Use of Pentyl Pyrone Extracted from Ultraviolet-induced Mutant Strain of *Trichoderma harzianum* for Control Leaf Spot of Chinese-kale

W. Intana[1,*], C. Chamswarng[2], K. Chantrapromma[3], P. Yenjit[1] C. Suwanno[1] and S. Sattasakulchai[1]

[1]School of Agricultural Technology, Walailak University, Tha Sala Nakhon Si Thammarat 80161, Thailand
[2]Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen Kasetsart University, Nakhon Pathom 73140, Thailand
[3]School of Science, Walailak University, Tha Sala, Nakhon Si Thammarat 80161, Thailand

**Corresponding author. Email: iwarin@wu.ac.th*

Abstract

The antifungal pentyl pyrone was purified from an ultraviolet-induced mutant strain of *Trichoderma harzianum* T-35-co4 by a chromatography procedure (Intana, 2003). The pentyl pyrone at the concentrations of 50 and 100 mg L^{-1} were tested for the inhibition of mycelial growth and spore germination of *Alternaria brassicicola*, a causal agent of leaf spot on Chinese-kale. The results showed that treatments with extracted pentyl pyrone gave high percentages of inhibition. Especially, complete (100%) inhibition of mycelial growth of *A. brassicicola* on PDA at room temperature after 5 days subculture was achieved from the treatment with 100 mg L^{-1} of pentyl pyrone. Furthermore, all mycelia and most of spores of the pathogen were demonstrated non-viable after being soaked in pentyl pyrone solution. These concentrations were used to control the leaf spot disease in laboratory and under glasshouse conditions. When Chinese-kale leaf was sprayed with 50 or 100 mg L^{-1} of pentyl pyrone, the disease severity of leaf spot was significantly decreased when compared with the control (2% methanol). Treatments with 100 mg L^{-1} of pentyl pyrone showed best efficacy. Disease severity found in laboratory and under glasshouse conditions were 6.5 and 19.0% respectively, while those of a control treatments (2% methanol) were 88.0 and 92.5%, respectively.

Keywords: pentyl pyrone, *Trichoderma harzianum*, mutant strain, leaf spot, *Alternaria brassicicola*, biological control

Introduction

Alternaria brassicicola, a causal agent leaf spot disease, is the major plant pathogen of Chinese-kale. It is responsible for the 20-50% yield loss found in infected plants. The disease appears on the leaves as black, sooty, velvety lesions with yellow halos around them (chlorotic zones) (Verma and Saharan, 1994). This pathogen can attack most part of Chinese-kale especially on leaf. During plants attack, this pathogen can produce bassicicolin A and has been implicated in *A. brassicicola*'s pathogenesis (Gloer et al., 1988).

Various methods are used to control the disease. At present, most farmers in Thailand have tried to control the *A. brassicicola* disease by using chemical fungicides such as benomyl. However, consumers are reluctant to buy crops treated with this potential oncogenic fungicide. Fungicide also causes resistance of diseases and destroys the ecological balance of microorganisms. Therefore biological control agents (BCAs) and their products such as antifungal

metabolites are the alternative methods to disease control. *Trichoderma harzianum* has been reported to produce large quantity of antifungal, antibacterial, antiviral and antibiotic metabolites. In 2003, Intana reported that bioactive compound, pentyl pyrone, from ultraviolet-induced mutant strain of *T. harzianum* T-35-co4 gave high values of control damping-off disease of cucumber caused by *Pythium aphanidermatum*. Similarly, experiments performed on another commercially important crop, the Chinese-kale were attempted investigate on the efficacy of the pentyl pyrone (a) in inhibiting mycelial growth and spore germination of *A. brassicicola* (b) on viability of *A. brassicicola* and (c) to control both in laboratory and under glasshouse conditions leaf spot of the Chinese-kale.

Materials and Methods

Pentyl Pyrone

Pentyl pyrone is an antifungal metabolite of ultraviolet-induced mutant strain of *Trichoderma harzianum* T-35-co4. This mutant strain was supported by Biological Laboratory, Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom. It gave high values for the control of damping-off on cucumber caused by *Pythium aphanidermatum* (Intana, 2003).

Pathogen and Pathogenicity Test

The pathogen (*Alternaria brassicicola*) was supported by School of Agricultural Technology, Walailak University, Nakhon Si Thammarat Province (Intana et al., 2004). For use in pathogenicity tests, the pathogen was cultured on potato dextrose agar (PDA) for 3 days. Then 0.7 cm agar plug contained with mycelia of *A. brassicicola* was placed on pierced area on Chinese-kale leaf (*Brassica alboglabra* Bailey) obtained from our glasshouse at Walailak University. The inoculated leaf was incubated in moist plastic chamber, kept at room temperature ($27\pm^{o}C$). Disease severity of leaf spot infection was recorded at 4 days after incubation by measuring diameter of diseased lesion on Chinese-kale leaf. The percentage of disease severity was calculated by using the formula: $((R_T-R_C)/ R_T)x100$, when R_T was the mean of diseased lesion radius on Chinese-kale leaf in the tested treatment and R_C was the mean of diseased lesion radius on Chinese-kale leaf in the control (placed with a agar plug without *A. brassicicola*). For each treatment, four replicates with five Chinese-kale leaves by replicate were made.

Mycelial Growth Inhibition

Aliquant of 0.1 mL of 50 or 100 mg L^{-1} of pentyl pyrone solution, using 2% methanol as a solvent (Intana, 2003), was spread on the surface of PDA. The activity of pentyl pyrone left in agar was tested by inoculating 0.7 cm plug of *A. brassicicola,* taken from the actively growing margin of colony. The diameter of mycelial growth of each colony of *A. brassicicola* was measured after incubation at room temperature for 5 days. The percentage of inhibition of growth was calculated using the formula: percent inhibition = $((D_C-D_T)/D_C)x100$, when D_C was a mean of diameters from all colonies of *A. brassicicola* growing on Petri-dish in the control, D_T was a mean of diameters from all colonies of *A. brassicicola* growing on Petri-dish in the tested treatment. This formula was modified from Intana and Chamswarng (2007). Each of five replicated dishes contained mycelial plug (0.7 cm) of *A. brassicicola* placed on surface of PDA, spread with 2% methanol, and was served as a control.

Inhibition of Spore Germination

Spore suspension of *A. brassicicola* was prepared and adjusted to 1×10^5 spores mL^{-1}. Then, 3.0 mL of spore suspension was mixed with 3.0 mL of 50 or 100 mg L^{-1} of pentyl pyrone in a test tube and kept for 18 h at room temperature. Then the mixed sample was stained with lactophenol cotton blue. Germinating spores were observed and recorded under compound light microscope compared with a control treatment (2% methanol) and treatments with 50 or 100 mg L^{-1} of benomyl fugicide (Yenjit et al., 2004).

Viability of *Alternaria brassicicola*

Mycelial viability of *A. brassicicola* test: The pathogen was subcultured on PDA which covered with dialysis membrane for 4 days. Then the membrane contained with mycelia was cut (0.5×0.5 cm), placed into sterile Petri-dish and flooded with 10 mL of 50 or 100 mg L^{-1} pentyl pyrone. At 12 h

after incubation, the membrane was rinsed with sterile water for three times before placing onto sterile soft paper. Then the membrane was put into the Petri-dish containing PDA added with 1 mg L^{-1} streptomycin before incubation at room temperature. Five days after incubation, the mycelial growth of *A. brassicicola* was observed. The design used for this experiment was a completely randomized design (CRD) with five replications, 10 membranes per a replicate.

Spore viability of *A. brassicicola* test**:** Spore suspension of the pathogen was prepared and adjusted to 1×10^5 spores mL^{-1}. The spore suspension (2.5 mL) was added into a test tube contained with 2.5 mL of 50 or 100 mg L^{-1} pentyl pyrone solution and kept for 12 h at room temperature. Then the suspension was diluted for 10 times with 2.5 mL of sterile water before 0.1 mL of the diluted suspension was spread on surface of PDA added with 1 mg L^{-1} streptomycin. The Petri-dish was sealed with plastic wrap and incubated at room temperature. At 18 h after incubation, germinating spores were observed under compound light microscope, compared with a control treatment (2% methanol) and treatments with 50 or 100 mg L^{-1} of benomyl fugicide (Yenjit et al., 2004).

Disease Control in Laboratory

Detached Chinese-kale (*Brassica alboglabra* Bailey) leaves collected from our glasshouse were disinfested with 0.525% sodium hypochlorite before a tiny wound was made on each leaf with a sterilized needle. The wounded Chinese-kale leaf was sprayed with 1 mL of 50 or 100 mg L^{-1} of pentyl pyrone and kept for an hour at room temperature. One mL of spore suspension of *A. brassicicola* (1×10^5 spores mL^{-1}) was dropped on the wounded area. The inoculated Chinese-kale leaf was kept in moistened plastic box and incubated for 5 days in plant growth cabinet (25^oC, 12 h of light and 80% of water capacity content). The percentage of disease severity was then observed and compared with the control treatment (wounded Chinese-kale leaf sprayed with 1 mL of 2% methanol). The treatments with 50 or 100 mg L^{-1} of benomyl fungicide were used as another control for efficacy comparison. Each treatment comprised of four replicates with ten leaves by each replicate. Disease severity was recorded by comparing diseased area with the total leaf area on the fifth day after inoculation.

Disease Control in Glasshouse

Three-wk-old Chinese-kale seedlings were used in this experiment. The seedlings grown in the pots were sprayed with 10 mL spore suspension of *A. brassicicola* (1×10^5 spores mL^{-1}). At 30 min. after inoculation, the seedlings were then sprayed with 10 mL of 50 or 100 mg L^{-1} pentyl pyrone solution. The pots were kept under glasshouse conditions. Disease severity was evaluated by comparing diseased area with the total leaf area at 14 days after inoculation. There were 10 pots per replication and five replications per treatment.

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

Pathogen and Pathogenicity Test

The result showed that *A. brassicicola* could cause disease on Chinese-kale leaf at 75.0% of infection at room temperature, while no infection was observed in a control.

Mycelial Growth Inhibition

From mycelial growth inhibition test, the results showed that the mycelial growth of *A. brassicicola* was completely inhibited (100%, no visible growth) on PDA previously spread with 0.1 mL of 50 or 100 mg L^{-1} of pentyl pyrone solution. This result was similar to treatments using 0.1 mL of 50 or 100 mg L^{-1} of benomyl fungicide. On the other hand, a control treatment, with 2% methanol, the mycelia of *A. brassicicola* grew normally and covered the whole agar surface in the Petri-dish within 8 days (Table 1).

Inhibition of Spore Germination

Two concentrations of 50 or 100 mg L^{-1} of pentyl pyrone extracted from ultraviolet-induced mutant strain of *T. harzianum* (T-35-co4) significantly inhibited spore germination of *A. brassicicola* as compared to the control. Spore germinations of *A.*

brassicicola were 9.0 and 2.5% when treated with 50 and 100 mg L^{-1} of pentyl pyrone, respectively, whereas the control was 93.5%. For treatments using 50 and 100 mg L^{-1} of benomyl fungicide, spore germinations of *A. brassicicola* were 4.5 and 3.5%, respectively (Table 1).

Viability of *Alternaria brassicicola*

There were no detectable mycelial viability (no growth of mycelia) from dialysis membranes contained with *A. brassicicola* mycelia which were soaked in 50 and 100 mg L^{-1} pentyl pyrone. Similar result was obtained from treatments using 50 and 100 mg L^{-1} benomyl fungicide while in a control treatment, with 2 % methanol, the mycelia of *A. brassicicola* grew normally, covered the whole agar surface in the Petri-dish within 8 days.

For spore viability of *A. brassicicola* test, the result showed that the viability (germination) of spores treated with pentyl pyrone and benomyl fungicide were significantly lower than in the control (2% methanol). Particularly, a treatment using 100 mg L^{-1} of pentyl pyrone the lowest spore germination (2.5%) was obtained, while a control treatment had 95.5% of spore germination (Table 2).

Control Disease in Laboratory

Disease severity on detached Chinese-kale leaf sprayed with 50 or 100 mg L^{-1} pentyl pyrone and 50 or 100 mg L^{-1} benomyl fungicide significantly decreased comparing with a control treatment (2% methanol). Pentyl pyrone and benomyl fungicide at concentration 100 mg L^{-1} gave the lowest disease severity with 6.5 and 5.5%, respectively, while the control treatment (2% methanol) provided 88.0% (Table 3).

Disease Control in Glasshouse

Pentyl pyrone from ultraviolet-induced mutant strain of *T. harzianum* T-35-co4 at 50 and 100 mg L^{-1}, significantly reduced severity of leaf spot on Chinese-kale leaf as comparing with a control treatment (2% methanol). In particularly, a treatment using 100 mg L^{-1} pentyl pyrone provided the lowest disease severity of 19.0%, whereas a control treatment (2% methanol) was 92.5% (Table 3). This concentration of pentyl pyrone provided non-significant disease control efficacy when compared to the use of 100 mg L^{-1} benomyl fungicide.

Table 1 Efficacy of pentyl pyrone from ultraviolet-induced mutant strain of *Trichoderma harzianum* T-35-co4 on the inhibition of mycelial growth and spore germination of *Alternaria brassicicola* at room temperature.

Treatment	Efficacy of pentyl pyrone[1/] (%)	
	Mycelial growth inhibition[2/]	Number of germinated spore[3/]
2% methanol (control)	0b	93.5c
Pentyl pyrone (50 mg L^{-1})	100a	9.0b
Pentyl pyrone (100 mg L^{-1})	100a	2.5a
Benomyl (50 mg L^{-1})	100a	4.5b
Benomyl (100 mg L^{-1})	100a	3.5a

[1/] Means in a column followed by the same letter are not significantly different according to DMRT (P=0.05).
[2/] Inhibition of mycelial growth of *Alternaria brassicicola* by pentyl pyrone on PDA in dual culture test.
[3/] Means of spore germination calculated from four replications (100 spores per replication).

Table 2 Efficacy of pentyl pyrone extract on mycelial and spore viability of *Alternaria brassicicola* at room temperature.

Treatment	Efficacy of pentyl pyrone on viability of *A. brassicicola*[1/] (%)	
	Mycelial viability	Spore viability
2% methanol (control)	100b	95.5c
Pentyl pyrone (50 mg L^{-1})	0a	9.5b
Pentyl pyrone (100 mg L^{-1})	0a	2.5a
Benomyl (50 mg L^{-1})	0a	7.0b
Benomyl (100 mg L^{-1})	0a	4.5a

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

Table 3 Disease severity on Chinese-kale leaf after applied with pentyl pyrone (50 and 100 mg L^{-1}) from ultraviolet-induced mutant strain of *Trichoderma harzianum* T-35-co4 in laboratory and under glasshouse conditions.

Treatment	Disease severity[1/] (%)[2/]	
	In laboratory	Under glasshouse condition
2% methanol (control)	88.0c	92.5c
Pentyl pyrone (50 mg L^{-1})	18.0b	28.5b
Pentyl pyrone (100 mg L^{-1})	6.5a	19.0a
Benomyl (50 mg L^{-1})	21.5b	31.0b
Benomyl (100 mg L^{-1})	5.5a	22.5a

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

Discussion

In inhibition of mycelial growth and spore germination testing, our results showed that pentyl pyrone completely inhibited (100%) mycelial growth and decreased number of spore germination of *A. brassicicola*. These results indicated that this extract was a high potential antifungal metabolite against *A. brassicicola*. This finding complies with the report which asserted that pentyl pyrone was very effective for inhibiting mycelial growth of fungal plant pathogens (Simon et al., 1988). In addition, many researchers reported that pentyl pyrone from *Trichoderma* spp. effectively inhibited mycelial growth, spore germination and sporangium production of other plant pathogens such as *Fusarium moniliforme*, *Pythium aphanidermatum* and *Ceratocystis paradoxa* (El-Hasan et al., 2007; Intana, 2003; Eziashi et al., 2006).

Concerning the effect of pentyl pyrone on viability of *A. brassicicola* test**,** the results showed that all mycelia and most of spores of pathogen were non-viable after being soaked in pentyl pyrone solutions. These results indicated that all mycelia and most of spores of *A. brassicicola* were destroyed/ killed by pentyl pyrone. Rey et al. (2001) found that mycelia and spores of fungal pathogens were very sensitive to a high concentration of antifungal metabolites.

For using in control disease in laboratory and glasshouse conditions, pentyl pyrone provided high efficacy in controlling leaf spot disease of Chinese-kale in both conditions. These results indicated that pentyl pyrone was an effective antifungal metabolite used for controlling leaf spot disease of Chinese-kale. Our reports are supported by others which were successful in using pentyl pyrone extracted from *T. harzianum* to control various plant diseases such as cucumber damping-off caused by *P. aphanidermatum* (Intana, 2003), Rhizoctonia root rot of wheat caused by *Rhizoctonia* sp. (Worasatit et al., 1994), black fruit rot of rambutan caused by *Lasiodiplodia theobromae* (Intana et al., 2005), postharvest rots in kiwifruit caused by *Botrytis cinerea* (Poole et al., 1998), disease on lentil seedlings caused by *Athelia rolfsii* (Dodd et al., 2000), damping-off of vegetables caused by *Rhizoctonia* sp. (Butt et al., 2001), wilt of cotton and watermelon caused by *P. aphanidermatum* (Ordentlich et al., 1992) and black seed rot in oil palm caused by *Ceratocystis paradoxa* (Eziashi et al., 2006).

T. harzianum was demonstrated to have great potential of applications in biological control programs of plant fungal diseases. The results of the efficacy to control the disease of pentyl pyrone antifungal metabolite extracted from ultraviolet-induced mutant strain of *T. harzianum* T-35-co4 were not significantly different statistically from those using benomyl. However penyl pyrone has the advantage to be a natural and biological product as well as environmental safe. Moreover, *T. harzianum* could produce many other kinds of high effective antifungal to plant pathogens such as glioviridin, gliotoxin, trichorzianines, oxazole, ferulic acid, 3,4-dihydroxycarotane and trichodermin (Ghisalberti and Sivasithamparam, 1991; Di Pietro et al., 1993; Lee et al., 1995; Intana, 2003; Dickinson et al., 1995; Watanabe et al., 1990; Yamamoto et al., 1969). Further evaluations of the efficacy of the other metabolites used alone or together with pentyl pyrone as well as improvement of antifungal metabolite production and field applications are warranted and must be the next research steps.

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