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LIGHT INTENSITY INFLUENCES VARIATIONS IN THE STRUCTURAL AND PHYSIOLOGICAL TRAITS IN THE LEAVES OF *IRIS PUMILA* L.

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Abstract — Ambient light significantly influences the structural and physiological characteristics of *Iris pumila* leaves. A random sample of *Iris* clones native to an exposed site at the Deliblato Sands, Serbia was partially covered with a neutral screen that transmitted 35% of daylight, so that each clone experienced reduced and full sunlight at the same time. The sun-exposed leaves were significantly thicker, had greater stomatal density, exhibited higher lipid peroxidation, increased activities of SOD, APX, CAT enzymes and higher contents of non-enzymatic antioxidants (anthocyanins and phenols) and water deficit relative to shade-leaves. The activities of GR, GPX, and GST enzymes was unaffected by the irradiance level.

Key words: Light intensity, antioxidants, functional leaf traits, *Iris pumila*

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INTRODUCTION

Although irradiance is essential to all photosynthetic organisms, it is one of the most unstable environmental factors, particularly on the daily scale (Larcher, 1995; Bazzaz, 1996; Lambers, 2008). Since photosynthesis, and therefore plant growth, primarily depends on light which is one of the most unstable environmental factors, the dynamic responses of plants to variations in irradiance levels deserve special attention (Larcher, 1995; Lambers, 2008). Plants can cope with their light environment at several levels: whole-plant, modular or cellular (Bazzaz, 1996; Evans and Poorter, 2001). In nature, the plasticity of plants to variations in abiotic factors, including light, is more effective at the modular than at the whole-plant level (Bazzaz, 1996; Evans and Poorter, 2001; Mittler, 2006). Because plant growth proceeds by the addition of new modules (e.g. leaves, stems, branches), each plant usually experiences different light conditions during its growth and development due to its sedentary lifestyle (Bazzaz, 1996).

Light-induced structural and physiological modifications in leaves influence the oxidative metabolism in chloroplasts, largely through the effects on light absorption and CO_2 diffusion (Fitter and Hay, 1981; Larcher, 1995; Bazzaz, 1996). In order to minimize excess light interception, many plants growing in full sunlight orient their leaves at a steep angle and produce thicker leaves (Mooney et al., 1977; Tucić et al., 1998). Such leaves have a higher content of most photosynthetic components per unit area, and consequently a higher rate of photosynthesis (Hanba et al., 2002; Oguchi et al., 2005). Leaf thickness is often used for screening plant strategies regarding resource acquisition and use, which, in turn, influence plant ecological performance (Wilson et al., 1999; Díaz et al., 2004; Vile et al., 2005; White and Montes-R, 2005). Since direct determination of leaf thickness is not always simple, it is often estimated indirectly, and a number of substitutes are proposed and used (Vile et al., 2005; White and Montes-R, 2005). One such estimate is the ratio of leaf dry mass to leaf surface area (LMA). In general, LMA increases with light intensity and

is positively related to leaf photosynthetic capacity per unit of leaf area (Lambers and Poorter, 1992; Ellsworth and Reich, 1993; Niinemets et al., 1998; Niinemets, 1999; Vendramini et al., 2002).

Besides LMA, stomatal density also plays an important role in the physiology of plants. Stomata regulate gas exchange, water loss, as well as leaf temperature (Casson and Gray, 2008). In addition, this trait is very sensitive to the irradiance level, and is in correlation with the increase in light intensity (Tucić et al., 1999; Al Afas et al. 2007; Poorter et al. 2009; Loranger and Shipley, 2010). Therefore, stomatal density is responsible for the trade-off between photosynthetic CO_2 gain and water loss due to transpiration (Gutschick, 1999). The impact of light intensity and CO_2 concentration on stomatal density has been documented in a number of plant species (Tucić et al., 1999; Thomas et al., 2003; Coupe et al., 2005; Miyazawa et al., 2006).

Since plants living in the wild are exposed to an array of abiotic factors, many of which are very unstable, they must adjust to them in order to maintain cellular homeostasis, and thus grow and reproduce. In addition, many of the abiotic factors including, for example excess light or extreme temperatures, are damaging to plants, because they can disrupt cellular metabolism and cause an enhanced production of reactive oxygen species or ROS (which includes hydrogen peroxide – H_2O_2, superoxide radical – $O_2{\cdot}^-$, hydroxyl radical – OH·and singlet oxygen $^1O_2$), that induce oxidative stress. The rapid and non-specific reactions of ROS cause damage to all classes of biomolecules, lead to lipid peroxidation and protein and nucleic acid denaturation (Asada and Takahashi, 1987; Mittler, 2002; Apel and Hirt, 2004; Suzuki and Mittler, 2006). Although ROS are a toxic by-product of aerobic metabolism, they also serve as secondary messengers involved in the stress-response signal transduction pathway. Hence, plants have developed a complex antioxidant defense system to regulate ROS cellular concentration.

The most important elements of the defensive response isare non-enzymatic and enzymatic antioxidants (Mittler, 2002; Mittler, 2006). The non-enzymatic components, or low-molecular weight antioxidants, include ascorbate, reduced glutathione (GSH), tocopherols, carotenoids, and phenol compounds. The non-enzymatic antioxidants, such as soluble phenols, appear to be essential for physiological functions associated with acclimatization to stressful environmental conditions (Grace, 2005). Phenols attenuate the high level of visible and UV light, protect plants against pathogens and the deleterious effects of oxidative stress (Grace et al., 1998; Grace, 2005; Neill and Gould, 2003). Anthocyanins are a group of phenolic compounds that can act as strong antioxidants, as well as screening agents against high irradiance levels that can produce photooxidative stress in vegetative tissues (Gould et al., 2002; Neill and Gould, 2003; Petrini et al., 2002).

Among enzymatic antioxidants, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.1), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), class III peroxidase (POD; EC 1.11.1.7), and glutathione-S-transferase (GST) (Bowler et al., 1994; Larcher, 1995; Noctor and Foyer, 1998; Mittler, 2002; Foyer and Shigeoka, 2011) assume key roles.

SOD catalyses the dismutation of superoxide to H_2O_2, and O_2, and is located in chloroplasts, mitochondria and cytosol (Scandelios, 1993; Bowler et al., 1994). In plants, SOD exists in three forms which are classified by their metal cofactors (Mn-SOD, Cu/Zn-SOD, and Fe-SOD). Catalase (CAT) converts H_2O_2 (largely produced in photorespiration) into water and O_2, and is predominantly located in the peroxisomes (Willekens et al., 1997; Fridovich, 1998; Sofo et al., 2004; Feierabend, 2005; Engel et al., 2006). APX detoxifies H_2O_2, and is mostly located in chloroplasts, though microsomal, peroxisomal, membrane-bound, soluble cytosolic and apoplastic isozymes exist as well (Scandalios, 1993; Asada, 1999; Logan et al., 2006). The class III peroxidases (POD) are a large family of multifunctional enzymes. These enzymes commonly use a wide range of different substrates such as phenolic

compounds, lignin precursors, auxin and various secondary metabolites to regulate H_2O_2 levels and ROS production in the vacuoles, cell wall and apoplast (Hiraga et al., 2001; De Gara, 2004; Passardi et al., 2005). GR is a homodimeric flavoprotein which catalyses the NADPH-dependent reduction of oxidized glutathione and theplays a central role in maintaining the cellular level of reduced glutathione. In photosynthetic tissue, GR is mostly located in the chloroplast, although different isoforms exist in the mitochondria and cytosol (Edwards et al., 1990; Lascano et al, 2003). The GST family of mainly cytosolic proteins has a large range of protective functions in the cell, including the detoxification of H_2O_2 and hydroperoxides, removal of some potentially cytotoxic compounds, regeneration of ascorbate from dehydroascorbate, and the transport of anthocyanins from the cytosol to the vacuoles (Cummins et al., 1999; Mueller et al., 2000; Collinson et al., 2002). The GPXs remove H_2O_2 as well as organic and lipid hydroperoxides, using glutathione as a reducing agent (Flohé and Gunzler, 1984; Kühn and Borchert, 2002; Yoshimura et al., 2004). GPXs are considered to be the main enzymatic defense mechanism against oxidative membrane damage (Kühn and Borchert, 2002; Yoshimura et al. 2004).

In this study, a field experiment was conducted to investigate the effect of light intensity on structural and physiological leaf traits in *Iris pumila* clones naturally growing in a population inhabiting a sun-exposed site. Within-clonal variation in light intensity was achieved by shading one half of each selected clone with a neutral PVC screen which reduced light intensity by about 65% without changing light quality. The other half of the same clone was exposed to full sunlight.

The aims of the study were: (i) to elucidate the impact of variations in ambient light intensity on the phenotypic expression of structural and physiological leaf traits in *I. pumila* plants within their natural habitat; and (ii) to determine the most important components of the antioxidative system in this taxon.

MATERIAL AND METHODS

Plant materials and experimental design

Iris pumila (L.) is a perennial rhizomatous monocot which is very abundant along the sun-exposed dune sites in the Deliblato Sands (44° 47' 39" N / 21° 20' 00" E to 45° 13' 10" N / 28° 26' 08" E) – an isolated complex of sand masses situated about 50 km northeast of Belgrade, Serbia. Less frequently, *I. pumila* populations inhabit the forest understory. Individual plants form circle-shaped clones composed of rhizome segments which spread from the center of a clone toward its periphery. The species exhibits a remarkable flower color polymorphism, which enables the identification and mapping of individual clone genotypes in the field (Tucić et al., 1988).

In April 2007, at the peak of the blooming phase of *I. pumila*, six large clones from a sun-exposed population were randomly selected, enumerated with a wooden tag and thereafter half of each of them was covered with a neutral PVC screen. In July 2000, the, leaf samples were harvested from the shaded (referred to as shade-leaves) and unshaded (sun-leaves) parts of the marked clones. For physiological analyses, the last fully expanded leaf was cut between 15:00 h and 16:00 h, immediately frozen in liquid nitrogen, transported to the laboratory, and stored at -70°C until preparation.

Leaf extract preparation

Frozen leaf material was individually grounded to a fine powder in liquid nitrogen and extracted using ice cold 100 mM potassium-phosphate buffer (pH 6.5) containing 0.1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5% (w/v) insoluble polyvinylpyrrolidine and 0.2% Triton X-100. For APX activity measurements 5 mM ascorbate was added to the extraction buffer. After centrifugation of the crude extracts at 10,000xg at 4°C for 20 min, the obtained supernatants were used for measuring enzymatic activity. The soluble protein content was determined according to Bradford (1976).

Antioxidative enzyme assays

The activities of all enzymes were measured using a Shimadzu UV-160 spectrophotometer (Shimadzu UV- 160, Kyoto, Japan) at 30°C in triplicate.

POD activity was measured as an absorbance increase at 430 nm in a 1 ml reaction mixture containing 50 µl of crude extract, 100 mM potassium-phosphate buffer (pH 6.5), 6 mM pyrogallol (A_{430} ε = 2.4 mM^{-1} cm^{-1}) as a hydrogen donor, and 10 mM H_2O_2. The specific activity of POD was expressed as µmol min^{-1} mg^{-1} soluble protein.

GR activity was measured as an absorbance decrease de ue to thGSSG-dependent oxidation of NADPH at 340 nm, in a reaction mixture consisting of 50 µl of crude extract, 100 mM potassium-phosphate buffer pH 7.5, 1 mM EDTA, 0.2 mM NADPH (A_{340} ε = 6.2 mM^{-1} cm^{-1}), and 0.2 mM oxidized glutathione (Foster and Hess, 1980). Specific GR activity was expressed as µmol NADPH min^{-1} mg^{-1} soluble protein.

GST activity was determined as an absorbance increase at 340 nm due to conjugation of the thiol group of GSH to the 1-chloro-2, 4-dinitrobenzene (CDNB) substrate. The reaction mixture consisted of 100 mM potassium-phosphate buffer (pH 6.5), and the required amount of crude extract and 0.8 mM CDNB (A_{340} ε = 9.6 mM^{-1} cm^{-1}) (Habig and Jacoby, 1981). GST specific activity was expressed as nmol of S-conjugate min^{-1} mg^{-1} soluble protein.

GPX activity was measured as described by Wendel (1980), following NADPH oxidation at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). The reaction mixture was constituted of 50 µl of crude extract, 50 mM potassium-phosphate buffer (pH 7.0), 1 mM EDTA, 0.24U GR, 1 mM GSH, 0.12 mM NADPH, 0.02 mM DL-dithiothreitol and 0.0007% H_2O_2. The specific activity of GPX was expressed as µmol NADPH min^{-1} mg^{-1}soluble protein.

Activity of antioxidative enzymes in native PAGE

Different isoforms of the analyzed antioxidative enzymes were separated electrophoretically using a discontinuous polyacrylamide gel and buffer system (PAGE) under non-denaturing conditions at 4°C (Laemmli, 1970).

SOD isoforms were separated on 10% gels using a Mini-Protean Tetra Cell (Bio-Rad Laboratories, Hercules, CA, USA) and visualized using the photochemical method of Beauchamp and Fridovich (1971). Tissue samples containing an equal amounts of protein (40 µg) were loaded per gel lane. After electrophoresis, the gels were incubated in a 50 mM potassium-phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.1 mM nitroblue tetrazolium and 0.05 mM riboflavin, washed in distilled water and subsequently illuminated until clear SOD bands appeared on a dark purple background. For identification of different SOD isoforms, the gels were preincubated for 15 min in 5 mM H_2O_2 (an inhibitor of Cu/Zn-SOD and Fe-SOD) or 2 mM KCN (an inhibitor of Cu/Zn-SOD) in 0.1 M potassium-phosphate buffer (pH 7.8), respectively.

The CAT isoforms were separated on 7 % gels, and thereafter equilibrated in 3.27 mM H_2O_2 for 20 min at room temperature, briefly rinsed in distillated water, and stained in a solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferrichloride (Woodbury, 1971).

APX isoforms were separated on 10% gels using buffer with 2 mM ascorbate. Following electrophoresis, the gels were incubated for 15 min in 0.1 M potassium-phosphate buffer (pH 6.5) containing 4 mM ascorbate and 4 mM H_2O_2, washed with distilled water, and then stained with 0.1% ferricyanide and 0.1% ferrichloride (w/v) in 0.125 N HCl at room temperature.

Band intensities were estimated by measuring their individual densities with ImageQuant image analysis software (Amersham Biosciences Ltd.). In order to make the quantitative comparisons between multiple gels reliable, an internal reference sample (foliar mixture of all analyzed genotypes) was run on each gel. The activities of different isoforms were

then standardized with the reference sample to obtain their relative intensity which was expressed in arbitrary units (AU).

Soluble phenols and anthocyanin content analysis

The content of soluble phenols was determined by the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). Leaf material was pulverized under liquid nitrogen, homogenized in 80% methanol (fw:v = 1:10) for 1h, and then centrifuged at 10,000xg for 30 min. The supernatant (50 µl) was mixed with 475 µl of 0.25 N Folin-Ciocalteu reagent and after 3 min 475 µl of 1 M sodium carbonate was added. The absorbance of the resulting blue-colored solution was measured at 724 nm after incubation at 30°C for 1h. Different concentrations of gallic acid in 80% methanol were used as the standard. The total soluble phenol content was expressed as gallic acid equivalents (GAE) in mmol g^{-1} of fresh material.

Anthocyanins were extracted according to Mancinelli et al. (1975). A 1 cm^2 part of each frozen leaf was pulverized under liquid nitrogen, and thereafter homogenized in 1 ml of 1% (w/v) HCl in methanol for 2 days at 5°C with continuous shaking. Absorbance of the extracts was measured spectrophotometrically at 530 and 653 nm (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). Anthocyanin absorbances were calculated as A_{530} - 0.24 A_{653} (Murray and Hackett, 1991). The anthocyanin content was computed as cyanidin-3-glucoside equivalents, using 26,900 L mol^{-1} cm^{-1} as the extinction coefficient and 445 as the molecular weight.

Lipid peroxidation

Lipid peroxidation, in terms of the malondialdehyde (MDA) content, was measured spectrophotometrically using a modified TBA-MDA method (Hodges et al., 1999). Leaf samples were homogenized in 80% ethanol, followed by centrifugation at 3,000×g for 10 min. One ml of the supernatant was vigorously mixed in a test tube with 1 ml of 20% TCA or 0.65% TBA in 20% TCA, and heated at 95°C for 25 min. After inserting the test tubes into crushed ice to stop reaction, the mixture was centrifuged at 3,000×*g* for 10 min. Absorbance at 440, 532, and 600 nm was read and used for calculation of MDA equivalents as follows:

(1) $[(\text{Abs } 532\text{nm}_{+\text{TBA}}) - (\text{Abs } 600\text{nm}_{+\text{TBA}}) - (\text{Abs } 532\text{nm}_{-\text{TBA}} - \text{Abs } 600\text{nm}_{-\text{TBA}})] = A$

(2) $[(\text{Abs } 440\text{nm}_{+\text{TBA}} - \text{Abs } 600\text{nm}_{+\text{TBA}})\ 0.0571] = B$

(3) MDA equivalents (nmol mL^{-1}) = $(A - B/157000) \times 10^6$

LMA, leaf water potential and stomatal density

Leaf mass per unit area (LMA; in g cm^{-2}) was calculated as the dry mass of a leaf divided by its projected fresh area. The fresh area of each leaf was first scanned by a manual scanner, oven dried at 60°C to constant mass and then weighed.

A Digital Plant Moisture System (SKPM1400, Skye Instruments Ltd, United Kingdom) was used for measuring leaf water potential (Ψ_{leaf}) in the last fully developed leaves sampled at midday (12:00–14:00 h). Water loss from the excised leaves was limited by humidifying the pressure chamber with a wet paper towel (Tyree and Hammel, 1972; Turner and Long, 1980). Leaf water potential was expressed in MPa.

Stomatal density (SD) was estimated by applying a micro-relief method (Pazourek 1970). Briefly, an area in the middle sections of the leaf adaxial surfaces was painted with a transparent nail polish. The dry polish was peeled from the leaf surface by a piece of sellotape and then fixed on a microscope slide. The stomata number was counted in 20 randomly selected microscopic fields (0.196 mm^2 at 40 x magnification).

Statistical analysis

All statistical analyses were conducted using a sample of six clonal genotypes. The statistical significance of the effects of different light intensities on the struc-

tural and physiological leaf traits was evaluated by applying a dependent *t*-test from the SAS statistical software (SAS Institute Inc., 2003). Statistical significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Impact of light intensity on leaf structure, membrane oxidative injury and water status

Leaf traits usually reflect the adaptive strategies of plants to essential environmental resources, including light, temperature, water, and nutrition (Lambers and Poorter, 1992; Vendramini et al., 2002). For example, a greater LMA is associated with a higher photosynthetic rate per unit leaf area. However, the value of LMA, and therefore the potential for photosynthesis, is traded-off with the size of leaf surface area for light interception. Consequently, LMA is highly responsive to abiotic environmental factors, including high light, drought and elevated temperature (Lambers and Poorter, 1992; Niinemets, 1999; Vendramini et al., 2002; Sack et al., 2003; Liu et al., 2008). A greater LMA is advantageous in high irradiance habitats because it allows a greater amount of photosynthetic apparatus components per unit leaf area, and thus a higher photosynthetic capacity, i.e., a higher carbon gain (Al Afas et al., 2007; Poorter et al., 2009). Conversely, larger and thinner leaves with lower LMA can intercept more light, and as such are required for shade tolerance in herbs inhabiting the forest understory (Tucić et al., 1998; Al Afas et al., 2007; Poorter et al., 2009). Table 1 presents variations in LMA and stomatal density expressed in the leaves of *I. pumila* clones growing under contrasting light conditions. In concurrence with other studies, our results provided evidence that sun-leaves had significantly higher LMA values and a greater number of stomata per unit area than their shaded counterparts (Hanba et al., 2002; Thomas et al., 2003; Coupe et al., 2006; Tanaka and Shiraiwa, 2009). Because stomata regulate gas exchange (mainly CO_2 and water vapor) variations in their number allows plants to optimize their photosynthetic performance in accordance with water availability and usage (Casson and Gray, 2008; Loranger and Shipley, 2010). Apart from osmotic adjustment, plants can maintain water balance by increasing the rigidity of cell walls. This kind of "elastic adjustment" contributes to the preservation of water flow throughout the leaves in spite of conditions that potentially induce osmotic stress. The thicker cell walls, enhanced lignification, larger LMA and higher number of SD per leaf area may cause a decrease in stomatal conductivity, leading to increased water use efficiency, and consequently to greater drought resistance (Hanba et al., 2002; Tanaka and Shiraiwa, 2009; Niinemets and Valldares, 2004). In our experiment, leaf water potential was more negative in the shaded leaves than in the sun-exposed leaves, indicating a greater water stress in the former compared to the latter.

The content of MDA (a measure of membrane oxidative damage) exhibited a greater value in the sun-exposed leaves than in the shaded leaves (16.4 vs. 13.3 nmol g_{fw}^{-1}, respectively; Table 1). The obtained results are in agreement with the *trade-off hypothesis* which predicts that shade tolerance increases when the allocation of resources is higher to the shoot than to the root, as well as when SLA (lamina area/total dry mass) is high, providing a greater irradiance capture under light-deprived conditions (Smith and Huston, 1989). On the other hand, a large evaporative leaf surface area combined with the reduced ability to acquire water make drought tolerance impossible in the shade (Smith and Huston, 1989). Valladares and Pearcy (2002) reported that plants living in the forest understory exhibited a much larger decrease in Ψ_{leaf} during the summer than plants from an open habitat, indicating a significantly greater water stress in the shaded as compared to the exposed sites. In our study, the water potential values indicated only a mild water stress (-0.83 MPa in sun-exposed leaves and -1.07 MPa in shaded leaves), although the soil water content was very low (~ 3%; data not shown), suggesting that *I. pumila* can override severe drought by storing water and/or assimilates in the rhizomes.

Table 1. Results of a dependent *t*-test exploring the effect of variation in light intensity on the activity of antioxidative enzymes (SODs, CAT, APX POD, GR, GST, and GPX), the content of low molecular antioxidants (anthocyanins and phenol), as well as structural (SD and LMA) and physiological (MDA and Ψ_{leaf}) leaf traits in *Iris pumila* clones naturally growing in the wild. For trait acronyms, see Material and Methods.

	Natural light intensity					
	Full daylight		Reduced daylight			
Trait	Mean	SE	Mean	SE	*t* (df=5)	P
Total SOD	4.636	0.269	3.732	0.156	4.49	0.006
Mn-SOD	3.396	0.197	2.768	0.104	3.68	0.014
Cu/Zn-SOD	1.240	0.074	0.964	0.056	8.74	0.000
CAT	1.550	0.119	1.235	0.149	4.50	0.006
APX	2.216	0.425	1.282	0.197	2.62	0.047
POD	0.651	0.065	1.055	0.101	-4.19	0.009
GST	0.047	0.005	0.054	0.005	-1.52	0.188
GPX	0.200	0.012	0.200	0.015	-0.06	0.955
GR	0.113	0.006	0.126	0.009	-1.19	0.286
Phen	5.352	0.517	4.563	0.130	1.49	0.197
Anth	22.490	2.745	9.550	0.687	5.38	0.003
MDA	16.400	1.075	13.300	1.148	1.46	0.203
LMA	0.006	0.001	0.005	0.001	2.75	0.041
SD	94.640	4.750	75.770	3.280	3.07	0.028
Ψ_{leaf}	-0.831	0.075	-1.075	0.152	1.41	0.218

Light-dependent variations in activities of antioxidative enzymes

Plants growing under strong light are more frequently faced with ROS generated in the chloroplasts than plants growing at low light intensity (Slooten et al., 1995; Grace and Logan, 1996). Consequently, sun-exposed plants might respond by accumulating a higher amount of antioxidants in the leaf tissue to protect them not only against oxidative stress, but also from other environmental stresses. A statistically significant effect of light intensity observed in the dependant *t*-test clearly indicates that the activity of the antioxidative enzymes SOD, APX, and CAT in *I. pumila* leaves was indeed higher in sun-exposed than in shaded leaves (Table 1). As was already reported, the total SOD activity was significantly greater in the sun-exposed leaves compared to the shaded leaves. When native gels were stained for SOD activity, five isoforms were detected (Figure 1). The SOD isoforms 1 and 4 were identified as Mn-SOD by their insensitivity to KCN and H_2O_2, whereas the SOD isoforms 2, 3 and 5 were recognized as Cu/Zn-SOD, because of their inhibition by both KCN and H_2O_2. The Fe-SOD isoform was not detected in the *I. pumila* leaves. Again, the activity of two SODs, Mn-SOD –predominantly located in mitochondria and peroxisomes, and Cu/Zn-SOD – distributed in all cell compartments, were higher in the sun-exposed leaves compared to the shaded leaves. Due to high H_2O_2 toxicity, any increase in SOD activity must be associated with a mechanism that controls the H_2O_2 content, as has been shown in experiments with transgenic tobacco plants overexpressing different SOD isoforms (Bowler et al., 1994,

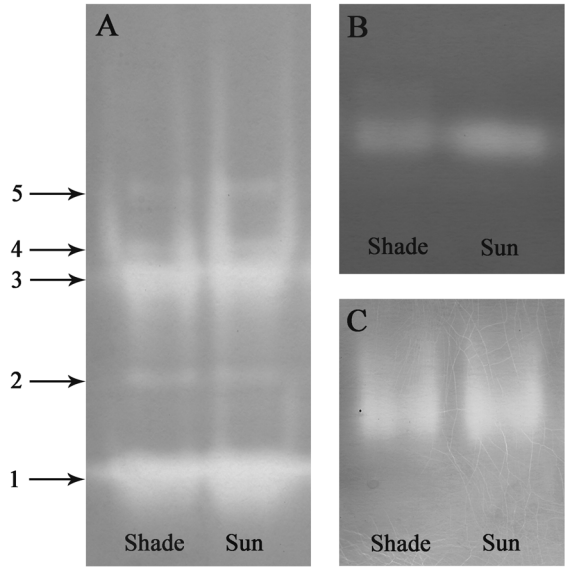

Fig. 1. The effects of light intensity on the pattern of isoenzymes (A) SOD; (B) APX; (C) CAT, in *I. pumila* leaves localized on the native gels. Bands 1 and 4 are Mn-SOD, while bands 2, 3 and 5 are Cu/Zn-SOD isoenzymes.

Alscher et al., 2002). Even a moderate increase in SOD activity can elevate the tolerance to oxidative stress, since it maintains an equilibrium between $O_2^{\cdot -}$ radicals and H_2O_2 (Bowler et al., 1994, Alscher et al., 2002). In contrast, the tobacco plants that exhibited approximately a 50-fold increase in SOD expression did not possessan elevated tolerance to oxidative stress (Tepperman and Dunsmuir, 1990). Our study provides evidence that the relative APX and CAT activities showed similar trends as SODs, displaying significantly higher values for the sun-exposed than for the shaded leaves (Table 1). Gels stained for CAT and APX activity did not reveal isoforms of these enzymes in *I. pumila* (Fig. 1). In the leaves of micropropagated *Phalaenopsis* raised in a culture room, CAT activity increased gradually with increasing light intensity (Ali et al., 2005). It was recently reported that in Arabidopsis and pea plants the expression of genes encoding antioxidative enzymes was also highly affected by light intensity, particularly those for *CAT, cytAPX* and *cytCu/Zn-SOD* transcripts (Hernandez et al., 2006; Murgia et al., 2006). Similar results were obtained in Arabidopsis and spinach, which accumulated *cytAPX* transcripts in response to high irradiance (Yoshimura et al., 2000; Fryer et al., 2003).

Since the enzyme CAT is involved in the scavenging of photorespiratory H_2O_2, there is a close association between the level of catalase activity and the rate of photosynthesis (Feierabend, 2005). This finding is in accordance with our data that showed a greater specific activity of CAT and a higher LMA value in the sun-exposed leaves than in the shaded leaves of the same *Iris* clones.

Light intensity affected POD activity in an opposite direction to APX and SOD, having a higher value in the shaded leaves than in the sun-exposed leaves of *I. pumila*. Enzymes included in glutathione metabolism like GR, GPX and GST exhibited similar specific activities in alternative light environments, suggesting that some other environmental factors may affect their regulation (Table 1). Gechev et al. (2003) analyzed GPX protein levels by Western blot analysis in tobacco leaves and revealed only a minor transient increase in protein level in different light treatments, but a stronger induction during chilling stress. The activity of GR and GST in the leaves of *Morus alba* cultivars increased significantly due to salinity stress (Sudhakar et al., 2001). In the *Phalaenopsis* plantlets, the GR activity in the leaves and roots did not vary with light intensity, in contrast to GST activity, which exhibited a pronounced increase under strong light conditions (Ali et al., 2005).

Impact of light intensity variations on the content of phenols and anthocyanins

The content of soluble phenols tended to be higher in the sun-exposed leaves of *I. pumila* than in the shaded leaves (Table 1). Conversely, the amount of foliar anthocyanins significantly differed between distinct light environments. Leaves developed in full sunlight accumulated more anthocyanins than those grown in lower light intensity under a neutral screen (Table 1). Anthocyanins can serve as screening agents that lower the high intensity

of daylight, thus protecting the chloroplasts from the photoinhibitory and photooxidative effects caused by strong radiation (Steyn et al. 2002; Neill and Gould, 2003; Hughes et al., 2005; Tucić et al., 2009; Vuleta et al., 2010). In addition, anthocyanins can act as powerful antioxidants (Gould, 2004; Hatier and Gould, 2008). Their capacity to scavenge free radicals and other ROS was found to be about four times greater than that of ascorbate and α-tocopherol (Wang et al., 1997; Grace, 2005). In summary, our results imply that in *I. pumila* leaves, high light intensity activates multiple photoprotective mechanisms, including physiological traits such as enzymatic and non-enzymatic antioxidants together with structural traits – LMA and SD.

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