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Research

Uncoupling of sodium and chloride to assist breeding for salinity tolerance in crops

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Summary

- The separation of toxic effects of sodium (Na^+) and chloride (Cl^-) by the current methods of mixed salts and subsequent determination of their relevance to breeding has been problematic.
- We report a novel method (Na^+ humate) to study the ionic effects of Na^+ toxicity without interference from Cl^-, and ionic and osmotic effects when combined with salinity (NaCl). Three cereal species (*Hordeum vulgare*, *Triticum aestivum* and *Triticum turgidum* ssp. *durum* with and without the Na^+ exclusion gene *Nax2*) differing in Na^+ exclusion were grown in a potting mix under sodicity (Na^+ humate) and salinity (NaCl), and water use, leaf nutrient profiles and yield were determined.
- Under sodicity, Na^+-excluding bread wheat and durum wheat with the *Nax2* gene had higher yield than Na^+-accumulating barley and durum wheat without the *Nax2* gene. However, under salinity, despite a 100-fold difference in leaf Na^+, all species yielded similarly, indicating that osmotic stress negated the benefits of Na^+ exclusion.
- In conclusion, Na^+ exclusion can be an effective mechanism for sodicity tolerance, while osmoregulation and tissue tolerance to Na^+ and/or Cl^- should be the main foci for further improvement of salinity tolerance in cereals. This represents a paradigm shift for breeding cereals with salinity tolerance.

Introduction

Global food requirements are expected to increase by *c.* 90% by 2050, and as land degradation, urban spread and seawater intrusion are increasing, gains in agricultural productivity must come from marginal land, including saline soils (Munns *et al.*, 2012). Of the 1.5×10^9 ha of cultivated land mass on Earth, 0.34×10^9 ha (23%) are saline (Shahid & ur Rahman, 2011). In general, there are two complementary approaches to improving crop production in saline soils: soil management and plant breeding. The soil management option (i.e. leaching salts below the root zone and gypsum application) for dryland cropping systems is not always practical and rarely cost-effective (Genc *et al.*, 2013), while the breeding option holds promise (Grewal *et al.*, 2004; Genc *et al.*, 2007; Munns *et al.*, 2012). With impressive intellectual and monetary investment over the last few decades, our understanding of plant biology under salinity stress has improved significantly, but progress in breeding salinity-tolerant cereal varieties has been slow. At present, there are a few salt-tolerant dryland cereal varieties developed via conventional breeding (Sharma, 2010), but no varieties have ever been released based on physiological mechanisms (Noble & Rogers, 1992; Colmer *et al.*, 2005; Munns, 2005; Rozema & Flowers, 2008; Cuin *et al.*, 2009). There are several possible reasons for this apparent slow progress, and these are discussed below.

Although researchers acknowledge the complexity of salinity tolerance and the need for combined mechanisms (Na^+ exclusion, osmotic tolerance and tolerance to high internal Na^+ concentration) to achieve it (Apse *et al.*, 1999; Munns, 2005; Genc *et al.*, 2007; Munns & Tester, 2008; Rozema & Flowers, 2008; Rajendran *et al.*, 2009), osmotic stress is the critical component of salinity stress (Bernstein, 1975; Epstein *et al.*, 1980; Munns *et al.*, 1995; Neumann, 1997; Husain *et al.*, 2003; Munns & Tester, 2008; Rengasamy *et al.*, 2010; Ul Haq *et al.*, 2014; Munns & Gilliham, 2015) and most plants are naturally efficient Na^+ excluders (Munns, 2005), Na^+ exclusion theory has for around two decades defined the salinity tolerance research paradigm. Despite this, there has only been one case where an Na^+ exclusion gene (*Nax2*, *HKT1;5*) resulted in improved grain yield: durum wheat Tamaroi possessing the *Nax2* gene (Tamaroi-*Nax2*) yielded, on average, 20% more than Tamaroi durum wheat at two high-salinity field sites in Australia from a total of 12 sites (Munns *et al.*, 2012). Although there was a gradient of salinity stress at the field site where the most significant yield advantage of the *Nax2* gene was observed, the other field sites with varying degrees of salinity did not show significant yield differences, and a salinity rate trial in a controlled environment to verify effects of

the *Nax2* gene on grain yield in isolation has not been reported. While recognizing the importance of this research, we believe that in the absence of controlled environment rate studies, it is difficult to conclude that the yield increase was the result solely of the *Nax2* gene. Tamaroi-*Nax2* was produced from a conventional cross between durum wheat Tamaroi and the diploid ancestral wheat relative *Triticum monococcum* (James *et al.*, 2006), and thus may have inherited other genes which could influence grain yield.

The lack of rapid and reliable screening methods for assessment of salinity tolerance has also contributed to slow progress in breeding (Genc *et al.*, 2007). To date, most studies have used short-term hydroponics or soil-based vegetative trials, with findings that tend to be poorly correlated with grain yield (Maas & Hoffman, 1977; Munns & James, 2003; Genc *et al.*, 2010a, 2013, 2014; Tavakkoli *et al.*, 2010). These recent studies suggest that hydroponics is not a good model for dryland salinity as soil moisture tension (i.e. the molecular attraction of the surface of the soil particles for water) (Richards, 1954) and rhizosphere ionic gradient (Vetterlein *et al.*, 2004) cannot be established in hydroponics. In addition, in hydroponics, nutrients are generally in excess, while in a farmer's field, they usually become less available as the soil dries. Therefore, future studies should consider soil-based pot assays where field testing is not possible if we are to make good progress in this area.

The difficulty in separating ionic effects from osmotic effects of salts has also impeded progress in breeding for salinity tolerance (Marx, 1979; Genc *et al.*, 2007; Rengasamy *et al.*, 2010). After half a century of experimenting, methods of using mixed salts to separate ionic effects of Na^+ ($CaCl_2$ or KCl vs NaCl) and Cl^- ($NaNO_3$ or Na_2SO_4 vs NaCl) have produced equivocal results (Munns & Tester, 2008; Munns, 2011). Mixed salts can produce Na^+ and Cl^- dominant solutions and soils, but it is difficult to alter the external concentration of one ion against another without modifying the osmotic pressure of the external solution or the rate of uptake of other ions (Richards, 1954; Bernstein & Pearson, 1956; Wright & Rajper, 2000; Sheldon *et al.*, 2006; Munns & Tester, 2008; Rengasamy *et al.*, 2010; Tavakkoli *et al.*, 2010; Munns, 2011). Even at equivalent osmotic pressure, mixed salts can generate high/toxic concentrations of other balancing ions (Kingsbury & Epstein, 1986; Martin & Koebner, 1995; Luo *et al.*, 2005; Tavakkoli *et al.*, 2011), which in turn can affect plant growth. Clearly, there is an urgent need for a novel method that is able to separate Na^+ toxicity from Cl^- toxicity without disturbing the balance of other ions. The following section gives a brief history of discovery and application of such a method.

Humic substances are natural organic compounds that originate from various sources (plant, peat, soil and coals such as lignite and leonardite), comprise 50–90% organic matter, and have been reported to ameliorate the effects of contaminants, adverse temperature, pH and also salinity (Kulikova *et al.*, 2005; Asik *et al.*, 2009; Katkat *et al.*, 2009). Of the humic substances, coal-derived potassium (K^+) and Na^+ humate are produced commercially for agricultural and industrial use, but surprisingly there is very little in the scientific literature on the effects of these types of commercial humates on plant growth or yield. The limited studies to date have reported mixed results (Sharif *et al.*, 2002; Van Tonder, 2008; Tahir *et al.*, 2011; Turan *et al.*, 2011; Leventoglu & Erdal, 2014). Our preliminary experiments investigating ameliorative effects of Na^+ humate on salinity-stressed barley, bread wheat and durum wheat showed that at all salinities, Na^+ humate (2–4 g kg^{-1} potting mix) reduced plant growth at heading by 10–20% depending on cereal species and degree of salinity stress (Supporting Information Fig. S1). Nutrient analysis of penultimate leaves of durum wheat revealed that at all salinities, Na^+ humate increased leaf Na^+ significantly, while leaf Cl^- concentrations remained low and similar to those of the control (data not shown). This prompted analysis of Na^+ humate itself (acid digest), which showed that it contained high concentrations of Na^+ (6%) with negligible Cl^- (0.15%) (Table S1), thus making it an ideal candidate to study the effects of Na^+ toxicity without interference from Cl^-. Following this serendipitous discovery, this study tested the hypotheses that Na^+ humate can be an ideal substrate to determine the ionic effects of Na^+ without interference from Cl^-; that ionic and osmotic effects can be studied more effectively when Na^+ humate is used in conjunction with NaCl; and that Na^+ exclusion is more effective under sodicity, while osmotic stress tolerance is more relevant to salinity.

Materials and Methods

Plant material

A barley (*Hordeum vulgare* L.) cv Clipper, a bread wheat (*Triticum aestivum* L.) cv Krichauff, a durum wheat (*Triticum turgidum* ssp. *durum*) cv Tamaroi and its sister line with Na^+ exclusion gene *Nax2* (Tamaroi-*Nax2*) were used in the present study. The candidate gene for *Nax2* (*TmHKT1;4-A*) was characterized and used to produce durum wheat Tamaroi-*Nax2* germplasm by James *et al.* (2006), and the actual gene was later identified by Byrt *et al.* (2007). These cereal species vary in their ability to exclude Na^+, and are historically ranked for salinity tolerance in the following order: barley > bread wheat > durum wheat (Munns & Tester, 2008). However, the cultivars used in this study do not necessarily represent the respective species.

Growth medium, treatments and seedling establishment

Following preliminary experiments, two experiments were conducted using University of California potting mix (UC mix). For 1000 kg of UC mix, 1200 l of course river sand sterilized at 100°C for 90 min, 660 l of peatmoss, 1.2 kg hydrated lime, 2.0 kg agricultural lime, and 4.5 kg mini-osmocote were added together and mixed thoroughly. A wide range of Na^+ humate (0, 0.1, 0.5, 1, 2, 4, 8 and 16 g kg^{-1} potting mix) and NaCl concentrations (0, 10, 25, 50, 75, 100, 150 and 200 mM) were used in the experiments (Table 1). Once the potting mix was air-dried and sieved, 4 kg of it was weighed into plastic bags. The potting mix in the bags was mixed with either Na^+ humate (Poultry Mate; Double Dragons Humic Acid Co. Ltd, Xinjiang, China) or NaCl. Sodium humate was mixed thoroughly in 4 kg potting

Table 1 Chemical properties of University of California potting mix supplied by varying degrees of salinity (NaCl) and sodicity (Na^+ humate) in the present study

				Saturation paste exchangeable cations (converted from mEq l^{-1} to mg kg^{-1})				
	pH	ECe	ESP%	Ca^{2+}	K^+	Mg^{2+}	Na^+	Cl^-
Salinity (mM NaCl)								
0	6.3 ± 0.0	1.5 ± 0.1	10.7 ± 0.3	108 ± 6	101 ± 2	21 ± 1	27 ± 2	16 ± 3
25	6.1 ± 0.0	2.0 ± 0.3	30.1 ± 0.3	137 ± 15	113 ± 10	27 ± 3	119 ± 10	170 ± 20
50	6.0 ± 0.0	2.2 ± 0.2	43.3 ± 0.6	134 ± 13	109 ± 8	26 ± 3	204 ± 15	310 ± 35
100	6.1 ± 0.0	4.4 ± 0.7	55.3 ± 0.8	214 ± 44	155 ± 21	39 ± 7	506 ± 76	458 ± 11
200	6.1 ± 0.0	5.6 ± 0.4	68.4 ± 0.1	203 ± 10	132 ± 5	35 ± 2	818 ± 38	1062 ± 78
Sodicity (g kg^{-1} Na^+ humate)								
0	5.8 ± 0.1	3.5 ± 0.6	10.4 ± 0.1	221 ± 32	216 ± 43	46 ± 8	54 ± 8	28 ± 7
1	6.0 ± 0.1	3.8 ± 0.5	25.4 ± 1.5	195 ± 12	233 ± 39	43 ± 5	149 ± 5	17 ± 3
4	6.4 ± 0.1	4.4 ± 0.5	60.5 ± 2.4	102 ± 11	213 ± 33	24 ± 3	438 ± 24	21 ± 2
8	7.0 ± 0.2	4.4 ± 0.4	82.1 ± 2.3	37 ± 6	156 ± 31	9 ± 2	679 ± 34	21 ± 3
16	7.5 ± 0.2	4.9 ± 0.4	86.0 ± 1.5	59 ± 21	116 ± 20	9 ± 2	940 ± 48	30 ± 2

Selected levels covering the ranges are presented. Means ± SE are presented ($n = 3$). ECe, electrical conductivity of paste extract; saline soils, ECe > 4.0 dS m; exchangeable sodium percentage, ESP = $(Na^+)/[Na^+ + Ca^{2+} + Mg^{2+} + K^+]$%; nonsodic soils, ESP < 6; strongly sodic soils, ESP > 15. For methods of soil analyses, see Genc *et al.* (2013).

mix portions, after which the potting mix was incubated for 4 wk at 18°C (Gaur & Bhardwaj, 1971). No supplemental Ca^{2+} was added as this would have altered exchangeable sodium percentages and possibly impacted on our ability to observe high-Na^+-associated responses in the present study. For salinity, 4 kg potting mix portions were divided into two halves. Sodium chloride together with a supplemental dose of $CaCl_2$ (molar Na : Ca = 30) was applied to half the potting mix (2 kg) as a general practice, mixed thoroughly and placed into the bottom half of the pot. This was to mimic salts generally present in South Australian subsoils (Genc *et al.*, 2013), and also to prevent NaCl-induced calcium deficiency (Genc *et al.*, 2010b). However, as the potting mix had a luxury supply of Ca^{2+}, as evident by high leaf Ca^{2+} in all treatments under salinity and sodicity except for very high sodicity (16 g kg^{-1} Na^+ humate), it is unlikely that the addition of a small amount of supplemental Ca^{2+} would have altered the results and interpretation. The other half of the potting mix (2 kg) with no NaCl treatment was placed on top of NaCl-treated potting mix in the pots. No incubation was conducted in the salinity experiment. The potting mix in plastic bags with respective treatments was placed into 8 inch plastic pots. Samples of the potting mix were analysed by CSBP Limited, Bibra Lake, Western Australia (for details of soil analytical methods, see Genc *et al.*, 2013). Salinity, sodicity, chloride and other chemical data for the growth media are provided in Table 1. As exchangeable sodium percentages (ESPs; a measure of sodicity) are all above 6, all of the treatments represent sodicity (or at least its high-Na^+ component), while NaCl treatments of 100 mM and above and Na^+ humate treatments of 4 and above represent salinity, having an electrical conductivity of paste extract (ECe) > 4.0 dS m^{-1}, and hence these represent saline-sodic conditions (Table S2).

Seeds of similar size of each species were surface-sterilized in 70% ethanol for 1 min, followed by soaking in 3% sodium hypochlorite for 5 min and three lots of rinsing with deionized water. Seeds were then germinated on filter paper in Petri dishes at room temperature for 4 d before transplanting into pots (three plants per pot). At 1 wk after transplanting, a 1 cm layer of white plastic beads (washed, rinsed and dried) was added to the surface of the potting mix to reduce evaporation. Additional pots with no plants were also included to determine actual evaporation so that daily plant water uptake (transpiration) could be accurately estimated.

Growth conditions

The experiments were conducted in a growth room maintained at 20 : 15°C with a 14 h photoperiod. The intensity of photosynthetically active radiation, measured with an LI 190SA quantum sensor (Li-Cor, Lincoln, NE, USA), was 250–300 μmol m^{-2} s^{-1} at the plant level, and relative humidity varied from 30–50% to 60–80% during light and dark periods, respectively (measured with a HOBO U12 data logger; http://www.onsetcomp.com).

Plant traits measured

The pots were weighed daily, and field capacity (7.4%) was maintained by watering with milli-Q water. The maximum daily water addition was 130 ml per pot under control at heading and thereafter, which amounted to less than half the field capacity (300 ml). However, as plants accumulated more mass over time, maintaining field capacity daily meant that less water was added to the pots, which somewhat mimicked field conditions where water availability is reduced as the season progresses. Daily watering ensured that plants were not water-stressed at any stage, except for water stress induced by salinity. The pots were shifted daily to minimize position effects. Plant daily water uptake was calculated as the weight of a planted pot minus evaporative water loss from an unplanted pot. The evaporative

water loss (4 ml d^{-1} per pot) was < 2% of the potting mix water content at field capacity. This type of daily plant water uptake is a nondestructive measure of transpiration, which is closely related to growth response (Harris *et al.*, 2010). Cumulative water use (CWU), a surrogate for biomass accumulation, was used to establish approximate growth curves nondestructively throughout the growth period. Incremental water use at weekly intervals up to heading (a well-defined growth stage beyond which correlation between water use and grain yield did not change) was used to calculate growth rates nondestructively. The number of days to heading was recorded when the head on the main culm was fully emerged. At heading, penultimate leaves (leaves immediately below the flag leaves) were sampled for elemental analysis. The leaf samples were oven-dried at 80°C, and ground before elemental analysis by inductively coupled plasma optical emission spectrometry (ARL 3580 B; Appl. Res Lab. SA, Ecublens, Switzerland) (Wheal & Palmer, 2010; Wheal *et al.*, 2011). At maturity, grain yield, grain number per plant, and grain weight were determined. For across-species comparisons, relative grain yield (the ratio of yield at an individual stress level to that under nil stress and expressed as a percentage) was also calculated.

Experimental design and statistical analysis

Both salinity and sodicity experiments were repeated, with similar results. Therefore, data from just one set are presented. The experiments were both set up as a completely randomized block design with four replicates of each species by treatment combination. To overcome the problem of nonhomogeneity of variances, leaf Na^+ data were log-transformed before ANOVA. For each of the traits, a least significant difference (LSD) at $P = 0.05$ was used in pairwise comparisons of means. All ANOVAs were conducted using the GENSTAT statistical package (v.15, VSN International Ltd).

There are statistical problems associated with the analysis of CWU over time (Mandel, 1957), and therefore incremental water use over time was chosen as a suitable candidate for assessing plant water uptake. To determine differences in the incremental water-use curves within the treatments of salinity or sodicity across species, a regression analysis was conducted using a linear mixed model. Specifically, the fixed component of the linear mixed model contained terms to individually model the intercept and slope of the linear regression for each of the species by treatment combinations. Additional nonlinearity of each of the curves was captured by including an appropriate random smoothing spline term (Verbyla *et al.*, 1999). To account for the increase in variation of the incremental water-use measurements over time, the residual component of the model contained individual variances for each day of measurement. Estimated linear regression slopes were then extracted from the fixed component of the fitted model, and an LSD at $P = 0.05$ was used for comparison of estimates. Linear mixed modelling was conducted using the package ASREML-R (Butler *et al.*, 2009) available in the R statistical computing environment (R Development Core Team, 2015).

Results

Given the inextricable link between plant transpiration and plant biomass accumulation and yield (Sinclair *et al.*, 1984), CWU was employed to nondestructively predict plant growth responses, and it showed that growth reduction occurred with exposure from 25 mM NaCl and continued in a linear dose–response manner with increasing NaCl, with growth reduction evident from as early as 2 wk after transplanting (Fig. 1). All species were similar in their growth response to salinity. Cumulative water use (CWU) under sodicity, however, showed less Na^+-associated growth reduction than under salinity (comparing equivalent ECe values), especially in the most efficient Na^+-excluders, the bread wheat Krichauff and the durum wheat Tamaroi-*Nax2* (Fig. 1). Overall CWU provided an accurate measure of osmotic stress and growth trends well before they appeared visually, and at heading (Zadoks stage 55; Zadoks *et al.*, 1974) it was strongly correlated with grain yield in all three species (Table 2). A regression analysis of incremental water use over time indicated that species had reduced water uptake and consequently reduced growth rates under increasing salinity (Fig. S2; Table S3). By contrast, at the highest sodicity (16 g kg^{-1} Na^+ humate), Na^+-excluding bread wheat Krichauff and durum wheat Tamaroi-*Nax2* had increased water uptake and consequently higher growth rates than Na^+-accumulating barley Clipper and durum wheat Tamaroi (Fig. S2; Table S3).

Chlorotic symptoms became visible first on the oldest leaves of bread and durum wheats (but were absent in barley) after 4 wk of growth at moderate to high NaCl doses (100–200 mM) and later on the second oldest leaves. No other leaf symptoms associated with NaCl toxicity were observed in any of the species. Under sodicity, foliar symptoms resembling those of Ca^{2+} deficiency, that is, emerging leaf blades tightly rolled and leaf tips severely withered and often necrotic (Genc *et al.*, 2010b), were observed first in young leaves after 2 wk of growth at the highest Na^+ humate dose (16 g kg^{-1}) and later in middle-aged leaves. These symptoms were severe in durum wheat Tamaroi, mild in barley Clipper, slight in durum wheat Tamaroi-*Nax2*, and not apparent in bread wheat Krichauff. Magnesium deficiency-like symptoms, that is, green-yellow plants with yellow interveinal chlorosis turning to brown necrosis on the middle leaves, became evident at 4 wk growth, especially in barley Clipper and durum wheat Tamaroi. Overall, while species looked visually similar in their responses to salinity, there were clear differences in their appearance under sodicity, with species being in reverse order (of increasing leaf symptoms) to Na^+ exclusion ability: bread wheat Krichauff > durum wheat Tamaroi-*Nax2* > barley Clipper > durum wheat Tamaroi (Fig. S3).

At heading, indicator (penultimate) leaves were sampled to determine the nutritional status of the plants. Salinity significantly increased leaf Na^+, especially in durum wheat Tamaroi and barley Clipper (Fig. 2; Tables S4, S5). At 100 mM NaCl, there was a 100-fold difference in leaf Na^+ between the species: bread wheat Krichauff < durum wheat Tamaroi-*Nax2* < barley Clipper < durum wheat Tamaroi (Fig. 2; Tables S4, S5). Leaf Cl^- was also increased by NaCl salinity but with smaller

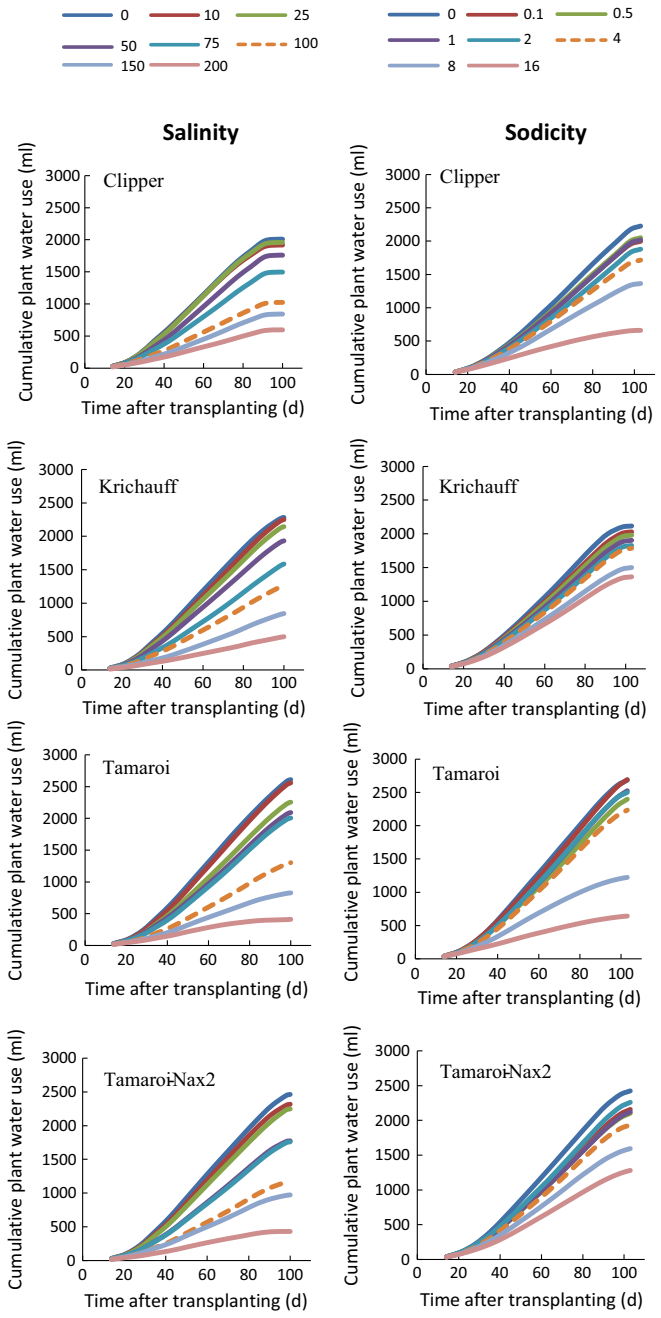

Fig. 1 Cumulative water use in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different salinity (mM NaCl; left panels) and sodicity (g kg^{-1} Na^+ humate; right panels) conditions applied to the potting mix. Owing to statistical problems associated with the analysis of cumulative water use (see the Materials and Methods section), only the means of four replications are presented. Salinity and sodicity treatments of similar electrical conductivity values (100 mM NaCl and 4 g kg^{-1} Na humate) are given as dashed lines.

differences between species (Fig. 2; Table S4): bread wheat Krichauff < barley Clipper < durum wheat Tamaroi-*Nax2* < durum wheat Tamaroi. Increases in leaf Na^+ and Cl^- were accompanied by significant reductions in K^+, Ca^{2+} and Mg^{2+}, especially in durum wheat Tamaroi and barley Clipper, but reductions were less than those seen under sodicity (Fig. 3; Table S4). At 100 mM NaCl, K^+ and Mg^{2+} concentrations were near adequacy in durum wheat Tamaroi, barley Clipper, bread wheat Krichauff and durum wheat Tamaroi-*Nax2* (Reuter & Robinson, 1997). However, the highest salinity (200 mM NaCl) caused further reductions in Mg^{2+} concentration of, in particular, durum wheat Tamaroi, indicating deficiency (Fig. 3; Table S4). Although reduced, leaf Ca^{2+} concentrations appeared adequate at all salinities. Similar to salinity, sodicity increased leaf Na^+, especially in durum wheat Tamaroi and barley Clipper, with much higher Na^+ occurring at 8 g kg^{-1} Na^+ humate (Fig. 2; Tables S4, S5). Unlike salinity, Cl^- concentrations under sodicity were low and similar in all species (Fig. 2; Table S4). Compared with nil, high sodicity (8 g kg^{-1} Na^+ humate) resulted in significant reductions in K^+, Ca^{2+} and Mg^{2+} in indicator leaves, especially in durum wheat Tamaroi and barley Clipper (Fig. 3; Table S4). Much lower concentrations were observed at the highest sodicity (16 g kg^{-1} Na^+ humate) (Fig. 3; Table S4). These K^+ and Mg^{2+} concentrations appear below the critical level for deficiency (cf. Genc *et al.*, 2010b). Na^+-excluding bread wheat Krichauff and durum wheat Tamaroi-*Nax2* maintained higher K^+, Ca^{2+} and Mg^{2+} than Na^+-accumulating (or less Na^+-excluding) barley Clipper and durum wheat Tamaroi (Fig. 3; Table S4).

As grain yield is the ultimate parameter, plants were grown to maturity, and yield and yield components were recorded. Salinity stress reduced grain yield with significant reductions occurring in durum wheats Tamaroi and Tamaroi-*Nax2* (10%) at 25 mM NaCl, and in barley Clipper and bread wheat Krichauff (15%) at 50 mM NaCl (Table 3; Fig. 2). While under moderate salinity (100 mM NaCl), irrespective of their Na^+ exclusion ability, all three species suffered 50% reduction, and at the highest salinity (200 mM NaCl), durum wheats Tamaroi and Tamaroi-*Nax2* showed slightly higher reduction (80–85%) than barley Clipper and bread wheat Krichauff (70–75%) (Table 3; Fig. 2). Sodium exclusion gene *Nax2* in durum wheat Tamaroi background was associated with a 13% increase in relative grain yield at 150 mM NaCl, but only 5% at 200 mM NaCl. Sodicity also reduced grain yield of all three species, and significant reductions occurred even at values as low as 1 g kg^{-1} Na^+ humate (14–17%; Table 3; Fig. 2). At high and very high sodicities (8 and 16 g kg^{-1} Na^+ humate, respectively), 30–70% and 40–90% reductions were recorded, respectively (Table 3; Fig. 2). However, there was little difference in yield reduction between the cereal species until the 8 g kg^{-1} Na^+ humate was reached. At this rate and beyond, Na^+-accumulating barley Clipper and durum wheat Tamaroi showed greater yield reduction than Na^+-excluding bread wheat Krichauff and durum wheat Tamaroi-*Nax2*. There was a clear yield advantage of Tamaroi-*Nax2* over Tamaroi (64 vs 29% and 50 vs 8% relative grain yield at 8 and 16 g kg^{-1} Na^+ humate, respectively).

Examination of grain yield components to determine the primary cause of yield differences showed that although both grain number per plant and grain weight were reduced by salinity and sodicity, reduction was greater on grain number per plant and under salinity; for example, 47% at 100 mM NaCl and 36% at 8 g kg^{-1} Na^+ humate (Table 3). However, reductions in grain weight at these salinity and sodicity values were 5 and 15%,

Table 2 Correlation coefficients (*r*) between grain yield per plant and cumulative water use over time in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff, and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under salinity and sodicity

	Salinity (NaCl)					Sodicity (Na^+ humate)			
DAT	Clipper	Krichauff	Tamaroi-*Nax2*	Tamaroi	DAT	Clipper	Krichauff	Tamaroi-*Nax2*	Tamaroi
14	0.8640	0.9183	0.8843	0.8849	14	0.0624	0.5543	0.6041	0.4718
21	0.9605	0.9808	0.9353	0.9437	21	0.6893	0.7658	0.7936	0.8829
28	0.9817	0.9844	0.9658	0.9664	28	0.8423	0.8354	0.8895	0.9406
35	0.9854	0.9861	0.9729	0.9724	35	0.8876	0.8810	0.9199	0.9544
42	**0.9869**	0.9884	0.9768	0.9758	42	**0.9074**	0.9045	0.9297	0.9625
49	0.9889	**0.9914**	**0.9802**	**0.9792**	49	0.9208	**0.9179**	**0.9378**	**0.9669**
56	0.9897	0.9926	0.9820	0.9821	56	0.9301	0.9323	0.9410	0.9709
63	0.9896	0.9935	0.9841	0.9842	63	0.9367	0.9389	0.9428	0.9738
70	0.9884	0.9944	0.9865	0.9855	70	0.9419	0.9428	0.9454	0.9767
77	0.9857	0.9952	0.9885	0.9851	77	0.9464	0.9440	0.9477	0.9798
84	0.9814	0.9952	0.9896	0.9881	84	0.9506	0.9446	0.9503	0.9826
91	0.9748	0.9952	0.9903	0.9887	91	0.9546	0.9431	0.9496	0.9844
98	0.9738	0.9945	0.9893	0.9894	98	0.9583	0.9374	0.9460	0.9863
100	0.9738	0.9941	0.9890	0.9895	103	0.9591	0.9334	0.9432	0.9866

Means of four replications were used in the analysis. DAT, d after transplanting. Heading times are indicated in bold. d.f. = 6, $P < 0.01$.

respectively. Much greater reduction occurred in both grain number and grain weight at the highest salinity (200 mM NaCl) and sodicity (16 g kg^{-1} Na^+ humate) (Table 3).

Discussion

A novel screening method

This paper describes a novel application of Na^+ humate to study ionic effects of Na^+ toxicity without interference from Cl^-, thus providing a model of the key determinant of sodicity. Before the application of the method is discussed, a few words on the technical side of Na^+ humate itself may be useful. Briefly, Na^+ humate is a humic acid salt obtained by sodium hydroxide extraction of black or brown coal (lignite or leonardite) and is used as a soil conditioner, as a stabilizer for ion exchange resins in water treatment, as a drilling additive in the drilling of wells for hydrocarbon exploration/extraction and geothermal drilling, and in the remediation of polluted environments (http://www.ahmadsaeed.com/sodium_Humate.html). Apart from its mainly industrial use, limited studies have also tested its effects on plant growth, with results ranging from none to stimulatory or negative effects (Van De Venter *et al.*, 1991; Sharif *et al.*, 2002; Iakimenko, 2005; Tahir *et al.*, 2011). However, the current use of Na^+ humate described here has not, to our knowledge, been reported in the literature. As mentioned in the introduction, mixed salts have been used to separate Na^+ from Cl^- for over 50 yr (Munns, 2011) but have produced ambiguous results (Munns & Tester, 2008; Munns, 2011). Sodium humate delivers high Na^+ but very little Cl^- in an inert, organic matrix, as shown by leaf and potting mix analyses (Fig. 2; Table 1), without altering the concentration of balancing ions, which has been a problem with mixed salts.

The leaf symptoms under sodicity demonstrate that Na^+ toxicity manifests not as Na^+ toxicity *per se* but rather as deficiencies of other exchangeable cations (Ca^{2+}, K^+ and Mg^{2+}) as a result of high Na^+ competing with their uptake. Of these cations, Ca^{2+} deficiency is frequently reported in pot/solution studies and sodic soils, and gypsum ($CaSO_4$) application in the field has been effective in correcting it (Pearson & Bernstein, 1958; Bains & Fireman, 1964; Bernstein, 1975; Qadir *et al.*, 2001). While low K^+ and Mg^{2+} induced by high sodicity are generally limited to pot/solution experiments (Pearson & Bernstein, 1958; Bains & Fireman, 1964; Sharma, 1991; Genc *et al.*, 2010b), there is the potential for K^+ and Mg^{2+} deficiencies to develop in sodic soils, and hence these elements should also be monitored. Given the wide occurrence of sodic soils worldwide (Rengasamy & Olsson, 1991), this method offers a rapid, reliable and cost-effective assessment of tolerance to high Na^+ for breeding programmes. There is clearly a need to screen large numbers of genotypes/accessions to identify sodicity-tolerant and sodicity-sensitive lines for genetic studies.

Unlike studies using NaCl alone, this method, when coupled with NaCl, also enables researchers to distinguish between toxicities of Na^+ and Cl^- and hence can assist in designing breeding programmes around the most relevant parameter, Na^+ or Cl^-, as plant species differ in their tolerance to Na^+ and Cl^-. For example, despite higher Na^+ in Na^+ humate-treated than in NaCl-treated plants, chlorosis of old leaves was only evident in NaCl-treated plants, indicating that the leaf chlorosis of old leaves was caused by high Cl^- but not high Na^+, supported by Slabu *et al.* (2009). This effect of high Cl^- can also be seen in grain yield. For instance, when salinity and sodicity treatments that result in similar soil osmotic potentials (measured as ECe) and exchangeable sodium percentages were compared (100 mM NaCl vs 4 g kg^{-1} Na^+ humate; Table 1), across species there was an average of 33% lower grain yield at 100 mM NaCl than at 4 g kg^{-1} Na^+ humate. This reduced grain yield is probably a result of higher Cl^- under salinity (15 400 mg kg^{-1} DW) than under sodicity (3700 mg kg^{-1} DW), as these high Cl^- concentrations

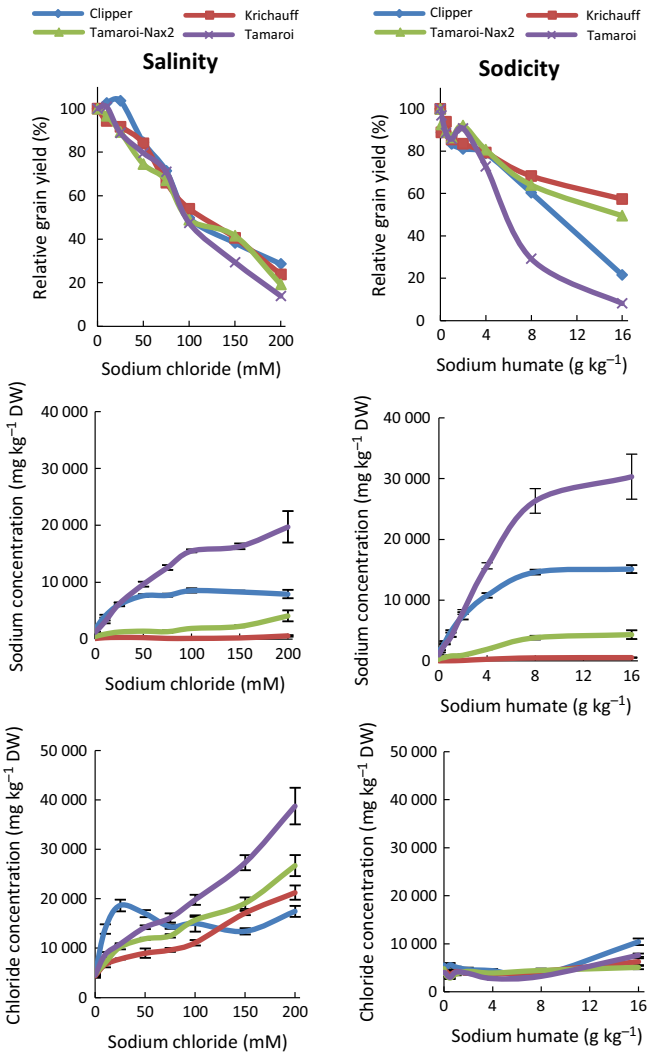

Fig. 2 Relative grain yield (%) (salinity or sodicity tolerance), and leaf sodium (Na^+) and chloride (Cl^-) concentrations in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different salinity (mM NaCl; left panels) and sodicity (g kg^{-1} Na^+ humate; right panels) conditions applied to the potting mix. As leaf Na^+ data required transformation before ANOVA, to assist comparisons across elements and other published data, only nontransformed data with SEM ($n = 3$) were presented here. See Supporting Information Table S4 for the least significant differences at $P = 0.05$ for species × treatment interaction and Table S5 for nontransformed leaf Na^+ concentration.

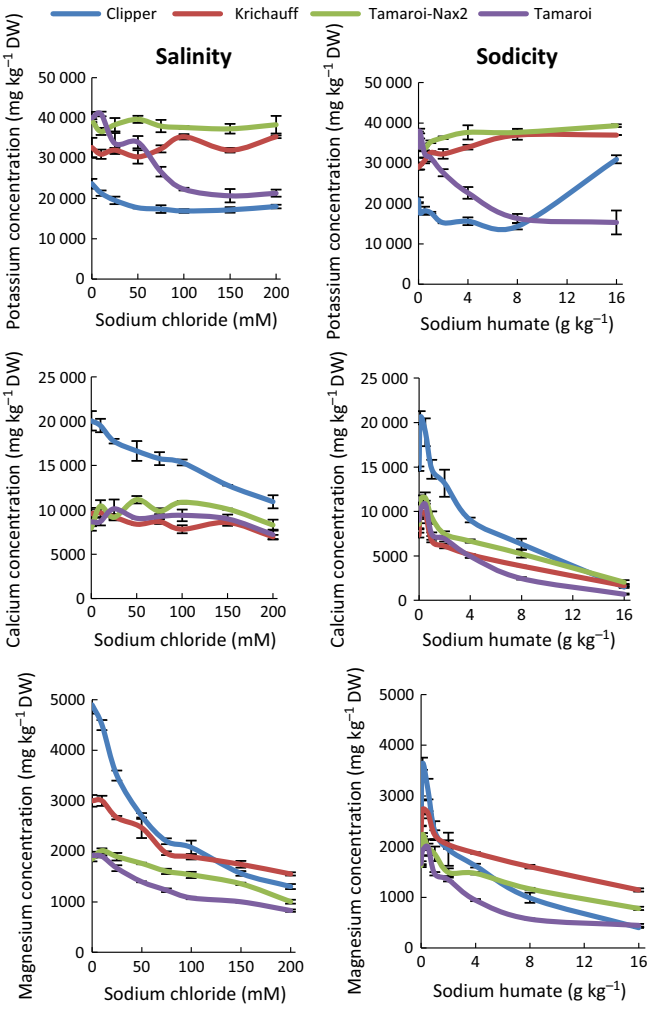

Fig. 3 Leaf potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) concentrations in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different salinity (mM NaCl; left panels) and sodicity (g kg^{-1} Na^+ humate; right panels) conditions applied to the potting mix. Data are means of three replicates and SEM. Refer to Table S4 for least significant differences at $P = 0.05$ for species × treatment interaction.

under salinity were above the critical concentration range for toxicity for Cl^--sensitive (4000–7000 mg kg^{-1} DW) and Cl^--tolerant plant species (15 000–50 000 mg kg^{-1} DW) (White & Broadley, 2001). Leaf Na^+, K^+, Mg^{2+} and Ca^{2+} concentrations were similar between the salinity and sodicity treatments compared (Figs 2, 3) (Ca^{2+} was lower under sodicity but still within the adequate range; Genc *et al.*, 2010b). Grain yield reduced by high Cl^- has also been reported for several crops under field conditions (Dang *et al.*, 2010). These results suggest that although at high concentration both Na^+ and Cl^- can be toxic to the plant, high Cl^- was more damaging than high Na^+. These findings agree with earlier mixed salt studies suggesting that Cl^- was more toxic but that Na^+ and Cl^- together were even more toxic (Martin & Koebner, 1995; Tavakkoli *et al.*, 2011), while disagreeing with NaCl studies associating leaf injury with high Na^+ where accompanying Cl^- was not measured (Ul Haq *et al.*, 2014).

Because of the strong correlation between CWU at heading and grain yield (Table 2), the assay could be concluded at heading rather than at maturity; the assay thus provides an inexpensive prefield screening method, as called for by researchers (Marx, 1979; Genc *et al.*, 2007; Rengasamy *et al.*, 2010). Ul Haq *et al.* (2014) found that biomarkers (leaf injury, tiller number and leaf Na^+) at 42 d were better correlated with shoot growth than those measured at 21 d. Effective, relatively inexpensive prefield trial screening is important, especially in view of the expense and variability inherent in field trials. Apart from intersite and intrasite variability in soil salinity, sodicity, pH and amounts of

Table 3 Grain yield, grain number and grain weight in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different salinity and sodicity conditions

	Grain yield per plant (g)				Grain number per plant				Grain weight (g)			
	Clipper	Krichauff	Tamaroi	Tamaroi-*Nax2*	Clipper	Krichauff	Tamaroi	Tamaroi-*Nax2*	Clipper	Krichauff	Tamaroi	Tamaroi-*Nax2*
Salinity (mM NaCl)												
0	4.766	7.128	7.455	6.891	85	199	136	138	0.056	0.036	0.055	0.050
10	4.890	6.727	7.522	6.665	85	188	154	133	0.057	0.036	0.049	0.051
25	4.939	6.535	6.624	6.152	86	178	121	125	0.057	0.037	0.055	0.050
50	4.030	5.993	5.938	5.137	71	165	104	101	0.056	0.036	0.057	0.051
75	3.404	4.704	5.317	4.627	63	129	101	93	0.054	0.036	0.053	0.050
100	2.379	3.882	3.542	3.375	44	112	70	72	0.054	0.035	0.051	0.047
150	1.826	2.898	2.196	2.869	34	82	54	60	0.054	0.035	0.041	0.048
200	1.368	1.700	1.039	1.329	28	52	33	35	0.050	0.033	0.032	0.037
LSD species × treatment		0.615				13				0.005		
Sodicity (g kg^{-1} Na^+ humate)												
0	5.691	6.337	7.082	5.924	111	191	140	125	0.051	0.033	0.051	0.047
0.1	5.057	5.634	6.863	5.487	93	165	138	113	0.055	0.034	0.050	0.049
0.5	5.041	5.951	6.275	5.277	96	177	111	101	0.053	0.034	0.052	0.048
1.0	4.748	5.421	6.075	5.123	90	165	123	104	0.053	0.033	0.050	0.049
2.0	4.613	5.293	6.429	5.457	86	159	125	112	0.049	0.033	0.052	0.049
4.0	4.493	5.029	5.156	4.782	83	157	103	101	0.050	0.032	0.051	0.047
8.0	3.436	4.327	2.069	3.793	73	132	69	87	0.047	0.033	0.030	0.044
16.0	1.227	3.638	0.577	2.935	37	112	30	78	0.033	0.033	0.017	0.038
LSD species × treatment		0.656				18				0.005		

LSD, least significant differences at $P = 0.05$ for species × treatment interaction. Values for grain number per plant were rounded off to whole numbers.

potentially toxic trace elements such as boron, there are differences across seasons in temperature and drought, which, particularly in dryland agriculture, will directly affect the build-up of salts around the roots (Munns *et al.*, 2006). But, of course, field trials are ultimately essential (El-Hendawy *et al.*, 2009).

Importance of osmotic stress

Despite similar leaf cation concentrations in sodicity- and salinity-stressed plants, greater reduction in yield under salinity than under sodicity indicated the dominance of osmotic stress, most probably driven by high Cl^-, under salinity (Fig. 2). Osmotic effects of salinity stress, manifested as reduced water uptake, tiller number and leaf expansion (Munns & Tester, 2008), commenced soon after sprouting and continued throughout the life of the plant (Fig. 1). This osmotic stress effect of salinity has been known for some time (Husain *et al.*, 2003; James *et al.*, 2012; Wang *et al.*, 2013), and its importance is noted in a recent review (Munns & Gilliham, 2015); however, it has not attracted the research attention afforded to Na^+ exclusion (Bernstein, 1975; Tavakkoli *et al.*, 2010; Genc *et al.*, 2014). The two-stage salinity stress hypothesis (Munns, 1993) posited a lack of genetic variation in osmotic stress tolerance (phase I) but significant genetic variation in ionic effects (phase II), thus suggesting that improving the ability of the plant to exclude specific ions such as Na^+ would lead to salinity tolerance. Measurement of Na^+ concentration in leaf 3 after 10 d growth and exposure to salt stress in hydroponics has found wide acceptance in salinity studies (Munns *et al.*, 2006) and has been shown to correlate with salinity tolerance in some studies (cf. Genc *et al.*, 2013). However, the present and other studies (Genc *et al.*, 2007; Ul Haq *et al.*, 2014) suggest that it is unlikely to correlate strongly with grain yield.

In addition, in contrast to earlier reports of minimal genetic variation in osmotic stress tolerance under salinity (Munns, 1993), several studies have found evidence of genetic variation in osmotic stress tolerance (Neumann, 1997; Vetterlein *et al.*, 2004; De Costa *et al.*, 2007; James *et al.*, 2008; Cuin *et al.*, 2009; Rajendran *et al.*, 2009; Rahnama *et al.*, 2010; Tavakkoli *et al.*, 2010). For example, a study of 25 bread and 25 durum wheat cultivars found genotypic variation and different mechanisms to counter osmotic stress: durum wheat fully adjusted its sap osmotic potential with inorganic ions only (K^+, Na^+, Cl^-), while bread wheat used inorganic ions and organic osmolytes. The overall impact of salinity was similar: yield reductions of 42 and 44% for bread and durum wheat, respectively, at 150 mM NaCl, with wide genotypic variation within each wheat species (Cuin *et al.*, 2009). This finding, along with the documentation of durum and bread wheats with similar salinity tolerance in other studies (Munns *et al.*, 1995) and the current study, does not support the general assertion that durum wheat is more salt-sensitive than bread wheat (Colmer *et al.*, 2005; Munns & Tester, 2008).

Moreover, barley's greater salt tolerance in the field may be partly attributed to its rapid growth and early maturity (Munns *et al.*, 2006). The current study found it to be only marginally more salt-tolerant than bread and durum wheat. In the light of these recent developments, it is timely to focus more on the osmotic component of salinity tolerance. A question to consider is whether Na^+ could have a role as an osmoticum. But if Na^+ exclusion *per se* is essential for salinity tolerance, as considered by many researchers, presumably it has no role in osmoregulation under salt stress. This will be explored later.

Na^+ exclusion under salinity and sodicity

During the last two decades, reports on Na^+ exclusion and salinity stress have dominated the literature, while little has been reported on the role of Na^+ exclusion in sodicity tolerance (Pearson & Bernstein, 1958; Sharma, 1986, 1991; Rajpar *et al.*, 2004). However, despite the extensive research on salinity tolerance, surprisingly few studies have investigated the relationships between Na^+ exclusion and grain yield, with reports ranging from moderate correlations in bread wheat and durum wheat ($r = 0.43$–0.49, $n = 25$ for both bread and durum wheats; Cuin *et al.*, 2009; $r = 0.43$–0.59, $n = 25$ and $n = 24$ for bread and durum wheats, respectively; Zhu *et al.*, 2015) to no correlations in the present study (Fig. 2) and other studies with bread wheat (Genc *et al.*, 2013, 2014). Even less is known about the effects of Na^+ exclusion genes on grain yield. The salinity rate study presented here demonstrates that the presence of the Na^+ exclusion gene *Nax2* in durum wheat Tamaroi background (Munns *et al.*, 2012) was associated with a 13% increase in relative grain yield at 150 mM NaCl, and 5% at 200 mM NaCl. When different cereal species are compared, at 100 mM NaCl, despite a 100-fold variation in leaf Na^+, they all had a relative grain yield of *c.* 50%, indicating limitation of Na^+ exclusion under salinity stress. As shown in this and previous studies (Genc *et al.*, 2007; Shavrukov *et al.*, 2009), commercial Australian bread wheats are mostly efficient Na^+ excluders, and hence it is unlikely that introgression of Na^+ exclusion genes would improve their salt tolerance.

Contrary to salinity, under sodicity Na^+ exclusion in general and the *Nax2* gene specifically were associated with significant yield increases. At high sodicity (8 g kg^{-1} Na^+ humate), where significant differences in yield occurred between species, the naturally Na^+-excluding bread wheat Krichauff had a relative grain yield of 68% (Na^+ concentration in the penultimate leaf of 477 mg kg^{-1} DW), while the durum wheat Tamaroi, a relatively high Na^+ accumulator, had a relative grain yield of only 29% (26 333 mg kg^{-1} Na^+ in the penultimate leaf) (Fig. 2). The *Nax2* gene was effective in limiting Na^+ accumulation in durum wheat Tamaroi-*Nax2*, with a relative yield of 64% (3800 mg kg^{-1} Na^+ in penultimate leaf) (Fig. 2). The barley Clipper, with an Na^+ accumulation around halfway between that of bread wheat Krichauff and durum wheat Tamaroi (14 600 mg kg^{-1} DW in penultimate leaf), had 60% relative grain yield at this sodicity (Fig. 2). The higher-yielding and Na^+-excluding bread wheat Krichauff and durum wheat Tamaroi-*Nax2* either maintained K^+ or achieved higher Ca^{2+} and Mg^{2+}, indicating the importance of maintenance of these cations under sodicity (Fig. 3). As for the yield reduction in Na^+-excluding bread wheat and durum wheat Tamaroi-*Nax2* despite their excellent Na^+ exclusion, it is tempting to speculate that this was the result of energy expended on Na^+ exclusion rather than of energy expended on osmoregulation (Epstein *et al.*, 1980), as by definition sodic soils contain low salts (ECe < 4 dS m^{-1}) and should therefore not have low osmotic potential. Taken together, these results clearly demonstrate that Na^+ exclusion is more relevant to sodicity than salinity, and there is scope for improving sodicity tolerance in durum wheat by using the *Nax2* gene.

A new paradigm for breeding salt-tolerant cereals

Is there scope for further improvement of salt tolerance in cereals in view of the dominance of osmotic stress under salinity? Given evidence of genetic variation in osmotic stress tolerance (Neumann, 1997; Vetterlein *et al.*, 2004; De Costa *et al.*, 2007; James *et al.*, 2008; Cuin *et al.*, 2009; Rajendran *et al.*, 2009; Tavakkoli *et al.*, 2010), large-scale screening for genetic variation in osmotic stress tolerance would provide a good start. As drought stress also induces osmotic stress, knowledge gained from drought studies can be utilized in salinity studies. As surprising as it sounds, Na^+, which is regarded as a problem by most authors, may in fact be part of the solution. As demonstrated by Cuin *et al.* (2009), certain cereal lines can employ Na^+ as a 'cheap osmoticum' to achieve osmotic adjustment as seen in salt-loving halophytes (Flowers *et al.*, 1977), while others accumulate organic solutes, which can be energetically expensive and result in reduced growth/yield. It is proposed that there may be an optimal level of Na^+ accumulation in plant tissue which may be much higher than that in 'Na^+ excluders', such as most Australian bread wheats, and this could improve osmotic adjustment and water uptake under salinity. This notion that high tissue Na^+ can play an important role in salinity tolerance is supported by other studies (Bower & Wadleigh, 1948; Greenway & Munns, 1980; Apse *et al.*, 1999; Rus *et al.*, 2006; Yong *et al.*, 2015) and by a recent review (Munns & Gilliham, 2015). A mechanism for this effect may involve the compartmentation of Na^+ into vacuoles via a vacuolar antiport such as Na^+/H^+ to avert the deleterious effects of Na^+ in the cytosol and maintain osmotic balance by using Na^+ accumulation in the vacuole to drive water into the cells (Apse *et al.*, 1999).

In conclusion, Na^+ humate provides a rapid and reliable assessment of sodicity tolerance in cereals. Na^+ exclusion appears to be effective in maintaining yield under sodicity, and therefore improving the Na^+ exclusion ability of Na^+-accumulating species such as durum wheat and barley should have priority over bread wheat, which is already an excellent Na^+ excluder (Genc *et al.*, 2007). To this end, significant progress has already been made in durum wheat with identification of the *Nax2* gene (Munns *et al.*, 2012). Contrary to sodicity, benefits of Na^+ exclusion are overcome by the osmotic effect under salinity. In a recent review of the costs associated with saline soils, the authors note that 'A role of prebreeding is to provide germplasm to breeders that produces significant increases in yield in stressful environments' (Munns &

Gilliham, 2015). Imaginative crossing programmes (which include germplasm with different mechanisms of osmotic adjustment) combined with relevant prefield screening and whole-plant evaluation, rather than reliance on biomarkers, is important in order to exploit genotypic variation in osmoregulation and Na^+ and/or Cl^- tissue tolerance to achieve progress in breeding salt-tolerant cereals. Most recently, application by our group of this strategy has revealed, in the form of an exciting new salt-tolerant bread wheat line, the probable role of Na^+ as an efficient osmoticum. Further research at physiological and molecular level is needed to elucidate mechanisms of osmoregulation and Na^+ and/or Cl^- tissue tolerance.

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Author contributions

Y.G. and G.H.L. were involved in experimental design and the conceptualization of the project, carried out phenotyping, performed statistical analysis of the data, and drafted the manuscript. J.T. performed statistical analysis of the data and drafted the manuscript. K.O. was involved in conceptualizing of the project, supervised genotyping of the Tamaroi and Tamaroi-*Nax2* lines, and drafted the manuscript. All authors read and approved the final manuscript.

References

Apse MP, Aharon GS, Snedden WA, Blumwald E. 1999. Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. *Science* **285**: 1256–1258.

Asik BB, Turan MA, Celik H, Katkat AV. 2009. Effects of humic substances on plant growth and mineral nutrients uptake of wheat (*Triticum durum* cv. Salihli) under conditions of salinity. *Asian Journal of Crop Science* **1**: 87–95.

Bains SS, Fireman M. 1964. Effect of exchangeable sodium percentage on the growth and absorption of essential nutrients and sodium by five crop plants. *Agronomy Journal* **56**: 432–435.

Bernstein L. 1975. Effects of salinity and sodicity on plant growth. *Annual Review of Phytopathology* **13**: 295–312.

Bernstein L, Pearson GA. 1956. Influence of exchangeable sodium on the yield and chemical composition of plants: I. Green beans, garden beets, clover, and alfalfa. *Soil Science* **82**: 247–258.

Bower CA, Wadleigh CH. 1948. Growth and cationic accumulation by four species of plants as influenced by various levels of exchangeable sodium. *Soil Science Society of America Proceedings* **13**: 218–223.

Butler DG, Cullis BR, Gilmour AR, Gogel BJ. 2009. *ASReml-R reference manual (release 3)*. Toowoomba, Qld, Australia: Queensland Department of Primary Industries.

Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R. 2007. HKT1;5-like cartion transporters linked to Na^+ exclusion loci in wheat, *Nax2* and *Kna1*. *Plant Physiology* **143**: 1918–1928.

Colmer TD, Munns R, Flowers TJ. 2005. Improving salt tolerance of wheat and barley: future prospects. *Australian Journal of Experimental Agriculture* **45**: 1425–1443.

Cuin TA, Tian Y, Betts SA, Chalmandrier R, Shabala S. 2009. Ionic relations and osmotic adjustment in durum and bread wheat under saline conditions. *Functional Plant Biology* **36**: 1110–1119.

Dang YP, Dalal RC, Buck SR, Harms B, Kelly R, Hochman Z, Schwenke GD, Biggs AJW, Ferguson NJ, Norrish S *et al.* 2010. Diagnosis, extent, impacts and management of subsoil constraints in the northern grains cropping region of Australia. *Australian Journal of Soil Research* **48**: 105–119.

De Costa W, Zörb C, Hartung W, Schubert S. 2007. Salt resistance is determined by osmotic adjustment and abscisic acid in newly develop maize hybrids in the first phase of salt stress. *Physiologia Plantarum* **131**: 311–321.

El-Hendawy SE, Ruan Y, Hu Y, Schmidhalter U. 2009. A comparison of screening criteria for salt tolerance of wheat under field and controlled environmental conditions. *Journal of Agronomy and Crop Science* **195**: 356–367.

Epstein E, Norlyn JD, Rush DW, Kingsbury RW, Kelley DB, Cunningham GA, Wrona AF. 1980. Saline culture of crops: a genetic approach. *Science* **210**: 399–404.

Flowers TJ, Troke PF, Yeo AR. 1977. The mechanism of salt tolerance in halophytes. *Annual Review of Plant Physiology* **28**: 89–121.

Gaur AC, Bhardwaj KKR. 1971. Influence of sodium humate on the crop plants inoculated with bacteria of agricultural importance. *Plant and Soil* **35**: 613–621.

Genc Y, McDonald GK, Tester M. 2007. Reassessment of tissue Na^+ concentration as a criterion for salinity tolerance in bread wheat. *Plant, Cell & Environment* **30**: 1486–1498.

Genc Y, Oldach K, Gogel BJ, Wallwork H, McDonald GK, Smith AB. 2013. Quantitative trait loci for agronomical and physiological traits for a bread wheat population grown in environments with a range of salinity levels. *Molecular Breeding* **32**: 39–59.

Genc Y, Oldach K, Verbyla A, Lott G, Hassan M, Tester M, Wallwork H, McDonald GK. 2010a. Sodium exclusion QTL associated with improved seedling growth in bread wheat under salinity stress. *Theoretical and Applied Genetics* **121**: 877–894.

Genc Y, Taylor J, Rongala J, Oldach K. 2014. A major locus for chloride accumulation on chromosome 5A in bread wheat. *PLoS ONE* **9**: e98845.

Genc Y, Tester M, McDonald GK. 2010b. Calcium requirement of wheat in saline and non-saline conditons. *Plant and Soil* **327**: 331–345.

Greenway H, Munns R. 1980. Mechanisms of salinity tolerance in nonhalophytes. *Annual Review of Plant Physiology* **31**: 149–190.

Grewal HS, Cornish P, Norrish S. 2004. Differential response of wheat cultivars to subsoil salinity/sodicity. In: Fischer T, Turner N, Angus J, McIntyre L, Robertson M, Borrell A, Lloyd D, eds. *Proceedings of the 4th International Crop Science Congress. Brisbane, Australia 26 September–1 October 2004*. Gosford, NSW, Australia: The Regional Institute Ltd.

Harris BN, Sadras VO, Tester M. 2010. A water-centred framework to assess the effects of salinity on the growth and yield of wheat and barley. *Plant and Soil* **336**: 377–389.

Husain S, Munns R, Condon AG. 2003. Effect of sodium exclusion trait on chlorophyll retention and growth of durum wheat in saline soil. *Australian Journal of Agricultural Research* **54**: 589–597.

Iakimenko OS. 2005. Commercial humates from coal and their influence on soil properties and initial plant development. In: Perminova IV, Hatfield K, Hertkorn N, eds. *Use of humic substances to remediate polluted environments: from theory to practice*. Dordrecht, the Netherlands: Springer, 365–378.

James RA, Blake C, Zwart AB, Hare RA, Rathjen AJ, Munns R. 2012. Impact of ancestral wheat sodium exclsuion genes *Nax1* and *Nax2* on grain yield of durum wheat on saline soils. *Functional Plant Biology* **39**: 609–618.

James RA, Davenport RJ, Munns R. 2006. Physiological characterisation of two genes for Na^+ exclusion in durum wheat: *Nax1* and *Nax2*. *Plant Physiology* **142**: 1537–1547.

James RA, von Caemmerer S, Condon AG, Zwart AB, Munns R. 2008. Genetic variation in tolerance to the osmotic stress component of salinity stress in durum wheat. *Functional Plant Biology* **35**: 111–123.

Katkat AV, Celik H, Turan MA, Asik BB. 2009. Effects of soil and foliar applications of humic substances on dry weigth and mineral nutrients uptake of wheat under calcareous soil conditions. *Australian Journal of Basic and Applied Sciences* **3**: 1266–1273.

Kingsbury RW, Epstein E. 1986. Salt sensitivity in wheat. *Plant Physiology* **80**: 651–654.

Kulikova NA, Stepanova EV, Koroleva OV. 2005. Mitigating activity of humic substances: direct influence on biota. In: Perminova IV, Hatfield K, Norbert Hertkorn N, eds. *Use of humic substances to remediate polluted environments: from theory to practice*. Dordrecht, the Netherlands: Springer, 285–309.

Leventoglu H, Erdal I. 2014. Effect of high humic substance levels on growth and nutrient concentration of corn under calcareous conditions. *Journal of Plant Nutrition* **37**: 2074–2084.

Luo Q, Bingjun Y, Liu Y. 2005. Differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *G. soja* under NaCl stress. *Journal of Plant Physiology* **162**: 1003–1012.

Maas EV, Hoffman GJ. 1977. Crop salt tolerance – current assessment. *ASCE Journal of Irrigation and Drainage Division* **103**: 115–134.

Mandel J. 1957. Fitting a straight line to certain types of cumulative data. *Journal of the American Statistical Association* **52**: 552–566.

Martin P, Koebner R. 1995. Sodium and chloride ions contribute synergistically to salt toxicity in wheat. *Biologia Plantarum* **37**: 265–271.

Marx JL. 1979. Plants: can they live in salt water and like it? *Science* **206**: 1168–1169.

Munns R. 1993. Physiological processes limiting plant growth in saline soils: some dogmas and hypothesis. *Plant, Cell & Environment* **16**: 15–24.

Munns R. 2005. Genes and salt tolerance: bringing them together. *New Phytologist* **167**: 645–663.

Munns R. 2011. Plant adaptations to salt and water stress: differences and commonalities. *Advances in Botanical Research* **57**: 1–32.

Munns R, Gilliham M. 2015. Salinity tolerance of crops – what is the cost? *New Phytologist* **208**: 668–673.

Munns R, James RA. 2003. Screening methods for salnity tolerance: a case study with tetraploid wheat. *Plant and Soil* **253**: 201–218.

Munns R, James RA, Lauchli A. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany* **57**: 1025–1043.

Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, Byrt CS, Hare RA, Tyerman SD, Tester M *et al.* 2012. Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. *Nature Biotechnology* **30**: 360–364.

Munns R, Schachtman DP, Condon AG. 1995. The significance of a two-phase growth response to salinity in wheat and barley. *Australian Journal of Plant Physiology* **22**: 561–569.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. *Annual Review of Plant Biology* **59**: 651–681.

Neumann P. 1997. Salinity resistance and plant growth revisited. *Plant, Cell & Environment* **20**: 1193–1198.

Noble CL, Rogers ME. 1992. Arguments for the use of physiological criteria for improving the salt tolerance in crops. *Plant and Soil* **146**: 99–107.

Pearson GA, Bernstein L. 1958. Influence of exchangeable sodium on yield and chemical composition of plants: II. Wheat, barley, oats, rice, tall fescue, and tall wheatgrass. *Soil Science* **86**: 254–261.

Qadir M, Schubert S, Ghafoor A, Murtaza G. 2001. Amelioration strategies for sodic soil: a review. *Land Degradation and Development* **12**: 357–386.

R Development Core Team. 2015. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rahnama A, James RA, Poustini K, Munns R. 2010. Stomatal conductance as a screen for osmotic stress tolerance in durum wheat growing in saline soil. *Functional Plant Biology* **37**: 255–263.

Rajendran K, Tester M, Roy SJ. 2009. Quantifying the three main components of salinity tolerance in cereals. *Plant, Cell & Environment* **32**: 237–249.

Rajpar I, Sial NB, Talpur U. 2004. Effect of soil sodicity on the growth, ion content and yield of wheat (*Triticum aestivum* L.). *Journal of Applied Sciences* **4**: 649–653.

Rengasamy P, North S, Smith A. 2010. *Diagnosis and management of sodicity and salinity in soil and water in the Murray Irrigation region*. Adelaide, SA, Australia: The University of Adelaide.

Rengasamy P, Olsson KA. 1991. Sodicity and soil structure. *Australian Journal of Soil Research* **29**: 935–952.

Reuter DJ, Robinson AD. 1997. *Plant analysis. An interpretation manual, 2nd edn*. Melbourne, Vic., Australia: CSIRO.

Richards LA. 1954. *Diagnosis and improvement of saline and alkali soils. Agricultural Handbook 60*. Washington, DC, USA: United States Department of Agriculture.

Rozema J, Flowers T. 2008. Crops for a salinized world. *Science* **322**: 1478–1480.

Rus A, Baxter I, Muthukumar B, Gustin J, Lahner B, Yakubova E, Salt DE. 2006. Natural variants of *AtHKT1* enhance Na^+ accumulation in two wild populations of *Arabidopsis*. *PLoS Genetics* **2**: e210.

Shahid SA, ur Rahman K. 2011. Soil salinity development, classification, assessment, and management in irrigated agriculture. In: Pessarakli M, Szabolcs I, eds. *Handbook of plant and crop stress*. Boca Raton, FL, USA: Taylor & Francis Group, CRC Press, 23–39.

Sharif M, Khattak RA, Sarir MS. 2002. Effect of different levels of lignitic coal derived humic acid on growth of maize plants. *Communications in Soil Science and Plant Analysis* **33**: 3567–3580.

Sharma SK. 1986. Mechanisms of tolerance in rice varieties differing in sodicity tolerance. *Plant and Soil* **93**: 141–145.

Sharma SK. 1991. Effect of exchangeable sodium on growth, yield and ionic relations in wheat genotypes differing in sodicity tolerance. *Indian Journal of Plant Physiology* **34**: 349–356.

Sharma SK. 2010. *Success stories on development, spread and impact of salt-tolerant varieties of rice, wheat and mustard in India*. [WWW document] URL http://www.plantstress.com/Files/ Salt_India.pdf [accessed 19 June 2015].

Shavrukov Y, Langridge P, Tester M. 2009. Salinity tolerance and sodium exclusion in genus *Triticum*. *Breeding Science* **59**: 671–678.

Sheldon AR, Dalal RC, Menzies NW. 2006. Toxicity of Na^+ and Cl^- to wheat and chickpea. In: *18th World Congress of Soil Science. International Union of Soil Science, 9–15 July, 2006*. Philadelphia, PA, USA.

Sinclair TR, Tanner CB, Bennett JM. 1984. Water-use efficiency in crop production. *BioScience* **34**: 36–40.

Slabu C, Zörb C, Steffens D, Schubert S. 2009. Is salt stress of faba bean (*Vicia faba*) caused by Na^+ or Cl^- toxicity? *Journal of Plant Nutrition and Soil Science* **172**: 644–650.

Tahir MM, Khurshid M, Khan MZ, Abbasi MK, Kazmi MH. 2011. Lignite-derived humic acid effect on growth of wheat plants in different soils. *Pedosphere* **21**: 124–131.

Tavakkoli E, Fatehi F, Coventry S, Rengasamy P, McDonald GK. 2011. Additive effects of Na^+ and Cl^- on barley growth under salinity stress. *Journal of Experimental Botany* **62**: 2189–2203.

Tavakkoli E, Rengasamy P, McDonald GK. 2010. The response of barley to salinity stress differs between hydroponic and soil systems. *Functional Plant Biology* **37**: 621–633.

Turan MA, Asik BB, Katkat AV, Celik H. 2011. The effects of soil-applied humic substances to the dry weight and mineral nutrient uptake of maize plants under soil-salinity conditions. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **39**: 171–177.

Ul Haq T, Akhtar J, Steele KA, Munns R, Gorham J. 2014. Reliability of ion accumulation and growth components for selecting salt tolerant lines in large populations of rice. *Functional Plant Biology* **41**: 379–390.

Van De Venter HA, Furter M, Dekker J, Cronje IJ. 1991. Stimulation of seedling root growth by coal-derived sodium humate. *Plant and Soil* **138**: 17–21.

Van Tonder JT. 2008. Effect of potassium humate on soil properties and growth of wheat. Magister Scientiae Agriculturae thesis, University of the Free State, Bloemfontein, South Africa.

Verbyla AP, Cullis BR, Kenward MG, Welham SJ. 1999. The analysis of designed experiments and longitudinal data by using smoothing splines. *Journal of the Royal Statistical Society Series C: Applied Statistics* **48**: 269–311.

Vetterlein D, Kuhn K, Schubert S, Jahn R. 2004. Consequences of sodium exclusion for the osmotic potential in the rhizosphere – comparison of two maize cultivars differing in Na^+ uptake. *Journal of Plant Nutrition and Soil Science* **167**: 337–344.

VSN International. 2012. *GenStat for Windows, 15th edn.* Hemel Hempstead, UK: VSN International.

Wang M, Zheng Q, Shen Q, Guo S. 2013. The critical role of potassium in plant stress response. *International Journal of Molecular Science* **14**: 7370–7390.

Wheal MS, Fowles TO, Palmer LT. 2011. A cost-effective acid digestion method using closed polypropylene tubes for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis of plant essential elements. *Analytical Methods* **3**: 2854–2863.

Wheal MS, Palmer LT. 2010. Chloride analysis of botanical samples by ICP-OES. *Journal of Analytical Atomic Spectrometry* **25**: 1946–1952.

White PJ, Broadley MR. 2001. Chloride in soils and its uptake and movement within the plant: a review. *Annals of Botany* **88**: 967–988.

Wright D, Rajper I. 2000. An assessment of the relative effects of adverse physical and chemical properties of sodic soil on the growth and yield of wheat (*Triticum aestivum* L.). *Plant and Soil* **223**: 277–285.

Yong H-Y, Wang C, Bancroft I, Li F, Wu X, Kitashiba H, Nishio T. 2015. Identification for a gene controlling variation in the salt tolerance of rapeseed (*Brassica napus* L.). *Planta* **242**: 313–326.

Zadoks JC, Chang TT, Konzak CF. 1974. A decimal code for the growth stages of cereals. *Weed Research* **14**: 415–421.

Zhu M, Shabala S, Shabala L, Fan Y, Zhou MX. 2015. Evaluating predictive values of various physiological indices for salinity stress tolerance in wheat. *Journal of Agronomy and Crop Science*. doi: 10.1111/jac.12122.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Effects of salinity and sodicity on shoot growth of barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheat (*Triticum turgidum* ssp. *durum*) Tamaroi at heading grown in a potting mix in a controlled environment room.

Fig. S2 Predicted incremental water-use curves over time in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different conditions of salinity and sodicity applied to the potting mix.

Fig. S3 Plant growth in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* at heading under control, sodicity and salinity in a potting mix in a controlled environment.

Table S1 Elemental analyses of Na^+ humate by inductively coupled plasma optical emission spectrophotometer

Table S2 Chemical properties of soils at six location–year combinations in South Australia

Table S3 Slopes of incremental water use over time (surrogate for growth rate) derived from linear regressions up to heading stage in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff, and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different salinity and sodicity conditions

Table S4 Leaf sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}) and chloride (Cl^-) concentrations in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different salinity and sodicity conditions

Table S5 Nontransformed leaf sodium (Na^+) concentrations in barley (*Hordeum vulgare*) Clipper, bread wheat (*Triticum aestivum*) Krichauff and durum wheats (*Triticum turgidum* ssp. *durum*) Tamaroi and Tamaroi-*Nax2* under different salinity and sodicity conditions

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