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The Physiological and Biochemical Responses of Eastern Purple Coneflower to Freezing Stress[1]

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Abstract—The freezing hardiness (expressed as LT_{50}) as well as changes in the antioxidant enzymes activity, total protein and lipid peroxidation (MDA content), total phenolic and flavonoid content, antioxidant capacity, chlorophyll fluorescence (F_v/F_m) of *Echinacea purpurea* (L.) Moench were investigated. Five-month-old purple coneflower seedlings were kept at 4°C for two weeks to induce cold acclimation. The acclimated seedlings were treated with freezing temperatures (0, −4, −8, −12, −16, and −20°C) for 6 h. The unfrozen control plants were kept at 4°C. The results with lowering freezing temperatures showed a sharp increase of ion leakage and MDA content at −20°C as compared to the nonfreezing temperature. Exposing seedlings to freezing temperatures were accompanied by decreasing dark-adapted chlorophyll fluorescence (F_v/F_m). Freezing stress significantly reduced superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) activity of seedling leaf except at 0°C. With lowering freezing temperature, peroxidase (POD) and polyphenol oxidase (PPO) activity showed a sharp decline to −20°C. Furthermore, total protein and antioxidant capacity of *Echinacea* leaves were declined significantly after exposure to freezing temperature, and thereafter reached to the highest at −8°C. Total phenolic content of freezing-treated seedlings was significantly lower than that of the nonfreezing seedlings. Total flavonoid content increased significantly with lowering freezing temperatures. It was found that percentage of freezing injury closely correlated to antioxidant enzymes activity (POD and PPO; $r = −0.93$) and F_v/F_m ratio ($r = −0.77$). Based on our results, the freezing tolerance (LT_{50}) of *Echinacea* seedlings under artificially simulated freezing stress in the laboratory was −7°C.

Keywords: *Echinacea purpurea*, chlorophyll fluorescence, antioxidative enzymes, ion leakage, freezing stress

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INTRODUCTION

The level and mechanisms by which plants resist freezing temperatures (<0°C) are related to the ambient temperatures that plants experience [1]. Freezing temperatures harmfully affects plant growth and development, limits their geographic distribution, and significantly reduces agronomic productivity. The main target of freezing injury is cell membranes, which are the primary cause of cellular dehydration in plants

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Abbreviations: APX—ascorbate peroxidase; CAT—catalase; DPPH—1,1-diphenyl-2-picrylhydrazyl; NBT—nitro blue tetrazolium; POD—peroxidase; PPO—polyphenol oxidase; PVPP—polyvinyl polypyrrolidone; SOD—superoxide dismutase; TBA—thiobarbituric acid.

exposed to freezing stress [2]. Like other abiotic stresses, exposure to freezing temperature leads to the accumulation of reactive oxygen species (ROS) in plant cells, followed by the increase in lipid peroxidation [3]. Plants have evolved both enzymatic and nonenzymatic antioxidant systems to prevent or alleviate membrane damage caused by ROS.

Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) are the major antioxidative enzymes that efficiently scavenge ROS, with SOD probably being central in the defense against toxic ROS [4]. SOD is a metal-binding enzyme that scavenges the toxic superoxide radicals and catalyzes the conversion of two superoxide anions into oxygen and H_2O_2. Then, POD and CAT convert H_2O_2 into H_2O and O_2, whereas APX decomposes H_2O_2 by oxidation

of co-substrates, such as phenolic compounds and/or antioxidants [5]. In addition, polyphenol oxidase (PPO) catalyzes the oxidation of *o*-diphenols to *o*-diquinones, as well as the hydroxylation of monophenols [6]. PPO is also an important enzyme in the response of plants against freezing stress, and it can help to avoid serious oxidative damage induced by freezing [6]. In order to accommodate the oxidative stresses, it is crucial that plants maintain the activities of these antioxidant enzymes.

Furthermore, under stress conditions, the possibility of overexcitation of photosystem II (PSII) increases and this reduces the photosynthetic rate and leads to an increase in the dissipation of absorbed energy through non-radiative processes [7]. Therefore, non-invasive measurement of photosynthesis by chlorophyll *a* fluorometry may potentially provide a means to determine plant viability and performance in response to stress. Researchers have shown chlorophyll fluorescence to be well correlated with foliar damage following freeze stressing [8, 9].

The genus *Echinacea*, commonly known as purple coneflower, is a member of the Asteraceae family comprising of nine species and four varieties, all native to North America. Many types of phytomedicine are commercially produced from the aerial portions of *Echinacea* for boosting the nonspecific immune system and treating common cold. *Echinacea purpurea* L. is one of the top selling medicinal plants that contain many active components such as alkamides, caffeic acid esters, polysaccharides, and polyacetylenes. It was found to possess antioxidant and high free radical scavenging properties making it a very promising medicinal botanical [10].

This plant has been reported to tolerate a wide range of environmental stresses such as salinity and drought [11]. However, up to now, there is hardly any report regarding freezing or chilling tolerance of *E. purpurea*. So, knowledge of the physiological and biochemical responses of *E. purpurea* seedlings to freezing temperatures will be crucial for planting at regions with colder climate.

In the present study, we investigated the effects of freezing temperatures on ion leakage, injury percentage, lipid peroxidation, total protein content, total phenol and flavonoid content, antioxidant capacity, antioxidative enzyme (SOD, POD, APX, CAT, and PPO) activities and the usefulness of F_v/F_m, as an indicator of seedlings freeze damage in five-month-old *Echinacea* leaves.

MATERIALS AND METHODS

Plant material. Five-month-old purple coneflower [*Echinacea purpurea* (L.) Moench] seedlings were used in this study. Seedlings were grown in 1-L polyethylene bags containing a sandy : vermiculite : loam substrate (2 : 1 : 1, v/v) in greenhouse during autumn (October to December). Before exposure to freezing tempera-

tures, seedlings were kept in controlled growth chamber at 4°C for two weeks to induce cold acclimation.

Freezing treatments. The bags were covered with a layer of glass wool to protect the roots from freezing damage. The seedlings were divided into two groups. One group was kept in a growth chamber at 4°C in the dark for 24 h as a unfrozen control and the second group was placed into a programmable test chamber for whole plant freezing treatment. The chamber temperature was decreased stepwise from 2°C/h until −4°C and at 5°C/h until −20°C. Seedlings were exposed to freezing temperatures (0, −4, −8, −12, −16, and −20°C) for 6 h. Relative humidity inside the chamber was kept at 45−50%, and darkness conditions were simulated. Then, the bags were removed from the glass wool, and recovery was performed by rising the temperature at the same rate until reaching again the temperature of 4°C for slow thawing in the dark for 24 h.

For freezing stress evaluation, the above ground parts were frozen in liquid nitrogen and kept at −80°C until further biochemical analysis. The rest of the plants were used to determine freezing injury and chlorophyll *a* fluorescence.

Determination of freezing injury. Ion leakage of leaves was measured as described by Dexter et al. [12] with some modifications. Samples were cut into equal pieces (10 mm in diameter), placed in the test-tube containing 10 mL of distilled water, and kept at 45°C for 30 min in a water bath. The initial conductivity of the solution was measured using a Mi 306 EC/TDS conductivity meter (Milwaukee Instruments, Hungary). The tubes were then kept in a boiling water bath for 10 min, and their conductivity was measured once again after cooling to room temperature. Percentage of ion leakage (IL) for each treatment was converted to percentage of injury as:

$$\text{Injury (\%)} = [(\text{IL(t)} - \text{IL(c)})/100 - \text{IL(c)}] \times 100,$$

where IL (t) and IL (c) are the percentage of IL from the respective freeze-treatment temperature and the unfrozen control, respectively.

LT_{50}, a measure of freezing tolerance, was derived for *E. purpurea* by determining the freeze test temperature at which 50% injury (midpoint of maximum and minimum percentage of injury) occurred as explained by Lim et al. [13].

Lipid peroxidation (MDA content). The MDA as the end product of membrane lipid peroxidation was measured to determine the level of membrane damage [14]. Leaves were weighed and homogenized in the solution containing 10% TCA and then centrifuged at 10 000 *g* for 10 min. 1.5 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA were added to 1.5 mL of the supernatant aliquot. The mixture was heated at 95°C for 60 min, cooled to room temperature, and centrifuged at 10 000 g for 10 min. The absorbance of the supernatant was read at 532 and 600 nm against TCA solution as a reagent blank. The content of MDA was

determined using the extinction coefficient of 1.55/(M cm) and expressed in nmol MDA per g fr wt.

Chlorophyll fluorescence. Chlorophyll **a** fluorescence was measured at room temperature (25°C), using a portable fluorometer (PAM-2500, Walz, Germany). Before fluorescence determining, the middle part of the leaves were fixed on a leaf-clip holder (2030-B, Walz) in the dark for 30 min. The minimal fluorescence level (F_0) with all PSII reaction centers opened was recorded by the measuring modulated light which was sufficiently low (<0.1 μmol/(m^2 s)) not to induce any significant variable fluorescence. The maximal fluorescence level (F_m) with all PSII reaction centers closed was determined by a 0.8 s saturating pulse at 8000 μmol/(m^2 s) in dark-adapted leaves. The leaf disc was then continuously illuminated with white actinic light at an intensity of 180 μmol/(m^2 s) which was equivalent to growth PPFD of *Echinacea* seedlings in the growth chamber [15].

Enzyme activities. *Echinacea* leaves (0.25 g) were homogenized in 1 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM of EDTA in the presence of PVP. The homogenate was centrifuged at 15000 *g* for 15 min at 4°C. The supernatant was used to measure the activities of SOD, POD, APX, CAT, and PPO and to determine total protein content. All assays were done at 25°C using a spectrophotometer (T80, PG Instrument, England).

SOD (EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) according to the methods of Beauchamp and Fridovich [16]. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 200 mM methionine, 1.125 mM NBT, 1.5 mM EDTA, 75 μM riboflavin, and 0–50 μL of the enzyme extract. Riboflavin was added as the last component. Reaction was carried out in test-tubes at 25°C under illumination supplied by two fluorescent lamps (20 W). The reaction was initiated by switching on the light and allowed to run for 15 min, and light switching off stopped the reaction. The tubes were then immediately covered with aluminum foil in order to stop the reaction, and absorbance of the mixture was then read at 560 nm. Identical tubes with complete reaction mixture containing no enzyme extract and developing maximum color served as a control. A non-illuminated complete reaction mixture with no color development served as a blank. Under experimental conditions, the initial rate of reaction, as measured by the difference in the increase of absorbance at 560 nm in the presence and absence of leaf extract was proportional to the amount of enzyme. One unit SOD activity was defined as the amount of enzyme required to inhibit 50% of the rate of NBT reduction measured at 560 nm. The SOD activity in the extract was expressed as activity units/mg protein.

POD (EC 1.11.1.7) activity in leaves was assayed by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance was recorded at 470 nm [17].

The reaction mixture contained 100 μL of crude enzyme extract, 500 μL of 5 mM H_2O_2, 500 μL of 28 mM guaiacol, and 1900 μL of 50 mM potassium phosphate buffer (pH 7.0). POD activity of the extract was expressed as activity units/(mg protein min).

APX (EC 1.11.1.11) activity was measured according to Nakano and Asada [18]. The reaction mixture contained 50 mM (pH 7.0) potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2, and 100 μL of the enzymes extract. H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm. The APX activity of the extract was expressed as activity units/(mg protein min).

CAT (EC 1.11.1.6) activity was assayed according to the method of Chance and Maehly [17]. The decomposition of H_2O_2 was monitored by the decrease in absorbance at 240 nm. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 400 μL of 15 mM H_2O_2, and 40 μL of enzyme extract. The CAT activity of the extract was expressed as activity units/(mg protein min).

PPO (EC 1.10. 3. 1) activity was assayed with 4-methylcatechol as a substrate as described in [19] with some modifications. The assay of the enzyme activity was performed using 2 mL of 0.1 mM sodium phosphate buffer (pH 6.8), 0.5 mL of 100 mM 4-methylcatechol, and 0.5 mL of the enzyme solution. The increase in absorbance at 420 nm was recorded. The PPO activity was expressed as activity units/(mg protein min).

Protein content. Protein content was determined according to Bradford [20] using BSA as a standard.

Total phenol and flavonoid contents. Leaf sample (1 g) was extracted with 10 mL of methanol and then total phenols were determined with a spectrophotometer using the modified Folin-Ciocalteu colorimetric method. The methanolic extract (125 μL) was mixed with 375 μL of distilled water in a test-tube followed by the addition of 2.5 mL of 10% Folin-Ciocalteu reagent and allowed to stand for 6 min. Then, 2 mL of 7.5% Na_2CO_3 was added. Each sample was incubated for 90 min at room temperature in darkness, and absorbance at 760 nm was measured. Then the contents of phenolic compounds of extracts as mg of the gallic acid equivalent (GAE) determined using an equation that was obtained from standard gallic acid graph.

Flavonoid content was determined by following colorimetric method [21]. Briefly, 0.5 mL of extracts in methanol were mixed with methanol, 10% aluminium chloride, 1 M potassium acetate and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 510 nm with a spectrophotometer. The results were expressed as mmol of catechin equivalents per kg of fresh weight.

DPPH scavenging capacity. The antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method. 1 g of leaf
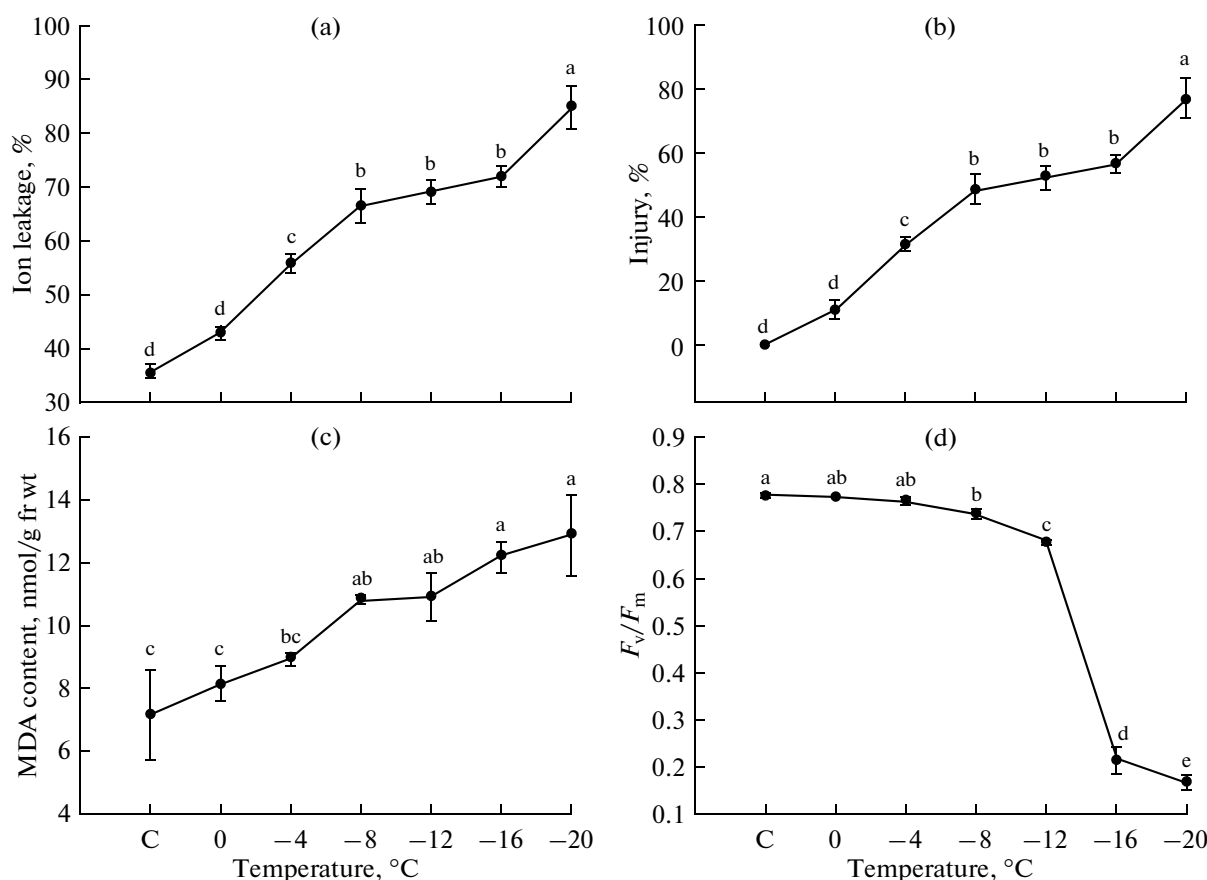
Fig. 1. Ion leakage (a), percentage of injury (b), lipid peroxidation (MDA content) (c) and F_v/F_m ratio (d) in *Echinacea purpurea* leaves under unfrozen control (C) and freezing temperatures for 6 h. Vertical bars indicate standard errors from means ($n = 3$). Values with different letters are significantly different, as determined by the LSD test ($P \le 0.05$).

sample was extracted with 10 mL of methanol. Then 50 μL of the methanolic extract was added to 950 μL of 0.1 mM DPPH radical, vortexed, and incubated at room temperature in darkness. The absorbance of the samples was measured at 517 nm using the spectrophotometer after 30 m

$$\text{Inhibition of DPPH (\%)} = [(A_{517\ \text{control}} - A_{517\ \text{sample}})/A_{517\ \text{control}}] \times 100.$$

Statistical analysis. The experiment was arranged in completely randomized design. Temperature treatments orders were randomly assigned for the growth chamber. Analysis of variance was performed on all experimental data using SAS software (v. 9.1, SAS Institute, United States). The figures were done using SigmaPlot software (v. 12). Means in the figures were subjected to LSD test at 0.05 probability level.

RESULTS

Ion Leakage

Ion leakage reflects the level of cell membrane injury as a result of oxidative damage. Freezing stress induced a significant increase in the ion leakage from *Echinacea* leaves (Fig. 1a). The lowest ion leakage was observed in the leaves exposed to nonfreezing control temperature (Fig. 1a). As shown in Fig. 1a, during freezing stress ion leakage gradually increased and reached to the highest at −20°C (up to 2.4 fold than unfrozen control temperature).

Freezing Injury in Leaf Tissues

Changes in freezing injury in *Echinacea* leaf tissues are shown in Fig. 1b. Freezing injury rate (expressed by reference to the control) gradually increased during freezing stress (Fig. 1b). The lowest and the highest freezing injuries were observed in the leaves exposed to 0°C and −20°C, respectively. LT_{50}, a measure of freezing tolerance for *E. purpurea* was determined −7°C (Fig. 1b).

Lipid Peroxidation

The production of MDA was used as an indicator of damage done by stress in plant membranes. In general, MDA content was the minimum in unfrozen control samples (7.15 nmol/g fr wt) (Fig. 1c), while it gradu-

ally increased in parallel to declining temperatures and reached to the maximum level (12.9 nmol/g fr wt) in leaves exposed to $-20°C$ (Fig. 1c). The percentage of increase was 80% at $-20°C$ as compared to nonfreezing control temperature.

Chlorophyll Fluorescence

To measure the chlorophyll fluorescence, fluorescence induction curve upon illumination of dark adapted leaves were recorded, where fluorescence is emitted from the chlorophyll *a* of photosystem II. From this induction curve, the maximum quantum use efficiency of photosystem II in dark-adapted leaves can be calculated as F_v/F_m. In this work, the F_v/F_m ratio was decreased significantly in freezing temperatures (Fig. 1d). Increase in freezing stress from 0 to $-20°C$ resulted in significant decrease in F_v/F_m (Fig. 1d); So, *Echinacea* plants grown at $-20°C$ showed remarkable decrease in F_v/F_m (79%) compared to unfrozen control seedlings.

Enzyme Activities

In general, plants activate a number of antioxidative enzymes when exposed to low temperature in order to protect them against potentially cytotoxic ROS. To find out the relation between antioxidative enzymes at freezing temperatures, the activities of antioxidative enzymes in *E. purpurea* seedlings at freezing temperatures were assayed. Results indicated that SOD activity tended to decrease when freezing stress was imposed (Fig. 2a). The highest SOD activity was observed at $0°C$ (92.8 units/mg protein), and thereafter its activity was decreased to $-20°C$ (Fig. 2a). When the seedlings were exposed to $0°C$ had 38% more SOD activity than at unfrozen control temperature (Fig. 2a).

With lowering freezing temperature, POD activity showed a sharp decline to $-20°C$ (Fig. 2b). When the seedlings were exposed to $-20°C$, POD activity reduced up to 94% as compared to the control (Fig. 2b). The data also showed that APX activity varied from 783.4 units/(mg protein min) at $0°C$ to 12.1 units/(mg protein min) at $-20°C$ (Fig. 2c).

As shown in Fig. 2d and Fig 2e, a similar trend was observed for CAT and PPO activities. At all temperatures, however, relative decrease was more in CAT activity than in PPO activity.

Protein Content

The data indicated that protein content varied from 216.5 mg/g fr wt in unfrozen control plants to 1.85 mg/g fr wt in $-20°C$ (Fig. 2f). Under freezing stress, protein content gradually increased to $-8°C$ (10% lower than unfrozen control) and thereafter decreased to $-20°C$ (Fig. 2f).

Correlation coefficients (*r*) between freezing injury and antioxidative enzymes activity, protein, MDA, phenolic and flavonoid contents, ion leakage and F_v/F_m in the leaf tissues of *Echinacea purpurea* under freezing stress

Variable	r
SOD activity	$-0.86**$
POD activity	$-0.93**$
APX activity	$-0.84**$
CAT activity	$-0.92**$
PPO activity	$-0.93**$
Antioxidant capacity	$-0.52*$
Protein content	$-0.71**$
MDA content	$0.81**$
Phenolic content	$-0.80**$
Flavonoid content	$0.61**$
Ion leakage	$0.99**$
F_v/F_m	$-0.77*$

Asterisks * and ** correspond to significance at $P < 0.05$ or $P < 0.01$, respectively.

Total Phenolic and Flavonoid Contents

Results showed that total phenolic content in freezing-treated seedlings was significantly lower than that of the nonfreezing control plants (Fig. 3a). Under stress conditions, the percentage of decrease was 87% at $-20°C$ as compared to seedlings at nonfreezing control temperature (Fig. 3a).

The total flavonoid content was significantly different among temperatures (Fig. 3b). According to the results presented in Fig. 3b, the flavonoid content decreased significantly until $-4°C$ and thereafter increased to $-16°C$ (20.9 mg quercetin/g fr wt).

Antioxidant Capacity

Freezing stress caused significant decrease in antioxidant capacity of *Echinacea* seedlings as compared to those plants at nonfreezing temperature (Fig. 4).

Correlation Between Percentage of Freezing Injury and Biochemical Characteristics

The percentage of freezing injury was negatively correlated with antioxidant enzymes activity in the leaf tissues of *E. purpurea* (table). Ion leakage, MDA and flavonoid content positively correlated with percentage of freezing injury ($r = 0.99$, 0.81, and 0.61 for ion leakage, MDA and flavonoid content, respectively; table). The correlations between percentage of freezing injury and protein and phenolic content were negatively ($P < 0.01$), and with antioxidant capacity and F_v/F_m ratio were negatively ($P < 0.05$) (table).
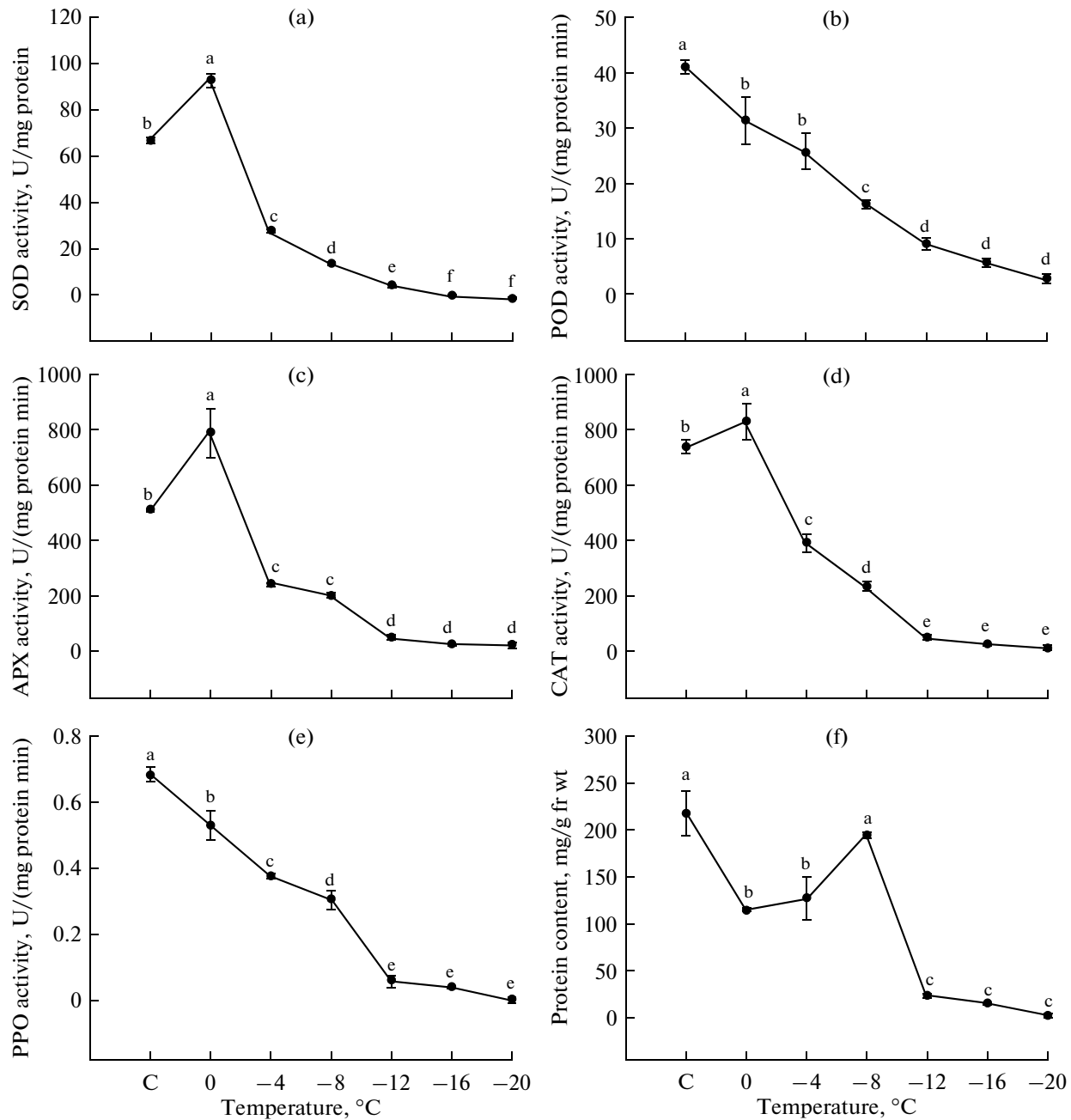
Fig. 2. Activity of SOD (a), POD (b), APX (c), CAT (d), PPO (e) and protein content (f) of *Echinacea purpurea* leaves under unfrozen control (C) and freezing temperatures for 6 h. Vertical bars indicate standard errors from means ($n = 3$). Values with different letters are significantly different, as determined by the LSD test ($P \leq 0.05$).

DISCUSSION

Up to date, there is hardly any report regarding freezing or chilling tolerance of purple coneflower. In the present study, we evaluated the physiological and biochemical responses of coneflower under freezing stress. Freezing temperatures as compared to non-freezing temperature caused a significant increase in ion leakage (Fig. 1a), freezing injury (Fig. 1b), and MDA content (Fig. 1c) in *Echinacea* seedlings, indicating that freezing stress could cause damages to the integrity of the cellular membranes and to cellular components, such as lipids. It has been shown that plant membrane damage during chilling is related to the peroxidation of membrane lipid due to the stress-induced accumulation of free radicals [22]. Lyons [23] proposed that membranes of plants under low temperature become less fluid, their protein components can no longer function normally, causing water and soluble materials to leak out into the intercellular spaces. This result is in agreement with Zhou et al. [24], who
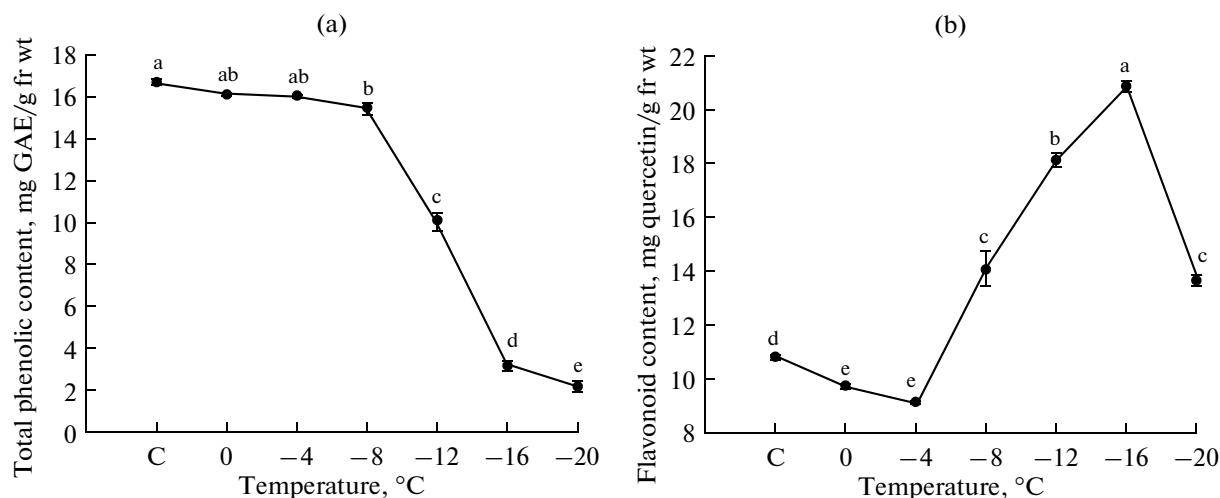
Fig. 3. Total phenolic (a) and flavonoid (b) contents in the leaves of *Echinacea purpurea* under unfrozen control (C) and freezing temperatures for 6 h. Vertical bars indicate standard errors from means ($n = 3$). Values with different letters are significantly different, as determined by the LSD test ($P \leq 0.05$).

reported that the membrane system of *Stylosanthes guianesis* was damaged under chilling stress due to the induction of oxidative damage related to the imbalance of ROS production.

The difference between F_m and F_0, called the variable fluorescence, F_v, and the ratio F_v/F_m (in most higher plants having usually the value in the range of 0.78−0.84) are used extensively; the parameter F_v/F_m reflects the maximal efficiency of excitation energy capture by "open" PSII reaction centers [15]. In our study, the reduction in F_v/F_m following freezing stress as compared to nonfreezing temperature (Fig. 1d) can be attributed to down-regulation of photosynthesis, inhibition of photophosphorylation due to photoinactivation of PSII centers "possibly attributable to D_1 protein damage" and enzymatic processes in the carbon reduction cycle resulting in a substantially reduced rate of CO_2 fixation as indicated by reduced O_2 evolution [8]. Decreases in F_v/F_m in response to freezing temperatures have been shown in red spruce [9] and spinach [25].

This study demonstrates that the activity of antioxidative enzymes decline in response to decrease in temperature. The data in our study about antioxidative enzyme activities showed in Figs. 2a−2e. Results showed that as *Echinacea* seedlings were exposed to freezing temperatures, the activities of antioxidant enzymes (SOD, APX, and CAT) increased at 0°C and thereafter their activities were declined to −20°C, while POD and PPO activity decreased continuously down to −20°C as compared to nonfreezing temperature (Figs. 2a, 2e). A large body of evidence has shown that the antioxidative enzyme systems are altered under abiotic stresses, including chilling and freezing [4]. Chilling could weaken the enzymatic antioxidant system of plants and induce an increase in H_2O_2 level, thereby exposing them to oxidative stress. The chilling

treatment decreased significantly both the CAT and the APX activities in rice [26] and *Stylosanthes guianesis* [24]. The mechanism of the decrease in CAT enzyme activity reported in the above mentioned studies has not yet been identified. Possible explanations include (a) decrease in CAT expression; (b) inhibition of enzyme activity by high H_2O_2 concentrations; (c) accumulation of endogenous CAT inhibitors [27]. Among these possible causes, the decrease in CAT enzyme activity is generally believed to be due to high H_2O_2 concentrations [27]. In addition, APX is believed to protect plants from the damage caused by low temperature stress more efficiently than CAT [27]. PPO enzyme also catalyses the oxidation of *o*-diphe-
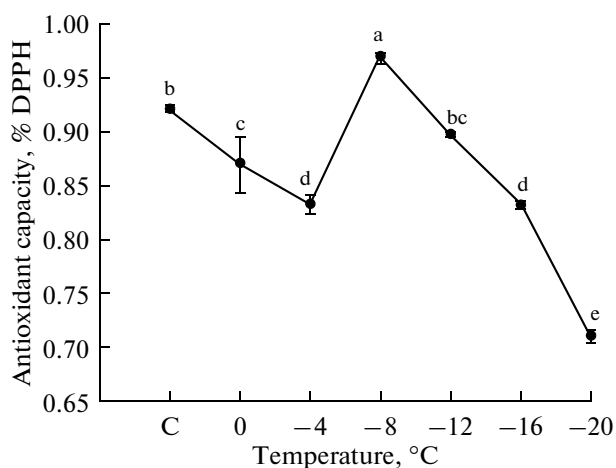

Fig. 4. Antioxidant capacity in the leaves of *Echinacea purpurea* under unfrozen control (C) and freezing temperatures for 6 h. Vertical bars indicate standard errors from means ($n = 3$). Values with different letters are significantly different, as determined by the LSD test ($P \leq 0.05$).

nols to *o*-diquinones, as well as the hydroxylation of monophenols. PPO is important in the response of plants against freezing stress and it can help avoid serious oxidative damage induced by freezing [6].

An accumulation of soluble and specific proteins, such as anti-freeze proteins and expression of specific genes may help to avoid the alteration of the permeability of the cell membranes, caused by dehydration during the formation of extracellular ice [29]. In the present work, total protein content gradually increased to −8°C. However, the protein content at this temperature was 10% lower than unfrozen control and then thereafter markedly decreased until −20°C (Fig. 2f). The increase in soluble protein content at −8°C may be associated with synthesis soluble and specific proteins, such as anti-freeze proteins and expression of specific genes [29]. Also, decline in protein content may be due to extensive damage of protein synthesizing system in various crops or synthesis and or activation of large quantities of proteolytic enzymes as protease [30].

Phenolics are diverse secondary metabolites and are abundant in plant tissues. They play several roles in plants; one of the most important roles is the protection on cells. In this work, exposing *Echinacea* seedlings to freezing temperatures reduced total phenolic content, whereas total flavonoid content was increased following freezing temperature treatment at −16°C and thereafter decreased at −20°C (Figs. 3a, 3b). The protection role of phenolics in plants under stress derived from their antioxidative properties. Another mechanism underlying the antioxidative properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modifying of the lipid packing order and to decrease fluidity of the membranes [31]. These changes could sterically hinder diffusion of free radicals and restrict peroxidative reactions. In addition, the phenolic compounds have been proven to have the ability to scavenge free radicals and inhibit membrane lipid peroxidation of seedlings.

In this work, the antioxidant capacity of seedlings under freezing stress decreased (Fig. 4) which may be due to the decreased PPO and PAL activity, phenolic compounds and antioxidant enzyme activities. Our results are in consistent with a previous report that the high level of DPPH-radical scavenging has been correlated with increased chilling tolerance [32].

In conclusion, this is the first study to determine freezing tolerance of purple coneflower seedlings and evolution of changes in their enzyme activities at freezing temperatures and shows that the freezing tolerance (LT_{50}) of *Echinacea* seedlings under artificially simulated freezing stress in the laboratory is −7°C. Freezing injury was closely related to ion leakage content ($r = 0.99$). In addition, freezing injury was negatively related to antioxidative enzymes activity (POD and PPO; $r = -0.93$) and F_v/F_m ratio ($r = -0.77$) in *E. purpurea* leaf tissues under freezing stress.

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