

Plant Growth-Promoting Rhizobacteria on Canola (Rapeseed)

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ABSTRACT

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During 1983 and 1984, more than 4,000 bacterial strains were collected in Canada from root zones of plants in diverse habitats. Strains were screened individually for Gram-stain reaction, growth at 4–14 C, metabolism of canola (rapeseed, *Brassica campestris* L. and *B. napus* L.) seed exudates, chemotaxis toward asparagine, and root colonization capacity. A total of 887 strains were tested in greenhouse assays for plant growth-promoting activity on canola. A marked increase in leaf area compared with the controls occurred with 222 strains in initial tests, and 35 strains enhanced growth in at least two of three repeating tests. Strains of plant growth-promoting rhizobacteria (PGPR) included *Pseudomonas putida*, *P. putida* biovar B, *P. fluorescens*, *Arthrobacter citreus*, and *Serratia liquefaciens*. Maximum yield promotion associated with PGPR in field trials in both 1985 and 1986 occurred when seeds were treated with bacterial suspensions; certain types of potential cell carriers produced some negative effects on plants. Plants treated with 13 of 28 strains of PGPR yielded up to 57% greater than the controls in individual trials with cell suspension inoculants in 1985. Three strains increased yields over a 2-yr period by 6 and 13% compared with the controls. Select PGPR strains also increased seedling emergence and vigor under field conditions.

The use of bacteria from the root zone to enhance plant growth has received renewed attention with the recent description and ecological characterization of plant growth-promoting rhizobacteria (PGPR) (7). Specific strains of PGPR have been associated with plant growth-promoting activity, which resulted in greater yields in field trials with potato (1,3,8,10) and sugar beet (15). When used as seed inoculants or soil amendments, PGPR metabolized seed exudates (12), which resulted in effective spermosphere colonization (5) followed by colonization of the developing root system. Root colonization by some PGPR strains displaced native root microflora (9,14) via production of siderophores by PGPR (4) and thereby enhanced plant growth.

The long-term goal of our work is to develop bacterial inoculants based on free-living rhizosphere microorganisms for agriculture. This group previously reported that certain rhizosphere bacteria

that actively metabolize seed exudates at cool temperatures can enhance seedling emergence in field soils (6). Therefore, we applied the same cool-temperature strategy for selection of beneficial bacteria in this report. We report the results of the isolation and screening of PGPR, including results from greenhouse and field trials on canola (rapeseed) (*Brassica campestris* L. and *B. napus* L.).

MATERIALS AND METHODS

Media and strain identification. Nutrient agar (NA), tryptic soy broth (TSB), and *Pseudomonas* agar F (PAF) were obtained from Difco Labs (Detroit, MI). Seed exudate broth was prepared by mixing 30% of canola seed exudate solution (12) with 70% sterile distilled water. Exudate agar consisted of 20% canola seed exudate solution (12) with 2% purified agar (Difco) that was previously washed three times with distilled water and dried. Asparagine soft agar (ASA) containing 1 g of L-

asparagine, 2 g of Bacto (Difco), and 1,000 ml of distilled water.

Strains that showed continued promise as PGPR were identified to genus (Table 1) by the following tests for confirmed gram-negative strains: reaction profiles on API 20E test strips (Analytapp Products, Ayerst Labs, Inc., Plainview, NY), growth on MacConkey medium, type of metabolism in OF glucose medium (OF test), production of DNase, fluorescent pigment production, gelatin hydrolysis, nitrate reduction, starch hydrolysis, oxidase reaction, and lipase production (Tween 80 hydrolysis). Strains confirmed as gram-positive were identified by the following tests: growth on MacConkey medium, type of metabolism in OF glucose medium, catalase test, gelatin hydrolysis, Voges-Proskauer reaction, indole production, citrate utilization, motility, urease production, endospore formation, and acid production from glucose, saccharose, and/or mannitol. All tests except the API 20E strips used methods and media recommended by the American Society for Microbiology (2).

Strain isolation. Soil samples were collected from 27 sites in eastern Canada in 1983. Five-gram samples were mixed with 100 ml of 30% canola seed exudate broth. After incubation for 10 days at 4 C and 50 rpm, the samples were diluted and plated onto NA and PAF, and plates were incubated for 7–10 days at 4 C. The fastest growing strains were purified and stored at –80 C in 50% glycerol.

During 1984, roots were collected from 300 native plants from 25 sites in eastern Canada. Root segments (2–5 cm long) were shaken vigorously for 5 min in sterile 0.1 M MgSO₄, removed, plated onto ASA, and incubated at 14 or 28 C. Plates were examined daily for chemotactic zones (halos around the

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Table 1. Identification of canola plant growth-promoting rhizobacteria strains^a

Identification	Strain
<i>Pseudomonas putida</i>	31-34, 63-36, G3-9, 25-71, 56-13, 54-26, 17-114
<i>P. putida</i> biovar B	25-33
<i>P. fluorescens</i>	63-49, 54-4, 39-8, 31-12, 34-36, 31-44, 63-28, 63-14, 61-16, 37-8, G8-17, 34-13, X, 46-8, 37-9, 36-43, 62-24
<i>Arthrobacter citreus</i>	44-9
<i>Serratia liquefaciens</i>	1-141, 2-16

^aStrains were identified using biochemical tests approved by the American Society for Microbiology (2).

roots). Agar at the edge of the chemotactic zones, which contained migrating bacteria, was mixed with 0.1 M MgSO₄ and plated onto PAF. The resulting colonies were purified and stored at -80 C in 50% glycerol.

Strains from the 1983 and 1984 collections were identified for Gram-strain reaction, and those identified initially as gram-negative were tested for rapid growth at 4, 10, and 14 C. Strains that produced a lawn within 24 hr at 14 C, 48 hr at 10 C, and 4-5 days at 4 C were then tested for growth on exudate agar.

Strains selected for greenhouse tests (described later) were stored at 80 C in glycerol. Those that showed promise as PGPR in early tests were rechecked for purity on PAF, and 10 subcultures of each strain were returned to storage (-80 C). A new vial of bacteria was used for each greenhouse or field test.

Greenhouse assays. Greenhouse screening assays for selecting canola PGPR used a 1:1 mix of perlite and field soil. Field soil, collected from the Allelix research center near Caledon, Ontario, was a clay loam with 2% organic matter, pH 7.0, total exchange capacity (M.E.) 14, and with the following nutrient concentrations in ppm: nitrate nitrogen 4, phosphorous 1, potassium 2, calcium 70, magnesium 16, sodium 0.5, boron 0.4, iron 550, manganese 130, copper 2, and zinc 7.

In initial tests, bacteria were grown on PAF plates at 10 C for 3 days, scraped off plates, and mixed with an aqueous solution of 0.1 M MgSO₄. Canola seeds were agitated in the bacterial suspensions for 10 min to 1 hr at 10 C. Control seeds were treated in the same manner with uninoculated PAF plates substituted for plates with bacterial lawns. Two seeds were planted in each of six 7.5-cm-square plastic pots. The pots were placed at 10 C for 1 wk, then transferred to a greenhouse with day and night temperatures of 22 and 15 C, respectively. Seedlings were thinned to one per pot after emergence.

The leaf areas of canola plants from bacterial treatments and controls were compared 3-4 wk after emergence (before bolting). A visual scale of 0-3 was used where 0 = leaf area equivalent to the control, 1 = slightly greater leaf area, 2 = moderately greater leaf area, and 3 = much greater leaf area than the control. Bacterial strains that were rated 2 or 3 in initial screens were retested at least twice. Most strains that then were rated 2 in two of three repeated tests were retested two more times.

Formulation of selected strains for field testing. Three experimental formulations were used for the field tests: lyophilized bacteria dispersed in oil (lyo/oil), peat, or alginate/oil. The lyo/oil inoculants were prepared from bacterial strains grown in TSB for 48 hr at 30 C (shaking at 150 rpm). The cultures were centrifuged for 10 min at 5,000 rpm,

the supernatant was discarded, the pellet was resuspended in 0.1 M MgSO₄, the tubes were recentrifuged, and the final pellet was resuspended in 2% mannitol (1:1, v/v). These suspensions (6 ml) were frozen (-20 C), lyophilized (Virtis model 24, the Virtis Co., Inc., Gardiner, NY) for 18 hr, and subsequently stored at -20 C. For seed treatment, 250 μ l of sterile canola oil was added to 0.13 g of finely ground lyophilized bacteria, and the mixture was shaken vigorously for 3 min. The lyo/oil mixture was pipetted onto and mixed with seeds at a rate of 7.5 μ l/g of canola seed. Treated seeds were stored at 4 C before planting (maximum 1 wk). Twenty-four hours before planting, 10 seeds were sampled from each bacterial treatment and control for determining the colony-forming units (cfu) per seed. Seeds were shaken with 10 ml of 0.1 M MgSO₄, and dilutions were plated onto PAF as previously described.

Preparation of bacterial pellets for the peat inoculants was the same as before. The pellet (9 ml) was mixed into 15 g of fine peat (at 20% moisture) in plastic bags and stored at 4 C before use. Seeds were coated with 1% methyl cellulose at a rate of 0.2 ml per gram of seed. The seeds were immediately mixed with 0.5 g of peat. Treated seeds were stored at 4 C up to 1 wk before planting. Seeds were sampled 24 hr before planting to determine cfu/seed as described.

The alginate/oil inoculants were prepared by growing bacterial strains as previously described. The culture was mixed (1:1, v/v) with 2% aqueous alginate (Scogin LV, Scotia Marine Products Ltd., Lower Woods Harbour, Nova Scotia, Canada). This suspension was mixed with canola oil (3:7, v/v) plus surfactant (Arlacel 186, ICI Americas Inc., Wilmington, DE), 0.2% final volume. The emulsion was thoroughly mixed and shaken onto canola seeds at a rate of 6 ml/kg of seed. When not in use, the emulsions were stored at 4 C for up to 2 wk. Seeds were again sampled 24 hr before planting to determine cfu/seed.

Field tests in 1985. Field trials were conducted in 1985 using a total of 28 canola PGPR strains. Three trials (trials 1-3) were planted mechanically using the peat formulation, and four trials (trials 4-7) were planted by hand using seed dipped in the bacterial suspensions (which was similar to the system used for the greenhouse assay). Included in the seven trials were two locations and two cultivars.

All mechanically planted trials were in a randomized complete block design with eight replicates per treatment and two controls per experiment (formulation control and untreated control). Each replicate consisted of two or four rows 4 m long. All hand-planted trials were of the Latin square design (8 \times 8) with seven bacterial treatments and one control, each at eight replicates of 4-m-long single

rows. Seeds for all bacterial treatments and controls were dipped into 0.1 M MgSO₄ before planting.

Seedling emergence of hand-planted trials was recorded at a 1- to 2-day frequency from the time emergence began until the counts stabilized. Vigor was rated visually on a scale of 1-10, 25 days after planting. Data of first flowering was recorded for each treatment in all experiments. Plots were harvested at maturity with a small-plot combine, seed was cleaned, and yield was based on 10% seed moisture. All collected data were analyzed for significance by analysis of variance. When a significant *F* value was detected, the treatment means were compared with control means using the LSD value.

Field trials in 1986. Nine bacterial strains were tested in eight trials (trials 9-15) in 1986. The strains included seven PGPR that increased yields in the 1985 trials: *Azospirillum brasilense* Cd, received from Y. Okon at the Hebrew University of Jerusalem, and one root-colonizing, nitrogen-fixing (diazotrophic) *Pseudomonas fluorescens* strain (GR12-2) (11). Trials 8, 9, 10, 12, and 13 had eight replicates per treatment; each replicate had six rows 6 m long and 18 cm apart. Trials 11, 14, and 15 had six replicates per treatment; each replicate had four rows 6 m long and 36 cm apart. Buffer plots consisting of untreated seed were planted between all plots to minimize lateral dispersal of bacteria between plots.

All formulations in 1986 used canola oil as a formulation system. For trial 9, the alginate/oil formulation system, described earlier, was used. For the remaining 1986 trials, the lyo/oil formulation was used.

RESULTS

Greenhouse assay. During 1983 and 1984, 887 bacterial strains were tested for PGPR activity on canola. In the first test, plants inoculated with 222 strains were larger and had greater leaf area than did control plants. Thirty-five strains were positive for PGPR activity in one of two additional tests. Some of the strains were tested 10 or 11 times with frequencies of growth promotion (rating 2 or 3) as great as 8/11 for two strains, 7/11 for two strains, 7/10 for three strains, and 8/9 for one strain.

Field trials. Normal rainfall patterns were recorded over the canola growing season for all sites in 1985 and 1986. Soils were either clay loams or sandy loams with organic matter ranging from 3.7 to 7.4% and pH from 6.8 to 7.5. Yield data for the 1985 mechanically planted (peat-treated) canola trials were skewed by a statistically significant negative effect of peat as a PGPR carrier. In trial 1, the peat-treated control (no bacteria) had 48% lower yield than the untreated control. Hence, when compared with

untreated controls, plants in most PGPR treatments had lower yield, although treatment with five strains resulted in yield increases from 10 to 23% greater than the untreated control. Most PGPR treatments resulted in increased yield compared with the stressed peat control in both trials 1 and 2 (cultivar Tobin), ranging as high as 146% over the control. In trial 3 (cultivar OAC Triton), peat alone did not cause a significant reduction in yield compared with untreated, and PGPR treatments resulted in yield increases ranging to 42% greater than the controls. Strains 63-49 (*P. fluorescens*) and 44-9 (*Arthrobacter*) induced significant ($P=0.05$) yield increases of 42 and 39%, respectively, compared with untreated controls and nonsignificant yield increases of 24 and 22%, respectively, compared with the peat controls. Peat treatments of seed resulted in bacterial

populations ranging from log 3.4 to 6.2 per seed.

No negative effects of canola PGPR treatment were observed in the 1985 suspension-treated trials (trials 4-7) (Table 2). Thirteen of 28 tested strains induced significant yield increases ranging from 24 to 57% compared with the controls. About half of the 28 PGPR strains induced increases in emergence rate of seedlings. Representative data for four strains are presented in Figure 1. Vigor of plants was significantly increased by 15 strains, 12 of which subsequently induced significant yield increases.

Combining the yield data for the peat-treated and the suspension-treated trials indicates superior performance by select strains in 1985. Even with the skewed data from trials 1 and 2, four strains induced average yield increases of 9% or

greater, compared with the controls, in three or more trials. These strains included one *Arthrobacter* (44.9), two *P. fluorescens* (63-49 and 34-36), and one *P. putida* biovar B (25-33).

Oil treatments used in 1986 resulted in bacterial populations ranging from log 2.9 to log 6.3 per seed. Oil formulations did not affect plant growth in the absence of bacteria as greatly as did the peat formulations in 1985. In six of nine trials, the yields of formulation controls were within 6% of yields for untreated controls. Addition of bacteria to the oil formulations in these six trials resulted in yield enhancement up to 17% greater than the oil controls. However, the oil formulation reduced yield at a non-significant level in eight of nine trials in 1986. Hence, only strain 44-9 resulted in a significant yield enhancement on cultivar Tobin compared with the untreated control.

Yield promotion capacity of individual PGPR strains generally was similar between cultivars Tobin and Westar. An exception occurred with strain 25-33, which resulted in a 1% yield reduction from the control on Tobin but a 7% yield increase on Westar. Yield promotion levels on Tribute (an atrazine-resistant cultivar) were markedly higher than those for Tobin and Westar, with statistically significant yield increases of 16 to 24% occurring with seven strains. These data were from a single experiment, and hence, they must be considered as preliminary. The 1986 all-trial yield averages were lower than the 1985 averages (Table 3). The six top-ranked strains have a 2-yr, all-site yield average of 6-13% over the controls.

DISCUSSION

These results constitute an extensive evaluation program for PGPR strains. Six specific strains, which demonstrated canola growth promotion in multiple greenhouse trials and in 2 yr of field trials, have been selected as the best of 887 screened strains (Table 3). The relative rankings of these six strains based on yield promotion in replicated field trials were consistent in the 1985 and 1986 trials. Three of the six strains induced a 2-yr average yield promotion response of 11-19%. In addition, some strains repeatedly induced demonstrable increases in emergence rates and vigor and increased yield up to 57% compared with the controls in individual field trials.

In previous PGPR studies with strain selection (1,10,15), the ability to detect PGPR activity was partially a numbers game because only 1-5% of tested rhizosphere strains promoted plant growth. Hence, our greenhouse selection assay was designed to maximize the number of tested strains. In addition, the emphasis of the greenhouse assay was placed on repeatability of strain performance rather than on the statistical

Table 2. Effects of plant growth-promoting rhizobacteria on canola yield using suspension treatments in 1985^a

Trial	Strain identification ^b	Strain	Yield (t ha ⁻¹)	Change from control ^c (%)
4	<i>Pf</i>	31-12	1.21*	42
	<i>Ac</i>	44-9	1.11*	30
	<i>Pf</i>	31-44	1.10*	29
	<i>Pp</i>	31-34	1.03	21
	<i>PpB</i>	25-33	0.94	10
	<i>Pf</i>	36-43	0.93	10
		Control	0.85	...
	<i>Pp</i>	G3-9	0.82	3
			LSD ($P=0.10$) = 0.25 ($P=0.05$) = 0.33	C.V. = 29
5	<i>Pf</i>	63-49	1.38**	41
	<i>Pf</i>	63-14	1.23*	25
	<i>Pf</i>	63-28	1.17	19
	<i>Pp</i>	25-71	1.13	15
	<i>Pf</i>	61-16	1.12	14
	<i>Pp</i>	63-36	1.08	10
	<i>Pf</i>	62-34	1.08	9
		Control	0.98	...
			LSD ($P=0.10$) = 0.23 ($P=0.05$) = 0.28	C.V. = 20
6	<i>Pp</i>	56-13	1.38*	24
	<i>Pf</i>	X	1.31	17
	<i>Sl</i>	2-16	1.30	16
	<i>Pf</i>	54-4	1.25	12
	<i>Sl</i>	1-141	1.16	4
	<i>Pf</i>	62-24	1.16	4
		Control	1.12	...
	<i>Pp</i>	54-26	1.11	0
			LSD ($P=0.10$) = 0.25 ($P=0.05$) = 0.28	C.V. = 22
7	<i>Pf</i>	34-13	1.67**	57
	<i>Pp</i>	17-114	1.51**	41
	<i>Pf</i>	37-9	1.42*	33
	<i>Pf</i>	G8-17	1.42*	33
	<i>Pf</i>	39-8	1.42*	33
	<i>Pf</i>	46-8	1.41*	32
	<i>Pf</i>	37-8	1.38	29
		Control	1.07	...
			LSD ($P=0.10$) = 0.33 ($P=0.05$) = 0.40	C.V. = 23

^a All values are means of eight replicates.

^b *Pp* = *Pseudomonas putida*, *Pf* = *P. fluorescens*, *PpB* = *P. putida* biovar B, *Ac* = *Arthrobacter citreus*, and *Sl* = *Serratia liquefaciens*.

^c * = Significant at $P=0.10$; ** = Significant at $P=0.05$ compared with the controls, which were treated with MgSO₄.

quantification of the magnitude of growth promotion. Only the strains with the highest levels of repeating growth promotion activity in the greenhouse assay were tested in the field.

As with past PGPR work with potato (1,3,8,10) and sugar beet (15), most of the PGPR strains detected were fluorescent *Pseudomonas* spp., although we also detected *Serratia liquefaciens*, *P. putida* biovar B, and *Arthrobacter citreus*. In our previous studies with canola and soybean EPR (emergence-promoting rhizobacteria) (6), one-fourth of the EPR-active strains were nonfluorescent bacteria.

Two challenges must be addressed in efforts to develop PGPR as agriculturally acceptable inoculants. First, there is an immediate need to develop an acceptable delivery system for applying PGPR to the seed. The yield increases presented here were consistently better when

PGPR were applied to seed as suspensions than with experimental formulations. The depressed yield that resulted from the peat formulation control compared with the untreated control in trials 1 and 2 may result from pathogen stimulation or from interference with emergence of the small-seeded canola.

The second challenge is to overcome or control the variation in the magnitude of plant response to a given PGPR strain. In 1986, for example, the average (of six to eight replicates) yield promotion compared with the formulation control for nine growing sites ranged from -3 to +24% for strain 63-49 and from -4 to +17% for strain 34-13. The main source of this variation does not appear to be host-related. Only one of nine PGPR strains showed a differential response between cultivars Tobin (*B. campestris*) and Westar (*B. napus*). Our recent results of greenhouse tests with wheat and corn

(unpublished) support this conclusion. Two strains, 63-49 and GR12-2, with positive PGPR activity in rapeseed improved wheat dry-weight accumulation by about 10%, whereas another strain, 25-33, increased corn seedling dry weight by 17%.

The major source of variation in the magnitude of the PGPR response appears to be related to PGPR activity per site. A determination of the factors that contribute to site conduciveness to PGPR can soon be attempted based on the accumulation of multiple years' field performance data. The most likely factors involved include the role of pathogenic and/or deleterious microorganisms, water or nutrient availability, and presence of toxic elements. Hence, it is imperative that future field trials be linked to mode of action studies aimed at determining the nature of site conduciveness.

These challenges should not overshadow the potential for using PGPR as beneficial crop inoculants. The yield enhancement that was previously demonstrated using PGPR on root crops such as potato (1,3,8,10), sugar beet (15), and radish (7) has potential for seed crops. In addition, certain high-performing strains can be selected. For example, in this study, strain 34-13 caused a numerical enhancement in yield in two of two trials in 1985, with an average of +29% compared with the controls and in eight of nine trials in 1986, with an average of +9% compared with the controls.

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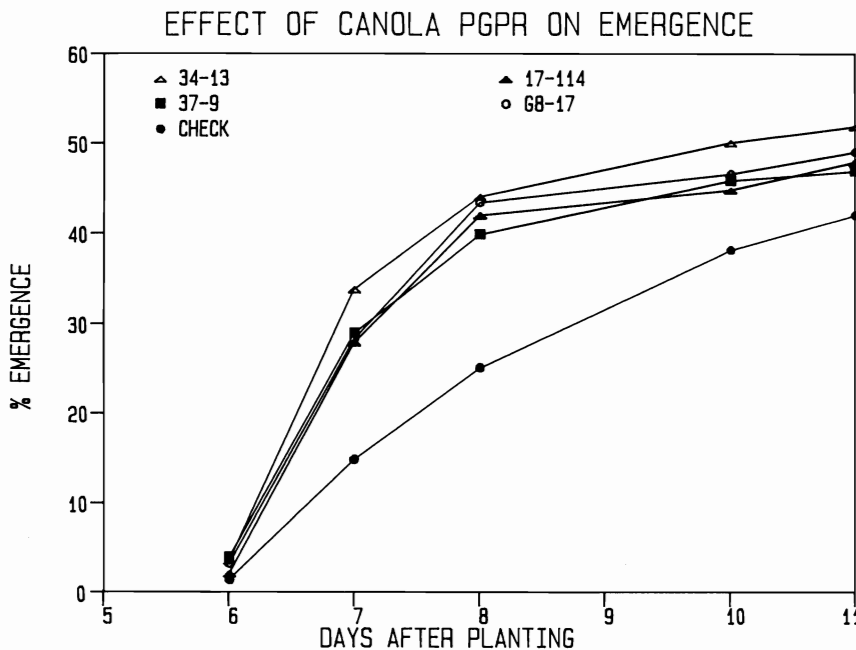


Fig. 1. Stimulation of seedling emergence rates by four plant growth-promoting rhizobacteria (PGPR) strains. Results are an average of eight replicates per treatment in a randomized block field trial (trial 7 in text). The four PGPR strains were applied by dipping canola seeds in bacterial suspensions just before planting.

Table 3. Summary of plant growth promotion activity for top-ranked PGPR strains^a

Strain	No. positive/total no. greenhouse assays ^b	1985		1986		Two-year yield promotion average (%)
		Yield promotion average (%)	Positive sites/sites tested ^c	Yield promotion average (%)	Positive sites/sites tested ^c	
34-13	6/10	29	2/2	9	8/9	19
63-49	5/9	23	4/4	7	7/9	15
44-9	8/11	15	3/4	8	6/9	11
X	7/11	10	2/3	7	7/9	8
34-36	5/9	10	2/3	5	6/9	7
25-33	5/9	9	3/4	5	6/9	7

^aPGPR = plant growth-promoting rhizobacteria.

^b"Positive" indicates a rating of 2 or 3, using a visual rating of 0-3 compared with the control. Six to eight replicate plants were used in each trial.

^c"Positive sites" are field trials where the mean yield of the PGPR treatment was numerically greater than the mean yield of the control (MgSO₄ control for 1985 and oil formulation control for 1986).

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