Screening of Fungi for the Control of *Aspergillus parasiticus*

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Abstract

Fungal isolates previously selected from soil [KMC 5, SRS 4, 480512 I08 (1) and 480519 I06 (3)] were screened for their ability to produce antifungal substances against aflatoxin producing *Aspergillus parasiticus* IMI 102566. The production of antifungal substances by these isolates was investigated in yeast extract sucrose broth under static condition. Culture filtrates of these isolates were subsequently tested against *A. parasiticus* on PDA. The filtrates of all isolates produced varying degrees of inhibition against *A. parasiticus* with the highest inhibition zone of 33.75 mm. These isolates were identified as *Trichoderma* and the other two isolates were *Penicillium* sp.

Keywords: *Trichoderma, Aspergillus parasiticus*, aflatoxin, biocontrol

1. Introduction

Aflatoxins are secondary metabolites produced by certain strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. tamarii*, *A. bombycis* and *A. pseudotamarii* [1-6]. Aflatoxins are one of the most potent toxic substances that occur naturally [7] and their contamination in economic crops is a major problem all over the world [8]. Aflatoxin is a serious human carcinogen and has been identified as a potential biological weapon for food and water contamination [9]. Ingestion of aflatoxins in contaminated food or feed can cause aflatoxicosis. Aflatoxin poisoning has been reported from all parts of world in almost all domestic and non domestic animals like cattle, horses, rabbits, and other non human primates [7]. Diet is the major way through which humans and animals are exposed to aflatoxins. Furthermore, exposure to aflatoxin can be through ingestion of contaminated milk containing aflatoxin M1. Occupational exposures to aflatoxins in agricultural workers, people working in oil mills, and granaries have been reported [10]. Experiments conducted in different areas especially in China and in the African countries have shown high incidence of liver cancer [11,12]. The outbreak of acute aflatoxicosis in Kenya in 2004 was one of the most severe case of human aflatoxin poisoning in history [13] with a case fatality rate of 39%. This epidemic resulted from the ingestion of contaminated maize [14]. Many countries have limitation of aflatoxin residue in agricultural products for safety of consumer. Current legislative level of aflatoxins permissible in Thailand is 20 ng/g or 20 ppb [15] whereas European limit for food is 1-5 µg/kg for aflatoxin B1 and 4-10 µg/kg for total aflatoxins [16]. FAO and WHO have reportd a high incidence of hepatitis B virus infection in areas where dietary exposure to aflatoxins

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was prevalent. Subsequent research proved that both hepatitis B virus and aflatoxins act synergistically in the imposed regulatory guidelines of 20 ppb of total aflatoxins as the maximum allowable limit in food or feed [17]. In Thailand, the contamination of aflatoxins ranging from 0.01-17.3 µg/kg was found in one hundred and twenty noodle dishes (51%) consumed in 3 areas of Bangkok [18] while 5 herbal medicinal products out of 28 products were also found to be contaminated with detectable amount of aflatoxins ranging from 1.7-14.3 ng/g [15].

Physical, chemical and biological methods have been investigated in order to prevent the growth of aflatoxigenic fungi, to reduce or eliminate the toxin levels, to degrade or detoxify the toxins in foods and feeds [19]. Many microorganisms have mechanisms to compete with others for nutrients and growth development. Fungi have many mechanisms such as growth competition, antibiotic production and endolysis, mycoparasitism and exolysis [20]. Many groups of fungi are well known as biocontrol agents. For example, *Trichoderma viride* and *T. harzianum* can produce amylolytic, pectinolytic, proteolytic and cellulolytic enzymes for growth inhibition of *A. flavus* and *Fusarium moniliforme* [21]. *Corynebacterium rubrum*, *A. niger*, *Mucor ambiguus* and *T. viride* can reduce aflatoxin B1 *in vitro* [22]. Nontoxigenic strains of *A. flavus* (NRRL 21882) and an orange–brown color mutant of *A. parasiticus* (NRRL 21369), each lacking the ability to produce aflatoxins, had competitive effect and reduced aflatoxin contamination occurred during storage [23]. The extract of *P. oxalicum* showed fungicidal activity which affected *A. candidus*, *A. versicolor*, *P. griseofulvum* , *Curvularia trifolii* and *Botrytis cinerea* [24].

The objective of this study is to screen fungal isolates previously selected from soil [KMC 5, SRS 4, 480512 I08 (1) and 480519 I06 (3)] with the ability to control *Aspergillus parasiticus* IMI 102566 on PDA.

2. Materials and Methods

2.1 Fungi

2.1.1 *Aspergillus parasiticus* IMI 102566 obtained from International Mycological Institute, U.K.

2.1.2 Fungal isolates KMC 5, SRS 4, 480512 I08 (1) and 480519 I06 (3) previously isolated from cultivated soil by Wongsariya *et al*.[25].

2.2 Cultivation of fungi

All fungal isolates were cultured on Potato Dextrose Agar (PDA) for 7 days at room temperature.

2.3 Preparation of spore suspension

Spores of fungal isolates were prepared by growing these fungi on PDA for 7 days before harvesting and filtering through sterile cotton wool by sterile distilled water plus 0.02 % tween 80. The numbers of spores were counted by haemacytometer.

2.4 Culture filtrate preparation

Yeast extract sucrose (YES) was used for culturing each fungal isolates. The medium was prepared by adding 45 ml broth into 125 ml flask and autoclaved. Spore suspension (10^6 spores/ml) of each isolate was inoculated into each flask and incubated at static condition for 14 days. Culture filtrates of these fungi were then filtered through sterile cotton wool and membrane filter (0.2 µm) under sterile condition to obtain cell-free filtrates [26].

2.5 Agar diffusion test

Twenty five ml of PDA were poured into a Petri dish (15 cm diameter) and left to set and 20 ml of PDA inoculated with spores of *A. parasiticus* IMI 102566 (10^6 spores/ml) were poured over the surface of PDA agar plate [27]. Sterile cylinder cups (6mm. diameter) containing 300 µl of each culture filtrate were placed onto the same plates. Cylinder cup containing broth without any culture was served as a control. The plates were then incubated for 4 days at room temperature and the inhibition zones were determined on days 2, 3 and 4 using vernier digital caliper. All experiments were repeated at least five times.

2.6 Identification of fungal isolates

Fungal isolates were identified by morphological studies using PDA and 2% malt extract agar according to the methods of Rifai [28] and Bissett [29-33].

3. Results and Discussion

The effects of antibiotic substances produced by 4 isolates of fungi, i.e, isolates KMC 5, SRS 4 480512 I08 (1) and 480519 I06 (3) on the growth of *A. parasiticus* IMI 102566 on agar plate were given in Table 1 and Figure 1. The results show that the culture filtrate of isolate 480519 I06 (3) gave the best inhibitory effect with the inhibition zone of 33.750 mm followed by the culture filtrates of isolates KMC 5, 480512 I08 (1) and SRS 4 with the inhibition zones of 15.079, 11.133 and 11.113 mm, respectively. The result suggested that these fungal isolates may produce some biological metabolites that affected other fungi. Isolates KMC 5 and SRS 4 were identified as *Trichoderma atroviride* whereas isolates 480512 I08 (1) and 480519 I06 (3) were identified as *Penicillium* sp. (Figures 2 and 3).

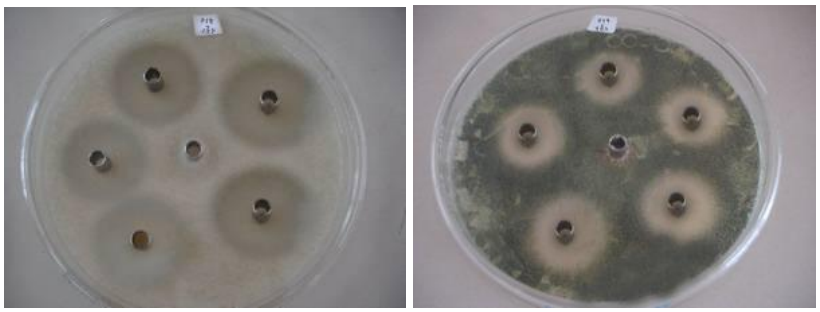
Our previous investigation showed that *Trichoderma* isolates TISTR 3167 and C1-1 selected from 16 isolates gave the best inhibitory effect on *A. flavus* IMI 242684 and aflatoxin production [34]. Reddy *et al.* [35] showed that rice seed treatment with *T. virens, Pseudomonas fluorescens, Rhodococcus erythropolis,* clove extract or carbendazim prevented or drastically reduced the contamination of *Aspergillus* and aflatoxin production. Among the biocontrol agents, culture filtrates of *Rh. erythropolis* completely inhibited the aflatoxin B1 production at 25 ml/kg concentration whereas the other biocontrol agents, *Ps. fluorescens, T. virens* and *Bacillus subtilis* showed 93, 80 and 68% reduction of *A. flavus* growth and 83.7, 72.2 and 58 % reduction of aflatoxin B1 at 200 mg/kg, respectively [36]. The undiluted culture filtrates of *T. harzianum* and *T.* pseudo-koningii completely inhibited spore germination of rot pathogens such as *Macrophomina phaseolina, Alternaria* sp., *Fusarium solani* and *Aspergillus niger* whereas 50% dilution gave varying degree of inhibition of spore germination [37]. Possible mechanisms of antagonism by *Trichoderma* spp. have been suggested to involve the production of antibiotics [38] and/or hydrolytic enzyme [39] as well as parasitism [40]. Production of volatiles and extracellular enzymes rather than mycoparasitism was the likely means by which *Trichoderma* species suppressed the growth of *A. flavus* and *F. monoliforme* [21, 41]. Culture filtrates of *Trichoderma viride* and *T. harzianum* were inhibitory of *F. moniliforme* and, to a lesser extent, *A. flavus*. The degree of inhibition was dependent on the carbon or nitrogen source incorporated into the medium [28]. Most strains of *Trichoderma* can produce volatiles and non volatile toxic substances such as harzianic acid, alamethicins and tricholin that hinder growth of other microorganisms [42]. Some aggressive *Trichoderma* spp. used as biocontrol agents are capable of producing either antibiotics or extracellular enzymes or both [43]. These findings are in agreement with our result. Thus the inhibitory activity of *Trichoderma atroviride* (isolates KMC 5 and SRS 4) on the growth of *A. parasiticus* IMI102566 on agar plate might be due to diffusible antibiotic substances or

extracellular enzymes or volatile compounds produced by *Trichoderma* isolates. However, their inhibition was less than *Penicillium* isolate 480519 I06 (3).

Penicillium spp. can produce secondary metabolites, e.g. *Penicillium griseofulvum* produces griseofulvin that can inhibit bacterial growth [44] while *P. citrinum* produces citrinin and

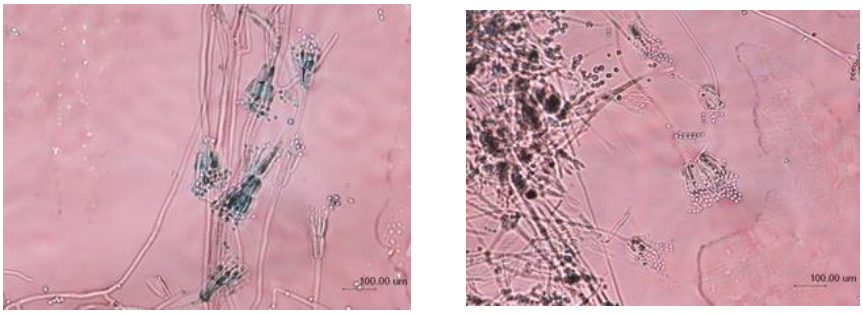
Table 1 Effect of culture filtrates of 4 fungal isolates on the growth of *A. parasiticus* IMI 102566.

Fungal isolate	Diameter of inhibition zone (mm) *
480519 I06 (3)	33.750 ± 7.172
KMC 5	15.079 ± 0.686
480512 I08(1)	11.133 ± 0.710
SRS 4	11.113 ± 0.597



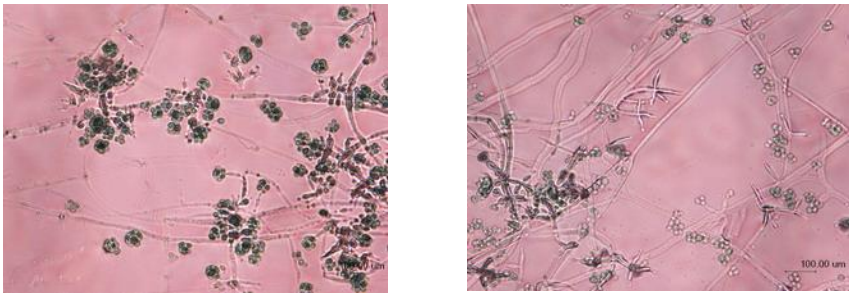
A. B.

Figure 1 Inhibition of *A. parasiticus* by culture filtrate (antibiotic substance) of *Penicillium*isolate 480519 I06 (3) in YES (static culture condition).
A. 2 day-old culture B. 4 day-old culture



A. B.

Figure 2 Conidiophores of *Penicillium*
A. Isolate 480512 I08 (1) B. Isolate 480519 I06 (3)



A. B.

Figure 3 Conidiophores of *Trichoderma*
A. Isolate KMC 5 (1) B. Isolate SRS

inhibits the root-rot pathogen, *Heterobasidion annosum* [45]. *Penicillium oxalicum* strain PY-1 isolated from soil produced antifungal substances effectively suppressed the mycelia growth of *S. sclerotiorum* and other tested plant pathogenic fungi[46]. Ma *et al*. [47] isolated *Penicillium striatisporum* Pst 10 from the rhizosphere of chilli peppers and tested for antagonistic effects on *Phytophthora* spp., *Cladosporium cucumerium* and *Sclerotinia sclerotiorum.* Pst10 could produce antifungal substances in liquid culture in PDB or solid fermentation on wheat barn. Sterilized liquid culture filtrate (SLCF) of Pst 10 grown in PDB completely inhibited mycelium growth of *Ph. capsici*. Production of antifungal substances in liquid cultures occurred for approximately 8-10 days when the inhibitory effects on mycelia growth of *Ph. capsici* reached a maximum.

4. Conclusions

This preliminary study showed the biological control mechanism of *Penicillium* sp. (isolate 480519 I06 (3)) and *Trichoderma atroviride* (isolate KMC 5) against *A. parasiticus* IMI 10256 may be the production of antifungal substances. Although there have been reports on the ability of *Trichoderma* isolates and their substances to inhibit the growth of *Aspergillus* sp. but the ability of antimicrobial substances of *Penicillium* sp. to inhibit the growth of aflatoxin producing fungi have not been reported so far. Thus this may lead to the discovery of new biological control agents for controlling the growth of aflatoxin producing fungi. Further studies have to be conducted in order to identify these antifungal substances.

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