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RESEARCH ARTICLE

Diversity of actinomycetes from fodder leguminous plants and their biocontrol potential

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Abstract

A total of 94 actinomycete isolates were obtained from 20 samples of rhizospheric soil and 20 samples of roots of fodder leguminous plants *viz*: *Vigna unguiculata* and *Trifolium alexandrinum*. Actinomycetes were more frequently recovered from rhizospheric soil (64% of all isolates) followed by roots (18%) and nodules (18%) respectively. The highest number and diversity of actinomycetes were isolated from *T. alexandrinum* rhizospheric soil (4.2×10^5 c.f.u/g). Forty one out of 94 isolates exhibited antagonistic activity against at least one of the three phytopathogenic fungi tested viz: *Fusarium oxysporum, Fusarium moniliforme and Sclerotinia sclerotiorum*. Scanning electron microscopy revealed rupture of the *F. moniliforme* mycelial cell wall at the area of interaction with CS16. It was observed that genus *Streptomyces* was dominant (63%) followed by *Micromonospora* (15%), *Nocardia* (10%), *Saccharopolyspora* (7%) and *Actinopolyspora* (5%). The isolates CS1 and CS44 were found to be promising in terms of seed germination and wilt control in cowpea crop under green house conditions. Therefore these isolates may be used as potential biocontrol agents against *Fusarium* wilt caused by *Fusarium oxysporum.*

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INTRODUCTION

Actinomycetes are the most widely distributed group of saprophytic soil inhabitants (Takizawa et al, 1993) and are prolific producers of diverse bioactive secondary metabolites (Kekuda et al, 2010). Plant rhizospheric soils are a major habitat for actinomycetes. These actinomycetes also gain entry inside host tissues via wounds or openings hence termed as endophytic actinomycetes. Streptomyces sp. have been previously described as rhizosphere colonizing bacteria (Miller et al, 1990), antifungal biocontrol agents useful in controlling fungal root diseases (Rothrock and Gottlieb, 1984; Aghighi et al, 2004), in vitro siderophore producers and in vitro producers of plant growth-promoting hormones (Ilic et al, 2007). This genus has also accounted for the production of 60% of antibiotics which are useful in agricultural industries (Mellouli et al, 2003; Fguira et al, 2005; Singh et al, 2006; Thakur et al, 2007). Actinomycetes living inside leguminous plant roots and soils are poorly studied. Sharma et al (2005) revealed high abundance of actinomycetes in rhizospheres of mature legumes which indicates their possible role in soil enrichment.

Vigna unguiculata and *Trifolium alexandrinum* are two important fodder leguminous crops known as milk multipliers. Being leguminous crops, they fix atmospheric nitrogen and thus are also good for soil fertility. Excessive use of chemical fertilizers is leading to diminishing reserves of high quality raw material and increasing energy costs. So actinomycetal bio-inoculants can be used to reduce dependence on chemical fertilizer which would lead towards sustainable agriculture. Present study was undertaken to isolate actinomycetes from rhizospheric soil and roots of fodder leguminous plants and to evaluate their potential as biocontrol agents.

MATERIALS AND METHODS

Collection of samples

Vigna unguiculata and *Trifolium alexandrinum* rhizospheric soil as well as root samples (twenty each) were collected from various locations of Punjab Agricultural University, Ludhiana. Root samples were obtained by digging the soil adjacent to the main trunk and collected sections of approximately 0.5cm diameter and 3–5cm in length. The samples were collected in polythene bags and transported to laboratory for immediate processing.

Procurement of standard fungal cultures

Fusarium oxysporum, Fusarium moniliforme and *Sclerotinia sclerotiorum* were obtained from Department of Plant Pathology, Punjab Agricultural University, Ludhiana. The fungal cultures were maintained on Glucose Yeast Extract Agar and stored at 4ºC.

Isolation of endophytic actinomycetes

Root and nodule samples were washed in running tap water to remove soil particles and sterilized by segmental immersion in 70% (v/v) ethanol for 5 minutes and sodium hypochlorite solution (0.9% available chlorine) for 20 minutes and then surface-sterilized samples were washed in sterile water three times to remove surface sterilization agents. The samples were soaked in 10% (w/v) sodium bicarbonate solution for 10 minutes to retard the growth of endophytic fungi. Then macerated tissue suspension (1 ml) was spread on to Petri plates containing starch casein agar (SCA) and incubated at 28^0C for 7-10 days. Isolated colonies were subcultured on slants and stored at 4^0C (Tian et al, 2004).

Isolation of actinomycetes from rhizospheric soil

Ten grams of soil samples were transferred to Erlenmeyer flasks containing 90 ml of sterile distilled water. The samples were serially diluted upto 10^{-5} levels. Aliquot (1ml) from serial logarithmic dilution of each suspension was pipetted onto the surface of duplicate Petri plates containing starch casein agar (SCA). The inoculum was spread evenly over the surface using glass spreader. Petri plates were incubated for 7-10 days at 28ºC. After the incubation period, the mean colony count was determined and recorded as colony forming unit (cfu/g) of each sample. The suspected colonies were picked up and purified on SCA medium and incubated at 30^0C for 5-7 days. Isolated colonies were streaked on Starch Casein Agar after purification and then subcultured on slants and stored at 4°C. Subculturing was done after every one month.

Antagonistic effect against phytopathogenic fungi

The actinomycetes isolates were evaluated for their antagonistic activity against the phytopathogenic fungi by dual-culture in vitro assay. Five days old fungal discs (8 mm in diameter) at 28ºC were placed at one end of the plates containing GYE (Glucose Yeast Agar) agar medium. 5 days old actinomycetes discs (8mm) grown on Starch Casein Agar at 28ºC were placed on opposite sides of the Petri plates. Plates without the actinomycetes disc serve as controls. All the plates were incubated at 28ºC for 14 days and colony growth inhibition (%) was calculated by using the formula: C – T/C x 100, where C is the colony growth of pathogen in control and T is the colony growth of pathogen in dual culture.

Scanning electron microscopy (SEM)

SEM was employed to evaluate the effect of actinomycetes isolate CS16 on the fungal cell wall of *Fusarium moniliforme* culture using chemical fixation and liquid osmium fixation technique (Bozzola and Russell, 1996).

Identification of actinomycetes isolates

Cultural and morphological characteristics, including presence of aerial mycelia, spore mass color, color of diffusible pigments and spore chain morphology were used as identification characters (Cao et al, 2005). Visual observation of both morphological and microscopic characteristics using light microscopy and Gram-stain properties were also performed. All morphological characters were observed on SCA and the criteria used for classification and differentiation was as follows: (i) Aerial mass color: The mass color of mature sporulating aerial mycelium was observed following growth on SCA plates. The aerial mass was classified according to Bergey's manual of systematic bacteriology. (ii) Distinctive colors of the substrate mycelium were recorded. (iii) The production of diffusible pigment was also considered. (iv) The shape of the spore chains observed under light microscope was also used as an important step in the identification. (v) Biochemical criteria such as ability to degrade casein, starch, esculin, Tween 20, tyrosine, xanthine and hypoxanthine as substrates by the various actinomycete strains were also used for identification.

Evaluation of effectiveness of actinomycete isolates (CS1 and CS44) as potential antagonists against *Fusarium oxysporum* in green house

Inoculum preparation of potential antagonists

The potential isolates were grown in broth medium for 5 days. The seeds of cowpea variety C22 were immersed overnight in the antagonist suspension containing 10^8 cfu ml^{-1}.

Fungal inoculum preparation

Fusarium oxysporum was grown on glucose yeast extract agar and discs of fungi were transferred to 250 ml Erlenmeyer flasks containing autoclaved wheat and sand. The flasks were incubated at 25ºC for 7 days. The rate of inoculum applied to the potting mixture was 10 gm of fungi in 9 kg of soil per pot.

Soil infestation

Soil was taken from cowpea field and sterilized by autoclaving at 121^0C for 1 hr for 3 consecutive days. Cowpea seeds variety C22 were grown in pots, using completely randomized block design (CRD) with 6 treatments and 3 replications. Ten seeds were sown per pot containing 9 kg of sterile soil. The treatments were: (A) Control without antagonists and *Fusarium oxysporum* (Negative control), (B) *Fusarium oxysporum* inoculation (Positive control), (C) Actinomycete isolate CS1 alone, (D) Actinomycete isolate CS44 alone, (E) Actinomycete isolate CS1 + *Fusarium oxysporum*, (F) Actinomycete isolate CS44 + *Fusarium oxysporum*.

RESULTS AND DISCUSSION

Isolation of actinomycetal diversity

A total of 94 isolates of actinomycetes (52 from cowpea and 42 from berseem) were obtained from the rhizospheric soil, roots and nodules. Out of 94 isolates, the majority (n=60) was recorded from rhizospheric soil, followed by roots (n=17) and nodules (n=17) respectively. Mingma et al (2014) also obtained seventy-seven isolates from root samples and 240 isolates from rhizospheric soil samples. The number of actinomycetes from rhizosphere soils was usually higher than that of non-rhizosphere samples as endophytes were considered to be a subset of the rhizosphere community (Qin et al, 2011; Zhao et al, 2012). Merckx et al (1987) suggested that the rhizosphere supports abundant and diverse saprophytic microorganisms due to a high input of organic materials derived from the plant roots and root exudates. In addition, the rhizosphere associated soils were reported to give almost twice as many isolates of actinomycetes as the non-rhizosphere-associated soils (Crawford et al, 1993).

The data on occurrence and enumeration of actinomycetes from rhizopheric soils showed that the population density of *V. unguiculata* and *T. alexandrinum* were recorded to be 3.1 x 10^5c.f.u g^{-1} and 4.2x 10^5 c.f.u g^{-1}, respectively. Ng and Amsaveni (2012) reported maximum of 6.7×10^5c.f.u g^{-1} and a minimum of 0.3×10^5c.f.u g^{-1} respectively from rhizospheric soils collected from various plants. In another study, a total of 100 actinomycetes isolates were obtained from 30 rhizospheric soil samples of *Catharanthus roseus* and *Withania somnifera* (Kamara and Gangwar, 2015).

In the present study, a total of 34 endophytic actinomycetes were isolated from both the crops (17 each). While 19 isolates were obtained from the nodules, 15 were acquired from the roots. Results are in agreement with Gangwar et al (2014), who reported that endophytic actinomycetes were most commonly recovered from roots (70% of all isolates) followed by stems (17.5%) and leaves (12.5%) from three medicinal plants namely *Aloe vera*, *Mentha arvensis* and *Ocimum sanctum*.

Antagonistic effect against phytopathogenic fungi

Out of the 94 isolates obtained from roots and rhizospheric soil of fodder leguminous plants, 28 from *Vigna unguiculata* (cowpea) and 13 from *Trifolium alexandrinum* (berseem) were displaying antagonistic activity against one or the other plant pathogenic fungi tested (Table 1). Out of 28 isolates of *Vigna unguiculata*, 22 were obtained from rhizospheric soil, 5 from nodules and one from roots. Similarly in case of *Trifolium alexandrinum*, nine isolates were from rhizospheric soil and 2 each from nodules and roots respectively.

CS1 an isolate from rhizospheric soil of cowpea exhibited antifungal activity against *Fusarium oxysporum* and *Fusarium moniliforme* with percent inhibition of 52.29% and 38.2% respectively. Eighteen isolates from *V. unguiculata* (CN1, CS15, CS16, CS18, CS20, CS22, CS23, CS24, CS26, CS28, CN35, CN36, CN41, CS43, CS44, CS50, CS51 and CS52) and eight from *T. alexandrinum* (BS4, BS6, BS13, BS15, BR20, BR21, BN25 and BS28) were observed to have antifungal activity against *Fusarium oxysporum*. Twenty isolates (12 from *V. unguiculata* and 8 from *T. alexandrinum*) were found to exhibit antifungal activity against *Fusarium moniliforme*. Five isolates from *V. unguiculata* were displaying antifungal activity against *Sclerotinia sclerotiorum*. Surprisingly, none of the isolate from *T.alexandrinum* was displaying antagonism against *Sclerotinia sclerotiorum*. Two isolates of *Vigna unguiculata* (CS1 and CS44) exhibited strong antagonistic activity against *Fusarium oxysporum* and *Fusarium moniliforme* (Fig.1). Isolate CS1 had maximum percent inhibition against *Fusarium oxysporum* (52.29%) while CS44 had maximum percent inhibition against *Fusarium moniliforme* (47.21%) and CS18 had maximum percent inhibition against *Sclerotinia sclerotiorum*.

Interactions between *Streptomyces griseus* strains and some soil borne plant pathogens like *Fusarium oxysporum, Alternaria alternata, Rhizoctonia solani* and *Fusarium solani* of tomato. *Streptomyces griseus* showed maximum percent inhibition of 61.1% against *F.oxysporum* f. sp. *lycopersici* (Anitha and Rebeeth, 2009). The

inhibition was due to the presence of some inhibitory substance, antibiotics and other enzymes such as glucanases, proteases essential for complete cell-wall lysis. Similarly, rhizospheric *Streptomyces* from Thai medicinal plants showed inhibitory activity against pathogenic fungi (Khamna et al, 2009).

Out of the 39 rhizospheric isolates from *Catharanthus roseus* and *Withania somnifera*, 9 showed antifungal activity against *Alternaria alternata*, 19 against *Fusarium oxysporum*, 20 against *Helminthosporium oryzae*, 14 against *Macrophomina phaseolina*, 10 against *Penicillium* sp. and 16 against *Rhizoctonia solani* (Kamara and Gangwar, 2015). Actinomycetes have the potential to control growth of several phytopathogens due to their ability to produce broad spectrum antimicrobial compounds. For example, tomato was protected against *Rhizoctonia solani* under greenhouse conditions by actinomycetes from rhizospheric soil of Indo-Gangetic plains in India (Patil et al, 2011).

Scanning electron microscopy (SEM)

Scanning electron micrographs showed brittle, disrupted and damaged hyphae of *Fusarium moniliforme* at the point of interaction with CS16 (Fig.2). The control plate on the other hand showed the presence of regular vegetative cells having smooth surface with overall intact morphology. Similar results were obtained by Tang-um and Niamsup (2012) who reported the breakage of the cell walls of *Fusarium oxysporum f.sp.lycopersici* mycelia growing towards *Streptomyces* sp. P4 as compared to control *Fusarium oxysporum*. He et al (2009) also reported that endophytic bacteria obtained from *Epimedium brevicornu* degraded hypha of *Sclerotinia sclerotiorum* and the cytoplasm was extravagated outside from the fungal walls.

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal agents that inhibit several plant pathogenic fungi (Khamna et al, 2009). The chitinolytic activity of *Streptomyces* sp. obtained from citrus and soybean plants showed high inhibition levels against fungi and the fungal hyphae exhibited a degraded appearance after chitinolytic A8 strain culture treatment. This indicated an inhibitory role of chitinase to plant pathogenic fungi. The *C. sublineolum* hyphae surface-treated with A8 culture filtrate contained many holes, possibly corresponding to lysis zones. However, the hyphal surfaces of both *C.sublineolum* and *Pythium* sp. treated with A8 culture filtrate exhibited a slightly roughened surface, indicating little or no effect of hydrolytic enzymes on these structures (Quecine et al, 2008).

Description of species isolated

Forty one isolates of actinomycetes from both the crops were found to display antagonistic activity against the three phytopathogenic fungi tested. These isolates were further identified presumptively by morphological and biochemical characteristics. Twenty six isolates out of 41 were identified as *Streptomyces* sp. based on colony and cultural characteristics. *Streptomyces* isolates were further characterized into various subgroups based on phenotypic and physiological characteristics. Out of 41 isolates, 9 belonged to *Streptomyces albosporus*, 7 to *S.roseosporus*, 4 to *S.viridis*, 3 to *S. griseorubroviolaceus*, 2 to *S.aureus* and one to *S.cinereus* (Table 2). Besides *Streptomyces* sp., other genera like *Actinopolyspora* sp. (n=2), *Saccharopolyspora* sp. (n=3), *Micromonospora* sp. (n=6) and *Nocardia* sp. (n=4) were also reported (Table 3). Out of 41 isolates, 31 were obtained from rhizospheric soil, 7 from nodules and 3 from roots.

Our results are in accordance with the observations of Verma et al (2009) who obtained a total of 55 isolates from 20 plants of *Azadirachta indica* A. Juss. The dominant genus was *Streptomyces*, followed by *Streptosporangium*, *Microbispora*, *Streptoverticillium*, *Saccharomonospora* sp. and *Nocardia*. In another study, *Streptomyces* sp. had 100% distribution in the rhizosphere of *Senna occidentalis* and 75% in the rhizosphere of *Musa sapientum* var *parasidiaca*. They found that *Micromonospora* and *Saccharomonospora* sp. each had 75% occurrence in the rhizosphere of both plants. At 25% each, S. globosus and S. rochei were the least distributed in the rhizosphere of *M. sapientum var parasidiaca* and *S. occidentalis* respectively (Grillo et al, 2013).

Evaluation of effectiveness of actinomycetes isolates (CS1 and CS44) as potential antagonists against *Fusarium oxysporum* in green house in cowpea

Maximum increase in seed germination, root length, shoot length, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight was observed in treatment with isolates CS1 and CS44. Similar parameters were observed more for CS1+*Fusarium oxysporum* and CS44+*Fusarium oxysporum* as compared to *Fusarium oxysporum* alone (Table 4). These phenomena may be related to the production of growth regulators by actinomycetes. Under greenhouse conditions in sorghum the *Streptomyces* strains significantly enhanced all the agronomic observations including root length (3-18%) over the un-inoculated control (Gopalakrishnan et al 2011). Aly et al (2012) observed that soil inoculation by *Azotobacter vinelandii* and *Streptomyces* sp. or both enhanced root depth, shoot length, dry weights of root and shoot and mineral and protein content which may due to nitrogen fixation, auxins, production or unidentified compounds.

Maximum wilt incidence (%) was observed in the treatment with *Fusarium oxysporum* (66.67%). There was no wilting in the treatments with inoculation of CS1 and CS44 isolates alone. Incidence of disease was reported to be maximum in CS1 with *Fusarium oxysporum* (16.67%) and CS44 with *Fusarium oxysporum* (13.33%). Maximum inhibition of disease was observed in CS44 with *Fusarium oxysporum* (80%) followed by CS1 with *Fusarium oxysporum* (75%) (Table 5). Costa et al (2013) reported two *Streptomyces* isolates for the control of *P. aphanidermatum* in cucumber (*Cucumis sativa* L.) under greenhouse conditions. Isolate 16R3B was able to reduce 71% damping-off incidence whereas isolate 14F1D/2 reduced the disease incidence by 36%. Damping off control in cucumber, mainly for the isolate 16R3B suggested for its use in greenhouse cucumber. The results found under greenhouse conditions with the isolates CS1 and CS44 proved their potential as a biocontrol agents to reduce the *Fusarium* wilt caused by *Fusarium oxysporum* in this planting system.

Table 1: Antifungal activity (% inhibition) of cowpea and berseem isolates

Isolates	*Fusarium oxysporum*	*Fusarium moniliforme*	*Sclerotinia sclerotiorum*
CN1	52.29±0.3	38.2±0.3	-
CS2	-	30.36±0.2	47.61±0.2
CS10	-	26.90±0.1	51.10±0.1
CS16	41.17±0.1	41.57±0.3	-
CS18	33.35±0.4	-	52.29±0.3
CS44	42.24±0.3	47.21±0.3	-
CS50	32.26±0.3	38.2±0.2	-
BS4	35.54±0.4	38.2±0.3	-
BS9	-	39.28±0.4	-
BS13	24.75±0.2	28.34±0.5	-
BR20	28.82±0.2	35.69±0.5	-

*Average ± standard error from three replicates

Table 2: Morphological and biochemical characterization of *Streptomycete* actinomycete isolates obtained from cowpea and berseem.

Property	*S. albosporus* (9)	*S. roseosporus* (7)	*S.viridis* (4)	*S. griseorubro violaceus* (3)	*S. aureus* (2)	*S. cinereus* (1)
Culture mass color	White	Orange, pink	Green, grey	Brown	Golden-yellow	Grey
Spore color	Yellow, orange	Pink	Green	Brown	Grey	Grey
Pigmentation	-	Pinkish orange	Green	Brown	Yellow	-
Spore arrangement	Chain	Chain	Chain	Chain	Chain	Chain

Hydrolysis of						
Casein	+	+	+	-	+	+
Starch	+	+	+	+	+	+
Decomposition of						
Esculin	-	+	+	+	-	-
Tween-20	+	-	-	-	+	+
Xanthine	-	+	-	+	+	+
Hypoxanthine	+	+	-	-	+	+
Tyrosine	+	+	-	+	+	+

Table 3: Morphological and biochemical characterization of non-Streptomycete actinomycetes isolated from cowpea and berseem.

Property	*Micromonospora* sp. (6)	*Nocardia* sp. (4)	*Saccharopolyspora* sp.(3)	*Actinopolyspora* sp. (2)
Culture mass color	Orange/white	Grey/brown/white	Yellow/pink	White
Spore color	White	-	Yellow/pink	White
Pigmentation	-	Brown	Pink	-
Spore arrangement	Single	-	Chain	Chain
Hydrolysis of				
Casein	+	-	+	-
Starch	+	+	+	+
Decomposition of				
Esculin	+	-	+	+
Tween-20	+	-	+	+
Hypoxanthine	+	-	+	+
Xanthine	+	-	+	+
Tyrosine	+	-	+	+

Table 4: Effect of actinomycetes isolates on cowpea under green house conditions

Treatments	Germination (%) 10 DAS	Root length (cm)	Shoot length (cm)	Root fresh weight (gm)	Shoot fresh weight (gm)	Root dry weight (gm)	Shoot dry weight (gm)
		60 DAS	60 DAS	60 DAS	60 DAS	60 DAS	60 DAS

Control	63.33	11.5	30.3	0.337	3.123	0.242	1.025
F. oxysporum	56.67	8.4	24.0	0.295	2.224	0.206	0.628
***Streptomyces albosporus* (CS1)**	96.67	14.2	40.2	0.442	4.976	0.383	1.160
***S.aureus* (CS44)**	90.00	16.3	44.2	0.454	5.237	0.404	1.232
CS1 + *F.oxysporum*	83.33	13.2	39.1	0.423	4.281	0.253	1.115
CS44+ *F.oxysporum*	76.67	12.8	34.3	0.417	4.330	0.246	1.156
$p \leq 0.05$	1.39	0.34	0.80	0.110	0.90	0.110	0.09

Table 5: Incidence of wilt and inhibition of disease development (%)

Treatments	Incidence of disease (%)	Inhibition of disease development (%)
CS1 + *Fusarium oxysporum*	16.67	75.00
CS44 + *Fusarium oxysporum*	13.33	80.00
Fusarium oxysporum	66.67	-

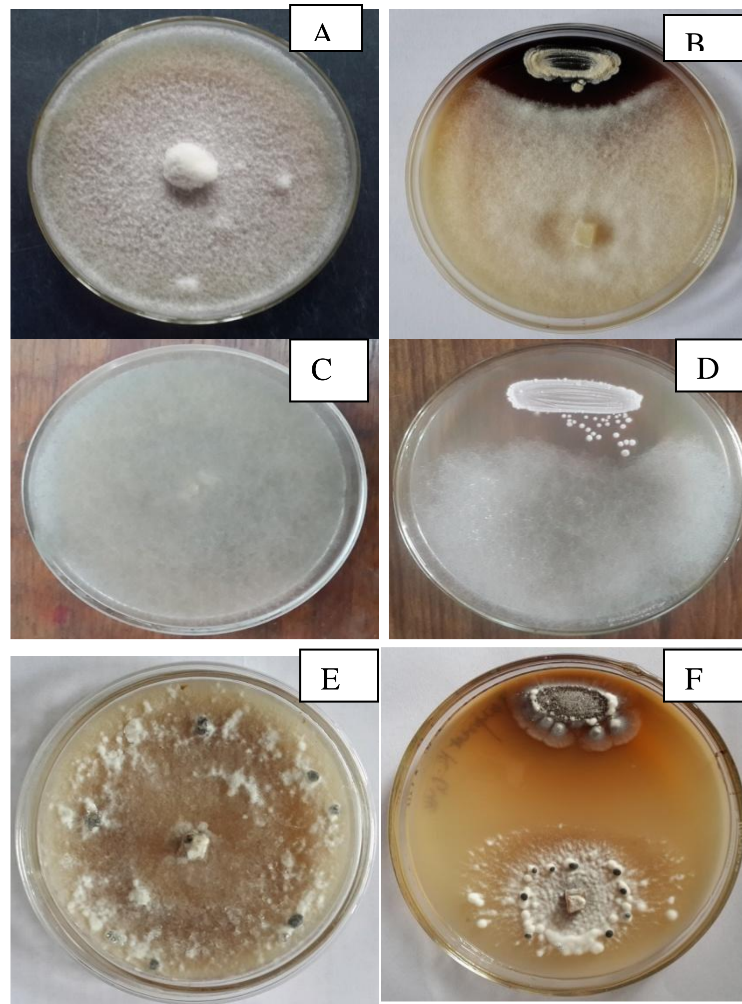


Fig 1: Antagonistic activity of actinomycete isolates against plant pathogenic fungi

Plate A= Control (*Fusarium oxysporum*)

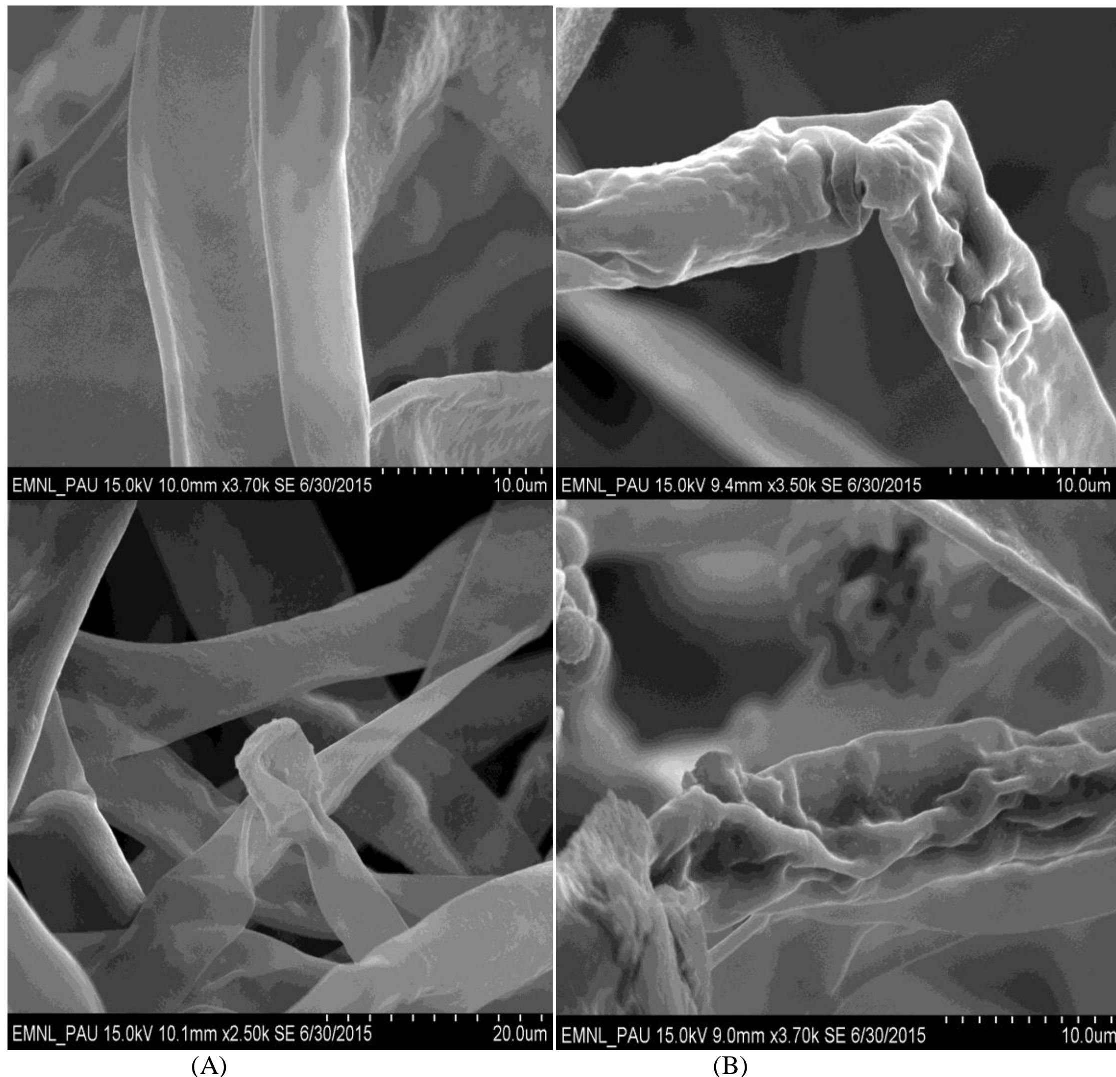
Plate B= CN1+ *Fusarium oxysporum*

Plate C= Control (*Fusarium moniliforme*)

Plate D= BS9+ *Fusarium moniliforme*

Plate E= control (*Sclerotinia sclerotiorum*)

Plate F= CS2+ *Sclerotinia sclerotiorum*



(A) (B)

Fig. 2 Fusarium moniliforme control (A), F.moniliforme infected with actinomycete isolate CS16 (B)

CONCLUSION

Actinomycetes strains can be considered for isolation of novel secondary metabolites which may be of importance for various biocontrol applications. Use of biocontrol agents such as actinomycetes will probably be one of the important tactics for plant disease management in the near future as they allow the reduced use of pesticides that are potential pollutants of the environment.

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