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ANTIOXIDATIVE ENZYMES IN ROOT AND LEAF OF *Rumex obtusifolius* L. GROWN ON ASH AMENDED SOIL

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ABSTRACT. Antioxidant enzymes are one of the most important links in the plant defense system to various types of environmental stress, so their response to a particular type of stress may indicate the sensitivity or tolerance of the plant species. Our paper studied the difference in antioxidative enzyme [catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1) and Class III peroxidases (POD, EC 1.11.1.7)], isoenzyme pattern and activities between *Rumex obtusifolius* L. (Polygonaceae) plants grown on ash amended and uncontaminated soil. Modified SDS-PAGE electrophoresis revealed the presence of a new POD isoform in leaf samples growing on ash-amended soil, although the activity of POD in the leaves did not change significantly compared to control plants. On the other hand, in the roots of ash-growing plants POD activity decreased by 90%. Single CAT isoform was detected in both leaf samples, and results indicate 47% higher CAT activity in leaves of ash growing plants. Native electrophoresis detected two SOD isoforms in leaves and roots from the control plant. SOD isoforms were inhibited in the roots of plants grown on ash. The paper indicates the possible role of CAT, SOD and POD in the adaptive response of *R. obtusifolius* plants on ash amended soil.

Key words: *Rumex obtusifolius*, peroxidases, catalase, superoxide dismutase, ash.

INTRODUCTION

Ash is the product of the combustion of wood, paper, coal, or some other materials. Wood ash contains 92% hydroxide and 8% carbonate, mostly potassium, sodium and zinc compounds (ETIÉGNI and CAMPBELL, 1991), as well as some heavy metals (TARUN *et al.*, 2003). Ash can also be derived from different inorganic minerals in the coal, such as quartz, clays and metal oxides, and the composition can be various, however in most cases is alkaline. Similar to other abiotic and biotic environment stresses, which lead to the increased synthesis of reactive oxygen species (ROS) at the cellular level, ash can be the cause of oxidative stress (SHARMA *et al.*, 2012). Plants have a complex antioxidative defense system which represents effective

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protection against reactive oxygen species (ROS). The antioxidative defense system includes non-enzymatic components, such as ascorbate (AsA), glutathione, tocopherol, carotenoids, and phenolic compounds, as well as enzymes: superoxide dismutase (SOD), catalase (CAT), Class III peroxideses (POD), enzymes of ascorbate-glutathione (AsA-GSH) cycle, ascorbate peroxidase (APX) (SHARMA *et al*., 2012). Oxidative stress implies a state in the cell when concentrations of ROS are increased. The most important ROS species are superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and one of the most reactive hydroxyl radicals ($OH^\cdot$) (HALLIWELL and GUTTERIDGE, 2006). Superoxide dismutase (SOD, EC 1.15.1.1) is one of the main defense components against oxidative stress (SCANDALIOS, 1993). SODs are enzymes that contain metals as cofactors (Fe, Mn, Cu) and catalyze the dismutation of O_2^- to O_2 and H_2O_2. SOD activity has been reported to increase in plants that respond to diverse environmental signals in order to survive stresses (ABEDI and PAKNIYAT, 2010). Catalase (CAT, EC 1.11.1.6) is a tetrameric enzyme in which each subunit contains *heme* as a cofactor. Although it has a high K_m for H_2O_2, it is most effective in removing elevated H_2O_2 concentrations. (SCANDALIOS, 1993; SOFO *et al*., 2015). Class III peroxidases (POD, EC 1.11.1.7) are *heme*-containing glycoproteins, which catalyze oxidoreduction reactions between H_2O_2 and various reductants. Peroxidases are enzymes whose increased activity can protect organisms from various types of abiotic stress, such as drought stress (DEVI *et al.*, 2012), salt stress (WANG *et al.*, 2008) and high light stress (MITTLER, 2002), heavy metal stress (GOMES-JUNIOR *et al.*, 2007; ROMERO-PUERTAS *et al.*, 2007). PODs are a class of enzymes, called "defense-responses", that protect the plant, either oxidizing phenolic monomers to form lignin, producing H_2O_2 in response to biotic stress, or metabolizing indole acetic acid (MATSUICHI and ENDO, 1968; HOSSEINI *et al*., 2015). An important component of the antioxidant metabolism of plants are phenolic compounds (flavonoids, tannins, hydroxycinnamate esters, and lignin) which can remove ROS in direct reactions or as substrates in enzymatically catalyzed reactions (GRACE and LOGAN, 2000; AGATI *et al*., 2012). Chelate complexes between transition metal ions and polyphenols can remove reactive oxygen species and prevent lipid peroxidation. Due to modification of lipid packing order and decreased fluidity of membranes, diffusion of free radicals and restricted peroxidative reactions have interfered. Moreover, it has been shown that especially flavonoids, act in H_2O_2-scavenging as part of the phenolic/AsA/POD system (MICHALAK, 2006).

Rumex obtusifolius L., commonly known as a bitter dock, broad-leaved dock, or bundle-leaf dock, is a perennial herb belonging to the family Polygonaceae (BRANDBYGE, 1993; RECHINGER and AKEROYD, 1993; EURO+MED 2006). This plant is erect and robust 40–150 cm high with a stout taproot. Basal leaves are large, ovate-oblong, cordate at the base, obtuse or subacute, twice as long as wide and form a rosette. Cauline leaves are alternate, narrow, ovate-lanceolate to lanceolate with acute apices. Flowers are arranged in whorls along the branched raceme inflorescence. Achene is enclosed within triangular valves (3–6 x 2–3 mm) with three to five distinct teeth (CAVERS and HARPER, 1964; RECHINGER and AKEROYD, 1993). *Rumex obtusifolius* is a highly variable species and hybridizes easily with other taxa of the subgenus *Rumex*. The native species range is confined to Europe (except the Mediterranean region), while in other parts of the world it is introduced (CAVERS and HARPER, 1964; RECHINGER and AKEROYD, 1993). *Rumex obtusifolius* is a "follower of man" and a common species of waste and disturbed sites, such as field borders and roadsides (CAVERS and HARPER, 1964; TAKAHASHI and HANYU, 2015).

Rumex obtusifolius was the subject of research in our work because it was the dominant species on ash-amended soil. Ash amended soil can lead to oxidative damage in plant cells while antioxidant enzymes may play a key role in defending against oxidative stress. The assumption that led us to the research is that the soil amended by ash caused changes in the metabolism of antioxidant enzymes (SOD, CAT, POD) and phenolic compounds as important non-enzymatic antioxidants that enable survival in amended environmental conditions.

MATERIALS AND METHODS

Plant material

We used *R.obtusifolius* plants for the experiments: plants grown on ash amended soil and plants grown in their native habitat. Both habitats were located in the courtyard of the Faculty of Natural Sciences and Mathematics (Banja Luka, B&H) (Figure 1). Plants were sampled in the morning, at 19ºC. We used mixed plant samples, made of three plants from both types of soil. Roots and leaves were powder in liquid nitrogen and homogenized in extraction buffer 0.1 M Na-phosphate pH 6.4 which contained 1 mM PMSF (phenylmethylsulfonyl fluoride) and 0.2% TWEEN-20 (ratio: 4 mL of buffer per 1 g of plant tissue). After centrifugation, 10 min at 10 000 rpm, at 4ºC, the supernatant was separated and used for further analysis. Lowry method (LOWRY *et al.*, 1951) was used for the determination of protein content using BSA as a standard.

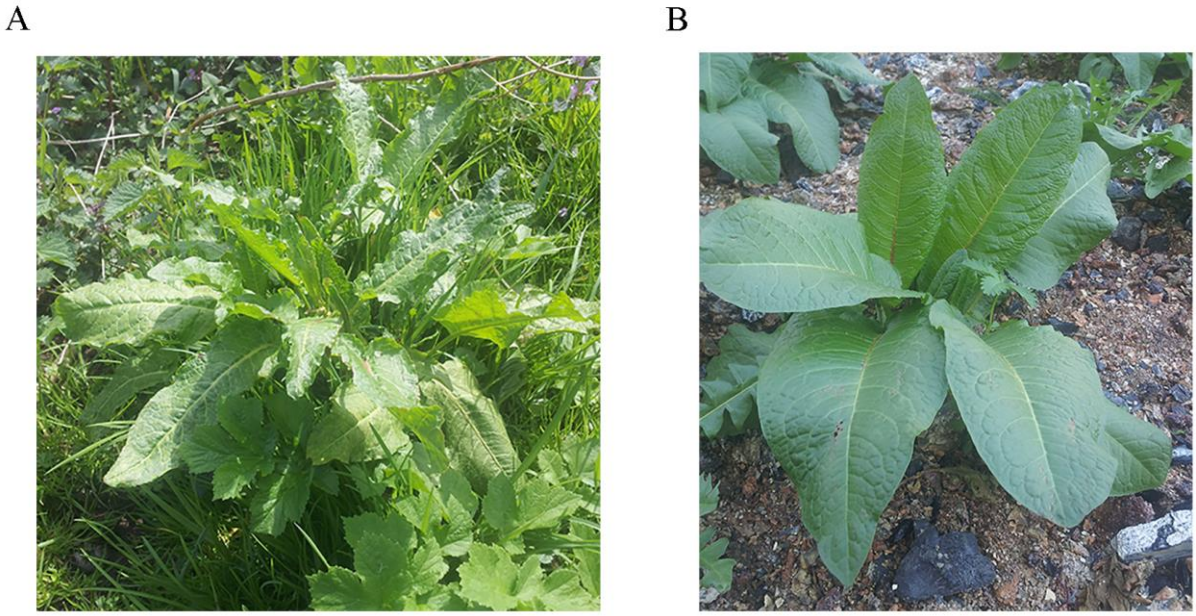


Figure 1. Individuals of *R. obtusifolius* grown on: (A) native habitat; (B) on ash amended soil

Peroxidase activity

Peroxidase activity was determined based on the increase in absorbance at 430 nm using a spectrophotometer (Shimadzu UV1800) according to the method of TEISSEIRE and GUY (2000). The reaction mixture consisted of 0.1 mM Na-phosphate buffer, pH=6.4, 100 µL of plant sample, 10 mM pyrogallol and 3.3 mM H_2O_2. The reaction was started by the addition of H_2O_2. The activity of POD was calculated on the basis of the extinction coefficient for purpurogalin (ε=12 $M^{-1}cm^{-1}$), a product of pyrogallol oxidation.

Native polyacrylamide gel electrophoresis

Isoenzyme patterns of POD, CAT and SOD were determined using polyacrylamide gel electrophoresis (PAGE, Mini-Protean Tetra Cell, Bio-Rad) according to the method of VELJOVIC-JOVANOVIC *et al*. (2006). For separation of POD and SOD isoforms 10% polyacrylamide gel was used and 8% polyacrylamide gel was used for separation of CAT isoforms. Buffer for electrophoresis (pH=8.3) contained 24.8 mM Tris and 192 mM glycine. Before loading on the gel, samples were mixed with loading buffer (50 mM Tris pH=6.8, 10%

glycerol and 0.1% bromophenol blue) in the ratio of 2:1 and on the gel, 10 µg of protein was applied. Peroxidase isoforms were determined with a special staining solution (0.01% 4-chloro-α-naphthol and 0.03% H_2O_2 in 100 mM Na-phosphate buffer, pH=6.5). After electrophoresis, the gel was incubated in the staining solution until the appearance of purple strips, which indicated the presence of POD isoforms. The gel was incubated in 0.003% H_2O_2 for 5 minutes for CAT determination. After washing several times with distilled water, the gel was incubated in a staining solution which contained 1% $FeCl_3$ and 1% $K_4[Fe(CN)_6]$. SOD isoforms were visualized after gel incubation in the solution consisting of 0.1 mM NTB; 2 mM TEMED; 0.03 mM riboflavin; 250 mM EDTA and 100 mM TRIS, pH=7.8 for 30 min in darkness and exposing the gel to UV light. White bands on violet gel detected the presence of SOD isoforms.

Quantification of SOD and CAT activity on the native gel was performed using Image Master Total Lab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA).

Modified sodium dodecyl sulfate–polyacrylamide gel electrophoresis

In the modified sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE electrophoresis) method 12% polyacrylamide running gel and 5% stacking gel were used according to MIKA and LÜHTJE (2003). Both, buffer for samples and buffer for electrophoresis, contained 0.1% SDS. For *heme* staining, gel was incubated in the specific mixture (40 mg 3,3',5,5'-tetramethylbenzidine (TMB), 30 mL methanol and 70 mL 0.02 M Na-acetate buffer, pH=5) for 2 hours. After that, 340 µL H_2O_2 (35%) was added and after 3 minutes gel was washed with 30 mL isopropanol, 70 mL 0.02 M Na-acetate buffer, pH=5. Bright blue strips on the gel indicated the presence of POD isoforms.

Content of phenolic compounds

The content of phenolic compounds was determined using the Folin-Ciocalteu reagent (SINGLETON and ROSSI, 1965) and by measuring the absorbance at 724 nm. As the standard, gallic acid (0.1-2 mM) was used.

Photosynthetic pigments

Photosynthetic pigments were extracted in 100% acetone (ratio of plant tissue and acetone was 0.5 g per 10 mL) and then filtered. The content of photosynthetic pigments was determined spectrophotometrically by measuring absorbance at 662, 644 and 440 nm (WETTSTEIN, 1957).

Soil pH

To determine the pH of the soil, 10 g of soil (combined with several parts of the rhizosphere) was suspended in 50 mL of distilled water. After intensive mixing, the suspended soil particles were precipitated. The pH of the supernatant was measured with a pH meter (WTW inoLab pH 7110) according to the KALRA (1995).

RESULTS AND DISCUSSION

In this paper, the activity of enzymatic antioxidants POD, CAT, SOD and phenolic compounds were estimated to describe the changes in the antioxidative system between control *R. obtusifolius* plants, grown in native habitat (soil pH=6.9) and plants grown on ash amended

soil (soil pH=9). To overcome various types of stress, plants are equipped with antioxidative mechanisms to eliminate or reduce their damaging effects (stress-induced ROS accumulation).

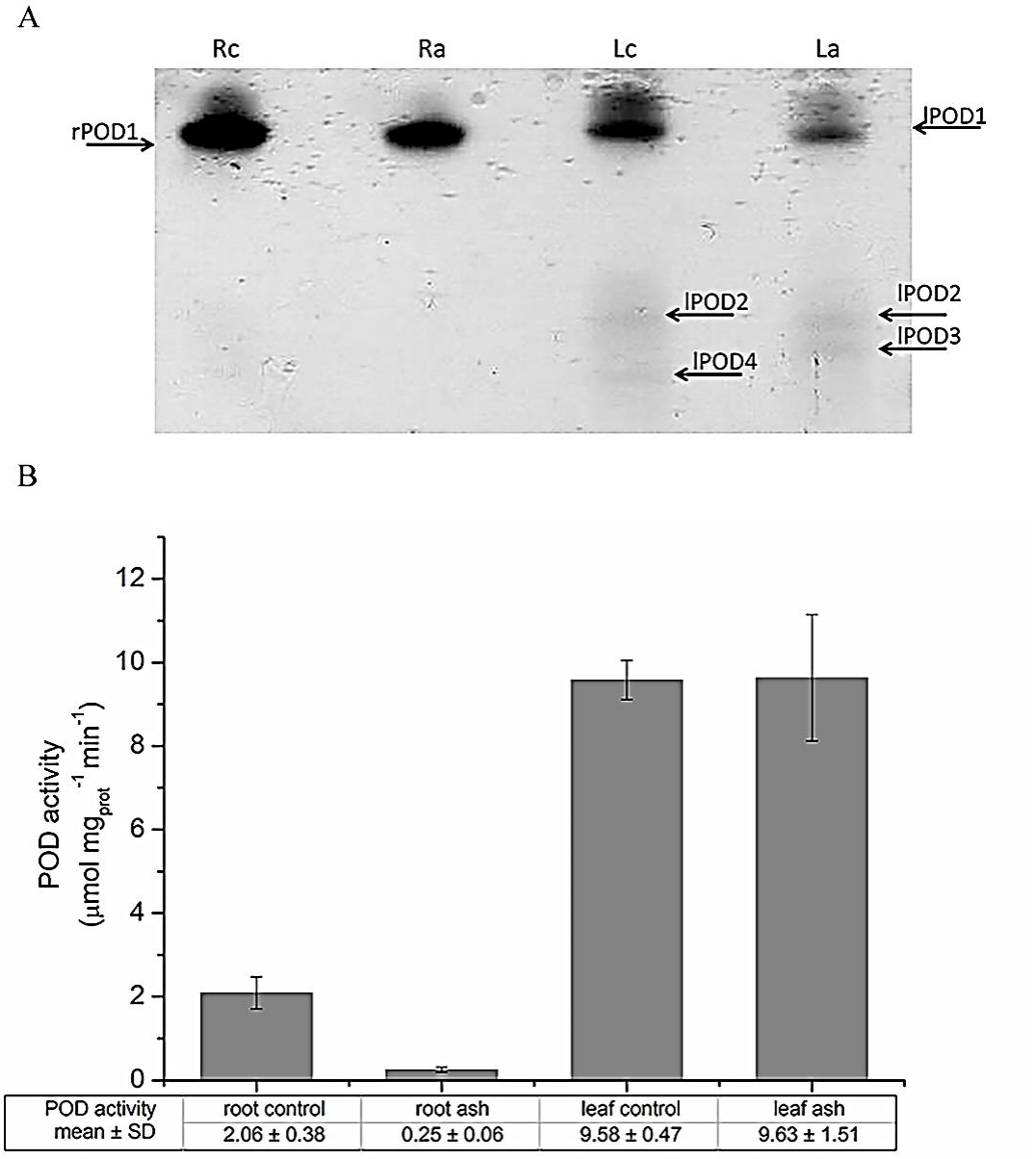


Figure 2. A) Native gel with POD isoforms detected with specific α-chloro-naphthol staining. The arrows indicate the different POD isoforms: in root samples rPOD1 and lPOD1, lPOD2, lPOD3 and lPOD4 in leaf samples. B) Peroxidase activity were determined spectrophotometrically by measuring the increase in absorbance at 430 nm.
Lc – control leaf; La – ash leaf; Rc – control root; Ra – ash root.

One peroxidase isoform was detected by native PAGE in the roots of control plants and plants grown on ash (Figure 2A). Isoenzyme profiles obtained by native electrophoresis in leaves of control plant and plant grown on ash amended soil was different: in control plants, isoforms marked with lPOD1, lPOD2 and lPOD4 were detected, while isoforms lPOD1, lPOD2 and lPOD3 were detected in ash plants (Figure 2A). POD activity, measured spectrophotometrically, in the root sample from ash, was significantly decreased, around 90%, while in the leaf sample, the change was negligible (Figure 2B).

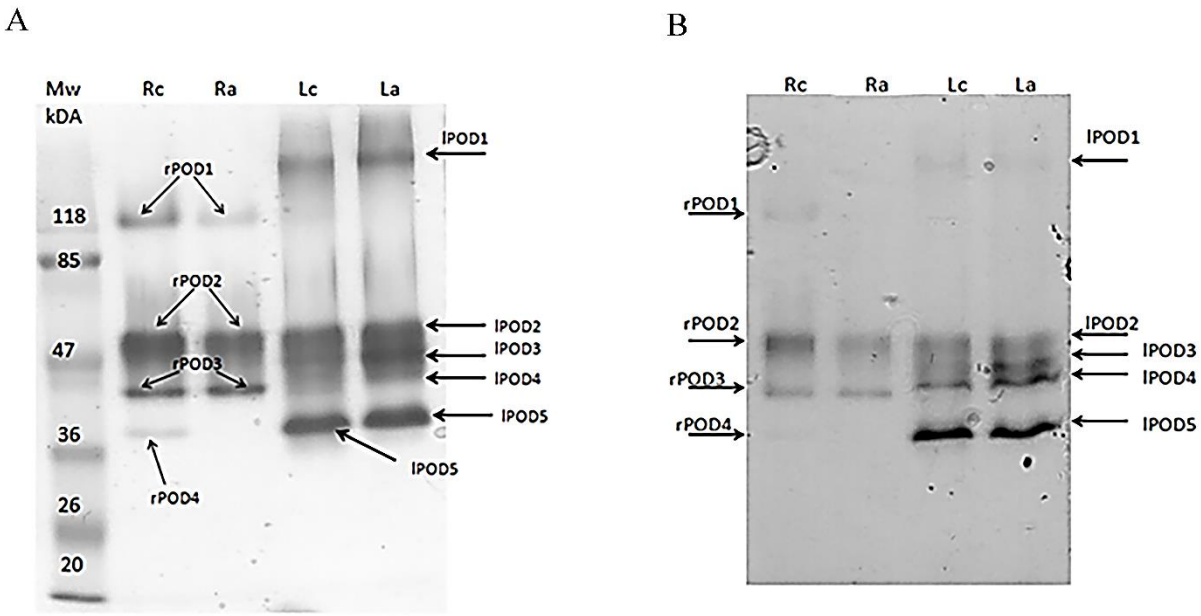


Figure 3. Isoenzyme pattern of POD isoforms detected after: (A) *heme* staining; (B) staining with α-chloro-naphthol. The molecular weight of standards: β-galactosidase (118 kDA), bovine serum albumin (85 kDA), ovalbumin (47 kDA), carbonic anhydrase (36 kDA), β-lactoglobulin (26 kDA), lysozyme (20 kDA). The arrows indicate the different POD isoforms: in root samples rPOD1, rPOD2, rPOD3, rPOD4 and lPOD1, lPOD2, lPOD3, lPOD4 and lPOD5 in leaf samples. Lc – control leaf; La – ash leaf; Rc – control root; Ra – ash root.

Modified SDS electrophoresis was used to separate enzyme isoforms in their 3D conformations, where isoform separation is affected by the shape and size of the isoforms. Protein markers used for SDS electrophoresis can also be used in modified SDS electrophoresis, which allows the determination of apparent molecular weights of isoforms in their 3D conformation The advantage of modified SDS electrophoresis over native is that it gives better separation of isoforms and enables the determination of their approximate molecular weights (MIKA and LÜHTJE, 2003). In our experiment modified SDS electrophoresis after staining with tetramethylbenzidine (TMB), resolved the presence of four POD (marked as rPOD 1-4) isoforms with an apparent molecular weight: rPOD1~122 kDa, rPOD2~61 kDa, rPOD~49 kDa and rPOD~38 kDa) (Figure 3; Table 1). POD isoform with molecular weight ~38 kDa was not detected in plants grown on ash.

Table 1. Molecular weights POD isoforms detected with modified SDS electrophoresis in roots and leaves of *R. obtusifolius.*

Samples	POD Isoforms	Apparent molecular weight (kDa)
Root	rPOD1	122
	rPOD2	61
	rPOD3	49
	rPOD4	38
Leaf	lPOD1	138
	lPOD2	61
	lPOD3	56
	lPOD4	48
	lPOD5	42

In leaves of control plants, also stained with TMB, five POD isoforms were separated on modified SDS gel with apparent molecular weight: lPOD1~138 kDa, lPOD2~61 kDa, lPOD3~56 kDa, lPOD4~48 kDa and lPOD5~42 kDa (Table 1). In contrast to the control plants, in leaves of plants that were grown on ash, isoform lPOD4 with a molecular weight 48 kDa is not detected, but there was an induction of new isoform Mw~51 kDa. Staining with 4-chloro naphthol, a specific substrate for peroxidase, showed that all isoforms identified after staining with TMB, in the leaves and roots of *R. obtusifolius* have peroxidase activity (Figure 3A). Every induction of a new stress-related isoenzyme is probably related to the level of ROS, which causes oxidative damage to the plant cells (ZHANG *et al*., 2013). According to literature, salt stress can stimulate or inhibit the expression of the isoforms of antioxidative enzymes (KIM *et al.,* 2003). Drought stress had indicated that higher activity levels of antioxidant enzymes may contribute to better tolerance (SHARMA and DUBEY, 2005). However, POD activities under the abiotic stress are dependent on plant species, cultivar, stress intensity and duration (ZHANG and KIRKHAM, 1996; SHIGETO and TSUTSUMI, 2015). In the study of temperature stress influence on POD, the lowest POD activity was found under the high temperature (RIVERO *et al.,* 2001). LIU *et al*. (2013) showed that low temperature affects the *Avena nuda* plants POD activity. Authors showed that decreased POD activity was the consequence of reduced synthesis of POD, because these temperatures can affect the RNA transcription and translation.

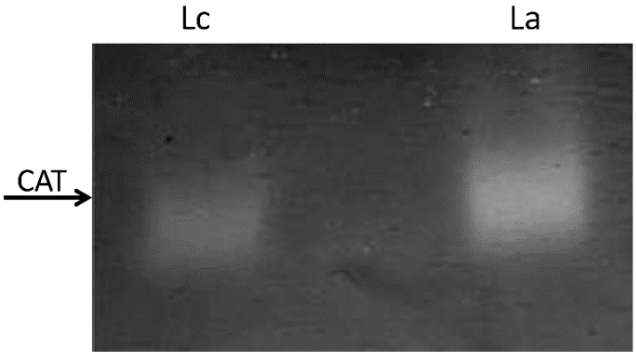
Table 2. Content of total proteins, chlorophyll *a*, chlorophyll *b* and phenolic compounds in roots and leaves of *R. obtusifolius* plants grown on uncontaminated area and ash amended soil.

Samples	Leaf control	Laf ash	Root control	Root ash
Total proteins (mg/g)	10.184±2.491	11.131±3.959	5.951±1.741	7.565±0.937
Content of phenolic compounds (mM)	10.269±0.484	10.3144±0.484	10.511±0.864	10.962±0.561
Chlorophyll *a* (mg/g)	0.759±0.062	0.801±0.086	-	-
Chlorophyll *b* (mg/g)	0.189±0.002	0.222±0.037	-	-
Carotenoids (mg/g)	0.278±0.024	0.300±0.001	-	-

Under oxidative stress, total soluble proteins are usually measured as an index of metabolic changes. Due to stress conditions, ROS can cause serious damage in the reactions with proteins, as well as with nucleic acids and lipids. Our results showed a significant increase in protein content (27%) in the roots of the plants growing on ash-amended soil. However, an increase in the protein content was 9% in the leaves (Table 2). The synthesis of stress proteins that participate in cellular detoxification was induced under heavy metals stress (RASTGOO and ALEMZADEH 2011; RASTGOO *et al*., 2014).

An increase in the content of photosynthetic pigments in the leaves of plants growing on ash amended soil was measured: Chlorophyll *a* for 5.5% and Chlorophyll *b* for 18% compared to control (Table 2). Higher concentrations of sodium, potassium and magnesium cause increased content of Chlorophyll *a* (RAI, 2002), while higher content of phosphorus increased Chlorophyll *b* concentration (CANJURA *et al*., 1991). Results showed a slightly higher concentration of carotenoids in plants grown on ash compared to control (Table 2).

Content of phenolic compounds in the leaves and roots of the plants growing on the ash did not significantly differ from the control plants (Table 2). These results may indicate the existence of other mechanisms involved in the adaptation of *R. obtusifolius* on ash amended soil. The induction of biosynthesis of phenolic compounds in response to abiotic stress can be considered an adaptation mechanism of plants. (RIVERO *et al.,* 2001; MARTINEZ *et al.,* 2016).

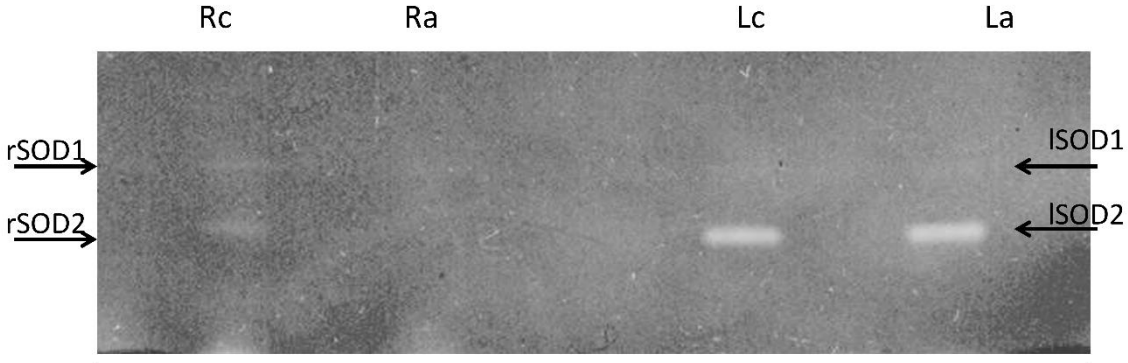


CAT activity (relative units)

Lc	La
676 096	1 282 040

Figure 4. CAT activity was determined using 8% native gel in leaves of control and ash-growing plants. CAT activity, obtained by densitometry after analysis of gels in the program TotaLab, was presented in the table below the gel. Lc – control leaf; La – ash leaf.

Native PAGE showed the presence of one CAT isoform in the leaves of all samples of *R. obtusifolius* (Figure 4). Results indicate 47% higher CAT activity in leaves of plants growing on ash-amended soil. In response to stress, the induction of CAT synthesis may be different depending on the plant species and the type of stress (SCANDALIOS, 2002). Salt stress (MITTLER *et al.*, 2011; SIMOVA-STOILOVA *et al.*, 2010) or drought (NADERI *et al.*, 2014) could increase CAT activity. UV-B light stress also led to the significant increase in CAT activity in some plants (AGARWAL *et al.*, 2007). However, that is not always the case and may depend on the plant species. Other cases showed decreased CAT activity under the same type of abiotic stress (SHARMA and DUBEY, 2005).



Total SOD activity (relative units)

Rc	Ra	Lc	La
12306	0	16247	20498

Figure 5. SOD activity was determined using 10% native gel in roots and leaves of control and ash-growing plants. SOD activity, obtained by densitometry after analysis of gels in the program TotaLab, was presented in the table below the gel. The arrows indicate the different SOD isoforms: rSOD1 and rSOD2 in root samples and lSOD1 and lSOD2 in leaf samples.

Obtained results indicated the presence of two SOD isoforms (rSOD1 and rSOD2) in the root of control plants, while in the root of plants grown on ash amended soil SOD isoforms

were inhibited (Figure 5). In both leaf samples, two SOD isoforms (lSOD1 and lSOD2) were detected. Total SOD activity in leaves of plants grown on ash amended soil was increased by 26% compared to the control plants. This can be a result of abiotic stress that affected plants grown on ash-amended soil. Literature data proves that different stress conditions such as salt (NaCl) stress (GAO *et al.,* 2008; TSANG *et al.,* 2014; NADERI *et al.,* 2014), drought stress in *Brassica napus* plants (ABEDI and PAKNIYAT, 2010) or high temperature (HE and HUANG, 2010) cause increased SOD activity.

CONCLUSIONS

Our results showed changes in the antioxidant metabolism of *R. obtusifolius* plants grown on ash amended soil compared to plants from their natural habitat. The most significant changes were observed at the level of antioxidant enzymes. New POD isoforms were detected in a plant leaves sample grown on ash amended soil. In addition, higher CAT and SOD activity was detected in a plant leaves sample growth on ash amended soil. On the other hand, the content of phenolic compounds, proteins and pigments did not change significantly, which may indicate the initiation of specific defense mechanisms in response to ash amended soil.

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