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Original scientific paper

Antioxidative response of *Melissa officinalis* L. and *Valeriana officinalis* L. leaves exposed to exogenous melatonin and excessive zinc and cadmium levels

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Abstract: Heavy metals disturb the redox homeostasis of the plant cell. The indolamine hormone, melatonin, protects plants from oxidative damage by directly scavenging reactive oxygen species or by stimulating the activity of antioxidant enzymes. The antioxidative role of melatonin in the leaves of two medicinal plants, lemon balm (*Melissa officinalis* L.) and valerian (*Valeriana officinalis* L.) that were treated with increased concentrations of Zn and Cd 24 h after sowing at an open field, were investigated. The plants were treated with Zn, Cd, melatonin and a mixture of melatonin with the mentioned metals. Exogenously added melatonin increased the endogenous melatonin concentration in lemon balm leaves. However, in the valerian leaves, lower or the same endogenous melatonin level was detected. The significantly higher concentration of endogenous melatonin in both plants was measured after treatment with Zn. As the results showed, changes in superoxide dismutase (SOD) and peroxidase (POD) activities are species-specific and change depending on the plant development phase, and the type of treatment. Melatonin pretreatment induced alternation in SOD isoenzyme profiles and activities as well as POD activity in both plant species treated with heavy metals.

Keywords: melatonin: lemon balm; valerian; superoxide dismutase; peroxidases; Zn; Cd.

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) is an ubiquitous hormone that was first identified in vascular plants in 1995.[1] Melatonin was detected in more than 20 dicotyledonous and monocotyledonous plant families in vegetative and generative organs in concentrations between several pg to several mg per g of

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tissue.[2] Various studies suggested specific physiological roles for melatonin in plant growth.[3]

A rapid change in environmental (light and temperature) and various biotic and abiotic stresses may increase or decrease the melatonin content. The effect of exogenously applied melatonin on plants is concentration-dependent: it ranges from significant growth improvement to being non-effective and even having toxic effects.[4]

It was shown that melatonin level increased 12-fold in *Lupinus albus* under different stresses (draught, anaerobic, cold, H_2O_2 and $ZnSO_4$) compared to control plants, which indicated that melatonin biosynthesis is upregulated under common stress situations.[5]

Heavy metals in plants can cause a series of disorders: inhibit photosynthesis and respiration, induce oxidative stress, inhibit growth, *etc*.[6] Cadmium causes photosynthesis inhibition after short and long-term treatment,[7] inhibits growth,[8] and interferes with the uptake and transport of Ca, Mg, P, K and water by plants.[9] Unlike Cd, Zn is an essential micronutrient involved in many vital processes necessary for normal plant growth and development. However, excessive Zn leads to inhibition of plant growth, reduction of photosynthetic pigments and leaf chlorosis, has a negative effect on the membranes permeability, electron transport chain uptake and translocation of nutrients and also has genotoxic effects.[8]

Hasan *et al.*[10] found that exogenous application of melatonin could mitigate phytotoxicity induced by Cd in *Solanum lycopersicum* L., modulating phytochelatins biosynthesis, vacuolar sequestration and anti-oxidative metabolism. The authors showed that the Cd and melatonin concentrations were gradually increased over time under Cd stress. However, such increase in endogenous melatonin was incapable to reverse the detrimental effects of Cd. The content of melatonin in rice increased under Cd stress, suggesting that melatonin plays a crucial role in modulating the responses to Cd stress in different plant tissues.[11] Additionally, a comprehensive genetic and molecular analysis of tomato HsfA1a and melatonin in response to Cd treatment was provided by Cai and co-workers.[12] Cd stress induced the expression of HsfA1a which acts as a positive regulator of COMT1 transcript levels and melatonin accumulation.[12]

Reactive oxygen species (ROS) are formed by a single-electron reduction of O_2 and include free radical types such as superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), and non-radical but highly reactive types, such as H_2O_2.[13] ROSs occur under normal physiological conditions in all plant cells and compartments as a result of photosynthesis, respiration, β-oxidation of fatty acids, *etc*. Different types of biotic and abiotic stress lead to an increase in ROS production (oxidative stress), which results in damage to biomacromolecules (proteins, lipids and DNA) and eventually could lead to cell death. Cd and Zn are non-redox active metals and do not produce ROS directly through Haber–Weiss reactions. Oxid-

ative stress could be the indirect consequence of Zn and Cd toxicity by interfering with the antioxidant defense system.[14] Since ROSs are continuously generated in aerobic metabolism, they must be permanently removed in order to maintain a redox balance and avoid cell damage. Removal and inactivation of ROSs are performed by an antioxidant defense system that includes enzymatic and non-enzymatic antioxidants.[13] Superoxide-dismutases (SOD: EC 1.15.1.1) are metalloenzymes that catalyze the degradation of $O_2^{\bullet-}$ to hydrogen peroxide and represent the primary antioxidants defense. According to the type of metal cofactors, SOD isoforms are classified as CuZnSOD, FeSOD and MnSOD that are located in different compartments of plant cells: CuZnSOD in chloroplasts, cytoplasm and apoplasts; MnSOD in mitochondria and peroxisomes and FeSOD in chloroplasts.[15] Plant Class III peroxidases (POD: EC 1.11.1.7), also called secretory peroxidases, are glycoproteins that contain heme as a prosthetic group. Besides numerous physiological functions, such as lignification, suberinization, indole-3-acetic acid catabolism, response to wounding and pathogens, peroxidases are antioxidant enzymes that remove hydrogen peroxide by reaction with a number of different phenolic substrates or *via* a POD/phenol/ascorbate mechanism in apoplast and vacuoles.[16] Melatonin has the ability to cross all morphophysiological barriers and it is widely distributed in tissues, cells and subcellular compartments.[17] There is broad evidence that melatonin protects plants from oxidative damage and harmful environmental conditions.[18,19] In addition to direct ROS scavenging, melatonin protects plant cells by chelating heavy metals and preventing the formation of free radical species.[20]

The available knowledge about the effect of melatonin on the antioxidant metabolism of plants exposed to various types of abiotic stress is summarized in Table S-I of the Supplementary material to this paper. Data on plant species, type and duration of stress, concentration of applied melatonin and their effects on the activities of SOD, POD, catalase (CAT) and ascorbate peroxidase (APX) are given.

It has been shown that melatonin acts as an activator of antioxidant enzymes in the case of different types of abiotic stress, such as salt stress[18,21] and Cd stress,[10,22] improving cellular redox homeostasis and protecting cells from oxidative stress.

Medicinal plants are the main source of natural antioxidants. Lemon balm (*Melissa officinalis* L.) and valerian (*Valeriana officinalis* L.) are two medicinal plants with sedative, depressant, spasmolytic and antibacterial properties and are widely used as calming and sleeping aid, as well as an additive in food, cosmetics, ornaments and medicine.[23] Antioxidative enzymes, SOD and POD, were not thoroughly explored in these plants. However, prompt activation of antioxidative enzymes is of great importance for the mitigation of the toxic effects of Zn and Cd.

The response of superoxide dismutase (SOD) and peroxidase (POD) activities in leaves of two medicinal plants under exogenous melatonin and oxidative stress conditions (induced with elevated concentrations of Zn and Cd) was evaluated for the first time through the present research. This study provides novel data on the role of melatonin in antioxidative defense in lemon balm and valerian leaves under oxidative stress.

EXPERIMENTAL

Reagents

Melatonin was purchased from Sigma–Aldrich (Steinheim, Germany) and was used as a 100 μmol L^{-1} solution in methanol. 4-Chloro-1-naphthol was purchased from Sigma–Aldrich (Steinheim, Germany). Methanol, acetonitrile, water (HPLC-grade), EDTA, glycine, Tris and all salts were purchased from Lach–Ner (Czech Republic). Poly(vinyl pyrrolidone) (PVP) was purchased from Acros (Belgium), phenylmethylsulfonyl fluoride (PMSF) and polyoxyethylene sorbitan monolaurate (TWEEN 20), nitro blue tetrazolium (NBT) and tetramethylethylenediamine (TEMED) from Fisher Bioreagents (USA), and riboflavin and glycerine from Semikem (Bosnia and Herzegovina). All the standard solutions were stored at 4 °C and brought to ambient temperature prior to use.

Plant material, treatments and growth conditions

Seeds of lemon balm (*Melissa officinalis* L.) were rinsed well under running water and twice with distilled water. The seeds were planted in plastic pots containing air-dried conventional substrate and daily sprayed with distilled water for the control and 1 mM melatonin solution, respectively, until germination was complete and plants had two formed cotyledons. The temperature was between 20 and 25 °C in the terms of the photoperiodic cycle. After 30 days of pretreatment with melatonin, the plants were sown under the open field conditions, and within 24 h treated with 3 g L^{-1} zinc sulfate and 15 mg L^{-1} cadmium sulfate solutions. The treatment was repeated three times. The temperature in the growth period varied from 15.5 to 22.9 °C, according to the data collected from the State Hydrometeorological Institute, Bosnia and Herzegovina, for 2016. Valerian was treated differently due to the rapid decline in germination. Valerian seedlings were submerged for 48 h in distilled water for the control and 1.0 mM melatonin solution for the experiments. The temperature was between 20 and 25 °C in the terms of the photoperiodic cycle after which the seedlings were sown in an open field, and within 24 h treated with 3 g L^{-1} zinc sulfate and 15 mg L^{-1} cadmium sulfate solution. The treatment was repeated three times. Samples of the lemon balm for the melatonin analysis were harvested in July, and valerian in October, lyophilized at –50 °C for 25–30 h (VaCo 2, ZIRBUS Technology, GmbH, Germany) and stored at 4 °C for extraction. For determination of proteins and antioxidant enzymes, the samples were harvested 12 h, and 15, 30 and 45 days after heavy metal treatment, frozen in liquid nitrogen for 15 days and stored at –30 °C for extraction (Figs. S-1 and S-2 of the Supplementary material to this paper).

Melatonin extraction and analysis

Melatonin was extracted with methanol as a solvent.[24] Sample preparations were performed under dark artificial light, due to the possible influence of light on analyte degradation. For melatonin analysis, 0.1–1.0 g of lyophilized and grounded leaves were weighed in test tubes with methanol in a total volume of 10 mL. The samples were left overnight (15–17 h) at 4 °C, with shaking, after which ultrasonic treatment on an ultrasonic bath (WiseClean WUC,

Witeg GmbH, Germany) was performed for 30 min at 4 °C (mixture of water and ice was used for cooling). The tubes with plant tissues were centrifuged at 6000 rpm for 30 min (Alresa Mod, Digicen). The supernatant was transferred to a second vial, and the remaining plant residue was washed three times with 0.5 mL of methanol. The extract of each sample was evaporated to dryness under vacuum (Rotavapor R-215, Buchi Switzerland). The residue was redissolved in 1 mL of methanol, transferred to vials and stored at 4 °C until analysis. Prior to analysis, the samples were filtered (0.45 µm) and analyzed by high-pressure chromatography coupled with a fluorescence detector (FLD). All extracts were stored in dark bottles protected from light at 4 °C.

Melatonin was identified and quantified by high performance liquid chromatography, HPLC (Agilent 1100 Series) with reversed phase C18 gravity column (Nucleodur, 3 µm particle diameter, 150 mm×4 mm, Macherey-Nagel, Germany) with an integrated pre-cell and programmed mobile phase 20 % methanol:80 % water. The retention time of melatonin was determined by the fluorescence (FLD) detector. The flow rate of the analyte was 1.5 mL min^{-1}, at room temperature.

Extraction and determination of the protein content

The leaf tissues (0.5 g) were powdered in liquid nitrogen, homogenized in 4 mL of 100 mM sodium phosphate buffer (pH 6.4) containing 1 mM PMSF, 0.2 % TWEEN 20 and 2 % PVP. The extract was centrifuged at 4 °C for 10 min at 10000 rpm and the supernatant containing soluble proteins was used to determine protein concentration and enzyme activity. The concentration of total proteins was determined by the Lowry method with bovine serum albumin as standard.[25]

Enzyme activity assay

A modified Teisseire and Guy method[26] (2000) was used for the peroxidase activity assay, in which the increase in absorbance at 430 nm (ε_{430} = 12 mM^{-1} cm^{-1}) was monitored. A Shimadzu UV-1800 spectrometer was used for the measurements. The reaction was initiated by adding 3.43 mM H_2O_2 to a mixture containing 50 µL of sample, 10.3 mM pyrogallol and Na-phosphate buffer (pH 6.4) at 37 °C. The activity of peroxidases is expressed as µmol min^{-1} mg^{-1} protein.

Native electrophoresis was performed on an 8 % running gel. The electrophoresis buffer contained 0.025 M Tris and 0.192 M glycine (pH 8.3) and an electric current intensity of 120–160 V was applied. Prior to loading on the gel, the samples (100 µL) were mixed with a loading buffer (20 µL) containing 50 mM Tris (pH 6.8), 10 % glycerol and 0.01 % bromophenol blue. A sample volume corresponding to the amount of 10 µg of proteins was applied to the native gel. SOD isoforms were determined after incubation of the gel in a solution for specific SOD staining that consisted of 0.02 % NBT, 2 mM EDTA, 0.004 % riboflavin, 0.004 % TEMED in 0.1 M Tris buffer (pH 7.8). After incubation for 30 min of, the SOD isoforms were detected as transparent bands on the violet gel. All gels were scanned and retention factor values (R_f) and total SOD activities were determined using Image Master TotalLab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA). The total SOD activity for each sample represents the sum of the activities of the individual SOD isoforms.

Statistical analysis

The data were analyzed using SPSS Statistics 23 (2013). Analysis of variance (ANOVA) was conducted and significance of differences among treatments and in time dependence were tested using the least significant difference (*LSD*). Differences were significant at the $*p < 0.05$ probability level. The SOD activity is expressed as U mg^{-1} protein.

RESULTS AND DISCUSSION

Melatonin quantification

The endogenous melatonin content in the leaves of two medicinal plants after pretreatment with melatonin, as well as treatment with Zn and Cd are presented in Fig. 1. Exogenous melatonin addition increased the endogenous melatonin concentration in lemon balm leaves, while in valerian leaves a decrease of the total melatonin concentration was found. The obtained results indicate a higher content of endogenous melatonin in the leaves of valerian compared to lemon balm (Fig. 1B). A significant effect of the chemical treatments on the melatonin content in both plants was observed ($p < 0.05$). Treatment with Zn had the highest stimulatory effect on the endogenous melatonin content in the leaves of both plants, which was particularly pronounced in the valerian leaves. Moreover, the addition of Cd induced an increased content of endogenous melatonin in the lemon balm and valerian leaves in relation to the control and treatment with exogenous melatonin. In the lemon balm leaves, the combined treatments increased the endogenous melatonin content compared to that of the control and melatonin treatment. On the other hand, the lowest content of endogenous melatonin was measured in valerian leaves pretreated with melatonin and treated with heavy metals.

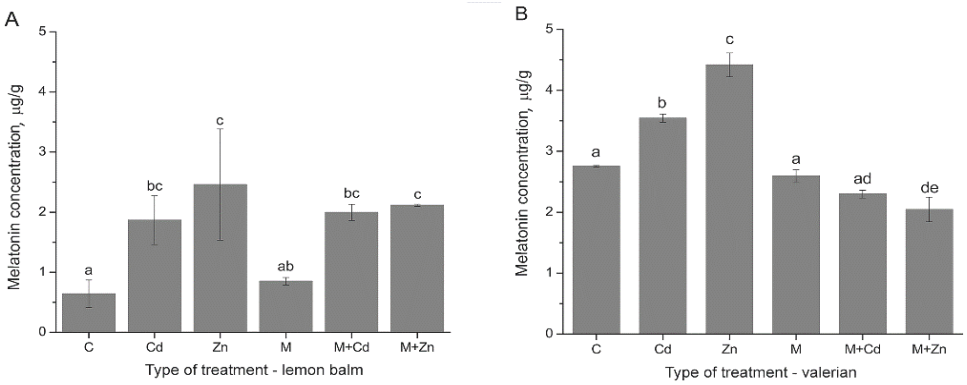

Fig. 1. Endogenous melatonin content measured in: A) lemon balm and B) valerian leaves after exogenous (100 µM) melatonin pretreatment, and Cd and Zn treatment (see Experimental). Different letters mark statistically significant differences. The data are expressed as µg g^{-1} dry weight and present the means of three replicates.
C – control; M – melatonin.

The research was performed on plants that were grown in the field and exposed to environmental influences. Field-grown plants are exposed to physical, chemical and biological agents in comparison with plants grown under artificial conditions. The differences in the content of endogenous melatonin in the control and the treated leaves of lemon balm and valerian may be due to specific plants

responses to environmental conditions. The lemon balm samples were harvested in summer time and the valerian in the fall, when the temperatures are not so devastating. Numerous papers state the effect of sunlight, among others, on the melatonin level in plants.[27] The present results are interpreted as a possible effect of sunlight on the steady state of melatonin utilization. Due to melatonin consumption in its role as antioxidant, it is likely that the higher the sunlight is, the lower the melatonin level. In addition, the decrease of melatonin in plant leaves may be a response of plants to re-direct this compound to another plant organ, which will be the subject of future research. More data are necessary to understand the physiological role of melatonin in plants under natural conditions. The difference could also be due to the diverse pretreatment of the two investigated plants. Additionally, the responses in valerian leaves may be dose or time dependent.

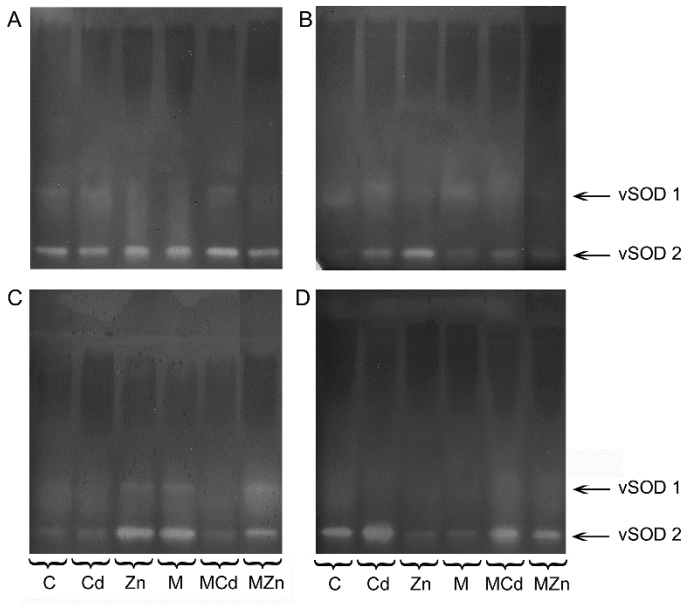

Fig. 2. Representative, 8 % native gel with separated SOD isoforms in valerian control plants and plants treated with Cd, Zn and melatonin 12 h (A) and 15 (B), 30 (C) and 45 days (D) after treatment (v – valerian). For each sample, 10 μg of protein was applied to the gel.

Arnao and Hernandez–Ruiz[5] found that plants cultivated outdoors contain several times more melatonin in the root and leaves than chamber-grown plants, which indicates that environmental conditions have an effect on the melatonin content in plant tissues. Previously, it was showed that treatment with 1 mM zinc sulfate in 9-day-old barley plants increased the endogenous melatonin content six-fold in barley root.[28]

Protein content

The content of total soluble proteins in the control and treated leaves of lemon balm and valerian is given in Table I. During development, the protein concentrations in the control lemon balm leaves did not significantly change, except for the increase (by 39 %) measured after 30 days. The most prominent increase of protein content was observed in the samples of lemon balms 30 days after heavy metal treatment, particularly in the samples treated with Zn and M+Cd. After 45 days, the protein content dropped significantly to the values almost the same as in the control. Protein content for valerian samples did not significantly change with respect to the treatment or time period. Apparently, the plant protection mechanism that involves protein accumulation in valerian differs from that of lemon balm and is not significantly affected by environmental factors such as heavy metals.

TABLE I. Effects of melatonin pretreatment and Cd and Zn treatment on the total content of soluble proteins in lemon balm and valerian leaves. The different letters indicate significance $p < 0.05$. The data are expressed as mg g^{-1} FW (fresh weight). C – control; M – melatonin

Plant species	Period after treatment	Protein content, mg g^{-1} FW					
		C	Treatment				
			Cd	Zn	M	M+Cd	M+Zn
Lemon balm	12 h	5.49^{a}	7.75^{b}	5.31^{a}	3.20^{a}	2.36^{a}	4.72^{a}
	15 days	5.51^{a}	4.89^{a}	2.92^{b}	2.67^{a}	7.04^{b}	12.75^{b}
	30 days	8.96^{b}	9.27^{b}	19.46^{c}	21.94^{b}	16.15^{c}	11.09^{b}
	45 days	5.64^{a}	4.28^{a}	4.39^{a}	6.03^{c}	4.37^{a}	6.63^{a}
Valerian	12 h	5.03^{a}	4.72^{a}	6.79^{b}	6.37^{a}	5.71^{a}	3.37^{a}
	15 days	5.71^{a}	5.33^{a}	5.93^{a}	6.02^{a}	4.75^{a}	4.13^{a}
	30 days	5.32^{a}	5.41^{a}	5.03^{a}	6.32^{a}	8.21^{b}	6.11^{a}
	45 days	3.31^{a}	6.33^{b}	4.84^{a}	4.54^{a}	4.23^{a}	5.91^{a}

It was shown that melatonin-treated plants exposed to cold stress had a higher concentration of soluble protein than control plants.[18] The authors suggested that melatonin stimulated *de novo* synthesis of antifreeze proteins in order to improve plant resistance to cold stress. The present results indicate that lower concentration of melatonin may lead to a reduction in the concentration of soluble proteins in the final development stages of lemon balm and in the first development stage of valerian plants treated with heavy metals after melatonin pretreatment.

Superoxide dismutase and peroxidase activities

Considering the fact that SOD represents the first line of plant cell defense against oxidative stress by removing highly reactive superoxide anion radicals,[29] the changes in the SOD isoenzyme pattern and activity in the lemon balm and valerian leaves under the influence of exogenous melatonin and increased Zn and

Cd concentration were examined. It was reported that melatonin could detoxify ROS by directly scavenging, and by attenuating free radical formation due to enhanced activities of antioxidant enzymes.[30] Superoxide dismutases isoenzyme patterns in the leaves of the control and treated valerian, and lemon balm leaves are presented in Figs. 2 and 3.

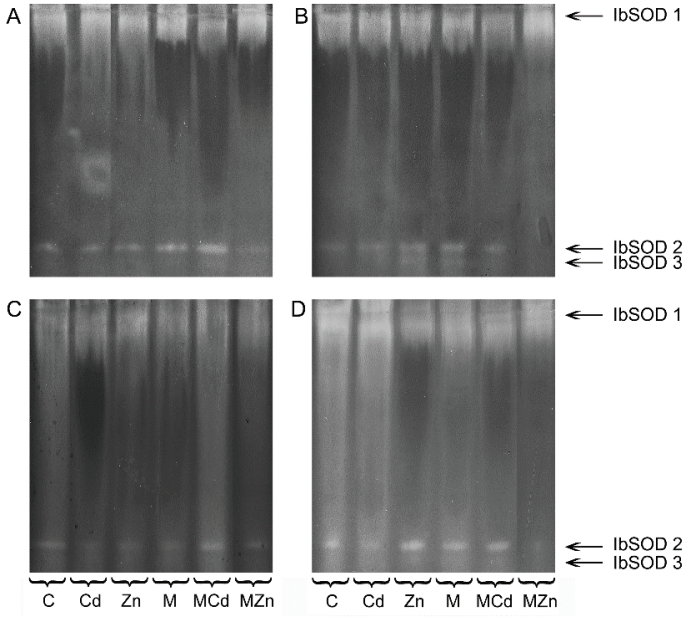

Fig. 3. Representative, 8 % native gel with separated SOD isoforms in the lemon balm control plants and plants treated with Cd, Zn and melatonin 12 h (A) and 15 (B), 30 (C) and 45 days (D) after treatment (lb – lemon balm). For each sample, 10 μg of protein was applied to the gel.

The obtained results of superoxide dismutases isoenzyme patterns show the presence of three SOD isoforms in the lemon balm leaves (marked as lbSOD 1, $R_f = 0.117$; lbSOD 2, $R_f = 0.834$ and lbSOD 3, $R_f = 0.886$; lb – lemon balm, Fig. 3). In the control and all treated lemon balm plants, the SOD isoform with a high molecular weight (lbSOD 1) was detected and this isoform is perhaps specific for these species. The lbSOD 2 isoform was present in all developmental stages of the plant and within all treatments. In the lemon balm leaves after 12 h, only treatment with M+Cd induced a new SOD isoform (lbSOD 3). In the subsequent stages of development in the leaves treated with M+Cd, this isoform does not occur. Moreover, treatment with Zn and melatonin 15 days after treatment induced the occurrence of the lbSOD 3 isoform. It is interesting that in the leaves of the plants treated with M+Zn, this isoform was not detected, and this treatment was inhibitory to the SOD isoform in all developmental phases. It is possible that

the lbSOD 3 isoform is specific to the developmental phase of the plant, as well as to the treatment, and this will be the subject of future research. The obtained results showed the presence of two SOD isoforms (marked as vSOD 1, R_f = = 0.640; vSOD 2, R_f = 0.856; v – valerian, Fig. 2) in the leaves of the valerian control and the treated plants. The isoform vSOD 2 was detected in all development stages of both the control and all the treated valerian leaves.

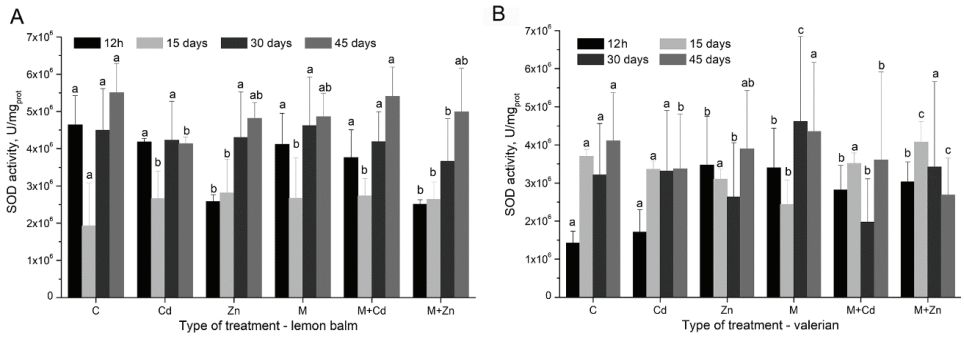

Fig. 4. Effect of melatonin pretreatment and Cd and Zn treatment on superoxide dismutase (SOD) activity in lemon balm (A) and valerian (B) leaves. The data are presented as means of three replicates. Different letters above the bars indicate significance at $p < 0.05$. C – control; M – melatonin.

On the other hand, the isoform vSOD 1 was detected in the valerian control leaves and its induction or inhibition also depended on the treatment and developmental phase. The studies showed that melatonin regulates gene expression of the antioxidant enzyme. In addition, up-regulation of the transcript levels of the genes encoding SOD, APX, CAT and POD by melatonin both under physiological and oxidative stress conditions has been reported.[31] In the present experiments, significant difference in SOD activity of lemon balm leaves was detected between the different sampling times, while between treatments significant difference was detected only 12 h after Zn treatment.

During development, in the control lemon balm leaves, the SOD activity significantly decreased 15 days after treatment and slightly 30 days after treatment, while it was increased after 45 days. The lemon balm leaves 12 h after heavy metal treatment, showed a slight decrease in SOD activity when treated with Cd, and a considerable decrease in the leaves treated with Zn. The same decrease in SOD activity was observed in the plants treated with melatonin and M+Cd and M+Zn (Fig. 4A). Most significant changes in the SOD activity were observed 45 days after treatment regardless of the type of the treatment. The measured SOD activity 30 days after treatment was not significantly different to that of the control. When these data are compared with the increase in the protein values at different periods after treatment, it is apparent that protein accumulation is the

first line of defense against external stress. Only after that does the enzyme antioxidant system take control, implying that a plant has a very specific highly organized defense system.

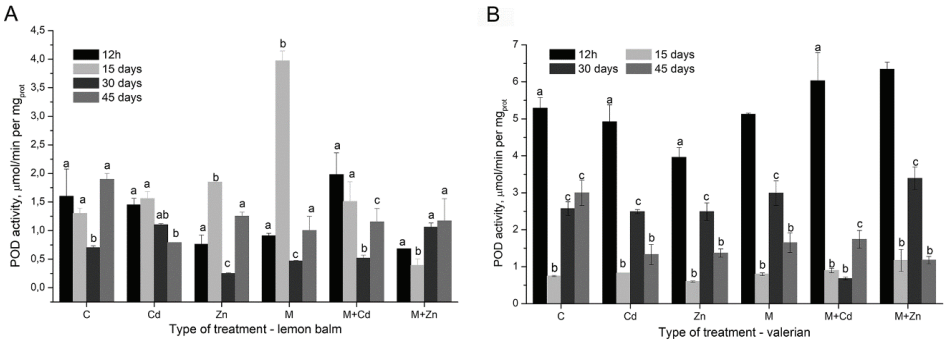

Fig. 5. The effects of melatonin pretreatment and Cd and Zn treatment on the POD activity in lemon balm (A) and valerian (B) leaves. Different letters above the bars indicate significance at $p < 0.05$. The data are expressed as μmol min^{-1} mg^{-1} protein and presented as means of three replicates. C – control; M – melatonin.

In valerian leaves, no statistically significant difference in SOD activity was observed at the different sampling times. The only significant difference in SOD activity was measured 15 days after Zn treatment (Fig. 4B) compared to the control. The valerian leaves treated with Cd and Zn showed an increase in SOD activity 12 h after heavy metal treatment compared to the control. Higher activity was also shown in the plants pretreated with melatonin, but lower in the plants treated with Cd, Zn and M. It seems that melatonin plays a role in protecting plants against oxidative stress in this early development stage. Different results in SOD activity 15 days after heavy metal treatment were obtained. Unlike in lemon balm leaves, in the valerian plants treated with Zn and Cd, the SOD activity was lower than in the control. A lower SOD activity was observed in the melatonin pretreated plants, but higher in combination with both heavy metals. As with the proteins, the obtained SOD activity may depend on the sampling time and applied dose of melatonin. Small differences in the SOD activity of the treated plants were measured in valerian 30 days after treatment in regards to the control. However, a significant decrease in the activity was observed in the melatonin pretreated plants treated with heavy metals compared to the only melatonin treated plants. A decrease in SOD activity was observed in valerian with elevated amounts of Zn and Cd compared to the control. As in the previous stage, the melatonin pretreated plants showed higher SOD activity, but lower in the same plants with elevated Zn and Cd concentrations.

In conclusion, melatonin pretreated plants show a better defense response after 12 h, as well as after 30 and 45 days after treatment with elevated Cd and

Zn concentrations. Different changes in the SOD response were observed in the two plants, lemon balm and valerian. An increase in the SOD activity was measured in lemon balm leaves, reaching its maximum at the end of the developmental period. In the melatonin pretreated lemon balm plants, the same phenomena were observed. However, in the valerian leaves, the SOD activity decreased in the melatonin pretreated plants in case of elevated Zn concentrations. Earlier, it was shown that melatonin treatment significantly enhanced SOD activity, and thus enhanced the ROS scavenging capacity of wheat leaves stressed with nano-ZnO.[32]

The effect of exogenous melatonin, Cd and Zn on the peroxidase activity in lemon balm and valerian leaves is presented in Fig. 5.

In the control lemon balm leaves, the POD activity decreased during plant development, while 45 days after treatment these activities increased by 19 % in relation to the control (Fig. 5A). In the plants treated with Zn, a higher POD activity was measured only 15 days after treatment, while in all other cases, Zn reduced the POD activity compared to the control. Melatonin pretreatment had no effect on the POD activity, except on the lemon balm plants 15 days after treatment. Cd treatment decreased the peroxidase activity 45 days after treatment compared to the control. In the plants treated with M+Cd, the POD activity was increased at 15 days, but decreased at 30 and 45 days after treatment. This might be attributed to melatonin antioxidative activity at the early plant development stage. Combined melatonin and Zn treatment show no significant difference in the POD activity compared to the control except at 15 days after treatment when the POD activity had decreased (Fig. 5A). There was no significant difference in POD activity between M+Cd or M+Zn treatment compared to treatment with Cd and Zn, except M+Cd treatment at the first developmental stage.

Changes in the POD activity in relation to treatments and developmental stages were different in the leaves of lemon balm and valerian (Fig. 5B). A significantly higher POD activity in valerian leaves was measured 12 h after heavy metal treatment. Both Cd and Zn decreased the POD activity, but melatonin had no effect on the POD activity. Interestingly, the POD activity was enhanced in M+Cd and especially M+Zn treated plants compared to treatments with Cd and Zn in the first developmental stage of valerian, but was the same or decreased in all other stages.

Induction of POD activities as one of the strategies for protecting plants from various heavy metals was shown.[33] In addition, increasing heavy metal concentration led to induction of oxidative stress in plants and changes in the antioxidant systems.[5,21] The results obtained in the present study indicated that the POD activity may be affected by plant species, type of treatment, treatment duration and the development stage of the plant. Previous studies showed higher POD and SOD activities in 45-day-old lemon balm seedlings treated with Fe for 24 h, and lower

catalase (CAT) activity.[21] Moreover, pretreatment with 0.1 μM melatonin stimulated SOD and CAT activities in cryopreservation process of *Rhodiola crenulata.*[30] In this paper, the authors showed that melatonin pretreatment affected the level of malondialdehyde (MDA) and antioxidant enzyme activity (POD and CAT).

CONCLUSIONS

In this paper, the contents of endogenous melatonin in leaves of lemon balm and valerian were determined. In addition, it was shown that Zn in relation to Cd induces a significant increase in the concentration of endogenous melatonin in the leaves of both plants. Both the analyzed plants showed an encouragingly high amount of melatonin, within the range of the highest amount previously reported for dried plants, with valerian having higher concentrations. The obtained results showed that the role of melatonin in the induction of SOD and POD activity, in addition to the type of stress, depends on the plant species and the plant developmental phase. The results supported the theory that melatonin might protect plants from stress conditions and prevent injuries induced by oxidative stress at the cellular level, especially in valerian, by elevating the activity of antioxidative enzymes in first developmental phases of the plant. Such a response could be explained in two ways: first, melatonin is a receptor-independent free-radical scavenger and a broad-spectrum antioxidant; second, it can stimulate antioxidant enzymes or augment the activities of other antioxidants to protect plant tissues from oxidative damage.

The existence of a correlation between heavy metals, increased melatonin concentrations and SOD and POD activity was shown, but the mechanism of their interaction is unknown and will be the subject of future research.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

АНТИОКСИДАТИВНИ ОДГОВОР ЛИСТОВА *Melissa officinalis* L. И *Valeriana officinalis* L. ПОД УТИЦАЈЕМ ЕГЗОГЕНОГ МЕЛАТОНИНА И ПОВИШЕНИХ КОНЦЕНТРАЦИЈА ЦИНКА И КАДМИЈУМА

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Тешки метали нарушавају редокс хомеостазу биљне ћелије и доводе до оксидативног стреса. Индоламински хормон, мелатонин, штити биљке од оксидативног оштећења тако што директно уклања реактивне врсте кисеоника или стимулише активност антиоксидативних ензима. У раду је испитивана антиоксидативна улога мелатонина у листовима две лековите биљке, матичњака (*Melissa officinalis* L.) и валеријане (*Valeriana officinalis* L.), третираних повишеним концентрацијама Zn и Cd 24 h након сејања на отворено поље.

Биљке су третиране са Zn, Cd, мелатонином и смешом мелатонина са наведеним металима. Егзогени мелатонин повећао је концентрацију ендогеног мелатонина у листовима матичњака. Међутим, у листу валеријане измерен је нижи или исти ендогени ниво мелатонина. Знатно већа концентрација ендогеног мелатонина у обе биљке измерена је после третмана са Zn. Наши резултати су показали да су промене у активностима супероксид-дисмутазе (SOD) и пероксидазе (POD) специфичне за биљну врсту, да зависе од фазе развоја биљака и врсте третмана. Мелатонин је индуковао промене у изоензимским профилима и активностима супероксид-дисмутазе, као и у активностима пероксидазе у листовима обе биљне врсте третиране са тешким металима.

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REFERENCES

1. R. Dubbels, R. J. Reiter, E. Klenke, A. Goebel, E. Schnakenberg, C. Ehlers, H. W. Schiwara, W. Schloot, *J. Pineal Res*. **18** (1995) 28 (https://doi.org/10.1111/j.1600-079X.1995.tb00136.x)
2. I. G. Hernández, F. J. V. Gomez, S. Cerutti, M. V. Arana, M. F. Silva, *Plant Physiol. Biochem*. **94** (2015) 191 (https://doi.org/10.1016/j.plaphy.2015.06.011)
3. M. B. Arnao, J. Hernández-Ruiz, *Plant Signaling Behav*. **1** (2006) 89 (https://doi.org/10.4161/psb.1.3.2640)
4. N. Zhang, Q. Sun, H. Zhang, Y. Cao, S. Weeda, S. Ren, Y.-D. Guo, *J. Exp. Bot.* **66** (2015) 647 (https://doi.org/10.1093/jxb/eru336)
5. M. B. Arnao, J. Hernández-Ruiz, *J. Pineal Res*. **55** (2013) 149 (https://doi.org/10.1111/jpi.12055)
6. C. Wang, S. H. Zhang, P. F. Wang, J. Qian, J. Hou, W. J. Zhang, J. Lu, *Chemosphere* **76** (2009) 938 (https://doi.org/10.1016/j.chemosphere.2009.04.038)
7. T. A. Tran, L. P. Popova, *Turk. J. Bot.* **37** (2013) 1 (https://doi.org/10.3906/bot-1112-16)
8. N. Rascio, F. Navari-Izzo, *Plant Sci.* **180** (2011) 169 (https://doi.org/10.1016/j.plantsci.2010.08.016)
9. P. Das, S. Samantaray, G. R. Rout, *Environ. Pollut. (Oxford, U.K.)* **98** (1997) 29 (https://doi.org/10.1016/S0269-7491(97)00110-3)
10. K. Hasan, G. J. Ahammed, L. Yin, K. Shi, X. Xia, Y. Zhou, J. Yu, J. Zhou, *Front. Plant Sci.* **6** (2015) 601 601 (https://doi.org/10.3389/fpls.2015.00601)
11. T. Ye, Y-H. Hao, L. Yu, H. Shi, R. J. Reiter, Y-Q. Feng, *Front. Plant Sci*. **8** (2017) 64 (https://doi.org/10.3389/fpls.2017.00064)
12. S. Y. Cai, Y. Zhang, Y. P. Xu, Z. Y. Qi, M. Q. Li, G. J. Ahammed, X. J. Xia, K. Shi, Y. H. Zhou, R. J. Reiter, J. Q. Yu, J. Zhou, *J. Pineal Res*. **62** (2017) e12387 (https://doi.org/10.1111/jpi.12387)
13. S. S. Gill, N. Tuteja, *Plant Physiol. Biochem*. **48** (2010) 909 (https://doi.org/10.1016/j.plaphy.2010.08.016)
14. U. Cho, N.-H. Seo, *Plant Sci*. **168** (2005) 113 (https://doi.org/10.1016/j.plantsci.2004.07.021)
15. R. G. Alscher, N. Erturk, L. S. Heath, *J. Exp. Bot*. **53** (2002) 1331 (https://doi.org/10.1093/jexbot/53.372.1331)
16. F. Minibayeva, R. P. Beckett, I. Kranner, *Phytochemistry* **112** (2015) 122 (https://doi.org/10.1016/j.phytochem.2014.06.008)
17. L. Ceraulo, M. Ferrugia, L. Tesoriere, S. Segreto, M. A. Livrea, V. T. Liveri, *J. Pineal Res*. **26** (1999) 108 108 (https://doi.org/10.1111/j.1600-079X.1999.tb00570.x)

18. H. Turk, S. Erdal, M. Genisel, O. Atici, Y. Demir, D. J. Yanmis, *Plant Growth Regul*. **74** (2014) 139 (https://doi.org/10.1007/s10725-014-9905-0)
19. X. Gong, S. Shi, F. Dou, Y. Song, F. Ma, *Molecules* **22** (2017) 1542 (https://doi.org/10.3390/molecules22091542)
20. A. Romero, E. Ramos, C. de Los Ríos, J. Egea, J. del Pino, R. J. Reiter, *J. Pineal Res*. **56** (2014) 343 (https://doi.org/10.1111/jpi.12132)
21. K. Esmaeilzadeh-Salestani, A. Riahi-Madvar, M. A. Maziyar, *Int. J. Food Allied Sci.***3** (2014) 562 (http://ijfas.com/wp-content/uploads/2014/05/562-565.pdf)
22. Y. Tang, L. Lin, Y. Xie, J. Liu, G. Sun, H. Li, M. Liao, Z. Wang, D. Liang, H. Xia, X. Wang, J. Zhang, Z. Liu, Z. Huang, Z. He, L. Tu, *Int. J. Phytoremediation* **20** (2018) 295 (https://doi.org/10.1080/15226514.2017.1374341)
23. J. Adinee, K. Piri, O. Karami, *J. Am. Biochem. Biotechnol*. **4** (2008) 277 (https://doi.org/10.3844/ajbbsp.2008.277.278)
24. M. B. Arnao, J. Hernández-Ruiz, *Phytochem. Anal*. **20** (2009) 14 (https://doi.org/10.1002/pca.1083)
25. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem*. **193** (1951) 265 (http://www.jbc.org/content/193/1/265.full.pdf)
26. H. Teisseire, V. Guy, *Plant Science* **153** (2000) 65 (https://doi.org/10.1016/S0168-9452(99)00257-5)
27. D. X. Tan, R. Hardeland, L. C. Manchester, A. Korkmaz, S. Ma, S. Rosales-Corral, R. J. Reiter, *J. Exp. Bot.* **63** (2012) 577 (https://doi: 10.1093/jxb/err256)
28. M. B. Arnao, J. Hernandez-Ruiz, *J. Pineal Res*. **46** (2009) 295 (https://doi.org/10.1111/j.1600-079X.2008.00660.x)
29. M. Garnczarska, L. Ratajczak, *Acta Physiol. Plant*. **22** (2000) 429 (https://doi.org/10.1007/s11738-000-0084-4)
30. Y. Zhao, L.-W. Qi, W.-M. Wang, P. K. Saxena, C. Z. Liu, *J. Pineal Res*. **50** (2011) 83 (https://doi.org/10.1111/j.1600-079X.2010.00817.x)
31. C. Rodriguez, J. C. Mayo, R. M. Sainz, I. Antolín, F. Herrera, V. Martín, R. J. Reiter, *J. Pineal Res.* **36** (2004) 1 (https://doi.org/10.1046/j.1600-079X.2003.00092.x)
32. Z. Zuo, L. Sun, T. Wang, T. P. Miao, X. Zhu, S. Liu, F. Song, H. Mao, H. Li, *Molecules* **22** (2017) 1727 (https://doi.org/10.3390/molecules22101727)
33. T. Bhattacharya, S. Chakraborty, D. K. Banerjee, *Environ. Monit. Assess*. **169** (2010) 15 (https://doi.org/10.1007/s10661-009-1146-8).