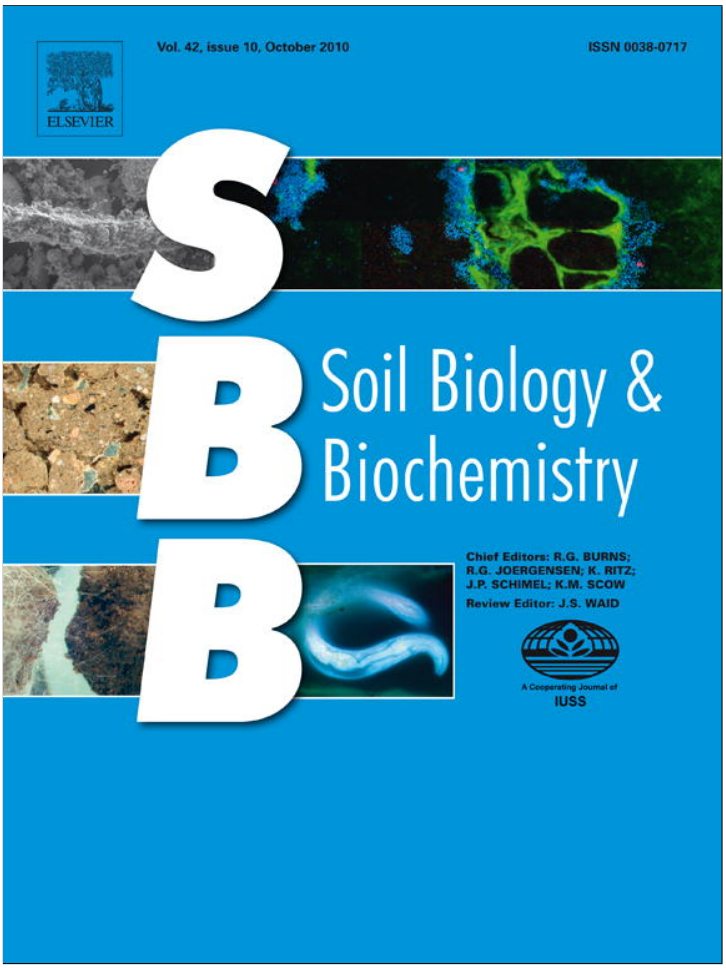

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Growth promoting effects of corn (*Zea mays*) bacterial isolates under greenhouse and field conditions

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ABSTRACT

Fertilizer costs are a major component of corn production. The use of biofertilizers may be one way of reducing production costs. In this study we present isolation and identification of three plant growth promoting bacteria that were identified as *Enterobacter cloacae* (CR1), *Pseudomonas putida* (CR7) and *Stenotrophomonas maltophilia* (CR3). All bacterial strains produced IAA in the presence of 100 mg l^{-1} of tryptophan and antifungal metabolites to several soilborne pathogens. *S. maltophilia* and *E. cloacae* had broad spectrum activity against most *Fusarium* species. The only strain that was positive for nitrogen fixation was *E. cloacae* and it, and *P. putida*, were also positive for phosphate solubilization. These bacteria and the corn isolate *Sphingobacterium canadense* CR11, and known plant growth promoting bacterium *Burkholderia phytofirmans* E24 were used to inoculate corn seed to examine growth promotion of two lines of corn, varieties 39D82 and 39M27 under greenhouse conditions. When grown in sterilized sand varieties 39M27 and 39D82 showed significant increases in total dry weights of root and shoot of 10–20% and 13–28% and 17–32% and 21–31% respectively. Plants of the two varieties grown in soil collected from a corn field had respective increases in dry weights of root and shoot of 10–30% and 12–35% and 11–19% and 10–18%. In sand, a bacterial mixture was highly effective whereas in soil individual bacteria namely *P. putida* CR7 and *E. cloacae* CR1 gave the best results with 39M27 and 39D82 respectively. These isolates and another corn isolate, *Azospirillum zeae* N7, were tested in a sandy soil with a 55 and 110 kg ha^{-1} of nitrogen fertility at the Delhi research Station of Agriculture and Agri-Food Canada over two years. Although out of seven bacterial treatments, no treatment provided a statistically significant yield increase over control plots but *S. canadense* CR11 and *A. zeae* N7 provided statistically significant yield increase as compared to other bacteria. The 110 kg rate of nitrogen provided significant yield increase compared to the 55 kg rate in both years.

1. Introduction

Corn (*Zea mays*) is the world's major cereal crop and is widely planted in Ontario and Quebec where it is grown for both grain and silage. To obtain an average yield of 7–9 t ha^{-1}, application of 110–150 kg ha^{-1} N, 20–50 kg ha^{-1} P_2O_5, and 30–80 kg ha^{-1} K_2O is recommended by Pioneer Hi-Bred Limited, Chatham, Ontario for cultivation in a medium fertile soil. In many areas of Ontario extensive spring rainfall results in loss of up to 70% of the nitrogen and phosphate fertilizers and this can result in eutrophication of lakes and rivers. In addition, the cost of fertilizer continues to increase production costs while decreasing grower revenues. For organic growers the use of such fertilizers is not permitted. In Ontario, and most of North America, there has been little attempt to harness naturally occurring soil bacteria and fungi that may play a role in plant fertility as a means of reducing the quantities of chemical fertilizers needed (Cleyet-Marcel et al., 2001). However, a variety of growth enhancing bacteria have been found to colonize the roots and aerial parts of cereals and other graminaceous plants including corn and they include *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Xanthomonas* species (Umali-Garcia et al., 1980; McInroy and Kloepper, 1995; Chelius and Triplett, 2000). Inoculation of corn with such bacteria has been shown to enhance crop yields (Jacoud et al., 1998; Riggs et al., 2001).

Use of microbial preparations for enhancement of plant production is becoming a more widely accepted practice in many countries including Australia, Belgium, Egypt, New Zealand, Russia, The Netherlands and USA (Rodriguez and Fraga, 1999). The commercial product "Azotogen" containing *Azotobacter* was

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introduced in Russia during 1937 and a biofertilizer named "Azogreen", is used commercially by corn farmers in France (Fages, 1994). In Cuba, several biofertilizers, comprised of isolates of *Azotobacter*, *Rhizobium*, *Azospirillum* and *Burkholderia* are commercially produced and employed with different crops (Rodriguez and Fraga, 1999).

Corn is rapidly becoming the base of biofuel industry as alcohol production increases in Ontario. To make corn a sustainable base for biofuels the cost of production must be lowered if growers are to earn a sustainable income. The primary objective of this study was to examine if plant growth promoting rhizobacteria (PGPR) can be isolated from corn plants grown in Southwestern Ontario and if such bacteria have the potential for use as biofertilizers. In this study, we present results on the isolation and identification of bacterial strains, isolated from corn roots that were selected on the basis of growth promotion (under greenhouse conditions) of two corn varieties planted in sterilized sand and in soils obtained from a corn field. These bacteria were also tested in field trials to assess increases in grain yields. To understand how these bacteria promote growth we examined their ability for production of the phytohormone indole acetic acid (IAA), nitrogen fixation, phosphate solubilization and release of antifungal metabolites.

2. Materials and methods

2.1. Isolation of strains

Local sweet corn (*Z. mays* convar. *saccharata*, var. *rugosa*) varieties growing in experimental plots at Agriculture and Agri-Food Canada, London, Ontario were used for isolation of PGPR. Root and shoot pieces were cut from four month old plants. They were then washed with sterilized water and cut into very small pieces with a sterile scalpel. The tissue slices were placed into semisolid malate medium (Mehnaz and Lazarovits, 2006) and combined carbon medium (CCM; 5 ml vial^{-1}; Rennie, 1981). Rhizosphere soil was directly inoculated in malate medium and CCM vials. The vials were incubated for 48 h at 30 °C. The bacteria growing out of the plant tissues were streaked onto malate medium and CCM plates according to origins. Purification of isolated colonies was done on Luria Bertani (LB) plates. Forty five phenotypes of colonies were obtained in total and eleven of these were from roots. For initial screening, plants of two corn varieties 39M27 and 39D82 were inoculated with root isolates in the presence or absence of nitrogen fertilizer. Four isolates, such as CR1, CR3, CR7 and CR11, showed increased weights of root or shoot of both varieties in the presence of nitrogen fertilizer as compared to the other strains. CR11 was subsequently identified as a new species *Sphingobacterium canadense* and is described in a separate study (Mehnaz et al., 2007a). The other three isolates were characterized in this study.

2.2. Bacterial identification

2.2.1. Physiological and biochemical tests

Physiological and biochemical tests were performed by using API 20E and API 20NE identification systems (BioMerieux, Marcy l'Etoile, France). Oxidase reagent was purchased from the same company and the manufacturer's instructions were followed for its use. Catalase activity was identified by the MacFadden (1980) method using H_2O_2 and colonies of pure cultures removed from agar plates.

2.2.2. Fatty acid analysis

Bacteria were subjected to analysis of fatty acid methyl esters using gas chromatography (Agilent Technology, CA, USA; Model #6890N) and a microbial identification system (Version 5.0 of the aerobe library, Microbial Identification System (1993) Operating Manual, MIDI, Inc., Newark, Del) following the manufacturer's recommended procedure.

2.2.3. PCR amplification and 16S rDNA sequence analysis

Bacterial DNA was isolated by using the QIAGEN blood and cell culture DNA Midi kit and the purified DNA was used as a template for PCR amplification of the 16S rDNA. The primers and PCR conditions were those previously described by Mehnaz et al. (2001). The purified PCR products were directly sequenced with an Applied Biosystems 3730 Analyzer. Amplification primers, as well as internal primers (Normand, 1995), were used for sequencing both strands of PCR products. The sequences were deposited in the GenBank (*Enterobacter cloacae* CR1, Accession No. AY787819; *Stenotrophomonas maltophilia* CR3, Accession No. AY785245; *Pseudomonas putida* CR7, Accession No. AY785244).

2.2.4. Antibiotic resistance

Antibiotic resistance of the isolates was tested with ampicillin, cycloheximide, gentamycin, kanamycin, rifampicin, spectinomycin, streptomycin and tetracycline at concentrations that ranged from 25 to 100 μg ml^{-1}, on plates of LB media incubated at 30 °C. All antibiotics were purchased from Sigma.

2.3. Assays for growth promoting abilities of isolates

All isolates were screened for indole acetic acid production, nitrogen fixation, phosphate solubilization and release of antifungal compounds using following assays.

2.3.1. Indole acetic acid (IAA) production

Bacterial cultures were grown in two batches in CCM medium with ammonium chloride (1 g l^{-1}) and L-tryptophan (100 mg l^{-1}). One batch was harvested after 3 days and second after 7 days. Medium without L-tryptophan was also used to determine IAA production. Cells were harvested at 10,000 rev min^{-1} (rpm) for 15 min. The pH of the supernatant was adjusted to 2.8 with hydrochloric acid and the supernatant was extracted three times with equal volumes of ethyl acetate (Tien et al., 1979). The extract was evaporated to dryness and resuspended in 1 ml of ethanol. The samples were analyzed using high performance liquid chromatography as described by Mehnaz and Lazarovits (2006).

2.3.2. Nitrogenase activity and phosphate solubilization assay

Nitrogenase activity was detected by acetylene reduction/ethylene production assay. Single bacterial colonies of *E. cloacae* CR1, *S. maltophilia* CR3, *P. putida* CR7 and *S. canadense* CR11 were inoculated into semisolid malate medium and CCM vials (5 ml vial^{-1}). After 24 h growth at 30 °C, acetylene (10%; v/v) was injected into all vials and these were further incubated at 30 °C for 20 h. Ethylene production was measured as described by Mehnaz and Lazarovits (2006). Bacterial protein estimation was carried out by using method of Lowry et al. (1951).

Phosphate solubilization was determined using calcium phytate agar medium (Rosado et al., 1998) and NBRIP medium (Nautiyal, 1999). The formation of a clear zone around the bacterial colony was considered as positive result.

2.3.3. Antifungal activity

To determine antifungal activity two media were utilized namely potato dextrose agar (PDA; Difco, MI, USA) and minimal medium (MM; Mehnaz and Lazarovits, 2006). The assay was carried out as described by Mehnaz and Lazarovits (2006). Antifungal activity was determined by the presence of an inhibition zone of mycelial growth on agar plates co-inoculated with the test

bacteria. A complete list of the fungal strains tested is given in Table 3. Strains of *Fusarium graminearum* and *Fusarium moniliforme* were kindly provided by Laboratoire de Diagnostic en Phytoprotection, Provincial Agriculture Ministry, Quebec. The remainder of the strains were from the culture collection of Dr. George Lazarovits.

2.4. Greenhouse experiments

The influence of the various bacterial strains on plant growth was examined on two corn varieties, i.e., 39M27 and 39D82. Seeds of these varieties were kindly provided by Pioneer Hi-Bred Limited, Chatham, Ontario. Initial experiments were carried out in sterilized industrial silica sand and in un-sterilized soil collected from a corn field at the Delhi research station (Ontario, Canada). All experiments were carried out under Greenhouse conditions.

2.4.1. Sand experiments

Seeds were washed with dish-washing detergent to remove the fungicide coating. They were then surface sterilized with 0.1% sodium hypochlorite for 5 min. After extensive washing with sterilized distilled water the seeds were germinated on wet filter paper in Petri plates at 30 °C. Three days old germinated seedlings were transferred into disposable coffee cups (225 ml) filled with autoclaved moist sand (250 g cup^{-1}). Two seedlings were planted in each cup. The bacterial strains *E. cloacae* CR1, *S. maltophilia* CR3, *P. putida* CR7 and *S. canadense* CR11 were grown in LB broth. Strain E24 of *Burkholderia phytofirmans* PsJN (Sessitsch et al., 2005) a known plant growth promoter of potato and tomato, was obtained by Tn5 mutagenesis and carries genes for rifampicin and kanamycin resistance as a marker (Conn and Lazarovits, unpublished data), was also included in this study. It was grown in NBY broth (g l^{-1}: nutrient broth 8.0; yeast extract 2.0; K_2HPO_4 2.0; KH_2PO_4 0.5; glucose 2.5; $MgSO_4.7H_2O$ 2.46; pH 6.5) with kanamycin (50 μg ml^{-1}) and rifampicin (100 μg ml^{-1}). Cultures were grown for 24 h and cells were harvested by centrifugation at 10,000 rev min^{-1} for 5 min. The pellets were suspended in 0.85% sterilized saline. The concentration of each bacterial strain was individually adjusted at 10^8 cells ml^{-1} and number of cells for each strain was same (10^8 cells ml^{-1}) in all treatments. For mixture, 1 ml (10^8 cells) of each bacterial strain were mixed and then one ml of this mixture was applied, assuming that number of cells of each strain is same in this one ml. Individual strains, as well as mixture of strains, were inoculated at the time of transplantation. Two types of bacterial mixtures were used, one was mixture of *E. cloacae* CR1, *S. maltophilia* CR3, *P. putida* CR7 and *S. canadense* CR11 and in other mixture *B. phytofirmans* E24 was also included with these four strains. Control plants were provided with 1 ml sterile saline per seedling. Six cups (twelve seedlings) were used for each treatment. Two days after transplantation, 5 ml ammonium nitrate solution (=50 kg N ha^{-1}) was added in each cup. The greenhouse was supplemented with lights to provide a photoperiod of 14 h light, 10 h dark when required. Temperature ranged from 20 to 28 °C. Tap water was used to keep the sand moist. Plants were harvested after 30 days growth. Roots and shoots were excised and placed in paper bags for drying in an oven at 70 °C for 72 h.

2.4.2. Soil experiments

Soil used for these experiments was a sandy loam containing 30.8% clay, 43% sand, 26% silt; and an organic matter content of 1.6%. Its pH was 5.5. The soil was collected from a corn field located at the Delhi Research Station, Agriculture and Agri-Food Canada. This field had not received any fertilizer for corn production. Seeds were washed and sterilized in the same manner as described for the sand experiment but the seeds here were not pre-germinated. Before sowing, ammonium nitrate (@50 kg N ha^{-1}) was mixed into the soil. Two seeds were planted into each cup containing 200 g non-sterilized field soil. Inoculum was prepared in the same manner as mentioned previously and at the time of sowing, 10^8 bacterial cells per plant were applied. Growing conditions, number of replicates, and sampling were as previously described.

All experiments were repeated at least three times. The replicated experiments showed similar trends and there were no significant differences between the same treatments in each experiment; hence the data from the replicated experiments were combined and analyzed by using SAS statistical software (ver.8.2; SAS Institute Inc., Cary, NC; on windows XP 2000). A one way analysis of variance (ANOVA) was used with the ANOVA procedure in SAS and comparisons among treatments were done using Duncan's multiple range test (DMRT). Data for variety 39M27 (root and shoot weight − sand experiment; root weight − soil experiment) were transformed (natural log of [x + 0.5]) before further analysis to normalize the data (Steel et al., 1997). All analyses were performed at the $P = 0.05$ level.

2.5. Field experiments

On the basis of greenhouse studies five bacterial isolates (*E. cloacae* CR1, *S. maltophilia* CR3, *P. putida* CR7, *S. canadense* CR11, *and B. phytofirmans* E24) were selected for field trial at Delhi research station. A strain of *Azospirillum zeae* N7, from corn rhizosphere (Mehnaz et al., 2007b) was also included in the field trials. Data of greenhouse experiments for *A. zeae* N7 were previously published by Mehnaz and Lazarovits (2006).

Replicated field plots of corn (*Z. mays* L. variety Pioneer 39D82) were established in Southwestern Ontario at the Delhi research farm (42°52′N, 80°31′W; 182 m above sea level) in years 2005 and 2006. The growing season at this location averages 136 frost-free days per year with an average total annual precipitation of 951 mm of which about 52% is received from April through September. The soil is fox loamy sand with the following characteristics: 85% sand, 7.5% clay, 7.5% silt, 1.2% organic matter, pH 6.0–6.4, and bulk density of 1.4 g cm^{-3}. The plots were cropped to common fall rye (*Secale cereale* L.) in 2004. A two row plateless, finger pickup type planter (Pequea Planter, 361 White Horse Road, Gap, PA 17527), calibrated to deliver about 52 seeds per row, was used to plant corn on May 12 and 23 in years 2005 and 2006, respectively. After the seedlings emerged, all plots were thinned back to 40 plants per row. Field plots consisted of 4 rows spaced 0.75 m apart and 8 m long arranged in a strip-plot design with nitrogen rates of 55 and 110 kg N ha^{-1} and bacterial selection as the main factors. All plots received a broadcast application of granular fertilizer containing 55 kg N + 20 kg P_2O_5 + 110 K_2O ha^{-1} incorporated to a depth of about 10 cm prior to seeding. For those treatments scheduled to receive the 110 kg N ha^{-1} rate, an additional 55 kg N ha^{-1} was applied as liquid urea-ammonium nitrate (UAN) injected between the rows to a depth of about 15 cm on June 21 and 19 in years 2005 and 2006, respectively.

Seeds of corn variety 39D82 were washed to remove fungicide and sterilized as mentioned earlier. Bacterial cultures, i.e., *E. cloacae* CR1, *S. maltophilia* CR3, *P. putida* CR7 and *S. canadense* CR11 and *A. zeae* N7 were grown in LB, and *B. phytofirmans* E24 in NBY medium. Individual strains were used as inoculum. After overnight growth, cultures were harvested by centrifugation at 10,000 rpm for 10 min. Cell pellets were suspended in 0.85% saline with 1% polyvinyl pyrrolidone K30 (PVP; sticker). Seeds were soaked in this solution for 1 h. Later on, excessive inoculum was drained and seeds were air dried at room temperature. Approximately 10^6 cells per seed were applied. Before starting the field trial, to check the survival of bacteria, the number of cells on dry seeds was repeatedly counted. For control, seeds were coated only with sticker. In

year 2005, eight treatments, i.e., control, *E. cloacae* CR1, *S. maltophilia* CR3, *P. putida* CR7, *S. canadense* CR11 and *B. phytofirmans* E24, mixture of these five bacteria, and *A. zeae* N7 were used as individual treatments. Repeat trials, on the same field plots, were conducted in 2006.

In the fall of each year, grains from the centre two rows of each plot, excluding the four end plants, were harvested, the moisture content determined, and the 15% moisture content (MC) yield was calculated. Chlorophyll content and height of the plants were also noted at different growth stages. Data were analyzed by using SAS statistical software (ver.9.1; SAS Institute Inc., Cary, NC; 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 (DMRT).

3. Results

3.1. Identification of PGPR

3.1.1. Colony morphology

For all strains, colonies on LB plates were opaque and round with entire margins. CR1 produces off-white colonies, CR3 pale yellow, and CR7 colonies were beige and fluoresced under UV light.

3.1.2. Biochemical tests

Results of biochemical tests of the isolates are given in Table 1. CR1 and CR3 were oxidase negative whereas CR7 was oxidase positive. All strains were catalase positive. Results of biochemical tests were compared with identification tables provided by the manufacturers of the API kits. Based on these tests, CR1 was identified as *E. cloacae* and CR3 as *S. maltophilia*. CR7 was identified as *Pseudomonas* (*fluorescens/putida*) by API 20E kit but it was unable to distinguish the two species. Subsequent results from API 20NE tests identified the bacterium as *P. putida*. The manufacturer in fact recommends that both kits be used for non-enteric gram-negative rods. To confirm genus/species designations we carried out fatty acid and 16S rRNA sequence analysis.

3.1.3. Fatty acid analysis

Fatty acid analysis profiles identified CR1 as *E. cloacae* (similarity index (SI) = 0.859), CR3 as *S. maltophilia* (SI = 0.864), and CR7 as *Pseudomonas mucidolens* (SI = 0.821), with close similarity index for *P. putida* (0.807) and *P. fluorescens* (0.784).

3.1.4. 16S rDNA sequence analysis

Partial sequence identities (~1.1–1.43 kb) of 16S rRNA were obtained for all strains. On the basis of these sequences, CR1 was identified as *E. cloacae* with 99.7% homology with the sequence of *E. cloacae* (Accession No. AF157695), CR3 as *S. maltophilia* (99.8% homology; with Accession No. AJ306833). For isolate CR7 the sequence information was obtained for 1429 nucleotides and comparison of this sequence with the data bank showed 99.8% similarity with the sequence of *P. putida* (Accession No. AF291048).

3.1.5. Antibiotic resistance pattern

All isolates were resistant to ampicillin and cycloheximide up to 100 $\mu g\ ml^{-1}$. Growth of *E. cloacae* CR1 was inhibited by remainder of the antibiotics tested at all concentrations (25–100 $\mu g\ ml^{-1}$). *S. maltophilia* CR3 was resistant to gentamycin, kanamycin, spectinomycin and streptomycin, up to 100 $\mu g\ ml^{-1}$ whereas, for rifampicin and tetracycline resistance was observed only at 25 $\mu g\ ml^{-1}$. *P. putida* CR7 was resistant to gentamycin up to 50 $\mu g\ ml^{-1}$, spectinomycin and streptomycin up to 100 $\mu g\ ml^{-1}$ but it was sensitive to kanamycin, rifampicin and tetracycline. *S. canadense* CR11 was resistant to gentamycin, spectinomycin and streptomycin up to 100 $\mu g\ ml^{-1}$, kanamycin at 50 $\mu g\ ml^{-1}$ and sensitive to rifampicin and tetracycline.

Table 1
Identification of corn isolates by using API 20E and API 20NE[a] bacterial identification system.

Biochemical tests (API 20E)	CR1	CR3[b]	CR7[b]	Biochemical tests (API 20NE)	CR3	CR7
2-Nitrophenyl-βD-galactopyranoside (ONPG)	+	+	−	Potassium nitrate (NO_3)	+	−
L-arginine (ADH)	+	+	+	L-tryptophan (TRP)	−	−
L-lysine (LDC)	−	+	−	D-glucose (GLU)	+	−
L-ornithine (ODC)	+	−	−	L-arginine (ADH)	+	+
Trisodium citrate (CIT)	+	+	+	Urease (URE)	−	+
Sodium thiosulfate (H_2S)	−	−	−	Esculin ferric citrate (ESC)	+	+
Urease (URE)	−	−	−	Gelatin (GEL)	+	−
Tryptophan deaminase (TDA)	−	−	−	4-Nitrophenyl-βD-galactopyranoside (PNPG)	+	−
Indole production (IND)	−	−	−	D-glucose (GLU)	+	+
Sodium pyruvate (VP)	+	−	−	L-arabinose (ARA)	+	+
Gelatin (GEL)	−	+	−	D-mannose (MNE)	+	+
D-glucose (GLU)	+	−	−	D-mannitol (MAN)	+	−
D-mannitol (MAN)	+	−	−	N-acetyl-glucosamine (NAG)	+	−
Inositol (INO)	−	−	−	D-maltose (MAL)	+	+
D-sorbitol (SOR)	+	−	−	Potassium gluconate (GNT)	+	+
L-rhamnose (RHA)	+	−	−	Capric acid (CAP)	+	+
D-sucrose (SAC)	+	−	−	Adipic acid (ADI)	−	−
D-melibiose (MEL)	+	−	−	Malic acid (MLT)	+	+
Amygdalin (AMY)	+	−	−	Trisodium citrate (CIT)	+	+
L-arabinose (ARA)	+	−	−	Phenyl acetic acid (PAC)	+	+
Cytochrome-oxidase (OXIDASE)	−	−	+	Oxidase	−	+
Catalase	+	+	+	Catalase	+	+
Nitrate reduction:NO_2 production	+	+	−			
N_2 (reduction to nitrogen gas)	−	−	−			
Glucose API-OF medium: OF-fermentation	+	−	−			
OF-oxidation	+	−	+			

[a] API 20E is a standardized identification system (bioMerieux Inc., USA) for Enterobacteriaceae and other non-fastidious gram-negative rods which uses 21 miniaturized biochemical tests and a database. API 20NE is a standardized system (bioMerieux Inc., USA) for the identification of non-fastidious, non-enteric gram-negative rods, combining 8 conventional tests, 12 assimilation tests and a database.

[b] CR3 and CR7 are non-enteric bacteria but it is suggested by manufacturers that they should be identified by both (API 20E & API 20NE) systems.

Table 2
Indole acetic acid (IAA) production by corn isolates, after 3 and 7 days of inoculation.

Isolates	3 Days (ng/ml)	7 Days (ng/ml)
E. cloacae CR1	3614 ± 314	709 ± 96
S. maltophilia CR3	1183 ± 69	3867 ± 419
P. putida CR7	946 ± 42	5493 ± 752
S. canadense CR11	106 ± 16	277 ± 17

Results are average of three experiments, each with three replicates. ± indicates standard deviation (SD).

3.2. Assays for growth promoting abilities of isolates

3.2.1. Indole acetic acid (IAA) production

All strains produced IAA in the presence of tryptophan (Table 2). IAA production in the absence of tryptophan was negligible (data not shown). *P. putida* CR7 produced the highest amount of IAA (5.49 μg ml^{-1}) and *S. canadense* CR11 the lowest (0.28 μg ml^{-1}). The amount of IAA produced varied with the age of culture. *E. cloacae* CR1 culture yielded 3.6 μg ml^{-1} of IAA after three days of inoculation but a five fold reduction was observed after seven days. *P. putida* CR7, *S. maltophilia* CR3, and *S. canadense* CR11 in contrast produced much higher amounts of IAA after seven days of inoculation than after three days.

3.2.2. Nitrogenase activity and phosphate solubilization

E. cloacae CR1 produced 8.2 nmol ethylene h^{-1} mg^{-1} protein in CCM. No activity was observed in NFM. *P. putida* CR7, *S. maltophilia* CR3 and *S. canadense* CR11 did not show detectable level of nitrogenase activity in either NFM or CCM.

E. cloacae CR1 and *P. putida* CR7 produced a clear zone on NBRIP agar plates after 4–5 days of inoculation but clearing zone was not observed on calcium phytate agar plate. *S. maltophilia* CR3 and *S. canadense* CR11 did not show clearing zone formation on both media.

3.2.3. Antifungal activity

All bacterial isolates and fungal strains grew rapidly on PDA and slowly on minimal medium (MM). The spectrum of antifungal activity of the four bacterial strains was different on the two media (Table 3). On PDA, all bacteria produced zones of inhibition with *Fusarium oxysporum lycopersici* strains – Fol#172, Fol#038 and an isolate from tomato, whereas, no inhibition was observed with *Pythium aphanidermatum*, *Helminthosporium carbonum*, *F. graminearum* and *F. moniliforme*. *E. cloacae* CR1 was the only bacterium that inhibited the growth of *Pythium ultimum*. *S. maltophilia* CR3 inhibited the growth of *Fusarium sambucinum*, *Fusarium solani* and *F. oxysporum lycopersici* strains Fol 1997 and Fol 1836 as well as the above mentioned fungi. Growth inhibition of *Fusarium culmorum* was observed by all strains except *S. canadense* CR11. Growth of *F. solani phaseoli* (Fsp) was inhibited by *S. maltophilia* CR3 and *S. canadense* CR11.

On minimal medium, *E. cloacae* CR1 showed growth inhibition of 11 fungal pathogens. It did not inhibit the growth of *H. carbonum, P. aphanidermatum*, one strain of *F. moniliforme* (3487) and *F. graminearum* (2331). *S. maltophilia* CR3 was the slowest growing bacterium on minimal medium and thus was unable to inhibit the growth of any fungal pathogen on this medium. *P. putida* CR7 inhibited two *F. oxysporum lycopersici* strains (Fol 1836 and Fol tomato) and *S. canadense* CR11 inhibited *P. ultimum* and *F. oxysporum lycopersici* strain, Fol 1836.

3.3. Greenhouse experiments

3.3.1. Growth in sand

All bacterial strains significantly enhanced the shoot and root dry weights of varieties 39M27 and 39D82 when compared with non-inoculated control plants (Table 4). With variety 39M27 the maximum increase in root and shoot weight was found with the mixture of five strains (Mix + E24) and was 20% and 28% respectively. The lowest weight increases was seen with *E. cloacae* CR1 which corresponded to 10% and 13% for roots and shoots as compared to control plants. The maximum growth increase with variety 39D82 plants was seen with plants inoculated with the mixture of four bacteria (Mix) and increase in root were 32% and shoot 31% greater than tissues of control plants. The lowest increase in root weight (17%) was with *P. putida* CR7 and of shoot (21%) with the mixture of 5 bacteria (Mix + E24). All bacterial treatments showed significant growth enhancements with both varieties compared to control plants, but for a few exceptions, did not show significant difference when compared to each other.

3.3.2. Growth in soil

Significant increase in root and shoot weight of both corn varieties 39M27 and 39D82 was also observed with all bacterial inoculations except *B. phytofirmans* PsJN E24 which showed a non-significant increase in root weight for variety 39M27 (Table 5). *P. putida* CR7 inoculation resulted in the highest root weights (30% increase) and *B. phytofirmans* PsJN E24 the highest shoot weights

Table 3
Antifungal activity of corn isolates, on two different media.

Fungi	Strains/host	Potato dextrose agar (PDA)				Minimal medium (MM)			
		CR1	CR3	CR7	CR11	CR1	CR3	CR7	CR11
Fusarium oxysporium lycopersicum	Fol # 172	+	+	+	+	+	–	–	–
	Fol 1997	–	+	–	–	+	–	–	–
	Fol 1836	–	+	–	–	+	–	+	+
	Fol tomato	+	+	+	+	–	–	+	–
	Fol-038	+	+	+	+	+	–	–	–
F. solani	Cucumber	–	+	–	–	+	–	–	–
F. solani phaseoli	Fsp	–	+	–	+	+	–	–	–
F. sambucinum		–	+	–	–	+	–	–	–
F. culmorum		+	+	+	–	+	–	–	–
F. moniliforme	0606/Onion	–	–	–	–	+	–	–	–
	3487/Asparagus	–	–	–	–	–	–	–	–
F. graminearum	03878/Tomato	–	–	–	–	+	–	–	–
	2331/Tomato	–	–	–	–	–	–	–	–
Helminthosporium carbonum		–	–	–	–	–	–	–	–
Pythium aphanidermata	Cucumber	–	–	–	–	–	–	–	–
P. ultimum		+	–	–	–	+	–	–	+

CR1 = *E. cloacae*; CR3 = *S. maltophilia*; CR7 = *P. putida*; CR11 = *S. canadense*.

Table 4
Effect of corn isolates on root and shoot weight of two corn varieties after 30 days growth, in sterilized sand.

Treatments	Corn varieties			
	39M27		39D82	
	Tissue dry weight (mg/plant)		Tissue dry weight (mg/plant)	
	Root	Shoot	Root	Shoot
Control	399 ± 50 e	500 ± 46 d	304 ± 44 d	350 ± 52 c
E. cloacae CR1	425 ± 45 d	563 ± 68 c	371 ± 38 bc	426 ± 52 b
S. maltophilia CR3	448 ± 52 bcd	577 ± 86 c	370 ± 34 bc	434 ± 43 ab
P. putida CR7	450 ± 43 abcd	597 ± 67 bc	356 ± 42 c	428 ± 53 b
S. canadense CR11	461 ± 53 abc	618 ± 53 ab	367 ± 31 bc	454 ± 45 a
Burkholderia phytofirmans PsJN E24	440 ± 45 cd	588 ± 55 bc	384 ± 40 ab	448 ± 38 ab
Mix[a]	468 ± 54 ab	595 ± 66 bc	402 ± 48 a	459 ± 55 a
Mix + *B. phytofirmans* E24[b]	477 ± 58 a	641 ± 53 a	368 ± 40 bc	425 ± 46 b

Means followed by the same letter are not statistically different at 5% level according to Duncan's multiple range test (DMRT).
Values are average of three experiments, each experiment had 10–12 plants for each treatment.
[a] Inoculum prepared by mixing equal amounts of individual inoculum of four isolates (CR1, CR3, CR7, CR11).
[b] Inoculum prepared by mixing equal amounts of individual inoculum of five isolates (CR1, CR3, CR7, CR11, PsJN E24).

(35% increase) for corn variety 39M27 whereas, *S. maltophilia* CR3 and Mix + E24 provided the lowest significant increase in root (10%) and shoot weight (12%), respectively. With variety 39D82, inoculation with *E. cloacae* CR1 yielded the highest root and shoot weights (19 and 18%) as compared to control plants. All other treatments, *S. maltophilia* CR3, *P. putida* CR7, *S. canadense* CR11 and *B. phytofirmans* PsJN E24 equally increased root and shoot weights but in most cases they were comparable to each other. The lowest increase in root weight with this variety was seen with *S. maltophilia* CR3 (11%) and of shoots with *B. phytofirmans* PsJN E24 (10%) compared to control plants.

3.4. Field experiments

The bacterial selections *E. cloacae* CR1, *S. maltophilia* CR3, *P. putida* CR7, *S. canadense* CR11, *B. phytofirmans* E24, Mix and *A. zeae* N7 were compared to a control treatment using two rates of fertilizer in a field trial carried out at the same field site in both 2005 and 2006. In 2005 corn plants receiving 55 kg of nitrogen and treated with *S. canadense* CR11 showed statistically significantly higher yields compared to CR1, CR3, E24 and Mix but non-significant difference with *A. zeae* N7, *P. putida* CR7 and the control plants (Table 6). The difference in yield between the *S. canadense* CR11 treatment and that of control plants was 0.7 t. At the 110 kg rate in 2005 the *S. canadense* CR11 treatment had significantly higher yield than all treatments except *A. zeae* N7 and the mixture of bacteria. The difference between the yield from *S. canadense* CR11 and control plots was 1.08 t. In 2006, *A. zeae* N7 provided the highest yield in the 55 kg rate N fertilizer rate and this was significantly different form *B. phytofirmans* E24, *S. canadense* CR11 and *E. cloacae* CR1 but not from the control or the other bacteria treatments. The difference between the best treatment and the control was 0.44 t. At the higher rate of N in 2006 no statistically significant difference was found among all treatments. The difference between the best treatment (*E. cloacae* CR1) and the control was 0.85 t. ANOVA analysis of all combined data collected did not reveal any significant interactions over the 2 years as to bacterial treatment, or bacteria vs. nitrogen rates. However, a significant difference was found in the yield with nitrogen rate applied at the $P = 0.01$ level (7.6 vs. 8.9 t ha^{-1} for the 55 and 110 kg rates respectively). There was no statistically significant effect of bacterial inoculation on chlorophyll content and plant height (data not presented).

4. Discussion

In screening for bacterial strains isolated from corn roots using selective media and plant growth promotion of corn seedlings in greenhouse assays we identified four strains that were particularly active. Isolates designated as CR1 and CR3 were subsequently identified as *E. cloacae* and *S. maltophilia* respectively by all tests conducted for identity. Isolate CR7 was identified as *P. putida* by biochemical tests and 16S rRNA analysis but based on results of

Table 5
Effect of corn isolates on root and shoot weight of two corn varieties after 30 days growth, in non-sterilized corn field soil.

Treatments	Corn varieties			
	39M27		39D82	
	Tissue dry weight (mg/plant)		Tissue dry weight (mg/plant)	
	Root	Shoot	Root	Shoot
Control	402 ± 76 c	692 ± 115 e	452 ± 45 c	697 ± 88 c
E. cloacae (CR1)	489 ± 118 ab	814 ± 113 cd	536 ± 59 a	820 ± 94 a
S. maltophilia (CR3)	452 ± 65 b	860 ± 134 bc	505 ± 56 b	822 ± 108 a
P. putida (CR7)	521 ± 115 a	904 ± 105 ab	506 ± 39 b	802 ± 64 ab
S. canadense (CR11)	489 ± 95 ab	800 ± 126 d	500 ± 55 b	805 ± 102 ab
Burkholderia phytofirmans PsJN E24	442 ± 83 bc	932 ± 128 a	507 ± 57 b	770 ± 74 b
Mix[a]	490 ± 87 ab	825 ± 134 cd	522 ± 62 ab	791 ± 80 ab
Mix + *B. phytofirmans* E24[b]	459 ± 122 b	772 ± 156 d	525 ± 47 ab	759 ± 67 b

Means followed by the same letter are not statistically different at 5% level according to Duncan's multiple range test (DMRT).
Values are average of three experiments, each experiment had 10–12 plants for each treatment.
[a] Inoculum prepared by mixing equal amounts of individual inoculum of four isolates (CR1, CR3, CR7, CR11).
[b] Inoculum prepared by mixing equal amounts of individual inoculum of five isolates (CR1, CR3, CR7, CR11, PsJN E24).

Table 6
Effect of corn isolates on grain yield (t ha^{-1}) of corn variety 39D82, in filed experiments.

Nitrogen fertilizer @	Treatments	Year 2005	Treatments	Year 2006
55 kg N ha^{-1}	*S. canadense* CR11	8.49 ± 0.89 a	*A. zeae* N7	8.07 ± 0.41 a
	A. zeae N7	8.09 ± 1.16 ab	*P. putida* CR7	7.87 ± 0.52 ab
	Control	7.79 ± 0.51 ab	Control	7.63 ± 0.41 ab
	P. putida CR7	7.70 ± 0.43 ab	*S. maltophilia* CR3	7.61 ± 0.56 ab
	B. phytofirmans E24	7.53 ± 0.87 b	Mix	7.42 ± 0.43 ab
	Mix	7.45 ± 0.67 b	*B. phytofirmans* E24	7.36 ± 0.68 b
	S. maltophilia CR3	7.28 ± 0.49 b	*S. canadense* CR11	7.23 ± 0.29 b
	E. cloacae CR1	7.19 ± 0.60 b	*E. cloacae* CR1	7.22 ± 0.78 b
110 kg N ha^{-1}	*S. canadense* CR11	9.84 ± 0.65 a	*E. cloacae* CR1	9.38 ± 0.47 a
	A. zeae N7	9.08 ± 0.57 ab	*P. putida* CR7	9.06 ± 0.23 a
	Mix	9.06 ± 1.02 ab	*A. zeae* N7	8.99 ± 0.81 a
	P. putida CR7	8.86 ± 0.48 b	*S. canadense* CR11	8.87 ± 0.74 a
	E. cloacae CR1	8.83 ± 0.80 b	*S. maltophilia* CR3	8.79 ± 0.34 a
	B. phytofirmans E24	8.79 ± 0.31 b	Control	8.53 ± 1.02 a
	Control	8.76 ± 0.55 b	*B. phytofirmans* E24	8.50 ± 0.21 a
	S. maltophilia CR3	8.63 ± 0.36 b	Mix	8.25 ± 1.56 a

Means followed by the same letter are not statistically different at 5% level according to Duncan's multiple range test (DMRT).
Values are average of four replicates.

fatty acid analysis the bacteria had the highest similarity index to *P. mucidolens*. The 16S rRNA sequences however, did not show any homology with *P. mucidolens*. Based on the overall results of these tests we feel confident that we are working with an isolate of *P. putida* (CR7). All three bacteria, i.e. *E. cloacae*, *Pseudomonas* spp. (fluorescent pseudomonads) and *S. maltophilia* have been shown to be endophytes of corn (Lambert et al., 1987; McInroy and Kloepper, 1995). *Sphingobacterium* species (*multivorum/spiritivorum*) were recently isolated from barnyard grass (Sturz et al., 2001) and corn root (Mehnaz et al., 2007a).

Test of the isolates for characteristics commonly associated with plant growth promotion revealed that all the isolates were able to produce IAA in the presence of tryptophan. *S. maltophilia* CR3 has not been previously reported to be able to produce IAA but in our tests it had almost the same capacity to produce IAA as that produced by *E. cloacae* CR1 and *P. putida* CR7 which are well-known IAA producers. *S. canadense* CR11 produced ~300 ng ml^{-1} IAA and this organism has also not been previously shown to produce IAA. We detected only very low levels of nitrogenase activity with *E. cloacae* CR1 and none with the other isolates. *P. putida* CR7 and *E. cloacae* CR1 had exceptional rapid abilities to solubilize phosphate. All bacteria produced antifungal compounds that inhibited a large number of known soilborne plant pathogens. *S. maltophilia* CR3 is a well-known bio-control agent against *Pythium* spp., *Fusarium* and *Rhizoctonia solani* (Dunne et al., 1997; Giesler and Yuen, 1998; Nakayama et al., 1999; Dal Bello et al., 2001). Results of such tests suggest that the bacteria have the characteristics most commonly sought for use in growth enhancement of PGPR.

The four bacteria were consistent in their enhancement of root and shoot weights with the two corn varieties in sterilized sand and in non-sterile soil. Since all isolates produce IAA in the presence of tryptophan this hormone is considered to be the primary mechanism involved in plant growth promotion. Tryptophan is used as a metabolic precursor of IAA by most soil bacteria (Sarwar et al., 1992; Kravchenko and Leonova, 1993). Benizri et al. (1998) showed that in the presence of corn root exudates *P. fluorescens* (strain M.3.1), originally isolated from corn root, produced 16.4 pg ml^{-1} IAA after 24 h of growth. There are many other reports that implicate hormonal substances in the growth promotion caused by the inoculated strains (Tien et al., 1979; Umali-Garcia et al., 1980).

Sturz et al. (2001) used *S. maltophilia* as inoculum for potato plantlets in an *in vitro* bioassay and growth promotion was observed. Alsanius and Gertsson (2004) reported increased number of fruit and fruit weight of tomato plants when inoculated with a mixture of *S. maltophilia*, *P. fluorescens* and an un-identified gram positive strain. *S. maltophilia* however, has been primarily considered as a bio-control agent and any contribution to plant growth promotion was considered to be an indirect effect. In our experiments, as well as results reported by Sturz et al. (2001) and Alsanius and Gertsson (2004), plants were grown in conditions without any pathogens present and thus growth promotion was not likely related to biological control. Although the growth promoting effects of *B. phytofirmans* PsJN has been well documented on tomato, pepper, and potato (Lazarovits and Nowak, 1997) it was not previously examined for its effect on corn. The mechanism of growth promotion by this bacterium is still not understood (Wang et al., 2006) but it does not fix nitrogen and does not produce IAA.

Although the corn varieties 39M27 and 39D82 varied somewhat in their response to inoculation as to the type of bacteria and the substrate used for cultivation in the bioassays (i.e. sand or soil) all bacteria increased plant biomass to some extent. There were some differences in response of cultivars such that in sand 39M27 grew best with the mixture of five bacteria whereas 39D82 did best with the mixture of four bacteria. There are many published reports on the variable effects of inoculation with PGPR on different genotypes of same crop. de Salmone and Dobereiner (1996), found that only six of fifteen corn genotypes showed a consistently positive response to inoculation with a mixture of *A. brasilense* strains isolated from corn. Similar genotypic responses were found with twenty three PGPR strains, isolated from corn and other grasses, when tested on fifteen genotypes of corn (Riggs et al., 2001). 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 (Ladha et al., 1986).

We did not find any significant effects in yields of corn of treated plants compared to control plots in our field experiments. However, in year 2005, treatments with the *S. canadense* CR11 and *A. zeae* N7 provided the highest yield with both the 55 kg and the 110 kg N rate and in year 2006, *A. zeae* N7 was the best treatment at the 55 kg rate. It was also observed that the yields of both years, for control and inoculated treatments except CR11, were very close to each other/quite persistent, such as yields of control treatment with 55 kg N were 7.79 and 7.63 t. ha^{-1}, and with 110 kg N these were 8.53 and 8.76 t ha^{-1}. Similarly for the plants inoculated with *A. zeae* N7, the yields with 55 and 110 kg N were 8.09, 8.07/9.08, 8.99 respectively. One possibility for a different behaviour of *S. canadense* CR11 can be its poor survival in year 2006 than 2005.

As we did not check the survival therefore we cannot say anything for sure. Different response at two fertilizer rates was quite expected as increase in nitrogen concentration affects the survival of each strain in different way and therefore results varied at both fertilizer rates. We consider these field trials to be preliminary in nature as we were unable to follow a number of important factors required for growth promotion to occur including, to check the survival of bacteria after seed sowing, early colonization of roots and sufficient build up of the populations of the bacteria in/on the plant, etc., which can be responsible for lower yield or non-significant yield of inoculated plants. Even the conditions used for growing the bacteria in the laboratory may influence its field performance and certainly our method of applying the bacteria to the seed differed form that use in laboratory assays. We were however, interested in seeing how to best deploy these microorganisms for use by growers. Currently we are developing quantitative PCR assays for following the specific organism on roots and are optimizing applications to seed for use in future trials. We are also inoculating the plants and repeating the trial every year.

Getting statistically significant results under field conditions is always difficult. Lack of statistical significance however, does not always mean that growers cannot benefit from use of such microorganisms as they value income earned more than statistics. The total of the average yields obtained with strains *S. canadense* CR11 (2005) plus *A. zeae* N7 (2006) at the 55 kg N rate was 16.56 t vs. 15.42 for the controls and at the 110 kg N rate of *S. canadense* CR11 (2005) and *A. zeae* N7 (2006) was 18.83 t vs. 17.29 t for the controls. At the current selling price of $326 per ton this would have translated to an additional income of $372 and $573 per ha respectively. Such increases in income, even with the cost of production of the bacteria, would merit further study in large scale field trials, particularly when we gain a better understanding as to how to grow and apply such inoculants to seed. Similarly, some treatments i.e. *E. cloacae* CR1 at 55 kg and *S. maltophilia* CR3 + Mix at 110 kg in 2005–6 would have resulted in financial losses of $326 and $134 respectively.

One of the dangers of testing such bacteria at the field level is that we have only single isolates of each of the microorganisms to evaluate and we do not know if these are the best strains for field application. Kiers and Denison (2008) pointed out that typically, individual plants associate with multiple microbial genotypes that vary in their mutualistic benefit. They clearly show how *Rhizobium* strains may vary greatly in their ability to increase a plant's performance from having no impact to increasing yields by two to three folds over non-inoculated plants. For future field tests we need to collect many more isolates of these bacteria and determine if the same genetic controls as described by Kiers and Denison (2008) for *Rhizobium* also occur in the corn root-microbe interactions with these organisms. We also need to check for the parameters other than grain yield, such as the root length/root weight, total plant weight, fungal suppression and nitrogenase activity. These isolates have shown the antifungal activity against several pathogenic fungal strains, phosphate solubilization on plate assays and these are producers of auxins. Therefore, there is a possibility that they might improved the root and/or shoot growth of the plant as well as suppressed the fungal pathogen or contributed in phosphorous or nitrogen uptake (considering that *A. zeae* N7 is a very good nitrogen fixer). These factors are known to improve the plant growth (Lazarovits and Nowak, 1997; Rodriguez and Fraga, 1999) although it is not always reflected in terms of grain yield.

In conclusion, three PGPRs isolated from corn were characterized as *E. cloacae*, *S. maltophilia* and *P. putida*. These isolates, *S. canadense* (another isolate from corn root) and a well-known PGPR *B. phytofirmans* PsJN E24 showed beneficial effects on root and shoot weight of two corn varieties under sterilized and unsterilized conditions, in greenhouse experiments. In field experiments none of the bacteria increased grain yields in a statistically significant manner compared to un-inoculated control plants. However, strains of *A. zeae* N7 *E. cloacae* CR1, and *S. canadense* CR11 provided sufficiently promising results as to warrant further field trials.

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