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Selection and evaluation of potential biocontrol rhizobacteria from a raised bog environment

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ABSTRACT

Understanding the interactions of bacteria with plants and the role of microorganisms in the lifecycle of plants is becoming more and more important due to the social claim for a clean agriculture. Thus the significance of studying plant associated bacteria is increasing. The main aim of our study was to isolate and characterize bacteria associated with bryophytes from a natural raised bog ecosystem. Characterization of the isolates by their beneficial properties was realized with microbiological and molecular tools. Bacterial strains were identified taxonomically, isolates belonging to the genera *Pseudomonas*, *Bacillus*, *Serratia*, *Stenotrophomonas*, *Delftia*, *Cedecea*, *Lysinibacillus* and *Viridibacillus* have been found. *Pseudomonas*, *Serratia* and *Bacillus* were the dominant bacterial genera associated with bryophytes. *In vitro* study of antagonism showed that a high number of the isolates inhibited the growth of fungal and bacterial plant pathogens – *Fusarium oxysporum*, *Alternaria alternata*, *Erwinia carotovora* – or produced secondary metabolic substances with biocontrol properties. Bacterial strains identified as *Serratia fonticola BB17* and *Pseudomonas fluorescens BE8* proved to be the most efficient against plant pathogens, with biocontrol effectiveness up to 48.28% and 55.17% respectively, suggesting the potential use of these strains in biotechnological applications.

1. Introduction

Raised bogs being one of the oldest vegetation forms are specific ecosystems, where *Sphagnum* mosses play a key role in processes of production and degradation just like in accumulation of organic matter (Kulichevskaya et al., 2007). The degradation process of these substances is very slow. These ecosystems were formed after organic material filled up shallow open waters followed by a peat accumulation above this wet surface (Van Breemen, 1995; Vinichuck et al., 2010). The primary reasons for the reduced decomposition rate in raised bogs are the high acidity, low temperature, the lack of oxygen, the high amount of plant litter and the low concentration of mineral nutrients (Opelt et al., 2007a).

The decomposition is also delayed by the unique properties and chemical structure of *Sphagnum* mosses, namely the polyuronic acids (e.g. galacturonic) containing cell wall. The uronic acids are predominant both in the excretion products of live plants and in the products of *Sphagnum* degradation, causing acidification of peat and bog water and inactivating some important enzymes in microbial transformation processes (Clymo, 1963; Kulichevskaya et al., 2007). In the decomposition processes fungi and bacteria play a very important role. Fungi being aerobic microorganisms are found in the aerobic layer of the raised bogs, whereas bacteria are represented in both the aerobic and anaerobic layers (Bragazza et al., 2007). In a Siberian raised peat bog three dominant taxonomic groups of bacteria (spirilla, myxobacteria, bacilli) were recorded, the upper layer being dominated by bacilli (*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus sphaericus* and *Bacillus polymyxa*) (Golovchenko et al., 2005).

The study site, Borsáros natural reserve is an oligotrophic raised bog, where bryophytes and their representatives, *Sphagnum* sp. are available. Raised bogs dominated by bryophytes are unique habitats for numerous plants, animals and microorganisms (Opelt et al., 2007a). Numerous bacterial strains associated with *Tortula ruralis*, *Sphagnum rubellum* and *Aulacomnium palustre* were revealed by Opelt and Berg (2004) including *Burkholderia phenazinium*, *Burkholderia terricola*, *Burkholderia cepacia*, *Pseudomonas putida*,

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Pseudomonas reactans, *Pseudomonas fluorescens*. Opelt et al. (2007b) studied the diversity of microorganisms associated with *Sphagnum magellanicum* and *Sphagnum fallax*. A high number of different bacterial strains were found belonging to the genera *Serratia*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Pantoea* and *Delftia*. The results also indicate that moss associated bacteria are important sources of fungal and bacterial antibiotics and also have potential plant growth promoting properties.

Plant growth promoting bacteria (PGPB) are beneficial microorganisms that stimulate plant growth and control the damage of plants caused by phytopathogens through different direct or indirect mechanisms. Direct mechanisms include the ability to synthesize different compounds which facilitate the uptake of certain nutrients from the environment and plant growth promotion through nitrogen fixation, phytohormone production, phosphorous and iron solubilisation. The indirect mechanisms of plant growth promotion occur when bacteria control the deleterious effect of phytopathogenic organisms.

Mechanisms responsible for root disease suppression and control of soilborne pathogens appear to involve the production of antibiotics and other antibacterial compounds (2,4-diacetylphloroglucinol, phenazine derivatives), extracellular lytic enzymes or unregulated waste products such as hydrogen cyanide (HCN) or siderophores (Glick and Bashan, 1997; Lucas-Garcia et al., 2004; Pedras et al., 2003; Parmar and Dufrense, 2011; Raaijmakers et al., 2009). Release of HCN by bacteria into the soil can be toxic to soilborne pathogens by inhibition of the electron transport systems (Mallesh, 2008). Siderophores are small extracellular compounds that form complexes with available iron in the photosphere. Siderophore producing bacteria thus compete with phytopathogens which also require iron, and inhibit competitor growth (Oldal et al., 2002; Parmar and Dufrense, 2011).

Another important trait in pathogenic and root colonizing bacterial strains is the cell to cell communication among bacteria. Quorum sensing is one of the communication forms, mediated by signal molecules such as N-acyl-homoserine lactones. Bacterial populations are able to recognize these molecules and change their behaviour by induction of different gene transcriptions (Kuttler and Hense, 2008). Physiological activities such as symbiosis, virulence and pathogenicity are also controlled by Quorum sensing.

Fusarium wilt of cucumber is a worldwide problem in cucumber-growing areas; both chemical and biological methods are known to control this disease. Biological methods represent an alternative approach. Chen et al. (2010) and Li et al. (2012) isolated and characterized different *B. subtilis* strains, which are promising biocontrol agents against *Fusarium* wilt of cucumber.

In this study our aim was to isolate, analyse and characterize different groups of bacterial strains with PGPB properties associated with bryophytes. Bacterial isolates were screened for their plant growth promoting and antagonistic properties such as siderophore and hydrogen cyanide production. The ability of cell to cell communication with different signal molecules was also tested. Community composition of the isolated bacterial strains was analysed by amplifying 16S rDNA by polymerase chain reaction. Isolates with the best PGPB and antagonistic properties were identified taxonomically.

2. Materials and methods

2.1. Isolation of bacterial strains

We analysed different *Sphagnum* sp. samples from Borsáros raised bog natural reserve. To isolate the external bacteria and bacteria from the rhizosphere 0.5 g of each sample was shaken in physiological salt solution overnight, at room temperature and 150 rpm. Dilution series were made (up to 10^{-2} dilution) and 0.1 ml of each solution was plated on Nutrient agar (yeast extract 2 g, meat extract 1 g, peptone 5 g, sodium-chloride 5 g, agar–agar 20 g, distilled water 1000 ml), King's B medium (proteose peptone 20 g, glycerol 10 ml, $K_2H_2PO_4$ 1.5 g, $MgSO_4{}^*7H_2O$ 1.5 g, agar–agar 20 g, distilled water 1000 ml) and MacConkey agar (peptone from casein 17 g, peptone from meat 3 g, sodium-chloride 5 g, bile salts 1.5 g, agar–agar 20 g, distilled water 1000 ml). The plates were incubated at 28 °C for 24 h. Colonies showing different morphology were purified and kept on Nutrient agar.

To isolate bacterial strains from the tissues of the plants, plant surfaces were sterilized, using 5% sodium-hypochlorite solution. Plant tissues were cut into small pieces, homogenized in a sterile mortar and diluted in physiological solution. Dilution series were made (up to 10^{-2} dilution) and 0.1 ml of each solution was plated on the above mentioned culture media.

2.2. Classification of isolates with molecular tools

Amplified ribosomal DNA restriction analyses (ARDRA) method was used for the classification of the isolated bacterial strains. Genomic DNA was isolated using Promega Wizard Genomic DNA Isolation Kit, according to the manufacturer's protocol.

For the 16S ribosomal DNA amplification we used the following 27 Forward (5′AGAGTTTGATCMTGGCTCAG3′) and 1492 Reverse (5′TACGGYTAC CTTGTTACGACTT3′) universal primers. The amplification reaction was performed in a Corbett Palm-Cycler thermo cycler and included an initial denaturation at 94 °C for 3 min, which was followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. ARDRA analyses were conducted with two different restriction endonucleases: *HaeIII* and *MspI*. The amplicons that resulted from the PCR reaction and the products of the ARDRA analyses were separated in 1% and 2% agarose gel, respectively, and visualized using a BioRad transilluminator. The isolates with the same profile were considered to belong to the same bacterial phylotype.

2.3. Identification of the isolated bacterial strains

16S rDNA fragments were analysed by sequencing. The sequences were edited and aligned with Chromas; phylogenetic analyses were conducted using MEGA 4 system. The comparison of the sequences with those found in the NCBI database was done with a BLAST algorithm. Sequences were sent and deposited into the European Molecular Biology Laboratory – European Bioinformatics Laboratory (EMBL – EBI) European Nucleotide Archive (ENA) database.

2.4. Screening of plant growth promoting properties

2.4.1. Detection of hydrogen cyanide producing strains

Production of HCN from glycine was tested by growing bacteria on Nutrient agar plates, supplemented with glycine (4.4 g/L). Cyanogenesis was revealed using picric acid (0.5%) and Na_2CO_3 (2%) solution. Sterile filter paper, impregnated in picric acid solution was fixed to the underside of the Petri dish lids. Plates were incubated for 5 days at 28 °C. A change in colour of filter paper from yellow to orange, light brown or brown indicated different amounts of hydrogen cyanide production (Samuel and Muthukkaruppan, 2011).

2.4.2. Siderophore production properties

Production of ferric ion chelates was detected by using agar plates containing Chrome Azurol S dye (CAS), according to Schwyn