



Efficacy of *Arcopilus cupreus* as biological agent to control *Phytophthora* spp. causing root rot of mandarin citrus

Noireung P¹, To-anun C¹ and Nguanhom J^{2*}

¹Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

²Department of Agricultural Technology and Development, Faculty of Agricultural Technology, Chiang Mai Rajabhat University, Chiang Mai 50300, Thailand

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Abstract

Citrus root rot causes very high impact on quality and quantity of mandarin cultivation. *Phytophthora* spp. is root rot causing pathogens. The *Phytophthora* spp. were isolated from soil and diseased roots of mandarin plants in Chiang Mai province (northern Thailand) and confirmed pathogenicity using detached leaf technique. *Arcopilis cupreus* species with antifungal activity is an effective strategy for biological control of fungal pathogens. *Arcopilis cupreus* BN21 was isolated from peanut pods from agricultural waste materials and the isolate was studied *in vitro*, against the isolated root rot pathogen *Phytophthora* spp. On dual culture, clear zone of *Arcopilus cupreus* BN21 against *Phytophthora* spp. showed antagonistic activity against pathogens. Moreover, the mycelia of BN21 attached to the pathogen hyphae and caused the cell walls degradation of pathogen. Fractional ethanol extract also showed the growth inhibition of the pathogen as well. Adding *A. cupreus* BN21 suspension into the soil showed the reduction of the root infection form *Phytophthora* sp.

Key words – antagonistic effect – Biological control – *Chaetomium* – Citrus root rot – Rhizosphere pathogens

Introduction

Mandarin (*Citrus reticulata* L. cv. Sainampung) has been cultivated as an important economic fruit crop in Northern Thailand for more than two decades (Shutsrirung et al. 2013).

Phytophthora spp. cause the most serious and economically important soil borne diseases of Citrus crops. This fungus-like organism infects and causes the root rot in many different citrus types worldwide (Erwin & Ribeiro 1996, Timmer et al. 1998). With the prevalence of high humid conditions in Thailand, infection with the *Phytophthora* has become a major problem for the citrus industry, causing yield losses of approximately 6~12% (Drenth & Sendall 2004). In addition, the resistance of *Phytophthora* species to an important group of fungicides such as phenylamides (metalaxyl and related compounds) has become a serious problem in their chemical control. In order to control *Phytophthora* diseases, screening of bio-control agents has become a vital research aspect (Erwin & Ribeiro 1996, Naqvi 2004).

Chaetomium spp. and related species such as *Amesia nigricolor* and *Botryotrichum murorum* (Wang et al. 2016) exist mainly in soil, indoor environments, animals, and as endophytes in several

plants with symbiotic relationship without causing any adverse effect to their hosts (Fatima et al. 2016). *Arcopilus cupreus* (L.M. Ames) X. Wei Wang and Samson (Syn. *Chaetomium cupreum*) has a potential of biocontrol agent against a range of plant pathogens by producing antifungal metabolites, mycoparasitism, competition for space and nutrients, or varying combinations of these (Zhang & Yang 2007). *Arcopilus cupreus* has been isolated from many habitats and substrates such as indoor environment (Wang et al. 2016), litter soil (Tirumale & Wani 2018), soybean seed (Yeh 1980) and endophyte in *Macleaya cordata* (Mao et al. 2010), *Ginkgo biloba* (Xiao et al. 2013) and *Mussaenda luteola* (Shylaja & Sathiavelu 2017). Recently, more than 200 active metabolites have been isolated from *Chaetomium* spp. and related species. Some of these metabolites involve in biological control activities. As being an effective biocontrol agent for several phytopathogens, *C. globosum* produces several types of bioactive compounds such as azaphilones (Yamada et al. 2011), orsellides, globosumones A–C (Bashyal et al. 2005), Xanthenone (Pontius et al. 2008), steroids, chaetoviridins A and C (Qin et al. 2009), cytoglobosins A–G (Cui et al. 2010), chaetoglobosins (Zhang et al. 2010), pyrones and chaetoglocins A and B (Ge et al. 2011). Accordingly, the use of beneficial microorganisms as biocontrol agents or biofertilizer instead agrochemicals in citrus orchards significantly reduces the cost of production. Further the usage of biocontrol agents or biofertilizers is one of the Thai government's policies to reduce the use of agrochemicals in every plant production (Sruamsiri 2008).

Materials and Methods

Isolation of microorganisms

Root symptoms and soil samples around rhizosphere under citrus plant canopy were collected and root rot pathogens were isolated using baiting method modified from Soyong & Quimio (1989). Soil samples were air-dried and ground to fine particles. For the baiting, 10 g of soil was placed in sterilized petri dishes and 20 ml of sterilized distilled water was added and cleaned citrus leaf disks (10 mm x 10 mm) were floated on suspension. Soil suspensions with baits were incubated at 27°C for 2–3 days. The presence of sporangia on baits were observed using light microscopy. The sporangia on the baits were transferred to the WA. After 1–2 days, hyphal tips were transferred to PDA, CMA and V8A to obtain pure culture. Pathogenicity test was carried out as described by Soyong et al. (2005) with some modifications. Two-month-old citrus leaves were used for the assessment of the pathogenicity. *Arcopilus cupreus* were isolated from peanut pod composited in soil. Ascospores were directly picked from peanut pod and single spore isolation technique (Goh 1999) was used for getting pure culture.

In vitro antagonistic test

In vitro antagonistic test was done on PDA in 9 cm diameter Petri dishes. In each Petri dish, a mycelial disc (5 mm diameter) of pathogen and a mycelial disc of antagonist were placed opposite to each other. For the control, the mycelial disc of the pathogen was placed on PDA in 9 cm diameter Petri dishes without the mycelial disc of antagonist. Petri dishes with mycelial disc were incubated at 25°C for 30 days. Colony diameter of the pathogen was measured. Further the number of sporangia produced by the pathogen was counted. Numbers of sporangia were counted using the hemacytometer. Data were calculated in the form of inhibition percentage of mycelial growth and sporangial production of the pathogen by using the formula below:

$$\text{PIRG} = \frac{(R1-R2)}{R1} \times 100$$

Where: R1= colony diameter or numbers of sporangia of pathogen in the control plates; R2 = colony diameter or numbers of sporangia of pathogen in bi-culture plates. Finally, the variance and the treatment means were analyzed and compared using Duncan's multiple range tests at 0.05.

***In vitro* test of crude extracts from BN21 to inhibit growth of pathogen**

Crude extracts of the BN21 were produced by method described in Kanokmedhakul et al. (2006). Each fungal antagonist was grown in potato dextrose broth at 25–35°C. After 45 days, mycelial biomass was collected, air-dried and ground. The ground mycelia were extracted sequentially over hexane, ethyl acetate (EtOAc), and Methanol (MeOH). The crude extracts were obtained by evaporating the extracted-solvents in a vacuum. These 3 crude extracts from BN21 were weighed, dissolved in 2% dimethyl sulfoxide and filtered through bacterial filter before adding into the PDA to obtain the desired concentrations (10, 50, 100, 500, and 1,000 µg/mL). A mycelial disc of *Phytophthora* spp. (5 mm diameter) were placed on the center of PDA plates containing the crude extracts in different concentration and incubated at temperature of 25–28°C. After incubating at room temperature (25–28°C) for 10 days, colony diameter and numbers of sporangia production of the pathogen were collected and then expressed as inhibition percentage using the same formula above. Effective dose ED50 value on mycelial growth and sporangial production were calculated by probit analysis using the software SPSS Statistics ver. 0.19. (IBM Co., Armonk, NY, USA)

***In vivo* test of antagonistic fungus against pathogen in greenhouse**

Pathogenicity was tested by artificial inoculation of *Phytophthora* spp. Ph71, Ph84 and Ph85 into roots of citrus. The six-month olds citrus cutting were replanted in 12 inches plastic pots with sterile soil. The experiment was separated into 3 groups including non-inoculated (control), inoculated *Phytophthora* spp. (Ph71, Ph84, Ph85) and inoculated *Phytophthora* spp. (Ph71, Ph84, Ph85) with *Arcopilus cupreus* BN21 (biocontrol). Inoculation of *Phytophthora* sp. Ph84 was done by pouring 100 mL of zoospore suspension of 10⁴ spore/mL to the sterile soil medium. Spore suspension of *A. cupreus* BN21 was prepared with 10⁶ spores/mL and 100 mL of antagonistic spore suspension was poured into the soil every month with 100 g of bio-compost.

Results

Isolation of citrus root rot pathogens

Phytophthora spp. including strain Ph71, Ph84 and Ph85 were isolated from soils and rotten mandarin roots. All isolates grown rapidly and colonized within 4 days on PDA, CMA and V8A. A chrysanthemum pattern of the fungal colony on PDA of Ph71 produced pear-shaped papillate sporangia (Fig. 1A). However, sporangia of Ph84 and Ph85 are globose and ovoid respectively and both are papillate (Fig. 1B, 1C). These three isolates showed strong pathogenicity on citrus leaves by showing the symptoms of brown rot within 2 days (Fig. 1D).

Antagonistic fungi isolation

The antagonistic *Arcopilus cupreus* BN21 was isolated from peanut pod and distinguished through its abundant production of a copper-colored metabolite that diffused in to the PDA medium. The fungus was identified by its morphological characteristics such as ascomata hairs, appendage, asci and ascospores (Fig. 2).

As shown in Table 1. The strain BN21 is with effective antagonistic activity, which expressed over 50 % inhibition against *Phytophthora* spp. The percentage of growth inhibition (PGI) are as follows: Ph71, Ph84 and Ph85 is 73.25%, 63.34% and 62.50% respectively. (Fig. 3) The observation of the interface of *Arcopilus cupreus* BN21 and *Phytophthora* spp. showed that mycelia of *Arcopilus cupreus* BN21. Developed into the mycelia of *Phytophthora* spp. Hence this is suggested to be the biological control mechanism of the citrus decline pathogen.

In the dual culture of strain BN21 and *Phytophthora* spp., inhibition zone was appeared at the third day (Fig. 3A). After that, the BN21 started to grow over the *Phytophthora* spp. and covered the *Phytophthora* spp. colony (Fig. 3A). BN21 started producing the fruiting bodies on 20 days (Fig. 3A). Mycelia of BN21 attached with *Phytophthora* spp. (Fig. 3B, 3C) and caused the mycelial degradation of *Phytophthora* spp. (Fig. 3D).

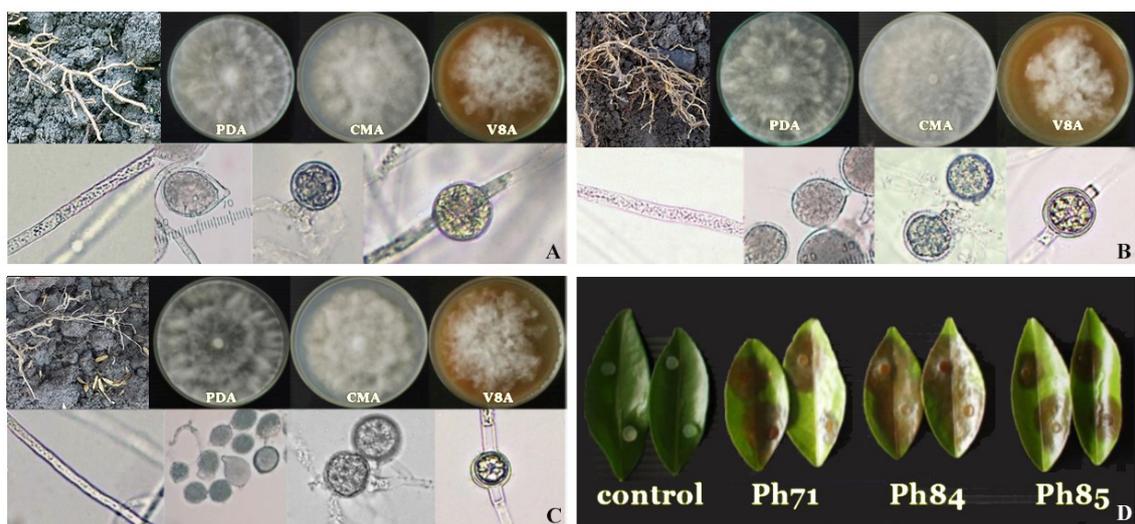


Fig. 1 – *Phytophthora* spp. characters isolated from root rot disease and pathogenicity test. A Isolate Ph71. B Isolate Ph84. C Isolate Ph85. D Disease symptom on inoculated leaf.

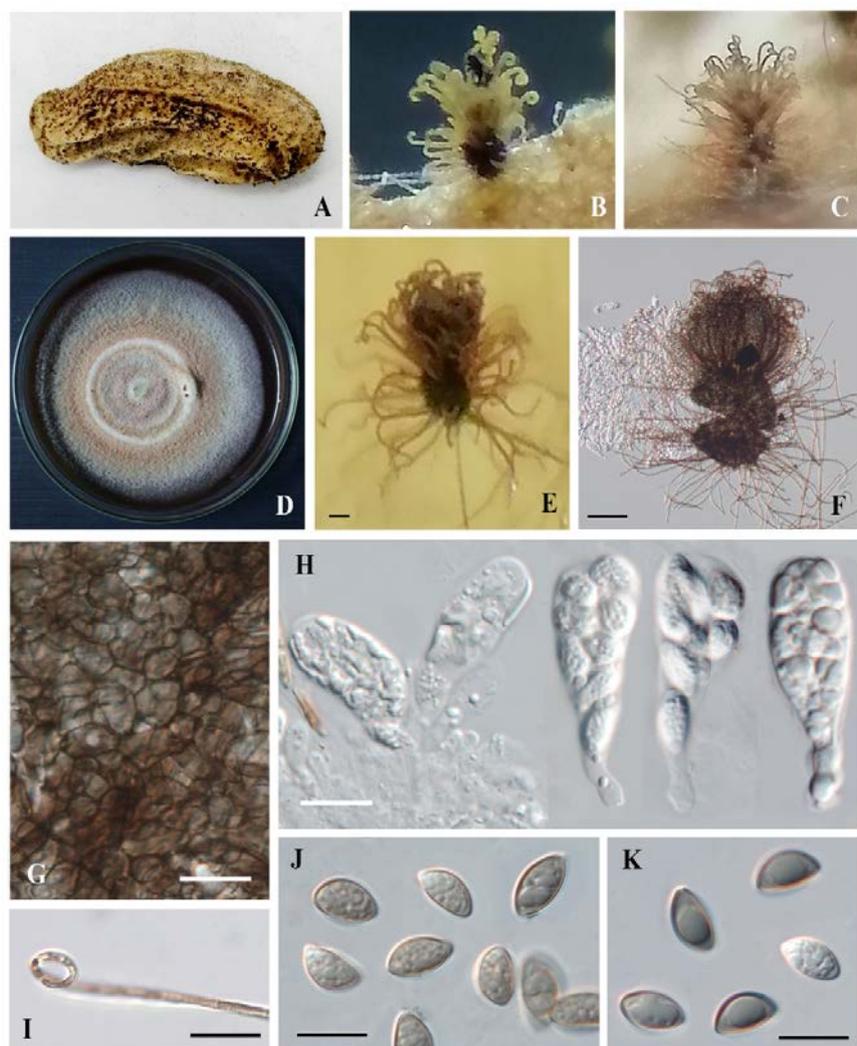


Fig. 2 – Morphological characteristics of *Arcopilus cupreus* BN21. A–C Ascomata on peanut pod. D Colony on PDA. E Ascoma on PDA. F Ascoma mounted in lactic acid. G Ascomatal wall. H Asci. I Ascomatal hair. J, K Ascospores. Scale bars: E–F = 50 µm, G–H, J–K = 10 µm, I = 20 µm.

Bi-culture test

Table 1 Percent inhibition of radial growth (%PIRG) in bi-culture antagonistic test against *Phytophthora* spp. isolate Ph71, Ph84 and Ph85.

<i>Arcopilus cupreus</i>	Percent inhibition of radial growth (%PIRG) ¹		
	Ph71	Ph84	Ph85
BN21	73.250 ^A	63.335 ^A	62.500 ^{AB}

¹Average from 4 repeatedly, means with the same uppercase letters in the same column are not significantly different ($p > 0.05$)

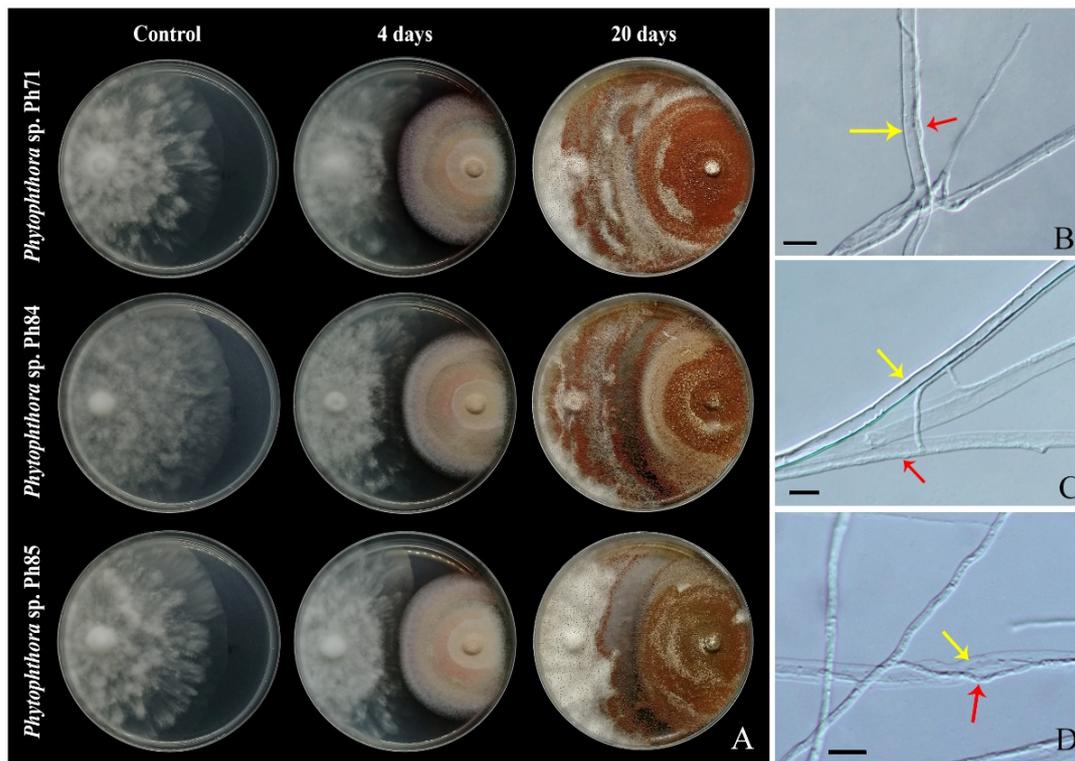


Fig. 3 – Antagonistic abilities of *Arcopilus cupreus* BN21 against *Phytophthora* spp. A Bi-culture test. B Mycelium of BN21 (red arrow) attached to mycelium of *Phytophthora* sp. Ph71 (yellow arrow). C Mycelium of BN21 (red arrow) attached to mycelium of *Phytophthora* sp. Ph84 (yellow arrow). D Mycelium of BN21 (red arrow) attached to degraded mycelium of *Phytophthora* spp. Ph85 (yellow arrow). Scale bars: B–D = 50 μ m.

Effects of crude extract on growth of *Phytophthora* spp.

The three fractional crude extracts in concentration series were tested for the growth inhibition of *Phytophthora* spp. Ph71, Ph84 and Ph85. As shown in Table 2, mycelial growth of the *Phytophthora* spp. was highly sensitive to the crude MeOH extracts of BN21. The MeOH extract was more effective than the EtAOc and hexane extract at concentrations from 100~1,000 μ g/mL effective against the growth of *Phytophthora* spp. Moreover, none of them showed significant colony inhibition of Ph71, Ph84 and Ph85 at 10 μ g/mL. The sporangial production of *Phytophthora* spp. was sensitive to all crude extracts (Fig. 4).

Greenhouse experiment

In this study, biocontrol products were compared to investigate their effectiveness on controlling citrus root rot disease under the greenhouse conditions. This experiment was used both chemical fertilizer and bio-compost to improve the soil condition and at the same time, to reduce disease incidence by applying both chemical fungicide and biological antagonist (Fig. 5).



Fig. 4 – Efficiency of each fractional extract ability of *Chaetomium* spp. to control *Phytophthora* spp.



Fig. 5 – Development of citrus after 18 months after inoculated. A citrus seedling and root of citrus seedling (control), B citrus seedling and root of citrus seedling inoculated *Phytophthora* spp. (Ph71, Ph84, Ph85), C citrus seedling and root of citrus seedling inoculated *Phytophthora* spp. (Ph71, Ph84, Ph85) with *Arcopilus cupreus* BN21 (biocontrol).

Table 2 Effectiveness of fractional crude extract of *Arcopilus cupreus* BN21 on mycelial growth and sporangial production of *Phytophthora* spp. caused of root rot of citrus.

Crude extracts	<i>Phytophthora</i> sp. Ph71		<i>Phytophthora</i> sp. Ph84		<i>Phytophthora</i> sp. Ph85	
	ED50 (µg/mL)		ED50 (µg/mL)		ED50 (µg/mL)	
	Colony inhibition	Spore inhibition	Colony inhibition	Spore inhibition	Colony inhibition	Spore inhibition
MeOH	99.588	86.110	2.1614	24.425	15.167	79.57
EtOAc	4.2582	99.279	8.1691	59.600	60.680	251.68
Hexane	9.1208	40.861	5.1825	8.1062	N/A	916.83

MeOH, methanol extract; EtOAc, ethyl acetate extract; Hexane, hexane extract.

Discussion

Arcopilus cupreus has been isolated from soil since 1949 and has been reported as cellulose degrading fungi (Ames 1949). *Arcopilus cupreus* was first isolated from soybean seeds and the culture extracts were tested against the inhibition of seed-borne fungi on soybean (Yeh 1980). In bioassays, inhibition zones developed between *A. cupreus* strain BN21 and *Phytophthora* spp. suggesting that BN21 produced a compound which is toxic to the mycelial growth and sporangial production of root rot pathogens. This was further supported by the colony inhibition of the pathogen on the media mixed with crude extracts of the antagonist. Inoculation of *Phytophthora* spp. causes the destruction of citrus roots. However, both biocontrol and chemical control reduced the symptoms and produced healthy leaves and roots. Hence, biocontrol and chemical control are able to reduce root rot disease of mandarin citrus in greenhouse conditions.

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