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Screening of Endophytic and Rhizospheric Actinomycetes with Potential Application for Biocontrol of Fusarium Wilt of *Gladiolus*

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A B S T R A C T

The present study was undertaken to isolate endophytic and rhizospheric actinomycetes from *Gladilous* and to evaluate their plant growth promoting traits. Out of 90 isolates, 40 were from rhizospheric soil and 50 from roots of *Gladiolus* plants. Sixteen isolates from rhizospheric and 14 from endophytic were observed to produce IAA ranged from 4.3-79.9 µg/ml. Eighteen (20%) isolates were observed to solubilize phosphate in the range of 6.1-64.5mg/100ml. Fifteen (16.6%) isolates displayed siderophore production. Twelve isolates were producing hydroxamate in the range of 5.5 to 39.4µg/ml and nine isolates produced catechol ranging between 12.1-35.3µg/ml. Fifty out of 90 isolates were observed to produce the gibberellic acid ranging from 12.5-50.6 µg/ml. Ten isolates were found to produce HCN and 5 isolates were able to show ACC deaminase activity. The antagonistic activity was displayed by twelve isolates against *Fusarium oxysporum*. On the basis of PGP traits and antifungal activity, isolate Sc9 was selected for green house study which was presumptively identified as *Streptomyces* sp. This actinomycete isolate could be used as potential plant growth promoting and biocontrol agent.

Introduction

Gladiolus (*Gladiolus hortulanus* L.H. Bailey) is an important seasonal and long-duration cut flower in many countries including India belonging to the family Iridaceae (Sinha and Roy, 2002). Corm rot and vascular wilt of *Gladiolus*, caused by *Fusarium oxysporum* f. sp. *gladioli* is one of the most serious diseases of *Gladiolus* affecting plants in the field and corms in storage (Riaz *et al.*, 2009, 2010). Vascular corm rot is also called "yellows" on infected plants in the field (Chandel and Bhardwaj, 2000). The infected corms show brownish to black dry rot symptoms which results in yellowing of leaves, stunted plant growth, discoloured flowers and even destruction of the corms which deteriorates its quality and market value (Ram *et al.*, 2004). Actinomycetes are a specific group of gram positive, saprophytic bacteria. Microorganisms living within plant tissues for all or part of their life without causing any visible symptoms of their presence are defined as Endophytes (Saikkonen *et al.*, 2004). Endophytic actinomycetes seem to be ubiquitous in most plant species and have been isolated from roots, leaves, and stems, and a few from flowers, fruits, and seeds (Lodewyckx *et al.*, 2002). Both endophytic and rhizospheric actinomycetes able to produce active compounds which can protect roots by inhibiting the growth of potential fungal pathogens, this may be achieved

through the production of enzymes which degrade the fungal cell wall or antifungal compounds (Errakhi *et al.,* 2007). Rhizodeposition of various exudates provide an important substrate for the soil microbial population (Marschner and Baumann, 2003). Interactions within the plants and microrganism increase the ability of plants to utilize nutrients from the soil by increasing root development, nitrate uptake, or solubilizing phosphorus, and to control soil-borne pathogens (Whipps, 2001).

Plant growth promoting actinomycete (PGP) inoculation has proven to be a promising agricultural approach that plays an important role in crop protection, growth promotion or biological disease control (Fatima *et al.,* 2009). The aim of this study was to isolate, characterize endophytic and rhizospheric actinomycetes for plant growth promoting (PGP) and biocontrol potential from *Gladiolus* plant.

Materials and Methods

Isolation of actinomycetes from rhizospheric soil

Twenty samples of rhizospheric soil of *Gladiolus* were collected from PAU, Ludhiana. One gram of each sample was transferred to a 30 ml test tube and suspended in 9 ml of sterile normal saline by shaking for 2 minutes on a vortex mixer. Aliquots (0.1ml) from serial logarithmic dilutions of each suspension were pipetted onto the surface of duplicate Petri dishes containing yeast malt extract agar (MYEA), both supplemented with cycloheximide (50µg/ml) to suppress fungal growth. The inoculum was spread evenly over the surface with glass applicator and incubated at 28°C for 10 days. Counts from the duplicate plates of the respective media are expressed as the mean number of colony forming units (cfu/gram).

Isolation of endophytic actinomycetes

Twenty plants of *Gladiolus* were selected randomly from different sites of Punjab Agricultural University, Ludhiana district (30.9° North and 75.85° East; Elevation, 247 m) of northern India. Root tissue surfaces were sterilized by using 70% (v/v) ethanol for 5 min and sodium hypochlorite solution (0.9% w/v available chlorine) for 20 min. Surface-sterilized tissues were washed thrice in sterile distilled water. In order to reduce the opportunity for emergence of endophytic fungi from the tissue, the samples were soaked in 10% $NaHCO_3$ solution for 10 min to disrupt the growth of the fungi.

Each root, shoot and leaf were cut into small pieces (0.5-1.0 cm) and placed on Starch casein agar (SCA) medium and incubated at 28°C for 7-10 days to record the microbial growth. Effectiveness of surface sterilization was tested by the method of Schulz *et al.,* (1993).

Characterization of actinomycete isolates for plant growth promoting traits

Indole acetic-acid (IAA) production

The production of IAA by actinomycete isolates was determined by colorimetric assay (Gordon and Weber, 1951). The isolates were inoculated into 100 ml of YME broth containing 0.2% L-tryptophan and incubated at 28°C with shaking at 125 rpm for seven days. Cultures were centrifuged at 11,000 rpm for 15 min.

One milliliter of the supernatant was mixed with 2 ml of the Salkowski reagent. Development of pink color indicated IAA production and optical density (OD) was read at 530nm using a spectrophotometer. The level of IAA produced was estimated by comparison with an IAA standard.

Siderophore production

The actinomycete isolates were inoculated on Chrome azurol S (CAS) agar medium and incubated at 28ºC for 5 days (Schwyn and Neilands, 1987). The colonies with orange zones were considered as siderophore-producing isolates. Catechol-type siderophores were estimated by Arnow's method (Arnow, 1937) and hydroxamate siderophores were estimated by the Csaky test (Csaky, 1948).

Phosphate solubilization

Phosphate solubilization test was conducted qualitatively by inoculating the endophytic actinomyctes isolates on National Botanical Research Institute's phosphate agar medium (NBRIP) containing $Ca_3(PO_4)_2$ according to Nautiyal (1999). The presence of halo clearing zone around growing colony after incubating at 28°C for 7 days was used as an indicator for positive P-solubilization. The ability of isolates to solubilize insoluble phosphate was also tested quantitatively in liquid culture. The cultures suspensions were centrifuged at 3000 rpm for 30 min. Soluble phosphate in supernatants were determined according to Jackson (1973).

ACC deaminase production

The qualitative estimation was done by the method prescribed by Govindasamy *et al.*, 2008. Actinomycetes isolates were spot inoculated on Petri plates containing DF (Dworkin and Foster) salt minimal medium supplemented with 3 mM ACC. Growth of isolates on ACC supplemented plates was compared to positive ((NH4)2SO4 as N-source) and negative controls (DF minimal medium without ACC) after 3-4 days incubation at 28°C. Isolates grown well on ACC plates were selected.

Gibberellic acid production

Cultures inoculated in their YME broth were incubated at 37ºC for seven days and then centrifuged at 8000rpm for 10 min. 15ml of the supernatant was pipetted out separately into the test tubes and 2 ml of zinc acetate solution was added. After 2 min, 2 ml of potassium ferrocyanide solution was added and centrifuged at 8000 rpm for 10 min. Five ml of supernatant was added to five ml of 30% hyperchloric acid. Absorbance was measured at 254nm in a UV-VIS spectrophotometer. From the standard graph prepared by using gibberellic acid solutions of known quantities, the amount of GA_3 produced by the culture was calculated and expressed as µg 25 ml^{-1} broth.

Hydrogen cyanide (HCN) production

On HCN screening medium, a Whatmann filter paper no. 1 soaked in 2% of sodium carbonate in 0.5% picric acid solution placed in the lid of the plate. Plates were sealed with paraffin and incubated at 28° C for seven days. Development of orange red color indicated HCN production (Lorck, 1948).

Screening of actinomycete isolates for antagonistic activity

The actinomycetes isolates were evaluated for their antagonistic activity against *Fusarium oxysporum* by dual-culture *in vitro* assay. Five days old fungal discs (8 mm in diameter) at 28ºC were placed at the center of the plates containing PDA media. Five days old actinomycetes discs (8mm) grown on SCA 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 microscopic (SEM)

SEM was employed to evaluate the effect of Sc9 on the fungal cell wall of *Fusarium oxysporum* culture using chemical fixation technique (Bozzola and Russell, 1996).

Evaluation of effectiveness of actinomycete isolate (Sc9) as potential antagonist against *Fusarium oxysporum* in green house

Inoculum preparation of potential antagonists

The potential isolate (Sc9) was grown in nutrient broth medium for 5 days. The surface sterilized corms of *Gladiolus* were immersed overnight in the antagonist suspension containing 10^8cfu/ml.

Fungal inoculum preparation

Fusarium oxysporum was grown on potato dextrose 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 at 200rpm. The rate of inoculum applied to the potting mixture was 10 gm of fungi per pot.

Soil infestation

Soil was taken from field and sterilized by autoclaving at 121^0C for 1 hr for 3 consecutive days. Corms were grown in pots, using completely randomized block design (CRD) with 6 treatments and 3 replications each. Five bulbs were sown per pot containing sterile soil. The treatments were: (A) T1- Control with sterile soil only (B) T2- Corms inoculated with fungal spore suspension (C) T3- Corms inoculated with potential actinomycete isolate (Sc9) (D) T4- Corms inoculated with potential actinomycete isolate (Sc9) + fungal spore suspension (E) T5- Recommended NPK (40:40:160) (F) T6- NPK+ potential actinomycete isolate Sc9.

Results and Discussion

Isolation of actinomycete diversity

A total of 90 isolates of actinomycete were obtained from the rhizospheric soil and roots of *Gladiolus* plant. Out of 90, forty were isolated from rhizospheric soil and 50 from roots of *Gladiolus* (Table 1) and labeled as prefix 'S' for rhizospheric and 'R' for endophytic actinomycetes. The data on occurrence and enumeration of actinomycetes from rhizopheric soil showed that *Gladiolus* has the population density of $2.5x10^5$c.f.u g^{-1}. These results are in agreement with the reports of Kamara and Gangwar (2015) who obtained 100 actinomycete isolates (50 from *Catharanthus roseus* and 50 from *Withania somnifera*) from 30 soil samples. The actinomycete population was found to be highest in *Withania somnifera* (3.9 $x10^5$ c.f.u g^{-1}) followed by *Catharanthus roseus* (3.2 $x10^5$ c.f.u g^{-1}).

Plant Growth Promoting (PGP) of the actinomycete isolates

Production of indole acetic acid (IAA)

Out of 90 isolates, thirty (16 from soil and 14 from roots) were observed to produce the phytohormone IAA. Indole acetic acid production ranged from 4.3-27.4 µg/ml in the absence and 8.1-79.9 µg/ml in the presence of precursor tryptophan (Figure 1). Maximum IAA production was reported in rhizospheric actinomycete isolate Sc9 (79.9 µg/ml), followed by Sb3 (68.9 µg/ml) in the presence of tryptophan. Minimum IAA production was showed by isolate Sc13 (8.1µg/ml) in the presence of tryptophan. Among endophytic

actinomycete isolates, maximum IAA production was observed in isolate Rm9 (60.7µg/ml), followed by Rm4 (59.4µg/ml) in the presence of tryptophan. Minimum IAA production was shown by Rc2 (12.6µg/ml) in the presence of tryptophan (Figure 2).

Our results are in accordance with Gangwar and Kataria (2013) who isolated 202 actinomycetes from rhizospheric soil of 25 medicinal plant, out of which fifty isolates were observed to produce the phytohormone indole acetic acid in the range of 10.2 to 93.8 µg/ml. Anwar *et al.,* (2016) isolated 98 rhizospheric actinomycetes from different wheat and tomato fields. The amount of indole acetic acid produced by actinomycete isolates, *Streptomyces nobilis* WA-3, *Streptomyces kunmingenesis* WC-3, and *Streptomyces enissocaesilis* TA-3 was found to be 79.5, 79.23, and 69.26 µg/ml IAA respectively at 500 µg/ml L-tryptophan.

Phosphate solubilization

Eighteen (20%) out of 90 isolates were observed to solubilize phosphate (Table 4 and Plate 3). The amount of phosphate solubilized by actinomycete isolates varied in the range of 6.1-64.5mg/100ml, after 10 days of incubation (Table 1). The maximum amount of phosphate solubilization was shown by Sc9 (64.5mg/100ml), followed by Sh2 (43.7mg/100ml). The results were in accordance with Gangwar and Kataria (2013), who reported that forty endophytic actinomycetes from medicinal plants solubilized phosphorus in the range of 0.02-0.68 mg/ml. The maximum amount of phosphate solubillized by *S. albosporus* A4 (16.5 mg/100 ml). Gangwar *et al.,* (2015) also reported high amount of phosphate solubilizing activity by *Streptomyces cinereus* AR3 (0.45±0.004 mg/ml), followed by *S. griseofuscus* strain AL4 and one *Micromonospora* isolate, AR15 (0.38±0.004mg/ml each).

Siderophore production

Fifteen isolates produced distinct orange halo on chrome-azurol S (CAS) plates indicating siderophore production (Table 1). Twelve isolates produced hydroxamate type of siderophore in the range of 5.5 to 39.4µg/ml and nine isolates produced catechol type of siderophore ranging between 12.1-35.3µg/ml. Maximum hydroxamate type of siderophore was produced by Sc9 (39.4µg/ml) followed by Sc16 (34.4µg/ml) and Sh2 (34.1µg/ml) and minimum was reported in Rc2 (5.5µg/ml). On the other hand, isolate Sc9 (35.3µg/ml) produced maximum catechol type of siderophore followed by Sc12 (30.7µg/ml). Our results are in accordance with Kanchanadevi *et al.,* (2013) who isolated six endophytic actinomycetes from agricultural crops and identified as *Propionibacterium acnes, Nocardia sp* and *Micromonospora sp*. All of these isolates produced siderophore in the range between 0.028µg/ml and 0.115µg/ml. Gangwar *et al.,* (2014) isolated 60 endophytic actinomycetes from leaf, root and shoot of *Musa acuminata*. Thirty isolates were able to produce hydroxamate type of siderophore ranging 4.1±0.5 to 128.6±1.1µg/ml and 16 isolates were able to produce catechol type of siderophore ranging between 9.2±0.7 and 147.3±0.5 µg/ml.

Production of gibberellic acid

Fifty out of 90 isolates were observed to produce the gibberellic acid. Out of which, 12 were from rhizosphere and 3 from roots of *Gladiolus.* Gibberellic acid production ranged from 12.5-50.6µg/ml. From these isolates, maximum gibberellic acid production was reported in Sc9 (50.6 µg/ml), followed by Sd10 (45.2µg/ml) and Sh2 (36.2µg/ml). Isolate Sc7 (8.1µg/ml) showed minimum gibberellic acid production as evident from table 1. These results are supported by Solans

(2011) who isolated 122 actinomycetes from *Ochetophila* plant. Among 122 isolates, maximum gibberellic acid production was reported in *Micromonospora sp.* (3.73µg/ml), followed by *Frankia sp.* (1.76µg/ml), *Actinoplanes sp.* (1.53µg/ml) and *Streptomyces sp.* (0.96µg/ml). Rashed *et al.*, (2015) tested 83 actinomycete isolates from marine sediment for gibberrellic acid production which ranged from 11.2-121.5 µg/ml.

Production of hydrogen cyanide and ACC deaminase activity

Ten isolates were found to produce HCN (Table 1). Out of which, Sc9, Sm6 and Rd9 were highest producers of HCN as evident from the reddish brown colour of filter paper. Five rhizospheric actinomycetes namely, Sc9, Sc16, Sb2, Sc20 and Sc7 showed ACC deaminase activity on DF medium (Table 1). Our results are supported by Shete and Kapdnis (2012) who isolated 10 actinomycetes from soil. Out of these 10 isolates, only two isolates showed HCN production. Cruz and Paterno (2014) screened 59 actinomycete isolates for its growth promoting activities, out of which fourteen showed ACC deaminase activity.

Antagonistic activity of actinomycete isolates against *Fusarium oxysporum*

Out of 90 isolates, 12 (13.33%) displayed antagonistic activity against *Fusarium oxysporum*. Isolate Sc9 exhibited maximum percent inhibition of 81.2±0.2% against *F. oxysporum*, followed by Sh2 (72.7±0.1%) and Sc16 (69.4±0.1%). Rm9 showed minimum inhibitory activity of 50.9±0.1% (Table 2).

Kamara and Gangwar (2015), 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.*

Scanning electron microscopic (SEM)

Scanning electron microscopy was performed by co-culturing *Fusarium oxysporum* and Sc9 an isolate of *Gladiolus* rhizospheric soil. Scanning electron micrographs showed degradation of *Fusarium oxysporum* cell walls due to secretion of diffusible compounds by Sc9 as compared to control. The control plate of *Fusarium oxysporum* showed the presence of regular vegetative cells exhibiting smooth surface with overall intact morphology whereas fungal colony inoculated with Sc9 showed brittle, disrupted and damaged hyphae at the edges of the inhibited fungal colonies on the GYE plates (Figure 3).

Tangum and Niamsup (2012) 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*. The effect was investigated and compared with the control. Gangwar *et al.*, (2015) reported that endophytic actinomycete *Streptomyces cinereus* AR16 isolated from *Emblica officinalis* degraded the cell wall of *Fusarium oxysporum* mycelia as compared t control *Fusarium oxysporum*.

Presumptive identification of actinomycetes isolates

On the basis of PGP characteristic such as IAA, P-solubilization, siderophore production, gibberelllic acid, HCN and ACC deaminase activity, two (Sc9 and Sh2) best performing isolates were presumptively identified.

Table.1 Plant growth promoting traits of actinomycete isolates

Isolates	Phosphate solubilization (mg/100ml)	Gibberellic acid (µg/ml)	Hydroxamate type of siderophore (µg/ml)	Catechol type of siderophore (µg/ml)	Zone of HCN	Growth on ACC
Sc9	64.5 ± 0.2	50.6 ± 0.1	39.4 ± 0.1	35.3 ± 0.1	+++	+++
Sd10	38.7 ± 0.1	45.2 ± 0.06	-	-	-	-
Sh2	43.7 ± 0.05	36.2 ± 0.05	34.1 ± 0.1	13.9 ± 0.1	++	-
Sc7	13.4 ± 0.1	12.5 ± 0.1	-	-	-	+++
Sc20	9.19 ± 0.1	15.9 ± 0.2	-	18.1 ± 0.2	-	+++
Sc11	42.6 ± 0.2	25.6 ± 0.2	-	-	-	-
Sc16	-	23.7 ± 0.1	34.4 ± 0.1	19.6 ± 0.1	++	+++
Sb2	-	45.2 ± 0.1	12.6 ± 0.2	-	+	+++
Sc12	-	15.3 ± 0.1	-	30.7 ± 0.1	-	-
Sb3	-	29.6 ± 0.1	16.2 ± 0.1	-	+	-
Rm5	35.8 ± 0.06	17.8 ± 0.1	-	-	-	-
Rm4	29.1 ± 0.06	34.5 ± 0.05	-	-	-	-
Rm9	35.4 ± 0.1	26.3 ± 0.2	23.9 ± 0.05	-	-	-

Values indicate mean of three replicates**.**

Table.2 Antifungal activity (%inhibition) of actinomycete isolates against *Fusarium oxysporum*

Isolates	Percent inhibition
Sc9	81.2±0.2
Sh2	72.7±0.1
Sc20	63.5±0.2
Sc16	69.4 ±0.1
Sb2	53.5±0.5
Sc7	62.3±0.2
Sm4	51.2±0.2
Sc3	58.2±0.1
Rd5	63.5±0.4
Rm9	50.9±0.1
Rxy	65.8±0.3
Rb3	57.6±0.1

Average ± standard error from three replicates
Values indicate mean of three replicates.

Table.3 Effect of actinomycete isolates on Gladiolus plant under green house conditions

Treatments	Corm germination @ 10 DAS	Root length (cm plant^{-1})		Shoot length (cm plant-1)		Fresh root weight (gm plant^{-1})	Fresh shoot weigh t (gm plant^{-1})	Dry root weight (gm plant-^{1})	Dry shoot weig ht (gm plant -^{1})
		30 DAS	60 DAS	30 DAS	60 DAS				
Control	60%	2.0	6	12.5	72	0.17	9.5	0.1	1.82
Fusarium oxysporum	40%	2.5	7.8	12.5	66	0.15	8.6	0.1	1.71
S. aureus (Sc9)	90%	7.5	10.5	44.1	70	0.24	18.3	0.5	7.73
S. aureus +Fusarium oxysporum	86%	4.5	9.7	37.5	63	0.21	20.1	0.6	4.68
NPK	100%	3.8	9.5	34.5	52.5	0.19	17.9	0.4	3.17
NPK + *S. aureus*	100%	4.2	11.8	27.4	82.5	1.77	22.2	0.8	5.06

Table.4 Effect of different treatments on inhibition of disease development (%)

Treatments	Incidence of Disease (%)	Inhibition of disease (%)
Fusarium oxyporum	60	-
S. aureus+ Fusarium oxyporum	26	80

Fig.1 Relative production of IAA by rhizospheric actinomycete isolates in the presence and absence of tryptophan

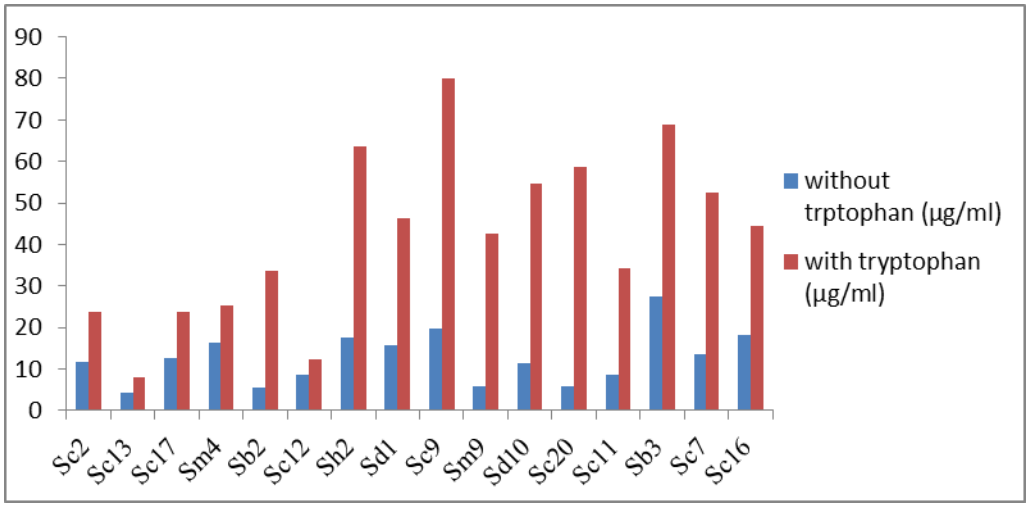


Fig.2 Relative production of IAA by endophytic actinomycete isolates in the presence and absence of tryptophan

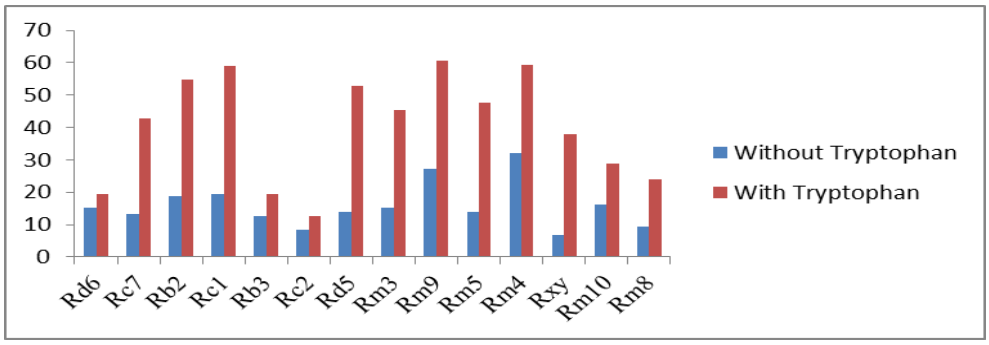
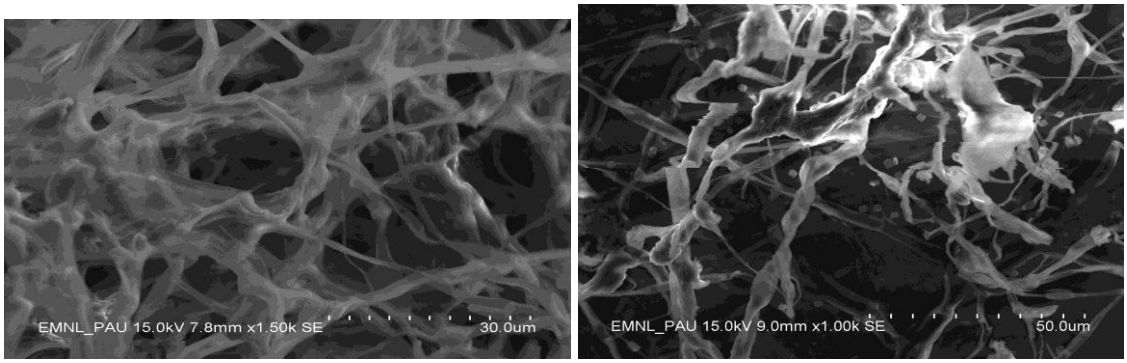


Fig.3 *Fusarium oxysporum* control (A), *F. oxysporum* infected with actinomycete isolate Sc9(B)

Presumptive identification of actinomycetes isolates



(A) **(B)**

Streptomyces aureus

The colony of isolate Sc9 was white in color. In slide culture elaborate primary and secondary mycelium was observed. Spores were arranged in chains. The strain was Gram positive. The strain decomposed casein, starch, Tween-80, esculin, xanthine, hypoxanthine and tyrosine.

Evaluation of effectiveness of actinomycete isolates as potential plant growth promoters and antagonists against *Fusarium oxysporum* in *Gladiolus* under green house

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 Sc9. Similar parameters were observed more for Sc9+*Fusarium oxysporum* as compared to *Fusarium oxysporum* alone and Sc9+NPK as compared to NPK (Table 3). These phenomena may be related to the production of growth regulators by actinomycetes. Results are in accordance with Kaur *et al.,* (2015) who observed that soil inoculation by Cs1and Cs44 isolates enhanced root depth, shoot length and dry weights of root and shoot which may due to nitrogen fixation, auxins, production or unidentified compounds.

There was no wilting in the treatments with inoculation of *S. aureus*, NPK and NPK + *S. aureus*. Incidence of disease was reported in *S. aureus* with *Fusarium oxysporum* (26%). Maximum inhibition of disease was observed in *S. aureus* with *Fusarium oxysporum* (80%) (Table 4). 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 isolate Sc9 proved their potential as a biocontrol agent to reduce the *Fusarium* wilt caused by *Fusarium oxysporum* in this planting system.

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