


Antifungal and antiaflatoxigenic effects of a fumigant, ethanedinitrile, on *Aspergillus flavus*

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Abstract Antifungal effects of ethanedinitrile (EDN) and ethyl formate (EF) on *Aspergillus flavus* were investigated using radial growth bioassay. *A. flavus* was inoculated in the center of potato dextrose agar plate and treated with 1, 5, and 10 g/m³ of EDN, or 5, 35, and 70 g/m³ of EF. EDN strongly inhibited fungal growth. At 1 g/m³ of EDN, the fungal growth reduced by 22.2% by the final days of culture. The growth was completely inhibited by EDN at the concentration of 5 g/m³. Antiaflatoxigenic activity of both the fumigants was also assessed. Aflatoxin formation was determined using high-performance liquid chromatography with a fluorescence detector. *A. flavus* did not produce aflatoxin B₁ and aflatoxin B₂ at EDN concentrations >5 g. EF had no inhibitory effect on *A. flavus* growth and the formation of aflatoxin. These results suggest that EDN can

be an alternative for currently used antifungal agents to control fungal and aflatoxin contamination of stored grains.

Keywords Aflatoxin B₁ · Aflatoxin B₂ · *Aspergillus flavus* · Ethanedinitrile · Ethyl formate

Introduction

Stored grain products are damaged by insect pests, mainly by those in Coleopteran insects [1, 2]. These insects live on all of the continents of the world. Insect pests of stored grains can be divided into two main groups based on their infesting patterns [3]. *Sitophilus* and *Rhyzopertha* are representatives of insect pests that infest intact stored grains and are classified as primary insect pests. *Oryzophilus* and *Tribolium* are examples of secondary pests as they attack only damaged grains [3]. Chemical treatments with fumigants such as phosphine and ethyl formate (EF) have been used to control these pests [4]. However, some of the insect pests develop resistance to these fumigants [5].

Many studies have reported that fungal infection was one of the reasons for the deterioration of the quality of stored grain [6, 7]. Recently, mycotoxin contamination has been found in stored grains such as maize, rice, and wheat [7]. *Fusarium*, *Aspergillus*, and *Penicillium* species contaminate stored grains with accompanying production of mycotoxins such as fumonisin, aflatoxin, and ochratoxins [6, 7].

Generally, fumigation has been used to kill insect pests, but recently, combined control of insect pests and fungal contamination by fumigation has been attempted using ethanedinitrile (EDN) or cyanogen [8]. The chemical structure of EDN comprises two carbons and two nitrogens with *sp* hybridization between carbon and nitrogen, and

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one sp^3 hybridization between carbons (Fig. 1). It has been developed for soil fumigation and is an alternative for methyl bromide [9].

In the current study, we examined the antifungal activity of EDN and EF against *Aspergillus flavus* and their ability to suppress aflatoxin biosynthesis. Antiaflatoxic activity was determined using high-performance liquid chromatography (HPLC) with a fluorescence detector (FLD).

Materials and methods

Chemicals and microorganisms

EF and EDN were formulated in Dongbu Hannong Co. (Daejeon, South Korea). *A. flavus* (ATCC 22546) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). It produces two types of aflatoxins, B1 and B2. However, in our study, we found it to produce a small amount of aflatoxin G1.

Preparation of the spore solution

Malt extract agar (MEA: Difco Laboratories, Sparks, MD, USA) was used to grow the fungi. Fungi were grown on MEA at 30 °C for 4 or 5 days until fungal spores were formed. Once there is steady formation of spores, a solution containing 0.05% (v/v) Tween 80 was used to collect the spores. The collected spores were stored in 20% glycerol solution (v/v) at –70 °C till further use.

Fumigation of fungal spores with EF and EDN

Fungal spore suspension (10 μ L) containing 10^6 spores/mL was inoculated in the middle of potato dextrose agar (PDA, Difco Laboratories) medium in a plastic plate. After inoculation, the spores were fumigated with either EF or EDN for 30 min in a glass vacuum desiccator at 25 °C. All fumigation experiments were performed in triplicate. After the treatments, fungal spores were grown for 8 days and the diameter of the growth zones was measured daily for

comparison. Fungal spores inoculated under the same conditions, but not subjected to fumigation were the controls.

Aflatoxin analysis by HPLC-FLD after fumigation

After the diameters of growth inhibition zones were measured, 10 mL of 0.1% Tween 80 was added to the PDA medium and mixed in an agitator at 180 rpm for 5 min. This fungal suspension (10 mL) was shaken in a separating funnel with 30 mL of ethyl acetate for 5 min. After good separation, the solvent layer was collected and dried using a rotatory evaporator (RV10, IKA, Staufen, Germany). After evaporation, 2 mL of 50% methanol was added and allowed to stand for subsequent aflatoxin analyses using an HPLC-FLD [10]. All the conditions for HPLC analysis were as described in an earlier report [10].

Average and standard deviation of three replicates were calculated. Controls and experimental groups were compared using one-way ANOVA. The differences were considered significant if p values were lower than 0.05.

Results and discussion

As can be seen from the results depicted in Table 1, EDN completely suppressed *A. flavus* growth at concentrations of 5 and 10 g/m^3 during 8-day incubation. Fungal growth was observed after 3 days of incubation in the presence of 1 g/m^3 of EDN. However, the growth was significantly reduced by day 8 of incubation, in comparison with that of control groups. On the other hand, fumigation with EF did not show any inhibitory effect on *A. flavus* growth for 8 days in culture (Table 2). Even at EF concentrations ranging from 5 to 70 g/m^3 , there was no growth inhibition. These results indicate that fumigation with EDN might be used to control insect pests and fungal contamination at 5 g/m^3 concentration.

As mentioned in previous reports, LD₉₉ of EDN is 12.6 g/m^3 for controlling burnt pine longhorn beetle (*Arhopalus fesus* [Mulsant]) [11] and 43.5 g/m^3 for the larvae of longhorn beetle (*Anoplophora glabripennis* [Motschulsky]) [8]. Recently, EDN at the concentration of 50 g/m^3 has been approved for fumigation by the Australian authorities [12]. It indicates that fumigation with the same concentration of EDN can control *A. flavus* growth. It is interesting that the same dose of fumigant can control two different pests.

In the case of aflatoxin production, the control group generated about 6.8 μ g/mL of AFB1, 0.38 μ g/mL of AFB2, and 0.079 μ g/mL of AFG1 after 8 days of incubation. EDN completely inhibited the production of AFB1

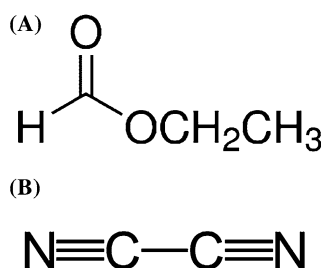


Fig. 1 Structures of tested fumigants, (A) ethyl formate and (B) ethanedinitrile

Table 1 Diameters (mm) of growth zones of *A. flavus* in a potato dextrose agar (PDA) medium after fumigation with ethyl formate or ethanedinitrile

Compound (g/m ³)	Time (days)							
	1	2	3	4	5	6	7	8
Control	5.4 ± 0.5 ^a	16.0 ± 1.0 ^a	23.0 ± 1.4 ^a	33.6 ± 1.1 ^a	41.6 ± 1.5 ^a	49.0 ± 1.0 ^a	58.2 ± 1.6 ^a	64.6 ± 0.5 ^a
Ethanedinitrile								
1	– ^b	– ^b	1.6 ± 3.6 ^b	11.6 ± 8.4 ^b	22.4 ± 7.6 ^b	31.2 ± 7.7 ^b	42.4 ± 7.5 ^b	50.2 ± 7.4 ^b
5	– ^b	– ^b	– ^c	– ^c	– ^c	– ^c	– ^c	– ^c
10	– ^b	– ^b	– ^c	– ^c	– ^c	– ^c	– ^c	– ^c
Ethyl formate								
5	8.2 ± 0.4 ^c	16.0 ± 0.7 ^a	22.6 ± 0.5 ^a	34.6 ± 1.1 ^a	43.0 ± 0.7 ^a	49.6 ± 0.5 ^a	58.0 ± 0.7 ^a	64.4 ± 0.9 ^a
35	6.6 ± 0.5 ^c	15.0 ± 1.0 ^a	23.2 ± 0.8 ^a	36.6 ± 1.1 ^a	44.4 ± 0.9 ^d	53.2 ± 2.0 ^d	59.0 ± 3.7 ^a	67.0 ± 2.9 ^d
70	5.4 ± 0.5 ^a	13.4 ± 0.5 ^c	20.8 ± 1.1 ^d	33.0 ± 1.2 ^a	42.8 ± 1.9 ^a	49.4 ± 0.9 ^a	57.8 ± 1.6 ^a	65.4 ± 2.1 ^a

Different superscript letters in the same column indicate significantly different from the control group ($p < 0.05$)

Table 2 Aflatoxin production in *A. flavus* fumigated with ethyl formate or ethanedinitrile

Compound (g/m ³)	Aflatoxin (μg/kg)			
	AFB1	AFB2	AFG1	AFG2
Control	6771.36 ± 1539.42 ^a	379.16 ± 113.93 ^a	79.32 ± 17.82 ^a	ND
Ethanedinitrile				
1	5740.70 ± 2568.25 ^a	418.08 ± 67.58 ^a	81.37 ± 9.92 ^a	ND
5	ND ^b	ND ^b	94.81 ± 7.35 ^a	ND
10	ND ^b	ND ^b	90.40 ± 4.62 ^a	ND
Ethyl formate				
5	7876.67 ± 2291.11 ^a	465.12 ± 101.07 ^a	91.76 ± 11.07 ^a	ND
35	7807.28 ± 1696.84 ^a	527.27 ± 168.25 ^a	80.99 ± 11.51 ^a	ND
70	6616.54 ± 2525.37 ^a	444.81 ± 134.84 ^a	79.61 ± 9.13 ^a	ND

Aflatoxins were expressed as ppb (μg/kg)

AFB aflatoxin B type, AFG aflatoxin G type

Different superscript letters in the same column indicate significantly different from the control group ($p < 0.05$)

and AFB2 at concentrations of 5 and 10 g/m³. There was no change in aflatoxin production at 1 g/m³ EDN concentration. In contrast, EF did not exhibit any inhibitory effect on the aflatoxin production. With these results, EDN will be considered to penetrate fungal membrane more strongly than EF. EDN will be slowly transformed to cyanides by cytochrome P450 species, and HCN will be a possible product by glutathione S-transferase [13]. However, EF will not be penetrated into the fungal membrane and it will not be metabolized in the fungal cell.

In our previous study, we showed that many natural products inhibited aflatoxin production via downregulation of aflatoxin biosynthetic genes [10, 14]. Among the natural products exhibiting antifungal and antiaflatoxic activities, methylenedioxy compounds such as piperine and piperonal are considered for suppressing aflatoxins in field grains [14]. In addition to the products mentioned in this

report, some essential oil constituents are also considered agents that can control *A. flavus* growth and production of aflatoxins [15]. Among the 20 constituents, geraniol, nerol, citronellol, cinnamaldehyde, and thymol completely inhibited fungal growth and aflatoxin formation at a concentration of 1000 ppm. Interestingly, they have been studied as fumigants to control stored grain insect pests [16]. With these possible candidates for their dual abilities to control insect and fungal pests of stored products, currently used fumigants should be studied for the dual insect and fungal pest control ability.

EDN use has been allowed for the control of stored product insect pests. Additionally, it has showed strong inhibitory effect on *A. flavus* growth and aflatoxin production. Therefore, EDN should be considered for dual-purpose use to control stored product pests in agricultural industries.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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