Middle-East Journal of Scientific Research 12 (11): 1459-1470, 2012
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2012.12.11.7

Development of Plant Growth Promoting Microbial Consortium Based on Interaction Studies to Reduce Wilt Incidence in *Cajanus cajan* L var. Manak

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Abstract: The present study was undertaken to develop a plant growth promoting microbial consortium on the basis of interaction studies to reduce the fusarial wilt in *Cajanus cajan*. All strains were exposed to interact under *in vitro* conditions. Three strains viz. *Pseudomonas fluorescens* LPK2, *Sinorhizobium fredii* KCC5 and *Azotobacter chroococcum* AZK2 did not inhibit each other. *In vitro* dual culture studies on the interaction of one strain to another, revealed no mutual growth inhibition among *S. fredii* KCC5, *P. fluorescens* and *A. chroococcum* AZK2. Spectrophotometric studies also showed that the individual growth of these strains was not affected in combined cultures, where strains were cultured together. The optical density values of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 were observed in present studies were at par with their treatments when incorporated into the broth and vice versa. A complete inhibition of the conidial and chlamydospores germination *F. udum* was observed by the culture filtrates of *S. fredii* KCC5 and *P. fluorescens* LPK2. The culture filtrates of *S. fredii* KCC5 and *P. fluorescens* LPK2 inhibited conidial germination significantly ($P>0.01$). The inhibition of germ tube was more pronounced than that of conidial germination. A highly significant effect ($P>0.01$) of the non-volatile metabolites was noted against the conidial germination and germ tube growth of the test pathogen. A corresponding amount of culture supernatant from *F. udum* grown in potato dextrose broth initiated a significant chemotactic response of *S. fredii* KCC5, *P. fluorescens* and *A. chroococcum* AZK2 cells within 6 h. In biocontrol pot assay, it was observed that the bacterial combinations resulted in enhanced disease suppression in comparison to individual strain. In biocontrol assay, the values from T2 to T6 were not significantly different from each other but the percentage of healthy plants were significantly ($P<0.05$) higher than control with pathogen. These three strains led to protocooperation as evidenced by synergism, aggressive colonization of roots and enhanced growth suggesting potential biocontrol active microbial consortium against wilt incidence in *Cajanus cajan*.

Key words: *Sinorhizobium* · *Pseudomonas* · Microbial consortium · Fusraium wilt · Microbial Interaction

INTRODUCTION

Pigeonpea (*Cajanus cajan* L var. Manak) is one of the important pulse crops and a very popular food in developing tropical countries. India is a principal pigeonpea-growing country contributing nearly 90% of total world's production. Pigeon pea is attacked by more than 100 pathogens including fungi, bacteria, viruses, nematodes and mycoplasma-like organisms, but only a few of them cause economic losses. Among them, wilt caused by *Fusarium udum* is considered the most important soilborne pathogen of pigeon pea.

Excessive use of synthetic chemicals for control of pathogens is effective and convenient but their use and abuse are causing serious ecological, economic and social problems. Alternative approaches are needed to substitute the chemicals with bacterial fertilizers and bio-pesticides because of the potential threat for development of chemical resistance, especially systemic fungicides, by fungal pathogens and non-target side effects on other plant pathogens and beneficial microorganisms. For sustainable fertility of the soil, blending of the chemical fertilizers with chemical-adaptive strains is one approach that may derive synergistic benefits [1].

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Biological control of *F. udum* has attracted attention throughout the world. Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents. Since the rhizosphere is the front line defense for roots against pathogenic fungi [2]. The premier example of bacterial biocontrol agents occur in many genera including *Actinoplanes, Agrobacterium, Alcaligens, Amorphosporangium, Arthrobacter, Azotobacter, Bacillus, Burkholderia, Cellulomonas, Enterobacter, Erwinia, Flavobacterium, Gluconacetobacter, Micromonospora, Pseudomonas,* Rhizobia, *Serratia, Streptomyces, Xanthomonas* etc. as stated by large number of microbiologists [3-5].

Most effective indirect mechanisms to be employed by antagonists to prevent proliferation of phytopathogens which are the synthesis of antibiotics [6], siderophores [7], hydrogen cyanide [8] and hydrolytic enzymes [9]. They have a role to play in inhibition of pathogens. Most approaches for biological control of plant diseases have used single biocontrol agents as antagonists against a single pathogen. This has partially been achieved by: (i) modifying genetics of the bio-control agent to add mechanism of disease suppression that are operable against more than one pathogen, (ii) alteration of environment to favour the biological control agent and to disfavour competitive microflora and (iii) to develop strain mixtures or consortia with superior bio-control activity [10]. A microbial consortium is a group of different species of microorganisms that act together as a community. For developing a consortium one can choose microorganisms that are resistant to environmental shock, fast acting, synergistically active, producing natural enzymatic activity, easy to handle, having long self life, good sustainability, non-pathogenic, non-corrosive of consistent quality and economical. Combinations of bio-control strains are expected to result in a higher level of potential to suppress multiple plant diseases.

The present work is focused on development of microbial consortium based on interaction studies to reduce the wilt incidence in *Cajanus cajan*.

MATERIALS AND METHODS

Isolation of F. udum: F. udum was isolated from infected roots of pigeon pea (*C. cajan*) following blotter techniques [11]. Two blotter papers (90 cm diameter) were moistened with sterile distilled water and placed in a Petri plate. Samples of diseased seeds and plant materials were placed between the blotting papers and incubated at 28 °C for one week. After incubation, fungal colonies were examined and a pure culture was maintained on PDA slants for further use.

Microorganisms: For the preparation of microbial consortium seven bacterial strains were selected based on their plant growth promoting characteristics. Among them, two rhizobacteria were isolated and characterized as potential antagonists of *F. udum* i.e. *Pseudomonas fluorescens* LPK2 and *Sinorhizobium fredii* KCC5 [12 13, 14]. *Azotobacter chroococcum* AZK2 was isolated as putative phosphate solublizing agent [12]. The other four reference strains were procured from authors laboratory which were *Pseudomonas aeruginosa* GRC2 [15], *Pseudomonas aeruginosa* 1934 (MTCC, IMTECH, Chandigrah), *Sinorhizobium meliloti* RMP1 [16] and *Bacillus licheniformis* 57 (MTCC, IMTECH, Chandigrah). These strains were maintained on culture media for further studies (Table 1).

Inter-strain Interaction Studies to Develop Microbial Consortia

Plate Assay: All seven bacterial strains were tested individually by plate assay. In this assay, pure cultures of *Pseudomonas fluorescens* LPK2, *Pseudomonas aeruginosa* GRC2 and *Bacillus licheniformis* 57 were inoculated individually in nutrient broth. Likewise, *Sinorhizobium fredii* KCC5 and *Sinorhizobium meliloti* RMP1 were inoculated in yeast extract mannitol broth and *A. chroococcum* AZK2 was inoculated in nitrogen-free medium. These strains were incubated at 28°C under shaking conditions. After 24 h of incubation 5 µl of each

Table 1: Plant growth-promoting and antifungal properties of bacterial strains used in this study

Strains	IAA	Phosphate solubilization	Siderophore	HCN	Chitinase	â-1,3-glucanase	Antagonism against *F. udum*	Intrinsic Antibiotic Resistant	Reference
S. fredii KCC 5	++	+	+++	-	-	-	++	Streptomycin (strp)	[13, 14]
P. aeruginosa LPK 2	++	++	+++	+++	+	+	++	Tetracyclin (tet)	[12, 14]
A. chrococcum AZK 2	++	+++	-	-	-	-	-	Ampicillin (amp)	[12]
Pseudomonas aeruginosa GRC2	+	+	+	+	-	-	-	Streptomycin (strp)	[15]
Pseudomonas aeruginosa 1934	+	+	+	+	-	-	-	Neomycin (neo)	MTCC
Sinorhizobium. meliloti RMP1	+	+	+	-	-	-	-	Ampicillin (amp)	[16]
Bacillus licheniformis 57	-	+	+	-	-	-	-	Bacitracin (bc)	MTCC

Abbreviations: -, negative, +, positive; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0cm wide surrounding colonies; MTCC, Microbial Type Culture Collection, IMTECH, Chandigrah (India)

culture was spotted on nutrient agar plates (1.5 cm from the edge). After 24 h of incubation, plates were sprayed with a 24 h old culture of a single strain using a chromatography sprayer and incubated at 28°C for 24 h and zone of inhibition (it present around each test strain) was measured. Each treatment was replicated thrice and the entire experiment was performed twice.

Spectrophotometric Method: The bacterial strains showing positive interaction in plate assay were further tested by spectrophotometric assay as described by Shanmungam *et al.* [17]. From the above experiment, three strains showed positive interaction. As described in above experiment, strains were grown in their respective media for 3 days at 28°C under shaking conditions and their cell free culture supernatants were obtained by passing the broth culture through membrane filter (0.45 µm). The individual culture filtrates were incorporated into sterile nutrient broth at 20% (v/v). After that, three combinations of mixtures of culture filtrates were prepared. In first combination, the mixture of culture filtrate and nutrient broth of *S. fredii* KCC5 was inoculated with 0.5 ml of bacterial inoculum (3×10^7 cfu ml^{-1}) of *P. fluorescens* LPK2 and *A. chroococcum* AZK2 individually. Likewise in second combination, the culture filtrate of *P. fluorescens* LPK2 was inoculated with *S. fredii* KCC5 and *A. chroococcum* AZK2. In third combination, the culture filtrate of *A. chroococcum* AZK2 was inoculated with *S. fredii* KCC5 and *P. fluorescens* LPK2. These three combinations of mixtures of cultures were then incubated at 28°C under shaking condition. The intensity of growth was assessed by observing the optical density of broth at 610 nm using UV-VIS spectrophotometer (Sigma-1401). The cultures without filter supernatant of bacteria served as control. Growth rate of the bacterial cultures was determined in control cultures as well as in supernatant inoculated cultures. The experiments were repeated twice and the data were presented as the means of three replicates.

Co-inoculation of Bacterial Strains: For this study antibiotic resistant marked strains of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 were used. The property of intrinsic antibiotic resistance was mutually exclusive, as no two strains were resistant for same antibiotic. The bacterial cultures were grown in nutrient broth until a cell density of 10^8 cell ml^{-1} was obtained. Then, they were inoculated in nutrient broth in four combinations such as (i) *S. fredii* KCC5^{sterp+} + *P. fluorescens* LPK2^{tet+}, (ii) *S. fredii* KCC5 $^{strep+}$ + *A. chroococcum* AZK2^{amp+}, (iii) *P. fluorescens* LPK2^{tet+} + *A. chroococcum* AZK2^{amp+} and (iv) *S. fredii* KCC5^{strep+}+ *P. fluorescens* LPK2^{tet+} + *A. chroococcum* AZK2^{amp+}. All culture broth was kept under shaking conditions at 150 rpm at 28°C. After every 6 h, samples were aseptically withdrawn for the determination of cell population. Serial dilutions of the samples of different combinations were poured into petri plates containing yeast extract mannitol agar medium supplemented with streptomycin (100 µg ml^{-1}) for *S. fredii* KCC5, nutrient medium with tetracycline (100 µg ml^{-1}) for *P. fluorescens* LPK2 and nitrogen-free agar medium containing ampicillin (100 µg ml^{-1}) for *A. chroococcum* AZK2. The plates were incubated at 28±1°C and colony forming units (cfus) were enumerated after every 24 h interval.

Biocontrol Studies

Chemotaxis: Chemotactic behaviour of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 towards culture filtrate of *F. udum* was studied. Chemotaxis experiments were performed using the "drop assay" as described by Grimm and Harwood [18]. Cells of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 were grown overnight in their respective growth medium. When cells reached the early logarithmic phase (OD_{600} of 0.12), 40-ml samples were gently spun down (3,000 rpm) and re-suspended in 12 ml of chemotaxis buffer (100 mM potassium phosphate, pH 7.0, 20 µM EDTA). The resulting cell suspension was transferred to a 60-mm-diameter petri dish in which it formed a layer approximately 3 mm thick. *Fusarium udum* was grown in potato dextrose broth and culture filtrate was obtained by passing the broth culture through membrane filter (0.45 µm). 10-µl of culture filtrate of *F. udum* was added to the center of the dish. During incubation for up to 6 hours at room temperature, the plates were analyzed for the appearance of a clear zone.

Effect of non-volatile metabolite containing culture filtrates of bacteria on germination of conidia and chlamydospores of the test pathogen [19]: The fruiting bodies of *F. udum* i.e. micro and macro conidia were harvested from 6 day old colony from PDA slants by rinsing with sterilized distilled water. The suspension was washed five times by centrifugation at 2000 rpm. The chlamydospore were obtained by the method described as follows: the conidia were allowed to germinate in liquid Czapek-Dox medium for 10 h. they were then washed with sterilized distilled water by centrifugation and were transferred to a basal salt medium without a carbon source. Spore germination test were carried out aseptically

in cavity slide. One ml spore suspension (0.20×10^4 spores) was added to 0.2 ml cell free metabolites of the test microorganisms (*S. fredii* KCC5 and *P. fluorescens* LPK2) and subsequently covered with a cover glass. They were kept in to moist chamber for 12 h for conidial germination and 16 h for chalmydospore germination. The germination accessed by examining 400 spores of the test pathogen in different microscopic fields and percent inhibition was calculated on the basis of control set in Czapek-Dox medium. The conidia and chlamydospores were considered to be germinated when they had produced a germ tube equal to spore diameter or more in length. The inhibition of germ tube growth by metabolite was also accessed.

Conidial and chlamydospore germination was observed after 6, 18, 24 and 48 h germination was defined as the conidium developing germ tube equal to its diameter. Percent germination of conidia was calculated as below:

$$\text{Germination of conidia (\%)} = \frac{\text{Total nos. of germinated conidia}}{\text{Total nos. of conidia counted}} \times 100$$

The length of the germ tube was also measured by ocular micrometer and germination was categorized in to the following three groups on the basis of germ tube length: (i) Germ tubes more than the diameter of conidia i.e. 6-20µm (Moderate germination), (ii) Germination tubes much longer than the diameter of conidia i.e. 21-60 µm (Good germination) and (iii) Germ tube forming hyphal mats i.e. >61 µm (Excellent germination).

Effect of volatile substances of *S. fredii* KCC5 and *P. fluorescens* LPK2 on *F. udum*: The method described by Dennis and Webster [20] was followed. The test organisms *S. fredii* KCC5 and *P. fluorescens* LPK2 were grown individually and in combination on Petri dishes containing 25 ml of nutrient agar medium. After the incubation at 28°C, lid of each Petri dish was replaced by the same size bottom plate 25 ml PDA containing 5 mm agar block of *F. udum*. The whole unit was taped with an adhesive tape. Control sets were prepared in the same way but with the test pathogen in place of the test organisms. The sets were incubated at 25±2°C and after 48, 72, 96 and 120 h colony diameter of the test pathogen was measured and percent inhibition of stimulation of growth was calculated.

Biocontrol of F. Udum in Pot Bioassay: Disease incidence of fusarium wilt in pigeon pea was further measured as described earlier but this time combinations of the antagonists were inoculated in the rhizosphere of the plants with their individual inoculants. In brief, antagonists were grown in their respective growth medium broth and incubated at 28°C under shaking conditions for 48 h. After incubation, cells were harvested, re-suspended in sterile distilled water and mixed in the 1% carboxymethyl cellulose slurry seeds of the pigeonpea so as to get a final population of 10^8 per seed. The bacterized seeds were sown in the earthen pots having *F. udum* infestation and observed for wilt diseases after 30 days of sowing.

Fungal pathogen and its inoculum preparation: *Fusarium udum* was maintained on potato dextrose agar (PDA) medium. For the production of fungal inoculum, the culture was grown on PDA for 5-6 days at 28°C. Growth on five to six plates were scrapped and mixed in sterile condition with sterile distilled water (25 ml) in blender for 2 min. Inoculum was adjusted to reach 10^{8-9} conidia ml^{-1}.

Treatments: *In vivo* studies of disease suppression were carried out in earthen pots containing 10 kg sterile sandy loam soil (77.3% sand, 13.6% silt, 11.7 clay, total organic C 0.0976%, pH 6.4 having 36% water holding capacity) during July 2005 to September 2005. Treatments for biocontrol experiment were: (T1) seeds inoculated with *A. chroococcum* AZK2, (T2) seeds inoculated with *P. fluorescens* LPK2, (T3) seeds inoculated *S. fredii* KCC5, (T4) seeds inoculated consortium 1 (*P. fluorescens* LPK2+ *A. chroococcum* AZK2), (T5) seeds inoculated consortium 2 (*P. fluorescens* LPK2 + *S. fredii* KCC5), (T6) seeds inoculated consortium 3 (*S. fredii* KCC5 + *P. fluorescens* LPK2 + *A. chroococcum* AZK2), (T7) positive control (seeds receiving no bacteria and pathogen) and (T8) negative control (unbacterized seeds inoculated with pathogen). In treatments (T1) to (T6) pathogen was also infested. Five healthy seeds of *Cajanus cajan* were sown in pot (30 cm diameter) in three replicates for each treatment. Fungal inoculum (1 ml $seed^{-1}$) was spread on each seed except the control (seeds inoculated without bacteria and pathogen) i. e. treatment (T7). One ml of inoculum consisted of 10^{8-9} conidia ml^{-1}.

RESULTS

In vitro interaction study of bacterial strains, seven different PGPR strains *Pseudomonas fluorescens* LPK2, *Sinorhizobium fredii* KCC5 and *A. chroococcum* AZK2, *Pseudomonas aeruginosa* GRC2, *Pseudomonas aeruginosa* 1934, *Sinorhizobium meliloti* RMP1 and *Bacillus licheniformis* 57, were selected for preparation of

Table 2: Inter-strain interactions among the PGPR used for consortium preparation *in vitro*

Strain No.	LPK2	GRC2	1934	KCC5	RMP1	AZK2	57
57	-	+	-	-	-	-	+
AZK2	+	-	-	+	+	+	
RMP1	+	+	+	+	+		
KCC5	+	-	-	+			
1934	-	+	+				
GRC2	-	+					
LPK2	+						

Abbreviations: LPK2, *Pseudomonas fluorescens*; GRC2, *Pseudomonas aeruginosa;* 1934, *Pseudomonas aeruginosa*; KCC5, *Sinorhizobium fredii*; RMP1, *Sinorhizobium meliloti*; AZK2, *Azotobacter chroococcum;* 57, *Bacillus licheniformis;* -, negative interaction; +, positive interaction.

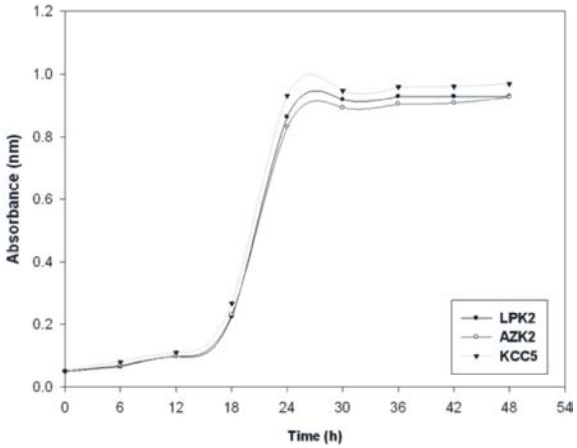

Fig. 1: Growth rate curves of *S. fredii* KCC5, *P. flurescens* LPK2 and *A. chroococuum* AZK2

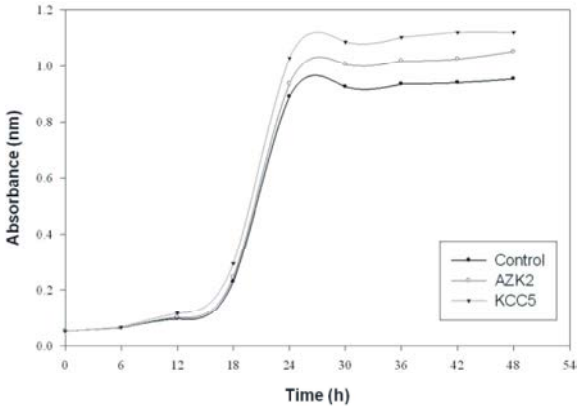

Fig. 2: Effect of culture filterates of *P. fluorescens* LPK2 on the growth of *S. fredii* KCC5 and *A. chroococcum* AZK2

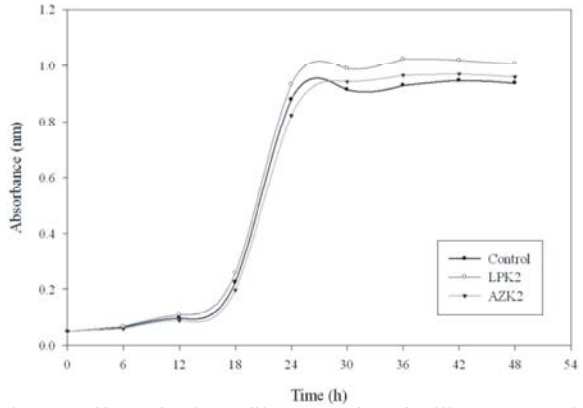

Fig. 3: Effect of culture filterates of S. fredii KCC5 on the growth of P. fluorescens LPK2 and A. chroococcum AZK2

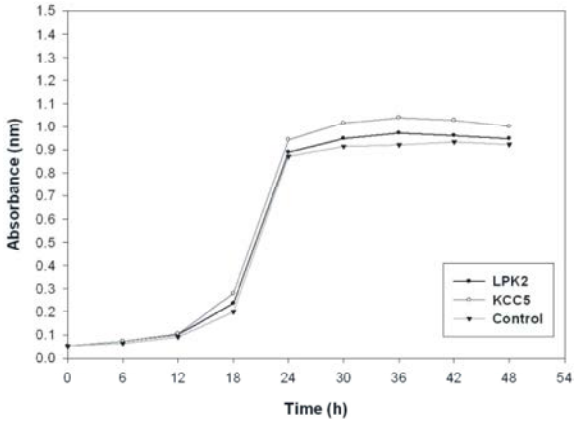

Fig. 4: Effect of Culture filterates of A. chroococcum AZK2 on the growth of S. fredii KCC5 and P. fluorescens LPK2

microbial consortium (Table 1). Initially, all strains were exposed to interact in *in vitro* conditions. After incubation the interaction plates for 24-72 h, only three strains viz. *P. fluorescens* LPK2, *S. fredii* KCC5 and *A. chroococcum* AZK2 did not inhibit each other, while the other strains viz. *Pseudomonas aeruginosa* GRC2, *Pseudomonas aeruginosa* 1934, *Sinorhizobium meliloti* RMP1 and *Bacillus licheniformis* 57, inhibited the growth of each other as revealed by growth suppressing activities (Table 2). Based on the preliminary studies and data obtained from previous experiment,

strains *S. fredii* KCC5, *P. fluorescens* LPK2and *A. chroococcum* AZK2 were tested further for mutual interaction. Dual culture studies revealed no mutual growth inhibition. Growth rate of individual strains were measured by spectrophotometric method based on turbidity (Fig. 1). The individual growth of strains *S. fredii* KCC5, *P. fluorescens* LPK2and *A. chroococcum* AZK2 were not affected significantly as evidenced by the similar optical density values of *S. fredii* KCC5, *P. fluorescens* LPK2and *A. chroococcum* AZK2 in their individual cultures (Fig. 2, 3, 4). *In vitro* dual culture studies for evaluating the interaction of one strain to another, revealed no mutual growth inhibition among *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 (Fig. 2, 3, 4). Further, spectrophotometric studies showed that the individual growth of these strains was not affected in combined cultures, where strains were cultured together. The optical density values of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 observed in present studies were at par with their treatments when incorporated into the broth and vice versa. The cell-free culture filtrate of one organism did not inhibit the growth of other bacteria and *vice versa* as compared with to their individual cultures (Fig. 2, 3, 4).

In the co-inoculation experiment of bacterial strains, the populations of *S fredii* KCC5^{sterp+}, *P. fluorescens* LPK2^{tet+} and *A. chroococcum* AZK2 $^{amp+}$ in different combinations were monitored by using serial dilutions of the samples. Different combinations were spread over the petri plates containing yeast extract mannitol agar medium supplemented with streptomycin (100 µg ml^{-1}) for *S fredii* KCC5, nutrient medium with tetracycline (100 µg ml^{-1}) for *P. fluorescens* LPK2 and nitrogen-free agar medium containing ampicillin (100 µg ml^{-1}) for *A. chroococcum* AZK2. The total numbers of CFU's were enumerated as 10^{7-8} ml^{-1} of broth in every combination of this experiment. Each combination showed substantial population of bacterial strains after different period of incubation as revealed by their appearance on respective growth

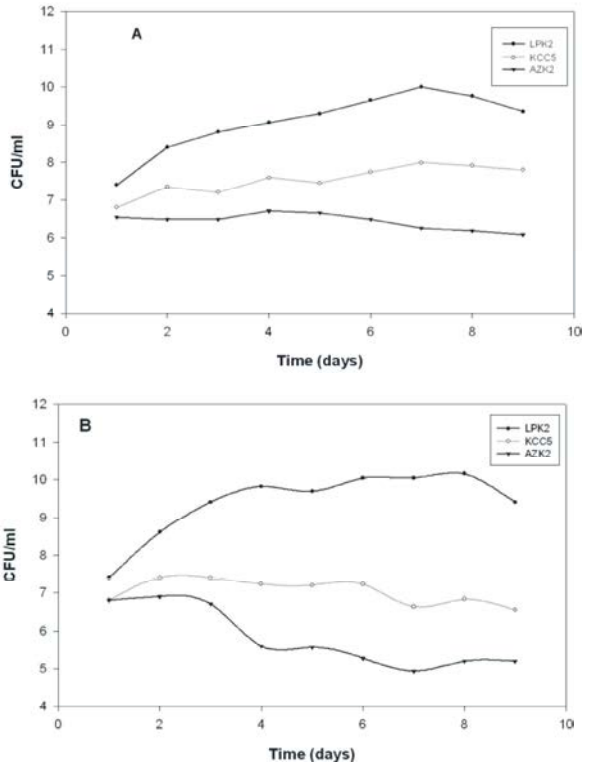

Fig. 5: Population of *P. fluorescens* LPK2, *S. fredii* KCC5 and *A. chroococcum* AZK2 in individual culture (A) and in consortium cultures (B)

medium. In this study, the population of *A. chroococcum* AZK2 showed a decline after 3 days that remained stationary till 9 days of incubation. The population of *P. fluorescens* LPK2 was found to be enhanced in consortium, while the population of *S fredii* KCC5 did not got changed from the initial population as in its individual culture (Fig. 5).

Effect of culture filtrates of *S. fredii* KCC5 and *P. fluorescens* LPK2 of germination of conidia and chlamydospores of *F. udum* showed complete inhibition of the conidial and chlamydospores germination *F. udum* (Table 3). The culture filtrates of

Table 3: Effect of non-volatile metabolite containing culture filtrates of *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 and their combinations on germination of conidia of *Fusarium udum*

	Conidia germination (%)				
	Number of hyphae per conidia				
Test	1-3	4-6	7-9	>10	Total
Control	-	0	0	92±4	92±4
Pseudomonas fluorescens LPK2	6±1	7±1	6±1	10±4	29±7
Sinorhizobium fredii KCC5	7±1	9±1	6±1	20±4	42±7
LPK2+KCC5	3±1	4±1	2±1	8±4	14±7

All values are means of three replicates; ±, standard error; 0, no growth.

Table 4: Effect of non-volatile metabolite containing culture filtrates of *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 and their combinations on germination of chlamydospore of *Fusarium udum*

	Chlamydospore germination (%) and hyphal development at different time (h) intervals										
	Moderate*			Good**				Excellent**			
	Time intervals (h)			Time intervals (h)				Time intervals (h)			
	6	18	24	6	18	24	48	6	18	24	48
Control	81	83	87	12	11	13	12	-	-	-	-
LPK2	NG	NG	2	-	-	NG	NG	-	-	-	-
KCC5	NG	NG	8	NG	-	NG	NG	NG	-	2	3
LPK2+KCC5	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG

Abbreviations: NG, no germination; *Moderate germination: Germ tubes more than the diameter of conidia i.e. 6-20µm; **Good germination: Germination tubes much longer than the diameter of conidia i.e. 21-60 µm; **Excellent germination: Germ tube forming hyphal mats i.e. >61 µm.

P. fluorescens LPK2 and *S. fredii* KCC5 inhibited conidial germination significantly (P>0.01). The inhibition of germ tube was more pronounced than that of conidial germination. A highly significant effect (P>0.01) of the non-volatile metabolites was noted against the conidial germination and germ tube growth of the test pathogen. It is apparent from the Table 3, that the conidia and chlamydospores of *F. udum* did not show germination due to non-volatiles produced by *S. fredii* KCC5 and *P. fluorescens* LPK2 after 6 h of incubation. All the germinated conidia and chlamydospores showed moderate germination in control and treatment with culture filtrate of *S. fredii* KCC5 and *P. fluorescens* LPK2. Conidia and chlamydospore germination was inhibited completely when they were inoculated in mixed culture filtrates of *S. fredii* KCC5 and *P. fluorescens* LPK2 (Table 3). It is obvious that 100% inhibition of germination of conidia and chlamydopores were recorded after 48 h of incubation due to mixed culture filtrates of *S. fredii* KCC5 and *P. fluorescens* LPK2 (Table 3). Numbers of hyphae per conidia were recorded (Table 4). Maximum numbers of hyphae per conidia were recorded as 92 in control. Combination of *S. fredii* KCC5 and *P. fluorescens* LPK2 inhibited maximum proliferation of number of hyphae from conidia of *F. udum* (Table 4).

Chemotaxis of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 toward culture filtrate of *F. udum,* the chemotaxis drop assay was used to get an impression of which fraction of the culture filtrate served as chemo-attractants for *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 cells. In contrast, a corresponding amount of culture supernatant (10 µl) from *F. udum* grown in potato dextrose broth initiated a significant chemotactic response of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 cells within 6 h (Fig. 6). In contrast, no chemotactic response

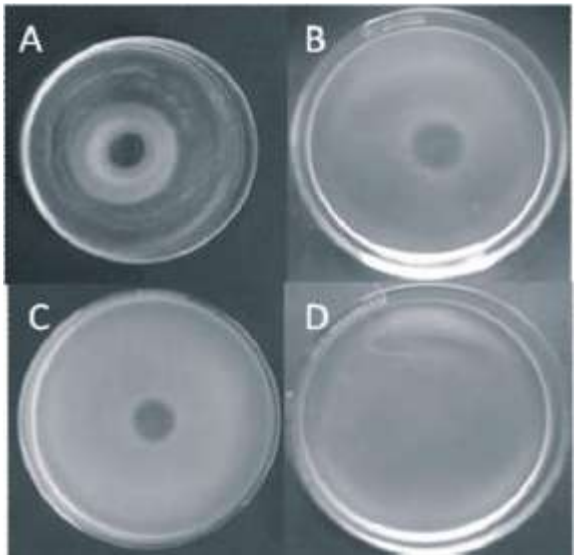

Fig. 6: Chemotaxis behavior of *P. fluorescens* LPK2 (A), *S. fredii* KCC5 (B), and *A. chroococcum* AZK2 (C), and towards culture filtrate of *F. udum*; control (D)

was observed toward sterile nutrient medium with culture supernatant of *F. udum* (control).

Effects of bacterial co-inoculation and consortium on the disease incidence and on early growth of pigeonpea plants were recorded. It was noted that the bacterial combinations showed enhanced disease suppression in comparison to individual strain (Fig. 7). *S. fredii* KCC5 and *P. fluorescens* LPK2 antagonized *F. udum* and inhibited its growth quite efficiently. Data of biocontrol experiment were collected 30 days after challenging the pathogen (Fig. 7). In positive control (T7), where seed were not inoculated with any bacteria and pathogen, 96% population of plants were healthy, which was significantly (P<0.05) higher the than negative control (T8), where only 25% plants were healthy (Fig. 7).

Table 5: Effect of *Sinorhizobium fredii* KCC5, *Pseudomonas fluorescens* LPK2, *Azotobacter chroococcum* AZK2 and their combinations on the growth of *Cajanus cajan* var. Manak under pot assays

	30 DAS				60 DAS		
Treatments	Seed germination (%)	Plant length (cm)	Plant fresh weight (g)	Plant dry weight (g)	Plant length (cm)	Plant fresh weight (g)	Plant dry weight (g)
Pseudomonas fluorescens LPK 2	84*	28.7*	24.5*	8.6*	102.5*	98.6*	38.6*
Sinorhizobium fredii KCC 5	83*	25.6*	23.1*	8.2*	98.6*	92.0*	37.0*
A.. chroococcum AZK 2	75ns	26.0ns	24.2*	8.8*	98.0*	93.2*	37.5*
Consortium 1 (LPK2 + KCC5)	87*	30.2*	28.0*	10.0**	110.6**	105.0**	41.5**
Consortium2 (LPK2 + AZK2)	84*	29.6*	27.4*	9.4*	109.2**	105.0**	41.5**
Consortium 3 (LPK2 + KCC5 + AZK2)	97**	36.2**	34.2**	12.8**	121.5**	118.6**	48.8**
Control (positive)	70	20.5	19.2	7.2	76.6	64.0	28.5
Control (Negative)	50	10.8	9.8	5.8	40.5	35.5	13.2

Abbreviation: Values are mean of 10 randomly selected plants from each set; ns, non-significant; * significant at 5% LSD; ** significant at 1% level of LSD as compared to control

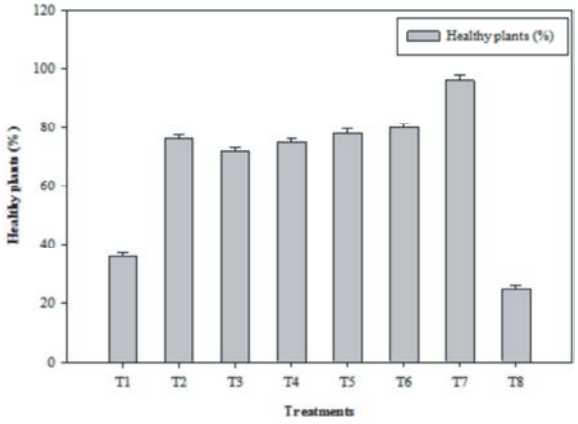

Fig. 7: Influence of plant growth promoting rhizobacteria on healthy plants (%) grown in *Fusarium udum*-infested soil. Lines above the bars indicate standard error. (Values are mean of three replicates). a, b Different letters indicate significant differences (P<0.05).

In T1 where *A. chroococcum* AZK2 resulted in non-significant increase in healthy plant as compared to control treated with pathogen (Fig. 7). The values from T2 to T6 were not significantly different from each other but the percentage of healthy plants was significantly (P<0.05) higher than control with pathogen (Fig. 7).

Bacterial co-inoculation and consortium showed positive effect on early growth of *C. cajan* plants (Table 5) in pot trial. *C. cajan* bacterized with *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 enhanced seed germination as compared to control at 15 days after sowing (DAS). The consortium of all strains and in co-inoculation of *P. fluorescens* LPK2 + *S. fredii* KCC5 treated seeds showed 97% and 87% seed germination, respectively that was 27% and 17% higher than that of control. Single inoculation, co-inoculation and consortium preparations applied to seed showed enhanced seed germination (Table 5). Such treated seed were used to raise seedlings which showed enhanced plant length, plant fresh weight and plant dry weight as compared to control. Maximum plant growth was found in consortium treated seeds in comparison to the single inoculation of the strains and co-inoculation. After 30 DAS under the influence of microbial consortium plant growth parameters (viz., plant length, plant fresh weight and plan dry weight) were enhanced by 77%, 78% and 78%, respectively in comparison to control. After 60 days plant growth got significant at 5% level of LSD. Again, in consortium-treated seeds maximum plant length, plant fresh weight and plan dry weight were enhanced (by 59%, 85% and 71%, respectively) in comparison to control (T7).

S. fredii KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 showed characteristic pattern of root colonization. LPK2 and KCC5 showed effective root colonizer, while in case of *A. chroococcum* AZK2 colonization process was not seen. This is the reason of getting lower population of AZK2 at different periods. On the other hand, co-inoculation with *A. chroococcum* AZK2 and in consortium (*S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2), *S. fredii* KCC5 and *P. fluorescens* LPK2 also colonized well the rhizosphere of *C. cajan*. The population of *P. fluorescens* LPK2 and *S. fredii* KCC5 was slightly increased from its initial inoculation after 30 and 60 DAS individually, in combinations and also in consortium. The log cfu of *P. fluorescens* LPK2 after 60 DAS was 7.813, 6.85 and 7.40 in individual,

co-inoculation with *S. fredii* KCC5 and in consortium, respectively. In individual and in consortium strain respective log cfu of *S. fredii* KCC5 was 7.65 and 7.79, while *A. chroococcum* AZK2 showed 5.70 and 4.3 log cfu in individual and in consortium, respectively.

DISCUSSION

Our approach was based on assembling microbial strain combinations strictly practical and based on the hypothesis by combining several effective strains which may lead to mutualistic effect. Currently, there are no *in vitro* tests that foretell which strain will have biocontrol activity and which one will be companionable in consortium? Thus, combinations must be screened *in situ* just as individual strain. We initially assumed that PGPR strains comprising effective combinations would be mutually non-inhibitory because of overlapping niche in the rhizosphere and the proven ability of PGPR strains to produce inhibitory secondary metabolites [21, 22]. Many of the strains that might be members of microbial consortium were either strongly inhibitory to or to strongly inhibited other members of the consortium in the *in vitro* assay. For example, *P. fluorescens* LPK2, *S. fredii* KCC5 and *A. chroococcum* AZK2 was inhibited by *P. aeruginosa* GRC2 out of 7 PGPR and also inhibited by the other 3 strains. Likewise, S. *fredii* KCC5 was inhibitory for *B. licheniformis* MTCC57 and itself inhibited by *P. aeruginosa* GRC2 and *P. aeruginosa* MTCC1934. The inhibitory actions of pseudomonads for the counterparts of the consortium have been reported by Pierson and Weller [23]. They found that some strains of pseudomonads inhibited 8 of 10 PGPR and was inhibited by 7 of 10 strains. Thus, for the preparation of microbial consortium of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 out of 7 strains were selected because of their non-inhibitory nature to each other.

Competition for substrate is considered to be one of the major evolutionary driving forces in the bacterial world and numerous experimental data obtained under well controlled conditions revealed how different organisms may effectively out-compete others because of better utilization of a given energy source. *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 were successfully grown as mixed cultures. The cooperation of *S. fredii* KCC5 and *P. fluorescens* LPK2 was due to the fact that both belong to the same group being Gram-negative, aerobic non-spore forming rods, fast-growing and having quite similar nutritional requirements. Earlier, it has been reported that *S. fredii* KCC5 and *P. fluorescens* LPK2 were produced IAA, siderophore, solubilized insoluble phosphate, showed chitinase and b-1,3-glucanase activities and strongly inhibited the growth of *F. udum* [14]. It also caused degradation and digestion of cell wall components, resulting in hyphal perforations, empty cell (halo) formation, shrinking and lysis of fungal mycelia along with significant degeneration of conidia [14]. These strains were showing synergistic growth and would be ideal due to reason that the microbial growth correspondents to their metabolic products which are beneficial to plants. Both *S. fredii* KCC5 and *P. fluorescens* LPK2 produced same type of siderophore (hydroxamate) in addition to IAA production and phosphate solubilization. The *in vitro* spectrophotometric interaction studies for growth parameters indicated the establishment of proto-cooperation among *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2. This is due to non-reactive nature of secondary metabolites produced by these strains.

Interaction among the three rhizobacteria showed that all were able to grow even in the presence of culture filtrates of one another. Proto-cooperative action of different groups of bacteria has been observed by number of workers [17, 24]. Earlier, Gera and Sharma [25] prepared a consortium of *Azotobacter, Azospirillum, Pseudomonas* and *Rhizobium* growing all the bacteria in a single medium and found that the bacteria did not affect the growth of other bacteria in the consortium. Hence, proto-cooperation behavior among *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 proved their significance in disease suppression and plant growth-promotion of *Cajanu cajan*.

In biocontrol experiment when seeds were inoculated with *A. chroococcum* AZK2 (T1), non-significant ($P<0.05$) increase in healthy plants was recorded as compared to that of pathogen control (T8). It might be due to the fact that *A. chroococcum* AZK2 was not an antagonist of *F. udum*. In the present study no significant differences ($P<0.05$) in T2 to T6 treatments were recorded because of the presence of *P. fluorescens* LPK2 and *S. fredii* KCC5 in different combinations which antagonized *F. udum* employing different types of mechanisms. Similar findings have been obtained by de Boer *et al*. [24] wherein each biocontrol agent may use different mechanism to counter the pathogen. This is the reason of achieving better performance of bacterial consortium. *S. fredii* KCC5 and *P. fluorescens* LPK2 had the ability to produce siderophore *in vitro*, which is a biocontrol related trait. Besides, *P. fluorescens* LPK2 also produced hydrocyanic acid (HCN) and chitinase enzyme (Kumar *et al*. 2010).

Due to important trait dependent consistent performance by *S. fredii* KCC5 and *P. fluorescens* LPK2 decreased wilt incidence in *Cajanus cajan* in comparison to control during pot study. Earlier, Leeman *et al.* [26] found decrease in *Fusarium* wilt by the inoculation of *P. fluorescens*.

In the present investigations, the application of microbial consortium increased early vegetative and reproductive plant parameters in comparison to individual strains of *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2. Effective root colonization is a pre-requisite attribute for the success of PGPR in plant growth and yield promotion. Further, for the protection of plants from soil-borne diseases, the crucial colonization level must be obtained to a level estimated at 10^5-10^6 CFU (colony forming unit) g^{-1} of root [27, 28]. Positive root colonization ability of *P. fluorescens* LPK2 obtained. This proved the successful colonization in the rhizosphere of pigeonpea. In our study, *S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2 evaluated for antibiotic resistance. The antibiotic resistant maker strains developed for carrying out studies on seed bacterization and root colonization. *S. fredii* KCC5 showed resistant to streptomycin (100 µg ml^{-1}), *P. fluorescens* LPK2 to tetracyclin (100 µg ml^{-1}) and *A. chroococcum* AZK2 for ampicillin (100 µg ml^{-1}). Presence of these marker strains revealed the effective root colonization and competence in the rhizosphere even in the presence of pathogens. Aggressive colonization by this consortium can be due to effect of secretion of root exudates. Secretion of root exudates enhanced rhizobacterial colonization [29] resulting in production of excess amount of siderophore and other compounds which may be involved in biocontrol of phytopathogens [30].

The greater genetic diversity of phenotypes within the consortium (*S. fredii* KCC5, *P. fluorescens* LPK2 and *A. chroococcum* AZK2) compared to single strains more likely due to greater variety of combined traits. All the three strains co-exist sin sufficient number in the rhizosphere of *Cajanus cajan* as revealed by their population size at different time periods during crop standing in the field. Thus, by applying microbial consortium, multiple biocontrol mechanisms are involved in biocontrol of *F. udum* in root region and genes expressed in a wider range of environmental conditions and cover a broader range of rhizosphere to reduce the wilt incidence as well as plant growth promotion is directly related to better nutrient uptake capability in *Cajanus cajan*.

The magnitude of plant growth promoting activities was better seen in the case of consortia or mixed cultures than single strain. Combined use of different biocontrol agents or integration of biocontrol agent with other disease management options, with identifiable differences in their mechanisms of action, has improved disease protection and the activity spectrum of biocontrol agents. From these results it was concluded that development of microbial consortium based on their interaction studies can reduce the possibilities of failure of potential microbial inoculants in the rhizosphere. These results have an agronomic importance for sustainable development of agriculture which may assist to solve problems encountered with phytopathogens.

ACKNOWLEDGEMENTS

Authors are grateful to CSIR-TMOP and M, New Delhi, India and IASE Deemed University, Sardarshahr-331401 (Rajasthan), India for providing financial assistance.

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