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Preliminary Investigation of *Trichoderma asperellum*'s Culture and Extracts Against Black Rot of Orchid

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Abstract

The effect of *Trichoderma asperellum* against black rot pathogen of orchid caused by *Phytophthora palmivora (*Butl.) Butl. was investigated. Eleven isolates of *P. palmivora* were isolated form leaves by tissue transplanting technique and were proved for their pathogenicity by detached leaves technique. The results showed that isolate V04 gave the highest virulence for disease incidence. *T. asperellum* was tested against mycelial growth of *P. palmivora* V04 by bi-culture test and crude extract methods. Bi-culture test showed that *T. asperellum* could inhibit mycelial growth at 71.67%. Crude extracts were extracted from *T. asperellum* with hexane, ethyl acetate and methanol. Crude extract which was extracted with methanol gave the best mycelial growth inhibition at the concentration of 1,000 µg/ml ($p \leq 0.05$) and the effective dose (ED_{50}) was 1,591.61 µg/ml.

Keywords: Black rot, *Phytophthora palmivora*, *Trichoderma asperellum* and Orchid

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การทดสอบใช้เชื้อราและสารสกัดจากเชื้อรา *Trichoderma asperellum* ในการต่อต้านเชื้อสาเหตุโรคเน่าดำของกล้วยไม้

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บทคัดย่อ

การทดสอบประสิทธิภาพของเชื้อรา *Trichoderma asperellum* ในการควบคุมโรคเน่าดำของกล้วยไม้ที่มีสาเหตุมาจากเชื้อรา *Phytophthora palmivora* (Butl.) Butl. โดยการแยกเชื้อราสาเหตุโรคจากใบด้วยวิธี Tissue transplanting technique ได้เชื้อราจำนวน 11 ไอโซเลท นำทั้ง 11 ไอโซเลท มาทดสอบการเกิดโรคโดยวิธี detached leaves พบว่า ไอโซเลท V04 สามารถทำให้เกิดโรครุนแรงที่สุด ในการทดสอบประสิทธิภาพของเชื้อรา *T. asperellum* ในการยับยั้งการเจริญเติบโตของเส้นใยเชื้อรา *P. palmivora* V04 ด้วยวิธี bi-culture test และ crude extract test ผลการทดสอบ bi-culture test พบว่า เชื้อรา *T. asperellum* สามารถยับยั้งการเจริญเติบโตของเส้นใยเชื้อรา *P. palmivora* V04 ที่ 71.67%. สารสกัดหยาบจากเชื้อรา *T. asperellum* ด้วย hexane ethyl acetate และ methanol โดยสารสกัดหยาบที่ได้จาก methanol ที่ความเข้มข้น 1,000 ไมโครกรัม/มิลลิลิตร สามารถยับยั้งการเจริญเติบโตของเส้นใยของเชื้อรา *P. palmivora* V04 ($p \leq 0.05$) โดยมีค่า ED_{50} เท่ากับ 1,591.61 ไมโครกรัม/มิลลิลิตร

คำสำคัญ: โรคเน่าดำ *Phytophthora palmivora* *Trichoderma asperellum* และ กล้วยไม้

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Introduction

Phytophthora spp. were reported as pathogenic fungi causing black rot disease of orchid including *P. cactorum* and *P. palmivora* which reported to cause black rot disease in many country (Cating *et al*., 2010; Khairum *et al.,* 2016). Black rot disease can reduce both quality and quantity of orchid. An initial symptom of the disease may include small black lesions on the roots or basal portion of the pseudobulbs and then enlarge and may engulf the entire pseudobulb and leaf. Ultimately, plant may die (Cating *et al*., 2010). This disease can be controlled by using chemical fungicides, which is the effective method to control black rot disease. However, the chemical fungicides have resulted in the residual toxic to the environment as well as induction of chemical resistant pathogen. These concerns have led to the desire to investigate other control methods, including biological control, an attendant decrease in the use of chemical fungicides. Currently, the researchers have been considerable efforts to find biological control agents for controlling plant diseases, including non-pathogenic microorganisms. These microorganisms can inhibit the growth of pathogen and reduce the disease incidences. Antagonistic fungi such as *Chaetomium* spp., *Gliocladium* sp., *Emericella* spp. and *Trichoderma* spp. are reported as effective bio-agent, especially *Trichoderma* spp. *Trichoderma* spp. are reported that can inhibit growth of many plant pathogens such as *Pythium myriotylum* causing cocoyam root rot, *Fusarium oxysporum* f. sp. *lycopersici* causing tomato Fusarium wilt, *Sclerotinia sclerotiorum* causing carnation stem rot, *P. capsici* in tomato and *C. gloeosporioides* in chilli (Mbarga *et al.,* 2012; Komy *et al.*, 2015; Vinodkumar *et al.,* 2017; Cruz-Quiroz *et al.,* 2018). This information would valuable to further study on biological control using antagonistic fungi. The objective of this study was to test antagonistic fungus, *T. asperellum* to inhibit growth of *P. palmivora* causing black rot disease of orchid.

Materials and Methods

1. Collection and isolation

Isolation of *P. palmivora* were obtained from infected leaves of orchid which were found in Maha Sarakham province. Isolation of causing agent was done by using tissue transplanting technique into pure cultures (Khairum *et al.*, 2016).

2. Pathogenicity test

The black rot pathogen was isolated from infected leaves. Pure cultures were multiplied on PDA for inoculation. All isolates were tested for pathogenicity test on leaves of orchid followed Koch's postulate. Inoculated leaves were kept in moist chamber at room temperature (28-32°C) in the dark. Data were collected as lesion diameter (cm.) on leaves, and analyses of variance using completely randomized design (CRD) with four replications.

3. Bi-culture antagonistic test

In this experiment, bi-culture antagonistic tests were proceeded to evaluate the antagonistic fungus, *T. asperellum* (TRICO), which provided by Agro-biomate Co., Ltd., against *P. palmivora.* Pathogen isolates used in bi-culture antagonistic test were obtained from pathogenicity test which was the most aggressive isolate. Hyphal plugs of *P. palmivora* and *T. asperellum* (TRICO) were placed to the middle of a half of petri dishes (9 cm diameter) and incubated at room temperature. Data were collected measuring colony diameter for 7 days of *P. palmivora* and computed as percent growth inhibition. Percentage of growth inhibition (PGI) of pathogen was evaluated in the formula (cc-cd)/cc × 100; cc = colony diameter of plant pathogenic fungus in control petri dish and cd = colony diameter of plant pathogenic fungus in bi-culture petri dish (Ratanacherdchai *et al.,* 2010). Data were computed analyses of variance using completely randomized design (CRD) with four replications.

4. Test for antifungal metabolites

The microbial antagonist, *T. asperellum* (TRICO) were cultured in Potato dextrose broth (PDB) for 30 days, then filtered and dried mycelium mats were collected for extraction method using rotary vacuum evaporator. The crude extracts were tested in petri dishes (5 cm diameter) which mixed to PDA at concentrations 0, 100, 250, 500 and 1,000 µg/ml. Cultures of plant pathogenic fungus, *P. palmivora* was grown for 7 days on PDA. Each pathogen was separately transferred the agar plug (0.3 cm diameter) to the center of petri dish containing crude extracts of microbial antagonist and incubated at room temperature. After 5 days of incubation, colony diameter was recorded and computed growth inhibition (GI), Effective dose (ED_{50}) and analyses of variance using completely randomized design (CRD) with four replications.

Results and Discussion

1. Collection and isolation

Eleven isolates of the black rot pathogen of orchid were isolated and identified as *P. palmivora.* With this, Cating *et al*. (2010) and Khairum *et al.* (2016) also reported that this pathogen causing black rot of orchid genera and it is the most common species affecting commercial orchid production.

2. Pathogenicity test

All isolates of *P. palmivora* were proved to be pathogenicity to the host species. The inoculated leaves showed the black rot symptoms within 5 days after inoculation. Symptoms were found as small black lesions on the basal portion of the pseudobulbs. As the lesions age, they enlarge and may engulf the entire pseudobulb and leaf (Cating *et al*., 2010). The result showed that isolate of *P. palmivora* V04 and V02 gave the most aggressive causing black rot symptom (Table 1 and Fig. 1).

Table 1 Pathogenicity test of *Phytophthora palmivora* isolates on orchid leaves using plug inoculation method for 5 days

Isolate	Lesion diameter (cm)
Control	0.00[e]
V01	1.20[cd]
V02	2.98[a]
V03	0.93[d]
V04	3.19[a]
V05	1.36[bcd]
V06	1.54[bc]
V07	1.43[bc]
V08	1.81[b]
V09	1.85[b]
V10	1.73[b]
V11	1.71[b]
C.V. (%)	18.48

[a-e] Average of four replications. Means followed by the same letter in a column were not significantly different by DMRT at P=0.05.

3. Bi-culture antagonistic test

Bi-culture showed that *T. asperellum* (TRICO) could inhibit mycelial growth of *P. palmivora* V04 (71.67%) (Fig. 2). There are several reports on the potential use of *Trichoderma* spp. as microbial antagonist for biological control of plant diseases such as cocoyam root rot caused by *P. myriotylum*, tomato Fusarium wilt caused by *F. oxysporum* f. sp. *lycopersici*, stem rot in carnation caused by *S. sclerotiorum*, as same as *P. capsici* in tomato and *Colletotrichum gloeosporioides* in chilli (Mbarga *et al.,* 2012; Komy *et al.*, 2015; Vinodkumar *et al.,* 2017; Cruz-Quiroz *et al.,* 2018). But this result was also expressed the potential of *T. asperellum* to control black rot disease of orchid caused by *P. palmivora*.

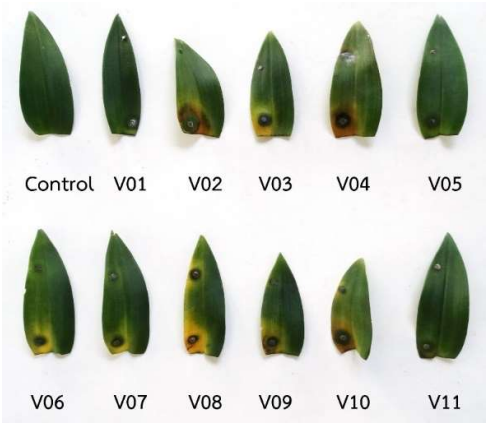

Fig. 1 Pathogenicity test of *Phytophthora palmivora* isolates on orchid leaves using plug inoculation method for 5 days

Fig. 2 Bi-culture antagonistic test between *Trichoderma asperellum* (TRICO) and *Phytophthora palmivora* V04

4. Test for antifungal metabolites

The inhibitory effect of antifungal metabolites from *T. asperellum* (TRICO) on mycelial growth of *P. palmivora* V04 in the medium amended with methanol crude extract showed that the concentration of 1,000 µg/ml strongly inhibited the mycelial growth as 31.50%. The ED_{50} value was 1,591.61 µg/ml. the antifungal metabolite extracted from ethyl acetate and hexane could inhibit the mycelial growth of 25 and 24%, respectively (Table 2 and Fig. 3). Similar results reported by Talubnak and Soytong (2010) that the methanol crude extract from *Emericella nidulans* strongly inhibited the mycelial growth of *C. gloeosporioides* VP8 causing vanilla anthracnose disease at the concentration of 1,000 µg/ml with 22.25%. Moreover, the methanol crude extract from *Gliocladium* sp. was reported that could inhibit the sporulation of *C. acutatum* J06 causing chilli anthracnose at the concentration of 500 µg/ml with 93.69%. (Ratanacherdchai *et al.*, 2017).

Fig. 3 Testing for antifungal metabolites from *Trichoderma asperellum* (TRICO) to inhibit *Phytophthora palmivora* V04 causing black rot of orchid

Table 2 Effect of antifungal metabolites form *Trichoderma asperellum* (TRICO) for inhibition of mycelial growth of *Phytophthora palmivora* V04

Treatment	Colony diameter (cm)	Mycelial growth inhibition (%)
Solvent (A)		
Hexane (A1)	4.27^{a}	14.70^{b}
Ethyl acetate (A2)	4.23^{a}	15.40^{b}
Methanol (A3)	4.09^{b}	18.25^{a}
F-test (A)	**	**
Concentration (B)		
0 µg/ml (B1)	5.00^{a}	0.00^{e}
100 µg/ml (B2)	4.35^{b}	12.92^{d}
250 µg/ml (B3)	4.08^{c}	18.50^{c}
500 µg/ml (B4)	3.88^{d}	22.33^{b}
1000 µg/ml (B5)	3.66^{e}	26.83^{a}
(A) × (B)		
A1B1	5.00^{a}	0.00^{j}
A1B2	4.45^{b}	11.00^{i}
A1B3	4.15^{d}	17.00^{fg}
A1B4	3.93^{e}	21.50^{cd}
A1B5	3.80^{f}	24.00^{bc}
A2B1	5.00^{a}	0.00^{j}
A2B2	4.33^{c}	13.50^{hi}
A2B3	4.10^{d}	18.00^{ef}
A2B4	3.98^{e}	20.50^{de}
A2B5	3.75^{f}	25.00^{b}
A3B1	5.00^{a}	0.00^{j}
A3B2	4.29^{c}	14.25^{gh}
A3B3	3.98^{e}	20.50^{de}
A3B4	3.75^{f}	25.00^{b}
A3B5	3.43^{g}	31.50^{a}
C.V. (%)	1.57	9.42

$^{a-i}$ Average of four replications. Means followed by the same letter in a column were not significantly different by DMRT at P=0.05.

Conclusion

T. asperellum showed the potential for against *P. palmivora* causing black rot disease of orchid. It proved that *T. asperellum* become the promising antagonistic fungus as a biological agent against plant pathogenic fungi.

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