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EMERGENCE-PROMOTING RHIZOBACTERIA:

DESCRIPTION AND IMPLICATIONS FOR AGRICULTURE

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INTRODUCTION

Specific strains of root-colonizing bacteria, termed plant growth-promoting rhizobacteria (PGPR), have recently been used as experimental inoculants to increase yield of sugar beet (Suslow and Schroth, 1982), radish (Kloepper and Schroth, 1978) and potato (Burr et al., 1978; Howie and Echandi, 1983; Kloepper et al., 1980). In each case, evidence of enhanced plant growth was observed sometime during the early-growth season prior to harvest. Increased early-season development of potato was manifested by increased stolon lengths on PGPR-treated plants (Kloepper et al., 1980; Kloepper and Schroth, 1981A; Kloepper and Schroth, 1981B). The earliest indication of PGPR-enhanced growth of sugar beet was a significant increase in seedling weight (Suslow and Schroth, 1982); seedling emergence was not affected by PGPR treatments.

During a field screening program for new PGPR strains for soybean, we observed that some bacteria induced increases in seedling emergence of 100% greater than controls (Kloepper and Scher, unpublished). This emergence promotion was repeated in the field, using the same bacterial strains, only when soil temperatures were below 20°C.

The objective of the work described herein was to expand upon our initial observations and determine if a specific group of PGPR could be identified which would increase emergence of seedlings in cold field soils. We termed such bacteria "EPR" (Emergence-promoting rhizobacteria). Our strategy was three fold: to develop an emergence assay in which we could screen phychrotrophic rhizobacteria for EPR activity; to determine the consistency of emergence enhancement by select EPR in the assay; and finally to field test strains which were consistent in the assay. We chose soybean and canola (rapeseed) as model crops.

METHODS

Isolation of Bacterial Strains

Two isolation procedures were used during the course of this study. For procedure one, canola and soybean seeds were surface-sterilized by rinsing for 5 minutes in 95% ethanol, rinsed in sterile water, soaked

5 min. in 1.5% sodium hypochlorite, and rinsed in sterile water again. Seeds were planted in various soil samples at 10 to 14°C. Roots from developing seedlings were removed, washed in sterile water to remove loosely adhering soil particles and ground in 5 ml sterile 0.1 M $MgSO_4$. Serial ten-fold dilutions were plated onto Pseudomonas Agar F (PAF)(Difco Labs, Detroit, MI, USA 48232) and plates were incubated 2 weeks at 14°C. Colonies were purified on PAF at 20°C.

For isolation procedure two, roots of plants collected in the eastern Northwest Territories, Canada, were washed to remove soil particles and placed directly onto asparagine soft agar (ASA). ASA contained 1g L-asparagine, 2g Bacto Agar and 1000 ml distilled water and was previously used to assess bacterial chemotaxis as an indicator of root-colonization capacity (Scher, unpublished). Bacteria which grew out from root segments on ASA were then purified on PAf plates at 20°C.

Strains isolated using procedures one and two were restreaked on PAF plates, and examined for rapid growth at 4, 10 and 14°C. Strains which developed an observable lawn in 24 h at 14°C, 48 h at 10°C and 4-5 d at 4°C were further tested for growth on exudate agar at 20°C. Exudate agar was prepared by mixing 10% soybean or 20% canola seed exudates with 2% washed purified agar (Difco). Exudates were prepared as described previously (Scher et al., 1985).

Identification and Storage of Bacteria

Purified bacterial strains were stored in glycerol at -80°C prior to being tested in the assays. Strains which induced emergence increases were rechecked for purity on PAF, and 10 copies of each strain were returned to -80°C storage. A new vial of bacteria was used for each emergence assay. Identification was done only for strains which repeated emergence-promoting activity. All strains were Gram-negative and were further tested for reaction profiles on API 20E test strips (Analytab Products, Ayerst Laboratories, Inc. Plainview, N.Y., U.S.A.). Additional tests included growth on MacConkey medium, type of metabolism in OF glucose medium, production of fluorescent pigment, gelatine hydrolysis, nitrate reduction, starch hydrolysis, oxidase reaction, production of DNase, and lipase production (Tween 80 hydrolysis). Methods for all of the above biochemical tests were those recommended by the American Society for Microbiology. The identifications of emergence-promoting strains are listed in Table 1.

Canola Emergence Assay

The following assay was developed to assess emergence of canola (*Brassica campestris* cv 'Tobin'). Field soil was collected from the Allelix Field Research Centre near Caledon, Ontario and consisted of a clay loam with 2% organic matter, pH 7.0, total exchange capacity (M.E.) 14, and with the following nutrient levels 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. Soil was thoroughly mixed in a 1:1 ratio with perlite and the resulting mix was used throughout the study.

Test bacteria were grown on PAF plates at 10°C for 3 days, scraped off plates, and mixed in 0.1 M $MgSO_4$. Canola seeds were agitated in the bacterial suspensions for 2 h at 10°C prior to planting 20 seeds in each of 8 replicate 15 cm pots. Seeds were planted 2 cm deep and pots were watered immediately and placed at 9°C. Each experiment consisted of 6 to 8 bacterial treatments with one control. The control consisted of canola seeds soaked in 0.1 M $MgSO_4$ which had been poured over an uninoculated PAF plate. Pots were examined daily and the number of emerged seedlings was recorded.

Table 1. Identification of emergence promoting bacteria

Soybeans	Strain designation	Identification
	1-104	*Pseudomonas putida*
	1-226	*Pseudomonas fluorescens*
	1-206	*Pseudomonas fluorescens*
	2-16	*Serratia liquefaciens*
	2-18	*Serratia liquefaciens*
	2-20	*Serratia liquefaciens*
	2-22	*Pseudomonas putida biovar B*
	2-67	*Serratia liquefaciens*
	2-114	*Enterobacter aerogenes*
	17-114	*Pseudomonas putida*
	17-29	*Pseudomonas fluorescens*
	17-76	*Pseudomonas putida*
	17-34	*Pseudomonas fluorescens*
	G25-25	*Pseudomonas fluorescens*
	G25-26	*Pseudomonas putida*
	G25-44	*Pseudomonas putida*
	G20-20	*Pseudomonas fluorescens*
	G23-34	*Pseudomonas putida*
	G24-16	*Pseudomonas putida*
	G24-14	*Pseudomonas putida*
	G24-3	*Pseudomonas putida*
	G20-18	*Pseudomonas fluorescens*
	1-102	*Serratia liquefaciens*
Canola	**Strain designation**	**Identification**
	G1-1	*Beijerinckia* spp.
	G1-3	*Pseudomonas fluorescens*
	G1-4	*Beijerinckia* spp.
	52-30	*Pseudomonas putida*

A total of 50 bacterial strains isolated using procedure one (see previous section) and 60 strains isolated using procedure two were tested for emergence promotion relative to controls. Strains which demonstrated significant (P=0.05) emergence promotion were retested 3 times to determine the consistency of emergence promotion.

Soybean Emergence Assay

The initial assay which was used for selection of soybean EPR strains was used from June 1983 through February 1984. Candidate EPR strains were grown for 48 h on PAF plates at 14°C and scraped into 50 ml 0.1 M $MgSO_4$. One hundred fifty soybean seeds (cv. 'Maple Presto' or 'Maple Arrow') were added to each 50 ml suspension and were shaken at 100 RPM at 10°C for 3 h. Typical experiments consisted of 6 bacterial treatments with one $MgSO_4$ control, each with 9 to 10 replications. Each replication consisted of 12 seeds planted in a 12-well plastic seeding tray (Plant Products Ltd., Bramalea, Ontario) with overall dimensions of 18 cm wide x 27 cm long x 6 cm deep and with dimensions of individual wells of 6 cm long x 5 cm

wide x 6 cm deep. Seeds were planted 3 cm deep in "conditioned field soil".

"Conditioned field soil" was prepared by mixing soil from the Allelix Field Research Centre (described in the previous section) in a 1:5 ratio with Promix C (Plant Products Ltd., Bramalea, Ontario). Soybean was seeded into flats containing the soil mixture and grown to the second true leaf stage when the plants were discarded. The same soil (termed "conditioned soil") was reblended and used in the soybean emergence assay.

After planting, each replicate seeding tray was watered and placed at 14°C. The number of emerged seedlings was recorded after 14 d, and data were analysed using a one-way analysis of variance to detect significant differences between treatment means. A total of 277 strains which were isolated using isolation procedure one, described above and 84 strains which were isolated using procedure two were tested for emergence-promoting activity in this assay. Strains which induced a significant increase in emergence in the first trial were retested twice using the same assay procedures.

A second assay was used from February 1984, through February 1985, for strains which demonstrated repeated EPR activity in the initial assay. Soybean seeds were shaken in bacterial suspensions or in 0.1 M $MgSO_4$ as described above and planted in a 1:1 mix of Allelix field soil:perlite. Five cm of the mix was placed in the bottom of 25 cm plastic azalea pots (Kord Plastics Ltd., Toronto); 20 seeds of the same treatment were placed on the soil: soil perlite mix was added to give a planting depth of 5 cm. Pots were immediately watered and placed at 12 to 14°C. Each pot of 20 seeds constituted a single replication, and 8 replications were used per treatment. Typical experiments consisted of 5 to 7 bacterial treatments with one 0.1 M $MgSO_4$ control. Emergence was recorded daily and strains were deemed "EPR" when they induced a 50% increase in emergence of controls for 3 consecutive days in 2 of 3 repeating experiments.

Preliminary Mode-of-Action Studies

Eleven soybean EPR strains were selected to test for possible siderophore action, which has been suggested to account for some plant growth-promoting rhizobacteria (PGPR) strains' mode-of-action. Emergence assays were set up as described above except that 16 replications were used instead of 8. Eight replications of each treatment and control were watered with 10^{-3} M $FeCl_3$, and 8 replications were watered with water immediately after seeding. Soil pH was recorded before and after addition of $FeCl_3$. Each experiment was repeated once.

RESULTS

Isolation of Bacterial Strains

Using isolation procedure one, 630 strains were obtained from soybean and 450 from canola. Of these strains, 277 soybean and 50 canola strains were found to grow on PAF in 4-5 days at 4°C and on exudate agars in 24 h at 20°C.

With isolation procedure two, 940 strains were obtained by direct isolation of chemotactic zones from roots on asparagine soft agar. Approximately 250 of these grew on PAF in 4-5 days at 4°C.

Canola Emergence Assay

Of 50 strains obtained using isolation procedure one, 3 induced

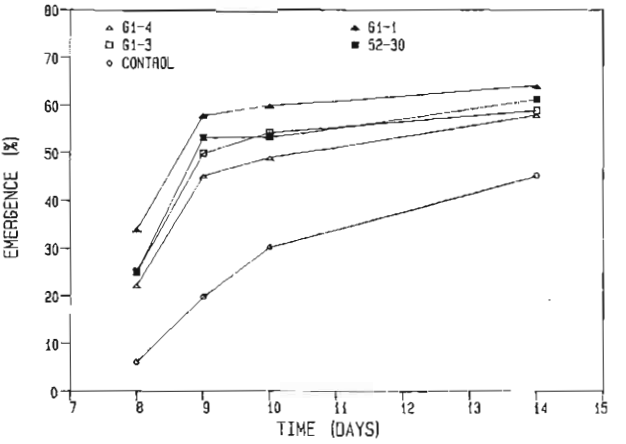

Fig. 1. Canola emergence promotion assay showing increases in emergence by 4 EPR. Assay was conducted in a field soil/perlite mix at 9°C with seeds shown to 2 cm depth. The percentage emergence values shown are the mean of 8 replications, each sown with 20 seeds. Similar results were obtained with the same 4 bacterial strains in 3 of 5 repeat experiments.

increases in emergence of 50% or greater than controls in first tests. None of these 3 increased emergence in 3 repeat experiments. Sixty strains obtained using isolation procedure two were tested in the canola assay and 10 increased emergence 40% or more in first trials. Four strains consistently increased emergence in 3 of 5 repeat trials (Fig.1). The percentage of emerged seedlings with bacterial treatments was 4 to 7 times greater than the percentage of emerged controls 8 days after seeding, 2 to 3 times greater at 9 days and 40 to 50% greater at 14 days.

Table 2. Soybean emergence assay initial selection untested strains*

	Number emerged/12 at 14 days										
	Replication										
Treatment	1	2	3	4	5	6	7	8	9	10	$\bar{x}$
1	2	4	2	3	2	5	3	3	2	4	3.0**
2	1	3	1	2	0	2	1	3	1	0	1.4
3	1	2	1	0	1	1	2	1	0	1	1.0
4	2	2	3	2	3	2	3	5	2	3	2.7**
5	1	2	2	1	0	2	1	1	1	0	1.1
6	0	1	1	2	3	1	1	2	0	1	1.2
Control	2	2	1	1	1	0	3	1	2	1	1,4

LSD 0.01 = 1.1 F = 8.1

* Assay was conducted at 14°C. The data shown are from one typical experiment. A total of 361 strains were tested over a 9 month period. See text for details.

Table 3. Soybean emergence assay - repeat testing of strains which induced a significant increase in emergence in the first test*

	Number emerged/12 at 14 days Replication										
Treatment	1	2	3	4	5	6	7	8	9	10	$\bar{x}$
2-16	1	0	7	3	3	3	1	3	2	1	2.4*
2-17	6	1	0	1	1	0	2	4	3	6	2.4*
2-18	3	1	5	2	9	3	6	9	5	8	5.1**
2-19	1	3	4	1	0	1	3	1	3	8	2.5*
2-20	5	6	1	1	2	4	2	1	4	0	2.6*
2-21	2	5	5	2	4	5	4	5	2	7	4.1**
Control	0	1	1	1	2	0	0	0	0	0	0.5

LSD 0.05 = 1.8 F = 4.94
0.01 = 2.5

* Assay was the same used for data shown in Table 2. A total of 62 strains were retested. Data shown here are from one typical experiment.

Soybean Emergence Assay

Over a 9 month period, 277 strains isolated using procedure one and 84 strains isolated using procedure two were tested in the initial soybean emergence assay using "conditioned field soil". Sixty-two strains induced significant increases in emergence at 14 days compared to controls. Raw data from one typical experiment in which 2 EPR strains were selected, are shown in Table 2. All 62 strains were retested twice with the same assay,

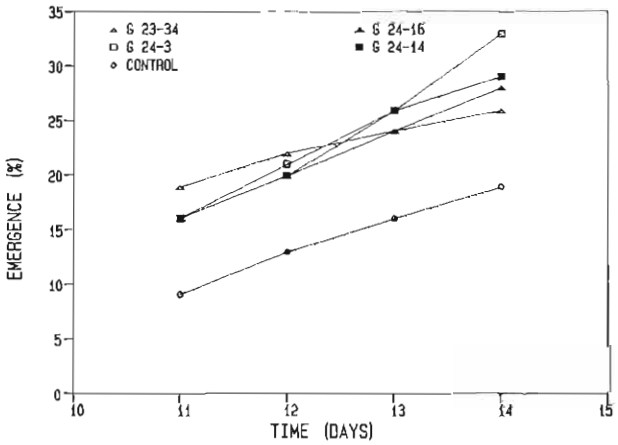

Fig. 2. Soybean emergence promotion showing increases in emergence by 4 EPR strains. The assay is the second soybean assay described in the text and was conducted in a field soil/perlite mix at 14°C. The percentage emergence values shown are the mean of 8 replications, each sown with 20 seeds.

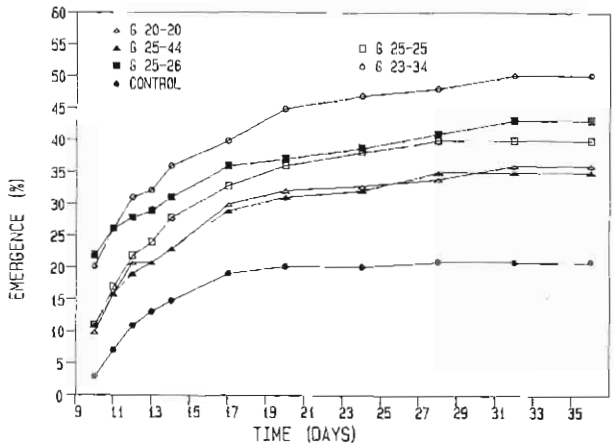

Fig. 3. Effect of 5 soybean EPR on early emergence rate and final percentage emergence. The assay is the same used in Fig. 2.

and 30 strains repeated emergence promotion in both repeat experiments (Table 3).

A second assay in field soil/perlite with 20 seeds per replication was used over a 12 month period to confirm the phenomenon of emergence promotion and to determine the effect of EPR on emergence on multiple days. Strains which induced a 50% increase in the percentage emergence of controls on each of 3 consecutive days were deemed to be EPR (Fig. 2). Twenty-three of the 30 strains which were originally selected for emergence-promoting activity repeated emergence promotion in at least 2 of 3 repeat experiments. Some of these strains increased both the rate of emergence and the final percentage emergence (Fig. 3) under the experimental conditions.

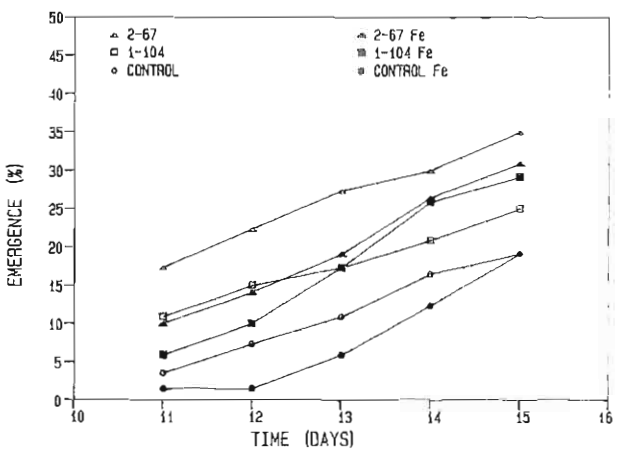

Fig. 4. Promotion of soybean emergence by 2 EPR strains with and without 10^{-3} M $FeCl_3$ amendments.

Preliminary Mode-of-Action Studies

Addition of 10^{-3} M $FeCl_3$ to soil in the soybean emergence assay changed the pH from 7.0 to 6.9. All 11 tested EPR retained emergence-promoting capacity in the presence of $FeCl_3$ amendments. Representative data for 2 of the 11 strains is shown in Fig. 4.

DISCUSSION

The discovery here of emergence-promoting rhizobacteria (EPR) which are operable at low soil temperatures represents a new, distinct class of microbial inoculants with potential use in agriculture. At suboptimal soil temperatures, seedling emergence is reduced (Acharya et al., 1983; Szyrmer and Szczepanska, 1982) and seed exudation is increased, (Schroth et al., 1966; Keeling, 1974; Hayman, 1969). The EPR strains reported herein were selected for growth on seed exudates at low temperatures, and hence may serve to reduce the total carbohydrates in the spermosphere which are available for growth of seedling pathogens.

The second procedure (described in the Methods Section) for isolation of candidate EPR strains, in which root segments were placed directly onto asparagine soft agar, allowed the direct isolation of motile strains which were chemotactically attracted to one of the major amino acids in seed exudates. (Scher et al., 1985). This procedure yielded a higher percentage of strains which were ultimately deemed to be EPR strains based on repeatable emergence promotion: for canola, 4 of 60 strains (7%) from procedure two, versus 0 of 50 from procedure one; for soybean, 14 of 277 strains (5%) from procedure one versus 9 of 84 (11%) from procedure two. It was surprising that EPR strains included 3 bacterial taxa not previously reported to be plant growth-promoting rhizobacteria (Table 1) i.e. *Enterobacter aerogenes, Serratia liquefaciens* and *Beijerinckia* spp.

The work reported here was concentrated on soybeans. However, the fact that a few canola EPR were also found suggests that EPR, like PGPR, can likely be found for any crop. The preferred method for detecting emergence promotion is the second soybean assay in which a field soil perlite mixture was used and emergence was recorded daily. We also found that it is important to conduct each assay 3 times and select strains which increase emergence in 2 of 3 tests.

The designation of a strain as an "EPR" can be made in several ways. We preferred to select strains which induced a 50% or greater increase in emergence relative to controls on 3 consecutive days. Alternative selection parameters are probably equally valid, as long as the emphasis is placed upon demonstrating the repeatability of emergence promotion.

The retention of emergence-promoting activity by EPR in the presence of ferric chloride amendments (Fig. 4) suggests that siderophores are not the primary operable compounds in mode-of-action. This conclusion is definitely preliminary, as our efforts to date have concentrated on the laborious establishment of the emergence-promoting phenomenon. Other possible modes of action are production of bacterial compounds which directly promote plant growth or antagonism against pathogens. *Pythium* is the major pathogen of soybean seeds at cool temperatures. To date we have not investigated interactions of EPR with *Pythium*.

While EPR may not be direct yield-enhancers under optimum plant growth conditions, as are PGPR, they will likely indirectly stimulate yields under adverse growing conditions. The development of plant

cultivars with increased emergence rates at low soil temperatures has been identified as a high priority for canola (Acharya et al., 1983) and soybean (Szyrmer and Szczepanska, 1982) breeders in order to increase yields. In addition, EPR should prove useful as one component in strategies to increase stands, since increased emergence rates at cold temperatures result in increased final stands (Hatfield and Egli, 1974) and sometimes in yield (Khan et al., 1983).

Results of field tests with EPR are required in order to determine the ultimate usefulness of EPR in agriculture. However, three possible use areas may be good targets for EPR. Firstly, EPR would be beneficial at the extreme northern perimeter of a crop zone. For example, canola on the Canadian Prairies is concentrated in areas with 90 or more frost-free days. If an EPR-induced acceleration in seedling emergence translates into crop maturity of even 5 days sooner, the total hectarage available for canola growing would be increased substantially. Secondly, EPR are reasonable candidates for including in an integrated control strategy for some disease situations in which the host is most susceptible at the early season stages. For example, tests at the Agriculture Canada Research Centre at Harrow, Ontario, indicate that a reduced incidence of disease caused by *Phytophthora megasperma* var. *glycinea* occurs following treatments which accelerate seedling emergence and growth (T. Anderson, personal communication). EPR also have potential usefulness in non-irrigated crop lands where post-seeding rainfall is often limited. Under these conditions, accelerated emergence would likely result in increased root mass prior to the drought stress period.

The EPR strains described here are currently being tested in field trials. Multiple planting dates have been used on 2 canola and 2 soybean cultivars at 2 locations. The results of these tests will help clarify the potential for EPR in agriculture.

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