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Evidence of endophytic diazotrophic bacteria in lodgepole pine and hybrid white spruce trees growing in soils with different nutrient statuses in the West Chilcotin region of British Columbia, Canada

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ABSTRACT

The West Chilcotin is a remote region located in the Sub-Boreal Pine-Spruce biogeoclimatic zone of British Columbia, Canada where cold climate and low annual precipitation have resulted in relatively dry and weakly developed soils. Lodgepole pine is the most widely found tree species in this region followed by hybrid white spruce. Their ability to grow on such nutrient-poor soils raise questions regarding nitrogen (N) inputs to these forest stands. A rarely evaluated but possible source of N could be N-fixing bacteria living in the internal tissues of pine and spruce trees (known as 'endophytic diazotrophic bacteria'). To examine this possibility, we selected two sites with different soil moisture contents in a predominantly spruce stand and collected soil and plant samples from each site. Similarly, soil and plant samples were also collected from two sites with different soil moisture contents in a predominantly pine stand. Analyses of soil samples revealed that overall nutrient content of soils of low-moisture (LM) sites was significantly lower than high-moisture (HM) sites in both stands, particularly, available and mineralizable N in soil. We isolated 55 (LM: 27 and HM: 28) and 48 (LM: 20 and HM: 28) bacteria from internal tissues of pine and spruce trees, respectively on N-free media. N-fixing ability of these isolates was evaluated using the acetylene reduction assay and 18 isolates from spruce (LM: 10 and HM: 8) and 23 isolates from pine (LM: 13 and HM: 10) were tested positive in this assay. These endophytic diazotrophic bacteria were identified as mainly belonging to genera: *Bacillus*, *Caballeronia*, *Paenibacillus*, and *Pseudomonas*. These results indicate that pine and spruce trees growing on different sites in this region harbour naturally occurring N-fixing bacteria in their tissues, possibly to gain fixed N.

1. Introduction

The importance of microbes for plant health and growth promotion has been known for a long time but internal tissue colonization was largely perceived as being related to the spread of disease. However, now it is widely accepted that microbes can colonize internal tissues of plants and establish beneficial symbiotic associations with the host plant (Puri et al., 2017b). Such microbes are known as 'endophytes'. Although there is considerable literature available regarding endophytic fungi in forest ecosystem (Doty, 2011), studies of endophytic bacteria of forest tree species are rather limited. However, these limited studies have indicated that the importance of endophytic bacteria should not be underestimated in forest ecosystem (Puri et al., 2017a). Principal mechanisms through which these endophytic bacteria can enhance the growth of trees include nitrogen (N) fixation (Padda et al., 2017a), production of phytohormones like cytokinins (Pirttilä, 2011), auxins (Taghavi et al., 2005) and gibberellins (Bottini et al., 2004), and suppression of pathogens in addition to improving the mutualistic relationship of mycorrhizae with roots of the host tree (Anand et al., 2006). Endophytic bacteria that possess the ability to fix N while living inside the tissues of a plant are known as 'endophytic diazotrophic bacteria' (Döbereiner, 1992). Although such bacteria have been widely studied in agricultural crops like sugarcane (*Saccharum officinarum*) (Boddey et al., 1991), corn (*Zea mays*) (Puri et al., 2015), canola (*Brassica napus*) (Puri et al., 2016a), rice (*Oryza sativa*) (Baldani et al., 2000), and tomato (*Solanum lycopersicum*) (Padda et al., 2016a); but very few studies have reported their presence in forest trees like, black cottonwood (*Populus trichocarpa*) (Knoth et al., 2014), Sitka willow (*Salix sitchensis*) (Doty et al., 2009), lodgepole pine (*Pinus contorta*) (Tang et al., 2017), and limber pine (*Pinus flexilis*) (Moyes et al., 2016). However, their existence and function in tree species require further evaluation.

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British Columbia (BC) has some of the most diverse terrestrial ecosystems in North America with 14 different biogeoclimatic zones. The Sub-Boreal Pine-Spruce (SBPS) biogeoclimatic zone is a montane zone in the west central interior of BC characterized by cold, dry winters and cool, dry summers due to its location in the strong rain-shadow of the Coast Mountains (Steen and Demarchi, 1991). It is divided into four subzones. The driest and coldest one is the SBPS xeric cold (xc) subzone. West Chilcotin is a remote region in this subzone, located about 200 km west of Williams Lake. Soils in most parts of BC are young and started developing only 10,000 years ago after the last glacial period. This coupled with the harsh climate (cold and dry with very low annual precipitation) of the West Chilcotin region has led to weakly developed soils and typically, very thin or even no surface organic layer with very slow rates of decomposition (Steen and Coupé, 1997). All these factors have severely limited the productivity of forests in this region. Lodgepole pine is the only tree species present in many extensive forest stands in this region, with hybrid white spruce (*Picea glauca* x *engelmannii*) occurring mostly on relatively moist sites (Steen and Coupé, 1997). Most of the stands in the SBPS xc subzone are naturally occurring secondary forests that are > 60 years old including one-third of the stands that are > 120 years old (Coupé, 2012). Considering the sustained growth of pine and spruce trees on such nutrient-poor soils, N inputs in forests of this region have been a long-standing conundrum (Steen and Demarchi, 1991; Steen and Coupé, 1997). Bal et al. (2012) isolated endophytic diazotrophic bacteria from lodgepole pine trees in the SBPS zone and reported that they could be involved in fulfilling a significant portion of N requirements of pine trees. Subsequently, one of the isolates, *Paenibacillus polymyxa* P2b-2R, was reported to fulfil as much as 79% of N requirements of young pine seedlings (Anand et al., 2013). However, to the best of our knowledge, there are no reports in the literature regarding the presence of endophytic diazotrophic bacteria in hybrid white spruce trees. Other species of spruce, such as Engelmann spruce (*Picea engelmannii*) (Carrell and Frank, 2014) and Norway Spruce (*Picea abies*) (Cankar et al., 2005), have been reported to harbour such endophytic diazotrophic bacteria.

In this study, we investigated the presence of endophytic diazotrophic bacteria in pine and spruce trees growing at sites with distinct nutrient contents in the West Chilcotin region. The objective behind selecting sites with distinct nutrient contents was based on the results of previous studies (Bal et al., 2012; Yang et al., 2016, 2017) that reported that endophytic diazotrophic bacteria perform significantly better under nutrient-poor conditions. As per Blouin et al. (2008), soil moisture content is directly related to the nutrient content of the soil, this led to our first hypothesis that sites with high moisture content in the West Chilcotin region are nutrient-rich and sites with low-moisture content are nutrient-poor. Our second hypothesis was that pine and spruce trees growing in this region harbour endophytic diazotrophic bacteria to fulfil their N requirements. Our third hypothesis was that bacteria originating from trees at nutrient-poor sites have higher N-fixing ability as compared to those originating from trees at nutrient-rich sites.

The main objectives of this study were to: (i) evaluate soils of the West Chilcotin region and compare the nutrient status of soils from high-moisture (HM) sites with those from low-moisture (LM) sites in this region; (ii) isolate and identify potential endophytic diazotrophic bacteria from lodgepole pine and hybrid white spruce trees growing at these HM and LM sites; and (iii) evaluate the N-fixing ability of isolated bacteria and draw comparisons between bacteria isolated from trees at HM sites with those at LM sites.

2. Materials and methods

2.1. Site description

A sampling area (52°00′ N, 125°00′ W) was selected in the West Chilcotin region with a granitic type of parent material, which is generally regarded as a coarse-textured parent material with very low nutrient content. This area was located about 250 km west of Williams Lake, BC, Canada on the Chilcotin-Bella Coola highway. In this area, two sites were selected to sample lodgepole pine trees in a primarily pine stand, one at a lower elevation (52°00′04.2″ N, 124°59′44.7″ W, 1003 m a.s.l.) and the other at a higher elevation (52°00′09.1″ N, 124°59′25.2″ W, 1035 m a.s.l.). Similarly, two sites were selected to sample hybrid white spruce trees in a primarily spruce stand at lower elevation (52°00′23.8″ N, 125°00′17.4″ W, 966 m a.s.l.) and higher elevation (52°00′19.5″ N, 125°00′17.8″ W, 993 m a.s.l.). As reported by Qiu et al. (2001), soil moisture content and elevation have an inverse relation (i.e. a site at higher elevation have relatively lower soil moisture content than a site at lower elevation). Therefore, we designated the lower elevation sites as high-moisture (HM) sites and higher elevation sites as low-moisture (LM) sites in each stand.

2.2. Soil and plant sampling

Soil samples were collected from the aforementioned four sites by using the following sampling design so as to cover spatial variability. At each site, a mature tree was chosen, and soil samples were collected in the four cardinal directions both inside and outside its dripline. Samples were collected from the forest floor and top mineral layer (0–10 cm). Samples collected in all four cardinal directions from both inside and outside the dripline were pooled to obtain one forest floor sample and one mineral layer sample near each tree. In this way, soil samples were collected near 10 trees at each of the four sites. Subsequently, a young tree (height: < 25 cm and age: < 5 years) growing near each mature tree (around which soil samples were collected) was uprooted and collected, which resulted in 10 spruce tree samples from each of the two sites at spruce stand and 10 pine tree samples from each of the two sites at pine stand.

2.3. Soil analyses

To determine the overall nutrient status of the LM site and the HM site at each stand, soil samples were analyzed for total C; total N; Available N (NH_4^+ and NO_3^-); Mineralizable N; Available P; total S; Available S; pH in H_2O; cation exchange capacity (CEC); base saturation; organic matter (OM); percent sand, silt and clay; and macro- and micro-nutrients (Al, B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, S, and Zn). These analyses were performed at the Analytical Chemistry Services Laboratory, BC Ministry of Environment and Climate Change Strategy, Victoria, BC, Canada.

2.4. Isolation of potential endophytic diazotrophic bacteria

Potential endophytic diazotrophic bacteria were isolated from stem, needle and root tissues of each seedling by using a surface sterilization-trituration-plating technique (Bal et al., 2012). Briefly, about 0.5 g (fresh mass) of each tissue was surface sterilized by immersion in 2.5% (w/v) sodium hypochlorite for 2 min, followed by three 30-sec rinses in 10 mmol/L phosphate buffered saline (PBS) (pH 7). To check for surface contamination, tissue samples were imprinted on tryptic soy agar (TSA) and incubated for 24 h. Tissues found to be free of surface contamination were triturated in 1 mL PBS using a sterile mortar and pestle. Triturated tissue suspensions were serially diluted, and 0.1 mL of each dilution was plated on N-free combined carbon medium (CCM) (Rennie, 1981) supplemented with 100 mg/L cycloheximide to suppress fungal growth. Following incubation for 3 days at 30 °C, representative bacterial colonies were selected from dilution plates based on colony size, shape, morphology, and colour and were purified by streaking onto fresh CCM plates amended with cycloheximide (100 mg/L). Purified isolates were grown in CCM broth amended with cycloheximide (100 mg/L) until turbid and stored frozen at −80 °C in cryovials containing 2 mL CCM amended with 20% (v/v) glycerol.