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Selection of antagonistic rhizobacteria against soil borne pathogens

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Soil borne pathogens, *Fusarium oxysporum* f.sp. *lycopersici* and *Sclerotium rolfsii*, cause wilt and collar rot respectively, are the major limiting factors of tomato cultivation. To structure an eco-friendly management of these diseases, two isolates, *viz.* Chilli-1 and Chilli-2 were screened from 142 bacterial isolates for their superior efficacy of inhibiting the two pathogens through dual culture studies. In addition of being potential biocontrol agents based on siderophore production, these two isolates also manifested growth promoting activities due to their ability to produce Indole acetic acid and solublization of phosphate. Under green house conditions, talc based formulation of both Chilli -1 and Chilli-2 recorded 95 % and 96.6 % reduction in incidence of *F. oxysporum* f.sp. *lycopersici* respectively as compared to pathogen inoculated control. Similarly they also gave appreciable control against *S. rolfsii* where the reduction in disease severity was 90.5 % by Chilli 1 and 98.1 % by Chilli 2. Observations on field experiments also gave similar results in terms of disease control, plant growth promotion and yield.

Key words : Plant growth promotion, antagonist action, rhizosphere

INTRODUCTION

Rhizobacteria are rhizosphere living microorganism which may be beneficial or inhibitory to the plant growth (Beauchamp 1993; Chen *et al.*, 2000). Among the rhizobacteria, few are beneficial to plant by promoting growth through direct or indirect means, are called as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1980). The mechanism of plant growth promotion associated with PGPR can be broadly classified into two major groups. The first group involves the direct promotion of plant growth by production of phytohormones (auxin, cytokinin, and gibberellin) or increase the level of nutrients by siderophore production, phosphate solubilization, and nitrogen fixation and assimilation. Indirect promotion occurs when PGPR promotes the growth conditions mainly by acting as biocontrol agents for controlling the pest and diseases. Many modes of action of PGPR biocontrol have been documented including production of antibiotics, fungal cell wall lysing enzyme and hydrogen cynide (Saravanakumar *et al.*, 2006). Similarly, PGPRs have the ability to compete with pathogens for the nutrients and making the macro and micro nutrient unavailable to pathogens and that action is called as competition. Also, PGPRs induced systemic resistance (ISR) has been reported against many diseases in wide range of crops (Kloepper and Beauchamp, 1992; Liu *et al.*, 1995; Chen *et al.*, 2000). Recent trends, an eco-friendly management of agriculture and horticulture crop diseases have paved the way to explore the potentiality of the beneficial microorganisms such as PGPRs. Hence, the present study focuses on utilization of PGPR for the management of major fungal diseases of tomato.

MATERIALS AND METHODS

Isolation of rhizobacterial isolates and fungal pathogens

Plants with high vigour were uprooted and the soil adhered to the roots were collected and sieved. About 10 g of soil was mixed in 100 ml of sterile distilled water and the supernatant was serially diluted up to 10^{-6}. From the final dilution, 1 ml suspension was mixed with Kings B medium and poured in the Petri dish. After 48 hrs, the bacterial

colonies were selected on the basis of morphological characteristics and purified through subculturing. The pure cultures were subcultured on slants and stored in glycerol stock at -70°C for further use. Fungal pathogens viz., *Fusarium oxysporum* f.sp *lycopersici* (*FOL*) and *Sclerotium rolfsii* were isolated from the infected plants using potato dextrose agar (PDA) medium. The fungi growing on plates were identified based on the morphological characters (Ellis, 1971; Nelson *et al.*, 1983). Sand-maize (19:1) inoculum was prepared and inoculated with 7 days old pathogen culture (s) and allowed to multiply for 15 days.

In vitro vigour test

Initially, all the bacterial isolates were cultured for 48 hrs in 5 ml liquid medium separately and later they were multiplied in 50 ml liquid medium upon inoculation of one ml of the initial culture. The cultures were kept on rotary shaker at 150 rpm for 48-72 hrs. After that the cultures were centrifuged at 4000 rpm for 10 min and the pellets were collected. Seeds of tomato (cv. DVRT-1) were treated with pellets (approximately 3 xIO^8 cfu/ml) and shade dried for overnight. The treated seeds were arranged on the vigour test paper and rolled from one end to other side as per the standard procedure (ISTA, 1993) and the each treatment was replicated thrice. The experimental materials were kept at 28±2°C and 70% RH with continuous light. After 7 days, the observations on germination (%), root and shoot lengths were recorded. The seedling vigour index was calculated as described by Abdul Baki and Anderson (1973) using the formula. Vigour index = (Mean root length + Mean shoot length) x germination percentage

In vitro dual culture experiment

PGPRs were tested for their inhibitory action on the mycelial growth of the pathogens (*FOL* and *S. rolfsii*) by dual culture plate method (Dennis and Webster, 1971). Per cent inhibition of pathogen over control was calculated using the formula : Per cent inhibition over control = (C-T/C) x 100 where, C, mycelial growth of the pathogen in control, T, mycelial growth of the pathogen in dual culture

Siderophore production

Individual isolates were grown in the broth culture for 48 hrs. The cultures were spot inoculated on CAS (Chrome Azurol S) medium and incubated for 5 days at 28° C (Schywan and Nieland, 1987). Observations were made on the inhibition zone and the diameter of the zones.

Phosphate solubilization

Pikovaskya's medium was prepared with known amount of inert phosphorus source and the medium was inoculated with rhizobacterial isolates and incubated at 28° C for 3-4 days. Observations were taken for phosphate solublization by observing the inhibition zones on the medium (Haider *et al.*, 2005).

IAA production

Nutrient broth amended with tryptophan (50 µg/ml) was inoculated with PGPR cultures and incubated for 24 hrs at 25°C on rotary shaker at 150 rpm. The cultures were centrifuged at 10,000 g for 15 min. To 2 ml of supernatant, 4 ml of reagent (1 ml of 0. 5 M $FeCl_3$ in 50 ml of 35% $HCl0_4$) followed by 2-3 drops of O-phosphoric acid were added and incubated for 25 min. After the incubation, absorbance was measured at 530 nm.

Effect of PGPR on collar rot and wilt diseases of tomato under greenhouse conditions

Talc formulation of PGPR isolates Chilli 1 and Chilli 2 were prepared as per the standard protocol (Vidhyasekaran and Muthamilan, 1995).The seeds of tomato (DVRT-1) treated with 48 hrs old broth culture of the bacterial isolates, dried for overnight and sown in pots containing sterilized soil. After 24 days, the seedlings were uprooted and dipped the root portions of the seedlings in 10 % talc bioformulatiuon solution for 30 min. The treated seedlings were transplanted into the pot containing sterilized soils treated with different PGPR talc formulation (@ 10 g/kg of soils) and the sand maize pathogen culture (@ 10 g/kg soil). Observations on disease incidence and yield were calculated 45 days after transplanting.

Effect of PGPR on collar rot and wilt diseases of tomato under field conditions

Similar to greenhouse experiments the seedlings were raised in pot soil amended with different talc formulation. After 30 days, seedling roots were treated with 10 % talc formulation solution for 30 min and transplanted into field amended with talc formulation @ 2.5 kg/ha mixed with 50 kg FYM. The each treatment was replicated four times and randomized block design (RBD) was followed. Observations on disease control and plant height were measured 45 days after transplanting.

RESULTS AND DISCUSSIONS

About 142 rhizobacterial isolates were isolated from rhizospheric regions of different vegetable crops and identified based on their morphological characteristics. All the bacterial isolates were checked for their induction of growth promotion on tomato crop, among them 96 isolates showed

Table 1 : Effect of seed treatment with PGPR isolates on vigour index of tomato

Isolate name	Host	Germination (%)	Shoot length (cm)	Root length (cm)	Vigour index
T2-7	Tomato	95.0	12.3	13.9	2508.0^{g}
Tomato-8	Tomato	95.0	13.5	16.0	2783.5^{s}
T3-4	Tomato	90.0	14.5	14.4	2637.0^{f}
Tomato- H6	Tomato	100.0	14.1	14.0	2800.0^{d}
Tomato- 17	Tomato	100.0	12.3	13.2	2540.0^{g}
T3-6	Tomato	90.0	14.0	13.8	2502.0^{g}
Chilli-2	Chilli	100.0	12.2	14.6	2680.0^{ef}
Brinjal-5	Brinjal	100.0	15.7	17.2	3290.0^{b}
T2-2	Tomato	100.0	11.3	14.9	2610.0^{f}
Chilli-1	Chilli	100.0	12.4	14.8	2710.0^{6}
Tomato -H9	Tomato	100.0	15.4	15.3	3080.0^{c}
Tomato- H4	Tomato	100.0	17.3	16.7	3400.0^{3}
Chilli-5	Chilli	100.0	14.1	14.2	2820.0^{d}
Tomato-14	Tomato	100.0	12.9	15.5	2830.0^{d}
Brinjal-7	Brinjal	100.0	14.6	14.0	2860.0^{d}
Tomato-6a	Tomato	100.0	12.7	16.7	2900.0^{d}
Tomato-1a	Tomato	100.0	13.8	13.3	2700.0^{e}
T2-8	Tomato	100.0	12.8	14.1	2680.0^{6}
C.P2.-7	Cowpea	100.0	6.5	9.1	15,00.0*
B-I	Brinjal	80.0	6.4	8.8	12,08.0'
Tomato-1	Tomato	100.0	5.8	8.6	14,35.0'
CP1-16	Cowpea	90.0	5.9	8.2	12,60.0'
CP1-18	Cowpea	100.0	3.8	6.5	10,30.0^{m}
TI-4	Tomato	100.0	5.8	7.2	1300.0^{k}
BI	Brinjal	90.0	5.2	8.6	1737.5^{h}
BS. D	IIVR	100.0	5.1	7.6	1270.0'
C5b	Chilli	90.0	5.9	8.7	1309.5^{k}
BI-C	Brinjal	100.0	5.0	9.0	1400.O'
Tomato-6	Tomato	90.0	6.0	10.6	1494.0^{1}
CI a	Chilli	100.0	7.9	9.9	1780.0^{11}
Tomato-12	Tomato	100.0	13.7	15.1	2890.0^{d}
Control	-	80.0	5.4	6.5	9,53.6"

* Values in the column are mean ± SE. In a column a mean followed by common letters are not Significantly different at 5% level by DMRT

positive growth promoting activity compared to control and remaining were inhibitory to the crop growth. However, 31 isolates showed significantly higher vigour index (Table 1). Many workers have evidenced that *Pseudomonas* spp. have the ability to produce IAA, which is effective for plant growth promotion (Kumar Dileep and Dube, 1992; O'Sullivan and O'Gara ,1992; Patten and Glick, 2002). In addition, PGPR increased plant growth directly by mediating the production of secondary metabolites and phytohormones such as auxins, cytokinins or gibberellic acid (Arshad and Frankenberger, 1991; Beyeler *et al.*, 1999) or indirectly either by suppression of well-known diseases caused by major pathogens or by reducing the deleterious effects of minor pathogens (Schippers *et al.*, 1987). *In vitro* results of antagonistic activity of PGPR against *FOL* and *S. rolfsii* revealed that the PGPR isolates viz. Chilli-1, Chilli-2, Chilli-5 and B.s.D showed maximum inhibitory action against the fungal pathogens (Table 2). *In vitro* inhibition of mycelial growth of the pathogen might be due to induction of antibiotics or cell wall degrading enzymes or competition or through combination of all mechanisms (Weller, 1988; Pierson and Thomashow, 1992; Kloepper *et al.*, 1980; Kloepper *et al.*, 1992; Lim *et al.*, 1991; Frindlender *et al.*, 1991; Potgieter and Alexander, 1996). Apart from biocontrol activity, these isolates also showed positive reactions for production of IAA, phosphate solublization and siderophore production (Table 3) and this could be reason for high level growth promotion action of tomato seedlings *in vitro*. Based on the performance of the *in vitro* tests (Tables 1, 2 and 3), two potential isolates Chilli 1 and Chilli 2 were identified for further greenhouse and field level experiments. Among these two isolates, Chilli 2 recorded significantly less wilt and collar rot incidences under greenhouse and field conditions (Table 4a, 4b and Table 5). Similarly, yield of the crop was significantly enhanced in Chilli 2 treated plants under greenhouse conditions (Table 4a and 4b). Observations on field trials also recorded similar results in terms of enhancing the plant height (Table 5) and the yield records could not be made since the experiment are still in progress. Many of the past reports demonstrated that the enhancement of plant growth and yield is due the collective action of both biocontrol action through ISR and induced plant growth promotion by PGPR (Leeman *et al.*, 1995; Vidhyasekaran and Muthamilan, 1995; Rabindran and Vidhyasekaran, 1996; Wei *et al.*, 1996; Vidhyasekaran *et al.*, 1997). Hence in the present study the enhanced yield might be due to the above said mechanisms. Based on morphological characters the potential isolate Chilli 2 was identified as species of *Pseudomonas* and further confirmation through molecular approach is in progress.

Table 2 : *In vitro* effect of the PGPR isolates on mycelial inhibition of *FOL* and *S. rolfsii*

Treatment /isolate	Inhibition (%)*	
	FOL	*S. rolfsii*
CP1-16	0.0^{a}	0.0^{a}
Tomato-6	7.6^{d}	0.0^{a}
Tomato-6a	53.0^{n}	50.0^{i}
C5b	44.0^{k}	0.0^{a}
T2-7	49.9^{m}	13.0^{e}
Tomato-H4	41.5^{j}	19.0^{f}
Tomato-H6	39.9^{j}	11.0^{d}
Tomato-H9	40.7^{j}	0.0^{a}
Tomato-17	0.0^{a}	8.0^{c}
Tomato-8	0.0^{a}	0.0^{a}
Chilli-2	62.1^{q}	99.5^{o}
Tomato-Ia	5.9^{c}	44.6^{l}
Tomato-12	40.7^{j}	4.1^{b}
Tomato-14	46.1^{l}	55.I^{k}
Chilli-1	64. I^{r}	99.4^{o}
CP2-7	48.4^{lm}	69.9^{m}
T3-4	7.6^{d}	14.9^{e}
Blc	7.6^{d}	30.8^{g}
Tomato-1	11.4^{e}	9.1^{c}
Brinjal-5	11.2^{e}	0.0^{a}
T2-2	3.6^{b}	0.0^{a}
T3-6	3.8^{b}	0.0^{a}
CP1-18	34.5^{h}	0.0^{a}
T2-8	13.0^{f}	0.0^{a}
Brinjal-7	7.2^{d}	0.0^{a}
B-I	38.0^{i}	41.5^{h}
CP1-15	19.9^{g}	63.0^{i}
TI-4	7.6^{d}	29.5^{g}
B.s.-D	59.8^{p}	74.2^{n}
Chilli-5	61.5^{q}	100.0^{o}
Cla	56.9^{o}	49.3^{j}
Control	0.0^{a}	0.0^{a}

*Values were arc sine transformed before the analysis. In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 3 : Selected rhizobacteria showing potentialities in production of IAA, siderophore and phosphate solublization

Treatment	IAA production (µg/ml)	Phosphate solublization	Siderophore production (zone of inhibition in mm)
Chilli-1	23.0[b]	+	4.0[a]
Chilli-2	26.0[a]	+	4.0[a]
Chilli 5	24.0[b]	+	3.2[a]
B.sD	23.0[b]	+	1.8[a]

In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 4a : Effect of PGPR on wilt disease of tomato under greenhouse conditions

Treatment	Wilt (%)*	Reduction over control (%)*	Plant height (cm)	Fruit yield (g/plant)
Chilli-1 alone	0.0[C]	100.0[3]	47.3[C]	65.2[U]
Chilli-2 alone	0.0[C]	100.0[a]	51.4[b]	70.6[C]
Chilli-1+ *FOL*	4.1[b]	95.0[b]	51.2[b]	77.9[b]
Chilli-2 + *FOL*	2.8[b]	96.6[b]	57.6[a]	81.0[a]
FOL alone	82.5[a]	0.0[e]	25.4[e]	45.4[f]
Captof	22.5[b]	72.7[d]	34.8[d]	52.3[g]
Carbendazim	16.6[C]	79.8[c]	47.0[C]	67.0[d]
Healthy control	0.0[C]	100.0[a]	45.3[d]	57.8[e]

*Values were arc sine transformed before the analysis. In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 4b : Effect of PGPR on collar disease of tomato under greenhouse conditions

Treatment	Collar rot (%)*	Reduction over control (%)*	Plant height (cm)	Fruit yield (g/plant)
Chilli-1 alone	0.0[d]	100.0[a]	42.0[d]	62.5[e]
Chilli-2 alone	0.0[d]	100.0[3]	43.4[C]	68.7[C]
Chilli-1+ *S. rolfsii*	4.1[b]	90.5[b]	45.2[b]	86.0[b]
Chilli-2+ *S. rolfsii*	0.8[d]	98.1[a]	47.7[a]	90.9[a]
S. rolfsii alone	43.3[a]	0.0[e]	32.0[e]	30.9[g]
Captof	22.4[b]	48.2[d]	34.0[d]	62.0[C]
Carbendazim	18.3[C]	57.7[C]	41.6[C]	67.0[b]
Healthy control	0.0[d]	100[a]	42.0[d]	57.8[f]

*Values were arc sine transformed before the analysis. In a column a mean followed by common fetters are not significantly different at 5% level by DMRT

Table 5 : Effect of PGPR on wilt and collar rot diseases of tomato under field conditions

Isolate	* Disease incidence %		Plant height (cm)
	Wilt	Collar rot	
Chilli-1	5.0[d]	4.1[c]	46.8[b]
Chilli-2	3.0[e]	1.0[d]	53.2[a]
Captof	37.5[b]	20.8[a]	26.1[d]
Carbendazim	20.0[C]	15.5[b]	30.1[c]
Control	40.0[a]	21.6[a]	24.1[e]

*Values were arc sine transformed before the analysis. In a column a mean followed by common letters are not significantly different at 5% level by DMRT

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