Symbiosis, 14 (1992) 427–438
Balaban, Philadelphia/Rehovot

Phytoeffective Combination Effects of Symbiotic and Associative Microorganisms on Legumes

GISELA HÖFLICH, FRANK GLANTE[1], HANS-HOLGER LISTE[4],
INA WEISE, SILKE RUPPEL[2], and CORNELIA SCHOLZ-SEIDEL[3]
Centre of Agrar Landscape and Land Use Research Müncheberg
Institute of Ökophysiology, W. Pieck Strasse 72, 0-1278 Müncheberg, Germany
Tel. 49 (033432) 82282, Fax 49 (033432) 82212
[1]*Federal Environmental Agency, Bismarckplatz 1, Berlin 33, Germany*
[2]*Institute of Vegetable Research, Theodor-Echtermeyer-Weg, 0-1722, Großbeeren, Germany*
[3]*Research Centre of Agriculture Braunschweig-Völkenrode, Institute of Agrarian-relevant Climatic Research, W. Pieck-Strasse 72, 0-1278 Müncheberg, Germany*
[4]*Humboldt-University, Faculty of Agriculture and Food Production Invalidenstrasse, 1040 Berlin, Germany*

Received November 20, 1991; Accepted July 10, 1992

Abstract

The effect of *Rhizobium* inoculation on lucerne was improved by a combined inoculation of selected *Rhizobium* bacteria and fast infecting VA mycorrhizal fungi or *Pseudomonas fluorescens* respectively. In pea, positive combination effects were obtained by inoculation of host plant specific *Rhizobium* bacteria and *Pseudomonas fluorescens* or *Rhizobium leguminosarum* bv. trifolii. Inoculation by VA mycorrhizal fungi or *Pantoae agglomerans* increased the effectiveness of the wild *Rhizobium* population and was more effective in pea than *Rhizobium* inoculation. Plant growth stimulation may be caused by: direct improvement of nutrition (N-fixation, P-mobilization), increase of the root system's nutrition uptake by microbial phytohormones, antagonistic effects against soil-born root pathogens. Inoculum types and inoculation methods affect the survival of microorganisms and their effectiveness. Suitable for field experiments are: preparation based on peat as the carrier of bacteria (symbiotic, associative), and preparation with peat-bentonite mixture for VA-mycorrhizal fungi. The inoculation can be done by coated seeds or as granules in the seed row.

0334-5114/92 /$03.50

Keywords: combination effects, rhizosphere microorganisms, phytohormone, N_2-fixation, P-mobilization

1. Introduction

Rhizosphere microorganisms are able to affect plant growth by improving the nutrition and by phytohormone-like effects (Weihs, 1989). Positive results in stimulating biological nitrogen fixation and plant growth without additional fertilization of legumes are possible by using *Rhizobium* bacteria (Afanasewa et al., 1982; Höflich et al., 1989). There are also publications of no or differing inoculation effects (Giddens et al., 1982). By combined inoculation of *Rhizobium* and associative bacteria (*Pseudomonas* spp., *Azospirillum* spp., *Azotobacter* spp.) or VA-mycorrhizal fungi the nodulation, nitrogenase activity and plant growth can be increased in comparison to *Rhizobium* inoculation alone (Yaholom and Okon, 1990; Li and Alexander, 1990; Patterson et al., 1990). Most of the experiments were carried out under sterile conditions and in pot trials. The aim of this work was to examine:

- Is it possible to increase the yield of pea and lucerne by inoculation of microorganisms (symbiotic and associative bacteria and VA mycorrhizal fungi) with different physiological abilities in field experiments under moderate climatic conditions?
- Which processes do cause these positive combination effects.

2. Materials and Methods

Phytoeffective *Rhizobium*, associative bacteria, and VA-mycorrhizal fungi, which vary in their ability for nitrogenase activity (acetylen reduction method) (Rennie, 1981), phytohormone production (cytokinines, auxines) (Ruppel and Wache, 1990; Scholz and Ruppel, 1992), P-mobilization (Domey, 1987) and antagonism (lysis zones of phytopythogenic fungi – *Fusarium* spp., *Rhizoctonia* spp., *Ascochyta* spp.), nitrate reductase (Drews, 1968), osmotolerance (growth on nutrient agar with 0.8 mol NaCl per 1) and plant affinity (bacterial growth on the root surface) respectively, were inoculated single and in combination in growth experiments with pea (var. Grapis) and lucerne (var. Verko). Field experiments were done in Müncheberg loamy sand pH 5.6–6.5, total carbon content 560–600 mg per 100 g soil, total nitrogen 50–60 mg per 100 g soil; Bad Lauchstädt chernozem pH 6.8–7.2, total carbon content 1800–2100 mg per 100 g soil, total nitrogen 190–220 mg per 100 g soil. The size of the field plots was 15 to 20 m^2 with 6 replicates. Fertilization was done with

60 kg P and 100 kg K per ha without nitrogen. Pot experiments were carried out with loamy sand (1 kg per pot) under greenhouse conditions (8 replicates).

For inoculation bacterial culture suspensions (10^6 cfu per plant) were used in pots but peat preparates of 400 to 800 g per ha were used in field experiments (Höflich et al., 1987b). Mycorrhizal fungi were inoculated as coated seeds or as granules (50 kg per ha for pea and 20 kg per ha for lucerne) respectively (Höflich and Glante, 1991).

Effectivity criteria were dry matter at the time of flowering and seed yield, raw protein (Kjeldahl method), P-uptake (lactic acid method) nitrogenase activity (Höflich and Hickisch, 1986; Wolf and Hölflich, 1986), nodulation, root surface (Lehfeldt et al., 1986), contamination level of roots infected with fungal pathogens (relative proportion of brownish roots per plant) and survival of the inoculated bacterial strains in the rhizosphere with rifampicine-resistant mutants (Höflich, 1989). Statistical analysis was made on the ANOVA basis.

3. Results

Yield effects in field experiments

Combined inoculations of Rhizobium *and associative bacteria*

Rhizobium-specific for pea (E163 = IMET 11417) and *Rhizobium*-specific for lucerne (mel8 = IMET 11287) led to extra yields in several previous field experiments (Höflich et al., 1987a, 1989). Three out of 24 associative rhizosphere bacterial isolates with nitrogenase activity and phytohormone or antagonistic effects gave an extra yield effect on pea, one isolate was also effective on lucerne (Table 1).

The strain *R39* = IMET 43726 (*Rhizobium leguminosarum* biovar *trifolii*) originally isolated from red clover increased especially in combination with *Rhizobium leguminosarum* biovar *viceae* the dry matter and seed yield of pea.

The strain *PsIA12* = IMET 11446 (*Pseudomonas fluorescence*) – origin from wheat (Höflich, 1992) – partly increased the seed yield of pea also by noneffective *Rhizobium* inoculation and the dry matter yield of lucerne and lupine-pea-pot mixture by combined inoculation (Table 1).

The strain *D5/23* = IMET 11328 (*Pantoae agglomerans*) – isolated from wheat, increased the dry matter and seed yield of pea without additional *Rhizobium* inoculation (Table 1).

Combined Rhizobium *and VA-mycorrhizal inoculation*

The positive effect on lucerne of single inoculations of the *Rhizobium meliloti* (strain mel8) and the fast invading VAM isolates VAM 3 and VAM 4 on dry

Table 1. Combined inoculation of symbiotic and associative bacteria on pea, lucerne and lupine-pea-oats mixture (field experiments 1989 and 1990)

Crop species field site		Pea loamy sand	Lupine-pea-oats loamy sand	Pea loamy sand	chernozem	Lucerne loamy sand	
Inoculated organisms		Dry matter	Extra yield	Seed extra yield		Dry matter extra yield	
						1. year	2. year
Rhizobia	Associative bacteria	t per ha	t per ha	t per ha	t per ha	t per ha	t per ha
–	–	(0.97)[1]	(3.52)[1]	(3.36)[1]	(2.49)[1]	(2.61)[1]	(19.35)[1]
+	–	0.20	0.00	0.00	0.29+	0.88+	1.38
+	R 39	0.47+	0.49	0.82+	0.54+		
+	PsIA12	0.16	0.92+	0.92+	0.59+	1.20+	2.59+
+	D5/23		0.34				
–	D5/23	0.47+		0.96+	0.48+		
LSD at 5%		0.36	0.68	0.78	0.29	0.39	1.90

[1] Yield of non-inoculated control (t per ha)
+ significantly to the control

matter yield, raw protein yield and P-uptake could be improved by combined inoculation (Table 2).

The seed yield of pea was increased due to inoculation with the VAM 5 isolate, either as coated seeds or as granules in the seed row (Table 3). Combination with *Rhizobium leguminosarum* (strain E163), was not more effective. This strain was effective as single inoculant.

Metabolic and physiological abilities of the microbes and their interrelationships with the host plant

The three associative plant growth promoting bacterial strains (D 5/23, PsIA12, R39) used, differed from the plant specific rhizobial strains in their ability to produce cytokinins (zeatin, N^6-isopentenyladenosine, N^6-isopentenyladenine) (Tables 4, 5). PsIA12 had additionally the ability to mobilize phosphate on nutrient agar and had antagonistic effects against soil-born plant pathogens in the petri dish tests but it had not nitrate reductase activity.

The plant growth effect may be due to greater nodulation and higher nitrogenase activity of the inoculated plant stimulated by the specific rhizobia R39 and PsIA12 respectively (Fig. 1). VAM fungi (Table 3) and D5/23 (unpublished) affected also without additional *Rhizobium* inoculation the nodulation and effectivity of the indigenous rhizobial population (Table 3). Due to inoculation with VAM 3 the contamination of root pathogens was decreased (Table 3). The increased microbial N-fixation and phytohormone production may be the reason for the greater root surface (Fig. 2). An increased root surface improves nutritional uptake.

The most important condition for a phytoeffective symbiosis or association is a strong interrelationship between microorganisms and the host plant. The associative strain PsIA12 was determined on pea and lucerne roots 4 weeks after sowing (Table 6). Its survival rate was lower than that of the plant specific rhizobia. The survival rate of the inoculated organisms could be influenced by the inoculum type. Peat-based preparates were more suitable for survival of seed inoculated bacteria than inoculation with bacterial suspensions.

4. Discussion

Combined inoculation of microorganisms (*Rhizobium* bacteria, VA mycorrhizal fungi, or associative bacteria) which complement in their metabolic physiological activities may improve the effectivity of a single inoculation on lucerne or pea under moderate climatic conditions.

Table 2. Combined inoculation of rhizobia and VA-mycorrhizal fungi on lucerne in two cropping yields

Inoculated organisms		Dry matter extra yield		Raw protein extra yield		P-uptake
Rhizobium strain	VAM isolate	$t \cdot ha^{-1}$	%	$h \cdot ha^{-1}$	%	$kg \cdot ha^{-1}$
–	–	(11.63)[1]	0	(2.09)[1]	0	42
mel8	–	2.92^{+}	25	0.517^{+}	25	52^{+}
–	VAM3	1.91^{+}	16	0.348^{+}	17	47^{+}
–	VAM4	2.32^{+}	20	0.200	10	48^{+}
mel8	VAM3	4.95^{+}	43	0.882^{+}	41	58^{+}
mel8	VAM4	5.26^{+}	42	0.693^{+}	33	57^{+}
LSD at 5%		1.44		0.280		5

[1] yield of noninoculated control ($dt \cdot ha^{-1}$)
$^{+}$ significantly to the control

Table 3. Combined inoculation or rhizobia and VA-mycorrhizal fungi on pea (seed extra yield, nodulation, contamination of roots with soilborn plant pathogens; field experiment 1990, loamy sand)

Inoculated organisms		Inoculum type	Extra seed yield		No. of nodules	Root contamination
Rhizobia strain	VAM-isolate		$t \cdot ha^{-1}$	%	%	%
–	–	–	$(3.36)^1$	0	100 $(32.1)^2$	21
E163	–	preparation	0.00	0	127	9^+
–	VAM5	seed coating	0.73	22	185	6^+
E163	VAM5	seed coating	0.78^+	23	124	16
–	VAM5	granules	0.69	21	212^+	9^+
E163	VAM5	granules	0.89^+	26	225^+	15
LSD at 5%			0.78		92	10

^{1} yield of control ($t \cdot ha^{-1}$)
^{2} no. of nodules per plant

Table 4. Physiological abilities of selected microorganisms

	Pantoea agglomerans	*Pseudomonas fluorescens*	*Rhizobium trifolii*	*Rhizobium meliloti*	*Rhizobium leguminos*	VA-*Mycorrhiza Glomus* sp.
	Strain: D5/23	PsIA12	R39	mel8	E163	VAM3
Nitrogenase activity	+	–	+	+	+	–
Phytohormone production						
cytokinins	+	+	+	–	–	
auxins	+	+	+	+	+	
P-mobilization	–	+	–	–	–	+
Antagonism	–	+	–		–	
Nitrate reductase	+	–	+	+	+	
Plant affinity	+	+	+	+	+	+
Osmotolerance	+	+	+	+	+	
Stimulation of root length	+	+	+	+	–	+
Increase of nodules per plant	+	+	+	+	+	+

Table 5. Phytohormone spectra (HPLC-analysis and indirect ELISA)

Bacterial strain	Cytokinins	Auxins
D5/23	N^6 – (isopentenyl) adenosine N^6 – (isopentenyl) adenine	Indole-3-acetic acid Indole-3-lactic acid
PsIA12	Zeatin	Indole-3-acetic acid Indole-3-lactic acid
R39	Zeatin	

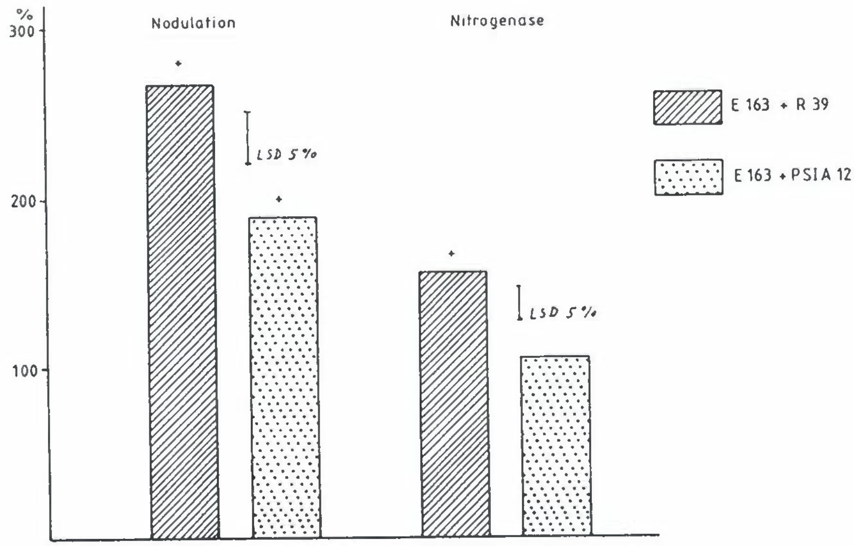

Figure 1. Nodulation and nitrogenase activity on pea after combined inoculation (sterile experiment, *Rhizobium* inoculation = 100%).

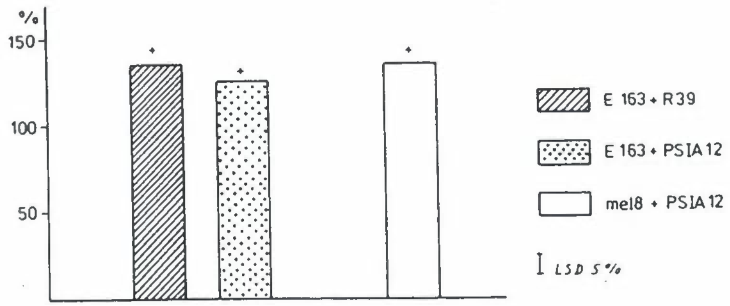

Figure 2. Root surface of pea and lucerne after combined inoculation (non-sterile, non-inoculated, control = 100%).

VA mycorrhizal fungi (VAM 5) or associative bacteria (D5/23), respectively, may increase the effect on peas too and affect plant growth without additional rhizobial inoculation. The ability of the bacterial isolates to produce phytohormone-like substances was not a guarantee for a growth stimulation of different crops. Suitable host plants and specific combinations have to be determined in vegetation experiments. It is important, that the microorganisms survive in the rhizosphere. The survival rate can be favoured by suitable

Table 6. Survival rate of *Rhizobium* strains (E163, mel8) and *Pseudomonas fluorescence* in the rhizosphere of pea and lucerne (1000 cfu per g root) depending on inoculum type (pot exp.; 150 ppm rifampicine resistant mutants)

	Bact. strain	Inoculum type[1]	Root length in cm 0–3	4–8	> 8
Pea	E163	culture suspension	52.0	10.0	0.3
		preparate	390.0	104.0	8.3
	PsIA12	culture suspension	2.0	0.3	0.3
		preparate	112.0	26.0	16.8
Lucerne	mel8	preparate	568.0	320.0	123.0
	PsIA12	preparate	32.0	72.0	20.0
	LSD at 5%		51		

[1] 10^6cfu per seed

inoculum types. The ability to produce antibiotics may improve the bacterial survival.

Positive inoculation effects may be the consequence of better nutrition (N_2, P), an increase of nutrient uptake and by protection against root pathogens.

Acknowledgements

This work was supported by the German Ministry of Research and Technology.

REFERENCES

Afanasewa, L.M., Dorosinskij, L.M., and Kozemjakov, A.P. 1983. Sel'skochoz. *Biol.-Moskva* **4**: 5–10.

Domey, S. 1987. Untersuchungen zur mikrobiellen Phosphatmobilisierung in der Rhizosphäre von Winterweizen, Winterroggen und Luzerne. Dissertation, Akademie der Landwirtschaftswissenschaften der DDR. 96 pp.

Drews, G. 1968. *Mikrobiologisches Praktikum*. Springer-Verlag, Berlin. 214 pp.

Giddens, J.E., Dunigan, E.P., Weaver, R.W. 1982. Legume inoculation in the Southeastern USA. *Southern Cooperative Series Bull.*, Spec. Vol. 283 pp.

Höflich, G. 1989. Einfluß einer Inokulation von *Rhizobium*-Bakterien auf das Wachstum von Getreide. *Zbl. Mikrobiol.* **144**: 73–79.

Höflich, G. 1992. Interrelationships between phytoeffective *Pseudomonas* bacteria and the growth of crops. *Zbl. Mikrobiol.* **147**: 182–191.

Höflich, G. and Glante, F. 1991. Inoculum production and inoculation of effective VA-Mycorrhizae fungi. *Zbl. Mikrobiol.* **146**: 247–252.

Höflich, G. and Hickisch, B. 1986. Isolierung und Effektivitätsprüfung von *Rhizobium*-Bakterien unter den Bedingungen der DDR. *Zbl. Mikrobiol.* **141**: 401–408.

Höflich, G. 1989. Erfahrungen und neue Ergebnisse zum Einsatz von *Rhizobium*-Präparaten zur Erhöhung Leguminosen. *Feldwirtschaft Berlin*, **30**: 354–355.

Höflich, G., Wolf, H.J., and Schiefer, C. 1987a. Effektivität von zu Luzerne inokulierten Biopräparaten aus selektierten *Rhizobium*-Stämmen auf Standorten der DDR. *Arch. Acker-, Pflanzenbau, Bodenkd., Berlin* **31**: 763–769.

Höflich, G., Wolf, H.J., and Rupprich, A. 1987b. Bereitstellung und Sterilisation von Torf als Trägersubstrat für *Rhizobium*-Präparate. *Zbl. Mikrobiol.* **142**: 581–586.

Lehfeldt, J., Groth, R., and Clausnitzer, J. 1986. Methode zur Bestimmung der Wurzellänge unter Verwendung des Bildauswertesystems "Densitron 3". *Arch. Acker-, Pflanzenbau, Bodenkd., Berlin* **30**: 91–94.

Li, De-Ming, and Alexander, M. 1990. Factors affecting co-inoculation with antibiotic-producing bacteria to enhance rhizobial colonization and nodulation. *Plant Soil* **129**: 195–201.

Patterson, A., Chet, I., and Kapulnik, Y. 1990. Effect of mycorrhizal inoculation on nodule initiation, activity and contribution to legume productivity. *Symbiosis* **8:** 9–20.

Rennie, R.J. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen fixing) bacteria from soils. *Can. J. Microbiol.* **27:** 8–14.

Ruppel, S. and Wache, H. 1990. Isolation und Selektion phytoeffektiver Bakterien. *Zbl. Mikrobiol.* **145:** 599–603.

Scholz, C. and Ruppel, S. 1992. Nitrogenase and phytohormone activities of *Pantoae agglomerans* in culture and its reflection in combination with wheat plants. *Zbl. Mikrobiol.* (in press).

Weihs, V. 1989. *Bacillus polimyxa* und andere Rhizosphärenbakterien von Getreide und Gräsern: Verbreitung, ökophysiologische Eigenschaften und Wirkungen auf die Entwicklung junger Weidelgraspflanzen. Dissertation, Braunschweig. 188 pp.

Wolf, H.J. and Höflich, G. 1986. Einsatz der Acetylenreduktionsmethode zur Ermittlung der Nitrogenaseaktivität bei Luzerne und Rotklee. *Zbl. Mikrobiol.* **141:** 251–257.

Yaholom, E. and Okon, Y. 1990. Possible mode of action of *Azospirillum brasilense* strain Cd on the root morphology and nodule formation in burr medic (*Medicago polymorpha*). *Can. J. Microbiol.* **36:** 10–14.