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Antioxidant response of the invasive alien species *Parthenium hysterophorus* L. under abiotic stress conditions with special emphasis on boiling-stable antioxidant enzymes

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ABSTRACT: Invasive alien plants have broad distribution throughout the world, displacing the indigenous vegetation, stunting the growth or development of native plants and diminishing their establishment in agricultural and natural areas. *Parthenium hysterophorus* is a noxious potential invader of a region whose harmful status can be attributed to its allelopathic effects and prolific seed production. However, there has been little research on the physiological and biochemical mechanisms governing its abiotic stress tolerance. Plants growing under natural conditions were sampled at random in the vicinity of Jalandhar. Samples were subjected to analysis for abiotic stress-induced changes in reactive oxygen species and free radical-scavenging boiling-stable antioxidant enzymes. Indices of oxidative stress such as malondialdehyde (MDA) and the membrane injury index (MII) were also studied. In order to discover a possible reason for the biological invasiveness of *P. hysterophorus*, in the present investigation we studied enzymatic and non-enzymatic biochemical changes that might explain it. Malondialdehyde and MII, indices of stress, increased with a rise in hydrogen peroxide and superoxide anion content in an organ- and abiotic condition-dependent manner, affirming oxidative stress to the plant. An elevated level of the antioxidant metabolite GSH was observed in June, which played a positive role in minimising the oxidative stress. Antioxidative enzymes such as BsSOD, BsPOD, BsGST, BsMDAR, BsPDI, BsTRx exhibited activities that increased in an organ- and abiotic condition-dependent manner. On the basis of the obtained results, we conclude that *P. hysterophorus* has the potential to cope with abiotic stress by accumulating abiotic stress-related metabolites and proteins.

Keywords: *Parthenium hysterophorus,* oxidative stress, ascorbate, glutathione, antioxidant enzymes, boiling-stable proteins

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INTRODUCTION

Invasive alien species (IAS) are non-native plants which have been introduced into ecosystems by accidental escape from their natural habitat and have broad distribution throughout the world in agricultural and natural areas, having an impact on the native species by diminishing their growth and establishment (Jung 2003; Henkel *et al*. 2009). Abiotic stress conditions such as temperature extremes, heat, light, drought, salt, heavy metals, ultraviolet radiation and nutrient deprivation lead to imbalance of cellular homeostasis and contribute to oxidative stress for a plant individually or in combination (Mittler 2002). Adverse abiotic conditions ultimately lead to production of reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide),

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the superoxide anion (O_2^-) and hydroxyl radicals ($OH^\cdot$), exerting a detrimental effect on biological systems by causing oxidation of lipids, proteins, carbohydrates and deoxyribonucleic acid (Kavas *et al*. 2013). It was previously noticed that the severity and duration of stress and growth conditions are responsible for the amount of ROS produced (Miller *et al*. 2010). The efficient removal of ROS is made possible by non-enzymatic [glutathione (GSH), ascorbate (AsA), phenolics, carotenoids] and enzymatic [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), monodehydroascorbate reductase (MDAR), thioredoxin reductase (TRX.R), glutathione reductase (GR)] components of antioxidative systems (Gill & Tuteja 2010). The protective role of these antioxidant systems in making plants tolerant to extreme environments has already been established (Yan *et al*. 2003; Gill & Tuteja 2010). Hydrophilins, the majority of which belong to the HSP and LEA families, are highly hydrophilic and remain soluble during boiling in aqueous solution. For this reason, they have been termed "boiling-stable proteins" (BSPs) (Jacobsen & Shaw 1989). Hydrophilins are able to protect enzymes from being inactivated under water (Garay-Arroyo *et al*. 2000; Posas *et al*. 2000; Yale & Bohnert 2001) and temperature (Henkel & Hofmann 2008; Gong *et al*. 2010; Zerebecki *et al*. 2011) stress.

Parthenium hysterophorus L. (Asteraceae) is a plant species that is native to tropical America, but is widespread as an invasive, alien plant in North America, Africa, Australia and India (Revero *et al*. 2001). This weed grows in overgrazed pastures, forests, wastelands and agricultural areas, as well as along roadsides. It has a high reproductive potential, tolerates a variety of soil types and possesses great adaptability in hot and dry environments. *Parthenium hysterophorus* has an allopathic effect on other plants, causes biodiversity depletion and lowers crop productivity (Davis & Swanson 2001). Little is known about the physiological and biochemical mechanisms that enable *P. hysterophorus* to tolerate stressful environmental conditions. Knowledge of the tolerance mechanisms of such invasive plants may shed light on their ability to grow and spread in different areas and under various abiotic conditions, which can be important for controlling and predicting the distribution of *Parthenium* species. We hypothesise that adverse abiotic stress conditions can lead to the overproduction of reactive oxygen species and oxidative stress in leaves and flowers of *P. hysterophorus*. At the same time, this species can experience activation of antioxidant protection through increased biosynthesis of non-enzymatic and enzymatic components. The objectives of the present study were as follows: a) determination of reactive oxygen species, lipid peroxidation and the membrane injury index; b) assessment of ascorbate and glutathione content; and c) determination of antioxidant enzymes (GST, MDAR, TRX.R and PDI) and boiling-stable proteins (BSPs).

MATERIAL AND METHODS

Sampling site. Samples of *P. hysterophorus* were collected in the vicinity of Jalandhar, which is located at 71° 31' east latitude and 30° 33' north longitude. The Jalandhar area has three seasons a year, viz., summer (March-June), the rainy season (July-September) and winter (October-February). The climate of the area is characterised by extremes of cold and heat. The mean maximal and minimal temperatures and daylength are shown in Table 1. Experiments were conducted from June of 2014 to March of 2015. Sample selection was random, leaves and flowers being collected and pooled together for further analysis. Throughout the year the sampling sites were regularly visited with a gap between visits of no more than 10 days.

Extraction of boiling-stable proteins (BSPs). Boiling-stable proteins were extracted according to Sharma *et al*. (2006). Leaves and flowers were homogenised with a chilled mortar and pestle in extraction buffer [50 mM Tris buffer (pH 7.0)], after which centrifugation was conducted at 10,000 x g for 10 min. The extract as obtained above was boiled for 15 min in order to get boiling-stable protein fractions. Boling-soluble proteins in the supernatant were precipitated with 5 volumes of ice-cold acetone, kept at -20°C overnight and centrifuged. The pellet was washed thrice with ice-cold 80% acetone. After centrifugation (10,000 x g at 4°C for 25 min), the obtained protein pellet was dissolved in minimum volumes of Tris buffer (50

Table 1. Temperatures and daylength in different months of the year in the Jalandhar area.

Month	Max. temperature	Min temperature	Daylength
December	4°C	0°C	10h 07m
March	29°C	10°C	11h 40m
June	42°C	24°C	14h 11m

mM, pH 7.0). With BSA as a standard, the protein content in the concentrated extracts was determined according to Lowry (1976). Samples of BSPs were resolved on NATIVE-PAGE on 12% (w/v) polyacryalamide gel and visualised with Coomassie brilliant blue according to Sambrook *et al.* (1989).

Lipid peroxidation. The level of lipid peroxidation was estimated from the content of malondialdehyde (MDA) in leaves and flowers according to Heath & Packer (1968). Fresh protein samples (120 µg) were mixed with 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) and heated at 95°C for 35 min, then quickly cooled on an ice bath and centrifuged at 10 000 x g for 15 min. Absorbance of the supernatant was measured spectrophotometrically at 532 nm and 600 nm. Content of malondialdehyde was calculated using the formula: MDA (µmol/g FW) = $[(A_{532} - A_{600})/156] \times 10^3 \times$ dilution factor.

Membrane injury index (MII). As a measure of ion leakage from flowers and leaves, the membrane injury index was determined according to Sairam *et al.* (1997). Samples of the same weight were taken in test tubes containing distilled water and kept in a water bath for 45 min at a constant temperature of 40°C. Electrical conductivity in samples at 40°C (C1) was recorded with a Model LT-26 Deluxe Conductivity Meter (Labtronics). The samples were then placed in a boiling water bath (100°C) for 15 min and their electrical conductivity was again recorded (C2). The membrane injury index was expressed in percentages (%) using the formula [1-(C1/C2)] x 100.

Determination of hydrogen peroxide (H_2O_2) content. Hydrogen peroxide was determined according to Chakrabarty *et al.* (2009). The reaction mixture was as follows: 0.5 ml of Tris-HCl buffer, pH 7.0, 0.5 ml of 0.1% trichloroacetic (TCA), 125 µg of protein and 2 ml of 1 M KI. After 1 h of reaction in the dark, absorbance was measured at 390 nm. The amount of hydrogen peroxide (µmoles/g FW) was calculated using a standard curve prepared with a known concentration of H_2O_2.

Determination of superoxide anion (O_2^-) content. Superoxide anion content was estimated according to Elstner & Heupel (1976). Consisting of protein equivalent to 120 µg, 0.9 ml of 50 mM Tris-HCl (pH 7.0) and 0.1 ml of 10 mM hydroxylamine hydrochloride, the assay mixture (2 ml) was incubated at 25°C for 30 min. Thereafter 1 ml of 17 mM sulphanilamide and 1.0 ml of 7.0 mM α-naphthylamine were added and the resulting mixture was incubated at 25°C for 2 h. Absorbance of the samples was recorded spectrophotometrically at 530 nm, and generated O_2^- was calculated from a standard curve of nitrite salt.

Determination of glutathione (GSH) content. The glutathione level was determined with Ellman's reagent according to Tukendorf & Rauser (1990). The following components were added to the reaction mixture: protein equivalent to 120 µg, 50 mM Tris–HCl buffer (pH 7.0) and 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid (DTNB) in a total volume of 3 ml. The reaction mixture was incubated at 37°C for 15 min and absorbance was measured at 412 nm. The amount of glutathione (µM/g FW) was calculated using a standard curve prepared with a known concentration of GSH.

Determination of ascorbate (AsA) content. Ascorbate content was determined according to Oser (1965). The reaction mixture contained 2 ml of 2% Na-molybdate, 2 ml of 0.15 N H_2SO_4, 1 ml of 1.5 mM Na_2HPO_4 and protein equivalent to 120 µg, bringing the total volume to 6 ml with buffer. It was mixed and incubated at 60°C in a water bath for 45 min. Then it was cooled and centrifuged at 3000 x g for 15 min, after which absorbance was measured at 660 nm. The amount of ascorbate (µM/g FW) was calculated using a standard curve prepared with a known concentration of ascorbate.

Determination of glutathione-S-transferase (GST) activity. Glutathione-S-transferase activity was assayed based on the GST-catalysed reaction between glutathione and 1-chloro-2,4- dinitrobenzene according to Habig & Jacoby (1981). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.0), 0.1 mM EDTA, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 mM reduced glutathione and protein equivalent to 120 µg in a volume of 3 mL. The enzyme activity was measured at 340 nm. It was calculated using the conjugate's extinction coefficient of 9.6 mM^{-1} cm^{-1} and expressed as units min^{-1} mg^{-1} of protein.

Determination of monodehydroascorbate reductase (MDAR) activity. The activity of MDAR was determined from the decrease in absorbance at 340 nm in a spectrophotometer (Leterrier *et al.* 2005). The assay mixture (1 mL) contained 0.2 mM NADH, 50 mM Tris-HCl (pH 7.0), 1 mM sodium ascorbate and an amount of the sample equivalent to 120 µg of protein. The reaction was initiated by adding 0.4 units of ascorbate oxidase (EC 1.10.33 from *Cucurbita*, Merck, Germany). Specific enzyme activity was expressed as mmol of NADH oxidised min^{-1} mg of $protein^{-1}$ (extinction coefficient = 6.2 mM^{-1} cm^{-1}).

Determination of thioredoxin reductase (TRx.R) activity. Thioredoxin reductase activity was analysed according to Luthman & Holmgren (1982). The assay mixture contained 50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.2 mM NADPH, 0.2 mg/mL bovine serum albumin and 5 mM 5,5′(dithiobis-(2-nitrobenzoic) acid with an amount of enzyme equivalent to 120 µg of protein

at 25°C in a total volume of 1 mL. Increase in absorbance (measured at 412 nm) was due to the reduction of DTNB with NADPH, producing a yellow-coloured product (5-thio-2-nitrobenzoic acid; TNB). Specific activity was expressed as nmol of NADPH oxidised min $^{-1}$ mg of protein $^{-1}$ or nmol of TNB produced min^{-1} mg of protein^{-1} (extinction coefficient = 13.6 mM^{-1} cm^{-1}).

Determination of protein disulphide isomerase (PDI) activity. Protein disulphide isomerase activity was assayed according to Holmgren (1979). The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.0), 5 mM DTT, 2 mM EDTA, 0.14 mM insulin and an amount of the sample equal to 120 µg of protein. The reaction shows an increase in absorbance due to aggregation of insulin, which is a result of the reduction of insulin disulphides and can be measured at 650 nm. It was monitored continuously at 30°C for 1 h. Reading of the control was subtracted. The activity was calculated as specific activity (units/mg protein/min) = (A_{650}/min)/(0.01×mg of protein in the reaction mixture).

Zymogram analysis. Extracted boiling-stable proteins were subjected to zymogram analysis according to Sharma *et al.* (2013). Proteins were separated by non-denaturing 12% polyacrylamide gel electrophoresis.

To detect superoxide dismutase activity, the gel was first soaked in 25 ml of 1.23 mM NBT for 15 min, briefly washed with water, then soaked in the dark in 30 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 2.8 x 10^{-2} mM riboflavin for another 15 min. The reaction was stopped by briefly washing the gel with water and illuminating it under white light to initiate the photochemical reaction. All the procedures were carried out at room temperature. The regions of SOD activity appeared as colourless bands on a purple background. The activity-stained gels were analysed by densitometry using Quantity One software (Bio-Rad, USA). In-gel SOD activity was expressed in terms of total pixel density of activity-stained gel lanes per mg of total soluble protein according to Solti *et al.* (2014).

For peroxidase activity analysis, when electrophoresis was complete, the gel was washed three times in 50 mM sodium acetate buffer (pH 5.0). Peroxidase activity was visualised by incubating the gel in 50 ml of a solution containing 50 mM sodium acetate buffer (pH 5.0), 3.3 ml of guaiacol and 1.5 ml of 6.6 % H_2O_2. The gel was incubated at room temperature in the dark until reddish-brown bands appeared, after which it was washed in distilled water and used for further analysis.

Monodehydroascorbate reductase activity was determined according to Kaplan & Beutler (1967). After electrophoresis, the gel was immersed in a solution of 1.3 mM NADH, 1.2 mM 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide and 0.06 mM 2,6-dichlorophenol-indophenol in 0.25 M potassium phosphate buffer (pH 8.0) for 10 min in the dark. The bands appeared dark on a blue background.

Glutathione-S-transferase activity was determined according to Ricci *et al.* (1984). After electrophoresis, the gel was equilibrated in 0.1 M potassium phosphate buffer (pH 6.5) for 10 min and transferred to a reaction mixture containing 4.5 mM GSH, 1 mM CDNB and 1 mM NBT in 0.1 M potassium phosphate buffer (pH 6.5) at 37°C for 10 min. The gel was then incubated at room temperature in 0.1 M Tris/HCl, pH 9.6, containing 3 mM phenazine methosulphate (PMS). The activity band appeared as an achromatic zone against a dark background.

SOD isoenzyme analysis. Isoenzymes of SOD were identified and characterised using inhibitory assays based on selective inhibition by KCN or H_2O_2 according to Biemelt *et al.* (2000) and Lee & Lee (2000). The gel was incubated for 25 min in 50 mM phosphate buffer (pH 7.8) containing either 3 mM KCN or 5 mM H_2O_2 before staining for activity. The Fe-SODs present were inhibited by H_2O_2, but resistant to KCN; Cu/ Zn-SODs were inhibited by KCN and H_2O_2; and Mn-SODs were resistant to both inhibitors.

Statistical analysis. Data obtained were analysed by one-way analysis of variance, followed by Tukey's test. Results with P values < 0.05 were considered significantly different. All values reported are means ± SE of three replicates.

RESULTS AND DISCUSSION

Changes in malondialdehyde (MDA), the membrane injury index (MII) and reactive oxygen species (ROS). Abiotic stresses can lead to overproduction of reactive oxygen species, which results in lipid peroxidation and thereby causes membrane injury (Tian *et al.* 2012). In the present study, great amounts of MDA were noted in leaves and flowers of *P. hysterophorus* in December and June (Fig. 1A), indicating the maximum oxidative stress. Similarly, Lu *et al.* (2008) found increased lipid peroxidation in *Eupatorium* sp. The membrane injury index is a measure of electrolyte leakage from cells as a consequence of an oxidative burst that leads to membrane peroxidation. The results obtained in our study showed that the membrane injury index in leaves and flowers of *P. hysterophorus* increased in June and December, which can be linked with lipid peroxidation (Fig. 1B). The present findings are in line with those of other authors who recorded that membrane stability decreased with increase of lipid peroxidation under stress conditions (Bajji *et al.* 2002; Sharifi *et al.* 2012). Production of hydrogen peroxide and the superoxide anion in leaves and flowers of *P. hysterophorus* was higher in June than in March (Fig. 1C, D). However, in December production of hydrogen peroxide was lower than that of the superoxide anion

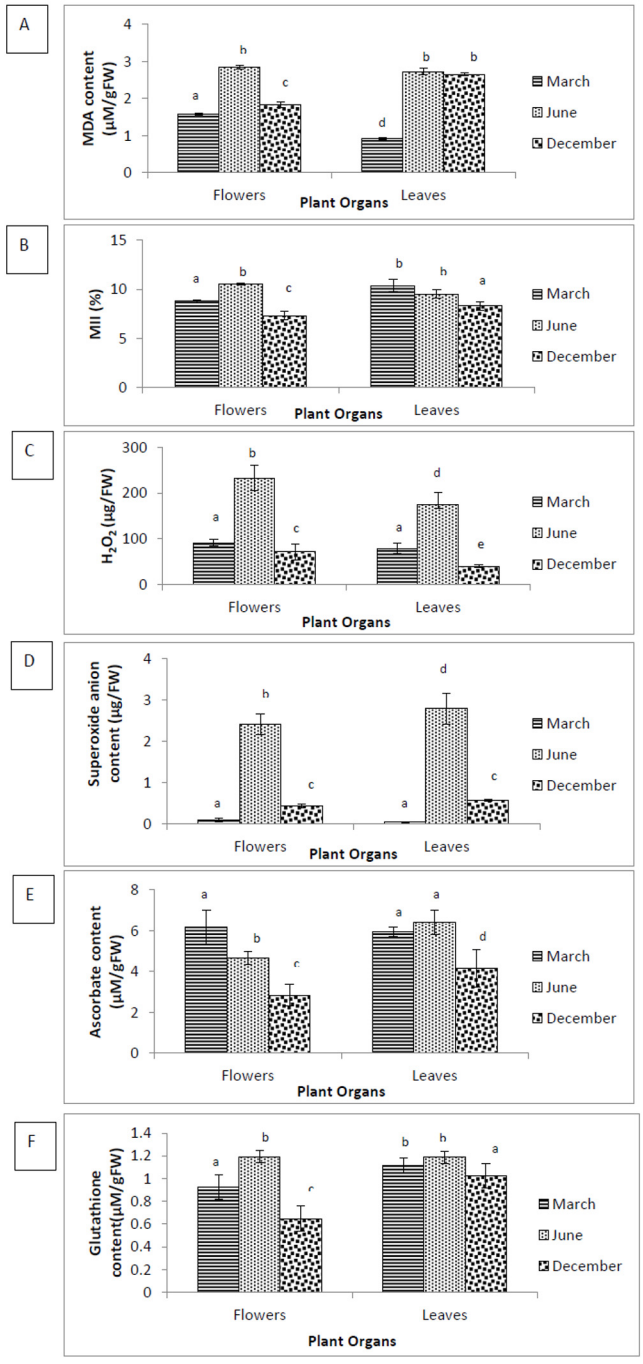

Figure 1. Changes of MDA content (A), MII (B), H_2O_2 content (C), superoxide anion content (D), ascorbate content (E) and glutathione content (F) in leaves and flowers of *P. hysterophorus* under conditions of different temperature regimes. Values are the means of three replicates ± SE. Means with different letters are significantly different at $P < 0.05$ using Tukey's multiple range test.

(Fig. 1C, D). Great production hydrogen peroxide and the superoxide anion has been reported in *Leucanthemum maximum* L. and *Cucumis melo* L. under conditions of drought and temperature extremes (high and low) (Zhou *et al.* 2005; Kavas *et al.* 2013). Taken together, the enhanced reactive oxygen species production in leaves and flowers of *P. hysterophorus* under adverse abiotic conditions may pose a threat to cells. According to Li & Yi (2012), reactive oxygen species can serve as signal molecules for activating antioxidant stress pathways (Shigeoka *et al.* 2002).

Changes in ascorbate and glutathione content. Ascorbate has been considered an important non-enzymatic antioxidant due to its ability to directly scavenge ROS, while preserving activities of enzymes and actively taking part as an electron donor in a number of enzymatic and non-enzymatic reactions (Gill & Tuteja 2010). Flowers of *P. hysterophorus* showed a high amount of ascorbate in March. However, in leaves ascorbate content was higher at high temperatures in June than in March and December (Fig. 1E).

A non-enzymatic component of antioxidative systems, glutathione is important for maintaining the redox balance of cells (Blokhina *et al.* 2003). It also plays a key role in the antioxidative defence system by regenerating ascorbate (Rausch & Wachter 2005). Glutathione concentrations in leaves and flowers were high in June (Fig. 1F). As in our study, increased glutathione content enhancing the plant's ability to overcome oxidative stress was also reported under severe conditions of stress by other investigators (Metwally *et al.* 2005; Gill & Tuteja 2010).

Changes in activities of boiling-stable antioxidant enzymes. Glutathione-S-transferases (GSTs) have the potential to reduce peroxides together with glutathione and remove compounds that are potentially cytotoxic (Gill & Tuteja 2010). In the present study, BsGST activity in leaves of *P. hysterophorus* was the highest in June, whereas in flowers BsGST activity then showed minimal values (Fig. 2A), suggesting organ- and abiotic stress-dependent regulation. No visible activity of BsGST was found in leaves and flowers in December. Other authors have reported that plant tolerance to abiotic stresses rose with increase of GST activity in *Lycopersicon esculentum* and *Phragmites australis* (Iannelli *et al.* 2002; Gapinska *et al.* 2008).

Monodehydroascorbate reductase (MDAR) is an essential enzyme of the ASC-GSH cycle that maintains the constant level of ascorbate required for a stabilised redox state in the plant (Huang *et al.* 2005). Under low temperature conditions (in December), BsMDAR activity was found only in the leaves (Fig 2B). Flowers of *P. hysterophorus* showed an increased level of BsMDAR activity in March, with minimal activity in December (Fig. 2B). No significant BsMDAR activity was found in June in leaves and flowers (Fig. 2B).

Protein disulphide isomerases (PDIs) are thiol disulphide oxidoreductases mainly involved in folding of the polypeptides arising as chaperones (Gruber *et*

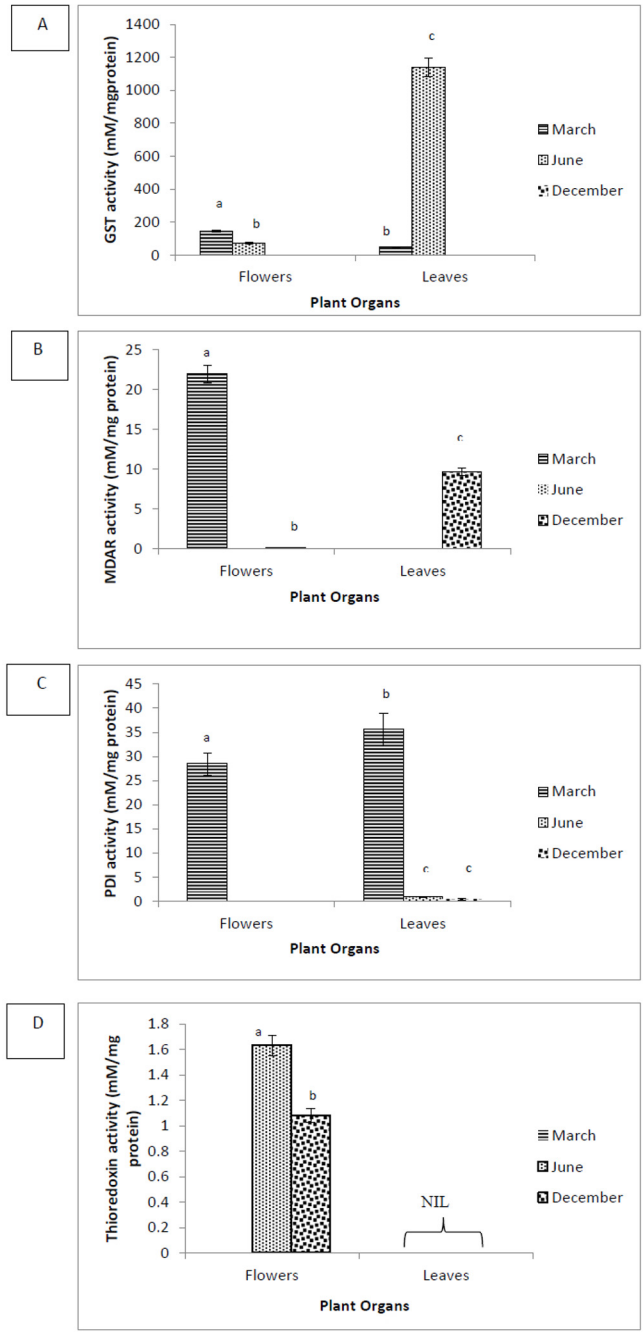

Figure 2. Changes in the activities of boiling-stable (Bs) antioxidant enzymes: glutathione-S-transferase (GST) (A); monodehydroascorbate reductase (MDAR) (B); protein disulfide isomerase (PDI) (C); and thioredoxin reductase (TRX.R) (D) in leaves and flowers of *P. hysterophorus* under conditions of different temperature regimes. Values are the means of three replicates ± SE. Means with different letters are significantly different at $P < 0.05$ using Tukey's multiple range test.

al. 2007). Activity of PDIs was found to be negligible in June and December, whereas a high level of activity was detected in March in leaves and flowers (Fig. 2C). Maintenance of cellular redox homeostasis is achieved through subtle changes in the levels of PDIs, but their stress-related functions still need to be investigated.

Thioredoxin reductases (TRX.Rs) are NADPH-dependent thiol-disulphide oxidoreductases that regulate numerous thiol- and non-thiol proteins via thiol/disulphide exchanges, thus playing a prominent role in stress protection (Reichheld *et al*. 2007). In the present study, flowers of *P. hysterophorus* showed high BsTRX.R activity in June and December (Fig. 2D). However, leaves showed no activity in any month. All these observations imply abiotic condition- and organ-specific regulation of BsTRX.R activity in order to detoxify ROS.

Analysis of isoenzymes of antioxidative enzymes. Superoxide dismutase (SOD) represents the first line of defence against toxic levels of ROS through removal of O_2^-, thereby decreasing the risk of $OH^·$ formation (Biemelt *et al*. 2000; Zhou *et al*. 2005; Gill & Tuteja 2010). In flowers and leaves of *P. hysterophorus*, three and four SOD isoforms, respectively, were detected in March. However, in June, only one SOD isoform was detected in leaves and flowers (Fig. 3A). Different content of SOD isoforms was observed in leaves and flowers of *P. hysterophorus*, indicating different regulatory antioxidant pathways over which free radicals are scavenged in an organ-dependent manner. Furthermore, our results showed that flowers had an increased level of BsSOD activity in December and June, which can be due to accumulation of SOD 1-2 isoenzymes. However, in leaves of *P. hysterophorus* BsSOD activity was the highest in March. Taken altogether, higher SOD activities were observed at low temperatures than at high temperatures.

As the most abundant SOD in higher plants, the Cu/Zn isoenzyme is localised in the cytosol and chloroplast; Mn-SOD is found in the mitochondria and glyoxysomes; and Fe-SOD is localised in the chloroplasts and peroxisomes (Gill & Tuteja 2010). Total SOD activity represents the combination of Cu/Zn-, Mn- and Fe-SOD activities (Asada & Kiso 1973). Table 2 presents the overall content of SOD isoenzyme(s) detected by selective inhibitor assays in all variants under different environmental conditions. In December bands of Fe- and Mn-SOD activity were detected in flowers and Cu/Zn- and Mn-SOD activity in leaves. However, higher temperatures in June led to the appearance of Cu/Zn-SOD activity in flowers and leaves. In March SOD isoenzymes were identified as Fe-SOD in flowers and Fe- and Mn-SOD in leaves. The high activity of SODs in *P. hysterophorus* could be related to high ROS generation under conditions of temperature extremes. The presented results suggest that cytosolic compartments are crucial in SOD enzyme protection of plants against ROS formation under adverse abiotic conditions.

Peroxidases are potential enzymes of the antioxidative system for reduction of H_2O_2 to water (Shigeoka *et al*. 2002). In the present study, two BsPOD isoforms were detected in flowers and one in leaves under conditions of high temperature in June (Fig. 3B), implying specific

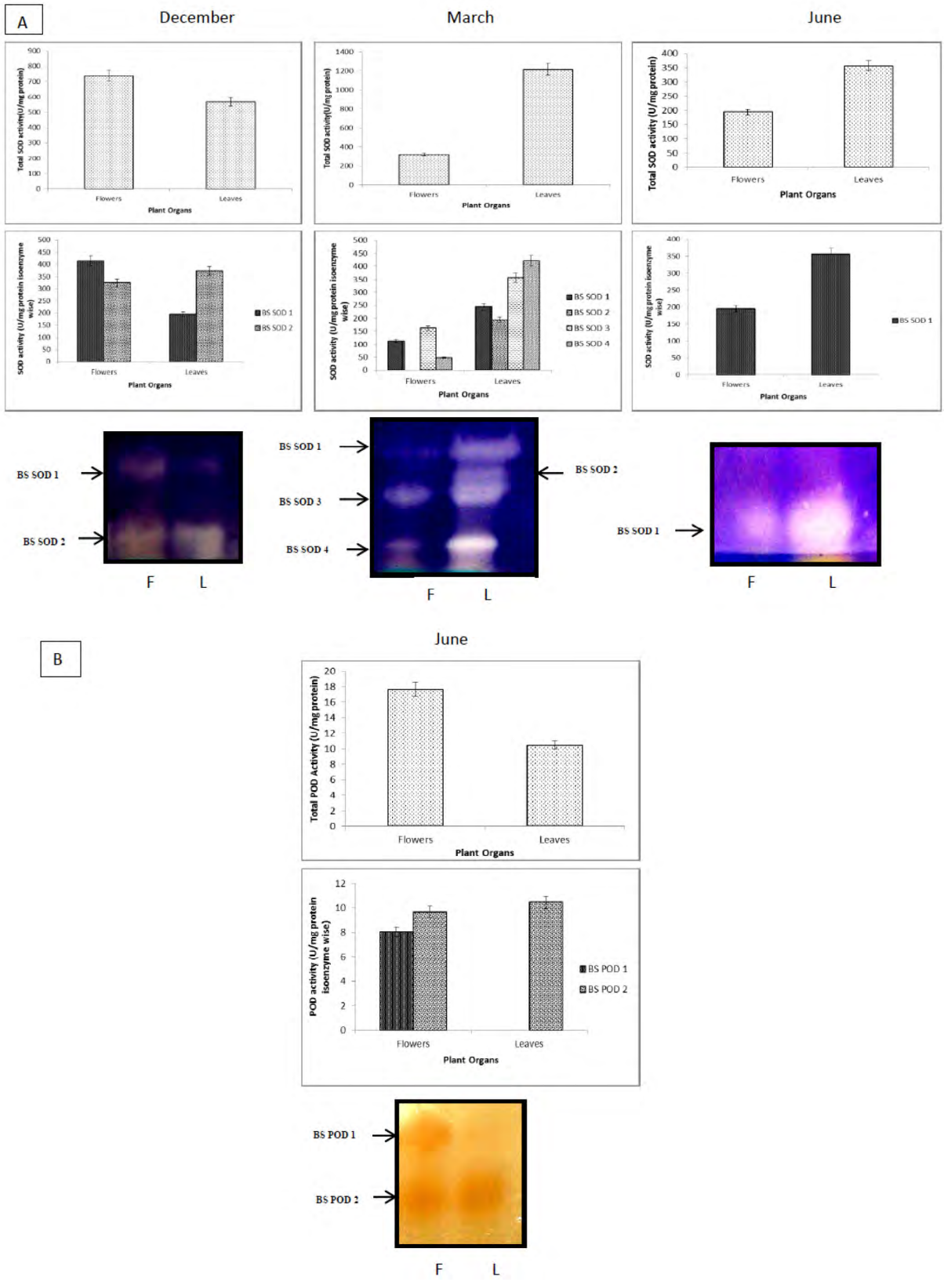

Figure 3. In-gel BsSOD activity (total and isoenzyme) of boiling-stable SOD (A); and in-gel BsPOD activity (total and isoenzyme) of boiling-stable POD (B). BSPs (120 µg) were separated by NATIVE- PAGE and stained for activity. Total SOD and POD activities were quantified with Quantity One software (Bio-Rad) using purified SOD and POD from bovine erythrocytes and horseradish peroxidase, respectively, as standards. F= Flowers, L=Leaves.

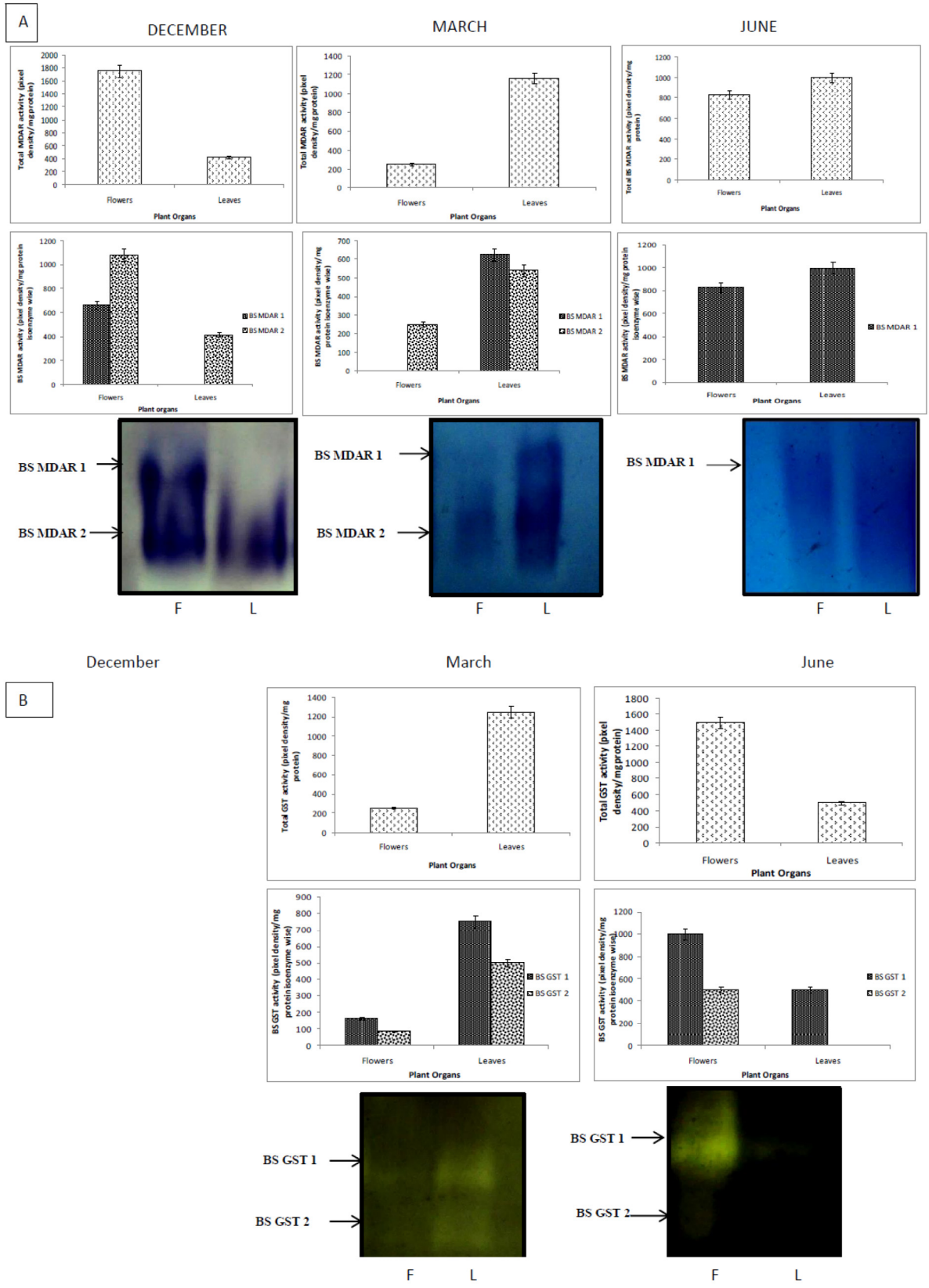

Figure 4. In-gel BsMDAR activity (total and isoenzyme) of boiling-stable MDAR (A); and in-gel BsGST activity (total and isoenzyme) of boiling-stable GST (B). BSPs (120 µg) were separated by NATIVE- PAGE and stained for activity. Total BsMDAR and BsGST in-gel activity was quantified with Quantity One software (Bio-Rad). In-gel activity was expressed in total pixel density of activity-stained gel lanes per mg of total soluble protein (Solti *et al.* 2014). F= Flowers, L=Leaves.

Table 2. Summary of isoenzyme profiles in leaves and flowers of *P. hysterophorus*. The numbers in brackets indicate the specific isoenzyme as observed in zymogram analysis of boiling-stable SOD in Fig. 3 (A).

Month	December	March	June
Tissue			
FLOWERS	Fe-SOD (1) Mn-SOD (2)	Fe-SOD (1,3,4)	Cu-SOD (1,2)
LEAVES	Cu-SOD (1) Mn-SOD (2)	Fe-SOD (2,4) Mn-SOD (1,3)	Cu-SOD (1,2)

regulation of hydrophilic antioxidant peroxidases. These observations suggest that ROS can be removed by boiling-stable peroxidase, decreasing the accumulation of H_2O_2 at high temperatures.

In flowers of *P. hysterophorus*, we were able to identify two isoforms of BsMDAR in December, as opposed to one isoform in March and June (Fig. 4A). However, in leaves enhanced activity (two isoforms of BsMDAR) was observed in March. Plants are able to biosynthesise different isoforms of antioxidant enzymes with altered kinetic properties in an organ- and stress-dependent manner (Edwards *et al*. 1994), which is in accordance with the results obtained in our study.

In the present study, BsGST activity in flowers was the highest in June, whereas in leaves a greater content of BsGST was observed in March (Fig. 4B). No activity could be found in December.

CONCLUSION

We can conclude from the data presented here that malondialdehyde and the membrane injury index are bio-indicators of oxidative stress related to the production of H_2O_2 and O_2^- at high temperature. The results of our study suggest that abiotic conditions can induce ROS production, which is accompanied by increased antioxidant enzyme activity. The content of antioxidant metabolites such as ascorbate and glutathione increased in June, which had a positive role in reducing oxidative stress. Increased activities of boiling-stable antioxidative enzymes such as BsGST, BsMDAR and BsTRX.R were observed in leaves and flowers, implying that different antioxidant regulatory pathways operated to provide protection against abiotic stress. The high BsSOD, BsPOD and BsMDAR activities in *P. hysterophorus* accompanied by isoenzyme accumulation confirm the existence of enhanced antioxidant enzyme activity in response to conditions of abiotic stress. Detailed analysis of the regulation of gene expression of hydrophilic enzymes should in the future elucidate the mechanisms governing oxidative stress tolerance in invasive plant species.

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Botanica SERBICA

REZIME

Antioksidativni odgovor invazivne vrste *Parthenium hysterophorus* L. u uslovima abiotičkog stresa sa posebnim osvrtom na antioksidativne enzime otporne na ključanje

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Invazivne vrste su široko rasrostranjene širom sveta, zamenjujući autohtonu vegetaciju, smanjujući im rast i razvoj u poljoprivrednim i prirodnim staništima. *Parthenium hysterophorus* je potencijalno štetna invazivna vrsta, čija se štetnost može pripisati njenim alelopatskim efektima i proizvodnji značajne količine semena. Međutim, do sada nije bilo mnogo istraživanja fizioloških i biohemijskih mehanizama koji regulišu toleranciju na abiotički stress. Nasumično su odabrane biljke koje rastu u prirodnim uslovima u blizini Jalandhara. Analiziran je uticaj abiotičkog stresa na produkciju ROS i aktivnost termostabilnih antioksidativnih enzima. Indeksi oksidativnog stresa kao što su malondialdehid (MDA) i indeks oštećenja membrane (MII) su takođe proučavani. Kako bi otkrili moguće razloge biološke invazivnosti *P. hysterophorus*, istraživali smo i enzimatske i neenzimatske biohemijske promene. Malondialdehid i MII, indeks stresa, rastu sa povećanjem vodonik-peroksida i superoksidnog anjonskog sadržaja, zavisno od organa i abiotičkih uslova, potvrđujući oksidativni stress u biljci. Povećan nivo antioksidativnog metabolita GSH je uočen u junu, što je dovelo do smanjenja oksidativnog stresa. Antioksidativni enzimi, kao što su BsSOD, BsPOD, BsGST, BsMDAR, BsPDI, BsTRx pokazuju povećanu aktivnost zavisno od organa i abiotičkih uslova. Na osnovu ovih rezultata, možemo zaključiti da *P. hysterophorus* ima potencijal da toleriše abiotički stress uzrokovan akumulacijom metabolita i proteina povezanih sa stresom.

Ključne reči: *Parthenium hysterophorus,* oksidativni stress, askorbat, glutation, antioksidativni enzimi, termostabilni antioksidativni enzimi