Soil Biology & Biochemistry 42 (2010) 1848–1856

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

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

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ARTICLE INFO

Article history:
Received 15 September 2009
Received in revised form
6 July 2010
Accepted 8 July 2010
Available online 23 July 2010

Keywords:
Corn
S. maltophilia
S. canadense
B. phytofirmans
P. putida
E. cloacae
A. zeae

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.

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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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doi:10.1016/j.soilbio.2010.07.003

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

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