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RESEARCH ARTICLE

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Modification of antioxidant systems in cell walls of maize roots by different nitrogen sources

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Abstract

Antioxidant systems of maize root cell walls grown on different nitrogen sources were evaluated. Plants were grown on a medium containing only NO_3^- or the mixture of NO_3^-+NH_4^+, in a 2:1 ratio. Eleven-day old plants, two days after the initiation of lateral roots, were used for the experiments. Cell walls were isolated from lateral roots and primary root segments, 2-7 cm from tip to base, representing zones of intense or decreased growth rates, respectively. Protein content and the activity of enzymes peroxidase, malate dehydrogenase and ascorbate oxidase ionically or covalently bound to the walls, as well as cell wall phenolic content and antioxidant capacity, were determined. Cell walls of plants grown on mixed N possess more developed enzymatic antioxidant systems and lower non-enzymatic antioxidant defenses than cell walls grown on NO_3^-. Irrespective of N treatment, the activities of all studied enzymes and protein content were higher in cell walls of lateral compared to primary roots. Phenolic content of cell walls isolated from lateral roots was higher in NO_3^--grown than in mixed N grown plants. No significant differences could be observed in the isozyme patterns of cell wall peroxidases isolated from plants grown on different nutrient solution. Our results indicate that different N treatments modify the antioxidant systems of root cell walls. Treatment with NO_3^- resulted in an increase of constitutive phenolic content, while the combination of NO_3^-+NH_4^+ elevated the redox enzyme activities in root cell walls.

Additional key words: nitrate; ammonia; antioxidant enzymes; phenolic compounds.

Abbreviations used: AO (ascorbate oxidase); DPPH (2,2-diphenyl-1-picrylhydrazyl); EAA (equivalent of ascorbic acid); FAE (ferulic acid equivalent); FW (fresh weight); IAA (indole-3-acetic acid); IEF (isoelectric focusing); MDH (malate dehydrogenase); POD (peroxidases); ROS (reactive oxygen species).

Authors' contributions: Conceived and designed the experiments: VHTŠ. Performed the experiments: VHTŠ, MV and KM. Statistical analysis: NK. Wrote the paper: VHTŠ, MV and ŽV. Coordinating the research project: ŽV.

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Introduction

The plant cell is enclosed by the apoplast that consists of the cell wall fibrillar structure and the intercellular liquid and gas spaces. Generally, apoplastic enzymes and reactive oxygen species (ROS) play a role in cell wall synthesis and loosening, processes involved in plant growth regulation (Passardi *et al*., 2004). Also, apoplast, especially the root apoplast, as the exposed compartment of the plant cell, is important for all of the plant's interactions with its environment (Zhou *et al*., 2011). It can detect environmental changes and stress signals and transfer them into the cell interior to trigger a whole cell response (Zhou *et al*., 2011). Cell-wall-located enzymes are considered as active sites for apoplastic ROS production. Depending on the concentration, ROS (superoxide anion radical, hydrogen peroxide, hydroxyl radical and singlet oxygen), have a dual role. At high concentration ROS cause damage to biomolecules, whereas at low/moderate concentration they act as secondary messengers in intracellular signaling cascades that elicit various cellular responses. Cell wall enzymes, such as class III peroxidases (PODs) in their oxidative cycle, malate dehydrogenase (MDH), oxalate- and amine oxidases are related to H_2O_2-production necessary for polymerization pro-

cesses such as lignification and oxidative coupling of cell wall proteins or for direct defense against abiotic and biotic stresses (Gross *et al*., 1977; Vuletić & Hadži-Tašković Šukalović, 2000; Laurenzi *et al*., 2001; Mika *et al*., 2004; Ros-Barcelo *et al*., 2004). By enabling rapid induction of chemical defenses against stress, such enzymes represent a part of induced plant defense. Being exposed to various abiotic and biotic stresses, apoplastic production of ROS in many adverse environmental conditions has to be tightly controlled in order to avoid oxidative damage of plant cell. Therefore, root cell walls have evolved protective systems to eliminate or reduce the excess of ROS, including enzymes such as POD, superoxide dismutase, ascorbate oxidase (AO) and non-enzymatic low molecular weight antioxidants ascorbate and phenolics (Takahama & Oniki, 1992; Mika *et al*., 2004; Pignocchi *et al*., 2006; Kukavica *et al*., 2009). PODs and superoxide dismutase are directly involved in ROS scavenging, removing hydrogen peroxide, and dismutating superoxide anion radical to hydrogen peroxide, respectively. AO oxidizes ascorbate to water and ascorbate-free radicals, contributing to the regulation of the ascorbate redox state and coupled redox pairs and radical species. Apoplastic ascorbate is involved in the regulation of cell expansion and elongation due to its ability to impair the catalytic reaction of POD by re-reducing the intermediate phenoxyl radical produced during the peroxidative reaction (Takahama & Oniki, 1992).

Plant phenolics are secondary metabolites involved in the defense mechanism of plants and growth regulation. Their accumulation is a striking example of metabolic plasticity against abiotic and biotic stress factors, enabling plants to adapt to changing environments (Boudet, 2007). Phenolics esterified with cell wall polysaccharides, lignin and wall proteins, constitute cell walls, and in such a way are involved in mechanical system of defense, efficient in response to stress conditions (Santiago *et al*., 2013). As cell wall constituents, phenolics contribute to overall antioxidant capacity in many ways: as electron donors to H_2O_2 in reactions catalysed by class III POD, free radical scavengers (Rice-Evans *et al*., 1997) and regulators of H_2O_2 and ascorbate content through the ASC/phenolic/H_2O_2 system catalysed by POD (Takahama & Oniki, 1997; Hadži-Tašković Šukalović *et al*., 2008).

Nitrogen availability and the use of NO_3^- or NH_4^+ affects plant growth (Marschner, 1995; Stitt, 1999), intracellular pH (Raven & Smith, 1976), cation and anion content of plant tissues (Kirkby & Mengel, 1967), protein, organic acids, and carbohydrates content (Marschner, 1995; Raab & Terry, 1995; Stitt, 1999), as well as the activity of enzymes involved in the N assimilation and organic acid synthesis (Hadži-Tašković Šukalović & Vuletić, 1998). Increased N supply, favoring plant growth, reduces accumulation of total phenolic compounds (Leser & Treutter, 2005) while N deficiency increases the content of phenolic compounds (Kováčik & Bačkor, 2007). However, there are only a few reports, in which the state of the antioxidant enzymatic systems was studied as the function of N nutrition. Polesskaya *et al*. (2004) demonstrated that NH_4^+ induced increased antioxidant enzyme activities. Wang *et al*. (2008) in a study with *Vallisneria natans* leaves have shown that NH_4^+ induce generation of ROS and up-regulate antioxidant enzymes (superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase). Podgórska *et al*. (2013) showed that ammonium nutrition changes mitochondrial electron transport chain activity, increasing mitochondrial reactive oxygen species production. Fernández-Crespo *et al*. (2012) also reported that the ammonium-fed citrus plants showed increased levels of H_2O_2 accumulation.

Previously, we demonstrated a variable response of maize root soluble antioxidant enzymes and phenolic compounds to the presence of increased nitrate or nitrate plus ammonium content in the growth medium (Vuletić *et al*., 2010). The goal of this work was to study whether N nutrition could modify antioxidant systems of cell walls. The activity of enzymes involved in regulation of ROS level and cross linking of cell walls, protein content, total phenolic content, reducing capacity and free radical quenching activity of isolated walls were analysed. Since N metabolism is tightly coupled to root growth, we performed the experiments on basal non-growing root regions and intensely growing lateral roots, the aim being the determination of an eventual metabolic shift in the above mentioned enzyme and antioxidant systems in the early stages of response before any visible morphological changes in the appearance of the plant occur.

Material and methods

Plant material and growth conditions

Maize (*Zea mays* L.) inbred line Va35 seeds were germinated for 3 days on watered filter paper and then transferred to a constantly aerated ¼-strenght Knop solution (Hoagland & Arnon, 1950), modified in N content. Nitrogen was supplied as KNO_3, $Ca(NO_3)_2$ and $(NH_4)_2SO_4$, in two treatments, the concentrations of NO_3^- and NH_4^+ in the solution being 3.6:0 and 3.6:1.8 mM, respectively. The initial pH of the nutrient solutions was adjusted to 5.6. Plants were grown in 2.5 L plastic pots (30 plantlets per container) for 8 days in a growth

chamber with a photoperiod of 12h/12h (light/darkness) at 22/18 °C, illuminated with 190 µmol/m^2·s photosynthetically active radiation and relative humidity of 70%. Plant cultivation was done in at least three independent experiments. Eleven-day old plants were used for the experiments. Root and shoot length and fresh weight (FW) of 10 plants from each experiment were measured.

Preparation of root cell wall fractions

Comparative analysis of the antioxidant status and enzyme activities in the tissue of different developmental stage was performed. Since the growing zone of maize primary root is limited to a region of about 1.2 cm from the apex (Zhang *et al*., 2013), primary root segment, 2-7 cm from tip to base, was used as the sample with decreased growth rate. Lateral roots, with a maximal length of about 1.5 cm (two days after initiation), were taken as intensive growth zone. Cell wall isolate was prepared according to procedure described earlier (Hadži-Tašković Šukalović *et al*., 2011). To release ionically bound proteins, the cell wall isolate was incubated in 10 mL of 1 M NaCl for 60 min with continuous stirring at 4 °C. The supernatant obtained after centrifugation at 1,000 × *g* for 10 min, was considered as ionic fraction. The pellet was washed twice and resuspended in 10 mL of 50 mM Tris-HCl buffer, pH 7.2. This suspension, representing cell wall covalently bound fraction, was used for most of the experiments, except for isoelectric focusing (IEF). To solubilise the covalently bound peroxidase for the IEF experiments, the procedure of Matsuo *et al*. (2006) was used. Namely, suspension containing covalent bound proteins was incubated in 0.1 M NaOH added to Tris-HCl buffer pH 7.2 at room temperature with constant shaking for 1 h, followed by centrifugation for 10 min at 1,000 × *g*. The obtained supernatant containing solubilised covalent proteins, as well as ionical fraction were concentrated before the IEF experiments using an Amicon Ultra-4 Centrifugal Filter Device. All samples were stored at -40 °C until use.

Enzyme assay procedures

Peroxidative activity of POD (EC 1.11.1.7) was determined by measuring the initial rate of ferulic acid absorbance decrease at 286 nm (ε=16.800/(mM·cm)) in 1-mL assay mixture containing 0.1 mM ferulic acid, 1 mM H_2O_2 and root isolate in 50 mM potassium phosphate buffer (pH 6.5). Oxidative activity of POD with NADH as substrate, was determined by measuring NADH oxidation at 340 nm (ε=6.22/(mM·cm)) in 1-mL assay mixture containing 0.2 mM NADH, 0.25 mM $MnCl_2$ and 0.1 mM *p*-coumaric acid and cell wall isolate in 50 mM potassium phosphate buffer, pH 5.5. To evaluate the effect of malate and oxaloacetate on oxidative activity of POD, the experiments were performed with indole-3-acetic acid (IAA) as substrate. The absorbance changes were measured at 260 nm (ε=3.200/(mM·cm)) in 1-mL assay mixture containing 0.66 mM IAA, 1 mM $MnCl_2$, 0.05 mM *p*-coumaric acid and cell wall isolate in 50 mM potassium phosphate buffer, pH 5.5.

Ascorbate oxidase (EC 1.10.3.3) activity was determined by measuring oxygen consumption in the reaction mixture containing 1 mM Na-ascorbate and 0.5 mM EDTA and cell wall isolate in 100 mM potassium phosphate buffer, pH 5.6.

Malate dehydrogenase (EC 1.1.1.37) activity was determined as oxaloacetate reduction, estimated by the decrease in absorbance of NADH at 340 nm (ε=6.22/(mM·cm)) (Hayes *et al*., 1991).

Enzyme activity was determined in ionic fraction (solubilized with 1 M NaCl) and covalent fraction (cell wall suspension obtained after salt washing) of the isolated cell walls. Each of the above assays was used in a range in which the rate of reaction was proportional to the amount of cell wall isolate added. The reactions were initiated by adding the cell wall isolate or substrates. All enzyme activities were expressed per gram of root FW. Experiments were performed at 30 °C. For analyses, duplicate cell wall isolates of two independent plant growing experiments were used.

Determination of DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method of Serpen *et al*. (2007) was used to evaluate free radical scavenging activity of NaCl-washed cell wall suspension. The results were expressed in µg equivalent of ascorbic acid (EAA) per gram of root FW.

Determination of reduction power

Aliquot of salt-washed cell wall suspension was used to detect reduction power by the method described by Kim & Kim (2010), based on the Fe^{3+} reduction to Fe^{2+}. The data were presented in mg EAA per gram of root FW.

Phenolic content determination

Total phenolics were determined after alkaline hydrolysis of NaCl-washed cell wall material. Samples

(0.25 mL) were suspended in 0.5 M NaOH (final volume 2.5 mL), heated to 96 °C for 2 h and after acidification to pH 2 with 6 N HCl, phenolics were extracted 4 times with 2 volumes of diethyl ether. Combined extracts were dried, and resuspended in 2.5 mL of the 70% acetone for the analysis of total phenolics by using the Folin-Ciocalteu procedure (Hagerman *et al*., 2000). Total phenolic content was calculated as a ferulic acid equivalent (FAE) from the calibration curve obtained using ferulic acid. Results were expressed as FAE per gram of root FW.

Isoelectric focusing of peroxidase isoforms

Proteins were separated by IEF in order to determine POD isoforms according to isoelectric points. IEF was carried out in 7.5% polyacrylamide gel with 3% ampholyte on a pH gradient from 2 to 11. Markers for IEF of pI range 3.6-10.7 were purchased from Sigma (IEF-M1A). The amount of sample applied to each well was quantified according to the ability to oxidize 0.2 µmol ferulic acid/min. After separation, isoforms of POD were stained in 250 mM sodium acetate buffer, pH 5.0 containing 1% guaiacol and 0.03% H_2O_2 (Mika *et al*., 2008).

Protein estimation

Protein content was determined using the Bradford assay (Bradford, 1976), with bovine serum albumin as the standard. To detect covalently bound proteins, salt-washed cell wall suspension was treated with 2 N NaOH for 3 h at room temperature. Proteins were determined in supernatant, after centrifugation at 10,000 × *g* for 5 min.

Statistical analysis

All analyses were performed in at least triplicate measurements and the results were statistically analyzed and presented as mean ± standard error (SE). Statistically significant differences between the observed mean values were determined by Fisher's least significant difference (LSD) test at the 0.05 probability level, after the analysis of variance (ANOVA) using one-way factorial RCB design. Differences with $p \leq 0.05$ were considered as significant.

Results

The effect of nitrogen sources on plant growth

To examine the possible modification of antioxidant systems by different N sources, we used nutrient solutions containing only NO_3^- or the mixture of NO_3^-+NH_4^+ in a 2:1 ratio, in order to alleviate the effect of ammonia toxicity as a sole N source (Hadži-Tašković Šukalović & Vuletić, 1998). When NO_3^- was the only N source, maize plants produced longer roots and shoots, increased shoot FW and slightly decreased root FW (Table 1). However, the differences noted were not statistically significant.

Protein content of fraction ionically and covalently bound to the cell walls

The content of proteins ionically and covalently bound to the cell walls is shown in Table 2. Covalent fraction contained 4-5 fold more proteins than the corresponding ionic fraction. Plants grown on mixed N had higher protein content (up to 25%) in comparison with nitrate grown plants in all fractions, except the ionic fraction isolated from primary roots.

Activity of enzymes ionically and covalently bound to the cell walls

Since POD enzymes are closely coupled to the oxidative metabolism by utilizing and/or producing reactive oxygen species during their peroxidative and oxidative cycles, respectively, both activities were evaluated. Ferulic acid, as natural substrate for cell wall PODs, was used to measure the peroxidative activity. Oxidative activity was assayed with NADH (widely used substrate for the in vitro experiments) or IAA (generally accepted as a naturally occurring reductant in the apoplastic place). Activity measurements show

Table 1. Root length (RL), shoot length (SL) and fresh weight (FW) of maize plants grown on nutrient solution containing a NO_3^-+NH_4^+ or just NO_3^-.

Treatment	RL (cm)	SL (cm)	Root FW (g)	Shoot FW (g)
NO_3^-+NH_4^+	22.3±2.5 A	9.6±0.8 A	0.32±0.03 A	0.43±0.03 A
NO_3^-	24.6±1.2 A	10.2±0.5 A	0.30±0.03 A	0.48 ±0.02 A

Data are means ± SE of three independent experiments with at least 10 plants per treatment in each experiment. Mean values in a column followed by the same letter are not significantly different ($p \leq 0.05$).

Table 2. Protein content (mg/g FW) in cell walls isolated from maize roots grown on different nitrogen containing solutions.

	Sample	$NO_3^-+NH_4^+$	NO_3^-	$NO_3^-+NH_4^+/NO_3^-$
Ionically bound	Lateral roots	0.45±0.02 Aa	0.39±0.01Ba	1.15
	Primary root	0.18±0.01 Ab	0.19±0.01Ab	0.97
Covalently bound	Lateral roots	1.94±0.04 Aa	1.54±0.05 Ba	1.25
	Primary root	0.89±0.01 Ab	0.75±0.06 Bb	1.19

Data are means of three independent experiments ± SE. Mean values in a row (upper case) and means of ionically or covalently bound fractions in a column (lower case) followed by the same letter are not significantly different ($p \leq 0.05$).

Table 3. Peroxidative activity of peroxidase (μmol ferulic acid oxidized/(g FW·min)) ionically and covalently bound to the cell walls of maize roots grown on different nitrogen forms.

	Sample	$NO_3^-+NH_4^+$	NO_3^-	$NO_3^-+NH_4^+/NO_3^-$
Ionically bound	Lateral roots	5.6±0.50 Aa	4.4±0.09 Ba	1.26
	Primary root	3.5±0.071 Ab	2.7±0.10 Bb	1.29
Covalently bound	Lateral roots	4.3±0.15 Aa	2.0±0.40 Ba	2.09
	Primary root	2.5±0.10 Ab	1.9±0.20 Ba	1.30

Data are means of three independent experiments ± SE. Mean values in a row (upper case) and means of ionically or covalently bound fractions in a column (lower case) followed by the same letter are not significantly different ($p \leq 0.05$).

Table 4. Oxidative activity of peroxidase (μmol NADH oxidized/(g FW·min)) ionically and covalently bound to the cell walls of maize roots grown on different nitrogen sources.

	Sample	$NO_3^-+NH_4^+$	NO_3^-	$NO_3^-+NH_4^+/NO_3^-$
Ionically bound	Lateral roots	89.97±4.0 Aa	73.63±2.5 Ba	1.22
	Primary root	68.57±3.1 Ab	58.47±1.2 Bb	1.17
Covalently bound	Lateral roots	70.03±1.1 Aa	58.25±0.5 Ba	1.20
	Primary root	46.45±0.15 Ab	43.50±3.2 Ab	1.07

Data are means of three independent experiments ± SE. Mean values in a row (upper case) and means of ionically or covalently bound fractions in a column (lower case) followed by the same letter are not significantly different ($p \leq 0.05$).

that POD enzymes are predominately associated with cell walls by ionic interaction. The ratio between ionically and covalently bound peroxidative activities (calculated on the root FW basis) was almost constant (≈ 1.35), except in lateral roots of nitrate-grown plants (≈ 2.1). Both activities, peroxidative (Table 3) and oxidative (Table 4), were generally higher in plants grown in the presence of mixed N in comparison with nitrate-grown plants. Thus, in most isolates, the peroxidative activity was 30% greater, with exception of covalent fraction isolated from lateral roots where it was doubled. Oxidative activity increased 7-22%. Activity of MDH was predominately detected in the covalent fraction. In lateral roots isolated from covalent fraction, the activity was about 2.8 times higher than in ionic fraction, irrespective of the N treatment. Also, both cell walls fractions isolated from lateral roots of plants grown on mixed N had about 1.4 times higher MDH activity than the corresponding isolated from plants grown on nitrate only (Table 5). In both cell wall fractions, mixed N induced the increase of MDH activities (>40%) in lateral roots only, while in primary roots the activity was decreased (Table 5).

Ascorbate oxidase activity was found to be predominantly covalently bound (~ 95% of total activity) to the cell walls (Vuletić *et al*., 2014), and thus the effects of N forms were analyzed in NaCl-washed cell wall suspension. The activity was higher in nitrate-grown plants (Table 6), in both lateral (27% increase) and primary roots (24% increase).

Since one could assume that cell wall enzyme activities are regulated *in situ* by variation in the concentration of apoplastic metabolite, we analysed the effect of malate and oxaloacetate on POD activity, and phenolic POD substrates on MDH activity, as well. Peroxidative reaction was inhibited with oxaloacetate in a concentration-dependent manner (Fig. 1). Much higher inhibition was detected in ionic fraction than in the covalently

Table 5. Activity of cell wall ionically and covalently bound malate dehydrogenase (μmol NADH oxidized/(g FW·min)) in maize roots grown on different nitrogen forms.

	Sample	NO_3^-+NH_4^+	NO_3^-	NO_3^-+NH_4^+/NO_3^-
Ionically bound	Lateral roots	1.76±0.07 Aa	1.14±0.02 Ba	1.55
	Primary root	0.40±0.09 Ab	0.45±0.04 Ab	0.90
Covalently bound	Lateral roots	4.75±0.4 Aa	3.37±0.3 Ba	1.4
	Primary root	0.50±0.2 Ab	0.67±0.3 Ab	0.74

Data are means of three independent experiments ± SE. Mean values in a row (upper case) and means of ionically or covalently bound fractions in a column (lower case) followed by the same letter are not significantly different ($p \leq 0.05$).

Table 6. Activity of root cell wall covalently bound ascorbate oxidase (nmol O_2/(g FW·min)) in maize plants grown on different nitrogen forms.

Sample	NO_3^-+NH_4^+	NO_3^-	NO_3^-+NH_4^+/NO_3^-
Lateral roots	640±31 Ba	817±15 Aa	0.78
Primary root	589±27 Aa	733±18 Bb	0.80

Data are means of three independent experiments ± SE. Mean values in a column (lower case) and in a row (upper case) followed by the same letter are not significantly different ($p \leq 0.05$).

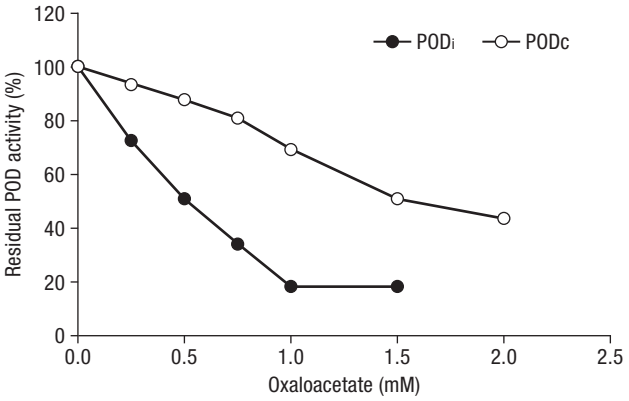

Figure 1. Inhibitory effect of oxaloacetate on peroxidative peroxidase activity measured with ferulic acid in both cell wall fractions (POD_i-ionic and POD_c-covalent). Residual POD activities are expressed as percent of maximal POD activity, obtained in the absence of oxaloacetate.

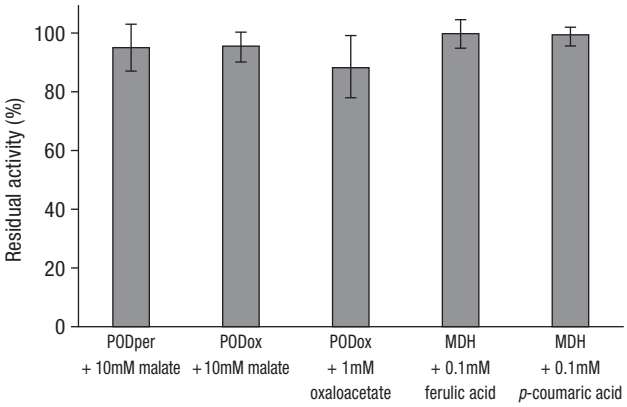

Figure 2. The effect of 10 mM malate on peroxidative peroxidase activity (PODper), 10 mM malate and 1 mM oxaloacetate on oxidative peroxidase activity (PODox) and the effect of 0.1 mM ferulic and 0.1 mM *p*-coumaric acid on MDH activity. Ionic fraction of cell walls isolated from lateral roots grown on NO_3^-+NH_4^+ nutrient solution was used for experiments. Residual enzyme activities are expressed as percent of maximal activities, measured without effectors added to the assay mixture. Data are means ± SE of three independent experiments.

bound fraction. Such a strong inhibitory effect of oxaloacetate on oxidative POD activity was not detected (the activity was measured as IAA oxidation). Also, 10 mM malate did not change POD activities. Activity of MDH was not changed in the presence of 0.1 mM ferulic or *p*-coumaric acid. Effects of oxaloacetate and malate on POD activities as well as the effect of phenolics on MDH activity are presented in Fig. 2.

Generally, the activities of all studied enzymes and protein content in both N treatments were higher in cell walls of lateral than in the investigated segment of primary roots, as presented in Fig. 3. The ratio of POD activities ionically bound to lateral and primary roots was similar irrespective of N forms. Slightly higher POD activity ratio was detected in the covalent fraction isolated from mixed N-grown plants. The difference between lateral and primary roots was most pronounced for MDH in both N treatments, especially in plants grown on mixed N.

Phenolic content, DPPH radical scavenging activity and reducing power of cell walls

The phenolic content, DPPH radical scavenging activity and reducing power of salt-washed cell walls isolated from lateral roots of plants grown solely on nitrate

Table 7. Total phenolic content, DPPH radical scavenging activity and reducing power of NaCl-washed cell wall suspension. Cell walls were isolated from maize roots grown on different nitrogen forms.

	Sample	NO_3^-+NH_4^+	NO_3^-	NO_3^-+NH_4^+/NO_3^-
Phenolic content (mg FAE/g FW)	Lateral roots	1.8±0.2 Ba	2.6±0.2 Aa	0.69
	Primary root	1.7±0.3 Aa	1.5±0.02 Ab	1.13
DPPH scavenging activity (µg EAA/g FW)	Lateral roots	34.1±4.8 Bb	67.2±3.6 Aa	0.51
	Primary root	44.1±4.4 Aa	40.2±3.2 Ab	1.10
Reducing power (mg EAA/g FW)	Lateral roots	0.10±0.006 Aa	0.12±0.009 Aa	0.83
	Primary root	0.12±0.01 Ab	0.13±0.02 Aa	0.92

Data are means of three independent experiments ± SE. Mean values in a row (upper case) and mean of phenolic content, DPPH scavenging activity or reducing power in a column (lower case) followed by the same letter are not significantly different ($p \leq 0.05$).

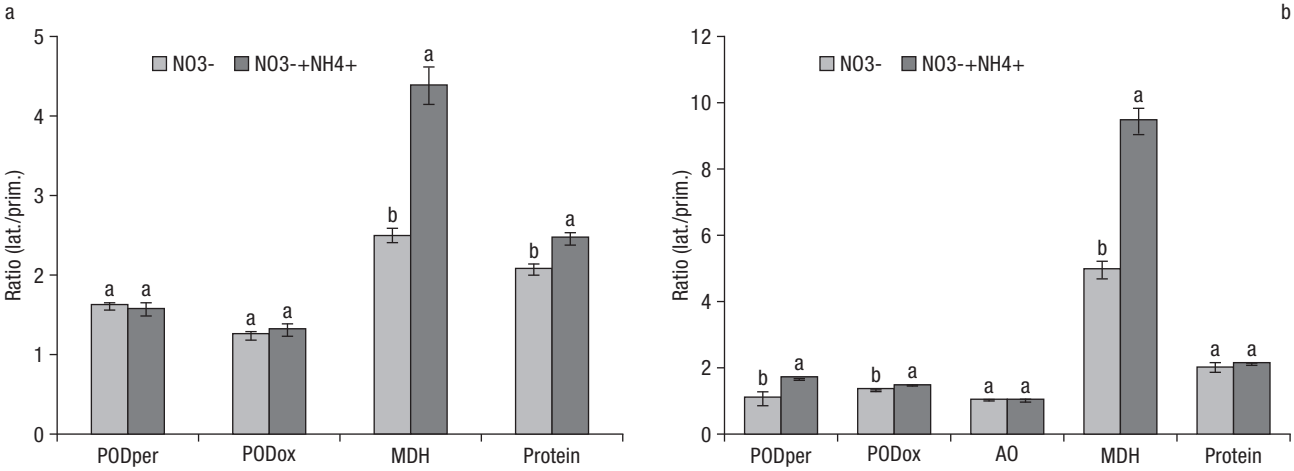

Figure 3. The ratio of enzyme activities and protein content between lateral and primary root cell walls of maize plants grown in the presence of NO_3^- or mixture NO_3^-+NH_4^+. Experiments were performed in ionic (a) and covalent fractions (b) of root cell walls. PODper, peroxidative peroxidase activity; PODox, oxidative peroxidase activity; MDH, malate dehydrogenase activity; AO, ascorbate oxidase activity. Data are means ± SE of three independent experiments. Different letters above the histogram bars indicate significant differences between groups ($p \leq 0.05$).

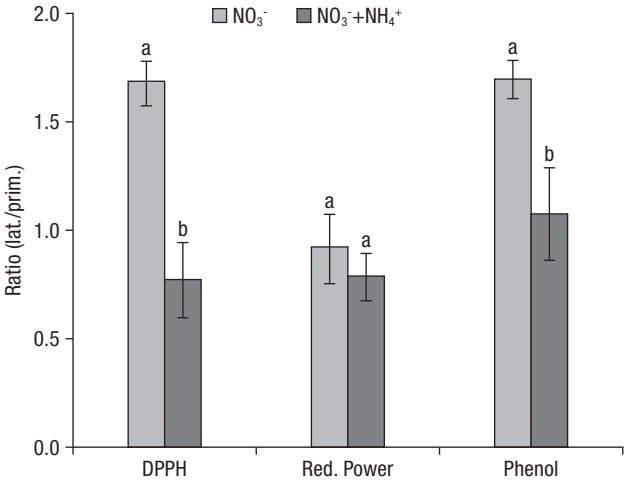

Figure 4. The ratio of DPPH radical scavenging activity, reducing power (Red. Power) and total phenolic content (Phenol) between lateral and primary root cell walls of maize plants grown in the presence of NO_3^- or NO_3^-+NH_4^+. Data are means ± SE of three independent experiments. Different letters above the histogram bars indicate significant differences between groups ($p \leq 0.05$).

were higher than of roots grown on mixed N (42, 97 and 22%, respectively), as opposed to enzyme activities and protein content. Phenolic content and radical scavenging activity were slightly higher (≈10%) in primary roots grown on mixed N or were unaffected in the case of reducing power, compared to nitrate-grown plants (Table 7). Cell walls of lateral roots grown on nitrate had much higher phenolic content and exhibited stronger free radical scavenging properties against DPPH radical than walls of primary roots (≈70%). Such a difference was not observed in mixed N-grown plants (Fig. 4).

Isoenzyme profiles of POD

IEF and activity staining with guaiacol showed that ionic and covalent protein fractions consisted of many POD isoforms. Isoelectric points (pI) of POD isoforms in covalent fraction showed a complete range, from pI 3.6 to 11, while in the ionic fraction acidic isoforms with pI<4.6 could not be observed (Fig. 5, lanes 1-8).

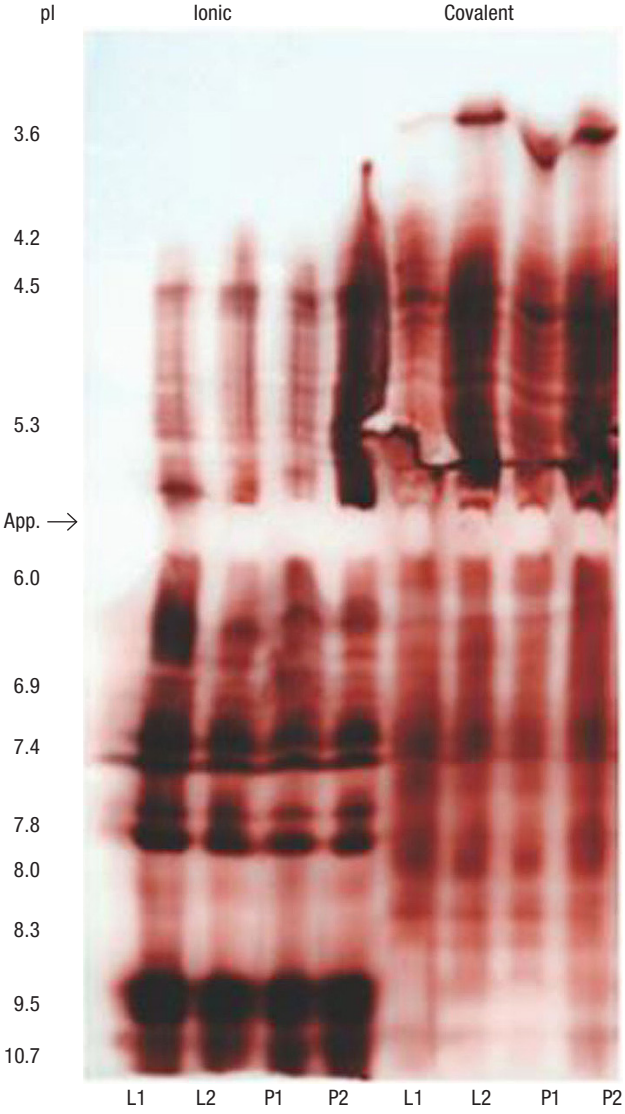

Figure 5. Isoelectric focusing of ionically and covalently bound cell wall proteins, isolated from lateral (L) or primary (P) maize roots and stained for peroxidase activity. Plants were grown in the presence of NO_3^-+NH_4^+ (1) or of NO_3^- (2). App, sample application place; pI, isoelectric point.

Although anionic and cationic POD isoforms existed in both fractions, anionic isoforms predominated in the covalent fraction, cationic in the ionic fraction. Anionic isoforms with pI from 7 to 4.6 and three cationic isoforms with pI>8.8 were present in both fractions. Nitrogen treatments did not influence the number of isoenzymes. Similar isozyme patterns were obtained with isolates from lateral and primary roots.

Discussion

Nitrogen, a major constituent of plant macromolecules, is available in the soil as nitrate (NO_3^-) or ammonium (NH_4^+) ions or as organic N derived from the degradation of plant and animal residuals. The degree of efficiency for two N forms on plant growth and nutrient uptake, when both sources of N are present in the solution, is dependent on plant species and NO_3^-/NH_4^+ ratio (Errebhi & Wilcox, 1990). Although ammonium was considered to be toxic when applied as the sole N source (Britto & Kronzucker, 2002), it has been shown that in combination with nitrate it provides maximal growth rates of maize plants (Schrader *et al*., 1972). Previous results of our laboratory indicated that the mixture of NO_3^-+NH_4^+ in a 2:1 ratio positively regulates root mitochondrial respiration and tricarboxylic acid metabolism (Hadži-Tašković Šukalović & Vuletić, 1998), root soluble antioxidant enzyme activities and proline content, but negatively total phenolics (Vuletić *et al*., 2010), when compared with plants grown on NO_3^- as the sole N source. Positive effect of mixed N on plant growth (longer roots with more developed lateral roots), manifested during prolonged cultivation was reported previously (Vuletić *et al*., 2010). However, in this work, in order to analyse the zone of intense growth rate, eleven-day old plants, 2 days after the initiation of lateral roots, were used for experiments. Since the experiments were performed in an early developmental stage of maize plants, during the shift from seedling heterotrophy to autotrophy (Enns *et al*., 2006), different N treatments exhibited no significant effect on growth parameters. Nevertheless, the effect of applied treatments on the enzymatic and non-enzymatic antioxidant systems of cell walls was evident, and point that at this stage of growth, the chemical analysis is a more reliable indicator of initial metabolic changes prior to first visible morphological parameters. An enhancement of protein content, POD and MDH activities, accompanied by the AO activity decrease, was observed in the cell walls isolated from mixed N-grown plants, as compared to that from nitrate-grown plants. On the other hand, cell wall fragments isolated from lateral roots grown on nitrate exhibited increased antioxidant capacity, detected as radical scavenging activity and reducing power.

Generally, higher POD and MDH activities and lower AO activity in plants grown on mixed N could point to the response against more intense ROS production. Although Wang *et al*. (2008) and Podgórska *et al*. (2013) indicated that ammonium ions induce an increase in ROS production, Domínguez-Valdivia *et al*. (2008) demonstrated that ammonium stress is not an oxidative stress, moreover, it diminishes oxidative damage to proteins. The increase in amino acid synthesis and protein turnover (resulting in C deficiency) in plants fed with ammonium (Domínguez-Valdivia *et al*., 2008), could be the explanation of increased cell wall protein content and consequently enzyme activities in

our experimental system. The increase of cell wall enzyme activities, as well as root soluble antioxidant enzyme activities (Vuletić *et al*., 2010) under similar growing conditions, point to the increased levels of preformed cell wall enzymes in symplast and their possible export to the apoplastic compartment.

Higher activity of POD in cell walls of plants grown on mixed N, suggests that this enzyme is not a limiting factor for phenolics cross-linking of cell wall polymers under mixed N conditions. It could be supposed that phenolic synthesis and/or delivering to the cell wall compartment could be one of the reasons for its lower content detected in the cell wall structure. Reduced accumulation of total phenolic compounds and higher protein content in cell walls of plant grown on mixed N could be explained by more intensive amino acid synthesis (Schrader *et al*., 1972), leading to the decrease of the C/N ratio and limited production of secondary metabolites, according to carbon/nutrient balance hypothesis (Bryant *et al*., 1983; Karowe & Grubb, 2011).

An additional reason for lower phenolics content in cell walls of mixed N-grown roots could be the *in situ* regulation of enzyme activities, implicated directly (POD) or indirectly (MDH and AO) in formation of phenolic cross-links between cell wall polymers. Although the regulation of POD activities in phenolic cross-linking to the cell wall structures could be closely and positively regulated with MDH activity through the providing NADH for H_2O_2 generation in oxidative POD reaction for lignification process (Gross, 1977; Gross & Janse, 1977), our results point to the possibility of inhibition of ferulic and *p*-coumaric acid oxidation with H_2O_2 in peroxidase reactions by oxaloacetate produced in MDH reaction. On the other hand, reductants such as ascorbate (regulated by the AO in the apoplast) or NADH (as a product of malate oxidation), as secondary electron donors, could generate ascorbate/phenolic/H_2O_2 or NADH/phenolic/H_2O_2 peroxidase system functioning solely as H_2O_2 scavengers (Hadži-Tašković Šukalović *et al*., 2008). In either case, the result would be the prevention of phenolic binding to cell wall structures. In the light of such considerations, lower phenolic content in lateral roots grown on mixed N compared to nitrate grown plants could be explained by suppression of POD activity by oxaloacetate, produced in MDH reaction, highly stimulated by mixed N, as well as higher ascorbate content in apoplastic compartment as a consequence of lower AO activity. As opposed to that, higher phenolic content in lateral roots of nitrate grown plants could be due to the increased AO activity in combination with decreased MDH activity that allows POD enzyme to function efficiently in the cross-linking of phenolics. Similar IEF patterns in both N treatments and almost constant ratio of ionically to covalently bound POD activities, indicating that POD enzyme binding to the cell wall was unaffected by N forms, argues in favor of POD regulation with metabolites.

The increased content of total phenolics together with increased radical scavenging activity and reducing power in nitrate-grown roots argue that phenolic esters bound to the wall polymers contribute significantly to the antioxidant capacity (Ohta *et al*., 1994). The most abundant phenolic constituents of maize root cell walls are ferulic and *p*-coumaric acids (Vuletić *et al*., 2014) esterified with arabinoxylan (Hatfield *et al*., 1999) and lignin (Hatfield & Chaptman, 2009), respectively. Much greater increase in DPPH scavenging activity than in reducing power in the same cell wall isolates could be ascribed to the presence of ferulic acid esters, being extremely potent as DPPH radical scavengers and possessing weaker reducing power than free ferulic acid (Szwajgier *et al*., 2005). The effect of *p*-coumaric acid could be minimal, due to its lower antioxidant activity in any form (Szwajgier *et al*., 2005). The difference in antioxidant capacity observed in roots grown on different N media was evident only in lateral roots, clearly demonstrating the differences in the structure of cell walls.

Nitrogen form slightly influenced the enzyme activity ratio between lateral and primary roots, with the exception of MDH, which increased in roots grown on mixed N. Increased MDH activity in intensively growing lateral roots, as demonstrated previously (Hadži-Tašković Šukalović *et al*., 2011), supports the proposal of Gross (1977) that MDH is implicated in growth regulation.

Our results confirm that in growing tissue mixed N stimulates metabolic processes leading to increased protein (and enzymes) availability, while nitrate highly improves antioxidative capacity by increasing constitutive phenolics content. It could be argued that plants with increased level of cell wall bound phenolics possess better mechanical defense, therefore exhibiting greater tolerance to biotic stress. On the other hand, increase in redox enzyme activities allows rapid induction of chemical defenses against biotic and abiotic stress.

The lack of such a difference within segments of primary roots may be explained by their formation during seedling heterotrophy.

It could be concluded that the use of nutrients, such as NO_3^- or NO_3^-+NH_4^+ enables an increase of constitutive phenolic content or redox enzyme activities, respectively, in root cell walls. Thus, different N treatments could be applied as a tool towards improved constitutive resistance or induced chemical defenses of plants.

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