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Assessment of population of pseudomonads inhabitant of tomato plant rhizosphere for searching of potential antagonist of *Fusarium udum*

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Abstract

Eight isolates of fluorescent pseudomonads were isolated from the disease suppressed and identified and characterized by physiological and biochemical and bacteriological tests. LPK2 aligned to same cluster (100% bootstrap value) with reference strains of *Pseudomonas fluorescens*. All the strains of *P. fluorescens* LPK2 showed IAA production, phosphate solubilisation, siderophore production and HCN production. Chitinase activity and β-1,3-glucanase activity was also pronounced in *P. fluorescens* LPK2. In dual culture technique, all the strains of *Pseudomonas* (LPK1 to LPK8) inhibited the *in vitro* growth of *F. udum*. Maximum inhibition of *F. udum* recorded in the case of *P. fluorescens* LPK2. Cell free culture filtrate of also restricted the hyphal growth of *F. udum*. Scanning electron microscopic studies on morphological features in the hyphae of *F. udum* in the form of fragmentation and degradation of mycelia and loss of structural integrity of conidia of *F. udum* were clearly observed. In iron supplemented (30 μM Fe^{+3}) medium inhibition of the pathogenic fungi was reduced. Under iron-deficient condition, *P. fluorescens* LPK2 inhibited *F. udum* by 65%. The inhibition of the pathogen by *P. fluorescens* LPK2 under iron-sufficient conditions was 51% .

Key words : Fusarium udum, pseudomonads, tomato.

Introduction

India is a principal *Cajanus cajan* growing country contributing nearly 90% of total world's production. Several biotic and abiotic factors constrain the productivity of pigeonpea under different cropping conditions responsible for its low productivity (700-800 kg/ha) against potential yield of 2500-3000 kg/ha. Pigeonpea, is attacked by more than 100 pathogens (Nene *et al.*, 1996). The disease of considerable economic importance at present is fusarium wilt (*Fusarium udum*). It infests the seeds and such seeds either failed to germinate or produce diseased seedlings of low survival potential.

The chemical fungicides and pesticides are known to be highly effective and promote in controlling fusarium wilt but have various constraints. An alternative to these chemicals is the use of certain bio-control agents which are inexpensive and eco-friendly and have no harmful effects on human population. The plant-associated, fluorescent pseudomonads have drawn considerable attention world wide because of their plant growth promoting and disease suppressive nature. Pseudomonads rapidly and aggressively colonize the root system and suppress pathogenic microorganisms improving plant growth and grain yield (Schippers *et al.*, 1987; Weller, 1988). In this study, the main aim is to investigate the potential fluorescent pseudomonads and their role in suppression of *F. udum* causing wilt in *C. cajan*.

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Materials and methods

Fluorescent bacteria were isolated from the disease suppressed rhizosphere of the tomato (*Lycopersicon esculentum*) plant cultivated at a farmer's field in Badgaon, Betul district (M.P.), India. Plants were gently uprooted and loosely adhering soil was carefully removed from the roots. The roots were cut into 2 cm long segments. Root segments (1g) with tightly adhering soil were shaken in 9 ml of sterile distilled water for 30 minutes on a rotary shaker. The suspension was serially diluted to spread on *Pseudomonas* isolation agar (Himedia; M406) plates. Plates

were incubated at 28± 1°C for 24-48 h and observed for fluorescent colonies. The blue green fluorescent pigment producing bacterial colonies were carefully picked up and maintained on King's B medium (KBM) slants at 4°C for further screening. Biochemical characterization of the bacteria strains was carried out according to Holt *et al.*, 1994.

Isolation and identification of fungal pathogen

Samples of diseased root parts and seeds were randomly collected and fungal pathogen *was* isolated from infected roots of pigeon pea (*Cajanus cajan*) following the water agar technique. After incubation at 28°C for one week, plates were observed for the appearance of fungal pathogen. The fungal isolates were grown on the potato dextrose agar (PDA) medium and cultures were maintained on potato dextrose agar (PDA) medium and characterized following illustration and description of standard mycological literature after comparing with standard cultures obtained from the Division of Plant Pathology, Indian Institute of Pulses Research (IIPR), Kanpur, India. The morphological characters of *F. udum* such as colony growth, colour pigmentation in substrate, mycelial characters, and micro and macro conidia production were considered for the identification of fungi as given by Booth (1971).

IAA Production

To observe IAA production exponentially grown cultures (10^8cells ml^{-1}) of the each bacterial isolate were strains were incubated separately. The 24 h cell free culture supernatants of the strains were analyzed for IAA production after measuring wavelength at 530 nm using UV-VIS spectrophotometer (Shimadzu, Model UV-1601. The calibration curve of pure IAA was used as a standard following the linear regression analysis.

Phosphate Solubilization

Phosphate solubilization ability was detected by spotting the culture (24 h) on Pikovskya's agar plates. The quantitative estimation of phosphate solubilization was carried out according to modified method of Jackson (1958). The data was recorded by comparing with the standard curve in the similar set of conditions.

Cyanogen Determination

HCN (hydrogen cyanide) production was determined by modified method of Bakker and Schippers (1987). For these exponentially grown bacterial cultures (10^8cells ml^{-1}) of each isolate was examined for putative HCN production.

Effect of HCN on fungal growth: The strains of fluorescent *Pseudomonas* were grown on Petri plates containing NAM for 12 h. An agar discs (5 mm diam) cut from 5 day old cultures of *F. udum* was placed at the center of bottom part of another plate containing PDA. The plates inoculated with fungi were placed in an inverted position over the other plate, containing already grown culture of the fluorescent *Pseudomonas*. The plates were made airtight by pasting with adhesive cellophane tape. The plates with NAM without fluorescent *Pseudomonas* served as control. Radial growth of the test fungi was recorded.

Siderophore Production

Siderophore production was determined on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). The bacterial isolates (24 h) spotted separately on CAS medium and incubated at 28±1°C for 48 h. The siderophores production was determined by evaluating the absorption spectrum of 48 h supernatant at 400 nm, spectrophotometrically.

Siderophore Assay: For siderophore production by bacteria, a special siderophore production liquid culture was prepared which contained MM9 salts, tris buffer, casamino acids (0.3%), L-glutamic acid (0.05%), (+) biotin (0.5 ppm) and sucrose (0.2%). Bacterial cultures were grown in this medium for 48 h at 28°C under rotating conditions and its cell density was monitored spectrophotometrically at 610 nm. After incubation, culture supernatant (0.5 ml) was mixed with 0.5 ml of CAS assay solution. An un-inoculated medium served as reference. After reaching colour equilibrium the absorbance of the mixture was measured at 630 nm. The concentration of siderophore in cultures was determined by using a calibration curve of hydroxalamine as a standard under the same conditions and following the linier regression analysis.

Time course of siderophore production: Following the above method of quantification of siderophore production, samples from the bacterial broth were withdrawn at every 24 h intervals up to 168 h for the quantitative determination of siderophore. A plot was prepared comparing siderophore production with respect to time.

Lytic Enzymes Production

Chitinase activity was observed by the method of Renwick *et al.* (1991) in a defined medium having colloidal chitin as sole carbon source. The bacterial cultures were spotted on the surface of defined medium and incubated at 28±1°C for 6-7 days. β-1,3-glucanase activity was assessed using the same medium except colloidal chitin was replaced by laminarin (Sigma). Bacterial cultures were streaked and/ or spot inoculated directly on to the plates. Chitinase activity was identified by the development of clear halo around the colonies or bacterial spots, while β-1,3-glucanase activity was confirmed with the bacteria growing on the medium having laminarin as a sole source of carbon (Rangel-Castro *et al.*, 2002).

For the preparation of colloidal chitin (Berger and Reynoldes, 1988), 10 g of crab shell chitin (Sigma) was slowly dissolved in one liter of concentrated HCl under stirring

conditions at 4°C temperature. The resultant viscous mixture was incubated on waterbath at 37°C until the viscosity of the mixture is decreased. To this mixture, 4 liters of sterile distilled water was added and left overnight at 4°C. The supernatant was slowly decanted and the precipitate was collected in a filter paper. This precipitate was then washed extensively with sterile distilled water to attain a natural pH (7.0). The saturated colloidal chitin was air dried and was dissolved in 250 ml sterile distilled water prior to use.

Biochemical quantification of chitinase: Extracellular chitinase activity was assayed by measuring the release of N-acetyl-D-glucosamine (Glc Nac) from colloidal chitin as a substrate. The assay mixture consisted of 0.3 ml of 1 M sodium acetate buffer (pH 5.3), 0.5 ml of 2% colloidal chitin and 0.25 ml of bacterial supernatant. The reaction mixture was incubated in a water bath at 37°C for 60 min and the reaction was stopped after boiling it for 15 min. The mixture was centrifuged at 5,000 rpm for 20 min and the concentration of released Glc Nac was assayed at 530 nm spectrophotometrically from the aliquots following DNS sugar estimation test using Glc Nac as standard. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µM of Glc Nac per h during these conditions.

Production profile of chitinase in P. fluorescens LPK2: Exponentially grown culture (10^8cells ml^{-1}) of strains were incubated separately on broth medium at 28 °C and 150 rpm on a rotary incubator shaker and the samples were aseptically withdrawn after every 24 h. Supernatant of the strains were collected by centrifugation at 10,000 rpm for 15 min at 4°C and quantity of chitinase in the supernatant in the form of Glc Nac was determined following above mentioned method, and the production profile was observed by correlating the quantity of Glc Nac with time.

DNA isolation

For the isolation of DNA from fluorescent *Pseudomonas* spp. were grown in Luria Bertani (LB) broth. The bacterial pellets were washed with 50 mM ethylenediamine tetraacetic acid (EDTA, pH 8.5). The washed cells were lysed by suspending in 0.5% sodium dodecyl sulphate (SDS) for 10 min at 60°C. The resulting lysate was cleared from cell debris by centrifugation at 10,000 rpm for 10 min. The supernatant was taken and protein was removed by the addition of proteinase K followed by addition of ethanol to collect the DNA. The DNA solution was prepared by the addition of 1 ml phenol-chloroform mixture. To the DNA extract 50 µg ml^{-1} RNase was added and incubated at 37°C for 30 min. This was followed by the addition of tris-phenol and centrifugation at 12,000 rpm for 15-20 min at 4°C. The supernatant was heat sterilized and 100 µl, 0.3 M sodium acetate and 1 ml cold isopropanol was added with continuous vortex. Finally, the DNA was purified by the addition of phenol-chloroform followed by the centrifugation at 15000 rpm.

16S rRNA Gene Amplification

Universal eubacterial primers FD1 5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTC AG3' and RD1 5'CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC3' were used for amplification of 1492 bp region of the 16S rRNA gene on PTC 100 (M.J. Research, USA) thermal cycler. A 50 µl reaction mixture included 5-10 ng of bacterial DNA as template, 1 µl of each primer, 1U of *Taq* DNA polymerase (Banglore Genei, India) and 100 µm dNTPs. The reaction conditions were: initial denaturation of 7 min at 94°C followed by 29 cycles of denaturation of 1 min at 94°C, extension of 1 min at 72°C and annealing temperatures 54°C for 7 cycles; 53°C and 52°C for one cycle each, 51°C for 20 cycles and a final extension of 10 min at 72°C. Amplified gene was visualized in 0.8% agarose after electrophoresis.

16s rRNA gene sequencing:

Partial 16S rRNA gene sequencing was performed in the same reaction mixture and following the same amplification conditions as described in full 16S rDNA. The only difference was in primers. In the partial gene amplification, primers f1 and r1 were used. The PCR products were analyzed on 1.2 % agarose gel in TAE buffer, run at 50 V for 2 h. Gels were stained with ethedium bromide and visualized as described above. The amplicons were purified with Banglore Genei, PCR purification kit and quantified spectrophotometrically at 260 nm compare with calf thymus DNA. The cleaned partial 16S rDNA amplicon was sequenced with DNA sequencing system.

Sequence analysis of 16S rDNA sequences using bioinformatics tools:

The PCR products were purified and sequenced. Theses all sequences were subjected for phylogenetic analysis. The homology of partial sequences were compared with the sequences from the DNA databases and similar sequences showing above 95% were retrieved by nucleotide BLAST (basic local alignment search tool) program at NCBI BLAST server (www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment of retrieved sequences was done by EBI ClustalW server (www.ebi.ac.uk.clustal/index.html). Phylogenetic tree constructed by using *genebee* server (www.genebee.msu.ru/services/phtree-reduced.html). Phylogenetic tree obtained with bootstrap values in cluster algorithm, phylip format and topological algorithm

Antifungal activities of Pseudomonas fluorescens LPK2

Antagonistic properties of *Pseudomonas fluorescens* LPK2 was tested against *F. udum* on PDA plates using dual culture technique (Skidmore and Dickinson, 1976). Five days old mycelial discs (5 mm diameter) were placed in four corners of solidified medium in plates containing modified PDA by addition of 2% sucrose.

Exponentially grown culture (108 ml-1) of LI :2 was spotted 2 cm juxtaposed from the fungal disc and incubated at 28±10C for 5 days. Growth inhibition was calculated by measuring the distance between the bacterial and fungal colonies as compared to control. Growth inhibition was calculated by measuring the distance between the edge of bacterial and fungal colonies. The zone of inhibition was recorded by using given below formula: Inhibition (%) = (C)−(T)/(C)× 100, where, C=Radial growth in control and T=Radial growth in dual culture.

Effect of culture filtrates on the inhibition of pathogenic fungi

Antagonistic bacteria were inoculated in their respective broth cultures and incubated in rotary incubator shaker at 150 rpm and 280C for 7 days. After incubation broth culture of the strains were collected by centrifugation at 10,000 rpm for 15 min at 40C and passed through Millipore filter (0.45 µm). Such culture supernatants were poured in the wells of the fungal challenged plates. After incubation at 280C for 5-7 days, inhibition of the pathogenic fungi was recorded.

Antagonism in iron-deficient and iron sufficient conditions

In vitro test for antagonism of *P. fluorescens* LPK2 was made under iron-deficient and iron-sufficient (30 and 60 µM Fe3+) conditions towards the *Fusarium udum*. Iron deficient medium was prepared by treating bacterial broth (250 ml) with 8-hydroxyquinoline solution in 100 ml chloroform (up to final concentration (0.1%). It was then vigorously shaken. Excess of hydroxyquinoline was removed by chloroform extraction until the disappearance of yellow color. Medium was boiled to remove the smell of chloroform. Agar (2%) was added and the content autoclaved at 15 lb /inch-2 (1210C) for 20 min. Antagonism was carried out by following the methodology mentioned as before. Bacterial broth supplemented with 30 and 60 µM Fe3+ was used to carry out *in vitro* fungal assay under iron sufficient condition. Methodology for antagonism study was adopted as mentioned above.

Compound microscopy of post-interaction events in fungal mycelia

Fungal mycelia growing towards the zone of inhibition were processed for microscopy. Fungal mycelium were picked up from the zone of inhibition with the help of sterile needle and transferred to a drop of lacto-phenol on the clean glass slide. Specimens were examined under a light compound microscope (Olympus BX 51 TRF) for fungal morphological abnormalities occurred due to antagonism mediated by the rhizobial strains. Images of fungal deformities were captured by using Image Analyser (Biovis).

Scanning electron microscopy (SEM) of post-interaction events in fungal mycelia

The antagonist and the test organism are grown in pure cultures. Agar disc (5mm diameter) are to be cut from the actively growing margin of the colonies and placed 3cm apart on the surface of the Petri dishes containing freshly prepared, sterilized and solidified potato Dextrose agar medium. Incubated Petri dishes at 25°C with continuous light after incubation of 4 days collect mycelial sample from the region of interaction. For preparation of SEM samples, the mycelia were collected with the help of micro-niddle in sterile environment from the zone of interaction with bacteria on the surface of gelled agar in Petri plate. Then it were fixed overnight at 4°C in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and washed three times (10 min each) in phosphate buffer. After three 10 min rinses in distilled water, samples were dehydrated through 70, 80, 90 and 100% ethanol (5 min in each stage) and three changes in 100% ethanol at room temperature. Ethanol was then replaced by liquid CO_2 and the samples were air dried. Then samples were mounted on stubs and coated with gold. These coated specimens were observed at 15 kV in a *LEO 485 VP* Scanning Electron Microscope. Photo-micrographs were recorded by the same machine.

Results

Mycelia were hyaline, branched and felted. Aerial mycelia were usually less developed and with profuse development of sporodochia (macroconidiophores), long creeping, slender, septate and measured 3-5 µm in width on PDA. Conidia were formed from simple or verticillately branched conidiophores and each conidiophore had monophialides on the ultimate tips of which conidia were produced. Microconidia were single or double celled hyaline, ovoid, fusoid, reniform, mostly curved and scattered, whereas macroconidia were produced in masses on pionnotes sporodochia. Macroconidia were hyaline, thin walled, falcate, septate slightly curved or sickle shape.

Eight isolates of fluorescent pseudomonads were obtained from the disease suppressed rhizosphere of the tomato (*Lycopersicon esculentum*) based on production of water soluble fluorescent pigment on *Pseudomonas* isolation agar (PIA). The isolates were abbreviates as LPK1 to LPK8. All the isolates were Gram-negative, non-spore forming, non-capsulated, motile and rod shape structures. They were fast growers with average mean generation time of 1.2 to 1.4 hours (Table 1). Although the optimum temperature was 28°C but growth occurred at wide range of temperatures from 5°C to 42°C. On NAM the colonies were smooth, translucent, large, low convex, 2-4 mm in diameter with regular spreading edge. Greenish blue fluorescent pigment was produced by the isolates which turned into brown colour in old cultures. On MacConkey's agar, pale colourless colonies were formed. The isolates were positive

for catalase, oxidase, and urease activities but negative for MR, VP tests and H_2S production. PHB accumulation was absent while citrate utilization was shown by all the fluorescent pseudomonad isolates (Table 1).

After amplification of 16S rRNA gene with universal fd1 and rd1 primers, the amplified products were compared with low range ruler DNA (Banglore Genei, India) and observed that total length of amplicons was approximately 1500 bp (1.5 kb) which shows the amplification of nearly full length 16S rRNA gene from all pseudomonads. The size of the PCR product corresponded to the size of the 16S rRNA genes among all eight isolates.

Neighbour-joining dendograms were generated (based on cluster algorithm) using 16S rDNA sequences of isolated pseudomonads and representative *Pseudomonas* spp. sequences from EMBL/Gen Bank/DDBJ/PDB (Fig. 1). The 16S rDNA sequence of the following strains (type strains unless otherwise indicated) were obtained from GenBank: *Pseudomonas aeruginosa* PT121 (EF515832), *Pseudomonas aeruginosa* PAW (EF434506), *Pseudomonas fluorescens* 2R37 (EF178447), *Pseudomonas fluorescens* ost5 (DQ439976), *Pseudomonas fluorescens* AE1 (AY247063), *Pseudomonas aeruginosa* ATCC 15692 (EF178445), *Pseudomonas fluorescens* ATCC 17386, *Pseudomonas fluorescens* MM-B16, *Pseudomonas fluorescens* AU2039A, *Pseudomonas aeruginosa* MCCB103 *Pseudomonas aeruginosa* PT121 (EF515832). Phylogenetic analysis of 16S rDNA sequences of pseudomonads (LPK1 to LPK5) with other reference strains revealed LPK1 and LPK3 belong to same cluster with 100% bootstrap value and maximum similarity with LPK4 (100%), *P. aeruginosa* PT121 (94%) and *P. aeruginosa* PAW (94%) (Fig.1). LPK2 and LPK5 showed maximum similarity with *P. fluorescens* strains (Fig. 1). A neighbour-joining dendogram was generated using the sequence from *Pseudomonas* sp. LPK2 and representative *Pseudomonas fluorescens* sequences from GenBank (Fig. 2). Form this analysis, LPK2 aligned to same cluster (100% bootstrap value) with reference strain *Pseudomonas fluorescens* 2R37 (Fig. 2). This analysis has proven that LPK2 is strain of *Pseudomonas fluorescens* and can be designated as *Pseudomonas fluorescens* LPK2.

All the strains of *Pseudomonas* spp. (LPK1 to LPK8) were positive to IAA production with the development of pink colour with and without the addition of tryptophan into the culture media (Table 2). Tryptophan supplementation in the medium induced IAA production. This trend was common in all the strains of Pseudomonads. Most of the *Pseudomonas* strains produced HCN as evidenced by the change of the colour of filter paper from yellow to moderate and reddish brown (Table 2). *Pseudomonas* sp. LPK2 produced deep reddish colour as well as good activity of HCN, while LPK7 was negative for HCN activity (Table 2). In the presence of glycine and $FeCl_3$, the deep brown colour of filter paper was observed, that was clear indication of induced HCN production by bacterial strains. All the strains of *Pseudomonas* spp. were screened for siderophore production on CAS agar. All the strains of *Pseudomonas* spp. produced siderophores forming orange halos around their spots on the CAS agar (Table 2). Maximum size of orange halo was produced by *P. fluorescens* LPK2. Secretion of siderophore by *P. fluorescens* LPK2 started after 12 h of incubation and lasted up to 5 days but on further incubation the production was declined (Fig. 2a). To know the type of siderophore, 48 h old supernatant of the strain LPK2 scanned at 400 nm showed major peak, corresponded with hydroxmate type of siderophores.

Chitinase activity was determined by the development of clear halo around the colonies or bacterial spots, while β-1,3-glucanase activity was confirmed by appearance of bacterial presence in the medium supplemented with laminarin as a sole source of carbon. *Pseudomonas* spp. LPK2, LPK4 and LPK8 showed chitinase activity (Table 2). *Pseudomonas* sp. LPK2 showed the maximum chitinase activity followed by LPK8 (Table 2). Chitinase formation was started after 24 h of incubation by *Pseudomonas* sp. LPK2 which reached maximum at 120 h and on further incubation its activity was declined (Fig. 2b).

In dual culture technique, all the strains of *Pseudomonas* (LPK1 to LPK8) inhibited the *in vitro* growth of *F. udum* (Table 2). Maximum inhibition of *F. udum* recorded in the case of *P. fluorescens* LPK2. It was recorded as 83% higher in comparison to control after 168h of incubation (Fig 3). Cell free culture filtrate of *Pseudomonas* spp. LPK2 also restricted the hyphal growth of *F. udum* (Fig. 3). Morphological abnormalties in the hyphae of *Fusarium udum* obtained from the zone of interaction during dual cultures was observed by light microscopy and in scanning electron microscopy, revealed loss of structural integrity of the mycelium of *F. udum*. Twisting, hyphal perforations, lysis and empty cell formation, septation, fragmentation and degradation of mycelia of *F. udum* was observed under light microscopy (Fig. 4). Fragmentation and degradation of mycelia and loss of structural integrity of conidia of *F. udum* were clearly observed under Scanning electron microscopic analysis (Fig. 5). These features of mycelia may be due to production of lytic enzymes by strains LPK2.

Volatile compounds produced by *Pseudomonas* sp. LPK2 exhibited antagonistic activity when challenged against *F. udum* after 5 days of incubation. This strain caused 45% reduction in vegetative growth of *F. udum* (Fig. 6). On further incubation zone of inhibition of fungal pathogen decreased. Mycelial growth of both the pathogens decreased significantly in dual cultures by the antagonists with respect to control in iron deficient conditions (Fig. 7). In iron supplemented (30 μM Fe^{+3}) medium inhibition of the pathogenic fungi was reduced. In this case, *Pseudomonas* sp. LPK2 reduced fungal growth

maximally. However, percent inhibition was lowered in 60 µM Fe^{+3} supplemented medium in comparison to normal conditions of the medium. Under iron-deficient conditions remarkable inhibition of *F. udum* was recorded. It was found that under iron-deficient condition *Pseudomonas* sp. LPK2 inhibited 65% vegetative growth of *F. udum* (Fig. 7). On the other hand, the inhibition of the pathogen by *Pseudomonas* sp. LPK2 under iron-sufficient conditions was 51% (Fig. 7).

Discussion

Fusarium is a group of filamentous fungi widely distributed in various soil types. This is a large cosmopolitan genus of imperfect fungi and is of primary interest because numerous species are important plant pathogens (Nelson, 1991). *Fusarium* spp. occur frequently among the fungal microflora associated with seedling disease, and wilt is a major cause of plant mortality in many important crops (Minton and Garber, 1983). Isolates of *Fusarium* produce colony growth with fluffy, appressed or intermediate types of mycelium. Key macroconidial characters are shape, size and septation that distinguish the isolates from each other.

Due to wide distribution of different *Pseudomonas* spp. in the soil environment and relatively easy cultivation, genus *Pseudomonas* is among the best-studied bacterial group worldwide (reviewed by Kumar *et al.*, 2005). Some noble strains of *Pseudomonas aeruginosa* were isolated from our group from the rhizosphere of potato (Gupta *et al.*, 2002) and Sunflower (Bhatia *et al.*, 2005). Keeping this fact, more strains of fluorescent pseudomonads having PGP characters, were isolated from the rhizosphere of tomato (*Lycopersicon esculentum*).

A number of workers (Gupta *et al.*, 2001; Validov *et al.*, 2005) isolated, identified and characterized fluorescent pseudomonads from the rhizosphere of agricultural crops such as potato, tomato, cotton and tea. Initially, Kloepper *et al.* (1988) identified it as plant growth promoting rhizobacteria (PGPR) due to its rapid and aggressive rhizosphere colonization that proved as most powerful inoculants to improve plant growth and grain yield (Lucy *et al.*, 2004).

The morphological, physiological, and biochemical characteristics for the identification of rhizospheric pseudomonads (LPK1 to LPK8) followed the characteristics mentioned by Palleroni (1984) and Holt *et al.* (1994). The strains identified as fluorescent pseudomonads belong to *Pseudomonads fluorescens* and *P. aeruginosa* showed blue green fluorescent colour due to the presence of pyocynin pigment (Paulitz and Loper, 1991). The organism losses its pigment production under iron sufficient condition due to inhibition of siderophores production such as pyoverdin (Paulitz and Loper, 1991).

Five strains of *Pseudomonas* spp. (LPK1 and LPK5) were characterized according to 16S rDNA sequence homology. All the strains were showing maximum sequence homology with their respective cluster of reference strains. In Phylogenetic tree based on 16S rDNA homology *Pseudomonas* sp. LPK2 is placed in *Pseudomonas fluorescens* clade. The use of 16S rDNA gene analysis has become promising. This is realized that 16S rDNA homology allows a first Phylogenetic affiliation of new isolate. Molecular microbiological characteristics have been carried out to characterize different groups of bacteria by using 16S rDNA sequencing.

The direct plant growth occurs due to Indole Acetic Acid (IAA) production (Chabot *et al.*, 1998), phosphate solubilisation (Nautiyal *et al.*, 2000), siderophores production (Gupta *et al.*, 2001) and HCN production (Antoun *et al.*, 1998), whereas indirect plant growth-promotion occurs due to growth restricting conditions by producing antagonistic substances such as antibiotics (Kang *et al.*, 2004), lytic enzymes (Lim and Kim, 1995). A bacterium can affect plant growth by one or more of these mechanisms and used different abilities for growth-promotion at various times during the life cycle of plants (Sessitsch *et al.*, 2005).

Interestingly, all the strains of *Pseudomonas* spp. produced siderophore which was agreement with the findings of Bhatia *et al.* (2005). The study further supported by Sindhu *et al.* (1999) who found 83% the fluorescent pseudomonads as siderophores producer while their quantities, in the form of orange halo vary. *P. fluorescens* LPK2 showed hydroxamate type of siderophores production. In case of LPK2 absorption maxima was recorded at 400 nm. Similar observations especially from fluorescent pseudomonads have been made by several workers (Gibson and Magrath, 1969). Iron in the soil is unavailable for direct assimilation by microbes because of its unavailable form i.e., ferric iron or Fe^{3+}, which is prominent in the soil and sparingly soluble about 10^{-18} M at pH 7.4 (Neilands and Nakamura, 1987). Siderophores secreted by PGPRs have been shown to have a very high affinity for iron and bind most of the Fe^{3+} that is available in the rhizosphere, and prevent the pathogens present in immediate vicinity from proliferation because of lack of iron (O' Sullivan and O' Gara, 1992).

Inhibitory effect of *P. fluorescens* LPK2 was observed against *F. udum* under iron-sufficient. It was found that inhibitory activity of strains of *P. fluorescens* LPK2 was partially neutralized when iron was supplemented to their respective medium. Significant reduction in the fungal inhibition has also been evidanced by Fuhrmann and Wollum (1989). Microbial siderophore production generally seems to be switched on when the Fe^{+3} concentrations is less than 20µM (Lynch, 1990). In the present study, LPK2 examined for inhibition of *F. udum* under iron (30 µM Fe^{+3}) supplemented conditions showed reduction in inhibition against both the fungal pathogens. On increasing the concentration of iron to 60 µM Fe^{+3}, more inhibition was recorded in comparison to normal iron condition. Similar

findings were obtained by Derylo and Skorupska (1993) who reported that 60 µM Fe^{+3} suppressed the siderophore production in *Pseudomonas* sp. This gives evidence that one of the mechanisms for the inhibition of *F. udum* was due to the involvement of siderophores produced by *P. fluorescens* LPK2

Harrison *et al.*, (1995) observed HCN production in *Pseudomonas aureofaciens* and one strain of *P. putida* from take-all suppressive soil. HCN is formed stoichiometrically in an oxidative reaction catalyzed by HCN synthase and its activity induced by glycine the precursor of HCN (Castric, 1981). Glycine amendment in the culture media of *P. fluorescens* LPK2 enhanced the quantity of HCN production showed its induced behaviour. HCN is a secondary metabolite produced by some Gram-negative bacteria such as *P. fluorescens* and *P. aeruginosa*, *Chromobacterium violaceum* (Askeland and Morrisom, 1983) and have a deleterious effect on growth of pathogens.

HCN and other volatile compounds produced by *P. fluorescens* LPK2 exhibited antagonistic activity on the growth of *F. udum* and caused growth inhibition of *F. udum*. The mode of action of HCN in disease control is partially understood but it is believed that cyanide ions derived from HCN are the potent inhibitor of many matelo-enzymes, especially copper-containing cytochrome C oxidase (Blumer and Haas, 2000) which are critical in respiration of living beings.

In our observations, *P. fluorescens* LPK2 released extracellular chitinase and β-1,3-glucanase enzymes. Chitinolysis plays an important role in biological control of plant diseases and has been substantiated with increased disease control by chitin-supplemented application of chitinolytic biocontrol agents. Chitinase and β-1, 3-glucanase are key enzymes in the decomposition of fungal hyphal wall (Rangel-Castro *et al.*, 2002). In the present study, It is thought that cell wall lysis occurred by concerted action of both chitinase and β-1,3-glucanase. chitinase since chitin and β-1,3-glucan are known to be the major components of most fungal cell walls (Lim and Kim, 1995). It has been also demonstrated that extracellular chitinase and laminarinase synthesized by *Pseudomonas stutzeri* digest and lyse mycelium of *F. solani* (Lim *et al.*, 1991).

Microscopic abnormalities in fungal pathogens under the influence of *P. fluorescens* LPK2 clearly showed degeneration and degradation of the hyphae of *F. udum*. Abnormal hyphal swelling, digestion of fungal cell wall, cytoplasm coagulation, hyphal perforation, hyphal tip degradation and halo cells formation appeared in mycelia of *F. udum* were caused by *P. fluorescens* LPK2 mediated by HCN and chitinolytic enzymes. Losses of structural integrity of conidia were also observed. Thus, it showed the hyphal wall interaction with bacterial cells rapidly lysed the hyphae, causing leakage of the fungal cytoplasm. Gupta *et al.* (2001) have been demonstrated the loss of sclerotial integrity, hyphal shriveling, mycelial and sclerotial deformities and hyphal lysis in *M. phaseolina* by *Pseudomonas* GRC_2. Microscopic studies revealed the mode of the action of antagonistic activity and help to localize site of interaction between antagonists and fungal pathogens. *In vitro*, fungal growth inhibition assay suggested that antagonistic nature of *P. fluorescens* LPK2 against *F. udum* mainly due to production of antimicrobial substances, such as chitinolytic enzymes, HCN, antibiotics, siderophore and nutrient competition. However, role of other inhibitory metabolites such as toxins and proteolytic enzymes in the inhibition process of fungal pathogens can not be ruled out (Dunne *et al.*, 1998). *P. fluorescens* LPK2 showed inhibition of *F. udum* in cell free culture filtrates. There are several factors involved in the microbial antagonism which including staling growth products, pH of media, growth rate of interacting microbes, competition for nutrients and space, competitive colonization, nature and chemical composition of growth media, ionic or nutrient imbalance caused due to nutrient impoverishment and physical factors. Many researchers have attempted the development of biocontrol agents against soil-borne plant pathogens since the discovery of the PGPR by Kloepper *et al.* (1980).

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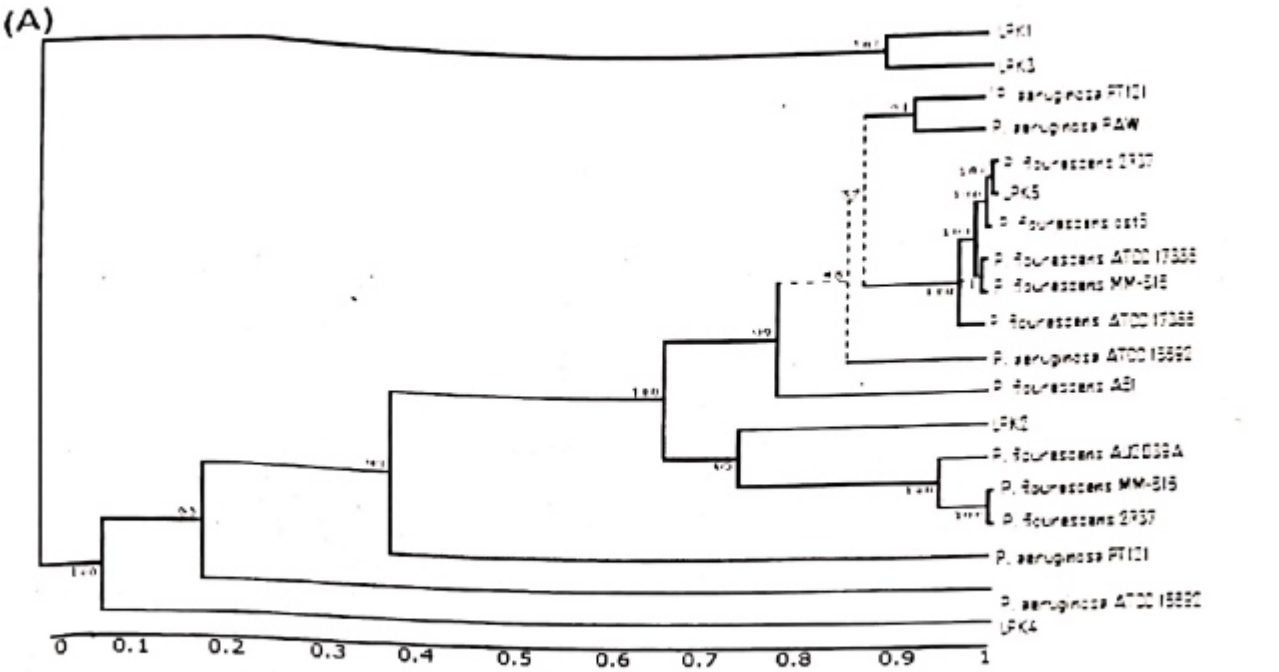
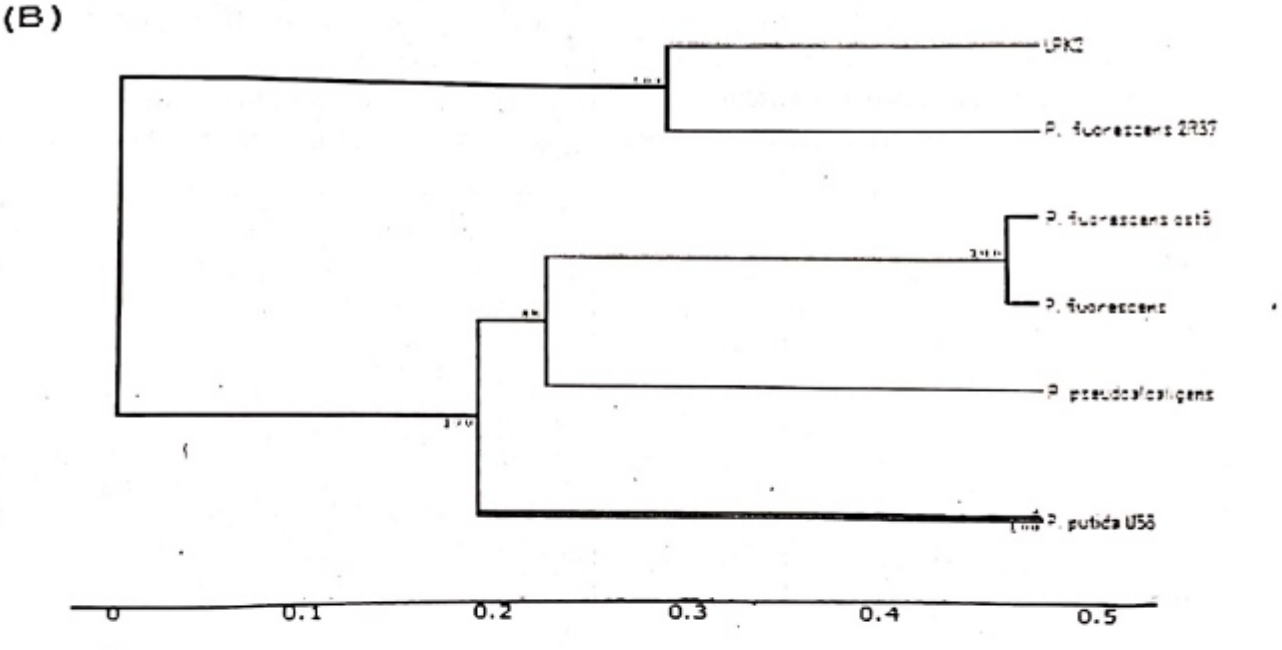
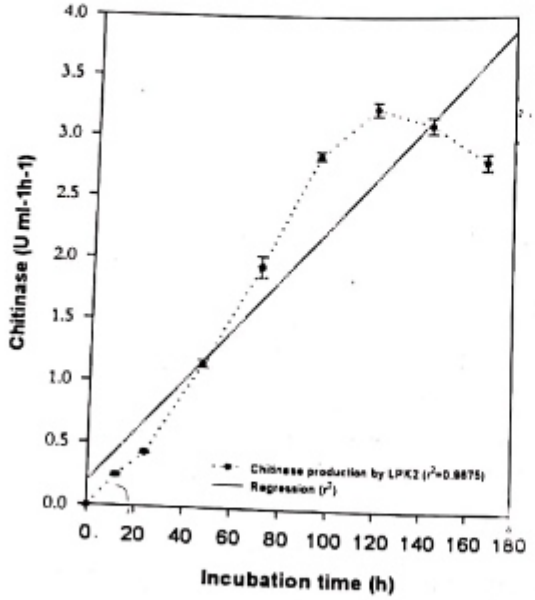


Fig 1: Time course analysis of siderophore production (A) and chitinase production (B) by *Pseudomonas* sp. LPK2 (Values are means of standard deviation of three replications)



Accession numbers represented: Pseudomonas fluorescence 2R37 (EF178447); *Pseudomonas fluorescens* ost5 (DQ439976); *Pseudomonas fluorescens* (Ab091837); *Pseudomonas pseudoalcaligens* (AF181570); *Pseudomonas putida* U58 (Ef194102)



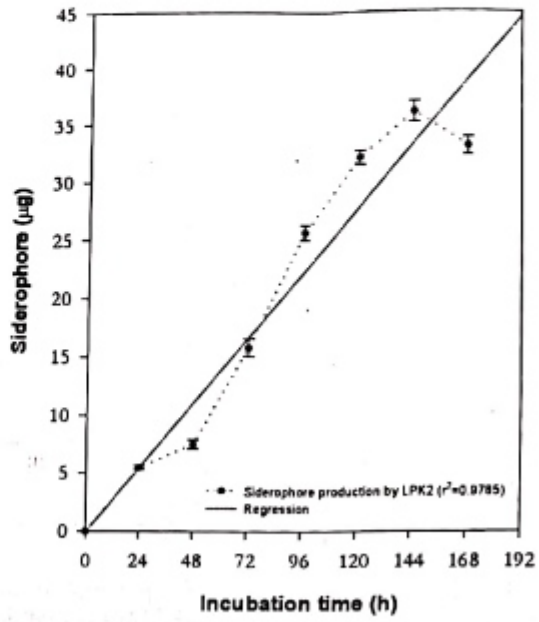


Fig 2: (A) Phylogenetic relationship between bacterial strains of Pseudomonads and representing species based on partial length 16S rDNA sequences constructed using cluster algorithm. Bootstrap values are indicated (from 1000 replication). Accession

(B) Phylogenetic relationship between *Pseudomonas* sp. LPK2 and representing species based on partial length 16S rDNA sequences constructed using cluster algorithm. Bootstrap values are indicated (from 1000 replication). Accession numbers are reported in text

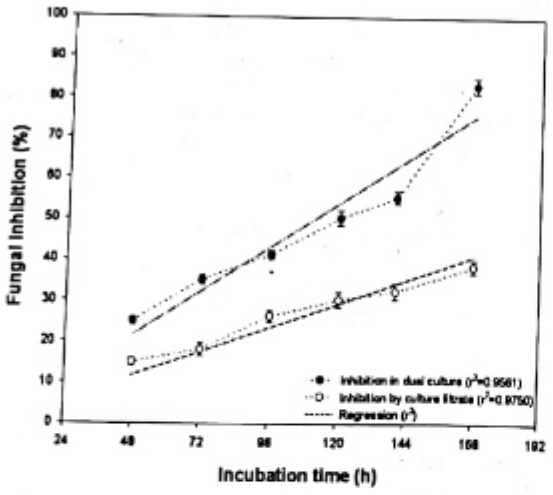


Fig 3: Inhibition of *F. udum* by *P. fluorescens* LPK2 (B) under dual culture and by cell free culture filtrate (Values are means of standard deviation of three replications)

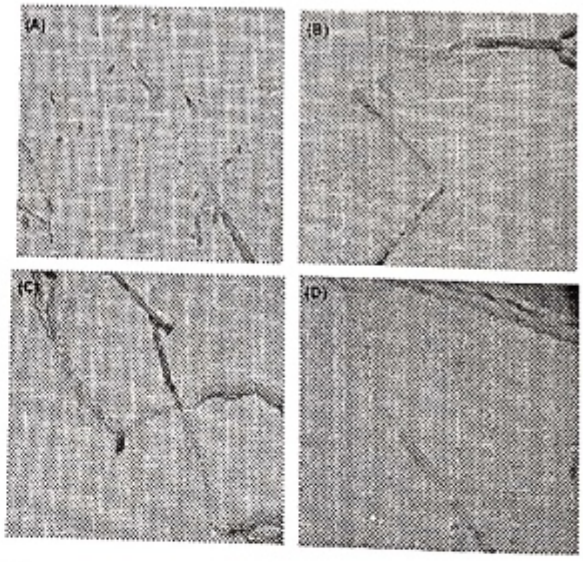


Fig 4: Compound microscopic photographs of post interaction events in *F. udum* due to *P. fluorescens* LPK2; halo cell formation and hyphal perforation (A), hyphal tip degradation (B), digestion of fungal cell wall (C) and halo cell formation (D) (see arrows)

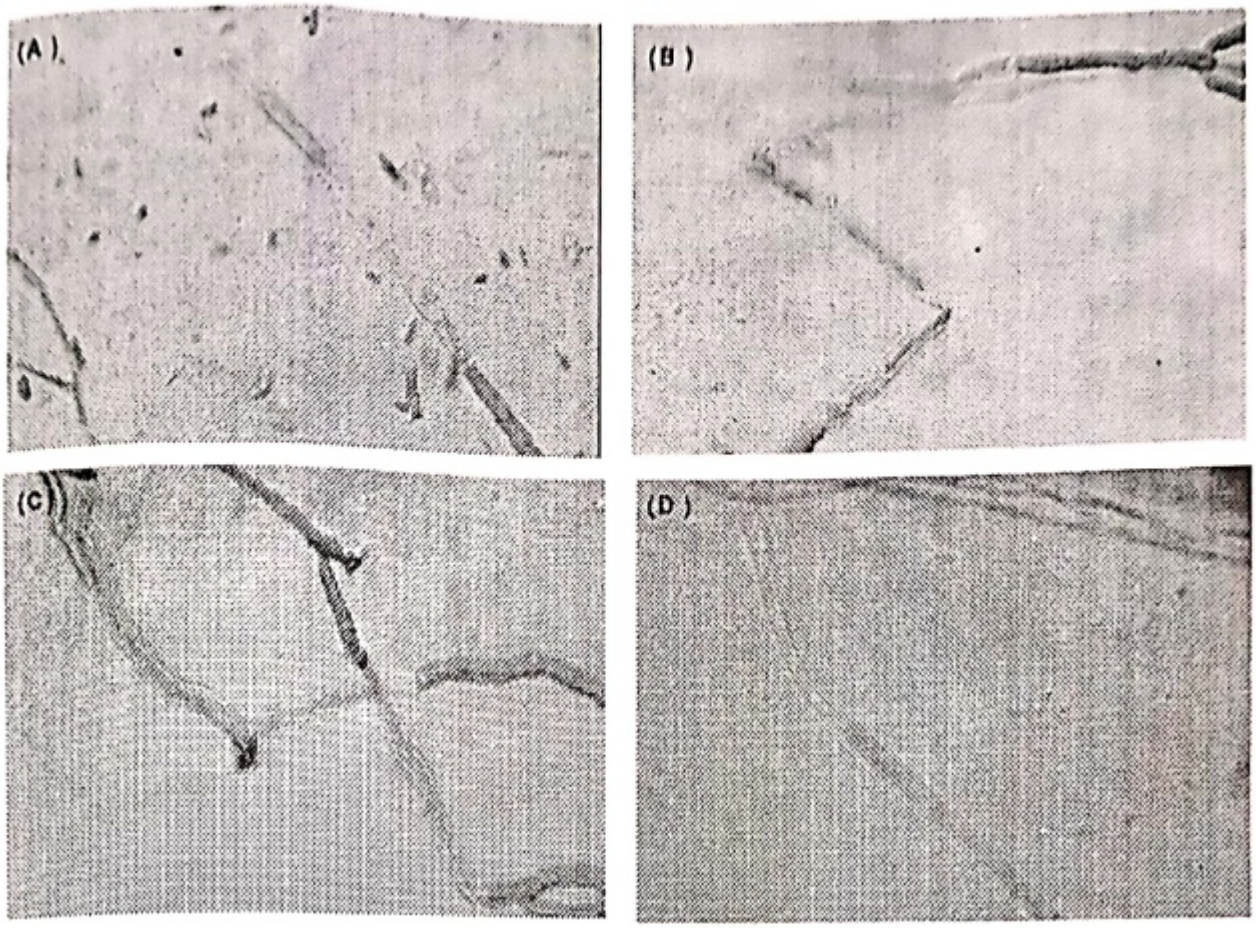


Fig 5: Scanning electron microscopic photographs of mycelial and conidial destruction by antagonistic interaction effects of *P. fluorescens* LPK2 control (A), hyphal lysis (B), hyphal destruction (C), hyphal and conidial lyses (D) (see arrows)

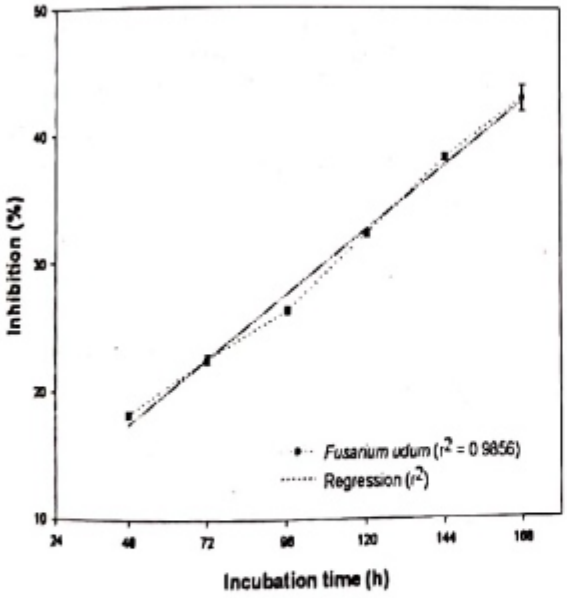


Fig 6: Effect of volatile compounds of *P. fluorescens* LPK2 on the growth inhibition of *F. udum* [Values are means of standard deviation of three replications]

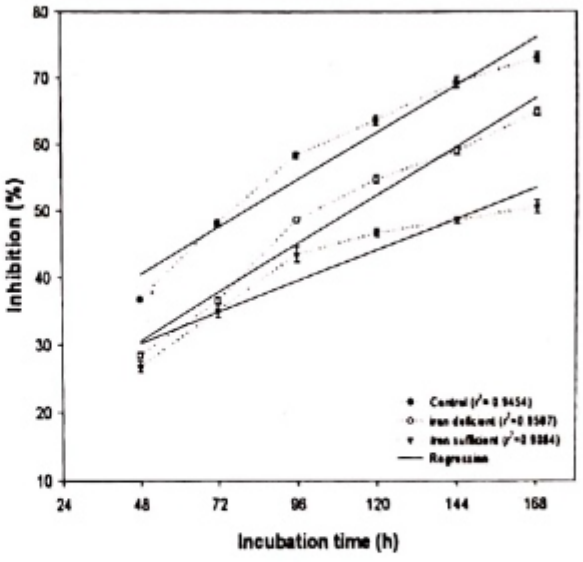


Fig 7: Antagonism between *F. udum* and *P. fluorescens* LPK2 under iron sufficient and iron deficient conditions (Values are means of standard deviation of three replications)

Table 1 Morphological, physiological and biochemical characters of fluorescent pseudomonads

Characteristics	LPK 1	LPK 2	LPK 3	LPK 4	LPK 5	LPK 6	LPK 7	LPK 8	1934	102	103
Gram reaction	-	-	-	-	-	-	-	-	-	-	-
Growth at: 4°C	-	-	-	-	-	+	-	-	-	+	+
41°C	+	+	+	+	+	-	+	+	+	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+
Fluorescent diffusible pigment	+ (bg)	+ (bg)	+ (bg)	+ (bg)	+ (y)	+(y)	+ (bg)	+ (bg)	+ (bg)	-	+(y)
Non-fluorescent non-diffusible pigment	-	-	-	-	-	-	-	-	-	-	-
Capsule	-	-	-	-	-	-	-	-	-	-	-
Endospore	-	-	-	-	-	-	-	-	-	-	-
PHB accumulation	-	-	-	-	-	-	-	-	-	-	-
Generation time (h)	1.4	1.3	1.3	1.4	1.4	1.3	1.4	1.2	1.2	1.3	1.3
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-
M. R. test	-	-	-	-	-	-	-	-	-	-	-
V. P. test	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+
Utilization of:											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Meso-Inositol	-	-	-	-	-	-	-	-	-	-	+
Maltose	+	+	+	-	+	+	+	+	+	-	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Mannose	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-	-	-	+	+

Abbreviations: +, positive; -, negative; PHB- poly hydroxyl butyrate; H_2S- hydrogen sulfide; MR- methyl red; VP-vogas proskaur, bg- blue green; y- yellow; *Pseudomonas aeruginosa* MTCC-1934; *P. putida* MTCC-102 and *P. fluorescens* MTCC-103

Table 2 Plant growth-promoting and antifungal properties of *Pseudomonas* spp. strains

Strains	IAA [A]	Phosphate solubilization [B]	Siderophore [C]	HCN [D]	Chitinase [E]	β-1,3-glucanase	Antagonism against *F. udum*
LPK 1	+	+	+	+	+	+	+
LPK 2	++	++	+++	+++	+	+	++
LPK 3	+	+	+	-	-	+	+
LPK 4	+	+	+	-	+	-	-
LPK 5	+	++	+	++	-	+	-
LPK 6	-	+	+	-	-	-	-
LPK 7	++	++	++	-	++	+	+
LPK 8	-	+	+	+	-	+	+
Standard strains							
MTCC 1934	-	-	+	-	-	-	-
MTCC 102	-	+	-	-	-	+	-
MTCC 103	-	-	-	-	-	+	-

Abbreviations: A -, IAA negative, +, IAA positive; B -, Phosphate solubilization negative; +, phosphate solubilization positive, C -, Absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0cm wide surrounding colonies; D -, HCN negative, +, HCN positive; E -, chitinase negative, + chitinase positive small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; F -, β-1,3-glucanase negative, +, β-1,3-glucanase positive; *Pseudomonas aeruginosa* MTCC-1934; *P. fluorescens* MTCC-103; *P. putida* MTCC-102