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RESEARCH ARTICLE

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF DIFFERENT BACILLUS SP. FROM THE RHIZOSPHERE SOIL OF OCIMUM BASILICUM

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ABSTRACT

Ocimum basilicum commonly known as Basil is one of the promising herb with important multiple medicinal properties. In the present study, *Bacillus sp.* possessing multiple plant growth promoting activities was isolated from the rhizospheric soil of *Ocimum basilicum* collected from Gandhi Krishi Vigyan Kendra, University of Agricultural Sciences, Bangalore, on Luria Bertini media. In Biochemical characterization, Plant Growth Promoting Rhizobacteria (PGPR) – *Bacillus sp.* was screened for their plant growth promoting activities like phosphate solubilisation, production of indole acetic acid (IAA), ammonia, hydrogen cyanide (HCN), catalase, cellulase, biofilm, siderophore and organic acid. Further, species level identification was done by performing specific tests like Tyrosine agar test and growth on 3%, 5% and 10% Nacl was checked. These isolates also showed heavy metal tolerance. Different *Bacillus* species like *Bacillus amyloliquefaciens, Bacillus cereus, Bacillus megaterium,* and *Bacillus sonorensis* were identified by doing comparative study using biochemical characterization. From their PGPR activity, it was concluded that *Bacillus cereus* showed maximum amount of PGPR traits, *Bacillus sonorensis* showed optimum and *Bacillus amyloliquefaciens* showed minimum amount of PGPR traits, therefore, *Bacillus cereus* is the best plant growth promoting rhizobacteria (PGPR) with maximum PGPR traits. In Molecular characterization, 8 *Bacillus sp.* isolates having multiple plants growth promoting activities was selected and genomic DNA was isolated from bacterial cultures. DNA samples from this different *Bacillus* species were subjected to RAPD analysis to differentiate them at the species level and the RAPD primer OPG-8 (5/-TCACGTCCAC-3/) was used for amplifications. The expected ladder was not observed in the result, instead single amplicon of each isolate were observed therefore, this primer cannot be used for molecular characterization of different *Bacillus* species. Further analysis using different RAPD primers should be tested for ladder formation or molecular characterization.

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INTRODUCTION

Over the last few decades, the high increase in crop yield was achieved through high input of inorganic fertilizers and pesticides. However, currently emphasis is on sustainable agriculture, which uses less of chemical inputs like fertilizers/pesticides having adverse effects on soil and environment. Use of organic inputs including microbial inoculants has been emphasized in order to reduce the dependency on chemical fertilizers. Recently there has been resurgence of interest in the use of sustainable agriculture, as there has been overuse of chemical fertilizers and pesticides which has led to environmental problems. The multiplying population is exerting immense pressure on agricultural lands for higher crop yields, which results in ever increasing use of chemical fertilizers.

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These agents are costly and create environmental problems. Consequently, there has recently arisen a renewed interest in environmental friendly agricultural practices (Karlidag, 2007). Long-term studies show that intensive application of chemical fertilizers lead to reduction in crop production. This reduction is mainly due to increasing soil acidity, decreasing biological activities and changing the soil physical characteristics and diminished microelements (Adediran, 2004). Microbial inoculants can fulfill diverse beneficial interactions in plants leading to promising solutions for sustainable and environment-friendly agriculture. Microorganisms have a vital role in agriculture as they promote the exchange of plant nutrients and reduce application of chemical fertilizers as much as possible (Dastager, 2011).

Rhizosphere of *Ocimum basilicum*

There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root

activity and metabolism which is known as rhizosphere. The rhizosphere concept was first introduced by Hiltner to describe the narrow zone of soil surrounding the roots where microbe populations are stimulated by root activities. A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology to a greater extent (Ganzera, 2009).

Plant growth promoting rhizobacteria (PGPR)

Kloepper and Schroth introduced the term rhizobacteria to the soil bacterial community that competitively colonize plant roots and stimulate growth and thereby reducing the incidence of plant diseases. Kloepper and Schroth (1981) termed these beneficial rhizobacteria as plant growth promoting rhizobacteria (PGPR). It is well known that PGPR play an important role in maintaining crop and soil health through nutrient cycling and uptake, suppression of plant pathogens, induction of resistance in plant host and direct stimulation of plant growth (Nehra, 2015). A large number of bacteria like *Azospirillum, Azotobacter, Bacillus, Enterobacter, Pseudomonas, Klebsiella and Paenibacillus* have been isolated from rhizosphere of various crops and used as PGPR. PGPR can be a best alternative to chemical fertilizer for sustainable and eco-friendly agriculture.

***Bacillus* species in rhizospheric soil**

Bacillus is a genus of Gram-positive, rod-shaped (bacilli), bacteria and a member of the phylum Firmicutes. *Bacillus* species can be obligate aerobes or facultative anaerobes. Ubiquitous in nature, *Bacillus* includes both free-living and parasitic pathogenic species. Multiple species of *Bacillus* are known to promote plant growth. The principle mechanisms of growth promotion include production of growth stimulating phytohormones, solubilisation and mobilisation of phosphate, siderophore production, antibiosis and induction of systemic resistance to pathogens (Ambrosini, 2012).

Bacterial genomic DNA

Genomic deoxyribonucleic acid is chromosomal DNA, in contrast to extrachromosomal DNAs like plasmids. It is often abbreviated as gDNA. Most bacteria have a genome that consists of a single DNA molecule (i.e., one chromosome) that is several million base pairs in size and is "circular" (doesn't have ends like chromosomes of eukaryotic organisms). In addition, bacteria may have one or more smaller circular DNA molecules, called plasmids that contain (usually) non-essential genes. Thus, bacteria are able to grow and divide much faster than eukaryotic cells.

RAPD

The random amplified polymorphic DNA (RAPD) technique is a PCR-based method that uses a short primer (usually 10 bases) to amplify anonymous stretches of DNA. With this technique, there is no specific target DNA, so each particular primer will adhere to the template DNA randomly. As a result, the nature of the obtained products will be unknown. The DNA fragments generated are then separated and detected by gel electrophoresis (Fani, 1993).

MATERIALS AND METHODS

Sample Collection

Rhizospheric soil of *Ocimum basilicum* was collected from Gandhi Krishi Vigyan Kendra (GKVK), University of Agricultural Sciences, Bellary Road, Bangalore, Karnataka, South Western India. Isolation was done from the rhizospheric region soil of *Ocimum basilicum.*

Isolation of bacteria from soil sample

The principle of quantitative estimation is the concentration of microorganisms in the given soil sample must be reduced and for this, serial dilution technique was used. For isolation of organisms, pour plate technique was used (Subba Rao, 1977). Identification of organisms was done as per the standard methods such as Gram staining and Biochemical tests.

Biochemical tests for identification of bacteria in the species level

The tests performed are (Margaret M Palmisano, 2003):

Tyrosine Agar Test: It is used for the differentiation of *Bacillus* species based on L-tyrosine utilization. Zone of clearance and colour appearance around the colony indicates tyrosine hydrolysis. The medium was inoculated by streaking the isolate to be tested onto the agar surface with a sterile inoculating loop. The medium was incubated for up to 3 weeks to allow positive hydrolytic reactions to develop. Plates were examined at regular intervals for growth and hydrolysis.

Growth on 3%, 5% and 10% Nacl was checked.

PGPR TESTS

Indole Acetic acid production (IAA) (Brick, 1991): Bacterial cultures were grown in NB amended with tryptophan (100µg/ml) at 30°C for 48 h on shaker (120 rpm). The cultures were centrifuged at 3000 rpm for 30 minutes. The supernatant (2ml) was mixed with two drops of *o*-phosphoric acid and 4ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1ml 0.5 M $FeCl_3$ solution). Absorbance at 530nm was recorded.

Production of HCN (Lorck, 1948): NA was amended with glycine (4.4 g/l) and bacteria were streaked on modified agar plates. Whattman's filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid was placed at the inner surface of the lid of the petriplates. Plates were sealed with parafilm and incubated at 30°C for 4 days.

Production of Ammonia (Bhattacharyya, 2012): Bacterial isolates were grown in peptone water. 1% inoculum was added to 5 ml of peptone water in each tube and incubated for 72 h at 30°C. Nessler's reagent (0.5 ml) was added in each tube.

Qualitative Phosphate Solubilisation (Nautiyal, 1999): Phosphate solubilizing activity of the isolates was evaluated on Pikovskaya's agar for 72 hours at 30°C. Bacterial isolates changed colour of methyl red pH indicator (added at a concentration of 0.03%), from yellow (pH 8.0) to red (pH 5.0 or below) on Pikovskaya's agar plates.

Table 1. Analysis of Biochemical PGPR characterization of different samples

Sample No.	Tyrosine Agar Test	3% Nacl	5% Nacl	10% Nacl	Indole Acetic Acid	HCN Production
1	Reddish brown	+	-	-	+	+ (Dark Red)
2	White	+	-	-	+	+ (Light Red)
3	Pale Yellow	-	+	+	+	+ (Dark Yellow)
4	Cream	-	+	+	+	+ (Dark Red)
5	Pale Yellow	+	+	+	+	+ (Dark Orange)
6	Yellow	+	-	-	+	+ (Light Red)
7	Yellow	+	-	-	+	+ (Dark Red)
8	Cream	-	+	+	+	+ (Dark Red)

Table 2. Analysis of Biochemical PGPR characterization of different samples

Sample No.	Ammonia Production	Zone of Phosphate Solubilisation	Cellulase Production	Biofilm Production	Siderophore Production
1	-	-	+	+	+
2	-	-	+	+	+
3	-	-	+	+	+
4	+	+	+	+	+
5	-	-	+	+	+
6	-	-	+	+	+
7	-	-	+	+	+
8	+	+	+	+	+

(+) Positive reaction; (-) Negative reaction

Table 3. Analysis of Organic Acid

Sample No.	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)	Volume of burette reading (ml)
1	0	0.7	0.7	1.4	1.4	2.1	0.7
2	2.0	2.8	2.8	3.6	3.6	4.4	0.8
3	4.8	5.6	5.6	6.5	7.0	7.9	0.5
4	8.0	8.9	8.9	9.8	9.8	10.7	0.9
5	10.0	10.6	10.6	11.2	11.2	12.0	0.6
6	12.0	12.5	12.5	12.8	12.8	13.1	0.3
7	13.1	13.6	13.6	14.1	14.1	14.4	0.5
8	14.4	14.9	14.9	15.3	15.3	15.7	0.4

Table 4. Details of Heavy Metals Production

Sample No.	Concentration (µg/ml)	Copper (cm)	Cobalt (cm)	Zinc (cm)
1	100	1.0	1.1	1.9
2	100	1.5	0.9	1.6
8	100	No Zone	No Zone	1.3
1	200	0.9	1.3	2.3
2	200	2.0	1.1	2.0
8	200	No Zone	No Zone	2.0
1	300	0.9	1.6	2.3
2	300	1.3	1.3	2.1
8	300	1.7	No Zone	2.5
1	400	1.0	1.8	2.5
2	400	2.0	1.6	2.2
8	400	1.6	0.5	2.3

Table 5. Optical Density measurement at 540 nm

Sample No.	Indole Acetic Acid	Biofilm Production	Siderophore Production
1	0.14	1.26	2.40
2	0.47	0.81	1.40
3	1.87	0.92	0.80
4	1.96	1.93	2.60
5	0.85	1.05	1.80
6	1.89	1.76	2.00
7	2.01	1.85	2.80
8	1.00	0.71	2.50

Cellulase Production (Capuccino, 2001): PGPR strains were grown on CMC (Carboxyl methyl cellulose) agar and were incubated at 30°C for 5 days. After incubation, agar medium was flooded with an aqueous solution of Congo red (1% w/v).

Organic acid analysis (Moumita, 2011): 50 ml NB in 250 ml Erlenmeyer flask was inoculated (1%) and incubated at 30°C for 72 hrs on shaker (120 rpm) followed by titration.

Biofilm Production (Yousef, 2008): 10 ml culture of *Bacillus* was grown overnight in LB medium. Biofilms were grown for 6 hrs. at 30^0C. Adherent cells were stained with a 0.1% (wt/vol) solution of crystal violet in water and the tubes were rinsed with water. 2 ml of a solution containing 30% methanol and 10% acetic acid was added. 1 ml was transferred into a cuvette and the absorbance was read at 540 nm.

Siderophore Production (Schwyn, 1987): The sample (0.5 ml) was mixed with 0.5 ml 0.5N HCl then, 0.5 ml 10g $NaNO_2$ and 10 g $NaMoO_4.2H_2O$ in a final volume of 100 ml water and finally 0.5 ml 1N NaOH and the absorbance was read at 540 nm.

Heavy Metal Production (Burd, 2000): Agar plates were amended with heavy metal salts like Zn, Co and Cu at various concentrations ranging from 100 to 400 µg/ml and were inoculated with overnight grown cultures. The plates were incubated at room temperature for 24-48hrs.

Bacterial Genomic DNA Isolation

Steps involved in isolation using chromous kit (Thankamani, 2011):

- Suspension of the Lyophilised cells.
- Lysis of cell wall using salts.
- Precipitation of the DNA.
- Running genomic DNA on agarose gel.
- Analyse the results.
- DNA purification was done by TE BUFFER, RNase A, CHLOROFORM, ISOAMYL ALCOHOL.
- DURATION: - 2 to 3 hrs.

PCR-RAPD PRIMER REACTION

- dNTP mix = 40 µl of dNTP +160 µl of MQ H_2O.
- RAPD-PRIMER =690.54 µl of MQ H_2O [diluted the primer in 1:9].
- Take 1 µl of RAPD-PRIMER mix with 9 µl of MQ H_2O in a PCR tube vial.
- The primer should be incubated for 1 hr at room temperature.
- PCR REACTION
- AGAROSE = 2%.
- 40 CYCLES.
- To standardise the reaction, only sample 8 and control was taken.
- NO DILUTION OF DNA (directly 1 of prepared DNA was used).
- Primer (2:8) was made that is 2µl of RAPD -PRIMER and 8 µl of MQ H_2O.

RESULTS

Isolation of Bacteria from soil sample

The *Bacillus* species were isolated from the Rhizosphere soil of *Ocimum basilicum*. By the serial dilution technique, the microbial population of the Rhizosphere soil sample was enumerated and recorded. The separated colonies were enumerated in a LB (Luria-Bertini) medium.

Gram Staining

On performing of staining with culture, using Crystal violet stain, Gram's iodine, 70% alcohol, and Saffranin it showed gram positive rods along with endospores or spores.

Bacterial Genomic DNA Isolation

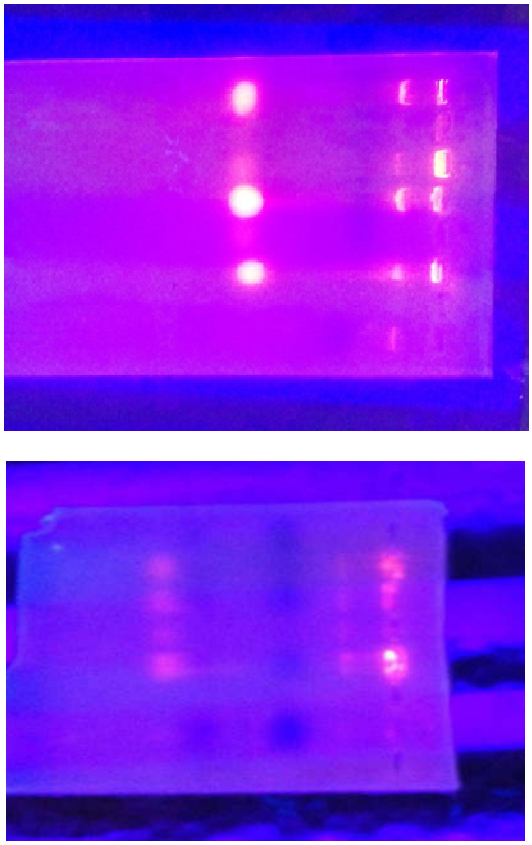

Figure 1. The *Bacillus* species DNA (sample 1 to 8) in agarose gel from the Rhizosphere soil of *Ocimum basilicum*

PCR-RAPD Primer Reaction

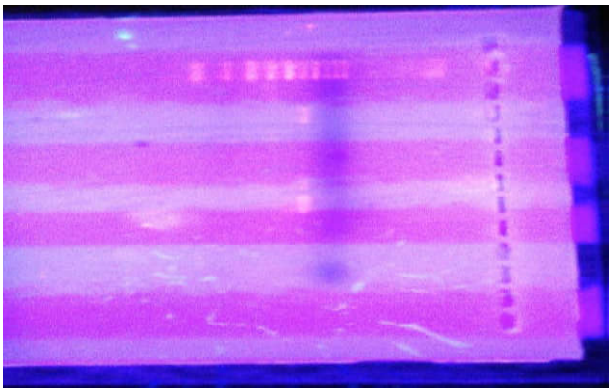

Figure 2. We got only one amplicon in all samples instead of ladder

DISCUSSION

Different *Bacillus* species were isolated from the Rhizosphere region of *Ocimum basilicum* on Luria Bertini media; confirmed by Gram staining and Endospore staining. These *Bacillus* species were subjected to different biochemical tests for confirmation and specific tests for identification of different species. Genus level identification was done by performing 15 biochemical tests which confirmed that the genus isolated were *Bacillus* species. Species level identification was done by performing specific tests like Tyrosine agar test where *Bacillus sonorensis* appeared reddish-brown (Sample 1), *Bacillus cereus* appeared cream (Sample 8), *Bacillus amyloliquefaciens* appeared white (Sample 2). Growth

on 3%, 5%, 10% Nacl was checked; in 3% Nacl *Bacillus sonorensis* showed growth (Sample 1), in 5% and 10% Nacl *Bacillus cereus* showed growth (Sample 8). *Bacillus sonorensis* will not grow in 5% and 10% Nacl and *Bacillus cereus* will not grow on 3% Nacl; similar work was done by (Subba Rao, 1977). They require more amount of salt concentration for their growth in environment and soil. *Bacillus amyloliquefaciens* answered positive for Gelatine liquefaction test, 10% Nacl, oxidase test, catalase test, nitrate reduction test, starch hydrolysis test and negative for citrate utilization test, indole test and urease test. No gas is formed in carbohydrate fermentation test, and acid is produced by glucose, mannitol, and fructose; similar work was done by (Francisco Javier Gutierrez-Manero, 2008). Sample 3 gave same results therefore; it was confirmed to be *Bacillus amyloliquefaciens. Bacillus cereus* answered positive for nitrate reduction test, starch hydrolysis test, methyl red test and citrate utilization test and negative for urease test, indole test, and Voges proskauer test. No gas is formed in carbohydrate fermentation test, and acid is produced by glucose, fructose, mannitol; similar work was done by (Cristiana Felici, 2007). Sample 4 gave same results therefore it was confirmed to be *Bacillus cereus.* Therefore, the eight samples that we isolated were sample 1, 7 – *Bacillus sonorensis*, sample 2, 3 – *Bacillus amyloliquefaciens*, sample 5, 6 – *Bacillus megaterium*, and sample 4, 8 – *Bacillus cereus.*

In Biofilm production test, optical density was measured at an absorbance at 540 nm. *Bacillus cereus* showed maximum absorbance capacity of 1.93 nm, *Bacillus sonorensis* showed optimum absorbance capacity of 1.85 nm, and *Bacillus amyloliquefaciens showed* minimum absorbance capacity of 0.92 nm compared to other test samples. Similar work was done by [16]. In Siderophore production test, the optical density was measured at an absorbance at 540nm. *Bacillus sonorensis* showed maximum absorbance capacity of 2.8nm and *Bacillus cereus* showed optimum absorbance capacity of 2.6 nm compared to other test samples. Similar work was done by (Schwyn, 1987). In Indole acetic acid production test, the optical density was measured at an absorbance at 540nm. *Bacillus sonorensis* showed maximum absorbance capacity of 2.01nm and *Bacillus cereus* showed minimum absorbance capacity of 1.96 nm compared to other test samples. Similar work was done by (Burd, 2004). In Heavy metals production test, we used copper, cobalt and zinc as heavy metals to observe the zone of inhibition at different concentration. In *Bacillus sonorensis*, zinc showed 2.3 cm zone of inhibition at 200 and 300µg/ml. In *Bacillus cereus*, zinc showed 2.5 cm zone of inhibition at 300µg/ml. In *Bacillus amyloliquefaciens*, zinc showed 2.2 cm zone of inhibition at 400µg/ml.; similar work was done by (Thankamani, 2011).

In Organic acid production*, Bacillus cereus* showed maximum production of organic acid which is 0.9/50 ml*. Bacillus amyloliquefaciens* showed minimum production of organic acid which is 0.8/50 ml in comparison to other test samples. Similar work was done by (Moumita, 2011). In hydrogen cyanide production test, *Bacillus cereus* and *B. sonorensis* showed dark red colour that confirms the production of more amount of hydrogen cyanide. Similar work was done by (Lorck, 1948). In phosphate solubilisation test, only *Bacillus cereus* showed zone of phosphate solubilisation. Similar work was done by (Capuccino, 2001). In cellulase and catalase production test, *Bacillus cereus, Bacillus megaterium*, *Bacillus sonorensis* and *Bacillus amyloliquefaciens* showed positive results. Similar work was done by (Capuccino, 2001). In ammonia production test, *Bacillus megaterium, Bacillus sonorensis* and *Bacillus amyloliquefaciens* showed negative results. Only *Bacillus cereus* showed positive result. Similar work was done by (Bhattacharyya, 2012). Genomic DNA was isolated from bacterial cultures by using the genomic DNA isolation kit; similar work was done by (Thankamani, 2011). Genomic DNA of 8 samples was observed under UV Transilluminator. DNA samples from this different *Bacillus* species were subjected to RAPD analysis to differentiate them at the species level and the RAPD primer OPG-8 (5/-TCACGTCCAC-3/) was used for amplifications. In the result, the expected ladder was not observed; therefore it cannot be used for molecular characterization instead single amplicon of each test sample were observed. Therefore, other RAPD primers are to be tested for ladder formation.

Conclusion

Different *Bacillus* species were isolated from *Ocimum basilicum* rhizosphere region and tested for their PGPR traits. Different *Bacillus* species like – *Bacillus sonorensis, Bacillus cereus, Bacillus amyloliquefaciens, Bacillus megaterium* were identified by biochemical characterization. From their PGPR activity, we concluded that *Bacillus cereus* showed maximum amount of PGPR traits, *Bacillus sonorensis* showed optimum and *Bacillus amyloliquefaciens* showed minimum PGPR traits and therefore, *Bacillus cereus* is the best plant growth promoting rhizobacteria with maximum PGPR traits. Application of such microbes as a biofertilizer may contribute to minimize the use of expensive fertilizers which can lead to create change in soil physiology. Beneficial plant-microbe interactions in the rhizosphere can influence soil fertility. Isolates may improve fertility of heavy metals contaminated sites as they showed tolerance against heavy metals.

PGPR have emerged as an important and promising tool for sustainable agriculture. They can function as Biofertilizers, Phytostimulators and Biopesticides which proves their societal values in the field of agriculture. It has been always felt the need and greed for increasing the crop yield due to the uncontrolled expansion in population. PGPR exhibits positive influence on crop productivity. With better research & development, these microbial populations will become a reality and instrumental to fundamental process that drive stability and productivity of agro-ecosystems, thus leading us towards an ideal agricultural system which is sustainable, maintains and improves human health, benefits environment and produces enough food for the increasing world population. We should encourage their successful implementation in the main agriculture system. *Bacillus sp*. accelerates the growth and development of the plant, enhancing root function. The capability to survive at higher temperatures makes our isolate a suitable inoculant for the crop as it can sustain harsh environments. Its ability to exhibit various PGPR traits at higher temperature shows that the isolate may prove effective as an inoculant. The additional feature of motility of the isolated bacterium leads to suppose that they can survive better in the environment.

In molecular characterization by RAPD OPG-8 primer, we observed single amplicons from sample 1 to 8 instead of ladder. Therefore, it cannot be used for molecular characterization of different *Bacillus* species. Further analysis using different RAPD primers should be done for molecular

characterization. Accordingly, further investigation is needed to improve the performance and use of the bacterial inoculants. Greater attention should be paid to studies and application of new combinations of bacterial inoculants for improved results. Future research should also investigate the stability and performance of the *Bacillus sp.,* once the bacteria have been inoculated in soil. It is necessary to extend these types of experiments to other types of bacteria. This would help in developing a potential inoculant for use in agriculture in the future.

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