Final Report Submitted to The Canola Council of Canada
Period: April 1, 1995 - March 1, 1998
Project nº CARP AG #97-95-13

"Nitrogen Fixing Rhizobacteria as Biofertilizers for Canola"

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Abstract

Eight hundred-ninety bacteria isolated from the rhizosphere, root surface and interior of canola (*Brassica napus* L.) grown at 3 field locations were screened for the ability to fix N_2 (nitrogenase activity - ARA) *in vitro*. Only 4% of these isolates (n=36) exhibited nitrogenase activity. Based on fatty acid methyl-ester identification, the most common N_2-fixers were bacilli (63%), with *Bacillus polymyxa* being the most common species (83% - 19/23 identified). Some *B. polymyxa* isolates from the rhizosphere of canola plants at two locations were related at the subspecies level. The other N_2-fixing species identified included: *Cellulomonas biazotea*, *Erwinia herbicola*, *Pseudomonas savastanoi* and *Xanthomonas maltophilia*, but the similarity index was low (<0.3) thus these names are suspect. The N_2-fixers were assessed for canola growth promotion in a Typic Cryoboralf (Choiceland) soil and isolates that exhibited ARA activity in the rhizosphere or enhanced the growth of canola seedlings compared to the uninoculated controls were identified. In a subsequent study, these isolates were assessed for their ability to promote canola growth and N-uptake in the Choiceland and Allan (Typic Haploboroll) soils. *Bacillus polymyxa* strain ES600A and RSN17 increased (P<0.05) shoot-N in the canola plants grown in the Choiceland soil. Similarly, *B. polymyxa* strain RSS4 and RSN17 increased (P<0.10) shoot-N content, shoot biomass or plant height of canola plants grown in the Allan soil. Subsequently, these three diazotrophs were tested in a field study for their ability to increase canola grain yield. Of the three strains tested, *B. polymyxa*-RSN17 increased (non-significant) canola yield by 20% over the uninoculated controls. These results demonstrate that diazotrophic bacteria such as *B. polymyxa*-RSN17 are promising biofertilizer for canola, thus deserve further attention.

Background

Chemical fertilizers have a substantial impact on food production, and are an indispensable part of modern agricultural practices. Modern agriculture is based on maximum out-put in the short term, with inadequate concern for input efficiency or stock maintenance (Odum, 1989). Nitrogen fertilizer ranks first among the external inputs to maximize output in agriculture. However, input efficiency of N-fertilizer is one of the lowest among plant nutrients. Biological alternatives which can optimize, and/or replace N-fertilizers must be sought.

The process of biological nitrogen fixation offers this alternative (Bohlool *et al.*, 1992). Nitrogen fixing plants may reduce external inputs and improving the quality and quantity of internal resources. Associative nitrogen fixation is carried out by a number of bacterial species living on the roots of non-leguminous plants and a diverse number of genus and species of N_2-fixing bacteria have frequently been isolated from the roots of various plants (Haahtela *et al.*, 1981; Haahtela & Korhonen, 1985; Rennie, 1980). Hence, BNF can be a major source of N in agriculture when N_2-fixing systems are used. Inoculant technology is well-developed for legume, but is still being developed for other biological nitrogen fixing systems (Torrey, 1978).

We hypothesized that specific bacteria isolated from canola rhizosphere might be used as seed-applied inoculants to enhance the growth and yield of canola through dinitrogen fixation. Therefore, the objective of this research project was to assess the potential use of N_2-fixing bacterial inoculants to promote canola growth and reduce dependency on N-fertilizers.

Objectives

The objectives of this research project were:

- Isolate, characterize and assess bacteria found in the rhizosphere of canola for their ability to reduce atmospheric nitrogen (N_2-fixing rhizobacteria *i.e.*, diazotrophs);
- Validate candidate diazotrophic strains for their ability to exhibit nitrogenase activity in the rhizosphere of canola plants in growth chamber assays;
- Develop a suitable method for the introduction and establishment of these diazotrophic agents in the rhizosphere of canola.

Study #1:

Isolation, characterization and assessment of bacteria found in the rhizosphere of canola for their ability to reduce atmospheric nitrogen *in vitro* (putative N_2-fixing rhizobacteria *i.e.*, diazotrophs)

Introduction

In recent years, rhizobacteria (bacteria from the root zone) have been used as biofertilizers to enhance plant growth (Kloepper & Schroth, 1978). For example, recent field trials on canola inoculated with plant growth-promoting rhizobacteria (PGPR) resulted in a 57% increase in yield (Kloepper *et al.*, 1988). However, the exact mechanism of growth stimulation by some rhizobacteria is unknown. Several mechanisms have been proposed for the beneficial effects observed on canola due to inoculation with PGPR. Lifshitz *et al.*, (1987) reported that PGPR inoculation enhanced root and shoot elongation, seedling emergence and phosphate uptake in canola plants, so the mechanism could be linked to nutrient uptake. Another possibility is that PGPR stimulate plant growth through N_2 fixation. In fact, Lifshitz *et al.*, (1986) isolated several pseudomonad rhizobacteria capable of reducing acetylene to ethylene (ARA activity) from various native plants grown in the Canadian High Arctic. In addition to colonizing roots of canola and exhibiting high levels of ARA activity, all of these pseudomonad strains demonstrated a competitive advantage for root colonization over other rhizosphere bacteria at low temperatures. Thus, the combined capabilities of nitrogen fixation and root colonization by diazotrophic pseudomonads suggest that a biofertilizer inoculant for canola might be developed.

The objectives of this study were to:

- Isolate and screen rhizobacteria of canola for nitrogenase activity;
- Identify diazotroph bacteria using whole-cell cellular fatty acids;
- Establish a bacterial culture collection of diazotrophic rhizobacteria associated with canola.

Materials and Methods

Isolation of rhizobacteria:

Rhizobacteria were isolated from the rhizosphere (root region), rhizoplane (root surface), endo-rhizosphere (root interior) and leaves of 100 to 115 day-old canola plants grown at three field sites (Bellevue, Allan and Watrous municipalities) in Saskatchewan. Plant shoots, roots and soil were separated, and putative N_2-fixers, pseudomonads and total heterotrophic bacteria enumerated using selective culture media.

Root samples (*ca.* 20 g) were collected and divided into two sub-samples; one for isolation of rhizoplane bacteria and the other for endo-rhizobacteria (*i.e.*, bacteria colonizing the root interior) (Foster & Rovira, 1976). Plant material (shoots or roots) was soaked in sterile phosphate buffered saline-PBS (pH 7.3) for 10 min to equilibrate osmotic pressure, and chopped into small pieces (3 cm). For endo-rhizobacteria, root samples were surface sterilized by soaking roots in 95% ($v \cdot v^{-1}$) ethanol and 0.1% ($w \cdot v^{-1}$) acidified $HgCl_2$ for 1 min, respectively and then washed (10 ×) with sterile tap H_2O (Vincent, 1970). Plant material (10 g) was suspended 1/10 ($w \cdot v^{-1}$) in PBS, blended in a sterile Waring blender at high speed for 1 min and then serially diluted 1/10 in PBS. Aliquots (0.1 mL) of appropriate dilutions were spread plated onto Combined carbon (CC), King's B (KB) and Trypticase soy agar (TSA) media for isolation of putative N_2-fixers, pseudomonads and total heterotrophs, respectively (King *et al.*, 1954; Rennie, 1981). Inoculated plates (4 replicates) were incubated at 28°C and bacterial colony-forming units (cfu) counted after 24, 48, and 72 h of incubation.

Selection of of putative N_2-fixing rhizobacteria:

Putative N_2-fixing rhizobacteria were selected based on their ability to exhibit nitrogenase activity *i.e.*, reduce atmospheric nitrogen using the acetylene reduction assay—ARA (De Freitas & Germida, 1990; Postgate, 1971). During this initial phase of the project, exactly 890 unknown rhizobacteria isolates *i.e.*, 390 isolated from rhizoplane (*i.e.*, root surface), 196 endophytic (from root interior), and 314 from the rhizosphere (the root region) were selected from CC plates. These isolates were randomly selected to represent a diverse group of colony morphologies. In addition to the rhizobacteria selected from the canola crop, two known diazotrophs (N_2-fixing bacteria) *Azospirillum brasilense* and *Azotobacter chroococcum*, were used as controls for nitrogenase activity. Isolates from root surface and/or interior, soil or those colonizing the leaves of canola were transferred to test tubes containing semi solid CC medium and incubated for 24 h at 28°C. Tubes were then sealed with sterile serum stoppers and acetylene injected to give a 1% ($vol \cdot vol^{-1}$) atmosphere. After 1h, 0.25-cc samples were analyzed with a Hewlett Packard gas chromatograph equipped with flame ionization detector and

a stainless steel (1/8" O.D.) Porapak R (80/100 mesh) column. The oven and detector temperatures were 45°C and 50°C, respectively.

Identification of putative N_2-fixing rhizobacteria using fatty acid methyl-ester (FAME) profiles:

Rhizobacteria which exhibited nitrogenase activity were identified based on whole-cell cellular fatty acids, derivatized to methyl esters - FAMEs (Miller, 1982) and analyzed by gas chromatography (GC), using the MIDI system (Microbial Identification System, Inc., Newark, USA). The procedures and protocols used for growing the cultures and instrument specifications are described by MIDI (1990). Briefly, isolates were grown on TSA plates at 28°C for 24 hs and bacterial cells collected. One ml of a methanolic NaOH (15% [wt·vol^{-1}] NaOH in 50% [vol·vol^{-1}] methanol) was added and cells were saponified at 100°C for 30 min. Esterification of fatty acids was performed with 2 ml of 3.25*N* HCl in 46% (vol·vol^{-1}) methanol at 80°C for 10 min. The FAMEs were extracted into 1.25 ml of 1:1 (vol·vol^{-1}) methyl-*tert*-butyl ether-hexane, and the organic extract washed with 3 ml of 1.2% (wt·vol^{-1}) NaOH before analysis by gas chromatography. The gas chromatograph (Hewlet-Packard 5890A) was equipped with a flame ionization detector and a capillary column Ultra 2-Hewlett Packard nº 19091B-102 (Cross-linked 5% phenyl-methyl silicone; 25 m × 0.22 mm ID; film thickness, 0.33µm; phase ratio, 150) with hydrogen as the carrier gas. FAME peaks were automatically integrated by a Hewlet-Packard 3365 ChemStation and bacterial isolates named using the MIDI Microbial Identification Software (Sherlock TSBA Library version 3.80; Microbial ID, Inc.). The FAME profile of *Xanthomonas maltophilia* ATCC 13637 was used as a reference for the MIDI determinations.

In addition to being characterized using the FAME profiles, each rhizobacteria isolate was assessed for carbon substrate utilization using the Biolog microplate panel (Biolog, Inc., Hayward, USA). The Biolog microplates comprise pre-filled and dryed panels with 95 different carbon substrates for both Gram + and Gram - isolates. Briefly, isolates were grown on liquid medium at 28°C for 24 hs and a bacterial suspension inoculated in each panel. After a 24 h incubation, carbon utilization reactions were assessed.

Results

Isolation of rhizobacteria:

Results for the enumeration and isolation of rhizobacteria associated with the canola crop are reported in Table 1. Populations of total heterotroph bacteria were similar for the three sites, but varied for the plant part or soil factor studied. In general the populations of heterotrophs exhibited the following trend (from the highest to the lowest): rhizosphere soil> rhizoplane>leaves>endo-

rhizosphere (root interior) (Table 1). Numbers of putative N_2-fixers varied from 4.0×10^2 to 2.8×10^6 cfu·g^{-1} of plant material or soil in the three sites. The highest populations of putative N_2-fixers were detected in rhizosphere soil from Bellevue (Table 1), whereas the lowest were detected in the endo-rhizosphere of plants grown in Allan and Bellevue. The number of pseudomonads also varied from site to site and number of cfu·g^{-1} of soil or canola plant material in all three sites ranged from 0 to 4×10^5 (data not shown).

Nitrogenase activity (N_2-fixation ability):

Results from the nitrogenase activity assay are reported in Table 2. Of the 890 isolates assessed for nitrogenase activity, only 37 demonstrated nitrogenase activity. Of these, 2 isolates were obtained from the rhizoplane and 9 isolates from the rhizosphere of canola plants grown at the Allan site. Six isolates were obtained from the rhizosphere soil from Bellevue. Three isolates were obtained from the endorhizosphere and 16 isolates from the rhizosphere of canola plants grown in Watrous soil. Isolates RSN17 and RSN18 exhibited the highest nitrogenase activity *i.e.*, 139.7 - 146.3 ppm C_2H_4·h^{-1}, respectively (Table 2). The remaining isolates reduced acetylene at levels ranging from 0.1 to 25.6 ppm·h^{-1}. All N_2-fixing rhizobacteria were re-streaked (2 ×) on CC plates, checked for purity, stored liquid CC medium using glycerol as a cryo-protectant agent at -80°C and identified.

Bacterial identification:

The 37 putative N_2-fixing isolates were comprised of two distinct groups (27 Gram + and 10 Gram - rhizobacteria). The most common N_2-fixing bacteria associated with canola were bacilli (62.1%) with pseudomonads (10.8%) being next most abundant (Table 2). The FAME profiles indicated that the bacilli group was comprised of several species with the most abundant being *Bacillus polymyxa*. Other rhizobacteria identified included *Xanthomonas maltophilia*, *Pseudomonas savastanoi*, *Cellulomonas biazotea*, *Erwinia herbicola* and *Ochrobactrum anthropi*. With the exception of *Bacillus* spp. and *E. herbicola*, none of these genera have been previously reported to fix nitrogen. Six N_2-fixing isolates could not be identified using the MIDI system.

Most of the N_2-fixing rhizobacteria metabolized between 66 - 72% of the carbon substrates tested (Table 3). There was no significant differences in the total numbers of substrates used by the Gram - and Gram + isolates. However, these two groups varied in their ability to utilize different types of compounds. For example, the Gram - isolates more readily used carboxilic acids than the Gram + group (74% *vs.* 44%) whereas the Gram + species metabolized more of the simple carbon chain carbohydrates (77% *vs.* 54%). These results indicate that diazotroph bacteria isolated from canola plants can utilize a wide range of carbon compounds. It is well known that inadequate supply of carbon and energy sources in the rhizosphere are limiting factors for the N_2-fixation process

(Postgate and Hill, 1979). Thus the ability of these diazotrophs to utilize a wide range of carbon sources might be important for N_2-fixing efficiency in the canola rhizosphere.

Table 1. Number (cfu·g^{-1}) of total heterotroph and putative N_2-fixing bacteria in leaves, roots (interior and surface) and soil from canola grown at three sites in Saskatchewan.

Site	Bacterial population	Leaves[1] ($\times 10^5$)	Root interior[1] ($\times 10^3$)	Root surface[1] ($\times 10^5$)	Root soil[2] ($\times 10^6$)
Bellevue	Total heterotrophs	5.8	1.3	26.0	7.4
	Putative N_2-fixers	3.1	0.4	5.9	2.8
Allan	Total heterotrophs	0.8	0.4	4.8	5.9
	Putative N_2-fixers	0.4	0.4	0.5	0.2
Watrous	Total heterotrophs	19.0	2.6	19.0	7.8
	Putative N_2-fixers	3.9	2.0	3.7	1.2

[1,2] Fresh and dry weight basis, respectively.

Conclusions

Our results demonstrate that N_2-fixing bacteria can be isolated from rhizosphere soil and several parts of canola plants grown in the field, thus N_2-fixers are active members of the bacterial community in canola fields. However, this N_2-fixing community represented only *ca.* 5% of the over 800 isolates screened. These rhizobacteria have been identified and characterized for substrate utilization. Bacterial identification indicated that *Bacillus polymyxa* strains were the most abundant (14/29 identified) N_2-fixing bacteria associated with canola plants. However, statistical analyses indicated that some *B. polymyxa* found in canola plants from Allan and Watrous were related to the subspecies level. Thus, these strains were associated with plants at all three field locations. These results indicate that some strains of *Bacillus* spp. form close association with canola and can be readily isolated from several field sites. These diazotrophs were subsequently tested in growth chamber assays for their ability to fix N_2 in association with canola grown in soil.

Table 2. Nitrogenase activity (ethylene formed per hour) and FAME identification and source of putative N_2-fixing bacteria isolated from field grown canola plants.

Isolate code	C_2H_4 formed (ppm h^{-1})	Identification (FAME)	MIDI ID[a] (similarity index)	Origin (source - site)
RPC18	0.1	Unknown	-	Rhizoplane - Allan
RPC19	1.8	Unknown	-	Rhizoplane - Allan
EC408A	6.8	*Bacillus polymyxa*	0.270	Endorhizosphere - Allan
EC409B	2.6	*Xanthomonas maltophilia*	0.162	Endorhizosphere - Allan
EC460A	5.6	*Bacillus brevis*	0.207	Endorhizosphere - Allan
RSC465A	10.3	*Bacillus polymyxa*	0.325	Rhizosphere soil - Allan
RSC465B	6.7	*Bacillus polymyxa*	0.233	Rhizosphere soil - Allan
RSC465C	5.4	*Bacillus polymyxa*	0.224	Rhizosphere soil - Allan
RSC466A	7.6	*Xanthomonas maltophilia*	0.081	Rhizosphere soil - Allan
RSC466B	7.5	Unknown	-	Rhizosphere soil - Allan
RSC487	7.8	*Ochrobactrum anthropi*	0.579	Rhizosphere soil - Allan
RSN17	139.7	*Bacillus polymyxa*	0.192	Rhizosphere soil - Bellevue
RSN18	146.3	*Bacillus polymyxa*	0.246	Rhizosphere soil - Bellevue
RSN19	0.6	*Variovorax paradoxus*	0.226	Rhizosphere soil - Bellevue
RSN172A	12.7	*Bacillus megaterium*	0.210	Rhizosphere soil - Bellevue
RSN229A	10.4	*Bacillus polymyxa*	0.122	Rhizosphere soil - Bellevue
RSN229B	8.0	*Cellulomonas biazotea*	0.184	Rhizosphere soil - Bellevue
ES597A	7.4	*Bacillus polymyxa*	0.332	Endorhizosphere - Watrous
ES600A	3.9	*Bacillus polymyxa*	0.613	Endorhizosphere - Watrous
ES600B	11.4	*Bacillus pumilus*	0.332	Endorhizosphere - Watrous
RSS4	16.1	*Bacillus polymyxa*	0.251	Rhizosphere soil - Watrous
RSS20	9.7	*Bacillus polymyxa*	0.769	Rhizosphere soil - Watrous
RSS21	6.8	*Bacillus polymyxa*	0.783	Rhizosphere soil - Watrous
RSS875-1	10.5	Unknown	-	Rhizosphere soil - Watrous
RSS875-2	11.5	*Bacillus polymyxa*	0.387	Rhizosphere soil - Watrous
RSS876-2	25.6	*Pseudomonas savastanoi*	0.072	Rhizosphere soil - Watrous
RSS879-1	10.6	*Erwinia herbicola*	0.290	Rhizosphere soil - Watrous
RSS879B-2	8.0	*Xanthomonas maltophilia*	0.033	Rhizosphere soil - Watrous
RSS882A	0.1	*Bacillus polymyxa*	0.456	Rhizosphere soil - Watrous
RSS885A	0.2	Unknown	-	Rhizosphere soil - Watrous
RSS886A	13.9	Unknown	-	Rhizosphere soil - Watrous
RSS905-1	14.4	*Bacillus polymyxa*	0.307	Rhizosphere soil - Watrous
RSS905-2	7.5	*Bacillus polymyxa*	0.527	Rhizosphere soil - Watrous
RSS910A	7.3	*Bacillus macerans*	0.080	Rhizosphere soil - Watrous
RSS910A-1	13.4	*Bacillus polymyxa*	0.015	Rhizosphere soil - Watrous
RSS913A-2	2.2	*Bacillus polymyxa*	0.479	Rhizosphere soil - Watrous
RSS901A-2	5.1	*Bacillus polymyxa*	0.373	Rhizosphere soil - Watrous
Control A	35.6	*Azospirilum brasilense*[b]	-	Lab culture collection
Control B	54.0	*Azotobacter chroococcum*[b]	-	Lab culture collection

[a]After overnight on TSA medium. Values of > 0.5 are considered a good match. [b]Known diazotroph.

Table 3. Carbon utilization by N_2-fixing rhizobacteria associated with canola crop.

Gram (-) MicroPlate	% isolates (n=10)	Gram (+) MicroPlate	% isolates (n=27)
CARBON SOURCE		CARBON SOURCE	
Polymers		**Polymers**	
α-Cyclodextrin	0	α-Cyclodextrin	0
Dextrin	40	β-Cyclodextrin	37
Glycogen	40	Dextrin	96
Tween 40	70	Glycogen	37
Tween 80	70	Inulin	3.7
Carbohydrates		Mannan	0
N-Acetyl-D-galactosamine	40	Tween 40	0.7
N-Acetyl-D-glucosamine	50	Tween 80	0.7
Adonitol	0	**Carbohydrates**	
L-Arabinose	0	N-Acetyl-D-glucosamine	3.7
D-Arabitol	0	N-Acetyl-D-mannosamine	0.7
Cellobiose	40	Amygdalin	22
I-Erythritol	0	L-Arabinose	0.7
D-Fructose	40	Arabitol	0
L-Fucose	0	Arbutin	48
D-Galactose	0	Cellobiose	81
Gentiobiose	30	D-Fructose	96
α-D-Glucose	40	L-Fucose	0
m-Inositol	0	D-Galactose	48
α-Lactose	30	D-galacturonic acid	26
Lactulose	30	Gentiobiose	81
Maltose	40	D-gluconic acid	26
D-Mannitol	0	α-D-Glucose	96
D-Mannose	40	*m*-Inositol	0
D-Melibiose	40	α-D-Lactose	0.7
β-Methyl-D-glucoside	40	Lactulose	0
Psicose	0	Maltose	78
D-Raffinose	0	Maltotriose	48
L-Rhamnose	0	D-Mannitol	3.7
D-Sorbitol	0	D-Mannose	78
Sucrose	30	D-melezitose	3.7
D-Trehalose	40	D-Melibiose	33
Turanose	40	α-Methyl D-galactoside	0
Xylitol	0	β-Methyl-D-galactoside	0.7
Esters		3-methyl glucose	81
Methyl puryvate	70	α-Methyl D-glucoside	0.7
Mono-methyl succinate	70	β-Methyl D-glucoside	52
Carboxylic acids		α-Methyl D-mannoside	0.7
Acetic acid	70	Palatinose	33
cis-Aconitic acid	50	D-Psicose	96
Citric acid	60	D-Raffinose	0.7
Formic acid	40	L-Rhamnose	0
D-galactonic acid lactone	30	D-Ribose	59
D-Galacturonic acid	0	Salicin	81
D-Gluconic acid	40	Sedoheptulosan	0.7
D-Glucosaminic acid	0	D-Sorbitol	15
D-Glucuronic acid	0	Stachyose	0
α-Hydroxybutyric acid	60	Sucrose	41
β-Hydroxybutyric acid	50	D-Tagatose	0
γ-Hydroxybutyric acid	60	D-Trehalose	0
p-Hydroxyphenylacetic acid	30	Turanose	81
Itaconic acid	40	Xylitol	0
α-Ketobutyric acid	70	D-Xylose	33
α-Ketoglutaric acid	70	**Carboxylic acids**	
α-Ketovaleric acid	0	Acetic acid	15
D,L-Lactic acid	60	α-Hydroxybutyric acid	0
Malonic acid	20	β-Hydroxybutyric acid	0
Propionic acid	70	γ-Hydroxybutyric acid	0
Quinic acid	0	p-Hydroxyphenylacetic acid	0
D-Saccharic acid	0	α-Ketoglutaric acid	0
Sebacic acid	30	α-Ketovaleric acid	3.7
Succinic acid	70	Lactamamide	0
Brominated chemicals		D-Lactic acid Methyl Ester	0
Bromosuccinic acid	70	L-Lactic acid	3.7
Amides		D-Malic acid	0
Succinamic acid	50	L-Malic acid	3.7
Glucuronamide	0	Methyl Pyruvate	78
Alaninamide	70	Mono-methyl succinate	0
Amino acids		Propionic acid	3.7
D-alanine	70	Pyruvic acid	96
L-Alanine	70	**Amides**	
L-Alanyl-glycine	70	Succinamic acid	0
L-Asparagine	70	Succinic acid	0
L-Aspartic acid	70	N-acetyl L-glutamic acid	0.7
L-Glutamic acid	60	Alaninamide	0
Glycyl-L-aspartic acid	70	**Amino acids**	
Glycyl-L-glutamic acid	70	D-alanine	0.7
L-Histidine	60	L-Alanine	0.7
Hydroxy-L-proline	20	L-Alanyl-glycine	0.7
L-Leucine	60	L-Asparagine	0.7
L-Ornithine	0	L-Glutamic acid	0.7
L-Phenylalanine	30	Glycyl-L-glutamic acid	0
L-Proline	70	L-Pyroglutamic acid	0
L-Pyroglutamic acid	0	L-Serine	0.7
D-Serine	50	**Amine**	
L-Serine	70	Putrecine	0
L-Threonine	60	**Alcohols**	
D, L-Carnitine	0	2,3-Butanediol	0
γ-Aminobutyric acid	0	Glycerol	15
Aromatic chemicals		**Aromatic chemicals**	
Urocanic acid	50	Adenosine	22
Inosine	40	2-Deoxy adenosine	3.7
Uridine	40	Inosine	15
Thymidine	20	Thymidine	26
Amines		Uridine	26
Phenylethylamine	30	**Phosphorilated chemicals**	
Putrecine	0	Adenosine-5-monophosphate	0
2-Aminoethanol	0	Thymidine-5-monophosphate	0
Alcohols		Uridine-5-monophosphate	0
2, 3-Butanediol	30	Fructose-6-phosphate	22
Glycerol	20	Glucose-1-phosphate	0.7
Phosphorilated chemicals		Glucose-6-phosphate	15
D, L-α-Glycerol phosphate	4	D, L-α-Glycerol phosphate	0
Glucose-1-phosphate	20		
Glucose-6-phosphate	30		

Study #2:

Validation of candidate diazotrophic strains for their ability to exhibit nitrogenase activity in the rhizosphere of canola plants in growth chamber assays (bacteria × canola screening).

Introduction

Some bacteria species found in the rhizosphere of plants, exhibit nitrogenase activity thus having the potential to fix N_2 from the atmosphere (Postgate and Hill, 1979). One way to exploit the potential of N_2-fixation is to screen and select plant host and N_2-fixing bacteria that exhibits significant amounts of nitrogenase activity (Rennie, 1981). Measurement of nitrogenase activity is a simple method when using the acetylene reduction assay - ARA (Hardy *et al.*, 1973). The intact plant assay technique have been used successfully to screen cultivars × diazotrophs, thus diazotroph rhizobacteria may be promising plant growth promoting rhizobacteria (Jain and Rennie, 1986). However, few attempts have been made to determine the influence of diazotrophic rhizobacteria on the development of canola plants through N_2-fixation. We have isolated several putative N_2-fixing bacteria from canola plants and rhizosphere soil. These diazotrophic bacteria have been tested for N_2-fixation activity *in vitro* (acetylene reduction activity) and identified to the species level. The objective of this study was to screen diazotrophs for their ability to exhibit acetylene reduction in the rhizosphere and identify potential canola-diazotroph associations.

Materials and Methods

A Dark Gray Luvisol (Typic Cryoboralf) soil from the A horizon of the Choiceland association was used for the diazotrophic screening. Soil characteristics (Table 4) indicated that the nutrient levels of this field soil were very low. The soil was sieved (<0.5 cm mesh), 500 g added to plastic pots and then watered with distilled water. Preliminary germination tests indicated that a soil moisture level of 40% of field capacity was adequate for seed germination and plant growth.

Table 4. Nutrient content* of the soils used in the screening study.

Soil location	pH (H_2O)	Cond. ($mS \cdot cm^{-1}$)	O.M. (%)	NO_3	P	K ($kg \cdot ha^{-1}$)	SO_4	sand	silt (%)	clay	texture
Choiceland	7.8	0.3	2.3	15	30	91	21	86.4	8.2	5.0	sand loam
Allan	8.5	0.2	0.8	8	5	335	26	69.8	18.6	11.6	clay loam

**Plains Innovative Laboratory Services*

Seed inoculation:

Bacterial isolates screened for ARA activity were assessed for diazotrophic association with canola. Isolates were grown on combined carbon (CC) medium plates at 25°C for 48 h, scraped off plates with a sterile glass rod, washed three times in phosphate buffered saline (PBS) and re-suspended in sterile tap H_2O. Canola seeds were inoculated with a bacterial suspension (*ca.* 10^7 cfu·ml^{-1} sterile tap water) and coated with talc. Seed titers were performed by adding 10 inoculated canola seeds to a test tube containing 9 ml sterile tap water. The seeds were vortexed and serial dilutions (1:10) made to 10^{-2} to 10^{-6} and plated in triplicate on CC agar plates. Plates were incubated at 25°C and colonies counted after 48-72 hs. Our results indicated that inoculated canola seeds contained *ca.* 10^2–10^5 cfu·seed^{-1} at seeding.

Plant growth:

Five inoculated canola (*Brassica napus* L.), *var.* Legend, seeds were planted in each of 5 replicate plastic pots. After germination, plants were thinned to 2 per pot and the soil surface covered with a 2-cm layer of sterile polyethylene beads (Dupont Inc., Mississauga, Ontario, Canada) to prevent cross contamination and excessive losses of moisture. Plants were allowed to grow in the growth chamber (photosynthetic irradiance of *ca.* 450-500 μE·m^{-2} s^{-1}) at 24°C under a 14/10 h day/night cycle. Four trials were established, each trial containing 9 to 11 bacterial treatments and a control. Each potential N_2-fixing candidate which demonstrated a positive response was selected for a final screening. The control consisted of wheat seeds inoculated with an autoclaved suspension of isolate RSN172A. *Azospirillum brasilense* ATCC 29729 and *Azotobacter chroococcum* ATCC 9043 were tested in trials 3 and 4, respectively as "positive N_2-fixing isolates". Trials 5A and 5B constituted a final screening of all putative diazotroph candidates identified in trials 1 to 4. The final screening was carried out using two soils of different textures and nutrient levels (Table 4); the Choiceland soil and the Allan soil (collected from an eroded shoulder of a Dark Brown Chernozemic soil - Typic Haploboroll from the Allan association in Saskatchewan). The growth chamber experiments were carried out in a complete randomized block design. The plants were harvested at 46 to 55 days after planting (DAP) and plant height, biomass, and nitrogenase activity - acetylene reduction assay (ARA) of roots determined (Hardy *et al.*, 1973). Plant material (shoot and root) was dried at 65°C in a forced-air oven for 72 h. Shoot material was digested in H_2SO_4-H_2O_2 (Thomas *et al.*, 1967) and total-N in the digests determined using an Autoanalyzer II Technicon™ system (Technicon Industrial Systems, Tarrytown, NY, USA). Plant biomass data were analyzed using the Systat statistic package for ANOVA (Systat, Inc., Evanston, Il, USA). When a significant *F* value was detected, least significance difference (Fisher LSD) was used to separate treatment means from the control (Steel and Torrie, 1960).

Results

Bacteria x canola screening:

Thirty-seven rhizobacteria isolates and 2 known N_2-fixing strains (*A. brasilense* ATCC 29729 and *A. chroococcum* ATCC 9043) were screened for plant growth promotion and N_2-fixation (nitrogenase activity) in the rhizosphere of canola plants grown in the Choiceland soil (Trials 1 to 4). Of the 37 candidate N_2-fixing isolates tested, 14 were inhibitory to canola growth *i.e.*, inhibited total plant biomass by at least 5%, as compared to the uninoculated control (Tables 5 to 8), and only 5 isolates significantly (P<0.05 or 0.10) increased plant biomass. However, different rhizobacteria isolates stimulated growth of different plant parts (*e.g.*, roots, shoots). For example, strain ES 600B produced significant increases in shoot, root and total dry weight (Table 6), whereas four other isolates (RSN17, RSN19, RPC19 and ES600A) produced increases in shoot dry weight (Table 8). In addition to these significant effects on plant biomass, inoculation of canola seeds with other isolates *e.g.*, RSC466B, RSS913A2, RSC466A, E600A or RSN17 also produced non-significant increases ranging from 8% to 80% in shoot, root, or total plant biomass (Tables 5 to 8). With the exception of isolates RSN229B, RSC466A and RSN18 (Tables 6, 7 & 8, respectively), most of the putative N_2-fixing isolates reduced acetylene (0.01 ppm to 73.6 ppm of C_2H_2 reduced per hour) when the roots of canola were assessed for the nitrogenase activity. However, these results were variable *i.e.*, sometimes inoculated plants exhibited higher, sometimes they exhibited lower nitrogenase activity, as compared to the uninoculated controls (Tables 5 to 8).

Based on the results obtained in the above four trials, 10 N_2-fixing isolates were selected for further testing. These isolates were re-tested using the Choiceland and Allan soils (final screening). Some isolates enhanced some plant growth parameter in both soils (Tables 9 & 10). For example, in the Choiceland soil, inoculation of canola seed with isolates ES600A and RSN17 only produced non-significant 11% - 18% increases in shoot and total plant dry biomass (Table 9). In contrast, in the less fertile and eroded Allan soil (Table 10), isolates RSN17 and RSC466A stimulated (P<0.05 & 0.10) plant height or shoot biomass when compared to the uninoculated controls (Table 10).

Total-N analysis indicated that of the 10 candidate N_2-fixing isolates tested, 5 isolates significantly (P<0.05 or 0.10) increased percentage or total-N in the canola plants. For example, strains ES 600A, RSN17 and RSN19 produced significant increases in the shoot-N of plants grown in the Choiceland soils. Similarly, isolates RPC19, 466A and RSS4 increased shoot-N of plants grown in the Allan soil (Table 11).

Conclusions

These growth chamber studies indicated that screening N_2-fixing bacteria for the ability to exhibit nitrogenase activity and enhance the growth of canola plants is possible. For example, of the 36 candidate N_2-fixing rhizobacteria tested, three isolates were able to reduce acetylene and to enhance the growth of canola seedlings during the six growth chamber trials. These isolates were identified using fatty acid methyl-ester (FAME) profiles as: (*i*) *Bacillus polymyxa* - ES600A isolated from the endorhizosphere of canola grown in Watrous; (*ii*) *Bacillus polymyxa* - RSN17, isolated from rhizosphere of canola grown in Bellevue; and, (*iii*) *Xanthomonas maltophilia* - RSC466A, isolated from rhizosphere soil of canola grown in Allan. The ability of these three diazotrophs to influence canola growth under field conditions was assessed in a field experiment.

Table 5. Effect of inoculation with N_2-fixing bacteria on nitrogenase activity, plant height and biomass of canola (*var.* Legend) plants grown in Choiceland soil. Data are average of 2 plants from 5 replicate pots from trial 1 at 48 DAP.

Treatment (strain)	Seed titer (cfu·seed^{-1}) ($\times 10^2$)	N-ase act. C_2H_2 (ppm·h^{-1})	Plant height (cm)	Plant biomass [dry weight (g·pl.$^{-1}$)] Shoot	Root	Total
Control	-	1.76	38.4	1.25	0.27	1.52
RSN 172a	5.8	5.39	39.8	1.16	0.16	1.32
RSN 229a	8.1	0.40	39.5	1.20	0.18	1.38
EC 408a	8.2	0.46	34.1	1.19	0.24	1.43
EC 409b	1.7	0.15	37.6	1.11	0.21	1.32
EC 460a	2.6	0.10	31.9	1.16	0.29	1.45
RSC 465a	1.7	0.46	28.3	1.24	0.29	1.53
RSC 465b	2.0	0.63	36.3	1.26	0.23	1.49
RSC 465c	5.6	0.34	43.7	1.28	0.25	1.53
RSS 905-2	3.0	0.94	35.5	1.24	0.22	1.46
LSD (5%)	-	n.s.	n.s.	n.s.	0.10	n.s.
(10%)	-	n.s.	n.s.	n.s.	0.09	n.s.

n.s. not statistically significant.

Table 6. Effect of inoculation with N_2-fixing bacteria on nitrogenase activity, plant height and biomass of canola (*var.* Legend) plants grown in Choiceland soil. Data are average of 2 plants from 5 replicate pots from trial 2 at 47 DAP.

Treatment (strain)	Seed titer (cfu·seed^{-1}) ($\times 10^2$)	N-ase act. C_2H_2 (ppm·h^{-1})	Plant height (cm)	Shoot biomass [dry weight (g·pl.$^{-1}$)] Shoot	Root	Total
Control	-	0.00	20.7	0.73	0.16	0.89
RSC 466b	16.1	8.96	25.8	0.86	0.19	1.05
RSS 901A2	4.9	7.46	19.9	0.62	0.14	0.76
RSC 487	9.1	3.82	18.9	0.64	0.12	0.76
RSS 886a	1.3	23.24	24.2	0.68	0.12	0.80
ES 597a	9.1	9.99	25.3	0.83	0.15	0.98
RSN 229B	22.0	0.00	22.8	0.76	0.14	0.90
RSS 913a2	7.3	4.36	30.9	0.90	0.16	1.06
RSS 875-2	9.8	53.47	15.3	0.66	0.14	0.80
ES 600b	22.4	18.74	19.3	0.96*	0.23*	1.19*
LSD (5%)	-	n.s.	n.s.	0.27	0.09	0.33
(10%)	-	n.s.	n.s.	0.22	0.07	0.28

* Denotes statistical significance from the controls at $P<0.10$; n.s. not statistically significant.

Table 7. Effect of inoculation with N_2-fixing bacteria on nitrogenase activity, plant height and biomass of canola (*var.* Legend) plants grown in Choiceland soil. Data are average of 2 plants from 5 replicate pots from trial 3 at 46 DAP.

Treatment (strain)	Seed titer (cfu·seed^{-1}) ($\times 10^2$)	N-ase act. C_2H_2 (ppm·h^{-1})	Plant height (cm)	Plant biomass [dry weight (g·pl.$^{-1}$)] Shoot	Root	Total
Control	-	5.7	21.2	0.75	0.24	0.99
A. brasilense	34.8	17.9	21.5	0.71	0.24	0.95
RSS 905-1	61.0	15.6	16.7	0.70	0.23	0.93
RSS 875-1	44.5	19.3	18.3	0.58	0.19	0.77
RSS 879-1	4.4	73.6	12.4	0.60	0.17	0.77
RSC 466a	18.9	2.0	26.9	0.79	0.28	1.07
RSS 910a	2.0	15.3	20.1	0.69	0.20	0.89
RSS 885a	30.4	0.0	23.4	0.61	0.19	0.80
RSS 876-2	111.7	10.2	22.5	0.81	0.21	1.02
RSS 879b2	46.0	0.0	13.9	0.46	0.15	0.61
LSD (5%)	-	n.s.	18.7	0.53	0.23	0.74
(10%)	-	n.s.	17.0	0.48	0.21	0.67

n.s. not statistically significant.

Table 8. Effect of inoculation with N_2-fixing bacteria on nitrogenase activity, plant height and biomass of canola (*var.* Legend) plants grown in Choiceland soil. Data are average of 2 plants from 5 replicate pots from trial 4 at 55 DAP.

Treatment (strain)	Seed titer (cfu·seed^{-1}) ($\times 10^2$)	N-ase act. C_2H_2 (ppm·h^{-1})	Plant height (cm)	Plant biomass [dry weight (g·pl.$^{-1}$)] Shoot	Root	Total
Control	0.0	0.4	24.8	0.71	0.05	0.76
RSS 4	85.0	1.2	14.9	0.81	0.07	0.88
A. chroococcum	820.0	3.5	15.5	0.74	0.06	0.80
RSS 21	65.0	3.7	8.7	0.79	0.08	0.87
RSS 910A1	6.0	3.2	16.3	0.77	0.08	0.85
RSN 18	360.0	0.0	25.9	0.81	0.06	0.87
RSN 19	100.0	0.3	35.1	1.37**	0.12	1.49**
RSN 17	64.0	0.1	23.0	1.17*	0.10	1.27*
RPC 19	70.0	0.4	25.9	1.26*	0.10	1.37**
RSS 20	120.0	0.3	15.4	1.03	0.09	1.12
ES 600A	460.0	0.9	24.7	1.28**	0.09	1.37**
RSS 882A	380.0	0.2	26.6	0.91	0.06	0.97
LSD (5%)	-	n.s.	14.6	0.55	n.s.	0.56
(10%)	-	n.s.	12.2	0.45	n.s.	0.49

*, ** Denotes statistical significance from the controls at $P<0.10$ and 0.05, respectively; n.s. not statistically significant.

Table 9. Effect of inoculation with N_2-fixing bacteria on nitrogenase activity, plant height and biomass of canola (*var.* Legend) plants grown in Choiceland soil. Data are average of 2 plants from 5 replicate pots from the final trial at 49 DAP.

Treatment (strain)	Seed titer (cfu·seed^{-1}) ($\times 10^2$)	N-ase act. C_2H_2 (ppm·h^{-1})	Plant height (cm)	Plant biomass [dry weight (g·pl.$^{-1}$)] Shoot	Root	Total
Control	0.0	100.0	14.94	0.43	0.24	0.67
RPC 19	33.0	26.4	13.06	0.35	0.20	0.55
913A-2	1.4	36.7	20.24	0.47	0.20	0.67
RSS 876-2	26.0	20.4	19.34	0.32	0.16	0.48
ES 600A	9.4	19.6	15.56	0.48	0.31	0.79
RSN 17	10.0	34.2	16.26	0.51	0.25	0.76
RSC 466A	9.8	16.5	16.32	0.36	0.24	0.60
ES 600B	11.0	18.5	14.32	0.36	0.22	0.58
RSN 19	21.0	104.2	16.62	0.34	0.25	0.58
RSC 466B	6.4	34.3	18.18	0.32	0.19	0.51
RSS 4	4.6	92.4	19.64	0.31	0.16	0.47
LSD (5%)	-	n.s.	n.s.	0.15	n.s.	0.23
(10%)	-	n.s.	n.s.	0.13	n.s.	0.19

n.s. not statistically significant.

Table 10. Effect of inoculation with N_2-fixing bacteria on nitrogenase activity, plant height and biomass of canola (*var.* Legend) plants grown in Allan soil. Data are average of 2 plants from 5 replicate pots from the final trial at 46 DAP.

Treatment (strain)	Seed titer (cfu·seed^{-1}) ($\times 10^2$)	N-ase act. C_2H_2 (ppm·h^{-1})	Plant height (cm)	Plant biomass [dry weight (g·pl.$^{-1}$)] Shoot	Root	Total
Control	0.0	57.2	8.7	0.17	0.08	0.25
RPC 19	54.0	30.9	7.8	0.13	0.07	0.20
913A-2	33.0	71.9	8.7	0.15	0.09	0.24
876-2	220.0	232.1	8.6	0.16	0.07	0.23
600A	27.0	72.0	8.1	0.14	0.05	0.19
RSN 17	3.5	289.2	13.1**	0.20*	0.09	0.29
466A	14.0	21.0	12.6**	0.17	0.10	0.27
600B	15.0	0.0	9.3	0.16	0.08	0.24
RSN 19	63.0	0.0	9.0	0.14	0.07	0.21
466B	30.0	421.8	9.5	0.16	0.07	0.23
RSS 4	4.0	0.0	9.7	0.14	0.05	0.19
LSD (5%)	-	n.s.	3.7	0.04	n.s.	0.06
(10%)	-	n.s.	3.1	0.03	n.s.	0.05

*, ** Denotes statistical significance from the controls at $P<0.10$ and 0.05, respectively; n.s. not statistically significant.

Table 11. Effect of inoculation with N_2-fixing bacteria on N-content of canola (*var.* Legend) plants grown in Choiceland and Allan soils. Data are average of 2 plants from 5 replicate pots from the final trial.

Treatment (strain)	Choiceland total - N		Allan total - N	
	(%)	(mg·pl.$^{-1}$)	(%)	(mg·pl.$^{-1}$)
Control	1.08	4.65	1.00	1.69
RPC 19	1.13	4.00	1.10*	1.41
913A-2	1.05	4.90	1.07	1.62
RSS 876-2	1.12	3.64	1.06	1.64
ES 600A	1.18*	5.66	1.06	1.51
RSN 17	1.26**	6.29**	1.00	1.96
RSC 466A	1.11	3.97	1.09*	1.86
ES 600B	1.11	4.01	1.06	1.65
RSN 19	1.18*	3.82	1.03	1.46
RSC 466B	1.03	3.33	1.08	1.66
RSS 4	1.08	3.29	1.12**	1.61
LSD (5%)	1.11	1.60	0.11	n.s.
(10%)	0.09	1.33	0.09	n.s.

*, ** Denotes statistical significance from the controls at $P<0.10$ and 0.05, respectively; n.s. not statistically significant.

Study #3:

Development of a inoculation method for the introduction and establishment of diazotrophic rhizobacteria in the rhizosphere of canola - Field validation and impact of diazotrophic inoculant for the canola crop.

Introduction

Some rhizobacteria form beneficial associations with plant roots and increase plant growth and yield. Some of the mechanisms behind plant growth promotion include biological nitrogen fixation. We have shown above that diazotrophic bacterial inoculants increased plant biomass and total-N contents of canola in growth chamber studies. The combined capabilities of N_2-fixation and root colonization by diazotrophic bacteria suggest that a biofertilizer inoculant for canola may be developed. We hypothesized that specific bacteria associated with canola plants can be used as seed-applied inoculants to enhance the growth and yields of field grown canola through N_2-fixation.

The objective of this study was to assess N_2-fixing bacteria isolated from plant and rhizosphere of canola for their ability to reduce atmospheric nitrogen and increase canola yield under field conditions.

Materials and methods

Bacteria growth and seed inoculation:

The bacteria assessed as inoculants for canola in the field study were selected during the screenings. Diazotroph isolates included: *Bacillus polymyxa*-ES600A, *B. polymyxa*-RSN17 and *Xanthomonas maltophilia*-RSC466A. For inoculant production, diazotrophs were grown on Petri plates containing Combined Carbon medium (Rennie, 1981) at 25°C for 72 hr. Cells were scraped from the plates, washed three times in phosphate buffered saline (PBS), and suspended in 50 ml of sterile tap water to yield a concentration of approximately 10^{10} cfu·ml^{-1}. Canola (*Brassica napus* L., *cv.* Legend) seeds were inoculated by soaking 10.0 g of seeds in 7.0 ml of the bacterial suspension for 1 min at 25°C. Inoculated seeds were placed in sterile plastic bags containing 7.0 g of talc. Seeds were rolled in this formulation until a uniform layer of talc was formed, air-dried (*ca.* 10 min.) and seeded into soil within 2 hs. This procedure yielded *ca.* 10^2–10^5 cfu·seed^{-1} at seeding.

Plant growth and plot set-up:

The canola microplots (Photo 1) were established on a sand clay Orthic Brown Chernozemic soil - Haploboroll - Haverhill association (soil chemical characteristics: pH, 6.7; conductivity, 0.2 $mS \cdot cm^{-1}$; organic matter (%), 1.6; and ($kg \cdot ha^{-1}$ - NO_3^-, 50; P, 108; K, 1340; S, 24) located in Central Butte - Saskatchewan. Inoculated canola seeds were planted at a rate of 6.0 $kg \cdot ha^{-1}$ during the spring of 1997. Each block (1.0 m^2) consisted of 6 rows of canola 1m in length. No fertilizers were applied in the field study. Treatments were set-up in a randomized complete block design with six replicates: (*i*) Control - autoclaved isolate RSN17, (*ii*) *B. polymyxa*-RSN17, (*iii*) *B. polymyxa*-ES600A; and (*iv*), *X. maltophilia*-RSC466A.

Plant Sampling:

Plants were harvested at maturity from the entire microplot (1.0 m^2 total harvest area). Plant material was dried at 65°C for 72 h, straw and grain separated and weighed. Data of plant biomass were analyzed for ANOVA [Systat, Inc., Evanston, IL, USA] (Steel and Torrie, 1960).

Photo 1. General view of canola field microplot located at Central Butte, Saskatchewan.

Results

Plant growth:

Three diazotrophs were tested as seed inoculants for canola during the 1997 field trial. These strains were chosen based on their performance for the ability to exhibit nitrogenase activity *in vitro* and in the growth chamber studies (above). In general, inoculation with the three diazotrophs, produced no significant effect on canola grown in the field (Table 12). However, a non-significant seed yield increase of *ca.* 20% (230 $kg \cdot ha^{-1}$) was observed for strain RSN17 when compared to the uninoculated controls (Table 12).

Table 12. Effect of inoculation with N_2-fixing bacteria on biomass of canola (*var.* Legend) plants grown in the field. Data are average of 6 replicate $1m^2$ microplots at harvest.

Treatment (strain)	Tillering ($nº \cdot m^{-2}$)	Plant biomass [dry weight ($g \cdot m^{-2}$)] Seed	Stem	Total
Control	154	98	371	469
ES600A	131	90	350	440
RSN17	131	121	394	515
RSC466A	158	97	363	460

Summary

Candidate N_2-fixing rhizobacteria *i.e.*, diazotrophs were assessed for canola growth promotion in growth chamber studies. Isolates that exhibited ARA activity in the rhizosphere or enhanced the growth of canola seedlings were then assessed for their ability to promote canola growth and N-uptake in the two different soils. Three candidate N_2-fixing rhizobacteria *i.e.*, a *Bacillus polymyxa*, strains ES600A and RSN17 and a *Xanthomonas maltophilia*-RSC466A increased ($P<0.10$) percentage shoot-N content of canola plants grown in these soils. These three diazotrophs were tested subsequently in a field study for their ability to increase canola yield. Of the three strains tested, *B. polymyxa*-RSN17 was a promising biofertilizer for canola, thus it deserves further attention.

Summary and general conclusions

The results obtained from this research project indicate that putative nitrogen-fixing bacteria are active members of the bacteria community in canola fields, and that they can be readily isolated from several parts of canola plants and rhizosphere soil. Over 800 isolates obtained from canola plants were screened for N_2-fixing activity associated with canola and a collection of 37 N_2-fixing rhizobacteria isolates has been established. Of this bacterial collection, the most promising diazotrophs were two *Bacillus polymyxa* strains (ES600A and RSN17) and a *Xanthomonas maltophilia*, strain RSC466A. These diazotrophs were tested for association with canola plants, and estimates of C_2H_2-reducing activity with excised roots indicated a potential for significant N_2-fixation. In fact, seed inoculation with these N_2-fixing rhizobacteria promoted significant accumulation of N in the shoot of canola plants. This suggests that the quantity of N_2 fixed by the rhizobacteria may be significant. Thus, a small-plot field study was established to assess the ability of strains ES600A, RSN17 and RSC466A to enhance the growth and yields of field grown canola through N_2-fixation. Results from this field study indicated that seed inoculation with the three diazotrophs, produced neutral and/or no significant effect on grain yield of canola grown in the field. However, seed inoculation with *B. polymyxa*-RSN17 produced a non-significant grain yield increase of *ca.* 20% when compared to the uninoculated controls. Hence, this *B. polymyxa*-RSN17 strain constitutes a promising diazotroph rhizobacteria that may be economically important for canola growers. Replicated field studies should be performed to validate the usefulness of this diazotroph as a biofertilizer for canola.

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