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Mannose regulates water balance, leaf senescence, and genes related to stress tolerance in white clover under osmotic stress

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Abstract

Mannose (MAN), an important monosaccharide, contributes to coping with abiotic stresses in plants. Objectives of this study were to examine whether exogenous MAN (30 mM) could significantly increase drought tolerance and further to reveal MAN-regulated tolerance mechanism in white clover under osmotic stress induced by 18 % (m/v) polyethylene glycol 6000 for 10 d in controlled growth chambers. Results show that the application of MAN significantly alleviated stress damage and the inhibition of growth and photosynthesis in white clover under osmotic stress. The MAN-induced increase in endogenous MAN content and the accumulation of organic osmolytes (proline and water soluble sugars) could be responsible for a lower osmotic potential (OP) in white clover. The exogenous application of MAN also enhanced antioxidant enzyme (superoxide dismutase, peroxidase, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase) activities and maintained ascorbic acid content in white clover during osmotic stress. As concern chlorophyll (Chl) metabolism, the MAN-treated plants showed significantly higher transcription of genes involved in Chl synthesis *Mg-chelatase* and *protochlorophyllide reductase* and lower transcription of *pheophorbide a oxygenase* and *chlorophyllase* related to Chl degradation and also a *senescence associated gene 101* than untreated plants. In addition, the MAN application increased transcription of *SK2*-, *Y2K*-, and *Y2SK*-type *dehydrin* genes, and *dehydrin b* in leaves of white clover under osmotic stress. These results indicate that MAN plays important roles in drought tolerance not only acting as a compatible solute for OP but also delaying leaf senescence through enhancing antioxidant metabolism, decreasing Chl degradation, and increasing transcription of dehydrin genes contributing to enhanced drought tolerance in white clover.

Additional key words: antioxidants, dehydrins, osmotic potential, photosynthesis, senescence, *Trifolium repens*.

Introduction

Plants are often exposed to different environmental stresses under natural conditions. Drought has become one of the most critical factors limiting plant growth and development due to global warming and persistent decrease in available irrigation water worldwide (Zlatev and Lidon 2012). For the improvement of stress resistance in plants, the application of plant growth regulators (PGRs) has many advantages such as simple operation, quick effect, and high practical value. For example, the PGR application

on ornamental plants can increase their stress resistance (Chu *et al.* 2004). Indolebutyric acid (IBA) and gibberellic acid (GA) can alleviate negative effects of water deficit on photosynthesis and dry matter accumulation in cotton (Zhao and Oosterhuis 1997, Biles and Cothren 2001). Exogenous γ -aminobutyric acid (GABA) could significantly improve heat and drought tolerance in creeping bentgrass associated with the alteration of transcriptions of stress-protective genes including transcription factors and genes encoding antioxidant enzymes, heat shock proteins, and metallothionein (Li *et al.* 2018). Previous studies have also

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Abbreviations: APX - ascorbate peroxidase; ASA - ascorbic acid; C - control conditions; CAT - catalase; Chl - chlorophyll; CHLASE - chlorophyllase; DHAR - dehydroascorbate reductase; EL - electrolyte leakage; F_v/F_m - variable to maximum fluorescence ratio (maximum quantum yield of photosystem II photochemical efficiency); GR - glutathione reductase; GSH - glutathione; MAN - mannose; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; Mg-CHT - Mg-chelatase; OP - osmotic potential; PGRs - plant growth regulators; PIABS - performance index on absorption basis; P_N - net photosynthetic rate; POD - peroxidase; PAO - pheophorbide a oxygenase; PBGD - porphobilinogen deaminase; POR - protochlorophyllide reductase; Pro - proline; RGR - relative growth rate; ROS - reactive oxygen species; RWC - relative water content; SAG101 - senescence-associated gene 101; SK2 - SK2-type dehydrin; SOD - superoxide dismutase; TAC - total antioxidant capacity; WSS - water soluble saccharides; WUE - water use efficiency; Y2K - Y2K-type dehydrin; Y2SK - Y2SK-type dehydrin.

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shown that application of abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) could improve the drought tolerance in various plant species (Ali *et al.* 2017, Li *et al.* 2017a), but the price of these PGRs is relatively high. In addition, the application effects and advantages of different PGRs vary with plant species.

Mannose (MAN), an important monosaccharide, contributes to tolerance to abiotic stresses as a compatible solute in plants. For example, exogenous GABA, ABA, and SA could significantly enhance the MAN accumulation associated with increases in osmotic adjustment (OA) and drought tolerance in creeping bentgrass (Li *et al.* 2017a). Under drought, MAN priming could effectively increase biochemical processes in wheat seeds, thereby increasing drought tolerance during seeds germination (Hameed and Iqbal 2014). The transgenic *Arabidopsis* plants overexpressing a gene encoding *Medicago sativa* GDP-mannose-3',5'-epimerase effectively enhanced acid, drought, and salt tolerance associated with the regulation of ascorbate accumulation (Ma *et al.* 2014). The overexpression of a tomato *GMP* (a gene related to GDP-mannose biosynthesis) improved antioxidant capacity leading to the enhancement of heat and cold tolerance in tobacco (Wang *et al.* 2011). These studies confirm that MAN not only acts as an important osmolyte, but also plays other multiple roles in regulating abiotic stress tolerance in plants. In addition, the MAN is characterized by low price, non-toxic side effects, and low concentration regulation as compared to other PGRs. The further investigation into MAN function in growth regulation and stress tolerance has high practical and scientific value.

White clover (*Trifolium repens*) is one of the most important leguminous forage worldwide due to strong biological nitrogen fixation function and high nutrient value. In addition, white clover is also applied to ornamental turf because of its better creeping growth characteristics, fast regeneration speed, and graceful leaf shape (Zhao *et al.* 2004). However, white clover is a cool-season and shallow-root plant and prefers cold and moist climate. Drought has become the main limiting factor for its production and utilization, especially in the subtropical and temperate regions (Frame and Newbould 1986). Objectives of this study was to investigate whether exogenous MAN could significantly enhance drought tolerance of white clover and further to reveal the MAN-regulated mechanism possibly involved in osmotic protection, antioxidant and chlorophyll metabolism, leaf senescence, and expression of dehydrin-encoded genes in white clover under control and osmotic stress conditions.

Materials and methods

Plants and treatments: White clover (*Trifolium repens* L.) drought-tolerant cv. Haifa was used and seeds were purchased from Barenbrug, Beijing, China. Seeds were uniformly sprinkled in the seedling-raising plate (15 cm length, 15 cm width, and 20 cm height) filled with moistened quartz sands in controlled growth chamber set at day/night temperatures of 23/19 °C, an irradiance

of 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (photosynthetically active radiation, PAR), and a relative humidity of 70 %. After 7 d of seed germination, seedlings of white clover were watered by Hoagland's solution (Hoagland and Arnon 1950) and after 30 d, they were treated by 30 mM MAN for 3 d. Then MAN-treated or untreated plants were carefully removed from quartz sands and subjected to PEG-induced osmotic stress. The concentration of MAN was chosen based on a preliminary test with a range of concentrations (0, 10, 20, 30, 40, and 50 mM) for the most effective concentration on phenotypic changes. Four treatments were set: 1) controls were grown in Hoagland's solution (C); 2) plants were pretreated by Hoagland's solution containing 30 mM MAN for 3 d and then grown in standard Hoagland's solution (C+MAN); 3) plants were grown in Hoagland's solution containing 18 % PEG 6000 (m/v) (PEG); and 4) plants were pretreated by Hoagland's solution containing 30 mM MAN for 3 d and then grown in Hoagland's solution containing 18 % PEG 6000 (PEG+MAN). The Hoagland's and PEG solutions were refreshed every day to avoid the change in concentration and aerated by pumps (115 V, 60 Hz, Tetra®, Blacksburg, VA, USA). Leaves were sampled at 0, 4, 6, 8, and 10 d of control cultivation or osmotic stress. Each treatment had four independent biological replicates.

Relative growth rate (RGR) was determined by using the formula: $\text{RGR} [\text{mg g}^{-1}(\text{d.m.}) \text{d}^{-1}] = (\ln M_f - \ln M_i) / \Delta t$, where M_i is initial and M_f final dry masses (d.m.) of plants and Δt means the elapsed time between two measurements (Ma *et al.* 2012).

For electrolyte leakage (EL) determination, fresh leaves (0.1 g) were immersed in 20 cm³ of deionized water at 4 °C for 24 h. The initial conductivity (C_{initial}) was detected by using a conductivity meter (DDS-307A, Shanghai Precision and Scientific Instrument, Shanghai, China). Leaves were autoclaved in an oven at 105 °C for 90 min and the maximum conductivity (C_{max}) was measured. $\text{EL} [\%] = C_{\text{initial}}/C_{\text{max}} \times 100$ (Blum and Ebercon 1981).

Leaf relative water content (RWC) was determined as: $\text{RWC} [\%] = [(FM - DM)/(WSM - DM)] \times 100$, where FM, DM, and WSM are fresh, dry, and water saturated masses, respectively. Leaves were collected from plants and FM immediately measured. Then they were wrapped in gauze and kept in distilled water at 4 °C for 12 h for determining WSM. After that, leaves were dried in an oven for 72 h and weighed for DM (Barrs and Weatherley 1962).

Osmotic potential (OP) was determined according to the method of Blum (1989). Fresh leaves were collected and immersed in deionized water at 4 °C for 8 h. Leaves were blotted dry and pressed. Osmolarity (c) of cell fluid was measured using an osmometer (Wescor, Logan, UT, USA); $\text{OP} [\text{MPa}] = -c \times 2.58 \times 10^{-3}$.

Water soluble saccharides (WSS) were detected based on the method of Buysse and Merckx (1993) with some modification. The 20 mg of dry leaf powder was mixed

with 10 cm³ of 80 % (v/v) ethanol and then extracted in boiling water bath for 15 min. Extract was then centrifuged at 3 600 g for 10 min to get the supernatant. Absorbance was read at 625 nm using a spectrophotometer (*Spectronic 601*, *Spectronic Instruments*, Rochester, NY, USA).

For determination of free proline (Pro), fresh leaves (0.1 g) were immersed in 5 cm³ of 3 % (m/v) aqueous sulfosalicylic acid for 1 h at room temperature. Homogenate was centrifuged at 3 000 g for 10 min and 2 cm³ of supernatant was mixed with 2 cm³ of glacial acetic acid and 2 cm³ of acid ninhydrin solution and then the mixture was heated in a water bath (100 °C) for 1 h and cooled in ice water. Then 5 cm³ of toluene was added. Absorbance was read at 520 nm using a spectrophotometer (Bates *et al.* 1973).

Content of MAN in leaves was determined by using mannose assay kits (*Suzhou Comin Biotechnology Company*, Suzhou, China).

Antioxidants: Fresh leaves (0.1 g) were ground with cold phosphate buffer (4 cm³, 50 mM, pH 7.6). The homogenate was centrifuged at 12 000 g and 4 °C for 30 min. The supernatant was used for assays of antioxidant enzyme activities and malondialdehyde (MDA) content. Superoxide dismutase (SOD) activity was measured by recording the rate of *p*-nitroblue tetrazolium chloride reduction according to changes in absorbance at 560 nm (Giannopolitis and Ries 1977). The activities of catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were determined by following the changes in absorbance at 240, 470, 290, 340, 265, and 340 nm, respectively (Nakano and Asada 1981). For each enzyme and total antioxidant capacities, a unit (U) means the amount of enzyme catalyzing reaction of 1 µmol of a substrate in 1 min. Protein content was determined according to Bradford (1976). For the determination of MDA content, enzyme extract (0.5 cm³) was mixed with reaction solution (1 cm³) containing 20 % (m/v) trichloroacetic acid and 0.5 % (m/v) thiobarbituric acid. The mixture was heated in a water bath at 95 °C for 15 min and cooled quickly in ice water. The homogenate was centrifuged at 8 000 g for 10 min. The absorbance of supernatant was measured at 532, 600, and 450 nm (Li *et al.* 2015b). The formation rate of O₂⁻ was measured by using sulfanilamide and the absorbance was measured at 530 nm (Elstner and Heupel 1976). Content of H₂O₂ was assayed by potassium iodide method (Velikova *et al.* 2000). Total antioxidant capacity (TAC), ascorbic acid (ASA), and glutathione (GSH) content were determined by using assay kits purchased from *Suzhou Comin Biotechnology Company*.

Determination of photosynthetic characteristics: For determination of chlorophyll (Chl) content, fresh leaves (0.1 g) were immersed in 10 cm³ of 80 % (v/v) acetone and 95 % (v/v) alcohol mixture (1:1, v/v) and left in the dark for 48 h. The extract was measured at 663 and 645 nm

with a spectrophotometer. Content of Chl was calculated using the formula described in Arnon (1949). Maximum quantum yield of photosystem II photochemical efficiency (F_v/F_m) and performance index on absorption basis (PIABS) were measured by using a chlorophyll fluorescence meter (*Pocket PEA*, *Hansatech*, Norfolk, UK). Individual leaves were adapted to darkness for 30 min using leaf clips. F_v/F_m ratio and PIABS were recorded. Net photosynthetic rate (P_N) and water use efficiency (WUE) were determined by using a portable photosynthesis system (*CIRAS-3*, *PP Systems*, Amesbury, MA, USA). This instrument provided a CO₂ concentration of 400 mm³ dm⁻³ and an red and blue irradiation of 800 µmol(photon) m⁻² s⁻¹.

Gene transcriptions were performed using real-time quantitative qPCR (Table 1 Suppl.). For total RNA, fresh leaves (0.1 g) were extracted by using *RNeasy Mini* kit (*Qiagen*, Düsseldorf, Germany). An RNA was reverse-transcribed to cDNA using a *Revert Aid First Stand* cDNA synthesis kit (*Fermentas*, Vilnius, Lithuania). The cDNA was subjected to quantitative PCR using primers of genes involved in Chl synthesis and degradation (*Mg-chelatase* - *Mg-CHT*, *protochlorophyllide reductase* - *POR*, *porphobilinogen deaminase* - *PBGD*, *pheophorbide a oxygenase* - *PAO*, *chlorophyllase* - *CHLASE*), a senescence marker gene (*senescence-associated gene 101* - *SAG101*), and dehydrin-encoded genes (*SK2*-, *Y2K*-, and *Y2SK-type dehydrins* - *SK2*, *Y2K*, and *Y2SK*). β -Actin was used as internal control. Conditions of PCR protocol for all genes were as follows: at 94 °C for 5 min, at 95 °C for 30 s (40 repeats), at 56 - 60 °C for 45 s. The formula $2^{-\Delta\Delta Ct}$ was used to calculate the transcription of all genes at the end of PCR cycle (Xia *et al.* 2009).

Statistical analysis: The data was analyzed by one-way ANOVA using *SPSS 20* (*IBM*, Armonk, NY, USA). The significant differences among treatments were tested based on the least significant difference (LSD) at $P \leq 0.05$.

Results

Exogenous MAN (30 mM) did not significantly affect RGR, RWC, and EL in white clover under control conditions (Fig. 1A-D). The osmotic stress significantly decreased RGR and RWC in leaves of white clover treated with or without MAN relative to control conditions (Fig. B,C). Exogenous MAN significantly improved RGR under osmotic stress (Fig. 1B); MAN-treated white clover had a significantly higher leaf RWC than untreated plants at 8 and 10 d of osmotic stress (Fig. 1C). Osmotic stress caused a significant increase in EL in both MAN-treated and untreated plants (PEG+MAN and PEG), but the PEG+MAN had lower EL than PEG at 6, 8, and 10 d of osmotic stress (Fig. 1D). OP and WSS, MAN, and free Pro content in leaves are not significantly affected by MAN treatment under control conditions (Fig. 2). PEG application significantly decreased OP in both MAN-treated and untreated plants and the MAN-treated white clovers had lower OP than untreated plants (Fig. 2A).

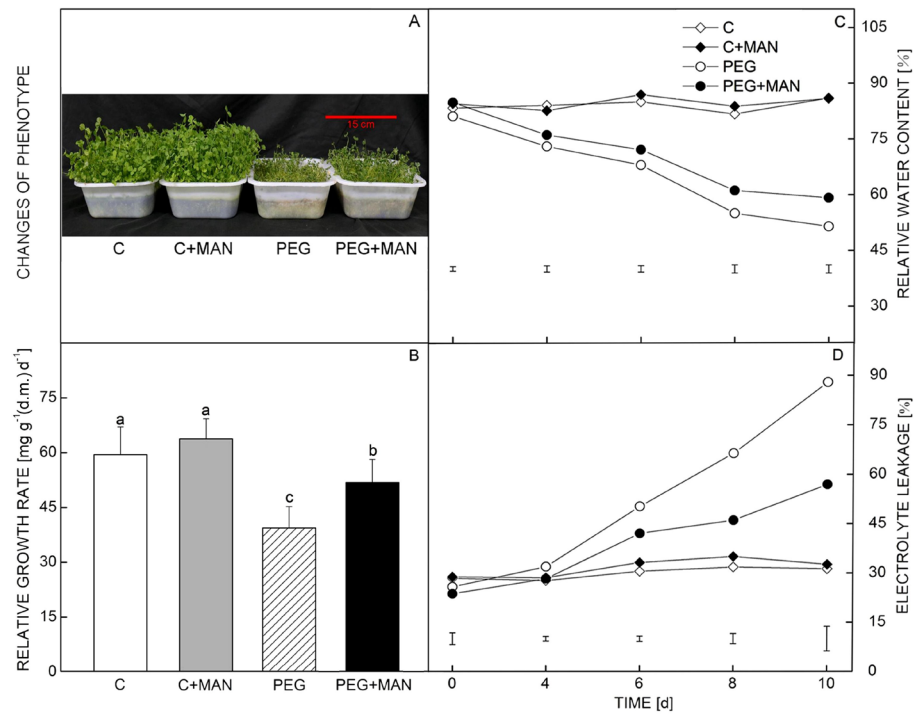


Fig. 1. Effects of mannose (MAN) on the phenotypic response (A), relative growth rate (B), relative water content (C), and electrolyte leakage (D) in leaves of white clover under control and osmotic stress conditions. Treatments: control (C), mannose under control conditions (C+MAN); polyethylene glycol-induced osmotic stress (PEG), PEG-induced osmotic stress in plants treated with MAN (PEG+MAN). Means \pm SEs, $n = 4$; different letters indicate significant differences among treatments based on the least significant difference (LSD) at $P \leq 0.05$. Vertical bars below curves represent LSD values at a given day of treatment.

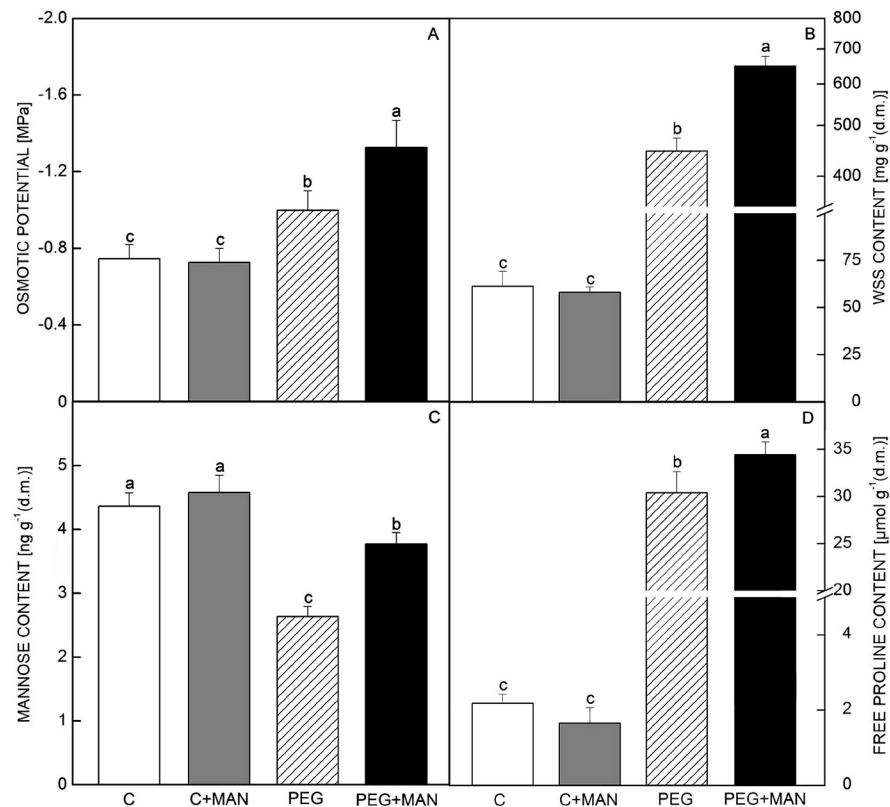


Fig. 2. Effects of mannose (MAN) on osmotic potential (A), water soluble saccharides, WSS (B), mannose content (C), and free proline content (D) in leaves of white clover under control and osmotic stress conditions. For other abbreviations, see Fig. 1. Means \pm SEs, $n = 4$; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

The MAN-treated white clovers exhibited 45, 43, and 1.33 % increases in WSS, MAN, and free Pro content than untreated plants under PEG treatment (Fig. 2B-D).

Osmotic stress induced by PEG caused significant increases in O_2^- , H_2O_2 , MDA, and TAC in leaves as compared to well-watered control (Fig. 3) but these parameters were not significantly affected by MAN treatment under control conditions. Under osmotic stress, the MAN-treated white clover had 42, 21, and 24 % lower O_2^- , H_2O_2 , and MDA content, respectively, than untreated plants (Fig. 3A-C) and TAC in leaves of MAN-treated white clover was 82 % higher than that in leaves of white clover without MAN treatment under osmotic stress (Fig. 3D). The activities of SOD, CAT, POD, APX, DHAR, GR, and MDHAR, and ASA and GSH content are not significantly affected by exogenous MAN under control conditions (Fig. 4). Application of MAN also had no effects on CAT activity, MDHAR activity, and GSH content under osmotic stress (Fig. 4B,G,I). The MAN-treated white clover exhibited 14, 17, 44, 22, and 32 % higher SOD, POD, APX, and GR activities and ASA content, respectively, than untreated plants under osmotic stress (Fig. 4A,C,D,F,H). DHAR activity in leaves of plants treated with MAN was twice higher than that those treated only by PEG (Fig. 4E).

The application of MAN did not significantly affect F_v/F_m , PIABS, P_N , and WUE in leaves during 10 d under control conditions (Fig. 5). Osmotic stress significantly

decreased F_v/F_m , PIABS, and P_N in leaves of MAN-treated and untreated white clovers at 8 and 10 d of osmotic stress, but MAN-treated plants exhibited significantly higher F_v/F_m , PIABS, and P_N than untreated plants under osmotic stress (Fig. 5A-C). MAN-treated plants had twice and seven times higher PIABS than untreated plants at 8 and 10 d of osmotic stress, respectively (Fig. 5B). The MAN-treated plants also exhibited 32 and 51 % increases in WUE than untreated plants at 8 and 10 d of PEG-induced stress, respectively (Fig. 5D).

Chlorophyll content and transcriptions of five different genes (*Mg-CHT*, *POR*, *PBGD*, *PAO*, and *CHLASE*) taking part in Chl synthesis and degradation were not affected significantly by MAN treatment under control conditions, but exogenous MAN caused a significant increase in *PBGD* transcription under control conditions (Fig. 6). The MAN-treated leaves exhibited 48 % increase in Chl content than untreated leaves under osmotic stress (Fig. 4J). Under osmotic stress, MAN-treated plants had 33 and 47 % higher *Mg-CHT* and *POR* transcriptions than untreated plants (Fig. 6B,C). Exogenous MAN had no significant effect on the transcription of *PBGD* in leaves under osmotic stress (Fig. 6D). However, application of MAN significantly inhibited stress-induced *PAO* and *CHLASE* expressions (Fig. 6E,F). Osmotic stress significantly enhanced the senescence marker gene *SAG101* transcription in both of MAN-treated and untreated white clovers (Fig. 6A), but the *SAG101* transcription in leaves of MAN-treated

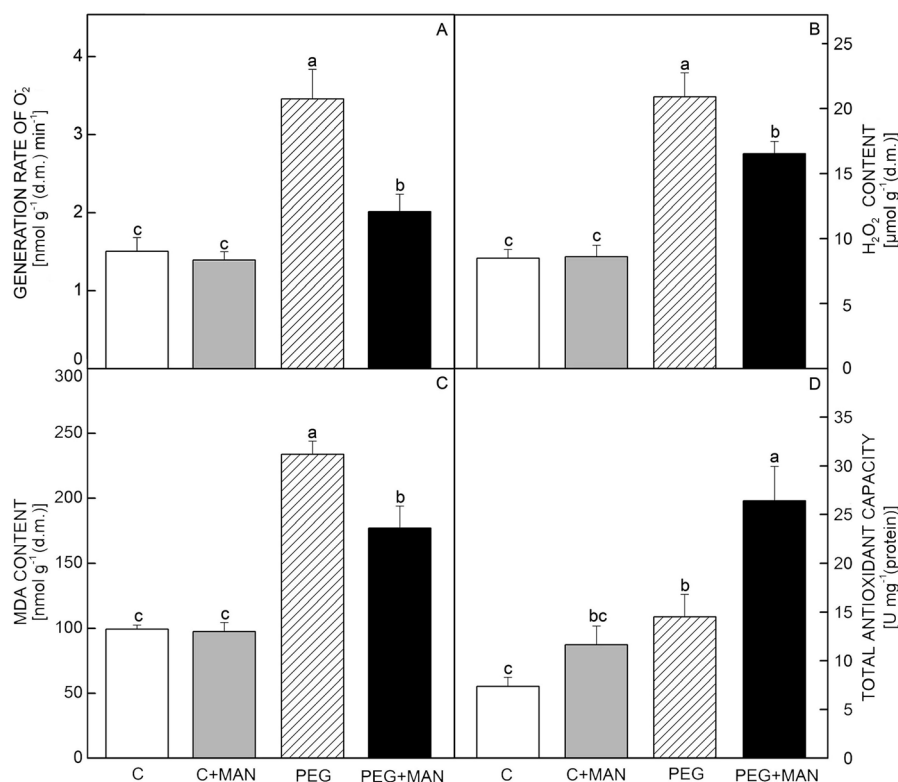


Fig. 3. Effects of mannose (MAN) on superoxide anion (O_2^-) generation (A), H_2O_2 content (B), malondialdehyde (MDA) content (C), and total antioxidant capacity (D) in leaves of white clover under control and osmotic stress conditions. For other abbreviations, see Fig. 1. Means \pm SEs, $n = 4$; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

plants was significantly lower than in those without MAN treatment under osmotic stress (Fig. 6A).

Transcriptions of four genes (*SK2*, *Y2K*, *Y2SK*,

and *dehydrin b*) encoding different dehydrins were examined in this study. Osmotic stress significantly up-regulated expressions of these genes (Fig. 7). Although

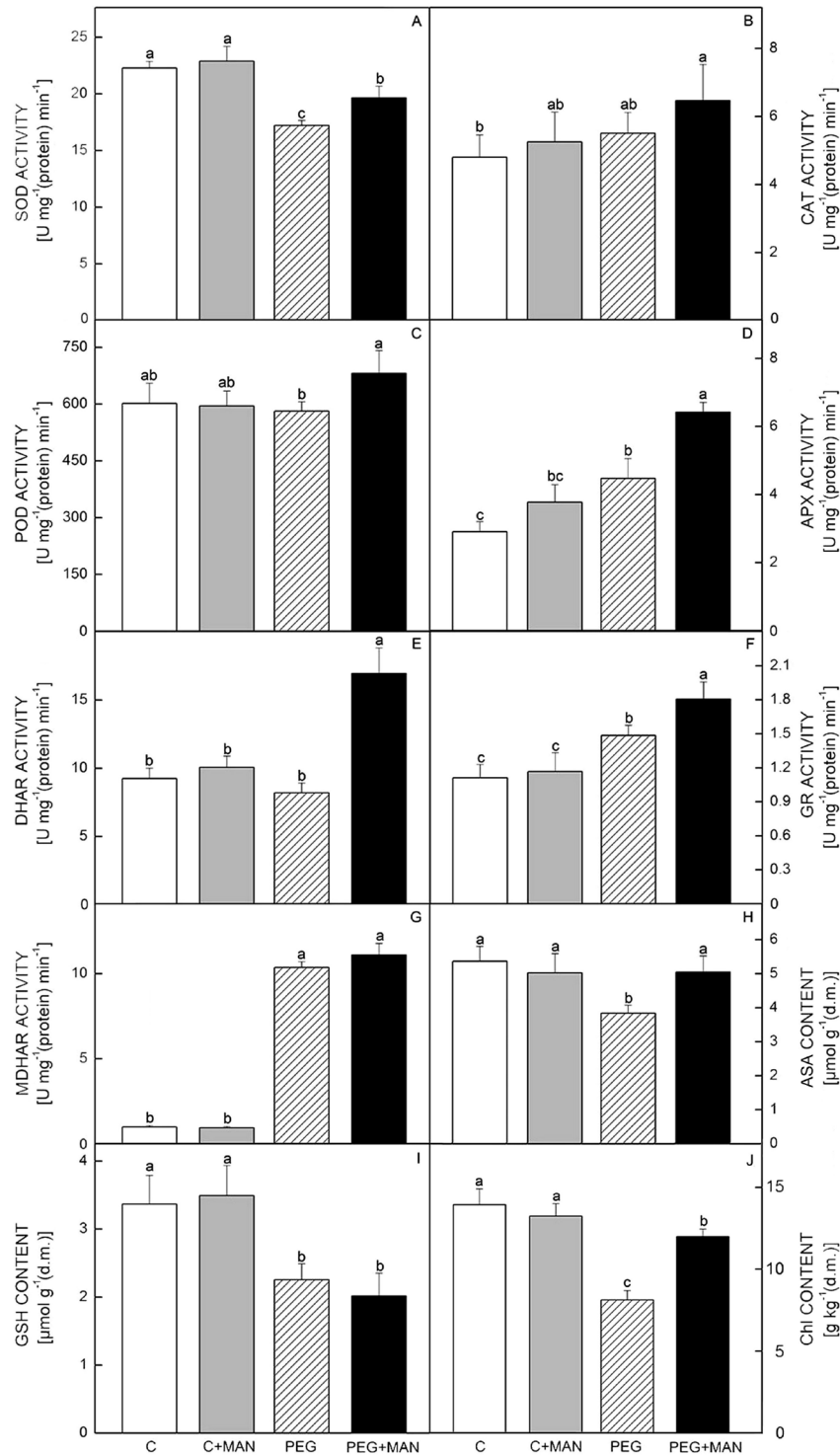


Fig. 4. Effects of mannose (MAN) on superoxide dismutase (SOD) activity (A), catalase (CAT) activity (B), peroxidase (POD) activity (C), ascorbate peroxidase (APX) activity (D), dehydroascorbate reductase (DHAR) activity (E), glutathione reductase (GR) activity (F), monodehydroascorbate reductase (MDHAR) activity (G), ascorbic acid (ASA) content (H), glutathione (GSH) content (I), and chlorophyll (CHL) content (J) in leaves of white clover under control and osmotic stress conditions. For other abbreviations, see Fig. 1. Means \pm SEs, $n = 4$; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

exogenous MAN did not cause significant effects on their transcriptions under control conditions, the application of MAN significantly enhanced their transcriptions under osmotic stress. Transcription of *SK2* in leaves of MAN-treated white clover was twice as high as that in leaves without MAN treatment under osmotic stress. The 59, 73, and 45 % increases in *Y2K*, *Y2SK* and *dehydrin b* transcriptions were observed in MAN-treated white clovers as compared to that in untreated plants under osmotic stress, respectively (Fig. 7).

Discussion

Osmotic stress usually results in wilting and growth inhibition. To alleviate plant damage, the osmotic adjustment is necessary to maintain water absorption, and the osmoprotection to maintain structure and function of plant cells under decreased water content (Hasegawa *et al.* 2000, Lambers *et al.* 2006). Our data showed that the increase in endogenous MAN content induced by MAN application caused significant increases in WSS and free pro accumulation in leaves of white clover under osmotic stress. It is well-known that sugars contribute to osmotic adjustment in response to water deficit and also they are sources of available energy for plant growth (Di Fonzo *et al.* 2000). Previous studies have shown that the accumulation of soluble sugars in wheat contributed to better drought tolerance (Hakimi *et al.* 1995, Kamaljit *et al.* 2007). The application of spermine (Spm) significantly increased accumulation of sucrose and fructose in two white clover cultivars, thereby alleviating drought-induced water deficit (Li *et al.* 2015a). Proline exhibits positive function in osmotic adjustment, osmoprotection,

and the detoxification of reactive oxygen species (ROS) in various plant species; and plant cells can accumulate a large amount of proline without detrimental side effects (Yancey *et al.* 1982, Ashraf and Foolad 2007, Kocsy *et al.* 2005). Proline is also an important source of nitrogen or energy in plants (Verslues and Sharp 1999). In this study, exogenous MAN-induced increases in MAN, WSS, and free pro content help to decrease OP and so better water absorption and also provide more available energy for growth of white clover under osmotic stress.

A long period of osmotic stress disturbs the balance of ROS metabolism leading to severe oxidative damage in plants. Both enzymatic and non-enzymatic antioxidants are involved in alleviating oxidative damage in plant tissue (Soares *et al.* 2016). The SOD catalyzes transformation of O_2^- into H_2O_2 , which is the first step of ROS scavenging (McKersie *et al.* 1993). Other antioxidant enzymes such as APX, POD, and CAT are responsible for transformation of H_2O_2 into H_2O and O_2 (Farooq *et al.* 2009). Previous studies have proved that higher antioxidant enzyme activities are directly related to higher protections and better stress tolerance in different plant species under drought stress (Bermejo *et al.* 2006, Hameed *et al.* 2013). Hameed *et al.* (2009) found that MAN could modulate activities of POD and APX which contribute to detoxifying ROS in wheat leaves. For non-enzymatic antioxidants, ASA-GSH cycle is mainly responsible for scavenging H_2O_2 in different cellular compartments (Asada 1992, Ai *et al.* 2016). The accumulation of ASA and GSH or enhanced ASA-GSH cycle can improve drought tolerance in white clover or other plant species (Eltayeb *et al.* 2006, Hasanuzzaman and Fujita 2011, Li *et al.* 2016). In our study, exogenous MAN significantly promoted SOD, POD, APX, DHAR, and GR activities, maintained significantly higher ASA

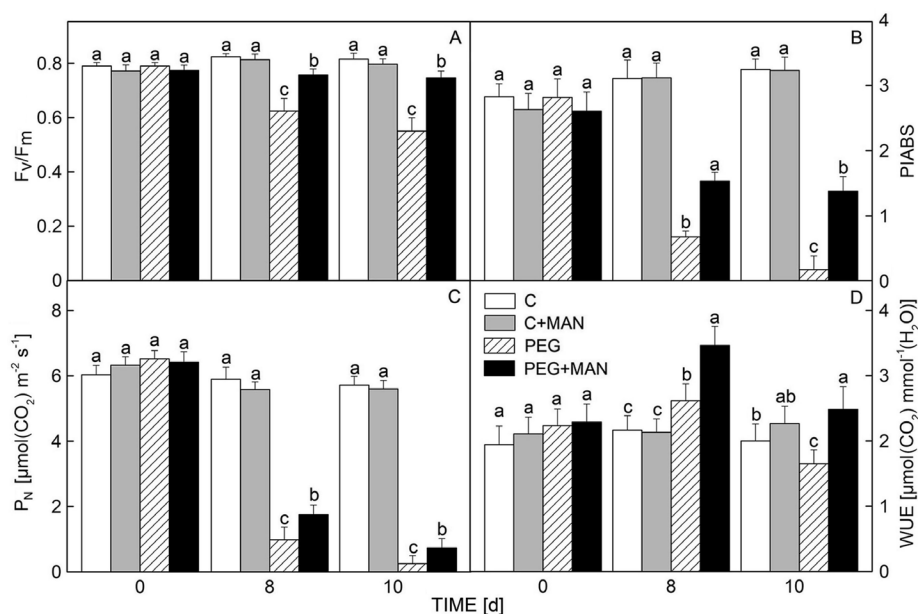


Fig. 5. Effects of mannose (MAN) on photochemical efficiency (F_v/F_m) (A), performance index on absorption basis (PIABS) (B), net photosynthetic rate, P_n (C), and water use efficiency (WUE) (D) in leaves of white clover under control and osmotic stress conditions. For other abbreviations see Fig. 1. Means \pm SEs, $n = 4$; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

content and also TAC, thereby inhibiting O_2^- , H_2O_2 , and MDA accumulation in leaves of white clover under osmotic stress. This data indicate that exogenous MAN improves the tolerance to osmotic stress associated with the enhancement of antioxidant defense and effective alleviation of oxidative damage in white clover.

Under abiotic stress, the overaccumulation of ROS damages cell membrane and photosynthetic organs leading to Chl degradation and senescence in plants (Rosenvasser *et al.* 2006). *PAO* and *CHLASE* are two main enzymes involved in Chl degradation in plants. It has been proved that drought- or heat-induced Chl loss was closely related to the up-regulation of *PAO* and *CHLASE* expression in grass species (Jespersen *et al.* 2016, Li *et al.* 2017b, Zhang *et al.* 2016). The inhibition of Chl degradation could

obviously suppress leaf senescence of perennial ryegrass (Zhang *et al.* 2015). Our current study showed that MAN pretreatment could significantly down-regulate stress-induced increases in transcriptions of *PAO* and *CHLASE* in leaves of white clover. Interestingly, MAN-treated white clover also could maintain significantly higher transcriptions of *Mg-CHT* and *POR* in response to osmotic stress. *PBGD*, *Mg-CHT*, and *POR* are three major enzymes regulating chlorophyll synthesis. *PBGD* can combine four porphobilinogen subunits to generate a porphyrin ring, *Mg-CHT* is responsible for inserting a magnesium ion into the porphyrin ring, and *POR* controls the final step in chlorophyll synthesis to generate chlorophyllide (Bollivar 2006). Our previous study has found that suitable concentration of Na^+ (30 mM) pretreatment

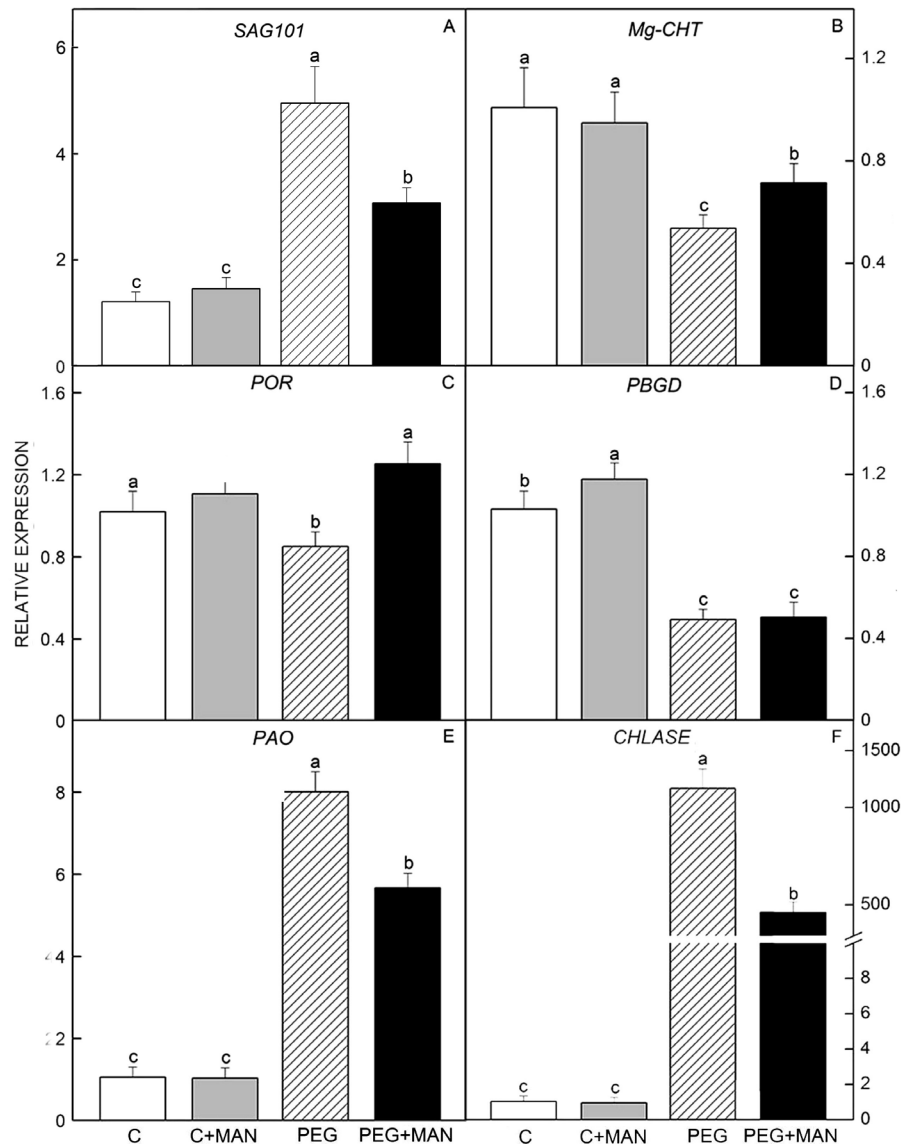


Fig. 6. Effects of mannose (MAN) on *senescence-associated gene 101 (SAG101)* relative expression (A), *Mg-chelatase (Mg-CHT)* relative expression (B), *protochlorophyllide reductase (POR)* relative expression (C), *porphobilinogen deaminase (PBGD)* relative expression (D), *pheophorbide a oxygenase (PAO)* relative expression (E), and *chlorophyllase (CHLASE)* relative expression (F) in leaves of white clover under control and osmotic stress conditions. For other abbreviations see Fig. 1. Means \pm SEs, $n = 4$; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

could significantly up-regulated *POR* and *Mg-CHT*, but inhibited drought-induced *PAO* expression, which effectively decreased Chl degradation and suppressed leaf senescence in white clover (Li *et al.* 2017b). In this study, the application of MAN maintained Chl synthesis and inhibited Chl degradation, which contributed to delaying leaf senescence and maintaining higher photosynthetic performance in white clover under osmotic stress.

In higher plants, the increase in dehydrin accumulation is another key adaptive mechanism in response to various abiotic stresses including drought or osmotic stress. Dehydrins have multiple positive function such as cell membrane and structures protection, osmotic adjustment, prevention of cell dehydration, and antioxidant activity when plants respond to harsh environment (Bian *et al.* 2002, Heyen *et al.* 2002, Hanin *et al.* 2011). It was reported that the drought-tolerant white clover had higher transcriptions of dehydrin genes than sensitive cultivar under osmotic stress (Vaseva *et al.* 2011). Transcriptions of genes encoding dehydrins in *Dendrobium candidum* contributed to dehydration and freezing tolerance (Bian *et al.* 2002). The study of Li *et al.* (2015a) also found that exogenous Spm could significantly alleviate PEG-induced osmotic stress damage in white clover through enhancing dehydrin accumulation and dehydrin-encoded genes expression. Our current findings showed that osmotic stress caused significant increases in transcriptions of dehydrin genes (*SK2*, *Y2K*, *Y2SK*, and *dehydrinb*), which

were important adaptive responses to osmotic stress. Interestingly, the MAN application could further enhance transcriptions of *SK2*, *Y2K* and *Y2SK* and *dehydrinb* in leaves of white clover under osmotic stress. Thus, MAN-regulated tolerance to osmotic stress in white clover is closely related to expressions of dehydrin genes.

Conclusions

Applications of suitable dose of MAN could significantly improve tolerance to osmotic stress in white clover. Physiological analyses showed that exogenous MAN induced significant increase in endogenous MAN, proline, and WSS content contributing to lower OP and better water balance in white clover. The application of MAN also activated antioxidant enzymes (SOD, POD, APX, DHAR, and GR) and maintained enhanced ASA content in white clover under osmotic stress, thereby effectively alleviating oxidative damage. In response to osmotic stress, the MAN-treated white clover could maintain significantly higher photosynthesis and transcription of genes involved in Chl synthesis (*Mg-CHT* and *POR*) and decrease stress-induced transcription of *PAO* and *CHLASE* genes relating to Chl degradation as well as the senescence marker gene *SAG101*. In addition, the MAN application further improved transcriptions of genes encoding dehydrins (*SK2*, *Y2K*, *Y2SK*, and *dehydrinb*) in leaves of white clover

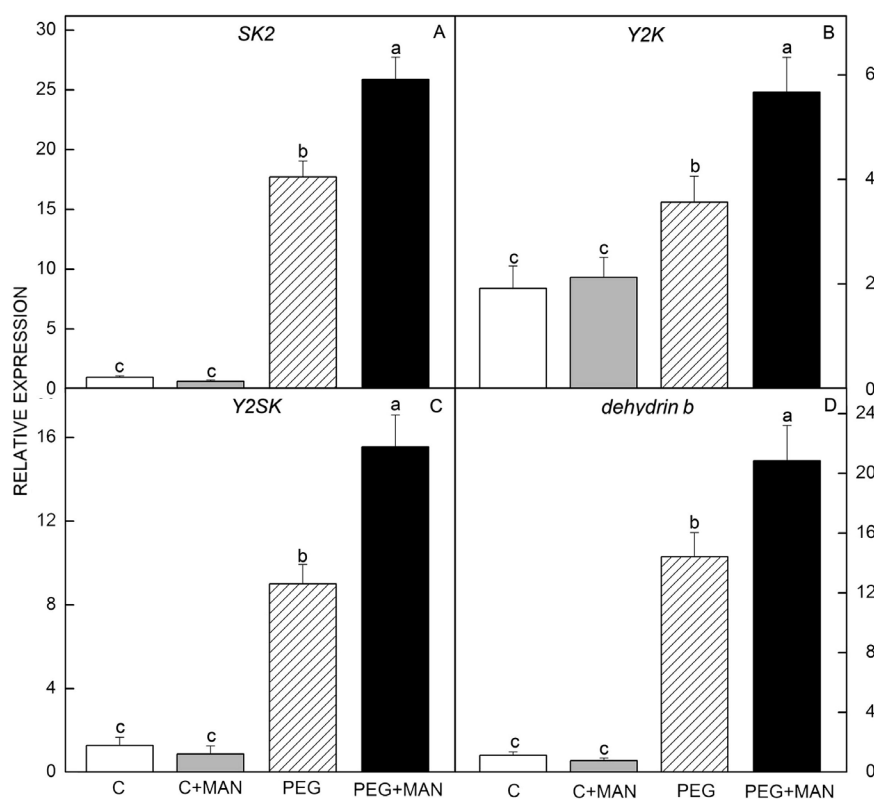


Fig. 7. Effects of mannose (MAN) on *SK2*-type dehydrin (*SK2*) relative expression (A), *Y2K*-type dehydrin (*Y2K*) relative expression (B), *Y2SK*-type dehydrin (*Y2SK*) relative expression (C), and *dehydrin b* relative expression (D) in leaves of white clover under control and osmotic stress conditions. For other abbreviations, see Fig. 1. Means \pm SEs, $n = 4$; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

under osmotic stress. These results indicate that MAN not only acts as an important compatible solute for OP, but also delays leaf senescence through enhancing antioxidant metabolism, decreasing Chl degradation, and increasing transcriptions of dehydrin genes contributing to enhanced drought tolerance in white clover. Current results provide new evidence for better understanding beneficial function of MAN when plants suffer from harsh environmental conditions.

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