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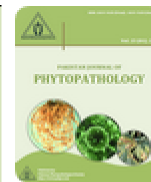

ISOLATION AND *IN-VITRO* SCREENING OF POTENTIAL ANTAGONISTIC RHIZOBACTERIA AGAINST *PYTHIUM DEBARYANUM*

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A B S T R A C T

A number of rhizobacterial isolates were isolated from the rhizospheric soil that was collected from infected chilli fields during survey. Main objective of the study was to identify the antagonistic bacterial isolates which have potential to restrict or suppressed the growth of *Pythium debaryanum*, the cause of chilli damping off. Thirty seven bacterial isolates were identified by their colony morphology and biochemical identification tests. Duel culture technique was applied to check the antagonistic behaviour of bacterial isolates. Among thirty seven bacterial isolates in which eleven isolates were shown significance antagonism towards the pathogenic fungal organism. Thirteen bacterial isolates (RB-17,RB-20,RB-21,RB-23,RB-25,RB-26,RB-27,RB-28,RB-31,RB-32,RB-34,RB36,RB-37) rendered between 77 and 96% inhibition while twenty four isolates rendered between 6 and 40%. Maximum inhibition was calculated in case of RB-37 (96%) and RB-34 (77%). Molecular identification was made by isolating bacterial DNA and amplification with 16SrRNA universal primers. PCR products were sequenced and phylogenetic analysis was done. *Bacillus subtilis* and *Pseudomonas fluorescence* were finally identified as potential antagonists against *P.debaryanum.*

Keywords: Antagonism, Biological Control, Damping off, Duel culture technique

INTRODUCTION

The primary screening strategy to isolate potential antagonistic bacterial species is integral to ensure the presence of most effective biological control agent. Moreover, screening through culture collections is not that much effective as the organisms are adapted to controlled conditions and remain unable to survive under natural conditions. Antagonistic organisms also should not be isolated from heavily infected plant parts (Campbell, 1986). Knowledge about the conditions required for antagonism gives idea about those factors which are responsible in triggering the potential or efficacy of antagonistic organisms. The antagonistic potential of an antagonistic organism is fully dependent upon its potential to survive in the same ecological niche as pathogen (Volksch and May, 2001).

According to an estimate about 60% losses have been reported in chili under nursery as well as field conditions due to damping off infection caused by the species of *Pythium* (Jadhav and Ambadkar, 2007). Biological control strategies such as of antagonistic organisms or plant growth promoting rhizobacteria (PGPR) instead of chemical pesticides are a better replacement in order to counter different plant pathogens (Schmidt *et al*., 2004). Species of *Pseudomonas* and *Bacillus* are root colonizer bacteria among the PGPR (Manikanda *et al*., 2010; Joseph *et al*., 2007), and also trigger the defence mechanism of host plant (Kloepper *et al*., 2004). These bacterial isolates espoused a variety of mechanism like production of siderophore, cyanide hydrogen, antibiotics as well as their struggle to get nutrients and space, induction of resistance and enrichment of plant growth development (Intana *et al*., 2008). *Pseudomonas* and *Bacillus* strains have great potential in control of Fusarium wilt disease of chickpea (Anjajah *et al*., 2003; Hervas *et al*., 1997; Landa *et al*., 1997). Modern research has been shifted towards the use of biological control agents as an alternative to chemical pesticides in order to ensure the

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safety and health of environment as well as farmers (Klopper *et al*., 1992; Ramesh *et al*., 2009).

MATERIALS AND METHODS

Isolation of rhizobacteria from roots: Rhizobacteria were isolated from the rhizosphere of healthy chilli plants from different chilli growing areas of district Lahore. Rhizospheric soil was separated from roots of the plants and roots were then excised into small pieces. Chopped root parts were dipped in 1%NaCl solution and vortexed at 8000rpm for ten minutes in order to remove the rhizospheric soil. Serial dilutions were prepared from this root wash. 500µl of the dilution was then spread on Luria Bertani Agar medium (Peptone 10g, Yeast extract 5g, Sodium chloride 5g, Agar 12g) supplemented with antifungal (Nystatin 50mg/L) (Simon and Ridge, 1974). Inoculated plates were incubated at 28ºC for 48 hours after that colonies were tested for their fluorescence under UV light at 354 nm at UV transilluminator.

Isolation of rhizobacteria from soil: One gram rhizospheric soil from each sample was mixed in 90 ml distilled water for the isolation of aboriginal rhizobacterial species. Dilution units were made and 100 µl from three dilution units (10^2, 10^4 and 10^8) were spread on Luria-Bertani (LB) plates with sterilized glass spreader. Inoculated plates were then incubated at 28°C for 24 hours (Aneja, 2002). After the first isolation all different colonies were further purified on fresh LB media by streaking method. For preservation the bacterial cultures were maintained at 4ºC and a bacteria glycerol stock was prepared by diluting the bacterial cultures with sterilized glycerol solution for a 25-50% v/v concentration. After same incubation period the purified cultures were preserved at -20ºC by adding 80% glycerol solution (Cappuccino and Sherman, 2002).

Primary screening for antagonistic potential: Thirty seven bacterial isolates were tested for their ability to produce antifungal substance against *Pythium debaryanum* using a dual culture *in-vitro* assay on potato dextrose agar (Sigma-Aldrich@19g/L). 5 µl of each bacterial suspension (10^8cfu/ml) was inoculated on plate and incubated at 28ºC for 48 hr. A single 6mm diameter mycelial disc was placed at the centre of plates. Then, plates were then incubated at 27-30ºC and after five days the growth diameter of the pathogen was measured. Percentage of growth inhibition was calculated by the formula of Skidmore and Dickinson, 1976.

$$\text{Percentage growth inhibition} = \frac{(\text{R1} - \text{R2})}{\text{R1}} \text{x}100$$

Where,

R1= Growth radius of pathogenic fungi on control plate

R2= Growth radius of pathogenic fungi with antagonistic organism

Identification of potential antagonistic rhizobacterial isolates

Phenotypic characterization of rhizobacteria: Classification and categorization of various bacterial colonies on the basis of appearance and morphology allows us to make a selection for the transfer of more relevant colonies from a mixed culture. Exact morphology of a bacterial cell as well as colony is accomplished after purification. Phenotypic profile of rhizobacterial isolates were recorded on LBA medium plates. 48 hr old pure cultures were used in order to study the features such as size, elevation, margin, colour, shape, texture, surface, smell and pigmentation of bacterial isolates. Moreover, rhizobacterial isolates were also visualized under UV light at 360nm wavelength in order to check the motility as well as fluorescence of isolated species.

Colony Morphology: Appearance and morphology of purified bacterial colonies was observed by streaking on LBA medium and incubated at 37ºC for 24 hr. various morphological features of purified bacterial colonies such as size, shape, shape of colony margins and elevation were observed with naked eye as well as under stereoscope (Barthalomew and Mittewar, 1950).

Biochemical characterization of bacteria

Oxidation Fermentation Test: Hugh and Leifsom medium (sodium chloride 5g, di-potassium phosphate 0.3g, bromothymol blue 0.08g, pancreatic digest of casein 2g, agar 3g) was ready and 6 ml of it was added in every culture tube and autoclaved. As the medium become solidified the bacterial inoculum from 24 hour old pure cultures was given while leaving the control tube. Thereafter, two test tubes of each isolate were sealed to provide anaerobic conditions and in the same way two tubes were remained unsealed for aerobic fermentation. Culture tubes were then incubated at 35ºC for 5 days. After incubation period the colour change from blue-green to yellowish was the sign of oxidative potential of the bacteria while change in the colour of medium and production of gas was indication of fermentative breakdown of carbohydrates by bacterial isolates.

Catalase Test: In order to determine the catalase activity of bacterial isolates, a minute fraction of inoculum was taken and placed on glass slide. Two or three drops of 3% H_2O_2 were added. After adding the

solution the emission of O2 designated by bubbling was the indication of catalase potential of test bacteria.

Citrate Utilization Test: For this test Simmon's citrate agar (sodium chloride 5g, sodium citrate 2g, ammonium dihydrogen phosphate 1g, di-potassium phosphate 1g, magnesium sulphate 0.2g, bromothymol blue 0.08g, agar 15g) was made, distributed in test tubes. Tubes amended medium were then autoclaved and kept for solidification in a slanting position. Rhizobacteria were then streaked from twenty four hour old fresh colonies. Inoculated tubes were incubated at 34ºC. Bromothymol blue was used as indicator for the presence of catalase enzyme in the medium. Change of medium colour from green to blue was the indication of positive result.

H2S Production Test: For H_2S production test SIM agar medium was made and dispensed in culture tubes. These tubes were autoclaved and kept for solidification of medium in tube. Bacterial isolates were inoculated in all culture tubes and the tubes were incubated at 34°C for 48 hours. After incubation period presence of black precipitates was the indication of positive result and no precipitation was the indication of negative result.

Indole Production Test: Culture tubes were made with SIM agar media. Tubes were then autoclaved and cooled to 45°C. Every culture tube was added with 50 $\mu g\ ml^{-1}$ tryptophane solution which was previously filtered as well as sterilised. Culture tubes were then inoculated with 24 hour old bacterial colonies and incubated at 34°C for 24 hours. After incubation period Kovac's reagent was added to each tube and production of cherry red layer was the indication of positive indole production test.

Hydrogen Cyanide Production: Bacterial isolates were screened for the production of hydrogen cyanide (HCN) adapting the method of Lorck (1948) described by Ahmad *et al.* (2008). Bacteria were streaked on N-agar plate and a filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution was placed on the top of agar surface. Plates were incubated at 30°C for 4 days. Development of orange to red colour indicated HCN production.

Phosphate Solubalization: Phosphate solubilization ability of plant associated bacteria was determined qualitatively by plating the bacteria in Pikovskaya agar medium (Glucose 10g, $Ca_3(PO4)_2$ 5g, $(NH_4)_2SO_4$ 0.5g, NaCl 0.2g, $MgSO_4.7H_2O$ 0.1 g, KCl 0.2g, Yeast Extract 0.5g, $MnSO_4$ 0.05g, $FeSO_4.7H_2O$ 0.05g, Agar 15g). Bacterial culture was streaked on the surface of Pikovskaya agar medium in duplicate. The presence of clearing zone around bacterial colonies after one week incubation period at 30°C was used as indicator for positive phosphate solubilisation.

Siderophore Production: Siderophore production was determined qualitatively using Chrome azurol S (CAS) agar as described by Alexander and Zuberer (1991). Bacterial cultures were streaked on the CAS agar plates with two replicates. Orange halos around the colonies after overnight incubation indicated siderophore production.

Preservation of selected rhizobacterial species: Different techniques were followed to preserve the best antagonistic bacterial species. Mineral oil preservation was done by pouring the sterile paraffin oil on the surface of nutrient agar tube containing bacterial cultures. The level of oil was made above the agar and cultures were incubated at 4°C in order to slow down the bacterial metabolism. 15% glycerol preservation method was also adopted by adding glycerol solution to the vials containing bacterial cultures and incubated at -10°C for preservation (Lelliott, 1966; Sleesman, 1982).

MOLECULAR IDENTIFICATION

PCR and Sequence analysis by using 16S rRNA universal primers: DNA was isolated from 48 hour old bacterial cultures in L-broth by using the Genomic DNA isolation kit (Thermo Fisher Scientific) by following the instructions. Isolated DNA was then subjected to PCR in order to amplify the 16SrRNA gene with primers f (AGAGTTTGATCCTGGCTC) and r (GGTTACCTTGTTACGACT) (Lane, 1991). PCR reaction was performed in 25µl reaction with 1µl of bacterial genomic DNA (Sambrook, 2001). PCR products were then subjected to gel electrophoresis for separation on 1 % agarose gel.

Sequencing and Phylogenetic Analysis:

Gene cleaned samples of both bacterial isolates were sent to commercial labs for sequencing analysis. Initial sequencing was done by using 16S rRNA forward primer. After the sequencing of samples, obtained sequences were blasted on NCBI for finding similar sequences. Multiple alignments of the sequences were performed. Only those sequences were included that showed highest sequence similarities with our query sequence. Phylogenetic analysis following the Maximum Likelihood method (Tamura and Nei, 1993) were conducted using software MEGA6 (Tamura *et al.,* 2013).

RESULTS

Isolation of rhizobacteria:

The total of thirty seven bacterial isolates were isolated and selected on the basis of their morphological features such as colony colour, colony size and texture. Out of these thirty seven bacteria 62% were originated from soil while other 38% were originated from roots. (Table 1).

Table1. Different Bacterial species isolated from Chili growing areas of Lahore.

Locality	No. of Samples Processed	No. of Bacterial colonies	Isolated Bacterial Species
Manawan	13	7	*Paraecoccus, Rhizobacter, Bordetella, Bacillus, Aeromonas, Streptococcus, Micrococuss*
Marl Mari	15	6	*Bordetella, Streptococcus, Pseudomonas, Aeromonas, Azotobacter, Acinetobacter*
Ganji Sindhuwan	21	7	*Rhizobacter, Bacillus, Bordetella, Aeromonas, Acinetobacter, Micrococcus, Streptococcus*
Dhoop Sari	23	6	*Aeromonas, Acinetobacter, Azotobacter, Bacillus, Pseudomonas, Bordetella*
Dhokywali	19	7	*Parococcus, Rhizobacter, Bordetella, Bacillus, Aeromonas, Streptococcus, Micrococuss*
Khwaja Faiq	22	6	*Bordetella, Streptococcus, Pseudomonas, Aeromonas, Azotobacter, Acinetobacter*
MoslleyWal	27	5	*Bacillus, Pseudomonas, Acinetobacter, Aromonas, Streptococcus*
Hadiara	18	7	*Rhizobacter, Bacillus, Bordetella, Aeromonas, Acinetobacter, Micrococcus, Streptococcus*
Chappa	15	6	*Aeromonas, Acinetobacter, Azotobacter, Bacillus, Pseudomonas, Bordetella*
Shamki Bhattian,	19	5	*Paraecoccus, Rhizobacter, Bordetella, Bacillus, Aeromonas*
Sunder	17	6	*Bordetella, Streptococcus, Pseudomonas, Aeromonas, Azotobacter, Acinetobacter*
Baath	22	4	*Bacillus, Pseudomonas, Azotobacter, Streptococcus*
Manga Mandi	23	5	*Bacillus, Pseudomonas, Acinetobacter, Aromonas, Streptococcus*
Echo Gill	19	6	*Bordetella, Streptococcus, Pseudomonas, Aeromonas, Azotobacter, Acinetobacter*
Jandiala	24	7	*Rhizobacter, Bacillus, Bordetella, Aeromonas, Acinetobacter, Micrococcus, Streptococcus*

***In-vitro* antagonism assay:** Thirty seven bacterial isolates were screened for antagonism or antifungal activity against *P.debaryanum.* Out of thirty seven bacterial isolates eleven had shown significant antagonism (77 and 96%) against pathogenic fungi. The isolates RB-17(89%), RB-20(86%), RB-21(89%), RB-23(90%), RB-25(81%), RB-27(79%), RB-28(87%), RB-31(91%), RB-32(82%), RB-34(77%), RB-36(80%) and RB-37(88%)were among the most potential antagonists. Other twenty six bacterial isolates have not shown significant antagonism against pathogenic fungi (Figure 1&2).

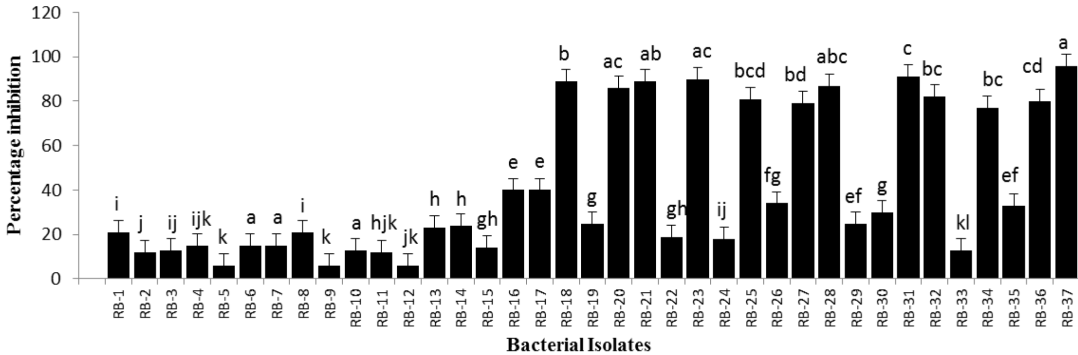


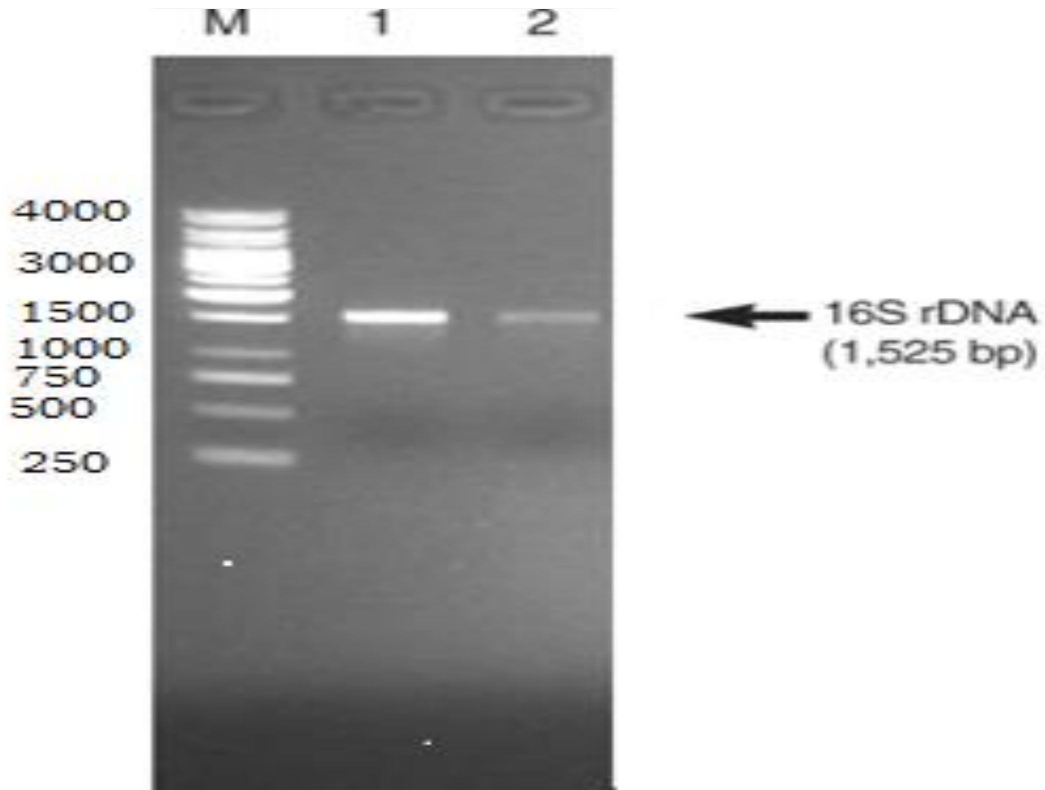
Figure 1. *In-vitro* antagonistic assay of bacterial isolates against *P.debaryanum.*

Figure 2. *In-vitro* antagonism. A: Growth of *P.debaryanum*in control; B & C: Inhibition caused by *P.flourescence*; D & E: Inhibition caused by *B.subtilis*.

Table 2. Biochemical characterization of bacteria.

Bacterial Isolates	Shape	Gram Test	Spore	UV test	NaCl	Catalase	H_2S	Indole	Citrate	Color	Name
RB1	Coci	-	+	Dull	-	+ W	-	-	+	Yellow	*Paracoccusdenitrificans*
RB2	Coci	-	-	Dull	-	+ M	-	+	+	Orange	*Azotobacternigricans*
RB3	Coci	-	+	Bright	-	+ S	-	-	+	Off white	*Bordetella pertussis*
RB4	Coci	+	+	Bright	+	+ S	+	-	+	Dull white	*Peptostreptococcussp*
RB5	Coci	+	+	Bright	-	+ S	-	+	+	Dull white	*P. Anaerobius*
RB6	Coci	-	+	Bright	+	+ W	-	-	+	Off white	*Phenylobacterium immobile*
RB7	Coci	-	+	Dull	-	+ W	-	-	+	Off white	*Phenylobacterium immobile*
RB8	Coci	-	+	Dull	+	+ W	-	-	-	Dull white	Could not identified
RB9	Coci	+	+	Bright	-	+ M	+	+	+	Off white	*Staphylococcus arseus*
RB10	Rod	-	-	Bright	-	+ M	-	+	-	Yellow	*Acinetobactercalcoaceticus*
RB11	Coci	+	+	Bright	-	+ S	-	+	+	Off white	*Streptococcus canis*
RB12	Rod	-	-	Bright	+	+ S	-	-	+	Off white	*Aeromonassp*
RB13	Coci	+	+	Dull	+	+ M	+	-	+	Off white	*Micrococcus sp*
RB14	Rod	-	-	Bright	+	+ S	+	+	-	Off white	*Acetobacteraceti*
RB15	Coci	+	+	Bright	-	+ S	-	+	+	White	*Streptococcus sp*
RB16	Rod	+	+	Bright	+	+ W	-	-	-	Off white	*Bacillus thuringensis*
RB17	Rod	+	+	Bright	+	+W	-	-	-	Off white	*Bacillus thuringensis*
RB18	Rod	+	+	Bright	-	+	+	-	-	Off white	*Bacillus sp*
RB19	Rod	+	+	Dull	-	+	-	-	+	Off white	*Baillusfortis*
RB20	Rod	+	+	Bright	-	+S	+	+	-	White	*Bacillus subtilis*
RB21	Rod	+	+	Dull	+	+	-	-	-	Off white	*Bacillus sp*
RB22	Rod	+	+	Bright	+	+	-	-	+	Off white	*Bacillus fortis*
RB23	Rod	+	+	Bright	-	+S	+	+	-	White	*Bacillus subtilis*
RB24	Rod	+	+	Dull	+	+W	-	-	-	Off white	*Bacillus thuringensis*
RB25	Rod	-	-	Bright	-	+S	-	-	+	Off white	*Pseudomonas fluorescence*
RB26	Rod	-	-	Dull	+	+	+	-	-	Off white	*Pseudomonas maiophillia*
RB27	Rod	-	-	Bright	-	+S	-	-	+	Off white	*Pseudomonas fluorescence*
RB28	Rod	+	+	Bright	-	+S	+	+	-	White	*Bacillus subtilis*
RB29	Rod	+	+	Bright	-	+	-	-	-	Off white	*Bacillus sp*
RB30	Rod	-	-	Dull	+	+	-	-	-	Off white	*Pseudomonas mailophilla*
RB31	Rod	+	+	Bright	-	+S	+	+	-	White	*Bacillus subtilis*
RB32	Rod	-	-	Bright	-	+S	-	-	+	Off white	*Pseudomonas fluorescence*
RB33	Rod	+	+	Dull	-	+	-	-	-	Off white	*Bacillus sp*
RB34	Rod	-	-	Bright	-	+S	-	-	+	Off white	*Pseudomonas fluorescence*
RB35	Rod	+	+	Dull	-	+	+	-	-	Off white	*Bacillus farraginis*
RB36	Rod	-	-	Bright	+	+	+	+	-	Off white	*Pseudomonas calcaligenes*
RB37	Rod	+	+	Bright	-	+S	+	+	-	White	*Bacillus subtilis*

DNA isolation and PCR



Above are the results of agarose gel electrophoresis (1% Agarose gel). DNA obtained from the *Bacillus subtillus* and *Pseudomonas flourescnes* strains amplified with 16srRNA forward primer of sequence “AGA GTA TTG ATC CTG GCTC” and reverse primer “GGT TAC CTT GTT ACG ACT”. Bands appeared near the size of 1600 bp indicates the right amplification of the target gene. DNA marker of 10 Kb was used to check the size of amplifies PCR product provided by Fermantas.

Sequence analysis and Phylogenetic Analysis

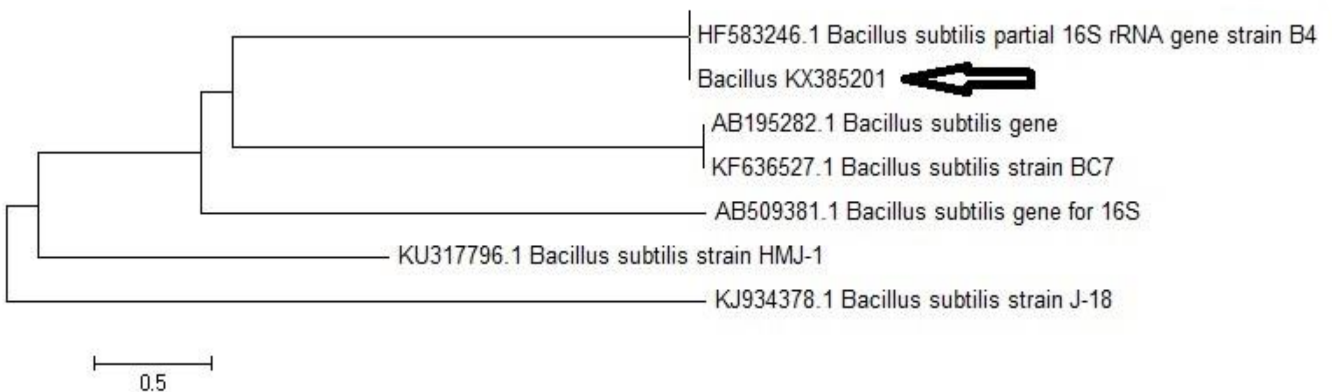


Figure 3. Phylogenetic dendrogram of *Bacillus subtillus* amplified with the 16S rRNA universal primers to confirm the phenotypic identification. Tree was made by using MEGA 6 software with the 1000 replicates in bootstrap. GenBank accession numbers are given with the names of sequences and arrow in the figure shows our isolated strain from roots of the green chillies (*Capsicum annum*).

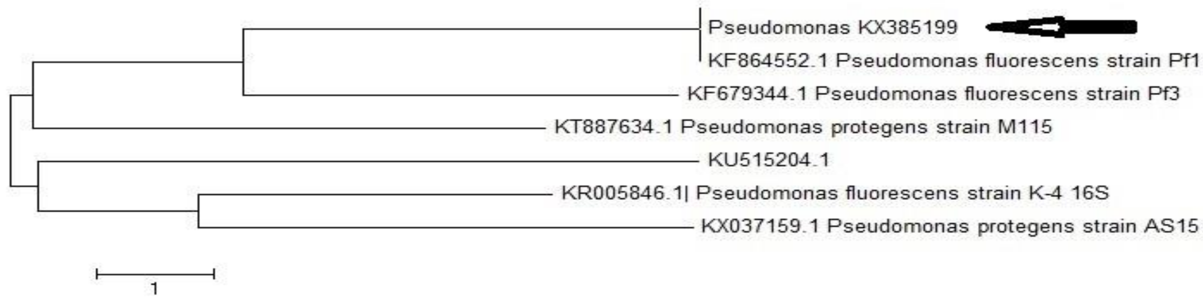


Figure 4. Phylogenetic dendrogram of *Pseudomonas fluorescens* amplified with the 16S rRNA universal primers to confirm the phenotypic identification. Tree was made by using MEGA 6 software with the 1000 replicates in bootstrap. GenBank accession numbers are given with the names of sequences and arrow in the figure shows our isolated strain from roots of the green chillies (*Capsicum annum*).

DISCUSSION

Present study was conducted to evaluate the potential of *Bacillus subtilis* and *Pseudomonas fluorescence* to supress the growth of *Pythium debaryanum.* In current study total of the thirty seven (37) bacterial strains were isolated among which twelve (12) strains were isolated from the roots of green chillies (*Capsicum annum*) while other twenty one (21) strains were isolated from the rhizosphere of the green chillies (*Capsicum annum*). Most appropriate interpretation of the bacterial antagonism against pathogenic fungus is beyond the other mechanisms such as competition or depletion of nutrients and an indication for the presence of highly potential antagonists in the rhizosphere (Figure 1). The strategies involved in the present research work are solely artificial in comparison with natural infection. Thus, it is quite difficult to estimate the required amount of antagonist inoculum in natural condition to suppress the disease. In the present study all bacterial isolates were isolated from rhizospheric region. A few bacterial isolates have shown significant antagonism against pathogen whereas, other showed weak or no antagonism in *in-vitro*. Most isolates had exhibited weak antagonistic behaviour against pathogen. Similar findings have been reported (Zheng, *et al*., 2011) while finding the potential of PGPR strains applied as soil drench or seed treatment in supressing the pathogen. *In-vitro* antagonistic assay has shown that two (2) strains out of total thirty seven (37) showed the significant potential to control the *P.debaryanum*. These strains were identifies as *B. subtilis* and *Pseudomonad fluorescence*, both isolated from the roots of *Capsicum annum*. *B. subtilis* have shown more potential as compared to the isolates of *P. fluorescence* which indicated the secretion of more extracellular metabolites by *Bacillus* (Figure 2). These findings are in agreement with the results of Romanenko *et al*., 2000 and kamal*et al*., 2009. These both strains were further analysed by using polymerase chain reaction amplified with 16S rRNAprimers to confirm their identification. Phylogenetic dendrogram of *B. subtilis* showed the maximum taxonomical similarity with the *B. subtilis* strain HF583246.1 isolated from South Korea while *P. fluorescence* showed maximum similarity with the strain KF864552.1 reported from Bangalore, India. It can be concluded that bacteria *B. subtilis* and *P. fluorescence*; isolated from the root of green chillies (*Capsicum annum*) can be better bio-control agents again the *P. debaryanum* and this can be landmark in the field of plant pathology.

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