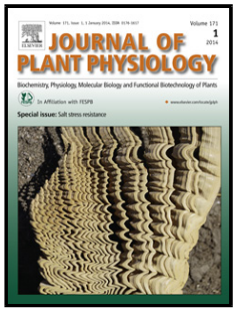

Title: Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: physiological responses

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Table 1

Osmotic adjustment (MPa) in the grafted pepper plants (cultivar 'Verset') onto the pepper accessions 5, 8, 12 and 14. Ungrafted 'Verset' plants were used as controls. Determinations were performed after 7 (T1) and 14 (T2) days under water stress conditions by PEG addition (3.5% and 7%). Each value is the mean of six independent determinations.

		Cultivar	5	8	12	14
T1	3.5% PEG	0.81*	0.12	0.25	0.27	1.17*
	7% PEG	0.07	-0.30	-0.41	2.12*	1.38*
T2	3.5% PEG	0.23	0.04	-0.09	0.61*	1.25*
	7% PEG	0.06	-0.27	-0.41	0.98*	1.71*

Significant differences in relation to controls (0% PEG and full turgor) (P<0.05) are indicated by asterisks

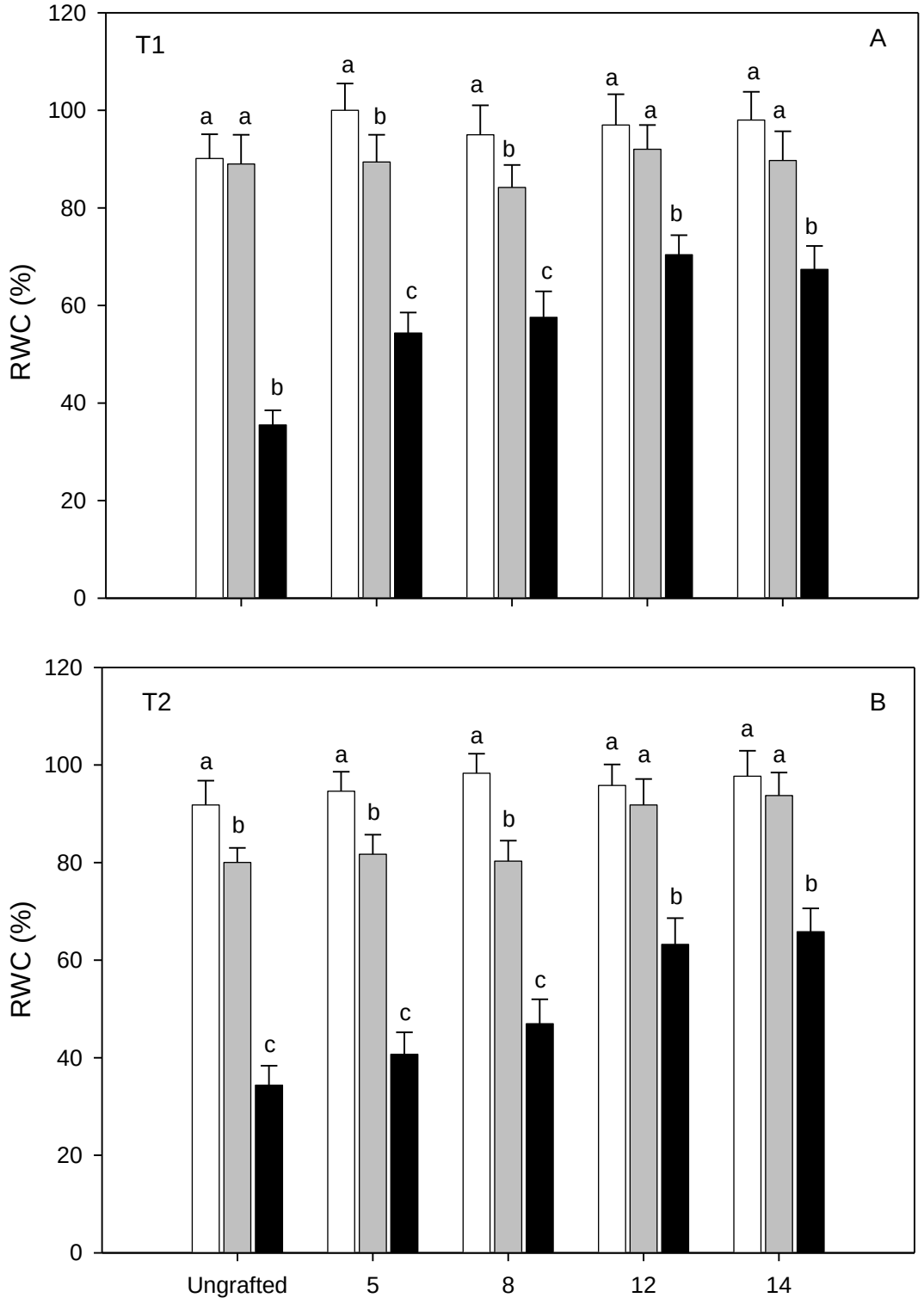

Fig. 1. Effect of PEG addition at 0% (□), 3.5% (▒) and 7% (■) on relative leaf water content (RWC %) during 7 day (A) and 14 day exposure (B) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14. Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at $P<0.05$ (LSD test).

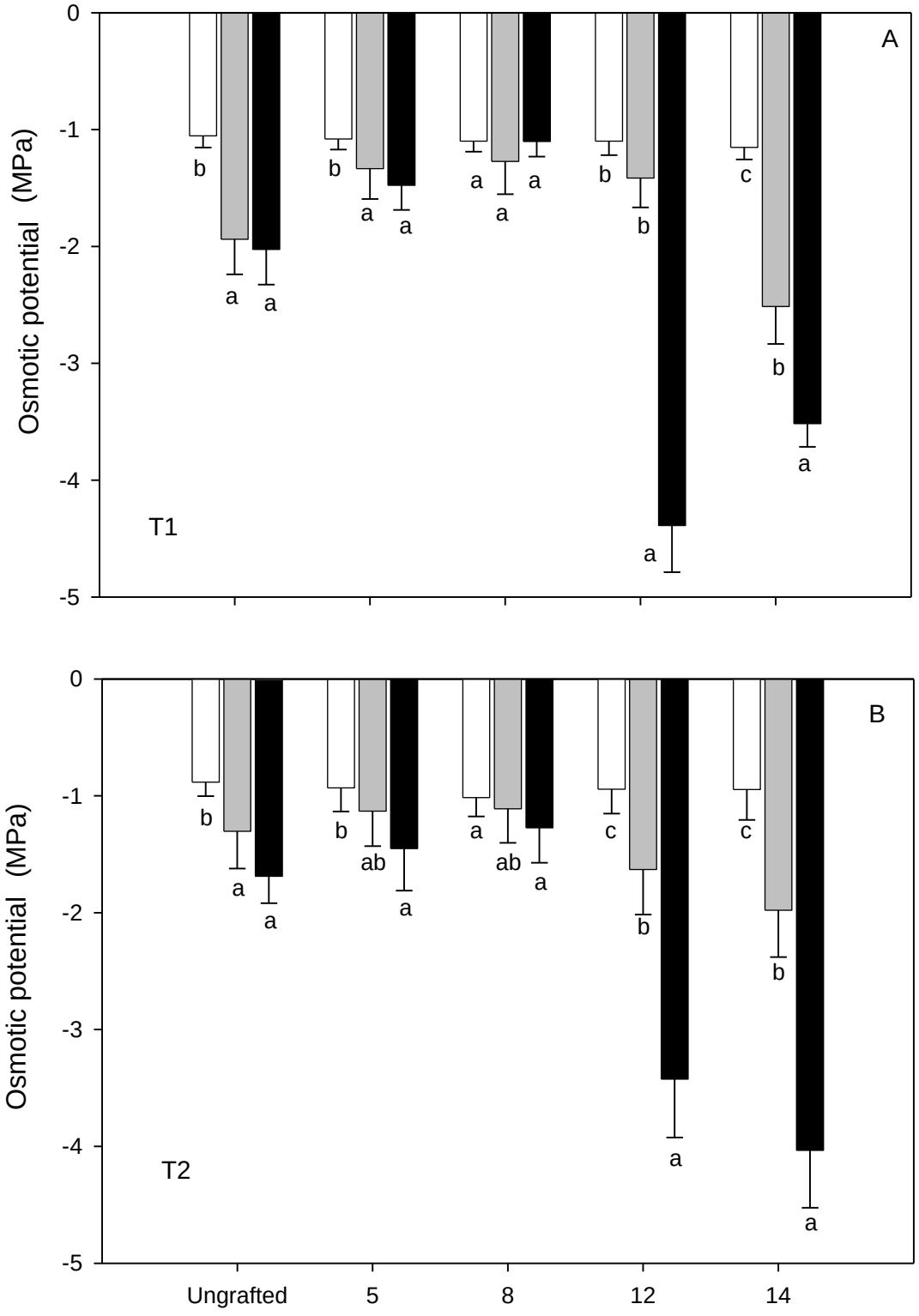

Fig. 2. Leaf osmotic potential (MPa) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during 7 day (A) and 14 day exposure (B). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at $P<0.05$ (LSD test).

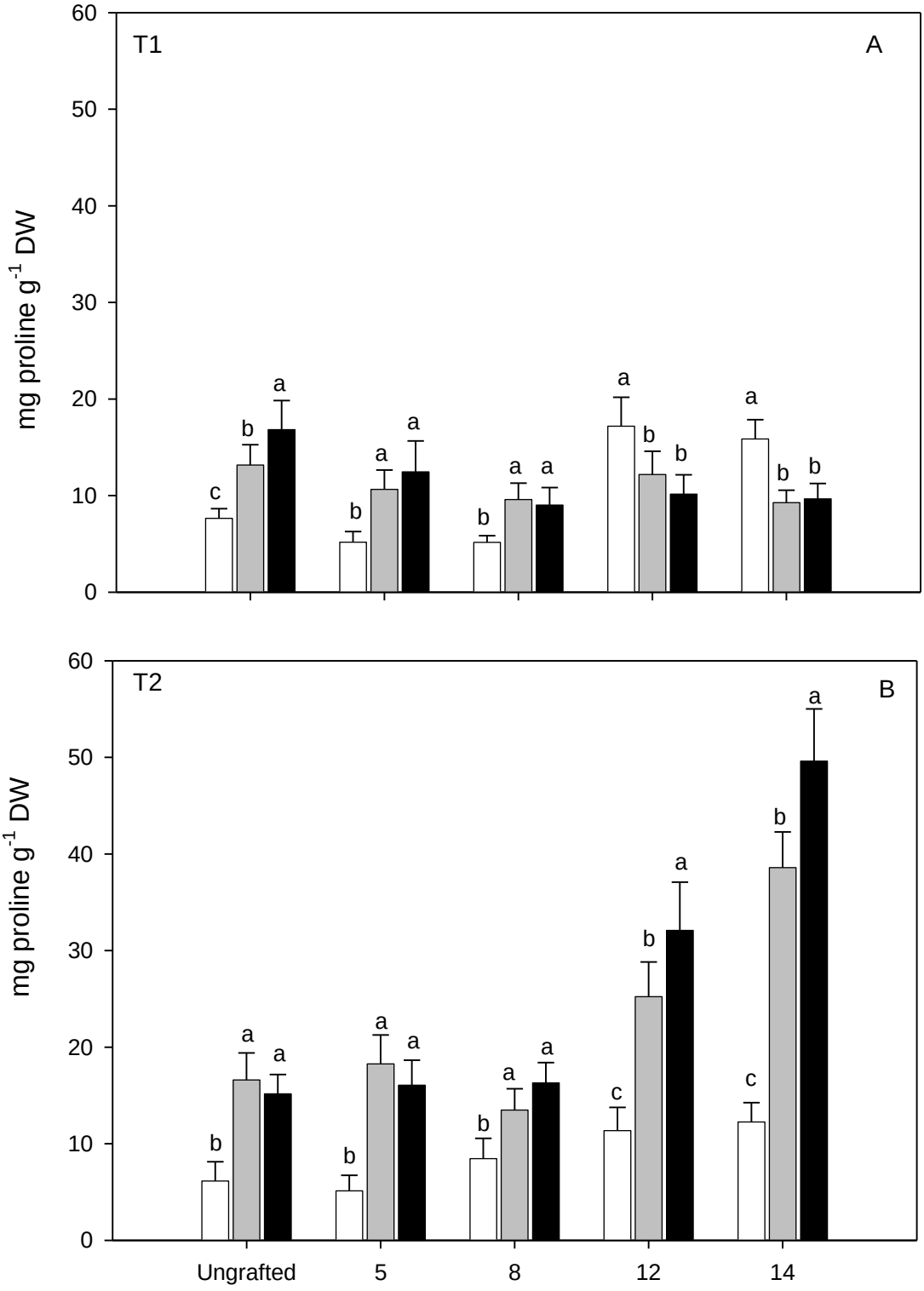

Fig. 3. Changes in proline concentration (mg proline /g DW) from ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during 7 day (A) and 14 day exposure (B). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at *P*<0.05 (LSD test).

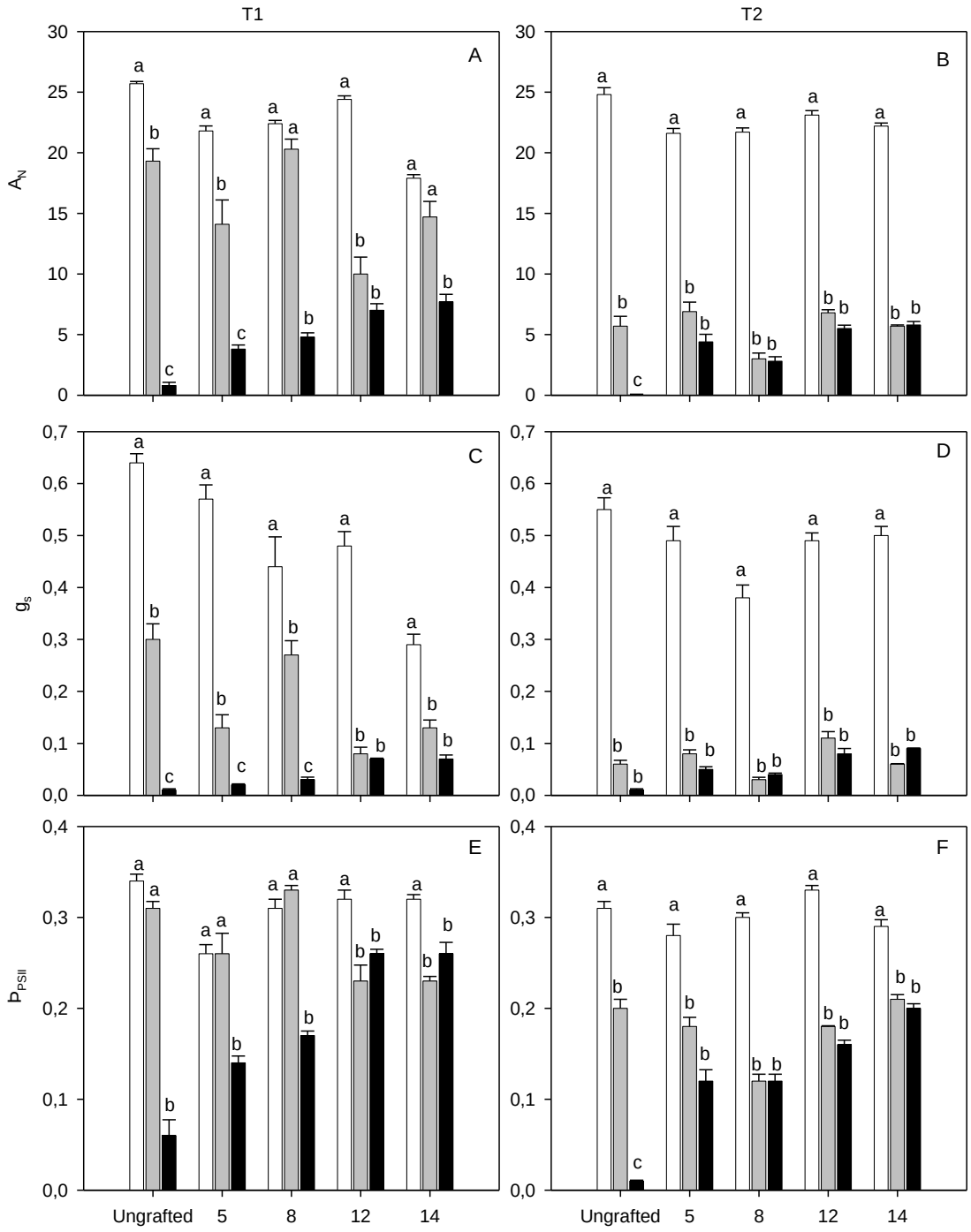

Fig. 4. Net CO_2 assimilation rate (A_N; μmol CO_2 m^{-2} s^{-1}) (A, B); leaf stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) (C, D) and actual quantum efficiency of PSII (ϕPSII) (E, F) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■)during 7 day (A, C, D) and 14 day exposure (B, D, F). Dates are mean values ± SE for n= 10. Within each plant combination different letters indicate significant differences at P<0.05 (LSD test).

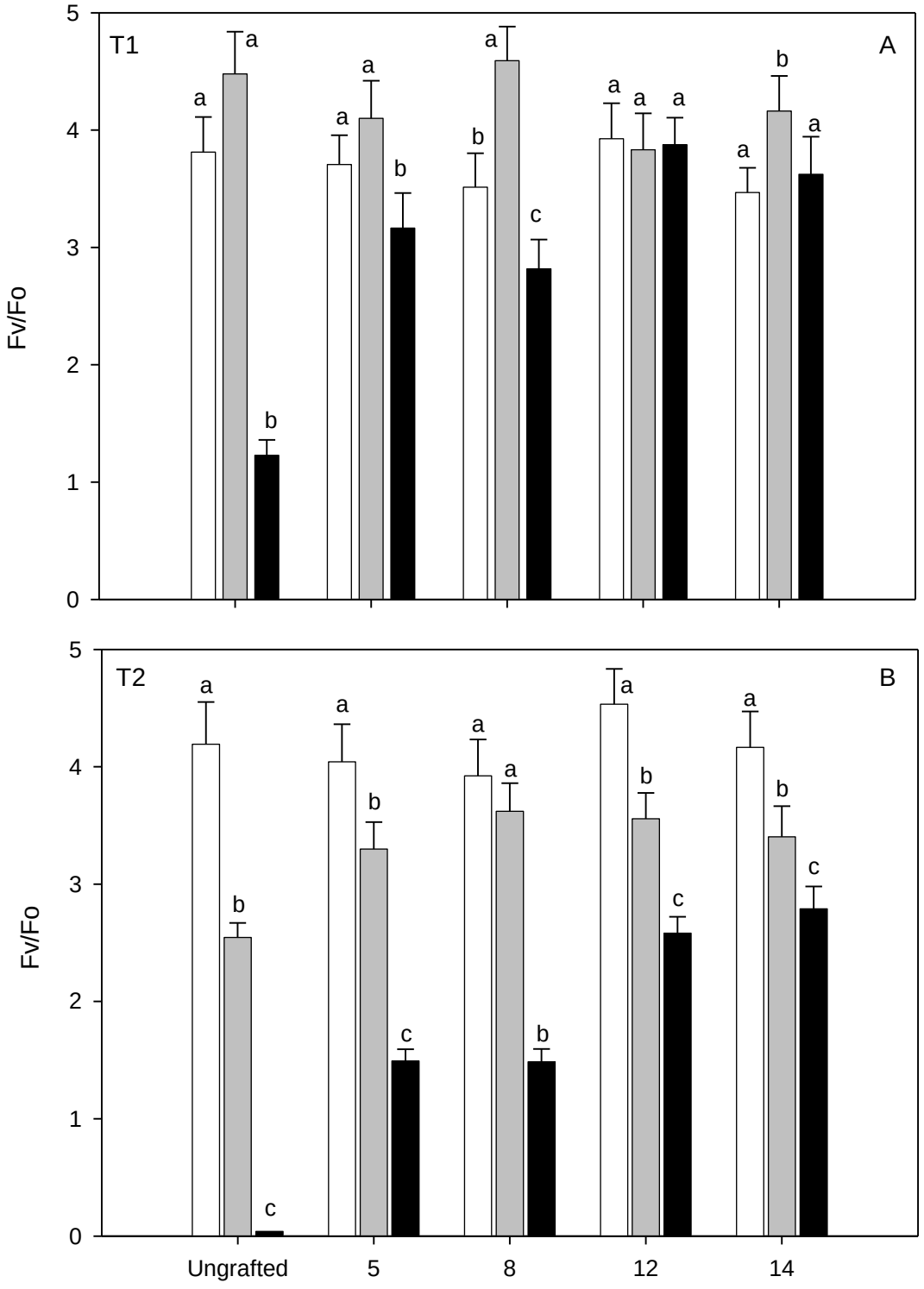

Fig. 5. Variations in dark-adapted Fv/Fo ratio in leaves of ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during 7 day (A) and 14 day exposure (B). Dates are mean values ± SE for n= 10. Within each plant combination different letters indicate significant differences at $P<0.05$ (LSD test).

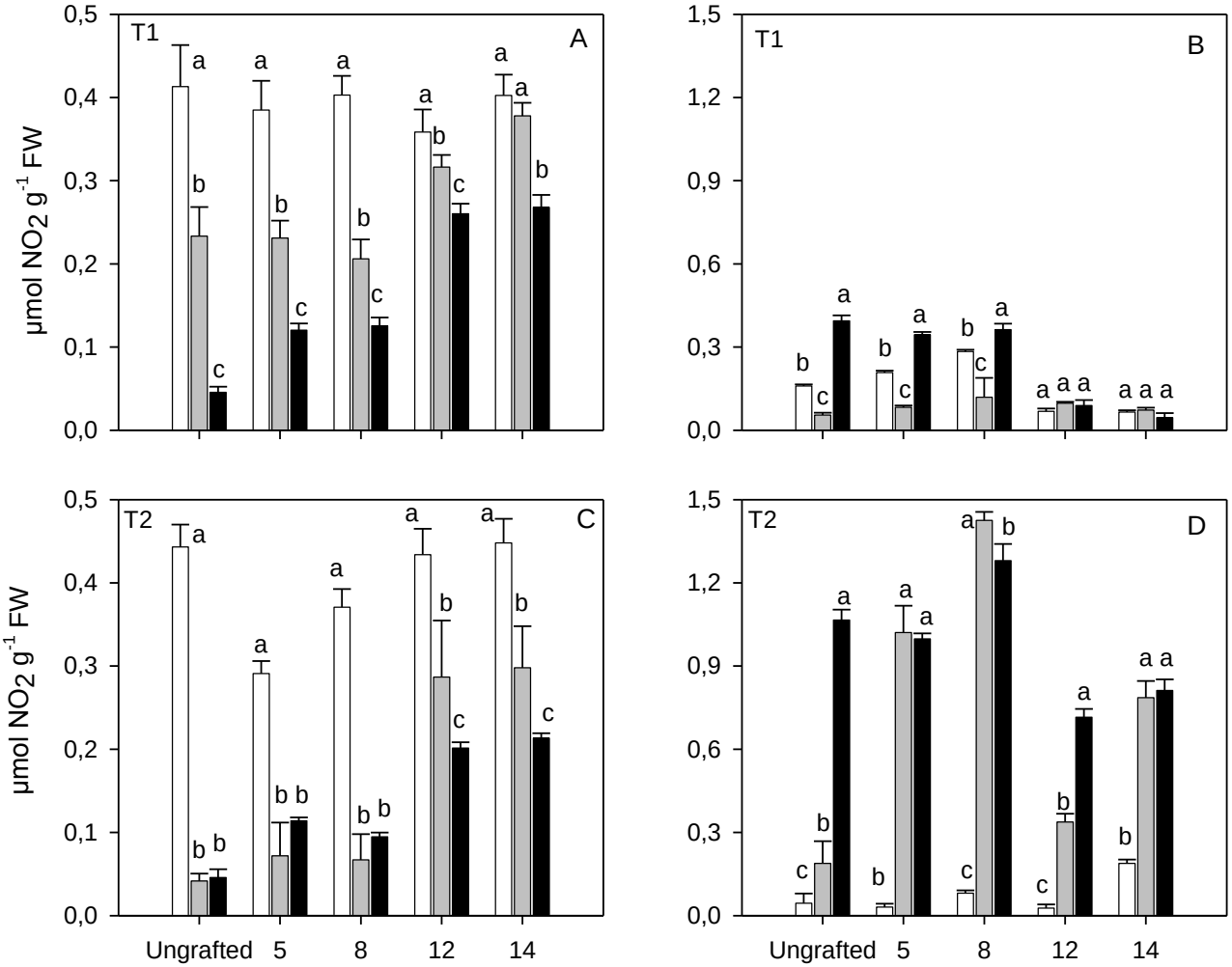

Fig. 6. Nitrate reductase activity (µmol NO_2 g^{-1} FW) in leaf (A, C) and roots (B, D) of ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▬) and 7% (■) during 7 day (A, B) and 14 day exposure (C, D). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at *P*<0.05 (LSD test).

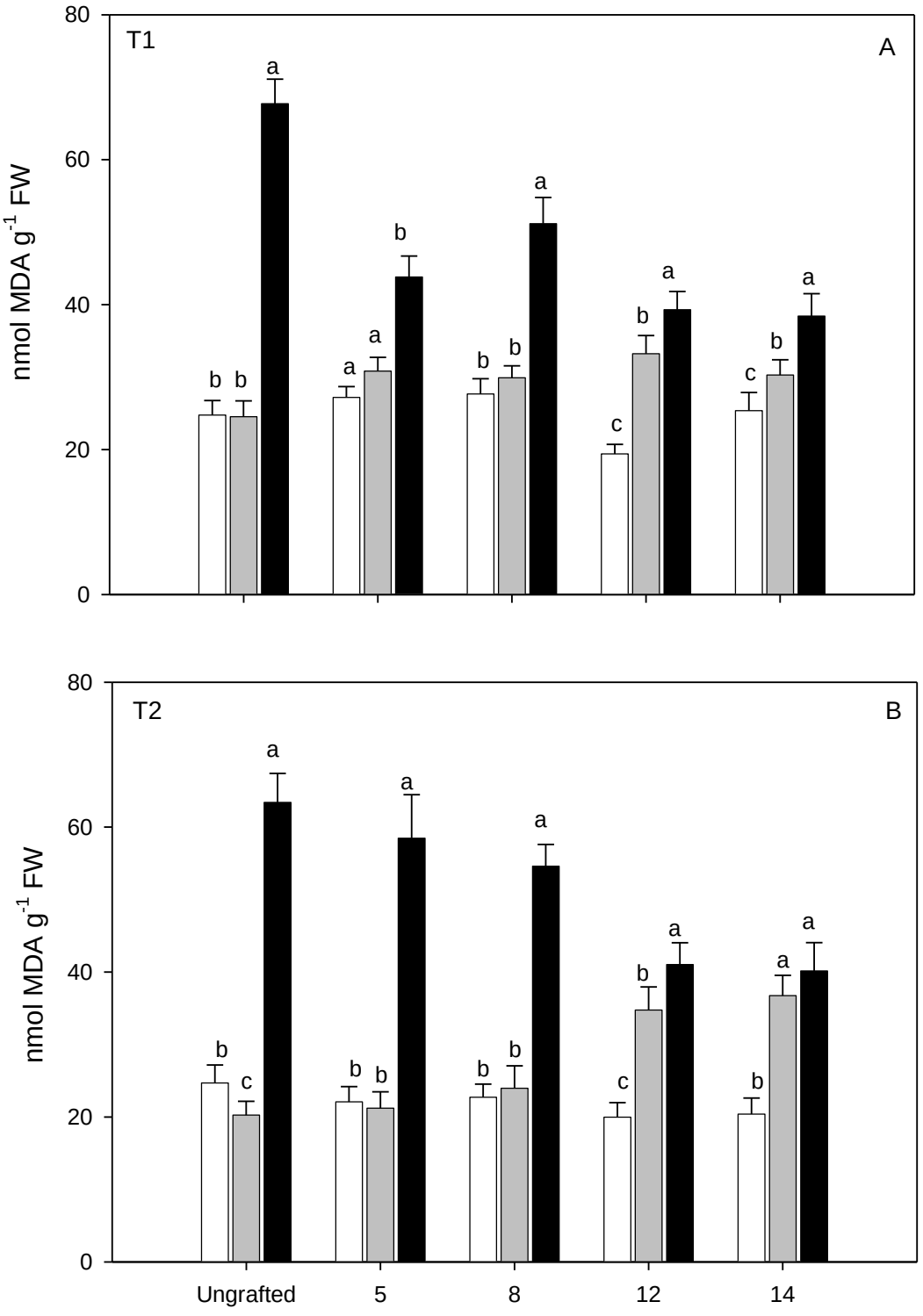

Fig. 7. Leaf malondialdehyde content (nmol MDA g^{-1} FW) in leaves of ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during 7 day (A) and 14 day exposure (C). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at *P*<0.05 (LSD test).

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Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: physiological responses

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ABSTRACT

Recent studies have shown that tolerance to abiotic stress, including water stress, is improved by grafting. In a previous work, we took advantage of the natural variability of *Capsicum* spp and selected accessions tolerant and sensitive to water stress as rootstocks. The behavior of commercial cultivar 'Verset' seedlings grafted onto the selected rootstocks at two levels of water stress provoked by adding 3.5 and 7% PEG (polyethylene glycol) was examined over 14 days. The objective was to identify the physiological traits responsible for the tolerance provided by the rootstock in order to determine if the tolerance is based on the maintenance of the water relations under water stress or through the activation of protective mechanisms. To achieve this goal, various physiological parameters were measured, including: water relations; proline accumulation; gas exchange; chlorophyll fluorescence; nitrate reductase activity; and antioxidant capacity. Our results indicate that the effect of water stress on the measured parameters depends on the duration and intensity of the stress level, as well as the rootstock used. Under control conditions (0% PEG) all plant combinations showed similar values for all measured parameters. In general terms, PEG provoked a strong decrease in the gas exchange parameters in the cultivar grafted onto the sensitive accessions, as also observed in the ungrafted plants. This effect was related to lower relative water content in the plants, provoked by an inefficient osmotic adjustment that was dependent on reduced proline accumulation. At the end of the experiment, chronic photoinhibition was observed in these plants. However, the plants grafted onto the tolerant rootstocks, despite the reduction in photosynthetic rate, maintained the protective capacity of the photosynthetic machinery mediated by

osmotic adjustment (based on higher proline content). In addition, water stress limited uptake and further NO_3^- transfer to the leaves. Increased nitrate reductase activity in the roots was observed, mainly in plants grafted onto the sensitive rootstocks, as well as the ungrafted plants, and this was associated with the lessened flux to the leaves. This study suggests that PEG-induced water stress can be partially alleviated by using tolerant accessions as rootstocks.

Key words: graft; osmotic potential; pepper; photosynthesis; water stress

Introduction

Pepper is one of the most important cultivated crops in the Mediterranean climate, where water shortage is a major problem limiting productivity. An improvement of plant yield under drought is one of the main scientific and economic challenges in these areas. Plants exposed to water stress may have different types of response: susceptibility, resistance mediated by avoidance, or tolerance. Water stress plant tolerance involves biochemical, physiological, and morphological mechanisms that enable plants to function during periods with decreased water availability (Nio et al., 2011) and prevent or alleviate damage. One of the important pathways to enhance water stress tolerance is through osmotic adjustment (OA), which maintains the leaf turgor necessary for stomatal opening and thus sustains photosynthesis and growth (Huang et al., 2010; Nio et al., 2011). Various types of compatible solutes accumulate: such as sugars, proline, gycinebetaine, or potassium (Munns et al., 1979; Morgan, 1992; Nio et al., 2011). These compounds can be added to a list

of non-enzymatic antioxidants that plants need to counteract the inhibitory metabolic effects of reactive oxygen species (ROS) provoked by stress (Gill and Tuteja, 2010). They also play a role in the stabilization of enzymes and proteins, as well as in the protection of membrane integrity (Patade et al., 2012).

Photosynthesis is extremely sensitive to water stress. The effects of water stress can be direct: such as decreased CO_2 availability caused by diffusion limitations through the stomata and/or the mesophyll (Flexas et al., 2007); or by alteration in CO_2 fixation reactions (Lawlor and Cornic, 2002). Photosynthetic responses to water stress are complex since they involve the interplay of limitations taking place at different parts of the plant (Chaves et al., 2009). Alterations in the photosynthetic process can provoke alteration in the uptake and translocation of mineral nutrients (Calatayud et al., 2008). Nitrate reductase (NR) is a key enzyme responsible for nitrogen (N) assimilation and is connected with carbon metabolism (Masclaux-Daubresse et al., 2010): N assimilation requires NADH to drive NR, as well as carbon skeletons derived from photosynthesis for synthesis of amino acids (Yousfi et al., 2012). A large fraction of leaf N is allocated to the photosynthesis apparatus. NR activity has been reported to decrease under water stress (Foyer et al., 1998), but the effect on grafted pepper has not been previously studied.

Mechanisms for plant adaptation to and survival of water stress have been favored by natural selection. Taking advantage of drought-resistant accessions is an important gateway for obtaining tolerant crops (although in pepper these accessions have a poor commercial value). A new perspective to improve resistance to water stress is the use of these tolerant accessions as rootstocks for a desirable commercial cultivar. Grafting has become a valid

strategy to increase tolerance in plants under several abiotic stresses (Huang et al., 2010; Martínez-Ballesta et al., 2010; Colla et al., 2010). The interactions between graft, vegetable plants, and water stress have been mostly studied in tomato (Sánchez-Rodríguez et al., 2013) and melon (Rouphael et al., 2008); and there are no reports on physiological alterations of pepper after grafting and exposure to water stress. Water scarcity is a major problem in arid and semi-arid regions and limited information exists regarding water stress tolerance in pepper grafted plants using accessions as rootstock. Our study offers promising results that could improve the understanding of several physiological mechanisms involved in scion and pepper rootstock interaction under water stress conditions.

In previous experiments we selected four accessions: two that were resistant and two that were sensitive to water stress (Calatayud et al., 2011). The aim of the present work is to study the responses to water stress of a commercial pepper cultivar grafted onto these rootstocks in order to identify the physiological traits responsible for the tolerance to this stress. Furthermore, we want to assess if this tolerance is based on the ability to maintain the water relations under low water availability little water is available; or through the activation of protective mechanisms in the scion – and if these effects depend on intensity of the water stress. For this purpose, several physiological parameters were determined, including: photosynthesis; chlorophyll (Chl) fluorescence; lipid peroxidation levels; relative water content (RWC); proline concentration; osmotic potential; and NR activity. We present evidence that grafting plants onto appropriate (tolerant) rootstocks is a good tool against water stress mediated by an efficient osmotic adjustment. Furthermore, these

physiological parameters could be useful for screening processes when selecting tolerant plants.

Materials and methods

Plant material and greenhouse conditions

Based on previous studies (Calatayud et al., 2011), the drought tolerant accessions 'ECU-973' of *Capsicum chinense* Jacq. (code 12) and 'BOL-58' of *Capsicum baccatum* L. var. *pendulum* (code 14), and the water stress susceptible accessions 'Piquillo de Lodosa' (code 8) and 'Serrano' of *Capsicum annuum* L. (code 5) were chosen as rootstocks in this study. The pepper cultivar 'Verset' (California type; Rijk Zwaan) was grafted onto these four pepper accessions. The pepper seeds were sown on 1 December 2011 in 100-cell polystyrene trays filled with peat-based substrate and kept under a Venlo-type glasshouse. The plants were transplanted to 54-cell trays. 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 the scion, previously cut at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip). Ungrafted 'Verset' plants were used as controls.

One month after grafting, the plants were placed in 5 L polyethylene pots covered with aluminum sheets (the root system having been previously washed clean of substrate). Pots were filled with a nutrient solution containing (in mmol L^{-1}): 12.3 NO_3^-; 1.02 $H_2PO_4^-$; 2.45 SO_4^{2-}; 3.24 Cl^-; 5.05 K^+; 4.23 Ca^{2+}, 2.55 Mg^{2+} and micronutrients (15.8 µM Fe^{2+}, 10.3 µM Mn^{2+}, 4.2 µM Zn^{2+}, 43.5 µM B^{5+}, 1.4 µM Cu^{2+}) that had been artificially aerated. The electrical conductivity and pH of this nutrient solution was 2.1 dS m^{-1} and 6.5, respectively. Nutrient solution was

added daily to compensate for absorption. After 7 days of seedling acclimation to the pots, PEG 8000 (Sigma Co) was dissolved in a nutrient solution for inducing osmotic stress at 3.5% and 7% PEG. The osmotic potential of the solutions, measured with a vapor osmometer (Digital osmometer, Wescor, Logan, USA), were -0.35 and -0.77 MPa respectively. Nutrient solution (0% PEG) was approximately -0.05 MPa due to the presence of the nutrient salt.

The treatments were defined by three PEG levels (0%, 3.5%, and 7%) and four plant combinations (the cultivar 'Verset' grafted onto rootstock accessions 5, 8, 12 and 14). The grafted combinations (rootstock/cultivar) were labeled as: 5/cultivar, 8/cultivar, 12/cultivar and 14/cultivar. The ungrafted cultivar was used as control. The layout was completely randomized with three replications for each combination and six plants per replication.

All physiological measurements were performed at 7 (T1) and 14 (T2) days after PEG addition on a fully expanded mature leaf (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}) and temperature ranges were 21-24 ºC; and relative humidity was 52-72%.

Water relations

The osmotic potential of leaf sap (Ψ_s in MPa) was measured using an osmometer (Digital osmometer, Wescor, Logan, USA). Two independent determinations were performed on each replicate and plant combination, obtained from 6 plants per treatment and combination.

The leaves were tightly wrapped in aluminum foil, frozen at -70 ºC, and stored in liquid nitrogen. After thawing, sap was collected from syringes at 25 ºC and placed in the osmometer (Rodríguez-Gamir et al., 2010). Osmolyte content (mmol kg^{-1}) was converted to MPa using the Van't Hoff equation. The osmotic adjustment (OA) was determined as the difference between the osmotic potential of the leaves at full turgor for control plants and the stressed plants (Garcia-Sanchez et al., 2007). Full turgor was achieved by rehydrating the leaves with distilled water in darkness for 24 h.

Six other similar leaves from two independent plants of each plant combination, PEG 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.

Proline determination

Proline content was determined as described by Bates et al. (1973). Leaf pepper tissue (0.05 g) was ground in 3% sulfosalicylic acid, the homogenate was filtered, and 0.75 mL glacial acetic acid, and 0.75 mL 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 hour, and readings were taken at a wavelength of 520 nm in a spectrophotometer. Three independent determinations were performed in three different extracts, obtained from 18 plants per treatment and combination (one leaf per plant or 500 mg (FW) of roots, and six plants per extract).

Photosynthetic activity and chlorophyll fluorescence

175 CO_2 fixation rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor
176 (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and substomatal
177 CO_2 concentration (C_i, μmol CO_2 mol^{-1} air) were measured at steady-state while
178 maintaining the plants at 1000 μmol m^{-2} s^{-1} during 10-15 min and 400 ppm CO_2
179 with a LI-6400 (LI-COR, Nebraska, USA). Light curves were previously
180 performed (data not shown) and A_N was saturated at 900 μmol m^{-2} s^{-1}. Current
181 fluorescence yield (Fs) and the maximum light adapted fluorescence (Fm') were
182 determined with the LI-6400 in the presence of an actinic illumination of 1000
183 μmol photons m^{-2} s^{-1}, and photochemical PSII efficiency (ϕ_{PSII}) was computed
184 as the quotient (Fm' – Fs)/Fm' (Genty et al., 1989).

185 To evaluate the presence of chronic photoinhibitory processes, the
186 variable fluorescence ratio Fv/Fo= Fm-Fo/Fo (Babani and Lichtenthaler, 1996)
187 was measured on leaves after 15 minutes in darkness using a portable pulse
188 amplitude modulation fluorometer (PAM-2100, Walz, Effeltrich, Germany). The
189 background fluorescence signal for dark adapted leaves (Fo) was determined
190 with a 0.5 μmol photon m^{-2} s^{-1} measuring light at a frequency of 600 Hz. The
191 application of a saturating flash of 10000 μmol photon m^{-2} s^{-1} enabled
192 estimations of the maximum fluorescence (Fm).

193 Gas exchange and fluorescence determinations were performed from
194 9:00 am to 11:00 am (GMT). One measurement per plant was performed, and
195 ten different plants were used (n=10) for each PEG treatment and plant
196 combination.

197

198 *Nitrate reductase activity*

Nitrate reductase activity (EC 1.6.6.1) was determined *in vivo* following the methods described by Hageman and Hucklesby (1971) and Jaworki (1971). Discs of 1 cm diameter in mature fresh leaves, or pieces of 1 cm in roots, were punched out. 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 to vacuum infiltration three times in order to induce anaerobic conditions in the incubation medium. Plant samples were incubated in a water bath at 30 ºC for 60 min in the dark and placed in a boiling water bath for 5 min to stop enzymatic reaction. Nitrite released from 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 contained in the samples (Calatayud et al., 2008). Sampling and replicates were used as described for proline determination.

Lipid peroxidation

Lipid peroxidation was estimated through malondialdehyde (MDA) determinations using thiobarbituric acid reaction, according to the protocol reported by Heath and Parker (1968)**,** and modified in Dhindsa et al. (1981). The non-specific background absorbance reading at 600 nm was subtracted from specific absorbance reading at 532 nm. Sampling and replicates used as described for proline determination.

Statistical analyses

The results were subjected to multifactor variance analysis (Statgraphics Centurion for Windows, Statistical Graphics Corp.). The effect of the genotype and stress level was estimated and significant interactions (genotype x stress level) were observed for all the analyzed parameters. The mean comparisons were performed using Fisher's least significance difference (LSD) test at $P < 0.05$.

Results

Plant water status

Seedling under control conditions maintained RWC leaf values above 90% during the experiment (Fig. 1). The presence of PEG in the nutrient solution reduced the RWC of the leaves (Fig. 1). At T1 this effect was more dramatically observed at 7% PEG, and the ungrafted cultivar was the most sensitive (37%; Fig. 1A). The 12/cultivar and 14/cultivar plants were less affected (70% and 68%, respectively; $P < 0.05$). After 14 days (T2) RWC fell, even at 3.5% PEG (Fig. 1B). The ungrafted plants, as well as the 5/cultivar and 8/cultivar plants had lower RWC values at 80% ($P < 0.05$). These genotypes showed the lowest reductions at 7% PEG (Fig. 1B), and the ungrafted plants had the lowest RWC values (35%), followed by the 5/cultivar and 8/cultivar plants ($P < 0.05$). The 12/cultivar and 14/cultivar plants maintained RWC values near 90% under 3.5% PEG without significant differences with respect to their controls and between 63%-65% at 7% PEG, respectively ($P < 0.05$).

Leaf osmotic potential

Leaf osmotic potential values at T1 and T2 are shown in Fig. 2. The Ψ_s remained unchanged in control conditions during the experimental period, with values near -1 MPa. The osmotic potential decreased in relation to time exposure and PEG concentration. At 3.5% PEG, the 14/cultivar plants showed the largest decreases ($P < 0.05$) in Ψ_s at T1 and T2 (Fig. 2A,B). This effect was also observed at T1 in the ungrafted plants and in the 12/cultivar plants at T2. At higher PEG concentrations, the 12/cultivar and 14/cultivar plants showed the lowest Ψ_s values during the experiment ($P < 0.05$). Furthermore, the 5/cultivar and 8/cultivar as well as the ungrafted plants showed significant but less intense decreases (Fig. 2).

Osmotic adjustment was observed at T1 in ungrafted plants and in 14/cultivar plants at 3.5% PEG, and in 12/cultivar and 14/cultivar plants at 7% PEG (Table 1). After 14 days, the highest OA was induced in the 12/cultivar and 14/cultivar plants at both PEG concentrations (Table 1).

Accumulation of proline

Proline accumulation was induced in pepper seedlings by drought and PEG exposure (Fig. 3). No effect of stress level was observed in the accumulation of proline. At T1 (Fig. 3A) a slight increase ($P < 0.05$) was observed in all genotypes irrespective of the PEG concentration in the culture medium, except for 12/cultivar and 14/cultivar plants where the proline concentration decreased with respect to the controls. Proline levels increased after 14 days (T2) (Fig. 3B) of water stress treatment. Two to three-fold increases were observed in the cultivar and 5/cultivar and 8/cultivar plants. The

maximum increase was found for 12/cultivar and 14/cultivar plants ($P < 0.05$), with increases from 12 mg/ g DW at 0% PEG to 32 and 49 mg/ g DW under 7% PEG conditions, respectively.

Photosynthetic parameters

PEG provoked a significant reduction in the photosynthetic rate (Fig. 4A, B), stomatal conductance (Fig. 4C,D), and photochemical PSII efficiency (Fig. 4E,F) in the studied pepper genotypes.

At T1 the A_N progressively diminished with the drought stress level in the ungrafted plants and 5/cultivar plants (Fig. 4A). In the 8/cultivar and 14/cultivar plants no significant effect of 3.5% PEG was observed; and in the 12/cultivar plant, the photosynthetic rate fell at 3.5% PEG; but did not fall further at 7% PEG. In the ungrafted plants, the photosynthetic rate reached null values at T2 in the 7% PEG media (Fig. 4B). At this concentration, the 12/cultivar and 14/cultivar plants showed smaller reductions ($P < 0.05$) in the photosynthetic rate. No effect for PEG concentration was observed in the grafted plants at T2 (Fig. 4B).

Differences in the stomatal conductance to drought were observed among genotypes (Fig. 4C,D). At T1, the ungrafted plants, 5/cultivar, and 8/cultivar plants maintained higher stomatal openings at 3.5% PEG when compared to 12/cultivar and 14/cultivar plants ($P < 0.05$). In addition, g_s fell to values near zero at 7% PEG in these genotypes. By contrast, stomata closed to values near 0.1 mol m^{-2} s^{-1} in 12/cultivar and 14/cultivar plants, irrespective of the stress level (Fig. 4C), and did not change at T2 (Fig. 4D). Stomatal

conductance was also strongly reduced in the ungrafted, 5/cultivar, and 8/cultivar plants at T2.

Substomatal CO_2 concentration (Ci) decreased with stomatal closure in all grafted plants (data not shown). In contrast in the ungrafted cultivar, Ci increased ($P < 0.05$) at low stomatal conductances under water stress.

No effect for 3.5% PEG on the ϕ_{PSII} was observed at T1 in the ungrafted, 5/cultivar, and 8/cultivar plants (Fig. 4E). By contrast, this parameter fell by more than 55% of the control values at 7% PEG in these genotypes. In 12/cultivar and 14/cultivar plants, the reduction provoked by PEG ranged from 75 to 81% of control values at T1, irrespective of the stress level. At T2, the response of the photochemical PSII efficiency was similar to that observed for the photosynthetic rate (Fig. 4B).

Similar Fv/Fo values were observed for all genotypes under control conditions (Fig. 5A,B). No changes were produced at T1 by 3.5% PEG, except for the 8/cultivar plants (where Fv/Fo increased with respect to its control). However, at 7% PEG, Fv/Fo fell in the ungrafted plants (32% of control value) and, to a lesser extent in the 5/cultivar and 8/cultivar plants (Fig. 5A). At T2, the decrease in Fv/Fo increased with the stress level (Fig. 5B). The ungrafted plants showed the lowest values, being zero at 7% PEG; while 12/cultivar and 14/cultivar plants showed the smallest reduction ($P < 0.05$) in Fv/Fo at 7% PEG (Fig. 5B).

Changes in nitrate reductase activity

Differing responses of NR activity to drought were observed in leaves and roots (Fig. 6). NR activity increased in roots (Fig. 6B,D) in all the water stress treatments when compared to control conditions – the highest values ($P < 0.05$) being for ungrafted plants, 5/cultivar, and 8/cultivar plants at 7% PEG and T2 (Fig. 6D). By contrast, water stress decreased NR activity in the leaves, and the lowest value ($P < 0.05$) was observed for ungrafted plants at 7% PEG followed by 5/cultivar and 8/cultivar plants (Fig. 6A, C). In the leaves, after 7 and 14 days of severe water stress, 12/cultivar and 14/cultivar plants showed the highest NR activity levels – while the lowest values were observed in the ungrafted plants.

Lipid peroxidation

Lipid peroxidation in pepper leaves increased with time and PEG levels (Fig. 7). At T1 MDA content increased with higher PEG levels (Fig. 7A) in all plants. The increase was highest in the ungrafted plants. After 14 days of exposure, lipid peroxidation increased significantly at 7% PEG in all plants and 12/cultivar and 14/cultivar plants at 3.5%. It is noteworthy that no further MDA accumulation was produced in these genotypes at 7%, whereas MDA accumulated to higher levels in 5/cultivar, 8/cultivar, and ungrafted plants (Fig. 7B).

Discussion

Water stress induced by PEG led to significant changes in physiologic parameters in pepper seedlings. The effect depended on the duration and the

intensity of the stress level. Moreover, consistent differences were observed between susceptible (5 and 8) and tolerant accessions (12 and 14) when used as rootstocks, although such differences vanished in the absence of water stress. The following discussion aims to establish which physiological processes could explain the different responses among grafted plants, including tolerant and sensitive accessions such as rootstocks and ungrafted plants.

Water status in a plant is highly sensitive to water stress and therefore is dominant in determining plant responses to stress. Leaf RWC decreased under water stress, but its effects were significantly dramatic only under the 7% PEG treatment. The highest RWC values (62-67%) were observed in the 12/cultivar and 14/cultivar plants after 14 days, when compared with ungrafted plant values (34%) ($P < 0.05$). Similarly, the leaves of tomato plants grafted onto *Solanum mammosum* – (with a greater ability for passive water uptake) maintained higher leaf water potential than self-grafted plants – despite greater water loss through transpiration under water stress conditions (Weng, 2000).

An alteration in the relationship between RWC and ψ_s was found. In this sense, the leaf ψ_s was lowest in 12/cultivar and 14/cultivar plants, compared with 5/cultivar, 8/cultivar, and ungrafted plants; although the RWC values at 3.5% PEG in T1 and T2 remained unchanged. This can be explained by the fact that the relationship between ψ_s and RWC is not unique (Acevedo et al., 1979), and other factors such as the rate of transpiration, stomatal aperture, or development of the root system can modulate this relation (Weng, 2000). Nevertheless, decreases in ψ_s may have contributed to the ability of these accessions (12 and 14) to uptake more water from the nutrient solution and could have minimized the harmful effects of water stress (Nio et al., 2011; Ming

et al., 2012). Significant correlations were demonstrated between ψ_s and the tolerance to drought in different crops, i.e. PEG-tolerant chilli pepper clones (Santos-Díaz and Ochoa-Alejo, 1994); tomato PEG-adapted cell lines (Handa et al., 1982); or barley after 36 days without irrigation (González et al., 2008).

Although the decrease in ψ_s could be a consequence of a reduction in the water content of tissues, active osmotic adjustment was observed in the studied genotypes, and mainly in the plants grafted onto the tolerant genotypes (12 and 14). The osmotic adjustment may have involved the accumulation of a range of osmotically active molecules, including organic compounds such as sugars, free amino acids, glycinebetaine, soluble proteins, and organic acids (Chaves et al., 2003) and with macronutrients such as inorganic components (Patakas et al., 2002). Free proline is considered an important osmoprotectant and accumulation following salt, drought, and heavy metal exposure is well documented (Gill and Tuteja, 2010). In our work, a strong correlation between ψ_s decrease and proline content increase was observed at T2 (ψ_s = -0.752 [proline] - 0.205; r^2 = 0. 87; $P < 0.05$) for all plant combinations and treatments; and at T1 for 5/cultivar, 8/cultivar, and ungrafted plants (ψ_s = -0.087 [proline] - 0.540; r^2 = 0. 79; $P < 0.05$). Nevertheless, the decrease at T1 in ψ_s was not related to the increase in proline in the 12/cultivar and 14/cultivar plants (ψ_s = 0.318 [proline] - 6.288; r^2 = 0.62; $P < 0.05$). At this earlier period, other components such as glycinebetaine, carbohydrates, amino acids, and macronutrients could have contributed to reducing the osmotic potential (Munns et al., 1979; Morgan, 1992; Navarro et al., 2003) in these plant combinations. Similar time-dependent behavior was reported in wheat (Nio et al., 2011), where K^+ was mainly involved in the osmotic responses to water stress during earlier

periods; whereas proline was mainly accumulated after long exposures. Alternatively, pepper plants (12 and 14) could have used the mineral components of the nutrient solution to produce the decrease in osmotic potential, such as described for sugarcane cells (Patade et al., 2012) during the first seven days of water stress.

The osmotic adjustment, mainly through the increase in proline content, and related to the duration and severity of the water stress, helped the 12/cultivar and 14/cultivar plants maintain tissue water status and avoid drought-induced damage. Similar results were obtained by Anjum et al. (2012) in pepper plants.

Moreover, osmolyte proline accumulation was proposed to act as a protein stabilizer, a metal quelator, an inhibitor of lipid peroxidation, and a scavenger of radical oxygen species (ROS) under salt, drought, and metal stress (Gill and Tuteja, 2010). Production of these species at higher levels may damage cellular membrane and other biologically vital components such as chlorophylls, DNA, proteins, and lipids (Blokhina et al., 2003). Lipid peroxidation is considered to be one of the most damaging processes as its decreases membrane fluidity; increases the leakiness of the membranes, and inactivates receptors, enzymes, and ion channels. The final product of lipid peroxidation is MDA – which is used as an index of oxidative membrane damage (Calatayud et al., 2002; Ozkur et al., 2009). In our work, improvement in proline accumulation under water stress helped maintain osmotic potential; and may also be involved in protection against oxidative damage as indicated by lower levels of MDA in the 12/cultivar and 14/cultivar plants (mainly at the end of the experiment under 7% PEG). These results indicate that these genotypes when used as rootstocks

provide protection to the scion. By contrast, the ungrafted plants and 5/cultivar and 8/cultivar plants showed less capacity to retain water in their cells: a minor decrease of $\psi_{S,}$ was associated with a minor increase in proline concentration, and as a consequence, a higher level of lipid peroxidation.

The oxidative stress provoked by water stress had a direct effect on proper PSII function. The Fv/Fo parameter, a sensitive Chl fluorescence ratio is related to the maximum quantum yield of PSII photochemistry (Babani and Lichtenthaler, 1996). A decline in Fv/Fo indicates a disturbance or damage of the photosynthetic apparatus, and has been frequently used as an indicator of photoinhibition (Calatayud et al., 2004). A decrease in the Fv/Fo ratio occurs under water stress, and the most dramatic decrease occurred in ungrafted plants at T2 under 7% PEG, where the values were zero. According to our observations (see above), the Fv/Fo ratio suggested a higher resistance for 12/cultivar and 14/cultivar plants to water stress. The decrease in Fv/Fo in ungrafted plants, 5/cultivar, and 8/cultivar plants may be as a result of an increase in protective non-radiative energy dissipation associated with a regulated decrease in photochemistry – described as down-regulation and/or chronic photodamage of the PSII centers (Genty et al., 1989; Osmond, 1994). The Fv/Fo ratio seems a robust parameter, and several authors have concluded that PSII photochemistry cannot be impaired by relatively severe water stress; although A_N and gs can decrease significantly (Lawlor and Tezara, 2009). In our experiment, all plant combinations, regardless of the Fv/Fo values, showed a significant decrease in the net carbon gain, due in part to stomatal closure that restricts water losses. The decrease in the rate of photosynthesis may be due to the chronic water stress effect of metabolic inhibition, or the down-regulation of

442 photosynthesis as described by Chaves et al. (2003) and Cornic (2000).
443 Distinguishing between these alternatives is difficult (Flexas et al., 2004).
444 Acclimation to water stress requires responses that enable essential reactions
445 of primary metabolism to continue for the plant to tolerate water deficit (Foyer et
446 al., 1998). The ability to maintain the functionally, or protective capacity of the
447 photosynthetic machinery under water stress, is of major importance for drought
448 tolerance in pepper plants (del Amor et al., 2010). Our results indicate that
449 rootstocks 12 and 14 provide the variety with the ability to maintain water
450 relations and protective mechanisms that enable the maintenance of a residual
451 photosynthetic rate (on 'stand-by'). The robust behavior of the cultivar 'Verset'
452 grafted onto accessions 12 and 14 was in accordance with our previous results
453 in field conditions where water availability was reduced by 50% compared to the
454 control treatment (Calatayud et al., 2013). In this experiment, pepper cultivar
455 grafted onto these genotypes showed higher marketable fruit production when
456 compared with ungrafted plants and 'Verset' grafted onto 5 and 8 (Calatayud et
457 al., 2013).

458 Maintenance of tissue water status helps the plants to avoid the
459 dehydration and protects the carboxylation and other enzymes from inactivation
460 and denaturation (Anjum et al., 2012). By contrast, a strong decrease in the
461 photosynthetic rate in 5/cultivar, 8/cultivar plants, and ungrafted plants, along
462 with a decrease in RWC (a weak osmotic adjustment), and a decrease in Fv/Fo
463 was observed under water stress. In the absence of protective mechanisms, an
464 increase in oxidative damage was produced (measured as lipid peroxidation)
465 and chronic photoinhibition of metabolic machinery limiting photosynthesis. The
466 degree of oxidative stress has been described as being closely associated with

467 the resistance/susceptibility of a genotype to water stress (Mittler, 2002; Anjum
468 et al., 2012).

469 At the whole plant level, water scarcity induces complex changes in C
470 and N metabolism resulting from modifications in the availability of nutrients
471 (Foyer, 1998; Imsande and Touraine, 1994). In addition to the discussed
472 changes in carbon assimilation, water stress may restrain nitrate acquisition by
473 the roots, as well as restrict the ability of plants to assimilate and reduce
474 nitrogen (Yousfi et al., 2012; Kocheva et al., 2007). In most herbaceous plants,
475 NR activity takes place predominantly in the leaves (Scheurwater et al., 2002;
476 Reda et al., 2011). In our results under control conditions, where the plants
477 have free access to nutrients, NR activity was higher in leaves than in roots in
478 all plant combinations at T1 and T2. The reduction of NO_3^- in the leaves may
479 provide the advantage of enabling the direct use of excess reductants produced
480 by photosynthesis (Pate, 1983). In our work, the predominant site of NO_3^-
481 reduction (leaves or roots) was dependent on the water stress intensity and
482 time of exposure. NR activity in leaves decreased considerably in all plant
483 combinations under drought, but especially in ungrafted plants, as well as
484 5/cultivar and 8/cultivar plants. However, since NR activity was calculated on a
485 FW basis, and PEG treatment affected the RWC of the leaves, the absolute
486 value of NR activity could be overestimated in these treatments. The utilization
487 of nitrate in the leaves is governed by CO_2 fixation (Larsson et al., 1989). In our
488 results, a decrease in NR activity in the leaves can be linked to a decline in the
489 rate of photosynthesis due to stomatal closure, according to Fresneau et al.
490 (2007); or due to a decrease in the NO_3^- transport from root to leaves due to
491 loss of turgor and lower transpiration flow (Sharma and Dubey, 2005; Yousfi et

al., 2012). Water stress would limit the uptake and further the transfer of NO_3^- to upper plant parts (Yousfi et al., 2012), and subsequently, a part of the nitrate uptake could be reduced in the roots. Observed differences in NR activity may depend on PEG concentration, time exposure, and plant combinations. After 7 days under 3.5% PEG with moderate photosynthesis inhibition, NR activity was located mainly in the leaves. This could be interpreted as that the rate of carbon fixation was not a limiting factor for NO_3^- reduction (Larsson et al., 1989). When the water stress was severe (7% PEG), or when the time exposure with PEG was longer (14 days), photosynthetic activity was compromised, and under this extreme situation the behavior between rootstocks differed. Sensitive genotypes (5 and 8) with lower NR activity in the leaves showed low levels of photosynthetic activity, i.e. when internal CO_2 concentration was reduced due to stomatal closure (Fresneau et al., 2007) and greater root NR activity (irrespective of PEG concentration). Tolerant rootstocks (12 and 14) showed increased root NR activity at only T2 in 7% PEG, although to a lesser extent. This could be because the remaining water transpiration flux (highest E values) enables reductions through the NO_3^- transport to the leaves. The significant increase in root NR activity may indicate that nitrate flux to roots was not restricted by water stress and that active NO_3^- reduction occurs in the roots, possibly due a minor transpiration flux to leaves.

Considering the overall results of this study, we can conclude that the response of commercial pepper cultivar to water stress can be improved by grafting when using appropriate accessions as rootstocks. It seems that grafting methods could be a useful tool for increasing resistance to water stress. Under these experimental conditions, accessions 12 and 14 grafted onto cultivar

alleviate the water stress effect. This effect may be attributed to enhanced osmotic adjustment because of active proline accumulation (as reflected by the lower reduction in RWC) which may protect leaves from excessive dehydration caused by damaged photosynthesis systems. In addition, the methods used in this work appear to be suitable for testing the water stress resistance of pepper rootstocks.

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694 **Legends of figures**

695

696 **Fig. 1.** Effect of PEG addition at 0% (□), 3.5% (▒), and 7% (■)
697 on relative leaf water content (RWC %) during 7 day (A) and 14 day exposure
698 (B) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto
699 accessions 5, 8, 12 and 14. Dates are mean values ± SE for n= 6. Within each
700 plant combination different letters indicate significant differences at $P < 0.05$
701 (LSD test).

702

703 **Fig. 2.** Leaf osmotic potential (MPa) in ungrafted pepper plants (cultivar
704 'Verset') and cultivar grafted onto accessions 5, 8, 12, and 14 after PEG
705 addition at 0% (□),3.5% (▒) and 7% (■) during 7 day (A) and 14
706 day exposure (B). Dates are mean values ± SE for n= 6. Within each plant
707 combination different letters indicate significant differences at $P < 0.05$ (LSD
708 test).

709

710 **Fig. 3.** Changes in proline concentration (mg proline /g DW) from ungrafted
711 pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12
712 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during
713 7 day (A) and 14 day exposure (B). Dates are mean values ± SE for n= 6.
714 Within each plant combination different letters indicate significant differences at
715 $P < 0.05$ (LSD test).

716

717 **Fig. 4.** Net CO_2 assimilation rate (A_N; μmol CO_2 m^{-2} s^{-1}) (A, B); leaf stomatal
718 conductance (g_s; mol H_2O m^{-2} s^{-1}) (C, D) and actual quantum efficiency of PSII

719 (ϕPSII) (E, F) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted
720 onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5%
721 (▒) and 7% (■) during 7 day (A, C, D) and 14 day exposure (B, D, F).
722 Dates are mean values ± SE for n= 10. Within each plant combination different
723 letters indicate significant differences at $P < 0.05$ (LSD test).
724
725 **Fig. 5.** Variations in dark-adapted Fv/Fo ratio in leaves of ungrafted pepper
726 plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14
727 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during 7 day
728 (A) and 14 day exposure (B). Dates are mean values ± SE for n= 10. Within
729 each plant combination different letters indicate significant differences at $P <$
730 0.05 (LSD test).
731
732 **Fig. 6.** Nitrate reductase activity (μmol NO_2 g^{-1} FW) in leaf (A, C) and roots (B,
733 D) of ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto
734 accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒)
735 and 7% (■) during 7 day (A, B) and 14 day exposure (C, D). Dates are
736 mean values ± SE for n= 6. Within each plant combination different letters
737 indicate significant differences at $P < 0.05$ (LSD test).
738
739 **Fig. 7.** Leaf malondialdehyde content (nmol MDA g^{-1} FW) in leaves of ungrafted
740 pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12
741 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during
742 7 day (A) and 14 day exposure (C). Dates are mean values ± SE for n= 6.

743 Within each plant combination different letters indicate significant differences at

744 $P < 0.05$ (LSD test).