Biological Forum – An International Journal **14(4a): 29-32(2022)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Characterization of *Bacillus* spp. Isolated from different Rhizosphere Soils

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(Received 25 September 2022, Accepted 02 November, 2022)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: In agriculture, *Bacillus* species are widely used to stimulate plant growth and to protect the plants from pathogens and other environmental stresses. In this present study, six *Bacillus* spp. were isolated from the rhizosphere soil of grapes and tomato and identified based on the morphological and biochemical features such as, Starch hydrolysis, Catalase test, Voges – Prokaeur test, Methyl red test, Gelatin hydrolysis test and KOH test and confirmed through Molecular characterization by using universal primers such as 27F and 1492R and got the amplicon size of 1500bp. Moreover, the confirmation of *Bacillus* spp. is done byusing 16S rRNA sequencing. This made to understand the nature of the bacteria present in the soil microbial community.

Keywords: Bacillus species, Biochemical tests, Characterization, Universal 27F and 1492 R.

INTRODUCTION

Plant diseases caused by various microorganisms such as fungi, bacteria, viruses, nematodes and protozoa affect the agricultural production and results in major yield losses (Chakraborty & Newton 2011). Approximately 20–40 % of crop loss is due to the fungal pathogenic infections (Savary *et al*., 2012). Different strategies have been employed to reduce these problems, such as insecticides, cultivars that are less vulnerable, crop rotation, and other control measures, but their efficacy is usually insufficient due to the endurance and resistance of soil-borne pathogens (Syed Ab Rahman *et al*., 2018). Moreover, the over use of synthetic pesticides harms both the environment and living things, disrupts the functioning of ecosystems, and reduces the sustainability of agriculture. Now-a-days, research is directed towards environment friendly alternatives as an integral part of integrated crop management system for controlling plant pathogens and improving crop production.

Bacillus species are the widely investigated biocontrol agents or biopesticides, which help to suppress plant pathogens by competition and/or antagonism. *Bacillus* spp. have different ways to prevent the growth of pathogens *viz*., competition for nutrients and space, production of antibiotic substances and hydrolytic enzymes, production of side rophores and induction of systemic resistance to the plants.

MATERIALS AND METHODS

Isolation of native *Bacillus* spp.

Bacterial antagonists were isolated from the rhizosphere soil samples. The *Bacillus* isolates were isolated using Luria Bertani (LB) medium. One gram of rhizosphere soil samples was transferred to sterile test tubes containing 9 ml of sterile distilled water. After shaking the test tubes for 15 min in a shaker, the suspension was serially diluted in sterile distilled water. One ml each from 10^{-5} and 10^{-6} dilutions was pipetted out and poured into sterile petri plates. Later 15 ml of LB medium was poured , and gently rotated in clock and anti-clock wise direction and incubated at room temperature (28 ± 2°C) for 24 hour. After incubation, the selected pure *Bacillus* colonies were streaked on selective medium and transferred to LB slants.

Biochemical tests for *Bacillus* spp.

KOH test. A loopful of bacterial culture was put on a clean glass slide. One drop of 3% KOH solution was placed over it and thoroughly mixed with the help of a

needle. Bacterial chromosomes separated out as thin threads, indicated gram negative bacteria.

Starch hydrolysis. Nutrient agar containing 0.2 per cent soluble starch was used. The test cultures were spotted on the petri plates. Starch hydrolysis was tested after 48 h of incubation by flooding the agar surface with Lugol's iodine solution. A colorless zone around the bacterial growth in contrast to the blue background of the medium indicated positive reaction.

Catalase test. Smears of 24 h old bacterial cultures were prepared on clean slide and added with a few drops of three percent hydrogen peroxide. Effervescence indicated the presence of catalase in the culture.

Voges – Prokaeur test. Forty eight hour old cultures were inoculated into five ml of the nutrient agar (NA) broth dispersed in test tubes. After an incubation period of seven days, 0.6 ml alpha napththnol solution (5% in 95% alcohol) and 0.2 ml of 40 % aqueous solution of KOH were added into one ml of the culture. The mixture was shaken for few minutes and allowed to stand for two hours. A crimson colour indicated positive test.

Utilization of citrate. The bacterial culture was streaked on the surface of Simmon's citrate agar slant. Blue color indicated that utilization of citrate. Original green colour indicated non – utilization of citrate.

Methyl red test. Methyl red test (MR test), the test bacteria is grown in a broth medium containing glucose. If the bacteria has the ability to utilise glucos**e** with production of a stable acid, the color of the methyl red changes from yellow to red**,** when added into the broth culture.

Gelatin hydrolysis test

The bacterial culture was inoculated into nutrient gelatin medium (peptone (5.0g/liter), beef extract (3.0 g/liter) and gelatin 120.0 g/liter) in test tube. After the tubes were kept in refrigerator (4^{o} C) and incubate overnight. the gram positive bacteria produce gelatin liquefying layer on nutrient gelatin slant.

Molecular characterization of *Bacillus* spp. The genomic DNA from the isolates of *Bacillus* spp. were isolated using the procedure given by William *et al.* (2012). Twenty five ml of actively grown broth culture was taken in a centrifuge tube and centrifuged at 6,000 rpm for 5 min at 4˚C. The supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged at 6,000 rpm at 4˚C for 5 min to remove all traces of butanol. Again the pellet was re-suspended in 1 ml TE buffer added with 100 µl of lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 µl of 10 per cent SDS and 25 µl of 100 µg ml^{1} proteinase K were added, mixed well and incubated at 37˚C for one hour. To the above mixture 200 µl of 5M NaCl was added and mixed well. To it 150 µl of CTAB solution was added, mixed well and incubated at 65˚C for 10 min. the mixture was extracted with 1 ml of phenol: chloroform mixture in the ratio of 25: 24, mixed well and centrifuged at 6,000 rpm for 15 min at 4˚C. the aqueous layer was transferred carefully to a 2 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold- propanol, incubated overnight at -20˚ C. the DNA was pelleted by centrifugation at 12,000 rpm for 15 min at 4˚C. The pellet was washed with 70 per cent ethanol and dried under vacuum for 10 min and re-suspended in 50 µl of TE buffer. One µl DNAse and free RNAse (10 mg ml^{-1}) was also added by swirling and incubated at 37˚C for 30 min. the DNA was stored at -20˚C for further use.

PCR amplification of *Bacillus* spp. The amplification of 16S rRNA region was done using the universal primers 27 F (5'-AGAGTTTGATCCTGGCTCAAG-3') and 1492 R (5'- GGTTACCTTGTTACGACTT-3'). The reaction was carried out in thermocycler with PCR conditions as 96˚C of initial denaturation for 4 minutes, 40 cycles of denaturation of 94˚C for 30 sec. annealing of 57˚C for 30 sec. and extension at 72˚C for 10 minutes. For this reaction, 10 µl of PCR mixtures was prepared which contains 5 µl of master mix, 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA and 2 µl of sterile water.

RESULT AND DISCUSSION

Cultural and morphological character of *Bacillus* spp. *Bacillus* spp. is a bacterial biocontrol agent, which is rod-shaped and Gram-positive. When cultured on ordinary nutrient agar, the morphology of bacteria is observed as rough, opaque, fuzzy white or slightly yellow coloured circular colonies (Plate 1).

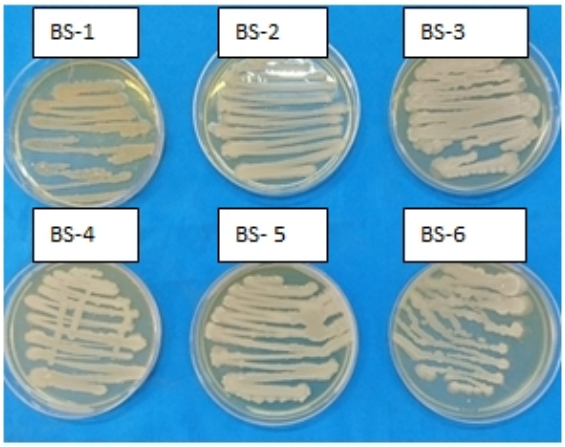

Plate 1. Cultural characterization of *Bacillus* spp. Isolates.

Biochemical characterization of *Bacillus* spp. The results of various biochemical tests revealed that, six bacterial isolates have shown positive reaction with starch hydrolysis, gelatin hydrolysis, catalase, vogesproskaeur test methyl red and utilization of citrate and negative reaction with KOH test. From these observations, it is evident that the bacterial isolates are gram positive and belonging to *Bacillus* spp. Various

biochemical methods have been developed and used for characterizing the bacterial isolates. In the present study, biochemical characterization of bacterial isolates was carried out to study the basic characters *viz.*, gram reaction, side rophore production, metabolites utilization of different nutrient sources. Based on the biochemical test, a total of five isolates (MzPP1, MzPP2, MzPP7, MzPP9 and MzPP10) were identified as *B. subtilis* and one as *B. licheniformis*. Similarly, Narasimhan *et al.* (2021) isolated bacteria from papaya phylloplane and identified the effective one as *Bacillus* sp. and *Pseudomonas* sp. based on the results of several biochemical tests *viz.*, gram character, ability to ferment sugars, Methyl red, catalase, citrate and gelatin liquefaction tests. The bacteria isolated from rice phylloplane were identified using the biochemical tests *viz.*, catalase, methyl red, vogesproskauer, nitrate reduction etc (Akter *et al.*, 2014).

Similarly, Singh and Singh (2020) isolated eight bacterial antagonists from rhizosphere region and confirmed the identification of bacterial isolates as *Bacillus* spp. using biochemical characterization *viz.*, Citrate utilization, Vogues Proskauer, Motility, Catalase, Nitrate reduction, TSI, Urease and Oxidase tests. Similar results were reported by Medina salazer *et al.* (2020); Gupta *et al.* (2015) who had isolated bacteria from lettuce and *Prosopis cinerea* and identified them as *Pseudomonas* sp. and *Bacillus* sp.

Table 1: Biochemical characterization of *Bacillus* spp.

Bacterial isolates	Starch hydrolysis	Gelatin hydrolysis	catalase	Vogesproskaeur test	Utilization of citrate	Methyl red	KOH test
1	+	+	+	+	+	+	-
2	+	+	+	+	+	+	-
3	+	+	+	+	+	+	-
4	+	+	+	+	+	+	-
5	+	+	+	+	+	+	-
6	+	+	+	+	+	+	-

Molecular characterization of *Bacillus* spp. The genomic DNA was isolated from the bacterial isolates which were found effective during *in vitro* studies. The molecular identification of these isolates was done by PCR amplification of 16S rRNA using the universal primers pair 27F and 1429R. The products were amplified at around 1500 bp and they were identified as *Bacillus subtilis*. The partially sequenced nucleotides of the isolate, BST 8 was deposited in NCBI Gen bank data base and given the accession number Op271691 (Fig. 1). Parvathi *et al*. (2009) used the fatty acid methyl ester (FAME) analysis and partial 16S rDNA sequencing to identify the *Bacillus pumilus.*

Phylogenetic analysis. The phylogenetic tree was constructed for the effective isolate *B. subtilis* BST 8 using MEGA 7.0 software. From the tree, it is evident that the isolate had 56 per cent similarity with the other *B. subtilis* retained from NCBI data base and compared with out group of *Ochrobactrum intermedium* (kp259604) (Fig. 2). Jongsik Chun and Bae (2000) reported the partial *gyrA* sequences were determined for the twelve strains of Bacillus amyloliquefaciens, *B. atrophaeus, B. licheniformis, B. mojavensis, B. subtilis* subsp. *subtilis, B. subtilis subsp. spizizenii,* and *B. vallismortis* have partial gyrA sequences identified the seven type strains had an average nucleotide similarity of 83.7 and a translated amino acid similarity of 95.1%, compared to a 16S rRNA sequence similarity of 99.1%. The closest link was discovered between *B. atrophaeus* and *B. mojavensis*, which had a nucleotide similarity of 95.8%. All of the type strains were severely separated from one another.

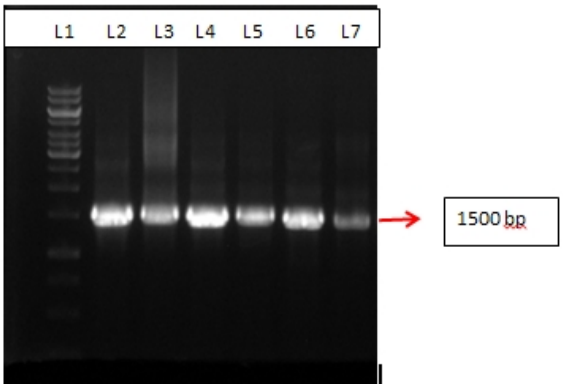

Fig. 1. Molecular characterization of the bacteria isolated from the soil by using 27 F and 1492 R primers. Lane 1:1kb ladder, Lane 2- *Bacillus* sp1, lane3- *Bacillus* sp-2, lane3- *Bacillus* s sp-3, lane3- *Bacillus* sp-2, lane 4- *Bacillus* sp-4, lane5- *Bacillus* sp-5, lane6- *Bacillus* sp-6, lane7- *Bacillus* sp-7.

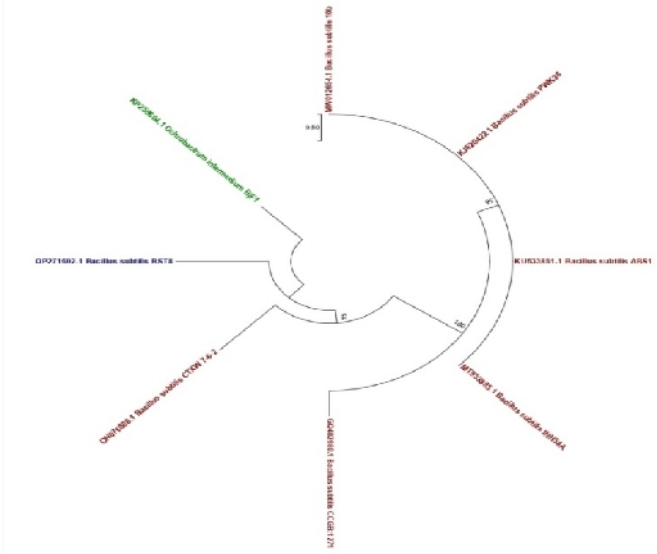

Fig. 2. Phylogenetic tree exhibits the grouping of partial sequences of BST 8 (1500 bp) with other *Bacillus subtilis* on NCBI database using Mega 7.0 software.

CONCLUSION

Bacillus spp. perform well in the rhizosphere and phylloplane through different mechanisms viz., competition for nutrient uptake from the soil and induction of plant defense response against the plant diseases. However, the applicability of *Bacillus* spp. was limited due to their inconsistent efficiency in disease reduction under different field conditions. Thus, better formulation strategies have to be developed for the bacterial biocontrol agent, *Bacillus* spp. to make them more durable with improved biocontrol efficacy.

Acknowledgment. The authors acknowledge the Department of Plant Pathology, Centre for Plant Protection studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.
Conflict of Interest. None.

REFERENCES

Ab Rahman, S. F. S., Singh, E., Pieterse, C. M. and Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Science*, *267*, 102-111.

Akter, S., Kadir, J., Juraimi, A. S., Saud, H. M. and Elmahdi, S. (2014). Isolation and identification of antagonistic bacteria from phylloplane of rice as biocontrol agents for sheath blight. *Journal of environmental biology*, *35*(6), 1095.

Chakraborty, S. & Newton, A. C. (2011). Climate change, plant diseases and food security: an overview. *Plant pathology*, *60*(1), 2-14.

Chun, J. and Bae, K. S. (2000). Phylogenetic analysis of Bacillus subtilis and related taxa based on partial gyrA gene sequences. *Antonie Van Leeuwenhoek*, *78*(2), 123-127.

Gupta, R. M., Kale, P. S. and Rathi, M. L. (2015). Isolation, characterization and identification of endophytic bacteria by 16S rRNA partial sequencing technique from roots and leaves of Prosopis cineraria plant. *Asian Journal of Plant Science and Research*, *5*(6): 36-43.

Medina-Salazar, S.A., M. Rodríguez-Aguilar, M.R. Vallejo-Pérez, R. Flores-Ramírez, J. Marín-Sánchez, G. Aguilar-Benítez, R. Jarquin-Gálvez, and J.P. Lara-Ávila. (2020). "Biodiversity of epiphytic *Pseudomonas* strains isolated from leaves of pepper and lettuce." *Biologia, 75*(5), 773-784.

Narasimhan, A. and Banerjee, K. (2021). Isolation and Characterization of Phylloplane Bacteria from Papaya Plant for the Biocontrol of post-harvest Diseases in Papaya. *International Journal of Environment, Agriculture and Biotechnology*, *6*(1).

Parvathi, A., Krishna, K., Jose, J., Joseph, N. and Nair, S. (2009). Biochemical and molecular characterization of Bacillus pumilus isolated from coastal environment in Cochin, India. *Brazilian Journal of Microbiology*, *40*, pp, 269-275.

Savary, S., Ficke, A., Aubertot, J. N. and Hollier, C. (2012). Crop losses due to diseases and their implications for global food production losses and food security. *Food security*, *4*(4), 519-537.

Singh, J. & Singh, S. P. (2020). Isolation and Identification of Bacillus Species from Soil for Phosphate, Potassium Solubilisation and Amylase Production. *Int. J. Curr. Microbiol. App. Sci*, *9*(5), 415-426.

Syed Ab Rahman, S. F., Singh, E., Pieterse, C. M. J. and Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Sci., 267*, 102–111.

William, S., Feil, H. and Copeland, A. (2012). Bacterial genomic DNA isolation using CTAB. *Sigma*, *50*(6876).

How to cite this article: Veerathilagam D., Kannan R., Parthiban V.K., Vincent S. and Bhuvaneswari K. (2022). Characterization of *Bacillus* spp. Isolated from different Rhizosphere Soils. *Biological Forum – An International Journal, 14*(4a): 29-32.