

ORIGINAL ARTICLES

Use of Antifungal Metabolite from *Trichoderma virens* for Controlling Chinese Kale Leaf Spots Caused by *Alternaria brassicicola*

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ABSTRACT

A Total of 13 isolates of *Trichoderma virens* were isolated from soil samples collected from 9 different sites. All isolates inhibited the mycelial growth of *Alternaria brassicicola* that causes Chinese kale leaf spot. There were two strains, T-ST-01 and T-NST-01, that gave high values of inhibition, 72.5 and 67.0%, respectively. Strains T-ST-01 and T-NST-01 were used to evaluate antifungal properties using dialysis membrane technique testing with *A. brassicicola*. The results showed that mycelial growth of *A. brassicicola* was completely inhibited. Antifungal metabolite was extracted and tested against germination of *A. brassicicola* spores. The results showed that spores of *A. brassicicola* germinated 9.4% compared with 98.5% for the control. Use of antifungal metabolite was effective both in laboratory and glasshouse conditions. Severity of the disease was significantly ($P<0.05$) reduced compared with the control treatment (2% methanol). Disease severity of Chinese kale treated with antifungal from extracted T-ST-01 were 22.5 and 32.0% when measured in laboratory and glasshouse conditions, respectively, while the severity in the control treatment was found to be 89.5 and 84.5%, respectively.

Key words: Antifungal metabolite - *Alternaria brassicicola* - Chinese kale disease - Biological control - *Trichoderma virens*

INTRODUCTION

Alternaria brassicicola (Schw.) Wilts. is a necrotrophic fungal pathogen that causes black leaf spots in several economically important *Brassica* species, including Chinese kale. It is responsible for the 20-50% yield loss found in infected plants. The disease manifests itself visibly on the leaves where it appears as black, sooty, velvety lesions with yellow halos around them (chlorotic zones) (1). During attack, this pathogen can produce brassicicolin A which has been implicated in *A. brassicicola*'s pathogenesis (2).

Farmers in Thailand have tried to control the *A. brassicicola* disease by using benomyl chemicals. However, fungicided crops are unpreferable among consumers. Fungicide potentially risks to cause oncogenic to consumer. Fungicide also causes

disease resistance and destroys the ecological balance of microorganisms. Therefore biological control agents (BCAs) and their products such as antifungal metabolite are alternative methods to disease control. *Trichoderma virens* has been reported to produce large quantities of antifungal, antibacterial, antiviral and antibiotic metabolites. This paper attempts to (a) isolate and screen promising strains of *T. virens* showing mycelial growth inhibition of *A. brassicicola* (b) screen for promising strains of *T. virens* producing antifungal metabolite (c) investigate efficacy of antifungal metabolite in inhibiting spore germination of *A. brassicicola* and (d) extract and use the selected antifungal metabolite to control black leaf spot in Chinese kale both in laboratory and glasshouse conditions.

MATERIALS AND METHODS

Isolation of *Trichoderma virens* and Pathogen

For *T. virens*, the upper 10 cm of the field planting soil was collected after removing the surface plant material. The soil samples were air dried for 2 days and sieved through a 2 mm mesh screen to remove coarse debris. Ten grams of each sieved sample was added into a 250 ml flask containing 90 ml of sterilized water and mixed by a shaker at 1,000 rpm for 30 min. Then, the soil suspension was diluted 10^{-2} and 10^{-3} fold or at the appropriate concentration. 0.1 ml of the diluted solution was dropped onto the surface of Martin's agar (3) in a Petri dish. The soil suspension was spread on the surface of the medium with a sterile glass rod. The dishes were sealed with plastic wrap and incubated at room temperature ($27\pm 2^{\circ}\text{C}$) until the result was measured.

For pathogen, the isolation was performed by a tissue transplanting technique modified from Agrios (4). Chinese kale with leaf spot symptoms were cut into small pieces (0.5×0.5 cm), dipped in 2% sodium hypochlorite solution for 30 s and rinsed with sterilized water 3 times. The plant samples were dried by placing on sterilized tissue paper before transferring to a Petri dish containing potato dextrose agar (PDA). The Petri dish was enfolded with plastic wrap and incubated at room temperature for 2 days. The growing colony was subcultured in PDA slant before being kept at 10°C and used as a stock culture.

Dual Culture Testing

All of strains *T. virens* and *A. brassicicola* were cultured on PDA for 2 days. Agar discs 7 mm in diameter were cut from the growing margin of fresh cultures using a cork borer. Agar discs of *T. virens* and *A. brassicicola* were placed 6 cm apart on a surface of PDA in a Petri dish. The dishes were incubated at room temperature and the mycelial growth of these fungi was monitored. The percentage of mycelial growth inhibition of the *T. virens* against *A. brassicicola* was measured by comparison with the control treatment. Calculation of growth inhibition was performed using the formula $((G_1 - G_2)/G_1) \times 100$, where G_1 is a mean of mycelial radius of *A. brassicicola* in a Petri dish without *T. virens* and G_2 is a mean of mycelial radius of *A. brassicicola* in a Petri dish with *T. virens*. There were four replications (Petri dishes) for each treatment.

Dialysis Membrane Technique Testing

The dialysis membrane overlay technique (5,6,7) was used in this experiment. Dialysis membrane discs were placed over the surface of PDA. Seven mm agar plugs of PDA from the growing margin of *T. virens* and *T. harzianum* strain T-CB-Pin-01 (commercial strain in Thailand) were inoculated in Petri dishes. The dish was tightly enclosed in plastic wrap and incubated at room temperature before removing the dialysis membrane. The activity of metabolite on agar was tested by inoculating 7 mm plugs of *A. brassicicola* taken from the actively growing margin of a colony in the position initially occupied by the inoculum plug of *T. virens*. The diameters of each colony of *A. brassicicola* were measured after incubation for 24 h. The percentage of inhibition of growth was calculated using the formula: % inhibition = $((G_1 - G_2)/G_1) \times 100$, where G_1 is a mean diameter of all colonies of *A. brassicicola* growing on the Petri dish prior to growth of *A. brassicicola* and G_2 is a mean of all colonies of *A. brassicicola* growing on the Petri dish prior to growth of the *T. virens* strain. This formula was modified from Royse and Ries (8).

Extraction of Antifungal Metabolite

Seven mm mycelial plugs obtained from margins of the growing colonies of promising *T. virens* strains and T- CB-Pin-01 grown on PDA were inoculated into a 3 L flask containing 1 L of 1/5 strength potato dextrose broth (PDB). Then, the flask was incubated at room temperature. Twenty-eight days after inoculation, spores and mycelia of *T. virens* strains were removed from broth culture by filtration. The filtrates were extracted with ethyl acetate (EtOAc) before evaporation at 40°C on a rotary evaporator. Concentrated metabolites were recorded for dry weight before mixing with 100 ml of 2% methanol.

Efficacy to Inhibit Spore Germination Testing

A 1 ml spore suspension of *A. brassicicola* (1.0×10^8 spores/ml) and 1 ml of 1,000 ppm metabolites from T-ST-01 and T-CB-Pin-01 strains were added into a test tube containing 1 ml of 1/5 PDB. The tube was shaken at 80 rpm and incubated at room temperature for 18 h. One hundred spores were observed under a light microscope and the amount of germination determined.

Disease Control in the Laboratory

Pathogenicity of *A. brassicicola* on Chinese kale leaf was conducted in laboratory conditions. The spore suspension (1.0×10^5 spores/ml) was sprayed on disinfected Chinese kale leaf (5 ml/leaf) before the leaf was placed in plastic box. The box was incubated in an incubator chamber ($25 \pm 2^\circ\text{C}$, 85% of water holding capacity and 12 h. light) for 7 days before the disease severity was measured.

For the disease control test, disinfected Chinese kale leaf was sprayed with a 5 ml *A. brassicicola* spore suspension (1.0×10^5 spores/ml) and incubated for 30 min. The leaf was then sprayed with 5 ml of 1,000 ppm metabolite solution and put in a plastic box. The box was incubated as described above. Disease severity was recorded by comparing the diseased area with the total leaf area 7 days after incubation. There were 5 leaves per replication and 5 replications per treatment.

Disease Control in the Glasshouse

Chinese kale seedlings (3 weeks after germination) were used in this experiment. The seedlings in the growing pots were sprayed with a 10 ml spore suspension of *A. brassicicola* and incubated for 30 min before spraying with 10 ml of a 1,000 ppm metabolite solution. The pots were incubated under glasshouse conditions. Disease severity was recorded by comparing the diseased area with the total leaf area 14 days after inoculation. There were 10 pots per replication and 5 replications per treatment.

Statistic Analysis

All data were analyzed by ANOVA using the GLM procedure in a SAS system for Windows. The model used was $Y_{ij} = \mu + \text{treatment}_i + e_{ij}$, where Y_{ij} =measured value such as numbers of leaf showing pathogenic symptoms, μ =general mean, treatment_i =various treatments, and e_{ij} =residual error. Duncan's Multiple Range Test was used to determine the differences of the responding values between treatment groups.

RESULTS

Isolation of *Trichoderma virens* and Pathogen

Thirteen isolates of *T. virens* were isolated from 9 field soil samples used for growing 5 different species of plants, tomato, pepper, Chinese kale, durian and rambutan (**Table 1**). On Martin's agar (3), all isolates grew slowly and produced yellow colonies. However, the colony turned to yellow green color after the conidia were produced. On PDA, all isolates grew rapidly at room temperature. *A. brassicicola* also grew rapidly and showed grey colonies on PDA at room temperature.

Dual Culture Testing

All *T. virens* strains showed differences in their ability to inhibit mycelial growth of *A. brassicicola* on PDA at room temperature. Seven strains provided better inhibition rates of mycelial growth of *A. brassicicola* than T-CB-Pin-01. Strains T-ST-01 and T-NST-01 provided 72.5 and 67.0% inhibition, respectively, while T-CB-Pin-01 provided 47.0% growth inhibition (**Table 1**).

Dialysis Membrane Technique Testing

In dialysis membrane technique testing, strains T-ST-01, T-NST-01 and T-CB-Pin-01 (*T. harzianum*) totally (100%) inhibited mycelial growth of *A. brassicicola* on PDA at room temperature after subculturing for 5 days. No growth inhibition was observed in the control treatment when a disinfected PDA plug was inoculated on dialysis membrane (**Table 2**).

Table 1. Isolates, places of collected filed soil samples, plant species and percent inhibition on mycelia of *Alternaria brassicicola* by *Trichoderma virens* and *Trichoderma harzianum* isolates at room temperature

Isolates	Places	Plant species	Inhibition (%)
T-NST-01	Nakhon Si Thammarat	Tomato	67.0 ab ¹
T-NST-02	Nakhon Si Thammarat	Tomato	38.5 f
T-NST-03	Nakhon Si Thammarat	Pepper	45.5 d
T-NST-04	Nakhon Si Thammarat	Pepper	42.5 de
T-NST-05	Nakhon Si Thammarat	Chinese kale	55.5 c
T-NST-06	Nakhon Si Thammarat	Durian	60.0 b
T-ST-01	Surat Thani	Rambutan	72.5 a
T-ST-02	Surat Thani	Rambutan	42.5 de
T-ST-03	Surat Thani	Chinese kale	60.5 b
T-ST-04	Surat Thani	Durian	62.0 b
T-CB-01	Chanthaburi	Rambutan	45.5 d
T-CB-02	Chanthaburi	Rambutan	40.5 de
T-CB-03	Chanthaburi	Durian	63.5 b
T-CB-Pin-01 ²	Prachuap Khiri Khan	Pineapple	47.0 d

¹ Values followed by the same alphabet are not significantly different from each other analyzed by Duncan's Multiple Range Test at P<0.05.

² *Trichoderma harzianum* commercial strain in Thailand.

Table 2. Inhibition rate of antifungal metabolite from *Trichoderma virens* strains (T-ST-01 and T-NST-01) and *Trichoderma harzianum* (T-CB-Pin-01) on mycelial growth of *Alternaria brassicicola* in a Petri dish using the dialysis membrane technique

Treatment	Inhibition (%)
T-ST-01	100 a ¹
T-NST-01	100 a
T-CB-Pin-01 ^{2/}	100 a
Benomyl	100 a
Control	0 b

¹ Values followed by the same alphabet are not significantly different from each other analyzed by Duncan's Multiple Range Test at P<0.05.

² *Trichoderma harzianum* commercial strain in Thailand.

Extraction of Antifungal Metabolite

Only strains T-ST-01 and T-CB-Pin-01 were used to determine metabolites produced in 1/5 strength PDB. The result showed that strains T-ST-01 and T-CB-Pin-01 produced 2.04 and 2.56 g dry weight of metabolites, respectively, after incubation for 28 days.

Efficacy to Inhibit Spore Germination Testing

Antifungal metabolites (1,000 ppm) from T-ST-01, T-CB-Pin-01 and benomyl chemicals showed spore inhibition of *A. brassicicola* under light microscope observation. Spores of *A. brassicicola* germinated only 7.6, 9.4 and 17.0% when treated with benomyl, antifungal metabolite extracted from T-ST-01 and T-CB-Pin-01, respectively, while spores of *A. brassicicola* in the control treatment germinated 98.5% (Table 3).

Table 3. Spore germination rate of *Alternaria brassicicola* and leaf spot disease severity after applying 1,000 ppm antifungal metabolites from *Trichoderma virens* strain T-ST-01 and *Trichoderma harzianum* strain T-CB-Pin-01 in laboratory and glasshouse conditions

Treatment	Spore germination (%)	Leaf spot disease severity (%)	
		Laboratory	Glasshouse
2% methanol	98.5 c ¹	89.5 c ¹	84.5 c ¹
T-ST-01	9.4 a	22.5 a	32.0 a
T-CB-Pin-01	17.0 b	36.0 b	46.5 b
Benomyl	7.6 a	19.5 a	28.5 a

¹ Values followed by the same alphabet are not significantly different from each other analyzed by Duncan's Multiple Range Test at P<0.05.

Disease Control in the Laboratory

All treatments with antifungal metabolites (1,000 ppm) and benomyl chemicals showed a significant decrease in disease severity compared with the control treatment (2% methanol) after spraying with a spore suspension of *A. brassicicola* for 5 days. Treatments with benomyl chemicals and antifungal metabolites from T-ST-01 and T-CB-Pin-01 gave disease severities of 19.5, 22.5 and 36.0%, respectively, while the control treatment (2% methanol) provided 89.5% (Table 3).

Disease Control in the Glasshouse

All treatments with antifungal metabolites (1,000 ppm) and benomyl chemicals showed a significant decrease in disease severity compared with the control treatment (2% methanol) after spraying with a spore suspension of *A. brassicicola* for 14 days. Treatments with benomyl chemicals and antifungal metabolites from T-ST-01 and T-CB-Pin-01 provided disease severities of 28.5, 32.0 and 46.5%, respectively, while the control treatment (2% methanol) provided 84.5% (Table 3).

DISCUSSION

There were 13 isolates of *T. virens* in total from 9 field soil samples used for growing 5 different species of plants. The results indicated that *Trichoderma* spp.

could grow and survive in various soil conditions. This result complies with the report of Lo et al (9) claiming that *Trichoderma* spp. had equal ability to colonize roots in both alkaline and acidic soils. Some species of *Trichoderma*, such as the *T. harzianum* strain T-22 could survive in various soil types ranging from sandy to heavy and with a wide variation in organic content (10). Intana (11) also reported that 165 isolates of *Trichoderma* spp. from 148 soil samples used for growing 22 species of plants could grow in differing pH of soil, 5.4-7.2.

In dual culture tests, the results showed that all strains of *T. virens* inhibited mycelial growth of *A. brassicicola* on PDA at room temperature. This finding complies with many reports which asserted that *Trichoderma harzianum*, *T. virens* and *T. hamatum* are very effective at inhibiting mycelial growth of soilborne, seedborne, phyllosphere and storage plant pathogens on PDA (12,13,14). During plant pathogens attack, *T. virens* secreted many cell wall-degrading enzymes such as endochitinase, chitobiosidase, N-acetyl- β -glucosaminidase and glucan 1,3- β -glucosidase. These enzymes strongly inhibit spore germination (or cell replication) and germ tube elongation (15).

The result of dialysis membrane technique testing showed that *T. virens* strains T-ST-01 and T-NST-01 totally inhibited mycelial growth of *A. brassicicola* on PDA. This result indicates that these strains produced antifungal metabolite, and the dialysis membrane technique is an effective technique to screen promising strains of fungal antagonist that produce antifungal metabolites. Worasatit et al (16) and Intana (11) have also reported a successful screening of the promising strains of *T. harzianum* that produce antifungal metabolite using this technique.

In *A. brassicicola* efficacy test, the result showed that antifungal metabolite of *T. virens* strain T-ST-01 reduced the percentage of spore germination of *A. brassicicola*. This antifungal metabolite provided high efficacy in controlling Chinese kale leaf spot disease in both laboratory and glasshouse conditions. These results indicated that *T. virens* produce effective antifungal metabolites for controlling leaf spot disease in Chinese kale. These results complied with many reports asserting that *T. virens* could produce many kinds of high effective antifungal, agents such as ferulic acid (17), 3,4-dihydroxycarotane (18) and trichodermin (19).

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บทคัดย่อ

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การใช้สารต่อต้านเชื้อราจาก *Trichoderma virens* สำหรับควบคุมโรคใบจุดคะน้าที่เกิดจากเชื้อรา *Alternaria brassicicola*

จากการแยกเชื้อรา *Trichoderma virens* รวม 13 ไอโซเลท จากดินปลูกพืช 9 แหล่ง นำมาทดสอบความสามารถในการยับยั้งการเจริญของเชื้อรา *Alternaria brassicicola* ที่เป็นสาเหตุโรคใบจุดของคะน้าในระดับห้องปฏิบัติการ พบว่าทุกไอโซเลทสามารถยับยั้งการเจริญของเส้นใยเชื้อรา *A. brassicicola* ได้ โดยเฉพาะอย่างยิ่งไอโซเลท T-ST-01 และ T-NST-01 ที่สามารถยับยั้งการเจริญได้มากถึง 72.5 และ 67.0 เปอร์เซ็นต์ ตามลำดับ เมื่อทำการทดสอบประสิทธิภาพการสร้างสารต่อต้านการเจริญของเส้นใยของเชื้อรา *A. brassicicola* ด้วยเทคนิค dialysis membrane พบว่าไอโซเลท T-ST-01 และ T-NST-01 สามารถสร้างสารต่อต้านการเจริญของเส้นใยของเชื้อรา *A. brassicicola* ได้อย่างสมบูรณ์ เมื่อทำการสกัดและศึกษาประสิทธิภาพของสารต่อต้านเชื้อราของไอโซเลท T-ST-01 ในการยับยั้งการงอกของสปอร์เชื้อรา *A. brassicicola* พบว่าสามารถยับยั้งการงอกของสปอร์เชื้อรา *A. brassicicola* ได้ โดยพบว่ากรรมวิธีที่ใช้สารต่อต้านเชื้อราจาก *T. virens* มีสปอร์เชื้อราโรคพืชงอกเพียง 9.4 เปอร์เซ็นต์ ในขณะที่กรรมวิธีควบคุม (เมทานอลความเข้มข้น 2%) มีสปอร์เชื้อราโรคพืชงอก 98.5 เปอร์เซ็นต์ เมื่อประยุกต์ใช้สารต่อต้านเชื้อราในการควบคุมโรคใบจุดของคะน้าในระดับห้องปฏิบัติการและโรงเรือนปลูกพืชทดลอง พบว่าสารต่อต้านเชื้อราสามารถลดความรุนแรงของโรคลงได้อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) เมื่อเปรียบเทียบกับกรรมวิธีควบคุม โดยพบว่าความรุนแรงของโรคในกรรมวิธีที่ใช้สารต่อต้านเชื้อราจาก *T. virens* สายพันธุ์ T-ST-01 ในระดับห้องปฏิบัติการและโรงเรือนปลูกพืชทดลอง มีค่า 22.5 และ 32.0 เปอร์เซ็นต์ ตามลำดับ ในขณะที่ความรุนแรงของโรคในกรรมวิธีควบคุมทั้งในระดับห้องปฏิบัติการและโรงเรือนมีค่าเท่ากับ 89.5 และ 84.5 เปอร์เซ็นต์ ตามลำดับ

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