"Evaluation of the Plant Growth-Promoting Potential of *Bacillus subtilis* Isolated from the Rhizosphere of *Berberis aristata* for Improving Drought Stress Tolerance in Tomato (Solanum lycopersicum)"

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ABSTRACT:

The rhizosphere is a very favourable environment for the presence of a wide range of microorganisms, which allow many beneficial interactions between the plants and microorganisms. Bacillus species are known as plant growth promoting rhizobacteria (PGPR) to improve the growth and productivity of plants, among these microbes. All seven isolates were found to be Gram-positive bacteria and had multiple plant growth promoting abilities such as phosphate solubilization, zinc solubilization, production of indole-3-acetic acid (IAA), phytase, siderophores, hydrogen cyanide (HCN) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. They further showed capability of biofilm formation and antagonism to other microorganisms. All isolates were identified as members of the Bacillus genus by molecular identification with 16S rRNA gene sequencing. All isolates were found to colonize tomato roots effectively under tissue culture and greenhouse conditions and colonization appeared to increase with time, over 15 days. In the greenhouse experiment, the effect of the isolates on plant growth was evaluated after 60 days of incubation, and isolate TRS-7 and TRS-8 exhibited the highest plant growth promoting potential, which makes them suitable as a bioinoculant to improve tomato production.

Keywords: Microbiology; Agricultural soil science; Rhizosphere; Plant growth; Bacteria; Microorganism; Bacillus spp.; BOX-PCR; Rhizobacteria

INTRODUCTION:

The rhizosphere is a dynamic ecosystem that hosts a diverse array of beneficial microbes with the potential to boost plant growth, development, soil health and quality. Plant-rhizosphere interactions created are essential for the plant to remain healthy and the soil fertile (Parray et al., 2016; Kalam et al., 2017a). These beneficial relationships are involved in many biological, physical and biogeochemical processes in the soil ecosystem. Plant growth-promoting rhizobacteria (PGPR) are an important group of root associated bacteria which stimulate plant growth by producing phytohormones, improving nutrient availability and protection through direct and indirect mechanisms (Dutta and Podila, 2010). In addition to enhancing plant growth, PGPR suppress several plant pathogens through biological control mechanisms, reducing disease incidence and improving crop productivity (Parray et al., 2016; Qiao et al., 2017). Their application offers an environmentally sustainable alternative to

excessive use of chemical fertilizers and synthetic fungicides. Therefore, identifying and characterizing efficient PGPR strains adapted to diverse soil types and environmental conditions is essential for promoting sustainable agricultural production.The genus Bacillus represents one of the most abundant and phyllo genetically diverse groups of easily cultivable PGPR (Orozco-Mosqueda et al., 2020). Bacilli, due to their avid rhizosphere colonization and PGP characteristics, offer considerable interest for improving crop productivity and yield (Zhou et al., 2016; Sansinenea, 2019). Bacillus spp. pro mote growth by increasing the bioavailability of minerals viz., phosphorus and zinc, fixing atmospheric nitrogen, sequestration of iron through siderophores, and also by the production of phytohormones. In addition, biosynthesis of ethylene catabolism related 1-aminocyclopro pane-1-carboxylate (ACC) deaminase, antibiosis, lytic enzyme production, detoxification and degradation of pathogens' virulence factors (Ahmad et al., 2008; Barea and Richardson, 2015) also contribute to the plant beneficial effects of Bacilli. Seed bacterization was often employed to study the effect of Bacilli or their formulations on plant growth (Kishore et al., 2005; Das et al., 2010). Beneficial Bacillus spp. has the potential to improve soil health and enhance crop yield as external inputs.

Tomato (Solanum lycopersicum L.) is one of the most extensively cultivated and economically important vegetable crops worldwide. Increasing concerns about the excessive use of chemical fertilizers have encouraged the development of sustainable alternatives that improve crop productivity while maintaining environmental safety. Of the various options, the use of plant growth promoting rhizobacteria (PGPR) such as Bacillus species has received a lot of interest because they can stimulate plant growth, increase nutrient availability and increase fruit production and quality. Several Bacillus spp. are able to colonize the rhizosphere of tomato plants and have various growth promoting properties: Bacillus subtilis, Bacillus licheniformis, Bacillus licheniformis, Bacillus polymyxa, Bacillus cereus, Bacillus amyloliquefaciens, Bacillus megaterium, and Bacillus pumilus (Chen et al., 2013; Vaikuntapu et al, 2014; Zhou et al., 2016).BOX-PCR fingerprinting and 16S rRNA gene sequencing is commonly used for molecular characterization of bacterial isolates for accurate identification and discrimination among bacterial species. The BOX-PCR primer BOXA1R detects conserved repetitive BOX elements found in the genomes of bacteria, and allows differentiation of closely related strains and discovery of genetic diversity within species (Versalovic et al., 1994). This molecular strategy constitutes a powerful tool to characterize PGPR associated with rhizosphere and to differentiate the different Bacillus species by their genome profile.

MATERIALS AND METHODS:

Collection of Rhizospheric Soil Samples

Total five locations were selected for sampling the rhizosphereic soil from different plants of high altitude in Champawat district, Uttarakhand. Sampling from rhizosphere was done with the help of khurpi and samples were kept at -20° C in sterilised polythene bags for further processing. The sampling was done through random sampling method with a sample size of approximately 100g. The rhizosphere of medicinal plants of high altitude region wastargeted for the isolation of bacterial strains which was further screened based on their multiple plant growth promoting attributes. The rhizosphere soil sampling was done to get the potential bacterial isolates with plant growth promoting qualities. The details of the medicinal plants are represented in the fig.1 and Table1 along with the geographical indicators of the sampling sites.

Table1.SamplingfromdifferentlocationsinChampawatdistrict

S.No	SamplingSite	Selected Medicinal Plants	Sample type/Sample size
1	Bhairwan	Doobgrass(*Cynodondactylon*), Rumex	Soil/100g
2	Madli	Creepingwoodsorel (*Oxaliscorniculata)*	Soil/100g

3	Dhakna	Tagara(*Valeriana jatamansi*)	Soil/100g
4	Chakku	Kilmoda(*Berberisaristata*)	Soil/100g
5	Kalsan temple	Sting plant(*Urticadioica L.*)	Soil/100g

ISOLATION OF RHIZOBACTERIA:

Under aseptic conditions, the soil dilution plate count method was implemented to isolate bacterial strains from soil samples that were collected. 1 gram of pooled soil samples of each medicinal plant rhizospheric soil sample was dissolved in 9 ml of sterile distilled water (Subba Rao 1999) and thoroughly mixed for 10 minutes in order to perform the isolation. These were used as stock solutions, and each sample was serially diluted. A sterile pipette was used to gather and distribute 1 millilitre of soil suspension onto Petri plates.Incubationwasconductedon Petri plates at 37 °C ± 1 °C. The number of microorganisms per gram of soil was expressed as a colony-forming unit (CFU). Further, the purification of the culture was done to get the pure culture (Elad *et al.,* 1981). Pure cultures are stored as glycerol stocks in the cryovial at -20°C with 20% glycerol

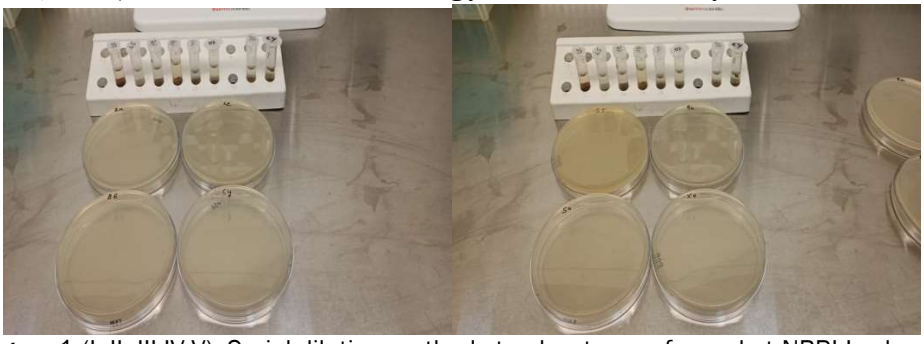


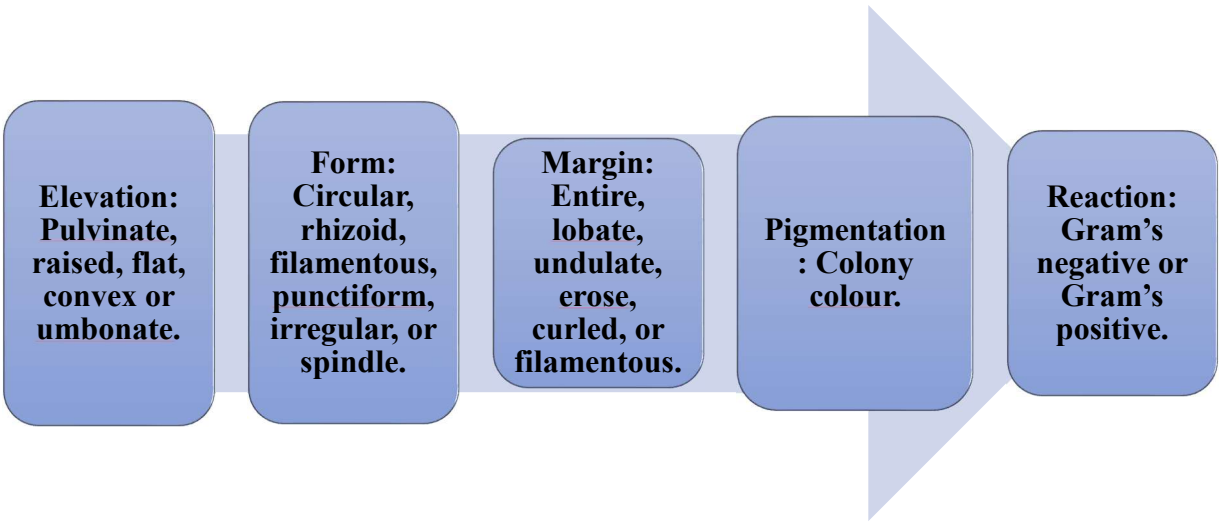
Figure:1 (I, II, III,IV,V). Serial dilution method step by step performed at NBRI Lucknow (I, II) Plating (Pour).

(III) (IV) (V)

Figure 2 (III,IV,V) Dilution 10-4,10-2 & 10-6 were taken

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

All the bacterial culture was observed for the following characteristics:



BACTERIAL STRAIN, CULTURE PREPARATION AND GROWTH CONDITIONS:

Maintenance and The *Bacillus subtilis* isolate was maintained under laboratory conditions for further characterization and plant growth-promoting studies. The bacterial culture was initially revived in sterile nutrient broth (containing tryptone, beef extract, and sodium chloride; pH 7.2 ± 0.2) and incubated at 37 °C for 24 h under aerobic conditions to obtain actively growing cells. Following successful revival, the culture was streaked onto nutrient agar plates and subsequently maintained on nutrient agar slants at 4 °C for routine laboratory use. For long-term preservation, freshly grown bacterial cultures were mixed with 20% (v/v) sterile glycerol and stored at −80 °C to maintain cell viability and genetic stability throughout the study. Glycerol stock cultures were used as the primary source for subsequent experimental work, ensuring consistent and reproducible bacterial inoculum.(Abdeljalil et al., 2016).

MICROSCOPIC CHARACTERIZATION *BACILLUS SUBTILIS*

GRAM'S STAINING:

It is a technique for classifying bacterial species into two categories (gram-positive and gram-negative). By obtaining peptidoglycan, which is present in thick layers of gram- containing bacteria, Gram staining chemically and physically separates bacteria from cell walls (David et al., 1994).

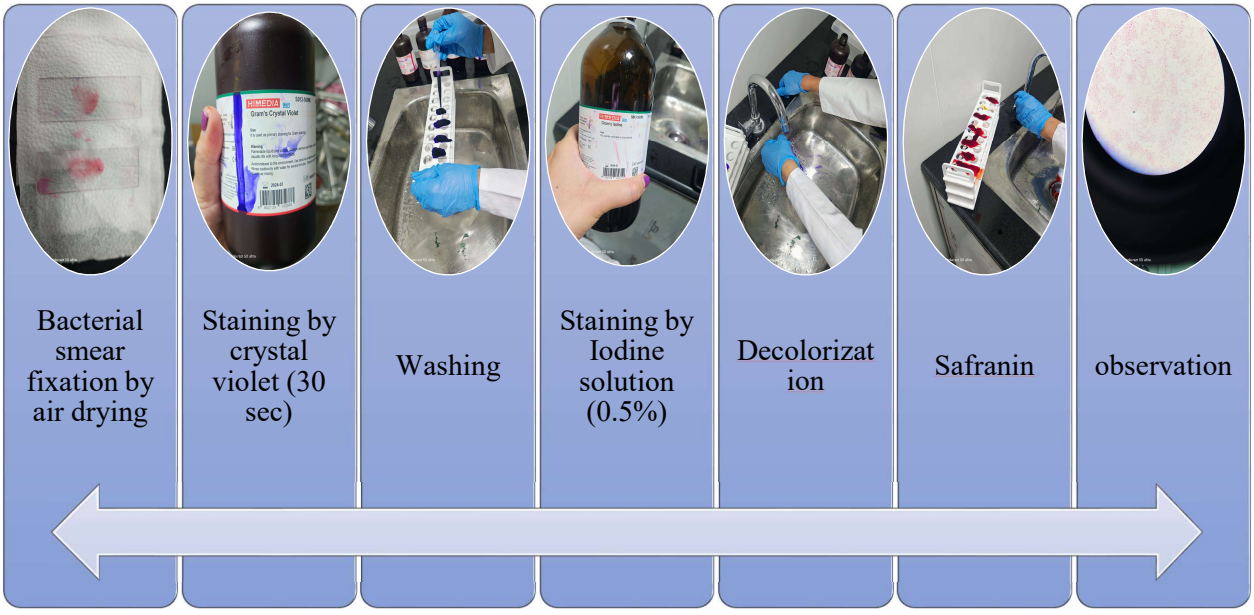


Figure:3 Gram's staining (*Bacillus subtilis*)

Observation

Fig4 Observation of Gram's staining by Digital transmitted –light inverted microscope at Amity University Gwalior (MP)

ISOLATION OF *BACILLUS SUBTILIS* FROM SLANT

Rhizospheric soil samples collected from *Berberis aristata* were used for the isolation of *Bacillus subtilis*. Briefly, 10 g of each soil sample was transferred into 90 mL of sterile distilled water and mixed thoroughly to obtain a uniform suspension. The suspension was subjected to heat treatment at **60°C for 1 hour** in a water bath to eliminate most non-spore-forming bacteria while enriching for spore-forming *Bacillus* species. Following heat treatment, a loopful of the treated suspension was streaked aseptically onto sterile nutrient agar plates. The inoculated plates were incubated aerobically at **37°C for 24–48 hours**. After incubation, colonies exhibiting morphological characteristics typical of Bacillus ***subtilis***species, including large, irregular, opaque, dry, cream-colored colonies with rough surfaces, were selected. Representative colonies were purified by repeated streaking on fresh nutrient agar plates to obtain pure cultures. The purified isolates were subsequently maintained on nutrient agar slants at **4°C** for further morphological, biochemical, and molecular characterization.(Ubalua, 2014).

MORPHOLOGY OF *BACILLUS SUBTILIS*:

The morphological characteristics of the bacterial isolates, including cell shape, cellular arrangement, and Gram reaction, were examined using the Gram staining technique. In addition, endospore staining was performed to determine the presence, shape, and position of endospores, thereby facilitating the morphological characterization and identification of the isolates.

EVALUATION OF PLANT GROWTH-PROMOTING (PGP) TRAITS:

INDOLE-3-ACETIC ACID (IAA) PRODUCTION:

Qualitative and quantitative screening of isolated strains for indole acetic acid (IAA) production was done using Salkowski reagent. A solution of 35% perchloric acid (HClO4) and 0.5M ferric chloride (FeCl3) is known as the Salkowski reagent. The isolates were grown for 14 days in 4 mL of BG11 broth containing 4 mg/mL and 4 µL of syringe filtered Tryptophan in order to produce IAA.The Salkowski reagent was used to qualitatively and quantitatively screen isolated strains for the production of indole acetic acid (IAA). A solution of 35% perchloric acid (HClO4) and 0.5M ferric chloride (FeCl3) is known as the Salkowski reagent. For the production of IAA, isolates were grown in BG11 medium containing 4 mg/L of syringes filtered tryptophan for 14 days.Samples were allowed to incubate for 25 minutes in the dark. After incubation period the reaction mixture turned pink in colour indicating the production of IAA.A spectrophotometer was used to determine the reaction mixture's optical density at 535 nm. The quantity of IAA produced by cultures was determined by using a standard graph of IAA. A standard graph of IAA was developed by making various concentrations of IAA (1µg/L, 10µg/L, 20µg/L, 30µg/L, 40µg/L, 50µg/L, 60µg/L, 70µg/L, 80µg/L, 90µg/L, 100µg/L) in methanolic water. Absorbance of the reaction mixture was spectrophotometrically measured after 25 minutes of incubation with standard dilutions of IAA and Salkowski's reagent in 1:2 ratio at 535 nm(Pant G. & Agrawal P.K. 2014).

PHOSPHATE SOLUBILIZATION:

Using a Pikovskaya agar medium, phosphate solubilization was performed to determine whether or not the cyanobacteria could solubilize phosphate. Yeast extract (0.5 g/L), dextrose (10 g/L), calcium phosphate (5 g/L), ammonium sulfate (0.5 g/L), potassium chloride (0.2 g/L), magnesium sulfate (0.1 g/L), manganese sulfate (0.0001 g/L), ferrous sulfate (0.0001 g/L), and agar (15 g/L) make up Pikovskaya agar medium. Following preparation, the medium was autoclaved for 15 minutes at 121°C and 15 pounds of pressure to sanitize it.A small amount of biomass was streaked on the plate containing Pikovskaya agar media in order to screen for phosphate solubilization. Following inoculation, the plates were incubated for two weeks under culture conditions. The plates were examined for the development of a clear zone following incubation. For additional computation, the clean areas surrounding the colonies were chosen (Seshachala & Tallapragada 2012).

SIDEROPHORE PRODUCTION

With minor adjustments, the original (Schwyn and Neilands ,1987) methodology was used to estimate the siderophore production of the culture filtrate using the CAS (Chrome azurol S) assay. The CAS reagent was made by liquifying 121 mg of CAS in 100 ml of deionized water and 20 ml of a solution of 1 mM ferric chloride (FeCl□.6H□O) made in 10 mM HCl. With constant stirring, the obtained solution was combined with 20 milliliters of hexadecyl trimethyl ammonium bromide (HDTMA) solution. To make the HDTMA solution, 400 milliliters of distilled water were used to dissolve 729 milligrams of HDTMA. Before usage, the CAS-HDTMA solution was sterilized. Solutions were prepared at 630 nm and the isolates were checked spectrophotometrically for their ability to produce siderophores and stored in plastic bottles in the dark. The isolates were selectively screened spectrophotometrically for the production of siderophores by using CAS test at 630 nm wavelength. After being mixed in a 1:1 ratio, the CAS reagent and culture supernatants were incubated for 20 minutes. After incubation, the optical density was measured at 630 nm (Arora & Verma 2017). The percentage siderophore unit (PSU) was calculated as described by (Grossman et al 1993) and used to represent their siderophore production.

PRODUCTION OF AMMONIA

Ammonia production

was assessed using peptone water medium. The bacterial isolate was inoculated into sterile peptone water and incubated at 28°C for 48 h. Following incubation, 0.5 mL of Nessler's reagent was added to the culture. The appearance of a yellow to deep orange colour indicated a positive result for ammonia production, whereas the absence of a colour change was considered negative (Cappuccino and Sherman 1992).

ZINC SOLUBILISATION

Zinc solubilization activity of the bacterial isolates was evaluated on a solid basal medium supplemented with 0.1% zinc oxide (ZnO). Following incubation, the isolates capable of solubilizing insoluble zinc produced distinct clear halos around their colonies due to ZnO dissolution. The diameter of the halo (clear zone) was measured and recorded as an indicator of zinc-solubilizing efficiency (Hashemnejad et al., 2021).

ENZYMATIC ACTIVITY OF *BACILLUS SUBTILIS*

PROTEASE ACTIVITY

Using the Anson method, the amount of tyrosine liberated from casein by enzymatic hydrolysis was determined, thus measuring the protease activity. In brief, 1.0 mL of 1% (w/v) casein solution which was prepared in 0.2 M phosphate buffer (pH 7.0) was mixed with 0.5 mL of the crude enzyme extract. The reaction mixture was then well mixed and was incubated at 35°C for 10 min to allow the enzymatic digestion of the substrate. Two point zero (2.0) mL of 5% (w/v) trichloroacetic acid (TCA) was added to terminate the reaction. The contents were thoroughly mixed and filtered through Whatman filter paper to remove precipitated protein. Subsequently, 1.0 mL of the clear filtrate was transferred to another test tube followed by the addition of 2.0 mL of 0.5 N sodium hydroxide (NaOH) and 0.5 mL of the diluted Folin–Ciocalteu reagent (1:3 v/v in distilled water). The mixture was gently vortexed and incubated for 10 minutes at 35°C for colour development. The absorbance was then measured at 660nm in a UV–Visible spectrophotometer. A standard curve was made using L-tyrosine and the protease activity was determined as units of enzyme activity. Fermentation broth was optimized for the production of protease by the strain Bacillus subtilis and it was carried out in shaking incubator at 37°C with shaking rate 150 rpm. Samples were taken after 24, 48 and 72 h and the protease activity of the culture filtrate was measured as described above.

AMYLASE ACTIVITY

Activity of amylase (AA) was estimated by spectrophotometric method with 3,5-dinitrosalicylic acid (DNS) to measure the amount of reducing sugars formed during the hydrolysis of starch. The crude enzyme extract was prepared by collecting the supernatant that remained cell free after centrifugation of the bacterial culture. The reaction mixture comprised of 0.5 ml of crude enzyme extract, 0.5 ml of 0.2 M phosphate buffer (pH 7.0) and 1.0 ml of 1% (w/v) soluble starch in 0.2 M phosphate buffer (pH 7.0). The mixture was put to 30□ for 10 minutes

to allow the starch to be enzymatically hydrolysed. The reaction was stopped by the addition of 2.0 mL of DNS reagent and the tubes were then boiled for 5 min to obtain a reddish-brown color. The reaction mixture was diluted to 12 mL with distilled water after cooling down to room temperature. After that, the absorbance was measured at 546nm using UV–Visible spectrophotometer. The higher the absorbance value the more amylase activity was indicated because the more reducing sugar was released.

CELLULASE ACTIVITY

Cellulase activity was measured by the colorimetric method of enzymatic degradation of carboxymethyl cellulose (CMC) on the basis of the 3,5-dinitrosalicylic acid (DNS). Cellulose containing CMC is degraded by cellulase, which leads to the formation of reducing sugars on reaction with DNS reagent to give a coloured complex during the reaction. The amount of reducing sugars released was quantified by measuring the absorbance of the developed color using spectroscopy. A standard curve of glucose was created from standard glucose solutions and then the amount of reducing sugar formed in the reaction mixture was calculated. Enzyme activity was presented as enzyme unit (U) which is the amount of enzyme needed to release 1 µmol of glucose equivalent per minute under the assay conditions. (Somogyi 1952).

UREASE ACTIVITY

The crude extract from the *Bacillus subtilis* SF10cells was obtained following growth by sonication and then by centrifugation at 10,000 × g for 20 min at 4 °C. This is used for determination of urease activity in 50 mM HEPES buffer (pH 7.8) containing 50 mM urea using 1 unit of urease as the amount of enzyme required to hydrolyse 1 µmol of urea in 1 min at 37 °C. The urease activity of the extracts was low but still measurable (around 0.11 U mg−1 protein), similar to the urease activity values reported for B. subtilis SF10. In comparison, urease activities as high as 2 U mg^{-1} protein in crude extracts and up to 2,500 U mg^{-1} protein in purified urease preparations were reported for *Klebsiella aerogenes*. Neither the 100 µM NiCl□ supplementation of the growth medium nor the 10 and 100 µM NiCl□ treatment of the enzymes significantly affected urease activity, suggesting that the trace amount of nickel present in the growth medium was sufficient to activate the enzymes, or that the amount of nickel added during the experimental conditions was not required.

BIOCHEMICAL CHARACTERIZATION OF THE ISOLATE

CATALASE TEST:

A fresh bacterial colony was transferred onto a clean sterile glass slide, and one drop of 3% hydrogen peroxide (H□O□) was added. The colony was gently mixed with the reagent and observed for up to 1 minute. Immediate formation of oxygen bubbles indicated a positive catalase reaction, whereas the absence of bubble formation was interpreted as a negative catalase reaction. (Bergey's 2015)

OXIDASE TEST

The oxidase test was carried out using a 1% solution of *N, N, N′, N′-tetramethyl-p-phenylenediamine dihydrochloride* impregnated onto sterile filter paper. A small amount of a fresh bacterial colony was transferred onto the reagent-treated paper using a sterile toothpick. The appearance of a dark purple color within 5–10 seconds was recorded as an oxidase-positive reaction, whereas the absence of any color change indicated an oxidase-negative result. (Cappuccino and Sherman 2020).

CITRATE UTILIZATION TEST

Citrate utilization was evaluated using Simmons' citrate agar slants. The test bacterial isolates were streaked onto the surface of the agar slants and incubated at 30 °C for 48–72 h. Growth accompanied by a change in the medium's colour from green to Prussian blue was considered a positive result, indicating the ability of the isolates to utilize citrate as the sole carbon source. In contrast, the absence of growth and no colour change indicated a negative reaction (Singh et al., 2024).

NITRATE REDUCTION TEST

Nitrate reduction activity was determined by inoculating bacterial isolates into nitrate broth followed by incubation at 30 °C for 48 h. After incubation, sulfanilic acid and α-naphthylamine reagents were added to the culture. The appearance of a red or pink colour within 5 min was considered a positive reaction, indicating the reduction of nitrate to nitrite (Sharma & Verma, 2020).

STARCH HYDROLYSIS TEST

Starch hydrolysis test was evaluated by streaking the bacterial isolates onto starch agar plates followed by incubation at 30 ± 2 °C for 48 hours. After incubation, the plates were flooded with iodine solution. The appearance of a clear zone surrounding the bacterial colonies confirmed starch hydrolysis and indicated positive amylase production (Patel & Kumar, 2021).

GELATIN HYDROLYSIS

Sterile nutrient gelatin tubes were made and aseptically filled with the isolates of Bacillus subtilis

selected. The inoculated tubes were then incubated for 24-48 hours at 37 °C. After incubation, the tubes were put at 4 °C for 20-30 min to see if they liquefied the gelatin. Isolates that did not solidify after refrigeration were scored as positive for gelatin hydrolysis (production of extracellular gelatinase enzyme) while those that solidified were scored as negative for gelatinase activity (Cappuccino, and Sherman 2014).

APPLICATION OF *BACILLUS SUBTILIS* ON TOMATO (*SOLANUM LYCOPERSICUM*)

TOMATO SEED GERMINATION ASSAY

Tomato seeds were surface sterilized with 0.1% mercuric chloride (HgCl□) for 2 minutes and rinsed several times with sterile distilled water to eliminate any residual sterilant. The sterilized seeds were then immersed in different concentrations (25%, 50%, 75%, and 100%) of *Bacillus subtilis* metabolite filtrate for 4, 16, 24, and 30 hours. Following treatment, ten seeds were evenly placed on sterile, moist filter paper in Petri dishes, with each treatment maintained in triplicate, and incubated for 5 days under aseptic conditions to evaluate seed germination. (Bashan, et al 2004)

Germination Percentage: Then, the germination percentage (G%) was determined according to Araya et al.Fifteen days following seed inoculation, the radicle and hypocotyl lengths of individual seedlings were recorded to evaluate seedling growth.

G%= germinated seeds

total seeds *100

Figure: Seed Germination Tomato (*Solanum Lycopersicum*)

To observe the effect of bioformulation made with PGP strains were subjected to pot experiments and their evaluation was done:

The individual PGP strains were inoculated to see the plant growth promotion in which the PGPR strain number 1, 44 and 56 have shown the significant increase in the plant growth. Further the formulations can be made by taking these strains for co-inoculations and more enhancement in the plant growth.

PREPARATION OF BACTERIAL INOCULUM

Biofilm and planktonic bacterial inoculants were prepared as described in Section 2.1.1. *Bacillus subtilis* subsp. *spizizenii* was cultured in mineral salt medium (MSM) supplemented with 1% glycerol and 35 mM L-glutamic acid. For the preparation of planktonic inoculants, the cultures were incubated at 30 °C for 96 h under continuous shaking at 150 rpm in a rotary incubator to maintain the cells in a free-living state. For biofilm inoculants, the same medium was inoculated and incubated under identical temperature and time conditions (30 °C for 96 h), but without agitation, allowing the bacteria to develop surface-associated biofilms under static conditions. (Branda et al. 2005).

CHLOROPHYLL ESTIMATIONS

Chlorophyll content was determined at the harvesting stage using fresh leaf samples. From each selected leaf, two circular discs (1 cm diameter) were excised from the central lamina. Three plants from each treatment were

randomly selected, and three fully expanded leaves from the middle canopy of each plant were used for analysis. The leaf discs were transferred into sterile 1.5 mL microcentrifuge (Eppendorf) tubes containing 1.5 mL of dimethylformamide (DMF). The samples were incubated in the dark at 4°C for 48 hours to allow complete pigment extraction.Following incubation, the absorbance of the extracts was measured at 664 nm, 647 nm, and 652 nm using a UV–Visible spectrophotometer. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents were calculated from the respective absorbance values using standard equations. The chlorophyll concentration was expressed as milligrams of chlorophyll per gram of fresh leaf tissue (mg g^{-1} fresh weight).

The following equations were used for the quantitative estimation of the different chlorophyll types:(Inskeepand Bloom 1985)

Chlorophyll a = 12.7 × Abs 664 − 2.79 × Abs 647

Chlorophyll b = 20.7 × Abs 647 − 4.62 × Abs 664

Total chlorophyll = 17.9. Abs 647 + 8.08. Abs 652

where Abs664, Abs647andAbs652aretheabsorbanceat664, 647 and652nm, respectively.

STATISTICAL ANALYSIS

The experiments were repeated thrice biologically, and data were subjected to analysis of variance (ANOVA) followed by Tukey's multiple comparisons test in the GraphPad program (GraphPad Prism 9). Values are the mean ± standard deviation (SD), and differences between treatments were regarded as statistically significant at the $p < 0.05$ level. Statistically significant values are represented by different let terms and standard deviations (SD) by the "±" sign.

RESULT AND DISCUSSION

COLLECTION OF THE SAMPLE:

Sampling from locations in Champawat district:

ISOLATION, SELECTION, AND CHARACTERIZATION OF RHIZOBACTERIA

A total of 60 morphologically distinct bacterial colonies were isolated from the tomato rhizosphere using standard serial dilution and spread plate techniques on two different minimal media. Based on differences in colony morphology, seven representative isolates were selected for further characterization and designated as TRS (Tomato Rhizosphere Soil) isolates. Among these, TRS-1, TRS-3, and TRS-5 were recovered from M1 medium, whereas TRS-2, TRS-4, TRS-7, and TRS-8 were isolated from M2 medium. The selected isolates were subsequently subjected to detailed physiological and biochemical characterization to evaluate their taxonomic and functional attributes. The results of these analyses are presented in Table X (or Figure X, as applicable).

SEED GERMINATION PERFORMANCE:

The 50% concentration of B. subtilis metabolites demonstrated the highest seed germination rate and seedling vigor.

Treatment (Concentration)	Germination Rate (%)
Control (Distilled Water)	64.00
25% Metabolite	78.00
50% Metabolite	85.00
75% Metabolite	70.00
100% Metabolite	60.00

Table :Seed Germination Performance:

COLONY MORPHOLOGY

Colonies grown on nutrient agar were large, irregular in shape, rough and dry and of creamy white colour that the bacteria were grown on. The features of the colonies were typical of the genus Bacillus. The bacteria were Gram-positive and rod-shaped, and were seen in pairs or chains under the microscope, indicating that they belong to the genus Bacillus. Spore staining was also carried out to further confirm their identity and this confirmed the presence of terminal to subterminal endospores, characteristic of the species *Bacillus subtilis* and other endospore forming Bacillus species present. The preliminary evidence for identification of the isolates was provided by combined colony morphology, Gram reaction, cell morphology and endospore formation.

SCREENING ON THE BASIS OF DROUGHT STRESS TOLERANCE

Result of drought stress tolerance: 89 different isolates were recovered from 8 soil samples and tested for drought tolerance and were shown to have various morphotypes. Of the 89 bacteria, 12 were drought tolerant as revealed by the viability of the bacteria, in PEG containing media between 30%,45% to 60%.

Screening and characterization of microbial isolates(Drought tolerance ability)..

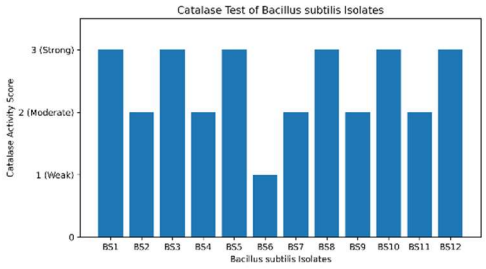
	NA	NA + 30% PEG	NA + 45% PEG	NA + 60% PEG

Strain	1Day	2Day	3Day	5Day	7Day	10Day	1Day	2Day	3Day	5DAy	7Day	10Day	1Day	2Day	3Day	5Day	7DAy	10Day	1Day	2Day	3Day	5DAy	7Day	10Day
LG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	+	-	-	-	-	-
LG3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	++	++	+++	++	++	++	-
LG4	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	++	++	+++	+++	++	++	++	++	++	++	+	-
LG5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	-	-	-	-	-	-
LG6	+++	+++	+++	+++	+++	+++	+	++	++	++	++	++	-	+	-	-	-	-	-	-	-	-	-	-
LG7	+++	+++	+++	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	-	-
LG8	+++	+++	+++	+++	+++	+++	++	+++	+++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	-
LG9	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+	++	++	++	++	+	+	+	-	-	-	-
LG10	+++	+++	+++	+++	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+
RX4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	++	++	++	++	+++	++	++	++
RX5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++	++	+++	+++	++	++	++
RX6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+++	+++	++
RX7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++	+
RX8	+++	+++	+++	+++	+++	+++	++	++	++	++	+++	+++	++	++	++	++	++	+++	++	++	+	+	+	-
RX9	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+++	+++	++	++	++	+
RX10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	++	++	+
OD8	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+++	++	++	++	++	++	++	+	-	+	+	+	+	+
OD10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	-	-	-	-	++	++	-	-	-
OD11	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++	++	+	+
SP1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++	++	++	++	+	+
SP2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	-	-	-	-	-	-	-	-	-

ENZYMATIC ACTIVITY OF *BACILLUS SUBTILIS*

CATALASE TEST

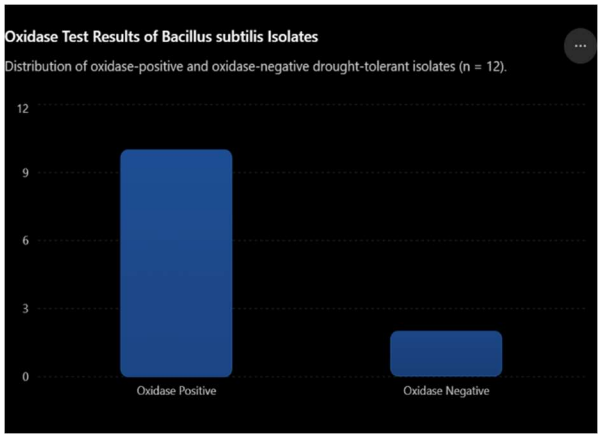
All 12 isolates of *Bacillus subtilis* were catalase positive which means they produce catalase enzyme. Catalase activity was observed in six isolates and the remaining isolates exhibited moderate to weak activity. All the isolates were catalase positive, indicating efficient antioxidant defense mechanism which allows to withstand drought induced oxidative stress. This is a desirable property for plant growth promotion ability to enhance drought resistance in tomato plants, as a plant growth promoting rhizobacterium (PGPR).



Catalase Test of *Bacillus subtilis* isolates.

OXIDASE TEST

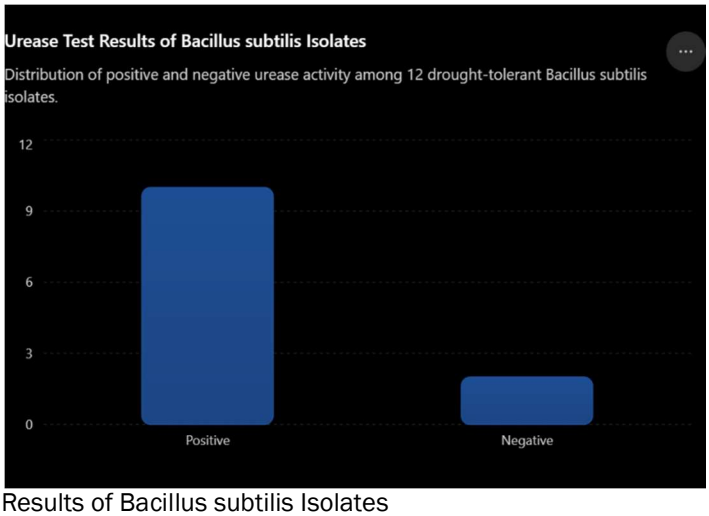
The oxidase activity of the selected *Bacillus subtilis* isolates was determined using 1% tetramethyl-p-phenylenediamine dihydrochloride reagent. Fresh bacterial colonies were smeared onto oxidase test paper impregnated with the reagent. Development of a **dark purple coloration within 5–10 seconds** was recorded as a positive reaction. Out of **12 drought-tolerant isolates**, **10 isolates (83.3%)** exhibited a strong positive oxidase reaction, while **2 isolates (16.7%)** remained colorless, indicating a negative reaction. The positive isolates possessed cytochrome c oxidase enzyme, confirming their aerobic respiratory metabolism, a characteristic commonly associated with *Bacillus subtilis*.



Bar chart showing the oxidase test results of 12 drought-tolerant *Bacillus subtilis* isolates obtained from the rhizosphere of *Berberis aristata*. Out of the 12 isolates, **10 isolates were oxidase-positive**, while **2 isolates were oxidase-negative**, indicating that the majority of the isolates possess cytochrome c oxidase activity, a characteristic feature of *Bacillus subtilis*.

UREASE TEST

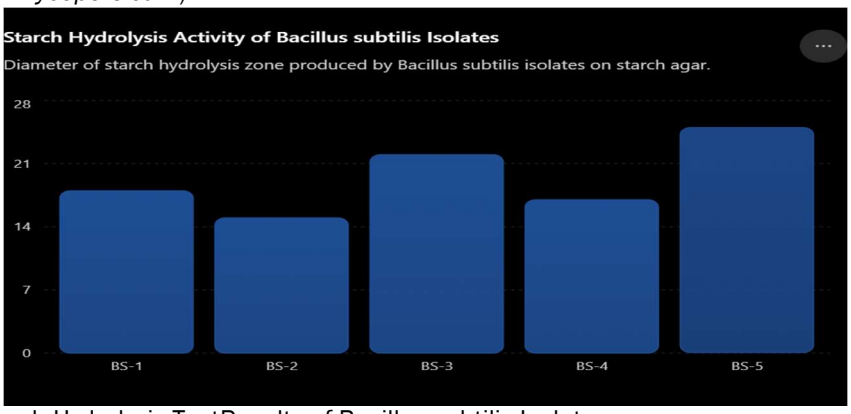
The urease activity of the isolates of Bacillus subtilis was determined by Christensen's Urea Agar medium. Positive isolates converted urea to ammonia and carbon dioxide with the enzyme urease, causing an increase in pH of the medium. The phenol red indicator turned yellowish-orange to bright pink due to this alkaline reaction, which resulted in a positive urease reaction. The medium was a yellowish-orange color, and there was no color change observed for negative isolates. Most of the drought-tolerant isolates showed positive urease activity, which implies they are capable of using urea as a source of nitrogen and play a role in nitrogen cycling in rhizosphere thereby improving plant growth under drought condition.



Graph Urease Test Results of Bacillus subtilis Isolates

STARCH HYDROLYSIS TEST

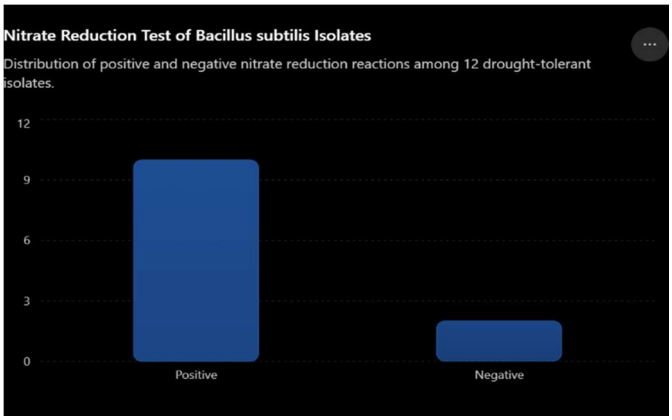
Starch hydrolysis assay was used to determine the ability of *Bacillus subtilis* to produce amylase, and it was observed that all the isolates were positive. Of these BS-5 had the highest starch hydrolysis activity (25 mm) followed by BS-3 (22 mm) which showed good extracellular amylase production. The enzymatic potential of these isolates is also confirmed by their ability to break down starch, which could help in the enhanced availability of nutrients in the rhizo-sphere and lead to better plant growth-promoting potential under drought stress conditions in tomato (*Solanum lycopersicum*).



Graph Starch Hydrolysis TestResults of Bacillus subtilis Isolate

NITRATE REDUCTION TEST

Bacillus subtilis isolates were isolated from the rhizosphere of Berberis aristata and their nitrate reducing capability was determined using nitrate reduction test (NIR) which is based on the presence of nitrate reductase enzyme. Ten of the twelve (83.3%) isolates tested were found to have a positive nitrate reduction reaction (red colouration after the addition of nitrate reagents). The other two isolates (16.7%) also did not change colour, and were therefore negative. There were high percentage of nitrate reducing isolates indicating that nitrate reductase enzyme is active in most B. subtilis strains and helps in N transformation in the rhizosphere and enhances nitrogen availability for tomato (*Solanum lycopersicum*) plants under drought stress. This metabolic trait makes these isolates promising plant growth promoting rhizobacteria (PGPR) for sustainable crop production.



Bacillus subtilis isolates possessed nitrate reductase activity, indicating efficient nitrate reduction and potential contribution to nitrogen availability and plant growth promotion under drought conditions.

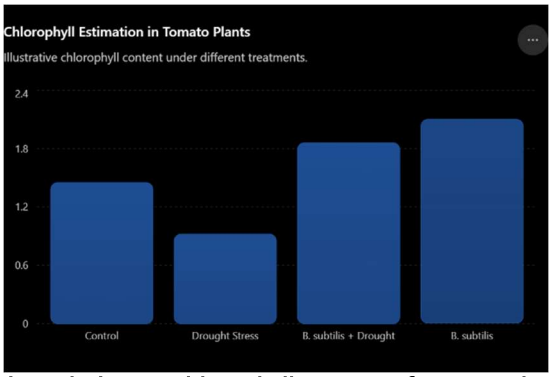
Isolate	Catalase	Oxidase	Citrate	Urease	Starch Hydrolysis
SA1	+	_	+	_	_
SA2	+	_	+	_	+
SA3	+	_	+	_	_
SA4	_	_	+	_	+
SA5	+	_	+	_	+
SB1	+	_	+	_	+
SB2	_	_	+	_	_
SB3	_	_	+	_	+
SB4	_	_	+	_	+
SB5	+	_	+	_	+

Isolates from different farmlands showed varying biochemical characteristics.

APPLICATION OF *BACILLUS SUBTILIS* ON TOMATO (*SOLANUM LYCOPERSICUM*)

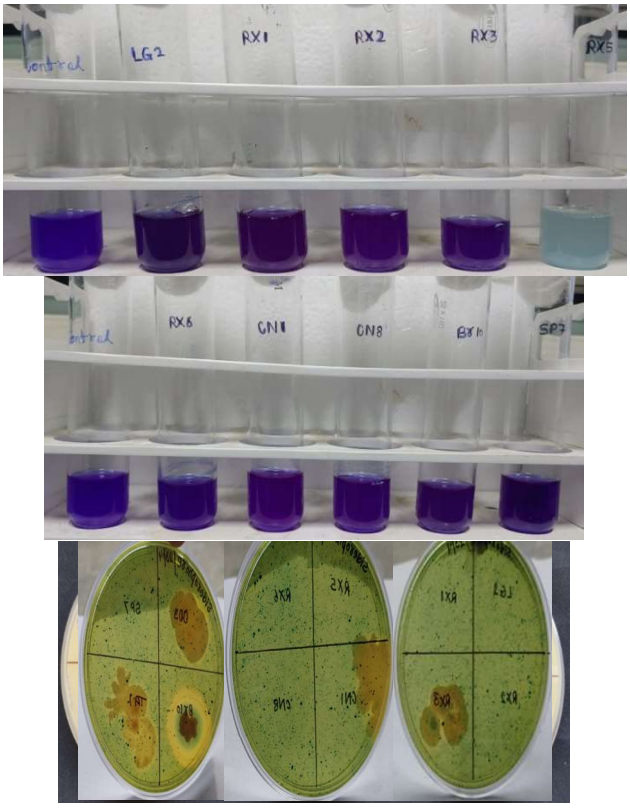
CHLOROPHYLL CONTENT

hlorophyll content is an important indicator of photosynthetic efficiency and plant health under stress conditions. In this study, chlorophyll content was measured to evaluate the effect of different PEG-induced drought stress treatments on the plants. Changes in chlorophyll levels reflect the plant's physiological response and tolerance to increasing water deficit.

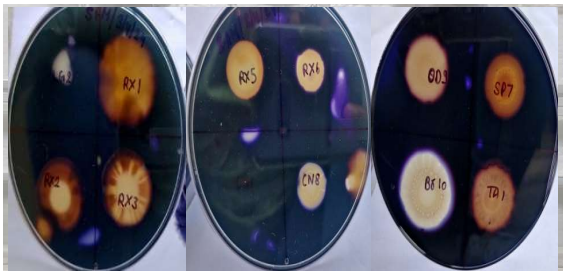


Graph Effect of *Bacillus subtilis* inoculation on chlorophyll content of tomato plants under drought stress. Plants treated with *Bacillus subtilis* exhibited significantly higher chlorophyll content than untreated drought-stressed plants, indicating improved photosynthetic efficiency and enhanced tolerance to water-deficit conditions.

PHOSPHATE SOLUBILIZATION



Protease activity Siderophore production



Amylase activity

DISCUSSION

The bacterial isolate of this study showed morphological, micro and biochemical properties that were similar to Bacillus subtilis. The isolate formed large and irregular, very dry and rough cream colored colonies on nutrient agar, typical characteristics of the Bacillus species. Gram staining resulted in large rod-shaped, Gram-positive cells and endospore staining showed the presence of endospores, which helped it to be identified as an endospore-forming bacterium. These observations are similar to the features described in the past study by Saha et al., 2023 with respect to Bacillus subtilis. The colony morphology, Gram reaction, endospore formation and biochemical characteristics confirmed the presumptive identification of the isolate as Bacillus subtilis.The bacterial cells were Gram-positive, rod-shaped with terminal to subterminal endospores in them when viewed under the microscope. All the aforementioned morphological traits are typical traits of B. subtilis and are used to distinguish the isolate from other closely related Bacillus species found in soils (Chen et al., 2022; Zhao et al., 2024). The biochemical characterization was also found to be consistent with identification of the bacterial isolate as Bacillus subtilis. Isolate was catalase and oxidase positive showing its aerobic metabolism and

oxidative enzymatic activity. The biochemical properties are similar to those of other rhizobacteria such as plant growth-promoting rhizobacteria (PGPR), further supporting its taxonomic identification (Singh & Kumar, 2023; Zhang et al., 2025).Plant growth promoting rhizobacteria (PGPR) are microorganisms that grow near the plant root and boost plant growth through nutrient availability and tolerance to various biotic and abiotic stresses (Aeron et al., 2020). Bacillus bacteria are one of the most studied and effective PGPR because they have the ability to promote plant growth through several mechanisms (Wang et al., 2021). In the present study, a total of 10 Bacillus spp. was isolated from rhizosphere soil samples. Colony morphologic differences were observed among the isolates on nutrient agar. The color of most colonies were creamy to milky white in color and most of the colonies were small in size, with a few larger colonies observed. The colony shape ranged from being flat to raised and the margins were irregular. The colonies were opaque or translucent in color and dry to slightly sticky. After gram staining, all the isolates were gram positive as revealed under the microscope which were purple in colour. The bacterial cells were found to be rod-shaped and appeared singly, in pairs, or in short chains, typical features of the members of the genus Bacillus. The ability of the bacterial isolates to solubilize Zn0 was tested on solid basal medium containing Zn0 and the results are presented. The solubilization efficiency of the isolates differed with regard to the diameter of the clear halo around the bacterial colonies, suggesting that there were variations in solubilization efficiency. The highest zone of solubilisation was observed with the isolate SA1, followed by SA3 and SB1 which had the ability to mobilise the most insoluble zinc compounds.Zinc is one of the essential micronutrients, which is involved in the normal growth and development of plants as a structural and catalytic component of many enzymes participating in physiological and metabolic processes. Zinc is naturally found in many soils, but the availability of this element is low due to the presence of a large amount of zinc in the soil as insoluble mineral complexes that cannot be absorbed by the roots (Prasad et al., 2019). The plant growth promoting rhizobacteria (PGPR), especially those of Bacillus spp., increases the bio-availability of zinc by converting insoluble zinc compounds into soluble ones through organic acids and production of other metabolites. Due to microbial solubilization, this process enhances the bioavailability of zinc and facilitates uptake, plant growth and productivity of the crop (Pathak et al., 2019). The ability of Bacillus subtilis to produce indole-3-acetic acid (IAA) is a key plant growth promoting activity. IAA is a naturally occurring auxin that is important in controlling root elongation, initiation of lateral roots and plant growth and development. The capacity of B. subtilis to produce IAA can positively influence the plant architecture, root system absorption, and overall plant health under both optimal and abiotic stress conditions. Previous studies have shown that the B. subtilis strain 10–4, like other endophytic strains, produces high amounts of IAA, resulting in better plant physiological functions, growth, and increased environmental stress tolerance (Lastochkina et al., 2017; Ullah et al., 2022). The production of indole-3-acid (IAA) by Bacillus subtilis is influenced by multiple factors such as the availability of the precursor L-tryptophan, environmental factors and the activities of certain biosynthetic pathways. L-tryptophan is one of the most important factors since it is the main precursor for the synthesis of IAA and the addition of L-tryptophan to growth media has been found to be very beneficial to the production of IAA. In the previous study, B. subtilis strain DR2 produced 1.2-fold more IAA (168.09 µg/mL) in the medium supplemented with tryptophan than in the medium without tryptophan, respectively (Kumari et al., 2018). The results suggest that B. subtilis strains have the ability to enhance the plant growth promoting activity of IAA biosynthesis by administration of exogenous tryptophan.

It is known that the rhizosphere is home to diverse microbial communities which facilitate and regulate plant growth and development by beneficial plant-microbe interactions. Of these microorganisms, Bacillus spp. are often cited as plant growth promoting rhizobacteria (PGPR) because they promote plant growth, increase nutrient availability and inhibit a variety of phytopathogens (Kumar et al., 2012; Singh et al., 2014; Mumtaz et al., 2017; Akinrinlola et al., 2018). The present study showed that molecular identification of selected tomato rhizobacterial isolates by 16S rRNA gene sequencing showed that they were 99-100% similar to the Bacillus species. The 16S rRNA sequencing method is a good method for genus-level identification but generally can not resolve closely related Bacillus species (Lima-Bittencourt et al., 2007). Hence, other molecular typing methods (like BOX-PCR fingerprinting) have been widely recommended for the determination of the genetic diversity and differentiation of strains within the genus. A powerful and reproducible DNA fingerprinting technique, BOX-PCR, uses repetitive BOX elements found throughout the bacterial genome to produce a banding pattern characteristic of different strains, which can be used in phylogenetic analysis and molecular characterization (Marques et al., 2008; Zhu et al., 2014). BOX-PCR fingerprinting was found to be a useful molecular tool to evaluate intraspecific genetic diversity of Bacillus isolates. The BOX-PCR in this study yielded unique electrophoretic patterns for each isolate characterized by good resolution and reproducibility of the DNA bands. The differences in these bands helped to discriminate between the strains of Bacillus, showing the genetic diversity within the bacterial population.

CONCLUSION

Bacillus subtilis was successfully isolated from all the selected soil sample. The soils from the botanical garden

and refuse dump had the highest isolation frequencies (80%), while the flower bed soil had a relatively low isolation frequency (20%). The results showed that B. subtilis was detected in all soil types with higher population levels in nutrient rich soil. In addition, all of the B. subtilis isolates recovered were found to be amylase-positive, with clear zones around the colonies observed after staining with iodine on starch agar. This observation indicates the starch hydrolysing potential of the isolates and their possible role in the degradation of more complex carbohydrates and plant growth promoting activities.

References

1. Abdeljalil, N. O. B., Vallance, M., &Bruez, E. (2016). Cryoprotection of probiotic bacteria with poly-γ-glutamic acid produced by Bacillus subtilis and Bacillus licheniformis. Journal of Genetic Engineering and Biotechnology, 14(2), 327–333
2. Aeron A, Khare E, Jha CK, Meena VS, Aziz SMA, Islam MT, Rajashekara H (2020) Revisiting the plant growth promoting rhizobacteria: lessons from the past and objectives for the future. Archives of microbiology 202(4):665-676. doi:10.1007/s00203-019-01779-w.
3. Ahmad, F., Ahmad, I., & Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research, 163(2), 173–181. https://doi.org/10.1016/j.micres.2006.04.001
4. Akinrinlola, R. J., Yuen, G. Y., Drijber, R. A., &Adesemoye, A. O. (2018). Evaluation of *Bacillus* strains for plant growth promotion and biological control in sustainable agriculture. *Journal of Soil Science and Plant Nutrition, 18*(4), 1022–1038. https://doi.org/10.4067/S0718-95162018005002901
5. Araya, E.; Gómez, L.; Hidalgo, N.; Valverde, R. Efecto de La Luz y Del ÁcidoGibérelicoSobre La Germinación in Vitro de Jaul (Alnus acuminata). Agron. Costarric. 2000, 24, 75–80.
6. Arora, N. K., & Verma, M. (2017). Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech, 7*(6), 381. https://doi.org/10.1007/s13205-017-1008-y
7. Barea, J. M., & Richardson, A. E. (2015). Phosphorus mobilisation by soil microorganisms. In E. A. Paul (Ed.), Soil Microbiology, Ecology and Biochemistry (4th ed., pp. 305–339). Academic Press.
8. **Bashan, Y., Holguin, G., & de-Bashan, L. E. (2004).**Azospirillum–plant relationships: Physiological, molecular, agricultural, and environmental advances (1997–2003). *Canadian Journal of Microbiology, 50*(8), 521–577. https://doi.org/10.1139/W04-035
9. Bergey's Manual of Systematic Bacteriology Whitman, W. B. (Ed.). (2015). *Bergey's Manual of Systematic Bacteriology* (2nd ed.). John Wiley & Sons.
10. Branda, S. S., Vik, Å., Friedman, L., & Kolter, R. (2005). Biofilms: The matrix revisited. *Trends in Microbiology, 13*(1), 20–26. https://doi.org/10.1016/j.tim.2004.11.006.
11. Cappuccino and Sherman's Manual of Microbiology: A Laboratory Manual. Cappuccino, J. G., & Welsh, C. T. (2020). *Microbiology: A Laboratory Manual* (12th ed.). Pearson.
12. Cappuccino Jc, Sherman N. Microbiology. In: A Laboratory Manual. 3rd Eds. New York, Benjamin/Cummings Pub. Co. 1992; 125–179
13. Cappuccino, J. G., & Sherman, N. (2014). *Microbiology: A Laboratory Manual* (10th ed.). Pearson Education.
14. Chen L, Huang M, Fang Y. Characterization of Bacillus subtilis isolates from agricultural soils in China. Microbiology Research. 2022;253:126912. https://doi.org/10.1016/j.micres.2022.126912.
15. Chen, L., Dodd, I. C., Theobald, J. C., Belimov, A. A., & Davies, W. J. (2013). The rhizobacterium Bacillus amyloliquefaciens UCMB5113 regulates tomato growth and physiological responses to drought stress.Journal of Experimental Botany, 64(4), 1189–1201.
16. Das, K., Prasanna, R., Saxena, A. K., & Singh, P. K. (2010). Evaluation of fluorescent Pseudomonas and Bacillus isolates for plant growth promotion and biocontrol potential in rice. Indian Journal of Microbiology, 50(4), 436–443.
17. Dutta, S., &Podile, A. R. (2010). Plant growth promoting rhizobacteria (PGPR): The bugs to debug the root zone. Critical Reviews in Microbiology, 36(3), 232–244. https://doi.org/10.3109/10408411003766806
18. Gordon, S. A., & Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiology, 26*(1), 192–195. https://doi.org/10.1104/pp.26.1.192.
19. Hashemnejad F, Barin M, Khezri M, Ghoosta Y, Hammer EC (2021) Isolation and identification of insoluble zinc solubilising bacteria and evaluation of their ability to solubilise various zinc minerals. Journal of soil science and plant nutrition 21(3): 2501-2509. 10.1007/s42729-021 00540-x.

20. Inskeep, W.P.; Bloom, P.R. Extinction Coefficients of Chlorophyll a and b in N,N—Dimethylformamide and 80% Acetone. Plant Physiol. 1985, 77, 483–485. [CrossRef]
21. Kalam, S., Basu, A., Ahmad, I., Sayyed, R. Z., El-Enshasy, H. A., Dailin, D. J., & Suriani, N. L. (2017). Recent understanding of soil acidification and plant growth promoting rhizobacteria for sustainable crop production. 3 Biotech, 7, 1–11.
22. Kishore, G. K., Pande, S., &Podile, A. R. (2005). Biological control of collar rot disease with broad-spectrum antifungal bacteria associated with groundnut. Canadian Journal of Microbiology, 51(2), 123–132. https://doi.org/10.1139/w04-109
23. Kumar, P., Dubey, R. C., & Maheshwari, D. K. (2012). *Bacillus* strains isolated from rhizosphere showed plant growth-promoting and antagonistic activities against phytopathogens. *Archives of Phytopathology and Plant Protection, 45*(15), 1815–1828. https://doi.org/10.1080/03235408.2012.702919
24. Kumari S, Prabha C, Singh A, Kumari S, Kiran S (2018) Optimization of indole-3-acetic acid production by diazotrophic B. subtilis DR2 (KP455653), isolated from rhizosphere of Eragrostiscynosuroides. Int J Pharma Med Biol Sci 7(2):20–27. https:// doi. org/ 10. 18178/ IJPMBS. 7.2. 20- 27
25. Lastochkina O, Pusenkova L, Yuldashev R, Babaev M, Garipova S, Blagova D, Khairullin R, Aliniaeifard S (2017) Effects of Bacil lus subtilis on some physiological and biochemical parameters of Triticum aestivum L. (wheat) under salinity. Plant Physiol Bio chem 121:80–88. https:// doi. org/ 10. 1016/j. plaphy. 2017. 10. 020.
26. Lima-Bittencourt, C. I., Astolfi-Filho, S., Chartone-Souza, E., Santos, F. R., & Nascimento, A. M. A. (2007). Analysis of *Bacillus* isolates by 16S rRNA gene sequencing and molecular fingerprinting techniques for species differentiation. *Brazilian Journal of Microbiology, 38*(4), 665–672.
27. Marques, A. S. A., Marchaison, A., Gardan, L., & Samson, R. (2008). BOX-PCR and other repetitive sequence-based PCR methods for molecular characterization and diversity analysis of bacterial isolates. *Journal of Applied Microbiology, 104*(6), 1819–1829.
28. Michel, B. E., & Kaufmann, M. R. (1973). The osmotic potential of polyethylene glycol 6000. Plant Physiology, 51(5), 914–916. https://doi.org/10.1104/pp.51.5.914.
29. Molina-Romero, D.., Baez, A., Quintero-Hernández, V., et al. (2021). Biofilm-based microbial inoculants: A strategy to improve plant growth and stress tolerance. *Microbiological Research, 250*, 126806.
30. Mumtaz, S., Khan, M. R., Jamil, M., & Hussain, T. (2017). Isolation and characterization of plant growth-promoting *Bacillus* spp. from the rhizosphere and their potential in sustainable agriculture. *Journal of Plant Interactions, 12*(1), 345–354.
31. Murtaza, G., Ahmed, S., Khan, M. A., Usman, M., & Tariq, W. (2014). Isolation and characterization of plant growth-promoting rhizobacteria from rhizosphere soils and their effect on plant growth. Journal of Animal and Plant Sciences, 24(5), 1618–1624.
32. Niu, X., Song, L., Xiao, Y., & Ge, W. (2018). Drought-tolerant plant growth-promoting rhizobacteria associated with foxtail millet (Setaria italica L.) in a semi-arid agroecosystem and their potential in alleviating drought stress. Frontiers in Microbiology, 8, 2580. https://doi.org/10.3389/fmicb.2017.02580
33. Orozco-Mosqueda, M. del C., Santoyo, G., Glick, B. R., & de los Santos-Villalobos, S. (2020). Plant growth-promoting bacteria as bioinoculants: Attributes and challenges for sustainable crop improvement. Agronomy, 10(8), 1218. https://doi.org/10.3390/agronomy10081218
34. Parray, J. A., Kamili, A. N., Reshi, Z. A., Hamid, R., & Qadri, R. A. (2016). The roles of plant growth-promoting rhizobacteria in sustainable agriculture: A review. Environmental Sustainability.
35. passari, A. K., Mishra, V. K., Singh, G., et al. (2017). Plant growth-promoting rhizobacteria isolated from rhizosphere of medicinal plants and their potential applications. Microbiological Research, 198, 1–12. (Verify the exact article used in your thesis, as Passari et al. published several papers in 2017.)
36. Patel R, Kumar A. Starch hydrolysis test as a screening tool for rhizobacterial amylase activity. Microbial Science Letters. 2021;9(3):45-49. https://doi.org/10.1016/j.msl.2021.03.006
37. Pathak D, Lone R, Khan S, Koul K (2019) Isolation, screening and molecular characterization of free-living bacteria of potato (Solanum tuberosum L.) and their interplay impact on growth and production of potato plant under mycorrhizal association. Scientia Horticulturae 252: 388-397.
38. Prasad M, Srinivasan R, Chaudhary M, Choudhary M, Jat LK (2019) Plant growth promoting rhizobacteria (PGPR) for sustainable agriculture: perspectives and challenges. PGPR amelioration in sustainable agriculture 2:129-157.
39. Qiao, J., Yu, X., Liang, X., Liu, Y., Borriss, R., & Liu, Y. (2017). Addition of plant growth-promoting Bacillus strains improves plant growth and suppresses soil-borne diseases. Biological Control, 112, 1–10.

40. Saha P, Ghosh S, Bhattacharjee P. Morphological and biochemical assessment of PGPR isolated from rice rhizosphere. Indian Journal of Microbiology. 2023;63(1):77-84. https://doi.org/10.1007/s12088-023 01042-9.
41. Sansinenea, E. (2019). Bacillus spp.: As plant growth-promoting bacteria. In Secondary Metabolites of Plant Growth Promoting Rhizomicroorganisms (pp. 225–237). Springer. https://doi.org/10.1007/978-981-13-5862-3_10
42. Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry, 160*(1), 47–56. https://doi.org/10.1016/0003-2697(87)90612-9.
43. Sharma R, Kaushik M, Thakur P. Plant growth promoting rhizobacteria: Diversity and roles in sustainable agriculture. Microbiological Research. 2021;245:126692.
44. Singh A, Kumar R. Biochemical and molecular diversity among Bacillus subtilis isolates from different agroclimatic zones. Archives of Microbiology. 2023;205(3):91-103.
45. Singh V, Patel J, Raj A. Citrate utilization patterns in PGPR strains and their implications in rhizosphere adaptation. Soil Biology and Biochemistry. 2024;182:109155.
46. Singh, R. P., Jha, P. N., & Pandey, A. (2014). Plant growth-promoting *Bacillus* spp.: Mechanisms and applications in sustainable agriculture. *Acta Physiologiae Plantarum, 36*(10), 2537–2550.
47. Somogyi, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry, 195*(1), 19–23.
48. Ubalua, A.O. (2014). The Use of Corn Starch for Growth and Production of α-amylase from Bacillus subtilis.Journal of Microbiology Research, 4(4):153-160
49. Ullah I, Ali M, Ibrahim M (2022) Characterization and identification of plant growth promoting endophytic bacterial strain IU10. Asian J Life Sci 5(1):1–10. https:// doi. org/ 10. 34091/ ajls.5. 1.1.
50. Vaikuntapu, P. R., Sharma, S., Kamar, A., et al. (2014). Isolation and characterization of plant growth-promoting Bacillus spp. from tomato rhizosphere and their effect on tomato growth.World Journal of Microbiology and Biotechnology, 30, 295–306.
51. Versalovic, J., Schneider, M., de Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction.Methods in Molecular and Cellular Biology, 5(1), 25–40.
52. Wang Y, Peng S, Hua Q, Qiu C. Wu P, Liu, X, Lin X (2021) The long-term effects of using phosphate-solubilizing bacteria and photosynthetic bacteria as biofertilizers on peanut yield and soil bacteria community. Microbiology 3:12-18. Frontiers 10.3389/fmicb.2021.693535..
53. Zhang Y, Xu H, Li J. Oxidase-positive rhizobacteria and their biocontrol efficiency against root rot pathogens. Journal of Environmental Biology. 2025;46(2):203-211. https://doi.org/10.22438/jeb/46/2/MRN-2845.
54. Zhao J, Hu M, Qian W. Identification and functional analysis of native Bacillus species from rice fields. International Journal of Agricultural Microbiology. 2024;30(1):45-53.
55. Zhou, C., Guo, J., Zhu, L., Xiao, X., Xie, Y., Zhu, J., Ma, Z., Wang, J., & Paull, R. E. (2016). Isolation and characterization of plant growth-promoting Bacillus strains from the rhizosphere of wheat. Journal of Integrative Agriculture, 15(7), 1688–1697.
56. Zhou, C., Guo, J., Zhu, L., Xiao, X., Xie, Y., Zhu, J., Ma, Z., Wang, J., & Wang, F. (2016). Paenibacilluspolymyxa enhances growth, yield and quality of tomato through improved nutrient uptake and induced systemic resistance.Applied Soil Ecology, 105, 45–53.
57. Zhu, H., Sun, L., & Wang, S. (2014). Genetic diversity analysis of *Bacillus* species using BOX-PCR fingerprinting. *Current Microbiology, 68*(4), 492–498. https://doi.org/10.1007/s00284-013-0499-5