Symbiosis, 23 (1997) 35–42
Balaban, Philadelphia/Rehovot

Nitrogen Fixation, Nodulation and Yield of Clover Plants Co-Inoculated with Root-Colonizing Bacteria

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Received October 10, 1996; Accepted February 8, 1997

Abstract

The potential of three putative plant growth-promoting rhizobacterial strains in enhancing nitrogen fixation, nodulation and growth of subclover plants (*Trifolium subterraneum* cv. Clare) inoculated with one selected indigenous *Rhizobium* strain was assessed under greenhouse conditions. *Azospirillum brasilense* Sp7 and a local rhizobacteria increased nodulation, nitrogenase activity and clover yield. Stimulatory effects were affected by cell ratio in mixed inocula. A unfavourably effect was observed with one of the local rhizobacteria.

Keywords: Nitrogenase activity, nodulation, clover, *Rhizobium*, PGPR

1. Introduction

Increased yield of several important crops have been observed following inoculation with soil bacteria able to colonize plant roots. These bacteria have been termed plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980). It has been suggested that production of plant hormones, enhancement of plant nutrient uptake or suppression of pathogenic or deleterious organisms may be involved with mechanisms by which PGPR increase plant growth (Cohen et

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0334-5114/97/$05.50

al., 1980; Zimmer and Bothe, 1988). Various studies indicate that these rhizosphere micro-organisms can also have a positive effect on *Rhizobium*-legumes symbiosis. Most of the research on the effect of combined inoculation has indicated a trend of growth promotion in leguminous plants, an increase in nodule number as well as earlier nodulation (Li and Alexander, 1988; Gallo and Fabbri, 1990; Höflich et al., 1992).

In a study on symbiotic effectiveness and diversity of rhizobial strains in Portugal, one strain was selected for its effectiveness on *Trifolium subterraneum* cv. Clare (Ferreira and Marques, 1992), the cultivar most widely sown. In the present communication we studied the synergistic effects of the application of Azospirillum and two local rhizobacteria on the *Rhizobium*-clover symbiosis.

2. Material and Methods

Bacterial strains and growth conditions

Strains used in this study included *Azospirillum brasilense* Sp7, two local rhizobacteria (ISA 019 and ISA 030) and *Rhizobium leguminosarum* biovar *trifolii* 123 Ts2. *Azospirillum brasilense* Sp7 was kindly supplied by Dr. J. Döbereiner. Strains ISA 019 and ISA 030 were isolated from maize roots growing in Portuguese soils and exhibited high nitrogenase activity in pure culture. Strain 123 Ts2 is a highly effective and competitive *Rhizobium* strain, largely used as an inoculant in Portugal.

The associative N_2-fixers (Sp7, ISA 019 and ISA 030) were grown prior to inoculation in malate liquid medium (Döbereiner and Day, 1976) supplemented with 0.05% NH_4Cl for 20 h at 30°C. *Rhizobium leguminosarum* 123Ts2 was grown for two days in YMB (yeast mannitol broth) at 25°C. Cells were harvested at the late logaritmic growth phase by centrifugation (1,000 xg) and were washed twice in potassium phosphate buffered saline, pH 7.

Cell densities were related to viable cell numbers, measured as colony forming units per ml (CFU/ml) by standard dilution plate counting procedures on appropriate solid media. Mixed cultures were prepared in K-phosphate buffer (pH 7.0) by diluting concentrated cell suspensions to the appropriate concentration (CFU/ml) by spectrophotometer measurements at 540 nm.

Seed inoculation and plant growth

Seeds of *Trifolium subterraneum* cv. Clare were rinsed with ethanol, surface sterilized in acidified mercuric chloride for five minutes, washed with six changes of sterile water and placed to germinate in sterile petri dishes

containing water agar. Two-day old seedlings, with straight radicles about one cm long, were aseptically transferred to large cotton wool plugged tubes (300 mm by 32 mm) containing 60 ml of Fahraeus agar medium (Fahraeus, 1957) and were inoculated with one ml of single (10^8 CFU/ml of *Rhizobium* 123Ts2) and mixed cultures of bacteria prepared as described. For mixed inoculation, the cell suspensions of each organism were combined in order to give *Rhizobium*: *Azospirillum* (or the local strain, ISA 019) proportions of 10^8:10^8, 10^8:10^7 and 10^8:10^6 CFU/ml. In each treatment 20 plants were grown for seven weeks in a controlled environment growth cabinet (18°C/12 h of light period, 15°C/12 hours of dark period) with a mean photosynthetic photon flux density of 250 $\mu mol/m^2/s$.

Plants were periodically observed for nodule formation and after the seven weeks they were removed from the tubes and assayed for nodule number and dry and weight (root and shoot).

C_2H_2 reduction activity

For this experiment two-day old seedlings were transferred to 25 ml serum vials, filled with quartz sand and five ml of Fahraeus medium. These vials were capped with plastic bags after the seedlings were inoculated with one ml of single (10^8 CFU/ml *Rhizobium* 123Ts2) or mixed inocula (10^8:10^8 CFU/ml *Rhizobium*:rhizobacteria).

The acetylene reduction assay was performed periodically, after the inoculation (9th day) until the date of harvest (27th day). Plants shoots were removed and the vials were closed with serum stoppers. The gas phase in the vials was replaced with acetylene (10% v/v) and the vials were incubated for one hour at 28°C. From each vial a 250 µl sample was removed and analysed for ethylene production, using a gas chromatograph (PU 4500) fitted with a hydrogen flame ionization detector and a Porapak R column of 80–100 mesh.

Statistical analysis

Statistical assessment was carried out using analysis of variance (Anova) and the mean separation procedure of LSD. Logaritmic transformation was performed whenever necessary.

3. Results and Discussion

The effect of mixed inoculation on acetylene reduction activity (ARA) was measured periodically beginning on the 9th day after the inoculation. The

acetylene reduction bioassay was used only to assess N-fixation along the different treatments. Although there are conflicting views on the reliability of the acetylene reduction technique, this method was used to provide relative estimates in nitrogenase activity, and to complement the nodulation and plant biomass data.

Clover plants inoculated with mixed inocula (*Azospirillum* Sp7 or the local isolate ISA 019 and *Rhizobium*) showed an increase in ARA when compared with *Rhizobium* alone (control plants) (Table 1). This effect was already significant at 9th day. In contrast strain ISA 030 did not stimulate but even suppressed nitrogenase activity. Twelve days after inoculation this effect was pronounced and the difference is stressed at the end of the experiment. At harvest date (27 days after inoculation) we noticed that strain ISA 030 reduced or prevented nodule formation and had an unfavourable effect on root development. This adverse effect of ISA 030 on nitrogenase specific activity may be the result of one or several compound(s) produced by this bacterium which affected root elongation and, with this, nodule formation. Negative effects have also been observed by other workers (Raverkar and Konde, 1988; Freitas et al., 1993). The significant effect of co-inoculation of clover plants with strains Sp7 and ISA 019 on ARA (earlier and higher), may probably be due to an early appearance of nodules.

The positive effect of co-inoculation with these two associative N_2-fixers (Sp7 and ISA 019) was confirmed in a subsequent experiment conducted in agar medium, in which experiment we could evaluate both nodule appearance or plant development under different mixture concentrations. Strain ISA 030 was not included due to negative effects observed in ARA assay.

Table 1. Effects of mixed inoculation on acetylene reduction activity (ARA) of clover plants*

Treatment	Days after inoculation				
	9	12	15	19	27
123TS2 (Control)	0.39 a	0.88 b	1.12 b	1.18 b	1.16 b
123TS2 + Sp7	0.88 c	1.38 c	1.90 c	1.84 c	1.69 c
123TS2 + 019	0.62 b	1.24 c	1.88 c	1.78 c	1.65 c
123TS2 + 030	0.39 a	0.42 a	0.47 a	0.53 a	0.39 a

Values followed by different letters in a column differ significantly at $P<0.05$. *Data are expressed as $\log_{10}$ ARA (nmoles C_2H_4/hour/plant).

Table 2. Effect of mixed inoculation on clover nodule formation*

Treatment	Days after inoculation											
	7	10	14	19	24	27	32	35	40	44	48	53
123TS2 (Control)	0.49a (85)**	0.86a	1.08a	1.13a	1.16a	1.16a	1.17a	1.18a	1.19a	1.20a	1.20a	1.21a
123TS2 : SP7 (10^8 : 10^8)	0.66b (95)	1.02b	1.21b	1.31b	1.33b	1.36b	1.36b	1.38b	1.40b	1.42b	1.44c	1.45c
123TS2 : SP7 (10^8 : 10^7)	0.94 c (100)	1.17c	1.26b	1.30b	1.32b	1.35b	1.36b	1.37b	1.37b	1.38b	1.39b	1.39b
123TS2 : SP7 (10^8 : 10^6)	0.92c (100)	1.15c	1.24b	1.28b	1.30b	1.32b	1.33b	1.35b	1.37b	1.37b	1.38bc	1.38b
123TS2 : 019 (10^8 : 10^8)	0.59ab (90)	0.97ab	1.11a	1.14a	1.17a	1.18a	1.19a	1.22ab	1.22ab	1.22ab	1.23ab	1.23ab
123TS2 : 019 (10^8 : 10^7)	1.69b (100)	0.98b	1.11a	1.14a	1.17a	1.19a	1.21a	1.23b	1.24b	1.25b	1.26b	1.27b
123TS2 : 019 (10^8 : 10^6)	0.64ab (100)	0.96ab	1.08a	1.10a	1.13a	1.13a	1.15a	1.16a	1.17a	1.18a	1.19a	1.20a

Values followed by different letters in a column differ significantly at $P<0.05$. *Values are expressed as $\log_{10}(x+1)$. **Percentage of nodulated plants.

Mixed inocula with *Rhizobium* and Sp7 or ISA 019, at any ratio (10^8:10^7; 10^8:10^7 or 10^8:10^6), affected clover nodulation by anticipating nodule formation and increasing nodule number (Table 2). The increased nodule formation was more marked with *Azospirillum brasilense* Sp7. This phenomenon of increased nodulation by strain Sp7 was not proportionately reflected on root and shoot dry weights (Table 3). Results given in Table 3 show a significant increase in shoot dry weight of plants inoculated with mixed inocula, either Sp7 or 019. However this stimulatory effect was affected by inoculum concentration. At a ratio 100:1 (*Rhizobium* : Sp7 or *Rhizobium* : 019) the promoting effect on shoot dry weight was increased, showing that the appropriate concentration of mixed inocula could significantly influence this symbiotic interaction. In the other hand, root dry weight was not affected by mixed inoculation (Table 3). At harvest date a beneficial effect on root morphology was observed, namely an increase in lateral root number.

Table 3. Effect of mixed inoculation on yield of clover plants 53 days after inoculation

Treatment	Root dry weight (mg)	Shoot dry weight (mg)
123TS2 (Control)	21.4 bc	87.1 a
123TS2 : SP7 (10^8 : 10^8)	20.9 ab	105.3 b
123TS2 : SP7 (10^8 : 10^7)	18.9 a	109.9 bc
123TS2 : SP7 (10^8 : 10^6)	23.2 c	113.2 bc
123TS2 : 019 (10^8 : 10^8)	22.5 bc	121.2 cd
123TS2 : 019 (10^8 : 10^7)	20.4 ab	114.7 bc
123TS2 : 019 (10^8 : 10^6)	21.4 bc	132.4 d

Values followed by different letters in a column differ significantly at $P<0.05$.

The results of this study indicate that root colonizing bacteria can interfere with several different important determinants of clover growth, leading to an increased growth response. Beneficial effects were observed on nodulation, plant growth and ARA. Increased ARA may be originated by an improved energy supply to the nodule as a result of PGPR inoculation (Itzigsohn et al., 1993). Some authors attributed the effect of enhanced nodulation to the production of nod products by these bacteria (Elmerich et al., 1991) or to an heightened secretion of root exudates containing flavonoids (Itzigsohn et al.,

1993), which in turn increase the level of nod gene inducer signals, as some studies suggested (Burdman et al., 1996).

The stimulatory effects of mixed clover inoculation, dependent on cell concentration, have been confirmed by other workers (Kapulnik et al., 1985; Plazinski and Rolfe, 1985, 1985a), who found that *Azospirillum* cell concentrations containing 10^5–10^7 CFU/ml increased root development, mineral uptake and nodulation in legumes. However, this effect of inoculum concentration was not observed under nonsterile conditions (Itzigsohn et al., 1993). Probably under gnotobiotic conditions, the inoculum concentration applied is critical where there is no competition from other bacteria.

These observations suggest the possibility of screening and selecting soil micro-organisms for enhancing the effectivity of a single inoculation under our moderate climatic conditions. Further studies are necessary in order to incorporate local PGPR strains into rhizobial inoculant formulations.

Acknowledgements

We thank Professor Artur V. Garcia, Technical University of Lisbon for constructive critical comments on the manuscript.

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