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Enhancement of Plant Growth in Tomato by Inoculation with Plant Growth Promoting *Bacillus* spp

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Abstract Four bacterial isolates selected from among 200 obtained from different source samples were evaluated for plant growth promoting (PGP) traits. These are found to be good for P solubilization, IAA, HCN, siderophore and NH_3 production with antifungal activity on phytopathogenic fungi and abiotic stress tolerance. Tomato plant growth was enhanced by these isolates at seed germination (14-19%) and pot culture (increase in biomass 47-76%). These isolates are identified as *Bacillus siamensis* RS8, *Bacillus tequilensis* MS3, *Bacillus subtilis subsp stercoris* MS19 and *Bacillus velezensis* MS20 having potential for developing as bioinoculants to enhance the tomato plant growth and productivity.

Keywords: *PGPB, PGPR, Bacillus spp, bio-inoculants, tomato plant*

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1. Introduction

Tomato (*Lycopersicon esculentum*), one of the most important vegetables, has worldwide nutritive and economic importance and considered as protective vegetable crop. It gives high yield in short duration and wide spread in production. Presence of carotenoid lycopene, which is effective against human diseases like cancer, cardiovascular disorders and anti ageing properties, makes it more important [1]. Nutrients are essential for healthy growth of crops which in turn feed ever expanding population globally [2]. Agricultural producers have become dependent on use of agrochemicals as reliable method of crop protection. However, increased use of chemical inputs can cause development of pathogens resistant to applied agents and can adversely affect the environment. Therefore alternative treatments for control of plant diseases are needed. Use of microorganisms to control plant pathogens is a method of biological control. It is accepted as an alternative or supplemental way to reduce use of chemicals against plant diseases [3].

Plants are evolved organisms present in association with different microorganisms as commensals, symbionts, pathogens and growth promoters. Such bacteria are referred to as plant growth promoting bacteria (PGPB) [4]. Increasing demand for crop production with significant reduction in use of synthetic chemical fertilizers and pesticides is a big challenge. Use of PGPB has been proven to be an environmentally sound way of increasing crop yields by facilitating plant growth through direct or indirect mechanisms [5]. Mechanisms of PGPB include solubilizing nutrients for easy uptake by plants, regulating hormonal and nutritional balance and inducing resistance against plant pathogens to improve plant growth. In addition, PGPB show synergistic and antagonistic interactions with other microorganisms within rhizosphere and beyond in bulk soil, which indirectly boosts up plant growth [6]. PGPB are not only associated with root to exert beneficial effects on plant development but also have positive effects on controlling phytopathogenic microorganisms. Therefore, PGPB serve as active ingredients in biofertilizer formulations [7]. Plant nutrients are vital component of sustainable agriculture [8] and increased crop production largely relies on the type of fertilizers used to supplement essential nutrients for plant growth. Objective of this study was to isolate PGPB from rhizosphere soil and marine sources. Potential PGPB were further characterized and assessed on tomato plant for enhancement of seed germination and growth promotion in pot culture.

2. Materials and Methods

2.1. Isolation of Plant Growth Promoting Bacteria

Soil samples were collected from rhizosphere of different crops viz., tomato, chilli, sorghum, maize, red gram, brinjal, green gram and soybean across Mahabubnagar dist, Telangana and marine water from Krishnapatnam port, Andhra Pradesh, India, as per standard protocols. Samples were serially diluted and plated onto nutrient agar and incubated at 30±2 °C for 24

hr. Isolated colonies were transferred onto nutrient agar slants, stored at 4°C for further studies.

2.2. Phosphate Solubilization

Actively growing bacterial cultures were spot inoculated on National Botanical Research Institute's (NBRI) phosphate agar medium amended with 0.5% tricalcium phosphate and incubated for 4-7 days at 30±2°C. Isolates with good zone of clearance around the colonies were subjected for quantitative estimation of phosphate as per the method [9] in brief: 1ml bacterial culture inoculated into 50 ml NBRI phosphate broth and incubated for 5 days at 30±2 °C, 200 rpm. Cell suspension was centrifuged at 10,000 rpm for 15 min and available phosphorus in supernatant was determined by ammonium phosphate-molybdate method and blue color absorbance was recorded at 660nm.

2.3. Test for Indole Acetic Acid (IAA) Production

10 µl of actively growing bacterial cultures were spotted onto Luria Bertani (LB) agar medium amended with 5mM L-tryptophan, covered with nitrocellulose membrane and incubated at 30±2°C for 24-48h followed by saturation of membrane with Salkowski reagent. Cultures showing positive results were subjected for quantitative estimation of IAA in broth culture by colorimetry as described by Gordon and Webber [10].

2.4. Hydrogen Cyanide (HCN) Production

Bacterial cultures were inoculated by spread plate method on nutrient agar amended with glycine (0.44%) and overlaid with Whatman No.1 filter paper pre-saturated with 0.5% picric acid and 2% sodium carbonate solution (w/v) and incubated at 30±2°C for 4 days. Change in color of filter paper from yellow to brown (light, moderate and deep) is indicative of HCN production.

2.5. Ammonia (NH_3) Production

100 µl of actively growing bacterial culture was inoculated into 10 ml peptone water and incubated at 30±2 °C for 48 hr. After incubation, 1ml broth culture was added with 1ml Nessler's reagent. Development of yellow to deep orange color was considered positive for ammonia production.

2.6. Siderophore Production

10 µl of actively growing bacterial culture was inoculated onto chrome azural S (CAS) agar plate and incubated for 3 to 5 days at 30±2°C. Development of orange/yellow halo around the colonies is considered positive for siderophore production [11].

2.7. Zinc Solubilisation

10 µl of actively growing bacterial culture was inoculated onto tris mineral salts agar medium (D-glucose–10 g, Tris HCl-6.06 g, NaCl-4.68 g, KCl-1.49 g, NH_4Cl-1.07 g, Na_2SO_4-0.43 g, $MgCl_2.2H_2O$-0.2 g, $CaCl_2.2H_2O$–30 mg, and Agar 15 g) amended with 0.2% insoluble zinc compounds [ZnO, $Zn_3(P0_4)_2$] followed by incubation for 3-5 days at 30±2°C. Appearance of clear zone around the colony was considered positive for Zn solubilization.

2.8. Antifungal Activity

Dual culture plate technique was used to test antifungal activity against three phytopathogens (*Sclerotium rolfsii, Macrophomina phaseolina and Fusarium oxysporum*) as per the method described [12]. Inhibition of fungal growth was calculated by using the given formula:

$$I\% = \left[(C\text{-}T)/C\right] x100$$

Where,
I = Inhibition % of mycelial growth (growth reduction over control)
C = Radial growth of fungus on control plate (mm)
T = Radial growth of fungus on plate inoculated with bacteria (mm)

2.9. Invitro Biofilm Formation

In vitro biofilm formation by bacterial isolates was quantified as per the method of O'Toole and Kolter [13] by inoculating 1% culture into 10 ml LB broth and incubation at 30±2°C for 15 days. Adhered biomass to growth tube was thoroughly washed with 70% ethanolic solution of 0.1% crystal violet and absorbance was detected at 590 nm.

2.10. Stress Tolerance Testing

Stress tolerance was tested by growing at salinity (2-10% NaCl), drought (2-10% PEG6000) and temperature (30-50°C) [14]

2.11. Seed Germination Testing

Selected bacterial isolates RS8, MS3, MS19 and MS20 were assessed for their influence on tomato seed germination by paper towel method [15]. Seeds were surface sterilized with 0.1% $HgCl_2$ (5 min) followed by washing thrice with sterile distilled water. Seeds were soaked in cell pellet suspension in 1% CMC solution for 1h. Each seed was placed onto wet paper towel using sterile forceps, rolled and kept in BOD incubator in wetting for 12 days. Seeds treated with sterile nutrient broth served as control. Germination percentage was assessed as per *International Seed Testing Association* (ISTA, 1985).

2.12. Green House Experiments

Pots (15×10 cm) were filled with optimum quantity of unsterile soil, sowed with seeds treated with selected bacterial isolates and grown in mesh house for 30 days during July-August. Untreated seeds served as control. Growth parameters like shoot, root length and total biomass (dry weight) were recorded.

2.13. Identification of Bacterial Isolates

Bacterial isolates were identified morphologically by Gram's staining and biochemical tests according to the Bergey's manual (1994). Molecular identification was done by 16S rRNA gene sequencing using universal primers at MACROGEN (Seoul, Korea). Further BLAST was performed with Ez BioCloud server. Based on analysis result, similarity of bacterial isolates was detected, sequence was deposited at EMBL, accession numbers obtained and Dendrograms for identity of isolates were constructed using Mega-6 software.

2.14. Statistical Analysis

All the experiments were performed in triplicates and repeated twice and data is expressed as mean standard deviation using IBM SPSS statistics 20.

3. Results & Discussion

3.1. PGP Characteristics of Bacterial Isolates

Two hundred bacterial isolates were obtained from different source samples and tested for PGPB traits qualitatively. Four potential isolates labelled as RS8, MS3, MS19 and MS20 showing appreciable P and Zn solubilisation, IAA, HCN, ammonia and siderophore production were selected for further evaluation (Table 1).

Table 1. Invitro qualitative plant growth promoting properties of selected bacterial isolates

PGPR trait observed	Bacterial isolates			
	RS8	MS3	MS20	MS19
P solubilisation (zone in mm)	12	16	16	14
IAA Production	+++	+++	+++	+++
HCN production	+++	++	+++	+++
Ammonia production	+++	+++	+++	+++
Siderophore	+++	+	++	++
Zn solubilisation (zone in mm)	11.4	7.3	8.6	10.6

+: slight, ++: medium, +++: good.

IAA is an important phytohormone for plant growth promotion as it enhances root formation and uptake of nutrients which is beneficial for overall plant growth. Isolate RS8 produced highest IAA (128.3±0.04 µg/ml) followed by MS19 (124.2±0.06 µg/ml), MS20 (121.3±0.07µg/ml) and MS3 (117.1±0.06 µg/ml) (Table 2). These results are superior to recent reports on IAA production by *Bacillus* sp., *Pseudomonas* spp. and *Acinetobacter* sp. (45-111.9 µg/ml) [16]. Phosphorus is a macro nutrient required for overall plant physiological activities. P solubilizing bacteria play vital role and its mineralization makes it available to plant by secreting organic acids. Highest release of phosphate was observed in RS8 (309.6±0.02 µg/ml) followed by MS20 (276.5±0.04 µg/ml), MS19 (271.6±0.04 µg/ml) and MS3 (257.8±0.03 µg/ml) (Table 2) which are superior to recent report on rhizobacteria (30-246 µg/ml) [17]. Plant growth promoting rhizobacteria (PGPR) with efficient biofilm formation perform well under stress in field condition due to increased root colonization compared to planktonic PGPR [18]. In our study these 4 isolates showed significant biofilm formation. Highest biofilm formation was by RS8 followed by MS3, MS20 and MS19 (Table 2). Neelam et al (2010) reported 6% NaCl tolerance by PGPR *P. fluorescence* and *P. aeruginosa* associated with tomato plant [19]. Present isolates RS8, MS20, MS3 and MS19 were tolerant to 8-10% NaCl (Table 2). These results are superior to previous reports on biofilm formation and salt tolerance by *Bacillus* spp. [20]. Drought tolerance is an important property for PGPR [21]. Present bacterial isolates RS8, MS3, MS20 and MS 29 were drought tolerant at highest concentration of 10% PEG6000 (Table 2) which is significant. Thermal stress tolerance is an important aspect of competitiveness among rhizobacterial isolates [22]. When tested at different temperatures (30 to 50°C) isolates RS8, MS20 and MS3 and MS19 were able to tolerate highest temperature at 45° C (Table 2).

Biological control of fungal pathogens are given more prominanace to protect the soil health and environment from chemical fungicides [23]. Antifungal activity of PGPR is an important character for bacterial inoculants. Present isolates were found to be good to inhibit soil born phytopathogens S.*rolfsii*, *M.phaseolina and F.oxysporum* (Table 2). S.*rolfsii* was inhibited more by RS8 (79%) followed by MS20 (75%), MS19 (72%) and MS3 (70%). Inhibition of M. *phaseolina* was more by RS8 (76%) followed by MS20 (69%), MS3 (66%) and MS19 (65%). Inhibition of *F.oxysporum* was high for RS8 (66%) followed by MS20 (63%), MS19 (61%) and MS3(52%) (Table 2). Inhibition of phytopathogenic fungi by the pressent isolates is better than reports on *Bacillus* strains [24].

Table 2. Quantitative PGPR characteristics and biotic stress tolerance of selected bacterial isolates

Isolate label	IAA production (µg/ml)	P solubilisation (µg/ml)	Biofilm formation (OD at 590 nm)	Abiotic stress tolerance (growth as OD at 600 nm)			Antifungal activity (% growth inhibition)		
				Highest growth at (%) NaCl	PEG6000 (10%)	Temperature 45°C	*Sclerotium rolfsii*	*Macrophomina Phaseolina*	*Fusarium oxysporum*
RS8	128.3±0.04	309.6±0.02	0.89±0.01	8 (0.73±0.02)	0.88±0.03	0.83±0.12	79.3±0.15	76.5±0.15	66.8±0.3
MS3	117.1±0.06	257.8±0.03	0.84±0.005	10 (0.84±0.005)	0.61±0.01	0.68±0.02	70.6±0.2	66.7±0.2	52.6±0.2
MS20	121.3±0.07	276.5±0.04	0.82±0.005	10 (0.86±0.005)	0.79±0.04	0.75±0.01	75.4±0.2	69.5±0.25	63.2±0.35
MS19	124.2±0.06	271.6±0.04	0.75±0.02	9 (0.82±0.01)	0.71±0.02	0.71±0.0.1	72.5 ±0.2	65.6±0.25	61.0±0.8

Note: Numerical values are mean ± SD.

3.2. Growth Enhancement in Tomato

Bacterial isolates of present study were found to be exhibiting appreciable PGPR characteristics compared to reports available. Therefore these were further evaluated for tomato plant growth promotion at seed germination and total plant growth in pot culture (Table 3 and Table 4). Seed germination of tomato was found to be highly stimulated by all these four isolates (Table 3 and Figure 1A). Maximum seed vigor index (SVI) was by isolate RS8 followed by MS20, MS3 and MS19. The highest increase in seed germination was 19% by RS8 followed by MS20 (17%), MS3 (17%) and MS19 (14%) which are much higher than earlier report on *Bacillus subtilis* (7.5%) [25]. Impressive increase in tomato plant root length, shoot length and total biomass (dry weight) was observed when inoculated with these 4 bacterial isolates (Table 4 Figure 1B). Highest increase in tomato plant growth (dry biomass) was by RS8 (71%) followed by MS20 (62%), MS3 (57%) and MS19 (47%) (Table 4) which are higher than 19 – 22% increase in tomato plant growth by *Bacillus subtilis* [26].

Table 3. Effect of potential bacterial isolates on tomato seed germination

Treatment	Root length (cm)	Shoot length (cm)	Total Biomass (dry weight mg)	Seed germination (%)	Seed vigour index (SVI)
Control	4.5±0.3	5.6±0.3	10.0±0.4	82±4.4	839±64.01
RS8	7.2±0.1	9.6±0.4	19.1±0.4	98±4.4	1648±43.84
MS20	6.9±0.3	9.2±0.2	17.9±0.4	96±5.47	1554±105.0
MS3	6.6±0.4	8.6±0.3	16.5±0.3	96±5.4	1473±148.2
MS19	6.4±0.3	8.4±0.4	15.1±0.5	94±5.4	1401±113.2

Note: Numerical values are mean ± SD.

Table 4. Enhancement of tomato plant growth by inoculation with potential PGP bacterial isolates

Isolate treatment	Root length (cm)	Shoot length (cm)	Total biomass (dry weight in mg)
Control	8.6±0.3	11.2±0.7	21.3±0.6
RS8	14.5±0.4	19.0±0.5	36.7±0.4
MS20	13.7±0.3	17.8±0.2	34.9±0.1
MS3	13.2±0.5	16.4±0.2	33.5±0.5
MS19	11.9±0.3	16.0±0.2	31.2±1.2

Note: Numerical values are mean ± SD.

Figure 1. Enhancement of plant growth in tomato A. Seed germination by paper towel method. B. Pot study by inoculation with potential plant growth promoting bacterial isolates, RS8, MS20, MS3 and MS19

There is a continuous search for better PGPB to improve crop produtivity. PGPR traits are primary characteristics for selecting bacterial inoculants. Among different bacteria known to promote plant growth, Pseudomonas spp. and Bacillus spp. have been identified as the potential inoculants for commercialization [27]. Gram positive sporulating *Bacillus* spp. are prefered to non spore forming Gram negative bacteria, since sporulating ability of *Bacillus* spp. makes them more robust to resistant adverse condition [28]. First commercial bacterial fertilizer, 'Alinit', was developed from Bacillus sp. resulted that 40% increase in peanut crop yield [29]. Present 4 bacterial isolates primarily identified as Bacillus spp., are found to have potential characteristics to be developed as bioinoculants to promote tomato plant growth. Therefore these were further identified by 16S rRNA gene sequence.

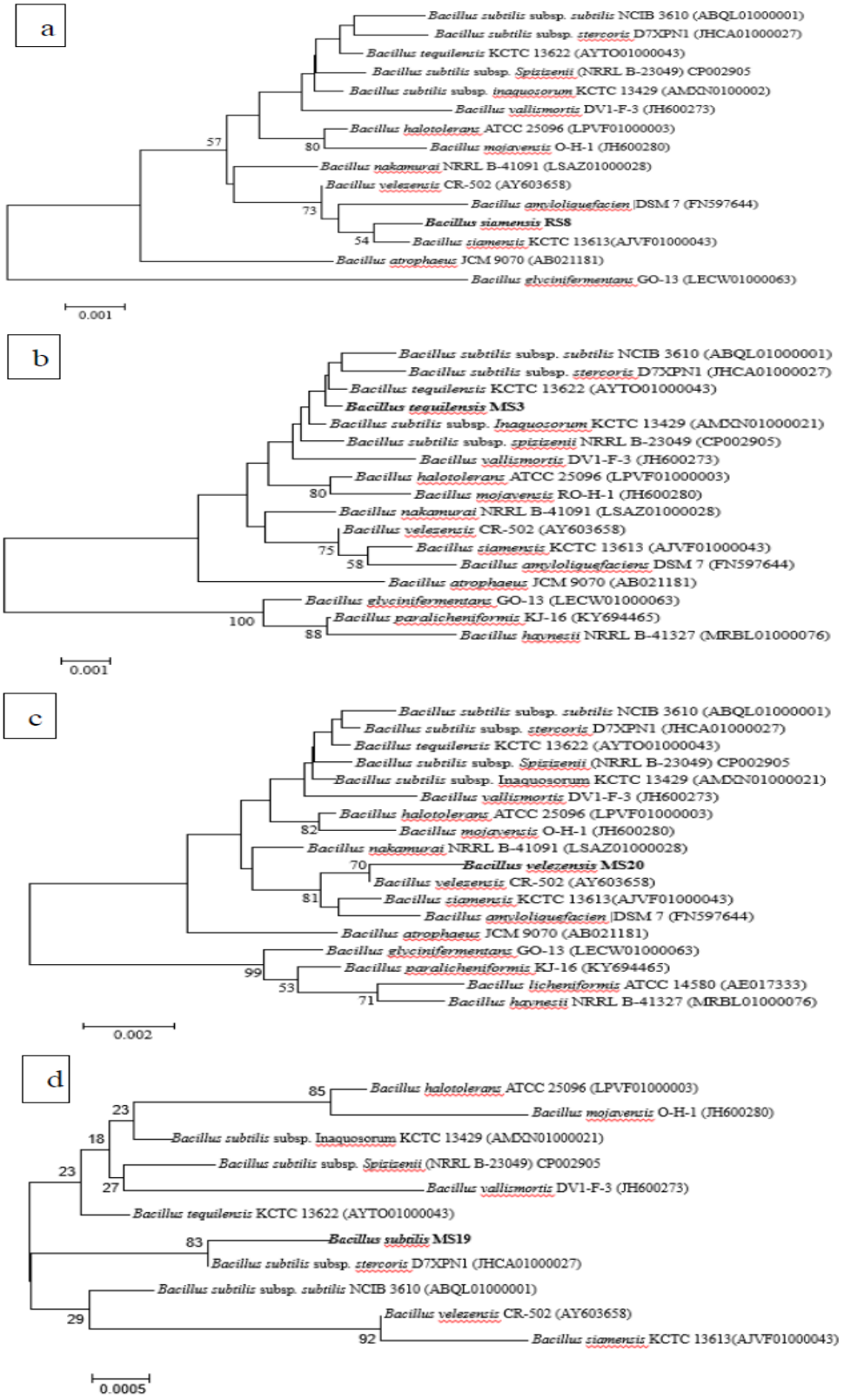

Figure 2. Phylogenetic tree of isolates, a. RS8, b. MS3, c. MS20, d. MS19

Table 5. Molecular identification of potential plant growth promoting bacterial isolates based on 16S rRNA sequence

Isolate label	Source	Cell morphology	16S rRNA Sequence Length	Hit strain with accession number	Similarity (%)	GenBank accession number [EMBL]
RS8	Rhizosphere	Gram +ve rods	1485bp	*Bacillus siamensis* AJVF01000043	99.93%	LR535796
MS3	Marine water	Gram +ve rods	1487bp	*Bacillus tequilensis* AYTO01000043	99.93%	LR536530
MS19	Marine water	Gram +ve rods	1503bp	*Bacillus subtilis subsp stercoris* JHCA01000027	99.92%	LR535810
MS20	Marine water	Gram +ve rods	1273bp	*Bacillus velezensis* AY603658	99.92%	LR535811

3.3. Molecular Identification

Isolates RS8, MS20, MS3 and MS19 were primarily identified as Gram positive, rod shaped, motile sporulating bacteria based on Bergey's manual of determinative bacteriology (1994). Molecular identification of these isolates was done and phylogenetic trees were constructed by neighbor-joining method based on 16S rRNA gene sequence, submitted to EMBL and accession numbers were obtained. These isolates are identified as *B. siamensis* RS8, *B. velezensis* MS20, *B. tequilensis* MS3 and *B. subtilis* MS19 (Figure 2 and Table 5).

4. Conclusion

Four bacterial isolates from rhizosphere and/or marine origin are found to be having good PGP properties and enhanced tomato plant growth at seed germination and pot culture. These can be developed as bioinoculants for improving tomato plant growth and crop yield.

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