

Selection of entomopathogenic fungus for biological control of chili anthracnose disease caused by *Colletotrichum* spp.

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Abstract The literature on the entomopathogenic fungi in the genus *Cordyceps* describing its use in the agricultural area as a biocontrol agent is limited. In this study, a total of 47 isolates of entomopathogenic fungi were isolated from dead cicada nymphs obtained from various locations in the northeast of Thailand. These isolates were primarily screened for antagonistic activity to inhibit the mycelial growth of one isolate of *Colletotrichum gloeosporioides* and one isolate of *C. capsici*. The screen revealed that five isolates of entomopathogenic fungi showed good inhibitory effects on the fungal mycelial growth and were chosen for further confirmation of their antagonistic effects against five isolates of *C. gloeosporioides* and five isolates of *C. capsici* by the dual culture method. After investigation, the isolate Cod-NB1302 had the best inhibitory effect.

Moreover, the mycelium extract and culture filtrate of isolate Cod-NB1302 also had inhibitory effects on the mycelial growth and conidial germination of all isolates of plant pathogenic *Colletotrichum* spp. under in vitro conditions. Interestingly, the mycelium extract and culture filtrate effectively reduced the size of the disease lesion and disease severity on chili fruits after inoculation with the plant pathogenic fungi. However, the mycelium extract exhibited greater antifungal activity than the culture filtrate. Finally, the isolate Cod-NB1302 was identified as *Ophiocordyceps sobolifera* based on the sequence of three ribosomal nuclear DNA genes and two protein-coding genes. These findings suggest that the isolate Cod-NB1302 is a potential candidate, with antagonistic activity, for use as a source of antifungal agents to control anthracnose disease caused by the plant pathogenic *Colletotrichum* spp.

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Introduction

Chili (*Capsicum annuum* L.) is an important crop in tropical and subtropical areas (Ratanacherdchai et al. 2007). During the process of production, chili is attacked by many plant diseases and caused there yield lose (Pakdeevaporn et al. 2005; Poonpolgul and Kumphai 2007; Than et al. 2008). One major disease in chili production areas is anthracnose (Shin et al. 2000; Sharma et al. 2005). In Thailand, anthracnose of chili is

caused by the fungi *Colletotrichum gloeosporioides* (Penz.) Sacc. and *C. capsici* (H.Syd.) E. Butl. & Bisby, which are the most serious fungal pathogens (Sangchote et al. 1998; Oanh et al. 2004). These fungal pathogens can produce disease symptoms on leaves, stems and both young and mature fruits (Hong and Hwang 1998; Kim et al. 1999), and it reduces the chili yield from 10 % to 80 % in some developing countries (Poonpolgul and Kumphai 2007).

The method to protect against these fungal pathogens is usually done through the integration of cultural management practices and chemical treatments (Asthana et al. 1989), but these ways possess a risk to human health and the environment (Montri et al. 2009). Therefore, an alternative and effective method to control the anthracnose pathogen is the use of biological control agents (Harman et al. 2004), as alternative methods that could potentially be less harmful to human health and the environment as chemical pesticides. For example, *Trichoderma* spp. are ubiquitous in the soil and has been known for many years as potential biological control agents (Bae and Knudsen 2005). *T. harzianum* (Th-F, Th-G, Th-I and Th-N) has been applied to control *C. capsici* in pepper (Ekefan et al. 2009). Interestingly, some entomopathogenic fungi, such as *Cordyceps sobolifera*, have been reported for use as a biocontrol agent against *C. gloeosporioides* and *C. miyabeanus* (Imtiaj and Lee 2007).

Cordyceps species are an entomopathogenic macrofungi that are well known for their use in traditional Chinese medicine (Cha et al. 2006; Wang et al. 2011). The various species in this genus produce many bioactive compounds that have only been used in medical and pharmacological areas. For example, Imtiaj and Lee (2007) found that a bioactive compound from *C. sobolifera* could inhibit the growth of the human pathogenic bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* under in vitro conditions. Wong et al. (2011) reported the anti-microbial properties of the peptide ‘cordymin’ isolated from *C. militaris* that could inhibit the mycelial growth of *Candida albicans*. Meanwhile, the literature describing agricultural uses of the biocontrol agents is limited. However, previous reports indicated that *Cordyceps* species are a potential candidate with broad antagonistic activity that could be used as a biocontrol agent. Therefore, the present study aimed to isolate the entomopathogenic fungi from dead cicada nymphs in the northeast of Thailand and to investigate their

potential to inhibit the growth of the plant pathogenic fungi *Colletotrichum* spp., which is the causal agent of chili anthracnose disease under in vitro and in vivo conditions. The potential isolate was identified by its particular genome using the PCR sequencing technique.

Materials and methods

Isolation of entomopathogenic fungus

The entomopathogenic fungus was isolated from dead cicada nymphs collected from the mixed deciduous forest in the northeast of Thailand using the tissue transplanting technique as described by Sangdee and Sangdee (2013). The mycelium growing out from the cicada larvae tissue was sub-cultured on potato dextrose agar (PDA) and incubated at 28 °C for further study.

Isolation of *Colletotrichum* spp. causal agent of chili anthracnose disease

The plant pathogenic fungi *Colletotrichum* spp. were isolated from infected chili with typical disease symptoms using the tissue transplantation technique as described by Sangdee et al. (2011). The mycelium growing out of the plant tissue were sub-cultured to PDA medium, and incubated at 25–28 °C. After confirming as *Colletotrichum* species by microscope examination based on conidia morphological characteristics, a pathogenicity test was done strictly with Koch’s postulates before being used in this study.

Primary screening of antagonistic entomopathogenic fungus

Forty-seven isolates of the entomopathogenic fungi were initially screened for their antagonistic activity against two isolates of *Colletotrichum* spp. by the dual culture method in 90 mm Petri dishes containing 20 mL of PDA. Fungal hypha tips of all isolates of the entomopathogenic fungi were cut with a 7 mm diameter cork borer and placed on PDA at a 10 mm from the plate periphery, while a mycelium plug of the pathogen was placed on the opposite side at a 70 mm distance. The dual culture plates were incubated at 28 °C for 14 days. The control plates consisted of individual cultures of the pathogen. After 14 days, the dual culture plates were evaluated for antagonistic activity that reduced pathogen

colony expansion. Each dual culture had three replicates. The top five isolates that inhibited the mycelial growth of the plant pathogenic fungi and produced inhibition zones were selected for further confirmation of their antagonistic activity.

Confirmation of antagonistic activity against the plant pathogenic *Colletotrichum* spp.

The selected isolates of antagonistic entomopathogenic fungi were tested for their antagonistic activity against five isolates of *C. gloeosporioides* (CgC) and five isolates of *C. capsici* (CcC) using the dual culture method as described in the primary screening test. After 14 days, the dual culture plates were evaluated for antagonistic activity that reduced the pathogen colony expansion. The percentage of mycelial growth reduction (PGI-1) was calculated using the formula:

$$\text{PGI-1}(\%) = \text{KR-R1}/\text{KR} \times 100,$$

where KR represents the fungal growth radius (mm) of the control culture and R1 represents the fungal growth radius distance (mm) in the direction of the entomopathogenic fungal growth (Korsten et al. 1995). The data from each experiment were analyzed with an analysis of variance, and means were compared by Duncan's Multiple Range Test (DMRT) (at $P = 0.05$). The best isolates that inhibited the mycelial growth of the plant pathogenic fungi were selected as candidate antagonistic biocontrol agents for further investigation of their antagonistic activity.

Effect of culture filtrate and cultured mycelium extract on mycelial growth of *Colletotrichum* spp.

Preparation of culture filtrate and cultured mycelium extract

A 20 day old disk of the mycelium of the best selected antagonistic biocontrol agent was inoculated in 25 mL of induced medium (Huang et al. 2009) without shaking at 28 °C for 20 days. The mycelium on the surface of the induced culture was collected and dried at 50 °C for 2–3 days, while the culture filtrate was also collected and then filtrated through a 0.2 µm filter before being used. The dried mycelium was powdered using a pestle and mortar. Then the mycelium powder was suspended in

50 % ethanol with the final concentration at 100 mg/mL (w/v). The mycelium suspension was sonicated with a High Intensity Ultrasonic Processor (Model VCX 750, Newtown, CT, USA) on ice for a total of 5 min in 10 s bursts with 2 s gaps for cooling. The sonicated solutions were centrifuged (Tomy MX-301, Tokyo, Japan) at 9100×g for 5 min and filtered through a 0.2 µm filter before being used.

Tube dilution assay

The sterile culture filtrate, mycelium extract and 50 % ethanol were diluted with two fold dilutions in PDB medium for a 5 mL total volume. The diluted culture filtrate, mycelium extract and 50 % ethanol were incubated with a mycelial disk of the plant pathogenic fungi *Colletotrichum* spp. for seven days at 28 °C under a static condition. The control tube contained 5 mL PDB that was incubated with the individual plant pathogenic fungi. The mycelial growth was categorized on a scale of 0 to 2, where 0 = no mycelial growth, 1 = growth limited around mycelial disk and 2 = mycelia overgrows into liquid medium (Alvandia and Natsuaki 2008). The experiment was done with three replications.

Pour plate technique

Five milliliter of the sterile culture filtrate, mycelium extract and 50 % ethanol were mixed in 100 mL PDA medium before being plated to 90 mm Petri dishes. Seven-day-old mycelial discs of each pathogen were cut with a sterilized cork borer to a diameter of 7 mm under aseptic conditions and placed onto the 25 mL PDA plates containing sterile culture filtrate, mycelium extract and 50 % ethanol. The plates were incubated at 28 °C. The mycelium growth was determined at 14 days. The fungus grew on the PDA plate that was used as a control plate. The experiment was done with three replications. The percentage of mycelial growth reduction (PGI-2) was calculated using the formula:

$$\text{PGI-2}(\%) = \text{R-R1}/\text{R} \times 100,$$

where R represents the fungal growth radius (mm) of the control culture and R1 represents the fungal growth radius (mm) in the treatment culture (Kumer et al. 2007). The PGI-2 was categorized from 0 to 4, where 0 = no growth inhibition; 1 = 1–25 % growth inhibition;

2 = 26–50 % growth inhibition; 3 = 51–75 % growth inhibition; and 4 = 76–100 % growth inhibition (Korsten et al. 1995). The data from each experiment was analyzed with an analysis of variance and means were compared by DMRT (at $P = 0.05$).

Effect of mycelium extract and culture filtrate on conidial germination of *Colletotrichum* spp.

Inoculum of the *Colletotrichum* spp. was prepared by culturing on PDA medium until sporulation. Conidia were harvested by flooding the cultures with distilled water. The concentrations of the propagules in suspension were standardized with the aid of a hemocytometer to 1×10^4 conidia mL^{-1} for each fungus. The conidial

germination test was determined on PDA plates containing 10 % sterile mycelium extract, 10 % culture filtrate and 10 % of 50 % ethanol. The plates were incubated at 28 °C. Then 100 spores of all treatments were determined within 24 h under light microscope. The inhibition rate of conidial germination (ICG) was calculated using the formula:

$$\text{ICG (\%)} = (1 - T/C) \times 100,$$

where T represents the germination rate of the treatment and C represents the germination rate of the control (Kwak et al. 2012). The experiment was done with five replicates.

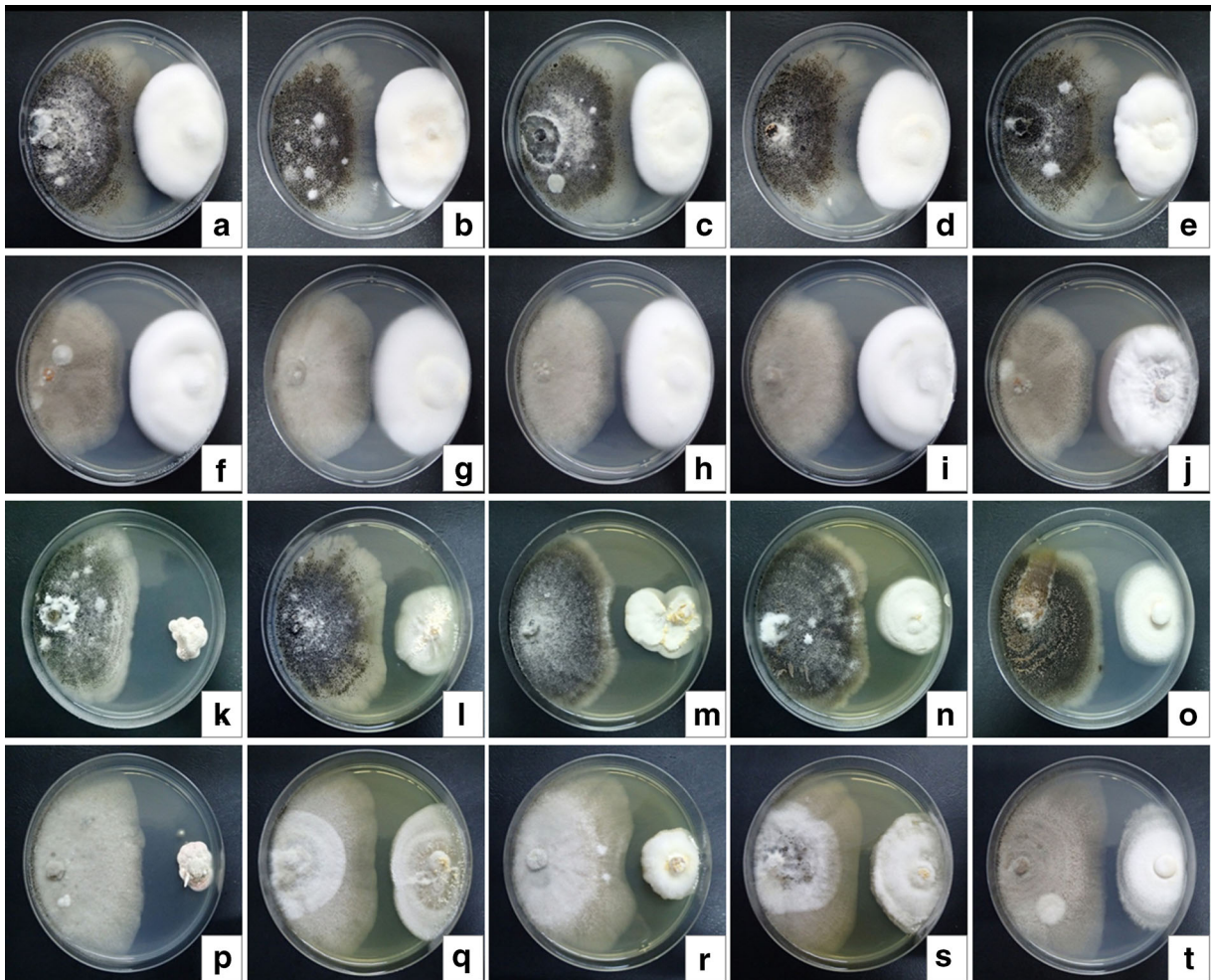


Fig. 1 Interaction between mycelial growth of entomopathogenic fungi and plant pathogenic *Colletotrichum* spp. (*C. capsici* a-e and k-o; *C. gloeosporioides* f-j and p-t) by dual culture method. Entomopathogenic fungi group 1 consisted of isolates Cod-

MK1301 (a, f), Cod-MK1311 (b, g), Cod-MK1321 (c, h), Cod-NN1303 (d, i) and Cod-NB1307 (e, j). Group 2 consisted of Cod-NB1302 (k, p), Cod-NB1305 (l, q), Cod-NN1307 (m, r), Cod-MK1208 (n, s) and Cod-Loei1301 (o, t)

Table 1 Inhibitory effect of five isolates of entomopathogenic fungi on mycelial growth of ten plant pathogenic *Colletotrichum* spp. by dual culture method

| Isolate | Location | Percentage of mycelial growth reduction (%)* | | | | | | | | | |
|--------------|----------|--|----------------------------|---------------------------|---------------------------|---------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| | | <i>C. capsici</i> | | | | | <i>C. gloeosporioides</i> | | | | |
| | | CcC1 | CcC2 | CcC4 | CcC5 | CcC6 | CgC6 | CgC7 | CgC10 | CgC11 | CgC12 |
| Cod-MK1208 | MK | 36.11 ± 3.47 ^b | 38.33 ± 2.88 ^b | 36.92 ± 2.66 ^b | 38.17 ± 2.46 ^b | 36.01 ± 0.92 ^a | 30.43 ± 0.51 ^{ab} | 30.91 ± 0.00 ^{bc} | 23.08 ± 0.00 ^{bc} | 28.57 ± 0.00 ^b | 24.66 ± 4.16 ^b |
| Cod-NB1302 | NB | 40.55 ± 1.92 ^a | 42.77 ± 1.92 ^a | 41.35 ± 1.81 ^a | 43.55 ± 0.00 ^a | 37.63 ± 1.86 ^a | 32.91 ± 0.31 ^a | 34.45 ± 1.25 ^a | 25.02 ± 1.92 ^a | 30.83 ± 2.77 ^{ab} | 25.52 ± 1.84 ^a |
| Cod-NB1305 | NB | 33.33 ± 0.00 ^{bc} | 31.11 ± 1.92 ^d | 36.40 ± 1.77 ^b | 36.01 ± 0.92 ^b | 35.48 ± 0.00 ^a | 27.27 ± 3.63 ^{bc} | 30.30 ± 2.10 ^{bc} | 23.08 ± 0.00 ^{ab} | 23.81 ± 2.06 ^c | 22.66 ± 2.30 ^{ab} |
| Cod-NN1307 | NN | 33.33 ± 0.00 ^{bc} | 33.33 ± 0.00 ^{cd} | 38.46 ± 0.00 ^b | 37.63 ± 2.46 ^b | 37.29 ± 1.93 ^a | 26.66 ± 1.05 ^c | 26.66 ± 1.05 ^d | 21.15 ± 0.00 ^b | 19.64 ± 2.06 ^d | 18.00 ± 2.30 ^b |
| Cod-Loei1301 | LO | 32.22 ± 1.92 ^c | 36.11 ± 2.54 ^{bc} | 38.46 ± 0.00 ^b | 36.55 ± 1.86 ^b | 35.48 ± 0.00 ^a | 30.36 ± 0.47 ^{ab} | 32.91 ± 0.66 ^{ab} | 23.08 ± 0.00 ^{ab} | 32.19 ± 1.62 ^a | 24.00 ± 4.00 ^{ab} |

Abbreviation of location: MK Maha Sarakham, NB Nong Bua Lam Phu, NV Nakhon Phanom, LO Loei

*Different lower case letters denote significant differences ($P < 0.05$) between the same isolate of *Colletotrichum* spp. in the same column

Control of anthracnose disease on chili fruit by detached fruit bioassay

Mature chili fruits were surface sterilized in 70 % alcohol for five minutes before being washed several times with sterile distilled water and then blotted dry on sterilized filter paper. The sterilized chili fruits were pin pricked gently with a sterilized needle prior to inoculation. Inoculums of the *Colletotrichum* spp. were prepared by culturing on PDA medium until sporulation. The mycelium extract and culture filtrate of the best selected antagonistic isolates were applied to a pin point wound on the mature chili fruits one day before the conidia suspensions of the pathogen were applied. The inoculated fruits were incubated in a moist chamber and kept at room temperature. The disease severity was observed daily for seven days. The severity index (SI) of the anthracnose symptoms was categorized based on the lesion diameter, SL = no lesion or symptomless, HR = highly resistant (1.0–4.9 mm), MR = moderately resistant (5.0–9.9 mm), MS = moderately susceptible (10.0–19.9) and HS = highly susceptible (>20.0 mm) (Hartman and Wang 1992).

Molecular identification of entomopathogenic fungal isolate Cod-NB1302

The entomopathogenic fungus was cultured on PDB medium at 28 °C for 20 days. Mycelia were harvested from the PDB medium before being homogenized with liquid nitrogen. The mycelium powder was transferred to a microcentrifuge tube and genomic DNA was extracted using the DNA extraction kit (Vivantis, Malaysia). The DNA samples were analyzed by 1 % agarose gel electrophoresis and stored at –20 °C.

Three regions of ribosomal nuclear DNA, the internal transcribed spacers of nuclear ribosomal DNA repeats (ITS), the partial small subunit of (*nrSSU*) rDNA, the partial large subunit of (*nrLSU*) rDNA, two protein-coding regions, the elongation factor 1 α (*EF-1 α*) and the largest subunit of the RNA polymerase II (*rpb1*) gene were used for fungal identification. The ITS region was amplified using the primers ITS1 (5'-TCCGTAGGTG AACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTAT TGATATGC –3') (White et al. 1990). The partial *nrSSU* rDNA was amplified using the primers NS1 (5'-GTAGTCATATGCTTGCTC-3') and NS2 (5'-GGCTGCTGGCACCAGACTTGC-3') (White et al.

1990). The partial *nrLSU* rDNA was amplified by PCR using the primers LROR (5'-ACCCGCTGAACTTAAGC-3') and LR7 (5'-TACTACCACCAAGATCT-3') (Vilgalys and Hester 1990). The *EF-1 α* was amplified using the primers EF-983F (5'-GCYCCYGGHCAAYGGTGAYTTYAT-3') and EF-2218R (5'-GACTTGACTTCRGTGVTGAC-3') (Currie et al. 2003), and the *rpb1* gene was amplified using the primers CRPB1 (5'-CCWGGYTTYATCAAGAARGT-3') and RPB1Cr (5'-CCNGCDATNTCRTRTCCATRTA-3') (Castlebury et al. 2004). The PCR reactions of all genes were performed as described by Sangdee et al. (2015).

The PCR products obtained were purified with a Gel/PCR DNA Fragments extraction kit (Geneaid, USA). Sequencing was performed by Macrogen Advancing through Genomics (Macrogen Inc., Korea). The sequence data of the partial ITS, *nrSSU*, *nrLSU*, *EF-1 α* and *rpb1* were compared with sequences in the National Center for Biotechnology Information data bank using the BLAST program (www.ncbi.nih.gov/blast). The novel partial sequences were deposited in the GenBank nucleotide sequence database, and reference sequences of related species were downloaded and aligned using ClustalW (www.genome.jp/tools/clustalw/). Phylogenetic analyses of the ITS and combined data sets of the ITS, *nrSSU*, *nrLSU*, *EF-1 α* and *rpb1* were performed using Mega 6 (Tamura et al. 2013) and a Neighbor Joining tree (NJ tree) was constructed (bootstrap replicates = 1000) using the Kimura 2 parameter method for pairwise deletion at

uniform rates. *Bionectria ochroleuca* was used as the outgroup.

Results

Isolation and primary screening of antagonistic entomopathogenic fungi

Within 7–14 days, a total of 47 isolates of the entomopathogenic fungi produced cottony colonies with white cream synnemata on cicada nymph tissues, were isolated and used to evaluate their antagonistic activity against the plant pathogenic fungi *C. gloeosporioides* and *C. capsici*. The results showed that the interaction between the mycelial growth of the entomopathogenic fungi and plant pathogenic *Colletotrichum* spp. were categorized into two groups. The first group, the mycelium of the entomopathogenic fungi and plant pathogenic fungi grow well and inhibited each other without an inhibition zone. The second group, the mycelial growth of plant pathogenic *Colletotrichum* spp. was inhibited by the mycelium of the entomopathogenic fungi with an inhibition zone (Fig. 1). The high percentage of mycelial growth reduction and large inhibition zone were used as criteria for selection of the antagonistic strain. Therefore, the top five isolates of the entomopathogenic fungi consisted of the isolates Cod-NB1302, Cod-NB1305, Cod-NN1307, Cod-MK1208 and Cod-Loei1301, which showed a high percentage of mycelium reduction in the range from 16.67 to

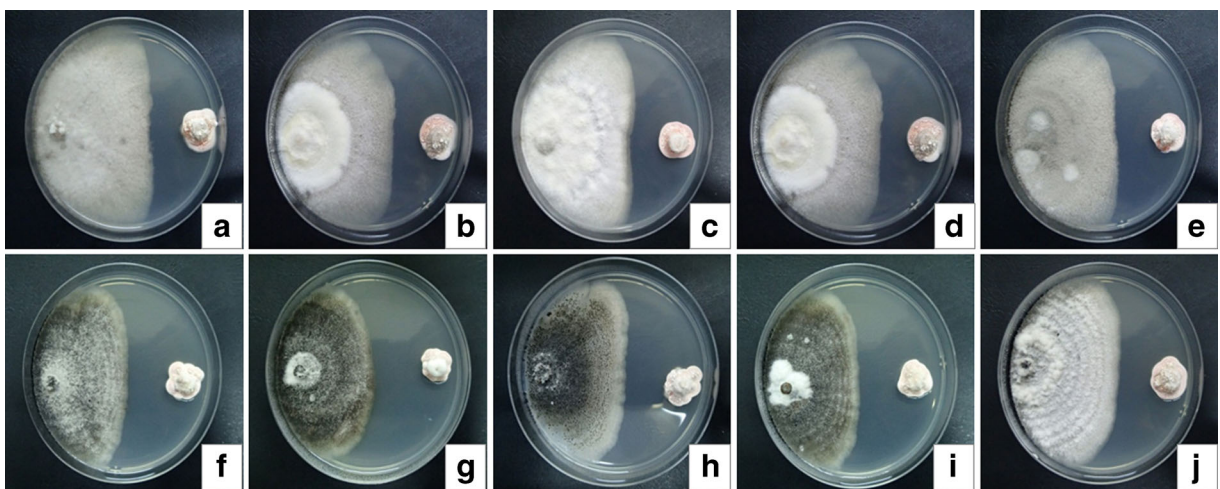


Fig. 2 Antifungal activities of entomopathogenic fungi isolate Cod-NB1302 against *C. gloeosporioides* isolates CgC6 (a), CgC7 (b), CgC10 (c), CgC11 (d) and CgC12 (e) and *C. capsici* isolate CcC1 (f), CcC2 (g), CcC4 (h), CcC5 (i) and CcC6 (j) by dual culture method

Table 2 Antifungal activity of dilutions of mycelium extract and culture filtrate from entomopathogenic fungal isolate Cod-NB1302 against plant pathogenic *Colletotrichum* spp. compared with 50 % ethanol

| <i>Colletotrichum</i> spp. | PDB | 50 % ethanol | | | | Mycelium extract | | | | Culture filtrate | | | |
|---------------------------------|-----|--------------|-----|-----|-----|------------------|-----|-----|-----|------------------|-----|-----|-----|
| | | 1:1 | 1:2 | 1:4 | 1:8 | 1:1 | 1:2 | 1:4 | 1:8 | 1:1 | 1:2 | 1:4 | 1:8 |
| <i>C. capsici</i> CcC1 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| <i>C. capsici</i> CcC2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| <i>C. capsici</i> CcC4 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| <i>C. capsici</i> CcC5 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| <i>C. capsici</i> CcC6 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| <i>C. gloeosporioides</i> CgC6 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| <i>C. gloeosporioides</i> CgC7 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| <i>C. gloeosporioides</i> CgC10 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| <i>C. gloeosporioides</i> CgC11 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| <i>C. gloeosporioides</i> CgC12 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |

54.55 % and produced inhibition zones of at least 3 mm, and they were selected for further studies.

Confirmation of antagonistic activity against the plant pathogenic *Colletotrichum* spp.

The top five isolates of entomopathogenic fungi were assayed against the five isolates of *C. gloeosporioides* and five isolates of *C. capsici*. The results showed that all the selected isolates inhibited the growth of *C. gloeosporioides* and *C. capsici* in the range from 18.00 to 34.45 % and 31.11 to 43.55 %, respectively. The

percentage of fungal mycelial growth reduction varied depending on the isolate of entomopathogenic fungi and plant pathogenic *Colletotrichum* spp. However, the selected entomopathogenic fungi isolate Cod-NB1302 showed the highest percentage of mycelial growth reduction in all the tested fungi, which was significantly greater than with the other isolates (Table 1). The inhibition zone produced by the isolate Cod-NB1302 varied from 12 to 19 mm and 16 to 21 mm for *C. gloeosporioides* and *C. capsici*, respectively (Fig. 2). Therefore, isolate Cod-NB1302 was chosen for further evaluation of the antagonistic activity.

Table 3 Antifungal activities of entomopathogenic fungal isolate Cod-NB1302 against plant pathogenic *Colletotrichum* spp.

| <i>Colletotrichum</i> spp. | Percentage of mycelial growth reduction (14 days) | | | | | | Inhibition of conidial germination (%)* | | |
|---------------------------------|---|----------------|------------------|----------------|------------------|----------------|---|---------------------|---------------------|
| | 50 % EtOH | PGI-2 category | Mycelium extract | PGI-2 category | Culture filtrate | PGI-2 category | 50 % EtOH | Mycelium extract | Culture filtrate |
| <i>C. capsici</i> CcC1 | 47.25 ± 1.93 | 2 | 71.09 ± 3.48 | 3 | 0 ± 0.00 | 0 | 41.61 ^{ab} | 43.04 ^{cd} | 28.84 ^{ab} |
| <i>C. capsici</i> CcC2 | 38.56 ± 2.99 | 2 | 57.95 ± 3.35 | 3 | 0 ± 0.00 | 0 | 43.94 ^a | 45.15 ^{ab} | 26.03 ^{bc} |
| <i>C. capsici</i> CcC4 | 45.77 ± 4.01 | 2 | 51.33 ± 2.00 | 3 | 0 ± 0.00 | 0 | 38.11 ^{bc} | 40.72 ^{cd} | 31.42 ^a |
| <i>C. capsici</i> CcC5 | 39.82 ± 1.35 | 2 | 69.26 ± 3.79 | 3 | 0 ± 0.00 | 0 | 41.65 ^{ab} | 46.11 ^{ab} | 26.18 ^{bc} |
| <i>C. capsici</i> CcC6 | 40.17 ± 6.42 | 2 | 70.51 ± 3.39 | 3 | 0 ± 0.00 | 0 | 40.91 ^{ab} | 48.25 ^a | 32.33 ^a |
| <i>C. gloeosporioides</i> CgC6 | 40.71 ± 9.66 | 2 | 70.47 ± 0.81 | 3 | 0 ± 0.00 | 0 | 33.98 ^c | 38.63 ^d | 28.05 ^{ab} |
| <i>C. gloeosporioides</i> CgC7 | 48.81 ± 1.09 | 2 | 50.47 ± 2.06 | 3 | 0 ± 0.00 | 0 | 39.58 ^b | 39.99 ^{cd} | 22.09 ^{cd} |
| <i>C. gloeosporioides</i> CgC10 | 34.89 ± 1.68 | 2 | 69.77 ± 3.35 | 3 | 0 ± 0.00 | 0 | 38.76 ^{bc} | 40.06 ^{cd} | 23.25 ^{cd} |
| <i>C. gloeosporioides</i> CgC11 | 42.73 ± 0.73 | 2 | 61.54 ± 6.41 | 3 | 0 ± 0.00 | 0 | 37.81 ^{bc} | 39.25 ^{cd} | 22.66 ^{cd} |
| <i>C. gloeosporioides</i> CgC12 | 57.35 ± 0.75 | 3 | 54.33 ± 0.38 | 3 | 0 ± 0.00 | 0 | 39.49 ^b | 44.79 ^{ab} | 20.16 ^d |

*Different lower case letters denote significant differences ($P < 0.05$) between the same isolate of *Colletotrichum* spp. in the same column

Table 4 Effect of mycelium extract and culture filtrate of entomopathogenic fungal isolate Cod-NB1302 on size of anthracnose disease lesion and severity index after inoculation with plant pathogenic *Colletotrichum* spp. compared with 50 % ethanol

| Isolate | 50 % ethanol | | Mycelium extract | | Culture filtrate | | Positive control | |
|---------------------------------|---------------------|----------------|---------------------|----------------|---------------------|----------------|---------------------|----------------|
| | Size of lesion (mm) | Severity index | Size of lesion (mm) | Severity index | Size of lesion (mm) | Severity index | Size of lesion (mm) | Severity index |
| <i>C. capsici</i> CcC1 | 16.4 ± 2.2 | MS | 3.0 ± 1.2 | HR | 3.6 ± 0.9 | HR | 18.8 ± 2.3 | MS |
| <i>C. capsici</i> CcC2 | 16.0 ± 1.4 | MS | 3.6 ± 1.5 | HR | 4.0 ± 1.0 | HR | 15.4 ± 3.8 | MS |
| <i>C. capsici</i> CcC4 | 15.6 ± 2.3 | MS | 3.0 ± 1.2 | HR | 4.8 ± 0.4 | HR | 15.6 ± 3.3 | MS |
| <i>C. capsici</i> CcC5 | 16.4 ± 3.0 | MS | 3.2 ± 1.1 | HR | 7.4 ± 1.8 | MR | 15.2 ± 2.8 | MS |
| <i>C. capsici</i> CcC6 | 14.6 ± 1.1 | MS | 5.8 ± 1.6 | MR | 8.0 ± 2.1 | MR | 13.8 ± 3.9 | MS |
| <i>C. gloeosporioides</i> CgC6 | 13.8 ± 2.8 | MS | 7.0 ± 2.1 | MR | 13.0 ± 4.4 | MS | 17.6 ± 3.7 | MS |
| <i>C. gloeosporioides</i> CgC7 | 14.8 ± 3.6 | MS | 11.2 ± 4.9 | MS | 15.6 ± 2.8 | MS | 15.6 ± 3.3 | MS |
| <i>C. gloeosporioides</i> CgC10 | 15.0 ± 3.1 | MS | 8.6 ± 2.1 | MR | 12.4 ± 2.5 | MS | 16.4 ± 4.7 | MS |
| <i>C. gloeosporioides</i> CgC11 | 15.4 ± 1.1 | MS | 8.6 ± 2.1 | MR | 12.8 ± 3.1 | MS | 17.6 ± 2.5 | MS |
| <i>C. gloeosporioides</i> CgC12 | 16.0 ± 4.2 | MS | 6.8 ± 2.7 | MR | 13.4 ± 2.3 | MS | 16.0 ± 2.2 | MS |

Effect of culture filtrate and mycelium extract on mycelial growth of *Colletotrichum* spp.

The mycelium extract of isolate Cod-NB1302 exhibited greater antifungal activity against all 10 isolates of the tested plant pathogenic fungi than the culture filtrate and 50 % ethanol (Table 2). The mycelium extract completely controlled the mycelial growth of *C. gloeosporioides* and *C. capsici* at the dilutions of 1:1 up to 1:8, whereas the culture filtrate showed partial effects on the growth of all 10 isolates of the tested fungi at the dilutions of 1:1 to 1:4. The 50 % ethanol also completely inhibited the fungal mycelial growth at the dilutions of 1:1 and 1:2, while at the dilution of 1:4 it inhibited the mycelial growth of some isolates (Table 2). Similar results were obtained with the pour plate technique. The percentage of mycelial growth reductions ranged from 50.4 to 71.09 % (PGI-2 = 3) for all 10 isolates of the tested fungi, and they were more sensitive to the mycelium extract than the other treatments with culture filtrate and 50 % ethanol. Interestingly, the mycelium extract exhibited greater antifungal activity than the 50 % ethanol in the range from 1.0 to 2.0-fold; whereas, the culture filtrate had no effect on the mycelial growth (Table 3).

Effect of mycelium extract and culture filtrate on conidial germination of *Colletotrichum* spp.

The effect of the mycelium extract and culture filtrate of the selected entomopathogenic fungal isolate Cod-NB1302 on conidial germination was investigated. The results showed that the culture filtrate suppressed conidial germination of all 10 isolates of the tested fungi ranging from 20.16 to 32.33 %, while the mycelium extract and 50 % ethanol exhibited suppression of the conidial germination that ranged from 38.63 to 48.25 % and 33.98 to 43.94 %, respectively (Table 3). Moreover, the effect of the mycelium extract and culture filtrate on the length of conidial germ tube were observed in this study. The mycelium extract had a greater effect on the conidial germ tube than the other treatments with culture filtrate and 50 % ethanol. The length of the germ tube on PDA plus mycelium extract ranged from 90 to 200 µm. While, germ tube lengths of 170 to 400 µm, 100 to 230 µm and 350 to 620 µm were observed from PDA plus culture filtrate, 50 % ethanol and control group, respectively. In addition, abnormally shaped mycelium was observed from the mycelium extract and culture filtrate treatments.

Control of anthracnose disease on chili fruit by detached fruit bioassay

The potential of the selected entomopathogenic fungus isolate Cod-NB1302 to inhibit *C. gloeosporioides* and *C. capsici* was successfully evaluated by the detached fruit technique. The mycelium extract showed generally lower disease severity on the inoculated chili fruits than the culture filtrate, 50 % ethanol and positive control. The disease lesion sizes for the mycelium extract ranged from 6.8 ± 2.7 to 11.2 ± 4.9 mm and 3.0 ± 1.2 to 5.8 ± 1.6 mm for *C. gloeosporioides* and *C. capsici*, respectively. While, the culture filtrate generally had larger disease lesion sizes than the mycelium extract. The disease lesion sizes for the culture filtrate ranged from 12.4 ± 2.5 to 15.6 ± 2.8 mm and 3.6 ± 0.9 to 8.0 ± 2.1 mm for *C. gloeosporioides* and *C. capsici*, respectively. In the 50 % ethanol treatment, *C. gloeosporioides* and *C. capsici* induced disease lesion sizes that ranged from 13.8 ± 2.8 to 16.0 ± 4.2 mm and 14.6 ± 1.1 to 16.4 ± 2.2 mm, respectively. While, the disease lesion sizes ranging from 15.6 ± 3.3 to 17.6 ± 3.7 mm and 13.8 ± 3.9 to 18.8 ± 2.3 mm were observed in the positive control after inoculation of chili fruits with *C. gloeosporioides* and *C. capsici*, respectively (Table 4 and Fig. 3).

Molecular identification of entomopathogenic fungal isolate Cod-NB1302

The ITS, *nrSSU*, *nrLSU*, *EF-1 α* and *rpb1* regions of the selected entomopathogenic fungal isolate Cod-NB1302

were amplified using universal primers. The DNA fragments were purified and sequenced. The DNA sequences of the ITS, *nrSSU*, *nrLSU*, *EF-1 α* and *rpb1* regions, which consisted of 508, 510, 1001, 437 and 662 nucleotides, respectively, were submitted to GenBank (Table 5). A BLAST search in NCBI (www.ncbi.nih.gov/blast) showed that the ITS sequence was most similar to *Ophiocordyceps sobolifera* (= *Cordyceps sobolifera*) with 91 % homology. A phylogenetic tree of the ITS region was generated from 27 aligned sequences with similar characters that indicated the isolate Cod-NB1302 was located in the same clade as *O. sobolifera* with a 6–7 % genetic distance and 93 % NJ bootstrap support (Fig. 4). These similar data findings were obtained with alignment of the combined data set consisting of ITS, *nrSSU*, *nrLSU*, *EF-1 α* and *rpb1* sequences (3096 nucleotides) for 28 taxa. The NJ tree of the combined data set confirmed that the isolate Cod-NB1302 was also placed within the same clade as *O. sobolifera* with 3–8 % genetic distance and 79 % NJ bootstrap support (Fig. 5). Based on the molecular data we suggest that the isolate Cod-NB1302 be designated *O. sobolifera* isolate Cod-NB1302.

Discussion

The entomopathogenic macrofungi in the genus *Cordyceps* have been known as important ingredients in Chinese medicine for thousands of years (Zhu et al. 1998; Cha et al. 2006). Information on the bioactivities of these macrofungi have been reported in the medical and



Fig. 3 Effect of mycelium extract (c and g) and culture filtrate (d and h) of the entomopathogenic fungal isolate Cod-NB1302 and 50 % ethanol (b and f) on chili fruits after inoculation with plant

pathogenic *C. gloeosporioides* isolate CgC12 (a-d) and *C. capsici* isolate CcC6 (e-h) compared with control group (a and e)

pharmaceutical areas, including antitumor, antioxidant, immunomodulatory, anti-inflammatory and antimicrobial activities (Tuli et al. 2014). While, the information on the bioactive compounds that use in the agricultural area, such as a biocontrol agent, is limited. In the present study, antifungal activity against the plant pathogenic fungus *Colletotrichum* spp. by an entomopathogenic macrofungus isolated from a dead cicada nymph was observed. Five of 47 isolates from the entomopathogenic fungi showed good inhibition of the mycelial growth of the plant pathogenic fungi. However, the antifungal effects of the entomopathogenic fungi depended on the

strain and species of the entomopathogenic fungi and the plant pathogenic *Colletotrichum*. For example, entomopathogenic fungal isolate Cod-NB1302 showed the highest percentage of mycelial growth reduction of *C. capsici* isolate CcC4 at 43.55 %, while the mycelial growth reduction of *C. gloeosporioides* isolate CgC10 was 25.02 %. In another case, a different isolate of entomopathogenic fungi showed variations in the percentage of mycelial growth reduction when tested with the same isolates of the plant pathogenic *Colletotrichum* spp. These findings correlate with those of Chen and Huang (2010) who found that the antimicrobial effect depended on the

Table 5 Sequences of entomopathogenic fungal isolate Cod-NB1302 and related species and their NCBI accession numbers used in this study

| Species | NCBI accession number | | | | |
|--------------------------------------|-----------------------|--------------|--------------|--------------------------------|-------------|
| | ITS | <i>nrSSU</i> | <i>nrLSU</i> | <i>EF-1α</i> | <i>rpb1</i> |
| Isolate Cod-NB1302 | KT281884 | KT281885 | KT281886 | KT281887 | KT281888 |
| <i>C. bifusispora</i> | AJ786553 | EF468952 | EF468806 | EF468746 | EF468854 |
| <i>C. brongniartii</i> | AB235200 | JN941759 | JN941382 | DQ376244 | JN992493 |
| <i>C. crinalis</i> | EF495104 | - | - | - | - |
| <i>C. gunnii</i> | JX402623 | HM135160 | HM140633 | HM140636 | HM140639 |
| <i>C. kanzashiana</i> | AB027371 | AB027325 | - | - | - |
| <i>C. lianzhouensis</i> | EU149921 | - | - | - | - |
| <i>C. militaris</i> | AJ309329 | JN941385 | JN941385 | HM140638 | JN992490 |
| <i>C. nipponica</i> | JN943301 | JN941754 | JN941387 | - | JN992488 |
| <i>C. pleuricapitata</i> | JN943306 | JN941749 | JN941392 | - | JN992483 |
| <i>C. pseudomilitaris</i> | JN943310 | JN941746 | JN941395 | - | JN992480 |
| <i>C. ramosopulvinata</i> | AB027372 | AB027326 | DQ118742 | DQ118753 | DQ127244 |
| <i>C. scarabaeicola</i> | JN049827 | AF339574 | AF339524 | DQ522335 | DQ522380 |
| <i>Cordyceps</i> sp. | EF495106 | KF226249 | KF226250 | KF226252 | KF226251 |
| <i>C. tuberculata</i> | JN943309 | JN941744 | JN941397 | DQ522338 | JN992478 |
| <i>C. valliformis</i> | AY245640 | AY245648 | - | - | - |
| <i>Ophiocordyceps cuboidea</i> | JN943333 | JN941722 | JN941419 | - | JN992456 |
| <i>O. longissima</i> | AB968406 | AB968394 | AB968421 | AB968585 | JX896450 |
| <i>O. neovolkiana</i> | HM856642 | KJ878930 | KJ878896 | KJ878976 | KJ879010 |
| <i>O. nutans</i> | AB968408 | JN941712 | JN941429 | AB968589 | JN992446 |
| <i>O. paracuboidea</i> | JN943337 | JN941711 | JN941430 | - | JN992445 |
| <i>O. prolifica</i> | AB378677 | JN941708 | JN941433 | - | JN992442 |
| <i>O. ryogamiensis</i> | AB378678 | JN941702 | JN941439 | - | JN992436 |
| <i>O. rubiginosiperitheciata</i> | JN943344 | JN941704 | JN941437 | AB968582 | JN992438 |
| <i>O. sobolifera</i> | AB027374 | - | - | - | - |
| <i>O. sobolifera</i> NBRC:106,967 | AB968409 | AB968395 | AB968422 | AB968590 | - |
| <i>Polycephalomyces sinensis</i> | HQ832885 | HQ832887 | HQ832886 | HQ832890 | HQ832888 |
| <i>Bionectria ochroleuca</i> | KF055399 | DQ862044 | AY283558 | DQ862029 | DQ842031 |

strains and species of mushroom and the species of test microorganism. Moreover, these suggest that different antagonistic strains from the same species produce different amounts of antimicrobial compounds (Suay et al. 2000).

In this study, the entomopathogenic fungal isolate Cod-NB1302 was identified as *O. sobolifera* based on

three ribosomal nuclear DNA genes and two protein-coding genes, and this showed the best inhibitory effect against the mycelial growth of all the test fungi using the dual culture method, as this isolate was chosen for confirmation of the antagonistic activity. The mycelium extract appeared to completely inhibit the mycelial growth of all test fungi using the tube dilution assay (Table 2) and

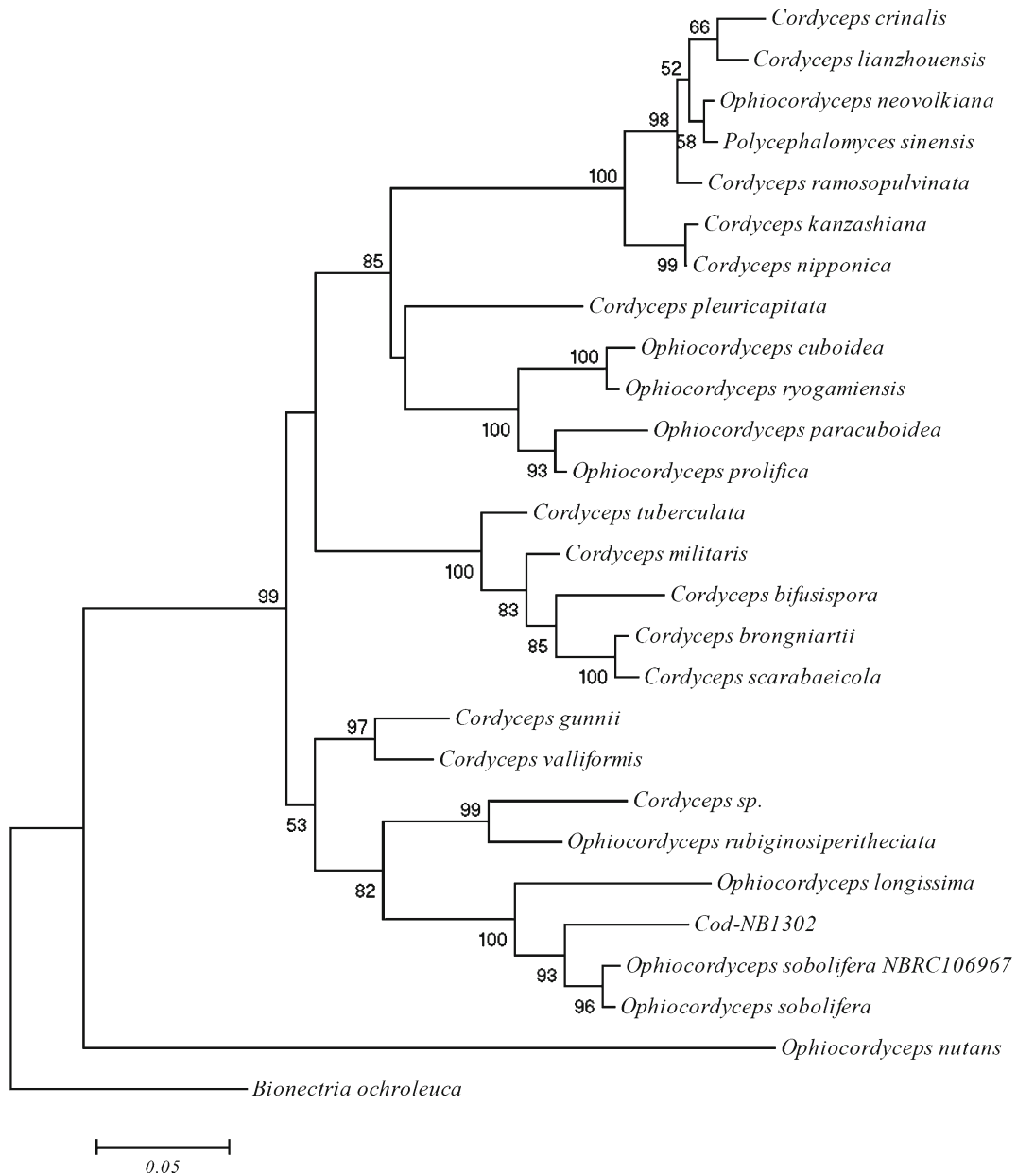


Fig. 4 Phylogenetic relationship of entomopathogenic fungal isolate Cod-NB1302, the 25 related *Cordyceps* species and one out group based on partial ITS gene sequences. Neighbor Joining (NJ) tree was constructed using Mega 6. Percentages expressed above the

branches are frequencies with which a given branch appeared in 1000 bootstrap replications when using the NJ method (branches corresponding to partitions reproduced in <50 % were collapsed)

there were partially inhibited by the pour plate assay (Table 3). Unfortunately, the inhibitory effect of the mycelium extract was interfering by 50 % ethanol that used as an organic solvent which is shown in Tables 2 and 3. A similar inhibition effect was found in the conidial germination test. However, we suggest that the mycelium extract has some bioactive compounds that affect the mycelial growth because the mycelium extract exhibited greater antifungal activity than the 50 % ethanol. Interestingly, the fungal effect on mycelial growth and conidial germination were also found in the culture filtrate treatments. These findings correlate with those of Intiaj

and Lee (2007) who reported that the culture filtrate from *O. sobolifera* showed good inhibitory effects against the mycelial growth of three plant pathogenic fungi: *Botrytis cinerea*, *C. gloeosporioides* and *C. miyabeanus*. Vesely and Koubova (1994) found that the entomopathogenic fungi *Beauveria bassiana* could inhibit the mycelial growth of the plant pathogenic *Pythium ultimum*, *P. debaryanum* and *Septoria nodorum* by a cell lysis mechanism. Chen and Huang (2010) also found that the culture filtrate of the mushroom *Lentinula edodes* could inhibit the mycelial growth and zoospore germination of the plant pathogenic fungi *Phytophthora capsici*.

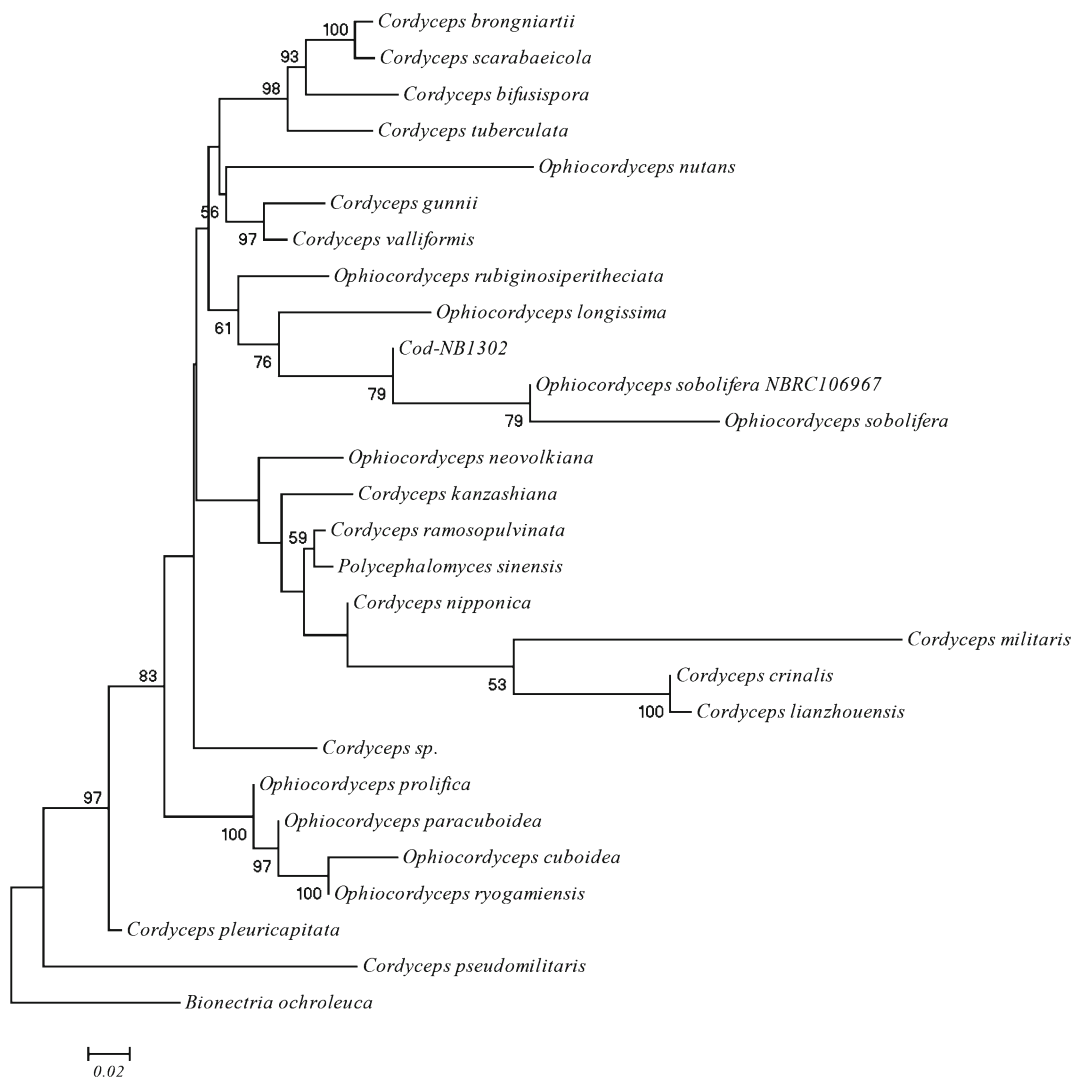


Fig. 5 Phylogenetic relationship of entomopathogenic fungal isolate Cod-NB1302 and 27 related species based on partial ITS, *nrSSU*, *nrLSU*, *EF-1 α* and *rpbl* sequences. Neighbor Joining (NJ) tree was constructed using Mega 6. Percentages expressed

above the branches are frequencies with which a given branch appeared in 1000 bootstrap replications by NJ method (branches corresponding to partitions reproduced in <50 % were collapsed)

Therefore, the mycelium extract and culture filtrate that contained some bioactive compounds, such as chitinase (Lee and Min 2003) and β -1-3, glucanase enzyme, may degrade the cell and lead to the lysis of the hypha of the pathogen (Wu et al. 1986). These possible reasons indicate that the isolate Cod-NB1302 had bioactive compounds that effected the mycelial growth and conidial germination of the plant pathogenic *Colletotrichum* spp. under in vitro conditions.

For the detached chili fruit bioassay, the chili fruits treated with the mycelium extract and culture filtrate of the isolate Cod-NB1302 effectively reduced the size of the disease lesion and disease severity of all test plant pathogenic *Colletotrichum*. These results indicated that the mycelium extract could reduce the disease severity of the pathogen without interference from the 50 % ethanol (Table 4). These findings correlate with those of Chen and Huang (2010) who found that pepper leaves treated with the culture filtrate of the mushroom *Clitocybe nuda* (LA82) and *C. aureus* effectively reduced the disease severity of phytophthora blight caused by *P. capsici*. Ownley et al. (2010) reported that the endophytic fungal entomopathogen *B. bassiana* could produce bioactive metabolites to reduce the disease severity caused by soil borne plant pathogens. To our knowledge, we suggest that the *O. sobolifera* isolate Cod-NB1302 could be used as a source of antifungal agents to control anthracnose disease caused by the plant pathogenic *Colletotrichum* spp.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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