

Plant Growth Promoting *Bacillus Sps* Suppressing Phytopathogens In-Vitro that Infect Groundnut Crop

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ABSTRACT:

Plant growth-promoting rhizobacteria [PGPR], revitalize the soil quality naturally and influence soil characteristics transforming wasteland to fertile land, not immediately, but progressively. Their crucial role as a bio-control agent as well as bio-fertilizer makes them unique to achieve high yields in crop production. Techniques used during this immediate research for isolation and screening the PGPR of the soil were most adaptive and traditional. Within this existing research, two bacterial strains did get toward being influential after vetting in vitro. Their compatibility with each other and the mode of action against certain pathogens was discussed in the present study. To meet the demands of the present farmer for sustainable agricultural production, further study on bio-formulation using these strains is potentially significant. On the other hand, the efficacy of these strains against groundnut pathogens especially on *Sclerotium*, *Fusarium* is noticeable.

Keywords:- Bio-control, Sustainable, Bio-fertilizer, Synergistic, Antagonist.

INTRODUCTION:

Hydrogen, Oxygen, Carbon [from water and air], Phosphorus, Chlorine, Potassium, Calcium, Nitrogen, Sulphur, Magnesium, are some essential macronutrients in soil, and micronutrients include Iron, Zinc, Manganese, Silicon, Boron, Copper, Nickel, Molybdenum, and Selenium [Traces]. The composition of the soil is regulated by organic carbon content, moisture, nutrients, and other biotic and abiotic agents. Though, chaotic management of fertilizers especially nitrogen plus phosphorus will begin abundant contamination in the earth, making nutrients unavailable to crops by reducing pH and exchangeable gases (Gupta et al, 2015). Also, the oversupply of nutrients of inorganic and organic content in extra to satisfy plant requirements also into that lack of a device to wrap the nutrients into the earth can guide environmental pollution (Serpil, 2012). For sustainable cultivation, the subsistence of soil and its changing environment is of top concern (Paustian et al, 2016). Therefore it is important to consider the adverse effects of these fertilizers beforehand applying them onto the field. The possible and progressively promising approach can be made by using soil microbiota (bacteria, fungi, algae, etc.) that can replenish soil remarkably but these are unfortunately diminishing in the soil by the extensive usage of synthetic fertilizers.

ROLE IN AGRICULTURAL SUSTAINABILITY:

PGPR are a colony of rhizosphere bacteria (near throughout the root sections of the soil). Their method of operation is as regards

1. Enhance solubilization of nutrient and fix atmospheric nitrogen, leading to nutrient availability to plants.
2. Suppression of soil-borne pathogens (by compounds like HCN, Iron chelating-siderophores, antibiotics by PGPR, and/or competition for nutrients);
3. Production of phytohormones such as indole-3-acetic acid (IAA) and
4. Enhancing plant stress sensitivity to drought, salinity, and metal toxicity; (Ibiene et al,2011).

Few studies expressed that, the thus referred to as PGPR will elicit changes in root catalyst activity concerned within the generation of metabolites, especially flavonoids, occurring in diversity inside the model of exudation of roots (Lavanaia et al, 2006). Furthermore, mixtures of microbial sources, such as phenazines and 2,4-diacetyl phloroglucinol (DAPG), could improve the absolute net efflux of amino acids in plant variations. (Phillips et al, 2004). The fore mentioned feature is significant for creating bio-formulation using blended inoculants. In addition to outcomes on root exudates, PGPR can trigger changes in metabolite formation of the complete plant that can never be achieved by using chemical fertilizers alone. PGPR, though has a significant role as a bio-control agent in disease resistance to crop, it is not accepted and applied in the farmer's field due to various limiting factors, one

amongst is lack of awareness. The modern study largely centres on the separation, classification, and characterization and the adaptability of the local PGPR to enhance the soil of the earth.

SIGNIFICANCE :

Bacteria at the proximity of the root surrounding regions promotes plant growth on one hand by enhancing nutrient uptake., on the other hand, suppresses phytopathogens, thereby holds significance in influencing the whole plant growth promotion. These can mend atmospheric nitrogen and mobilize phosphorus in the form in which plants can't utilize without environmental contamination. Some of the varieties of PGPR are studied and are commercialized worldwide as bio-fertilizers. They are used in agricultural fields to replenish soils for successful crop yields. The utilization of plant growth-promoting rhizobacteria [PGPR] is accustomed to its endurance, the communication capacity with native microflora in the ground, and environmental circumstances. According to Nakkeeran et al, 2005, an excellent PGPR should maintain great rhizosphere ability, improve plant growth capacities, have a wide spectrum of performance, be protected for the environment, UV radiation, receptive to heat, and the oxidizing agent, be cooperative with other rhizobacteria.

MATERIALS AND METHODS

1 Collection of the soil:

Rhizospheric soil of three different regions were selected for the current study. one amongst is from ten to fifteen-day old groundnut crop plants of the settur region from Anantapur, second from rhizospheric soil of wild plants from Tirupati (where the study is carried out) and another from the marine plant root-soil from Pulicat lake. Soil attached to the roots is gently separated and was subjected to serial dilutions and plated on the nutrient agar plates followed by incubation at 30°C for 48 hrs. After incubation, morphologically distinct colonies were selected for the screening of the potent microbes and are maintained at 4° C for further use.

SCREENING OF THE ISOLATES INVITRO

I Nitrogen fixation:

Nitrogen-free jensen's media is used for culturing bacterial isolates to test whether they can fix atmospheric nitrogen or not. Isolates were inoculated onto a petri plate containing solidified jensen's media and incubated for 2 -3 days. If everyone succeeds in the media, then the organism is capable of fixing nitrogen of atmosphere (Rokhzadi et al, 2008).

II. Phosphate solubilization assay:

Pikovskaya agar medium is used for screening the isolates that solubilize phosphate. The bacterial isolates were spread on the pikovskaya agar medium. Those dishes remained incubated for three to four days at 30±5°C. Following incubation time, clear distinct area encircling the colony registers the appearance of phosphate solubilizing bacteria (Karpagam et al, 2014).

III. Ammonia Production:

Peptone water is utilized to examine the composition of ammonia by bacterial isolates. Empty 5 ml of peptone water in 15-20 ml of sterilized test tubes. 1% inoculum is mixed in every tube that was filled with 5 ml of peptone water and incubated at 30°C for 72 hours. Following incubation,

0.5 ml of nessler's reagent is combined with each tube. The progress of brown to yellow colour designates a positive test. (Radziah et al, 2014).

IV. Organic Acid detection:

Organic acids produced by the isolates can be estimated by growing on pikovskaya's medium for 72 hours at 30°C. Organic acid-producing bacterial isolates were distinguished by their capacity to deliver a change in colour of methyl red pH indicator at a concentration of 0.03% of methyl red which turns, yellow on the pikovskaya agar plate (Karpagam et al, 2014) (Dey et al, 2014).

V. HCN Production:

Nutrient agar corrected with glycine (4.4 g/l) is utilized to examine the effect of HCN from bacteria. On qualified agar plates, bacterial isolates were streaked. Whatman filter paper no.1 immersed in 2 percent sodium carbonate in 0.5 percent picric acid was placed at the inner facade of the top lid that covers the petri plate containing the media. For four days, the plates were incubated at 30°C sealed by paraffin. The addition of orange to red colour denotes the composition of HCN (Pawar et al, 2013).

VI. Phytohormones by isolates:

To evaluate the phytohormone IAA composition from bacterial cultures, After incubation, bacterial isolates were directed at 30°C for 48 hours on a shaker (120 rpm) and centrifuged at 3000 rpm for 30 minutes in nutrient broth amended with tryptophan (100g/ml). Two drops of o- phosphoric acid and four millilitres of Salkowski reagent (50 ml, 35% perchloric acid, 1 ml 0.5 M FeCl₃) were added to two millilitres of supernatant. The rise of pink colour symbolizes IAA production (Etesami et al, 2014).

VII. Siderophore production

To detect the production of siderophores from bacterial cultures, assay proposed by Schwyn and Neilands 1987 has been used. The glassware to be used must be thoroughly washed with water and then with 3 mol/l hydrochloric acid (HCl) and deionized iron to exclude iron. CAS reagent preparation as per the standard protocol of Schwyn and Neilands (1987) is to be followed. Control is maintained without inoculating. The dishes were incubated at 28°C for 5–7 days. The orange zone around the bacterial colonies is a positive test for the generation of siderophores (Louden et al, 2011).

Rapid strip assay method using dye impregnated strip for detection of siderophores production from bacterial isolates:

The traditional method that is used so far from centuries for the detection of siderophore production from bacteria is tedious and is time-consuming, as it requires many ingredients. An alternate method of siderophore production detection can be made possible using dye impregnated strips which is a rapid test to detect siderophore production in a short span. The test is as follows:

Whatman filter no.1 filter paper strips (1 cm X 5 cm dimension) were taken and each strip is dipped completely in freshly prepared 0.5 ml chrome azurol sulfonate (CAS) solution, (a siderophore detecting indicator dye-prepared in the same way as mentioned above), for 4 hours in dark condition and, air-dried at room temperature. The dry strip turns blue in appearance, is a correct preparation. To avoid moisture problems and photo-deactivation of dye impregnated strips, these strips were kept in a sealed dark carter (Kadam et al, 2010).

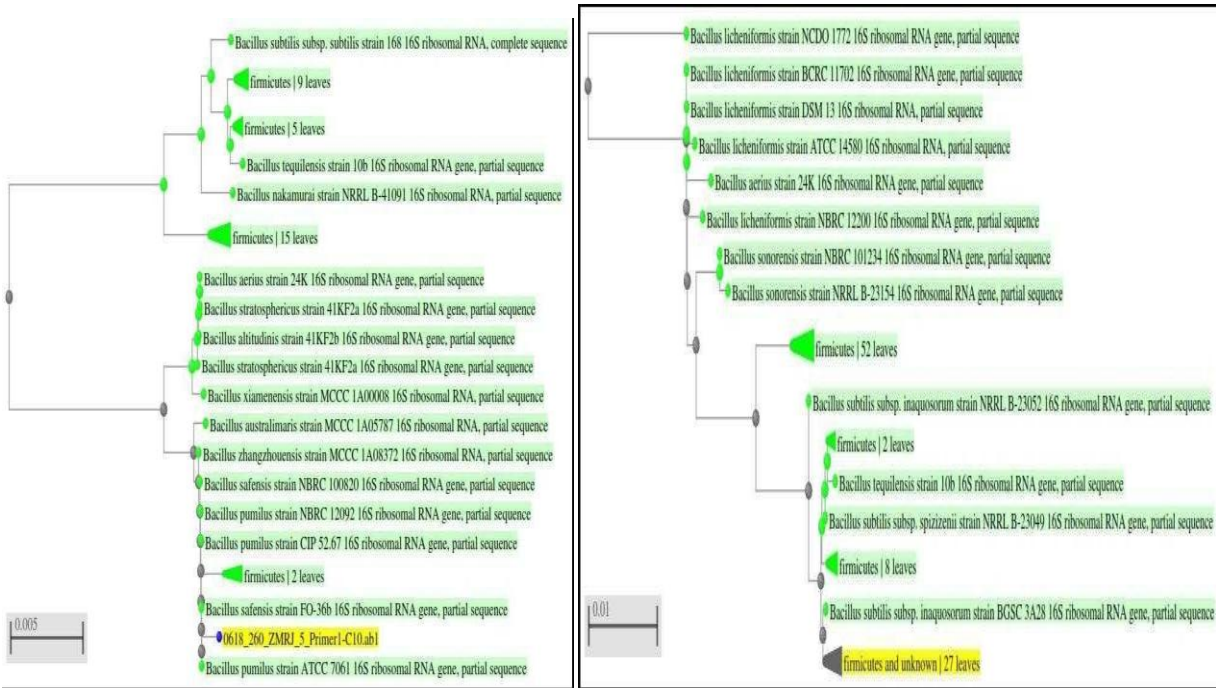
Siderophore detection using dye impregnated strip: A drop of test solution or broth culture as a spot is placed on dye impregnated strip and observed for change in the colour of the spot. The strip was then observed for a minute at room temperature. The brown-yellow colour versus the original blue colour of the strip symbolizes the positive result, whereas no change in colouration of strip indicates a negative result i.e. the absence of siderophore production. Using this method, multiple strains that produce siderophores can be detected at a time.

C IDENTIFICATION

Two isolates were selected based on screening tests as potent plant growth promoters and were sent to 3B biotech bioprocess laboratory, Vadodara, Gujarat for identification. Based on 16S rRNA gene sequencing of the bacteria with morphological analysis and biochemical tests, they were identified as *Bacillus Zhangzhouensis* and *Bacillus velezensis*. GenBank has provided the following accession number(s) for nucleotide sequence(s) of the submitted organisms:

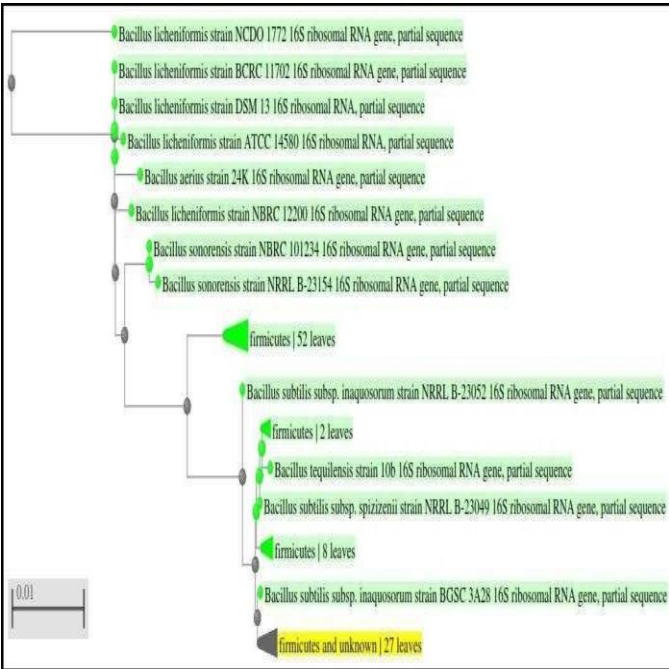
C.1.*Bacillus zhangzhouensis*-Seq1-MH612945 and

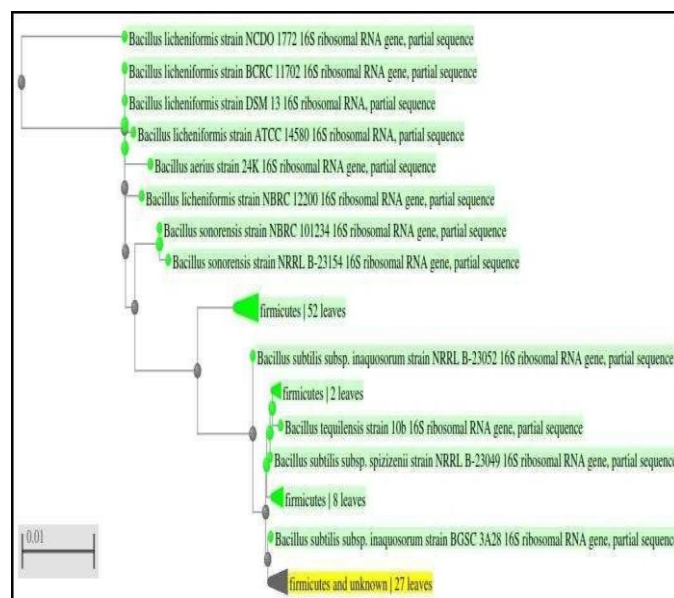
C.2. *Bacillus velezensis*-Seq2- MH612946.



1. Figure 1-Phylogenetic tree of *Bacillus Zhangzhouensis* based on 16s Sequencing
2. Figure 2-Phylogenetic tree of *Bacillus velezensis* based on 16s Sequencing

D COMPATIBILITY STUDY





COMPATIBILITY AMONG EACH OTHER:

The bacterial strains resulted streaked on nutrient agar media plates horizontally and vertically to each other. The plates were produced at room temperature and incubated at $28 \pm 2^\circ\text{C}$ for 72h. A cleared zone of restraint shows the animosity of the strains. Nevertheless, the lack of restraint zone showed the compatibility with individual bacterial strains in the present study (Fukui et al. (1994)).

2. **COMPATIBILITY WITH TRICHODERMA:** (using dual culture method) *Bacillus* and *Trichoderma* adaptability can also be evaluated using the dual culture plate method (Siddiqui and Shaukat (2003)). On one side of a petri dish containing PDA, a fresh overnight culture of *Bacillus* sps that is cultured on nutrient broth is streaked. The opposite side of the petri-dish was treated with a 5mm disc of *Trichoderma* (9 days old). The dishes were then incubated at $25 \pm 1^\circ\text{C}$ and are kept for a zone of inhibition. The vacancy of the inhibitory zone means both the fungi and bacillus are fit with each other.

IN VITRO SCREENING OF THE IDENTIFIED ISOLATES AGAINST PATHOGENS

Agar well diffusion method is widely used to assess the antimicrobial activity of microbes. the existing study shows the activity of the recognized effective bacterial strains against *Sclerotium*, *Fusarium*, and *A.flavus* (Magaldi, 2004; Valgas, 2007). Twenty ml of PDA media is utilized for discharging on petri plates and is permitted to solidify. Then, the plates were inoculated by covering pathogenic suspension across the entire agar surface. Holes were made with a width of 5 mm aseptically with a sterilized cork borer also these bacterial bio-agents (suspension having cfu $2 \times 10^8/\text{mL}$) were imported into the wells. Control is maintained using pathogen alone. The plates were incubated at $25 + 10^\circ\text{C}$ for 5 days before being examined for the inhibition zone. The bacterial bio-agent spreads in the agar medium and inhibiting the growth of the pathogen. A free visible zone of inhibition can be seen.

F RESULTS AND DISCUSSION

From the three distinct locations, 15 isolates from the soil were obtained. All were subjected to screening tests. Based on screening and biochemical tests conducted, two isolates among all were found to be more potent as the plant growth promoters and these two isolates were identified based on 16S RNA sequencing. Their role in suppressing phytopathogen has also been studied in the present study especially on *Sclerotium*, *Fusarium* and *Aspergillus flavus* in vitro. Greenhouse and field trials are being conducted to evaluate the efficacy of these isolates under normal, and dryland regions (Abiotic stress conditions).

Isolates	Nitrogen fixation	Phosphate solubilization	Organic acid	Ammonia Production	HCN	IAA	Siderophore production	Strip assay Siderophore
ZMRJ1	+++	+	+	+	+	+++	++	+
ZMRJ2	-	+	+	+	+++	+	-	-
ZMRJ3	+	-	-	+	+++	+	-	-
ZMRJ4	-	-	-	+		+	-	+++
ZMRJ5	++	+++	+++	+	+++	+	+	++
ZMRJ6	+	-	-	+		+	-	+
ZMRJ7	-	++	++	+	+	+	-	+
ZMRJ8	-	+	+	+		+	-	++
ZMRJ9	+++	+	+	++	+	+	+++	++
ZMRJ10	-	-	-	++	-	+	-	+
ZMRJ11	+	++	++	+++	+	+	+++	+
ZMRJ12	+	-	-	++	-	+	-	+
ZMRJ13	+	+	+	+	-	+	-	+++
ZMRJ14	+	++	++	+		+	-	++
ZMRJ15	+	+	+	+	+	+	-	++

Table 1: Overall Screening Results + Low*: ++Medium*: +++ High**COMPATIBILITY RESULTS**

Fig D.1 Compatible with each other

Fig D.2 Compatible with *Trichoderma* (dual culture)**ANTAGONISTIC ACTIVITY OF ISOLATES AGAINST PATHOGENS**

Fig E.1.1

Fig E.1.2

Fig E.1.3

Fig E.1 Antagonistic activity of *Bacillus sps* against *Sclerotium*

Fig E.1.1: Effect of *Bacillus Zhangzhouensis* on *Sclerotium* (right): control with *Sclerotium* alone(left)

Fig E.1.2: Effect of *Bacillus velezensis* on *Sclerotium* (right): control with *Sclerotium* alone(left)

Fig E.1.3: Effect of *Bacillus Zhangzhouensis* and *Bacillus velezensis* on *Sclerotium* (right): control with *Sclerotium* alone(left).



Fig E.2.

Fig E.2 Antagonistic activity of *Bacillus* sps against *Fusarium*

Upper: Control *Fusarium* alone

Down left: Effect of *Bacillus zhangzhouensis* on *Fusarium*:

Down Middle: Effect of *Bacillus velezensis* on *Fusarium*

Down Right: Effect of *Bacillus zhangzhouensis* and *Bacillus velezensis* on *Fusarium*



Fig E.3.

Fig E.3 Antagonistic activity of *Bacillus* sps against *Aspergillus flavus*

Upper: Control *Aspergillus flavus* alone

Down left: Effect of *Bacillus zhangzhouensis* on *Aspergillus flavus*:

Down Middle: Effect of *Bacillus velezensis* on *Aspergillus flavus*

Down Right: Effect of *Bacillus zhangzhouensis* and *Bacillus velezensis* on *Aspergillus flavus*

Bacterial growth in the rhizosphere is determined by the availability of nutrients in the soil. Due to the regular distribution of nutrients from plant roots, soil microorganisms help in plant growth development by different devices. Because PGPR is used as an inoculant to promote the growth and yield of various crops, screening for effective PGPR strains must be extensive.

In this study, seven screening tests were carried out to determine the distinguishing features of two potent PGPR isolates among 15 isolates and identified as *Bacillus zhangzhouensis* and *Bacillus velezensis*. During the study on their compatibility with each other and with the most accepted and widely used *Trichoderma*, the bacteria

are compatible with each other and with *Trichoderma*, bacterial isolates are well able to proliferate but are not suppressed.

Later on, the antagonistic activity of these microbes was studied against plant pathogens of groundnut. Bacterial isolates, when used as a consortium, were able to effectively inhibit the growth of *Sclerotium*.

The effect of bacterial isolates as an antagonist over *Fusarium* is effective and satisfactory, as the growth of *Fusarium* was suppressed but not completely inhibited. Effect of bacterial isolates, when used as a consortium on *Aspergillus flavus*, is low as the pathogen proliferates within no period, on the media that has been used in the study. But, the growth of the *Sclerotium* is completely been inhibited by the synergistic effect of both the isolates than the individual ones on the same media (PDA-Fungal media). In this case, *Bacillus Zhangzhouensis* is more effective than *Bacillus velezensis*. But, when used as mixed culture their antagonistic activity is noted to be very effective and inhibition took place at its earliest in-vitro. It can be concluded from the above discussion that the synergistic effect of two strains of *Bacillus* isolated from soil and identified is able to promote plant growth while inhibiting phytopathogen growth.

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