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Full Length Research Paper

In vitro inhibition of tomato *Fusarium* wilt causative agent by zearalenone from a soil inhabiting fungus

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To find naturally occurring compounds for the control of *Fusarium* wilt disease of tomato in the farming fields, 200 fungal strains were cultured in liquid media and screened for antifungal activity against *Fusarium oxysporium* f. sp. *Lycosperci*. The screening was carried out using *in vitro* antifungal assays and coincidentally a strain of *Fusarium* species was found to produce secondary metabolites, which were prepared as crude extracts that showed strong antifungal activity. The responsible compound was purified using column chromatography and the chemical structure of the purified compound was determined using nuclear magnetic resonance (NMR) spectroscopic techniques. A known compound, zearalenone, was found to be responsible for the antifungal activity, with a minimum inhibitory concentration (MIC) of 550 ± 10.5 ppm. This is a sufficient inhibition for *F. oxysporium* f. sp. *Lycosperci,* which is the causative agent for *Fusarium* wilt disease to tomato. Given the global abundance of zearalenone and its implication to human health, the results from this study suggest a sustainable manner of disposing of the mycotoxin and simultaneous benefit in control of the *Fusarium* wilt disease. It is recommended from this finding that it can be used to suppress fungal attack onto the roots of tomato plant to manage the *Fusarium* wilt disease.

Key words: *Fusarium oxysporium* f. sp. *Lycosperci*, tomato, submerged cultures, column chromatography, zearalenone.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most economically valuable horticultural crops globally (U.S. Department of Agriculture, 2008; Srivastava et al., 2010). However, production of this important crop is constrained in tropical countries with the major disease *Fusarium* wilt contributing to the loss (Jarvis, 1998). This is a soil-borne disease, which is widely distributed and difficult to control (Saremi and Burgess, 2000; Flood, 2003; Fawzi et al., 2009). Pathogenic strains of the fungus *Fusarium oxysporium* (Sacc.) W.C. Synder and H.N. Hans are the chief causative agents of the disease, and have specific host range in grasses, legumes and horticultural crops. *F. oxysporium* f. sp*. Lycopersici* is specific to tomato plant, and is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease, which is prevalent in tomato growing zones in Kenya, causes serious losses because soil and air temperatures are rather high during most of the season. The main challenge to production of tomatoes therefore is finding affordable and effective methods of controlling *Fusarium* wilt (Muthomi et al., 2002).

Synthetic chemical fungicides have been used for decades to control fungal diseases. Fungicidal treatment can keep tomato crops healthy or suppress the infection sufficiently (Song et al., 2004; Allen et al., 2004). However, the effectiveness of fungicides is threatened by development of resistance by the pathogen and in some

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instances, there are cases of efficacy concerns. Large scale agricultural systems depend on chemical control of *Fusarium* wilts, to a large extent on the fumigant methyl bromide that has various hazardous environmental implications (Fuchs et al., 1999). However, the use of methyl bromide is already restricted due to environmental and food quality considerations (Santos et al., 2006; Taylor, 1994). In addition to environmental concerns, there are high chances of development of multi-resistant strains of the fungal phytopathogens. Currently, no effective control measure is available for the control of *Fusarium* wilt, except only prevention strategies (Nel et al., 2006).

In the last two to three decades, efforts have been reported that involve the control of *Fusarium* wilt, using antagonistic fungi (Sabuquillo et al., 2006, 2009; De Cal et al., 2009). These anatagonistic interactions with other fungi typically have been classified as based on antibiosis, mycoparasitism and competition for nutrients (Hjeljord and Tronsmo, 1998). Certain soils are naturally suppressive to *Fusarium* wilt, a property due to the role of non-pathogenic *F. oxysporum* in the indigenous micro-biota of these soils. However, the spectrum of activity of microorganisms when they are used as biological control agents is usually narrower than that of synthetic pesticides (Baker, 1991; Janisiewicz, 1996). In addition, the inconsistent performance of microorganisms in commercial agriculture has limited their use as agents for controlling plant pathogens. Another major setback in biological control strategy stems from the fact that these biological control agents have their activities dictated by various soil conditions such as soil moisture; pH, and even temperature (Copping and Menn, 2000). Accor-dingly, control of a wide spectrum of pathogens using fungi still remains an unfulfilled goal in commercial agriculture (De Cal et al., 2009).

In this study, soil-borne fungal strains were isolated from soils collected from undisturbed habitats within Mount Kenya Forest. The soil samples were collected from sites that had different species of mushrooms sprouting under thick tree canopy. The main objective of this work was to search for strong antifungal secondary metabolites from defined fermentation cultures of the isolated fungal strains. The approach has been proven as a consistent source of chemical innovation in natural product screening programs (Getha et al., 2009). From a collection of fungal strains, a *Fusarium* species (accession number JO51525) was found to produce antifungal compounds from fermentation cultures, and the activity has been traced to zearalenone, as reported in this paper.

MATERIALS AND METHODS

Isolation of *F. oxysporium* f. sp. *Lycopersici*

The infected roots and stems of the tomato plant were washed using distilled water and then sliced into small pieces, targeting the inner tissues. The pieces from the inner portions were sterilized with 10% of sodium hypochlorite and rinsed with sterilized distilled water. The sterilization of these pieces was repeated three times before the pieces were placed on freshly prepared PDA plates and left to grow at room temperature until the hyphal strands emerged. The cultures were periodically checked for purity and successively sub-cultured, until pure cultures were obtained.

Isolation of the *Fusarium* species

The producing organism (*Fusarium* species) was isolated from a soil sample collected from an undisturbed site in Mount Kenya Forest that was heavily infested with freshly sprouted mushrooms. The producing organism and the soil samples were preserved in the culture collection in Integrated Biotechnology Research Laboratory (IBRL), Egerton University (accession number JO5125). The strain was cultured in 35 replicates of liquid media. The media was prepared by dissolving 10.0 g of molasses, 4.0 g glucose, and 4.0 g of yeast extract in 1.0 L of tap water. The pH of the media was adjusted to 5.5 using 1.0 M NaOH and 1.0 M HCl. The media was immediately sterilized by autoclaving at 121°C and 1.5 bars for 15 min, and left to cool on the sterile working bench. From a well-grown plate of the strain of the producing organism, 20 pieces of agar plugs (1 × 1 cm), with evident mycelia colony, were cut and used to inoculate all the sterilized media. Build-up of the biomass was used to monitor the rate of growth, as well as the antifungal activity against the test organism *F. oxysporium* was followed until peak and then the growth was stopped.

Preparation of crude extracts from the culture broth

Once growth was stopped, the mycelium was separated from the culture filtrate using Buchner filtration system under vacuum. From both the mycelium and culture filtrate, in each case, crude extract was prepared separately to give the mycelial crude extract (Kex) and culture filtrate crude extract (Kex). The combined mycelium from all the replicate cultures was soaked in 1.0 L of acetone for 4 h with constant stirring to extract intracellular secondary metabolites. The mycelia residues were filtered off and discarded, while the acetone extract was concentrated under reduced pressure using rotary evaporator. Once acetone was recovered, the remaining aqueous concentrate was extracted thrice with equal volume of ethyl acetate. The combined volume of ethyl acetate extract was dried using anhydrous sodium sulphate, before concentration to dryness. The dried crude extract was transferred into screw-capped vials, weighed, and always kept at 4°C, awaiting analysis and further processing.

Crude extract from culture filtrate, targeting extracellular secondary metabolites was prepared using liquid-adsorption technique. This involved adsorbing the secondary metabolites onto a reverse phase resin (Mitsubishi HP21 DIAION) packed in a glass column. The resin was pre-equilibrated in 1.0 M HCl for 72 h and thoroughly washed with distilled de-ionized water to neutral pH. The combined culture filtrate obtained above was then passed three times through the column packed with the resin. The extract-laden resin was rinsed with distilled de-ionized water before elution with organic solvents. The column was eluted with 1.0 L of acetone, followed in succession by 1.0 L of methanol, and the eluents collected. The eluents were separately concentrated under reduced pressure using rotary evaporator to remove acetone and methanol, respectively. The aqueous remains from each of the portions were separately extracted thrice with ethyl acetate. In each case, the combined volume of ethyl acetate extract was dried with anhydrous sodium sulphate and concentrated to afford a crude extract, which

was transferred into screw-capped vials and stored at 4°C awaiting further analysis and processing.

In vitro antifungal activity testing

Potato dextrose agar (PDA) was used as solid medium for agar diffusion assay. Test plates were prepared by autoclaving 39 g of PDA in 1.0 L of distilled water. The sterilized PDA media was allowed to cool to 40°C in a water bath. Meanwhile, 10 ml of sterilized distilled water was used to wash and pick spores of the test organism, *F. oxysporium* from fully-grown culture on PDA plates. This was repeated three or four times, and the combined volume of the spore suspension was thoroughly mixed using a Vortex machine. The concentration of the spores in the mixture was determined, and a definite volume of the mixture added to 250 ml of cooled PDA in the conical flask. The PDA mixture was swirled thoroughly to ensure uniformity and immediately, 16 ml was dispensed into sterile Petri dishes to give about 2.0×10^6 spores per plate. The antifungal test was carried out using agar diffusion assay test and incubated for duration of 96 h at room temperature (Rugutt et al., 2006).

Determination of minimum inhibitory concentration (MIC)

The crude extracts and pure compounds were tested for minimum inhibitory concentration (MIC) in a serial dilution assay using modified published methods. This was done by setting up an array of sterile test tubes, in which 2 ml of a suspension having 1×10^6 spores/ml in 2% (w/v) malt extract were added in each tube. A negative control contained the medium only (methanol), which is the solvent used to dissolve the crude extract and pure compounds. The concentration of the crude extracts and the pure compound ranged from 50 to 1500 ppm, and were incubated for 96 h at room temperature. The lowest concentration whose optical density corresponded to the blank was taken as the MIC.

Column chromatography

Chromatographic column was packed with silica gel 60 as slurry in cyclohexane. The column was then eluted with a mobile phase introduced as discrete solvent gradient system, with increasing polarity. The second solvent used was ethyl acetate (EtOAc), and finally the column was eluted with methanol (MeOH) or ethanol (EtOH). The pattern of separation in the collected eluent fractions were pooled into main fractions by spotting each of the eluent fractions onto TLC plate (20 × 20 cm, Macherey-Nagel). The developed TLC plate was viewed under dual fixed wavelength UV lamp (λ = 254 and 365 nm), and the spots were visualized by spraying with freshly prepared *p*-anisaldehyde solution before being heated to 115°C. The pooled main fractions were further subjected to antifungal activity testing to detect the fraction(s) with the active secondary metabolites. The described silica gel column chromatography procedure was repeated until the antifungal compound was purified.

Nuclear magnetic resonance (NMR) spectroscopy

The chemical structure of the purified antifungal compound was determined by performing 1-D and 2-D NMR experiments using Bruker ARX300 spectrometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were recorded at room temperature with NMR on Bruker AV300 spectrometer. The purified compounds were dissolved in deuterated chloroform ($CDCl_3$), and the solvent signals (δ_H 7.26 and δ_C 77.0) were used as reference. The two-dimensional NMR experiments (COSY, HSQC and HMBC) were recorded with gradient enhancements, using sine-shaped gradient pulses. The raw data was acquired, transformed, and the spectra evaluated with the standard Bruker software.

Statistical analysis

The data for the antifungal activity are presented as mean ± SD. For agar diffusion assay, statistical analysis was performed on five replicates in addition to controls. Minimum inhibitory concentration (MIC) was calculated for nine replicates. Differences between the control and treated replicates were analyzed using Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

The *Fusarium* wilt causative agent

The tomato fusarial causative agent was identified as *F. oxysporum* f. sp. *Lycopersici* from the cultures isolated from the infected plant. The taxonomic identification of *F. oxysporum* was performed based on morphological and conidial characteristics. The mycelium was colorless at first but after 7 days, it turned cream, then pale yellow and finally pale pink. The spores were found to be 3 to 5 celled, septate, and with gradually pointed and curved ends. The isolated *F. oxysporum* f. sp. *Lycopersici* was used as the test organism in the antifungal tests.

The zearalenone producing organism

From a screening of 200 strains of fungi isolated from soil samples collected from habitats located in undisturbed parts of Mt. Kenya and mimicking intense fungal-fungal interactions, *Fusarium* species (accession number JO5125) showed very strong antifungal activity of its crude extracts against the test organism *F. oxysporium* f. sp. *Lycopersici* (Table 1). They were further purified to target the extracellular secondary metabolites. The morphological and microscopic analysis of the strain of the producing organism showed that it is *Fusarium* specie. The mycelium gave a pink coloration when grown on a PDA plate, and gave characteristic microscopic features of *Fusarium* species. Further investigation using 18S RNA technology confirmed the strain as *Fusarium* species but the genus could not be unequivocally assigned.

Production of zearalenone from submerged cultures of *Fusarium* species (JO5125)

The cultivation of the producing organism in liquid still cultures took 21 days, and when growth was stopped, the culture broth gave 950 g of mycelium and 30 L of culture

Table 1. Antifungal activity results for crude extracts from the 20 strains of fungal cultures.

S/N	Strain (code)	Collection location	Crude extract	Screening results	Reproducibility
1	JO5057	Mt. Kenya	Kex	26±1.7	<10
			Mex	20±0.6	<10
2	JO5064	Mt. Kenya	Kex	14.3±1.2	Not observed
	JO5106	Mt. Kenya	Kex	20±1.3	12±2.6
3	JO5115	Mt. Kenya	Kex	10±1.2	Not observed
4	JO5125	Mt. Kenya	Kex	32±1.4	20±1.7
			Mex	20±1.5	10±1.8
5	JO5185	Mt. Kenya	Mex	20±1.4	15±1.4
6	JO5301	Mt. Kenya	Kex	10±1.6	Not observed
7	JO5304B	Mt. Kenya	Kex	33±1.6	20±0.8 diffuse
8	JO5315B	Mt. Kenya	Kex	38.7±1.2	14±1.2
9	JO5319	Mt. Kenya	Kex	19±0.8	22±1.6
10	JO5447A	Londiani	Kex	12±1.6	<10
11	JO5447B	Londiani	Kex	23±1.0	Not observed
12	JO5460A	Londiani	Kex	32±0.6	Not observed
13	JO5460B	Londiani	Kex	32±1.4	Not observed
14	JO5469	Londiani	Mex	15±1.6	<10
15	JO5505A	Londiani	Kex	30±1.2	Not observed
16	JO5506	Londiani	Kex	13.5±1.3	Not observed
17	JO5511A	Londiani	Kex	30±0.6	<10

filtrate (Figure 1). From the mycelium, 2.1 g of crude extract (Mex) was prepared, which was active in the antifungal test. The crude extract was eluted with solvent ratio 1:1 (v/v) of cylcohexane and ethyl acetate. The column was eluted with solvent system of increasing polarity from cyclohexane to ethyl acetate. The crude extract was subjected to silica gel chromatography, which afforded 560 mg of intermediate fraction (referred to as Intermediate Product I) (Figure 1). The intermediate product I was further subjected to silica gel chromatography twice and 40 mg of zearalenone was finally eluted with 9:1 cylcohexane/ethyl acetate. Zearalenone was found to be the main compound in the mycelia crude extract responsible for the observed activity. From the 30 L of culture filtrate, 2.4 g of crude extract was obtained from acetone eluent from liquid-solid adsorption on Mitsubishi HP21 DIAION resin (Figure 1). Meanwhile, the subsequent methanol eluent afforded 5.5 g of crude extract. However, only the acetone-eluted crude extract showed significant activity, as compared to the methanol-eluted crude extract, which had weak activity; hence the latter was not investigated further.

The acetone-eluted crude extract was subjected to silica gel chromatography using gradient solvent system of increasing polarity in cyclohexane/ethyl acetate/ ethanol mixture. The eluents from the solvent ratio 1:1 (v/v) cyclohexane/ethyl acetate afforded 680 mg intermediate product II, and the eluents from 100% ethanol gave 420 mg of intermediate product III (Figure 1). Intermediate product II eluted with ratio of 1:1 (v/v) cyclohexane/ethyl acetate, while intermediate product III eluted with 100% ethanol. Intermediate product II on further silica gel chromatography gave 12.5 mg of zearalenone that eluted with 100% ethyl acetate. Intermediate product III gave 15 mg of zearalenone when subjected to further silica gel chromatography that eluted with 100% ethyl acetate. At every stage during purification, the antifungal activity was tested to track the active compound, zearalenone (Figure 2). The crude extracts were tested in both agar diffusion assay and serial dilution assay. The results from both assays are given in Tables 2 and 3, respectively. The intermediate products I, II and III were tested in agar diffusion assay and the results are summarized in Table 4. The purified compound, zearalenone, was tested in serial dilution assay giving a MIC value of 550 ± 10.5 ppm.

Nuclear magnetic resonance (NMR) spectroscopy and structure elucidation of zearalenone

The identity of the purified antifungal compound was established by comparison of ^{1}H and ^{13}C NMR spectroscopic data with those reported from literature (Cordier et al., 1990). Two-dimensional experiments, COSY, HSQC and HMBC helped to piece up the molecule. The NMR spectrocscopic data corresponded perfectly with the data from the literature that led to

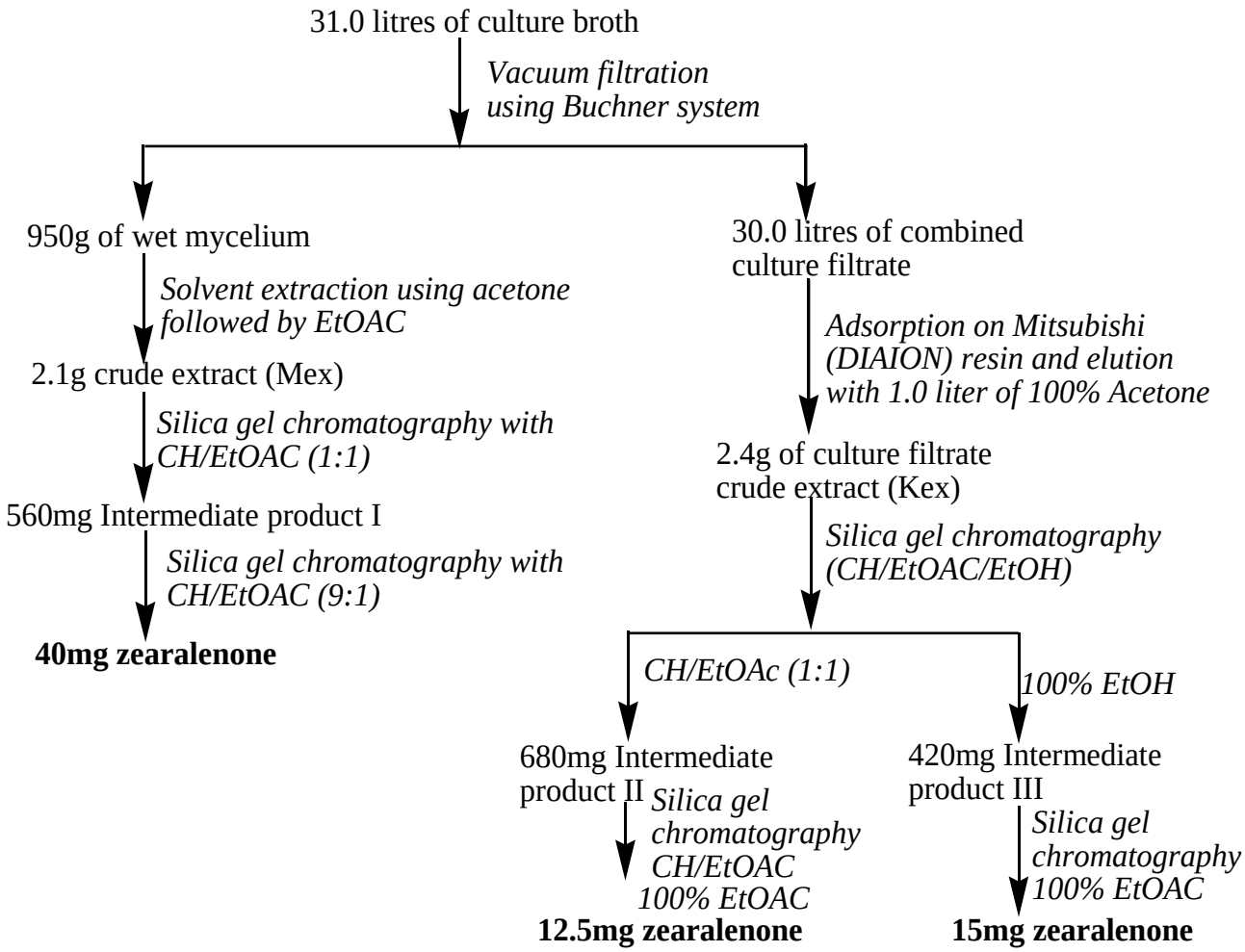

Figure 1. Purification scheme for zearalenone from submerged cultures of the producing organism (*Fusarium* species)

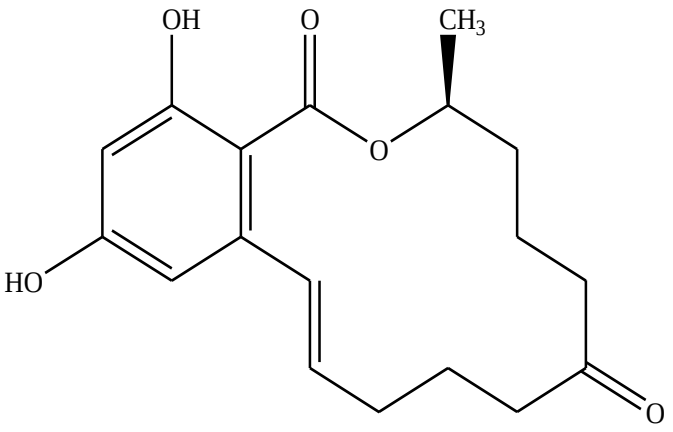

Figure 2. Chemical structure of zearalenone

Table 2. Diameters of inhibition zones in agar diffusion assay for the crude extracts from the cultures of Fusarium species (JO5125) against the F. oxysporium f. sp. lycopersici.

Crude extract	Diameter of inhibition zone (mm)
JO5125 Mex	14±1.0
JO5125 Kex (Acetone-eluted)	15±1.6
JO5125 Kex (Methanol-eluted)	10±1.2

Table 3. Minimum inhibitory concentration (MIC) for the crude extracts tested in serial dilution assay against *F. oxysporium* f. sp. *lycopersici*

Crude extract	MIC (Mean ± SD)
JO5125 Mex	430.3±15.0
JO5125 Kex (Acetone-eluted)	220±9.0
JO5125 Kex (Methanol-eluted)	455±15.0

Table 4. Diameters of inhibition zones for the intermediate products in agar diffusion assay against the *F. oxysporium* f. sp. *lycopersici*.

Intermediate product	Diameter of inhibition zone (mm)
I	15±1.2
II	12±1.1
III	15±1.3

unequivocal conclusions that the compound was zearalenone.

DISCUSSION

The intense antagonism of the *Fusarium* species (JO5125) and the test phytopathogen *F. oxysporum* was exploited, and the compound responsible for the activity was purified and the structure was elucidated to be that of zearalenone. Zearalenone effectively inhibited *F. oxysporum in vitro*. Such antagonistic interaction between strains of fungi belonging to the same genera has been reported, and has been a main focus of intense investigation (Fuchs et al., 1999). Fravel et al. (2005) found that non-pathogenic *F. oxysporum* CS-20 could effectively suppress *Fusarium* wilt of the tomato through a host-mediated mechanism. It provides biocontrol of *Fusarium* wilt through a host-mediated mechanism.

On cultivation of the *Fusarium* species (JO5125) in submerged cultures, the crude extracts prepared from mycelium (Mex) and culture filtrate (Kex) demonstrated antifungal activity against the test organism *F. oxysporum*. From the Kex crude extract, only acetone-eluted had significant activity, compared to the methanol-eluted. The acetone eluent of Kex was found to be more active than the mycelium crude extract (Mex). The acetone-eluent Kex had an activity of 220.2 ± 9.0 ppm, while the methanol-eluent was 455 ± 15.0 ppm, and for the activity for Mex, it was 430 ± 15.0 ppm (Table 2). The MIC from these extracts was quite significant as compared with other crude extracts such as from the essential oils from *Curcuma longa* that gave complete mycelia inhibition at 2000 ppm (Gurdip et al., 2002), and from *Flourensia* species, which gave total inhibition at 1500 ppm. Based on these observations, the antifungal activity was traced to zearalenone, a known fungal secondary metabolite. The MIC for zearalenone was found to be 550 ± 10.5 ppm, which was less than the antifungal activity observed for the crude extracts. The diminution may be attributed to concentration of zearalenone in the crude extract, and partly to synergistic effects of other compounds present. The method of processing may be responsible for the reduction, as well given that from the structure of zearalenone, stability factors are evident.

Zearalenone is a white crystalline solid produced by fermentation of various *Fusarium* species including *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense* and *F. semitectum*, which are common soil fungi in temperate and warm countries, and are regular contaminants of cereal crops worldwide (Bennett and Klich, 2003). Zeralenone is classified as a mycotoxin generally known for strong estrogenic effects in mice, pigs and rabbits. The exact mechanism of zearalenone is not completely established. Globally, it is ubiquitous and presents a potential danger for animal and human health only when absorbed in higher amounts or over a long time of exposure (Zinedine et al., 2007; Monbaliu et al., 2010). The *F. oxysporum* pathogen infects the tomato plant by penetrating through root tips or wounds on roots. Therefore, if zearalenone has to be applied to control the pathogen, it must be applied onto the soil. Therefore, there is a high probability that bioaccumulation of the mycotoxin might not appear in the fruits of the crop, since it is not sprayed directly on the plant. The plant system also has a mechanism of degrading the toxins, since the pesticide residual found in the harvested crop are of low concentration.

In conclusion, the antifungal activity found for zearalenone is significant and can be of scientific value in the control *Fusarium* wilt in tomato farming. This can lead to concurrent benefit in detoxifying and disposing zearalenone-contaminated food and feeds. However, detailed studies on the uptake by the tomato plant and bioaccumulation of zearalenone in the fruits should be investigated.

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Nicholas Karubiu of Egerton University. Andy Foster of University of Kaiserslautern, Germany identified the producing organisms (*Fusarium* species) using molecular techniques.

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