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Can a diazotrophic endophyte originally isolated from lodgepole pine colonize an agricultural crop (corn) and promote its growth?

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ABSTRACT

Several diazotrophic *Paenibacillus* strains were isolated from extracts of surface-sterilized lodgepole pine seedling and tree tissues. One strain, *Paenibacillus polymyxa* P2b-2R, was found to fix high amounts of nitrogen when reintroduced to the gymnosperms, lodgepole pine and western red cedar. We wanted to determine if *P. polymyxa* P2b-2R could colonize rhizosphere and internal tissues, fix N and promote growth of corn (*Zea mays* L), an important agricultural crop. We inoculated corn seeds with *P. polymyxa* strain P2b-2R and grew seedlings for 30 days. Corn seedlings were harvested 10, 20 and 30 days after inoculation for evaluation of endophytic and rhizospheric colonization as well as nitrogen fixation and growth promotion. P2b-2R successfully colonized the rhizosphere as well as internal root tissues of corn (*i.e.*, endophytically) with population densities near 10^6 cfu. Corn seedling growth was promoted significantly by inoculation with P2b-2R with an increase of up to 35% in length and up to 30% in biomass after 30 days of inoculation. Seedlings inoculated with P2b-2R derived up to 20% of foliar nitrogen from atmosphere after 30 days of inoculation, which is significant considering the fact that this was a short growth trial. These results suggest that *P. polymyxa* P2b-2R might have a broad range of plant hosts and is able to fix N and promote the growth of at least one important agricultural crop *i.e.* Corn.

1. Introduction

For plants, nitrogen is necessary as a primary constituent of nucleotides, proteins, and chlorophyll (Robertson and Vitousek, 2009). Many agricultural scientists see the availability of fixed nitrogen (nitrate or ammonium converted from dinitrogen) as the most yield-limiting factor related to the crop production (Muthukumarasamy et al., 2002). An inexpensive and natural way of providing plants with fixed nitrogen is by biological nitrogen fixation (BNF). Approximately 80% of all BNF is accomplished through the symbiotic interaction between legumes and α-proteobacteria in the order Rhizobiales, family Rhizobiaceae (Garg and Geetanjali, 2007). However, non-specific nitrogen-fixing bacteria also exist and their discovery has opened up the possibility of biological nitrogen fixation in a wide array of agricultural crops like corn.

Endophytic bacteria have been defined as 'bacteria that live within living plant tissues without doing substantive harm or gaining benefit other than securing residency' (Bressan and Borges, 2004). In contrast to free-living, rhizosphere or phyllosphere microorganisms, bacterial endophytes are better protected from abiotic stresses such as extreme variations in temperature, pH, nutrient, and water availability as well as biotic stresses such as competition (Chanway et al., 2014). Diazotrophs comprise certain bacteria and archaea that fix atmospheric nitrogen into a biologically usable form such as ammonia. The term "endophytic diazotrophic bacteria" was introduced in the area of BNF by Döbereiner (1992) to designate all diazotrophs able to colonize primarily the root interior of graminaceous plants, survive very poorly in soil and fix nitrogen in association with these plants. Since the discovery of diazotrophic endophytes in sugarcane (*Saccharum officinarum* L.) (Ruschel et al., 1975), several other agriculturally important crop species including rice (*Oryza sativa*) (Shrestha and Ladha, 1996), maize (*Zea mays* L.) (Montañez et al., 2009), and kallar grass (*Leptochloa fusca* L.) (Malik et al., 1997) have been postulated to receive significant amounts of fixed N_2 in this way.

Paenibacillus polymyxa is a Gram-positive, rod-shaped, endospore-forming bacterium that is non-pathogenic and found in environments such as plant roots in soil and marine sediments (Timmusk et al., 2005; He et al., 2007; Ravi et al., 2007). The wide

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range of potential plant growth promoting characteristics this bacterium possesses include the ability to fix nitrogen and to produce hormones that promote plant growth as well as hydrolytic enzymes and antibiotics that protect against harmful microorganisms (Lal and Tabacchioni, 2009). Górska et al. (2015) isolated two nitrogen-fixing microorganisms from agricultural soil and identified them as *P. polymyxa* [laboratory names EG2 and EG14] based on 16S rRNA sequence. The genome of these bacterial strains was found to carry *nif* genes coding the individual components of the nitrogenase complex. Their nitrogen fixing ability was confirmed by studying nitrogenase activity in cultures of the studied bacteria in N-free medium supplemented with carboxymethylcellulose (CMC).

Bal et al. (2012) isolated several *Paenibacillus* strains that possessed significant acetylene reduction activity from extracts of surface-sterilized lodgepole pine seedling and tree tissues (Bal and Chanway, 2012a,b). When one of the strains, *P. polymyxa* strain P2b-2R (GU132543), was reintroduced to lodgepole pine and grown in a very N-limited soil for 35 weeks, seedlings were found to derive more than half (66%) of their foliar N from biological nitrogen fixation (BNF) (Bal and Chanway, 2012a). In a long-term experiment (13-month growth trial), P2b-2R showed even better results as the inoculated lodgepole pine seedlings derived 79% of their foliar N from BNF and doubled their biomass (Anand et al., 2013). Anand and Chanway (2013c) characterized *nif* gene structure of *P. polymyxa* strain P2b-2R to determine the arrangement and sequences of genes in the *nif* operon. This strain was found to possess a single copy of the *nif*H gene with *nif*B located directly upstream of *nif*H and D. Phylogenetic analyses of the full *nif*H, partial *nif*B, and *nif*D, and 16S rDNA (rrs) gene sequences indicated that P2b-2R was part of a monophyletic cluster with other members of the genus *Paenibacillus* (Anand and Chanway, 2013c). These results provided confirmation of the ability of *P. polymyxa* strain P2b-2R to colonize gymnosperms (lodgepole pine and western red cedar) and fix nitrogen when associated with them. However, we were interested in determining if this bacterial strain could also fix nitrogen and promote plant growth if introduced into an agricultural crop? We chose corn for our study, as it is an important agricultural crop grown on a large scale worldwide. In terms of global cereal crop production, corn is second to wheat with an annual production of 868 million metric tonnes in the year 2012–13, which increased to 990.64 million metric tons in 2013–14. Globally, the total area under corn crop was 177.44 million hectares in 2012–13, which is also second to wheat (USDA FAS, June 2015).

In this study, we test the hypothesis that the endophytic diazotroph *P. polymyxa* P2b-2R isolated from lodgepole pine is capable of colonizing rhizosphere and internal tissues (endophytically) of an important agricultural crop, corn (*Zea mays*) and stimulating biomass production through BNF.

2. Materials and methods

2.1. Seed and bacteria

Corn seeds (*var.* Golden Bantam) were obtained from West Coast Seeds (Delta, British Columbia, Canada). *P. polymyxa* strain P2b was isolated from surface-sterilized stem tissues of a lodgepole pine seedling naturally regenerating near Williams Lake, British Columbia, Canada (52°05′ N lat., 122°54′W long, elevation 1300 m, Sub-Boreal Pine Spruce, SBPSdc Zone) (Bal et al., 2012). *P. polymyxa* P2b-2R (GU132543) was a spontaneous antibiotic-resistant derivative of strain P2b, capable of growing on combined carbon medium (CCM) (Rennie, 1981) agar amended with 200 mg/L rifamycin (Bal and Chanway, 2012a). Strain P2b-2R was stored at −80 °C in CCM amended with 20% (v/v) glycerol.

2.2. Seed inoculation and plant growth

Seedling growth assays were performed in small pots (12 cm × 8cm × 4 cm) filled to 67% capacity with sterile Sand–Turface (montmorillonite clay, Applied Industrial Materials Corporation, Deerfield, Ill., USA) mixture (69% w/w silica sand; 29% w/w Turface; 2% w/w $CaCO_3$). Each pot was fertilized with 50 mL of a nutrient solution (Chanway et al., 1988), which was modified by replacing KNO_3 and $Ca(NO_3)–4H_2O$ with $Ca(^{15}NO_3)_2$ (5% ^{15}N label) (0.0576 g/L) and sequestrene 330 Fe (CIBA-GEIGY, Mississauga, Ont., Canada) with Na_2-FeEDTA (0.02 g/L). Other nutrients in the nutrient solution included (in grams per liter): KH_2PO_4, 0.14; $MgSO_4$, 0.49; H_3BO_3, 0.001; $MnCl_2–4H_2O$, 0.001; $ZnSO_4–7H_2O$, 0.001; $CuSO_4–5H_2O$, 0.0001; and $NaMoO_4–2H_2O$, 0.001.

Corn seeds were surface-sterilized by immersion in 30% hydrogen peroxide for 90 s, followed by three 30-s rinses in sterile distilled water. To confirm the effectiveness of surface sterilization, seeds were imprinted on tryptic soy agar (TSA) and checked for microbial contamination 24 h later. Three surface sterilized seeds were aseptically sown in each pot.

Two seed inoculation treatments – live P2b-2R and control (phosphate buffered saline (PBS)) were evaluated using corn seedlings, replicated 60 times. Bacterial inoculum was prepared by thawing a frozen culture of strain P2b-2R and streaking a loopful onto combined carbon medium (CCM) agar amended with 200 mg/L rifamycin, and incubating at 30°C for 2 days. A 1 L flask containing 500 mL of CCM broth amended with rifamycin was then inoculated with a loopful of bacterial growth from the agar plate, secured on a rotary shaker and agitated (150 rpm) at room temperature for 2 days. Bacterial cells were harvested by centrifugation (3000× *g*, 30 min), washed twice in sterile PBS (pH 7.4) and resuspended in the same buffer to a density of 10^6 cfu/mL. Immediately after sowing the seeds, 5 mL of the P2b-2R – PBS suspension was pipetted directly into each pot designated for live P2b-2R. This process was repeated using 5 mL of sterile PBS without bacteria for uninoculated (control) pots. Pots were placed in a growth chamber (Conviron CMP3244, Conviron Products Company, Winnipeg, MB, Canada) under an 18-h photoperiod with an intensity of at least 300 μmol s^{-1} m^{-2} and a 25/18 °C day/night temperature cycle. Seedlings were thinned to the largest single germinant per pot 2 days after sowing and were watered as required with sterile distilled water. Seedlings received modified nutrient solution without $Ca(^{15}NO_3)_2$ only once in the entire experiment which was on the 20th day from inoculation. The entire experiment was repeated to confirm treatment effects.

2.3. Evaluation of endophytic colonization

To evaluate endophytic colonization by P2b-2R in corn, 3 randomly chosen seedlings of each treatment were harvested destructively 10, 20 and 30 days after inoculation. Seedlings were rinsed in a 2 L flask containing 1 L sterile distilled water for removal of loosely adhering growth media. In the first growth trial, seedlings were surface-sterilized in 1.3% (w/v) sodium hypochlorite for 5 min, rinsed three times with sterile distilled water and imprinted on TSA plates for 24-h to check for surface contamination. Samples of stem, root and leaf tissues were triturated separately in 1 mL of sterile PBS using a mortar and pestle. Triturated tissue suspensions were serially diluted on CCM plates supplemented with 100 mg/L cycloheximide and 200 mg/L rifamycin. Plates were incubated at room temperature for 7 days and colonies were counted after incubation. Data from seedlings that showed contamination after surface sterilization were excluded from further analysis.