Botanica SERBICA 39 (2): (2015) 121-128

Original Scientific Paper

Paraquat-mediated oxidative stress in *Nepeta pannonica* L.

Jelena Cvetković, Milica Milutinović, Jelena Boljević, Neda Aničić, Jasmina Nestorović Živković, Suzana Živković and Danijela Mišić*

Institute for Biological Research 'Siniša Stanković', University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

ABSTRACT: The toxicity of bipyridynium herbicide paraquat (PQ) is related to its rapid generation of superoxide in a chain reaction and the production of reactive oxygen species (ROS) in chloroplasts under light conditions. The nature and magnitude of the cellular responses to oxidative stress effects of five paraquat concentrations (0.05-20 μM) on *in vitro* grown *Nepeta panonnica* shoots were investigated. The results revealed that relative abundances of Mn SOD and Cu/Zn SOD isoforms changed dose-dependently, whereas catalase (CAT) and peroxidase (POX) activities increased along with paraquat concentrations. Polyphenol oxidase (PPO) activity was progressively inhibited, and, consequently, phenolic compounds were accumulated. Rosmarinic acid (RA) and an unidentified caffeic acid derivative (UCAD) were found to be the most abundant phenolics in shoots of this species. Paraquat was shown to be a potent elicitor that can stimulate accumulation of potent bioactive compounds in *Nepeta pannonica*, such is rosmarinic acid.

Key words: *Nepeta pannonica,* paraquat, oxidative stress, rosmarinic acid.

Received: 31 March 2015 Revision accepted 29 May 2015

UDK 581.1

INTRODUCTION

The non-selective herbicide paraquat (1, 1'-dimethyl-4, 4'-bipyridinium chloride, PQ) has been used worldwide since the 1960s and remains an important herbicide for broad-spectrum weed control. Paraquat is a redox active compound that exhibits a phytotoxic effect by diverting electrons from photosystem I (PSI) in the thylakoid membrane of chloroplasts to molecular oxygen generating the superoxide radical ($O_2^{\bullet-}$). In turn, highly reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are produced (Fuerst & Vaughn 1990). ROS interact with unsaturated lipids of membranes causing irreversible lipid peroxidation, destruction of cellular organelles, proteins and nucleic acids, and depletion of NADPH (Aksakal 2013). Scavenging of excess ROS is achieved by an efficient antioxidative system comprising enzymatic and non-ezymatic antioxidants and each plant cellular compartment contains more than one that detoxifies a particular ROS. Previous studies have demonstrated enhanced activity of active oxygen-scavenging enzymes, particularly superoxide dismutase (SOD) and catalase (CAT), as a response to paraquat-mediated oxidative stress (Liu *et al.* 2009; Sood *et al.* 2011; Aksakal 2013; Tsuji *et al.* 2013).

The genus *Nepeta*, one of the largest genera of the *Lamiaceae* family, comprises ca. 300 herbaceous perennial, rarely annual species native to the larger part of central and southern Europe, the Near East, central and southern Asia, and some areas of Africa (Formisano *et al.* 2011). In the flora of Serbia, *Nepeta* is represented by three species: *N. cataria* L., *N. pannonica* L. (syn. *nuda*) and *N. rtanjensis* Diklić and Milojević (Chalchat *et al.* 1998).

The main secondary metabolites in most *Nepeta* species are iridoid monoterpene nepetalactones. However, in a number of *Nepeta* species nepetalactones are either present as minor components or are not produced at all (Gkinis *et al.* 2003). Excepting terpenes,

*correspondence: dmisic@ibiss.bg.ac.rs, drdanela@gmail.com

another important group of secondary metabolites in this genus are phenolic compounds, which are regarded as potent nonenzymatic antioxidants. Among phenolics, rosmarinic acid (hydroxycinnamic acid ester) is known to be a compound characteristic of the whole *Nepetoideae* subfamily. Phenolic compounds and especially rosmarinic acid were previously reported to be dominant constituents of *N. pannonica* (Janicsák *et al.* 1999; Mišić *et al.* 2015), and therefore total phenolic content and rosmarinic acid content alterations, as a response to paraquat induced oxidative stress, were analyzed.

The present study is an exploration of the nature and magnitude of the cellular responses to paraquat-mediated oxidative-stress in *Nepeta pannonica* L. shoot cultures, with special attention to antioxidant enzyme activities.

MATERIALS AND METHODS

Plant material and *in vitro* culture establishment. *Nepeta pannonica* L. seeds were surface sterilized in a 20% solution of commercial bleach for 10 min and rinsed five times with sterile deionized water. Seeds were transferred to Petri dishes containing half-strength MS medium (Murashige & Skoog 1962) pH 5.8, supplemented with 20 g l^{-1} sucrose, 7 g l^{-1} agar (Torlak, Serbia). Seeds were kept in a growth chamber under long-day conditions (16 h light/8 h dark regime) at 25±2°C and relative humidity of 60-70%. Seedlings were aseptically transferred to 350 ml glass jars closed with polycarbonate caps, each containing 70 ml of the same medium.

Two-month-old plants were transferred to solid half-strength MS medium supplemented with paraquat (Sigma-Aldrich) at the following concentrations: 0.5, 1, 5, 10, and 20 μM. PQ was filter sterilized and added to the culture medium previously sterilized by autoclaving at 114°C for 25 min. After two weeks of PQ treatment, above-ground parts of plants were harvested, weighed, and stored at -80°C until use.

Extraction of soluble proteins. Plant tissue (1 g) was homogenized with liquid nitrogen (LN2) and soluble proteins were extracted in 3 ml of 100 mM potassium phosphate buffer (pH 6.5) containing 5 mM ascorbic acid, 2 mM ethylenediaminetetraacetic acid (EDTA), 2% (w/v) insoluble polyvinylpyrrolidone (PVPP), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 12000*g* for 20 min at 4°C and the resulting supernatant was used further. Protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

Native PAGE. Proteins were separated by native polyacrylamide gel electrophoresis (native PAGE) using a 12% running gel and 5% stacking gel (Mini PROTEAN Tetra Cell system, Bio-Rad, Richmond, CA, USA). The reservoir buffer consisted of 25 mM TRIS and 192 mM glycine (pH 8.3). Electrophoresis was performed at +4°C for 2 h, at a constant current of 120 V. The amount of total proteins loaded was 20 μg per well.

Active SOD isoforms were localized on gels using the nitroblue tetrazolium–riboflavin reaction (Beauchamp & Fridovich 1971). After electrophoresis, gels were incubated in a reaction mixture containing 100 mM EDTA, 98 mM nitroblue tetrazolium (NBT), 30 mM riboflavin, and 2 mM N,N,N',N'-tetramethylethylenediamine (TEMED) in potassium phosphate buffer (pH 7.8) for 30 min in the dark, washed in distilled water and subsequently illuminated to start the photochemical reduction of NBT to blue formazan. To determine Cu/Zn-, Mn- and Fe SOD isoforms, gels were incubated with 5 mM KCN and/or 5 mM H_2O_2 before staining (Yamahara *et al.* 1999).

SDS PAGE and immunoblotting. Samples were prepared by mixing the crude protein extracts with loading buffer to a final concentration of 62 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.002% (w/v) bromophenol blue and 0.5% (w/v) β-mercaptoethanol. Samples were heated at 95°C for 3 min, quickly cooled to 4°C, and centrifuged at 10000*g* for 3 min. Proteins were separated by denaturing SDS-PAGE with an acrylamide concentration of 12% (w/v) for the resolving gel and 4% (w/v) for the stacking gel, using the Mini-Protein II system (Bio-Rad, Richmond, CA, USA). 20 μg of the protein equivalent was loaded into each well. The transfer of proteins onto PVDF membranes (0.2 μm; Bio-Rad, Richmond, CA, USA) was conducted in transfer buffer containing 25 mM Tris–HCl (pH 8.3) and 192 mM glycine, using a Bio-Rad transfer apparatus (Richmond, CA, USA). The blotted membranes were pre-incubated overnight at 4°C, in blocking TPBS buffer (PBS supplemented with 0.05% (w/v) Tween 20) containing 10% non-fat dry milk (NFDM; Nestle, USA). Membranes were rinsed in TPBS buffer and subsequently incubated with commercial Cu/Zn SOD antibodies (Agrisera, Vännäs, Sweden) diluted in TPBS containing 5% NFDM, for 2 h at room temperature with gentle shaking. After extensive washing with TPBS, membranes were incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma-Aldrich) diluted in TPBS containing 5% NFDM (1:20000 dilution). Protein signals were scored using an enhanced chemiluminescence detection system and densitometric quantification was performed by ImageJ 1.32j software (W. Rasband, National Institute of Health, USA).

Enzyme activity assay. CAT activity was determined spectrophotometrically (Agilent 8453, Agilent Technologies, Waldbronn, Germany) using 50 mM sodim potassium phosphate buffer (pH 7), and 30% H_2O_2. Decomposition of H_2O_2 was measured at 240 nm during 3 min (Aebi 1984). For POX activity determination, pyrogallol was used as hydrogen donor

and the absorbance was measured at 430 nm. The reaction mixture consisted of 20 mM pyrogallol, 10 mM H_2O_2, 50 mM potassium phosphate buffer (pH 6.5) and an aliquot of diluted extract. PPO activity was determined according to (Jiménez-Atiénzar *et al.* 2007) with slight modifications. The reaction mixture contained 10 mM pyrocatechol, 2 mM sodium dodecyl sulfate (SDS) and 50 mM potassium phosphate buffer (pH 6.5). Enzyme activity was determined spectrophotometrically at 400 nm, at room temperature.

Preparation of plant methanol extract. Aerial parts of plants were ground in liquid nitrogen and extracted for 15 min in 99.8% methanol (w:v=1:10) in an ultrasonic bath (RK 100, Bandelin, Berlin, Germany). After centrifugation at 10000*g* for 10 min at 4°C, supernatants were filtered through 0.2 µm cellulose filters (Agilent Technologies, USA) and stored at 4°C until use.

Determination of total phenolic and total flavonoid concentrations. Total phenolics (TP) were quantified using a modified Folin-Ciocalteu assay (Singleton & Rossi 1965). Briefly, 50 µl of plant extract and 475 µl of 5% Na_2CO_3 were incubated at room temperature for 5 min. Following the addition of 475 µl of Folin-Ciocalteu reagent, samples were kept for one hour in darkness. Reduction of Folin-Ciocalteu reagent was measured using a UV-visible spectrophotometer (Agilent 8453, Agilent Technologies, Waldbronn, Germany) at 724 nm. Rosmarinic acid was used as a standard for the calibration curve and results are expressed as rosmarinic acid equivalents per gram of plant fresh weight (g ERA g^{-1} FW). Total flavonoid (TF) content was determined according to Kardeniz *at al.* (2005) with slight modifications. 50 µl of plant methanol extract and 600 µl of ddH_2O were well shaken and mixed with 40 µl of 5% KNO_2. The mixture was allowed to stand at room temperature for 6 min and subsequently 70 µl of 4.26% $AlCl_3$ solution was added. After 5 min of incubation at room temperature, 240 µl of 1M NaOH was added to the mixture and the absorbance was measured at 510 nm using an Agilent 8453 spectrophotometer. TF content was calculated from the standard curve based on rutin, and the results are expressed as gram of rutin equivalents per gram of plant fresh weight (g ER g^{-1} FW).

UHPLC/DAD/+HESI-MS/MS analysis. Identification and quantification of rosmarinic acid and an unidentified caffeic acid derivative in methanol extracts of *N. pannonica* shoots was performed using a Dionex Ultimate 3000 UHPLC system equipped with a diode array detector (DAD) and connected to a Quantum Access Max triple-quadrupole mass spectrometer (Thermo Fisher Scientific). Separations were performed on a Hypersil gold C18 column (50 × 2.1 mm, 1.9 µm particle size). The mobile phase consisted of (A) water + 0.1% formic acid, and (B) acetonitrile, which were applied in the gradient elution previously described by Mišić *at al.* (2015). The flow rate was set to 0.4 ml min^{-1} and the detection wavelength to 260 and 320 nm. The injection volume was 2 µl. A triple-quadrupole mass spectrometer, equipped with an heated electrospray ionization (HESI) source, was used with vaporizer temperature kept at 450°C, and ion source settings as follows: spray voltage 4000 V, sheet gas pressure 50 AU, ion sweep gas pressure 0 AU and auxiliary gas pressure at 20 AU, capillary temperature at 320°C, skimmer offset 0 V. Product ion scanning (PIS) and selected reaction monitoring (SRM) scanning modes of the mass spectrometer were conducted for the qualitative analysis.

Rosmarinic acid (RA) was identified by direct comparison with a commercial standard and with the literature data. Quantification of RA and UCAD in samples was based on a calibration curve of pure rosmarinic acid. The total amount of each compound was evaluated by the calculation of peak areas and expressed as mg g^{-1} FW.

Statistical analyses. Statistical analyses were performed using STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation 1985-1989, USA). The data were subjected to one-way analysis of variance (ANOVA). Differences between means were evaluated by Fischer's LSD test calculated at a confidence level of $P<0.05$.

RESULTS AND DISCUSSION

Reactive oxygen species, such as superoxide free radical anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), react with plant cellular components and cause damage that could result in cellular death. In plant cells, a wide range of antioxidant mechanisms have evolved to remove ROS generated in stress conditions such as light, drought, extreme temperature, heavy metals, salinity, UV radiation, and herbicide treatments (Sood *et al.* 2012).

Superoxide dismutase (SOD) catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and oxygen (O_2) and is the key enzyme in the ROS-scavenging system in plant cells (Gupta *et al.* 1993). Under stress conditions, the activity of SOD increases as the concentration of $O_2^{\cdot-}$ increases. The increase of SOD activity induces higher tolerance to oxidative stress (Bowler *et al.* 1991). Based on the metal co-factor used by the enzyme, SODs can be separated in three groups: manganese SOD (Mn SOD), iron SOD (Fe SOD) and copper-zinc SOD (Cu/Zn SOD). These enzymes are located in different compartments of the cell. Fe SODs are located in the chloroplast, Mn SODs in both the mitochondrion and peroxisome, and Cu/Zn SODs in the chloroplast, cytosol, and possibly the extracellular space (Alscher *et al.* 2002). To determine whether there were PQ-mediated differences among individual SOD isoforms, SOD activity assays were performed on the control and

PQ-treated samples separated in non-denaturing gels. In this study, two forms of SOD were visible on the activity gels - Mn SOD and Cu/Zn SOD. Fe SOD was not detected in the samples. Mn SOD showed increasing activity with increasing PQ concentrations from 5 µM to 20 µM, where as Cu/Zn SOD activity decreased as the concentration of PQ increased, compared to the control samples (Fig. 1A). Immunoblot analysis revealed two Cu/Zn SOD isoforms in the control samples and samples treated with 0.5µM PQ. One isoform was recorded at higher PQ concentrations (Fig. 1B). The decrease of enzyme activity can be ascribed to substrate depletion. At the same time, the activity of POX and CAT increased, which is in accordance with the proposed role of these enzymes in removing the products of the SOD catalyzed reaction.

In addition to SOD, CAT and POX are considered to play an important role in antioxidant defense. CAT catalyzes the decomposition of H_2O_2 to H_2O and O_2 and participates in the main defense system against accumulation and toxicity of H_2O_2 in plant cells (Aksakal 2013). However, besides its role in eliminating H_2O_2, CAT activity appears to be critical for maintaining the redox balance during oxidative stress (Zimmermann & Zentgraf 2005). H_2O_2, resulting from the action of SOD, can rapidly diffuse across membranes (Foyer *et al.* 1997). POX catalyzes H_2O_2-dependent oxidation of substrate incorporating an oxygen molecule into ROH and could play a role in alleviating oxidative stress induced by toxic pollutants (Aksakal 2013). Previous research demonstrated that PQ treatments induce consequential activation of these enzymes (Liu *et al.* 2009; Sood *et al.* 2011; Aksakal 2013; Tsuji *et al.* 2013). Under the current experimental conditions, the application of PQ (0.5–20 µM) induced a 1.18 to 1.51-fold increase in CAT (Fig. 2A) and 1.59 to 3.08-fold increase in POX activity compared to the control (Fig. 2B). Increased activities of these enzymes may be considered as circumstantial evidence for enhanced production of ROS induced by herbicide application, as noted by Sood *et al.* (2011).

The metabolism of phenolic compounds involves the action of the enzyme PPO, which catalyzes the oxidation of phenols to quinones. Some studies have reported that PPO activity increases in response to different types of stresses (Agarwal & Pandey 2004; Ruiz *et al.* 1998; Rivero *et al.* 2001). In contrast to these findings, in our experiments, PPO activity declined concomitant with increasing paraquat concentrations. The supplementation of increasing PQ concentrations (0.5–20 µM) inhibited PPO activity by 1.83 to 4.69 fold, in comparison with the control (Fig. 2C). Consequently, phenolic compounds accumulated, probably as a result of the plant's defense mechanism to overcome oxidative stress damage caused by paraquat. In agreement with our results, literature data also show that some plants decrease their PPO activity while encountering stress conditions (Balakumar *et al.* 1997; García *et al.* 2001; Llorente *et al.* 2014).

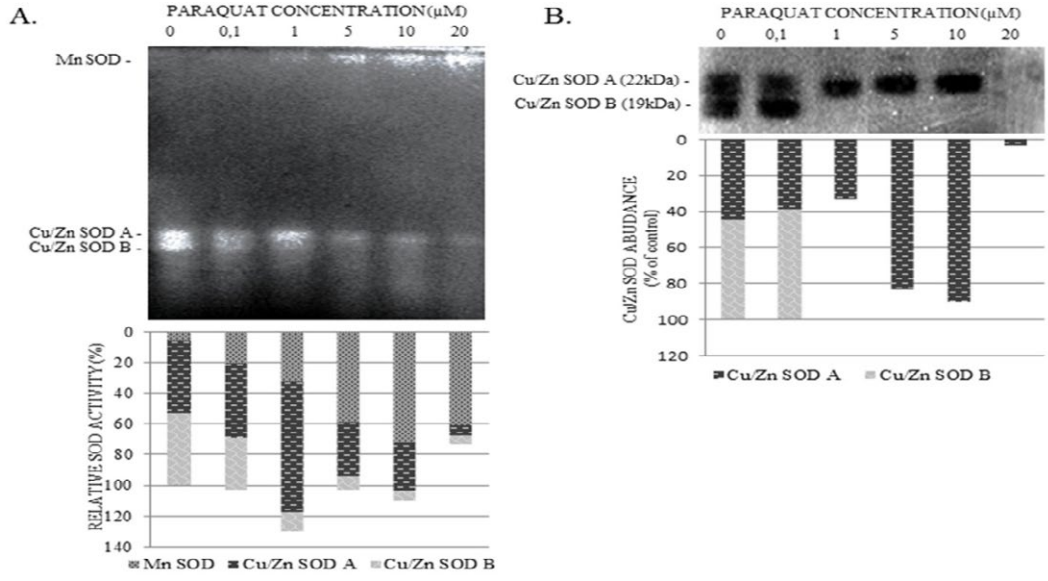

Fig 1. A. Superoxide dismutase (SOD) activity in above-ground parts of *in vitro*-grown *Nepeta pannonica* shoots treated with five PQ concentrations (0-20 µM). **A.** Total soluble proteins were separated by Native PAGE and stained for SOD activity. SOD isoform identification was performed by pre-incubation of gels with 5 mM KCN or 5 mM H_2O_2 for 30 min prior to activity staining. The detected activities were evaluated densitometrically and presented as means of 3 replicates. **B**. Immunoblot detection of Cu/Zn SOD isoforms in samples of *N. pannonica* treated with different PQ concentrations. Total soluble proteins were separated by SDS-PAGE, and transferred onto PVDF membrane and immunoblotted using anti-rabbit chloroplastic Cu/Zn-superoxide dismutase. Bar diagrams show densitometric values expressed as a percentage of the control values.

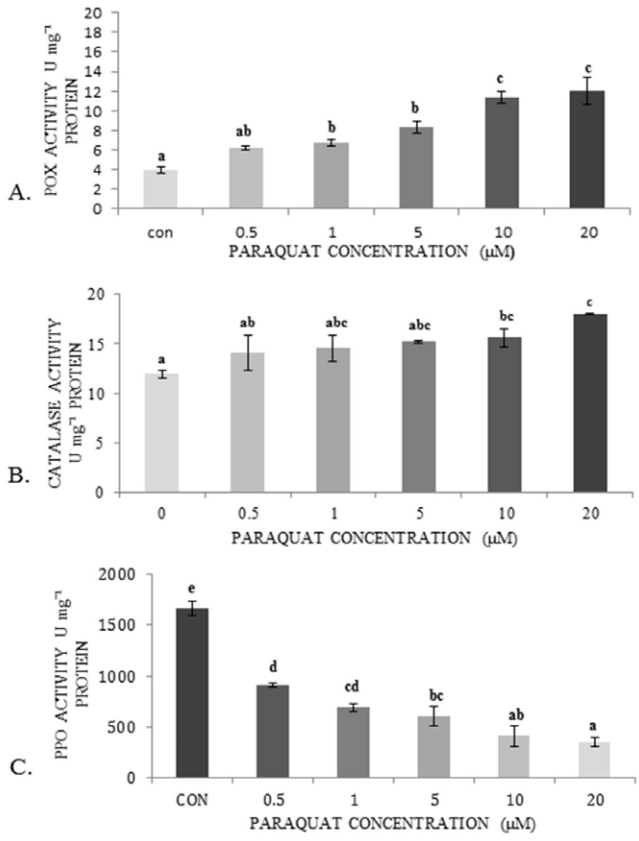

Fig 2. Activity of catalase (A), peroxidase (B) and PPO (C) of *Nepeta pannonica* shoots grown *in vitro* at different PQ concentrations. The results represent the mean ± SE of 3 replicates. Within each parameter, values with different letters are statistically different at the p≤0.05 level according to Fisher's LSD test.

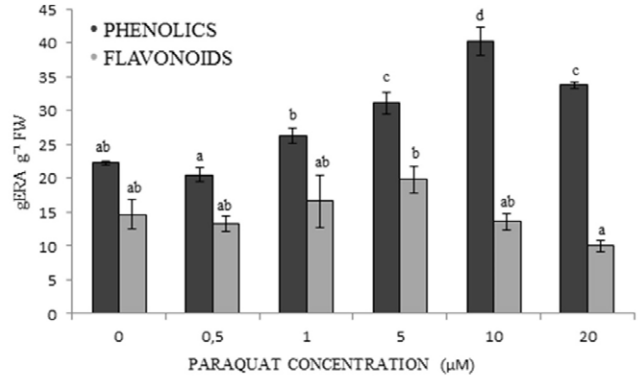

Fig 3. Total phenolic and total flavonoid contents (g ERA g^{-1} FW) of *Nepeta pannonica* shoots grown at different PQ concentrations. The results represent the mean ± SE of 3 replicates. Within each parameter, values with different letters are statistically different at the p≤0.05 level according to Fisher's LSD test.

This observation substantiates the hypothesis that, apart from the enzymatic antioxidants (SOD, CAT, and POX), phenolic compounds may also act as antioxidants (Balakumar *et al.* 1997).

TP concentrations in methanol extracts of *Nepeta pannonica* increased with PQ concentrations up to 10 μM (Fig. 3). However, at 20 μM PQ the concentration of TP decreased (Fig. 3). Concurrently, at 10 μM PQ the POX activity showed a sudden increase compared with the activity of this enzyme at 5 μM PQ (Fig. 2B). A possible explanation for this occurrence was provided by Takahama & Oniki (2000). Phenolic compounds and some phenol POX are present in vacuoles (Takahama 1992; Pedreño *et al.* 1993; Wink 1999). The coexistence of phenolics and POX indicates that if large amounts of H_20_2 are formed in or diffused into vacuoles, oxidation products of phenolics are accumulated. When plants are treated with PQ, which stimulates the production of H_2O_2 in chloroplasts, such accumulation should be observed. Increased phenol POX activity leads to the accumulation of phenolic oxidation products and, finally, to a decrease of TP concentration. Furthermore, it was shown that vacuoles rapidly oxidize esters of hydroxycinnamic acids, which are also present in the vacuoles (Takahama & Oniki 1998). This could be a possible explanation for the simultaneous decrease of RA content and sudden enhancement of the POX activity at 5 μM PQ.

A significant influence of increasing herbicide concentration on TF content in *N. pannonica* samples was not observed (Fig. 3).

As previously reported by Mišić *et al.* (2015), RA and an unidentified caffeic acid derivative (UCAD) were determined as the dominant phenolic compounds in shoots of *Nepeta pannonica*. The UHPLC/(–)HESI–MS total ion chromatogram (TIC) of *N. pannonica* (Fig. 4) showed peaks at t_R 3.65 and 4.83 min, which were assigned to UCAD and RA, both showing *m/z* $[M–H]^-$ of 359. The UHPLC/DAD analysis corroborated the presence of these compounds in samples (Fig. 4). The –HESI–MS/MS spectrum of UCAD exhibited major fragments *m/z* $[M–H]^-$ at 197 (fragment intensity <5), 179 (<5), 153 (15), 135 (100), 121 (<5), and 109 (5). Major MS/MS fragments of RA are at *m/z* $[M–H]^-$ of 197 (10), 179 (20), 161 (100), 135 (50), 133 (75), 123 (30). Both compounds exhibited a fragment *m/z* $[M–H]^-$ of 179, assignable to a caffeic acid moiety (Fig. 4). Results have shown that the amount of RA and UCAD gradually increased with increasing PQ concentrations from 0.5 μM to 5 μM by 1.04 to 1.52-fold. Further increase of herbicide concentration resulted in decreases of RA and UCAD amounts - by 1.06-fold at 10 μM and 0.88-fold at 20 μM PQ, compared with the control (Fig. 5). Thus, the stimulating effect of PQ on RA accumulation is detectable at lower doses of herbicide. Some previous reports showed that production of RA and related phenolics can be induced by various biotic and abiotic elicitors, whereby biotic stressors were much more effective in stimulating accumulation of RA.

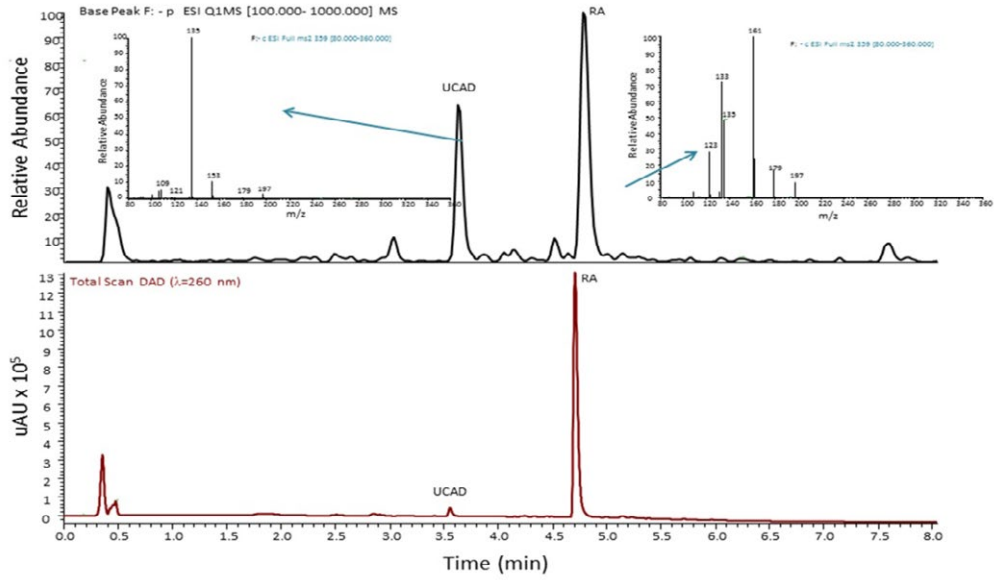

Fig. 4. UHPLC/(–) HESI–MS total ion chromatogram (TIC) of *Nepeta pannonica* methanol extracts, showing peaks between *m/z* 100 and 1000 in negative ion mode (upper chromatogram). Extracted are MS2 spectra of an unidentified caffeic acid derivative (UCAD) and rosmarinic acid (RA). UHPLC-DAD chromatogram at λ= 260 nm of *N. pannonica* methanol extracts confirmed the presence of UCAD and RA (lower chromatogram).

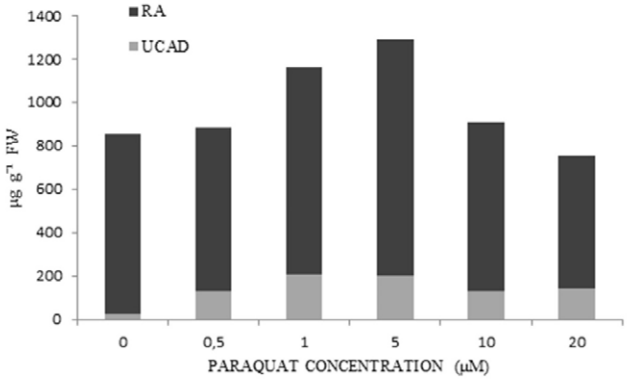

Fig 5. RA and UCAD concentrations (μg g^{-1} FW) in *Nepeta pannonica* shoots grown *in vitro* and treated with different PQ concentrations.

(Yan *et al.* 2006). On the other hand, results presented by Fletcher *et al.* (2005) suggest that heat stress conditions lead to significant loss of RA. Generally, phenolics are considered to play an important role in plant resistance and defense against microbial infections, which are intimately connected with ROS (Maher *et al.* 1994; Grassmann *et al.* 2002), and to have high antioxidative potential (Tepe *et al.* 2007; Erkan *et al.* 2008).

CONCLUSION

As a response to oxidative stress induced by PQ in *Nepeta pannonica* shoot cultures, the activities of oxygen scavenging enzymes CAT and POX were gradually enhanced by increasing PQ concentrations. Increased activities of these enzymes may be considered as circumstantial evidence for elevated production of ROS induced by the herbicide, as previously noted by Sood *et al.* (2011). Significant differences in the activities of Mn SOD and Cu/Zn SOD isoforms were observed, as influenced by applied PQ concentrations. PPO activity was progressively inhibited, and, consequently, radical scavengers - phenolic compounds accumulated, most probably as a result of the plant's defense mechanism to overcome oxidative stress damage. PQ was demonstrated to be a potent elicitor, which can stimulate accumulation of medically-important RA in *Nepeta pannonica*.

REFERENCES

Aebi H. 1984. Oxygen radicals in biological systems. *Methods Enzymol.* **105**: 121–126.

Agarwal S & Pandey V. 2004. Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. *Biol. Plant.* **48**: 555–560.

Aksakal O. 2013. Assessment of paraquat genotoxicity on barley (*Hordeum vulgare* L.) seedlings using molecular and biochemical parameters. *Acta Physiol. Plant*. **35**: 2281–2287.

Alscher RG, Erturk N & Heath LS. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot*. **53**: 1331–1341.

Balakumar T, Gayathri B & Anbudurai PR. 1997. Oxidative stress injury in tomato plants induced by supplemental UV-B radiation. *Biol. Plant*. **39**: 215–221.

Beauchamp C & Fridovich I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem*. **44**: 276–287.

Bowler C, Slooten L, Vandenbranden S, De Rycke R, Botterman J, Sybesma C, Van Montagu M & Inzé D. 1991. Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J*. **10**: 1723–1732.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**: 248–254.

Chalchat J-C, Petrovic SD & Gorunovic MS. 1998. Quantity and composition of essential oil of the wild plant *Nepeta nuda* L. from Yugoslavia. *J. Essent. Oil Res*. **10**: 423–425.

Erkan N, Ayranci G & Ayranci E. 2008. Antioxidant activities of rosemary (*Rosmarinus officinalis* L.) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. *Food Chem*. **110**: 76–82.

Fletcher RS, Slimmon T, McAuley CY & Kott LS. 2005. Heat stress reduces the accumulation of rosmarinic acid and the total antioxidant capacity in spearmint (*Mentha spicata* L). *J. Sci. Food Agric*. **85**: 2429–2436.

Formisano C, Rigano D & Senatore F. 2011. Chemical constituents and biological activities of *Nepeta* species. *Chem. Biodivers*. **8**: 1783–1818.

Foyer CH, Lopez-Delgado H, Dat JF & Scott IM. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiol. Plant*. **100**: 241–254.

Fuerst EP & Vaughn KC. 1990. Mechanisms of paraquat resistance. *Weed Technol*. **4**: 150–156.

García PC, Rivero RM, López-Lefebre LR, Sánchez E, Ruiz JM & Romero L. 2001. Response of oxidative metabolism to the application of carbendazim plus boron in tobacco. *Funct. Plant Biol*. **28**: 801–806.

Gkinis G, Tzakou O, Iliopoulou D & Roussis V. 2003. Chemical composition and biological activity of *Nepeta parnassica* oils and isolated nepetalactones. *Zeitschrift Fur Naturforsch. - Sect. C J. Biosci*. **58**: 681–686.

Grassmann J, Hippeli S & Elstner EF. 2002. Plant's defence and its benefits for animals and medicine: Role of phenolics and terpenoids in avoiding oxygen stress. *Plant Physiol. Biochem*. **40**: 471–478.

Gupta AS, Webb RP, Holaday AS & Allen RD. 1993. Overexpression of superoxide dismutase protects plants from oxidative stress (induction of ascorbate peroxidase in superoxide dismutase-overexpressing plants). *Plant Physiol*. **103**: 1067–1073.

Janicsák G, Máthé I, Miklóssy-Vári V & Blunden G. 1999. Comparative studies of the rosmarinic and caffeic acid contents of Lamiaceae species. *Biochem. Syst. Ecol*. **27**: 733–738.

Jiménez-Atiénzar M, Pedreño MA, Caballero N, Cabanes J & García-Carmona F. 2007. Characterization of polyphenol oxidase and peroxidase from peach mesocarp (Prunus *persica* L. cv. Babygold). *J. Sci. Food Agric*. **87**: 1682–1690.

Karadeniz F, Burdurlu HS, Koca N & Soyer Y. 2005. Antioxidant activity of selected fruits and vegetables grown in Turkey. *Turkish J. Agric. For*. **29**: 297–303.

Liu ZJ, Zhang XL, Bai JG, Suo BX, Xu PL & Wang L. 2009. Exogenous paraquat changes antioxidant enzyme activities and lipid peroxidation in drought-stressed cucumber leaves. *Sci. Hortic. (Amsterdam)*. **121**: 138–143.

Llorente B, López MG, Carrari F, Asís R, Di Paola Naranjo RD, Flawiá MM, Alonso GD & Bravo-Almonacid F. 2014. Downregulation of polyphenol oxidase in potato tubers redirects phenylpropanoid metabolism enhancing chlorogenate content and late blight resistance. *Mol. Breed*. **34**: 2049–2063.

Maher EA, Bate NJ, Ni W, Elkind Y, Dixon RA & Lamb CJ. 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proc. Natl. Acad. Sci. U. S. A*. **91**: 7802–7806.

Mišić D, Šiler B, Gašić U, Avramov S, Živković S, Nestorović Živković J, Milutinović M & Tešić Ž. 2015. Simultaneous UHPLC/DAD/(+/–)HESI-MS/MS analysis of phenolic acids and nepetalactones in methanol extracts of *Nepeta* species: A possible application in chemotaxonomic studies. *Phytochem. Anal*. **26**: 72–85.

Murashige T & Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant*. **15**: 473–497.

Pedreño MA, Bernal MA, Calderon AA, Ferrer MA, Lopez-Serrano M, Merino de Caceres F, Munoz R & Barcelo RA. 1993. A general pattern for peroxidase isoenzyme localization and function in Vitaceae, Solanaceae and Laguminoseae. In: Welinder KG, Rasmussen SK, Penel C, Greppin H. (eds.), Plant Peroxidases. Biochemistry and Physiology, Rochat-Baumann, Imprimerie Nationale, Geneva, pp. 307-314.

Rivero RM, Ruiz JM, García PC, López-Lefebre LR, Sánchez E & Romero L. 2001. Resistance to cold and heat stress: Accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci*. **160**: 315–321.

Ruiz JM, Bretones G, Baghour M, Ragala L, Belakbir A & Romero L. 1998. Relationship between boron and phenolic metabolism in tobacco leaves. *Phytochemistry* **48**: 269–272.

Singleton VL & Rossi, JA Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic*. **16**: 144–158.

Sood A, Kalra C, Pabbi S & Uniyal PL. 2012. Differential responses of hydrogen peroxide, lipid peroxidation and antioxidant enzymes in *Azolla microphylla* exposed to paraquat and nitric oxide. *Biologia (Bratisl)*. **67**: 1119–1128.

Sood A, Pabbi S & Uniyal PL. 2011. Effects of paraquat on lipid peroxidation and antioxidant enzymes in aquatic fern *Azolla microphylla*. *Russ. J. Plant Physiol*. **58**: 667-673.

Takahama U. 1992. Hydrogen peroxide scavenging systems in vacuoles of mesophyll cells of *Vicia faba*. *Phytochemistry* **31**: 1127–1133.

Takahama U & Oniki T. 1998. 3,4-Dihydroxyphenylalanine is oxidized by phenoxyl radicals of hydroxycinnamic acid esters in leaves of *Vicia faba* L. *J. Plant Res*. **111**: 487–494.

Takahama U & Oniki T. 2000. Flavonoids and some other phenolics as substrates of peroxidase: Physiological significance of the redox reactions. *J. Plant Res*. **113**: 301–309.

Tepe B, Eminagaoglu O, Akpulat HA & Aydin E. 2007. Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of *Salvia verticillata* (L.) subsp. verticillata and *S. verticillata* (L.) subsp. *amasiaca* (Freyn & Bornm.) Bornm. *Food Chem*. **100**: 985–989.

Tsuji K, Hosokawa M, Morita S, Miura R & Tominaga T. 2013. Resistance to paraquat in *Mazus pumilus*. *Weed Res*. **53**: 176–182.

Wink M. 1999. Introduction: biochemistry, role and biotechnology of secondary metabolites. In: Wink M (ed.), Biochemistry of Plant Secondary Metabolism, Sheffield Acad. Press Ltd., Sheffield, pp. 1-16.

Yamahara T, Shiono T, Suzuki T, Tanaka K, Satoh T & Chem JB. 1999. Isolation of a germin-like protein with manganese superoxide dismutase activity from cells of a moss , *Barbula unguiculata*. *J. Biol. Chem*. **274**: 33274–33278.

Yan Q, Shi M, Ng J & Wu JY. 2006. Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Salvia miltiorrhiza* hairy roots. *Plant Sci*. **170**: 853–858.

Zimmermann P & Zentgraf U. 2005. The correlation between oxidative stress and senescence during plant development. *Cell. Mol. Biol. Lett*. **10**: 515–534.

REZIME

Oksidativni stres izazvan parakvat herbicidom kod *Nepeta pannonica* L.

Jelena Cvetković, Milica Milutinović, Jelena Boljević, Neda Aničić, Jasmina Nestorović Živković, Suzana Živković, Danijela Mišić

Toksičnost bipiridinskog herbicida parakvata (PQ) pripisuje se njegovom brzom generisanju superoksida u lančanoj reakciji i produkciji reaktivnih kiseoničnih vrsta (ROS) u hloroplastima. Istražene su priroda i opseg ćelijskog odgovora *in vitro* gajenih izdanaka vrste *Nepeta pannonica* L. na efekte oksidativnog stresa izazvanog različitim koncentracijama parakvata (0.05-20 μM). Rezultati su pokazali da su se relativne zastupljnosti Mn SOD i Cu/Zn SOD izoformi menjale zavisno od doze parakvata, dok su aktivnosti katalaza (CAT) i peroksidaza (POX) rasle uporedo sa primenjenim koncentracijama parakvata. Smanjenje aktivnosti polifenol oksidaze (PPO) je dovelo do akumulacije fenolnih jedinjenja. Pokazano je da su ruzmarinska kiselina (RA) i neidentifikovani derivat kafeinske kiseline (UCAD) najzastupljenije fenolne kiseline kod ove vrste. Parakvat je ispoljio snažnu elicitorsku aktivnost i stimulisao akumulaciju značajnih bioaktivnih jedinjenja vrste *Nepeta pannonica*, kao što je ruzmarinska kiselina.

Ključne reči: *Nepeta panonnica*, parakvat, oksidativni stres, ruzmarinska kiselina.