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Molecular differentiation of plant beneficial *Bacillus* strains useful as soil agro-inoculants

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Abstract

Plant rhizosphere is rich in bacterial cells of *Bacillus* species. This bacterial genus includes a large number of strains with beneficial properties for plant protection and growth promotion. For this study we selected seventeen beneficial strains of *Bacillus* spp., isolated from food products, legume rhizosphere and agricultural soils from South and South-East areas of Romania. The selection was based on their ability to inhibit the growth of several plant pathogens and to produce different enzymes and metabolic compounds with beneficial traits for plant protection and growth promotion, as it was presented in our previous studies. For a successful use of such beneficial bacteria as agro-inoculants, their survival in natural ecosystems should be in sufficiently high levels to produce the intended purpose. The best way considered for the evaluation of their fate is the analysis *in situ* of the inoculated bacterial strains. For differentiating the inoculated *Bacillus* beneficial strain from other indigenous bacilli present in the environment, the molecular techniques seem to be the proper way, if we refer to the accuracy, time consumption and financial resources. In our study, RAPD analysis was used for the molecular analysis of selected strains. According to our results, the use of RAPD-PCR technique with OPE-02, OPE-20 and OPM-15 primers can offer a good differentiation of *Bacillus* strains recommend as agro-inoculants. Using strain specific markers, inoculated bacteria could be accurately detected after their application in soil.

Keywords: beneficial bacteria, *Bacillus*, RAPD

INTRODUCTION

Bacillus species are widely spread in the soil and rhizosphere. Their presence in such substrates was recorded in concentrations as high as 10^7cfu/g of rhizosphere soil (Pandey et al., 1997). Inside *Bacillus* genus the species are clustered based on their phylogeny (Xu and Côté, 2003). Five of these groups, *Bacillus cereus*, *B. megaterium*, *B. subtilis*, *B. circulans* and *B. brevis* groups are intensively studied and used for biotechnological and industrial purposes (Sicuia et al., 2015).

Plant beneficial traits described in *Bacillus* refer to growth promotion, biological control and increased resistance to abiotic factors (Siddiqui, 2006; Lim and Kim, 2013). Inoculation

with beneficial *Bacillus* strains increases plant vigor and productivity and, in some cases, can contribute to plant earliness (Mia, 2002) or delay the senescence (Freitas et al., 2015). For the biocontrol, *Bacillus* can produce and release different metabolic compounds like antibiotics, lytic enzymes or volatile and non-volatile compounds with inhibitory effect against phytopathogenic agents (Ahmad et al., 2008; Aftab, 2010; Martínez-Viveros et al., 2010; Wahyudi et al., 2010a,b). The niche and nutritional competition are also responsible for the suppression of plant pathogens (Pal and McSpadden Gardener, 2006; Yu et al., 2011). Another mechanism involved in plant protection is the Induced Systemic Resistance in plants inoculated with biocontrol strains (Compant et al., 2005; Yang et al., 2009). Beside the beneficial effects that protect plants against harmful biotic factors, several studies reveal plant increased tolerance to abiotic stress, such as drought, salt and nutrient deficiency or excess when inoculated with *Bacillus* stains (Yang et al., 2008; Bianco and Defez, 2011). Therefore, *Bacillus* species have great potential for agricultural improvement. They are reported as attractive candidates for biological control of different plant pathogens and have the advantage of endospore forming useful in formulation and surviving under adverse environmental conditions (Saidi et al., 2009). However, the identification of such beneficial strains should be completed in advance formulation and use at large scale.

For species belonging to *Bacillus subtilis* group, which are sharing morphological and biochemical characteristics, the differentiation and identification are particularly difficult (Freitas et al., 2008). Within this group there are mentioned twelve closely related species, *Bacillus amyloliquefaciens*, *B. atrophaeus*, *B. axarquiensis*, *B. licheniformis*, *B. malacitensis*, *B. mojavensis*, *B. pumilus*, *B. subtilis, B. sonorensis*, *B. tequilensis*, *B. vallismortis* and *B. velezensis* (Rooney et al., 2009; Jeyaram et al., 2011), with ≥ 99% similarity inside the 16S rRNA sequence, which is a highly conserved region (Sicuia et al., 2015). The molecular approaches used for accurate identification and differentiation of closely related species are the Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Internal Transcribed Spacers – PCR – RFLP (ITS-PCR-RFLP), Amplified Ribosomal DNA Restriction Analysis (ARDRA), repetitive elements sequence-based PCR (rep-PCR), and Sequence Characterized Amplified Region (SCAR). These methods provide molecular fingerprinting and could reveal differences between closely related species or strains (Versalovic et al., 1994; Cihan et al., 2012; Felici et al., 2008; Gajbhiye et al., 2007; Kathleen et al., 2014; Sen et al., 2015).

The aim of our study was to evaluate RAPD technique for the fingerprinting of biocontrol bacterial strains, belonging to "*Bacillus subtilis* group", previously selected for agricultural purposes after *in vitro* and *in vivo* experiments.

MATERIALS AND METHODS

Bacterial biocontrol strains

Seventeen bacterial strains isolated from food products (BPA, BIR), plant rhizosphere (Usa2, Cpb5, Cpb6, OS15, OS17) and agricultural soil from the South (Bucharest area) and South-East (Bărăgan area) of Romania (BW, Icpc, 50.4, 63.3, 70.1s, 73.4, 76.2, 77.1s, 77.3, 83.2s) were used in this work. These strains were previously selected for their beneficial characteristics (Constantinescu et al., 2010), mostly in plant protection, but also for plant growth promotion and environmental applications. Italian native bacterial strains were also used to compare the genetic variability inside *Bacillus* group. Two strains, *Bacillus* sp. BATT102 and *Bacillus cereus* BATT197, were kindly provided from the Department of Agricultural, Forestry and Food Sciences (DISAFA), University of Torino, Italy. Two *Bacillus subtilis* strains

(ATCC6633 and ATCC11774), and *Bacillus licheniformis* ATCC14580 were used as reference for the molecular fingerprinting.

Bacterial DNA extraction

Two milliliters of bacterial culture obtained over night in aerated Luria Bertani broth medium were centrifuged at 5000 rpm for 5 min. The harvested bacterial biomass was subjected to DNA extraction using a Macharey-Nagel Kit for genomic DNA according to the manufacturer protocol.

Bacterial identification procedures

Microbial identification was achieved by two different approaches. First, the bacterial strains were identified using the Biolog GEN III system, according to the manufacturer protocol B for spore-forming Gram-positive bacilli. The procedure concerned in cultivating the bacterial strains on Biolog Universal Growth (BUG) media, suspending single colonies in Biolog B type inoculation fluid up to 97% turbidity (in 590 nm light), and inoculating the bacterial suspension in Biolog GEN III microplate. After over night incubation at 33°C the microbial strains were identified by reading the microplate with the semi-automat Biolog Microstation Plate Reader. The system analyses the ability of the cell to metabolize major classes of biochemicals, in addition to determining other important physiological properties such as pH, salt, and lactic acid tolerance, reducing power, and chemical sensitivity. The identification procedure was completed with molecular studies. The 16S rDNA PCR technique was performed using the universal primers for bacteria, 27F and 1492R. The PCR products were visualized on 1.5% agarose gel by electrophoresis, purified and sequenced at BMR Genomics. The partial sequences obtained with forward and reverse primers were aligned using the Molecular Evolutionary Genetics Analysis (MEGA) program. The sequences obtained were further subjected to online BLAST (Basic Local Alignment Search Tool) software available from the National Center for Biotechnology Information (NCBI) for taxonomic identification based on the sequence similarity with other microorganisms found in the NCBI database.

RAPD-PCR

RAPD analysis was performed with 24 primers with arbitrary nucleotide sequence, from five different series: OPA (OPA-11, OPA-12, OPA-15, and OPA-20), OPB (OPB-12, OPB-17, and OPB-19), OPE (OPE-02, OPE-03, OPE-10, and OPE-20), OPM (OPM-07, OPM-09, OPM-10, OPM-11, OPM-14, OPM-15, and OPM-19) and OPT (OPT-05, OPT-11, OPT-14, OPT-16, OPT-18, and OPT-20). The RAPD primers used in this study are listed in table 1. The reaction mixture included 1X Coloral Load buffer (Qiagen); 0.2 mM dNTPs; 2.5 mM $MgCl_2$; 1 µM primer; 1 U Taq polymerase (Promega) and 20 ng genomic ADN in 20 µl reaction volume. The PCR program consisted of: 3 min at 94°C to denature the template; 44 cycles of 94°C for 45 sec, 36°C for 1 min and 30 sec and 72°C for 2 min; and final incubation at 72°C for 10 min. Finally, the amplification products were loaded on 1.5% agarose gel supplemented with SYBR Safe DNA Gel Stain and subjected to electrophoresis in 1X TAE buffer, for 5 h at 35 V. The electrophoretic profiles were revealed in UV light using the GelDoc XR system (BioRad).

Analyzing the molecular fingerprints

The molecular fingerprints generated by the electrophoretic profiles of RAPD, PCR reactions were visually compared and analyzed with TREECON software for Windows.

RESULTS AND DISCUSSION

Bacterial identification

The Biolog GEN III analysis revealed the phenotypic fingerprint of beneficial strains isolated in Romania. Based on their biochemical profile these bacterial strains were identified with the MicroLog3 software. This identification analysis showed that all studied bacterial strains, except for *Bacillus cereus / thuringiensis* 50.4 strain, belong to closely related species (table 2[A]). There were identified 9 strains of *Bacillus subtilis* (Usa2, Cpb5, Cpb6, BPA, Icpc, 63.3, 70.1s, 73.4, 77.3), two strains of *B. amyloliquefaciens* (OS17, BW), four strains of *B. pumilus* (OS15, BIR, 76.2, 83.2s) and one strain of *B. licheniformis* (77.1s). These species are considered closely related and, as proposed by Jeyaram et al. (2011), are included in *Bacillus subtilis* group.

Highly conserved region corresponding to 16S rDNA was amplified with 27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492r (5'-CGG TTA CCT TGT TAC GAC TT-3') primers, and the nucleotide sequence of the amplicons was determined. Bacterial strains were identified with the NCBI BLAST on-line program for taxonomic identification, based on their sequence similarity with other microorganisms found in the NCBI database. This identification method generated comparable results to those of Biolog GEN III analysis. Only five of the seventeen strains were differently identified when the two methods were applied. However, the tested strains are closely related and belong to *B. subtilis* group (Jeyaram et al., 2011), except for the 50.4 strain, which was found to be from *B. cereus* group (table 1).

Table 1. Bacterial strain identification

Bacterial strain	Biolog GEN III – biochemical identification	16S rDNA partial sequencing
Usa2	*Bacillus subtilis*	*Bacillus subtilis*
Cpb5	*Bacillus subtilis*	*Bacillus amyloliquefaciens*
Cpb6	*Bacillus subtilis*	*Bacillus subtilis*
OS15	*Bacillus pumilus*	*Bacillus amyloliquefaciens*
OS17	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens*
BPA	*Bacillus subtilis*	*Bacillus amyloliquefaciens*
BIR	*Bacillus pumilus*	*Bacillus amyloliquefaciens*
BW	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens*
Icpc	*Bacillus subtilis*	*Bacillus subtilis*
50.4	*Bacillus cereus / thuringiensis*	-
63.3	*Bacillus subtilis* ss. *subtilis*	*Bacillus subtilis*
70.1s	*Bacillus subtilis* ss. *subtilis*	*Bacillus subtilis*
73.4	*Bacillus subtilis*	*Bacillus subtilis*
76.2	*Bacillus pumilus*	*Bacillus pumilus*
77.1s	*Bacillus licheniformis*	*Bacillus licheniformis*
77.3	*Bacillus subtilis*	*Bacillus subtilis*
83.2s	*Bacillus pumilus*	*Bacillus amyloliquefaciens*

Eleven of the tested strains were similarly identified by both methods as *B. subtilis* (Usa2, Cp.b6, Icpc, 63.3, 70.1s, 73.4, 77.3), *B. amyloliquefaciens* (OS17, BW), *B. pumilus* (76.2) and *B. licheniformis* (77.1s). The other strains (Cp.b5, OS15, BPA, BIR, 83.2s) were affiliated to different species by the Biolog system comparing with the 16SrDNA technique. However, these two identification methods revealed closely related species that are included in the same group, "*Bacillus subtilis* group", (according to Ash et al., 1991; Freitas et al., 2008; Jeyaram et al., 2011).

Considering that the 16SrDNA sequencing is a more accurate method, these six strains were further referred as *B. amyloliquefaciens*.

Molecular characterization of the selected bacterial stains

The RAPD techniques were chosen to describe the genetic diversity within bacterial strains of "*Bacillus subtilis* group" along with two strains of *B. cereus* (BATT 197 and 50.4). PCR reactions were carried out using 24 RAPD primers primers. The electrophoretic profiles with these PCR reactions revealed 403 amplified bands. These molecular characteristics generated different dendrograms that were subjected to be analyzed with TREECON software for Windows. The dendrograms were generated using UPGMA clustering analysis (figure 1). Reproducibility of the groupings below each node of the dendrogram was verified by analyzing 2000 multiple data sets from bootstrapping.

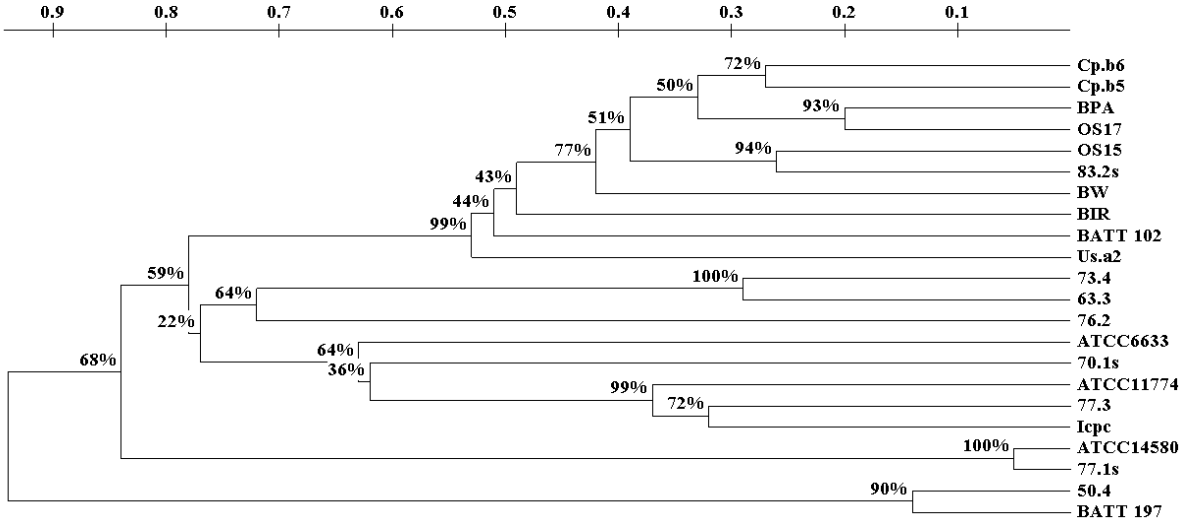

Figure 1. Phylogenetic tree performed with TREECON software for Windows on the matrix resulted from the RAPD- PCRs performed on the bacterial strains analyzed in this study.

The phylogenetic tree obtained using the RAPD primers tested for the mentioned strains complied with the taxonomic relationship between the identified species, along with their origin.

The use of RAPD-PCR technique with OPA-11, OPA-20, OPB-12, OPB-17, OPB-19, OPE-02, OPE-10, OPE-20, OPM-11, OPM-15, OPM-19 and OPT-20 primers (Figure 2) offered a good differentiation between *Bacillus* strains recommend as agro-inoculants. These 12 primers induced 71.7% of the differentiation registered with all oligonucleotide primers used during this study.

Regarding the differentiation between the analyzed beneficial *Bacillus* strains, the use of OPE-02, OPE-20 and OPM-15 primers revealed the highest number of amplification bands of distinct molecular weight. However, no strain specific marker was fount with the analyzed RAPD primers.

According to our results, the use of RAPD-PCR technique with OPE-02, OPE-20 and OPM-15 primers can offer a good differentiation of *Bacillus* strains recommend as agro-inoculants. Using strain specific markers, inoculated bacteria could be accurately detected after their application in soil.

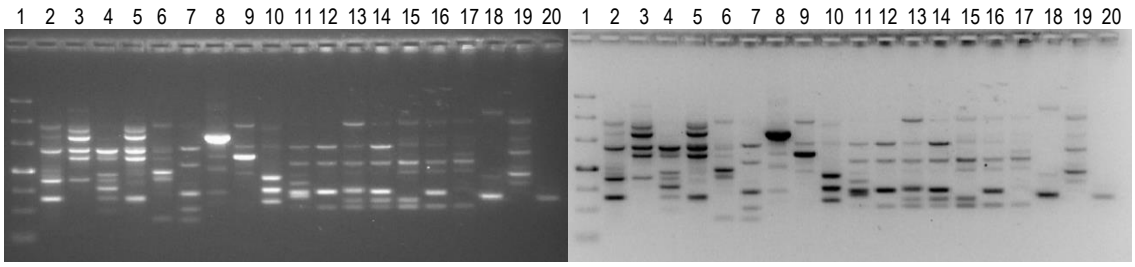

Figure 2. RAPD-PCR profile of the beneficial strains of *Bacillus* species obtained with OPM-15 oligonucleotide primer.
Lanes: 1- Molecular weight marker (Wide Range); 2- *Bacillus subtilis* ATCC 6633, 3- *B. subtilis* ATCC11774, 4- *B. subtilis* 77.3, 5- *B. subtilis* Icpc, 6- *B. subtilis* Us.a2, 7- *B. subtilis* Cpb6, 8- *B. subtilis* 73.4, 9- *B. subtilis* 63.3, 10- *B. amyloliquefaciens* BATT 102, 11- *B.amyloliquefaciens* Cpb5, 12- *B. amyloliquefaciens* BPA, 13- *B. amyloliquefaciens* BW, 14- *B. amyloliquefaciens* OS17, 15- *B. amyloliquefaciens* OS15, 16- *B. amyloliquefaciens* 83.2s, 17- *B. amyloliquefaciens* BIR, 18- *B. pumilus* 76.2, 19- *B. licheniformis* ATCC14580, 20- BATT 197.

CONCLUSIONS

Microbiological and biochemical characteristics are not always sufficient to distinguish between closely related species within the group, or between the strains of the same species. However, for genetic diversity evaluation RAPD techniques could be successfully used in differentiating among strains from *B. subtilis* group. The results obtained in the experiments could be good arguments for the application of such molecular differentiating methods for the analysis of plant beneficial microorganisms like biocontrol bacteria.

Since the interest for using biological control agents is increasing, a particular attention should be paid for the microbial strains recommended regarding their efficiency, the benefits for plants and the harmless for the non-target organisms. For this reason, in order to restore the phytosanitary balance in crop protection, we sustain the idea of the use of indigenous microbial strains. Moreover, in order to determine the ability of agro-inoculated strains to accommodate in the soil, the identification of some molecular fingerprints is very important for monitoring the biocontrol strains at different moments after application.

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