

Efficacy of *Chaetomium* Species as Biological Control Agents against *Phytophthora nicotianae* Root Rot in Citrus

Phung Manh Hung^{1,2,*}, Pongnak Wattanachai¹, Soy tong Kasem¹ and Supattra Poeaim³

¹Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand

²Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phutho 293840, Vietnam

³Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand

Abstract Thailand is one of the largest citrus producers in Southeast Asia. Pathogenic infection by *Phytophthora*, however, has become one of major impediments to production. This study identified a pathogenic oomycete isolated from rotted roots of pomelo (*Citrus maxima*) in Thailand as *Phytophthora nicotianae* by the internal transcribed spacer ribosomal DNA sequence analysis. Then, we examined the *in vitro* and *in vivo* effects of *Chaetomium globosum*, *Chaetomium lucknowense*, *Chaetomium cupreum* and their crude extracts as biological control agents in controlling this *P. nicotianae* strain. Represent as antagonists in bi-culture test, the tested *Chaetomium* species inhibited mycelial growth by 50~56% and parasitized the hyphae, resulting in degradation of *P. nicotianae* mycelia after 30 days. The crude extracts of these *Chaetomium* species exhibited antifungal activities against mycelial growth of *P. nicotianae*, with effective doses of 2.6~101.4 µg/mL. Under greenhouse conditions, application of spores and methanol extracts of these *Chaetomium* species to pomelo seedlings inoculated with *P. nicotianae* reduced root rot by 66~71% and increased plant weight by 72~85% compared to that in the control. The method of application of antagonistic spores to control the disease was simple and economical, and it may thus be applicable for large-scale, highly effective biological control of this pathogen.

Keywords Biological control, *Chaetomium*, Citrus root rot, *Phytophthora nicotianae*

Phytophthora nicotianae Breda de Haan (syn. *P. parasitica* Dastur) is known as a typical root pathogen that can infect hundreds of plant genera. This fungus-like organism infects and causes root rot in many different citrus types worldwide [1, 2]. According to the Food and Agricultural Organization of the United Nations Statistics Division (FAOSTAT), in 2013, approximately 0.1 million ha were dedicated to citrus cultivation in Thailand, resulting in the production of 1.2 million tons of fruit, which was slightly less than that in Indonesia in Southeast Asia [3]. With the prevalence of

wet climatic conditions in Thailand, however, infection with *Phytophthora* has become a major problem for the citrus industry, causing yield losses of approximately 6~12% and economic losses of at least 37 million USD/yr [4].

Current practices for controlling *Phytophthora* diseases are largely based on cultivation management in fields and application of synthetic fungicides [1, 5]. Intensive use of chemical pesticides for the control of plant pathogens may lead to be harmful accumulation of toxins in the environment and adverse effects on human health. Moreover, the resistances of *Phytophthora* species to fungicides are the topical questions in controlling the pathogens [1, 2]. In particular, many fungicides targeting chitin and sterol synthesis are ineffective against *Phytophthora* species because of the differences in their cell wall composition compared to that of true fungi [1, 5].

To reduce the dependence of agriculture production on noxious synthetic pesticides, the search for effective biological control agents (BCAs) against plant pathogens has been carried out worldwide. *Chaetomium* species are ubiquitous fungi of which more than 350 species exist. Some of these fungi can act as antagonists against various plant pathogens through the production of lytic enzymes and metabolites [6-8]. In our efforts to control *Phytophthora* root rot in *Citrus* plants, some potent strains of *Chaetomium* species

Mycobiology 2015 September, 43(3): 288-296
<http://dx.doi.org/10.5941/MYCO.2015.43.3.288>
pISSN 1229-8093 • eISSN 2092-9323
© The Korean Society of Mycology

***Corresponding author**

E-mail: manhhungnomafsi@gmail.com

Received June 7, 2015

Revised June 10, 2015

Accepted July 21, 2015

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

have been used as BCAs, and we have recently succeed in controlling *P. palmivora* *in vitro* using *Chaetomium* [9]. However, the applications of these BCAs for the control of *Phytophthora* species in citrus production are limited because our inadequate understanding of the mechanisms through which *Chaetomium* species control *Phytophthora* species.

In our recent investigation, we isolated a *Phytophthora* sp. (denominated as KA1) from rotted roots of a pomelo orchard near Bangkok, Thailand affected by root rot. Therefore, in this study, we characterized this isolate and examined the capacity of *Chaetomium* species and their crude extracts for biological control of this strain.

MATERIALS AND METHODS

Characterization and identification of *Phytophthora* sp. KA1. For morphological studies, the following media were used to culture *Phytophthora* sp. KA1: V8 juice agar (200 mL/L V8 juice [Campbell Soup Co., Camden, NJ, USA], 3 g/L CaCO₃, 20 g/L agar, and 800 mL/L water) and potato dextrose agar (PDA; HiMedia Laboratories Pvt. Ltd., Mumbai, India). A camera with associated software attached to an Olympus light microscope (CH40; Olympus Optical Co. Ltd., Tokyo, Japan) was used for observation and measurement of sporangia and other structures of *Phytophthora* sp. KA1.

Sporangia of the studied isolate were produced by floating some mycelial discs (obtained from margins of a 3-day-old culture on V8 agar) in 10 mL of double distilled water. The discs were then incubated under fluorescent light, at temperature of 25~28°C for 3~4 days. To determine the caducity of sporangia, the floating mycelial discs (bearing sporangia) were raised in a drop of distilled water several times, and the length of pedicels was measured under a light microscope. Sporangia caducity was determined based on the uniformity of the pedicel length [1]. *Phytophthora* sp. KA1 was identified into species level based on sequence analysis of the internal transcribed spacer (ITS) ribosomal DNA. Primers ITS6 (5'-GAA GGT GAA GTC GTA ACA AGG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify ITS1, ITS2 and the 5.8S ribosomal DNA fragments by polymerase chain reaction under previously described conditions [10]. The sequencing of the cloned fragments then was performed at First Base Laboratory (Selangor, Malaysia), using the same primers. The full-length determined ITS nucleotide sequences of *Phytophthora* sp. KA1 then were used as queries for BLAST searches in GenBank (<http://www.ncbi/blast/>). Subsequently, the sequences of *Phytophthora* sp. KA1 and related taxa (obtained from GenBank database) were aligned and analyzed to construct a phylogenetic tree using software MEGA ver. 5.2 [11].

Fungal isolates and *in vitro* antagonism tests. *Phytophthora* sp. KA1 was the target for biological control in this study. Meanwhile, *C. globosum* (CG05), *C. lucknowense*

(CL01), and *C. cupreum* (CC3003), which have all been proven to control *P. palmivora*-induced root rot in *Citrus* sp. [9], were used as antagonists and to produce antagonistic crude extracts.

Each of the *Chaetomium* species was tested for antagonism against *Phytophthora* sp. KA1 using bi-culture techniques [9]. A 5-mm-diameter mycelial disc from the active growing area of a 5-day-old colony of *Phytophthora* sp. KA1 was placed alone (as a control) or opposite a mycelial disc from one of the above antagonists on PDA plates (9 cm diameter). All plates were then maintained at temperature of 28°C in the dark, and the colony diameters of the pathogen were measured after 10 and 30 days. The inhibition of mycelial growth of the pathogen was then calculated as a percentage using the formula below:

$$\text{Inhibition (\%)} = 100 \times (1 - B/A) \quad (1)$$

, where A and B are the colony diameters of *Phytophthora* sp. KA1 in the control and bi-culture plates, respectively. The experiment was repeated twice, and performed in a completely randomized design with 4 replicates.

***In vitro* effects of antagonistic crude extracts on the growth of *Phytophthora* sp. KA1.**

Crude extracts of the tested antagonists were produced by method of Kanokmedhakul *et al.* [12]. Each fungal antagonist was cultivated in potato dextrose broth (200 g/L potato infusion and 20 g/L dextrose) at 25~28°C for 45 days. Fungal biomass then was collected, air-dried, ground, and extracted sequentially with hexane, ethyl acetate (EtOAc), and methanol (MeOH) to produce crude hexane, crude EtOAc, and crude MeOH extracts, respectively. The crude extracts were obtained by evaporating the extracted-solvents in a vacuum.

These 3 crude extracts from each antagonist were tested at different concentrations (10, 50, 100, 500, and 1,000 µg/mL; untreated samples [0 µg/mL] were used as controls) for their potential to inhibit the growth of *Phytophthora* sp. KA1. A 5-mm-diameter mycelial disc of *Phytophthora* sp. KA1 was placed on the center of PDA plates (5 cm in diameter) containing the crude extracts. To obtain the desired concentrations, stock crude extract was weighed, dissolved in 2% dimethyl sulfoxide, mixed into molten PDA, and then autoclaved for 20 min at 121°C (15 psi). All plates were then incubated at temperature of 28°C in the dark, and colony diameters were measured when the pathogen colonies developed fully on the control plates (about 5 days). Finally, the inhibition of mycelial growth of the tested pathogen was calculated as a percentage using the same formula Eq. 1 above. In which, A and B are the colony diameters of *Phytophthora* sp. KA1 (after subtracting the diameter of the inoculum disc) in the control and crude extract plates, respectively. Effective dose (ED₅₀) values for inhibition of mycelial growth were also computed by probit analyses using SPSS Statistics ver. 19.0 software (IBM Co., Armonk, NY, USA). All experiments were repeated twice,

and performed in completely randomized designs with 4 replicates.

Preparation of inocula. For morphological studies and inoculum production, chlamydospores of *Phytophthora* sp. KA1 were produced as described by Tsao [13]. Mycelia of *Phytophthora* sp. KA1 were first grown at 28°C for 1 wk in 250-mL bottles containing 25 mL of cleared V8 broth. Subsequently, 100 mL of sterile-distilled water was added to each bottle, and then the bottles were maintained at temperature of 18°C in the dark for 3 wk. For use as the inoculum, chlamydospores were separated from the mycelium by repeated blending and low-speed centrifugation.

The pathogen inoculum was prepared by mixing chlamydospores of *Phytophthora* sp. KA1 into sterilized fine sand before dark-moist incubation at 28°C. After 3 days, the propagule density of the inoculum was then determined as described by Timmer *et al.* [14] using selective agar medium pimarin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol (PARPH). Infested soil was then prepared by mixing the inoculum with potting media (sterilized clay soil, sand, and compost at a v/v/v ratio of 3 : 1 : 1) to give concentrations of 5 or 2.5 propagules of *Phytophthora* sp. KA1 per cubic centimeter of soil; these samples were used in the pathogenicity and greenhouse tests, respectively.

To provide BCAs for the greenhouse test, each antagonist was separately cultured on PDA plates for 30 days, and spores were then collected by scraping of the agar surface of the plates and ground before addition to 300 mL of potato dextrose broth in a 500-mL bottle for semi-germination. The bottle was incubated at 30°C for 8 hr, and then propagule density was determined under a microscope using a haemocytometer. The crude MeOH extract of each antagonist was weighed and then dissolved in 2% dimethyl sulfoxide before being added to sterilized distilled water to give a 50 µg/mL solution.

Pathogenicity and greenhouse tests. For the first experiment, pathogenicity was examined by artificial inoculation of *Phytophthora* sp. KA1 into roots of pomelo seedlings. Seedlings (3-mon-old) were washed the roots under running water to remove potting mix, then were transferred into either the infested soil or the sterilized potting media only (as controls) in plastic tubes (10 × 15 cm). Each treatment was performed with 5 seedlings and repeated once.

For the second experiment, spores and crude MeOH extracts of each antagonist were used as BCAs to control *Phytophthora* sp. KA1 under greenhouse conditions. For crude treatments, 5-wk-old pomelo seedlings were planted in plastic tubes containing infested soil. The prepared solutions of crude MeOH were used to treat the seedlings via bi-weekly watering of 100 mL per tube. For each spore treatment, the infested soil was mixed with the prepared spores of a single antagonist at a concentration of 100 propagules per cubic centimeter of soil, and these soils were then used for planting the seedlings. In addition,

seedlings were also planted in infested soil as controls and in sterilized potting media as references. Whenever the crude treatments were applied, the plants in the other groups were also watered with the same volume of fresh water. Each treatment was performed with 4 plants (with 1 plant considered as 1 replication). The experiment was performed in a completely randomized block design, under greenhouse conditions (temperature of 28–33°C), and repeated twice.

All plants in the experiments were weekly flooded with fresh water for 24 hr, and the root rots were rated after 6 wk. The potting mix was washed away, then up to 100 root tips on each seedling were visually rated as healthy or rotted, and the root data were expressed as the percent root rot. Plants from the second experiment were then dried at 65°C for 12 hr before being weighed. The selective agar medium PARPH was used throughout the experiments to isolate the pathogen from the infected roots.

Data analysis. All data were subjected to analysis of variance (ANOVA) using SPSS Statistics ver. 19.0 software (IBM Co.). Because there was no significant differences between the experiments based on preliminary analysis of variance, data from the repeated experiments were combined. Means were compared using least significant differences with analysis of Duncan's multiple range test (DMRT).

RESULTS

Pathogenicity of *Phytophthora* sp. KA1. At the inoculum level of 5 chlamydospores of *Phytophthora* sp. KA1 per cubic centimeter of soil, an average of 45.3% of root tips were rotted, with very few new roots in pomelo seedlings. In contrast, the non-inoculated seedlings had no root rot and produced an abundance of new roots. Importantly, the tested organism was re-isolated from newly infected roots of the inoculated plants. This result indicates that *Phytophthora* sp. KA1 was the causal agent of the pomelo root rot observed in Thailand.

Morphological characteristics and identification of *Phytophthora* sp. KA1. Colonies of *Phytophthora* sp. KA1 grown on PDA showed aerial, arachnoid, and branched mycelia with hyphal swelling. Sporangia were produced abundantly when the pathogen was flooded with the distilled water, but no sporangia formed on the tested agar media. The isolate produced papillate, caducous sporangia with very short pedicels (mean length: 3.1 µm) (Fig. 1). The sporangial shape was predominantly subspherical and turbinate, with an average length-to-breadth ratio of 1.3 : 1 (Table 1). Chlamydospores formed abundantly in a globose shape when the method of Tsao [13] was applied; however, chlamydospores did not form on the agar media. No sexual organs were observed in single cultures of this isolate.

Nucleotide sequences of the ITS ribosomal DNA fragments of *Phytophthora* sp. KA1 were determined and deposited in

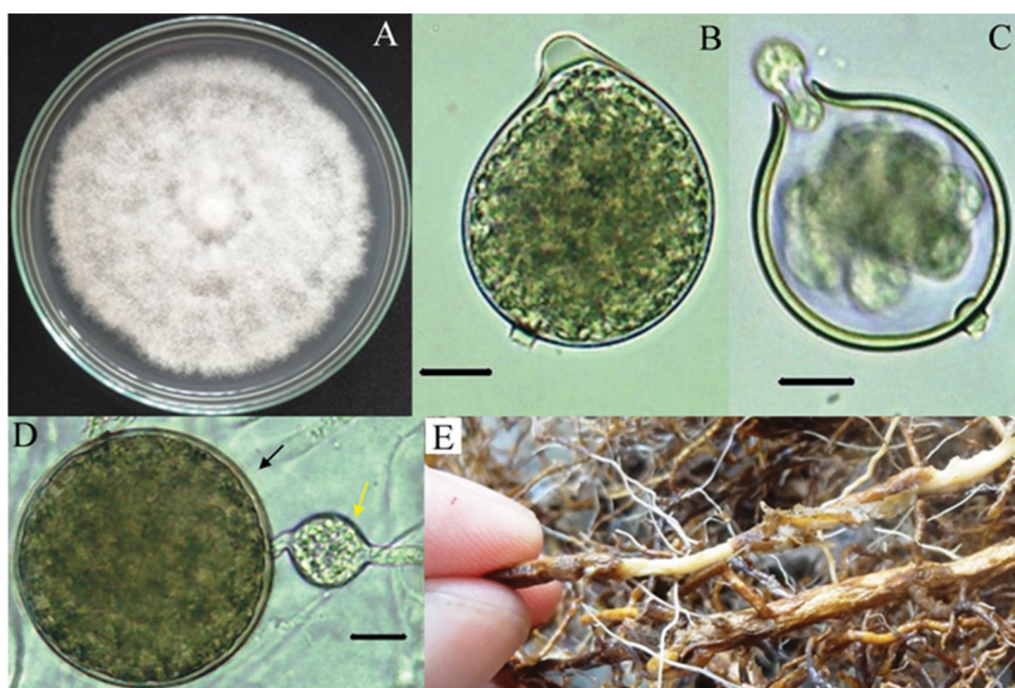


Fig. 1. Morphological characteristics of *Phytophthora nicotianae* KA1. A, Seven-day-old culture on potato dextrose agar; B, C, Caducous sporangium with a short pedicel; D, Chlamydospore (black arrow) and hypha swelling (yellow arrow) (scale bars: B~D = 10 μ m); E, Root rot symptoms in a pomelo plant near Bangkok, Thailand.

Table 1. Characteristics of *Phytophthora nicotianae* KA1 from pomelo

Structure	Mean of size
Sporangia ^a	
Length (μ m)	49 \pm 8.6 ^b
Breadth (μ m)	37 \pm 4.7
Length/Breadth ratio	1.3 \pm 0.2
Papilla length (μ m)	4.8 \pm 1.0
Pedicle ^c length (μ m)	3.1 \pm 1.0
Chlamydospore ^d diameter (μ m)	37 \pm 4.6

^aData collected from 150 separate sporangia.

^bMean \pm standard deviation.

^cData collected from 85 separate detached sporangia.

^dData collected from 85 separate chlamydospores.

the GenBank under accession No. KT175508. The DNA sequences of *Phytophthora* sp. KA1 were used as queries to search GenBank using the BLAST function. This analysis demonstrated that the nucleotide sequences of *Phytophthora* sp. KA1 shared 100% identity with those of *P. nicotianae* (accession Nos. GU111681 and GU111670 from *Citrus* spp. in Taiwan; JF792541 and JF792530 from citrus soils in India; and many other isolates existing in the GenBank database). Phylogenetic analysis confirmed the relationships between *Phytophthora* sp. KA1 and the related taxa (Fig. 2).

Antagonism of *Chaetomium* species to *P. nicotianae* KA1. The 3 potent strains CG05, CL01, and CC3003 of *Chaetomium* spp. were tested *in vitro* for antagonism to

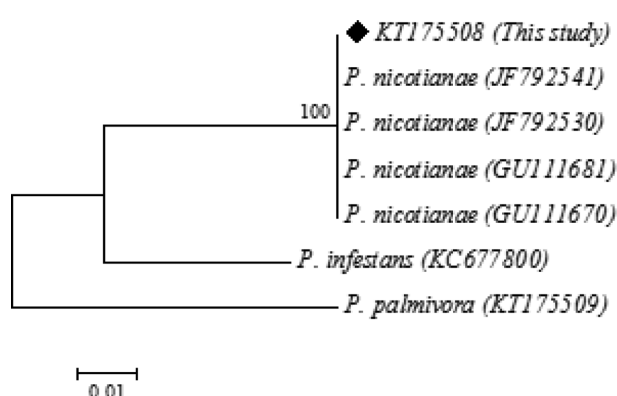


Fig. 2. Phylogenetic tree showing relationship between *Phytophthora nicotianae* KA1 and related taxa base on the internal transcribed spacer ribosomal DNA sequences, using the neighbor-joining method with 5,000 bootstrap replicates. *P. infestans* (KC677800) (the species placed in the same clade 1 with *P. nicotianae*) was isolated from a potato in India, and *P. palmivora* (KT175509) was isolated from a pomelo in Thailand [9].

P. nicotianae KA1 using bi-culture techniques. Ten days after inoculation with these fungi in bi-culture plates, *P. nicotianae* KA1 growth was inhibited to 50.6%, 52.5%, and 59.7% of that of the control, respectively, with further reductions to 49.7%, 51.7%, and 56.4% of that of the control after 30 days. The growth of *P. nicotianae* KA1 was prevented by substances that were released into the agar medium by CC3003 colonies, resulting in a clear zone of

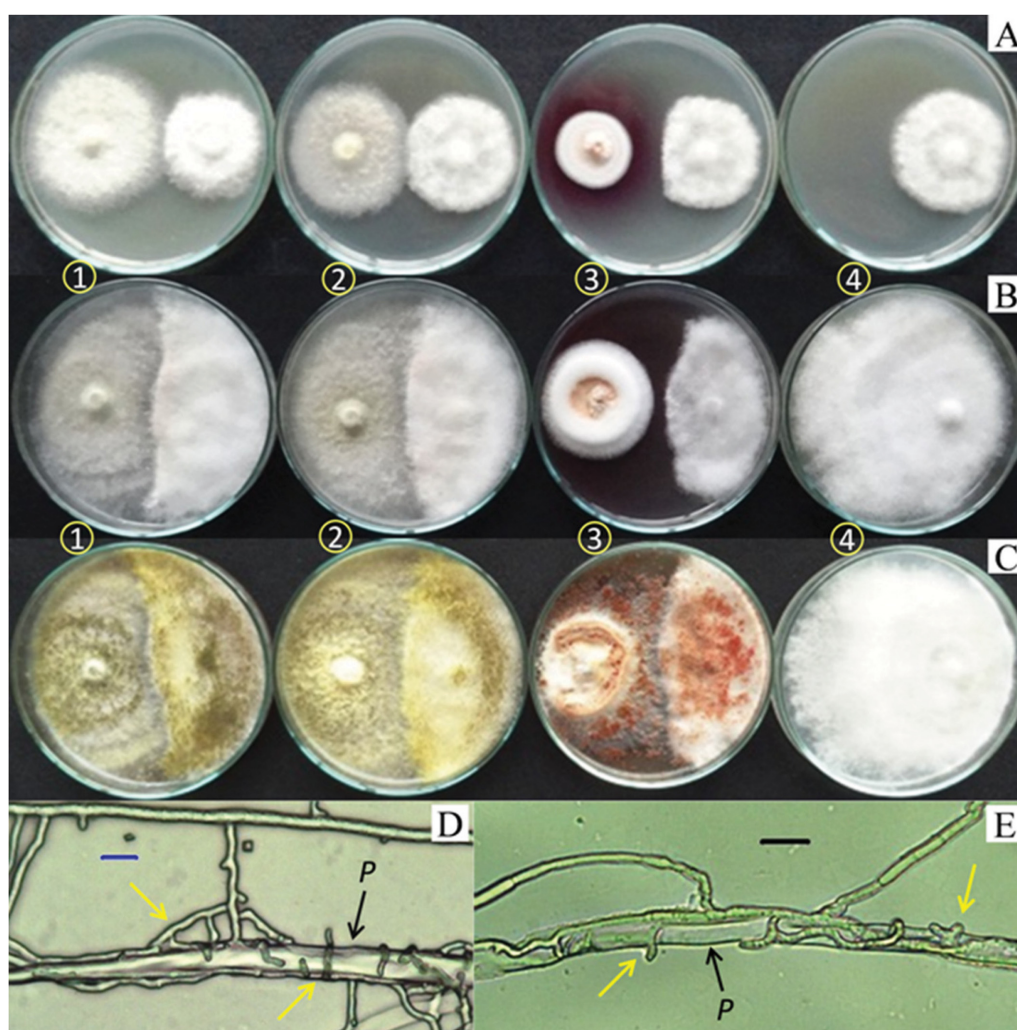


Fig. 3. Growth of *Phytophthora nicotianae* KA1 in bi-culture tests at 4 (A), 10 (B), and 30 days (C) after inoculation (1, 2, and 3: *P. nicotianae* KA1 [placed on the right side of the plates] inoculated with CG05, CL01, and CC3003, respectively; 4: *P. nicotianae* KA1 alone); D, E, *Chaetomium* hyphae (yellow arrows) coiled around and grew inside hypha of *P. nicotianae* KA1 (P) (scale bars: D, E = 10 µm).

inhibition between the antagonist and pathogen before contact was made. In contrast, CG05 and CL01 colonies made contact with pathogen colonies soon after inoculation without the clear zone of inhibition (Fig. 3A and 3B).

At 30 days after inoculation, all of the tested antagonists grew over colonies of *P. nicotianae* KA1 in bi-culture plates (Fig. 3C). Hyphae of the antagonists were shown to penetrate or coil around hyphae of *P. nicotianae* KA1 (Fig. 3D and 3E), resulting in the degradation and discoloration of pathogenic colonies (from white to light yellowish-brown). According to DMRT, at both points of measurement (10 and 30 days), CC3003 caused significantly higher growth inhibition of *P. nicotianae* KA1 than CG05 and CL01 ($p = 0.05$).

Effects of antagonistic crude extracts on the growth of *P. nicotianae* KA1. Nine antagonistic crude extracts were tested at different concentrations for growth inhibition

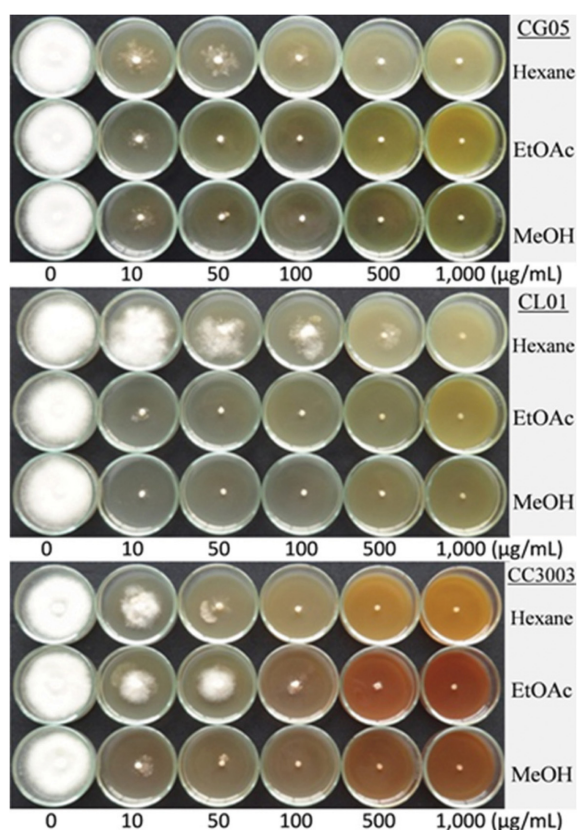
of *P. nicotianae* KA1. As shown in Table 2 and Fig. 4, mycelial growth of the tested pathogen was highly sensitive to the crude MeOH extracts of all 3 antagonists and to the crude EtOAc extracts of CG05 and CL01, with very low ED_{50} values (2.6–4.6 µg/mL). In particular, in the presence of these crude extracts at a concentration of 100 µg/mL, the growth of *P. nicotianae* KA1 was inhibited completely (the colony diameter did not increase significantly). In contrast, the mycelial growth of *P. nicotianae* KA1 was moderately sensitive to other tested crude extracts (ED_{50} values ranging from 20.8 to 101.4 µg/mL).

According to DMRT, the effects of crude MeOH and EtOAc extracts of CG05 on mycelial growth of *P. nicotianae* KA1 were not significantly different at all tested concentrations. However, when the tested concentrations were from 10 to 500 µg/mL, greater inhibition of mycelial growth was observed with these extracts than with the crude hexane extract. Similarly, whereas the crude MeOH

Table 2. Inhibition of *Phytophthora nicotianae* KA1 growth at different concentrations of antagonistic crude extracts

Crude extract		Inhibition of <i>P. nicotianae</i> KA1 growth (%)					Effective dose (ED ₅₀ , µg/mL)
		Concentration (µg/mL)					
		10	50	100	500	1,000	
CG05	Hexane	21 ± 2.9 f ^a	36 ± 3.6 e	66 ± 2.6 d	91 ± 2.3 b	97 ± 2.8 a	67.2
	EtOAc	71 ± 3.1 c	93 ± 2.4 b	98 ± 2.1 a	100 ± 0 a	100 ± 0 a	4.6
	MeOH	71 ± 2.1 c	93 ± 2.3 b	98 ± 2.0 a	100 ± 0 a	100 ± 0 a	4.6
CL01	Hexane	6 ± 2.3 g	15 ± 4.3 f	38 ± 4.0 e	78 ± 2.6 d	94 ± 2.0 b	101.4
	EtOAc	81 ± 3.8 cd	94 ± 2.1 b	99 ± 1.7 a	100 ± 0 a	100 ± 0 a	2.9
	MeOH	84 ± 2.6 c	95 ± 2.3 b	99 ± 1.0 a	100 ± 0 a	100 ± 0 a	2.6
CC3003	Hexane	34 ± 2.1 e	67 ± 2.1 d	86 ± 2.1 c	99 ± 1.1 a	100 ± 0 a	20.8
	EtOAc	26 ± 2.1 f	41 ± 2.1 e	70 ± 2.9 d	95 ± 2.3 b	100 ± 0 a	41.2
	MeOH	69 ± 4.1 d	91 ± 2.8 c	99 ± 1.6 a	100 ± 0 a	100 ± 0 a	4.5

^aMean standard deviation of 4 replicates. For crude extracts of each antagonist, values indicated by the same letter are not significantly different according to Duncan's multiple range test at $p = 0.05$.

**Fig. 4.** Growth of *Phytophthora nicotianae* KA1 in the presence of different concentrations of antagonistic crude extracts.

and EtOAc extracts of CL01 did not cause significantly different inhibition at all tested concentrations, the growth inhibition caused by both of these extracts was higher than that caused by the crude hexane extract. In contrast, among the CC3003 extracts, the crude EtOAc extract of CC3003 had the lowest inhibitory effect on mycelial growth of *P. nicotianae* KA1 when the tested concentrations were from 10 to 500 µg/mL.

Because the MeOH extracts exhibited strong inhibitory

Table 3. Effects of biological control agents on root rot and plant weight of pomelo seedlings inoculated with *Phytophthora nicotianae* KA1

Treatment	Root rot (%)	Dry plant weight (mg)
Non-inoculated	0 ± 0 d ^a	998 ± 46 a
KA1 ^a (control)	49.8 ± 1.8 a	483 ± 34 d
Me ^b -CG05	15.6 ± 1.4 bc	840 ± 47 bc
Me-CL01	13.7 ± 1.1 c	893 ± 29 b
Me-CC3003	15.4 ± 1.7 bc	833 ± 41 bc
S ^c -CG05	14.7 ± 1.6 c	865 ± 37 bc
S-CL01	17.1 ± 1.2 b	828 ± 45 c
S-CC3003	14.2 ± 1.7 c	885 ± 37 bc

^aInoculated plants were treated with water.

^bInoculated plants were treated with crude MeOH extracts of antagonists.

^cPlants were inoculated with both chlamydospores of *P. nicotianae* KA1 and spores of antagonists.

^dMean standard deviation of 4 replicates. For each parameter, values indicated by the same letter are not significantly different according to Duncan's multiple range test at $p = 0.05$.

effects against the tested pathogen, we chose these extracts as BCAs to control the pathogen in greenhouse experiments.

Efficacy of BCAs from *Chaetomium* antagonists against *P. nicotianae* KA1 infection under greenhouse conditions.

Applications of spores and MeOH extracts of the antagonists showed highly significant effects on root rot rates and plant weights of pomelo seedlings inoculated with *P. nicotianae* KA1. As shown in the Table 3, in the absence of BCAs, inoculated seedlings (controls) exhibited a root rot rate of 49.8%. Following inoculation with *P. nicotianae* KA1, plant weight was reduced by 51.6% compared with that of non-inoculated seedlings (998 mg in non-inoculated seedlings versus 483 mg in inoculated seedlings). However, in the presence of BCAs, the plant weight of seedlings was reduced by only 11~17% compared with that in non-inoculated plants and increased by 72~85% compared with the inoculated controls. Plants treated with BCAs

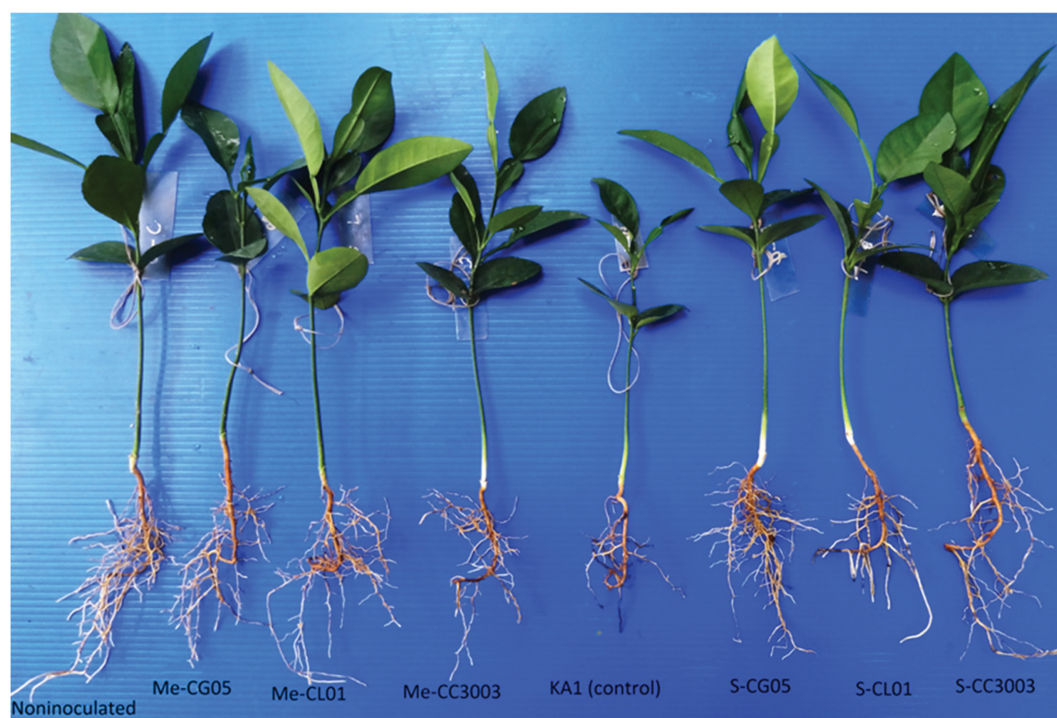


Fig. 5. Pomelo plants at 6 weeks after application of different biological control agents.

exhibited root rot rates of 13.7~17%, which was 66~71% lower than that observed in inoculated controls. Non-inoculated plants did not exhibit root tip rot (Fig. 5). The pathogen *P. nicotianae* KA1 was detected in infected roots in all treatments except for non-inoculated controls.

Among the 6 treatments involving application of BCAs, little difference in root rot percentage and plant weight was observed according to DMRT. For BCAs from CG05 and CC3003, no significant difference in root rot percentage and plant weight was observed when comparing crude MeOH extracts and spores. However, for CL01, seedlings treated with crude MeOH extracts had slightly higher plant weight and lower root rot percentage than seedlings treated with spores.

DISCUSSION

In this study, we identified a pathogenic oomycetous isolate obtained from rotted roots of pomelo as *P. nicotianae*. The high virulence of this pathogen in pomelo, as shown in this study, supported the conclusion that “*P. nicotianae* is the main causal agent of root rot in all types of citrus worldwide” [1, 2]. We also clearly demonstrated that the tested *Chaetomium* species and their crude extracts strongly inhibited the growth of *P. nicotianae* KA1 *in vitro* and caused a dramatic reduction in the rate of root rot in plants infected with *P. nicotianae* KA1 under greenhouse conditions. Thus, our data provide important insights into the use of these BCAs in the control of *P. nicotianae* root rot of citrus plants.

All morphological characteristics of *P. nicotianae* KA1 were similar to those of *P. nicotianae* Breda de Haan, which has been described in detail in previous studies. According to extensive reviews by Erwin and Ribeiro [1], *P. nicotianae* is a non-caducous sporangia species. However, Cacciola *et al.* [15] found that isolates identified as *P. nicotianae* obtained from affected *Forsythia* plants had caducous sporangia with a very short pedicels (less than 5 μm). From lavender (*Lavandula angustifolia* Mill.), Álvarez *et al.* [16] also obtained 5 isolates identified as *P. nicotianae* that had caducous sporangia with short pedicels (2.1~3.8 μm). Their descriptions are consistent with our observations for *P. nicotianae* KA1 isolated from pomelo. Despite the unusual characteristics of the sporangia, the ITS sequences of this isolate were identical to those of many other isolates of *P. nicotianae* found in GenBank.

The inhibition of *P. nicotianae* KA1 growth at both 10 and 30 days in the bi-culture test demonstrated that the BCAs applied in this study could maintain inhibition of the pathogen over time. The coiling, penetration, and degradation of *P. nicotianae* KA1 mycelia in response to treatment with all tested *Chaetomium* species are typical of mycoparasitism. Similarly, all of the *Chaetomium* species used in this study have been reported to parasitize hyphae and to grow over and degrade *P. palmivora* colonies [9]. Hyphae of *P. cinnamomi* and *P. nicotianae* can be parasitized and lysed by *C. globosum* [17]. However, other studies also indicated antibiosis as the mechanism of the action of *C. globosum* and *C. cupreum* against *Colletotrichum gloeosporioides*, *Pyricularia oryzae*, *Rhizoctonia solani*, and

Curvularia lunata [8] and of *C. lucknowense* against *Fusarium* spp. [18, 19]. In these studies, parasitism was not detected. Interestingly, parasitism of *Chaetomium* spp. has only been reported on species within genus *Phytophthora*. The interactions between *C. cupreum* CC3003 and *P. nicotianae* KA1 found in this study showed characteristics typical of both of these mechanisms.

Mycoparasitism relies on various lytic enzymes for degradation of the cell wall of the host, causing “death of the target organism”, which “results in a decrease in inoculum density”, whereas antibiosis is a type of “antagonism resulting from the production of secondary metabolites toxic to other microorganisms” [7, 20]. Indeed, *Chaetomium* species are known to produce lytic enzymes and many other secondary metabolites, which may be involved in their antagonistic activity [6]. Degrading enzymes such as chitinase and beta-1,3-glucanase often are secreted by *Chaetomium* spp. in culture substrates and under mycoparasitism conditions [7, 21]. Additionally, various other compounds with antifungal activity against plant pathogens, such as chaetoviridins A and B [22] and chaetoglobosins A and C [23] have been isolated from *C. globosum*. The *C. lucknowense* CL01 has also known to produce chaetoglobosin C, which exhibited antifungal activity against both mycelial growth and formation of conidia in *Fusarium oxysporum* f. sp. *lycopersici* that is the causal agent of tomato wilt [19]. From crude EtOAc and MeOH extracts of *C. cupreum* CC3003, Kanokmedhakul *et al.* [12] isolated 3 compounds (rotiorinols A and C and rubrorotiorin), having strong antifungal activities against mycelial growth of fungus *Candida albicans* [12]. These findings suggested that multiple metabolites may contribute to the antifungal activity of a single crude extract. The growth inhibition of *P. nicotianae* KA1 observed in this study may have resulted from the combined antifungal activities of various metabolites within the crude extracts.

It is particularly notable that the mycelial growth of *P. nicotianae* KA1 in this study responded more sensitively to the crude extracts of tested antagonists (ED_{50} values of 2.6~101.4 µg/mL) than the mycelial growth of *P. palmivora* did (ED_{50} values of 26.5~2,495 µg/mL), which was reported in our previous study [9].

Our results above indicate that *Chaetomium* species and their crude extracts could provide sufficient control of *P. nicotianae* root rot in the pomelo, under greenhouse conditions. The introduction of *Pythium nunn* [24] and *Penicillium funiculosum* [25] into soil for controlling *Phytophthora* root rot in citrus has been successful under greenhouse conditions in prior studies. Moreover, a bio-product named Ketomium® (Strong Crop Co. Ltd., Samatprakaru Province, Thailand), developed from 22 strains of *C. globosum* and *C. cupreum* has showed ability to control *Phytophthora* sp. causing citrus root rot in the field [8]. In our study, in plants inoculated with *P. nicotianae* and treated with the BCAs, the plant weight was less than that of the non-inoculated seedlings. Thus, although these antagonists and their MeOH extracts did provide protection

against pathogen infection, they did not promote the growth of pomelo seedlings. Similarly, Fang and Tsao [24] found that *Pythium nunn* reduced significantly the root rot rate caused by *P. parasitica* in citrus, but did not favor plant growth. The pathogen was still detected after the BCAs were applied, suggesting that the antagonists and their crude MeOH extracts could not remove *P. nicotianae* KA1 from the soil, despite their strong inhibition of pathogen growth *in vitro*. Thus, these crude extracts are fungistatic rather than fungicidal. The ability of the tested crude extracts to control different plant pathogens *in vitro* has been well documented, but has never been reported under *in vivo* conditions.

Approximately 300 g of dry biomass of *Chaetomium* species will yield approximately 20 g of crude MeOH [12]. In contrast, a single PDA plate harboring 4-wk-old *C. globosum* yields hundreds of millions of spores [26]. Thus, mixing of spores from these antagonists with potting media to control root rot in pomelo plants caused by *P. nicotianae* may provide a simple and economical approach, and may be easier to use in large-scale applications than crude extracts. The effectiveness of the tested *Chaetomium* strains and their crude extracts in the control of *P. nicotianae* in this study provides a convincing reason to promote the application of these strains to control *Phytophthora* root rot in citrus plants.

ACKNOWLEDGEMENTS

This work is supported by King Mongkut's Institute of Technology Ladkrabang Research Fund (Grant number: KREF125602).

REFERENCES

1. Erwin DC, Ribeiro OK. *Phytophthora* diseases worldwide. St. Paul (MN): APS Press; 1996.
2. Timmer LW, Graham JH, Zitko SE. Metalaxyl-resistant isolates of *Phytophthora nicotianae*: occurrence, sensitivity, and competitive parasitic ability on citrus. Plant Dis 1998;82:254-61.
3. Food and Agriculture Organization of the United Nations, Statistics Division. Browse data, production crops [Internet]. Rome: Economic and Social Development Department; 2015 [cited 2015 Feb 15]. Available from: <http://faostat3.fao.org/browse/Q/QC/E>.
4. Drenth A, Sendall B. Economic impact of *Phytophthora* diseases in Southeast Asia. In: Drenth A, Guest DI, editors. Diversity and management of *Phytophthora* in Southeast Asia. ACIAR Monograph No. 114. Canberra: Australian Centre for International Agriculture; 2004. p. 10-28.
5. Latijnhouwers M, De Wit PJ, Govers F. Oomycetes and fungi: similar weaponry to attack plants. Trends Microbiol 2003;11: 462-9.
6. Zhang Q, Li HQ, Zong SC, Gao JM, Zhang AL. Chemical and bioactive diversities of the genus *Chaetomium* secondary metabolites. Mini Rev Med Chem 2012;12:127-48.

7. Sun H, Yang J, Lin C, Huang X, Xing R, Zhang KQ. Purification and properties of a beta-1,3-glucanase from *Chaetomium* sp. that is involved in mycoparasitism. *Biotechnol Lett* 2006;28:131-5.
8. Soyong K, Kanokmedhakul S, Kukongviriyapa V, Isobe M. Application of *Chaetomium* species (Ketomium®) as a new broad spectrum biological fungicide for plant disease control: a review article. *Fungal Divers* 2001;7:1-15.
9. Hung PM, Wattanachai P, Kasem S, Poaim S. Biological control of *Phytophthora palmivora* causing root rot of pomelo using *Chaetomium* spp. *Mycobiology* 2015;43:63-70.
10. Cooke DE, Drenth A, Duncan JM, Wagels G, Brasier CM. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genet Biol* 2000;30:17-32.
11. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;28:2731-9.
12. Kanokmedhakul S, Kanokmedhakul K, Nasomjai P, Louangysouphanh S, Soyong K, Isobe M, Kongsaree P, Prabpai S, Suksamrarn A. Antifungal azaphilones from the fungus *Chaetomium cupreum* CC3003. *J Nat Prod* 2006;69: 891-5.
13. Tsao PH. Chlamydospore formation in sporangium-free liquid cultures of *Phytophthora parasitica*. *Phytopathology* 1971;61: 1412-3.
14. Timmer LW, Sandler HA, Graham JH, Zitko SE. Sampling citrus orchards in Florida to estimate populations of *Phytophthora parasitica*. *Phytopathology* 1988;78:940-4.
15. Cacciola SO, Belisario A, Pane A, Magnano di San Lio G. Forsythia: a new host of *Phytophthora nicotianae* in Italy. *Plant Dis* 1994;78:525-8.
16. Álvarez LA, Pérez-Sierra A, Armengol J, García-Jiménez J. Characterization of *Phytophthora nicotianae* isolates causing collar and root rot of lavender and rosemary in Spain. *J Plant Pathol* 2007;89:261-4.
17. Heller WE, Theiler-Hedtrich R. Antagonism of *Chaetomium globosum*, *Gliocladium virens* and *Trichoderma viride* to four soil-borne *Phytophthora* species. *J Phytopathol* 1994;141:390-4.
18. Charoenporn C, Kanokmedhakul S, Lin FC, Poaim S, Soyong K. Evaluation of bio-agent formulations to control *Fusarium* wilt of tomato. *Afr J Biotechnol* 2010;9:5836-44.
19. Sibounnavong P, Charoenporn C, Kanokmedhakul S, Soyong K. Antifungal metabolites from antagonistic fungi used to control tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici*. *Afr J Biotechnol* 2011;10:19714-22.
20. Alabouvette C, Olivain C, Steinberg C. Biological control of plant diseases: the European situation. *Eur J Plant Pathol* 2006;114:329-41.
21. Liu ZH, Yang Q, Hu S, Zhang JD, Ma J. Cloning and characterization of a novel chitinase gene (*chi46*) from *Chaetomium globosum* and identification of its biological activity. *Appl Microbiol Biotechnol* 2008;80:241-52.
22. Park JH, Choi GJ, Jang KS, Lim HK, Kim HT, Cho KY, Kim JC. Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. *FEMS Microbiol Lett* 2005;252:309-13.
23. Zhang G, Wang F, Qin J, Wang D, Zhang J, Zhang Y, Zhang S, Pan H. Efficacy assessment of antifungal metabolites from *Chaetomium globosum* No.05, a new biocontrol agent, against *Setosphaeria turcica*. *Biol Control* 2013;64:90-8.
24. Fang JG, Tsao PH. Evaluation of *Pythium nunn* as a potential biocontrol agent against *Phytophthora* root rots of azalea and sweet orange. *Phytopathology* 1995;85:29-36.
25. Fang JG, Tsao PH. Efficacy of *Penicillium funiculosum* as a biological control agent against *Phytophthora* root rots of azalea and citrus. *Phytopathology* 1995;85:871-8.
26. Fogle MR, Douglas DR, Jumper CA, Straus DC. Growth and mycotoxin production by *Chaetomium globosum*. *Mycopathologia* 2007;164:49-56.