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Ecological Diversity of *Lycopersicon esculentum* (Tomato) Root Associated Plant Growth Promoting Rhizobacteria (PGPRs)

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Abstract

Tomato member of family *Solanaceae* is one amongst the foremost important vegetable crop worldwide. It has its significance due to its nutritive, therapeutic and antioxidant properties. An ecofriendly approach to improve the crop yield is the use of PGPRs which improves the growth of plant through nitrogen fixation, phosphorus solubilization and phyto-hormone production. The present study is to evaluate the biodiversity of such PGPRs and their potential role as biofertilizer for tomato crop. A total eight bacteria were isolated and purified from soil and rhizosphere of tomato plant collected from temperate and tropical rainfed regions of Pakistan including Rawalakot and Attock respectively. Soil texture of Rawalakot and Attock varied from sandy loam to loamy. Plant growth promoting traits like N_2 Fixation, P-solubilization and IAA production were determined for all the eight isolates. Maximum P-solubilization was shown by isolates from Attock, AS4 (129.72 µg mL^{-1}) and Rawalakot, RS3 (132.73 µg mL^{-1}) and maximum IAA production was observed in Rawalakot isolates, RS2 (22.237 µg mL^{-1}) followed by Attock isolates, AS3 (49.63 µg mL^{-1}) and AS2 (62.86 µg mL^{-1}). PGPRs were selected with multifunctional properties and were used in plant inoculation experiment to study enhanced growth of tomato plants. Bacterial isolates showed remarkable increase in all growth parameters as compare to uninoculated control. These PGPRs can be best developed for improved development of tomato plants with less dependence on chemical fertilizers.

Keywords: Nitrogen fixation, Phosphorus solubilization, Indole acetic acid

Introduction

Tomato is one among foremost important vegetable worldwide belongs to *Solanaceae* family and contains about 3,000 to 4,000 species in almost 90 genera (Knapp *et al.* 2004). Tomato is a diploid plant with 24 numbers of chromosomes (Bhatia *et al*., 2004). The uniqueness of tomatoes is that they contain an astounding blend of antioxidants like vitamin A, E and C, a few polyphenols, (Tyssandier *et al*., 2004) , β-carotene, carotenoids and flavonoids (Kousar *et al*., 2020). Several soil microorganisms have the ability to get associated with the roots of the plants and stimulate plant growth (Naqqash *et al*., 2020). Several microbial products either directly promote growth or indirectly protect them from diseases. Root colonizing bacteria (rhizobacteria) that exert advantageous effect on plant development via direct or indirect mechanisms have been defined as plant growth promoting rhizobacteria (PGPR) Etesami and Maheshwari (2018). In order to control the chilli bacterial wilt, plant growth-promoting rhizobacteria increase defense-related enzymes and restrict pathogen growth in chilli plants, respectively (Kashyap *et al*., 2021). (Ali *et al*., 2011), P-solubilization (Ganeshan and Manoj, 2005), creation of siderophores which helps in concealment of plant pathogenic microorganisms, (Ahmad *et al*., 2008). In ecosystem, nitrogen (N_2) is the predominant nutrient for all living organisms and mainly exists in dinitrogen form in atmosphere (Ahmad *et al*., 2009). NF (Nitrogen fixing) bacterium can exist in advantageous interaction and in either case captures atmospheric nitrogen or changes over the inert N_2 to NH_3, a structure that is promptly used by plants (Staccone *et al*., 2020). Nitrogen fixers upgrade the development and efficiency as well as result in more advantageous plant that is significantly fit for fighting diseases and microbes just as ready to get by under pressure conditions (Kannapiran and Ramkumar, 2011). Phosphorus (P) is second significant plant macronutrient next to nitrogen (Santana *et al*., 2016). Minute living beings that solubilize phosphorus are insinuated as phosphate solubilizing Bacteria (PSB); these PSBs convert insoluble organic and inorganic phosphorus into soluble form (Alori *et al*., 2017). Phosphorus plays a vital role in each part of the plant development and improvement and numerous physiological and biochemical plant processes including photosynthesis (Kalayu, 2019), advancement of roots, fortifying the stalks and stems, development of seeds (Satyaprakash *et al*., 2017), crop development, root development, cell division and growth (Walpola & Yoon, 2012). The indole acetic acid is the commonest endogenous auxin (Cakmakci *et al*., 2020) normally helpful in improvement and development of plant (Kumla *et al*., 2020). Zinc (Zn) is a basic and essential micronutrient needed for an ideal plant growth (Mahmood *et al*., 2005). Microorganisms that are involved in solubilization of zinc are powerful alternative for conversion of inorganic zinc to accessible form (Kamran *et al*., 2017). Zinc is a basic micronutrient that is necessary for many different physiological and biochemical functions in plants. Lack of it impacts plant growth and development, resulting in lower yields and lower-quality nutrients (Jalal *et al*.,2024). So the PGPR effect on growth parameters of plant is the objective of this study.

Materials and Methods

Isolations and morphological studies: Isolates AS1, AS2, AS3, AS4 and isolates RS1, RS2, RS3, RS4 were isolated from rhizosphere of tomatoes cultivated in agro-ecologically different zones of Pakistan, Attock (33.7660°N,

72.3609°E) and Rawalakot (33.853406°N, 73.751475°E) by serial dilution method. Rhizospheric soil and plant samples were collected and harvested from tomato plants cultivated in Rawlakot and Attock. Rhizospheric soil samples were stored in polybags before transported to UW lab complex and stored at 4°C. The Roots were then shaken gently in the sterile distilled water to detach the loosely adhering soil. One gram of strictly bound soil was added in 9ml 0.85% (w/v) NaCl solution and serially diluted to isolate bacteria on Luria-Bertani (LB) agar media by spreading 20µl from each of the dilutions (10^{-3}, 10^{-5}, 10^{-7}). Selection of eight isolates from huge number of rhizotype obtained was based on morphological and phenotypic characterization. Colony morphology and gram reaction were studied under light microscope. Electrical conductivity (ECe) and pH of soils were measured on EC and pH meters.

Nitrogen Fixation: For estimation of nitrogen fixing microorganisms NFM (Nitrogen Free Malate) medium (Okon et al., 1977).) was prepared and 5ml of NFM medium is shifted to each vial. Then freshly grown bacterial colony was picked and inoculated in each vial. These vials were kept in thermal incubator at 28° C for 9-10 days. Color change from green to blue or yellow was observed and also recorded the bacterial growth. Growth and change in color showed positive result of nitrogen fixation. Color change indicated that they were either alkali or acid producer.

Phosphate Solubilization: Phosphate solubilization was done by both qualitative and quantitative method.

Qualitative assay: Pikovskaya′s medium was used to screen bacterial isolates in vitro for their phosphate solubilizing activity. Pure and freshly grown bacterial isolates from LB broth were spotted on Pikovskaya's agar plates and incubated at 28°C for 7-10 days. Clear zones around bacterial isolates were indicated a positive results for P-solubilization. Zone diameter was measured with the help of ruler and p- solubilization index was calculated by following formula: :(Edi-Premono et al., 1996).

$$\text{PSI} = \frac{\text{Colony Diameter} + \text{Holo zone Diameter}}{\text{Colony Diameter}}$$

Quantitative assay: Pikovskaya′sbroth of 800ml was prepared and transferred 25mL to each Erlenmeyer flask in triplicates and then autoclaved. The bacterial strains were inoculated in flasks containing broth and incubated them in shaking incubator at 28°C for 7-10 days. After 7 days of incubation, broth containing bacterial strain was transferred to sterile falcon and centrifuged for 10 minutes at 13000 rpm. After 10 minutes of centrifugation, supernatant was transferred to other sterile falcons. Glass tubes were washed with ethanol and again with distilled water. One ml of supernatant, 4ml of reagent B (1.056gof Ascorbic acid in 200ml of mixed reagent) and 20ml of distilled water in each test tube were poured. In order to adjust UV Spectrophotometer reading at zero, control was prepared (1ml of distilled water and 4ml of B-reagent). Phosphate solubilization activity of bacterial isolates was determined by usinphosphomolybdate blue color method with the help of M 350 double beam UV-visible spectrophotometer at the wavelength of 882nm.

Production of Indole-3-Acetic Acid (IAA)

Qualitative test for IAA production: First prepared the LB broth, after autoclaving the broth, added 0.1 g/L tryptophan, 1000 µL was transferred to each 1.5 mL Eppendorf tube. Freshly grown bacterial colonies were taken from LB agar plates and inoculated in each Eppendorf tube and then incubated in shaking incubator at 28° C for 7 days. After 7 days of incubation the Eppendorf tubes were centrifuged for 10 minutes. Now sterile Eppendorf tubes were taken and transfer 100 µL of supernatant from each inoculated Eppendorf tube was transferred followed by addition of 100 µL of salkowski reagent in it. Change of color was clearly observed in Eppendorf tube. Samples were kept for 20-25 minutes in dark to observe the change in color. Pink color indicated the positive result for IAA production. (Kamnev *et al.,* 2001)

Quantitative test for IAA production: LB broth was prepared with tryptophan and inoculated with isolates of bacteria. Then growth cultures were placed in shaking incubator at 28±2° C for 7 days. After 7 days of incubation the bacterial cultures were transferred to sterilized falcon and then centrifuged for 30 minutes at 3000 rpm. After centrifugation, the supernatant was separated in another sterilized falcon and then 5ml of salkowski reagent and 2 drops of orthophosphoric acid were added to 2ml of sample supernatant. The samples were kept in dark for 30 minutes. On UV spectrophotometer OD of samples was recorded at 530 nm.

Zinc solubilization test: For confirmation of zinc solubilizing microorganism, zinc media plates were prepared. The pure bacterial colonies were transferred on zinc agar plate with the help of sterile aluminum loop. The plates were than incubated for 5 days at 28°C. Formation of clear zones indicates the positive result for zinc solubilization. BR basal media plates were prepared (Bunt and Rovira, 1955)

Pot Experiment: A pot experiment was conducted to check diversity of PGPRs isolated from rhizosphere of tomato plant using a completely randomized design (CRD). Soil of two different areas Rawalakot and Attock was used for this purpose. The seeds of tomato were sown in moist, sterile petri plate. These plates were placed in a glass house at the temperature of 24-25°C. Germination of seeds took place almost after ten days and tomato seedlings were ready to transplant to selected soils. Eight pots were filled with Rawalakot soil and Attock soil. Tomato seedlings were carefully transferred to the pots (seedlings were dipped in the LB broth inoculum for 10-15-min before transplating). each pot was transplanted with 2 seedings, than placed under controlled conditions in green house. After two days when seedlings were settled in soil they were inoculated by adding 10ml of inoculm with selected bacterial culture. For inoculum preparation all strains were grown in LB broth. The plants were harvested after the twenty (20) days and different growth parameters were determined, height and weight were determined and analyzed by T test.

Results and Discussion

Morphological characteristics and Soil Analysis: Soil sample of Attock is Sandy loam while rawalakot soil is silt loam and loamy in nature. Electrical Conductivity of sample collected from Attock and Rawalakot was 270 µS/cm and 339 µS/cm, soil pH was 4.2 and 8.0 respectively (Table 1). Most of bacterial colonies were irregular and opaque in nature, yellowish and creamy white in color. Few PGPR colonies were also in circular form. All of the bacterial strains were gram negative. Morphological and physiological characteristics of rhizobacterial isolates were shown in (Table 2). AS1-4 indicates bacterial isolates from Attock soil and RS1-4 indicates bacterial isolates from Rawalakot soil while C indicates uninoculated control.

Table 1. Soil Analysis of soil samples collected from Attock and Rawalakot

Sr. No	Parameters	Attock	Rawalakot
1	Texture	Sandy loam	Silt loam, Loamy
2	pH	4.2	8.0
3	Electrical conductivity (EC e)	270 µS/cm	339 µS/cm
4	Total amount of Sodium	23.3 mg/L	26.6 mg/L
5	Total amount of Potassium	8.6 mg/L	6.7 mg/L
6	Amount of Nitrogen	1.152%	0.64%

Table 2: Morphological Characterization of isolates from Rawalakot and Attock,

Isolates	**Colony Morphology**	**Cell Morphology**	**Gram (+ive/-ive)**
AS1	Large, Yellow, Round	Long rods	Gram negative
AS2	Small, Pale yellow, round	Round	Gram negative
AS3	Large, round, creamy white	Short rods	-ive
AS4	Small , Round, orange	Small rods	Gram negative
RS1	Large, round, creamy white	Small rods	Gram negative-ive
RS2	Very small, round, whitish	Short rods	Gram negative-ive
RS3	Large, round, white,	Long rods	Gram negative-ive
RS4	Small , round, whitish	Long rods	Gram negative-ive

Plant beneficial traits: All the eight bacterial isolates were tested for nitrogen fixation ability. Seven bacterial isolates showed excellent growth and color change in medium (AS1, AS2, AS3, AS4, RS1, RS2, RS4) except (RS3). In our study inorganic P-solubilization was shown by six isolates (AS2, AS3, AS4, RS1, RS3, and RS4). Maximum phosphorus solubilization was shown by isolates from Attock, AS4 (129.72 µgmL-1) and Rawalakot, RS3 (132.73 µgmL-1). (Table 3, Figure.1). Ability of phytoharmones (Indole acetic acid) production was observed in five bacterial isolates (AS1, AS2, AS4, RS2, and RS4). Quantitative analysis of IAA production was determined by taking OD at 530 nm on UV – Visible spectrophotometer. Maximum IAA production was observed in Attock isolates, AS3 (49.63 μgmL^{-1}) and AS2 (62.86 μgmL^{-1}) followed by Rawalakot isolates, RS2 (22.237 μgmL^{-1}). All the eight bacterial isolates showed positive result for solubilization of zinc oxide. Two isolates (RS2, RS4) from Rawalakot showed big halo zone as compared to other isolates. (Table.4, Figure. 2 Figure. 3).

Table 3: Quantitative analysis of phosphate solubilization activity by five (5) bacterial isolates

Isolates	**Phosphate solubilization µg mL^{-1}**
AS2	50.91
AS3	29.43
AS4	129.72
RS3	132.73
RS4	63.72
Control	13.11

Table 4: Qualitative analysis of IAA production by 5 bacterial isolates

Isolates	Production of IAA (µg mL^{-1})
AS1	62.86
AS2	49.63
AS4	13.189
RS2	22.237
RS4	16.972
Control	3.686

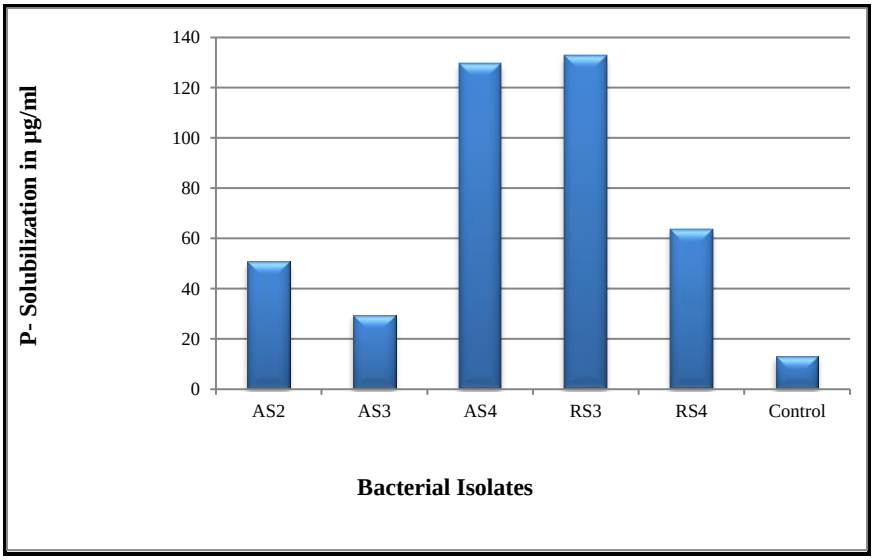

Figure 1: Quantitative analysis of P-solubilization by 5 bacterial isolates

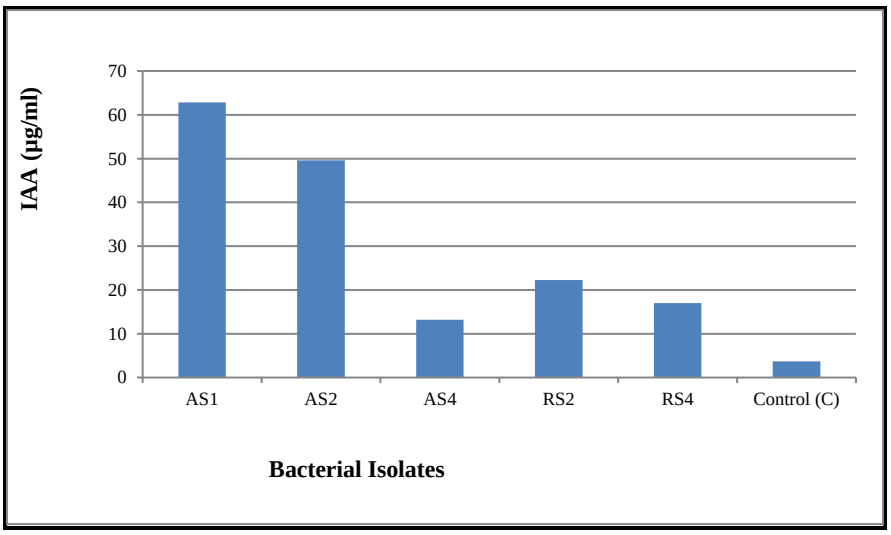

Figure. 2: Intensity of IAA production by bacterial isolates

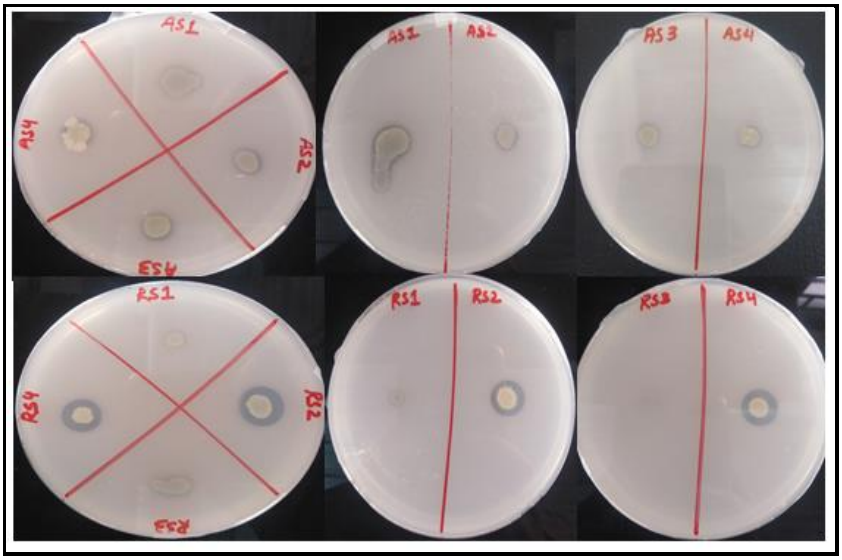

Figure 3: Zinc solubilizing ability of eight (8) bacterial isolates. Arrow head indicates halo zone around bacterial colony. Zone formation shows positive result for solubilization of Zinc oxide.

Pot experiment: Tomato plants inoculated with selected isolates (AS1, AS2, AS3, AS4, RS1, RS2, RS3, and RS4) showed significantly higher root and shoot length, total height and number of leaves as compare to uninoculated control. Inoculation response of isolate from Attock (AS3) and isolates from Rawalakot (RS1 and RS2) was found to be significantly higher in all growth parameters than uninoculated control in tomato plants grown in Attock soil (Table 5). Inoculation response of isolate from Attock (AS1 and AS4) and isolates from Rawalakot (RS2 and RS4) was found to be significantly higher in all growth parameters than uninoculated control in tomato plants grown in Rawalakot soil RS2 has shown maximum height, maximum fresh and dry weight by AS2 and AS3 has more number of leaves as compare to control in all cases. (Table 6)

Table 5: Cumulative Growth parameters of tomato plants grown in Attock soil

Treatments	Total Height (cm)	Total Fresh Weight (g)	Total Dry Weight (g)	Number of Leaves
Control	16.2 ± 1.30	0.3529 ± 0.07	0.0386 ± 0.004	8 ± 2
AS1	19.3 ± 2.22	0.5208 ± 0.11	0.0559 ± 0.006	12 ± 1
AS2	18.2 ± 1.60	0.6028 ± 0.07	0.0720 ± 0.003	14 ± 2
AS3	17.6 ± 2.53	0.5820 ± 0.15	0.0536 ± 0.01	12 ± 3
AS4	19.1 ± 2.18	0.4280 ± 0.10	0.0486 ± 0.001	9 ± 1
RS1	18.3 ± 2.23	0.6060 ± 0.06	0.0528 ± 0.003	13 ± 2
RS2	20.2 ± 2.12	0.5630 ± 0.11	0.0509 ± 0.002	11 ± 2
RS3	18.1 ± 3.02	0.6291 ± 0.16	0.0510 ± 0.005	15 ± 1
RS4	19.2 ± 2.1	0.5908 ± 0.14	0.0509 ± 0.004	14 ± 3

Table 6: Cumulative Growth Parameters of Tomato Plants grown in Rawalakot soil

Treatments	Total Height (cm)	Total Fresh Weight (g)	Total Dry Weight (g)	Number of Leaves
Control	14.2 ± 2.34	0.5223 ± 0.08	0.0461 ± 0.003	9 ± 2
AS1	15.3 ± 1.2	0.5363 ± 0.01	0.0336 ± 0.002	5 ± 2
AS2	14.1 ± 2.12	0.3289 ± 0.13	0.0432 ± 0.005	10 ± 3
AS3	18.2 ±1.49	0.5750 ± 0.07	0.0736 ± 0.007	14 ± 2
AS4	15.3 ± 2.12	0.5189 ± 0.01	0.0226 ± 0.006	8 ± 3
RS1	17.3 ± 1.45	0.4622 ± 0.16	0.0706 ± 0.003	15 ± 2
RS2	18.3 ± 1.89	0.5822 ± 0.11	0.0537 ± 0.003	16 ± 2
RS3	15.4 ± 2.88	0.5336 ± 0.02	0.0402 ± 0.002	9 ± 3
RS4	15.8 ± 2.45	0.3863 ± 0.01	0.0498 ± 0.003	8 ± 2

Discussion

Lycopersicon esculentum is the second most important vegetable crop in Pakistan because of its significance in nutritive, therapeutic and antioxidant properties (Knapp *et al*., 2004). A collection of PGPRs is currently being utilized worldwide with the point of upgrading plant efficiency, for example, Bacillus, Enterobacter (Cocking, 2003), Burkholderia, Acinetobacter, Alcaligenes, Azospirillum, Erwinia, Rhizobium, Flavobacterium and Serratia (Burd et al., 2000). For some farming crops, the advantageous impacts of PGPRs have been shown, for example, Triticum aestivum (Khalid et al., 2004), Nicotiana tabacum, leaf mustard, Lycopersicon esculentum (Kidoglu et al., 2007), chime peppers and cucumbers (Cakmakci et al., 2007). The objective of present study was to evaluate the biodiversity of tomato root associated PGPRs and their potential role as bio-fertilizer for the crop. Henceforth, the researchproject was aimed to isolate, identify and characterize the nitrogen fixing, phosphorus solubilizing and phytohormone producing rhizobacteria of tomato plants growing under temperate and tropical rainfed regions of Pakistan. Some selected PGPRs were then cross checked on soils from tropical and temperate rainfed regions to establish their diversity. Most of bacterial colonies were irregular and opaque in nature, yellowish and creamy white in color. Few PGPR colonies were also in circular form. Many of the bacterial isolates were found to be motile with many small rods and some long rods. All of the bacterial isolates were gram negative. Sakai et al., (1996) reported that the bacterial motility was significant factor in colonization of roots. Bano and Fatima (2009) reported that the inoculation of phosphate solubilizing microbes had ideal impact on saltiness stress resilience of *Zea mays* under stress of sodium chloride. Son et al., (2006) have reported that the P-solubilizing *Pseudomonas* species improved the nodules mass, quantity, yield of grain, accessibility and uptake of nutrients in soybean. In our study inorganic P-solubilization was shown by six isolates. Maximum phosphorus solubilization was shown by isolates from Attock, AS4 (129.72 µgmL-1) and Rawalakot, RS3 (132.73 µgmL-1). Indole-3-acetic acid (IAA) is a significant and most bountiful phytohormone normally helpful in improvement and development of plant (Kumla *et al*., 2020). It has been reported that more than 80% of bacteria present in rhizosphere are potential indole acetic acid producers which directly improve the cell enlargement, biosynthesis of various metabolites, development of root elongation and stimulation of nitrogen fixation (Khan *et al*., 2014). Ability of phytohormones (Indole acetic acid) production was seen in five bacterial isolates. Quantitative analysis of IAA production was determined by taking OD at 530 nm on UV – Visible spectrophotometer. Maximum IAA production was observed in Rawalakot isolates, RS2 (22.237 μgmL^{-1}) followed by Attock isolates, AS3 (49.63 μgmL^{-1}) and AS2 (62.86 μgmL^{-1}). Naqqash *et al*. (2020) reported the bacteria from rhizosphere of

potato that produces highest amount of indole acetic acid measured by UV- spectrophotometer method that was in the range of 312.14 μgmL^{-1}. Among different bacterial species production of indole acetic acid varies as influenced by condition of culture, different growth stages and substrate (s) availability (Malik and Sindhu, 2011). Zinc is a basic and essential micronutrient needed for an ideal plant development (Mahmood et al. 2005). Saravanan et al (2003) has reported the Zn solubilizing capacity of other microbes including Bacillus spp. that can be used instead of chemical fertilizers. All the eight (8) bacterial isolates showed positive result for solubilization of zinc oxide. Two isolates (RS2, RS4) from Rawalakot showed big halo zone as compared to other isolates. Various microorganisms have ability to fix atmospheric nitrogen (Mirza *et al*., 2006), and the most widely recognized among these incorporate Acetobacter, Burkholderia, Enterobacter, Pseudomonas and Cynobacteria (Vessey 2003). Naqqash *et al*. (2016) isolated five PGPRs from rhizosphere of potato on Nitrogen free media and these inoculated isolates exhibited increased fresh and dry weight along with nitrogen content in roots and shoots. They concluded that TN10 was the potential bacteria that could raise the uptake of nitrogen in potato plant.

Conclusion and Recommendations

In the present study total eight bacterial isolates were screened for nitrogen fixing ability by using NFM medium. Seven (7) bacterial isolates showed excellent growth color change in medium (AS1, AS2, AS3, AS4, RS1, RS2, RS4) except (RS3). The pot experiment was conducted to check the biodiversity of selected bacteria in two types of soil from Rawalakot and Attock. Overall eight (8) PGPRs were selected with multifunctional properties and were used in plant inoculation experiment to observe the enhanced growth of tomato plants. There is a remarkable increase in all growth parameters like root and shoot length, root and shoot fresh weight, root and shoot dry weight and number of leaves as compare to uninoculated control. Bacterial isolates (AS3, RS1 and RS2) showed remarkable increase in all growth parameters when inoculated in tomato plants grown in Attock soil while bacterial strains (AS1, AS3, RS1 and RS2) showed increase in all growth parameters when inoculated in tomato plants grown in Rawalakot soil. On the basis of results of in vitro bacterial characterization and in vivo growth studies in various soils, it can be concluded that isolates from Attock (AS2, AS3 and AS4) and from Rawalakot (RS2 and RS4) are potential Plant Growth Promoting Rhizobacteria. These PGPRs can be best developed for improved development of tomato plants with less dependence on chemical fertilizers. In diverse agro climatic nature of soil, these isolates therefore, can be used as biofertilizers for crop of *Lycopersicon esculentum.* In future detailed morphological, biochemical and genetic analysis can be performed to identify the bacterial strains.

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Novelty Statement

Bacterial isolates (AS3, RS1 and RS2) showed remarkable increase in all growth parameters when inoculated in tomato plants grown in Attock soil while bacterial strains (AS1, AS3, RS1 and RS2) showed increase in all growth parameters when inoculated in tomato plants grown in Rawalakot soil ,

Author's contribution

JR and SH conceived and designed the experiments. JR performed the experiments. NM performed the inoculation experiment in the field. JR analyzed the data and wrote the manuscript. NM reviewed the manuscript. All authors have read and approved the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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