Interrelations between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa in sterile, AMF-free or normal soil conditions

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Abstract

Co-inoculations of the alfalfa (*Medicago sativa* L.) plants with the associative- and/or the obligate nitrogen-fixing bacteria (*Azospirillum brasilense*, *S*; *Rhizobium meliloti*, *R*) and/or the vesicular arbuscular mycorrhiza fungus (*Glomus fasciculatum*, *M*) were evaluated in a pot experiment under controlled conditions. The effect of these beneficial microbes, as single- (*M, R, S*), dual- (*MR, MS*) or multilevel (*MRS*) inoculation-treatments were assessed in a calcareous loamy chernozem soil, originating from a grass-type natural ecosystem. A range of substrates were used to separate the influence of the indigenous microbes: C, untreated original soil (i.e. including all of the usual microflora); G, gamma-sterilised soil (no competitive microbes); GB, sterile soil (re-suspension of a mycorrhiza-free soil extract). The weight of the host, nodule-number, macro- and microelement contents and the colonisation by the inoculated bacterial and fungal microsymbionts were recorded.

In the gamma-sterilised substrate all of the mono- (*M*), dual- (*MR, MS*) or multilevel (*MRS*) co-inoculations with the selected, *Glomus fasciculatum* M 107 strain were effective in improving plant growth, nutrient-uptake and abundance of the microsymbionts in the rhizosphere of alfalfa. In contrast a competition from the indigenous microflora in the non-sterilised soil, greatly reduced the functioning of the applied mycorrhizal inoculum. Although the associative *Azospirillum* bacteria (*S*) slightly reduced effects relative to single mycorrhizal inoculation (*M*), the multilevel treatments, with both of the diazotrophs (*MRS*), showed a further enhancement (a synergistic effect) for almost all of the tested parameters and substrates. The functional compatibility of the obligate- and associative diazotrophs in the mycorhizosphere are discussed.

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1. Introduction

The contribution of arbuscular mycorrhizal fungi (AMF) and of associative or obligate nitrogen-fixing microsymbionts (*Azospirillum* and *Rhizobium* bacteria) to soil fertility, productivity and crop yield has been well-documented (Boddey et al., 1991; Jeffries and Dodd, 1991; Biró et al., 1993a; Bethlenfalvay and Schüepp, 1994). The organisms are therefore used to evaluate the functioning of ecosystems (Giller and Cadish, 1995), especially under nutrient unbalanced conditions.
conditions (Barea and Azcon-Aguilar, 1983; Barea et al., 1983). Well-known effects of AMF include improved uptake of water (Bethlenfalvay et al., 1987; Sanchez-Diaz et al., 1990; Tobar et al., 1994), and of phosphorus or other macro- and microelements in non-optimal situations (Pacovsky et al., 1985). Rhizosphere–mycorrhizosphere systems, can therefore be tailored to help plants to establish and survive in nutrient-deficient, or degraded habitats or during periods of stress (Smith and Bowen, 1979; Sanchez-Diaz et al., 1990).

As a result of the above, seed and soil inoculations are common agricultural practices (Subba Rao, 1985; Champawat, 1990). The success of these technologies, however, depends on the effectiveness and infectivity of indigenous microbes and on the interactions between the main participants, such as mycorrhizal fungi, in the rhizosphere (Lindermann, 1983; Puppi et al., 1994). Compatible combinations of the inoculated microbes, such as nitrogen-fixing rhizobium bacteria and AMF, may result in an enhanced effect on plant development (Ames and Bethlenfalvay, 1987; Barea et al., 1988a; Paula et al., 1992; Biró et al., 1993b) in the various microsymbiont–legume systems. Positive influence of the associative Azospirillum diazotrophs on AM fungal activity has been reported (Subba Rao, 1985; Garbaye, 1994) especially for the monocotyledonous host plants. In contrast differences in total dry weight were not found in the Glomus+Azospirillum tripartite system studies by Pacovsky (1988).

Reports on the dual co-inoculations of the AMF with both of the nitrogen-fixing bacteria Rhizobium- and Azospirillum are uncommon. Spores and the hyphosphere of the AMF may, however, harbour so-called “helper bacteria”, such as Burkholderia or associative diazotrophs (Minerdi et al., 1999; J. Döbereiner, pers. comm.), which can result in beneficial effect in a range of soil–plant systems.

The aim of this study was to screen microbial interactions, which would take place in the rhizosphere of a target plant, by assessing the functional compatibility of three groups of microorganisms, Rhizobium and/or Azospirillum nitrogen-fixers and/or AMF. The concept of single- dual- and/or the multilevel microsymbiont co-inoculations was studied in relation to mycorrhizosphere effect, and the impact of soil conditions (e.g. including or excluding the indigenous endomycorrhizal fungal populations).

2. Materials and methods

2.1. Soil treatments and characteristics

In vitro pot experiment was carried out to study the effects of various co-inoculations using alfalfa (Medicago sativa L.) and the following soil treatments: C, untreated loamy chernozem soil; this served as an original control soil, where all of the usual rhizosphere microflora (all microbes) were present. G, gamma-irradiated sterile soil; the normal rhizosphere microflora was excluded (no microbes), through gamma-sterilisation (15 000 J Co kg$^{-1}$ soil). GB, gamma-sterilised soil, with the re-addition of a soil extract (80 ml per pot of a soil/water mixture, 2:1 (v/v), filtered through a Jena G 5 (MN, 5 μm pore-sized paper) to reintroduce the native microbial community, with the exception of propagules of AMF (no indigenous AMF). Some of the main physical and chemical characteristics of the soil before and after the gamma-sterilisation are shown in Table 1.

2.2. Microbial inoculations

Using the above substrates (C, G, GB) plants were grown with or without associative and symbiotic

### Table 1

The physical and chemical characteristics of a calcareous chernozem soil originating from a natural, grass-type ecosystem (Erd, Hungary) before (C) and after (G) the gamma irradiation (15 000 J Co kg$^{-1}$ soil)

<table>
<thead>
<tr>
<th>Soil characteristics</th>
<th>Original (C)</th>
<th>Gamma-sterilised (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil texture (plasticity)$^a$</td>
<td>39.0</td>
<td>39.0</td>
</tr>
<tr>
<td>Organic matter (g kg$^{-1}$)</td>
<td>28.7</td>
<td>29.0</td>
</tr>
<tr>
<td>CEC (cmol kg$^{-1}$)</td>
<td>16.0</td>
<td>16.4</td>
</tr>
<tr>
<td>PH_{H_2O}</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td><strong>Macronutrients (mg kg$^{-1}$)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P$_2$O$_5$</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>K$_2$O</td>
<td>208.0</td>
<td>182.0</td>
</tr>
<tr>
<td>NH$_4$</td>
<td>40.0</td>
<td>40.0</td>
</tr>
<tr>
<td>NO$_3$+NO$_2$</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td><strong>Micronutrients (mg kg$^{-1}$)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>6.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Fe</td>
<td>20.5</td>
<td>20.9</td>
</tr>
<tr>
<td>Mg</td>
<td>98.2</td>
<td>99.0</td>
</tr>
<tr>
<td>Cu</td>
<td>4.9</td>
<td>4.8</td>
</tr>
<tr>
<td>S</td>
<td>44.6</td>
<td>45.0</td>
</tr>
<tr>
<td>Ca (%)</td>
<td>6.1</td>
<td>6.2</td>
</tr>
</tbody>
</table>

$^a$ According to Arany (Buzás, 1998).
microorganisms, the *Azospirillum* and *Rhizobium* N₂-fixers and the AMF (S, R and M, respectively). There were three replicates. The strains used (*Azospirillum brasilense* Km 5, *Rhizobium meliloti* S 5/7 + K 4/1 + Lu 41 mixture and *Glomus fasciculatum* M 107) originated from the Commercial Inoculum Collection of the RISSAC, Budapest. The effect of six *R. meliloti* strains, as candidate inocula was evaluated in a preliminary seedling experiment on alfalfa (*M. sativa* L.). Sterilised seeds (in 3% chloramin T, washed with five changes of sterile tap water), were germinated on Thorton agar and inoculated with 1 ml of rhizobium inoculum (approximately 10⁸ CFU ml⁻¹) (Vincent, 1970). Nodule-number and the acetylene reduction activity (ARA) were measured in six replicates using the method of Hardy et al. (1973). After 8 weeks of growth, the three most effective rhizobia were selected for further inoculations, as 1:1:1 (v:v:v) mixture. Alfalfa plants were inoculated after emergence with 5 ml cell-suspension per pot (approximately 10⁸ CFU ml⁻¹). The AMF inoculations, 3% of a root–soil mixture (*G. fasciculatum* and red clover host) for each pot with application made below the seed-layer, prior to sowing.

2.3. Plant growth and measurements

Eight alfalfa seeds (*M. sativa* L.) were sown into pots containing 250 g of the experimental soil. These were thinned after emergence to five seedlings. The pots were arranged in a randomised block design and supplied with tap water whenever needed. Plants were grown for 90 days under the controlled conditions at 22°C during the day and 18°C during the night, with a 18 h photo-period (photosynthetic photon flux density of 600 µmol m⁻² s⁻¹, using metal halide lamps) and a relative humidity of 70–80%. The dry weights of shoots at the 6th, 9th and 12th weeks of growth and the roots (at the final harvest) were recorded after drying in an oven at 70°C.

2.4. Estimation of the microbial colonisation and efficiency

Both the total nitrogen content and the nodule-number were used to estimate the efficiency of the rhizobium nitrogen-fixers. Nitrogen-free semisolid Nfb medium (Döbereiner and Day, 1976) was used for the MPN counts of the *Azospirillum* bacteria (Okon et al., 1977; Tarrand et al., 1978). Washed and cleared 1 g root samples were taken from each treatment and after grinding with sterilised quartz-sand a dilution series was prepared for inoculating the MPN tubes (10 ml of suitable semisolid media in three replicates). Growth records (development of the white, subsurface pellicle) were assessed after 2 days of incubation in 33°C. Parallel root samples of 1 g (wet weight) were also measured and dried in an oven at 70°C to give the root moisture percentage.

To estimate the root-colonisation of AMF an additional subsample of 1 g fresh lateral root was randomly taken and cut for approximately 1 cm segments. They were cleared and stained with acid glycerol trypan blue (Phillips and Hayman, 1970), and mounted on a microscopic slide to estimate AMF colonisations (30 segments in three replicates). The frequency and intensity of the mycorrhizal infection (F %; M) and the arbusculum content of the infected parts (a %) were recorded and calculated (Trouvelot et al., 1985), using a five-class system.

2.5. Chemical analysis

Soil physical characteristics were estimated by the method of Buzás (1998). The total element content of the soil was determined for both original (C) and gamma-sterilised (G) samples which were digested with cc. HNO₃ at 80°C in a microwave oven. Plant element contents (Ca, Mg, K, P and S) were assessed after wet digestion with HNO₃ + H₂O₂ of air-dried ground plant samples. Elements were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES, type: FY-238). Total N content in the shoot biomass was estimated by a modified Kjeldahl method after the wet digestion with cc. H₂SO₄ + H₂O₂.

2.6. Statistical analysis

Mean data on the MPN counts of the *Azospirillum* bacteria were calculated using the McGrady tables (Postgate, 1969) and transformed as the logarithmic values (n=3). The effects of mycorrhizal and bacterial treatments on element uptake, the root-colonisation of AMF and dry matter production were tested using
analysis of variance (two-way ANOVA). Comparison of means was made by using the least significant differences (LSD = \(p < 0.05\)). Regression analysis of the nodulation data and the ARA measurements used the Statgraphics 5.0 program.

3. Results

3.1. Selection of the rhizobium inoculum

Table 2 shows the result of a preliminary experiment, where six candidate \(R.\) meliloti strains were used to inoculate sterile alfalfa seedlings, grown in a Thornton agar with six replicates (Vincent, 1970). After 8 weeks of growth, the number of root nodules and the ARA were estimated (Hardy et al., 1973). The number of root nodules on the alfalfa seedlings varied between 4.5 and 12. The data mostly positively correlating with that of ARA (10.7–35.6 ppm ethylene h\(^{-1}\) per plant). Correlation coefficient \((r^2) = 0.998\) (at \(p = 0.0017\) level). The equation of the correlation curve was \(y = \log 1.293x + 137\). With strain Bk 5/1, some small and white nodules developed. This was not significantly related to performance (the ARA measurements). The strains Lu 41, K 4/1, S 5/7 showed the highest efficiency. These strains were selected as candidates for further inoculations as a mixed population.

Table 2
A sterile seedling experiment for estimating the efficiency (ARA and nodulating ability) of some \(R.\) meliloti strains, as potential candidates for the inoculation of alfalfa in pot experiments \((n=6)^a\)

<table>
<thead>
<tr>
<th>Laboratory code of rhizobia</th>
<th>Root nodules (number per plant)</th>
<th>ARA (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bk 5/1</td>
<td>12±2.0</td>
<td>28.7±6.2</td>
</tr>
<tr>
<td>Lu 41^b</td>
<td>8.6±2.5</td>
<td>35.6±4.1</td>
</tr>
<tr>
<td>K 4/1</td>
<td>6.3±1.0</td>
<td>25.8±4.7</td>
</tr>
<tr>
<td>S 5/7</td>
<td>5.7±1.2</td>
<td>20.9±3.2</td>
</tr>
<tr>
<td>Kt 1/2</td>
<td>5.0±2.9</td>
<td>18.5±3.6</td>
</tr>
<tr>
<td>Kt 4/1</td>
<td>4.5±1.5</td>
<td>10.7±1.8</td>
</tr>
</tbody>
</table>

^a Strains originated from the Commercial Inocula Collection of the Research Institute for Soil Science and Agricultural Chemistry (RISSAC-MTA TAKI) of the Hungarian Academy of Sciences, Budapest.

^b Strains set as italics were selected for the pot experiment, as 1:1:1 (v:v:v) mixed inoculum (approximately \(10^6\) CFU ml\(^{-1}\)).

3.2. Effect of mono- or multilevel inoculations on the dry matter accumulation

All diazotrophs as dual- (MR, MS) or tripartite (MRS) combinations with the AMF (M) increased dry matter production in the sterilised (G) and in the re-suspended (GB) treatments, where the other competitive AMF populations were excluded (Table 3). \(G.\) fasciculatum M 107, as single inoculum also significantly enhanced growth. This was further increased in the RM dual-inoculated (tripartite) systems. The \(R.\) meliloti (R) strain-mixture, which was selected for great effectivity also resulted in the same beneficial effect on the dry matter accumulation in the non-sterilised soil. A much higher increase was found, however, in the multilevel co-inoculation treatments, where the AMF was accompanied both by the associative and the symbiotic (\(Azospirillum\) and \(Rhizobium\)) nitrogen-fixers (synergistic effect of the MRS treatments).

Re-inoculation of the gamma-sterilised soils with the AM-free soil extract (GB) reconstructed the original (C) microflora in the rhizosphere. The same dry matter yield was found in the controls in comparison with the non-treated C soil.

In the original non-sterilised soil (C) a uniform beneficial effect was found for the plant growth as a result of the single inoculation of microsymbionts, both by the diazotrophs (R, S) and by the AMF (M). The tripartite and the multilevel systems with the \(Azospirillum\) and the \(Rhizobium\) diazotrophs (MR, MS and MRS treatments), however, have resulted in the same dry matter accumulation, as the non-inoculated controls (Table 3).

3.3. Nutrient uptake of the inoculated plants among the various soil conditions

Both the macro- and microelement content was affected by the microsymbiont co-inoculations, and influenced by the soil substrates (C, G, GB) used. The most positive effect of the single- or multilevel microbial treatments occurred with the sterilised substrates, where the indigenous AMF were excluded. The inoculated strain of the mycorrhizal fungi (\(G.\) fasciculatum M 107) was effective in increasing, both the N-, P- and the K-content of the alfalfa shoot, in the G, GB treatments.
Table 3
Shoot dry weight (Sh), root dry weight (Rt) and the total dry matter production (Sh+Rt) of alfalfa* (*M. sativa L.)*

<table>
<thead>
<tr>
<th>Strains</th>
<th>Substrates and dry matter yield (g per pot)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>Sh</td>
</tr>
<tr>
<td>Control</td>
<td>1.56a</td>
</tr>
<tr>
<td>M</td>
<td>1.72b</td>
</tr>
<tr>
<td>MR</td>
<td>1.60a</td>
</tr>
<tr>
<td>MS</td>
<td>1.71b</td>
</tr>
<tr>
<td>MRS</td>
<td>1.59a</td>
</tr>
<tr>
<td>R</td>
<td>1.73b</td>
</tr>
<tr>
<td>S</td>
<td>1.73b</td>
</tr>
<tr>
<td>RS</td>
<td>1.74b</td>
</tr>
</tbody>
</table>

a Plants were grown for 3 months in either the original- (C), gamma-sterilised- (G) or AM-free-resuspended (GB) chernozem soil with single-, dual- or multilevel microbial treatments. Dry matter was collected at the 6th, 9th and 12th weeks and is summarised here (g DW per pot). Each value is the mean of three pots. n.d.: not determined.

b M: mycorrhiza (G. fasciculatum M 107), R: rhizobium (R. meliloti S 5/7+Lu-41+K 4/1), S: spirillum (A. brasilense Km5) or their combinations, respectively.
c Not determined.

d In relation to nitrogen uptake among the original (non-sterilised) soil conditions (C), positive effects of rhizobium inoculation were found in all of the combinations (R, RS or MRS). In case of the Azospirillum diazotroph bacteria an enhanced N content was found in the MS treatment; the mycorrhizal inoculation resulted in an additive positive effect.

Uptake of P and K were also increased by the inoculated R. meliloti strain-mixture as single- (R), or dual systems with the other nitrogen-fixing bacteria (A. brasilense Km 5-RS). With the mycorrhizal (M, G. fasciculatum M 107) partner (Table 4) the effect was smaller.

Difference between the mono- and multilevel co-inoculations were not observed for the concentrations of elements such as the Ca, Mg, Fe, Zn, Cu and S in the shoot biomass. In the non-mycorrhizated (G, GB) control substrates, however, an increased element-content

Table 4
The quantity of the main nutrients (N, P, K) in the shoot biomass of alfalfa in the pot experiment*

<table>
<thead>
<tr>
<th>Strain combinations</th>
<th>Substrates and macro-elements§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Control</td>
<td>39.8a</td>
</tr>
<tr>
<td>M</td>
<td>38.2a</td>
</tr>
<tr>
<td>MR</td>
<td>39.1a</td>
</tr>
<tr>
<td>MS</td>
<td>49.6b</td>
</tr>
<tr>
<td>MRS</td>
<td>50.7b</td>
</tr>
<tr>
<td>S</td>
<td>42.4a</td>
</tr>
</tbody>
</table>

* Plants were grown in a non-sterilised calcareous chernosem soil (C: all microbes), in a gamma-sterilised soil (G: no microbes) and in a sterilised substrate, to which a mycorrhiza-free soil extract was added (GB: no mycorrhiza). The effect of the single- (M, R, S), dual- (MR, MS, RS) and multilevel (MRS) inoculations were assessed by using bacterial and fungal microsymbionts. M: G. fasciculatum M 107, R: R. meliloti S 5/7+Lu-41+K 4/1, S: A. brasilense Km5. n.d.: not determined.

§ Values denoted by the same letter are not significantly different at p≤0.05 level. Significant differences are given in italics.
was found, as a result of the gamma-sterilisation. The effect of the additional AM-free rhizosphere microflora depended on the microelement, studied. Any microbial re-inoculation (GB) decreased the Mg, Zn and Cu and S content of the alfalfa shoot-biomass, compared to the gamma-sterilised substrate (G). The concentration of Fe was increased by the re-suspension of the AM-free soil extract in the control substrates.

In the original normal soil (C), the content of some elements was unaffected by the inoculated microsymbions. With Ca slightly increased, but not significant, uniform uptake occurred with the single- or dual diazotroph treatments C+R, C+S and C+RS, respectively (Table 5).

3.4. Abundance of the inoculated microbes in the rhizosphere of the alfalfa

For the AMF, the infection frequency (F, %) in the targeted roots was high (all near to the 100%) and was not significantly changed for in the different microbial treatments (data not shown). The arbuscular content of the infected parts (a, %), however, was a more variable as a function of the single-, dual- or multilevel co-inoculations (Fig. 1). Addition of the AM-free soil suspension (the normal rhizosphere constituents) to the gamma-sterilised substrates (G, GB) increased the arbusculum content of the alfalfa root system. A synergistic effect was achieved, when all of the inoculated microbes were present (MRS). The non-sterilised original soil showed significantly, the lowest colonisation (arbusculum content=14.6%) by the mycorrhiza

![Fig. 1. Nodule-number (I), arbusculum content (II) and abundance of the associative diazotrophs (III) on the alfalfa roots after 3 months of growth in a calcareous loamy chernozem soil (C: all microbes), in the same gamma-sterilised soil (G: no microbes) and in the sterilised substrate, resuspended with mycorrhiza-free soil extract (GB: no mycorrhiza). The effect of the single- (M, R, S), dual- (MR, MS, RS) and multilevel (MRS) co-inoculations were assessed after 3 months of growth in a pot experiment among controlled conditions. M: vesicular AMF (G. fasciculatum M 107), R: R. meliloti S 5/7+K 4/1+Lu 41 mixture), S: A. brasilense Km 5 and their combinations, respectively.](image-url)
For nodule-number, after the 3 months of plant growth, a beneficial effect was found for all microbial inoculations in the sterile or AM-free soils (Fig. 1). A further enhancement was developed with the multilevel co-inoculations (MRS). In the original soil (C) a significantly increased nodule-number was also recorded with the mycorrhizal (G. fasciculatum M 107) inoculation. Dual treatments with the obligate- and the associative diazotrophs (Rhizobium + Azospirillum bacteria, RS) also resulted in a synergistically increased nodule-number (nn = 27 per pot, data not shown). This was the highest rate in the control soil.

The most probable number method (MPN) for calculating the abundance of the associative nitrogen-fixing bacteria was also carried out for the rhizosphere of the alfalfa host. These data showed that the cell-number of the Azospirillum diazotrophs increased due to the mono- or multilevel inoculations. The highest rates were found in case of the single- or dual mycorrhizal co-inoculations (M, MS, MRS) especially in the sterile or in the AM-free soils (G, GB). In the original substrate (C) the lowest cell numbers were recorded in the Azospirillum-non-amended combinations, such as the M, R and MR treatments. All of the mycorrhizal co-inoculations increased the abundance of the associative diazotrophs. In the GB substrate, only the dual- or multilevel co-inoculations of the MS or (MRS) treatments were effective.

4. Discussion and conclusions

Associative and symbiotic nitrogen-fixing bacteria and AMF are common beneficial microbes of the monocotyledonous- and leguminous- plants; about 80–90% of the higher plants, respectively. Artificial seed and soil inoculation techniques were, therefore, started over 100 years ago; as a simple application of a mixed nodule extracts (Hiltner, 1895). Inoculated microbes, however, compete with the native microflora of the soil. This is the main reason for failed inoculation experiments (Graham, 1992; Giller and Cadish, 1995). Effects of abiotic environmental stress factors (temperature, drought, acidity, etc.) are also common (Smith and Bowen, 1979; Graham, 1992; Bayoumi et al., 1995). The final effect of any microbial inoculation of plant-rhizosphere functioning is, therefore, the result of a complex of interactions between the plants, the rhizosphere inhabitants and the different microbial and environmental components involved (Barea et al., 1988b; Postma et al., 1989). To study antagonistic and synergistic effects, especially among the so-called beneficial microorganisms is crucial to plant growth and sustainability (Bethlenfalvay et al., 1985; Höflich, 1993).

In the present study the effect of the separated single-, dual- or multilevel mycorrhizal co-inoculations have been demonstrated for alfalfa, grown in normal, sterilised and AM-free soil conditions. Detection of the impact of associative and symbiotic nitrogen-fixers and of AMF on the plant growth and development was possible with this technique. Sterilisation procedures have been used to study the effect of some abiotic factors on the introduced microbes (Bayoumi et al., 1999), or to eliminate the influence of soil-borne plant pathogens (Postma et al., 1989). Separation of the AMF and the other usual rhizosphere components (mainly bacteria and other micromycetes) is possible using a sterilisation and re-inoculation procedure. In the GB treatments a successful reconstruction of the original rhizosphere microflora occurred; evidence by plant growth and the element-content of the host (Table 2).

In all of the applied substrates (C, G, GB) beneficial, synergistic effect of the multilevel co-inoculations of the obligate- and associative diazotrophs and the AMF (MRS) were found. Other measurements, such as chlorophyll-fluorescence analysis also indicated a stress buffer effect of AMF (Strasser, 1996).

Positive effect could be directly correlated with the more efficient colonisation of the inoculated nitrogen-fixing bacteria and the indigenous AMF (Fig. 1). G. fasciculatum M 107, used as mycorrhiza inoculum, was selected because of its beneficial effect on alfalfa growth. Single treatment (M) of this strain resulted in a stimulation of (especially in the G, GB substrates) growth. In the non-sterilised soil (C), the introduced strain of the AMF was not sufficiently competitive. Mycorrhizal co-inoculations on the other hand enhanced the nodulation and the MPN counts of the obligate and the associative diazotrophs, respectively. The arbusculum content of the infected roots was not correlated with the colonisations data.
The dry matter production and *Rhizobium* nodulation data showed a significant synergistic effect between the AMF (M) and the separately applied diazotrophs (MR or MS), and between the obligate and associative nitrogen-fixing bacteria (RS) used. The effects of diazotroph bacteria are rarely considered in studies of legumes. *Azospirillum* bacteria are used mainly for mono-cotyledonous hosts (Döbereiner and Day, 1976; Subba Rao, 1985; Pacovsky, 1988; Belimov et al., 1999). In the other dual-inoculated systems the AMF and the *Rhizobium* nitrogen-fixing bacteria are normally used for inoculating the leguminous hosts (Ames and Bethlenfalvay, 1987; Champawat, 1990). Synergistic effect of the associative and obligate nitrogen-fixers should be a consequence of the clear separation of their functioning (e.g. by the root-nodules) inside the plant-rhizosphere.

Several experiments have been published detailing the success and failure of *Azospirillum* inoculations for agricultural crops, such as the wheat and the maize (Biró, 1992a,b). Simultaneous AMF treatment seems to result in better establishment of the nitrogen-fixers. It is less common for co-inoculated microorganisms (such as *Azospirillum* or *Agrobacterium* or *Flavobacterium* sp.) (Barea et al., 1988b; Belimov et al., 1999).

All of the *R. meliloti* isolates used originated from the root-nodules of the alfalfa. This is grown widely in Hungary. Strains were selected for their performance as single treatments with sterile substrates. Under non-sterile-conditions (C), however, a high competitive ability with the indigenous populations is needed. A mixture of three efficient nodulating *R. meliloti* strains were therefore used. The microsymbionts have distinct habitats in the rhizosphere, such as the nodules (for the *Rhizobium*) and the root-interior for the associative diazotrophs and the AMF. Both nitrogen-fixers inhabit the interia of the root-systems or the mycelial network. Functional interrelations must exist between them and the AMF (Bethlenfalvay et al., 1985; Subba Rao, 1985; Champawat, 1990; Paula et al., 1992). In case of the *Rhizobium* bacteria the nitrogen-fixing ability is separated to the inside of the root nodules. Generally competition seems not to occur between rhizobia and AMF (Ames and Bethlenfalvay, 1987). Associative diazotrophs, on the other hand are not separated from the mycelia of the endomycorrhizal fungi. These nitrogen-fixers can function both inside and outside AMF structures. This may result in a competition for nutrients between the two microsymbionts. The success and the failure of the *Azospirillum* and AM fungal co-inoculations may therefore depend on the physiological stage of the host, the time of the infections or on the nutrient demands of the microsymbiont partners.

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References


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