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# Inhibition of Fungal Growth Isolated from three Economic Plants of North Sumatra by Chitinolytic Bacterial Isolates

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**ABSTRACT**

*A study on the inhibition of growth of fungi isolated from three economic plants i.e. coffee, passion fruit, and orchid showing plant disease symptoms with chitinolytic bacterial isolates has been done. Since it produces chitinase, chitinolytic bacterial isolates can be used to inhibit the fungal growth. The purpose of this study was to find out the ability of chitinolytic bacterial isolates in inhibiting the growth of the fungi.*

*A total of 12 isolates of suspected pathogenic fungi were isolated from orchid, coffee, and passion fruit tree. Most of the isolated fungi were* Fusarium spp.*, but one* Alternaria sp. Fusarium *usually causes tree death due to obstruction of the stem vessel of the affected plant. Chitinolytic bacterial isolates varied in inhibiting the fungal growth. Microscopic studies of the hyphae showed morphological abnormalities i.e. broken, lytic, rolled, twisted, and curled hyphae after antagonistic assay to the bacterial isolates.*

***Key words:*** *chitinolytic bacteria, fungal growth inhibition, coffee, passion fruit, orchids*

## INTRODUCTION

Many economic plants are growing in North Sumatra. Coffee, passion fruit and orchids are few plants that contribute to the economic growth of the region. Fungal phytopathogens may cause serious problems by destroying these economically important plants. One disease in coffee is caused by *Fusarium*. *Fusarium* constitute some of the most important and extensive commercial losses in many crops throughout the world by reducing both quality and yield. This disease causes economic losses to coffee plantations in Africa [1]. Purple passion fruit is often infected with fungal pathogens such as *Alternaria passiflorae*, causal agent of brown spot. *Phythophthora cinnamomi* causes stem rot disease, and *F. oxysporum* inhibit the growth of the plant. Black rot is the most destructive diseases attacking orchids, *Cattleya* sp., caused by *Pythium ultimum*. This disease infects the leaves, then spread to the root [2].

Conventional practice to overcome this problem has been the use of chemical fungicides, which have adverse environmental effects affecting non-target organisms and causing health hazards to humans, besides demanding high costs. Therefore, an alternative control of fungal plant diseases should be considered. Biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and the ecosystem; it neither eliminates the pathogen nor the disease, but brings them into natural balance [3]. Therefore, microbe-based biocontrol method is an alternative way to control diseases in place of agrochemicals [4].

Biological control using bacteria and fungi is based on the ability of microbes to produce antifungal metabolites such as chitinase, β-1,3-glucanase that lyse fungal cells [5, 6]. Chitinolytic bacteria such as *Aeromonas hydrophila*, *A. caviae*, *Pseudomonas maltophila*, *Bacillus licheniformis*, *B. circulans*, *Vibrio furnissii*, *Xanthomonas* spp., and *Serratia marcescens* may play an important role in biological control of plant pathogenic fungi [9]. Anand & Reddy [5] and Prapagdee et al. [6] showed that chitinolytic bacterial isolates inhibited the growth of plant pathogenic fungi. Bacteria as biocontrol agents have several advantages since they are easy to isolate. Mass production is also much easier and faster than other microorganisms.

The aim of this study was to find out the ability of chitinolytic bacterial isolates to inhibit the growth of fungi isolated from some economic plants such as coffee, passion fruit, and orchids. These were done by following isolation of pathogenic fungi, assay of antagonism between the fungi and the chitinolytic bacterial isolates, and examine fungal hyphae abnormalities after antagonistic test.

## MATERIALS AND METHODS

### Chitinolytic bacterial isolates

Bacterial isolates BK08, *Bacillus* sp. BK13, BK14, *Enterobacter* sp. BK15, BK16, and *Bacillus* sp. BK17 used in this study are collection of Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara. These isolates originated from soil of Bangka, Indonesia. The isolates were kept at 30°C in a modified salt medium (0.7 g $K_2HPO_4$, 0.3 g $KH_2PO_4$, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.001 g $ZnSO_4$, and 0.001 g $MnCl_2$ in 1000 ml) containing 2% (w/v) chitin colloidal (MSMC) agar, with a pH of 6.8.

### Isolation of pathogenic fungi of coffee, passion fruit, and orchids

Fungal isolates obtained from direct isolation of the coffee plants, passion fruit from Berastagi of North Sumatra, and orchids from the orchid farm of Tiga Dolok of Dolok Panribuan of Simalungun. The fungi were isolated from the plants showing symptoms of disease from the leaves, shoots, twigs and fruits of coffee plants, from the leaves, stem and base of the stem passion fruit, and from the roots, leaves, stems and flowers of orchid plants. A small piece of the infected parts were surface disinfected for few seconds in 70% alcohol and in 1% aqueous sodium hypochlorite (NaOCl) for 5 minutes, and then rinsed thoroughly with sterile distilled water (SDW). Surface-disinfected plant parts were grown on potato dextrose agar media (PDA) for 72 hours at 28-30°C. Growing fungal colonies were purified and stored in PDA for further study. Observation and identification of suspected pathogenic fungi were carried out macroscopically and microscopically. Determination of fungal species isolated using the book of Barnes [8].

### Antagonistic assay of chitinolytic bacterial isolates with isolated fungi

The ability of the chitinolytic bacterial isolates to inhibit pathogenic fungal growth was conducted in vitro. Fungal cultures were grown at the center of the media MSMC. Two pieces of paper discs immersed with bacterial suspension (≈ $10^8$ cells/ml) placed in the opposite direction about 3.5 cm from the fungal culture. Cultures were incubated at 28-30°C. Inhibitory activity was determined based on the inhibition zone formed around bacterial colonies on medium MSMC. Inhibition zone was measured from 2 to10 days of incubation as the radius of the normal fungal growth subtracted the radius of the inhibited fungal growth.

### Observation of fungal hyphae abnormality

Inhibited hyphae of the fungi were cut by 1 $cm^2$. The hyphae were examined under light microscope and compared with normal ones.

## RESULTS

### Identification of fungi

Fifteen isolates of fungi were successfully isolated from all plants. However, only twelve were suspected to cause disease in the plants. Most of them were *Fusarium* (Table 1.). Isolation of fungi of orchids of *Cattleya* sp., *Vanda* sp., and *Phalaenopsis* sp. with disease symptoms found five isolates of suspected fungal pathogens. Isolation of fungi from coffee plants found four isolates of *Fusarium* species of suspected pathogens. *Fusarium* attacks the coffee plant vessels causing plants to wilt [1]. This disease attacks both seedlings and mature plants [2]. *Aspergillus* sp., *Penicillium* sp., *Mucor* sp. and three species of common pathogenic fungi of passion fruit plant (two isolates of *Fusarium* spp. and one isolate of *Alternaria* sp.) were isolated from passion fruit plant.

### Assay of antagonism of chitinolytic bacterial isolates to isolated fungi

The ability of the bacterial isolates to inhibit growth of *C. gloeosporioides* hyphae was examined by growing chitinolytic isolates next to the fungi in chitin containing media. All bacterial isolates showed different ability to inhibit the fungal growth (Table 2.). Bacterial genera *Achromobacter, Bacillus, Chromobacterium, Pseudomonas* and *Vibrio,* along with bacteria from the *Flavobacterium-Cytophaga* group and the *Enterobacteriaceae* family [9], and *Streptomyces* [6, 10] are reported to produce chitinase. Chitinolytic bacteria were often characterized by their ability to produce a clear zone around their colony in chitin containing media.

Inhibition zone was observed on 6-days of incubation and continued to 10-days of incubation (Table 2.). This indicated that chitinase was still produced and diffused to the media to degrade fungal hyphae. Kim et al. [11] showed that chitinase activities of the bacterial strains reached the maximum level between 10 and 20 days after bacterial inoculation. The lytic activity of bacteria is one of a

number of mechanisms that has been implicated in biocontrol [5, 7, 10, 12].

**Hyphae abnormalities of antagonistic assay**

In vitro antagonistic assay of chitinolytic isolates against fungal isolates showed occurrence of hyphal abnormality. Microscopic observation was to determine morphological changes of impaired growth of fungi occur in the hyphae. Abnormal hyphae were marked with broken, lytic, rolled, twisted, and curled hyphae (Figure 1). Fungal cell wall degrading enzymes produced by an antagonist were thought to be involved in mechanism of cell wall lytic resulted in hyphae impaired that should grow normally.

**Table 1.** Fungal isolates from disease symptoms plants of coffee, passion fruit, and orchids

| Plant host | Species | Properties | Plant host | Species | Properties |
|---|---|---|---|---|---|
| **Orchid** | *Fusarium* sp.1 | White cottony colony, macroconidia with 2-4 septate, light orange at the base of medium and smooth at the surface. | Coffee | *Fusarium* sp.2 | White cottony hyphae, Macroconidia with 2-4 septate |
| **Orchid** | *Fusarium* sp.2 | Grey colony, macroconidia with 2-4 septate, black at the base and smooth cottony at the surface | Coffee | *Fusarium* sp.3 | White colony with light brown at the center, macroconidia with 2-4 septate |
| **Orchid** | *Fusarium* sp.3 | White colony and yellowish at the center, macroconidia with 3-8 septate, orange at the base of medium, colony grows slightly separated into four parts and has rough edges | Coffee | *Fusarium* sp.4 | Purple whitish colony, macroconidia with 2-8 septate |
| **Orchid** | *Fusarium* sp.4 | Colony has white, macroconidia 2-4 septate, orange at the base of medium, smooth and white at the surface | Passion fruit | *Fusarium* sp.1 | Thin light purple colony, macroconidia with 2-4 septate |
| **Orchid** | *Fusarium* sp.5 | Colony has white, macroconidia with 3-8, septate, orange at the base, rough white at the surface and has rough edges | Passion fruit | *Fusarium* sp.2 | Thick, light purple colony, macroconidia with 2-5 septate |
| **Coffee** | *Fusarium* sp.1 | Rough and light orange colony on the surface, macroconidia with 3 to 8 septate | Passion fruit | *Alternaria* sp. | Thick, whitish-yellowish colony, ellipse microconidia |

**Table 2**. Inhibition zone (cm) of antagonism assay of chitinolytic bacterial isolates to isolated fungi

| Plant host | Bacterial isolates | Fungal isolates | Incubation days | | | | |
|---|---|---|---|---|---|---|---|
| | | | 6 | 7 | 8 | 9 | 10 |
| **Orchids** | BK08 | *Fusarium* sp.1 | 0,66 | 1,50 | 1,54 | 1,95 | 0,49 |
| | | *Fusarium* sp.2 | 0,36 | 1,04 | 1,49 | 1,45 | 1,61 |
| | | *Fusarium* sp.3 | 0,94 | 1,99 | 2,00 | 1,33 | 2,11 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Fusarium* sp.4 | 1,34 | 1,30 | 1,36 | 1,60 | 1,81 |
| | | *Fusarium* sp.5 | 1,22 | 1,43 | 1,55 | 1,45 | 2,10 |
| | BK13 | *Fusarium* sp.1 | 0,34 | 0,41 | 0,60 | 0,60 | 2,63 |
| | | *Fusarium* sp.2 | 0,44 | 0,65 | 1,79 | 0,80 | 0,42 |
| | | *Fusarium* sp.3 | 0,91 | 1,40 | 1,44 | 1,45 | 1,46 |
| | | *Fusarium* sp.4 | 1,54 | 1,66 | 2,09 | 1,85 | 2,51 |
| | | *Fusarium* sp.5 | 0,83 | 1,78 | 2,26 | 1,95 | 2,49 |
| | BK14 | *Fusarium* sp.1 | 1,23 | 1,59 | 1,90 | 1,76 | 2,29 |
| | | *Fusarium* sp.2 | 0,49 | 0,04 | 0,70 | 1,09 | 1,29 |
| | | *Fusarium* sp.3 | 1,30 | 1,26 | 1,60 | 1,39 | 1,90 |
| | | *Fusarium* sp.4 | 1,15 | 1,22 | 1,40 | 1,20 | 1,81 |
| | | *Fusarium* sp.5 | 0,65 | 1,41 | 1,41 | 1,81 | 2,09 |
| | BK15 | *Fusarium* sp.1 | 0,63 | 0,73 | 1,44 | 1,15 | 1,71 |
| | | *Fusarium* sp.2 | 0,78 | 0,15 | 0,86 | 1,25 | 0,90 |
| | | *Fusarium* sp.3 | 1,06 | 1,46 | 1,46 | 1,54 | 1,99 |
| | | *Fusarium* sp.4 | 1,13 | 1,49 | 1,70 | 1,35 | 2,40 |
| | | *Fusarium* sp.5 | 1,60 | 1,60 | 1,54 | 2,16 | 2,82 |
| | BK16 | *Fusarium* sp.1 | 1,22 | 1,77 | 1,79 | 1,60 | 2,20 |
| | | *Fusarium* sp.2 | 0,21 | 0,03 | 0,31 | 0,45 | 0,46 |
| | | *Fusarium* sp.3 | 0,84 | 1,41 | 1,30 | 1,35 | 1,70 |
| | | *Fusarium* sp.4 | 1,09 | 1,35 | 1,45 | 1,71 | 2,30 |
| | | *Fusarium* sp.5 | 1,61 | 1,79 | 2,00 | 1,76 | 2,61 |
| | BK17 | *Fusarium* sp.1 | 1,30 | 1,37 | 1,74 | 1,65 | 2,00 |
| | | *Fusarium* sp.2 | 1,15 | 1,39 | 1,97 | 1,80 | 2,40 |
| | | *Fusarium* sp.3 | 0,65 | 1,19 | 1,30 | 1,54 | 1,98 |
| | | *Fusarium* sp.4 | 0,50 | 0,64 | 0,85 | 1,10 | 0,67 |
| | | *Fusarium* sp.5 | 1,29 | 1,83 | 1,84 | 1,98 | 2,30 |
| **Coffee** | BK08 | *Fusarium* sp.1 | 0,05 | 0,15 | 0,20 | 0,28 | 0,60 |
| | | *Fusarium* sp.2 | 0,08 | 0,55 | 0,93 | 1,10 | 1,65 |
| | | *Fusarium* sp.3 | 0,33 | 0,34 | 0,46 | 0,60 | 0,95 |
| | | *Fusarium* sp.4 | 0,35 | 0,35 | 0,80 | 0,80 | 1,10 |
| | BK13 | *Fusarium* sp.1 | 0,98 | 1,09 | 1,13 | 1,25 | 1,33 |
| | | *Fusarium* sp.2 | 0,73 | 1,25 | 1,48 | 1,73 | 2,23 |
| | | *Fusarium* sp.3 | 0,45 | 0,90 | 1,25 | 1,50 | 1,89 |
| | | *Fusarium* sp.4 | 0,14 | 0,98 | 1,92 | 2,00 | 2,25 |
| | BK14 | *Fusarium* sp.1 | 1,40 | 1,47 | 2,08 | 2,15 | 2,40 |
| | | *Fusarium* sp.2 | 0,65 | 1,40 | 1,44 | 1,70 | 2,17 |
| | | *Fusarium* sp.3 | 0,59 | 0,95 | 1,19 | 1,40 | 1,92 |
| | | *Fusarium* sp.4 | 1,05 | 1,39 | 1,60 | 1,75 | 2,40 |
| | BK15 | *Fusarium* sp.1 | 1,50 | 1,52 | 1,53 | 1,86 | 2,10 |
| | | *Fusarium* sp.2 | 0,60 | 1,27 | 1,43 | 2,00 | 2,27 |
| | | *Fusarium* sp.3 | 0,60 | 1,30 | 1,52 | 1,65 | 1,85 |
| | | *Fusarium* sp.4 | 1,30 | 1,30 | 1,55 | 2,18 | 2,50 |
| | BK16 | *Fusarium* sp.1 | 1,15 | 1,25 | 1,25 | 1,30 | 1,38 |
| | | *Fusarium* sp.2 | 0,70 | 1,10 | 1,70 | 1,70 | 2,10 |
| | | *Fusarium* sp.3 | 1,05 | 1,25 | 1,50 | 1,53 | 1,80 |
| | | *Fusarium* sp.4 | 1,08 | 1,08 | 1,60 | 1,90 | 2,07 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | BK17 | *Fusarium* sp.1 | 1,50 | 1,50 | 1,75 | 1,88 | 2,20 |
| | | *Fusarium* sp.2 | 1,23 | 1,28 | 1,38 | 1,83 | 2,18 |
| | | *Fusarium* sp.3 | 0,07 | 0,45 | 0,80 | 1,00 | 1,40 |
| | | *Fusarium* sp.4 | 1,27 | 1,27 | 1,97 | 2,03 | 2,42 |
| **Passion fruit** | BK08 | *Fusarium* sp.1 | 0,95 | 1,95 | 1,31 | 2,17 | 2,21 |
| | | *Fusarium* sp.2 | 0,90 | 1,35 | 1,94 | 2,24 | 2,40 |
| | | *Alternaria* sp. | 1,05 | 1,52 | 1,89 | 1,91 | 1,68 |
| | BK13 | *Fusarium* sp.1 | 1,06 | 1,31 | 1,45 | 1,40 | 2,32 |
| | | *Fusarium* sp.2 | 1,24 | 2,20 | 2,30 | 2,17 | 2,26 |
| | | *Alternaria* sp. | 0,79 | 1,15 | 1,34 | 0,84 | 1,73 |
| | BK14 | *Fusarium* sp.1 | 0,92 | 1,60 | 1,82 | 1,50 | 1,62 |
| | | *Fusarium* sp.2 | 1,09 | 2,33 | 1,48 | 1,99 | 2,27 |
| | | *Alternaria* sp. | 1,66 | 2,06 | 1,40 | 1,79 | 1,90 |
| | BK15 | *Fusarium* sp.1 | 0,80 | 1,56 | 2,05 | 2,33 | 2,67 |
| | | *Fusarium* sp.2 | 1,57 | 1,95 | 1,97 | 2,10 | 2,37 |
| | | *Alternaria* sp. | 1,72 | 1,82 | 1,97 | 2,47 | 2,78 |
| | BK16 | *Fusarium* sp.1 | 0,87 | 1,35 | 2,33 | 2,56 | 2,56 |
| | | *Fusarium* sp.2 | 1,27 | 1,92 | 2,17 | 2,50 | 2,69 |
| | | *Alternaria* sp. | 1,72 | 2,00 | 2,03 | 2,16 | 2,03 |
| | BK17 | *Fusarium* sp.1 | 1,75 | 1,90 | 2,03 | 2,66 | 2,60 |
| | | *Fusarium* sp.2 | 1,37 | 2,06 | 2,81 | 2,56 | 2,15 |
| | | *Alternaria* sp. | 0,67 | 2,25 | 2,37 | 2,47 | 2,60 |

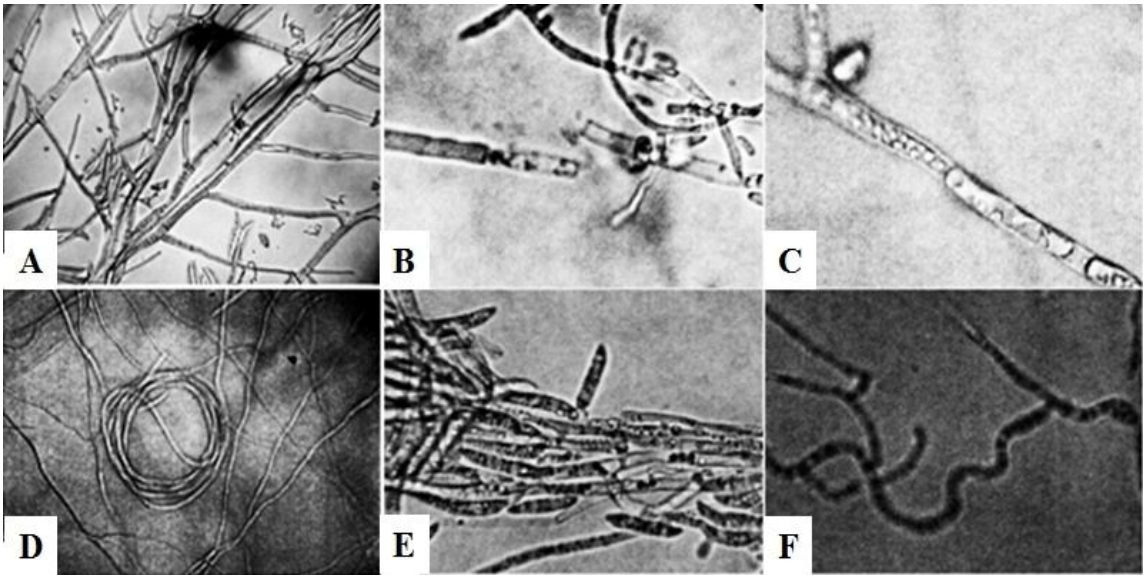


**Figure 1**. Microscopic observation of a normal hyphae (A) with abnormal ones (B: broken, C: lytic, D: rolled, E. twisted, and F: curled hyphae) after antagonistic assay

**DISCUSSION**

Early symptoms of Fusarium wilt disease could be seen in *Cattleya* sp. as wilt disease of plants with brown and shriveled stem, in *Vanda* sp. showed black spots on leaves, whereas in *Phalaenopsis* sp. the symptom was necrosis around the leaves. Although direct observation showed the variation of color and structure of the hyphae of the isolates, microscope observation showed that all isolates were *Fusarium*. *P. ultimum* causal agent of black rot of *Cattleya* sp. was not found during the isolation. More specific isolation medium might be necessary to isolate this type of fungi.

One of destructive disease in coffee is caused by *Fusarium*, both in the tropics and sub-tropics [1].

Fusarium attacks the plant vascular system, penetrate through the roots, without showing outward symptoms directly and then clog the vascular system, causing plants to wilt [1]. This disease can attack plants in the nursery and adult [2]. At a very young plant disease causes plants to die suddenly, whereas in adults infected plants may produce few and small fruit [2].

Passion fruit plant part used for the isolation of the fungus showed symptoms of the disease such as yellowing of leaves and petiole and eroded of the base of the stem with white threads of fungus mycelium. Severe infected plant showed plant wilted. *Alternaria passiflorae* causes brown spots and *F. oxysporum* var. *passiflorae* causes *Fusarium* wilt in passion fruit plant [13]. *F. oxysporum* var. *passiflorae* is a soil borne pathogens so that all types of passion fruit susceptible to *Fusarium*. Disease symptoms of *Fusarium* wilt can be observed as pale leaves, brown stem vessels, and plants wilt [14].

Microbial antagonism implies direct interaction between two microorganisms sharing the same ecological niche [12]. Three main types of direct interactions may be involved i.e. parasitism, antibiosis and competition for nutrients. Antagonistic effects responsible for disease suppression results either from microbial interactions directed against the pathogen, mainly during its saprophytic phase, or from an indirect action through induced resistance of the host plant [12]. Antibiosis is considered as a type of antagonism mediated by specific or nonspecific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds or other toxic substances [12]. Fungal cell wall degrading enzymes produced by an antagonist were, therefore, thought to be involved simultaneously in parasitism and antibiosis.

Parasitism involves specific recognition between the antagonist and its target pathogen and several types of cell wall degrading enzymes to enable the parasite to enter the hyphae of the pathogen [12]. A number of fungi are particularly susceptible to be degraded by microorganisms [11]. Investigations on lytic activity of biocontrol agents have focused mainly on the characterization of enzyme systems capable of degrading fungal cell wall components, of which chitinases are among the most intensively studied [10]. Therefore, chitinase is known as one of the antifungal proteins.

The ability of chitinolytic isolates to control fungal growth varied. This variation might be due to species specific, different bacterial chitinase activity, chitin composition of the fungal mycelium, the growth rate of the bacterial and the fungi, and other antifungal metabolites. Though the fungal cell wall is made up of mainly of glucan and chitin, the β-1,3-glucanase and chitinase are key enzymes responsible for fungal cell wall lytic and degradation [5, 7]. The presence of other metabolites in addition to chitinase is also responsible for inhibiting fungal growth [6].

Degradation of cell wall component to some extent results in hyphal abnormality. Khikmah showed that the chitinase enzyme application caused abnormal hyphae of *Rhizoctonia solani* after 24 hours of the enzyme application [15]. The hyphal tips were segmented, swollen, shrink, and curled. Hyphal tip lytic was followed. Prapagdee et al. showed hyphal swelling and abnormal shapes in *Colletotrichum gloeosporioides* or *Sclerotium rolfsii* grown on potato dextrose agar that contained the culture filtrates of *Streptomyces hygroscopicus* after 3 days of incubation [6]. Hyphal distortion, like swelling, bulbous growth, abnormal branching of hyphae and, formation of hyphal protuberances were also seen. By the fourth day, distortion and lytic of the hyphae were more frequently noted.

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