ISSN 1992-4917. ВІСНИК ХАРКІВСЬКОГО НАЦІОНАЛЬНОГО АГРАРНОГО УНІВЕРСИТЕТУ СЕРІЯ БІОЛОГІЯ, 2015, вип. 1 (34), с. 22-27

УДК 581.1

INFLUENCE OF EXOGENOUS CALCIUM AND ITS ANTAGONISTS ON NITRIC OXIDE CONTENT IN ROOTS OF WHEAT PLANTLETS

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The influence of treatment of roots of etiolated plantlets of wheat (*Triticum aestivum* L.) with exogenous calcium (50 mM $CaCl_2$), chelator of calcium EGTA (100 μM), blocker of calcium channels lanthanum chloride (2 mM), inhibitors of phospholipase C neomycin (100 μM) and ADP-ribosylcyclases nicotinamide (5 mM), and also inhibitor of protein biosynthesis cycloheximide (20 μM) on the content of nitric oxide (NO) have been investigated. The intensifying of formation of nitric oxide in roots of plantlets occurred under the influence of calcium chloride with the maximum within 1-2 hours after the treatment beginning. Antagonists of calcium EGTA, lanthanum chloride and neomycin to some extent reduced the nitric oxide content in cells of roots. At the combined action of $CaCl_2$ and lanthanum chloride or neomycin partial leveling of intensifying of NO generation, caused by exogenous calcium, was observed. At the same time nicotinamide in itself did not render the significant effect on the nitric oxide content in wheat roots and did not influence almost on the effect of intensifying of its formation, invoked by the exogenous Ca^{2+}. Cycloheximide reduced the nitric oxide generation in cells of wheat roots. However at the cotreatment with the cycloheximide and calcium chloride the nitric oxide content in roots was considerable above, than in the variant with one only antibiotic. The conclusion is made that the generation of nitric oxide by cells of wheat roots essentially depends on the calcium homeostasis.

Key words: *Triticum aestivum L., calcium, nitric oxide, enzyme similar to animal NO-synthase*

Calcium and nitric oxide (NO) are important signal mediators in plant and animal cells between which one there is the difficult functional interplay (Khan et al., 2014). It is shown that exogenous calcium in low concentration influences the rhythmic dynamics of content of nitric oxide in cells of roots of pea plantlets (Glyan'ko et al., 2014). It is established that one of the main enzymatic producers of nitric oxide in plants cells – the enzyme similar to animal NO-synthase – is activated with involvement of calcium and/or calmodulin (Lamotte et al., 2004; Courtois et al., 2008; Neill et al., 2008). Also it is known that the nitrate reductase, capable to generate nitric oxide, is activated with the participation of protein kinases dependent on calcium (Weiner, Kaiser, 1999; 2000). At the same time it is established that oxidation of L-arginine in chloroplasts of green seaweed by enzyme, homologous to animal NO-synthase, does not depend on calcium, though the similar process, which is taking place in peroxisomes, is calcium-dependent (Roster, 2014). In general, information about the influence of calcium on the NO content in various plant objects and on the activity of appropriate enzymatic systems remain inconsistent enough till now.

With use of methods of chemiluminescence it is shown the presence of the enzyme, similar to animal NO-synthase, in cytoplasm, chloroplasts, peroxisomes and mitochondria (Neill et al., 2008; Wilson et al., 2008). It is reported that the greatest pool of this enzyme is localized in chloroplasts and peroxisomes (Gas et al., 2009; Gupta et al., 2011). On the other hand, it is shown that in the cells of Arabidopsis roots the nitric oxide synthesis was suppressed by the inhibitors of animal NO-synthase (Zhao et al., 2007). In our experiments it is established that the treatment of intact roots of

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etiolated wheat plantlets with the exogenous calcium and/or calcium ionophore A23187 caused the intensifying of formation of nitric oxide, which was substantially suppressed with the inhibitor of enzyme, similar to animal NO-synthase, L-NAME (N^G-nitro-L-arginine methyl ester) (Karpets, Kolupaev, 2015). It is possible to suppose that the activation of animal-like NO-synthase by calcium ions takes place at their influx in the cytosol both from extracellular and from intracellular compartments.

The participation of different pools of calcium in the activation of NO formation in plant cells can be established with the use of calcium chelators and blockers of calcium channels, different by relative specifity. The purpose of present study was to investigate the action of exogenous calcium and its antagonists, influencing the influx of Ca^{2+}ions in cytosol from various compartments, on the nitric oxide content in roots of wheat plantlets.

MATERIALS AND METHODS

For investigations the etiolated plantlets of wheat (*Triticum aestivum* L.) variety Elegiya were used. After 30-minute disinfecting in the 5% solution of hydrogen peroxide the seeds were germinated during four days on the cleared tap water at the temperature of 20°C. After that plantlets of experimental variants transferred for one days on the solutions of 50 mM calcium chloride, 500 μM chelator of external calcium EGTA, 5 mM lanthanum chloride, 50 μM neomycin, 5 mM nicotinamide, 20 μM cycloheximide. Also the effects of combination of calcium and blockers of calcium channels or inhibitor of protein biosynthesis cycloheximide on the nitric oxide content in roots were investigated. In this case the EGTA, lanthanum chloride, neomycin, nicotinamide and cycloheximide were added in the media of incubation of roots for 2 hours before the addition of calcium chloride into it. The concentrations of indicated compounds were chosen on the basis of preliminary experiments.

Through certain time after the beginning of incubation of plantlets on the solutions of investigated compounds, the nitric oxide content in roots was quantified by the method described Zhou et al. (2005) with modifications. The method is based on the transformation of NO containing in plants tissues into the nitrite and quantification of its concentration on Griess reaction. The weighed portion of plant material was homogenized on ice in the 50 mM acetate buffer (pH 3,6) with addition of 2% zinc acetate. The homogenate was centrifuged at the temperature not above 4°C at 8000 g within 15 minutes and then to the 10 ml of supernatant was added the 250 mg of charcoal. Further, the filtration through the paper filter was conducted, and then 2 ml of filtrate and 1 ml of 1% Griess reagent, dissolved in 12% acetic acid, was mixed. Within 30 minutes the solution light absorption was quantified at the wavelength of 530 nanometers. As the standard the solutions of sodium nitrite were used.

Experiments were conducted in triple biological replicate and each experiment was reproduced independently three times. The figure shows the average values and their standard errors. Except where specifically noted, the differences significant at $p \leq 0{,}05$ are discussed.

RESULTS AND DISCUSSION

The influence of 50 mM calcium chloride on the plantlets roots invoked the increase of nitric oxide contents in them (fig. 1). The maximum effect was observed during 1-2 hours after the treatment beginning, within 6-24 hours it decreased, however the NO content in variant with calcium exceeded the values of control.

In the further experiments there was investigated the nitric oxide generation in wheat roots within 1 and 24 hours after the beginning of their treatment with calcium salt in the combination with blockers of calcium channels or else within 3 and 27 hours after the beginning of influence of calcium antagonists.

Under the influence of "external" calcium chelator EGTA the small, but significant at $p \leq 0{,}05$, decrease of NO content in plantlets roots was registered (fig. 2). More essential reduction of generation of nitric oxide by wheat roots took place at their treatment with the lanthanum chloride blocking calcium channels of various types. NO formation was also a little suppressed by neomycin, which one through the binding of phosphatidylinositolbiphosphates can inhibit the phosphatidylinositol-specific phospholipase C (PI-PL C) (Liu et al., 2006) and by that can prevent the accumulation of product of reaction – inozitol-1,4,5-phosphate (IP_3). It is considered that the last one influences the calcium influx in cytosol from the intracellular compartments and by that activates many calcium-dependent processes (Lee, Lee, 2008).

In the opening of intracellular calcium channels along with IP_3 the cADP-ribose can also participate. For the studying of its contribution to the regulation of processes of formation NO by wheat roots the antagonist of cADP-ribose synthesis (an inhibitor of ADP-ribosylcyclases) nicotinamide (Allen et al., 1995) was used. The treatment of

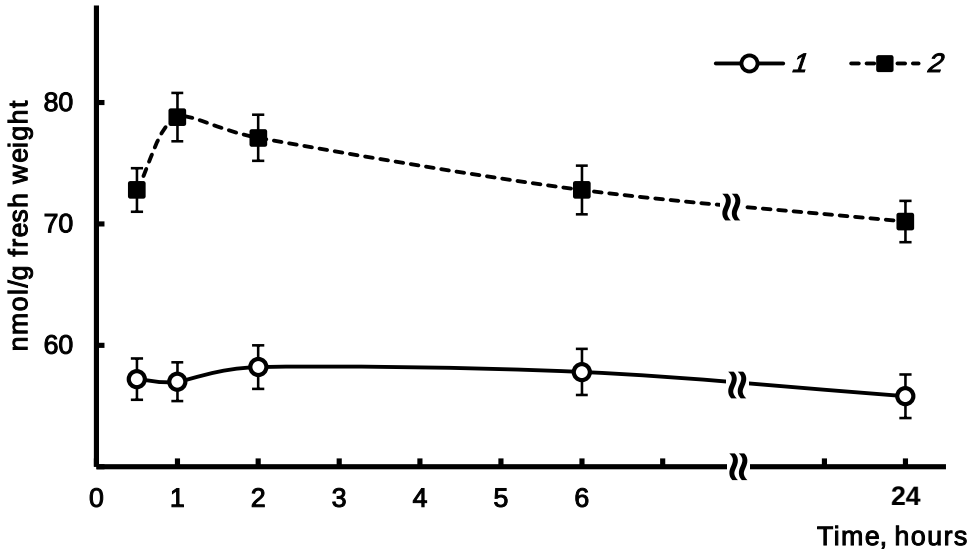

Fig. 1. The dynamics of content of nitric oxide (nmol/g fresh weight) in the roots of etiolated wheat plantlets under the calcium chloride influence.
1 – control; 2 – $CaCl_2$ (50 mM).

roots with this compound did not influence almost on the content of nitric oxide in them (fig. 2).

The influence of lanthanum chloride and neomycin on plantlets roots partially removed the effect of rising of NO content in them, caused by exogenous calcium (fig. 2). These effects were more appreciable within 1 hour after the beginning of influence of exogenous calcium. Nicotinamide almost did not change the action of calcium on the nitric oxide content in roots.

As it was already noted, in the literature there are evidences on the activation by calcium ions of enzyme similar to animal NO-synthase (Lamotte et al., 2004). It is possible to suppose that in this case the increase of enzyme activity by calcium ions does not depend on the protein biosynthesis. In our experiments the inhibitor of protein biosynthesis on 80S ribosomes cycloheximide reduced the NO formation in the roots of wheat plantlets (fig. 2). This result will be agreed with the data received on the protoplasts of Arabidopsis and rape, cultivated *in vitro* (Tewari et al., 2013). Authors have concluded that the synthesis of enzyme similar to animal NO-synthase, which can be localized in various compartments, depends on the biosynthesis of proteins in cytoplasm. In the conditions of our experiments in variant with the cotreatment of plantlets roots with cycloheximide and calcium chloride the NO content was above, than in variant with one only inhibitor of protein synthesis (fig. 2). In this connection it is possible to suppose that influence of calcium on the enzymatic systems, generating nitric oxide, is not bound to the induction of their synthesis, and determined by activation of already existing enzymatic molecules.

Thus, in our experiments dependence of formation of nitric oxide in roots cells from the calcium homeostasis is shown. This process was enhanced under the influence of exogenous calcium and suppressed with some its antagonists. Reduction of NO generation in roots cells under the influence of EGTA gives evidence to importance of influx of calcium into the cytosol from the extracellular compartments in the regulation of enzymatic systems, that generate the nitric oxide. The blocker of calcium channels of various types lanthanum in itself lowered the nitric oxide formation in roots cells and leveled the effect of increase of NO content, caused by treatment with the exogenous calcium (fig. 2).

Some decrease of NO content in cells of wheat roots took place under the influence of neomycin which is used as the inhibitor of PI-PL C. It is difficult to interpret unequivocally the effect of neomycin to level of intensifying of NO formation, caused by calcium in cells of wheat roots. Phenomenological similarity of neomycin effects with influence of EGTA and lanthanum indicates possibility of its action on the calcium homeostasis. As it is known, in cells of animals the PI-PL C, catalyzing process of formation of IP_3 and diacylglycerol, stimulates calcium influx into the cytosol. It is bound to opening of intracellular calcium channels under the influence of IP_3, and also with the activation by diacylglycerol of protein kinase C, which participates in the regulation of potential-dependent calcium channels of plasmalemma. De-

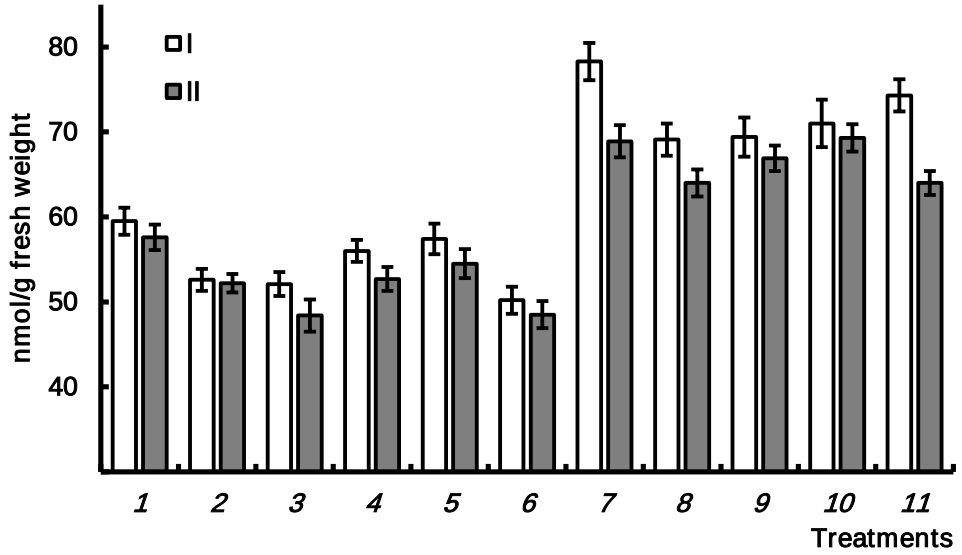

Fig. 2. The influence of calcium antagonists and cycloheximide on the nitric oxide content (nmol/g fresh weight) in the roots of wheat plantlets.

I – within 1 hour after the beginning of influence of calcium chloride or within 3 hours after the beginning of influence of calcium antagonists and cycloheximide; II – within 24 hours after the beginning of influence of calcium chloride or within 26 hours after the beginning of influence of calcium antagonists and cycloheximide.

1 – control; 2 – EGTA (500 μM); 3 – $LaCl_3$ (5 mM); 4 – neomycin (100 μM); 5 – nicotinamide (5 mM); 6 – cycloheximide (20 μM); 7 – $CaCl_2$ (50 mM); 8 – $CaCl_2$ (50 mM) + $LaCl_3$ (5 mM); 9 – $CaCl_2$ (50 mM) + neomycin (100 μM); 10 – $CaCl_2$ (50 mM) + nicotinamide (5 mM); 11 – $CaCl_2$ (50 mM) + cycloheximide (20 μM).

spite the fact that in plants have not yet detected the homologues of targets of IP_3, it is known that neomycin can reduce the calcium influx into the cytosol of plant cells. For example, this compound removed the effect of rising of calcium concentration in the tobacco cells, invoked by the influence of elicitor cryptogein (Lecourieux et al., 2002). Thus, it is possible to suppose that the NO formation in cells of wheat roots, at least, partly depends on processes of calcium-fosfolipid signalling.

On the other hand, the NO formation by roots in the conditions of our experiments was almost not influenced by the treatment with the inhibitor of ADP-ribosylcyclase, suppressing calcium influx in the cytosol through the calcium channels, dependent on the cADP-ribose and localised mainly in the tonoplast.

Thus, apparently, the generation of nitric oxide by wheat roots depends on the calcium homeostasis, namely on the influx of calcium ions in the cytosol through specific calcium channels. With calcium participation, probably, the activation of enzyme similar to animal NO-synthase occurs. At the same time, as it was already noted, activity of nitrate reductase (other enzyme, possessing ability to generate NO) also depends on calcium (Weiner, Kaiser 1999; 2000; Neill et al., 2003). However in our experiments the plantlets grew up in the absence of the special nitrogen nutrition, and the nitrate reductase, as it is known, refers to the enzymes which synthesis is induced by substrate. Earlier with the use of nitrate reductase inhibitor sodium wolframate it has been shown by us that the contribution of this enzyme to nitric oxide synthesis in roots of wheat plantlets at their cultivation on water is comparatively low (Karpets et al., 2014). In this regard, within the model used by us it can be considered that the pathway of NO formation with the participation of enzyme similar to animal NO-synthase, serves as the main one.

In our experiment, the total generation of nitric oxide in the root cells was determined, which does not allow talking about that, in which exactly compartments its formation took place. At the same time, there are grounds to believe that this process is calcium-dependent. Now the enzymatic systems (similar to NO-synthase of animals) located in peroxisomes are considered as the important source of NO in plant cells (Roszer, 2014). Remarkable, in plant peroxisomes the calcium oscillations, which resemble the changes of calcium concentration in cytosol, are revealed (Costa et al., 2013). Is not excluded that the similar mechanism

may be involved in the regulation of nitric oxide production in the cells of non-green parts of plants. Naturally, to confirm this assumption the special investigations are required for the establishing of subcellular localization of nitric oxide production under the conditions of changing of calcium homeostasis.

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Received
10.02.2015

ВПЛИВ ЕКЗОГЕННОГО КАЛЬЦІЮ ТА ЙОГО АНТАГОНІСТІВ НА ВМІСТ ОКСИДУ АЗОТУ В КОРЕНЯХ ПРОРОСТКІВ ПШЕНИЦІ

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Досліджували вплив обробки коренів етіольованих проростків пшениці (*Triticum aestivum* L.) екзогенним кальцієм (50 мМ $CaCl_2$), хелатором кальцію ЕГТА (100 мкМ), блокатором кальцієвих каналів хлоридом лантану (2 мМ), інгібіторами фосфоліпази С неоміцином (100 мкМ) і АДФ-рибозилциклази нікотинамідом (5 мМ), а також інгібітором біосинтезу білка циклогексимідом (20 мкМ) на вміст оксиду азоту (NO). Під впливом хлориду кальцію відбувалося посилення утворення оксиду азоту в коренях проростків з максимумом через 1-2 год після початку обробки. Антагоністи кальцію ЕГТА, хлорид лантану і неоміцин тією чи іншою мірою знижували вміст оксиду азоту в клітинах коренів. При одночасній дії $CaCl_2$ і хлориду лантану або неоміцину відзначалося часткове нівелювання посилення генерації NO, спричинюваного екзогенним кальцієм. Водночас нікотинамід сам по собі не чинив достовірної дії на вміст оксиду азоту в коренях пшениці і майже не впливав на ефект посилення його утворення, спричинюваний екзогенним Ca^{2+}. Циклогексимід зменшував генерацію оксиду азоту в клітинах коренів пшениці. Однак при комбінованій обробці коренів циклогексимідом і хлоридом кальцію вміст оксиду азоту в них був значно вищим, ніж у варіанті з самим антибіотиком. Зроблено висновок, що генерація оксиду азоту клітинами коренів істотно залежить від кальцієвого гомеостазу.

Ключевые слова: *Triticum aestivum L., кальцій, оксид азоту, фермент, подібний до NO-синтази тварин*

ВЛИЯНИЕ ЭКЗОГЕННОГО КАЛЬЦИЯ И ЕГО АНТАГОНИСТОВ НА СОДЕРЖАНИЕ ОКСИДА АЗОТА В КОРНЯХ ПРОРОСТКОВ ПШЕНИЦЫ

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Исследовали влияние обработки корней этиолированных проростков пшеницы (*Triticum aestivum* L.) экзогенным кальцием (50 мМ $CaCl_2$), хелатором кальция ЭГТА (100 мкМ), блокатором кальциевых каналов хлоридом лантана (2 мМ), ингибиторами фосфолипазы С неомицином (100 мкМ) и АДФ-рибозилциклазы никотинамидом (5 мМ), а также ингибитором биосинтеза белка циклогексимидом (20 мкМ) на содержание оксида азота (NO). Под влиянием хлорида кальция происходило усиление образования оксида азота в корнях проростков с максимумом через 1-2 ч после начала обработки. Антагонисты кальция ЭГТА, хлорид лантана и неомицин в той или иной степени снижали содержание оксида азота в клетках корней. При сочетанном действии $CaCl_2$ и хлорида лантана или неомицина отмечалось частичное нивелирование усиления генерации NO, вызываемого экзогенным кальцием. В то же время никотинамид сам по себе не оказывал достоверного влияния на содержание оксида азота в корнях пшеницы и почти не влиял на эффект усиления его образования, вызываемый экзогенным Ca^{2+}. Циклогексимид уменьшал генерацию оксида азота в клетках корней пшеницы. Однако при комбинированной обработке корней циклогексимидом и хлоридом кальция содержание оксида азота в них было значительно выше, чем в варианте с одним антибиотиком. Сделано заключение, что генерация оксида азота клетками корней существенно зависит от кальциевого гомеостаза.

Ключові слова: *Triticum aestivum L., кальций, оксид азота, фермент, подобный NO-синтазе животных*