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Antifungal activity of a new *Streptomyces griseoruber* isolated from banana Rhizosphere soil

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Abstract
Fusarium wilt is a disease reducing significantly on banana productivity. The disease is caused by *Fusarium oxysporum* f.sp. *cubense* that attacks most of banana variety in the world. Biocontrol of the disesase has been being developed within the last decades such as the use of Actinomycetes. This research is aimed to isolate and identify antagonistic actinomycetes against *Fusarium oxysporum* f.sp. *cubense*. Actinomycetes were isolated from rhizospheric soils of banana plants with and without *Fusarium* wilt symptom. Antagonistic activity was carried out by dual culture method for selecting among isolates. Actinomycetes isolates showing higher antagonistic activity were subjected for morphological and molecular identification. Molecular identification was performed by sequencing of 16S rDNA gene. The research also found three isolates having higher antagonistic activity rather than the others. The three isolates, AcB60, AcB21and AcB32 was identified as *Streptomyces griseoruber*, *Streptomyces chrysomallus* and *Streptomyces alanosinicus* respectively. *Fusarium* wilt of banana, caused by *Fusarium oxyspoum* f. sp. *cubense* (Foc), is one of the most important and destructive diseases of banana, and is known to be a major biotic limiting factor for the development of the present banana industry. Biocontrol on the destructive disease, the use of antagonist as biocontrol agents (BCAs) against *Foc*, constitutes an effective option for the management of the disease.

Keywords: *Fusarium* wilt of banana, *Fusarium oxysporum* f. sp. *cubense, Streptomyces*

1. Introduction
Ecologically, actinobacteria and, particularly, the *Streptomyces* spp. are generally saprophytic, soil-dwelling microorganisms that spend the majority of their life cycle as spores. It has also been demonstrated that actinomycetes are important microorganisms in the rhizosphere and their antagonism to phytopathogenic fungi has been demonstrated (Valois *et al*., 1996) [14]. Actinomycetes are known as producers of antibiotics and other biologically active substances with high commercial value such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors (Tanaka and Omura, 1993) [12]. Approximately two-thirds of naturally occurring antibiotics, including some of agricultural importance, have also been isolated from these soil microorganisms. Evidences indicated that actinomycetes are important in the rhizosphere because they can influence plant growth and protect plant roots against invasion by root pathogenic fungi (Crawford *et al*., 1993; Tokala *et al*., 2002) [13].
Banana (*Musa* spp.) is the fourth most important global food commodity after rice, wheat and maize in terms of gross value production. Banana is one of the main fruits cultivated in subtropical and tropical regions. Panama disease, which is also known as *fusarium* wilt, is regarded as one of the most destructive diseases of banana production in those regions (Moore *et al*., 2001) [7]. The disease is ranked as one of the top 6 important plant diseases in the world (Ploetz & Pegg, 1997). The pathogenic fungus (*Fusarium oxysporum* f. sp. *cubense*, *Foc*) enters through the roots and blocks the vascular system causing the plant to wilt, followed by death of the whole plant (Moore *et al*., 2001) [7]. Several existing disease management strategies such as crop rotation with rice, and injection of rhizomes with 2% carbendazim, are tedious. A cost-effective measure of control for the disease is still not available. A complementary approach for managing fusarium wilt is biological control and the search for antagonistic microorganisms has identified several antagonistic fungi and bacteria with high activity (Saravanan *et al.,* 2003; Getha *et al*., 2005) [10, 3]. But the banana plant is a giant perennial herb, so the introduction of suppressive soils and antagonistic bacteria around the rhizosphere will be time- and labor- consuming.

Panama disease, which is also known as fusarium wilt, is one of the most important diseases. Evidence indicated that nearly 100% in the incidence of the Panama disease of banana were caused by *Fusarium oxysporum* f. sp. *cubense* (Green 1981). Control of this pathogen by chemical methods has been shown to be ineffective. In order to search for effective biocontrol agents of Panama disease of banana, an effective method to isolate actinobacteria from banana rhizosphere soil and the antifungal activities of these actinomycetes *in vitro* have been studied.

Materials and Methods

Survey, isolation and identification of *Fusarium* wilt pathogen in banana

Field survey was conducted throughout the Tamil Nadu to collect different isolates of *Fusarium oxysporum* f. sp *cubense* (*Foc*) from various cultivars of banana in banana growing region Thoothukudi, Tiruchirapalli, Coimbatore, Tirunelveli, Karur, Erode and Kanniyakumari. The *Foc* isolates were identified based on morphological characters as well as PCR based methods. The isolated fungus was multiplied in sand maize medium and incorporated in potting soil for proving the Koch postulates theory. During the survey, the banana roots and rhizosphere soil collected for the isolation of native actinobacteria.

Isolation of *Foc* culture

The fungus *Foc* was isolated from wilt infected rhizomes of different varieties using half strength potato dextrose agar (PDA) medium amended with streptomycin (Ainsworth, 1971) and the single spore cultures obtained were maintained on PDA. Pathogenicity was tested for all the *Foc* isolates under pot culture conditions. It was done in the two month old rasthali variety by giving a corm injection of spore suspension@ 3 ml/plant (10^6cfu/ml) plus sand maize inoculum @ 10 % w/w/kg soil.

Identification of vascular wilt pathogen (*Fusarium* spp.)

The pure cultures on PDA were used for the observation of phenotypic characters such as colony features, growth rate and pigmentation. The conidial characters were studied by microscopic observation following slide culture of the fungus (Riddell, 1950). Publications of Leslie and Summerell (2006) was used for the identification of *Fusarium* isolates on a species level based on morphological features.

PCR amplification of ITS (Internal Transcribed Spacer) region of *Fusarium oxysporum* f. sp *cubense* isolates

Genomic DNA of different isolates of *Fusarium oxysporum* f. sp *cubense* were subjected to PCR amplification of complete ITS/5.8s rDNA region for molecular detection (Dutta and Lal, 2013 and Babu *et al*., 2007). ITS1 F (5'-GTCCTAACAAGGTTTCCGTA-3) and ITS4 R (5'-TTCTCCGCTTATTGATATGC-3) primers were used for the amplification of conserved region of 5.8s/18s rDNA with the expected size of 650 base pair. PCR amplification was performed using a Mastercycler. The PCR reaction volume 20 µl, contained 2.0 U of Taq polymerase (Sigma-Aldrich), 2 µl of 10X buffer, 1.5 µl of 2.5 mM $MgCl_2$, 1 µl of 2.5 mM dNTP, 2 µl of 10 µM primer, 4 µl of genomic DNA and sterile distilled water. The PCR was performed with an initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 46°C and 1 min extension at 72°C, with final extension of 10 min at 72°C.

Pathogenicity confirmation test

Foc culture multiplied on maize meal was thoroughly hand mixed with sterilized field soil separately at 1:20 w/v ratio. This mixture was filled in big earthen pots (1.5 × 1 feet), previously surface sterilized by two per cent formalin solution. The sterilized pots filled with the mixture were incubated for ten days under glass house conditions. On the 11th day, banana rhizome drenched in *Foc* cultures overnight were planted in pots. Pots were watered regularly so as to maintain 50% holding capacity of the soil. After 45 days of inoculation (DAI) root bits was placed on PDA and incubated, reisolated to prove Koch's postulates and presence of *Fusarium* was confirmed. Isolation and characterization of actinobacteria and testing their antimicrobial activity against *Foc* isolate under *in-vitro* conditions.

Isolation of actinobacteria from banana rhizosphere soil

The soil samples surrounding the banana rhizosphere region were taken for this study collected from different locations in Tamil Nadu. The actinobacteria were isolated by pour plate technique following the serial dilution (10^{-3} and 10^{-4}) of soil samples and further purified in Ken knights agar medium. Actinomycetes were isolated based on the colony morphology, colour, size, texture and powdery growth exhibited by the isolated organism after 7 days of incubation. Selected colonies (rough, chalky, dry) of actinomycetes were further isolated in pure form on the solidified Ken Knight Agar medium by streak plate method. Totally one twenty eight isolates of actinomycetes were isolated and named as AcB1-AcB128.

Identification of the actinobacteria isolates

The isolates were initially characterized by preliminary microscopic examination based on Gram staining. Observations on morphological and colony cultural characters of the isolates were used for further grouping. For biochemical characterization the actinomycetes culture will be subjected to following tests such as Starch hydrolysis, Gelatin hydrolysis, Casein hydrolysis, Indole production test, Hydrogen sulfide production test, Methyl red Voges proskauer test and Urease test. The elite actinobacteria isolates were further subjected to 16S rDNA sequencing studies for its identification.

In vitro antagonism of Actinobacteria isolates against *Fusarium oxysporum* f. sp. *cubense* –in Dual Culture Technique (Dennis and Webster, 1971)

Banana rhizospheric actinobacteria isolates were tested for their antagonistic activity against mycelial growth of *Fusarium oxysporum* f. sp *cubense* by following the dual culture technique (Dennis and Webster, 1971). Mycelial disc (5mm diameter) of seven-day old culture of the pathogens were placed at one side of the Petri plate containing PDA medium

10 mm away from the periphery. Actinobacteria cultures were streaked onto the medium exactly opposite to the mycelial disc 10 mm away from the periphery. The control plates were inoculated with the pathogen alone. The plates were incubated at room temperature (28 ± 2 °C) for seven days. The radial growth of the pathogen was measured both in control and treatment plates. Efficacy of the Actinobacteria isolates against the pathogens were assessed by calculating per cent inhibition (PI) over control.

$$PI = [(C - T)/C] \times 100$$

Where, C is the growth of test pathogen (mm) in the absence of the antagonist strain; T is the growth of test pathogen (mm) in the presence of the antagonist strain (Vincent, 1927).

Sequencing analysis (Sanger *et al.,* 1977)

The actinomycetes isolates were subjected to sequencing using enzymatic method proposed by Sanger *et al.,* 1977. PCR products were sequenced through single pass analysis from forward and reverse direction. Sequence data was compared with already available sequence data by BLAST analysis in NCBI sequence data bank. Relevant sequences were collected and data was plotted with PHYLIP software. Selected isolates were identified at genus and species level from the phylogenetic tree.

Results and Discussion

Actinomycetes are useful biological tools for the production of antimicrobials against fungi and bacteria. In general, *Streptomyces* are saprophytic and are commonly associated with soils, were they contribute significantly to the turnover of complex biopolymers and antibiotics. These antibiotics are considered as valuable ingredients for controlling plant diseases caused by bacteria and fungi. The present study was aimed at isolation and screening of actinobacterial for their antimicrobial activity against *Fusarium* wilt of banana. Among the isolated strains of actinomycetes only one strain exhibited very good antimicrobial activity against *Fusarium oxysporum* f. sp *cubense*. Further studies were conducted to evaluate the broad spectrum activity against *Fusarium* wilt. The results obtained in the study are described below.

Isolation of *Fusarium oxysporum* f. sp *cubense* and phenotypic characters of *Foc* isolates

The pathogen associated with wilt of banana was isolated from infected rhizome samples collected from different banana growing regions of Tamil Nadu *viz*., Thoothukudi, Tiruchirapalli, Coimbatore, Tirunelveli, Karur, Erode and Kanniyakumari. A total of 12 isolates were collected from the above regions (Fig. 1). Colonies in culture showed variations in morphological characters. The colour of the all isolates varied from pink to violet except two which were pure white in colour. Microscopic observations revealed that the microconidia of all the isolates were oval to reniform in shape, whereas the macroconidia were falcate to straight with the pointed apical cell (Fig. 2 and Table 1).

Fusarium wilt of banana (*Musa* sp.) caused by *fusarium oxysforum* f. sp. *cubense* was first reported in Hawaii. It is also known as panama disease, due to the prevalence of the diseases in panama (Central America) since 1910. It is considered to be one of the six most devastating plant diseases in recorded history (Simmonds, 1966). This disease kills susceptible banana plants and there is no cure. The fungus infects banana plants through the roots and invades the plants water conducting tissue. Once Foc is introduced into banana gardens, it remains in the soil making it impossible to grow susceptible cultivars vessels, which in turn leads to yellowing of leaves (progressing from older to younger leaves) and wilting of plants. Distinctive symptoms appear inside the pseudostem. Brown, red or yellow lines are seen in vertical section, which appear as rings in cross section. Splits appear in the pseudostem and infected plants usually do not produce fruits. Management of fusarium wilt of banana is very difficult to achieve and there is no effective individual technology. So, an integrated approach comprising of biocontrol agents, botanical formulation and synthetic fungicides is needed for managing this diseases.

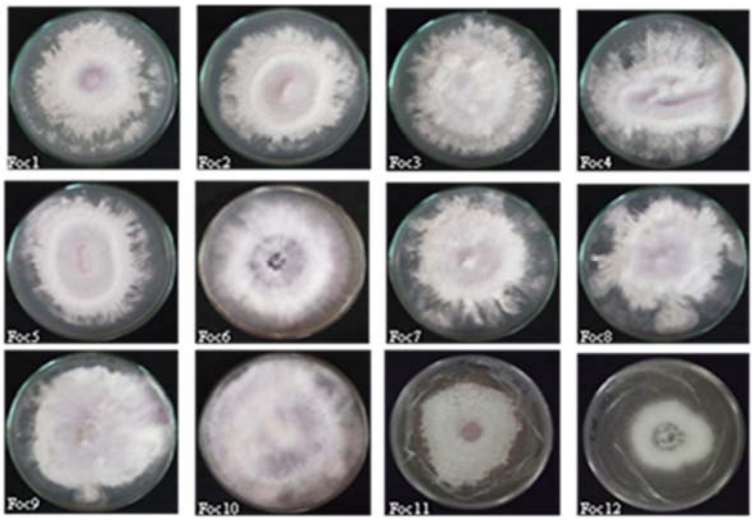
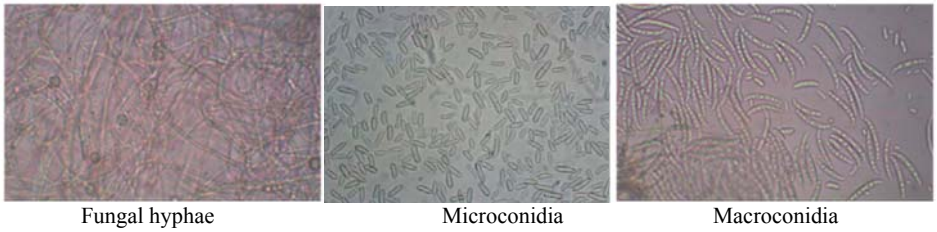


Fig 1: *Foc* isolates from wilt infected banana plants



Fungal hyphae Microconidia Macroconidia

Fig 2: Microscopic observations of *Foc* isolates

Table 1: Morphological features of *Fusarium oxysporum* f. sp. *cubense* isolates from banana

Sl. No	Place of collection	*Foc* isolates	Colony diameter (mm) in 7 days	Colony morphology	Mycelial colour
1	Coimbatore	Foc1	90	Fluffy growth	Milky White
2	Erode	Foc 2	70	Adherent smooth	Creamy white
3	Kanniyakumari	Foc 3	64	Adherent smooth	Creamy white
4	Kanniyakumari	Foc 4	78	Fluffy growth	Milky white
5	Trichy	Foc5	90	Fluffy growth	Light pink
6	Trichy	Foc 6	80	Fluffy growth	Creamy white
7	Tirunelveli	Foc 7	85	Fluffy growth	Creamy white
8	Thoothukudi	Foc 8	62	Adherent smooth	White
9	Karur	Foc 9	73	Adherent smooth	Cream
10	Mettupalayam	Foc 10	85	Adherent smooth	Salmon pink
11	Sirumugai	Foc 11	70	Fluffy growth	Creamy white
12	Authour	Foc 12	62	Adherent smooth	White

Molecular characterization of wilt pathogen of banana

The 12 *Fusarium* isolates were subjected to PCR using the universal fungal primers ITS 1 and ITS 4 for the amplification of ITS region and the intervening 5.8s coding rDNA. All the isolates yielded approximately 650 bp amplification of rDNA gene cluster indicating the isolates belongs to wilt causing *Fusarium oxysporium* f sp. *cubense* (Fig. 3).

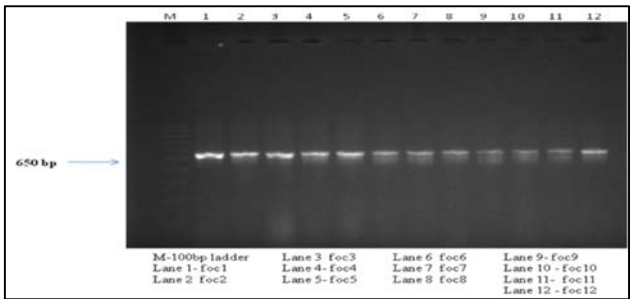


Fig 3: Amplification of ITS region in *Foc* isolates

Pathogenicity test

Pathogenicity test was carried out for all the isolates. This test resulted in the typical wilt symptoms such as yellowing from older to younger leaves and buckling of petioles (Fig. 4).

Fig 4: Pathogenicity test of *Foc* isolates in potted banana plants

Isolation of actinobacteria from banana rhizospheric soil

Soil is the commonest habitat for Streptomycetes, it is found in sub-surface layer of the soil, where organic matter is higher (Kutzner, 1986; Williams *et al*., 1989) [4, 5]. Hence the soil samples were collected from sub-surface of selected area in the present study. The diversity of terrestrial actinomycetes are of extraordinary significance in several areas of science and agriculture, particularly in antibiotic production (Magarvey *et al.,* 2004) [5]**.** A total 128 isolates of actinobacteria were isolated from rhizosphere soils collected from different parts of Tamil Nadu. Actinobacteria were isolated at 10^{-4} dilution by pour plate method. Isolates were selected based on the colony morphology, colour, size, texture and powdery growth exhibited by the isolated organism after 7 days of incubation. Totally hundred and twenty eight isolates of actinobacteria were isolated and named as (Actinobacteria) AcB1 to AcB128 (Fig. 5). In searches for bioactive antibiotics, *Streptomyces* isolates have been isolated from various types of soils, including rice, lake mud and water, deciduous forest, tropical forest, wasteland, and cave soils (Bhattacharya *et al.,* 2007) [1]. In addition to antibacterial components *Streptomyces* also produce secondary metabolites with biological activities which account for 80 percent of the total production by actinomycetes (Cragg and Newman, 2005) [2]. Actinobacteria with chitinolytic activity was isolated from Jordan soil by Tahtamouni *et al*. (2006) [11].

Fig 5: Actinobacteria isolates from banana rhizospheric soil

Characterization of the actinobacteria isolates

The actinobacteria isolates were characterized primarily by morphological observations. Further the elite isolates exhibiting antifungal activity subjected to biochemical and molecular characterization. The antifungal activity against banana wilt pathogen was evaluated for all the actinobacteria isolates under *in vitro* dual culture plate studies. Morphological and cultural characters of the actinobacteria were determined. The taxonomic position of the antibiotic producing unknown actinomycetes was studied using moist chamber technique. Based on the mycelia and cellular morphology observed under phase contrast microscope (100X). Microscopic observation of cultures grown under moist chamber conditions revealed the characteristics branched mycelium (Fig. 6).

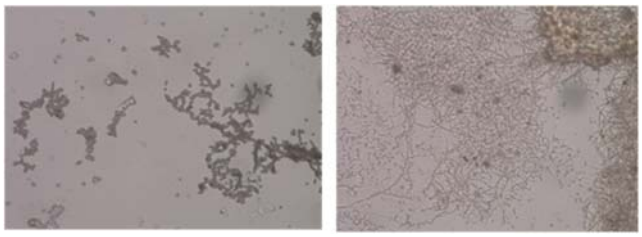


Fig 6: Microscopic observation of actinobacteria isolates

Screening of inhibitory activity of actinobacteria isolates against *Foc*

The antifungal activity of actinobacteria isolates against *Foc* was evaluated by dual culture plate technique. Both the cultures were allowed to grow in a single petriplate and the growth of the fungus was measured as observation. The growth of the fungal mycelium compared with the control fungus plate and the percent inhibition by the actinobacteria isolates expressed in percentages (Table 2 and Fig. 7).

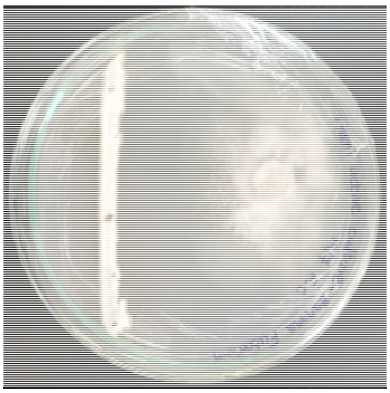


Fig 7: Antifungal activity of actinobacteria isolates against wilt causing pathogen

Cultural and Biochemical characteristics of actinobacteria cultures on different media

The growth of actinomycetes isolates AcB1- AcB5 on different media was studied. It was found that Ken Knight's agar, Starch casein agar, AF/MS medium and Crawford's agar medium supported the growth of AcB1- AcB5 isolate. Kuster's agar medium showed profuse growth but Nutrient agar and Yeast extract agar showed very low growth compared to other media. Growth characteristics of culture AcB1- AcB5 *viz.,* growth, colour of mycelium, sporulation on these media were recorded. The screened actinobacteria isolates AcB1- AcB5 produced amylase and protease enzyme that were confirmed by starch and casein hydrolysis test. Negative results were observed in Indole, Vogues Proskauer and Methyl red test of AcB1- AcB5 isolates. The effective hydrolysis of urea and nitrate reduction was obtained for AcB1- AcB5 isolate. Phenotypic characteristics of the strain AcB1- AcB5 is shown in Table 3.

Based on the cultural, physiological and biochemical characteristics of the isolated strains Moncheva *et al*. (2002) [6] characterized soil actinomycetes from Antartica and reported that most of the isolates showed no inhibitory effect against *E.coli.* Oskay *et al.,* (2004) [9] sampled a large number of isolates from farming soil of Turkey and confirmed the isolated strain as streptomycetes as per Bergey's manual of determinative Bacteriology. Narayana *et al.* (2004) [8] isolated eight different strains of *streptomycetes* sp. from virgin soil and characterised them with different biochemical tests.

Table 2: Biochemical and Cultural characterization of actinobacterial isolates from banana rhizosphere

Test	AcB1	AcB2	AcB3	AcB4	AcB5
Cultural characterization					
Aerobic growth	+	+	+	+	+
Colour of aerial mycelium	White	Dark grey	Whitish pink	Pinkish white	Yellow
Colour of substrate mycelium	Cream	Grey	Creamy white	Greyish pink	Creamy white
Melanin pigment	+	+	-	+	-
Soluble pigment	-	-	-	-	-
Texture of colony	P	P	P	P	P
Earthy odour	+	+	+	+	+
Siderapore production	+	+	+	+	+
Biochemical characterization					
Starch hydrolysis	+	-	+	-	+
Gelatin hydrolysis	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+
Indole production test	+	+	+	+	+
Hydrogen sulfide production test	+	+	+	+	+
Methyl red	-	-	-	-	-
Voges proskauer test	+	+	+	+	+
Urease test	+	+	+	+	+
Gram staining	+	+	+	+	+

(+) Positive (-) Negative (P) Powdery

Identification and phylogenetic analysis of antagonistic microorganisms by 16s rDNA studies

Based on the 16S rDNA sequencing studies, the antifungal actinomycete isolates were identified as *Streptomyces griseoruber*. The phylogenetic tree obtained by applying the neighbour-joining method is shown in Fig. 8.

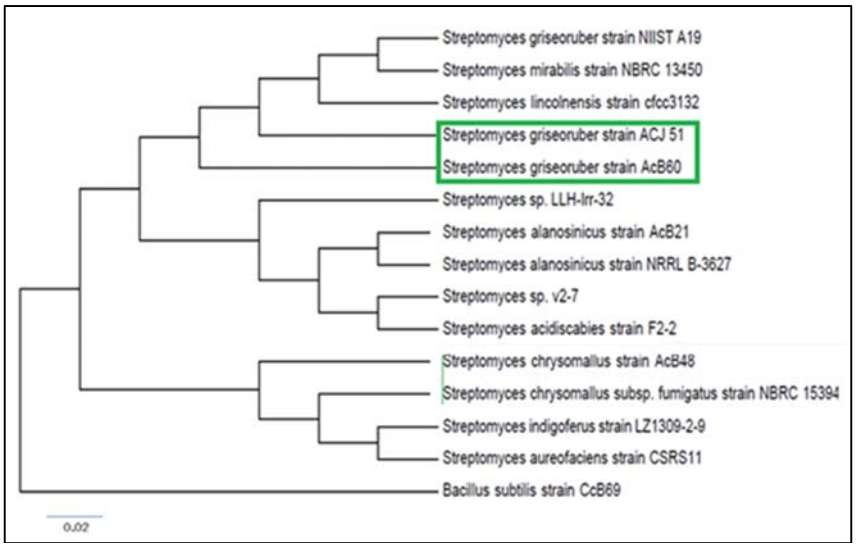


Fig 8: Phylogenetic tree of elite actinobacteria isolates

Antimicrobial activity of *Streptomyces* have been documented by several workers. A commercial product containing *Streptomyces griseoviridis* is applied through the irrigation system to control important plant pathogens, such as *F. oxysporium*, *Botrytis cinera* and *Alternaria brassicicola* (Tuomi *et al.*, 2001)

Sivakumar *et al.,* (2005) reported that *S. gibosonii* strains isolated from mangroove environment have less inhibitory activity against *Staphylococcus aureus.* His findings showed similarity to this present work as *Streptomyces polychromogenes* does not showed inhibitory activity against *Staphylococcus aureus.* Antimicrobial and cytotoxic activity of *Streptomyces* sp. active against *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris, Salmonella typhi* and human lung carcinoma cell was shown by Kharat *et al.,* (2009). Several actinomycetes produced antibiotics which are being routinely used in an agricultural setting such as streptomycin and terramycin for fire blight control (Kuepper and Preston, 2004). Streptomyces have demonstrated both *in vitro* and *in vivo* activity against plant pathogens such as *Fusarium, pythium, Phytophthora megasperma, Colletotrichum* (Lim, 2005)

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