Microbial Ecology

Inoculation Effects of *Pseudomonas putida*, *Gluconacetobacter azotocaptans*, and *Azospirillum lipoferum* on Corn Plant Growth Under Greenhouse Conditions

Samina Mehnaz and George Lazarovits

Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, Canada N5V4T3

Received: 30 September 2005 / Accepted: 18 October 2005 / Online publication: 6 April 2006

Abstract

Alcohol production from corn is gaining importance in Ontario, Canada, and elsewhere. A major cost of corn production is the cost of chemical fertilizers and these continue to increase in price. The competitiveness of alcohol with fossil fuels depends on access to low-cost corn that allows growers to earn a sustainable income. In this study we set out to determine if we can identify root-associated microorganisms from Ontario-grown corn that can enhance the nutrient flow to corn roots, directly or indirectly, and help minimize the use of extraneous fertilizer. Bacteria were isolated from corn rhizosphere and screened for their capacity to enhance corn growth. The bacteria were examined for their ability to fix nitrogen, solubilize phosphate, and produce indole acetic acid (IAA) and antifungal substances on potato dextrose agar. Bacterial suspensions were applied to pregerminated seed of four corn varieties (39D82, 39H84, 39M27, and 39T68) planted in sterilized sand and unsterilized cornfield soil. The plants were grown under greenhouse conditions for 30 days. Three isolates were identified as having growth-promoting effect. These bacteria were identified as to species by biochemical tests, fatty acid profiles, and 16S rDNA sequence analysis. Corn rhizosphere isolates, *Gluconacetobacter azotocaptans* DS1, *Pseudomonas putida* CQ179, and *Azospirillum lipoferum* N7, provided significant plant growth promotion expressed as increased root/shoot weight when compared to uninoculated plants, in sand and/or soil. All strains except *P. putida* CQ179 were capable of nitrogen fixation and IAA production. *Azospirillum brasilense*, however, produced significantly more IAA than the other isolates. Although several of the strains were also able to solubilize phosphate and produce metabolites inhibitory to various fungal pathogens, these properties are not considered as contributing to growth promotion under the conditions used in this study. These bacteria will undergo field tests for their effect on corn growth.

Introduction

The use of rhizosphere-associated microorganisms as biofertilizers is now being considered as having potential for improving plant productivity [37]. Vessey [37] defines biofertilizers as substances that contain living microorganisms that when applied to seed, plant surfaces, or soil colonize the plant and promote its growth by increasing the nutrient availability. Rhizosphere-associated nitrogen fixing and phosphate-solubilizing bacteria have been used as inoculum for nonlegume crop species such as corn, rice, wheat, and sugarcane [3, 31]. Many of the bacteria that increase plant growth were shown to possess the ability to solubilize phosphate, increase the efficiency of biological nitrogen fixation, improve the availability of Fe and Zn, and alter the growth of roots or shoots by production of plant hormones [14]. However, the actual mechanisms have rarely been clearly identified except for some bacteria that act as biological control agents. Strains of *Pseudomonas putida* and *Pseudomonas fluorescens* were particularly effective in increasing root and shoot elongation in canola, lettuce, and tomato and yield of potato, radish, rice, sugar beet, tomato, lettuce, apple, citrus, bean, ornamental plants, and wheat [29]. Asymbiotic nitrogen-fixing bacteria replaced 60% of the nitrogen requirements of sugarcane amounting to 200 kg N/ha [36]. *Gluconacetobacter diazotrophicus*, a species found in high numbers in roots and stems of sugarcane in Brazil and Australia [33], was the only known nitrogen-fixing species

Correspondence to: George Lazarovits; E-mail: lazarovitsg@agr.gc.ca

DOI: 10.1007/s00248-006-9039-7 • Volume 51, 326–335 • © Springer Science+Business Media, Inc. 2006

of this genus until Jimenez-Salgado *et al.* [13] isolated two other acetic-acid-producing, nitrogen-fixing bacteria (*Gluconacetobacter azotocaptans* and *Gluconacetobacter johannae*) from the rhizosphere of coffee plants [7]. *G. diazotrophicus* has numerous properties associated with enhanced plant growth including nitrogen fixation at low pH (5 or less), which is only partially inhibited by ammonia, production of growth hormones, phosphate solubilization, and antagonistic potential against plant pathogens [22]. These properties make it highly suitable as a candidate for a biofertilizer. *G. azotocaptans* has also been shown to fix nitrogen at low pH, even in the presence of 10 mM NO_3 [7], but we could not find any reports concerning its ability to produce plant hormones, solubilize phosphate, or produce antifungal metabolites.

Azospirillum spp. are commonly isolated bacteria from the rhizosphere of various grasses and cereals and are well characterized as plant-growth-promoting rhizobacteria (PGPR). Many published reports exist on the use of *Azospirillum* spp. for inoculation of cereals [18]; in France, *Azospirillum lipoferum* is used as commercial fertilizer for maize under the trade name AzoGreen-m [12]. Plant-growth promotion by *Azospirillum* is not fully understood. Initially, it was known only as a nitrogen fixer, but the current opinion is that the primary mechanism is related to production of growth-promoting substances such as cytokinins, gibberellins, and auxins [32].

Alcohol production from corn has seen tremendous growth in Ontario, and the pace is accelerating. To keep the cost of alcohol competitive with fossil fuels, it is necessary to produce corn as cheaply as possible. As fertilizer costs are a major component of corn production, the use of biofertilizers may be one way of reducing production costs. The primary objective of this study was to examine the rhizosphere residents of corn plants grown in southwestern Ontario for potential use as biofertilizers. In this study, we present the isolation and identification of nitrogen-fixing and indole acetic acid (IAA)-producing bacteria from corn rhizosphere. These isolates and three *Gluconacetobacter* strains (*G. diazotrophicus* Wt and *nif* D, kindly provided by Agribiotics, Canada; *G. azotocaptans* DS1, previously isolated by our laboratory from corn rhizosphere) were screened for their growth-promoting abilities on four corn varieties under greenhouse conditions.

Materials and Methods

Isolation. Rhizosphere soil (collected in 2003) from local sweet corn varieties, i.e., Extra Super Sweet and Precious Gem, was used for isolation of nitrogen-fixing bacteria (soil properties: loam; sand 45%, clay 40%, silt 15%; organic carbon 0.9%, organic matter 1.7%, pH 6.3). These varieties were found growing in experimental plots at the farm owned by Agriculture and Agri-Food Canada (London, Ontario, Canada). Soil was directly inoculated into glass vials containing semisolid malate medium (in grams per liter: KH_2PO_4 0.4; K_2HPO_4 0.1; $MgSO_4{\cdot}7H_2O$ 0.2; NaCl 0.1; $CaCl_2$ 0.02; $FeCl_3$ 0.01; $Na_2MoO_4{\cdot}2H_2O$ 0.002; sodium malate 5.0;yeast extract 0.05; agar 2; pH 7.2–7.4). The vials were incubated for 48 h at 30°C. The bacteria were then streaked onto malate medium plates. Purification of isolated colonies was done on Luria–Bertani (LB) plates. Seventeen phenotypes of colonies were obtained from rhizosphere soil. All isolates were screened for nitrogen-fixing activity. Two isolates, i.e., N7 and N8, showed nitrogenase activity. These two isolates and an unidentified isolate from corn rhizosphere (CQ179, Agribiotics Inc., Cambridge, Ontario, Canada) were characterized and used for detailed study. In addition, *G. azotocaptans* strain DS1 (isolated on LGI medium, from corn rhizosphere soil collected from Delhi Research Station, Ontario, Canada, and identified in a separate study on the basis of: 16S rRNA gene sequence, Accession No.AY958232, 16S rDNA amplification with species specific primer, Accession No. for this sequence: DQ073427; restriction of amplified 16S rDNA with *Rsa*I enzyme, colony morphology on LGI, Potato dextrose agar, GYC plates, Carbon and nitrogen source utilization pattern; manuscript submitted) and *G. diazotrophicus* Wt isolated from sugar cane in Brazil and its mutant *nif* D (kindly provided by Agribiotics, Canada) were also tested.

Characterization of Isolated Bacteria. For identification of N7, N8, and CQ179 isolates, morphological and biochemical tests, fatty acid analysis, and analyses of the gene sequence encoding 16S rRNA were completed.

Morphological and Biochemical Tests. Colony morphology was observed on nitrogen-free malate medium (NFM) (N7, N8) and LB plates (CQ179). Cell morphology was observed in LB broth. Biochemical tests were performed by using API 20E and API 20NE bacterial identification kits (bioMèrieux, Durham, NC, USA). Cell and colony morphology of two strains (N7, N8) were very similar to each other and *Azospirillum*. Therefore, *A. lipoferum* and *A. brasilense* ATCC strains were used for comparison. API 20NE kit was used for N7, N8, *A. lipoferum* ATCC 29707, and *A. brasilense* ATCC 29729. In addition, N7, N8, *A. brasilense*, and *A. lipoferum* ATCC strains were plated on NFM with 1 mg/L biotin and malate replaced by filter-sterilized 1% glucose [34]. CQ179 was analyzed by both kits (API20E

and 20NE). Oxidase reagent was purchased from the same company and manufacturer's instructions were followed. Catalase was identified by the MacFadden [19] method using H_2O_2 and pure culture colonies from agar plates.

Antibiotic Resistance Pattern. Antibiotic resistance pattern was checked for ampicillin, cycloheximide, gentamycin, kanamycin, rifampicin, spectinomycin, streptomycin, and tetracycline, from 25 to 100 μg/mL, on LB plates. All antibiotics were purchased from Sigma (St. Louis, MO, USA).

Fatty Acid Analysis. Bacteria were subjected to analysis of fatty acid methyl esters, using a microbial identification system equipped with a gas chromatograph (model 6890N, Agilent Technologies, Foster City, CA, USA) and version 5.0 of the aerobe library [Microbial identification system (1993) Operating manual. MIDI, Inc., Newark, Delaware, USA] following the manufacturer's recommended procedures. MIDI is a microbial identification system and similarity index (SI) of 0.6 to 1 indicates excellent match. This analysis was done at University of Guelph, Ontario, Canada.

Polymerase Chain Reaction Amplification and 16S rDNA Sequence Analysis. Pure colonies of isolates N7, N8, and CQ179 were inoculated in LB broth. After overnight growth at 30°C, DNA was isolated from these bacterial cultures by using the Qiagen (Valencia, CA, USA) blood and cell culture DNA Midi kit. The DNA was dissolved in 100 μL TE buffer and used as a template for polymerase chain reaction (PCR) amplification of 16S rDNA. The amplification primers and PCR conditions were the same as those previously described by Mehnaz *et al.* [20]. Each 50-μL reaction mixture contained 0.5 μL *Taq* polymerase (5 U/μL), 5 μL PCR buffer, 2.5 μL $MgCl_2$ (50 mM), 1 μL dNTPs (10 mM), 1 μL (10 μM) of each primer (FGPS4-281 and FGPS1509-153), 37 μL filter-sterilized MilliQ water, and 2 μL of template DNA. The PCR products were purified by using QIAquick PCR purification kit (Qiagen) and sequenced on an Applied Biosystems 3730 Analyzer (Foster City, CA, USA) at Robarts Research Institute (London, Ontario, Canada). Amplification primers as well as internal primers [24] were used for sequencing both strands of PCR products. Additional primers were designed for *Azospirillum* strains 16S-489 (5′–GCCGAACCGAAGAGCCTGA–3′ for N7) and 16S-530 (5′–CGCAAGGTTAAAACTCAAAG–3′ for N8). The sequences were deposited at GenBank (*A. lipoferum* N7, accession no. AY998242; *A. brasilense* N8, accession no. AY958234; *P. putida* CQ179, accession no. AY958233).

Assays For Growth-Promoting Abilities of Isolates

All bacterial isolates (N7, N8, CQ179, DS1, Wt, *nif* D) were screened for nitrogenase activity, IAA production, phosphate solubilization, and antifungal activity by using the following assays.

Nitrogenase Activity. Nitrogenase activity was detected by an acetylene reduction/ethylene production assay. Single bacterial colonies of N7 and N8 were inoculated in vials containing NFM semisolid medium (5 mL/vial). Single bacterial colonies of CQ179, DS1, Wt, and *nif* D were inoculated in semisolid LGI vials. After 24 h growth of N7, N8, CQ179 and 5 days' growth of DS1, Wt, and *nif* D at 30°C, acetylene (10% v/v) was injected into all vials, which were then reincubated at 30°C for 20 h. Ethylene production was measured on an HP 5890 Series II gas chromatograph (Agilent Technologies) fitted with a Carboxen 1006 plot column (Supelco Canada, Oakville, Ontario, Canada) using flame ionization detection. The carrier gas was helium. Ten microliters of the headspace of each vial was sampled using a gastight syringe (Hamilton, Reno, NV, USA) and injected into the gas chromatograph. Ethylene standards in helium (93.5 and 984 ppm; Alltech Associates, Inc., Deerfield, IL, USA) were used for quantification of ethylene in samples. Nitrogenase activity was described in terms of nanomoles ethylene per hour per milligram bacterial protein. Bacterial protein estimation was carried out by using the method of Lowry *et al.* [17].

IAA Production. Bacterial cultures were grown for 7 days in liquid NFM (N7, N8), and DS1, CQ179, Wt, and *nif* D were grown in rich medium (in grams per liter: glucose 2.0, glutamic acid 1.5, peptone 1.5, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, yeast extract 2.0; pH 6). L-Tryptophan (100 mg/L) was added to the NFM and rich medium as a precursor of IAA biosynthesis. For DS1, Wt, and *nif* D, rich medium was also provided with ammonium chloride (100 mg/L) as suggested by Fuentes-Ramirez *et al.* [6]. Cells were harvested at 10,000 rpm for 15 min. The pH of the supernatant was adjusted to 2.8 with hydrochloric acid; then the supernatant was extracted three times with equal volumes of ethyl acetate [35]. The extract was evaporated to dryness and resuspended in 1 mL of ethanol. The samples were analyzed by high-performance liquid chromatography (HPLC) on an HP 1100 system (Agilent Technologies) with diode-array UV detection using a Symmetry C18 column (4.6 × 250 mm; Waters Canada, Mississauga, Ontario, Canada). The mobile phase consisted of methanol/acetic acid/water (30:1:70, v/v/v) and the flow rate was 1.2 mL/min [26]. Standard solutions were prepared with pure indole-3-acetic acid (Sigma) for identification and quantification of IAA produced by the bacterial strains.

Solubilization of Organic Phosphate. Aliquots (10 μL) of bacterial cultures grown overnight in their specific media were spot inoculated onto NBRIP medium [23] and plates were incubated for 14 days at 28°C. Formation of a clear zone around the bacterial growth spot was considered as a positive result.

Antifungal Activity. Antifungal activity was observed by the formation of inhibition zone of mycelial growth, based on agar diffusion of extracellular bacterial metabolites. Two different media were used for this assay, namely, potato dextrose agar (PDA, Difco, Sparks, MD, USA) and minimal medium (MM; in grams per liter: ammonium sulfate 1.0, dipotassium phosphate 7.0, monopotassium phosphate 3.0, magnesium sulfate 0.1, sucrose 10.0, agar 15; pH 7). A small plug cut from a fresh agar culture of target fungus was seeded at the center of medium in a petri plate. Aliquots (10 μL) of bacterial cultures grown in their specific media overnight were spotted 2 cm away from the center (one isolate per plate). They were allowed to air-dry in a laminar-flow cabinet before incubation. Plates were incubated for 10–15 days at 24°C. For comparison, all fungal strains were also grown on PDA and MM media. Bacterial isolates were checked against fungal pathogens, *Helminthosporium carbonum*, *Pythium aphanidermata*, *Pythium ultimum*, and *Fusarium* spp. (*F. culmorum*, *F. graminearum*, *F. moniliforme*, *F. oxysporium*, *F. solani*, *F. sambucinum*). *F. graminearum* and *F. moniliforme* strains were kindly provided by Laboratoire de Diagnostic en Phytoprotection, Provincial Agriculture Ministry, Quebec, Canada. The other strains were from the culture collection of Dr. George Lazarovits. A complete list of the fungal strains tested is given in Table 3.

Plant Experiments. The effects of these bacteria were studied on four corn varieties, i.e., 39D82, 39H84, 39M27, and 39T68. Seeds of these varieties were kindly provided by Pioneer Hi-Bred International, Inc. (Chatham, Ontario, Canada). All varieties were grown in sterilized industrial silica sand and unsterilized cornfield soil of the Delhi Research Station (Ontario, Canada) under greenhouse conditions.

Sand Experiments. Seeds were thoroughly washed with water to remove fungicide. Sodium hypochlorite (0.1%) was used as sterilizing agent. Seeds were soaked in this solution for 5 min and washed three times with autoclaved distilled water. Seeds were grown on wet filter papers in petri plates at 30°C in an incubator. Three-day-old seedlings were transferred into disposable coffee cups (8 oz) and the cups were filled with autoclaved moist sand (~250 g/cup). In addition, 25 mL sterilized water was added to each cup. Two seedlings were planted in each cup. N7 and N8 were grown in LB broth, whereas Wt, *nif* D, CQ179, and DS1 were grown in rich medium. After overnight growth, cultures were harvested at 10,000 rpm for 5 min and pellets were suspended in 0.85% sterilized saline to get a final concentration of 10^8 cells/mL. Individual strains were inoculated at the time of transplantation, at 10^8 bacterial cells per seedling. Control plants were provided with 1 mL sterilized saline per seedling. P_2O_5 (~19 mg P/cup at 20 kg P/ha) was used for phosphorus fertilizer, and K_2O (~7 mg K/cup at 10 kg K/ha) for potassium fertilizer. Both fertilizers were ground to fine powder, suspended in water, and added to moist sand before transplantation of seedlings. Two days after seedling transplantation, ammonium nitrate (~22 mg N/cup at 50 kg N/ha) was applied by dissolving in water. A photoperiod of 14 h light/10 h dark and thermoperiod of 25/20°C was used. Sterilized water was used to keep the sand moist throughout the study. Plants were harvested after 30 days of growth and placed in paper bags for drying in an oven at 70°C for 72 h.

Soil Experiments. Soil used for these experiments (loamy sand; 7.6% clay, 85.6% sand, 6.8% silt; organic matter 1.9%; pH 5.8) was collected from the upper 15 cm of a cornfield located at the Delhi Research Station, Ontario. Seeds were washed and sterilized in the same way as mentioned for sand experiments. Ammonium nitrate (~22 mg N/cup, at 50 kg N/ha), P_2O_5 (~13 mgP/cup at 20 kg P/ha) and K_2O (~7 mg K/cup at10 kg K/ha) were mixed in soil before sowing. Two seeds were planted in each cup containing ~200 g, unsieved, unsterilized soil. Before sowing, 20 mL unsterilized tap water was added to make the soil moist. Soil moisture was maintained by using unsterilized tap water throughout the study. Inoculum was prepared in the same way as mentioned above. At the time of sowing, 10^8 bacterial cells per seed were inoculated. Growing conditions and harvesting were the same as previously described.

Statistical Analyses. All experiments were repeated at least three times. All treatments had six replicates (12 plants; 2 plants per cup). The repeated experiments showed similar trends and there were nonsignificant differences between the same treatments in each experiment. The data were analyzed by using SAS statistical software (ver.8.2, SAS Institute Inc., Cary, NC, USA) on windows XP 2000. One-way analysis of variance (ANOVA) was done with the ANOVA procedure in SAS and comparison among treatments was done by using Duncan's multiple-range test. All analyses were performed at the $P = 0.05$ level.

Table 1. Identification of corn rhizosphere isolates by using API 20NE[a] bacterial identification kit and comparison of *Azospirillum*-like isolates (N7, N8) with *A. lipoferum* ATCC 29707 and *Azospirillum brasilense* ATCC 29729

Tests	*A. lipoferum*	*Isolate N7*	*Isolate N8*	*A. brasilense*	*Isolate CQ179*
Potassium nitrate (NO_3)	+	+	+	+	−
L-arginine	−	−	−	−	+
Urease	−	+	−	+	−
Gelatin	−	−	−	−	+
Esculin ferric citrate	+	+	+	+	+
4-nitrophenyl-βD-galactopyranoside (PNPG)	+	+	+	+	−
Assimilation					
D-glucose	+	+	−	−	+
L-arabinose	+	+	−	−	+
D-mannose	−	−	−	−	+
D-mannitol	+	+	−	−	+
N-acetyl-glucosamine	+	+	+	−	+
D-Maltose	+	−	+	−	−
Potassium gluconate	+	+	−	+	+
Capric acid	+	+	−	−	+
Malic acid	+	+	+	+	+
Trisodium citrate	+	−	−	−	+
Phenyl acetic acid	+	+	−	−	−
Cytochrome-oxidase	+	+	+	+	+
Catalase	+	+	+	+	+

All isolates showed negative results for the presence of indole production, fermentation of D-glucose and assimilation of adipic acid.
[a]API 20NE is a standardized system (bioMèrieux) for the identification of nonfastidious, non-enteric Gram-negative rods, combining eight conventional tests, 12 assimilation tests, and a database.

Results

Characterization of Isolated Bacteria

Morphological and Biochemical Tests. The cells of all three isolates (N7, N8, CQ179) were Gram-negative, motile rods. Cells of N7 and N8 had a semihelical shape, a characteristic of *Azospirillum* spp. Colonies of these strains were flat, dried, and wrinkled on NFM plate. N7 showed a light pink colony and N8 had a deep pink colony on NFM plate. N7 and *A. lipoferum* ATCC strains showed good growth on glucose-containing NFM plates, but N8 and *A. brasilense* ATCC strains did not grow on these plates. CQ179 produced gummy off-white colonies on LB plate, and these emitted fluorescence under UV light.

Biochemical data for N7 and N8 are given in Table 1. Both strains were oxidase and catalase positive. Based on biochemical tests (API NE20 kit) N7 was identified as *A. lipoferum* and N8 as *A. brasilense*. N7 showed similar results to that of *A. lipoferum* except that it was positive for urease and assimilated phenyl acetic acid but did not assimilate D-maltose and trisodium citrate. *A. lipoferum* ATCC strain was negative for urease and showed positive response for D-maltose and weak response for phenyl acetic acid and trisodium citrate. N8 showed similarity with *A. brasilense* ATCC strain, although it was positive for assimilation of *N*-acetyl glucosamine and D-maltose and negative for urease and assimilation of potassium gluconate, as compared to *A. brasilense* ATCC strain. CQ179 was identified as *Pseudomonas* (*fluorescens*/*putida*) by API 20E kit (data not shown). It was unable to distinguish between two species of *Pseudomonas*, but results from API 20NE (Table 1) identified it as *P. putida* (manufacturers recommend use of both kits for non-enteric Gram-negative rods). Fatty acid profile and 16S rDNA sequence analysis were used for further confirmation.

Table 2. Nitrogenase activity, IAA production, and phosphate solubilization by bacterial isolates

Isolates	*Nitrogenase activity (nmol ethylene h^{-1} mg $protein^{-1}$)*	*IAA (ng/mL)*	*Phosphate solubilization*
A. lipoferum N7	6,600 ± 338	6,490 ± 358	−
A. brasilense N8	7,950 ± 3,870	16,400 ± 2,390	−
P. putida CQ179	ND	105 ± 6	+
G. azotocaptans DS1	40 ± 2	106 ± 10	+
G. diazotrophicus Wt	240 ± 11	122 ± 12	+
G. diazotrophicus nif D	120 ± 12	47 ± 3	+

Results are averages of three experiments, each with three replicates. ± indicates standard deviation (SD). IAA: indole acetic acid.

Table 3. Antifungal activity of bacterial isolates on Potato dextrose agar medium (PDA)

Fungi	*Strains/host*	*Wt*	*nif D*	*DS1*	*CQ179*
F. solani	Cucumber	+	+	+	−
F. solani phaseoli	Fsp	+	+	+	+
F. sambucinum		+	+	+	+
F. culmorum		+	+	−	−
F. moniliforme	0606/onion	+	+	+	+
	3487/asparagus	+	+	+	+
F. graminearum	0-3878/tomato	+	+	+	+
	2331/tomato	+	+	+	+
H. carbonum		+	+	−	−
P. aphanidermata	Cucumber	−	−	−	−
P. ultimum		−	−	−	−

A. lipoferum N7 and *A. brasilense* N8 did not show growth on PDA. PDA: potato dextrose agar; Wt: *G. diazotrophicus*; *nif* D: mutant; DS1: *G. azotocaptans*; CQ179: *P. putida*.

Antibiotic Resistance Pattern. N7 and N8 were resistant to ampicillin and cycloheximide up to 100 μg/mL and sensitive to the rest of the antibiotics at 25 μg/mL. CQ179 showed resistance to ampicillin, cycloheximide, and spectinomycin up to 100 μg/mL and for gentamycin and streptomycin up to 50 μg/mL. It showed sensitivity to kanamycin and tetracycline at 25 μg/mL.

Fatty Acid Analysis. Fatty acid analysis identified N7 as *A. brasilense* with a similarity index (SI) of 0.689. N8 was identified as *Roseomonas fauriae* (a bacterium known to be very closely related to *A. brasilense*) with a very high SI of 0.859. CQ179 was identified as *Pseudomonas aureofaciens* (SI = 0.624) with a close similarity to *P. putida* (SI = 0.618).

16S rDNA Sequence Analysis. Partial sequences (~1.3–1.5 kb) of 16S rDNA of three isolates (N7, N8, CQ179) were obtained and compared with the NCBI data bank through BLAST search. N7 showed 98.2% homology (1180 identities/1202 positions) with the sequence of *A. lipoferum* (accession no. X79736.1), 95.9% homology (1247 identities/1300 positions) with the sequence of *A. brasilense* ATCC 29145 (accession no. AY324110). N8 showed 98.8% homology (1284 identities/1300 positions) with *A. brasilense* ATCC 29145 (accession no. AY324110) and 98.6% homology (1287 identities/1305 positions) with *R. fauriae* ATCC 49958 (accession no. AY150046). For the isolate CQ179, comparison of its partial sequence with the NCBI data bank showed 99.3% similarity (1453 identities/1464 positions) with the sequence of *P. putida* (accession no. AF095892).

Assays for Growth-Promoting Abilities of Isolates

Nitrogenase Activity. All strains except *P. putida* CQ179 produced ethylene from acetylene (Table 2). *A. lipoferum* N7 and *A. brasilense* N8 showed very high nitrogenase activity, i.e., 6.6 and 7.9 μmol ethylene h^1 mg^1 protein, respectively. *G. azotocaptans* DS1 showed lowest ethylene production (40 nmol ethylene h^1 mg^1 protein) as compared to the rest of the strains. *G. diazotrophicus* strains (Wt and *nif* D) produced 240 and 120 nmol ethylene h^1 mg^1 protein, respectively. *nif* D is a mutant of Wt strain, but it seems that it still has a part of the *nif* D gene.

IAA Production. IAA production was observed for all strains (Table 2). *A. brasilense* N8 produced the highest amount, 16.8 μg/mL IAA, whereas *A. lipoferum* strain N7 produced 6.5 μg/mL IAA. *P. putida* CQ179 and *G. azotocaptans* DS1 produced similar amounts of IAA, i.e., 105 and 106 ng/mL. *G. diazotrophicus* Wt strain produced 122 ng/mL and its *nif* D mutant produced 47 ng/mL IAA.

Phosphate Solubilization Test. *P. putida* CQ179, *G. azotocaptans* DS1, *G. diazotrophicus* Wt, and its *nif* D mutant showed quick growth and produced a clear zone by solubilizing insoluble phosphates in NBRIP medium (Table 2). *A. lipoferum* and *A. brasilense* strains took long to grow on NBRIP and did not solubilize phosphate.

Antifungal Activity. *P. putida* CQ179, *G. azotocaptans* DS1, and *G. diazotrophicus* (Wt, *nif* D) showed antifungal activity on PDA (Table 3), but *A. lipoferum* N7 and *A. brasilense* N8 did not grow on PDA

Table 4. Effect of bacterial isolates on root weight of four corn varieties after 30 days' growth in sterilized sand

Treatments	*39D82 (mg/plant)*	*39H84 (mg/plant)*	*39M27 (mg/plant)*	*39T68 (mg/plant)*
Control	260 ± 40 bc	210 ± 56 a	270 ± 50 a	220 ± 60 c
A. lipoferum N7	300 ± 42 a	220 ± 36 a	270 ± 47 a	255 ± 52 abc
A. brasilense N8	290 ± 41 ab	200 ± 59 a	265 ± 63 a	270 ± 64 ab
P. putida CQ179	250 ± 34 c	215 ± 39 a	250 ± 46 a	270 ± 64 ab
G. azotocaptans DS1	250 ± 32 c	220 ± 65 a	260 ± 41 a	280 ± 90 a
G. diazotrophicus Wt	260 ± 33 bc	210 ± 57 a	290 ± 42 a	230 ± 49 bc
*G. diazotrophicus nif*D	250 ± 40 c	200 ± 42 a	250 ± 42 a	240 ± 50 abc

Values are averages of 12 replicates. Letters indicate a statistically significant difference between treatments according to Duncan's multiple range test at $P \leq 0.05$. Mean separation within a column followed by the same letters do not differ significantly.

Table 5. Effect of bacterial isolates on shoot weight of four corn varieties after 30 days' growth in sterilized sand

Treatments	*39D82 (mg/plant)*	*39H84 (mg/plant)*	*39M27 (mg/plant)*	*39T68 (mg/plant)*
Control	400 ± 62 bcd	525 ± 108 ab	310 ± 60 b	510 ± 98 d
A. lipoferum N7	430 ± 41 abc	510 ± 124 ab	315 ± 54 b	590 ± 66 abc
A. brasilense N8	450 ± 58 ab	490 ± 124 b	330 ± 66 b	590 ± 130 ab
P. putida CQ179	360 ± 54 d	560 ± 100 a	350 ± 60 b	570 ± 74 abcd
G. azotocaptans DS1	445 ± 71 ab	540 ± 86 ab	400 ± 71 a	630 ± 122 a
G. diazotrophicus Wt	455 ± 65 a	510 ± 98 ab	330 ± 57 b	530 ± 88 cd
*G. diazotrophicus nif*D	450 ± 55 a	535 ± 105 ab	320 ± 51 b	560 ± 85 bcd

Values are averages of 12 replicates. Letters indicate a statistically significant difference between treatments according to Duncan's multiple range test at $P \leq 0.05$. Mean separation within a column followed by the same letters do not differ significantly.

medium. *P. putida* CQ179, *G. azotocaptans* DS1, and *G. diazotrophicus* strains were unable to inhibit the growth of *P. ultimum* and *P. aphanidermata*. *G. diazotrophicus* strains, Wt and *nif* D, successfully inhibited growth of the rest of the fungal strains. *G. azotocaptans* DS1 did not show antifungal activity for *F. culmorum* and *H.* carbonum, in addition to *Pythium* spp. *P. putida* CQ179 inhibited the growth of *Fusarium* spp. (*F. solani phaseoli*, *F. sambucinum*, *F. moniliforme*, *F. graminearum*). All bacterial strains showed growth on minimal medium (M-M), but none of them showed antifungal activity against fungal isolates used in this study (data not shown).

Plant Experiments

Sand Experiments. The response to bacterial inoculum was different for all corn varieties. For corn variety 39D82, significant increases in root weight was found with *A. lipoferum* N7, whereas *G. diazotrophicus* strains Wt and *nif* D resulted in significant increases in shoot weight as compared to control (Tables 4 and 5). For corn variety 39H84, bacterial inoculum showed nonsignificant difference in root and shoot weight as compared to control. For corn variety 39M27, *G. azotocaptans* DS1 significantly increased shoot weight as compared to control but no increase in root weight was found with any treatment. For corn variety 39T68, *G. azotocaptans* DS1 and *A. brasilense* N8 significantly increased root and shoot weight as compared to control. In addition, *P. putida* CQ179 significantly increased root weight and *A. lipoferum* N7 significantly increased shoot weight.

Soil Experiments. For corn variety 39D82, *A. lipoferum* N7 and *P. putida* CQ179 significantly increased root and shoot weight as compared to control (Tables 6 and 7). For corn variety 39H84, *G. azotocaptans* DS1 and *P. putida* CQ179 significantly increased root and shoot weight, respectively. For corn variety 39M27, *P. putida* CQ179 showed significant increase in root and shoot weight. *A. lipoferum* N7 and *G. diazaotrophicus nif* D also significantly increased shoot weight. For corn variety 39T68, nonsignificant increase in root and shoot weight was observed for all bacterial treatments as compared to control.

Discussion

In this study we identified three bacterial isolates from corn rhizosphere with potential benefits to corn production, namely, *A. lipoferum* N7, *A. brasilense* N8, and *G. azotocaptans* DS1. These isolates and three bacterial strains obtained from Agribiotics (*P. putida* CQ179, *G. diazotrophicus* Wt, *nif* D) were tested for their biochemical properties and for their effect on the growth of four corn varieties under greenhouse conditions.

CQ179, a corn isolate provided by Agribiotics, was identified as *P. putida* by biochemical tests, fatty acid

Table 6. Effect of bacterial isolates on root weight of four corn varieties after 30 days' growth in nonsterilized cornfield soil

Treatments	*39D82 (mg/plant)*	*39H84 (mg/plant)*	*39M27 (mg/plant)*	*39T68 (mg/plant)*
Control	240 ± 30 c	290 ± 45 bc	240 ± 45 bc	320 ± 61 a
A. lipoferum N7	290 ± 48 ab	310 ± 53 abc	260 ± 47 abc	340 ± 74 a
A. brasilense N8	250 ± 34 bc	310 ± 70 abc	255 ± 33 abc	330 ± 79 a
P. putida CQ179	300 ± 39 a	330 ± 50 ab	290 ± 52 a	335 ± 63 a
G. azotocaptans DS1	250 ± 57 bc	345 ± 53 a	220 ± 69 c	320 ± 65 a
G. diazotrophicus Wt	240 ± 37 c	280 ± 66 c	260 ± 64 abc	340 ± 75 a
*G. diazotrophicus nif*D	240 ± 25 c	290 ± 75 bc	280 ± 53 ab	340 ± 63 a

Values are averages of 12 replicates. Letters indicate a statistically significant difference between treatments according to Duncan's multiple range test at $P \leq 0.05$. Mean separation within a column followed by the same letters do not differ significantly.

Table 7. Effect of bacterial isolates on shoot weight of four corn varieties after 30 days' growth in nonsterilized cornfield soil

Treatments	*39D82 (mg/plant)*	*39H84 (mg/plant)*	*39M27 (mg/plant)*	*39T68 (mg/plant)*
Control	500 ± 67 b	570 ± 87 b	450 ± 111 b	690 ± 99 a
A. lipoferum N7	570 ± 64 a	610 ± 92 b	560 ± 66 a	710 ± 127 a
A. brasilense N8	520 ± 46 ab	590 ± 120 b	450 ± 109 b	670 ± 97 a
P. putida CQ179	560 ± 59 a	675 ± 88 a	560 ± 63 a	720 ± 131 a
G. azotocaptans DS1	480 ± 42 b	570 ± 149 b	470 ± 111 b	700 ± 144 a
G. diazotrophicus Wt	500 ± 72 b	595 ± 130 b	505 ± 81 ab	710 ± 118 a
*G. diazotrophicus nif*D	520 ± 49 ab	580 ± 117 b	590 ± 99 a	710 ± 120 a

Values are averages of 12 replicates. Letters indicate a statistically significant difference between treatments according to Duncan's multiple range test at $P \leq 0.05$. Mean separation within a column followed by the same letters do not differ significantly.

analysis, and 16S rDNA analysis. Based on results of biochemical tests and 16S rDNA sequence analysis, N7 was identified as *A. lipoferum*. Fatty acid profile and the pink color of N7 colonies suggested that it was *A. brasilense*. Okon *et al.* [25] and Eskew *et al.* [4], however, have reported deep pink strains of *A. lipoferum*. Biochemical tests, particularly the use of glucose as a sole carbon source, clearly differentiated N7 from *A. brasilense* [34]. For further confirmation, we digested amplified 16S rDNA of *A. brasilense* ATCC 29729, of *A. lipoferum* ATCC 29707, and of isolate N7 with the restriction endonuclease *Alu*I and compared the fragments obtained using gel electrophoresis (data not shown), as suggested by Grifoni *et al.* [10]. The restriction fragment patterns for N7 and *A. lipoferum* were identical and, therefore, we are convinced that N7 is *A. lipoferum*. Results of fatty acid profiles of N8 identified it as *R. fauriae*. However, 16S rDNA sequence analysis showed a 99.2% homology (1408 identities/1420 positions) of isolate N8 with genus *Azospirillum* (accession no. AY118225), 98.8% homology with *A. brasilense* ATCC 29145, 98.6% homology with *R. fauriae* ATCC 49958, and 98.2% homology (1281 identities/1305 positions) with *Roseomonas genomospecies* 6 strain ATCC 49961 (accession no. AY150050). Cohen *et al.* [2] also identified *R. fauriae* strain as *A. brasilense* on the basis of morphological and biochemical tests and reported 99.4% (1430 identities/1439 positions) 16S rDNA sequence homology of *R. fauriae* (*R. genomospecies* 3) with *A.* brasilence ATCC 29145 strain and 98% (1450/1481) homology with *R. genomospecies* 6. They suggested that their results, combined with previous studies [30, 38, 27], indicated that establishment of *R. genomospecies* 3 and 6 was not justified and the isolate designated as such should be reclassified into the genus *Azospirillum*. Our results also support their conclusions.

In this study, *P. putida* CQ179, *G. azotocaptans* DS1, and *A. lipoferum* N7 were shown to be highly promising strains for use in corn production. *P. putida* CQ179 significantly increased root weight (39T68 in sand, 39D82 and 39M27 in soil) and shoot weight (39D82, 39H84, and 39M27 in soil) of corn varieties. Fluorescent pseudomonads are aggressive root colonizers and are capable of moving from seeds to root and of promoting plant yield under field conditions [16]. Initially, the mechanism of growth promotion by these pseudomonads was believed to be primarily by antagonism or exclusion of pathogenic and deleterious microorganisms from the rhizosphere. Lifshitz *et al.* [16], however, found that the growth promotion of canola by a nitrogen fixer strain and a nonfixer mutant of *P. putida* GR12-2, under gnotobiotic conditions, was due to phosphate solubilization, not nitrogen fixation. Xie *et al.* [39] reported two- to threefold increase in root length of canola seedlings by the same strain of *P. putida* GR12-2 due to IAA production. *P. putida* CQ179 does not fix nitrogen, but it is an IAA producer and a phosphate solubilizer, and it releases antifungal compounds. Therefore, growth enhancement by this organism may involve more than one mechanism.

G. azotocaptans DS1 significantly improved root weight of corn varieties 39T68 (in sand) and 39H84 (in soil) and shoot weight of corn varieties 39M27 and 39T68 (in sand). Isolate DS1 is a nitrogen fixer, an IAA producer, and a phosphate solubilizer, and it possess antifungal activity. It carries all of the growth-promoting abilities found for *G. diazotrophicus*. Thus, it also appears to be a suitable candidate as a biofertilizer. *G. azotocaptans* is a new, nitrogen-fixing species of *Gluconacetobacter* and was recently isolated from coffee in Mexico [13]. There are no published reports about its plant-growth-promoting activity or use as inoculum. Its discovery in the corn rhizosphere indicates it may play a role in the growth promotion of corn plants.

A. lipoferum N7 promoted root growth of corn variety 39D82 (in sand and soil) and shoot growth of corn varieties 39T68 (in sand) and 39D82 and 39M27 (in soil). Growth promotion by isolate N7 could have involved IAA production and/or nitrogen fixation. Our plants received 50 kg N/ha, and therefore additional nitrogen fixation would have provided only minimal benefits under these conditions. As we did not subject the plants to nitrogenase activity, we cannot completely eliminate this possibility. Auxin production by *Azospirillum* spp. is currently believed to play a major role in plant growth promotion. Although *A. brasilense* N8 showed high nitrogenase activity and IAA production,

it promoted plant growth of only one variety under conditions where the substrate was virtually microorganism-free. *A. lipoferum* N7 had lower nitrogenase activity than *A. brasilense* N8 and produced 2.5-fold less IAA but promoted plant growth of three varieties under both sterilized and unsterilized conditions. Fages and Mulard [5] isolated *A. lipoferum* CRT1from maize rhizosphere and developed it in France as a commercial fertilizer (Azogreen-m) for maize. Field and greenhouse trials conducted by Jacoud *et al.* [11, 12] showed that Azogreen-m enhanced maize root development under both growing conditions.

G. diazotrophicus nif D significantly promoted shoot growth of two corn varieties, 39D82 (in sand) and 39M27(in soil), and *G. diazotrophicus* PAL 5 (Wt) promoted shoot growth of 39D82 (in sand). Riggs *et al.* [28] used the same *G. diazotrophicus* PAL 5 (Wt) strain as inoculum for maize, under greenhouse and field conditions, in the presence and absence of nitrogen fertilizer. They repeated the experiments for 3 years and reported reproducible yield increases. They suggested that these increase are probably caused by a growth regulator response rather than nitrogen fixation since chlorosis was not reduced in plants when nitrogen fertilizer was not applied.

Two corn varieties, 39M27 and 39D82, showed positive response for bacterial inoculum in soil and sand, although the response was variable in sterilized and unsterilized conditions. 39T68 did not show any significant response with all the bacterial strains tested in soil, and 39H84 was nonresponsive to bacterial inoculum in sand. Such variable response to bacterial inoculation with different genotypes or cultivars of the same crop has been commonly observed. Riggs *et al.* [28] studied the effect of a number of diazotrophic bacteria, including *A. brasilense* and *G. diazotrophicus*, on different maize genotypes and observed variable effects. There are several reports about the effect of maize genotypes on the response to *Azospirillum* inoculation [1, 8, 9]. Mirza *et al.* [21] reported the variable response of *Azospirillum*, *Herbaspirillum*, and *Pseudomonas* inoculum to two rice varieties. It has been postulated that these genotypic differences may be due to specificity of plant–bacterial associations, difference in root exudation, and gaseous diffusion efficiency [15]. It would be interesting to work with plant breeders to see if such associations can be increased by breeding selections.

In conclusion, three bacterial strains isolated from corn rhizosphere were characterized as *A. lipoferum* (N7), *A. brasilense* (N8), and *P. putida* CQ179. These bacteria and isolates *G. azotocaptans* DS1 from corn rhizosphere, *G. diazotrophicus* Wt and *nif* D from sugarcane were tested for their ability to fix nitrogen, produce IAA, solubilize phosphate, and produce antifungal agents, and for their effect on the growth of four corn varieties under sterilized and unsterilized conditions. The results show that under greenhouse conditions, *P. putida* CQ179 significantly promoted root and shoot growth of two corn varieties, 39D82 and 39M27, in soil, root weight of 39T68 in sand, and shoot weight of 39H84 in soil. *G. azotocaptans* DS1 significantly increased root and shoot weight of corn variety 39T68 in sand, shoot weight of 39M27 in sand, and root weight of 39H84 in soil. *A. lipoferum* strain N7 showed significant increase in root and shoot weight of 39D82 in soil and root weight of 39D82 in sand as well. Shoot weight of 39T68 (in sand) and 39M27 (in soil) was also significantly enhanced by *A. lipoferum* N7. IAA production was considered as the major mechanism responsible for the growth promotion by these three strains. Future studies will examine the effect of these bacteria under field conditions and examine their impact on other corn varieties as well as horticultural plants.

Acknowledgments

The authors are grateful to Jackie Hill for her help in statistical analysis of data, Brian McGarvey for gas chromatography, Robert Pocs for analysis of indole-3-acetic acid, Brian Weselowski for sequencing reactions, Tom Kowalik for laboratory work and greenhouse experiments, and Alex Richman for primer design. This work was supported by grants from Commercial Alcohols Inc. (Brampton, Ontario, Canada) and Agriculture and Agri-Food Canada.

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