Increased Efficacy for Controlling Anthracnose of Chili Using Antifungal Metabolites from Mutant Strains of *Trichoderma harzianum*

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Abstract

Three mutant and two wild type strains of *Trichoderma harzianum* were tested for efficacy to inhibit and overgrow mycelia of *Colletotrichum capsici*, a causal agent of anthracnose of chili on potato dextrose agar (PDA) at room temperature. All strains effectively inhibited and overgrew mycelia of the pathogen, especially two mutant strains (T-35-co4 and T-35-co5) providing the high percent of inhibition of 83.00 and 75.50%, respectively. In addition, another mutant strain, T-50-co4, overgrew mycelia of the pathogen with the highest percentage of 69.50. By using dialysis membrane technique, three mutant strains (T-35-co4, T-35-co5 and T-50-co4) could produce antifungal metabolites, which completely inhibited mycelial growth of the pathogen. Extracted antifungal metabolites from these strains were tested for their efficacy to inhibit spore germination and mycelial growth of the pathogen in 1/5 potato dextrose broth (PDB) amended with equal volume of extracted antifungal metabolites. The results showed that 1000 mg L^{-1} of antifungal metabolites completely inhibited both the mycelial growth and spore germination of the pathogen. When chili fruits were soaked in 1000 mg L^{-1} of antifungal metabolites from three mutant strains to control anthracnose disease, the severity of anthracnose was significantly decreased as compared with the control (2% methanol). The inhibition percentage of disease severity in treatments with antifungal metabolites from strains T-35-co4, T-50-co4 and T-35-co5 were 84.5, 80.0 and 77.5%, respectively, while a control treatment (2% methanol) was 12.5% of disease severity.

Keywords: antifungal metabolite, *Trichoderma harzianum*, biological control, anthracnose of chili

Introduction

Anthracnose disease caused by *Colletotrichum capsici* is one of the major diseases for chili. This fungus causes severe damage on chili fruits in both pre and post harvest stages. The black wound found on infected fruits will expand very quickly under high moisture condition, especially in tropical countries. Fruiting bodies and spores of *C. capsici* were abundantly produced on those black lesions. There are various methods in controlling the disease, but the two common ones are chemical using benomyl and biocontrol. Nowadays, consumers become more aware on food safety, especially pesticide residues which should not be detected on final products. Biocontrol is thus a promising and safe way for management of anthracnose disease. *Trichoderma harzianum* and its products have been studied and used for biocontrol in many countries. New strains of better properties or higher yield of antifugal metabolite are always reported. Some mutant strains induced by ultraviolet irradiation

provided higher biocontrol properties than wild types. Successful examples of using *T. harzianum* mutant strain as a biocontrol agent were damping-off of cucumber caused by *Pythium aphanidermatum* (Intana et al., 2003) and black rot of rambutan caused by *Lasiodiplodia theobromae* (Rajappan et al., 1996). Most of effective strains produced both cell wall lytic enzymes and secondary metabolites against the disease causing fungi. The purposes of this research were (1) to evaluate the efficacy of antifungal metabolites extracted from wild type and mutant strains of *T. harzianum* for controlling anthracnose on chili fruits and (2) to study the mechanisms of antifungal metabolites for inhibition of spore germination and germ-tube growth.

Materials and Methods

Strains of *Trichoderma harzianum*

Wild type strains of *T. harzianum* including T-35-WT and T-50-WT were isolated from Durian growing soil in Surat Thani and Songkhla provinces, Thailand, respectively. Three mutant strains used in this study, T-35-co4, T-35-co5 and T-50-co4, derived from the ultraviolet induction of those wild type strains (T-35-WT and T-50-WT) were came from previous research of Intana (2003). The mutant strains could grow on potato dextrose agar (PDA) amended with 1000 mg L^{-1} benomyl, while wild type strains failed to grow. All mutant strains produced higher amount of β-1,3-glucanase and β-1,4-glucanase than wild types, while antifungal oxazole and pentyl pyrone were also detected (Intana, 2003).

Isolation and Pathogenicity Test

Colletotrichum capsici, a causal agent of anthracnose was isolated from eight anthracnose infected chili fruits obtained, from the chili planting areas in Nakhon Si Thammarat and Nakhon Prathom provinces, Thailand. Anthracnose fungus was isolated by tissue transplanting method described by Agrios (2005). For pathogenicity test, each of eight isolates of *C. capsici* was cultured on PDA for 3 days. Then 0.7 cm agar plug contained with mycelia of *C. capsici* was placed on pierced area on chili fruit (*Capsicum anuum* L. var. annuum) obtained from chili plantations at Pak Phanang, Nakhon Si Thammarat province. All inoculated fruits were incubated in moist plastic chamber, kept at room temperature (27$\pm^{o}$C). Disease severity of anthracnose infection was recorded at 5 days after incubation by measuring size of diseased lesion on chili fruit. The percentage of disease severity was calculated by using the formula; $((R_T-R_C)/R_T)\times100$, when R_T was the mean of diseased lesion radius on chili in the tested treatment and R_C was the mean of diseased lesion radius on chili in the control (placed with a agar plug without *C. capsici*). For each treatment, there were four replicates and 5 chili fruits was used in each replicate. Then a most pathogenic isolate was used for further.

Mycelial Growth Inhibition and Over-growth Test

For mycelial growth inhibition and overgrowth test, dual culture test was performed. *T. harzianum* strains and *C. capsici* were subcultured onto PDA for 4 days. The margin of colony of *C. capsici* was cut with sterile cork borer (0.7 cm diameter) and placed on agar surface at 1.5 cm from a margin of 9 cm diameter Petri-dish. At 4 days after placing the plug of *C. capsici*, a plug of the *T. harzianum* was inoculated at the opposite direction, 6 cm apart from the *C. capsici* plug. The dishes were incubated for 5 days at room temperature, then mycelial growth inhibition and the ability of *T. harzianum* strains to overgrow the colony of *C. capsici* were observed and compared with the control treatment (*C. capsici* grown on PDA without a *T. harzianum*) and the treatment of commercial *Trichoderma* strain sold in Thailand, *T. harzianum* CB-Pin-01 (Chamswarng and Tanangsa-nakul, 1996). The inhibition levels were calculated by using the formula; $((G_C-G_T)/G_C)\times100$, when G_C was the mean of colony radius of *C. capsici* in the control dish and G_T was the mean of colony radius of *C. capsici* in Petri-dish of dual culture test. Each treatment was performed with four replicates, one dish per a replicate. The overgrowth rates of *T. harzianum* were calculated by using the formula; $((D_1-D_2)/T_d)\times100$, when D_1 was the mean of colony radius of the *T. harzianum* strain on the day of recording, D_2 was the mean of colony radius of the *T. harzianum* strain on the day before recording and T_d was the time (d) between before and after

recording. Each treatment was performed with 4 replicates.

Dialysis Membrane Technique Test of Antifungal Metabolite

The cork borered-plug (0.7 cm) of 3-day-old *T. harzianum* strain grown on PDA was placed on sterile dialysis membrane which was aseptically placed over surface of PDA. Five replicates (one Petri-dish for each replicate) were used for each strain. The Petri-dishes were tightly sealed with a plastic wrap and incubated at room temperature for 36 h before the dialysis membrane covered with the mycelia of *T. harzianum* was removed. The activity of secondary metabolite left in agar after removal of dialysis membrane was tested by inoculating 0.7 cm plug of *C. capsici*, taken from the actively growing margin of colony, at the position previously occupied by the inoculum plug of a *T. harzianum* strain. The diameter of mycelial growth of each colony of *C. capsici* was measured after incubation at room temperature for 5 days. The percentage of inhibition of growth was calculated using the formula: percent inhibition = $((D_C-D_T)/D_C)\times100$, when D_C was a mean of diameters from all colonies of *C. capsici* growing on Petri-dish with previously grown with *C. capsici* on dialysis membrane, D_T was a mean of diameters from all colonies of *C. capsici* growing on Petri-dish with previously grown with *T. harzianum* strain on dialysis membrane. This formula was modified from Intana (2003). In each of five replicated dishes contained with mycelial plug (0.7 cm) of *C. capsici* placed on dialysis membrane was served as a control.

Extraction of Antifungal Metabolites

T. harzianum strains were cultured on PDA for 2 days, before the margin of colony of each strain was cut by 0.7 cm cork borer. Twenty-five plugs of each *T. harzianum* strain were put into 3 L flask containing 1 L of 1/5 strength potato dextrose broth (PDB) and incubated at room temperature. Twenty-eight days after incubation, conidia and mycelia of *T. harzianum* strains were removed from broth culture by filtration. The culture filtrate was used for extraction of antifungal metabolites. The extraction of antifungal metabolites was repeated for three times in sequences with 350 mL of ethyl acetate (EtoAc). Separation of the two phases was facilitated by the addition of 5 g of sodium chloride to the first extraction. Then EtoAc was evaporated at 40^{o}C in a rotary evaporator. Dry weights of antifungal metabolites were recorded (Intana, 2003).

Inhibition of Spore Germination

Spore suspension of *C. capsici* was prepared from 7-day-old culture growing on PDA, using 1/5 strength PDB as a diluent. Concentration of spores in a suspension was determined and adjusted with haemacytometer to $1x10^5$ spores mL^{-1}. Then, 5.0 mL of spore suspension was mixed with 5.0 mL of 1000 mg L^{-1} of each antifungal metabolite in a test tube and kept for 12 h at room temperature before the sample was stained with lactophenol cotton blue. Germinating spores were observed and recorded under compound light microscope compared with a control treatment and a treatment with 1000 mg L^{-1} of antifungal from commercial strain (Yenjit et al., 2004).

Inhibition of Germ-tube Elongation

The spore suspension of *C. capsici* (1×10^5 spores mL^{1}) was incubated at room temperature for 12 h for most spores to germinate. Then the 5.0 mL of germinating spore suspension was mixed with 5.0 mL of 1000 mg L^{-1} of each antifungal metabolite and incubated at room temperature for 12 h. The length of germ-tube was measured with the help of lactophenol cotton blue staining under light microscope and compared with a control treatment and a treatment with 1000 mg L^{-1} of antifungal from commercial strain (Yenjit et al., 2004).

Control of Anthracnose on Chili Fruit

The chili fruits (*Capsicum anuum* L. var. annuum) collected from chili field at Pak Phanang, Nakhon Si Thammarat province were disinfested with 0.525% sodium hypochlorite before making a tiny wound on each chili fruit with a sterilized needle. Then, the wounded chili fruit was immersed in 1000 mg L^{-1} of each antifungal metabolite for an hour at room temperature. Then, 0.5 mL of spore suspension of *C. capsici* was dropped on the wounded area on chili fruit. The inoculated chili fruits were kept in plastic box and incubated for 4 days in plant growth cabinet (25^{o}C, 12 h of light and 80% of water capacity content). The percentage of disease

inhibition was then observed, recorded and compared with the control treatment (wounded chili fruits soaking in 2% methanol). The treatment with 1000 mg L^{-1} of antifungal from commercial *Trichoderma* strain and a benomyl chemical fungicide were used as other controls for efficacy comparison. Each treatment comprised of four replicates with ten fruits in each replicate. The percentage of disease inhibition was calculated by using the formula; $((R_C-R_T)/R_C)\times100$, when R_C was the mean of diseased lesion radius on chili in the control and R_T was the mean of diseased lesion radius on chili in the treatment with antifungal metabolite.

Statistical Analysis

All data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute Inc., 2000) system for Windows and were considered significant when $P\leq0.05$.

Results

Isolation and Pathogenicity Test

The results showed that all strains of *C. capsici* could cause disease on chili fruits at various levels of percent infection. Isolates Cc-NST-01 and Cc-NST-02 collected from Pak Phanang, Nakhon Si Thammarat provided 75.5 and 78.0% of infection. While isolate Cc-DAT provided only 47.5% of infection (Table 1). Therefore isolate Cc-NST-02 was used in further experiments.

Growth Inhibition Test and Overgrow Test

All strains of *T. harzianum* could inhibit mycelial growth of *C. capsici* Cc-NST-02 with the inhibition percentages ranging from 52.0-83.0. Strains T-35-co4 showed the highest percentage of mycelial growth inhibition of 83.0%. All mutant isolates provided significant higher percent inhibition than their wild type strains as well as the commercial strain, T-CB-Pin-01 (Table 2).

All strains of *T. harzianum* overgrew mycelia of *C. capsici* Cc-NST-02 with overgrowth percentages ranging from 41.5 to 69.5. Strains T-50-co4 provided the highest percent overgrowth of 69.5. In addition, all mutant isolates provided significant higher percent mycelial overgrowth than their wild type strains and the commercial strain, T-CB-Pin-01 (Table 2).

Dialysis Membrane

From dialysis membrane technique, the results showed that all strains of *T. harzianum* produced antifungal metabolites left on PDA. Therefore, the mycelial growth of *C. capsici* Cc-NST-02 was completely inhibited on PDA previously cultured with *T. harzianum* over dialysis membrane (Table 3).

Table 1 Percent infection on chili fruits by *Colletotrichum capsici* isolated from anthracnose infected chili fruits in various locations.

Strain	Location	Infection (%)[1/]
Cc-NST-01	Pak Phanang, Nakhon Si Thammarat	75.50 a
Cc-NST-02	Pak Phanang, Nakhon Si Thammarat	78.00 a
Cc-NP-01	Khampheang Saen, Nakhon Prathom	46.00 c
Cc-NP-02	Khampheang Saen, Nakhon Prathom	51.50 bc
Cc-NP-03	Khampheang Saen, Nakhon Prathom	63.00 b
Cc-NP-04	Khampheang Saen, Nakhon Prathom	65.00 b
Cc-NP-05	Khampheang Saen, Nakhon Prathom	26.00 d
Cc-NP-06	Khampheang Saen, Nakhon Prathom	46.00 c
Cc-DAT	Department of Agriculture of Thailand	47.50 c

[1/] Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).

Table 2 Efficacy of wild type and mutant strains of *Trichoderma harzianum* on mycelial growth inhibition and overgrowth of *Colletotrichum capsici* Cc-NST-02 in Petri-dish at room temperature.

Strain	Efficacy of *Trichoderma harzianum* (%)[1/]	
	Mycelial growth inhibition[2/]	Mycelial overgrowth[3/]
T-35-WT[4/]	64.50 c	55.00 c
T-35-co4	83.00 a	61.50 b
T-35-co5	75.50 b	62.00 b
T-50-WT[4/]	52.00 d	43.00 d
T-50-co4	69.50 bc	69.50 a
T-CB-Pin-01	65.00 c	41.50 d

[1/] Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).
[2/] Inhibition of mycelial growth of *Colletotrichum capsici* by *Trichoderma harzianum* on PDA in dual culture test.
[3/] Percent of mycelial overgrowth of *Trichoderma harzianum* on colony of *Colletotrichum capsici*.
[4/] Wild type isolates.

Table 3 Dry weight of antifungal metabolites from *Trichoderma harzianum* and the inhibition on mycelial growth of *Colletotrichum capsici* Cc-NST-02.

Treatments	Mycelial growth inhibition (%)	Dry weight of antifungal metabolite (g L^{-1})
T-35-WT[1/]	100	4.63
T-35-c04	100	4.74
T-35-co5	100	4.28
T-50-WT[1/]	100	4.31
T-50-co4	100	4.83
T-CB-Pin-01	100	4.39
Control (without *T. harzianum*)	0	-

[1/] Wild type isolates.

Extraction of Antifungal Metabolites

Antifungal metabolites with coconut milk smell were successfully extracted from all strains *T. harzianum* using ethyl acetate as a solvent. Dried extracts were white-yellow in color and their weights were in the range of 4.28-4.83 g per 1 L PDB. Strain T-50-co4 gave the highest amount (4.83 g L^{-1}) of the extract, while strain T-35-co5 gave the lowest amount (4.28 g L^{-1}) of the extract. However, the amounts of extracts from all strains were not significantly different (Table 3).

Inhibition of Spore Germination

Antifungal metabolites extracted from all strains of *T. harzianum* significantly inhibited spore germination and germ tube growth of *C. capsici* as compared to the control. Spore germination of *C. capsici* Cc-NST-02 were 5.0-29.5% when treated with the antifungal extract, whereas the control was 86.0%. Strains T-35-co4, in particular, provided the strongest spore inhibition which only 5.0% of *C. capsici* Cc-NST-02 spore could germinate (Table 4).

Inhibition of Germ-tube Elongation

Antifungal metabolites extracted from all strains of *T. harzianum* inhibited germ-tube growth of *C. capsici* Cc-NST-02. Growth of germ-tubes were 17.46-27.40 μm when treated with the antifungal extracts while the control was 61.14 μm (Table 4). Again, strains T-35-co4 provided the strongest inhibition of *C. capsici* Cc-NST-02 germ-tube growth (Table 4).

Control of Anthracnose on Chili Fruit

Antifungal metabolites extracted from all strains of *T. harzianum*, at 1000 mg L^{-1}, significantly reduced anthracnose disease severity on chili fruit by at 63.50-84.50%, whereas the control (2% methanol) was at 12.50%. All mutant strains yielded significantly better inhibition of anthracnose disease than wild types, especially strain T-35-co4 which was the most effective strain with 84.50% of inhibition. However, there were no statistically different to the use of benomyl (87.00%), while treatment with antifungal metabolite from commercial strain provided 63.50% of inhibition (Table 5).

Discussion

The results showed that both wild types and mutant strains of *T. harzianum* could inhibit growth and overgrow on mycelia of *C. capsici*. Moreover, the efficacy study of antifungal metabolites by dialysis membrane technique indicated that all strains of *T. harzianum* produced highly effective antifungal

Table 4 Efficacy of antifungal metabolites (1000 mg L^{-1}) from *Trichoderma harzianum* on the inhibition of spore germination and germ-tube elongation of *Colletotrichum capsici* Cc-NST-02 within 12 h of incubation[1/].

Treatment	Spore germination[2/] (%)	Length of germ-tube[3/] (μm)
T-35-WT[4/]	14.0 c	20.34 bc
T-35-c04	5.0 d	17.46 c
T-35-co5	10.5 cd	20.17 bc
T-50-WT[4/]	29.5 b	26.48 b
T-50-co4	12.0 c	24.31 b
T-CB-Pin-01	26.5 b	27.40 b
2% methanol	86.0 a	61.14 a

[1/] Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).
[2/] Means of spore germination calculated from four replications (100 spores per replication)
[3/] Means of germ-tube's length from four replications (100 germ-tubes per replication).
[4/] Wild type isolates.

Table 5 Inhibition of disease severity on chili fruits after applied with antifungal metabolites (1000 mg L^{-1}) from four mutant strains of *Trichoderma harzianum* in growth chamber at 25°C for 5 days[1/].

Treatment	Disease severity[2/] (%)	Inhibition of disease severity[3/] (%)
T-35-WT[4/]	30.0 d	70.0 c
T-35-c04	15.5 f	84.5 a
T-35-co5	22.50 e	77.5 b
T-50-WT[4/]	28.50 d	71.5 c
T-50-co4	20.0 ef	80.0 ab
T-CB-Pin-01	36.5 c	63.5 d
benomyl	13.0 f	87.0 a
2% methanol	87.5 b	12.5 e
Control1 (with pathogen)	100.0 a	0.0 f
Control2 (without pathogen)	0.0	-

[1/] Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).
[2/] Percentage of disease severity in the treatments as compared to the control.
[3/] Percentage of inhibition of disease severity in the treatments as compared to the control.
[4/] Wild type isolates.

metabolites that suppressed growth of this pathogenic fungus. There have been shown by Howell (2003) that *Trichoderma* spp. are successful antagonistic fungi. *Trichoderma*'s biocontrol properties include successful competition, myco-parasitism and antibiosis. In addition, *Trichoderma* spp. promotes plant growth and induces resistance in plant.

Comparison of the efficacy of *T. harzianum* in mycelial growth inhibition and overgrowth on *C. capsici* between wild types and mutant strains in laboratory revealed that all mutant strains provided higher efficacy. The results suggested the potential of mutant strains of *T. harzianum* to produce better and more effective antifungal metabolites. Intana (2003) reported that these mutant strains provided higher efficacy than their wild type strains to produce some antifungal metabolites, pyrone and oxazole. Moreover these mutant strains produced higher product of β-1,3-glucanase and β-1,4-glucanase than wild types. This report may support our results. Examples of UV-induced mutant strain of *T. harzianum* that showed similar findings of better growth inhibition and better overgrowth rate were those of Rajappan et al. (1996); Baby (1998) and Intana (2003) in controlling *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium aphanidermatum*, respectively. This suggested that the genetic manipulation of biological control fungi could be achieved with UV mutagenesis as pointed out by Lewis and Papavizas (1991). In another reported Baby (1998) induced mutation of *T. longibrachiatum* using UV irradiation and obtained mutant strains tolerant to Bavistin, that were able to control *R. solani* in rice and to survive in soil better than parental strains.

Ghisalberti and Sivasithamparam (1991) and Intana (2003) found that *T. harzianum* both wild type and mutant strains produced antifungal metabolites in 1/5 concentration of normal PDB under room temperature for 28 days. The antifungal metabolites produced were successfully extracted by ethyl acetate, especially pyrone which had a distinct smell of coconut milk. Our results were in accordant to the previous mentioned. There was a possibility that the extract might contain pyrone. The dried antifungal metabolites extracted were in the range of 4.28-4.83 g L^{-1} PDB.

Antifungal metabolites extracted from all strains of *T. harzianum* 1000 mg L^{-1} provided markedly high inhibitive activities on spore germination and germ-tube growth of *C. capsici*. These inhibition efficacies were significantly different from those of a control. Antifungal metabolites produced in this study were probably similar to those previous identified such as glioviridin, pentyl pyrone, gliotoxin, trichorzianines and oxazole (Ghisalberti and Sivasithamparam, 1991; Di Pietro et al., 1993; Lee et al., 1995; Intana, 2003).

There are many reports of successful use of antifungal metabolite extracted from *Trichoderma* spp. to control disease causing fungi such as *S. rolfsii* causing disease on vegetables (Maiti et al., 1991), *P. aphanidermatum* causing wilt of cotton and watermelon (Ordentlich et al., 1992) and damping-off of cucumber (Intana, 2003) and *Phytophthora* sp. causing various plant diseases (Wilcox et al., 1992). This research indicated an additional successful use of antifungal metabolites from both *T. harzianum* wild type and mutant strains in controlling anthracnose on chili fruits caused by *C. capsici*.

Higher efficacy of metabolites to inhibit of spore germination and germ-tube growth of *C. capsici* usually resulting in better control of chili's anthracnose. However, the efficacy of antifungal metabolites obtained from mutant strains of *T. harzianum* was not significantly different statistically to the one using benomyl. It is an expectation possible that mutant strains which produced more effective antifungal metabolites will provided higher yield than wild type. The antifungal metabolites mentioned in this study have not been purified. Therefore, it is very interesting to proceed further researches on the mass production and application of the antifungal metabolites for the control of other plant diseases in the fields. More research are needed to modify or simplify the metabolites extraction procedure as well as the information of metabolite concentration and stability in order to develop the metabolite products for further effective application.

Acknowledgments

This work was supported by Thailand Research Fund and National Center for Genetic Engineering and Biotechnology, under the BRT's Bioresources Ultilization Program grant (grant number BUP014G-48) Thanks for Scientific and Technology Equipment Center and Tropical Fruits Research Unit at Walailak University for the use of their facilities.

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Manuscript received 17 March 2007, accepted 21 December 2007