The intensity of drought-induced oxidative processes in soybeans depends on symbiosis with *Bradyrhizobium* strains

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ABSTRACT

The intensity of drought-induced oxidative processes by the degree of superoxide anion radical generation and the activity of NADPH oxidase in soybean associated with different activity and virulence of *Bradyrhizobium* strains were studied. The importance of active *Bradyrhizobium japonicum* for the formation of effective symbiosis with soybean to improve the realization of the adaptive potential of plants during drought and post-stress period has been established. Increased NADPH oxidase activity in nodules (by 3.5 – 4.7 times), as well as excessive production of superoxide radical in nodules (by 1.5 – 2 times) and roots (from 113 to 323%) of soybean in symbiosis with efficient *Bradyrhizobium* strains were observed during drought. However, the development of oxidative processes in effective soybean-rhizobial symbioses slowed down after exposure to stress. This indicates the adaptation of soybean plants associated with active *Bradyrhizobium* strains to growing conditions. In symbiotic systems with ineffective *Bradyrhizobium* strains, a significant increase in NADPH oxidase activity in nodules (by 3.3 – 6.0 times) and intensification of superoxide radical formation in nodules (by 2 – 3 times) and roots (from 260 to 330%) recorded both under drought and post-stress period. This indicates a significant development of oxidative processes and the inability to realize the adaptive potential of soybean plants in symbiosis with ineffective *Bradyrhizobium* strains. Thus, excessive formation of the superoxide radical and an increase in the activity of NADPH oxidase indicate the appearance of oxidative processes, which are a typical reaction in response to drought in soybean-rhizobial symbioses of varying efficiency. At the same time, the development of oxidative processes depends on the ability of soybean plants in symbiosis with *Bradyrhizobium japonicum* to realize their adaptive potential in during drought and post-stress period.

Keywords: *Glycine max* (L.) Merr., *Bradyrhizobium japonicum*, strain, NADPH oxidase, superoxide anion radical, drought

INTRODUCTION

The study of molecular mechanisms in plant resistance to drought is one of the important problems today with climate change. Its solution will help to improve existing and develop new ways to increase the realization of adaptive potential and tolerance of plants in drought conditions.

Drought is one of the most spread stress factors that has a negative impact on all systems of plant functioning, including the formation of mutualistic symbioses (Dong et al., 2019, Nadeem et al., 2019). This initiates a cascade of biochemical reactions leading to specific rearrangements of plant metabolism (Guo et al., 2018). The signal for the development of these changes is a violation of the optimal balance of prooxidant-antioxidant status due to increased levels of reactive oxygen species (ROS) production in plant cells (Singh et al., 2019). As a result, there is a rapid increase in intracellular ROS levels, inducing an "oxidative burst" (Hasanuzzaman et al., 2020).

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ROS generation occurs in various cell compartments, where they are used to transduce signals into the cell nucleus and induce reprogramming of cell function, including gene expression (Mhamdi and Breusegem, 2018). Therefore, their formation is a complex process that requires clear biochemical control involving enzymatic and non-enzymatic detoxification mechanisms in plants (Laxa et al., 2019). The main processes of ROS formation are direct photoreduction (photoreduction) of the oxygen molecule to the superoxide anion radical in photorespiration and respiration reactions in chloroplasts involving Rubisco and glycolatoxidase, as well as catalase and peroxidase reactions in peroxisomes (Noctor et al., 2018).

ROS affect the plant response to stresses in two ways (Mittler et al., 2017). They react with a large number of biomolecules and can cause irreversible damage that leads to tissue necrosis and plant death. At the same time, ROS affect the expression of a number of genes and signal transduction pathways, so they can be specific indicators of the environment and at the same time biological signals that activate various programs of genetic responses to stress.

It was found that the accumulation of ROS can activate various mitogen-activated protein kinase signaling cascades (MAPKs), in particular, MAPKKK MEKK1, MPK4 and MPK6 (Takahashi et al., 2011). Although the mechanisms of this activation are unknown, ROS-induced MAPK is probably the main response of cells to stress.

The enzymes responsible for the formation of the whole spectrum of ROS in plants have not been thoroughly studied. The effect of adverse factors on plants is primarily associated with increased NADPH oxidase activity. This enzyme is involved in the reduction of molecular oxygen with the formation of superoxide anion radical with the participation of NADPH (Khafi et al., 2020). The important role of NADPH-oxidase enzyme complex in the generation of ROS, as well as in the regulation of protective reactions of plant cells is known (Marino et al., 2011; Yu et al., 2020). In particular, NADPH oxidase is the main generator of ROS in plant cells under

the action of pathogenic bacteria and the development of hypersensitivity reactions and systemically acquired resistance (Segal and Wilson, 2018). In contrast to the pathogenic effect on the plant organism, the participation of NADPH oxidase in legume-rhizobial symbiosis has not been definitively studied.

ROS have been shown to play an important role in signaling processes in establishing bean-rhizobial symbiosis (Berrabah et al., 2015; Nadeem et al., 2020). This interaction involves complex molecular signaling between the macrosymbiont (host plant) and the microsymbiont (nodule bacteria), which leads to infection of root hairs and the formation of nitrogen-fixing nodules (Wang et al., 2018). It is believed that rhizobial infection of legumes significantly modifies the metabolism of the host plant (Gourion et al., 2015), which determines the effectiveness of the formed symbiotic system under appropriate growing conditions.

Thus, one of the mechanisms of stressors on plants is excessive production of ROS, which can lead to damage and death of plant cells. At the same time they can induce the activation of protective systems and the development of plant tolerance to stress. Research in this field is promising for the involvement of ROS in the regulation and maintenance of plant cell homeostasis in symbiotic relationships legumes with nodule bacteria under the action of stressors, including drought. This may be important for the creation of effective soybean symbioses with *Bradyrhizobium japonicum* that will be able to maximize their adaptive and symbiotic potential under drought conditions.

The aim of the study was to investigate the intensity of drought-induced oxidative processes by the degree of superoxide anion radical generation and NADPH oxidase activity in soybeans associated with *Bradyrhizobium* strains of different activity and virulence. To establish the importance of active *Bradyrhizobium* strains for the formation of effective symbiosis with soybeans to improve the realization of plants adaptive potential under drought conditions and post-stress.

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MATERIALS AND METHODS

Plant material and drought modelling

The studies were conducted under controlled vegetation conditions using soybeans plants (*Glycine max* (L.) Merr.) of Almaz variety – an early ripening variety, recommended for cultivation in the Forest-Steppe of Ukraine, obtained by hybridization of a Moldavian line 3/86 and a Swedish variety Fiskeby 840-5-3 at the Poltava State Agrarian Academy (Biliavska, 2007).

Prior to sowing, the soybean seeds were inoculated with suspension of rhizobia for one hour. The culture of slow-growing nodule bacteria was cultivated on mannitol-yeast medium for 9 days at 26–28 °C (the titer of bacteria was 10^8 cells in 1 ml). The inoculation titre was 200–300 thousand cells of rhizobia per one seed.

The work was done using *Bradyrhizobium* strains of different efficiency: 646 (active, virulent), B1-20 (active, virulent), 107 (inactive, virulent), 604k (inactive, highly virulent) from the museum collection of nitrogen-fixing microorganisms at the Institute of Plant Physiology and Genetics, the NAS of Ukraine. Inactive highly virulent strain *B. japonicum* 604k is a mutant, isolated from the nodule while passaging strain 604k through soybeans plants in the high radiation area – "cesium spot" which lost its ability of effective symbiosis (Tolkachev et al, 1995). Active virulent strain *B. japonicum* 646 was isolated from the soybean nodule by analytic selection. Active virulent Tn5-mutant B1-20 created by transposon mutagenesis involving Esherichia coli S17-1 with plasmid pSUP5011, which contains transposon Tn5 (pSUP5011 :: Tn5mob). Inactive virulent Tn5-mutant 107 created from the original strain 646 by transposon mutagenesis.

The plants were cultivated in pots on sandy medium with the addition of Herligel nutrient solution (0.25 nitrogen norm) under natural illumination and optimal water supply. Drought stress treatments were measured and applied based on the field capacity (FC). The plants were exposed to three water regimes – 60% FC as control (optimal water supply), 40% FC (moderate drought) and 30% FC (severe drought). Drought was created by stopping watering of plants for 10 days in the stages of the third true leaf (40% FC) and budding (30% FC). Watering of plants was resumed (60% FC) in the flowering stage.

Extraction and measurement of NADPH oxidase

To obtain an enzyme extract, a weighed portion of plant material in a 1:3 ratio was homogenized with chilled 50 mM sodium-phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1% Triton X-100, 0.04% sodium thiosulfate and 1% PVP. The samples were incubated on ice for 30 minutes, then centrifuged at 10,000 rpm (4 °C) for 20 minutes. The supernatant was used to determine the activity of NADPH oxidase (EC 1.6.99.9) by the oxidation of NADPH (Pinton et al., 1994). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 1 mM sodium azide, 0.12 mM NADPH. The reaction was initiated by adding 100 µl of supernatant. The rate of NADPH oxidation was measured using a spectrophotometer (Shimadzu UV-1900, Japan) at 340 nm for 5 min and calculated from the extinction coefficient (ε=6,2/mM/cm). The content of total soluble protein in the enzyme extract was determined according to Bradford (1976).

Determination of superoxide anion radical

The generation of the superoxide radical was determined using the Shoring method (Shoring, 2000), based on the reduction of nitro blue tetrazolium (NTB) upon interaction with superoxide radicals. A sample of plant material was placed in tubes with 0.1 M phosphate buffer (pH 7.6) containing 0.05% NTB, 0.1 mM EDTA, 0.1% Triton X-100. The samples were incubated on a shaker (120 rpm) for an hour. After this the optical density of the incubation solution was determined at 530 nm using a spectrophotometer (Shimadzu UV-1900, Japan). To check the specificity of the generation of superoxide radicals in special experiments, superoxide dismutase (50 U/ml) was added to the samples. Superoxide dismutase inhibits the generation of superoxide molecules at least 90%. In this connection, it was believed that the amount of reduced by NTB is determined by the amount of superoxide molecules formed.

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Data analysis

Data analysis was performed using Statistica 6.0 (StatSoft Inc., USA, 2001). The data are presented in the figures as x ± SD (x ± standard deviation). Differences between values in the control and experimental groups were determined using the Tukey test, where they were considered significant at $P<0.05$ (taking into account the Bonferroni correction).

RESULTS AND DISCUSSION

ROS are products of normal aerobic respiration resulting from a specific free radical oxidation process (Slesak et al., 2007). The primary radical formed is the superoxide anion radical, the production of which is carried out by enzymatic and non-enzymatic pathways with the participation of specialized and non-specialized systems (Khafi et al, 2020). The only specialized enzymatic system is NADPH oxidase, whose main function is to generate ROS (Zhu et al., 2018). NADPH oxidase is a member of a large family of NOX proteins homologous to phagocyte oxidase encoded by the *Rboh* (*Respiratory Burst Oxidase Homologues*) genes (Kim et al, 2019). NOX protein is a transmembrane redox chain that allows the electron donor NADPH to interact on the cytoplasmic side of the membrane with the electron acceptor – oxygen on its outer side, accompanied by the formation of superoxide (Khafi et al, 2020).

At optimum cultivation of plants in the third true leaf stage, no differences in the levels of NADPH oxidase activity in soybean nodules inoculated with different *Bradyrhizobium* strains were detected (Figure 1). In the budding stage, an increase in the total level of NADPH oxidase in soybean nodules was recorded, compared to the previous stage of ontogenesis. This is especially noticeable in ineffective symbiosis of soybeans with *Bradyrhizobium* strain 604k and Tn5 mutant 107. In optimal conditions for growing plants in the flowering stage, the highest NADPH oxidase activity was detected in soybean nodules formed by ineffective *Bradyrhizobium* Tn5 mutant 107, compared to other symbiotic systems (Figure 1).

NADPH oxidase has been shown to play an important role in the generation of ROS and the regulation of protective responses of plant cells (Yu et al, 2020), as well as in the formation of legume-rhizobial symbiosis (Lohar et al, 2007). Thus, one hour after treatment of *Medicago truncatula* roots with Nod-factor, a decrease in ROS production was observed, which was accompanied by inhibition of the expression of *MtRbohs* genes encoding NADPH oxidase (Lang and Long, 2015). In addition, the deterioration of *MtRbohA* gene expression in nodules by RNA interference methods led to a decrease in biological nitrogen fixation (Marino et al, 2012).
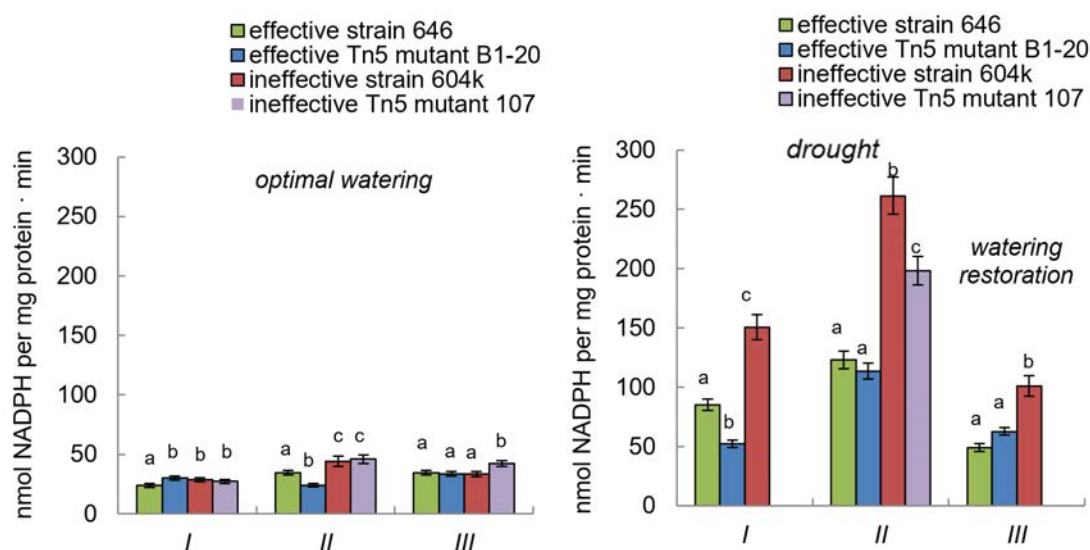
Stages of ontogenesis: *I – the third true leaf, II – budding, III – flowering*; (x ± SD, n = 6)

Figure 1. NADPH oxidase activity in soybean nodules associated with different activity and virulence of *Bradyrhizobium* strains

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Prolonged drought in the third true leaf and budding stages induced an increase in NADPH oxidase activity by 3.5 times in soybean nodules formed by effective *Bradyrhizobium* strain 646 (Figure 1). NADPH oxidase activity increased during exposure to stress in soybean nodules inoculated with effective *Bradyrhizobium* Tn5 mutant B1-20. In particular, in moderate drought there was an increase of enzyme activity in nodules by 1.7 times, while in prolonged dehydration – by 4.7 times (Figure 1).

In soybean nodules formed by inefficient *Bradyrhizobium* strain 604k was recorded intensification of NADPH oxidase activity by 5.2–6.0 times under the action of drought in the third true leaf and budding stages (Figure 1). Prolonged exposure to drought revealed an increase in NADPH oxidase activity in soybean nodules associated with ineffective *Bradyrhizobium* Tn5 mutant 107 by 4.3 times (Figure 1).

After resumption of watering plants to the control level (60% of FC) there was a slight increase in the activity of NADPH oxidase in soybean nodules inoculated with effective *Bradyrhizobium* strain 646. Whereas the activity of the enzyme increased by 1.8 times after stress in soybean nodules formed by effective *Bradyrhizobium* Tn5 mutant B1-20 (Figure 1). In the post-stress period, the highest NADPH oxidase activity was recorded in soybean nodules associated with ineffective *Bradyrhizobium* strain 604k. It was three times higher than control (60% FC) (Figure 1). The soybean symbiotic system with inefficient *Bradyrhizobium* Tn5 mutant 107 proved to be very sensitive to drought. This manifested itself in considerable depression of the nodulation process (Mamenko et al., 2018) and limited the collection of sufficient nodules for research in the third true leaf and flowering stages.

Despite their toxic potential, ROS are key components of signal transduction pathways that trigger stress responses (Czarnocka and Karpiński, 2018; Singh et al, 2019), are involved in plant development (Hasanuzzaman et al., 2020) and plant microbial interactions (Nanda et al., 2010; Segal and Wilson, 2018).

With the optimal water supply of plants in the third true leaf stage, the lowest level of superoxide radical production was found in soybean nodules infected with ineffective *Bradyrhizobium* Tn5 mutant 107. Whereas during the budding stage of soybeans the lowest level of its production was recorded in nodules formed by ineffective *Bradyrhizobium* strain 604k (Figure 2). In the flowering stage of plants, an increase in the overall level of superoxide generation in soybean nodules was observed in all studied symbioses. This could be due to ontogenetic changes in metabolism through the transition to the generative stage of plant growth and development.

ROS, such as superoxide anion radical and hydrogen peroxide, were detected in the early stages of symbiotic relationships legumes with nodule bacteria (Nanda et al., 2010; Tsyganov and Tsyganova, 2020). They have been found in infectious filaments that are invasive plant inclusions that allow bacteria to invade cortical cells (Miyahara et al., 2010; Oldroyd et al., 2011). The production of ROS, in particular superoxide radical and hydrogen peroxide, was observed in infectious filaments and nodules of alfalfa (*Medicago truncatula*), peas (*Pisum sativum* L.) and legume *Sesbania rostrata* (Santos et al., 2000; D'Haeze et al., 2003; Rubio et al., 2004). In the later stages of the symbiotic relationships, a high concentration of ROS in the nodules is generated, which is due to the increased level of respiration intensity and autooxidation of leghemoglobin (Matamoros et al, 2003). It is believed that ROS play an important role during infection, nodule development, active nitrogen fixation, and also during nodule aging (Hérouart et al., 2002; Puppo et al., 2005; Oldroyd and Downie, 2013).

It was found that ROS are necessary for the optimal formation of legume-rhizobial symbiosis, while increasing the generation of ROS can play a dual role: to suppress the protective reactions of plants in the penetration of rhizobia and simultaneously activate its defense mechanisms under unfavorable conditions for symbiosis (Schwem et al., 2019; Laxa et al., 2019).

In soybean nodules associated with effective *Bradyrhizobium* strain 646 the superoxide anion

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radical production increased by 2 times at moderate drought whereas with prolonged dehydration – by 1.5 times (Figure 2). An effective symbiotic system with *Bradyrhizobium* Tn5 mutant B1-20 recorded an increase in the generation of superoxide radical in nodules by 1.9 times during prolonged drought (Figure 2).

When exposed to drought, an increase in the superoxide anion radical generation by 3 times was determined in soybean nodules associated with the *Bradyrhizobium* strain 604k (Figure 2). During dehydration the production of the superoxide anion radical increases twice in soybean nodules inoculated with ineffective *Bradyrhizobium* Tn5 mutant 107 (Figure 2).

After watering the plants to the control level (60% FC) there is a decrease in the production of superoxide radical in soybean nodules formed by effective *Bradyrhizobium* strain 646 and Tn5 mutant B1-20. In symbiotic system with inefficient *Bradyrhizobium* strain 604k, the production of superoxide anion radical in soybean nodules increased by 59.1% after resumption of plant watering (Figure 2).

Modulation of macrosymbiont (legume) metabolism under the influence of microsymbiont (rhizobia) is accompanied by increased ROS production, which indicates a hypersensitive response of cells (Wang et al., 2018). Nodule bacteria are more sensitive to ROS than other species of bacteria and, as symbiotic

microorganisms, can use the protective reactions of the host plant against toxic forms of oxygen (Glyanko, 2016). In the formation of legume-rhizobial symbiosis, ROS can be involved in the regulation of rhizobia root infection both by direct antibacterial action and by regulating the functional activity of the host plants defense systems (Vasilyeva et al., 2007).
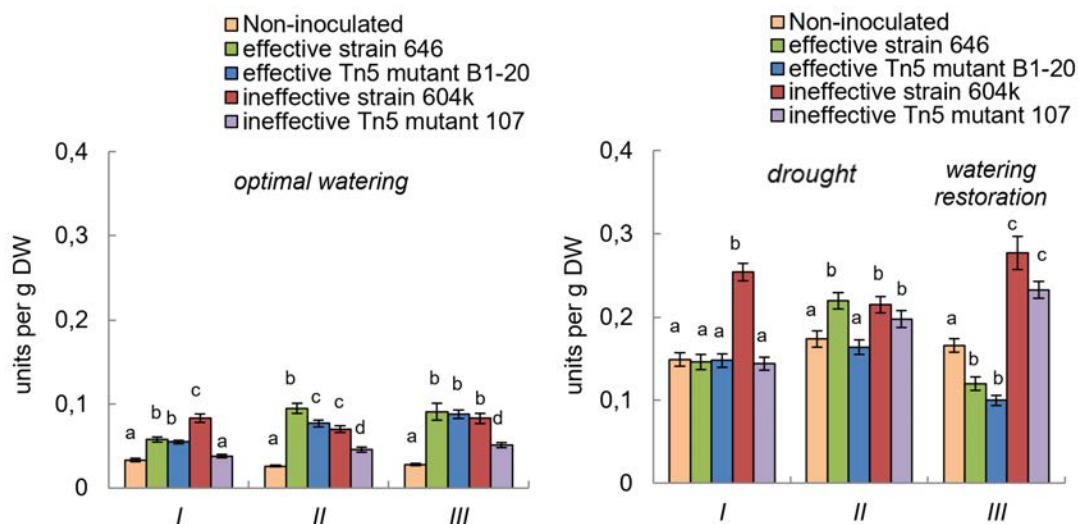
It was found that the inoculation of soybean seeds with rhizobia leads to an increase in the total level of superoxide radical production in plant roots under optimal growing conditions. This is evidenced by data indicating higher levels of superoxide radical generation in roots of soybean symbiosis with different activity and virulence of *Bradyrhizobium japonicum* (Figure 3). The highest level of superoxide production was recorded in soybean roots associated with effective *Bradyrhizobium* strain 646 and Tn5 mutant B1-20 during the budding and flowering stages of plant development. This indicates changes in plant metabolism induced by rhizobia invasion, which lead to higher levels of superoxide radical production in soybean roots under optimal growing conditions.

It is investigated that the drought induces excessive production of superoxide anion radical by 351.5 – 569.2% in soybean roots without seeds bacterization of rhizobia (Figure 3). In soybeans associated with effective *Bradyrhizobium* strain 646 recorded an increase
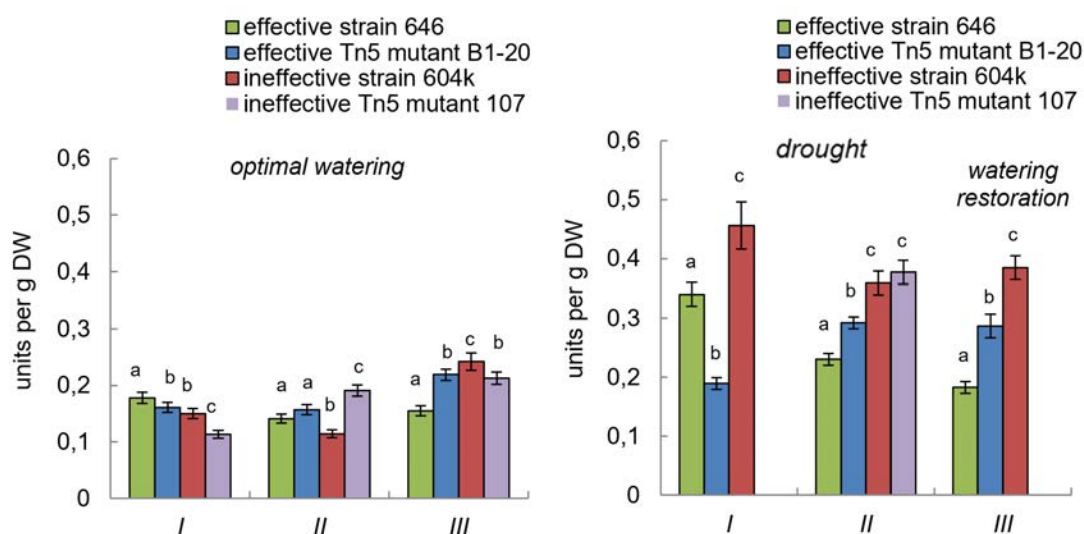
Stages of ontogenesis: *I* – the third true leaf, *II* – budding, *III* – flowering; (x ± SD, n = 6)

Figure 2. Superoxide generation in soybean nodules associated with different activity and virulence of *Bradyrhizobium* strains

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Stages of ontogenesis: *I* – the third true leaf, *II* – budding, *III* – flowering; (x ± SD, n = 6)

Figure 3. Superoxide generation in soybean roots without the use of bacterization and associated with different *Bradyrhizobium* strains

in the formation of superoxide anion radical in the roots by 204.2% and 131.5% at moderate and prolonged dehydration, respectively (Figure 3). In soybeans inoculated with effective *Bradyrhizobium* Tn5 mutant B1-20 recorded an increase in the generation of superoxide radical in the roots by 322.8% and 112.9% during drought (Figure 3).

In an inefficient symbiotic system with the *Bradyrhizobium* strain 604k, an increase in the production of superoxide radical in the roots by 206.1 – 258.3% was detected during drought (Figure 3). Whereas in soybeans inoculated with inactive *Bradyrhizobium* Tn5 mutant 107 there was an increase in the production of superoxide radical in the roots by 278.9 – 330.4% during drought (Figure 3).

After watering plants to the optimal level (60% FC), a decrease in superoxide radical generation in roots was recorded in soybean symbiosis with effective *Bradyrhizobium* strain 646 and Tn5 mutant B1-20 (Figure 3). In symbiotic systems with ineffective *Bradyrhizobium* strain 604k and Tn5 mutant 107, as well as in soybeans without the use of bacterization, an increased level of superoxide radical production was observed in the roots of the post-stress period (Figure 3).

Research today focuses on aspects of the role of ROS, such as the dynamics and specificity of their signaling inside and outside cells, and the transduction of these signals in the plants (Mittler et al, 2017). Despite the widespread recognition of the role of ROS as regulatory and signaling molecules in plant cells, it remains unclear how exactly these signals are perceived, transmitted and elicit a response (Mittler et al, 2017). Research on the involvement of ROS in the formation and functioning of symbiotic relationships legumes with nodule bacteria in drought conditions is of great scientific interest. This field is not well understood and requires further research.

CONCLUSIONS

Soybean symbiotic systems with inefficient *Bradyrhizobium* strain 604k and Tn5 mutant 107 are characterized by increased activity of NADPH oxidase in nodules and excessive of superoxide anion radical generation in nodules and roots during drought and post-stress. This indicates a significant development of oxidative processes induced by drought in inefficient soybean-rhizobial symbioses, which are unable to adapt to growing conditions even after stress.

In soybeans symbiotic systems with effective *Bradyrhizobium* strain 646 and Tn5 mutant B1-20 increase in oxidative processes in nodules and roots was detected during prolonged drought, the development of which slows down after the resumption of plant watering. Effective soybean-rhizobial symbioses with active *Bradyrhizobium* strains can be used as an alternative means to provide plants with environmentally friendly sources of nitrogen, which at the same time will increase plant tolerance to drought. This is important for preserving and reproducing the quality of the environment.

Excessive formation of the superoxide radical and an increase in the activity of NADPH oxidase indicate the appearance of oxidative processes, which are a typical reaction in response to drought in soybean-rhizobial symbioses of varying efficiency. At the same time, the development of oxidative processes depends on the ability of soybean plants in symbiosis with *Bradyrhizobium japonicum* to realize their adaptive potential in during drought and post-stress period.

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