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Physiological characterization of a pepper hybrid rootstock designed to cope with salinity stress

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ABSTRACT

In pepper crops, rootstocks that tolerate salt stress are not used because available commercial rootstocks offer limited profits. In this context, we obtained the hybrid NIBER®, a new salinity-tolerant rootstock that has been tested under real salinity field conditions for 3 years with 32%-80% higher yields than ungrafted pepper plants. This study aimed to set up the initial mechanisms involved in the salinity tolerance of grafted pepper plants using NIBER® as a rootstock to study root-shoot behavior, a basic requirement to develop efficient rootstocks. Gas exchange,

Na^+/K^+, antioxidant capacity, nitrate reductase activity, ABA, proline, H_2O_2, phenols, MDA concentration and biomass were measured in ungrafted plants of cultivar Adige (A), self-grafted (A/A), grafted onto NIBER® (A/N) and reciprocal grafted plants (N/A), all exposed to 0 mM and 70 mM NaCl over a 10-day period. Salinity significantly and quickly decreased photosynthesis, stomatal conductance and nitrate reductase activity, but to lower extent in A/N plants compared to A, A/A and N/A. A/N plants showed decreases in the Na^+/K^+ ratio, ABA content and lipid peroxidation activity. This oxidative damage alleviation in A/N was probably due to an enhanced H_2O_2 level that activates antioxidant capacity to cope salinity stress, and acts as a signal molecule rather than a damaging one by contributing a major increase in phenols and, to a lesser extent, in proline concentration. These traits led to a minor impact on biomass in A/N plants under salinity conditions. Only the plants with the NIBER® rootstock controlled the scion by modulating responses to salinity.

Keywords: antioxidant capacity; graft; H_2O_2; pepper; photosynthesis; rootstock

1. Introduction

New scenarios due to climatic change are affecting crop yield and quality. In this context, salinity is one of the most important environmental factors that limits plant growth, productivity, quality and the increasing demand for food crops (Ashraf, 2004; Srivastava and Kumar, 2015). More than 20% of cultivated land worldwide is affected by salt stress and this amount is increasing daily (Srivastava and Kumar, 2015). At the same time, the global population is expected to reach 9 billion by 2050. Thus increasing of agriculture productivity will be needed to meet food demands (Shelden and Roessner, 2013). To achieve the increased food production under salinity conditions, it is necessary to identify naturally occurring genetic variations within a crop species by screening varieties, wild genotypes and landraces that could provide salt tolerance (Roy et al., 2011).

Pepper is an important crop that grows in most countries on our planet, and covers 1.93 million ha of crop-growing surface area (Penella and Calatayud, 2018). As a spice and fruit, the world's pepper production was 34 million tons in 2017 (Penella and Calatayud, 2018). Generally speaking, commercial pepper varieties need friable, well-drained, sandy loam soil with a pH of 6.5–7.5 for optimum production. Salt content in soil and irrigation water should be low. There are reports of a salinity resistance threshold of 1.5 dS m^{-1}, below which no effect on growth occurs, and a 14% drop in biomass production per additional 1 dS m^{-1} has been reported (Maas, 1973). Pepper and *Capsicum annuum* species in particular are highly susceptible to salt stress by showing blossom end rot (BER), lower yields and more unmarketable fruits (Penella et al., 2015). Physiological changes have

been analyzed in pepper under salt stress like membrane permeability and water channel activity alterations, ion imbalance, reduced total photosynthesis and stomatal conductance, and increasing reactive oxygen species production, which modify the carbon balance required to maintain both productivity and growth (Penella and Calatayud, 2018).

To minimize salinity damage in pepper crops, graft technology is an agronomic practice that can improve plant tolerance by using rootstocks capable of reducing the negative effect of external stress on the scion. In addition, grafted plants can avoid the problem associated with the "building or design" of tolerant varieties due to complexity of salinity traits and lack of practical selection tools; one example is genetic markers, which have made these tasks slow and inefficient (Flowers, 2004; Ashraf and Foolad, 2007; Schwarz et al., 2010). Grafting can combine suitable commercial fruit quality characteristics and high production of a scion and tolerance traits to environmental factors from rootstock by working together like a single plant. Nevertheless, rootstocks that tolerate salt stress are not used in pepper plants because available commercial rootstocks offer limited profits (Lee et al., 2010; Penella et al., 2013; Kyriacou et al., 2017).

There is a need to perform rigorous screenings to find *Capsicum* plants that tolerate salt stress so they can be used as pepper rootstocks. In this context, we screened physiological and phenotypically characterized accessions of pepper from gene banks before selecting those for their tolerance to salinity and then using them as rootstocks in grafted pepper plants (Penella et al., 2013, 2015; López-Serrano et al., 2017; Penella and Calatayud, 2018). The obtained results

have allow to confirm that the tolerance to salinity of these grafted plants was expressed by maintaining scions presenting better physiological performance and, consequently, by increasing yields (Penella et al., 2015, 2016, 2017). Afterward, a classic breeding program was applied to salinity-tolerant pepper accessions (*C. annuun* x *C. annuun*) have allowed obtain more uniform hybrids in terms of germination, growth and highest vigor to be used as rootstocks under salinity conditions. One of them, NIBER®, has been tested under real salinity field conditions for several years (Calatayud et al., 2016) and showed higher yields (range of 32%-80%) than ungrafted plants or other tested commercial pepper rootstocks.

The aim of the present work was to evaluate the early physiological response of a tolerant rootstock under salt stress conditions using the hybrid NIBER®. To date, information about the initial mechanisms involved in the tolerance to of grafted pepper plants remains limited. The initial evaluation of root-shoot to physiological evolution is a basic requirement to help develop improved efficient rootstocks with the ability to cope with salinity and to ensure a better understanding of the response mechanisms of grafted pepper plants to imbalanced salinity.

To fulfill this objective, we compared the relative tolerance responses of ungrafted, self-grafted, grafted and reciprocal grafted pepper plants under both control and salinity conditions. Gas exchange, proline, phenols, hydrogen peroxide, radical scavenging capacity and nitrate reductase activity were measured in the leaves of all the pepper plants combinations. Na^+/K^+, Cl^- concentration and ABA levels were determined in both leaves and roots. In addition, biomass parameters

(stem and root length and total dry weight) were measured. All those information has been analyzed to identify the mechanisms by which the NIBER® rootstock enhances tolerance to salinity.

2. Material and methods

2.1. Plant material

A new hybrid pepper salinity-tolerant rootstock, NIBER® *(Capsicum annuum x C. annuum)* (abbreviated herein as N), and the salt-sensitive pepper cultivar 'Adige' (abbreviated as A) (Lamuyo type, Sakata Seeds, Japan), were used as either a scion or rootstock. Four plant combinations were herein used: ungrafted A plants (A), self-grafted A plants (A/A), A grafted onto N (A/N) and N grafted onto A (N/A). Early in March, the seeds of A and N were sown in 96 seedling trays filled with a peat-based substrate for germination. After 2 months, the grafted plant combinations were performed by the tube-grafting method (Penella et al., 2015). They were maintained in a chamber with relative humidity above 95% and air temperature around 28-29º C for a 4-6 day period (Penella et al., 2014). The grafted plants were then placed in a greenhouse until transplanted. The ungrafted (A) plants were sown 2 weeks later to obtain plants with a similar biomass to that of the grafted plants upon transplantation (10-12 development leaves). The plants obtained by the above-mentioned procedure were utilized in greenhouse experiments at the end of May.

2.2. Hydroponic greenhouse experiment

The root systems of the plants were washed to clean the substrate and plants were placed in 5 L polyethylene pots, which were previously covered with

aluminum sheets. Pots were filled with a standard nutrient solution for pepper (Sonneveld et al., 1994) containing (in mmol L^{-1}): 12.3 NO_3^-, 1.02 H_2PO_4, 2.45 SO_4^{2-}, 3.24 Cl^-, 0.6 NH_4^+, 5.05 K^+, 4.23 Ca^{2+}, 2.55 Mg^{2+}, 2.2 Na^+ and micronutrients (15.8 µM Fe^{2+}, 10.3 µM Mn^{2+}, 4.2 µM Zn^{2+}, 43.5 µM B^+, 2.14 µM Cu^{2+}), which were artificially aerated with an air pump. The electrical conductivity (EC) and pH of this nutrient solution were 1.7 dS m^{-1} and 6.5, respectively. Nutrient solution was added daily to compensate for uptake. After of leaving seedling plants for 7 days to acclimatize to pots, the salinity treatment was initiated by adding NaCl (70mM) to the nutrient solution to obtain an EC of 8.5 dS m^{-1} and a pH of 6.1.

While the experiment was underway, plants were grown in a Venlo-type greenhouse under natural light conditions (610-870 µmol m^{-2} s^{-1}). Temperature and relative humidity ranges were 21-25ºC and 52-72%, respectively.

The layout was a completely randomized design with four replications of six plants per combination (A, A/A, A/N and N/A).

All the physiological measurements were taken in days 1, 2, 4, 7 and 10 after the salt treatment (DAT) had started, except for ABA concentration and nitrate reductase activity, which were measured on 1DAT and 10DAT, and ion determination on 10DAT. Measurements were taken in fully and expanded mature leaves (3^{rd}-4^{th} leaf from the shoot apex) and in lateral roots for Na^+/K^+, Cl^- and ABA. They were taken in random order in three plants per replication (12 measurements per plant combination and treatment) for the gas exchange measurement, and in four plants (1 plant per replication) in the other analysis of the physiological parameters.

2.3. Ion determination

Leaves and roots were dried in a laboratory oven at 70°C for 72 h before being burnt in a muffle furnace for 12 h at 550ºC. Ions were extracted with 2% nitric acid in an ultrasonic bath for 30 min at 40ºC. The Na^+ and K^+ concentrations were measured by ICP emission spectrometry (iCAP 6000, Thermo Scientific, Cambridge, UK). The chloride concentration (Cl^-) in the dry plant material was extracted with 0.1 N HNO_3 in 10% (v/v) acetic acid and was determined by potentiometric titration with $AgNO_3$ in a chloride analyzer (Sherwood, MKII 926, Cambridge, UK).

2.4. Gas exchange measurements

The CO_2 assimilation rate (A_N, µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) were determined on fully expanded leaves (3^{rd}-4^{th} leaf from the apex) in the steady state under saturating light conditions (1000 µmol m^{-2} s^{-1}) and with 400 ppm CO_2 by a LI-6400 infrared gas analyzer (LI-COR, Nebraska, USA) at 24ºC (24 ±2ºC) and 65% relative humidity (65±10%). The gas exchange measurements were taken from 9 am to 11 am (GMT).

2.5. Abscisic acid analysis

The thoroughly ground leaves and roots (about 0.1mg fresh weight) on 1DAT and 10DAT were suspended in 80% methanol-1% acetic acid containing internal standards, and were mixed by shaking for 1 h at 4ºC. The extract was kept a 20ºC overnight and was then centrifuged. The supernatant was dried in a

vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through a reverse phase column (HLB Oasis 30 mg, Waters), as described in Seo et al. (2011). The final residues were dried and dissolved in 5% acetonitrile-1% acetic acid and hormones were separated by UHPLC with a reverse Accucore C18 column (2.6 µm, 100 mm long; Thermo Fisher Scientific) with a 2-55% acetonitrile gradient containing 0.05% acetic acid at 400 µL/min for 21 min.

Abscisic acid (ABA) was analyzed by a Q-Exactive mass spectrometer (Orbitrap detector, Thermo Fisher Scientific) by targeted Selected Ion Monitoring (tSIM; capillary temperature 300ºC, S-lens RF level 70, resolution 70.000) and electrospray ionization (spray voltage 3.0 kV, heater temperature 150ºC, sheath gas flow rate 40 µL/min, auxiliary gas flow rate 10 µL/min) in the negative mode.

The concentration of ABA in the extracts were determined using embedded calibration curves and the Xcalibur 4.0 and TraceFinder 4.1 SP1 programs. The internal standards for the quantification of all the different plant hormones were deuterium-labeled hormones.

2.5. Nitrate reductase activity

Nitrate reductase activity (NR) in leaves (Enzyme Code 1.7.1.1) was determined *in vivo* on 1DAT and 10DAT following the methods described by Hageman and Hucklesby (1971) and Jaworski (1971). Discs (1 cm in diameter) were collected from mature fresh leaves. Samples (0.2 g of fresh weight) were suspended in plastic vial containing 10 mL of 100 mM potassium phosphate buffer (pH 7.5), 1% (v/v) n-propanol and 100 mM KNO_3. Plant samples were incubated in a water bath at 30°C for 60 min in the dark and 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-Naphthyl-ethylenediamine and 1% (w/v) sulfanilamide. A standard curve with KNO_2 was prepared to calculate the amount of NO_2 contained in the samples. NR activity was expressed as a percentage of NR in the salt treatment *versus* the control on 1DAT and 10DAT.

2.6. Proline determination

Proline content was determined as described by Bates et al. (1973). Dry leaves (20 mg) were ground in 3% sulfosalicylic acid, the homogenate was filtered, and glacial acetic acid and ninhydrin reagent were added to an aliquot of the filtrate. The reaction mixture was boiled at 100ºC for 1 h, and readings were taken in a spectrophotometer at a wavelength of 520 nm.

2.7. Total phenolic content

Total phenolic content was determined according to Koç et al. (2010) with modifications. Fresh leaf samples (0.1 g) were frozen in liquid nitrogen and stored at -80°C. They were mixed with 1.5 mL of extraction solution (50% (v/v) methanol and 1% (v/v) HCl). Samples were extracted in a boiling bath at 80°C for 15 min. Next 0.02 mL of the leaf extracts (diluted in 0.08 mL extraction solution) were mixed with 0.7 mL of Folin–Ciocalteu solution (Sigma-Aldrich®), and diluted at the proportion of 1:10, and 0.7 mL of 6% (w/v) Na_2CO_3. Samples were incubated at room temperature and in the dark for 1 h before being subjected to absorbance measurements at 765 nm. Gallic acid was used as a standard.

2.8. Hydrogen peroxide determination

H_2O_2 content was determined according to Sergiev et al. (1997) and Velikova et al. (2000) with slight modifications (López-Serrano et al., 2019). First 0.25 g of fresh leaves was frozen in liquid nitrogen and kept at -80°C. Samples were ground in a mortar and 2 mL of 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 g and 4°C for 8 min. Then 0.4 mL of the supernatant was diluted with 0.6 mL of 0.1 % (w/v) TCA. Finally, 0.5 mL of 100 mM potassium phosphate buffer (pH= 7) and 2 mL of 1M of KI were added. Samples were incubated for 1 h at room temperature and in the dark and absorbance was measured at 390 nm. H_2O_2 content was given by a H_2O_2 standard curve.

2.9. Lipid peroxidation determination

Lipid peroxidation was estimated by malondialdehyde (MDA) determinations using a thiobarbituric acid reaction, according to the protocol reported by Heath and Packer (1968) and modified in Dhindsa et al. (1981). First 0.1 g of fresh leaves was frozen in liquid nitrogen and kept at -80°C. Samples were ground in a mortar and 2 mL of 0.1% (w/v) TCA. Later the homogenate was centrifuged at 10000 g and 4°C for 5 min. Afterward, 2 mL of reaction buffer (TCA 20% + TBA 0.5%) were added and heated at 95°C for 30 min. The non specific background absorbance reading at 600 nm was subtracted from the specific absorbance reading at 532 nm.

2.10. DPPH radical-scavenging capacity

Radical scavenging capacity (RSA) was determined by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, proposed by Brand-Williams et al. (1995) with modifications. Namely, 0.1 g of fresh leaves was frozen in liquid nitrogen and stored at -80°C. Samples were ground in a mortar with the addition of 80% (v/v) methanol. After 12 h at 4°C in a mixer, samples were centrifuged for 10 min at 10000 g and 4°C. A 10-µL volume of sample and 990 µL of 0.065 mM DPPH were taken and incubated for 30 min in the dark at room temperature. Absorbance was measured at 515 nm. The percentage of the inhibition of the DPPH radical was measured by this equation: [(DPPH absorption – Sample absorption)/ DPPH absorption] x 100 (López-Serrano et al., 2019).

2.11. Biomass measurements

The roots length (maximum root depth) and stems length and total dry weight of biomass (roots+leaves+stems) were measured at the end of the experiment (10DAT). The plants were dried at 65º C for 72 h to determine the dry weight.

2.12. Statistical analysis

The results were subjected to a two-way ANOVA analysis (Statgraphics Centurion for Windows, Statistical Graphics Corp.) with treatment and plant combinations used as factors of the analyses. Each time of measurement (DAT) was separately analyzed. In all the parameters where the interaction was significant, the plant combinations and treatment were analyzed together by a one-way ANOVA. In the

case of biomass parameters (root length, stem length and total dry biomass), interaction was not significant, but the genotype was, so a one-way ANOVA was performed separating both treatments. In the case on nitrate reductase, since the values were referenced by the percentage of salt with respect to control, one-way ANOVA considering only the plant combinations was carried out. Means were compared by the Fisher's least significance difference (LSD) test at $P < 0.05$. No significant differences were found among the four replicates for each measured parameter.

3. Results

3.1. Ions determination

The Na^+/K^+ ratio at the end of experiment (10DAT) was higher in roots than in leaves for all the plant combinations and treatments (Fig. 1). In leaves, Na^+/K^+ significantly decreased ($P<0.05$) in the A/N plants under salt applications (Fig. 1A). Under the control conditions of leaves, A/N showed a decrease with significant differences compared to N/A (Fig. 1A). In the root compartment (Fig. 1B), the Na^+/K^+ values increased in all the plant combinations under salt treatment. Na^+/K^+ were significantly higher in the ungrafted (A) and N/A plants, and the lower values were measured in A/N with significant differences. In the control treatment, all grafting combinations had significantly lower Na^+/K^+ values compared to ungrafted plants (Fig. 1B).

The Cl^- concentration in both leaves and roots (Fig. 1C, D) increased with NaCl addition in all the plant combinations, although the Cl^- concentration was

higher in roots. The highest Cl^- levels in leaves were obtained for N/A plants (Fig. 1C), whereas no significant differences were found among all the plant combinations in roots (Fig. 1D). In the control treatment, no significant differences appeared among the plant combinations in both leaves and roots (Fig. 1C-D).

3.2. Photosynthetic rate and stomatal conductance

The figures 2 and 3 showed the changes along the experiment for A_N (Fig. 2 A-D) and g_s (Fig. 3 A-D) under control and salt treatment. The photosynthetic rate (Fig. 2) significantly dropped in all the plants ($P<0.05$) in response to salt stress and reached null values at the end of experiment, except for A/N (Fig. 2C) where the A_N values were higher with significant differences between salt and the control conditions from 4DAT to 10DAT.

A drop in g_s under salt treatment was observed in all the plants with almost total stomatal closure (Fig. 3). A minor drop in g_s was noted for A/N plants under salt stress showing the highest values of g_s respect the other salt-plant combinations with a significant difference at the end of experiment (Fig. 3C).

3.3. ABA analysis

After 1DAT and 10DAT, the average ABA concentration values (Fig. 4) were higher in leaves than in roots. In leaves, significantly higher ABA concentrations were found under salinity conditions compared to the control ones in all the plant combinations (Fig. 4A, C). Under the salinity conditions, the minimum ABA values in leaves belonged to A/N plants (Fig. 4A, C). In roots (Fig. 4B, D), the ABA levels were different depending on both plant combinations and salt time exposure. On

1DAT, the maximum values were found for A and A/N plants (Fig. 4B). However on 10DAT, the highest ABA values were found in A and A/A plants (Fig. 4D).

3.4. Percentage of nitrate reductase activity in the salt treatment vs. the control

On 1DAT and 10DAT, the effect of salt addition induced reduction in the percentage of NR activity in leaves compared to their control in all plants combinations (Fig. 5). Nevertheless, in A/N the reduction was lower compared to other plant combinations with significant differences observed between them both 1DAT and 10DAT (Fig. 5).

3.5. Proline analysis

The proline concentration in leaves (Fig. 6) was always higher under salinity compared to the control condition from 1DAT to 10DAT. The maximum proline values appeared on 7DAT in all plant combinations. Afterward the drop in concentration became more emphasized in A/N (Fig. 6C) and N/A (Fig. 6D) until 10DAT, but with higher values compared to A and A/A.

3.6. Total phenols analysis

The phenol concentrations in leaves under the salinity conditions (Fig. 7) were higher with significant differences for all the plant combinations compared to the controls on each day after the start of treatment application. From 1DAT to 7DAT, A/N (Fig. 7C) was the plant combination with the highest phenol concentrations compared to other plant types. The lowest phenol levels were found for N/A plants (Fig. 7D).

3.7. H_2O_2 determination

The hydrogen peroxide concentration in the A plant leaves (Fig. 8) increased after salt exposure (Fig. 8A) on 1DAT. In the other plant combinations, the increase in H_2O_2 was observed on 2DAT (except for A/N plants). A/N plants (Fig. 8C) showed the highest H_2O_2 concentration, which increased from 2DAT to 7DAT, after which time the H_2O_2 levels remained constant until 10DAT.

3.8. Lipid peroxidation

The MDA concentration in leaves (Fig. 9) was higher in all the plant combinations under the salinity conditions and increased during the exposure time. At the end of experiment (10DAT), A/N plants (Fig. 9C) displayed the smallest differences between control and salt stress compared to the other plants, followed by A/A (Fig. 9A), N/A (Fig. 9D) and A (Fig. 9A), with the highest lipid peroxidation levels for salt treatment.

3.9. DPPH-Radical Scavenging Activity

The leaves of the plants grown under salt stress obtained an increased percentage of inhibition radical DPPH compared to their control plants (Fig. 10). Maximum activity was found for A/N (Fig. 10C) plants on 7DAT and 10DAT under the salinity conditions.

3.10. Biomass measurements

At the end of the experiment (10DAT), the root length (Fig. 11A), the shoot length (Fig. 11B) and the total dry weight (Fig. 11C) were significantly higher in A/N under salinity conditions compared to all other plant combinations. Under control conditions, the highest root length was measured in A/N and N/A; however for the other biomass parameters significant differences were not observed between plant combinations.

4. Discussion

Vegetable grafting is an effective technique in increasing salt tolerance (Colla et al., 2010). Some rootstocks, mainly hybrids for tomato, melon and cucumber, have demonstrated tolerance to salinity (Colla et al., 2006; Savvas et al., 2011; Huang et al., 2013). To date, grafting onto pepper rootstocks has not been a feasible solution to cope with salinity given the unsatisfactory performance of available rootstocks (Kyriacou et al., 2017; Penella and Calatayud, 2018). In previous field studies conducted under water salinity conditions, the hybrid NIBER®, obtained for that purpose, has been demonstrated as an effective rootstock in overcoming salinity and improved production compared to ungrafted or other commercial rootstocks (Calatayud et al., 2016). The high yield obtained under the salinity conditions has been reported in other grafted vegetables, such as melon, watermelon or cucumber, grafted onto the hybrid *Curbita maxima* x *C. moschata* (Romero et al, 1997; Alan et al., 2007; Colla et al., 2012), or tomato grafted onto *S. lycopersicum* x *S. habrochaites* (Savvas et al., 2009). These findings demonstrate that grafting directly and positively affects plant production.

For many crops, a significant factor that contributes to salinity tolerance is the ability to manage concentrations of toxic ions inside the plants (Munns and Tester, 2008). Of all the different strategies, the capacity of salt ions exclusion and/or retention in roots, better maintenance of potassium homeostasis, or compartmentation of salt ions in the vacuole are available (Fernández-García et al., 2004; Colla et al., 2010). Moreover in grafted plants, the graft itself can act as a barrier to limit salt ions from the rootstock to the scion (Edelstein et al., 2011). In this study, the Cl^- concentration under salinity did not show any significant differences among the plant combinations in roots, and only N/A-leaves exhibited the highest Cl^- levels on 10DAT. This result suggests that the graft effect itself does not act as a selective barrier to limit Cl^- movement from root to leaves by showing a uniform Cl^- concentration-distribution between root and leaves. Similar results were obtained by Edelstein et al. (2011) in melon grafted onto pumpkin. Further Cl^- accumulation exceeded that of Na^+ in all the plant combinations. This agrees with the results obtained by Navarro et al. (2002) in 'Orlando', and also with Chartzoulakis and Klapaki (2000) in 'Sonar' pepper varieties or in grafted pepper plants (Penella et al. 2015). A higher external Cl^- concentration could be linked to a major passive uptake root component, which might occur when the membrane potential is less negative than Cl^- equilibrium potential allowing for a passive influx and a very slightly active Cl^- uptake system (Altman and Mendel, 1973; Skerrett and Tyerman, 1994). However, for many vegetables like cucumber, melon, watermelon, tomato, eggplant and pepper, Na^+ ion is the primary cause of ion-specific damage (Tester and Davenport, 2003; Varlagas et al., 2010, Penella et al., 2015). Na^+ is largely a result of its capacity to compete with K^+ for essential binding

sites for cellular function; moreover, regulation of ion homeostasis and selectivity of Na^+/K^+ discrimination are closely linked to a lower Na^+ concentration and its relation to salt tolerance (Volkmar et al., 1998; Munns and Tester, 2008). The significant depletion of the Na^+/K^+ ratio occurred only in the plants grafted onto N (A/N plants) in both leaves and roots to reduce the Na^+ load due to a higher K^+ concentration and/or lower Na^+ uptake compared to another plant combinations. Furthermore, the Na^+/K^+ ratios in roots undergoing the salinity treatment were higher compared with leaves (average of all plant combinations Na^+/K^+ was 24 fold higher in roots than leaves), regardless of the plant combination. The lowest Na^+ concentration in leaves to favor K^+ levels could be due to Na^+ retention and accumulation in roots (Edelstein et al., 2011). Grafted plants had a higher K^+ content, which is apparently related to higher salt tolerance showing lower inhibited extent of stem and root and plant growth under salinity conditions (Zhu et al., 2008; Huang et al., 2009; Colla et al., 2010, Nawaz et al. 2016) as just occurred in A/N plants in contrast to the other plant combination. In fact in the tomato-grafted plants, salt tolerance was associated with K^+, but not with Na^+ concentration (Albacete et al., 2009).

The lowest foliar Na^+/K^+ ratio in the grafted A/N plants could possibly diminish the phytotoxic effect of salinity on photosynthesis (Ruiz et al,. 2005), facilitating the maintenance of growth (Rouphael et al., 2012). The net CO_2 assimilation rate dropped in all the plant combinations under the salinity conditions, and this decrease was accompanied by a significant reduction in stomatal conductance. A/N plants showed a higher A_N than the self-grafted (A/A), non grafted (A) and reciprocal self-grafted (N/A) plants and the correlation analysis

suggest that total DW was positively related with A_N (R^2 = 0.886 at 10DAT) indicating that plant growth was directly linked to photosynthesis. These results agree with previous findings which revealed that tolerant rootstocks can improve photosynthesis performance and growth under the salt treatment (Moya et al., 2002; Massai et al., 2004; He et al., 2009, Rouphael et al., 2012). However, g_s significantly decreased and more markedly compared to A_N, with values close to zero and reduced plants' ability to supply CO_2 to the photosynthetic apparatus (Piñero et al., 2014). These results coincide with another finding which showed that g_s was very sensitive to salt stress (Jiang et al., 2006; He et al., 2009); although the least stomatal closure at the end of experiment was observed in A/N plants under the salinity conditions. A decrease in g_s has been observed in melon-, cucumber-, pepper- and tomato-grafted plants in response to salinity when tolerant rootstocks were also used (He et al., 2009; Rouphael et al., 2012; Penella et al., 2015).

According to our results, salinity induced ABA accumulation, which could cause stomatal closure (Zhu, 2001; Finkelstein et al., 2002), regardless of the root genotype (Holbrook et al., 2002). In our experiment, the ABA concentration in leaves affected g_s and a linear correlation was found for both parameters on 1DAT and 10DAT (R^2= 0.84 and 0.90, respectively). This observation falls in line with the results for sweet pepper under salinity stress observed by Piñero et al. (2014). The A/N plant leaves showed a lower ABA concentration with higher stomatal opening, but the reciprocal grafted N/A plants exhibited a similar ABA concentration to plants A/A and A. This situation indicates that the ABA levels in leaves were dependent on rootstock. There is evidence to show that a reduction in g_s is

associated with an increased in ABA in roots prior to a detectable increase in leaf ABA (Davies and Zhang, 1991). The relation between both parameters was consistent on 1DAT (R^2= 0.72), but not on 10DAT (R^2= 0.40), according to our results. It is possible that the control of stomata conductance for a longer time (10DAT) was exerted by leaf metabolic activity (leaf water status, change in ion transport or transpiration stream) rather than by the ABA produced by roots, and/or ABA could be synthesized in leaves (Munns and Cramer, 1996; Holbrook et al., 2002; Manzi et al., 2017).

Nitrate reductase is sensitive to g_s and becomes less active when the stomata are closed (Kaiser and Huber, 2001; Yousfi et al., 2012) limiting the assimilation of nitrate into organic compounds inducing visible effects on biomass (López-Serrano et al., 2019). In this study, we observed diminished NR activity *versus* its control on 1DAT and 10DAT in all the plant combinations. However, only A/N plants maintained 50% (1DAT) and 30% (10DAT) enzyme activity, and both sustained the highest g_s and growth. This trend has been observed in pepper-, tomato- and cucumber-grafted plants in earlier studies (Liu et al., 2013; Penella et al., 2015, Ruiz et al., 2005).

The low photosynthesis rate increased ROS formation in a very early response stage (Formentin et al., 2018). The accumulation of an excessive ROS level may react with proteins, DNA and lipids, which could lead to redox imbalance and oxidative stress to cause metabolic dysfunction (Gill and Tuteja, 2010; Hossain et al., 2015). To prevent ROS oxidative damage, plants up-regulate antioxidant enzymes and molecules to strike a balance between the formation rate and ROS removal (Munns and Tester, 2008). Salt-induced ROS are predominantly

represented by H_2O_2 (Pang and Wang, 2008). Although H_2O_2 has been described to play a signaling role to plant processes related with abiotic stress acclimation in the last decade: antioxidative defense, up-/down-regulation of ABA, promotion of gibberellic acid biosynthesis or improvement of the K^+/Na^+ ratio in seedlings (Kim et al., 2008; Shu et al., 2016; Formentin et al., 2018; Niu et al., 2018). Furthermore, H_2O_2 has been considered a second messenger as it mediates adaptive responses to abiotic stress (Neill et al., 2002; Yu et al., 2003; Liu et al., 2010; Baxter et al., 2014; Hossain et al., 2015). H_2O_2 accumulation has also been found to precede signaling activation, or has even been found to be the consequence of signaling (Hossain et al., 2015). Under our salinity conditions, all the plant combinations increased the H_2O_2 concentration to show significant differences with its control. Particularly in A/N plants, H_2O_2 levels were the highest (with significant differences) compared to other plant combinations. The increased H_2O_2 in A/N plants has been associated with higher total antioxidant capacity and lower lipid peroxidation, with a less marked effect on the photosynthetic system (Zandalinas et al., 2016). In tomato, an enhanced H_2O_2 level has been found to modulate the expression of stress and to up the defense genes related with antioxidant capacity (Zhou et al., 2014). A, A/A and N/A plants showed minor antioxidant capacity (and significant differences compared to A/N plants) and a major MDA concentration, which tends to show greater lipid peroxidation in salt-sensitive than salt-tolerant cultivars under salt stress (Zhu et al., 2008; Penella et al., 2015) thereby inhibiting biomass production (MDA concentration-total DW, R^2= 0.70 at 10DAT). These results indicate that H_2O_2 could be positively used by A/N plants to activate antioxidant capacity to help fight against salt stress (Hossain et al., 2015) by acting as a signal

molecule rather than a damaged plant system (Bose et al., 2014; Rejeb et al., 2015; Formentin et al., 2018). Other molecules like proline and phenols could work well for salinity protection (Parida and Das, 2005; Ashraf and Foolad, 2007; Szabados and Savouré, 2010). Some studies suggest that proline may play a role as an enzyme-stabilizing agent under NaCl stress (Demir and Kocaçaliskan, 2001), reduce peroxidative damage to lipid membranes due to salt-dependent oxidative stress (Huang et al., 2009), and play an important role as a compatible osmolyte (Szabados and Savouré, 2010). A_N-enhanced proline biosynthesis has been described to help prevent photosynthetic apparatus damage (Ashraf et al., 2008). The increase in proline (2-fold times) herein observed was detected in the plant combination in which N was used as both a scion and rootstock compared to plants A or A/A. These results could indicate that N is implicated in more proline transport from roots to leaves for A/N plants by contributing to proline accumulation in leaves and/or could stimulate proline synthesis in N/A plant leaves (An et al., 2013). Although proline metabolism has long since been studied in several crops, very little is known about the signaling pathways, biosynthesis, degradation and transport that regulate stress-induced accumulation, and this knowledge is vital to develop plants for stress tolerance (Kishor et al., 2005; Szabados and Savouré, 2010).

Another metabolic process to be associated with tolerance responses to salinity stress in plants involves phenolic compounds (Parida and Das, 2005). Increasing phenolic content has been correlated with salt stress tolerance in watermelon plants grafted onto squash (Evrenosoğlu et al., 2010) or in tomato-grafted plants (Ali and Ismail, 2014). Phenol compounds help avoid ROS

formation, display antioxidant action and protect the photosynthetic apparatus (Harborne and Williams, 2000). According to our results, a significant increase in total phenols was detected in A/N plants under salinity treatment compared to the other plant combinations, which coincides with antioxidant capacity stimulation, minor lipid peroxidation formation and higher photosynthetic rates.

Grafting is an integrative reciprocal process in which both the scion and rootstock can influence salt tolerance (Etehadnia et al., 2008). The importance of root characteristics in regulating salinity has been documented mainly in terms of the role in the control of toxic ions, water uptake, biomass and molecules signaling from root to leaves that modulate plant responses to salinity (Albacete et al., 2009; He et al., 2009; Colla et al., 2010; Niu et al., 2018; Penella and Calatayud, 2018). In contrast, other authors (Santa-Cruz et al., 2002; Chen et al., 2003; Zhu et al., 2008) have suggested that salt tolerance in grafted plants is attributed to the scion genotype. This might be due to either differences in the salt tolerance of both the rootstock and scion used in the experiments or the applied salinity dose (Huang et al., 2013). In this study, the reciprocal graft (N/A) was done to examine whether plant tolerance to salinity can be helped by the rootstock or scion. In response to salinity N/A plants showed dramatically reduced photosynthesis and biomass, similarly to that obtained in A plants and A/A, which was associated with other physiological factors like greater stomatal resistance and higher ABA leaf concentration, minor phenol levels and lower antioxidant activities with major lipid peroxidation. These results suggest that A roots are less able to adapt to changes under salinity. Similar results have been found in cucumber grafted onto luffa under drought stress (Liu et al., 2013) or in cucumber grafted onto pumpkin under salinity

(Huang et al., 2013), where reciprocal grafted plants showed no salinity tolerance. However, different tolerant mechanisms to cope with salinity can be used in grafted plants (Colla et al., 2010).

This work has led to a better understanding of the response mechanisms of grafted plants to imbalanced salinity. We demonstrate that the new pepper rootstock, NIBER®, could influence scion behavior by preserving its plant physiology performance and growth. The time-course analysis showed that the reduction in ABA leaf content in the plants grafted on to NIBER® under salinity allowed to keep stomata open, strike an appropriate photosynthesis balance and lead to NR activation. The increases in endogenous H_2O_2 in these plants acted as a signaling molecule by activating the defense mechanism (increase in total antioxidant capacity, proline and phenols), which tips the balance to ROS scavenging. The least damage caused to the metabolism in the plants grafted onto NIBER® was strengthened to maintain ion homeostasis in relation to the ability to lower the Na^+/K^+ ratio, all of which mitigating the reduction of the biomass imposed by salt stress. This ability is a cost-effective trait of salt tolerance in plants.

Authors`contributions

LLS, CP and AC conceived and designed the experiments. LLS, GCS, GVS, CP and AC performed the experiments. AS, SLG, LLS and AC analyzed the data and discussed the study results. AC and SLG wrote the paper. All the authors read and approved the manuscript.

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Figure legends

Fig. 1. The Na^+/K^+ ratio (A, B) and Cl^- concentration (C, D) in the leaves and roots of ungrafted pepper plants (cultivar Adige, A), self-grafted (A/A), A grafted onto N (A/N) and N grafted onto A (N/A) after addition of NaCl at 0 mM (Control) and 70 mM (Salt) for 10-day exposures. Data are the mean values for n=4. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 2. The net CO_2 assimilation rate (A_N; µmol CO_2 m^{-2} s^{-1}) of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=12. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 3. Leaf stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) of the ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=12. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 4. ABA contents (ng ABA g^{-1} DW) in the leaves (A, C) and roots (B, D) of the ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0

mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each plant combination and time, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 5. Nitrate reductase activity expressed as a percentage compared to its control (% vs. control) in the leaves of the ungrafted pepper plants (cultivar Adige, A), self-grated plants (A/A), A grafted onto N (A/N) and N grafted onto A (N/A) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each time, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 6. Proline concentration (mg Pro g-1 DW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

Fig. 7. Changes in phenolic content (mg GA g^{-1} FW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4.

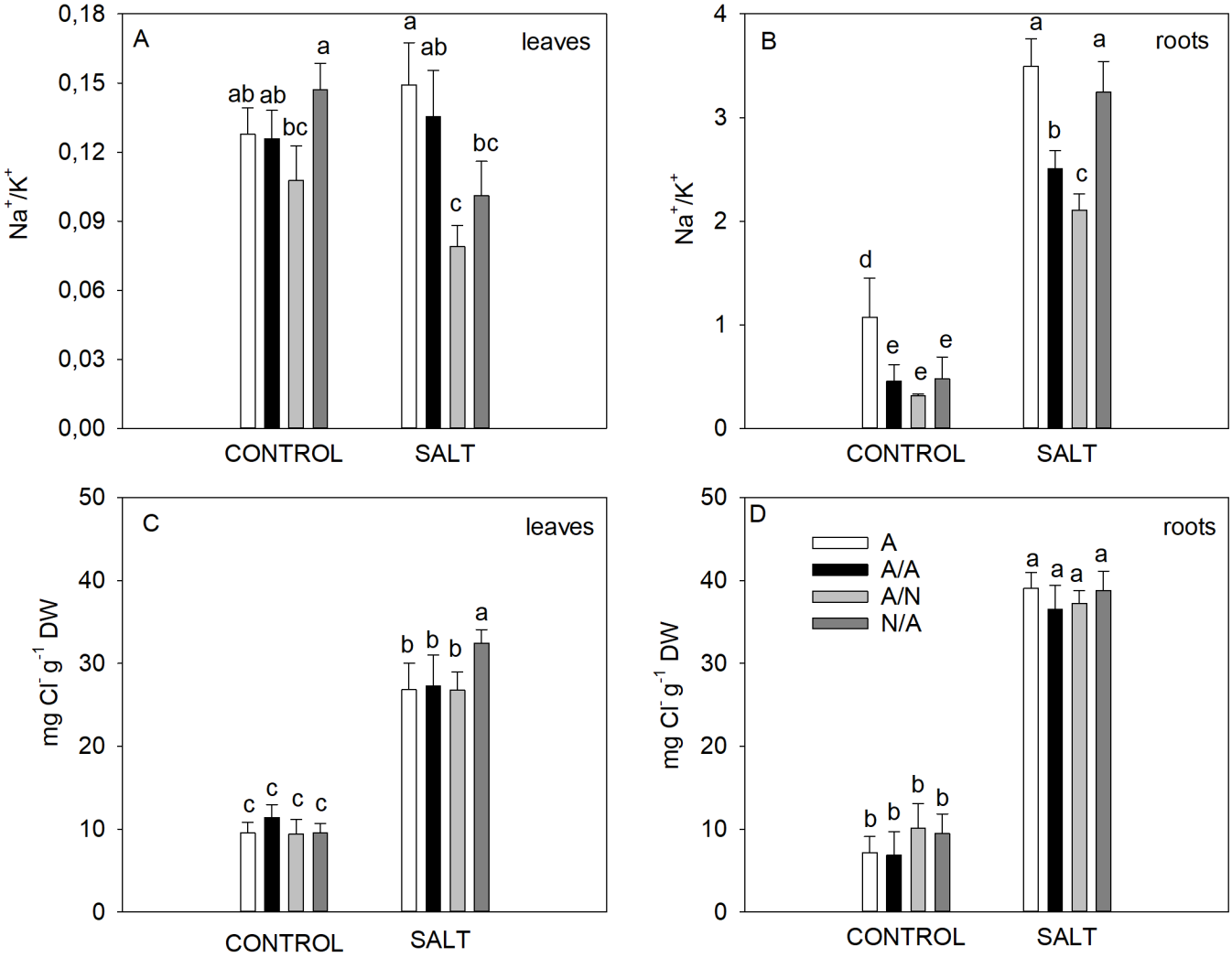
For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

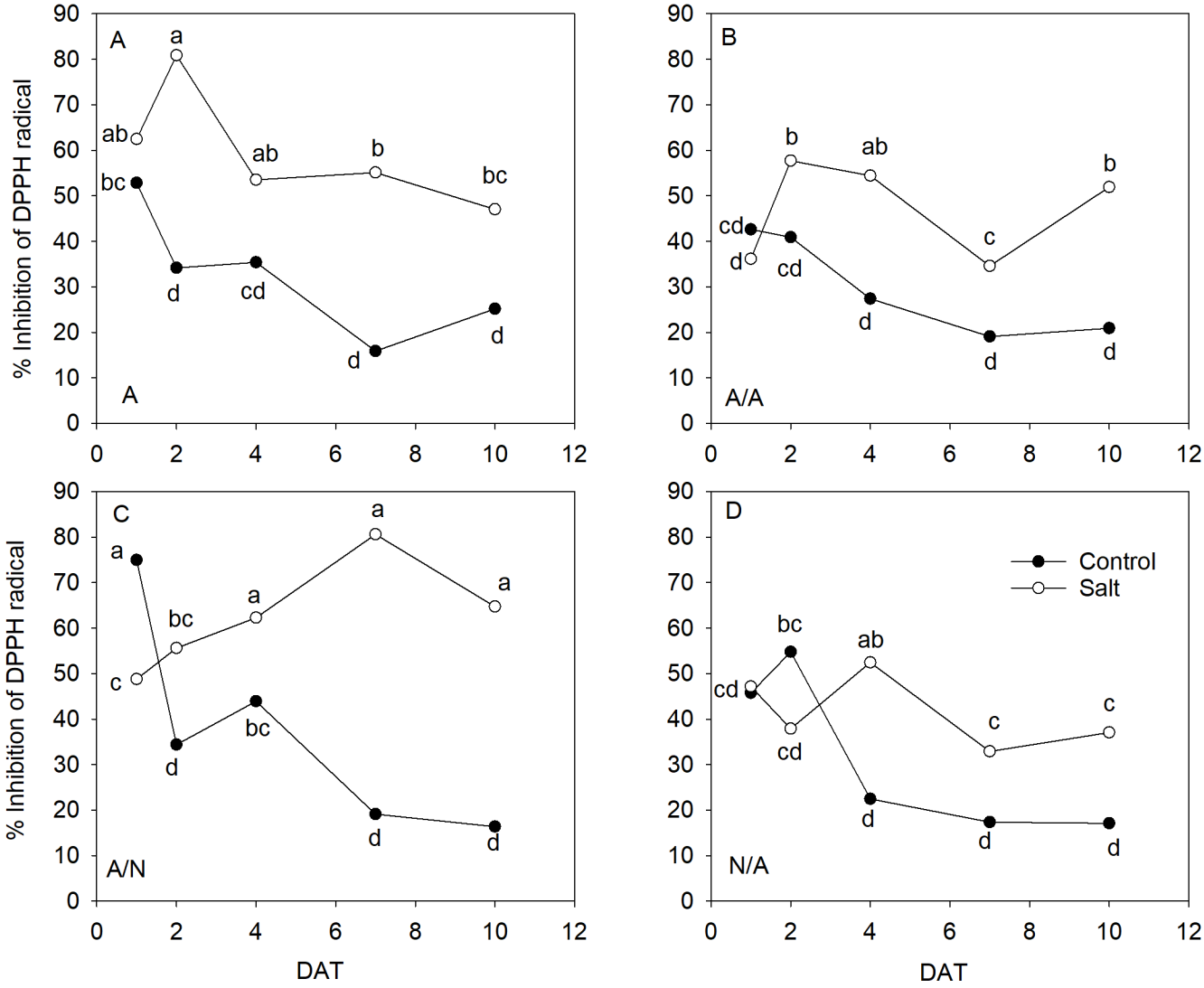
Fig. 8. Hydrogen peroxide concentration (nmol H_2O_2 g^{-1} FW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

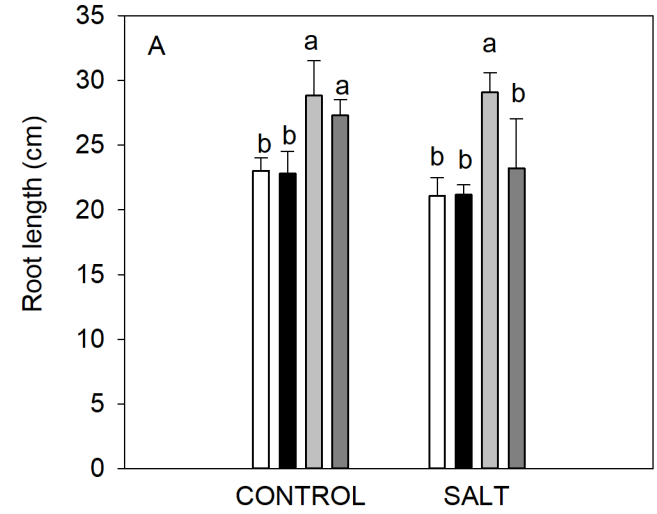
Fig. 9. Malondialdehyde (MDA) content (nmol MDA g^{-1}FW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

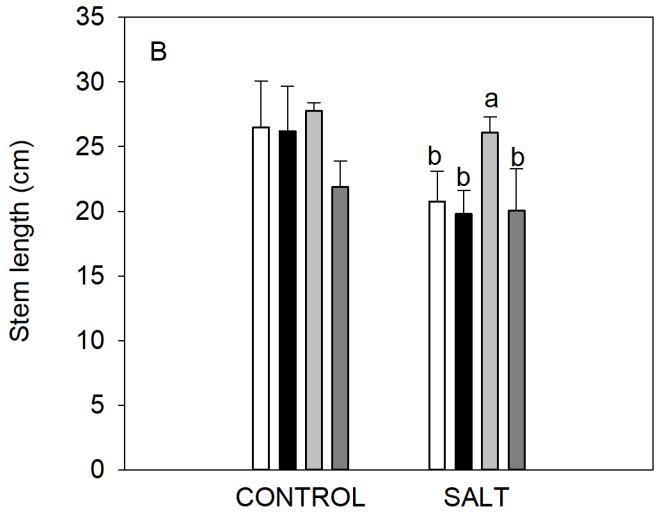
Fig. 10. Percentage of inhibition of DPPH radical in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

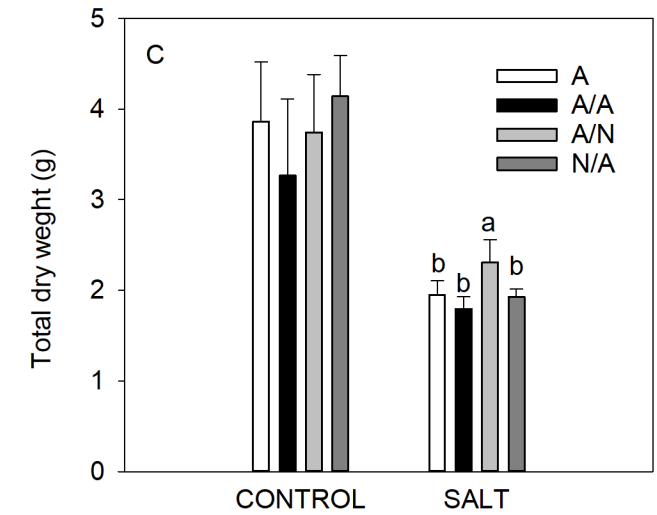
Fig. 11. Root length (A), stem length (B) and total dry weigh (roots + stem + leaves) (C) of the ungrafted pepper plants (cultivar Adige, A), self-grated plants (A/A), A grafted onto N (A/N) and N grafted onto A (N/A) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each plant combination and treatment, different letters indicate significant differences at $P < 0.05$ (LSD test). Not significant differences for stem length and total dry weight under control conditions are denoted with the absence of the letters above the bars.

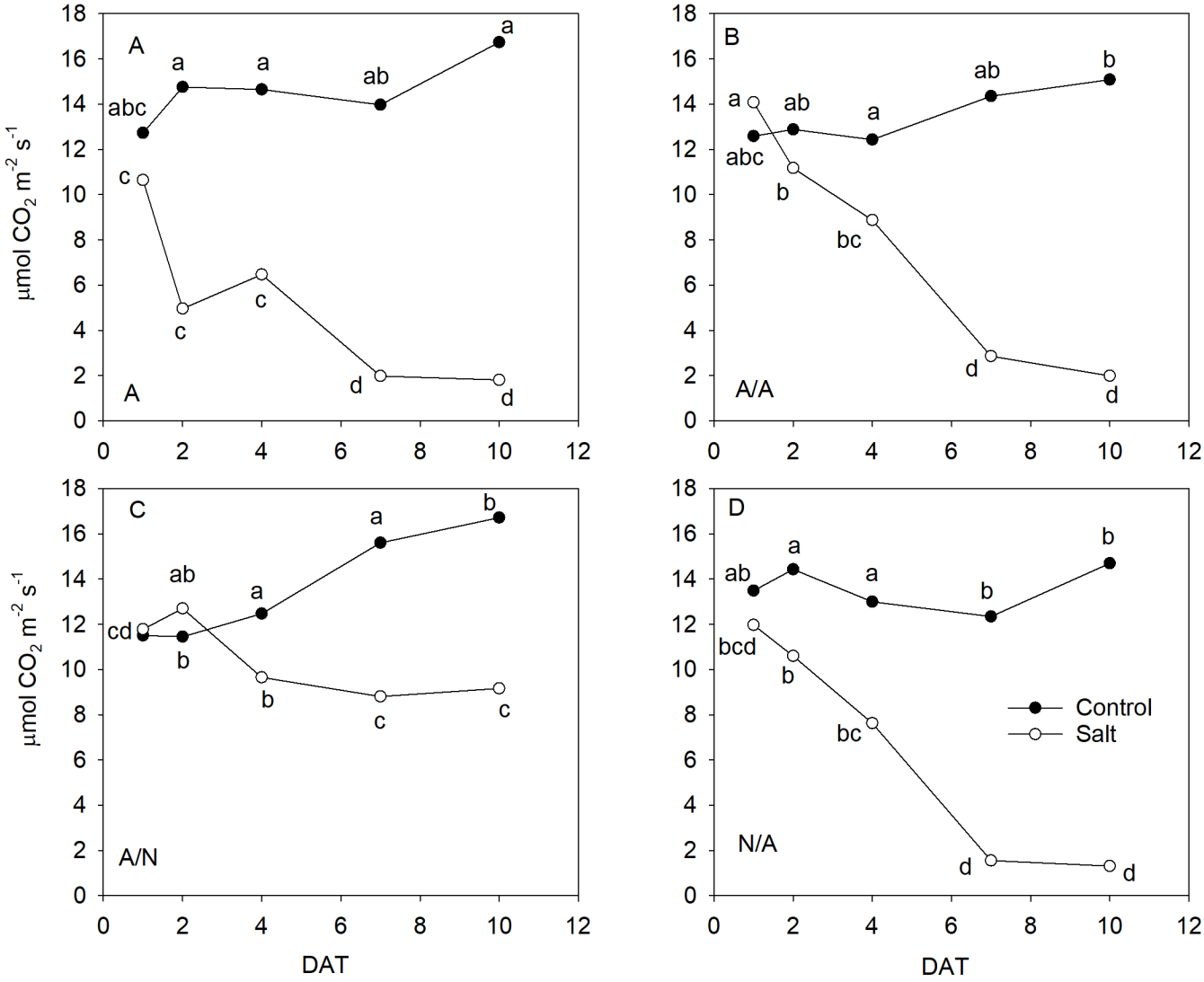

A
leaves
Na+/K+
0,18
0,15
0,12
0,09
0,06
0,03
0,00
ab ab bc a
a ab c bc
CONTROL
SALT
B
roots
Na+/K+
4
3
2
1
0
d e e e
a b c a
CONTROL
SALT
C
leaves
mg Cl- g-1 DW
50
40
30
20
10
0
c c c c
b b b a
CONTROL
SALT
D
roots
A
A/A
A/N
N/A
mg Cl- g-1 DW
50
40
30
20
10
0
b b b b
a a a a
CONTROL
SALT

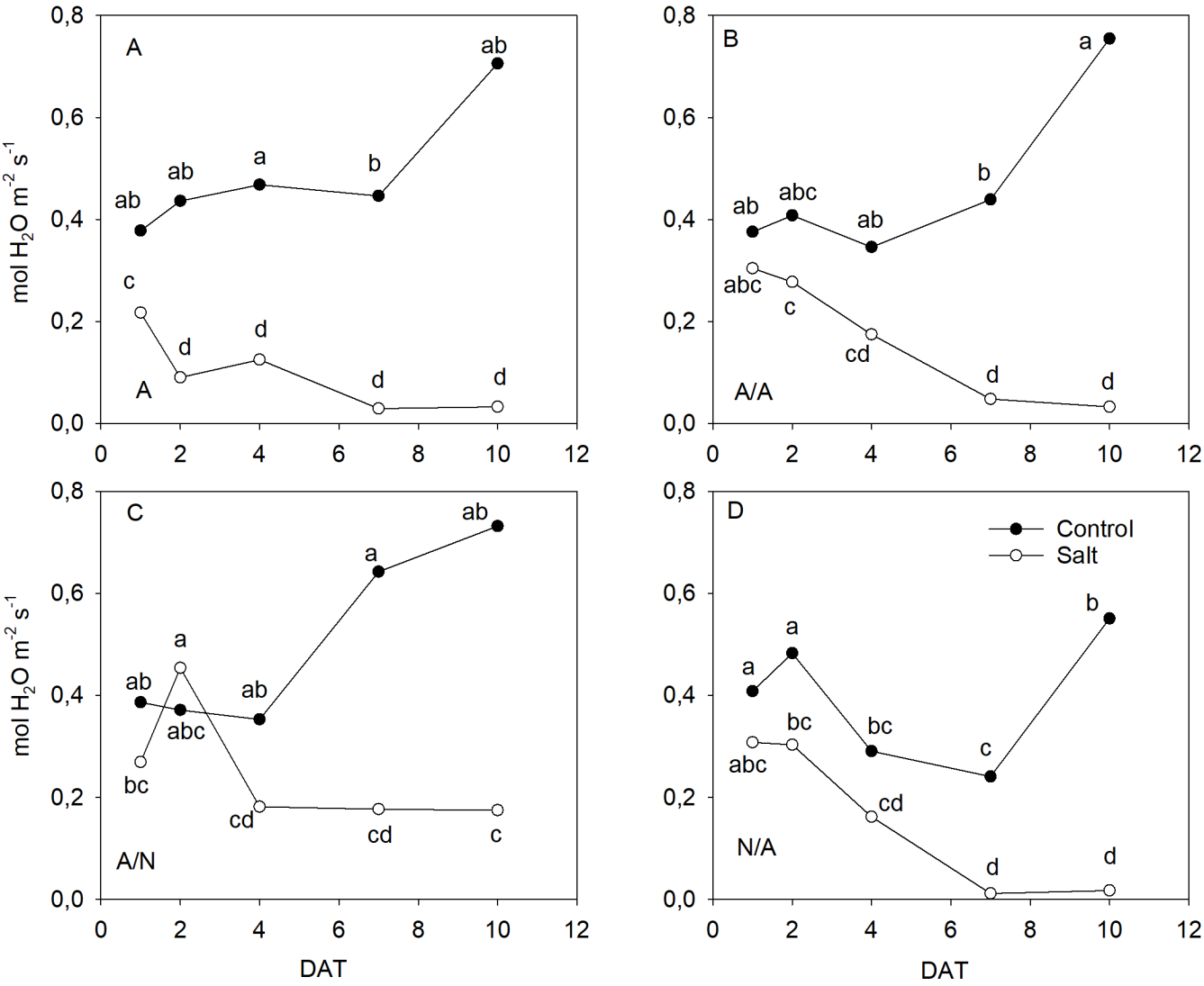

A
A
B
A/A
C
A/N
D
N/A
Control
Salt
% Inhibition of DPPH radical
DAT

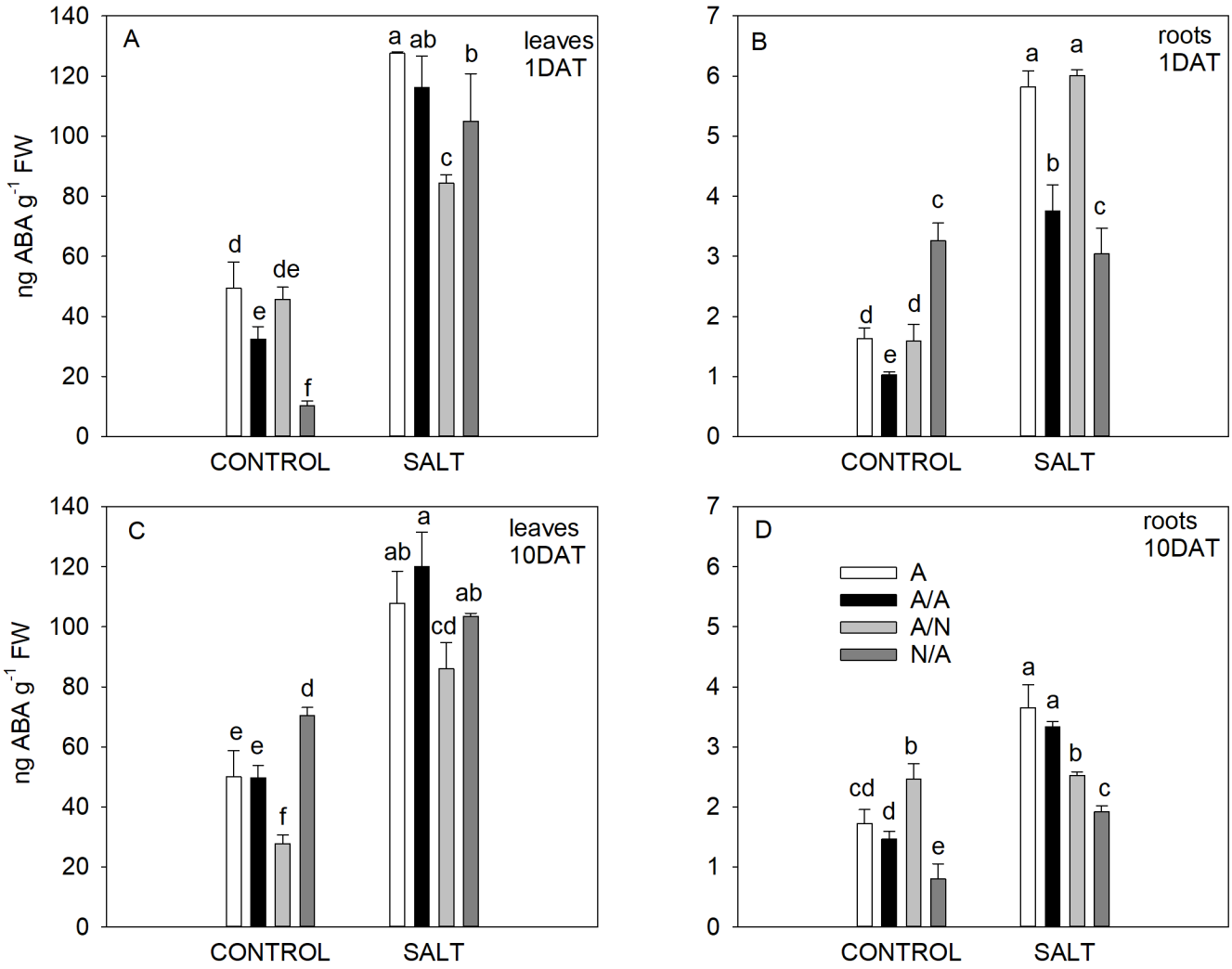

A
Root length (cm)
35
30
25
20
15
10
5
0
b
b
a
a
b
b
a
b
CONTROL
SALT

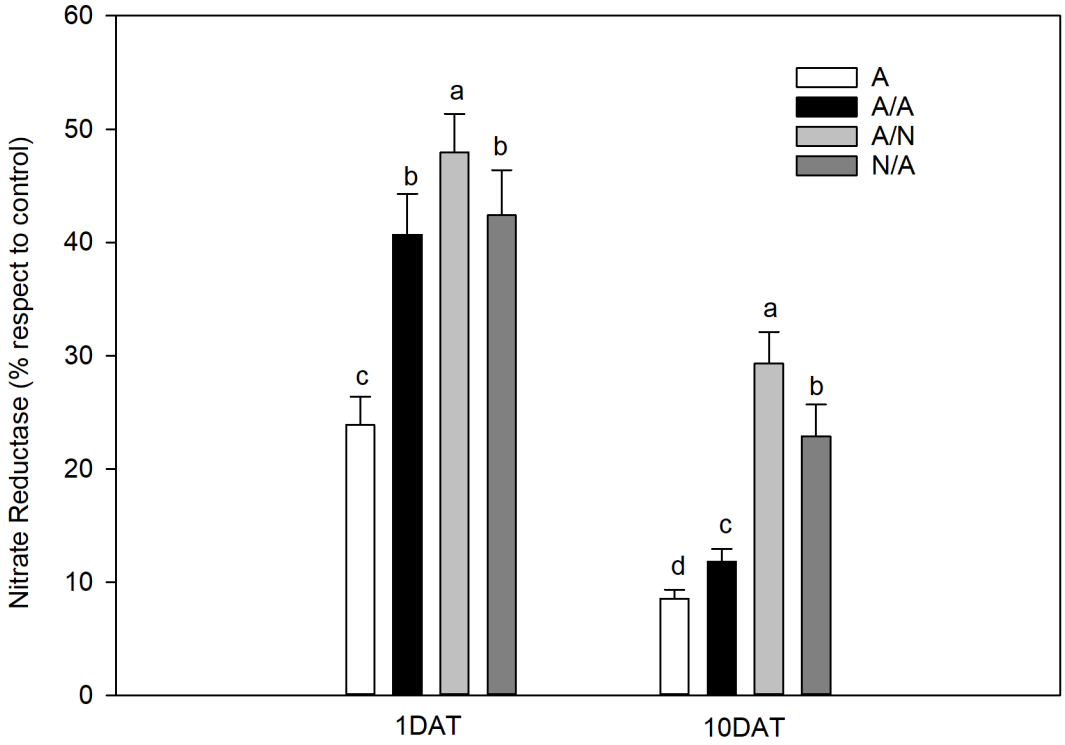

B
Stem length (cm)
35
30
25
20
15
10
5
0
b
b
a
b
CONTROL
SALT

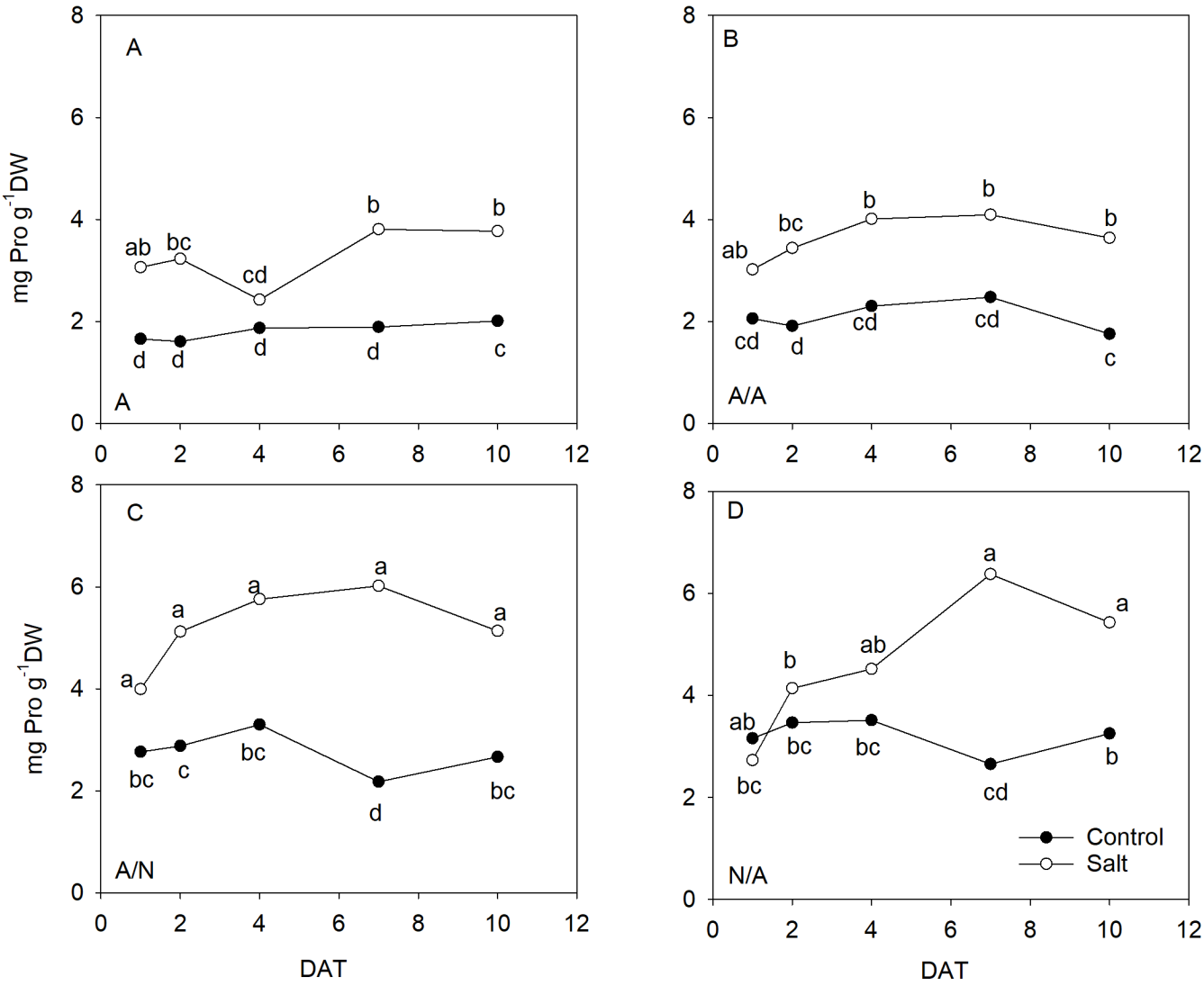

C
Total dry weght (g)
5
4
3
2
1
0
b
b
a
b
A
A/A
A/N
N/A
CONTROL
SALT

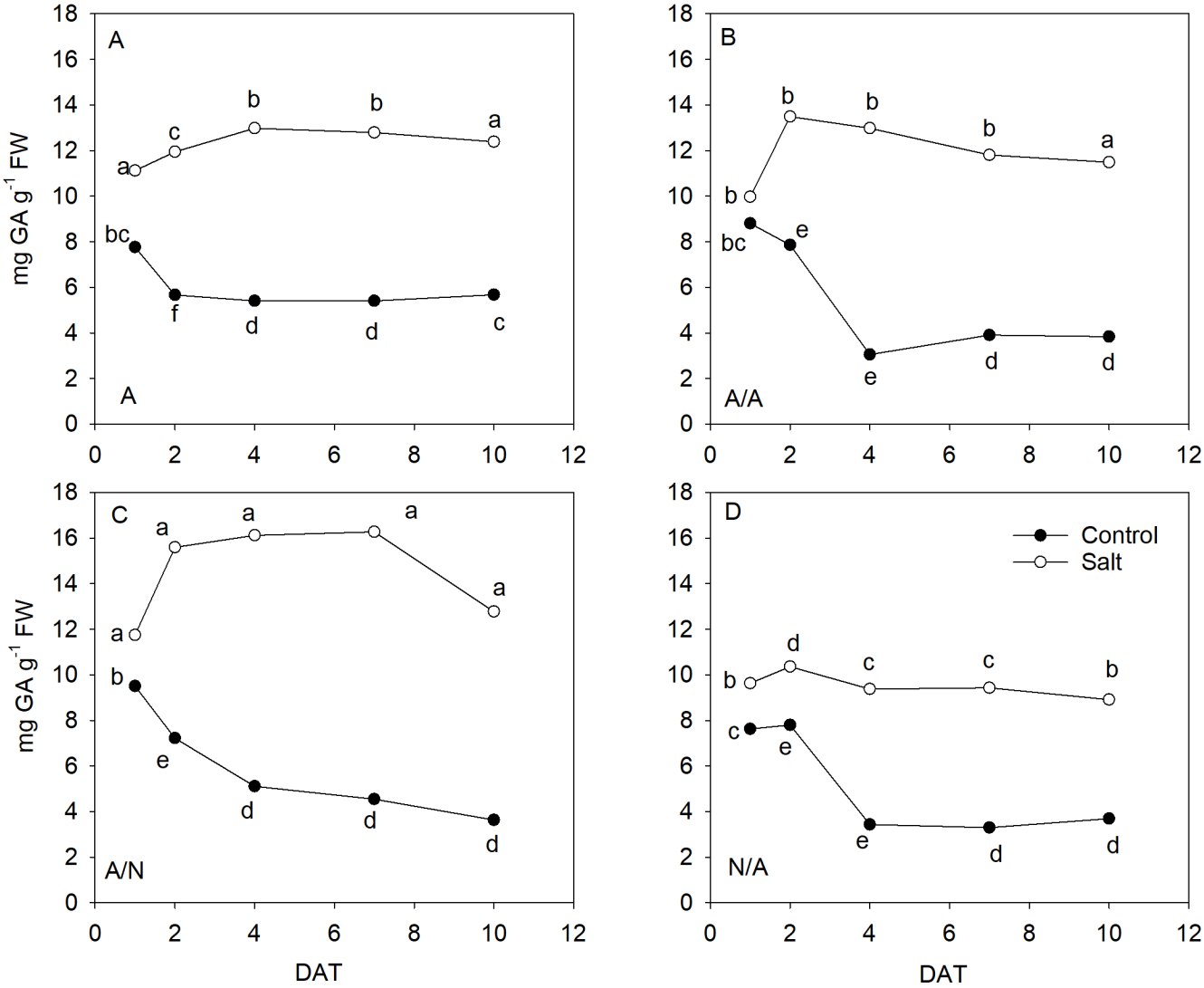

A
B
C
D
A
A/A
A/N
N/A
µmol CO2 m-2 s-1
DAT
Control
Salt

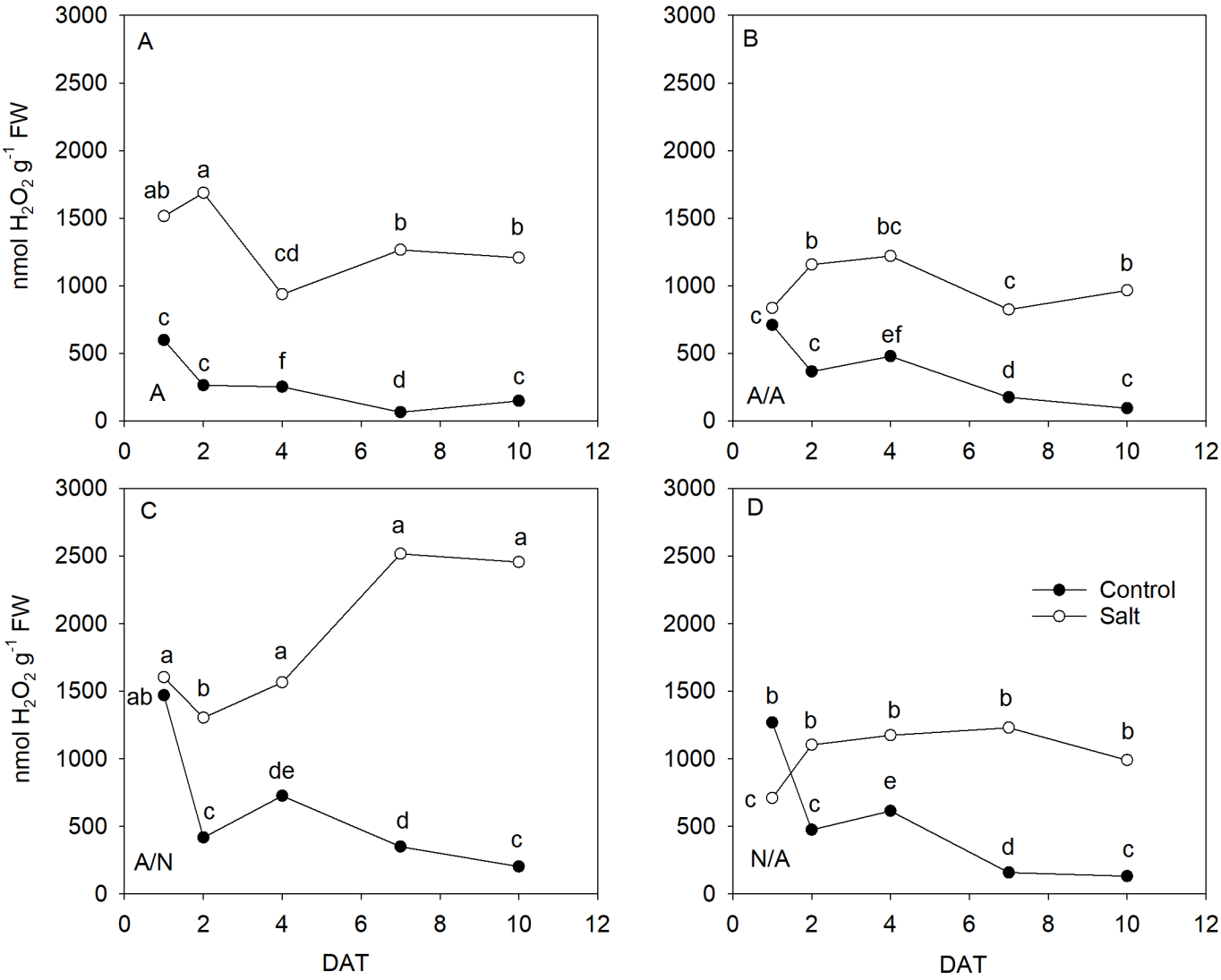

A
B
C
D
A
A/A
A/N
N/A
Control
Salt
mol H2O m-2 s-1
DAT
0,0
0,2
0,4
0,6
0,8
0
2
4
6
8
10
12


A
leaves
1DAT
ng ABA g-1 FW
140
120
100
80
60
40
20
0
d
e
de
f
a
ab
c
b
CONTROL
SALT
B
roots
1DAT
7
6
5
4
3
2
1
0
d
e
d
c
a
b
a
c
CONTROL
SALT
C
leaves
10DAT
ng ABA g-1 FW
e
e
f
d
ab
a
cd
ab
CONTROL
SALT
D
roots
10DAT
A
A/A
A/N
N/A
cd
d
b
e
a
a
b
c
CONTROL
SALT


Nitrate Reductase (% respect to control)
60
50
40
30
20
10
0
A
A/A
A/N
N/A
c
b
a
b
d
c
a
b
1DAT
10DAT


A
B
C
D
A
A/A
A/N
N/A
mg Pro g-1DW
DAT
Control
Salt


A
B
C
D
A
A/A
A/N
N/A
Control
Salt
mg GA g-1 FW
DAT


A
B
C
D
A
A/A
A/N
N/A
Control
Salt
nmol H2O2 g-1 FW
DAT
0
500
1000
1500
2000
2500
3000
0
2
4
6
8
10
12
ab
a
cd
b
b
c
c
f
d
c
c
b
bc
c
b
c
c
ef
d
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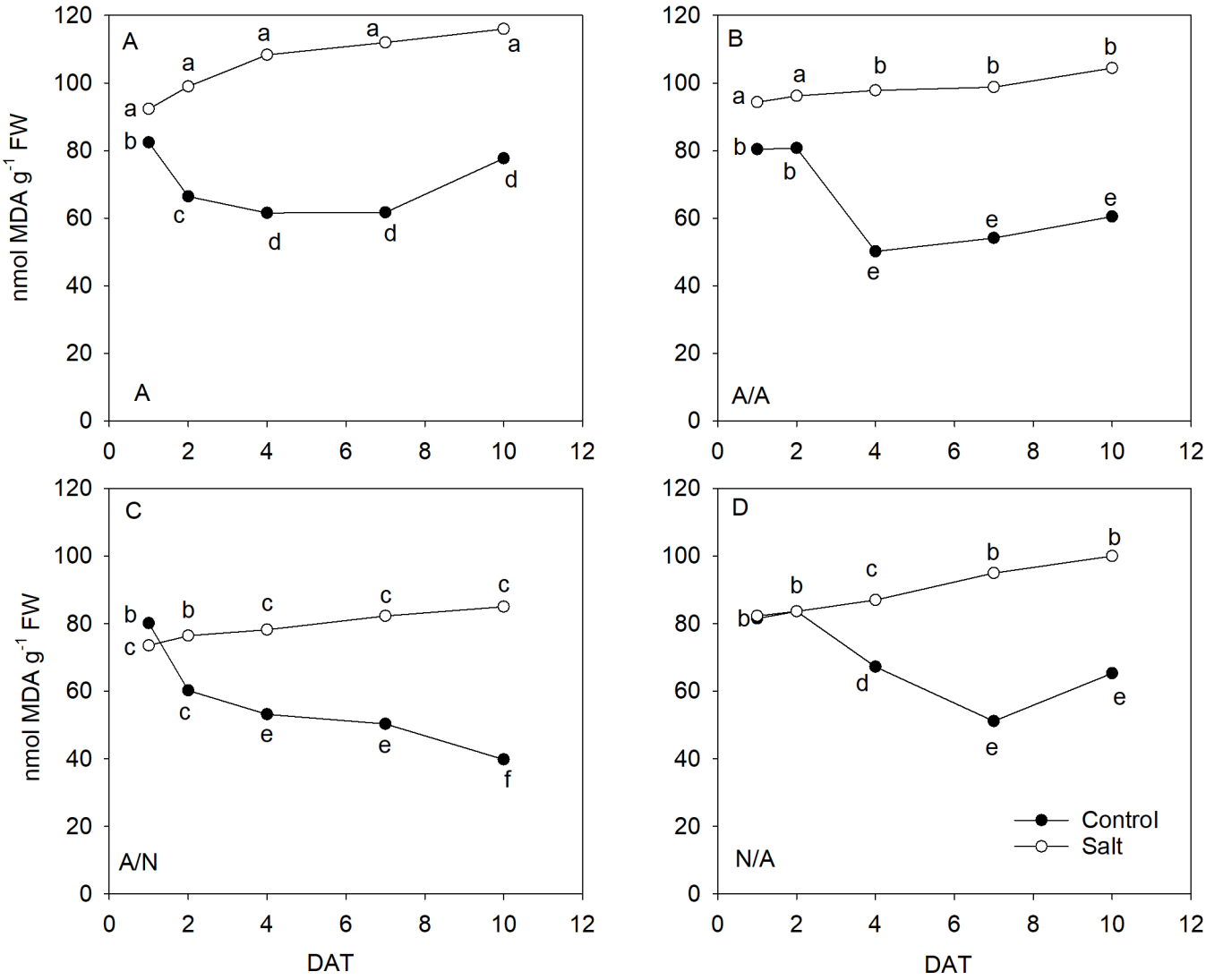
b
b
b
b
b
c
c
e
d
c


A
B
C
D
A
A/A
A/N
N/A
nmol MDA g-1 FW
DAT
Control
Salt

HIGHLIGHTS

NIBER® is a hybrid pepper rootstock tolerant to salinity with high yields in this condition.

Using NIBER® as rootstock improve physiological mechanisms in the scion, inducing its tolerance.

NIBER®-grafted pepper plants exhibit a decreased Na^+/K^+ ratio, ABA content and lipid peroxidation activity.

The tolerance was achieved activating the antioxidant capacity, enhancing H_2O_2 and increasing phenols and proline contents.

Authors`contributions

LLS, CP and AC conceived and designed the experiments. LLS, GCS, GVS, CP and AC performed the experiments. AS, SLG, LLS and AC analyzed the data and discussed the study results. AC and SLG wrote the paper. All the authors read and approved the manuscript.

Interest statement

I certify that all authors have seen and approved the final version of the manuscript being submitted.

Any authors in this manuscript any financial and personal relationships with other people or organizations that could inappropriately influence their work.

The manuscript has not been published and is not under consideration in the same or substantially similar form in any other journal.