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Full Paper

Growth inhibition of *Penicillium digitatum* by antagonistic microorganisms isolated from various parts of orange tree

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Abstract: *Penicillium digitatum* (Pers.:Fr) Sacc. is the major cause of green mold disease in orange trees grown for orange production industry in Thailand. In this study, antagonistic microorganisms were isolated from various parts of healthy orange trees. A total of 121 microbial isolates were recovered from which 15 isolates showed inhibitory effect against *P. digitatum* by spot test technique. The inhibitory effect of these 15 isolates was also tested by agar disc diffusion method using culture filtrate from nutrient broth. It was found that isolate W1L1, D1L2 and W1L9 gave the best inhibition with a clear zone of 21, 15 and 14 mm in diameter, respectively. The effect of culture filtrate on spore germination was also investigated. The result showed that isolate W1L1 gave the highest inhibition after 8 hours of incubation with the lowest germination rate of 2.4%. Morphological and biochemical characterizations revealed that these isolates were members of the genus *Bacillus* and identified as *B. pumilus*.

Keywords: *Penicillium digitatum*, antagonistic microorganisms, oranges, *Bacillus*, biological control

Introduction

Orange (*Citrus sinensis*) is one of the economic fruits in Thailand. Most production areas are in the northern part of the country especially Chiang Mai. In 2004, Thailand exported 2,189 tons of oranges to Asian countries with a value of 50.8 million Baht [1]. Fungal diseases are one of the major problems facing the citrus production in all areas of the world and resulted in enormous economic losses [2]. Green mold is the most important postharvest disease of citrus in many Asian countries including Thailand. The responsible agent is the fungus, *Penicillium digitatum* (Pers.:Fr) Sacc. [3]. This fungus survives in the orchard from season to season mainly in the form of conidia and causes infection by airborne spores where there are injuries or blemishes [4]. The disease is generally controlled by fungicides such as imazalil, sodium ortho-phenyl phenate, or thiabendazole [5,6]. The use of these chemical agents has been applied for many years with few or limited success due to the development of resistance by the fungus [7,8]. In addition, the accumulation of hazardous chemicals in the environment raises public concern about their effect on human health. There is an urgent demand for new methods to supplement the existing regimes to achieve better disease control. Biological control offers an environment-friendly alternative to the use of chemicals for controlling plant diseases [2,9,10]. The increasing interest in the development of such control has led to our investigation of microorganisms associated with orange trees as potential microbial agents for the control of *P. digitatum*. In this study, we reported the isolation of antagonistic microorganisms from various parts of healthy orange trees and their inhibitory effect on growth and spore germination of *P. digitatum* in vitro.

Materials and Methods

Isolation of antagonistic microorganisms

Samples of branches, flowers, leaves, and young fruits of healthy orange trees were collected from 4 orange plantations in Chiang Mai and antagonistic microorganisms were isolated from these samples. Approximately 25 g of each sample were added to 225 ml of sterile distilled water in a 500 ml conical flask and incubated in an ultrasonic bath for 10 minutes. The resultant suspension (0.1 ml) was used to inoculate nutrient agar plates. All plates were incubated at room temperature and microbial colonies appearing after 24 hours were picked and restreaked until pure cultures were obtained. The isolates were maintained on nutrient agar slant for routine use and in 20% glycerol at -20^{o}C for long term preservation.

Pathogen

P. digitatum was isolated from an infected orange and cultured on a potato dextrose agar (PDA). The spore suspensions were prepared from a 5 day old culture by adding sterile distilled water on the surface of the agar slant and scraping the growth off the surface using sterile cotton swab.

Tween 20 (0.04%) was added to the resultant spore suspension to help dispersion. The concentration of spores was determined by direct count using a haemacytometer and adjusted as required.

Preliminary screening for antifungal activity

The antagonistic potential of isolated microorganisms against *P. digitatum* was determined in triplicate by spot test technique [11] on PDA for each isolate. The tested isolates were grown on nutrient agar for 24 hours and used to inoculate at the periphery of PDA plates seeded with *P. digitatum* spore suspension. Five isolates were inoculated on each plate. All plates were incubated for 3 days at room temperature before recording of the inhibition zone. Isolates that showed inhibitory effect against *P. digitatum* were selected for further screening using agar diffusion method.

Screening of antifungal activity by agar disc diffusion method

Subsequent screening of promising isolates from preliminary study was performed using Kirby-Bauer method [12]. For this purpose, the antagonistic isolates were grown on nutrient agar and incubated for 24 hours at room temperature. Two loops were taken from the resulting colonies from each isolate and transferred to 50 ml of nutrient broth. The cultures were incubated on a rotary shaker (150 rpm) for 24 hours at 28^{o}C. The bacterial cells were harvested by centrifugation (6000 rpm for 20 min) at 4^{o}C and the supernatant collected. The supernatant was filtered through a 0.22 μm-pore cellulose acetate filter and stored at 4^{o}C until use. Three 5-mm sterile paper discs were placed on PDA plates seeded with *P. Digitatum* spore suspension and 25 μl of supernatant were applied to each disc. Antifungal activity was determined on PDA after 4 days incubation at room temperature and expressed as diameter (mm) of the inhibition zone.

Inhibitory effect of culture filtrate on the spore germination of P. digitatum

An equal volume of *P. digitatum* spore suspension ($3x10^6$ spores/ml) was mixed with the culture filtrate of isolates D1L2, W1L1 and W1L9 in sterile test tubes. The germination of spore was observed microscopically at 0, 4 and 8 hours. The spore was considered to be germinated only when the length of the germ tube was twice that of the diameter [12], and the results were presented as percentage of germination. In addition, another set of experiment was prepared by incubating the mixture of spore suspension and culture filtrate (1:1, v/v) at room temperature for 7 days and spore germination observed. A control set using nutrient broth in place of culture filtrate was included in all experiments.

Identification of bacterial isolates

Identification of the three most effective isolates was carried out according to Bergey's Manual of Systematic Bacteriology volume 2 [14]. The dichotomous key of Guerra-Cantera and Raymundo [15] was used to assist in the assignment of these isolates to the species level.

Results and Discussion

A total of 121 microorganisms were isolated from various parts of healthy orange trees. Among these, 68 isolates were recovered from branches, 38 isolates from leaves, 9 isolates from fruits and 6 isolates from flowers. Preliminary screening of these isolates by spot test technique showed that 15 isolates were inhibitory to *P. digitatum* on PDA with the diameter of clear zone ranging between 12.5-28 mm (Table 1). Further screening by agar disc diffusion method showed that only 7 isolates gave positive results when their culture filtrates were tested. Among these, isolates W1L1, W1L9 and D1L2 gave the largest clear zones on the test media (Table 2). In addition, the culture filtrates of these three isolates were found to inhibit the germination of *P. digitatum*'s spores (Table 3). No growth was

Table 1. Antagonism of bacterial isolates against P. digitatum by spot test assay

Bacterial isolate	**Zone of inhibition (mm)**
D1L2	26
D2S2	12.5
K4L3	28
K6L1	22
WC2	16.5
W1L1	21
W1L2	22
W1L4	19
W1L7	15
W1L8	26
W1L9	27.5
W2F3	18
W3L2	28
W3L4	15
W3S1	21.5

observed in the tube containing a mixture of the spore suspension ($3x10^6$ spores/ml) and the culture filtrate (1:1, v/v) after incubation at room temperature for 7 days. On the contrary, the fungal hyphae were clearly visible in the control set. Identification tests showed that these isolates were Gram positive, rod shaped and produced endospores. They were catalase and oxidase positive and did not grow under anaerobic condition, thus belonging to the genus *Bacillus*. According to the identification key of Guerra-Cantera and Raymundo [15], these isolates were assigned to *B. pumilus* species.

Table 2. Antagonism of bacterial isolates against *P. digitatum* by agar disc diffusion method

Bacterial isolate	**Zone of inhibition (mm)**
D1L2	15
WC2	10
W1L1	21
W1L4	12
W1L8	12
W1L9	14
W3S1	7.5

Table 3. Germination rate (%) of *P. digitatum* spore in culture filtrate of antagonistic microorganisms

Time (hr)	**Germination rate (%)**			
	control	**W1L1**	**W1L9**	**D1L2**
0	0	0	0	0
4	1.5	0	1.9	1.9
8	17.0	2.4	3.0	4.3

Several antagonistic microorganisms have been shown to suppress decay of citrus by green mold [8,16-19]. Moreover, a commercial biocontrol product containing the yeast *Candida oleophila* has been available under the name Aspire (Ecogen Corporation, Langhorne, PA) in the United States and Israel for use against postharvest decay of citrus [20]. However, Aspire alone was found to be insufficient to reduce the decay to commercially acceptable levels. Later, Brown et al. [3] reported that the efficacy of Aspire was also affected by the type of injury, and cells of *C. oleophila* were sensitive to volatiles of orange peel oil. They also showed that citrus oil and its volatiles not only exhibited no activity against *P. digitatum* but also stimulated the germination of the fungus.

The use of *Bacillus* occurring naturally on the surface of fruits or vegetables as biocontrol of postharvest diseases has also been reported previously. Many surveys of antagonistic bacteria support the potential of *Bacillus* for controlling various plant pathogenic fungi [21-26]. Members of the genus

Bacillus have a natural advantage over Gram negative bacteria for use as biocontrol agents. Particularly, this is their ability to produce heat- and dessication-resistant spores, which offers an ease of formulation and storage of the products [9]. The formulation and application methods play key issues for the efficacy and successful outcome of the commercial products [10]. In addition, there are currently 2 biological control products approved by U.S. Environmental Protection Agency that contain *B. pumilus* as an active ingredient and currently available in the market, namely GB34 concentrate or technical biological fungicide (Bayer Cropscience LP, NC) and Sonata ASO or QST2808 technical (Agraquest Inc., OR) [27].

Conclusion

In conclusion we have demonstrated that strains assigned as *Bacillus pumilus* isolated from healthy orange trees can inhibit the growth of *Penicillium digitatum* in vitro, thus contributing to the literature on biological control of *P. digitatum.* The results of the present study also provide further evidence that antagonistic microorganisms are good sources of potential biocontrol agents against plant pathogenic fungi.

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