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THERMAL DEPENDENCE OF THE ANTIOXIDANT ENZYMES SUPEROXIDE DISMUTASE, CATALASE, AND PEROXIDASE IN FOLIAGE OF *IRIS PUMILA* L.

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Abstract — Thermal dependence of the enzymes SOD, CAT, and POD was investigated in leaves of *Iris pumila* plants inhabiting two contrasting light environments, a sun-exposed dune site and a woodland understory. At the same assay temperature, both the specific activity and the activation energy of SOD and CAT were higher in plants inhabiting vegetation shade than in those experiencing full sunlight. Conversely, the temperature optima for the two enzymes did not differ between alternative radiation environments. The specific activity of POD increased with temperature increase, and was always greater in plants growing under full sunlight than in those from vegetation shade. The activation energy of POD was higher than that of SOD or CAT, being lower in sun- than in shade-exposed plants.

Key words: Thermal curves, SOD, CAT, POD, specific activity, activation energy, *Iris pumila*, natural populations

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INTRODUCTION

Temperature is one of the major abiotic factors in determining the rate of enzyme activity. Theoretically, enzymatic catalysis increases as temperature rises until a maximum velocity is achieved, and with further increases in temperature, the rate of enzymatic reaction declines owing to some inactivation process. The events taking place during the catalytic process are complex, but it has been emphasized that the key determinant of the speed of an enzymatic reaction is the velocity of rate-limiting conformational changes (Hochachka and Somero, 2002). There is strong experimental evidence for the presence of an inverse correlation between enzyme conformational stability and specific activity, mediated via molecular flexibility (Jaenicke, 1991; Daniel et al., 1996). As might be expected from this flexibility/stability/activity association, “thermophilic” enzymes (tolerant to high temperatures) have been shown to be more rigid and less active at room temperature than their mesophilic orthologs (Jaenicke, 1991; Daniel et al., 1996). There is growing evidence, however, that the way in which enzymes respond to temperature may depend not only upon their intrinsic biochemical properties (Daniel et al., 1996; Thomas and Scopes, 1998; Lu et al., 2008), but also upon the environmental growth conditions to which the organisms were exposed (Hull et al., 1997; Peltzer et al., 2002).

The aim of this study was to investigate the thermal behavior of three key antioxidant enzymes, viz., superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and class III peroxidase (POD; EC 1.11.1.7), in leaves of naturally growing *Iris pumila* plants at sun-exposed and shaded sites in the Deliblato Sands, Serbia. Since within its natural ecological niches *I. pumila* regularly faces a variety of abiotic stresses, such as elevated temperatures, scarcity of water, and high irradiance – particularly during the summer – and because abiotic environmental stress can operate as a strong selective force (Sørensen et al., 2003), we expect that the thermal behavior of the three analyzed antioxidant enzymes (SOD, CAT, and POD) will differ between *I. pumila* plants growing naturally under full sunlight and ones inhabiting the woodland understory.

MATERIALS AND METHODS

Studied species and *experimental setup*

Iris pumila L. (Iridaceae) is a rhizomatous perennial monocot native to the Deliblato Sands (44º 47' 39" N/ 21º 20' 00" E to 45º 13' 10" N/ 28º 26' 08" E), an isolated complex of sand dunes situated between the Danube River and the western Carpathian slopes in the southern part of Banat (Serbia).

For this study, we selected two natural populations of *I. pumila* living in habitats with contrasting ambient light conditions: one occupying an exposed site along the top and south-facing slope of a dune, and the other residing in the understory of a *Pinus sylvestris* stand. In April 2004, at the peak of the *I. pumila* blooming phase, we randomly selected 10 individual plants (clones) with distinct flower colors (five from each habitat type) and marked each with a wooden peg.

Leaf sample collection

Leaf samples were collected from each of the 10 marked clones once during each of three seasons – spring (15-16 April), summer (1-2 July), and autumn (29-30 September) – in 2004. More specifically, a fully developed leaf was harvested from each plant between 15:00 and 16:00 h and immediately frozen in liquid nitrogen. In the laboratory, leaf samples were stored at -70˚C until preparation.

Tissue extract preparation

Foliar tissue extracts were prepared by pulverization of frozen leaves under liquid nitrogen, followed by sonification in cold 100 mM K-phosphate buffer, pH 6.5, containing 2 mM phenylmethylsulfonyl fluoride and 5% (w/v) insoluble polyvinylpyrrolidine. After centrifugation of the homogenates at 10000 x g at 4ºC for 15 min, the supernatants were used for enzyme assays and soluble protein determination (Bradford, 1976).

Thermal dependence of SOD, CAT, and *POD enzyme activities*

The thermal dependences of antioxidant enzymes were investigated by measuring enzyme activities at 15, 20, 30, 40, and 50ºC. For each temperature, control assays were carried out with samples including all of the reagents, but lacking leaf tissue extracts. The leaf tissue samples were kept on ice, whereas substrates and buffers were kept at measurement temperatures. Separate analyses were done for each population using a mixture of 15 plants (five per season) harvested over the entire growth period. All measurements were conducted in triplicate, with the exception of SOD, whose activity was assayed once per temperature point. The reaction time was 3 min for POD and 1 min for CAT.

Analytical assayes

Peroxidase activity (POD, in μmoles · min^{-1} · mg^{-1} of soluble protein) was assayed by measuring the increase in absorbance at 430 nm and 15, 20, 30, 40, and 50˚C of a reaction mixture containing 1 ml of 100 mM K-phosphate buffer (pH 6.5), 50 μl of enzymatic extract, 60 mM pyrogallol (A_{430} ε=2.4 mM^{-1} · cm^{-1}) as a hydrogen donor, and 10 mM H_2O_2 (Kukavica and Veljović-Jovanović, 2004), using a UV/visible light spectrophotometer (Shimadzu UV-160, Kyoto, Japan).

Catalase activity (CAT, in mmoles of H_2O_2 · min^{-1} · mg^{-1} of soluble protein) was determined spectrophotometrically by measuring the decrease in absorbance at 240 nm and 15, 20, 30, 40, and 50˚C of a reaction mixture containing 1 ml of 100 mM K-phosphate buffer (pH 7.5), 50 μl of enzymatic extract, and 30 mM H_2O_2 (A_{240} ε=0.04 mM^{-1}·cm^{-1}) (Aebi, 1984).

Superoxide dismutase activity (SOD, in units of enzyme activity · mg^{-1} soluble protein) was determined according to Beyer and Fridovich (1987). A reaction mixture containing 3 ml of 100 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 12 mM L-methionine, 75 μM nitroblue tetrazolium chloride (NBT), 2 μM riboflavin, and 0-50 μl of enzymatic extract was exposed to illumination from a 30-W fluorescent lamp for 15 min at 15, 20, 30, 40, and 50˚C to start the photochemical reduction of NBT to blue formazan, which was measured as the increase in absorbance at 540 nm using an ELISA microplate reader. One SOD unit was defined as the amount of

enzyme required to inhibit 50% of the NBT photo reduction in comparison with tubes without the tissue extract that were kept in the dark.

Statistical analyses

The dependent variables used for statistical analyses are individual values of the specific activity of antioxidant enzymes CAT and POD quantified repeatedly using the same mixture of 15 plant for each population. Repeated ANOVA was employed to test for differences in the level and shape of the enzyme thermal curves (von Ende, 2001).

RESULTS AND DISCUSSION

In nature, temperature is a major driving force of enzyme evolution because of its direct effect on the key enzyme properties of specific activity and thermal stability (McNaughton, 1974; Hochachka and Somero, 2002). Since under conditions of reduced thermal energy, the activation energy barriers to conformational changes may slow the rate of an enzymatic reaction, it has been hypothesized that in cold habitats, the optimal adaptive strategy would be development of enzymes with the lowest possible energy barriers to catalytic conformational changes, whereas in warm habitats, enzymes with enhanced resistance to heat denaturation (less structurally flexible) would be more selectively advantageous than others (Hochachka and Somero, 2002).

To understand more fully how temperature influences the behavior of antioxidative enzyme activities, foliar extracts obtained from *I. pumila* plants naturally growing at exposed or shaded sites were assayed separately over a temperature range of from 15 to 50°C. Figure 1 shows plots of the specific enzyme activity of SOD, CAT, and POD against the assay temperature. Using the data acquired from the effects of temperature on enzymatic activity, the apparent energy of activation for each of these reactions was calculated according to the Arrhenius equation (Arrhenius, 1889):

$$\ln (k) = \ln (k_o) - E_a/R \times T^{-1},$$

where k refers to the reaction velocity, k_o is a proportionality constant, E_a is the activation energy, R is the

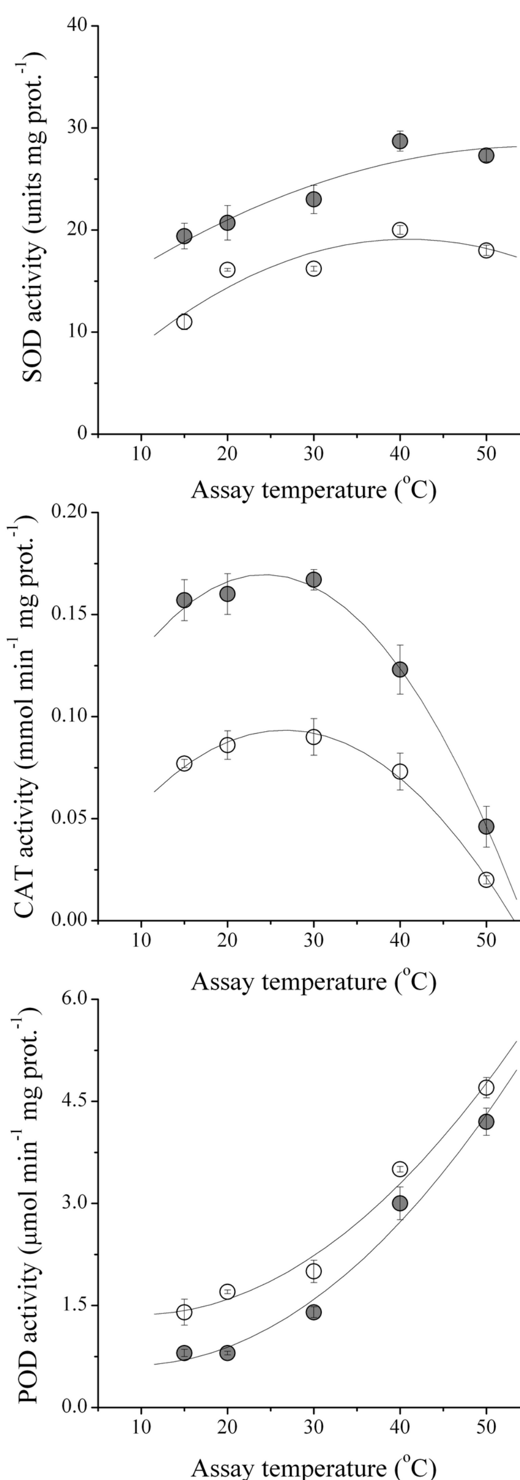

Fig. 1. Activities of SOD, CAT, and POD in leaves of *I. pumila* plants naturally growing in an exposed (○) and a shaded (●) population determined at different assay temperatures. Vertical bars represent the SD of the mean.

Table 1. Activation energies (E^a) of three antoxidant enzymes in *Iris pumila* plants from an exposed and a shaded population, calculated for the temperature range within which each of the enzymes obeyed the Arrhenius equation. SOD = superoxide dismutase, CAT = catalase, POD = peroxidase.

Enzyme	Habitat type	Temperature range (°C)	E^a (kJ · mole^{-1})
SOD	Exposed	15-40	14.9
	Shaded	15-40	11.4
CAT	Exposed	15-30	6.8
	Shaded	15-30	3.2
POD	Exposed	15-50	27.6
	Shaded	15-50	40.6

ideal gas law constant, 8.314 x 10^{-3} kJ · mole^{-1} · K^{-1}, and T is the temperature (in degrees Kelvin).

The key result that emerges from these data is that at a common measurement temperature, the enzymatic activity of SOD and CAT was always higher in plants inhabiting vegetation shade than in ones experiencing full sunlight. The temperature optima of each of these enzymes were similar across alternative light habitats, approaching 40°C for SOD and 30°C for CAT (Fig. 1).

The energy of activation for SOD was only slightly higher in sun-exposed plants than in ones living in vegetation shade (E_a = 14.9 vs. 11.4 kJ · mole^{-1}, respectively; Table 1; Fig. 1). In contrast to SOD, the activation energy of CAT appeared to be two times greater in plants experiencing full sunlight in comparison to those native to the forest understory (E_a = 6.8 vs. 3.2 kJ · mole^{-1}, in exposed and shaded habitats, respectively; Fig. 1). The specific activity of POD generally increased with increasing temperature up to 50°C, and its average level was higher in the exposed relative to the shaded plants (Fig. 1). The activation energy of POD was comparatively greater than that of either SOD or CAT, especially in plants inhabiting vegetation shade (E_a = 40.6 kJ · mole^{-1}; Table 1; Fig. 1).

In naturally growing *I. pumila* plants, the variation of enzyme activity with temperature was found to be strongly protein- as well as habitat-specific. In general, the specific activities of all three examined enzymes, SOD, CAT, and POD, increased gradually with increase of assay temperature; however, the shapes of their temperature response curves appeared to be dissimilar (Fig. 1). In contrast to individual enzymes, the sun and shade ortholog of the same enzyme exhibited comparable thermal behavior, which was reflected in equivalent temperature optima and similar forms of their temperature response curves. However, when the levels of the temperature response curves of each pair of ortholog enzymes were compared, it appeared that the amounts of SOD and CAT specific activities were continuously greater in forest-understory plants than in those experiencing full sunlight. Results of repeated ANOVA corroborated that the CAT temperature response curves were significantly different in their levels (between subjects habitat effect: F = 165.8, df = 1, P = 0.006), but had comparable shape (within subject temperature x habitat effect: F = 2.16, df = 4, P = 0.165) between plants from contrasting light habitats. The temperature response curves of POD orthologs were inversely related, differing in both level (between subjects habitat effect: F = 365.9, df = 1, P = 0.0027) and shape (within subject temperature x habitat effect: F = 76.8, df = 4, P < 0.0001) between shade-exposed plants and ones growing along an open dune site.

Our study provides evidence that in both radiation environments, the amount of activation energy (E_a) for CAT was lower compared to that estimated for either SOD or POD, as well as that the E_a value of CAT in sun-exposed plants was greater than that in forest-understory plants. Because the rate of a chemical reaction reflects the underlying activation energy (Lehninger, 1982), the smaller activation energy of CAT observed in plants located in vegetation shade corresponds to the greater velocity of catalytic activity detected in that habitat (Hochachka and Somero,

2002). An inverse trend was revealed for the thermal behavior of POD kinetic parameters. When all three enzymes were contrasted, the activation energy of POD was the highest, regardless of the environmental light conditions, being, for example, more than 12 times greater in forest-understory plants than the activity estimated for CAT (40.6 vs. 3.2, respectively; Table 1). This exceptionally high E_a value calculated for POD, in conjunction with its resistance to activity loss at high temperatures (50°C), rendered POD a thermally stable enzyme capable of working across a relatively wide range of temperatures (Fig. 1) (Trasar-Cepeda et al., 2007).

Since our preliminary studies on *I. pumila* plants raised under similar ambient conditions in an environment-controlled growth room (100 μmoles · m^{-2} · s^{-1} of PAR; 16 h photoperiod; 21/17°C day/night T) failed to detect significant differences in leaf specific activity of any of the three analyzed enzymes between plants originating from an exposed and a shaded light habitat (data not shown), a significantly higher level of SOD and CAT thermal curves, as well as a lower level of the POD thermal curve, observed in shade-exposed *I. pumila* plants relative to those experiencing full sunlight could be the outcome of acclimatization of these plants to the different thermal and/or light conditions of their habitats, which likely occurred through alteration of the amount of these enzymes in their cells, rather then due to distinct kinetic properties of their sun and shade orthologs (Hull et al., 1997; Hochachka and Somero, 2002; Peltzer et al., 2002).

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ТЕМПЕРАТУРНА ЗАВИСНОСТ АНТИОКСИДАЦИОНИХ ЕНЗИМА СУПЕРОКСИД ДИСМУТАЗЕ, КАТАЛАЗЕ И ПЕРОКСИДАЗЕ У ЛИСТОВИМА *IRIS PUMILA* L.

АНА ВУЛЕТА и БРАНКА ТУЦИЋ

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У овоме раду испитивана је температурна зависност ензима SOD, CAT и POD у листовима биљака *Iris pumila* које насељавају отворена и засенчена природна станишта. Утврђено је да су специфична активност, као и енергија активације SOD и CAT биле ниже код биљака изложених сунцу него код оних које насељавају сенку, док се температурни оптимуми ових ензима нису разликовали између станишта. Специфична активност POD повећавала се са порастом температуре, и била је увек виша код биљака изложених сунцу у поређењу са биљкама из сенке. Активациона енергија ензима POD је била виша од активационе енергије SOD и CAT, али релативно нижа код биљака са отвореног него са засенченог станишта.