African Journal of Biotechnology Vol. 9(36), pp. 5836-5844, 6 September, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 ©2010 Academic Journals

*Full Length Research Paper*

# Evaluation of bio-agent formulations to control *Fusarium* wilt of tomato

**C. Charoenporn[1]*, S. Kanokmedhakul[2], F. C. Lin[3], S. Poeaim[4] and K. Soytong[5]**

[1]International College, King Mongkut's Institue of Technolog, Ladkrabang, Chalongkrung Road, Ladkrabang, Bangkok 10520, Thailand.
[2]Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.
[3]College of Agriculture and Biotechnology, Biotechnology Institute, Zhejiang University, Kaixuan Road, Hangzhou 310029, P.R. China.
[4]Faculty of Science, King Mongkut's Institue of Technology, Ladkrabang, Bangkok, Chalongkrung Road, Ladkrabang, Bangkok 10520, Thailand.
[5]Faculty of Agricultural Technology, King Mongkut's Institue of Technology, Ladkrabang, Bangkok, Chalongkrung Road, Ladkrabang, Bangkok 10520, Thailand.

Accepted 18 June, 2010

**The $ED_{50}$ value of antagonistic substance from *Chaetomium globosum* N0802 extracted with hexane was 157 µg/ml. This gave the highest inhibition of conidial production of *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt var Sida. Crude hexane from *Chaetomium lucknowense* CLT and crude methanol from *Trichoderma harzianum* PC01 gave $ED_{50}$ values of 188 and 192 µg/ml. It clearly demonstrated that antagonistic substances from all tested fungi could be deformed and this could break the conidial cells. The bio-agent formulations namely N0802, CLT and PC01 gave significantly highest disease reduction of tomato wilt which were 44.68, 36.28 and 41.01%, respectively, followed by prochoraz (21.95%). All tested bio-agent formulations could significantly increase the yield of tomato when compared to prochoraz and inoculated control. It is concluded that *C. globosum*, *C. lucknowense* and *T. harzianum* developed as bio-agent formulations namely N0802, CLT and PC01 and showed their abilities to control tomato wilt.**

**Key words:** *Fusarium oxysporum* f. sp. *lycopersici*, *Chaetomium globosum*, *Chaetomium lucknowense*, *Trichoderma harzianum*, bio-agent formulations.

## INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the world's most cultivated vegetable crop. Fusarium wilt is one of the most serious disease in tomato throughout the world, especially in upland. This disease is caused by *Fusarium oxysporum* f. sp. *lycopersic*i (Sacc.) leading to serious economic losses (Snyder and Hansen, 1940). It becomes one of the most prevalent and damaging disease wherever tomatoes are grown intensively because the pathogen can persist indefinitely in infested soils (Agrios, 1997). The excessive misuse of a wide range of fungicides has led to it being harmful to the environment and increases the resistant pathogen populations (Özgönen et al., 2001). *F. oxysporum* f. sp. *lycopersici* becomes resistant to those chemical fungicides. For this reason, alternative methods to control the disease had been studied with emphasis on biological control using fungi or bacteria to reduce fungicide application and decrease cost of production. Biological control has the potential to manage this disease which occurred through different mechanisms such as antibiosis, competition, suppression,

*Corresponding author. E-mail: chamai1611@hotmail.com, chamai1611@yahoo.com. Fax: 66-44-272939.

**Abbreviations: DSI,** Disease severity index; **ITS,** internal transcribed spacer; **PDA,** potato dextrose agar; **PDB,** potato-dextrose broth; **PB,** phosphate buffer; **PCR,** polymerase chain reaction; **DMSO,** dimethyl sulfoxide.

direct parasitism, induced resistance, hypovirulence and predation. The antagonistic activity has often been associated with production of secondary metabolites (Haggag and Mohamed, 2007; Larkin and Fravel, 1998). There were many reports of biological control agents to control Fusarium wilt pathogen such as *Trichoderma harzianum*, *Pythium oligandrum*, *Achromobacter xylosoxydans*, *Penicillium oxalicum* and non-pathogenic *F. oxysporum* (Mohamed and Haggag 2006; Floch et al., 2003; De Cal et al., 2000; Moretti et al., 2008; Silva and Bettiol, 2005). Soytong (1992) and Kanokmedhakul et al. (1993) reported that crude extract of *Chaetomium cupreum* KMITL-N 4320 inhibited spore production of *F. oxysporum* f. sp. *lycopersici* at 85.14%. Moreover, there are many reports on bioactive com-pounds which were extracted from antagonistic fungi to inhibit Fusarium wilt of tomato pathogen (Kanokmedhakul et al., 2003; Kanokmedhakul et al., 2006). These bioactive compounds such as Trichotoxin A50 extracted from *T. harzianum* PC01, and Chaetoglobosin C extracted from *Chaetomium globosum* KMITL-N0802 have been reported to elicit the resistant or immunity in plant (Soytong et al., 2001). The research project was to eva-luate the potential of antagonistic fungi, *C. globosum* KMITL-N0802, *Chaetomium lucknowense* CLT and *T. harzianum* PC01 which are formulated as biological fungicides to control Fusarium wilt of tomato.

## MATERIALS AND METHODS

### Isolation of pathogen

The diseased samples of tomato wilt were collected from infested fields in Bangkok, Pathumthani, Nakhon Ratchasima, Burirum, Nongkhai, Sakonnakhon, Khon Kaen, and Mukdahan provinces, Thailand. *F. oxysporum* f. sp. *lycopersici* was isolated from diseased plant using tissue transplanting method according to the method of Agrios (1997). Single spore isolation was performed on each isolate to pure culture on potato dextrose agar (PDA).

### Pathogenicity test

To confirm the identification of species and form a speciales of *Fusarium* isolates, pathogenicity test was performed using root-dipped method (Marlatt et al., 1996) with conidial suspension of pathogen $2 \times 10^6$ conidia/ml. Disease severity index (DSI) was scored at 21 days after inoculation based on the modified disease severity scale of Silva and Bettiol (2005). They were as follows: 1 = no symptom; 2 = plant showed yellowing leaves and wilting 1 - 20%, 3 = plant showed yellowing leaves and wilting 21 - 40%, 4 = plant showed yellowing leaves and wilting 41 - 60%, 5 = plant showed yellowing leaves and wilting 61 - 80% and 6 = plant showed yellowing leaves and wilting 81-100% or die. Pathogenicity test was conducted twice for each isolate. All tested isolates were recorded for non-pathogenic and pathogenic isolates. Virulent group was categorized according to DSI as non-pathogenic (DSI =1), low (DSI ≤ 3.50), moderate (DSI > 3.50 - 4.50), and high (DSI > 4.50). The most aggressive isolate was selected for further experiment.

### Molecular and phylogenetic analysis

Since there have been variations and doubts in morphological characters, rDNA sequencing analysis of the internal transcribed spacer (ITS) regions were performed to determine the phylogenetic relationship among isolates of *Fusarium* species.

### DNA extraction

Stationary cultures were grown in potato-dextrose broth (PDB) for 3 days at room temperature (28 - 32°C). DNA extraction was prepared as the protocol of GF-1 Plant DNA Extraction Kit (Vivantis Co., Ltd., Selangor DE, Malaysia). 0.2 g of fresh weight mycelium was ground to fine powder with mortar and pestle using liquid nitrogen and mixed with 280 µl of lysis buffer. Protinase K (20 mg/ml) was added and mixed before being incubated at 60°C for 1 h in a water-bath. The mixture was kept on ice for 3 min and then centrifuged for 5 min at 10,000 g. The supernatant was added with 20 µl of RNase A (20 mg/ml) and was incubated at 37°C for 5 min. The samples were homogenized by adding equal volumes of a mixture of phosphate buffer (PB) (600 µl) and were incubated for a further 10 min at 60°C. DNA was precipitated by adding 200 µl of absolute ethanol and then the sample was transferred into a column and centrifuged at 10,000 g for 1 min. DNA pellets were washed with 750 µl wash buffer and the procedure repeated again. DNA was eluted by adding 50 µl of 10 mM Tris-HCL, pH 8.5 and centrifuged for 1 min at 10,000 g.

### Polymerase chain reaction (PCR) and DNA sequencing

The region of the ribosomal, including a small portion of 18S rDNA, ITS 1, 5.8S rDNA, ITS2, and a small portion of the 28S rDNA, was amplified by PCR. The primers used were, PN3: 5´-CGTTGGT GAACCAGCGGAGGGATC-3´ and PN16: 5´-TCCCTTTCAACAA TTTCACG-3´, as described by Neuvéglise et al. (1994). PCR reactions were conducted using modified method of Yasushi and Tsutomu (2006) in a 25 µl reaction mixture containing 200 ng of DNA template, 4 µl (1.25 mM) dNTPs, 1 µl of each primer (20 *p*mol/µl), 1.5 µl of $MgCl_2$, 0.2 µl of *Taq* DNA polymerase (Vivantis Co., Ltd., Malaysia) and 2.5 µl of 10X PCR buffer. Amp-lification was performed using a thermal cycler. A cycle was composed of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. 5 µl of PCR products were electrophoresed in a 1.5% agarose gel in 1xTAE buffer, which was stained with ethidium bromide for observation of the amplicons. PCR products were sent for purification and sequence at Tech Dragon Limited, Hong Kong.

### Molecular phylogeny analysis

DNA sequences of tested *Fusarium* sp. were edited and aligned with BioEdit, version 7.0.5 program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were multiple aligned with Clustal X version 1.83 before the analysis was performed using maximum parsimony methods with PAUP 4.0b (Swofford, 1998). For maximum parsimony analyses, bootstraps of 1,000 replicates were performed to examine the relative of each isolate. Maximum parsimony trees were calculated via fast step-wise addition with the representative isolates of *Fusarium* sp. from GenBank (http://www.ncbi.nlm.nih.gov) as shown in Table 1 and *Verticillium dahliae* was used for analysis as outgroup. Genetic relative among *Fusarium*

**Table 1.** Sequences of *Fusarium* sp. from GenBank used in this study.

| *Fusarium* species | Locality | ITS GenBank accession No. |
|---|---|---|
| *F. oxysporum* f.sp. *lycopersici* | India | EU214564.1 |
| *F. incarnatum* | Japan | AY633745.1 |
| *F. chlamydosporum* | P.R. China | AB369435.1 |
| *F. redolens* | P.R. China | FJ441013.1 |
| *F. solani* | USA | AF161222.1 |
| *F. sporotrichioides* | P.R. China | FJ238107.1 |

sp. isolates in ITS regions of rDNA seq-uences were determined as a cluster in the phylogenetic tree.

**Dual culture test**

The antagonistic fungi were tested to inhibit the growth of *F. oxysporum* f. sp. *lycopersici* by using dual culture test. The antagonistic fungi e.g. *C. globosum* N0802, *C. lucknowense* CLT and *T. harzianum* PC01 were provided by Assoc. Prof. Dr. Kasem Soytong of King Mongkut of the Institute of Technology Ladkrabang, Thailand. Dual culture test was conducted using the method of Soytong (1992). The experiment was designed in completely randomized design (CRD) with four replications. The most aggressive isolate of *F. oxysporum* f. sp. *lycopersici* was used in the experiment. The tested dual culture plates were incubated at room temperature (28-32˚C). The data were collected as colony diameter and conidial number of pathogenic fungus. The colony diameter and conidia of pathogen were measured and calculated using the following formula:

% Inhibition = [(colony diameter or conidial number of pathogen in control – colony diameter or conidial number of pathogen in dual culture plate) / (colony diameter or conidial number of pathogen in control)] x 100.

The experiment was repeated two times.

**Testing on antagonistic substances to inhibit *F. oxysporum* f. sp. lycopersici**

The crude extracts from antagonistic fungi were performed using the method of Kanokmedhakul et al. (2006). Antagonistic fungi (*C. globosum* N0802, *C. lucknowense* CLT, and *T. harzianum* PC01) were cultured in PDB at room temperature (28 - 30°C) for 30 days. Mycelial mats were removed from PDB, filtered through cheesecloth and air-dried overnight. Dried mycelial mats were ground and extracted with 200 ml hexane and shaken for 24 h at room temperature. The ground mycelia were separated by filtration through Whatman No. 4 filter paper. The marc was extracted again with hexane using the method described above. Then the filtrates were evaporated in *vacuo* to yield the crude extract. The marc was further extracted with ethyl acetate (EtOAc) and methanol (MeOH) respectively using the same procedure as hexane. The crude extracts of antagonistic fungi were tested for inhibition of the most aggressive isolate of *F. oxysporum* f. sp. *lycopersici* obtained from the previous experiment. The experiment was conducted by using 3x6 factorial in CRD with four replications. Factor A represented crude extracts which consisted of crude hexane, crude ethyl acetate and crude methanol and factor B represented concentrations of 0, 10, 50, 100, 500, and 1,000 µg/ml. Each crude extract was dissolved in 2% dimethyl sulfoxide (DMSO), and then mixed into PDA before autoclaving at 121°C, 15 lbs/inch$^2$ for 30 min. The tested pathogen was cultured on PDA and incubated at room temperature for 5 days; then colony margin was cut by 3 mm diameter sterilized cork borer. The agar plug of pathogen was transferred to the middle of PDA plate (5.5 cm diameter) in each concentration and incubated at room temperature (28 - 30°C) for 4 days. Data were collected as colony diameter and number of conidia. Percentage of inhibition was computed as described above. The effective dose ($ED_{50}$) was computed by using inhibitory probit analysis.

**Testing bio-agent formulations to control *Fusarium* wilt of tomato *in vivo***

**Preparation of bio-agent formulations**

Bio-agent formulations were separately formulated as oil formulation according to the method of Soytong (2001) by using spores of antagonistic fungi namely N0802 (*C. globosum*), CLT (*C. lucknowense*), and PC01 (*T. harzianum*). Each antagonistic fungus was cultured in PDB and incubated at room temperature (28 - 32°C) for 30 days. The culture was filtered to get spore masses, and then put in the electrical mixer and each bio-agent formulation was adjusted to 2.5 × $10^6$ spores/ml before been added in sterilized palm's oil. These bio-agent formulations were periodically checked for their shelf life at every month for 1 year.

**Testing bio-agent formulations to control Fusarium wilt of tomato**

Bio-agent formulations of N0802, CLT and PC01 were tested for their abilities to control tomato wilt caused by *F. oxysporum* f. sp *lycopersici in vivo*. Tomato seedlings var. Sida at 30 days old were inoculated with conidial suspension of *F. oxysporum* f. sp. *lycopersici* at concentration of 2 × $10^6$ conidia/ml by dipping root for 15 min and transplanting them into plastic pot containing sterilized mix soil (soil: sand: compost, 4:1:1). The mix soil was sterilized at 121°C, 15 lbs/inch$^2$ for 1 h in two consecutive days. Randomized completely block design (RCBD) was performed with four replications. Treatments were designed as follows: Bio-agent formulations namely N0802, ($T_1$); CLT ($T_2$); Bio-agent formulation namely PC01 ($T_3$); Chemical fungicide (prochoraz 50% WP) ($T_4$); inoculated with pathogen and non-treated bio-agent formulation ($T_5$); and non-inoculated control ($T_6$). Bio-agent formulations were separately applied in each treatment at the rate of 10 ml/ 20 L of water and prochoraz 50% WP chemical fungicide was applied at the rate of 20 g/ 20 L of water at every 2 weeks by spraying rhizosphere around soil and above plants. Data were collected as DSI, fresh plant and dry weights (g), and fruit weight (g) at 30 days and 60 days. DSI was scaled as previous experiment. Percentage of disease reduction was analyzed using the formula:

% disease reduction = [(Disease severity index of control – Disease severity index of treatment)/ (Disease severity index of control)] x 100.

Fresh plant and dry weights (g) and fruit weight (g) were recorded at harvest day. Percent increase in yield was analyzed using the formula:

[(Yield per plant in treatment – yield per plant in control)/ (yield per plant in treatment)] x 100.

All data were subjected to analysis of variance (ANOVA). Treatment means were statistically compared with Duncan's new multiple range test (DMRT) at P≤0.05 to separate means. The experiment was repeated two times.

## RESULTS

### Isolation of pathogen and pathogenicity test

The 45 isolates were yielded and confirmed species by using morphological characters and molecular phylogeny. 12 isolates of SSoC04, BRC03, SSoC03, KSoC02, NKRC09, KK2, NKSC01, NKSC02, NKRC02, SRC02, NKRC04 and NSC01 proved to be *F. oxysporum* f.sp. *lycopersici*. These isolates were tested for pathogenicity which clearly showed that all isolates were pathogenic to tomato var. sida. The 15 isolates were proved to be *F. solani* as follows: - MSC02, SSoC02, BRC02, BRC01, NSoC01, NSC09, BKFC03, MSoC03, BKRC02, MSoC02, MSC02, KSoC01, BKSC02 and KSoC04. Out of these, 14 isolates were showed to be *Fusarium incarnatum*; they were: PSC01, PSC04, BKFC12, BKFC06, BKFC04, MSoC01, NSC07, NSC02, NRC04, PSC03, PSC02, KSoC03, PSC05 and BKFC01. With this, 2 isolates were showed to be *Fusarium chlamydosporum* as NSoC04 and BKFC07 and the other 2 isolates of NKRC11 and NKSoC01 were confirmed as *Fusarium redolens.*

Disease severity index was recorded and virulent group was categorized from pathogenicity test on tomato seedlings var. *sida* which were inoculated with conidial suspension of *Fusarium* sp. in each isolate at $2 \times 10^6$ conidia/ml. The results revealed that tomato seedlings did not show any wilt symptom after inoculation with isolates of *F. incarnatum*, *F. chlamydosporum*, *Fusarium solani*, *Fusarium redolens* and *Fusarium sporotrichiodes*. Twelve isolates were found to be *F. oxysporum* and expressed wilting symptom, they were then categorized into 3 virulent groups. High virulent group was shown in the isolates NKSC01, NKSC02, and KK2 presented to be the most aggressive isolates whose DSI were 6.00, 5.88, and 5.75, respectively. While, the moderate virulent group were the isolates SRC02, NKRC04, NSC01, and NKRC02 whose DSI were 4.38, 4.38, 4.31, and 4.13, respectively. Moreover, low virulent group was the isolates KSoC02, NKRC09, BRC03, SSoC03, and SSoC04 whose DSI were 3.38, 3.38, 3.19, 3.13, and 3.06, respectively. The isolate NKSC01 was classified as *F. oxysporum* f.sp. *lycopersici* according to molecular study and then selec-ted for testing in further experiment (Table 2).

### Molecular phylogeny analysis

Forty-five isolates of *Fusarium* spp. were identified by morphological characters but some isolates did not show clear results under the compound microscope. Therefore, the sequences were studied to confirm morphological identification by using ITS sequences with the length of the complete ITS1, 5.8S and ITS2 including a small portion of 18S rDNA and a small portion of the 28S rDNA. This was clearly demonstrated to identify and confirm the species of *Fusarium* spp. as a valid identification. The phylogenetic tree presented a cluster of the *Fusarium* species into 5 major groups (Figure 1). The Group I belonged to *F. solani* which consisted of isolates BKFC03, BKRC02, BKSC02, KSoC01, KSoC04, MRC02, MSC04, MSoC02, MSoC03, NSC09, NSoC01, BRC01, BRC02, MSC02, SSoC02. Group II was identified as *F. incarnatum* which belonged to the isolates BKFC01, BKFC04, BKFC06, BKFC12, KSoC03, MSoC01, NRC04, NSC02, NSC07, PSC01, PSC02, PSC03, PSC04, and PSC05. While, group III was *F. chlamydosporum* which were classified as BKFC07 and NSoC04. Group IV was confirmed to be *F. oxysporum* which consisted of NKSC01, NKSC02, KK2, NKRC02, NKRC04, SRC02, NSC01, BRC03, KSoC02, SSoC03, SSoC04, and NKRC09. Group V was classified as *F. redolens* which was arranged in the isolates NKRC11 and NKSoC01.

### Dual culture test

The inhibition on mycelial growth and conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC01 in dual culture test are shown in Table 3. *T. harzianum* PC01 and *C. lucknowense* CLT gave higher significant inhibition of the mycelial growth of the pathogen than *C. globosum* N0802 which were 90.56, 88.89 and 71.11%, respectively. But, all tested antagonists were not significantly inhibited, the conidia production were 99.99, 92.54 and 92.14%, respectively.

### Testing antagonistic substances to inhibit *F. oxysporum* f. sp. *lycopersici*

Crude extracts of tested antagonists could inhibit conidial production of the pathogen at the concentrations ranged from 10 to 1,000µg/ml as shown in Table 4. Crude extracts of tested antagonists showed the highest properties

**Table 2.** Isolates of *Fusarium* sp. and their pathogenicity results.

| Provinces | Isolates | DSI[1] | Virulent group[3] | Provinces | Isolates | DSI | Virulent group |
|---|---|---|---|---|---|---|---|
| Nong Khai | NKSC01 | 6.00a[2] | H | Burirum | BRC01 | 1.00d | NP |
| | NKSC02 | 5.88a | H | | BRC02 | 1.00d | NP |
| | NKRC02 | 4.13b | M | | BRC03 | 3.19c | L |
| | NKRC04 | 4.38b | M | Bangkok | BKFC01 | 1.00d | NP |
| | NKRC09 | 3.38c | L | | BKFC03 | 1.00d | NP |
| | NKRC11 | 1.00d | NP | | BKFC04 | 1.00d | NP |
| | NKSoC01 | 1.00d | NP | | BKFC06 | 1.00d | NP |
| Sakon Nakhon | SRC02 | 4.38b | M | | BKFC07 | 1.00d | NP |
| | SSoC02 | 1.00d | NP | | BKFC12 | 1.00d | NP |
| | SSoC03 | 3.13c | L | | BKRC02 | 1.00d | NP |
| | SSoC04 | 3.06c | L | | BKSC02 | 1.00d | NP |
| Khon Kaen | KK2 | 5.75a | H | Pathumthani | PSC01 | 1.00d | NP |
| | KSoC01 | 1.00d | NP | | PSC02 | 1.00d | NP |
| | KSoC02 | 3.38c | L | | PSC03 | 1.00d | NP |
| | KSoC03 | 1.00d | NP | | PSC04 | 1.00d | NP |
| | KSoC04 | 1.00d | NP | | PSC05 | 1.00d | NP |
| Nakhon | NRC04 | 1.00d | NP | Mukdahan | MRC02 | 1.00d | NP |
| Ratchasima | NSC01 | 4.31b | M | | MSC02 | 1.00d | NP |
| | NSC02 | 1.00d | NP | | MSC04 | 1.00d | NP |
| | NSC07 | 1.00d | NP | | MSoC01 | 1.00d | NP |
| | NSC09 | 1.00d | NP | | MSoC02 | 1.00d | NP |
| | NSoC01 | 1.00d | NP | | MSoC03 | 1.00d | NP |
| | NSoC04 | 1.00d | NP | | Control | 1.00d | |

[1]Disease severity index (DSI) was scored at 21 days after inoculation. 1= No symptom; 2 = plant showed yellowing of leaves and wilting 1 - 20%, 3 = plant showed yellowing of leaves and wilting 21 - 40%, 4 = plant showed yellowing of leaves and wilting 41 - 60%, 5 = plant showed yellowing of leaves and wilting 61 - 80%, and 6 = plant showed yellowing of leaves and wilting or die 81 - 100%.
[2]Average of four replications. Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.[3] Virulent group of the isolates was determined according to DSI; NP = Non-pathogenic, L = low, M = moderate, H = high.

to inhibit conidial production of *F. oxysporum* f. sp. *Lycopersici* NKSC01 at 1,000µg/ml. The highest conidial inhibition was presented by using crude hexane and crude ethyl acetate of *C. globosum*, crude hexane, crude ethyl acetate and crude methanol of *C. lucknowense*, crude ethyl acetate and crude methanol of *T. harzianum* which were 63.13, 62.58, 74.85, 69.55, 72.62, 96.93 and 97.50%, respectively. Crude extract of *C. globosum* N0802 which was extracted with hexane showed the highest inhibition of conidial production of the pathogen in which $ED_{50}$ value was 157 µg/ml while crude ethyl acetate and crude methanol presented their abilities to inhibit conidial production at the $ED_{50}$ values 339 and 302 µg/ml, respectively. Crude hexane of *C. lucknowense* CLT gave the highest inhibition of $ED_{50}$ value which was 188 µg/ml followed by crude ethyl acetate and crude methanol in which the $ED_{50}$ values were 209 and 212 µg/ml, respectively. Crude extracts of *T. harzianum* PC01 which was extracted from methanol showed the highest inhibition of conidial production of which the $ED_{50}$ value was 192µg/ml while crude ethyl acetate and crude hexane were 232 and 861 µg/ml, respectively.

## Testing bio-agent formulations to control *Fusarium* wilt of tomato *in vivo*

Result showed that bio-agent formulations namely N0802, CLT and PC01 gave significantly, high disease reduction of tomato wilt which were 44.68, 36.28 and 41.01 %, respectively, followed by prochoraz treatment (21.95%). This resulted to increase in yield of tomato, bio-agent formulations of N0802, CLT and PC01. There were also significant increased in yield of tomato which were 88.53, 83.74 and 87.24%, respectively, followed by prochoraz treatment (41.57%). With this, disease severity index (DSI) of tomatoes which were treated with bio-agent formulations of developed N0802, CLT, PC01 and

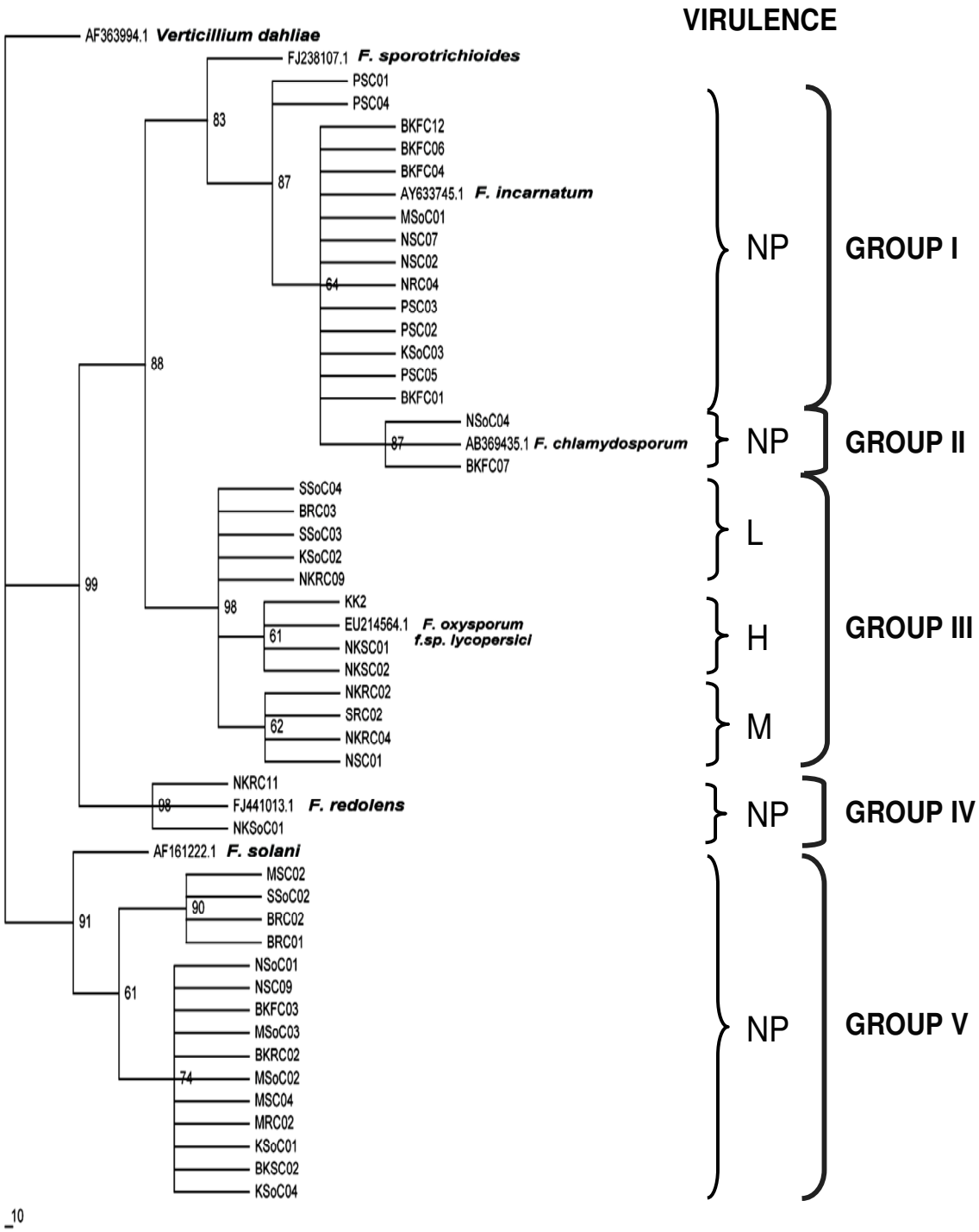


**Figure 1.** Phylogenetic relationships of *Fusarium* sp. inferred by the parsimony analysis of rDNA sequences. Phylogenetic tree was obtained from analysis by the parsimony method using the PAUP*4.0b program. *Verticillium dahliae* was used as an outgroup. The numbers above the lines represent the 1000 replicates parsimony bootstrap values. Virulence group; NP = non pathogenic, L = low virulent, M = moderate virulent, H = high virulent.

prochloraz at 60 days were not significantly different in DSI which were 2.6, 3.0, 2.8, and 3.7, respectively when compared to tomato inoculated with pathogen, the DSI was 4.7 (Table 5). Moreover, the tested bio-agent formulations gave significantly higher plant growth parameters than prochloraz and non-treated control. Treated

**Table 3.** Mycelial and conidial inhibition of antagonistic fungi against *Fusarium oxysporum* f.sp. *lycopersici* isolate NKSC01 in dual culture test at 30 days.

| Antagonistic fungi | Mycelial inhibition[1] (%) | Conidial inhibition (%) |
|---|---|---|
| *Chaetomium globosum* N0802 | 71.11b[1] | 92.14a |
| *Chaetomium lucknowense* CLT | 88.89a | 92.54a |
| *Trichoderma harzianum* PC01 | 90.56a | 99.99a |

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

**Table 4.** Testing antagonistic substances at concentration of 1,000 µg/ml to inhibit *Fusarium oxysporum* f.sp. *lycopersici* isolate NKSC01 at 7 days.

| Antagonistic fungi | Crude extract | Conidial inhibition[1] (%) | $ED_{50}$ (µg/ml) |
|---|---|---|---|
| *Chaetomium globosum* N0802 | CG/Hexane | 63.13c | 157 |
| | CG/Ethyl acetate | 62.58c | 339 |
| | CG/Methanol | 56.57d | 302 |
| *Chaetomium lucknowense* CLT | CL/Hexane | 74.85b | 188 |
| | CL/ Ethyl acetate | 69.55b | 209 |
| | CL/Methanol | 72.62b | 212 |
| *Trichoderma harzianum* PC01 | Thz/Hexane | 52.06d | 861 |
| | Thz/Ethyl acetate | 96.93a | 232 |
| | Thz/Methanol | 97.50a | 192 |

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

**Table 5.** Testing bio-agent formulations to control Fusarium wilt of tomato *in vivo* for 60 days.

| Treatments[1] | DSI | DR[3] (%) | Plant height (cm) | Plant fresh weight (g) | Plant dry weight (g) | Yield/plant (g) | Increase in yield[4] (%) |
|---|---|---|---|---|---|---|---|
| N0802 | 2.60b[2] | 44.68a | 37.40b | 67.55a | 11.53a | 133.81a | 88.53a |
| CLT | 3.00b | 36.28a | 35.65b | 54.90ab | 8.68a | 94.40ab | 83.74a |
| PC01 | 2.80b | 41.01a | 40.20ab | 54.90ab | 12.11a | 120.32ab | 87.24a |
| prochoraz | 3.70ab | 21.95b | 21.10c | 32.5bc | 4.90b | 26.27c | 41.57b |
| Fol | 4.70a | - | 14.80c | 22.20c | 3.80b | 15.35c | - |
| No-Fol | 1.00c | - | 49.50a | 50.35ab | 9.89a | 82.47b | 81.39a |

[1] N0802 = *C. globosum* N0802, CLT = *C. lucknowense* CLT, PC01 = *T. harzianum* PC01, Fol = inoculated with *F. oxysporum* f sp *lycopersici* only, No-Fol= non-inoculated with pathogen and non-treated bio-agent formulation. [2]Average of four replications (5 plants/rep.). Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.05. [3]% disease reduction (DR) = disease severity index (DSI) of control – disease severity index of treatment/ disease severity index of control x 100. [4]% increase in yield = Yield per plant of treatment – Yield per plant of control/ Yield per plant of treatment x 100.

bio-agent formulations of N0802, CLT and PC01 gave non-significant difference in plant height as 37.40, 35.65 and 40.20 cm, respectively but there was significant difference when compared to prochloraz and inoculated control which showed plant height as 21.10 and 14.80cm, respectively. Moreover, it showed that bio-agent formulations of N0802, CLT PC01 and non-inoculated with pathogen could be significantly higher in fresh plant and dried weights than prochloraz and inoculated control.

Interestingly, bio-agent formulation treatments of N0802 gave significantly high yields as 133.81 g/plant, followed by bio-agent formulations treatments of CLT, PC01 and non-inoculated treatment which were 94.40, 120.32 and 82.47 g/plant, respectively. Prochloraz treatment and inoculated control gave non-significant differrence in yields as 26.27 and 15.35 g/plant, respectively.

## DISCUSSION

*F. oxysporum* f. sp. *lycopersici* NKSC01 was isolated from infested tomato fields in Thailand and it proved to be

the most aggressive isolate of tomato var. Sida as confirmed by Sibounnavong et al. (2010). The sequences were confirmed through morphological identification by using ITS sequences with the length of the complete ITS1, 5.8S and ITS2 including a small portion of 18S rDNA and a small portion of the 28S rDNA. It was clearly demonstrated that the isolate NKSC01 is *F. oxysporum* f.sp. *lycopersici* with a valid identification. There are many reports showing that this technique has been validly used to confirm the species of *F. oxysporum* (Paplomatas, 2004; Hirano and Arie, 2006; Kawabe et al., 2005).

Dual culture test showed that *C. globosum* N0802, *C. lucknowense* CLT and *T. harzianum* PC01 could control Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici*. Based on the results, they exhibited high properties to inhibit conidial production over 90%. The results were similar to the report of Srinon et al. (2006) who reported that *T. harzianum* WS01 showed efficacies of more than 90% to inhibit conidial production of *F. oxysporum* f. sp. *lycopersici and F. oxysporum* f. sp. *Cucumerinum* (wilt of cucumber). In this study, the antagonistic substances (crude extracts) of *C. globosum* N0802, *C. lucknowense* CLT and *T. harzianum* PC01 at 1,000 µg/ml could inhibit conidial production of pathogen over 50%. With this, crude extracts of *T. harzianum* PC01 at 1,000 µg/ml inhibited conidial production over 90%. These results are similar to many works that showed that antagonistic substances from *C. globosum* CG extracted with ethyl acetate and *T. harzianum* PC01 extracted with ethyl acetate at 500 µg/ml could inhibit conidia production of *Colletotrichum gloeosporioides* WMF01 causing anthracnose disease of grape in which $ED_{50}$ values were 2 and 7 µg/ml, respectively (Soytong et al., 2005). The result of this study showed that the $ED_{50}$ values of crude extracts of *C. globosum* N0802 and *T. harzianum* PC01 were 157 and 192 µg/ml, respectively with effective inhibition to Fusarium wilt pathogen. These tested antagonistic fungi showed antibiotic mechanism to inhibit growth of Fusarium wilt pathogen which was supported by Soytong (1992) who stated that antagonistic substance from *C. cupreum*, *C. globosum* and T. *harzianum* could inhibit growth and also break the cells of *F. oxysporum* f. sp. *lycopersici*. Moreover, Park et al. (2005) stated that liquid culture of *C. globosum* F0142 could suppress the development of disease more than 80% and can exhibit antifungal activity against *Phytophthora infestans* in tomato at moderate level *in vivo*. *C. globosum* N0802 was also reported to produce antibiotic substances namely chaetomanone that exhibited antitubercular activity against *Mycobacterium tuberculosis*, a major human disease (Kanokmedhakul et al., 2002). Besides, Suwan et al. (2000) has reported that *T. harzianum* PC01 can also be used to produced trichotoxin A50, an antibiotic polypopeptides, which could inhibit the pathogen, stimulate plant growth and induce plant immunity. Moreover, Haggag and Mohamed (2007) reported that *T. harzianum* salt-tolerant mutants produced antifungal metabolites (trichodermin, gliotoxin and gliovirin) as antibiotics that could reduce the growth rate of *F. oxysporum*. These results were supported by the work of Yiğit and Dikilitas (2007) which stated that *T. harzianum* has multi mechanism of actions for controlling plant pathogens that is mycoparasitism via production of chitinase, β-1-3 glucanase, β-1-4 glucanase, antibiotic, competition, induced resistance and inactivation of enz-yme produced by pathogen in the process of infection.

*In vivo* testing, bio-agent formulations N0802 (*C. globosum*), CLT (*C. lucknowense*) and PC01 (*T. harzianum*) clearly demonstrated that these bio-agent formulations gave a highly effective control of Fusarium wilt of tomato caused by *F. oxysporum* f. sp *lycopersici*. The bio-agent formulations could reduce disease incidence of tomato wilt, leading to increase in yield. The study showed that bio-agent formulation of N0802 could decrease wilt incidence of 44.68% and increases the yield by 88.53%. The bio-agent formulation of PC01 decreased disease incidence of 50.97% and increased the yield by 87.24%. This result is similarly reported by Soytong (1992) who stated that registered bio-fungicide formulated from *C. cupreum* could decrease disease incidence of tomato wilt and also increase its yield. The result from bio-agent formulation of PC01 in this study is also supported by the work of Yiğit and Dikilitas (2007). It was reported that *T. harzianum* T-22 could reduce disease incidence of tomato Fusarium wilt at 42% and also improve yield and mineral contents of tomatoes.

As a result of bio-agent formulations N0802 (*C. globosum*), CLT (*C. lucknowense*) supported the previous work of Soytong et al. (2005) which showed that *Chaetomium* bio-products formulated from *C. globosum* and *C. cupreum* as powder formulation could control bud rot and basal stem rot of bottle palms caused by *Thielaviopsis paradoxa* in the field and reduce disease incidence by 75%. These new bio-agent formulations which act as concentration suspension in oil form were of different formulation but are effective in controlling Fusarium wilt of tomato as seen in the report of Soytong et al. (2001) who showed that the biological products consist of *Chaetomium* sp. (22 strains of *C. cupreum* and *C. globosum*) in biopellet and biopowder formulations which when applied to the soil could suppress the growth of *F. oxysporum* f. sp. *lycopersici* and reduce infection rate in tomato.

## ACKNOWLEDGMENTS

This research was a part of Ph.D. thesis and was supported by Nakhon Ratchasima Rajabhat University. We thank Mr. Sowat Mardwangsaeng and Mrs. Sukrittaya Dannarong for their help in collecting samples and Ms.

Kanchalika Rattanacherdchai for her help with this experiment.

**REFERENCES**


Agrios GN (1997). Plant Pathology. The 4th edition. Academic Press, San Diego.

De Cal A, Garcia-Lepe R, Melagarejo P (2000). Induced resistance by *Penicillium oxalicum* against *Fusarium oxysporum* f. sp. *lycopersici*: histological studies of infected and induced tomato stems. Phytopathology, 90: 260-267.

Floch GL, Rey P, Déniel F, Benhamou N, Picard K, Tirilly Y (2003). Enhancement of development and induction of resistance in tomato plants by antagonist, *Pythium oligandrum*. Agronomie, 23: 455-460.

Haggag WM, Mohamed HAA (2007). Biotechnological aspects of microorganisms used in plant biological control. Am.-Eurasian J. Sustain. Agric. 1: 7-12.

Hirano Y, Arie T (2006). PCR-based differentiation of *Fusarium oxysporum* ff. sp. *lycopersici* and *radicis-lycopersici* and races of *F. oxysporum* f. sp. *lycopersici*. J. Gen. Plant Pathol. 72: 273-283.

Kanokmedhakul K, Kanokmedhakul S, Soytong K (1993). Testing of Chaetomium and Some plant extracts for controlling Fusarium wilt of tomato (*Fusarium oxysporum* f. sp. *lycopersici*). J. Agric. Res. Exten. 10: 5-9.

Kanokmedhakul S, Kanokmedhakul K, Nasomjai P, Louangsysouphanh S, Soytong K, Isobe M, Kongsaeree P, Prabpai S, Suksamrarn A (2006). Antifungal azaphilones from the fungus *Chaetomium cupreum* CC3003. J. Nat. Prod. 69: 891-895.

Kanokmedhakul S, Kanokmedhakul K, Phonkerd N, Soytong K, Kongsaeree P, Suksamrarn A (2002). Antimycobacterial antraquinone-chromanone compound and diketopiperazine alkaloid from the fungus *Chaetomium globosum* KMITL-N0802. Planta Med. 68: 834-836.

Kanokmedhakul S, Kanokmedhakul K, Prajuabsuk T, Soytong K, Kongsaeree P, Suksamrarn A (2003). A bioactive triterpenoid and vulpinic acid derivatives from the mushroom *Scleroderma citrinum*. Planta Med. 69: 568-571.

Kawabe M, Kobayashi Y, Okada G, Yamaguchi I, Teraoka T, Arie T (2005). Three evolutionary lineages of tomato wilt pathogen, *Fusarium oxysporum* f.sp. *lycopersici*, based on sequences of IGS, MAT1, and pg1, are each composed of isolates of a single mating type and a single or closely related vegetative compatibility group. J. Gen. Plant Pathol. 71: 263-272.

Larkin RP, Fravel DR (1998). Efficacy of various fungal and bacterial biocontrol organisms for control of Fusarium wilt of tomato. Plant Dis. 82: 1022-1028.

Marlatt ML, Correll JC, Kaufmann P (1996). Two genetically distinct populations of *Fusarium oxysporum* f.sp. *lycopersici* Race 3 in the United States. Plant Dis. 80: 1336-1342.

Mohamed HAA, Haggag WM (2006). Biocontrol potential of salinity tolerant mutants of *Trichoderma harzianum* against *Fusarium oxysporum*. Braz. J. Microbiol. 37: 181-191.

Moretti M, Gilardi G, Gullino ML, Garibaldi A (2008). Biological control Potential of *Achromobacter xylosoxydans* for suppressing *Fusarium* wilt of tomato. Int. J. Bot. 4: 369-375.

Neuvéglise CY, Brygoo Y, Riba G (1994). Comparative analysis of molecular and biological characteristics of strains of *Beauveria brongniartii* isolated from insects. Mycol. Res. 98: 322-328.

Özgönen H, Biçici M, Erkiliç A (2001). The effect of salicyclic acid and endomycorrhizal fungus *Glomus etunicatum* on plant development of tomatoes and Fusarium wilt caused by *Fusarium oxysporum* f.sp. *lycopersici*. Turk. J. Agric. For. 25: 25-29.

Paplomatas EJ (2004). Molecular diagnostics for soilborne fungal pathogens. Phytopathol. Mediterr. 43: 213-220.

Park JH, Choi GJ, Jang KS, Lim HK, Kim HT, Cho KY, Kim JC (2005). Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. FEMS Microbiol. Lett. 252: 309-313.

Sibounnavong P, Keoudone C, Soytong K, Divina CC, Kalaw SP (2010). A new mycofungicide *Emericella nidulans* against tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici*. J. Agric. Technol. 6:19-30.

Silva JC, Bettiol W (2005). Potential of non-pathogenic *Fusarium oxysporum* Isolates for control of Fusarium wilt of tomato. Fitopatologia Brasileira, 30: 409-412.

Soytong K (1992). Biological control of tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* by using *Chaetomium cupreum*. Kasetsart J. 26: 310-313.

Soytong K, Kanokmedhakul S, Kukongviriyapa V, Isobe M (2001). Application of *Chaetomium* (Ketomium®) as a new broad spectrum biological fungicide for plant disease control: A review article. Fungal Div. 7: 1-15.

Soytong K, Pongnak W, Kasiolarn H (2005). Biological control of *Thielaviopsis* bud rot of *Hyophorbe lagenicaulis* in the field. J. Agric. Technol. 1: 235-245.

Soytong K, Srinon W, Rattanacherdchai K, Kanokmedhakul S, Kanokmedhakul K (2005). Application of antagonistic fungi to control anthracnose disease of grape. J. Agric. Technol. 1: 33-41.

Srinon W, Chuncheen K, Jirattiwarutkul K, Soytong K Kanokmedhakul S (2006). Efficacies of antagonistic fungi against Fusarium wilt disease of cucumber and tomato and the assay of its enzyme activity. J. Agric. Technol. 2: 191-201.

Suwan S, Isobe M, Kanokmedhakul S, Lourit N, Kanokmedhakul K, Soytong K, Koga K (2000). Elucidation of high micro-heterogeneity of an acidic-neutral trichotoxin mixure from *Trichoderma harzianum* by electrospray ionization quadrupole time-of-flight mass spectrometry. Mass Spectrometry, 35: 1438-1451.

Swofford DL (1998). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Yiğit F, Dikilitas M (2007). Control of Fusarium wilt of tomato by combination of fluorescent *Pseudomonas*, non-pathogen *Fusarium* and *Trichoderma harzianum* T-22 in greenhouse condition. Plant Pathol. 6: 159-163.

Yasushi H, Tsutomu A (2006). PCR-based differentiation of Fusarium *oxysporum* f. sp. *lycopersici* and radicis-*lycopersici* and races of *F. oxysporum* f.sp. *lycopersici*. J. Gen. Plant Pathol. 72: 273-283.