ORIGINAL ARTICLE

Control of Chinese-kale damping-off caused by *Pythium aphanidermatum* by antifungal metabolites of *Trichoderma virens*

Warin Intana[1] and Chiradej Chamswarng[2]

Abstract

Intana, W. and Chamswarng, C.

Control of Chinese-kale damping-off caused by *Pythium aphanidermatum* by antifungal metabolites of *Trichoderma virens*

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Seven strains of *Trichoderma virens* were isolated from Chinese-kale planting soil in Nakhon Si Thammarat province. Efficacy of those isolates to inhibit mycelial growth and overgrow on mycelia of *Pythium aphanidermatum*, a causal agent of damping-off on Chinese-kale, were determined by a dual culture test. All strains significantly inhibited growth and overgrew on mycelia of *P. aphanidermatum* on potato dextrose agar (PDA) as compared with the control. Strains T-NST-01, T-NST-05 and T-NST-07 gave high values of inhibition by 85.5, 82.5 and 78.5%, respectively. For efficacy to overgrow on mycelia of pathogen test, strains T-NST-05, T-NST-07 and T-NST-01 provided 48.3, 47.0 and 46.1% of mycelial overgrowth, respectively. Antifungal metabolites were extracted from three promising strains and tested against mycelial growth and sporangium production of *P. aphanidermatum*. The results showed that 1,000 mg/L of all metabolites completely inhibited mycelial growth and sporangium production. Under laboratory condition, all metabolites (1,000 mg/L) significantly increased the number of Chinese-kale seedling germination, especially

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[1]Ph.D. (Plant Pathology), Asst. Prof., School of Agricultural Technology, Walailak University, Thasala, Nakhon Si Thammarat, 80161 Thailand. [2]Ph.D. (Plant Pathology), Assoc. Prof., Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

Corresponding e-mail: iwarin@wu.ac.th

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the metabolites from T-NST-01 and T-NST-07 provided germination of 92.5 and 87.5%, respectively. Under glasshouse conditions, Chinese-kale seedlings treated with 1,000 mg/L of metabolites from strains T-NST-01 and T-NST-07 survived by 90.5 and 87.5%, respectively, while the control 1 (sterile water) and control 2 (2% methanol) had 19.0 and 18.5% of survived seedlings, respectively. In *P. aphanidermatum* viability test, mycelia of *P. aphanidermatum* treated with antifungal metabolites from three strains of *T. virens* showed no visible growth, while the control with 2% methanol or sterile water, mycelia of *P. aphanidermatum* rapidly grew and covered whole surface of PDA in of the Petri dish within 4 days.

Key words : antifungal metabolite, damping-off, Chinese-kale, *Trichoderma* spp., biological control

บทคัดย่อ

วาริน อินทนา[1] และ จิระเดช แจ่มสว่าง[2]

การควบคุมโรคเน่าระดับดินของคะน้าที่เกิดจากเชื้อ *Pythium aphanidermatum* โดยสารต่อต้านเชื้อราจาก *Trichoderma virens*

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นำเชื้อรา *Trichoderma virens* จำนวน 7 สายพันธุ์ ที่แยกได้จากดินปลูกคะน้าจังหวัดนครศรีธรรมราช มาทดสอบประสิทธิภาพการยับยั้งการเจริญและคลุมทับเส้นใยเชื้อรา *Pythium apahanidermatum* สาเหตุโรคเน่าระดับดินของคะน้าในระดับห้องปฏิบัติการ พบว่า ทุกสายพันธุ์มีประสิทธิภาพยับยั้งการเจริญและคลุมทับเส้นใยของเชื้อรา *P. aphanidermatum* บนอาหาร potato dextrose agar (PDA) แตกต่างจากกรรมวิธีควบคุมอย่างมีนัยสำคัญ โดยสายพันธุ์ T-NST-01, T-NST-05 และ T-NST-07 มีประสิทธิภาพสูงที่ 85.5, 82.5 และ 78.5% ตามลำดับ สำหรับประสิทธิภาพการเจริญคลุมทับเส้นใยเชื้อราโรคพืช พบว่า สายพันธุ์ T-NST-05, T-NST-07 และ T-NST-01 มีประสิทธิภาพการคลุมทับที่ 48.3, 47.0 และ 46.1% ตามลำดับ เมื่อสกัดสารต่อต้านเชื้อราจากสายพันธุ์ที่ผ่านการคัดเลือก 3 สายพันธุ์ และทดสอบการยับยั้งการเจริญของเส้นใยและการสร้างสปอร์แรงเจียม (sporangium) ของเชื้อรา *P. aphanidermatum* พบว่า สารสกัดทุกกรรมวิธีที่มีความเข้มข้น 1,000 มก./ลิตร สามารถยับยั้งการเจริญของเส้นใยและการสร้างสปอร์แรงเจียมได้สมบูรณ์ ทดสอบประสิทธิภาพของสารสกัดในการควบคุมโรคพืชในระดับห้องปฏิบัติการ พบว่า สารสกัด (1,000 มก./ลิตร) ทุกกรรมวิธีเพิ่มจำนวนการงอกของเมล็ดคะน้าได้อย่างมีนัยสำคัญ โดยเฉพาะสารสกัดจากสายพันธุ์ T-NST-01 และ T-NST-07 ที่มีจำนวนการงอกที่ 92.5 และ 87.5% ตามลำดับ ส่วนการทดสอบในระดับโรงเรือน พบว่า กรรมวิธีที่แช่เมล็ดคะน้าในสารสกัดความเข้มข้น 1,000 มก./ลิตร จากสายพันธุ์ T-NST-01 และ T-NST-07 มีการรอดตายของต้นกล้าที่ 90.5 และ 87.5% ตามลำดับ ในขณะที่กรรมวิธีควบคุม 1 (น้ำนึ่งฆ่าเชื้อ) และกรรมวิธีควบคุม 2 (เมทานอลความเข้มข้น 2%) มีจำนวนการรอดตายของต้นกล้าที่ 19.0 และ 18.5% ตามลำดับ ในการตรวจสอบการมีชีวิตของเชื้อรา *P. aphanidermatum* พบว่า เส้นใยเชื้อรา *P. aphanidermatum* ที่แช่ในสารต่อต้านเชื้อราจากเชื้อรา *T. virens* ทั้ง 3 สายพันธุ์ ไม่สามารถเจริญได้ ในขณะที่กรรมวิธีควบคุมที่แช่ในเมทานอลความเข้มข้น 2% หรือน้ำนึ่งฆ่าเชื้อ เส้นใยเชื้อรา *P. aphanidermatum* เจริญรวดเร็วและเจริญคลุมทับอาหาร PDA ในจานเลี้ยงเชื้อได้หมดภายใน 4 วัน

[1]สำนักวิชาเทคโนโลยีการเกษตร มหาวิทยาลัยวลัยลักษณ์ อำเภอท่าศาลา จังหวัดนครศรีธรรมราช 80161 [2]ภาควิชาโรคพืช มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จังหวัดนครปฐม 73140

Pythium aphanidermatum is a major pathogen causing both pre- and post emergence damping-off disease of Chinese-kale in Thailand. This disease can be controlled by some fungicides and biological control agents such as *Trichoderma* spp. and *Bacillus* spp. (Wolfhechel and Jensen,

1992; Hong *et al.*, 2004). However, side effects from fungicide usages were found concerning fungicide-resistance of this pathogen and the potential oncogenic risks to the consumer. Therefore, the application of biological control agents (BCAs) and their products (antifungal metabolites) to control this disease is an interesting alternative topic. *Trichoderma* spp. are soil-borne fungi and most commonly used as biocontrol agents throughout the world. They have been successfully used for disease control in a variety of important crops e.g., carrot, cotton, grape, lettuce, onion, peas, pepper, tomato, wheat, cucumber and radish (Tronsmo and Hjeljord, 1998) durian, tangerine, soybean, asparagus, barley, cucumber etc. (Chamswarng and Tanangsnakool, 1996; Intana, 2003). The mechanisms underlying their antagonism for plant disease control involve mycoparasitism, antibiosis, competition for nutrients or space, enhancement of root and plant development, induction of plant resistance, solubilization and sequestration of inorganic nutrients and/or inactivation of the pathogen's enzymes (Harman, 2000). However, the biological control abilities of *Trichoderma* spp. have been more or less related to potential antifungal activities and lytic enzyme extracted from the fungus (Ghisalberti and Sivasithamparam 1991; Intana, 2003). The aims of this investigation were (a) to determine *in vitro* inhibition and overgrow on mycelia of *P. aphanidermatum* by *T. virens* (b) to determine the effect of antifungal metabolites from *T. virens* in the Petri dishes and on disease severity under the glasshouse conditions and (c) to determine the mechanisms of antifungal metabolites of *T. virens* to control *P. aphanidermatum*.

Materials and Methods

Microorganisms

Seven strains of *T. virens* used in this experiment were isolated from Chinese-kale field soil in Nakhon Si Thammarat by surface soil dilution technique using Martin's agar medium (Intana, 2003). They were purified by single spore isolation technique before kept in sterile soil. Spore suspension of *T. virens* was prepared by flooding 7-day-old cultures grown on potato dextrose agar (PDA) with sterile distilled water. Spore mass was scraped with a sterile spatula. The concentration of spore was adjusted to $1.0x10^5$ spores/ml, then 0.5 ml of spore suspension was added into a bottle containing 15 g of sterilized soil-sand (soil 85%, sand 15%), and moistened with sterile distilled water to 65% water holding capacity (WHC). The bottles were then incubated at room temperature (27 ± 2^oC) for seven days, during which each bottle was thoroughly shaken.

Isolation of a pathogen from Chinese-kale with damping-off symptom was performed by tissue transplanting technique modified from Agrios (2005). The plant materials were cut to small pieces (0.5x0.5 cm) and dipped in 2% of sodium hypochlorite for 30 sec before washing with sterilized water for 3 times. Then, the plant samples were dried by placing on sterilized tissue before transferring to a Petri dish containing PDA. The Petri dish was sealed with plastic wrap and incubated at room temperature for 2 days. The growing colony was subcultured to PDA slant, then kept at 10^oC as a stock culture for further study.

Dual culture test

T. virens strains and *P. aphanidermatum* were cultured on PDA for 2 days. Agar discs 7 mm in diameter were cut from the growing margin of fresh cultures by using sterilized cork borer. Agar discs of *T. virens* and *P. aphanidermatum* were placed 6 cm apart on the surface of PDA in a Petri dish. The dishes were incubated at room temperature and the mycelial growth of these fungi was monitored. Percentage of mycelial growth inhibition of the *T. virens* against *P. aphanidermatum* was measured and calculated to compare with a treatment of *T. harzianum* strain CB-Pin-01 (commercial strain in Thailand). The calculation formula was $((R_C-R_T)/R_C) \times 100$, when R_C was a mean of mycelial radius of *P. aphanidermatum* in Petri dish without *T. virens*; R_T was a mean of mycelial radius of *P. aphanidermatum* in Petri dish with *T. virens* (Intana, 2003). The design used

for this experiment was a complete randomized design (CRD) with four replications.

The overgrow levels were calculated by using the formula; $((C_1-C_2)/T_d) \times 100$, when C_1 was the mean of colony radius of the *T. virens* strain on the day of recording, C_2 was the mean of colony radius of the *T. virens* strain on the day before recording and T_d was the time (d) between C_1 and C_2 (Intana, 2003). The design used for this experiment was a CRD with 4 replicates.

Extraction of antifungal metabolites

Mycelial plugs (7 mm in diameter) from the margins of colonies of three strains of *T. virens* (T-NST-01, T-NST-05 and T-NST-07) and *T. harzianum* CB-Pin-01 grown on PDA were separately inoculated into 3 L flask containing 1 L of 1/5 strength potato dextrose broth (PDB) and incubated at 27±2°C. Twenty-eight days after incubation, spores and mycelia of *Trichoderma* spp. strains were removed from broth culture by filtration. The filtrate was extracted with ethyl acetate (EtoAc) and EtoAc was evaporated at 40°C in a rotary evaporator. Concentrated antifungal metabolites as crude extracts were recorded before mixed with 25 ml of 2% methanol and kept as a stock.

Disease control in laboratory

Pathogenicity of *P. aphanidermatum* on Chinese-kale seeds was conducted in the laboratory as described below. Sterile paper discs 1.3 cm in diameter were placed onto PDA Petri dish inoculated with *P. aphanidermatum*. These dishes were sealed with plastic wrap before incubation at 27±2°C until discs were well colonized with *P. aphanidermatum*. The discs were removed and placed on sterile moistened filter paper (Whatman No. 1) in Petri dishes, 10 discs per Petri dish. Disinfested Chinese-kale seeds (dipped in 2% of sodium hypochlorite for 30 sec and rinsed with sterile water 5 min for 3 times) were placed on filter paper discs, one seed per disc. After placing seeds for 7 days, percent of seedling germination was recorded and compared with the control which comprised of surface sterilized Chinese-kale seed placed on sterile filter paper discs (Intana, 2003).

For disease control test, filter paper discs infested with *P. aphanidermatum* were covered with sterile filter paper disc imbibed with 1,000 mg/L of crude extracts of *Trichoderma* spp. face to face before placing the discs on sterile moistened filter paper (Whatman No. 1) in Petri dishes, 10 pairs per dish. Disinfested Chinese-kale seed soaked over night in water were placed one each on every paired disc. These Petri dishes were incubated in plastic box at 27±2°C, the percent of disease severity was recorded at 14 days of incubation. For the control 1 and control 2, sterile filter paper discs colonized with *P. aphanidermatum* were covered with sterile discs dipped in sterile water and 2% methanol for 10 min., respectively. Paper disc imbibed with 1,000 mg/L of metalaxyl, a systemic fungicide was also used for disease control comparison (Intana, 2003). The design used for this experiment was a CRD with five replications, 10 seeds per a replicate.

Disease control under glasshouse conditions

In glasshouse test, the pots contained sterilized potting mix (Mai Long Mai Loo potting mix autoclaved at 121°C for 1 h in two consecutive days). The paired discs precolonized with *P. aphanidermatum* and treated with 1,000 mg/L crude extracts of *Trichoderma* spp. were then transferred into potting mix (one pair per pot) at 0.5 cm depth from surface. Then disinfested Chinese-kale seed immersed in sterile water over night was dipped in crude extracts of *Trichoderma* spp. for 10 min before placing over the paired discs approximately 0.3 cm from surface (1 seed per a pair of discs). Fourteen days after planting, the number of germinated Chinese-kale seeds was recorded. For the control 1, Chinese-kale seeds were dipped in sterile water for 10 min before placing above the paired colonized discs. Chinese-kale seeds dipped in 2% methanol before placing above paired colonized discs were used for the control 2. The design used for this experiment was a CRD with five replications, 10 seeds per a replicate.

Inhibition of mycelial growth and sporangium production

Crude extracts of *Trichoderma* spp. were evaluated for the efficacy to inhibit mycelial growth and sporangium production of *P. aphanidermatum*. For mycelial growth inhibition test, *P. aphanidermatum* was sub-cultured on PDA which covered with dialysis membrane for 2 days. Then the membrane contained with mycelia was cut (0.5x 0.5 cm), placed into sterile Petri dish and flooded with 10 ml of 1,000 mg/L crude extracts of *Trichoderma* spp.. At 24 h after incubation, the membrane was rinsed with sterile water for 3 times, and then they were put on the surface of PDA medium. Two days after incubation, a mycelial growth of *P. aphanidermatum* was determined to compare with a control 1 and control 2, which the Pythium inoculated membrane were added with 10 ml of sterile water and 2% methanol, respectively.

For the inhibition of sporangium production, small pieces of membrane contained with mycelia of *P. aphanidermatum* were put into a Petri dish before 10 ml of sterile water was added. The sterile water was changed daily, then at day 2 after incubation it was replaced with crude extracts of *Trichoderma* spp.. These crude extracts were replaced daily until the experiment was terminated. Sporangium of *P. aphanidermatum* was observed under compound microscope compared with control 1 (sterile water) and control 2 (2% methanol). The design used for this experiment was a CRD with five replications, 10 membranes per a replicate.

Viability of *Pythium aphanidermatum*

The mycelial growth inhibition and sporangium production tests were further studied for viability, the mycelia of *P. aphanidermatum* from all treatments were observed for viability. The membrane with mycelia of *P. aphanidermatum* was washed with sterile water for three times before placing onto sterile tissue. Then the membrane was put into the Petri dish containing PDA added with 1 mg/L streptomycin before the Petri dish was incubated at room temperature. Four days after incubation, the mycelial growth of *P. aphanidermatum* was observed.

Statistical analysis

All data were analyzed by ANOVA using the GLM procedure of SAS (SAS, 1995) system for Windows and were considered significant when $P<0.05$.

Results

Dual culture test

At 4 days after incubation, all *T. virens* strains showed differences in their ability to inhibit mycelial growth of *P. aphanidermatum* on PDA at room temperature. Five strains inhibited mycelial growth of *P. aphanidermatum* significantly higher than *T. harzianum* CB-Pin-01. Especially, strains T-NST-01, T-NST-05 and T-NST-07 gave 85.5, 82.5 and 78.5% inhibition, respectively, while *T. harzianum* CB-Pin-01 gave 67.5% of growth inhibition (Table 1).

The efficacy to overgrow on mycelia of *P. aphanidermatum*, all strains of *T. virens* showed different levels of mycelial overgrowth. Three out of seven strains of *T. virens* showed significantly higher in overgrowth efficacy than *T. harzianum* CB-Pin-01, a commercial strain in Thailand. Especially, strains T-NST-05, T-NST-07 and T-NST-01 gave 48.3, 47.0 and 46.1% overgrowth, respectively, while 35.4% overgrowth was observed from *T. harzianum* CB-Pin-01.

Extraction of antifungal metabolites

The crude extracts were extracted from *T. virens* strains T-NST-01, T-NST-05, T-NST-07 and *T. harzianum* CB-Pin-01 which cultured in 1 L of 1/5 strength PDB gave 2.24, 2.41, 2.34 and 2.56 g of dried crude extract, respectively.

Disease control in laboratory

All treatments using 1,000 mg/L crude extracts of *T. virens* stains T-NST-01, T-NST-07, T-NST-05 and *T. harzianum* CB-Pin-01 significantly increased seed germination of Chinese-kale by 92.5, 87.5, 85.0 and 60.5%, respectively when compared with a control (water). Per cent of seed

Table 1. Percent of mycelial growth inhibition and mycelial overgrowth of *Pythium aphanidermatum* on PDA by *Trichoderma virens* (seven strains) and *Trichoderma harzianum* CB-Pin-01 at 4 days after inoculation

Strains	Efficacy to inhibit and overgrow (%)	
	Mycelial growth inhibition (%) [1/]	Overgrowth rate[2/]
T. virens T-NST-01	85.5 a[3/]	46.1 a[3/]
T. virens T-NST-02	75.5 b	39.5 b
T. virens T-NST-03	70.0 c	37.1 b
T. virens T-NST-04	70.0 c	30.5 c
T. virens T-NST-05	82.5 ab	48.3 a
T. virens T-NST-06	75.5 b	43.9 ab
T. virens T-NST-07	78.5 ab	47.0 a
T. harzianum CB-Pin-01	67.5 c	35.4 bc

[1/] **Inhibition of mycelial growth of *Pythium aphanidermatum* on PDA as compared to the control.**

[2/] **Overgrowth of *Trichoderma* spp. mycelia on mycelia of *Pythium aphanidermatum* on PDA as compared to the control.**

[3/] **Values followed by the same alphabet in each column are not significantly different from each other according to Duncan's Multiple Range Test (P=0.05).**

germination in the control 1 (sterile water) and control 2 (2% methanol) were 9.5 and 11.5, respectively, whereas seeds treated with 1,000 mg/L metalaxyl gave 95.5% seed germination (Table 2).

Disease control under glasshouse conditions

In the pot test, when Chinese-kale seeds were placed over filter paper discs precolonized with *P. aphanidermatum* and treated with 1,000 mg/L of Trichoderma crude extracts, seed germination percentages (14 days after planting) in all treatments were significantly higher seed germination than in the control 1 (sterile water) and control 2 (2% methanol). Percent seedling germination of treatments with the seeds treated with crude extracts of *T. virens* stains T-NST-01, T-NST-07, T-NST-05 and *T. harzianum* CB-Pin-01 was 90.5, 87.5, 67.5 and 62.5%, respectively, while in the control 1 (sterile water) and control 2 (2% methanol) was 19.0 and 18.5%, respectively. In a treatment using metalaxyl, percentage of seed germination was 92.5% (Table 2).

Inhibition of mycelial growth and sporangium production

Crude extracts (1,000 mg/L) of *T. virens* strains T-NST-01, T-NST-05, T-NST-07 and *T. harzianum* CB-Pin-01 provided complete inhibition of mycelial growth of *P. aphanidermatum* (no visible growth of mycelia) as compared with the control 1 (sterile water) and control 2 (2% methanol).

For the inhibition of sporangium production test, the result revealed that 1,000 mg/L of crude extracts of *T. virens* strains T-NST-01, T-NST-05, T-NST-07 and *T. harzianum* CB-Pin-01 totally inhibited sporangium production. While sporangia were observed under compound microscope in the treatments with sterile water and 2% methanol, especially a treatment with sterile water provided the highest number of sporangia with high density of mycelial mats.

Viability of *Pythium aphanidermatum*

The result indicated that there was no visible growth of mycelia of *P. aphanidermatum* in all

Table 2. Seed germination percentages of Chinese-kale at 14 days after placing seeds over paired discs of filter paper precolonized with *Pythium aphanidermatum* and treated with 1,000 mg/L crude extracts from three strains of *Trichoderma virens* and *T. harzianum* CB-Pin-01 in laboratory and glasshouse test

Treatments	Seed germination (%)	
	laboratory	glasshouse
Water (control 1)	9.5 d [1/]	19.0 c [1/]
2% methanol (control 2)	11.5 d	18.5 c
T. virens T-NST-01 (1,000 mg/L)	92.5 a	90.5 a
T. virens T-NST-05 (1,000 mg/L)	85.0 b	67.5 b
T. virens T-NST-07 (1,000 mg/L)	87.5 ab	87.5 a
T. harzianum CB-Pin-01 (1,000 mg/L)	60.5 c	62.5 b
1,000 mg/L benomyl	95.5 a	92.5 a

[1/] **Values followed by the same alphabet in each column are not significantly different from each other according to Duncanís Multiple Range Test (P=0.05).**

treatments with 1,000 mg/L crude extracts. While a control treatment, with 2% methanol or sterile water, the mycelia of *P. aphanidermatum* grew rapidly, covered the whole agar surface in the Petri dish within 4 days.

Discussion

Tronsmo and Hjeljord (1998) reported that isolates of *T. harzianum, T. virens* and *T. hamatum* were generally used against diseases in a wide variety of economically important crops. Many researchers have also reported that *Trichoderma virens* (*Gliocladium virens*) was used effectively against diseases in many different crops, e.g., cotton, grape, sweet, cucumber, lettuce, onion, pea, plum, apple and carrot caused by pathogens such as *Pythium* spp., *Phytophthora* spp., *Rhizoctonia* spp., *Sclerotinia* spp., *Botrytis* spp. and *Fusarium* spp. (Nelson, 1991, Di Pietro *et al.*, 1993). These reports supported our results which indicated that all strains of *T. virens* inhibited mycelial growth of *P. aphanidermatum* in dual culture test at room temperature.

In our study, all strains of *T. virens* were highly effective to overgrow mycelia of *P. aphanidermatum*. This result indicated the potentiality of *T. virens* to parasitize mycelia of *P. aphanidermatum* through the production of some of cell wall lytic enzymes and antifungal metabolites such as β-1,3 and β-1,4-glucanase (Intana, 2003), chitinase and laminarinase (Bruce *et al.*, 1995), cellulase (Abrahao-Neto *et al.*, 1995), xylanase I (Mach *et al.*, 1996) and β-glucosidase (Mach *et al.*, 1995).

Both in laboratory and glasshouse conditions, 1,000 mg/L crude extracts produced by all *Trichoderma* strains gave high efficacy to control damping-off disease by increasing percent germination of seeds. The result was supported by the report previously described by of Intana (2003) which showed that antifungal metabolites of mutant and wild type strains of *T. harzianum* could control damping-off of cucumber caused by *P. irregulare*. In 1992, Lumsden *et al.* reported that gliotoxin of *T. virens* effectively inhibited mycelial growth of fungal pathogens while pyrone produced from *T. koningii* significantly reduced root rot when applied into soil and incubated for 14 days with *Rhizoctonia solani* (Worasatit *et al.*, 1994).

Several reports indicated that *T. virens* produced many antifungal metabolites to inhibit mycelial growth and spore germination of plant pathogenic fungi (Lumsden *et al.*, 1992). Hence, it can be expected from our studies that a mechanism of *T. virens* (three strains) and *T. harzianum*

CB-Pin-01 to control damping-off caused by *P. aphanidermatum* was attributed to the production of antifungal secondary metabolites which effectively inhibited mycelial growth and sporangium production. Ghisalberti and Sivasithamparam (1991) also reported the production of volatile fungal metabolite 6-pentyl-α-pyrone (6PAP) from a number of *Trichoderma* species and this was thought to have significant role in the biological control activity against many plant pathogens.

Application of antifungal secondary metabolites derived from antagonistic microorganisms, especially fungal antagonists for control of plant disease has received more attention. Isolation purification and structural characterization of secondary metabolites from our crude extracts will be further conducted. Biological control of *P. aphanidermatum* by using the isolated metabolites will be studied.

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