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SALINITY STRESS

# Salt Stress Delayed Flowering and Reduced Reproductive Success of Chickpea (*Cicer arietinum* L.), A Response Associated with $Na^+$ Accumulation in Leaves

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## Abstract

Salinity is known to reduce chickpea yields in several regions of the world. Although ion toxicity associated with salinity leads to yield reductions in a number of other crops, its role in reducing yields in chickpea growing in saline soils is unclear. The purpose of this study was to (i) identify the phenological and yield parameters associated with salt stress tolerance and sensitivity in chickpea and (ii) identify any pattern of tissue ion accumulation that could relate to salt tolerance of chickpea exposed to saline soil in an outdoor pot experiment. Fourteen genotypes of chickpea (*Cicer arietinum* L.) were used to study yield parameters, of which eight were selected for ion analysis after being grown in soil treated with 0 and 80 mM NaCl. Salinity delayed flowering and the delay was greater in sensitive than tolerant genotypes under salt stress. Filled pod and seed numbers, but not seed size, were associated with seed yield in saline conditions, suggesting that salinity impaired reproductive success more in sensitive than tolerant lines. Of the various tissues measured for concentrations of $Cl^-$, $Na^+$ and $K^+$, higher seed yields in saline conditions were positively correlated with higher $K^+$ concentration in seeds at the mid-filling stage ($R^2 = 0.55$), a higher $K^+/Na^+$ ratio in the laminae of fully expanded young leaves ($R^2 = 0.50$), a lower $Na^+$ concentration in old green leaves ($R^2 = 0.50$) and a higher $Cl^-$ concentration in mature seeds. The delay in flowering was associated with higher concentrations of $Na^+$ in the laminae of fully expanded young leaves ($R^2 = 0.61$) and old green leaves ($R^2 = 0.51$). We conclude that although none of the ions appeared to have any toxic effect, $Na^+$ accumulation in leaves was associated with delayed flowering that in turn could have played a role in the lower reproductive success in the sensitive lines.

## Introduction

Salinity affects an arable land area of 100 million ha worldwide, and this area is increasing (Rengasamy 2006). Chickpea (*Cicer arietinum* L.) is considered very sensitive to salinity (Flowers et al. 2010), but variation in salinity tolerance has been observed among chickpea accessions (Vadez et al. 2007, Krishnamurthy et al. 2011, Turner et al. 2013). However, little is known about the mechanisms of salt tolerance in chickpea. Adverse water relationships, excess $Na^+$ accumulation (Munns and Tester 2008), interference with $K^+$ homoeostasis, production of reactive oxygen species (ROS) (Abogadallah 2010, Bose et al. 2014, Pottosin et al. 2014) in plant tissues are reportedly causes for crop sensitivity under exposure to salinity, but the influence of shoot $Na^+$ concentration in chickpea sensitivity/tolerance is

 

equivocal. Although shoot $Na^+$ concentration was low and was not associated with yield under saline conditions in a study with 263 accessions (Vadez et al. 2007), higher $Na^+$ concentrations in the youngest fully expanded leaves were associated with lower yields under saline conditions in a second study with several of the same genotypes (Turner et al. 2013). The salt-sensitive genotypes also had higher concentrations of $Na^+$ in the seed than salt-tolerant genotypes (Turner et al. 2013). Salt sensitivity was not significantly associated with the accumulation of $Na^+$ in other tissues or the accumulation of $Cl^-$ in any vegetative or reproductive tissues (Turner et al. 2013). Moreover, based on apparent critical concentrations for $Cl^-$ and $Na^+$ in chickpea shoots (as reported in the literature), Samineni et al. (2011) hypothesised that $Cl^-$ toxicity might be of importance in chickpea. On exposure to stresses such as drought and salinity, the plant cells are affected by osmotic stress and osmotic adjustment takes place to maintain normal turgor pressure by uptake of inorganic ions (Wyn Jones and Pritchard 1989, Bohnert et al. 1995). Shabala and Lew (2002) showed that turgor recovery, along with increased uptake of $K^+$, $Cl^-$ and $Na^+$, occurred in Arabidopsis root cells within a few minutes after a hyperosmotic stress treatment.

Yield per plant of chickpea in saline soil has been associated with more tertiary branches and flowers, as well as the capacity to maintain filled pods (Vadez et al. 2007, 2012). However, seed size was maintained under salinity, suggesting that seed set was more sensitive than the rate of seed filling under salinity (Vadez et al. 2007, 2012). Although pollen viability and germination were not affected by salinity, pod abortion was higher in sensitive genotypes (Turner et al. 2013), suggesting that reduced seed numbers may be due to failed fertilisation or early seed development (Samineni et al. 2011, Turner et al. 2013).

Tissue concentrations of $Na^+$ and $Cl^-$ in chickpea increased under saline conditions (Samineni et al. 2011, Turner et al. 2013). In white clover and white lupin, adverse changes in tissue ion homoeostasis led to cellular damage, cessation of growth and tissue death, and eventually to plant death (Munns and Termatt 1986, Manchanda and Sharma 1989, Zhu 2001). On the other hand, exposure of plants to salinity has been shown to induce osmotic adjustment by uptake of ions (Bernstein 1961, 1963, Shabala and Lew 2002) and synthesis of organic solutes (Greenway and Munns 1980), result in increased production of abscisic acid (Wolf et al. 1990) and other hormones, and increase ROS and activate antioxidant defence mechanisms (Bose et al. 2014, Pottosin et al. 2014). In their review, Munns and Tester (2008) concluded that exclusion of $Na^+$ and $Cl^-$ by the roots and sequestering the ions in old tissues helped to avoid ion toxicity in young leaves and reproductive organs. Turner et al. (2013) found an association between higher $Na^+$ concentration in young leaves and seeds and salt sensitivity in chickpea, but no association in older tissues. This finding suggests that limiting ion accumulation in young tissues is important for salt tolerance, but may not relate to storing of salt ions in older tissues. In addition, there was no association under saline conditions between yield and the accumulation of $Cl^-$ in leaves or pod shells and accumulation in the seed (Turner et al. 2013). This result for a larger number of genotypes suggests that $Cl^-$ does not play a major part in salt tolerance/sensitivity in chickpea, and this finding furnishes an important broader understanding of the earlier physiological work of Samineni et al. (2011) on only one variety. Our present hypothesis is that reproductive success is the key factor in attaining a higher yield under salinity and that this linkage may relate to a particular pattern of ion accumulation in both reproductive and vegetative tissues.

In this study, seven reportedly tolerant and seven reportedly sensitive genotypes (Krishnamurthy et al. 2011) were exposed to salinity, and their salinity tolerance, based on yield or relative yield, was confirmed. In an adjacent experiment, four salt-sensitive and four salt-tolerant genotypes were sampled for a systematic analysis of $Na^+$, $K^+$ and $Cl^-$ concentrations in leaves, stems, floral and seed tissues during reproductive development. We focused on (i) confirming yield-related traits that discriminate tolerant and sensitive genotypes for salinity and (ii) determining whether $Na^+$, $Cl^-$ and/or $K^+$ concentrations in vegetative and reproductive tissues were associated with salt tolerance/sensitivity among genotypes. The results of these investigations could yield a better understanding of salt tolerance in chickpea. The analysis of $Na^+$, $K^+$ and $Cl^-$ concentrations in different vegetative and reproductive tissues helps to test the hypothesis that higher accumulation of the above-mentioned ions, in particular in reproductive tissues, under saline conditions compared with non-saline conditions will cause a disturbance of ion homoeostasis and thus affect plant growth and yield.

## Materials and Methods

### Plant material, growth and treatment conditions

This study was conducted in pots buried in the ground at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India (17°30′N; 78°16′E; altitude 549 m); the system enables soil salinity treatments to be imposed in outdoor conditions but with controlled soil salinity. Fourteen chickpea genotypes, contrasting for sensitivity to salinity based on yield, were selected from a larger study (Krishnamurthy et al. 2011). The seven sensitive (S) genotypes had low yields when exposed to salinity (ICC3421, ICC6263, ICC7315, ICC15510, ICC10755,


 

ICC13283, ICC15518), and the seven tolerant (T) genotypes had high yields when exposed to salinity (ICC11121, ICC1431, ICC4495, ICC8950, ICC456, ICC9942, ICC12215) (Krishnamurthy et al. 2011). The first four tolerant and sensitive genotypes listed were also used for ion analyses of different vegetative and reproductive tissues.

The experiment was sown on 15 November 2011 and harvested in March 2012. The average maximum and minimum air temperatures ranged from 29 to 36.5 °C and 12–20 °C, respectively. Pots (0.27 m diameter) containing 7.5 kg of a vertisol (fine montmorillontic isohyperthermic typic pallustert) soil were buried in the soil so that the outer rim of each pot and outside soil surface were at the same level to avoid direct heating of the pots by solar radiation. The vertisol soil (pH = 8.1, cation exchange capacity (CEC)/clay ratio = 0.87, electrical conductivity (EC) = 0.1 mM) (El Swaify et al. 1985) was taken from the ICRISAT farm and fertilised with di-ammonium phosphate at a rate of 300 mg $kg^{-1}$ soil. One-half of the pots were artificially salinised with 1.17 g NaCl $kg^{-1}$ soil, equivalent to 80 mM NaCl in sufficient volume (1.875 l) to wet the vertisol to field capacity. The control pots received tap water containing no significant amounts of NaCl, in the same volume to bring the soil to field capacity. Subsequent watering of both treatments was performed with tap water. The bottoms of the salinised pots were sealed to avoid any salt leaching.

In both treatments, six seeds were planted in each pot and later (14 days after sowing (DAS)) thinned to four similar-sized plants per pot. The plants for the evaluation of $Na^+$, $Cl^-$ and $K^+$ concentrations in vegetative and reproductive tissues were adjacent to those for the evaluation of yield and yield components. The experimental design was a randomised block design (RBD) with two treatments, a non-saline control (0 mM NaCl) and a saline treatment (80 mM NaCl) as main factors and genotypes as subfactors with four replications per treatment (each replicate was a single pot containing four plants).

### Measurements

In the plants used for yield and yield components, time (days) to first flower (two plants per pot had commenced flowering) and to maturity (all plants in the pot had yellowed) was recorded. At maturity, all plants were harvested and oven dried at 65 °C for 48 h. After drying, the number of filled pods, empty pods and seeds was counted, and total shoot dry matter, pod weight and seed yield (seed weight) were measured on a pot basis and calculated on a per plant basis. The 100-seed weight (seed size) was calculated from seed yield/seed number per pot.

When plants used for ion analysis reached the mid-podding stage (60–65 DAS for the genotypes used), tissue samples were collected for analyses of $Na^+$, $Cl^-$ and $K^+$. The tissues were as follows: (i) old green leaves from the bottom 2–3 nodes, (ii) laminae of the youngest fully expanded leaves, (iii) petioles of the youngest fully expanded leaves, (iv) other leaves, that is all leaves between the oldest green leaves and the youngest fully expanded leaves, (v) unopened flower buds from the top nodes and (vi) seeds at the filling stage (developing seeds). At maturity, mature seeds and pod shells were also sampled for ion analyses. Each tissue sample was placed into a paper envelope and oven dried at 60 °C for 48 h. Tissues were weighed, ground and transferred (with appropriate export/import and quarantine permissions) to the laboratory at The University of Western Australia, Perth, Australia. Each sample was extracted in 0.5 M nitric acid in 10-ml tubes placed on a shaker for 48 h (Munns et al. 2010). The samples were then diluted as appropriate and analysed for $Na^+$ and $K^+$ on a Sherwood flame photometer (Model 410, Sherwood Scientific, Cambridge, UK), and $Cl^-$ was measured using a chloridometer (SLAMED, model 50CL l-50, Frankfurt, Germany). Reference plant tissue with known ion concentrations was measured along with the samples and showed that the analyses recovered 95 % of the $Na^+$, 98 % of the $Cl^-$ and 83 % of the $K^+$; no adjustments were made to the measured values.

### Statistical analysis

The data were analysed using GENSTAT 12.0 (VSN International Ltd., Hemel Hempstead, UK). An unbalanced analysis of variance was performed for all observed parameters individually. As the number of genotypes differed for ion analysis and yield components, the two data sets were analysed separately. Differences between mean values of treatments were evaluated using a least significant difference (LSD) test at a 0.05 probability level. Linear regressions were fitted using MICROSOFT EXCEL 2007 (Microsoft Corp., Redmond, WA, USA). A cluster analysis was performed using PAST software (version 1.9).

## Results

### Agronomic assessment

All parameters differed significantly for genotype, treatment and genotype × treatment interaction at the 5 % level of significance except the interaction for total shoot dry matter (Table 1). In the control treatment, genotypes differed significantly ($P < 0.001$) for days to flower, days to maturity, filled pod number, empty pod number, 100-seed weight, seed number and seed yield, but not for total shoot dry matter (Fig. 1, Table 2).

The salinity treatment (80 mM NaCl) induced a delay in flowering and maturity compared with the control.

**Table 1** *F* probability, least significant difference (LSD) and standard error (SE) values for genotype, treatment and genotype × treatment interaction for total shoot dry matter, days to first flower, days to maturity, filled pod number per plant, empty pod number per plant, seed number, seed yield and 100-seed weight of 14 chickpea genotypes grown in soil with 0 or 80 mM NaCl. Each pot had four plants

| Parameter | Total shoot dry matter (g per plant) | Days to first flower | Days to maturity per plant | Filled pod number per plant | Empty pod number per plant | Seed number per plant | Seed yield (g per plant) | 100-seed weight |
|---|---|---|---|---|---|---|---|---|
| Genotype | | | | | | | | |
| *F* probability | 0.003 | <0.001 | <0.001 | <0.001 | 0.045 | <0.001 | <0.001 | <0.001 |
| LSD | 2.485 | 3.460 | 2.996 | 3.896 | 4.717 | 5.108 | 0.652 | 1.209 |
| SE | 1.249 | 1.742 | 1.506 | 1.954 | 2.371 | 2.560 | 0.327 | 0.854 |
| Treatment | | | | | | | | |
| *F* probability | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| LSD | 0.939 | 1.308 | 1.132 | 1.473 | 1.783 | 1.931 | 0.246 | 2.402 |
| SE | 0.472 | 0.658 | 0.569 | 0.739 | 0.896 | 0.968 | 0.124 | 1.697 |
| Genotype × treatment | | | | | | | | |
| *F* probability | 0.136 | <0.001 | <0.001 | <0.001 | 0.091 | 0.001 | <0.001 | 0.023 |
| LSD | 3.514 | 4.893 | 4.237 | 5.510 | 6.670 | 7.224 | 0.922 | 1.697 |
| SE | 1.767 | 2.463 | 2.130 | 2.764 | 3.352 | 3.621 | 0.463 | 0.854 |

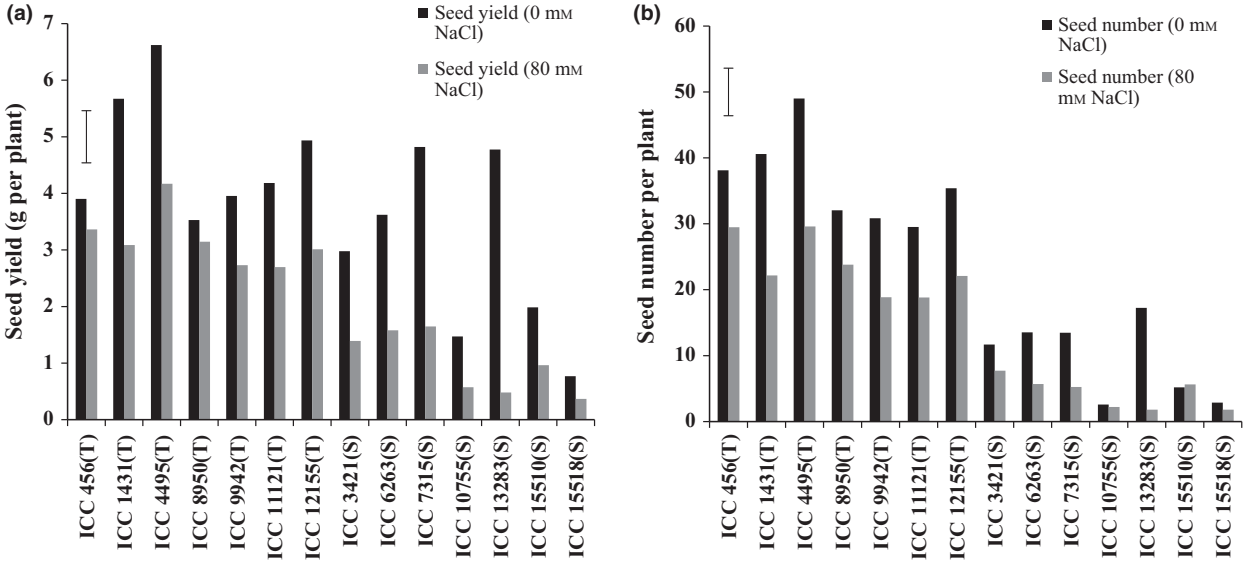


**Fig. 1** Seed yield (a) and seed number per plant (b) of 14 genotypes of chickpea, salt-tolerant (T) and salt-sensitive (S), when grown in control (0 mM NaCl, black bars) and saline (80 mM NaCl, grey bars) soil in an outdoor pot system. The bar gives the least significant difference (LSD) at P = 0.05 for the genotype × treatment interaction.

However, the delay in flowering and maturity in sensitive genotypes varied more (12–20 days for flowering except for ICC10755 (4 days); 1–23 days for maturity) than in tolerant genotypes (1–3 days for the delay in flowering; 1–5 days for the delay in maturity). Salinity reduced total shoot dry matter by 30 % in tolerant and 38 % in sensitive genotypes (Table 2). The salt treatment reduced pod number per plant less in tolerant (59–96 %) than in sensitive genotypes (78–99 %) (Table 2). Similarly, the salt treatment reduced filled pod number per plant by 13–43 % in tolerant and 48–89 % in sensitive genotypes except for ICC10755 (S) and ICC15510 (S), which increased by 15 % and 18 %, respectively. Empty pod number was less in the salt treatment compared with the control plants (Table 2), reflecting that salt-treated plants produced smaller numbers of pods. At 80 mM NaCl, the seeds of the tolerant genotypes were similar in size to those in the controls, while several sensitive genotypes had smaller seeds than the controls (Table 2).

Yield in the 80 mM NaCl treatment varied more than 10-fold among the genotypes, ranging from 0.36 to 4.1 g per plant; all sensitive genotypes had lower yields than tolerant


 

**Table 2** Mean values of total shoot dry matter (g plant$^{-1}$), days to first flower, days to maturity, filled pod number per plant, empty pod number per plant, seed number per plant and 100-seed weight. F probability value at the 5% level of significance and least significant difference (LSD) of 14 chickpea genotypes grown in soil with 0 or 80 mM NaCl. Each pot had four plants

| Genotype | Total shoot dry matter (g plant$^{-1}$) | Days to first flower | Days to maturity | Filled pod number per plant | Empty pod number per plant | 100-seed weight |
|---|---|---|---|---|---|---|
| 0 mM NaCl | | | | | | |
| ICC 456(T) | 8.3 | 46 | 82 | 22.1 | 6.9 | 10.2 |
| ICC 1431(T) | 9.3 | 45 | 77 | 30.2 | 17.3 | 13.9 |
| ICC 4495(T) | 12.4 | 44 | 78 | 38.0 | 5.6 | 13.5 |
| ICC 8950(T) | 9.3 | 44 | 80 | 29.8 | 5.3 | 11.0 |
| ICC 9942(T) | 10.9 | 36 | 75 | 20.4 | 5.1 | 12.8 |
| ICC 11121(T) | 12.3 | 46 | 80 | 22.3 | 9.9 | 14.6 |
| ICC 12155(T) | 9.1 | 40 | 77 | 23.4 | 5.0 | 13.9 |
| ICC 3421(S) | 10.9 | 36 | 85 | 14.3 | 5.5 | 25.5 |
| ICC 6263(S) | 14.7 | 35 | 77 | 15.3 | 4.5 | 26.8 |
| ICC 7315(S) | 15.0 | 32 | 86 | 12.7 | 6.9 | 35.8 |
| ICC 10755(S) | 12.7 | 35 | 76 | 2.0 | 4.3 | 56.8 |
| ICC 13283(S) | 10.1 | 46 | 87 | 14.8 | 4.7 | 27.7 |
| ICC 15510(S) | 7.9 | 32 | 84 | 4.5 | 5.3 | 38.3 |
| ICC 15518(S) | 10.8 | 35 | 78 | 3.4 | 1.7 | 26.8 |
| Mean tolerant | 10.2 | 43 | 78 | 26.6 | 7.9 | 12.8 |
| Mean sensitive | 11.7 | 36 | 82 | 9.6 | 4.7 | 33.9 |
| F probability | 0.08 | <0.001 | <0.001 | <0.001 | 0.08 | <0.001 |
| LSD | NA | 3.6 | 3.4 | 6.1 | 3.8 | 2.1 |
| 80 mM NaCl | | | | | | |
| ICC 456(T) | 6.1 | 53 | 86 | 18.1 | 2.9 | 11.4 |
| ICC 1431(T) | 5.9 | 48 | 82 | 17.1 | 0.6 | 13.9 |
| ICC 4495(T) | 9.8 | 47 | 78 | 23.4 | 1.7 | 14.1 |
| ICC 8950(T) | 8.2 | 46 | 85 | 19.6 | 1.8 | 13.2 |
| ICC 9942(T) | 6.0 | 40 | 76 | 17.7 | 0.3 | 14.5 |
| ICC 11121(T) | 5.9 | 48 | 82 | 16.3 | 2.6 | 14.3 |
| ICC 12155(T) | 7.4 | 44 | 78 | 19.7 | 1.9 | 13.6 |
| ICC 3421(S) | 8.1 | 48 | 86 | 6.7 | 1.2 | 18.1 |
| ICC 6263(S) | 8.7 | 53 | 92 | 5.9 | 0.6 | 27.8 |
| ICC 7315(S) | 7.2 | 42 | 87 | 6.2 | 1.1 | 31.4 |
| ICC 10755(S) | 6.4 | 39 | 84 | 2.3 | 0.4 | 25.9 |
| ICC 13283(S) | 5.7 | 60 | 98 | 1.6 | 0.03 | 26.8 |
| ICC 15510(S) | 6.4 | 44 | 89 | 5.3 | 0.5 | 17.1 |
| ICC 15518(S) | 6.9 | 55 | 101 | 1.8 | 0.2 | 20.4 |
| Mean tolerant | 7.1 | 47 | 81 | 18.8 | 1.7 | 13.6 |
| Mean sensitive | 7.1 | 49 | 91 | 4.3 | 0.6 | 23.9 |
| F probability | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| LSD | 1.6 | 6.08 | 5.11 | 4.13 | 0.42 | 2.7 |

genotypes (Fig. 1). Salinity decreased seed number and seed yield per plant compared with the non-saline control, although the reduction was less in tolerant genotypes (23–45 % for seed number, 10–46 % for seed yield) than in sensitive genotypes (34–90 % for seed number, 52–90 % for seed yield) except for seed number in ICC10755 (S), which decreased by 14 %; in ICC15510 (S), it increased by 8 % (Fig. 1). These results could be related to the relatively low seed number in the control treatment of both of these sensitive genotypes. The highest seed number and seed yield in both treatments were recorded in ICC4495 (T) and the lowest in ICC15518 (S). The delay in flowering under 80 mM NaCl compared with 0 mM NaCl treatment was significantly associated with the reduced relative yields ($R^2 = 0.21$) (Fig. 2).

Seed number, filled pod number (Table 2) and seed yield (Fig. 1) in the 80 mM NaCl treatment clearly discriminated tolerant from sensitive genotypes. Even under the 0 mM NaCl treatment, sensitive genotypes had significantly fewer seeds compared to tolerant genotypes, but the seed yield of tolerant and sensitive genotypes did not differ in the non-saline treatment because the low seed number in sensitive genotypes was compensated for by a larger seed size. Thus, to account for the variation in seed parameters in the 0 mM

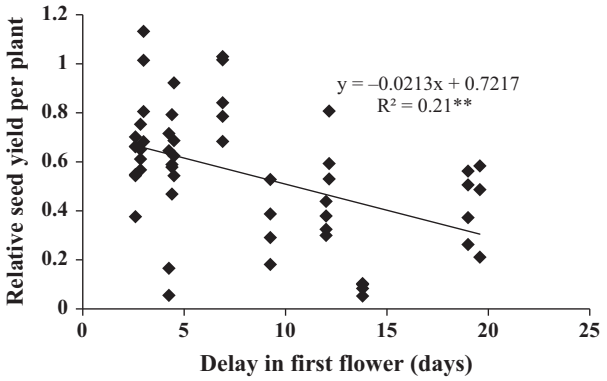


**Fig. 2** Relationship between delay in flowering (days) under 80 mM NaCl treatment compared to 0 mM NaCl and relative seed yield per plant (each replicate value of salt stressed in a genotype divided by corresponding non-saline control mean) for 14 genotypes of chickpea. The data are values in 80 mM NaCl relative to those in 0 mM NaCl. **- significant at $P < 0.01$.

NaCl treatment, parameters were expressed as relative values, calculated as the ratio of values in 80 mM NaCl to the mean value of the trait under 0 mM NaCl for each genotype. The relative values were calculated only for the yield parameters but not for the ion concentrations as no significant relationship was found between ion concentration under control and saline treatment. Relative filled pod number ($R^2 = 0.93$) and relative seed number ($R^2 = 0.96$), but not relative seed size ($R^2 = 0.028$), were associated with relative yield (Fig. 3). Several replicates of both tolerant and sensitive genotypes had higher yields in saline pots than in non-saline controls, and this outcome was always associated with higher pod and seed numbers (Fig. 3).

### Ion concentrations in various tissues

The concentration of $Cl^-$ in tissues differed between the 0 and 80 mM NaCl treatments, but although salt treatment increased the $Cl^-$ concentration in all tissues (Fig. 4) no genotypic differences were observed for any ion concentration in the assessed tissues (data not shown, but box-and-whisker plots in Figures 4, 5 and 6 show the ranges of tissue ion concentrations for the eight genotypes measured). In both treatments, old green leaves had the highest concentration of $Cl^-$, followed by other leaves, with

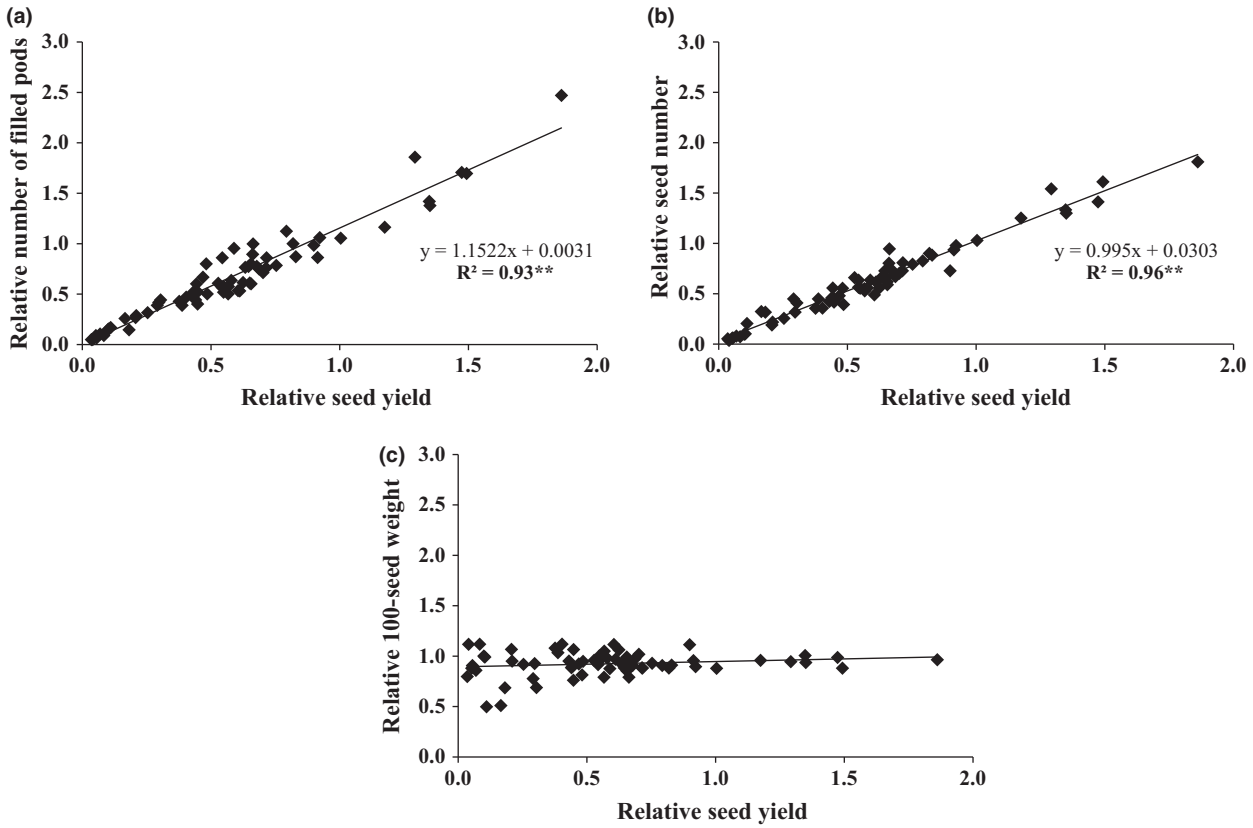


**Fig. 3** Relationship between relative seed yield (each replicate value of salt stressed in a genotype divided by corresponding non-saline control mean) and relative filled pod number (a), relative seed number (b), and relative 100-seed weight (c) (not significant) for 14 genotypes of chickpea. The data are values in 80 mM NaCl relative to those in 0 mM NaCl.


 

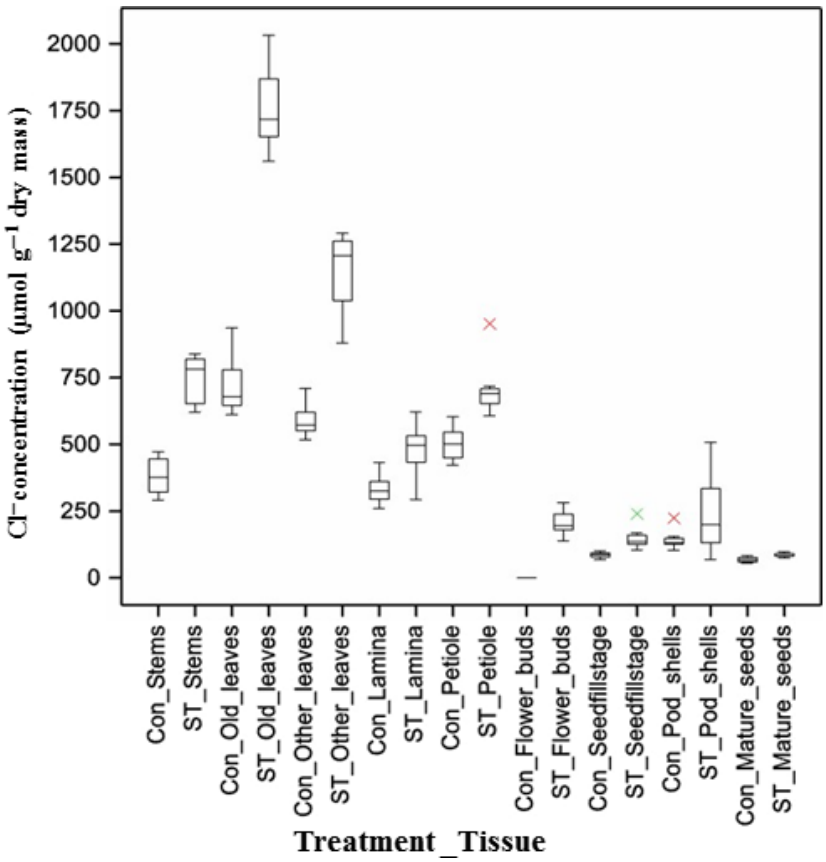


**Fig. 4** Chloride concentrations in nine tissues from plants grown under 0 mM NaCl (Con) and 80 mM NaCl (ST). The whiskers show the lower and upper limit of ion concentration among the eight genotypes: four sensitive - ICC3421, ICC6263, ICC7315, ICC15510 and four tolerant- ICC11121, ICC1431, ICC4495, and ICC8950. The (*x*) represents outliers and the line within the box represents the median. The upper and lower horizontal line in the box represents the quartiles 1 and 3. No flower buds under 0 mM NaCl were available for ion analysis.

the lowest in mature seeds; the increase more than twofold in old green leaves and other leaves compared to the control.

The $K^+$ concentration was highest in the petioles, stems and laminae of fully expanded young leaves and lowest in mature seeds in both saline and non-saline treatments (Fig. 5). In no tissue was the $K^+$ concentration able to significantly discriminate the tolerant from the sensitive genotypes under saline conditions (data not shown). The concentration of $Na^+$ increased markedly in tissues of plants in 80 mM NaCl, in the stems, in the laminae and petioles of fully expanded young leaves, in seeds at the mid-pod filling stage and in mature seeds (Fig. 6). In most of the tissues, the $Na^+$ concentration was higher in the sensitive genotype ICC3421 than in all other genotypes (data not shown). However, the $Na^+$ concentrations in the various tissues did not discriminate the group of tolerant and sensitive genotypes, except for the old green leaves which contained higher $Na^+$ in the sensitive genotypes.

### Relationships between tissue ions and seed yield in the 80 mM NaCl treatment

There were only a few associations between ion concentrations in tissues and seed yield. The accumulation of $Cl^-$ in mature seeds, of $K^+$ in seeds at the filling stage and a higher $K^+/Na^+$ ratio in the laminae of fully expanded young leaves under 80 mM NaCl treatment was positively associated with higher seed yield. The accumulation of $Na^+$ in old green leaves under saline treatment was negatively correlated with seed yield. Additionally, the mean $Na^+$ concentration in old green leaves differed between the tolerant (79 $\mu$mol $g^{-1}$ dry mass) and sensitive (117 $\mu$mol $g^{-1}$ dry mass) genotypes at $P < 0.01$ (LSD = 20.50) except for ICC8950 (T), where the accumulation difference was not significant (Fig. 7, Figure S1).

### Relationship between accumulation of ions and delay in flowering

The accumulation of $Na^+$ in laminae of fully expanded young leaves ($R^2 = 0.61$), of $K^+$ in old green leaves ($R^2 = 0.57$) and of $Na^+$ in old green leaves ($R^2 = 0.51$) was all significantly ($P < 0.05$) correlated with delayed flowering. The more the $Na^+$ and $K^+$ accumulated in corresponding tissues, the longer the delay in flowering (Fig. 8).

## Discussion

The main findings from the present study are as follows: (i) exposure to 80 mM NaCl delayed flowering to a greater

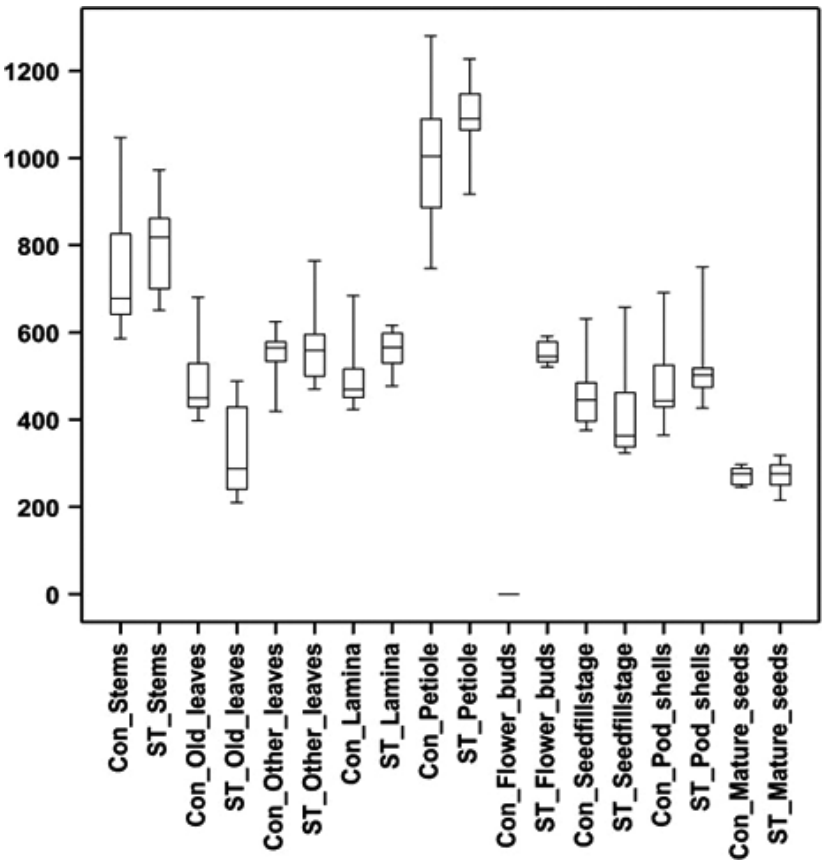


**Fig. 5** Potassium concentrations in nine tissues from plants grown under 0 mM NaCl (Con) and 80 mM NaCl (ST). The whiskers show the lower and upper limit of ion concentration among the eight genotypes, i.e. four sensitive - ICC3421, ICC6263, ICC7315, ICC15510 and four tolerant- ICC11121, ICC1431, ICC4495, and ICC8950. The line within each box represents the median value. The upper and lower horizontal line in the box represents the quartiles 1 and 3. No flower buds under 0 mM NaCl were available for ion analysis.

extent in sensitive genotypes than tolerant ones, was related to lower seed yield and was positively correlated with the accumulation of $K^+$ and $Na^+$ in leaf tissues, (ii) yield of chickpea under saline stress was determined by seed number, but not seed size, (iii) in none of the tissues did ion accumulation discriminate between tolerant and sensitive genotypes except for the slight increase in $Na^+$ concentration in old green leaves in the 80 mM NaCl treatment, (iv) the accumulation of $Cl^-$ in mature seeds and $K^+$ in developing seeds was positively associated with seed yield as was the $K^+/Na^+$ ratio in the laminae of fully expanded young leaves, while the accumulation of $Na^+$ in old green leaves was negatively associated with the seed yield.

### Effect of salinity on yield and yield components

Reduced seed yield under salinity was highly correlated with a reduction in filled pod and seed numbers, in agreement with previous reports (Vadez et al. 2007, 2012, Samineni et al. 2011, Turner et al. 2013). The greater reduction in filled pod number in sensitive genotypes could be associated with higher levels of pod abortion than those that occur in tolerant genotypes. Similarly, in tomato, Albacete et al. (2014) showed that fruit set and development was affected under salinity and in turn caused yield reduction. While the reason for the pod abortion under saline treatment is not clear, salinity could decrease sink activity and impaire sucrose metabolism by reducing the apoplastic and cytoplasmic sucrose-cleaving enzyme activity. These changes could be mediated by changes in carbon supply or hormone concentration. Increased cytokinin concentration and/or metabolic activity have been linked to increase fruit sink strength, growth and yield in tomato under salinity (Albacete et al. 2014). In chickpea salinity could impair sucrose metabolism, increase abscisic acid production and/or decrease the production of cytokinins, and/or influence other metabolic factors. More research is needed to ascertain a possible role of hormonal changes or carbon supply in pod abortion under salinity.

### Effect of salinity on phenological development

Exposure to salinity delayed flowering and delayed flowering to a greater extent in the sensitive than tolerant genotypes. The plants adjust their physiology to survive under salt stress, drought, high temperature and extending darkness by accelerating the vegetative growth combined with leaf senescence and enters rapidly into the reproductive


 

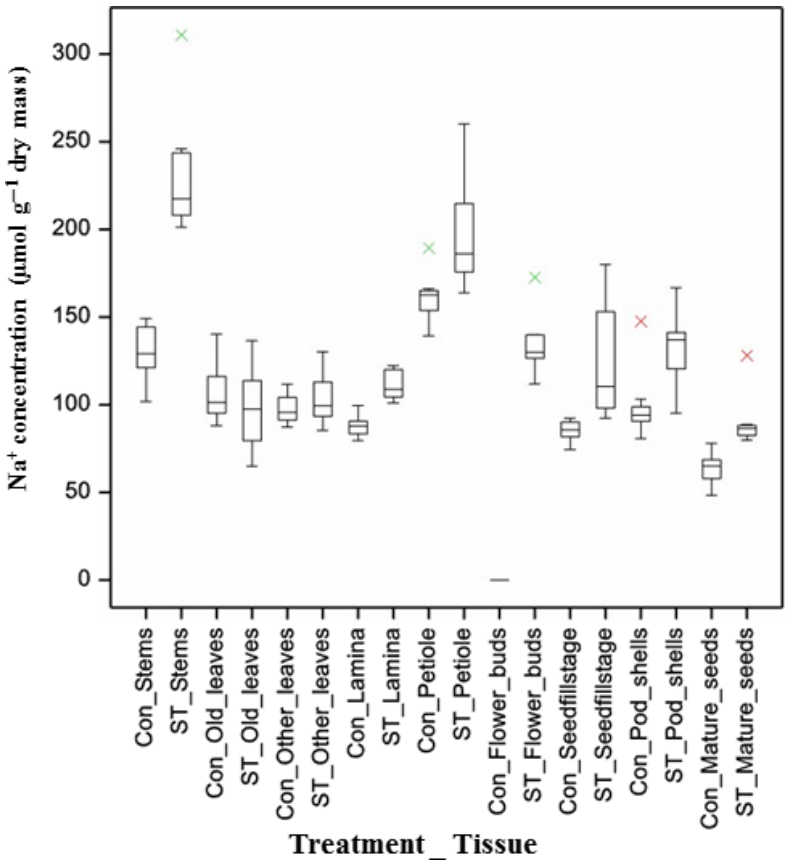


**Fig. 6** Sodium concentrations in nine tissues from plants grown under 0 mM NaCl (Con) and 80 mM NaCl (ST). The whiskers show the lower and upper limit of ion concentration among the eight genotypes: four sensitive - ICC3421, ICC6263, ICC7315, ICC15510 and four tolerant- ICC11121, ICC1431, ICC4495, and ICC8950. The (*x*) represents outliers and the line within the box represents the median. The upper and lower horizontal line in the box represents the quartiles 1 and 3. No flower buds under 0 mM NaCl were available for ion analysis.

phase, that is flowering and podding (Allu et al. 2014). In contrast, in our study, although we found stunted growth in plants under saline treatment earlier leaf senescence was not observed. High salinity has been observed to delay the onset of flowering in many plant species (Van Zandt and Mooper 2002). However, we are not aware of any reported delay in flowering arising from salinity in chickpea. Indeed, Turner et al. (2013) reported that salinity did not affect the time to first flower. The delay in flowering was much shorter in tolerant genotypes (1–3 days) than in sensitive genotypes (12–20 days), and this difference in the delay of flowering could have been the cause of the higher reproductive failure of the sensitive genotypes in this late-sown trial. Indeed, a negative relationship between time to flowering and seed yield under salinity was found earlier, although exclusively in late-sown trials, at the same location as this current work (Krishnamurthy et al., 2011). In such a situation, a delay in flowering would result in pods and seeds developing in warmer conditions, particularly in the short-season southern Indian environment where the study was conducted. Later flowering in the sensitive genotypes would have forced pod and seed development into a period of increasing temperatures at the beginning of summer when heat stress shortens the period of flower production, induces pod and seed abortion and reduces yields.

The delay in flowering was associated with the accumulation of $Na^+$ in the laminae of fully expanded young leaves and the accumulations of $Na^+$ and $K^+$ in old green leaves, and this association was stronger in sensitive than in tolerant genotypes. However, whether the greater reduction in seed yield in the sensitive genotypes was associated with the greater delay in flowering *per se* or the greater $Na^+$ concentrations in the leaves in the sensitive than tolerant genotypes is not clear. Further study is needed to determine the causes of the delay in flowering. There is a possibility that salinity could have impaired the N nutrition of the crop by impairing symbiotic $N_2$ fixation, which could have delayed flowering in plants as a result of N deficiency (Nord and Lynch 2008). Therefore, more research would be need to test the hypothesis of a higher $N_2$ fixation impairment in sensitive lines, then leading to a delayed flowering and a lower yield, itself potentially related to two negative influences: (i) a delayed flowering that would expose flower to warmer temperature in the conditions of the trials reported here; (ii) more pod abortion related to less N availability.

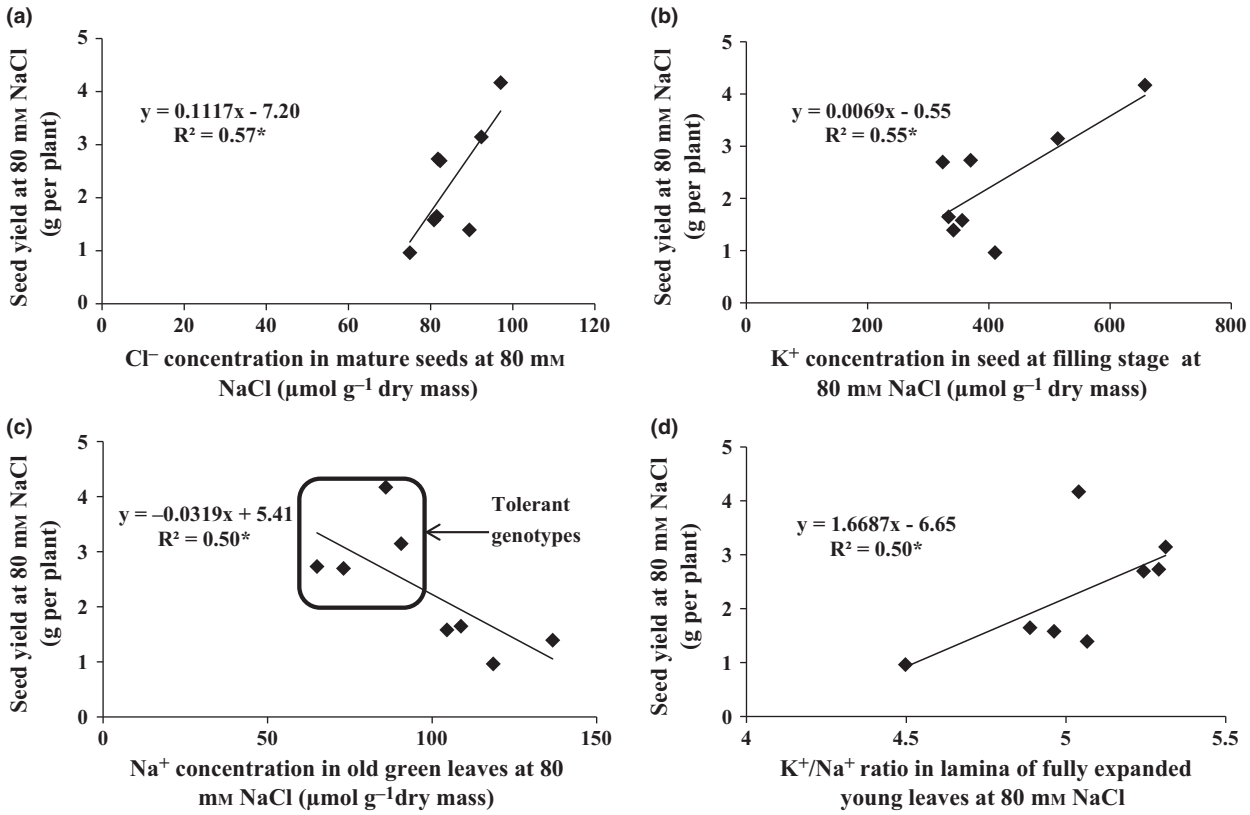


**Fig. 7** Relationship between $Cl^-$ concentration in mature seeds (a), $K^+$ concentration in seeds at the filling stage (b), $Na^+$ concentration in old green leaves (c), $K^+/Na^+$ ratio in lamina of fully-expanded young leaves (d) and seed yield per plant (*-significant at $P < 0.05$) at 80 mM NaCl treatment. The accumulation of $Cl^-$, $K^+$, and $Na^+$ in all other tissues had no significant effect on seed yield.

### Ion concentrations and the association with yield

Salinity reduced yield and there were clear genotypic differences among genotypes for seed yield and relative seed yield under salinity. Ion accumulation in plant tissues has been proposed as a simple explanation for the deleterious effect on yield under salt stress. The accumulation of $Na^+$ or $Cl^-$ in leaves may lead to dehydration of cells; the accumulation of these ions in the cytoplasm could inhibit enzymes in metabolism; and accumulation in the chloroplast may exert a direct toxic effect on photosynthetic processes (Munns and Tester 2008). $Cl^-$ accumulated to higher concentrations compared to $Na^+$, but the greater accumulation of $Cl^-$ in the seed was associated with greater seed yield. By contrast, $Na^+$ accumulation in the old green leaves was associated with lower seed yields in the sensitive genotypes. The toxic effects of $Na^+$ are related to its competition with $K^+$ for binding sites of over 50 enzymes (Tester and Davenport 2003), whereas the effects of $Cl^-$ on metabolism have been found to be much smaller. In this study, it is also possible that the $Cl^-$ preferentially accumulated in the epidermal cells of leaves, thus reducing the $Cl^-$ toxicity in the mesophyll cells that play an important role in photosynthesis (Teakle and Tyerman 2010). These results contrast with previous findings. For instance, Manchanda and Sharma (1989) reported that accumulation of $Cl^-$ concentration beyond 5 % w/w dry mass in tissues, equivalent to 1410 $\mu$mol $g^{-1}$ dry mass, disturbed plant metabolic processes, nutrient absorption and its utilisation, thus decreasing chickpea yield. Dua (1998) observed higher $Na^+$ concentrations in roots than shoots in sensitive genotypes, but similar amounts in tolerant genotypes; in our study, the $Na^+$ concentration in old green leaves was negatively associated with seed yield with the tolerant genotypes having lower $Na^+$ concentrations and higher yields than the sensitive genotypes. The higher accumulation of $Na^+$ in sensitive genotypes may have induced necrosis in older leaflets and thus shortened the lifetime of individual leaflets and in turn affected the yield (Tester and Davenport 2003).

Salinity decreased total shoot dry mass. This finding might be explained by reduced photosynthesis and higher leaf necrosis (Dua and Sharma 1997, Maliro et al. 2008) resulting from the destruction of chlorophyll in cells due to the increased accumulation of $Na^+$ or $Cl^-$ in leaves.


 

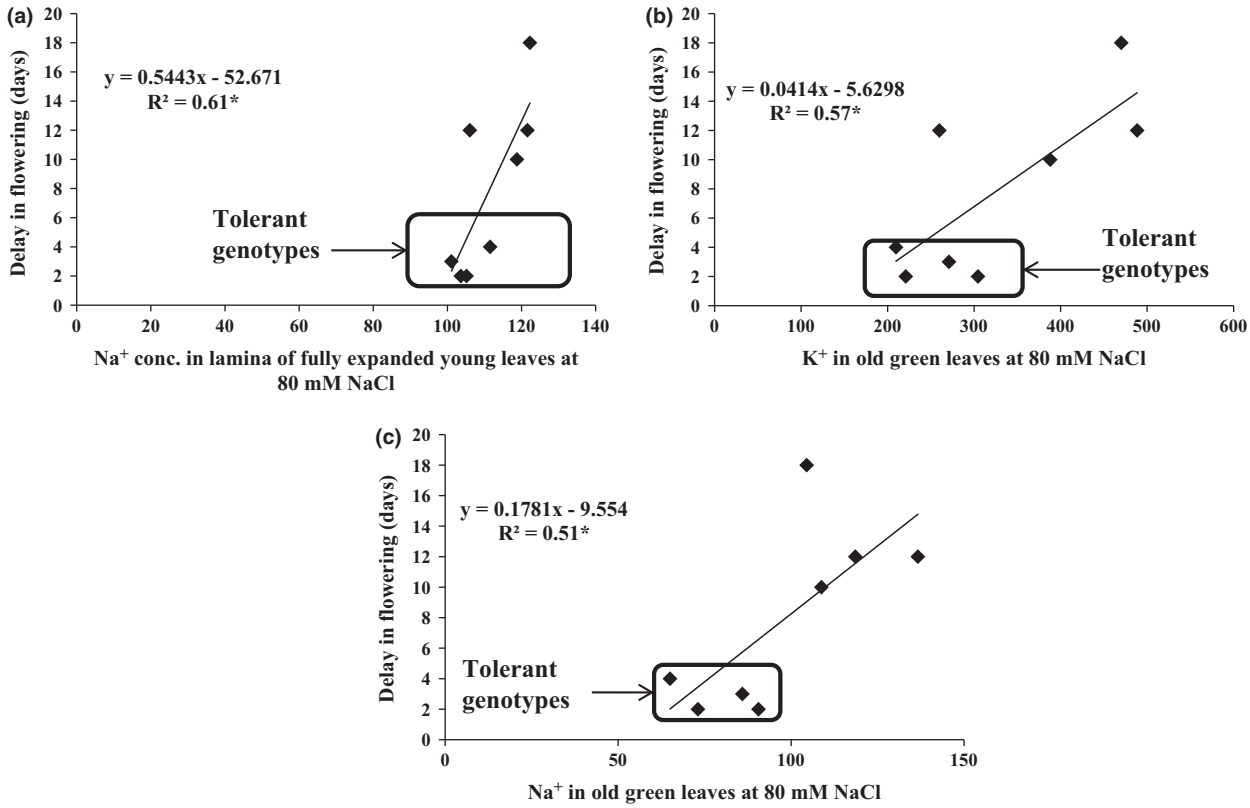


**Fig. 8** Relationship between the delay in 50 % flowering and $Na^+$ concentration in lamina of fully-expanded young leaves (a), $K^+$ concentration in old green leaves (b), $Na^+$ in old green leaves (c) at 80 mM NaCl treatment. The delay in flowering at 80 mM NaCl treatment is compared to 0 mM NaCl treatment (*-significant $P < 0.05$). The accumulation of $Cl^-$, $K^+$, and $Na^+$ in all other tissues had no significant effect on delay in flowering.

However, the decrease in shoot weight did not differ between tolerant and sensitive lines, and we observed little accumulation of $Cl^-$ and $Na^+$ in reproductive tissues relative to vegetative tissues. Interpretations of ion concentrations against critical concentrations in tissues derived from other studies can be relatively crude, as these thresholds may vary with the type of plant tissue and with various other growth conditions. In addition, the ion measurements that most studies did, including this one, consider whole tissue concentrations and do not distinguish between cytosolic and vacuolar concentrations, so that high tissue concentration may not necessarily indicate high cytosolic concentration. Nevertheless, in view of the critical concentrations in tissues from other studies (Reuter and Robinson 1986, Lauter and Munns 1987) albeit in vegetative tissues, the data reported here support the idea that none of the ions analysed here ($Na^+$, $K^+$ and $Cl^-$) reached toxic concentrations that could have explained the reproductive failure.

The lower $Na^+$ concentration in the lower old green leaves of tolerant genotypes compared with sensitive genotypes may be a result of a reduced $Na^+$ uptake rate (Ding and Zhu 1997). If so, this finding would justify an investigation of possible differences in $Na^+$ exclusion in root tissues in chickpea. In almost all of the tissues, the $Na^+$ concentration was much lower than those of $K^+$ and $Cl^-$. In addition, the level of $Na^+$ may be lower in shoots if $Na^+$ is sequestered in the roots, as less $Na^+$ would then enter the xylem and reach the shoot (Munns and Tester 2008). $K^+$/$Na^+$ homoeostasis was maintained in the laminae, but a higher retention of $K^+$ was observed in seeds at the filling stage. This outcome may be a result of better $Na^+$ exclusion, helping to avoid $Na^+$ toxicity and improve yield (Zepeda-Jazo et al. 2008). The concentration of $Na^+$ was only 10–40 % that of $Cl^-$ in tissues, except in pod shells, seeds at the filling stage and mature seeds. This finding suggests that the exclusion of the cation $Na^+$ is better regulated than that of the anion $Cl^-$ in ion translocation to reproductive tissues, possibly because $Cl^-$ is an essential micronutrient that regulates enzyme activities in the cytoplasm, is an essential co-factor in photosynthesis, acts as a counter anion to stabilise membrane potential and is involved in turgor regulation (Teakle and Tyerman 2010). Ion transport across cellular membranes is also largely determined

by membrane potential, and root $Na^+$ uptake results in a massive membrane depolarisation. From this point, a concurrent uptake of negatively charged $Cl^-$ may be essential to attenuate (or completely overcome) this salt-induced plasma membrane depolarisation (Anschütz et al. 2014). Therefore, the role of $Cl^-$ here, initially thought to be harmful, could actually have a beneficial role to play. In addition, the beneficial effect of chloride ion could have been in terms of osmotic adjustment to maintain turgor pressure and growth and development processes, as it has been shown to be responsible for 30 % of the osmotic adjustment under salt treatment (Shabala and Lew 2002). Indeed, it is known that a drought effect hastens flowering in chickpea (Soltani et al. 2001). Therefore, a delay in flowering would suggest that our salt treatment did not create any osmotic effect on the crop, possibly because of the higher accumulation of chloride ions playing the role of osmoticum here.

The present study showed that higher $K^+$ retention in laminae of young leaves and seeds at the filling stage and higher accumulation of $Cl^-$ in the mature seeds were all associated with higher grain yield. A recent report by Wu et al. (2014) showed that higher retention of $K^+$ in leaf mesophyll cells in barley was found to be an important trait that was closely associated with higher levels of salinity tolerance. High cytosolic $K^+$ level was reported to be important to suppress activity of caspase-like proteases and endonucleases and loss of cytosolic $K^+$ homoeostasis leads to programmed cell death. In present study, as we measured the whole leaf tissues, differentiation of cytosolic and vacuolar compartmentation of ions was not possible. The higher yield/salinity tolerance in the present study may be due to higher retention of cytosolic $K^+$ and thus better $K^+/Na^+$ homoeostasis (Anschütz et al. 2014). Shabala and Lew (2002) showed that accumulation of $Cl^-$ in Arabidopsis can be beneficial under saline conditions and also showed in direct single-cell pressure-probe measurements that 30 % of total root osmotic adjustment was achieved solely by increased $Cl^-$ concentration. In sugarcane, Gandonou et al. (2011) showed that genotypes that accumulated more $Cl^-$ and maintained higher $K^+$ concentration in young leaves had higher levels of tolerance under saline conditions.

## Conclusions

Exposure to 80 mM NaCl throughout the life of the plant resulted in a delay in flowering, and this delay was greater in the sensitive than the tolerant genotypes. To best of our knowledge, this is the first report in chickpea where delay in flowering significantly differentiated the sensitive and tolerant genotypes under saline conditions. The delay in flowering was significantly associated with a decrease in seed yield which in turn was associated with the greater accumulation of $Na^+$ in the leaves. However, whether the greater increase in $Na^+$ in the leaves of the sensitive genotypes was the cause of the greater reduction in yield or whether the delay in flowering and consequent pod and seed development in the hotter conditions of summer was the cause of the reduction in yield in this late-sown experiment is not clear. What is clear is that filled pod number, seed number and seed yield can be used to distinguish salt-tolerant chickpea genotypes from salt-sensitive genotypes because reproductive failure clearly discriminated tolerant from sensitive entries. While ions accumulated primarily at concentrations that might not be considered as toxic levels, the ion homoeostasis disturbance ($Na^+$ and $K^+$) that occurred in certain tissues was associated with altered plant yield. Further research is needed to determine the causes of flowering delay and consequent pod abortion and lower yields; possible causes are the effect of salt stress on carbon assimilation and symbiotic $N_2$ fixation, changes in level of hormones involved in stomatal control and signalling pathways or seed development, and the activity of floral repressor genes.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Eight genotypes contrasting for salinity tolerance were grouped based on $Na^+$ concentration in old green leaves and seed yield in the 80 mm NaCl treatment using PAST software.



 