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Bioprospecting of Endophytic Actinomycetes for Antiphytofungal Activity

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Abstract: Antiphytofungal activity of selected endophytic actinobacterial strains was investigated. Totally 19 actinomycetes were isolated from neem, eucalyptus and coffee seeds and screened for their antiphytofungal activity. Strain NEK5, EE9 and CE1 showed good antiphytofungal activity. The ethyl acetate extract obtained from agar media inoculated with NEK5, EE9 and CE1 inhibited the growth of phytofungal pathogens such as *Fusarium sp, Pythium sp, Curvularia sp* and *Cercospora sp*. Good growth of these three endophytic actinomycete strains on plant product based media supported its endophytic nature. These endophytic actinomycete strains will be a candidate for the development of biocontrol agents to fight against phytofungal diseases.
Key words: endophytes, actinomycetes, antiphytofungal, biocontrol.

Introduction

Plants always playing a key role in fulfilling human needs. However, as like animals, they are also prone to various infectious agents including fungal pathogens. Phytofungal pathogens pose serious problems worldwide and cause a number of diseases such as rusts, smuts, rots, and may cause severe damage to crops. Excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of fungicide resistance among the pathogens. Because of these problems in fungal disease control, a serious search is needed to identify alternative methods for plant protection[1]. Biological control of plant diseases has received worldwide attention in recent years mainly as a response to public concern about the use of hazardous chemicals in the environment. Several microbial antagonists are widely used as biocontrol agents in agriculture. Actinomycetes are the group of Gram positive bacteria which are ubiquitously present in nature. They are the major group of bioactive organisms with the ability to synthesize numerous secondary metabolites many of which are in clinical and agriculture applications[2]. Actinomycetes like *Streptomyces fradiae* isolated from soil was used as biocontrol agents to fight against plant pathogens[3,4].

Nowadays scientists are also searching actinomycetes from alternate ecological sources. The existence of endophytes has been known for over the hundred years and they may produce a plethora of substances of potential use to modern medicine, agriculture and industry. Both pharmaceutical industry and plant breeders are interested in endophytes for production of enzymes, medicines and biocontrol agents. Actinomycetes are filamentous bacteria that live in widely diverse ecological settings including in plants[5]. Endophytic

actinomycetes have attracted interest of microbiologists, agrochemists and pharmacologists as the promising producers of novel antibiotics, growth promoters, and lead compounds to develop new medicines and agrochemicals[6].

In India, though various works have been carried out in endophytic bacteria and fungi[7], works on endophytic actinomycetes were not much reported[8,9]. With this view the present study concentrated on bioprospecting of endophytic actinomycetes from selected plants like neem, eucalyptus and also from coffee seeds for antiphytofungal activity.

Materials and Methods

Sample Collection and pretreatment

Healthy leaves of two different medicinal plants *viz., Eucalyptus melliodora* and *Azardirachta indica* (Neem) were collected from Kanchipuram district (Lat: 12° 83' N, Long: 79° 72' E) and transported aseptically to the laboratory. Coffee seeds (*Coffea arabica*) were collected from Wayanad district of Kerala (Lat: 11° 25' N, Long: 75° 77' E) in sterile plastic bags and stored at 4° C until further studies. The leaves and seeds were excised and subjected to a three step surface sterilization procedure, a 60 second wash in 99 % ethanol followed by a 6 minutes wash in 1 % sodium hypochlorite solution, a 30 second wash in 99 % ethanol and a final rinse in sterile water[10].

Isolation of endophytic actinomycetes

The neem leaves were crushed and serially diluted up to 10^5 dilutions using sterile distilled water. After sterilizing the starch casein agar (SCA), filter sterilized nystatin (100 µg/ml) and nalidixic acid (20 µg/ml) was added in to it in order to retard the growth of fungal and bacterial population, respectively[11]. About 100 µl of aliquot from 10^{-3} to 10^{-5} dilutions was taken and spreaded on SCA medium. Plating was done in triplicate and all the plates were incubated at 28°C for 1 month. Morphologically different actinomycete colonies were selected from SCA plates and purified on yeast extract malt extract (YEME) agar medium. Cultural characteristics of actinomycetes on YEME agar were visually observed. The presence or absence of substrate and aerial mycelium was detected by observing under bright field microscope with 40 X magnification. Viability of morphologically different isolates was maintained as slant culture on ISP2 medium as well as in 15% glycerol broth[12].

Screening for antiphytofungal activity

Antiphytofungal activity of endophytic actinomycete strains were assessed by cross spot method[13]. Phytofungal pathogens such as include *Fusarium spp, Pythium spp, Curvularia spp* and *Cercospora spp.* were obtained from Department of Microbiology, Sri Sankara Arts & Science College, Kanchipuram. Viability of fungal cultures was maintained on Sabouraud's dextrose agar. Endophytic actinomycete strains were inoculated as a spot at the centre of modified nutrient glucose agar and incubated at 28°C for 5 days. After incubation, the phytofungal cultures were spotted on either sides of the endophytic actinomycete growth and incubated at 28°C for 3 to 5 days.

Production of bioactive compounds by submerged and agar surface fermentation

Actinomycete strains NEK5 from neem leaves, EE9 from eucalyptus leaves and CE1 from coffee seeds, which showed good antiphytofungal activity was selected as potential strains for further studies. The actinomycete strains were inoculated into 50 ml of soybean meal inoculation medium and incubated in rotary shaker at 120 rpm for 48 hours at 28°C. Then 10 % of inoculum from was transferred to soybean meal production medium and kept in rotary shaker with 120 rpm for 120 hours at 28°C. The pH of the fermentation medium was observed and recorded[14]. After fermentation, actinomycete mycelium from the fermentation broth was separated by centrifugation at 5000 rpm for 30 minutes at 4°C. To extract the extracellular compounds, culture supernatant was extracted by liquid–liquid extraction method using equal volume of ethyl acetate at pH 4, 7 and 9[12].

The potential endophytic actinomycete strains NEK5, EE9 and CE1 was inoculated in to each five YEME agar by continuous streaking and incubated at 28°C for 120 hours. After removing the mycelial growth, the agar medium was cut into pieces. Then the agar blocks were put in to ethyl acetate and kept at 4°C for 24 hours. Then the crude compound was obtained by concentrating the solvent portion[15].

Detection of antimicrobial activity

The crude extracts obtained through submerged and agar surface fermentation were tested against phytofungal pathogens by disc diffusion method. Two fifty microgram of crude extract was impregnated on sterile filter paper disc with 5 mm diameter and placed over the SDA plates inoculated with phytofungal pathogens. The diameter of the inhibition zone was measured after 72 hours incubation at 28ºC[12].

Characterization of potential Streptomyces species

Microscopic and cultural characteristics of potential Streptomycetes were studied by adopting the methods described by Shirling and Gottileb[16]. Growth on different agricultural substrate media was also studied. pH and temperature tolerance was studied using ISP2 agar medium. The potential actinomycetes were also screened for extracellular enzymatic activities such as lipase, amylase, protease[11], asparaginase, glutaminase, and cellulase[17].

Results & Discussion

Isolation of endophytic actinomycetes

Starch casein agar plates showed powdery colonies with different aerial mass colour. From the 40 actinomycete colonies recovered from SCA plates, 19 actinomycete strains including 5 strains from neem, 6 strains from eucalyptus and 8 strains from coffee seeds were selected based on their morphological differences. All the 19 actinomycete strains were suspected as Streptomyces based on their microscopic and cultural characteristics. Neem and eucalyptus leaves were selected for the study because they are widely used in Indian traditional medicine for various therapeutic purposes as well as the source of agrochemical for many centuries[18]. Coffee seeds were selected due to its important as consumer products of larger people and also due to the reports on its antioxidant activity. According to Strobel[19], all these plants may have microorganisms that mimic the chemistry of their respective host plants and make the same bioactive natural products or derivatives that are more bioactive than those of their respective host. In this study, the endophytic actinomycetes were isolated after a surface sterilization protocol as described by Coombs and Franco[10] and was found to be effective in removing the surface adhering microorganisms, as no other organisms were seen to grow other than actinomycetes. Thus the isolates obtained after surface sterilization can be considered to be a true endophyte. Further, the isolation medium were also supplemented with two antibiotics, nalidixic acid and nystatin (antibacterial and antifungal), which also favoured the isolation of endophytic actinomycetes.

Screening for antiphytofungal activity

About 12 out of 19 actinomycete strains showed good antiphytofungal activity in cross spot method. Three strains namely NEK5 from neem leaves, EE9 from eucalyptus and CE1 from coffee seeds, which showed predominant activity against all the phytofungal pathogens tested, were selected potential strains for further studies. Sardi *et al*.,[20] reported that an endophytic *Streptomyces* strain from surface sterilized roots showed prominent activity in biological control of plant diseases by producing antifungal substances.

Production of bioactive compounds and antiphytofungal activity

After 96 hours of incubation, the production medium was changed into brown colour. The intensity of the brown colour was increased up to 120 hours of incubation. The pH of the medium was found to be 7-8. In liquid-liquid extraction, compounds from none of the strains were extracted in the solvent ethyl acetate. All the three isolates showed good mycelial growth with diffusible pigment production on YEME agar medium. Sambamurthy and Ellaiah[14] reported the use of soybean meal medium for the production of bioactive compound from actinomycete strains. In this present study also, the fermentation was carried out in soybean meal medium for 7 days. Only one strain (EE9) produced compound by submerged fermentation. As by the reports of Ezra *et al*.,[21], the antibiotic was produced in the fermentation medium only after 3-4 weeks of incubation. So a further incubation may be suggested for the extraction of compound by submerged fermentation. Due to the problems in extraction of compounds by submerged fermentation, production of the compound was carried out on solid media and the compound was extracted using ethyl acetate after 10 days of incubation. The reasons may be that some microorganisms produce antibiotics when grown on agar but not in submerged culture[22]. In disc diffusion method, the ethyl acetate extract obtained from all the three strains by agar surface fermentation inhibited all the four phytofungal pathogens (table 1).

Table 1. Antimicrobial activity of ethyl acetate extract of potential endophytic actinomycetes

	Endophytic actinomycetes		
Test pathogens	**Strain NEK5**	**Strain EE9**	**Strain CE1**
Fusarium species	8	9	10
Pythium species	12	11	9
Curvularia species	10	8	11
Cercospora species	13	12	9

Table 2. Characteristics of potential endophytic actinomycetes

Characteristics	**Strain NEK5**	**Strain EE9**	**Strain CE1**
Micromorphology			
Aerial mycelium	+	+	+
Substrate mycelium	+	+	+
Spore chain	Rectusflexibile (RF)	Rectusflexibile (RF)	Rectusflexibile (RF)
Fragmentation	-	-	-
Cultural morphology			
Colony consistency	Powdery	Powdery	Powdery
Aerial mass colour	Gray	Dirty white	Dirty white
Reverse side pigment	+	-	-
Soluble pigment	-	-	-
Growth characteristics			
ISP1 agar	Moderate	Poor	Moderate
ISP2 agar	Moderate	Poor	Moderate
ISP3 agar	Moderate	Moderate	Poor
ISP4 agar	Moderate	Moderate	Good
ISP5 agar	Poor	Poor	Poor
ISP6 agar	Moderate	Good	Moderate
ISP7 agar	Good	Good	Moderate
Chitin agar	+	+	+
Cellulose agar	-	-	+
Inulin agar	+	+	+
Pectin agar	-	-	+
Utilization of sugars			
Glucose	+	+	+
Sucrose	+	+	+
Xylose	-	+	+
Inositol	-	-	-
Mannitol	+	+	+
Fructose	-	+	+
Raffinose	-	-	-
Cellulose	+	+	+
Rhamnose	+	-	+
Arabinose	-	+	-
Temperature tolerance			
20	Poor	Moderate	Poor
30	Good	Good	Good
40	Good	Good	Good
50	-	-	-
pH tolerance			
5	Moderate	Moderate	Moderate
7	Good	Good	Good
9	Moderate	Moderate	Good

11	Moderate	Poor	Moderate
Enzymatic activities			
Protease	+	+	+
Amylase	-	+	-
Lipase	+	+	+
Asparaginase	+	+	-
Glutaminase	+	+	-

Characterization of potential strains

Characteristics of all the three actinomycete strains were given in table 2. Under microscopic observation all the three strains showed the presence of both aerial ans substrate mycelium without any fragmentation. All the three strains showed growth on media supplemented with glucose, sucrose, mannitol and cellulose. Strains EE9 and CE1 showed growth on xylose and fructose containing medium. None of the strains showed growth on inositol and raffinose containing media. Good growth was produced at pH 7 and temperature ranges between 30 and 40^0C and moderate growth at anaerobic conditions by all the three strains. The endophytic actinomycete strains showed good lipase, protease and cellulolytic activity

The potential endophytic actinomycete strains grown on different solid media showed poor to moderate growth on most of the media tested. These strains also utilized different sugars and indicated that it is possible to carryout the fermentation process with a wide range of available substrates. Reports about *Streptomyces* strains producing hydrolytic cell wall degrading enzymes such as cellulase and amylases were given by Trejo *et al*.,[23]. Till now only a few strains producing hydrolytic enzymes are of plant origin. *Streptosporangium spp.* isolated from leaves of maize was reported to produce glucoamylase[24]. In this present study, all the potential endophytic actinomycete strains showed good enzymatic activity which suggests that they may be a source for industrial enzymes.

The effect of cultural parameters on secondary metabolite production has been well documented by James and Edwards[25] and they stated that the yield of antibiotic from *Streptomyces* was greater at 45°C and synthesis was more rapid at 37°C. The endophytic actinomycete strains used in this study also showed good growth at 30 and 40°C. Hence further studies concerning the effect of temperature and pH on antibiotic production from these potential strains may pave the way to improve the productivity. The potential endophytic actinomycetes were also found to grow in anaerobic condition.

Strobel[19] reported that the minimum contribution of plant to the endophyte is by the way of providing nutrition. However, it is also possible that the plant provide to the endophytes compounds critical for the completion of its life cycle or essential for growth or self defense. By keeping this in view, the growth of endophytic actinomycetes on different plant product based media was tested and good growth was observed on media supplemented with chitin, cellulose and potato dextrose agar.

To conclude, certain endophytic Streptomyces sp isolated in this study from selected plants are the potential candidates for further investigation in the line of agricultural biocontrol agents.

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