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ANALYSIS OF THE EFFECTS OF JOINT INOCULATION BY ARBUSCULAR MYCORRHIZAL FUNGI AND RHIZOBIA ON THE GROWTH AND DEVELOPMENT OF PEA PLANTS *Pisum sativum* L.

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Abstract

Co-inoculation of plants with arbuscular mycorrhizal (AM) fungi and nitrogen-fixing bacteria of the order *Rhizobiales* (rhizobia) can have a stimulating effect on plant growth and development. This influence can be considered as the synergistic effect of two microorganisms on a plant in a multicomponent system and as the result of the mutual influence of microorganisms on each other. However, the mechanisms underlying the mutual influence of microorganisms remain insufficiently understood. In the presented work, it was shown for the first time that in the case of joint inoculation of pea plants with fungi of arbuscular mycorrhiza and rhizobia, the method of introduction microorganisms may be important. The results may indicate the presence of competition of microorganisms for a niche in the plant during sequential inoculation. The purpose of our research was to study the possibility of selection of the effective combinations of AM and rhizobia strains for inoculation of such important agricultural crop as *Pisum sativum* L. as well as estimation of influence on the productivity of plants. In this work, we analyzed the effects of joint inoculation of pea plants *Pisum sativum* L. with the fungus *Rhizophagus irregularis* BEG144 and the rhizobial strain *R. leguminosarum* bv. *viciae* RCAM 1026. For this purpose, the level of induction of markers development of two types of symbiosis, the degree of root mycorrhization were assessed, as well as the biometric parameters of plants. The research was performed using pea seedlings of the cultivar Frisson grown under sterile conditions. The isolate of the fungus *Rhizophagus irregularis* BEG144 was used for inoculation. An inoculum was obtained from the mycorrhized roots of *Plecthrantus australis.* The inoculum was introduced into a moisture substrate before planting the pea seedlings. The scheme of sequential inoculation was used, according to which pea plants were first inoculated with AM fungi *R. irregularis* BEG144 and 7 days after cultivation, the rhizobial strain *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 were introduced into the system. The experimental scheme included the following options: without inoculation (control), *R. leguminosarum* (Rlv), *R. irregularis* (AM), *R. irregularis* + *R. leguminosarum* (AM + Rlv). 9 and 21 days after planting (2 and 14 days after bacterial inoculation), the material was collected to analyze the expression of genes - markers of the legume-rhizobial symbiosis development. On day 21 after planting, the plants were collected in order to determine their biometric parameters, as well as the markers of the symbiosis development. The lateral roots of the plants were cut off and frozen in liquid nitrogen. After isolation of the total RNA, cDNA was synthesized on the RNA template using reverse transcriptase using oligo(dT) primers. For the analysis, quantitative PCR combined with reverse transcription (RT-PCR) was performed (a CFX96 Real-Time instrument, «Bio-Rad Laboratories», USA). The total weight of plants and the weight of the root system significantly increased in case of monoinoculation with rhizobia, monoinoculation with AM fungi, as well as joint inoculation, compared with the control variant. However, no significant differences in these biometric parameters between the variant with monoinoculation with rhizobia or AM fungi and double inoculation were found. Probably, upon inoculation with several endosymbionts, competition arose between them at the stage of penetration into the plant, which led to a decrease in the intensity of plant infection with rhizobia. This was evidenced

by the absence of additional stimulation of the *Enod5* and *Sym37* gene expression during double inoculation, which are activated in the plant during the development of rhizobial infection. Upon double inoculation, we also did not reveal additional stimulation of the expression of marker genes of symbiosis with AM fungi — *PT4, TI, RAM1* and *DELLA3*. This correlated with the absence of significant differences in all biometric parameters between the variant with monoinoculation with rhizobia or AM fungi and double inoculation, which does not allow us to conclude about the positive effect of double inoculation on the growth and development of pea plants under the conditions of this experiment. The results of data analysis using the experimental scheme used may indicate the presence of competition of microorganisms for a niche in the plant, leading to a decrease in intra-root mycorrhizal colonization and the level of induction of markers that are activated during the development of rhizobial infection.

Keywords: plant-microbe interactions, rhizosphere, symbiosis, arbuscular mycorrhiza, inoculation, rhizobia, *Rhizophagus irregularis*, gene expression, *Pisum sativum*

Currently, world agriculture is facing the problem of transition from the intensive to a sustainable type aimed at environmentally friendly farming [1, 2]. The use of mineral fertilizers, which are costly and severely damaging to the environment, should be reduced [3]. The ability of most agricultural crops to form endosymbioses with mycorrhizal fungi of the phylum *Glomeromycota* (the arbuscular mycorrhiza, AM) plays a significant role in improving plant mineral nutrition, primarily phosphorus nutrition, and productivity. An alternative to the use of mineral nitrogen fertilizers can be the biological nitrogen. Symbiosis between legumes and bacteria of the order *Rhizobiales* called rhizobia plays a significant role in biological nitrogen fixation.

Co-inoculation with AM fungi and rhizobia also positively affects plants, mainly due to the improvement of mineral nutrition and inhibition of fungal pathogens [4-7]. Since the lack of phosphorus and nitrogen in the soil is one of the limiting factors, co-inoculation stimulates plant growth. In this case, the stimulating effect can be due both to the synergistic effect of microorganisms on a plant in a multicomponent system and to the influence of microorganisms on each other. However, the mechanisms underlying the mutual influence of microorganisms remain poorly understood.

It is known that AM fungi provide plants with available phosphorus which plays an important role in energy metabolism and especially in nitrogen fixation as an energy-consuming process [8]. Mobilization of phosphorus can stimulate the activity of nitrogenase and nitrogen fixation by rhizobia [9-11]. Indeed, upon co-inoculation of *Phaseolus vulgaris* L. and *Medicago arborea* L. with AM fungi and rhizobia, a significant increase in the number and weight of nodules occurred as compared to monoinoculation [12, 13]. Co-inoculation led to a greater accumulation of phosphorus and nitrogen in the bean shoots and to a better phosphorus utilization compared to control plants [12]. The positive effect of double inoculation with AM fungi and rhizobia on growth, nutrient uptake, and nitrogen fixation occurred in soybeans [4], cowpea [14], and peas [15]. The double inoculation of pea plants leads to an increase in their biomass, the number and weight of nodules, the efficiency of nitrogen fixation and transport of bound forms of nitrogen [16, 17].

The mutual influence may be due to signaling molecules that AM and rhizobia fungi exchange with the plant, i.e., Myc and Nod factors. Co-inoculation of soybean *Glycine max* (L.) Merr. with *Bradyrbizobium japonicum* 61-A-1O1 significantly enhanced the colonization of plant roots by the fungus *Glomus mosseae* [18]. The Nod factors of rhizobia can influence stimulation of the mycorrhiza formation, since plant inoculation with strains defective in the synthesis of these signaling molecules did not lead to an increase in root colonization by the fungus. The positive effect can be associated with the structural similarity of the Nod and Myc factors, since the structure of Myc factors is similar to that of Nod factor with minimal substitutions on the molecular backbone [19]. In addition, Nod

factors activate the synthesis of flavonoids in plant roots which also have a stimulating effect on root colonization by AM fungi [18].

Co-inoculation also increases in resistance to pathogens. In plants inoculated with AM fungi the incidence and severity of symptoms caused by *Rhizoctonia*, *Fusarium*, or *Verticillium*, as well as oomycetes *Phytophthora*, *Pythium*, and *Aphanomyces*, decrease [20]. The biocontrol properties of rhizobia can be associated with the release of lytic enzymes and antimicrobial secondary metabolites that suppress the pathogens [21]. With co-inoculation, the effect of AM and rhizobia fungi can be enhanced. Thus, in soybeans, double inoculation significantly reduced the signs of red crown rot. This effect correlated with the high expression of the *PR2*, *PR3*, *PR4*, and *PR10* genes which control defense reactions [22]. However, it remains unclear how AM and rhizobia fungi in a multicomponent system avoid mutual negative influence caused by the release of lytic enzymes and antimicrobial compounds.

Probably, due to the peculiarities of the mutual influence, the effect of AM fungi and rhizobia on a plant during co-inoculation varies greatly depending on the strains used. In this case, both a significant stimulation of plant growth and the absence of a pronounced effect can occur [15, 23]. For example, the yield and nitrogen content in pea plants were maximum when co-inoculated with the fungus *Glomus clarum* NT4 and the highly efficient strain *Rhizobium* LX43 [15]. On the contrary, the fungus strain *Glomus mossae* NT6 increased the yield of peas when co-inoculated only with the ineffective rhizobia strain 175P4 [15]. Consequently, through the selection of effective strain combinations of fungi and rhizobia, it is possible to influence crop yields but it is necessary to understand what mechanisms underlie this interaction.

The presented work shows for the first time that the mode of introducing fungi of arbuscular mycorrhiza and rhizobia when co-inoculating pea plants is important. Namely, with sequential inoculation, the microorganisms compete for a niche in the plant.

Our goal was to investigate the possibility and effect of co-inoculation of pea *Pisum sativum* L. seed with a combination of the arbuscular mycorrhiza fungus *Rhizophagus irregularis* BEG144 and rhizobia *Rhizobium leguminosarum* bv. *viciae* RCAM 1026, to assess the induction of markers of two types of symbiosis, the degree of root mycorrhization, and biometric parameters of plants.

Material and methods. Pea (*Pisum sativum* L., cv. Frisson) seeds were sterilized for 10 min with concentrated H_2SO_4, washed 3 times with sterile distilled water, and germinated in Petri dishes with 1% agar (4 days at room temperature in the dark).

For inoculum of *Rhizophagus irregularis* BEG144 (provided by the International Bank for the Glomeromycota, Gidon, France), the mycorrhized roots of *Plectranthus australis* were washed in running tap water, rinsed 3 times with distilled water, cut into fragments ~ 1 cm long, and examined under a microscope to detect fungal propagules (vesicles and spores). The inoculum (1.3 g per pot) was introduced into a moistened substrate to a depth of 3 cm before planting pea seedlings. In 7 days, *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 was introduced. For the inoculum, the bacteria were cultured at 28 °C on solid TY medium [24] with streptomycin (500 μg/ml) and washed off the plates with autoclaved tap water. The resultant suspension was diluted to $OD_{600} = 0.5$ and used for inoculation (2 ml per pant).

The plants were grown under the controlled conditions using clay marl added with 1 g/l $CaCO_3$ as a substrate (a MLR-352H phytotron, Panasonic, Japan; 16 h day/8 h night, 21 °C, and 60% relative humidity). Pots with the substrate

were pre-sterilized by autoclaving (60 min, 134 °C, 0.22 MPa). A designed nutrient solution (without phosphorus and with a reduced nitrogen concentration) was used for plant feeding. The solution contained macroelements (mmol per l of the substrate) NH_4NO_3 0.16, or 1/10 of the norm, $MgSO_4 \cdot 7H_2O$ 0.51, and K_2SO_4 0.72, and microelements (μmol per l of the substrate) H_3BO_3 9.19, $MnSO_4 \cdot 5H_2O$ 2.28, $ZnSO_4 \cdot 7H_2O$ 0.19, $CuSO_4 \cdot 5H_2O$ 0.08, $(NH_4)_2MoO_4$ 0.03, $CoCl_2 \cdot 6H_2O$ 0.03, and NaFe-EDTA 8.36. Top dressing was carried out once during planting pea seedlings; watering was carried out with distilled water as needed.

The experimental design included no treatment (control without inoculation), inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 (Rlv), inoculation with *Rhizophagus irregularis* BEG144 (AM), and co-inoculation with *R. irregularis* + *R. leguminosarum* (AM + Rlv).

Nine and twenty-one days after planting (2 and 14 days after bacterial inoculation), plant material was collected to analyze the expression of markers of legume-rhizobial symbiosis (genes *Sym10*, *NIN*, *Enod5*, and *Sym37*). On day 21 after planting (day 14 after inoculation with rhizobia), the plants were collected to measure their biometric parameters, to analyze the expression of markers of AM fungus inoculation (genes *PT4*, *TI*, *RAM1*, and *DELLA3*), and to assess the development of symbiosis. The development of AM was assessed using light microscopy as described previously [25].

The lateral roots were cut off and frozen in liquid nitrogen for isolation of total RNA using NucleoSpin® RNA columns as per the manufacturer's method (Macherey-Nagel, Germany). cDNA on the RNA template was synthesized using RevertAidH minus reverse transcriptase (Thermo Scientific, USA) and oligo(dT) primers (Evrogen, Russia). For the analysis, quantitative PCR combined with reverse transcription (RT-qPCR) (CFX96 Real-Time PCR Detection System, Bio-Rad Laboratories, USA) was run as follows: 30 s at 95 °C, 30 s at 54 °C, 40 s at 72 °C (40 cycles); the primers are

PsSym10-F — 5′-GTACTTCATTGGCGGAGACTG-3′;
PsSym10-R— 5′-CCATAAGTTTCACAAGATTTCCAT-3′;
PsNIN-F — 5′-CCGCAAAGAGCATCGGTGTATG-3′;
PsNIN-R — 5′-GCATAGAAAGATCCAATCTGTATAGC-3′;
PsPT4-F — 5′-CTTCACGTGCCATGTTCATC-3′;
PsPT4-R — 5′-GCGTCGGAAACAGCTCC-3′;
PsTI-F — 5′-ACCTTACAGCGTGAGCCTATAAGA-3′;
PsTI-R — 5′-GCGGCCGAGGTACGAAAGGTG-3′;
PsRAM1-F — 5′-GTCCATGATAAGAGACCAAGCACC-3′;
PsRAM1-R — 5′-GGAGGAAGATAATGGAAGGGAAAG-3′;
PsDELLA3-F — 5′-GCAATATAGAATTAACCGCCACAAC-3′;
PsDELLA3-R — 5′-CGGATGAGCGGGACAACC-3′.

The amount of mRNA was normalized to two constitutively expressed genes encoding ubiquitin and actin. Three independent biological replicates were analyzed.

The results were statistically processed (SigmaPlot 12.0 software, SPSS Inc., USA). The means (*M*) and standard errors of the means (±SEM) were calculated. One-Way ANOVA with multiple pairwise comparisons using the post hoc Tukey test (ANOVA post hoc test) were used to assess the significance of differences between the treatments. Differences were considered statistically significant at $p < 0.05$.

Results. We used *Sym10* and *NIN* genes activated in plant roots on days 1-2 in response to inoculation [26] as markers of early stage of establishing symbiosis in pea plants upon inoculation with rhizobia. The *Sym10* gene encodes a LysM-containing receptor-like kinase involved in the recognition of the signaling

molecules of rhizobia — lipochito-oligosaccharides (Nod factors). The activation of the signaling cascade under the influence of the *SYM10* receptor leads to the development of nitrogen-fixing nodules on the plant roots. In the signal transduction, the expression of the *NIN* (Nodule Inception) gene which encodes a transcription factor and thus participates in the initiation of the development of symbiosis of peas with rhizobia, significantly increases.

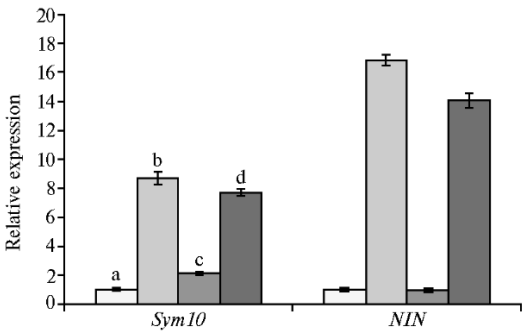

Fig. 1. Expression of the genes *Sym10* (receptor of rhizobia signaling molecules) and *NIN* (the main transcription factor of the signaling pathway) in pea (*Pisum sativum* L., cv. Frisson) roots 9 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 2 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: a — without inoculation (control), b — *R. leguminosarum*, c — *R. irregularis*, d — *R. irregularis* + *R. leguminosarum*. Bars show standard errors of the mean (±SEM) for three analytical replications. The experiment was arranged in three biological replicates (the data of one replicate are shown).

Monoinoculation with rhizobia caused a significant increase in the expression of the *Sym10* and *NIN* genes as compared to the non-inoculated control on day 9 (Fig. 1). In plants inoculated with AM fungi *R. irregularis* BEG144, no significant changes occurred in the *Sym10* and *NIN* expression which indicates the specific activation of these genes only in response to the recognition of signaling molecules of rhizobia (see Fig. 1). The expression of *Sym10* and *NIN* genes remained high upon co-inoculation with Rlv and AM fungi (see Fig. 1). In our opinion, this indicates that at the initial stages of formation and development of the multicomponent system, the plant effectively distinguishes the signals of rhizobia. They stimulate early responses in plants, including changes in ion fluxes across the plasma membrane and its depolarization, the production of reactive oxygen species, and the activation of channels that regulate the calcium flow into plant cells (Ca^{2+} influx).

The expression of genes *PT4*, *TI*, *RAM1*, and *DELLA3* that we used as markers for symbiosis with AM fungi increases most significantly on days 21-28 after inoculation with AM fungi [27]. The *PT4* gene encodes a phosphate transporter [17] the activation of which is associated with the later steps of the development of symbiosis with AM fungi when formation of arbuscules and vesicles occur. Transcriptomic profiling of the roots of pea plants inoculated with the AM fungus revealed a transcriptional inhibitor encoded by the *TI* gene [17]. Of transcription factors, the expression of RAM1 regulator was found to increase during the symbiosis with AM fungi [27]. DELLA proteins are regulators of plant response to gibberellins; they are directly involved in the control of the development of both nitrogen-fixing symbiosis and symbiosis with AM fungi [28, 29]. Proteins interact with the main transcription factors of signaling pathways — NSP2, IPD3, and RAM1, which stimulates the activation of target genes [29]. Earlier, we showed that in pea plants, symbiosis with AM fungi increases the expression of one of the genes of this family, DELLA3 [27].

On day 21 after planting, we revealed a significant increase in the expression of the *PT4* and *TI* genes upon monoinoculation by *R. irregularis* BEG144 and co-inoculation (AM + Rlv) (Fig. 2). Similarly, the expression of the *RAM1* and *DELLA3* genes increased with AM and AM + Rlv treatments. This indicated the ability of plants to respond to inoculation with AM fungi when applied together with rhizobia; however, we did not detect any additional stimulation of marker

gene expression during co-inoculation.

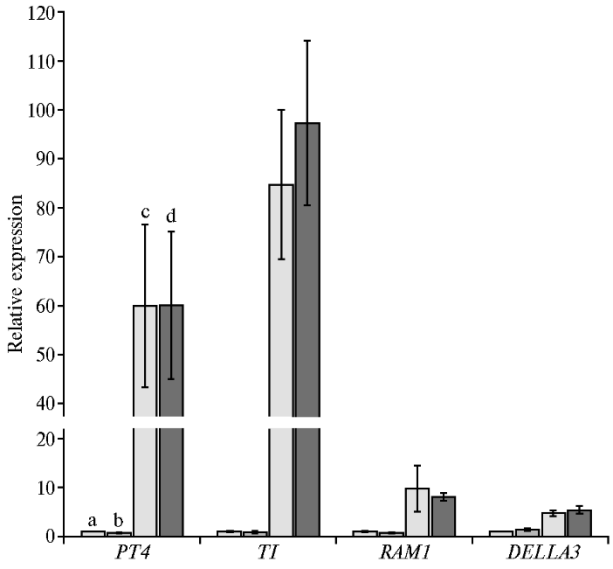

Fig. 2. Expression of genes *PT4* (a phosphate transporter), *TI* (a transcriptional inhibitor), *RAM1* (a transcription factor), and *DELLA3* (a regulator of plant response to gibberellins) in pea (*Pisum sativum* L., cv. Frisson) roots 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: a — without inoculation (control), b — *R. leguminosarum*, c — *R. irregularis*, d — *R. irregularis* + *R. leguminoussarum*. Bars show standard errors of the mean (±SEM) for three analytical replications. The experiment was arranged in three biological replicates (the data of one replicate are shown).

On day 21, we assessed expression of the genes *Enod5* and *Sym37* that can be activated during legume-rhizobial symbiosis in peas. *Enod5* encodes nodulin which is activated in a specific way in pea root cells after penetration of infection threads [30, 31]. The *Sym37* gene encodes a receptor that is necessary for the development and spread of infection threads in pea roots [32].

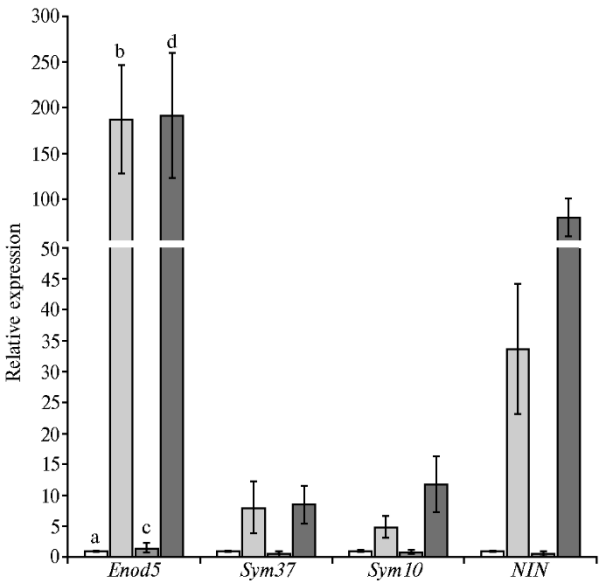

Fig. 3. Expression of genes *Enod5* and *Sym37* that can be activated during development of legume-rhizobial symbiosis in pea (*Pisum sativum* L., cv. Frisson) roots 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: a — without inoculation (control), b — *R. leguminosarum*, c — *R. irregularis*, d — *R. irregularis* + *R. leguminoussarum*. Bars show standard errors of the mean (±SEM) for three analytical replications. The experiment was arranged in three biological replicates (the data of one replicate are shown).

On day 21 after planting, the expression of the *Enod5* and *Sym37* genes, as well as *Sym10* and *NIN* genes increased upon monoinoculation with rhizobia (Fig. 3). No changes occurred in the expression of these genes with *R. irregularis* BEG144 inoculation (see Fig. 3). With AM + Rlv, the expression of the *NIN* gene increased compared to the Rlv monoinoculation. However, upon the co-infection, the expression of the genes *Enod5* and *Sym37* associated with the control of the infection development remained similar to that upon monoinoculation with rhizobia. Expression of the *Sym10* gene also did not change significantly upon co-inoculation of pea plants with the fungus and rhizobia.

The development of infection in legume-rhizobial symbiosis and symbiosis with AM fungi is under the host plant control. Probably, upon successive inoculation with several endosymbionts, competition arises between them at penetration into the plant, which leads to a decrease in the intensity of infection with rhizobia. In our opinion, the evidence to support this assumption is that co-inoculation did not additionally stimulate the expression of the genes *Enod5* and *Sym37* activated during the rhizobial infection.

With Rlv monoinoculation, there was a pronounced tendency to an increase in the total biomass of plants compared to the control ($p = 0.072$) (Fig. 4).

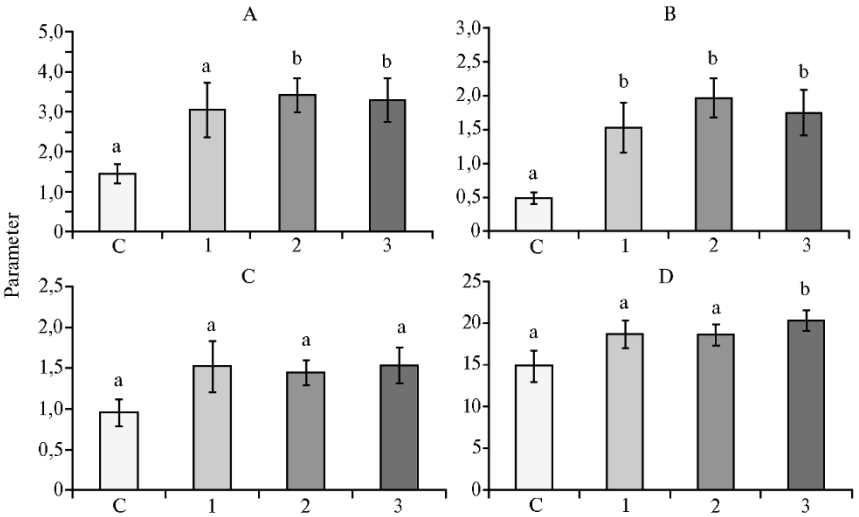

Fig. 4. Total biomass, g (A)**, root weight, g** (B)**, aboveground biomass, g** (C)**, and root length, cm** (D) **in pea (*Pisum sativum* L., cv. Frisson) plants 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026:** C — without inoculation (control), 1 — *R. leguminosarum*, 2 — *R. irregularis*, 3 — *R. irregularis* + *R. leguminosarum*. The arithmetic mean values for n = 12-15 are presented. Bars show standard errors of the means (±SEM). Different letters indicate statistically significant differences ($p < 0.05$). To assess the significance of differences between the treatments, one-way ANOVA post hoc test was used.

A significant change ($p < 0.05$) of the total plant biomass occurred both upon monoinoculation with AM fungus and co-inoculation ($p < 0.05$) (see Fig. 4, A). The root weight significantly ($p < 0.05$) increased upon inoculation with Rlv, AM, and AM + Rlv as compared to the control. However, Rlv, AM, and AM + Rlv did not differ significantly in root weight (see Fig. 4, B). Weighing of the aerial parts did not reveal significant differences between the control and mono- (Rlv or AM) or co-inoculation (AM + Rlv) (see Fig. 4, C).

Upon inoculation with Rlv and AM, there was a tendency to an increase in the plant root length compared to the control ($p = 0.08$ and $p = 0.09$, respectively); however, only co-inoculation led to a significant increase ($p < 0.05$) (see Fig. 4, D). Any inoculation treatment had no significant effect on the length of the aerial parts.

The obtained results allow us to conclude that in our experiment, monoinoculation with rhizobia or AM fungi was effective. Rlv and AM monoinoculation increased the biomass of the plant roots but not the aerial parts. Upon co-inoculation, a slight increase in the root length ocurred. However, the absence of significant differences in plant weight and root weight between the Rlv or AM monoinoculation and co-inoculation does not allow us to draw a conclusion about the positive effect of double inoculation on the growth and development of pea plants.

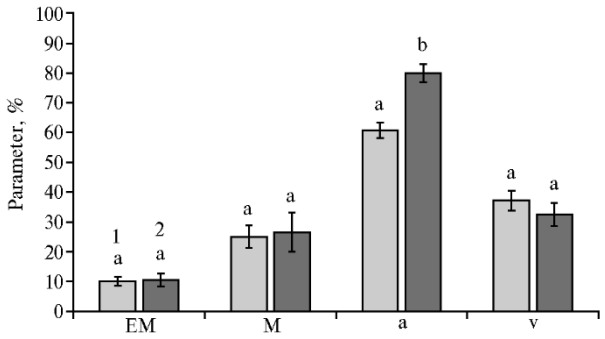

Fig. 5. Developemt of arbuscular mycorrhiza in pea (*Pisum sativum* L., cv. Frisson) plants 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: EM — intensity of external mycorrhization, M — intensity of in-root mycorrhization, a — abundancy of arbuscules in mycorrhized root fragments, v — abundancy of vesicles in mycorrhized root fragments; 1 — *R. irregularis*, 2 — *R. irregularis* + *R. leguminosarum*. The arithmetic mean values for n = 12-15 are presented. Bars show standard errors of the means (±SEM). Different letters indicate statistically significant differences ($p < 0.05$). To assess the significance of differences between the treatments, one-way ANOVA post hoc test was used

On day 21, all plants inoculated with rhizobia formed pink nodules (on average, 180±35 per plant with Rlv monoinoculation and 228±55 per plant with AM + Rlv). There were no statistically significant differences in the number of nodules between these two treatments.

In both fungal inoculation (AM and AM + Rlv), the plants had approximately the same intensity of external root colonization (EM%) with *R. irregularis* hyphae (approximately 10%) (Fig. 5). This testified to the good quality of the mycorrhizal inoculum. The intra-root mycelium developed quite intensively in both variants, however, for AM and AM + Rlv, no significant differences in the intensity of intra-root colonization (M%) occurred (see Fig. 5). In the mycorrhized root segments, arbuscules intensively developed. However, the relative number of arbusules (a%) was significantly higher ($p < 0.05$) for co-inoculation than for AM monoinoculation, which indicates a more intensive exchange of nutrients between fungal and plant cells (see Fig. 5). Inoculation with rhizobia did not affect the relative number of vesicles (v%) serving as storage organs of AM fungi (see Fig. 5). Without fungal inoculation (in control and in Rlv inoculation), no structures of AM fungi were found, which indicates the absence of cross-contamination with these microorganisms.

Overall, based on the analysis performed, it can be concluded that legume-rhizobial symbiosis and symbiosis with AM fungi developed in the system under study. In this regard, the data obtained on the expression of genes markers of symbiosis are adequate. However, the analysis of the double symbiosis showed that when using the AM + Rlv sequential inoculation, there is no significant increase in intra-root mycorrhizal colonization, which is probably due to the competition between microorganisms for a niche in the plant or to stimulation of a systemic response from plants.

Numerous publications show that AM fungi and rhizobia act as synergists, stimulating plant growth via improved mineral nutrition and inhibition of fungal pathogens [4-7]. However, several works revealed that competition for a niche in a plant can be the reason for a decrease in the efficiency of root colonization by AM fungi. AM *Glomus* sp. R-10 commercial inoculum used to increase yields of soybean *Glycine max* (L.) Merrill had a negative effect on the root colonization by endogenous strains of fungi well adapted to the conditions of plant growing [33]. Similarly, in carrot roots which were cultivated in vitro, competition for a niche was the reason for a decrease in intra-root colonization upon co-inoculation with AM *Rhizophagus irregularis* and *Glomus aggregatum* as compared to monoinoculation [34]. Therefore, increased competition between symbionts can

affect the intensity of intra-root colonization by AM fungi. This determines the interest in studying the mutual influence of symbionts on plants.

The mutual influence of AM fungi and rhizobia in a multicomponent system may be quite important, especially with the sequential inoculation we used. Our data indicate that not only combinations of certain strains of fungi and rhizobia are important to achieve a positive effect on plants but also the scheme of co-inoculation. In the future, it is necessary to identify the conditions, decreasing competition, and to assess whether such changes in mycorrhizal colonization can be a marker of the effectiveness of plant—AM fungi interaction during co-inoculation.

Thus, the level of the induced expression of markers for two types of symbiosis, the root mycorrhization intensity, and the plant biometric parameters draw us to the conclusion that a two-step inoculation of pea plants (cv. Frisson), first with *Rhizophagus irregularis* BEG144 and then with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 failed to reach the stimulating effect characteristic of co-inoculation if compared to monoinoculation. Our findings may indicate the competition of microorganisms for a niche in the plant or the activation of systemic mechanisms that prevent the enhancement of intra-root colonization by symbionts.

REFERENCES

1. Lal R. Soils and sustainable agriculture: a review. In: *Sustainable agriculture*. E. Lichtfouse, M. Navarrete, P. Debaeke, S. Veronique, C. Alberola (eds.). Springer, 2009: 15-23 (doi: 10.1007/978-90-481-2666-8_3).
2. Conway G.R., Barbier E.B. *After the green revolution: sustainable agriculture for development*. Routledge, London, 2013 (doi: 10.4324/9781315066820).
3. Mahanty T., Bhattacharjee S., Goswami M., Bhattacharyya P., Das B., Ghosh A., Tribedi P. Biofertilizers: a potential approach for sustainable agriculture development. *Environmental Science and Pollution Research International*, 2017, 24(4): 3315-3335 (doi: 10.1007/s11356-016-8104-0).
4. Bethlenfalvay G.J., Brown M.S., Franson R.L. Glycine-Glomus-Bradyrhizobium symbiosis: X. Relationships between leaf gas exchange and plant and soil water status in nodulated, mycorrhizal soybean under drought stress. *Plant Physiology*, 1990, 94(2): 723-728 (doi: 10.1104/pp.94.2.723).
5. Artursson V., Finlay R.D., Jansson J.K. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environmental Microbiology*, 2006, 8(1): 1-10 (doi: 10.1111/j.1462-2920.2005.00942.x).
6. Ballesteros-Almanza L., Altamirano-Hernandez J., Peca-Cabriales J.J., Santoyo G., Sanchez-Yacez J.M., Valencia-Cantero E., Macias-Rodriguez L., Lopez-Bucio J., Cardenas-Navarro R., Farias-Rodriguez R. Effect of co-inoculation with mycorrhiza and rhizobia on the nodule trehalose content of different bean genotypes. *The Open Microbiology Journal*, 2010, 4(1): 83-92 (doi: 10.2174/1187428580100401008 3).
7. Bhattacharjee S., Dutta Sharma G. Effect of dual inoculation of arbuscular mycorrhiza and *Rhizobium* on the chlorophyll, nitrogen and phosphorus contents of pigeon pea (*Cajanus cajan* L.). *Advances in Microbiology*, 2012, 2(4): 561-564 (doi: 10.4236/aim.2012.24072).
8. Dilworth M.J. Dinitrogen fixation. *Annual Review of Plant Physiology*, 1974, 25(1): 81-114 (doi: 10.1146/annurev.pp.25.060174.000501).
9. Stewart W.D.P., Alexander G. Phosphorus availability and nitrogenase activity in aquatic blue-green algae. *Freshwater Biology*, 1971, 1(4): 389-404 (doi: 10.1111/j.1365-2427.1971.tb01570.x).
10. Kondo M., Kobayashi M., Takahashi E. Effect of phosphorus and temperature on the growth and nitrogenase activity in *Azolla-Anabaena* association. *Soil Science and Plant Nutrition*, 1989, 35(2): 217-226 (doi: 10.1080/00380768.1989.10434754).
11. Crews T.E. Phosphorus regulation of nitrogen fixation in a traditional Mexican agroecosystem. *Biogeochemistry*, 1993, 21(3): 141-166 (doi: 10.1007/BF00001115).
12. Tajini F., Trabelsi M., Drevon J.J. Combined inoculation with *Glomus intraradices* and *Rhizobium tropici* CIAT899 increases phosphorus use efficiency for symbiotic nitrogen fixation in common bean (*Phaseolus vulgaris* L.). *Saudi Journal of Biological Sciences*, 2012, 19(2): 157-163 (doi: 10.1016/j.sjbs.2011.11.003).
13. Valdenegro M., Barea J.M., Azcón R. Influence of arbuscular-mycorrhizal fungi, *Rhizobium meliloti* strains and PGPR inoculation on the growth of *Medicago arborea* used as model legume for re-vegetation and biological reactivation in a semi-arid mediterranean area. *Plant Growth Regulation*, 2001, 34(2): 233-240 (doi: 10.1023/A:1013323529603).
14. Islam R., Ayanaba A., Sanders F.E. Response of cowpea (*Vigna unguiculata*) to inoculation with

VA-mycorrhizal fungi and to rock phosphate fertilization in some unsterilized Nigerian soils. *Plant and Soil*, 1980, 54(1): 107-117 (doi: 10.1007/BF02182003).
15. Xavier L.J.C., Germida J.J. Selective interactions between arbuscular mycorrhizal fungi and *Rhizobium leguminosarum* bv. *viceae* enhance pea yield and nutrition. *Biology and Fertility of Soils*, 2003, 37(5): 261-267 (doi: 10.1007/s00374-003-0605-6).
16. Stancheva I., Geneva M., Zehirov G., Tsvetkova G., Hristozkova M., Georgiev G. Effects of combined inoculation of pea plants with arbuscular mycorrhizal fungi and *Rhizobium* on nodule formation and nitrogen fixing activity. *General and Applied Plant Physiology*, 2006, 1: 61-66.
17. Grunwald U., Nyamsuren O., Tamasloukht M., Lapopin L., Becker A., Mann P., Gianinazzi-Pearson V., Krajinski F., Franken P. Identification of mycorrhiza-regulated genes with arbuscule development-related expression profile. *Plant Molecular Biology*, 2004, 55(4): 553-566 (doi: 10.1007/s11103-004-1303-y).
18. Xie Z.P., Staehelin C., Vierheilig H., Wiemken A., Jabbouri S., Broughton W.J., Vogeli-Lange R., Boller T. Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. *Plant Physiology*, 1995, 108(4): 1519-1525 (doi: 10.1104/pp.108.4.1519).
19. Maillet F., Poinsot V., André O., Puech-Pagés V., Haouy A., Gueunier M., Cromer L., Giraudet D., Formey D., Niebel A., Martinez E.A., Driguez H., Becard G., Dénarié J. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature*, 2011, 469(7328): 58-64 (doi: 10.1038/nature09622).
20. Whipps J.M. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. *Canadian Journal of Botany*, 2004, 82(8): 1198-1227 (doi: 10.1139/B04-082).
21. Volpiano C.G., Lisboa B.B., Granada C.E., José J.F.B.S., de Oliveira A.M.R., Beneduzi A., Perevalova Y., Passaglia L.M.P., Vargas L.K. Rhizobia for biological control of plant diseases. In: *Microbiome in plant health and disease*. V. Kumar, R. Prasad, M. Kumar, D. Choudhary (eds.). Springer, Singapore, 2019, 1(1): 315-336 (doi: 10.1007/978-981-13-8495-0_14).
22. Gao X., Lu X., Wu M., Zhang H., Pan R., Tian J., Li S., Liao H. Co-inoculation with rhizobia and AMF inhibited soybean red crown rot: From field study to plant defense-related gene expression analysis. *PLoS ONE*, 2012, 7(3): e33977 (doi: 10.1371/journal.pone.0033977).
23. Azcón R., Rubio R., Barea J.M. Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2-fixation (^{15}N) and nutrition of *Medicago sativa* L. *New Phytologist*, 1991, 117(3): 399-404 (doi: 10.1111/j.1469-8137.1991.tb00003.x).
24. Orosz L., Sváb Z., Kondorosi A., Sik T. Genetic studies on rhizobiophage 16-3. I. Genes and functions on the chromosome. *Molecular and General Genetics MGG*, 1973, 125(4): 341-350 (doi: 10.1007/BF00276589).
25. Shtark O.Y., Sulima A.S., Zhernakov A.I., Kliukova M.S., Fedorina J. V., Pinaev A.G., Kryukov A.A., Akhtemova G.A., Tikhonovich I.A., Zhukov V.A. Arbuscular mycorrhiza development in pea (*Pisum sativum* L.) mutants impaired in five early nodulation genes including putative orthologs of *NSP1* and *NSP2*. *Symbiosis*, 2016, 68(1-3): 129-144 (doi: 10.1007/s13199-016-0382-2).
26. Kirienko A.N., Porozov Y.B., Malkov N.V., Akhtemova G.A., Le Signor C., Thompson R., Saffray C., Dalmais M., Bendahmane A., Tikhonovich I.A., Dolgikh E.A. Role of a receptor-like kinase K_1 in pea *Rhizobium* symbiosis development. *Planta*, 2018, 248(5): 1101-1120 (doi: 10.1007/s00425-018-2944-4).
27. Leppyanen I.V., Shakhnazarova V.Y., Shtark O.Y., Vishnevskaya N.A., Tikhonovich I.A., Dolgikh E.A. Receptor-like kinase LYK9 in *Pisum sativum* L. is the CERK1-like receptor that controls both plant immunity and AM symbiosis development. *International Journal of Molecular Sciences*, 2017, 19(1): 8 (doi: 10.3390/ijms19010008).
28. Jin Y., Liu H., Luo D., Yu N., Dong W., Wang C., Zhang X., Dai H., Yang J., Wang, E. DELLA proteins are common components of symbiotic rhizobial and mycorrhizal signalling pathways. *Nature Communications*, 2016, 7: 12433 (doi: 10.1038/ncomms12433).
29. Fonouni-Farde C., Tan S., Baudin M., Brault M., Wen J., Mysore K.S., Niebel A., Frugier F., Diet A. DELLA-mediated gibberellin signaling regulates Nod factor signalling and rhizobial infection. *Nature Communications*, 2016, 7: 12636 (doi: 10.1038/ncomms12636).
30. Scheres B., van Engelen F., van der Knaap E., van de Wiel C., van Kammen A., Bisseling T. Sequential induction of nodulin gene expression in the developing pea nodule. *The Plant Cell*, 1990, 2(8): 687-700 (doi: 10.1105/tpc.2.8.687).
31. Dolgikh E.A., Leppyanen I. V., Osipova M.A., Savelyeva N.V., Borisov A.Y., Tsyganov V.E., Geurts R., Tikhonovich I.A. Genetic dissection of *Rhizobium*-induced infection and nodule organogenesis in pea based on *ENOD12A* and *ENOD5* expression analysis. *Plant Biology*, 2011, 13(2): 285-296 (doi: 10.1111/j.1438-8677.2010.00372.x).
32. Zhukov V., Radutoiu S., Madsen L.H., Rychagova T., Ovchinnikova E., Borisov A., Tikhonovich I., Stougaard J. The Pea *Sym37* receptor kinase gene controls infection-thread initiation and nodule development. *Molecular Plant-Microbe Interactions*, 2008, 21(12): 1600-1608 (doi: 10.1094/MPMI-21-12-1600).
33. Niwa R., Koyama T., Sato T., Adachi K., Tawaraya K., Sato S., Hirakawa H., Yoshida S.,

Ezawa T. Dissection of niche competition between introduced and indigenous arbuscular mycorrhizal fungi with respect to soybean yield responses. *Scientific Reports*, 2018, 8: 7419 (doi: 10.1038/s41598-018-25701-4).
34. Engelmoer D.J., Behm J.E., Toby Kiers E. Intense competition between arbuscular mycorrhizal mutualists in an in vitro root microbiome negatively affects total fungal abundance. *Molecular Ecology*, 2014, 23(6): 1584-1593 (doi: 10.1111/mec.12451).