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MOLECULAR IDENTIFICATION OF FIVE *BACILLUS* STRAINS WITH PLANT GROWTH POTENTIAL ABILITIES

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Abstract: Plant growth promoting rhizobacteria (PGPR) are a group of bacteria typically isolated from rhizosphere where they can enhance the growth of plants directly or indirectly. The aim of this study was to identify five rhizosphere strains from genus *Bacillus* by complex of phenotypic and molecular methods for taxonomy characterization. Belonging of five investigated strains to *Bacillus* genus were confirmed by their biochemical profile, estimated by API 50CHB. On the base of analysis of 16S rDNA sequences two of the studied strains, 6VR and 8VR, were identified as Bacillus subtilis. The other strains belong to three different species: 7VR to *Bacillus cereus*, 9VR to *Bacillus pumilus* and 13VR to *Bacillus thuringiensis* respectively.

INTRODUCTION

Various bacterial species from *Bacillus, Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia* and *Serratia* genera have been reported to enhance plant growth (Saharan BS., 2011) and they were defined as plant growth promoting rhizobacteria (PGPR). *Bacillus* is the most abundant genus in the rhizosphere with known plant growth promoting activities (Probanza A. *et al.,* 2002; Gutierrez M. FJ. *et al.,* 2003). Thus, the identification and taxonomical characterization of each new selected plant growth promoting microorganism at species level is becoming more important. Modern polyphasic taxonomy combines various phenotypic and molecular methods for typing and sub-typing of bacteria. Different molecular techniques have been applied to detect newly isolated bacterial strain. The nucleotide base sequences of bacterial 16S ribosomal DNA provides an accurate basis for phylogenetic analysis and identification. Analysis of 16S rDNA sequences

is a simple, commonly used method for the identification of microorganisms. PCR methods for identification on *Bacillus* strains are used by many authors to classify new isolates (Naveed M. *et al.,* 2013, Zahid M. *et al.,* 2015, Nemeckova *et al.,* 2011). The NCBI database contains over a million full-length 16S gene sequences that are useful for identification of bacterial species from diverse samples (Ntushelo K., 2013).

The present work aimed to identify five rhizosphere strains from genus *Bacillus* by complex of phenotypic and molecular methods for taxonomy characterization.

MATERIALS AND METHODS

Bacterial strains

Five rhizosphere Bacillus sp. strains: 6VR, 7VR, 8VR, 9VR and 13VR were taken from the culture collections of the Department of Biotechnology, Biological Faculty, Sofia University and ROMB Ltd.

Phenotypic characterization

Carbohydrate fermentation profile was obtained by the commercial API 50 CHB test according to the manufacturer specification (BioMérueux, France). The Apiweb^{R} identification software was used for interpretation of the results.

Molecular identification

DNA from the *Bacillus* strains was isolated with NucleoSpin® tissue isolation kit (Macherey-Nagel) according to the description the manufacturer and was amplified with universal primers (27F or 1492R) by using Ready-To-Go PCR Beads Kit (GE Healthcare Live Sciences). Obtained PCR product was used as a template DNA for standard sequencing procedure (Macrogen Inc, office Netherlands). The sequences were edited to exclude the PCR primer-binding sites and were compared to the available nucleotide database from the NCBI GenBank using BLAST algorithm. Phylogenetic trees were constructed by neighbor - joining method using MEGA 7 (Molecular Evolutionary Genetics Analysis) software (Kumar S. *et al,* 2016).

RESULTS AND DISCUSSION

In the present study, five *Bacillus* strains, isolated from rhizosphere were identified, according to polyphasic taxonomy. On the basis of the results from carbohydrate utilization, estimated by API 50 CHB system, the belonging of the five investigated strains to the *Bacillus* genus was confirmed, but the level of the determination to the species level was considered as low (Table 1).

Table 1 Biochemical profile of five *Bacillus* strains (API 50 CHB)

Characteristics	**VR6**	**VR7**	**VR8**	**VR9**	**VR13**
Catalase	+	+	+	+	+
Oxidase	-	+	-	-	+
Glycerol	+	-	+	+	+
Erythritol	-	-	-	-	-
D - arabinose	-	-	-	-	-
L- arabinose	+	-	+	+	-
D- ribose	+	+	+	+	+
D - xylose	+	-	+	+	-
L- xylose	-	-	-	-	-
D- adonitol	-	-	-	-	-
Methyl β-D-Xylopyranoside	-	-	-	-	-
D - galactose	-	-	-	+	-
D - glucose	+	+	+	+	+
D- fructose	+	+	+	+	+
D - mannose	+	+	+	+	+
L - sorbose	-	-	-	-	-
L - rhamnose	-	-	+	-	-
Dulcitol	-	-	-	-	-
Inositol	+	-	+	-	-
D - mannitol	+	-	+	+	-
D - sorbitol	+	-	+	-	-
Methyl–αD-Mannopyranoside	-	-	-	-	-
Methyl–αD-Glucopyranoside	-	-	+	-	-
N- Acetylglucosamine	+	+	+	+	+
Amygdalin	+	-	+	+	-
Arbutin	+	+	+	+	+
Esculin ferric citrate	+	+	+	+	+
Salicin	+	-	+	+	+
D - celiobiose	+	-	+	-	-
D - maltose	+	+	+	-	+

Characteristics	VR6	VR7	VR8	VR9	VR13
D – lactose (bovine origin)	-	-	-	-	-
D- melibiose	+	-	+	-	-
D – sucrose	+	-	+	+	+
D - trehalose	+	+	+	+	+
Inulin	+	-	+	-	-
D - melezitose	-	-	-	-	-
D - raffinose	+	-	+	-	-
Amidon(starch)	+	-	+	-	+
Glycogen	+	-	+	-	+
Xylitol	-	-	-	-	-
Gentiobiose	+	-	+	+	-
D- turanose	+	-	+	-	-
D - lyxose	-	-	-	-	-
D - tagatose	-	-	-	+	-
D - fucose	-	-	-	-	-
D - arabitol	-	-	-	-	-
L - arabitol	-	-	-	-	-
Potassium gluconate	+	+	+	-	-
Potassium 2- KetoGluconate	-	-	-	-	-
Potassium 5- KetoGluconate	-	-	-	-	-
Citrate	+	-	+	-	-

For correct identification of rhizosphere *Bacillus sp.* isolates, one of the widely accepted methods of 16S rDNA gene sequencing and comparison of the obtained sequences with global databases was used (Table 2). Two of the studied strains, 6VR and 8VR, were identified as *Bacillus subtilis*. The other strains belong to three different species: 7VR to Bacillus cereus, 9VR to *Bacillus pumilus* and 13VR to *Bacillus thuringiensis* respectively. The obtained sequences of each of the five strains and similar sequences of closely related *Bacillus* species were used to construct the phylogenetic tree using neighbor - joining method (**Fig. 1**).

Table 2. Molecular Identification of isolated strains on the base of 16S rDNA sequence

Strain	Species	Similarity %
6VR	*Bacillus subtilis*	99
7VR	*Bacillus cereus*	99
8VR	*Bacillus subtilis*	98
9VR	*Bacillus pumilus*	98
13VR	*Bacillus thuringiensis*	99

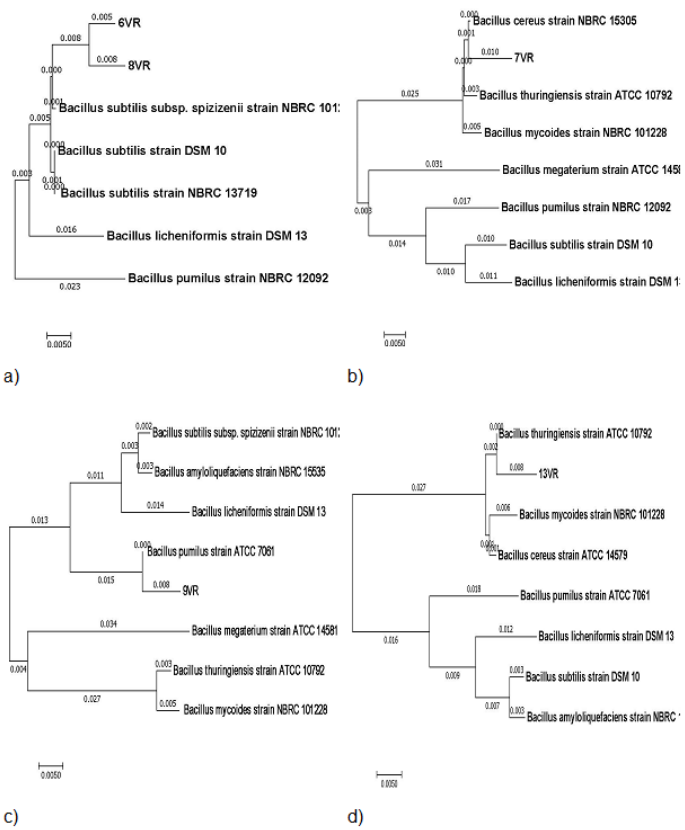

Fig. 1. Phylogenetic tree of **a)** *Bacillus subtilis* 6VR and *Bacillus subtilis* 8VR, **b)** *Bacillus cereus* 7VR, **c)** *Bacillus pumilus* 9VR, **d)** *Bacillus thuringiensis* 13VR, constructed via neighbor - joining method.

CONCLUSION

Different *Bacillus* strains with plant growth-promoting activity are mostly used in commercial bio formulas because their endospores are tolerant to extremes of the abiotic environments. Molecular characterization and typing of the five rhizosphere strains confirmed their belonging to species from genus *Bacillus* with special reference as PGRR. All studied strains show a plant growth promotion potential in series of experiments (unpublished data). The correct identification of the rhizosphere *Bacillus subtilis* 6VR, *Bacillus cereus* 7VR, *Bacillus subtilis* 8VR, *Bacillus pumilus* 9VR and *Bacillus thuringiensis* 13VR in the present study make them suitable to be involved in new commercial plant growth promoting products and to improve safety in specific technological uses.

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