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REGULAR ARTICLE

Salinity tolerance and ion accumulation in chickpea (*Cicer arietinum* L.) subjected to salt stress

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Abstract
Aims Chickpea (*Cicer arietinum* L.) is considered a salt sensitive species, but some genetic variation for salinity tolerance exists. The present study was initiated to determine the degree of salt tolerance among chickpea genotypes, and the relationship between salt tolerance and ion accumulation in leaves and reproductive tissues.
Methods Three experiments were conducted in a glasshouse in Perth, Western Australia, in which up to 55 genotypes of chickpea were subjected to 0, 40 or 60 *mM* NaCl added to the soil to determine the variation in salt tolerance, and the association between salt tolerance and reproductive success. Pod and seed numbers, seed yield and yield components, pollen viability, in vitro pollen germination and in vivo pollen tube growth, were used to evaluate reproductive success. Leaves, flowers and seeds were sampled in the reproductive phase to measure the concentrations of sodium, potassium and chloride ions in these organs.
Results When grown in soil with 40 *mM* NaCl, a 27-fold range in seed yield was observed among the 55 chickpea genotypes. The increased salt tolerance, as measured by yield under salinity or relative yield under saline conditions, was positively associated with

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higher pod and seed numbers, and higher shoot biomass, but not with time to 50 % flowering nor with the number of filled pods in the non-saline treatment. Pod abortion was higher in the salt sensitive genotypes, but pollen viability, in vitro pollen germination and in vivo pollen tube growth were not affected by salinity in either the salt tolerant or salt sensitive genotypes. The concentrations of sodium and potassium ions, but not chloride, in the seed were significantly higher in the sensitive (106 μmol g^{-1} DM of sodium and 364 μmol g^{-1} DM of potassium) than in the tolerant (74 and 303 μmol g^{-1} DM, respectively) genotypes. Sodium and potassium, but particularly chloride, ions accumulated in leaves and in pod wall, whereas accumulation in the seed was much lower.

Conclusions Considerable genotypic variation for salt tolerance exists in chickpea germplasm. Selection for genotypes with high pod and/or seed numbers that accumulate low concentrations of salt in the seed will be beneficial.

Keywords Salinity · Salt sensitivity · Reproductive success · Leaf sodium · Leaf chloride · Pollen viability · Pollen tube growth · Genotypic variation

Abbreviations

DAS	Days after sowing
DM	Dry mass
FDA	Fluorescein diaceteate
G x E	Genotype by environment

Introduction

Salinity is an increasing problem in many regions worldwide. Chickpea is particularly sensitive to salinity (Flowers et al. 2010); attempts at finding differences in salinity tolerance have been undertaken (Vadez et al. 2007; Krishnamurthy et al. 2011). Vadez et al. (2007) found a six-fold range in the yield under salinity in the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) chickpea mini-core collection when exposed to 80 *mM* sodium chloride (NaCl) in the soil from sowing. While Krishnamurthy et al. (2011) reported that there was considerable genotype by environment (G x E) interaction for yield under salinity among the 294 accessions from the ICRISAT mini-core and reference collections (Upadhyaya and Ortiz 2001; Upadhyaya et al. 2008) exposed to NaCl in the same way as Vadez et al. (2007), there were 12 genotypes that were consistently tolerant of salinity in both a Vertisol (uniform-textured, deep black soil containing more than 30 % clay-sized panicles) and an Alfisol (red soil with low content of clay-sized particles enriched in aluminum- and iron-bearing minerals particularly at the surface and clay content increasing with depth) soil (El-Swaify et al. 1985). However, the question remains whether the genotypic variation is maintained when the tolerant and sensitive genotypes are grown in other soil types and when grown in a cool Mediterranean-type winter climate such as in south-west Australia (daily mean growing-season pan evaporation=~3.6 mm) rather than a cool-season sub-tropical winter climate such as at ICRISAT (daily mean growing-season pan evaporation=~4.5 mm).

The present experiments were initiated to determine whether salinity tolerant and sensitive genotypes selected in pots of artificially-salinized soil outdoors at the short-season, sub-tropical environment at ICRISAT near Hyderabad, India, had similar responses to salinity when grown in pots of a different soil salinized by addition of NaCl, but under longer-season glasshouse conditions in Australia. Consistency in salt tolerance rankings would save screening time in each local environment, but such consistency may be thwarted by any G x E interaction for salinity tolerance. Fifty lines selected for differences in salinity tolerance in India were transferred to Australia and evaluated for their salinity tolerance in a local soil using a similar system to that used at ICRISAT, but in a glasshouse in Perth, Western Australia. The 50 lines were augmented by a number of local check cultivars. As previous research had shown that the seed yield under salinity was correlated with the number of pods and seeds (Vadez et al. 2007, 2012a, b), the effect of salinity on reproductive success was also measured.

Plants can tolerate salinity by excluding sodium and chloride at the roots, sequestering the ions in lower leaves so that ion toxicity is avoided in the young growing leaves and developing reproductive tissues, or by tolerating the ions in the young and developing tissues (Munns and Tester 2008). Sodium, potassium and chloride in the youngest fully-expanded leaves, flower parts, pod walls, and developing seeds were measured to determine whether the reproductive success associated with salinity tolerance was linked to ion concentrations in the tissues.

In the three experiments conducted in this study we aimed: (i) to determine whether there was variation for salt tolerance, measured as yield under salinity or yield under saline conditions relative to yield under non-saline conditions, among genotypes of chickpea; (ii) to determine whether the yield under saline conditions arose from differences in reproductive success; and (iii) to determine whether the effect of salinity on seed set arose from its influence on pollen viability, pollen germination, pollen tube growth or ion concentrations in the plant tissues.

Materials and methods

Three experiments were conducted in 2009 and 2010 in evaporatively-cooled glasshouses at The University of Western Australia, Crawley, Perth, Western Australia (31°57′ S, 115°47′ E). In 2009 the glasshouse day/night temperature (± range) and relative humidity varied from 22.5±2.5 °C/15±2 °C and 40/90 %, while in 2010 day/night temperature and humidity varied from 22±2 °C/12±4 °C and 40/90 %, respectively.

Experiment 1

Fifty-five genotypes of chickpea, 50 imported from ICRISAT and reportedly either salt tolerant or sensitive (Table 1), including CSG 8962 a cultivar released in India for its salt tolerance, and five local check genotypes, were grown in non-draining, plastic pots (20 cm diameter, 19 cm high) containing 4.5 kg soil. The genotypes included desi (small seeded, thick testa) and kabuli (large seeded, thin testa) types with a range of phenologies and seed sizes (Table 1). The soil was a self-mulching cracking clay (Department of Agriculture and Food, Western Australia 2002), also known as a self-mulching Vertosol (Isbell 1996), with 50 % clay, 22 % silt, 27 % sand and 1 % organic matter (pH=8 in 0.01 M $CaCl_2$, electrical conductivity (1:5)=0.5 μS m^{-1}=~5 *mM* in the soil solution, CEC=34.3 mequiv.100^{-g}) from the upper 0.1 m of a field site near Bindi Bindi (30°37′S, 116°28′E), Western Australia. Nutrients were added (g pot^{-1}containing 4.5 kg soil) as: 0.675 KNO_3, 0.963 $Ca(NO_3)_2$, 0.859 KH_2PO_4, 0.112 $MgSO_4$, and 3 mL pot^{-1} of Hoaglands micronutrient stock solution. The water content (w/w) at field capacity (i.e. freshly drained pot) was 34.5 %. For each pot, four seeds of a particular genotype were planted on 13 May 2009 along with a granular chickpea inoculum *Bradyrhizobium* (Nodulator®, Becker-Underwood, Somersby, NSW, Australia). There were four replicate pots of each genotype in each treatment. Before sowing, 1.814 g pot^{-1} of NaCl was applied to each of the 220 pots in the saline treatment by adding the NaCl in the solution also containing the nutrients and watering the pots up to field (pot) capacity (to give an initial salinity of 20 *mM* NaCl in the soil solution), while the non-saline controls were watered up to field capacity with an equivalent volume of solution only containing the nutrients. After 28 days, the pots were left for 2 days to transpire about 400 mL of water and then a further 1.814 g pot^{-1} of NaCl in 400 mL of de-ionised water per pot was added to each saline pot to increase the salt concentration to 40 *mM* NaCl, while an equivalent volume of de-ionised water was added to the non-saline control pots. Throughout the study, the plants were watered every 2–3 days to bring the water content back to field capacity. Each Monday, all pots were weighed and water added to bring the water content to 100 % field capacity, while on Wednesdays and Fridays, 10 representative pots per treatment were weighed and used to judge the approximate volume of water to be added to return the pots to near 100 % field capacity.

From 6 to 16 days after sowing (DAS), the number of seeds that had emerged was recorded daily, with a final count being made 29 DAS after which the plants were thinned to two similarly-sized plants per pot. The date of first flower (flower fully open) was recorded on each of the two plants per pot. When all genotypes were podding (98 DAS), one or two of the youngest fully-expanded leaves per pot were sampled for analyses of sodium, potassium and chloride. Each leaf was placed into a labelled paper envelope, oven-dried at 60 °C for 48 h, weighed and then the sample was extracted in 0.5 M nitric acid in 10 mL tubes placed on a shaker for 48 h. The samples were then analysed for sodium and potassium on a Sherwood flame photometer (Model 410, Sherwood Scientific, Cambridge, UK) and chloride was measured using a chloridometer (SLAMED, model 50CL 1–50, Frankfurt, Germany). Measurements on reference plant tissue of known ion concentration showed sodium, potassium and chloride concentrations within 5 %, 17 % and 2 %, respectively, of the expected values; no adjustments were made to the data presented.

Table 1 Genotypes of chickpea grown in the glasshouse in Experiment 1, classified as salt tolerant or salt sensitive when selected at ICRISAT or local check with no salinity ranking, and as desi or kabuli types. Also presented is the mean time to first flower in days after sowing (DAS), seed size (100-seed weight) in non-saline conditions, number of filled pods and seed yield (g per plant) at maturity in 40 *mM* NaCl from the ANOVA. The genotypes are listed from the highest to the lowest seed yield per plant. Genotypes differed significantly ($P<0.001$, ***); least significant differences (LSD, $P=0.05$) for the genotype means are given. The genotypes used in Experiment 2 are shaded in grey and those in Experiment 3 are shaded in grey with an asterisk

GENOTYPE	ICRISAT ranking	TYPE	Time to first flower (DAS)	100-seed weight (g)	Filled pods plant^{-1}	Seed yield plant^{-1} (g)
ICC 15518	Sensitive	Kabuli	95.0	33.73	47.0	16.44
ICC 4533	Tolerant	Desi	65.9	18.74	68.9	11.23
ICC 9942*	Tolerant	Desi	74.4	21.93	39.6	10.57
GENESIS 836*	Check	Desi	78.6	15.97	174.9	9.66
ICC 7819	Tolerant	Desi	85.6	18.35	72.9	9.26
ICCV 95423	Tolerant	Kabuli	71.2	29.11	49.0	8.56
ICC 6263	Sensitive	Desi	67.0	32.32	30.6	8.08
ICC 283	Tolerant	Desi	70.5	12.09	89.2	8.00
ICC 5879	Tolerant	Desi	69.0	22.69	45.6	7.32
ICC 32	Tolerant	Kabuli	67.7	59.96	57.2	6.70
ICCV 8855	Tolerant	Kabuli	81.2	22.60	49.8	6.39
ICC 13357	Sensitive	Kabuli	81.8	19.05	30.0	6.21
ICC 15868	Tolerant	Desi	72.6	12.21	59.1	6.04
GENESIS 510	Check	Desi	79.0	16.52	33.5	6.02
ICC 14799	Tolerant	Desi	71.9	17.41	48.8	5.77
ICC 2580	Tolerant	Desi	63.0	18.83	49.2	5.73
ICC 11121	Tolerant	Desi	69.4	13.05	50.5	5.45
JG 11*	Tolerant	Desi	63.5	30.84	33.8	5.28
ICC 6306	Sensitive	Desi	85.8	13.95	29.2	5.08
ICC 13077	Sensitive	Kabuli	96.7	21.36	39.2	5.08
ICC 1915	Sensitive	Desi	92.8	15.23	26.0	4.95
ICC 10399	Tolerant	Desi	67.4	15.96	38.9	4.90
ICC 12866	Tolerant	Desi	73.4	15.09	35.4	4.79
K 850	Tolerant	Desi	71.6	26.28	29.9	4.41
WR 315	Tolerant	Desi	60.8	16.46	33.4	4.34
ICC 6816	Tolerant	Desi	71.9	14.64	37.9	4.31
ICC 8522	Sensitive	Desi	95.0	13.84	31.2	4.13

Table 1 (continued)

GENOTYPE	ICRISAT ranking	TYPE	Time to first flower (DAS)	100-seed weight (g)	Filled pods plant^{-1}	Seed yield plant^{-1} (g)
ICC 15888	Tolerant	Kabuli	71.5	17.85	34.0	4.10
ICC 15606	Tolerant	Desi	67.6	18.14	34.0	4.10
ICC 10885	Sensitive	Desi	113.2	25.85	17.8	3.84
ICC 1083	Tolerant	Desi	60.2	16.81	35.2	3.82
ICC 12964	Check	Kabuli	104.3	16.74	24.6	3.75
ICC 14595	Tolerant	Desi	60.8	23.65	27.5	3.68
CSG 8962	Tolerant	Desi	79.6	14.07	32.5	3.67
ICCV 2	Sensitive	Desi	51.3	21.83	25.0	3.64
WALR 50	Check	Desi	85.4	17.54	31.8	3.53
ICC 2263	Tolerant	Desi	76.2	15.46	37.4	3.46
ICC 12155	Tolerant	Desi	62.1	11.48	33.4	3.28
JG 62	Tolerant	Desi	62.1	16.74	43.1	3.26
ICCV 96029	Sensitive	Desi	63.2	10.21	33.0	3.20
ICC 1431	Tolerant	Desi	74.5	14.81	27.3	3.08
SONALI	Check	Desi	59.2	18.27	28.0	3.03
ICC 12824	Tolerant	Desi	75.2	13.85	35.2	3.03
ICC 7272	Tolerant	Kabuli	72.8	25.12	30.7	2.88
ICC 14669	Tolerant	Desi	73.1	19.07	27.9	2.85
ICC 5337	Sensitive	Kabuli	91.2	19.08	11.4	2.24
ICC 11284*	Tolerant	Desi	72.6	16.18	26.9	2.11
ICC 5003	Tolerant	Desi	78.8	18.63	14.3	2.06
ICC 8318*	Tolerant	Desi	56.5	26.54	12.5	1.72
ICCV 10	Tolerant	Desi	76.0	18.71	9.5	1.71
ICC 14778	Tolerant	Desi	70.7	12.69	15.5	1.38
L 550 (ICC 4973)	Tolerant	Kabuli	88.2	22.39	20.2	1.33
ICC 2242	Sensitive	Desi	107.4	9.78	2.2	0.18
RUPALI*	Check	Desi	66.1	15.20	1.4	0.12
ICC 5845	Tolerant	Desi	94.8	16.32	0.0	0.03
LSD ($P = 0.05$)			**11.50*****	**11.98*****	**32.55*****	**6.03*****

At maturity, the number of plants per pot was recorded, the plants were then cut off at ground level, pods removed, and the remainder of the shoot was dried (with pod shells added back to the shoot sample) in a forced-draught oven at 70 ° C for 48 h and weighed. The total number of pods was counted, the number of empty pods recorded, before the seeds were removed, counted and weighed after drying in a fan-forced drier at 30 ° C for 2 days.

Experiment 2

The second experiment was similar to Experiment 1 except that it was conducted in 2010 with 10 chickpea genotypes. The 10 genotypes were chosen to represent a range of seed yields when grown with 40 *mM* NaCl in 2009 (Table 1). One hundred and twenty of the same size of pots were filled with the same soil and the same protocols were used as in Experiment 1, except that there were three treatments: (i) a non-saline treatment, (ii) 40 *mM* NaCl added to the soil, and (iii) 60 *mM* NaCl added to the soil. Half of each saline treatment was applied at seeding and half at 28 DAS. Four seeds were sown in each pot on 20 May 2010 and the number of seedlings per pot that had emerged was recorded every 1–4 days from 4 to 27 DAS at which time the plants were thinned to two plants per pot. The date of first flower (flower fully open), and pod (pod ~3 mm long) was recorded for each plant in each pot. When all the plants had at least one flower (84 DAS), two of the youngest fully-expanded leaves of one of the two randomly-chosen plants per pot were sampled and analysed for sodium, potassium and chloride as described for Experiment 1. Measurements on reference plant tissue of known ion concentration showed sodium, potassium and chloride concentrations within 2.5 %, 12 % and 1 %, respectively, of the expected values; no adjustments were made to the data presented. Sampling at maturity for biomass and yield components was as in Experiment 1.

Experiment 3

The third experiment was grown adjacent to Experiment 2 at the same time and in the same glasshouse. Six of the 10 chickpea genotypes (Table 1) grown in Experiment 2 were selected, three that were salt tolerant and three that were salt sensitive in Experiment 1. Each of the three salt tolerant genotypes was paired with a salt sensitive genotype with similar phenology. Forty-eight pots were filled with the same soil and the same protocols as in Experiment 1, with two treatments: (i) a non-saline treatment, and (ii) 40 *mM* NaCl added to the soil. Half of the NaCl was applied at seeding and half at 28 DAS, as in Experiment 2. Four seeds were sown in each pot on 20 May 2010 and thinned to two plants at 27 DAS.

In two of the four replicate pots, flowers were tagged twice per week from first flower to 100 DAS in both plants per pot; the date when the flower opened, and when a 3-mm long pod was observed was recorded on the tag. At maturity the plants were cut at the soil surface, and pods were removed and sorted into flowering date. The number of tags (flowers), pods, empty pods and seeds for each flowering date was counted before oven drying (30 °C for 48 h) and weighing the seeds. Untagged pods were also counted, the number of empty pods recorded before hand-threshing and seed number and oven-dry mass recorded. Finally, the pod shells, stems and leaves of the bulk sample were again oven-dried (70 °C for 48 h) before weighing. Flower abortion was calculated from tags where no podding date was recorded. Pod abortion was calculated from tags where a podding date was recorded, but at maturity no pod was present or, if a pod was present, it had a small or no seed (Leport et al. 2006).

In the other two replicate pots, when flowering had been established for about 10 days in one of the three pairs of genotypes, 20 hooded buds from each of the saline and non-saline treatments were collected at around 10.00 h; half for the determination of pollen viability and half for the determination of pollen germination in vitro (Fang et al. 2010). To determine the influence of salinity on pollen tube growth, flowers from the top branches in the saline and non-saline treatments of the salt tolerant genotype Genesis 836 and the salt sensitive genotype Rupali were reciprocally-pollinated by hand from 17.00 to 18.00 h at the stage when petals were visible and slightly smaller than the sepals (Clarke and Siddique 2004). Stigmas of saline plants were pollinated with pollen from non-saline plants, while stigmas of non-saline plants were pollinated with pollen from saline plants (10 flowers per combination). The flowers were harvested 24 h later to measure pollen germination and pollen tube growth in the pistil in each treatment (Fang et al. 2010).

Pollen was collected in an Eppendorf tube by squeezing the keel of the flower from the base upwards with forceps until most pollen exuded through the tip. Pollen viability was assessed using the fluorochromatic reaction adapted from Heslop-Harrison and Heslop-Harrison (1970). Fluorescein diacetate (FDA, 2 mg) was dissolved in 1 mL of acetone and a drop of the acetone-FDA solution put on a microscope slide and allowed to evaporate. Pollen was mixed with a 10 % sucrose solution and a drop of the solution placed on the stain of evaporated acetone-FDA and covered with a cover slip. Pollen grains with a grey colour under a fluorescence microscope (Zeiss Axiocam MRm, Oberkochen, Germany) were assessed as having lost viability. The percentage of viable and unviable pollen was measured by examining 300 grains (10–15 microscopic fields of view). Pollen in vitro

germination was tested by spreading pollen on to growth media containing $CaCl_2 \cdot 2H_2O$ (300 mg/L), H_3BO_3 (100 mg/L) and 15 % sucrose (Brewbaker and Kwack 1963) placed on cellophane and incubated for 4 h in darkness at 20 °C. The cellophane was then lifted and decolourised aniline blue stain was added before observing under the fluorescence microscope (Zeiss, Oberkochen, Germany) (Samineni et al. 2011). A pollen grain was scored as germinated when the length of the pollen tube exceeded the diameter of the pollen grain. Previously-pollinated pistils were excised from flowers 24 h after pollination and fixed for 24 h in the acetic alcohol described previously, then cleared with 6 N NaOH overnight, and thoroughly rinsed before being stained with decoloured aniline blue. The preparation was observed and photographed under the same Zeiss fluorescence microscope as above, linked to a Dell computer with Axiovision software to view images under the fluorescence microscope.

Leaves, flowers, pods and seeds were also sampled for analysis of sodium, potassium and chloride, using the methods described in Experiment 1. During flowering, hooded buds, open flowers and the young leaf subtending the flower were sampled in two of the pairs of genotypes JG 11 (salt tolerant, see Table 1) and ICC 8318 (salt sensitive) which began flowering 45 DAS, and ICC 9942 (salt tolerant) and ICC 11284 (salt sensitive) which began flowering 72 DAS, in both the saline and non-saline treatment. When pods were developing seeds (about 20 days after the flowers were sampled), the pod wall, seeds and subtending leaf were sampled and at maturity the pod wall and seeds were sampled in the same two pairs of genotypes and treatments. In all cases the samples were placed in small envelopes, oven dried and weighed. Additionally, flowers of Genesis 836 (salt tolerant) and Rupali (salt sensitive) were divided into sepals, and petals plus pistils, placed in Eppendorf tubes, oven dried and analysed for sodium, potassium and chloride. The small quantities of sepals and petals plus pistils from the replicates had to be combined and so these analyses were unreplicated.

Statistical analyses

For each experiment, a two-way ANOVA in Genstat 12th edition was used to assess the effect of genotype, salt treatment and genotype x treatment interactions. Results are presented on a per plant basis. Regressions were fitted and analysed in SigmaPlot version 12.1.

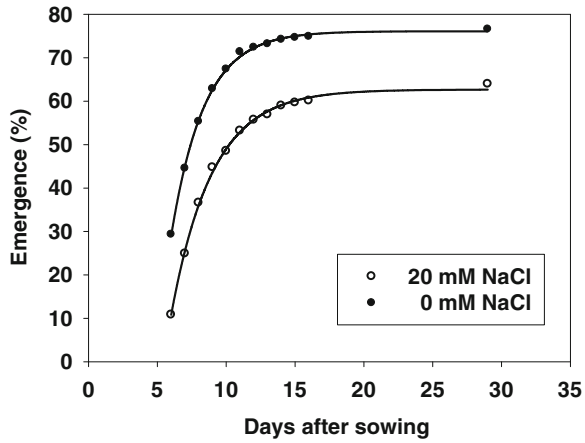

Fig. 1 Percentage of seeds planted that emerged with time after sowing (days) in chickpea genotypes in the non-saline soil (0 *mM* NaCl, *Black circle*) and saline soil (20 *mM* NaCl, *White circle*). The data are the pooled means of 55 genotypes of chickpea; the lines are fitted exponential curves. An additional 20 *mM* NaCl was added and the plants thinned to two plants per pot after the last observation. Experiment 1

Results

Experiment 1

Emergence differed significantly among genotypes ($P<0.001$) and salinity treatments ($P<0.001$), but the interaction was not significant. Compared with the non-saline control treatment, the application of 20 *mM* NaCl at sowing delayed the initial emergence and also reduced the final emergence of the seedlings. At the first time of measurement 6 DAS, the mean percentage emergence in the saline pots was 11 % compared to 29 % in the non-saline pots, increasing steadily to reach a maximum of 60 % compared to 75 % in the controls at 16 DAS (Fig. 1). Final emergence in 20 *mM* NaCl (before the second application of NaCl) varied from 19 % in ICC 95423 to 100 % in Genesis 836. The plants were then thinned to two plants per pot for the remainder of the experiment.

The genotypes varied markedly ($P<0.001$) in the time to first flower from 51 DAS in ICCV 2 to 107 DAS in ICC 2242 (Table 1), but the level of salinity in the soil had no effect ($P=0.19$) on the time to flowering (mean time to first flower was 74 DAS in the non-saline control and 75 DAS in 40 *mM* NaCl).

Seed yield varied significantly ($P>0.001$) among genotypes, with salinity treatment and their interaction. The mean seed yield in the saline treatment (40 *mM*

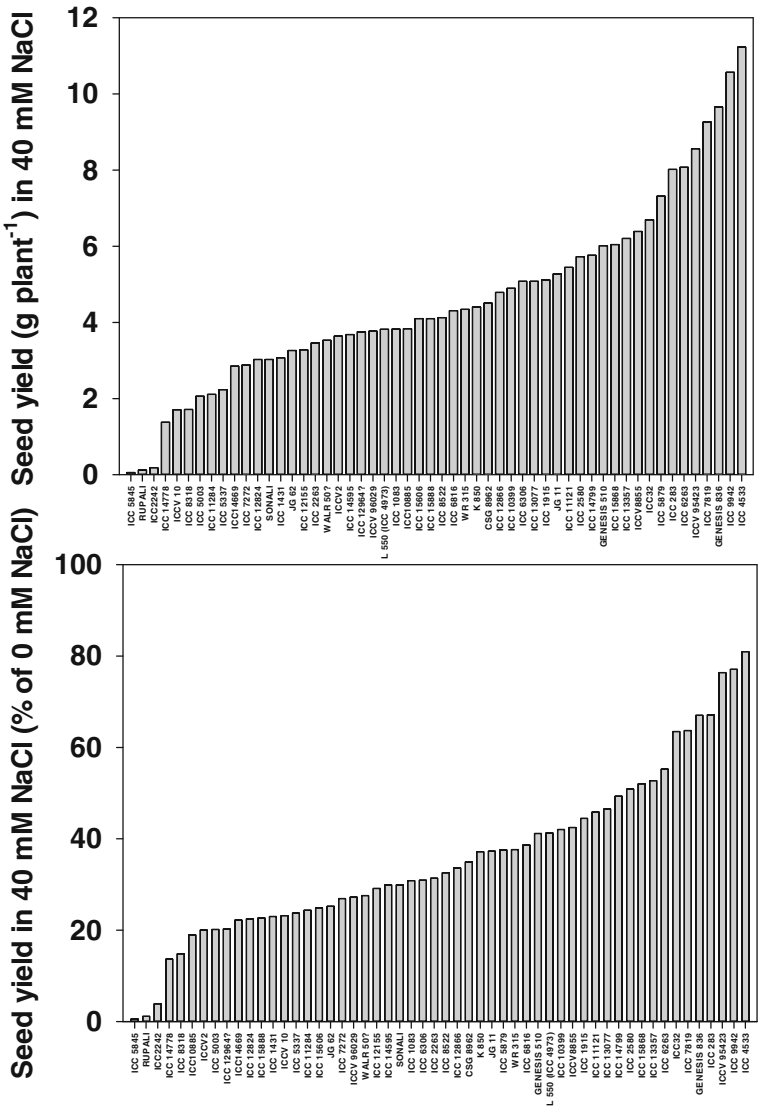

Fig. 2 **a** Seed yield per plant of 55 genotypes of chickpea in 40 *mM* NaCl ranked from lowest to highest, and **b** yield per plant of the genotypes in 40 *mM* NaCl relative to the non-saline (0 *mM* NaCl) yield per plant, also ranked from lowest to highest. Experiment 1

NaCl) was significantly smaller at 4.6 g per plant than in the non-saline controls at 13 g per plant. The seed yield in the saline soil varied significantly ($P<0.001$) among genotypes from 0.03 g per plant in ICC 5845 to 16.4 g per plant in ICC 15588, a 27-fold range (Table 1). Figure 2 shows the distribution of seed yield from the most sensitive genotypes at the left to the most tolerant genotypes on the right. The rankings were similar ($r^2=0.99$***) when the seed yield was calculated as a percentage of the yield in the non-saline plants (Fig. 2b) and when ranked for the seed yield in the saline treatment alone (Fig. 2a). Seed yield in the saline pots was poorly correlated with the seed yield in the non-saline pots ($r^2=0.15$**). Therefore, the seed yield in the 40 *mM* NaCl is used as a measure of salt sensitivity/tolerance.

Seed yield was not correlated with the time to flowering in either the saline ($r^2=0.010^{n.s.}$) or the non-saline ($r^2=0.029^{n.s.}$) treatments (Fig. 3), but the seed yield of the genotypes grown in the saline soil was correlated ($P<0.001$) with filled pod number per plant and seed number per plant, and correlated ($P<0.01$) with seed size and shoot biomass per plant (Table 2). However, the seed yield in 40 *mM* NaCl was not correlated with the filled pod number per plant in the controls (Table 2), even though the seed yield in the non-saline plants was correlated with the filled pod number in the controls ($r^2=0.328$*).

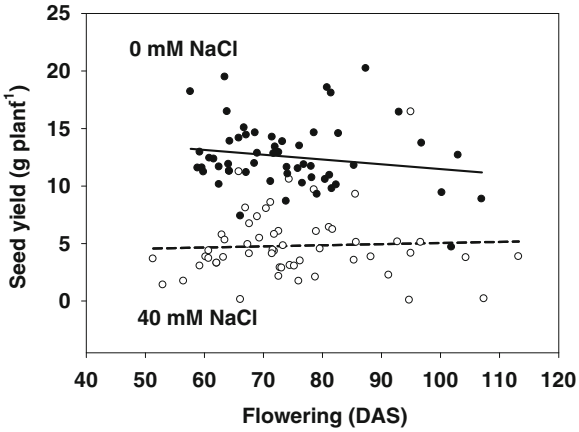

Fig. 3 Relationship between time to first flower in days after sowing (DAS) and seed yield in the non-saline controls (*Black circle*, 0*mM* NaCl, r^2=0.029$^{n.s.}$) and the saline treatment (*White circle*, 40*mM* NaCl, r^2=0.010$^{n.s.}$) in the 55 genotypes of chickpea. Experiment 1

When all genotypes were podding, the mean concentrations of sodium, potassium and chloride, and particularly chloride ions, in the youngest fully-expanded leaf were significantly higher in the saline treatment than the non-saline treatment (Table 3). The genotypes differed significantly (P<0.001) in the concentration of sodium and potassium, but not chloride. As there was a significant (P<0.001) genotype x treatment interaction for sodium, the leaf sodium concentration was plotted against the seed yield under 40*mM* NaCl. The seed yield decreased as the sodium concentration increased (Fig. 4) indicating that the salinity tolerant lines had lower sodium concentration in the leaves. Seed yield in 40*mM* NaCl did not vary with leaf potassium concentration (r^2=0.044 $^{n.s.}$) or with chloride concentration (r^2=0.070 $^{n.s.}$).

Table 2 Relationship between seed yield (g per plant) in 40*mM* NaCl (SY_{40}) and filled pod number per plant (PN_{40}), seed number per plant (SN_{40}), mean individual seed mass (mg) or seed size (SS_{40}), shoot dry weight (g per plant) (SDM_{40}) in the saline treatment and pod number in the non-saline treatment (PN_0) for 55 genotypes of chickpea. The equations are the fitted linear regressions with the correlation coefficients and level of significance (***=P<0.001, **=P<0.01; *=P<0.05; *n.s.* non-significant). Experiment 1

Factor	Linear relationship
Pod number plant^{-1} (PN_{40})	SY_{40}=0.768+0.114 PN_{40}, r^2=0.638***
Seed number plant^{-1} (SN_{40})	SY_{40}=0.860+0.099 SN_{40}, r^2=0.600***
Seed size (SS_{40})	SY_{40}=2.01+0.023 SS_{40}, r^2=0.169**
Shoot dry mass plant^{-1} (SDM_{40})	SY_{40}=-0.04+0.399 SDM_{40}, r^2=0.169**
Pod number plant^{-1} (PN_0)	SY_{40}=2.20+0.040 PN_0, r^2=0.073$^{n.s.}$

Table 3 Mean concentration of sodium, potassium and chloride ions in the youngest fully-expanded leaf of 55 chickpea genotypes grown in a non-saline (0*mM* NaCl) and a saline (40*mM* NaCl) soil 98 days after sowing when all genotypes were podding. All ions varied significantly (***, P<0.001) between treatments. The least significant difference (LSD, P=0.05) for the treatment means are also given. Experiment 1

Ion (μmol g^{-1} DM)	Non-saline (0*mM* NaCl)	Saline (40*mM* NaCl)	LSD
Sodium	77.3	121.3	8.9***
Potassium	671.8	715.7	26.8***
Chloride	90.5	1341.5	151.8***

Experiment 2

Among the 10 lines grown in 2010, there was a delay in seedling emergence in the 20*mM* NaCl (half the final) treatment and no emergence in the 30*mM* NaCl (half the final) treatment at 4 DAS, but by 7 DAS 60 % of seedlings had emerged in all treatments and thereafter there was no difference among the three treatments in emergence (data not shown). There were significant differences in percentage emergence among genotypes, but these were removed by thinning each pot to two plants at 27 DAS. ICCV 2 was the earliest to flower (39 DAS for first flower), while ICC 11284 was the last to flower (73 DAS for first flower). Salinity had no effect on the time to first flower and pod.

Seed yield varied from 8.5 g per plant to 26.5 g per plant when grown in 40*mM* NaCl and from 0.4 to 18.6 g per plant when grown in 60*mM* NaCl. In both saline treatments, the relationship between seed yield in 2010 and the seed yield of the same lines in 2009 grown in the glasshouse at 40*mM* NaCl was positive and linear (Fig. 5). As in Experiment 1, seed yield in the saline plants was positively associated with the number of filled pods and seeds per plant, and with shoot biomass per plant (data not shown). Despite large differences in yield between the three treatments there was no significant

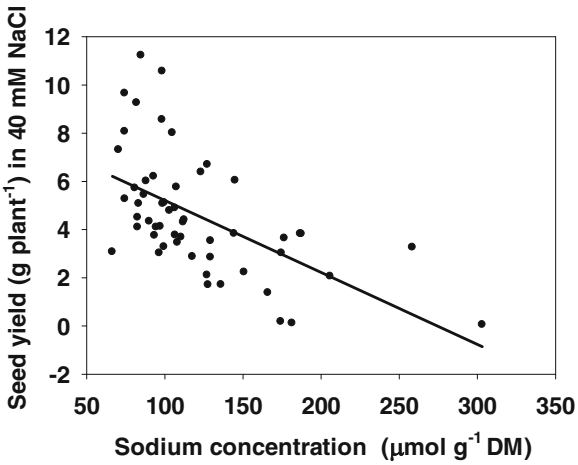

Fig. 4 Relationship between seed yield per plant (SY_{40}) and sodium concentration (Na) in the youngest fully-expanded leaf of chickpea grown in the 40*mM* NaCl treatment. SY_{40}=8.194–0.030 Na, r^2=0.321***. Experiment 1

difference in the sodium and chloride concentrations in the youngest fully-expanded leaves when measured at 84 DAS when all genotypes had at least one flower, but there was an 8 % decrease in potassium in the leaves at both salt concentrations in the soil compared with the plants grown in the non-saline soil (Table 4).

Experiment 3

The plants in Experiment 3 were grown adjacent to those in Experiment 2, but only at 0 and 40*mM* NaCl.

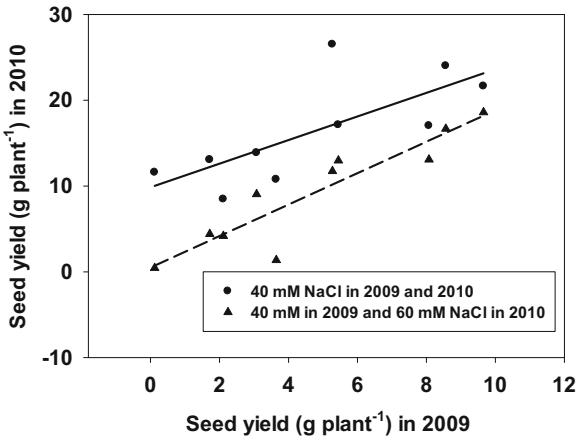

Fig. 5 Relationship between the seed yield per plant (SY) in the saline treatments (40*mM* and 60*mM* NaCl) in the same genotypes in 2009 (Experiment 1) and 2010 (Experiment 2). SY_{2-40}=9.866+1.374 SY_{1-40}, r^2=0.536*; SY_{2-60}=-4.900+0.862 SY_{1-40}, r^2=0.658***

Table 4 Mean concentration of sodium, potassium and chloride ions in the youngest fully-expanded leaf of 10 chickpea genotypes grown in a non-saline soil (0*mM* NaCl) and two saline soils (40*mM* and 60*mM* NaCl) 84 days after sowing when all genotypes had at least one flower. The least significant difference (LSD, P=0.05) for the treatment means that differed significantly (**, P<0.01, *n.s.* non-significant) is also given. Experiment 2

Ion (μmol g^{-1} DM)	Non-saline (0*mM* NaCl)	Saline (40 *mM* NaCl)	Saline (60 *mM* NaCl)	LSD
Sodium	142.5	136.4	139.7	n.s.
Potassium	768.8	701.0	715.6	45.2**
Chloride	489	497	502	n.s

In Experiment 3 the first flower to emerge on each plant per pot was tagged, revealing a difference in flowering between treatments. In the non-saline soil, JG 11 was the first genotype to flower with the first flower appearing 76 DAS, while ICC11284 and ICC 9942 were the last to flower with first flowers at 86 DAS. In the 40*mM* NaCl treatment, ICC 8318 flowered earlier at 57 DAS, but flowering was delayed in ICC 11284. This significant interaction (P=<0.001) can be seen in the data in Table 5 with the saline treatment decreasing the time to first flower in the early genotypes, JG 11 and ICC 8318, and having no effect or even increasing the time to first flower in the later genotypes, ICC 9942 and ICC 11284 (Table 5). The seed yields of the plants in Experiment 3 were similar (r^2=0.60**) to those in Experiment 2 in all the six common lines when grown in 40*mM* NaCl and the number of filled pods was positively associated with seed yield (r^2=0.32**) as in Experiment 1. The tagging of flowers and noting which flowers produced a pod and which pods survived and produced a seed or seeds, enabled the critical stages in seed production to be identified. From first flower to 100 DAS, a mean of 103 flowers $plant^{-1}$ was produced and tagged. There were no significant differences in flower number between the sensitive and tolerant lines in both the non-saline and saline soil, Almost 50 % (47.4 %) of the flowers aborted and did not produce a pod in both the sensitive and tolerant genotypes. Of the pods that were observed to have initiated (3 mm long), 17.5 % aborted in the sensitive genotypes, significantly (P<0.05) higher than the 6.1 % in the tolerant genotypes, so that the number of surviving pods in the tolerant genotypes was almost double the number in the sensitive genotypes.

Table 5 Time to first flower in days after sowing (DAS), pollen viability and in vitro pollen germination of six genotypes of chickpea grown in either a non-saline soil (0 *mM* NaCl) or a saline (40 *mM* NaCl) soil. The least significant differences (LSD, $P=0.05$) for the genotype and treatment means and the genotype by treatment interaction are also given when statistically significant (***, $P<0.001$, *n.s.* non significant). Experiment 3

Genotype	First flower (DAS)		Pollen viability (%)		In vitro pollen germination (%)	
	0 *mM* NaCl	40 *mM* NaCl	0 *mM* NaCl	40 *mM* NaCl	0 *mM* NaCl	40 *mM* NaCl
Rupali	82.0	70.1	99.17	99.10	99.33	99.47
Genesis 836	81.8	75.8	99.57	99.43	99.80	99.73
JG 11	76.5	57.1	99.63	99.33	99.53	99.53
ICC 8318	78.1	67.9	99.60	99.67	99.33	99.30
ICC 9942	86.4	85.5	99.67	99.53	99.50	99.57
ICC 11284	85.4	91.4	99.53	99.47	99.37	99.20
LSD:						
Genotype	5.53***		n.s		0.23***	
Treatment	3.20***		n.s		n.s.	
Interaction	7.82***		n.s		n.s.	

There were no significant differences in the number of empty pods and hence no significant differences in seed abortion, but the number of seeds produced from the ~100 flowers was 46 in the tolerant genotypes, but only 32 in the sensitive genotypes. Despite the reduction in yield in both the tolerant and sensitive genotypes with 40 *mM* NaCl, pollen was almost 100 % viable and almost 100 % of the pollen germinated in vitro (Table 5). Pollen tube growth was also not affected in vivo when the pollen was placed on the stigma of saline and non-saline plants in lines that were salt tolerant and salt sensitive (Fig. 6), as identified by seed yield in the saline treatment in Experiment 1(Table 1).

In seeds at maturity, there were significantly ($P<0.05$) higher concentrations of sodium (106 μmol g^{-1} DM) and potassium (364 μmol g^{-1} DM) in the three sensitive genotypes than in the three tolerant genotypes (74 and 303 μmol g^{-1} DM, respectively), but no significant difference in the chloride concentrations. With the exception of the seeds, however, there were no statistically significant differences between the tolerant and susceptible genotypes in the concentrations of the sodium, potassium or chloride in other tissues and therefore the ion concentrations in the tolerant and susceptible genotypes were combined to identify differences resulting from exposure to salinity. In the young fully-expanded leaves and flowers there was no significant difference in sodium concentration between plants grown in the saline and non-saline treatments, but the concentrations of chloride and potassium were significantly higher under saline conditions in both the young fully-expanded leaves and the leaves subtending a bud, while chloride was significantly higher in the sepals of the flowers (Table 6). However about 20 days later, the leaves subtending a pod at the mid-filling stage had continued to accumulate ions and had significantly higher concentrations of sodium and chloride at this stage (Table 6). At mid-filling, the developing seeds also had higher concentrations of all three ions in the plants in the saline soil, but by maturity the seeds had lower concentrations of all three ions on a dry mass basis than at mid-filling, presumably because of the rapid accumulation of dry matter in the maturing seed (Davies et al. 1999). Nevertheless, at maturity the concentration of sodium and potassium, but not chloride, was higher in the seeds from saline plants than in those from plants in non-saline soil (Table 6). In the saline soil, the concentration of all three ions in the pod wall also increased, and as the pod wall did not increase in dry mass (data not shown), there was clearly an accumulation of ions in this maturing phase of pod development.

Discussion

Excluding three genotypes that had extremely low yields in the saline conditions, the range of salt tolerance, measured as yield in saline conditions, or as yield in saline relative to yield in non-saline conditions, was approximately 10-fold, which is similar to or greater than that observed by Vadez et al. (2007) and Krishnamurthy et al. (2011) with many more genotypes than in this study. This confirms that there is a wide range of salt tolerance in the chickpea germplasm that can be exploited in breeding for improved salt tolerance in this salt-sensitive grain legume species. As in Vadez et al. (2007), the seed yield under saline conditions in the present study was positively associated with both the number of pods and seeds produced by the salinized plants in all three experiments. However, the reduction in the number of filled pods was associated with an increase in pod abortion, but not with a decrease in pollen viability, the germination of pollen, or pollen tube growth in vivo. This suggests that the reduction in pods with seeds and in

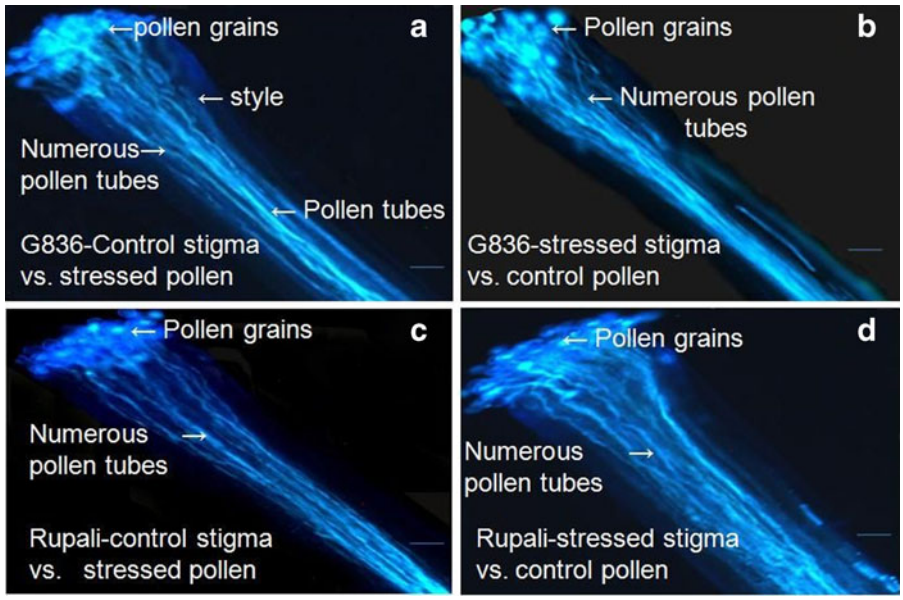

Fig. 6 Styles of chickpea showing pollen grains and pollen tube growth in (**a**) a style (♀) from Genesis 836 grown in non-saline (0*mM* NaCl) soil and pollen (♂) from a plant grown in saline (40*mM* NaCl) soil, (**b**) a style (♀) from Genesis 836 grown in saline (40*mM* NaCl) soil and pollen (♂) from a plant grown in soil with 0*mM* NaCl, (**c**) a style (♀) from Rupali grown in soil with 0*mM* NaCl and pollen (♂) from a plant grown in soil with 40*mM* NaCl, and (**d**) a style (♀) from Rupali grown in soil with 40*mM* NaCl and pollen (♂) from a plant grown in soil with 0 *mM* NaCl. Experiment 3

seed numbers arose either from the lack of seed fertilization, despite the growth of the pollen tubes to the ovary, or from the failure of the fertilized seeds to thrive, possibly because of a lack of assimilates for seed growth. The reduction in seed size with salinity treatment may reflect the shortage of assimilates for seed filling in the salt sensitive genotypes.

Table 6 Mean concentration of sodium, potassium and chloride ions in the youngest fully-expanded leaf (young leaf), hooded bud, the leaf subtending the hooded bud, open flower, petals and stamen, sepals, the leaf subtending a pod at the filling stage, pod wall at the filling stage, seed at the filling stage, pod wall at maturity and seed at maturity in chickpea genotypes grown in a non-saline (0*mM* NaCl) and a saline soil (40*mM* NaCl). The data are the pooled genotype means. The least significant differences (LSD, P=0.05) for the treatment means that differed significantly (*, P<0.05, **, P<0.01, ***, P<0.001, *n.s.* non-significant) are also given. Experiment 3

	Sodium (μmol g^{-1} DM)			Potassium (μmol g^{-1} DM)			Chloride (μmol g^{-1} DM)		
Salinity	0*mM*	40*mM*	LSD	0*mM*	40*mM*	LSD	0*mM*	40*mM*	LSD
Young leaf	96.3	104.3	n.s	377.4	549.3	151.1*	89.2	304.2	47.2***
Hooded bud	82.4	197.8	n.s.	468.2	587.7	n.s.	75.4	241.8	n.s.
Leaf subtending hooded bud	99.8	105.1	n.s.	398.6	536.5	164.3*	92.4	351.4	123.7**
Open flower	77.0	124.8	n.s.	390.7	495.3	n.s.	91.6	242.6	n.s.
Petal and stamen	162.0	172.0.	n.s.	506.6	488.2	n.s.	146.4	158.3	n.s.
Sepal	175.0	198.5	n.s.	604.3	619.6	n.s.	72.2	190.7	102.1*
Leaf subtending pod at filling	69.9	85.8	15.0*	348.2	405.2	n.s	92.6	716.1	359.8**
Pod wall at filling stage	60.4	80.6	n.s.	238.9	281.5	n.s.	168.7	147.6	n.s
Seed at filling stage	76.8	130.9	53.4*	354.1	454.9	77.9*	75.1	176.0	37.1**
Pod wall at maturity	85.5	143.7	n.s.	391.6	386.4	n.s.	66.9	303.5	134.1*
Seed at maturity	67.8	102.4	16.9**	285.0	362.2	60.6*	101.3	160.1	n.s.

The plants growing in the saline soil had markedly less chloride in the developing seed and in the seed at maturity than in the ageing leaves and in the pod wall at maturity. Chickpea appears to be able to limit the accumulation of chloride in the flowers and seeds during development by sequestration of ions in the leaves and pod walls. For example, while chloride in the mature seed was similar in the chickpea in the saline and non-saline soil, the leaf subtending the bud accumulated over 300 % more chloride in the plants subjected to salinity than plants in non-saline soil, and about 20 days later when the seeds were filling, the chloride in the leaves had increased to more than 750 % of that in the non-saline plants (Table 6). We conclude that the lower chloride concentration in the leaves in Experiment 2 (Table 4) than in Experiment 1 (Table 3) was a consequence of the 14-day earlier sampling in our second experiment, and is consistent with the accumulation of chloride in the older leaves during seed filling (Table 6). Surprisingly, salinity tolerance did not appear to be associated with accumulation of chloride as there were no significant differences between sensitive and tolerant genotypes in the accumulation of chloride in the leaves or seeds, but does appear to be associated with the accumulation of sodium in the leaves and in the seeds at maturity. At maturity, the seeds of the sensitive and tolerant genotypes did not differ in chloride concentration, but the tolerant genotypes accumulated less sodium in the leaves during podding (Fig. 4) and in the seeds at maturity. Interestingly, the sensitive genotypes not only contained higher tissue sodium, but also slightly more potassium. Moreover, sodium and chloride did not accumulate in the petals and stamens, while chloride only significantly accumulated in the sepals (Table 6). While we were not able to collect sufficient pollen to measure its ion content, the accumulation of sodium and chloride in the flowers of the plants in saline soil was not statistically different from that in the non-saline soil (Table 6). This is consistent with the observations that salinity did not affect pollen viability, in vitro pollen germination (Table 5) *or* in vivo pollen tube growth in the style (Fig. 6). While we have observed that the main ion accumulating in chickpea as a result of exposure to salinity is chloride and that the chloride accumulates in the older leaves and pod wall, it remains to be determined whether these high chloride concentrations in the subtending leaf and pod wall impact on photosynthesis and thus assimilate provision to the developing seeds. Singh and Pandey (1980) showed that 40–65 % of the carbon fixed by the subtending leaf of adequately-watered chickpea was transported to the associated pod, while the pod wall has been shown to photosynthesise and refix carbon dioxide respired by the developing seed (Ma et al. 2001; Furbank et al. 2004; Turner et al. 2005). Thus, the accumulation of sodium and chloride in the subtending leaves and pod wall may create ion toxicity that may limit assimilation and translocation of assimilates to the developing seed. Ghanem et al. (2009) showed that high levels of salinity (150 mM NaCl) imposed during early reproductive development induced sodium accumulation in the style and ovaries, but not pollen grains, of tomato (*Solanum lycopersicum* L.). However, they concluded that it was not the accumulation of toxic ions, but the decrease in soluble carbohydrates in the inflorescence that induced flower abortion.

While there were clear differences in salt tolerance, as yield under saline conditions, among the genotypes in this study, the sensitive/tolerant ranking of the genotypes differed from those observed in the same genotypes in India (Table 1). When the yields of 50 genotypes measured under saline conditions in the glasshouse in Perth were compared with the saline yields of the same genotypes measured in an Alfisol at ICRISAT in 2006–2007 (Krishnamurthy et al. 2011), there was no association between the yields in the two environments ($r^2=0.024^{n.s.}$). This is consistent with the G x E interaction for salt tolerance observed between soil types at ICRISAT (Krishnamurthy et al. 2011) where application of 80 mM NaCl in an Alfisol had a much greater reduction of yield than the same concentration applied to a Vertisol (Krishnamurthy et al. 2011). In the present study 40 to 60 mM NaCl applied to the soil was found to reduce yields by the same degree as 80 mM in the Vertisol soil in India, while at Punjab Agricultural University only 20 mM NaCl applied to loamy sand was adequate to reduce yields by 50 % (J. Kaur and G. Singh, personal communication, 2011). Additionally, the ranking in the warmer and less humid conditions in the outdoors at ICRISAT may also have induced the genotypes to respond differently to the salinity stress under the cooler and more humid conditions in the glasshouse in Perth. Vadez et al. (2007) showed that in the very short season at ICRISAT (growing season November to February), the late-flowering genotypes (above 80 DAS) had lower apparent salt tolerance than mid-

season flowering types (50–80 DAS), possibly due to long-season genotypes not being able to express their salt tolerance in the short-season environment. Perth has a cooler and longer growing season than at ICRISAT, particularly when kept adequately watered in the glasshouse, and enabled some of the salt tolerant, late-maturing cultivars to yield well under saline conditions so that there was no effect of flowering time on yields in both the saline and non-saline soil (Fig. 3). Nevertheless, in all the studies at both locations, the yield in the saline soil was correlated with the number of pods and seeds, indicating that salinity similarly affected the reproductive success of chickpea in both environments (Vadez et al. 2007, 2012a, b). In Vadez et al. (2012b), the more salt tolerant genotypes at ICRISAT were ones that produced a large number of tertiary branches and flowers, in both saline and non-saline conditions. However, in the present study salt tolerance was not associated with the constitutive trait of the production of a large number of flowers under non-saline conditions, but exposure to salinity did significantly increase pod abortion and decreased both pod and seed number.

In conclusion, the present study has demonstrated significant differences in salt tolerance among chickpea genotypes, with consistent variation from year to year when grown in the same environment. However, the study has shown that there is G x E interaction for salt tolerance so that the tolerance rankings differed between experiments conducted outdoors in a warm short-season environment at ICRISAT near Hyderabad in southern India, and the cooler long-season environment of the glasshouse in Perth, Western Australia. Chickpea has been shown to exhibit considerable G x E interaction for dryland yields related to phenological adaptation from southern to northern India and across Australia (Berger et al. 2004, 2006). Nevertheless, there are some genotypes that were salt tolerant in both our studies and at ICRISAT. ICC 9942 was consistently high yielding under saline conditions in all studies at ICRISAT (Vadez et al. 2007; Krishnamurthy et al. 2011) and was high yielding in the three experiments in the present study. The large G x E interaction observed in this study mandates that putative sources of salt tolerance need to be evaluated under local conditions, and eventually in field conditions. The present study has also highlighted that salt tolerance in chickpea is associated with high reproductive success (Samineni et al. 2011; Vadez et al. 2012a, b) and the maintenance of pod and seed numbers when exposed to salinity. The study also demonstrated that chickpea accumulates ions, particularly chloride and to a lesser extent sodium, in the leaves and pod wall during seed filling, reducing the accumulation of chloride in the seed. Further research is needed, using a wide range of genotypes, to elucidate whether differences in salt tolerance in chickpea may be associated with differences in ion accumulation in leaves and pods at the time of seed-filling and whether the accumulation of chloride and sodium reduces the leaf and pod wall photosynthesis and starves the young developing seed of assimilates, or whether the accumulation of ions directly affects the fertilisation of the ovaries.

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