



Original Research Article

Exploration on native actinomycetes strains and their potential against fungal plant pathogens

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ABSTRACT

Keywords

Actinomycetes,
Phytophthora infestans,
Sclerotium rolfsii,
Streptomyces plicatus.

Actinomycetes have the ability to inhibit and reduce the incidence of fungal plant pathogens due to their enzymatic activity and through antifungal secondary metabolites produced by them. A potential native strain of *Streptomyces plicatus* was isolated from the soil habitat applied with horse dung compost and characterized. The enzymatic activity studies carried out suggested the presence of chitinase, cellulase, gelatinase and lipase activity of the actinomycetes. The actinomycetes isolates varied considerably in their ability to inhibit plant pathogenic fungi. The metabolite extract from *S. plicatus* could inhibit plant pathogenic fungi *Phytophthora infestans* and *Sclerotium rolfsii* to the tune of 80–100 per cent at a concentration of 2000–5000 ppm.

Introduction

Actinomycetes have great potential in plant disease management. They are Gram +ve filamentous bacteria having high genomic (74%) G+C content. Actinomycetes are basically the degraders of organic matter and also very good producers of antibiotics and diverse medically and economically important secondary metabolites (Saugar *et al.*, 2002; Basilio *et al.*, 2003). They are also found to be a source of novel and very powerful agents which could be used in the control of a variety of pests and parasites (Wraight and Roberts, 1987). Approximately 23,000 bioactive microbial secondary metabolites have been reported of which over

10,000 are produced by actinomycetes, which is almost 45% of all bioactive microbial metabolites. The *Streptomyces* spp. is conspicuous among actinomycetes in terms of research studies and this alone is known to produce nearly 7,600 compounds (Berdy, 2005). They are also the important producers of enzymes like chitinase, cellulases, peptidase, protease, xylanase, ligninase, amylases, sugar isomerase, pectinase, hemicellulase and keratinase which have varied roles. Actinomycetes also produce secondary metabolites like Siderophores, hydrocyanic acid (HCN), Indole acetic acid (IAA) (Gopalakrishnan *et al.*, 2011) and so on.

Many researchers have studied biological control of fungal plant diseases using *Streptomyces* spp. (Prapagdee *et al.*, 2008; Oskay, 2009; Degtyareva *et al.*, 2009) and reported their potential. Antagonistic effect of actinomycetes against range of plant pathogens such as *Alternaria*, *Fusarium*, *Macrophomina*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Verticillium* has been reported (Trejo-Estrada *et al.*, 1998; Valois *et al.*, 1996).

The *Streptomyces* spp. strain 5406 has been used in China from past 35 years to protect cotton crops against soil borne plant pathogens (Valois *et al.*, 1996). The commercial product, Mycostop, based on strain K61 of *S. griseoviridis* and *S. lydicus* WYEC108 were found useful in the control of root rot and wilt diseases caused by *Pythium* spp., *Fusarium* spp., *Rhizoctonia* spp. and *Phytophthora* spp. (Mahadevan and Crawford, 1997).

In the present investigation, attempts were made to isolate a potential native strain of actinomycetes to exploit and develop further in plant disease management. The enzymatic activities like chitinase, cellulase, gelatinase and lipase activity of the strain were studied to understand the mechanism of action against the plant pathogens. The experiments were carried out to establish the antagonist activity of the metabolite extract *in-vitro* against fungal plant pathogens *Phytophthora infestans* (Mont.) de Bary and *Sclerotium rolfsii* (Sacc.) to understand its fungicidal ability.

Materials and Methods

Isolation, identification and characterization of actinomycetes strains

This study was carried out at Vittal Mallya Scientific Research Foundation (VMSRF),

Bengaluru during the year 2012–13. Soil samples were collected from different rhizospheres of Karnataka and Kerala and pretreated by drying in open for a week. One gram of each sample were mixed with 10 ml of sterile distilled water and vortexed properly and serially diluted till 10^{-6} concentration. Starch casein agar (SCA) and actinomycetes isolation Agar (AIA) containing antibiotic cycloheximide (100 µg/ml) were used for isolation. 100µl of each concentration were spread and kept for incubation at 28°C for 7 days. Colonies of actinomycetes were picked up by using sterile tooth pick and placed onto fresh SCA and AIA plates and incubated at 28°C for 7 days for getting pure colonies. The isolates with varied colours and pigmentation were subjected further characterization by colony hardness (hard colonies), Odor (earthy/geosmin smell), spore production and gram staining (Gram +ve). Different colonies satisfying these parameters were used further screening against the plant pathogenic fungi. Further based on the assay results, selected strain (ATMY-1) was physiologically and biochemically characterized.

The DNA was isolated following modified CTAB method. PCR amplification of 16S rDNA was carried out with a set of universal primers 27F 5'-AGAGTTTGGATCMTGGC TCAG and 1525R 5'-AAGGAGGTGW TCCARCC. Purified PCR product was sequenced and subjected to BLAST analysis with the NCBI database. The Sequence was aligned with representative actinomycetes 16S rDNA sequences in the database and found to match with *Streptomyces* spp. Further the ATMY-1 strain was identified at Institute of Microbial Technology, Chandigarh. The sequence information has been submitted to GEN bank. (<http://www.ncbi.nlm.nih.gov/nuccore/HG515374>)

Screening of isolates for antifungal activity

Dual culture technique was adopted to screen the potent strains based on their ability to inhibit the wilt causing plant pathogenic fungus *P. infestans* and root rot fungus *S. rolfsii*. Actinomycetes strains were streaked on potato dextrose agar plates at one (left) side of the plate and kept for incubation at 28°C for 7 days. Once the strains grew properly, a disc of test fungus from a fresh culture was inoculated on the right side of the plate with help of sterile cork borer (5mm) and incubated at 28°C for 5 days. The antagonistic activity was observed by measuring the colony growth of each pathogen (radius towards the actinomycetes strain) and the growth inhibition (%) was worked out in comparison with untreated control.

Enzymatic screening

Assay experiments were carried to estimate chitinase, cellulase, gelatinase and lipase activity of selected strain ATMY-1. Chitinase activity of the microorganism was studied using colloidal chitin agar (Hsu and Lockwood, 1975) and observed for a halo zone around colonies suggesting chitin degradation by chitinase produced. The Cellulase activity was studied using minimal medium agar with Carboxy methyl cellulose in the media as active substrate for the enzyme (Ponnambalam *et al.*, 2011) and observed for a clear zone around the colony.

Both plate and stab methods were carried out to understand the Gelatinase activity (Frazier, 1926). The lipase activity was studied with Tween 80 as substrate using method for determination of esteratic activity (Sierra, 1957) indicated by opaque zone around the colony.

Secondary metabolite extraction

Evaluation of extract with secondary metabolites was the primary focus of the research work with an idea developing scalable system to produce the metabolites to use in plant disease management. Based on the initial screening for fungal inhibition, the selected strain (ATMY-1) was precultured in potato dextrose broth and further inoculated (10%) into fermentation broth (glucose 1%, peptone 2% and pH 7.2) and kept for fermentation at 28°C for 7 days in an incubator shaker. The fermented broth was centrifuged at 10000 rpm for 10 mins. The cell-free supernatant obtained was added with equal volume (1:1) of ethyl acetate and shook vigorously for two hours. Ethyl acetate layer containing secondary metabolites was separated. Ethyl acetate was evaporated in rotavapor (bath temperature 40°C, pressure 200psi) and the residue obtained after evaporation was collected, weighed and used for its antagonist effect against plant pathogenic fungi.

Assays against plant pathogenic fungi

The metabolite extract from strain ATMY-1 was assayed *in-vitro* for its potential against fungal plant pathogens *P. infestans* and *S. rolfsii* through poison plate method. Known quantity of the extract was mixed to get the test concentrations with media (carrot agar for *P. infestans* and potato dextrose agar for *S. rolfsii*) and poured into plates. For all treatments, three replications were maintained. A disc of test fungus from a fresh culture was inoculated in poison plate with help of sterile cork borer (5mm) and incubated at 28°C for 5 days. Colony diameter was measured 4 times across the colony randomly and averaged. Per cent growth inhibition was measured vis-à-vis untreated control for both the fungi and MIC₅₀ was worked out.

Results and Discussion

Isolation, identification and characterization of actinomycetes strains

The soil samples obtained from varied rhizospheres from Karnataka and Kerala were found to contain morphologically different types of actinomycetes strains, especially varying in colony colour (pigmentation) and morphology. A total of 62 sunken, hard colonies were identified and named as ATMY-1 to ATMY-62. Further, all these strains were confirmed through earthy smell (geosmin smell) and gram reaction (Gram +ve).

All the isolates were screened *in-vitro* against *P. infestans* and *S. rolf sii* following dual plate technique. The growth inhibition of the wilt fungus *P. infestans* by the different actinomycetes strains ranged between 5 and 70 per cent. The strain ATMY-1 (Fig. 1) showed the maximum inhibition (70%) followed by ATMY-18, ATMY-22 and ATMY-48 (50%). The growth inhibition against the rot fungus *S. rolf sii*, by different strains of actinomycetes was to the tune of 10–60 per cent. The maximum inhibition was by the strain ATMY-48 followed by ATMY-1 and ATMY -22 (50 %). When grown together with plant pathogenic fungi without having any physical contact, ATMY-1 (Fig. 2) and few other actinomycetes strains did not allow the test fungus to grow towards them suggesting their growth inhibiting ability.

The 16S rRNA gene sequence of ATMY-1 matched 99% to genus *Streptomyces*. Further phenotypic identification at Institute of Microbial Technology, Chandigarh confirmed the identity as *Streptomyces plicatus*. All the details of the isolate were submitted to GEN bank (accession no HG515374, <http://www.ncbi.nlm.nih.gov/nucleotide/HG515374>).

Enzymatic activity

Actinomycetes are Gram+ve microorganisms and have the ability to utilize variety of substrates due to the range of enzymes they produce. The studies for enzymatic activity of the actinomycetes strain ATMY-1 (*S. plicatus*) to utilize specific substrates to establish chitinase, cellulase, gelatinase and lipase activity, showed differential response of the microorganism (Fig. 3). The results suggested that the strain has good chitinase, cellulase, gelatinase and lipase activity. However, looking at the zone formation around the colony, the cellulase activity is very clear and considerable followed by gelatinase and lipase activity. Though chitin utilization was observed as expressed with the halo zone around the colony, the extent was less compared to other substrates.

Activity of metabolites produced by *S. plicatus* against plant pathogenic fungi

The secondary metabolites produced by the actinomycetes *S. plicatus* were tested *in-vitro* against the plant pathogenic fungi, *P. infestans* and *S. rolf sii* (Fig. 4 & 5). The dose response is evident with higher inhibition at higher dosages against both the plant pathogens. At 5000 ppm of the metabolite extract dosage, growth of *P. infestans* was completely inhibited (Table 1) in 5 days and it was 94 per cent inhibition against *S. rolf sii*. The MIC₅₀ was 1853 and 2033 ppm for *P. infestans* and *S. rolf sii*, respectively.

Different types of actinomycetes inhabit diverse plant ecosystems. The organic matter and the rhizosphere soil are supposed to be very good habitat for these microorganisms (Watve *et al.*, 2001). The strains differ in their colony morphology, colour and pigmentation. Actinomycetes are

Gram +ve microorganisms and have the ability to utilize variety of substrates due to the range of enzymes they produce.

In the present study, among 62 actinomycetes isolated, 12 are from cultivated soil habitats which gets horse dung based compost and remaining 50 are from rhizosphere soil of arecanut, coconut, betel wine, pepper, bamboo and undisturbed forest soil, where organic matter is generally high. These ecosystems encourage diverse microorganisms with variety of roles to play. The actinomycetes strain ATMY-1, which is further identified as *S. plicatus* (<http://www.ncbi.nlm.nih.gov/nuccore/HG515374>.) was isolated from the crop habitat applied with horse dung based compost.

All the strains of actinomycetes isolated were screened for their antifungal activities. ATMY-1 and few other strains emerged potential in inhibiting the growth of wilt causing pathogen *P. infestans* and rot causing pathogen *S. rolfisii* to the tune of 50–70 per cent. When grown together in same media plate with plant pathogenic fungi without having any physical contact, ATMY-1 (*S. plicatus*) and few other strains did not allow the test fungus to grow towards them suggesting their inhibiting ability, most probably through production of certain enzymes or specific metabolites released in the media. Antifungal substance production may be reason for inhibition without physical contact in dual plate assay as opined by Gopalakrishnan *et al.*, (2011). Colony growth of the strain and its growth inhibition ability against both the plant pathogens, the isolate ATMY-1 proved suitable compared to other strains. The enzymatic studies with *S. plicatus* have proven that it is having chitinase, gelatinase, cellulase and lipase activity. Earlier studies have reported that *Streptomyces* spp. are

very good producer of extracellular enzymes capable of degrading organic compounds such as cellulose, lignin, chitin and proteins. The enzymes produced by them were also reported to degrade fungal cell wall composed of chitins (Goodfellow and Williams, 1983, El-Tarabily *et al.*, 2000, Getha *et al.*, 2005, Errakhi *et al.*, 2007). It has been reported that protease and chitinase-producing microorganisms have proven to be one of good biocontrol agents on protein cell wall-bearing pathogens such as *Phytophthora* and *Pythium* (Lima *et al.*, 1998). The metabolite mixture extracted from fermenting medium using *S. plicatus* showed significant growth inhibition against fungal pathogens *P. infestans* (80–100 % at 2000–5000 ppm) and *S. rolfisii* (86–94 % at 2000–5000 ppm). Certain metabolites produced by the actinomycetes could be antifungal, inhibiting the hyphal growth of fungal pathogens, as reported by Gopalakrishnan *et al.* (2011) and Kavitha *et al.* (2010). In general, almost all antimicrobial substances produced are extracellular secondary metabolites (Hacene *et al.*, 2000) which may be in present case also. Specific metabolite responsible for inhibition could not be ascertained in the present study.

The potential of actinomycetes in general and *Streptomyces* spp. in particular in the management of plant diseases and pests has been well documented. Few commercial products are also in the market using specific strains of the microorganism. While it is cumbersome to mass produce the potential microorganisms like *S. plicatus* to use in biocontrol programmes harnessing their enzymatic properties, it seems practical to develop methods for production and extraction of secondary metabolites in bulk. Further suitable formulations can also be developed to suit varied crop ecosystems to contain and manage fungal plant pathogens.

Fig.1 Growth inhibition of *Phytophthora infestans* and *Sclerotium rolfsii* by actinomycetes strains

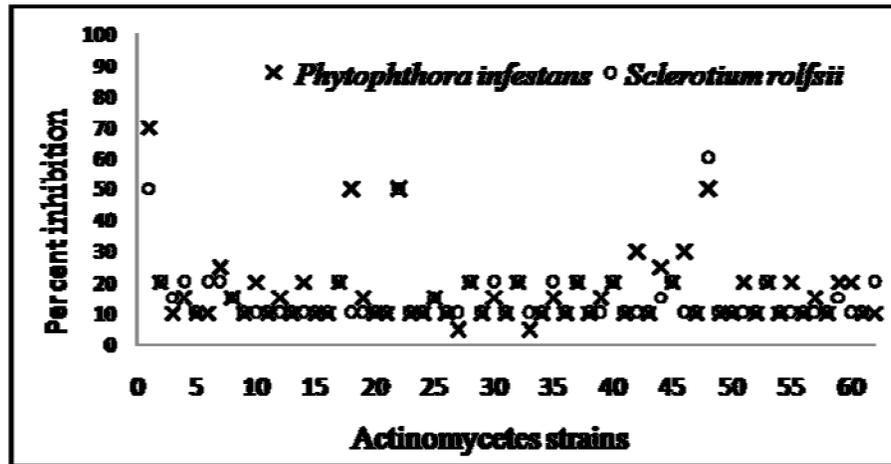


Fig. 2 Inhibition of *Phytophthora infestans* by actinomycetes strain ATMY-1

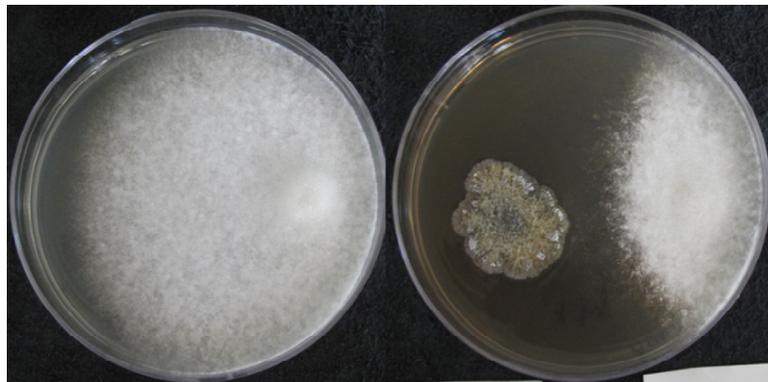


Fig.3 Enzymatic activity by the actinomycetes *Streptomyces plicatus*
 A. Chitinase activity B. Cellulase activity C. Gelatinase activity D. Lipase activity

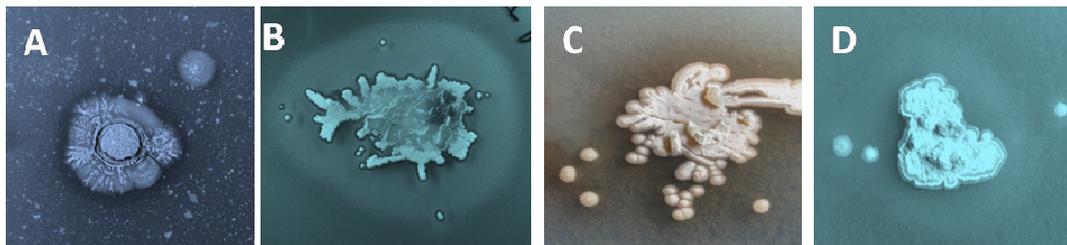
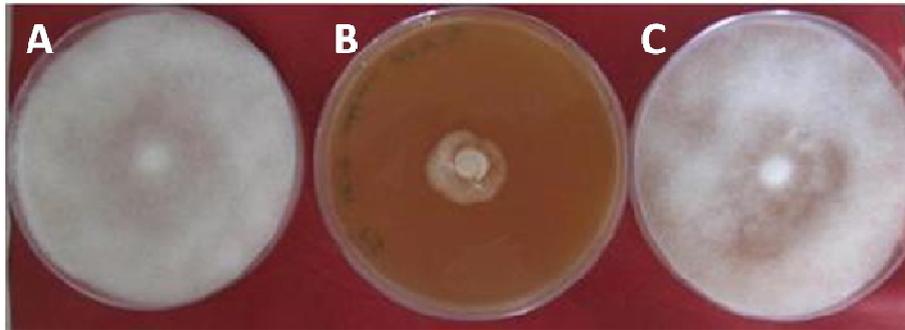
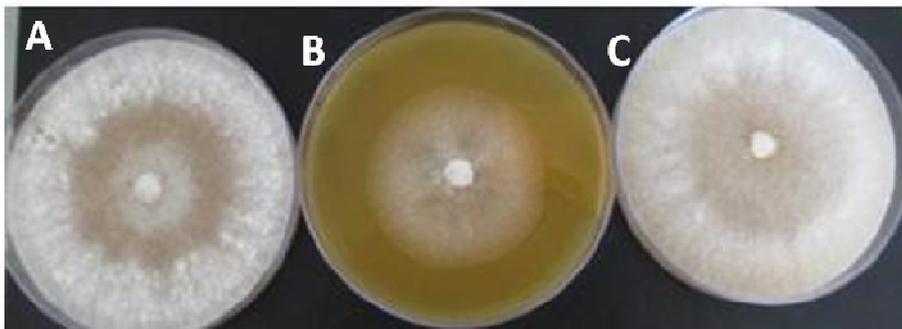


Fig.4 Growth inhibition of *Phytophthora infestans* through metabolites produced by *Streptomyces plicatus*



A. Untreated Control B. 1000ppm of metabolites C. Ethyl acetate control

Fig.5 Growth inhibition of *Sclerotium rolfsii* through metabolites produced by *Streptomyces plicatus*



A. Untreated Control B. 1000ppm of metabolites C. Ethyl acetate control

Table.1 *In-vitro* efficacy of metabolites produced by *Streptomyces plicatus* against fungal plant pathogens

Metabolite Concentration (ppm)	Inhibition of <i>P. infestans</i> (%)	Inhibition of <i>S. rolfsii</i> (%)
0	0	0
50	2.68	5.01
100	5.93	10.30
500	33.41	14.69
1000	50.09	38.21
2000	80.26	86.34
5000	100.00	93.95
MIC₅₀ (ppm)	1853.18 +/- 49.92	2033.35 +/- 84.84

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