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In – vitro biological activities of *Emericella nidulans*, a new fungal antagonist, against *Fusarium oxysporum* f. sp. *lycopersici*

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Biculture test for antagonism showed that *E. nidulans* exerted inhibitory activity against *F. oxysporum* f. sp. *lycopersici*. In slide biculture, the conidia and hyphae of the pathogen were deformed, abnormal in shape and showed protoplast plugs inside. The crude extract of *E. nidulans* applied at the rate of 1000 ug/ml gave significantly higher inhibition of mycelial growth and spore formation while 10 ug/ml gave the largest mycelial diameter and lowest growth inhibition ($p<0.01$). Hexane and ethyl acetate extract registered significantly higher mycelial diameter and spore formation and lower mycelial growth and spore production inhibition while methanol extract produced significantly lower mycelial growth and spore production but higher mycelial growth and spore inhibition ($p<0.01$).

Introduction

Tomatoes (*Lycopersicon esculentum* Mill.) are one of the most widely cultivated, popular and important vegetable crops in the world. There is increasing demand in developed countries for organic tomatoes, as well as heirloom tomatoes to make up for flavor and texture faults in commercial tomatoes. However, the tomato crop is usually attacked by many kinds of diseases such as *Fusarium* wilt, bacterial wilt, and early blight. Among these diseases, *Fusarium* wilt is one of the most serious that can result in tremendous economic losses. It is caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen. In general, this pathogenic fungus is a limiting factor in the production of many crops and accounts for 10 – 20 percent yield losses annually which can reach as high as 100 per cent (USDA, 2008). It has become one of the most prevalent and damaging diseases wherever tomatoes are grown

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intensively because the pathogen persists indefinitely in infested soils. The methods used to control vascular wilt are either not very efficient or are difficult to apply. The best recommended way to control the disease is selecting resistant varieties of tomato (Silva and Bettiol, 2005).

Recently, biological control of plant pathogens has been the concern of most plant pathologists and many researchers. There are many new species of promising antagonists that can be used to control *Fusarium* wilt of tomatoes. Bioactive compounds extracted from different species of fungi were reported to inhibit the growth of many plant pathogenic fungi, including *Fusarium* that causes wilt of tomato (Kanokmedhakul *et al.,* 2006 and 2003; Thongsri and Soytong, 2004; Srinon *et al*., 2002, Suwannapong and Soytong, 2002). The bioactive compounds are Tricotoxin A50 extracted from *Trichoderma harzianum* PC01; and Chaetoglobosin C extracted from *Chaetomium globosum.* These compounds have been reported to elicit resistance or immunity in plants by inducing oxidative burst in plant cells (Nuchdonrong *et al*., 2004; Soytong *et al*., 2001). *E. nidulans* is one of the promising fungal antagonists that can be used to control the fungal wilt of tomato.

Materials and methods

Source of pure culture

Pure cultures of *F. oxysporum* f. sp. *lycopersici* were obtained from the laboratory of Biocontrol Research Unit and Mycology Section, Department of Plant Pest Management, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Thailand. The cultures were transferred into Potato Dextrose Agar (PDA) and incubated at room temperature. The morphological characteristics of *Fusarium* were studied under compound microscope

Biculture

The antagonistic fungus, *E. nidulans* strain EN was provided by Dr. Kasem Soytong of KMITL, Ladkrabang. Biculture test was done following the methods of Soytong (1989). A virulent isolate of *F. oxysporum* f. sp. *lycopersici* was used in bi-culture test with antagonistic fungus. An agar disc taken from the edge of radial growth of *F. oxysporum*, in PDA plate was obtained using a sterile cork borer. This was placed in one side of a potato dextrose agar (PDA) plate about 2.0 cm from the center. An agar disc of *E. nidulans*, the antagonistic fungus, was placed on the other side of the plate. For

control treatment, the agar plug of only pathogen or antagonist was placed on PDA plates.

The bi-culture plates were incubated at room temperature until colony of control grew to full plate. At this point, colony diameter was measured using ruler and spores were counted using hemacytometer. Percentage of growth inhibition and spore inhibition of pathogen was calculated using the formula below:

$$\% \text{ inhibition} = \frac{A - B}{B} \times 100$$

Where:

A = colony diameter or conidia number of pathogen in control

B = colony diameter or conidia number in biculture

Slide biculture

Agar blocks of *E. nidulans* and *F. oxysporum* were grown side by side with a distance of approximately 1 cm in a glass slide. The slides were then placed into a sterilized Petri dish lined with moist sterile filter paper as moist chamber. It was periodically observed for almost two weeks under the compound microscope.

Bioactivity assay of crude extracts of *E. nidulans*

F. oxysporum f. sp. *lycopersici* was subcultured from a previous experiment and was transferred onto potato dextrose agar and incubated at room temperature for five to seven days before the experiment. Crude extracts were obtained from *E. nidulans* strain EN 01 by Dr. Somdej Kanokmedhakul, Department of Chemistry of Khon Khan University, Thailand. The bioactive compounds were extracted using hexane (EN/H), ethyl acetate (EN/E) and methanol (EN/M). According to Kanokmedhakul (2008), the isolated compounds were from the 30-day old mycelial culture of *E. nidulans* in potato dextrose broth.

These crude extracts were tested to determine whether they could inhibit the growth of *F. oxysporum* f. sp. *lycopersici*. The different concentrations such as 0, 10, 50, 100, 500 and 1,000 µg/ml were evaluated. The bioactive compounds in each concentration were dissolved by using 2% dimethylsulphide (DMSO), and then autoclaved at 121^{o} C, 15 psi for 20 minutes, and poured into sterilized petri dishes about 5 ml/plate.

The pathogen was transferred into the test plates by cutting agar discs from actively growing colony by a cork borer. The disc was placed at the center of

Petri dishes with each concentration and incubated at room temperature. The colony diameter (cm) was measured every 24 hours until the control plates were fully ramified. The spores were counted using haemocytometer.

Results and discussion

Emericella nidulans perfect stage of Aspergillus nidulans

Colony is growing at room temperature, dark green and brown. Fruiting structure is globose, 125-150 um (Domsch *et al*., 1993) surrounding with hullee cells, ascospores purple red, lenticular, smooth-walled, with two equatorial crests (Fig.1).

Anamorph (Imperfect state) is *Aspergillus nidulans* according to the report of Domsch *et al.* (1993). The imperfect stage is classified into *A. nidulans* which produces conidia on philalide, phialides borne in head or vesicle that stand on phialophore and foot cell (Domsch *et al*., 1993).

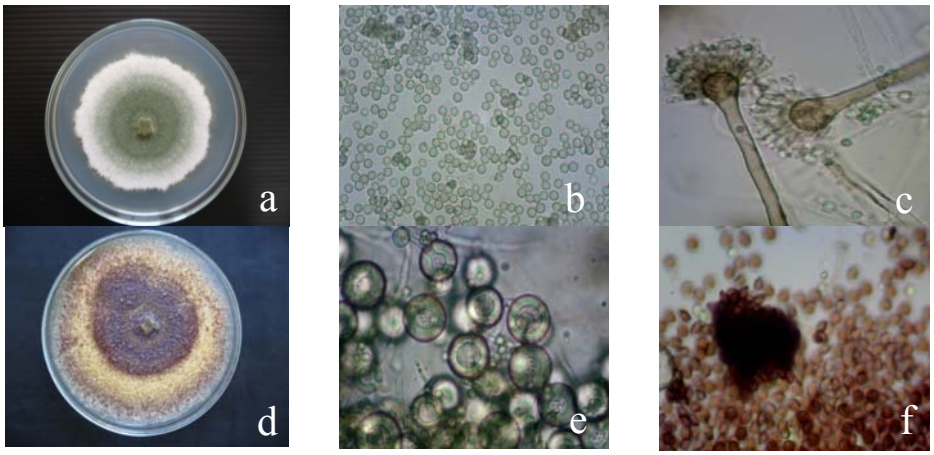


Fig. 1. Characteristics of *Emericella nidulans* perfect stage of *Aspergillus nidulans*. Imperfect stage on PDA (a), conidia (100x) (b), Thalli of imperfect staged (c). perfect stage on PDA (d), hullee cells (400 X) (e) and ascospores (400x) (f).

Plate biculture of E. nidulans and F. oxysporum f. sp. lycopersici

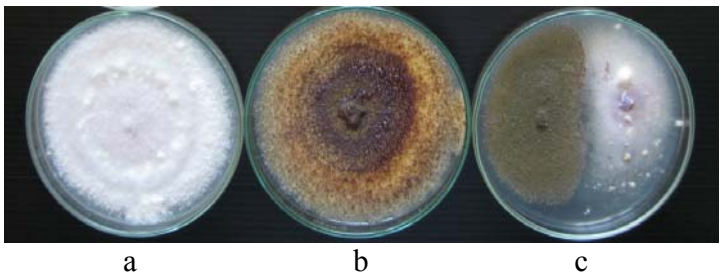
Biculture of *E. nidulans* and *F. oxysporum* f. sp. *lycopersici* was prepared to determine the possible interaction between the biological antagonist and the pathogen. Table 1 and Fig. 2 present the colony diameter and number of spores formed by *F. oxysporum* f. sp. *lycopersici.* The pathogen grown in the control plate grew faster and significantly formed larger colony diameter with a mean of 8.97 cm while those in the biculture plate produced smaller colony with a mean diameter of 6.17 cm. The *E. nidulans*, the fungal antagonist, caused 31.18% inhibition of mycelial growth of *F. oxysporum.*

Meanwhile, the mean number of spores produced by *F. oxysporum* f. sp. *lycopersici* in control plate was 4.43 x 10^8 spore/ml which was significantly higher than in biculture plate with a mean of 1.23 x 10^8 spore/ml. The spore formation of *F. oxysporum* was inhibited by 71.23% (b). This result suggests that *E. nidulans* can exert inhibitory effect on the mycelial growth and sporulation of the test pathogen. Soytong (1988) reported that in testing antagonistic fungus against fungal pathogen in biculture plate, one should consider the ability of the antagonist to inhibit spore production. Spores served as inoculum of the pathogen. The result of the present study clearly demonstrated that *E. nidulans* is a promising antagonistic fungus against *F. oxysporum* f. sp. *lycopersici.*

Table 1. Mean colony diameter (cm) and mean number of spore of *Fusarium oxysporum* f. sp. *lycopersici* at 21 days in biculture**.**

Treatment	Colony Diameter	Number of Spores (x 10^8)
Pathogens Alone	8.97[a]	4.43[a]
Pathogen in Biculture	6.17[b]	1.23b
Percent Inhibition	31.18	71.23

Means followed by a common letter in each column are not significantly different by DMRT at $P < 0.01$.



a b c

Fig. 2. Biculture antagonistic test between *Emericella nidulans* and *Fusarium oxysporum* f. sp. *lycopersici* at 30 days, *Fusarium oxysporum* f. sp. *lycopersici* (a),, *Emericella nidulans* (b), and biculture (c).

Slide biculture of E. nidulans and F. oxysporum f. sp. lycopersici

Chang and Kommedahl (1968) described a similar reaction in some strains of *C. globosum* and *C. cochliodes.* Similar observation was reported by Soytong (1992) which showed bioactivity between *C. cupreum* and *F. oxysporum* f. sp. *lycopersici.* However, in this experiment, it was also observed that macroconidia, microconidia and hypha of the tested pathogen were broken, and the shape of conidia was normal. (Fig. 3) Morover, the protoplast plug was inside the cells and some were released from the fungal cells.

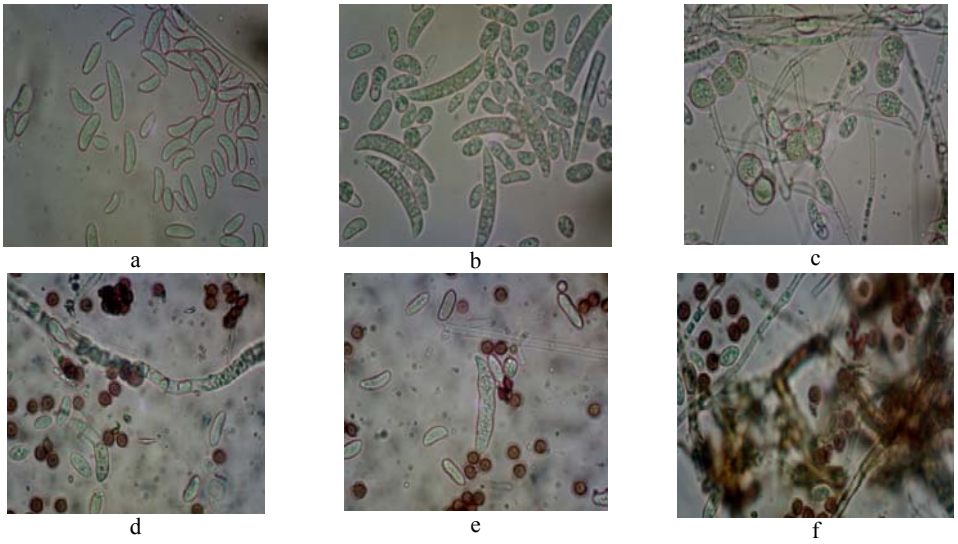


Fig 3. *Emericella nidulans* and *Fusarium oxysporum* f . sp. *lycopersic* under binocular compound microscope: *E. nidulans*, normal microconidia (a), normal micro- and macroconidia (b), normal chladomyspores (c), protoplast plug inside the mycelium of *F. oxyporum* f. sp. *Lycopersici* (d), abnormal macroconidia (f), and broken hyphae of *F. oxyporum* f. sp. *Lycopersici* (e).

Mycelial growth and inhibition

The diameters of mycelia and percent inhibition of mycelial growth as influenced by crude extract from *E. nidulans* at different concentrations are shown in Figs. 4 and 5. Generally, all the bioactive compounds extracted from *E. nidulans* inhibited the mycelial growth and proliferation of *F. oxysporum* f. sp. *lycopersici*. Hexane and ethyl acetate extracts registered significantly higher diameter and lower mycelial growth inhibition while those applied with methanol extract produced significantly lower mycelial growth but higher

mycelial growth inhibition. This implies that methanol extract is better than hexane and ethyl acetate extract. Probably, more bioactive compounds in *E. nidulans* were extracted using methanol as solvent.

The crude extract of *E. nidulans* applied at the rate of 1000 µg/ml caused significantly higher inhibition of radial mycelial growth and smaller mycelial diameter of pathogen with mean values of 35.26% and 3.20 cm, respectively. On the other hand, crude extract applied at the rate of 10 µg/ml gave the largest mycelial diameter with a mean of 4.82 cm and lowest growth inhibition with a mean of 3.83%. The result suggests that crude extract from *E. nidulans* could significantly inhibit the colony growth of the pathogen even at lower concentrations. Moreover, the percent inhibition of mycelial growth increases with an increase in the concentration of the extract.

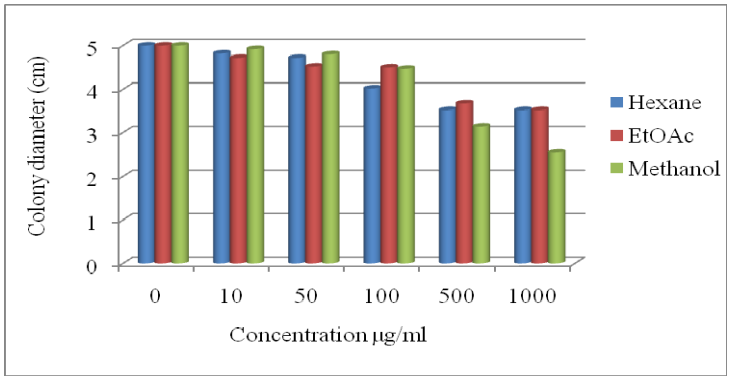


Fig. 4. Mean diameter (cm) of mycelial growth of *F. oxysporum* f. sp *lycopersici* at different concentrations of *E. nidulans* crude extract.

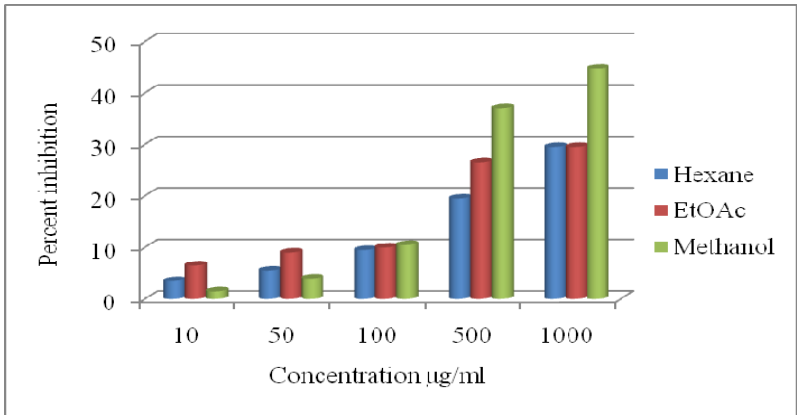


Fig. 5. Mean percent inhibition of mycelial growth of *F. oxysporum* f. sp. *lycopersici* at different concentrations of *E. nidulans* crude extract

Methanol extract of *E. nidulans* applied at the rate of 1000µg/ml gave significantly lower mycelial diameter and higher per cent mycelial growth inhibition. The result indicates that this is the most effective treatment combination that could give maximal mycelial growth inhibition.

Spore formation and inhibition

The mean number of spores produced by *F. oxysporum* and the percent inhibition of spore formation are presented in Figs. 6 and 7. The number of spores produced by the pathogen was significantly affected by the extract used. The highest number of spores formed and lowest percent inhibition was observed in those applied with ethyl acetate extract with mean values of 0.86 x 10^8 per ml and 8.30%, respectively. On the other hand, hexane and methanol extracts caused significantly lower spore production and higher percent spore inhibition. This finding implies that methanol and hexane extracts are better than ethyl acetate which could be attributed to the ability of these solvents to extract more bioactive compounds from *E. nidulans*.

Meanwhile, results further showed that a decrease in the diameter of mycelial colony was observed as the concentration of the crude extract increased. The widest diameter was observed in *F. oxysporum* treated with 10 µg/ml while the smallest diameter was noted in those applied with 1000 µg/ml. However, the percent inhibition of spore formation increased with an increase in the concentration of the crude extract. Extract applied at the rate of 500 µg/ml and 1000 µg/ml significantly inhibited the spore formation compared to other concentrations suggesting that higher concentration of the extract is more affective.

Methanol extract of *E. nidulans* applied at the rate of 1000 µg/ml gave the highest inhibition of spore formation with a mean of 86.13% which implies that this is the best treatment combination.

Conclusion

E. nidulans can exert inhibitory activity against the mycelial growth and spore production of *F. oxysporum* f. sp. *lycopersici*. Methanol extract of *E. nidulans* is more effective than hexane and ethyl acetate crude extract in inhibiting the mycelial growth and spore production. Moreover, as the concentration of the crude extract increases, its effectiveness also increases.

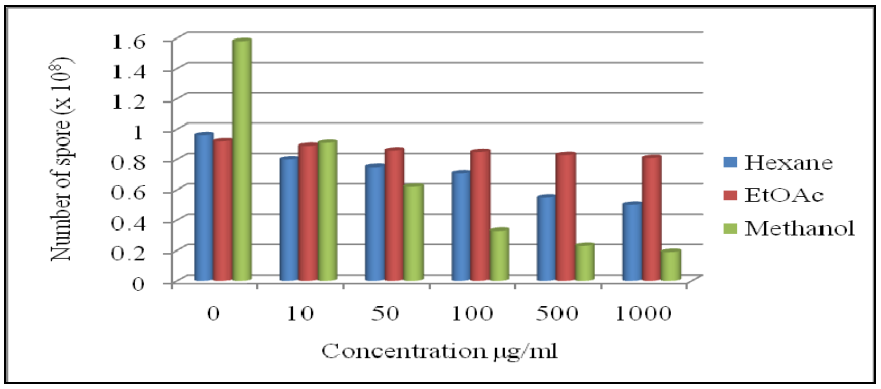


Fig. 6. Mean number of spores (x 10^8) of *F. oxysporum* f. sp. *lycopersici* at different concentrations of *E. nidulans* extract.

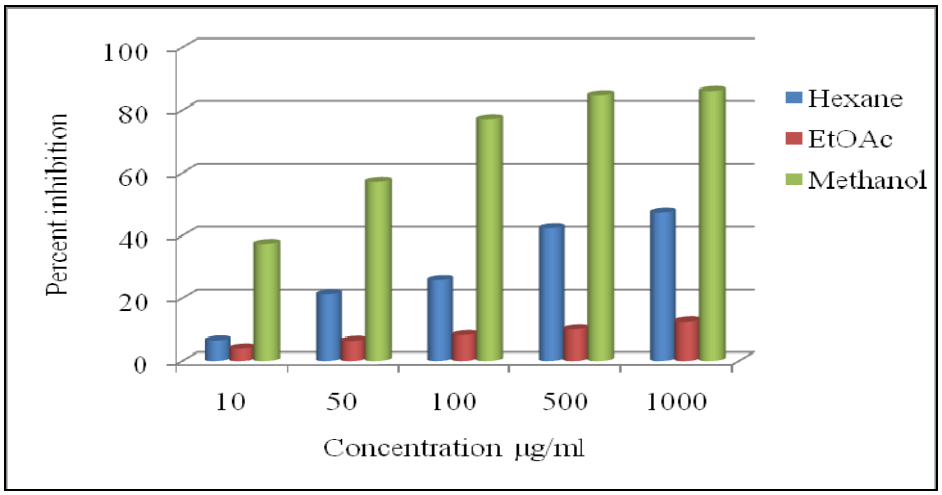


Fig. 7. Mean percent spore inhibition *F. oxysporum* f. sp. *ycopersici* at different concentrations of *E. nidulans* extract.

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