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Generation of nitric oxide in roots of *Pisum sativum*, *Triticum aestivum* and *Petroselinum crispum* plants under osmotic and drought stress

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ABSTRACT The concentration-, time- and tissue-dependent generation of nitric oxide (NO) was investigated in roots of *Pisum sativum* L. and *Triticum aestivum* L.. under osmotic stress, as well as *in Petroselinum crispum* L. under drought stress. Osmotic stress for pea and wheat was induced by polyethylene glycol (PEG) treatments in nutrient solution, while drought stress was caused by withdrawal of watering of soil-grown parsley. NO was detected by the NO-specific fluorescent dye, 4,5-diaminofluorescein-diacetate (DAF-2DA), using Zeiss Axiowert 200 M type fluorescent microscope. Changes in nitrate reductase activity was determined in the same series of treatments. Our results show that NO generation was proportional to the osmotic concentration of PEG both in pea and wheat roots and to the severity of drought in parsley root. The sites of NO production were in the regions of meristemic and elongation zones, and in case of wheat, root cap was also involved. In parsley root, the exodermis and the central cylinder showed the most intensive NO accumulation. In wheat and pea, time course revealed a fast transient (several hours) and a slow permanent increase in NO production. It is suggested that the fast kinetics may be due to non-enzymatic, while the constant increase was caused by enzymatic reactions. In parsley, long term experiments were carried out including the regeneration process after rewatering. It is concluded that NO plays a role as signaling molecule under osmotic and drought stress conditions.
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KEY WORDS

nitric oxide
Pisum sativum
Triticum aestivum
Petroselinum crispum
osmotic stress
drought stress

Nitric oxide (NO) is an inorganic free radical that acts as a signaling molecule with different kinds of physiological functions. In plants, it can be generated by different enzymes, like nitric oxide synthase (NOS; Wendehenne et al. 2003), nitrate reductase (NR; Deshikan et al. 2002), xantine oxidase (XOR) and nitrit:NO reductase (NiNOR). Non-enzymatic processes also play role in NO genesis in plants. For example, NO can be generated with a spontaneous reduction of nitrite at acidic pH (Stöhr et al. 2002) or with the light catalyzed conversion of NO_2 to NO (Cooney et al. 1994). NO plays role in plant-pathogen interaction, hypersensitive reaction, senescence (Leshem et al. 1996) programmed cell death, activation of protein kinases, phosphatases (Neill et al. 2002) and depending on it can have a prooxidative or antioxidative function under abiotic stresses (Neill et al. 2003).

In this work we hypothesize that NO plays role in acclimatization to osmotic stress conditions. Our results show that kinetics of NO production is composed of two phases in time. We support previous hypothesis that besides a non-enzymatic reaction, NR takes part in the synthesis of NO.

Materials and Methods

Plant materials

Fourteen-day-old *Pisum sativum* L. and *Triticum aestivum* L. plants were grown up in complete nutrient solution, supplemented with different concentrations of polyethylene-glycol (PEG; 0, 50, 100, 200, 400 mOsm) modeling drought stress. *Petroselinum crispum* L. plants were grown in standard garden soil for nine week. Drought stress was exposed by withdrawal of watering. In the green house, 12/12 day/night period was used at 240 μmol m^{-2} s^{-1} light intensity, at 25/20°C temperature and 55-60% relative humidity.

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Detection of NO, measurement of NR activity

In pea and wheat, NO content was measured in roots and stems of plants after1, 2, 3, 4, 5, 6, 12, 24 and 48 hours after the onset of PEG treatments. The „0 hour sample" was regarded as control. For parsley, samples for microscopy were taken at the last day of watering (zero time control), 4 and 7 days after the onset of drought treatment, and 1 week after rewatering as recovery time.

For the visualization of NO, NO-specific fluorescent dye, 4,5-diaminofluorescein-diacetate (DAF-2DA) was used. The dyeing was carried out in dark for 20 minutes. The prepared root and stem segments were investigated with the help of a Zeiss Axiowert 200 M type fluorescent microscope. Pixel intensity was measured with Axiovision 4.0. software. The pixel intensity of the dyed plant material was in directly proportional to NO content. Nitrate reductase activity was measured by *in vivo* method using. 0,5 g of roots incubated

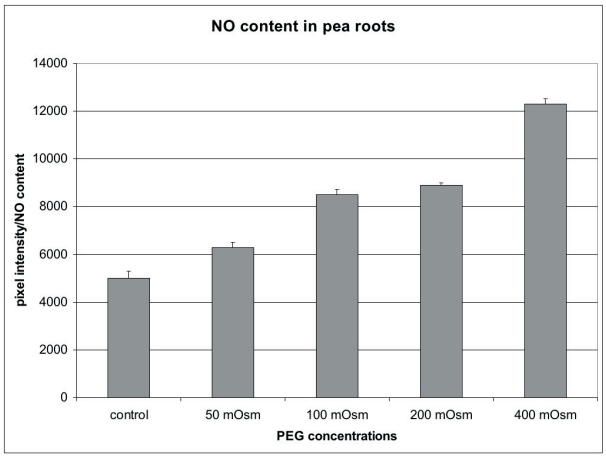

Figure 1. Effects of PEG concentrations on NO levels in pea roots.

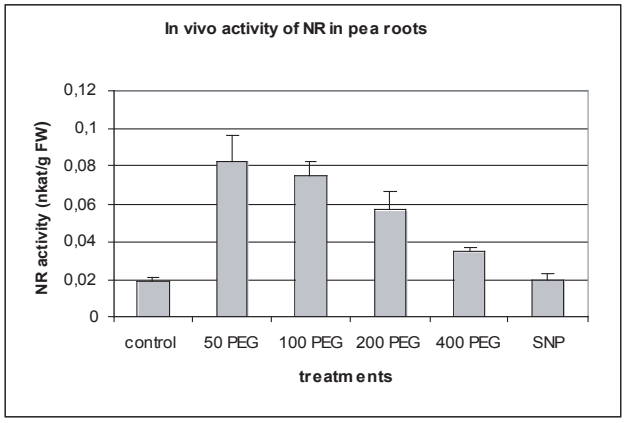

Figure 2. Effects of increasing PEG concentrations on NR activity in pea roots.

in a buffer (pH 6.5) containing 50 mM K_2HPO_4 x $2H_2O$, 50 mM KH_2PO_4, 50 mM KNO_3, 0,1 mM Triton x-100, at 33°C for 2 hours. The reaction mixture contained 100µl sample, 426 µl water, 240 µl 0.5% sulfanilamide, 240 µl N-(1-naphtyl) ethylenediamine dihydrochloride. The *in vivo* activity of nitrate reductase was calculated with the help of the following formula: NR activity (nkat/g FW) = Ext. x 1,17583 (Pécsváradi and Zsoldos 1996).

Results

Effect of increasing PEG concentrations on the NO contents in plant roots

NO generation was detected after 24-hour PEG treatment. Samples were: control, 50 mOsm, 100 mOsm, 200 mOsm, 400 mOsm PEG treated pea and wheat plants. We established that every PEG treatment enhanced the NO content in both cases of pea and wheat roots. The increase in NO levels in roots was PEG concentration-dependent (Fig. 1) and approached three-fold enhancement in case of 400 mOsm PEG as related to the control value.

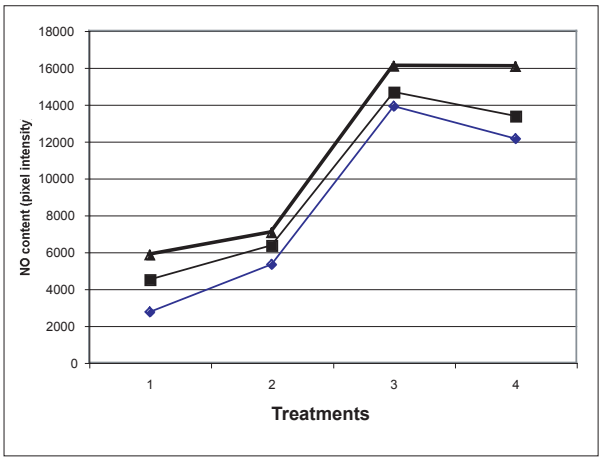

Figure 3. Effects of water withdrawal and re-watering on NO production and its tissue-specific localization in parsley roots. Quantitative estimation was measured on the series of cross sections as shown in Figure 4. Symbols: ▲, vascular cylinder; ■, exodermis; ♦, secondary root. Treatments: 1, last day of watering (zero time control), 2, 3, four and seven days after the onset of drought treatment, 4, after 1 week of recovery time.

Time-dependence of NO production in roots of pea and wheat plants

As the function of time, NO levels were determined as described above. The time-dependent kinetics showed a fast and smaller transient (with maximum value at 4 h) followed by a slow but higher (after 24 to 48 hours) increase in NO generation at all osmotic concentrations examined. Tentatively we consider the early phase as non-enzymatic, and the later phase as enzymatic mechanisms of NO production (data not shown).

Effect of osmotic stress on the in vivo activity of NR

It is known from literature that the NR activity is one of the NO sources in plant roots. NR activities in PEG treated pea and wheat roots. In control roots the activity of the enzyme was very low and it was enhanced significantly by all the PEG concentrations showing a maximum activity at 50 mOsm PEG in case of both plant species. Sodium-nitroprusside (SNP), as NO donor did not influence NR activity (Fig. 2). These results indicate that the NR, besides the nitrate reductase function, through nitrite, contributes to the production of NO. Under osmotic stress conditions, the second activity of the enzyme comes to the front and by *in vitro* NR activity determination, when nitrite is detected in the assay, increasing nitrite consumption is seen.

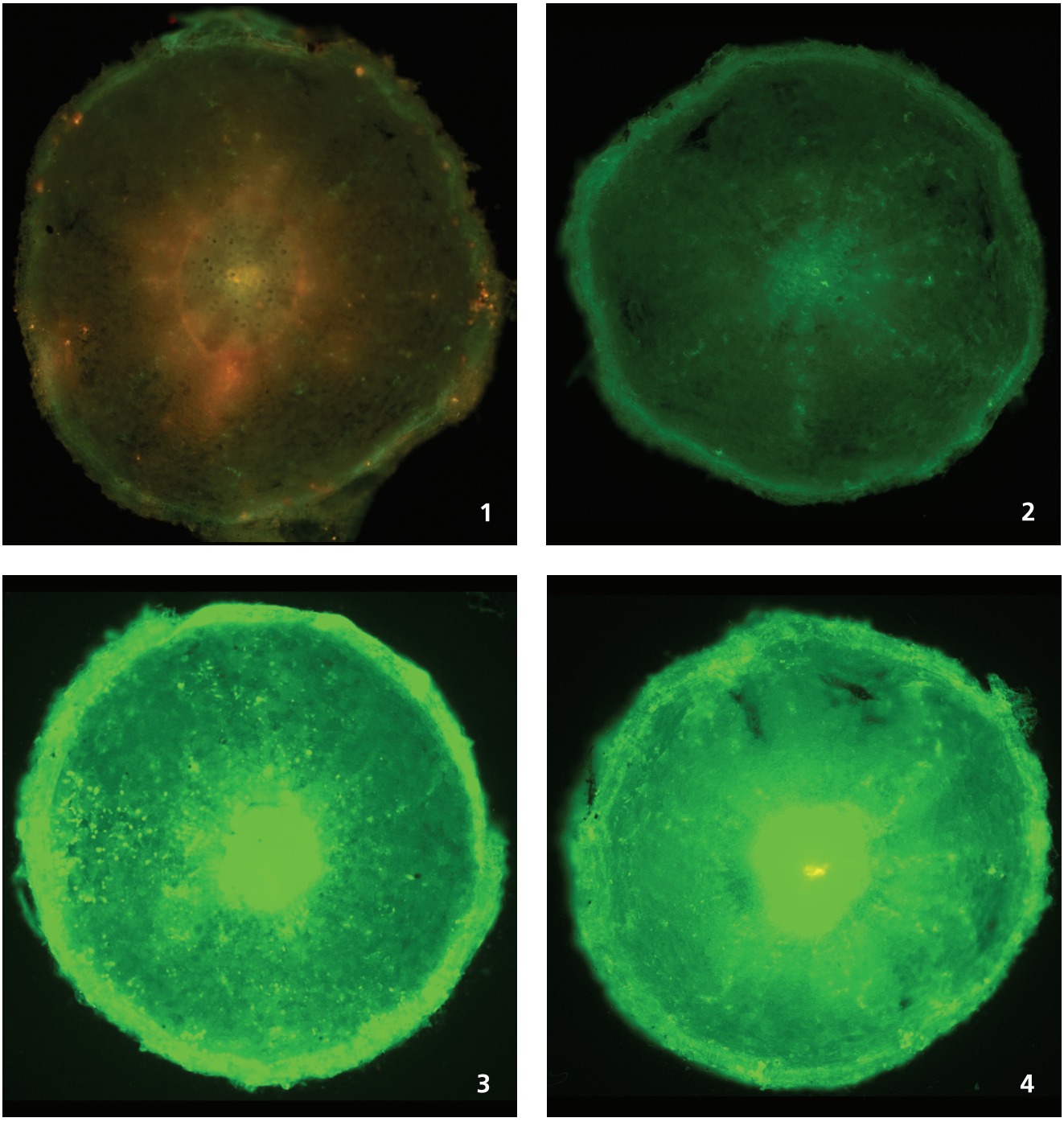

Figure 4. Fluorescence microscopic visualization and localization of NO production in cross sections of the taproot of *Petroselinum crispum*. NO was detected by fluorescence microscopy at 485 nm for excitation and 530 nm for emission by Axiovert 200M fluorescence microscope (Karl Zeiss, Germany) using the NO-specific 4,5-diaminofluorescein-diacetate (DAF-2DA) green fluorescent dye. Treatments as in Figure 3.

Effect of drought on the NO contents in parsley roots

Figure 3 shows that NO was intensively accumulated on the course of drought treatment and culminated after one week, under already severe drought at that time. The highest NO levels were detected in the vascular cylinder followed by the exodermis in the taproot. Secondary roots showed similar pattern. After re-watering, the recovery process was slow and the low level of NO, comparable to the original control status,

was re-established after 4 to 5 weeks growing in wet soil (data not shown). The fluorescence microscopic visualization of these changes in NO production and localization within the taproot is shown by Figure 4.

Discussion

The key role of reactive nitrogen compounds in plant stress responses is receiving increasing evidence. It can be foreseen that NO fulfills multiple functions in signal transduction *via* direct *S*-nitrosylation of iron-sulfur proteins or cysteines to form *S*-nitrosothiols. Nitric oxide appears to be present in most of the stress reactions (Gould et al. 2003). Hyperosmotic environment is one of the common abiotic stressors, component of drought and salt stresses, and induces reactive oxidative components. Although it was already shown that a fast response of tobacco cell suspension culture to sorbitol and salinity involved nitric oxide production (Gould et al. 2003), so far the relation between NO generation and hyperosmotic stress responses were not investigated.

In this study we found that in pea and wheat roots NO production was enhanced and NO levels in the root tips were proportionally related to PEG concentrations. Kinetics of time-dependence revealed two periods in NO production: a fast, transient, probably non-enzymatic, and a slower, probably enzymatic period confirming these different possibilities for NO generation (Bethke et al. 2004). Similarly, a fast transient and a slow increase in NO production were found under Cu^{2+} treatment in *Pisum* and *Brassica* roots (Bartha et al. 2005).

Our data concerning changes in NR activity suggest that NR is one of the sources of NO. In roots, NR activity generally is low (Jiang et al. 1998) as it was in the control samples, however it was increased by osmotic treatments suggesting its role in osmotic reactions (Rockel et al. 2002). The sites of NO production in root meristemic and, in a decreasing extent in the elongation zones, suggest that NO participates in the control of cell division cycle and tissue differentiation (Ötvös 2003).

In case of parsley, long term reactions were studied since the first sampling for NO measurement was carried out after 4 days of water withdrawal. In this way, the fast response, even if occurred, was missed. There is, however, a sharp difference between the development of NO production until its maximum levels at around the 7th day, and the slow decline during the recovery process, which lasted through 4 to 5 weeks. The presence and maintenance of high NO levels in the recovery period is well illustrated by Figure 3 and 4, but its role still remains to be elucidated. The localization of NO may, however, reflect its multiple role in the sensing of drought (exodermis) and long distance signaling (central cylinder; Stöhr and Ullrich 2002).

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