

## Original Article

# Salt sensitivity in chickpea (*Cicer arietinum* L.): ions in reproductive tissues and yield components in contrasting genotypes

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## ABSTRACT

**The reproductive phase in chickpea (*Cicer arietinum* L.) is affected by salinity, but little is known about the underlying cause. We investigated whether high concentrations of Na<sup>+</sup> and Cl<sup>-</sup> in the reproductive structures influence reproductive processes. Chickpea genotypes contrasting in tolerance were subjected to 0, 35 or 50 mM NaCl applied to soil in pots. Flower production and abortion, pod number, percentage of empty pods, seed number and size were evaluated. The concentrations of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> were measured in various plant tissues and, using X-ray microanalysis, in specific cells of developing reproductive structures. Genotypic variation in reproductive success measured as seed yield in saline conditions was associated with better maintenance of flower production and higher numbers of filled pods (and thus seed number), whereas seed size decreased in all genotypes. Despite the variation in reproductive success, the accumulation of Na<sup>+</sup> and Cl<sup>-</sup> in the early reproductive tissues of developing pods did not differ between a tolerant (Genesis836) and a sensitive (Rupali) genotype. Similarly, salinity tolerance was not associated with the accumulation of salt ions in leaves at the time of reproduction or in seeds at maturity.**

**Key-words:** genotypic variation; ovule; pod wall; reproductive success; salinity; salt tolerance; tissue ions; tissue K<sup>+</sup> and Na<sup>+</sup>; X-ray microanalysis.

## INTRODUCTION

Chickpea is grown mainly in arid and semi-arid regions; the soils in these areas are susceptible to salinization, yet chickpea is relatively salt sensitive (Vadez *et al.* 2007; Flowers *et al.* 2010). Salinity adversely affects chickpea germination (Khalid *et al.* 2001), plant establishment (Al-Mutata 2003) and vegetative growth (Lauter & Munns 1986; Dua &

Sharma 1997); however, the reproductive processes are considered the most salt sensitive (Vadez *et al.* 2007, 2012; Samineni *et al.* 2011; Turner *et al.* 2013). Reproductive success (i.e. seed yield per plant) is a function of flower number and successful fertilization of the ovule, pod development, pod retention, seed number per pod and seed growth (Flowers *et al.* 2010). A few studies have reported that salt-tolerant chickpea lines tend to produce more flowers (Datta *et al.* 1987; Vadez *et al.* 2012) and maintain more pods, indicating that the conversion of flowers into pods is a salt-sensitive process (Vadez *et al.* 2007; Samineni *et al.* 2011; Turner *et al.* 2013).

The processes of reproductive development most affected by salinity are not known for chickpea and have rarely been studied for plants generally (other species are briefly considered in the next paragraph below). However, it is well established in chickpea that there is a specific sensitivity to salinity around reproduction as yields were similar when the salt was applied at sowing or at the beginning of flowering (Vadez *et al.* 2012). Samineni *et al.* (2011) showed that the reproductive stage was not affected by changes in pollen viability, but *in vitro* pollen germination was severely inhibited by addition of NaCl to the germination media indicating that if Na<sup>+</sup> and/or Cl<sup>-</sup> accumulate to high levels in floral tissues this may compromise pollen tube growth and fertilization. Turner *et al.* (2013) documented increased pod abortion in salt-sensitive genotypes, but pollen viability, *in vitro* pollen germination and *in vivo* pollen tube growth were not affected by salinity, suggesting that the reduction of number of filled pods arose either from the lack of ovule fertilization or from the failure of the fertilized ovule to develop and grow into a seed (and/or associated structures). The mechanism(s) by which salinity affects reproductive processes in chickpea remains unclear: do high concentrations of Na<sup>+</sup> or Cl<sup>-</sup> in reproductive structures, or in other parts of the plant, influence reproductive processes?

A negative relationship between shoot Na<sup>+</sup> accumulation and grain yield has been observed in chickpea (Manchanda

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& Sharma 1989), but a more recent study of numerous genotypes found no relationship (Vadez *et al.* 2007). Little is known about the effects of ion accumulation in reproductive structures of chickpea. Analysis of pod walls and seeds of chickpea (cv. Chafa) grown with 50 mM NaCl in sand culture identified potentially toxic levels of Na<sup>+</sup> and Cl<sup>-</sup> in both of these tissues (Murumkar & Chavan 1986). Samineni *et al.* (2011) reported that Na<sup>+</sup> concentrations in floral parts (498  $\mu\text{mol g}^{-1}$  dry mass) were similarly high to those in the whole shoot of plants exposed to 60 mM NaCl in nutrient solution. By contrast, differential distribution of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> in seeds, pod walls and subtending leaves has been described for chickpea grown in a saline soil by Turner *et al.* (2013); these had significantly lower concentrations (dry mass basis) of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> in seeds than in pod walls and subtending leaves. According to Turner *et al.* (2013), salinity tolerance appears to be associated with lower Na<sup>+</sup> accumulation in seeds. In other salt-sensitive plant species, a reduction in fruit or seed yield has been associated with poor flower fertilization, which has been attributed to the accumulation of Na<sup>+</sup> and Cl<sup>-</sup> in pollen grains and stigmas (rice; Khatun & Flowers 1995) or in the style, ovaries and anther intermediate layer (tomato; Ghanem *et al.* 2009). In addition to these direct effects of potentially toxic ions and the possible salt-induced reduction in K<sup>+</sup> or Ca<sup>2+</sup>, other factors that may influence reproductive processes include hormones (chickpea, Dhingra & Varghese 1997; sorghum, Amzallag 2005) or a low carbon supply as a result of reduced leaf area and/or low rates of net photosynthesis (rice, Abdullah *et al.* 2001; tomato, Ghanem *et al.* 2009). However, a recent assessment of transpiration (used as a proxy for photosynthesis) upon salt application showed that although some tolerant lines had slightly higher transpiration rates than sensitive lines, the rate of transpiration did not discriminate all tolerant from sensitive chickpea lines when significant differences were small, indicating that differences in carbon supply to the reproducing structures were likely not a major cause for reproductive failure in chickpea under salt stress (Vadez *et al.* 2012). As a step towards understanding ion relations in reproductive tissues of salinized chickpea, in the present study we have applied quantitative X-ray microanalysis for ion-specific localization in cells of reproductive organs.

The overall aim of this study was to investigate whether the effect of salinity on reproductive processes in chickpea is associated with ion concentrations in specific tissues. The study was performed with two relatively tolerant and two sensitive chickpea genotypes subjected to two NaCl treatments applied to soil at the time of sowing in pots. The specific objectives were to determine for the contrasting genotypes: (1) the effect of salinity on flower production and abortion, pod number, percentage of empty pods, seed number and size; (2) the concentrations of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> in various plant tissues and using X-ray microanalysis, in specific cells of developing reproductive organs. Understanding salt tolerance traits related to reproductive processes may help to identify key factors responsible for the reduction in seed yield under saline conditions.

## MATERIALS AND METHODS

### Plant growth and treatment conditions

Two desi-type chickpea (*Cicer arietinum* L.) varieties that are reportedly either salt tolerant (Genesis836) or salt sensitive (Rupali) (Turner *et al.* 2013) and two desi-type breeding lines that have been classified as salt tolerant (DICC0442) and salt sensitive (DICC0478) based on previous screening work for salinity tolerance in the field and under controlled conditions (own unpublished data) were used. The pedigrees of the two varieties (Genesis836 and Rupali) are diverse, coming from a range of genotypic backgrounds. The two breeding lines DICC0442 and DICC0478 are similar in their genetic background having one parent cultivar that is the same. They are not genetically related to either Genesis836 or Rupali. The experiment was carried out between June and November 2013 in a controlled temperature glasshouse, with average day/night temperatures of 21/13 °C, in Perth, Western Australia (31°57'S, 115°47'E). Plants were grown in non-draining plastic pots (200 mm in diameter, 190 mm high) filled with 4.75 kg of red-brown sandy clay loam soil (Calcic Haploxeralf) collected from a farm approximately 25 km northeast of Mukinbudin (30°78'S, 118°31'E), Western Australia. The soil (pH 8.2, electrical conductivity = 0.4 dS m<sup>-1</sup> in 1:5 soil : water extract) was fertilized with (g pot<sup>-1</sup> containing 4.75 kg soil) 0.713 KNO<sub>3</sub>, 1.016 Ca(NO<sub>3</sub>)<sub>2</sub>, 0.906 KH<sub>2</sub>PO<sub>4</sub>, 0.119 MgSO<sub>4</sub> and 3.3 mL of half-strength Hoagland solution micronutrients. The water content (w/w) at field capacity (i.e. pot capacity when fully drained) was 17.8%.

Prior to sowing, seeds of the four genotypes were imbibed in 0.5 mM CaSO<sub>4</sub> for 3 h, pre-germinated in Petri dishes containing 5 mL of 0.5 mM CaSO<sub>4</sub> and covered with aluminium foil to ensure darkness. For each pot, five seeds of a particular genotype were sown on 12 June 2013 along with a peat-based *Rhizobia* inoculum (3 g pot<sup>-1</sup>; Group N, New Edge Microbials Pty Ltd, Albury, New South Wales, Australia). Twenty-two days after sowing (DAS), seedlings were thinned to two per pot.

Three treatments were used: a non-saline control, 0.36 g NaCl and 0.52 g NaCl kg<sup>-1</sup> soil. The experimental design was: 4 genotypes × 3 treatments × 4 replicates = 48 pots (with two plants per replicate pot). Pots were re-randomized weekly to minimize positional effects. The added NaCl corresponded to solution concentrations in the pot at field capacity of 0, 35 and 50 mM NaCl, respectively. The two NaCl treatments were applied in two doses. The first dose was applied before sowing by adding 0.208 g NaCl kg<sup>-1</sup> soil to pots assigned to the 35 mM NaCl treatment, and 0.364 g NaCl kg<sup>-1</sup> soil to pots assigned to the 50 mM NaCl treatment. NaCl was added to pots along with the nutrients (listed above) in a sufficient solution volume to wet the soil to field capacity. The non-saline controls were watered up to field capacity with an equivalent solution volume only containing the nutrients. The second salt dose was applied 30 DAS by adding, in de-ionized water, 0.156 g NaCl kg<sup>-1</sup> soil to each saline pot to increase the NaCl concentration to the final 35 or 50 mM NaCl in the soil. The equivalent volume of de-ionized water was added to the non-saline control pots. Pots were weighed every 2 or 3 days

and de-ionized water added to bring the water content to 90% field capacity for duration of the experiment. Use of non-draining pots and watering to weight ensured that no salts were leached and that plants received adequate water. Pot watering ceased 131 DAS when all plants had reached maturity (stopped flowering and with many filled pods).

### Flower tagging and harvest procedure

The beginning and end of flowering and podding was recorded for each plant and new flowers were tagged twice per week from the first flower to maturity. Ninety-seven DAS (34 days before maturity) one or two of the young fully expanded leaves (YFEL) and the oldest green leaves (OGL) of each plant were sampled, placed into labelled paper envelopes, oven dried at 60 °C for 48 h and analysed for Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> (see below). At maturity, the number of tags (flowers) was counted, plants were cut at the soil surface and shoots (with pods) were dried in an oven at 60 °C for 48 h. Pods (pod >5 mm long) were then collected and counted, and the numbers of empty pods (infertile pods, small or no seed present, i.e. seed abortion) and seeds were recorded for each plant. As we did not note the flowers that developed a pod that ultimately aborted and abscised, flower abortion also includes pod abortion as measured in Turner *et al.* (2013). Flower plus pod abortion percentage was calculated as: [(total number of flowers produced – total number of pods at maturity (filled + empty))/total number of flowers produced] × 100. All seeds were collected and weighed and the remainder of the shoot (including pod walls) was also weighed. Average-sized seeds of each plant were selected for germination test and for analyses of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup>.

### Seed germination test

Seed germination was tested using 15 average-sized seeds per plant (when available) from one tolerant (Genesis836) and one sensitive (Rupali) genotype (two plants per pot were pooled for one replicate of 30 seeds in total) placed in plastic boxes containing two sheets of Whatman filter paper moistened with 0.5 mM CaSO<sub>4</sub>, placed in a phytotron (day/night temperatures of 20/15 °C) and covered with aluminium foil to ensure darkness. Germination was recorded every 24 h for 21 days and expressed as a percentage of the 30 seeds in each replicate. Seeds were considered germinated when the radicle had emerged 1 mm from the seed. Some of the seed had not imbibed after 6 days, so the seed coats were punctured using a needle and seeds placed on new moistened filter papers in plastic boxes and germination was monitored.

### Ion analyses

Oven-dried leaves were cut into small pieces, weighed and extracted in 5 mL (20–50 mg sample) or 10 mL (50–100 mg sample) of 0.5 M nitric acid by shaking for 2 days at room temperature (Munns *et al.* 2010). Dried seeds were ground to a fine powder and 100 mg subsamples were extracted in 10 mL of 0.5 M nitric acid by shaking for 2 days at room

temperature. The concentrations of Na<sup>+</sup> and K<sup>+</sup> in dilutions of extracts (seed extract dilution factor was 10 for both Na<sup>+</sup> and K<sup>+</sup>; leaf extract dilution factor was 5 for Na<sup>+</sup> and 25 for K<sup>+</sup>) were determined using a flame photometer (PFP7, Jenway, Essex, UK) and Cl<sup>-</sup> (no dilutions were required) using a chloridometer (SLAMED, model 50CL 1–50, Frankfurt, Germany). Data were verified by taking reference plant material of known ion concentration through the same procedures; no adjustments were made to the data presented.

### Sample preparation for X-ray microanalysis

Two contrasting chickpea genotypes, Genesis836 (salt tolerant) and Rupali (salt sensitive), were selected for energy-dispersive X-ray microanalysis experiments. When flowering and podding were well established (90 DAS; Supporting Information Table S1), new flower buds from control and 50 mM NaCl treatment plants were tagged as soon as they appeared and their development followed at daily intervals. After the flower petals had faded in colour, when the early pod was only just visible (pod setting stage, about 3 mm long, 7–8 days after bud appearance), faded flower petals and sepals were removed, the early pods were excised and immediately plunge frozen into liquid N<sub>2</sub> slush. Frozen early pods were subsequently freeze substituted in a 10% acrolein in diethyl ether mixture over 3 Å molecular sieve, and embedded in Araldite 502 epoxy resin as outlined in Kilburn & Clode (2014). All solutions were anhydrous and once embedded, samples were kept desiccated. Araldite 502 is the preferred epoxy resin as it contains negligible levels of elements detectable by energy-dispersive microanalysis (Pålsgård *et al.* 1994).

This particular substitution method, when performed under anhydrous conditions, maximizes the retention and immobilization of diffusible ions (Marshall 1980a,b; Condrón & Marshall 1990; Pålsgård *et al.* 1994; Orlovich & Ashford 1995). Clear demonstration of the suitability of this method for retaining and preserving diffusible salts and ions is shown by Marshall *et al.* (2007), where seawater is preserved in the coelenteric cavities of coral polyps prepared in this manner. This substitution method has been reliably used to prepare animal, plant and algal tissues (Altus & Canny 1985; Hyatt & Marshall 1985; Orlovich & Ashford 1995; Mostaert *et al.* 1996; Crawford *et al.* 1998; Clode & Marshall 2002, 2003, 2004; Bidwell *et al.* 2004; Lozić *et al.* 2014) for cellular element analysis. This substitution procedure is preferential to freeze-drying and embedding plant material, which may result in a number of structural artefacts and redistribution of cellular ions (Echlin 1992; Grovenor *et al.* 2006). Although it is possible that not all cellular compounds may be fully preserved by this substitution procedure, any losses would affect all samples equally; thus, comparisons between treatments remain valid (Hyatt & Marshall 1985).

Resin-embedded mounts were prepared to create a cross section of the early pod in the block face. Cutting was performed on an EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) using a dry glass knife. Dry-cut 1 µm-thick sections were initially collected and placed on glass

slides for toluidine blue staining to check the quality of the sample and to identify suitable regions of the early pod for analysis, in particular we were interested to target the ovule. Optical images from stained sections were collected using an Axioskop microscope (Zeiss, Oberkochen, Germany) fitted with a digital camera. Once a suitable region was identified, the block face was finely planed using a dry knife to create a perfectly flat cross-sectional surface. The block was then mounted on a metal stub with double-sided carbon tape and the edges painted with conductive carbon paint before being coated with 20 nm of carbon.

### Quantitative X-ray microanalysis

Planed blocks were analysed in a Supra 55 field emission SEM (Zeiss) fitted with an X-Max80 SDD X-ray detector (80 mm<sup>2</sup>) interfaced to Oxford Instruments AZtecEnergy software (Oxford Instruments, Oxfordshire, UK). The microscope was operated at 15 kV in high current mode. Immediately prior to each map acquisition, the instrument was calibrated and the beam current measured and recorded using a pure copper standard. Elemental maps were acquired at a resolution of 1024 × 768 pixels, for >400 frames with a dwell time of 50–100 μs per pixel. Drift correction and pulse-pile up correction were activated. For such analyses of bulk samples, the analytical resolution approximates a 2–3 μm sphere, with detection limits around a few mmol kg<sup>-1</sup> (Roomans & Dragomir 2007).

Using the Oxford Instruments AZtecEnergy software, quantitative numerical data were extracted from cellular regions of interest drawn on the element maps, with individual spectra from each pixel summed and processed to yield concentration data. Summed spectra from regions of interest were quantified using the AZtec XPP model for matrix corrections. Standards comprised polished microprobe standards of pure elements or minerals of well-defined composition. Matrix corrections were performed using fixed concentrations of 10% C, 10% H and 3.3% N, with O calculated by difference, reflecting a generalized protein matrix. However, even when these fixed concentrations were set to reflect the extreme (100% resin matrix = ~75% C, 10% H), the calculated Na, Cl and K concentrations did not change (results not shown).

All elemental concentrations are given in μmol g<sup>-1</sup> embedded tissue, which, if the resin fully occupies the space previously occupied by water in the living tissue, closely reflects the concentration on a wet-weight basis. The regions analysed were pod wall mesocarp (six to eight layers of parenchyma cells), pod wall endocarp (three to four layers of sclerenchymatous cells adjacent to the pod cavity) and cells in the outer part of the ovule and inner part of the ovule.

### Statistical analyses

Data are presented as mean ± SE. Two plants per pot were pooled for one replicate data, where *n* = number of pots. Two-way analysis of variance (ANOVA) was used to assess the

effects of genotype, salt treatments and genotype × treatment interactions. Means were compared at the *P* < 0.05 level using least-significant difference test. Three-way ANOVA was used to assess the genotype × treatment × leaf interaction (for leaf tissue ions). X-ray microanalysis data are presented as mean ± SE, where *n* = the number of cells analysed. These data were collected from two different sites in one cross section. Two-way ANOVA was used to assess the effects of treatment and tissue × genotype interaction.

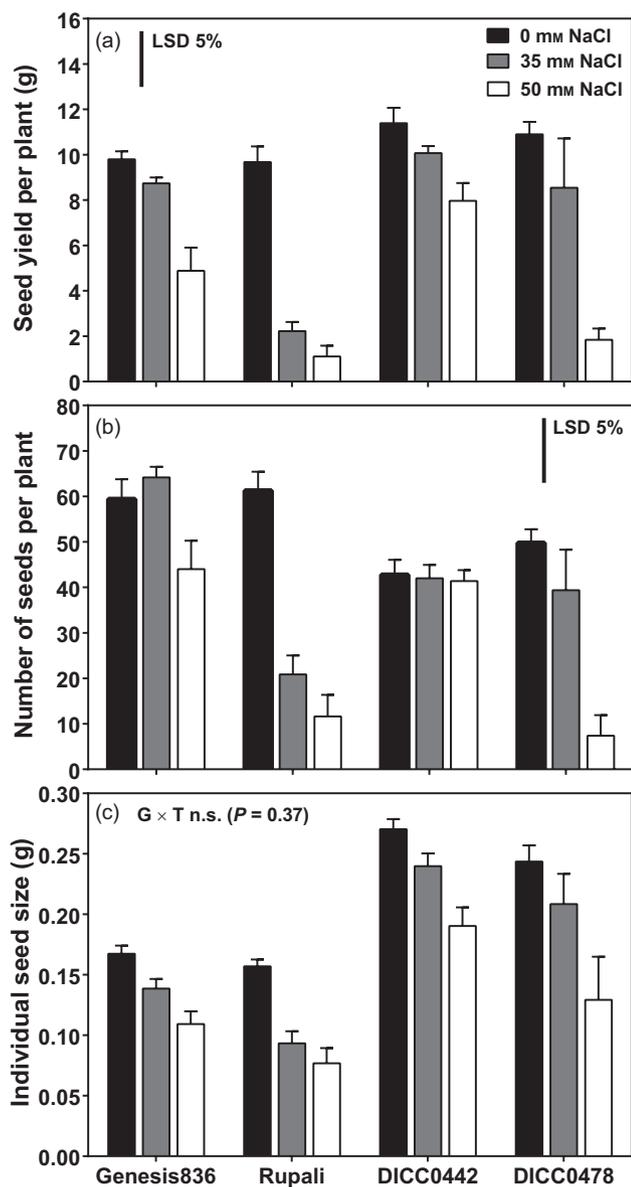
## RESULTS

### Effect of salinity on seed yield and its components

The total seed yield produced by the four genotypes in the non-saline soil was similar at 9.6–11.4 g per plant (Fig. 1a). Seed yield components differed among genotypes; Rupali produced the most seeds per plant (61 ± 4) and DICC0442 produced the least (43 ± 3), whereas the reverse was true for individual seed size with DICC0442 having the largest seeds (0.27 ± 0.008 g) and Rupali the smallest (0.15 ± 0.006 g). The 35 mM NaCl treatment did not affect productivity in three genotypes, but had a significant adverse impact on Rupali with seed yield, seed number and seed size reduced by 77, 56 and 41%, respectively. The 50 mM NaCl stress reduced productivity in all four genotypes, with Rupali and DICC0478 (sensitive genotypes) more severely affected than Genesis836 and DICC0442 (tolerant genotypes). Seed yields in the 50 mM NaCl treatment were decreased relative to the non-saline controls by 89 and 83% in Rupali and DICC0478, respectively, and by 50 and 30% in Genesis836 and DICC0442, respectively (Fig. 1a). As a result, there was a significant genotype × treatment interaction for seed yield (*P* < 0.005). Interestingly, the 50 mM NaCl treatment significantly reduced seed number per plant in sensitive genotypes (by 81 and 85% for Rupali and DICC0478, respectively), but had no effect on seed number in tolerant genotypes when compared with controls (significant genotype × treatment interaction at *P* < 0.0001) (Fig. 1b). The 50 mM NaCl treatment reduced seed size in sensitive (by 51 and 47% for Rupali and DICC0478, respectively) and tolerant (by 35 and 30% for Genesis836 and DICC0442, respectively) genotypes; there was no significant genotype × treatment interaction for individual seed size (*P* = 0.37) (Fig. 1c).

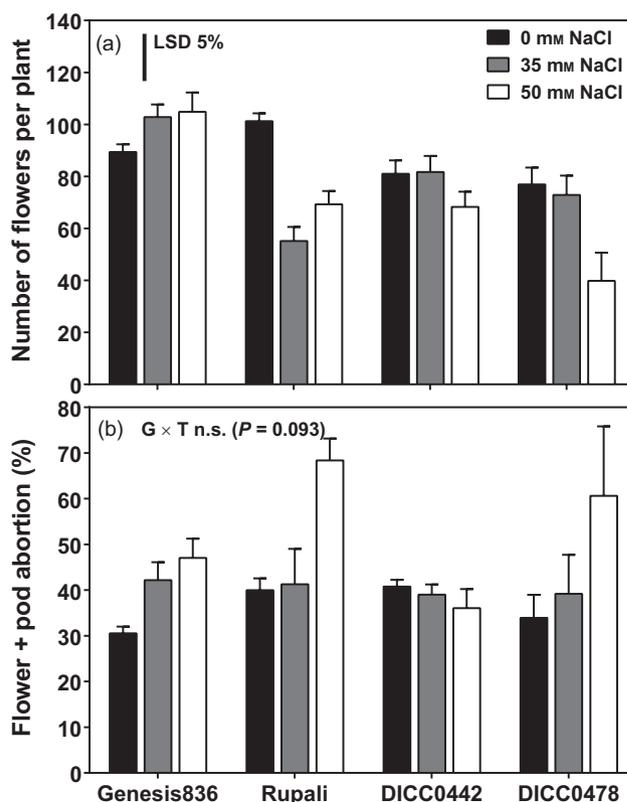
### Effect of salinity on flower production, flower plus pod abortion, pod production and percentage of empty pods

In the non-saline controls, flowering commenced at 52, 55, 65 and 67 DAS for Rupali, Genesis836, DICC0478 and DICC0442, respectively (Supporting Information Table S1). The salinity treatments had no significant effect on time to first flower in three genotypes, but in DICC0442 it increased in the 35 and 50 mM NaCl treatments to 72 ± 1 and 75 ± 2 DAS, respectively. In the non-saline soil Rupali produced the most flowers (101 ± 3) and DICC0478 produced the least



**Figure 1.** Seed yield per plant (a), number of seeds per plant (b) and individual seed size (c) of four chickpea genotypes grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. To achieve these concentrations, NaCl was added in the volume of water held in pots at field capacity. Bars represent least-significant differences (LSD) at  $P = 0.05$  for the genotype  $\times$  treatment interaction ( $G \times T$ ). For individual seed size, there was no significant  $G \times T$  interaction but there was a significant treatment effect (LSD = 0.02). Data are means  $\pm$  SE of four replicate pots. Two plants per pot were pooled and the mean per plant used for one replicate data.

(77  $\pm$  6) (Fig. 2a). The imposition of NaCl treatments did not significantly affect flower number in the two tolerant genotypes (Genesis836 and DICC0442) and interestingly in Genesis836 flower number was maintained despite the significant reduction in shoot dry mass (Supporting Information Table S2). In Rupali, the 35 and 50 mM NaCl treatments reduced flower numbers to 55 (54% of control) and 69 (68%



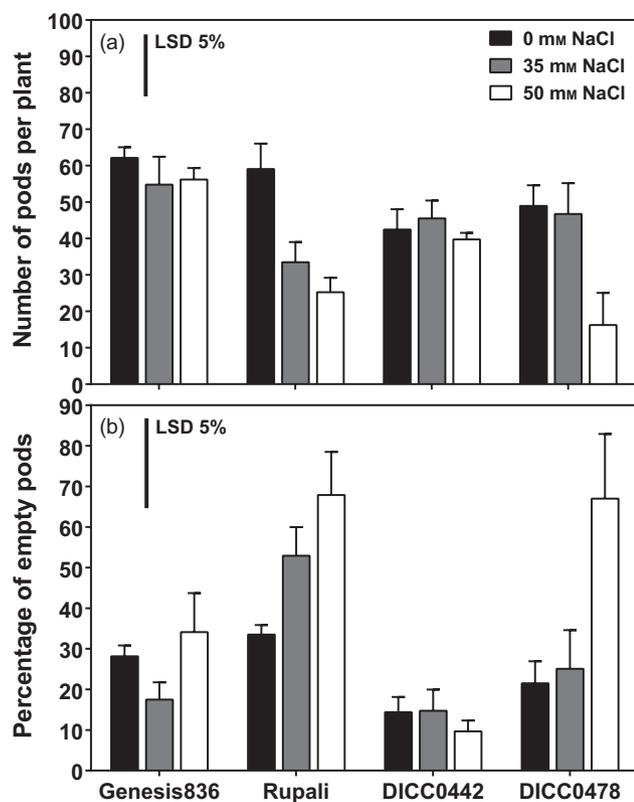
**Figure 2.** Number of flowers (a) and percentage of flower plus pod abortion (b) of four chickpea genotypes grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. Bars represent least-significant differences (LSD) at  $P = 0.05$  for the genotype  $\times$  treatment interaction ( $G \times T$ ). For percentage of flower plus pod abortion, there was no significant  $G \times T$  interaction but there was a significant treatment effect (LSD = 8.4). Data are means  $\pm$  SE of four replicate pots. Two plants per pot were pooled and the mean per plant used for one replicate data.

of control), respectively. In DICC0478, the 35 mM NaCl treatment had no effect on flower number, but the 50 mM NaCl reduced flower number to 40 (52% of control) (Fig. 2a). There was a significant genotype  $\times$  treatment interaction for flower number ( $P < 0.0001$ ).

In non-saline soil, flower plus pod abortion (see Material and Methods) was similar for all genotypes ranging from 30 to 41% (Fig. 2b). The 35 mM NaCl treatment had no effect on flower plus pod abortion except for Genesis836 where it significantly increased from 30 to 42%. In 50 mM NaCl treatment, the percentage of aborted flowers plus pods in the sensitive genotypes was 68% in Rupali and 61% in DICC0478 and in the tolerant genotypes 47% in Genesis836 and 36% in DICC0442; there was no significant genotype  $\times$  treatment interaction for flower plus pod abortion ( $P = 0.093$ ) (Fig. 2b).

In the non-saline soil, the first pods set at 64, 71, 77 and 79 DAS for Rupali, Genesis836, DICC0478 and DICC0442, respectively (Supporting Information Table S1). The number of pods per plant at maturity varied across genotypes from 42 in DICC0442 to 62 in Genesis836, while the percentage of

empty pods (i.e. seed abortion) was highest in Rupali (33%) and lowest in DICC0442 (14%) (Fig. 3a,b). The salinity treatments did not affect the time to first pod set in three genotypes, but in DICC0442 the 35 and 50 mM NaCl treatments delayed first pod set by 5 and 8 days, respectively (Supporting Information Table S1). The salinity treatments did not affect the number of pods at maturity or percentage of empty pods (i.e. seed abortion) in the two tolerant genotypes (Fig. 3a,b), but they were affected in the two sensitive genotypes. As a result, there was a significant genotype  $\times$  treatment interaction for both pod production ( $P < 0.05$ ) and percentage of empty pods ( $P < 0.05$ ). In Rupali, pod number decreased to 33 (57% of control) and 25 per plant (43% of control) in the 35 and 50 mM NaCl treatments, respectively. The percentage of empty pods (i.e. seed abortion) increased in Rupali to 53% in the 35 mM NaCl treatment (~1.6 times the control) and 68% in the 50 mM NaCl treatment (~2.0 times the control). In DICC0478, the number of pods and percentage of empty pods was not affected by the 35 mM NaCl treatment, but at 50 mM NaCl the pod number had decreased to 16 per plant (33% of control) and 67% of the pods were empty (3.1 times the control).



**Figure 3.** Number of total (filled + empty) pods (a) and percentage of empty pods (b) at maturity of four chickpea genotypes grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. Bars represent least-significant differences (LSD) at  $P = 0.05$  for the genotype  $\times$  treatment interaction ( $G \times T$ ). Data are means  $\pm$  SE of four replicate pots. Two plants per pot were pooled and the mean per plant used for one replicate data.

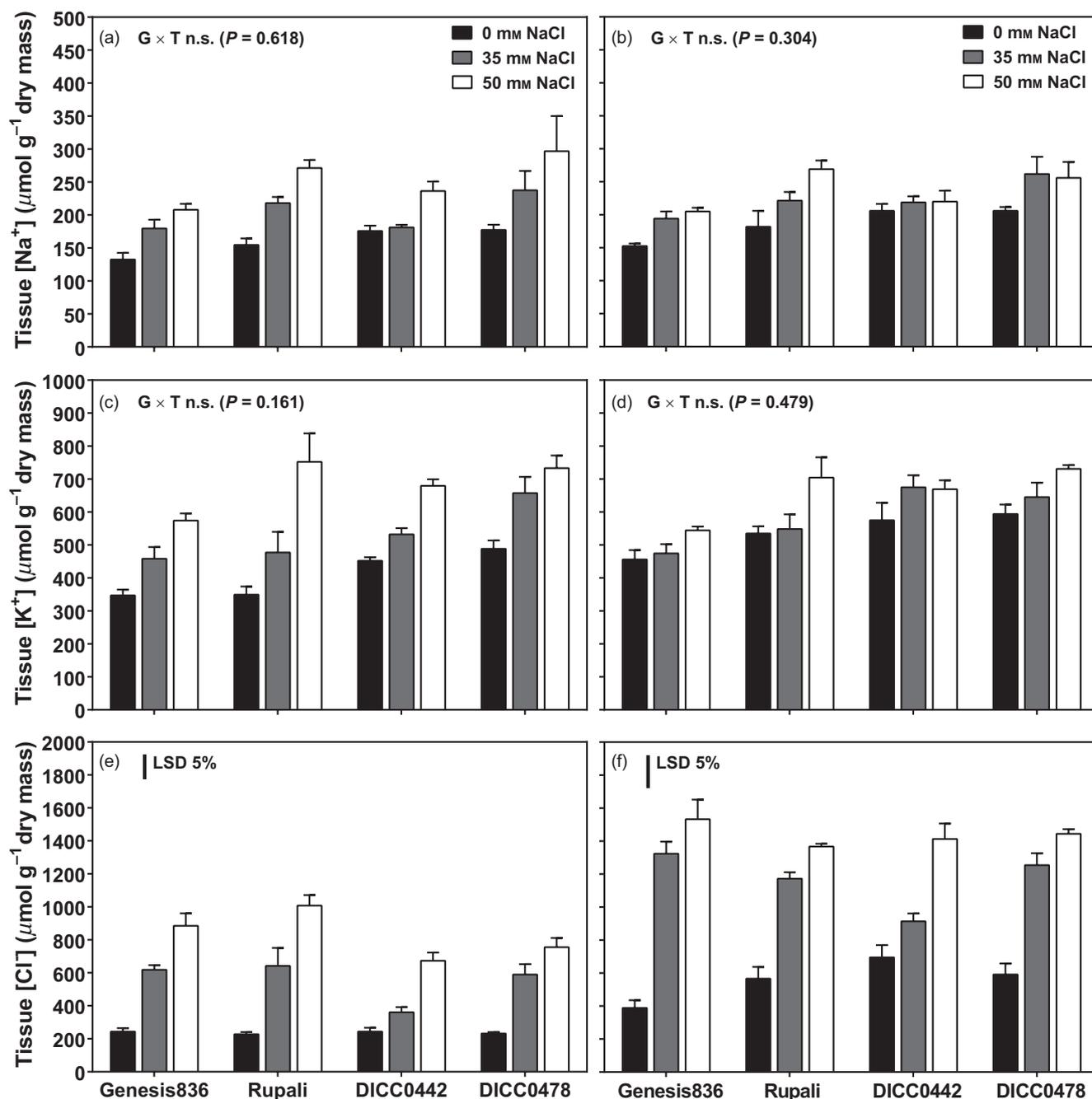
### Effects of salinity on Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> concentrations in leaves

NaCl stress increased Na<sup>+</sup>, K<sup>+</sup> and particularly Cl<sup>-</sup> concentrations in YFEL and the OGL. There was no significant genotype  $\times$  treatment interaction for the Na<sup>+</sup> and K<sup>+</sup> concentrations in YFEL ( $P = 0.618$  for Na<sup>+</sup> and  $P = 0.161$  for K<sup>+</sup>) or OGL ( $P = 0.304$  for Na<sup>+</sup> and  $P = 0.479$  for K<sup>+</sup>), but there was a significant interaction for Cl<sup>-</sup> concentration ( $P < 0.05$  for YFEL and  $P < 0.001$  for OGL). The mean Na<sup>+</sup> concentration in YFEL in the control, 35 and 50 mM NaCl treatments was 160, 204 and 253  $\mu\text{mol g}^{-1}$  dry mass, respectively (Fig. 4a). Interestingly, there were no significant differences in Na<sup>+</sup> concentration between YFEL and OGL (no significant genotype  $\times$  treatment  $\times$  leaf interaction;  $P = 0.894$ ) (Fig. 4a,b). The mean K<sup>+</sup> concentration in YFEL in the control, 35 and 50 mM NaCl treatments was 409, 531 and 684  $\mu\text{mol g}^{-1}$  dry mass, respectively (Fig. 4c). As with Na<sup>+</sup>, K<sup>+</sup> concentration did not differ between YFEL and OGL ( $P = 0.638$ ) (Fig. 4c,d). The mean Cl<sup>-</sup> concentration in YFEL in the control, 35 and 50 mM NaCl treatments was 236, 553 and 830  $\mu\text{mol g}^{-1}$  dry mass, respectively (Fig. 4e). OGL had significantly higher Cl<sup>-</sup> concentrations than YFEL (significant genotype  $\times$  treatment  $\times$  leaf interaction;  $P < 0.05$ ). In OGL, Cl<sup>-</sup> concentrations were 560, 1166 and 1439  $\mu\text{mol g}^{-1}$  dry mass for the control, 35 and 50 mM NaCl treatments, respectively (Fig. 4f).

### X-ray microanalysis of the developing pod

Concentrations of Na, K and Cl were measured in cells in an early stage (7–8 days after flower bud appearance and about 5 days after self-fertilization) pod wall and ovule of the tolerant genotype Genesis836 and the sensitive genotype Rupali grown in the non-saline control and the 50 mM NaCl treatment (Fig. 5a). The anatomical structures of the pod wall where the analyses were made included parenchyma layers of the mesocarp and the inner thickened layers of cells of the endocarp. Structures analysed in the ovule consisted of densely packed cell layers of the outer coat and large parenchyma cells occupying the interior of the ovule (Fig. 5b).

When grown in non-saline soil, the Na concentration in these reproductive tissues at an early pod developmental stage in both Genesis836 and Rupali was low in all tissues analysed (no significant tissue  $\times$  genotype interaction;  $P = 0.395$ ) (Fig. 6a). In Genesis836, the Na concentration in all tissues was relatively constant with a mean of 13  $\mu\text{mol g}^{-1}$  (mmol kg<sup>-1</sup>) equivalent wet mass. In Rupali, the highest Na concentration was in cells of the mesocarp ( $14.2 \pm 1.9 \mu\text{mol g}^{-1}$  equivalent wet mass) and the lowest in cells of the ovule's coat ( $4.3 \pm 2.2 \mu\text{mol g}^{-1}$  equivalent wet mass). The imposition of 50 mM NaCl significantly increased Na concentration in both tolerant and sensitive genotypes ( $P < 0.0001$ ); however, on average, early stage pod tissues of the salinity-tolerant genotype Genesis836 had higher Na than those of the salinity-sensitive genotype Rupali (significant tissue  $\times$  genotype interaction,  $P < 0.05$ ). In Genesis836, Na concentrations ( $\mu\text{mol g}^{-1}$  equivalent wet mass) ranged

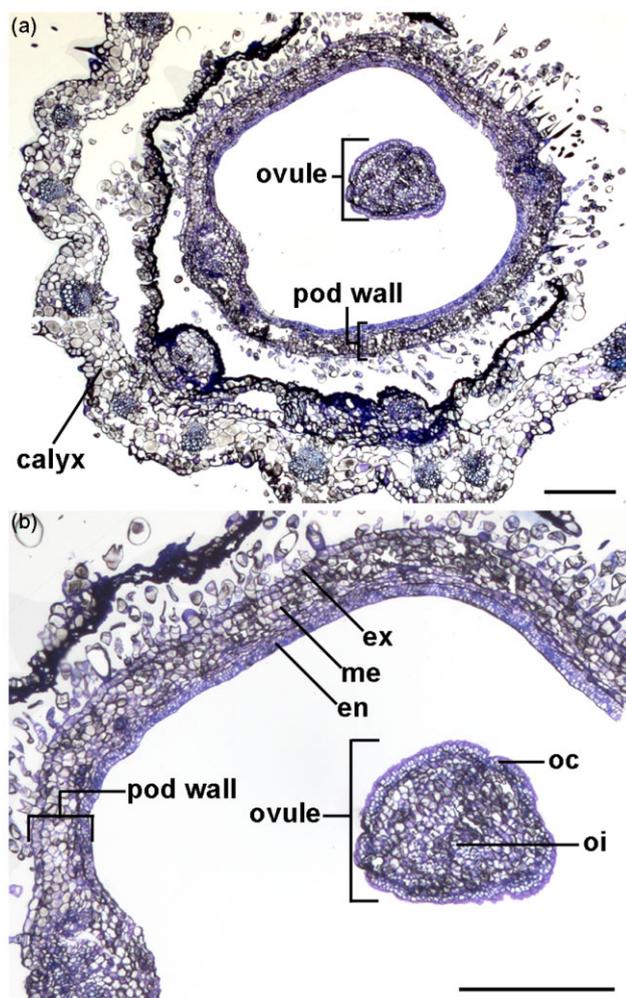


**Figure 4.** Mean concentration of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> in the youngest fully expanded leaf (left column; a, c, e) and the oldest green leaf (right column; b, d, f) of four chickpea genotypes grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. Leaves were sampled 97 days after sowing (34 days before maturity). Bars represent least-significant differences (LSD) at  $P = 0.05$  for the genotype  $\times$  treatment interaction (G  $\times$  T). G  $\times$  T was not significant for Na<sup>+</sup> and K<sup>+</sup> in both young fully expanded leaves and oldest green leaves. Data are means  $\pm$  SE of four replicate pots. Two plants per pot were pooled and the mean per plant used for one replicate data.

from 24 in cells of the ovule's interior to 45 in the mesocarp, whereas in Rupali, Na concentrations ( $\mu\text{mol g}^{-1}$  equivalent wet mass) ranged from 12 in the ovule's coat to 37 in the mesocarp.

Genesis836 had significantly higher K concentrations than Rupali in all corresponding tissues, with the exception of the ovule's interior, for plants grown in non-saline soil (significant tissue  $\times$  genotype interaction,  $P < 0.0001$ ) (Fig. 6b). In

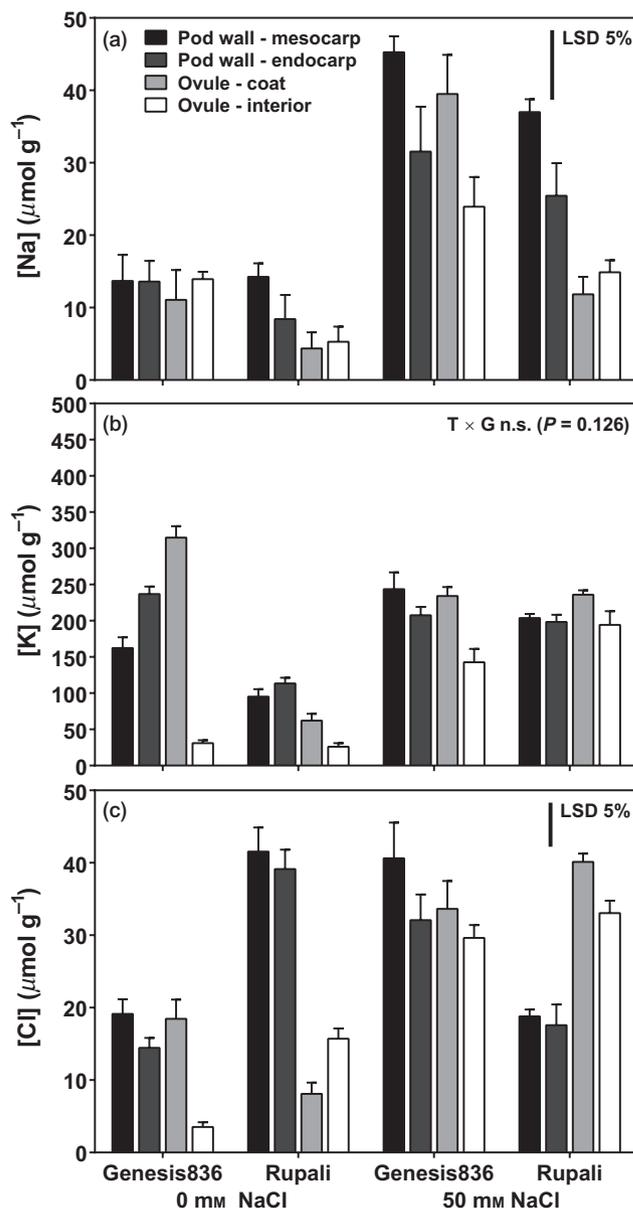
Genesis836, the ovule's coat had the highest K concentration ( $314 \pm 15 \mu\text{mol g}^{-1}$  equivalent wet mass; being five times higher than in Rupali), and the ovule's interior had the lowest K ( $31 \pm 4 \mu\text{mol g}^{-1}$  equivalent wet mass; not significantly different to the value for Rupali). In Genesis836, the 50 mM NaCl treatment reduced the K concentration in cells of the endocarp and ovule's coat, but increased in cells of the mesocarp and ovule's interior. In Rupali, the 50 mM NaCl



**Figure 5.** Cross sections of a typical early pod (pod setting stage) used for X-ray microanalysis. Resin-embedded sections were cut with a glass knife, stained with Toluidine Blue O and viewed with bright field. Photographs were taken at different magnifications 5 $\times$  (a) and 10 $\times$  (b). Bars = 200  $\mu\text{m}$ . ex, exocarp; me, mesocarp; en, endocarp of pod wall; oc, coat of ovule; oi, interior of ovule.

treatment significantly increased the K concentration in all cells analysed ( $P < 0.0001$ ). As a result, the concentration of K in the 50 mM NaCl treatment did not significantly differ between Genesis836 and Rupali in all corresponding tissues (no significant tissue  $\times$  genotype interaction;  $P = 0.126$ ). In both genotypes, the ovule's interior had the lowest K concentration being  $143 \pm 18$  and  $194 \pm 19 \mu\text{mol g}^{-1}$  equivalent wet mass in Genesis836 and Rupali, respectively, although these values were substantially higher than in the same cell types of the non-saline controls of both genotypes.

Cl concentration differed significantly between Genesis836 and Rupali in all corresponding tissues when grown in non-saline soil (significant tissue  $\times$  genotype interaction;  $P < 0.0001$ ) (Fig. 6c). In Genesis836, cells of the mesocarp, endocarp and ovule's coat had similar Cl concentrations, with a mean of  $17.3 \mu\text{mol g}^{-1}$  equivalent wet mass, but cells of the ovule's interior had less ( $3.5 \mu\text{mol g}^{-1}$  equivalent wet mass) than the other tissues. In Rupali, cells of the pod wall



**Figure 6.** Concentrations ( $\mu\text{mol g}^{-1}$  of wet weight equivalent) of Na (a), K (b) and Cl (c) in cells of the mesocarp and endocarp of the pod wall, and the coat and interior of the ovule at the pod setting stage (7–8 days after bud appearance) of two chickpea genotypes grown in soil with 0 (non-saline control) or 50 mM NaCl. Samples were collected about 97 days after sowing. Elemental concentrations of Na, K and Cl were measured using X-ray microanalysis. Bars represent least-significant differences (LSD) at  $P = 0.05$  for the tissue  $\times$  genotype ( $T \times G$ ) interaction at 50 mM NaCl.  $T \times G$  was not significant for K at 50 mM NaCl. Data are means  $\pm$  SE of 3–16 cells. Note scale differences on vertical axes.

(mesocarp and endocarp) accumulated on average more Cl ( $40.3 \mu\text{mol g}^{-1}$  equivalent wet mass) than cells of the ovule ( $11.9 \mu\text{mol g}^{-1}$  equivalent wet mass). The 50 mM NaCl treatment significantly increased Cl concentration in all tissues in Genesis836 ( $P < 0.0001$ ). In Rupali, the 50 mM NaCl treatment unexpectedly reduced the Cl concentration in cells

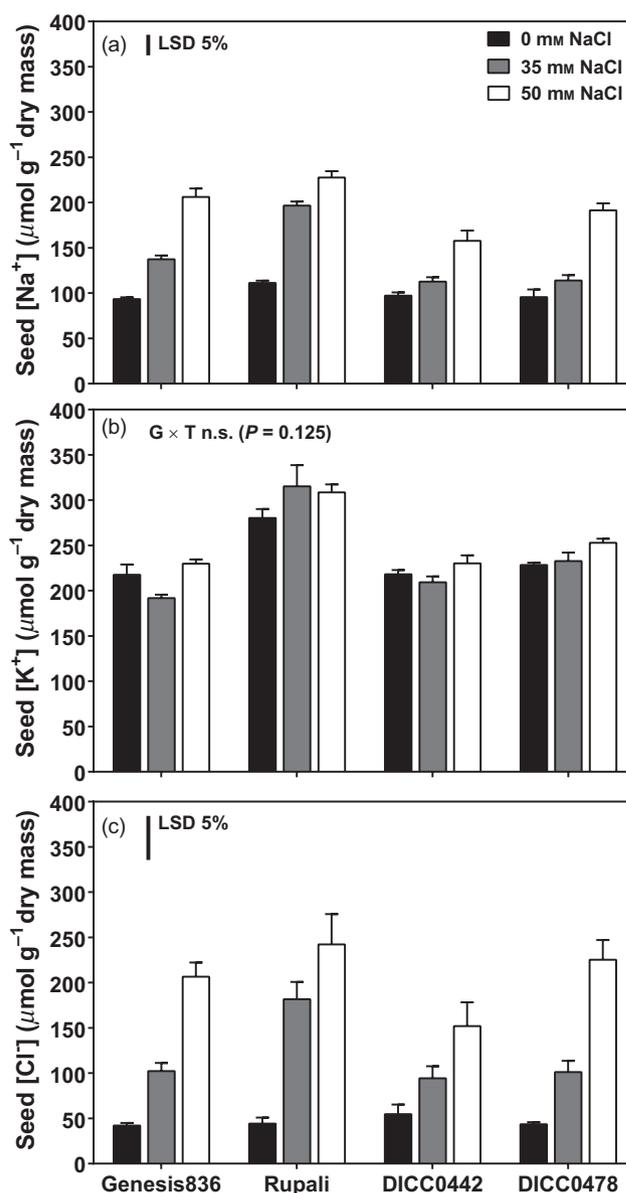
of the mesocarp and the endocarp, but it increased in cells of the ovule's coat and interior. As a result, the Cl concentration in cells of the pod wall of Rupali was lower than those in Genesis836 being on average 18.2 and 36.3  $\mu\text{mol g}^{-1}$  equivalent wet mass, respectively, whereas the Cl concentrations in cells of the ovule were similar in Rupali and Genesis836 being on average 36.5 and 31.6  $\mu\text{mol g}^{-1}$  equivalent wet mass, respectively. There was a significant tissue  $\times$  genotype interaction for Cl in cells of plants in the 50 mM NaCl treatment ( $P < 0.0001$ ).

### Effects of salinity on $\text{Na}^+$ , $\text{K}^+$ and $\text{Cl}^-$ concentrations of the mature seeds

In the non-saline control, all four genotypes had similar seed  $\text{Na}^+$  concentrations ranging from 93 to 111  $\mu\text{mol g}^{-1}$  dry mass (Fig. 7a). The 35 mM NaCl treatment did not affect  $\text{Na}^+$  concentration in seeds of DICC0442 or DICC0478, but significantly increased that of Genesis836 and Rupali. The 50 mM NaCl treatment affected the seed  $\text{Na}^+$  concentration in all four genotypes. Compared with the non-saline controls, seed  $\text{Na}^+$  concentration increased by 1.6, 2, 2 and 2.2 times in DICC0442, DICC0478, Rupali and Genesis836, respectively. As a result there was a significant genotype  $\times$  treatment interaction for seed  $\text{Na}^+$  concentration (Fig. 7a); however, the genotype  $\times$  treatment interaction was not significant between tolerant (Genesis836 and DICC0442) and sensitive (Rupali and DICC0478) genotypes ( $P = 0.40$ ) when data were pooled into these two groups.

Seed  $\text{K}^+$  concentration differed among genotypes grown in non-saline soils, ranging from 217 (Genesis836) to 280 (Rupali)  $\mu\text{mol g}^{-1}$  dry mass (Fig. 7b). The 35 mM NaCl treatment reduced seed  $\text{K}^+$  concentration in Genesis836 (12% reduction compared with control), but it was unaffected in the other three genotypes. In the 50 mM NaCl treatment, seed  $\text{K}^+$  concentration had increased by 10% in DICC0478 and was unaffected in Genesis836, Rupali and DICC0442. As a result, there was no a significant genotype  $\times$  treatment interaction for seed  $\text{K}^+$  concentration ( $P = 0.125$ ).

All four genotypes had similar and low seed  $\text{Cl}^-$  concentrations when grown in non-saline soil ranging from 42 to 55  $\mu\text{mol g}^{-1}$  dry mass (Fig. 7c). The 35 mM NaCl treatment did not significantly affect seed  $\text{Cl}^-$  concentration in DICC0442, but increased it in DICC0478, Genesis836 and Rupali by 2.3, 2.4 and 4.1 times, respectively. The 50 mM NaCl treatment significantly increased seed  $\text{Cl}^-$  concentration in all four genotypes. Compared with non-saline controls, seed  $\text{Cl}^-$  concentrations increased by 2.8, 4.9, 5.2 and 5.5 times in DICC0442, Genesis836, DICC0478 and Rupali, respectively. As a result there was a significant genotype  $\times$  treatment interaction for seed  $\text{Cl}^-$  concentration (Fig. 7c); however, the genotype  $\times$  treatment interaction was not significant between tolerant (Genesis836 and DICC0442) and sensitive (Rupali and DICC0478) genotypes ( $P = 0.078$ ) when data were pooled into these two groups.



**Figure 7.** Mean concentration of  $\text{Na}^+$  (a),  $\text{K}^+$  (b) and  $\text{Cl}^-$  (c) in seeds of four chickpea genotypes grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. Bars represent least-significant differences (LSD) at  $P = 0.05$  for the genotype  $\times$  treatment interaction ( $G \times T$ ).  $G \times T$  was not significant for  $\text{K}^+$ . Note that there was no significant  $G \times T$  interaction between tolerant and sensitive genotypes for  $\text{Na}^+$  and  $\text{Cl}^-$  (see Results). Data are means  $\pm$  SE of four replicate pots. Two plants per pot were pooled and the mean per plant used for one replicate data.

### Germination test

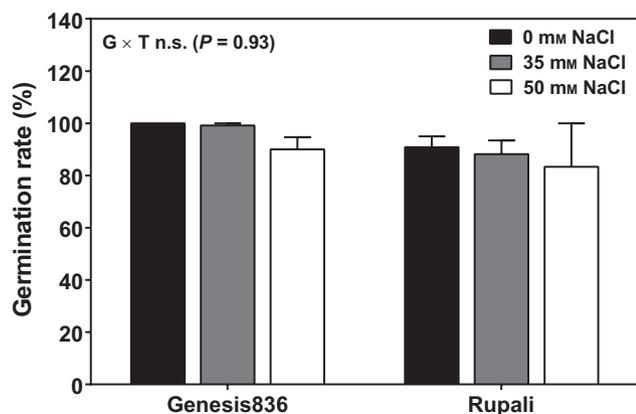
This experiment was undertaken to determine whether the accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in seeds had any negative effect on seed germination in non-saline conditions. The germination test was conducted using one tolerant (Genesis836) and one sensitive (Rupali) genotype. Seeds of both Genesis836 and Rupali taken from plants grown under non-saline conditions had high germination rates of 100 and 93%,

respectively (Fig. 8). Germination of seeds from plants from the 35 and 50 mM NaCl treatments did not significantly change except for seeds from Genesis836 in the 50 mM NaCl treatment where it was reduced by 10%. There was no significant genotype  $\times$  treatment interaction ( $P = 0.93$ ). In Rupali, more than 90% of seeds from all treatments germinated on the second and third days. In Genesis836, only 10–50% of seeds imbibed and germinated within the first 6 days (with most on the second and third days). To assist imbibition of the remaining seeds, seed coats were punctured and subsequently these seeds germinated 2–3 days later.

## DISCUSSION

Several studies have indicated adverse effects of salinity on reproductive processes in chickpea (see Introduction) and have suggested that their sensitivity during reproductive stages may be due to the accumulation of Na<sup>+</sup> and Cl<sup>-</sup> in reproductive structures (e.g. Samineni *et al.* 2011). This paper reports that despite genotypic variation in reproductive success expressed as seed yield per plant under saline conditions, no differences were observed in the accumulation of Na or Cl in early reproductive tissues of developing ovules and pods between a tolerant (Genesis836) and a sensitive (Rupali) genotype.

Genotypic variation for seed yield in saline soil was associated with better maintenance of flower production and higher numbers of filled pods (and thus seed number) in tolerant genotypes, whereas seed size decreased in all genotypes. The maintenance of seed numbers (saline versus control) for the present genotypes was not related to flower production under non-saline conditions, as was found for other chickpea genotypes by Vadez *et al.* (2012). These earlier authors reported that tolerant lines produced more flowers than sensitive lines under non-saline conditions and suggested that this is a constitutive trait contributing to the



**Figure 8.** Germination rate (%) of seeds from two chickpea genotypes grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. There was no significant genotype  $\times$  treatment interaction ( $G \times T$ ) for germination rate. Data are means  $\pm$  SE of four replicates. Seeds from two plants per pot were pooled for one replicate (typically with 30 seeds per replicate).

maintenance of seed numbers under salt stress, but the genotypes in the present study produced similar numbers of flowers in non-saline control plants. Phenology (time to first flower) can also influence seed yield of chickpea in saline conditions (Katerji *et al.* 2001; Vadez *et al.* 2007), but the present study used pairs of tolerant and sensitive genotypes of similar phenology and neither time to first flowering nor time to first podding discriminated tolerant from sensitive genotypes (Supporting Information Table S1). Under saline conditions, flower number decreased in sensitive genotypes (on average by 40% in 50 mM NaCl) whereas salinity had no effect on the flower number of tolerant genotypes (Fig. 2a). These results show that the number of flowers produced under non-saline conditions does not discriminate the two tolerant from the two sensitive genotypes; the tolerant genotypes produced a similar number of flowers in the saline treatment despite a reduction in shoot dry mass. Large genotype  $\times$  treatment differences in flower production corresponded to large differences in pod number per plant as flower plus pod abortion levels were relatively similar in all genotypes (no significant genotype  $\times$  treatment interaction).

Significant differences among genotypes in response to salinity occurred at pod filling. The percentage of empty pods (i.e. seed abortion) in the 35 mM NaCl treatment increased about 1.6 times in Rupali, when compared with the non-saline control, and in the 50 mM NaCl treatment it increased 2.0 and 3.1 times in Rupali and DICC0478, respectively. The number of empty pods remained unaffected in tolerant Genesis836 and DICC0442 in both salinity treatments. These results suggest that a decline in the ability to produce seeds contributes to the reduction of seed numbers in salt-sensitive chickpea genotypes. It is interesting to note that the two sensitive genotypes, Rupali and DICC0478, differed in their salt sensitivity. In 35 mM NaCl treatment, productivity of Rupali dropped sharply (seed yield declined to 23% compared with control; see Results) whereas the same concentration did not affect seed yield or its components in DICC0478. It appears that genotypes differ in their expression of resistance at different levels of salinity. Taken together, the higher seed yield under saline conditions in the tolerant than the sensitive chickpea genotypes used in this study was attributable to higher flower production in saline conditions and the ability to produce pods with fully developed seeds.

X-ray microanalysis of developing pod walls and ovules after fertilization (7–8 days after flower bud appearance) of plants grown in 50 mM NaCl revealed that the salinity treatment affected cellular elemental concentrations of Na, K and Cl in both Genesis836 and Rupali when compared with control plants. It did not, however, explain the large differences in seed yield between tolerant and sensitive genotypes. K concentration was similar between the two genotypes whereas Na and Cl were either not different or in some cases lower in the sensitive genotype (Rupali) than the tolerant genotype (Genesis836) in corresponding cells of the pod wall and ovule. As far as we are aware, this is the first detailed analysis of ion concentrations in specific cells of reproductive structures of plants subjected to salt stress; future work could

evaluate reproductive structures during the fertilization process (especially in pollen, stigma and style) to add to the data presented here for the early pod stage.

Our results do not support a previous hypothesis/speculation that accumulation of either  $\text{Na}^+$  and/or  $\text{Cl}^-$  in reproductive structures of chickpea, at least for the ovule, might inhibit reproductive processes and thus reduce seed yield (Samineni *et al.* 2011). For plants exposed to 50 mM NaCl, the Na concentration varied between 12 and 45  $\mu\text{mol g}^{-1}$  equivalent wet mass in cells of the ovule's coat and the mesocarp, respectively, and Cl ranged from 18 to 41  $\mu\text{mol g}^{-1}$  equivalent wet mass in cells of the endocarp and mesocarp, respectively (Fig. 6). The Na and Cl concentrations in all cells analysed were relatively low and would not be expected to be toxic at these levels (see Munns & Tester 2008) and so could not explain the high percentage of empty pods observed for Rupali. These ion concentrations are unlikely to adversely affect metabolism; *in vitro* studies have shown that  $\text{Na}^+$  levels over 100 mM have substantial inhibitory effects on enzyme activity (Greenway & Osmond 1972). Moreover, the K : Na ratio was maintained well above 1:1 in the cells analysed, being about 6:1 in Genesis836 and 9:1 in Rupali, which also supports the conclusion that Na levels likely did not affect metabolism (see Greenway & Munns 1980). Further, these Na and Cl concentrations are unlikely to affect fertilization processes. The present data on ovule ion concentrations add to the study by Turner *et al.* (2013) on chickpea, which showed that pollen viability, *in vitro* pollen germination and *in vivo* pollen tube growth were not affected by salinity. A different situation may occur in rice, where pollen viability, pollen germination and stigma receptivity decreased in saline conditions and concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in these floral parts substantially exceeded those found in the present study (Khatun & Flowers 1995; Khatun *et al.* 1995). When rice was grown in 25 mM NaCl, pollen  $\text{Na}^+$  and  $\text{Cl}^-$  were, respectively, 1.11 and 1.47 mmol  $\text{g}^{-1}$  dry mass.  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations in stigmas were even higher (Khatun & Flowers 1995).

Ion analysis of the mature seeds of chickpea grown in saline soils revealed an increased concentration of  $\text{Na}^+$  and  $\text{Cl}^-$  compared with control plants whereas the  $\text{K}^+$  concentration did not change (Fig. 7a,b,c). This agrees with Murumkar & Chavan (1986) who showed that in chickpea (cv. Chafa) grown at 50 mM NaCl in sand culture, seed  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations increased in comparison with non-saline controls, but  $\text{K}^+$  was not affected. Mamo *et al.* (1996) demonstrated that in three chickpea genotypes grown at 2 dS  $\text{m}^{-1}$  NaCl, seed  $\text{Na}^+$  concentration increased on average 1.5-fold and  $\text{Cl}^-$  concentration 4.5-fold. This is comparable with the present study, where the 50 mM NaCl treatment increased the average seed  $\text{Na}^+$  concentration twofold and  $\text{Cl}^-$  concentration 4.6-fold, compared with seeds from non-saline controls. The changes in  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations for mature seeds were similar between tolerant (Genesis836 and DICC0442) and sensitive (Rupali and DICC0478) genotypes (no significant genotype  $\times$  treatment interaction), indicating that salinity tolerance was not associated with the accumulation of ions in seeds at maturity. Germination rates remained high

for seeds from both Genesis836 and Rupali that matured in the saline treatment, indicating no adverse effects of the ions that had accumulated in the seeds on (short-term) seed viability and germination.

Young developing pods had lower concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  than in mature seeds and leaves of plants grown in soil with 50 mM NaCl. Interestingly, mature seeds, YFEL and OGL had similar  $\text{Na}^+$  concentrations (dry mass basis), being consistent with the study by Mamo *et al.* (1996) who also showed that the  $\text{Na}^+$  concentration in the seeds was approximately equal to that in vegetative tissues for other chickpea genotypes. In contrast, plants grown in soil with 50 mM NaCl had less  $\text{Cl}^-$  in mature seeds than in YFEL, and the  $\text{Cl}^-$  concentration in YFEL was lower than in OGL. For instance, the  $\text{Na}^+$  concentration in mature seeds, YFEL and OGL were relatively similar with means of 195, 250 and 237  $\mu\text{mol g}^{-1}$  dry mass, respectively, while the  $\text{Cl}^-$  concentrations in YFEL and OGL was about four- and sevenfold higher than in mature seeds, respectively. These differences in  $\text{Cl}^-$  concentrations between tissues support the previous finding by Turner *et al.* (2013) that chickpea can limit  $\text{Cl}^-$  accumulation in developing seeds relative to its sequestration in leaves. The  $\text{Na}^+$  concentrations in YFEL and OGL of plants grown in soil with 50 mM NaCl were similar to critical  $\text{Na}^+$  concentration reported for chickpea shoots (200–270  $\mu\text{mol g}^{-1}$  dry mass; Lauter & Munns 1987), whereas the  $\text{Cl}^-$  concentrations exceeded the critical concentrations (~450  $\mu\text{mol g}^{-1}$  dry mass; Lauter & Munns 1987). Salinity tolerance was not, however, associated with genotypic differences in the concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in both YFEL and OGL between tolerant (Genesis836 and DICC0442) and sensitive (Rupali and DICC0478) genotypes ( $P = 0.30$  for YFEL and  $P = 0.412$  for OGL). There was also no relationship between the leaf  $\text{K}^+:\text{Na}^+$  ratio and salinity tolerance among genotypes; the ratio was between 2.2 and 3.1 in both tolerant and sensitive genotypes.  $\text{K}^+$  concentration in both the YFEL and OGL increased on average by 67 and 22%, respectively, in plants exposed to 50 mM NaCl treatment. Increased  $\text{K}^+$  concentrations in the YFEL of chickpea grown in soil with 40 mM NaCl was also observed by Turner *et al.* (2013) with the mean  $\text{K}^+$  concentration of 55 genotypes being 7% higher ( $P < 0.001$ ). These results for chickpea are contrary to the generalized expectation that salinity reduces  $\text{K}^+$  in plants (Greenway & Munns 1980; Marschner 1995), although other exceptions have also been reported, for example, in durum wheat that showed an increased  $\text{K}^+$  concentration in the flag leaf at high, but not at intermediate, salinity, which the authors suggest was due to induction of a high affinity  $\text{K}^+$  uptake mechanism only at high  $\text{Na}^+$  concentrations (Dvořák *et al.* 1994). In addition to the mechanism speculated on by Dvořák *et al.* (1994), some plants might also have enhanced  $\text{K}^+$  remobilization from senescent leaves and/or less 'dilution' if growth is inhibited more than that of  $\text{K}^+$  net uptake at some levels of salinity, both which could also enhance  $\text{K}^+$  supply to the remaining green leaves.

In conclusion, genotypic variation exists for seed yield of chickpea in saline soil, with Genesis836 and DICC0442

proving tolerant to salt stress and Rupali and DIC0478 being considerably more salt sensitive and producing lower yields under saline conditions. Large genotypic variation for seed yield, the determinant of salinity tolerance, correlated with the maintenance of seed numbers. X-ray microanalysis revealed that Na and Cl accumulation in cells within specific tissues of developing pod walls and ovules after fertilization (early pod stage) was relatively low and presumably would not explain large differences in seed yield between tolerant and sensitive genotypes. Similarly, salinity tolerance was not associated with the accumulation of salt ions in seeds at maturity and was not associated with ion concentrations in leaves. Research is needed to elucidate whether a wider range of chickpea genotypes respond similarly to salinity, or differ in terms of their ion relations in reproductive tissues. Future studies are also needed to elucidate whether reductions of seed yields in salt-sensitive genotypes are related to assimilate supplies, as suggested might occur in tomato under saline conditions (Ghanem *et al.* 2009). In the wider context of salinity tolerance in plants, data on Na, K, Cl and Ca relations in reproductive tissues and cells of various species should be beneficial for understanding the influence of ion relations on seed and fruit development, to build on the present chickpea data and the earlier studies (e.g. Khatun & Flowers 1995) of rice.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Time to first flower and pod in days after sowing (DAS) of four genotypes of chickpea grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. Two-way ANOVA was used to compare genotype (G), treatment (T) and genotype  $\times$  treatment ( $G \times T$ ) effects. Least-significant differences (LSD) at  $P = 0.05$  for each G, T and  $G \times T$  effect are provided (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., non-significant). Data are means  $\pm$  SE of four replicate pots. Two plants per pot were pooled and the mean per plant used for each replicate.

**Table S2.** Shoot dry mass (g) of four genotypes of chickpea grown in soil with 0 (non-saline control), 35 and 50 mM NaCl. It should be noted that many older dry leaves were lost during harvest. Two-way ANOVA was used to compare genotype (G), treatment (T) and genotype  $\times$  treatment ( $G \times T$ ) effects. Least-significant differences (LSD) at  $P = 0.05$  for each G, T and  $G \times T$  effect are provided (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Data are means  $\pm$  SE of four replicate pots. Two plants per pot were pooled per pot and the mean per plant used as each replicate.