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*Research Paper*

# Evaluation of Plant Growth Promoting Rhizobacteria to Control Bacterial Wilt of Groundnut Plants

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## A B S T R A C T

A total number of 118 bacterial isolates were isolated from the infected groundnut soil and plant samples. These isolates have undergone biochemical characterization and were identified as *Ralstonia solanacearum*. Among 118 isolates, ninety isolates belonged to Race 2 biovar III. The remaining 28 isolates belonged to biovars I and V. Twenty-five isolate belonging to the Race 2 biovar III were shown positive in pathogenicity studies; out of these three molecular methods identified highly virulent isolates. Between December 2017 and January 2018, 80 PGPRs were isolated from vegetable crops and groundnut rhizospheres in Andhra Pradesh and Karnataka regions. The isolates were characterized by morphological and molecular methods and evaluated for antagonistic activity against Groundnut's bacterial wilt (*Ralstonia solanacearum*). Out of 80 PGPR, PGB24 (MH285273), PGB28 (MH283866), PGP24 (MH290482) were showed significant activity against *Ralstonia solanacearum*. The rest of the other PGPR showed less activity against *Ralstonia solanacearum* when tested in *in-vitro*.

***Key words*:** Rhizosphere, Biocontrol agents, Groundnut, Bacterial wilt, *Ralstonia solanacearum*

Agriculture is one of an essential human activity that contributes to the increasing amount of chemical pollutants via excessive use of chemical fertilizers, pesticides, and fungicides, that cause the environmental damage and potential risk to the human health to the outcome of this environmental and health hazardous, the use of microbial inoculants plays a vital role in plant growth promotion, crop production through biological disease control and sustainable soil fertility [1]. PGPR has gained considerable interest in research because of the stimulation of plant growth, high yield production, crop protection, and being less harmful to the environment, thus maintaining ecological balance. Based on the relationship with the plant, the PGPR are classified into:

· Intracellular plant promoting rhizobacteria (iPGPR)
· Extracellular plant growth-promoting rhizobacteria (ePGPR) [2].

iPGPR is also known as symbiotic bacteria. They live inside plants and exchange the metabolites with them directly; on the other hand, ePGPR is also known as free-living rhizobacteria that live outside the plant cells and facilitates the plant growth indirectly by the production of antibiotics. Plant growth rhizobacteria is a promising, sustainable, and environmentally friendly approach to obtain sustainable soil fertility and plant growth by direct and indirect mechanisms. The natural means of PGPR involved straightforwardly supporting plant growth. The direct agents are bio fertilization, stimulation of root growth, rhizoremediation, nitrogen fixation, phosphate solubilization, plant stress control, and Phyto stimulation activity. PGPR increases soil fertility and plant growth indirectly by producing antibiotics, siderophores, hydrogen cyanide, hydrolytic enzymes, etc. [3]. The rhizobacteria like *Acetobacter*, *Actinoplanes*, *Agrobacterium*, *alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradirhizobium*, *Cellulomonas*, *Clostridium*, *Erwinia*, *Flavobacterium*, *Pasteuria*, *Rhizobium*, *Serratia* and *Xanthomonas* are ideal for the use of biocontrol agents as they can provide the front-line defense for plant roots against the attack of various plant pathogens [4]. Our study mainly focused on Rhizobacteria *Bacillus* sp.; these are also referred to as biocontrol agents that comprise a significant role in the induction of plant pathogens, crop production, and antibiotic production against various plant pathogens.

We have screened these biocontrol agents for their antagonistic activity against three highly virulent strains known as *Ralstonia solanacearum*, which has been responsible for the Bacterial wilt of Groundnut plants. *R. solanacearum* is an anaerobic, non-spore-forming, gram-negative, soilborne pathogen with a single polar flagellum. Size varies from 0.5 – 0.7 × 1.5 – 2.0µm in size. It has a large host range of 200 species in 50 families [5], including important crops such as Groundnut, potato, tobacco, eggplant, banana, etc. Bacterial wilt causes 15 to 55% of crop loss worldwide [6]. *R. solanacearum* inhabits the vascular tissue of its hosts by colonizing the xylem that prevents water movement into the upper portion of the plant tissue [7]. The first symptoms are wilting of young leaves during the heat of the day, infected leaves turn yellow and remain wilted and yellow-brown discoloration of vascular tissue. Rhizobacteria are the most abundant organisms present in the rhizosphere

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that may, directly and indirectly, contribute to plant growth and crop production by suppressing the plant pathogens. Many species of the *Bacillus* genus are potential biocontrol agents and versatile weapons for the various plant pathogens. Plant growth-promoting *Bacillus* strains have been widely studied to develop plant growth [8). *Bacillus* spp., such as *Bacillus subtilis, Bacillus cereus, Bacillus amyloliquifaciens, Bacillus pumilus, Bacillus pasteurii,* and *Bacillus mycoides,* etc., influence the growth and development of the infected plants by bio fertilization, production of lytic enzymes, siderophore production, antibiotic production, phosphate solubilization and elicitation of induced systemic resistance. Several species of *Bacillus* are known to produce toxins that are inhibitory to the growth and activities of fungal pathogens of plants, wherein most thoroughly studied species include *Bacillus subtilis* [9). *Bacillus* sp., used as biofertilizers probably, have the major effects on plant growth through the synthesis of growth hormones [10).

## MATERIALS AND METHODS

*Groundnut field survey and sample collection*

The Field surveys were conducted in July and August 2015 to know the status of bacterial wilt of Groundnut in Andhra Pradesh and Karnataka in terms of its incidence and severity in major Groundnut growing places in India such as Tumkur district (Pavagada) of Karnataka and Anantapur district (Penukonda, Madakasira, Hindupur, and Acharya N. G. Ranga Agricultural Research University, Extension center, Kadiri) of Andhra Pradesh. The plants were observed with typical wilt symptoms viz., droopy leaves yellowing, full plant wilted and vascular browning and farmers field for each growing area were surveyed to record the bacterial wilt incidence and severity. Collect at least ten samples of diseased plants and rhizosphere soil from sterile polyester bags in each survey area and bring them into the laboratory to isolate different types of *R. solanacearum.*

*Isolation of Ralstonia solanacearum from infected samples*

The stored soil and plant samples were used for the isolation of bacterial wilt, causing organisms. *R solanacearum* using a specific medium, Triphenyl tetrazolium chloride (TTC). The bacteria in infected soil samples were isolated by dilution plate on modified semi selective South Africa (SMSA) agar medium [11). Use 1% NaOCl solution to surface sterilize (0.5-1cm) plant sections for 1 to 2 minutes, then rinse thoroughly with sterile distilled water and blot dry. The fixed plant sections were plated on 2, 3, 5 Triphenyl Tetrazolium chloride (Kelman's TZC agar) medium [12). The plates were incubated at 28°C for 24–48 h. The identification of *R. solanacearum* isolates from the groundnut plants was based on the morphological, physiological, biochemical, and cultural [13], [14]. The Biovars of *R. solanacearum* were differentiated based on their ability to oxidize disaccharides and hexose alcohols [15].

*Sample collection*

A total of sixty rhizosphere samples were collected from healthy vegetable crops during the field survey in the months of Jan-March (2017-18) from different agroclimatic regions of Karnataka and Andhra Pradesh. The collected soil samples were placed individually in plastic bags and stored in a refrigerator to isolate rhizobacteria.

*Isolation and identification of Rhizosphere bacteria*

The collected samples were used for the isolation of Rhizosphere bacteria was carried out on Nutrient agar, and peptone yeast extract dextrose medium. Ten grams of soil samples were suspended in a tube and mixed well. Incubate the test tube in an 80ºC water bath, transfer the heat-treated soil samples were added to the 90 ml of sterile distilled water and mixed thoroughly by shaking the flask on a rotary shaker for 5 minutes. After serial dilution, 0.1 suspensions were spread over pre-sterilized and cooled down on peptone yeast extract dextrose plates and nutrient agar plates in triplicates. The inoculated plates were incubated at 30°C for 24-48h. Rough and abundant colonies with waxy growth (1-4 mm diam) and irregular spreading edge were obtained. The resulted colonies were sub cultured on Nutrient agar medium and maintained as pure cultures for further study. Physiological and biochemical characterization of isolates was performed using Bergey's Manual of Determinative Bacteriology [16].

*Characterization of bacterial isolates for different plant growth-promoting activities*

*Root colonization bioassay*

According to the procedure of [17]. The bioassay was performed by the surface-sterilized groundnut seeds and was immersed in 250 ml rhizobia suspension for about 24 hours, and then it will be transferred to a sterile 0.6% water agar test tube. Let the seedlings grow at room temperature. Perform regular visual observations to detect the growth of bacteria around the roots.

*Production of indole acetic acid (IAA)*

Quantitative estimation of indole-3-acetic acid (IAA) [18] for the production of auxins, bacterial cultures were grown in Luria Bertani broth for 48hrs at 37°C under shake conditions. The supernatant was prepared by centrifugation of cultures at 15,000rpm for 20 minutes and was stored at 4°C. Pipette 3ml of the supernatant into a test tube, and add 2ml of Salkowski reagent to it. The tubes were incubated in a dark region for about 30 minutes for the development of pink color.

*Phosphate solubilization*

Many soil microorganisms can solubilize the unavailable form of bound phosphorus. The solubilization of phosphate was tested using Pikovaskaya's agar medium. One loop full of the 24 h broth culture was spot inoculated on the Pikovaskaya's culture plate. The plates were incubated at 28°C for 96 h. The plates were observed for the zone of clearance around the bacterial colony, which indicated phosphate's solubilization.

*Production of HCN*

Production of HCN was measured by the qualitative method of [19] with the bacterial cultures were streaked on pre-poured plates of King's B medium amended with 4.4g/L glycine. The single strip of Whatman No. 1 filter paper was immersed in 0.5% picric acid in 0.2% sodium carbonate solution and placed between Petri plates. Petri plates were sealed with parafilm and were incubated at 37°C for 1-4 days. Un-inoculated control was kept for comparison. Plates were observed for change of color of filter paper from yellow to orange-brown to dark brown.

*Production of ammonia*

The isolates were tested for ammonia production in peptone water. Freshly bacterial cultures were inoculated to

10ml peptone water and incubated for 48-72 hours at 36±2°C Nessler's reagent was (0.5ml) added to the tubes. A positive test is indicated by developing brown to yellow color [20].

*Antagonism assay of PGPR isolates*

The *Bacillus* sp., antimicrobial activity was assessed against the highly virulent strain *(R. solanacearum*) on Muller Hinton's Agar media (MHA) by the Disc Diffusion method. The discs were soaked in the cell suspension (incubated for about 12-24 hours); each disc was placed on the MHA media and incubated in the refrigerator for about one hr., then plates were kept for incubation at $28^0$C for 24hrs. The incubated plates were observed, and the diameter of the zone inhibition was measured.

## RESULTS AND DISCUSSION

*Isolation of Ralstonia solanacearum from infected samples*

After incubation, the *R. solanacearum* colonies on TZC media were observed as cream color or off-white color with pink centered colonies on isolation plates. A total of eighty isolates of *R. solanacearum* were obtained from the wilted groundnut plant samples, i.e., 65 isolates from Andhra Pradesh (Biovar- I, III & V) and 15 isolates from Karnataka (Biovar- I, III&V). Previous studies showed that the pathogen was isolated and identified as an *R. Solanacearum* [21].

*Identification of isolates*

*Morphological, Microscopic and Physiological identification*

Virulent *R. solanacearum* colonies appeared as white or cream-colored with a light pink centered irregularly shaped, highly fluidal, and opaque on TZC media. On the other hand, avirulent colonies were round deep red [22]. Microscopic studies revealed that bacterial isolates were Gram -ve, rod-shaped, non-capsulated, and non-spore-forming, strictly aerobic. All strains were able to grow at 37°C but failed to produce at 40°C. All the *R. solanacearum* isolates were motile, which was confirmed by the hanging drop method [23]. The morphological and cultural characteristics of specific TZC agar medium results confirmed the isolates [24].

*Isolation and selection of rhizosphere bacteria*

A total of 80 rhizosphere isolates were obtained from different groundnut soil samples from Andhra Pradesh and Karnataka. These isolates were used for further studies.

*Morphological, microscopic and physiological identification*

Morphological and physiological characters of the PGPR isolates were examined according to Bergey's manual of determinative bacteriology, 2005. Morphological, Microscopic, and Physiological Identification of *Bacillus* isolates. Morphological characteristics of each *Bacillus* isolates colony were examined on Peptone yeast extract dextrose (PYE) agar media. All the 80 isolates were streaked on Nutrient Agar plates. After three days of incubation, different colonies' characteristics such as shape, size, elevation, surface, margin, color, etc. were recorded; Gram staining was performed as per standard procedures. These microscopic studies revealed that *Bacillus* isolates were Gram-positive, rod-shaped, capsulated, and spore-forming bacteria.

*Antagonism assay of PGPR isolates*

A total of 80 isolates *Bacillus* were screened for their antagonistic activity against 3 highly virulent *R. solanacearum* by disc diffusion and well diffusion method. Not all the rhizobacterial isolates inhibited the growth of *R. solanacearum*. Among 80 isolates, two isolates of *Bacillus subtilis* have recorded the maximum zone of inhibition screened against three highly virulent *R. solanacearum* by the Disc diffusion method and well diffusion method.

The plant's rhizosphere represents the thin layer of soil around the plant roots and the soil occupied by the roots, supporting a large number of active bacterial communities [25], and is called plant growth rhizosphere bacteria (PGPR) [26]. These organisms can also benefit the plant by stimulating growth. and it will produce secondary metabolites, such as antibiotics, plant hormones [27], volatile compounds, and so on. Plant growth-promoting rhizobacteria (PGPR) are a group of bacteria that can enhance plant growth using the mechanisms mentioned earlier and protect roots from pathogens' invasion by producing induced systemic resistance [28].

This research involves the isolation, screening, and characterization of plant rhizobia promoting bacteria (PGPR) obtained from rhizosphere samples of different plants. In our research, 80 *Bacillus* spp. Isolates were isolated from rhizosphere soil of varying vegetable crop plants collected from other agro-climatic regions of Karnataka and Andhra Pradesh. *In vitro* assays like antagonistic assay against highly virulent strain *R. solanacearum*, physiological, morphological, biochemical, and molecular characterization was conducted to screen the rhizobacterial isolates. All the isolates of *Bacillus* spp., designated as B1 to B80, were found to be irregular colonies, rod-shaped, gram-positive bacteria.

Biological control, using microorganisms to suppress plant disease, offers a powerful alternative to the use of synthetic chemicals. The abundant diversity of the microbial world provides a seemingly limitless resource for this. Although a variety of microorganisms contribute to the biological control of plant pathogens, most studies have utilized species of Bacillus, Pseudomonas, and Trichoderma [29]. There are eight species of microorganisms registered by the U. S. Environmental protection agency for commercial use against soilborne plant pathogens in the U. S. [30]. In most biocontrol investigations, Pathogen inhibition and root colonization do not always correlate with biocontrol efficacy under natural conditions [31]. Biological control accepts special significance in being ecology conscious, cost-effective alternative strategy for bacterial wilt management. The antagonist's *Bacillus* spp. were evaluated under in vitro condition for their effect on highly virulent strain *R. solanacearum*. *Bacillus* spp. Produces various compounds that suppress the growth of *R. solanacearum* and also induce systemic resistance in the plant. *Bacillus subtilis* is well known to generate systemic resistance in the plant by various secret kinds of lipopeptides and secondary metabolites. This agent also improves plant growth [32]. Among the antagonists tested, seven isolates of *Bacillus* were showed maximum inhibition diameter <18mm;>11mm.

The biochemical characteristics of selected seven isolates of *Bacillus* gave a negative result for the KOH loop test because gram-positive bacteria, by contrast, possess a thicker, more rigid cell wall that resists the disruptive effect of KOH. All the seven isolates of *Bacillus* spp. were positive for the catalase test. Catalase is an enzyme that can decompose hydrogen peroxide into water and oxygen [33]. Kovac's oxidase test is used to detect the presence of cytochrome C oxidase (Oxidase positive) [34]. All the isolates of *Bacillus* spp. Showed positive results for the oxidase test. All the isolates of *Bacillus* spp. were negative for the methyl red

test—only *Bacillus* spp. Isolates were showed a negative result for the Voges Proskauer test because of their neutral nature. All the isolates of *Bacillus* spp. Showed positive results for the Gelatinase test. All the isolates of *Bacillus* spp. were showed positive results for the Casein hydrolysis test, starch hydrolysis test, Arginine test, and Simon's citrate agar test.

All the selected seven isolates of *Bacillus* spp. were positive for IAA production. It has been previously reported that IAA production by microbial isolates varied greatly within species and or strains of the same species. Increased plant growth was correlated to rhizobacterial isolates with traits such as IAA production phosphorous solubilization [35]. The characteristics of isolates of *Bacillus* spp. Showed positive results for phosphate solubilization, the second significant plant growth-limiting nutrient after nitrogen, is abundantly available in soils in organic and inorganic forms [36]. All the selected Bacillus spp. were positive for ammonia production and Hydrogen cyanide production [37].

The genus Bacillus was established by Cohen in 1872 and covered more than 200 species, and subspecies belonging to Salmonella described above. The species differentiation is difficult because of their large number and often incomplete descriptions of a newly reported species. In this study, molecular identification of *Bacillus* and *Trichoderma* isolates based on 16S rRNA sequencing technology was performed using the PCR amplification technique. Among seven isolates of *Bacillus*, only one culture (B28), which recorded the maximum inhibition zone, was selected for molecular identification. Differences in the *Bacillus-related* community structures in different soil types and management regimes suggest environmental factors influence the diversity of endospore-forming bacteria in soil [38]. The identification of *Bacillus* isolates also showed incredible biodiversity among soil samples distributed among different species [39].

## CONCLUSIONS

A successful outcome of biological control in the field demands a better understanding of plants' complex microbial interactions. The research was proposed to study Groundnut's bacterial wilt, its occurrence, distribution, losses due to disease, diagnostic symptoms, and epidemiology. Our result validated to development of a biological strategy against the pathogen *R. solanacearum*. The selected biocontrol agents such as Rhizosphere bacteria were characterized and directed towards the susceptible groundnut variety by treating them with the chosen biocontrol agents observed for its disease incidence in *in-vitro* conditions.

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