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Alleviation of osmotic stress in *Brassica napus*, *B. campestris*, and *B. juncea* by ascorbic acid application

M.M. ALAM[1], K. NAHAR[1,2], M. HASANUZZAMAN[3], and M. FUJITA[1]*

Laboratory of Plant Stress Responses, Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan[1]
Department of Agricultural Botany[2] *and Department of Agronomy*[3], *Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh*

Abstract

The roles of ascorbic acid (AsA, 1 mM) under an osmotic stress [induced by 15 % (m/v) polyethylene glycol, PEG-6000] were investigated by examining morphological and physiological attributes in *Brassica* species. The osmotic stress reduced the fresh and dry masses, leaf relative water content (RWC), and chlorophyll (Chl) content, whereas increased the proline (Pro), malondialdehyde (MDA), and H_2O_2 content, and lipoxygenase (LOX) activity. The ascorbate content in *B. napus, B. campestris*, and *B. juncea* decreased, increased, and remained unaltered, respectively. The dehydroascorbate (DHA) content increased only in *B. napus*. The AsA/DHA ratio was reduced by the osmotic stress in all the species except *B. juncea*. The osmotic stress increased the glutathione (GSH) content only in *B. juncea*, but increased the glutathione disulfide (GSSG) content and decreased the GSH/GSSG ratio in all the species. The osmotic stress increased the activities of ascorbate peroxidase (APX) (except in *B. napus*), glutathione reductase (GR) (except in *B. napus*), glutathione *S*-transferase (GST) (except in *B. juncea*), and glutathione peroxidase (GPX), and decreased the activities of catalase (CAT) and monodehydroascorbate reductase (MDHAR) (only in *B. campestris*). The osmotic stress decreased the glyoxalase I (Gly I) and increased glyoxalase II (Gly II) activities. The application of AsA in combination with PEG improved the fresh mass, RWC, and Chl content, whereas decreased the Pro, MDA, and H_2O_2 content in comparison with PEG alone. The AsA addition improved AsA-GSH cycle components and improved the activities of all antioxidant and glyoxalase enzymes in most of the cases. So, exogenous AsA improved physiological adaptation and alleviated oxidative damage under the osmotic stress by improving the antioxidant and glyoxalase systems. According to measured parameters, *B. juncea* can be recognized as more drought tolerant than *B. napus* and *B. campestris*.

Additional key words: abiotic stress tolerance, antioxidants, AsA-GSH cycle, methylglyoxal, oxidative stress, polyethylene glycol.

Introduction

Among different abiotic stresses, drought is considered as the most complex and devastating due to the multifaceted nature of its damage to plants (Pennisi 2008). Drought has various detrimental effects on plant growth and development that limits the yield potential of crop plants (Jaleel *et al*. 2009, Hasanuzzaman and Fujita 2011, Deeba *et al*. 2012, Hasanuzzaman *et al*. 2012a, 2014, Alam *et al*. 2013). A stress imposed by a shortage of water can result in changes in chlorophyll (Chl) content, chloroplast ultrastructure, damage to the photosynthetic apparatus,

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Abbreviations: AO - ascorbate oxidase; APX - ascorbate peroxidase; AsA - ascorbic acid (ascorbate); BSA - bovine serum albumin; CAT - catalase; CDNB - 1-chloro-2,4-dinitrobenzene; Chl - chlorophyll; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; DTNB - 5,5´-dithio-bis-(2-nitrobenzoic acid); EDTA - ethylenediaminetetraacetic acid; Gly I - glyoxalase I; Gly II - glyoxalase II; GPX - glutathione peroxidase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; GST - glutathione *S*-transferase; LOX - lipoxygenase; MDA - malondialdehyde; MDHA - monodehydroascorbate; MDHAR - monodehydroascorbate reductase; MG - methylglyoxal; NTB - 2-nitro-5-thiobenzoic acid; PEG - polyethylene glycol; Pro - Proline, ROS - reactive oxygen species; RWC - relative water content; SLG - *S*-D-lactoylglutathione; TBA - thiobarbituric acid; TCA - trichloroacetic acid.
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* Corresponding author; fax: (+81) 87 8913021, e-mail: fujita@ag.kagawa-u.ac.jp

and cell death (Hameed *et al*. 2013). Drought stress usually promotes the accumulation of reactive oxygen species (ROS) like a superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) which then cause further oxidative damage to plant tissues (Lindahl *et al*. 1995, Foyer and Noctor 2000, Hasanuzzaman *et al*. 2012a, Hasanuzzaman and Fujita 2013). These ROS can oxidize or peroxidize macromolecules like lipids, proteins including enzymes, and nucleic acids including DNA (Wang *et al*. 2009, Desai *et al*. 2010).

To counteract the toxicity of ROS, plants have highly efficient antioxidative systems composed of both non-enzymatic antioxidants and antioxidant enzymes. The non-enzymatic antioxidants include ascorbate (AsA), glutathione (GSH), carotenoids, flavanones, and anthocyanins, whereas antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) (Gupta *et al*. 2009, Hasanuzzaman *et al*. 2012a, Hasanuzzaman and Fujita 2013). Different studies have shown that adaptation and improved tolerance to drought stress in plants correlates with an enhanced antioxidative capacity (Singla-Pareek *et al*. 2003, Hoque *et al*. 2007).

Another toxic compound that is produced during abiotic stress is methylglyoxal (MG) which damages proteins, lipids, and saccharides similarly as ROS (Yadav *et al*. 2005a,b, Singla-Pareek *et al*. 2006, 2008, Hasanuzzaman *et al*. 2011a,b, 2012b,c). Plants also possess a detoxification system for MG which consists of two enzymes, glyoxalase I (Gly I) and glyoxalase II (Gly II) (Yadav *et al*. 2005a,b). Gly I uses GSH to convert MG to *S*-D-lactoylglutathione, whereas Gly II converts *S*-D-lactoylglutathione to D-lactic acid. GSH is recycled at the end of these reactions. Upregulation or overexpression of Gly I and Gly II helps to alleviate the effect of MG under different abiotic stresses including drought (Hoque *et al*. 2007, Hasanuzzaman and Fujita 2011).

Reduced ascorbate (AsA) is a vital component of the AsA-GSH cycle and therefore plays an important role in plant stress tolerance (Sharma and Dubey 2005, Hasanuzzaman *et al*. 2011a). AsA is an efficient electron donor that can directly react with a range of ROS, including H_2O_2, $O_2^{\bullet-}$, and $^1O_2$. In the AsA-GSH cycle, AsA is utilized by APX to reduce H_2O_2 to water. This reaction generates MDHA which is then readily converted into DHA and AsA (Del Río *et al*. 2006, Wu *et al*. 2007). AsA is also thought to maintain other antioxidants, like α-tocopherol, in their reduced states. It also maintains the reduced state of metal ions that are required for the activity of various antioxidant enzymes (De Tullio 2004). An enhanced synthesis of AsA in plants has been confirmed to enhance drought tolerance (Hasanuzzaman and Fujita 2011, Alam *et al*. 2013). Similarly, an exogenous application of AsA to plants can also influence the antioxidant defense system and has been shown to confer tolerance to several abiotic stresses (Shalata and Neumann 2001, Pignocchi and Foyer 2003, Athar *et al*. 2008, Bybordi *et al*. 2012, Darvishan *et al*. 2013).

AsA also upregulates the content of GSH in plants exposed to osmotic stress. Since GSH is a vital metabolite used in the glyoxalase system, we hypothesize that an exogenous application of AsA could also upregulate the activities of glyoxalases. However, few studies have investigated the effects of AsA on antioxidant defense or the glyoxalase system in plants under osmotic stress. *Brassica* species are important oil crops and several species are cultivated worldwide. *Brassica juncea*, *B. napus*, and *B. campestris* are three widely cultivated species and these species often face drought and osmotic stresses. So, in the present study, these three species were selected to examine their responses to osmotic stress as well as to exogenous AsA.

Materials and methods

Seeds of three *Brassica* spp. (*Brassica napus* L. cv. BARI Sharisha 13, *Brassica campestris* L. cv. BARI Sharisha 9, and *Brassica juncea* L. cv. BARI Sharisha 11) were surface-sterilized with 70 % (v/v) ethanol for 10 min, washed, placed in Petri dishes lined with filter paper moistened with distilled water, and germinated in the dark for two days. Then seedlings were grown in 10 000-fold diluted *Hyponex* (Kagawa, Japan) solution under a steadily irradiance of 100 μmol(photon) m^{-2} s^{-1}, a temperature of 25 ± 2 °C, and a relative humidity of 65 - 70 %). After 10 d, one set of seedlings of each species was subjected to an osmotic stress by adding 15 % (m/v) PEG-6000 (*Wako*, Kagawa, Japan) to the *Hyponex* solution to create the osmotic potential of -0.30 MPa. Another set of seedlings was supplied with 1 mM AsA (L-ascorbic acid, *Wako*) and 15 % PEG. Control plants were grown in the *Hyponex* solution only. Data were collected after 48 h. The experiment was repeated three times.

Ten randomly selected fresh seedlings from each treatment were weighed to record the fresh mass. The dry mass was determined after drying the seedlings at 80 °C for 48 h. A relative water content (RWC) was measured according to Barrs and Weatherly (1962). For Chl determination, leaves were extracted with 80 % (v/v) acetone, centrifuged at 5 000 *g*, and the absorbances of supernatant were taken at 663 and 645 nm with a UV-visible spectrophotometer (*UV-1700*, *Shimadzu*, Kyoto, Japan). The content of Chl *a* and Chl *b* was calculated according to Arnon (1949).

Proline (Pro) was appraised according to Bates *et al*. (1973). Leaves were homogenized in 3 % (m/v)

sulfosalicylic acid and centrifuged at 11 500 *g* for 12 min. Two cm^3 of supernatant was mixed with 2 cm^3 of acid ninhydrin, 2 cm^3 of glacial acetic acid, and 6 M phosphoric acid. After incubating the mixture at 100 °C for 1 h and cooling to 4 °C, the absorbance was read at 520 nm.

Lipid peroxidation was measured by estimating the malondialdehyde (MDA) content using thiobarbituric acid (TBA) according to Heath and Packer (1968) with modifications (Hasanuzzaman *et al*. 2011a).

Hydrogen peroxide (H_2O_2) was assayed according to Yu *et al*. (2003) by homogenizing leaves in a potassium-phosphate buffer (pH 6.5) and centrifuging at 11 500 *g* for 15 min. Then $TiCl_4$ in 20 % (m/v) H_2SO_4 was added and the absorbance was read at 410 nm.

For determination of ascorbate and glutathione content, leaves (0.5 g) were homogenized in 5 % (m/v) meta-phosphoric acid containing 1 mM EDTA, centrifuged at 11 500 *g* and 4 °C for 15 min and the supernatant was collected. The ascorbate content was determined following the method of Huang *et al*. (2005) with some modifications as described by Hasanuzzaman *et al.* (2011a). The glutathione pool was assayed according to Yu *et al.* (2003) with modifications described by Paradiso *et al*. (2008) and Hasanuzzaman *et al.* (2011a). The protein content of each sample was determined following the method of Bradford (1976) using BSA as standard.

Leaves were homogenized in a potassium-phosphate (K-P) buffer (pH 7.0) containing 1 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol, and 10 % (v/v) glycerol. Homogenates were centrifuged at 11 500 *g* and 4 °C for 12 min and supernatants were used for enzyme assays. The APX (EC: 1.11.1.11) activity was measured according to Nakano and Asada (1981). A reaction mixture contained a 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and the enzyme extract (the final volume of 700 mm^3). The reaction was started by adding H_2O_2. The absorbance was measured at 290 nm for 1 min. A coefficient of absorbance (ε) was 2.8 mM^{-1} cm^{-1}. The MDHAR (EC: 1.6.5.4) activity was determined according to Hossain *et al*. 1984. A reaction mixture contained a 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of ascorbate oxidase (AO), and the enzyme extract (the final volume of 700 mm^3). The reaction was started by adding AO. The absorbance was taken at 340 nm and the activity was calculated from a change during 1 min (ε = 6.2 mM^{-1} cm^{-1}). For the DHAR (EC: 1.8.5.1) activity assay, the method of Nakano and Asada (1981) was used. A reaction mixture contained a 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, 0.1 mM DHA, and the enzyme extract. The activity was calculated from a change in absorbance at 265 nm for 1 min using ε = 14 mM^{-1} cm^{-1}. The GR (EC: 1.6.4.2) activity was measured according to Hasanuzzaman *et al*. (2011b). A reaction mixture contained a 0.1 M K-P buffer (pH 7.0), 1 mM Na_2EDTA, 1 mM GSSG, 0.2 mM NADPH, and the enzyme extract (the final volume of 1 cm^3). The reaction was initiated with GSSG and a decrease in absorbance at 340 nm was recorded for 1 min (the activity was calculated using ε = 6.2 mM^{-1} cm^{-1}). The GST (EC: 2.5.1.18) activity was determined according to Hossain *et al*. (2006). A reaction mixture contained a 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and the enzyme extract (the final volume of 700 mm^3). The reaction was initiated by CDNB, an increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using ε = 9.6 mM^{-1} cm^{-1}. The GPX (EC: 1.11.1.9) activity was measured as described by Elia *et al*. (2003) with slight modifications as described by Hasanuzzaman *et al*. (2011a). A reaction mixture consisted of a 100 mM K-P buffer (pH 7.0), 1 mM Na_2EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 unit of GR, 0.6 mM H_2O_2 (as substrate), and 20 mm^3 of the enzyme extract. The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using ε = 6.2 mM^{-1} cm^{-1}. The CAT (EC: 1.11.1.6) activity was measured according to Hasanuzzaman *et al*. (2011a) in the reaction mixture containing 50 mM potassium-phosphate buffer (pH 7.0), 15 mM H_2O_2 and enzyme extract in a final volume of 0.7 cm^3. A decrease of absorbance (by decomposition of H_2O_2) at 240 nm was recorded for 1 min. The reaction was initiated with the enzyme extract; the activity was calculated from ε = 39.4 $M^{-1}cm^{-1}$. The glyoxalase I (EC: 4.4.1.5) activity was measured according to Hasanuzzaman *et al*. (2011a). An assay mixture contained a 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH, 3.5 mM methyl-glyoxal (MG), and the enzyme extract (the final volume of 700 mm^3). The reaction was started by adding MG and an increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using ε = 3.37 $mM^{-1}cm^{-1}$. The glyoxalase II (EC: 3.1.2.6) activity was determined according to Principato *et al*. (1987). A formation of GSH at 412 nm was monitored for 1 min in a reaction mixture containing 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, 1 mM *S*-D-lactoylglutathione (SLG) (the final volume of 1 cm^3) The activity was calculated using ε =13.6 $mM^{-1}cm^{-1}$. The LOX (EC 1.13.11.12) activity was estimated according to the method of Doderer *et al*. (1992) by monitoring the increase in absorbance at 234 nm using linoleic acid as substrate. The reaction mixture contained potassium-phosphate buffer (50 mM), NBT (2.24 mM), catalase (0.1 units), xanthine oxidase (0.1 unit), xanthine (2.36 mM) and the enzyme extract. The activity was calculated using ε = 25 mM^{-1} cm^{-1}. All the enzyme activities were measured using the above mentioned spectrophotometer.

All data obtained were subjected to the analysis of variance (*ANOVA*) and the mean differences were compared by the Duncan's multiple range test (DMRT) using the *XLSTAT v.2010* software (*Addinsoft 2010*). Differences at $P \leq 0.05$ were considered as significant.

Results

Exposure to the osmotic stress resulted in significant decreases in seedling fresh masses: 23 % in *B. napus,* 37 % in *B. campestris,* and 34 % in *B. juncea* when compared to the unstressed controls. The application of AsA in combination with PEG resulted in fresh mass increases of 24 % in *B. napus,* 51 % in *B. campestris,* and

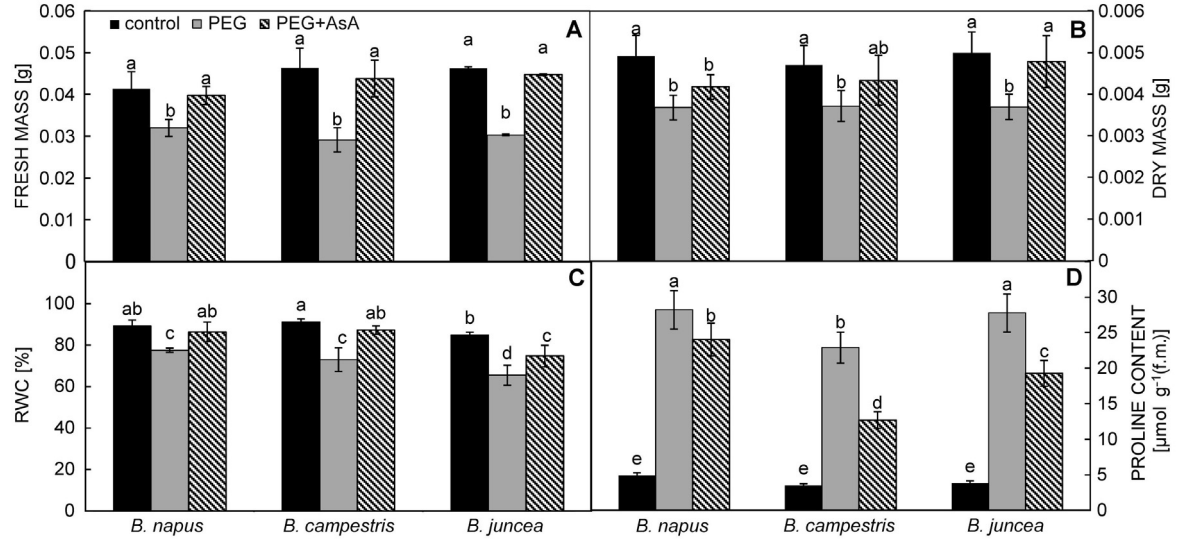

Fig. 1. The fresh mass (*A*), dry mass (*B*), leaf relative water content (RWC; *C*), and proline content (*D*) of *Brassica* seedlings under ascorbic acid (1 mM AsA) and osmotic stress (15 % PEG) treatments. Means ± SE were calculated from three replicates for each treatment. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

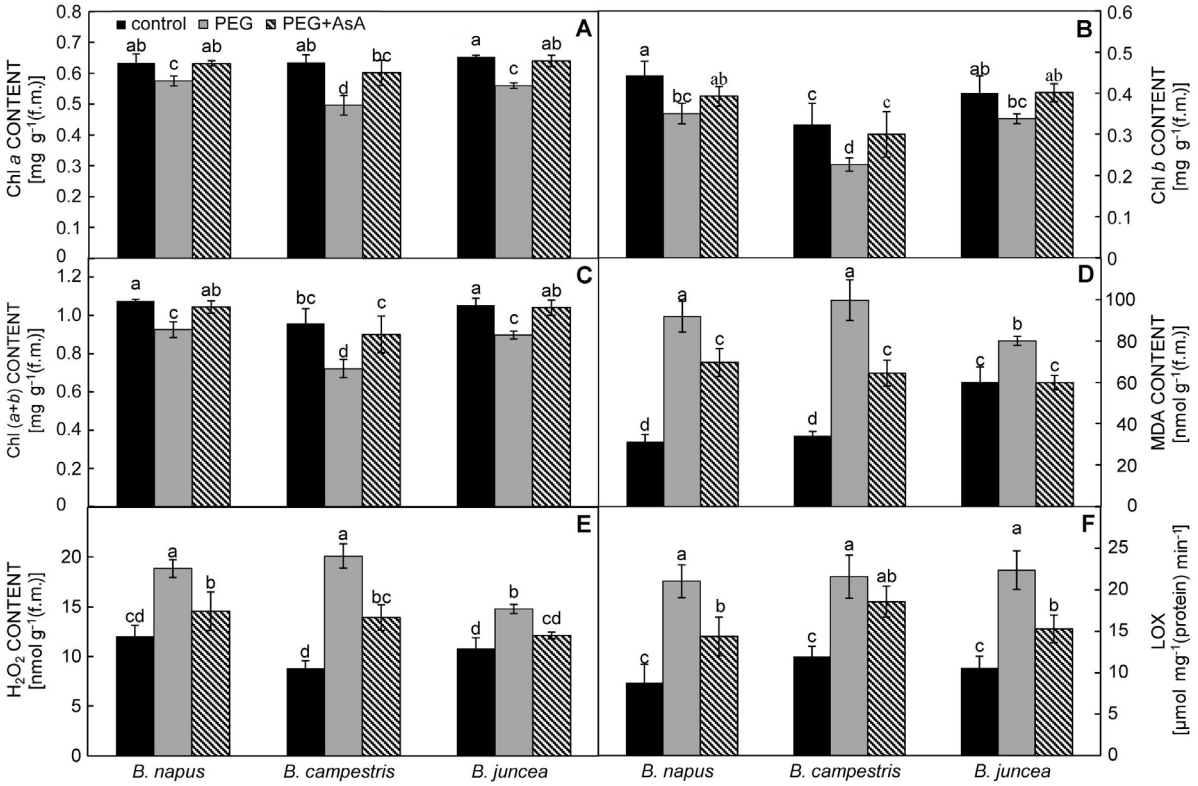

Fig. 2. The content of Chl *a* (*A*), Chl *b* (*B*), Chl (*a*+*b*) (*C*), MDA (*D*), and H_2O_2 (*E*), and the LOX activity (*F)* in *Brassica* seedlings under 1 mM AsA and 15 % PEG treatments. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

48 % in *B. juncea* (Fig. 1*A*) in comparison with PEG alone. The seedling dry mass showed similar changes, however, only osmotic-stressed *B. campestris* showed increases in dry mass in response to AsA (compared to the PEG treatment alone) (Fig. 1*B*).

The leaf relative water content (RWC) was reduced by 13, 20, and 23 % in PEG-treated *B. napus*, *B. campestris,* and *B. juncea,* respectively, compared to the unstressed controls. The application of exogenous AsA with osmotic stress resulted in noticeable improvements in RWC in all *Brassica* species, when compared to the seedlings grown under PEG alone (Fig. 1*C*).

The proline content was noticeably increased in all three *Brassica* species upon exposure to the osmotic stress. The Pro content of *B. napus, B. campestris,* and *B. juncea* increased by 478, 557, and 637 %, respectively, compared to the unstressed controls. The addition of AsA to the PEG solution resulted in a lower Pro content (15, 44, and 31 %) in *B. napus, B. campestris* and *B. juncea,* respectively than that found in seedlings exposed to the osmotic stress alone (Fig. 1*D*).

The leaf Chl *a* and Chl *b* content was significantly reduced by the osmotic stress irrespective of *Brassica* species. The Chl *a* content in *B. napus, B. campestris,* and *B. juncea* was reduced by 9, 22, and 14 %, respectively, compared to the unstressed controls (Fig. 2*A*). The Chl *b* content decreased by 21, 30, and 16 % in *B. napus, B. campestris,* and *B. juncea,* respectively (Fig. 2*B*). The total Chl therefore significantly declined in all three species (Fig. 2*C*). The addition of AsA improved Chl *a* content in all three species, whereas Chl *b* content significantly enhanced only in *B. campestris,* compared to its drought stress treatment. The Chl (*a*+*b*) content was improved in all three species when AsA was added (Fig. 2*A*-*C*).

The osmotic stress caused lipid peroxidation as indicated by the increased MDA content. The highest MDA content was observed in *B. napus* and *B. campestris*. Upon the osmotic stress treatment, the content of MDA increased by 194, 192, and 33 %, in *B. napus, B. campestris*, and *B. juncea,* respectively, as compared to their respective controls. The addition of AsA in combination with the osmotic stress significantly decreased the MDA content by 24, 35, and 25 % in *B. napus, B. campestris,* and *B. juncea*, respectively, when compared to the seedlings exposed to the osmotic stress alone (Fig. 2*D*).

The content of H_2O_2 was also significantly increased in all three species after exposure to the osmotic stress, being 55, 109, and 37 % higher in *B. napus, B. campestris*, and *B. juncea*s, respectively, compared to the control. In the case of the exogenous AsA application in osmotic-stressed seedlings, the H_2O_2 content decreased by 22, 31, and 18 % in *B. napus, B. campestris*, and

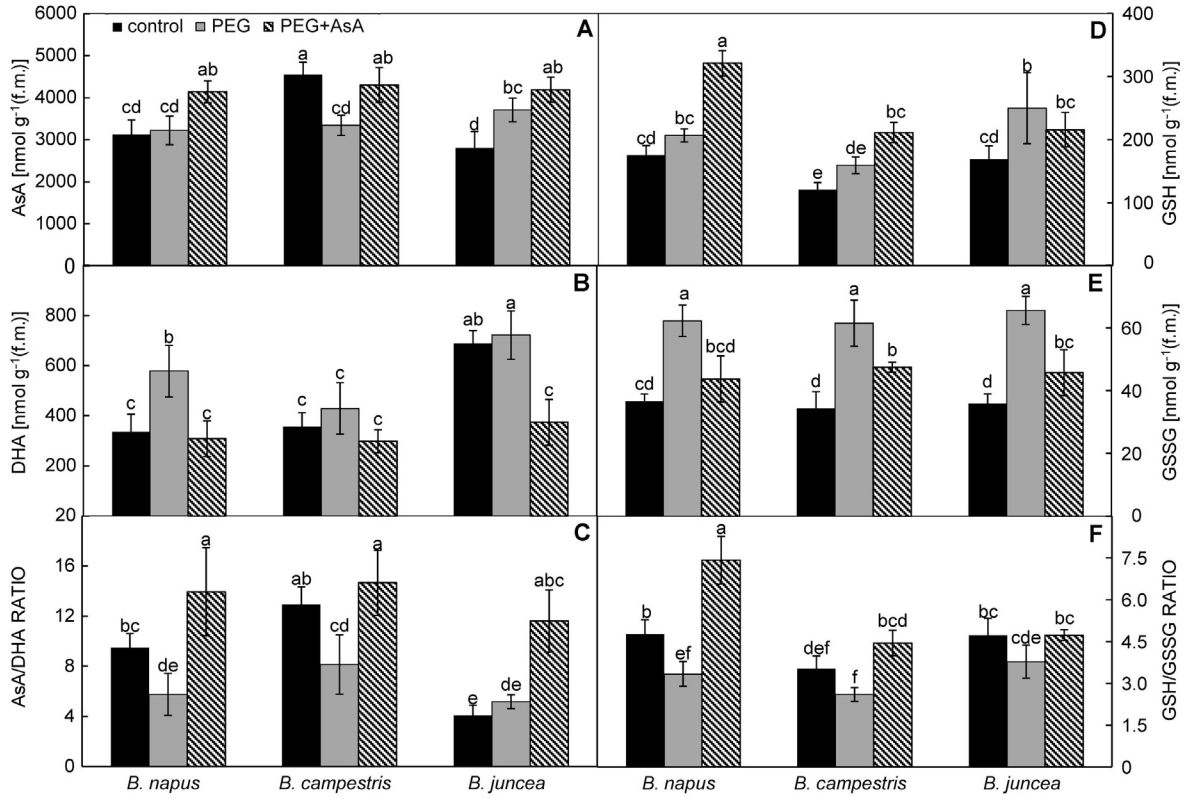

Fig. 3. The AsA content (*A*), DHA content (*B*), AsA/DHA ratio (*C*), GSH content (*D*), GSSG content (*E*), and GSH/GSSG ratio (*F*) in *Brassica* seedlings under 1 mM AsA and 15 % PEG treatments. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

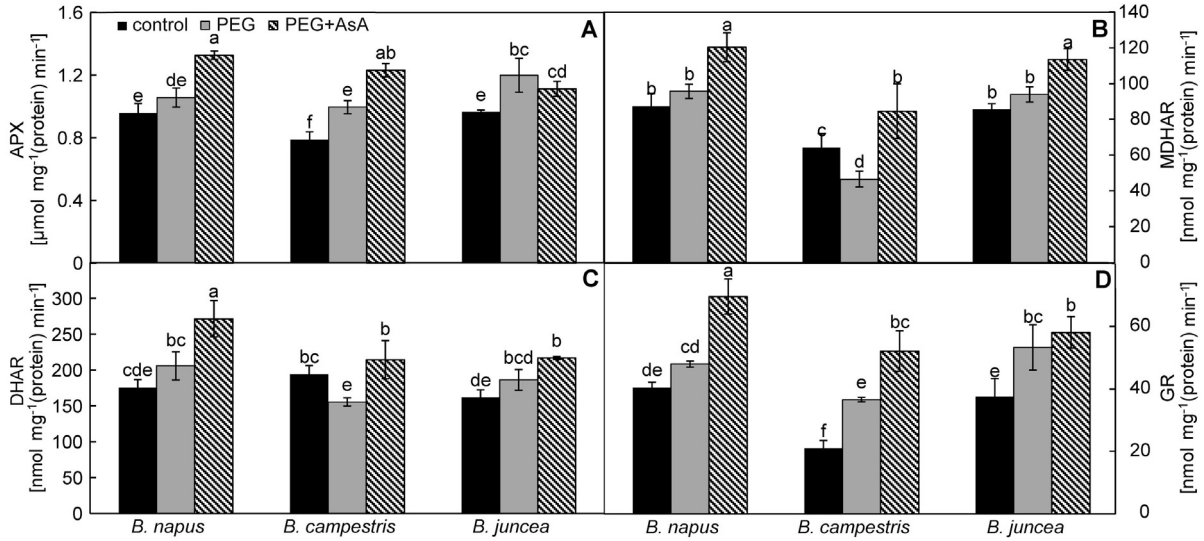

Fig. 4. The activities of APX (*A*), MHAR (*B*), DHAR (*C*), and GR (*D*) in *Brassica* seedlings under 1 mM AsA and 15 % PEG treatments. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

B. juncea, respectively, in comparison with seedlings treated with PEG alone (Fig. 2*E*).

The lipoxygenase (LOX) activity of PEG treated *B. napus, B. campestris* and *B. juncea* seedlings were increased by 77, 81, and 112 %, respectively, as compared to their respective controls. The exogenous AsA supplementation caused a reduced LOX activity by 32 % in both *B. napus* and *B. juncea*, whereas the activity was not changed in *B. campestris* (compared to the PEG treatment alone) (Fig. 2*F*).

The endogenous AsA content of *B. napus* remained unchanged under the osmotic stress, but the addition of AsA in combination with the osmotic stress resulted in the significantly enhanced AsA content (28 %) in these seedlings. The endogenous AsA content in *B. campestris* decreased by 27 % under the osmotic stress but the addition of AsA in combination with the osmotic stress increased the endogenous AsA content. The osmotic stress increased the endogenous AsA content by 33 % in *B. juncea*; however, no further significant increases were noted in response to the combination of the osmotic stress and exogenous AsA (Fig. 3*A*).

The DHA content was markedly increased in *B. napus* (73 %) exposed to the osmotic stress, but it was unchanged in *B. campestris* and *B. juncea*. The combination of the exogenous AsA and osmotic stress resulted in a reduction in DHA content in *B. napus* and *B. juncea* by 47 and 30 %, respectively, compared to the osmotic-stressed conditions alone (Fig. 3*B*). The AsA/DHA ratio decreased significantly by the osmotic stress in both *B. napus* and *B. campestris* compared to their respective unstressed controls, but it was unchanged in *B. juncea*. When compared to the osmotic stress alone, the addition of AsA in combination with the osmotic stress improved the AsA/DHA ratios by 142, 80, and 124 % in *B. napus*, *B. campestris,* and *B. juncea*, respectively (Fig. 3*C*).

The GSH content was enhanced by 19, 33, and 48 % in *B. napus, B. campestris,* and *B. juncea,* respectively, under the osmotic stress compared to the unstressed controls. The addition of exogenous AsA in combination with the osmotic stress further increased the GSH content in *B. napus* and *B. campestris* by 56 and 33 %, respectively, but no additional effect was observed in *B. juncea* (Fig. 3*D*). The GSSG content was increased by 67, 79 and 83 %, respectively, in PEG-treated *B. napus*, *B. campestris,* and *B. juncea*, respectively, compared to the unstressed controls. The addition of AsA in combination with PEG decreased the GSSG content in all three species with the greatest decrease in B. *juncea* (Fig. 3*E*). The GSH/GSSG ratio showed a decreasing trend under the osmotic stress, whereas this ratio increased by 122, 71, and 25 % in *B. napus*, *B. campestris*, and *B. juncea*, respectively, when AsA was combined with PEG (Fig. 3*F*).

The APX activities were higher in the PEG-treated *Brassica* seedlings than in the controls. The combination of AsA and PEG further increased the APX activities by 26 % in *B. napus* and 24 % in *B. campestris,* but had no effect on the *B. juncea* activity (Fig. 4*A*). The MDHAR activity was unchanged in response to the osmotic stress imposition except in *B. campestris,* where the activity was significantly reduced. The combination of AsA and PEG increased the MDHAR activity markedly (Fig. 4*B*). The DHAR activity decreased (20 % compared to the controls) in *B. campestris* in response to the osmotic stress but remained unchanged in the other two species. The DHAR activity was increased by 32 % in *B. napus* and 38 % in *B. campestris* in response to the combination of AsA and PEG. No changes were noted in this activity in *B. juncea* (Fig. 4*C*).

The GR activity increased by 74 % in *B. campestris* and 42 % in *B. juncea* but was unchanged in *B. napus* when osmotic-stressed. The addition of AsA increased

the GR activities by 45 % in *B. napus* and 42 % in *B. campestris* but no change was noted in *B. juncea* (Fig. 4*D*).

The osmotic stress decreased the CAT activity by 35 % in *B. napus* and 27 % in *B. campestris* but had no effect in *B. juncea*. The addition of AsA in combination with PEG increased the CAT activity in all three species compared to the PEG treatment alone (Fig. 5*A*).

The GST activity in *B. napus* increased by 51 % under the osmotic stress, compared to the unstressed controls, and by 35 % when AsA was combined with the osmotic stress compared to the osmotic stress alone. The GST activity in *B. campestris* increased by 36 % due to the PEG treatment but the AsA supplementation had no effect on this activity. No significant change was observed in the GST activity in *B. juncea* in response to either treatment (Fig. 5*B*).

The GPX activity was significantly higher in PEG-treated seedlings of all three species compared with the controls. The combination of AsA and PEG further increased the GPX activity by 26 % in *B. napus*, 42 % in *B. campestris,* and 35 % in *B. juncea* (Fig. 5*C*).

The osmotic stress increased the Gly I activity by 42 % in *B. campestris* and 23 % in *B. juncea* compared to the controls, but it had no effect in *B. napus.* The combination of AsA and PEG increased the Gly I activity by 27 % in *B. napus,* 25 % in *B. campestris,* and 31 % in *B. juncea* when compared to the osmotic stress alone (Fig. 6*A*). The Gly II activity dropped in response to the osmotic stress in all three *Brassica* species. However, the Gly II activity significantly increased when the osmotic stress was combined with the AsA addition compared to seedlings exposed to the osmotic stress alone (Fig. 6*B*).

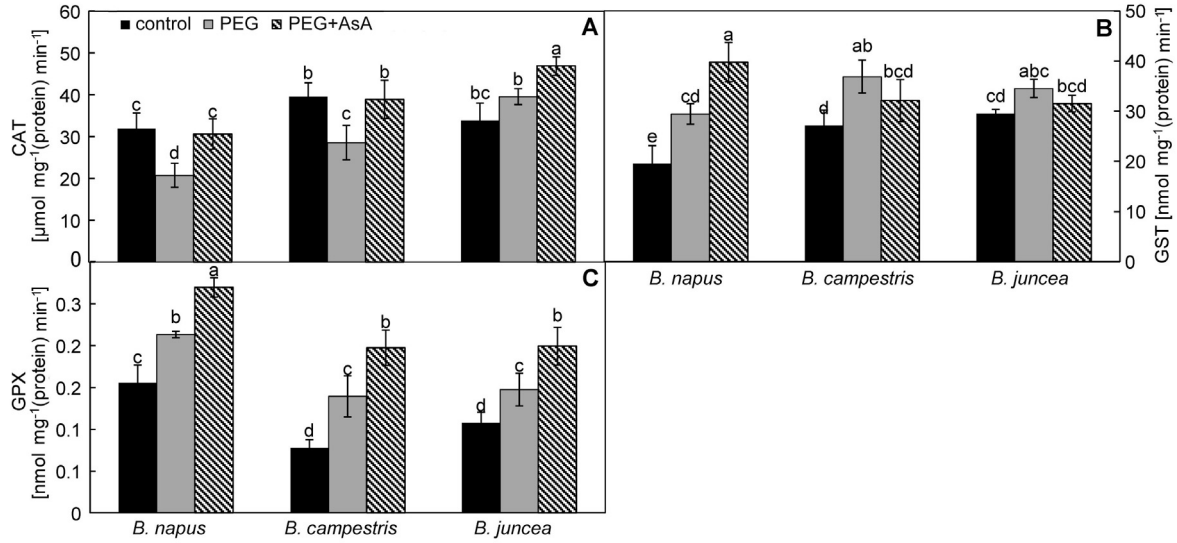

Fig. 5. The activities of CAT (*A*), GST (*B*), and GPX (*C*) in *Brassica* seedlings under 1 mM AsA and 15 % PEG treatments. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

Discussion

Drought or osmotic stress reduces cell expansion by limiting the entrance of water which subsequently affects cell division and growth (Pendall *et al*. 2004, Dreesen *et al*. 2012). In the present study, the osmotic stress markedly reduced the fresh and dry masses of *Brassica* seedlings (Fig. 1*A,B*) in agreement with many earlier reports (*e.g*., Alves and Setter 2004, Kahmen *et al*. 2005). When the osmotic stress was combined with the exogenous AsA treatment, a significant improvement in the fresh and dry masses was noted over those seen in the PEG treatment alone; these results are also similar to previous findings. For example, exogenous AsA improved radical and plumule lengths and fresh and dry masses of okra seedlings (Baghizadeh and Hajmohammadrezaei 2011) and alleviated the growth reduction in rice seedlings in response to water stress (Guo *et al*. 2005). Multiple roles for AsA are evident in plant growth and developmental processes; for example, AsA improves cell division and cell wall expansion that are of vital importance for plant growth (Smirnoff 1996, Asada 1999, Pignocchi and Foyer 2003). Under limited water supply, plants cannot take up sufficient water for growth requirements. Moreover, the disruption of osmotic balance between the plant and its growth medium disrupts the normal water transport. A reduction in water uptake reduces a plant water content and indeed the tissue water content decreases linearly with the severity of drought (Costa *et al*. 1997, Liu *et al*. 2004, Hussain *et al*. 2009). The present study revealed similar reductions in the leaf water content in osmotic-stressed *Brassica* species. The leaf RWC was markedly reduced in all three *Brassica* species by the PEG treatment (Fig. 1*C*) which is

consistent with other reports (*e.g.*, De Souza *et al*. 1997). Alam *et al*. (2013) also observed reductions in leaf RWC by 10 and 20 % in response to lower and higher PEG concentrations, respectively. The combination of exogenous AsA and the osmotic stress restored the leaf RWC to levels closer to the control unstressed plants supporting the proposed role for AsA in osmotic regulation. In previous studies, AsA has been documented to modulate the content of osmoprotectants and hormones that are important in maintaining osmotic balance under reduced water availability (Baghizadeh *et al*. 2009, Dolatabadian and Jouneghani 2009, Darvishan *et al*. 2013, Halimeh *et al*. 2013). Restoration and maintenance of leaf RWC might be due to balancing Pro content (Fig. 1*D*) or various other regulations that were not examined in the present study.

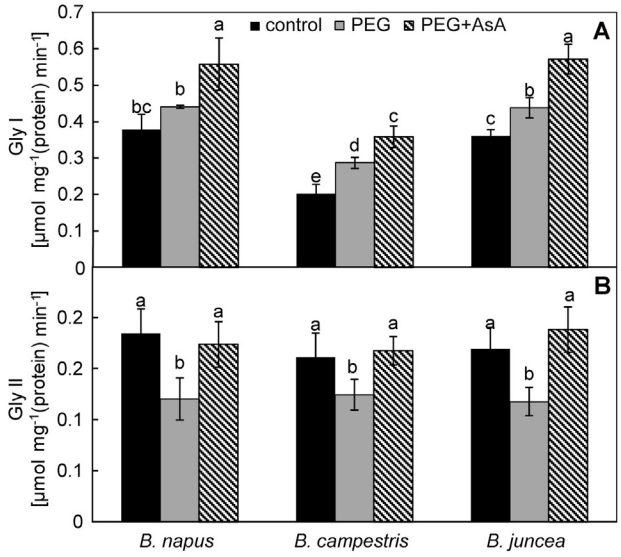

Fig. 6. The activities of Gly I (*A*) and Gly II (*B*) in *Brassica* seedlings under 1 mM AsA and 15 % PEG treatments. Means ± SE from three replicates for each treatment. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

A protective effect of Pro accumulation under different stress conditions has been documented in higher plants, where this amino acid stabilizes proteins, scavenges hydroxyl radicals, regulates cytosolic pH, and maintains pressure potential (Fabro *et al*. 2004, Ashraf and Foolad 2007, Yang *et al*. 2009, 2013). The PEG-induced increase in the Pro content in *Brassica* species (Fig. 1*D*) probably contributed to osmotic adjustment. Similar increases in Pro content are documented in mustard (Alam *et al*. 2013), in *Dracocephalum moldavica* (Halimeh *et al*. 2013) and in wheat (Sakhabutdinova *et al*. 2003). A reduction in Pro content in the three *Brassica* species studied here in response to the exogenous AsA application during the osmotic stress suggests that AsA alleviated the stress and prevented the biosynthesis of extra Pro (Dolatabadian *et al*. 2009). Similar reductions in Pro content after AsA application have been reported in *Zea mays* (Dolatabadian *et al*. 2009), *Dracocephalum moldavica* (Halimeh *et al*. 2013), maize (Darvishan *et al*. 2013), and okra (Baghizadeh *et al*. 2009).

Chlorophylls are key components in the photosynthetic apparatus (Taiz and Zieger 2006). Osmotic stress leads to an increased ROS generation in chloroplasts, which can destroy Chl. Our research indicated a significant decrease in the Chl *a* and Chl *b* content under the osmotic stress (Fig. 2*A-C*). Similar results were observed in mustard (Alam *et al*. 2013), sunflower (Manivannan *et al*. 2007), rice (Pattanagul 2011), okra (Amin *et al*. 2009), and wheat (Moaveni 2011). The application of AsA in combination with PEG improved the Chl content (Fig. 2*A-C*) in agreement with previous studies. For example, leaf Chl content in *Zea mays* is reduced by osmotic stress and then it is restored by exogenous AsA (Dolatabadian *et al*. 2009). AsA enhances Chl content in *Pimpinella anisum* (Asadi *et al*. 2010) and in *Phaseolus vulgaris* (Dolatabadian and Jouneghani 2009).

Under osmotic stress, photorespiration may account for about 70 % of total H_2O_2 production (Noctor *et al*. 2002). Lipid peroxidation caused by ROS is also very common under osmotic stress. The H_2O_2 content increased under the osmotic stress in all *Brassica* species and an increased H_2O_2 content was associated with a higher content of MDA, a product of lipid peroxidation. The highest MDA content was in *B. campestris* and the lowest in *B. juncea*. The AsA application caused reductions in the H_2O_2 and MDA content (Fig. 2*D,E*) in comparison with the osmotic stress alone. AsA also reduces MDA content in water-stressed *Zea mays* plants probably due to inactivation and scavenging free radicals (Dolatabadian *et al*. 2009). Lipoxygenase is an oxidative enzyme responsible for oxidation of polyunsaturated fatty acids (Kabiri *et al*. 2012, Sánchez-Rodríguez *et al*. 2012). Higher LOX activity is deduced as reason for increased lipid peroxidation under environmental stresses (Aziz and Larher 1998, Molassiotis *et al*. 2006). In our study, we found a higher LOX activity in the osmotic-stressed seedlings (Fig. 2*F*) in comparison with the control ones. This higher activity might be correlated with a higher MDA content which is consistent with other studies (Kabiri *et al*. 2012, Sánchez-Rodríguez *et al*. 2012). However, the combination of the AsA application and osmotic stress reduced the LOX activity significantly. Reduction in LOX activity together with reduced H_2O_2 and $O_2^{\bullet-}$ content and MDA content implies an improvement in dehydration or drought tolerance (Kabiri *et al*. 2012, Sánchez-Rodríguez *et al*. 2012).

Plants with their antioxidant systems readily scavenge ROS, thereby protect cells from oxidative stress. However, under stressful conditions, the ROS production may overwhelm the capacity of antioxidant system, thereby resulting in oxidative stress symptoms. An efficient antioxidant system means high activities of antioxidant enzymes and a high content of non-enzymatic components. Antioxidants AsA and GSH together with enzymes APX, MDHAR, DHAR, and GR constitute the AsA-GSH or Halliwell-Asada cycle (Gill and Tuteja 2010, Hasanuzzaman *et al*. 2012a). AsA is an antioxidant and

cellular reductant that has roles in plant growth and development and in the response to environmental stresses (Khan *et al*. 2011). AsA functions coordinately with glutathione and several enzymatic antioxidants to detoxify ROS (Noctor and Foyer 1998, Dolatabadian and Jouneghani 2009). Under the osmotic stress, the *Brassica* seedlings showed a decreased AsA content and an increased DHA content resulting in a decreased AsA/DHA ratio (Fig. 3*A,B,C*). A reduced AsA content under water deficit was previously recorded (Sharma and Dubey 2005, Hasanuzzaman and Fujita 2011). Dolatabadian *et al*. (2009) reported that AsA content is reduced and DHA content is increased in *Zea mays* under water deficit. The application of AsA under the osmotic stress enhanced the endogenous AsA content (except in *B. juncea*) and the AsA/DHA ratios in all three species (Fig. 3*A,C*). Al-Ghamdi (2009) also recorded a higher AsA/DHA ratio in response to AsA application which is correlated with higher oxidative stress tolerance under limited water supply.

AsA is regenerated and recycled by MDHAR and DHAR (Gill and Tuteja 2010, Hasanuzzaman *et al*. 2012a). Thus, the improved AsA content, even after the PEG treatment, were thought to be due to higher MDAR and DHAR activities. Compared to the osmotic stress alone, both the activities of MDAR and DHAR were improved by the combination of PEG with exogenous AsA (Fig. 4*B,C*). Previous reports also expressed a similar relationship between MDAR and DHAR activities and AsA regeneration (Hasanuzzaman and Fujita 2011, Alam *et al*. 2013). The other enzyme of the AsA-GSH cycle, APX, catalyzes the AsA-dependent reduction of H_2O_2 to H_2O. Its activity in the *Brassica* seedlings increased after the osmotic stress imposition and further after the combined AsA and PEG treatment except in *B. juncea* (Fig. 4*A*). The combination of exogenous AsA with osmotic stress improved APX activity in *Triticum aestivum* (Hameed *et al*. 2013) and *Glycine max* (Bashor and Dalton 1999).

Glutathione acting as substrate for GPX is considered to be the most important component of the AsA-GSH cycle for maintaining intracellular defenses against ROS induced oxidative damage (Mullineaux and Rausch 2005, Gill and Tuteja 2010) and several research findings have shown that GSH content increases under osmotic stress (Sharma and Dubey 2005, Kadioglu *et al*. 2011, Hasanuzzaman and Fujita 2011). In our study, the osmotic stress induced an elevation of GSH content and this was further increased in *B. napus* and *B. campestris* by the addition of AsA (Fig. 3*D*). GPX with the help of GSH reduces content of H_2O_2 and lipid hydroperoxides (Noctor *et al*. 2002). An enhanced GPX activity induced by exogenous AsA might improve the antioxidant system of *Brassica* species (Fig. 5*C*). Another important enzyme, GST, not only catalyzes the detoxification of lipid peroxides but also detoxifies xenobiotics (Navari-Izzo *et al.* 1997, Gill and Tuteja 2010). In this process, GSH plays a vital role (Noctor *et al*. 2002). The activity of GST increased by the osmotic stress in all the three *Brassica* species, but its activity remained unchanged after the AsA addition except in *B. napus* (Fig. 5*B*). Similar improvement in GST and GPX activities correlate with the improvement of osmotic stress tolerance (Hasanuzzaman and Fujita 2011, Alam *et al*. 2013). Exogenous AsA modulates GPX activity in maize and together with the modulation of other antioxidants reduces oxidative stress induced by drought (Darvishan *et al*. 2013).

The GR is involved in regeneration and recycling GSH (Fig. 4*D*). The higher GR activity was correlated with the higher GSH content in the seedlings treated with PEG alone or with AsA and PEG. In the absence of enhanced regeneration, an increased GSH content may be explained by the increased GSH biosynthesis (Mittova *et al*. 2003). In our study, the osmotic stress resulted in an increase in GSSG content (Fig. 3*E*), however, the AsA application reduced the PEG-induced increase in GSSG (Fig. 3*E*). The ratio of GSH/GSSG expresses the redox status of the cell and is considered more important than their individual content. A higher GSH/GSSG is desirable for better stress tolerance (Sharma and Dubey 2005, Gill and Tuteja 2010, Hasanuzzaman *et al*. 2012 b). An enhanced GSH/GSSG ratio in response to combined AsA and osmotic stress treatments is an indication of improved drought tolerance (Hasanuzzaman and Fujita 2011, Alam *et al*. 2013).

CAT converts H_2O_2 into H_2O. In the *Brassica* seedlings, the CAT activity was reduced under the osmotic stress in *B. campestris* and *B. napus*, but increased in *B. juncea*. The CAT activity increased in all the three species when the osmotic stress was combined with exogenous AsA (Fig. 5*A*). An elevated CAT activity was also recorded in wheat (Hameed *et al*. 2013), maize (Dolatabadian *et al*. 2009) and *Carthamus tinctorius* (Razaji *et al*. 2012) in response to exogenous AsA under water deficit. An improved CAT activity can improve osmotic stress tolerance in *Dracocephalum moldavica* (Halimeh *et al*. 2013) and rapeseed (Hasanuzzaman and Fujita 2011).

Efficient activities of Gly I and Gly II detoxify methylglyoxal (MG), thereby reducing its potential adverse effects (Hossain *et al*. 2010, Hasanuzzaman and Fujita 2011). These enzymes have an impact on GSH pool, as GSH is the cofactor in the step by step reaction of MG detoxification and is regenerated at the end of the reaction sequence. The maintained content of GSH with improved activities of Gly I and Gly II are indications for an efficient glyoxalase system that is vital under stress conditions (Hoque *et al*. 2007, Hossain *et al*. 2010). Activities of Gly I and Gly II help to detoxify MG, hydrolyze thioesters, and hydrolyze *S*-D-lactoyl glutathione to D-lactate and GSH (Vander 1993). The activity of Gly I increased after the osmotic stress in *Brassica* species (Fig. 6*A*). An increase in Gly I activity has been observed under different stress conditions including drought (Hasanuzzaman and Fujita 2011). This may be due to an increase in the plant own MG detoxification capacity under stress conditions. In the current study, this capacity was apparently higher after the

exogenous AsA application as evidenced by a higher Gly I activity
(Fig. 6*A*). Thus, the AsA application supposedly increased the MG detoxification capacity by increasing Gly I activity (Vander 1993). In this study, the Gly II activity decreased by the osmotic stress; similar findings were reported previously (Hasanuzzaman and Fujita 2011, Alam *et al*. 2013). Similarly to Gly I, the Gly II activity was also improved significantly in response to the combination of AsA and PEG (Fig. 6*B*). Improved Gly I and Gly II activities and a higher GSH content thus increased tolerance to the osmotic stress. The relationship between glyoxalase system components and drought tolerance has been previously confirmed (Hasanuzzaman and Fujita 2011, Alam *et al*. 2013).

In conclusion, the present study provides evidence for a protective role of exogenous AsA against the adverse osmotic stress in three *Brassica* species.

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