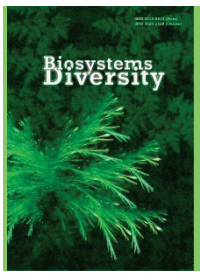


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# Key role of phenol enzymes metabolism in the legume-rhizobial symbiosis under different water supply regimes

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The legume-rhizobium interaction induces formation of specific reactions that take metabolism in the host plant up to a new functional level, increasing its tolerance to unfavourable cultivation conditions. Our objective was to study the participation of key enzymes – phenylalanine ammonia lyase, guaiacol peroxidase, and polyphenol oxidases – in the phenol-metabolism processes and synthesis of a broad spectrum of secondary metabolites in soybean plants that have established symbiotic interactions with rhizobia of varying effectiveness during optimal and insufficient water supplies. In our studies, we used symbiotic systems of soybean and rhizobia (*Bradyrhizobium japonicum*) that varied in efficiency and virulence. In the period of active nitrogen fixation by soybean, from the third-true-leaf stage until budding, we created different water-supply regimes for the plants, including optimal watering at the level of 60% of full field capacity (control) and insufficient, at the level of 30% (drought). When the soybean was flowering, we recovered the optimal level of water supply (resumed watering). In the studies, we employed microbiological, biochemical, and physiological approaches. We determined the specificity of how key enzymes of the phenol metabolism such as phenylalanine ammonia lyase, polyphenol oxidase and guaiacol peroxidase in the nodules, roots, and leaves of the soybean reacted to different levels of water supply, depending on the functional efficiency of the symbiotic system involving strains of *B. japonicum*, varying in effectiveness and virulence. In the effective soybean-rhizobium symbiosis, there occurred insignificant changes in the activity of phenol-metabolism enzymes in the nodules, roots, and leaves during drought and after action of the stress. This evidence is that in symbiosis with effective rhizobia B1-20, soybean could realize its own defensive systems that regulate optimal functioning of phenol metabolism in dehydration conditions. In the low-effective 107 and ineffective 604k symbiotic systems of soybean, there was observed unstable dynamics of the activity of enzymes in leaves and roots, manifested in intensification or inhibition of their activity levels during drought or post-stress period. This indicates malfunctioning of the processes associated with phenol metabolism in the soybean plants. We concluded that tolerance of legume-rhizobium symbiosis to water deprivation depends on mutual involvements of the both symbiotic partners – host plant and rhizobia, their ability to fully realize the defensive systems for activation of the key enzymatic complexes taking part in regulation of phenol metabolism in plants.

*Keywords*: *Glycine max* (L.) Merr.; *Bradyrhizobium japonicum*; polyphenol oxidases; guaiacol peroxidase; phenylalanine ammonia lyase; symbiotic system; strain.

## Introduction

Secondary phenol metabolites play an important role in the quality of plant-derived foods, because they affect characteristics such as appearance, taste, and beneficial qualities. Their content in foods depends on many factors that cause disturbances in phenol stability, their biosynthesis, and breakdown (Xu et al., 2021). From the perspective of their biosynthesis, an especially important key enzyme is phenylalanine ammonia lyase, which can be induced by various stressful (ecological) conditions (Jun et al., 2018; Gho et al., 2019; Fan et al., 2022). Furthermore, polyphenol oxidases and peroxidases are the main enzymes responsible for loss of quality due to phenol degradation (Araji et al., 2014; Omiadze et al., 2018; Chen & Vierling, 2022). The literature considers various factors that have effects on quality of food products, which are related to changes in phenol composition in plants (Dicko et al., 2006). They include internal (genetic) and ecological (agronomic) factors, technological processing used while harvesting fruits and vegetables, and also treatment and storage of the processed products (Tomás-Barberán & Espín, 2001). Researchers consider various phenol-related strategies to support or increase the quality of food products (Chon, 2013; Cosme et al., 2020 Singh & Yadav, 2022).

One of the most broadly cultivated and used oil crops is soybean. Its use varies from food products for people to food for animals, industrially-made products, and ingredients to precursor materials (Gaonkar & Rosentrater, 2019). Currently, soybean is grown on around 119 M ha around the world, with the total annual production accounting for 319 M t (www.fao.org). The global production of soybean has increased from 155.1 M metric tons in 1999 to 284 M metric tons in 2013 (www.soystats.com). At the same time, the share of soybean in the global production of oil crops equals around 55%, and over recent 10 years, its production has been increasing by over 5% per year. Soybeans comprise the greatest share of production among all oil crops (53%), whereas rape, cotton, and peanut account only for 15%, 10%, and 9%, respectively.

Legumes (Fabaceae) are able to form symbiotic relations with nodule bacteria (Rhizobiaceae) that fixate atmosphere nitrogen as organic compounds. This fixation occurs during vital periods of growth of plants, supporting the development of stable and ecologically stable crops (Wang et al., 2018; Khatun et al., 2021). Such a biological fixation is a natural source of nitrogen for plants, which poses no threat to ecological processes in the environment (Raza et al., 2019; Sousa et al., 2022). Therefore, the development of effective legume-rhizobium symbiotic systems with active strains of nodule bacteria can play an important role in increasing the effectiveness of nitrogen fixation by plants, and also in formation of their tolerance to implications of the climate change.

Our studies were focused on participation of the key enzymes of phenol metabolism in providing optimal functioning of legume-rhizobium symbiosis in optimal conditions of cultivation of plants and drought. Phe-

nolic compounds function as signal inductors of virulence genes during interaction between micro- and macrosymbionts (Mamenko, 2021). At the same time, metabolism of a macrosymbiont (legume plant) is modulated under influence of the microsymbiont (rhizobia). This manifests in accumulation of active oxygen species and indicates oversensitive response of the plant cells. It is believed that rhizobial infection of legumes substantially modifies metabolism of a host plant, creating optimal conditions for the infection and nodulation (Hawkins & Oresnik, 2021) and can be a precondition for further effective functioning of the legume-rhizobium symbiosis.

Phenylalanine ammonia lyase (EC 4.3.1.5) is a key enzyme in the reaction of plants to biotic stress, including the rhizobacteria-induced systemic resistance (Tonelli et al., 2021). Expression of the gene of phenylalanine ammonia lyase has been demonstrated to be activated by the ethylene / jasmonate signal pathway, which leads to development of rhizobacteria-induced systemic resistance. Expression of the gene and activity of phenylalanine ammonia lyase enzyme can be considered as markers of this defensive function (Jun et al., 2018). This enzyme is important for biosynthesis of a broad spectrum of secondary metabolites: polyphenols, phenylpropanoids, lignin monomers, salicylic acid, and other compounds necessary for the development of plants and their protection from external factors (Zhang & Liu, 2015).

In the literature, the peroxidase reaction is considered a response to rhizobia entering the plant cells (Pandey et al., 2017). At the same time, it is believed that peroxidases can provide enough information about the physiological condition of a plant and be a criterion of resistibility to stress factors of biotic and abiotic nature (Minibayeva et al., 2015; Nogales, 2021). This is so-called class III of peroxidases that use phenol derivatives as substrates. They are also called non-specific, or classic, which are in the cytoplasm vacuoles. They are involved in a large number of processes in the plant cells, in particular lignifications, suberization, auxin catabolism, protection from pathogens and oxidative stress. They include – among others – guaiacol peroxidase (POD) (EC 1.11.1.7) (Almagro et al., 2009).

Polyphenol oxidases (EC 1.10.3.1) is one of enzymes participating in the defense of plants against pathogens and abiotic-stress factors. According to a number of authors, increased expression of genes of polyphenol oxidase when subject to stress factors is directly associated with resistibility of the plants (Taranto et al., 2017; Nogales, 2021). At the same time, studies of activity of polyphenol oxidase in the root nodules of legumes have suggested possible relationship between the enzyme and virulent properties of rhizobia (McCormick, 2018).

Formation of legume-rhizobium interaction is accompanied by a coordinated participation of the both partners, macro- and microsymbionts, which exchange specific signals, thereby activating a complex of molecular processes and the main symbiotic pathways in the host plant, while inhibiting its defensive systems (Hawkins & Oresnik, 2021; Mamenko, 2021). Aspects of molecular interaction of symbiotic partners at the initial stages of interaction are generally well known (Gourion et al., 2015; Mamenko et al., 2019). Of great importance are complex studies accounting for specificity of the formation of legume-rhizobium symbiosis and its functioning at late stages when a host plant has high demand for nitrogen, which is satisfied by a successful symbiotic interaction with the strains of rhizobia. A relevant topic to study is the stress-tolerance of legume-rhizobium symbiosis, i.e. its ability to realize its own protective systems, including activation of enzymatic complexes to regulate optimal functioning of metabolism and adaptation to action of stress factors, including drought. In this regard, special attention should be paid to enzymes that partake in metabolic processes to provide synthesis of secondary phenol metabolites as protective compounds of the plant tissue. The objective of this study was to examine the participation of key enzymes – phenylalanine ammonia lyase, guaiacol peroxidase, and polyphenol oxidases – that are involved in phenol metabolism and synthesis of a broad spectrum of metabolites in soybean plants during formation of symbiotic interaction with rhizobia with various efficiency during optimal and insufficient water supply.

## Materials and methods

The objects of the study were varying-efficiency symbiotic systems of soybean (*Glycine max* (L.) and rhizobia bacteria (*Bradyrhizobium japonicum*): an effective symbiotic system with active virulent rhizobia B1-20; a less-effective one, with participation of low-active virulent rhizobia 107; and an ineffective one, involving inactive, highly virulent rhizobia 604k (Fig. 1). In the study, we used *B. japonicum* from the museum collection of nitrogen-fixating microorganisms of the Department of Symbiotic Nitrogen Fixation of the Institute of Plant Physiology and Genetic of the National Academy of Sciences (NAS) of Ukraine.

Prior to sowing, we sterialized the seeds with 70% ethanol and rinsed them in tap water for 1 h. Then, we inoculated them with suspensions of nodule bacteria (titer $10^8$ cells in 1 mL). The culture of slowly growing nodule bacteria was grown on mannitol-yeast environment for 7 days at 26–28 °C. The bacteria were cultivated in Erlenmeyer's flasks containing 200 mL of growth medium using the method of periodic incubation on shaking orbital incubator at 220 rpm velocity. The inoculation material was introduced to laboratory flasks in the concentration of 2% of the growth medium. The suspension that was introduced to the growth medium contained rhizobia in the amount of $10^8$ cells/mL. Purity of the rhizobium cultures was checked by inoculating them on meat-peptone agar, on which rhizobia do not grow.

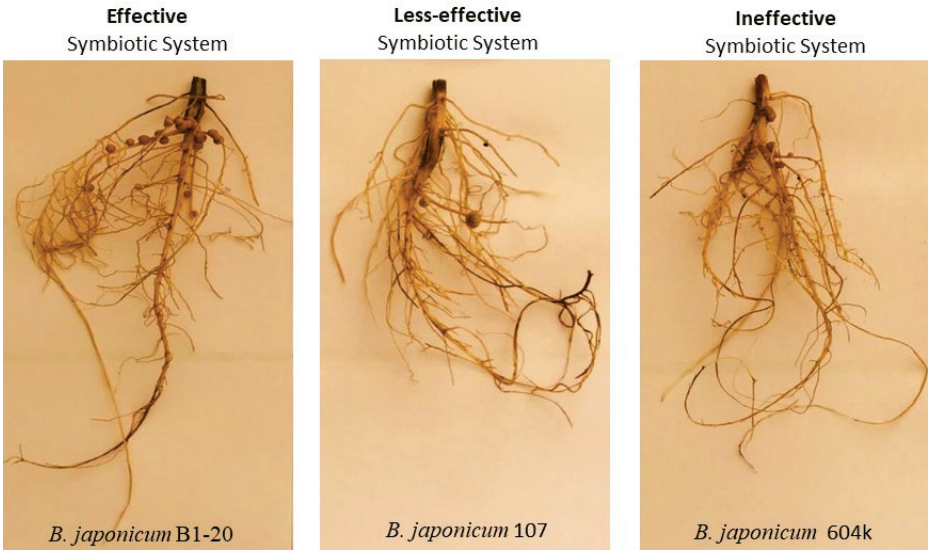


**Fig. 1.** Formation of nodules on the roots of soybean in different-efficiency symbiotic systems during optimal watering in the flower-bud stage

The plants were grown in 10 kg containers on a sterile substrate (sand) with introduction of a nutrition mixture of Herligel (0.25 of the nitrogen norm) in the conditions of natural light and optimal water supply (60% of full field capacity, FFC, Fig. 2). From the stage of third true leaf to the flower-bud stage, we subjected the plants to various regimes of watering for 12 days: optimal water supply (60% of FFC) – control,

insufficient water supply (30% of the FFC) – drought. During the flowering stage, moisture of the substrate in all containers was adjusted to 60% of FFC by resumption of the watering for 7 days. To conduct the studies, we collected the nodules, roots, and leaves of the soybean when the plants were actively securing nitrogen – stages of the three true leaves, flower bud, and flowering.

**Fig. 2.** Soybean during the stage of third true leaf at the vegetation area of the Institute of Plant Physiology and Genetic of NAS of Ukraine

To obtain enzymatic extract, a weighed plant material (1 : 2) was homogenized with 60 μmol/L of cooled phosphate buffer (pH 7.5), which contained 2 μmol/L of ethylenediaminetetraacetic acid, 1 μmol/L of phenylmethylsulfonyl fluoride, and 5 μmol/L of β-mercaptoethanol and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 ºC. The supernatant was used for identification of enzymes using a UV-1900 two-ray spectrometer (Shimadzu, Japan).

The activity of guaiacol peroxidase was measured according to increase in the optical density at 470 nm for a minute as a result of guaiacol oxidation (extinction coefficient = 26.6 μmol/L*cm) (Egley, 1983). The reactive mixture contained 60 μmol/L of potassium-phosphate buffer (pH 7.0), 0.1 μmol/L of ethylenediaminetetraacetic acid, 0.5% guaiacol, and 100 μmol/L of hydrogen peroxide. The reaction was initiated by adding the supernatant. The results are presented in μmol of oxidated guaiacol per concentration of protein (mg) in the supernatant.

The activity of polyphenol oxidase was measured according to increase in the optical density at 420 nm for a minute in presence of pyrocatechol (Panadare & Rathod, 2018). The reactive mixture contained 60 μmol/L of potassium-phosphate (pH 7.4), 0.1 μmol/L of ethylenediaminetetraacetic acid, and 0.05 M pyrocatechol. The reaction was initiated by addition of the supernatant. The results are presented in units of enzyme activity (units of activity) per concentration of protein (mg) in the supernatant. Content of the general soluble protein in the enzyme extract was determined using the Bradford's method (Bradford, 1976).

To obtain enzymatic extract when determining the activity of phenylalanine ammonia lyase, the plant material was homogenized with 0.2 M solution of borate buffer (pH 8.8) in 1:2 ratio (weight/volume), which contained 1 μmol/L of ethylenediaminetetraacetic acid, 5 μmol/L of β-mercaptoethanol and 1% (weight/volume) polyvinylpyrrolidone (Mamenko et al., 2021). The homogenate was centrifuged at 12,000 rpm for 20 min at 4 ºC. The supernatant was used to measure the activity of phenylalanine ammonia lyase spectrophotometrically at 290 nm according to formation of trans-cinnamic acid in 0.1 M borate buffer (pH 8.8) in presence of 50 μmol/L of L-phenylalanine. The reactive mixture was incubated for 1 h at 40 °C. The results are presented in units of enzyme activity (activity units) per concentration of protein (mg) in the supernatant.

The data were analyzed using the Statistica 6.0 software (www.statsoft. pl/textbook/stathome.html). The data are given in the tables as x ± SD (mean ± standard deviation). The differences between the control and experimental groups were determined using the Tukey test, where the differences were considered significant at $P < 0.05$ (accounting for the Bonferroni's correction).

## Results

In the variant with cultivation in optimal water-supply conditions, during the stages of the third true leaf and flower bud, the highest level of the activity of phenylalanine ammonia lyase was observed in the nodules formed by ineffective highly virulent rhizobia 604k, compared with its activity level in soybean in the effective (*B. japonicum* B1-20) and low-effective (*B. japonicum* 107) symbioses (Table 1).

Long drought induced insignificant increase in the activity of phenylalanine ammonia lyase in the nodules in soybean in the effective symbiotic system with participation of rhizobia B1-20 (Table 1). In the symbiotic systems of soybean formed involving ineffective and low-effective rhizobia, we observed decrease in activity of the enzyme in the nodules subject to drought, measuring 27.0% (*B. japonicum* 604k) and 18.2% (*B. japonicum* 107), compared with the control (60% of full field capacity). After resuming watering of the plants in the flower-bud stage, the activity of phenylalanine ammonia lyase in the nodules of soybean increased up to the control level in all the examined symbiotic systems (Table 1).

**Table 1**
Activity of phenylalanine ammonia lyase in nodules of soybean subject to different water supply (x ± SD, n = 6)

| Variant | Stage of plant development | | |
|---|---|---|---|
| | three true leaves | flower bud | flowering |
| Watering level | optimal watering, 60% of full field capacity (FFC) | | |
| *B. japonicum* B1-20 | 103.4 ± 7.1$^{a}$ | 133.4 ± 9.8$^{a}$ | 183.5 ± 12.1$^{a}$ |
| *B. japonicum* 604k | 193.8 ± 13.4$^{b}$ | 190.3 ± 12.1$^{b}$ | 206.3 ± 15.6$^{b}$ |
| *B. japonicum* 107 | 104.6 ± 11.3$^{a}$ | 133.4 ± 11.9$^{a}$ | 183.2 ± 11.9$^{a}$ |
| Watering level | drought, 30% of FFC | | resumption of watering, 60% of FFC |
| *B. japonicum* B1-20 | 115.1 ± 7.5$^{ab}$ | 159.9 ± 8.2$^{ab}$ | 194.1 ± 13.2$^{a}$ |
| *B. japonicum* 604k | 127.8 ± 8.9$^{ab}$ | 138.9 ± 9.3$^{a}$ | 262.6 ± 18.7$^{c}$ |
| *B. japonicum* 107 | no nodules | 109.2 ± 10.6$^{c}$ | 229.5 ± 22.1$^{bc}$ |

*Note*: different letters indicate values that are significantly different ($P < 0.05$ accounting for the Bonferroni's correction) within one column according to results of the Tukey's test.

In optimal conditions of water supply to the plants, from the third-true-leaf stage to flowering, the highest activity of polyphenol oxidase took place in the root nodules formed by low-effective rhizobia 107, compared with the other symbiotic systems (Table 2).

In the soybean the seeds of which had been inoculated with effective rhizobia Tn5-mutant B1-20, we saw 29.1% increase in the activity of polyphenol oxidase in the nodules during drought and recovery of the enzyme's activity up to the level of control (Table 2). In the ineffective symbiotic system of soybean with rhizobia 604k, we observed significant 4.5-fold increase in the activity of polyphenol oxidase in the nodules during the drought (the stage of three true leaves) and 3.4-fold increase (flower-bud stage) (Table 2). Following inoculation of soybean seeds with low-effective rhizobia 107, the activity of polyphenol oxidase in the root nodules increased by 1.4 times when moisture was in deficit for a long time. After resumption of the optimal watering conditions, the activity of polyphenol oxidase in the soybean nodules in the ineffective and low-effective symbioses was 4.7 times (604k) and 2.2 times (107) higher than the control.

**Table 2**
Activity of polyphenol oxidase in nodules of soybean subject to different water supply (x ± SD, n = 6)

| Variant | Stage of plant development | | |
|---|---|---|---|
| | three true leaves | flower bud | flowering |
| Watering level | optimal watering, 60% of full field capacity (FFC) | | |
| *B. japonicum* B1-20 | 3.54 ± 0.24$^{a}$ | 4.22 ± 0.26$^{a}$ | 5.67 ± 0.36$^{a}$ |
| *B. japonicum* 604k | 4.53 ± 0.32$^{a}$ | 3.35 ± 0.23$^{a}$ | 5.43 ± 0.38$^{a}$ |
| *B. japonicum* 107 | 14.65 ± 1.12$^{b}$ | 13.39 ± 0.86$^{b}$ | 13.21 ± 0.94$^{b}$ |
| Watering level | drought, 30% of FFC | | resumption of watering, 60% of FFC |
| *B. japonicum* B1-20 | 4.28 ± 0.31$^{a}$ | 5.44 ± 0.38$^{ab}$ | 5.61 ± 0.31$^{a}$ |
| *B. japonicum* 604k | 20.26 ± 1.41$^{c}$ | 11.31 ± 0.74$^{b}$ | 25.66 ± 1.71$^{c}$ |
| *B. japonicum* 107 | no nodules | 19.14 ± 0.80$^{c}$ | 29.51 ± 2.11$^{c}$ |

*Note*: see Table 1.

In the optimal watering conditions, during the stages of third true leaf, flower bud, and flowering, we saw the highest activity of guaiacol peroxidase in the root nodules formed by ineffective rhizobia 604k, compared with the other symbiotic systems (Table 3).

During drought, from the stage of the third true leaf to flowering, we observed significant 4.3-fold increase, compared with the control, in the activity of guaiacol peroxidase in the nodules of soybean in the symbiosis with effective rhizobia B1-20. After resumption of watering of the plants, the activity of guaiacol peroxidase in the root nodules of soybean in the effective symbiosis was 2.7 times higher than in the control (Table 3).

In the ineffective symbiotic system, formed with the participation of rhizobia 604k, the activity of guaiacol peroxidase in the root nodules increased during drought by 2.3 (stage of three true leaves) and 1.8 times (flower-bud stage). At the same time, in soybean in the symbiosis with low-effective rhizobia 107, the activity of guaiacol peroxidase in the nodules decreased by 40% during the long drought (Table 3). As the plants were being watered again, the enzyme's activity in the root nodules formed by ineffective and low-effective rhizobia increased, by 159.7% and 62.4%, respectively, compared with the control (60% of the full field capacity).

**Table 3**

Activity of guaiacol peroxidase in the nodules of soybean subject to different water supply (x ± SD, n = 6)

| Variant | Stage of plant development | | |
|---|---|---|---|
| | three true leaves | flowering bud | flowering |
| Watering level | optimal watering, 60% of the full field capacity (FFC) | | |
| *B. japonicum* B1-20 | $0.369 \pm 0.091^{a}$ | $0.263 \pm 0.063^{a}$ | $0.172 \pm 0.042^{a}$ |
| *B. japonicum* 604k | $2.512 \pm 0.223^{b}$ | $2.364 \pm 0.223^{b}$ | $1.541 \pm 0.136^{b}$ |
| *B. japonicum* 107 | $0.264 \pm 0.081^{a}$ | $0.213 \pm 0.052^{a}$ | $0.314 \pm 0.081^{ab}$ |
| Watering level | drought, 30% of FFC | | resumption of watering, 60% of FFC |
| *B. japonicum* B1-20 | $1.071 \pm 0.162^{bc}$ | $1.121 \pm 0.172^{bc}$ | $0.468 \pm 0.121^{ab}$ |
| *B. japonicum* 604k | $5.823 \pm 0.573^{c}$ | $4.362 \pm 0.332^{c}$ | $4.121 \pm 0.233^{c}$ |
| *B. japonicum* 107 | no nodules | $0.128 \pm 0.031^{d}$ | $0.512 \pm 0.081^{ab}$ |

*Note*: see Table 1.

In the optimal conditions of water supply of the plants, starting from the third-true-leaf stage to flowering, soybean in the variant without seed bacterization had the highest activity of polyphenol oxidase in the roots, compared with the variants with rhizobial inoculation of the seeds (Fig. 3a). At the same time, among the studied soybean-rhizobium symbioses, the highest activity of polyphenol oxidase was seen in the soybean roots inoculated with low-effective rhizobia.

We determined that moderate drought in the stage of third true leaf induced no significant changes in the activity of polyphenol oxidase in the roots of soybean that had not been subject to seed bacterization (Fig. 3a, 3b). During long water deprivation, when the plants were in the flower-bud stage, the level of enzyme activity in roots of the non-inoculated soybean plants decreased by 52.1%, compared with its activity in the control (60% of full field capacity). After drought stress in the flowering stage, the activity of polyphenol oxidase in the roots of soybean increased by 48.3%, compared with the roots of the control plants.

In the effective symbiotic system, formed with participation of the soybean plants and strain *B. japonicum* B1-20, we observed 183.8% increase in the activity of polyphenol oxidase in the roots in the conditions of moderate drought stress and 104.9% increase during long dehydration, compared with the control (60% of the full field capacity, Fig. 3a, 3b). After watering of the plants had been resumed, the activity of polyphenol oxidase in the soybean roots remained 105.6% higher than in the control. In the ineffective symbiotic system with participation of highly virulent rhizobia 604k, we detected 146.8% increase in the activity of polyphenol oxidase in the soybean roots during moderate dehydration and 66.5% increase in conditions of drought stress (Fig. 3a, 3b). After resumption of watering of the soybean plants, this symbiosis was observed to have significant increase in the activity of polyphenol oxidase in the roots, measuring 432.7%, compared with the control (60% of the full field capacity). In the soybean inoculated with low-effective rhizobia 107, we found higher, compared with the control (60% of the full field capacity), level of the activity of polyphenol oxidase in the roots during the drought and after resumption of watering, by 148.1% and 106.2%, respectively (Fig. 3a, 3b).

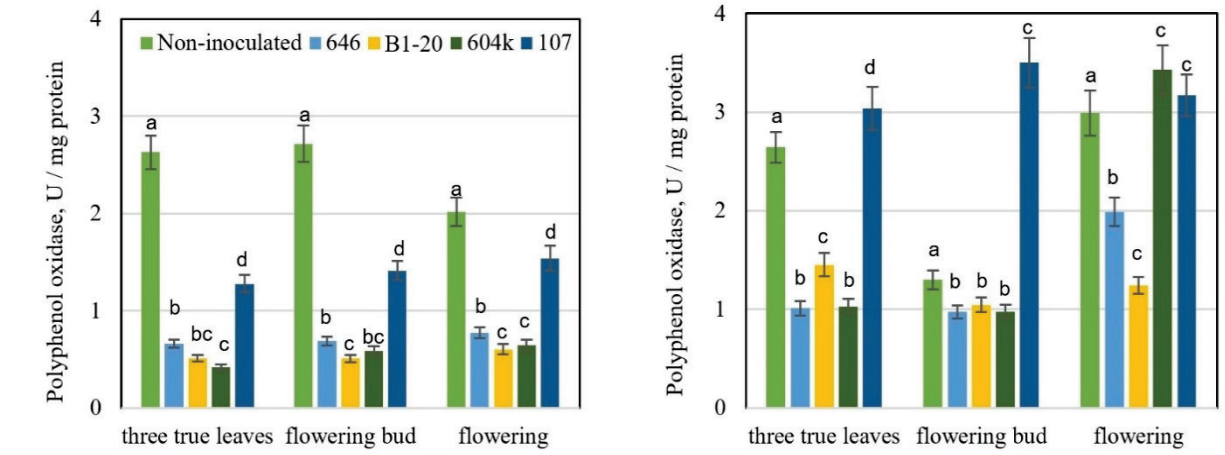


**Fig. 3.** Activity of polyphenol oxidase in the roots of soybean subject to different levels of water supply: *a* – optimal, 60% of full field capacity, *b* –drought, 30% of full field capacity in the stages of three true leaves-flower bud and watering resumption, 60% of full field capacity during flowering (x ± SD, n = 6); different letters indicate values that are significantly different one from another within a diagram column compared with the Tukey test $P < 0.05$ (accounting for the Bonferroni correction); no inoculation; polyphenol oxidase, units of activity/mg of protein; three true leaves; flower bud; flowering

In the optimal conditions of water supply during the flower bud and flowering stages, the highest level of guaiacol-peroxidase activity was detected in the roots of the soybean plants not subject to seed bacterization, compared with the enzyme activity in soybean in the variants using inoculation (Fig. 4a). Among the studied symbiotic systems, soybean the seeds of which had been inoculated with low-effective rhizobia 107 had the highest level of the enzyme activity in the roots in cases of optimal watering in the stage of third true leaf (Fig. 4a). We found that moderate drought in the stage of third true leaf had no effect on the activity of guaiacol peroxidase in soybean roots in the variant with no seed bacterization. At the same time, long drought induced inhibition of the enzyme activity in the roots of non-inoculated soybean, which was 74.8% lower than the enzyme activity in the control-plant roots with optimal watering (Fig. 4a, 4b). After resumption of the watering of the plants, the activity of guaiacol peroxidase in the soybean roots significantly increased by 214.8%, compared with the control plants (60% of the full field capacity).

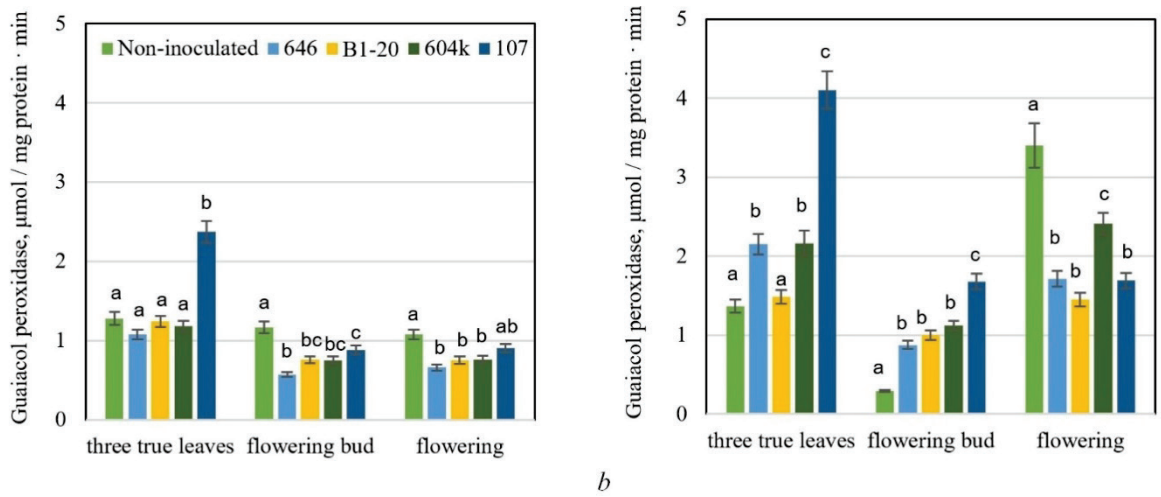


**Fig. 4.** Activity of guaiacol peroxidase in the roots of soybean subject to different levels of water supply: *a* – optimal, 60% of full field capacity, *b* – drought, 30% of full field capacity in the stage of three true leaves-flower bud and watering resumption, 60% of full field capacity during flowering (x ± SD, n = 6); different letters indicate values that are significantly different one from another within one column of the diagram compared with the Tukey test, $P < 0.05$ (accounting for the Bonferroni correction); no inoculation; guaiacol peroxidase, µmol of guaiacol /mg of protein * min; three true leaves; flower bud; flowering

Drought induced insignificant increase in the activity of guaiacol peroxidase in roots of soybean in the symbiosis with effective rhizobia B1-20 (Fig. 4a, 4b). At the same time, in the post-stress period, we saw 92.5% increase in the enzyme activity in the roots, compared with the control (60% of full field capacity). During drought and after resuming watering of the plants, it was found that in the symbiosis of soybean and low-effective rhizobia 107, dynamics of the activity of guaiacol peroxidase in the roots increased by 72.7% and 90.3%, compared with the control (Fig. 4a, 4b). In the ineffective symbiotic system of soybean with strain of *B. japonicum* 604k, we saw 83.0% increase in the enzyme activity in the dehydration conditions and 216.5% increase after stress (resumption of watering, Fig. 4a, 4b).

In optimal watering conditions of soybean, in all variants with inoculation with rhizobia of various effectiveness, we found lower activity of polyphenol oxidase in the leaves, compared with the enzyme activity in the variant with no seed bacterization (Fig. 5a). At the same time, between the examined symbiotic systems of soybean, we found no significant difference in the enzyme activity in leaves in the optimal watering conditions. Long drought induced 50% decrease in the activity of polyphenol oxidase in the leaves of soybean in the variant with no seed bacterization, compared with the enzyme level in the variant with optimal water supply (Fig. 5a, 5b). After the stress, the enzyme activity in the leaves of non-inoculated soybean did not recover up to the optimal level the way it did in the control plants (60% of the full field capacity).

In the symbiotic system of soybean plants and effective rhizobia B1-20, the activity of polyphenol oxidase in the leaves increased by 158.8% during the flower-bud stage as the drought continued (Fig. 5a, 5b). In the post-stress period, the enzyme activity in the soybean leaves was 86.9% higher than in the control (60% of full field capacity). In the non-effective symbiotic system of soybean (*B. japonicum* 604k), we saw significant 4.6-fold increase in enzyme activity in the leaves against the background of long dehydration, the level of which increased 4.9-fold in the post-stress period, compared with the control (Fig. 5a, 5b).

In the soybean inoculated with low-effective rhizobia 107, we observed the activity of polyphenol oxidase in the leaves increased by 81.2% during drought and 313.6% in the flower-bud stage (Fig. 5a, 5b). After resuming watering in the flowering stage, the enzyme activity in the leaves dropped 44.5%, compared with the control (60% of full field capacity).

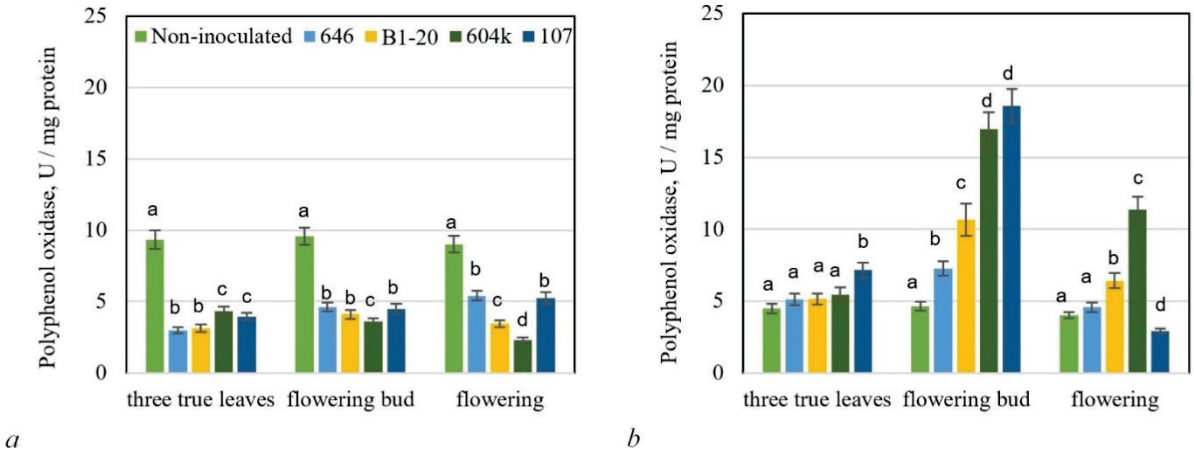


**Fig. 5.** Activity of polyphenol oxidase in the leaves of soybean subject to different levels of water supply: *a* – optimal, 60% of full field capacity, *b* – drought, 30% of full field capacity in the stage of three true leaves-flower bud and watering resumption, 60% of full field capacity during flowering (x ± SD, n = 6); different letters indicate values that are significantly different one from another within one column of the diagram compared with the Tukey test, $P < 0.05$ (accounting for the Bonferroni correction); no inoculation; polyphenol oxidase, units of activity/mg of protein; three true leaves; flower bud; flowering

In the optimal cultivation conditions, activity of guaiacol peroxidase was the highest in the leaves of soybean inoculated with ineffective and low-effective rhizobia, compared with its activity in the leaves in the symbiosis with effective rhizobia and the variant without bacterization (Fig. 6a). The studies revealed that in soybean plants of the variant with no bacterization of seed with rhizobia, the activity of guaiacol peroxidase in the leaves increased during drought (by 106.9%) and after the stressor action (by 83.6%), compared with the control (optimal watering) (Fig. 6a, 6b).

During moderate drought, there was 47.9% increase in the activity of guaiacol peroxidase in the leaves of soybean inoculated with effective *B. japonicum* B1-20 rhizobia, and 134.1% increase during long dehydration, compared with the control (60% of full field capacity, Fig. 6a, 6b). After drought stress, the enzyme activity in the leaves of soybean approached the level of the control plants with optimal watering. In the soybean inoculated with low-effective rhizobia 107, the guaiacol-peroxidase activity in leaves decreased 30.1% during dehydration in the flower-bud stage. At the same time, in the ineffective symbiotic system with rhizobia 604k, the activity of guaiacol peroxidase in leaves increased by 88.2% during drought in the flower-bud stage (Fig. 6a, 6b). In the post-stress period, the enzyme activity in leaves in those symbiotic systems (604k and 107) increased by 176.6 and 240.0%, compared with the control (60% of full field capacity).

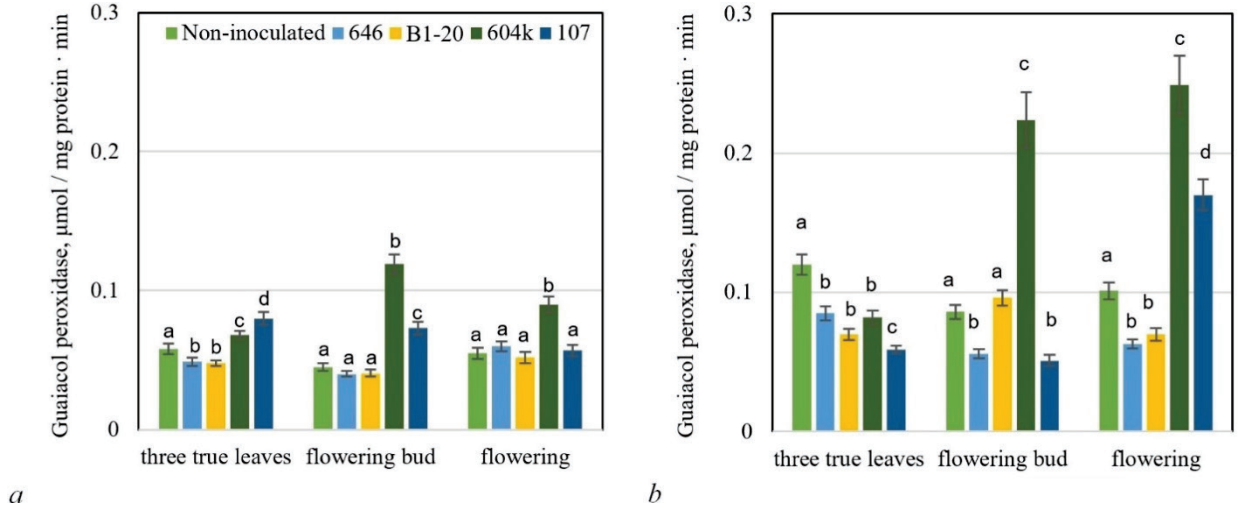


**Fig. 6.** Activity of guaiacol peroxidase in the leaves of soybean subject to different water supply: *a* – optimal, 60% of full field capacity, *b* – drought, 30% of full field capacity in the stage of three true leaves-flower bud and watering resumption, 60% of full field capacity during flowering (x ± SD, n = 6); different letters indicate values that are significantly different one from another within one column of the diagram compared with the Tukey test, $P < 0.05$ (accounting for the Bonferroni correction); no inoculation; guaiacol peroxidase, µmol of guaiacol /mg of protein * min; three true leaves; flower bud; flowering

## Discussion

Legume-rhizobium interaction induces the formation of specific reactions in a plant host, which may include changes in the functioning (inhibition or activation) of the defensive enzymatic complexes of peroxidase, catalase, polyphenol oxidase, superoxide dismutase, etc, which can prevent the ruining action of free radicals (Damiani et al., 2016, Mittler, 2017). Such changes can promote realization of the protective mechanisms of the plants and elevating metabolism to a new functional level, which helps the plant to adapt to stress factors. At the same time, the functional effectiveness of the symbiotic systems in stress conditions depends on the ability of macro- and microsymbionts to realize their own defensive mechanisms and symbiotic potential to the fullest.

Analysis of the obtained data demonstrates the specificity of how phenol-metabolism enzymes function in the nodules of soybean inoculated with rhizobia with different symbiotic properties in optimal cultivation conditions. In the optimal conditions of water supply, the soybean inoculated with ineffective rhizobia 604k was observed to have higher levels of activity of phenylalanine ammonia lyase and guaiacol peroxidase in the nodules, whereas low-effective symbiosis with *B. japonicum* 107 had high level of the polyphenol-oxidase activity, compared with the effective symbiosis with rhizobia B1-20. The enzyme activation we observed in the soybean nodules can be a reaction in response to inoculation with ineffective and low-effective rhizobia, accompanied by changes in metabolism, specifically those enzymes involved in the functioning of phenol cycle in the soybean nodules.

In the optimal conditions of plant cultivation, there was found lower level of the activity of polyphenol oxidase in the roots and leaves of soybean in the variants with inoculation with various rhizobia, compared with enzymatic activity in the plants without seed bacterization. At the same time, the level of activation of guaiacol peroxidase was higher in the leaves of the soybean inoculated with low-effective and ineffective rhizobia, compared with other variants of the experiment (without bacterization and effective-rhizobia inoculation). This confirms significant changes in phenol metabolism in the roots and leaves of the host plants after inoculation with rhizobia of different efficiency and virulence. During drought, there were seen differences in the levels of activation of phenol-metabolism enzymes in the nodules formed by rhizobia of different effectiveness. In particular, the effective symbiotic system (*B. japonicum* B1-20) was seen to have higher level of guaiacol-peroxidase activity and insignificant changes in phenylalanine ammonia lyase and polyphenol oxidase. The low-effective symbiotic system (*B. japonicum* 107) had significant increase in the activity of polyphenol oxidase and decrease in phenylalanine ammonia lyase and guaiacol peroxidase. In the ineffective symbiosis (*B. japonicum* 604k), the levels of activity of guaiacol peroxidase and polyphenol oxidase increased and phenylalanine ammonia lyase underwent insignificant changes. After stress, in the soybean inoculated with rhizobia 107 and 604k, the level of the activity of all enzymes in the nodules increased, especially polyphenol oxidase. Other studies demonstrated high mobility of enzymes of phenol metabolism in response to different stress factors (Kuvalekar et al., 2011; Mrázová et al., 2017). The research results indicate specificity of phenol metabolism in the nodules of soybean, which is related to peculiarities of formed symbiotic system, first of all symbiotic properties of rhizobia, their activity and virulence. Soybean is known to be very sensitive to cultivation conditions, especially to moisture deficit during critical ontogenesis stages, when molecular atmosphere nitrogen is being actively fixated (Cerezini et al., 2016). During drought, soybean plants in the low-effective and ineffective symbioses underwent double stress because they could not realize their own needs in nitrogen fixation, i.e. nitrogen-fixating functions. Complex action of those factors obviously determines changes in metabolism in the soybean nodules during drought, particularly phenol enzymes.

Long drought induced disturbances in phenol metabolism in the soybean roots in the variant without using bacterization. This suggests unstable activity of redox enzymes of phenol metabolism – sharp increase (polyphenol oxidase) or inhibition (guaiacol peroxidase) of the activity in the roots subject to drought. At the same time, there occurred significant inhibition of the activity of polyphenol oxidase, increase in guaiacol peroxidase in the leaves of plants in the variant without seed bacterization, and the level of activity of both enzymes did not recover up to the control (60% of full field capacity) after stress. This indicates malfunctioning of the phenol-metabolism enzymes in the leaves of soybean and obvious stress-induced changes in phenol metabolism in the plants. In all the studied symbiotic systems of soybean, we observed similar dynamics of the acti-

vities of polyphenol oxidase and guaiacol peroxidase in the roots – increase during drought and intensification of their levels after watering of the plants had been resumed. In the symbiosis of soybean and low-effective rhizobia 107, activity levels of polyphenol oxidase and guaiacol peroxidase in the roots subject to drought were the highest, whereas in soybean in the symbiosis with ineffective rhizobia 604, the activity of those enzymes significantly increased after the stress. In the effective symbiotic B1-20 system, we saw the most stable dynamics of activity of enzymes in the roots during drought and after stress (when watering had been resumed). Drastic increase in activity of the enzymes in soybean roots in the ineffective symbiosis (604k) indicates its excessive sensitivity to the conditions of water supply compared with other symbiotic systems (B1-20 and 107).

During drought, in the effective symbiotic system (*B. japonicum* B1-20), the activities of guaiacol peroxidase and polyphenol oxidase in the leaves increased, and approached the control (60% of full field capacity) after watering resumption. This confirms adaptive changes in enzymes of soybean leaves as a response to stress. In the low-effective and ineffective symbiotic systems of soybean, we observed unstable dynamics of activity levels of polyphenol oxidase and guaiacol peroxidase in the leaves during drought. In particular, the low-effective symbiotic system had significant increase in the activity of polyphenol oxidase and decrease in guaiacol peroxidase in the leaves during long dehydration, whereas after resumption of watering, those changes in activity of those enzymes reversed. In the ineffective symbiotic soybean system, we saw increased activity level of the both enzymes in leaves, especially polyphenol oxidase, both during and after stress, when the watering was resumed. Such data indicate drought-induced disturbance of functioning of the phenol pool in the leaves of plants and also inability of the low-effective and ineffective symbioses to realize their own stress-protection mechanisms for regulation of phenol homeostasis when subject to a stressor action and after its action stopped.

Phenol compounds play an important role in legume-rhizobium symbiosis. Not only do they induce emergence of symbiosis as signals – mediators when subject to Nod-factors, but also influence re-distribution of auxin, thus controlling the formation of nodules, while also remaining the regulators of physiological condition of a symbiotic system, both during the stages of the active nitrogen-fixation and aging of the nodules (Keet et al., 2017). The studies revealed that high level of peroxidase activity in rhizobia-inoculated legumes can indicate possible change in phytohormonal balance of the symbiotic system (Ryu et al., 2012). The literature has data about identification of correlation between the content of auxins and peroxidase activity in the roots and young nodules of plants. In particular, increase in the activity of peroxidase as a polyfunctional enzyme has been considered – among other functions in cell – a response to increase in the content of endogenous auxin, and by level of the peroxidase activity in plants, one can conclude on concentrations of auxins in them (Kots & Hryschuk, 2019). The special role of peroxidase in plant metabolism is evidenced by its ability to perform not only oxidative degradation of auxin but also its ability to participate in a number of other reactions: photosynthesis, catabolism of phenol compounds (oxidated flavonoids), lignified cellular walls, and also general processes of biological oxidation (Rosa, 2017).

Scientists found that in symbiotic systems formed with participation of highly virulent rhizobial mutants, the activity of polyphenol oxidase was higher than in systems with average-virulent mutants (Kots et al., 2010). At the same time, the enzyme activity in the root nodules of soybean formed by low-virulent mutants had the lowest level of enzymatic activity. It is assumed that high level of polyphenol-oxidase activity in nitrogen-fixating nodules may suggest similarities between some reactions – response of plants to an infection by pathogenic microorganisms and rhizobial inoculation (Pradhan et al., 2018). However, result of those reactions in the nodules was not inactivation of the microorganism but regulation of its reproduction and metabolic activity in symbiosomes.

The activity of phenylalanine ammonia lyase correlates with the content of phenol compounds and lignin, which play an important role in induction of resistance of plants to stress factors (Jun et al., 2018). Currently, this enzyme is considered to be a possible marker of induced resistance to plant diseases caused by phytopathogens. Experiments with inhibitors of transcription and translation revealed that increased activity of phenylalanine ammonia lyase depends on RNA and protein biosynthesis, and its synthesis and repression took place in all induced systems (Alunni et al., 2003). At the same time, inactivation of the enzyme is not a universal mechanism because its synthesis precedes the synthesis of protein inhibitor, induced by trans-cinnamic acid. Regulation of the enzyme/inhibitor ratio had developed during evolution as an important means of regulatory mechanisms of live cell and its specific response to a stressor (Yu et al., 2018). Despite the fact that biochemical function of phenylalanine ammonia lyase has been extensively studied, the functional value of its genes in symbiotic processes has been studied insignificantly.

## Conclusions

Inoculation of soybean with rhizobia induced changes in the activity level of phenol-metabolism enzymes in the roots and leaves of soybean in optimal cultivation conditions. This confirms that metabolism of the host plant can be modified by seed inoculation with rhizobia of different effectiveness. We identified the specificity of reactions of phenol-metabolism enzymes in the nodules, roots, and leaves of soybean to conditions of water supply, which is first of all determined by functioning of the symbiotic system with participation of *B. japonicum* strains of various effectiveness and virulence.

The effective symbiotic system (*B. japonicum* B1-20) in the conditions of drought was characterized by insignificant increases in the activities of the key enzymes of phenol metabolism (guaiacol peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase) in the nodules, roots, and leaves, and also recovery of their level to the optimal after the stress. This indicates adaptability of the symbiotic system to changes in the water-supply conditions and also its ability to realize the defensive capabilities during drought stress.

The less-effective symbiotic system (*B. japonicum* 107) was observed to have heightened levels of the activities of polyphenol oxidase and guaiacol peroxidase in the nodules, roots, and leaves of soybean, and also decrease in the activity of phenylalanine ammonia lyase in the nodules during drought, and partial recovery of activities of those enzymes to the control level (60% of full field capacity) after the stress.

In the ineffective symbiotic system (*B. japonicum* 604k), we observed unstable reaction of the phenol-metabolism enzymes, which was either intensified or inhibited during drought and in the post-stress period, indicating disturbance in phenol metabolism in the plants.

Our studies confirm the importance of coordinated participation of the both partners of symbiosis, macro- and microsymbionts, in the formation of tolerance of legume crops to drought, which is attained by maximal realization of their protective systems, including key enzymatic complexes for supporting plant homeostasis.

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