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CROP PRODUCTION

CALCIUM DEPENDENT RHYTHMIC CHANGES IN THE CONTENT OF NITRIC OXIDE (NO) IN THE ROOTS OF ETIOLATED PEA SEEDLINGS (*PISUM SATIVUM* L.)

Conflict of Interest

None declared.

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Abstract
The temporal dynamics (during 30 and 60 min) of nitric oxide generation (NO) in the roots of 2-day etiolated seedlings of pea seeds has been studied. During the exposure of seedlings on water and $CaCl_2$ solution (100 μM), fluctuations in the level of nitric oxide in the roots (its increase and decrease) have been shown. The physiological role of fluctuations in the level of nitric oxide in the root and the participation of calcium ions in this process are discussed.

Keywords: *Etiolated pea seedlings, calcium ions, nitric oxide (NO), fluorescent probe.*

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1 Introduction

Nitric oxide (NO) is a multifunctional signal molecule that plays a key role in a wide range of physiological and biochemical processes in plants, animals and microorganisms (Glyan'ko, Ischenko, 2010; Gupta et al., 2011). In plants, NO acts as a signal molecule and one of the most important components of a protective system under the action of stress factors (Kolupaev, Karpets, 2009; Karpets et al., 2015). NO is known to control the homeostasis of calcium ions (Ca^{2+}) in cells of organisms (Clementi, 1998) and almost all types of calcium channels and transporters are regulated by nitric oxide via S-nitrosylation of proteins or with secondary messengers NO – cyclic GMP (cGMP), ADPR (cADPR) and protein kinases (PK) (Besson-Bard, 2008; Jeandroz et al., 2013). Endogenous and exogenous sources of NO activate the influx of Ca^{2+} into the cytoplasm from the extracellular space (Garcia-Mata et al., 2003; Lamotte et al., 2004). On the other hand, an increase in the concentration of cytosolic Ca^{2+} under the influence of external factors affects NO synthesis, leading to enhanced physiological and biochemical processes and a cascade of signal reactions accompanied by gene expression (Courtois et al., 2008). Thus, activation of calcium calmodulin - dependent kinase (CaMK) by rhythmic changes in calcium concentration in the cytoplasm and organelles is shown, which is accompanied by an increase in NO generation (Courtois et al., 2008). Nitric oxide, in turn, can affect calcium channels by S-nitrosylation of the calcium calmodulin sensor, which blocks the influx of Ca^{2+} into the cytoplasm (Jeandroz et al., 2013). Based on this, one can talk about the cross-over and mutual influence of two signal compounds on metabolic processes, especially when transducing abiotic and biotic signals (Vandelle et al., 2006; Kolupaev, 2010; Jeandroz et al., 2013). However, the mechanisms by which NO modulates the Ca^{2+} flow in cells are not fully understood.

On the other hand, the question of ways of NO synthesis in plants remains unsolved. At present, the way to NO generation in plant cells involving cytosolic nitrate reductase (NR) is undeniably recognized. However, it should be noted that the functional connection between Ca^{2+} ions and NO synthesis with the participation of NR remains doubtful (Courtois et al., 2008). The second way of NO synthesis, connected with the use of L-arginine as a substrate, has been confirmed by numerous studies (Besson-Bard, 2008; Glyan'ko, 2013), but has not been proved by the presence in the plants of the enzyme identical to NO-synthase (NOS) of mammals catalyzing the oxidative synthesis reaction of NO from arginine in animal cells. As for the relationship between Ca^{2+} and NO generation on the L-arginine-dependent way, there are some evidences of the need for calcium ions in a reaction catalyzed by plant NO-synthase similar to NOS of animals (Vandelle et al., 2006). Thus, the dependence of the activity of the constitutive isoform of cNOS of mammals on CaM and Ca^{2+} ions (Bogdan, 2001) was also confirmed in experiments with tissues of various plant species (Corpas et al., 2006). Though these data do not establish a direct interaction of calcium ions with the NOS-like plant enzyme, they appear to indicate the role of fluctuations in the concentration of extracellular and intracellular calcium in NO synthesis in plant cells and the participation of NO in the signaling cascade initiated by Ca^{2+} (Jeandroz et al., 2013). But the sequence of signal transmission in the NO / Ca^{2+}-bonded way also remains unclear.

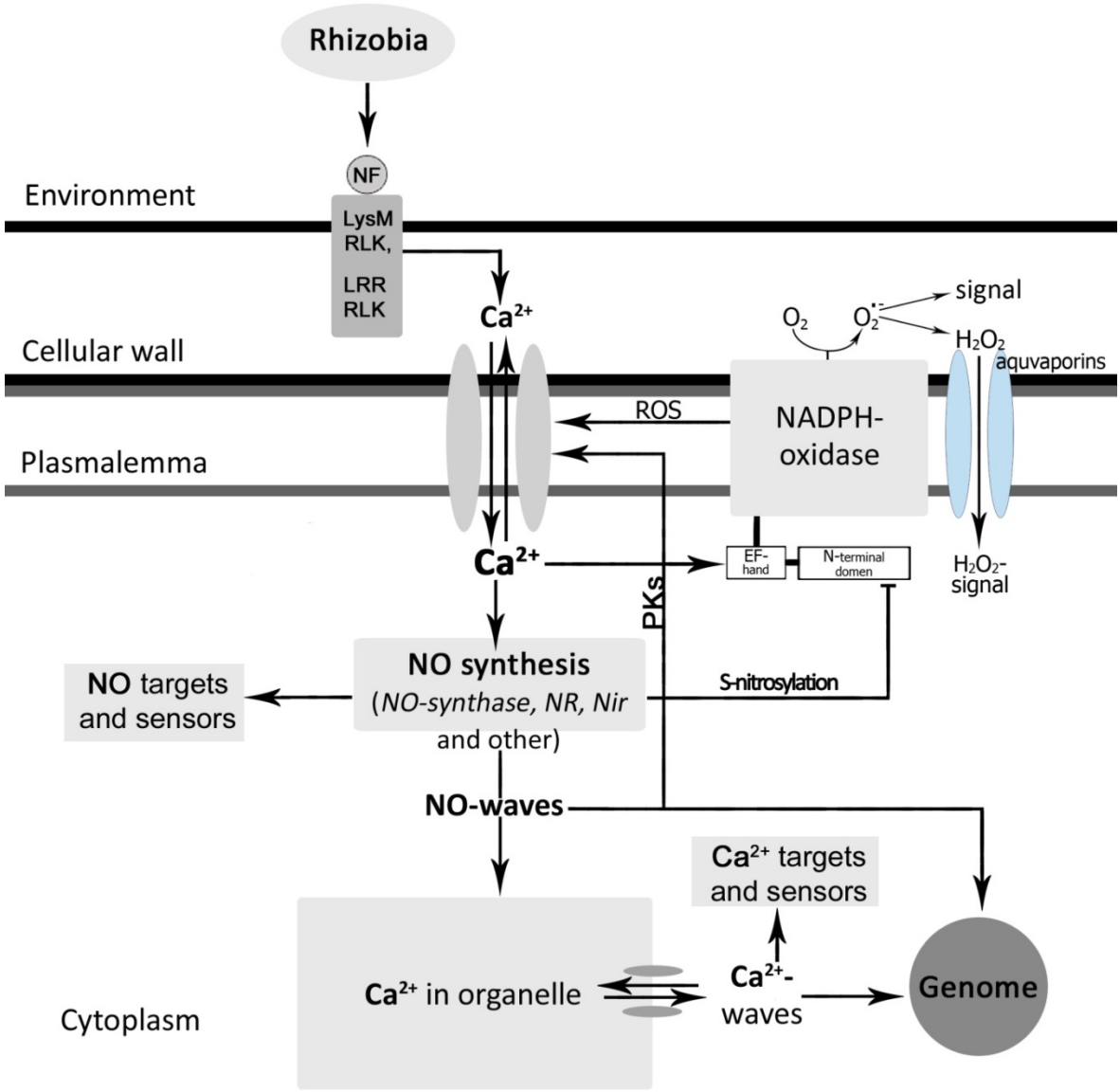

Fig. 1 – Hypothetical scheme of interaction of signaling molecules in the cells of plant roots

NF, Nod-factor (lipochitooligosaccharide); LysM RLK, Lysine Motiv-Receptor-Like Kinase; LRR RLK, Leucine-rich Repeat Receptor-Like Kinase; PKs, pyruvate kinases; NO targets and sensors (S-nitrosylation, metal nitrosylation, hemoglobin, nitrosilglutathione reductase, cytochrome *c* oxidase, ets.); Ca^{2+} targeta and sensors (Ca^{2+}-dependent protein kinase-CDPKs, mitogen-activated protein kinases-MAPKs, Ca^{2+}-sensitive channels, Ca^{2+}-ATPase, ets.); NADPH oxidase (EC 1.6.3.1) catalyzing the reaction of $O_2^{\bullet-}$ formation and containing membrane subunit RbohD (respiratory burst oxidase homologs); aquvaporins, water channels.

In connection with the foregoing, it is possible to present the following simplified scheme of mutual influence of Ca^{2+} and NO in the initial stages of transduction processes (Fig. 1). Endogenous nitric oxide, generated by enzymatic (or non-enzymatic) plant systems, has a direct or indirect effect on Ca^{2+} channels, enhancing or repressing the flow of extracellular Ca^{2+} into the cytoplasm through the plasma membrane. On the other hand, Ca^{2+} stimulates the generation of plant nitric oxide cells, at least through a reaction with L-arginine as a substrate. The accumulation of intracellular NO will lead to the stimulation of processes that affect the homeostasis of Ca^{2+} in intracellular organelles by creating calcium waves (oscillations) in the cytoplasm that constitute links in the transduction of information into the genome (Downie, 2004; Medvedev, 2005). Calcium waves are carried out due to membrane transport, Ca^{2+}-ATPase, kinases, protein-sensors (Tarchevsky, 2002). The effect of NO on the generation of ROS (Reactive Oxygen Species), which also affect Ca^{2+} channels, can manifest in repressing the activity of NADPH oxidase by S-nitrosylation of cysteine (Cys890) enzyme (Yun et al., 2011).

It should also be noted that the increase in Ca^{2+} concentration in the cytoplasm occurs both from

extracellular calcium and from its release from the intracellular components of the cytoplasm – endoplasmic reticulum, vacuoles and other organelles (Downie, 2004; Denisenko, Kuzmina, 2013).

In this study, the effect of endogenous (exposure of seedlings on water) and exogenous calcium (exposition of seedlings on $CaCl_2$ solution) on the temporal dynamics of NO generation in roots of pea seedlings was studied in short-term experiments with etiolated pea seedlings in order to evaluate the nature of nitric oxide synthesis by the root cells.

2 Methodology

The object of research is etiolated pea seedlings (*Pisum sativum* L.) of Yamalsky variety, grown in plastic cuvettes on wet filter paper at 22°C. To maintain the set temperature, an electric thermostat with a water-jacket ZTS-1125M (Russia) was used, accurate to ± 0.5°C. Before soaking, the seeds were washed with warm running water with soap and disinfected with 3% hydrogen peroxide solution for 15 minutes. After that, the seeds were poured with boiled tap water (60°C) and placed into a thermostat for swelling at 22°C for 48 hours. For the studies, homogeneous seedlings with a root length of 25-30 mm (initial plant material) were selected.

The NO content of the root tissues was determined using a 4,5-diaminofluorescein-diacetate (DAF-2DA) fluorescent probe. This compound permeates the cell membrane and deacetylates via intracellular esterases in 4,5-diaminofluorestsein (DAF-2) which forms fluorescent compound with NO - diaminotriazolefluorescein-triazole (DAF-2T) (Nakatsubo et al, 1998.). Before staining, the initial 2-day-old seedlings were exposed to the appropriate media (H_2O and $CaCl_2$) for the required time: 5, 10, 15, 20, 25, and so on minutes. Then, the root segments (5-15 mm from the apex) were incubated in a staining medium containing 10 μM DAF-2DA and 10 mM Tris-HCl (pH 7.4), for 20 minutes on a shaker at 26°C. Fluorescence intensity was determined on cross-sections (thickness 100-150 μm) of roots using an Axio Observer Zl fluorescent microscope (Germany) with Axio Cam MRm3 digital monochrome camera and Axio Vision Rel.4.6 image capture and analysis software package. Filter unit No. 10, excitation wavelength – 450-490 nm, emission – 515-565 nm. The results of the analysis of the cross-cuts are presented in relative units of fluorescent intensity.

The content of overall calcium in seedling roots was determined with an atomic absorption spectrometer of Perkin Elmer (USA) model 403 in acetylene-air flame. Dried to air-dry state (in a thermostat at 110°C) roots were used for determination. They were thoroughly washed before drying with distilled water. Dry root samples (0.05 g) were decomposed (mixture of HNO_3 and H_2O_2) using an autoclaved Ancon-AT complex (Russian). The mixture was left at room temperature for 1 hour. The autoclaves were then sealed and placed into an electric heater for 2 hours at 160°C (1 h) and 200°C (1 h). After cooling, the contents of the autoclaves were quantitatively transferred to calibrated flasks and the calcium content was determined instrumentally.

The values are arithmetic means with a standard error of three independent experiments performed in triplicate analytical replication. The number of root sections to be analyzed in microscopic studies is no less than 10. The reliability of differences in mean values was estimated by Student's *t*-test. Statistical data processing was carried out using the Microsoft Excel software package.

3 Results and discussion

As it follows from Fig. 2, the temporal dynamics of NO content in samples of pea seedlings root cuts against the background of boiled tap water and taken every 10 minutes is characterized by fluctuations: an increase in NO level after 10, 30 and 50 minutes, and a decrease in 20, 40 and 60 minutes. Fluctuations in NO level are also observed against the background of the action of an exogenous calcium source (100 μM $CaCl_2$). However, the time dependence of the fluctuations of NO level in this case is different: an increase in NO level after 20, 40, 60 minutes, a decrease – in 10, 30, 50 minutes. A similar pattern was obtained in the experiment, when root samples were taken after 5 minutes (Fig. 3). However, as it follows from this figure, a clear picture of the maxima and minima in NO synthesis is also observed after 10 min, that agrees with the data presented in Fig. 2. Thus, one can talk about a certain rhythm in the synthesis of nitric oxide in the roots of etiolated pea seedlings. In this case, exogenous calcium affects the synthesis of nitric oxide, changing the time amplitude of its synthesis and preserving the rhythmicity of fluctuations. This effect of exogenous calcium is believed to be associated with an increase in intracellular and apoplastic Ca^{2+}. This can be indirectly judged by the content of overall calcium in the roots of the initial (2-day) etiolated pea seedlings: 860 ± 36 μg / g of dry matter (21.5 ± 0.9 μmol) in the variant with water and 1043 ± 28 μg / g of dry matter (26.0 ± 0.7 μmol) in the variant with exogenous calcium. That is, against the background of $CaCl_2$, the calcium content in the roots tissues is by 21% reasonably higher than against the background of water.

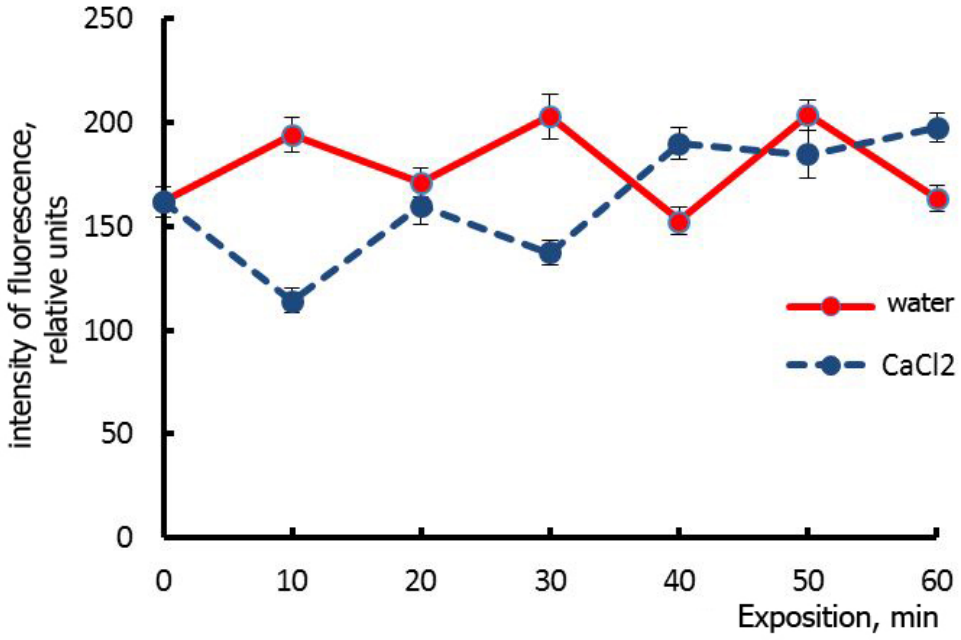

Fig. 2 – Changes level nitric oxide (NO) in roots etiolated seedlings of pea (sampling in 10 minutes).

Considering exogenous calcium as an environmental factor, it can be assumed that some other external factors, both abiotic and biotic can affect NO synthesis. It may also be associated with a certain rhythm in its generation. The data obtained suggest that there is a mechanism in the roots regulating NO synthesis and, apparently, associated with calcium ions (Karpets, Kolupaev, 2017). Moreover, an increase in Ca^{2+} content in the roots due to exogenous calcium alters the nature of the rhythmicity of NO synthesis, reducing the level of nitric oxide and changing the maxima and minima in NO synthesis in comparison with the variant with water. It is noteworthy that in our earlier experiments we obtained results indicative of fluctuations in the activity of microsomal NADPH oxidase localized on the cells plasmalemma and on the pea root membranes organelles (Glyan'ko et al., 2012). Thus, it was found that the activity of the enzyme against the background of exogenous $CaCl_2$ increased after 5 and 20 minutes and decreased after 10 and 30 minutes. The endogenous rhythm of the activity of NADPH oxidase and NO generation in the roots, in all probability, indicates the presence of a cell mechanism regulating both the formation of nitric oxide and the ROS-products of the functioning of NADPH oxidase and superoxide dismutase (SOD). It is confirmed by literature data that in response to various factors, NO generation and ROS in cells occur simultaneously and changes in the concentration of one component affect the level of the other (Neill et al., 2008). Such a mechanism appears to be related to the homeostasis of Ca^{2+} ions, expressed in a pulsating change in the concentration of this ion in the cytoplasm (Ca^{2+} wave). This phenomenon in the literature is denoted by the term "Ca^{2+}-signature" (calcium signatures), which characterizes fluctuations in the intracellular calcium concentration according to such parameters as amplitude, frequency, pulse duration (Whalley, Knight, 2013; Granqvist et al., 2015). Discussing the possible mechanism regulating both physiological processes, it is first of all urgent to note the necessity of Ca^{2+} ions for the reactions that generate NO. We have already discussed literature data on the possible dependence of the activity of NOS-like plant enzyme on calcium ions. The necessity of calcium for the manifestation of NADPH oxidase activity has been proved (Glyan'ko et al., 2009). On the other hand, high NO concentrations repress NADPH oxidase (AtRBohD) by S-nitrosylation of cysteine (Cys890) enzyme (Yun et al., 2011). It can be concluded that the activation and repression of the studied physiological processes is apparently associated with a change in the concentration of cytosolic and apoplastic calcium due to the effect of NO and, apparently, ROS on the calcium channels – on their opening and closing (Mori, Schroeder, 2004; Demidchik, Maathuis, 2007).

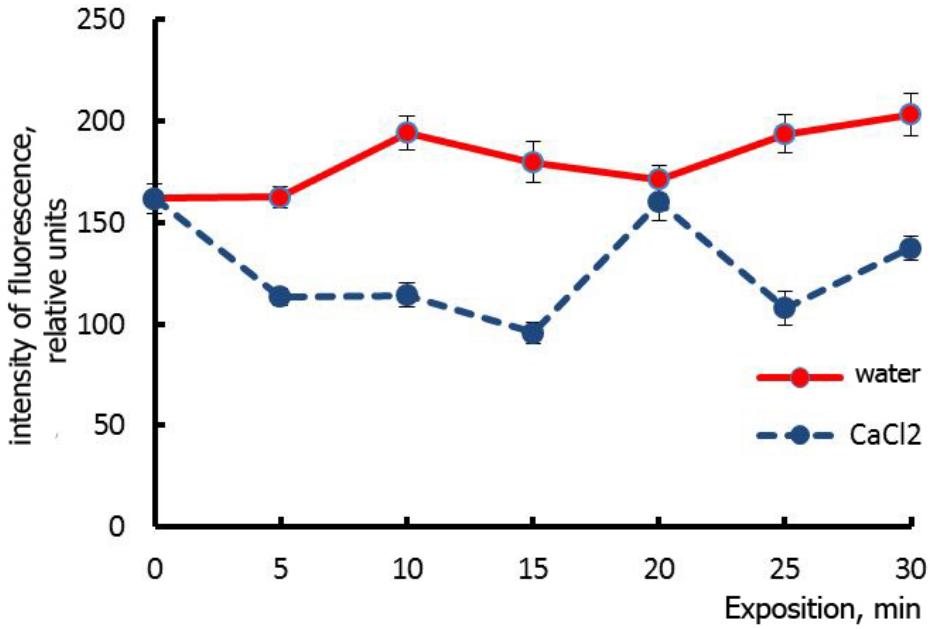

Fig. 3 – Changes level nitric oxide (NO) in roots etiolated seedlings of pea (sampling in 5 minutes)

The wave nature of the functioning of ROS (H_2O_2) in plant cells is reported in Miller et al. (2009), Mittler et al. (2011) and Montiel et al. (2016), who proved the presence of ROS-waves in plant cells, which function as a system signal for long distances, from one cell to another and from one organ to another, associated with the formation of systemic resistance to the action of the stressor. The main role in these processes is played by NADPH oxidase (RBohD oxidase). It is ROS generator which activity is associated with Ca^{2+} signal and Ca^{2+}-regulated kinases (Steinhorst, Kudle, 2013). The results of the key role of NADPH oxidase at various stages of legume-rhizobia symbiosis (formation of an infection thread, organogenesis of the nodule, N_2-fixation, nodule aging) are given in the article (Montiel et al., 2016).

The main conclusion to be drawn from the work is that, in short-term experiments, an endogenous rhythm of NO generation in root tissues of pea seedlings has been observed. This rhythm is characterized in time dynamics by an increase and decrease in the level of nitric oxide and is subject to the influence of exogenous calcium. The physiological role of NO (NO-wave) fluctuations in plant cells has not been studied, although there is no doubt that this is due to the signal function of nitric oxide.

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