Some rootstocks improve pepper tolerance to mild salinity through ionic regulation

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Some rootstocks improve pepper tolerance to mild salinity through ionic regulation

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ABSTRACT

Grafting has been proposed as an interesting strategy that improves the responses of pepper cultivars under salinity. However, very little is known about the physiological mechanisms underlying the increased tolerance provided by rootstocks on the scions. With this aim, we performed this experiment. The commercial 'Adige' pepper cultivar was grafted onto three different pepper accessions that showed differential salt tolerance (accessions 5, 12 and 14). Responses to salinity (40mM NaCl) were studied for 30 days by determining water relations, mineral content, proline accumulation, photosynthetic parameters, nitrate reductase activity and antioxidant capacity. The responses observed were depended on salinity treatment duration and the rootstock used. Higher salt tolerance was achieved when the 'Adige' cultivar was grafted onto the 12 genotype, which allowed not only lower Na^+ and Cl^- accumulation in the scion, but also ion selectivity maintenance, particularly Na^+/K^+ discrimination. These traits led to a minor negative impact on photosynthesis, nitrate reductase activity and lipid peroxidation in grafted scion leaves. This work suggests that using tolerant pepper rootstocks that maintain the scion's ion homeostasis is a promising strategy to provide salinity tolerance and can consequently improve crop performance when faced with farmland salinity.

Key words: Graft; NaCl; Ions; Pepper; Photosynthesis; Water relations

1. Introduction

Grafting plants onto tolerant rootstocks is one of several approaches that can reduce the impact of salinity [1], one of the most serious problems of horticultural crops in arid and semi-arid regions [2].

Pepper is one of the most important vegetable crops in these areas and is considered sensitive to salinity [3], even though salt tolerance can vary between pepper genotypes [4]. Some pepper accessions have been identified as salinity-tolerant and have been successfully used as pepper rootstocks under saline conditions [5].

Several studies have been conducted in tomato and melon to elucidate the mechanisms involved in increased salinity tolerance of grafted plants. This increased tolerance of grafted plants is generally associated with their capacity to exclude or retain and/or accumulate toxic ions, Na^+ and Cl^- in rootstock roots, thus limiting their transport to leaves rather than through the synthesis of osmotically active metabolites or the induction of antioxidant systems [6–8]. Other authors have indicated that influence of rootstock on the salt tolerance of the scion is due to a more efficient control of stomatal functions (changes in stomatal regulation and water relations), which indicate that the grafting incision may alter hormonal signalling between roots and shoots [9]. In other cases, this raised tolerance has been explained by the re-establishment of ionic homeostasis [10].

Nevertheless, the mechanism of resistance against salinity in grafted plants displays great complexity in association with specific rootstock/scion interactions [11,12], and can vary among species. As far as we know, very few studies of this type have been conducted in pepper to elucidate whether or not

salt tolerance conferred by rootstocks is also due to exclusion and/or retention mechanisms, as in tomato or melon given their better capacity to alleviate the toxic effects of salts or other processes; e.g., maintenance or water relations or antioxidant capacity. Guifrida et al. [13] found that stunted growth due to salinity was attenuated in pepper-grafted plants when compared to non-grafted plants associated primarily with reduced uptake of salt ions and, therefore, with a lower concentration of these ions in the grafted plants instead of maintaining leaf turgor by osmotic adjustments.

To answer this question, in previous experiments we selected three pepper accessions with different degrees of salinity tolerance [5] under mild salt stress. In this study, we aimed to identify the physiological responses to salinity stress involved in increased tolerance of pepper-grafted plants using these accessions as rootstocks and to elucidate if mechanisms to tolerance are related with the role of roots rootstocks in altering the stress perception by the scion. To fulfil these objectives, we discussed differences in pepper-grafted plants adaptation mechanisms in response to mild salt stress by comparing some physiological parameters: photosynthesis; lipid peroxidation levels; relative water content (RWC); proline concentration; osmotic potential (Ψ_S); ions concentration; nitrate reductase activity (NR). We present evidence that grafting plants onto appropriate (tolerant) rootstocks is a good tool against salinity stress, which is mediated mainly by reducing ionic toxicity to the scion.

2. Materials and methods

2.1. Plant material and greenhouse conditions

Based on previous studies, we selected three pepper accessions (wild types) with a different salinity tolerance [5]: 'ECU-973' of *Capsicum chinense* Jacq. (code 12) as being tolerant; 'BOL-58' of *Capsicum baccatum* L. var. *pendulum* (code 14) as being moderately tolerant; and 'Serrano' of *Capsicum annuum* L. (code 5) as being less tolerant. These accessions were chosen as rootstocks and the pepper cultivar 'Adige' (Lamuyo type, Sakata Seeds, Japan) was grafted onto these three pepper accessions in this study. Pepper seeds were sown on 1 December in 100-cell polystyrene trays filled with peat-based substrate and kept in a Venlo-type glasshouse. The graft was performed on 12 February using the tube-grafting method (cutting the growing tip of the rootstock at a 45º angle below the cotyledons, attaching to the scion, previously cut at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip).

One month after grafting, the root system of the plants was washed to clean the substrate and plants were placed in 5 L polyethylene pots covered with aluminium sheets. Pots were filled with a standard nutrient solution for pepper [14]. The electrical conductivity (EC) and pH of this nutrient solution was 1.7 dS m^{-1} and 6.5, respectively. Nutrient solution was added daily to compensate for uptake. After 7 days of leaving seedling plants to acclimatise to pots, salinity treatment was initiated by adding NaCl (40mM) to the nutrient solution to reach an EC of 5.2 dS m^{-1} NaCl.

Treatments were defined by two salinity levels (0 and 40mM NaCl) and four plant combinations: the cultivar 'Adige' grafted onto rootstock accessions 5, 12 and 14, and ungrafted 'Adige' plants were used as the controls.

The grafted combinations (cultivar/rootstock) were labelled as A/5, A/12 and A/14. The layout was completely randomised with three replications per combination and six plants per replication.

All the physiological measurements were taken on 14 (T1) and 28 (T2) days after NaCl addition on fully expanded mature leaves (third or fourth leaf from the shoot apex).

During the culture, plants were grown in a Venlo-type greenhouse under natural light conditions (610-870 μmol m^{-2} s^{-1}), temperature ranges were 21-24ºC, and relative humidity was 52-72%.

2.2. Water relations

The osmotic potential of leaf sap (Ψ_s in MPa) was measured with an osmometer (Digital osmometer, Wescor, Logan, USA). Two independent determinations were made on each replicate and plant combination, obtained from six plants per treatment and combination at T1 and T2.

Leaves were tightly wrapped in aluminium foil, frozen in liquid nitrogen and stored at -80ºC. After thawing, sap was collected from syringes at 25ºC and placed in the osmometer. Osmolyte content (mmol kg^{-1}) was converted into MPa using the Van't Hoff equation [15].

Six other similar leaves from two independent plants of each plant combination, salinity treatment and replicate were collected to determine the (RWC) as (FW-DW)/(TW-DW) x 100, where FW is fresh weight, DW is dry weight, and TW is turgid weight [15].

2.3. *Ion analysis*

The leaves and roots collected at T1 and T2 for $n \geq 5$ samples of each treatment and plant combination were dried at 70ºC for 4 days. Dried samples were digested in a mixture at 70% of HNO_3-$HClO_3$ (2:1). Macronutrients (K^+, Ca^{2+}, Mg^{2+} and Na^+) were measured by ICP emission spectrometry (iCAP 6000, Thermo Scientific. Cambridge, United Kingdom).

The chloride concentration (Cl^-) in the dry plant material was extracted with 0.1N HNO_3 in 10% (v/v) acetic acid and was determined by potentiometric tritation with $AgNO_3$ in a chloride analyzer (Sherwood, MKII 926). The results were expressed as mg g^{-1} DW.

2.4. *Proline determination*

Proline content (mg g^{-1} DW) was determined as described by [16]. Leaf pepper tissue (0.05 g) was ground in 3% sulphosalicylic acid, the homogenate was filtered, and 0.75 mL of glacial acetic acid and 0.75 mL of ninhydrin reagent (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6N phosphoric acid) were added to an aliquot of the filtrate. The reaction mixture was boiled for 1 h, and readings were taken at a wavelength of 520 nm in a spectrophotometer. Three independent determinations were made in three different extracts obtained from 18 plants per treatment and combination (one leaf per plant, and six plants per extract).

2.5. *Photosynthetic activity and chlorophyll fluorescence*

The CO_2 fixation rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and

substomatal CO_2 concentration (C_i, μmol CO_2 mol^{-1} air) were measured in the steady state while maintaining plants at 1,000 μmol m^{-2} s^{-1} for 10-15 min and 400 ppm CO_2 with a LI-6400 (LI-COR, Nebraska, USA). Light curves were previously performed (data not shown) and A_N was saturated at 900 μmol m^{-2} s^{-1}. The gas exchange and fluorescence determinations were made from 9 am to 11 am (GMT). One measurement per plant was taken, and ten different plants were used (n=10) for each treatment (control and salinity stress) and plant combination.

2.6. Nitrate reductase activity

Nitrate reductase activity (EC 1.6.6.1) in leaves was determined *in vivo* following the methods described by [17,18]. Discs, 1 cm in diameter, were punched out of mature fresh leaves. Samples (200 mg) were suspended in a glass vial containing 10 mL of 100 mM potassium phosphate buffer (pH 7.5), 1% (v/v) *n*-propanol and 100 mM KNO_3. The glass vial was subjected 3 times to vacuum infiltration in order to induce anaerobic conditions in the incubation medium. Plant samples were incubated in a water bath at 30ºC for 60 min in the dark and were placed in a boiling water bath for 5 min to stop the enzymatic reaction. The nitrite released from the plant material was determined colorimetrically at 540 nm (spectrophotometer PerkinElmer, Lambda 25) by adding 0.02% (w/v) N-naphthylethylenediamine and 1% sulphanilamide. A standard curve with KNO_2 was prepared to calculate the amount of NO_2 that the samples contained. Sampling and replicates were used as described for proline determination.

2.7. Lipid peroxidation

Lipid peroxidation in leaves was estimated through malondialdehyde (MDA) determinations using the thiobarbituric acid reaction following the protocol reported by [19], and modified in [20]. The non-specific background absorbance reading at 600 nm was subtracted from the specific absorbance reading at 532 nm. The sampling and replicates used were those described for proline determination.

2.8. Statistical analyses

The results were subjected to a variance analysis (ANOVA; Statgraphics Centurion for Windows, Statistical Graphics Corp.). The mean comparisons were made using Fisher's least significance difference (LSD) test at $P < 0.05$. The data obtained in some measurement parameters were subjected to linear regression and analyses to identify the relationships between the parameters.

3. Results

3.1. Water relations

Plant water relations were assessed by the determination of RWC and Ψ_s (Figs. 1 and 2). No changes in RWC were observed in the experiment in any plant combination, except for ungrafted plants (Fig. 1A, B), where RWC diminished ($P< 0.05$) after salt treatment.

The Ψ_s of all the plant combinations reduced significantly ($P< 0.05$) under salinity at T1 and T2 (Fig. 2). At T1, no significant interaction was found. At T2, differences between treatments were greater in ungrafted and A/5 than in A/12 and A/14 ($P< 0.05$).

3.2. Ion partitioning

The Na^+ concentration in leaves and roots increased under NaCl (Fig. 3A) in all the plant combinations. The Na^+ concentration in leaves was higher in ungrafted and A/5 plants (Fig. 3A) if compared with A/12 and A/14 ($P<0.05$) at T1 and T2 under salinity. In general terms, the Na^+ concentration in the roots under salinity was higher than in leaves (Fig. 3B), with a lower concentration found in A/12 and A/14.

Chloride content was approximately 4 times higher than Na^+ in leaves. The Cl^- concentration in leaves (Fig. 3C) increased with a higher NaCl concentration and time exposure, but this incident did not occur in roots (Fig. 3D) and in none of the plant combinations. Ungrafted and A/5 obtained the highest Cl^- levels in leaves, whereas A/12 and A/14 plants showed a greater accumulation in roots ($P<0.05$) (Fig. 3D).

In general terms, a consistent K^+ content reduction trend was observed in leaves at T1 under saline conditions in all the plant combinations (Fig. 3E). This decrease occurred at T2 only in ungrafted and A/5 plants, but not in A/12 and A/14, where no significant differences in the K^+ levels were found if compared with their controls (Fig. 3E). In roots, a marked increase in K^+ content was observed in A/12 at T1 (Fig 3F). In contrast, the K^+ concentration at T2 did not change in A/5 and A/14 under salinity (Fig. 3F).

The Na^+/K^+ ratio increased significantly depending on salt application and the exposure time in the ungrafted and A/5 leaves (Fig. 3G). The lower values ($P<0.05$) in leaves were observed for 12/cultivar and 14/cultivar. In the root compartment (Fig. 3H) under salt treatment at T1, the Na^+/K^+ values increased

in ungrafted and A/5. At T2, the Na^+/K^+ ratio in roots lowered under salt conditions if compared to the values obtained at T1 in these plant combinations due to a sharp drop in the Na^+ content in roots at T2.

The Ca^{2+} (Fig. 4A) and Mg^{2+} levels (Fig. 4C) were similar in leaves for the tandem ungrafted and A/5 plants, with reduced plant exposure to NaCl (Fig. 4). In A/12 and A/14, the Ca^{2+} and Mg^{2+} concentrations in leaves showed minor variations between the control and treated samples (Fig. 4 A, C). In roots, the Mg^{2+} levels (Fig. 4D) lowered in all the plant combinations with time, while the Ca^{2+} levels lowered in A/5 at T1 and T2, but increased in ungrafted, A/12 and A/14 at T2 (Fig. 4B).

3.3. Proline content in leaves

Under the control conditions, no significant differences were found in the proline leaf content between plant combinations with time. Salinity gave rise to increased leaf proline content ($P<0.05$). This increase was similar for all the plants at T1 (Fig. 5A). At T2 (Fig. 5B) under 40mM NaCl, proline content substantially increased in ungrafted and A/5 if compared with their control values, but not in 12/cultivar and 14/cultivar ($P< 0.05$), which showed similar values to T1.

3.4. Gas exchange parameters

As shown in Figure 6, the A_N (Fig. 6A, B) and g_s (Fig. 6C, D) of the grafted plants did not differ from those of the ungrafted plants under the control conditions. The photosynthesis rate significantly lowered in all the plants

(P<0.05) in response to salt stress, except 12/cultivar at T2, when the A_N values did not significantly differ from those of the control (Fig. 6B).

A decrease in g_s under salt treatment was observed in all the plants (Fig. 6C, D). Significant differences were found for the ungrafted, A/5 and A/14 plants if compared to 12/A at T1 and T2. A minor decrease, but with a significant difference compared to its control, was noted for 12/cultivar.

Instantaneous carboxylation efficiency, estimated by the A_N/C_i ratio (Fig. 6E, F), reduced in ungrafted, A/5 and A/14 at T1 and T2. Interestingly at T2, minor differences were seen in the A_N/C_i values in A/12, followed by A/14, if compared to their controls, but no significant differences were observed between them.

3.5. Nitrate reductase activity in leaves

Salt stress resulted in diminished NR activity in leaves after 14 (Fig. 7A) and 28 (Fig 7B) days of mild NaCl treatment. Under salinity, the greatest NR activity at T1 and T2 was seen for A/12 plants, with significant differences (P< 0.05) if compared to ungrafted and A/5. Nevertheless, the inhibition percentages due to salt application at T2 were not associated with the NR control values: 74% for ungrafted, 50% for 5/cultivar, 22% for 12/cultivar and 32% for 14/cultivar (Fig. 7B).

3.6. Lipid peroxidation

At T1 (Fig. 8A), MDA content increased and significant differences were observed only in the ungrafted plants. After 28 days of salt exposure, lipid peroxidation increased significantly in the ungrafted and 5/cultivar plants

(P<0.05). It is noteworthy that no further MDA accumulation occurred in any of the plant combinations (Fig. 8B).

3.7. Relationship between osmotic potential, ions and proline concentrations and photosynthesis in leaves

Regression analyses were performed with the physiological study parameters. Only the significant linear relations that contribute to understanding tolerance mechanisms to salinity (Na^+, Cl^- and K^+ concentration and proline level *vs.* osmotic potential and A_N) are shown in Table 1.

At T1 and T2, the data gave an inverse linear relationship among Ψ_S and Na^+, Cl^- and proline, but a positive correlation with the K^+ level in leaves. Proline was the parameter that obtained the steepest slope values to modify Ψ_S.

A_N at T1 correlated negatively with the Na^+, Cl^- and proline concentrations, but not significantly only for the last parameter (P> 0.05). The regression analysis indicated inhibition of A_N with greater dependency of Na^+ and Cl^-. Nevertheless at T2, A_N lowered, which was due mainly to an increased proline concentration. Although A_N showed a positive dependency with the K^+ levels, no significant influence was found, not even at T1 and T2.

4. Discussion

NaCl addition is associated with differential responses in physiological parameters in ungrafted and grafted pepper plants. We demonstrate that tolerance to moderate salt stress can be improved by grafting. The best salt acclimation was obtained when accession 12 was used as a rootstock (A/12),

based on the minor negative effects caused by salt treatment on photosynthesis, NR activity and lipid peroxidation. Furthermore, some favourable physiological characteristics for salt acclimation, such as higher K^+ Ca^{2+} and Mg^{2+} levels in leaves and a lower Na^+/K^+ ratio, were seen in this plant combination. The latter parameter has been demonstrated as a good indicator of salt tolerance [21].

Salt tolerance in plants is usually associated with the ability to restrict the uptake and/or transport of saline ions from roots to leaves and their compartmentalisation [22]. In this study, more Cl^- was withheld in the roots of rootstocks in A/12 and A/14, and less Cl^- was transported to their leaves if compared with the ungrafted and A/5 plants under NaCl stress ($P<0.05$). This suggests either maximised Cl^- retrieval to the rootstock or a retention mechanism in the roots of these plant combinations. Unlike Cl^-, rootstocks 12 and 14 showed a reduced Na^+ net uptake, consequently their leaves gave a lower Na^+ concentration value if compared with the others ($P<0.05$). Two mechanisms can explain the lower Na^+ concentration in roots: firstly, as suggested by Aktas et al. [4], in salt-tolerant pepper genotypes, a plasma membrane Na^+/H^+ antiporter protein is activated in root cells upon NaCl exposure. This mechanism has been reported in different grafted plants, such as melon [23–25], tomato [22,26,27], watermelon [28] and cucumber [29]. Alternatively, the root system of rootstocks 12 and 14 might be able to control Na^+ influx, as reported for pumpkin roots [8].

Regarding concentration; leaf Cl^- accumulation exceeded that of Na^+ in all the plant combinations. This is in accordance with the results obtained by Navarro et al. [30] and Chartzoulakis and Klapaki [31] in the ‘Orlando’ variety

and the ‘Sonar’ pepper variety, respectively. The higher Cl^- concentration, if compared to Na^+ (mainly in roots), can be linked to a higher passive uptake root component and a very feebly active Cl^- uptake system [32]. However, it is unknown whether some rootstocks are capable of regulating the transport of Na^+ or/and Cl^- to leaves [33]. Based on our results, the capacity to regulate Na^+ and Cl^- uptake and transport was linked to the ability of rootstocks, and not to the grafting process itself (comparing A/5 *vs.* A/12 and A/14), indicating that the physiological and biochemical mechanisms of these salts operate at the rootstock level, as observed in grafted melon plants [7] or cucumber plants [8].

Regulation of ion homeostasis and selectivity, particularly Na^+/K^+ discrimination, is closely linked to the lower Na^+ concentration and its relation to salt tolerance [34]. Given the similar physico-chemical structure between Na^+ and K^+, a high Na^+ concentration in the external solution can lower the K^+ level in the tissues of many plants species [35]. In our study, the Na^+/K^+ ratio in leaves of the ungrafted and A/5 pepper plants under salinity was significantly higher ($P< 0.05$) than those of the plants grafted onto rootstocks 12 and 14, and the latter is able to select, use and transport K^+ to leaves, as in many vegetable-grafted plants exhibiting salinity tolerance; e.g., tomato [6], melon [9] or cucumber [11,29]. However, the direct relation between K^+ homeostasis and salinity tolerance has not been well-established [1]. In some species, Na^+ can be balanced by a higher K^+ concentration [36], while in other plants, tolerance is due to the capacity of roots to maintain K^+ transport in the xylem, as in tomato-grafted plants [37,38].

Despite the negative effect on plant growth derived from its toxic effect, accumulation of ions under salinity can help maintain the turgor pressure of

plants [30,39]. In addition, different osmolytes can be involved in the reduction of ψ_S, including organic compounds such as sugars, free amino acids, glycinebetaine, soluble proteins, proline and organic acids [40–42], and/or macronutrients such as inorganic components [43]. According to our results, a strong negative correlation between the reduction in leaf ψ_s and salt ions content for all the plant combinations was observed in the experiment. The linear regressions equations showed that Na^+ and Cl^- display a different response on ψ_s. The lower osmotic potential seems to be achieved mainly by Na^+ and, to a lesser extent by Cl^-. This can be explained by a more marked change in Na^+ accumulation if compared to Cl^- between the ungrafted and A/5 *vs.* A/12 and A/14 plants, rather than by the absolute concentration of both ions. The reduced osmotic potential assigned to Na^+ was consistent with pepper plants [44], and salt-tolerant species such as *Centarurea ragusina* [45], *Atriplex nummularia* [46] or *Aster tripolium* [47]. The contribution of K^+, Mg^{2+} and Ca^{2+} to ψ_s under the salinity conditions in our study was more relevant in the A/12 and A/14 plants at T2, where K^+, Mg^{2+} and Ca^{2+} represented 30-35% of the total ions if compared to 15% in the ungrafted and A/5 plants.

The adjustment of the osmotic potential through inorganic ion uptake supposes a much lower energy cost than that conferred by the organic molecules synthesised in the cell [48]. However in order to reduce $\psi_{s,}$ our plants required proline synthesis to produce sufficient osmotics under salt stress conditions. The synthesis and accumulation of proline depended on plant combinations and time exposure. At T1, when the ionic-osmotic phase was predominant, proline accumulation contributed less. In contrast at T2, strong proline synthesis took place in the ungrafted and A/5 plants when compared

with A/12 and A/14 (P<0.05), and as result, the reduction in ψ_s strongly related with proline accumulation in these plant combinations (r^2= 0.95), but more weakly in the latter ones (r^2 = 0. 36). An larger amount of proline or other compatible solutes may protect plants by scavenging the oxygen-free radicals caused by salt stress [1,29], which has been observed in different grafted plants like tomato [49], cucumber [29] or tobacco [50]. Conversely, the concomitant increase in proline with a prolonged exposure time in the ungrafted and A/5 plants was consistent with the higher leaf proline concentrations in salt-sensitive genotypes reported for other species such as wheat [51], barley [52], *Centaurea ragusina* [45] or rice [53]. This indicates that significant proline accumulation generally occurs only beyond the salt stress threshold [54]. The energy cost imposed by ion exclusion and/or compartmentalisation mechanisms are relatively low when compared with the synthesis of organic molecules [52,55] but, conversely, accumulation of saline ions may interfere with the normal biochemical activities taking place within the cell [56].

Plants respond to lower water availability under salinity by reducing their leaf transpiration, stomatal conductance, and by adjusting their root water uptake [57]. Under prolonged periods of exposure to salt, root conductivity can be partially recovered, mainly through the accumulation of compatible solutes and/or ions in roots. These responses should be involved in the maintenance of the relative water content in the leaves of grafted pepper genotypes in the experiment. Despite the reduction of the leaf osmotic potential and stomatal conductance described in the ungrafted plants, no root conductivity recovery should occur in the experiment since RWC was significantly lower under salinity. According to this relation, a reduction in either the functionality or the

amount of aquoporins has been reported to occur in pepper plants under salinity [30,58].

In this experiment, the Na^+ and Cl^- concentrations did not provoke salt toxicity symptoms in our pepper plants, and only minor leaf chlorosis and small necrotic areas was/were observed in ungrafted plants. These results agree with the lipid peroxidation levels reported in ungrafted plants when compared with grafted plants. Lower MDA concentrations were found in A/12 plants, followed by A/14. Nevertheless, gas exchange parameters were affected after a 2-week salt exposure and extended to T2. Excessive Na^+ and Cl^- accumulation is harmful and may disrupt the integrity of the photosynthetic apparatus [24]. Reduced photosynthetic capacity can be related to higher leaf Na^+ or Cl^- concentrations [22,28,59,60]. In our experiments, the highly significant correlation found between A_N and Na^+ and Cl^- foliar concentrations suggested that both ions can be involved in reduced photosynthesis, although the regression analyses indicated a predominant inhibition effect by Na+. This effect can be linked to the concentration level in leaves and/or a major toxic power to promote inhibition. In contrast to the reductions observed in the other plant combinations, maintenance of A_N in the A/12 plants can be attributed, at least in part, to increased K^+ levels or to other beneficial macronutrients, such as Ca^{2+} and Mg^{2+}, which contribute to better regulate stomata regulation under salinity [35]. Notwithstanding, g_s significantly lowered under mild salt stress in all the plant combinations and for the time exposures, which corroborates a previous finding that g_s are very sensitive to salt [49,61]. In addition, the diminished instantaneous carboxylation efficiency (A_N/C_i) noted at T1 and T2 in the ungrafted, A/5 and A/14 plants suggests that salt stress affects

photosynthesis by metabolic limitations, probably in association with reduced Rubisco carboxylase activity [62]. In contrast, stomatal limitations to photosynthesis should occur in A/12 at T1 and T2 since no changes in A_N/Ci were observed under salinity [63].

There is evidence that photosynthesis regulates nitrate reduction by modulating NR activity [64,65], which agrees with the results presented herein, which indicate that salt application diminishes A_N and NR activity. The most tolerant rootstock (A/12) in A_N terms exhibited lower NR inhibition if compared with the others. A drop in NR by salt can be due to: reduced nitrate transport to leaves, mainly because of nitrate/chloride competition [66]; inactivation of NO_3^- transporters by toxic effects of salt ions [67]; the disruption of root membrane integrity [58]; diminished NO_3^- transport from roots to leaves due to a lower transpiration flow [15] and, consequently, low NO_3^- loading into the root xylem, which affects NR activity [68]. Accordingly, and in accordance with the results obtained, the more marked decrease noted in NR activity (ungrafted and A/5 plants) in leaves can be associated with higher Cl^- and Na^+ accumulations and/or lower carbon fixation rates.

In conclusion, the greater salt tolerance of grafted plants, mainly the A/12 (and A/14) combinations, can be attributed to their ability to restrict Cl^- transport to leaves and to diminished Na^+ loading in roots, thus favouring K^+ (Ca^{2+} and Mg^{2+}) uptake and allowing a smaller osmotic potential with a lower energy cost. These traits led to a minor inhibitory effect on photosynthesis and NR activity, which favourably affected yield [5] when compared with the A/5 and ungrafted plants. Knowledge of the physiological and biochemical processes that promote salt stress tolerance can improve our understanding of not only the mechanisms

involved in the scion and rootstock interaction, but also of the selection of robust rootstocks to be used under field salinity conditions.

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Legends of figures

Fig. 1. Effect of NaCl addition at 0 mM (□) and 40mM (■) on relative leaf water content (RWC %) for exposures of 14 days (A) and 28 days (B) in ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14. Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 2. Leaf osmotic potential (MPa) in ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12, and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig.3. Concentrations of Na^+ (A, B), Cl^- (C, D), K^+ (E, F) in mg g-1 DW and the Na^+/K^+ ratio (G, H) in the leaves and roots of ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12, and 14 after addition of NaCl at 0mM and 40mM for exposures of 14 days (□, ■) and 28 days (▨, ▬), respectively. Dates are mean values±SE for n=6. In each

plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

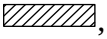
Fig. 4. Ionic concentration of Ca^{2+} and Mg^{2+} in the leaves (A, C) and roots (B, D) in mg g^{-1} DW of ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12, and 14 after addition of NaCl at 0mM and 40mM for exposures of 14 days ( , ) and 28 days ( , ), respectively. Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 5. Changes in the proline concentration (mg proline g^{-1}DW) from ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM () and 40mM () for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 6. The Net CO_2 assimilation rate (A_N; μmol CO_2 m^{-2} s^{-1}) (A, B); leaf stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) (C, D) and instantaneous carboxylation efficiency (A_N/C_i; E, F) in ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM () and 40mM () for exposures of 14 days (A, C, E) and 28 days (B, D, F). Dates are mean values±SE for n=10. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 7. Nitrate reductase activity (µmol NO_2 g^{-1} FW h) in the leaves of ungrafted pepper plants (cultivar ‘Adige’) and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 8. Leaf malondialdehyde (MDA) content (nmol MDA g^{-1}FW) in the leaves of ungrafted pepper plants (cultivar ‘Adige’) and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Table 1.

Linear regression and statistical analysis between mineral ions concentration (mg g-1 DW) in the leaves of the cultivar “Adige” ungrafted and grafted onto different pepper genotypes (5, 12 and 14), and proline (mg g^{-1} DW), as related to the osmotic potential (Ψ_s s in MPa) and CO_2 fixation rate (AN, μmol CO_2 m^{-2} s^{-1}).

Salt treatment time	Regression equations	*P**	R^2
T1	ψ_S= -0.021[Na^+] -1.05	0.0035	0.782
	ψ_S= -0.006 [Cl^-] – 0.99	0.0003	0.898
	ψ_S= 0.02 [K^+] – 1.61	0.008	0.716
	ψ_S= -0.22 [Proline] – 0.64	0.0229	0.616
	A_N = -0.641 [Na^+] + 21.35	0.0002	0.919
	A_N = -0.1616 [Cl^-] + 22.47	0.0017	0.828
	A_N = 0.642 [K^+] + 2.48	0.1333 ns	0.701
	A_N = -7.856 [Proline] + 37.28	0.0548 ns	0.593
T2	ψ_S= -0.029 [Na^+] -1.13	0.0001	0.923
	ψ_S= -0.0065 [Cl^-] – 1.105	0.0031	0.792
	ψ_S= 0.021 [K^+] – 1.87	0.0514 ns	0.495
	ψ_S= -0.143 [Proline] – 1.02	0.0332	0.986
	A_N = -0.647 [Na^+] + 21.81	0.0004	0.897
	A_N = -0.144 [Cl^-] + 22.46	0.0032	0.788
	A_N = 0.537 [K^+] + 4.88	0.0794 ns	0.635
	A_N = -2.943[Proline] + 24.38	0.0018	0.910

Determinations were made after 14 (T1) and 28 (T2) days. Fisher’s least significance difference (LSD) test at $P < 0.05$ was used.

*For all the linear regressions, the degrees of freedom are n= 4.

Figure 1

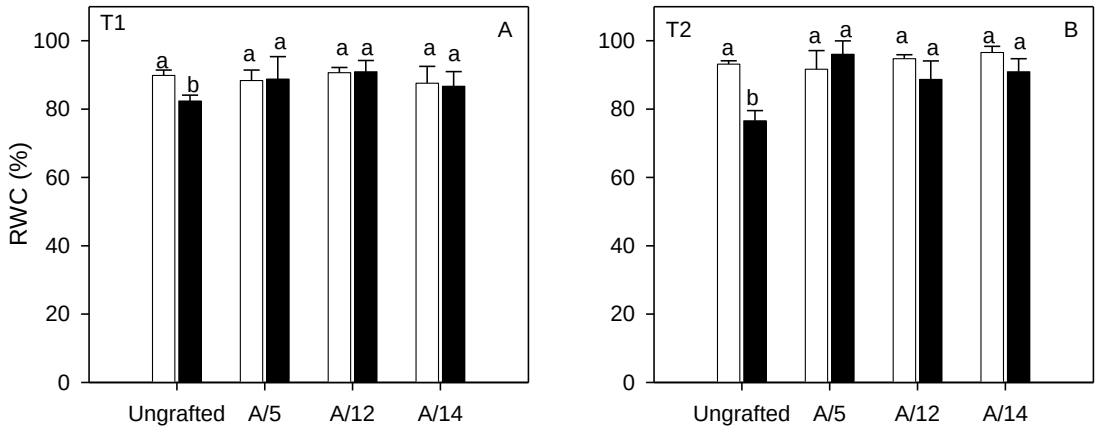

T1
A
RWC (%)
100
80
60
40
20
0
a
b
a
a
a
a
a
a
Ungrafted
A/5
A/12
A/14
T2
B
a
b
a
a
a
a
a
a
Ungrafted
A/5
A/12
A/14

Figure 2

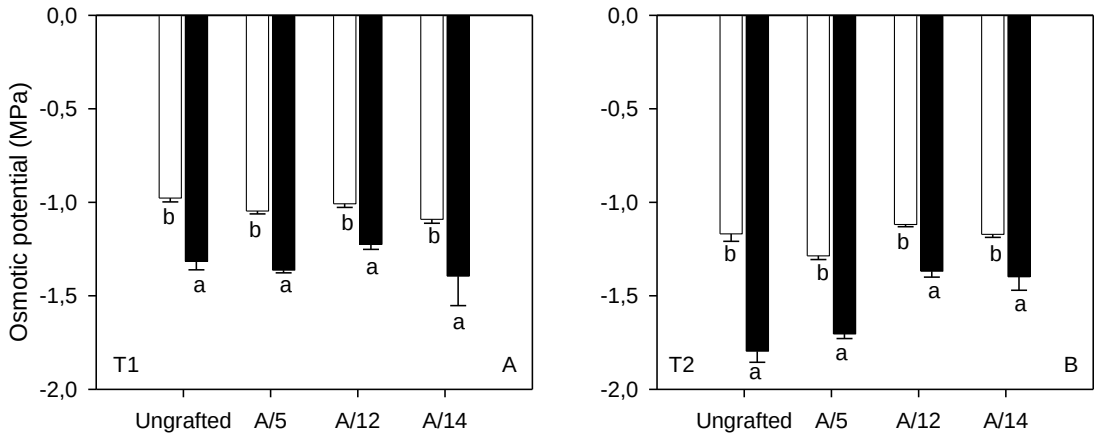

Osmotic potential (MPa)
0,0
-0,5
-1,0
-1,5
-2,0
T1
A
T2
B
b
a
Ungrafted
A/5
A/12
A/14

Figure 3

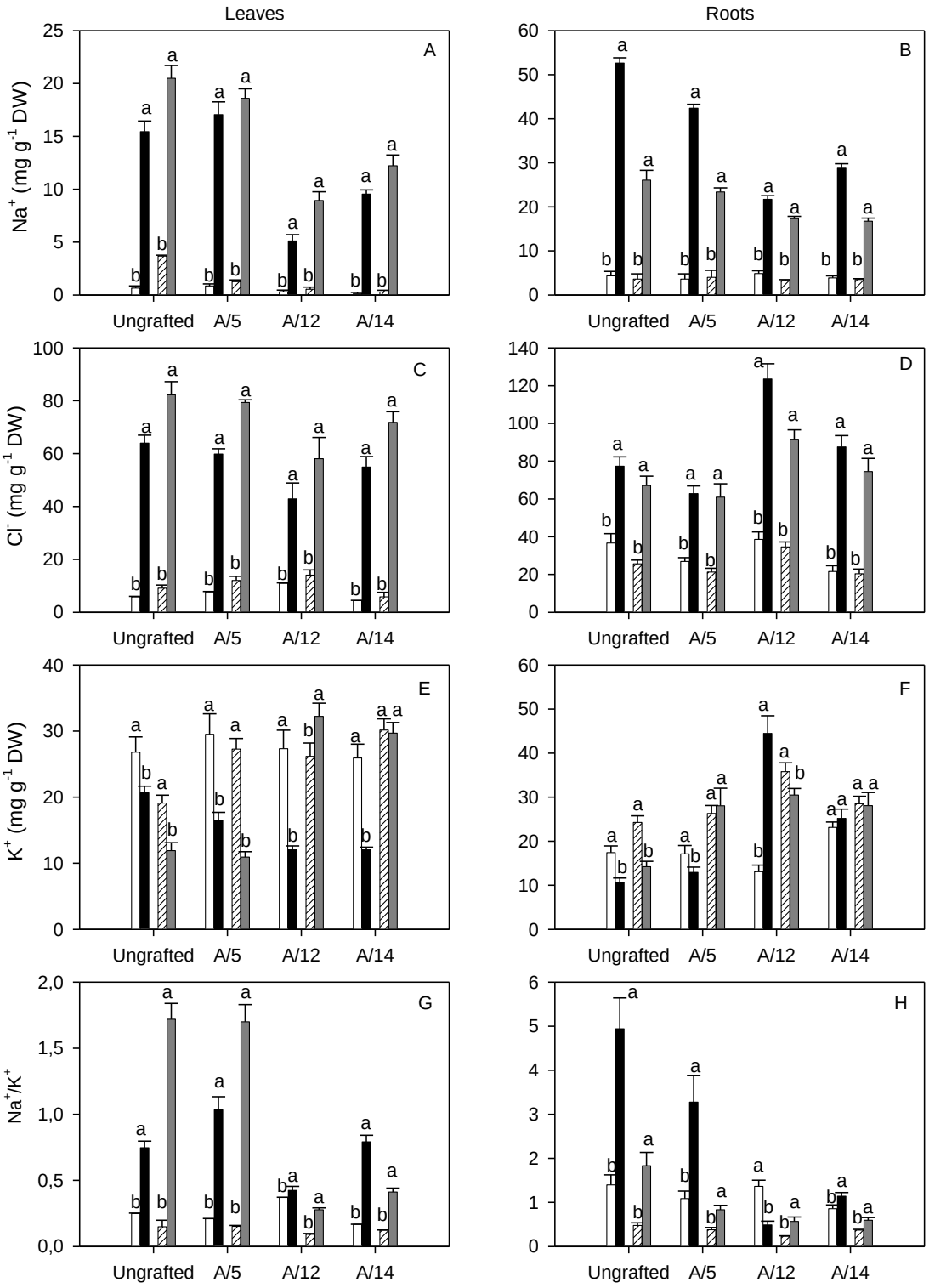

Leaves
Roots
A
B
C
D
E
F
G
H
Na+ (mg g-1 DW)
Cl- (mg g-1 DW)
K+ (mg g-1 DW)
Na+/K+
Ungrafted
A/5
A/12
A/14

Figure 4

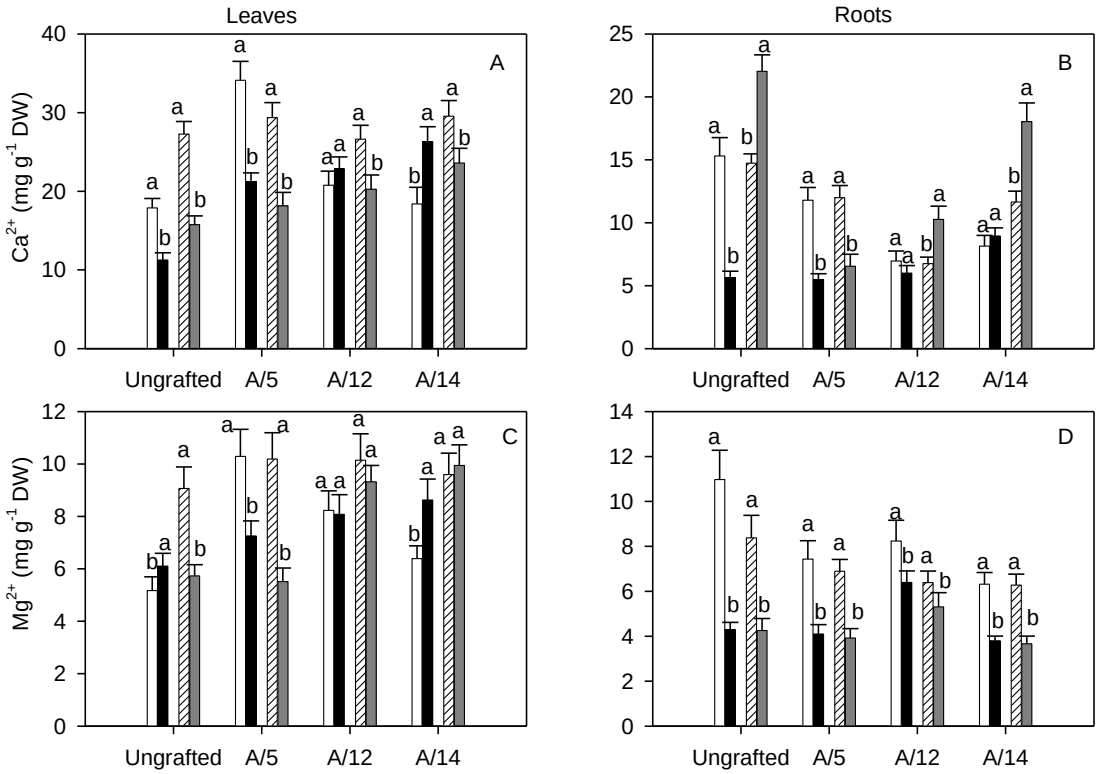

Leaves
Roots
A
B
C
D
Ca2+ (mg g-1 DW)
Mg2+ (mg g-1 DW)
Ungrafted
A/5
A/12
A/14

Figure 5

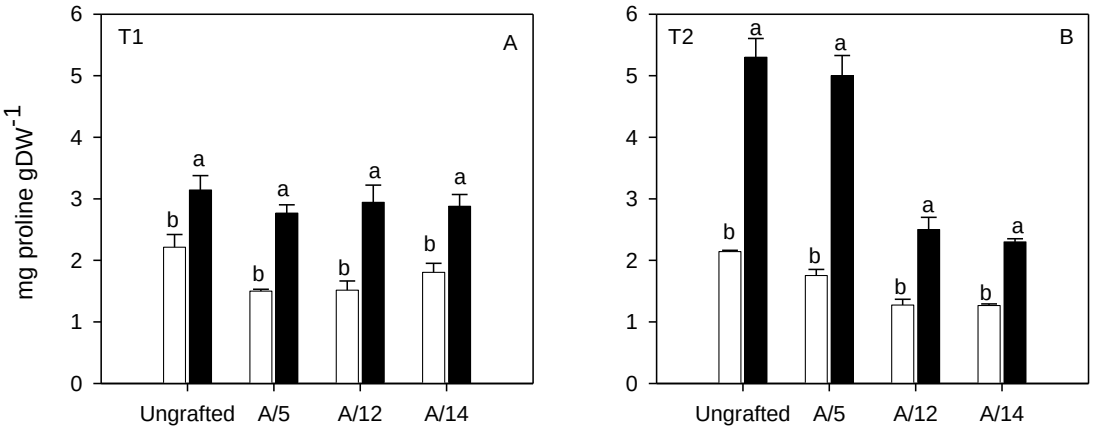


Figure 6

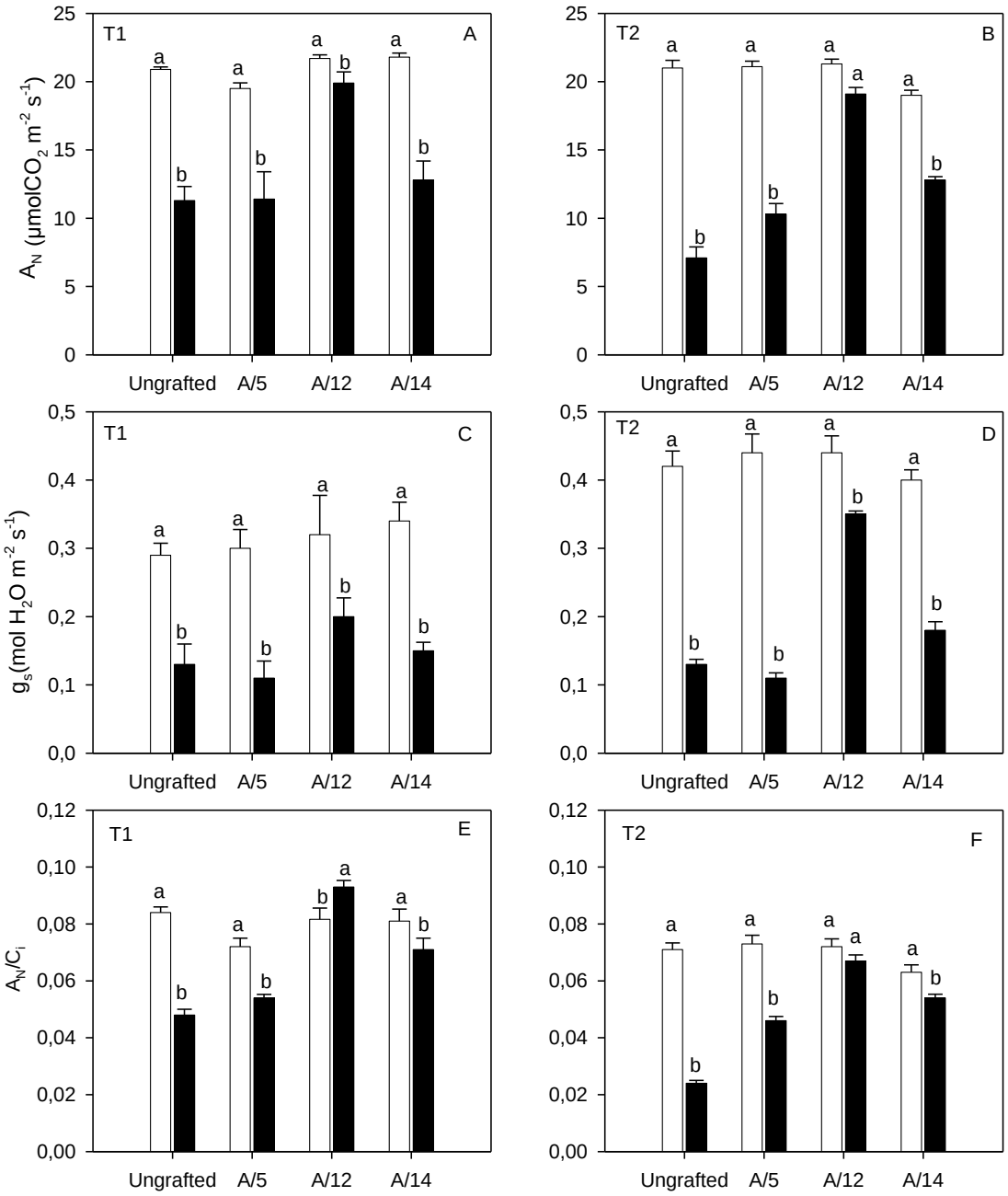

T1
A
T2
B
C
D
E
F
A_N (µmolCO$_2$ m^{-2} s^{-1})
g_s(mol H$_2$O m^{-2} s^{-1})
A_N/C_i
Ungrafted
A/5
A/12
A/14

Figure 7

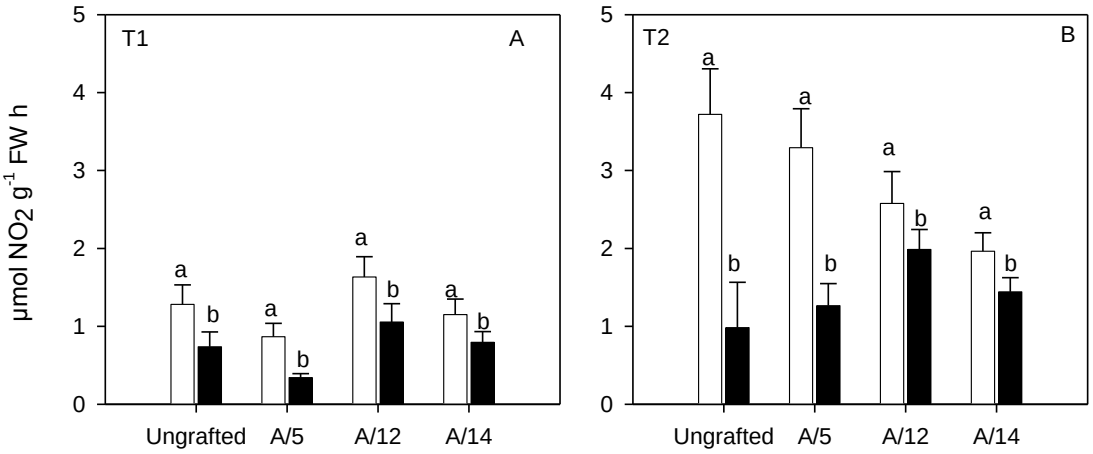
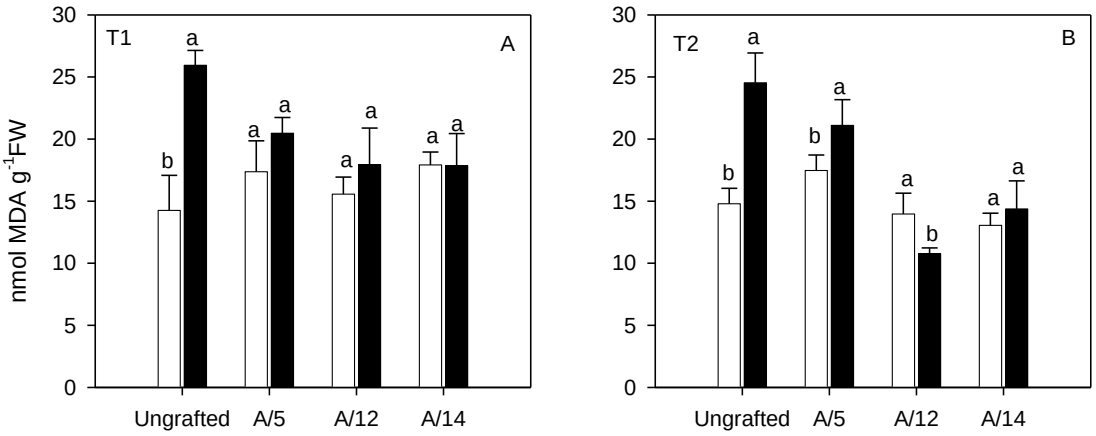


Figure 8


T1
A
T2
B
nmol MDA g-1FW
30
25
20
15
10
5
0
a
b
a
a
a
a
a
a
a
b
a
b
a
a
Ungrafted
A/5
A/12
A/14