THE EFFECT OF DOUBLE INOCULATION ON THE BROAD BEANS (*VICIA FABA* L.) YIELD QUALITY

Laila Dubova, **Alise Šenberga**, **Ina Alsiņa**
Latvia University of Agriculture
Laila.Dubova@llu.lv

Abstract
Legumes (*Leguminosae*) are one of the most widely grown crops in the world after cereals (*Poaceae*). They are not only an important source of protein in food and feed, but also a significant component of different agrosystems. The N_2 fixation by legumes is of great importance in nutrient management and sustainable economy of nitrogen. Legume productivity largely depends on a successful formation of symbiosis between the plant and soil microorganisms. The most important among those are rhizobia and mycorrhiza fungi. The field experiment was carried out at the Institute of Soil and Plant Sciences, Latvia University of Agriculture in 2014 to evaluate the influence of double inoculation using *Rhizobium leguminosarum* and mycorrhiza fungi preparation on yield formation of broad bean *V. faba* L. var. *major* Harz 'Bartek'. The bean seeds were treated with rhizobia and/or mycorrhiza fungi before sowing. Seeds were treated with rhizobia by soaking in bacteria suspension for 30 minutes directly before sowing. Mycorrhiza fungi preparation was added in soil under seeds before sowing. Plant height, fresh and dry weight and the weight of nodules were measured at the beginning of broad bean flowering (BBCH 60-61). *Rhizobium leguminosarum* response to double inoculation differed between the strains. *Rhizobium leguminosarum* strain RL407, isolated from *Vicia faba*, was shown to be the most appropriate strain used for inoculation of broad bean seeds. Bean seed double inoculation increases the protein content significantly comparing to single inoculation using mycorrhiza fungi preparation.
Key words: legumes, *Rhizobium leguminosarum*, mycorrhiza fungi, nodulation, protein.

Introduction

Legumes (*Leguminosae*) are economically important crop, the growing areas of which increase yearly not only in Latvia. The sowing area of legumes during the period from 2007 to 2013 increased approximately four times. Beans (*Vicia faba* L.) in these volumes averaged about a quarter (Central Statistical Bureau of Latvia). Legumes are a high-quality, protein-rich animal feed and wholesome source of nutrition for people; moreover, they are a valuable crop in different crop rotation systems. Legumes are able to fix atmospheric nitrogen up to 250 kg ha^{-1}, thus reducing the load on the agro fertilizer.

In order to develop sustainable farming systems with reduced inputs of manufactured fertilizers, more attention should be given to the use of microorganisms that could have beneficial effects on crop production. Symbiotic N_2 fixation by legumes and the potential N_2 transfer to other non-legume plants has great importance in nutrient management and sustainable economy of nitrogen (Høgh-Jensen and Schjoerring, 1994; Chalk et al., 2006). In the *Rhizobium*-legume symbiosis, the process of N_2 fixation depends not only on the physiological state of the host plant (Mabrouk and Belhadj, 2012; Franzini et al., 2013), but is limited by a competitive and persistent rhizobial strain and soil microorganism association activity too (Goel et al., 2001; Barea et al., 2005).

For the development of effective symbiotic system it is necessary to ensure the host and rhizobia not only with carbon and nitrogen, but also with other mineral elements and water. The uptake of mineral elements can be improved through mycorrhizal fungi. Therefore, a growing interest in the world is given to mycorrhiza fungi and rhizobia symbiotic association, which contributes not only to the supply of the plant with certain mineral elements, but also improves plant stress tolerance. Symbiotic associations' effectiveness is affected by each of the association components and interaction between the soil and climatic conditions (Xavier and Germida, 2002; Chalk et al., 2006; Jia and Gray, 2008).

Soil arbuscular mycorrhiza fungi (AMF) colonize roots of host plants and promote plant growth, which is generally attributed to the improved uptake of nutrients with particular emphasis on P nutrition (Franzini et al., 2010; Farzaneh et al., 2011). However, other studies indicate that AMF inoculation increases dry matter accumulation and nutrient uptake compared with indigenous AMF communities (Pearson et al., 1993; Chalk et al., 2006; Farzaneh et al., 2011). R. Porcel et al. (2003) observed that the inoculation of soybean plants with AMF enhances protein accumulation as well as increases the nodule activity. Different legume species have shown the development of symbiotic associations with both phosphorus–acquiring arbuscular mycorrhizal fungi and nitrogen fixing rhizobia. Suitable symbiotic association provides legume with essential mineral elements for good protein accumulation in the seeds (Xavier and Germida, 2002; Scheublin et al., 2004; Scheublin and van der Heijden, 2006).

Research confirms the importance of the formation of the tripartite symbiosis, but there is no clear mechanism known to ensure that. It has been shown that the response of legume host to *Rhizobium* can be

Table

Microorganisms used in the experimental variants

Label of variants	Treatment
K	control without treatment
RL23	inoculated with *Rhizobium leguminosarum* strain RL23
RL23M	inoculated with *Rhizobium leguminosarum* strain RL23 and mycorrhiza fungi
RL407	inoculated with *Rhizobium leguminosarum* strain RL407
RL407M	inoculated with *Rhizobium leguminosarum* strain RL407 and mycorrhiza fungi
M	inoculated with mycorrhiza fungi
KN	used addition mineral nitrogen fertilizers, without microorganism preparations

modified by the AMF species involved in the tripartite association. It is not clear whether the presence of AMF influences nodule formation and functioning (Scheublin and van der Heijden, 2006). Formation of the tripartite symbiotic association can be determined and regulated by a series of biochemical signals. A.K. Goel et al. (2001) found out that some rhizobium strains can produce bacteriocin. I. Sampedro et al. (2007) pointed out the significance of xylogenase activity, which characterizes microsymbiont ability to penetrate plant root cell wall. Another, even more important biochemical signal between legume and rhizosphere microorganisms are flavonoids. They can be released from plant cells in response to biotic and abiotic signals in the rizosphere and exudation from the root changes during the symbiosis (Maj et al., 2010; Abdel-Lateif et al., 2012; Hassan and Mathesius, 2012).

The aim of the experiment was to evaluate the influence of double inoculation with *Rhizobium leguminosarum* and mycorrhiza fungi preparation on yield formation of broad bean *V. faba* L. var. *major* Harz.

Materials and Methods

Experiments were carried out at the Institute of Soil and Plant Sciences (56º39′ N, 23º45′ E), Latvia University of Agriculture in 2014. Field experiment was conducted in a loamy sand soil characterized by ph kcl 7.6, ec ms cm^{-1} 0.73 and mineral element content in 1m hcl solution (mg l^{-1}): n-78, p-523, k-170, ca-161450, mg-3850, s-65, fe-1920, mn-170, zn-10.5, cu-6.5, mo-0.08, b-0.4.

The field experiment was carried out in four replications. Size of each experimental plot was 1 m^2. Plants were sown in rows by hand. Distance between rows was 30 cm and 10 cm between seeds. Experimental plots were separated by one meter buffer zones.

Broad bean *Vicia faba* L. var. *major* Harz 'Bartek' was grown in the experiments. Seeds were treated with rhizobia – soaked in bacteria suspension for 30 minutes directly before sowing. Mycorrhiza fungi preparation was added in soil under seeds before sowing. The experimental variants are summarized in Table.

Rhizobium leguminosarum strains were obtained from the Collection of Rhiziobia of Latvia University of Agriculture. Strains RL23, isolated from *Pisum* sp., and RL407, isolated from *Vicia faba*, were used in the experiment. All used strains are streptomycin resistant. Suspensions for seed inoculation contain 10^6 to 10^8 cells per mL.

Mycorrhiza fungi inoculum was obtained from company Symbiom Ltd. in the frame of EU 7th Research Framework Programme of the European Union project 613781, EUROLEGUME (Enhancing of legumes growing in Europe through sustainable cropping for protein supply for food and feed). Mycorrhiza inoculums contain a mixture of at least three species of mycorrhiza fungi.

Meteorological conditions during all vegetation period of 2014 were characterized by larger precipitation than average (123-220% from long term data); however, at the beginning of the flowering stage water deficit in the soils was observed. After warm beginning, the end of June was relatively cool. Temperature on average was 0.9 °C lower in comparison with the long term average. July was warm and dry. Temperature on average was 2.7 °C higher in comparison with the long term average but precipitation was only 74% of the long term average. August was warm, but with higher precipitation in comparison with July. Precipitation was 178% of the long term average[1].

The plant height, fresh and dry weight and the weight of nodules were measured at the beginning of broad bean flowering (BBCH 60-61). The bean yield parameters – 100 bean weight and protein content of the seeds was determined at the end of vegetation period. Protein content of the bean seeds was determined using mature, dry seeds. For protein analyses the average sample of replicates was used. Protein content was determined in two replicas by

[1] www.meteo.lv

Kjeldal method (LVS EN ISO 5983-2:2009) in the Analytical laboratory for agronomic research, Latvia University of Agriculture.

All statistical analyses were done using Excel (Microsoft Corporations, Redmond, Washington, USA).

Results and Discussion

Efficient symbiotic relationships can have a positive impact on the formation of leguminous plants and crop production. The obtained results show that inoculation of bean seed with symbiotic microorganisms influences plant shoot and root growth (Fig. 1.). The results show different effects of double inoculation of bean seeds. *Rhizobium leguminosarum* strain RL407 significantly stimulated the growth of shoot in the variants with double inoculation. However, no significant differences between variants inoculated with RL23 were observed.

The activity of symbiotic microorganisms in the plant root and rhizosphere influenced the growth intensity of roots. Bean shoot and root ratio ranged between 2.4 and 2.9. The lowest ratio was obtained in the variant with *Rhizobium* strain RL407 but the highest one with strain RL23 and double inoculated variant RL407M. Plant shoot and root ratio in the control variant was 2.7. Broad beans with double inoculation were characterized by more extensive root system. The average root weight in the variants RL23M and RL407M in comparison with control were about 28 and 30% higher, respectively. Single inoculation using only *Rhizobium leguminosarum* or mycorrhiza fungi increased the average root weight from 12 to 16%. These results are consistent with data in literature where different responses of legume inoculation are reported (Xavier and Germida, 2002; Barea et al., 2005). L.J.C. Xavier and J.J. Germida (2002) concluded that the influence of different strains of rhizobia on shoot and root dry weight of lentil (*Lens culinaris*) were altered by mycorrhiza fungi.

Effective symbiosis between legume and rhizobia is characterised by the number and size of nodule on plant roots. As pointed out by T.R. Scheublin and M.G.A. van der Heijden (2006), it is not yet clear whether the presence of mycorrhiza fungi influences the nodule functioning. In our experiment the nodule formation was observed at the beginning of flowering (BBCH – 60-61) (Fig. 2). Some nodules were found on the control plant roots too, suggesting that experimental soil contains indigenous rhizobia. These bacteria can compete with experimental rhizobia strains. Goel et al. (2001) found out that some rhizobium strains can produce bacteriocin which inhibits the growth of homologous *Rhizobium* strains.

The weight and size of nodule depends on specific activity of rhizobia. The most efficient bacterium stimulates the formation of nodule which provides legumes with nitrogen, so the plants can develop a greater weight. The various numbers of nodules on experimental plant roots may indicate the competitiveness of *Rhizobium* strains. Results showed significant differences ($p = 0.018$) between the average

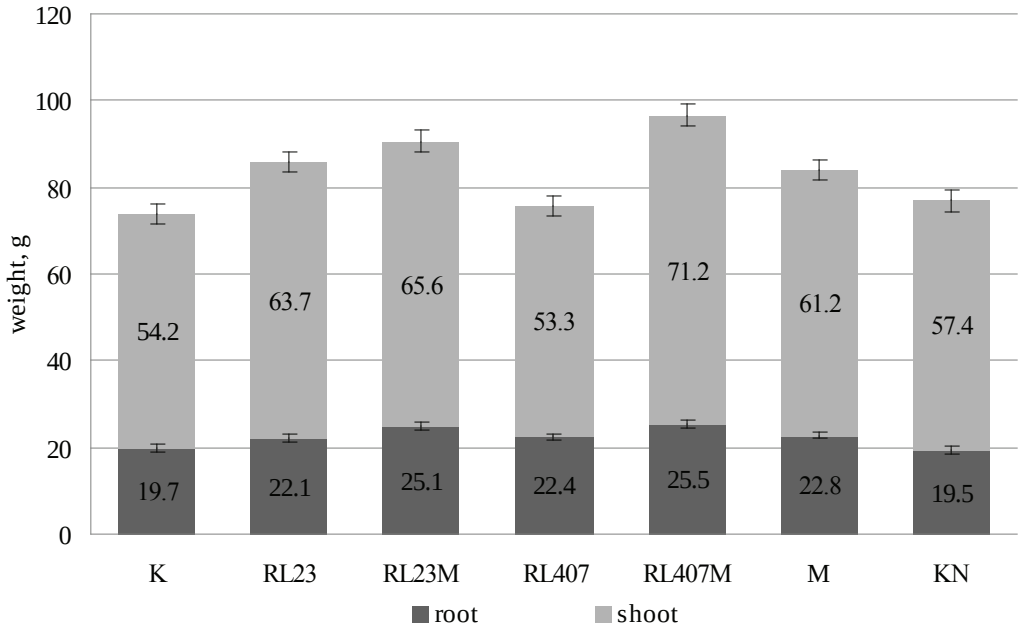

Figure 1. Average shoot and root weight at the flowering stage (BBCH – 60-61) of broad beans: K – control, RL23 and RL407 – *Rhizobium leguminosarum* strains, M – mycorrhiza preparation, KN – with additional nitrogen, without microorganism preparations.

number of nodules compared to control (K). Results show that both mycorrhiza alone and an additional N fertilizer have contributed to the indigenous rhizobia activity, as nodule is significantly heavier than in controls.

The effect of double inoculation on broad bean yield was analysed by seed weight and protein content in the seeds. An increase of 100 bean weight was detected only in the variant with double inoculation with *Rhizobium leguminosarum* strain RL407 and mycorrhiza fungi (Fig. 3.). A significant difference was observed between single inoculation variants with RL407 and double inoculated RL407M. For all other variants no significant differences of 100 bean weight were detected.

Protein accumulation in the seeds depends not only on plant biosynthetic activity but can be affected by microbial symbionts. A significant increase ($p>0.005$) of protein content was observed in all treatments comparing with control (Fig. 4). The highest protein content in the seeds was determined in the double inoculated plant (R23M and R407M).

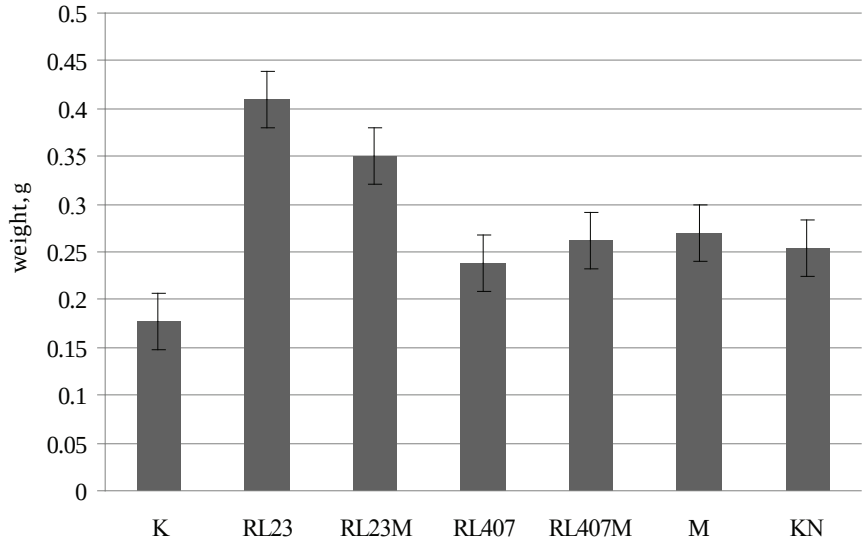

Figure 2. Weight of 10 nodules from broad beans root at the beginning of flowering stage (BBCH – 60-61) of broad beans: K – control, RL23 and RL407 – *Rhizobium leguminosarum* strains, M – mycorrhiza preparation, KN – with additional nitrogen, without microorganism preparations.

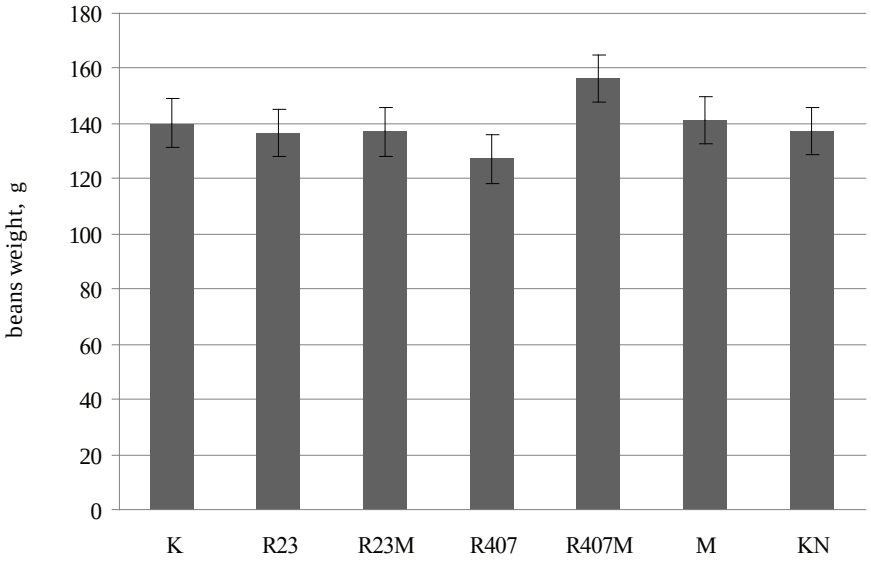

Figure 3. 100 bean weight (g), depending on the treatment: K – control, RL23 and RL407 – *Rhizobium leguminosarum* strains, M – mycorrhiza preparation, KN – with additional nitrogen, without microorganism preparations.

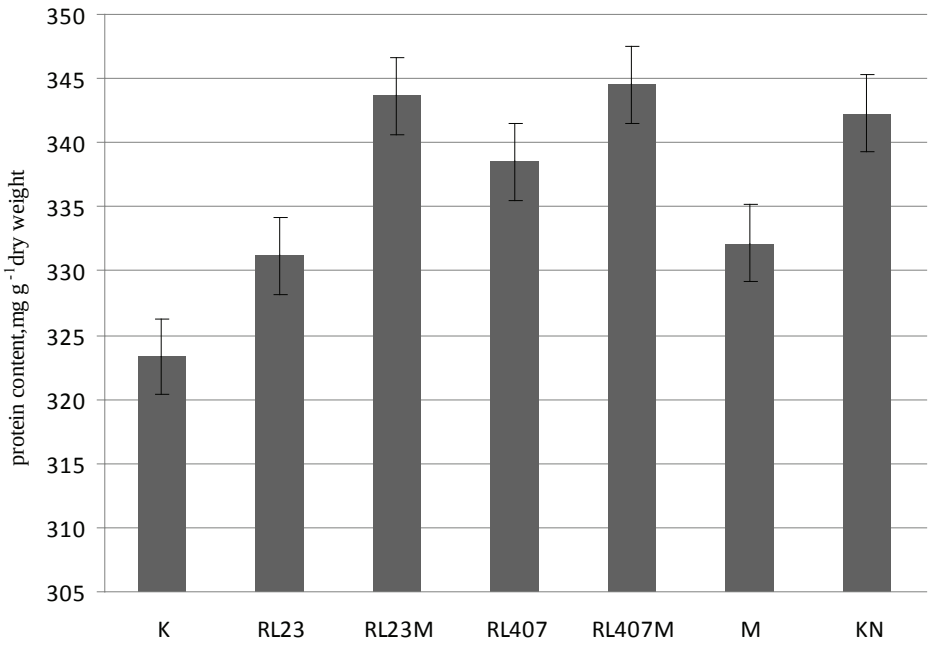

Figure 4. Protein content in bean seeds, mg g^{-1} dry weight, depending on the treatment: K – control, RL23 and RL407 – *Rhizobium leguminosarum* strains, M – mycorrhiza preparation, KN – with additional nitrogen, without microorganism preparations.

Single inoculation with mycorrhiza fungi (M), in comparison with double inoculation (RL23M and RL407M), did not increase the protein content in seeds. Inoculation of seeds with single microsimbiont gave the increase of protein content in broad been seeds from 7.9 to 15.2 mg g^{-1}. Double inoculation increases protein content in seeds on average by 20.3-21.2 mg g^{-1} in comparison with control. The obtained results are consistent with some literature data. P.M. Chalk et al. (2006) pointed that inoculation with rhizobia and mycorrhiza fungi has beneficial effects on legume dry matter or grain yield. P.M. Chalk et al. (2006), Y. Jia and V.M. Gray (2008), T.R. Scheublin and M.G.A. van der Heijden (2006) explained this effect as follows – that mycorrhiza fungi support legume plant with phosphorus uptake and another immobile nutrients, including microelements.

Conclusions

Bean seed double inoculation with *Rhizobium leguminosarum* and mycorrhiza fungi preparation increases the protein content significantly comparing to single inoculation using mycorrhiza fungi preparation.

Rhizobium leguminosarum strain RL407, isolated from *Vicia faba,* is more appropriate for inoculation of broad bean seeds, while stimulates protein accumulation more than strain RL23.

The selectivity of mycorrhiza fungi to Rhizobia strains was observed. More suitable for triple symbiosis was the combination of broad beans-mycorrhiza fungi – *Rhizobium* strain RL407.

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