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EFFECT OF *TRICHODERMA* sp. TC1 AND ITS EXTRACT ON GROWTH INHIBITION AND AFLATOXIN PRODUCTION OF *Aspergillus flavus* AND *Aspergillus parasiticus*

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ABSTRACT

Aflatoxins are toxic carcinogenic secondary metabolites produced predominantly by two fungi: *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi are a health risk and are responsible for losses and contamination of processed foods and feeds. It is very important to find methods to inhibit the growth of *A. flavus and A. parasiticus,* and degrade aflatoxin. In laboratory conditions, the Percentages of Inhibition of Radial Growth (PIRG %) of *Trichoderma* sp. TC1 against *A. flavus* LA21 and *A. parasiticus* NG10 after 7 days' dual culture were 63.51% and 60.22%, respectively. However, the use of *Trichoderma* sp.TC1 mycelium has restrictions in agricultural products due to the risks of consuming the substrate and degrading the products' quality. Therefore, its extracts are more preferred in application. In the PD media, when up to 4% *Trichoderma* sp. TC1 extract was added, the biomass rate for *A. flavus* LA21 and *A. parasiticus* NG10 reduced to 74.00% and 60.30%, respectively, after 5 days of incubation. The toxin levels of corn samples deliberately infected by aflatoxin producing fungi were negatively correlated with sprayed on *Trichoderma* sp. TC1 extract. Seven days after spraying the extract 4%, the aflatoxin in samples from more than 60 ppb at the initial time reduced down to 16 ppb for *A. flavus* LA21 and 21.33 ppb for *A. parasiticus* NG10.

Keywords: Aflatoxin, antifungus, *A. flavus, A. parasiticus*, *Trichoderma*.

Hiệu quả của *Trichoderma* sp. TC1 và dịch chiết của chủng này tới sự ức chế phát triển và sản sinh aflatoxin của nấm mốc *Aspergillus flavus* và *Aspergillus parasiticus*

TÓM TẮT

Aflatoxin là độc tố thứ cấp gây ung thư được sản sinh bởi hai chủng nấm chính là *Aspergillus flavus* và *Aspergillus parasiticus.* Đây là những loài nấm gây nguy hiểm tới sức khỏe con người và là nguyên nhân chính gây tổn thất và ô nhiễm trong quá trình chế biến thực phẩm và thức ăn chăn nuôi. Việc nghiên cứu tìm ra phương pháp ức chế sự phát triển của *A. flavus, A. parasiticus* và làm giảm hàm lượng aflatoxin là rất quan trọng. Trong điều kiện nghiên cứu, tỷ lệ phần trăm ức chế sinh trưởng (PIRG,%) của *Trichoderma* sp. TC1 lên hai chủng *A. flavus* LA21 và *A. parasiticus* NG10 được xác định thông qua phương pháp đồng nuôi cấy. Lượng aflatoxin được xác định bằng phương pháp Elisa. Kết quả cho thấy, giá trị PIRG của *Trichoderma* sp. TC1 ức chế *A. flavus* LA21 và *A. parasiticus* NG10 sau 7 ngày tương ứng là 63,51% và 60,22%. Tuy nhiên, việc sử dụng trực tiếp nấm *Trichoderma sp*. TC1 lên nông sản sẽ dẫn đến việc tiêu thụ cơ chất và ảnh hưởng đến chất lượng sản phẩm. Ứng dụng dịch chiết nấm sẽ được ưu tiên nghiên cứu. Trên môi trường PD, khi tăng nồng độ dịch chiết *Trichoderma* sp. TC1 lên tới 4%, tỷ lệ giảm sinh khối khô của *A. flavus* LA21 và *A. parasiticus* NG10 đạt 74.00 % và 60.30% sau 5 ngày nuôi cấy. Lượng aflatoxin của các mẫu ngô được nhiễm nấm tỷ lệ nghịch với nồng độ dịch chiết *Trichoderma* sp. TC1 được phun. Từ hơn 60 ppb ở thời điểm ban đầu, với nồng độ 4% dịch chiết, sau 7 ngày, lượng aflatoxin trong các mẫu ngô nhiễm *A. flavus* LA21 giảm còn là 16 ppb và 21.33 ppb cho *A. parasiticus* NG10.

Từ khóa: Aflatoxin, *A. flavus, A. parasiticus*, kháng nấm, *Trichoderma*.

1. INTRODUCTION

Corn is the third most important grain worldwide and the most important raw material for feedstuff. In tropical countries like Vietnam, the warm and humid climate creates a favourable condition for fungal growth and increases the risk to humans and animals through the production of mycotoxins (especially aflatoxins).

Aflatoxins are produced by two closely related fungi, *Aspergillus flavus* and *A. parasiticus*. They are mutagenic and carcinogenic in animals and humans. Many strategies, including physical, chemical, and biological controls, have been investigated to reduce aflatoxins. Among them, biological control appears to be the most promising approach for the control of aflatoxin. Besides lactic acid bacteria, the use of *Trichoderma* strains to manage aflatoxins from *Aspergillus* spp. in India had good results (Verma *et al.*, 2007).

The antagonistic ability of *Trichoderma* is mainly through the activity of extracellular enzymes. The study of Gachomo *et al.* (2008) showed that *Trichoderma* can be exploited as a potential target for antibacterial fields, anti-fungus, and reducing aflatoxin content. However, the application of *Trichoderma* in controlling fungi producing aflatoxin and reducing the levels of aflatoxin in agriculture has been limited (Thanh *et al.*, 2014). The objective of this study is to test the antagonistic ability of *Trichoderma* sp. TC1 and its extract at various concentrations on the growth and production of aflatoxin by *A. parasiticus* and *A. flavus* in PDA media and corn.

2. MATERIALS AND METHODS

2.1. Materials

Corn samples were provided by CP Vietnam Feedstuff Joint Stock Company. The samples were aseptically collected in sterile polyethylene bags, taken to the laboratory, and kept at 4°C. *Trichoderma* sp. TC1 strains were derived from the Department of Plant Pathology, Faculty of Agronomy - Vietnam National University of Agriculture (VNUA). Fungal strains producing aflatoxin, *A. parasiticus* NG10 and *A. flavus* LA21, came from the collection of the Faculty of Food Science and Technology - VNUA. The culture media included: potato dextrose agar (PDA), coconut agar (CA), and potato dextrose (PD).

2.2. Methods

2.2.1. Tests for fungi static effect of antagonists by dual culture method

In this method, the toxin-producing fungi and the antagonist fungi were cultured in petri dishes containing PDA and incubated at 28°C for 5 - 7 days. The medium was poured into 100 mm Petri dishes. After agar solidification, an agar disc of the antagonist, *Trichoderma* sp. TC1, was placed 2 cm away from the periphery of the Petri dish, and an agar disc of an aflatoxin producing strain was similarly placed 2 cm away from the edge of the Petri plate but on the side opposite of the *Trichoderma* sample (Disk 2). Plates without antagonist fungi were used as controls (Disk 1). All pairings were incubated at 28°C for 7 days. The ability of the antagonist to inhibit the toxic fungi was evaluated. Three replications were used for each experiment. The diameter growth of fungi was measured to evaluate the antagonistic ability.

The Percentage of Inhibition of Radial Growth (PIRG) was calculated using the formula (Siddquee *et al.,* 2009):

$$PIRG = \frac{R1 - R2}{R1} \times 100\%$$

Whereas:

R1 - Radius of the radial growth of the pathogen towards the opposite side in control plates

R2 - Radius of the radial growth of the pathogen towards the opponent antagonist in test plates

Antagonistic ability was evaluated using the scale: very high (PIRG > 75%); high (PIRG = 61 - 75%); medium (PIRG = 50 - 60%); and low (PIRG < 50%).

2.2.2. Antagonistic effectiveness of Trichoderma sp. *TC1 extract on controlling A. flavus LA21 at different concentrations*

The extract of *Trichoderma* sp. TC1 was obtained by the following procedure. *Trichoderma* sp. TC1 were cultured in PD broth media at 28 - 30°C for 5 - 7 days and shaken at 200 rpm. Then, the broth was filtered out and the extracellular extract was centrifuged at 6000 rpm for 30 minutes. The supernatant was filtered through filter paper. Ethyl acetate solvent was used with the ratio of 1:1 to separate the extract. A rotary evaporator was used to remove the solvent from the *Trichoderma* sp. TC1 extract.

In order to evaluate the antagonistic ability, *Trichoderma* sp. TC1 extract was added at different concentrations into PD broth media according to the description of Soytong *et al.*, (2001). *A. flavus* LA21 and *A. parasiticus* NG10 were cultivated on PD media containing *Trichoderma* sp. TC1 extract at 28 - 30°C for 5 days with shaking at 200 rpm. Fungal biomass was filtered through filter paper and dried at 27°C for 2 days, and then, the percentage inhibition of mycelium was calculated according to the following formula:

$$X = \frac{(dc - dt)}{dc} \times 100\%$$

Where as: dc is the mass of mycelium at 0% extract content

dt is the mass of mycelium at additional extract contents

2.2.3. Aflatoxin infection in corn

Corn samples (without disease) were broken down to 3 - 4 pieces, and then crushed through 0.5 mm mesh.

The infection by aflatoxin fungi was conducted by taking a fungal sample from a PDA medium plate, diluting it in sterile saline, and injecting it into the corn samples. The quantitative measurement of aflatoxin was carried out by the ELISA (Enzyme Linked Immuno Absorbant Assay) method after 5 days. After that, TC1 strain extract was sprayed at the various concentrations of 1, 2, 3, and 4% onto samples. Aflatoxin was also determined by the same method after 2 days.

2.2.4. ELISA analysis of aflatoxin content

Sample preparation and total aflatoxin ELISA test kit protocol was implemented by the MaxSignal Total Aflatoxin ELISA Test Kit, Reference #: 1030-02 (BIOO Scientific Corp. 2008)

A standard curve was constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration in ng/mL on a logarithmic curve.

$$Relative\,absorbance = \frac{absorbance\,standard\,(or\,sample) \times 100}{absorbance\,zero\,standard}$$

The mean relative absorbance values for each sample were used to determine the corresponding aflatoxin concentrations of the tested TC1 strain concentrations in ng/ml from the standard curve. ELISA data were analyzed using the MaxSignal ELISA Analysis Program in Excel.

2.2.5. Data analysis

By Minitab 16 software.

3. RESULTS

3.1. Antagonistic ability of *Tricoderma* against toxin producing fungi

For *A. flavus* LA21, after 2 days' incubation, both colonies developed evenly in the control plate. Whilst in the antagonistic plate, *Tricoderma* developed strongly and inhibited the growth of *A. flavus* LA21. The PIRG value was 63.51% after 7 days of incubation (Figure 1 and Table 1).

For *A. parasiticus* NG10, after 3 days of incubation, the fungus producing aflatoxin came into contact with *Tricoderma* sp. TC1 in the antagonistic plate. During development on PDA medium, the PIRG value was 60.22% after 7 days of incubation (Figure 2 and Table 1).

Control

A. flavus LA21 -
A. flavus LA21

Experiment

Trichoderma sp. TC1 -
A. flavus LA21

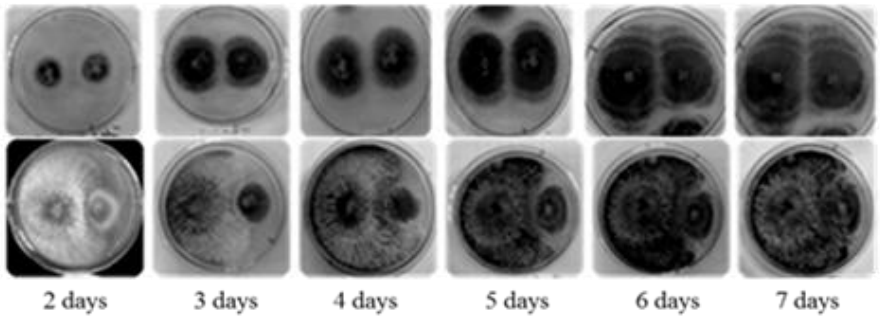

Figure 1. The antagonistic ability of *Tricoderma* sp. TC1 against *A. flavus* LA21

Where as: Left: Trichoderma colonies, Right: Aspergillus flavus LA21

Control

A. parasiticus NG10 -
A. parasiticus NG10

Experiment

Trichoderma sp. *TC1*-
A. parasiticus NG10

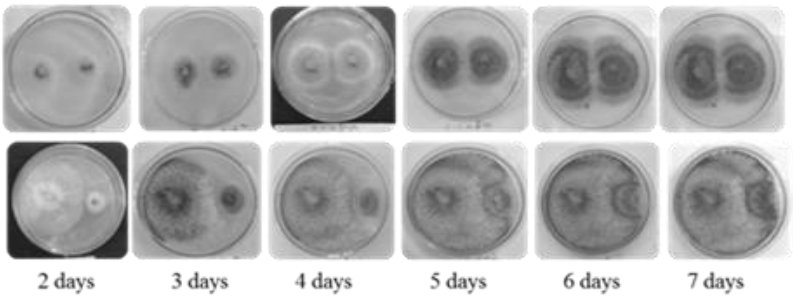

Figure 2. The antagonistic ability of *Tricoderma* sp. TC1 against *A. parasiticus* NG10

Note: Left: Trichoderma colonies, Right: Aspergillus parasiticus NG10

Table 1. The percentage inhibition of radial growth (PIRG) of *Trichoderma* sp. TC1 against fungi producing toxins during 7 days of incubation

Fungi producing toxins	Percentage inhibition of radial growth (PIRG, %)					
	2 days	3 days	4 days	5 days	6 days	7 days
Aspergillus flavus LA21	36.71^{e}	50.66^{d}	53.75^{c}	55.03^{c}	59.69^{b}	63.51^{a}
Aspergillus parasiticus NG10	31.18^{e}	36.53^{d}	45.43^{c}	50.99^{b}	58.01^{a}	60.22^{a}

Note: Means with different letters in each row are significantly different at α = 0.05.

The inhibition reached 50.66% (after 3 days) and 50.99% (after 5 days) for *A. flavus* and *A. parasiticus* NG10, respectively. The velocity of inhibition for *A. flavus* LA21 was higher compared to A. *parasiticus* NG10 in the first few days of incubation. After that, the inhibition increased gradually for *A. flavus* LA21 and rapidly for *A. parasiticus* NG10. Finally, the inhibitory effect of *Trichoderma* sp. TC1 against *A. flavus* LA21 was 63.51% and against *A. parasiticus* NG10 was 60.22% after 7 days.

3.2. Inhibitory effect of *Tricoderma* sp. TC1 extract on toxin-producing fungi

The inhibitory effect of *Trichoderma* sp. TC1 extract against *A. flavus* LA21 and *A. parasiticus* NG10 in PD medium is shown in Table 2.

For *A. flavus* LA21, when the *Trichoderma* sp. TC1 extract concentration increased from 0 to 4%, the inhibitory effect increased from 0 to 74.00%. With higher extract concentrations up to 3%, the yield of fungal biomass decreased down to 0.224 mg/ml which equaled a biomass reduction rate of 73.64%. When the extract concentration increased from 3 to 4%, the yield of fungal biomass changed insignificantly (0.224 to 0.221 mg/ml) and the reduction rate fell slightly (from 73.64 to 74.00%).

For *A. parasiticus* NG10, when the *Trichoderma* sp. TC1 extract concentration increased from 0 to 4%, the effectiveness of inhibition rose from 0 to 60.30%. As Table 2 shows, added extract concentrations up to 3% led the yield of biomass to drop down to 0.283 mg/ml and the biomass reduction rate decreased 60.03%. When the concentration increased from 3 to 4%, the yield of biomass (0.283 to 0.285 mg/ml) and the reduction rate (from 60.03 to 60.30%) varied inconsiderably.

3.3. Aflatoxin reduction of *A. flavus* LA21 and *A. parasiticus* NG10 by *Trichoderma* sp. TC1 extract

Aflatoxin levels of infected samples were measured after spraying the samples with the extract of *Trichoderma* at different concentrations. These extracts had effects on both strains, which were clearly indicated after 5 days.

Table 2. Dry biomass of *A. flavus* LA21 and *A. parasiticus* NG10 harvested in PD medium supplemented with extract of *Trichoderma* sp. TC1

Extract concentration (%)	*A. flavus* LA21		*A.parasiticus* NG10	
	Yield of biomass (mg/ml)	Biomass reduction rate (%)	Yield of biomass (mg/ml)	Biomass reduction rate (%)
0 (control)	0.850	0.00	0.713	0.00
1	0.435	48.82	0.431	39.55
2	0.350	58.82	0.378	46.98
3	0.224	73.64	0.285	60.03
4	0.221	74.00	0.283	60.30

Table 3. Aflatoxin content in experimental corn (ppb)

Trichoderma sp. TC1 extract (%)	1 day	3 days	5 days	7 days
Samples containing *A. flavus* LA21				
0	-	40.00	60.00	66.67
1	-	26.67	33.33	37.50
2	-	20.00	23.33	26.67
3	-	16.67	18.66	20.00
4	-	13.33	15.33	16.00
Samples containing *A. parasiticus* NG10				
0	-	41.30	62.00	68.00
1	-	28.66	36.67	40.00
2	-	22.00	26.67	29.33
3	-	20.00	24.60	26.66
4	-	19.33	20.00	21.33

The result shows that for both strains, when the *Trichoderma* sp. TC1 extract concentration went up from 0 to 2%, at the same time, the amount of aflatoxin in all infected samples was reduced 2 to 2.5 times when compared to the control sample. At the concentration of 4%, aflatoxin reductions were up to 3 times for *A. parasiticus* NG10 and 4 times for *A. flavus* LA21 5 and 7 days after spraying.

4. DISCUSSIONS

Trichoderma is a beneficial and non-toxic microorganism for plants and has been shown in many studies to be highly antagonistic towards fungi from soil microorganisms. *Trichoderma* decomposes organic matter and provides nutrients for plants, and it is these biological agents that have antagonistic abilities against fungal phytopathogens such as *Rhizoctonia solani*, *Fusarium*, *Rolfsii sclecrotium*, and *Verticillium* (Thanh *et al.,* 2014).

There are many studies about inhibiting fungi producing aflatoxin. According to Al-Othman *et al.* (2013), *T. harzianum*'s extract can inhibit *A. flavus* approximately 68.8 to 100%. The study of Baig *et al.,* (2012) stated that *T. harzianum* inhibited the growth of *A. flavus* about 67% after 5 days in dual culture tests.

Anita *et al.* (2012) showed that *T. harzianum* is an effective antagonistic fungus against plant pathogens, and their results showed the range of inhibition rate to be from 56.52 to 71.01% for *A. flavus*, and from 50.00 to 64.00% for *A. parasiticus*. Some strains of *Trichoderma* spp. can reduce aflatoxin B1 content from 65 - 99% in 5 days at 28 ± 2°C (Tsitsigiannis *et al.,* 2012). The use of *Trichoderma* strains with antagonistic abilities to control aflatoxin content produced by *Aspergillus* spp. has had good results in India (Verma *et al.,* 2007).

The results presented here also show the inhibitory ability of *Trihcoderma* sp. TC1 against *A. flavus* LA21, which was similar to Bagwan's study (2011) for *Tricoderma* strains T093, TC116, TC127, and G221.

In preliminary experiments, very positive results were obtained. *Trichoderma* sp. TC1 was capable of inhibiting fungi producing aflatoxin and the fungal extracts reduced aflatoxin levels in corn.

5. CONCLUSIONS

Trichoderma sp. TC1 was used to inhibit *A. flavus* LA21 and *A. parasiticus* NG10 with PIRG values of 63.51% and 60.22%, respectively, after 7 days in culture. Moreover, *Trichoderma* extract significantly reduced the levels of aflatoxin produced by these pathogenic fungi. These results support the development and promotion of the application of *Trichoderma* extract in the agro products preservation field.

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