

# Single and combined applications of arbuscular mycorrhizal fungi and *Enterobacter radicincitans* affect nutrient uptake of faba bean and soil biological characteristics

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## Abstract

Microorganisms like arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) can contribute significantly to plant nutrition and thus may help to reduce chemical inputs in agricultural systems.

In two pot experiments under semi-controlled conditions the effects of applications with AMF and an *Enterobacter radicincitans* strain were evaluated regarding shoot yield and nutrient uptake (P and N) of faba bean (*Vicia faba*) as well as on soil characteristics (basal respiration, microbial biomass, and the number of P solubilizing bacteria). The first experiment (2007) was established to investigate the single and combined effects of AMF and *E. radicincitans*. The second experiment (2008) was established on a nutrient poor soil to evaluate the efficacy of PGPR and AMF with or without mineral fertilizer (P, K and Mg) application.

For the experiment with suboptimal soil nutrient contents (2008) higher nutrient uptakes of bean was found after application of AMF in comparison to the control. The effect of AMF was comparable to the positive effect of mineral nutrient application. Under better nutrient status of soil (experiment 2007) none of the applied microorganisms affected the growth and nutrient uptake of bean. However, AMF application alone or with mineral nutrient supply increased the soil respiration and soil microbial biomass. The studies showed the potential of AMF applications for plant growth and nutrition mainly under nutrient deficient conditions.

**Keywords:** *Vicia faba*, arbuscular mycorrhizal fungi, *Enterobacter radicincitans*, phosphorus, nitrogen, soil microbial parameters

## Zusammenfassung

### Einzel- und Kombinationseffekte von Mykorrhizapilzen und *Enterobacter radicincitans* auf die Nährstoffaufnahme von Bohnen und bodenbiologische Parameter

Mikroorganismen wie arbuskuläre Mykorrhizapilze (AMF) und Bakterien können zur Stärkung des Pflanzenwachstums und zur Nährstoffaufnahme von Pflanzen beitragen. In zwei Gefäßversuchen wurden AMF sowie ein Bakterienstamm der Art *Enterobacter radicincitans* appliziert und bezüglich ihrer Effekte auf den Ertrag und die Nährstoffaufnahme (N und P) von Bohnen (*Vicia faba*) sowie auf bodenbiologische Charakteristika untersucht. Im ersten Experiment wurden Einzel- und Kombinationseffekte von AMF und *E. radicincitans* geprüft. Das zweite Experiment wurde durchgeführt, um auf einem nährstoffarmen Boden die Wirksamkeit von AMF und des Bakterienstammes mit und ohne mineralische Düngung (P, K und Mg) zu prüfen. Bei suboptimaler Nährstoffversorgung des Bodens wurden nach Ausbringung der AMF höhere Nährstoffaufnahmen der Bohne ermittelt als in der Kontrolle. Der Effekt der Pilze war hierbei vergleichbar mit dem der mineralischen Düngung. Bei guter Nährstoffversorgung des Bodens konnte keine Auswirkung der Mikroorganismen auf Ertrag und Nährstoffaufnahme nachgewiesen werden. Jedoch führte die Ausbringung von AMF zur Erhöhung der Bodenatmung und der mikrobiellen Biomasse im Boden. Die Ergebnisse deuten auf eine bessere Wirkung der AMF auf den Ertrag und die Nährstoffaufnahme von Bohnen bei schlechterer Nährstoffversorgung des Bodens hin.

**Schlüsselworte:** *Vicia faba*, Mykorrhizapilze, *Enterobacter radicincitans*, Phosphor, Stickstoff, bodenbiologische Parameter

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## Introduction

An efficient use of phosphorus (P) and mobilization of P by crops and microorganisms has special importance in cropping systems, since P often is the limiting nutrient in soils. The integration of P mobilizing crops in crop rotation is one of the most effective methods to improve the P supply in cropping systems (Eichler et al., 2004; Eichler-Löbermann et al., 2008). Along with peas, faba bean is the most important grain legume in Europe followed by lupine, soya, chickpea and lentil (Metayer, 2004). Besides the positive effect of nitrogen (N<sub>2</sub>) fixation and following N input into the cropping system legumes were also often described to be efficient in utilization of less available soil P. The mobilization of P is mainly due to excretion of organic acids and subsequent acidification, chelation, and exchange reactions (Vassilev et al., 1995; Dakora and Phillips, 2002).

The application of microbial inoculants as so called biofertilizers is a promising method to promote plant growth by increasing the availability of nutrients to plants, mainly N and P (Eichler et al., 2004; Yasmin et al., 2007; Shaharoon et al., 2008; Richardson et al., 2011). Main sources of biofertilizers are plant growth-promoting rhizobacteria (PGPR), beneficial fungi and cyanobacteria (blue-green algae). In addition to the improvement of nutrient uptake microbial inoculants can promote the growth of crops by protecting plants against soil-borne pathogens (Thrane et al., 2000) and the production of phytohormones (Kannan and Sureendar, 2009).

*Enterobacter radicincitans* is a PGPR which was shown to increase growth of winter wheat, corn and beans (Ruppel, 2008). These bacteria are able to colonize different parts of plants and to survive both on the surface and within internal tissue of the plant (Ruppel et al., 2006). In pure culture, the bacterial cells can fix atmospheric N, produce phytohormones (auxin-like and cytokinin-like compounds) and solubilize calcium phosphate (Schilling et al., 1998).

Arbuscular mycorrhizal fungi (AMF) are known to improve the bio-availability of nutrients, in particular P (Jakobsen, 1999; Vassilev et al., 2001). Additionally, AM fungi may increase plant N uptake as a result of mineralizing organic soil N and/or producing phytohormones (Mohammadi et al., 2008). Despite the high importance of native AM fungi for plant nutrition, the application of AM fungi as inoculant is often limited due to the disability to grow in artificial media and the inconsistent effect of those inocula in field (Richardson et al., 2011). A joint inoculation of AMF with other microorganisms however, might be more promising due to additive effects on plant growth and plant nutrition. For example, positive effects of dual inoculation of a P-solubilizing fungus (*Mortierella* sp.) together with AMF were described by Zhang et al. (2011). Optimistic results of the interactions of bacteria and AMF were found by Gamalero et al. (2004) with *Pseudomonas* sp., by Singh and Kapoor (1999) with *Bacillus circulans*, and by Ruiz-Lozano and Bonfante (1999) with *Burholderia* sp..

It was shown, that AM fungi can increase the spread of PGPR throughout the rhizosphere (Morrissey et al., 2004; Toljander et al., 2007) as well as so called mycorrhiza helper

bacteria may promote mycorrhizal development or interact positively with the functioning of the symbiosis (Frey-Klett et al., 2007; Dames and Ridsdale, 2012).

However, antagonism between AM fungi and bacteria might be also possible (Adesemoye et al., 2009) and the combined application of different microorganisms did not always show advantages. All microbial inoculants alone and in association with the plant affect the native soil microbial biomass and their activities. These complex microbial-plant interactions need additional research especially in native soils. Therefore, the objective of the present study was to investigate the effect of single and combined inoculations of AM fungi (*Glomus etunicatum*, *Glomus intraradices* and *Glomus claroideum*) and the bacterial strain *E. radicincitans* DSM 16656 under different soil nutrient conditions on shoot dry weight, P and N uptake of faba bean. To evaluate the impacts of microbial inoculants on the native soil microflora we monitored changes in the soil microbial biomass and microbial activity. We assumed that the effect of single and combined application of AM fungi and *E. radicincitans* will depend on chemical soil characteristics (nutrient supply and pH value).

## Material and Methods

### Experimental setup

The experiments were established under semi controlled conditions in a greenhouse at the University of Rostock, Northern Germany. The soil utilized was loamy sand originating from the upper soil layer (0 to 30 cm) of two field experiments established in the experimental station of the Rostock University (soil characteristics are given in Table 1). The dominant soil type on the field site was a Stagnic Cambisol. The trials were carried out in a complete randomized design with four replications using Mitscherlich pots containing 6 kg air-dried soil. Soil was sieved (10 mm) but not sterilized to allow competition by indigenous microorganisms and colonization by the soil indigenous mycorrhizal spores and soil bacteria. Ten *Vicia faba* seeds (Scirocco cultivar) were cultivated per pot. The seeds were obtained from SAATEN-UNION GmbH in Germany.

Table 1

Chemical properties of soils used in the experiments in 2007 and 2008

	pH	C org (%)	P* mg kg <sup>-1</sup> soil	K* mg kg <sup>-1</sup> soil	Mg* mg kg <sup>-1</sup> soil
2007	5.8	1.41	53	82	281
2008	6.6	1.76	40	57	305

\* Nutrients extracted with double lactate solution represent the plant available nutrients

The first experiment was established in 2007 to study the effect of inoculation with *E. radicincitans*, AM fungi and their co-inoculation. It comprised the following four treatments:

I Control (without inoculation), II Inoculation with *E. radicitans*, III Inoculation with AMF, IV Combined inoculation *E. radicitans* + AMF.

The second experiment was performed in 2008 to investigate the effect of the microbial inoculants in dependence of nutrient status of soil, which usually has an impact on the efficacy of microbial inoculants. The soil had suboptimal P and K contents (according to German soil test classification) and the impact of AMF and *E. radicitans* was tested with and without nutrient addition. Six treatments were established: I Control without inoculation and without nutrient addition, II Inoculation with *E. radicitans*, III Inoculation with AMF, IV Nutrient addition, V Nutrients + AMF, VI Nutrients + *E. radicitans*. The amounts of nutrients in the respective treatments comprised 1 g  $\text{KH}_2\text{PO}_4$  (= 0.23 g P and 0.28 g K) 1.46 g  $\text{MgSO}_4$  (= 0.14 g Mg and 0.19 g S) and 3 g  $\text{KH}_2\text{SO}_4$  (= 0.70 g S and 0.85 g K) per pot. In both experiments plants were harvested after 51 days of growth.

### Microbial inoculants

The mycorrhizal commercial product (INoQ Top) consisted of *Glomus etunicatum*, *Glomus intraradices* and *Glomus claroideum* with a spore number of  $10^5$  per l. The carrier material was expanded clay with a grain size of 2 to 4 mm and a pH of 7.5. Twenty five ml product was incorporated into the 6 kg soil per pot.

The bacterial strain *Enterobacter radicitans* DSM 16656 was isolated from winter wheat phyllosphere and selected for its plant growth-promoting abilities (Ruppel, 2008). *E. radicitans* cells were grown in standard nutrient solution (MERCK 1) at 29 °C in a rotary incubator at 100 rpm for 48 h (Ruppel et al., 2006). Faba bean seeds were treated with *E. radicitans* by coating the seeds with the bacterial suspension of  $10^8$  cells  $\text{ml}^{-1}$  for 5 to 10 minutes, and then dried in the dark at room temperature. Additionally, after seeding in the stage of two leaves a bacterial suspension was sprayed onto young plants ( $10^8$  cell per plant) using a hand pump to increase the chances of colonization and establishment of the bacterial cells.

### Plant and soil analyses

After plant harvest the shoot dry yield was measured. Shoot-biomass P concentrations were measured after dry-ashing using the vanadad-molybdate method (Page et al., 1982). Shoot biomass N concentration was measured by using a modified Kjeldahl digestion method (Jones et al., 1991). Nutrient uptake was calculated by multiplying shoot weight with nutrient concentration.

The soil samples were taken after plant harvest and the soil was sieved to 2 mm and stored in aliquots at -20 °C until microbial analysis. Microbial biomass carbon (Cmic) content of the soil samples (100 g wet soil, 50 % water holding capacity) was determined using substrate-induced respiration activity measurements (SIR) with an infrared gas analyser (Heinemeyer et al., 1989). Microbial biomass C, which

encompasses all respiratory active soil organisms, are able to metabolise glucose, is expressed as  $\mu\text{g Cmic g}^{-1}$  dry soil. Soil basal respiration activity (R) was measured by an infrared gas analyser without addition of substrates (4 - 16 h, 20°C ± 1 K) and expressed as  $\mu\text{g CO}_2\text{-C g}^{-1}$  dry soil  $\text{h}^{-1}$ .

To enumerate P solubilizing bacteria, bacterial cells were separated from soil samples by shaking (290 rpm) 10 g soil with 90 ml 0.05 M NaCl + 10 glass beads at 4 °C for 1 h. Bacterial numbers were determined applying the MPN (most probable number) dilution and plating technique (Bast, 1999). Therefore, separated microorganisms were 5 times tenfold diluted in sterile 0.05 M NaCl. Hundred  $\mu\text{l}$  of succeeding dilutions were streaked onto solid Murovec nutrient medium in three replicates and incubated at 29 °C for two weeks delete (Deubel, 1996). The medium consisted of ( $\text{g l}^{-1}$ )  $\text{K}_2\text{SO}_4$  0.2,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.4, agar agar 20, glucose 10, L-asparagine 1 (both separately filter sterilized and added after autoclaving and cooling down the medium to 60 °C), simultaneously  $\text{CaCl}_2$  2.2,  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$  3.8 were mixed by consistent shaking the medium to precipitate Calciumphosphate. After one and two weeks colonies inducing transparent zones in the medium (indicating P-solubilizing activity) were counted. Numbers per g dry soil were calculated according the MPN method. P solubilizing bacteria were estimated for all treatments in 2007 and for 4 treatments in 2008 (control, mineral fertilization, *E. radicitans*, AM fungi).

### Statistical Analyses

The results of the experiments were analysed using Statistica 6.0 software (StatSoft, 2001). The effects of experimental factors were evaluated by the analysis of variance (ANOVA), and comparisons between means were carried out using Tukey HSD test at the significance level of  $P \leq 0.05$ .

### Results and discussion

#### Shoot weight and nutrient uptake

The effects of the applied microorganisms on shoot weight and plant nutrient uptake differed between the both experiments.

In 2007 when soil with higher P and K contents but lower pH values was used, the yield and nutrient uptake into the shoots of bean did not differ significantly between the treatments (Table 2). However, a tendency for higher yields and N uptakes became observable when microorganisms were applied. For the experiment in 2008 with suboptimal soil P and K contents but relatively high pH values the application of AMF raised the plant P and N uptake significantly in comparison to the non-inoculated control. Here, the AMF had a similar positive effect like the mineral nutrients (Min treatment). No treatment affected the biomass yield significantly. We did not measure the root weight and length. However, nutrient supply may affect the root:shoot ratio within a short time span (Peek et al., 2003).

Often, the effect of MO was found to be higher under sub-optimal nutrient supply. This can be confirmed by results of Krey et al. (2011), where a strain of *P. fluorescens* increased plant P uptake and P contents in soil only when it was not combined with organic fertilizers. Similarly, for AMF higher efficacy was usually detectable when applied on P poor soils (Schweiger et al., 1995; Richardson et al., 2011). Here, the AMF with their extensive hyphal length and density have a spatial advantage to explore P-sources.

However, sometimes positive impacts on yield after AMF application were also found when it was combined with mineral fertilizers. For example, for a sandy soil deficient in P small doses of nutrients showed good results regarding the efficacy of AMF (Bagayoko et al., 2000). This was related to enhanced root growth which appears to be a precondition for mycorrhizal infections and a subsequent significant contribution of AMF to plant growth. This improved root growth and mycorrhization rate can have especial importance in short term pot experiments, and might be the explanation for the relatively high yields and nutrient uptakes in the AMF + Min treatment in our experiment.

The added mineral fertilizers could also have served as an energy source to the applied microorganisms. This was reported by El-Ghandour et al. (1996) when applied AMF increased dry weight of faba bean plants in presence of an additional P source in pot experiments.

**Table 2**

Shoot yield (dry matter), P and N uptake of faba bean in dependence of the treatments applied in the pot experiment 2007 (n = 4, SD values in brackets)

Treatment	shoot yield g pot <sup>-1</sup>	P uptake mg pot <sup>-1</sup>	N uptake mg pot <sup>-1</sup>
Control	19.8 a (1.29)	55.6 a (6.57)	468 a (44.9)
ER	20.8 a (1.94)	55.8 a (3.65)	488 a (43.4)
AMF	21.7 a (1.07)	57.4 a (5.02)	508 a (59.4)
ER + AMF	21.4 a (2.26)	54.7 a (3.15)	492 a (38.9)

ER = *E. radicitans*,

AMF = arbuscular mycorrhizal fungi.

Different letters show significant statistical differences between the treatments according to Tuckey's Test ( $p \leq 0.05$ ), values within columns with the same letter are not significantly different.

Due to the immobility of P in soil the AMF inoculation has special importance for plant P nutrition. In comparison to the control, P uptake in 2008 increased about 57 % when AMF was applied. The N uptake increased about 37 % and the yield only about 15 % (not significant different from the control). Due to the higher P concentration in bean seeds compared to the whole bean plant the P effect would have been even higher with running experimental time and the formation of the bean seeds.

The inoculation of beans with the PGPR strain *E. radicitans* did not result in higher yields and nutrient uptakes, neither in 2007 nor in 2008.

Since, the activity of microbes also depends on the soil reaction (Vassilev et al., 2012) the pH value may also have been contributed to the differences in the efficacy of microorganisms. Generally the soil respiration and soil microbial biomass was higher in the second experiment in 2008, independently of whether nutrients were applied or not. Therefore we assume that the pH values had a considerable effect and that the pH of 6.8 was better for the development of the soil micro biota in soil and probably also for the applied microorganisms. However, also other soil features, like quality and quantity of organic matter influence the microflora in soil and further experiments would be necessary to evaluate the impact of pH on the efficacy of the tested microorganisms.

**Table 3**

Shoot yield (dry matter), P and N uptake of faba bean in dependence of the treatments applied in the pot experiment 2008 (n = 4, SD values in brackets)

Treatment	shoot yield g pot <sup>-1</sup>	P uptake mg pot <sup>-1</sup>	N uptake mg pot <sup>-1</sup>
Control	15.0 a (0.74)	30.8 a (2.51)	399 a (77.9)
Min	17.3 ab (2.24)	49.4 bc (4.77)	516 abc (47.9)
ER	15.9 a (1.45)	33.6 a (5.40)	430 ab (38.8)
AMF	17.4 ab (0.35)	48.4 b (3.95)	549 bc (46.6)
Min + ER	19.4 bc (2.10)	50.0 bc (6.51)	590 c (65.0)
Min + AMF	21.4 c (0.55)	57.3 c (2.49)	634 c (69.9)

ER = *E. radicitans*,

AMF = arbuscular mycorrhiza fungi,

Min = mineral nutrients applied.

Different letters show significant statistical differences between the treatments according to Tuckey's Test ( $p \leq 0.05$ ), values within columns with the same letter are not significantly different.

## Soil microbial biomass, microbial respiration activity, microbial metabolic quotient and P solubilizing bacteria

The microbial inoculants affected the soil microbial parameters. Highest values of microbial biomass and microbial respiration were found after application of AMF alone or AMF in combination with mineral fertilizers (Table 4 and 5). The inoculation with *E. radicitans* alone did not increase these parameters, whereas the combined application with AMF in 2007 resulted in a higher soil microbial biomass than the control. Considering the values of the single application it seems however, that this increase was mainly due to the appli-

cation with AMF. Promoting effects on the soil microbial community and their activity after mycorrhizal application was observed by Cavagnaro et al. (2006). Similar effects were recently reported by Vassileva et al. (2010) analysing the multifunctional properties of plant growth-promoting, particularly P solubilizing microorganisms after inoculation with AMF.

**Table 4**

Soil basal respiration, soil microbial biomass, number of P solubilizing bacteria in soil in dependence of the treatments applied in the pot experiment 2007 (n = 4, SD values in brackets)

Treatment	Basal Respiration CO <sub>2</sub> -C, µg g <sup>-1</sup> soil h <sup>-1</sup>	Soil Microbial Biomass C, µg g <sup>-1</sup> soil	Metabolic quotient µg CO <sub>2</sub> -C/ mg C <sub>mic</sub> h <sup>-1</sup>	P Solubilizing Bacteria cells g <sup>-1</sup> soil
Control	15.6 ab (3.24)	148 a (7.00)	105	1.441E+06 a (2.40947E+05)
ER	17.1 ab (1.07)	186 ab (26.3)	92	3.608E+06 b (1.08572E+06)
AMF	19.1 b (1.42)	193 b (28.5)	99	2.691E+06 ab (1.20412E+06)
ER + AMF	14.4 a (1.44)	192 b (13.6)	75	1.744E+06 ab (4.52565E+05)

ER = *E. radicitans*,

AMF = arbuscular mycorrhiza fungi.

Different letters show significant statistical differences between the treatments according to Tuckey's Test (p ≤ 0.05), values within columns with the same letter are not significantly different.

The metabolic quotient describing the value of CO<sub>2</sub> emission per unit microbial biomass is often discussed as an efficiency parameter of microbial metabolic processes (Anderson, 1994). This quotient was lower after microbial inoculation in comparison to the control since the microbial biomass was stronger increased than the basal respiration (Table 4 and 5). The organic matter application in the AMF treatment as an additional carbon (C) source might have been contributed to the increased values of the soil microbial biomass C. The plant nutrition effects obtained in 2008 after AMF inoculation could be induced by the inoculated organisms themselves, their P-solubilizing or atmospheric N fixing activities, or they could also be induced by affecting the native soil microbial population. Such changes could be very versatile and they are hard to prove.

The number of P solubilizing bacteria was significantly increased after *E. radicitans* application in 2007 in comparison to the non-inoculated control experiment. In 2008 the application of *E. radicitans* also increased the number of P solubilizing bacteria in the soil, however, due to high standard deviation the differences were not significant. Surprisingly, the combination of *E. radicitans* with AMF in 2007 resulted in lower numbers of P solubilizing bacteria than the single treatments. One has to consider that the effects of inoculants are highly variable (Avis et al., 2008; Adesemoye and Kloepper, 2009; Fini et al., 2011). The complex interaction

between microbial inoculants, plants and environmental factors can turn out in synergistic, mutualistic or even antagonistic effects.

**Table 5**

Soil basal respiration, soil biomass, number of P solubilizing bacteria in soil in dependence of the treatments applied in the pot experiment 2008 (n = 4, SD values in brackets)

Treatment	Basal Respiration CO <sub>2</sub> -C, µg g <sup>-1</sup> soil h <sup>-1</sup>	Soil Microbial Biomass C, µg g <sup>-1</sup> soil	Metabolic quotient µg CO <sub>2</sub> -C/ mg C <sub>mic</sub> h <sup>-1</sup>	P Solubilizing Bacteria cells g <sup>-1</sup> soil
Control	20.6 a (3.79)	299 a (49.5)	69	8.833E + 06 a (2.44192E + 06)
Min	20.7 a (1.47)	338 a (94.2)	61	1.025E + 07 a (4.17890E + 06)
ER	20.9 a (1.93)	338 a (19.3)	61	1.817E + 07 a (3.35499E + 06)
AMF	26.1 ab (2.46)	433 ab (52.2)	60	1.842E + 07 a (3.29421E + 06)
Min + ER	20.6 a (1.18)	304 a (46.2)	67	n.d.
Min + AMF	29.5 b (3.81)	495 b (101.7)	59	n.d.

ER = *E. radicitans*,

AMF = arbuscular mycorrhiza fungi,

Min = mineral nutrients applied,

n.d. = not determined.

Different letters show significant statistical differences between the treatments according to Tuckey's Test (p ≤ 0.05), values within columns with the same letter are not significantly different.

The results showed the potential of microbial inoculants but also made clear that further investigation of these processes would be necessary for obtaining optimal benefits to plant growth and plant nutrition.

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