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Full Length Research Paper

Antifungal metabolites from antagonistic fungi used to control tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici*

P. Sibounnavong[1]*, Charoenporn, C.[2], S. Kanokmedhakul[3] and K. Soytong[4]

[1]International College, King Mongkut's Institute of Technology Ladkrabang, Ladkrabang, Bangkok, 10520, Thailand.
[2]Department of Agriculture, Faculty of Science and Technology, Nakhonratchsima Rajabhat University, Thailand.
[3]Natural Products Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand.
[4]Major of Plant Pest Management Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Ladkrabang, Bangkok, 10520, Thailand.

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***Chaetomium elatum* strain ChE01, *Chaetomium lucknowense* strain CLT01 and *Emericella rugulosa* strain ER01, which were isolated from soil in Thailand, effectively controlled the most virulent isolate of *Fusarium oxysporum* f. sp. *lycopersici* NKSC02 causing wilt of tomato (*Lycopersicon esculentum* var Sida). Two antifungal substances were purified: Chaetoglobosin-C from *Ch. elatum* and *Ch. lucknowense* and tajixanthone from *E. rugulosa*. Chaetoglobosin-C showed greater antifungal activity against *F. oxysporum* f. sp. *lycopersici*, with an effective dose (ED_{50}) of 5.98 µg/ml, compared with tajixanthone (ED_{50} of 167 µg/ml). These results suggest that the disease control mechanism of these antagonistic fungi involves antibiosis. The inoculating tomato seedlings var. Sida with conidia of *F. oxysporum* f.sp. *lycopersici,* mixed with either a solution of chaetoglobosin-C or tajixanthone showed no wilt symptom. The conidia of *F. oxysporum* f. sp. *lycopersici* treated with these two compounds appeared abnormal and lost pathogenicity.**

Key words: Bioactivity test, *Chaetomium elatum*, *Chaetomium lucknowense*, *Emericella rugulosa*, *Fusarium oxysporum*.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely cultivated, popular and important vegetable crops in the world. *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen is one of the most common pathogen that causes wilt of tomato in areas of upland cultivation which can cause economic losses. *F. oxysporum* f. sp. *lycopersici* has become one of the most damaging and difficult to control wherever tomatoes are grown intensively, because it grows endophytically and persists in infested soils (Agrios, 1997). The disease control measures for this vascular wilt are either inefficient or difficult to apply the chemical fungicides. Over time tomatoes may develop resistance to some races of the pathogen; however, the pathogenic fungus may also develop resistance to chemical fungicides (Silva and Bettiol, 2005). *Chaetomium* spp. belong to the Ascomycota, and have been reported as antagonists against several plant pathogens (Soytong and Quimio, 1989; Soytong et al., 2001; Dhingra et al., 2003; Aggarwal et al., 2004; Park et al., 2005). Many species of *Chaetomium* with the potential to be biological control agents suppress the growth of bacteria and fungi through competition (for substrate and nutrients), mycoparasitism, antibiosis, or various combinations of these (Marwah et al., 2007; Zhang and Yang, 2007). *Chaetomium globosum* and *Ch. cupreum* in particular have been extensively studied and

*Corresponding author. E-mail: sone_lp@hotmail.com. ajkasem@gmail.com Tel: 66-84-4258286. Fax: 66-02-3266267.

successfully used to control root rot disease of citrus, black pepper and strawberry, and have been shown to reduce damping off disease of sugar beet (Soytong et al., 2001; Tomilova and Shternshis, 2006). The plant disease control mechanism may involve in antibiosis, with the antagonistic fungus releasing antibiotic substances (Soytong et al., 2001; Kanokmedhakul et al., 2002, 2006; Park et al., 2005). Cullen and Andrews (1984) reported evidence of antibiosis by *Ch. globosum* against *Venturia inaequalis*, which causes apple scab.

Di Pietro et al. (1992) reported that *Ch globosum* can produce chetomin, which effectively inhibited *Pythium ultimum*, which caused damping-off of sugar beet. *Ch. globosum* strain KMITL 0802 has been shown to produce chaetoglobosin – C, which inhibits some pathogens (Kanokmedhakul et al., 2002). Park et al. (2005) also reported that *Ch. globosum* F0142 can produce chaetoviridin A to control rice blast, wheat leaf rust and tomato late blight. Soytong (1992) and Soytong et al. (2001) showed that a specific isolate of *Chaetomium cupreum* produced secondary metabolites that significantly suppressed tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* in the tomato fields in Thailand, and later found that this isolate of *Ch. cupreum* produced rotiorinols A to C and rotiorin, which exhibited antifungal activity against *Candida albicans* (Kanokmedhakul et al., 2006). *Chaetomium cochlioides*- strains VTh01 and CTh05 have been shown to exhibit antimicrobial activity against a *Phytophothora* sp. that causes root rot, the anthracnose fungus *Colletotrichum gloeosporioides*, and *F. oxysporum* f. sp. *lycopersici.*

Among the compounds isolated from *Ch. cochlioides* strains VTh01 and CTh05, Phonkerd et al. (2008) identified four new dimeric spiro-azaphilones; (cochliodones A to D), two new azaphilones; (chaetoviridines E and F), and a new *epi*-chaetoviridin A. The isolate of the *Chaetomium elatum* strain ChE01 used in this study has been reported to produce a chaetoglobosin V, prochaetoglobosin III and prochaetoglobosin III_{ed}, chaetoglobosins B to D, F and G, and isochaetoglobosin D, which have been shown to exhibit cytotoxicity against a human breast cancer cell line. It should be noted that *Ch. elatum* ChE01 can also produce chaetoglobosin-C as a major compound, comprising up to 2% of the dried mycelial mat when grown in liquid culture (Thohinung et al., 2010). Moreover, the isolate of Ascomycete *Emericella rugulosa* used in this study has been shown to produce five prenylxanthones, rugulox-anthones A to C, 14-methoxytajixanthone,-tajixanthone ethanoate, a bicycle(3.3.1)–nona-2, 6-diene derivative named rugulosone, shamixanthone, tajixanthone, 14-methoxytajixanthone-25-acetate, tajixanthone hydrate, tajixanthone methanoate, isoemericellin and ergosterol. Among these, the bicycle (3.3.1)–nona-2,6-diene derive-ative has been shown to exhibit antimalarial and antimycobacterial activity and cytotoxicity against three cancer cell lines (Moosophon et al., 2009). Another species of *Emericella* (*Emericella nidulans*) has also been reported to antagonize *F. oxysporum* f. sp. *lycopersici* (Sibounnavong et al., 2009). The antimicrobial activity of chaetoglobosin-C, which is produced by *Ch. elatum* ChE01, and *Ch. lucknowense* CLT01, and of tajixanthone, which is produced by *E. rugulosa* ER01, could be involved in the disease control mechanism of these antagonistic fungi against the tomato wilt fungus *F. oxysporum* f. sp. *lycopersici*. The objectives of research findings were to isolate *F. oxysporum* f. sp. *lycopersici* causing tomato wilt and test for it pathogenicity and to investigate antagonistic fungi namely Ch. elatum strain ChE01, Ch. lucknowense strain CLT01 and *E. rugulosa* strain ER01 for antagonism of *F. oxysporum* f. sp. *lycopersici*. Crude extracts and pure compounds, chaetoglobosin-C from Ch. elatum and Ch. lucknowense and tajixanthone from *E. rugulosa* were tested for their antibiosis. The effects of chaetoglobosin-C and tajixanthone for pathogenicity loss of *F. oxysporum* f. sp. *lycopersici* were also investigated.

MATERIALS AND METHODS

Isolation and pathogenicity test

Disease samples were collected from infested soil from tomato fields in Bangkok, Pechaboon, Tak, and Chaingmai provinces in Thailand. The pathogen was isolated by transferring surface-sterilized plant tissue to a potato dextrose agar (PDA) medium and using soil plate techniques following the methods used by Agrios (1997). Pure cultures of *F. oxysporum* f. sp. *lycopersici* were identified by morphological characteristics under a binocular compound microscope, maintained on PDA slants and deposited at the Biocontrol Research Unit and Mycology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Eleven isolates of *F. oxysporum* f. sp. *lycopersici*, which were isolated from Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakon-nakhon provinces in Thailand were obtained from Asst. Prof. Dr. Chamaiporn Charoenporn (Nakhonratchasima Rachabhat University, Nakhonrat-chasima, Thailand) as follows: isolates BRCO3, KK2, KSoC02, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04. The morphological identification of the 11 isolates has been confirmed previously by Charoenporn et al. (2010) by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA as well as a small portion of the 28S rDNA.

All *F. oxysporum* f. sp. *lycopersici* isolates were tested for pathogenicity to tomato seedlings using Koch's postulates. Briefly, all isolates were sub-cultured and multiplied on PDA and incubated for 7 to 10 days at room temperature approximately (30 to 32°C). The inoculum of pathogen was adjusted to1×10^7 spores/ml before inoculating to 20–day–old tomato seedlings var. Sida. The roots of tomato seedlings were washed under running sterilized water and cut at five points on the root tips before dipping the roots into a 20 ml spore suspension for 15 min. A control was performed by dipping seedling roots into sterile distilled water. The seedlings were then potted in sterilized soil. After 10 days, symptoms of

disease were recorded using the Disease Severity Index (DSI) and rated according to Sibounnavong et al. (2009, 2010) as follows: 1 = no symptoms, 2 = 1 to 20% of leaves yellow and wilted, 3 = 21 to 40% of leaves yellow and wilted, 4 = 41 to 60% of leaves yellow and wilted, 5 = 61 to 80% of leaves yellow and wilted, and 6 = 81 to 100 % of leaves yellow and wilted. The experiment was conducted using a completely randomized design (CRD) with six replications of each treatment. The experiment was repeated twice. Virulence was categorized according to the DSI, following the method used by Charoenporn et al. (2010) as follows: non-pathogenic (DSI =1), low virulence (DSI ≤ 3.50), moderate virulence (DSI > 3.50 to 4.50), and highly virulence (DSI > 4.50). The most virulent isolate was selected for further experiments.

Antagonism test

Antagonistic fungi were isolated from soil in Thailand, namely *Ch. elatum* strain ChE01, *Ch. lucknowense* strain CLT01 and *E. rugulosa* strain ER01. These isolates were tested to determine their ability to antagonize the *F. oxysporum* f. sp. *lycopersici* isolate identified as the most virulent in the pathogenicity test. The test was conducted using the methods of Soytong (1992), Sibounnavong et al. (2009) and Charoenporn et al. (2010). The antagonistic fungi and pathogen were separately cultured on PDA at room temperature (30 to 32°C) for seven days. A 0.5 cm diameter sterilized cork borer was used to remove agar plugs from the actively growing edge of cultures of the pathogenic fungus and of the antagonistic fungi and used to inoculate 9 cm diameter PDA plates: an agar plug of the pathogen was placed on one side of the plate opposite an agar plug of an antagonistic fungus. Plates inoculated with a single plug of an antagonistic fungus or of the pathogen acted as the controls. The plates were incubated at room temperature (30 to 32°C) for 30 days. The experiment was performed using a completely randomized design (CRD) with four replications. Data were collected regarding colony diameter (cm) and the number of conidia produced by the pathogen. A haemcytometer was used to count the number of conidia. Percentage inhibition of pathogen colony growth and of conidia production was calculated using the following formula:

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

Where, A is the colony diameter or number of conidia produced by the pathogen on the control plate and B is the colony diameter or number of conidia produced by the pathogen when inoculated opposite an antagonistic fungus. Analysis of variance was statistically analyzed and treatment means were compared using Duncan's Multiple Range Test (DMRT) at p = 0.05 and 0.01. The experiment was repeated twice.

Crude extracts and pure compounds from antagonistic fungi

Crude extracts from each antagonistic fungus were obtained from the method used by Kanokmedhakul et al. (2006), Moosophon et al. (2009) and Thohinung et al. (2010). The fungi were cultivated in potato dextrose broth at room temperature (30 to 32°C) for 30 days. The dried fungal biomass of each antagonistic fungus was ground and sequentially extracted with hexane, ethyl acetate, and methanol. The solvents were then evaporated *in vacuo* to yield crude hexane, crude ethyl acetate (EtOAc), and crude methanol (MeOH) extracts, respectively. The extracts were separated and purified using chromatographic methods to obtain the compounds. The structures of these compounds were identified by spectroscopic methods, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR (COSY, HMQC, HMBC, and NOESY).

Crude extract bioassay

The crude extracts were assayed for inhibition of the most virulent isolate of *F. oxysporum* f. sp. *lycopersici*. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented the different solvents: A1 = crude hexane, A2 = crude ethyl acetate and A3 = crude methanol. Factor B represented the different concentrations: B1 = 0 µg/ml (control), B2 = 50 µg/ml, B3 = 100 µg/ml, B4 = 500 µg/ml and B5 = 1,000 µg/ml. Each crude extract was dissolved in 2% dimethyl sulfoxide and added to PDA before autoclaving at 121°C (15 psi) for 30 min. To perform the assay, a sterilized 3 mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5 cm diameter Petri dishes of PDA containing crude extract at each concentration and incubated at room temperature (30 to 32°C) until the pathogen on the control plates had grown over the plate. Data were collected regarding the number of conidia produced by the pathogen and used to calculate the percentage of conidia inhibition. The effective dose (ED_{50}) was calculated using Probit analysis. The experiment was repeated twice.

Bioactive compound assay

Pure compounds of chaetoglobosin-C from *Ch. elatum* and *Ch. lucknowense* and tajixanthone from *E. rugulosa* (Figure 1) were separately tested for their antifungal activities against *F. oxysporum* f. sp. *lycopersici*. To perform the assay, a sterilized 3 mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5 cm diameter Petri dishes of PDA containing either pure compounds of Chaetoglobosin–C or tajixanthone at each concentration and incubated at room temperature (30 to 32°C) until the pathogen on the control plates grows over the plate. The experiment was performed using a CRD with four replications. Treatments comprised four different concentrations: 0, 10, 50 and 100 µg/ml. The experiment was repeated twice. Data were collected regarding the number of conidia produced by the pathogen and calculated for percentage conidial inhibition. The ED_{50} was calculated using Probit analysis

Effect of fungal metabolites on disease incidence

The roots of 20– day– old tomato seedlings var. Sida were washed under running sterilized water and cut at five points on the root tips before dipping the roots into each treatment a 20 ml spore suspension of 1×10^{7} spores/ml mixed with different concentration of pure compounds for 15 min. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented the pure compounds: A1 = tajixanthone, and A2 = chaetoglobosin –C. Factor B represented the different concentrations: B1 = 0 (control), B2 = 10, B3 = 50, and B4 = 100 µg/ml. A control was performed by dipping seedling roots into sterile distilled water. The seedlings were then planted in pots which contained sterilized soil. The experiment was repeated twice. Disease incidence was recorded using the Disease Severity Index used previously in the pathogenicity test.

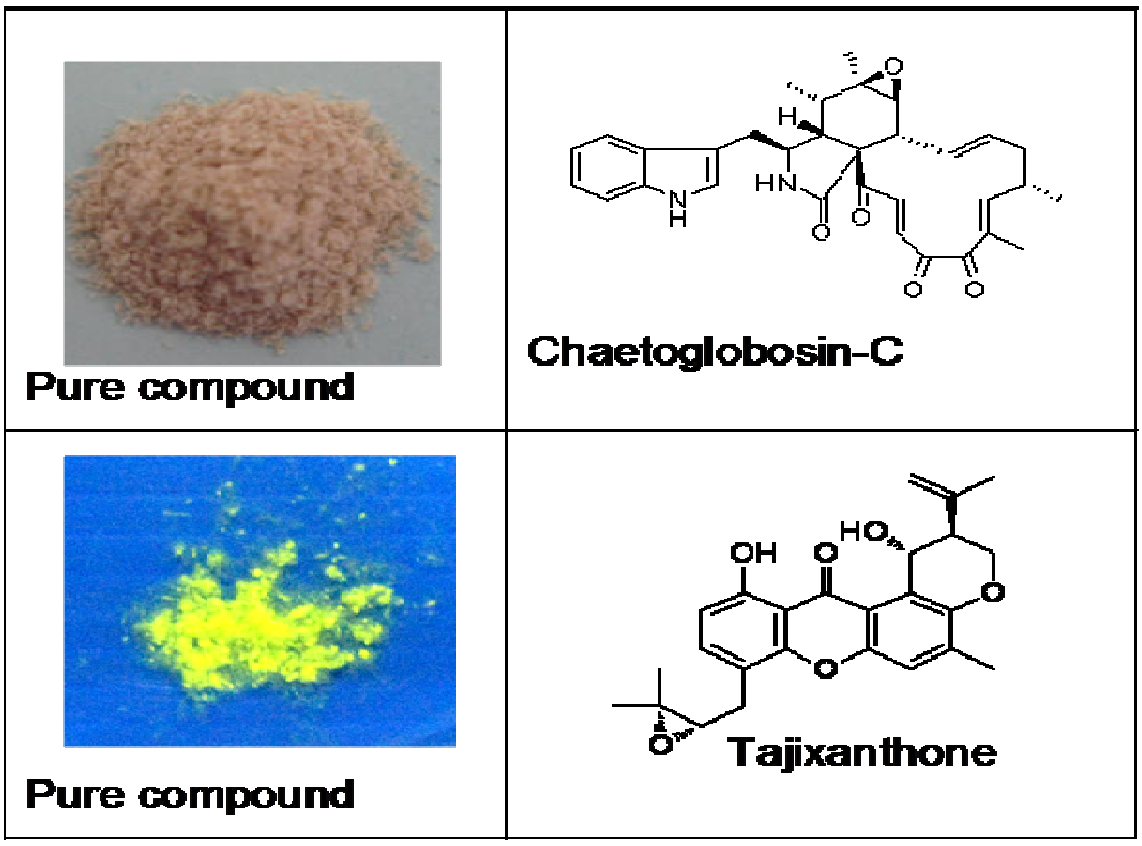

Figure 1. The pure compounds chaetoglobosin-C and tajixanthone: powder (left) and chemical structures (right).

Table 1. Isolates of *Fusarium* spp. and their pathogenicity group.

Provinces	Isolates	DSI[1]	Pathogenicity group[3]
Bangkok	BKRS01	1.87^{cde2}	L
	BKKRS02	1.25^{cde}	L
Pechaboon	PBRS01	1.62^{cde}	L
	PBRS02	1.50^{cde}	L
	PBRS03	1.50^{cde}	L
	PBRS04	1.62^{cde}	L
	PBrS01	2.12^{cd}	L
	PBrS02	1.50^{cde}	L
	PBSS01	1.87^{cde}	L
Tak	MSRS01	1.50^{cde}	L
	MSrS01	1.62^{cde}	L
	TrS01	2.50^{c}	L
	TRS01	2.63^{c}	L
	TSS01	2.37^{c}	L
Chaingmai	CMRS01	1.50^{cde}	L
	CMRS02	1.62^{cde}	L

Table 1. Continue

Burirum	BRC03	1.00[e]	A
	KK2	4.25[b]	M
KhonKaen	KSoC02	1.00[de]	A
Nongkhai	NKSC02	5.25[a2]	H
	NKRC02	1.00[e]	A
	NKRC04	1.00[e]	A
	NKRC09	1.00[e]	A
Nakhonratchasima	NSC01	1.25[d]	L
Sakon-nakhon	SRC02	1.00[e]	A
	SSoC03	1.50[cde]	L
	SSoC04	1.75[cde]	L
	control	1.00[e]	A

[1]Tomato plants were assessed for disease symptoms 21 days after inoculation using the Disease Severity Index (DSI): 1 = No symptoms; 2 = Plant showed 1 to 20% yellowing leaves and wilting, 3 = Plant showed 21 to 40% yellowing leaves and wilting, 4 = Plant showed 41 to 60% yellowing leaves and wilting, 5 = Plant showed 61 to 80% yellowing leaves and wilting, and 6 = Plant showed 81 to 100% yellowing leaves and wilting or death. [2]Average of four replications. Means with the same common letters in each column were not significantly different according to Duncan's multiple range test at p = 0.01. [3]The pathogenicity group of the isolates was determined according to the DSI: A = Avirulent (DSI = 1), L = Low virulence (DSI ≤ 3.50), M = Moderate virulence (DSI ≥ 3.50 to 4.50), H = High virulence (DSI > 4.50).

RESULTS

Isolation and pathogenicity test

Sixteen isolates of *Fusarium* spp. (BKRS01, BKKRS02, PBRS01, PBRS02, PBRS03, PBRS04, PBrS01, PBrS02, PBSS01, MSRS01, MSrS01, TrS01, TRS01, TSS01, CMRS01 and CMRS02) were obtained from isolation work, and 11 isolates of *F. oxysporum* f. sp *lycopersici* (BRCO3, KK2, KSoC02, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04) were grouped for pathogenicity according to disease severity index as shown in Table 1. The results showed that six of the isolates were avirulent (NKSC02, NKRC04, NKRC09and SRC02), 19 isolates showed a low level of pathogenicity (BKRS01, BKKRS02, PBRS01, PBRS02, PBRS03, PBRS04, PBrS01, PBrS02, PBSS01, MSRS01, MSrS01, TrS01, TRS01, TSS01, CMRS01 and CMRS02, NSC01, SSoC03 and SSoC04), one isolate was moderately virulent (KK2). Only isolate NKSC02 was highly virulent (Table 1 and Figure 2). Therefore, the highly virulent isolate NKSC02 was chosen for further studies.

Antagonism test

The result shows that *Ch. elatum* ChE01 significantly inhibited mycelial growth and the production of conidia by *F. oxysporum* f. sp. *lycopersici* NKSC02 of 74.95 and 77.12% inhibition, respectively followed by *Ch. Lucknowense* CLT01 and *E. rugulosa* ER01, which inhibited mycelial growth by 48.64 and 40.20%, respectively and inhibited conidial production by 58.41 and 63.28%, respectively (Table 2).

Crude extract bioassay

All tested crude extracts of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 significantly inhibited the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 as shown in Table 3. Among the three types of crude extracts, methanol extract from all antagonistic fungi showed the highest inhibitory activity with ED50 ranged from 1.50 to 7.66 µg/ml. With this, the crude hexane, crude ethyl acetate (EtOAc) and crude methanol (MeOH) extracted from *Ch. elatum* ChE01 inhibited the conidial production of isolate NKSC02 with the ED_{50} of 9.10, 8.90 and 1.50 µg/ml respectively. The crude hexane, crude EtOAc and crude MeOH extracted from *Ch. lucknowense* CLT01 inhibited the conidial production of isolate NKSC02 with the ED_{50} of 9.1, 18.0and 1.63 µg/ml, respectively. Moreover, the crude hexane, crude EtOAc and crude MeOH extracted from *E. rugulosa* ER01 inhibited the conidial production of isolate NKSC02 with the ED_{50} of 20.8, 9.8 and 7.6 µg/ml,

Figure 2. Pathogenicity test. (a) Uninoculated tomato seedlings (control), (b). tomato seedlings inoculated with *Fusarium oxysporum* f sp. *lycopersici* isolate NKSC02.

Table 2. Mycelial and conidia inhibition of *Fusarium oxysporum* f. sp. *lycopersici* by antagonistic fungi at 30 days.

Antgonistic fungi	Mycelial inhibition (%)[1]	Conidia inhibition (%)[1]
Ch. elatum ChE01	74.95[a]	77.12[a]
Ch. lucknowense CLT01	48.64[b]	58.41[c]
E. rugulosa ER01	40.20[b]	63.28[b]

[1]Average of four replications. Means with the same common letters in each column were not significantly different according to Duncan's multiple range test at p = 0.05.

Table 3. Bioassay of crude extracts against *Fusarium oxysporum* f. sp. *lycopersici.*

Antagonistic fungi	Crude extracts	Conidial inhibition (%)	ED_{50} µg/ml
	Hexane	92.00[ab1]	9.10
Ch. elatum ChE01	Ethyl acetate	94.00[a]	8.90
	Methanol	97.00[a]	1.50
	Hexane	90.29[b]	9.13
Ch. lucknowense CLT01	Ethyl acetate	90.00[b]	18.10
	Methanol	95.00[a]	1.63
	Hexane	79.00[c]	20.83
E. rugulosa ER01	Ethyl acetate	92.00[ab]	9.86
	Methanol	91.00[ab]	7.66

[1]Average of four replications. Means with the same common letters in each column were not significantly different according to Duncan's multiple range test at p = 0.01.

respectively (Table 3). The blue highlight should be deleted.

Bioactive compound assay

Chaetoglobosin-C was isolated from *Ch. elatum* ChE01 and *Ch. luckowense* CLT01 and tajixanthone isolated from *E. rugulosa* ER01 which elucidated using chromatographic methods to obtain these compounds. The structures were identified by spectroscopic methods, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR (COSY, HMQC, HMBC, and NOESY) as shown in Figure 1. Chaetoglobosin-C, a pure compound produced by *Ch. elatum* ChE01 and *Ch.*

Table 4. Assay of bioactive compounds against *Fusarium oxysporum* f. sp. *lycopersici.*

Pure compound	Conidial inhibition (%)	ED_{50} µg/ml
Chaetoglobosin-C	89.00	5.94
Tajixanthone	68.37	167

Table 5. Effect of fungal metabolites on disease incidence of tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici*

Pure compound	Concentrations µg mL^{-1}	DSI[1]
Chaetoglobosin-C	0	6^{a2}
	10	1^{b}
	50	1^{b}
	100	1^{b}
Tajixanthone	0	6^{a}
	10	1^{b}
	50	1^{b}
	100	1^{b}

[1]Tomato plants were assessed for disease symptoms 21 days after inoculation using the Disease Severity Index (DSI): 1 = No symptoms; 2 = Plant showed 1 to 20% yellowing leaves and wilting, 3 = Plant showed 21 to 40% yellowing leaves and wilting, 4 = Plant showed 41 to 60% yellowing leaves and wilting, 5 = Plant showed 61 to 80% yellowing leaves and wilting, and 6 = Plant showed 81 to 100% yellowing leaves and wilting or death. [2]Average of four replications. Means with the same common letters in each column were not significantly different according to Duncan's multiple range test at p = 0.01.

luckowense CLT01, significantly inhibited conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED50 value of 5.94 µg/ml. Moreover, tajixanthone, a pure compound produced by *E. rugulosa* ER01, significantly inhibited conidia production of *F. oxysporum f. sp. lycopersici* NKSC02 with an ED50 value of 167 µg/ml (Table 4).

Effect of fungal metabolites on disease incidence

Inoculum of *F. oxysporum* f. sp. *lycopersici* (1×10^7 spores/ml) treated with pure compounds of chaetoglobosin-C and tajixanthone before inoculating to tomato seedlings caused no symptoms at day 21 while the treatment with pathogen alone showed significantly high disease severity index as shown in Table 5. No disease incidences were observed at all tested concentration of 10, 50 and 100 µg/ml of either chaetoglobosin-C or tajixanthone which significantly differed from the control. It revealed that the antibiotic substances of chaetoglobosin-C and tajixanthon affected directly to the pathogen conidial inoculum which implies antibiosis mechanism of control. Moreover, the occurrences of ruptured cells and abnormal conidia thereafter mixing with each pure compound of chaetoglobosin-C and tajixanthon were observed under the microscope (Figure 3).

DISCUSSION

The pathogenicity tests performed on tomato seedlings in this study showed that the *F. oxysporum* f. sp. *lycopersici* NKRC02, NKRC04, SRC02 and NSC01 isolates were avirulent whereas Charoenporn et al. (2010) reported in a previous study as moderately virulent to the same variety of tomato. It revealed in this study that isolate NKRC09, KSoC02, BRC03, SSoC03 and SSoC04 were also found to be avirulent, whereas a previous study showed low virulence (Charoenporn et al., 2010). This suggested that repeatedly sub-cultured of *F. oxysporum* f. sp. *lycopersici* affected the stability of pathogenicity. *F. oxysporum* f. sp. *lycopersici* isolate NKSC02 was the most virulent isolate to cause wilt of tomato var. Sida. This observation is supported by *in vitro* studies of virulence by Soytong et al. (2001), Sibounnavong et al. (2009) and Charoenporn et al. (2010).

The antagonism test demonstrated the antagonistic activity of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 to inhibit the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 between 63 to 77%. The result was in accordance with the study from Charoenpoen et al. (2010) who reported that *Ch lucknowense* CLT significantly inhibited the mycelial growth and conidial production of *F. oxysporum* f. sp. *lycopersici* as 88.89 and 92.54%, respectively. Further-

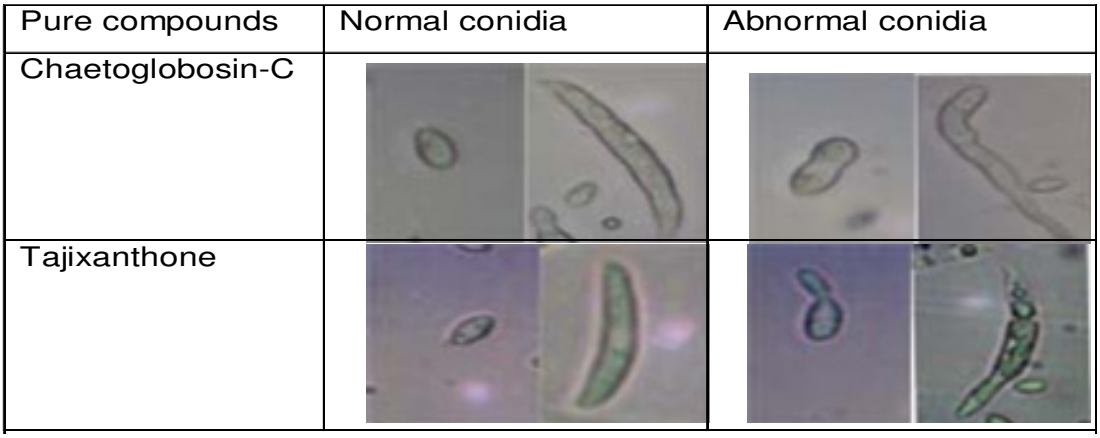

Figure 3. Abnormal conidial lysis of *F.oxysporum* f. sp. *lycopersici* owing to chaetoglobosin-C and tajixanthone

more, Sibounnavong et al. (2009) reported that *E. nidulans* strongly inhibited colonial growth and sporulation of *F. oxysporum* f. sp. *lycopersici* in antagonism and crude extract tests.

To elucidate the control mechanism involved in the inhibition of *F. oxysporum* f. sp. *lycopersici*, crude extracts of these antagonistic fungi were tested for antifungal activity against *F. oxysporum* f. sp. *lycopersici* NKSC02. The control mechanism of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 revealed releasing antibiotic substances to inhibit *F. oxysporum* f. sp. *Lycopersici*.

All tested crude extracts of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 significantly inhibited conidial production of *F. oxysporum* f. sp. *lycopersici*. This result is similar to the report of Charoenpoen et al. (2010) who stated that crude hexane, crude ethyl acetate and crude methanol from *Ch. lucknowense* CLT inhibited *F. oxysporum* f. sp. *lycopersici* NKSC01 with the ED_{50} of 188, 209 and 212 µg/ml, while in this study, those crude extracts inhibited the conidial production of different isolate of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} of 9.13, 18.10 and 1.63 µg/ml which were lower than those from previous report. Similar results were also reported by Srinon et al. (2006) and Sibounnavong et al. (2009), who stated that crude hexane, ethyl acetate and methanol extracts from *E. nidulans* inhibited the colony and sporulation of *F. oxysporum* f. sp. *lycopersici*. Moreover, Soytong et al. (2005) reported that crude ethyl acetate extract of *Ch. globosum* CG at 1000 µg/ml inhibited conidial production of this pathogen.

The crude extracts were further isolated to pure compounds and the chemical structures were elucidated. It is clearly demonstrated that chaetoglobosin-C, a pure compound produced by *Ch. elatum* ChE01 and *Ch. lucknowense* CLT01, and tajixanthone, a pure compound produced by *E. rugulosa* ER01. Both pure compounds significantly inhibited conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} of 5.94 and 167 µg/ml, respectively which were more effective than their crude extracts. It is suggested that chaetoglobosin-C and tajixanthone are expressed as an antibiotic substances to destroy the pathogen cells implies antibiosis. As previously reported by Soytong et al. (2001), chaetoglobosin-C from *Ch. globosum* inhibited several plant pathogens including *F. oxysporum* f. sp. *lycopersici*. Thohinung et al. (2010) also reported that *Ch. elatum* ChE01 produce chaetoglobosin-C that showed cytoxicity against the human breast cancer and cholangiocarcinoma cell lines. In this study, we found that tajixanthone from *E. rugulosa* ER01 inhibited the tested plant pathogen. *E. rugulosa* ER01 was also reported by Moosophon et al. (2009) to produce tajixanthone. It is concluded that *Ch. elatum* ChE01 and *Ch. lucknowense* CLT01 are confirmed to produce chaetoglobosin-C and *E. rugulosa* ER01 produce tajixanthone. In this study, these two compounds exhibited antifungal activity against *F. oxysporum* f. sp. *lycopersici* NKSC02 at low concentration. In addition, Park et al. (2005) reported that chaetoviridin-A purified from *Ch. globosum* F0142 exhibited moderate control of tomato late blight at 125 µg/ml. In addition, Chaetoglobosin-C produced by *Ch. elatum* ChE01, was not only shown to exhibit cytotoxicity against the human pathogens (Thohinung et al., 2010) but also inhibited the tomato wilt pathogen; *F. oxysporum* f. sp. *lycopersici* in this study.

However, this study demonstrated that either tajixanthone or chaetoglobosin-C mixed in a solution with pathogen cells of *F. oxysporum* f. sp. *lycopersici* caused cells rupture and abnormal conidia. It is suggested that these pure compounds can lyse the cell wall of the pathogen and the protoplast becomes a plug inside the cells. These observations were similar to those reported by Sibounnavong et al. (2009) and Soytong (1992) who showed that the crude extracts of these antagonists

ruptured the cells of the *F. oxysporum* f. sp. *lycopersici* inoculums. In this study, the abnormal conidia of pathogen cells affected by tajixanthone or chaetoglobosin-C leading to loss of its pathogenicity when inoculated to tomato seedlings var Sida and no symptoms were observed. Moreover, formulated *E. nidulans* as a biological fungicide significantly reduced *Fusarium* wilt of tomato var. Sida in a pot experiment (Sibounnavong et al., 2010). It is questioned that these bioactive compounds should be further evaluated to determine their ability to control tomato wilt in pot and field trials. Tajixanthone and chaetoglobosin-C may possible develop to be microbial elicitors to induce immunity in tomato plants against *F. oxysporum* f. sp. *lycopersici.*

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