Enhanced salt-induced antioxidative responses involve a contribution of polyamine biosynthesis in grapevine plants

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Summary

The possible involvement of polyamines in the salt stress adaptation was investigated in grapevine (*Vitis vinifera* L.) plantlets focusing on photosynthesis and oxidative metabolism. Salt stress resulted in the deterioration of plant growth and photosynthesis, and treatment of plantlets with methylglyoxal-bis(guanylhydrazone) (MGBG), a S-adenosylmethionine decarboxylase (SAMDC) inhibitor, enhanced the salt stress effect. A decrease in PSII quantum yield (Fv/Fm), effective PSII quantum yield (Y(II)) and coefficient of photochemical quenching (qP) as well as increases in non-photochemical quenching (NPQ) and its coefficient (qN) was observed by these treatments.

Salt and/or MGBG treatments also triggered an increase in lipid peroxidation and ROS accumulation as well as an increase of superoxide dismutase (SOD) and peroxidase (POX) activities, but not ascorbate peroxidase (APX) activity. Salt stress also resulted in an accumulation of oxidised ascorbate (DHA) and a decrease in reduced glutathione. MGBG alone or in combination with salt stress increased monodehydroascorbate reductase (MDHAR), SOD and POX activities and surprisingly no accumulation of DHA was noticed following treatment with MGBG. These salt-induced responses correlated with the maintaining of high level of free and conjugated Spd and Spm, whereas a reduction of agmatine and putrescine levels was observed, which seemed to be amplified by the MGBG treatment.

These results suggest that maintaining polyamine biosynthesis through the enhanced SAMDC activity in grapevine leaf tissues under salt stress conditions could contribute to the enhanced ROS scavenging activity and a protection of photosynthetic apparatus from oxidative damages.

Key words: antioxidative metabolism, grapevine, photosynthesis, polyamines, salt stress

Abbreviations

APX, ascorbate peroxidase; Fv/Fm, PSII quantum yield; MDHAR, monodehydroascorbate reductase; MGBG, methylglyoxal-bis(guanylhydrazone); NPQ, non-photochemical quenching; PA, polyamine; POX, peroxidase; Put, putrescine; qN, coefficient of non-photochemical quenching; qP, coefficient of photochemical quenching; SOD, superoxide dismutase; Spd, spermidine; Spm, spermine; Y(II), PSII quantum yield.

Introduction

Salinity is one of the most important stress factors which limits the growth and development of plants by altering their morphological, physiological and biochemical attributes. In addition to osmotic stress and ionic toxicity, salt stress also results in an oxidative stress, mediated by reactive oxygen species (ROS), all three factors contributing to its deleterious effects (Hernández et al., 2001; 2003; López-Gómez et al., 2007). ROS production is kept under tight control by an efficient antioxidative system, which includes both enzymatic and non-enzymatic compounds. Among these non-enzymatic scavengers, low molecular weight compounds, including ascorbic acid (ASC) and glutathione (GSH) are involved, while the main enzymatic arsenal of ROS scavengers includes enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7) and the ascorbate-glutathione cycle enzymes [ascorbate peroxidase (APX, EC 1.11.1.11), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC. 1.6.4.2)] (Noctor and Foyer, 1998; Asada, 1999).

Plant polyamines (PAs) have been suggested to play important roles in morphogenesis, growth, embryogenesis, organ development, leaf senescence, and abiotic and biotic stress responses (Kusano et al., 2008). Therefore, homeostasis of cellular PA levels is also a defensive strategy that plants have developed to cope with adverse situations (Chinnusamy et al., 2005; Groppa and Benavides, 2008). Putrescine (Put), spermidine (Spd), and spermine (Spm) are the major PA pools commonly present in higher plants and known as active oxygen scavenging compounds being considered as mediators in protective reactions against different stresses (Kovacs et al., 2010). Higher level of PAs is of paramount importance since it correlates with several important physiological functions, including protein regulation (Takahashi and Kakehi 2010), ion channels (Zepeda-Jazo et al., 2011), membrane fluidity, control of nitrogen-carbon balance (Moschou et al., 2012) and stress and defense responses (Hatmi et al., 2013).

In plants, Put is synthesized by the decarboxylation of arginine and ornithine catalyzed by arginine decarboxylase (ADC; EC 4.1.1.19) and ornithine decarboxylase (ODC; EC 4.1.1.17), respectively. The following addition of two aminopropyl groups to Put in two reactions catalyzed by Spd synthase (SPDS; EC 2.5.1.16) and Spm synthase (SPMS; EC 2.5.1.22) leads to the formation of Spd and Spm, respectively. The

aminopropyl moieties arise from the decarboxylation of S-adenosylmethionine by the enzyme S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) (Slocum, 1991). In this sense, methylglyoxal-bis(guanylhydrazone) (MGBG) is a potent inhibitor of SAMDC and thus affects endogenous PAs levels (Boucherau et al., 1999).

Changes in plant PA metabolism occur in response to a variety of abiotic stresses (Bouchereau et al. 1999; Groppa and Benavides 2008). Although it is known that the antioxidative effect of PAs in response to stress is due to a combination of their anion- and cation-binding properties (Groppa and Benavides, 2008), the precise molecular mechanism(s) by which PAs control plant responses to stress stimuli are largely unknown (Kusano et al. 2008; Alcázar et al., 2010; Gill and Tuteja, 2010). Nevertheless, a PA role in ROS production has been suggested. Spermine, Spd and Put all reduce level of superoxide radicals generated by senescing plant cells (Drolet et al., 1986). In addition, PA catabolism produces hydrogen peroxide (H_2O_2), a signaling molecule that can modulate the stress signal transduction chain promoting an activation of an antioxidative defense response, but can also act as a pro-oxidant agent (Groppa and Benavides, 2008).

The involvement of PAs in the tolerance of higher plants to abiotic stress has been widely demonstrated in crops of relevant economic importance, such as tomato, rice (Roy et al., 2005) and barley (Zhao and Qin, 2004). The effect of salinity on PA metabolism is not always clear, and differences in PAs (Put, Spd and Spm) response under salt-stress have been reported among and within species (Boucherau et al., 1999). In rice, root plasma membranes of salt-tolerant cultivars were rich in Spm/Spd, whereas the root plasma membranes of sensitive cultivars were rich in Put only (Roy et al., 2005). Zapata et al. (2004) studied the potential role of PAs in response to salt stress in several plant species such as spinach, lettuce, melon, pepper, broccoli, beetroot and tomato. With the exception of beetroot, Put concentration was lower in seedlings grown under saline conditions. However, salinity caused a significant increase in Spd and Spm in almost all the plant species studied. Accordingly, in rice, Spm and Spd significantly prevented the leakage of electrolytes and amino acids from roots and shoots of rice subjected to salinity and a positive correlation between salt tolerance and accumulation of higher levels of PAs was found (Chattopadhayay et al., 2002).

In this work, we studied the effect of salt stress in the presence and the absence of MGBG, an inhibitor of SAMDC activity, in order to investigate the effects of both

treatments on photosynthesis and oxidative metabolism providing new information about the contribution of PA metabolism to salt stress adaptation in grapevine plantlets.

Material and Methods

Plant material and chemical treatments

In vitro rooted plants of grapevine [*Vitis vinifera* (L.) var. Doukkali] were routinely subcultured every four weeks on half Murashige and Skoog (Murashige and Skoog, 1962) medium containing 3% sucrose, 0.7% agar, 1 µM 6-benzylaminopurine, 0.2 µM indole butyric acid (pH 5.7). The cultures were maintained at 25 °C under cool white fluorescent tubes (55 µmol m^{-2} s^{-1}) and a 16/8h photoperiod.

To carry out the experiments one-month-old plantlets were divided into four groups with four replications. For the first group, 100 mM NaCl was added to the subculture medium. For the second group 1 mM of filter sterilized MGBG was added to the medium. For the third group a combination of 100 mM NaCl and 1 mM MGBG was added. The fourth group consisted in control plants that grown in the absence of NaCl and MGBG. The different analyses were performed on plantlets 10 days after the treatments were applied.

Measurement of chlorophyll fluorescence

The fluorescence of chlorophyll was measured with a chlorophyll fluorometer (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany) in detached leaves from controls and salt-treated grapevine plantlets, grown in the presence or the absence of 1 mM MGBG. After dark-incubation of plants (5 min), the minimum and the maximal fluorescence yields were monitored. Kinetic analyses were carried out with actinic light (81 µmol quanta m^{-2} s^{-1} PAR) and repeated pulses of saturating light at 2700 µmol quanta m^{-2} s^{-1} PAR for 0.8 s at intervals of 20 s. The effective PSII quantum yield (Y(II)), the non-photochemical quenching (NPQ), the maximal PSII quantum yield (Fv/Fm) and the coefficients of non-photochemical quenching (qN) and the photochemical quenching (qP) were analyzed.

Lipid peroxidation

The extent of lipid peroxidation was estimated by determining the concentration of thiobarbituric acid-reactive substances (TBARS). Leaf material (200 mg) was homogenized in 2 mL of 0.1% TCA solution. The homogenate was centrifuged at 15000 g

for 10 min and 0.5 mL of the supernatant obtained was added to 1.5 mL 0.5% TBA in 20% TCA. The mixture was incubated at 90º C in a water bath for 20 min, and the reaction was stopped by placing the reaction tubes in ice. Then, the samples were centrifuged at 10000 g for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted (Cakmak and Horst, 1991). The amount of TBARS was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} according to Cakmak and Horst (1991).

Polyamine extraction and analysis

Polyamines were extracted from lyophilized leaves (250 mg) in 1 M HCl at 4ºC according to the method of Flores and Galston (1982). After crushing in 1 M HC1, samples were filtered and the supernatant, containing the "free" PA fraction, was evaporated to dryness and re-dissolved in (3 ml) 1 M HCl and stored at -20°C. The conjugated PAs were extracted after acid hydrolysis of the pellet (bound polyamines, PAs-I) and an aliquot (2 ml) of the filtrate (conjugated polyamines, PAs-S) was treated with 6 M HCl at 110°C during 12h.

All PA fractions were dansylated as described in Aziz et al. (2001). Dansylated PAs were then analyzed using an Acquity UPLC system (Waters), with an Acquity UPLC BEH C18 1.7 mm 2.1 × 100 mm column heated at 30 °C. Dansyl- PAs were eluted with a mixture of water and pure acetonitrile gradient at a flow rate of 0.5 ml min^{-1} according to Hatmi et al. (2013). Dansyl-PAs were detected using Acquity fluorimeter (Waters) with an excitation wavelength of 365 nm and an emission wavelength of 510 nm, and quantified after calibration with external standards (Sigma).

Enzyme extraction and assays

All operations were performed at 0-4ºC. Leaf samples (about 0.5 g fresh weight) were homogenized with an extraction medium (1/2, w/v) containing 50 mM Tris-acetate buffer (pH 6.0), 0.1 mM EDTA, 2 mM cysteine, 1% PVP, 1% PVPP and 0.2% (v/v) Triton X-100. For the APX activity, 20 mM sodium ascorbate was added to the extraction buffer. The extracts were filtered through two layers of nylon cloth and centrifuged at 10,000 g for 15 min. The supernatant fraction was filtered on Sephadex G-25 NAP columns (GE Heathcare) equilibrated with the same buffer used for the homogenization. For the APX activity, 2 mM sodium ascorbate was added to the equilibration buffer.

The activities of the ASC-GSH cycle enzymes, POX, CAT, and SOD were assayed as described in Diaz-Vivancos et al. (2006, 2008). Protein content was estimated according to method of Bradford (1976). All these analyses were performed using a UV/Vis V-630 Bio spectrophotometer (Jasco, Tokyo, Japan).

SOD and POX isoenzymes were identified by non-denaturing PAGE performed on 15% and 10% acrylamide gels respectively, using a Bio-Rad Mini-protean III dual slab. SOD isoenzymes were localized by the photochemical method of Weissiger and Fridovich (1973). Isoenzyme identification was performed by selective inhibition with KCN or H_2O_2 (Hernández et al., 1999).

Staining of peroxidase isoenzymes with 4-methoxy-α-naphtol (4MN) was performed as described by Ros Barceló (1998).

Ascorbate and glutathione analyses

Leaf samples were snap-frozen in liquid nitrogen and then ground to a fine powder, and the powder was extracted in 1 mL of 1 M $HClO_4$. Homogenates were centrifuged at 12 000 g for 10 min, and the supernatant was neutralized with 5 M K_2CO_3 to pH 5.5–6. The homogenate was centrifuged at 12 000 g for 1 min to remove $KClO_4$. The obtained supernatant was used for ascorbate and glutathione contents (Diaz-Vivancos et al., 2010). The glutathione and ascorbate determination was performed according to the method described in Pellny et al. (2009).

Histochemical ROS detection

Histochemical H_2O_2 and $O_2^{\cdot -}$ staining was carried out according to Hernández et al. (2001), with some modification. Briefly, the histochemical detection of H_2O_2 in grapevine leaves was performed using an endogenous, peroxidase-dependent *in situ* histochemical staining, in which whole leaves were infiltrated with 0.05 mg mL^{-1} of 3,3'-diaminobenzidine (DAB) in 50 mM Tris-acetate buffer (pH 5.0) and incubated at 25ºC, in the dark, for 2 h. Controls were performed in the presence of 10 mM ascorbic acid. The histochemical detection of $O_2^{\cdot -}$ was performed by infiltrating grapevine leaves directly with 0.05 mg mL^{-1} nitroblue of tetrazolium (NBT) in 25 mM K-Hepes buffer (pH 7.6) and incubation at 25 ºC in the dark for 4 h. Controls were performed in the presence of 10 mM $MnCl_2$ ($O_2^{\cdot -}$ removing reagent). In both cases, grapevine leaves were cleared in 50% (v/v) ethanol and photographed directly using an Olympus SZX PT stereomicroscope (Hernández et al., 2001).

Statistical analysis

The effects of NaCl and/or MGBG treatments on the different parameters monitored in grapevine plantlets were analyzed by Duncan's test, calculated at $P<0.05$. The statistical procedures were carried out with the SPSS software package.

Results

Effect of salt stress and MGBG on growth and chlorophyll fluorescence

Salt stress applied in the culture medium of *in vitro* grapevine plantlets disturbed the growth rate. The application of MGBG, an inhibitor of SAMDC, resulted in further deterioration of plant growth, especially under salt stress conditions. Leaves from salt treated plantlets developed chlorotic symptoms in the leaf margins; this effect was more evident in the presence of both treatments (Fig. 1).

Different chlorophyll fluorescence parameters were recorded in leaves from grapevine plantlets grown in the presence and the absence of 100 mM NaCl and/or the PA synthesis inhibitor MGBG (Fig. 2). Salt stress increased qN and NPQ parameters (Fig. 2). However, a decrease in qP and Y(II) was occurred in response to salt stress. The presence of the inhibitor MGBG had no important effect on qN, but it decreased NPQ values, as well as qP and Y(II) (Fig. 2).The effect of NaCl and MGBG on Fv/Fm was less pronounced when the measure was performed in the middle of the leaves. However, when Fv/Fm were recorded near the chlorotic areas (in the leaves margins) the effect of NaCl and/or MGBG was more noticeable (Fig. 2). Finally, plants grown in the presence of both treatments also exhibited an increase of qN and NPQ, although to a lower level than in plant subjected only to NaCl stress. Regarding Fv/Fm parameter a higher decrease was displayed in the presence of both treatments than in plantlets treated independently with salt or MGBG (Fig. 2).

Effect of salt stress and MGBG on oxidative stress markers

Both NaCl and MGBG treatments induced an oxidative stress as shown by the increase in lipid peroxidation level, measured as TBARS. A synergistic effect on lipid peroxidation was observed in salt-treated plantlets grown in the presence of MGBG (Fig. 3). The increase in lipid peroxidation, and therefore the damage to membrane was parallel with ROS accumulation (H_2O_2 and $O_2^{\cdot -}$) detected by histochemical staining with DAB, or NBT, respectively (Figs. 4 and 5). The H_2O_2 and $O_2^{\cdot -}$ staining was more clearly

observed in plantlets grown in the presence of both NaCl and MGBG. A different staining pattern was observed for H_2O_2 and $O_2^{\cdot -}$. Whereas H_2O_2 staining appeared as small spot throughout the leaf surface (Fig. 4E, F), $O_2^{\cdot -}$ staining was observed mainly as an intense staining in the veins (Fig. 5E, F). The incubation of leaves in the presence of 10 mM ascorbate or 10 mM $MnCl_2$ removed the H_2O_2 or the $O_2^{\cdot -}$ staining, respectively (Fig. 4C, 5C), indicating the specificity of staining. In this sense, ascorbate is an effective H_2O_2 scavenger whereas $MnCl_2$ is a highly effective dismutating catalyst agent of $O_2^{\cdot -}$ (Hernández et al., 2001).

Effect of salt stress and MGBG on polyamine levels

Salt treatment affected the PA contents in grapevine plantlets, especially the free and conjugate forms of agmatine (Agm) and Put. In this sense, salinity reduced the free and conjugate forms of Agm up to 33% and 41%, respectively (Fig. 6). The effect of salt stress was even more conspicuous on Put levels. In this case, salinity produced a drop in the three forms of Put. The reduction in free, conjugate and bound Put was about 35%, 50% and 47%, respectively (Fig. 6). No significant effects on the others PAs, such as Spd and Spm (Fig. 6), were observed in NaCl-treated plantlets.

MGBG induced also a small rise in Agm content, whereas Put, Spd and Spm levels remained relatively unchanged in non-salinized plantlets (Fig. 6). The effect of salt-stress on Agm and Put was intensified in the presence of MGBG, mainly in their free forms. In the presence of both treatments, a 56% of decrease in free Agm and a 63% in free Put were observed. Surprisingly, the level of Spd remained unaffected by MGBG whatever its form, while, a 27% decrease in bound Spm was observed in the same conditions (Fig. 6).

Effect of salt stress and MGBG on antioxidative metabolism

The activity of different antioxidant enzymes was examined in leaf extracts from grapevine plantlets. Among the all analyzed enzymes only APX, MDHAR, SOD and POX were detected. The activity of neither the rest of the ASC-GSH cycle enzymes (DHAR and GR) nor of catalase was detected. Salt-stress induced a decrease in APX activity up to 29%, whereas no significant effect in MDHAR was recorded (Fig. 7). However, significant increases in SOD and POX activities were induced by NaCl. In this case, increases were up to 1.7-fold and 2.3-fold for SOD and POX, respectively (Fig. 7). The incubation of grapevine plantlets in the presence of MGBG produced no

effects in APX activity, whereas significant increases in MDHAR, SOD and POX were observed, and a similar situation was recorded in the presence of both treatments (NaCl plus MGBG) (Fig. 7).

SOD activity was also determined qualitatively using native PAGE (Fig. 8A, B). Three different bands displaying SOD activity were detected in grapevine leaves. According to their sensitivity to the inhibitors KCN and H_2O_2 they were classified as Cu,Zn-SOD isoenzymes, named I, II and III in order of increasing electrophoretic mobility (Fig. 8A, B). We also detected two main bands with POX activity by native PAGE (Fig 8C). Their intensity was correlated with the increase in POX activity detected by kinetic analysis (Fig. 7).

The effect of salt-stress and/or MGBG on the levels of non-enzymatic antioxidants ascorbate (ASC) and glutathione was analyzed (Table 1). Salt-stress slightly affected the reduced ASC contents, although a strong accumulation in oxidized ascorbate (DHA), in relation to control plants, was recorded. This effect resulted in a strong decrease in the redox state of ascorbate in NaCl-treated plants (Table 1). No effect in the reduced ASC contents was observed when grapevine plantlets were incubated with MGBG. However, a significant decrease (up to 42%) was noticed after simultaneous incubation with NaCl and MGBG (Table 1).Surprisingly, in plants treated with MGBG, in absence or presence of NaCl, no accumulation of DHA was noticed. Even a decrease in DHA in relation to control plants occurred, and accordingly, an increase in the redox state of ascorbate (Table 1).

Salt-stress also produced a decrease in reduced glutathione (GSH) both in the absence (about 55%) and in the presence of MGBG (about 30%) (Table 1). In contrast, the treatment with MGBG alone had no effect in GSH contents. No significant change in oxidised glutathione (GSSG) was produced, but due to the negative effect of NaCl in GSH, a decrease in the redox state of glutathione was observed in salt-stressed grapevine plantlets (Table 1).

Discussion

Salinity is one of the most important abiotic stresses that limit crop production, affecting plant growth, development and productivity (Flowers, 2004). *In vitro* micropropagated plants seem to be a useful tool for the study of responses to environmental stress at the cellular level. In our work, we showed that salinity disturbed

the growth of *in vitro* grapevine plantlets inducing chlorotic symptoms in the leaves margins, and this effect was even more evident when MGBG (an inhibitor of SAMDC) was present in the culture media. This result suggests that MGBG enhances the toxicity of NaCl which was visibly manifested by a chlorosis on the margins of old leaves. It has been described that the presence of this inhibitor affects PA synthesis in different plants (Bouchereau et al., 1999). In our experimental conditions salt stressed plants displayed lower PA content than control plants throughout the stress period, with Spd and Spm contents being particularly low compared to the level of Put. The content of Spd in stressed plants treated with MGBG was similar to that in the control plants, but the contents of free and conjugated Put, and bound Spm were still lower under the stress conditions. These results suggest that PA homeostasis under salt stress conditions involves not only a rise in Put oxidation, but also a constant conversion of Put to Spd and Spm which is also regulated at the level of SAMDC. Experiments performed with MGBG support the view that the biosynthesis of Spd from Put and SAM in grapevine plantlets exposed to salt stress is not blocked. This is in variance with most studies showing that MGBG-treated plants had a much lower level of Spd, but a higher level of Put than the control (He et al., 2002). MGBG effect might also be related to stimulation of both ACC synthesis and the conversion of ACC to ethylene. Some studies have also reported that exogenous Spd increased ethylene production in some plant systems by stimulating the activities of both ACC synthase and ACC oxidase (Downs and Lovell, 1986). Under stress conditions, ethylene could behave as a growth inhibitor and exert a positive effect on the senescence processes.

A large body of study has shown that plant PAs are involved in the acquisition of tolerance to abiotic stresses as high and low temperatures and salinity (Gill and Tuteja, 2010). Polyamine levels were measured by Zapata et al. (2004) in several plant species (spinach, lettuce, melon, pepper, broccoli, beetroot and tomato) subjected to salt stress (100 or 150mM NaCl). With the exception of beetroot, Put concentration was lower in seedlings grown under saline conditions. They found that PA levels changed with salinity and in most cases Put decreased while Spd and/or Spm increased. This meant that the pool of Put was directed to Spd and Spm synthesis. The (Spd + Spm)/Put ratio increased with salinity which would be in agreement with the idea of a protective role of higher PAs (Spd and Spm) against salt stress (Zapata et al., 2004; Groppa and Benavides, 2008; Gill and Tuteja, 2010). In this work 62% increase in the (Spd +

399 Spm)/Put ratio was recorded, but under our experimental conditions this increase was
400 due to a strong decrease in Put because Spd and Spm levels remained unaffected.
401 Furthermore, NaCl stress can also stimulate the catabolism of Put via Cu-
402 containing amino oxidase (CuAO), and this effect seemed to be amplified in the
403 presence of MGBG. This protein is a H_2O_2-generating enzyme (Bolwell and Wojtaszek,
404 1997), and can be involved in a H_2O_2 accumulation, contributing to the salt-induced
405 oxidative stress observed in grapevine plantlets subjected to both treatments. In
406 addition, CuAO remained active even under high salt concentration (Campreste et al.,
407 2011). Several authors reported that salt-stress promoted PA oxidation by stimulating
408 CuAO and polyamine oxidase (PAO) activities (Smith, 1985), generating H_2O_2
409 (Bolwell and Wojtaszek, 1997). PA oxidationmay contribute partially to hypocotyl
410 growth under salinity through the production of H_2O_2, reinforcing the importance of PA
411 oxidation and H_2O_2 production in salt tolerance in plants (Campestre et al., 2011). In
412 this work, we observed that salt stress induced an oxidative stress, as revealed by the
413 increase in lipid peroxidation and the accumulation of ROS, mainly in in the presence of
414 the inhibitor MGBG. Lipid peroxidation also increased in grapevine plantlets treated
415 with MGBG. It is known that PAs are able to bind with negatively charged
416 phospholipid groups providing a positive effect on membrane stability and permeability
417 (Slocum et al., 1984). It has been reported that Put levels are elevated in a drought-
418 resistant wheat variety as well as in an oxidant-stress-resistant *Conyza bonaerensis*
419 biotype (Ye et al., 1997) and among the possible mechanisms of Put and others PAs
420 conferring stress resistance, these authors included their function as free radical
421 scavengers and their role stabilizing membranes (Ye et al., 1997).
422 The H_2O_2 accumulation observed in salt treated grapevine plantlets correlated
423 with a decrease in APX (a H_2O_2-scavenging enzyme) as well as an increase in SOD (a
424 H_2O_2-producer enzyme) and POX activities. Some authors point out that POXs can also
425 participate in H_2O_2 generation (Bolwell and Wojtaszek, 1997). In NaCl+MGBG-treated
426 plantlets the accumulation of H_2O_2 did not correlate with a decrease in APX activity. In
427 this case, the H_2O_2 accumulation could be due to a decrease in other H_2O_2-scavenging
428 enzymes such as CAT and glutathione peroxidase. However, this assumption remains to
429 be elucidated because under our experimental conditions no CAT activity was detected.
430 Neither DHAR nor GR were detected in grapevine plantlets, suggesting that in those
431 plants ascorbate can be recycled mainly by MDHAR via NADH. Interestingly, using
432 native PAGE only Cu,Zn-SOD isoenzymes were detected in grapevine. The same

situation has been reported recently in plum plantlets (Faize et al., 2013). Cu,Zn-SODs are sensitive to H_2O_2, and they can be located in cell organelles that have an active H_2O_2 generation, such as chloroplast, peroxisomes and mitochondria.

The effect of salt stress on the antioxidative metabolism has been widely studied (Hernández et al., 1999; 2000; Elkahoui et al. 2005). However, data regarding the effect of PA biosynthesis inhibitors on the antioxidative metabolism are scarce. Nevertheless, treatment of barley seedlings with Spm, before water stress application, contributed to an improvement of the functioning of ASC-GSH cycle, decreasing the intensity of oxidative stress induced by the water deficit (Kubis, 2001). In contrast, the treatment with 1 mM Spm imposed an oxidative stress as well as the root growth inhibition in maize seedlings due to the H_2O_2 generation in the apoplast as result of Spm degradation. As consequence, gene expression and activity of APX, a key enzyme for H_2O_2 scavenging, were induced (de Agazio and Zacchini, 2001).

Although grapevine plantlets treated with MGBG, in the presence or the absence of NaCl, displayed increased SOD, POX, MDHAR and maintained APX activities, an oxidative stress was observed. The increase in enzymatic antioxidants not always guarantees resistance or tolerance to a given stress. In loquat plants sensitive to NaCl, increases in APX, MDHAR and DHAR were reported under saline conditions (Hernandez et al., 2003). In salt-adapted *Catharanthus roseus* cells, salinity induced SOD and APX activities, as occurred in non-adapted cells grown in the presence of 50 or 100 mM NaCl, but in all cases, salinity enhanced lipid peroxidation (Elkahoui et al., 2005). These authors suggested that the increase in antioxidant enzymes could also be a response to the cellular damage produced by salt stress, and probably this increase could not stop the deleterious effects of salt, but reduced the severity allowing cell growth (Elkahoui et al., 2005).

In grapevine salt stress was accompanied by the oxidation of ascorbate to DHA. However, no significant GSSG accumulation was produced by salinity, but a decrease in GSH was noticed, suggesting a negative effect of NaCl in glutathione biosynthesis. The oxidation of ascorbate and glutathione under salt stress conditions has been reported in different plant species, both in leaves as well as in different cell compartments (apoplast, symplast, soluble fractions…) (Hernández et al., 2000; 2001; Barba-Espín et al., 2011), producing a decrease in the redox state of ascorbate and glutathione. Surprisingly, no accumulation of DHA took place in plants grown with 100 mM NaCl in the presence of MGBG. This could be due to the induction of MDHAR

activity in MGBG-treated plantlets. Despite the increase of antioxidant defences, salinity and/or MGBG treatments caused damage to membranes in grapevine plantlets, displayed by the increase in lipid peroxidation. Damage to membrane was parallel with a ROS accumulation (H_2O_2 and $O_2^{\cdot-}$) detected by histochemical staining with DAB, or NBT, respectively. The decrease in Put and Spm levels by MGBG and/or NaCl treatments could contribute to this enhanced ROS production. In this sense, it has been described that PAs can reduce the levels of superoxide radicals generated by senescing plant cells (Drolet et al., 1986). Moreover, PAs catabolism produces H_2O_2 (Groppa and Benavides, 2008). A different staining pattern was observed for H_2O_2 and $O_2^{\cdot-}$ in grapevine leaves. Whereas H_2O_2 staining appeared as small spot throughout the leaf surface, $O_2^{\cdot-}$ staining was observed mainly as an intense staining in the veins (Fig. 4E, F). The treatment of the salt-sensitive pea cv. Lincoln with 90 mM NaCl also produced the accumulation of ROS. However, in this case, the pattern of staining was somewhat different to that observed in grapevine plantlets. In pea leaves, $O_2^{\cdot-}$ first appears on the cell wall of mesophyll cells, whereas H_2O_2 was also localized on the cell wall of mesophyll cells, but then H_2O_2 diffuses rapidly to the minor veins of the leaf (Hernandez et al., 2001). The DAB-stainable ROS production was also observed in a relatively salt-tolerant pea cultivar (cv. Puget), where the lesions were smaller in size (Hernandez et al., 2001). This differential response may be related to the different behavior of the apoplastic and symplastic antioxidant systems in these two pea cultivars, which increased in the NaCl-resistant cultivar and decreased in the salt-sensitive cultivar, and was also parallel with a higher increase in lipid and protein oxidation (Hernandez et al., 2001).

Finally, salt stress as well as MGBG treatment, induced some changes in chlorophyll fluorescence parameters. The increase in qN and NPQ in salt stressed plantlets can be regarded as a protective mechanism, because maintenance or increase in these parameters are associated with a protective response in order to avoid or minimize photoinhibitory damage to the reaction centers (Rahoutei et al., 2000). NPQ and qN measure changes in heat dissipation relative to the dark-adapted state. Increases in NPQ and qN can occur as a result of either of process that protects the leaf from light-induced damage or the damage itself (Maxwell and Johnson, 2000). Y(II) measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry, whereas qP gives an indication of the proportion of PSII reaction centers that are open. Y(II) and qP are interrelated with Fv/Fm, which is a measure of

the maximum efficiency of PSII (i.e., the quantum efficiency if all PSII centers were open) (Maxwell and Johnson, 2000). However, a decrease in qP was observed in plants treated with salt and/or MGBG. It has been described that a drop in qP is accompanied by an increase in the lifetime of the exciton in PSII, which can increase the probability of chlorophyll triplet formation and the associated formation of singlet oxygen ($^1O_2$) (Foyer et al., 1994). The decrease in qP was parallel with a reduction in Y(II) in leaves from plantlets treated with 100 mM and/or MGBG. When Fv/Fm was analyzed, leaves from salt-treated plantlets in the presence of MGBG showed a drop in this parameter mainly in leaves margins, which correspond to the chlorotic areas.

In conclusion, results showed that MGBG treatment contribute to the deleterious effect of oxidative stress in grapevine plantlets grown in presence of NaCl, affecting different physiological and biochemical processes, including plant growth, PA levels, photosynthesis and redox state of the cells, highlighting a possible protecting role of PA homeostasis in plants subjected to salt stress.

Acknowledgements

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References

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio AF. Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta 2010;231:1237–49.

Asada K. The water–water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Ann Rev Plant Mol Biol 1999;50:601-39.

Aziz A, Brun O, Audran JC. Involvement of polyamines in the control of fruitlet physiological abscission in grapevine (*Vitis vinifera*). Physiol Plant 2011;113:50-8.

Barba-Espín G, Clemente-Moreno MJ, Álvarez S, García-Legaz MF, Hernández JA, Diaz-Vivancos P. Salicylic acid negatively affects the response to salt stress in pea plants: effects on PR1b and MAPK expression. Plant Biol 2011;13:909–17.

Bolwell GP, Wojtaszek P. Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. Physiol Mol Plant Pathol 1997;51:347-66.

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J. Polyamines and environment challenges: recent advances. Plant Sci 1999;140:103-25.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54.

Cakmak I, Horst WJ. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soybean (*Glycine max*). PhysiolPlant 1991;83:463-68.

Campestre MP, Bordenave CD, Origone AC, Menéndez AB, Ruiz OA, Rodriguez AA, Maiale SJ. Polyamine catabolism is involved in response to salt stress in soybean hypocotyls. J Plant Physiol 2011;168:1234-40.

Chattopadhayay MK, Tiwari BS, Chattopadhayay G, Bose A, Sengupta DN, Ghost B. Protective role of exogenous polyamines on salinity-stressed rice (*Oryza sativa*) plants. Physiol Plant 2002;116:192-99.

Chinnusamy V, Jagendorf A, Zhu JK. Understanding and improving salt tolerance in plants. Crop Sci 2005;45:437–48.

de Agazio M, Zacchini M. Dimethylthiourea, a hydrogen peroxide trap, partially prevents stress effects and ascorbate peroxidase increase in spermidine-treated maize roots. Plant Cell Environ 2001;24:237-44.

567 Diaz-Vivancos P, Rubio M, Mesonero V, Periago PM, Ros Barceló A, Martínez-Gómez
568 P, Hernández JA. The apoplastic antioxidant system in Prunus: Response to plum
569 pox virus. J Exp Bot 2006;57:3813-24.
570 Diaz-Vivancos P, Clemente-Moreno MJ, Rubio M, Olmos E, Garcia JA, Martinez-
571 Gomez P, Hernandez JA. Alteration in the chloroplastic metabolism leads to ROS
572 accumulation in pea plants in response to Plum pox virus. J Exp Bot 2008;59:2147-
573 60.
574 Diaz-Vivancos P, Dong YP, Ziegler K, Markovic J, Pallardó FV, Pellny Tet al.
575 Recruitment of glutathione into the nucleus during cell proliferation adjusts whole
576 cell redox homeostasis in Arabidopsis thaliana and lowers the oxidative defence
577 shield. Plant J 2010;64:825–38.
578 Downs CG, Lovell PH. The effect of spermidine and putrescine on the senescence of
579 cut carnations. Physiol Plant 1986;66:679–82.
580 Drolet G, Dumbroff EB, Legge R, Thompson JE. Radical scavenging properties of
581 polyamines. Phytochemistry 1986;25:367–71.
582 Elkahoui S, Hernández JA, Abdelly C, Grhir R, Limam F. Changes induced by NaCl in
583 lipid peroxidation, lipoxygenase and antioxidant enzyme activities of *Catharanthus*
584 *roseus* suspension cells. Plant Sci 2005;168:607-13.
585 Faize M, Faize L, Petri C, Barba-Espin G, Diaz-Vivancos P, Clemente-Moreno MJ et
586 al. Cu/Zn superoxide dismutase and ascorbate peroxidase enhanced in vitro shoot
587 multiplication in transgenic plum. J Plant Physiol 2013;170:625-32.
588 Flores HE, Galston AW. Analysis of polyamines in higher plants by high performance
589 liquid chromatography. Plant Physiol 1982;69:701-06.
590 Flowers TJ. Improving crop salt tolerance. J Exp Bot 2004; 396: 307-19.
591 Foyer CH, Lelandais M, Kunert KJ. Photooxidative stress in plants. Physiol Plant
592 1994;92:696-17.
593 Gill SS, Tuteja N. Polyamines and abiotic stress tolerance in plants. Plant Signal Behav
594 2010;5:26-33.
595 Groppa MD, Benavides MP. Polyamines and abiotic stress: recent advances. Amino
596 Acids 2008;34:35–45.
597 Hatmi S, Trotel-Aziz P, Villaume S, Couderchet M, Clément C, Aziz A. Osmotic stress-
598 induced polyamine oxidation mediates defence responses and reduces stress-
599 enhanced grapevine susceptibility to *Botrytis cinerea*. J Exp Bot 2013;
600 doi:10.1093/jxb/ert351.

He LX, Nada K, Kasukabe Y, Tachibana S. Enhanced susceptibility of photosynthesis to low-temperature photoinhibition due to interruption of chill-induced increase of S-adenosylmethionine decarboxylase activity in leaves of spinach (*Spinacia oleracea* L.). Plant Cell Physiol 2002;43:196-06.

Hernández JA, Campillo A, Jiménez A, Alarcón JJ, Sevilla F. Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. New Phytol 1999;141:241-51.

Hernández JA, Jiménez A, Mullineaux PM, Sevilla F. Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ 2000;23:853-62.

Hernández JA, Ferrer MA, Jiménez A, Ros-Barceló A, Sevilla F. Antioxidant systems and $O_2^{\cdot-}/H_2O_2$ production in the apoplast of *Pisum sativum* L. leaves: its relation with NaCl-induced necrotic lesions in minor veins. Plant Physiol 2001;127:817-31.

Hernández JA, Aguilar A, Portillo B, López-Gómez E, Mataix Beneyto J, García-Legaz MF. The effect of calcium on the antioxidant enzymes from salt-treated loquat and anger plants. Funct Plant Biol 2003;30:1127-37.

Kovacs Z, Simon-Sarkadi L, Szücs A, Kocsy G. Different effects of cold, osmotic stress and abscisic acid on polyamine accumulation in wheat. Amino Acids 2010;38: 623–31.

Kubis J. Polyamines and "scavenging system": influence of exogenous spermidine on Halliwell-Asada pathway enzyme activity in barley leaves under water deficit. Acta Physiol Plant 2001;23:335-341.

Kusano T, Berberich T, Tateda C, Takahashi Y. Polyamines: essential factors for growth and survival. Planta 2008;228:367–81.

López-Gómez E, Sanjuán MA, Diaz-Vivancos P, Mataix Beneyto J, García-Legaz MF, Hernández JA. Effect of salinity and rootstocks on antioxidant systems of loquat plants (*Eriobotrya japonica* Lindl.): Response to supplementary boron addition. Environ Exp Bot 2007;160:151-58.

Maxwell K, Johnson GN. Chlorophyll fluorescence - a practical guide. J Exp Bot 2000;51:659-68.

Moschou PN, Wu J, Cona A, Tavladoraki P, Angelini R, Roubelakis-Angelakis KA. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. J Exp Bot 2012;63:5003-15.

634 Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco
635 tissue culture. Plant Physiol 1962;15:473-97.
636 Noctor G, Foyer CH. Ascorbate and glutathione: keeping active oxygen under control.
637 Ann Rev Plant Physiol Plant Mol Biol 1998;49:249-79.
638 Pellny TK, Locato V, Vivancos PD, Markovic J, De Gara L, Pallardó FV, Foyer CH.
639 Pyridine nucleotide cycling and control of intracellular redox state in relation to
640 poly (ADP-ribose) polymerase activity and nuclear localisation of glutathione
641 during exponential growth of Arabidopsis cells in culture. Mol Plant 2009;2:442-
642 56.
643 Rahoutei J, Garcia-Luque I, Baron M. Inhibition of photosynthesis by viral infection:
644 Effect on PSII structure and function. Physiol Plant 2000;110:286-92.
645 Ros Barceló A. The generation of H2O2 in the xylem of Zinnia elegans is mediated by
646 an NADPH-oxidase-like enzyme. Planta 1998; 207:207-16.
647 Roy P, Niyogi K, SenGupta DN, Ghosh B. Spermidine treatment to rice seedlings
648 recovers salinity stress-induced damage of plasma membrane and PM-bound Hþ-
649 ATPase in salt-tolerant and salt-sensitive rice cultivars. Plant Sci 2005;168:583-91.
650 Slocum RD. Polyamine biosynthesis in plants. In: Slocum RD, Flores HE, editors. The
651 biochemistry and physiology of polyamines in plants. Boca Raton: CRC Press,
652 1991. p.23–40.
653 Slocum RD, Kaur-Sawhney R, Galston AW. The physiology and biochemistry of
654 polyamines in higher plants. Arch Biochem Biophys 1984;235:283-03.
655 Smith TA. The di- and poly-amine oxidases of higher plants. Biochem Soc Trans
656 1985;13:319-22.
657 Takahashi T, Kakehi JI. Polyamines: ubiquitous polycations with unique roles in
658 growth and stress responses. Ann Bot 2010;105:1-6.
659 Ye B, Müller HH, Zhang J, Gressel J. Constitutively elevated levels of putrescine and
660 putrescine-generating enzymes correlated with oxidant stress resistance in *Coniza*
661 *bonaerensis* and wheat. Plant Physiol 1997;115:1443-51.
662 Weissiger RA, Fridovich I. Superoxide dismutase: Organelle specifity. J Biol Chem
663 1973;248:3582-92.
664 Zapata PJ, Serrano M, Pretel MT, Amorós A, Botella MA. Polyamines and ethylene
665 changes during germination of different plant species under salinity. Plant Sci
666 2004;167:781–88.

667 Zepeda-Jazo I, Velarde-Buendia AM, Enriquez-Figueroa R, Bose J, Shabala S, Muniz-
668 Murguia J, Pottosin II. Polyamines Interact with Hydroxyl Radicals in Activating
669 Ca^{2+} and K^{+} Transport across the Root Epidermal Plasma Membranes. Plant
670 Physiol 2011;157:2167-80.
671 Zhao FG, Qin P. Protective effect of exogenous polyamines on root tonoplast function
672 against salt stress in barley seedlings. Plant Growth Reg 2004;42:97–103.
673

Tables

Table 1. Effect of MGBG on ascorbate and glutathione contents in *in vitro* grapevine plantlets growing in the presence and the absence of 100 mM NaCl. Data are expressed as nmol g^{-1} FW.

	ASC	DHA	ASC/ ASC+DHA	GSH	GSSG	GSH/ GSH+GSSG
Control	0.43±0.02a	0.21±0.02b	0.668	0.40±0.01a	0.23±0.01a	0.629
100 mM NaCl	0.38±0.01ab	1.05±0.01a	0.264	0.18±0.01c	0.28±0.04a	0.378
MGBG	0.44±0.02a	0.15±0.01c	0.750	0.40±0.03a	0.22±0.01a	0.644
NaCl + MGBG	0.34±0.03b	0.12±0.01c	0.736	0.28±0.02b	0.26±0.03a	0.520

Data represent the mean ± SE of at least four repetitions. Different letters indicate statistical significance according to Duncan's test ($P<0.05$).

684 **Legends of figures**

685 Fig. 1.- Picture showing the effect of salt stress on the growth of grapevine plantlets in
686 the presence and the absence of MGBG. Plantlets were treated with 100 mM NaCl, or
687 1mM MGBG, or a mixture of 100 mM NaCl and 1mM MGBG and the picture as
688 representative of at least four replicates was taken 10 days after treatment.

689

690 Fig.2.- Effect of salt stress on chlorophyll fluorescence parameters in leaves of
691 grapevine plantlets grown in the presence or the absence of MGBG. Images of the
692 coefficient of non-photochemical quenching (qN), non-photochemical quenching
693 (NPQ), coefficient of photochemical quenching (qP), the effective PSII quantum yield
694 [Y(II)] and the maximal PSII quantum yield (Fv/Fm). An average of the qN, NPQ, qP,
695 Y(II) and Fv/Fm values are shown in brackets (C, central part of the leaves; M, leaves
696 margins).

697

698 Fig. 3.- Effect of salt stress on lipid peroxidation in grapevine plantlets in the presence
699 and the absence of MGBG. Data represent the mean ± SE of at least four repetitions.
700 Different letters indicate statistical significance according to Duncan's test ($P<0.05$).

701

702 Fig. 4.- Effect of salt stress on H_2O_2 accumulation in grapevine plantlets growing in the
703 presence or the absence of MGBG, detected by histochemical staining with DAB. (A)
704 Control plantlets; (B) NaCl-treated plantlets; (C) NaCl-treated plantlets stained in the
705 presence of 10 mM ascorbate; (D), MGBG-treated plantlets; (E) MGBG-treated
706 plantlets in the presence of 100 mM NaCl; (F) Magnification from E.

707

708 Fig. 5.- Effect of salt stress on superoxide accumulation in grapevine plantlets growing
709 in the presence or the absence of MGBG, detected by histochemical staining with NBT.
710 (A) Control plantlets; (B) NaCl-treated plantlets; (C) NaCl-treated plantlets stained in
711 the presence of 10 mM $ClMn_2$; (D) MGBG-treated plantlets; (E) MGBG-treated
712 plantlets in the presence of 100 mM NaCl; (F) Magnification from E.

713

714 Fig.6.- Effect of salt stress on free, conjugated and bound polyamine (PA) contents in
715 grapevine plantlets growing in the presence or the absence of MGBG. Agm: Agmatine;
716 Put: Putrescine; Spd: Spermidine; Spm: Spermine. Data (nmol g^{-1} DW) represent the

mean ± SE of at least two repetitions. Different letters indicate statistical significance according to Duncan's test ($P<0.05$).

Fig. 7.- Effect of salt stress on APX, POX, SOD and MDHAR activities in grapevine plantlets growing in the presence or the absence of MGBG. APX and MDHAR are expressed as nmol min^{-1} g^{-1} FW; POX is expressed as µmol min^{-1} g^{-1} FW, and SOD is expressed as U g^{-1} FW. Data represent the mean ± SE of at least four repetitions. Different letters indicate statistical significance according to Duncan's test ($P<0.05$).

Fig. 8.- Effect of salt stress on isoenzyme patterns of SOD (A, B) and POX (C) in grapevine plantlets grown in the presence or the absence of MGBG. SOD and POX were detected by PAGE. Aliquot of 5 µg of proteins was used. (A) Identification of the different SOD isoenzymes; (B) Effect of MGBG (M) and/or NaCl (S) on SOD activity; (C) Effect of MGBG (M) and NaCl (S) on POX activity.

Figure 1

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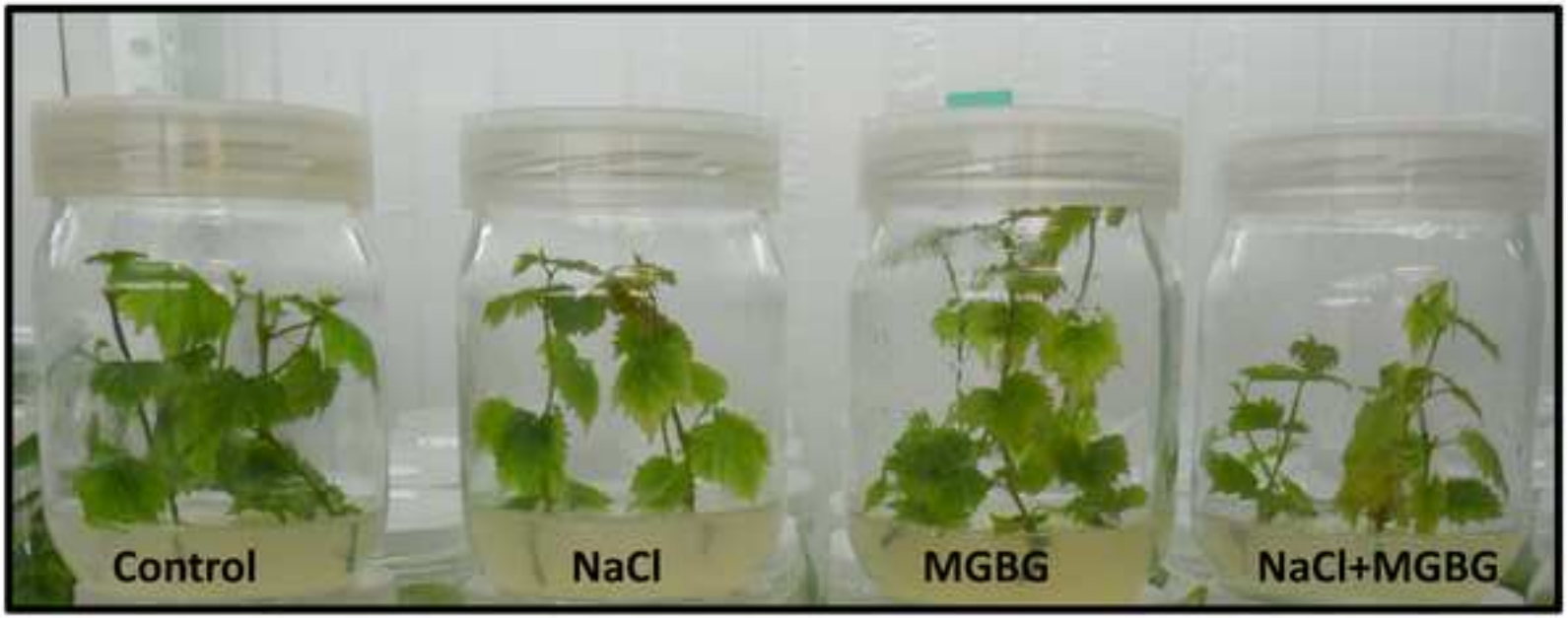

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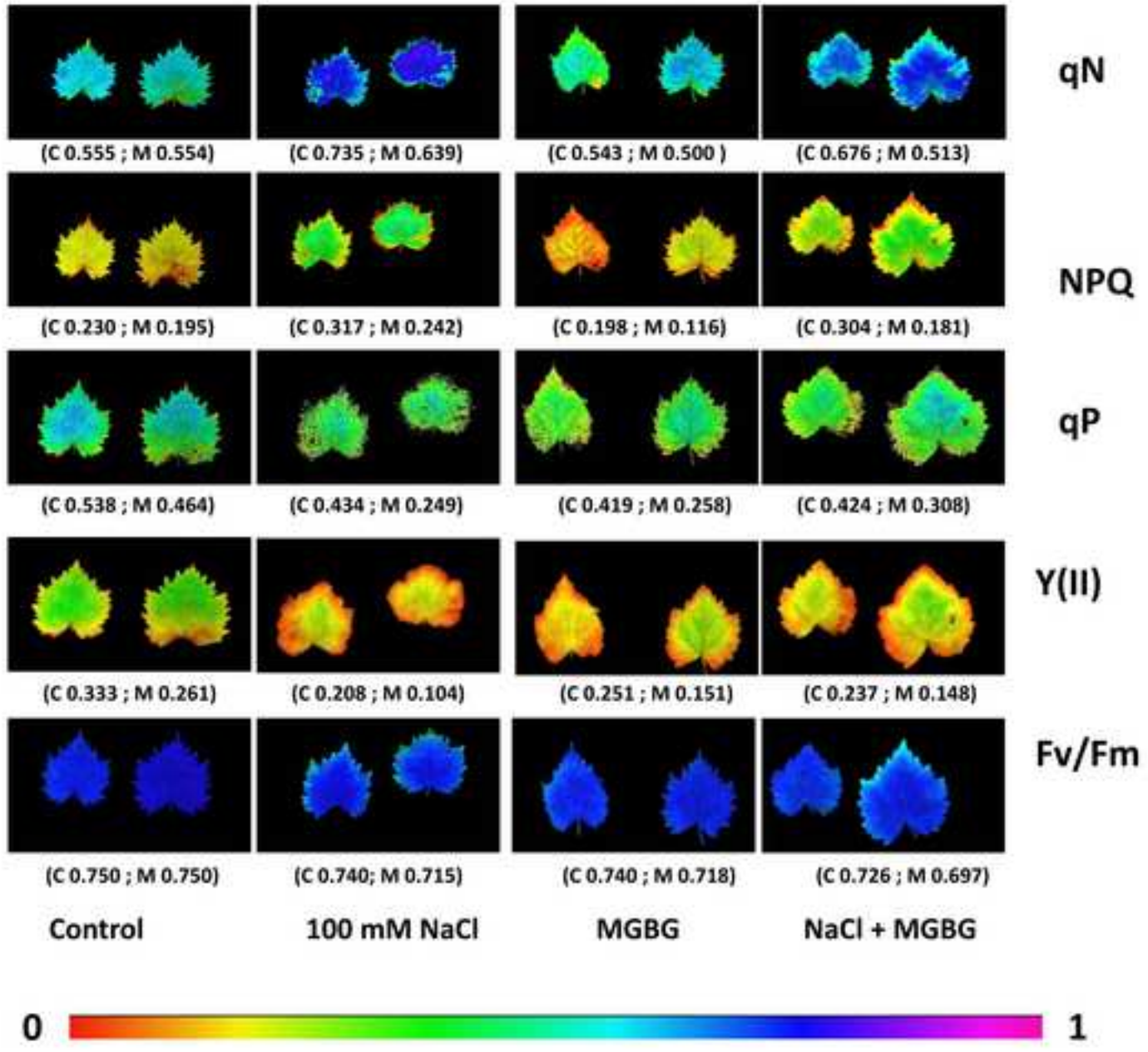

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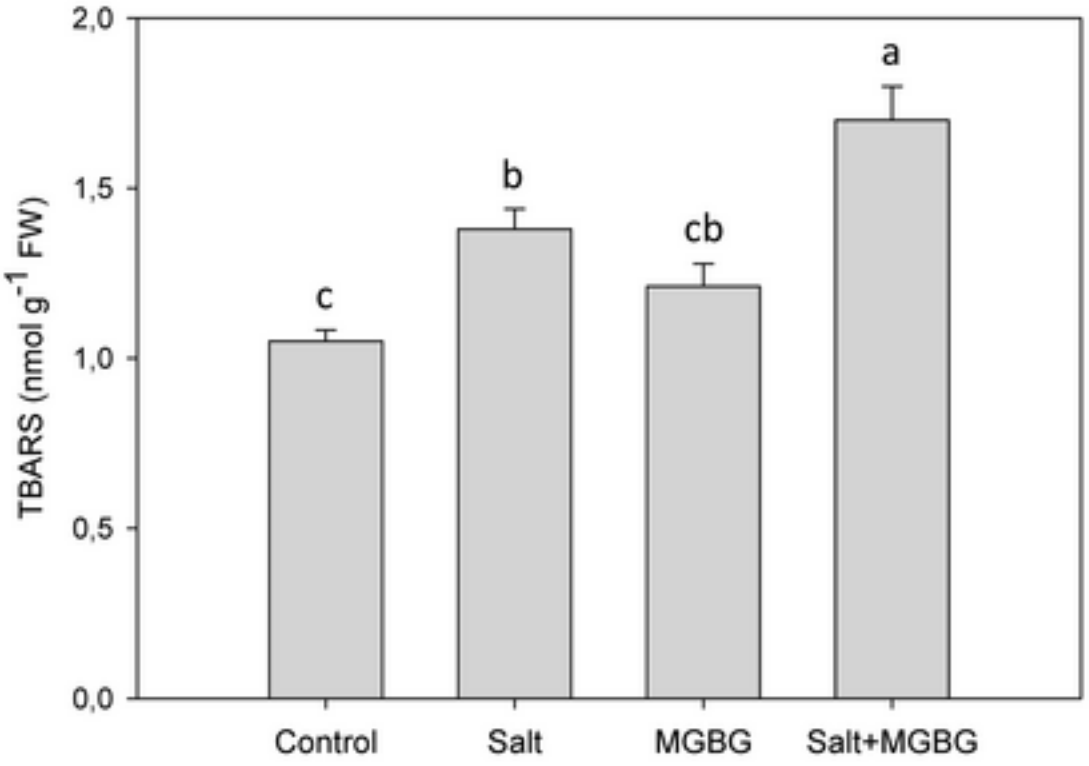

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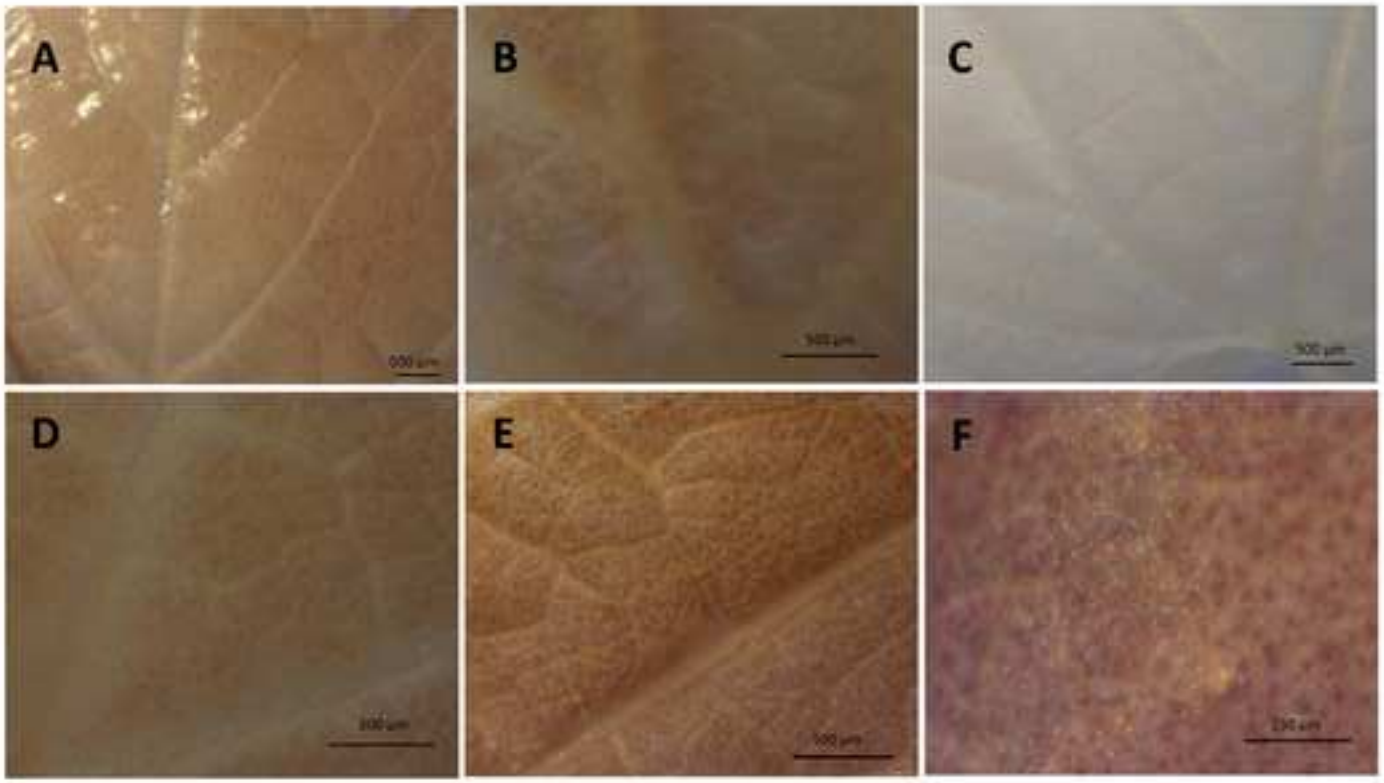

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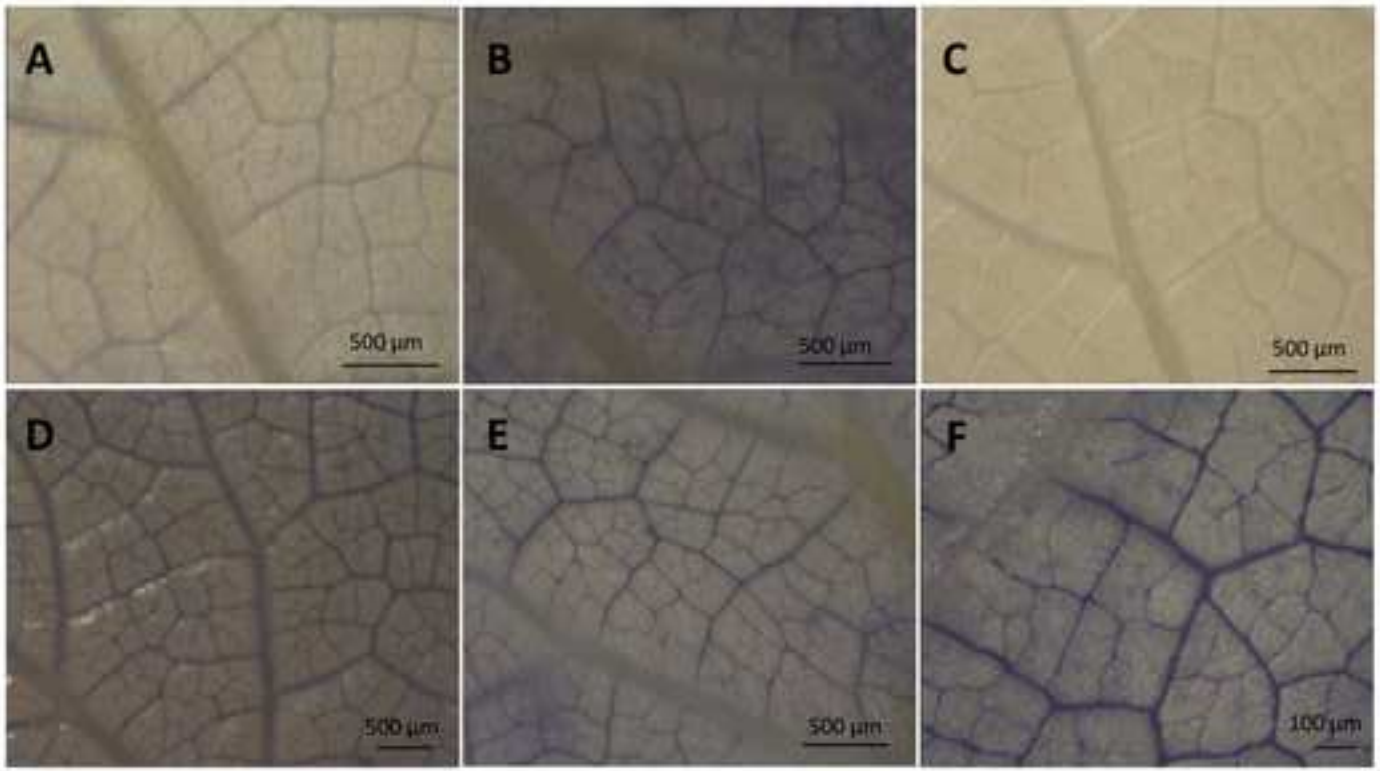

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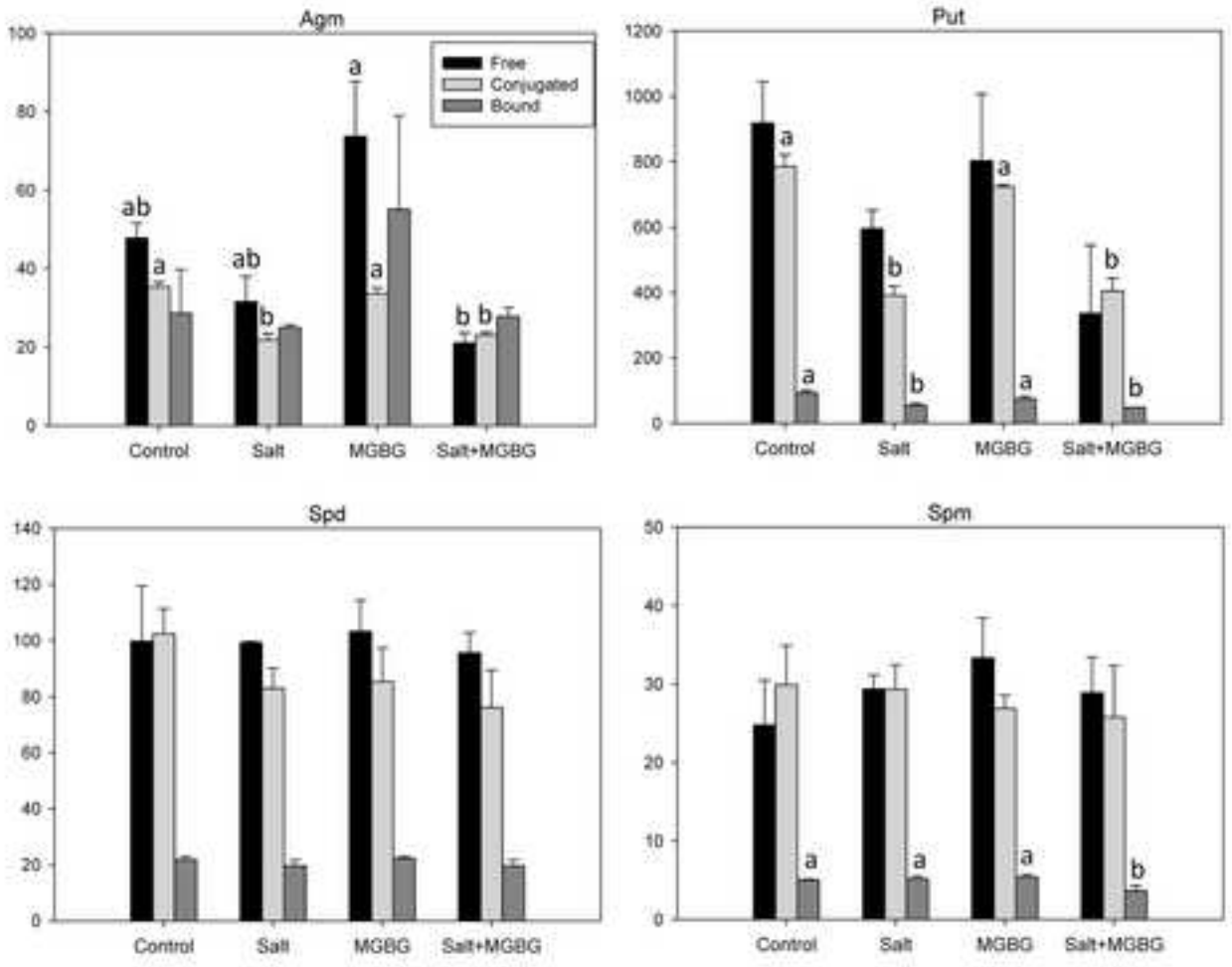

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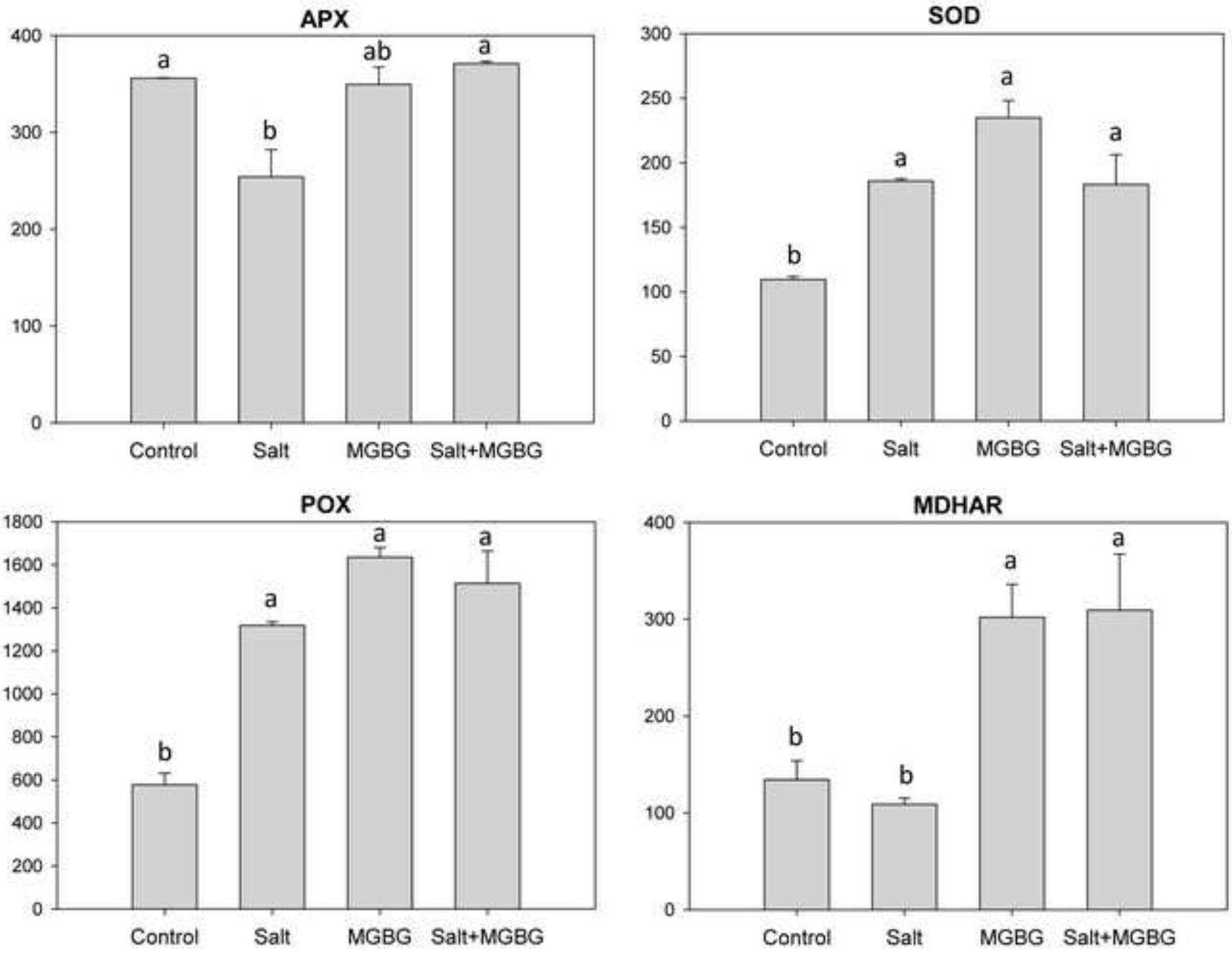

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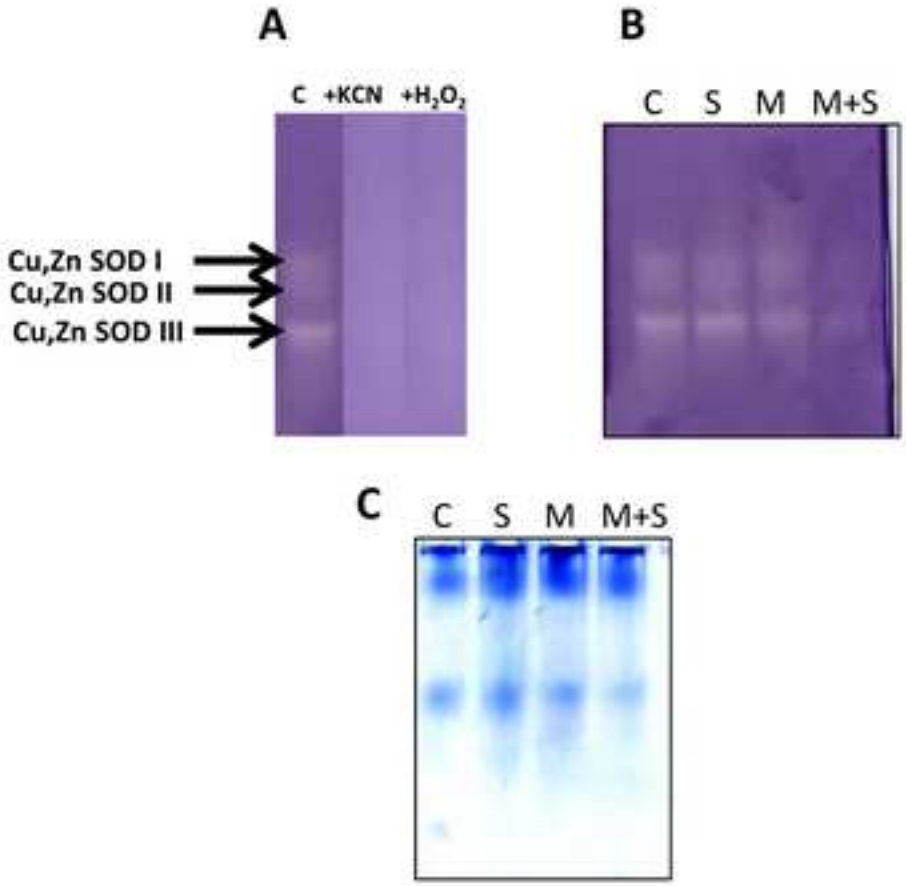

Fig. 8.