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ISOLATION, SCREENING CHARACTERIZATION, OF PGPR AND THEIR ANTAGONISM TEST AGAINST ALTERNARIA SOLANI

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are a group of beneficial bacteria that can be found in the rhizosphere, in association with root which can enhance the growth of plant directly or indirectly by several mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticide and supplements. In the present study, A total six bacterial isolates in vitro screening was done for different plant growth promotion activities i.e. phosphate solublization, IAA production, Siderophore production. Six isolates of bacterial, designated as TR25, TR21, TR22, TR23, TR24, and TR15, were successfully isolated and characterized. In the present work five bacterial isolates were positive for IAA production and siderophore production and one (TR25) isolates were found to be positive for phosphate solublization. Most of the isolates grown best under the temperature of 200C & 280c when compared to 100c & 370C and very few can grown at 450c, Furthermore, most of the PGPR isolates shown antifungal activity against Alternaria solani. The present study, there for, suggest that the use of PGPR isolates TR25, TR21, TR15, and TR22 as inoculants /biofertilizers might be beneficial for tomato cultivation as they enhanced growth of tomato due to production of Indole acetic acids, phosphate solublization and also having antifungal activity against phytopathogenic fungi.

KEYWORDS: *Plant growth promoting rhizobacteria, antagonism assay, siderophore*

production, phosphorous production, IAA production.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a group of microorganism in the rhizosphere that promote plant growth by increasing nutrient availability and may be used as inoculants for biofertilization phytostimulation and biocontrol and can be classified according to their beneficial effect.(Bloemberg & Lugtenber., 2001., Gwyn, 2006). The use of PGPR is steadily increasing in agricultural and offers an attractive way to replace chemical fertilizer pesticide, and supplement. PGPR have been applied to various crop to enhance growth .seed emergence and crop yield and some have been commercialized. Application of some PGPR strain to seed or seedling has also been found to lead to a state of induced system resistance in the treated plant Kloepper et al., 1999) In addition to improvement of plant growth of PGPR are dinitrrectly involved increased uptake of nitrogen synthesis of phytohormones, solubilizing of minerals such as phosphorous. And production of siderophores that chelate iron and make and it available to plant root. Inoculants development has been most successful to deliver biological control agent of plant disease i.e. Organism capable of killing other organism pathogenic or disease causing to crop. Various bacteria which are predominantly studied and increasingly marketed as the biological control agent includes as the genera Bacillus, Pseudomonas, Agrobacterium, Streptomycin. They suppress plant disease through at least one mechanism induction of systemic resistance and production of siderophores or antibiotic. Exposer to the PGPR triggers a defense response by the crop as if attacked by pathogenic organism Joseph et al., 2007.

2 MATERIALS AND METHODS

2.1 Isolation of PGPR from tomato rhizosphere

Soil sample were collected from the rhizosphere of tomato plant in the areas of Indian Institute of Vegetable Research in Varanasi district. The rhizosphere was dugout with interact root system. The sample were placed in plastic bags and stored at 4C0 in refrigerator. Ten grams of rhizosphere soil were taken in a 250 ml of conical flask. And 90 ml of sterile distilled water was added to it. The flask was shaken for 10 min on rotatory shaker. One milliliter of suspension was add to 10 ml vial and shaken for 2 min. Serial dilution technique was performed up to 10-7 dilution. An aliquot (0.1) of this suspension was spread on the plates of Luria –Bertany (LB) agar medium. Plates were incubated for 3 days at 28 0C to observe the colonies of bacteria. Bacteria colonies were streaked to other LB agar plates and

the plates were incubated at 28 0C for 3 days. Typical bacterial colonies were observed over the streak well isolated single colony was picked up and re-streaked to fresh LB agar plates and incubated similarly. The technique was perpetuated thrice and cultures were made single colony.

2.2Growth under different temperature conditions

The culture of 15 isolates was streaked on LB agar plates and incubated at 10, 28, 37 0C and 45 0 the change in growth and color was observed and recorded after 3days of incubation.

3 In vitro screening of isolates for plant growth promoting activities

3.1 Phosphate solublization

The culture of Six isolates were streaked on Pikovskaya's agar plates for phosphate solublization under aseptic conditions and incubated for 3 days at 30 0C. The plates were then examined and data were recorded .visual detection and semi quantitative estimation of phosphate solublization ability of microorganism is possible by plate screening methods that show clear halo zone around the microbial colonies in media containing insoluble minerals phosphates (tricalcium phosphate) as sole P source.

3.2 I AA Production

Indole acetic acids (IAA) production was carried out as described by Brick et al.(1991) The culture of 15 isolates was incubated in the nutrient broth enriched with 50mg/ml of L-tryptophan at 30 0C for 48h. Fully grown cultures were centrifuge at 8000rpm for 10 min. The supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Solkowski reagent (50ml.35% of perchloric acids, 1 ml 0.5 M FeCl3 solution). The tubes were kept at room temperature for 20 minutes. Development of pink color indicates IAA indicates IAA production.

3.3 Siderophore production

Siderophore production was tested qualitatively using chromo azurol S medium (CAS-medium The culture of 15 isolates were streak on the surface of CAS agar medium and incubated at room temperature for 1 to 3 days siderophore production was indicates by orange halo around the colonies after the incubation and these was done in two replications**.**

3.4 Antagonism activity against A. solani fungi

All the Six isolates were assayed for antifungal activities against Alternaria solani by using

potato Dextrose Agar (PDA) medium. The isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at the centre of the medium. The barrier between isolates and fungi indicated antagonistic interaction between them. Antagonist activity was investigated for 4 to 7 days after incubation at room temperature. The value of inhibition zone was measured using the formula described by Kumar et al., 2002. Which is 1-(a/b)×100% (a ; distance between fungi in the centre of Petri dish to test isolates, b; distance between fungi in the centre of Petri dish to blank are without isolate).

4 In vitro efficacy of biocontrol agent on growth parameters of tomato seed bacterization

Seed (one gram) of tomato of tomato –DVRT-1 surface sterilized with 2% sodium hypo chloride for 20 second; rinsed in sterile distilled water and driedovernight were treated with Ten ml of biocontrol inoculums containing not less than 3×108 cfu ml-1. One hundred mg of Carboxyl Methyl Cellulose (CMC) was added as an adhesive material. The treated seeds were kept for two hours and air – dried overnight in a sterile Petri dish and used for sowing.

5. Plant growth promotion

The plant growth- promoting activity of biocontrol agent was assessed based on the seedling vigor index by the standard roll towel method.[12] Seed bacterization was done as described above. Twenty five treated seeds were placed on a pre –soaked germination paper. The seed were placed on a pre-soaked germination paper. The seeds were held in position with another germination Paper. The seed were held in position with another germination paper strip and gently pressed. The polythene sheet along with the seeds was then rolled up and incubated in a growth chamber for 10 days. The replication of each treatment and a suitable control were also maintained. After incubation the root length and shoot length of individual seedling were measured and percent germination was calculated using the formula. Vigor index = (mean root length ÷ mean shoot length) × %germination.

6 Preparation of selected PGPR bioformulation

A loopful of selected bacterial isolates were inoculated in to the sterilized KB and Nutrient broth respectively and incubated in a at shaker at 150 rpm for 48 h of incubation, the broth containing 9×108 cfu /ml was used for the preparation of talc-based formulation, to 400 ml of bacterial suspension. 1 kg of talc powder (sterilized at 12h), calcium carbonate 15 g (to adjust the PH to neutral) and carboxyl Methyl Cellulose (CMC) 10 gm Co adhesive) were mixed under sterile conditions, following method described by.[14] After shade drying overnight, it

was packed in polypropylene bag and sealed, at the time of application the population of bacteria in talc formulation was not less than 2.5 - 3×108 cfu/g. For bacterial stain mixture, the bacterial strains were grown separately and the strain that are going to make up the mixture were add equally (v/v) and finally mixed with talc powder CaCo3 and CMC.[14]

7 Efficacyofbioformulation mixture on the incidence of A. solani under greenhouseconditions.

A pot culture experiment was conducted with individual and combination of rhizosphere and entophytic bacteria treatment. Seed of tomato DVR-1 were sown in earthen pots (size -0.35 m diameter, 0.50 m high, volume of soil: 0.04 m3) filled with sterilized potting soil @ five seeds per pot. The talc based product was dissolved in water (20 g/l) and allowed to settle for 1 h, filled through muslin cloth and the filtrate was used for spray.The fungicide macozeb 0.2 % spray was used as a positive control. The talc based bioformulation mixtures were sprayed at 45 days after sowing and one day after spray, the plants were inoculated with the spore suspension (5×106 spore /ml) of A solani. The plant inoculated with the pathogen alone served as inoculated control. Ten days of inoculation, observation on development of early blight symptoms were maintained for each treatment; each replication consisted of five pots and in each pots four plants were maintained in completely a randomized design under glasshouse conditions. The trials wererepeated one and the data presented were pooling of two glasshouse trials. The intensity of disease was recorded in each treatment following the 0-9 scale score chart (0- Healthy; 1-1 to 5% 3-6 to 10% 3-6 101; 5-1 ‘25%; 7- 26 to 50% and 9- 51% leaf area infected) proposed by Ramakrishna et al.[15] The percent disease incidence (PDI) was estimated using the formula suggested by Mickinney.[16]

8 RESULTS

Six bacterial isolates were successfully isolated from the rhizosphere soils of tomato field from different areas in Indian Institute of Vegetable Research in Varanasi district. (Table 1). They are designated as TR25, TR21 TR24, TR18, and TR15, TR23.

Table Description of the PGPR isolates.

S.NO	Isolates the field	Location of rhizosphere	Variety of tomato in
1	TR25	Indian Institute of Vegetable Research	DVRT-1
2	TR23	Indian Institute of Vegetable Research	DVRT-2
3	TR24	Indian Institute of Vegetable Research	DVRT-1
4	TR15	Indian Institute of Vegetable Research	DVRT-6

5	TR18	Indian Institute of Vegetable Research	DVRT-2
6	TR21	Indian Institute of Vegetable Research	DVRT-

9 Microscopic Observation of PGPR isolates

Microscopic observations were performed to investigate the some characteristics of PGPR isolates such as shape. Gram reaction and motility (Table-3) each isolates were rod shaped while two TR25, TR21 showed ellipsoidal shape. All the isolates were motile and Gram negative in reaction. It was also noted that the Growth of isolates on LB agar plates varied in temperature.

Table 3: Cell shape, Motility and Gram reaction of PGPR isolates.

Isolates reaction	cell shape	Motility	Gram
TR21 negat	Ellipsoidal	Motile	Gram-
TR24 nega	Rod	Motile	Gram –
TR25 -neg	Rod	Motile	Gram
TR18 -ne	Ellipsoidal	Motile	Gram
TR23 ne	Rod	Motile	Gram -
TR22 -ne	Rod	Motile	Gram

Table 4: Growth of PGPR isolates at different temperature conditions.

Isolates	Temperature				
10 0C	20 0C	280C	370C	450C	
TR21	+	++	++	+	-
TR25	+	+ +	++	++	-
TR22	+	++	++	++	-
TR15	+	++	++	++	-
TR23	++	++	++	+	-
TR24	++	++	++	++	_

10 Production of IAA and Solublization of phosphorous

We investigated the IAA production and phosphorous solublization of PGPR isolates. As shown in table out of 6 isolates 5 isolates TR25, TR21, TR24, TR15, TR22 induced the production of IAA. Isolates TR25, TR21 were found to be good producers of IAA. On the other hand only TR25 isolates had ability to solubilize the phosphorous.

PGPR isolates Isolates	IAA production	Phosphorous solubilization	Siderophore production
TR21	+	Not solubilizing	Positive
TR22	+	Not solubilizing	Positive
TR23	+	Not solubilizing	Negative
TR18	+++	Not solubilizing	Positive
TR25	+	solubilizing	Positive
TR24	+	Not solubilizing	Positive

Siderophore production

Out of six isolates 5 isolates TR25, TR21, TR22, TR18 and TR24 were able to produced siderophore and it is confirmed by the development of orange halossurrounding to those colonies. (Table 4).

Antagonistic assay against phytopathogenic fungi

Opposition assay was used to determine the isolates that inhibit the growth of Alternaria solani. In this study among six PGPR isolates that significant promoted plant growth of tomato seedling there were all isolates TR25, TR21, TR22, TR24, TR15, TR23) that were able to inhibit Alternaria solani. However the application of TR22 and TR18 show highest mycelial growth with 53.28 to 62.17.

Dual culture experiment with different isolates of selected PGPR and *Alternaria solani (ALT-3)*

	Culture + PGPR	% of Inhibition			
		R_1	R_2	R_3	Mean
1	A.S.+TR21	28.00	32.58	43.20	34.59
2	A.S.+S-TR15	16.00	32.58	23.45	24.01
3	A.S.+TR22	48.00	55.05	56.79	53.28
4	A.S.+TR24	12.00	28.08	19.75	19.94
5	A.S.+TR25	20.00	38.20	50.56	36.25
6	A.S.+TR18	14.66	32.58	24.69	23.98
7	Control	0	0	0	0.00

Effectiveness of seed treatment with PGPR strain on plant growth promotion under in vitro condition

Treatment no	Biocontrol strain	% of germination	Shoot length	Root length	Vigour index
T1	TR21	93.01	16.6	18.2	3236.748
T2	TR22	88.1	14.9	17.8	28808.7
T3	TR24	89.5	15.5	17.9	2989.30
T4	TR15	90.5	16.2	18.4	3131.30
T5	TR25	93.5	18.6	21.2	3721.30
T6	TR23	90.5	15.3	16.3	2802859.
7	Control	80.0	14.3	16.4	2456.00

Efficacy of PGPR and PGPE strains on early blight incidence of tomato under greenhouse conditions

Treatment	Biocontrol strain	Disease severity	Reduction
1.	TR21	32.1	45.3
2.	TR22	32.8	44.1
3.	TR15	34.8	40.2
4.	TR24	39.4	36.5
5.	TR23	30.4	48.2
6.	TR25	22.6	61.5
7.	Mancozeb(0.2%)	18.5	69.5
8	control	54.1	00

Effectiveness of PGPR strain in plant growth promotion

The treatment with biocontrol agent TR25, TR21, TR15, TR24 Produced significant higher vigor index (TR25) of tomato than other biocontrol strain TR25, TR21, TR22, TR15- whose vigor index 3721.30,3236.748,28808.7, 3131.30, recorded respectively. The untreated control seedling had the lowest vigor index (-) Table-2.

Effectiveness of PGPR strain on early blight incidence under green house condition

All the treatment with biocontrol strain significant reduced the PDI (by35 -61 %) compared to untreated control, and increased the seed germination (by 88%-93 in Table) conspicuously; the treatment of TR25 resulted in a significantly lower early blight disease severity than any of the strains treated individually.

DISCUSSION

PGPR colonize plant root and exert beneficial effect on plant growth and development by a wide variety of mechanism. To be an effective PGPR. Bacteria must be able to colonies roots because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects. The exact mechanism by PGPR stimulate plant growth is not clearly established. Although several growth is not clearly established. Although several hypothesis such as production of phytostohormones, suppression of deleterious organism .activation of phosphate solublization and promotion of the mineral nutrient uptake are usually believed to be involved Liu et al 1992 and Joseph et al 2007.

IAA, a member of the group of phytohormones, is generally considered to be the most important native auxin. IAA may the function as important signal molecules in the regulation of plant development of six isolates are positive for IAA production (Table No,) Among them, two isolates TR25, TR21 are found to be good producers of IAA (Table) (Mirza et al

2001) has been reported that IAA production by PGPR can vary among different species and strains, Sarwar et al 1991. Found that moreover isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soils.

Phosphorous is one of the major nutrient second only to nitrogen in requirement for plants. Most of phosphorous in soil is present in the form of insoluble Phosphates and cannot be utilized by the plants Pradhan et al reported that the ability of bacteria to solubilizing minerals phosphates has been of interest to agricultural microbiologist as it can enhance the availability of phosphorous and iron for plant growth. PGPR Verma et al 2001 have been shown to solubilized precipitate phosphate and enhance phosphate availability to tomato that represents a possible under field conditions. In our experiment, only TR25 isolate was able to solubilized phosphate in the rhizosphere soil (Table 5) Further this isolate was found to noted that several phosphate solubilizing bacilli occurs in soils.

Siderophore is one of the biocontrol mechanism belong to PGPR group under iron chelating condition. PGPR produce a range of siderophore which have a very high affinity for iron .Therefore the low availability of iron in the environment would suppress the growth of pathogenic organism including plant pathogenic fungi.

Whipps JM 2001 have found that in addition to siderophore there are other mechanism of biocontrol including antibiotics compound, elicitation of induced systemic resistance (ISR) of plant. This has been demonstrated that the 6 PGPR isolates classified as plant growth promoter and produced siderophore such as TR25, TR21, TR24, TR22, TR23 and TR15 and these isolates already found to be IAA producer.

Based on the siderophore produced by those isolates. It has been determined that all the isolates which produced the bioactive compound siderophore were able to inhibit the phytopathogenic fungi (Table). The result of this study suggests that siderophore produced by those bacteria function as suppressor to that the growth of phytopathogenic fungi such as Alternaria solani.

Earlier it has been reported that the biocontrol agent such as Trichderma viride and Pseudomonas fluorescence significance reduced the mycelial growth, spore germination, spore production and germ tube formation of A solani and A. Alternata.[19] Result from the present study clearly indicates maximum reduction in mycelial growth due to the biocontrol

strain in reducing the mycelial growth of the pathogen .result in reducing the disease severity. Thus the ecofriendly nature of antagonist bacterial formulation is advantage over the use of the tomato plants.

CONCLUSION

The result of the present study demonstrated that isolated strain e.g. PGPR () (Rhizosphere bacterial strains.) is a promising approach for the ecofriendly management of early blight disease caused by A. solani and enhancing the growth of the tomato plants.

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