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In Vitro Study on Actinomycetes Extracts Against the Stem Bleeding Disease of Coconut

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Abstract

Several compounds produced by Actinomycetes group of bacteria have proven potential in inhibiting plant pathogenic fungi. In the present study, two microbial ethyl acetate extracts from Streptomyces were tested for their antifungal properties against *Thielaviopsis paradoxa*, the causal organism of stem bleeding disease in coconut. The cultural filtrates of 2 strains of Streptomyces (AFL-1 and AFL-2) were extracted with Ethyl acetrate. The resulting ethyl acetate extract of AFL-1 showed complete *in-vitro* inhibition of fungal growth at relatively low concentrations (0.25%) while Ethyl ecetate extract of AFL-2 needed higher concentrations (over 0.5%) to eliminate pathogen growth. This finding indicate their potential and further exploitation as biopesticides for the control of deadly pathogen
T. paradoxa.

Keywords: antifungal effects, coconut, phytopathogen, streptomyces extracts, stem bleeding, *Thielaviopsis paradoxa*

Introduction

Thielaviopsis paradoxa (de Seyness) Von Hohnel, is a soilborne plant pathogen that causes diseases in diverse economically important crop plants. Stem bleeding disease of coconut caused by *Thielaviopsis paradoxa* mainly infects wounds and openings in the coconut stem. However, the fungus can infect multiple parts of a palm and therefore cause several infection points. As decay progresses, a dark pigmented liquid bleeds down the trunk from the point of invasion covering the stem surface with a superficial black layer of stem fluids. The sap flow may extend several feet down the trunk, blackening the trunk as it dries. In advanced cases, the interior of affected trunks becomes hollow due to the decay of internal tissues. Severely affected or untreated palms may rot out completely inside and die. Basal stem invasion may occur in wet areas, producing a black collar of diseased-stem tissue at the plant-soil interface. Roots may also show blackening and decay.

The disease is prevalent in all soil types in tropical coconut growing regions. In Karnataka the disease was reported as a serious concern, while infestation turned out rampant in Appangala (Coorg district, Karnataka State) and Vittal (Karnataka State). Copius gushing of brown liquid was noticed on palms at Appangala, the coolest among the places. The gummy exudates contained conidia of *T. paradoxa*. The disease was equally distributed in all tall, dwarf and hybrid varieties (Rajesh Muthu, *et al*., 2013). The disease is most common where wounds occur to palm stems in highly irrigated or moist landscapes. The wounds exposed to infested soil are prone to infection. The pathogen can spread from soil to open wounds by splashing rain or irrigation water. Growth cracks on the coconut trunk, severe downpours, water stagnation, imbalances in nutrition, excess salinity, and plant stress can act as predisposing and aggravating factors.

Control of *T. paradoxa* is achieved by cultural practices and chemical treatment with triadimefon. However, it is desirable to integrate biological control agents into disease management plans to minimize the environmental impact of agrochemicals (Sánchez, *et al*., 2007).

In recent years, compounds from different microbial groups have been described as having antifungal activity, among others are the Actinomycetes group and their constituent (Kekuda,

et al., 2015) A wide range of species have been explored for their secondary metabolites in the past few decades. Secondary metabolites are complex volatile compounds synthesized naturally during the process of secondary metabolism (Hayakawa, *et al*., 2004).

The metabolites produced are generally a high molecular weight group of compounds, which directly acts as antifungal agents or have structural biopolymers which have localized action especially at the sites of infection which restricts fungal development in plants either through the formation of lignin, cellulose and other such kinds of structures (Gebreyohannes, *et al*., 2013). These compounds may be active by themselves or by the production of enzymes like peroxidases which are antifungal (Harman, 2000). In general, the interest in such antifungal compounds depends upon the concentration required for activity and the biological spectrum of activity (Peela & Porana, 2016). The presence of different types of aldehydes, phenolics, terpenes, and other antimicrobial compounds justifies that these compounds are effective against a diverse range of plant pathogens (Goodfellow *et al*., 2012 and Crawford *et al*., 1993). Today technology has evolved many folds and different chemical/synthetic products are available in the market to deal with fungal plant pathogens. But instances of chemical residues in soil and plant produce posing serious health and environmental hazard has again jeopardized the use of chemical methods to control infections caused by notorious plant pathogens. Understanding the need of the hour, many research has studied and indicated the potential of different naturally available compounds which can be harnessed from a wide range of microbial diversity present around us.

The objective of this study was to examine the antifungal activity solvent extracts of two strains of Actinomycetes derived through a microbial fermentation process.

Materials and Methods

Sample collection

Ten soil samples were collected from the foot of Nandi Hill region of Bangalore Rural, Karnataka, India. The samples were collected from 5-25 cm depth by sterile method from four locations in Nandi Hills area and transported aseptically to the microbiology laboratory of Agferm Innovations Private Limited, Bangalore. The pathogen *T. paradoxa* was collected from tissues of stem bleeding infected palms in Hassan district.

Isolation of pathogen

Infested stem portion where bleeding symptoms were conspicuous was chiseled out carefully. This was surface sterilized with 0.1% sodium hypochlorite followed by 3 washes in sterilized distilled water (SDW). The stem bits were plated on Potato Dextrose Agar (PDA) media plates under aseptic condition. The plates were incubated for three days at 29 + 1°C and observed for any growth.

The incubated plated showed black pigmentation with profuse mycelial growth and high degree of sporulation. Microscopic observation confirmed to the characteritics of *T. paradoxa*, the conidiophores were straight, hyaline to pale brown, up to 200 µm long, with a terminal conidia-bearing cells in chains. The conidia were cylindrical with square ends, hyaline to pale brown, 7-12 × 3-5 µm. The chlamydospores were borne terminally in chains from short hyphal branches with pale brown to brownish-black, smooth, oval and 10-20 × 5-10 µm.

Isolation of Actinomycetes

Calcium carbonate enrichment methods were used to isolate Actinomycetes. The soil samples were mixed with $CaCO_3$ at the ratio of 10:1 and were incubated under moisture-rich conditions for seven days at room temperature (Hayakawa *et al*., 2004). The soil was further held in a water bath at 50°C to destroy other vegetative microorganisms. Isolation and enumeration of Actinomycetes were performed by the soil dilution plate technique using starch case, in agar medium (g/l: Starch 10 gr, Casein 0.3 gr, KNO_3 2 gr, NaCl 2 gr, K_2HPO_4 2 gr, $MgSO_4.7H_2O$ 0.05 gr, $CaCo_3$ 0.02 gr, $FeSO_4.7H_2O$ 0.01 gr, and Agar 18 gr). To minimize the fungal and bacterial growth, actdione 20 mg/L, and nalidixic acid 100 mg/L were added. The plates were incubated at 30°C for 10 days. After the incubation period, plates were examined for the presence of Actinomycetes colonies. The colonies likely to be the fungus

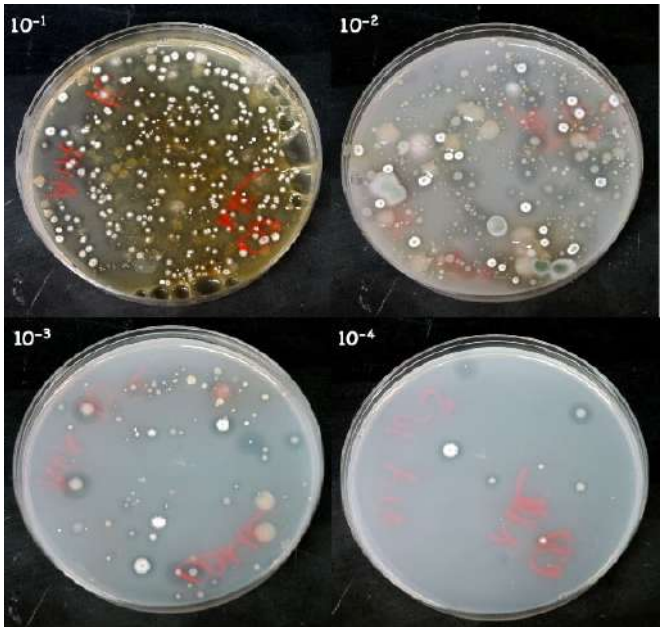

Figure 1. Plate 1, showing Dilution plates up to 10-4 colonies of Actinomycetes on SCA media

were picked up and purified on media and incubated at room temperature for about 7 days (Plate 1).

Characterization of the Isolates

Morphological, physiological, and biochemical characterization of Actinomycetes

Identification of Actinomycetes to genus level was conducted by first using morphological and chemical criteria according to Bergey's Manual of Determinative Bacteriology (Goodfellow *et al*., 2012 and Crawford *et al*., 1993). Cultural characteristics of the isolates were studied based on the intensity of the growth, growth pattern, colony color along with the color of aerial mycelia on Tryptone yeast agar (ISP Medium 1), yeast extract malt extract agar (ISP Medium 2), Oatmeal agar (ISP Medium 3), Inorganic salt starch agar (ISP Medium 4), Glycerol Asparginine agar (ISP Medium 5), Peptone yeast extract iron agar (ISP Medium 6), Tyrosine agar (ISP Medium 7) as described by Shirling and Gottlieb (1966) and also on Starch casein agar, Potato Dextrose agar, Kuster's agar and CzapekDox agar. Gram staining and spore surface morphology was examined by scanning electron microscopy (SEM). The arrangement of spore and sporulating structures were examined microscopically by using the coverslip culture method (Mitchell & Britt, 1981). The mycelium structure, color and arrangement of conidiophores and arthrospore on the mycelium were observed through the oil immersion (1000×) microscope (Plate 2).

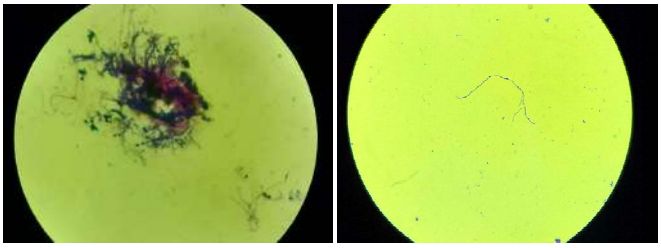

Figure 2. Image of Streptomyces species on Gram staining in Oil immersion microscopy

Biochemical characterization of Actinomycetes

The isolates were inoculated into an ISP-2 medium M1 medium. Incubation of the slide was done at 37°C for 7 days. Staining with methylene blue was done followed by observation of the slides under the microscope after staining with methylene blue stain (Plate 3). Based on physical parameters two isolates were selected and were studied further.

Diagnostic tests of Actinomycetes strains were performed based on macro and microscopic features according to Bergey's Manual of Systematic Bacteriology (Goodfellow *et al*., 2012 and Crawford *et al*., 1993). The genus of two selected Actinomycetes was ascertained using the above methods and were found to be of genus Streptomyces. These suspected pure Actinomycetes cultures (AFL-1 and AFL-2) were inoculated

Figure 3. Streptomyces growth on Actinomycetes agar on ISP-2 slants and after the incubation period, the slants were taken for antifungal screening. The stock culture was preserved in 50% glycerol.

Fermentation and extraction of secondary metabolite

Spores (107/ml) of the two isolates were used to inoculate 1,000 ml Erlenmeyer flasks containing 200 ml of Actinomycetes media each. After six days of incubation at 30°C in an orbital incubator shaker at 200 rpm the flask was opened, and Ethyl Acetate was added to the broth in the ratio of 1:1 and was again shaken for 24 hrs. This whole solution was evaporated at 50°C in a rotary vacuum evaporator to eliminate the solvent fully and concentrate the extracted metabolite broth up to 10 folds. A final volume of 20 ml of secondary metabolite concentrate was retrieved and was tested against *T. paradoxa*.

Fungal growth inhibition test/poisoned food technique

To determine the effect of different concentrations of the two microbial extracts on the growth of fungus it was diluted with acetone in a 1:1 ratio and was added into potato dextrose agar media at 0.1, 0.25, 0.5, 0.75, 1, 2.5, and 5% concentration. Treated media (20 ml) was then poured into the petri plates and allowed to solidify. Mycelial plugs (3 mm in diameter) of pure culture of *T. paradoxa* were placed in the center of each PDA plate (9 cm diameter). All the experimental transfers were performed aseptically under a laminar airflow cabinet. These plates inoculated with fungus were incubated at 28°C and 70% RH for 5-7 days. Mycelial growth was measured every day until plates were completely colonized with mycelium. Plates with only culture media and with extracts were also placed with *T. paradoxa* mycelial plugs and used as control. A solvent control was also set up with media and solvent. The experiments were done in triplicates.

Statistical analysis

All statistical analyses were carried out using Analysis of Variance (ANOVA). ANOVA was performed on all experimental data and means were compared using Duncan's multirange test. The significance level was $p<0.05$.

Table 1. Growth (mean ± SD) of *T. paradoxa* mycelium in different concentrations of results of Extract 1

Conc. of media in %	Colony Diameter (cm)			
	After 1 day	After 3 days	After 5 days	After 6 days
0.1	1 ± 0b	1.2 ± 0.1b	2.8 ± 0.11b	3.17 ± 0.15b
0.25	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
0.50	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
0.75	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
1.00	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
2.50	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
5.00	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
Control	1.7 ± 0.34c	2.88 ± 0.4c	4.2 ± 0.12d	7.03 ± 0.15d
Solvent	1.78 ± 0.2b	2.79 ± 0.12c	3.73 ± 0.25c	6.57 ± 0.15d

Table 2. Growth (mean ± SD) of *T. paradoxa* mycelium in different concentrations of Extract 2

Conc. of media in %	Colony Diameter (cm)			
	After 1 day	After 3 days	After 5 days	After 6 days
0.1	3.07 ± 0.12c	3.87 ± 0.16c	4.23 ± 0.25b	6.30 ± 0.4b
0.25	2.87 ± 0.19b	3.23 ± 0.22b	3.93 ± 0.3b	6.50 ± 0.1b
0.50	2.50 ± 0.10b	3.16 ± 0.08b	4.10 ± 0.1b	6.30 ± 0.1b
0.75	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
1.00	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
2.50	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
5.00	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
Control	2.83 ± 0.13c	3.80 ± 0.15b	4.70 ± 0.1d	7.10 ± 0.15d
Solvent	2.33 ± 0.12b	3.50 ± 0.15b	4.23 ± 0.1d	6.50 ± 0.25c

Results and Discussion

Diagnostic tests of different isolates of Actinomycetes strains were performed based on macro and microscopic features according to Bergey's Manual of Systematic Bacteriology. The genus of two selected Actinomycetes (AFL-1 and AFL-2) was ascertained and found to be of genus Streptomyces. The two strains were morphologically different in terms of pigmentation. AFL-1 showed pinkish pigmentation on agar plate with irregular and serrated edges characteristics. While the other isolate AFL-2 exhibited no such pigmentation on agar plate. The pigmentation is highly coorelated to the presence of various potent bio active agents.

Biochemical characteristics, Mycelia color phenotypes and sugars utilization were highly similar in both the strains isolated. When compared to AFL-2 strain the appearance of substrate mycelia of AFL-1 was pinkish. Indole production was noted in the AFL-2. Most of the sugars tested as carbon source were commonly used by both the strains. Differences were situated in the case of xylose which was used by the strain1 but not by AFL-2. In addition, AFL-1 were able to use mannose, glycerol galactose; sugars with no indication found for the AFL-2.

In the present study the two Streptomyces strain AFL-1 and AFL-2 active against *T. paradoxa* was evaluated. The level of inhibition of pathogen and the toxin produced were strain specific. Streptomyces strain produces several bio control agents/compounds which inhibit spore germination of variety of fungal pathogens. The cultural filtrate of AFL-2 were extracted with ethyal acetate. The resulting crude extractexhibited antifungal property against *T. paradoxa*. The results of the antifungal tests showed that AFL-1 extracted with ethyl acetate showed high inhibition rate at lower concenteration compared to AFL-2 extract. The effects of AFL-1 and AFL-2 are shown in Tables 1 and 2 respectively.

AFL-1 was found to be the most effective in inhibiting fungal growth. All the concentrations, except 0.1% were effective in controlling the growth of the fungal pathogen (Table 1).

AFL-2 was not effective when compared to AFL-1 in inhibiting fungal growth. Specifically, at lower concentrations, the inhibition was almost nil (Table 2).

The growth of pathogen was not restricted completely with AFL-2 at lower concentrations of 0.1 – 0.5%. The pathogen grew in both control and solvent treated.

In this study, 2 different extracts of Actinomycetes sp. were investigated against phytopathogenic fungus *Thielaviopsis paradoxa*. Growth inhibition studies indicated that AFL-2 was found to be the most potent against the fungus. They completely inhibited the growth of fungus in all the concentrations tested. These may be further tested in field conditions and formulated to be used as an environmentally safe alternative to chemical fungicides.

Conclusion

Secondary metabolites through microbial derivation serves as an important source for plant disease management. From the study, it can be concluded that the two streptomyces strains isolated, harbours many important volatile secondary metabolites that can inhibit spore germination of *T. paradoxa* at various degrees of infection. The level of inhibition of pathogen and toxin produced were strain-specific. AFL-1 strain could be an important sources of new compounds or analogues with possible interesting use as fungicide. The comparison between different streptomyces strains in terms of their metabolite production showed consistent differences. Thus, there seems to be constitutive metabolites responsible for the difference in efficacy on plant pathogen *T. paradoxa*, this could be further exploited for commercialization and more field evaluation.

References

Bergey's Manual of Systematic Bacteriology (Goodfellow *et al*., 2012) and Crawford *et al*. (1993).

Gebreyohannes, G., Moges, F., Sahile, S., & Raja, N. (2013). Isolation and characterization of potential antibiotic-producing Actinomycetes from water and sediments of Lake Tana, Ethiopia. *Asian Pacific Journal of Tropical Biomedicine, 3*(6), 426–435.

Harman, G. E. (2000). *Plant Disease*, April 2000 377.

Hayakawa, M., Yoshida, Y., & Iimura, Y. (2004). Selective isolation of bioactive soil Actinomycetes belonging to the Streptomyces violaceusniger phenotypic cluster. *Journal of Applied Microbiology, 96*(5), 973–981.

Kekuda, P., Onkarappa, R., & Jayanna, N. (2015). Characterization and Antibacterial Activity of a Glycoside Antibiotic from Streptomyces variabilis PO-178. *Science, Technology and Arts Research Journal, 3*(4), 116.

Mitchell, J. L., & Britt, E. M. (1981). A coverslip culture technique for preparing permanent fungus mounts. *Mycopathologia, 76*(1), 23-25.

Peela, S., & Porana, S. (2016). Isolation and Screening of novel Streptomyces from sediments of Bay of Bengal Near Srikakulam Coast. *International Journal of Current Pharmaceutical Research, 9*(1), 40.

Muthu, M. R., Subbaiya, R., Balasubramanian, M., Ponmurugan, P., & Selvam, M. (2013). Isolation and identification of Actinomycetes Isoptericola variabilis from Cauvery River soil sample. *Int. J. Curr. Microbiol. App. Sci, 2*(6), 236-245.

Sánchez, V., Rebolledo, O., Picaso, R. M., Cárdenas, E., Córdova, J., González, O., & Samuels, G. J. (2007). In vitro antagonism of Thielaviopsis paradoxa by Trichoderma longibrachiatum. *Mycopathologia, 163*(1), 49–58.