Biochemical and physiological responses of two grapevine rootstock genotypes to drought and salt treatments

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Abstract

Background and Aims: In the light of possible climate change, a crucial aspect for future Mediterranean Europe viticulture is the selection of new rootstocks exhibiting adaptability to drought and salt stress. In this context and considering recent literature, it is fundamental to increase our knowledge of the biochemical and physiological events that characterise stress responses in grapevine roots. In this study, leaf and root responses induced by water stress (WS) and sodium chloride (NaCl) exposure in a new selected genotype, named M4 (*Vitis vinifera* × *V. berlandieri*) × *V. berlandieri* cv. Resseguier n. 1), were compared with those of the commercial rootstock 101.14.

Methods and Results: The effect of progressive WS and NaCl exposure was studied under controlled environmental conditions on ungrafted plants grown in pots. Shoot growth, plant water status and leaf photosynthetic parameters were measured. The concentration of sugars, amino acids and total proteins, as well as the concentration of the more abundant ions, was determined in both leaf and root organs. The M4 genotype showed greater capacity to maintain photosynthetic activity, to accumulate osmotic compounds as well as to counteract Na and Cl accumulation.

Conclusion: The M4 genotype exhibited a greater capacity to tolerate both WS and exposure to an increasing concentration of Na and Cl, maintaining photosynthetic activity also under severe stress conditions. The root system appeared to play a central role in sustaining biochemical and physiological responses experienced under these adverse conditions.

Significance of the Study: This study showed that the tolerance to abiotic stress conditions, such as WS and NaCl exposure, depends on root integrity/functionality, confirming that these aspects must be considered in further selection programs.

Keywords: *drought, grapevine rootstock, NaCl stress, osmotic adjustment, photosynthesis*

Introduction

The selection of new grapevine genotypes, especially in view of climate change events occurring in recent years, is a crucial factor for the development of sustainable agricultural models (moderate irrigation, fertilisation and recovery of marginal soils) and for ensuring optimal maturation profiles of grapes. As a result, recurrent drought spells and the increase in salt concentration in soils represent the most common environmental factors that will have a strong negative impact on Mediterranean viticulture (Jones et al. 2005, Cramer 2010, Schultz and Stoll 2010).

Although grapevines are relatively tolerant to water deficit, severe drought can affect crop quality and yield (Cramer 2010, Flexas et al. 2010). Stomatal closure is among the earliest responses to water deficit adopted by plants to reduce evaporative water loss and maintain a safe water balance (Chaves 1991, Chaves et al. 2010). In this context, an important aspect to be considered is the role of roots in sensing water availability in the soil, as well as the chemical and/or hydraulic root-to-shoot signalling under drought (Christmann et al. 2007, Schachtman and Goodger 2008, Lovisolo et al. 2010). As the stress becomes severe, net CO_2 assimilation (A_n) and other metabolic processes operating in the mesophyll are inhibited, and water use efficiency thus declines (Chaves et al. 2010). Under these conditions, an increased photorespiration has also been observed to reduce the risks brought about by an increase in reactive oxygen species (Medrano et al. 2002, Pinheiro and Chaves 2011). A typical response of plant tissues allowing adjustment of osmotic potential under these stress conditions is the accumulation of organic solutes and ions (Schultz and Matthews 1988, Patakas et al. 2002, Cramer et al. 2007). Moreover, a central role of aquaporins in maintaining an adequate cellular water balance is now emerging (Tyerman et al. 2009, Vandeleur et al. 2009, Lovisolo et al. 2010). Nevertheless, it is important to emphasise that the decrease of stomatal conductance (g_s), as well as the molecular and physiological responses described as activated in grapevines under water deficiency, also depend on both the growth conditions and the specific genotypic characteristics (Chaves et al. 2010, Lovisolo et al. 2010, Tomás et al. 2012).

doi: 10.1111/ajgw.12071

Grapevines are classified as having medium salt tolerance (Tattersall et al. 2007). Considering that under high salt exposure a decrease in water availability also occurs, several responses to salt are similar to those observed under water deficit (i.e. osmotic stress), while others are salt-specific (i.e. ionic stress) (Tattersall et al. 2007, Munns and Tester 2008, Chaves et al. 2009). According to this view, the method by which NaCl is added during experiments is a crucial aspect that must be considered, causing 'salt shock' or 'salt acclimation'. In the first instance, the osmotic effect represents the main driver (Shavrukov 2013). Many studies emphasise that plant salt tolerance appears mainly related to the capacity to minimise NaCl influx, as well as to the maintenance of a lower concentration of NaCl in the cytoplasm (Zhu 2002, Munns and Tester 2008). Thus, a better elucidation of the mechanisms involved in the root Na uptake represents a central point to understand salt tolerance (Zhang et al. 2010). Moreover, the emerging literature suggests the role of sodium homeostasis in the regulatory network implicated in salt stress responses, such as root system development (Hongtao et al. 2013).

Under salt exposure, in order to balance the osmotic pressure of the ions in the vacuole, an accumulation of compatible solutes in the cytoplasm and in other organelles has been observed (Hasegawa et al. 2000, Munns and Tester 2008). In this context, but also to counteract Na toxic effects, an increase of K concentration was also suggested, even though a strong relationship between leaf K and salt tolerance has not yet been described (Munns and Tester 2008). Another important aspect is that the concentration of Na and Cl attained in leaf cells appears to be linked with specific genetic characteristics, suggesting that the two ions could impose differing degrees of toxicity (Munns and Tester 2008).

In cultivated grapevines, commonly derived from the union of a rootstock with a scion, both genotypes influence the final phenotype and its physiological performance (Cortell et al. 2008, Kodur et al. 2010, Cookson et al. 2012, Gambetta et al. 2012).

Considering that the rootstocks used in Europe exhibit a relatively narrow genetic background, because their phenotypic selection was essentially based on only a few traits (rooting ability, phylloxera resistance and scion-induced vigour), the discovery of new genotypes better able to cope with unfavourable environmental conditions is a key asset for future viticulture in this region (Chaves et al. 2007, Schultz and Stoll 2010). As previously pointed out, the achievement of this goal should also be a good strategy to prevent negative effects on scion behaviour, which remains the major determinant of grape features and quality (Vivier and Pretorius 2002, Marguerit et al. 2012). Nevertheless, it has to be considered that rootstock behaviour could be influenced by scion genotypes, and this could happen under both favourable and unfavourable environmental conditions (Swanepoel and Southey 1989, Zhang et al. 2002, Tandonnet et al. 2010).

Novel candidate genotypes to be used as a rootstock were established from 1985 by the DiSAA research group operating at Milan University. In a preliminary screening, one of these, named M4 [(*V. vinifera* × *V. berlandieri*) × *V. berlandieri* cv. Resseguier n. 1], was selected for its relatively high tolerance to water deficiency and salt exposure. The aim of the present study was to assess biochemical and physiological responses of this genotype under these stress conditions. The performance of M4 was compared against the 101.14 commercial rootstock. The study, which was carried out under semi-controlled environmental conditions on ungrafted plants grown in pots, was focused on both leaf and root organs. Specifically, several biometrical, plant water status and leaf gas exchange parameters were measured, such as internode elongation, leaf area expansion, leaf water potential (Ψ_{leaf}), osmolality, net CO_2 assimilation (A_n) and g_s. Moreover, the concentration of total sugars, amino acids and total proteins, as well as that of the more abundant ions, K, Ca and Mg, was determined. Finally, to investigate the possible role of K in response to NaCl exposure, the tracer rubidium (Rb) was used to study K accumulation in the shoot. Taken together, the results showed a significant difference among the two genotypes in response to water and salt stresses, as well as highlighting that the greater tolerance of M4 to these unfavourable growth conditions involves adaptive responses occurring in the root.

Materials and methods

Sample material

Two-year-old grapevines (genus *Vitis*) of commercial rootstock 101.14 Millardet et de Grasset (*V. riparia* x *V. rupestris*) and a new genotype obtained by crossing (*V. vinifera* × *V. berlandieri*) × *V. berlandieri* cv. Resseguier n. 1, named M4, were grown in pots filled with a sand–peat mixture (7:3 in volume). The experiment was conducted in a greenhouse sited in Milan (Italy) equipped with supplementary light and a cooling system, with a 16 h light [~PPFD of 600 µmol of photons/(m^2 • s)] and an 8-h dark photoperiod. Plants were grown in 3-L pots fertilised monthly with 100 mL of solution containing 0.54 g KNO_3, 0.084 g NH_4HPO_4, 0.42 g $MgSO_4$ and 0.01 g of a microelement mixture (OligoGreen, GREEN Italia, Canale d'Alba, Italy). During this period, plants were managed by shoot thinning and lateral shoot removal to provide uniform material. The experiment was conducted from the end of June to July of 2011. A total of 108 plants of each genotype was randomised to obtain six pools that were used as: (i) control (C), plants that were maintained at 80% of soil field capacity; (ii) water stress (WS), plants in which water supply was progressively reduced until 30% of field capacity; and (iii) salt stress (NaCl), plants to which 5 mmol of NaCl were added daily and a field capacity of 80% was maintained. The effect of WS and NaCl was studied for an experimental period of 10 and 21 days, respectively. In order to attenuate the fluctuation in soil water content (SWC), pots were weighed and then the quantity of water adequate to reach/maintain the desired soil field capacity was added. The procedure was repeated twice a day, at 8:00 am and at 6:00 pm.

According to the experimental scheme (Figure 1), at the start of the experiment (T_0) and at each of the following times [at 2 days (T_1), 4 days (T_2), 7 days (T_3) and 10 days (T_4)] and [at 4 days (T_1), 10 days (T_2) and 21 days (T_3)] for WS and NaCl stress, respectively, at least six randomly chosen plants were destructively sampled. The plants were sampled immediately after the in vivo measurements. The leaf samples were collected from leaves that were fully expanded and of approximately equivalent physiological stage and condition (i.e. from the fourth to the seventh node of the primary shoot). Differently, root samples were obtained by harvesting the whole root system. The soil was removed from roots by a gentle shaking action, after which the sample was rinsed twice in distilled water and immediately blotted with paper towels. The samples were weighed, frozen in liquid nitrogen and stored at −80°C.

Biometrical measurements of plant growth

Internode elongation rate (cm/d) was evaluated through daily measurement of the length of internodes, which were in the growing phase. An average of four shoot internodes for plants was measured.

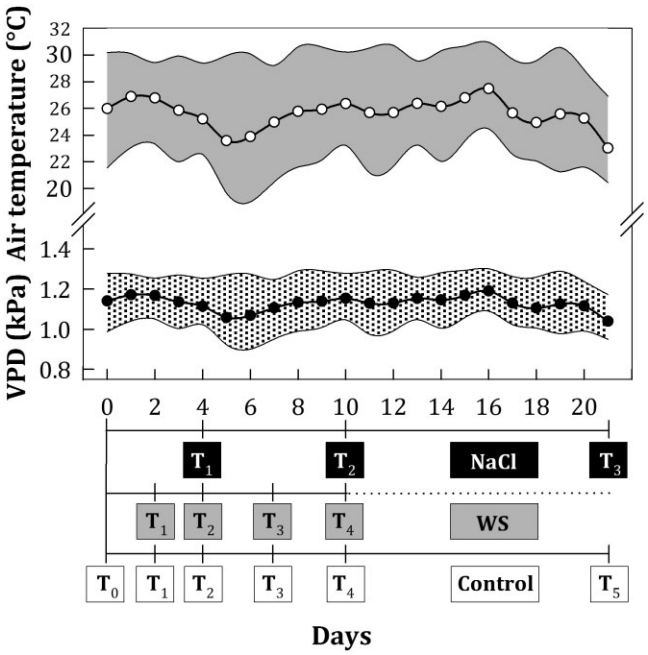

Figure 1. Experimental scheme and climatic condition data. T_0, start of experiment. The plants of the 101.14 and M4 rootstock genotypes were sampled after 2 days (T_1), 4 days (T_2), 7 days (T_3) and 10 days (T_4) for water stress (WS), and after 4 days (T_1), 10 days (T_2) and 21 days (T_3) for NaCl stress. Control plants were maintained in soil with a field capacity of 80%. Water stress was induced by progressively reducing soil water content down to a field capacity of 30%. Salt stress was induced by adding 5 mmol of NaCl daily, maintaining a field capacity of 80%. The range of the daily change (■) of air temperature (○) and the rate of daily change (⊡) of vapour pressure deficit (VPD) (●) are shown.

Leaf expansion rate was determined by daily measurement of both leaf length (L) and maximum leaf width (W) to determine leaf area (LA). The leaf L was measured from the tip of the leaf to the base. The leaf W was measured from end to end between the widest lobes of the lamina perpendicular to the longitudinal axis of the leaf. After physiological measurements, for each plant sample, all leaves were removed and the specific area determined using image analysis software (ImageJ 1.44p, US National Institutes of Health, Bethesda, MD, USA, public domain software: http://rsbweb.nih.gov/ij/). Image analysis enabled the development of a relationship to estimate LA through $L \times W$. The dependent variable (LA) was regressed with the product $L \times W$. Linear regression methods were used to develop the models. The values of the linear regression coefficients (b) and constants (a) were 2.515, 0.762 and 1.959, 0.781 for M4 and 101.14, respectively, with a coefficient of determination (R^2) of 0.97 for both genotypes. Leaf expansion rate was evaluated using at least six leaves for plants.

Leaf physiological measurements

Single-leaf gas exchange measurements were performed with a LI-6400 portable photosynthesis system (Li-Cor Inc. Lincoln, NE, USA). Measurements were made on two fully expanded leaves per plant comprising at least six to eight leaves per treatment at regular times during the experimental period, between 11:00 am and 2:00 pm solar time. The leaves were analysed with the circular 2 cm^2 leaf cuvette equipped with the LI-6400-40 fluorometer (Li-Cor Inc.) as the light source. The leaves were subjected to a 10-min acclimation at a constant saturating photosynthetic photon flux density (PPFD) of 600 µmol of photons/(m^2 • s), a CO_2 concentration of 380 µmol/mol, and relative humidity between 60 and 70%, allowing ~1.5 kPa of vapour pressure deficit (VPD) inside the chamber. Block temperature was maintained at 25°C, allowing leaf temperature to range between 26 and 31°C. The parameters used were net CO_2 assimilation rate [A_n, µmol CO_2/(m^2 • s)] and stomatal conductance [g_s, mmol H_2O/(m^2 • s)]

Leaf water potential and tissue osmolality

Leaf water potential (Ψ_{leaf}, MPa) was measured in leaves using a Scholander-type pressure chamber (model PMS-1000, PMS Instruments, Corvallis, OR, USA). Measurements were performed on the same fully expanded leaves immediately after gas exchange measurements. Two leaves per plant were selected. Each leaf was excised from the shoot with a scalpel blade and then placed into the pressure chamber with the petiole protruding from the chamber lid. The chamber was pressurised using an air pressure tank, and Ψ_{leaf} was recorded as soon as the xylem sap was observed emerging from the cut end of the petiole.

Leaf and root tissue osmolality (mOsm) was measured by a semi-micro osmometer (model K-7400, Knauer GmbH, Berlin, Germany) on the extracts obtained by homogenising the frozen samples with five volumes of distilled water and centrifuging at 14 000 *g* for 20 min. Solute potential (Ψ_s) was deduced from osmolality using the van't Hoff equation.

Amino acids, total sugars and total proteins

Amino acids and total sugars were extracted by homogenising frozen tissues in four volumes of ice-cold 0.5 M perchloric acid (PCA). The homogenate was centrifuged for 10 min at 13 000 *g* at 4°C, and the resulting pellet was washed with the same volume of PCA and then centrifuged again under the same conditions. Potassium hydroxide was added to the collected supernatant (up to pH 7.6) to remove excess PCA.

Total amino acids were measured by the ninhydrin method (Moore and Stein 1954). Total soluble sugars were determined by the same method of boiling an aliquot of the PCA extract for 1 h before neutralisation. Sugar concentration was then measured according to the colorimetric method of Nelson (1944). Total proteins were extracted as previously described by Martínez-Garcia and co-workers (Martínez-Garcia et al. 1999) by homogenising the samples, previously powdered in liquid nitrogen, in four volumes of a 125 mmol pH 8.8 Tris-HCl buffer containing 1% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol and 50 mmol $Na_2S_2O_5$. The homogenate was centrifuged at 13 000 *g* for 20 min to obtain a clarified supernatant. The protein content was measured by using the 2-D Quant Kit (GE Healthcare Europe GmbH, Freiburg, Germany).

Ion concentration

Samples of approximately 0.5 g of dried tissue were digested by a microwave digestor system (Anton Paar Multiwave 3000, Anton Paar, Graz, Austria) in Teflon tubes filled with 9.5 mL of 65% HNO_3 and 0.5 mL of H_2O_2 by applying a two-step power ramp (step 1: at 500 W for 10 min, maintained for 5 min; and step 2: at 1200 W for 10 min, maintained for 15 min). After 20 min of cooling time, the mineralised samples were transferred to polypropylene test tubes. Samples were then diluted 1:40 with Milli-Q water (EMD Millipore Corporation, Billerica, MA, USA), and the concentration of ions was measured with a Varian 820 ICP-MS (Varian, Inc., Palo Alto, CA, USA). A 2 mg/L aliquot of an internal standard solution (^{45}Sc, ^{89}Y, ^{159}Tb) was

Table 1. Leaf area, total leaf area, leaf and root biomass of grapevine rootstock genotypes 101.14 and M4 at the start of the experiment.

Genotype	Leaf area (cm^2)	Total leaf area (cm^2)	Leaf biomass (g/plant)	Root biomass (g/plant)
101.14	44.38 ± 1.53	1531 ± 129	25.20 ± 1.95	17.68 ± 1.52
M4	24.94 ± 0.67	909 ± 97	14.09 ± 1.60	9.67 ± 1.72

Values are the mean ± SE of three independent biological samples analysed in triplicate ($n = 9$).

added both to samples and the calibration curve to give a final concentration of 20 µg/L. Typical analysis interferences were removed by using the collision–reaction interface of the ICP-MS with an H_2 flow of 40 mL/min.

In the pool of plants exposed to NaCl treatment, Rb was used as a tracer for K (Kochian et al. 1985, Cocucci and Sacchi 1993, Kodur et al. 2010, 2011). At the start of the experiment, Rb was added to reach a soil final concentration of 0.1 mmol, (approximately 1% of K concentration). The leaf concentration of Rb was measured at the end of the experimental period by ICP-MS as described above.

Statistical analyses

Regression coefficients, correlations and box plots were obtained using the 10.0 Sigma Plot software package (SPSS, Chicago, IL, USA). The significance of differences between means within each cultivar and measured parameter were assessed by univariate analysis of variance, followed by the Duncan multiple range test ($P < 0.01$) using Statistica 8.0 (Statsoft, Tulsa, OK, USA).

Results

Experimental conditions

Air temperature and VPD were measured within the greenhouse using a humidity and temperature probe (model HMP-45A, Vaisala, Vantaa, Finland) housed inside a solar radiation shield; their trend and range of variation are presented in Figure 1. An average of 26.5 ± 3.1°C and 25.5 ± 4.1°C was measured in the light and dark periods, respectively, while the mean daily VPD was 1.1 kPa ± 0.1.

Some plant parameters, such as leaf area, total leaf area, leaf biomass and root biomass, were evaluated on a preliminary basis (Table 1). The dimension of leaves of the 101.14 genotype was greater than that of the M4 genotype (+ 56%). The average number of leaves for each plant chosen for the experiments was about 35, so the total leaf area, leaf biomass and root biomass were greater in 101.14 compared with M4.

Changes in SWC were monitored before the start of the experiment. These preliminary measurements verified that, under the control conditions (i.e. 80% of soil field capacity), SWC decreased during the diurnal period from 8:00 am to 6:00 pm to field capacity values of about 70%. From 6:00 pm to 8:00 am, the SWC reduction did not exceed 4%. These changes were quite similar for both the studied genotypes and did not affect leaf gas exchange.

After this preliminary monitoring period, a progressive WS or salt stress was induced by reducing the daily water supply or adding 5 mmol/day of NaCl, respectively (Figure 1). The variation in SWC in pots of plants subjected to WS conditions is shown in Figure 2. For both genotypes, the SWC measured before water addition was lower than the target SWC imposed in the first phase of the experiment (80–60% of soil field capacity), while afterwards SWC depletion rate progressively slowed down compared with the planned curve. In this view, it was observed that, to achieve the lowest SWC value planned in this experimental design (i.e. from 40 to 30%), about 2 days were required. In this last phase of the experiment, the M4 genotype showed a higher water loss compared with that of 101.14. Finally, considering all experimental times, the water loss did not exceed 15% of the water content of the estimated trend.

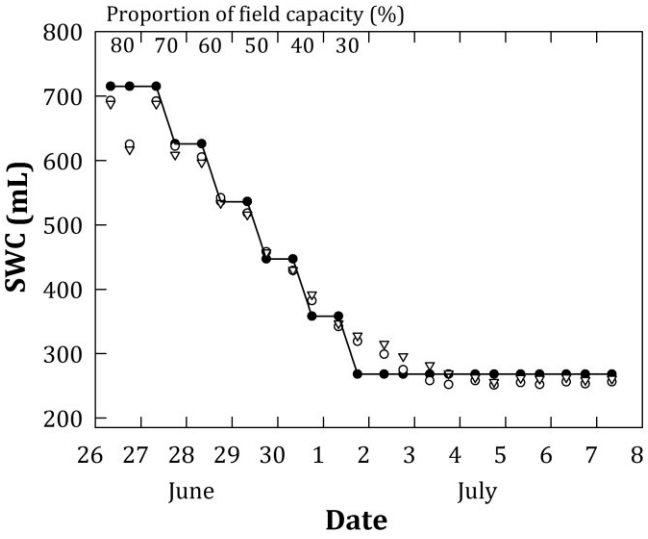

Figure 2. Effect of water stress on the soil water content (SWC) in pots of 101.14 (▽) and M4 (○) grapevine rootstock genotypes. The values were measured before the soil water adjustment. No water addition was made if SWC was higher than the chosen trend (estimated trend) (●).

Leaf expansion and internode elongation

At time T_0, the rate of leaf area expansion for the 101.14 and M4 genotypes was 1.16 and 0.42 cm^2/day, respectively (Figure 3a). These rates were ascribable to the difference in the mean leaf area (see Table 1). At the first two time points, the effect of WS on leaf area expansion rate was higher for 101.14 than for M4, while later leaf expansion was completely inhibited in both genotypes (Figure 3a). Similar results were observed under NaCl stress, but in this case the lesser effect on M4 was also observed after a longer period (Figure 3b).

Initially, internode growth rate was similar in both genotypes (1.67 ± 0.08 and 1.43 ± 0.16 cm/day in 101.14 and M4, respectively). This parameter was affected by WS in a way similar for both genotypes (Figure 3c). On only the second day the effect was higher for 101.14 than for M4. Both genotypes exhibited a similar trend of internode elongation under NaCl

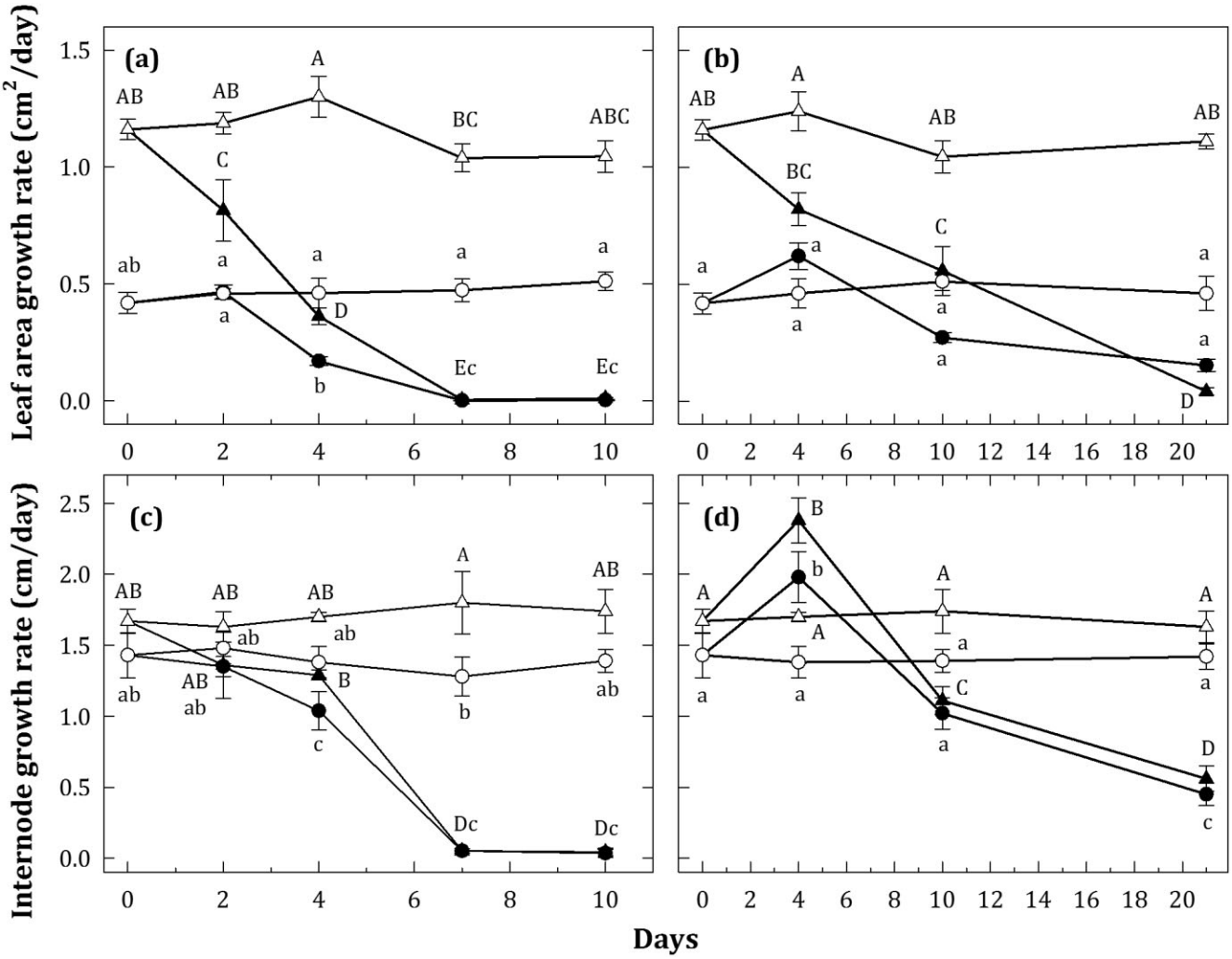

Figure 3. Effect of (a,c) water deficit (WS) and (b,d) salt stress (NaCl) on (a,b) leaf expansion rate and (c,d) internode elongation rate of M4 (○, ●) and 101.14 (△, ▲) grapevine rootstock genotypes. Values are the means ± SE (n = 9). Values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

exposure. Increased internode elongation was observed after 4 days, while a progressive decrease occurred at later time points (Figure 3d)

Plant water status and leaf gas exchange

Leaf water potential (Ψ_{leaf}) measured in the control condition was −0.89 ± 0.03 and −0.86 ± 0.03 MPa for the M4 and 101.14 genotypes, respectively. As a consequence of WS and NaCl stresses, Ψ_{leaf} decreased in both genotypes. These results are shown in Figure 4, expressed as an increasing proportion of control. The effect was progressively higher in M4 than in 101.14 under both stresses, becoming evident and statistically significant in respect to previous points (i.e. control vs stress conditions) with time. In detail, the drop of Ψ_{leaf} was −1.45 MPa under both the stresses in M4, while in 101.14 a decrease of −1.22 and −1.28 MPa under WS and NaCl, respectively, was measured.

Under the same experimental conditions, A_n was 6.8 ± 0.4 and 5.28 ± 0.7 μmol CO_2 /(m^2 • s) for M4 and 101.14 genotypes, respectively. At the same time, g_s was 0.15 ± 0.02 and 0.07 ± 0.01 mmol H_2O/(m^2 • s) for M4 and 101.14, respectively. Under WS, A_n decreased in both genotypes, but to a different extent when the stress became more severe. After 6 days, almost complete inhibition was measured for 101.14, while M4 showed A_n values of approximately 40% with respect to its control (Figure 5a). At later time points, a partial recovery (approximately 20%) was observed in M4.

Under NaCl exposure, the difference between the two genotypes was durably significant after 14 days of treatment (Figure 5c). In the last stage, while the A_n value for M4 was similar to those measured previously, the inhibition detected in 101.14 was about 80% of that of its control (Figure 5c).

Under both stress conditions, a concurrent decrease of g_s took place (Figure 5b,d). Almost complete stomatal closure was induced by WS in 101.14, while in M4 it reached a value of about 20% of the control (Figure 5b). Under NaCl conditions, the decrease of g_s was more evident after a shorter period, while it was affected to a lesser extent at later time points with respect to those observed under WS (Figure 5d). Also in this case, M4 was able to maintain a higher value of g_s than that observed for 101.14.

The dependence of g_s on Ψ_{leaf} as well as of A_n on g_s (i.e. their ratio or leaf intrinsic water use efficiency, $iWUE_{leaf}$) was analysed (Figure 6). Data comprising well watered (C), water (WS) and salt (NaCl) stressed plants for both genotypes are presented, and the best-fitting regression curves are shown, all of these being highly significant ($R^2 \approx 0.8$). When g_s is plotted against Ψ_{leaf} (Figure 6a,b) and against A_n (Figure 6c,d), a logistic and a single

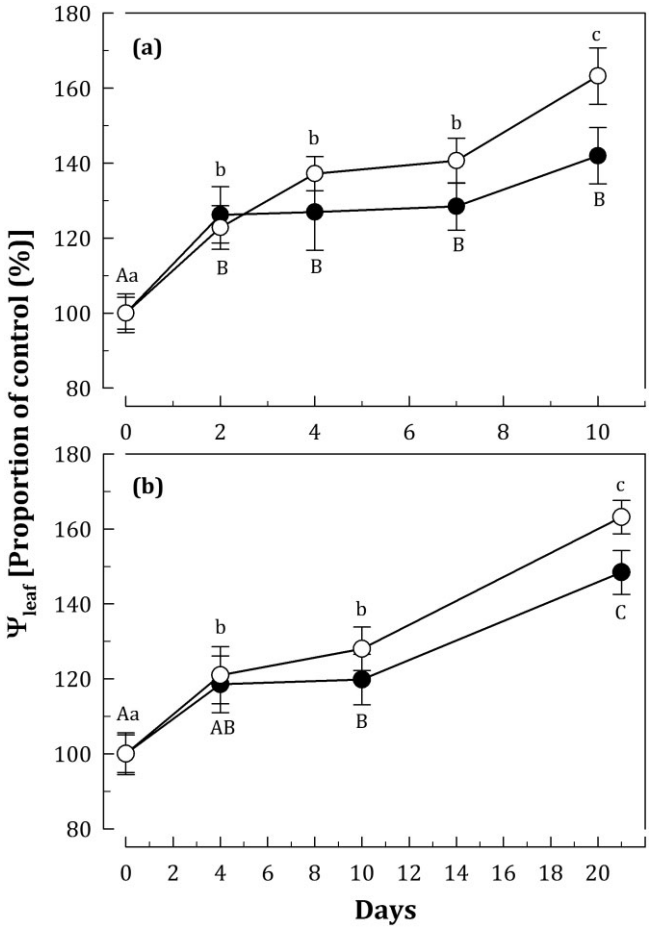

Figure 4. Effect of (a) water stress (WS) and (b) salt stress (NaCl) on the leaf water potential (Ψ_{leaf}) of 101.14 (●) and M4 (○) grapevine rootstock genotypes. Mean ± SE values are expressed as a proportion of the control (i.e. Ψ_{leaf} values of −0.89 ± 0.03 and −0.86 ± 0.03 MPa for M4 and 101.14 genotypes, respectively at T_0). Values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

hyperbolic function, respectively, satisfactorily fitted data from both genotypes.

The relationship between g_s and Ψ_{leaf} was characterised using a four-parameter logistic model of the form:

$$y = y_0 + \frac{a}{1 + \left(\frac{\psi}{\psi_0}\right)^b}$$

where 'a' represents the upper asymptote, and in this case is the maximum theoretical g_s. The Ψ_0 is associated with the point of symmetry of the logistic curve (inflection point), and in this case is the Ψ required to reach 50% of a, while 'b' is a curvature parameter related to the slope of the curve.

The evaluation of these regressions enabled the detection of three distinct phases, which were characterised by a 'mild or no stress', a 'moderate drought' and a 'severe stress' phase, respectively (Figure 6). These phases were selected on the basis of the Ψ_0 threshold for g_s reduction [inflection points: Ψ_0 of −0.88 (101.14) and Ψ_0 of −1.04 (M4)] and on the pattern of response of photosynthesis to drought using g_s as reference parameters, as reported by Medrano et al. (2002). A profound difference was observed between the two genotypes. For 101.14, the reduction of g_s occurred already at a Ψ_{leaf} value lower than −0.6 MPa, being halved at −0.88 MPa and completely inhibited at a Ψ_{leaf} value lower than −1.2 MPa (Figure 6a). In contrast, for M4 the decrease in g_s occurred at a Ψ_{leaf} value lower than −0.9 MPa, reaching a lower inflection point at −1.04 MPa, and was not completely inhibited once the Ψ_{leaf} value reached −1.5 MPa (Figure 6b).

The results revealed a similar pattern of photosynthetic response for both WS and NaCl stress but with different ranges between the two genotypes. In the early stages of the mild or no stress phase, A_n values for 101.14 were higher than those detected for M4 (Figure 6c,d). After an early effect of drought resulting in partial stomatal closure (phase 2, see Figure 6a,b, moderate stress), further reduction of g_s was evident as drought gradually proceeded leading to severe stress conditions, with a simultaneous dramatic reduction of g_s (see phase 3) and an almost complete inhibition of A_n for 101.14 (Figure 6c). In contrast, under the same conditions, only a partial stomatal closure occurred for M4, leading to a lesser inhibition of the A_n value (Figure 6d).

Osmolality, metabolites and inorganic ions

Foliar and root tissue cell osmolality and the concentration of metabolites and inorganic ion were measured at the last time point of treatments (Figures 7–9). Under WS and NaCl stress conditions, leaf osmolality increased in both genotypes (Figure 7a). This increase was similar in the two genotypes under WS, while it was slightly higher for M4 than for 101.14 under NaCl stress (28 and 44% in 101.14 and M4, respectively). An increase in osmolyte concentration was observed in root tissue (Figure 7b). Under WS, the extent of this enhancement was higher for M4 compared with that for 101.14 (70 and 106% for 101.14 and M4, respectively), while a similar increase was measured under NaCl stress conditions (121 and 124% for 101.14 and M4, respectively).

The concentration of total sugars, amino acids and total proteins in the leaf and root tissues is shown in Figure 8. In the leaves, a non-significant increase in all metabolites measured was observed for M4 under WS, while for 101.14 both stress conditions induced only a slight increase in the concentration of amino acids (Figure 8a,c,e). Nevertheless, the most important changes occurred in the roots. Under stress conditions, a significant increase in the concentration of total sugars and amino acids occurred in both genotypes (Figure 8b,d). This effect, which was more marked under WS, was greater for M4. Water stress induced a significant decrease in protein concentration for 101.14, while under the same experimental conditions only a slight effect was observed in M4 (Figure 8f). A slight decrease in protein concentration was observed for 101.14 under NaCl exposure.

The concentration of K, Mg and Ca in both leaf and root tissues is shown in Figure 9. Leaf K concentration increased under NaCl exposure in both genotypes, but this increase was significantly higher in M4 (Figure 9a). In the root tissue of 101.14, the concentration of K tended to decrease under WS, while a significant upsurge in the same experimental treatment was detected for M4. Under NaCl treatment, the concentration of K in roots increased only for M4 (Figure 9b).

The leaf concentration of Mg and Ca did not show significant change under all conditions tested (Figure 9c,e). In the root tissue, the concentration of Mg increased under WS only for M4 (Figure 9d,f).

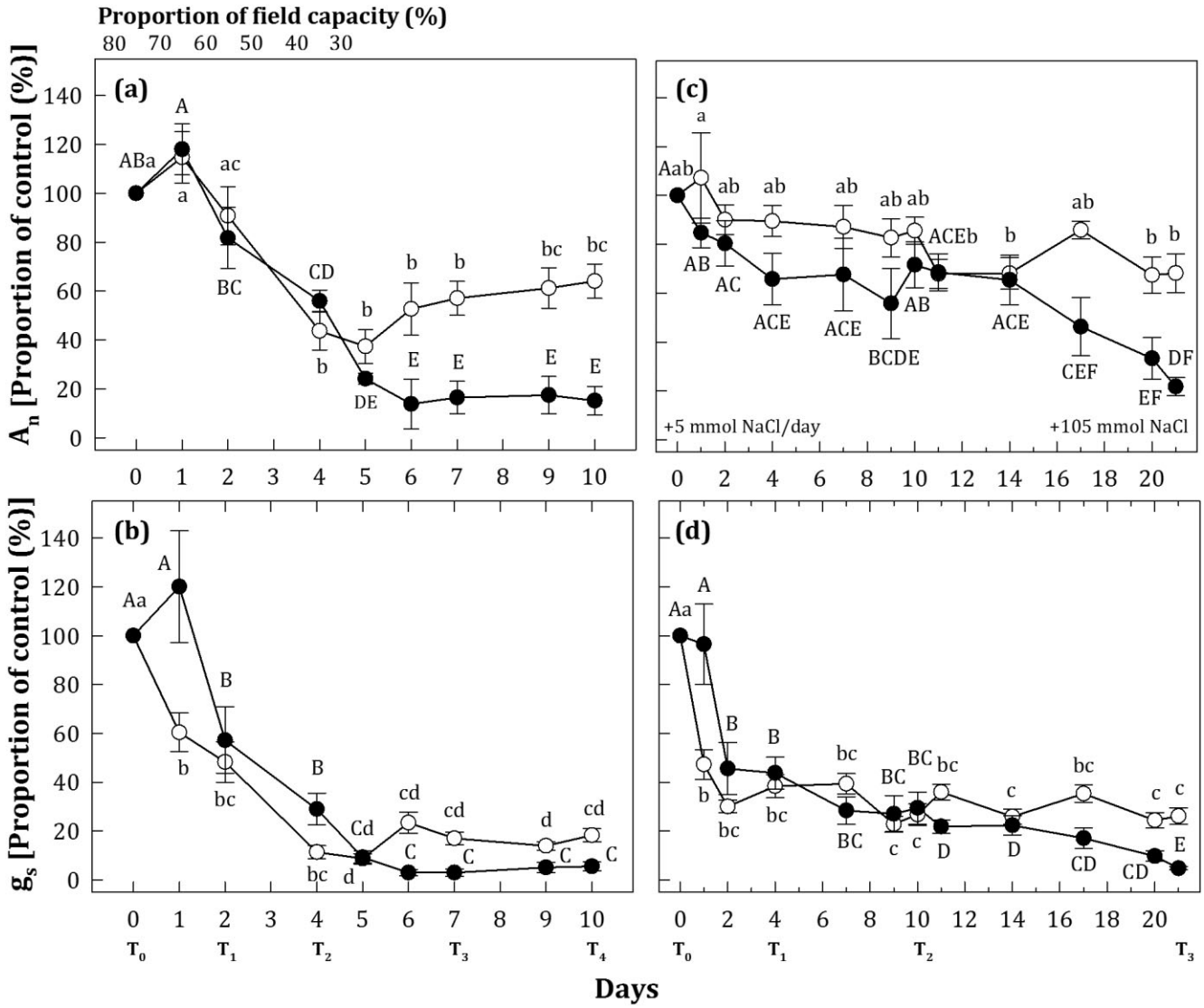

Figure 5. Effect of (a,b) water stress (WS) and (c,d) salt stress (NaCl) on the net CO_2 assimilation (A_n) and stomatal conductance (g_s) for 101.14 (●) and M4 (○) grapevine rootstock genotypes. Average ± SE values of A_n and g_s are expressed as a proportion of the control [i.e. A_n values of 6.8 ± 0.4 and 5.28 ± 0.7 μmol $CO_2/(m^2 \cdot s)$ for M4 and 101.14 genotypes, respectively, at T_0, and g_s values of 0.15 ± 0.02 and 0.07 ± 0.01 mmol $H_2O/(m^2 \cdot s)$ for M4 and 101.14, respectively, at T_0]. T_{1-4} represent sampling times throughout the experimental period after control (T_0). Values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

Leaf sodium, chloride and Rb concentration

Table 2 reports the concentration of Na, Cl and Rb in the leaves of plants grown for 21 days in the absence of NaCl or under salt stress conditions. A strong increase of both Na and Cl was detected in the leaves of plants exposed to NaCl, and this effect was approximately twice as great for both ions in M4. The accumulation of Rb in leaf tissue was significantly higher for M4 (+56% compared with control conditions), while the change in 101.14 was not significant.

Net CO_2 assimilation recovery in WS plants after re-watering

In a preliminary experiment, plants previously grown under WS conditions for 10 days were watered back to 80% of the soil field capacity, after which the recovery in A_n was evaluated (Figure 10). After 24 h of recovery, the A_n value increased in the WS treatment for both genotypes, but to a different extent (32 and 75% of the control for 101.14 and M4, respectively). The difference between the control and recovered samples was significant only in M4.

Discussion

The aim of this work was to study the biochemical and physiological responses of the rootstock candidate M4 under WS and NaCl exposure. The study was also conducted on the commercial rootstock 101.14, generally considered to be relatively susceptible to WS (Alsina et al. 2011, Pavlusek 2011, Gambetta et al. 2012). Moreover, considering that 101.14 is a genotype representative of the many hybrids derived from V. *riparia* x V. *rupestris*, and that M4 was obtained from crossing V. *vinifera* x V. *berlandieri*, the comparison is of interest for future investigations aimed at understanding better the molecular basis of stress responses also from the genetic point of view.

Apart from peculiar genotypic characteristics, the stress responses might be influenced by many environmental factors (Lovisolo et al. 2010, Alsina et al. 2011, Tomás et al. 2012). The experiment adopted a protocol under controlled environmental conditions suitable to induce a WS characterised by a strong decrease in soil water availability, thereby provoking a clear effect early in the experiment (Figures 1,2). Otherwise, NaCl

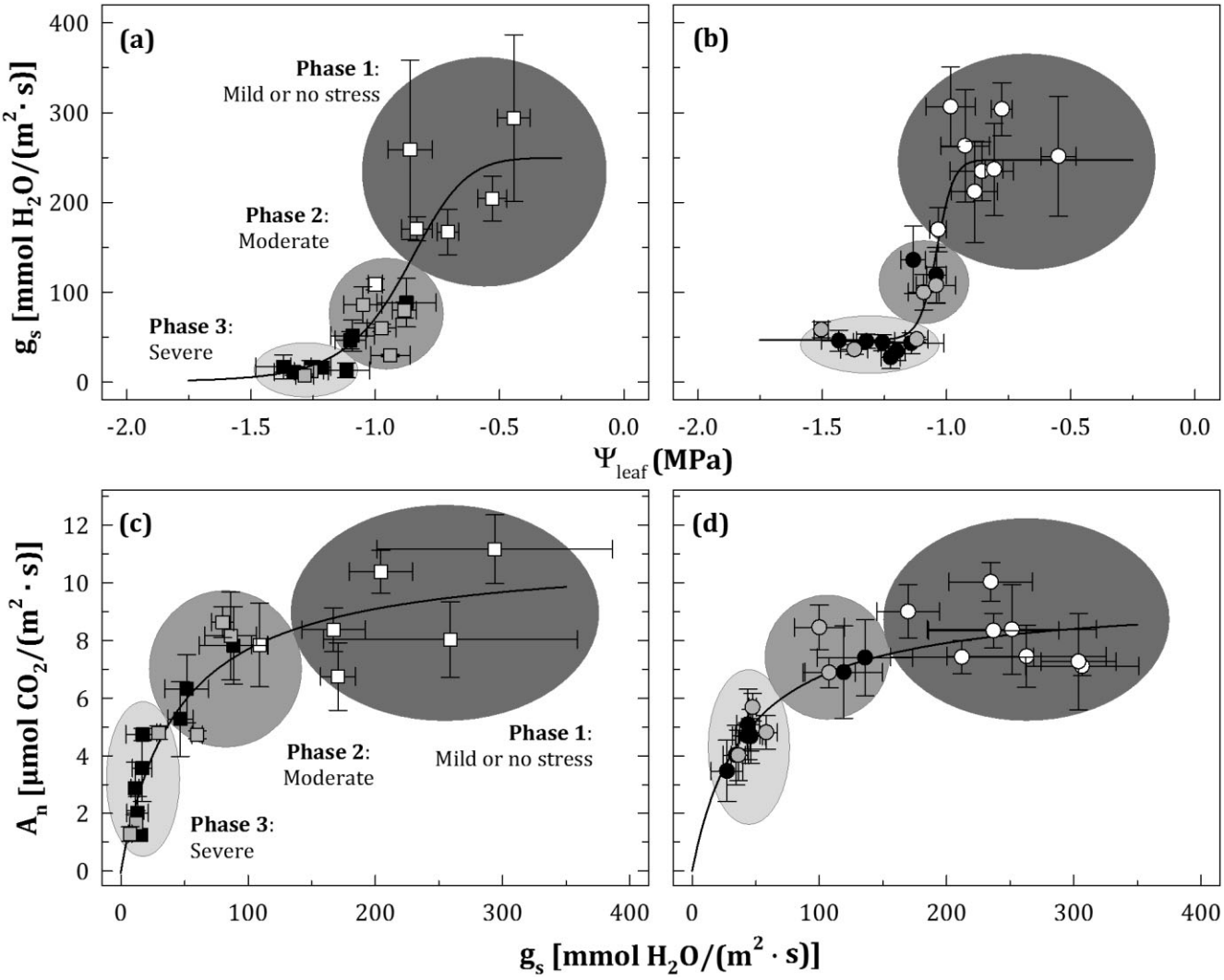

Figure 6. Stomatal conductance (g_s) in (a) 101.14 {$y = 4.42 + 249.6/[1 + (x/–0.88)^{7.08}]$, $R^2 = 0.78$} and in (b) M4 {$y = 47.47 + 203.66/[1 + (x/–1.04)^{31.85}]$, $R^2 = 0.86$} grapevine rootstock genotypes as a function of leaf water potential (Ψ_{leaf}), or of net CO_2 assimilation rate (A_n) in (c) 101.14 ($y = 11.1x/44.25 + x$, $R^2 = 0.86$) and in (d) M4 ($y = 9.579x/42.14 + x$, $R^2 = 0.80$) in well-watered (□, ○), water-stressed (■, ●) and salt-stressed (■, ●) plants of the two genotypes, 101.14 (□, ■, ■) and M4 (○, ●, ●). Each point corresponds to measurements on different sampling days (0, 2, 4, 7, 10 and 21). Data represent the mean ± SE for both *x* and *y* axes of six replicates for control (□, ○), drought (■, ●) and salt loading (■, ●) plants for each genotype. The curve of best fit for (a,b) and (c,d) plots was a four-parameter logistic and a single rectangular hyperbola, respectively. Three main regions are distinguished along the curves using g_s as a reference parameter: mild or no stress (Phase 1), moderate (Phase 2) or severe stress (Phase 3).

stress was induced by adding 5 mmol of NaCl every day, a condition that progressively affected plant performance. The tolerance capacity was studied for 10 and 21 days for WS and NaCl, respectively (Figure 1), because at these times strong stress conditions were reached, as suggested from the comparison of growth and leaf gas exchange parameters (Figures 3–5) with those present in the literature (Lovisolo et al. 2002, Beis and Patakas 2010, Chaves et al. 2010, Williams et al. 2010, Pou et al. 2012, Williams 2012).

Differences in growth as well as in stomatal conductance emerged between the two genotypes. The observed discrepancy in total leaf area (Table 1) did not determine differences in water loss in the control condition (Figure 2). These data fitted well with g_s values higher in M4 than in 101.14. Preliminary investigations on stomatal distribution indicated that stomatal density was higher in M4, so confirming the peculiar morphological characteristics of the two genotypes (Dr Massimo Galbiati, pers. comm., 2013).

The inhibition of leaf expansion rate measured for M4 was of lesser extent during both the initial phase of WS and throughout the NaCl treatment (Figure 3).

Under both stress conditions, a progressive decrease of Ψ_{leaf} occurred in both genotypes (i.e. increase in proportion, Figure 4), thereby suggesting a typical anisohydric response (Lovisolo et al. 2010, Pou et al. 2012). The drop of Ψ_{leaf} detected in M4 showed that this genotype experienced a more severe WS, even higher than –1.5 MPa, a value suggested to be the threshold for severe cavitation described for grapevines (Salleo and Lo Gullo 1989, Lovisolo et al. 2010).

According to previous studies (Medrano et al. 2002, Chaves et al. 2009, Lovisolo et al. 2010, Pou et al. 2012), the progressive reduction of g_s measured under WS and NaCl exposure (Figure 5b,d), in combination with its relationship with water status conditions and photosynthetic activity, suggested three different stages to be defined.

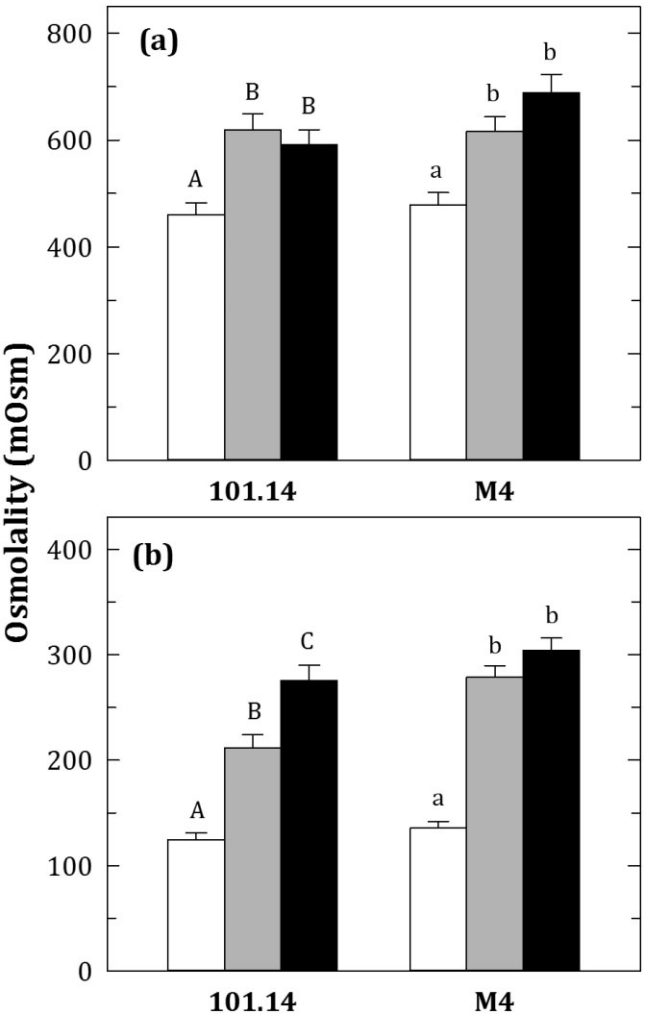

Figure 7. Effect of water stress (■) and salt stress (■) and control (□) on the osmolality of (a) leaves and (b) roots collected from M4 and 101.14 grapevine rootstock genotypes. Osmolality values are mean ± SE ($n = 6$), and those values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

Although a dramatic decrease of g_s occurred in both genotypes, when the stress conditions became more severe, the values remained significantly higher in M4 than in 101.14. This behaviour suggested that, under these conditions of severe drought and almost complete stomatal closure, photoinhibition probably occurred in the 101.14 genotype. This conclusion was supported by preliminary measurements indicating that the maximum efficiency of PSII (Fv′/ Fm′) was reduced by about 20% under WS in 101.14 (data not shown). Analysing the response of g_s as a function of changes in Ψ_{leaf}, differences in the water use strategies adopted by the two genotypes clearly emerged (Figure 6a,b). In 101.14, g_s started decreasing when Ψ_{leaf} declined at a value under −0.6 MPa, while this effect was observed in M4 at a Ψ_{leaf} value less than −0.9 MPa. These results suggest a more drought-avoiding behaviour for 101.14 compared with that of M4, which appears to maintain a partial stomatal aperture even at the more severe stress conditions tested (Chaves et al. 2010).

The dependence of A_n on g_s and their ratio (i.e. $iWUE_{leaf}$) are potential physiological targets for improving water use strategies of grapevines (Flexas et al. 2010, Williams 2012). A close relationship between A_n and g_s was observed in both genotypes (Figure 6c,d). Considering such a relationship, it can be noted that while under the control conditions, the CO_2 carboxylation efficiency appeared to be higher for 101.14 compared with that of M4; under stress treatments, the opposite behaviour was evident. Furthermore, although the possibility of changes in mesophyll CO_2 conductance cannot be excluded, these results supported the idea that under these experimental conditions the variation in photosynthesis essentially depended on photosynthetic stomatal limitations (Escalona et al. 1999). Accordingly, a reduction of A_n was measured in both stress conditions at early stages, but significant differences were observed over the longer term (Figure 5a,c). In 101.14, A_n was almost completely inhibited under WS and deeply affected under NaCl exposure, in contrast to a partial recovery of this parameter in M4, suggesting that this genotype was able to acclimate.

A typical response occurring under WS and during NaCl exposure is the increase in compatible solutes that participate in osmotic adjustment (Morgan 1984, Munns and Tester 2008). Evaluation of the contribution of different osmolytes is an important aspect for the clarification of the strategies adopted by different species because these can determine profound differences in primary metabolism, carbon fluxes and energy requirements (Hummel et al. 2010). In other words, under drought and saline conditions, photosynthesis is strongly affected, and at the same time the adaptive responses, which have a cost in terms of the carbon and energy, must be sustained (Yeo 1983, Raven 1985, Medrano et al. 2002, Chaves et al. 2009). Accordingly, previous studies conducted on the grapevine at the leaf scale revealed changes in metabolite and ion profiles, as well as showing that these responses also involve substantial changes in gene expression (Patakas et al. 2002, Cramer et al. 2007).

Although the central role played in the perennial species by roots in the responses to WS and NaCl exposure is coming to light, until now studies performed in the grapevine on this organ essentially concentrated on the transport activities involved in water and ion uptake and movement, as well as on root anatomy (Storey et al. 2003, Addington et al. 2006, Nardini et al. 2006, Galmés et al. 2007, Vandeleur et al. 2009, Alsina et al. 2011, Kodur et al. 2011, Gambetta et al. 2012).

In the present work, osmotic responses were analysed in both leaf and root tissues. Under stress conditions, an increase in osmolyte concentration was measured in both organs, but this response was greater for roots (Figures 7,8). The increase in osmolality measured in the leaves of plants grown under WS was similar among the two genotypes. The calculation of Ψ_s by the van't Hoff equation revealed only a partial adjustment in M4, which by maintaining a greater stomatal aperture enabled a higher water transpiration rate. Furthermore, the Ψ_{leaf} values of M4 leaves were similar to their Ψ_s, suggesting that the mesophyll cells were near to incipient plasmolysis, indicating that the water driving force was dependent essentially on Ψ_s.

No significant change in the concentration of total soluble sugars, amino acids and ions was measured in the leaves (Figures 8a,c, 9a,c,e), showing a behaviour different from that of previous studies, in which a decrease in total soluble sugars and a more consistent enhancement of the concentration of ions were found in this organ (Patakas et al. 2002). Although greater information should be available to clarify this discrepancy, it can be assumed that the reason could be attributed to differences in the experimental conditions adopted as well as to the specific genetic background of the genotype (Tomás et al. 2012).

The roots appeared to be markedly different, as a greater increase in osmolytes was measured as well as a significant difference in the extent of the responses between the two genotypes (Figures 8b,d, 9b,d,f). In particular, under WS, M4 showed a greater increase in amino acids, K and Mg when

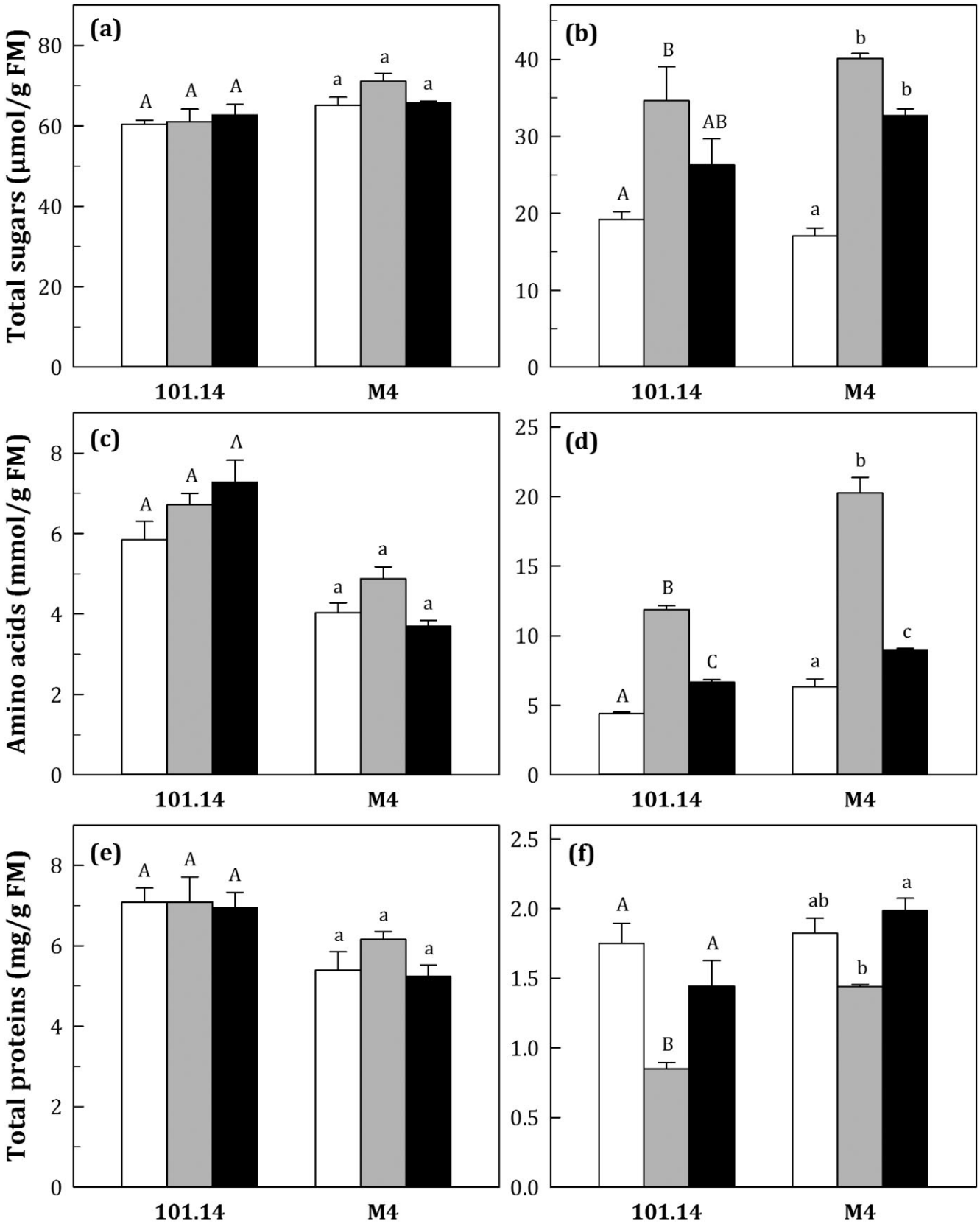

Figure 8. Effect of water stress (▒) and salt stress (■) and control (□) on the concentration of (a,b) total soluble sugars, (c,d) amino acids and (e,f) total proteins of (a,c,e) leaves and (b,d,f) roots collected from M4 and 101.14 grapevine rootstock genotypes. The values of the concentration of total soluble sugars, amino acids and total proteins are the mean ± SE ($n = 8$), and those values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

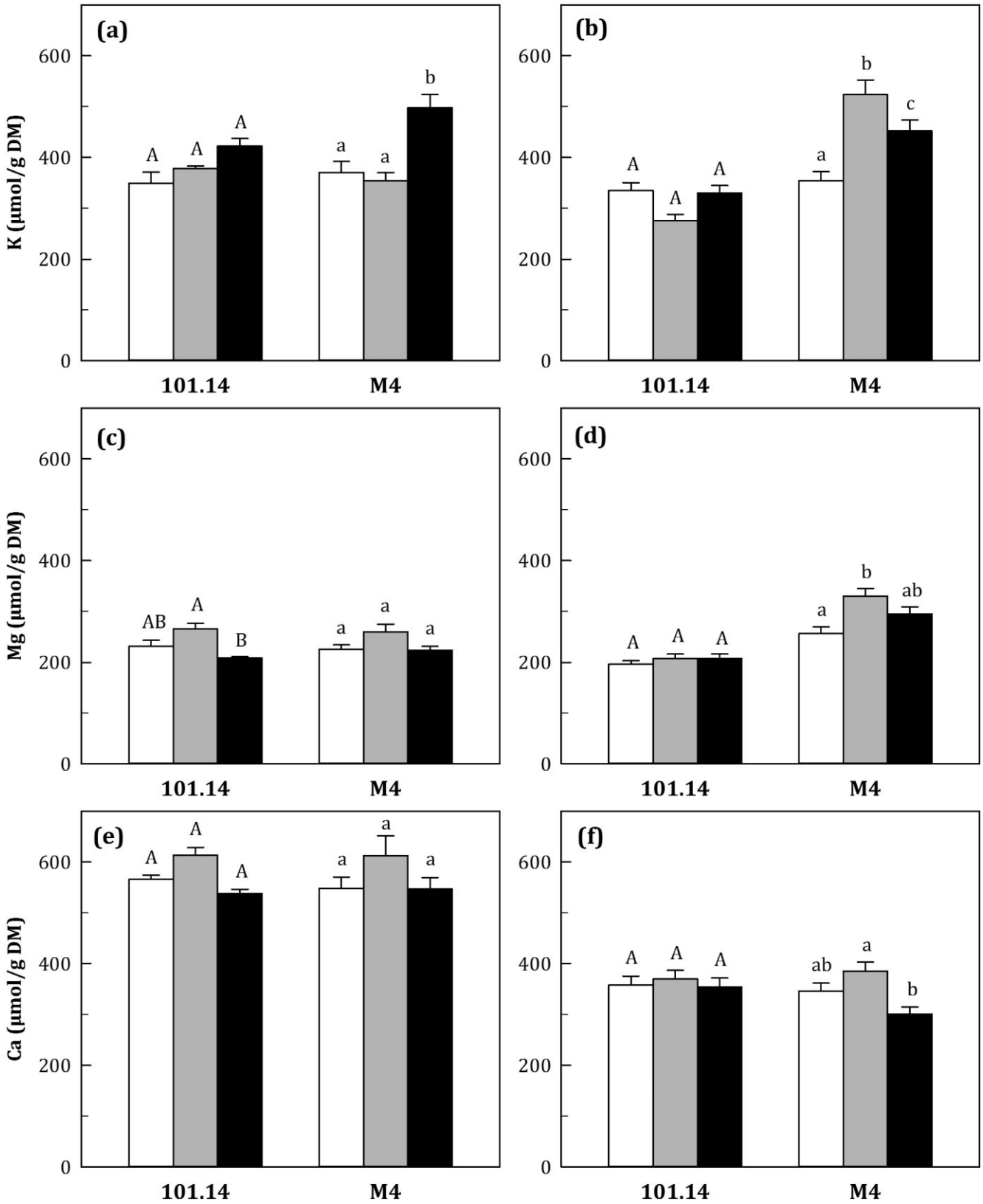

Figure 9. Effect of water stress (▨) and salt stress (■) and control (□) on the concentration of (a,b) K, (c,d) Mg and (e,f) Ca of (a,c,d) leaves and (b,d,f) roots collected from M4 and 101.14 grapevine rootstock genotypes. The values of the concentration of K, Mg and Ca are the mean ± SE values ($n = 9$), and those values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

compared with that of 101.14. At the same time, 101.14 showed a dramatic decrease in total proteins (−51%) compared with that of M4 (−21%), suggesting that the roots of two genotypes were affected differently under WS (Figure 8f). These results could be ascribable to cellular damage, but could also be explained as a consequence of a different ratio between metabolically active and highly lignified (differentiated) tissues. Moreover, even if in this work only the short-term responses

Table 2. Concentration of sodium, chloride and Rb in the leaves of the grapevine rootstock genotypes 101.14 and M4 grown for 21 days in the absence of or under salt stress. Rubidium was used as a K tracer and was added at the onset of salt treatment.

Genotype	Treatment	Na (μmol/g DM)	Cl (μmol/g DM)	Rb (μmol/g DM)
101.14	Control	$2.27^{A} \pm 0.17$	$2.47^{A} \pm 0.11$	$1.14^{A} \pm 0.05$
	NaCl	$21.0^{B} \pm 1.06$	$16.9^{B} \pm 0.87$	$1.25^{A} \pm 0.11$
M4	Control	$4.06^{a} \pm 0.27$	$2.48^{a} \pm 0.17$	$1.06^{a} \pm 0.07$
	NaCl	$43.8^{b} \pm 2.08$	$31.4^{b} \pm 1.77$	$1.65^{b} \pm 0.10$

Values are the mean ± SE of three independent biological samples analysed in triplicate ($n = 9$). Values indicated with the same letters do not significantly differ according to Duncan's multiple range test ($P < 0.01$). Cl, chloride; DM, dry mass; Na, sodium; Rb, rubidium.

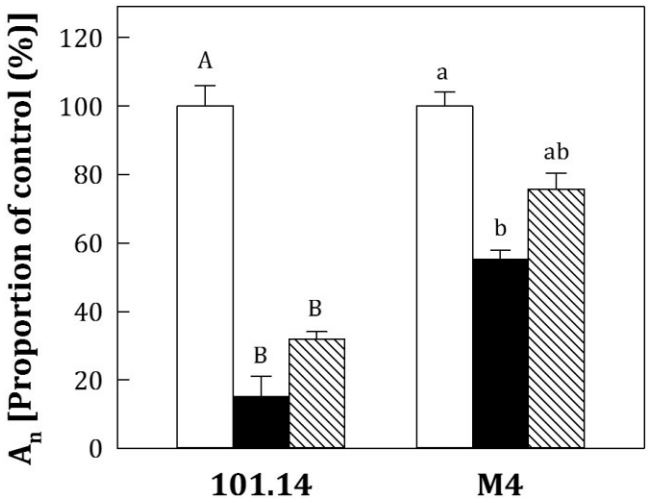

Figure 10. Effect of severe water stress (■) and recovery (▧) on changes of net CO_2 assimilation (A_n) for 101.14 and M4 grapevine rootstock genotypes. The values of A_n are expressed as a proportion of the control (□) and represent the mean ± SE of three replicates (absolute values of control plants are reported in the Results section and in Figure 6). Values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

were studied, this result could suggest a difference in root plasticity between the two genotypes (Alsina et al. 2011). Nevertheless the difference between the two genotypes suggested that an effective counteracting response to WS occurred in the root system of M4, while a stress state (e.g. decrease in total proteins) was emerging in 101.14.

Taken together, these results support the idea that the higher capacity of M4 to respond to WS conditions appears to be dependent on root responses, in which the accumulation of osmolytes plays a central role in the rate of water uptake under stress conditions (Aroca et al. 2012).

Conservation of cell integrity, adjustment of transport activities (in particular to sustain water and ion transport) and energy availability represent further crucial factors that contribute to WS acclimation (Yeo 1983, Raven 1985, Medrano et al. 2002, Keller 2005, Chaves et al. 2009, Lovisolo et al. 2010, Szabados et al. 2011). Although in the present study none of the above-mentioned aspects were studied in detail, some data, such as the content of total proteins and the greater ion accumulation, support the idea that in M4 these typical responses were activated. Indeed, in the re-watering experiments, the recovery shown by M4 after 24 h was in agreement with this conclusion (Figure 10). The A_n recovery, in fact, is not ascribable only to the status of photosynthetic machinery, but also to other factors like hydraulic conductance (Vandeleur et al. 2009). Recent studies have shown that in the grapevine, the root surface area is one of the factors determining root hydraulic conductance in well-watered and WS conditions (Alsina et al. 2011, Gambetta et al. 2012). In the present study, it was found that under WS the root fresh mass at T_4 was 61and 63% of the control condition in 101.14 and M4, respectively (data not shown), indicating that root system biomass was not affected differently in the two genotypes. This result further supported the idea that the strategy adopted by M4 under WS was dependent on its capacity to better preserve root tissue integrity and therefore its functionality (see above).

Salt stress response is characterised by two main components, osmotic and toxic ion effects (Munns and Tester 2008, Shavrukov 2013). Nevertheless, their predominance is strictly linked to the manner by which NaCl is applied to the plants (Shavrukov 2013). In the present study, salt was gradually applied and the experiment was conducted for 21 days (Figure 1). Salt exposure produced similar responses to those observed under WS, even though specific differences were found. The effects induced by salt treatment on internode growth, leaf expansion, Ψ_{leaf}, A_n and g_s were quite similar to those observed under WS, although they were of lesser extent after longer exposure times (Figures 3b,c, 4, 5c,d). Taken together, these results showed that, also in this case, M4 had a significantly greater capacity to tolerate NaCl exposure. In this genotype, an enhancement of leaf tissue osmolality was observed (Figure 7a), and this was essentially related to an increase in K concentration (Figure 9a).

The increase in leaf concentration of Na and Cl was markedly higher in M4 than in 101.14, and this could be a direct consequence of higher transpiration rates maintained by this genotype (Table 2 and Figure 5d). Nevertheless, despite the fact that M4 accumulated approximately twice as much Na and Cl in the leaves compared with 101.14, it exhibited greater functionality, suggesting greater tolerance to salt exposure. In this context, the higher concentration of K measured for M4 should not only play an osmotic role but also have an important function in counteracting the toxic effects of Na (Munns and Tester 2008, Fozouni et al. 2012). In order to investigate this, the ability of K accumulation in the shoot was studied by using Rb as tracer (Kochian et al. 1985, Cocucci and Sacchi 1993, Kodur et al. 2010, 2011). This experiment showed that the exposure to NaCl induced only in M4 a significant increase of Rb in the leaves (+56%), thereby confirming that an increase of K translocation occurred in this genotype (Table 2).

Nevertheless, it was observed that the values measured in this organ at the final time point (i.e. 21 days of NaCl treatment) were not high enough to induce severe Na and/or Cl toxicity (Munns and Tester 2008, Fozouni et al. 2012, Shavrukov 2013). Although information on Na and Cl distribution in the leaf tissue should be gathered to clarify this point in detail (Storey et al. 2003), it should be remembered that the inhibitory effects detected in the leaves could also be determined by long distance signals originated from roots exposed to NaCl (Munns and Tester 2008).

For some species, Cl is considered to be the most toxic ion because it is accumulated to a greater concentration in the leaves, although this characteristic appears to be dependent on genotype (Munns and Tester 2008, Gong et al. 2011, Fozouni et al. 2012). In both genotypes, leaf Cl accumulation appeared to parallel the increase in Na, reaching a value that was, however, of lesser extent with respect to this cation (Table 2). These results, however, did not highlight a difference between 101.14 and M4 for the ability to exclude/translocate Cl.

In the roots, a greater enhancement of total soluble sugars, amino acids, K and Mg was measured under NaCl for M4 compared with that of 101.14, while only slight but not significant difference was observed for total protein concentration (Figures 8b,d,f, 9b,d). Taken together, these observations indicated that in M4 a different activation of metabolism implicated in response to NaCl occurred. As observed for WS, the tolerance to NaCl showed by M4 was also dependent on its capacity to maintain an adequate integrity/functionality of the root system. In this context, it was observed that after 21 days, root biomass was affected to a greater extent for 101.14 than for M4 (−34 and −13%, respectively; data not shown).

Although further studies are required to clarify the relationship between roots and leaves in the grapevine under stress conditions, this work highlights the central role of the root organ in response to WS and NaCl exposure. In this context, the importance of the rootstock role in the adjustment to adverse environmental conditions, in addition to the selection of genotypes that can tolerate combinations of stresses, is emerging as a key hint for future viticulture (Storey et al. 2003, Mittler and Blumwald 2010, Alsina et al. 2011, Gambetta et al. 2012, Marguerit et al. 2012). It should be stressed, however, that these data were obtained on ungrafted plants, so they did not permit an in-depth clarification of the possible performance of M4 as a rootstock, and as well as they did not consider that in grafted plants the scion could also influence its characteristics (Swanepoel and Southey 1989, Zhang et al. 2002, Tandonnet et al. 2010).

The data obtained in this study should encourage further work aimed to investigate the molecular responses occurring in M4 compared with that of 101.14 to research putative molecular markers that will assist genetic selection within the genus *Vitis*.

Acknowledgements

This work was supported by AGER-SERRES Project, grant n°2010–2105. We thank Dr Simon Pierce (University of Milan) for polishing the English in the final version of the manuscript.

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Manuscript received: 21 June 2013
Revised manuscript received: 20 September 2013
Accepted: 13 October 2013