Salt sensitivity of the vegetative and reproductive stages in chickpea (*Cicer arietinum* L.): Podding is a particularly sensitive stage

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ABSTRACT

Soil salinity is an increasing problem, including in regions of the world where chickpea is cultivated. Salt sensitivity of chickpea was evaluated at both the vegetative and reproductive phase. Root-zone salinity treatments of 0, 20, 40 and 60 mM NaCl in aerated nutrient solution were applied to seedlings or to older plants at the time of flower bud initiation. Even the reputedly tolerant cultivar JG11 was sensitive to salinity. Plants exposed to 60 mM NaCl since seedlings, died by 52 d without producing any pods; at 40 mM NaCl plants died by 75 d with few pods formed; and at 20 mM NaCl plants had 78–82% dry mass of controls, with slightly higher flower numbers but 33% less pods. Shoot Cl exceeded shoot Na by 2–5 times in both the vegetative and reproductive phase, and these ions also entered the flowers. Conversion of flowers into pods was sensitive to NaCl. Pollen from salinized plants was viable, but addition of 40 mM NaCl to an *in vitro* medium severely reduced pollen germination and tube growth. Plants recovered when NaCl was removed at flower bud initiation, adding new vegetative growth and forming flowers, pods and seeds. Our results demonstrate that chickpea is sensitive to salinity at both the vegetative and reproductive phase, with pod formation being particularly sensitive. Thus, future evaluations of salt tolerance in chickpea need to be conducted at both the vegetative and reproductive stages.

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1. Introduction

Worldwide, about 20% of cultivated land is affected by salinity (Ghassemi et al., 1995). Cool season food legumes such as chickpea, lentil and faba bean, are relatively sensitive to salinity (Stoddard et al., 2006). Saline soils occur mainly in arid and semi-arid regions. Chickpea is the major grain legume in these regions (Ali et al., 2002) so saline soils constrain chickpea production in many parts of the world (Ryan, 1997). In chickpea, salinity leads to leaf necrosis and reduced vegetative growth (Maliro et al., 2008) and impedes flower and pod formation (Manchanda and Sharma, 1989; Katerji et al., 2001; Vadez et al., 2007). Salinity of only 3 dS m^{-1} in field soils was the threshold for reduced shoot growth and yield in chickpea (Rao et al., 2002; Katerji et al., 2005), although this exceeds the even lower salinity threshold (<1.3 dS m^{-1}) in some tropical legumes like cowpea, soybean and pigeon pea (Keating and Fisher, 1985).

Plant responses to salinity differ depending upon growth/developmental stage, and length of exposure (Munns and Tester, 2008). Salinity tolerance in chickpea has been evaluated at different growth stages, but each individually and with a focus to identify tolerant genotypes (Flowers et al., 2010). For example, salinity tolerance has been evaluated at germination (Garg and Gupta, 1998; Khalid et al., 2001; Singh, 2004), seedling growth up to 3 weeks (Al-Mutata, 2003; Karajeh et al., 2003), vegetative stage up to 8 weeks (Elsheikh and Wood, 1990), and some studies have extended into the reproductive stage (Sadiki and Rabih, 2001; van Hoorn et al., 2001; Bruggeman et al., 2003; Katerji et al., 2005; Vadez et al., 2007). Germination is less sensitive to salinity than early vegetative growth (Garg and Gupta, 1998; Zurayk et al., 1998; Sekeroglu et al., 1999; Al-Mutata, 2003) and the reproductive phase is considered to be even more sensitive than vegetative growth (Vadez et al., 2007). In contrast with chickpea, cowpea is most salt sensitive during the vegetative stage (Maas and Poss, 1989). Thus, the present experiments directly assessed salt sensitivity of chickpea at the reproductive phase, using NaCl dose-response experiments with treatments applied not only during the vegetative phase but also at flower initiation,

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to enable evaluation of the sensitivity of the reproductive phase without carry-over effects of prior exposure during the vegetative phase. Furthermore, ion (Na, K and Cl) regulation in roots, shoots, and floral organs, as well as pollen viability and sensitivity to NaCl, were all evaluated as potential contributors to salt sensitivity in chickpea.

Tissue ion regulation is a key trait for salt tolerance in plants (Munns and Tester, 2008), but whether Na or Cl 'exclusion' contributes to tolerance in chickpea remains uncertain. Two reputedly tolerant genotypes displayed contrasting responses: CM 663 accumulated higher levels of Na ($95\,\mu\text{mol}\,\text{g}^{-1}$ fresh mass) in leaves compared with roots ($20\,\mu\text{mol}\,\text{g}^{-1}$ fresh mass) whereas ICC 10572 accumulated higher Na in roots ($60\,\mu\text{mol}\,\text{g}^{-1}$ fresh mass) than in leaves ($35\,\mu\text{mol}\,\text{g}^{-1}$ fresh mass), although the Cl concentration was always more in leaves than in roots for both genotypes (Ashraf and Waheed, 1993). These authors could not separate the toxic effect of Na and/or Cl and concluded that leaf Na and/or Cl concentrations cannot simply be used as selection criteria for identification of salt tolerance in chickpea. Other studies have found tolerant chickpea genotypes display preferential accumulation of Na in roots rather than in shoots (Sleimi et al., 2001; Baalbaki et al., 2000). Similarly, lower shoot ion concentrations were observed in a tolerant genotype (i.e., half the Na and Cl in a sensitive genotype), but only when ion concentrations were expressed on a tissue water content basis; these differences were not evident on a dry mass basis (Dua, 1998). Dua and Sharma (1997) also reported that growth reductions were associated with higher concentrations of Na and Cl in chickpea. However, in a salinity screening experiment no relation was found between final yield and Na (% dry mass) in shoots at the vegetative stage (Vadez et al., 2007). It therefore appears that in chickpea a combination of mechanisms (e.g. ion exclusion and tissue tolerance of excess ions) are likely to contribute to salt tolerance.

Understanding salt tolerance and its component traits related to ontogenic stages would facilitate development of salt tolerant genotypes. The present study evaluated the effect of NaCl (three concentrations) applied at two stages (seedling or early reproductive stages) on growth, tissue ion concentrations, and flowering/podding in chickpea. A similar approach of first adding salinity to chickpea at the early flowering stage in sand culture was used by Dhingra and Varghese (1993) to study flower numbers and pollen production, but growth, tissue ion concentrations, and pod production were not reported. The present study imposed three NaCl treatments either on seedlings or on plants at the start of the reproductive phase (flower bud initiation). Salinity treatments continued for both these sets of plants, and in addition, to assess recovery, a third set of plants that had been exposed to NaCl since seedlings were transferred to NaCl-free conditions at the start of the reproductive phase. This experimental approach enabled the hypothesis to be tested that the reproductive phase is the most salt sensitive stage in chickpea, without carry-over effects of prior salt exposure during the vegetative phase.

2. Materials and methods

The chickpea desi cultivar 'JG 11', widely grown in southern India, has been identified by Vadez et al. (2007) as relatively salt tolerant (ranked 13th in salt screening of the ICRISAT mini-core plus reference collection). It is a medium-maturity cultivar; flowering commences 37–40 d after sowing; and it matures within 90 d at ICRISAT, Patancheru, India.

2.1. Plant growth

The experiment was conducted at The University of Western Australia, Perth, with a completely randomized design in a phy-

totron (natural light) with 20/15 °C day/night temperatures during winter and spring 2007. Plants were grown in continuously aerated nutrient solution within 4.5 L plastic pots covered with Al-foil to prevent entry of light.

The nutrient solution contained (in mM): $5.0\,\text{Ca}^{2+}$, $3.75\,\text{K}^+$, $3.125\,\text{NH}_4^+$, $0.4\,\text{Mg}^{2+}$, $0.2\,\text{Na}^+$, $5.4\,\text{SO}_4^{2-}$, $6.875\,\text{NO}_3^-$, $0.2\,\text{H}_2\text{PO}_4^-$, $0.1\,\text{SiO}_3^{2-}$, 0.1 Fe-sequestrene, $0.05\,\text{Cl}^-$, $0.025\,\text{BO}_3^{3-}$, $0.002\,\text{Mn}^{2+}$, $0.002\,\text{Zn}^{2+}$, $0.0005\,\text{Cu}^{2+}$, $0.0005\,\text{MoO}_4^{2-}$, $0.001\,\text{Ni}^{2+}$ and 1.0 MES buffer. KOH was used to adjust the pH to 6.5 every second d. The nutrient solution was renewed at 10-d intervals, at which time pots were re-randomized to minimize the potential influence of environmental heterogeneity in the phytotron.

Seeds were surface sterilized with 0.04% bleach for 300 s, rinsed with tap water and germinated in darkness on plastic mesh floating on aerated 0.1-strength nutrient solution. After 48 h, germinating seeds were transferred to 0.25-strength aerated nutrient solution and grown until emergence of the first leaf. Nine-d-old seedlings were transferred to full strength aerated nutrient solution and allowed to grow for another 4 d, at which time treatments were imposed (i.e., 13 d after imbibition).

2.2. NaCl treatments

Three concentrations of NaCl (20, 40 and 60 mM) were applied at two stages of plant growth, along with controls without NaCl (but with 0.2 mM Na$^+$ from Na$_2$SiO$_3$ and 0.05 mM Cl$^-$ in the micronutrient stock). Each treatment was replicated seven times, with six plants in each pot to enable six samplings (an initial sample was also taken from extra pots, giving seven sampling times).

Treatments were categorized into three sets based on the time of NaCl application at different growth stages. Set I was with continuous NaCl treatments (0, 20, 40 and 60 mM), applied 13 d after imbibition. Set II was without NaCl until flower bud initiation, treatments were then imposed ($0 \rightarrow 20$ mM, $0 \rightarrow 40$ mM, and $0 \rightarrow 60$ mM). In all cases, NaCl was added in steps of 20 mM NaCl per d. Set III was with NaCl from 13 d after imbibition until flower bud initiation and then NaCl was removed (20 mM $\rightarrow$ 0, 40 mM $\rightarrow$ 0, and 60 mM $\rightarrow$ 0). This second phase of the experiment, with NaCl either added for the first time or removed, occurred at flowering (50% of plants showed flower bud initiation; 48 d after imbibition). The experiment was continued until 111 d after imbibition.

2.3. Plant samplings

Initial samples were taken at the time treatments were imposed, 13 d after imbibition. The remaining samplings were at 23, 33, 47 d after imbibition (these three samplings were during the vegetative phase), 65, 87, and 111 d after imbibition (these three samplings were during the reproductive phase).

At each sampling, plants were separated into roots and shoots. To remove the external treatment solutions, roots were washed three times in 5 mM CaSO$_4$, for 20 s each time. Surface water was blotted off using paper towels and fresh mass measured. Tissues were oven dried at 65 °C for 48 h and dry mass was determined.

Numbers of flower buds, flowers and developing pods were recorded over the final 50 d, at 10 d intervals. At the final sampling, these reproductive structures were also separated and oven-dried.

2.4. Ion analyses

Na, K and Cl in tissues were extracted in 0.5 M HNO$_3$ by shaking for 2 d. Na and K in extracts were analyzed using a flame-photometer (Model PFP7, Jenway, Essex, UK) and Cl using a Buchler-Cotlove chloridometer (Model 4-2000, Buchler Instruments, Fortlee, USA). A certified reference plant tissue taken
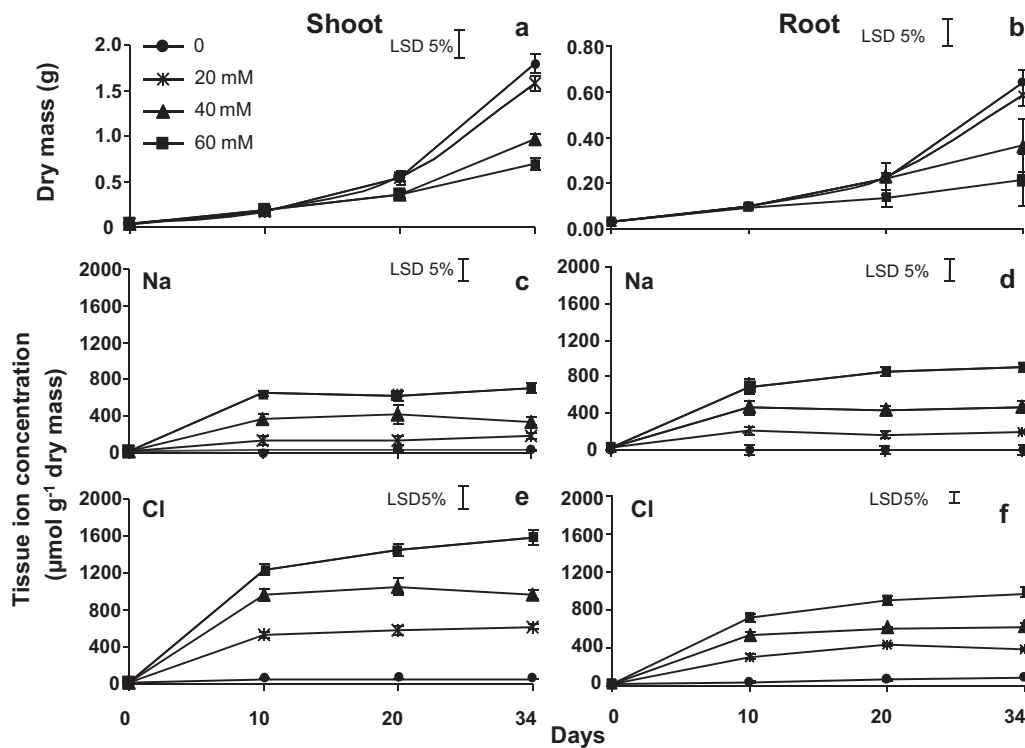
Fig. 1. Effect of NaCl with time on (a) shoot and (b) root dry mass per plant, and concentrations of Na and Cl in shoots (c and e, respectively) and roots (d and f, respectively) of chickpea during the vegetative stage. Plants were grown in nutrient solution in a phytotron (20/15 °C). Values given are means ± SE of 7 replicates. LSD at P = 0.05 was calculated for the data at 34 d of treatments (i.e., 47 d after imbibition).

through the same procedures recovered 99% K, 104% Na and 92% Cl. No adjustments were made to the data presented.

2.5. Pollen viability test

Pollen was collected from flowers of plants after 35-40 d in the continuous NaCl treatments (Set I). Two flowers on each plant of seven replicates were used. Pollen germination was tested on cellophane placed on growth media containing $CaCl_2 \cdot 2H_2O$ ($300\,mg\,L^{-1}$), H_3BO_3 ($100\,mg\,L^{-1}$) and sucrose 15% (Brewbaker and Kwack, 1963; Alexander and Ganeshan, 1989) and incubated for 8 h in darkness at 20–25 °C. Cellophane was lifted and aniline blue fluorescence stain was added before observing under a fluorescence microscope (Carl Zeiss, Germany). Pollen was considered to have germinated when tube length was more than the diameter of the pollen grain. To estimate pollen viability, observations were taken on ten random locations on each slide (approx. 350–1600 pollen grains).

2.6. Statistical analyses

Data were analyzed using GENSTAT (Version 10.2) software. Statistical significances for dry mass and ion concentrations in different tissues were evaluated using one-way ANOVA with Duncan's multiple comparison test at 5% level of significance. Skewed data sets were log_e transformed prior to ANOVA.

3. Results

3.1. Vegetative phase

3.1.1. Symptoms of leaf damage

Plants exposed to NaCl treatments developed symptoms of salt damage to leaves, characterized initially by yellowing and subsequent necrosis of margins of older leaves; with time these leaves suffered complete necrosis. These symptoms appeared first in the oldest leaves and then progressively in successively younger leaves (i.e., from base upwards). Initial symptoms appeared in the 60 mM treatment after about 10 d, then 2 d later in the 40 mM treatment, and another 2 d later in the 20 mM treatment. The rate of symptom spread was fastest in the 60 mM treatment and slowest in the 20 mM treatment.

3.1.2. Growth

Shoot dry mass did not differ among the four NaCl treatments up to 10 d after salinization, but by 20 d plant dry mass in 40 and 60 mM treatments was less than the control and 20 mM treatment (Fig. 1a). After 34 d of NaCl treatments, just prior to the commencement of flower bud initiation, shoot dry mass (% of controls) was 88% in 20 mM NaCl, 54% in 40 mM NaCl and 39% in 60 mM NaCl.

Root dry mass also did not differ across the four NaCl treatments up to 10 d after salinization, but by 20 d root dry mass in 60 mM NaCl was less than the other treatments (Fig. 1b). After 34 d of NaCl treatments, just prior to the commencement of flower bud initiation, root dry mass (% of control) was 92% in 20 mM NaCl, 59% in 40 mM NaCl and 33% in 60 mM NaCl.

3.1.3. Ion concentrations in roots and shoots

Within 10 d of salinization, Na in shoots had increased 7.5-fold in the 20 mM NaCl treatment, 14-fold in the 40 mM treatment, and 28-fold in the 60 mM treatment (Fig. 1c). In roots, Na concentration progressively increased in each higher NaCl treatment (Fig. 1d). Interestingly, after these large increases in tissue Na concentrations during the first 10 d, levels then remained relatively constant in both roots and shoots in all treatments for the next 24 d (Fig. 1c and d).

Within 10 d of salinization, Cl in shoots had increased 12-fold in the 20 mM NaCl treatment, 18-fold in the 40 mM treatment, and 30-fold in the 60 mM treatment (Fig. 1e). In roots, Cl concentration
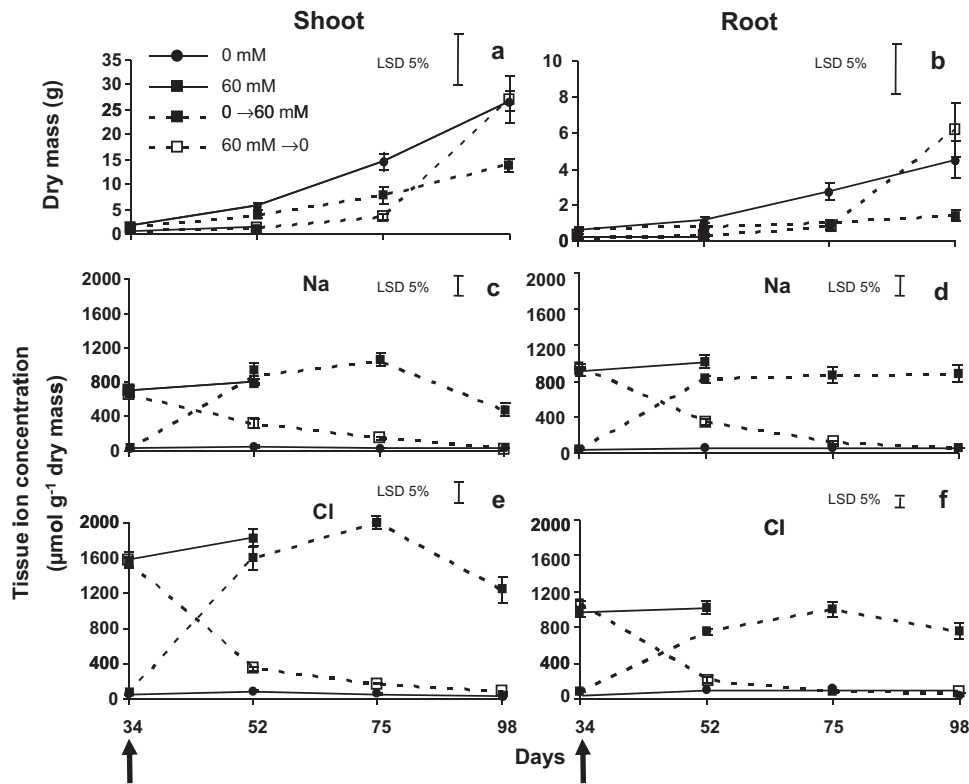
Fig. 2. Effect of NaCl with time on (a) shoot and (b) root dry mass per plant, and concentrations of Na and Cl in shoots (c and e, respectively) and roots (d and f, respectively) of chickpea during the reproductive stage. Arrow at 34 d on the horizontal axis indicates when treatments were shifted—i.e., NaCl continued, removed, or added for the first time to different batches of plants). Plants were grown in nutrient solution in a phytotron (20/15 °C). Values given are means ± SE of 7 replicates. Plants in continuous 60 mM NaCl concentration died at 52 d of treatment. LSD at *P* = 0.05 was calculated for data at the final sampling (98 d of treatments; i.e., 111 d after imbibition).

also progressively increased in each higher NaCl treatment but was much lower than those in shoots (Fig. 1f). After these initial, large increases in tissue Cl concentrations, levels then remained relatively constant in both roots and shoots of plants in all treatments for the next 24 d (Fig. 1e and f).

By contrast with the changes in tissue Na and Cl described in the preceding paragraphs, shoot K concentration did not differ significantly between the control and 60 mM NaCl treatments (data not shown). Root K concentration, however, had decreased in the 60 mM NaCl treatment to 74% of the control, and was 85% of the control at 40 mM NaCl (data not shown). The average K concentrations (μmol g^{-1} dry mass) in plants across four treatments were: 920 in shoots and 1670 in roots.

3.2. Reproductive phase

For clarity of presentation, and since the responses of plants exposed to 20 or 40 mM NaCl followed similar patterns, but intermediate to those in controls and 60 mM NaCl, time-series data on growth and tissue ion concentrations are only shown for the controls and 60 mM treatments (Fig. 2). Data for all treatments are given for the final sampling (i.e., 111 d after imbibition; Figs. 3–5).

3.2.1. Growth

Shoot (Fig. 2a) and root (Fig. 2b) dry mass continued to increase in controls during the reproductive phase. Plants continued at 60 mM NaCl were much smaller than controls (Fig. 2a and b) and although these plants initially increased in dry mass, all leaves suffered complete necrosis (i.e., no green leaves remained) by 52 d of treatment and no further observations were taken in 60 mM treatment (Fig. 2a and b). Plants that remained in 40 mM NaCl continued to grow, but by 75 d of treatment all leaves suffered complete necro-
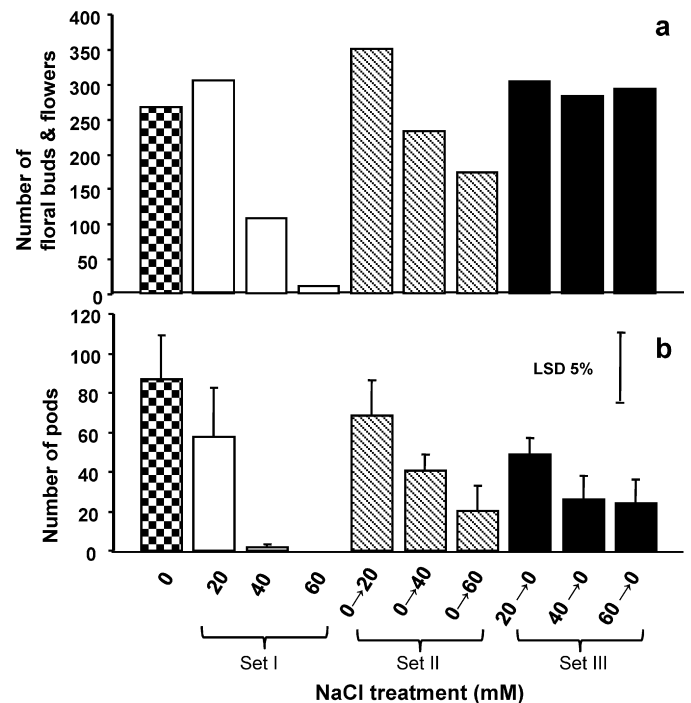
Fig. 3. Total number of buds and flowers per chickpea plant (a) observed over 50 d, at 10 d intervals, and (b) total numbers of pods per plant at the final sampling (98 d of treatments). Set I—continuous NaCl treatments; Set II—NaCl applied at flower bud initiation stage; Set III—NaCl removed at flower bud initiation stage. No pods were formed in continuous 60 mM NaCl treatment, as plants died. Values given are means ± SE of 7 replicates. LSD at *P* = 0.05 is shown. SE values were not given for flower numbers as these data were cumulative.
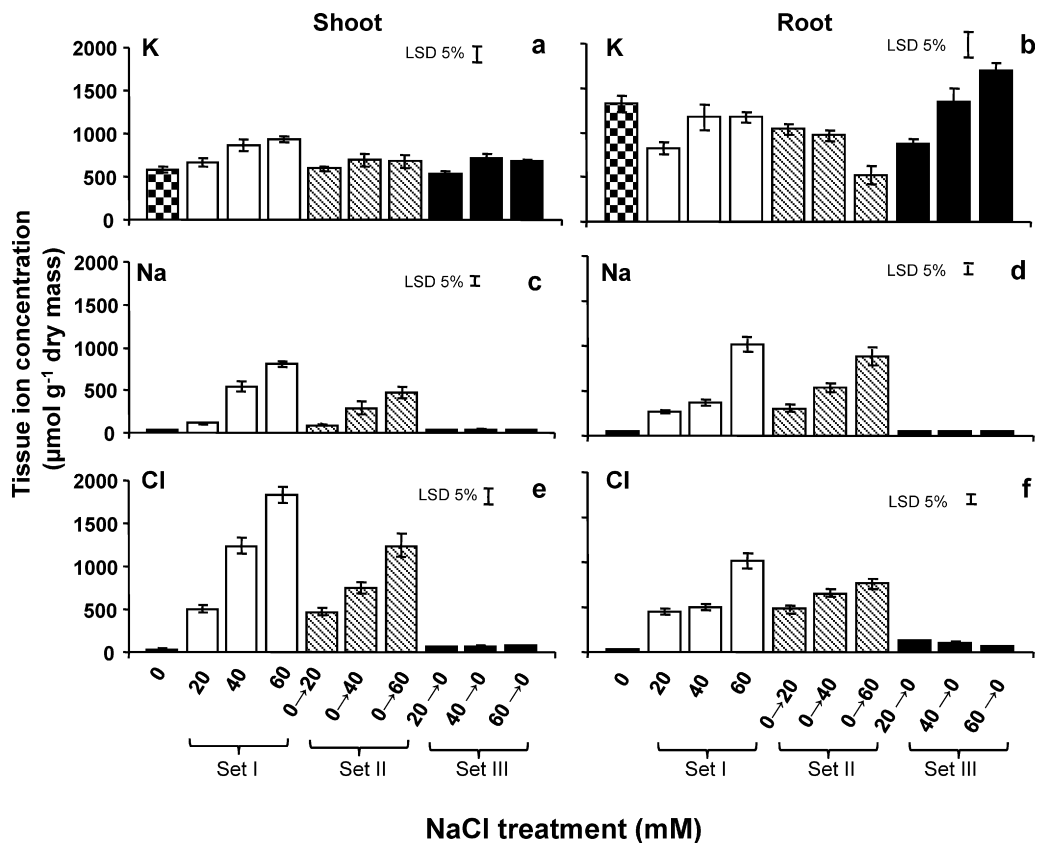
Fig. 4. K, Na and Cl concentrations at the final sampling (98 d of treatments) in the shoot (a, c and e) and roots (b, d and f) of chickpea exposed to different concentrations and timing of NaCl treatments. Set I—continuous NaCl treatments; Set II—NaCl applied at flower bud initiation stage; Set III—NaCl removed at flower bud initiation stage. Values given are means ± SE of 7 replicates. LSD at P = 0.05 is shown.

sis. Plants in 20 mM NaCl survived; shoot and root dry mass at the end of the experiment were 78–82% of controls (Fig. 6). As described below, controls and 20 mM NaCl treated plants produced numerous pods, but plants treated with 40 mM NaCl produced few pods prior to death, and with 60 mM NaCl plants died without producing any pods (Fig. 3).

For plants first exposed to NaCl treatments at the reproductive stage (Set II), growth was severely stunted at 60 mM, such that final dry mass of shoots was 53% of controls (Fig. 2a) and roots was 32% (Fig. 2b). At 40 mM NaCl, shoot and root dry mass were 56% and 34%, respectively, of controls (data not shown). At 20 mM NaCl, shoot and root dry mass were 73% and 66%, respectively, of controls (data not shown). All plants in treatments first imposed at the reproductive stage produced pods, but numbers declined with increasing NaCl concentration (see below).

For plants initially in 60 mM NaCl and then removed at flowering (Set III), shoot and root dry mass increased modestly up to d 75, but then plants had a remarkable recovery in shoot (Fig. 2a) and root (Fig. 2b) growth by the final sampling. Following removal of the NaCl, plants previously in 20 mM and 40 mM NaCl up to the reproductive stage had both fully recovered shoot and root dry mass by the final sampling (data not shown).

3.2.2. Flower and pod numbers

The numbers of flowers (including buds) and pods were counted every 10 d, during the final 50 d of the experiment. For plants in continuous NaCl treatments (Set I), the number of flowers was not affected by 20 mM, but at 40 mM had decreased to 40% and at 60 mM to 4% of controls (Fig. 3a). Although flower numbers at 20 mM NaCl did not differ from the control, pod number had declined to 33% of the control. At 40 mM NaCl, most flowers

dropped off so that very few pods were formed, and at 60 mM NaCl plants died so that none of the very few flowers formed developed into pods (Fig. 3a and b).

For plants first exposed to NaCl at flower bud initiation (Set II), flower number increased by 31% at 20 mM, but declined progressively at each higher NaCl treatment (Fig. 3a). In contrast to the stimulation of flowering by 20 mM NaCl, pod number was less than in the controls (Fig. 3b). Flower and pod numbers were both reduced progressively as NaCl was increased to 40 and then 60 mM (Fig. 3b). The reductions in pod numbers in plants first exposed to NaCl at flower bud initiation were, however, much less than those for plants continuously in NaCl from the early vegetative stage (Fig. 3b). Plants exposed to NaCl in the vegetative stage and then without NaCl during the reproductive phase (Set III), all recovered such that flower numbers were equal to those in the control (Fig. 3a). Pod numbers, however, were still reduced in these plants at the final sampling, although this was not a direct on-going effect of the previous NaCl exposure as many of the flowers formed later in the experiment did not have time to develop into pods before the end of the experiment.

3.2.3. Ion concentrations in shoots and roots

Concentrations of Na (Fig. 4c and d) and Cl (Fig. 4e and f) remained low in shoots and roots of control plants during the reproductive phase. Plants that continued in the 60 mM NaCl treatments already contained high tissue concentrations of Na and Cl, and these only had modest increases prior to death. Plants that remained in 40 mM NaCl, increased in Na and Cl concentrations during the reproductive phase and the values reached as much as 15- and 29- times those in controls, respectively (Fig. 4c–f). These plants died after 75 d of NaCl treatment. Plants that continued in 20 mM NaCl
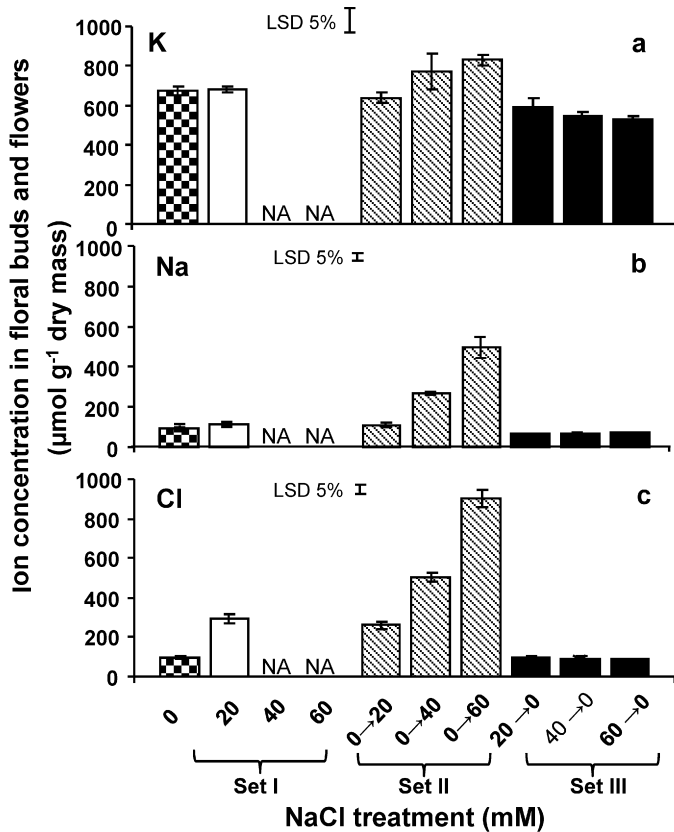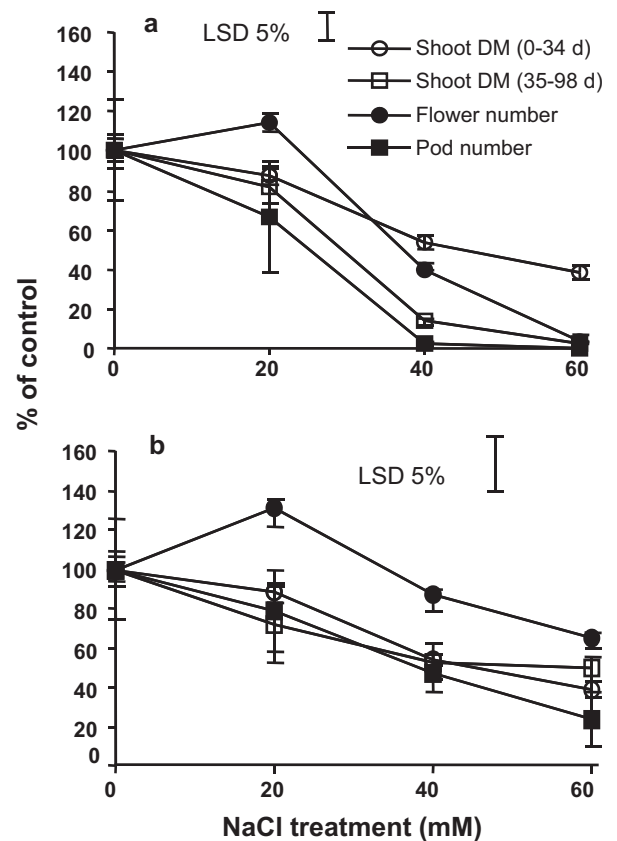
Fig. 5. K, Na and Cl concentrations (a, b and c, respectively) in buds and flowers of chickpea exposed to different concentrations and timing of NaCl treatments. These floral structures were sampled at the final sampling (98 d of treatments). Set I—continuous NaCl treatments; Set II—NaCl applied at flower bud initiation stage; Set III—NaCl removed at flower bud initiation stage. Data not available (NA) for continuous 40 mM and 60 mM treatments, as plants died before the final sampling. Values given are means ± SE of 7 replicates. LSD at P = 0.05 is shown.

Fig. 6. Responses of chickpea to increasing NaCl concentrations applied during the (a) vegetative (Set I) and (b) reproductive (Set II) stages. Responses (as % of control) are shown for vegetative growth (shoot dry mass) and reproductive components (flower and pod numbers). In continuous 40 mM and 60 mM NaCl (Set I) plants died prior to maturity; only a few flowers, but no pods were formed at 60 mM NaCl. Values given are means ± SE of 7 replicates. LSD at P = 0.05 is shown.

also had Na and Cl concentrations higher than controls; Na and Cl were, respectively, 3.4- and 12-times higher in shoots and 5.4- and 13.5-times higher in roots (Fig. 4). In continuous NaCl treatments (Set I), K concentration did not change in shoots or roots, even though plants soon died in the two highest NaCl concentrations. By contrast, K concentration decreased significantly (38%) in roots of plants grown in 20 mM NaCl (Fig. 4a and b).

For plants first exposed to NaCl treatments at the reproductive stage (Set II), shoot Na (Fig. 2c) and Cl (Fig. 2e) increased by the next sampling time (18 d) to levels approximately equal to those in plants continuously in the same NaCl concentrations. Similarly, root Na (Fig. 2d) and Cl (Fig. 2f) increased to 80% of the concentrations in plants continuously exposed to NaCl. Like the vegetative stage (Fig. 1), after the initial increase in tissue Na and Cl upon first exposure at flower bud initiation, concentrations in roots remained relatively constant; but in shoots, Na and Cl declined modestly by the final sampling. K concentration did not change significantly in shoots or roots of plants with NaCl treatments imposed at flower bud initiation (Set II) (Fig. 4a and b). General observations across all treatments, for plants near a new steady state in NaCl treatments (Set I and II), were that Na concentrations were similar in shoots and roots (Fig. 2c and d), whereas Cl concentrations in shoots were higher than in roots (Fig. 2e and f). Thus, shoot Cl always exceeded shoot Na, in the vegetative (Fig. 1) and reproductive phase (Fig. 2).

Plants initially in 60 mM NaCl, but removed at flowering (Set III), had large reductions in Na and Cl in shoots (Fig. 2a and e) and roots (Fig. 2d and f). The reductions were greatest in the first 18 d, but then continued such that, by the end of the experiment, tissue Na

and Cl concentrations did not differ from controls. After removing NaCl at flowering, shoot K did not differ at the final sampling irrespective of the previous presence or absence of NaCl (Fig. 4a). By contrast, in roots the K concentration did differ at the final sampling; being 28% higher in plants previously at 60 mM NaCl, but not different to the control when previously at 40 mM NaCl, and 34% lower than in the control for plants previously at 20 mM NaCl (Fig. 4b).

3.2.4. Ion concentrations in floral buds/flowers

In the continuous NaCl treatments, plants died in the 40 and 60 mM treatments prior to the final sampling at which floral structures were collected, so floral buds and flowers could only be sampled for the 20 mM treatment and control (Set I). Floral buds and flowers did not show significant differences in tissue Na concentration between controls and the 20 mM NaCl treatment (Fig. 5b). Tissue Cl, however, increased 3-fold in these floral tissues of plants at 20 mM NaCl (Fig. 5c). For plants first exposed to NaCl at flowering (Set II), Na in floral tissues increased progressively at each higher NaCl treatment (Fig. 5b), as did Cl (Fig. 5c). Cl in floral tissues was equal to that of Na in controls, but was 2.6, 1.9 and 1.8 times higher than Na in plants at 20, 40 and 60 mM NaCl, respectively (Fig. 5b and c). For plants with NaCl treatments removed at flower bud initiation (Set III), Na and Cl concentrations in floral tissues were low by the final sampling, being equivalent to those in controls (Fig. 5b and c). K concentrations in floral buds and flowers (Fig. 5a) were affected much less than those of Cl (Fig. 5c); no effect was seen for plants continuously in 20 mM NaCl (Set I). Interestingly, K increased slightly with higher NaCl treatments applied at

flowering (Set II), whereas K declined to 88–78% of control values in floral buds/flowers of plants exposed to NaCl during the vegetative phase and without NaCl in the reproductive phase (Set III).

3.2.5. Pollen viability

Pollen grain germination *in vitro* was determined to assess viability, as reduced pollen viability was one possible cause of reduced conversion of flowers to pods. Pollen collected from NaCl treated plants (Set I after 35–40 d of treatments) showed *in vitro* germination of 99% (20 mM), 96% (40 mM), and 91% (60 mM). The influence of NaCl added to the *in vitro* test solution was also determined for pollen taken from control plants; germination was inhibited by addition of NaCl, being only 30% (40 mM), 27% (80 mM), and 19% (120 mM) of control values.

4. Discussion

Imposition of NaCl treatments at flower bud initiation enabled evaluation of the sensitivity of the reproductive phase of chickpea without carry-over effects of prior exposure during the vegetative phase. Such carry-over effects would be large in chickpea exposed to 40 and 60 mM NaCl, owing to high tissue concentrations of Na and Cl, severe symptoms of shoot damage, and reduced biomass before flowering commenced (see Section 3). Plants exposed to 40 mM NaCl from the seedling stage produced 2% of the pods compared with the control, and those at 60 mM did not pod (Fig. 6a). For plants first exposed to NaCl at flower bud initiation, flower production was affected less by salinity than vegetative growth, but pod formation was more sensitive than vegetative growth (Fig. 6b). Although flower production was inhibited by NaCl at 40 mM and above, at 20 mM NaCl flower numbers increased by 14% in continuous treatments applied to seedlings, and by 31% when NaCl was applied at flower bud initiation, compared with the controls (Fig. 6). Similarly, salinity (Na:Ca:Mg 4:1:3 and Cl:SO$_4$ 7:3) at 20 meq L^{-1} increased flower numbers 2-fold in earlier studies (Dhingra and Varghese, 1993; Dhingra et al., 1996). In summary, our approach enabled us to test the hypothesis that in chickpea the reproductive phase is more salt-sensitive than vegetative growth, and further strengthened the idea of Vadez et al. (2007) that conversion of flowers to pods appears to be a salt sensitive process. Therefore, screening of chickpea for tolerance at this stage between flowering and seed development under salinity should be a priority for future work.

Sensitivity of flower conversion into pods under saline conditions might be related to high concentrations of Na and Cl in floral tissues (Fig. 4). For plants exposed to 60 mM NaCl starting at flower initiation, Na on a tissue water basis in flowers reached 151 mM (498 µmol g^{-1} dry mass) and Cl was 274 mM (903 µmol g^{-1} dry mass); these are considered to be relatively high concentrations for plant tissues (cf. Munns and Tester, 2008). Interestingly, Na on tissue water basis in flowers (151 mM) did not differ to that in the whole shoot, whereas flower Cl concentration (274 mM) although still higher than Na, was just under half of the Cl concentration in the whole shoot (637 mM) (calculated from Fig. 4 and 5 and data on tissue water contents). This situation of high Na concentrations in reproductive tissues of chickpea is similar to the situation in rice (Khatun et al., 1995); rice is also very sensitive to salinity during the reproductive phase (Heenan et al., 1988; Khatun et al., 1995). Like chickpea (present study), Na concentrations in floral parts of rice were also similar to, or even exceeded, those in leaves (Khatun et al., 1995). In rice, high Na concentrations even occur in the stigma and pollen (Khatun et al., 1995), so it is also possible that in chickpea high Na (and possibly Cl) concentrations might also occur in these floral parts, in addition to the likely accumulation in the transpiring

outer parts of flowers. Future research should elucidate the delivery pathways of Na and Cl into floral tissues of salt-sensitive species such as chickpea and rice.

It seems likely that accumulation of Na and/or Cl to toxic levels in floral tissues might compromise flower to pod conversion in chickpea in saline conditions, as high tissue ions can cause toxicities and/or disturb tissue water relations (cf. Munns and Tester, 2008). Pollen from salt-treated chickpea (even at 60 mM NaCl) was viable, giving 91% germination *in vitro*, but pollen germination *in vitro* was severely inhibited by addition of 40 mM NaCl to the germination medium. Similar to chickpea, salinized tomato retained pollen viability (Grumberg et al., 1995) and pollen germination was inhibited *in vitro* by NaCl of 34 mM and above (Foolad, 2004). In rice, both pollen viability and stigma receptivity were reduced in saline conditions, and by most in genotypes with the highest Na concentrations in these floral parts (Khatun et al., 1995). Moreover, in chickpea experiencing water deficits, stigma receptivity declines markedly (Fang et al., 2010). So, if Na and Cl had accumulated in the stigma of salinized chickpea, then it seems reasonable to expect that *in vivo* pollen germination and pollen tube growth would have been inhibited, thereby reducing conversion of flowers to pods and thus seed numbers. Although not evaluated in the present study, seed size in chickpea can also be reduced under saline conditions (e.g. by 20%, Vadez et al., 2007), and entry of Na and Cl into seeds (Murumkar and Chavan, 1986; Mamo et al., 1996) might have contributed to these declines if these ions adversely affected metabolism, in addition to possible declines in photosynthate available for seed-filling in salinized plants (cf. suggested for rice, Khatun et al., 1995).

Na and Cl concentrations in vegetative (present study and see also Lauter and Munns, 1987) and reproductive phases (present study) reached relatively steady levels after 10 and 18 d of treatment, respectively; after which, more or less the same tissue concentrations were maintained. The growth reduction and leaf damage could have resulted from an interaction of time with the concentration of Na and/or Cl in the tissue (cf. Wilson et al., 1970; Munns et al., 1995). With time some leaves died; leaf death was most likely caused by ion toxicity, although it is difficult to separate individual effects of Na and Cl (cf. Munns and Tester, 2008). When the capacity of cells to store ions is exceeded, ion toxicity occurs in the cytoplasm and/or ions build up in intercellular spaces, leading to cell dehydration and death (Munns, 1993), and although Na is most commonly regarded as the toxic ion in many species suffering salt damage, Cl toxicity occurs in several salt-sensitive species (Munns and Tester, 2008). For chickpea, Lauter et al. (1981) and Dua (1998) considered the predominance of Cl in salt injury, whereas in other work based on studies using NaCl (50 mM) and Na$_2$SO$_4$ (25 mM) Na damage was considered to be more than that of Cl (Lauter and Munns, 1986, 1987). In chickpea shoots, critical Na concentrations have been reported as 200–270 µmol g^{-1} dry mass (Lauter and Munns, 1987) and critical Cl concentrations at ~450 µmol g^{-1} dry mass (Reuter and Robinson, 1986). In the present experiment, plants at 20 mM NaCl, that suffered growth reductions of 17%, had shoot Na and Cl concentrations of 115 and 500 µmol g^{-1} dry mass, respectively; as Cl exceeded the critical concentration, whereas Na did not, Cl might have predominately caused the toxicity. At higher external NaCl (60 mM) shoots also contained 2.25 (vegetative phase) and 2.65 (reproductive phase) higher Cl than Na; being consistent with other studies of chickpea reporting that shoot concentrations of Cl exceed those of Na (e.g. Mamo et al., 1996). Further, shoot Na concentration was lower than in roots in all treatments whereas the opposite occurred for Cl, i.e., shoot Cl exceeded root concentrations (Fig. 4). Thus, the present data indicate Cl toxicity in shoots as a likely contributing mechanism causing salt sensitivity in chickpea.

Chickpea exposed to 60 mM NaCl died after 52 d, whereas those transferred back to non-saline solutions at flower initiation (after 34 d at 60 mM NaCl) recovered (Fig. 2). These plants had 60% less shoot dry mass when transferred back to non-saline conditions, but recovered by producing new branches and, although later, the same numbers of flowers as the non-saline control. This response of chickpea contrasts with that of cowpea (also with indeterminate growth); for cowpea, when salinity was removed after 20 d of salinization in the vegetative phase, vegetative shoot growth and seed yields were still significantly reduced compared with when salinity was applied at flowering and pod filling stages (Maas and Poss, 1989). In the present study, ion concentrations in shoots and roots declined during recovery, back to control levels. Ions present when transferred to non-saline conditions would have been diluted by new growth, lost in old leaves that dropped, and some efflux from the roots could also have occurred. The capacity to recover after saline levels decrease might be an important adaptation in field situations with fluctuating salinity levels, such as in southern Australia (Rengasamy, 2006) and presumably other Mediterranean cropping environments.

4.1. Conclusions

NaCl at 40 mM (or above) applied to chickpea seedlings or to plants at flower initiation stage, in both cases reduced root and shoot growth and flower and pod numbers, eventually causing death when 40 or 60 mM was applied at the seedling stage and continued. Both vegetative and reproductive stages of chickpea were sensitive to continuous NaCl exposure; but conversion of flowers to pods appears to be particularly salt sensitive. Sensitivity occurred even for a reputably 'tolerant' cultivar (JG 11; Vadez et al., 2007) at NaCl levels (viz. 20 and 40 mM) that would be considered as relatively mild for many crops (e.g. wheat, Colmer et al., 2005) with which chickpea might be grown in rotation. Upon removal of NaCl, however, chickpea showed excellent recovery with substantial new shoot growth, presumably aided by an indeterminate growth habit. Sensitivity during the reproductive stage was not caused by changes in pollen viability but was potentially due to toxic accumulation of Na and Cl in flowers, and possibly the sensitivity of pollen tube growth if NaCl entered the stigma. Evaluations of salt tolerance in chickpea need to include reproductive, as well as earlier growth stages.

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