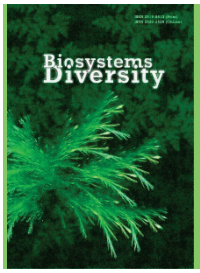

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Peculiarities of soybean-rhizobial systems subject to different levels of water supply following treatment with succinic acid and epibrassinolide

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All around the world, one of the leading – according to area of cultivated fields – oleic crops is soybean, which has a high demand for moisture. Given the significance of this crop and negative impact of drought on its yield, integrated research of the influence of insufficient water supply on the intensity of physiological-biochemical processes in those plants is necessary for identifying and understanding the drought-tolerance mechanisms of soybean, as well as symbiotic systems created with its participation, and also for search for ways to adapt it to this stressor. Therefore, our objective was determining the specifics of formation and functioning of the symbiotic systems of soybean and *Bradyrhizobium japonicum*, following treatment with succinic acid (0.01 g/L) and 24-epibrassinolide (0.00001 g/L), subject to different levels of watering. Our studies revealed that pre-sowing treatment of the seeds with a solution of 24-epibrassinolide with their subsequent inoculation with *B. japonicum* T21-2 resulted in the most pronounced stimulation of formation and functioning of the symbiotic systems of soybean in the optimal growing conditions. At the same time, during water shortage, the intensity of nitrogen fixation was the highest in the plants grown from seeds that had been successively treated with the acid and the inoculant. We confirmed that water deficit led to significant increase in the overall content of phytohormones of cytokinin nature in the soybean root nodules, depending on the way the seeds were treated. However, the largest pool of cytokinins was seen in the plants that had been treated with succinic acid against the background of both optimal and insufficient water supply. Treatment of the seeds with 24-epibrassinolide caused significant excess of content of zeatin riboside over the content of zeatin during the flowering stage, whereas in the stage of pods formation it led to an opposite effect – excess of zeatin over zeatin riboside. Fourteen days-long water deficit decreased the content of chlorophylls in the leaves and grain productivity of the plants of all variants of the experiment. The use of growth regulators managed to alleviate the negative impact of stress and protect the pigment complex from ruination. Treatment of the seeds with solutions of succinic acid and 24-epibrassinolide provided the growth of soybean grain productivity regardless on water-supply level. The most efficient was 24-epibrassinolide. Therefore, use of 24-epibrassinolide for pre-sowing treatment of the soybean seeds provided formation of effective symbiotic systems with high nitrogen-fixing activity and caused a number of specific changes in the pattern of accumulation of free and complex forms of cytokinins in the root nodules of those plants. At the same time, the treatment provided the highest concentration of photosynthesis pigments in the soybean leaves, and as a result produced the greatest increase in grain productivity of plants of all the variants, regardless of levels of water supply. In turn, use of succinic acid produced the highest level of nitrogen-fixing activity in the case of the lowest number of root nodules in the conditions of insufficient water supply, and also caused significant accumulation of cytokinins in the nodules, compared with other studied variants against the background of both optimal and insufficient water supply. Therefore, it did result in increase in soybean grain productivity, but this was lower than in the plants treated with 24-epibrassinolide.

Keywords: drought; symbiotic nitrogen-fixation; cytokinins; zeatin; zeatin riboside; photosynthetic pigments.

Introduction

Among legume crops, soybean (*Glycine max* L.) is one of the most effective biological fixers of atmosphere nitrogen, being inferior only to perennial legume crops. It is a valuable food and oil crop worldwide (Frederick et al., 2001; Islam et al., 2022). Furthermore, it is one of the most profitable crops. Production of legumes in many regions of the world is impacted by drought (Daryanto et al., 2015). Water deficit in soil impedes the flowering, leading to decrease in the number of beans, and thus decrease in yield (Sadeghipour & Abbasi, 2012). The main cause of reduced productivity of plants in such conditions is inhibition of photosynthetic processes, which are the first to react to water deficiency (Arya et al., 2021; Yang et al., 2021). During drought, photosynthesis is first of all inhibited due to closure of the stomates (Mak et al., 2014). Lack of water in soil induces the signal from the roots to shoots, which is transported across the xylem and inhibits the stomatal transpiration. As a result, the plant looses less water. At the same time, CO_2 deficit, inhibition of the Calvin cycle and the activity of photosystem II were seen, which entailed formation of surplus active oxygen species. Also, the stress causes decrease in the content of photosynthetic pigments as a result of inhibition of their biosynthesis or ruination. The result of such changes is impairment of transport of electrons and photooxidation (Kangasjärvi et al., 2012). Plants reduce the rates of electron transport, converting the surplus of absorbed light into heat (Tikkanen et al., 2011) or producing active oxygen species, which cause damage to the membrane systems and ruination of proteins of the plant.

Plants react to oxidative disorders through antioxidant systems, enhancing the activity of antioxidant enzymes and increasing the content of saccharose, protein, and proline so as to reduce water losses (Manavalan et al., 2009; Mahmood et al., 2020). This mechanism maintains a sufficient content of saccharose during short drought stress in order to provide the plants with energy to grow and develop. Nitrogen metabolism often decreases significantly, inhibiting the protein synthesis and transport ability of photosynthesis products (Gil-Quintana et al., 2013).

During drought, respiration processes undergo a number of changes as well. In particular, the share of the pentose phosphate pathway increases. The extra-mitochondrial oxidation systems become more active as a result of weakening of electron transport in the electron-transporting chain

of mitochondria, which is related to detoxification of breakdown products. Moreover, drought is one of the main factors impacting nitrogen fixation in the soybean-rhizobial symbiotic system. Researchers found that there are several mechanisms through which symbiotic systems regulate N_2 fixation when subject to drought-induced stress, in particular carbon deficiency and impairment of its metabolism in the root nodules, limited ingress of oxygen and reverse regulation as a result of accumulation of nitrogen bound. Those physiological processes inhibit nitrogenase activity of the nodules, imposing a negative impact on the plants and their productivity (Collier & Tegeder, 2012; Larrainzar et al., 2014; Kunert et al., 2016). In particular, it was revealed that enrichment of soybean with CO_2 during drought provides increase in photoassimilation and, as a result, enhances the distribution of carbon in the nodules, the main effect of which is support of their growth and activity, which allows N_2 to be maintained at a sufficient level in the conditions of water deficit in soil. Studies of interrelation between the processes of permeability of the nodules for O_2 and fixation of N_2 concluded that oxygen deficit inhibited the activity of the nodules only at the initial stages of the stress action. A number of studies revealed participation of ureides in reaction of nitrogen-fixation process to drought, because their significant accumulation in the shoots during stress action caused negative feedback response between ureides and activity of the nodules (Serraj, 2003). Thus, it was confirmed that all three physiological mechanisms are important for understanding the regulation of N_2 fixation and its reaction to soil drought (Serraj et al., 1999).

Work of the symbiotic apparatus of soybean subject to unfavorable environmental factors can be optimized by introducing growth regulators to the technique of cultivation of this crop. Perhaps, an advantage of those compounds is their ability to realize the genetic and physiological potentials of the plants to the fullest by making them use nutrients more effectively and stabilizing their adaptive reactions to unfavorable factors of natural and anthropogenic origins: critical temperature fluctuations, water deficit, toxic action of pesticides, diseases, and infestations with pests (Gaspar et al., 1996; Gruznova et al., 2018).

Growth regulators can directly affect the hormonal status of the plants. Such regulators include plant hormones or their synthetic analogues, inhibitors of biosynthesis or translocation of hormones, and blockers of hormonal receptors. Moreover, they can cause local and transient phytotoxic effects (Rademacher, 2015). However, it was confirmed that the use of growth regulators in techniques of cultivating plants managed to increase their yield by 10–25%, shorten the maturation period, reduce the content of nitrates in them, as well as pesticides and heavy metals, increase nutritional value of the products, etc.

Among the broad range of growth regulators, we should note succinic acid and epibrassinolide, because those compounds are currently actively used in agriculture, and at the same time they pose no threat to people and the environment (Lieshchova et al., 2020). Succinic acid is an intermediate product of tricarboxylic acid cycle in many organisms and is produced by plants, animals, and microorganisms. It is also one of the end products of anaerobic digestion. It can be produced by the plant roots during their growth and is accumulated in soil during decay of plant remaining (Zeikus et al., 1999; Hayat et al., 2010). Succinic acid is used in protection of plants as a growth regulator and stress adaptogen. It also partakes in the cellular respiration of aerobic organisms and promotes increase in the count of chlorophyll.

Epibrassinolide is a representative of another group of phytohormones – brassinosteroids, which are present in any plant cell, activate cell stretching, and can regulate the seed formation and increase stress-tolerance and phytoimmunity (Krishna et al., 2017). The effects of brassinosteroids on the formation of α-amylases and germination rate of seeds of grain crops have been confirmed. Experiments revealed that treatment of plants with preparations that contain brassinosteroids increased plant growth in general and some plant organs in particular, and also strengthening of the stems through intensified calcium accumulation. Furthermore, those phytohormones increased tolerance of the plants to negative environmental factors and improved qualitative and quantitative yield parameters. During lack of water in soil, they are able to regulate its content in plants through stimulation of water-retaining ability of the leaves, which ultimately has a positive effect on crop yield. It was found that treatment of wheat sprouts with 24-epibrassinolide significantly increased the expression of dehydrin gene TADHN – a protein that performs osmoprotective functions (Kumari et al., 2018; Basit et al., 2021). Accumulation of dehydrin proteins is also known as a marker of drought tolerance of plants (Riyazuddin et al., 2022). Moreover, increase in drought tolerance can be a consequence of activation of antioxidant system by brassinosteroids.

Besides regulation of physiological processes in the plant organism, growth regulators positively influence the development and functioning of soil microflora (Kots et al., 2010), which is especially important for growing legumes, particularly soybean. As is known, they activate the microbiological processes in the area of the root system. Furthermore, they are able to stimulate organogenesis of the root nodules and provide the formation of a more powerful symbiotic apparatus. At the same time, the effect of the growth regulators is determined by macrosymbiont genotype, degree to which of complementarity the varieties with homologous rhizobia and the nature of growth regulators.

Therefore, drought is a serious climatic risk that necessitates development of effective strategies of alleviation of its impact. Therefore, it would be important and practical to study the protective effect of growth regulators and rhizobial seed inoculation when growing soybean in one technological process in order to increase the nitrogen-fixing activity and tolerance of the soybean-rhizobial system under stress. Accordingly, our objective was identifying the specifics of formation and functioning of symbiotic soybean-*Bradyrhizobium japonicum* systems following treatment with succinic acid in the concentration of 0.01 g/L and 24-epibrassinolide in 0.00001 g/L concentration, against the background of different levels of water supply.

Materials and methods

The objects of the study were symbiotic systems formed with participation of soybean of the Titan variety (selective breeding of the Plant Breeding & Genetics Institute – National Centre of Seed & Cultivar Investigation; Institute of Feed Research and Agriculture of Podillya of National Academy of Agrarian Sciences of Ukraine) and active strain of nodule bacteria *B. japonicum* T21-2 (the strain was obtained by inter-species conjugation of *Escherichia coli* S17-1 and *B. japonicum* 646). The genome of the cells of nodule bacteria of this strain contained a fragment of gene of neomycin phosphotransferase transposon Tn5 of 517 bp length, which makes it different from the initial strain and provides tolerance to kanamycin sulfate in the concentration of 200 mg/mL. Strain T21-2 is characterized by increased production of exopolysaccharides, it is non-pathogenic, with high ecological plasticity and competitive ability (Kots et al., 2016). To treat the seeds, we used succinic acid in 0.01 g/L concentration and 24-epibrassinolide in 0.00001 g/L concentration.

The bacterial culture was grown at 26–28 °C on mannitol-yeast agar of the following composition (g/L): KH_2PO_4 – 0.5, $MgSO_4$ – 0.2, NaCl – 0.1, yeast extract – 1.0, and mannitol – 10.0. The bacteria were cultivated using the method of periodic incubation on orbital shakers in Erlenmeyer flasks that contained 200 mL of growth medium, at 220 rpm shaker velocity. Purity of the culture was tested by its inoculation onto meat-peptone agar, on which rhizobia do not grow. Prior to inoculation, seeds were sterilized using 70% ethanol solution and rinsed with tap water. Treatment of the seeds with growth regulators and inoculation were performed in 1 h according to the following scheme of the experiment:

– seeds + *B. japonicum* T21-2, 60% of full field capacity (control 1);

– seeds + succinic acid + *B. japonicum* T21-2, 60% of full field capacity;

– seeds + 24-epibrassinolide + *B. japonicum* T21-2, 60% of full field capacity;

– seeds + *B. japonicum* T21-2, 30% of full field capacity (control 2);

– seeds + succinic acid + *B. japonicum* T21-2, 30% of full field capacity;

– seeds + 24-epibrassinolide + *B. japonicum* T21-2, 30% of full field capacity.

The studies were carried out at the vegetative area of the Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine. Six soybean plants were cultivated in each 7 kg container, in natural light and temperature, optimal (60% of full field capacity) and

insufficient (30% of full field capacity) watering, using rinsed river sand as a substrate. The source of mineral nutrition was a Hellriegel's nutrient medium, enriched with microelements: molybdenum, boron, manganese, and copper, and nitrogen-impoverished – 0.25 of the norm (1 norm of nitrogen corresponds to 708 mg of $Ca(NO_3)_2 \cdot 4H_2O$ per 1 kg of substrate). Drought was created by controlled watering for two weeks, starting from the stage of the three true leaves, after which watering was resumed up to 60% full field capacity.

To analyze the formation and functioning of the symbiotic apparatus, we gathered samples during the stages of flower bud formation (7-day effect of insufficient watering), flowering (14-day impact of water deficit), and pods formation (5 days of water resumption). Measuring was carried out in 5–6 biological repetitions. The nodulation activity of the rhizobia was examined by counting the number of nodules on the plant roots and identifying their mass. Content of photosynthetic pigments and cytokinins was studied in the stages of flowering and pods formation.

The nitrogen-fixing activity was measured using the acetylene method (Hardy et al., 1968). For this purpose, the roots with nodules were placed in hermetically closed 75 cm^3 glass vials, containing 10% concentration of acetylene. The incubation lasted for 1 h. Afterwards, the gas mixture that contained ethylene, formed as a result of reduction of acetylene nitrogenase, was analyzed on a gas chromotograph Agilent GC system 6850 (USA). The nitrogen-fixing activity was expressed in micromoles of ethylene formed in 1 h (μmol of C_2H_4/(plant•hour)). The measurement was carried out in 4 repetitions.

Content of photosynthetic pigments (carotenoids, chlorophylls *a* and *b*) were measured spectrophotometrically in the soybean leaves of the third tier (Wellburn, 1994) on the instrument of Shimadzu UV-1900 (Japan) at the wavelengths of 480, 649, and 665 nm, respectively, and expressed in mg/g of fresh mass of leaves. The extracts were diluted in dimethyl sulfoxide (1:9), which was accounted for during the final count of content of pigments.

The content of endogenous cytokinins in the soybean nodules was measured during the flowering stage and pods formation according to the methods (Shcherbatyuk et al., 2020). Weighed amounts (500 mg) were ground in liquid nitrogen and homogenized in cooled extracting solution (methanol, double-distilled water, and formic acid in 15/4/1 ratio). The extraction was carried out at –20 °C for an hour followed by centrifugation for 30 min at 15,000 rpm per minute and the temperature of +4 °C in a centrifuge, after which the supernatant fluid was removed, and an extracting solution was repeatedly added to the sediment. Extraction was performed for 30 min followed by centrifugation. The extracts were blended and evaporated on a rotational evaporator (HEIDOLPH Laboporata 4000 Efficient, Germany). Purification of the extracts was performed using the method of solid-phase extraction (SPE) using two types of cartridges: C18 (cartridge with solid silica-gel phase – to remove most lipophilic compounds) and Oasis MCX (cartridge with MCX phase, in which hormones bind strongly, and then subsequently eluted by respective solvents).

Qualitative and quantitative parameters of zeatin and zeatin riboside were identified using the method of highly productive liquid chromatography using an Agilent 1260 Infinity 3D LC System liquid chromatograph (USA). For elution of the extracts, we used Zorbax SB-C18 reversed-phase column (4.6 · 150 mm, 5 μm) (Agilent Technologies, USA) at 25 °C. Detection of zeatin and zeatin riboside was performed in a UV absorption region at the analytical wavelength of 269 nm by the system of solvents, containing methanol, ultrapure water (distilled, and then deionized), acetic acid in the volumetric proportion (35.0 : 64.5 : 0.5%; v/v) with isocratic elution with the flow rate of 0.5 mL/min. The number of individually selected phytohormones was determined according to the peak area. For the control, we used the standard synthetic phytohormones manufactured by Sigma (Germany) Z – Zeatin; ZR – trans-Zeatin-riboside.

The data are presented in the tables and figures as x ± SD (mean ± standard deviation). Differences between the control and experimental groups were determined according to the Tukey test, where the differences were considered significant at $P < 0.05$ (accounting for Bonferroni's Correction).

Results

We found that regardless of the watering level, the stage-by-stage presowing treatment of the seeds with 24-epibrassinolide and *B. japonicum* T21-2 led to 23.2% and 21.3% increases in the number of nodules on the roots of the soybean in the flower bud phase, compared with the plants treated only with inoculants (Table 1). At the same time, the nitrogen-fixing activity in the symbiotic systems of plants of this variant was significantly dependent on water-provision level. In the conditions of the optimal water supply, it was 28.8% higher than control 1, whereas in the conditions of water deficiency it was 40.0% lower than in control 2 (Fig. 1). We should note that during the flower bud phase, the nitrogen-fixing activity of the root nodules in soybean across all the variants we studied in the conditions of drought was significantly lower than in the plants grown under the optimal water supply. We found that in the same development stage, the soybean that had been treated with succinic acid and the inoculants formed 16.0% less nodules in the conditions of optimal water supply than the plants treated with rhizobia alone, but at the same time those nodules weighed 24.2% more. Subject to water deficit, the number and mass of nodules on plants of this variant were at the level of plants bacterized without prior use of growth regulators, but the nitrogen-fixing activity was 30.8% higher (Table 1, Fig. 1).

Table 1

Nodulation activity *B. japonicum* T21-2 under the influence of growth regulators and different water supply (x ± SD, n = 6)

Variant	Stage of plant development					
	flower bud		flowering		pods formation	
	number of nodules per plant	mass of nodules per plant	number of nodules per plant	mass of nodules per plant	number of nodules per plant	mass of nodules per plant
Watering level	optimal watering, 60% of full field capacity (FFC)					
seed + *B. japonicum* (control 1)	24.4 ± 2.3^{b}	0.033 ± 0.003^{b}	21.2 ± 0.5^{b}	0.051 ± 0.006^{b}	27.0 ± 3.0^{bc}	0.050 ± 0.004^{b}
seed + succinic acid + *B. japonicum*	20.5 ± 1.3^{c}	0.041 ± 0.002^{a}	26.0 ± 2.6^{a}	0.051 ± 0.003^{b}	32.5 ± 1.1^{b}	0.078 ± 0.008^{a}
seed + 24-epibrassinolide + *B. japonicum*	30.0 ± 2.1^{a}	0.037 ± 0.006^{ab}	27.0 ± 3.0^{a}	0.060 ± 0.009^{a}	24.4 ± 1.8^{bc}	0.067 ± 0.005^{ab}
Watering level	drought, 30% of FFC				resumption of watering, 60% of FFC	
seed + *B. japonicum* (control 2)	20.3 ± 0.7^{c}	0.019 ± 0.002^{c}	16.3 ± 1.4^{bc}	0.027 ± 0.004^{d}	22.3 ± 1.4^{c}	0.049 ± 0.005^{bc}
seed + succinic acid + *B. japonicum*	20.0 ± 1.1^{c}	0.025 ± 0.004^{bc}	14.0 ± 1.1^{c}	0.029 ± 0.003^{d}	37.0 ± 2.4^{a}	0.056 ± 0.008^{b}
seed + 24-epibrassinolide + *B. japonicum*	24.7 ± 1.5^{b}	0.016 ± 0.002^{c}	21.3 ± 1.3^{b}	0.056 ± 0.004^{ab}	35.0 ± 2.1^{ab}	0.083 ± 0.002^{a}

Note: different letters indicate values that are significantly different ($P < 0.05$) within one column according to the results of the Tukey test.

Compared with the plants that had been treated only with nodule bacteria, root nodules in the plants treated with succinic acid and epibrassinolide increased 22.9% and 27.6%, respectively, during the flowering stage, in the conditions of optimal watering (Table 1). Furthermore, in the variant with 24-epibrassinolide, we observed a significant increase (118.7%) of the nitrogen-fixing activity of symbiotic soybean systems compared with the system in the same control (Table 2). After treating seeds with solution of succinic acid, this parameter only had an upward tendency. In the plants cultivated subject to water deficit, epibrassinolide provided the formation of a strong symbiotic apparatus on the soybean roots, compared with the plants treated only with the inoculant and the plants treated with succinic acid alone. Number and mass of the root nodules in plants of this variant were 30.4% and 107.4% higher than control 2, respectively, though their nitrogen-fixing activity was at the level of control. The succi-

nic acid-treated plants developed the symbiotic systems with the number and mass of the nodules at the level of the plants that had been treated with the inoculant alone. However, their nitrogen-fixing activity was significantly (125.4%) greater than the control.

In the pods formation stage, the symbiotic systems of plants that had optimal watering throughout the vegetation period were superior by nitrogen-fixing activity to the plants bacterized without being previously subjected to the growth regulators. Accordingly, succinic acid and 24-epibrassinolide treatments of seeds resulted in 16.0% and 31.2% higher nitrogen-fixing activity, respectively. Moreover, the symbiotic systems developed following succinic acid treatment were higher than control 1 according to the number and mass of the root nodules by 20.2% and 56.0%, respectively. In this development phase, in the epibrassinolide pre-sowing treatment soybean the watering of which had been resumed, we saw increase in the number and mass of the root nodules, and also their nitrogen-fixing activity by 57.0%, 69.4%, and 73.4%, compared with the plants treated only with rhizobia (Table 1). As is known, cytokinins are involved in regulation of drought-tolerance. We found that in the flowering stage, in the succinic acid-treated plants grown in the optimal conditions, the contents of zeatin and zeatin riboside in the root nodules were 78.2% and 54.0% higher than in the plants treated only with the inoculants. Treatment with 24-epibrassinolide produced significant increase (67.5%) in zeatin riboside, whereas zeatin content only had a tendency towards increase (Table 2).

Treatment of the seeds with succinic acid led to significant increase in zeatin content (55.9%) and zeatin riboside (150.2%) in soybean nodules compared with control 1 in the stage of pods formation. Treatment with 24-epibrassinolide, on the contrary, led to 22.5% and 20.9% decreases in those phytohormones in the given period of plant development (Table 2). We found that 14 day water deficit caused increase in zeatin and zeatin riboside in the soybean root nodules in the flowering stage, compared with the plants of similar variants grown with optimal watering (Table 2). In the stage of pods formation, we saw an opposite effect: the plants that had their watering resumed mostly had reduced contentsof phytohormones of cytokinin nature in the soybean nodules, compared with the plants grown in 60% full field capacity.

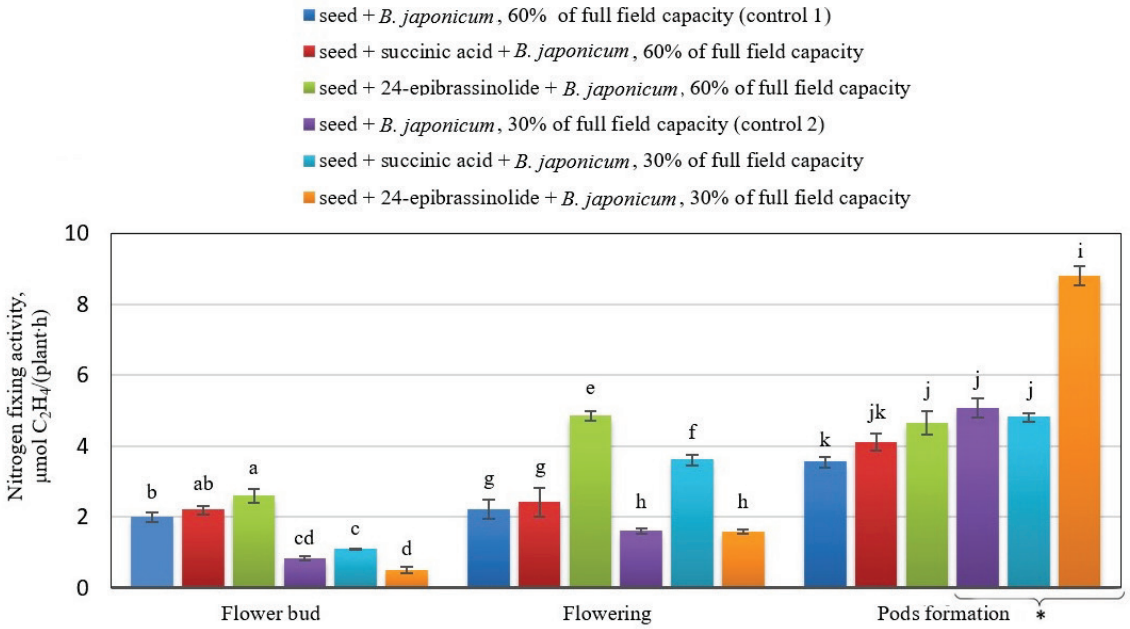

Fig. 1. Nitrogen-fixing activity of symbiotic systems soybean – *B. japonicum* T21-2 subjected to growth regulators and different water supply (x ± SD, n = 4): different letters in columns indicate values that significantly differed one from another between the variants according to the Tukey test ($P < 0.05$); letters *a, b, c, d* – significantly between the variants in the flower bud stage; letters *e, f, g, h* – significantly differed between variants in the flowering stage; letters *i, j, k* – significantly differed between variants in the pods formation stage; * – resumption of watering, 60% of full field capacity

Table 2

Content of cytokinins (ng/g of fresh mass) in soybean root nodules formed following treatment growth regulators and subject to different water supply (x ± SD, n = 6)

Variant	Stage of flowering		Stage of pods formation	
	zeatin	zeatin riboside	zeatin	zeatin riboside
Watering level	optimal watering, 60% of full field capacity (FFC)			
seed + *B. japonicum* (control 1)	3.49 ± 0.24^{d}	2.65 ± 0.11^{e}	3.24 ± 0.28^{b}	2.35 ± 0.20^{b}
seed + succinic acid + *B. japonicum*	6.22 ± 0.35^{b}	4.08 ± 0.08^{d}	5.05 ± 0.34^{a}	5.88 ± 0.11^{a}
seed + 24-epibrassinolide + *B. japonicum*	3.90 ± 0.23^{cd}	4.44 ± 0.25^{d}	2.51 ± 0.17^{bc}	1.86 ± 0.27^{bc}
Watering level	drought, 30% of FFC		resumption of watering, 60% of FFC	
seed + *B. japonicum* (control 2)	4.58 ± 0.13^{c}	6.53 ± 0.25^{b}	0.91 ± 0.01^{cd}	2.15 ± 0.10^{b}
seed + succinic acid + *B. japonicum*	7.06 ± 0.19^{a}	5.59 ± 0.14^{c}	1.74 ± 0.11^{c}	1.72 ± 0.13^{c}
seed + 24-epibrassinolide + *B. japonicum*	4.23 ± 0.22^{c}	8.21 ± 0.40^{a}	3.39 ± 0.32^{b}	0.91 ± 0.06^{d}

Note: see Table 1.

Treatment of the soybean seeds with solution of succinic acid caused 54.1% (flowering stage) and 91.2% (pods formation stage) increases in the content of zeatin in root nodules of the plants experiencing water deficit, compared with the plants treated only with the inoculant. At the same time, we observed 14.4% (flowering stage) and 20.0% (pods formation stage) decreases in zeatin riboside compared with the control. Cultivated during drought, the plants the seeds of which had been treated with 24-epibrassinolide prior to sowing had 25.7% higher content of zeatin riboside than control 2. In the watering resumption period (pods formation stage), the situation was the opposite – with 272.5% increase in zeatin content, compared with the plants of the same control, there was seen 57.7% decrease in the content of zeatin riboside (Table 2).

Measurement of content of photosynthetic pigments revealed that in the flowering stage, the lowest content of chlorophyll a in the leaves was in the plants of variant with alternately treatment with succinic acid and the inoculant. The highest parameters were in the plants that had been treated

with 24-epibrassinolide (Fig. 2). Fourteen-day drought led to decrease in the content of chlorophyll a in the leaves in all studied variants compared with the plants with optimal watering. At the same time, the examined parameter was only 7.9% higher in the plants the seeds of which had been treated with solution of 24-epibrassinolide with subsequent inoculation with *B. japonicum* T21-2, as compared with the plants bacterized without prior use of growth regulators. Treatment of seeds with 24-epibrassinolide produced the highest content of chlorophyll *b* in the leaves of soybean during the flowering stage, depending on watering. In the conditions of optimal water supply, succinic acid caused insignificant decrease (6.4%) in the studied parameter in the leaves, compared with control 1, while in the conditions of drought it increased 16.2% in 24-epibrassinolide-treated plants compared with plants of control 2. We also observed a tendency towards its increase in the succinic acid-treated plants (Fig. 2).

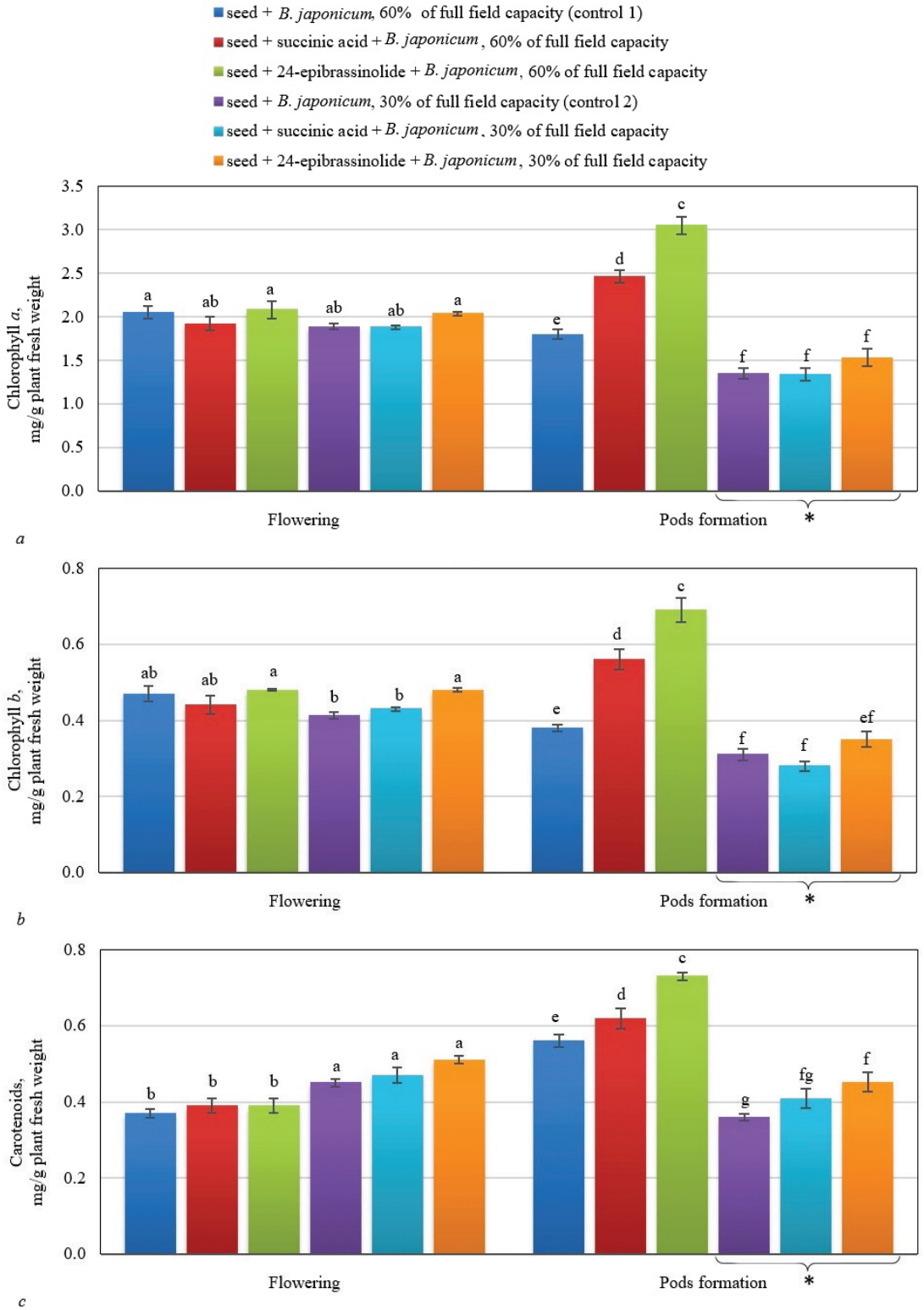

Fig. 2. The content of photosynthetic pigments in soybean plants treated with growth regulators and subject to different water supply (x ± SD, n = 6): different letters in columns indicate values that significantly differed one from another between the variants according to the Tukey test ($P < 0.05$), letters *a*, *b* – significantly differ between variants in the flowering stage; letters *c*, *d*, *f*, *g* – significantly differ between variants in the pods formation stage; *a* – Chl *a*, *b* – Chl *b*, *c* – carotenoids; * – resumption of watering, full field capacity

According to carotenoids, we found no significant differencess between plants of the variants grown in the optimal conditions. We determined that 14-day impact of water deficit led to increase in the number of those pigments in the leaves of soybean plants. In particular, this parameter increased by 13.3% in the soybean treated with 24-epibrassinolide and tended to increase following treatment with succinic acid.

Similarly to the previous stage of plant development, during the pods formation stage, the highest content of photosynthesizing pigments was in plants of the variant with subsequent treatment of seeds with 24-epibrassinolide and *B. japonicum* T21-2 (Fig. 2). In the plants that had not been subject to stress factor but had been treated with the growth regulators, the content of chlorophyll *a* was higher than in the plants treated only with nodule bacteria. It was 36.7% higher in the succinic acid-treated plants and 69.4% higher in the 24-epibrassinolide-treated plants. Furthermore, chlorophyll *b* was higher by 47.4% and 81.6%, and carotenoids by 10.7% and 30.4% higher, respectively. In the plants that had watering resumed, the effect from using growth regulators was somewhat different. In particular, we found that succinic acid provided 13.6% increase only in the content of carotenoids, compared with plants of control 2, whereas content of chlorophyll *a* underwent no changes, and the content of chlorophyll *b* decreased by 9.7%. Action of 24-epibrassinolide provided 13.3% and 25.6% increases in the content of chlorophyll *a* and carotenoids, respectively, compared with the same control.

We observed that the growth regulators we used led to increase in the productivity of soybean plants grown in the conditions of optimal water supply (Table 3). The best results were produced by 24-epibrassinolide. Treatment of soybean seeds with this growth regulator and their subsequent inoculation with *B. japonicum* T21-2 resulted in 9.8% higher grain productivity than the plants treated only with the inoculant. Succinic acid was less effective, though provided increase in yield at the level 7.3%, compared with the same control.

We determined that the soybean grown subject to water deficiency had significantly lower productivity than the plants cultivated in the optimal conditions (Table 3). However, use of growth regulators we tested soothed the negative impact of the stress factor on the 24-epibrassinolide-treated plants and promoted 10% increase in their productivity, compared with the plants treated only with the inoculants, and also caused tendency towards increase in case of treatment with succinic acid.

Table 3

The grain yield of soybeans, cultivated under different water supply conditions, with pre-sowing, step-by-step treatment of seeds using growth regulators and nodule bacteria (x ± SD, n = 6)

Variant	Mass of grain, g per plant	Mass of grain, % increase to control
seed + *B. japonicum*, 60% of full field capacity (control 1)	2.591 ± 0.062^{b}	–
seed + succinic acid + *B. japonicum*, 60% of full field capacity	2.780 ± 0.087^{ab}	7.3
seed + 24-epibrassinolide + *B. japonicum*, 60% of full field capacity	2.844 ± 0.109^{a}	9.8
seed + *B. japonicum*, 30% of full field capacity (control 2)	1.704 ± 0.056^{d}	–
seed + succinic acid + *B. japonicum*, 30% of full field capacity	1.813 ± 0.049^{cd}	6.4
seed + 24-epibrassinolide + *B. japonicum*, 30% of full field capacity	1.874 ± 0.071^{c}	10.0

Note: see Table 1.

Discussion

Legume-rhizobial symbiosis following treatment with growth regulators and subject to different water supply. Intensification of the process of symbiotic fixation of atmosphere nitrogen by the plants produced maximum yields of soybean at minimal costs and energy expenditures. Today, this process becomes especially relevant because increase in yield of this crop around the world in general is associated with a broad introduction of highly-productive varieties into the agriculture. However, involvement of such varieties in agricultural production is only possible in presence of large amounts of easily available nitrogen compounds in the soil. Specifically symbiotic nitrogen fixation is the most optimal, ecologically safe, and economically practical way of solving this issue. Treating seeds of legumes with high-quality inoculants with high titer of nitrogen-fixing microorganisms is currently necessary because it allows modern varieties and hybrids of agricultural crops to fully realize their genetic potential. As a result, it provides the highest possible yields, maximizing the return on investments. At the same time, it has to be noted that effective functioning of symbiotic systems is substantially impeded by drought. Since droughts have been becoming longer and more frequent over the recent several years, it is relevant to focus research on how the process of symbiotic fixation of nitrogen can be adapted to this stressor (Khatun et al., 2021). Solving this issue is what are research has been aiming for. The results revealed that treatment of seeds with 24-epibrassinolide with subsequent inoculation with *B. japonicum* T21-2 led to the highest parameters of nodulation activity, compared with the plants bacterized without the previous treatment with the growth regulators and the plants treated with succinic acid, in almost all plant development stages that we studied, against the background of water deficit (Table 1, Fig. 1). At the same time, in the stage of flower bud and flowering of soybean, the highest level of nitrogen-fixing activity, compared with the control plants and after treatment with 24-epibrassinolide, was observed in the soybean treated with succinic acid. Small doses of succinic acid are believed to activate the course of the Krebs cycle in symbiotic and associative diazotrophs. In general, this favors the energy balance of bacterial cells and obviously provides its efficient functioning in symbiotic systems with legume plants. The stimulating effect of succinic acid on nitrogen fixation, which we observed, only confirms the assumption made by a team of authors that succinic acid and its salts can be an energy source for nitrogen-fixing process (Finan et al., 1981). Further evidence of this fact may be the study by Bergersen et al. (1967) which revealed that N_2 fixation by isolated bacteroids of soybean is strongly stimulated by succinate and fumarate. At the same time, stimulation of the nitrogen-fixing activity in plants during stress also suggests its role of an anti-stressor. We saw opposite results in the period of watering resumption (stage of pods formation). In particular, the plants grown from seeds treated with succinic acid were characterized by reduced nitrogen-fixing activity, compared with the plants treated only with the inoculants, whereas 24-epibrassinolide led to a significant increase in the studied parameter, compared with the control plants.

Positive effect from using 24-epibrassinolide was seen in the plants cultivated subject to 14-days-long water deficit, and also in the plants grown in the optimal conditions. In particular, we determined that use of 24-epibrassinolide for pre-sowing treatment of seeds with subsequent inoculation with *B. japonicum* T21-2 led to the formation of more effective symbiotic systems on soybean roots in the stages of flower bud and flowering. Therefore, the plants had greater number and mass of root nodules than the plants treated only with the inoculants and the plants treated only with succinic acid. According to the nitrogen-fixing activity, those symbiotic systems were more effective throughout the vegetative period (Table 1, Fig. 1). Thus, we can state that the most distinct difference between the parameters of nodulation activity of rhizobia, as well as the nitrogen-fixing activity of symbiotic systems developed with their participation of this variant, and the parameters of control 1 manifested in the stages of flower bud and flowering. High nitrogen-fixing activity in this period is crucial for soybean as it absorbs the greatest amount of nitrogen starting from the flower bud stage until flowering, which is when its vegetative mass grows intensively.

The literature contains a large amount of data from conflicting results regarding the influence of brassinosteroids on symbiotic apparatus of plants: from decrease in number of nodules and length of roots to increase in number and mass of nodules and their nitrogenase activity. In particular, soaking seeds of pea in a solution of 24-epibrassinolide led to increases in the number of nodules and nitrogenase activity (Shahid et al., 2011). Treatment of leaves with epibrassinolide also increased the number and mass of nodules in groundnut (Vardhini et al., 1999) and French beans (Upreti et al., 2004). At the same time, treatment of soybean roots with it

decreased the nodule formation and nitrogen fixation (Hunter et al., 2001). The results of our studies confirm the positive effect of exogenous 24-epibrassinolide in low concentration on the processes of formation and functioning of root nodules of soybean.

Thus, we determined that compared with the control plants and plants treated with succinic acid, pre-sowing treatment of soybean seeds with 24-epibrassinolide in 0.00001 g/L concentration with subsequent inoculation with *B. japonicum* T21-2 produced the most pronounced stimulation of processes of formation and functioning of symbiotic systems of soybean in optimal cultivation conditions and caused formation of a powerful symbiotic apparatus with high nitrogen-fixing activity on the roots of those plants. However, subject to water deficit, despite substantial stimulation of nodulation parameters by 24-epibrassinolide, the intensity of nitrogen-fixation was the highest in the plants cultivated from seeds treated with succinic acid and then the inoculant.

Content of free and complex forms of cytokinins in the root nodules of soybean treated with succinic acid and epibrassinolide against the background of various water supply. Cytokinins play a key role in organogenesis of nitrogen-fixing root nodules in legumes (Heckmann et al., 2011). At the same time, researchers assume that processes of cellular division which initiate the formation of those structures are regulated with participation of cytokinins in both the roots and the nodules. In the nodules, compared with the roots, the level of those hormones can increase in common species, such as *Pisum sativum*, *Phaseolus mungo*, *Myrica gale*, and *Vicia faba*. It is believed that it is cytokininin that activate the cellular division and the group of genes associated with it, and also a number of early-nodulation genes, particularly ENOD2 (Suzaki et al., 2013), ENOD12A (Liu et al., 2018), and ENOD40 (Gamas et al., 2017). Such an ability of those phytohormones indicates their active involvement in formation and growth of root nodules.

Analysis of the plant samples revealed a number of changes in accumulation and ratio of the cytokinininisoforms we identified in the root nodules of soybean treated with succinic acid and 24-epibrassinolide against the background of various water supply of the plants, compared with the plants of respective control variant. It was found that in the flowering stage, in the root nodules of the plants grown from seeds treated with succinic acid, the content of zeatin was higher than the zeatin riboside content, whereas after treatment with 24-epibrassinolide, on the contrary, content of zeatin riboside was higher than zeatin. The observed effect did not depend on the level of water provision to the plants (Table 2). Brassinosteroids are known to generally increase the endogenous content of cytokinins and synergistically affect physiological processes (Bajguz & Piotrowska-Niczyporuk, 2014). Our studies of the content of cytokinins in the root nodules did not confirm this observation, though it allowed us to assume that because the excess of zeatin riboside content over the content of zeatin following the treatment with 24-epibrassinolide was seen in the plants cultivated in both optimal and stress conditions, the treatment of the seeds with this regulator caused a specific inactivation of those phytohormones, because complex forms of cytokinins, to which definitely zeatin riboside belongs, are biologically low-active and mainly perform an accumulating function. Obviously, the protective effect of 24-epibrassinolide on the symbiotic apparatus of soybean in the conditions of water deficit was not associated with the influence of this growth regulator on the functioning of phytohormones of cytokinin nature in the root nodules of soybean, but was rather caused by its effect on other specific protective systems.

In the flowering stage, in the optimal conditions and, the content of zeatin and zeatin riboside in the root nodules of the succinic acid-treated soybean significantly exceeded such in the plants treated only with the inoculant. While having notably heightened zeatin, the plants that had undergone similar treatment and were subject to the 14 day stress had zeatin riboside at the level of the plants of the same variant, cultivated in the optimal conditions, and this was much lower than in the plants treated only with rhizobia. Perhaps, this is related to active conversion of zeatin riboside conjugant into active form – zeatin. Ribosides are known to be quickly convertible into active forms of cytokinins. We should note that two week-long water deficiency led to increase in total content of cytokinins in the root nodules of soybean of all the studied variants, compared with the plants of the similar variants, grown in the conditions of optimal watering. Increase in the content of those phytohormones, in our opinion, indicates activation of protective functions in the tissues enriched with them and also activation of a number of reparative processes. It was confirmed that cytokinins, similarly to other phytohormones, are mediators connecting the stress action and physiological reaction of plant organism. Furthermore, it is known that the effects of cytokinins depend on their concentration in the plant tissues: low concentrations of them activate mitosis, whereas high concentrations inhibit it (Schaller et al., 2014). Based on this assumption, we in turn may assume that the observed decrease in the number of root nodules after a two-week effect of water deficit can be a consequence of inhibition of cellular division in the studied tissues that was related specifically to increase in the content of cytokinins.

In the optimal cultivation conditions, in stage of pods formation, similarly to the previous stage of plant development, treatment of seeds with succinic acid produced the highest content of cytokinins in the root nodules, compared with the plants treated only with the inoculant and the variant where 24-epibrassinolide was used. At the same time, treatment with 24-epibrassinolide decreased the contents of zeatin and zeatin riboside, compared with the plants bacterized without prior use of the growth regulators. Furthermore, while 24-epibrassinolide led to excess of zeatin content over zeatin riboside during flowering, in this plant development stage we saw an opposite effect. The content of zeatin was higher than the content of zeatin riboside in both conditions of soybean cultivation (Table 2), indicating growth of biological activity of those phytohormones in this period.

In the plants the watering of which was resumed, the content of cytokinins in the root nodules was mostly much lower than in the previous development stage. The same tendency took place in the plants cultivated in the optimal conditions, though less pronounced (Table 2). The effect that we observed correlates well with the established fact that cytokinin levels are the highest in young nodules and the lowest in aging ones (Syono et al., 1976). However, the effects that we found indicate that the impact of water deficiency enhanced the decrease in the content of cytokinins in the root nodules of soybean at the late growth stages. Compared with control 2, the content of zeatin was higher both after treatment with succinic acid and 24-Epibrassinolide, whereas the content of zeatin riboside decreased in both variants. If we consider the abovementioned fact, that low level of cytokinins in the tissues activates mitosis, and also taking into account excess of zeatin over zeatin riboside in this development stage, we can state activation of cellular division in the tissues of root nodules and intense activation of reparative processes in them, as indicated by the increased nodulation activity of rhizobia and nitrogen-fixing activity of the root nodules. This was especially notable for the variant with 24-epibrassinolide (Tables 1, 2, Fig. 1).

Therefore, we found that the level of water supply caused differentiation effect on the content of free and complex forms of cytokinins in the root nodules of the soybean, causing a number of specific changes in the pattern of accumulation of those phytohormones, depending on pre-sowing treatment of the seeds, presence or absence of the stressor, and plant development stage. Water deficit led to significant increase in the total content of phytohormones of cytokinin nature in the root nodules of the soybean, regardless of method by which the seeds were treated. At the same time, the pre-sowing treatment of seeds with succinic acid provided the best pool of cytokinins in the root nodules, compared with the control plants and the plants treated with 24-epibrassinolide against the backgrounds of both optimal and insufficient water supply. We found that treatment of the seeds with 24-epibrassinolide caused significant excess of the content of reserving form of cytokinins, zeatin riboside, over zeatin during the flowering stage and an opposite situation in the stage of pods formation – content of zeatin exceeded such of zeatin riboside.

Content of photosynthetic pigments in the leaves of soybean plants treated with growth regulators and subject to various water supply. Important physiological features reflecting the condition of plant organism, peculiarities of course of the productive process, and degree of adaptation of the plants to stress factors are contents and ratios of photosynthetic pigments (chlorophylls *a* and *b*, carotenoids, anthocyanins, etc.) (Croft & Chen, 2017). Moreover, changes in the pigment complex, their dynamics and rates of accumulation serve as one of the indirect indicators of efficiency of legume-rhizobial symbiosis.

Drought induces changes in the quantitative and qualitative compositions of pigments in the plants. Some authors observed increase in the content of green pigments following decrease in soil moisture down to a certain level. This effect is considered as a protective growth slowdown stage, during which cellular structures, including chlorophyll, intensively recover. Strong impact of drought was observed to ruin plastids, entailing decrease in chlorophyll content. Carotenoids perform the protective role, and their amount can rise during drought (Mak et al., 2014; Jingnan et al., 2021).

It was determined that the pre-sowing treatment of seeds with 24-epibrassinolide with alternately rhizobial inoculation led to increase in the content of photosynthetic pigments in the soybean leaves, compared with other studied variants, both against the background of optimal and insufficient water supply. Treatment of the seeds with succinic acid was less effective. The existing data regarding the influence of 24-epibrassinolide on the content of photosynthetic pigments in the leaves of plants, during both optimal and stressful conditions of their cultivation are quite conflicting. Some studies suggest increase in the content of photosynthetic pigments after treatment with 24-epibrassinolide (Janeczko et al., 2007; Eleiwa et al., 2011; Shkliarevskyi et al., 2019). Others report that this growth regulator had no effect on the content of photosynthetic pigments (Kočová et al., 2010). Results of our studies correlate with the former. Given increases in photosynthetic pigments after treatment with 24-epibrassinolide in both optimal and stress conditions of soybean cultivation, such a reaction of the plant can be considered a specific response to its treatment with this growth regulator, rather than adaptive response of the plant to stress. That means, use of 24-epibrassinolide for pre-sowing treatment of seeds promoted formation of a powerful pigment complex in the leaves of the soybean, regardless of their water provision. Considering the role of pigments in the formation of protective mechanisms of photosynthetic apparatus of plants, oriented at increase in their resilience to unfavorable environmental factors and drought in particular, the 24-epibrassinolide-induced increase in content of chlorophylls and carotenoids in the soybean leaves can indicate not only the formation of a powerful photosynthetic apparatus in such plants, which positively influences their productivity, but also a partial alleviation of the negative impact of water deficit those plants experienced. Thus, we determined that treatment of the soybean seeds with 24-epibrassinolide produced the highest content of photosynthetic pigments in the leaves of all the variants we studied. Two-week impact of water deficit led to decrease in the chlorophyll content. At the same time, use of the growth regulators mitigated the negative effect of the stress and protected the pigment complex from ruination, perhaps because of the increase in the content of carotenoids, which, as known, protect the light-harvesting complex or activate synthesis of enzymes that partake in biosynthesis of chlorophyll.

Grain productivity of the soybean treated with succinic acid and epibrassinolide when subject to different water supply. The criterion of photosynthetic activity and efficiency of legume-rhizobial symbiosis can be grain productivity. The degree of realization of biological potential of various crops, including soybean, depends heavily on weather conditions. Soybean is a crop that is especially sensitive to water deficit during flowering and ripening of beans (Petrychenko et al., 2016). Plants suffering drought in this period lose mass, number of beans, number of grain, and their mass, which results in decrease in grain yield per unit area (Ghassemi-Golezani & Lotfi, 2012). Our studies revealed that impact of 14-day drought in the stages of three true leaves – flowering led to almost two-fold decrease in grain productivity of the soybean plants of all variants we studied, compared with the plants of similar variants grown in optimal conditions (Table 3). Treatment of seeds with solutions of succinic acid and 24-epibrassinolide provided increase in the studied parameter regardless of level of water provision. At the same time, the most efficient pre-sowing treatment of seeds was use of 24-epibrassinolide, resulting in the greatest increase in the soybean productivity.

Conclusion

Therefore, provision of plants with water during vegetation and treatment of seeds with growth regulators in complex with inoculants influenced the composition of the pigment complex of soybean-rhizobial symbiosis, the intensity of nitrogen assimilation by the root nodules, and the number of cytokinins they accumulated.

Lack of water in the period between three true leaves and flowering caused differentiation impact on the Titan variety of soybean in symbiosis with nodule bacteria *B. japonicum* T21-2, including a complex of specific changes in the quantitative composition of pigments in the leaves and regulation of formation processes and functioning of root nodules, which in turn impaired nitrogen and carbon metabolisms of the plants and, as a result, reduced grain productivity.

Use of 0.00001 g/L concentration of 24-epibrassinolide for pre-sowing treatment of the seeds provided formation of effective symbiotic systems with high nitrogen-fixing activity on the soybean roots, caused a number of specific changes in the pattern of accumulation of free and complex forms of cytokinins in the root nodules of those plants, leading to significant excess of the content of reserving form of cytokinins, zeatin riboside, over the content of zeatin in the flowering stage and the opposite effect in the stage of pods formation – excess of zeatin content over the content of zeatin riboside. Following this treatment, concentration of photosynthetic pigments in soybean leaves was the highest in all variants we studied, both in the optimal conditions and when subject to water deficiency. As a result, the grain yield increased ten percent, regardless of watering level.

Use of succinic acid in 0.01 g/L concentration for pre-sowing treatment of seeds, similarly to 24-epibrassinolide, stimulated the processes of formation and functioning of symbiotic systems of soybean regardless of watering level. At the same time, in the conditions of water deficit, it caused the highest level of nitrogen-fixing activity against the background of the lowest number of root nodules of all the studied variants. Treatment of the seeds with succinic acid produced the highest pool of cytokinins in the root nodules compared with the control plants and the plants treated with 24-epibrassinolide against the background of both optimal and insufficient water supply. Also, it led to significant increase in grain productivity of the soybean, though it was lower than in the variant where 24-epibrassinolide was used.

Further, it would be practical to carry out studies in field conditions, focusing on effects of succinic acid and epibrassinolide on the legume-rhizobial symbioses formed with participation of various soybean genotypes in regions with different soil and climatic conditions. This would expand the knowledge of the role growth regulators play in the formation of adaptive reactions of rhizobia-bacterized plants to various watering condition in order to ensure maximum realization of their symbiotic and productive potentials.

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