Chitinase and Glucanase Activities of Antagonistic Streptomyces spp Isolated From Fired Plots under Shifting Cultivation In Northeast India

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Abstract

Antagonistic Streptomyces spp (Streptomyces sp NEA55 and Streptomyces cavourensis NEA5), isolated from fired plots under shifting cultivation in northeast India, are studied for their chitinase and glucanase activities. The species showed strong antagonism against test fungi (Rhizoctonia solani and Cladosporium sp.) in plate assays. Maximum % inhibition was observed due to the effect of diffusible compounds produced by these species. Streptomyces sp. NEA55 showed 54.83 % inhibition against R. solani while S. cavourensis NEA5 showed up to 50.00 % inhibition against Cladosporium sp. The inhibitory effect of volatile compounds by Streptomyces sp. NEA55 was recorded up to 50.7 % against R. solani and 37.50 % against Cladosporium sp. While S. cavourensis NEA5 showed 49.23 % inhibition against R. solani and 34.37 % inhibition against Cladosporium sp. S. cavourensis NEA5 and Streptomyces sp. NEA55 produced 0.13±0.006 µg/ml and 0.1±0.004 µg/ml chitinase, 0.2±0.001 µg/ml and 0.2±0.002 µg/ml β-1,3 glucanase, respectively. Both the species showed maximum chitinase activity at pH 6 and temperature 50 ºC, while minimum enzyme activity was observed at pH 10 and temperature 20 ºC. Both the species showed glucanase activity maximum at pH 7 and temperature 40 ºC and minimum activity at pH 10 and temperature 20 ºC. Both the species hydrolyzed glycol-chitin as a substrate in denaturing conditions showing variable amount of different isoforms. This study demonstrates that the antagonistic species of Streptomyces survive the fire operations under shifting cultivation.

Keywords: Streptomyces; Antagonism; Chitinase; Glucanase; Shifting cultivation

Introduction

Actinobacteria are most widely distributed and distinct group of microorganisms in nature. Actinobacteria, Streptomyces species in particular, have been a broadly exploited group of microorganisms for the production of important secondary metabolites and enzymes in the field of medicine and agriculture [1, 2]. Streptomyces are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi [3]. The antagonistic activity of Streptomyces to fungal pathogens is usually related to the production of antifungal compounds and extracellular hydrolytic enzymes [4, 5]. Many species of Streptomyces are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi e.g., Phytophthora capsici Sclerotinia rolfsii, Fusarium sporotrichiodes, Rhizoctonia solani and Sclerotium rolfsii, Alternaria alternata and Phomopsis archeri [6-9]. Furthermore, Streptomyces produce bioactive compounds such as antimicrobial, antiparasitic and immune-suppressing compounds via secondary metabolism. Streptomycetes have been found in beneficial associations with plants where they improve plant growth and protect against pests; this has attracted the attention of researchers worldwide [10].

Chitinase and β-1,3-glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls [11]. Chitinolytic enzymes have been identified in several Streptomyces spp. including Streptomyces sp. M-20, S. venezuelae P10, and S. anulatus CS242 [12-14]. Glucanase has been known to be produced by several microorganisms and playing important role in biocontrol [15]. Several Streptomyces have been studied for antifungal properties along with the production of glucanase, some of the examples are Streptomyces sp. S27 and Streptomyces sp. Mo [16, 17]. Shifting cultivation, refers to ‘slash and burn’, is a predominant form of agricultural practice in hills of northeast India. The microbiological aspects, basically survival of bacterial, fungal and actinobacterial communities after fire events have been studied in recent times [18-20]. The focus of present study is on the antagonistic potential of two Streptomyces species that were isolated after the fire events. These species have been studied with respect to production of diffusible and volatile compounds.
against test fungi along with the chitinase and β-1, 3-gluconase activities. In addition, both the species are also studied with hydrolyzed glycol–chitin as a substrate in denaturing conditions.

**Material and Methods**

**Study site and isolation of actinobacteria**

Actinobacteria were isolated from the soil samples that were collected after the completion of fire events in Papumpare District, Itanagar, Arunachal Pradesh under shifting cultivation. The details of the study sites have been described in Pandey et al. [18]. Among the two actinobacterial isolates used in the present study, isolate no. NEA5 showed maximum similarity with *Streptomyces cavourensis* NR 043851 and NEA55 with *Streptomyces* sp. YIM8 AF389344 [19]. The test fungi (*Rhizoctonia solani* and *Cladosporium* sp.) were also originally isolated from shifting cultivation site [20].

**Scanning electron microscopy**

In order to see the deep morphological pattern (substrate and aerial mycelia) of *Streptomyces* spp., scanning electron microscopy was performed. Glutaraldehyde (2.5 %) was added to the culture and then centrifuged. After centrifugation, washing was given with phosphate buffer saline (PBS) twice and centrifuged. The sample was dehydrated with CPD (Critical dry point), and then the samples were coated with gold by auto fine coater (JFC-1600). After coating, the sample was viewed under scanning electron microscope (JSM-6610LV).

Dual culture technique for determination of the production of diffusible antifungal compounds

To test the ability of the *Streptomyces* spp. to inhibit the phytopathogens, the test fungal culture and *Streptomyces* sp. was spot inoculated off-center on each potato carrot agar (PCA) plate. After 7 days of incubation at 28 °C, the zone of inhibition was measured. Per cent growth inhibition was calculated using the following formula: (R1-R2)/R1 × 100, where R1 represents the diameter of the test fungus on control plate and R2 is the diameter of the growth on inoculated plate.

**Quantitative estimation of production of chitinase and glucanase on different temperature and pH**

Quantification of chitinase and glucanase has been done following prescribed procedures [22]. The *Streptomyces* species were cultured at 28 °C for 5 days on a rotary shaker in 250.0 ml of chitin peptone medium for chitinase production and peptone medium containing laminarin (0.2 %) (from Laminaria digitata, Sigma) for glucanase production [23]. The cultures were centrifuged at 12,000 g for 20 min at 4 °C and the supernatants were used as enzyme source. The reaction mixture contained 0.25 ml of enzyme solution, 0.3 ml of pH buffer (1.0 M citrate buffer pH 5, phosphate buffer pH 6 to 8, and glucose buffer pH 9). The reaction mixtures were incubated at different temperatures (20, 30, 40, 50 and 60 °C) for 4 h in a water bath. One unit of chitinase was determined as 1 µmol of N acetyl glucosamine (GlcNAc) released min-1 and β-1, 3-gluconase activity was determined as 1 µmol of glucose released min-1. Protein content was determined as described by Lowry method [24].

**SDS-PAGE analysis for chitinase**

*Streptomyces* spp. was grown as shake culture in chitin-peptone medium at 28 °C for 7 days. The supernatant was centrifuged at 12000 g for 20 min at 4 °C and filtered through 0.22 µl sterile filter (Millipore) and collected in conical flask. Protein content was analyzed by SDS-PAGE [25]. Polyacrylamide slab gel consisted of 4 % stacking gel and 10 % separating gel containing 0.01 % glycol–chitin. Electrophoresis was carried out at a constant voltage of 65 V; gel was stained with coomassie brilliant blue R-250 and analyzed under gel documentation system (Alpha Imager 2200). Glycol chitin was prepared as described by Trudel and Asselin [26].

**Results and Discussion**

The scanning electron microscopy of both the species is presented in Figure: 1A and Figure: 1B. Scanning electron microscopy revealed definite structures of mycelium and spores of both the *Streptomyces* spp. Two of the isolates that were used for detailed studies based on their antagonistic properties were subjected to scanning electron microscopy. The scanning electron microscopy has been referred to provide perfect characterization of *streptomyces* [27]. The effect of diffusible compounds produced by the *Streptomyces* spp., evaluated in terms of reduction in radial growth of two test fungi viz. *Rhizoctonia solani* and *Cladosporium* sp., following 7 days of incubation at 28 °C, are presented in Figure:2 & Table 1. The results showed that volatile compounds produced by *Streptomyces* spp. were inhibitory to the growth of the test fungi, viz. *R. solani* and *Cladosporium* sp. Inhibition (%) in fungal growth by *Streptomyces* species is presented in Table 1. Microscopic observations revealed that the diffusible as well as volatile compounds produced by
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Figure 1A: Scanning electron micrograph of Streptomyces cavourensis NEA5

Figure 1B: Scanning electron micrograph of Streptomyces sp. NEA55

Figure 2: Production of antifungal compounds by Streptomyces cavourensis NEA5 & Streptomyces sp. NEA55 (A&B) respectively: Inhibition of R. solani and Cladosporium sp. due to diffusible compounds produced. Morphological deformities: (A1&B1) Normal structures of R. solani and Cladosporium sp. (A2&B2) deformed structures of respective fungus. (Bar = 5 µm)

Table 1: In vitro percent inhibitory effect of diffusible and volatile metabolites of Streptomyces species on growth of test fungi

<table>
<thead>
<tr>
<th>Pathogenic test fungi</th>
<th>% Inhibition in fungal growth by Streptomyces spp. after 7 days of incubation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Streptomyces cavourensis NEA5</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>51.61</td>
</tr>
<tr>
<td>Cladosporium sp.</td>
<td>50</td>
</tr>
</tbody>
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Table 2: Comparative account of structures of normal and antagonized pathogen

<table>
<thead>
<tr>
<th>Test fungi</th>
<th>Structure</th>
<th>Normal</th>
<th>Antagonized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizoctonia</td>
<td>Mycelium</td>
<td>Somatic and fertile hypha well distinguished</td>
<td>Irregular</td>
</tr>
<tr>
<td>Solani</td>
<td>Somatic hypha</td>
<td>Septate, dia. 2.5-3.5 µm</td>
<td>Irregular septate, dia. 3.0-6.0 µm</td>
</tr>
<tr>
<td></td>
<td>Fertile hypha</td>
<td>Septate and dark in colour, dia. 3.0-4.5 µm</td>
<td>Irregular septate, dia. 3.0-7.5 µm</td>
</tr>
<tr>
<td></td>
<td>Hyphal wall</td>
<td>Present</td>
<td>Lysed</td>
</tr>
<tr>
<td>Cladosporium sp.</td>
<td>Mycelium</td>
<td>Somatic hypha septate, dia. 2-3.5 µm</td>
<td>Somatic hypha septate, dia. 2-5 µm</td>
</tr>
<tr>
<td></td>
<td>Conidia</td>
<td>Curved or straight width 2.0-2.5 µm</td>
<td>Irregular width 2-4 µm</td>
</tr>
</tbody>
</table>

Streptomyces species induced morphological abnormalities in the fungal structures (Table 2). Deformation was observed in mycelial, hyphal or conidial structures. The longitudinal septae completely disappeared and the conidia became thick walled and spherical or irregular in shape. Lysis of fungal hyphae and vacuolization as well as granulation in mycelium was observed in R. solani. Size of the somatic and fertile hypha of R. solani increased due to the antagonistic effect of actinobacteria. Due to the antifungal activity of Streptomyces sp., hyphal wall of R. solani was completely lysed. In case of Cladosporium sp., size of mycelium and conidia increased with irregular shape of conidia. The inhibition of growth of the test pathogenic fungi continued to increase with increasing incubation time. A clear inhibitory area and dense sporulation ring was observed near the growth of the isolate which antagonized respective test fungi. No physical contact was observed between the Streptomyces species and the test fungi that were observed to be antagonized. Moreover, the formation of the inhibitory halo suggested the presence of fungicidal metabolites secreted by Streptomyces. Similar observations have been reported by Aghigni et al. [28] from a number of actinobacterial isolates from Iranian soil. These isolates formed inhibition zones in dual culture based assays inhibiting the growth of Alternaria solani, A. alternata, Fusarium solani, Phytophthora megasperma, Verticillium dahlia and Saccharomyces cerevisiae. The antifungal potential of actinobacteria against Colletotrichum gloeosporioides and Sclerotium rolfsii was assessed by dual culture technique [10]. Al-Askar et al. [29] reported that Streptomyces spororaveus RDS28 produces antifungal compounds against some phytopathogenic fungi, viz., Rhizoctonia solani, Fusarium solani, F. verticillioides, Alternaria alternata and Botrytis cinerea. There was a change in colour of the test fungi in inoculated plates, indicative of the inhibitory effect of volatile compounds. The test fungi, inhibited by the Streptomyces, under present study exhibited morphological abnormalities due to the production of diffusible and volatile antifungal compounds. Abnormal hyphal swelling, degradation and lysis of mycelia were observed by Joo [6] when Phytophthora capsici was grown with the high or low molecular fraction of Streptomyces halstedii AJ-7.

Quantitative estimation of production of chitinase and β 1, 3 glucanase activities is presented in Figure 3 (A&B) showing maximum activity of chitinase and glucanase. Effect of different pH and temperature on chitinase activity of both the species is presented in Figure 4 (A&B). Both the species showed maximum activity of chitinase at pH 6 and temperature 50 ºC, while minimum enzyme activity was observed at pH 10 and temperature 20 ºC. In case of glucanase activity both the species showed maximum enzyme activity at pH 7 and temperature 40 ºC and minimum activity at pH 10 and temperature 20 ºC (Figure 5A&B). Kim et al. [12] reported maximum activity of chitinase
between pH 5.0 to 6.0 and it was relatively stable at pH 4.0 to 8.0 when kept at 4 ºC. Beyond this pH range there was a rapid loss in the activity. Glucanase activity of the two isolates was found maximum at pH 7 and temperature 40 ºC. Highest activity was found between pH 5 to 8 and temperature 30 to 50 ºC, in previous studies. The optimum activity for short term incubation is often seen at temperature in the range of 30 to 50 ºC, while many fungal glucanases appear stable between 50 to 60 ºC [30].

SDS-PAGE exhibited the chitinase activity of the two *Streptomyces* species (NEA5 & NEA55). *Streptomyces* species hydrolyzed glycol-chitin as a substrate in denaturing conditions. Species showing variable amount of different isoforms with molecular weights between 31 to 40 kDa are presented in Figure 6. Majority of bacterial chitinases has been reported to be in the range of 20 to 60 kDa. Chitinases from various *Streptomyces* were found to possess molecular weights as 20 kDa from *Streptomyces*.
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Figure 5B: Glucanase activity of Streptomyces sp NEA55 at different pH & temperature

Figure 6: Detection of chitinase activity after SDS-PAGE using glycol chitin as substrate, lane 1 (Molecular weight markers), lane 2 and 3 sample Streptomyces cavourensis NEA5 and Streptomyces sp NEA55, respectively

sp. M-20 [13] and some strains of Streptomyces varying from approximately 25 kDa to 200 kDa [31].

The aim of this work is to prove the potential of Streptomyces species to survive the fire operations retaining their potential to reduce or eliminate the plant pathogenic fungi. Such robust isolates may prove to be the potential biocontrol agents for field applications. Further studies are needed in order to determine the nature of Streptomyces species metabolites and their mechanism of action.

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References

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