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Antagonistic Effect of *Pseudomonas Mendocina* Isolate against Phytopathogenic Fungi.

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ABSTRACT

Use of *Pseudomonas* as a biofungicide is one of the best ways to treat plants diseases thereby reducing the amount of chemical fungicides used. In the present studies, potential use of *Pseudomonas* as a biofungicide against random fungal isolates from Indian crops was explored**.** Bacteria were isolated from different soil samples on selective medium. Morphological characterization showed all the bacterial isolates to be Gram negative, rod shaped, fluorescent and motile. Biochemical tests indicated them to be catalase and citrate positive and TSI, IMViC and Indole negative. Molecular characterization of bacteria confirmed that the bacterial isolate was *Pseudomonas mendocina.* Five phytopathogenic fungi were isolated from various plants and studied morphologically. Evaluation of the antifungal activity by Lawn culture technique and Agar diffusion method showed 100 per cent inhibition of radial growth of fungal isolates in presence of the isolated *Ps. Mendocina* indicating its potential as a potent biofungicide.

Keywords: Biofungicide, Lawn culture technique*, Pseudomonas mendocina*, Phytopathogenic fungus

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INTRODUCTION

Fungal plant diseases are one of the major concerns to agricultural production. Conventional practice to overcome this problem has been the use of fungicides [1] which include chemical fungicides and biofungicides. Though effective in many cases, chemical fungicides are also harmful for environment as their excessive use causes pollution and also harms the productivity of crop over time [2].

Biofungicide is an alternative to mitigate the harmful effects of chemical fungicide on both plant and host. Biofungicides are the microorganisms and natural extracts that kill or inhibit the plant pathogenic fungi. Biofungicides are natural, bio-rational biological extracts (modified) that reduce disease risk and may be protective or curative in nature. Moreover, the use of biofungicide is a potential non-chemical mean. It is an eco-friendly and inexpensive method [3]. Use of biofungicides reduces use of toxic chemicals and prevents the development of chemical resistant varieties of pathogens. Biofungicides are safer to use as well as more stable than chemicals when the storage issue comes. Moreover they are harmless to the host plant even when used for several times. Effectiveness of biofungicides depends on the way in which disease causing organisms are attacked through the production of a chemical compound that interferes with and kills the disease causing organism. Direct competition, predation and parasitism are some of these methods. Alternately, biofungicides may also produce natural antibiotics that 'beef up' a plant's resistance to disease [2].

There are number of evidences demonstrating the ability of certain bacteria to suppress the diseases caused by phytopathogenic fungi [4]. Those which have potential to be used as biofungicides include *Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens, Pseudomonas aeroginosa, Pseudomonas putida, Pseudomonas syringae* etc. [5]. Genus *Pseudomonas* is the genus of bacteria that comprises a large group of active biocontrol strains as a result of their general ability to produce vast range of antifungal metabolites such as 2, 4-diacetylphloroglucinol phenazine-1-carboxylic acid as well as complex macrocyclic lactone [1]. *Pseudomonas* spp. especially *Ps. putida* is widely used as biofungicide because of its non pathogenic nature. *Pseudomonas* induces plant growth and also protects the plants from pathogens. Because *Pseudomonas* assists in promoting plant development, researchers have been exploring its use in bioengineering research to develop biofungicides to improve the plant health.

MATERIAL AND METHODS

Experimental organism

Bacterial strains were isolated from the soil samples collected from various regions across Jalandhar, Punjab and Himachal Pradesh. Appropriately diluted soil samples were inoculated on selective *Pseudomonas* isolation agar media. After the incubation of 24 hours, isolated colonies were counted, randomly selected and sub cultured on fresh medium in order to get axenic cultures. Slants cultures of the isolates were preserved in refrigerator at 4°C.

Morphological Characterization of the bacterial isolates

The shape, texture, margins, color and size of the bacterial colonies was observed and compared using the manual Colony morphology protocol by Breakwell *et al* [6]. Petri plates were observed in UV illuminator and plates were checked for any fluorescence [7]. The bacterial isolates were subjected to Gram staining in order to determine the gram reaction of the isolates and determining shape of the bacterial cell [8]. The motility of bacteria was observed using cavity slide by the standard hanging drop procedure.

Biochemical Characterization of bacterial isolates

The bacterial isolates were characterized by subjecting them to standard biochemical tests including catalase test, citrate test [9], indole test [10], Methyl red-Voges Proskaeur test [11] and Triple-sugar iron Test [12].

Molecular characterization of Bacterial Isolate

Isolation of genomic DNA from the 24 hour old bacterial culture was performed by agarose gel electrophoresis and quantification of DNA was carried out on 1.2% Agarose Gel. Fragment of 16S rDNA gene was amplified by PCR from the isolated DNA. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF primers and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA gene sequence was used to carry out BLAST with the nucleotide database of NCBI genbank database. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

Isolation of Phytopathogenic fungi

Phytopathogenic fungus was isolated by inoculating fungal spores or teased fungal hyphae from infected vegetables on Potato Dextrose Agar. Inoculated plates were kept for incubation at 28°C for 5 days, subcultured and preserved. The fungal hyphae were stained with lacto-phenol cotton blue dye and observed microscopically. Average length of fungi was also determined using micrometry.

Antagonistic effect of isolated bacterial strain on phyto-pathogenic fungi

Antagonistic activity of bacterial strains against fungal pathogens was studied by two methods: Agar diffusion technique [13] and Lawn Culture method. For testing their antimicrobial activity, 1 ml suspension of isolated bacteria was inoculated evenly on the surface of nutrient agar medium in petriplate. A plug disc of 5 mm diameter received from actively growing fungal culture was placed in the center of each petridish to test the inhibition activity of tested bacterial species [14]. Mycelial growth of each fungal species was measured after 7 days of incubation at 28°C. Fungal growth without bacterial inoculum was taken as control. Inhibition was calculated using the formula [15]:

ISSN: 0975-8585

$$\% \text{ Inhibition } = 1 - \left[\frac{\text{Experimental Fungal Colony diameter}}{\text{Control Diameter}}\right] * 100$$

RESULT AND DISCUSSION

Isolation of Bacteria

Bacteria were isolated from appropriately diluted soil samples on Pseudomonas Isolation Agar medium. The number of colonies obtained were counted (data not given) and studied.

Morphological Characterization of Bacteria

Some colonies were yellowish in color and some were egg white in appearance (Figure 1). When bacteria were microscopically examined, it was found that bacteria were Gram negative in nature and rod shaped.

Figure 1: Bacterial colonies in *Pseudomonas* Isolation Agar. Yellow colored colonies were obtained having round margins.

Biochemical Characterization of Bacterial isolate

Bacterial culture was found to be Catalase positive, Citrate positive, Methyl red negative, VP negative, Indole negative and TSI negative. All the biochemical characterization results were similar to *Pseudomonas spp.*

Molecular Characterization

Electrophoretic studies showed a single discrete PCR amplicon band of 750 bp when resolved on Agarose Gel which was continued with purification of PCR amplicon to remove any undue contaminants. Size of DNA was found to be 1.5 Kb when tested against standard ladder (Figure 2)**.** Consensus sequence of 1456bp rDNA gene was generated from forward and reverse sequence data using aligner software. When the resultant sequences were

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aligned in BLAST [16], it was found that isolated bacterial culture was closely related to the *Pseudomonas* species having accession no DQ192041. The culture was found to be *Pseudomonas mendocina strain PC19* (GenBank Accession Number: DQ178226.1) based on nucleotide homology and phylogenetic analysis (Figure 6).

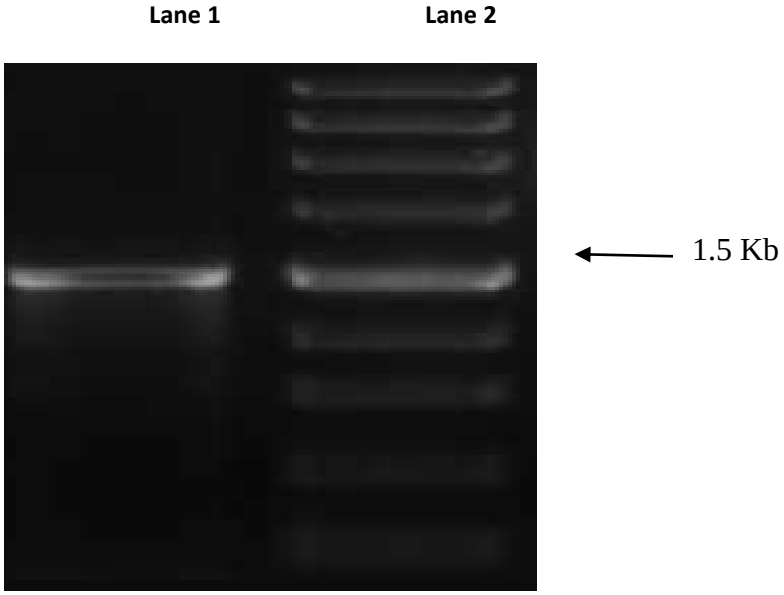

Figure 2: DNA band obtained by Agrose Gel Electrophoresis
Lane 1: 16S rDNA amplicon band,
Lane 2: DNA marker.

Isolation of fungus

Fungus was isolated from infected plants by inoculation from vegetable crops on PDA medium. The resultant pure cultures of fungi were used for further study (Figure 3). White and green colored pure culture of fungus was obtained. Table 2 shows the morphological characteristics of the isolated fungal colonies. Average length of fungal hyphae was found to vary between 48 and 56 µm (Table 1). When fungi were examined microscopically, fruiting body, and conidiospores were observed and all the isolated fungi were found to be septate in nature.

Table 1: Colony Characteristics of isolated fungi

Fungus isolates	Source of Fungus	Colony color	Margins	Colony Shape	Spore Colors	Elevation Of Colony	Average Length of Fungus Hyphae (mm)
A1	Tomato	White	Curled	Circular	White	Convex	55.83
V	Sugarcane	White	Filiform	Circular	White	Convex	48.5
A	Cauliflower	Green	Entire	Circular	Green	Convex	48.33
S	Soil sample	White	Filiform	Irregular	Brown	Convex	52.5

Media Used "Potato Dextrose Agar" Incubation Temperature "28°C"

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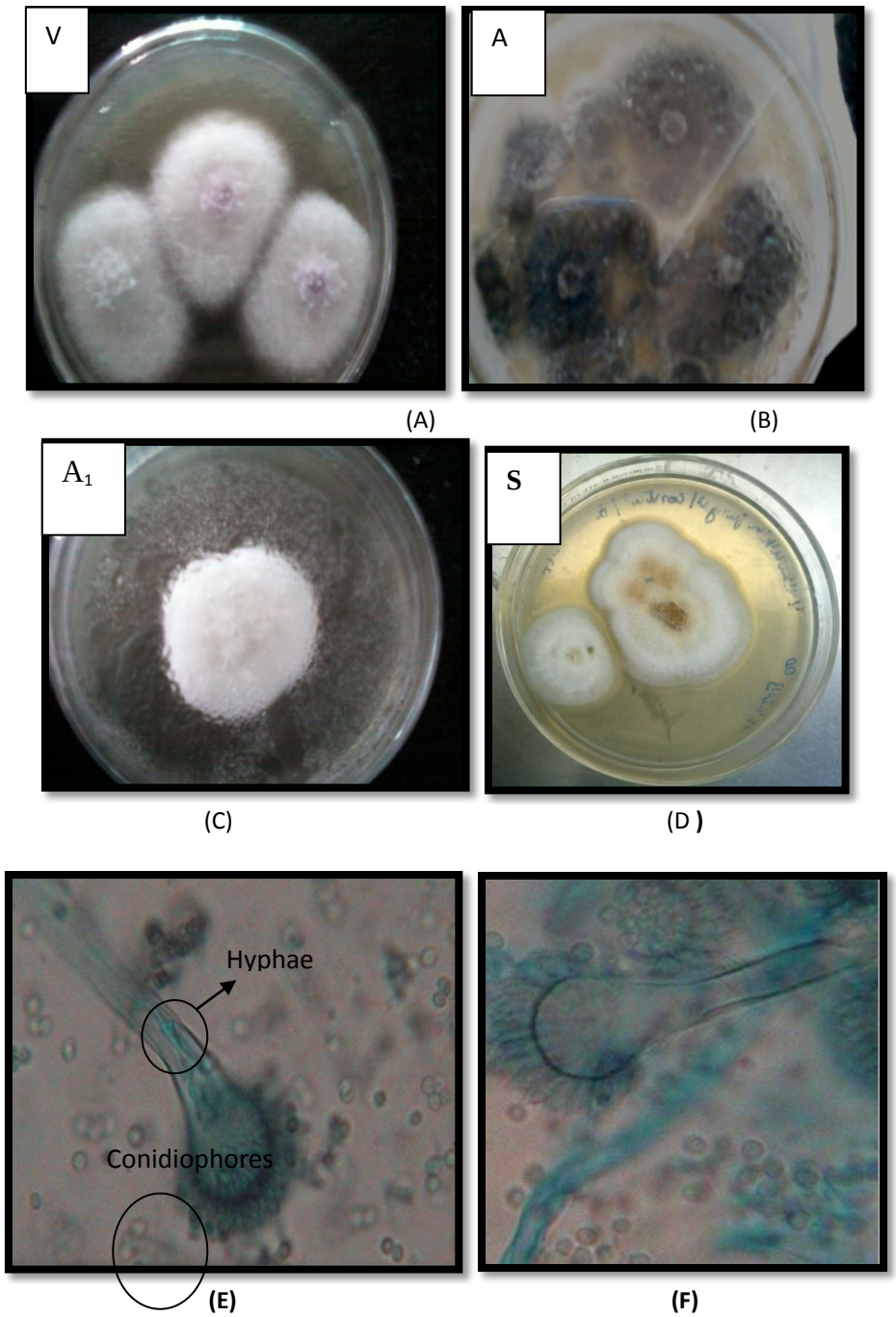

Figure 3: Fungus Isolates: Pure culture of Fungus was obtained from different vegetables in Potato dextrose agar, Fungus V (A) from Sugarcane, A (B) from Cauliflower, Fungus A_1 (C) from Tomato, Fungus S (D) from soil, Fungus. (E) Microscopic Structure of Fungus A, (F) Microscopy Structure of isolated Fungus A1.

Biofungicidal effect of bacteria

Antifungicidal activity of bacteria by Agar diffusion method

When the Biofungicidal effect of bacteria was observed using agar diffusion method, the growth of fungus was found to be around the *Ps. mendocina* colonies but not over the bacterial colonies. This indicated that bacteria was inhibiting the growth of fungus and hence preventing its growth (Figure 4).

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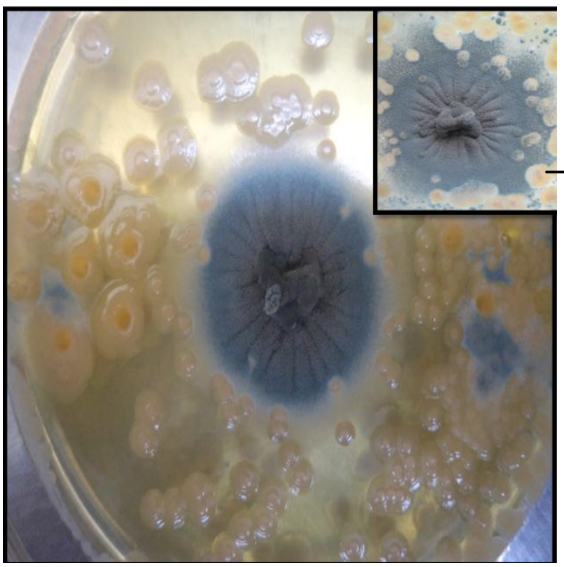


(A)

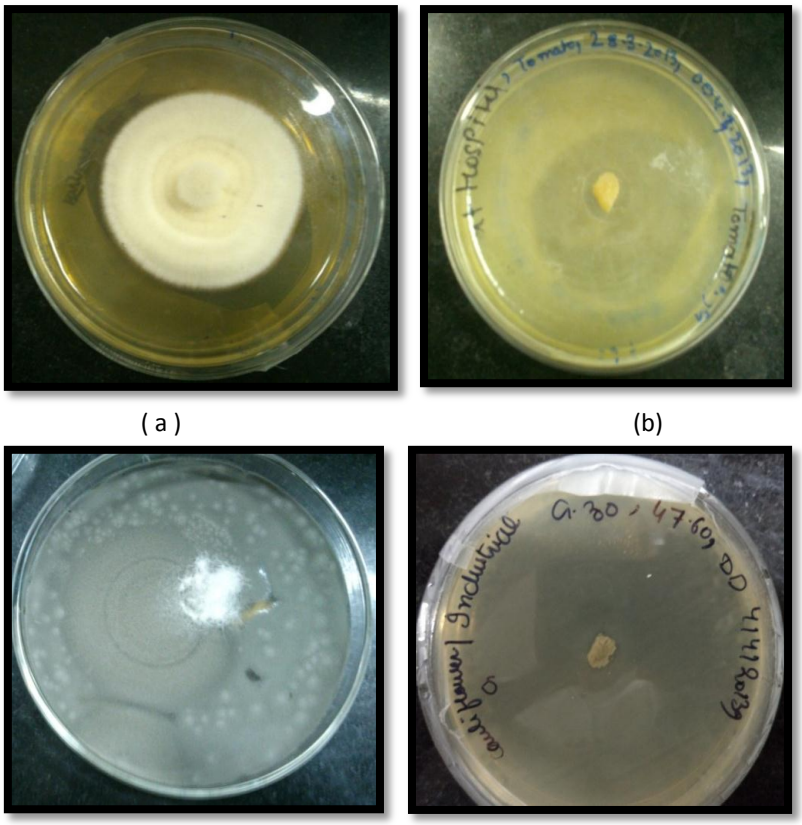
(B)

Figure 4: Antagonistic activity of *Pseudomonas mendocina*: Bacterial growth around the fungus colony (A) was observed. In (B) diameter of fungus was observed, diameter was more in area where there was no growth; but at bacteria and fungus interface, growth of fungus was inhibited.

Antifungicidal activity of bacteria by lawn culture technique.

Agar disc carrying fungal mycelium was incubated in the centre of petriplate containing *Ps. mendocina* lawn. A control without bacterial lawn was also run simultaneously. It was observed that there was absolutely no growth in petriplates in which fungus was grown in the presence of bacteria. When evaluated, inhibition of fungus by bacteria was found to be 100% (Table 2). Similar studies was carried by Khokar *et al* [15] and they also found that *Pseudomonas* possess higher antagonistic activity against fungi than *E.coli* and *Bacillus spp.*

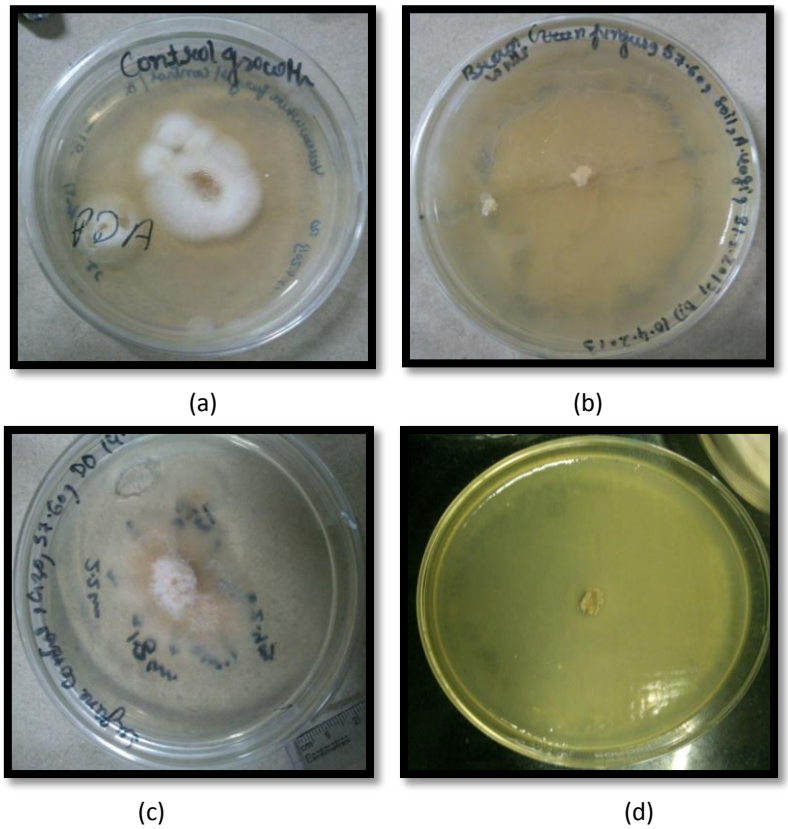
ISSN: 0975-8585



(a) (b)

(c) (d)

Figure 5: Antagonistic activity of bacteria by lawn culture technique: growth of fungal isolates (A1 and A) was observed in control without bacterial lawn in (a and c). But no growth was observed when fungus was grown with bacteria (c and d).



(a) (b)

(c) (d)

Figure 6: Fig: (a) and (b) Growth of Fungus S with and without *Pseudomonas mendocina*. (b) Growth of Fungus V with and without *Pseudomonas mendocina*.

ISSN: 0975-8585

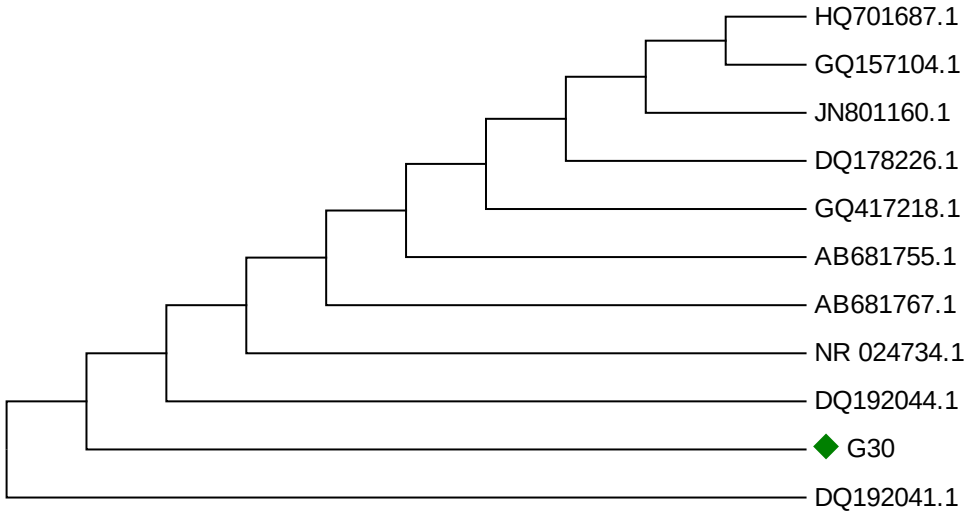
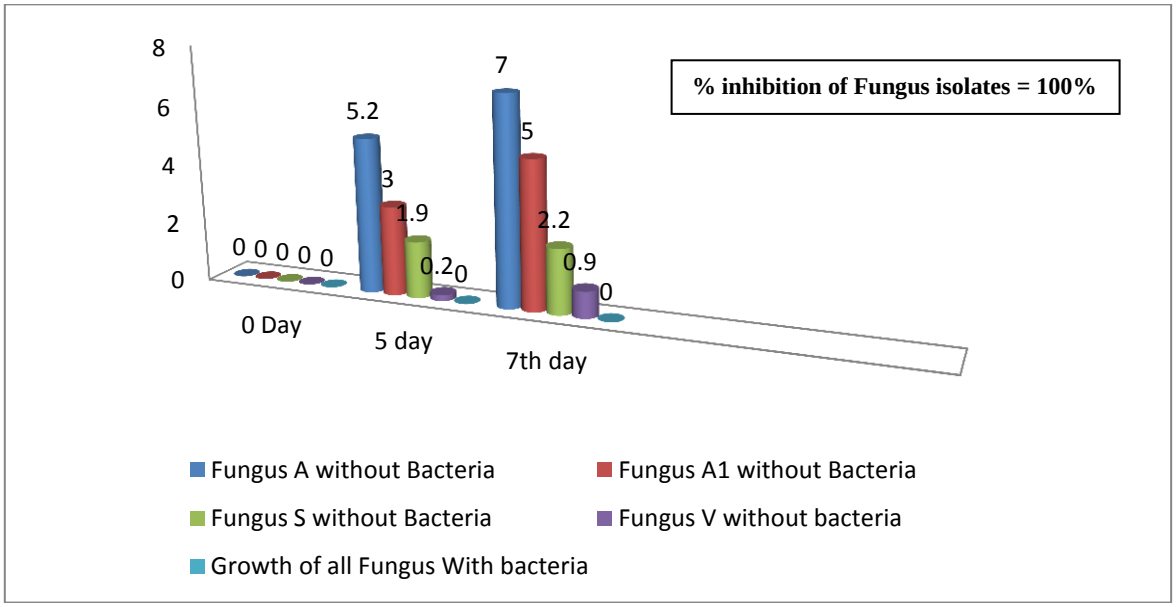


Figure 6: Dendogram showing evolutionary relationship of isolated bacteria. G30 is *Pseudomonas mendocina.*



Graph 1: Inhibition of fungal isolates in presence of *Ps. mendocina*

Table 2: Radial growth of isolated fungi in the presence and absence of *Ps. mendocina* by lawn culture method.

Fungal Strain	Radial growth of fungi* (cm)						
	0 Day		5 Day		7 Day		Inhibition (%)
	With *Ps. mendocina*	Without *Ps. mendocina*	With *Ps. mendocina*	Without *Ps. mendocina*	With *Ps. mendocina*	Without *Ps. mendocina*	
A	0	0	0	5.2	0	7	100
A1	0	0	0	3	0	5	100
S	0	0	0	1.9	0	2.2	100
V	0	0	0	0.2	0	0.9	100

Media Used " Potato Dextrose Agar" Incubation Temperature "28°C"

ISSN: 0975-8585

CONCLUSION

Ps. mendocina culture isolated from soil showed 100 per cent antagonistic activity against four phytpathogenic fungal isolates. *Ps. mendocina* can be used as Biofungicide not only because of its antifungal activity but also because of its non virulence and low pathogenic nature for humans. There are very rare cases of infection that are being caused by *Ps. mendocina*.. This strain is being further explored in order to evaluate its antagonistic activity against fungi causing diseases in standing crops. If *Ps. Mendocina* not only have biofungicidal property bit it can used for the process of bioremediation.

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