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Germination and growth of primary roots of inoculated bean (*Vicia faba*) seeds under different temperatures

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Abstract. Temperature stress strongly affects legumes, rhizobia, and the efficiency of legume-rhizobia interaction. An experiment in 2016 was developed to test the seed germination in Petri dishes using different microorganism inoculation under several temperature treatments (4, 8, 12 and 20 °C). The goal of this study was to test the effect of rhizobium inoculation under low root zone temperature, and to examine whether the addition of mycorrhiza fungi could enhance rhizobia resistance to abiotic stress and improve faba bean (*Vicia faba*) germination. Four faba bean cultivars were selected for the experiment ('Lielplatone', 'Fuego', 'Bartek' and 'Karmazyn'). Four different seed inoculation variants were included in this experiment – 1) with rhizobium inoculation; 2) with a commercial preparation containing mycorrhiza fungi; 3) inoculation with both rhizobium and the mycorrhiza fungi preparation; 4) control variant. The number of germinated seeds, the length of the primary root and the primary root weight ratio were determined. The effect of inoculation was found out to be dependent not only on the temperature treatment, but it also significantly varied between the bean cultivars. Variants where seeds were inoculated with both mycorrhiza and rhizobia resulted in the highest results (length and weight ratio of primary roots), comparing with other inoculation variants, regardless of temperature. Variants where seeds were treated only with rhizobia mostly showed the lowest results – both length and weight ratio of primary roots, especially under treatment of 4 °C. Faba bean inoculation with only rhizobia might not be efficient, when sowing seeds under a low temperature stress. Inoculation with both rhizobia and mycorrhiza fungi could be a potential solution, when the root zone temperature is still below the optimal temperature.

Key words: rhizobia, mycorrhiza, abiotic stress, low root zone temperature, legumes, *Vicia faba*.

INTRODUCTION

Legume growers often use the bacteria from the genus *Rhizobia* (further – rhizobia) for seed inoculation, as rhizobia helps to supply legumes with the necessary amount of nitrogen. Legume inoculation with rhizobia results in an increased yield quantity and/or quality (Dash & Gupta, 2011; Ahemad & Kibret, 2014; Pawar et al., 2014; Voisin et al., 2014). Optimal growth conditions for both legumes and rhizobia are of great importance to achieve the optimal yield quality and quantity that can be achieved by this symbiosis. Root zone temperature (RZT) affects this symbiotic relationship even before nodule formation, including rhizobia survival in soil, competitiveness, legume root infection and subsequent nodule development, and nitrogen fixing ability (Paulucci et al., 2011;

Alexandre & Oliveira, 2013). An optimal temperature that fits most rhizobia has been suggested to be between 25–30 °C (Zhang et al., 1995). Nevertheless, there is no general temperature that can fit to all legume–rhizobia symbioses (Alexandre & Oliveira, 2013).

Farmers in Latvia tend to sow faba bean seeds early in the spring – starting from the end of March until the end of April (Balodis et al., 2016) to avoid the insufficient moisture necessary for bean seed germination in the soil. The average air temperature in March is -1 °C, in April the average air temperature is 5 °C (data obtained from the database of 'Latvian Environment, Geology and Meteorology Centre'; https://www.meteo.lv/); therefore, faba beans are initially exposed to low RZT.

Extensive research on the effect of temperature on the symbiosis between leguminous plants and rhizobia bacteria has been done in southern regions, where the problem mostly is high temperature and insufficient soil moisture, which disturbs rhizobia and legume symbiosis. Fewer studies have looked at the low root zone temperature's impact on legume-rhizobia symbiosis and legume growth (Zahran, 1999; Drouin et al., 2000). Several studies suggest that low RZT (2–15 °C), common in early spring in Northern Europe, can have a negative effect on legume nodule development and nitrogen fixation, leading to a subsequent reduced growth of legumes (Lynch & Smith, 1994; Zhang & Smith, 1994; Zhang et al., 1995; Ahlawat et al., 1998; Воробьев, 1998; Lira Junior et al., 2005). In the case of soybeans, nitrogen fixation can be reduced up to 40% if exposed to low RZT (Lynch & Smith, 1994). Faba bean seeds, germinated using Petri dishes, had normal seed germination at 15 °C, with significantly lower results at 4 °C and 10 °C (Rowland & Gusta, 1977). Slightly decreased RZT (10–12 °C) resulted in nodule formation up to 13 days later than normal temperature (18–22 °C), RZT of 5–7 °C delayed nodule formation up to 28 days compared to control; strong delay of nodule formation (up to 34 days compared to normal RZT) was caused by the RZT of 4–5 °C (Воробьев, 1998). In addition to delayed and reduced nodulation, low root zone temperature can also completely inhibit legume nodule formation (Graham, 1992; Lira Junior et al., 2005). In-depth knowledge about the low RZT induced stress affecting legumes is still required to provide the market with the most suitable legume seed inoculum. Moreover, compared to soybean, there is not enough research done on the effect of low RZT on faba bean early development.

To ensure optimal early development and growth of legumes, as well as to improve currently inhibited nodulation under suboptimal root zone temperatures, there is a need for new solutions that could be used by farmers. For this purpose, we tested the potential of a commercial rhizobia preparation that would also contain additional mycorrhiza fungi. In addition to rhizobia, mycorrhiza fungi are also used when growing legumes, as they both have been suggested to have a positive effect on the quality and quantity of legume yield (Kantar et al., 2003). Arbuscular mycorrhiza fungi formation on roots improves water and nutrient supply (i.e., P, Zn, and Cu) for plants, thus providing a healthier and denser root system and promoting growth of the whole plant, which is particularly important when growing in poor soil conditions (Ruotsalainen & Kytöviita, 2004; Dash & Gupta 2011; Grover et al., 2011). A consortium of rhizobia and mycorrhiza as a bioinoculant, instead of single inoculation, has been previously recommended, as these microorganisms have shown to complement each other. This consortium has been suggested to improve rhizobia induced nodulation, nitrogen fixation, mycorrhizal colonization, and the growth of the whole plant (Dash &, Gupta, 2011). A higher faba bean yield was obtained, when seeds were treated with both rhizobia and mycorrhiza before sowing, compared to single rhizobia inoculation (El-Wakeil & El-Sebai, 2007). However, mycorrhiza fungi activity, like rhizobia, can be adversely affected by low RZT. For instance, mycorrhization of pine seedlings was reduced at low RZT (5 °C) and it was reported that longer pine seedling exposure to lower soil temperature (starting only from week 6 and further) results in reduced mycorrhization (Domisch et al., 2002).

It is important to develop a recommendation for legume seed inoculation with microorganisms and germination for farmers, as cold stress is a widespread problem, which can significantly reduce the yield of important agricultural legume crops (Grover et al., 2011). Knowledge of the temperature impact on efficient microorganism activity would help farmers to choose more efficient sowing time, as well as to consider the appropriate microorganism preparation inoculation. Until now no previous study has been carried out testing Latvian rhizobia activity and the nodulation ability of legume roots under the temperature stress. In addition, no recent study has researched the effect of low root zone temperature on the efficiency of rhizobia and mycorrhiza double inoculation. The goal of this study is to determine which is the most suitable microorganism inoculum for faba bean seed treatment, that would improve the resistance to low RZT and ensure normal seed germination. An experiment was developed to test seed germination using different microorganism inoculation treatments under several temperature treatments.

MATERIALS AND METHODS

Experimental setup

Experiments were carried out using glass Petri dishes. Four bean (*Vicia faba*) cultivars were used: two *V. faba* var. *minor* Beck – 'Lielplatone' (obtained from State Priekuli Plant Breeding Institute, Latvia) and 'Fuego' (obtained from Norddeutsche Pflanzenzucht Hans–Georg Lembke KG, Germany), and two *V. faba* var. *major* Harz – 'Bartek' and 'Karmazyn' (obtained from Torseed®, Poland) For *V. faba* var. *minor*– 10 seeds per Petri dish; for *V. faba* var. major – 5 seeds per Petri dish were placed. Seeds were not surface sterilized, and no growth media was used in the Petri plates. Four variants of seed treatment were used in experiments: 1) *Rhizobium sp.* ('Rh'), 2) mycorrhiza fungi containing preparation ('M'), 3) rhizobia and mycorrhiza fungi preparation ('Rh + M') and 4) control without any symbiont ('control'). Experiment was done in three replicates with each Petri dish as a replicate.

Seed treatment

A *Rhizobium leguminosarum* strain (RV407), isolated from beans (*Vicia faba*), was used for 'Rh' variant. This strain was isolated in Latvia and is included in the 'Rhizobium Collection' of the Institute of Soil and Plant Sciences, Latvia University of Agriculture. Rhizobia suspension was prepared by washing off *Rhizobium* bacterium pure culture, grown on Yeast Mannitol Agar media, with 20 mL of distilled water (not sterilized). The final concentration of inoculation suspension contained 10^6 bacteria cells per millilitre. This rhizobium strain was chosen as it is the most commonly used strain in various experimental trials and has proven itself to be the most efficient when growing faba beans.

Commercial granulated mycorrhiza fungi preparation, used for 'M' and 'Rh+M' variants, was obtained from Symbiom® (Czech Republic). According to the mycorrhiza fungi preparation producer, mycorrhiza inoculum contains a mixture of tree AMF (arbuscular mycorrhizal fungi) strains: *Glomus claroideum, G. intradices* and *G. mosseae*. For 'M' variant, one gram of mycorrhiza fungi preparation was added to 20 mL of distilled water in the Petri dish. For 'Rh+M' variant, one gram of the commercial granulated mycorrhiza fungi preparation was added in 20 mL of rhizobia bacteria suspension. In the case of control variant, 20 mL of distilled water was poured in the Petri dish.

Seed germination

Seed germination was done in dark conditions. Four different temperature treatments were tested under controlled conditions: 4, 8, 12 and 20 °C. Three days after seed inoculation, the number of germinated seeds was counted, the exudate was removed for further biochemical measurements (not discussed in this article) and additional 10 mL of distilled water was added. This step (removal of the exudate and addition of 10 mL distilled water) was repeated twice a week until the end of the experiment. The length of the experiment varied between the temperature treatments, as the germination in higher temperatures was much faster than in lower ones. Seeds under 20 °C treatment were germinated for 6 days; under 12 °C – for 15 days; under 8 °C – for 30 days; under 4 °C – for 40 days.

Measurements

At the end of temperature treatment experiment, the fresh weight of the whole seedling was measured, the root of the seedling was removed right below the seed, the length and the fresh weight of the primary root was measured. The primary roots in most cases had not yet developed lateral roots, therefore lateral roots were not considered for the measurements. The primary root weight ratio (ratio of the root weight to the total plant weight, expressed in percentage) was calculated. In addition, percentage of germinated seeds, out of the total number of seeds used, was recorded. Seeds were considered as germinated when the primary root could be visible.

Data analyses

All statistical analyses were done using Excel (Microsoft Corporations, Redmond, Washington, USA). Obtained data were processed using Analyses of Variance (ANOVA). Differences were considered statistically significant when $p < 0.05$. Error bars for figures show the Least Significant Difference (LSD).

Data was transformed in the case of length of faba bean seedlings, to meet the requirements for homogeneity of variance. Data transformation was conduced as follows: each measured seedling length result was multiplied with a coefficient, which was obtained by dividing the population mean with the sample mean.

RESULTS

Primary root length

Under normal faba bean seed germination temperature treatment (20 °C), most of the cultivars had significantly longer primary roots ($p < 0.001$), when seeds were treated with a microorganism inoculum ('Rh', 'M', and 'Rh+M'), compared with control variant (Fig. 1). An exception was 'Fuego', where 'Rh' treatment had no significant root length promoting effect, compared with control. Mycorrhiza preparation (‘M’) appears to have a stronger promoting effect on the early growth of primary roots under the temperature treatment of 20 °C. Only faba bean cultivar ‘Bartek’ had significantly ($p < 0.01$) longer primary roots under the combined rhizobia and mycorrhiza preparation treatment (‘Rh+M’), compared to other treatments.

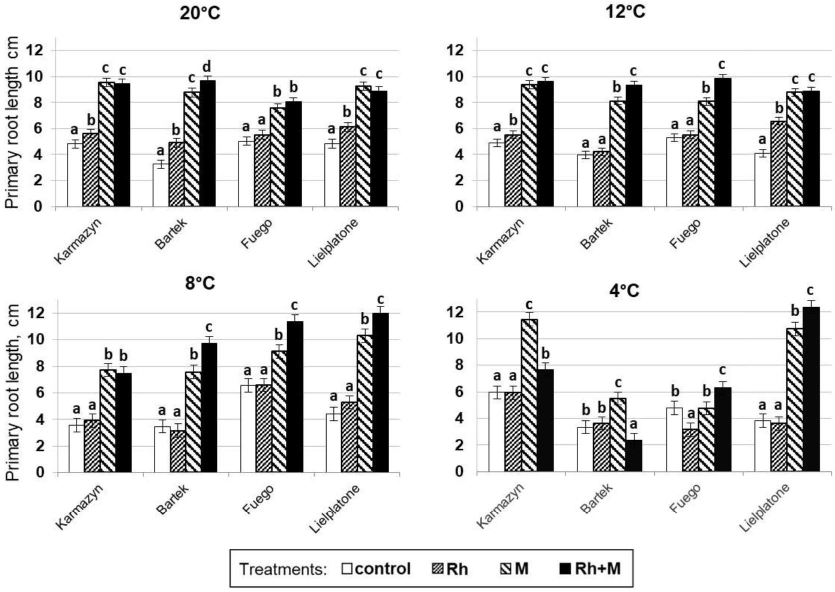

Figure 1. Length of faba bean seed primary roots depending on different microorganism inoculations under various temperature treatments. Different letters indicate significant differences between the different treatments within one cultivar at a certain temperature (LSD; $p < 0.05$).

When the germination temperature was decreased to 12 °C, only ‘Karmazyn’ and ‘Lielplatone’ seed inoculation with ‘Rh’ treatment resulted in significantly ($p < 0.05$) longer primary roots, compared to the control variant. The use of mycorrhiza fungi preparation – both alone or when added to rhizobia, significantly promoted primary root growth for all cultivars. Moreover, ‘Bartek’ and ‘Fuego’ seedlings had significantly longer primary roots ($p < 0.05$) under ‘Rh+M’ treatment, compared with single ‘M’ treatment.

At an even lower seed germination temperature (8 °C), none of the cultivars with ‘Rh’ treatment, had significantly longer primary roots, compared with control variant. The treatment ‘M’ and ‘Rh+M’, resulted in significantly longer primary roots for all bean cultivars. Furthermore, ‘Bartek’, ‘Fuego’ and ‘Lielplatone’ had significantly ($p < 0.001$) higher results of primary root growth under ‘Rh+M’ treatment, in comparison with ‘M’ preparation treatment alone.

When seedlings were exposed to a cold stress during germination (4 °C temperature treatment), primary root length strongly differed depending on the bean cultivar used. ‘Karmazyn’ seedlings had significantly longer primary roots compared with control and ‘Rh’ variant, when treated with ‘Rh+M’ ($p < 0.001$); however, the promoting effect was much larger when mycorrhiza was used alone (‘M’). Like ‘Karmazyn’, also for ‘Bartek’ ‘M’ treatment achieved the longest primary roots ($p < 0.05$), compared to control, ‘Rh’ and ‘Rh+M’ treatments. Primary roots for ‘Bartek’ under ‘Rh+M’ treatment resulted in significantly lower ($p < 0.05$) results compared to all the other treatments. ‘Fuego’ seeds treated with ‘Rh’ resulted in significantly ($p < 0.05$) lower results in comparison with other treatments, while ‘Rh+M’ variant had the longest primary roots ($p < 0.05$). For the faba bean cultivar ‘Lielplatone’ – ‘M’ and ‘Rh+M’ treatments were the most successful for promoting primary root length. In addition, primary root length under ‘Rh+M’ treatment significantly ($p < 0.05$) exceeded the results achieved with ‘M’ treatment.

Primary root weight ratio

The ratio of the primary root weight of the total plant weight was calculated (Fig. 2). Under the germination temperature of 20 °C primary root weight ratio for cultivars ‘Karmazyn’, ‘Bartek’ and ‘Lielplatone’ was significantly ($p < 0.05$) higher than control when treated with any of the bio-stimulants (‘Rh’, ‘M’ and ‘Rh+M’). For these three cultivars, single treatment with rhizobia (‘Rh) had lower primary root weight ratio results than seed treatment with ‘M’ or ‘Rh+M’. For ‘Fuego’ primary root weight ratio results did not differ significantly between the seed treatment variants at 20 °C. Seed germination temperature reduction to 12 °C resulted in significantly higher primary root weight ratio for all the cultivars under seed treatment variants ‘M’ and ‘Rh+M’, compared with control and ‘Rh’.

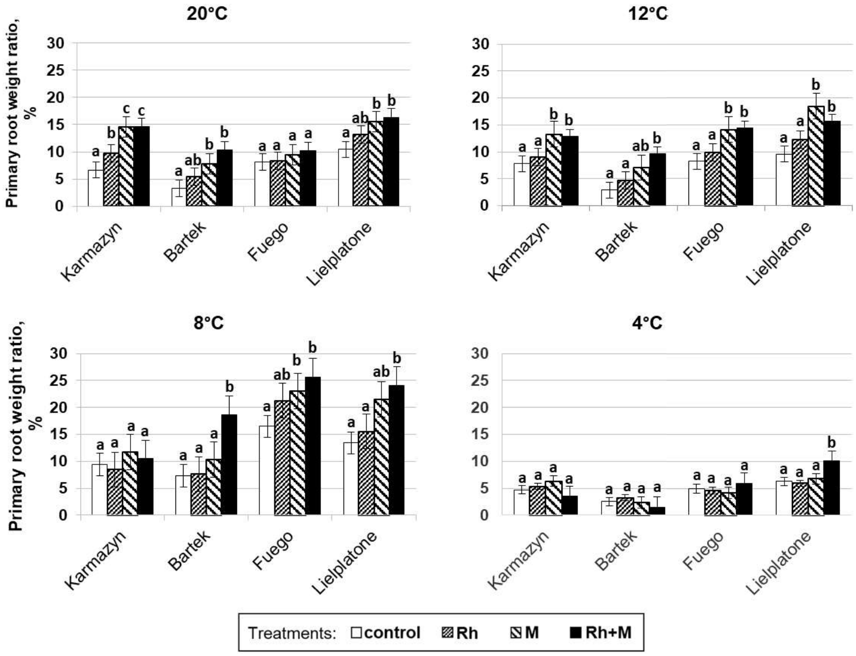

Figure 2. Primary root weight ratio in faba bean seedling depending on different microorganism inoculation under various temperature treatments. Different letters indicate significant differences between the different treatments within one cultivar at a certain temperature (LSD; $p < 0.05$).

When germination temperature was lowered to 8 °C, primary root weight ratios remained significantly higher compared to control and ‘Rh’ for cultivars ‘Bartek’ – variant ‘Rh+M’, ‘Fuego’ and ‘Lielplatone’ – variants ‘M’ and ‘Rh+M’. At 8 °C ‘Rh’ treatment has a significant promoting effect on the primary root growth only for cultivar 'Fuego'. Unlike primary root length (Fig. 1), seed germination at 4 °C dramatically affected the primary root weight ratio. Almost no promoting effect of microorganisms can be observed under 4 °C treatment. 'Lielplatone' alone had significantly higher primary root weight ratio under 'Rh+M' treatment.

Percentage of germinated seeds

During the experiment it was observed that microorganism treatment has an important effect of on seed germination at temperatures ≤ 12 °C (data not shown). Percentage of germinated seeds mostly depended on bean cultivar. 'Karmazyn' had the highest number of germinated seeds (Fig. 3) under 'M' treatment; however, it was not significantly higher than control plants or variant 'Rh+M'. The use of rhizobia slowed germination process significantly ($p < 0.05$) for cultivar 'Karmazyn'. Broad bean cultivar 'Bartek' treated with 'M' had the highest percentage of germinated seeds, compared to any other treatment ($p < 0.05$). For 'Fuego' the highest number of germinated seeds was obtained when treated with the combined microorganism inoculum 'Rh+M', while single inoculation with 'Rh' and 'M' separately reached significantly higher ($p < 0.05$) percentage of germinated seeds compared to control. 'Lielplatone' seeds had the highest germination energy when treated with 'Rh' inoculum, although it did not significantly differ from treatments 'M' and 'Rh+M'.

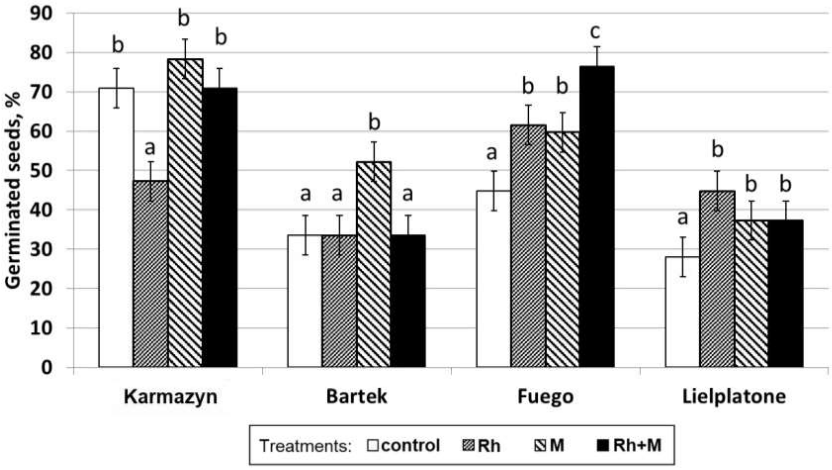

Figure 3. Percentage of germinated faba bean seeds depending on different microorganism inoculation. Different letters indicate significant differences between the different treatments within one cultivar (LSD; $p < 0.05$).

DISCUSSION

In this study the effect of different microorganisms at various temperatures (4, 8, 12 and 20 °C) was tested on the germination of seeds. Primary root length and primary root weight ratio were selected as indicators of seed germination activity. As it is stated by Fyson & Sprent (1982), root development indicates not only the plant growth potential, but also demonstrates the effect of microorganisms that the seedling is exposed to, as delayed nodulation due to low RZT has been associated with slower plant development.

As expected, the growth promoting effect of rhizobia strain RV407 ('Rh') reduced with lower temperatures. Lower primary root parameters for seedlings initially treated with rhizobia could be explained as rhizobia consumes energy for early nodule development (Fyson & Sprent, 1982). Low root zone temperature has previously shown to cause delayed nodule formation – RZT of 4–5 °C resulted in up to 34-day delay in nodule formation (Воробьев, 1998). In addition, decrease in nodulation has been recorded when faba beans are exposed to 10 °C, compared with 15 and 20 °C treatments (Herdina & Silsbury, 1989). Although our experiment was not set-up as to monitor seedling further growth and nodulation, results obtained in this study suggest that the activity of rhizobia decreases below 8 °C, resulting in a potentially lower nitrogen fixation ability that has been previously shown to lead to reduced plant growth, reflected in a lower final yield (Prévost et al., 2003; Lira Junior et al., 2005; Dash & Gupta, 2011). From the results obtained in our study, it can be suggested that seed inoculation with only rhizobia ('Rh') is not efficient practice in the field when sown in early spring, when the RZT often does not exceed 4 °C. Although rhizobia are tolerant to 4 °C temperature (Drouin et al., 2000) and the bacterial activity is expected to increase with increasing temperature later in spring, if the rhizobia treated seeds are initially exposed to low RZT for a longer time, it might increase the bacterial lag phase, therefore delaying or even decreasing the final rhizobia cell number, leading to reduced nodulation (Fyson & Sprent, 1982; Beales, 2004).

Results obtained in this study indicate that adding mycorrhiza preparation to rhizobia inoculum can significantly enhance the early growth of primary roots, compared to single inoculation with only rhizobia. In many cases the results obtained for variants of the double inoculation were not significantly different from the ones treated with just mycorrhiza fungi (e.g. 'Karmazyn' at 8, 12 and 20 °C; 'Lielplatone' at 12 and 20 °C). Nevertheless, seed inoculation with only mycorrhiza is not recommended, as rhizobia treatment is crucial for nitrogen supply at later plant developmental stages. Root growth promoting effect caused by mycorrhiza fungi has been previously reported on maize plants, with higher root dry weight under both – optimal and low RZT (25, 15 and 5 °C; Zhu et al., 2010). The promoting effect of the microorganism treatments on primary root development, observed in this study, often depended on the *V. faba* cultivar used.

It was observed that a low seed germination temperature of 4 °C resulted in no consistent microorganism treatment effect. This is in line with previous studies, showing that not only rhizobia, but also root colonization with mycorrhiza fungi and corresponding shoot and root biomass is negatively affected by low RZT (Ruotsalainen & Kytöviita, 2004; Zhu et al., 2010). It could be argued that the experimental duration of two to six weeks (depending on the germination temperature) is not enough to develop a symbiosis between mycorrhiza fungi and bean seedlings. Nevertheless, data obtained in the present study showed a significant stimulating effect of single mycorrhiza inoculation and combined rhizobia and mycorrhiza inoculation on the development of primary roots.

The commercial preparation used in this study may contain mycorrhizal metabolites that could have been released from the previous symbiosis with the host plant during the mycorrhiza cultivation for this commercial product. It has been shown by Dash & Gupta (2011) that organic compounds, excreted by rhizosphere microorganisms, can accelerate root growth; therefore, it could be possible that such organic compounds, left in the mycorrhiza preparation, could be directly used by the seedlings, stimulating primary root growth, as observed in the present experiment. As the commercial mycorrhiza fungi preparation used in this study is not sterile, another possibility could be that there are some other plant growth promoting rhizobacteria (PGPR) present in this preparation. However, the manufacturer does not provide the bacterial content of this mycorrhiza product, nor was it determined in this study. It has been previously shown that rhizosphere microorganisms, through various mechanisms, can effectively increase seed germination, including primary root development, leading to a better crop quality and quantity (Antoun et al., 1998; Dash & Gupta, 2011; Pérez-Montaño et al., 2014; Verbon & Liberman, 2016). This growth promoting effect is especially important when plants are exposed to abiotic stress, such as low root zone temperature (Grover et al., 2011; Souza et al., 2015). However, even if there are other PGPR present in the mycorrhiza preparation used in the present study, the significance of the seedling primary root growth promoting effect could not be attributed only to this possibility.

Combination of rhizobia and mycorrhiza fungi for seed inoculation has been suggested before. The synergic relationship between both microorganisms have been shown to have a plant growth promoting effect, including enhancing legume nodulation and providing mineral nutrition for the plant (Antoun et al., 1998; Dash & Gupta, 2011). An enhanced growth of soybeans has been observed due to the complementary interaction of rhizobia and mycorrhiza fungi, particularly in an environment with nitrogen and phosphorous deficiency (Wang et al., 2011). Soybean co-inoculation with rhizobia and mycorrhiza fungi preparation have showed an enhanced nitrogen fixation ability, compared with control plants and plants inoculated with only rhizobia (Mishra et al., 2011).

CONCLUSIONS

It can be concluded that seed germination significantly depends on bean cultivar and root zone temperature. *Vicia faba* var. *major* seeds require higher temperature compared to *Vicia faba* var. *minor*. For Latvian bean cultivar 'Lielplatone' the highest percentage of germinated seeds could be observed at 4 °C. Decrease in the percentage of germinated seeds was observed for *Vicia faba* var. major cultivars 'Bartek' and 'Karmazyn' because of single rhizobia treatment. The use of mycorrhiza preparation mitigated the effect of rhizobia. Inoculation of *Vicia faba* var. *minor* stimulated germination of seeds, and the most significant effect was obtained using of rhizobia. At temperatures higher or equal to 8 °C, stimulation of primary root growth was observed as a result of microorganism and plant interaction. In most cases the largest stimulating effect was obtained with combination of rhizobia and mycorrhiza fungi preparation. At 4 °C the stimulating effect persists only for *Vicia faba* var. *minor* cultivars, compared to the suppressive effect on *Vicia faba* var. *major*.

It can be concluded that seed inoculation with rhizobia should be supplemented with a mycorrhiza fungi preparation, especially when the sowing is done while the root zone temperature is still below the optimal. Double inoculation with both rhizobia and mycorrhiza fungi containing preparation is especially important for cultivars 'Bartek', 'Fuego' and 'Lielplatone'. In practice, as root zone temperature is crucial for seed germination and development of effective symbiotic system, faba bean sowing is recommended when the root zone temperature reaches at least 8 °C.

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