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EVALUATION OF LEAF 'TISSUE TOLERANCE' OF Na^+ AND Cl^- IN SOYBEAN (*GLYCINE MAX* (L.) MERR.), AND COMMON BEAN (*PHASEOLUS VULGARIS* L.)

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ABSTRACT

Evaluation of tissue tolerance of potentially toxic ions (Na^+ and/or Cl^-) can be complicated by differences in ion exclusion by the roots resulting in differences in tissue ion concentrations in the leaves. This research tested two approaches to 'deliver' NaCl to leaves to assay tissue tolerance: (i) injection of NaCl into the stem or petiole of intact plants and (ii) an excised leaf method (transpiring leaf with petiole in a saline solution) and determined whether measurements of leaf ion concentrations and leaf function can be used to assess 'tissue tolerance'. The infusion and excision methods were conducted on 18–day–old of soybean and common bean grown in aerated non–saline nutrient solution culture for over 5 days. The NaCl infusion system was not successful since Na^+ and/or Cl^- in lamina of soybean and common bean did not significantly increase. In contrast, the excised leaf system resulted in approximately equal increases in Na^+ and Cl^- in lamina in the two soybean genotypes, which was 20% lesser than that in common bean. The Na^+ concentrations in the lamina of excised leaves reached or exceeded the critical levels for toxicity; however, there was no relationship between leaf Na^+ and/or Cl^-) concentrations and leaf photosynthesis (P_n). It was likely that the cavitation of the xylem which would impede water flow along the petioles and impacted on P_n even in the non–saline controls.

Keywords: Cl^- toxicity, ion tissue tolerance, ion uptake, leaf gas exchange, Na^+ toxicity

1. INTRODUCTION

Under salt stress most plants can 'exclude' Na^+ from the roots so that its entry to the xylem is only about 10% of that in the external solution, thus restricting the amount of Na^+ transported in the xylem stream to above ground parts [1]. Ion exclusion is the ability of a plant to restrict ion

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movement from roots to shoots by: (i) minimising ion uptake by root cells and maximising ion efflux back to the soil solution, and/or (ii) restricting ion loading into the xylem, and/or (iii) retrieving of ions from the xylem stream as it moves along a root, stem, petiole or leaf sheath [2]. Under salt stress, ionic and osmotic stress both occur, however, it takes days or weeks for ionic stress to develop while osmotic stress can occur right away [3].

Ionic stress occurs when the high accumulation of Na^+ and/or Cl^- in a tissue leads to ion toxicity (impaired cellular function), which impairs plant growth. The most noticeable ion toxicity symptom in plants is necrosis in older leaves. High concentrations of Na^+ and/or Cl^- can disrupt enzymes in cytoplasm, damage the photosynthetic apparatus in chloroplasts and dehydrate cells due to accumulation in the apoplast [3]. For example, high Na^+ concentrations in leaves have been associated with damage to chlorophyll, decreased photosynthesis and increased necrosis in mungbean [4]. Research on barley showed an additive effect of Na^+ and Cl^-, since high Cl^- concentrations decreased chlorophyll activities and affected the photosynthetic capacity and quantum yield, while high Na^+ interfered with K^+ and Ca^{2+} uptake and impaired stomatal regulation, which reduced photosynthesis and growth [5]. However, in this study, the ion concentrations were measured in tissues not chloroplasts, so these conclusions require further testing.

'Tissue tolerance' is a term used to describe when plants can tolerate high Na^+ and/or Cl^- concentration in leaf tissues without, or with only minor, detrimental effects on tissue functioning [6]. Salt–tolerant plants can sequester much of the Na^+ and/or Cl^- that enters leaves into vacuoles and restrict the amount of Na^+ and/or Cl^- present in photosynthetically active mesophyll cells [3, 6, 7]. Under salt stress, plants can have similar amounts of Na^+ and/or Cl^- in the bulk tissue, but salt–tolerant plants can inter– and/or intracellularly partition these ions away from the most sensitive cells and metabolically active compartments [7].

Leaf Na^+ exclusion and leaf tissue tolerance, both contributed to salt tolerance in plants [6]. The ability of roots to restrict the amount of Na^+ reaching leaves was presumably why soybean had a lower leaf Na^+ concentration and was more tolerant to salt stress than common bean. This research tested the hypothesis that injection of NaCl into stem or petiole of intact plants, or the use of an excised leaf method (transpiring leaf with petiole in a saline solution), will deliver these ions to leaves, and that measurements of leaf function can be used to assess leaf 'tissue tolerance' in two soybean genotypes and one common bean genotype. As roots are not part of this system, any differences in root capacity to exclude ions is removed, so that the excised leaves of these three genotypes could receive similar amounts of ions entering with transpiration to facilitate comparisons of tissue tolerance. Leaf tissue tolerance was assessed as the ability to maintain photosynthesis and/or leaf chlorophyll (SPAD) when leaf tissues contain high Na^+

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and/or Cl^- concentrations. Thus, this research aimed to test for tissue tolerance in these (sub)tropical grain legumes.

2. MATERIALS AND METHODS

2.1. Plant Materials and Growth Conditions

The experiments were conducted in a phytotron (temperature–controlled glasshouse; 28/22 ± 2 °C day/night) September and October 2019 in Perth, WA, Australia (31°57'S, 115°47'E). Plants received natural sunlight transmitted through polycarbonate panels and no supplementary light was needed. The two tropical grain legumes chosen in this research are grown in a number of countries and so it was of interest to compare these to evaluate any common and/or different responses to salinity to increase knowledge of stress tolerance in this important group of crop plants.

Seeds of soybean (*Glycine max* (L.) Merr.) cvs. Bunya and Lee, and common bean (*Phaseolus vulgaris* L.) cv. Spearfelt were washed with 0.042% (w/v) sodium hypochlorite for 5 min, rinsed thoroughly with tap water and placed on wet tissue paper in Al-covered 20 L buckets. Germinated seeds were placed on plastic mesh floating on 10% (v/v) strength aerated nutrient solution in the dark for two days. On day 3, the nutrient solution was changed to 25% (v/v) strength and the seedlings were exposed to natural sun light., Seven-days-old seedlings were transferred to pots (four seedlings per 4.5 L plastic pot) containing 100% (v/v) strength aerated nutrient solution (Table 1) [8]. The plastic pots were covered with aluminium foil to exclude light from the root zone. Plants were grown in aerated non-saline nutrient solution for 18 days to obtain plants with three fully expanded leaves and then infusion and excision methods were conducted.

2.2. NaCl infusion system

The goal was to test whether infusion of NaCl into the stem and/or petiole can be used to modulate leaf tissue ion concentrations in two selected tropical grain legume species, and whether this provides more information on leaf Na^+ and/or $Cl^-$and their influence on P_n. This method might enable researchers to test for differences in leaf tissue tolerance to Na^+ and/or Cl^-. This study tested the hypothesis that the infused ions move into the stem/petiole and then into lamina tissue. Lamina with higher Na^+ and/or Cl^- concentrations would be expected to have reduced rates of P_n, but if genotypes differ in the P_n response to leaf ion concentrations then this would provide a measure of leaf tissue tolerance.

After 18 days of growing in aerated non saline nutrient solution culture, an 18–gauge needle (1.20 x 38 mm) was inserted into the petiole of the second youngest fully–expanded leaf, or the

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stem just below this petiole, to inject 50 mM NaCl into these organs. The using of 50 mM NaCl to inject directly to the petiole or stem was to avoid a great shock of salinity damage to the plants and was considered to be a relevant concentration for what might be expected to occur in the xylem of a transpiring plant. The control had deionised water (DI) injected into petiole or stem (Table S3.1). The objective was to manipulate leaf Na^+ and/or Cl^- to evaluate the possible relationships between leaf tissue ions (concentrations) and P_n.

The back end of the 18–gauge needle was connected to a tube, which in turn was connected to a 1.0 L reservoir of 50 mM NaCl or DI water hung on a rack 1.2 m above the plant providing a gravitational force of 0.012 MPa. The needle tip was inserted into the middle of a petiole or the stem (3 cm deep and about 5 cm away from the petiole). Parafilm was wrapped around the injection site to avoid evaporation or leaking. The weight of the reservoir was recorded daily to assess the volume of solution injected. Leaf SPAD values and gas exchange measurements were also recorded. The weight of the injection solution in the reservoir hardly changed indicating that this system was not effective for injecting NaCl; indeed, leaf SPAD values did not change. Thus, this trial experiment ended after 5 days of treatment. Lamina of injected plants were harvested for ion analysis. The ion analysis showed that the infusion system did not work as there was no significant flow into the plant as the weight of the solution did not significantly change. The system had been checked to ensure flow prior to inserting the needle tip into the plant. The pressure head might not have been set high enough to 'push' the water/solution into the plants. A manual injection of NaCl into the petiole might offer greater pressure, but this would be difficult to sustain over time. The reservoir bottle may need to be positioned at greater height (larger pressure head) or a pressure–regulated system developed but establishing the resistance to flow from the needle tip into these tissues and evaluating any physical damage should be considered first. Hence, the result and discussion in this research only briefly considers further these attempted 'injection' experiments and instead focuses on another method (the excised leaf system; see below).

2.3. Excised leaf system

The excised leaf system used plants grown for 18 days in aerated non-saline nutrient solution until plants had three fully-expanded leaves and the second youngest fully-expanded leaf was excised near the base of the petiole. The cut end of the petiole was placed in control or 5 mM NaCl treatment solutions, with the lamina in air in a controlled environment room (12 h light, 28/22± 2°C, RH 75%) so that transpiration would result in the solutions and ions moving into leaves.

The control treatment comprised 5% (v/v) strength of standard nutrient solution; and the NaCl treatment comprised 5 mM NaCl in the same 5% (v/v) strength nutrient solution.

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The following approach was used:

1. Initial measurements: SPAD and gas exchange were measured on the 2nd youngest fully-expanded leaf of four plants. The leaves were then sampled to measure initial ions in the petioles and lamina.

2. The nutrient solution and leaves were placed in 20 ml vial (28 mm × 61 mm). The weight of the nutrient solution was recorded at the same time each day to calculate daily water use.

3. To sample and transfer leaves into the holding vials, plastic cling film was used to cover the 2nd youngest fully-expanded leaf to prevent evaporation. A sharp razor blade was used to cut the base of the petiole, with the excised end immediately placed in the control solution and about 1 cm of the excised end of the petiole was removed in the solution (leaving about 5 cm of petiole) before immediately transferring the petiole (+leaf) to a vial containing the treatment solution (control or NaCl); the weight of the vial + nutrient solution + petiole/leaf was recorded.

4. The top of the vial with the petiole protruding was covered with parafilm to avoid evaporation (wrapped around petiole), and the vial was placed in a rack. The rack was wrapped with aluminium foil. The plastic cling film was removed from the leaf.

5. Measurements included SPAD daily and gas exchange after one and four days of treatment. Leaf gas exchange was measured on leaves exposed to light for about 30 minutes after the preceding 12 h dark period. Some trails with the photosynthesis measured at 10 to 11:30 AM, and after 30 minutes exposed to the light. The data of $P_n$at the after 30 minutes exposed to the light was more stable in this excised system.

6. Water use was measured each day and the solution renewed each day (i.e. measure weight of the solution at the start and the end of each 24 h period).

2.4. Other measurements

2.4.1. Leaf gas–exchange and chlorophyll measurements

Stomatal conductance (g_s), net photosynthetic rate (P_n), transpiration (T), and internal CO_2 concentration (C_i) were measured on the second youngest fully–expanded leaf at vegetative, podding and pod-filling stages using an LI–6400XT open gas–exchange system coupled with a 6 cm^2 chamber head (LI–COR Biosciences Inc., Lincoln, NB, USA). The measurements were conducted at photosynthetically active radiation of 1500 µmol photons m^{-2} s^{-1} (light–saturated) and CO_2 concentrations of 400 µmol mol^{-1} (ambient) and 800 µmol mol^{-1} (elevated; podding and pod-filling stages) [9]. The gas exchange measurements were taken on the same day between

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10:00 and 15:00 h. The leaf chamber temperature was 28°C with 60–70% relative humidity. Chlorophyll concentrations in leaves (lamina) were measured on the same day using a SPAD meter (Minolta, Osaka, Japan) on the same leaf that was used for the gas exchange measurements.

2.4.2. Tissue Ion Analysis

Oven–dried samples of different tissues (green leaves, damaged leaves, dead leaves, petioles, stems, roots, flowers, seeds and pods) of the same sampled plants were ground to a fine powder and analysed for Na^+, K^+ and Cl^- following the procedures of Munns et al. 2010. Tissues (100 mg) were extracted in 0.5 M HNO_3 (10 mL) by shaking for 48 h in darkness at room temperature. Diluted samples of the extracts were then analysed for Na^+ and K^+ using a flame photometer (Flame Photometer 410, Sherwood, Cambridge, UK) and for Cl^- with a chloridometer (Model 50CL, SLAMED ING. GmbH, Frankfurt, Germany). A reference tissue (broccoli, ASPAC no. 85) with known ion concentrations was taken through the same analyses to confirm the reliability of the methods.

2.4.3. Leaf sap osmotic potential and tissue water content

Part of the leaf lamina of the second youngest fully–expanded leaf was sampled into a 2 mL air–tight cryo–vial, quickly frozen in liquid N_2, and then stored at –20°C until required. Samples were thawed in the sealed vials and then crushed in a manual press to obtain tissue sap. The osmotic potential was measured using 20 µL of sap in a calibrated freezing point depression osmometer (Fiske Associates, Model One–Ten, Ma, USA). The readings were converted from mosmol L^{-1} to MPa using the formula (2.447*X)/1000; where X = is the value in mosmol L^{-1}, 2.447 is R×T where R is the universal gas constant (8.314472 J K^{-1} mol^{-1}) and T is temperature in Kelvin (293 at 20 °C). Tissue water content was calculated using the fresh and dry mass data to calculate mL g^{-1} dry mass.

2.4.4. Leaf area measurement

Leaves were photographed and the ImageJ 1.52a program used to measure leaf area [10].

2.4.5. Statistical analyses

Data were subjected to one–way, two–way, or three–way analysis of Variance (ANOVA) using Genstat Software (VSN International Ltd, Hemel Hempstead, UK) to observe differences between treatments and genotypes/species and to test for any genotype/specie × treatment interaction. The three–way ANOVA also included a time factor for some data sets. Means were

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compared for significant differences using LSD at the 5% probability level. Figures and scatter plots were graphed using Origin Pro 2019 (v.9.65).

3. RESULTS

For both injection methods (stem or petiole), the tissue ion analysis showed no significant differences between Na^+, Cl^- or K^+ concentrations in the lamina of any genotype/species in either treatment (Table 2). Neither the stem nor petiole injection method was successful, so another method (excised leaf system) was investigated.

3.1. Excised leaves soon ceased to transpire

In the excised leaf system (control and NaCl solution), leaves used the most water on the first day of treatment (about 1 mmol m^{-2} s^{-1}), being about 60–70% of the total water use for 4 days of treatment, followed by about 20% on day 2, and about 10% on days 3 and 4 (Fig. 1). There were no significant differences in leaf daily water use (per unit area) between genotypes (P = 0.1) or treatments (P = 0.8). There was no significant genotype × treatment interaction for water use (P = 0.4) on day 1 or day 4, but significant differences (P < 0.05) occurred on days 2 and 3. Common bean used less water than soybean.

3.2. Ion concentrations (Na^+, Cl^-, K^+) and K^+/Na^+ and Cl^-/Na^+ ratios in lamina and petioles of excised leaves

In the excised leaf system, Na^+ concentrations in lamina and petioles increased in the NaCl treatment, relative to the control. No significant differences occurred between genotypes for Na^+ concentration in lamina (P = 0.07) (Fig. 2e, Table 3), but significant differences occurred in petioles (P < 0.01) (Fig. 3e, Table 3). After 4 days of treatment, the petioles of soybean Lee and Bunya each had about 50% higher Na^+ concentrations than those in common bean (Fig. 3e). Lamina and petiole Na^+ concentrations in soybean Lee and Bunya did not differ in the NaCl treatment, but Na^+ concentrations in common bean Spearfelt lamina about 40% higher than that in petioles.

Cl^- concentrations in lamina and petioles in three genotypes also increased, relative to the control (Fig. 2f, Table 3), but Cl^- concentrations in petioles differed between genotypes (P < 0.001) (Fig. 3f, Table 3). Cl^- concentrations in petioles in common bean Spearfelt and soybean Lee each was about 40% higher than that in soybean Bunya (Fig. 3f). Soybean Bunya Cl^- concentrations in lamina was 40% higher than that in petioles, but common bean Spearfelt and soybean Lee had 20% lower Cl^- concentrations in lamina than petioles.

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Interestingly, soybean and common bean had slightly higher K^+ concentrations in lamina in the NaCl treatment than the control. There were no significant changes in K^+ concentrations between excised lamina and intact lamina (initial values at time of excision) (Fig. 2c and g, Table 3). In contrast, the K^+ concentrations in excised petioles in both the control and NaCl treatment was only about 50% of intact petioles (Fig. 3c and g).

The NaCl treatment decreased the K^+/Na^+ ratios in lamina and petioles, relative to the control (Fig. 2h, Fig. 3h, Table .3) in all three genotypes; for example, K^+/Na^+ was about 1.5 in lamina in NaCl treatment, compared with >100 in the control. The K^+/Na^+ ratios in lamina differed significantly between genotypes, but not in petioles (P = 0.1). However, the K^+/Na^+ ratios in excised lamina in the control did not significantly differ from intact lamina (Fig. 2d, h). In contrast, the K^+/Na^+ ratios in excised petioles in the control were about 50% of intact petioles (Fig. 3d, h).

The NaCl treatment also decreased the Cl^-/Na^+ ratios in lamina and petioles about 80% relative to the control (Table 4). The Cl^-/Na^+ ratios in lamina did not significantly differ between genotypes (P = 0.1), but significant differences were observed in petioles ($P < 0.01$). The Cl^-/Na^+ ratios in excised lamina in the control did not significantly differ from intact lamina (Table 4). The Cl^-/Na^+ ratios in excised petioles of soybean in the control were about 50% of the intact petioles (Table 4).

3.3. Leaf gas exchange and leaf SPAD readings of excised leaves

After being excised and exposed to the control or NaCl treatment solution, photosynthesis (P_n), stomatal conductance (g_s) and transpiration rate (T) of both soybean and common bean had declined by 80% to 90%, relative to intact leaves (Fig. 4a, c, d, Table 5). However, the excised leaf system did not affect internal CO_2 concentration (C_i) in any genotype, relative to the intact lamina (Fig.4b). There were no significant differences between genotypes or treatments, and genotype × treatment interaction for P_n, g_s, T or C_i on day 1 or 4 of treatment.

Similarly, the excised leaves in both the control and NaCl treatment had reduced SPAD values after 2 days (Fig. 5, with significant differences between genotypes and treatments ($P < 0.01$), but no genotype × treatment interactions (P = 0.7). The SPAD values for lamina in the control and NaCl treatment decreased gradually with time, with about 10–20% greater reductions in the NaCl treatment that the control, and greater reductions in soybean Bunya and common bean than soybean Lee.

3.4. Leaf sap osmotic potential (Ψπ) in excised leaves

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Leaf sap osmotic potential ($\Psi\pi$) decreased (i.e. more negative) in both soybean and common bean in the control and NaCl treatment, more so in NaCl–treated lamina than the control lamina (Fig.6).

3.5. Tissue water content in excised leaves

Lamina and petiole water contents differed significantly between genotypes and treatments, but there was only genotype × treatment interaction for petiole water content ($P < 0.05$) (Fig. 7a, b). Tissue water content declined in the excised leaves relative to the initial values (Fig. 7a, b). Soybean Bunya and common bean had similar lamina water contents, which were higher than soybean Lee (Fig. 7a). Both soybean and common bean had 30–50% lower water contents in lamina than petioles. Common bean had higher petiole water content than soybean (Fig. 7b).

3.6. Relationships between net photosynthetic rate (P_n) and stomatal conductance (g_s), intercellular CO2 (C_i) and transpiration rate (T)

Regression analyses were undertaken to assess for possible relationships between P_n and g_s, C_i, and T on day 1 (Fig. 8) and day 4 (Fig. 9) of treatment. The P_n had positive relationships with g_s for all genotypes at both sampling times. The P_n had no relationship with C_i in soybean Bunya, negative relationship in soybean Lee, and a positive relationship in common bean after 1 day of treatment (Fig. 8). However, after 4 days of treatment, P_n had a negative relationship with C_i in soybean Bunya and common bean, but no relationship in soybean Lee (Fig. 9). P_n had a positive relationship with T in soybean Bunya and common bean, but no relationship in soybean Lee after both 1 day and 4 days of treatment.

3.7. Relationships between net photosynthetic rate (P_n) and ion concentrations in lamina

The regression analyses did not identify any relationships between P_n and ion concentrations (Na^+, Cl^- and K^+), K^+/Na^+ ratio or Cl^-/Na^+ ratio in lamina on day 4 of treatment (Fig. 11) or between P_n and lamina ion concentration on day 1 of treatment (when P_n was higher than after the longer time since excision) (Fig. 10).

4. DISCUSSION

4.1. NaCl stem/petiole infusion system and an excised leaf system were not suitable for assessing leaf tissue tolerance

The stem/petiole infusion trial experiment was unable to assess leaf tissue tolerance in soybean and common bean because the Na^+ concentrations in lamina remained low and did not result in a substantial flow of Na^+ and/or Cl^- into leaves (Table 2).

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In the excised leaf system, the excised leaves of all genotypes in both the control and NaCl treatment had low transpiration, even on day 1 following excision. On day 1, P_n of excised leaves, even in the controls, was only 40% of that of intact leaves, which decreased further on day 4 to only 20% of intact leaves. Despite Na^+ and Cl^- concentrations increasing in lamina subjected to NaCl solution, there was no relationship between P_n and ion concentration in any genotype. The low P_n in leaves, even in the absence of NaCl, would have contributed to the difficulty in detecting Na^+ specific responses. Hence, the excised leaf system requires further development (see suggestions below) to determine whether it would be a useful approach for evaluating leaf tissue Na^+ tolerance.

4.2. Methods had limitations, as NaCl did not enter stem or petiole in infusion system, and leaf transpiration ceased for excised leaves

In the excised leaf system, leaves almost stopped transpiring on day 1 of treatment, despite care being taken to avoid 'cavitation' in the xylem. The lamina was covered in plastic cling film to prevent evaporation at the time of excision, the cut petiole end was placed into a control solution immediately, and the petioles were trimmed by about 5 cm when under solution to remove any air bubbles inside the xylem at the distal end of the petiole. However, embolism might have been present or formed after trimming back the petioles, which would have impeded water flow into and along the petioles to the lamina. The transpiration rate on the first day was only 30% of that of intact leaves. Perhaps the humidity of the controlled–environment chamber (RH 75%) needed to be higher during the excisions, the transfer and for the initial period, or even the whole experiment period. Future experiments could test the system at higher levels of air humidity to gauge its success for maintaining function of excised leaves.

4.3. Ion concentrations in soybean and common bean lamina

Salt–tolerant soybean genotypes can control Na^+ entering leaves better than salt–sensitive genotypes, for plants with roots in saline conditions [11, 12]. Soybean accumulated most Na^+ and/or Cl^- in roots, and Na^+ and/or Cl^- remained relatively low in leaves [11]. However, in this chapter, the excised leaves lost the ability to regulate the movement of ions (Na^+, Cl^-, and K^+) to lamina. Therefore, Na^+ concentration in lamina and petioles in the excised leaf system of soybean and common bean subjected to 5 mM NaCl solution increased greatly, and over 4 days, the lamina Na^+ concentration was about 30% higher than that in plants with roots exposed to 120 mM NaCl for 23 days in other study [11]. In contrast, the Cl^- concentration was similar to that in plants subjected to 120 mM NaCl for 23 days [11]. In addition, the Na^+ concentrations in soybean lamina of excised leaves exposed to NaCl exceeded the leaf tissue critical concentration for toxicity (>0.5% or 217 μmol g^{-1} dry mass) [13], but the Cl^- concentrations did not reach the threshold level for Cl^- toxicity (<2.6–5.0% or 713–1407 μmol g^{-1} dry mass) [13]. Hence, Na^+

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might have caused ion toxicity in soybean lamina, but since all leaves (including controls) would also have been adversely affected by partial desiccation (low tissue water content) and the P_n was already low even in controls, no significant relationship with Na^+ or Cl^- concentrations in the lamina could be detected using the leaf excised system after 1 or 4 days of treatment (Fig. 10, 11).

For common bean, previous study reported Na^+ concentrations in roots greater than in leaves, and high Cl^- concentrations in both lamina and roots [14]; Na^+ and Cl^- in common bean leaves was also reported to be greater than in other plant parts [15]. In this research, common bean lamina subjected to 5 mM NaCl solution for 5 days in the excised leaf system had very high Na^+ concentrations, being about five times higher than those exposed 100 mM NaCl solution for 15 days (Fig 2). In contrast, common bean lamina subjected to 5 mM NaCl solution in the excised leaf system had about four times lower Cl^- concentrations than those subjected to 90 mM NaCl solution for 20 days [14]. Besides lamina subjected to 5 mM NaCl solution accumulated 183 mmol Na^+ and 54 mmol Cl^- per kg lamina water (calculated from Fig. 3.2), higher than the critical toxicity levels for Na^+ and Cl^- in common bean shoots (20-25 mmol Na^+ and 30-40 mmol Cl^- per kg shoot water) [16, 17]. The extremely high accumulation of Na^+ and Cl^- in lamina of NaCl–treated excised leaves indicates that common bean could not control the movement of Na^+ and Cl^- to lamina. However, as for soybean, P_n had no significant relationship with Na^+ or Cl^- concentration in lamina in the excised leaf system; again, P_n was very low in the non–saline control excised leaves (Fig.10, 11).

Interestingly, K^+ concentrations in excised lamina remained the same as that in intact leaves. The capacity of plants to maintain high K^+ and high K^+/Na^+ contributes to salt tolerance [18, 19]. In this research, the lamina of excised leaves could maintain K^+ concentrations, despite considerable reductions in K^+ concentrations in petioles. However, there was no significant relationship between P_n and lamina K^+ concentration.

4.4. Further consideration of impaired photosynthesis (P_n) in soybean and common bean

In this study, P_n in soybean declined severely soon after the leaves were excised and exposed to the control solution; it is then not surprising that P_n had no significant relationships with Na^+ or Cl^- for either of the two soybean genotypes in NaCl solution. In this research, P_n had a negative relationship with C_i in soybean Bunya and common bean after 4 days of treatment, and a positive relationship with T in both day 1 and day 4 of treatment (Fig. 9). Hence, the reduction in P_n in excised leaves of soybean Bunya and common bean might be due to damaged chloroplasts exposed to water deficit in the control and NaCl treatment. Other possible effects such as low K^+/Na^+ ratio might combine with the high ions in these leaves, which is detrimental to metabolic activities [20, 21]. In the present study, excised soybean leaves exposed to NaCl had a low

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K^+/Na^+ ratio (about 1.5) in lamina, which could have negatively impacted the photosystems and/or photosynthetic metabolism in soybean (Fig.2). Other experiments conducted in greenhouses or growth chambers [22, 23, 24] and the field [25] have reported that salt stress significantly reduced P_n, g_s and Chl fluorescence parameters in soybean, with g_s being a primary limiting factor for the reduction in P_n. Unfortunately, these experiments did not measure tissue ion concentrations.

Under salt stress, a decline in P_n is often due to stomatal limitations when g_s decreases, which reduces C_i and decreases P_n[14, 20]. In this research, the lamina C_i of common bean subjected to 5 mM NaCl on day 4 of treatment had not declined (Fig. 4). While P_n had a negative relationship with C_i in common bean (r^2= 0.86), the C_i ranged from 260 to 310 µmol mol^{-1}, whereas the tissue ions had reached the apparent toxic threshold, so there may have been a negative impact of ions on photosystems and photosynthetic metabolism in common bean.

5. CONCLUSIONS

The attempt to infuse NaCl into stems/petioles of soybean and common bean failed because significant Na^+ and/or $Cl^–$ did not enter the lamina. Despite significant amounts of Na^+ and $Cl^–$ entering the lamina in the excised leaf system, and lamina Na^+ concentrations exceeded apparent toxic levels for soybean, and Na^+ and $Cl^–$ reached apparent toxic levels in common bean, P_n in lamina did not have a relationship with any tissue ion concentrations. In addition, excised leaves had low P_n, even in the non–saline controls, while the transpiration rate was low and tissue water content significantly decreased, which was likely due to cavitation of xylem that would impede water flow along the petioles. It is possible that partial desiccation of tissues adversely impacted the photosystems and photosynthetic metabolism of excised leaves. In conclusion, the two approaches of NaCl infusion into stems or petioles and the excised leaf system with exposure to NaCl need to be improved before they can be used to test for tissue tolerance in (sub)tropical grain legumes.

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FIGURES

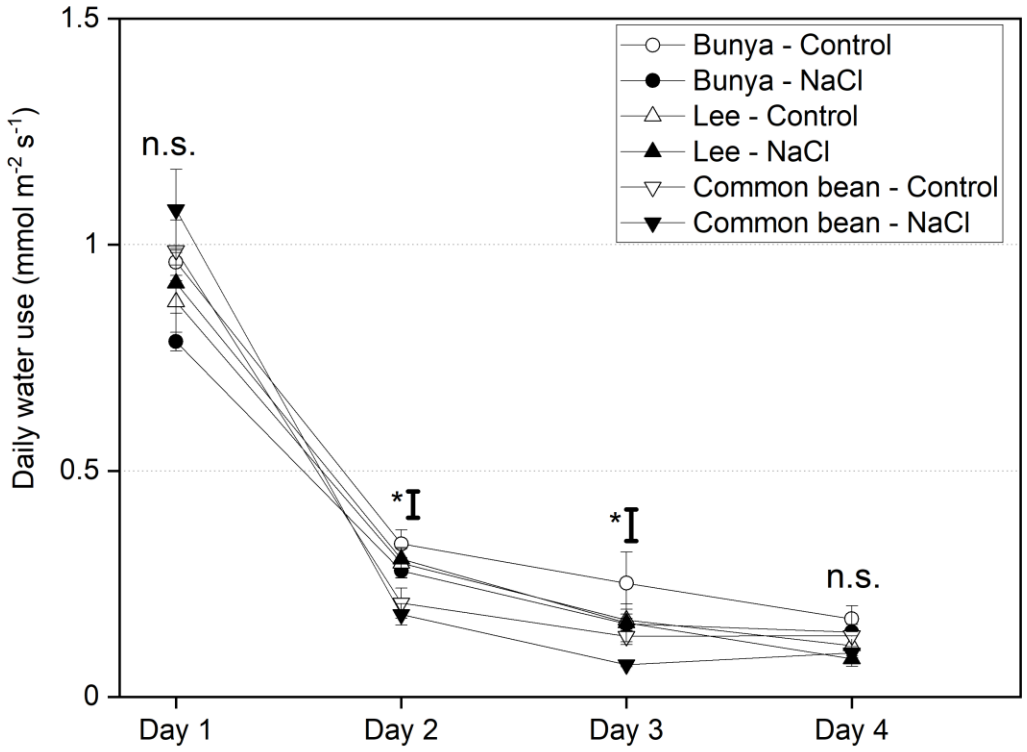

Fig. 1: Daily water use by leaves of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) when excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. The composition of the basal treatment solution is in Table 1. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). Daily water use was measured from 10 am to 10 am for each 24 h period. Values are means ± SE (n = 4). The probability levels for two–way ANOVA for genotype treatment means within each species are indicated by * P< 0.05 and n.s. = not significant. Bars represent least significant differences (LSD) at P = 0.05. The probability levels and LSD (5%) for genotype × treatment × day of treatment interaction are in Table 2.

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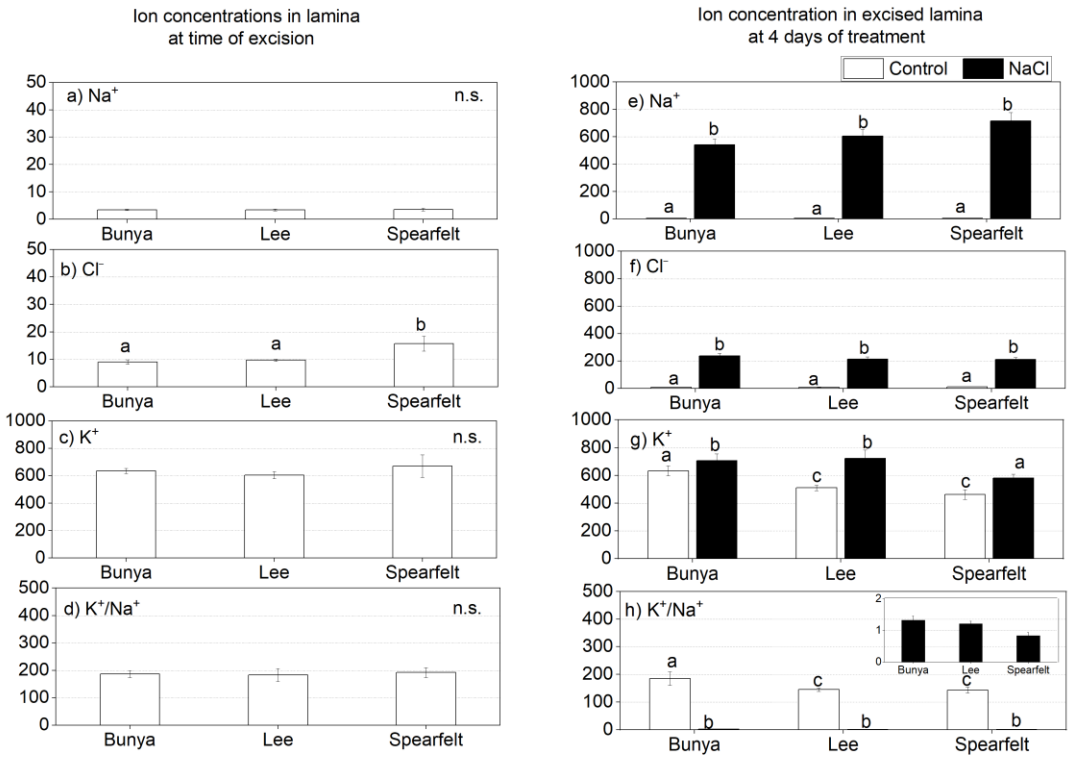

Fig. 2: Na^+, Cl^-, K^+ concentrations (µmol g^{-1} dry mass) and K^+/Na^+ ratio in lamina of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) at (a, b, c, d) excision (initial) and (e, f, g, h) after leaves wereexcised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. The composition of the basal treatment solution is in Table 1. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). Values are means ± SE (n = 4). Significant differences for (a, b, c, d) genotype means and (e, f, g, h) treatment means within each species are indicated by different letters (P = 0.05) and n.s. = not significant. The probability levels and LSD (5%) for two–way ANOVA for the data in each panel after 4 days of treatment are in Table 4. Note: the axis scale for the K^+/Na^+ ratio (d, h) differs from others. The small insets in panels h are the K^+/Na^+ ratio in lamina of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) after leaves wereexcised and the cut end of the petiole exposed to 5 mM NaCl for 4 days.

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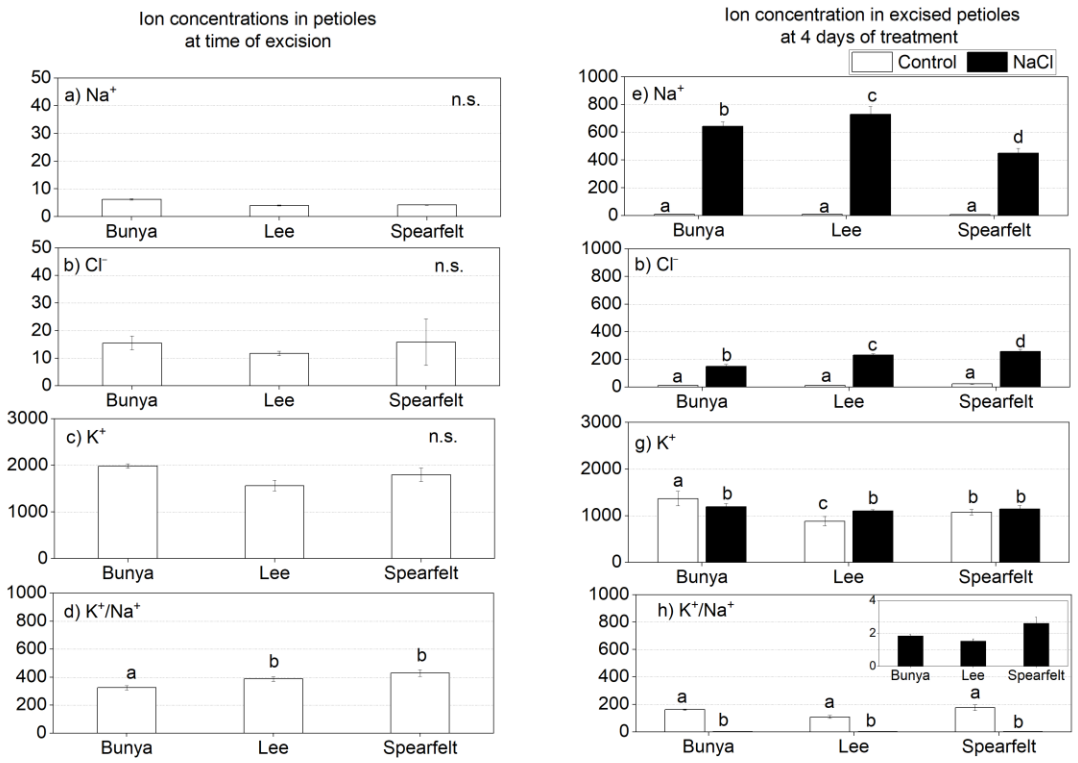

Fig. 3: Na^+, Cl^-, K^+ concentrations (µmol g^{-1} dry mass) and K^+/Na^+ ratio in petioles of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv Spearfelt) at (a, b, c, d) excision (initial) and (e, f, g, h) after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days of treatment (d, e, f). The composition of the basal treatment solution is in Table 1. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). Values are means ± SE (n = 4). Significant differences for (a, b, c, d) genotype means and (e, f, g, h) treatment means within each species are indicated by different letters (P = 0.05) and n.s. = not significant. The probability levels and LSD (5%) for two–way ANOVA for the data in each panel after 4 days of treatment are in Table 4. Note: the axis scale for K^+ concentration (c, g) differs from others. The small insets in panels h are the K^+/Na^+ ratio in petioles of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) after leaves wereexcised and the cut end of the petiole exposed to 5 mM NaCl for 4 days.

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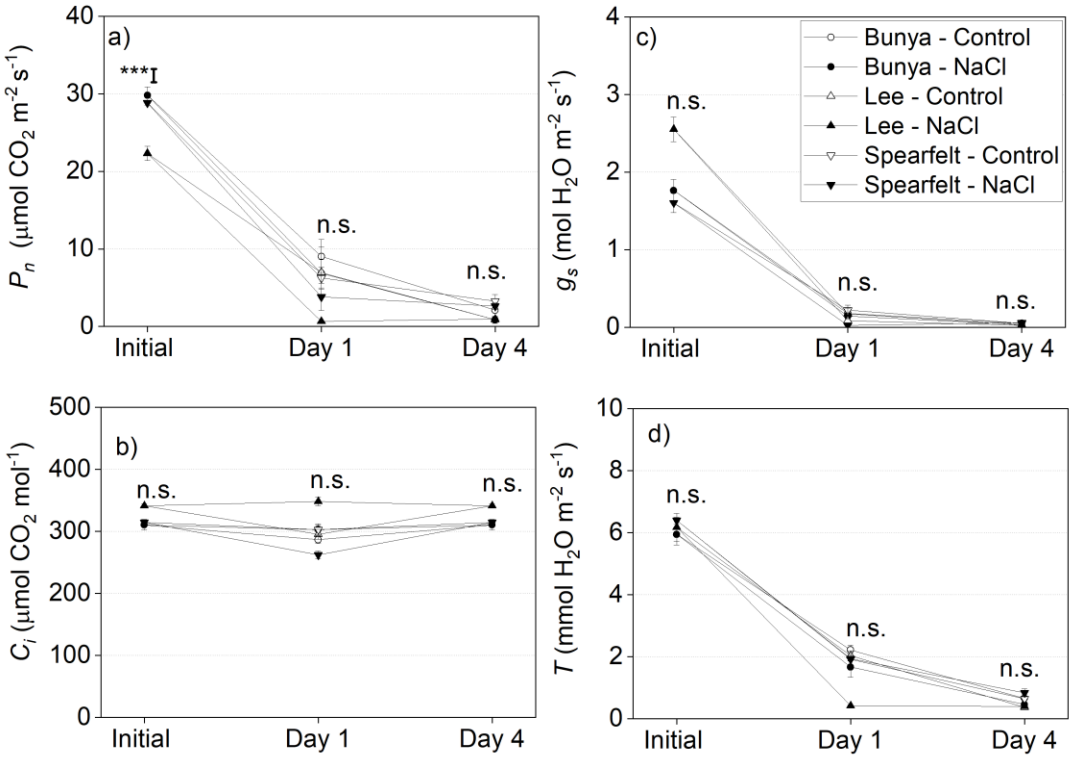

Fig. 4: Net photosynthetic rate (a) (P_n), (b) sub-stomatal CO_2 (internal CO_2concentration, C_i), (c) stomatal conductance (g_s), and (d) transpiration rate (T) of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) at the time of excision (initial) and after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. The composition of the basal treatment solution is in Table 1. Leaves were excised from 18-day-old plants grown in aerated nutrient solution, and the cut end of the petiole exposed to treatments in a controlled environment room (12 h light, 28/22±2°C, RH 75%). P_n was measured on leaves immediately prior to excision (initial) and after 1 and 4 days of treatment after being exposed to light for 30 minutes following the night-time dark period at photosynthetically active radiation of 1500 µmol photons m^{-2} s^{-1}, CO_2 concentration of 400 µmol mol^{-1}, leaf chamber temperature of 28° C and 60–70% relative humidity. Values are means ± SE (n = 4). The probability levels for two–way ANOVA for genotype means within each day are: *** P< 0.001 and n.s. = not significant. Bars represent least significant differences (LSD) at P = 0.05. The probability levels and LSD (5%) for genotype × treatment × day of treatment interaction at P = 0.05 are in Table 5. Note: the axis scales differ for (a) P_n, (b) g_s, (c) C_i, and (d) T.

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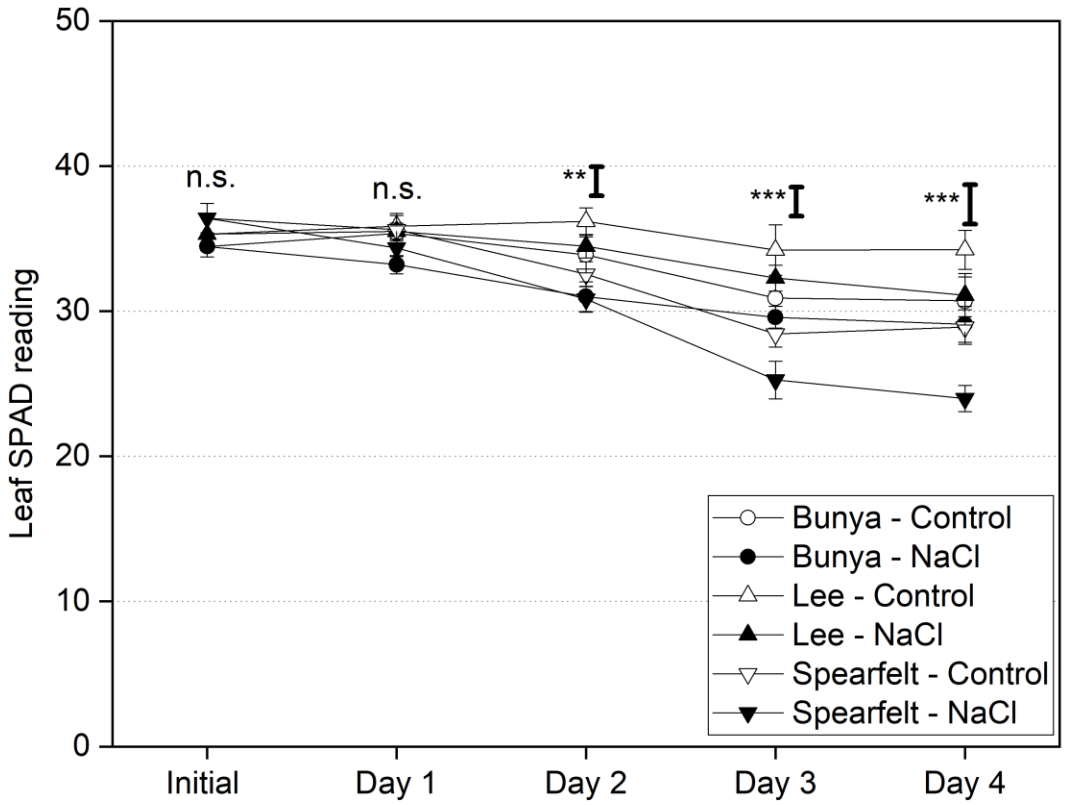

Fig. 5: Daily leaf SPAD reading of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) at the time of excision (initial) and after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. The composition of the basal treatment solution is in Table 1. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). SPAD values were measured at time of excision (initial) and every day of treatment between 09:00 and 14:00h. Values are means ± SE (n = 4). The probability levels for the two–way ANOVA for genotype means within each day are: ** P< 0.01, *** P< 0.001, and n.s. = not significant. Bars represent least significant differences (LSD) at P = 0.05. The probability levels and LSD (5%) for genotype × treatment × day of treatment interaction at P = 0.05 are in Table 5.

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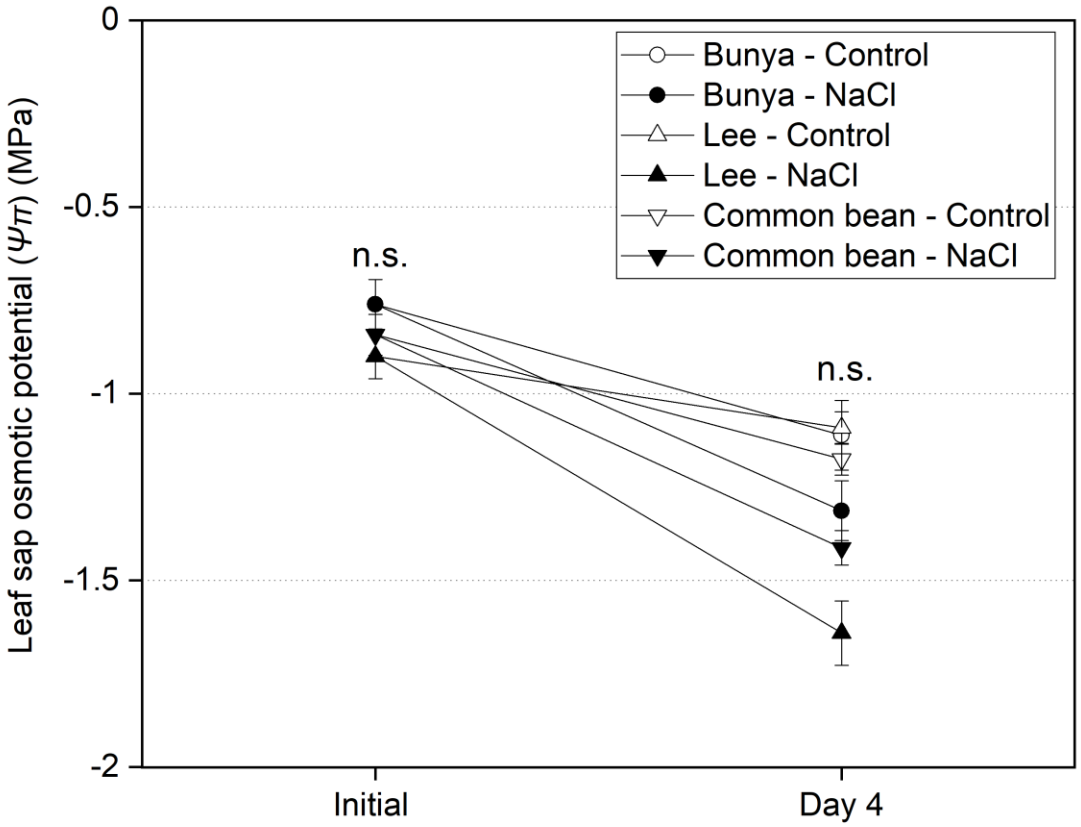

Fig. 6: Leaf sap osmotic potential (Ψπ) of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) at the time of excision (initial) and after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. The composition of the basal treatment solution is in Table 1. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). Values are means ± SE (n = 4). The probability levels for two–way ANOVA for genotype means within each day are: n.s. = not significant.

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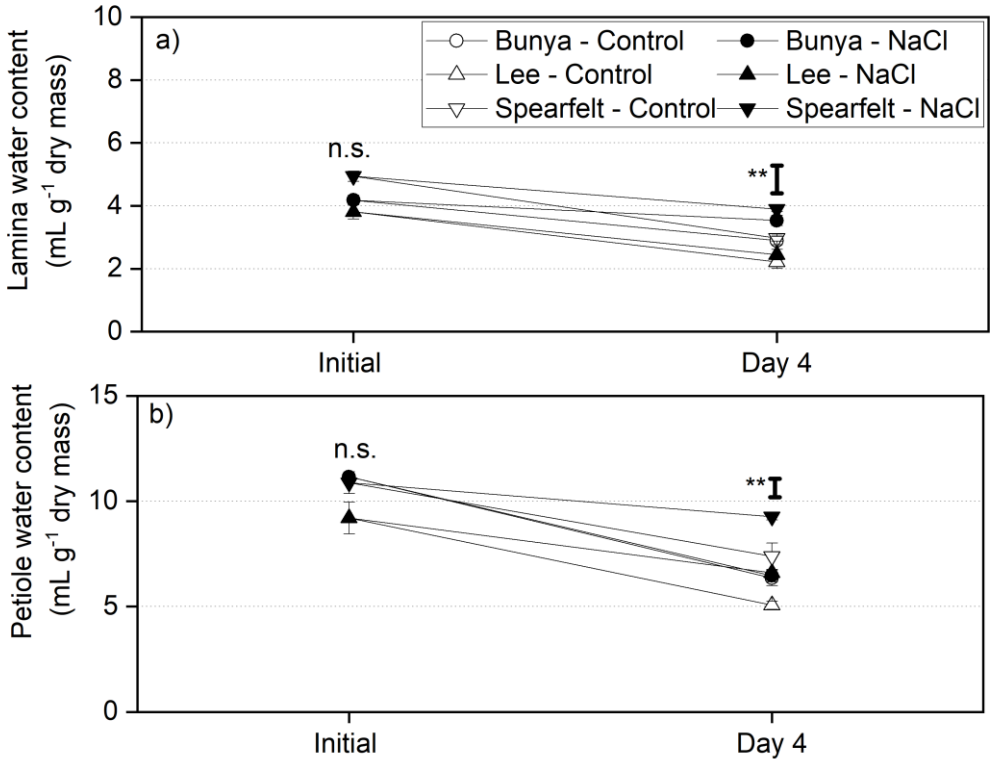

Fig. 7: Tissue water content of soybean (G. max cvs. Lee and Bunya) and common bean (P. vulgaris cv. Spearfelt) at the time of excision (initial) and after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. The composition of the basal treatment solution is in Table 1. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). Values are means ± SE (n = 4). The probability levels for two–way ANOVA for genotype means within each day are: ** P< 0.01 and n.s. = not significant. Bars represent least – significant differences (LSD) at P = 0.05.

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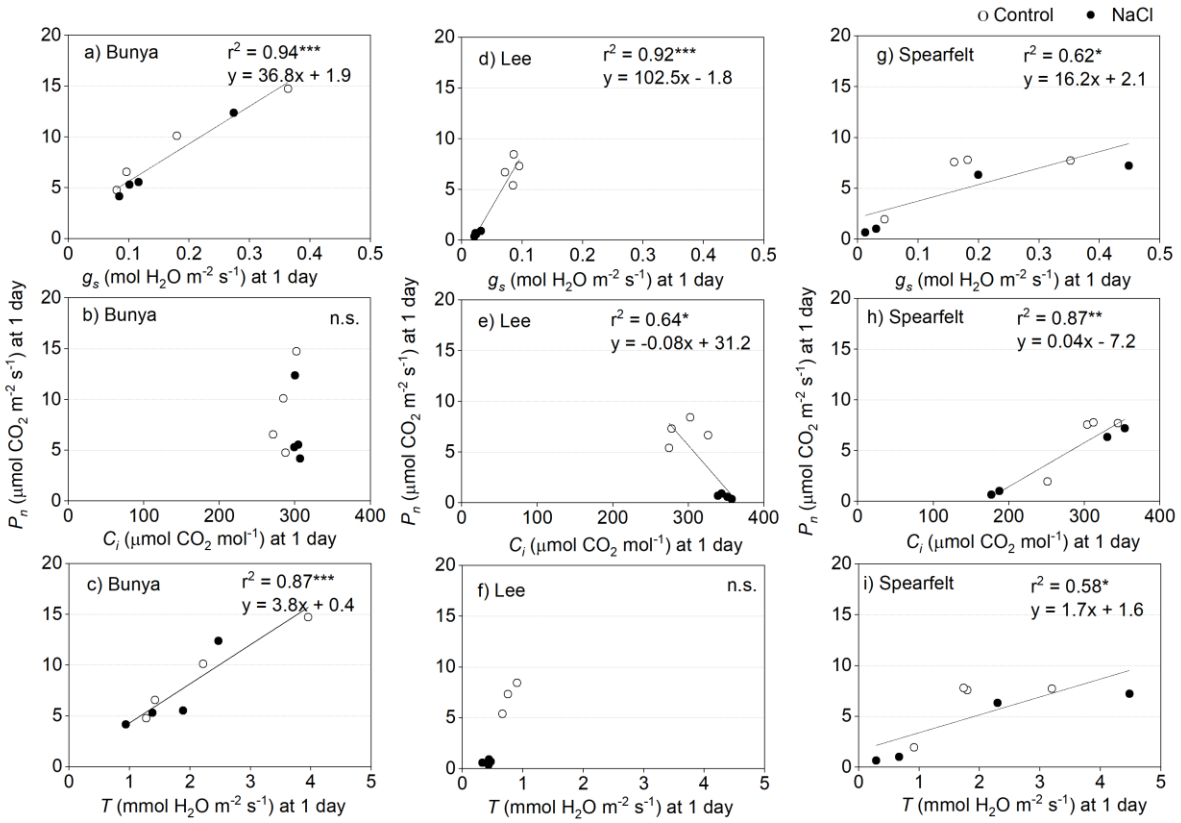

Fig. 8: Scatter plots of net photosynthesis rate (P_n) and stomatal conductance (g_s), sub-stomatal CO_2 (internal CO_2concentration, C_i), and transpiration rate (T) of (a, b, c) soybean (G. max cv. Bunya), (d, e, f) soybean (G. max cv. Lee) and (g, h, i) common bean (P. vulgaris cv. Spearfelt) after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 1 day of treatment. The composition of the basal treatment solution is in Table 1. Leaves were excised from 18–day–old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). P_n was measured on leaves after 1 day of treatment after being exposed to light for 30 minutes following the night-time dark period at photosynthetically active radiation of 1500 µmol photons m^{-2} s^{-1}, CO_2 concentration of 400 µmol mol^{-1}, leaf chamber temperature of 28° C and 60–70% relative humidity. Each value is an individual replicate and each replicate is a leaf excised from a plant grown in a different pot. * The probability levels are: P< 0.05, ** P< 0.01, *** P< 0.001, and n.s. = not significant.

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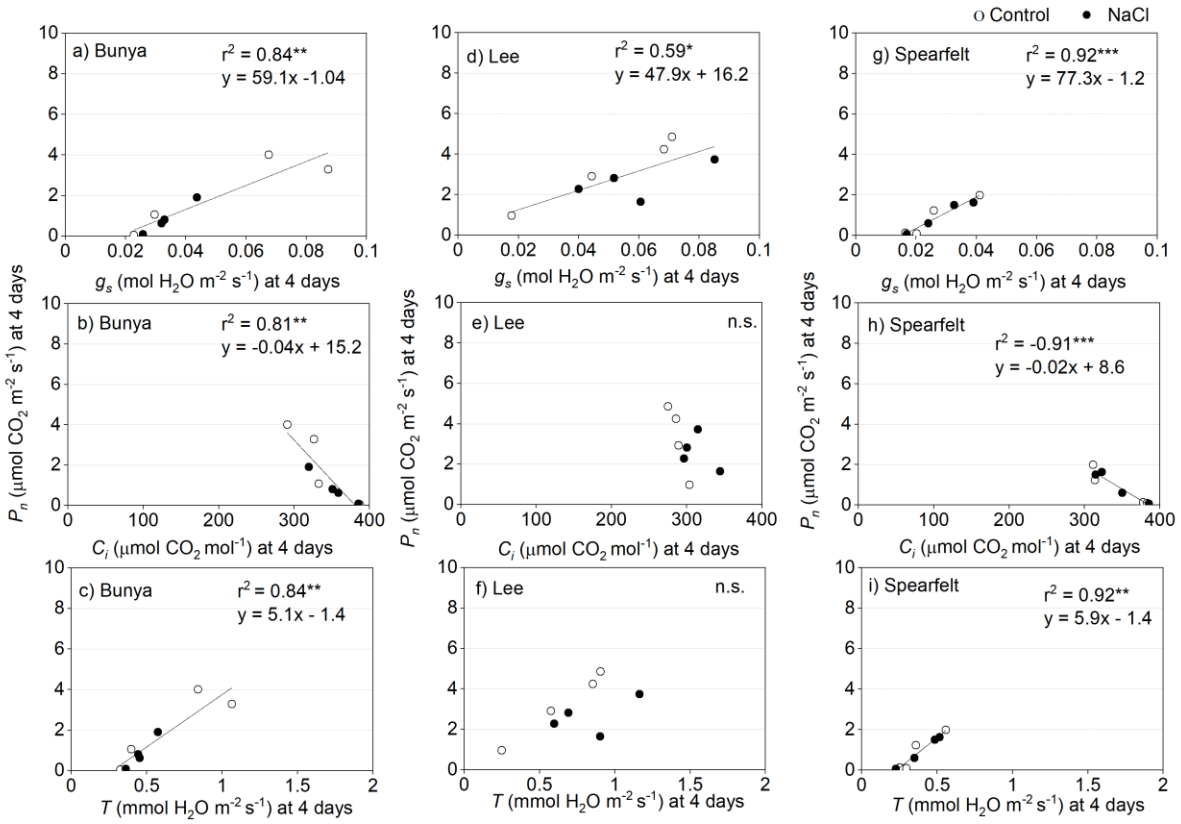

Fig. 9: Scatter plots of net photosynthesis rate (P_n) and stomatal conductance (g_s) and sub-stomatal CO_2 (internal CO_2concentration, C_i) of (a, b, c) soybean (G. max cv. Bunya), (d, e, f) soybean (G. max cv. Lee) and (g, h, i) common bean (P. vulgaris cv. Spearfelt) after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. Leaves was excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). P_n was measured on leaves after 4 days of treatment after being exposed to light for 30 minutes following the night-time dark period at photosynthetically active radiation of 1500 µmol photons m^{-2} s^{-1}, CO_2 concentration of 400 µmol mol^{-1}, leaf chamber temperature of 28° C and 60–70% relative humidity. Each value is an individual replicate and each replicate is a leaf excised from a plant grown in a different pot. The probability levels are: * P< 0.05, ** P< 0.01, *** P< 0.001, and n.s. = not significant.

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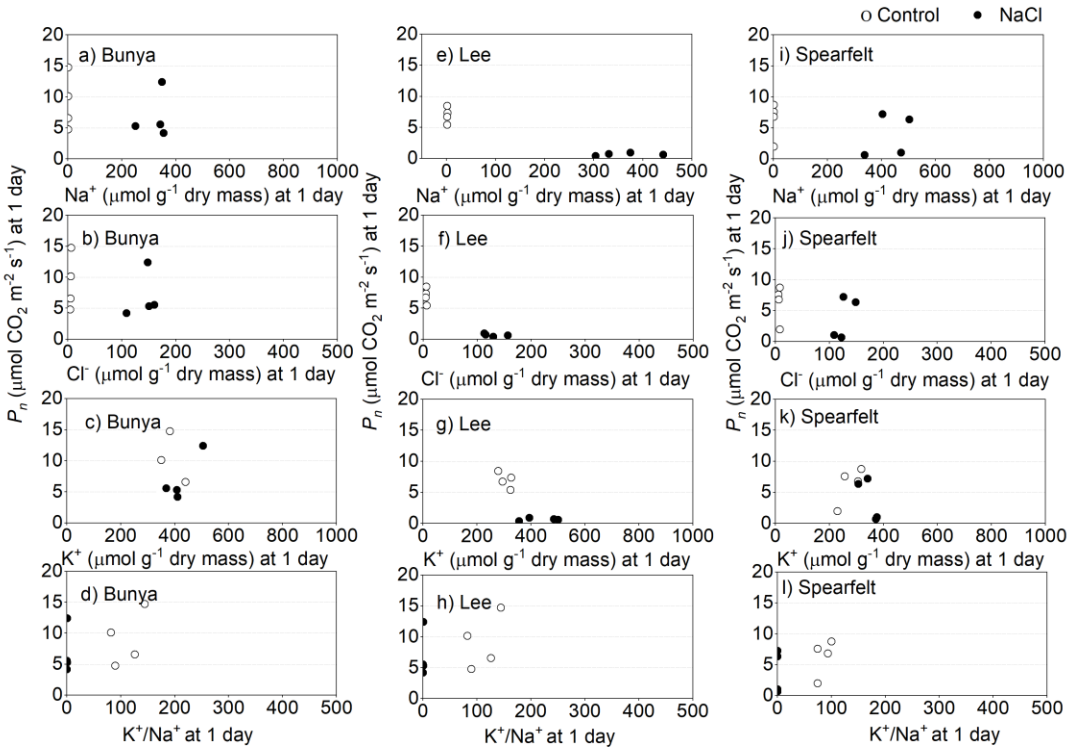

Fig. 10: Scatter plots of net photosynthesis rate (P_n) against Na^+, $Cl^–$, K^+, and K^+/Na^+ ratio of (a, b, c, d) soybean (G. max cv. Bunya), (e, f, g, h) soybean (G. max cv. Lee) and (i, j, k. l) common bean (P. vulgaris cv. Spearfelt) after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 1 day (Na^+, $Cl^–$, K^+, and K^+/Na^+ ratio calculated from daily water intake in Fig. 2 and ion concentration in lamina in Fig. 3). Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22±2°C, RH 75%). P_n was measured on leaves after 1 day of treatment (1 day) y after being exposed to light for 30 minutes following the night-time dark period at photosynthetically active radiation of 1500 µmol photons $m^{–2}$ $s^{–1}$, CO_2 concentration of 400 µmol $mol^{–1}$, leaf chamber temperature of 28° C and 60–70% relative humidity. Each value is an individual replicate and each replicate is a leaf excised from a plant grown in a different pot. At the 0.05 level, the slopes of all graphs do not significantly differ from zero.

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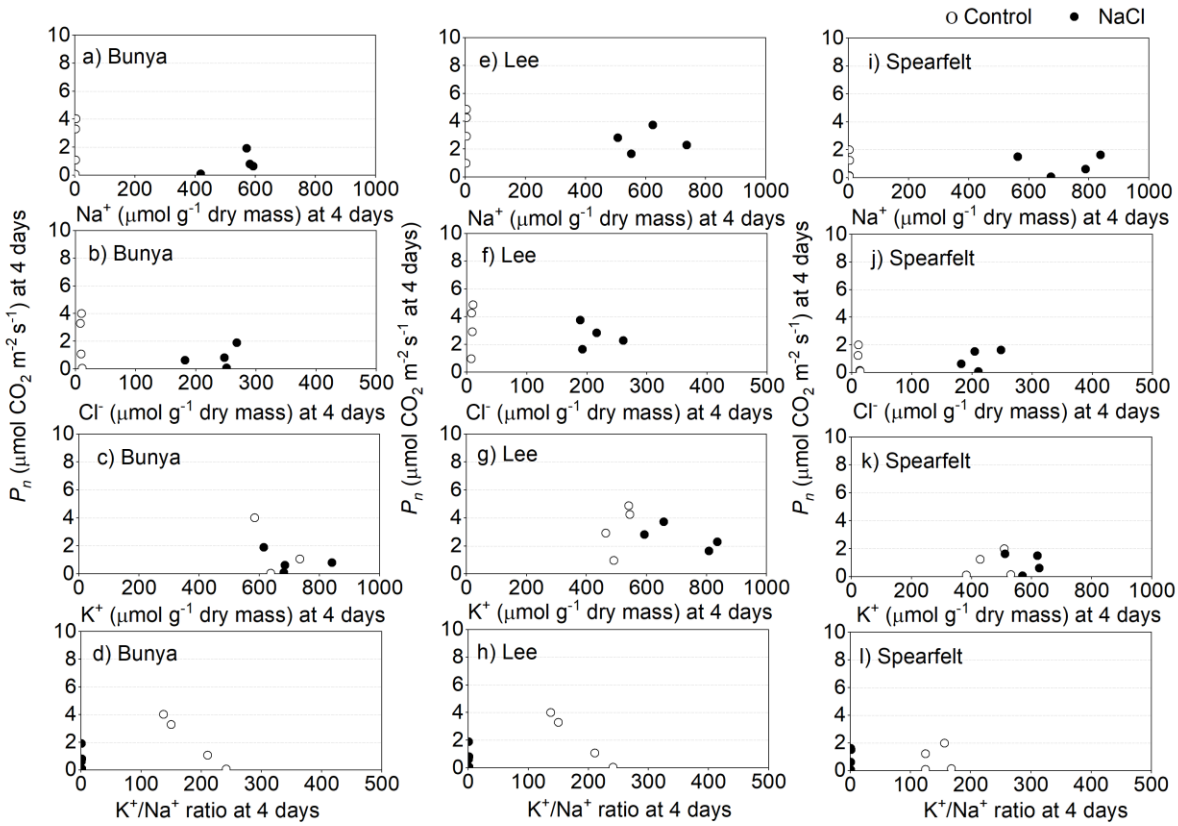

Fig. 11: Scatter plots of net photosynthesis rate (P_n) Na^+, Cl^-, K^+ concentrations and K^+/Na^+ ratio of (a, b, c, d) soybean (G. max cv. Bunya), (e, f, g, h) soybean (G. max cv. Lee) and (i, j, k. l) common bean (P. vulgaris cv. Spearfelt) after leaves were excised and the cut end of the petiole exposed to control (non–saline) and 5 mM NaCl for 4 days. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). P_n was measured on leaves after 4 days of treatment (4 days) after being exposed to light for 30 minutes following the night-time dark period at photosynthetically active radiation of 1500 µmol photons m^{-2} s^{-1}, CO_2 concentration of 400 µmol mol^{-1}, leaf chamber temperature of 28° C and 60–70% relative humidity. Each value is an individual replicate and each replicate is a leaf excised from a plant grown in a different pot. At the 0.05 level, the slopes of all graphs are not significantly different from zero.

Table 1: Ion concentrations, salts used and osmotic potential of the treatments in a complete basal (non-saline control) nutrient solution.

	Ψπ	Ion concentrations (mM)									
	(MPa)	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	NO_3^-	SiO_3^-	NH_4^+	$H_2PO_4^-$
Leaf and stem injection											
Control											
	-0.05	0.2	0.1	5.0	5.0	0.4	5.4	4.4	0.1	0.6	0.2
NaCl (50 mM)											
	-0.3	50.2	50.1	5.0	5.0	0.4	5.4	4.4	0.1	0.6	0.2
Leaf excised system											
Control											
	–0.005	0.02	0.005	0.5	0.5	0.04	0.54	0.44	0.01	0.06	0.02
NaCl (5 mM)											
	–0.025	5.01	5.0025	0.25	0.25	0.02	0.27	0.22	0.005	0.03	0.01

[1]For the basal nutrient solution, the macronutrient concentrations are shown in the table (Control). The micronutrients in all solutions were (µM): 10 Fe–sequestrene, 2.50 HBO_3^{2-}, 0.20 Mn^{2+}, 0.20 Zn^{2+}, 0.05 Cu^{2+}, 0.05 MoO_4^{2-}, 0.10 Ni^{2+}. The solution was buffered with 1.0 mM MES (2-[N-morpholino] ethane sulfonic acid) and adjusted to pH 6.5 using KOH.

Table 2: Na^+, Cl^-, K^+ concentration and K^+/Na^+, and Cl^-/Na^+ ratio in lamina of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) after injecting 50 mM NaCl or DI water in petioles or stems for 5 days. The injection was conducted on 18-day-old plants grown in aerated nutrient solution. Values are means ± SE (n = 4). The probability levels for two–way ANOVA was used to compare genotype (G), treatment (T), and (G × T) effects (* P<0.05, ** P < 0.01, *** P < 0.001, and n.s. = not significant).

		Na^+ (µmol g^{-1} dry mass)	K^+ (µmol g^{-1} dry mass)	Cl^- (µmol g^{-1} dry mass)	K^+/Na^+ ratio	Cl^-/Na^+ ratio
Petiole injection						
Bunya	50 mM NaCl	161.8±54.9	1170.3±65.9	73.3±4.4	115.2±16.9	2.6±1.4
	DI water	26.6±8.8	1080.5±183.5	50.75±11.0	86.5±13.8	5.6±2.9
Lee	50 mM NaCl	35.1±7.4	869.5±142.4	24.4±7.3	40.6±6.0	2.5±0.1
	DI water	38.7±6.8	709.1±144.3	104.1±7.9	18.3±2.2	3.1±1.1
Spearfelt	50 mM NaCl	25.9±8.9	1137.7±106.5	123.3±27.9	52.1±13.4	5.6±1.6
	DI water	29.7±5.3	1272.6±191.8	42.7±14.3	56.0±10.7	1.8±0.5
LSD (5%)	G	n.s.	n.s.	n.s.	n.s.	n.s.
	T	n.s.	n.s.	n.s.	n.s.	n.s.

	G × T	n.s.	n.s.	n.s.	n.s.	n.s.
Stem injection						
Bunya	50 mM NaCl	4.5±0.4	887.3±52.5	16.8±1.0	197.5±7.9	3.8±0.3
	DI water	2.4±0.4	956.0±131.7	13.5±0.9	403.3±43.3	6.0±1.4
Lee	50 mM NaCl	3.4±0.3	605.6±25.9	9.7±0.4	183.7±23.3	2.9±0.4
	DI water	36.9±8.4	789.3±97.5	94.4±21.7	29.4±8.8	2.8±0.5
Spearfelt	50 mM NaCl	11.2±1.1	585.3±57.7	47.7±3.5	148.4±13.4	9.9±1.5
	DI water	6.8±1.8	732.3±45.5	20.4±3.5	142.8±23.2	4.1±1.8
LSD (5%)	G	n.s.	n.s.	67*	116*	n.s.
	T	n.s.	n.s.	46.7*	n.s.	n.s.
	G × T	n.s.	n.s.	55.8***	n.s.	n.s.

Table 3: $LSD_{0.05}$ values and probability levels (P-values; * P< 0.05, ** P< 0.01, *** P< 0.001, n.s.=not significant) of the two-way ANOVA for ion concentrations (Na^+, Cl^-, K^+),and K^+/Na^+ and Cl^-/Na^+ in lamina and petioles of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) after leaves were excised and the cut end of the petiole exposed to control (non-saline) and 5 mM NaCl for 4 day. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22 ± 2°C, RH 75%). Values are means ± SE (n = 4). The probability levels for two–way ANOVA was used to compare genotype (G), treatment (T), and (G × T) effects (* P<0.05, ** P < 0.01, *** P < 0.001, and n.s. = not significant).

	Na^+ (µmol g^{-1} dry mass)	**K^+ (µmol g^{-1} dry mass)**	**Cl^-(µmol g^{-1} dry mass)**	**K^+/Na^+ ratio**	**Cl^-/Na^+ ratio**
Lamina					
G	n.s.	83**	n.s.	n.s.	n.s.
T	62***	67***	20***	19***	0.46***
G × T	n.s.	n.s.	n.s.	n.s.	0.8*
Petiole					
G	62***	189*	20***	20**	0.18***
T	50***	n.s.	16***	16***	0.14***
G × T	87**	n.s.	28***	29**	0.25***

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Table 4: Cl^-/Na^+ ratio in lamina and petioles of soybean (G. max cvs. Bunya and Lee) and common bean (P. vulgaris cv. Spearfelt) after leaves were excised and the cut end of the petiole exposed to control (non-saline) and 5 mM NaCl for 4 days. Leaves were excised from 18-day-old plants grown in aerated nutrient solution and exposed to treatments in a controlled environment room (12 h light, 28/22±2°C, RH 75%). Values are means ± SE (n = 4). Least significant differences (LSD) for treatment means within each genotype, treatment and genotype × treatments interaction are at the bottom of each column data (P = 0.05). The probability levels for one, and two–way ANOVA was used to compare genotype (G), treatment (T), and (G × T) effects (* P<0.05, ** P < 0.01, *** P < 0.001, and n.s. = not significant).

		Cl^-/Na^+ ratio in lamina	**Cl^-/Na^+ ratio in petioles**
At time of excision			
Bunya		2.6±0.1	2.5±0.4
Lee		2.9±0.4	2.9±0.3
Spearfelt		4.7±4.5	0.6±11.1
LSD (5%)		0.5*	0.01́**
Excised and after 4 days of treatment			
Bunya	Control	2.9±0.48	1.4±0.07
	NaCl	0.5±0.06	0.2±0.02
Lee	Control	2.6±0.24	1.3±0.06
	NaCl	0.4±0.03	0.3±0.01
Spearfelt	Control	3.9±0.38	3.5±0.17
	NaCl	0.3±0.03	0.6±0.07
LSD (5%)	G	n.s.	0.2***
	T	0.4***	0.1***
	G × T	n.s.	0.2***

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Table 5: $LSD_{0.05}$ values and probability levels (P-values; * P < 0.05, ** P< 0.01, *** P< 0.001 n.s.=not significant) for net photosynthetic rate (P_n), stomatal conductance (g_s), sub-stomatal CO_2 (internal CO_2concentration, C_i), leaf respiration rate (T), daily water use and SPAD value in soybean (G. max cvs. Bunya and Lee) and common bean (Phaseolus vulgaris cv. Spearfelt) at the time of excision and after excision exposed to control (non-saline) and 5 mM NaCl for 4 days of treatment. Leaves were excised from 18-day-old plants grown in aerated nutrient solution, before being exposed to the treatments. Gas exchange was measured on leaves immediately prior to excision (initial) and after 1 and 4 days of treatment after being exposed to light for 30 minutes following the night-time dark period at photosynthetically active radiation of 1500 µmol photons m^{-2} s^{-1}, CO_2 concentration of 400 µmol mol^{-1}, leaf chamber temperature of 28° C and 60–70% relative humidity. Values are means ± SE (n = 4). The probability levels for two, and three–way ANOVA was used to compare genotype (G), treatment (T), day of treatment (D), and (G × T), (G × D), (T × D), and (G × T × D) effects (* P<0.05, ** P < 0.01, *** P < 0.001, and n.s. = not significant).

	***P_n*(µmol CO_2 m^{-2} s^{-1})**	***g_s*(mol H_2O m^{-2} s^{-1})**	***C_i* (µmol CO_2 mol^{-1})**	***T* (mmol H_2O m^{-2} s^{-1})**	**Daily water use (mmol m^{-2} s^{-1})**	**SPAD value**
G	1.5***	ns.	n.s.	n.s.	n.s.	0.9***
T	1.2*	n.s	n.s.	n.s.	n.s.	0.7***
D	1.5***	0.3***	17.8**	0.5***	0.1***	1.1***
G × T	n.s.	n.s		n.s.	n.s.	n.s.
G × D	2.6***	n.s	30.9**	n.s.	0.2*	2.1***
T × D	2.1*	n.s	n.s.	n.s.	n.s.	n.s.
G × T × D	n.s.	n.s	n.s.	n.s.	n.s.	n.s.