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Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 01

Res. Jr. of Agril. Sci. (2022) 13: 291–299

CARAS

Res. Jr. of Agril. Sci. (Jan-Feb 2022)
13(1): 291–299

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

Contribution of Root Associated Zinc Solubilizing *Pseudomonas aeruginosa* on Plant Growth and Translocation of Zinc in Basmati Rice Variety

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Received: 16 Dec 2021 | Revised accepted: 03 Feb 2022 | Published online: 25 Feb 2022

A B S T R A C T

Root and rhizosphere associated Zinc solubilizing Bacteria (ZSB) prevailing in different agroecosystems reveal multiple attributes which could be exploited in various aspects of workable agriculture due to transfer of Zinc (Zn) from soil to sustainable plants. In an in-vitro experiment, Sh15 Shown maximum zone of 3.2 cm on $ZnSO_4$ ore, also showed promising solubilizing capacity of phosphorus (P) and potassium (K). Selected ZSB induced indole acetic acid in the absence of tryptophan and gave a positive response to the oxidase test, exhibited phosphate-solubilizing activity by the formation of clear zones on NBRIP agar plates and could produce siderophores. In the greenhouse study, Sh15 raised Zn translocation toward grains, which enhanced the production of basmati (PB -1637). Zinc transmission index (ZTI) was measured by atomic absorption spectroscopy (AAS). Sh15 (116 mg/g) treated plants showed the best results for carbohydrate estimation, as compared with control (82 mg/g) and for protein estimation, a maximum amount of protein was estimated in Sh15 (312 mg/g) bacterized plants as compared with control (201 mg/g). Throughout all the experiments, Sh15 expresses the highest potential as Zn solubilizer, biochemical and PGP strain. Based on molecular characterization like Matrix-Assisted Laser Desor.ption Ionization-Time of Flight–Mass Spectrometry (MALDI-TOF-MS) and 16S rRNA gene sequencing Sh15 is identified as *Pseudomonas aeruginosa*. Collectively, this study suggests that among four ZSB isolates, this potential *Pseudomonas aeruginosa* could be a potential ZSB PGPR candidate and demands a further investigation for developing a consortium with other PGPR for future on-site (infield) applications.

***Key words*:** ZSB, Zn ores, Basmati (PB-1637), 16S rDNA gene analysis, MALDI-TOF-MS, ZTI, AAS

Due to the impact of global warming and excess use of chemical fertilizer, the plants are subjected to a negative influence on the productivity and quality of crops which affect the food security of the world. Rice (*Oryza sativa* L.) is one of the most important consumed foods throughout the world specially in Asian countries. In 2012, three billion people relied on rice every day. Rice accounted for 40% of the nation's food production [1]. China is the premier consumer consuming 162.4 million metric tons of paddy, 28.7% of world consumption. After that, India is the second-largest consumer all over the world which consuming 130.4 million metric tons of paddy that equivalent to 23.1% of world consumption. In the current storyline, due to intensive farming, soils are continuously exhausting essential macro and micronutrients for the wheat-rice cropping system [2]. Zn is an important micronutrient that is required throughout the whole life cycle of plants as a mineral nutrient for growth and development [3]. Zinc finger transcription factor also helps in floral tissue development, flowering, fruiting [4]. Some crops like rice, barley, potato, soybean, corn, sorghum, and tomato are most sensitive to zinc deficiency [5]. Although Zn is present in the soil, it mostly remains in insoluble forms, which cannot enter inside the plant. In India, 48% of soils are affected by Zinc deficiency [6]. Zn deficiency is the universal micronutrient disorder in rice so efforts to develop cultivars with enhanced tolerance have been inhibited by inadequate understanding of genetic factors contributing to tolerance. Zinc deficiency shows multiple symptoms in plants like chlorosis, reduced leaf size, affects grain yield, water uptake and transport [7], leaves develop brown blotches, plants remain stunted, and reduce the number of tillers. Various methods have been applied to reduce zinc deficiency. Many zinc-containing chemical fertilizers are used

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to alleviate this problem but this type of chemical fertilizer creates a major problem both in the soil as well as to the humans and animals who consume it [8]. PGPR boosts soil with essential plant nutrients like nitrogen (N), phosphorus (P) and potassium (K). Among those, PGPR enhances soil quality by fixing N from the atmosphere but in the case of P and K, bacteria solubilizing P and K from their insoluble source present in the soil. They also accommodate the bioavailability of Zn by solubilizing various Zn. Plant uptake zinc in the form of divalent cations (Zn^{2+}) from soil solution; but in high pH zinc uptake mainly may be a valence ion form [9-10]. Generally, the beneficial soil or rhizospheric bacteria which exist in association with roots are referred to as plant growth-promoting rhizobacteria (PGPR) [11]. Plant's growth-promoting bacteria (PGPB) remain a symbiotic relationship without causing any harmful effect on the host. The group of beneficial microbes is designated as the plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting fungi (PGPF) due to their growth-promoting influence on Phyto-biota. PGPB mainly helps in plant growth and development by using two types of mechanisms such as direct mechanism and indirect mechanism. PGPB by producing antibiotics induce systemic resistance, cell wall degrading enzymes promote plant growth indirectly [12-13]. The direct mechanism for plant growth includes N_2 fixation, hormone synthesizing, enhancing phosphate solubilization, production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme, IAA production. Therefore, biofertilizer could be utilized as the best alternative way to enhance plant growth and yield production as compared with the chemical fertilizers and fungicides with environmental safety and lower production cost. Inoculation of plants with PGPR has reported promising results in improved nutrition, vigorous plant growth and higher yield. So many effective PGPR strains have been formulated as biofertilizers. In the current intensive agricultural practices use of registered biofertilizers and widely accepted microbial technologies to overcome all problems throughout the world. The purpose of this study was to evaluate the role of ZSB isolate in growth promotion in rice plants and transport Zn from soil to plants simultaneously. Initially, the Zn tolerance aerobic bacteria were isolated, screened and identified with the help of MALDI-TOF-MS and16S rDNA gene analysis. Eventually, the ability of the selected ZSB isolate was assayed to improve the health status of rice plants.

MATERIALS AND METHODS

Collection of soil samples and isolation of ZSB

Soil samples were collected from rice root-associated and rhizospheric soil at different locations of Malda and North Dinajpur, West Bengal, India. Four to five fields situated in the range of one kilometre cultivating in the similar soil type were recognized per location. At least four soil samples were collected from one field and placed individually in zipper bags, labelled and stored in the laboratory. After that take one gram of soil from each sample in a sterile test tube and then diluted with 10 ml sterile distilled water individually. Then vortex each sample for proper mixing and then the plant growth-promoting bacteria were isolated by serial dilution plating or sterilized on Luria Bertani (LB) (HiMedia, M1151) agar plates [14]. LB agar plates were kept in an incubator at 37 ºC for 24-36 h. Individual colonies appeared on the agar plate. Then picked the colonies and purified them by retreating on LB agar plates separately.

Screening of ZSB

Modified Pikovskaya's agar (HiMedia, M520) media was used to screen the ZSB containing various Zn ores such as $ZnCl_2$, ZnO, $ZnSO_4$, Zn $(NO_3)_2$. The ZSB isolates were grown in LB broth overnight. Then five microliter of each bacterial suspension with optical density (OD) normalized to 0.6 was inoculated on specific plates containing 0.1% of the respective ore and incubated at 37ºC for 24-36 h. Clear halo zones around the colonies showed potential to solubilize Zn which was calculated by measuring the zone diameter. There were three biological replicates. The zone diameter was calculated by the Zn solubilization index following by the formula [15]:

$$ZSI = \frac{\text{(Colony diameter + halo zone)}}{\text{Colony diameter}}$$

Phosphorus (P) and potassium (K) solubilizing activity

Pikovskaya agar containing plates were used to check the P and K solubilizing capacity of screened ZSB [17-18]. Each bacterial strain was spot inoculated on the centre of agar plates and was incubated at 37 ºC for 5–7 days and observed the halo zone around the colonies. This experiment was performed with three biological replicates.

Morphological and biochemical characterization of selected ZSB

Two strains were selected for morphological and biochemical characterization due to their maximum potentiality to solubilize Zn from various ores. For each experiment, the strains were freshly cultured in LB broth overnight and normalized to the O.D. of 0.6 (at 600 nm) before being used for further study.

Gram reaction

The overnight grown pure culture was transferred on a clean grease-free slide and morphological characters were observed under the microscope.

Biochemical characterization

ZSB isolate was characterized by biochemical analysis such as methyl red (MR) test, Voges Proskauer (VP) test, oxidase test, production of acid and gas from carbohydrate, citrate utilization, nitrate reduction, coagulase test, urease test, catalase test [19].

Identification of Zn solubilizing bacteria

Molecular identification by 16S rDNA sequencing

Selected ZSB strain, Sh15 was identified at the molecular level by 16S rDNA sequencing. The genomic DNA of the bacterial isolate was extracted by the CTAB extraction technique [20-21]. Around 1500 bp 16S rDNA gene was amplified by using the forward primers 27f (5′ GAGTTTGATCACTGGCTCAG 3′) and reverse primers 1492r (5′ TACGGCTACCTTGTTACGACTT 3′). The sequences obtained from PCR products were subjected to BLAST analyses in NCBI and identified based on the closest homologous strain. Then the sequences were deposited to NCBI GenBank through Bankit procedure and approved as the sequence after complete annotation and given accession numbers. The phylogenetic analyses were conducted in MEGA-6.0 software [22].

By MALDI-TOF analysis

Identification by using MALDI-TOF biotype relies primarily on mass spectrometry of bacterial ribosomal protein profiles and the mass spectral pattern of protein expression was compared with reference pattern in a database. Sh15 were

grown on LA plates for 24 h before analysis and stored at -40ºC. After that separate colony came onto the plate. Then colonies were removed from the Petri plates by using sterile disposable plastic loops. 5-10 mg of cells were suspended with 400 µl of distilled water in an Eppendorf tube and add 900 µl of absolute ethanol, vortex and keep at room temperature for 5 mins. After that, the suspension was centrifuged at 14000 rpm for 2 min and completely removed the residual ethanol by pipetting. Then add 50 µl of 70% formic acid, mix thoroughly by vortexing or pipetting and centrifuge for 2 min at 14000 rpm. Finally, place 1 µl of the supernatant onto the target and dry it in the air at room temperature. Overlay 1 µl of matrix solution and air-dried. Then the target was ready for loading to MALDI-Biotyper [23]. A short Formic acid extraction protocol was followed to extract proteins for the samples preparation for identification [24].

Antibiotic resistance

The antibiotic resistances of ZSB strains were tested on LA agar plates and spread by swab following the method of Vincent [25]. Antibiotic discs of Azithromycin (Himedia, SD204), Oxacillin (Biogram, C30-1813), Chloramphenicol (Biogram, OX1-1807), Rifampicin (Himedia, SD128) were placed on bacteria-containing agar plates and then incubated at 37 °C for overnight [26]. After incubation, the inhibition zone appeared around the antibiotic discs. Based on the diameter of inhibition zones, bacterial strains were classified as high or moderate resistance and highly or moderately susceptible.

Plant growth-promoting the profile and extracellular hydrolytic enzyme activity of ZSB

ZSB strains were screened for *in-vitro* plant growth-promoting activities such as indole acetic acid (IAA) production [27], production of hydrogen cyanide (HCN) [28], ammonia production [19], and siderophore production [29] (Schwyn and Neilands, 1987) and phosphate solubilization [18]. Furthermore, this selected four ZSB strains were used for four extracellular hydrolytic enzyme activity test like a qualitative assay of protease production [30], Chitinase production [31], amylase production [32] as well as cellulase production [33].

Preparation of the greenhouse experiment

Preparation of soil for treatment

The soil was collected for the pot trial experiment, from the experimental field of the Department of Botany, University of Gour Banga, Malda. By measuring the soil texture, pH, total organic carbon; the physical and chemical characteristics of soil were measured. Briefly, at first, the soil samples were air-dried and screened to a molecular size of 2 mm. The texture of this soil was examined by following the pipette method [34]. By suspending the soil particles with an equal volume of deionized water, the soil pH was estimated [35]. Presence of organic carbon was found by using the chromic acid wet digestion method by the help of $FeSO_4$ titration [36]. This soil was slightly alkaline (pH 7.5) with 0.75% of organic carbon and had a silty clay-loam texture having sand (6.9%), silt (71.2%) and clay (21.7%). Air-dried so il was selected with 2 mm nylon mesh and autoclaved twice at 121 ºC for 30 min with 15 lb pressure. Then 2 kg soils were poured into each separate polythene pot. The control set was amended with commercially available $ZnSO_4$ fertilizer.

Bacterial application on experimental seeds

Basmati (PB-1637) seeds were collected from State Seed Testing Laboratory, Malda. For seeds, bacterization selected ZSB (Sh15) was grown in LB broth in a rotary shaker incubator at 37°C for 48 h at 150 rpm. After that, the broth was centrifuged at 8000 rpm to get the pellet of the bacterial cell. Then the pellet was resuspended with sterile distilled water and the OD value was adjusted to 0.06 (10^8 cfu/mL). Then seeds soaked in bacterial suspension supplemented with 1% carboxymethyl cellulose (CMC) and then kept seeds on blotting paper containing Petri plate, whereas seeds soaked with sterile distilled water with CMC served as uninoculated control in the presence and absence of Zn in soils. After seeds germination, seeds were transferred to pots. Three sets of biological replicates were performed.

Plant maintenance

All pot containing plants were properly watered with sterile distilled water and maintained in sunlight with the natural environment (Temperature 25-30ºC, 11 h photoperiod, and proper aeration) for 30 days.

Effect of ZSB on rice plants

The plant growth-promoting activity was determined by the germination percentage of seeds, shoot length, root length, leaves count, shoot dry weight and root dry weight. The germination percentage of seeds was measured after 48–72 h of seed bacterization. But the shoot length, root length, leaves count, shoot and root dry weights were assessed after 7, 15 and 30 days of germination.

Estimation of total chlorophyll

Total chlorophyll was estimated by the Harborne method [37]. Collected fresh leaf tissue and homogenized in 80% acetone. The mixture was filtered using Whatman-1 filter paper and collected in a sterile test tube. Then the absorbance was recorded in UV–VIS spectrophotometer at 645 nm and 663 nm, respectively and calculated using the formula as given by Arnon [38].

Extraction and estimation of total proteins

Whole plant parts were collected for extraction and estimation of soluble protein by using this method [39]. The fresh plant was homogenized in a pre-chilled mortar pestle containing 50 mM sodium phosphate buffer (pH 7.2) and polyvinyl pyrrolidone (PVP) under ice-cold condition. Then the mixture was centrifuged at 4 °C for 15 min with 10,000 rpm and the supernatant was collected for estimation of protein by using the standard protocol [40].

Total carbohydrate extraction and estimation of tested plants

Plant fresh leaves were crushed with 95% ethanol for total carbohydrate estimation. The alcoholic fraction was evaporated off in a boiling water bath. Then the aqueous fraction was centrifuged at 4ºC and 10,000 rpm for 10 min. Collect the supernatant and total soluble sugar contents of plant leaves extract determined by using the Anthrones method by Plummer [41].

Determination of plant Zn content and Zn translocation index (ZTI)

The Zn analysis of tested plant shoot and root portion was carried out commercially by AAS (Atomic Absorption Spectroscopy) in the State Soil Testing Laboratory, Malda, West Bengal. Zn translocation index (ZTI) was calculated by using the formula [42-43].

$$ZTI = \frac{\text{Zn concentration in plants}}{\text{Zn concentration in shoot}} \times 100$$

Statistical analysis

All experimental results are expressed as mean ± SE (Standard error). The data were computed by one-way analysis of variance (ANOVA) with the help of Duncan's multiple range test in SPSS.

RESULTS AND DISCUSSION

About sixty-five bacterial isolates were procured from the root and rhizospheric soil of rice agricultural fields at different locations of West Bengal, India. Out of 65 isolates, 27 bacterial strains were capable of solubilizing Zn from different Zn ores like Zinc oxide (ZnO), Zinc chloride ($ZnCl_2$), Zinc sulphate ($ZnSO_4$) and Zinc nitrate ($Zn(NO_3)_2$) but only best of four isolates (based on solubilization zone, solubilization index and amount solubilized) were selected for further studies and selected most potent ZSB for identification.

Table 1 Zinc solubilization zone and Zn solubilization index of ZSB

Sample code	Solubilization zone by using different Zn ores (cm)				Zn Solubilization index (cm)			
	ZnO	$ZnSO_4$	$ZnCl_2$	$Zn(NO_3)_2$	ZnO	$ZnSO_4$	$ZnCl_2$	$Zn(NO_3)_2$
Sh1	1.9 ± 0.15^{d}	1.8 ± 0.2^{a}	2.2 ± 0.1^{d}	1.2 ± 0.26^{a}	1.2 ± 0.2^{c}	1.3 ± 0.25^{c}	1.8 ± 0.2^{b}	0.9 ± 0.15^{c}
Sh15	2.6 ± 0.15^{a}	3.2 ± 0.21^{a}	2.8 ± 0.15^{a}	2.4 ± 0.25^{a}	2.1 ± 0.2^{a}	2.6 ± 0.15^{a}	2.3 ± 0.3^{a}	2 ± 0.25^{a}
Sh28	2.1 ± 0.26^{c}	2 ± 0.25^{c}	2.3 ± 0.21^{c}	1.3 ± 0.31^{b}	1.7 ± 0.3^{b}	1.8 ± 0.20^{b}	1.7 ± 0.4^{c}	1 ± 0.15^{c}
Sh48	2.4 ± 0.12^{b}	2.8 ± 0.15^{b}	2.6 ± 0.3^{b}	1.6 ± 0.33^{b}	2 ± 0.2^{a}	2.5 ± 0.25^{a}	2.4 ± 0.2^{a}	1.4 ± 0.15^{b}

Here all the values are the mean of three replicative observations with ± SE. (Values bearing different letters in the same column are significantly different from each other according to the analysis of variance ($p < 0.05$)

In vitro assessment of isolation and screening of ZSB

In vitro, the Zn solubilization capacity of the selected bacterial isolates was assessed by determining the zone diameter in a plate assay. Among all isolates, only four isolates were selected based on solubilization of zinc, solubilization index and amount of Zn solubilized. ZSB exhibited a varying degree of Zn solubilization in four Zn ores supplemented medium. Maximum solubilization zone (3.2 cm) was exhibited by Sh15 followed by Sh48 (2.9 cm) in $ZnSO_4$ supplemented medium. On the contrary, $ZnCl_2$ supplemented medium, exhibited similar solubilization by Sh15 (2.8 cm) followed by Sh48 (2.6 cm) as shown in (Table 1). On the other side, these strains also solubilized P and K. In the case of P solubilization, the maximum zone (3.7 cm) was exhibited by Sh15 followed by Sh48 (3.5 cm) (Fig 1A). But in the case of K solubilization, the maximum zone (4.1 cm) was shown by Sh48 compared with Sh15 (3.8 cm) (Fig 1B).

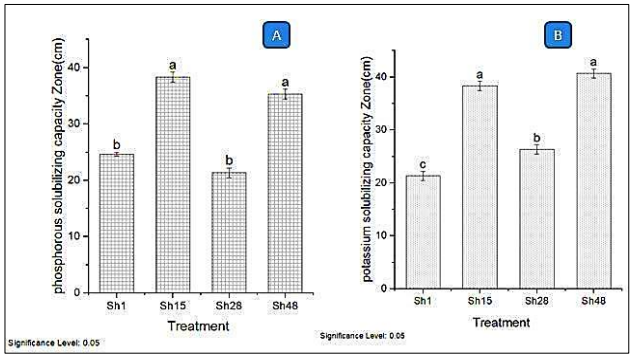

Fig 1 Other solubilizing capacities of ZSB, A) Phosphorus; B) Potassium

Morphological, biochemical and molecular identification of screened ZSB

Based on the morphology, gram staining and biochemical tests of selected ZSB were characterized as per Bergey's manual of determinative bacteriology. All selected ZSB isolates are Gram-negative in nature. Morphological, and biochemical characterization was also performed. Based on 16S rDNA sequence analysis, the most potential ZSB strains (Sh15) were identified as *Pseudomonas aeruginosa*. Nucleotide sequences of 1476 bp have been submitted to the GenBank nucleotide sequence database with the accession number (MN999950). A nBLAST search for this 16S rDNA nucleotide sequence of ZSB strain was phylogenetically 100% similar to *Pseudomonas aeruginosa* JCM5962 and the result is shown in (Fig 2). MALDI-TOF-MS analysis also confirmed that the isolate Sh15 belongs to the genus *Pseudomonas*. So, based on both the studies, Sh15 was considered as *Pseudomonas aeruginosa.*

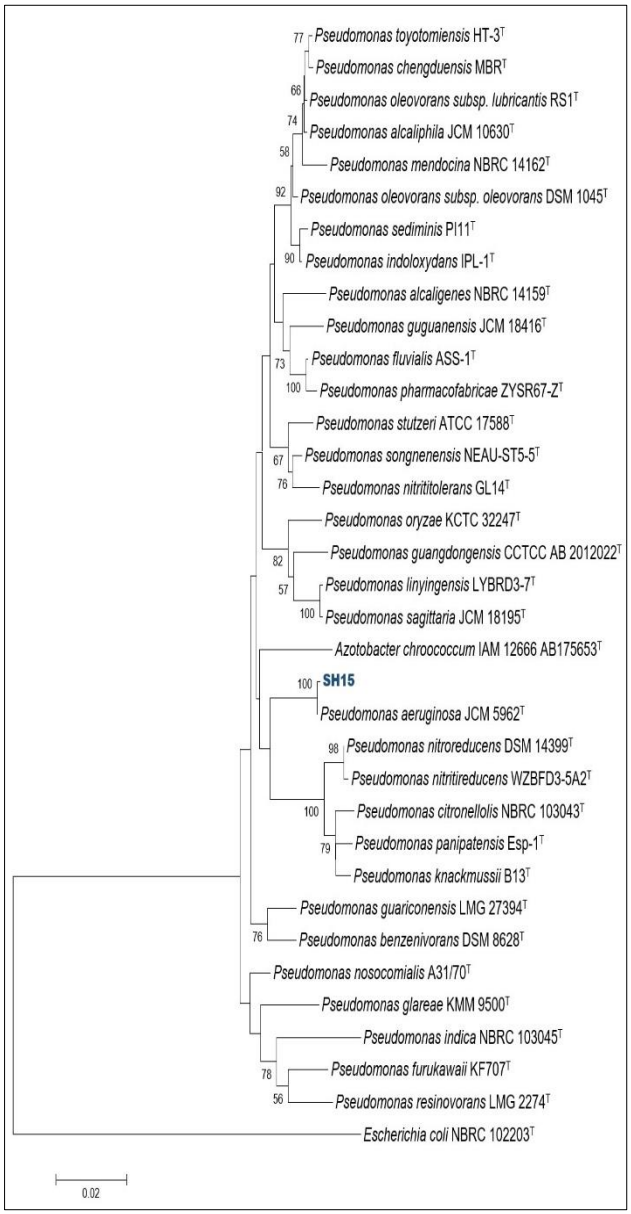

Fig 2 Phylogenetic analysis of Sh 15 isolates based on 16S rDNA gene sequencing

Antibiotic assay

Different antibiotics showed varying effects on selected ZSB by forming a clear zone around the colony. All ZSB strains revealed resistance against Chloramphenic, Azithromycin and Rifampicin antibiotics but in the case of oxacillin, Sh15 and Sh48 showed sensitivity followed by resistant strains such as Sh1 and Sh28 (Fig 3).

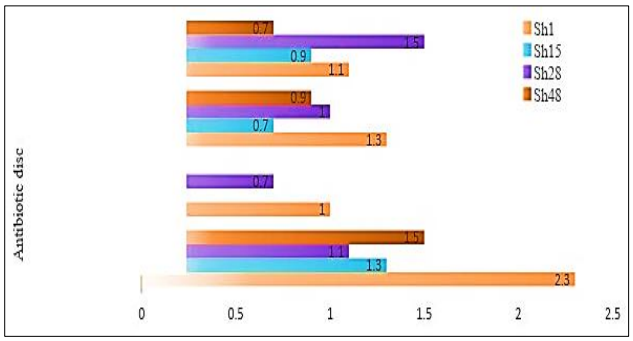

Fig 3 Antibiotic sensitivity assay by disc diffusion

Plant growth-promoting attributes of ZSB

All ZSB isolates were evaluated for PGP abilities assays. After 7 days of inoculation, the Chromo azurol S agar plates were revealed that three ZSB strains Sh1, Sh15, Sh28 were able to produce siderophores and another one Sh48 did not respond to this test. Besides, the IAA production assay showed that all ZSB strains exhibited the ability of IAA production, on the addition of Salkowski's reagent after 24–72 h incubation. In the case of ammonia production, only Sh1 showed a positive result. Similarly, Sh15 and Sh48 showed a positive result for the phosphate solubilizing activity test. The exponentially grown bacterial strains were inoculated on Kings B medium supplemented with 4.4 g glycine L^{-1} and the colour change of picric acid (0.5% picric acid in 1% Na_2CO_3) containing filter paper indicated HCN production. Extracellular hydrolytic enzyme assay of protease, cellulase, chitinase and amylase was performed. Moreover, Sh15 strain produced all hydrolytic enzymes but in the case of Sh1, Sh28 and Sh48 showed a negative result for amylase, protease and chitinase production respectively followed by other positive results (Table 2).

Table 2 Plant growth-promoting attributes and extracellular hydrolytic enzyme activity

Plant growth-promoting activities	Result of ZSB			
	Sh 1	Sh 15	Sh 28	Sh 48
IAA production	+ ve	+ ve	+ ve	+ ve
Ammonia production	+ ve	- ve	- ve	- ve
Phosphate solubilization	- ve	+ ve	- ve	+ ve
HCN production	+ ve	+ ve	+ ve	+ ve
Siderophore production	+ ve	+ ve	+ ve	- ve
Extracellular enzymes				
Protease activity	+ ve	+ ve	- ve	+ ve
Amylase activity	- ve	+ ve	+ ve	+ ve
Cellulase activity	+ ve	+ ve	+ ve	+ ve
Chitinase activity	+ ve	+ ve	+ ve	- ve

Here '+ ve' means positive and '- ve' means negative

Supplemental Table: Biochemical characterization

Biochemical characterization	Result of ZSB isolates			
	Sh 1	Sh 15	Sh 28	Sh 48
Catalase activity	+	+	+	+
Coagulase test	-	+	-	+
Methyl red test	-	-	+	-
Voges-Proskauer test	-	-	+	-
Production of acid gas	+	+	+	+
Nitrate test	+	++	+	+
Oxidase test	++	++	+	++

Here, (+) indicate positive result and (-) indicate negative result

Supplemental Table: Morphological characterization and gram reaction

Sample code	Morphological characterization					Gram reaction
	Colony colour	Surface	Shape	Pigmentation	Margin	
Sh 1	White	Rough	Rod	No	Erosive	Gram-negative
Sh 15	Greenish Yellow	Shiny	Small rod	Yellow	Undulate	Gram-negative
Sh 28	Milky white	Rough	Small rod	No	Plain	Gram-negative
Sh 48	Greenish	Shiny	Rod	Blue-Green	Undulate	Gram-negative

Pot experiment

Effect of ZSB on plant growth and development

The effects of all treatments on the shoot and root length, shoot and root fresh weight as well as dry weight and leaves count of rice plants were determined after 7 days, 15 days and 30 days of germination. Also observed Zn untreated pot considered as a blank and commercially available $ZnSO_4$ treated pot considered as a control. Sh15 showed better results against all combinations of treatment. All selected ZSB isolates showed promising results as compared to control and control with commercially available zinc preservatives [Fig 4-6].

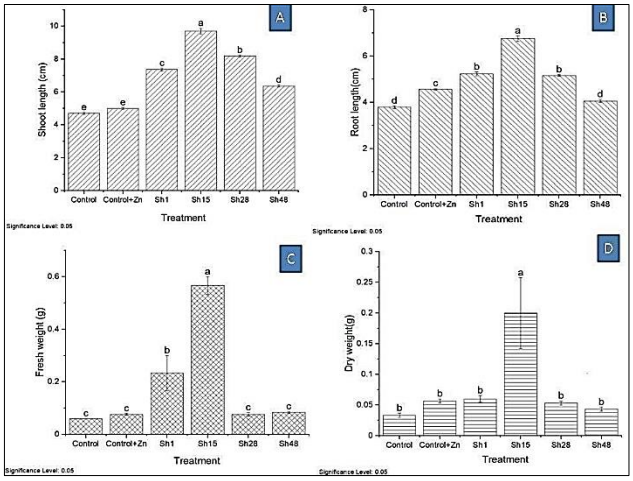

Fig 4 The effects of ZSB on plant growth after 7 days experiment A. Shoot length, B. Root length, C. Fresh weight, D. Dry weight. (Here, the values are the average of triplicate trials ± SE and Bar with different alphabet significantly different from each other according to the analysis of variance ($p < 0.05$))

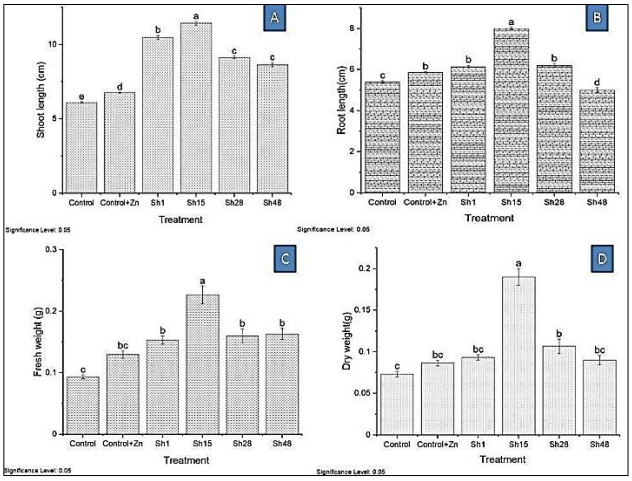

Fig 5 The effects of ZSB on plant growth after 15 days A. Shoot length, B. Root length, C. Fresh weight, D. Dry weight. (Here, the values are the average of triplicate trials ± SE. Bar with different alphabet significantly different from each other according to the analysis of variance ($P< 0.05$)

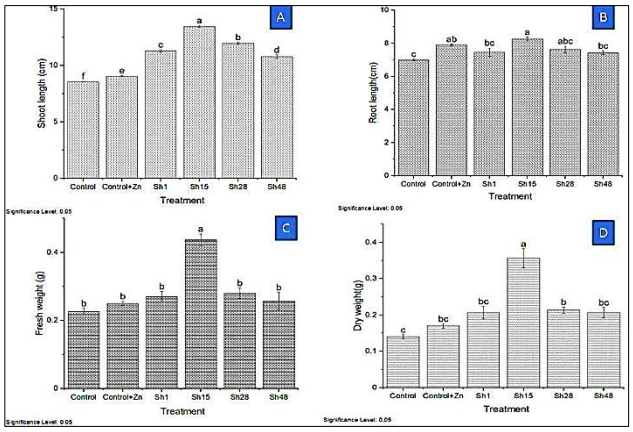

Fig 6 The effects of ZSB on plant growth after 30 days experiment, A. Shoot length, B. Root length, C. Fresh weight, D. Dry weight. (Here, the values are the average of triplicate trials ± SE. Bar with different alphabet significantly different from each other according to the analysis of variance ($p < 0.05$))

Total chlorophyll estimation of treated and untreated plant

Different treatments also showed varying effects on chlorophyll contents. Chlorophyll contents were maximum in Sh15 (0.63mg/ml) as compared to other ZSB isolates like Sh1 (0.53 mg/ml), Sh28 (0.45mg/ml) and Sh48 (0.41mg/ml). On the other side selected ZSB strains showed increased chlorophyll content with comparison to control (0.3mg/ml) and control with $ZnSO_4$ (0.38mg/ml) treated plant (Fig 7).

Total protein estimation of treated and untreated plant

It has been observed that the total soluble protein content increased significantly in all the treatments in comparison to the control. The highest amount of protein was estimated in the Sh15 bacterized plant is 315 mg/ml. Soluble protein content also increased in presence of others ZSB like Sh1, Sh28 and Sh48. Moreover, in all sets of bacterial treated plants, a significant increase in protein content was observed in comparison to both controls as well as zinc (Fig 8).

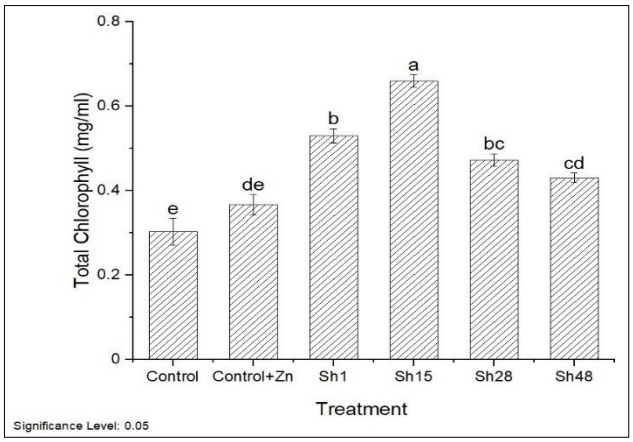

Fig 7 Total Chlorophyll Content (mg/ml) of treated plant. The data are displayed as mean ± standard error, Bar with different letters significantly different from each other according to the analysis of variance ($p < 0.05$)

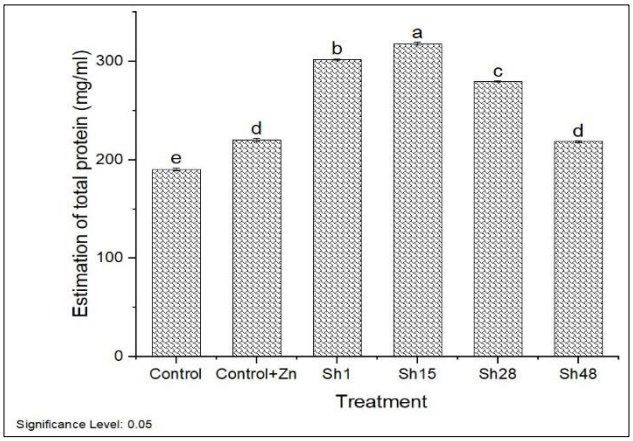

Fig 8 Graphical representation of viable protein (mg/ml) content. The data are displayed as mean ± standard error, Bar with different alphabet significantly different from each other according to the analysis of variance ($p < 0.05$)

Total carbohydrate estimation of treated and untreated plant

The effect of bacterial isolates on total soluble carbohydrate was estimated in the different treated plants. In the case of carbohydrate estimation, Sh28 treated plants showed the highest amount of total estimated carbohydrate that is 130 mg/ml. Sh1, Sh15 and Sh48 treated plant gives 113mg/ml, 121mg/ml and 96 mg/ml to total carbohydrate which were greater than control (93 mg/ml) and Control with $ZnSO_4$ (98 mg/ml) treated plant (Fig 9).

Effect of ZSB on yield

Potential ZSB strains significantly increased the yield after 3 months from seed bacterization for the pot experiment. Maximum effect on yield was given by Sh15 treated plants, such as 31gm/pot. Similarly, Sh1, Sh28 and Sh48 treated plant also given a better effect on yield like 25 gm/pot, 27 gm/pot and 23.5 gm/pot respectively that was greater than control or control with $ZnSO_4$ treated pot like 19 gm/pot and 22gm/pot respectively.

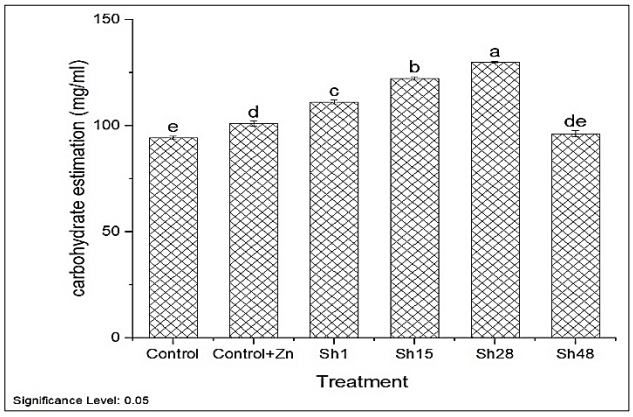

Fig 9 Quantitative determination of total carbohydrate content (mg/ml). The data are displayed as mean ± standard error, Bar with different alphabet significantly different from each other according to the analysis of variance ($p < 0.05$)

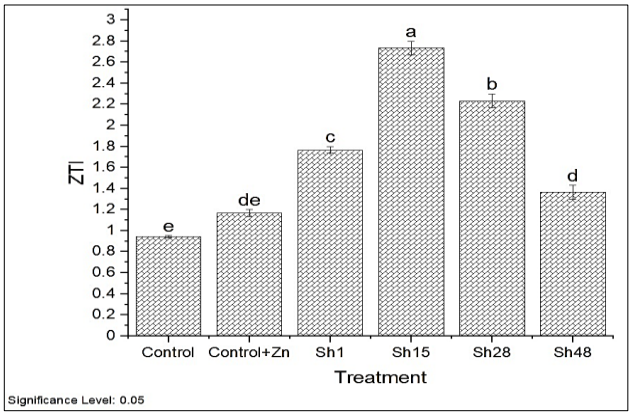

Fig 10 ZTI of ZSB isolates (Values are the mean of three replicates and bearing various letters in the similar column are significantly different from each other according to the investigation of difference ($p < 0.05$). Here, the values are the average of triplicate trials ± SE)

Estimation of plant Zn content and Zn translocation index (ZTI)

The Effect of ZSB on the total Zn transformation on plant and ZTI shown in figure 10. The Highest Zn content was measured in the plant treated with Sh15 (ZTI- 2.6). In this study, another potential ZSB such as Sh1(ZTI- 1.7), Sh28 (ZTI- 2.1) and Sh48 (ZTI- 1.3) treated plant also showed high Zn content than control (ZTI- 0.93) and control with $ZnSO_4$ (ZTI- 1.2).

Zinc amended crop yield, besides raising nutritional quality and safety. Zinc plays an important role in cellular functions, protein metabolisms, chlorophyll biosynthesis, and photosynthesis and seed maturation. To resolve these great issues, many different methods are utilized for several years like using chemical fertilizers, genetic engineering, and transgenic approaches. The use of those fertilizers that contain zinc has critically affected the soils as well as human and other animals, environment. Interference of soil microbes solubilizes unavailable zinc smattering that increases zinc uptake in plants but exploitation of ZSB for moderate insoluble zinc mite is least known still now. That's Why the application of ZSB can overcome the lack of zinc fractions, therefore, achieving zinc solubilization which improves crop production [44]. Present research reported that plant growth-promoting microorganisms have been proficient in zinc solubilization [45]. In this present investigation, ZSB was isolated from the agricultural field of Malda and North Dinajpur district, West Bengal. After preliminary screening, only four capable ZSB strains were selected as visualized by clear zone development around the colonies against maximum zinc ores. Among four bacterial isolates, Sh15 (*Pseudomonas aeruginosa)* showed remarkable zinc solubilization, were characterized by morphological, biochemical and PGP tests, as well as molecular identification by 16S rDNA sequencing. The main domain of this experimental study was to identify the strains which can be used as zinc bio-fertilizers. All selected ZSB isolates are Gram-negative bacteria. All strains showed positive results for carbohydrate production, protein estimation, and IAA production. IAA helps in cell division, expansion and differentiation of plant cells and tissues stimulate root elongation, seed germination, flowering and also affect photosynthesis. Multitudinous experiments have illustrated that for plant growth and development increases in certain factors such as PGP attributes are inevitable [46]. Siderophores also form some heavy metals such as Al, Cd, Cu, Zn. Siderophore is a low molecular iron chelator that helps to satisfy the nutritional requirements of iron. At the time of iron limitation condition, Siderophore acts as a solubilizing agent for iron from minerals [47]. Davies [68], observed that IAA production through PGP bacteria increases cell elongation and also enhances the growth of roots in plants, resulting in an increase in the nutrient value of the plant. Here, all ZSB strains produce IAA, and they also help to increase plant height, chlorophyll content, and total carbohydrate content. Sh15 strain showed a positive result for methyl red test and Voges-Proskauer test respectively. Sh15, Sh1 and Sh28 bacterial strains produced Siderophore. The production of ammonia helps to influence the plant's growth. The result of hydrogen cyanide production was positive in the case of all strains. Rhizobacteria can inhibit phytopathogens by hydrogen cyanide production. HCN also helps to lead to the death of an organism by inhibiting electron transport, energy supply to the cell [49].

Dubey *et al*. [50], revealed that Bacillus subtilis (BSK17) solubilizes inorganic phosphate and improves the production. In this present study, Sh15 and Sh48 bacterial strains can solubilize phosphate. Phosphate solubilizing microorganisms developed clear holo zones by solubilizing suspended TCP because it releases organic acids into the surrounding medium. All strains showed a positive result in the case of nitrate and oxidase tests. Aqueous ammonia is used by the plant as a source of nitrogen which is produced by microorganisms. Among all strains, Sh15 produced the maximum amount of carbohydrate and protein. Pots with bacterial treatments showed more length of shoot and root than control and zinc fertilizer containing pot. Shoot and root length was maximum in the case of A and D bacterial strains respectively. High growth performance indicates that bacterial strains uptake more nutrients and also promote the plant's growth. Four strains utilized in this research have the capacity to induce indole acetic acid and furthermore help to upgrade the shoot and root length through cell elongation and duplication. Zinc solubilization can be completed by different mechanisms including the production of chelating agents, organic acid production. The principal focus point of this work was to isolate, identify and characterize the

ZSB that have the capability to transfer Zn from soil to plant as well as improve PGP activity. Calculation of the ZSI and ZTI showed increased Zn content of roots and shoots as compared to uninoculated plants [51-52]. The most amazing aspect of this trial work is *Pseudomonas aeruginosa* (Sh15) as a zinc solubilizer, plant growth enhancer and supplement rises, proposing it as an option in contrast to synthetic or chemical composts and furthermore to convey the supplement lacks in experimental plants.

CONCLUSION

Zinc solubilizing bacteria solve many problems in the modern agriculture field. In developing countries like India, the necessity of chemical fertilizer for crop production is increased tremendously due to the required nutrition of the plants. But excessive fertilizer can cause many problems like soil fertility decrease, affects the environment, plant abnormality. To reduce excessive use of chemicals nowadays, one alternative process is present that provides eco-friend relationships without causing any problem. Rice is one of the staple foods throughout the World. Nowadays zinc deficiency is a major problem in the agricultural field. Rice is more susceptible to this problem. The major aim of this study was to evaluate four bacterial strains for increasing crop productivity, plant growth and development.

Acknowledgement

The authors acknowledge Dr. Sukhendu Mondol, CU for helping in gene analysis by 16s rDNA sequencing and Prof. Bidhan Roy, UBKV for supplying authentic seed.

Compliance with ethical standards

Conflicts of interest: The authors declare that there is no conflict of interest.

LITERATURE CITED

1. Vaid SK, Kumar B, Sharma A, Shukla AK, Srivastava PC. 2014. Effect of zinc solubilizing bacteria on growth promotion and zinc nutrition of rice. *Jr. Soil Sci. Plant Nutrition* 14: 889-910.
2. Rana A, Kabi SR, Verma S, Adak A, Pal M, Shivay YS, Prasanna R, Nain L. 2015. Prospecting plant growth promoting bacteria and cyanobacteria as options for enrichment of macro- and micronutrients in grains in rice wheat cropping sequence. *Cogent Food Agric*. 1: 1037379.
3. Höller S, Meyer A, Frei M. 2014. Zinc deficiency differentially affects redox homeostasis of rice genotypes contrasting in ascorbate level. *Jr. Plant Physiology* 171: 1748-1756.
4. Epstein E. 2005. *Mineral Nutrition of Plants : Principles and Perspectives*. 2nd ed. Sinauer Associates, Inc., Sunderland, Mass.
5. Vitosh M, Warncke DD, Lucas RE. 1973. Secondary-and Micro-nutrients for Vegetables and Field Crops. Cooperative Extension Service, Michigan State University.
6. Singh MV. 2009 Micronutrient nutritional problems in soils of India and improvement for human and animal health. *Indian Jr. Fertil*. 5: 11-16.
7. Tavallali V, Rahemi M, Eshghi S, Kholdebarin B, Ramezanian A. 2010. Zinc alleviates salt stress and increases antioxidant enzyme activity in the leaves of pistachio (*Pistacia vera* L. 'Badami') seedlings. *Turkish Jr. Agric. For*. 34: 349-359.
8. Bhardwaj D, Ansari MW, Sahoo RK, Tuteja N. 2014. Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microb. Cell Fact*. 13: 66.
9. Alam S, Khalil S, Ayub N, Rashid M. 2002. In vitro Solubilization of inorganic phosphate by phosphate solubilizing microorganisms (PSM) from maize rhizosphere. *Int. Jr. Agri. Biology* 4(4): 454-458.
10. Alloway BJ. 2008. Zinc in soils and crop nutrition. 2nd ed., published by IZA and IFA Brussels, Belgium and Paris, France.
11. Kloepper JW, Schroth MN. 1978. Plant growth-promotingrhizobacteria on radishes. In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, in: Station DePathologie Ve´ge´tale et de Phytobacte´riologie. pp. 879–882.
12. Lugtenberg B, Kamilova F. 2009. Plant-Growth-Promoting Rhizobacteria. Annu Rev Microbiol. 63:541–556.
13. Zahir ZA, Arshad M, Frankenberger WT. 2003. Plant growth promoting rhizobacteria: Applications and perspectives in Agriculture. *Advanced Agronomy* 81: 97.
14. Hassan MN, Afghan S, Hafeez FY. 2010. Suppression of red rot caused by *Colletotrichum falcatum* on sugarcane plants using plant growth-promoting rhizobacteria. *BioControl*. 55: 531-542.
15. Premono ME, Moawad AM, Vlek PLG. 1996. Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Jr. Crop Sci*. 11(1): 13-23.
16. Kumar P, Dubey RC, Maheshwari DK. 2012. Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiol. Research* 167: 493-499.
17. Nautiyal CS. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol Letters* 170: 265-270.
19. Cappuccino JG, Sherman N. 1992. *Microbiology*: A laboratory manual.
20. Nyssen GA, Lovell GS, Simon AA, Smith JG, Tolar BK, Wilson DJ. 1987. Removal of trace levels off phenols from aqueous solution by foam flotation. *Separation Science and Technology* 22(11): 2127-2141.
21. Borges A, Rosa MS, Recchia GH, Queiroz-Silva JR, Bressan ED, Veasey EA. 2009. CTAB methods for DNA extraction of sweetpotato for microsatellite analysis. *Scientia Agricola* 66(4): 529-534.
22. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evolution* 30: 2725-2729.
23. Schmitt BH, Cunningham SA, Dailey AL, Gustafson DR, Patel R. 2013. Identification of anaerobic bacteria by Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry with on-plate formic acid preparation. *Jr. Clin. Microbiology* 51: 782-786.
24. Kurli R, Chaudhari D, Pansare AN, Khairnar M, Shouche YS, Rahi P. 2018. Cultivable microbial diversity associated with cellular phones. *Front Microbiology* 9.
25. Vincent JM. 1970. A manual for the practical study of the root-nodule bacteria. A manual for the practical study of the root-nodule bacteria.

26. Chakraborty S, Afaq N, Singh N, Majumdar S. 2018. Antimicrobial activity of *Cannabis sativa*, *Thuja orientalis* and *Psidium guajava* leaf extracts against methicillin-resistant *Staphylococcus aureus. Jr. Integr. Med*. 16: 350-357.
27. Bric JM, Bostock RM, Silverstone SE. 1991. Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Environ. Microbiol*. 57: 535-538.
28. Bakker AW, Schippers B. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and Pseudomonas SPP-mediated plant growth-stimulation. *Soil Biol. Biochemistry* 19: 451-457.
29. Schwyn B, Neilands JB. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochemistry* 160: 47-56.
30. Walsh GA, Murphy RA, Killeen GF, Headon DR, Power RF. 1995. Technical note: detection and quantification of supplemental fungal beta-glucanase activity in animal feed. *Jr. Anim. Sci*. 73(4): 1074-1076.
31. Rodriguez-Kabana R, Godoy G, Morgan-Jones G, Shelby RA. 1983. The determination of soil chitinase activity: Conditions for assay and ecological studies. *Plant Soil* 75: 95-106.
32. Shaw JF, Lin FP, Chen SC, Chen HC. 1995. Purification and properties o-f an extracellular α-amylase from *Thermos* sp. *Bot. Bull. Acad. Sin.* 36: 165-200.
33. Lu WJ, Wang HT, Nie YF, Wang ZC, Huang DY, Qiu XY, Chen JC. 2004. Effect of inoculating flower stalks and vegetable waste with ligno-cellulolytic microorganisms on the composting process. *Jr. Environ. Sci. Heal - Part B Pestic. Food Contam. Agric. Wastes* 39: 871-887.
34. Day PR. 2015. *Particle Fractionation and Particle-Size Analysis*. John Wiley and Sons, Ltd. pp 545-567.
35. Rhoton FE, Bruce RR, Buehring NW, Elkins GB, Langdale CW, Tyler DD. 1993. Chemical and physical characteristics of four soil types under conventional and no-tillage systems. *Soil Tillage Res*. 28: 51-61.
36. Walkley A, Black IA. 1934 An examination of the degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Sciences* 37: 29-38.
37. Harborne JB. 1998. *Phytochemical Methods A Guide To Modern Techniques of Plant Analysis*. Third Edition. Chapman Hall. pp 58.
38. Arnon DI. 1949. Copper enzymes isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* 24: 1-15.
39. Chakraborty U, Chakraborty BN, Kapoor M. 1993. Changes in the levels of peroxidase and phenylalanine ammonia-lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia Microbiol*. (Praha). 38: 491-496.
40. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Jr. Biol. Chem*. 193: 265-275.
41. Plummer DT. 1978. *An Introduction to Practical Biochemistry*. Tata McGraw Hill Publications. New Delhi.
42. Rengel Z, Graham RD. 1996. Uptake of zinc from chelate-buffered nutrient solutions by wheat genotypes differing in zinc efficiency. *Jr. Exp. Bot*. 47: 217-226.
43. Sadeghzadeh B, Rengel Z. 2011. Zinc in soils and crop nutrition. *Mol. Physiology Basis Nutr Use Effic Crop*. pp 335-375.
44. Dinesha R, Srinivasana V, Hamza S, Sarathambal C, Ankegowda SJ, Ganeshamurthy AN, Divya VC. 2018. Isolation and characterization of potential Zn solubilizing bacteria from soil and its effects on soil Zn release rates, soil available Zn and plant Zn content. *Geoderma* 321: 173-186.
45. Ramesh A, Sharma SK, Sharma MP, Yadav N, Joshi OP. 2014 Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. *Applied Soil Ecology* 73: 87-96.
46. Kloepper JW, Ryum M, Zhang S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94: 1259-1266.
47. Patle P, Phule Krishi Vidyapeeth M, Navnage IN, Krishi Vishwavidyalaya G, Ramteke IP, Navnage N, Ramteke P. 2018. Endophytes in plant system: Roles in growth promotion, mechanism and their potentiality in achieving agriculture sustainability. *Int. Jr. Chem. Studies* 6: 270-274.
48. Davies PJ. 2010. The plant hormones: their nature, occurrence, and functions. Plant Hormones. Netherlands: Springer. pp 1-15.
49. Ma Y, Rajkumar M, Freitas H. 2009. Isolation and characterization of Ni mobilizing PGPB from serpentine soils and their potential in promoting plant growth and Ni accumulation by *Brassica* spp. *Chemosphere* 75: 719-725.
50. Dubey RC, Khare S, Kumar P, Maheshwari DK. 2014. Combined effects of chemical fertilizers and rhizosphere-competent *Bacillus subtilis* BSK17 on yield of *Cicer arietinum*. *Arch Phytopathol. Plant Protect* 47: 2305-2318.
51. Maleki M, Mokhtarnejad L, Mostafaee S. 2011. Screening of rhizobacteria for biological control of cucumber root and crown rot caused by *Phytophthora drechsleri*. *The Plant Pathology Journal* 27(1): 78-84.
52. Stafford WHL, Baker GC, Brown SA, Burton SG, Cowan DA. 2005. Bacterial diversity in the rhizosphere of Proteaceae species. *Environ Microbiology* 7: 1755-1768.