Differential Responses of the Activities of Antioxidant Enzymes to Thermal Stresses between Two Invasive *Eupatorium* Species in China

Ping Lu¹,², Wei-Guo Sang¹∗ and Ke-Ping Ma¹

¹Key Laboratory of Vegetation and Environment Change, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, China; ²Resources and Environmental Sciences College, Northeast Agricultural University, Harbin 150030, China

Abstract

The effect of thermal stress on the antioxidant system was investigated in two invasive plants, *Eupatorium adenophorum* Spreng. and *E. odoratum* L. The former is sensitive to high temperature, whereas the latter is sensitive to low temperature. Our aim was to explore the relationship between the response of antioxidant enzymes and temperature in the two invasive weeds with different distribution patterns in China. Plants were transferred from glasshouse to growth chambers at a constant 25 °C for 1 week to acclimatize to the environment. For the heat treatments, temperature was increased stepwise to 30, 35, 38 and finally to 42 °C. For the cold treatments, temperature was decreased stepwise to 20, 15, 10 and finally to 5 °C. Plants were kept in the growth chambers for 24 h at each temperature step. In *E. adenophorum*, the coordinated increase of the activities of antioxidant enzymes was effective in protecting the plant from the accumulation of active oxygen species (AOS) at low temperature, but the activities of catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), and monodehydroascorbate reductase (MDAR) were not accompanied by the increase of superoxide dismutase (SOD) during the heat treatments. As a result, the level of lipid peroxidation in *E. adenophorum* was higher under heat stress than under cold stress. In *E. odoratum*, however, the lesser degree of membrane damage, as indicated by low monodehydroascorbate content, and the coordinated increase of the oxygen. Detoxifying enzymes were observed in heat-treated plants, but the antioxidant enzymes were unable to operate in cold stress. This indicates that the plants have a higher capacity for scavenging oxygen radicals in heat stress than in cold stress. The different responses of antioxidant enzymes may be one of the possible mechanisms of the differences in temperature sensitivities of the two plant species.

Key words: antioxidant enzyme system; cold stress; *Eupatorium adenophorum*; *Eupatorium odoratum*; heat stress; invasive plants.


Available online at www.jipb.net
However, there has been little research on their physiological and biochemical mechanisms that confer different heat and/or cold resistance. A better understanding of these mechanisms is very important for controlling and predicting the potential distributions of the two species.

It has been proven that the response of antioxidant enzyme systems is one of the most important mechanisms of environmental acclimatization for plants. The role of these enzyme systems for making plants tolerant to extreme environments has already been demonstrated (Dhindsa and Matowe 1981; Schnier and Krause 1990; Hernández et al. 2000). It is currently assumed that the negative effect of various environmental stresses is at least partially due to the generation of active oxygen species (AOS) and/or the inhibition of the system that defends against them (Asada 1997; Alscher et al. 1997). AOS are highly reactive and, in the absence of any protective mechanism, can seriously disrupt normal metabolism through oxidating membrane lipids, protein and nucleic acids (Smirnoff 1993; Foyer et al. 1994a, 1994b). However, to mitigate and repair damage initiated by AOS, plants evolved cellular adaptive responses such as upregulation of oxidative stress protectors and accumulation of protective solutes (Horling et al. 2003). Antioxidant defense enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD), glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) are systems that minimize the concentrations of superoxide and hydrogen peroxide.

Therefore, in this paper, we compared the response of the antioxidant enzyme system to heat and cold stresses in E. adenophorum and E. odoratum. Specifically, we studied changes in lipid peroxidation and the antioxidant enzymes SOD, CAT, POD, APX, GR, MDAR and DHAR under heat and cold stress.

Results and Discussion

Superoxide dismutase catalyses the dismutation of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ and $\text{O}_2$ (Bowler et al. 1992). Both species exhibited an increase in SOD activity in both heat and cold stresses (Figure 2A, D). In leaves of E. adenophorum, the 38°C and 42°C treatments caused a 61% and 138% increase in total SOD activity in comparison to the control plants, respectively, and the highest total SOD activity was observed at 42°C. In leaves of E. odoratum, however, the highest total SOD activity was observed at 5°C. SOD has also been reported to increase on exposure to heat stress in mulberry (Chaitanya et al. 2002), and to cold stress in leafy spurge (Davis and Swanson 2001).

Catalase eliminates $\text{H}_2\text{O}_2$ by breaking it down directly to form water and oxygen. In leaves of E. adenophorum, CAT activity was depressed by high temperature, and increased to a high level in the low temperature treatment (Figure 2B). In leaves of E. odoratum, however, the response pattern of CAT activity was reversed, CAT activity was enhanced by high temperature and the plants were unable to maintain normal activity when subjected to low temperature, such as 10°C and 5°C (Figure 2E). An increase in SOD activity with a decrease in CAT activity has been reported when the plants were subjected to abiotic stress (Saruyma and Tanida 1995; Fu and Huang 2001; Jung 2003). Others have found that CAT activity
Antioxidant Enzymes Respond to Heat in *Eupatorium* Species

**E. adenophorum**

![Graph A](image)

**E. odoratum**

![Graph D](image)

**Figure 2.** Effect of temperature on superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD) activities of *Eupatorium adenophorum* (A–C) and *E. odoratum* (D–F).

Values are the means of three replicates with standard errors. Means with the same letters are significantly different at $P \leq 0.05$ using Duncan’s multiple range test.

is photoinactivated under low and high temperature stresses and CAT activity varies with plant species (Feierabend et al. 1992).

Guaiacol peroxidase catalyses $H_2O_2$-dependent oxidation of substrate. In leaves of *E. adenophorum*, POD activity increased remarkably with decreasing temperatures from 25 °C to 5 °C, enabling the plants to protect themselves against the oxidative stress, but in heat stress, POD activity was almost the same until the temperature was at 38 °C, and then declined (Figure 2C). In contrast, in leaves of *E. odoratum*, POD activity was significantly enhanced by the heat stress, but in cold stress, POD activity remained unchanged until the temperature was at 5 °C, and then decreased (Figure 2F). These results were consistent with those of Rivero et al. (2001), who reported differential responses of POD activity to thermal stress between tomato and watermelon plants. In watermelon plants, POD activity increased under high temperature, but decreased under low temperature. In tomato plants, POD activity decreased under high and low temperature, and the lowest POD activity was found under high temperature stress.

Ascorbate peroxidase uses ascorbate as the electron donor for the reduction of $H_2O_2$ and is well known to be important in the detoxification of $H_2O_2$. In leaves of *E. adenophorum*, the activity of APX increased with decreasing temperature from...
25°C to 5°C, but in heat stress, the activity of APX reached a peak value at 30°C and 35°C and then declined (Figure 3A). In leaves of *E. odoratum*, however, the activity of APX was increased by increasing the temperature from 25°C to 42°C, but in cold stress, the activity of APX reached a peak value at 15°C and then declined (Figure 3D). The drastic decreases of APX activities at 38°C or 42°C in leaves of *E. adenophorum*, suggested that APX can not compensate for the loss of CAT and POD activities at high temperatures, which seems to severely reduce the H₂O₂-scavenging capacity. However, during cold treatment, crofton weed exhibited an increased activity of H₂O₂ detoxifying enzyme APX, CAT and POD. Thus, the levels of membrane lipid peroxidation were relatively lower under cold stress, which seems to be related to its poor heat tolerance. The restriction of crofton weed to temperate climates (mainly on the Yunnan-Guizhou Plateau) (Lu and Ma 2004) suggests that poor heat-stress resistance may be a factor limiting the distribution of the plants. While in *E. odoratum*, the drastic decreases of APX activities at high temperatures suggested that APX can not provide sufficient protection against H₂O₂ accumulation.

Figure 3. Effect of temperature on ascorbate peroxidase (APX), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) activities of *Eupatorium adenophorum* (A–C) and *E. odoratum* (D–F).

Values are the means of three replicates with standard errors. Means with the same letters are significantly different at $P \leq 0.05$ using Duncan’s multiple range test.
activities were found at 10 °C or 5 °C, which seems to be related to its poor cold tolerance.

Glutathione reductase, another enzyme in the Asada-Halliwell pathway, can also remove cytosolic hydroperoxides such as lipid hydroperoxide, using reduced glutathione as a co-substrate (Ye and Gressel 2000). In leaves of *E. adenophorum*, the activity of GR was increased significantly by decreasing the temperature from 25 °C to 5 °C, but in heat stress, the GR activity was depressed (Figure 3B). In leaves of *E. odoratum*, however, the activity of GR was increased by increasing the temperature from 25 °C to 42 °C, but in cold stress, the activity of GR was unchanged (Figure 3E). According to Foyer and Halliwell (1976), H$_2$O$_2$ is mainly eliminated by the ascorbate-glutathione cycle. Besides the scavenging effect of CAT and POD, cytotoxicity of H$_2$O$_2$, which might be produced due to constitutive and induced levels of SOD in both species, might be disposed of with the aid of both APX and GR.

The ascorbate-glutathione cycle needs MDAR and DHAR in addition to APX and GR. MDAR and DHAR are enzymes responsible for ascorbate regeneration in plant tissues. The activity of MDAR decreased significantly in heat stress, whereas MDAR activity in leaves of the cold-treated *E. adenophorum* was not significantly different from the controls (Figure 3C). In leaves of *E. odoratum*, however, the activity of MDAR was increased by increasing the temperature from 25 °C to 42 °C, but in cold stress, the activity of MDAR was decreased (Figure 3F).

Dehydroascorbate reductase activity in leaves of the two species increased with both heat and cold stresses (Figure 4A, C). In both species, a greater increase in DHAR activity was found with heat treatment than in cold treatment, and the highest DHAR activity was recorded at 42 °C. DHAR is thought to play an important role in the oxidative stress tolerance of plants by regenerating ascorbate from dehydroascorbate (Foyer and Mullineaux 1998). The upregulation of DHAR activity in both cold-sensitive and heat-sensitive, and cold-tolerant and heat-tolerant invasive plants rather than MDHAR, suggests that in *E. adenophorum* and/or *E. odoratum* subjected to thermal stress conditions, ascorbate is regenerated via glutathione. It may be that DHAR activity could participate in ascorbate regeneration under conditions of severe stress when MDHAR activity is

![Figure 4](image_url)

*Figure 4.* Effect of temperature on dehydroascorbate reductase (DHAR) activity and Lipid peroxidation of *Eupatorium adenophorum* (A, B) and *E. odoratum* (C, D).

Values are the means of three replicates with standard errors. Means with the same letters are significantly different at $P \leq 0.05$ using Duncan’s multiple range test.
limited by the availability of NADH (Asada and Takahashi 1987; Hernández et al. 2000). This is in contrast to the situation described by Jahnke et al. (1991) who reported the DHAR losing almost all of its activity in cold stress of two Zea genotypes differing in cold tolerance, Jin et al. (2003) who reported the activities of DHAR were not sensitive to changes in temperature of five conifers with different cold tolerance.

Heat and cold stresses led to a significant increase in MDA content in both species (Figure 4B, D). MDA accumulation in E. adenophorum leaves was greater with heat treatment than with cold treatment, with greater increase at 42 °C than at 5 °C, indicating that a higher degree of lipid peroxidation occurred at higher temperatures. In leaves of E. odoratum, however, the MDA accumulation was greater with cold treatment than with heat treatment, with greater increase at 5 °C than at 42 °C, indicating a higher degree of lipid peroxidation induced by cold stress. Exposure to low temperature may increase the amount of active oxygen species not only in cold-sensitive E. odoratum, but also in cold-tolerant plants like E. adenophorum, although E. adenophorum showed elevated activities of SOD, CAT, POD, APX, GR, MDAR and DHAR on exposure to cold treatment, the capability of detoxification of AOS is limited. This is similar to some reports that have demonstrated the elevated activities of antioxidant enzymes in salt-tolerant rice varieties while the level of lipid peroxidation was increased under conditions of salinity stress (Vaidyanathan et al. 2003).

The interactions of APX, CAT, SOD and their involvement in scavenging AOS are very complex, and also involve other peroxidases (Scandalias 1997a, 1997b; Noctor and Foyer 1998). The extent of oxidative stress causing membrane and cellular damage might possibly differ depending on the stress imposed. Plants may respond to different environmental stresses in different ways, as shown in our experiment (Figures 2–4). SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, so the increase of SOD activity suggested an increased production of H₂O₂. H₂O₂ is eliminated by catalase and peroxidases, which include both enzymatic and non-enzymatic H₂O₂ degradation (Peltzer et al. 2002). Catalase decomposes H₂O₂ into water, whereas POD decomposes H₂O₂ by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Blokhina et al. 2003). The antioxidants such as ascorbate and glutathione are involved in scavenging H₂O₂ primarily via the Halliwell-Asada pathway (Horeman et al. 2000). It has been suggested that hydrogen peroxide must be effectively scavenged in order to minimize cytotoxicity by activated oxygen in the presence of elevated SOD activity (Finazzi-Agro et al. 1986; Scot et al. 1987).

In E. adenophorum, the coordinated increase of oxygen-detoxifying enzymes was effective in protecting the plant from the accumulation of AOS at low temperatures, thus averting cellular damage under unfavorable conditions. However, the activities of CAT, POD, APX, GR and MDAR were not accompanied with increased SOD during heat stress; thus in heat-treated E. adenophorum, cytotoxicity was likely due to the accumulation of H₂O₂, which can react with O₂· to produce hydroxyl free radicals via the Herbert-Weiss reaction (Bowler et al. 1992). As a result, the level of lipid peroxidation in E. adenophorum was higher under heat stress than under cold stress. In E. odoratum, however, the lesser degree of membrane damage (as indicated by low MDA content) and the coordinated increase of the oxygen-detoxifying enzymes was observed in heat-treated plants, but the antioxidant enzymes were unable to do this with cold stress. This indicates that the plant has a higher capacity for scavenging oxygen radicals in heat stress than in cold stress.

The diverse response of antioxidant enzymes such as SOD and other enzymes (CAT, POD, APX, MDAR, GR) due to thermal stress suggests the role of oxidative stress as a component of environmental stress on the two invasive species. It appears that the differences in SOD and other enzymes (CAT, POD, APX, MDAR, GR) has a direct relation to the sensitivity of the two plants to temperature. In rice, increased activity of SOD and decreased activities of CAT and POD were detected in cold-sensitive cultivars, whereas increased activities of these enzymes have been found in resistant cultivars exposed to chilling stress, while GR in both cultivars was stable to low temperature stress (Saruyama and Tanida 1995). Clear differences were found in the cold sensitivity of CAT and APX between the cold-sensitive and cold-tolerant rice cultivars. In maize, the activity of SOD and APX of the resistant variety of plants grown at low temperatures were higher than in controls. In contrast, in the sensitive variety of plants grown at low temperature, the activity of SOD was higher than in the controls and the activity of APX was lower than in the controls (Massacci et al. 1995). Our results suggested that a coordinated increase of the activity of oxygen-detoxifying enzymes could be necessary to protect E. adenophorum leaves from the accumulation of oxygen radicals at low temperatures, or protect E. odoratum leaves from the accumulation of oxygen radicals at high temperatures.

Although several environmental stresses in plants are known to induce the expression and/or increasing levels of antioxidative enzymes and their mRNAs (Pan and Yau 1992; Klieweinstein et al. 1998; Morita et al. 1999), the mechanism by which these activated oxygen-scavenging enzymes can work co-operatively, in response to temperature stress is not yet known. Further analysis of the regulation of gene expression in these enzymes should elucidate the mechanism of different temperature tolerances. Identifying the genes that regulate some of the enzymes involved in stress and finding ways to interfere with gene regulation may prove useful to aiding an economically viable control of persistent perennial weeds in difficult-to-reach locations or along waterways where the use of herbicides is undesirable or illegal (Davis and Swanson 2001). Our future goal also involves examining the long-term responses of the antioxidative system, to further elucidate their function in thermal tolerance mechanisms of the two invasive species.
Considering the different distribution patterns of *E. adenophorum* and *E. odoratum* in China, our data suggest that the differences of antioxidant enzyme activities and MDA content in the two species may be ascribed to differences in mechanisms underlying oxidative stress injury and subsequent tolerance to temperature stress.

**Materials and Methods**

**Plant material and culture conditions**

*Eupatorium adenophorum* Spreng. seedlings were collected from the Kunming Institute of Botany, the Chinese Academy of Sciences and *E. odoratum* L. seedlings were collected from the Xishuangbanna Tropical Botanical Garden, the Chinese Academy of Sciences. Seedlings were planted in plastic pots (19 cm diameter and 16 cm high) filled with a 1:1:1 mixture of soil:sand:peat. They were grown in a glasshouse with day/night (19 cm diameter and 16 cm high) filled with a 1:1:1 mixture of soil:sand:peat. They were grown in a glasshouse with day/night temperatures of 30 °C/20 °C. Maximal photosynthetic photo flux density was approximately 800 μmol/m² per s. Plants were watered daily and fertilized on a regular basis. After 4 weeks, when the plants of both species had achieved a steady state in their growth and reached heights of about 30–40 cm, they were subjected to the temperature treatments.

**Heat and cold stress treatments**

Plants of each invasive species were divided into two groups. For the heat stress treatment, one half of the plants were moved from the glasshouse and placed in growth chambers at a constant temperature of 25 °C and relative humidity of 70%, a 12/12 h light/dark photoperiod regime, and photon flux density of 250 μmol/m² per s for 7 days to acclimatize the plants to the growth chambers. After acclimatization, the temperature was increased stepwise to 30 °C, 35 °C, 38 °C and finally to 42 °C and exposed to each temperature at 24 h intervals. Simultaneously, for the cold stress treatment, the same acclimatization period of 7 days was used. Another group of plants were then exposed to progressively decreasing temperatures of 20 °C, 15 °C, 10 °C and 5 °C at 24 h intervals. Each treatment was repeated in three separate growth chambers. The soil was kept saturated at field capacity during both treatments to avoid possible drought. The plants that were kept at 25 °C for 24 h were used as controls. After each 24 h temperature treatment, fully expanded leaf material was collected from three plants per treatment, frozen, stored in liquid nitrogen and maintained at −70 °C. The tissue was ground and the enzymes extracted for assay.

**Lipid peroxidation**

Lipid peroxidation in *E. adenophorum* and *E. odoratum* was determined by measuring the malondialdehyde (MDA) content, a product of lipid peroxidation, in 1 g leaf fresh weight following Madhava Rao and Sresty (2000).

**Enzyme extraction**

One gram of frozen leaf material was homogenized in 8 mL of 50 mM phosphate buffer (pH 7.0) that included 1 mM ethylenediaminetetraacetic acid (EDTA) and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 13 000 g for 40 min at 4 °C. In the cases of APX and DHAR activities determination, 1 mM ascorbate and 2 mM 2-mercaptoethanol, respectively, were added to the homogenizing buffer to prevent enzyme inactivation. The supernatant was used for assays of enzyme activity and protein content. Total soluble protein contents of the enzyme extracts were determined following Bradford (1976), using bovine serum albumin (BSA) as a standard.

**Enzyme assays**

Superoxide dismutase activity was determined using the method of Beauchamp and Fridovich (1971) that measures the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. Measuring the activity of CAT followed Knörrer et al. (1996) by monitoring the rate of decomposition of H₂O₂ at 240 nm for 2 min. Measuring the activity of POD followed Chance and Maehly (1955) by monitoring the rate of the increase in the absorbance at 470 nm for 2 min due to guaiacol oxidation. The activity of APX was measured by following Nakanos and Asada (1981), and monitoring the rate of ascorbate oxidation at 290 nm for 3 min. The activity of GR was assayed according to the method of Halliwell and Foyer (1978) by following the decrease in absorbance at 340 nm for 3 min due to NADPH oxidation. The enzyme activity of MDAR was measured in the supernatant at 25 °C as described by Hossain et al. (1984). MDAR was assayed by following the decrease in absorbance at 340 nm for 5 min due to NADH oxidation. The activity of DHAR was measured in the supernatant at 25 °C as described by Hossain and Asada (1984) by monitoring the increase in absorbance at 265 nm for 3 min due to ascorbate formation.

**Statistical analysis**

The experiment was arranged in a completely randomized block design with three replicates. Duncan’s multiple range test for multiple comparisons was used for the analysis of statistical significance. Comparisons with *P* values ≤ 0.05 were considered significantly different. All values reported are means ± SE of three replicates.

**Acknowledgements**

Many thanks to Mark Williamson, Yulong Feng and F. Andrew Smith for their suggestions about this paper and for giving much
constructive advice. The authors also thank Nan Lu, Ping Lin, Lingfeng Chen, Nianwei Qiu, Junfeng Wang, Yanhong Bing, Li Zhu and Fan Bai for their assistance in this research.

**References**


Jung S (2003). The combined action of catalase (CAT) and superoxide dismutase (SOD) is critical in mitigating the effects of oxidative stress. Pesticide Biochem. Physiol. 75, 9–17.


