METHOD TO STUDY THE MICROBIAL INTERACTIONS BETWEEN THE INOCULATED MICROSYMBIONTS AND THE INDIGENOUS MICROBES IN THE RHIZOSPHERE

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Abstract
Three-substrate microcosm method was used to study the microbial interactions in the rhizosphere of alfalfa (*Medicago sativa*) grown in a loamy chernozem control soil. The effect of some beneficial microsymbionts, such as the nitrogen-fixing associative *Azospirillum* (*A. brasilense*-S) and root-nodulating *Rhizobium* (*R. meliloti*-R) bacteria and the arbuscular mycorrhizal fungi (*Glomus fasciculatum*-M) were also assessed as single- (M), dual- (MR, MS) or tripartite mycorrhizal co-inoculations (MRS). Beside the unsterilised, control soil (including the normal rhizosphere community-C), there were other substrates used, such as the b/ gamma-sterilised soil (G) and c/ bacterial reincubated mycorrhiza-free soil (GB). In these substrates the single-, dual- or tripartite inoculation of the diazotrophs were used, so as to get data about their interactions with the indigenous AM fungal populations.
Beside the mass production, nodule-number, and mycorrhizal colonisation was recorded.
In the gamma-sterilised soil, without the interacting microbial parameters in the rhizosphere, all of the mono- (M) or multilevel (MR, MS, MRS) microbial combinations were effective for improving either plant development or the rhizosphere-colonizations. The tripartite inoculation (MRS) of microsymbionts further enhanced the tested parameters, causing a synergistic effect in the gamma-sterilised soils. In the mycorrhiza-free, but bacterial re-inoculated substrate, however no further improvement could be realised, suggesting the importance of AM fungal colonisations in the mycorhizosphere. Among the dual inoculations the less compatibility was found between the applied *Rhizobium* and mycorrhizal strain (MR), while the opposite happened between the nitrogen-fixers in the unsterilized control soil (RS). The microcosm method, used in this study offers an appropriate tool to evaluate the specific relationships and the functional compatibility between the microbial inocula and the host-plants in the various soil-plant systems.
Key words: microbial interactions, inoculation, microsymbionts, associative diazotrophs, rhizobia, arbuscular mycorrhizal fungi.

1. Introduction

Microbial activities in the various soil-plant systems are considered as the main parameters in the ecosystem functioning. Beneficial diazotroph bacteria, such as the associative and root-nodulating N_2-fixers (*Azospirillum* and *Rhizobium* sp.) in the rhizosphere of the leguminous plants are key elements for plant establishment and growth (Barea and Azcon-Aguilar 1983).

Except them arbuscular endomycorrhizal fungi (AMF) are also mutualistic microsymbionts of about 90 % of the higher plants. Their possible effect is to improve plant water relations (Sanchez-Diaz et *al*. 1990), phosphorus and other element-uptake at suboptimal situations (Barea et *al*. 1987, Pacovsky 1988).

Seed- or soil inoculation for the above mentioned reasons is a common agricultural practice (Champawat 1990). The success, however is being highly dependent on the effectivity of the indigenious microbes and on the interactions between the other participants in the rhizosphere (Lindermann 1983, Puppi et *al*. 1994). There are also several abiotic environmental stress factors (temperature, drought, acidity..etc.), however which may influence on the growth and activity of the beneficial rhizosphere components (Graham 1992, Bayoumi et *al*. 1995). The final effect of microbes in the plant rhizosphere is the result of the interactions among the different microbial components involved. To study of the antagonistic and synergistic interactions between the beneficial microorganisms is a crucial step therefore considering the sustainability (Biró 1993).

The aim of this study was to develop an *in vitro* method, which would be appropriate to study the microbial interactions in the rhizosphere, concentrating on the functional compatibility of the beneficial microorganisms at single-, dual- or tripartite interactions.

2. Materials and methods

Soil treatments

Various soil-conditions were developed, by the elimination and the re-inoculation of certain microbial groups. Substrate **C:** the untreated loamy chernozem soil served as control, where all the usual microorganisms (both *bacteria+mycorhiza*) were present. Substrate **G:** the sterilised soil in which the original microbial components were excluded (*no any microbes*) due to the gamma irradiation (15 kGy Co kg^{-1} soil). Substrate GB**:** the gamma-sterilised soil, but reincubated by 10 ml/pot of the AM-free normal rhizosphere suspension (*only bacteria, no mycorrhiza*) – (500 ml distilled water kg^{-1} soil was adjusted through a 5 mm pore size sieve to exclude AM fungi).

Microbial inoculations

Using one of the three substrates (C, G, GB) in the pots, the plants of *Medicago sativa* were grown with or without the associative and symbiotic micro-organisms, such as the *Azospirillum* and *Rhizobium* N_2-fixers and the arbuscular endomycorrhizal fungi (S, R and M

respectively). Strains investigated (*Glomus fasciculatum* M107, *Rhizobium meliloti* Lu+5/7) were originating from a commercial strain-collection (RISSAC, Budapest). Regarding the AMF inoculation 3 % of a root-soil mixture was used in each pot below the seed-layer before the sawing. Diazotroph bateria, however were used after the emergence as 10 ml pot^{-1} inoculum (approximately10^8 CFU ml^{-1}).

Plant growth and microbial colonisation

Alfalfa (*Medicago sativa* L.) was cultivated (5 plants in each pot, with 200g dry soil) among the greenhouse conditions (18h/6h photo-period, $22^0C/18^0C$ temperature, 10.000 lx light intensity), for three months. After the vegetation period, fresh weight of the shoots and roots and also the dry matter-accumulation were determined. The colonisation values of the inoculated microsymbionts were assessed by the nodulation characteristics of rhizobia (Vincent 1970) and by the five-class system of tripan-blue stained (Phillips and Hayman 1970) mycorrhiza-colonised roots (Trouvelot et *al*. 1985).

Statistical analysis

Significant differences were calculated between the treatments by ANOVA analysis and least significant differences - $LSD_{5\%}$ ($P<0.05$) is considered at the evaluations.

3. Results

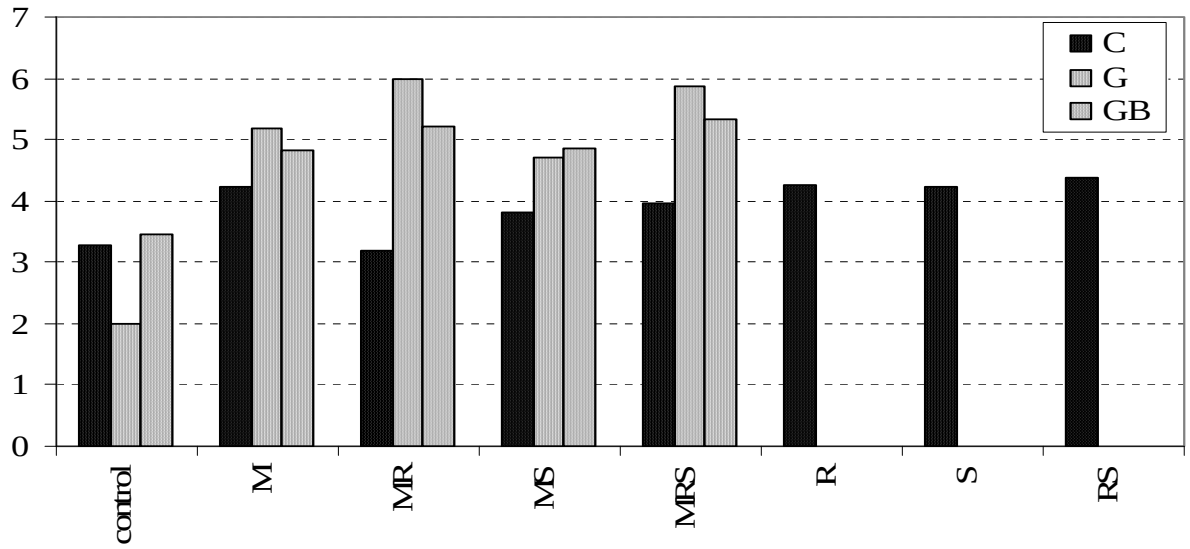
3.1 *Dry matter accumulation affected by the microsymbiont coinoculations*

All diazotrophs (R, S) as dual- or tripartite combinations with the arbuscular mycorrhizal fungi (MR, MS, MRS) increased the dry matter production in the sterilised (G) and in the bacterial re-inoculated AM-free (GB) treatments, where no competitive AMF population was present. A much higher increase of the dry matter yield was found in the tripartite systems (MRS), due to the additive beneficial effect of both the associative and symbiotic diazotrophs. Bacterial reincubation of the sterilised soil has re-constracted the original (C) rhizosphere microflora, and therefore a higher dry matter yield could be developed in comparison with the non-inoculated G treatment. Mono- or multi-level inoculation by the AM fungi of the gamma-sterilised (G) and the bacterium-amended substrates (GB) resulted the same plant development (Figure 1).

In the non-sterilised soil (C) a general beneficial effect was found on the plant growth by all the single-inoculated diazotrophs (R, S) and AM fungi (M). Dual systems, however, with AM fungi (MR, MS) was not producing better growth, than the control.

Figure 1.

Total dry matter yield (g/pot) of the inoculated alfalfa (*Medicago sativa*) in calcareous chernozem soil of Érd. Plants were grown for 3 months in the original- (C), in the gamma-sterilized- (G) or in the bacterium reincubated (GB) chernozem soil and affected by single-, dual- or tripartite microbial treatments (g DW pot^{-1})
M = mycorrhiza (*Glomus fasciculatum* M107), R = rhizobium (*R. meliloti* Lu+5/7), S = spirillum (*Azospirillum brasilense* Km5) or their combinations, respectively.



3.2 Nodulation and mycorrhizal colonisation in the rhizosphere

Among the colonisation data of the arbuscular mycorrhizal fungi, an increased arbusculum richness of the roots (a %) was found in the sterile- or in the bacterium-amended treatments (G, GB). This fact was also realised in case of the single-, dual- or tripartite inoculations (Table1). A synergistic effect was found on the colonisation data, when all of the inoculated microbes were present (MRS). In the non-sterilised soil on the other hand there was a beneficial influence recorded by the single or dual *Rhizobium* inoculations (R, RS). The lowest arbusculum content was found (a % = 14,6-22,1), when the AMF and the nitrogen-fixing bacteria were simultaneously interacting (MS and MR). That suggests a more efficient colonisation values by the inoculated nitrogen-fixing bacteria with the native mycorrhiza population, but not with the introduced AM fungal strain.

Regarding the nodule number, which has developed after the 3 months of plant growth, a beneficial effect was also found at all microbial inoculations in the sterile or in the AM-free soils. In the non-sterilised substrate (C) not only the diazotroph *Rhizobium* strain, but also the AM fungal inoculum could enhance the root-nodulation capacity considerable in the rhizosphere of alfalfa (Table 1).

Table 1

Root nodulation and colonisation of arbuscular mycorrhizal fungi in the rhizosphere of alfalfa grown for 3 months in untreated loamy chernozem soil (C), in gamma-sterilized soil (G) and in AM-free, bacterium-reincubated soil (GB). Plants were inoculated with symbiotic beneficial microbes, such as M = mycorrhiza,

Glomus fasciculatum M_{107}, R = *Rhizobium meliloti* Lu+5/7, S = spirillum, *Azospirillum brasilense* Km_5, and their combinations, respectively.

Treatments	Root-nodulation (N^0/pot)			Arbusculum richness (%)		
	Non-sterile (C)	Sterile (G)	AM-free (GB)	Non-sterile (C)	Sterile (G)	AM-free (GB)
Control	10,3 a	2,6 a	16,2 a	34,1 b	-	-
M	24,1b	13,5 b	26,2 b	31,2 b	23,1 a	33,4 b
MR	13,3a	18,4 b	20,0 a	22,1 a	23,2 a	38,5 b
MS	12,1a	19,5 b	27, b2	13,2 a	28,1 a	24,5 a
MRS	16,2a	32,3 c	34.1 c	34,1 b	48,2 b	54,3 c

The letters in each column are showing the significant differences between the treatments

Among these treatments being in a positive correlation with the arbusculum richness of mycorrhizal fungi, a further synergistic enhancement could develop at the multilevel inoculation treatments (MRS). Dual inoculation of the diazotrophs (*Rhizobium* and *Azospirillum* bacteria) resulted also an enhancement in the nodulation (data not shown). Although introduced rhizobium strain was found to increase the root-nodulation, the incompatibility with the AM fungal inoculum was also recorded in the experiment.

4. Discussion and Conclusions

A three-substrate microcosm method was used to study the effect of microbial inoculation of beneficial microbes in the rhizosphere of alfalfa. Due to the nutrient competitions, the effect of any introduced inoculum, is the outcome of the complex interactions in the rhizosphere. The measurements of the functioning of soil-plant-microbe interactions *in situ*, by the avoiding of high disturbance of the systems are having great importance recently (Strasser and Eggenberg 1995).

In the present study a microcosm experiment was designed to assess the effect of beneficial microbial inoculum strains *in situ*. The separated single-, dual- or tripartite microsymbiont co-inoculations are demonstrated on the alfalfa grown among normal-, sterilised and only bacterium-amended, AM-free soil conditions. Estimating the special role of the associative and symbiotic nitrogen-fixers and AM fungi (weather they are separated or combined) in the plant growth and development proved to be possible with this technique. The sterilised inoculation trial (G) clearly proved the beneficial, synergistic effect of the multilevel inoculations by the selected microbes, such as the symbiotic- and the associative nitrogen-fixers and the arbuscular mycorrhizal fungi (MRS). Although *Azospirillum* inocula are mainly used for the mono-cotiledonous, grass-type plants (Döbereiner et *al*. 1967, Pacovsky 1988), there was an influence recorded on the clover. Several experiments were published about the success and the failure of the *Azospirillum* inoculations of the main agricultural crops, such as the wheat and the maize (Biro 1992, 1993). In the present study a synergistic effect could be realised between the mycorrhizal fungi (M) and the diazotrophs (MR or MS),

and also between the two nitrogen-fixing bacteria (RS), which are rarely considered together in one study on the leguminous plants.

Although all of the used micro-symbionts were originating from the rhizosphere of the alfalfa not all proved to be effective regarding the tested parameters (i.e. dry-mass production). Usually the indigenous population are better adapted to the certain environmental conditions, which are the main reason of the failed inoculation experiments (Graham 1992). In this study a better compatibility was found between the nitrogen-fixing bacteria and the native AM fungal population in the non-sterilised control soil.

Using the demonstrated soil treatments (C, G or GB) the separation of the effects of single-, dual- or tripartite inoculations could be possible by the applied microcosm technique.

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