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BRIEF COMMUNICATION

Effect of diazotrophic bacteria isolated from a mycelium of arbuscular mycorrhizal fungi on colonization of maize roots by *Glomus fistulosum*

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Abstract

The inoculation of mycorrhizal maize plants with three isolates of microaerophilic diazotrophic bacteria obtained from the mycelium of arbuscular mycorrhizal fungi associated with three grasses (*Arrhenatherum elatius* - bacterial isolate ARR, *Agropyrum repens* - isolate AGR and *Poa annua* - isolate POA) caused no increase in nitrogen content in plant biomass. The inoculation with bacterial isolate ARR resulted in the decreased plant growth. Bacterial isolate AGR decreased the percentage of the root length colonized by arbuscular mycorrhizal fungus *Glomus fistulosum*. The inoculation with both mycorrhizal fungus and isolate POA increased significantly the concentration of phosphorus in plant shoots compared to uninoculated control.

Additional key words: bacterial isolates, grasses, mycorrhizal colonization, nitrogen, phosphorus.

Research on associations between the roots of gramineaceous plants and associative nitrogen-fixing bacteria (microaerophilic diazotrophs) suggested that this bacterial group may be important for plant ecology (*e.g.* Rai and Gaur 1982). Microaerophilic diazotrophs belong to the genera *Azospirillum*, *Herbaspirillum*, *Azoarcus* and *Acetobacter* and can be isolated in semi-solid, nitrogen free media, forming characteristic veil-like pellicles several millimetres below the medium surface, where their respiration rate is in equilibrium with the oxygen diffusion rate (Döbereiner 1995). The association of endophytic diazotrophs with cultivated maize has been

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reported several times (*e.g.* Albrecht *et al.* 1977) and there are the reports that the diazotrophic bacteria may interact with arbuscular mycorrhizal (AM) fungi in their effects on plant growth (Pacovsky *et al.* 1985, Pacovsky 1989, Paula *et al.* 1992, Subba Rao *et al.* 1985). Microaerophilic diazotrophs may be isolated mainly from plant tissues and are less abundant in the soil, but were always detected on the mycelium of AM fungi in the tropical soil (Gryndler, dos Reis, Döbereiner, unpublished observations).

The aim of our study was to find the effect of diazotrophic bacteria isolated from mycelium of AM fungi in the soil of temperate zone on the development of mycorrhizal symbiosis. We suspected a close physiological interaction of the mycorrhizal fungus and associative bacteria and positive synergistic effect on plant growth and nitrogen and phosphorus nutrition.

The cultures of diazotrophic bacteria were isolated and maintained as described by Döbereiner (1995). The soil samples were collected from three plots in Northern and Central Bohemia from *Agropyrum repens* (garden soil, locality Soběnice near Litoměřice), *Arrhenatherum elatius* (dystric cambisol, locality Prague 4) and *Poa annua* (garden soil, locality Soběnice near Litoměřice) dominated plant communities. The mycelium of arbuscular mycorrhizal fungi from these soil samples was extracted using wet sieving (250 and 90 μm sieve mesh), collected and cleaned using the finest forceps and washed several times in 10 cm^3 sterile 0.1 % $MgSO_4$ and examined microscopically. In all three samples, the large majority (> 80 %) of the hyphal length was represented by aseptate coarse hyphae, in morphology well corresponding with the typical hyphae of AM fungi. Washed mycelium (3 - 5 mm^3) was then homogenized using sterile mortar and pestle in 1 cm^3 0.1 % $MgSO_4$. Samples of homogenates (0.1 cm^3) were inserted into the bottom of 25 cm^3 test tubes with 10 cm^3 semi-solid JNFb isolation medium (Döbereiner 1995) containing (dm^{-3}): D,L-malic acid (5 g), K_2HPO_4 (1.5 g), $MgSO_4 . 7 H_2O$ (0.2 g), $CaCl_2 . 2 H_2O$ (20 mg), FeNaEDTA (66 mg), $CuSO_4 . H_2O$ (0.8 mg), $ZnSO_4 . 7 H_2O$ (0.24 mg), H_3BO_3 (2.8 mg), $Na_2MoO_4 . 2 H_2O$ (2 mg), $MnSO_4 . 4 H_2O$ (3 mg), *Biotin* (0.1 mg), *Pyridoxin*-HCl (0.2 mg), *Bromothymol blue* (2 cm^3 0.5 % solution in 0.2 M KOH) and agar (2 g). The inoculated test tubes were aseptically closed by a cotton wool plugs and incubated at 28 °C in the dark. Five tubes were incubated per source (collected sample of mycelium). The diazotrophic bacteria forming the clean, sharp pellicle in the column of nitrogen free JNFb medium were collected by Pasteur pipette and cleaned on the same medium. The purity of cultures was checked under the microscope (magnification 1000 ×).

Inoculum of an AM fungus *Glomus fistulosum* BEG23 was produced in soil based pot cultures of *Plantago lanceolata* for 3 months. Inocula of bacteria were produced in a liquid non-shaken cultivation medium containing the same ingredients as JNFb medium except agar and malic acid being replaced by the same amount of glucose. Bacterial inocula were prepared by washing and diluting the crude cultures by 0.1 % $MgSO_4$.

The experiment was designed as unifactorial, with an uninoculated control, another control inoculated with the mycorrhizal fungus only and with three treatments inoculated with the mycorrhizal fungus plus an isolate of diazotrophic

bacterium obtained from mycelium of the mycorrhizal symbionts of *Agropyrum* (AGR), *Arrhenatherum* (ARR) or *Poa* (POA). Maize plants were grown in *Perlite*:steamed soil (5:1) substrate with nutrient solution. Pre-germinated seeds were surface disinfected with 1 % hydrogen peroxide for 2 min before planting. Plants were inoculated with 10 g of soil inoculum (2 cm below the seed) containing 400 spores of *Glomus fistulosum* Skou & Jakobsen (isolate BEG 23) and 1 cm^3 of bacterial inoculum containing approx. 10^{10} bacterial cells. The plants were cultivated in plastic tubs 18 cm in height and 5 cm in diameter, covered at the bottom with canvas. The tubes of the same treatment were then put in a plastic tub and supplied with distilled water for the first two weeks of growth. After this two-week period, 2 dm^3 of a nitrogen free nutrient solution containing [dm^{-3}]: 541 mg $MgSO_4 . 7 H_2O$, 12.2 mg KH_2PO_4, 191 mg $CaCl_2 . 2 H_2O$, 162 mg $MgCl_2 . 6 H_2O$, 58 mg KCl, 157 mg K_2SO_4, 0.75 mg $MnCl_2 . 4 H_2O$, 0.75 mg KI, 0.75 mg $ZnSO_4 . H_2O$, 1.5 mg H_3BO_3, 0.001 mg $CuSO_4 . 5 H_2O$, 4.3 mg FeNaEDTA and 0.00017 mg $Na_2MoO_4 . 2 H_2O$ were added to each tub and renewed twice a week. Fourteen plants were cultivated in the tub per treatment.

Plants were harvested after 8 weeks. At harvest time, the plants were dried at 105 °C and the dry mass of shoot was assessed. The shoot dry matter was digested in concentrated sulfuric acid - hydrogen peroxide (30 %) mixture (1:2) at 360 °C. The samples were analyzed for phosphate content by phosphomolybdate complex spectrophotometry (Watanabe and Olsen 1965), and for total nitrogen by a distillation method (*Kjeltec Auto 1030 Analyzer*, *Tecator AB*, Höganäs, Sweden). The roots were stained with *Trypan blue* (Phillips and Hayman 1970) and mycorrhizal colonization was determined by a grid-line intersect method (Giovanetti and Mosse 1980). Results were statistically treated by analysis of variance and Duncan's multiple range test at $P < 0.05$.

All initial bacterial cultures in JNFb medium showed the growth of pellicule forming bacteria, indicating the occurrence of diazotrophs in the three field collected samples of mycelium. Observed under the microscope, the bacterial cells of all used isolates were motile, 0.6 - 1 by 0.8 - 4 μm, curved rods, forming the yellow colonies and utilizing malic acid and glucose as a sole source of energy. The bacteria were able of extensive growth on non-shaken nitrogen-free liquid medium, forming clearly yellow suspension culture.

Table 1. Effect of inoculation with three isolates of diazotrophic bacteria associated with mycelium of AM fungi on growth of mycorrhizal plants. Significant differences are marked by different letters.

Mycorrhizal inoculation	Bacterial inoculation	Shoot dry mass [g]	Shoot/root ratio
None	none	0.88 a	1.44 a
G. fistulosum	none	0.88 a	1.42 a
G. fistulosum	isolate ARR	0.68 b	1.16 b
G. fistulosum	isolate AGR	0.86 a	1.30 ab
G. fistulosum	isolate POA	0.87 a	1.32 ab

In the experiment with plants, the only significant response of plant growth to inoculation was observed (inhibition) in the treatment inoculated with *G. fistulosum* and isolate ARR (Table 1). The lowest shoot/root ratio was obtained in the same treatment and the effect was statistically significant, compared to both controls.

Percentage of the root length colonized by *G. fistulosum* was significantly decreased by inoculation with bacterial isolate AGR, compared to the treatment inoculated with the mycorrhizal fungus only (Table 2). There was no remarkable stimulation of intraradical phase of the AM fungus by other two bacteria either what confirmed the observation of Subba Rao *et al.* (1985) on *Azospirillum brasilense* associated with barley.

Table 2. Effect of inoculation with three isolates of diazotrophic bacteria associated with mycelium of AM fungi on mycorrhizal colonization (percentage of root lenth colonized, RLI) and on P and N concentration in dry plant biomass.

Mycorrhizal inoculation	Bacterial inoculation	RLI [%]	P [mg g^{-1}]	N [%]
None	none	0	2.22 b	1.44 a
G. fistulosum	none	11.52 ab	2.33 ab	1.42 a
G. fistulosum	isolate ARR	11.95 a	2.39 ab	1.16 b
G. fistulosum	isolate AGR	7.28 c	2.26 ab	1.30 ab
G. fistulosum	isolate POA	8.61 bc	2.59 a	1.32 ab

Inoculation with the isolate POA stimulated the accumulation of phosphorus by host plant, compared to the control treatment without inoculation, however no increase in the concentration of nitrogen in plant biomass was observed. The nitrogen content in shoots was significantly decreased by inoculation with bacterial isolate ARR.

Such results are in contradiction with those of Pacovsky *et al.* (1985), who observed promoted growth of *Sorghum* and mycorrhiza development by inoculation with diazotrophs in soil free substrates, comparable with our cultivation system. In our experiment we used the nitrogen-free cultivation system, the seed and the soil portion in the substratum being the only initial nitrogen sources (*Perlite* is essentially nitrogen free, the soil used for preparation of the substratum contained 0.08 % of total nitrogen). Such a system should support the nitrogen fixation because of very low nitrogen availability and existence of microaerobic spaces in the wet hydroponic substratum. The decrease in the N concentration in plants inoculated with diazotrophic bacterium is unexplained but it might be somehow linked to higher development of mycorrhizal colonization. The observation might be thus interpreted as competition of mycorrhizal fungus (stimulated by bacterial inoculation) with the host plant for nitrogen.

Our observation support the idea that isolates of diazotrophic bacteria associated with the mycelium of AM fungi in temperate ecosystem differ in their effects on the mycorrhizal symbiosis and plant nutritional status and that bacterial inoculation need

not necessarily cause the improvement of plant nitrogen supply. Further research of the effect of bacterial isolates particularly on extraradical mycelium is desirable.

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