Growth and Nutrient Uptake of Orchardgrass (Dactylis glomerata L.) and Meadow Fescue (Festuca pratensis Huds.) as Affected by Rhizobacteria

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Abstract

A diverse group of soil bacteria found in the rhizosphere which can colonize plant roots and improve plant growth are designated as plant growth promoting rhizobacteria. The aim of this study was isolation and screening of different rhizobacterial strains for plant growth promoting characteristics and their ability to improve growth of two grass species, orchardgrass (Dactylis glomerata L.) and meadow fescue (Festuca pratensis Huds.). The strains investigated, belonging to the genera Azotobacter, Bacillus, Pseudomonas and rhizobial bacteria, showed various plant growth promoting traits, such as phosphate solubilisation, siderophore production, and indole-3-acetic acid (IAA) production. Co-inoculation of meadow fescue with Azotobacter chroococcum A2 and Sinorhizobium meliloti or Pseudomonas sp., and A. chroococcum A5 with S. meliloti, significantly increased shoot dry weight (SDW)(25-33%), as well as total N (26-33%), P (24-31%) and K (26-28%) contents in plants (mg pot⁻¹), compared to uninoculated control. In addition, inoculation of orchardgrass with A. chroococcum strain A1, as well as co-inoculation with B. megaterium and A. chroococcum A1 or A31, significantly increased SDW (51-59%) and total N (54-59%), P (51-74%) and K (49-55%) contents, compared to uninoculated control. Nitrogen percentage in SDW was slightly higher than sufficiency ranges, while K percentage was optimal in all treatments in both species. Phosphorous percentage was lower than sufficiency ranges as a consequence of very low soil P content. The results emphasize the potential of particular rhizobacteria to improve the growth of forage grasses.

Keywords: grasses, inoculation, plant nutrition, plant growth promoting rhizobacteria, yield increase, soil fertility

Introduction

Perennial grasses include variety of widely spread species, adaptable to different agro-ecological conditions, which represent basis for sustainable livestock production as a main food for ruminants (Tomić et al., 2007). Among perennial grasses, orchardgrass (Dactylis glomerata L.) and meadow fescue (Festuca pratensis Huds.) are considered to be of great importance due to their high productivity and nutritive values, as well as durability, vitality, modest soil requirements and tolerance to moderate drought stress (Sosnowski et al., 2015). Both species are very productive, orchardgrass dry matter yields ranged from 5-6 t ha⁻¹ to 15 t ha⁻¹, while meadow fescue realised similar yield of 13.5 t ha⁻¹ (Tomić et al., 2007). They are suitable for mixed sowing with perennial legumes, alfalfa (Medicago sativa L.), red clover (Trifolium pratense L.) or white clover (Trifolium repens L.). The legume-grass mixtures are of great importance, considering that legumes perform the biological fixation of nitrogen, with consequent economic and ecological benefit. In addition, being deep-rooted, both grass species may be used as a ground cover, in order to control soil erosion and in rehabilitation programmes of sites disturbed by mining (Baran et al., 2015).

The use of plant growth promoting rhizobacteria (PGPR), to increase soil fertility and improve growth and yield of agronomically important crops is a significant alternative to chemical fertilizers in sustainable agriculture (Saia et al., 2015). Increases in growth and yield of crops in response to inoculation with PGPR have been repeatedly reported (Baris et al., 2014; Dinesh et al., 2015; Imran et al., 2015; Saia et al., 2015). PGPR exert positive effect on plant growth by direct mechanisms, such as biological N₂ fixation, phosphate solubilisation and production of growth regulators (phytohormones), or by indirect mechanisms, such as prevention of the deleterious effects of plant pathogens, production of inhibitory substances (siderophore, antibiotics), or increase of natural resistance of the host (Glick, 1995). Positive growth effects of non-symbiotic nitrogen fixing bacteria of the genera Azotobacter, Azospirillum, Bacillus, Klebsiella etc. associated with different cereals and some grasses have been reported (Bodley and Dobereiner, 1995; Steenhoudt and Vanderleyden, 2000; Kennedy et al., 2004). In these studies, plant growth enhancement is considered not only

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the result of biological nitrogen fixation, but also of other mechanisms, such as phytohormones, etc. (Steenhoudt and Vanderleyden, 2000). Therefore, rhizobacteria with multiple mechanisms of action could be successful in the production of forage grasses. Rhizobial strains, known as legume symbiotic nitrogen-fixing bacteria, can also promote non-legume plant growth by mechanisms independent of N fixation, which can be essential in legume-grass mixture inoculation (Hilali et al., 2001; Vargas et al., 2009; Souza et al., 2013). Soil physical and chemical properties, (such as pH, water availability, temperature, salinity, etc.) can affect plant growth and microflora and fauna in the rhizosphere (Antoun and Prevost, 2005). Therefore, the isolation of PGPR tolerant to these factors is also important.

Although there are some studies showing the potential of rhizobacteria inoculation to increase yield of perennial grasses, a limited number is available regarding PGPR rhizobacteria effects on the growth and nutrient accumulation in orchardgrass and especially in meadow fescue. The use of indigenous PGPR can be an added advantage since they can easily acclimatize to the natural conditions (Verma et al., 2013). Therefore, the aim of the research was to isolate native rhizobacterial strains with multiple plant growth-promoting traits and evaluate their effects on growth and nutrient uptake (N, P, K) of orchardgrass and meadow fescue.

Materials and Methods

Bacterial strains

Seven strains of Azotobacter chroococcum (A1, A2, A3, A4, A5, A31 and A136v), used in this study, were isolated from arable land of different localities. Strains of Bacillus megaterium 4148pk, LR1K and SNj, strains of Pseudomonas sp. luc2, LG, L1K and L2Cr previously isolated as root nodules endophytes of alfalfa (Stajković et al., 2009; 2011), as well as two rhizobial strains, Sinorhizobium meliloti 218 and Rhizobium leguminosarum bv. trifolii 459, were also used. Azotobacter strains were cultivated in the N free mannitol broth, Pseudomonas strains in King B medium (KB), Bacillus strains in nutrient broth (NB), and rhizobial strains in yeast mannitol (YM) medium.

Phosphate solubilisation

Strain phosphate solubilising ability was examined on Pikovskaya medium (1948). The appearance of clear zone around the colony (halo), after 15 days of incubation at 28 °C, indicated P solubilisation ability of strains. Halo size was calculated by subtracting colony diameter from the total diameter.

Siderophore production

Siderophore production was determined in plates with CAS-blue agar and appropriate medium according to the procedure Milagres et al. (1999). The distance of colour change from blue to orange was measured on the 7th, 14th and 21st day of incubation.

Indole-3-acetic acid production

For indole-3-acetic acid (IAA) detection, the strains were grown in appropriate liquid medium (YM, KB, NB or N free mannitol broth) supplemented with L-tryptophan (2 mg ml⁻¹). Cultures were centrifuged and 1 ml of supernatant was mixed with 2 ml of reagent, which consisted of 4.5 g of FeCl₃ per litre in 10.8 M H₂SO₄ (Glickmann and Dessaux, 1995). The appearance of pink colour indicates IAA production and IAA concentration was determined by spectrophotometer at 530 nm.

Tolerance to some environmental factors

Tolerance to NaCl, temperature and pH were examined according to Somasegaran and Hoben (1994).

Vegetative pot experiment

To evaluate plant promoting potential, pots were field with 2 kg of non-sterile soil with the following chemical characteristics: pH 7.0, EC 4.1 mmhos, 5.1 humus 2.83%, N 0.15%, P 28.1 mg kg⁻¹, K 15.94 mg kg⁻¹. Fifty seeds of each grass species were planted in every pot. The experiment was carried out with 6 replications in a completely randomised system and the pots were kept in greenhouse conditions. Orchardgrass (variety K24) and meadow fescue (variety K21) seeds were inoculated with single strains or with their mixture. Inoculums were prepared mixing 2 g of sterile peat with 0.5 ml of liquid single strain culture containing >10⁹ cells ml⁻¹ or with strains mixture in 1:1 ratio. For every grass species there were 6 different treatments with inoculation (single or co-inoculation) and uninoculated control plants (Ø). Plants were harvested after seven weeks.

Plant samples analyses

Plant shoots were separated from roots and dried in an oven at 70 °C till constant weight and the average dry weight per plant were calculated. The percentage of shoot N was determined from dried and ground plant samples, using the CNS analyzer, and the percentage was used to calculate total N content. In plant material samples, P and K contents were determined by dry ashing at 550 °C and acid digestion, after which, P was determined colorimetrically and K by flame photometry (Egner et al., 1960).

Statistical analysis

The effect of inoculation was evaluated using analysis of variance (ANOVA) (statistical program COSTAT), and differences between means were tested for significance by Duncan’s multiple range test.

Results and Discussion

Strains of A. chroococcum showed optimal growth in the pH range of 6 to 12, and weaker growth at pH 5, while strains of Pseudomonas sp. and B. megaterium grew well at pH 5, but they tolerated only pH 9 (Table 1). Similar results for Azotobacter strains were obtained by Ninawe and Paulraj (1997), who detected their growth in the pH range of 5-10, with the optimum about 7.5, while the growth and N-fixation declined with further pH increase or decrease. Detected pH tolerance for Pseudomonas and Bacillus also corresponds to the previous reports (Mishra et al., 2011; Peter and Pandey, 2014).

Strains of A. chroococcum showed the lowest NaCl tolerance, with the good growth in the presence of 0.5% NaCl, and visibly weaker growth going up to 2% NaCl. Pseudomonas sp. strains grew well up to 3% NaCl, while strains of B. megaterium grew up to 7% NaCl, and the most tolerant strain LR1K, showed moderate growth in the presence of 9% NaCl (Table 1). As expected, B. megaterium strains tolerated the highest temperature, showing moderate growth at 50 °C, while other strains could grow up to 35 °C. The lowest temperature for
moderate growth was 12 °C for *Azotobacter* and *Bacillus*, while *Pseudomonas* strains showed moderate growth at 4 °C. *Azotobacter* is likely more sensitive to salinity and high temperature with no growth at 45 °C and 1% or 2.5% of NaCl (Sharma and Rai, 2013; Nosrati et al., 2014). However, some literature data indicate that most *Azotobacter* strains grew slowly in the presence of 4% NaCl, while 6% NaCl completely stopped their growth. Moreover, some *Azotobacter* strains grew in the presence of even 10% NaCl (Akhter et al., 2012). *Bacillus* strains tolerated 50 °C, as well as salinity of 7% NaCl, in contrast to genera *Pseudomonas* with only few strains tolerant to these factors (Kumar et al., 2014).

Three *A. chroococcum* strains A2, A5 and A31 solubilized phosphates in PVK medium (7-10 mm halo) (Table 2). Among *Pseudomonas* strains, P solubilisation ability was detected for L1K, but it was also confirmed for strains L2Cr, LG, luc2 (Stajković et al., 2011; 2014). *B. megaterium* strains confirmed week ability of phosphate solubilisation previously detected (Stajković et al., 2009). Numerous literature data also indicate good phosphate solubilisation ability of *Azotobacter* strains (Garg et al., 2001; Farajzadeh et al., 2012; Nosrati et al., 2014) as well as *Pseudomonas* and *B. megaterium* strains (Bhaktavatchalu et al., 2013; Deshwal and Kumar, 2013). Siderophore production was not detected for *A. chroococcum* strains, while all *B. megaterium* strains showed this ability, but with lower efficiency (lower diameter of colour change) compared to *Pseudomonas* sp. strains (Table 2). Siderophore production is not such a common characteristic of *Azotobacter*; although some strains possessed this feature (Muthuselvan and Balagurunathan, 2013), research conducted by Joseph et al. (2007) indicated that none of the 40

### Table 2. Plant growth promoting traits of the strains

<table>
<thead>
<tr>
<th>Table 2. Plant growth promoting traits of the strains</th>
<th><em>Azotobacter</em></th>
<th><em>Bacillus</em></th>
<th><em>Pseudomonas</em></th>
<th><em>Rhizobia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>A4</td>
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<tr>
<td>P solubilisation (halo in mm)</td>
<td>-</td>
<td>-</td>
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<td>Siderophore production (color change in mm)</td>
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<td>7th day</td>
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<td>14th day</td>
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<tr>
<td>21st day</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IAA production (µg ml⁻¹)</td>
<td>2³</td>
<td>28</td>
<td>51</td>
<td>28</td>
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<tr>
<td>48h</td>
<td>56</td>
<td>143</td>
<td>56</td>
<td>76</td>
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<td>72h</td>
<td>105</td>
<td>141</td>
<td>105</td>
<td>97</td>
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<tr>
<td>96h</td>
<td>146</td>
<td>105</td>
<td>146</td>
<td>97</td>
</tr>
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</table>

- no production/solubilisation; nt not tested
tested Azotobacter strains produced siderophores. Indole-3-acetic acid (IAA) production, the most physiologically active auxin, is a major property of rhizobacteria which promote plant growth (Hayat et al., 2010). Production of IAA was detected in all Azotobacter strains and quantified in these and Bacillus and Pseudomonas strains. Strains of B. megaterium showed the lowest IAA production, while strains of A. chroococcum reached 146 μg ml⁻¹. Rhizobial strains (218 and 459) did not produce siderophores, but they solubilized phosphates and produced significant amount of IAA.

Inoculation of both grasses with some rhizobacterial strains, increased height, shoot dry weight and total N, P, and K contents significantly (Tables 3 and 4). Inoculation of meadow fescue with single A. chroococcum strains A2 and A5, did not influence any significant changes in the parameters investigated, compared to uninoculated control Ø. However, co-inoculation with A. chroococcum A2 + Pseudomonas sp. L2Cr, A2 + S. meliloti 218 and A5 + S. meliloti 218 significantly increased SDW and total N, P, and K contents (mg pot⁻¹) compared to uninoculated control Ø. The SDW increase was 25%, 33% and 31%, respectively, compared to control Ø (100%). Considering A. chroococcum did not increase SDW individually, it is possible that the increased SDW in co-inoculated plants is the result of S. meliloti or Pseudomonas sp. strain. Content of N (%) in SDW was increased only in the A5 treatment, which realised the lowest SDW, while content of P and K (%) did not significantly differ among treatments. Mineral nutrient contents in plants depend on plant genotype (cultivars), and to a lesser extent, on mineral nutrition and ecological factors.

Inoculation of orchardgrass with single A. chroococcum A1 strain and co-inoculation with A1 + B. megaterium LRIK and A31 + B. megaterium LRIK increased SDW for about 60% compared to control Ø (100%). In these treatments total N, P and K contents were also increased, compared to control Ø.

Contents of N, P and K (%) did not significantly differ among treatments.

Nitrogen percentage in SDW of meadow fescue and orchardgrass ranged between 4.10-4.61% and 3.67-3.95% respectively, which is slightly higher than sufficient ranges, 2.5-3.5% for both species (Schwab et al., 2007). The P content was 0.17-0.19% for meadow fescue and 0.20-0.23% for orchardgrass which is lower than sufficient ranges of 0.26-0.4% (actively growing plants of tall fescue) and 0.250-0.35% (for 5 week old plants) respectively. Higher N% could be the consequence of plant development early phase and limited plant growth, while lower P content was possibly caused by low P soil content. The K content was optimal in both plants in all treatments.

Number of reports have proven that Azotobacter application, as a free-living nitrogen-fixer, improves the yield of different plants, including grass species, wheat, English ryegrass, Italian ryegrass, etc. (Delić et al., 2012; Stamnenov et al., 2012; Miri et al., 2013). In this research we have identified only one (A1) of 7 isolated A. chroococcum strains, which is able to promote grass (orchardgrass) growth individually (about 60% SDW increase compared to control Ø).

It is well-known that rhizobial strains, besides N fixation with legumes, can promote growth of non-legume plants by mechanisms independent of biological N₂ fixation (mainly through phytohormones production). Previous studies showed that rhizobium can stimulate the growth of non-legumes, such as wheat (Hilali et al., 2001), rice (Vargas et al., 2009; Souza et al., 2013) and oats (Stajković-Srbinović et al., 2014). In this study, S. meliloti 218 strain showed significant growth promotion of meadow fescue in co-inoculation with A. chroococcum A2 and A5, but R. leguminosarum bv. trifoliis 459 did not have any positive effects on orchardgrass. Similarly, the inoculation of orchardgrass with Beijerinckia or Azospirillum did not significantly increase dry mass during a two-year period (Dragomir and Moisuc, 2007). However, in the same research,
when orchardgrass was grown in the mixture with alfalfa and co-inoculated with rhizobium *Beijerinckia* and *Azospirillum*, an increase in dry mass was noted in the second year, indicating a benefit inter-species relationship. The ability of *S. meliloti* strain 218 to promote grass growth could be of special importance, since grasses are often grown in mixture with alfalfa, a host plant of *S. meliloti*.

Strains of *Pseudomonas* and *Bacillus* are among the most efficient PGPR and promoted growth and yield of variety of plants (Hayat et al., 2010). Single inoculation with *P. fluorescens* or *B. subtilis* showed a statistically significant increase in the yield of fresh and dry mass of English ryegrass (Delić et al., 2012; Stamenov et al., 2012a). In our research *B. megaterium* LR1K showed some PGP potential, since co-inoculation with *A. chroococcum* A31 increased all parameters of orchardgrass in respect to control Ø, while single inoculation with *A. chroococcum* A31 strain did not. It is possible that the PGP effect of LR1K could not be visible in co-inoculation with A1, due to the good effect of A1 strain alone. Previously the strain *Pseudomonas* sp. L2Cr increased oats and barley growth in single inoculation (Stajković-Srbinović et al., 2014), while in this research co-inoculation of meadow fescue with *A. chroococcum* A2 and L2Cr also increased plant growth.

In the presented research it is difficult to connect positive influence of particular strains on growth promotion with their PGP mechanisms detected *in vitro*. All the strains used showed more mechanisms that might be involved in plant growth promotion, and within each genera (*Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*), there were almost no differences in PGP characteristics. Most of the strains belong to nitrogen-fixing bacteria, produced IAA, could solubilise phosphates and differed only in siderophore production ability. Therefore, the presence of specific growth promoting traits does not guarantee that an isolate will promote plant growth.

**Conclusions**

All rhizobacterial strains tested showed some PGP characteristics, with IAA production as a dominant characteristic. Different rhizobacterial strains improved the growth of both investigated forage grasses. Shoot dry weight of inoculated orchardgrass increased significantly up to 59%, compared to the uninoculated control plants, while the increase in SDW of inoculated meadow fescue went up to 33% over uninoculated control plants. Total content of N, P or K in shoot dry weight increased in some treatments, depending on the strains applied and plant species. The results demonstrate that grass seed inoculation could be of practical benefit in sustainable agricultural practices.

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**References**


