

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF MULTIFACETED HALOTOLERANT *BACILLUS LICHENIFORMIS* AND *BACILLUS WUDALIANCHIENSIS* FROM RHIZOSPHERIC SOILS OF BANGALORE

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<https://doi.org/10.55251/jmbfs.3553>

ARTICLE INFO

Received 7. 8. 2020
Revised 29. 1. 2022
Accepted 31. 1. 2022
Published 1. 6. 2022

Regular article



ABSTRACT

Plant growth promotion through versatile microorganisms inhabiting the rhizospheric region of the soil has emerged as a sustainable approach in improving the crop productivity and crop health management. The present study reports the isolation and characterization of halotolerant as well as drought tolerant isolates that possess exceptional plant growth promotional attributes. Out of 32 isolates obtained, 13 isolates exhibited excellent plant growth promotional attributes such as phosphate solubilization, phytohormone production, siderophore production etc. Among 13 isolates tested for stress tolerance studies, two isolates Mulberry -1 and Ragi-7 were able to tolerate both drought and saline stresses and were further identified as *Bacillus licheniformis* and *Bacillus wudalianchiensis* based on biochemical and molecular techniques. Antibiosis activity of both the isolates tested against two important plant pathogens *Fusarium oxysporum* and *Phomopsis vexans* was found to be positive. *Bacillus licheniformis* and *Bacillus wudalianchiensis* were further tested for their efficacy to solubilize five different inorganic phosphate sources. The amount of phosphate liberated was found to be highest (3562 mgml⁻¹ and 1894 mgml⁻¹) with diammonium phosphate followed by single super phosphate and tricalcium phosphate with respect to *Bacillus licheniformis* and *Bacillus wudalianchiensis*. *Zea mays* L. seeds treated with the culture filtrate of both the isolates were cultivated on Murashige-Skoog medium under *In-vitro* conditions. *Bacillus wudalianchiensis* was found to be better compared to *Bacillus licheniformis* with respect to plant growth promotional aspects as they were able to increase the root and shoot length of *Zea mays* L. under *In-vitro* conditions. Application of the multifaceted capabilities of unexplored halotolerant strain of *Bacillus wudalianchiensis* could open up a new avenue in plant biotechnology.

Keywords: *Bacillus wudalianchiensis*; *Bacillus licheniformis*; Halotolerant; Phosphate Solubilization Studies; *In-vitro* plant studies

INTRODUCTION

Rhizosphere is a dynamic environment surrounding plant roots which harbours diverse group of microbes. Some of the bacteria from this zone are termed as “rhizobacteria”, which effectively colonize the plant roots. Release of organic compounds from the plant roots serve as nutrients to the microbial community present in the rhizospheric soil. The microorganism inhabiting the rhizosphere, engineers the rooting pattern, activates the plant defense mechanism and improves nutrient uptake in plants (Cruz *et al.*, 2002; Barea *et al.*, 2005). Plant growth promoting rhizobacteria (PGPR) play a cardinal role in reducing the application of chemical fertilizers to a larger extent by extensively promoting the exchange of plant nutrients. Several mechanisms are being reported in the literature by which rhizospheric microorganisms stimulate the plant growth (Kiely *et al.* 2006).

Microorganisms play an imperative role in agriculture as they are very much essential for the decomposition of organic matter and recycling of old plant material. Some soil bacteria and fungi form symbiotic association with various plant roots there by helping in facilitating certain essential nutrients like nitrogen or phosphorous to plants.

Although soil microorganisms comprise <1% of the total mass of a soil, they play a vital role in supporting all living beings. Some of the functions of rhizobacteria are: (i) Break down of organic matter (ii) Nitrogen fixation (iii) Creation of soil structure (iv) Creation of humus (v) Recycling of soil nutrients (vi) Phosphate solubilization and (vii) Plant growth promotion.

Among several PGPR, *Bacillus* spp. have gained considerable attention and are most widely used as bacterial inoculants. *Bacillus* species possess wide metabolic capabilities allowing them to exhibit significant characteristics in soil ecosystem functions and processes. Due to their heterotrophic nature, *Bacillus* spp. are also important in fixing carbon (C), nitrogen and sulfur (S) in the soil along with transformation of certain other soil nutrients (Mandic- Mulec and Prosser 2011). Several studies have reported the beneficial role of various *Bacillus* species in plant

growth promotion, increased yield, phytohormone production, phosphorus (P) solubilization and nutrient uptake (Cakmakci *et al.* 2006, Khan 2010).

Maize is considered as the third most significant cereal crop and it has become staple nourishment in numerous parts of the world. It is mainly utilized as a food for human, feed for livestock and as a raw material for various industries (Khatoun *et al.*, 2010). It is cultivated widely in various soil and climatic conditions. It plays a magnificent role in the country’s economy by feeding malnourished people as well as in solving country’s food problems. Maize encompasses more digestible protein than any other cereals (Hassan *et al.*, 2018).

The present study focuses on isolation of an exceptional, halotolerant and drought tolerant rhizobacteria possessing plant growth promotional attributes from the rhizospheric soils of rural Bangalore.

MATERIAL AND METHODS

Soil sampling and isolation of plant growth promoting rhizobacteria

Soil samples from the rhizospheric region of various crops such as ragi, mulberry, corn, sugar cane and paddy were collected from the agricultural fields of rural Bangalore in the month of February 2019. Geographically the study area/ collection site falls in the longitude of 77° 23’ 45’’ E and 77° 38’ 20’’ E and latitude of 12° 38’ 55’’ N and 12° 52’ 20’’ N. A total of 10 samples were collected carefully in a sterilized container and sieved to remove any debris prior to isolation. 10 grams of soil sample from each crop was serially diluted and plated onto nutrient agar medium using standard microbiological techniques. Plates were incubated at 30°C for 48 hours.

In-vitro assessment of plant growth promoting attributes

Plant growth promotional attributes of bacterial isolates were assessed for inorganic phosphate solubilization, indole-3-acetic acid (IAA) production, siderophore, HCN, ammonia production and antifungal activity against two potent plant pathogens. All assays were carried out in triplicates.

Phosphate solubilization

Solubilization of inorganic phosphate by the isolates was detected by cultivating them on National Botanical Research Institute Phosphate- Bromophenol blue (NBRI-PBPB) medium containing tricalcium phosphate as a inorganic phosphate source. pH of solubilization medium was adjusted to 5.8±0.2 and incubated at 37±2°C for 5 days. Formation of clear zone around the colonies was taken as positive. Uninoculated NBRI-PBPB media served as a control (Nautiyal 1999).

Indole acetic acid production

IAA production by selected bacterial isolates was investigated by cultivating them in nutrient broth supplemented with 0.1 % Tryptophan and incubated at 37°C for 48h under continuous shaking conditions (100 rpm). Further, the culture broth was centrifuged at 5000rpm for 10 minutes. 1ml of the supernatant was mixed with 2ml of Salkowski's reagent (colour reagent) and kept at room temperature for 20 minutes. For negative control, un-inoculated nutrient broth containing 0.1 % tryptophan was mixed with the Salkowski's reagent. Development of pink colour upon addition of colour reagent indicated the production of IAA. Quantity of IAA produced was calculated against a standard curve prepared with different concentrations of standard IAA (Padmavathi et al., 2016a).

Siderophore production

Siderophore production capability of the isolates was evaluated by inoculating the test organisms on chrome azurol S (CAS) agar and incubated at 37° C for 4-5 days. Appearance of orangish brown coloured colony was considered as positive for siderophore production. The isolates that produced orange coloured colonies with clear zones around them were scored as siderophore producers (Schwyn and Neiland 1987).

Hydrogen cyanide production

A loop full of bacterial isolates was inoculated into sterile nutrient broth amended with 4% Glycine. Whatmann No. 1 filter paper strips soaked in 0.5% picric acid and 2% Na₂CO₃ solution were placed towards the end of the test tube and the tubes were incubated for 48h at 37°C. Change in the colour of the filter paper from yellow to orange brown colour was considered as positive for the HCN production (Baker and Schippers, 1987).

Ammonia production

For ammonia production, isolates were inoculated into 5ml of sterile peptone water and incubated at 37°C for 48 h. After the bacterial growth, 0.5ml of Nessler's reagent was added to the broth. Development of yellow to brown colour was observed and indicated as positive for ammonia production (Cappuccino and Sherman, 1992). Uninoculated medium served as control.

Stress tolerance studies

Drought tolerance

In order to study the ability of the isolate to endure drought stress, isolates were cultivated on nutrient agar supplemented with different concentrations of polyethylene glycol (PEG) ranging from 0 % to 16 % at 37°C for 48 hours (Swetha and Padmavathi, 2019).

Halotolerance assay

Bacterial isolates were screened for halotolerance by cultivating them on nutrient agar medium supplemented with various concentrations of sodium chloride (0mM to 240mM). The plates were incubated at 30±2°C for 48 h (Albdaiwi et al., 2019).

Biochemical profiling

The potential isolates that exhibited tolerance to drought and salt stress were further characterized using biochemical and molecular techniques (16srRNA sequencing). Potential isolates were screened for indole, methyl red, Voges-Proskauer test as per standard protocols (Harley and Prescott, 2002). For citrate utilization test, bacterial isolates were cultivated on Simmons citrate medium and incubated at 30±2 °C for 48 hours; change in the colour of the medium from green to blue was taken as positive. For catalase test, potential isolates were smeared on clean glass slide and few drops of 3% hydrogen peroxide were added on to the

smear. Appearance of bubbles confirmed the presence of catalase activity. For oxidase test, a loop full of test organism was streaked in a zigzag manner on the oxidase disk. Development of dark purple colour on the disk after 10-15 seconds indicated positive for oxidase. Carbohydrate utilization efficacy of isolates against various carbohydrates such as glucose, sucrose, mannitol and lactose was studied by inoculating potential isolates in triple sugar medium. The test cultures were stab inoculated onto hydrogen sulphide media and incubated at 37±2°C for 24-48h. The formation of black precipitate in the medium indicated positive for the test.

Molecular identification and phylogenetic tree analysis

The genomic DNA was isolated according to Sambrook and Russel (2001). Amplification of 16S rRNA gene of two potential isolates M-1 and R-7 was carried out using universal eubacterial primers FD1 5_ CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TC AG 3_ and RD1 5_ CCC GGG ATC CAA GCT TAA GGA GGT GAT CCAGCC 3_ (Weisburg et al., 1991). Similarity of 16S rRNA gene sequence was aligned using BLAST programme of GenBank database (NCBI).

Extracellular enzymes production by two potential isolates M1 and R7

Chitinase production

Potential isolates M1 and R7 were inoculated on to nutrient agar plate supplemented with colloidal chitin and incubated at 37°C for 24 h. After incubation, the plates were stained with 0.1% congo red and destained with 1% sodium chloride, clear zone around the colony indicated positive results.

Pectinase production

Pectinase production was investigated by inoculating M1 and R7 on to Vincent's agar plate for 24 hours at 37°C. Formation of clear zone around the colonies upon addition of iodine solution was considered as positive for pectinase production (Venkata et al., 2013).

Starch hydrolysis /amylase production

Bacterial isolates M1 and R7 were streaked on to starch agar plates and incubated for 24 h at 37°C. Further the plates were flooded with iodine solution. Appearance of clear zone around the colonies was considered as positive.

Casein/protease: M1 and R7 were subjected to screening for protease enzyme production by inoculating them on to skimmed milk agar medium and incubated at 37°C for 24 h. Appearance of clear zone around the colonies was taken as positive.

Antibiosis/ antifungal activity

An 8mm diameter agar plugs containing *Fusarium oxysporum* and *Phomopsis vexans* mycelium were placed in the centre of the plate containing potato dextrose agar medium and incubated at 25 °C for 48 h. Isolates M1 and R7 were cultured in nutrient broth and were centrifuged at 5000 rpm for 20 minutes. Two wells of 6 mm diameter on either side of the fungal culture were bored in the inoculated media with the help of a sterile cork-borer. 100 µl of the cell free supernatant was filled in each well. Further, the plates were incubated at 37°C for 18-24 hours. Formation of inhibition zone around the fungal colony was observed which in turn corresponds to the antifungal activity of the cell free supernatant (Kandel et al., 2017).

Phosphate Solubilization Studies by M1 and R7

Different phosphate sources such as Tricalcium phosphate (TCP), Di-ammonium phosphate (DAP), Aluminum phosphate (AIP), Zinc phosphate (ZnP) and Single super phosphate (SSP) were used in the present study.

Phosphate solubilization by M1 and R7 in Liquid medium

Phosphate solubilization in liquid medium was performed by inoculating M1 and R7 isolates in Pikovskaya's broth amended with five different phosphate sources tricalcium phosphate (TCP), diammonium phosphate (DAP), aluminium phosphate (AIP), zinc phosphate (ZnP) and single super phosphate (SSP). The initial pH of the medium was adjusted to 7. After sterilization of the media, M1 and R7 were inoculated and incubated at 37° C for 2 days under continuous shaking conditions. Amount of phosphate liberated, titratable acidity, production of organic acid and drop in pH were analyzed (Swetha and Padmavathi 2016).

Phosphate estimation

Amount of phosphate liberated upon solubilization of different inorganic phosphate sources by M1 and R7 isolates was assessed according to method as described by Fiske-Subbarow (Fiske and Subbarow, 1925).

Titrateable acidity

In order to determine the titrateable acidity, a known amount of culture filtrate was titrated with 0.1M NaOH in the presence of phenolphthalein as an indicator. Drop in pH of the media was also recorded using digital pH meter (Swetha and Padmavathi 2016).

Plant- microbe interaction studies

Zea mays L. seeds obtained from local farmers were sterilized with 70% ethanol for 30 seconds and 2% sodium hypochlorite for 1 min, followed by rinsing for several times with sterile deionized water. The bacterial cultures were grown in nutrient broth at 30°C for 24 h with 150 rpm. Bacterial cells were harvested by Fully grown culture was subjected to centrifugation at 5000 rpm for 5 min at 4°C in order to harvest bacterial cells. Cell pellets were resuspended in saline to achieve a cell density of 1.0×10^8 CFU mL⁻¹. Seed bacterization process was performed by soaking the *Zea mays* L. seeds in bacterial suspensions along with 1 % Carboxy Methyl Cellulose (CMC) for 4 hours whereas the control seeds were soaked only in sterile water. After 4 hours of seed treatment, the seeds were air dried and transferred to culture bottles containing Murashige and Skoog medium (Kiely et al., 2006). Treatments used in the present study are (i) Control (ii) Treated with M1 isolate (iii) Treated with R7 isolate (iv) Treated with Co-culture (M1 and R7). Culture bottles were placed in a growth chamber at 22 °C with a photoperiod of 16/8h (light/dark). Ten days after inoculation, the seed germination and phenotypic characterization was determined.

RESULTS AND DISCUSSION

Isolation of Rhizospheric Microorganisms

A total of 32 bacterial colonies were isolated from the rhizospheric region of various crops such as ragi, mulberry, corn, sugar cane and paddy. Out of these 32 isolates, 10 were Gram positive rods, 17 were Gram negative rods and six were

found to be Gram positive cocci based on the colony morphology and Gram staining technique.

Plant growth promotional attributes

All the 32 isolates were subjected to check their ability to exhibit plant growth promotional attributes, of these 13 isolates showed positive for all the tested plant growth promotional attributes such as phosphate solubilization, production of phytohormone, siderophore production, HCN and ammonia production (Table 1). Phosphorus is one of the essential macronutrient which plays a prominent role in the development of plant roots, flowers, helps in crop maturity, seed formation and offers resistance to plant diseases (Swetha and Padmavathi, 2016). Production of phytohormones such as auxins (IAA) and gibberellins is one of the mechanisms among several other mechanisms by which rhizospheric microorganisms stimulate the plant growth. IAA molecules serve as signalling molecules which are necessary for the development of plant organs (Swetha and Padmavathi, 2016b).

Out of 13 isolates, six isolates exhibited excellent siderophore production on CAS media. Two isolates did not exhibited siderophore production on CAS media. Siderophore production by rhizobacteria plays a vital role in stimulating plant growth and also in controlling several plant diseases. They are also responsible for inducing systemic resistance (ISR) in plants. Siderophores are directly involved in stimulating the production of certain antimicrobial compounds via increasing the availability of essential trace minerals to the bacteria (Solanki MK et al., 2013). Almost all the bacterial isolates showed positive result for ammonia production whereas only eight isolates were effective in producing HCN. HCN production by rhizobacteria is one of the important attribute which plays an important role in the biological control of several soil-borne pathogenic fungi (Ramette et al., 2003). Ammonia producing bacteria are involved in converting Nitrogen (N₂) to Ammonia (NH₃), making it an available nutrient for the plants (Hayat et al., 2010). Hence application of ammonia producing bacteria may effectively enhance/boost the plant growth.

Table 1 Screening of PGPR Traits

Sl. No.	Isolates	Phosphate	Siderophore	Ammonia	HCN	IAA
1	Ragi 10 ⁻² - 7	+++	++	+++	-	++
2	Ragi 10 ⁻² - 2	+++	+++	+++	-	++
3	Ragi 10 ⁻² - 3	+++	+++	++	-	-
4	Ragi 10 ⁻² - 4	+++	-	+++	+	-
5	Ragi 10 ⁻⁴ -1	+++	+++	+++	+	+++
6	Mulberry10 ⁻² - 3	+++	+	++++	+	+++
7	Mulberry 10 ⁻⁴ -2	+++	+	++++	+	-
8	Mulberry 10 ⁻⁴ -1	+++	+++	++++	+	-
9	Corn 10 ⁻² -1	+++	+++	++	-	-
10	Corn 10 ⁻² - 2	+++	++	++	+	++
11	Corn10 ⁻⁴ -1	+++	-	++	-	++
12	Paddy 10 ⁻² -7	+++	++	+++	+	-
13	Sugar cane 10 ⁻² -1	+++	+++	+++	+	++

Note: “+” indicates positive “-” indicates negative

Table 2 Biochemical characteristics of selected bacterial isolates

Sl. No.	Biochemical Tests	Mulberry 1	Ragi 7
1	Catalase test	+	+
2	Oxidase Test	+	-
3	Indole Test	+	+
4	Methyl Red Test	+	-
5	Voges-Proskauer Test	+	-
6	Gelatin Hydrolysis	+	-
7	Citrate Utilization Test	+	+
8	Hydrogen Sulfide Test	+	-
9	Carbohydrate Test		
	Glucose	+	-
	Sucrose	+	-
	Mannitol	+	-
	Lactose	-	-
10	Triple sugar iron test	+	-

Note: “+” indicates positive “-” indicates negative

Molecular identification

Based on 16s rRNA gene sequence analysis, isolates M1 and R7 were identified as *Bacillus licheniformis* and *Bacillus wudalianchiensis* respectively (Figure 1). The phylogenetic tree for M1 and R7 isolates was constructed using phylogeny.fr. 16s rRNA. The gene sequence of the isolates have been deposited in the gene bank database with accession numbers MK937815 and MK937834 for *Bacillus licheniformis* and *Bacillus wudalianchiensis* respectively.

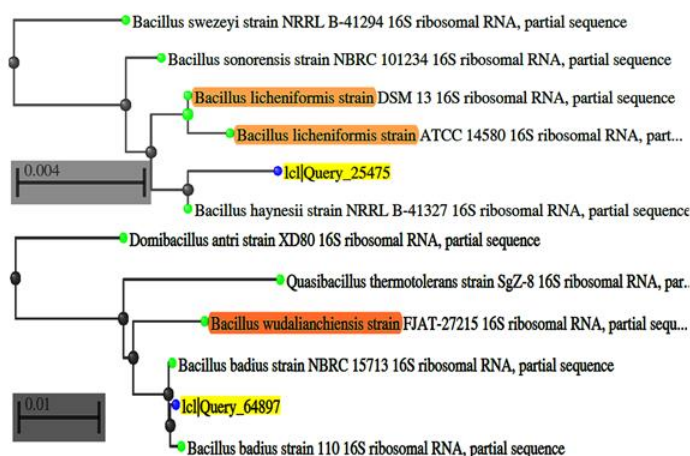


Figure 1 Phylogenetic tree of potential isolates was constructed using phylogeny.fr

Extracellular enzymes production by two potential isolates M1 and R7

Figure 2 illustrates the extracellular enzyme production by *Bacillus licheniformis* and *Bacillus wudalianchiensis*. Both the isolates showed positive for chitinase, pectinase, starch hydrolysis and casein hydrolysis. A hydrolytic enzyme like

protease, amylase, chitinase and cellulase are capable of degrading the fungal cell-wall. Most of the plant growth promoting rhizobacteria is capable of producing these extracellular enzymes that can hydrolyze the variety of complex polymeric compounds like cellulose, hemicellulose, chitin, proteins and pectin present in the cell wall of pathogenic fungi. These extracellular hydrolytic enzymes alter the structural integrity of the cell wall of the targeted pathogens (Budi et al., 2000). Chitinase enzymes are responsible for the degradation of chitin present in the cell wall of fungal phytopathogenes (Kim et al., 2003). These enzymes can either be used directly/ indirectly in the biocontrol activity of fungal pathogens. Proteases are a large group of enzymes that play a significant role in the lysis of cell wall of phytopathogenic fungi, as the major constituents of the fungal cell wall are embedded into the protein matrix. Protease enzyme helps in breaking down of major proteins of phytopathogens into peptide chains and/or their constituent amino acids (Jadhav and Sayyed, 2016). The biocontrol capability of plant growth promoting rhizobacteria is dependent on the production of the above mentioned hydrolytic enzymes (Kobayashi et al., 2002).

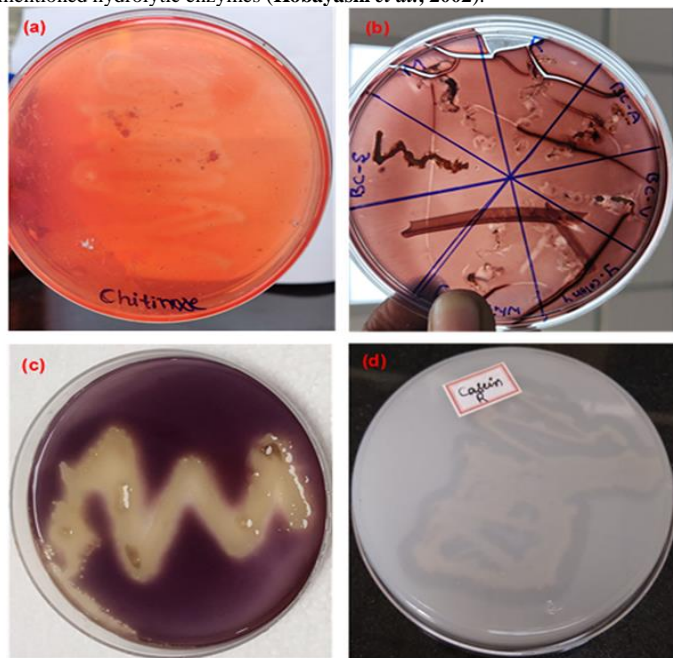


Figure 2 Screening of extracellular enzymes. A- Chitinase, B-Pectinase, C- Starch, D-Casein

Antibiosis/ Antifungal activity

Fusarium oxysporum and Phomopsis vexans are two important plant pathogens which are responsible for the destruction of various crops. Bacillus licheniformis and Bacillus wudalianchiensis exhibited excellent antifungal activity against both the fungal pathogens (Figure 3).

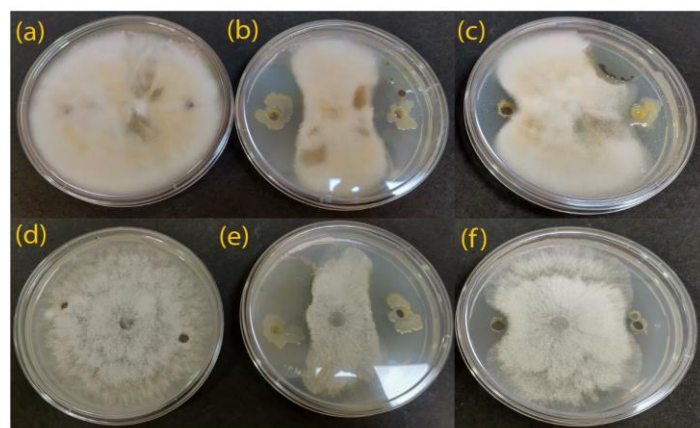


Figure 3 Production of diffusible antifungal metabolites by Bacillus licheniformis and Bacillus wudalianchiensis active against Fusarium oxysporum and Phomopsis vexans(a) Fusarium oxysporum Control (b) Fusarium oxysporum and Bacillus licheniformis (c) Fusarium oxysporum and Bacillus wudalianchiensis (d) Phomopsis vexans Control (e) Phomopsis vexans and Bacillus licheniformis (f) Phomopsis vexans and Bacillus wudalianchiensis .

Phosphate Solubilization Studies by M1 and R7

Figure 4 represents the amount of phosphate liberated by both the isolates Bacillus licheniformis and Bacillus wudalianchiensis for all the five substrates. The amount of phosphate liberated was found to be highest with DAP followed by SSP and TCP. Both the organisms were not able to solubilize ZnP and AIP. Bacillus licheniformis was found to be better in solubilizing all the five substrates compared to Bacillus wudalianchiensis. The obtained results are in conformity with the results of Selvi et al., (2011) who have also reported the highest phosphate solubilization in potassium di hydrogen phosphate followed by tricalcium phosphate, aluminum phosphate and iron phosphate by isolated Bacillus sp. Although the solubilization of different inorganic forms of phosphate by Bacillus licheniformis was found to be better compared to Bacillus wudalianchiensis; a detailed study on phosphate solubilization involving titratable acidity, drop in pH and organic acid production was performed with Bacillus wudalianchiensis as the isolate is not much explored in terms of plant growth promotional attributes. Percentage of titratable acidity was found to be the highest (0.47 %) with DAP (Figure 5). Titratable acidity of 0.11 and 0.18 % for SSP and TCP was obtained respectively. These results clearly explain that phosphate solubilization occurs via carboxylic acids released by microorganisms, resulting in decrease in the pH of the media. An increase in titratable acidity with decrease in pH was recorded with all the phosphate sources which concur with the results obtained by Swetha and Padmavathi (2016b). Similar studies on solubilization of TCP, RP and AIP by various microorganisms have been reported earlier. Likewise, Panhwar et al., (2011) have studied the solubilization of triple super phosphate by two species of Bacillus namely PSB 9 and PSB 16. Change in the pH of the medium is completely dependent on the type of phosphate source present in the medium and the mechanism includes modification of pH by the secretion of organic acids and protons or cation dissociation (Selvi et al., 2011).

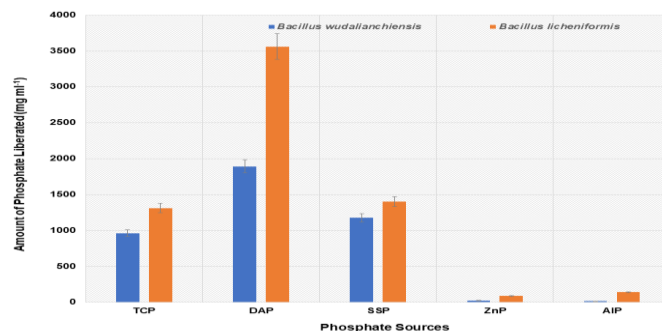


Figure 4 Plots depicting Solubilization of Five different Phosphate sources by Bacillus licheniformis and Bacillus wudalianchiensis

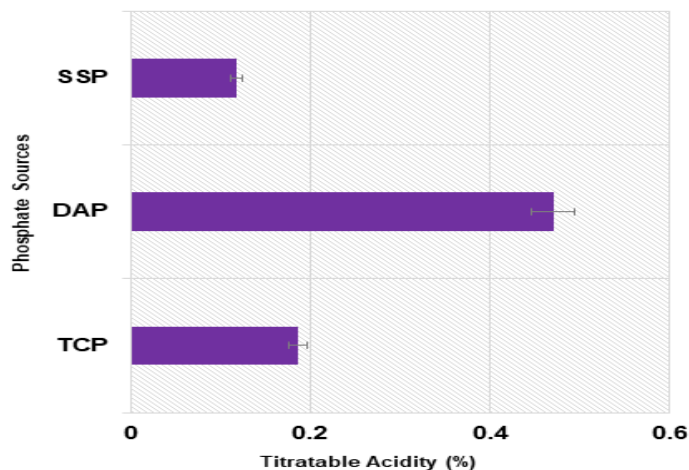


Figure 5 Plots depicting Titratable Acidity

Plant- Microbe Interaction Studies

Seeds of Zea mays L. were treated with cell suspensions of two different rhizobacteria Bacillus licheniformis (M1) and Bacillus wudalianchiensis (R7) under in vitro conditions (Omar et al.,2014). Figure 6 illustrates the effect of bacterial isolates on the germination of Zea mays L seeds under In-vitro conditions. The results revealed that treatment with both the bacterial isolates induced the plant growth promotion significantly over untreated plants. Among the two rhizobacteria used in the study, R7 isolate (Bacillus wudalianchiensis) was found to be better than the M1 (Bacillus licheniformis) in enhancing the plant growth with respect to increase in root length and shoot length.

The results revealed that dual inoculation of both *Bacillus wudalianchiensis* and *Bacillus licheniformis* increased the growth of *Zea mays* L seedlings significantly when compared to untreated control.

Several reports have indicated that bacterization of seeds with *Pseudomonas* and *Bacillus* spp. was found to promote the plant growth. Nagendran (2011) reported that bacterization of seed with bacterial endophytic *Bacillus* strains viz., EPB 18, EPB 11, EPCO 74, EPB 6 and FZB24 were found to enhance the vigour index of the rice seedlings under *In-vitro* conditions.

Similarly, co-culture studies involving strains of *Bacillus* possessing multiple plant growth promotional attributes were found to be more effective in enhancing the growth of wheat plants (Baig et al., 2012).



Figure 6 Effect of bacterial isolates on the germination of *Zea mays* L seeds under *In-vitro* conditions. (a)Control (b) *Bacillus licheniformis* (c) *Bacillus wudalianchiensis* (d) *Bacillus licheniformis* and *Bacillus wudalianchiensis*

Plant growth promoting rhizobacteria play an exceptional role in agriculture by promoting the exchange of plant nutrients and reducing the application of chemical fertilizers to a larger extent.

There are several mechanisms by which rhizospheric microorganisms stimulate the plant growth (Kiely et al. 2006). The direct mechanisms involved in PGPR mediated plant growth promotion are phosphorus solubilization, HCN production, nitrogen fixation, production of phytohormones such as auxins, cytokinins and gibberellins and lowering of ethylene concentration. Among all the other mechanisms; phosphate solubilisation is one of the most important plant growth promoting traits. Availability of phosphorus is also responsible for the functioning of certain key enzymes which are involved in the regulation of many metabolic pathways (Swetha and Padmavathi 2016). One of them includes the production of IAA which plays a prime role in the plant's life cycle by controlling many physiological processes such as cell enlargement and cell division, tissue differentiation, responses to light and gravity etc which are essential for the development of plant. According to the several reports available in literature, application of certain strains of PGPR to various crop plants at an initial stage of development improves the biomass production through direct effects on root and shoots growth. Some of the examples of PGPRs which have been widely exploited for plant growth promotion and suppression of crop diseases are fluorescent *Pseudomonas*, *Bacillus* spp., *Azotobacter* spp., *Azospirillum* spp etc. Among the PGPR, *Bacillus* spp are the most widely exploited bacterial inoculants, which possess wide metabolic capabilities allowing them to exhibit significant characteristics in soil ecosystem functions and processes.

Three endophytic bacterial isolates of *Bacillus* namely BECS3, BECS6 and BECS7 were proven to be very efficient as they exhibited both antagonistic as well as plant growth promoting activities in tomato and chilli plants upon seed bacterization (Amareesan et al., 2012). In a similar study, bacterial strains viz., EPC 5, EPC 8, EPC 15, EPC 29 and EPC 52 were found to increase the vigour index of rice seedlings significantly (Rajendran 2007).

Bacillus wudalianchiensis, a gram positive endospore forming aerobic rods are motile by means of lateral flagella and are rod-shaped bacterium with rounded ends. They possess ellipsoidal endospores which lie at a central position in slightly swollen sporangia. *Bacillus wudalianchiensis* possess a pronounced growth-promoting activity and also found to increase the resistance to plants under various environmental stresses including salinity, drought and plant pathogens (Liu et al., 2017). However, *Bacillus wudalianchiensis* is a novel and unexplored species of *Bacillus*, thus an in-depth study on this particular halotolerant versatile species of *Bacillus* can be very helpful in plant biotechnology field for amelioration of abiotic and biotic stresses in various plants. *Bacillus licheniformis* is a gram positive mesophilic bacteria commonly found in the soil. *Bacillus licheniformis* are beneficial bacteria which have the ability to colonize the roots and promote plant growth through either direct or indirect mechanism or via biological control of plant diseases. They are associated with many plant species and are generally present in varied environments (Hurek and Reinhold-Hurek, 2003).

In a nutshell, the present study has demonstrated the possibility of application of multifaceted *Bacillus licheniformis* and *Bacillus wudalianchiensis* as a biofertilizer in increasing the plant growth in *Zea mays* L. under *In-vitro* conditions. *Bacillus licheniformis* and *Bacillus wudalianchiensis* have well demonstrated the fungistatic/ fungitoxic nature alongside various plant growth promoting attributes. Both the strains have incredible/tremendous potential for inoculum production and exhibit a lot of competitive advantages in colonization of rhizosphere.

CONCLUSION

In the present study, 32 isolates were obtained from the rhizospheric soils of various crops. Out of 32 isolates, 13 exhibited excellent plant growth promotional attributes such as phosphate solubilization, phytohormone production, siderophore production, HCN and ammonia production. Among 13 isolates tested for stress tolerance studies, two isolates Mulberry -1 (M1) and Ragi-7 (R7) were able to endure both the stresses. M1 and R7 were identified as *Bacillus licheniformis* and *Bacillus wudalianchiensis* respectively based on 16s rRNA gene sequence analysis. Both the isolates exhibited antibiosis against most potent phytopathogens; *Fusarium oxysporum* and *Phomopsis vexans*. Production of extracellular hydrolytic enzymes such as chitinase, protease, pectinase and starch hydrolysis were observed with both the isolates. *Bacillus licheniformis* and *Bacillus wudalianchiensis* were tested for their efficacy to solubilize five different inorganic phosphate sources such as tricalcium phosphate, diammonium phosphate, aluminium phosphate, zinc phosphate and single super phosphate. The amount of phosphate liberated was highest with diammonium phosphate followed by single super phosphate and tricalcium phosphate. Both the isolates were not able to solubilize zinc phosphate and aluminium phosphate. Among the two rhizobacteria used in the study, *Bacillus wudalianchiensis* was found to be better compared to *Bacillus licheniformis* with respect to plant growth promotional aspects as they were able to increase the root and shoot length of *Zea mays* L. under *In-vitro* conditions. Dual inoculation studies of both the strains were found to be more effective than single strain.

Deeper understanding of the multifaceted capabilities of unexplored halotolerant strain of *Bacillus wudalianchiensis* could open up a new avenue/ horizon in plant biotechnology. Dual inoculation of the versatile species of *Bacillus* (*Bacillus wudalianchiensis* and *Bacillus licheniformis*) can be a promising approach in the improvement of plant growth and suppression of various diseases in several plants thereby helping the farmers to increase crop productivity in a better way.

Conflict of interest: The authors have no conflict of interest.

Acknowledgments: Authors wish to acknowledge SERB, Department of Science and Technology, Government of India for the financial support and Dr. Krishna Venkatesh (Director, CIIRC- Bengaluru) for his kind help and support during the entire study.

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