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THE PHYSIOLOGICAL ROLE OF NITRIC OXIDE (NO) IN PLANTS

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The data on physiological role of nitric oxide (NO) in plants have been generalized. The multifunctional character of NO resulting from high reactivity of this molecule and its capability to react with proteins and low-molecular substances are emphasized. The issues related to participation of NO in various physiological processes, the role of this molecule in legume-rhizobial symbiosis, possible mechanisms of NO synthesis in plants, mechanisms of interaction with other endogenous molecules, as well as some mechanisms preventing from toxic impact of nitric oxide have been considered.

Key words: *nitrogen oxide (NO), nitric oxide synthase (NOS), NOS-like reaction, proteins nitrosylation and nitration, nitrosative stress*

Nitric oxide (NO) is a gaseous neutral diatomic molecule (free radical), which easily penetrates through membranes of organisms' cells and has the 6 s period of half-disintegration in biological media. However, at low concentrations (below 1 μM) the period of its half-disintegration grows and may be of 1 min to 1 h (Stohr, Ullrich, 2002).

NO as a radical possesses a wide range of biological activity provoking activation and inhibition of chain free-radical reactions. It forms numerous low-molecular N-compounds with the oxidation degree of nitrogen atom from -3 to +5.

Some 30 to 40 years ago NO and other gaseous molecules of nitrogen were considered as atmospheric polluting agents. Revolution with respect of NO took place during the period from the late 1980s to the early 1990s, when it was discovered that the fundamental role of nitric oxide is bound up both with its signal function in mammals' cells and with regulation of various physiological processes (Ignarro et al., 1987; Palmer et al., 1987; Culotta, Koshland, 1992). In 1992, the international journal "Science" called NO "the molecule of the year" (Koshland, 1992).

Since then, a large number of publications have been dedicated to investigations of biological properties of this molecule both in animal living organisms and in plants. It has been acknowledged worldwide that NO is a multifunctional signal molecule, which is active in all organisms – from bacteria to animals and plants. In mammals, NO is involved in the processes of regulation of vascular homeostasis, neural signalling, organism's immune response to infections, inflammations and other processes (Schmidt, Walter, 1994; Men'shchikova et al., 2000). NO plays the key role in activation of macrophagues in animal cells and in cell protection from pathogenic bacteria. It also participates in the progress of a number of human diseases (Proskuryakov et al., 1999). The research has given evidence that mechanisms of NO activity in genetically different organisms demonstrate a high degree of affinity, what allows the researcher to make an assumption of the ancient roots of the biological role of NO in animals, plants, bacteria and other organisms (Durner et al., 1999).

Nevertheless, high reactivity and the janus-like character of NO hampers the development of the model related to its role in cell's signal paths

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(Menshchikova et al., 2000; Grün et al., 2006). When present in large concentrations, NO molecule is toxic for bacteria, fungi, tumor cells, viruses, animals, plants (Zumft, 1997). Nitrogen oxide in micromolecular concentrations is known to induce activity of caspases, cause disintegration of nucleic acids, and it reduces synthesis of ATP in tobacco cells (Dubovskaya et al., 2007). Toxicity of an NO molecule is bound up with its high reactivity with respect to the proteins containing metals having variable valences and to oxygen, as well as with its capability to form products with amines and thiols (Van der Vliet et al., 1996). In this connection, the term "nitrosative stress" (having resemblance to the term "oxidative stress") has been proposed and accepted in the scientific literature (Hausladen, Stamler, 1999; Klatt, Lamas, 2000).

NO in animal cells

In animal cells NO synthesis takes place via oxidation of aminoacid of α-arginin to citrullin and NO. The reaction is NADP-independent, and it is catalyzed by enzyme – synthase of nitric oxide (EC: 1.14.13.39). Three major isoforms of nitric oxide synthase (NOS) have been found in mammal cells. These isoforms have all been named according to the designations of the tissues from which these were extracted: neuronal (nNOS), inducible from macrophages (iNOS), endothelial (eNOS). An isoform similar to iNOS has been extracted from rat liver mitochondria (mtNOS) (Wendehenne et al. 2001). All NOS-isoforms demonstrate the 50–60% identity in their aminoacid succession. Each NOS is a double domain containing N-terminal oxygenase and C-terminal reductase. The domain of oxygenase has a haem centre and sites for co-factor of tetrahydrobiopterin. The domain of reductase contains NADPH, FAD and FMN-linking sites. Both domains interact with the help of calmodulin-linking site in the enzyme. Besides, each NOS has a different length of N-terminal end, which defines the intracellular localization of the enzyme.

nNOS and eNOS are constitutive isoforms, and iNOS is induced in macrophages and in some other types of animal cells in response to infection factors. Activity of nNOS and eNOS is to a substantial extent dependent on the increase in the concentration of intra-cellular free Ca^{2+}, which is linked into a complex Ca^{2+}–calmodulin. Activity of iNOS is independent of the concentration of intracellular Ca^{2+}, and calmodulin is linked to the enzyme even in the absence of cytosol Ca^{2+}. iNOS is characterized by stability and high activity. The amount of NO produced is 2 to 3 orders higher than that of constitutive NOS (Marletta, 1994). Large amounts of NO produced by macrophages NOS determine the cytotoxic and antibacterial effect in the immune system of mammals (Schmidt, Walter, 1994).

NO molecule has long been known in inorganic chemistry as a ligand in iron-haem complexes. It forms relatively stable complexes with iron in the haem of cytochrome P-450, hemoglobin, leghemoglobin and in the NOS itself. In the latter case, nitrogen oxide formed with the participation of NOS inhibits the enzyme activity (Marletta, 1994).

The biological effect of NO and NO-derivates (for example, peroxinitrite) is conditioned by the processes of chemical modification of biological molecules at the expense of linking with metals having variable valences in metal-proteins (metal-nitrosylation) and co-valent modification of the protein remains of cysteine (S-nitrosylation) and tyrosine (tyrosine-nitration).

These processes are regarded as specific post-translation modifications of the proteins. Metal-nitrosylation and S-nitrosylation are considered reversible, while tyrosine-nitration – as an irreversible process (Stamler et al., 2001; Schopfer et al., 2003). Over 100 proteins identified as targets for NO (Besson-Bard et al., 2008) are known. As a result of NO-modification, the proteins change their qualities, i.e. these are either activated or inhibited (Lindermayr et al., 2005). The change of conformation for these proteins under the influence of NO may be accompanied by either activation or inactivation of the transcription factors, and so this change may influence the expression of the genes. On the other hand, NO may activate signal processes, while including the process of synthesis of salicylic acid, cyclic guanosine-monophosphate (cGMP), the flows of Ca^{2+}, the process of reversible phosphorylation of proteins. All these processes in their turn influence the transcription factors resulting in expression of the genes (Neill et al., 2003).

The role of NO in plants

Investigation of NO in plants was started in the 1970s after the discovery of the phenomenon of NO emission by plant tissues (Anderson, Mansfield, 1979; Klepper, 1979). These and other authors (Wildt et al., 1997) established NO extraction from plant tissues under normal physiological conditions of plant growing and enhancement of NO emission for high concentrations of nitrogen nitrate

in soils, treatment of plants with herbicides, salicylic acid and other biologically active substances.

In 1998, Delledonne et al. and Durner et al. were the forst to characterize the role of NO in plants as the role of a signal molecule responsible for initiation of protective reactions of plants. During the recent few years, the results of investigations related to the role of nitric oxide in plants were regularly reported in scientific periodicals (Beck et al., 1999; Wojtaszek, 2000; Lamattina et al., 2003; Neill et al., 2003; Wendehenne et al., 2004; Dmitriev, 2004; Arasimowicz, Floryszak-Wiczorek, 2007; Molina-Favero et al., 2007; Besson-Bard et al., 2008; Hong et al., 2008; Kolupaev, Karpets, 2009b). NO was found to be involved in a number of metabolic processes in plants: in protective reactions (Hong et al., 2008), tropisms (Hu et al., 2005), blooming (He et al., 2004), stomatal mechanism (Neill et al., 2008), xylem formation (Gabaldon et al., 2005), adaptation and response to stress factors (Valderrama et al., 2007), root formation (Pagnussat et al., 2003; Correa-Aragunde et al., 2004;) and other physiological processes (Beligni, Lamattina, 2000).

The profile of *Arabidopsis* genes expressed by NO (sodium nitroprusside) was investigated (Polverari et al., 2003; Parani et al., 2004). It was demonstrated that – in response to plant treatment with the use of the 0.1 мМ solution of sodium nitroprusside (NO donor) – 124 genes are expressed, and in case of treatment with the use of the 1.0 мМ solution – 261 genes. During this process 43 genes underwent activation for both concentrations of NO (Parani et al., 2004). Plant treatment with scavenger NO – PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl) leveled the activating effect of NO on the expression of *Arabidopsis* genes (Parani et al., 2004). This speaks in favor of specificity of the influence of NO on the level of the transcripts. Most part of the genes, which are activated under the influence of NO, undergo activation also under the influence of other abiotic and biotic stress factors, what gives evidence that NO is involved into the processes of regulation of a wide range of physiological functions bound up with protection of the plant from diseases and pathogenic bacteria, with oxidizing stress, signal transduction and with the transcription factors (Parani et al., 2004). At the same time, according to the data published by Parani et al. (2004), 126 genes from the set of 342 *Arabidopsis* genes, which are activated by NO, encode proteins with unknown functions.

Substantial concentrations of NO were discovered in tissues of young pea plants. And as far as the subcellular level is concerned, intensive formation of NO was observed in peroxisomes with the participation of the enzyme like NOS, which was known to use arginine as the substrate of the reaction (Barroso et al., 1999; Corpas et al. 2004).

NOS was identified in animal mitochondria, and, as far as plant mitochondria are concerned, the NOS-like reaction was found in the roots of *Arabidopsis thaliana* (Guo, Crawford, 2005). The modulation of activity of alternative oxydase in mitochondria may take place owing to NO formed in peroxisomes (Barroso et al., 1999; Huang X. et al., 2002). However, NO of high concentration may cause oxidative-nitrosative stresses and death of the cells (Zottini et al., 2002). Under these conditions, the researchers observed an almost triple increase in expression of the gene of the alternative oxydase in *Arabidopsis* leaves (Parani et al., 2004), what may counteract and prevent from the inhibition of cytochrome oxydase and from the increase of its resistance to NO-toxicity (Huang X. et al., 2002).

NO interaction with other signal molecules

The signal system of NO tightly overlaps with other signal paths and with individual signal molecules (Tarchevskij, 2002). Particular attention in this respect is paid to reactive oxygen species (ROS), calcium ions (Ca^{2+}), salicylic acid (SA), cGMP and cADPR (Asai, Yoshioka, 2008; Courtois et al., 2008; Neill et al., 2008).

An enzyme, in this case – guanylate-cyclase, is the important target for nitric oxide as a signal molecule in mammal tissues. Its activity in the presence of NO increases by 10 to 50 times (Arnold et al., 1977). Cyclic guanylate-monophosphate (cGMP), which is formed in the process of linking NO to guanylate-cyclase haem, regulates many functions of cells (McDonald, Murad, 1995). cGMP of plants participates in the induction of synthesis of the metabolites involved in protective reactions (Bowler et al., 1994). Noteworthy, the tobacco genes, which are responsible for synthesis of protective metabolites, are induced by both NO and cGMP and even cyclic adenosine-diphosphate-ribose (cADPR). These two molecules act as secondary messengers in NO-signaling in animals. The process of increase of the level of cGMP in tobacco tissues under the influence of NO reminds of the behavior of cGMP level under

the influence of NO in the cells of mammalia unstriated muscles (Durner et al., 1998). These authors have made an assumption that organisms of plants and animals use common mechanisms for NO-signal transduction.

Treatment of tobacco suspension cell or leave cultivar by sodium nitroprusside resulted in temporary increase of endogenous cGMP content by several orders, expression of genes encoding *PR*1 (protein associated with pathogenesis) and phenylalanine-ammonium-lyase (*PAL*) (Durner et al., 1998). Therefore, NO, cGMP, cADPR may activate genes encoding synthesis of metabolites, which produce toxic effect on pathogenic bacteria. Their influence is interconnected and synergic. Mechanisms of this influence are currently being actively investigated in various organisms (Besson-Bard et al., 2008).

NO is one of key messengers responsible for regulation of Ca^{2+} homeostasis (Courtois et al., 2008). Almost all types of calcium canals and transporters are under control of nitric oxide. NO changes their activity via S-nitrosylation or via the secondary messengers – cGMP and cADPR. For example, cADPR fosters the process of release of Ca^{2+} from the intracellular space in animals and plants via activation of Ca^{2+}–penetrable canals (Fliegert et al., 2007). At the same time, exposition of the suspension cells in beans and tobacco on the solution with NO-donor provoked rapid increase of Ca^{2+} concentration in cytosol and its removal from the extracellular space (Lamotte et al., 2004). Appearance of calcium in cytosol activates ROS-generating enzymes including peroxidases and NADPH oxydase (Desikan et al., 1998; Kolupaev, Karpets, 2009a).

The protein-kinase of molecular weight 42 kD, which is identical to protein-kinase, which is activated by osmotic stress in the cultivar of tobacco suspension cells (*NtOSAK*), is probably involved into NO-signal cascade modulating activity of calcium canals (Lamotte et al., 2006). At the same time the osmotic stress results in rapid increase of NO synthesis in tobacco leaves (Gould et al., 2003). The evidence of participation of protein-kinases in NO-cascade bound up with Ca^{2+}–canals can be found in the survey by Courtois et al. (2008).

The physiological role of NO as a Ca^{2+}–modulating compound has been proved in the tests with suspension cells of tobacco and grapes (Gould et al., 2003; Lamotte et al., 2004, 2006; Vandelle et al., 2006). In particular, in these cells outflow of Ca^{2+} into cytoplasm at the background of the effects of both osmotic stress and the pathogenic elicitor reduced under the influence of the NO-scavenger (PTIO) and inhibitors of animal NOS activity. It has been supposed that any change of free Ca^{2+} concentration in the cytoplasm and in the extracellular space may be explained by direct or indirect influence of NO on signal proteins, which include Ca^{2+}-dependent protein-kinases (CDPKs) and mitogen-activated protein-kinases (MAPKs) (Besson-Bard et al., 2008). According to Parani et al. (2004), NO regulates the transcription level of 24 protein-kinases of various classes. The fact that protein-kinases are nowadays considered to be the principal compounds in signal network in the cell and between cells (Nakagami et al., 2005) explains the multifarious number of the organism responses to the effect of NO, such as expression of protection genes, closure of stomata, formation of lateral roots and so on (Lamattina et al., 2003).

Interaction between ROS and NO has been well studied under pathogenesis, when the systemic acquired resistance (SAR) is formed on the basis of the hyper-sensitive reaction of the cell and its further degradation (death). As obvious from the data of the investigations, death of a cell in the host plant under SAR is a result of simultaneous effect of H_2O_2 and NO (Zaninotto et al., 2006).

The NADPH oxydase producing superoxide-anion ($O_2^{\bullet -}$) and superoxide dismutase is involved into the process (Glyanko et al., 2008; 2009). In the course of interaction between NO and $O_2^{\bullet -}$ peroxinitrite ($OONO^-$) is formed, which, as well as NO may react with proteins (nitration, S-nitrosylation) and change their properties (Lindermayr et al., 2005). Correlation of NO, $O_2^{\bullet -}$, H_2O_2 and $OONO^-$ determines cell SAR to phytopathogen invasion or elicitor action on the plant (Delledonne et al., 2001; Zhao et al., 2007a).

Salicylic acid is well known to act as the most important signal molecule in plants under pathogenesis. In presence of SA (as the enhancer of reactions cascade initiated by nitric oxide) NO causes expression of the protection genes and, in particular, synthesis of *PR*1 and *PAL* (Durner et al., 1998). In this case, the influence of NO on the expression of protective genes in the plants is increased at the expense of ROS and SA, i.e. we encounter the synergetic character of the process (Delledonne et al., 1998). H_2O_2 may influence signal functions of NO via activation of synthesis of SA, which – being a competitive inhibitor of catalase – contributes in its turn to the process of accumulation of H_2O_2 (Rao et al., 1997).

So, NO is involved into many cell responses to any stresses impacts. However, some investigations of interaction between NO and other signal molecules distinctly have shown that it is insufficient to accumulate one signal component in order to induce any physiological changes (Zaninotto et al., 2006). Accumulation of reactive nitrogen species (RNS), which is initiated by unfavorable factors in plant cells, causes nitrosative stress in these plants, which is accompanied by accumulation of NO and some products of its interaction with other molecules, such as peroxinitrite, S-nitrosoglutation, nitrotyrosine, etc. in these plant cells.

These compounds may produce a toxic effect on organisms of plants (Valderrama et al., 2007). Obvious is close interconnection between the nitrosative stress and the oxidative stress (Valderrama et al., 2007). The fact of initiating the nitrosative stress in plant tissues in case of effect of the salt stressor has been proved. In this case, the data related to the level of RNS (the level of nitric oxide, S-nitrosothyols, S-nitrosoglutation) were the indicators of stress (Valderrama et al., 2007). However, the mechanisms of these processes are still unknown. According to Dubovskaya et al. (2007), in case of the oxidative stress in tobacco cells, which was caused by the effect of exogenous H_2O_2, NO in micromolar concentrations suppressed peroxidation of lipids and fragmentation of total DNA. NO in its millimolar concentration produced some toxic effect expressed in activation of the caspase-like activity, degradaton of DNA and cell proteins, in the decrease of ATP synthesis. According to Asai, Yoshioka (2009), NO plays the key role in protection of tobacco cells from necrotrophic pathogen known *Botrytis cinerea.*

Some author have indicated to the correlation between NO and phytohormonal exchange (Lamattina et al., 2003; Molina-Favero et al., 2007). In the auxin-dependent formation of the lateral roots, NO acts as a secondary messenger in the synthesis of indolylacetic acid (IAA) (Pagnussat et al., 2003). In these processes, auxin with the help of an unknown mechanism intensified NO synthesis (Lombardo et al., 2006). Close correlation between the process of phytohormonal exchange and the NO metabolism has been confirmed also in other publications. Hence, treatment of plants with the use of exogenous phytohormones – auxine, abscisic acid (ABA), kinetin – results in the enhancement of NO synthesis in plant cells (Tun et al., 2001, 2008; Neill et al., 2002). Believe, that nitric oxide, probably, is synchronizing chemical messenger activate phytohormones (Molina-Favero et al., 2007).

The role of NO in legume-rhizobial symbiosis

The role NO in the processes of symbiosis is still also insufficiently investigated (Glyan'ko, Vassil'eva, 2007). Pii et al. (2007) have reported about the fact that there is the need in NO for auxin-dependent formation of nodules in legumes characterized by a non-determinant type of differentiation for the nodules. One can find the data releted to presence of NO in root nodules of lucerne (Baudouin et al., 2006). The authors of that paper believe that synthesis of NO in the nodules takes place with participation of an enzyme, which possesses NO-synthase activity. Other authors emphasize that – under normal physiological conditions – the role of NO as a negative regulator of N_2-fixation in the nodules is hardly ever probable (Herouart et al., 2002). The presence of an NO complex with leghemoglobin in the processes going on in soy nodules in the absence of nitrate in the medium has been proved (Mathieu et al., 1998). According to Kanayama et al. (1990, 1990a), when the content of nitrate in soy is high, NO is synthesized in bacteroids and is further linked to leghemoglobin, while presuming further formation of nitrosyl-leghemoglobin. This may result in inhibition of the process of transfer of O_2 into bacteroids and, as a result, to reduction of the nitrogenase activity and further degradation of the nodules. Tests *in vitro* earlier showed the inhibiting impact of NO on nitrogenase of soy nodules (Trinchant, Rigaud, 1982). Kosmachevskaya (2008) has demonstrated that leghemoglobin *in vivo* may form NO-nitrosyl complexes with haem and non-haem iron. This, from the author's viewpoint, excludes nitric oxide from the redox-cycle of reactions of free-radical oxidation.

Therefore, one of the possible roles of NO in the processes going on in root nodules may consist in regulation of the O_2 transfer into bacteroids via formation of a complex with leghemoglobin (Herouart et al., 2002). Nevertheless, the level of NO in the nodules will be at a fairly low level, which does not give the possibility to inhibit the the activity of nitrogenase (Kanayama et al., 1990) and the delivery of O_2 to the bacteroids via formation of a complex with leghemoglobin. To ensure this there must be an efficient mechanism of activity in the nodules, which neutralizes the excess of NO. It may be supposed that – side by side with leghemoglobin – non-symbiotic hemoglobin may be an efficient scavenger of NO in the nodules (Shimoda et

al., 2005; Vieweg et al., 2005). According to Shimoda et al. (2005), the content of non-symbiotic hemoglobin (LbHg1) grows in the active nodules in response to the rhizobial infection combined with the growth of transient NO in the roots *of Lotus japonicus*. Our results have shown that the rhizobial infection reduces the content of NO in epidermal cells of roots seedlings in pea (Glan'ko et al., 2010).

There are practically no data on the influence of NO on the processes of infection and formation of the legume-rhizobial symbiosis. According the data obtained by Glayn'ko et al. (2009a), exogenous NO (in the form of Na nitroprusside) renders negative effect on the growth of nodular bacteria in the cultivar, on their adhesion and penetration into root tissues, as well as on the growth of root hairs and roots of pea seedlings. The degree of inhibition of the abovementioned processes depends on the concentration of sodium nitroprusside (from 0.019 to 0.67 mM) in the medium. NO is supposed to participate in the regulation of the number of nodules (Herouart et al., 2002). Stohr and Stremlau (2006) have presented data on generation of NO in plasmatic membranes of plant cells under the process of interaction between legume-rhizobial and arbuscular-mycorhiza. These authors have noted that an obvious increase in the process of generation of NO is observed at high doses of nitrate in the medium, what coincides with the increase in the activity of membrane (but not cytoplasmatic) nitrate-reductase. High concentrations of NO can contribute to triggering of protective reactions in the host-plant and, hence, preclude infecting of the plant by rhizobia and mycorhiza.

We are sure that one of the possible causes of the negative impact of high doses of nitrate nitrogen on the process of formation of legume-rhizobial symbiosis is the disturbances in the proportion of the balance of auxins and nitric oxide, which initiate the division of cortical cells and the formation of the primordium of nodules (Glyan'ko, Mitanova, 2008a). On the other hand, the increase in the process of accumulation of nitrate by plants under the influence of rhizobial infection is conditioned by activation of anion channels of plasmalemmas and by changes in metabolism of plants (Wendehenne et al., 2002; Mitanova et al., 2006). The possibility of influence of mineral nitrogen (represented in the form of ammonium (NH_4^+) on the interaction between the plants of pathogenic fungus and tomato is confirmed by the data published by Alkan et al. (2009). According to the data presented by this team of authors, ammonium secreted by the pathogenic fungus *Colletotrichum coccode* activates plant NADPH oxidase, what causes accumulation of ROS and death the celles of tomato fruit. With this in view, of interest is a hypothesis of possible influence of NO on the flows of extra- and intracellular Ca^{2+}, what in turn influences the activity of NADPH oxidase. The idea of this hypothesis is based on the assumption that NO can increase or inhibit the stress factors inducing the flow of Ca^{2+} to cytoplasm at the expense of variation of permeability of Ca^{2+}–channels on account of signal proteins, which are subject to post-translation modification by nitric oxide (Besson-Bard et al., 2008). This mechanism related to regulation of Ca^{2+} flows on the plasmatic membrane (with the participation of NO) may be used for explaining the mechanisms of functional activity for NADPH oxidase, in particular, during the symbiotic interactions, when the excessive accumulation of ROS may prevent the establishment of symbiosis (Shaw, Long, 2003).

Synthesis of NO in plant organisms

The issue of the ways related to synthesis of NO has been unequivocally solved for animal organisms: in this case, there takes place the process of NO formation from α-arginin, O_2 and reduced NADP participating in this process. The reaction is catalyzed by nitric oxide synthase (NOS) according to the following scheme:

$$\alpha\text{-arginin} + O_2 + NADPH \rightarrow \alpha\text{-citrullin} + NO.$$

All the NOS isoforms specifically utilize α-arginin as the substrate (Men'shchikova et al., 2000).

As far as plant organisms are concerned, this issue has not been solved yet. Presently, the researchers are actively discussing the two ways of NO synthesis in plants: nitrate/nitrite and α-arginin-dependent trends. The first trend presupposes restoration of nitrate and nitrite in leaves and roots up to NO with the participation of cytosol nitrate reductase (NR) (Yamasaki, Sakihama, 2000; Garcia-Mata, Lamattina, 2003; Meyer et al., 2005; Shi, Li, 2008) and nitrite-NO-reductase localized on the plasmatic membrane of tobacco roots (Stohr et al., 2001).

Participation of the assimilation NR in the process of generation of NO has been proved. Already in the early 1960s, Fewson and Nicholas (1960) identified NO as an intermediate link in the course of reduction of nitrate by plants and microorganisms. The problem to be solved is the amount in which NO may be formed with participation of NR. According to Rockel et al. (2002), production

of NO under saturating concentrations of the substrate is only 1% of the nitrate-reducing capability of NR. However, in case of creating definite conditions (anoxia, high doses of nitrogen fertilizer, etc.) the activity of NR increases and, respectively, both the level of nitrite and the process of NO generation increase in cytosol (Rockel et al., 2002). The authors conclude that exo- and endogenous conditions contributing to the variation of either the concentration of nitrate in cytosol or the speed of reduction of the nitrate may either increase or decrease the process of NO generation with the participation of NR. The functional state of NR is determined by phosphorylation or dephosphorylation of the enzyme (Lea et al., 2004).

It is believed that the amount of NO, which is formed in the plant cells with the participation of NR, is more than sufficient for its explication as a signal molecule (Meyer et al., 2005). However, as mentioned above, the expression of nuclear genome (exemplified by *Arabidopsis* under the influence of NO) is a process dependent on the level of nitric oxide: the higher is the concentration of NO in the cells the larger is the number of expressed genes and the higher is the intensity of formation of the transcripts (Parani et al., 2004). Participation of NR in the process of generation of NO has been confirmed in the tests with *Arabidopsis* mutants, which are deficient in the aspect of NR. It has been shown that synthesis of NO with NR the participation is the main link in signaling of ABA registered in the operations of regulation of processes of opening and closing of stomatal cells (Bright et al., 2006).

Another compartment for restoration of nitrate and nitrite may be the plasmatic membrane of plant cells. According to Stohr et al. (2001), NR (PM-NR), which is localized on the plasmatic membrane in association with nitrite-reductase (NiRNOR), reduces nitrate and nitrite and initiates formation of NO. High doses of mineral nitrogen, as well as biotic factors (rhizobia and fungal micorhiza) intensify the process bound up with generation of NO, what coincides with the increase of PM-NR activity, but not the activity of cytoplasmatic activity of NR (Stohr, Stremlau, 2006). Nevertheless, currently there is no data on identification of NiRNOR (Besson-Bard et al., 2008).

The most debatable is the issue of generation of NO in plants via oxidation of α-arginin with the help of NOS. No homologue, which would be identical to the animal NOS, has been identified in the genome of *Arabidopsis* (*Arabidopsis* Genome…, 2000). But presently one can find sufficient data giving evidence of the possibility of a NOS-like reaction in plant tissues and organelles (Cueto et al., 1996; Barroso et al., 1999; Ribeiro et al., 1999; Crawford, 2006). Inhibition of the increase in the process of NO generation in tissues and suspension cultivars in response to the effect of various exogenous factors with the help of animal NOS inhibitors is the confirmation of the presence of a NOS-dependent enzyme reaction in plants (Foissner et al., 2000; Tun et al., 2001; Guo et al., 2003; Lamotte et al., 2004; Vandelle et al., 2006; Zhao et al., 2007b; Arnaud et al., 2006). In investigations conducted by Corpas et al. (2004, 2006, 2008, 2009), the reality of NOS-like activity in pea leaves has been proved by various methods: confocal laser microscopy, inhibitor analysis, application of antibodies to animal iNOS and other methods. An enzyme (or an enzyme complex), which possesses NOS-activity and uses the same substrates as animal NOS for its reaction, has been extracted from peroxisomes of pea leaves and purified (Corpas et al., 2009).

Nowadays, there arise numerous questions related to *AtNOS,* the gene identified in *Arabidopsis thaliana* encoding the protein with NOS-activity. This protein (*AtNOS1* or *AtNOA1*) has an intense affinity to the snail protein participating in generation of NO (Guo, Crawford, 2005). There are quite a few studies proving the fact of participation of this protein (with NO-synthase activity) in various physiological processes: blooming (He et al., 2004), ABA-signal transduction (Guo et al., 2003), in liposacharide impact (Zeidler et al., 2004). The latest research (Shi, Li, 2008; Tun et al., 2008) has also drawn the attention to *AtNOS1/AtNOA1* as a generator of NO. However, the issue of capability of this protein to catalyze the reaction, which is bound up with formation of NO on the basis of arginin, remains undecided (Zemojtel et al., 2006; Crawford et al., 2006). Zemojtel et al. (2006) are sure that this protein is GTPase, which is involved in ribosome biogenesis in mitochondria and in the related translation processes. This assumption has been confirmed by Moreau et al. (2008) insisting that *AtNOA1* from *Arabidopsis* is not a NO-producing enzyme, but belongs to the family of small GTPases. A similar judgment about *AtNOA1* as GTPase is discussed in detail in Besson-Bard et al. (2008), where possible errors in the investigation of *AtNOA1* as a NO-producing enzyme are considered.

The researchers have lately focused their studies on the process of biosynthesis of NO with the participation of polyamines. It was shown that spermin and spermidin induce quick synthesis of

NO in various tissues of *Arabidopsis* (Tun et al., 2006; Yamasaki, Cohen, 2006; Flores et al., 2008). In this connection, the specialists presuppose the presence of unknown enzyme(s), which catalyze(s) the process of transformation of polyamines and formation of nitric oxide in tissues of *Arabidopsis*. These enzymes may be represented by arginase or arginindecarboxylase, which lose their activity under the influence of inhibitors of animal NOS and under these conditions may indirectly suppress the synthesis of NO with participation of polyamines (Besson-Bard et al., 2008).

There are data confirming that P-protein, which belongs to the glycine-decarboxylase complex of plants, possesses NOS-activity (Chandok et al., 2003). The same is true of peroxidase in horseradish, which catalyzes the formation of NO from hydroxyurea (Huang J. et al., 2002).

There is one more way of forming NO in plants. This is non-enzymatic reduction of nitrite in the acid medium in presence of some reducer. For example, it was shown that cells of the aleuron layer of barley in a fairly acididous apoplast compartment can reduce nitrite to NO in presence of ascorbic acid and phenols (Beligni, Lamattina, 2000; Bethke et al., 2004). Reduction of nitrite in apoplastic space of plant cells without enzymes is considered to be one of ways for formation of NO in plants, particularly, in the roots of plants (Stohr, Ullrich, 2002; Bethke et al., 2004).

We have to note a paradoxical fact. In the literature one can find indications to the mutants of *Arabidopsis* (deficient in NR and nitrite), which contain 10 times less of α-arginin, which is known to be the substrate for NOS-like enzymes (Modolo et al., 2006). This proves the fact that synthesis of NO via nitrate- and nitrite-reduction is somehow bound up with metabolism of α-arginin. According to other data, activity of arginase and the pool of arginin are rather high in the processes of (i) germination of the seeds of *Arabidopsis* and (ii) growing of seedlings of *Arabidopsis* (Zonia et al., 1995). In Filippovich et al. (2007), the mutants of fungus *Neurospora crassa*, which are characterized by deprived activity of nitrate- and nitrite-reductase and have been grown in the medium, which does not contain nitrogen salts, demonstrated the release of nitrate and nitrite into this medium. The authors believe that NO synthesized in cells of the fungus (without participation of NR and NIR) transforms into nitrate and nitrite, which are extracted into the medium, in which the mycelium grows.

According to our data, synthesis of NO, which is assessed by NO-specific fluorescent sampling (with 4.5-diaminfluorescein diacetate, DAF-2 DA) and application of fluorescence microscopy, is observed in epidermal cells of the roots of etiolated pea seedlings exposed in water. Addition of α-arginin (1 mM) into the medium with seedlings provokes a considerable increase in fluorescence in the surface roots of the cells (Glyan'ko et al., 2010). These results may give evidence that synthesis of NO takes place in the root cells under normal physiological conditions. This synthesis is stimulated by the mechanism using α-arginin.

So, the problem of the ways of NO synthesis in plants remains unsloved and open for further investigations. According to Flores et al. (2008), there may exist several sources of formation of NO in plants, and some of these may be regulated via signal paths (downstream).

The mechanisms preventing from the toxic effect of NO

As note above, high concentrations of NO produce a toxic effect on organisms. The nitrosative stress caused by unfavorable exogenous factors is accompanied by accumulation of free NO and its derivatives – peroxinitrite and other low-molecular N-compounds. Particularly toxic is the product of reaction of NO with $O_2^{\bullet -}$ –peroxinitrite ($ONOO^-$), which can oxidize thyol remains and nitrate thyrosine of proteins, what prevents their phosphorylation (Reiter et al., 2000). Nitration of secondary amins by NO, which causes formation of cancerogenic nitrosocompounds, has been proved in experiments conducted on animal tissues (Men'shchikova et al., 2000). As far as animal organisms are concerned, the researchers suppose that there functions the cycle of nitric oxide, in which – owing to cyclic transformations of NO and products of its interaction with other substances – efficient regulation of NO metabolism is provided (Reutov, 1995; Reutov et al., 2005).

Likewise in the case of oxidative stress, organisms probably possess some system protecting themselves from the toxic impact of reactive nitrogen species. However, the issue of protective mechanisms the against nitrosative stress in plant organisms remains practivally underinvestigated, and there are no distinct ideas on this issue. In the capacity of one of the mechanisms related to protection of plant cells from the toxic impact of NO the experts consider the emission of this gas from the cells into the environment, what results in the reduction of the concentration of nitric oxide in the

cells. The reality of this process has been proved for soils and for plants (Klepper, 1979, 1991; Wildt et al., 1997).

Another mechanism of rendering NO and its derivate – peroxinitrite – harmless is their interaction with protein molecules via nitrosylation and nitration of remains of cystein and tyrosin or via combining with metals into haem protein complexes. However, all these processes are the processes of post-translation modification of proteins and may modulate the exchange processes, while producing either a positive effect or a negative effect on the organisms. The issues of the use and the harm of such modifications of proteins have been discussed above.

During the recent years, the researchers have focused their attention on the so called nonsymbiotic forms of hemoglobin in plants. These forms of hemoglobin are synthesized in plants in response to various stress factors (Dordas et al., 2003; Perazzolli et al., 2004). The physiological role of these forms of hemoglobin is not quite clear, but their role as scavengers of NO is obvious (Dordas et al., 2003).

Nonsymbiotic hemoglobin and leghemoglobin can represent themselves as scavengers of NO in active been nodules (Shimoda et al., 2005; Vieweg et al., 2005). According to Schimoda et al. (2005), the content of nonsymbiotic hemoglobin (*LbHg*1) increases in the active nodules in response to the rhizobial infection bound up with the transient increase of NO in roots of *Lotus japonicus*.

Urates – purines destruction products – are known to be strong inhibitors of the toxic effect of peroxinitrite in animal tissues (Balavoine, Geletii, 1999). The content of urates in plants is insignificant, except for some legumes characterized by ureic type of NH_3 absorption. According to Alamillo and Garcia-Olmedo (2001), exogenous urates prevent from toxic effect of peroxinitrite in *Arabidopsis*. The mechanism of removal of the toxic effect caused by peroxinitrite is probably bound up with nitrosylation of urates (Vasquez-Vivar et al., 1998).

Toxicity of NO is substantially dependent on its concentration in plant cells. This fact must condition the dual role of NO in the animal or plant cell as (i) an antioxidant preventing from any destructive action of ROS and (ii) a pro-oxidant capable – in combination with ROS – of causing hypersensitive reaction or even programmed death of the cell. The toxicity of NO has to be assessed from the viewpoint of the type of stress arising in plant tissues under the impact of abiotic or biotic factors. For example, in the systems, where the toxicity is conditioned mainly by ROS, NO probably acts as a compound fixing oxygen radicals ($O_2^{\bullet -}$), and hence minimizing the damage possible (Wink et al., 1993). The compounds fixing NO may probably serve as a sort of "depot" for nitric oxide, which – under definite conditions – can release in its free state.

Conclusion

The fact that NO is a normal product of plant metabolism is without doubt, as well as the fact that the concentration of this compound may increase by several orders when there are some unfavorable factors acting on animal and plant organisms. The so called "NO-burst" is another term (lokewise "oxidative burst") confirming the importance of the investigations related to the role of nitric oxide and its derivatives in the aspect of resistance of organisms to biotic and abiotic factors (Foissner et al., 2000). At the same time, the capability of NO to produce different effects on various exchange processes is conditioned by its high capability to turn into other nitro-compounds (NO_3^-, NO_2^-, NO^-, NO^+, $NO_2^{\bullet}$-radical and others) and react with other endogenous substances. This is possibly one more mechanism, which participates in rendering NO harmless. Men'shchikova at al. (2000) state that unique physics-chemical properties of NO are used by the organisms for the purpose of efficient regulation of NO content in the tissues and participation in signalling mechanisms.

The concept of the cyclic character of NO and its derivatives in mammal cells and tissues may be fruitful – in our opinion – from the viewpoint of further investigations of the role of NO in plant organisms (Reutov, 1995; Reutov et al., 2005). As far as plant organisms are concerned, it is important to find out the mechanism activation of NO (and its derivatives) synthesis under various unfavorable influences. What plant receptors are related to activation of the process of NO production? It is difficult to answer this questing because there are probably several mechanisms of NO synthesis in plant organisms (unlike that in animal), and it is necessary to find out which of the mechanisms is the most important.

Is it possible to expect that as a result of investigations related to the role of NO in plant organisms we can obtain an effect similar to that of understanding of its role in mammals cells (including human)? To answer this question it is first of

all necessary to understand the role of nitric oxide for plants as a signal molecule, which trigger the mechanisms of protecting the plant under unfavorable impacts (Tarchevskij, 2002). Secondly, somen nitro-compounds including nitrate are the plant nutrient elements. In case of excessive doses, the plants "throw" nitrate to vacuole and, so, preclude their reduction to ammonia and the formation of intermediate compounds. The excess of nitrate is substantially more important for humans in case of consuming the food containing large amounts of nitrate. The formation of cancerogenic nitroso-compounds in the process of interactions between NO-derivatives and amins is a serious hazard to the health of people. The janus-face character of NO identified for animal cells holds for plant cells as well. Further investigations of the signal role of NO and the products of its interaction with other molecules, investigations of the antagonistic and synergetic influences on the exchange processes, investigations of the prooxidant and antioxidant effects – represent an incomplete list of problems, whose solution is quite important for understanding of the role of nitric oxide in the life of plant organisms.

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ФІЗІОЛОГІЧНА РОЛЬ ОКСИДУ АЗОТУ (NO) У РОСЛИН

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Узагальнені дані про фізіологічну роль NO у рослин. Підкреслюється багатофункціональність NO, зумовлена високою реакційністю цієї молекули, її здатністю реагувати з білками і низькомолекулярними речовинами. Розглянуті питання участі оксиду азоту в різних фізіологічних процесах, роль цієї молекули в бобово-ризобіальному симбіозі, можливі механізми синтезу NO у рослин, взаємодія з іншими ендогенними молекулами, а також механізми, що запобігають токсичному ефекту оксиду азоту.

Ключові слова: *оксид азоту (NO), NO-синтаза, NOS-подібна реакція, нітрозилювання і нітрування білків, нітрозативний стрес*

ФИЗИОЛОГИЧЕСКАЯ РОЛЬ ОКСИДА АЗОТА (NO) У РАСТЕНИЙ

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Обобщены данные о физиологической роли NO у растений. Подчеркивается многофункциональность NO, обусловленная высокой реакционностью этой молекулы, ее способностью реагировать с белками и низкомолекулярными веществами. Рассмотрены вопросы участия оксида азота в различных физиологических процессах, роль этой молекулы в бобово-ризобиальном симбиозе, возможные механизмы синтеза NO у растений, взаимодействие с другими эндогенными молекулами, а также механизмы, предотвращающие токсический эффект оксида азота.

Ключевые слова: *оксид азота (NO), NO-синтаза, NOS-подобная реакция, нитрозилирование и нитрование белков, нитрозативный стресс*