley genotypes differing in their salt tolerance in solution and soil-based systems. High Na⁺, Cl⁻, and NaCl separately reduced the growth of barley, however, the reductions in growth and photosynthesis were greatest under NaCl stress and were mainly additive of the effects of Na⁺ and Cl⁻ stress. The results demonstrated that Na⁺ and Cl⁻ exclusion among barley genotypes are independent mechanisms and different genotypes expressed different combinations of the two mechanisms. High concentrations of Na⁺ reduced K⁺ and Ca²⁺ uptake and reduced photosynthesis mainly by reducing stomatal conductance. By comparison, high Cl⁻ concentration reduced photosynthetic capacity due to non-stomatal effects: there was chlorophyll degradation, and a reduction in the actual quantum yield of PSII electron transport which was associated with both photochemical quenching and the efficiency of excitation energy capture. The results also showed that there are fundamental differences in salinity responses between soil and solution culture, and that the importance of the different mechanisms of salt damage varies according to the system under which the plants were grown.

Key words: Barley, chloride, salinity, sodium, specific ion toxicity, tolerance.

Introduction

The yield of grain crops over large areas of the world's farming land is limited by a number of physicochemical constraints in the subsoil including salinity and sodicity (Rengasamy, 2010). Attempts to develop viable management options to improve productivity of saline–sodic soils, such as irrigation and drainage, have met with minimal success to date. The use of breeding to develop better-adapted crops with improved physiological tolerance to saline–sodic soils offers a strategy for managing crop production on these soils. Improving salt tolerance of barley (*Hordeum vulgare* L.) has been of interest for a long time and has resulted in a considerable body of data from studies using physiological (Cramer *et al.*, 1990; Munns and

Rawson, 1999; Munns *et al.*, 2000; Tavakkoli *et al.*, 2010b), genetic (Mano and Takeda, 1997; Ellis *et al.*, 2002), and cytogenetic (Forster *et al.*, 1997) approaches.

The constituent cations of total soluble salts in soils are usually sodium (Na⁺), calcium (Ca²⁺), and magnesium (Mg²⁺) and the anions are chloride (Cl⁻), sulphate (SO₄²⁻) and carbonate (including bicarbonate; CO₃²⁻ HCO₃⁻). However, Na⁺ dominates the cations and Cl⁻ the anions in the majority of saline soils to the extent that NaCl comprises from 50–80% of the total soluble salts (Rengasamy, 2010). Salt stress has 3-fold effects on plant growth: it reduces soil water potential leading to osmotic stress, it induces ion imbalance in cells, especially lower concentrations of K⁺,

Ca^{2+}, and NO_3^-, and it causes ion (Na^+ and/or Cl^-) toxicity. Since salt stress involves both osmotic and ionic stresses, growth suppression is directly related to the total concentration of soluble salts and osmotic potential of the soil solution. The detrimental effect is observed at the whole-plant level as the death of plants or a decrease in productivity (Munns and Tester, 2008). Therefore, understanding the mechanisms of tolerance to high soil concentration of NaCl is essential to improve crop salt tolerance.

For most plants to tolerate salinity, Na^+ and Cl^- uptake must be restricted while maintaining the uptake of macronutrients such as K^+, NO_3^- and Ca^{2+}) The mechanisms of Na^+ and K^+ transport in plants under salt stress has been extensively researched and reviewed (Amtmann and Sanders, 1998; Maathuis and Amtmann, 1999; Tester and Leigh, 2001; Tester and Davenport, 2003; Rodriguez-Navarro and Rubio, 2006; Apse and Blumwald, 2007; Shabala and Cuin, 2008). Reduced Na^+ loading into the xylem is one of the main mechanisms of salinity tolerance and it is often considered one of the most crucial features of restricting Na^+ accumulation in plant tissues (Tester and Davenport, 2003; Munns and Tester, 2008).

Given that the dominant salt in saline soils is NaCl, both Na^+ and Cl^- ions will occur in high concentrations. However, the contribution of Cl^- to growth reduction under salt stress is less well understood than that of Na^+ in broadacre crops. This reflects the fact that most research on salt tolerance in cereals has focused on Na^+ with little regard to Cl^- toxicity (Teakle and Tyerman, 2010). Both Na^+ and Cl^- should be given equal consideration since they are both metabolically toxic to plants if accumulated at high concentrations in the cytoplasm (Xu *et al.*, 2000; White and Broadley, 2001; Tavakkoli *et al.*, 2010*a*, *b*; Teakle and Tyerman, 2010). For some species, shoot or root concentrations of Na^+, rather than Cl^- are negatively correlated with salt tolerance (Kingsbury and Epstein, 1986; Kinraide, 1999; Lin and Kao, 2001), while there are many examples of species for which control of Cl^- transport and Cl^- exclusion from shoots is correlated with salt tolerance (Martin and Koebner, 1995; Luo *et al.*, 2005; Islam *et al.*, 2007; Teakle *et al.*, 2007; Aydi *et al.*, 2008; Tavakkoli *et al.*, 2010*a*, *b*). There has also been some recent debate about the importance of soil Cl^- and by implication plant Cl^- uptake, as predictors of damage and yield loss at the field level. Based on analysis of a number of field trials, Dang *et al.* (2008) concluded that the Cl^- concentration in the soil was more important to growth and yield reduction than Na^+. They estimated the critical level (defined as the concentration that reduces the growth or yield by 10%) of subsoil Cl^- concentration to be 490 mg Cl^- kg^{-1} soil with Cl^- concentration in the youngest mature leaf of bread wheat, durum wheat, and chickpea showing greater variability with increasing levels of subsoil constraints than Na^+ concentration (Dang *et al.*, 2006).

Research to determine Na^+ toxicity relative to Cl^- has largely been based on solution culture (Kingsbury and Epstein, 1986; Martin and Koebner, 1995; Luo *et al.*, 2005; Slabu *et al.*, 2009) and there has been little research to examine the responses to Na^+ and Cl^- toxicity in soil-based systems (Tavakkoli *et al.*, 2010*a*). Given the inherent differences in plant growth interactions with solution and soil culture (Gregory *et al.*, 2009; Tavakkoli *et al.*, 2010*b*), a critical assessment of Na^+ and Cl^- ion toxicity in both systems is needed.

The aim of this study was critically to assess the extent to which Na^+ and Cl^- contribute to ion toxicity in barley and whether tolerant genotypes have a better ability to exclude Na^+ and/or Cl^-). Ion toxicity was assessed using a range of physiological techniques (tissue nutrient analysis, chlorophyll fluorescence, gas exchange) on selected barley varieties with different mechanisms of salt tolerance in both hydroponic and soil systems. It is argued that salt tolerance is related to the ability of a genotype to regulate both Na^+ and Cl^- transport in order to minimize cytoplasmic concentrations and to avoid toxicity.

Materials and methods

Barley varieties

The effects of Na^+ and Cl^- ion concentration on barley growth was assessed using four genotypes; Barque73, Clipper, Sahara, and Tadmor. In previous hydroponic experiments, Barque73 showed a higher salt tolerance compared with Tadmor (60% versus 42% relative growth at 150 mM NaCl) with similar Na^+ concentration (2207 versus 2150 mmol kg^{-1} DW) but a superior ability to exclude Cl^- (1341 versus 2440 mmol kg^{-1} DW) (E Tavakkoli, unpublished data). Clipper is a variety with an ability to exclude both Na^+ and Cl^- and Sahara accumulates Na^+ and Cl^- to high levels (Rivandi, 2009; Tavakkoli *et al.*, 2010*b*). Barque73 and Tadmor were only used in Experiment 1 and all genotypes were used in subsequent experiments.

Growth conditions

Experiment 1. The separate effects of Na^+ and Cl^- ions at different concentrations on growth of barley in solution culture: A solution culture experiment was conducted to assess the effect of different concentrations of Na^+ and Cl^- ions on the growth of Barque73 and Tadmor. Ten concentrations (0, 10, 20, 40, 60, 80, 100, 120, 140, and 160 mM) of Na^+-dominant and Cl^--dominant Hoagland's solutions were prepared by dissolving a mixture of Na^+ salts (Na_2SO_4, Na_2HPO_4, and $NaNO_3$) or a mixture of Cl^- salts ($CaCl_2$, $MgCl_2$, and KCl) in Milli-Q water (conductivity $\geqslant$18.2 MΩ cm^{-1}). All treatment solutions had a background of modified Hoagland's solution for nutrient supply, the composition of which (in mM) was: NH_4NO_3 (0.2), KNO_3 (5), Ca (NO_3)$_2$ (2), $MgSO_4$ (2), KH_2PO_4 (0.1), NaFe(III) hydroxyethyl ethylenediamine triacetic acid (HEDTA) (0.05), H_3BO_3 (0.01), $MnCl_2$ (0.005), $ZnSO_4$ (0.005), $CuSO_4$ (0.0005), and Na_2MoO_3 (0.0001). Solutions were changed every 10 d and the pH of the solution was monitored several times each week and maintained within a range of 6.0–6.5. The experiment was conducted in an air-conditioned growth chamber with day/night temperatures of approximately 25/18 °C. The intensity of photosynthetically active radiation was measured using a Li-Cor quantum sensor (model LI-1000, Li-Cor, Lincoln, NE, USA) and varied from 550 to 600 mmol m^{-2} s^{-1}. At 10–12 d after germination, when the third leaf was beginning to appear, the Na^+ and Cl^- treatments were introduced in increments of 20 mM over 8 d. Supplementary Ca^{2+} (5 mM) as $CaCl_2$ was added to the Na^+ treatment to prevent Ca^{+2} deficiencies in plants (Genc *et al.*, 2010).

The growth system of the plants was modified from Casey *et al.* (2003) and consisted of a polypropylene mesh supported at the

bottom of a flanged tube (4 cm diameter×8 cm depth) which, in turn, formed the bottom of the tube. This assembly was inserted into a lid which rested on top of a PVC pot (10.4 cm diameter×32 cm depth) filled with nutrient solution. Each lid had a 0.5 cm drilled hole for a capillary air tube (see Supplementary Fig. S1 at *JXB* online). 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. Four seeds were placed on the mesh and, after the seedlings were established, they were thinned to one seedling. The tubes were then filled with cylindrical black polycarbonate pellets (approximately 2–4 mm long and 1–2 mm in diameter) to support the plant stem and prevent light penetration to the nutrient solution. At this stage the level of the solution was allowed to drop to about 1.5 cm below the mesh. The experimental design was a factorial, completely randomized design comprising 19 salt treatments (control and 9 rates Na^+/Cl^-)×two barley genotypes with three replicates.

Experiment 2. The separate effects of Na^+ and Cl^- ions at high concentration on barley growth using solution culture: A solution culture experiment was conducted to assess the relative importance of Na^+ and Cl^- ions to salt toxicity. The varieties, Barque73, Clipper, Sahara, and Tadmor were used because of their different sensitivities to salt stress and abilities to exclude Na^+ and Cl^- ions. A factorial experiment consisted of six different treatments: control, 120 mM NaCl, 120 mM NaCl+0.07 mM 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), 120 mM Na^+-gluconate, 120 mM Cl^--dominant, and 120 mM Na^+-dominant Hoagland's solutions. The effect of Na^+ independently of Cl^- was examined in three ways: by using DIDS, which is a non-permeating amino acid that inhibits Cl^- transport (Lin, 1981; Lin and Kao, 2001); by using Na^+-gluconate because gluconate is an anion that is unable to permeate the cell membrane; and by using the Na^+-Hoagland solution. The solutions were designed to have similar EC and osmotic potentials (Table 1). The growth conditions were identical to those of Experiment 1. The experimental design was a factorial completely randomized design comprising six treatments×four barley genotypes with three replicates.

Experiment 3. The separate effects of Na^+ and Cl^- ions at high concentration on barley growth using soil culture: An experiment was conducted using field soil to assess the effect of high concentrations of Na^+ and Cl^- ions on the growth of Barque73, Clipper, Sahara, and Tadmor. The A horizon (topsoil) soil of a sandy loam red Chromosol (Isbell, 1996) collected from Rose-worthy (34.51 °S, 138.68 °E, South Australia) was used. 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.

The experiment was designed to compare growth in saline soils at similar soil electrical conductivities (EC) and soil osmotic potential (Ψ_O) but different ionic compositions. To achieve this, it compared the effects of 120 mmol kg^{-1} Na^+ (applied as a range of sulphate, nitrate, and phosphate salts), 120 mmol kg^{-1} Cl^- (applied as a range of calcium, magnesium, and potassium salts), and 120 mmol kg^{-1} NaCl at a similar EC in a soil-based system (Table 2). The Cl^-, Na^+, and NaCl-dominant saline soils were prepared by dissolving a mixture of Na^+ salts (20 mM Na_2SO_4, 20 mM Na_2HPO_4, and 40 mM $NaNO_3$), a mixture of Cl^- salts (20 mM $CaCl_2$, 20 mM $MgCl_2$, and 40 mM KCl), and NaCl salt (120 mM NaCl), respectively, in milliQ H_2O and spraying each solution on a 2 cm layer of soil to reach field capacity moisture content. Each soil was covered with plastic to control evaporation and left for 5 d at 25 °C to reach equilibrium, then mixed thoroughly and air-dried (Tavakkoli *et al.*, 2010a). Basal fertilizer was thoroughly mixed with the soil at the following concentrations (in mg pot^{-1}): 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). All soils with basal fertilizer were wetted to field capacity with deionized water and allowed to equilibrate for 4 d at 25 °C. Samples of the four synthesized soils 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 passed through 0.25 μm filter paper. Electrical conductivity, Ψ_O and ion concentrations of the solutions were measured (Table 2).

The plants were grown in pots, 10.4 cm in diameter and 32 cm deep in which there were two layers of soil; 2200 g of air-dried soil (subsoil) which contained the salt treatment with 800 g of untreated soil above (topsoil). Each layer was packed to a bulk density of 1.35 Mg m^{-3}. The subsoil and topsoil were separated by a 3 cm layer of plastic beads (120 g) to prevent salt rising to the topsoil through capillarity action. The top 3 cm of the pot was also covered by plastic beads to minimize the water evaporation from the soil surface. 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. Four barley seeds were sown in each pot and thinned to one per pot after 5 d. The experiment was conducted under the same growth conditions as described in Experiment 1. The pots were weighed and watered to field capacity regularly and daily water use calculated. The experimental design was a factorial, completely randomized design comprising four treatments×four barley genotypes with three replicates, giving a total of 48 pots.

Measurements

Experiment 1: Plants were harvested after 49 d, the fresh and dry weights of the shoots were recorded, and the whole shoot moisture content was calculated. 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). The concentration of Na^+ and K^+ in the digested samples was determined using a flame photometer (Model 420, Sherwood, Cambridge, UK). Chloride concentrations of the digested extracts were determined using

Table 1. The electrical conductivity and osmotic potential of hydroponic solutions used in Experiment 2

Treatment	Solution EC (dS m^{-1})	Osmotic potential (MPa)
Control	1.5	–0.05
NaCl	11.5	–0.85
NaCl+DIDS	11.4	–0.84
Na^+-gluconate	9.8	–0.84
Na^+-dominant Hoagland	11.4	–0.84
Cl^--dominant Hoagland	11.8	–0.86

Table 2. Osmotic potential (MPa), pH, electrical conductivity (dS m^{-1}) and ionic concentration (mM) of the treatment soil solutions extracted at field capacity in Experiment 3.

Values are means ($n=2$).

	Ψ_o	pH	EC_{FC}	Ca^{2+}	Mg^+	Na^+	K^+	P	S	Cl^-
Control	–0.01	7.8	1.2	9.0	2.7	1.9	1.9	0.12	4.2	1.9
Na^+-soil	–0.78	7.6	11.1	19.0	10.9	112.9	5.7	0.48	23.2	1.3
Cl^--soil	–0.73	7.7	11.0	33.6	18.6	2.4	41.2	0.12	4.0	125.0
NaCl-soil	–0.79	7.9	11.2	18.7	11.1	115.1	6.5	0.11	3.8	120.4

a chloride analyser (Model 926, Sherwood Scientific, Cambridge, UK). Recovery of Na^+, Cl^-, and K^+ from plant standards (Australasian Soil and Plant Analysis Council) were 94, 98, and 93%, respectively.

Experiments 2 and 3: Leaf chlorophyll content was measured using a hand-held SPAD 502 meter (Minolta, Osaka, Japan) in Experiment 3. Average SPAD chlorophyll readings were calculated from four measurements from the leaf tip to the leaf base of the fourth and the fifth leaf. An infrared, open gas exchange system LI-6400 (Li-Cor, Inc., Linclon, 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 made on the fifth leaf, 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), transpiration (T), 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 operating quantum efficiency of PSII photochemistry, ΦPSII, was calculated as $(F'm - F')/F'm$. The efficiency of open PSII reaction centres $(F'v/F'm)$ was calculated as $(F'm - F'o)/F'm$ and the photochemical quenching (qP) factor was determined as $(F'm - F')/(F'm - F'o)$. The non-photochemical quenching (NPQ) was expressed as $Fm/F'm - 1$ where $F'm$ is the maximal fluorescence during a saturating flash of light >7 mmol m^{-2} s^{-1}, and $F'o$ is the minimal fluorescence of a momentarily darkened leaf.

To measure the osmotic potential (Ψ_s) 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 stored until measurement. The frozen leaf was thawed to ambient temperature, 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).

Water use was determined as the difference between the water added to the pots to replace that lost and that retained in the plant (due to growth). Transpiration efficiency ($TE=A/T$) was determined by measurements of A and T on single leaves and water use efficiency (WUE) was determined by gravimetric determinations of growth and water loss on whole plants. Plants were harvested 49 d after sowing, 1 d after watering to field capacity. Fresh weight was measured before plants were dried at 70 °C for 72 h and dry weights were recorded. Shoot moisture content was estimated from the difference between fresh weight and dry weight. The nutrient concentration of the whole shoot was measured after digestion in nitric acid/hydrogen peroxide in a closed vessel (50 ml poly-propylene centrifuge tube) using a hot block digestion system. Elemental analyses of plant samples were performed using in-ductively coupled plasma-optical emission spectrometry (Zarcinas *et al.*, 1987). Chloride concentrations of the digested extracts were determined using a chloride analyser (Model 926, Sherwood Scientific, Cambridge, UK).

Statistical analysis

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 ($LSD_{0.05}$). Relationships between individual variables were exam-ined using simple linear correlations and regressions which were performed using SigmaPlot (version 10).

Results

Experiment 1. The effect of different Na^+ and Cl^- ion concentrations on plant biomass

The increase in shoot Na^+ concentration as a function of external Na^+ concentration followed a curvilinear pattern in both genotypes (Fig. 1a, b). In Na^+-dominant Hoagland's solution, maximum growth and production of shoot dry weight was attained at a Na^+ tissue concentration of 300–400 mmol kg^{-1} DW in both genotypes (Fig. 1c, d). The Cl^- tissue concentration of Tadmor followed a very similar pattern to its Na^+ uptake and accumulated to similar concentrations, whereas in Barque73 the Cl^- concentration increased linearly with an increase in the Cl^- concentration of the external solution and was always less than the Na^+ concentrations. In Cl^--dominant Hoagland's solutions, the growth of Barque73 reached maximum at a whole shoot concentration of 165–285 mmol kg^{-1} DW beyond which growth declined (Fig. 1c). In Tadmor, there was an increase in shoot dry weight up to tissue Cl^- concentration of 195–280 mmol kg^{-1} DW, and growth declined sharply beyond this concentration (Fig. 1d). Barque73 accumulated up to 35% less shoot Cl^- concentration compared with Tadmor. At the highest external Cl^- concentration, dry weight of Barque73 was reduced by 35% with a tissue Cl^- concentra-tion of 530 mmol kg^{-1} DW, whereas the dry weight of Tadmor was reduced by 61% with a tissue Cl^- concentration of 865 mmol kg^{-1} DW (Fig. 1c, d).

Experiment 2. The separate effects of Na^+ and Cl^- ions at high concentration on barley growth in solution culture

Whole shoot ion concentration and leaf osmotic potential: For plants grown in the control treatment, tissue Na^+ concentration was 4–10 mmol kg^{-1} DW and no difference was apparent among the four genotypes. The Na^+ concen-tration in all varieties increased with increasing concentra-tion of Na^+ in treatment solutions (Table 3). Sahara had the highest Na^+ concentration of all the genotypes ($\sim$1040 mmol kg^{-1} DW). The Na^+ concentration in shoots of Clipper was about 5% less than Sahara, while Barque73 and Tadmor had the lowest Na^+ concentration ($\sim$720 mmol kg^{-1} DW) (Table 3) which is consistent with previous hydroponic screening of these varieties (E Tavakkoli, un-published data). Potassium concentrations were not signif-icantly affected by NaCl and Na^+ treatments and did not differ significantly among the four varieties but were about 10% higher in Cl^--dominant Hoagland's solutions due to using KCl to prepare this salt solution. The K^+:Na^+ ratio never fell below 1.1, which is the ratio considered critical for
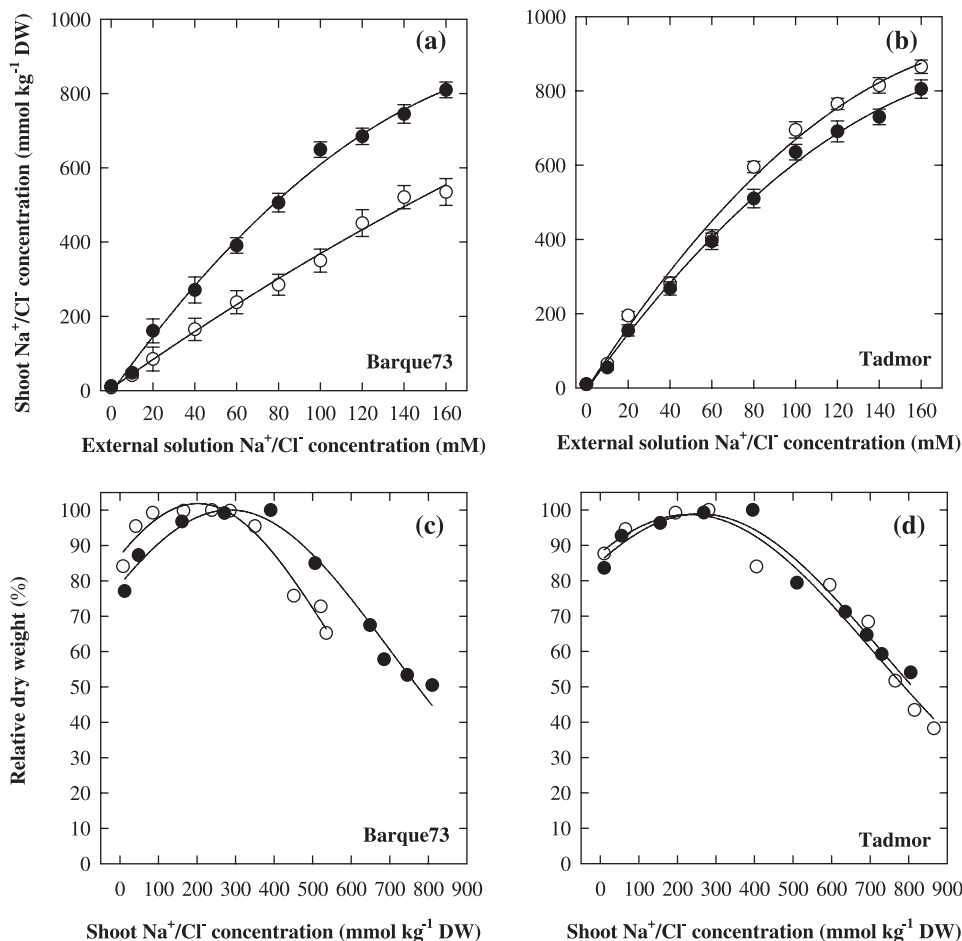
Fig. 1. The whole shoot concentration of Na$^+$ (black circles) and Cl$^-$ (open circles) of Barque73 and Tadmor as a function of the external concentration of Na$^+$ and Cl$^-$ in hydroponic solution (a, b) and the relationship between relative shoot dry matter (%) and shoot Na$^+$ and Cl$^-$ concentration. The dry matter weight of Barque73 and Tadmor in the control treatment was 4.45 g and 4.59 g, respectively. Bars indicate the standard error of the means. Fitted curves for (a) and (b) are derived from linear or exponential regressions, while the curves in (c) and (d) are the best fit of gaussian equation (three parameters). Values are means (n=3).

maintaining metabolic activity (Munns, 1985). Chloride concentrations in the shoot tissue of all genotypes were 10–15 mmol kg^{-1} DW in the control and 2 mmol kg^{-1} DW in the Na$^+$-dominant Hoagland's solutions and the Na$^+$-gluconate treatments. The concentration increased significantly when grown under Cl$^-$-dominant Hoagland's solutions and NaCl treatments, but the responses of genotypes differed significantly (Genotype×Treatment interaction, P <0.001). Consistent with our previous results, Barque73 showed greater Cl$^-$ exclusion (relative to Na$^+$) whereas the shoot Cl$^-$ concentration of the other three genotypes increased to levels similar to their shoot Na$^+$ concentration. Treatments with NaCl in the presence of DIDS resulted in about 75–83% reduction in shoot Cl$^-$ concentrations, but did not affect Na$^+$ concentrations (Table 3).

Plant biomass and water relations: The four genotypes did not differ significantly in shoot dry weight when grown in non-saline solution for 49 d. Increased levels of salts in the solution reduced shoot dry weights of all the varieties significantly but the responses of varieties differed (Fig. 2). In Barque73 the shoot dry weight was reduced by 24% in

Na$^+$ (NaCl+DIDS, Na$^+$-gluconate, and Na$^+$-Hoagland) and 10% in Cl$^-$ treatments (Cl$^-$-Hoagland), respectively, however, the reduction due to NaCl was about 34%. The dry weight of Clipper was reduced by 18%, 23%, and 47% in Na$^+$, Cl$^-$, and NaCl treatments, respectively. Sahara had a similar salt tolerance to Barque73 in the NaCl treatment but the growth reduction in Na$^+$ treatments was 10%, whereas it was reduced by 23% in the Cl$^-$ treatment. Tadmor had the lowest shoot dry weight under NaCl salinity compared with the other three (43%). It showed similar shoot dry weight under high Na$^+$ concentration as Barque73, however, the growth reduction under Cl$^-$ treatments was 40% indicating a significant effect of Cl$^-$ to shoot dry weight depression. Adding DIDS to NaCl increased the shoot dry weight of plants by 10%, 45%, 37%, and 62% in Barque73, Clipper, Sahara, and Tadmor, respectively, compared with NaCl in the absence of DIDS (Fig. 2). The pattern of growth of four genotypes in Na$^+$-gluconate was very similar to that obtained in Na$^+$-dominant Hoagland's solution. Leaf osmotic potential also decreased 2-fold in the saline treatments compared to control (Table 3). The reduction in Ψ_s was similar in Na$^+$,

Table 3. The leaf osmotic potential (MPa) and whole plant shoot concentrations of Na^+, K^+, and Cl^- (mmol kg^{-1} DW) of four genotypes of barley grown on solutions of NaCl (120 mM) in the presence and absence of DIDS (0.07 mM), Na^+-gluconate (120 mM), Na^+-Hoagland (120 mM), and Cl^--Hoagland (120 mM) for 49 d

Values are means (n=3).

	Leaf Ψ_s	Na^+	K^+	Cl^-
Barque73				
Control	−1.04	4	1133	10
NaCl	−2.46	780	1130	495
NaCl+DIDS	−2.40	714	1120	128
Na^+-gluconate	−2.34	710	1133	2
Na^+-Hoagland	−2.31	722	1140	2
Cl^--Hoagland	−2.35	5	1242	503
Clipper				
Control	−1.06	4	1145	12
NaCl	−2.55	974	1128	1139
NaCl+DIDS	−2.52	985	1139	155
Na^+-gluconate	−2.45	979	1122	2
Na^+-Hoagland	−2.47	971	1133	2
Cl^--Hoagland	−2.46	2	1253	1011
Sahara				
Control	−1.03	10	1132	15
NaCl	−2.11	1040	1123	1299
NaCl+DIDS	−2.10	1042	1121	209
Na^+-gluconate	−2.04	1039	1123	2
Na^+-Hoagland	−2.09	1051	1129	2
Cl^--Hoagland	−2.04	8	1267	1066
Tadmor				
Control	−1.06	5	1148	11
NaCl	−2.54	791	1122	759
NaCl+DIDS	−2.45	727	1131	225
Na^+-gluconate	−2.49	715	1149	3
Na^+-Hoagland	−2.50	730	1133	2
Cl^--Hoagland	−2.55	4	1263	751
$LSD_{0.05}$ (Genotype×Treatment)	0.07	15.7	24.3	10.2

Cl^-, and NaCl treatments. Sahara had significantly higher Ψ_s compared with the other three genotypes.

Experiment 3. The effects of Na^+ and Cl^- ions at high concentration on barley growth in saline soil

Soil properties: 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^- (Table 2). 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 (Tables 2, 4) and all were within the physiologically normal range for barley (Reuter and Robinson, 1997). While the effects of this variation in the other cations and anions on the growth of the plants in the different salt treatment can not be discounted completely, the much greater range in the final concentrations of Na^+ and Cl^- in the plants means that the responses to salt

that were observed in barley were largely due to the effects of Na^+, Cl^-, and NaCl.

Whole shoot ion concentration: For plants in the control treatments, the mean tissue Na^+ concentration was 88 mmol kg^{-1} DW and there was no significant difference among the four genotypes (Table 4). As soil Na^+ concentration increased, so too did tissue Na^+ concentration in all genotypes. Large genotypic variation was observed in shoot Na^+ concentration: Clipper had the lowest Na^+ concentration (450 mmol kg^{-1}) whereas Sahara had the highest (750 mmol kg^{-1}), and Tadmor and Barque73 were intermediate with shoot Na^+ concentration of about 685 mmol kg^{-1} DW. The concentration of shoot Cl^- increased in Cl^- and NaCl soils, and the Cl^- concentrations were consistently greater than the tissue Na^+ concentrations in control and NaCl treatments with the exception of Barque73 grown under NaCl. The responses of the four genotypes differed significantly (Genotype×Treatment interaction, P <0.001) (Table 4). The Cl^- concentrations in Tadmor and Sahara were over 2–3 times greater than Barque73 and Clipper. Potassium concentration of all genotypes decreased significantly when exposed to Na^+ or NaCl. Sahara consistently maintained a higher concentration of K^+ and a much higher K^+:Na^+ ratio (>1.1), whereas this ratio fell below 1.1 for the other varieties, with Clipper having the lowest ratio (Table 4). This negative effect of Na^+ on K^+ was indicated between treatments within each genotype, however, among the genotypes there is a positive relationship between Na^+ and K^+. In other words, genotypes that exclude Na^+ also seem to have lower K^+, and the competitive relationship operates within a genotype but not between genotypes. Changes in calcium and magnesium tissue concentration followed a very similar pattern as K^+, and it decreased in all genotypes when grown in Na^+ and NaCl-dominant soils.

Plant biomass and water use: Increases in Na^+, Cl^- or NaCl concentration of the soil solution significantly (P <0.05) reduced shoot dry weight in all genotypes but the genotypic responses were different (Table 4). The shoot dry matter of Barque73, Tadmor, and Clipper was reduced by up to 20–25% in the Na^+-dominant soil, whereas the reduction in Sahara was only 11%. The biomass reduction under Cl^--dominant soil was 10%, 30%, 22%, and 45% in Barque73, Clipper, Sahara, and Tadmor, respectively. The biomass reductions due to NaCl salinity reflected the additive effects of Na and Cl: they ranged from 30% in Barque73 and Sahara to 45% and 65% in Clipper and Tadmor, respectively.

Daily water use increased consistently throughout the experiment in the control treatments of all four genotypes (Fig. 3). Salinity reduced daily water use with the largest reduction occurring in NaCl treatments. The greater sensitivity of Tadmor to salt is evident as is the greater effect of Cl^- on water use. Similar to shoot dry weights (Table 4), the reduction in total water use due to the presence of NaCl was greater than that due to Na^+ and Cl^- individually, and was equal to the additive effect of the two.
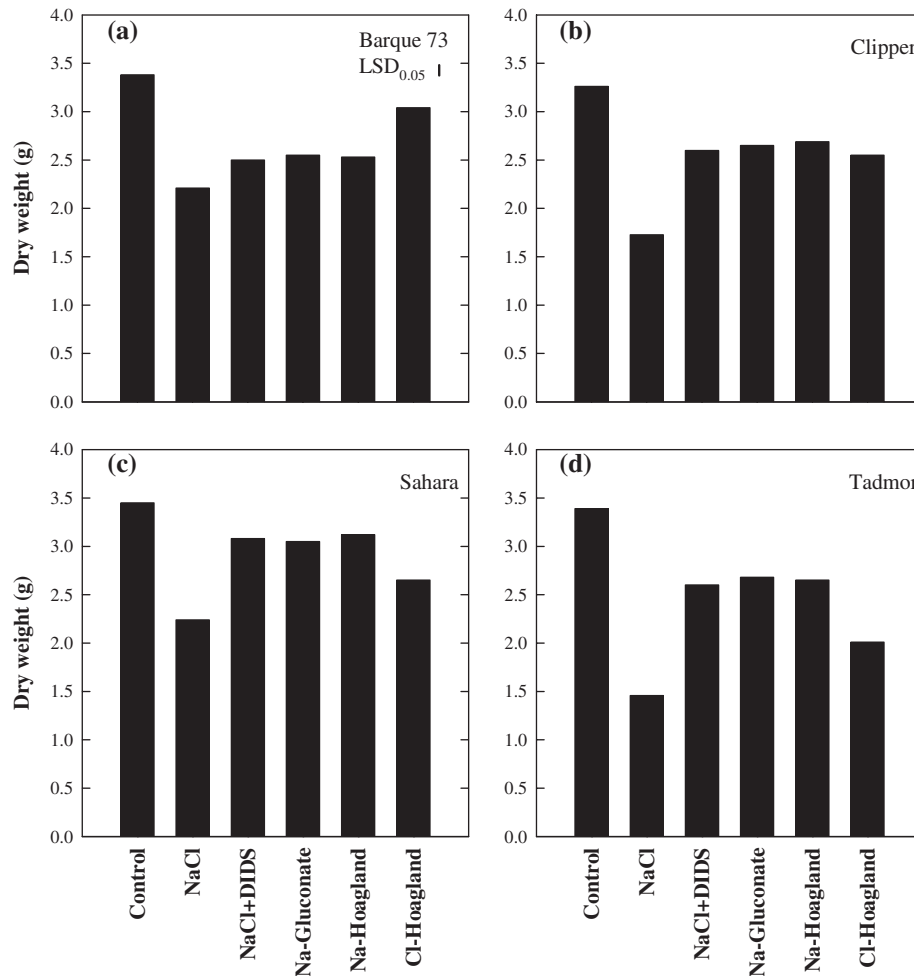
Fig. 2. Effects of NaCl (120 mM) in the presence and absence of DIDS (0.07 mM), Na^+-gluconate (120 mM), Na^+-dominated Hoagland (120 mM), and Cl^--dominated Hoagland (120 mM) on shoot dry weight of (a) Barque73, (b) Clipper, (c) Sahara, and (d) Tadmor grown for 49 d. Values are averages (n=3). $LSD_{0.05}$=0.089 and CV=2.1%.

The cumulative water use at the final measurement can be used to quantify the relative effects of Na^+, Cl^-, and NaCl to total plant water uptake and, consequently, growth. Total water use in Barque73 in the Na^+ and Cl^- and NaCl treatments was reduced by 21%, 10%, and 30%, respectively, whereas in Tadmor the reductions were 20%, 45%, and 60%, respectively (Fig. 3b, d). The cumulative water use of Clipper was reduced by 22% and 25% by Na^+ and Cl^- treatments, respectively, and a 45% reduction occurred under NaCl. Sahara was more sensitive to Cl^- than Na^+ as indicated by a 20% reduction in cumulative water use under Cl^- treatment compared with a 10% reduction under Na^+ treatment (Fig. 3f). Leaf osmotic potential also decreased in the salt treatments compared with the control. Sahara and Barque73 had inherently higher Ψ_s than Tadmor and Clipper in the control (Table 4). Sahara maintained a significantly higher Ψ_s than the other varieties under salt stress.

Chlorophyll content, gas exchange, and chlorophyll fluorescence parameters: The leaf chlorophyll content of all genotypes was increased in Na^+-dominant soil (15–19%. whereas it was reduced by between 20% and 36% in Clipper,

Sahara, and Tadmor in Cl^--dominant and NaCl-dominant soils and remained unchanged in Barque73 (Table 5). The gas exchange parameters of all genotypes decreased in all salt treatments. The rate of photosynthesis (A) of plants grown in Na^+-dominant soil decreased by 20–25% in Barque73, Clipper, and Tadmor, and by 10% in Sahara (Table 5). In Cl^--dominant soil A was reduced by 40% in Tadmor, 23% in Clipper and Sahara, and 10% in Barque73. The greatest reduction in A occurred in the NaCl treatment and followed a similar pattern to shoot dry weight and cumulative plant water use. Clipper and Tadmor had significantly lower A than Barque73 and Sahara in the Na^+ and NaCl treatments. Stomatal conductance (g_s) of all genotypes followed a similar pattern of reduction to A. The relative intercellular CO_2 concentration (C_i/C_a) of all genotypes decreased by 2.5–4% when grown in the Na^+ treatment. This value decreased by only 6% in Barque73 under Cl^- treatment, but there was a larger reduction of C_i/C_a in Tadmor (40%), Sahara (22%), and Clipper (16%). The changes of C_i/C_a under NaCl salinity was similar or just slightly less than Cl^- treatment which indicates the reduction of C_i/C_a under salinity was driven by Cl^- concentration (Table 5).

Table 4. The shoot dry matter (g), water use efficiency (WUE, g l^{-1}), leaf osmotic potential (MPa), and whole plant shoot concentration of Ca^{2+}, Mg^{2+}, Na$^+$, K$^+$, P, S, and Cl$^-$ (mmol kg^{-1}DW) of four genotypes of barley grown on soils treated with Na$^+$, Cl$^-$, and NaCl salts for 49 d. Values are means (*n*=3).

	Dry weight	WUE	Leaf Ψs	Ca^{2+}	Mg^{2+}	Na$^+$	K$^+$	K$^+$/Na$^+$	P	S	Cl$^-$
Barque73											
Control	3.805	1.46	−0.25	294	189	85	980	11.5	189	58	165
Na$^+$-soil	3.010	1.47	−0.75	144	155	688	648	0.94	210	95	162
Cl$^-$-soil	3.415	1.46	−0.72	335	214	95	1189	12.5	185	52	359
NaCl-soil	2.645	1.45	−0.77	156	160	685	650	0.95	150	51	367
Clipper											
Control	3.603	1.38	−0.30	285	186	90	915	10.1	193	58	146
Na$^+$-soil	2.755	1.35	−0.87	148	125	450	373	0.82	209	91	148
Cl$^-$-soil	2.555	1.33	−0.89	364	211	106	1184	11.1	196	51	411
NaCl-soil	1.975	1.37	−0.91	162	120	457	355	0.77	211	58	487
Sahara											
Control	3.504	1.33	−0.21	304	191	90	995	11.0	201	58	167
Na$^+$-soil	3.111	1.33	−0.59	253	160	750	881	1.1	220	89	153
Cl$^-$-soil	2.715	1.29	−0.57	397	215	110	1385	12.5	208	51	1100
NaCl-soil	2.338	1.29	−0.60	279	167	757	885	1.1	196	53	1112
Tadmor											
Control	3.903	1.46	−0.31	279	187	84	955	11.3	190	57	141
Na$^+$-soil	3.115	1.45	−0.84	149	112	670	657	0.98	208	79	153
Cl$^-$-soil	2.125	1.44	−0.80	351	208	102	1188	11.6	193	50	980
NaCl-soil	1.333	1.27	−0.85	132	116	675	651	0.96	150	52	999
LSD$_{0.05}$(Genotype×Treatment)	0.167	n.s	0.022	13.8	7.1	27.1	29.1	0.58	7.2	5.5	15.5

The barley varieties used in this study showed some significant differences in the instantaneous rates of net transpiration (*T*) by single leaves which followed a similar pattern to changes in the *A*. The transpiration efficiencies (*TE*) generated from these data on individual leaves therefore varied little (Table 5): the difference between the maximum and minimum values of the *TE* for the different cultivars was about 15% of the maximum (*P* <0.05). There was a significant increase in *TE* under Na$^+$ treatments in all genotypes and under Cl$^-$ only in Tadmor, however, the *TE* of Barque73 and Clipper was not affected by NaCl treatment while it increased in Sahara and Tadmor (*P* <0.05). The varieties also differed significantly in their water-use efficiencies (WUE) when these were estimated for whole plants (Table 4). The WUE was correlated with the growth among the four varieties, however, Na$^+$, Cl$^-$, and NaCl treatments did not significantly affect WUE.

The Cl$^-$ and NaCl salts had a marked effect on the four key fluorescence parameters measured but the effects varied with genotypes (Table 6). The presence of Cl$^-$ had a larger effect than Na$^+$ in all varieties. Values for the efficiency of light harvesting of PSII (*F*′v/*F*′m) of Clipper and Sahara were reduced by 20–25% in the Cl$^-$ treatment and by 40% in Tadmor; the reduction in Barque73 was smaller, but still significant (*P* <0.05). A similar pattern was observed for q*P* and ΦPSII under both treatments. Significant relationships between *A* and both (*F*′v/*F*′m) (*r*=0.89) and ΦPSII (*r*=0.92) were found for Clipper, Sahara, and Tadmor, indicating that these parameters may not be independent or are co-regulated (Tables 5, 6). As the proportion of PSII

reaction centres that remained open (q*P*) was reduced in Clipper, Sahara, and Tadmor, the portion of fluorescence quenching associated with thermal energy dissipation (NPQ) increased significantly, but the effect was larger in Tadmor. These values changed similarly for Barque73 under Cl$^-$ and NaCl treatments, but the changes are smaller in magnitude (Table 6).

Discussion

The work reported here is one of the few studies to have attempted to separate the toxic effects of Na$^+$ and Cl$^-$ and their individual and combined contributions to salt tolerance at the whole plant level. Previous studies comparing the effects of Na$^+$ and Cl$^-$ in cereals grown under salinity stress have mainly used short-term hydroponic experiments with a single salt of Na$^+$ or Cl$^-$ and one or two genotypes (Kingsbury and Epstein, 1986; Lin and Kao, 2001; Tsai *et al.*, 2004; Luo *et al.*, 2005). This has produced only equivocal results because of the difficulty in changing the external concentration of one ion versus another without changing the osmotic pressure of the external solution (Munns and Tester, 2008) or the rate of uptake and activity of other ions (Cramer *et al.*, 1986). Also, given the fact that the most convincing approach to test the toxicity of Na$^+$ versus Cl$^-$ is by comparing genotypes differing in their salt tolerance and ion uptake (Munns and Tester, 2008; Teakle and Tyerman, 2010), there is a need to integrate the use of suitable genetic material and treatments with a mixture of
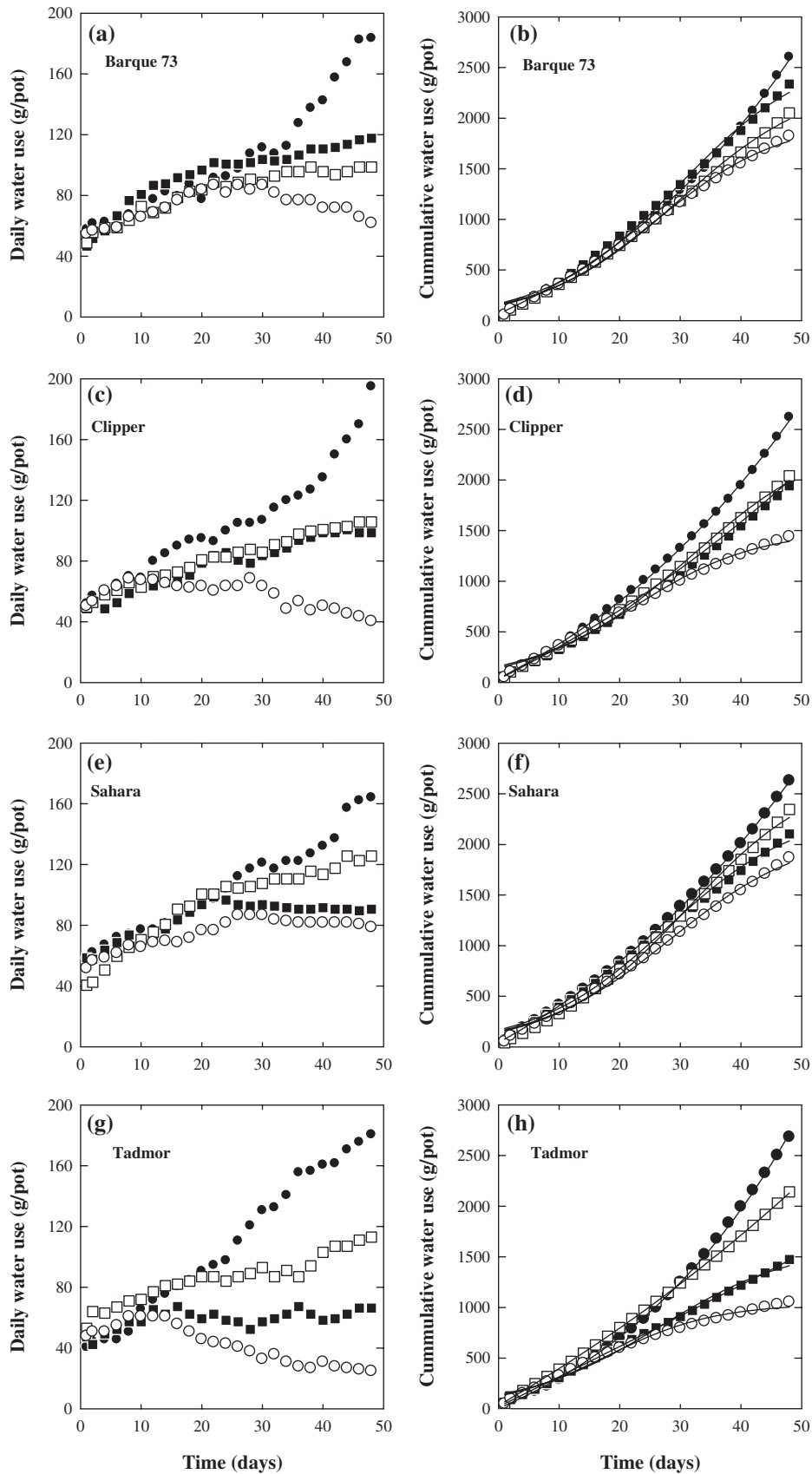
Fig. 3. The daily changes of water use (a, c, e, g) and cumulative water use (b, d, f, h) of four genotypes of barley under different levels of soil salinity generated from Cl⁻-salts (black squares), Na⁺-salts (open squares) or NaCl-salt (open circles) compared with the control (black circles) treatments. A sigmoidal curve fitted to the data of cumulative water use. Values are averages (n=3).

Table 5. Changes in leaf chlorophyll content (SPAD unit), CO_2 assimilation rate (*A*, $\mu mol\ CO_2\ m^{-2}\ s^{-1}$), stomatal conductance (g_s, $mol\ m^{-2}\ s^{-1}$), partial pressures of CO_2 in inside of leaf and in the air ($C_i:C_a$), transpiration (*T*, $mmol\ m^{-2}\ s^{-1}$), and transpiration efficiency (*TE*, $\mu mol\ CO_2\ mmol^{-1}\ H_2O$) of four varieties of barley grown in control conditions or Cl^--dominant soil, Na^+-dominant soil, and NaCl-dominant soil. Plants were grown for 49 d and values are from the last measurement at day 45. Values are means (*n*=3).

	Chlorophyll content	A	g_s	$C_i:C_a$	T	TE
Barque73						
Control	39.0	21.78	0.83	0.78	4.86	4.48
Na^+-soil	44.1	17.40	0.61	0.75	3.45	5.04
Cl^--soil	38.5	19.55	0.75	0.76	4.15	4.71
NaCl-soil	38.0	14.40	0.53	0.70	3.22	4.47
Clipper						
Control	38.7	20.24	0.80	0.74	4.51	4.49
Na^+-soil	46.1	16.25	0.60	0.71	3.35	4.85
Cl^--soil	30.5	15.51	0.61	0.58	3.46	4.48
NaCl-soil	33.7	11.14	0.41	0.55	2.60	4.28
Sahara						
Control	41.0	21.23	0.85	0.77	4.73	4.49
Na^+-soil	46.0	18.91	0.76	0.75	3.95	4.79
Cl^--soil	32.5	16.29	0.65	0.64	3.61	4.51
$NaCl^-$soil	35.3	14.62	0.56	0.65	2.89	5.06
Tadmor						
Control	39.3	20.70	0.83	0.74	4.62	4.48
Na^+-soil	44.7	15.87	0.58	0.71	3.06	5.18
Cl^--soil	25.7	12.19	0.47	0.44	2.50	4.88
NaCl-soil	27.3	8.18	0.31	0.45	1.73	4.74
$LSD_{0.05}$ (Genotype×Treatment)	2.36	0.800	0.045	0.040	0.095	0.255

the Na^+ and Cl^- salts to improve our understanding in this area. In this study, the Na^+-dominant and Cl^--dominant Hoagland's solutions were designed to give equimolar concentrations of the Na^+ and Cl^- ions generated from various salts of Na^+ and Cl^- to avoid increasing particular counter-anions/counter-cations. Using barley varieties with known genetic variation in salinity tolerance and in Na^+ and Cl^- uptake also assisted in distinguishing the toxic effects of Na^+ from Cl^-. As the responses to salinity in hydroponics and soil may also be fundamentally different (Tavakkoli *et al.*, 2010*b*), a soil-based experiment was designed to simulate the responses in field-grown plants. The method employed here maintained a constant EC and Ψ_O, but used a combination of different salts to produce soils enhanced with Na^+, Cl^-, and NaCl. It is essential to maintain the same Ψ_O over the treatments because changes in soil salt composition also affects soil Ψ_O and therefore plant growth. Comparisons of the effects of Na^+ and Cl^- on growth were possible due to the use of soils with similar EC and Ψ_O but different concentrations and combinations of Na^+ and Cl^-. Despite the utility of this approach, 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 2). These concentration differences may potentially affect the responses since the Ca^{2+} and K^+ availability can change with salinity responses (Genc *et al.*, 2010). However, the variation in concentrations of these ions in the plant tissue was considerably less that that measured in the soil solution (Table 4). As a result, the

buffering capacity of the soil may have, in part, affected the activity of the ions in solution and may have reduced their potential interactions with Na^+ and Cl^- ions on salt tolerance (Khasawneh and Copeland, 1973).

Growing barley under NaCl stress reduced the growth of barley in hydroponics and in soil with significant differences among genotypes in the sensitivity to salt stress. However, in both growth media, the reduction in growth was caused by the additive effect of the reductions due to Na^+ and Cl^-. Moreover, the responses to Na^+ and Cl^- stress were independent and so similar levels of tolerance to NaCl could be achieved by different combinations of responses to Na^+ and Cl^-. This result is clearly at odds with previous studies that have dismissed Cl^- toxicity as a contributing factor to salt damage (Kingsbury and Epstein, 1986; Kinraide, 1999; Lin and Kao, 2001; Tsai *et al.*, 2004). However, it is important to consider that these previous experiments either used sole counter-anions such as 121 mM nitrate (Kingsbury and Epstein, 1986), which at high concentrations can be phytotoxic (Chen *et al.*, 2004), or were short-term studies lasting less than a week (Kinraide, 1999). The two-phase model of salt injury (Munns *et al.*, 1995) suggests that specific ionic toxicity builds up over a longer period. Thus, a relationship which may arise under short-term studies may not necessarily be valid for long-term responses. Moreover, in the study by Lin and Kao (2001) where 50 mM Na^+-gluconate was compared with 50, 100, and 150 mM NaCl, the interpretation of the effects of Na^+ and Cl^- was confounded by differences in the EC of

Table 6. Changes in fluorescence parameters: efficiency of light harvesting $(F'v/F'm)$, actual quantum yield of PSII electron transport (ΦPSII), photochemical quenching (qP) and non-photochemical quenching (NPQ) of intact leaves of four varieties of barley grown in control conditions or Cl⁻-dominant soil, Na⁺-dominant soil, and NaCl-dominant soil. Plants were grown for 49 d and values are from the last measurement at day 45. Values are means (n=3).

	$(F'v/F'm)$	ΦPSII	qP	NPQ
Barque73				
Control	0.75	0.56	0.72	0.32
Na⁺-soil	0.73	0.55	0.70	0.33
Cl⁻-soil	0.70	0.50	0.65	0.36
NaCl-soil	0.71	0.51	0.62	0.35
Clipper				
Control	0.76	0.56	0.69	0.32
Na⁺-soil	0.74	0.51	0.68	0.34
Cl⁻-soil	0.60	0.42	0.52	0.38
NaCl-soil	0.59	0.41	0.50	0.38
Sahara				
Control	0.75	0.56	0.74	0.33
Na⁺-soil	0.73	0.54	0.75	0.37
Cl⁻-soil	0.57	0.41	0.58	0.39
NaCl-soil	0.56	0.40	0.55	0.40
Tadmor				
Control	0.76	0.55	0.73	0.32
Na⁺-soil	0.72	0.53	0.74	0.33
Cl⁻-soil	0.41	0.35	0.45	0.52
NaCl-soil	0.40	0.33	0.46	0.58
LSD$_{0.05}$ (Genotype×Treatment)	0.057	0.037	0.044	0.022

the solutions as well as the ion concentrations. The contribution of Cl⁻ toxicity to the effects of salt stress observed in the current experiments lends support to the arguments that high soil Cl⁻ can be an important limitation to the growth and yield of crops (Dang *et al.*, 2008, 2010).

The concentrations of Na⁺ and Cl⁻ increased in barley plants exposed to salt (Fig. 1; Tables 3, 4), with tissue Cl⁻ concentrations of soil-grown plants generally exceeding those of Na⁺ (Table 4). Increases in plant ion concentrations occur with all plants exposed to NaCl (Flowers *et al.*, 2010) but there are few, if any, reliable estimates of the critical concentrations for Na⁺ and Cl⁻ toxicity (Boursier *et al.*, 1987; Reuter and Robinson, 1997). When grown under Na⁺-dominant Hoagland's solutions, the dry weight of both genotypes started to decline at a tissue Na⁺ concentration of >400 mmol kg⁻¹ DW, however, Barque73 and Tadmor differed in their response to Cl⁻ treatments. The tissue Cl⁻ concentration of Barque73 increased up to 60 mM external Cl⁻ concentration, after which plant growth declined, which corresponded to concentrations above 250–300 mmol Cl⁻. This is similar to the pattern of salt response that was proposed by Maas and Hoffman (1977). On the other hand, a greater decline in the growth of Tadmor with higher tissue Cl⁻ concentration compared to Barque73 strongly suggests that the poor ability of Tadmor to exclude the Cl⁻ from shoots reduces its growth. Although a few studies in the past have shown the importance of partition-

ing of Cl⁻ in the leaf sheath (Boursier *et al.*, 1987; Boursier and Läuchli, 1989) and maintenance of low Cl⁻ concentrations in mesophyll cells of leaf blades of barley seedlings under salt stress (Huang and Van Steveninck, 1989), to our knowledge, this is the first report on a significant contribution of Cl⁻ exclusion to the salt tolerance of barley at the whole plant level. The other interesting point of Fig. 1 is that barley plants responded positively to Na⁺ and Cl⁻ at very low concentrations (up to 40 mM in external solution), which is the range found in the non-saline soil (Table 4).

A comparison between Barque73 and Tadmor with similar shoot Na⁺ concentrations but different levels of Cl⁻ accumulation (Table 3) in Cl⁻-dominant Hoagland's solution clearly showed that maintenance of low Cl⁻ concentration in Barque73 contributed to its higher Cl⁻-salt-tolerance (92%), whereas growth of Tadmor was reduced by 45%. The growth of Clipper was reduced by Na⁺ and Cl⁻ treatments to a similar extent, whereas Sahara showed a slightly higher sensitivity to high Cl⁻ compared with Na⁺. Together, these results strongly suggest that high concentrations of Na⁺ and Cl⁻ were contributing to a growth reduction under saline treatments. It was indicated that an ability of a variety to exclude Cl⁻ can be very important for improving salt tolerance similar to that showed for Barque73.

Maintenance of high K⁺ concentrations in salt-tolerant genotypes may be one of the mechanisms underlying their superior salt tolerance (Maathuis and Amtmann, 1999; Britto *et al.*, 2010; Tester and Davenport, 2003), but the expression of this trait in this study differed between hydroponics and soil systems. A significant reduction in plant K⁺ occurred with increasing Na⁺ concentration under Na⁺ and NaCl treatments in soil but, in the hydroponic experiment, the concentrations of Na⁺ and K⁺ were independent of one another (Tables 3, 4). Although barley exhibited a high selectivity for K⁺ (Table 4) similar to that found in highly salt-tolerant species (Ball *et al.*, 1987), the amount of K⁺ in leaf tissues declined with increasing salinity as Na⁺ increased. As Na⁺ cannot replace the specific functions of K⁺ required for protein synthesis (Leigh and Wyn Jones, 1984), it is very likely that changes in growth and photosynthetic capacity in association with increasing salinity in the present study are related to salinity-induced changes in K⁺ uptake by roots, and concomitantly related to the K⁺ concentration in the cytoplasm and chloroplasts. It is also important to note that the competitive relationship between Na⁺ and K⁺ operates within a genotype, but not between genotypes, and so it could not be extrapolated to salt tolerance among genotypes, an aspect that is frequently overlooked.

The effect of salinity on photosynthesis is complex. The positive relationship between stomatal conductance and net photosynthesis indicates that the primary limiting factor for net photosynthesis upon exposure to salinity is stomatal closure. Photosynthesis can be further limited by non-optimal metabolic conditions caused by ion toxicity or ion imbalances (Table 5). The main cause for the non-stomatal limitation of photosynthesis seems to be an accumulation

of the toxic ions in leaf cells (Cramer, 1992; Greenway and Munns, 1980; Huang and Van Steveninck, 1989). The Na^+ and Cl^- accumulation showed a high correlation with the progressive decrease in growth and most of the photosynthetic characteristics studied (Table 5). 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. Because stressed plants require high concentrations of K^+ in epidermal and guard cells for the regulation of stomata opening and closure, salt-induced K^+ loss leads to a closing of stomata (Chow *et al.*, 1990). The lack of stomatal closure causes high transpiration and loss of water. Humble and Raschke (1971) indicated that K^+ is the specific ion involved in stomatal opening. Shabala and Cuin (2008) also concluded that K^+ distribution in the leaf apoplast is one of the important factors determining stomatal patchiness and heterogeneity in leaf photosynthesis. Therefore, one of the reasons that Sahara was less affected by Na^+ may be due to its high K^+ concentration and greater control of stomatal regulations under such stress compared with other genotypes. However a large reduction of $C_i:C_a$ in parallel to g_s under Cl^- and NaCl in Clipper, Sahara, and Tadmor suggests specific damaging effects of Cl^- on photosynthetic machinery in addition to stomatal limitations (Table 5).

Long-term inhibition of plant growth by salinity is associated with the appearance of leaf chlorosis or necrosis (Strogonov, 1974). Chlorosis also develops from chloride injuries (Kozlowski, 1997). A significant decline in leaf chlorophyll content with increasing leaf Cl^- concentration was observed in Clipper, Sahara, and Tadmor. However, plants grown under Na^+-dominant soil not only showed no reduction but a slight increase in chlorophyll concentration (Table 5). Therefore, it may be assumed that high concentrations of Na^+ are not the primary reason for the degradation of chlorophyll under NaCl stress, and the decreased chlorophyll concentration may be induced by increased Cl^- concentrations.

A significant correlation between reduced leaf chlorophyll content and the parameters of chlorophyll fluorescence with increasing Cl^- concentration but not Na^+ was found (Tables 5, 6). Moreover, while a significant reduction in $(F'v/F'm)$, ΦPSII and qP of Tadmor, Clipper, and Sahara under Cl^- and NaCl stress was indicated, Barque73 (the Cl^--excluding variety) maintained a higher capacity of the PSII system (Table 6). Not only does this finding suggest that a high concentration of Cl^- is damaging the photosynthetic machinery, but also that Cl^- exclusion is an important mechanism under saline conditions where Cl^- concentrations are high. The decrease of $C_i:C_a$ along with stomatal closure seems more likely to cause losses in PSII efficiency, probably by reducing the CO_2 availability for photosynthesis. The decrease of $C_i:C_a$ (Table 5) with declining g_s, reduced $F'v/F'm$ which is often associated with unrepaired damage to PSII rather than just with reduced operating efficiency (Lawson *et al.*, 2002). The limited availability of CO_2 would be likely to depress the amounts of electron-accepting $NADP^+$ as the carbon reduction cycle slowed

(Else *et al.*, 2009). The quantum yield of PSII in the light results from the fraction of open centres that perform photochemistry (qP) and its reduction is indicative of a down-regulation of leaf photochemistry to match the reduced carbon acquisition under high Cl^- and NaCl accumulation (i.e. photo-inhibition as well as salt-induced photo-damage).

Despite the fact that it was frequently stated that photosynthetic capacity is weakly correlated with the amounts of PSII reaction centres, and even less correlated with the PSI reaction centres (Jiang *et al.*, 2006*a*, *b*; Linger and Brüggemann, 1999; Netondo *et al.*, 2004), our results demonstrate that ΦPSII is highly correlated with A and g_s. Similar results were obtained only in two other studies: in a study of grape leaves subjected to progressive drought (Flexas *et al.*, 2002) and one examining barley plants under saline treatments (Jiang *et al.*, 2006*b*). This study has provided estimates of the separate contributions of Na^+ and Cl^- to the effects of salt stress on yield of PSII electron transport. A reduced quantum yield, as found in the present experiment (Table 6) 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 surroundings or both is suggested by the increase of NPQ in plants grown at high salt concentration (Table 6). 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). 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. (Ziska *et al.*, 1990), which correlates with the known inhibitory effect of Cl^- on protein and enzyme synthesis (Wyn Jones and Pollard, 1983; Gimmler *et al.*, 1984). Thus it is certainly conceivable that excess Cl^- ions in the cytoplasm could act to reduce growth and photosynthesis of the plant. If these effects were combined with those generated by the loss of K^+ and Ca^{2+} homoeostasis, a substantial loss of plant viability could result.

Most of the studies described in the literature about the effects of salinity on the growth of cereals have used hydroponic systems to assess the importance of different mechanisms of salt tolerance and/or to develop selection criteria to improve salt tolerance. However, in this study, some important differences were observed between solution and soil culture which must be taken into account for future research. The maintenance of high K^+ concentrations in salt-tolerant genotypes is one of the mechanisms underlying their salt tolerance, but the expression of this trait differed between hydroponics and soil systems. In the hydroponic experiment, the concentrations of Na^+ and K^+ were independent of one another (Table 3). By contrast, in the soil experiment a significant K^+ and Na^+ discrimination was found. In addition, genetic differences in Na^+ exclusion and $Na^+:K^+$ discrimination between genotypes were not

expressed in hydroponics. In soil, however, genetic differences in Na^+ exclusion were expressed (Tables 3, 4). The leaf osmotic potential of plants grown under the hydroponic system was significantly lower than those grown in soil, which reflects the large difference between the two cultures in terms of the rate of ion uptake by plants (Tables 3, 4). Much of the research has, in the past, involved suddenly exposing plants to such high concentrations of NaCl (>100 mM) so as to cause osmotic shock rather than osmotic stress which induces major trauma that rarely if ever occurs in nature (Passioura 2010). An attempt was made to overcome the trauma of osmotic shock by increasing the concentration of salt gradually, in several small steps over a few days rather than in one large step (see the Materials and methods), but even this may uncover little useful genetic variation in salt tolerance. The important point is that it can take weeks for such variation to become evident in soil and especially under field conditions, and soil-grown plants will have more time to adapt to the salt concentration than plants in hydroponic systems (Passioura 2010). This is of a particular importance for adaptation mechanisms such as osmotic adjustment, which requires the uptake of ions and the formation of compatible solutes (Vetterlein et al. 2004).

Conclusion

The results of this study clearly indicated that the high Cl^- and Na^+ concentrations of saline soils can be a major cause of a reduction in barley growth. The greater reduction in growth under NaCl treatment compared with Na^+ and Cl^- separately, suggests that high concentrations of Na^+ and Cl^- have an additive and/or interactive effect. The results demonstrated that Na^+ and Cl^- exclusion are independent mechanisms, and different genotypes expressed different combinations of the two mechanisms. High Na^+ interferes with K^+ and Ca^{2+} nutrition and stomatal regulation, while high Cl^- concentration reduces photosynthetic capacity due to chlorophyll degradation. This study also demonstrated that solution culture may not allow differences in salt tolerance between genotypes to be discerned and the physiological responses are not the same when the same materials were grown in soil. The results of this work provided support for further studies to examine both Na^+ and Cl^- ions in parallel, which is of crucial importance for the understanding of salt damage and for the manipulation of salt tolerance.

Acknowledgements

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