# Isolation and Characterization of Actinomycetes with In-vitro Antagonistic Activity against Fusarium oxysporum from Rhizosphere of chilli

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ABSTRACT: This study was carried out to isolate and characterize antagonistic bacteria against wilt causing fungal pathogen i.e. Fusarium oxysporum, from the rhizosphere of chilli plant. Twenty bacterial strains were isolated from the rhizosphere soil samples from healthy chilli plant, collected from different locations of upm, Serdang, Malaysia. Out of these, seven isolates were found to be antagonistic against the tested fungal pathogen i.e. Fusarium oxysporum, under in-vitro conditions. On the basis of percentage inhibition of radial growth of Fusarium oxysporum, isolate At5 was found to be the most effective antagonistic rhizobacteria against the pathogen. Based on its morphological and biochemical properties along with 16s rRNA sequence analysis, it was identified as a Streptomycetes indiaensis with accession number KJ872546. Average percentage inhibition given by this isolate was 71%, and it was found to produce diffusible and volatile antifungal metabolites along with hydrogen cyanide and ammonia. Effect of physiological parameters on the growth and antagonistic behavior of the potential isolates was also examined. The present study, hence, provides a potential biocontrol agent for Fusarium oxysporum, however, field studies of this isolate as soil inoculants in chilli are required in order to establish its actual performance.

Keywords: Rhizobacteria, Biocontrol, Percentage inhibition, chilli wilt.

# **INTRODUCTION**

In the increase of developing sustainable agricultural practices and rising public awareness about the ill-effects of Agrochemicals, research directed towards the development of alternative and complementary pathogen control methodologies is extremely required. Exploring the inherent inhibitory potential possessed by many microbes against the phytopathogens can to be an alternative and an environment-friendly substitute of agrochemical methods. Utilizing antagonistic microorganisms associated with the plant rhizosphere has great potential for control of soil borne plant pathogens. Huge volume of literature has been generated in the last few decades that reports the efficient use of rhizosphere microflora to control fungal pathogens in a variety of plants, [1], [2],[3] (Anjaiah et al., 2006; Siddiqui and Akhtar, 2007; Sahu and Sindhu, 2011).

Chilli (Capsicum annuum L.) is a common crop and cultivated all over the world. It is known as economically very important and valuable cash crop of Malaysia. It belongs of the family Solanaceae, as are potatoes, tomatoes and eggplants [4] (Hussain and Abid, 2011). Several abiotic and biotic stresses affect the productivity of chilli crop worldwide. In addition to fungal, bacterial, nematodes and viral diseases are also responsible for significant production constraints affecting, both yield and quality, and are often difficult to control [5] (Nono-womdim, 2001). Fusarium wilt is the most important fungal disease affecting pepper plants.[6] (Kirankumar,2008),It is caused by a soil borne fungus, Fusarium oxysporum f. sp. lycopersici that affects the vascular system of plant and severely decrease the yield.

At present used means of controlling Fusarium oxysporum include The use of fungicidal chemicals and resistant cultivars, crop rotation with non-host varieties of the fungus, use of clean equipment, and raising bids to promote soil drainage and dry soil surface [7] (Smith et al., 1988).. The present study, therefore, aimed at isolation and characterization of antagonistic bacteria against wilt causing fungal pathogen Fusarium oxysporum from the rhizosphere of chilli, which can be further developed as biocontrol soil inoculum for chilli crop.

# METHODOLOGY

#### Soil sample collections

The specimens (actinomycetes) were used in this study was isolated from a different region of chilli roots (healthy plants). Soils about 10-20 cm depth from the surface and near to the root area were selected for sampling. [8] (Bonjar et al., 2005).

### **Isolation of Actinomycetes**

CaCO3 enrichment methods were used to isolate Actinomycetes, the soil samples were mixed with CaCO3 at the ratio of 10:1, and were incubated under moisture rich conditions for seven days at room temperature [9] (Hayakawa et al., 2004). One gram of the soil sample was serially diluted up to 10–7 dilution. Aliquots of 0.1 ml of each dilution was spread plated on Actinomycetes agar plates in triplicates and incubated at room temperature for seven days. After an incubation period, the plates were examined for the presence of actinomycetes colony. The suspected colonies were picked up and purified on International Streptomyces Project (ISP-2) agar by [10] Waksman (1961) media and incubated at room temperature for about 7 days. The suspected pure actinomycetes culture was inoculated on ISP-2 slants, after the incubation period the slants were taken for further identification and antifungal screening. The stock culture was preserved in 15% glycerol (v/v) at  $-20^{\circ}$ C [11] (Maniatis, 1989).

#### **Isolation of the Pathogen**

Root samples of 10 chilli pepper plants infected with wilting were collected from different locations of Cameron highland, discolored parts of the roots and stems samples were cut into small pieces up to 1.5 cm length and surface sterilized with 0.1% sodium hypochlorite (NaOCl) for two minutes and rinse in sterilized water dry between folds of sterilized filter paper [12] (Nightsarwar et al., 2005). The sterilize root pieces were transferred to four replicated petridishes containing sterilized (potato dextrose agar) 15 ml/plate and incubate seven days in (28°C). The fungal isolates purify following hyphal tip, technique [13] (Tuite, 1996). Repeated culture has been made from the tip of the single hyphae to obtain a pure culture of the identified Fusarium oxysporum the pure culture was stored in the PDA slants at 10°C for further use.

### DNA amplification and sequencing of F.oxysporium and PCR method

F.oxysporium isolates was used for DNA extraction. These isolates was cultured in 10 ml of potato dextrose broth (PDB) at 28°C for seven days. After incubation, the mycelial mat will be harvested by filtration and stored at  $-80^{\circ}$ C until use. Total genomic DNAs will be extracted from 50 mg of fresh mycelium . the region of the ribosomal repeat from 3 ends of 18S rDNA to 5 ends of the 28S rDNA with a partial sequence, spanning ITS1, 5.8S rDNA and ITS2. Primer sequence used were 5-TCCTCCGCTTATTGATATGC-3(ITS4) and 5- GGAAGTAAAAGTCGTAACAAGG-3 (ITS5) (White et al., 1990). The PCR was set up using the following components: 12.5µL Taq Polymerase (5U), 1µL Forward primer (10µM), 1µL Reverse Primer (10µM), 2µL DNA template and 8.5µL distilled water. Initial denaturation was at 94°C for 4 min denaturation, annealing and elongation was done at 94°C for 30 Sec, 55°C for 30 Sec and 72°C for 1 min, respectively, in 45 cycles. Final extension was at 72°C for 5 min and hold at 12°C. For the amplification of ITS-rDNA region, ITS4 and ITS5 primers were used according to the method described by [14] White et al. (1990). The PCR product, spanning approximately 500 – 600bp was checked on 1% agarose electrophoresis gel. It was then purified using quick spin column and buffers (washing buffer and elution buffer) according to the manufacturer's protocol (QIA quick gel extraction kit Cat No. 28706). DNA sequencing was performed using the above-mentioned primers in an Applied Biosystem 3130x1 analyzer.

### Screening of Rhizobacteria for Antagonism against Fusarium oxysporum

All the rhizobacterial isolates so obtained were evaluated for their antagonistic activity against mycelial growth of Fusarium oxysporum using The dual culture technique [15](Gupta et al., 2001). 5 mm agar disc of a five-day-old culture of the fungal pathogen was placed in the center of potato dextrose agar (PDA) plates. Twenty-four hour old culture of each isolate was streaked parallel on either side of the fungal disc at a distance of 2 cm. The plates with only centrally placed fungal disc, but without bacterial streaks, served as a control. The inoculated plates were incubated at  $28\pm2^{\circ}$ C for five days, and inhibition of the radial growth of the pathogen was measured. Each treatment was replicated three times. The colony diameter of the fungal pathogen was measured and compared with the control. Percentage inhibition of the pathogen by the rhizobacterial strain over the control was calculated by using the formula given by [16](Vincent,1947) as follows: Percent inhibition of mycelium =C- T ×100 /C

Where,

C = Growth of mycelium in the control,

T =Growth of mycelium in the treatment.

## Elucidation of Antagonistic Mechanism

Bacterial isolates showing antagonistic activities against tested pathogen i.e. Fusarium oxysporum were further examined for elucidation of the possible mechanism underlying their antagonistic behaviour.

# Hydrogen Cyanide (HCN) Production

HCN production by rhizobacterial isolates was tested according to the method described by[17] Wei et al. (1991). Plates with Whatman No.1 filter paper pads inside their lids were poured with glycine supplemented (4.4 g l-1) tryptic soy agar (TSA) medium and streak inoculated with twenty-four hours old bacterial isolates. The filter paper padding was soaked with a sterile picric acid solution, and the lid was closed. Inoculated plates were sealed properly and were given an incubation of five days at 30°C and then observed for color change of the filter paper padding. Degree of HCN production was evaluated according to the color change, ranging from yellow to dark brown.

# **Protease Production**

Proteolytic activity was determined using skim milk agar [18] (Kumar et al., 2005). Overnight activated cultures were spot inoculated on skim milk agar plates and given an incubation of five days at 30°C. Afterwards, plates were observed in the formation of a clear zone around the bacterial growth, which indicated a positive proteolytic activity.

# **Production of Diffusible Antifungal Metabolites**

The method described by [19] Montealegre et al. (2003) was used to determine the production of diffusible antifungal metabolites by antagonistic rhizobacteria isolated. Overnight activated bacterial cultures were stab inoculated in the center of PDA plates covered with a filter paper and incubated at 28°C for 72 hours. Afterwards, the filter paper with the bacterial growth was removed from the plate, and it was inoculated with a 5 mm disk of the test fungus (pathogen) in the center. Plates were further incubated at 28°C for five days, and the growth of fungus was measured. Filter paper covered PDA plates inoculated with sterile distilled water in place of bacteria and further inoculated with the test fungus served as a control. The colony diameter of the fungal pathogen was measured and compared with the control. Percentage inhibition of fungal growth was calculated, and production of diffusible antifungal metabolites was recorded as nil, low, medium, and high.

### **Production of Volatile Antifungal Metabolites**

Production of volatile metabolites having antagonistic activity against fungal pathogens was tested by pairing plate technique of [20] Fiddaman and Rossall (1993) with some modifications. A petri plate containing ISP2 medium was streaked inoculated with a loop full of 48 hours old rhizobacterial isolate. A second petri plate containing PDA was inoculated with a 5 mm plug of the activated test fungus (pathogen) at the center of the plate. Both half plates were sealed together, and the paired plates were incubated at 28 °C for seven days. Control set of paired plates was designed with only the test fungus on a PDA half plate inverted over the unstreak ISP2 half plate. The experiment was conducted in triplicates. After an incubation period, the paired plates were observed for inhibition of fungal growth as compared to the control. The colony diameter of the fungus was measured and compared with the control set. Percentage inhibition of radial growth of the fungus was calculated as mentioned before and production of volatile antifungal metabolites was recorded as nil, low, medium, and high.

### **Production of Ammonia**

Ammonia production was tested in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water and incubated for 48-72 hours at 30°C. Afterwards, 0.5 ml Nessler's reagent was added to each tube. Development of brown to yellow color was taken as a positive reaction for ammonia production [21] (Cappuccino and Sherman, 2010).

### Identification of Antagonistic Rhizobacteria

Isolated antagonistic strains were characterized on the basis of various morphological and biochemical features according to Bergey's manual of determinative bacteriology [22](Holt et al., 1994) as per the standard procedures [23], [21] (Aneja 2003, Cappuccino and Sherman, 2010). Further, the molecular characterization was carried out for the best antagonistic isolate (in terms of percentage inhibition of mycelial growth) i.e. At5 on the basis of the 16s rRNA sequencing. The 16s rRNA region was sequenced using universal primers and compared with sequences deposited at the National Center for Biotechnology Information (NCBI) using BLAST.

### Statistical analysis

Data were analyzed by using Analysis of Variance (ANOVA) technique, by SAS GLM (General Linear Model) procedure (SAS Inst. 2002-08, SAS V9.3) considering isolates and replication as fixed in RCBD.

# **Results and Discussions**

#### Molecular identification method using PCR

The presence of F. oxysporum in the isolates isolated from diseased plant was definite by comparing the amplified DNA fragments with the marker and positive control. The analyzed samples were about 500 bp in size, identified as Fusarium oxysporum f. sp. Lycopersici with Accession Number (KJ850251).

### Identification of Antagonistic Rhizobacteria

Amongst the seven antagonistic strains, all were isolated on Actinomycetes agar, (ISP2) and Triptic soy agar. All the strains isolated were Gram-positive, spore-forming and motile rods. Further identification of the best isolate At5 on the basis of 16s rRNA sequencing followed by BLAST showed that it had 100% nucleotide identity with seven strains of Streptomyces indiaensis and deposited in GenBank with Accession Number (KJ872546).

#### Isolation and Selection of Antagonistic Rhizobacteria

A total of Twenty isolates were obtained from the rhizosphere of healthy chilli plants from different locations of upm, Serdang, Malaysia, out of which seven isolates showed in-vitro antagonistic potential against Fusarium oxysporum when tested on PDA plates using dual culture technique (Table 1) (Figure 1). Average percentage inhibition of the pathogen was observed as 49.0%, and it varied significantly between the isolates (P < 0.05). On the basis of percentage inhibition of the radial growth of the test pathogen, isolate At5 was found to be the best antagonist (71.0% inhibition).

Table 1. III-viti o initibition of rusarium oxysporum by inizobacterial isolates from chim on the basis of dual culture technique
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S. No.	Isolate	Radial growth (in mm)	Percentage inhibition of radial growth
1	At1	35 <sub>b</sub>	50.0%
2	At5	20 <sub>a</sub>	<mark>71.0%</mark>
3	At6	40 <sub>d</sub>	42.0%
4	At8	40 <sub>d</sub>	42.0%
5	At11	37 <sub>c</sub>	47.1%
6	At17	40 <sub>d</sub>	42.0%
7	At18	35 <sub>c</sub>	50.0%

Mean percentage inhibition

A The values given are mean (n=3) with a standard deviation. Means in the same column followed by the same letter are not significantly different at P< 0.05 (Tukey's Honestly Significant Difference test).



control

# Figure 1: In vitro evaluation of antifungal activity assay. Actinomycetes were tested in a dual culture assay against pathogenic fungi on PDA agar plates.

The next two best isolates i.e. At1 and At18 were also identified as belonging to Streptomycetes sp. On the basis of their morphological and biochemical characteristics. All antagonistic strains isolated on Actinomycetes agar were Gram-positive, rod-shaped, motile and oxidase positive.

# Elucidation of Antagonistic Mechanism

All the seven antagonistic isolates were found to produce more than one kind of antifungal compounds under in-vitro conditions (Table 2). All the antagonistic isolates produced ammonia, except isolation At6, At17. and 30.0% of them produced HCN (Figure 6), and only At5 and At8 of the isolates produced proteases. Isolate At5 and At8 were found to produce all the tested . antifungal metabolites in medium to high range.

S. No	Isolate	HCN production	NH3 production	Protease production mm
1	At1	-	+	-
2	At5	+	+	+25
3	At6	+	-	-
4	At8	+	+	+16
5	At11		+	-
6	At17	and the second	and the second second	-
7	At18		+	-

#### Table 2. Production of various antifungal compounds by rhizobacteria isolates against Fusarium oxysporum.

Nil; +: production is positive; - production is negative

# **Diffusible Antifungal Metabolites**

As stated above, seven antagonistic isolates produced diffusible antifungal metabolites in PDA plates and the level of inhibition of the fungus under their effect varied significantly among the isolates (P<0.05). Isolate At5 showed maximum inhibition of 78.5% due to diffusible antifungal metabolites, while isolate At6 showed the least inhibition of only 40.0%. Average percentage inhibition by all the seven isolates, against Fusarium oxysporum, was calculated as 26.37% (Table 3)(Figure 2). While isolates At1, At8andAt11was77.1% Followed by isolates At17 and At18 was 45.7,57.1% Respectively.

Table 3. Inhibition of Fusarium oxysporum by diffusible antifungal metabolites produced by antagonistic rhizobacterial isolated

and the second s	The second se	Radial growth	Percentage inhibition of
S. No.	Isolate	(in mm)	radial growth
1	At1	16.0b	77.1 <sub>b</sub>
2	At5	15.0 <sub>a</sub>	78.5 <sub>a</sub>
3	At6	42.0 <sub>e</sub>	40.0 <sub>e</sub>
4	At8	16.0 <sub>b</sub>	77.1 <sub>b</sub>
5	At11	16.0 <sub>b</sub>	77.1 <sub>b</sub>
6	At17	38.0 <sub>d</sub>	45.7 <sub>d</sub>
7	At18	30.0c	57.1.

A The values given are mean (n=3) with a standard deviation. None of the means in the same column are similar at P< 0.05 (Tukey's Honestly Significant Difference test).



Figure 2: Antibacterial activity of the isolate (At5) with control by well diffusion technique

# **Volatile Antifungal Metabolites**

Seven antagonistic isolates produced volatile antifungal compounds against Fusarium oxysporum with significantly varied level of antagonistic potential (P< 0.05). An average percentage inhibition of 35.8% was observed. Isolate At5 gave maximum inhibition i.e. 56.2% Followed by isolate At1, 46.2%, while isolate At17 showed the least percentage inhibition of 18.7% (Table 4) (Figure 3) .then Isolates At6,At8,At11,At18 was 25.0%,37.5%,40.0,27.5 Respectively.

# Table 4. Inhibition of Fusarium oxysporum by volatile antifungal metabolites produced by antagonistic rhizobacterial isolates <sup>a</sup>

S. No.	Isolate	Radial growth (in mm)	Percentage inhibition of radial growth (%)
1	At1	40.3 <sub>b</sub>	46.2 <sub>b</sub>
2	At5	30.5 <sub>a</sub>	<mark>56.2</mark> a
3	At6	$60.0_{\rm f}$	25.0 <sub>f</sub>
4	At8	50.0 <sub>d</sub>	37.5 <sub>d</sub>
5	At11	40.8 <sub>c</sub>	40.0 <sub>c</sub>
6	At17	60.5 <sub>g</sub>	18.7 <sub>g</sub>
7	At18	50.8 <sub>e</sub>	27.5 <sub>e</sub>

A The values given are mean (n=3) with a standard deviation. Means with the same letter in the same column are not significantly different at P< 0.05 (Tukey's Honestly Significant Difference test).



Figure 3: Volatile Antifungal Metabolites

**Figure 4: HCN production** 

Fusarium wilt of chilli is an economically important disease that is found worldwide. At this time practiced methods to control the disease along with the need to develop sustainable methods of disease management has started the hunt for a suitable alternative. Plant Growth promoting rhizobacteria (PGPR) has emerged as the most promising choice in this direction. Antagonistic activities of PGPR have been reported against several soil borne fungal pathogens of plants like Phytophthora capsici [24] (Lee et al., 2008), Rhizoctonia solani [25] (Asaka and Shoda, 1996), Pythium ultimum [26] (Lee et al., 2000) and many others. Here, we report the antifungal properties of rhizobcaterial isolates of chilli, against Fusarium oxysporum. Amongst the seven antifungal strains isolated in this investigation, Streptomyces sp. was observed to have the strongest antagonistic potential against the tested pathogen. Rhizobacteria belonging to Streptomyces species have been previously reported to inhibit various wilt causing strains of Fusarium oxysporum in plants like chickpea [27], [28] (Landa et al., 2004; Karimi et al., 2012), eggplant [29], [30], [31], (Yildiz et al., 2012), lily (Chung et al., 2011), lentil (Akhtar et al., 2010) as well as tomato [32],[33] (Larkin and Fravel, 1998; Adebayo and Ekpo, 2004). However, Further, on the basis of the results obtained, it may be observed that a significant difference existed among the different strains of the same genera isolated from the same ecological niche in terms of their antagonistic behavior against a particular pathogen. It may be noted that percentage inhibition of radial fungal growth by different isolates of Streptomyces varied widely in the present study (P< 0.05). Divergence in their capacity to produce effective antifungal compounds may explain this disparity among the antagonistic isolates of the same genera. According to the observations made in this study, production of diffusible and volatile antifungal molecules along with compounds like ammonia and HCN seems to be the primary source of inhibition

of the tested fungal pathogens. Isolate At5 belonging to Streptomyces indiaensis with accession number (KJ872546). Was found as a strong producer of volatile and diffusible antifungal compounds, a character that has been previously well established for various strains of Streptomyces [25],[34],[35] (Asaka and Shoda, 1996; Wang et al., 2007; Dunlap et al., 2011).

Efficiency of volatile and diffusible antifungal compounds produced by different Streptomyces isolates also varied significantly (P < 0.05) and so was the case with other antifungal compounds as well. In order to exhibit their plant growth promotion and protection capabilities, the foremost requirement for the PGPR is to colonize the suitable sites in the rhizosphere so as to establish themselves in the soil. [36],[37] (Kumar, 2007; Sargaonkar et al., 2008). The present study has, therefore, provided a potential bacterial isolate suitable for controlling chilli wilt causing fungus Fusarium oxysporum. However, it is suggested that a detailed investigation must be carried out to evaluate isolate At5 for its field performance to control Fusarium oxysporum in chilli before it can be established as a biocontrol soil inoculant.

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