Optimizing legume production using sulfur oxidizing bacteria and *Rhizobium* consortia

Laila Yesmin[1], Ivan Oresnik[2] and Manas R. Banerjee[1]

[1]Research & Development Division, Brett-Young Seeds Limited, Box 99 St. Norbert Postal Station, Winnipeg, Manitoba, R3V
1L5, Canada; www.byseeds.com Email lailay@byseeds.com; manasb@byseeds.com
[2]Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, R3T 2N2, Canada; www.umanitoba.ca
Email oresniki@ms.umanitoba.ca

Abstract

Within the leguminosae family, soybean (*Glycine max* L.) and peas (*Pisum sativum*) are the two most predominant legume crops in Canada and the United States. The use of *Rhizobium* inoculant for better crop production is a common practice in the region. The use of an inoculant allows the legume plants to form root nodules within which atmospheric nitrogen is fixed and supplied to the plant. In the present study we are investigating a synergistic response that occurs when a sulfur-oxidizing plant growth promoting rhizobacteria (PGPR) *Delftia acidovorans* RAY209 is added to some *Rhizobium* inoculants. Preliminary experiments using a *Bradyrhizobium japonicum*/*D. acidovorans* consortium (SJ1) and *Rhizobium leguminosarum*/*D. acidovorans* consortia (SL1 and SL2) have yielded promising results on soybean and peas, respectively. These results suggest that if these legumes are inoculated with consortia SJ1, SL1 or SL2, the plants show enhanced seed emergence, increased biomass, and increased nodule numbers. By utilizing an inherent antibiotic resistant phenotype of RAY209 we have been able to determine that RAY209 strain can colonize peas roots and also capable of causing nodule-like outgrowths on these roots in some cases. It is hoped that by gaining an understanding of this association a consortia based inoculant can be developed.

Media summary

Potential development of inoculant consisting of a consortium of sulfur oxidizing bacteria and nitrogen fixing bacteria to enhance soybean and peas production in North America.

Key Words

Soybean, Peas, PGPR, Consortia, Nitrogen fixation.

Introduction

Plant growth promoting rhizobacteria (PGPR) can influence growth in a diverse range of host plants. A number of direct and indirect mechanisms have been identified those are responsible for plant growth promotion (Kloepper 1997). Nitrogen (N) is an essential mineral nutrient and is one of the limiting factors for plant growth and biomass production. Atmospheric N fixation by *Rhizobium* is one of the direct mechanisms where the bacteria that reside in the soil interact with the plant and cause the formation of a nodule structure by inducing localized proliferation of the plant root. Within this structure the *Rhizobium* reduce N and supply it to the plant. Due to this benefit of atmospheric N fixation, rhizobial inoculants for legumes have been used worldwide.

Besides N fixation, there are many other mechanisms that can be utilized for plant growth promotion. For example, studies with the sulfur (S)-oxidizing PGPR *Delftia acidovorans* RAY209 has been shown to be beneficial in seed emergence, root formation, acquisition of macro and micronutrients, and the overall yield of canola (*Brassica napus* L cv) crop (Yesmin and Banerjee 2001; Banerjee and Yesmin 2002). This PGPR has also been found to be compatible with certain *Rhizobium spp*. (Yesmin and Banerjee, unpublished). Since this PGPR is capable of co-existing with *Rhizobium*, it was of interest to investigate whether a synergistic effect would occur if both *Rhizobium* and *D. acidovorans* RAY209 were used as an

inoculant. To our knowledge these types of co-inoculation experiments have not been previously attempted. Hence, our approach is to study the association of both *Rhizobium* and S-oxidizing PGPR strain RAY209 to examine if they benefit the legume plants.

Methods

Bacterial consortia

Rhizobacterial consortia used in these experiments were:

- For soybean: *Bradyrhizobium japonicum* CT and the S-oxidizing *Delftia acidovorans* RAY209 (SJ1).
- For peas: Either *Rhizobium leguminosarum* 248 and *D. acidovorans* RAY209 (SL1) or *Rhizobium leguminosarum* 3841 and *D. acidovorans* RAY209 (SL2).

Seed inoculation

The consortia bacterial cultures were initially grown in tryptone and yeast extract broth (TY) (Fisher Biotech, New Jersey, USA) at 28^{O}C to 10^{9} colony forming units (cfu)/mL. Appropriate consortia cultures were then aseptically injected into packets containing sterilized peat. Inoculated peat packets were then incubated at 28^{O}C for 7 days to attain a population of 10^{8} cfu/g. The packets were then stored in a cool dry place (<22^{O}C) away from direct sunlight until use for inoculation purpose. For quality assurance, each packet was tested for bacterial quantity and survivability before use. The seed inoculation was done by moisten the seeds with sprinkle of water, and then applying the inoculated peat powder on the seeds, and stirred the seeds for uniform coating. The ratio of peat inoculant : seed was 4.5 g : 1 kg.

Growth room study

A total of 48 pots (10.5 cm width and 12 cm height) were prepared for the growth room study. Sterilized soilless medium (50% white silica sand + 50% vermiculite) was used as a potting mixture. Two different legumes, soybean (*Glycine max* L. cv Gentleman) and peas (*Pisum sativum* cv Hilight) were used. Prior to seeding, pots containing potting mixture were moisten with distilled water and seeded using an appropriate inoculant. After seedling emergence, plants were thinned to three plants per pot. The plants were watered daily with a N free nutrient solution (half strength Wood's nutrient solution with full strength Fe) (Walsh et al. 1987). For treatments with N (+N), 0.5 mM KNO_3 was added to the nutrient solution. Plants were grown at 22^{o}C with a 16 h photoperiod and an 8 h dark at 18^{o}C. The experiments were laid as completely randomized design (CRD) with five treatments for soybeans and seven treatments for peas, each treatment with 4 replications to give a total of 20 (5?4) pots for soybean and 28 (7?4) pots for peas. Pots were randomized twice each week.

The treatments used for soybean were:

- Control
- RAY209-N
- RAY209+N
- *Bradyrhizobium* CT
- *Bradyrhizobium* CT+RAY209

The treatments used for peas were:

- Control
- RAY209-N
- RAY209+N
- *Rhizobium* 248
- *Rhizobium* 3841
- *Rhizobium* 248+RAY209
- *Rhizobium* 3841+RAY209

All pots were harvested five weeks after seeding. Shoot length, nodule number, and shoot and root dry weights were measured.

Isolation of bacteria from nodules and nodule-like structures

Bacteria were isolated from nodule or nodule-like structures by first detaching the nodules from plants and washing with sterile distilled water. Structures were then surface sterilized using 1% sodium hypochlorite for one minute. These were then extensively washed with sterile water, crushed and plated onto tryptone-yeast extract (TY) agar. Bacteria were scored based on inherent antibiotic resistances as well as colony morphology and growth rate.

Root and potting mixture colonization assays

Overnight cultures of *D. acidovorans* were grown in TY broth, diluted in sterile water and used to inoculate pea seedlings. The total amount of bacteria inoculated into each pot was approximately 10^2 cfu/g potting mixture. To estimate the relative population, inoculated seedlings were harvested and the potting mixture was separated from the seedling. The relative population of *D. acidovorans* was determined by resuspending the potting mixture into 1 ml of sterile distilled water, vortexing for one minute, followed by incubation in a sonicating water bath. The bacteria were quantified by plating onto TY agar supplemented with appropriate antibiotics (Oresnik et al. 1998).

To enumerate the bacteria adhering to the pea root, a 2.5 cm root length was aseptically removed, and first washed to remove loosely associated bacteria. The root was then resuspended in 1 ml sterile distilled water, vortexed for one minute, and sonicated in a sonicating water bath for 10 minutes. The root was then crushed and quantified onto TY agar plate.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA), and pair wise comparisons were done using a student's *t*-test, using JMP IN 5.0 software package. All hypotheses were tested at the 95% confidence level.

Results

Table 1. Growth Room study of Soybean.

Treatment	Plant height (cm) /plant	Shoot wt. (g)/plant	Root wt. (g) /plant	Active nodule /plant
Control	31.22	0.24	0.05	0.00
RAY209-N	40.64	0.39	0.08	0.00
RAY209+N	43.15	0.38	0.09	0.00
Bradyrhizobium CT	44.16	0.39	0.08	14.75
Bradyrhizobium CT+RAY209	51.23	0.44	0.12	20.00

LSD (5%)	*6.71*	*0.11*	*0.03*	*2.88*

Table 2. Growth Room study of Peas.

Treatment	Plant height (cm) /plant	Shoot wt (g)/plant	Root wt. (g) /plant	Active nodule /plant
Control	34.00	0.25	0.10	0.00
RAY209-N	40.64	0.28	0.15	0.00
RAY209+N	40.32	0.32	0.15	0.00
Rhizobium 248	46.69	0.32	0.13	82.75
Rhizobium 3841	48.34	0.37	0.15	84.50
Rhizobium 248+RAY209	45.87	0.33	0.15	119.25
Rhizobium 3841+RAY209	49.74	0.41	0.16	111.50
LSD (5%)	8.75	0.07	0.03	16.65

Sulfur oxidizing PGPR strain RAY209 are able to proliferate in the rhizosphere and also able to colonize the roots of developing pea seedlings (Figure 1). Growth room studies showed a significant ($p \leq 0.05$) increase in biomass on both soybean and peas with all bacterial treatments when compared to the controls (Tables 1 and 2). Interestingly the PGPR/*Rhizobium* treatments (SJ1, SL1 and SL2) all show a significant ($p \leq 0.05$) increase in active nodule number when compared to plants inoculated with only rhizobia (Tables 1 and 2). Nodules however were spread throughout the root system and were smaller then those only inoculated with rhizobia. In the case of the pea root system, those inoculated with only RAY209 also showed small nodule-like outgrowths. These outgrowths were white to light brown in color. The results of this investigation have shown the feasibility of potential use of S-oxidizing PGPR and rhizobia association as legume inoculant for the benefit of soybean and peas production.

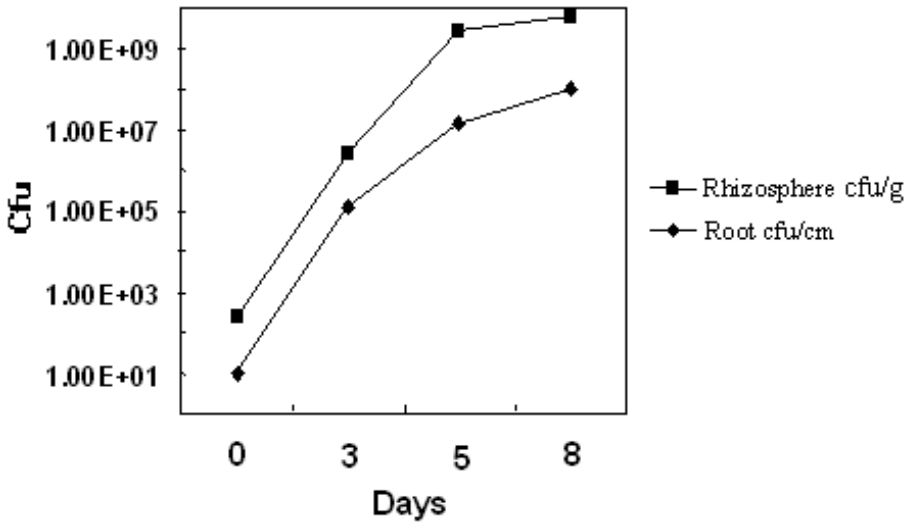

Figure 1. Colonization of pea root with RAY209.

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