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Original Article

Comparative study of symbiotic activity of legumes when using Risotorphin and Epin-extra

Estudo comparativo da atividade simbiótica de leguminosas em uso de Risotorfina e Epin-extra

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Abstract

In a vegetation experiment with soybean plants of the Svapa and Mageva varieties and in a field experiment with bean plants of the Geliada and Shokoladnitsa varieties, we studied the effect of pre-sowing treatment of the seeds of these plants with Rizotorfin and Epin-extra on the nitrogenase activity of the nodules of these plants and their ultrastructure. Analysis of the ultrastructure of the nodule tissue of beans and soybeans was carried out in the flowering phase. It was found that the highest indices of the mass and number of nodules and the activity of nitrogenase in them were found in bean plants of the Heliada cultivar when the seeds were treated with Epin-extra against the background of inoculation with Rizotorfin, where the largest area of symbiosomes, volutin and their number was noted in the nodules. Beans of the Shokoladnitsa variety showed the protective effect of Rizotorfin. In the nodules of soybean plants of the Svapa variety, the seeds of which were treated with Epin-extra against the background of inoculation with Rizotorfin, the presence of a large number of symbiosomes, bacteroids, volutin inclusions with a larger area and a minimum number of inclusions of poly-β-hydroxybutyric acid (PHB) was noted, and the highest indicators of symbiotic activity. Soybean plants of the Mageva variety showed the protective effect of Rizotorfin. The efficiency of the symbiotic system was determined by the number and weight of nodules and the activity of the nitrogenase enzyme.

Keywords: beans, soybeans, symbiotic activity, nitrogenase, Rizotorfin.

Resumo

Em um experimento de vegetação com plantas de soja das variedades Svapa e Mageva e em um experimento de campo com plantas de feijão das variedades Geliada e Shokoladnitsa, o efeito do tratamento pré-semeadura das sementes dessas plantas com Rizotorfin e Epin-extra sobre a atividade nitrogenase dos nódulos dessas plantas e sua ultraestrutura foi examinado. A análise da ultraestrutura do tecido do nódulo de feijão e soja foi realizada na fase de floração. Dessa forma, constatou-se que os maiores índices de massa, número de nódulos e a atividade da nitrogenase foram encontrados em plantas de feijão da cultivar Heliada quando as sementes foram tratadas com Epin-extra no contexto da inoculação com Rizotorfina, em que o maior área de simbiossomas, volutina e seu número foi anotado nos nódulos. O feijão da variedade Shokoladnitsa mostrou o efeito protetor do Rizotorfin. Nos nódulos de plantas de soja da variedade Svapa, cujas sementes foram tratadas com Epin-extra no contexto da inoculação com Rizotorfin, a presença de um grande número de simbiossomas, bacteróides, inclusões de volutina com uma área maior e um número mínimo de inclusões de ácido poli-β-hidroxibutírico (PHB) foi notado, bem como os indicadores mais altos de atividade simbiótica. As plantas de soja da variedade Mageva apresentaram o efeito protetor do Rizotorfin. A eficiência do sistema simbiótico foi determinada pelo número e peso dos nódulos e pela atividade da enzima nitrogenase.

Palavras-chave: feijão, soja, atividade simbiótica, nitrogenase, Rizotorfina.

1. Introduction

Currently, issues related to resource conservation are relevant. The main energy costs of the agro-industrial complex represent the costs of production and use of nitrogen fertilizers (Boswell et al., 1985; Hamid et al., 2021; Liu et al., 2021; Yao et al., 2021; Molajou et al., 2021a; Molajou et al., 2021b). Due to the high cost of nitrogen fertilizers, their use in agricultural production is no more than one third of the need. The use of alternative sources of nitrogen is an urgent task not only due to the lack of fertilizers, but also in connection with the need to

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introduce adaptive farming systems, where the capabilities of microorganisms can be used to meet the most varied needs of plants (Tikhonovich et al., 2012).

Leguminous plants have a unique ability to overcome the deficiency of bound nitrogen in the soil due to symbiotic nitrogen fixation in the nodules of these plants. Biological nitrogen fixation is the only clean and safe way of supplying plants with available nitrogen, in which contamination of soil, water and air is completely excluded. Biological nitrogen not only does not pollute the environment, but even significantly heals the ecological situation in nature, since it does not penetrate into groundwater, does not accumulate in wastewater, and does not violate the biological balance in the soil. Its use is one of the energy-saving, cost-effective technologies that reduce the consumption of nitrogen by plants from the soil and the need to apply expensive nitrogen fertilizers to the soil.

The practical use of symbiotic nitrogen fixation is carried out by bacterizing legumes with appropriate preparations based on highly efficient and competitive cells of nodule bacteria (Dobbelaere and Okon, 2007; Volobueva and Skorobogatova, 2010). Recently, in order to increase the efficiency of the legume-rhizobial symbiosis, growth regulators have been used (Oldroyd et al., 2011; Alemneh et al., 2020; Kshnikatkina et al., 2020; Mamenko et al., 2020; Paço et al., 2020). An increase in the yield of leguminous plants significantly depends on the efficiency of legume-rhizobial symbiosis. Therefore, in their work, we used the preparations Rizotorfin and Epin-extra to increase the efficiency of the symbiotic activity of legumes.

The purpose of this work is to carry out a comparative study of the symbiotic activity of bean plants of varieties Geliada and Shokoladnitsa and soybean plants of varieties Svapa and Mageva when the seeds of these plants are treated with Rizotorfin and Epin-extra.

2. Material and Methods

In the experiments, we used beans (*Phaseolus vulgaris* L.) varieties Geliada and Shokoladnitsa and soybean plants (*Glycine max* L.) varieties Mageva and Svapa. The seeds were obtained at the Federal State Budgetary Scientific Institution of the Federal Scientific Center of Legumes and Goat Crops (Oryol region). The description of the varieties was given earlier (Teixeira-Guedes et al., 2019; Aserse et al., 2020; Le et al., 2021). Studies with soybean plants were carried out in the conditions of a growing house of the nitrogen metabolism laboratory of the Institute of Plant Physiology named after K.A. Timiryazev, Russian Academy of Sciences (Moscow) in vessels with 6 kg of quartz sand on a modified nitrogen-free Knop nutrient mix. Nitrogen was introduced in the form of $Ca(NO_3)_2$ at a dose of 430 mg per vessel in the phase of a quaternary leaf, and then, after 2 weeks, Knop's mixture with microelements was added according to Rinkis. Seeds of soybean plants of varieties Svapa and Mageva were soaked for 3 hours in a solution of the growth regulator Epin-extra at a concentration of 10^{-6}M, immediately before sowing, they were treated with Rizotorfin strain 634, based on nodule bacteria of soybean *Bradyrhizobium japonicum*, according to the experimental scheme: 1 - treatment of plant seeds soybean varieties Swapa Rizotorfin (control); 2 - treatment of seeds of soybean plants of the Swapa variety with Epin-extra against the background of inoculation with Rizotorfin; 3 - treatment of seeds of Mageva soybean plants with Rizotorfin (control); 4 - treatment of seeds of soybean plants of the Mageva variety with Epin-extra against the background of inoculation with Rizotorfin (Dem'yanova-Roj and Bortsova, 2014). Repetition 5 times, each vessel contains 10 plants. Studies with beans plants were carried out under the conditions of a field experiment at the Federal State Budgetary Scientific Institution of the All-Russian Research Institute of leguminous and cereal crops (Oryol region). The conditions for the experiment were described earlier (Pavlyuchik et al., 2019; Matvienko et al., 2022). Seeds of bean plants of both varieties were soaked for 3 h in a solution of Epin-extra at a concentration of 10^{-6}M, then dried, and treated with Rizotorfin before sowing. Experimental options: 1 - control (without treatment), 2 - seed treatment with Rizotorfin, 3 - seed treatment with Epin-extra, 4 - seed treatment with Epin-extra against the background of inoculation with Rizotorfin. The repetition was 4-fold, the arrangement of the variants was randomized. Rizotorfin (*Rhizobium luguminosarum* bv. Phaseoli, strain 700). Rizotorfins were obtained at the All-Russian Research Institute of Agricultural Microbiology (St. Petersburg). Epin-extra is a growth regulator, the active ingredient is epibrassinolide (EPB). The drug was purchased from the D. N. Pryanishnikov VNIIA (Moscow) (Abril et al., 2007).

The efficiency of the legume-rhizobial symbiosis of leguminous plants was judged by the parameters of the ultrastructure of the nodules and the efficiency of the symbiotic system (Montanuci et al., 2013; Televičiūtė et al., 2020; Alashbayeva et al., 2021). The efficiency of the symbiotic system was determined by the number and weight of nodules and the activity of the nitrogenase enzyme (Maslennikov et al., 1996; Alemneh et al., 2020; Han et al., 2020).

Analysis of the ultrastructure of the nodule tissue of beans and soybeans was carried out in the flowering phase. Fresh nodules were analyzed (most often in the middle part). Before fixation, the nodules were cut with a razor into small 1 mm pieces and immersed in 0.1 M phosphate buffer (pH 7.4) in 10 replicates. For 2.5 - 3.0 h at 0 °C (in a cold room), they were fixed in a 2.5% solution of glutaraldehyde, after which the nodules were washed in phosphate buffer 3 times for 30 min at 0 °C and flooded with octal fixative overnight (in cold room). The nodules washed in phosphate buffer were dehydrated in alcohols of increasing concentrations and acetone, and then poured into a mixture of Epones. The nodules were fixed in glutaraldehyde according to the Sabatini method (Santos et al., 2019). The obtained sections were placed on grids with a formvar substrate, contrasted with 1% aqueous solution of uranyl acetate and 0.2% lead citrate. The preparations were viewed in a TEMSCAN 100CX2 electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV and an instrumental magnification of 300000x. Photographic plates for nuclear research were used for photography. Statistical processing of electron microscopic studies was also carried out using a MOP – VIDEOPLAN device from Reichert (Austria) (Volobueva and Skorobogatova, 2010).

3. Result and Discussion

Analysis of the data on the effect of the biological product Rizotorfin and the growth regulator Epin-extra on the symbiotic system of soybean plants of the Svapa and Mageva varieties showed that the highest nitrogen-fixing activity in the nodules of the Mageva and Svapa soybean plants was noted in the fruiting phase. In soybean plants of the Svapa variety, the highest indicators of nitrogen-fixing activity in the nodules were observed when the seeds of this variety were treated with Epin-extra against the background of inoculation with Rizotorfin. In the Mageva variety, the highest indicators of the nitrogen-fixing activity of nodules were noted under the influence of only Rizotorfin (Table 1).

The results of studies on the nitrogen-fixing activity of the nodules of soybean plants of the varieties Svapa and Mageva confirmed the data of our studies of the ultrastructure of the nodules of these plants. Thus, in the nodules of soybean plants of the Svapa variety, whose seeds were treated with Epin-extra, against the background of inoculation with Rizotorfin, the presence of a large number of symbiosomes, bacteroids, volutin inclusions was observed, which had a larger area and a minimum number of PHB inclusions (Table 2) (Volobueva and Skorobogatova, 2010). In soybean plants of the Mageva variety, under the influence of Rizotorfin, an increase in the area and number of symbiosomes, volutin inclusions is observed (Table 2). The area and amount of PHB in this variant was minimal, which indicated active nitrogen fixation.

Volutin - nitrogen and phosphorus-containing substance, is considered as a reserve substance, a reserve of inorganic phosphates. Volutin serves as a storage reservoir for phosphate, an important precursor to ATP. Symbiotic nitrogen fixation carried out by rhizobia is a rather energetically consuming process, as a result of which a

Table 1. Influence of Epin-extra and Rizotorfin on the symbiotic activity of nodules of soybean plants of varieties Svapa and Mageva.

Indicator	Phase development	Option			
		Swapa + Rizotorfin (control)	Swapa + Epin-extra	Mageva + Rizotorfin (control)	Mageva + Epin- extra
Weight roots with nodules, g / plant	budding	2.7±1.1	2.7±1.1	1.7±0.9	2.1±1.0
	bloom	3.7±1.3	4.9±1.5	2.6±1.2	2.8±1.2
	fruiting	3.1±1.2	4.3±1.5	4.9±1.5	3.0±1.2
Number nodules, pcs / plant	budding	26±3.6	30±3.9	24±3.5	16±2.8
	bloom	38±4.4	42±4.6	36±4.2	27±3.7
	fruiting	27±3.7	33±4.1	21±3.2	21±3.2
Weight nodules, mg / plant	budding	463±15.4	550±16.7	345±13.2	289±12.1
	bloom	620±17.7	724±19.2	660±18.4	460±15.3
	fruiting	882±21.2	1163±24.3	1403±26.7	1203±24.7

Table 2. Changes in the ultrastructure of symbiosomes and bacteroids of soybean plants.

Indicator	Option			
	Swapa + Rizotorfin (control)	Swapa + Epin-extra	Mageva + Rizotorfin (control)	Mageva + Epin- extra
Symbiosome area, µm2	1.55 ± 0.070	3.30 ± 0.240	1.89 ± 0.088	1.33 ± 0.049
Bacteroid area, µm2	0.34 ± 0.013	0.49 ± 0.054	0.33 ± 0.012	0.26 ± 0.008
PHB area, µm2	0.021 ± 0.002	0.016 ± 0.001	0.023 ± 0.001	0.026 ± 0.001
Volutin square, µm2	0.030 ± 0.002	0.034 ± 0.001	0.039 ± 0.002	0.026 ± 0.002
Number of symbiosome	5.64 ± 0.800	8.08 ± 0.87	8.43 ± 0.410	7.23 ± 1.430
Number of bacteroids	2.73 ± 0.140	3.63 ± 0.220	3.60 ± 0.18	2.57 ± 0.082
Number of PHB	1.64 ± 0.110	1.61 ± 0.098	1.19 ± 0.040	1.46 ± 0.074
Number of volutin	3.98 ± 0.230	6.30 ± 0.49	3.25 ± 0.130	2.51 ± 0.140

significant amount of energy is consumed, therefore, the presence of volutin granules can be considered as one of the possible sources of energy for this process. The area and number of volutin inclusions were greater in those variants where the highest nitrogen-fixing activity was observed in the nodules of leguminous plants (Volobueva and Skorobogatova, 2010).

PHB - it is a reserve nutrient, an endogenous store of energy and carbon for prokaryotes. Usually, with active nitrogen fixation, the content of PHB in bacterial cells is minimal, since the synthesis and decomposition of PHB are most intense in this case. The role of PHB is mainly in the regulation of the use of photoassimilates entering the bacteroids, and the polymer content can, to a certain extent, be judged on the supply of carbohydrate substrates to bacteroids. A low level of nitrogen fixation in nodules can be determined by the inability of the host plant to assimilate all nitrogen fixed by bacteroids, that is, insufficient transport of bound nitrogen from the nodules to the aboveground part of the plant. The content of PHB is usually insignificant in bacteroids precisely when the cells are most actively breathing and fixing nitrogen, which means that their need for energy and reduced equivalents is especially high in aerobic bacteria, which include nodule bacteria. SIP exchange is usually closely related to the functioning of the Krebs cycle. Therefore, the content of PHB and volutin inclusions can serve as a new additional characteristic of the activity of the symbiotic system for some rhizobia species.

Analysis of the effectiveness of the symbiotic system of beans of the varieties Geliada and Shokoladnitsa showed that the highest indices of the mass and number of nodules and the activity of nitrogenase in them were observed in the plants of the bean variety Geliada when the seeds were treated with Epin-extra against the background of inoculation with Rizotorfin (Volobueva and Skorobogatova, 2010). The bean variety Shokoladnitsa showed the protective effect of Rizotorfin. The highest indices of nodule weight and nitrogenase activity were observed when seeds were treated with Rizotorfin alone (Table 3). These data were confirmed by studies of the ultrastructure of their nodules.

Thus, in the nodules of bean plants of the Geliada variety, the largest area of symbiosomes, volutin inclusions and their number were noted in the variant with Epin-extra treatment against the background of Rizotorfin inoculation. In plants of the variety Shokoladnitsa, the largest area and number of bacteroids, volutin inclusions with a minimum amount of PHB, were observed in the variant with seed treatment with Rizotorfin only (Table 4).

Table 3. Influence of Epin-extra and Rizotorfin on the symbiotic activity of bean nodules of varieties Geliada and Shokoladnitsa.

Option	Root mass with nodules g / plant	Number nodules, pcs / plant	Mass of nodules, mg / plant	Nitrogenase Activity, NMol / plant / hour
Heliada (control)	1.92 ± 1.40	10 ± 3.20	41 ± 6.47	725 ± 27.2
Heliada + Rizotorfin	1.86 ± 1.38	16 ± 4.04	65 ± 8.14	761 ± 27.87
Heliada + Epin-extra	1.85 ± 1.37	17 ± 4.16	69 ± 8.45	1776 ± 42.57
Heliada + Epin-extra + Rizotorfin	1.89 ± 1.39	17 ± 4.16	78 ± 8.92	2827 ± 53.71
Chocolate girl (control)	2.20 ± 2.22	18 ± 4.29	31 ± 5.62	362 ± 19.22
Shokoladnitsa + Rizotorfin	2.03 ± 2.02	23 ± 4.84	90 ± 9.58	2319 ± 48.64
Shokoladnitsa + Epin-extra	1.89 ± 1.39	12 ± 3.50	36 ± 6.06	1123 ± 33.85
Shokoladnitsa + Epin-extra + Rizotorfin	1.67 ± 1.31	18 ± 4.29	43 ± 6.62	1087 ± 33.30

Table 4. Changes in the ultrastructure of symbiosomes and bacteroids of bean plants.

Option	Area, μm2				Quantity			
	S	B	PHB	V	S	B	PHB	V
Heliada (control)	–	0.061 ± 0.0029	0.40 ±	0.011 ± 0.0002	–	20.16 ± 2.26	3.21 ± 0.10	7.15 ± 0.35
Heliada (control)	–	0.091 ± 0.0027	0.033 ± 0.0019	0.014 ± 0.0002	–	23.56 ± 2.28	1.62 ± 0.08	9.51 ± 0.37
Heliada + Epin-extra	2.5 ± 0.10	0.39 ± 0.016	0.031 ± 0.0013	0.016 ± 0.003	12.13 ± 0.40	3.26 ± 0.13	1.42 ± 0.09	9.18 ± 0.30
Heliada + Epin-extra + Rizotorfin	2.7 ± 0.13	0.47 ± 0.017	0.023 ± 0.0013	0.018 ± 0.0004	14.22 ± 0.42	4.28 ± 0.14	0.62 ± 0.10	10.41 ± 0.28
Chocolate girl (control)	–	0.42 ± 0.026	0.40 ± 0.0026	0.024 ± 0.006	–	14.8 ± 1.97	1.69 ± 0.07	6.11 ± 0.22
Shokoladnitsa + Rizotorfin	–	0.56 ± 0.028	0.023 ± 0.0012	0.033 ± 0.007	–	24.70 ± 1.93	1.09 ± 0.06	9.08 ± 0.37
Shokoladnitsa + Epin-extra	–	0.48 ± 0.027	0.035 ± 0.009	0.024 ± 0.006	–	15.7 ± 1.96	1.60 ± 0.06	7.0 ± 0.31
Shokoladnitsa + Epin-extra + Rizotorfin	–	0.51 ± 0.013	0.031 ± 0.009	0.026 ± 0.0007	–	16.41 ± 1.05	1.52 ± 0.09	7.58 ± 0.37

Note: S = sibosomes; B = bacteroids; PHB = poly-ß-hydroxybutyric acid; V = valuntin.

4. Conclusion

Thus, comparing the symbiotic activity of soybeans and beans of different varieties when treated with Epin-extra and Rizotorfin, it can be noted that the variety-specificity of legumes on the effect of these drugs was manifested. In bean plants of the Geliada variety and soybean plants of the Svapa variety, the highest indicators of symbiotic activity in the nodules were observed after treatment with Epin-extra against the background of inoculation with Rizotorfin. The bean plants of the Shokoladnitsa variety and the soybean plants of the Mageva variety showed the protective effect of Rizotorfin, under the influence of which there was an increase in the number and weight of nodules and the activity of the nitrogenase enzyme in them. A correlation was noted between symbiotic activity and nodule ultrastructure. Thus, the processes of nitrogen fixation were most active in the variants with a larger amount of bacteroids, with a larger amount and a larger area of granules of volutin inclusions, while the content of PHB inclusions in these variants was minimal. Therefore, the content of inclusions of volutin and PHB in cells can serve as an additional characteristic of the activity of the symbiotic system for some species of nodule bacteria (Volobueva and Skorobogatova, 2010).

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