RESPONSES OF RED CLOVER TO INOCULATION WITH ENDOPHYTIC BACTERIA FROM ROOT NODULES

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ABSTRACT

The aim of this study was to identify endophytic bacteria isolated from the nodules of healthy red clover plants (*Trifolium pratense* L.) and to assess their effects, alone and in combination with *Rhizobium*, on the growth and development of red clover seedlings. Clover root nodules were host to 12 bacteria species other than rhizobia. In root bacterization experiments, root nodule bacteria often promoted *in vitro* growth and nodulation of red clover when applied in combination with *R. leguminosarum* BV *trifolii*. Instances of growth depression were fewer in mixed than in individual bacterial applications. Single isolate applications of *Rhizobium* species to roots always led to clover growth depression, but mixtures of *R. leguminosarum* BV *trifolii* and *R. leguminosarum* BV *phaseoli* resulted in growth promotion. The latter is considered further evidence of a beneficial allelopathic side-effect of strain competition for the same ecological niche.

KEY WORDS

Root nodule, endophytic bacteria, PGPR, clover, allelopathy

INTRODUCTION

Many studies have shown that simultaneous infection with rhizobia and rhizosphere bacteria can increase nodulation and growth in a wide variety of legumes (Bolton et al. 1990; Grimes and Mount 1984; Polonenko et al. 1987; Yahalom et al. 1988). However, the term 'rhizosphere' is restrictive, marginalizing bacterial populations into discrete ecological niches. Regardless of definition, a proportion of rhizobacteria are consistently found to colonize internal plant tissues (Old and Nicolson, 1978; McInroy and Kloepper 1995; Sturz, 1995) and the bacterial populations in these two juxtaposed ecological niches often overlap.

The aim of this study was to identify those endophytic bacteria isolated from the nodules of healthy red clover plants; and to assess their effects, alone and in combination with *Rhizobium*, on the growth and development of red clover seedlings.

MATERIALS AND METHOD

Fifteen undamaged, healthy root nodules of similar size and root location were sampled from the lateral roots of red clover plants (cv. AC Charlie) randomly chosen from a field at Harrington, Prince Edward Island, Canada. The clover plants had not been inoculated with rhizobia at planting and were in their second year of establishment.

Nodules were surface sterilized then suspended in a quarter-strength Ringer's solution and crushed individually in a mortar and pestle, previously sterilized in an autoclave. At this time nodule interiors were confirmed as being pink. The macerate was decanted into conical flasks, shaken on a wrist-action shaker for 45 min., a dilution series made and the diluent plated onto tryptic soy agar (TSA). Petri plates were incubated at 22 ^{o}C for 3-5 d at which time the number of colony forming units (c.f.u.) were counted. Ten replicates per dilution were made. To confirm that the sterilization process was successful, tissue was pressed onto, or rolled over TSA, and aliquots of water from the final rinse solutions were plated onto TSA and examined for contaminants.

Following incubation bacterial flora were taken at random from Petri plates. Differentiation of the bacterial genera was made after the protocol of Schaad (1988), Bergey's Manual of Determinative Bacteriology (Holt, 1994) and the Biolog MicroStation System - an automated bacterial identification system (RDG Laboratories, Hayward, California, USA).

To test what effect, if any, endophytic bacteria isolated from nodules had on plant growth, a root bacterization experiment was conducted. Clover plants, cv. AC Charlie, were grown from seed which had been surface sterilized. The seeds were germinated in magenta cubes in 'SGL medium' (Rupert and Collins, 1985) and grown at 22^{0}C under a 16 hr day until approximately 3-4 cm tall. Contaminated magenta vessels were discarded. Seedlings were removed from their respective growth medium, their roots washed in sterile distilled water and then dipped into one of the 15 endophytic bacteria isolates suspended in sterile distilled water, singly and in combination with *Rhizobium leguminosarum* BV *trifolii*. The concentration of bacterial suspensions was estimated using the plate dilution method and fell within a range of 5 - 6 ¥ 10^{7} c.f.u. per ml of diluent. There were a total of 30 treatments; 15 isolates applied singly, 14 applied in combination with *Rhizobium* and a sterile distilled water control. Following inoculation seedlings from each treatment were divided into two groups. Seedlings in group I were grown aseptically in magenta cubes containing 'modified CR2 medium' (after Phillips and Collins, 1979, 1982; Grosser and Collins, 1984); modified by substituting lactose for sucrose to discourage bacteria growth on the media surface. The magenta cubes were set out in the growth room, at 22 ^{o}C under a 16 hr day, in randomized complete blocks, with 20 replicates and 1 plant per treatment replicate. Experimental blocks were rotated about the growth room bench to minimize any environmental effects. After 6 weeks of growth, plant height (length of the longest leaf-bearing stem), root, shoot and total wet weight were measured. Clover seedlings in group II were immediately transplanted into individual 'Cone-tainers' (4 cm x 20 cm pots tapering to 1 cm; Ray Leach Cone-tainers, Stuewe and Sons Inc., Corvallis, Oregon, U.S.A.) of commercial potting compost. After 12 weeks growth, plants were removed from their pots, there root systems washed in running water and the number of nodules per clover root system estimated using a scale of 1-5, where 1 = 0 - 25; 2 = 26 - 50; 3 = 51 - 75; 4 = 76 - 100 and 5 = > 100 nodules respectively. The experiment was a randomized complete block design with eight replicates per isolate tested and one seedling per replicate. Pots were rotated every 1-2 d to remove any border effects.

All statistical analyses were performed using the Genstat statistical program (Genstat, 1993). Data was analyzed by analysis of variance and a protected Least Significant Difference test.

RESULTS

Several bacteria genera (7) and species (15) were recovered from the nodules of the red clover plants (Table 1). Nodule bacteria populations were estimated as 4.06 ¥ 10^{4} c.f.u. per g nodule fresh weight. Three species of rhizobia were identified: *R. leguminosarum* BV *phaseoli*, *Rhizobium loti* B and *Rhizobium leguminosarum* BV *trifolii*.

The bacteria significantly affected the growth of red clover, both alone and in combination with *Rhizobium leguminosarum* BV *trifolii* (Table 1). Increases in root weight were the most common beneficial effect; while root length was decreased in every case, compared to the controls. Individual applications of *Rhizobium* species always resulted in growth depression, but a mixture of *R. leguminosarum* BV *trifolii* and BV *phaseoli* promoted growth. *Bacillus megaterium* and *Curtobacterium luteum* consistently promoted growth when applied individually or in mixtures with *R. leguminosarum* BV *trifolii*. By contrast a mixture of *Pseudomonas corrugata* and *R. leguminosarum* BV *trifolii* caused growth depression effects despite the former promoting growth when applied individually (Table 1). Instances of growth promotion were more frequent in co-inoculation treatments with *R. leguminosarum* BV *trifolii* (20) than individually (10) (Table 1). Similarly, instances of growth depression were fewer in mixed (57) than in single bacterial applications (74).

Nodulation was promoted when *R. leguminosarum* BV *trifolii* was co-inoculated with either *Bacillus insolitus*, *B. brevis or Agrobacterium rhizogenes* (Table 1). Root inoculation with *Rhizobium* species, either alone or in combination with each other, did not result in significantly greater levels of nodulation from the controls.

DISCUSSION

Current Rhizobium taxonomic nomenclature is based upon host plant nodulation responses and suggests that the subsequent symbiosis is species-specific (van Rhijn and Vanderleyden, 1995). However, it is clear that the degree of host specificity among the rhizobia is extremely variable. (Eardly et

al., 1985; Lewin et al., 1987). Root nodules in the present study were host to species and biovars of bacteria other than *R. leguminosarum* BV *trifolii*; namely *R. loti* and *R. leguminosarum* BV *phaseoli*. Thus, the presence of multiple rhizobia colonists within legumes in general and clover in particular, may be more common than previously believed.

Legumes are already known to benefit from simultaneous infection with *Rhizobium* spp. and VAM (Barea and Azcon-Anguilar 1983; Ikram et al. 1993). In cowpea, *Vigna unguiculata* (L.) Walp., the presence of the VAM *Glomus pallidum* Hall enabled introduced strains of *Bradyrhizobium* spp. to become more competitive than the native rhizobia (Thiagarajan and Ahmad 1992). In our study, nodulation was enhanced by a combination of *R. leguminosarum* BV *trifolii* with certain species of *Agrobacterium* or *Bacillus*. When introducing a legume species to a new geographical area, nodulation is often lacking even though inoculation with rhizobia is carried out at seeding. Perhaps the absence of quantities of a suitable partner species is the limiting factor in such situations.

Various strains of *Azospirillum*, *Pseudomonas* and *Bacillus* species, have been shown to enhance legume growth under greenhouse and field conditions (Bolton et al. 1990; Nishijima et al. 1988; Polonenko et al. 1987; Raj et al. 1981; Yahalom et al. 1988). Our study identified bacteria from the genera *Agrobacterium*, *Bacillus*, *Curtobacterium* and *Pseudomonas*, as present in red clover nodules and capable of promoting red clover growth or nodulation. The total number of positive growth effects were greater in bacterial co-inoculations involving *R. leguminosarum* BV *trifolii* than without. This was especially so in mixtures *of R. leguminosarum* BV *trifolii* and *R. leguminosarum* BV *phaseoli*. Individually, these species depressed growth, but in combination growth was promoted. Such findings provide further evidence that a certain proportion of plant promotion or depression effects are in fact the allelopathic side-effect of the competition between endophytes for the same ecological niche (Sturz and Christie 1995).

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Table 1 Growth responses of red clover (*Trifolium pratense* L.) cv. AC Charlie to single strains of endophytic bacteria isolated from clover root nodules and co-inoculation with *Rhizobium leguminosarum* BV *trifolii*.

Bacteria species		Length (mm)			Weight (mg)			Nodules[¶]
		Total	Shoot	Root	Total	Shoot	Root	Rating
Agrobacterium rhizogenes A		12.0 a[+]	6.8	5.1 a	0.087 a	0.064 a	0.023 a	0.000 a
Bacillus insolitus		11.9 a	6.5 a	5.4 a	0.089 a	0.065 a	0.023 a	0.143 a
Bacillus megaterium		8.5 a	4.4 a	4.2 a	0.090 a	0.035 a	0.055 *[++]	0.000 a
Bacillus brevis		10.0 a	4.3 a	5.7 a	0.080 a	0.034 a	0.045 *	0.429 a
Bacillus subtilis		9.3 a	5.0 a	4.3 a	0.052 a	0.019 a	0.032	0.000 a
Bordetella avium		13.0 a	7.9 *	5.1 a	0.123	0.098 *	0.025 a	0.000 a
Curtobacterium citreum		14.9 a	7.2	7.6 a	0.100 a	0.081	0.019 a	0.000 a
Curtobacterium luteum		18.8	10.5 *	8.3 a	0.186 *	0.115 *	0.070 *	0.000 a
Curtobacterium flaccumfaciens								
PV oortii		15.6 a	7.6	8.0 a	0.120	0.089	0.031	0.001 a
Phyllobacterium myrsinacearum		14.6 a	7.3	7.3 a	0.089 a	0.073 a	0.015 a	0.001 a
Pseudomonas corrugata		14.6 a	7.8 *	6.8 a	0.124	0.064 a	0.060 *	0.857
Rhizobium loti B		14.1 a	7.1	7.0 a	0.106	0.082	0.023 a	0.667
Rhizobium leguminosarum								
BV *phaseoli*		10.1 a	5.7 a	4.4 a	0.073 a	0.050 a	0.022 a	0.400 a
Rhizobium leguminosarum								
BV *trifolii*		11.7 a	7.1	4.5 a	0.091 a	0.069 a	0.021 a	1.000
Agrobacterium rhizogenes A	plus§	10.8 a	6.1 a	4.7 a	0.080 a	0.064 a	0.016 a	4.000 *
Bacillus insolitus	plus	13.9 a	6.6 a	7.2 a	0.121	0.091	0.029	5.000 *
Bacillus megaterium	plus	13.6 a	7.8 *	5.8 a	0.116	0.058 a	0.057 *	1.333
Bacillus brevis	plus	9.4 a	5.8 a	3.5 a	0.089 a	0.054 a	0.035	2.140 *
Bacillus subtilis	plus	11.8 a	6.2 a	5.5 a	0.069 a	0.030 a	0.038	0.714
Bordetella avium	plus	13.3 a	7.5	5.7 a	0.194 *	0.080	0.114 *	0.429 a
Curtobacterium citreum	plus	12.3 a	7.4	4.9 a	0.093 a	0.046 a	0.046 *	0.000 a
Curtobacterium luteum	plus	18.8	9.9 *	8.9 a	0.188 *	0.135 *	0.052 *	0.286 a
Curtobacterium flaccumfaciens								
BV *oortii*plus		14.2 a	7.0	7.1 a	0.123	0.088	0.035	0.500a
Phyllobacterium								
myrsinacearum	plus	17.0 a	10.5 *	6.4 a	0.199 *	0.124 *	0.074 *	0.286 a
Pseudomonas corrugata	plus	14.0 a	7.3	6.6 a	0.115	0.079	0.035	0.000 a
Pseudomonas fragi	plus	9.2 a	5.3 a	3.8 a	0.071 a	0.049 a	0.022 a	0.000 a
Rhizobium loti B	plus	12.4 a	6.3 a	6.1 a	0.100 a	0.079	0.021 a	0.000 a
Rhizobium leguminosarum								
BV *phaseoli*	plus	15.6 a	8.0 *	7.5 a	0.139 *	0.095 *	0.043 *	0.000 a
Rhizobium leguminosarum								
BV *trifolii*	plus	10.8 a	6.6	4.2 a	0.084 a	0.066 a	0.017 a	0.714
Control (sterile distilled water)		18.5	7.2	11.3	0.118	0.085	0.033	1.298
Grand Mean		13.3	7.0	6.2	0.110	0.073	0.037	0.652
LSD[+++]		1.2	0.5	1.0	0.014	0.009	0.007	0.699

[¶] number of nodules per clover root system estimated using a scale of 1-5, where 1 = 0 - 25 nodules; 2 = 26 - 50 nodules; 3 = 51 - 75; 4 = 76 - 100 and 5 = > 100 nodules.

§ plus indicates a mixed bacterial suspension with *Rhizobium leguminosarum* BV *trifolii*

[+] number in columns followed by the letter 'a' are significantly lower than the control at the p= 0.05% level

[++] number in columns followed by an asterisk are significantly greater than the control at the p= 0.05% level

Table 1
Analysis of soil in June 1982, prior to the application of lime.

Test	Mean of six replicates	Range Min	Max
0-10 cm samples			
pH (1 soil : 5 water)	5.3	5.2	5.5
Electrical conductivity (umho/cm)	159	123	186
Skene Potassium (ppm)	72	60	92
Olsen Phosphorus (ppm)	8.1	6.9	9.0
Organic carbon (%)	2.9	2.3	3.7
Nitrogen (%)	0.228	0.193	0.303
CPC Sulphur (ppm)	15.0	11.4	19.4
Exchangeable cations (meq/100 gm)			
Ca++	2.0	1.6	2.5
Mg++	0.8	0.7	0.9
Na+	0.3	0.3	0.3
K+	0.3	0.2	0.3
Al+++	20	15	30
pH (1 soil : 5 water)			
0-15 cm5.3	5.1	5.5	
15-30 cm	5.6	5.5	5.7
30-45 cm	6.3	6.2	6.4
45-60 cm	6.6	6.4	6.7
60-90 cm	6.7	6.6	6.8

Table 2
Effect of 1982 lime incorporation on subsequent soil pH and exchangeable aluminium.

Lime (t/ha)	pH (water) August 1983	pH (water) January 1989	pH (CaCl2) January 1989	pH (CaCl2) August 1991	Exchangeable Al (ppm) August 1983	Exchangeable Al (log ppm) January 1989
0	5.1	5.0	4.6	4.5	27	2.13
0.5	5.3	5.1	4.7		16	1.95
1	5.3	5.2	4.8		14	1.69
2	5.5	5.3	4.8	4.8	7	1.62
5	5.9	5.6	5.2	5.1	3	1.39
10	6.3	6.3	6.0	5.6	3	1.39
LSD (P=0.05)	0.19	0.17	0.17	0.20	10.4	0.485

Table 3
Effect of lime on the density of established seedlings of sown species.

Lime (t/ha)	Plant density (no./m²) Lucerne 1/12/82	Lucerne 12/12/84	Lucerne 12/3/86	Lucerne 9/2/87	Sub. clover 6/12/82	Sub. clover 5/4/84	Phalaris 22/12/88
0	210	83	32	2	59	365	114
0.5	217	106	38	3	58	389	109
1	203	116	44	2	57	411	114
2	212	124	46	6	63	441	104
5	220	138	50	8	64	364	113
10	232	151	55	8	59	352	130
LSD (P=0.05)	22.3	30.1	10.2	3.7	10.7	10.5	24.8

Table 4
Effect of 1982 lime incorporation on nutrient concentrations in pasture species (dry weight basis).

Lime (t/ha)	N %	P %	K %	Ca %	Mg %	S %	Na %	Cu ppm	Zn ppm	Mn ppm	Mo ppm	B ppm	Fe ppm
						Lucerne 21/3/86							
0	3.1	0.16	1.5	1.9	0.48	0.43	0.21	5	28	40	0.2	57	
0.5	3.0	0.15	1.6	1.8	0.44	0.42	0.18	6	26	38	0.3	48	
1	3.1	0.16	1.6	1.8	0.46	0.42	0.20	6	27	39	0.4	57	
2	3.2	0.15	1.6	1.7	0.40	0.38	0.16	7	23	31	0.3	48	
5	3.3	0.16	1.5	1.8	0.44	0.39	0.21	6	24	30	0.4	44	
10	3.3	0.16	1.4	1.7	0.42	0.37	0.18	5	22	27	0.6	50	
LSD (*P*=0.05)	0.3	0.012	0.23	0.13	0.054	0.029	0.047	1.0	3.9	6.8	0.10	8.4	
						Subterranean clover 7/9/93							
0		0.26	2.20	1.39	0.29	0.19	0.58	10	31	86			169
0.5		0.25	2.13	1.39	0.28	0.18	0.62	9	29	76			157
1		0.26	1.97	1.44	0.29	0.19	0.72	9	30	77			164
2		0.25	1.94	1.45	0.29	0.18	0.69	8	28	70			157
5		0.26	1.73	1.54	0.29	0.18	0.78	8	27	58			147
10		0.26	1.73	1.59	0.29	0.18	0.76	7	24	47			150
						Phalaris 7/9/93							
0		0.28	2.27	0.26	0.26	0.30	0.72	11	21	143			117
0.5		0.28	2.35	0.22	0.22	0.30	0.66	10	22	107			108
1		0.30	2.47	0.24	0.27	0.34	0.68	10	25	134			119
2		0.30	2.47	0.26	0.27	0.34	0.77	11	24	131			110
5		0.34	2.55	0.28	0.28	0.36	0.76	9	24	101			119
10		0.35	2.46	0.32	0.29	0.38	0.82	10	24	72			132