

Original Research Article

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Antifungal Activity of Endophytic Actinomycetes against *Fusarium* Wilt (*Fusarium oxysporum*) of Banana Trees (*Musa acuminata*)

Khushboo* and Madhurama Gangwar

Department of Microbiology, 141004, Punjab, India

*Corresponding author

ABSTRACT

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A total of 60 endophytic actinomycete strains were obtained from root and leaf tissue of 30 banana trees. Each isolate was tested against phytopathogenic fungus *Fusarium oxysporum*. Thirty isolates (50%) were displaying antagonistic activity against *Fusarium oxysporum*, a pathogen of Panama disease. Out of 60 isolates, 41 belonged *Streptomyces* sp. (68.33%) followed by *Nocardia* sp. (11.7%), *Micromonospora* sp. (8.33%) and *Saccharopolyspora* sp. (5%) based on their morphological and culture characteristics. The extracellular chitinase activity of selected isolate i.e. BR9 came out to be 0.0115 and 0.065 U/ml with 0.6% and 1% colloidal chitin concentration respectively. This study revealed that endophytic actinomycetes isolated from banana roots and leaves tissue are potent natural sources and can be applied in agriculture.

Introduction

Actinomycetes represent a large portion of the rhizospheric microbial community and are prolific producer of diverse bioactive secondary metabolites with high commercial value, such as vitamins, alkaloids, plant growth factors, enzymes, and enzyme inhibitors. Approximately two-thirds of naturally occurring antibiotics, including some of agricultural importance, have also been isolated from these soil microorganisms (Kieser *et al.*, 2000).

Actinomycetes are found also as endophytes that colonize the plant tissues. Endophytes are microorganisms that live within healthy plant tissue causing no apparent disease symptoms.

Endophytic actinomycetes have been largely exploited mainly because their capability to produce bioactive compounds. These compounds have often been related as one of the most important tools to control the soil-borne diseases with low environmental impact and toxic effect for humans and animals, well-desired traits for new consumer's requirements (Cardoso *et al.*, 2010).

Earlier endophytic actinomycetes have been isolated from surface-sterilized wheat roots (Coombs *et al.*, 2003), leaves of maize (Araújo *et al.*, 2000) and *in vitro* and *in vivo* antagonistic activities of endophytic actinomycetes against plant pathogens have

been reported (Taechowisan and Lumyong, 2003; Tian *et al.*, 2004; Cao *et al.*, 2005).

Banana (*Musa* sp.) is the fourth most important global food commodity after rice, wheat and maize in terms of gross value production. At present, it is grown in more than 120 countries and it is the staple food for more than 400 million people. It is an important fruit crop of many tropical and subtropical regions of India. It is cultivated in India in an area of 830.5 thousand ha and total production is around 29,779.91 thousand tons.

In 2010 the National Horticulture Board, India surveyed that the global production of banana is around 102028.17 thousand tons of which India contributes 29.19% (Gangwar *et al.*, 2014). Global banana production is seriously threatened by the re-emergence of a *Fusarium* Wilt.

The disease caused by the soil-borne fungi *Fusarium oxysporum* f. sp. cubense (Foc) and also known as “Panama disease”. *F. oxysporum* is considered invasive because it can be distributed from location to location and from country to country with traditional planting material. Also, once established it can spread within plantations in runoff water and in soil.

Currently, there is an increasing public concern regarding the continued use of agrichemicals to control the phytopathogenic fungi. This awareness relies mainly in the noxious effects of the pesticides on the environmental and human health (Tang and Niamsup, 2012).

Several efforts have been made to find less hazardous options for controlling these plant pathogens among which the biological control using the microorganisms has been demonstrated to be a feasible alternative (Zucchi *et al.*, 2008) but it is not widely used

on commercial scale (Bressan 2003 and Medeiros *et al.*, 2012).

In the present study the isolated endophytic actinomycete from the banana root and leaf tissues have been tested for their bioactivities and quantitative production of extracellular chitinase against phytopathogenic fungi *Fusarium oxysporum* that cause economically important disease in banana crop and to identify potent antagonistic strain.

Materials and Methods

Sample collection and isolation of endophyticactinomycetes

A total of 60 endophytic actinomycete isolates and fungal culture of *Fusarium oxysporum* was procured from the department of Microbiology and department of Plant breeding and genetics respectively, Punjab Agricultural University.

***In vitro* antagonistic bioassay**

The actinomycetes isolates were evaluated for their antagonistic activity against phyto pathogenic fungi *Fusarium oxysporum* by dual-culture *in vitro* assay. Colony growth inhibition (%) was calculated by using the formula: $C - T/C \times 100$, 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) studies of the antagonistic effect of potential actinomycete isolates on fungal cell wall

The two actinomycetes BR9 and BL49 were selected for Scanning electron microscopy as these were exhibiting highest antagonistic potential against the tested fungus. This was performed using chemical fixation and liquid osmium fixation technique (Bozzola and Russell 1996) (Fig. 4).

Chitinase production

The test for chitinase production was performed by the procedure described by (Taechowisan and Lumyong, 2003, Tang-um and Niamsup, 2012).

Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin (Hi Media) by the modified method of Hsu and Lockwood (1975).

Quantitative production of extracellular chitinase

For the quantitative estimation of chitinase activity 0.6% and 1% colloidal chitin concentration was used. Colloidal chitin broth was used as a production medium with pH 7 and incubated at 30°C in the incubator shaker at 150- 160 rev min⁻¹ for 7 days.

Spores were inoculated to a concentration of 105 ml⁻¹. Chitinase activity in the supernatant was determined by the procedure of Taechowisan *et al.*, (2003), Tang-um and Niamsup (2012). The amount of N-acetyl glucosamine (GlcNAc) released in the supernatant was spectrophotometrically measured by the method of Somogyi-Nelson (Green *et al.*, 1989) on the 520-nm absorbance. One unit (U) of chitinase activity was defined as the amount of enzyme required to produce 1 mol of reducing sugar per min. under the conditions of the experiment.

DNA isolation, 16S rDNA gene amplification and phylogenetic analysis: Endophytic actinomycete isolate

Total DNA of BR9 isolate from cells, processed for genomic DNA extraction. The fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA, a single discrete PCR amplicon band of 1500 bp was

observed when resolved on agarose. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1366bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

The 16S rDNA gene sequence was used to carry out BLAST with the nr data base of NCBI gen bank 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.

Results and Discussion

Screening of isolates for hydrolytic enzymes production

Out of 60 endophytic actinomycete isolates, 39 and 50 displayed protease and amylase production potential.

Similar results were obtained by Tang-um and Niamsup (2012) who found that an endophytic *Streptomyces griseoflavus* P4 isolate from sweet pea root was capable of producing amylase enzyme using plate agar assay. Several proteases were obtained from *Streptomyces* and were biochemically characterized as serine protease produced by *Streptomyces pactum*, metallo and serine proteases from *Streptomyces exfoliatus* and amino peptidase from *Streptomyces rimosus* (Rifaat *et al.*, 2007). In another study, the endophytic actinomycete isolates obtained from tomato plant roots (*Lycopersicon esculentum*). The starch degrading capability was observed in 73.91% of isolates grown at 25°C and 28°C, and for 95.65% of isolates cultured at 30°C (Van *et al.*, 2014).

Antagonistic activity of endophytic actinomycete isolates against phytopathogenic fungus

Out of sixty isolates, 30 isolates (50%) were displaying antagonistic activity against *Fusarium oxysporum*. The antagonistic activity of endophytic actinomycete isolates against phytopathogenic fungus was observed to fall in a range of $49.57 \pm 0.82\%$ to $73.36 \pm 0.22\%$. It was observed that root endophytes were better antagonistic agents as compared to leaf isolates. The minimum and maximum zone of inhibition was displayed by BR38 (49.57 ± 0.19) and BR9 ($76.33 \pm 0.22\%$) respectively (Table 1).

Out of 20 leaf isolates, minimum percent inhibition was exhibited by BL48 (51.26 ± 0.4) while isolate BL49 showed the maximum percent inhibition against *Fusarium oxysporum* (73.36 ± 0.22) when compared with control. The results obtained in this study demonstrated that most of endophytic actinomycetes have potential for inhibiting the growth of *Fusarium oxysporum*.

Passari *et al.*, (2015) recovered forty-two endophytic actinomycetes from medicinal plants were evaluated for their antagonistic potential and plant growth-promoting abilities. Twenty-two isolates which showed the inhibitory activity against at least one pathogen were subsequently tested for their plant-growth promoting activities and were compared genotypically using DNA based fingerprinting.

Examination of antagonistic mechanism

Isolate BR9 was tested for antagonistic activity against *Fusarium oxysporum*. Antibiosis was observed directly by light microscopy and scanning electron microscopy.

Scanning electron microscopy (SEM)

Scanning electron microscopy was employed to evaluate the effects of BR9 on the fungal cell walls of *Fusarium oxysporum*. The co-culture containing *F. oxysporum* and endophytic actinomycete isolate BR9 as well as *Fusarium oxysporum* culture alone as a control was selected for experiment. Results obtained showed that control appeared sectorial regular vegetative cells along with large roughly spherical spores (Fig. 3A) whereas fungal colony co-cultured with BR9 showed aberrant vegetative cell structure of the hyphae. Further, the fungal hyphae appeared like flattened ribbons having several pits at the poles (Fig. 3B) as well as presence of bulbous structures at the edges of the inhibited fungal colonies on the PDA plates was evident (Fig. 3C). Our results are in conformity with several studies carried out by other investigators. Rawlinson *et al.*, (2010) observed that the bacterial cells examined using SEM, were totally deformed and exhibited severe destruction. The surfaces of the bacterial cells were damaged and had become rough and swollen, but unlysed.

Quantitative production of extracellular chitinase

On the basis of maximum antifungal activity as well as hydrolytic enzymes production, the endophytic actinomycete isolate BR9 was selected for qualitative production of chitinase enzyme by plate agar assay. A clear zone surrounding the actinomycete colony was observed, indicating that BR9 produced chitinase. Maximum chitinase activity was observed on 4th day 0.115 U/ml at 0.6% colloidal chitin concentration.

With 1% colloidal chitin substrate concentration, the maximum activity of BR9 was observed 0.065 U/ml on 5th day (Fig. 2) as compared to standard (Fig. 1). Similar

observations were reported by Young and Bell (1985) and Neugebour *et al.*, (1991) during production of chitinase from *S.*

marcescens and *S. lividans*, where by enzyme production was observed at exponential stage i.e. 84 h.

Table.1 Antagonistic activities of actinomycete isolates against *Fusarium oxysporum*

Isolates	Percentage(%) inhibition <i>Fusarium oxysporum</i>
<i>S.viridis</i> BR6	65.04 ±0.04
<i>S. albosporus</i> BR9	76.33±0.32
<i>S.cinereus</i> BR27	51.60 ±0.41
<i>S.cinereus</i> BR28	60.09±0.03
<i>Streptomyces albosporus</i> BR29	52.20±0.31
<i>S. albosporus</i> B42L	61.17±0.22
<i>S. albosporus</i> BR44	62.18 ±0.42
<i>S. albosporus</i> BR46	61.02±0.22
<i>S. albosporus</i> BL48	51.26±0.39
<i>S. albosporus</i> BR50	64.45 ±0.36
<i>S. albosporus</i> BR51	64.70 ±0.22
<i>S. albosporus</i> BR33	56.42±0.05
<i>S. albosporus</i> BR35	52.62±0.06
<i>S. albosporus</i> BR36	55.50±0.32
<i>S. griseorubroviolaceous</i> BR39	52.62±0.15
<i>S.viridis</i> BR42	70.83±0.62
<i>S.viridis</i> BL43	49.57±0.82
<i>S. albosporus</i> BR45	54.46±0.04
<i>S.aureus</i> BL45	58.40±0.17
<i>S.aureus</i> BL47	59.66 ±0.21
<i>Micromonospora</i> BR20	68.48±0.37
<i>Micromonospora</i> BR38	49.57±0.19
<i>Micromonospora</i> BR59	62.22 ±0.24
<i>Micromonospora</i> BR60	58.82 ±0.06
<i>Nocardia</i> BL49	73.36±0.22
<i>Nocardia</i> BR57	65.96±0.46
<i>Nocardia</i> BR58	72.24 ±0.39
<i>PseudoNocardia</i> BL3	58.82±0.26
<i>PseudoNocardia</i> BR54	60.71 ±0.44
<i>Saccharopolyspora</i> BR53	57.98±0.38

An Average ± standard error from triplicates samples

Fig.1 Standard curve of N-acetyl glucosamine

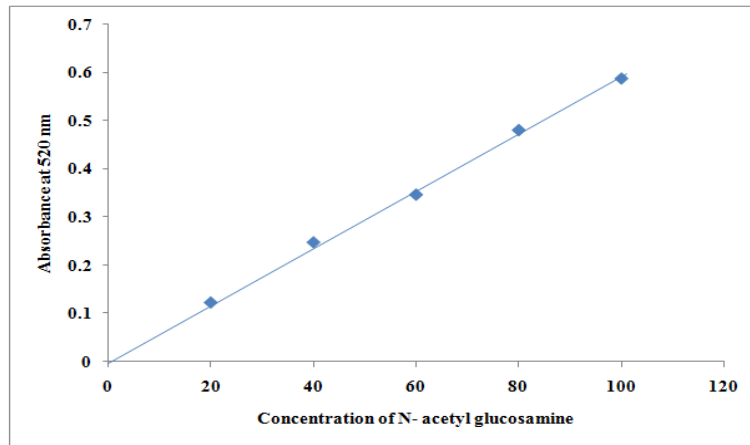


Fig.2 Quantitative production of chitinase by BR9 isolate

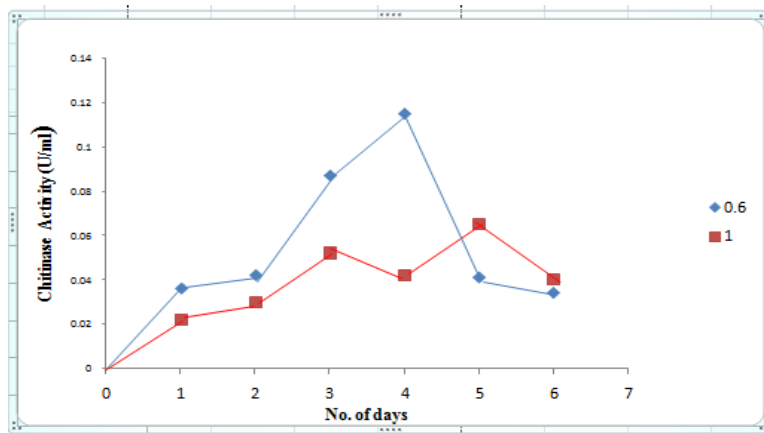


Fig.3 Phylogenetic tree of the isolate BR9 with the selected best Homologous known bacterial strains

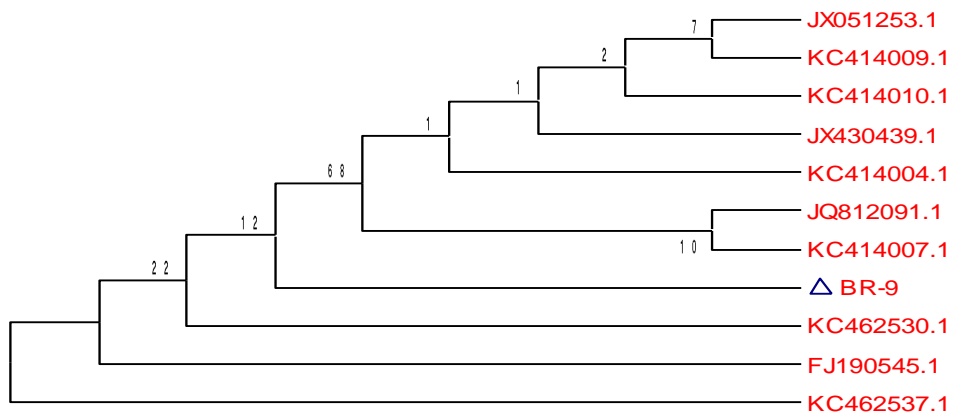


Fig.4 Scanning electron microscopic analysis of *Fusarium oxysporum* grown alone showed regular, radial growth (A) and co-cultured with *Streptomyces albosporus* BR9 hyphae (arrow) showing thickened (B) and bulbous structures (arrow) at the edges of the inhibited fungal colonies on the PDA plates (C)

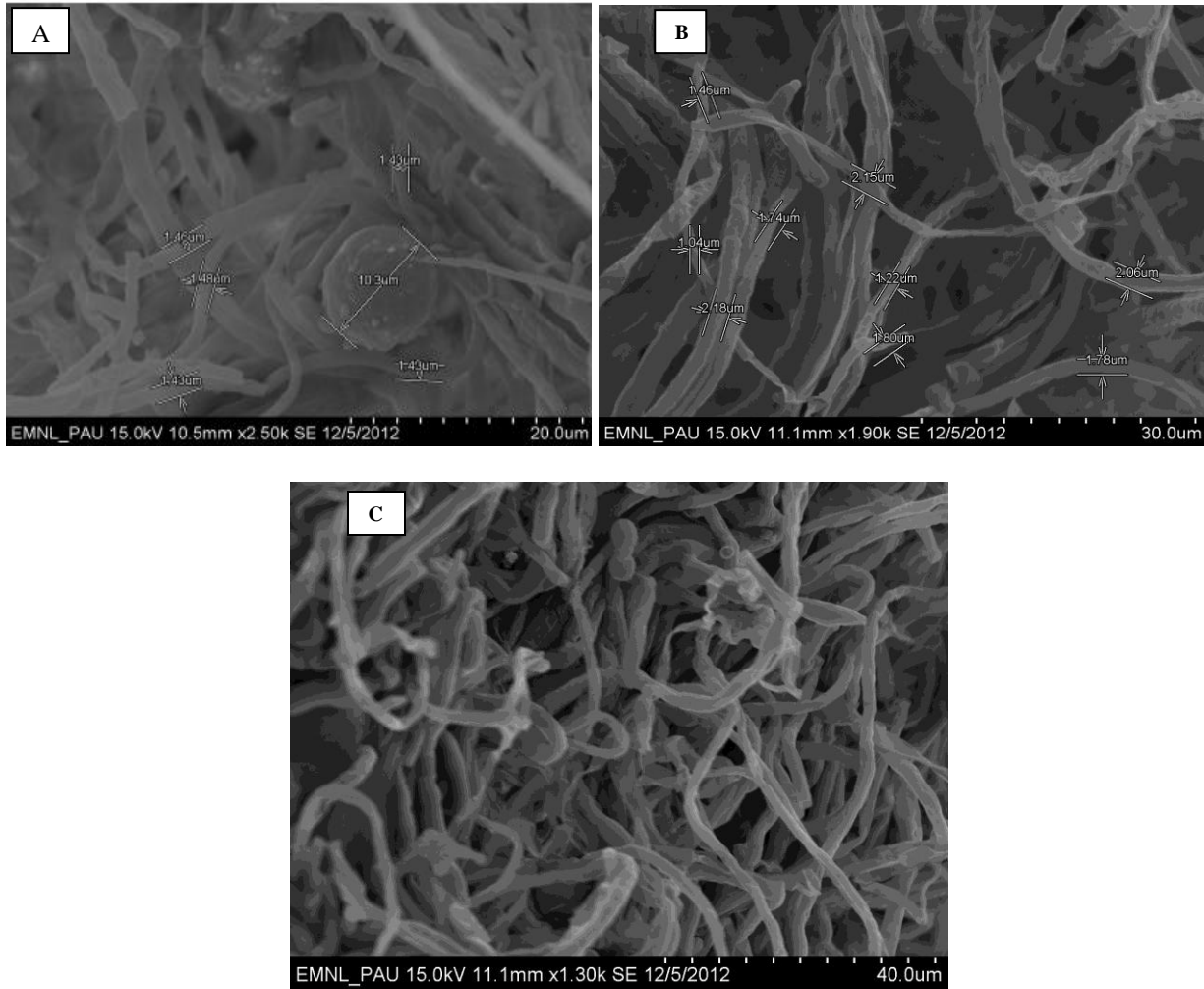


Plate.1 Hydrolysis of starch (BR9)

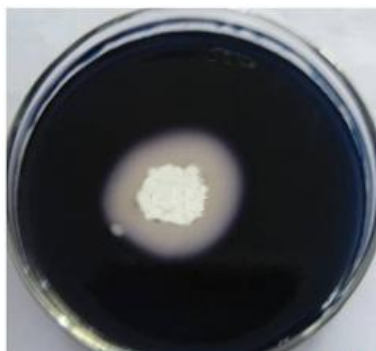


Plate.2 Hydrolysis of Casein (BR9)



Plate.3 Antifungal activity of endophytic actinomycetes (A) *Fusarium oxysporum* (B) *Streptomyces albosporus* (BR9) (C) *Nocardia* sp. (BL49)



Nineteen isolates were positive for chitinase production and formed clear halo zone around the colonies. Chitin degrading activity was found to be high in *Leifsonia xyli* 24 and *Microbacterium* sp. 21 which exhibited a colloidal chitin degradation zone of 15 and 17mm, respectively. All the 19 positive isolates were subjected to the amplification of the chitinase gene and an amplified fragment that was approximately 400 bp was obtained from these isolates (Passari *et al.*, 2015).

Phylogenetic analysis of endophyte rRNA gene sequences

16S rDNA gene sequence analysis

To further classify BR9 isolate, its 16S rRNA genes was PCR amplified and the 1500bp-long PCR fragment was sequenced. It was confirmed that isolate BR9 belongs to the *Streptomyces* species. The BR9 isolate was 99%-100% similar to the genus *Streptomyces* sp. D5 (Gen Bank Accession Number: KC414007.1) based on nucleotide homology and phylogenetic analysis. The phylogenetic tree was constructed with bootstrap values (Figure 3). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. A neighbor-joining tree based on the 16S rRNA gene sequences showed that BR9 occupied a phylogenetic position alongside *Streptomyces* sp. D5 (KC414007.1). Combining the

morphological, physiological and biochemical characteristics, BR9 was determined to belong to the species of *Streptomyces* sp. D5

In conclusion, the present investigation revealed endophytic actinomycetes could be isolated from not only inside banana roots but also inside leaves of banana plants (*Musa acuminata*). The presence of actinomycetes inside the plant confer many advantages to host plants such as the production of certain enzymes and displayed antagonistic activity against *Fusarium oxysporum* that further confirmed their role as biocontrol agents.

The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds and/or extracellular hydrolytic enzymes. The study revealed that genus *Streptomyces* has potential to reduce or eliminate *Fusarium* wilt of banana. Further studies are needed to determine the purity and nature of *Streptomyces* sp. metabolites and their mechanism of action. This biological agent could be an alternative to the synthetic fungicides used for management of *Fusarium* wilt.

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