Enzymatic and Genetic Variability in *Bacillus* spp. Strains with Plant Beneficial Qualities

Oana-Alina SICUIA^{1,2}, Iulian GROSU¹, Florica CONSTANTINESCU², Cătălina VOAIDEȘ¹, Călina Petruța CORNEA^{1,*}

¹ University of Agronomic Sciences and Veterinary Medicine – Bucharest, Faculty of Biotechnologies, 59 Marasti Blvd., PC 011464, Bucharest, Romania;

² Research and Development Institute for Plant Protection, 8Ion Ionescu de la Brad Blvd., Bucharest, Romania.

*corresponding author, e-mail: pccornea@yahoo.com

Bulletin UASVM series Agriculture 73(1)/2016

Print ISSN 1843-5246; Electronic ISSN 1843-5386

DOI 10.15835/buasvmcn-agr: 11570

ABSTRACT

Plant health is one of the issues that have to be maintained and closely monitored during cultivation and harvest. In this regard, prevention is the key factor in organic production. Biological control of plant pathogens and plant growth stimulation can be done through beneficial microorganisms. Different bacterial bio-preparates are available on the market, many of them based on selected strains of *Bacillus* species. In our previous studies, we isolated autochthones strains of *Bacillus* spp. with beneficial traits for plant protection and growth promotion. Considering the interest for biological production, and public concern for healthy products without significantly reduced yield, different biological control strains of *Bacillus* spp. that could be used as bio-inoculants for plant protection were analyzed. Thirteen biocontrol strains of *Bacillus* spp. were analyzed in comparison with three referent strains of *B.subtilis* and *B.licheniformis*. Qualitative and semi-quantitative analysis of these biocontrol *Bacillus* spp. strains was studied in order to characterize their enzymatic activity with implications either in cell wall degradation of plant pathogenic microorganisms, or in metabolism of various substrates. Genetic variability was studied by rep-PCR analysis compared with reference strains of *B.subtilis* and *B.licheniformis*. Microbiological studies performed in order to characterize the selected beneficial bacteria for their ability to produce lytic enzyme involved in plant pathogenic inhibition and plant growth stimulation revealed chitinase, cellulose, protease, lipase, amylase, decarboxylase, ACC-deaminase and phosphatase activity. The molecular techniques revealed significant genetic differences among the bacterial strains analyzed. The study allowed the detection of several enzymatic mechanisms involved in plant growth and protection, and revealed the potential of autochthon microbiota to be used for biotechnological purposes.

Keywords: *Bacillus*, lytic enzymes, genetic diversity

INTRODUCTION

Public concern for healthy and high quality foodstuffs oriented the agricultural farmers to increase consideration for organic production. In Romania, the subsidies for organic farming conversion have made more attractive this agricultural sector, although the organic yields are highly export-oriented (<http://www.ifoam-eu.org/en/romania>). Pest and disease management is

one of the most significant challenges in obtaining the estimated yield, also with grate implication in the quality of the product. In this regard, prevention is the key factor in organic production. Biological control of plant pathogens and plant growth stimulation could be completed with beneficial microorganism application (Borriss, 2011). Different bacterial bio-products are available on the market, many of them based on selected strains

of *Bacillus* species (Constantinescu and Siciua, 2013). However, it is assumed that native strains are more suitable as bio-inoculants (Ceccon et al., 2012), due to a better environmental integration of the inoculated microorganism. Such treatments will act as enhancers in restoring the natural balance of the micro-biota inside the cropland. Plant growth-promoting bacteria with biological control competences, refer to as biocontrol-PGPB, can release different enzymes involved in plant pathogens inhibition, ISR elicitation and plant growth promotion (Compant et al., 2005.).

In our previous studies, we isolated autochthonous strains of *Bacillus* spp. with beneficial traits for plant protection and growth promotion that could be used as bio-inoculants for plant protection. The spectrum of extracellular enzymes produced by these strains was evaluated in order to classify tested *Bacillus* strains based on their abilities and potential use. Genetic variability was also analyzed in order to reveal molecular differences between these strains.

MATERIAL AND METHODS

Selected Bacterial strains. Thirteen Romanian strains of *Bacillus* spp. (table 1) were analyzed in these studies, along with three referent strains of *B. subtilis* and *B. licheniformis*. All of these bacteria

were stored in microbial collections at -80°C, in 20 % glycerol, and routinely grown on Luria Bertani agar at 28°C. Different characteristics were noted between the strains on specified medium.

Bacterial genomic DNA extraction

Fresh bacterial cultures were used for DNA extraction. The nucleic acids were purified with High Pure PCR Template Preparation kit (Roche Life Science).

Rep-PCR analysis

The genetic variability among the bacterial strains was evaluated by rep-PCR technique, using BOXA1R primer 5'-CTA CGG CAA GGC GAC GCT GAC G-3' (Versalovic et al., 1994). The BOX primers are targeting the highly conserved repetitive DNA sequences from BOXA subunit of the BOX element (Xie et al., 2008). The genomic fingerprinting of previously described bacteria was done in comparison with *B. subtilis*, ATCC 6633 and ATCC11774, and *B. licheniformis* ATCC 14580 reference strains. PCR reaction was performed as described by Siciua et al. (2015). Molecular differences between the tested strains were examined through horizontal electrophoresis in 2% agarose gel stained with ethidium bromide, at 100V for 1h in 0.5X TBE buffer. The electrophoretic patterns were revealed in UV light with an UVP transilluminator,

Tab 1. Bacterial strains and corresponding collections

Bacterial name	Bacterial strain	Microbial collection
<i>B. subtilis</i>	Us.a2	RDIPP, Bucharest
10		Faculty of Biotechnologies
Icpc		
<i>B. amyloliquefaciens</i>	BW	Faculty of Biotechnologies
BN7		
B7.2		
B3		
OS17		RDIPP, Bucharest
<i>Bacillus</i> spp.	BIR	Faculty of Biotechnologies
BPA		
B5		
OS15		
83.2s		RDIPP, Bucharest
<i>Bacillus subtilis</i>	ATCC 6633	Reference strains
<i>Bacillus subtilis</i>	ATCC 11774	
<i>B. licheniformis</i>	ATCC 14580	

and their images were captured with the BioDoc-It™ Imaging System.

For the analysis of BOX-PCR results we manually determined, based on the electrophoretic pattern, the molecular weight and Rf values of each amplification. We transferred this information in binomial format, encoding with '1' each DNA band, and with '0,' when no amplification was present. The dendrogram was built using TreeCon software, for the cluster analysis by the unweighted-pair-group method with arithmetic average (UPGMA).

Estimation of cell wall degrading enzymes involved in plant-pathogens biocontrol

Cellulase activity

The bacterial strains were cultured at 28°C for 72h on carboxy-methyl cellulase (CMC) medium containing 10g/L CMC, 0.5 g/L NaCl, 1g/L KH_2PO_4 , 0.5g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.3g/L NH_4NO_3 , 0.01g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 12g/L agar. After incubation plates were treated with 0.1% Congo red for 15 min and later than the dye was fixed using 1M NaCl. Clear halo, indicating the CMC degradation, was measured compared with the colony area for semi-quantitative determination of cellulase activity.

Chitinase activity

Chitinolytic enzyme production was determined on Agrawal and Kotasthane (2009) medium with the pH of 5.5 and bromocresol purple as indicator dye. An identical test was performed on the same culture medium with the pH of 6.0 using phenol red as indicator dye. The colloidal chitin was prepared from crab shell using Roberts and Selitrennikoff (1988) method modified by Agrawal and Kotasthane (2009). However, the obtained colloidal chitin was brought to pH 5.0 using 1N NaOH solution in the distilled washing water. Chitinase activity was revealed by the change of color in the growth media, from yellow (at acid pH) to purple (at neutral pH) due to chitin degradation in the presence of bromocresol purple, or from yellow (at pH 6) to red (at pH 8), when phenol red dye was used.

Protease activity

Bacteria were grown for three days on gelatin agar medium containing 5g/L peptone, 3g/L beef extract, 4g/L gelatin, and 18g/L agar. After incubation, plates were flooded with 15% HgCl_2 in 20% HCl solution as protein precipitating agent. Mercuric chloride solution reacted with gelatin and produced a white precipitate,

revealing clearing zones of protein degradation. Enzyme activity was quantified by measuring the clearing zones indicated the extracellular protease activity.

Semi-quantitative estimation was possible using the following formula: $EA = D-d$; where D is the diameter of the clearing zone; and d is the microbial colony diameter (Hasan et al., 2013).

Lipase activity

Two lipolytic screening assays were performed, revealing Tween 80 and olive oil degradation. Modified Schoofs et al. (1997) medium was prepared with 10g peptone, 5g NaCl, 0.5g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 18g agar and 10ml Tween 80 in 1L of distilled water. Tween 80 hydrolysis is associated with the production of lipolytic enzymes and the appearance of a white precipitate (calcium soap) underneath and around the bacterial growth. Oil degrading bacteria were selected by growing on YOC medium supplemented with 0.0001% (w/v) rhodamine B (Boonmahame and Mongkolthanaruk, 2013). Pink-orange colony, observed under 350nm UV light, reveals lipase production.

Enzymes involved in plant growth promotion
Phosphorus solubilization

Bacterial strains were inoculated on Pikovskaya agar medium modified by Sundara Rao and Sinha (1963). Clear zone around the bacterial colony indicate phosphorus solubilization.

ACC-deaminase activity

Bacterial strains were tested for 1- aminocyclopropane -1- carboxylic acid (ACC) deaminase by growing on DF salt minimal medium (Dworkin and Foster, 1958) supplemented with 3mM ACC as sole nitrogen source. Plates containing DF medium supplemented with 2g/L ammonium sulphate were inoculated as positive control, and DF medium with no nitrogen source were used as negative control.

Arginin decarboxylase activity

Linear polyamines can be produced by two alternatives pathways. Arginine decarboxylase activity triggers one of these pathways using arginine as enzymatic substrate. For the evaluation of this enzyme biosynthesis, bacterial strains were grown on the following medium, containing 2g/L arginine, 5g/L bacto-peptone, 5g/L beef extract, 0.5g/L glucose, 5mg/L pyridoxine, 20mg/L phenol red and 18g/L agar, at pH 6.0. Positive reaction was seen due to the pH indicator dye that changed the color of the medium from yellow to red.

Amylase activity

Starch depolymerisation was screened on nutrient agar medium supplemented with 0.4% soluble starch. Amylase activity was revealed using Lugol solution, which results in a colorimetric reaction. Clear zone around the bacterial colony indicate starch hydrolysis, and the blue color is due to starch interaction with iodine.

RESULTS AND DISCUSSION

Genetic variability within *Bacillus* strains

The BOX-PCR profile obtained with BOX1A primer for the 15 *Bacillus* strains analyzed (including de three reference strains) revealed 17 polymorphic bands within a total of 111 amplified DNA fragments (figure 1).

The dendrogram derived from repetitive sequence-based PCR (rep-PCR) using BOX1A primer clustered the *Bacillus subtilis* Us.a2 and *Bacillus* spp. 83.2s strains along with the reference strain *B.subtilis* ATCC 11774. Likewise, *B. amyloliquefaciens* BN7 and *Bacillus* spp. OS15 were clustered as similar (figure 2).

Bacillus enzymatic characterization

Lytic enzymes, such as chitinase, proteases, or cellulases produced by antagonistic microorganisms, are responsible for the lysis or hyperparasitism expressed against deleterious fungal pathogens or insects. In the biological control mechanisms, these extracellular enzymes released by beneficial microorganisms are digesting the cell wall components of the phytopathogenic fungi (Illakkiam *et al.*, 2013). Such enzymatic activity was found in all *Bacillus* strains analyzed in this study (Table 2), suggesting these beneficial properties as additional mechanism in their biological control activity.

The biological control mechanisms triggered by cellulases involve the lytic activity that damages the cell wall of *Pythium* and *Phytophthora* plant pathogens, and the elicitation of plant defense. Such traits could be involved in the biocontrol activity of the selected strains, considering the cellulase activity expressed on the CMC substrate (figure 3). Similar cellulose degrading ability was also recently described in other *B. subtilis*,

Fig. 1. Electrophoresis pattern of the *Bacillus* spp. amplicons from BOX-PCR reaction

Fig. 2. Dendrogram showing the similarity between *Bacillus* strains analyzed through BOX-PCR technique

B. amyloliquefaciens, and *B. licheniformis* strains (Singh et al., 2013; Lambertz et al., 2014; Seo et al., 2013).

Several studies mention chitin hydrolysis due to *Bacillus* enzymatic activity, as for *B. subtilis* (Wang et al., 2006) or *B. amyloliquefaciens* (Songsiriritthigul et al., 2010). Chitinolytic enzymes are mentioned as potential bio-pesticides against agricultural pathogenic fungi and insects (Singh et al., 2014). In our study, intense chitinase

activity was expressed by *B. amyloliquefaciens* B3 and OS17 (figure 4 b and d), *B. subtilis* 10, and *Bacillus* spp. 83.2s strains.

Gelatin is a mixture of peptides and proteins derived from collagen, an abundant component in cuticular regions and peritrophic membrane of larvae and pupae of several pathogenic insects (Brammacharry and Paily; 2014). Considering these aspects, the gelatinase activity has an important role the pest biological control and plant

Tab. 2. *Bacillus* lytic enzymes involved in biocontrol

Bacterial strain	Cellulase	Chitinase	Protease
Us.a2	10mm	+	24mm
10	4mm	+	19mm
BW	11mm	+	24mm
BN7	2mm	-	13mm
B7.2	8mm	+	20mm
B3	7mm	+	27mm
OS17	8mm	+	25mm
BIR	6mm	±	27mm
BPA	8mm	+	25mm
lcpc	5.5mm	+	23mm
OS15	2mm	+	21mm
83.2s	6mm	+	10mm
ATCC 6633	6mm	+	16mm
ATCC 11774	-	+	16mm
ATCC 14580	3.5mm	+	7mm

Where: mm =clear zone of substrate hydrolysis, + =enzymatic activity, ± =poor enzymatic activity, - =negative reaction.

Fig. 3. Cellulase activity after 4 days of incubation on CMC-medium

protection. In our study, all tested *Bacillus* strains, including the references, expressed gelatinase activity (table 2). After three days of growth on gelatin containing medium, clear zones of protein hydrolysis, larger than 2 cm long were revealed

by nine of the strains analyzed: B7.2, OS15, Icpc, Us.a2, BW, OS17, BPA, B3 and BIR (figure 5).

Several others *Bacillus* spp. strains, belonging to *B.amyloliquefaciens*, *B. subtilis*, *B.licheniformis*, *B.cereus*, or *B.megaterium*, are described for their gelatin degrading activity. However, the maximal

Fig. 4. Chitinase activity on Agrawal and Kotasthane (2009) medium with bromocresol purple (a,b) and phenol red (c,d) indicator dye

Fig. 5. *Bacillus* spp. strains showing gelatinase activity on gelatin agar plates treated with mercuric chloride

gelatinolytic enzyme production in *Bacillus* spp. was found to be 3,5U/ml (Sai-Ut et al., 2014).

Lipase activity was analyzed after 7 days of incubation on Tween 80 containing medium, were two of the analyzed strains (OS15 and Icpc) and all three references were able to convert the substrate into saponins (table 3).

Due o the lipolytic enzymes, the triglycerides from the Tween 80 were hydrolyzed in glycerol and fatty acids that formed an white precipitate in the presence of calcium salts (figure 6).

Tab. 3. Lipase production revealed in tested *Bacillus* strains

Bacterial strain	Lipase activity	
	Tween 80	Olive oil
Us.a2	–	+
10	N.A.	+
BW	–	+
BN7	N.A.	+
B7.2	N.A.	+
B3	N.A.	+
OS17	–	+
BIR	–	+
BPA	–	+
Icpc	+	+
OS15	+	+
83.2s	–	+
ATCC 6633	+	+
ATCC 11774	+	+
ATCC 14580	+	+

Where: N.A. - not available, + positive reaction, – negative reaction

On YOC medium with rhodamine B, lipase production was revealed at all tested strains after 5 day of incubation. Bacterial colonies depicted a fluorescent orange-pink growth in 350nm UV light (figure 6), due to lipase activity.

The significance of microbial lipase activity is due to their implication in pest biological control, were lipolytic enzymes are contributing to the degradation of lipids contained by the insect epicuticle (Sandhu et al., 2012).

For plant growth promotion, some other enzymes are involved, increasing the opportunity of nutrients uptake. Phosphorus is one of the essential nutrients needed by the plants; however, it has very low solubility.

Regarding the analyzed *Bacillus* spp., B3, BIR, 10, Icpc, BN7, OS17, 83.2s and BPA strains have shown phosphatase activity on modified Pikovskaya agar medium (figure 7). Microbial phosphate solubilization ability being considered as the most important traits associated with plant P nutrition (Hemalatha et al., 2013).

Grown on minimal medium with ACC as sole nitrogen source, all the analyzed bacterial strains shown reduced bacterial growth compared with the same medium supplemented with ammonium sulphate; these being correlated with a low ACC-deaminase activity. The ACC deaminase is interfering in ethylene biosynthesis in plants, by converting its precursor, the ACC, into ammonia and α -ketobutyrate, and thereby lowering the levels of ethylene in plantlets or stressed plants (Glick, 2005).

It is also suggested that, in plants, ethylene biosynthesis is competed by polyamines (PAs) synthesis and application (de Dios et al., 2006).

Fig. 6. Lipolytic activity of *Bacillus* spp. OS15 and *B.licheniformis* ATCC 14580 showing Twen 80 hydrolysis

One of the PAs synthesis pathways is derived from arginine, as enzymatic substrate. Analyzing our bacterial strains, we found arginine-decarboxylase activity in all tested strains, except for the ATCC 14580 reference (figure 8), and a reduced enzymatic activity in OS17 and ATCC 6633 strains.

It is considered that inoculation with PAs producing bacteria, not only that increases plant growth and development, but it can increase plants tolerance to abiotic stresses.

Regarding starch hydrolysis, all tested bacterial strains, including the three references, expressed amylase activity. Clear hydrolysis zones, of up to 4mm long, being revealed at *B. amyloliquefaciens* BW, B3 and OS17, *B. subtilis* ATCC11774, 10 and

Icpc, and *Bacillus* spp. BIR and BPA strains, after over night incubation at 28°C.

CONCLUSIONS

The present study revealed the enzymatic and genetic variability within a Romanian native group of thirteen *Bacillus* biocontrol strains, compared with three reference strains of *B. subtilis* (ATCC6633 and ATCC11774) and *B. licheniformis* (ATCC14580). Eight types of lytic enzymes known to be involved in nutrients bioavailability and plant biological control mechanisms against pest and pathogens were analyzed. Qualitative and semi-quantitative analysis of these enzymes production revealed the phenotypic variability among these

Fig. 7. Lipase activity of bacterial colonies having fluorescent orange-pink color on YOC rhodamine B medium

Fig. 8. Phosphate solubilizing bacteria on Pikovskaya agar medium after 4 days of growth at 28°C

Fig. 9. Arginine-decarboxylase activity revealed by the change of color in the media, from yellow to pink and purple.

strains. Molecular diversity was studied by BOX-PCR analysis, and beside the genetic variability found inside the studied group; two clusters were assembled.

Acknowledgements. This work was published under the frame of European Social Found, Operational Program Human Resources Development 2007-2013, project no. POSDRU/159/1.5/S/132765.

REFERENCES

- Boonmahame P, Mongkolthanaruk W (2013). Lipase-producing bacterium and its enzyme characterization. JOLST, 1(4): 196-200.
- Borriess R (2011). Use of Plant-Associated *Bacillus* Strains as Biofertilizers and Biocontrol Agents in Agriculture. Chapter 3 in *Bacteria in Agrobiolgy: Plant Growth Responses*. DK Maheshwari (ed.), Springer-Verlag Berlin Heidelberg 2011.
- Brammacharry U, Paily K, (2014). Gelatinase activity of metabolites of *Pseudomonas fluorescens* Migula on larvae and pupae of culex *Quinquefasciatus* (Diptera: Culicidae). IJPBS, 5(3): 234-245.
- Ceccon E, Almazo-Rogel A, Martinez-Romero E, Toledo I (2012). The effect of inoculation of an indigenous bacteria on the early growth of *Acacia farnesiana* in a degraded area. CERNE, 18(1): 49-57.
- Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005). Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. Appl Environ Microbiol. 2005 Sep; 71(9): 4951-4959.
- Constantinescu F, Siciua OA (2013). Combaterrea biologica a bolilor plantelor, pp99.
- de Dios P, Matillab AJ, Gallardo M, (2006). Flower fertilization and fruit development prompt changes in free polyamines and ethylene in damson plum (*Prunus insititia* L.). J Plant Physiol, 163: 86-97.
- Dworkin M, Foster J (1958). Experiments with some microorganisms which utilize ethane and hydrogen. J. Bacteriol., 75: 592-601.
- Glick BR (2005). Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase – MiniReview. FEMS Microbiology Letters, 251: 1–7
- Hasan S, Ahmad A, Purwar A, Khan N, Kundan R, Gupta G (2013). Production of extracellular enzymes in the entomopathogenic fungus *Verticillium lecanii*. Bioinformation, 9(5): 238-242.
- Hemalatha N, Raja N, Jayachitra A, Rajalakshmi A, Valarmathi N, (2013). Isolation and characterization of phosphate solubilizing bacteria and analyzing their effect in *Capsicum annum* L. IJBPR, 4(3):159-167
- Illakkiam D, Ponraj P, Shankar M, Muthusubramanian S, Rajendhran J, Gunasekaran P (2013). Identification and structure elucidation of a novel antifungal compound produced by *Pseudomonas aeruginosa* PGPR2 against *Macrophomina phaseolina*. Applied Biochemistry and Biotechnology, 171: 2176-2185.
- Sai-Ut S, Benjakul S, Sumpavapol P, (2014). Optimization of gelatinolytic enzyme production by *B. amyloliquefaciens* sp. H11 through Plackett–Burman design and response surface methodology. Int Aquat Res, 6:59.
- Sandhu SS, Sharma AK, Beniwal V, Goel G, Batra P, Kumar A, Jaglan S, Sharma AK, Malhotra S (2012). Myco-Biocontrol of Insect Pests: Factors Involved, Mechanism, and Regulation. Journal of Pathogens, vol. 2012, Article ID 126819, 10 pages.
- Siciua OA, Voaides C, Grosu I, Constantinescu F, Cornea CP (2015). Genetic diversity of *Bacillus* genus strains antagonistic to various plant pathogenic fungi. Poster presentation in the International Conference “Agriculture for life, life for agriculture”, Section 6, Biotechnology.
- Singh G, Bhalla A, Bhatti JS, Chandel S, Rajput A, Abdullah A, Andrabi W, Kaur P (2014). Potential of chitinases as a biopesticide against agriculturally harmful fungi and insects. Research and Reviews: JMB, 3(1): 27-32.
- Singh S, Moholkar WS, Goyal A (2013). Isolation, Identification, and Characterization of a Cellulolytic *Bacillus amyloliquefaciens* Strain SS35 from Rhinoceros Dung. ISRN Microbiology, Article ID 728134, 7 pages.
- Songsiriritthigul C, Lapboonrueng S, Pechsrichuang P, Pesatcha P, Yamabhai M (2010). Expression and characterization of *Bacillus licheniformis* chitinase (ChiA), suitable for bioconversion of chitin waste. Bioresource Technol, 101: 4096–4103.
- Sundara Rao WVB, Sinha MK (1963). Phosphate dissolving microorganisms in the soil and rhizosphere Indian Journal of Agricultural Science 33: 272-278
- Versalonic J, Schneider M, de Bruijin FJ, Lupski JR (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth Mol Cel Biol, 5: 25–40.
- Wang SL, Lin TY, Yen YH, Liao HF, Chen YJ (2006). Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase. Carbohydr. Res., 341: 2507-2515.
- Xie M, Yin H-q, Liu Y, Liu J, Liu X-d (2008). Repetitive sequence based polymerase chain reaction to differentiate close bacteria strains in acidic sites. Trans Nonferrous Met Soc China, 18: 1392-1397.