Efficacies of antagonistic fungi against Fusarium wilt disease of cucumber and tomato and the assay of its enzyme activity

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Fusarium oxysporum f.sp. *cucumerinum* and *F. oxysporum* f.sp. *lycopersici* (plant pathogens) were isolated from cucumber and tomato wilt and those virulence isolate's activities were inhibited using several antagonisitic fungi by bi-culture test. The results obtained shown that most of antagonistic fungi were effectively inhibited the spore production of the plant pathogens where the growth inhibition (GI) of the spore production were greatly increased in some of the antagonistic fungi used. This was happen due to the activity of enzymes produced by the antagonistic fungi. To elucidate this evidence, the activity of the cellulose and hemicellulase (xylanase) produced by all isolates were assayed. It was found out that *T. hamatum* WS01 and *Penicillium* sp.WS01 shown a higher diameter of clear zone than the other fungi on cellulose activity. On the other hand, the highest hemicellulase activity was seen in *T. hamatum* WS01 and WS01.

Key words: Antagonistic Fungi, *Penicillium*, *Trichoderma*, *Gliocladium*, *Nigrospora*, *Fusarium oxysporum***,** enzyme

Introduction

Biological control agents have been reported to be an effective method to control plant pathogens. Among the plant pathogens, *F. oxysporum* were found to be the cause for the most serious disease of commercial plants in the world. The plant pathogens produce enzymes and toxin (mode of action) that degrade the plant cell wall components. (Moreira *et al.,* 2005; Omokolo *et al.,* 2003). Many studies had been conducted to inhibited the activity of the *F. oxysporum* f.sp. *cucumerinum* (wilt disease of cucumber) and *F. oxysporum* f.sp.

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lycopersici (wilt disease of tomato); the most importance pathogenic fungi. Recently, there were many reports about the application of antagonistic fungi in controlling plant disease such as the use *Penicillium* species studied by Soytong *et al.* (2005), *Trichoderma* species by Soytong *et al.* (2005), *Gliocladium virens* by Howell (1991), *Cheatomium* species by Soytong *et al.* (2005) *Bacillus* species by Sadfi *et al.* (2001), and *Paecilomyes* species by Kiewnick and Sikora (2006).

Hydrolytic enzymes of antagonistic microorganisms have been considered to play an important role in the biological control of plant pathogens. Many enzymes were isolated from various kind of strains and their activities were assayed such as the assay of enzyme isolated from *Trichoderma* species by Harman *et al.* (2004), *Gliocladium virens* by Di-Pietro *et al*. (1993), *Paenibacillus* and *Streptomyces* species by Singh *et al.* (1999) and *Stenotropmonas maltophilia* by Zhang and Yuen (2000).

In the present study, we investigated the efficacy of antagonistic fungi on the growth of *F. oxysporum* f.sp. *cucumerinum* (wilt of cucumber) and *F. oxysporum* f.sp. *lycopersici* (wilt of tomato) and the assay of its enzyme activity.

Materials and methods

Isolation and identification of antagonistic fungi and plant pathogens

F. oxysporum f.sp. *cucumerinum* and *F. oxysporum* f.sp. *lycopersici* (pathogenic strains) were isolated from wilt of cucumber and tomato, respectively. Pathogenicity test for all isolates of cucumber and tomato was done by Koch's Postulate method. Then, the most virulent isolate was selected for the further experiments. The antagonistic fungi used were *Nigrospora* sp. (WS01) isolated from *Dactyloctenium aegyptium* (L.) disease by tissue transplanting and *Penicillium* sp. (WS01), *T. harzianum* (WS01), *T. hamatum* (WS01), *T. hamatum* (WS01), *T. viride* (WS01) and *G. virens* (WS01) hich were isolated from forest soil in Thailand by soil plate technique.

Efficacy of Antagonistic Fungi on growth of plant pathogens by bi-culture test

The antagonistic fungi and the plant pathogens were cultured on potato dextrose agar (PDA) for 7 days at room temperature (28-30°C). Then, 0.3 cm diameter of antagonistic fungi colony was cut and transferred to the opposite colony of plant pathogens and further incubated at room temperature for 7-10 days. Four replicates were taken in each of the experiments. Data were

collected and the statistical analysis of variance (ANOVA) was determined using the Duncan's multiple range test (DMRT) at P=0.01.

Extracts of Antagonistic Fungi's Activity

Crude extracts from *Nigrospora* sp. (WS01), *Penicillium* sp. (WS01), *T. harzianum* (WS01), *T. hamatum* (WS01), *T. hamatum* (WS02), *T. viride* (WS01) and *G. virens* (WS01) were used. Hexane, ethyl acetate and methanol were used as solvent for the extraction. After extraction, the experiment was carried out in rotary evaporator to remove the solvents from the extracts. The crude extracts were then tested for inhibitory activity against the growth of plant pathogens on PDA at concentrations of 0, 10, 50, 100, 500 and 1000 µg/ml. The plant pathogens were grown on PDA for 7 days at room temperature (28-30°C). The 0.3 cm diameter plug of colony was placed in the centre of PDA mixing with each concentration of the extract, then incubated at room temperature for 7-10 days. The experiment was Completely Randomized Design with four replications. Data were collected as colony diameter (cm) and spore production. The statistical analysis of variance (ANOVA) was determined using the Duncan's multiple range test (DMRT) at P=0.01 and effective dose of ED_{50}.

Cellulase activity assays

The antagonistic fungi were grown on yeast extract peptone agar medium (yeast extract 0.1 g, peptone 0.5 g, agar 16 g, congo red 0.2%, distilled water 1000 ml) supplemented with 0.5% Na-carboxymethyl cellulose (CMC). The clear zone surrounding the colonies (cm) which indicated the cellulolytic activity was observed at 5 days. The experiment was done by using Completely Randomized Design with four replications.

Hemicellulase (xylanase) activity assays

The antagonistic fungi were cultured on xylan agar medium (xylan 1 g, rice bran 5 g, yeast extract 1 g, agar 16 g, distilled water 1000 ml). After incubation, xylan utilization on the medium was observed for clear zone. Dilute iodine solution was used to stain the agar plates and a yellow-opaque area around colonies indicated the xylan degradation while the reddish purple colour indicated for the undegraded xylan (Choi *et al*., 2005). The reaction was determined after day 4 and represented as follows; +++ = strong, ++ = medium, + = weak and - = no-reaction. The experiment was done by using Completely Randomized Design with four replications.

The experiment was further conducted to find the hemicellulose activity by growing the antagonistic fungi in liquid medium consist of KH_2PO_4 1 g, K_2HPO_4 0.4 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 0.013 g, L-asparagine 1.5 g, NH_4NO_3 0.5 g and rice bran 5 g, 1 ml of trace element solution ($ZnSO_4.7H_2O$ 0.0264 g, $MnCl_2.4H_2O$ 0.02 g, $CaCl_2.4H_2O$ 0.004 g, $CuSO_4.5H_2O$ 0.4 g, distilled water 1000 ml), at pH 7. The 0.3 cm diameter plug of fungal colony was transferred into the flask of liquid medium, then incubated for 9 days at room temperature (28-30°C) under static condition. Hemicellulase activity was analyzed at 3, 6 and 9 day intervals by using the method of Nelson (1944) and Somogyi (1952). The rate of release of reducing sugars was determined by using xylan as substrate and D-xylose as standard. Reducing sugars was determined using a spectrophotometer at 540 nm. One unit of enzyme was defined as the amount of enzyme releasing 1 μmole of glucose equivalent one minute (min^{-1}).

Results

Isolation and identification of antagonistic fungi and plant pathogens

F. oxysporum f.sp. *cucumerinum* and *F. oxysporum* f.sp. *lycopersici*, isolated from wilt of cucumber and tomato, respectively. Antagonistic fungi strains; *P. rubrum* (WS01), *T. harzianum* (WS01), T. *hamatum* (WS01; WS02), *T. viride* (WS01), and *G. virens* (WS01), isolated from forest soil and *Nigrospora* sp. (WS01), isolated from *Dactyloctenium aegyptium* (L.) disease.

Bi-culture test's result shown that *T. harzianum* WS01 was inhibited highest against spore production of *F. oxysporum* f. sp. *cucumerinum* whereas *Penicillium* sp. WS01 was inhibited highest against *F. oxysporum* f. sp. *lycopersici* (Table 1 and Fig. 1).

Extracts of Antagonistic Fungi's Activity

All the extracts of antagonistic fungi showed a good potency of an antifungal against spore production of plant pathogens. The percentage of growth inhibition of spore production (GI) was high concentration of crude extracts (Table 2 and Fig. 2).

Cellulase activity assays

Results shown that cellulase and hemicellulase produced by all antagonistic fungi tested. *T. hamatum* WS01 and *Penicillium* sp. WS01 was seen the highest diameter of clear zone (cm) on cellulase activity (Table 3).

Table 1. Effect of antagonistic fungi against spore production of *Fusarium oxysporum* f. sp. *cucumerinum* by bi-culture test.

Antagonistic fungi	Number spore production and growth inhibition of *Fusarium oxysporum* f. sp. *cucumerinum*[1/]					
	Macroconidia			Microconidia		
	Control	Bi-culture	GI [1/] (%)	Control	Bi-culture	GI(%)
Nigrospora sp. WS01	16.43 a[2/]	7.40 b	54.96 b[3/]	19.05 a	8.17 b	57.11 b
Penicillium sp. WS01	13.55 a	2.32 b	82.87 a	18.26 a	2.83 b	84.50 a
T. harzianum WS01	15.24 a	1.10 b	92.78 a	17.81 a	1.04 b	94.16 a
T. hamatum WS01	15.56 a	10.33 b	33.61 b	17.16 a	15.60 a	9.09 c
T. hamatum WS02	13.07 a	6.98 b	46.59 b	18.58 a	7.61 b	59.04 b
T. viride WS01	11.87 a	1.30 b	89.04 a	17.96 a	5.49 b	69.43 b
G. virens WS01	15.17 a	1.72 b	88.66 a	15.15 a	1.37 b	90.95 a

[1/]Growth Inhibition (GI) = R1-R2/R1*100; R1=Number spores of tested pathogen produced in control plate and R2 = Number spores of tested pathogen produced in bi-culture test.
[2/]Means followed by the same letter within a row are not significantly different as determined by Duncan multiple range test at $p = 0.01$.
[3/]Means followed by the same letter within a column are not significantly different as determined by Duncan multiple range test at $p = 0.01$.

Hemicellulase (xylanase) activity assays

From the results, it was observed that *T. hamatum* WS01 and *Penicillium* sp.(WS01) had the highest diameter of clear zone (cm) on cellulose activity that was showed the yellow-opaque area around colonies indicated highest xylan degradation (Table 3 and Fig. 3, 4).

Discussion

Bi-culture antagonistic tests of *Nigrospora* sp. WS01, *Penicillium* sp. WS01, *T. harzianum* WS01, *T. hamatum* WS02, *T. hamatum* WS02, *T. viride* WS01 and *G. virens* WS01 against *F. oxysporum* f.sp. *cucumerinum* and *F. oxysporum* f.sp. *lycopersici* were proved to be effective isolates to control the tested pathogens as similar reports by several workers. In general, there are many reports stated that the antagonistic fungi; like *P. oxalicum* could inhibit *F. oxysporum* f.sp. *lycopersici.*, *P. chrysogenum* protects cotton plants against wilt disease (*F. oxysporum* f. sp. *vasinfetum*) and increases yield under field condition (Dong and Cohen, 2001; Saidkarimov and Cohen, 2003), Trichoderma species control *F. oxysporum* f. sp. *melon* (Datnoff *et al*., 1995)

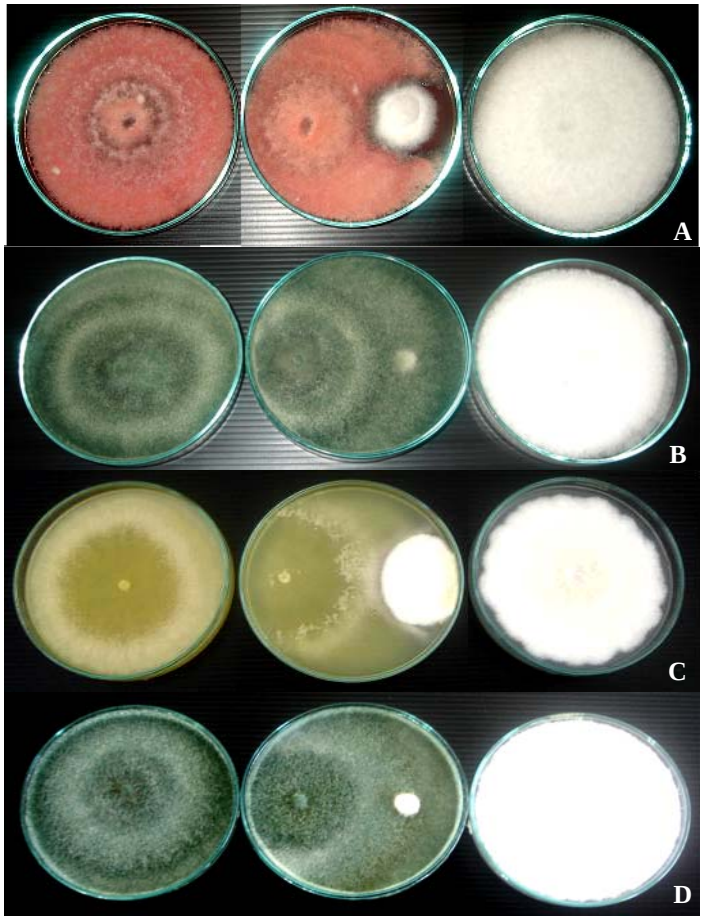

Fig. 1. Effect of antagonistic fungi on the growth of the *Fusarium. oxysporum* f.sp. *cucumerinum*; A: *Nigrospora* sp. WS01, B: *Trichoderma harzianum* WS01, C: *Trichoderma. hamatum* WS02 (MJUP02); D: *Gliocladium virens* WS01.

But we need to know the control mechanism, then the extraction of those promising antagonists had been cultured and extracted by methanol, ethyl acetate and hexane to get the different extracts for bioactivities against tested plant pathogens as the work of Kanokmedhakul *et al*. (2006) With this, in our experiments showed that the tested crude extracts from those promising antagonist could inhibit the tested pathogens.

Table 2. Spore production of *Fusarium oxysporum* f. sp. *cucumerinum* by crude extracts from antagonistic fungi.

Crude Extracts of Antagonistic fungi	Number macroconidia of *F. oxysporum* f.sp. *cucumerinum* (x 10^6 spore/ml) at each concentration (µg/ml)					
	0	**10**	**50**	**100**	**500**	**1000**
***Nigrospora* sp.WS01**						
Hexane	11.39a[1/]	5.93 b	4.82 b	2.61 c	1.86 c	1.72 c
Ethyl Acetate	9.19 a	8.16 ab	6.83 b	4.18 c	3.21cd	1.69 d
Methanol	10.56 a	4.67 b	4.45 b	1.86 c	1.51 c	1.48 c
***Penicillium* sp.WS01**						
Hexane	10.89 a	10.49 a	10.34 a	7.52 ab	4.96 b	4.13 b
Ethyl Acetate	10.95 a	13.40abc	10.41 ab	8.00 bc	7.37 c	3.78 d
Methanol	13.46 a	10.65 b	8.37 bc	7.22 c	6.24 c	5.87 c
***T. harzianum* WS01**						
Hexane	13.05 a	10.93 b	10.90 b	6.42 c	2.42 d	1.69 d
Ethyl Acetate	11.27 a	9.73 ab	9.24 b	5.09 c	3.16 d	2.20 d
Methanol	12.09 a	11.30 a	5.42 b	1.46 c	0.92 c	0.91 c
***T. hamatum* WS01**						
Hexane	8.77 a	8.68 a	5.77 b	3.42 c	1.86 c	1.32 c
Ethyl Acetate	8.25 a	6.61 b	5.06 c	3.94 cd	3.25 d	1.46 e
Methanol	9.66 a	7.76 b	5.66 c	3.86 d	2.23 de	1.49 e
***T. hamatum* WS02**						
Hexane	14.18 a	10.51 b	9.30 b	6.66 c	6.14 c	4.65 c
Ethyl Acetate	14.32 a	10.36 b	7.58 c	5.80 cd	5.69 cd	3.93 d
Methanol	15.01 a	11.27 b	8.90 c	8.57 c	6.25 d	5.45 d
***T. viride* WS01**						
Hexane	9.17 a	9.17 a	4.83 b	2.49 bc	2.12 bc	1.64 c
Ethyl Acetate	8.70 a	6.40 b	4.22 c	3.05 cd	2.90 cd	1.63 d
Methanol	10.14 a	9.53 a	4.47 b	3.33 bc	1.75 cd	1.13 d
***G. virens* WS01**						
Hexane	16.46 a	14.63 ab	13.13 b	10.17 c	7.17 d	3.62 e
Ethyl Acetate	16.38 a	14.61 a	14.15 a	9.15 b	8.99 b	8.25 b
Methanol	14.20 a	13.62 a	8.36 b	6.16 bc	5.12 c	5.02 c

[1/]Means followed by the same letter within a row are not significantly different as determined by Duncan multiple range test at $p = 0.01$.

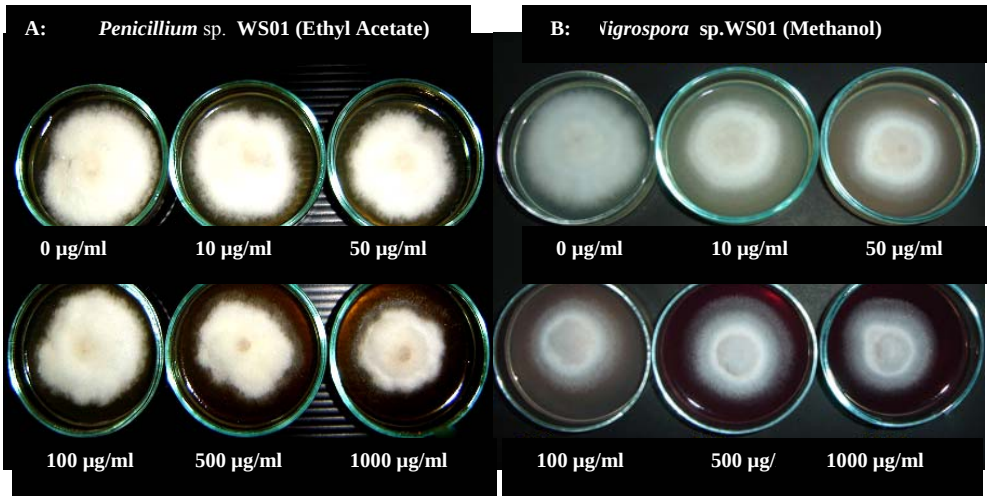

Fig. 2. Efficacy of crude extracts of antagonistic fungi on the growth of plant pathogens; *A: Penicillium* sp. WS01 and *Fusarium. oxysporum* f. sp. *cucumerinum*, B: *Nigrospora* sp. WS01 and *Fusarium. oxysporum* f.sp. *lycopersici.*

Table 3. Enzyme activity assays of antagonistic fungi on agar medium plates.

Antagonistic fungi	Enzyme activity assays	
	Cellulase (diameter of clear zone; cm)[1/]	Hemicellulase (xylanase)[3/]
Nigrospora sp. WS01	2.04 f [2/]	+
Penicillium sp. WS01	4.03 b	+
T. harzianum WS01	1.56 g	++
T. hamatum WS01	4.16 a	+++
T. hamatum WS02	3.58 c	+++
T. viride WS01	3.47 d	++
G. virens WS01	2.88 e	++

[1/]diameter of clear zone (cm) at 5 days.
[2/]Means followed by the same letter within a row are not significantly different as determined by Duncan multiple range test at $p = 0.01$.
[3/]reaction at 4 days +++ = strong ++ = medium + = weak

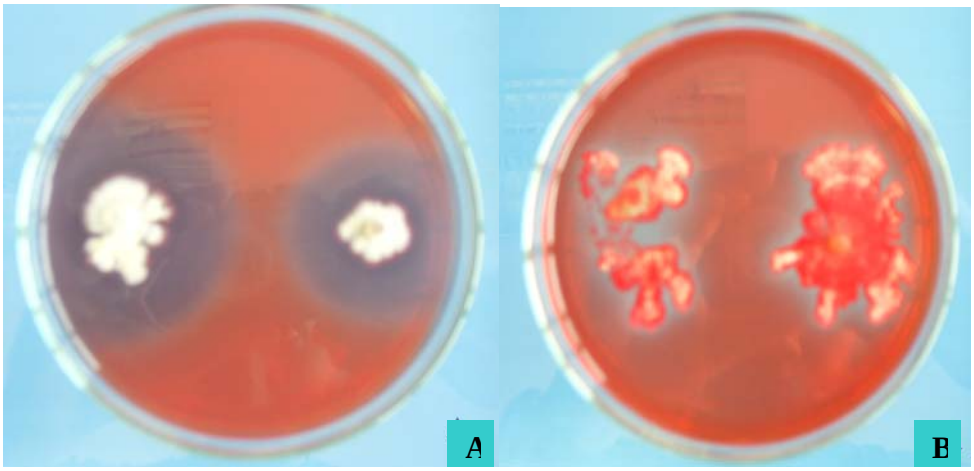

Fig. 3. Cellulase activity assay of antagonistic fungi on CMC agar plates
A: *Penicillium* sp. WS01, B: *Trichoderma viride* WS01.

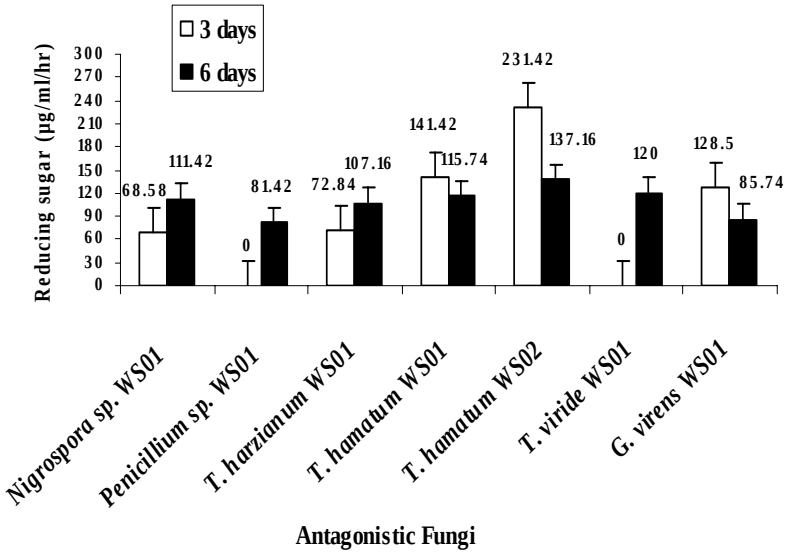

Fig. 4. Hemicellulase activity of antagonistic fungi.

However, in addition, the extract of *N. oryzae* to inhibit growth of some phytopathogenic fungi (Szewczuk *et al*., 1991). *G. virens* produced gliotoxin (Lumsden *et al*., 1992) and its properties against wood attacking fungi; *Postia placenta* and *Neolentinus lepideus* and *Trametes versicolor* and *Phlebia brevisspora* (Terry *et al*., 1996). These crude extracts could further elucidate the chemical structures that may play an important role of mechanism of biocontrol in term of antibiosis. Suwan *et al*., (2000) stated that *T. harzianum* PC01 can produce peptibols namely Trichotoxins that could inhibit plant pathogen and also promote plant growth. Moreover, our study for possible control mechanism of those promising antagonists in term of lysis, we have preliminary studied on enzymes that may released fromthose antagonists,them possible to play an important role of lysis and gave a good control of plant pathogens. As the report of Kucuk and Kivanc (2002) stated *T. harzianum* isolated from soil could inhibit *F. F.oxysporum* and produced chitinase to degrade cell wall of tested plant pathogen (Kucuk and Kivanc, 2004). It is suggested that biological control of plant pathogens may play an important role of antibiosis and lysis. Further studies need to be studied on the chemical elucidation of bioactive substances from those promising antagonistic fungi.

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References

Datnoff, L.E., Nemec, S. and Pernezny, K. (1995). Biological control of Fusarium crown rot and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. Biological Control 5: 427-431.

Di Pietro, A., Lorito, M., Hayes, C.K., Broadway, R.M. and Harman, G.E. (1993). Endochitinase from *Glioladium virens*: isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. Phytopathology 83: 308-313.

Dong, H.Z. and Cohen, Y. (2001). Induced resistance in cotton seedlings against Fusarium wilt by dried biomass of *Penicillium chrysogenum* and its water extract. Phytoparasitica 30: 77-87.

Kanokmedhakul, S., Kanokmedhakul, K., Nasomjai, P., Loungsysouphanh, S., Soytong, K., Isobe, M., Kongsaeree, P., Prabpai, S.S. and Suksamrarn, A. (2006). Antifubgal Azaphilones from the Fungus, *Chaetomium cupreum* CC300. Natural Products 69:891-895.

Kiewnick, S. and Sikora, R.A. (2006). Biological control of root-knot nematode Meloidogyne *nicognita* by *Paecilomyces lilacinus* strain 251. Biological control 38:179-187.

Kucuk, C. and Kivanc, M. (2002). Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. Turkey Journal of Biology 27: 247-253.

Lumsden, R.D., Locke, J.C. Adkins, S.T., Walter, J.F. and Ridout, C.J. (1992). Isolation and localization of the antibiotic gliotoxi produced by *Gliocladium virens* from alginate prill in soil and soilless media. Phytopathology 82: 230-235.

Moreira, F.G., Dos Reis, S., Costa, M.A.F., De Souza, C.G.M., and Peralta, R.M. (2005). Production of hydrolytic enzymes by the plant pathogenic fungus *Myrothecium verrucaria* in submerged cultures. [Online] Available: http:// www. scielo.br/ scielo.php?pid=s1517-83822005000100002&script=sci

Nelson, N. (1994). A photometric adaptation of the Somogyi method for the determination of glucose. Biological Biochemistry 152: 375-380.

Omokolo, N.D., Nankeu, D.J., Niemenak, N. and Boudjeko, T. (2003). Variation of β-1,3-glucanase, chitinase and polyphenoloxidase activities on cacao pods upon *Phytophthora megakarya* inoculation. African Crop Science 11: 97-106.

Sadfi, N., Cherif, M., Fliss, I., Boudabbous A. and Antoun, H. (2001). Evaluation of bacterial isolates from salty soils and *Bacillus thuringiensis* strains for the biocontrol of Fusarium dry rot of potato tubers. Plant Pathology 83: 101-118.

Singh, P.P., Shin, Y.C., Park, C.S. and Chung, Y.R. (1999). Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. Phytopathology 89: 92-99.

Somogyi, M. (1952). Note on sugar determinations. Biological Biochemistry 195: 19-23.

Soytong, K., Srinon, W., Ratanacherdchai, K, Kanokmedhakul, S., Kanokmedhakul, K (2005). Application of antagonistic fungi to control anthracnose disease of grape. Agricultural Technology 1: 33-42.

Suwan, S., Isobe, M., Kanokmedhakul, S., Lourit, N. Kanokmedhakul, K., Soytong, K. and Koga, K. (2000). Elucidation of great micro-heterogenecity of an acid-neutral Trichotoxin mixture from *Trichoderma harzianum* by ESI-QTOF. Mass Spectrometry 35:1438-1451.

Szewczuk, V., Kita, W., Jarosz, B., Truszkowska, W.and Seiwinski, A. (1991). Growth inhibition of some phytopathogenic fungi by organic extracts from *Nigrospora oryzae* (Berk&Br.) Pecth. Basic Microbiology 31: 69-73.

Terry L. Hightley, H.S. Anantha Padmanabha and C.R. Howell. (1996). Antagonistic properties of Gliocladium virens against wood attacking fungi. The International Research Group on Wood Preservation 1-10.

Zhang, Z. and Yuen, G.Y. (2000). The role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of *Bipolaris sorokiniana*. Phytopathology 90: 384-389.

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