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BRIEF COMMUNICATION

Antioxidative system in maize roots as affected by osmotic stress and different nitrogen sources

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Abstract

The activities of antioxidative enzymes and contents of proline and total phenolics were assayed in roots of two maize (*Zea mays* L.) genotypes grown in a medium containing nitrate (NO_3^-) or both nitrogen forms, nitrate and ammonium (NH_4^+/NO_3^-). An increase in the activities of class III peroxidases (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), ascorbate oxidase (AO) and proline content, and decrease in phenolic content were observed in NH_4^+/NO_3^- in comparison with NO_3^- grown plants. When polyethylene glycol (PEG) was added to both nitrogen treatments, the content of total phenolics and proline was increased, especially in NH_4^+/NO_3^- treatment. The PEG treatment decreased enzyme activities in NH_4^+/NO_3^- grown plants, but in NO_3^- grown plants activities of POD and SOD were increased, opposite to decreased APX and AO. Isoelectric focusing demonstrated increased activities of acidic POD isoforms in PEG treated NO_3^- grown plants, and lower activities of both, acidic and basic isoforms in NH_4^+/NO_3^- grown plants.

Additional key words: antioxidative enzymes, proline, phenolics, *Zea mays* L.

Reactive oxygen species (ROS) are important signals in the biosynthesis of complex organic molecules, polymerization of cell wall constituents, and defence against abiotic and biotic stresses. They are produced in both unstressed and especially in stressed plant cells. However, oxidative damage to lipids, proteins and DNA occurs under excess of ROS. Therefore, it is the balance between the production and the scavenging of ROS that is critical to the maintenance of the active growth and metabolism of the plant and overall stress tolerance (Foyer and Noctor 2005). To remove the excess of harmful ROS plant cells possess antioxidative systems, consisting of low-molecular mass antioxidants, as well as antioxidative enzymes. In addition to well known antioxidants such as ascorbate and glutathione, amino acid proline, besides its role in stress protection as an osmolyte, was shown to be a potent scavenger of ROS (Matysik *et al.* 2002). Antioxidant properties of phenolics were also reported (Rice-Evans *et al.* 1997). Superoxide dismutase (SOD), peroxidases and catalase are directly involved in ROS scavenging.

Nitrogen, an essential mineral nutrient for the plant growth, is mostly supplied in form of nitrate and ammonium, with different effect on many metabolic processes, including ROS production and antioxidative defence. The activities of antioxidative enzymes were significantly higher in the plants grown in NH_4^+ than NO_3^- medium (Polesskaya *et al.* 2004, Domínguez-Valdivia *et al.* 2008). Recent studies demonstrated that proline accumulates in the condition of nitrogen excess (Sánches *et al.* 2001) and that NH_4^+ increases proline accumulation in leaves (Rivero *et al.* 2004).

The joint influence of stress factors and nitrogen form on the plant antioxidative system was studied in a few laboratories (Rios-Gonzales *et al.* 2002, Polesskaya *et al.* 2006). Studies that combine NH_4^+ and salt stress suggest that NH_4^+ or its assimilation products may serve as a stress signal to activate antioxidative enzymes (Rios-Gonzales *et al.* 2002). Since the root is a primary plant organ involved in mineral acquisition, responding to

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Abbreviations: AO - ascorbate oxidase; APX - ascorbate peroxidase; IEF - isoelectric focusing; PEG - polyethylene glycol; POD - class III peroxidases; ROS - reactive oxygen species; SOD - superoxide dismutase.
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nitrogen form by modifications in the cellular metabolism, we have investigated whether such changes could influence the antioxidative system and its response to osmotic stress. In this work, we studied the influence of PEG-induced osmotic stress on the activities of SOD, class III peroxidases (POD), ascorbate peroxidase (APX), ascorbate oxidase (AO), as well as on contents of free proline and total phenolics in roots of maize plants grown in a medium with nitrate or with both nitrogen forms.

Seeds of maize (*Zea mays* L.) inbred lines VA35 and B73 were germinated for 3 d and then transferred into plastic pots containing Knop solution, with modified nitrogen content. For the first 7 d, plants were grown on ¼ strength nutrient solution and during the following 4 d on full strength solution. Nitrogen was supplied in the form of KNO_3, $Ca(NO_3)_2$ and $(NH_4)_2SO_4$ in two treatments, the concentrations of NO_3^- and NH_4^+ in the full strength solution being 10.9:0 and 10.9:7.2 mM, respectively. The initial pH of the solutions was adjusted to 5.6. Plants were kept in a growth chamber under a 12-h photoperiod at 22/18 °C, with irradiance of 40 W m^{-2} and relative humidity of 70 %. The last 48 h of the growing period plants were grown on the fresh aerated nutrient solution (control, C), or solution supplemented with 4 % polyethylene glycol (PEG, Mr 10 000) (treatment, T).

Free proline was determined according to Bates *et al.* (1973). Total phenolic content was determined from 40 % ethanol extract of roots by using Folin-Ciocalteu reagent (Hagerman *et al.* 2000). To assay enzyme activities, root tissue was homogenized with 10 volumes of 100 mM K-phosphate buffer, pH 7.5 and centrifuged at 20 000 *g* for 15 min. Proteins from supernatant were precipitated with ammonia sulfate (90 % saturation), dialysed overnight against the same buffer, and used for experiments. The oxidative activity of POD (EC 1.11.1.7) was determined as NADH oxidation, with *p*-coumaric acid and $MnCl_2$ as cofactors, while phenolic POD activity was determined as oxidation of ferulic acid and coniferyl alcohol with 1 mM H_2O_2 and 0.3 µg of protein (Hadži-Taškovic Šukalović *et al*. 2005). SOD (EC 1.15.1.1) activity was assayed by the method of Sutherland and Learmonth (1997). APX (EC 1.11.1.11) activity was determined as ascorbate oxidation by monitoring the absorbance decrease at 290 nm (coefficient of absorbance 2.8 mM^{-1} cm^{-1}) in a reaction mixture, consisting of 0.5 mM Na-ascorbate, 0.1 mM H_2O_2 in 50 mM K-phosphate buffer, pH 7.2 and about 10 µg of protein. Determination of AO (EC 1.10.3.3) activity was performed in assay mixture consisting of 0.5 mM EDTA, 0.1 mM Na-ascorbate and about 30 µg of protein in 50 mM K-phosphate buffer, pH 5.6, by monitoring ascorbate oxidation at 265 nm (coefficient of absorbance 14 mM^{-1} cm^{-1}). Calculations of specific enzyme activities were done on the protein basis, measured by the method of Lowry *et al.* (1951). All the assays were performed at 30 °C.

Proteins were separated by isoelectric focusing (IEF) and POD isoenzymes stained with 10 % 4-chloro-1-naphthol and 0.03 % H_2O_2 in 50 mM K-phosphate buffer, pH 6.5, for 10 min at 25 °C. IEF was carried out in a 7.5 % polyacrylamide gel with 3 % ampholite in a pH gradient from 3.5 to 10. Total amount of protein applied to each well was 2 µg.

Since NH_4^+ as sole nitrogen source is toxic to many plant species, high concentration of NH_4^+ was supplied in the mixture with NO_3^- in order to alleviate the effect of ammonia toxicity (Schortemeyer *et al*. 1997). Such plants did not show visual symptoms of NH_4^+ toxicity and produced longer roots with more developed lateral roots, and slightly higher fresh mass (FM) compared to those grown on NO_3^- (Table 1). However, in comparison to NO_3^- grown plants, root dry mass (DM) of plants grown on NH_4^+/NO_3^- was decreased for about 20 %. Lower root DM of such plants is in agreement with results obtained for different maize genotypes (Schortemeyer *et al*. 1997), as well as other plant species (Lasa *et al.* 2002, Domínguez-Valdivia *et al.* 2008) grown in the presence of sole NH_4^+. Treatment with PEG induced slight decrease of FM (about 20 %) and almost no change of DM of roots when grown on NO_3^- solution. In the presence of NH_4^+ decrease of FM was more pronounced (up to 40 %) and DM increased for about 30 %.

The content of free proline was increased by about 50 % in both genotypes grown on NH_4^+/NO_3^- in comparison with NO_3^- grown plants. This could imply the induction of aminating role of glutamate dehydrogenase (GDH) as a response to increased intracellular NH_4^+ content and the shift of glutamate toward proline synthesis. Stimulation of mitochondrial GDH activity in maize roots by exogenous NH_4^+ under the same experimental conditions was demonstrated in previous study (Hadži-Tašković Šukalović and Vuletić 1998). In addition, the incorporation of $^{15}NH_4$ into ^{15}proline by means of aminating activity of GDH *via* glutamate pathway in tobacco plants under stress conditions was confirmed (Skopelitis *et al*. 2006). PEG treatment induced further increase of proline, which was more pronounced in plants grown on NH_4^+/NO_3^- (~70 %), compared to plants grown on NO_3^- (10 - 20 %) in both genotypes. The increased proline accumulation could be due to the increased transport (Verslues and Sharp 1999), or to dominated proline synthesis over its breakdown (Rivero *et al.* 2004). Although the exact molecular mechanism of proline-induced protection of plants under stress is still unknown, its role in osmotic adjustment at low water potential was often reported (*e.g.* Voetberg and Sharp 1991). The increase of proline in heat stressed tomato plants, higher in NH_4^+ than in NO_3^- grown plants, coincided with higher tolerance to heat stress of NH_4^+-grown plants (Rivero *et al.* 2004), thus suggesting that NH_4^+ could confer tolerance to heat stress. It could be supposed that NH_4^+ assimilation stimulated alternative pathways of respiration in root mitochondria and so stimulated quick response to various biotic and abiotic stimuli (Eskobar *et al.* 2006).

Our experiments demonstrated that endogenous levels of antioxidative enzymes in roots were also influenced by nitrogen form applied in nutrient medium (Table 1). The

Table 1. Effect of nitrogen forms, NO_3^- or NO_3^-/NH_4^+, and 4 % PEG treatment on fresh mass (FM), dry mass (DM), content of free proline and total phenolics, and activities of SOD, POD, APX and AO in roots of two maize genotypes (Va35, B73). The results are means ± SE of duplicate assays from at least three isolations.

Parameter	PEG	Va35 NO_3^-	Va35 NO_3^-/NH_4^+	B73 NO_3^-	B73 NO_3^-/NH_4^+
Root FM	-	0.57 ± 0.07	0.58 ± 0.10	0.32 ± 0.12	0.35 ± 0.12
[g]	+	0.45 ± 0.10	0.35 ± 0.64	0.24 ± 0.06	0.25 ± 0.08
Root DM	-	6.3 ± 1.1	5.2 ± 0.4	6.5 ± 0.7	5.2 ± 0.4
[%]	+	6.0 ± 0.2	6.9 ± 0.5	6.8 ± 0.6	6.9 ± 1.1
Proline	-	79.0 ± 6.1	116.4 ± 14.8	59.9 ± 6.9	88.6 ± 13.0
[nmol g^{-1}(FM)]	+	92.9 ± 5.2	196.3 ± 19.9	64.3 ± 10.4	151.1 ± 16.5
Total phenolics	-	0.92 ± 0.02	0.81 ± 0.08	0.55 ± 0.005	0.44 ± 0.06
[mg(chlorogenic acid) g^{-1}(FM)]	+	0.90 ± 0.07	0.83 ± 0.03	0.76 ± 0.01	0.54 ± 0.02
SOD	-	272.1 ± 6.0	265.15 ± 5.0	298.2 ± 7.0	318.0 ± 20
[U mg^{-1}(prot.) min^{-1}]	+	344.0 ± 11	220.5 ± 17	339.8 ± 5.0	306.0 ± 6.0
POD	-	124.1 ± 2.0	144.4 ± 11	156.4 ± 5.0	255.9 ± 5.5
[μmol(conif. alc.) mg^{-1}(prot.) min^{-1}]	+	143.6 ± 0.6	70.2 ± 3.0	187.4 ± 2.0	150.2 ± 7.4
POD	-	26.99 ± 1.3	29.5 ± 0.3	34.17 ± 1.3	51.3 ± 0.4
[μmol(ferulic acid) mg^{-1}(prot.) min^{-1}]	+	36.95 ± 0.6	23.7 ± 0.2	38.9 ± 1.4	24.9 ± 0.7
POD	-	81.3 ± 0.9	93.8 ± 2.2	89.4 ± 1.2	165.8 ± 2.0
[μmol(NADH) mg^{-1}(prot.) min^{-1}]	+	103.9 ± 1.0	58.2 ± 1.6	104.3 ± 1.1	72.9 ± 1.5
APX	-	2.95 ± 0.0	5.03 ± 0.06	5.73 ± 0.1	6.49 ± 0.1
[μmol(ascorbate) mg^{-1}(prot.) min^{-1}]	+	3.53 ± 0.01	2.65 ± 0.03	4.33 ± 0.03	3.17 ± 0.003
AO (μmol mg^{-1} prot min^{-1})	-	0.24 ± 0.003	0.49 ± 0.012	0.23 ± 0.008	0.26 ± 0.005
[μmol(ascorbate) mg^{-1}(prot.) min^{-1}]	+	0.23 ± 0.002	0.33 ± 0.001	0.17 ± 0.0	0.19 ± 0.03

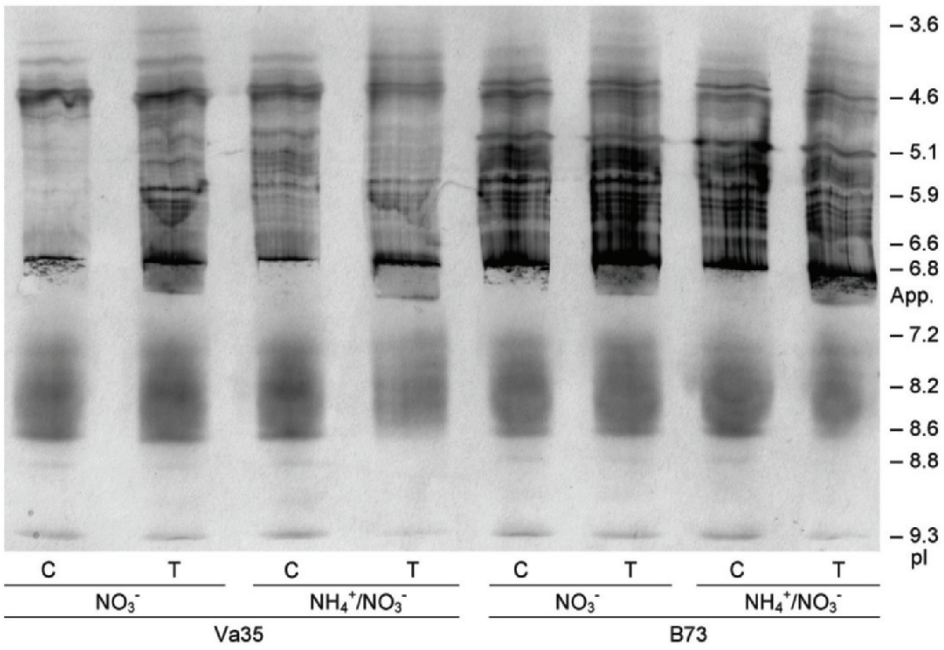

Fig. 1. IEF stained for POD activities in root of two maize genotypes (Va35 and B73) grown on different nitrogen forms, NO_3^- or NH_4^+/NO_3^- without (C) or in the presence of 4 % PEG (T).

activities of H_2O_2 scavenging enzymes POD (peroxidative and oxidative), APX and AO were increased by NH_4^+ supply and this increase exhibited genotype differences, POD activities being more increased in B73, and APX and AO in Va35. On the other hand, SOD was not significantly affected by nitrogen form. Stimulated POD and SOD activities in wheat (Polesskaya *et al.* 2004) and decreased in maize (Rios-Gonzales *et al.* 2002) were observed in roots of NH_4^+-fed plants, compared to NO_3^--plants. These

discrepancies could be explained by different experimental conditions. Also, the changes of POD and SOD activities under PEG treatment were influenced by nitrogen form (Table 1). Activities of these enzymes were increased due to PEG treatment when plants were grown on NO_3^-, while decreased in the presence of NH_4^+. The increase of POD activity was observed in different plant species under osmotic stress or water deficit (Lin and Kao 2002, Veljović-Jovanović *et al.* 2006) or other abiotic stresses (Rao *et al.* 1996, Liu *et al.* 2008).

PODs from maize root tissue use H_2O_2 to oxidize a variety of phenolics (Hadži-Tašković Šukalović *et al.* 2005). Besides, POD use conyferil alcohol and ferulic acid as substrates in the processes of lignification and cross-linking of cell-wall polymers (Ralph *et al.* 2004). Increased amount of POD acidic isoforms was observed by IEF in the presence of NH_4^+ (Fig. 1) and in PEG treated NO_3^- grown plants, especially in Va35 genotype. Since acidic PODs are involved in lignification (Penel and Castillo 1991), increased lignin production in plants grown on NH_4^+/NO_3^-, as well as in PEG treated plants could be supposed.

APX activities, which confer general resistance to an array of environmental stresses, were decreased under PEG treatment, especially in NH_4^+/NO_3^- grown plants (Table 1). The only exception was increase in APX activity in NO_3^--grown Va35 plants. Contrary to these results, decrease of APX demonstrated in wheat roots under salinity was more pronounced in NO_3^- than in NH_4^+-grown plants (Polesskaya *et al.* 2006). However, decrease of AO activity in PEG treated plants was not considerably dependent on nitrogen form.

Phenolics which are known as the substrates for PODs, thus being indirectly involved in antioxidative system, could also play direct antioxidative function due to their radical-scavenging properties (Rice-Evans *et al.* 1997). In our experiments it was shown that content of total phenolics in maize roots was 12 - 18 % lower when grown on NH_4^+/NO_3^- in comparison with NO_3^--grown plants (Table 1). Higher content of phenolics in roots of Va35 inbred line together with lower POD activities in comparison to B73 indicated that content of phenolics was not the limiting factor for POD activity. Although content of phenolics significantly varied depending on the genotype, similar decrease in the presence of NH_4^+ was shown in both genotypes. The treatment with PEG did not induce the change of content of phenolics content in Va35, irrespectively of N-form. However, in B73 plants PEG induced the increase of content of phenolics for about 40 and 20 % in NO_3^-- and NH_4^+/NO_3^- grown plants, respectively. According to carbon/nutrient balance hypothesis (Bryant *et al.* 1983) this increase could be explained by excess of carbon in limited growth conditions under stress which is shunted to the production of carbon-based secondary metabolites such as phenolics (Reichardt *et al.* 1991).

The present study showed that form of nitrogen influenced antioxidative systems in maize roots, as well as their sensitivity and response to osmotic stress. The increased proline content and activities of antioxidative enzymes were mainly induced by NH_4^+. The joint influence of osmotic stress and NH_4^+ stimulated only proline synthesis, not protective enzymatic systems. It could be supposed that two mechanisms coping with osmotic stress are active depending on nitrogen form supply, NO_3^- up-regulating antioxidative enzymes and NH_4^+ increasing proline content.

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