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Isolation and evaluation of rhizospheric bacteria for biological control of chickpea wilt pathogens

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ABSTRACT: **Three hundred rhizospheric isolates of bacteria collected from different regions of Karnataka were screened for *in vitro* antagonism in dual culture on Tryptic Soya Agar (TSA) against five fungal pathogens *viz.*, *Botrytis cinerea, Macrophomina phaseolina*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *ciceris*. Four isolates selected as potential antagonists were identified as *Pseudomonas putida* (PDBCAB 19), *P. fluorescens* (PDBCAB 2), *P. fluorescens* (PDBCAB 29) and *P. fluorescens* (PDBCAB 30) and their root colonizing ability was tested. The total rhizosphere population was the highest (log cfu 6.4) for *P. fluorescens* (PDBCAB 29) after four days of germination. The rhizosphere population stabilized (log cfu 4.0 to 5.0) after eight days of germination. Three pathogens namely *F. oxysporum* f. sp. *ciceris* (wilt pathogen), *R. solani* and *M. phaseolina* (root rot pathogens) were targeted by the four selected antagonists under greenhouse conditions. The maximum plant stand (100%) was observed with *P. fluorescens* (PDBCAB 29 and 30) treated pots for *R. solani* and *M. phaseolina*. *P. putida* (PDBCAB 19) and *P. fluorescens* (PDBCAB 30) were able to fully control *F. oxysporum* f. sp. *ciceris*. All the four antagonists promoted growth of chickpea. *P. fluorescens* isolates (PDBCAB 29 and 30) produced the maximum growth. Survival of *P. fluorescens* (PDBCAB 29) in a talc based formulation was monitored over a period of 90 days at room temperature.**

KEY WORDS: Antagonism, chickpea, plant growth promotion, rhizospheric bacteria, survival, talc, wilt

Rhizosphere bacteria like the fluorescent pseudomonads, *Bacillus subtilis* and *Streptomyces* spp. have been established as plant disease suppressors and plant growth promoters (Weller, 1988; Ryder *et al.*, 1994; Vidhyasekaran, 1998). The application of these rhizosphere bacteria as biocontrol agents for crop protection is an important alternative to the use of chemical fungicides. Isolation of rhizosphere bacteria is easy as they are ubiquitous but selection is difficult since only a few of them will be disease suppressors (Cook, 1981; Vidhyasekaran, 1998). Selection of potent strains of rhizosphere bacteria must be based on rhizosphere competence, antagonistic ability and root colonizing ability (Lugtenberg and Dekkers, 1999). Chickpea is a very important pulse crop of India and is cultivated in more than 9 million hectares. But its production is limited by wilt/root rot disease caused by *Fusarium oxysporum* f. sp. *ciceris*, *Macrophomina phaseolina* and *Rhizoctonia solani* (Rangaswami, 1993; Vidhyasekaran and Muthamilan, 1995).

A study was undertaken to isolate and evaluate rhizosphere bacteria for the supression of the above mentioned three pathogens and also their growth promoting ability.

MATERIALS AND METHODS

Isolation of rhizosphere bacteria

Rhizosphere soil samples were collected from different regions of Karnataka. Special emphasis was laid on collecting soils from sites where chickpea was one of the crops that was raised during that year and also where soil-borne diseases were present. Healthy plants were chosen randomly and rhizosphere soil collected. Three hundred rhizosphere bacterial isolates were collected and pure cultures obtained. The cultures were stored in nutrient broth and glycerol mixture (1:1) at – 80°C for further studies.

Pathogen isolates

Chickpea pathogens *viz.*, *F. oxysporum* f. sp. *ciceris*, *M. phaseolina* and *R. solani* were isolated from infected chickpea plants of Dharwad area, pure cultured and maintained on Potato Dextrose Agar (PDA). Two other pathogens namely *Botrytis cinerea* and *Sclerotium rolfsii* were from the culture collection of Project Directorate of Biological Control (PDBC) and were used for *in vitro* screening only.

In vitro antagonistic studies

All the three hundred rhizosphere isolates were screened for *in vitro* antagonism in dual culture on TSA against five fungal pathogens *viz.*, *B. cinerea, M. phaseolina, S. rolfsii, R. solani* and *F. oxysporum* f. sp. *ciceris*. *In vitro* antagonism of rhizosphere bacteria was tested by streaking the bacteria as a 4cm line on TSA. Mycelial plugs, 5mm in diameter of the fungal pathogens were transferred to the most distal point from the bacteria on the plate. Plates were replicated thrice and repeated once. Plates were incubated at 28 ± 2 °C, and zones of inhibition were recorded after 8 days. Inhibition zone (in mm) was calculated by taking the difference of pathogen growth in control plate and growth in treated.

Identification

The isolates were tested for fluorescent pigment on King's B (KB) medium and $FeCl_3$ - amended KB medium. All the isolates were screened for Kovac's oxidase, arginine dihydrolase and gelatin liquefaction (Valasubramanian *et al.*, 1996).

HCN Production

Production of HCN was determined as described by Wei *et al.* (1991). Bacteria were grown on TSA supplemented with 4.4 g/L of glycine, white filter paper strips soaked in picric acid solution (2.5 g of picric acid, 12.5 g of Na_2CO_3, 1 L of water) were placed in the lid of each Petri-dish. Petri-dishes were sealed with parafilm and incubated for 2-4 days. After incubation at 28 °C for 48h, HCN production was indicated by the presence of a coloured zone around the bacteria. Reactions were scored as weak (yellow to light brown), moderate (brown), or strong (reddish brown) for each of the test strains. The experiment was repeated once.

Assay for root colonizing ability

Talc based formulations of selected antagonists were prepared as described by Vidhyasekeran *et al.*(1996). Its pH was adjusted to 7.0 using calcium carbonate. Ten grams of carboxy methylcellulose (CMC) per kg of carrier was used as adhesive. Selected rhizosphere bacteria were grown in Nutrient Broth (NA) for 48 to 72h in a rotary shaker to obtain a minimum population of 9×10^8 cfu/ml. The suspensions were mixed with sterile carrier (400 ml/kg) and air-dried. Chickpea seeds were surface sterilized with sodium hypochlorite (1.05%) for 3 minutes and rinsed four times in sterile tap water. The seeds were wetted with carboxy methyl cellulose (1%) and uniformly coated with the respective formulations (5 g/kg seed) and sown in sterile sand: vermiculite (1:1) mixture in pots. Three replications were maintained for each antagonist and repeated once. Total rhizosphere population/ colony forming units (cfus) per plant was estimated by serial dilution at 4 and 8 days.

In vivo assay

Three pathogens namely *F. oxysporum* f. sp. *ciceris, R. solani* (wilt pathogens) and *M.*

phaseolina (root rot pathogen) were targeted by the four selected antagonists, which had the potential to inhibit the pathogens under dual culture (*in vitro*), in greenhouse conditions in unsterilized soil mixture. The pathogens were grown in wheat bran -vermiculite (1:1) mixture containing sucrose @ 10 g/kg in one litre conical flasks for 10 days. Ten grams of pathogen inoculum containing actively growing mycelium was used as inoculum for each pot.

The antagonists were first allowed to colonize the roots of chickpea plants (cv: Annegiri) in sterile soil: vermiculite (1:1 w/w) mixture containing the bacterial antagonist (about 10^6 to 10^8 cfu/g of soil mixture). The seedlings were replanted in pathogen infected unsterile field soil (three parts loam and one part of well-decomposed farm yard manure) filled in plastic pots of 15cm diameter (2 kg/pot). Three plants were maintained per pot. Control pots having fungicide (mancozeb @ 2.5 g/kg of seed) and only pathogen was used for comparative analysis. Each treatment was replicated four times. Disease incidence was monitored over a 60-day period.

Plant growth promoting ability

Surface disinfected chickpea seeds were treated with the talc based formulations of four selected antagonists and sown in plastic pots containing autoclaved field soil (2 kg/pot). After germination, one plant was maintained per pot. Four replications were maintained for each treatment. Plant shoot and root weight parameters were recorded after 60 days of growth and compared with untreated the control. Vigour index was calculated by multiplying per cent plant stand with sum of shoot and root length.

Survival studies

Survival of *P. fluorescens* (PDBCAB29) was monitored over a period of 60 days at room temperature (25 ± 2^0 C) in the talc-based formulation. Samples were drawn every 15 days to determine viable colony forming units by serial dilution and expressed as cfus/g formulation.

RESULTS AND DISCUSSION

Out of three hundred rhizosphere isolates screened for *in vitro* antagonism in dual culture on TSA against five fungal pathogens *viz.*, *B. cinerea, M. phaseolina, S. rolfsii, R. solani* and *F. oxysporum* f. sp. *ciceris*, four of the isolates were found to be potential antagonists of the five

Table 1. Inhibition of fungal pathogens under dual culture by antagonistic bacteria

Pathogen	*P. putida* (PDBCAB19)	*P. fluorescens* (PDBCAB 2)	*P. fluorescens* (PDBCAB29)	*P. fluorescens* (PDBCAB 30)
		Inhibition Zone (mm)		
Botrytis cinerea	32	28	48	60
Macrophomina phaseolina	38	32	35	54
Sclerotium rolfsii	70	42	23	38
Rhizoctonia solani	35	28	33	67
Fusarium oxysporum f. sp. ciceris	45	42	45	52
CD (P = 0.05)	13.2	3.8	3.8	3.4
HCN production	+++*	++**	+++	++

* = strong, ** = moderate reaction

pathogens. These were identified as *Pseudomonas putida* (PDBCAB 19), *P. fluorescens* (PDBCAB 2), *P. fluorescens* (PDBCAB 29) and *P. fluorescens* (PDBCAB 30).

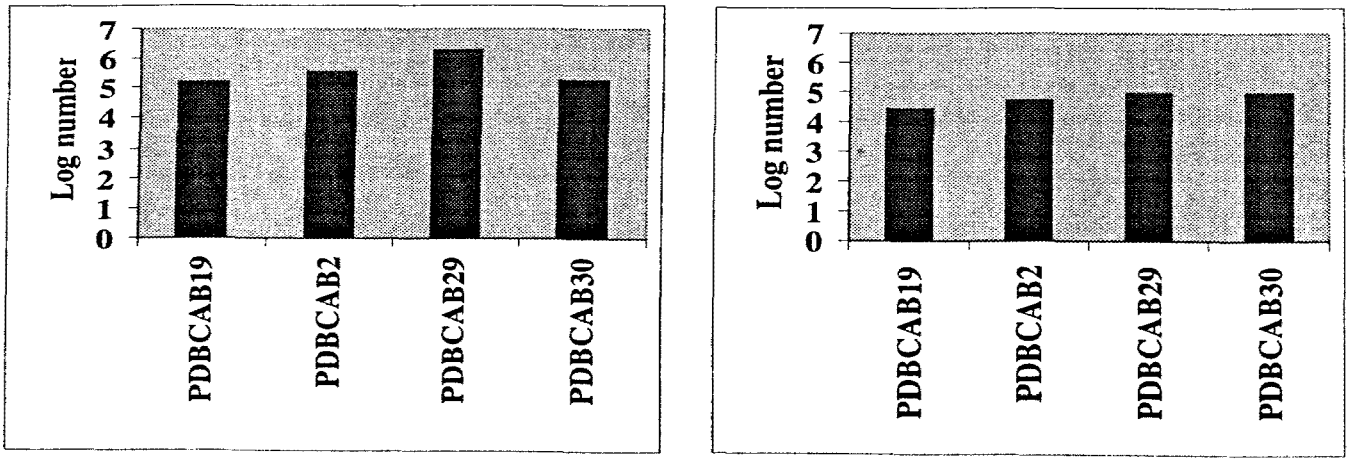
All the four antagonists showed good inhibition zones against the five pathogens (Table 1). *Pseudomonas putida* (PDBCAB 19) and *P. fluorescens* (PDBCAB 2) were most effective against *S. rolfsii* and *F. oxysporum* f. sp. *ciceri* (42 to 70mm inhibition zones). *Pseudomonas fluorescens* (PDBCAB 29) was effective against *B. cinerea* (inhibition zone of 48 mm). *Pseudomonas fluorescens* (PDBCAB 30) was found to be effective against all the five pathogens. Inhibition zones ranging from 38 to 67 mm was observed in plates inoculated with *P. fluorescens* (PDBCAB 30). Wei *et al.* (1991) observed that inhibition of *R. solani* on TSA by plant growth promoting rhizobacteria (PGPR) was higher when compared on PDA or green bean agar.

The four selected antagonists tested were positive for HCN production (Table 1) with *P. putida* (PDBCAB 19) and *P. fluorescens* (PDBCAB 29) exhibiting good response, the other two antagonists reacted moderatively. HCN production is reported to play a role in disease suppression (Wei *et al.*, 1991). However, Haas *et al.* (1991) have reported that HCN production by strains of *P. fluorescens* helped in suppression of black root rot of tobacco (caused by *Thielaviopsis basicola*) but a HCN$^-$ mutant was also able to suppress the disease. Hence, HCN production could be one of the contributing factors in disease suppression.

The root colonizing ability of the above four potential antagonists was highest (log cfu 6.4) for *P. fluorescens* (PDBCAB 29) after four days of germination (Fig. 1) indicating it's ability as a good root colonizer. The rhizosphere population stabilized (log 4.0 to 5.0) after eight days of germination for all the four antagonists. Nautiyal (1997) selected one bacterium from 386 isolates based on root colonizing ability and inhibition of chickpea pathogens. It is possible to predict the rhizosphere competence of *Pseudomonas* bacteria based on their root colonizing ability (Lugtenberg and Dekkers, 1999) since the root colonizing ability of a bacterium will determine its rhizosphere competence.

Three pathogens namely *F. oxysporum* f. sp. *ciceris* (wilt pathogen), *R. solani* and *M. phaseolina* (root rot pathogens) targeted by the four selected antagonists in greenhouse conditions in unsterile soil mixture revealed maximum plant stand (100%) with *P. fluorescens* (PDBCAB 29 and 30) treated pots for *R. solani* and *M. phaseolina* (Table 2). *Pseudomonas fluorescens* (PDBCAB 30) was also able to fully control *F. oxysporum* f. sp. *ciceris*. *Pseudomonas putida* (PDBCAB 19) was able to inhibit only *F. oxysporum* f. sp. *ciceris*. *Pseudomonas fluorescens* (PDBCAB 2) was marginally effective against *R. solani*.

Fig. 1. Root colonizing ability of bacterial antagonists after 4 and 8 days



The plant growth promoting ability of the four potential bacterial antagonists determined by recording the root and shoot weights of inoculated chickpea plants revealed that all the four antagonists were able to support better growth of chickpea (Table 3). Maximum shoot and root dry weights were observed with *P. fluorescens* (PDBCAB 2) and *P. fluorescens* (PDBCAB 29) treated plants. Vigour index was the highest for *P. fluorescens* (PDBCAB 29) but *P. fluorescens* (PDBCAB 30) was also able to show good vigour index. Alstrom (1994) has stressed that rhizosphere bacteria could improve plant productivity.

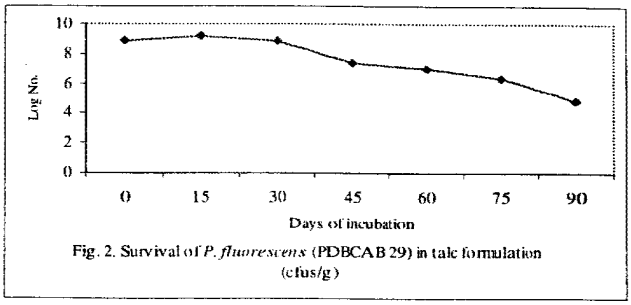


Fig. 2. Survival of *P. fluorescens* (PDBCAB 29) in talc formulation (cfus/g)

Survival of *P. fluorescens* (PDBCAB 29) monitored at room temperature in a talc-based formulation showed viable population upto 60 days (Fig 2). The population was drastically reduced after 60 days of storage. However, Vidhyasekaran and Muthamilan (1995) were able to store *P. fluorescens* (*Pf1*) up to 120 days at room temperature in a talc-based formulation. Whether the difference is due to strain variation or changes in room temperature during storage will have to be further ascertained.

The results indicate that certain rhizosphere competent bacteria are involved in plant protection and growth promotion and such antagonistic/beneficial rhizosphere bacteria must be isolated from healthy plants growing in diseased soils. Selection of antagonistic bacteria must be based on effective root colonizing ability (ability to compete in rhizosphere), disease suppressing ability in soil and plant growth promotion. The selected antagonists are currently being field tested for further recommendation.

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Table 2. Inhibition of chickpea pathogens in soil

Treatment	Pathogens tested and per cent plants surviving after 60 days		
	F. oxysporum f. sp. ciceri	*R. solani*	*M. phaseolina*
P. putida (PDBCAB19)	100 (90)	33 (35)	22 (23)
P. fluorescens (PDBCAB2)	44 (42)	67 (55)	22 (23)
P. fluorescens (PDBCAB29)	56 (48)	100 (90)	100 (90)
P. fluorescens (PDBCAB30)	100 (90)	100 (90)	100 (90)
Control (Fungicide)	89 (78)	100 (90)	100 (90)
Control (infected)	0 (0)	0 (90)	11 (12)
Control (plain)	100 (90)	100 (90)	100 (90)
CD (P = 0.05)	17.16	0.04	24.84

Figures in parentheses are angular transformations.

Table 3. Plant growth promotion of chickpea by bacterial antagonists in *R. solani* infected soil

Treatment	Per cent plant stand	Shoot length (cm)	Root length (cm)	Vigour Index	Dry weight/plant	
					Shoot	Root
Pseudomonas. putida (PDBCAB 19)	33 (35)	20.6	7.3	687.0	145.0	38.0
Pseudomonas fluorescens (PDBCAB 2)	67 (55)	22.6	11.5	1625.7	162.7	46.0
Pseudomonas fluorescens (PDBCAB 29)	100 (90)	25.0	14.0	2517.0	201.0	53.0
Pseudomonas fluorescens (PDBCAB 30)	100 (90)	19.4	9.7	1949.7	158.0	37.0
Control (fungicide)	100 (90)	18.7	10.4	1877.0	145.0	35.7
Control (plain uninoculated)	100 (90)	14.0	6.5	1409.8	108.0	27.3
CD (P= 0.05)	0.04	1.83	2.12	223.6	20.19	8.43

Figures in parentheses are angular transformations.

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