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Co-inoculation of *Pseudomonas* spp. with *Rhizobium* improves growth and symbiotic performance of fodder galega (*Galega orientalis* Lam.)

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ABSTRACT

The effect of *Rhizobium galegae* alone and in combination with root colonising *Pseudomonas* strains on the growth of fodder galega (*Galega orientalis* Lam.) was studied under greenhouse conditions in potting soil containing low levels of nitrogen. Eight weeks after sowing combined inoculations of fodder galega with *R. galegae* bv. *orientalis* HAMBI 540 and *Pseudomonas trivialis* 3Re27 or *Pseudomonas extremorientalis* TSAU20 had increased shoot and root dry matter, as compared with inoculation with *R. galegae* HAMBI 540 alone. Both *Pseudomonas* strains produced indole-3-acetic acid (IAA) in culture but *R. galegae* did not. While the cellulase producing strain *P. trivialis* 3Re27 was able to significantly increase nodule numbers and nitrogen content of the co-inoculated plants, the cellulase-negative *P. extremorientalis* TSAU20 showed no significant stimulation of nodule numbers and nitrogen content in roots. We conclude that *P. trivialis* 3Re27 improve rhizobia–legume interactions, acting as "rhizobium helper bacteria". The production of IAA and/or cellulase by *Pseudomonas* strains may contribute to such a positive effect.

1. Introduction

Fodder galega (*Galega orientalis* Lam.) is a perennial leguminous plant indigenous to the sub-alpine regions of the Caucasus. It has been introduced to the Baltic countries, Scandinavia and north-west Russia as a forage crop and green manure. After a slow initial development fodder galega grows fast, producing excellent quality forage for all kinds of livestock and poultry, being rich in essential amino acids (38–47%), especially proline and arginine [20,25]. Cultivation of fodder galega improves soil fertility and structure by accumulating nitrogen and organic matter and by decreasing soil permeability and erosion [23,27]. The symbiosis between fodder galega and rhizobium is very host specific. Only *Rhizobium galegae* bv. *orientalis* forms nitrogen-fixing nodules on fodder galega. *R. galegae* bv. *officinalis* fixes nitrogen in symbiosis with *Galega officinalis* and induces inefficient nodules on fodder galega [18]. Neither host plant is nodulated by other rhizobial species [16,26]. Inoculation of seeds with effective *R. galegae* bv. *orientalis* strains is necessary in soils where fodder galega is not native.

In the rhizosphere the synergism between various bacterial genera such as *Bacillus, Pseudomonas* and *Rhizobium* has been demonstrated to promote plant growth and development [5,14,39]. Compared to single inoculation, co-inoculation has improved the absorption of nitrogen, phosphorus and mineral nutrients by plants [2]. However, some *Pseudomonas* strains reduced nodule numbers and their capacity for nitrogen fixation for example on beans [6] and fodder galega [33]. Therefore, before practical applications it is important to investigate, how inoculation with a root-colonising and plant-growth promoting *Pseudomonas* strain influence nodulation and nitrogen fixation of leguminous plants.

The aim of our study was to find out if co-inoculation of fodder galega seeds with specific rhizobia and plant growth promoting *Pseudomonas* strains has a positive effect on plant growth and symbiotic performance, when growing plants in potting soil low in nitrogen. *P. trivialis* 3Re27 and *Pseudomonas extremorientalis* TSAU20 were selected for the study based on their documented excellent root colonising capability, plant growth promoting activity and antagonism towards plant pathogenic fungi [3,10,11].

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2. Materials and methods

The root colonising *Pseudomonas trivialis* strain 3Re27 was obtained from the culture collection of Graz University of Technology, Graz, Austria and *P. extremorientalis* strain TSAU20 from the culture collection of the Department of Microbiology and Biotechnology, National University of Uzbekistan. *P. trivialis* strain 3Re27 was originally isolated from the endosphere of potato [3] whereas, *P. extremorientalis* strain TSAU20 was isolated from the rhizosphere of wheat grown in salinated soil by an enrichment procedure which selects for enhanced root colonizers [10]. *R. galegae* bv. *orientalis* strain HAMBI 540 was obtained from the culture collection of University of Helsinki, Finland (HAMBI). The *Pseudomonas* strains were grown on King's B agar (KB) [17], and *R. galegae* strain HAMBI 540 on tryptone yeast extract agar (TY) [4] at 28 °C.

The production of indole-3-acetic acid (IAA) was determined according to the method of Bano and Musarrat [1]. The *R. galegae* strain was grown in TY broth and *Pseudomonas* strains in KB broth and incubated for three days at 28 °C. After cultivation, 2-ml aliquots of bacterial cultures were centrifuged at 13 000 g for 10 min. One ml of supernatant fluid was added to a tube with 100 μl 10 mM orthophosphoric acid and 2 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ + 50 ml of 35% $HClO_4$). After incubation for 25 min, the absorbance of the developed pink color was read at 530 nm. The IAA concentration (in culture) was determined from the calibration curve using standard indole-3-acetic acid (IAA) (Sigma–Aldrich, St. Louis, MO, USA) solution.

Cellulase activity was determined on KB agar plates containing the substrate carboxymethylcellulose [15]. Bacterial strains were grown for three days on agar plates, and a halo appearing around colonies indicated cellulase activity.

Plant tests in the greenhouse were conducted in plastic pots (16 cm diameter, 18 cm height, with drainage holes on the bottom) filled with 1.5 l of potting soil with low nitrogen content (Kekkilä Oyj, Mellilä, Finland). The nutrient content and other properties of potting soil were as follows: N 70 mg l^{-1}, P 21 mg l^{-1}, K 140 mg l^{-1}, and pH 6.0, electrical conductivity 20 mS/m. Before sowing, seeds of fodder galega (Naturcom Oy, Ruukki, Finland) were surface-sterilized for 5 min with concentrated sulphuric acid followed by 70% ethanol for 3 min and rinsed five times with sterile, distilled water. The sterility of seeds was tested on KB and TY agar after incubation for three days. Sterilized seeds were germinated on 1% water agar in the dark at 28 °C [30].

For seed inoculation *R. galegae* was grown overnight in TY broth and *Pseudomonas* strains in KB broth. One ml of each culture was pelleted by centrifugation and the supernatant was discarded. Cell pellets were washed with 1 ml phosphate buffered saline (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and suspended in PBS. Cell suspensions were diluted to an optical density of 0.1 at 620 nm, corresponding to a cell density of 10^8 cells/ml. Cell suspension with two strains were mixed in a ratio 1:1 and vortexed vigorously to yield a homogenous suspension. Germinated seeds were placed in the bacterial suspension with sterile forceps and shaken gently for a few seconds. After standing for 10 min the inoculated seeds were planted in the plastic pots. Six inoculated germinated seeds were sown per pot at a depth of approximately 1.5 cm. After germination plants were thinned to two per pot. Five replicate pots were used for each treatment, each of them containing two plants. The treatments were as follows: i) seeds inoculated with *R. galegae* HAMBI 540 alone, ii) *R. galegae* HAMBI 540 combined with *P. trivialis* 3Re27 and iii) *R. galegae* HAMBI 540 combined with *P. extremorientalis* TSAU20. After plant emergence plants were watered from the bottom manually to saturation as needed, and no fertilizers were applied. Fodder galega is a perennial plant, the initial growth of which is slow. About two months of growth is normally required to observe clear differences in plant yield, nodulation and especially in nitrogen content between different treatments. In the present work fodder galega was grown for eight weeks (57 days) in a greenhouse with a 16-h light period at 23 °C. The relative humidity of the air was adjusted to 40%. At harvest, shoots were separated from roots, and roots were washed. Shoots and roots of each individual plant were dried to constant weight at 100 °C and weighed, each plant separately. The number of nodules per plant root was determined using a stereomicroscope.

For the determination of total N content oven-dried shoots and roots were homogenized, and powders of three shoots and roots were combined, resulting in three shoot and root samples per treatment ($N = 3$). Two replicate measurements were made from each sample. Total nitrogen in the shoots and roots was determined by dry combustion on a LECO® CNS-1000 Carbon, Sulphur and Nitrogen Analyser (Leco Instrument, St. Joseph, Michigan).

Analysis of variance was performed using the Excel program package version 11 for Windows 98 (Microsoft Corporation), Student's *t*-test and least significant differences (LSD) were applied to compare means at $P < 0.05$.

3. Results and discussion

The greenhouse experiment showed that co-inoculation of fodder galega either with *R. galegae* HAMBI 540 and *P. trivialis* 3Re27 or with *R. galegae* HAMBI 540 and *P. extremorientalis* TSAU20 improved plant growth, nodulation and N content compared to plants inoculated with *R. galegae* HAMBI 540 alone in potting soil containing low levels of nitrogen. Co-inoculation of plants with *P. trivialis* 3Re27 and *R. galegae* HAMBI 540 showed the highest stimulatory effect, by increasing statistically significantly shoot growth by 69% and root growth by 63% in comparison to inoculation with *R. galegae* HAMBI 540 alone (Fig. 1A).

Furthermore, plants co-inoculated with *R. galegae* HAMBI 540 and *Pseudomonas* strain 3Re27 showed a significantly greater number of nodules than plants inoculated with *R. galegae* HAMBI 540 alone (Fig. 1C), increasing the number of nodules up to 32%. A majority of the nodules were located in the upper part of the root and were pink, i.e. being capable of fixing nitrogen. The cytoplasm of nodule cells contains an oxygen-carrying compound, leghemoglobin, which gives active nodules a pink color [24]. The lateral roots had white, small inefficient nodules. The greater number of active nodules can be expected to contribute to more nitrogen being fixed which increases plant growth under nitrogen deficient conditions.

Because the aim of this work was to test whether the combination of a growth-promoting *Pseudomonas* strain, either *P. trivialis* strain 3Re27 or *P. extremorientalis* strain TSAU20, with a rhizobium strain improves or disturbs nodulation and nitrogen fixation on fodder galega, we did not include uninoculated controls in the greenhouse experiment. However, when the same potting soil with low nitrogen content was used in another experiment with fodder galega, no plant out of ten uninoculated pots (two plants per pot) was nodulated (Räsänen, unpublished results).

A significant increase in N content of root and shoot of fodder galega was also observed after co-inoculation of *Pseudomonas* strains with *R. galegae* HAMBI 540. *P. trivialis* 3Re27 combined with *R. galegae* HAMBI 540 significantly increased the N content of the roots by 20% and of the shoots by 52% compared to *R. galegae* HAMBI 540 alone, whereas *P. extremorientalis* TSAU20 increased only the N content of shoots, but not of roots (Fig. 1B).

The positive effect of combined bacterial treatments on plant growth, nodulation and nitrogen fixation has already been described for other leguminous crops. Co-inoculation with *Pseudomonas* spp. and *Rhizobium* spp. enhanced nodulation and

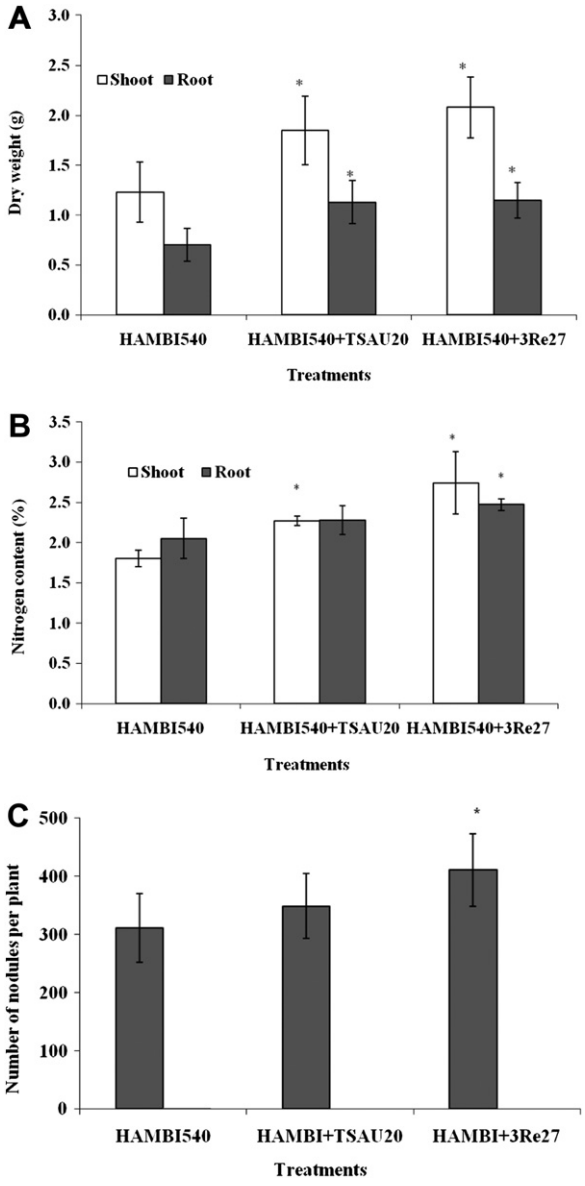

Fig. 1. Dry weight (A) and nitrogen content (B) of shoots and roots, and number of nodules (C) formed on fodder galega roots when seedlings were inoculated with *Rhizobium galegae* strain HAMBI 540 alone and together with *Pseudomonas extremorientalis* strain TSAU20 or *P. trivialis* strain 3re27. Plants were grown in the greenhouse for eight weeks in potting soil containing low levels of nitrogen. Values of dry weight and number of nodules represent means for two plants from five replicate pots ($N = 10$) and values of nitrogen content represent means for three plants ($N = 3$) with error bars showing standard deviation. Columns marked with an asterisk differed significantly from plants inoculated with *Rhizobium* alone at $P < 0.05$ (Student's *t* tests).

nitrogen fixation, plant biomass and grain yield in various leguminous species including chickpea [13], pigeonpea [35] and pea [9].

However, the mechanisms behind this effect are only partly understood. One of the mechanisms used by plant growth promoting bacteria to alter nodule formation is the production of phytohormones such as auxin, gibberellins, and cytokinins [16,21,37]. In our study both *Pseudomonas* strains produced the auxin phytohormone indole-3-acetic acid (IAA) in culture; *P. trivialis* strain 3Re27 produced 12 μg ml^{-1} and *P. extremorientalis* strain TSAU20 10.1 μg ml^{-1}. In previous studies *P. trivialis* strain 3Re27 was able to produce IAA *ad planta* along the whole root (Berg, unpublished results). *R. galegae* strain HAMBI 540 did not produce IAA in our experiment.

The endogenous IAA level in plant regulates growth of shoots and roots, and in the case of legumes formation of nodules [34,38]. In our recent study we observed that low concentrations of exogenously given pure IAA stimulated shoot and root growth of wheat in nonsaline and saline conditions, and similar effects were induced by IAA producing bacteria [11]. A similar phenomenon has been observed among legumes, including those co-inoculated with *Azospirillum brasilense* and rhizobia. Low concentrations of pure IAA or inoculation with a low titer of IAA producing bacteria enhanced root growth and nodulation of legumes [7,28,36] whereas high IAA concentration or inoculation of legumes with high titers of IAA producing bacteria inhibited root growth and nodulation [12,22]. It has been suggested that inhibition of nodulation is due to both strains being IAA producers. However, for example inoculation of soybean with two IAA producing bacteria, *A. brasilense* and *Bradyrhizobium japonicum,* singly or in combinations promoted seed germination and early seedling growth [8]. Apparently, bacterial IAA biosynthesis alone cannot explain improved plant growth [31]. The role of IAA is more complex because IAA of bacteria can also act as a signal molecule in bacteria–bacteria communication and perhaps in bacteria-plant communication [31]. Taken together, stimulation of the growth of the root system observed both in previous studies and in our present work may result in better absorption of water and nutrients from the soil.

Another explanation for enhancement of nodule formation by the rhizobia in legumes might be the production of hydrolytic enzymes such cellulases by root colonising *Pseudomonas* strains [32]. One of the key events during the infection process is a highly localized complete erosion of the legume cell wall, through which the bacterial symbiont penetrates to establish nitrogen-fixing nodules [32]. Co-inoculation of cellulase producing strain *P. trivialis* 3Re27 with *R. galegae* HAMBI 540 significantly increased nodulation and nitrogen content of fodder galega, whereas cellulase-negative *P. extremorientalis* TSAU20 showed no significant stimulation.

Co-inoculations of five cellulase producing *Pseudomonas* strains with *Mesorhizobium* also increased the number of nodules and especially nodule biomass in chickpea (*Cicer arietinum*) [32]. Robledo et al. [29] showed that both the cell-bound cellulase enzyme from *Rhizobium leguminosarum* bv. *trifolii* and the purified enzyme could erode the tip of root hair wall of the host white clover, making a localized hole of sufficient size to allow rhizobial cell penetration. The pretreatment of wheat roots with cellulase was found to increase root-colonisation of *A. brasilense*, regardless of the strain-wheat cultivar combination [19]. Perhaps the cellulase enzyme produced by *P. trivialis* 3Re27 enhanced colonisation of *R. galegae* HAMBI 540 on fodder galega roots, leading to the increased number of infected root hairs and, subsequently, improving the nodulation probability, i.e. more nodule initials successfully developed to nitrogen-fixing nodules. However, an ultimate proof of the role of cellulase in the stimulation of nodulation and increase in nitrogen content by rhizobia would be to use mutants of the *P. trivialis* strain 3Re27 impaired in cellulase production.

In conclusion, this study suggests that root colonising *Pseudomonas* strains improve rhizobia–legume interactions. Combined inoculations could be an option to improve plant growth and increase nodule numbers and N content of fodder galega. For strains able to improve the rhizobium symbiosis, we suggest the term “rhizobium helper bacteria”. The production of IAA and/or cellulase by *Pseudomonas* strains may contribute to such a positive effect. Combined inoculants could be developed in order to improve the establishment and yield of forage legumes. This hypothesis must however be tested under field conditions and the mode of action studied at the molecular level.

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