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Original research article

Vermicompost Contained Fungi Act as Biocontrol Agents Against Fungal Pathogens of Chili

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ABSTRACT

The present study aimed to isolate fungi found in vermicompost that exhibit antifungal activity against fungal pathogens of chili. The fungal pathogens were isolated from chili and their pathogenicity was tested and identified by morphology with sequence analysis of Internal Transcribed Spacer (ITS), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), and Beta-tubulin2 (TUB2) genes. The results indicated that fungal isolate B1 was identified as *Colletotrichum siamense,* a causative agent of chili anthracnose. The total fungi in the vermicompost was 2.64 x10^5 CFU/g with 32 fungal colonies of different morphology being isolated. The antifungal activity was observed with fungal isolates from vermicompost. Among these fungal isolates, 13 fungal isolates (40.63%) revealed antifungal activity against *C. siamense* with the percent inhibition in the range of 46.85-72.64%. The E10, E8 and F5 isolates revealed the highest percent inhibition of 72.75±4.81%, 71.70±5.45%, and 70.65±4.80%, respectively. The identification of fungal isolates E10, E8, and F5 by morphological study and sequence analysis of ITS, RPB2 (DNA-directed RNA polymerase II subunit 2), and TEF1 (Translation elongation factor 1) were examined. These isolates were identified as *Trichoderma breve* (E10) and *T. zerobreve* (E8 and F5). The findings of this study suggest that the fungal isolates from vermicompost with inhibitory activity against chili pathogen could support the use of

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vermicompost in chili plantations, not only for promoting plant growth but also for controlling fungal pathogens of chili anthracnose.

Keywords: Anthracnose; Chili; Fungi; Inhibition; Vermicompost

1. Introduction

Chili (*Capsicum* spp.) is an important economic crop worldwide; however, agriculturists face problems with chili diseases that result in yield losses and low prices [1]. Among chili diseases, anthracnose is the most common, found very often in chili plantations including Chanthaburi province, Eastern Thailand. This disease is caused by *Colletotrichum* spp., a fungal pathogen that destroys chili plants including stem, leaf, and especially chili fruits. Moreover, during the pre- and post-harvest of chili, anthracnose can be observed. The dissemination of anthracnose is caused by infected seeds, contaminated soil, and wasted plant. In addition, environmental factors also affect the transmission of this pathogen. Conditions such as 27 ºC and 80% relative humidity support *Colletotrichum* spp. growth, leading to high dissemination and infections rates during the rainy season [2, 3] .

Chili anthracnose causes high economic loss and poses a problem that is difficult to solve. Several methods to prevent and control anthracnose are used [2]; however, agriculturists typically use chemical fungicides due to its greater ease of use and effectiveness as compared to other methods. However, chemical fungicides are toxic and affect the health of people through direct contact, and unsuitable usage results in acute and chronic diseases. Additionally, chemical fungicides affect the environment and ecology, resulting in chemical contamination of soil and water. In light of these drawbacks, other methods such as biological control, organic substances, and organic compost are being developed to replace them.

Vermicompost is the product of the decomposition process in the organic waste that is associated with earthworms and microbe activities. It promotes plant growth by allowing the proliferation of microbes that produce beneficial hormones and enzymes. In parallel, these microbes can inhibit plant pathogens [4]. Therefore, the activity of the microbial community results in the production of bioactive compounds in the vermicompost, the fungi play an especially important role in these activities [5].

Chili anthracnose was disseminated and observed in Pong Nam Ron District, Chanthaburi Province. The agriculturist of community enterprises in this area produces vermicompost for use in their chili plantations to replace chemical fungicides and to control anthracnose.

This study aimed to isolate fungi contained in vermicompost that exhibited antifungal activity against fungal pathogens of chili. The fungal isolates from vermicompost with inhibitory activity against chili pathogens could support the use of vermicompost in chili plantations, not only for promoting plant growth but also for controlling chili anthracnose pathogens.

2. Materials and Methods

2.1 Fungal pathogen isolation

Chili plants with disease symptoms were collected from an agricultural plantation in Pong Nam Ron district, Chanthaburi province. For sampling, a piece of chili was cut and suspended in 1% sodium hypochlorite for 3 min. After that, the chili tissue was washed with sterile distilled water 3 times. Subsequently, a piece of chili tissue was placed on Potato Dextrose Agar (PDA) and incubated at room temperature for 3-5 days. The colony of fungal growth from chili tissue was observed and point inoculated on PDA again

for preparation of pure fungal culture. The colony morphology was observed and a slide was prepared for investigating the fungal structure under a microscope.

2.2 Pathogenicity test

To investigate the pathogenicity of the fungal pathogen isolated from chili according to Koch's postulation, the fungal pathogen was cultured on PDA for 7 days and a cork borer with a 5 mm diameter was used to drill at the edge of the colony. A piece of the mycelial disc was placed on uninfected chili in a moisture chamber and incubated at room temperature. The symptoms of fungal infection in chili were investigated. The infected chili was cut and the fungi were isolated from chili tissue as described above.

2.3 Determination of total fungi in vermicompost

Vermicompost, a product of community enterprise in Pong Nam Ron district, Chanthaburi province, was used in this study. Total fungal components in vermicompost were determined by total plate count on dichloran rose bengal chloramphenicol agar (DRBC agar) according to standard protocol [6].

2.4 Isolation of fungi in vermicompost

The different morphological colonies of fungi on DRBC agar were selected to determine antifungal activity with the fungal pathogen of chili. The fungi were point-inoculated on PDA again for pure culture. The colony morphology was observed and fungal structures were investigated from slide culture under a microscope.

2.5 Determination of antifungal activity

To determine the antifungal activity of fungi isolated from vermicompost, the dual culture technique was used to investigate this property as previously described [7]. Briefly, all fungal isolates were cultured on PDA for 7 days. A cork borer with a 5 mm diameter was used to drill at the edge of the colony. A piece of the pathogen mycelial disc was placed where 2 cm from the edge of the PDA plate, and another piece of fungi isolated from vermicompost was placed on the opposite side. A plate containing only the fungal pathogen on the PDA plate was used as a control. The experiment was done in triplicate and the inhibition percentage was calculated by [(R1-R2)/R1x100], with R1 being the radius of the fungal pathogen colony in the control plate, and R2 being the radius of the fungal pathogen colony in a dual culture plate.

2.6 DNA extraction and PCR amplification

All fungal samples were DNA extracted by commercial kit following the manufacturer protocol (Flavogen, Taiwan). To identify the fungal pathogen isolated from chili, PCR amplifications were performed in 3 positions including ITS (Internal Transcribed Spacer), GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase), and TUB2 (Beta-tubulin2) with specific primers. The ITS region was amplified with primer ITS1 and ITS4 [8], GAPDH was amplified with primer GDF-F and GDF-R [9] while TUB2 was amplified with primer T1-F and T2-R [9] (Table 1).

The PCR reaction was performed in a total volume of 20 µl consisting of distilled water (Apsalagen, Thailand), 1x PCRmaster mix (Apsalagen, Thailand), 0.5 µM of each primer, and 10 pg-1 µg of DNA template at the final concentration. The DNA target amplification by PCR techniques was carried out in a thermal cycler. PCR conditions used were as follows.

2.6.1 The ITS region

An initial denaturation at 95°C for 3 min, subsequently the 35 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1

min, and a final extension at 72°C for 10 min.

2.6.2 GAPDH

An initial denaturation at 95°C for 3 min, then 35 cycles of denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 45 sec, and final extension at 72°C for 10 min.

2.6.3 TUB2

An initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 10 min.

To identify fungi isolated from vermicompost with antifungal activity, the PCR amplifications were performed in 3 positions including ITS, RPB2, and TEF1 (Translation elongation factor 1). The ITS region was amplified as described above. Additionally, the RPB2 gene was amplified with primer fRPB2-5f and fRPB2-7cR [10], whereas TEF1 was amplified with primer EF1-983F and EF1-1567R [10] (Table 1). PCR was performed as described previously and the PCR amplification was carried out in a thermal cycler. PCR conditions used were as follows.

2.6.4 RPB2

An initial denaturation at 95°C for 3 min, then the 35 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 10 min.

2.6.5 TEF1

An initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 10 min.

PCR products were analyzed by gel electrophoresis (2% gel with RedSafe (iNtRONbiotechnology, Korea) at 100V for 30 min.

Table 1. Primers used in this study.

Target	Primer	Sequence (5' to 3')
ITS	ITS1	TCCGTAGGTGAACCTGCGG
	ITS4	TCCTCCGCTTATTGATATGC
GAPDH	GDF-F	GCCGTCAACGACCCCTTCATTGA
	GDF-R	GGGTGGAGTCGTACTTGAGCATGT
TUB2	T1-F	AACATGCGTGAGATTGTAAGT
	T2-R	TAGTGACCCTTGGCCCAGTTG
RPB2	fRPB2-5f	GAYGAYMGWGATCAYTTYGG
	fRPB2-7cR	CCCATRGCTTGYTTRCCCAT
TEF1	EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT
	EF1-1567R	ACHGTRCCRATACCACCRATCTT

2.7 Phylogenetic tree analysis

Purification of PCR products and DNA sequencing were performed at ATGC company (Pathum Thani, Thailand). The obtained sequences were determined for the percent similarities by BLASTn in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were analyzed using the Neighbor-Joining method to generate an evolutionary tree [11, 12]. The evolutionary distances were computed using the Maximum Composite Likelihood method [13]. Evolutionary analyses were conducted in MEGA X [14].

3. Results and Discussion

3.1 The fungal pathogen in chili

The fungal pathogens causing disease in chili plants in the agricultural plantation in Pong Nam Ron district, Chanthaburi province were isolated and identified. Five fungal strains were isolated from chili and tested for pathogenic properties. Only isolate B1 was identified by morphology

(Fig. 1) and sequence analysis of ITS, GAPDH, and TUB2. The results found that fungal isolate B1 was identified as *Colletotrichum siamense,* (Fig. 2) and was thus used in further experiments.

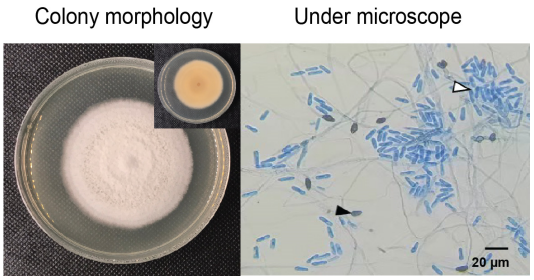

Fig. 1. Colony morphology and structure under the microscope of the isolated fungal pathogen B1. Black and white arrow head indicates appressorium and conidia, respectively.

C. siamense is a causative agent of chili disease which has previously been described. *Colletotrichum* spp.were isolated from chili with symptoms of anthracnose in Thailand [15]. Forty-eight isolates of *Colletotrichum* spp. were identified based on morphology and sequence analysis of the ITS and actin gene (ACT). They were identified as 4 species, comprising *C. gloeosporioides*, *C. siamense*, *C. acutatum,* and *C. capsici*. Additionally, the study of chili anthracnose in Asia (including Thailand) revealed *Colletotrichum* spp. isolates (260 isolates) that were identified by the morphological study and muti-gene phylogenetic analysis (including ITS, GAPDH, TUB2). The collected isolates were identified as *C. endophyticum*, *C. frucicola*, *C. karsti*, *C. plurivorum*, *C. scoveillei*, *C. siamense*, *C. tropicale*, *C. javanense*, *C. makassarense*, and *C. tainanense* [16].

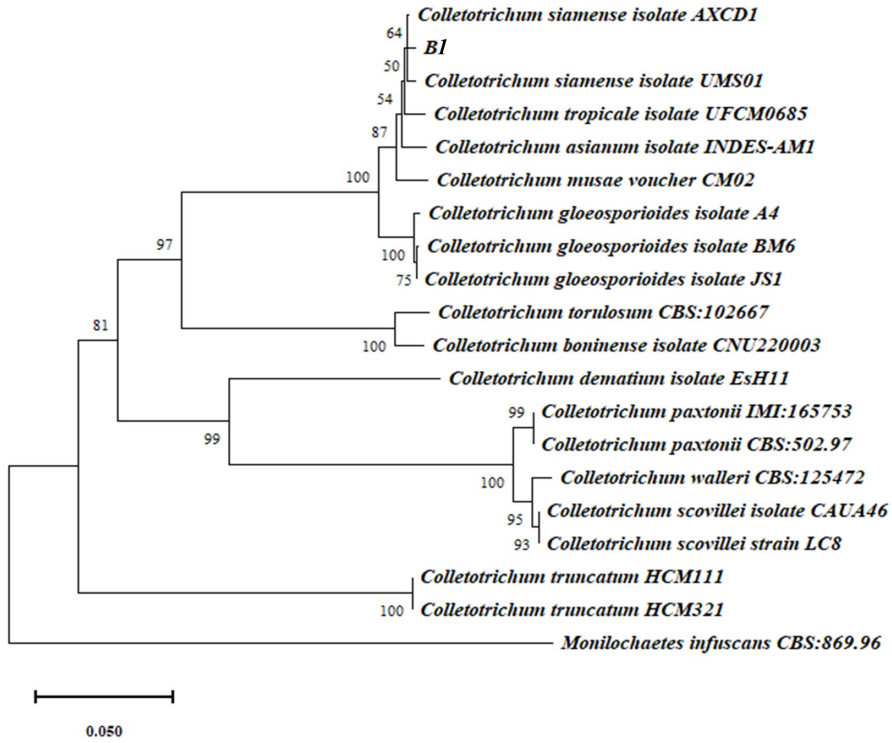

Fig. 2. Phylogenetic tree of nucleotides from fungal pathogen of chili isolate B1 base on ITS, GAPDH, TUB2.

3.2 Fungi contained in vermicompost

Vermicompost was produced by agriculturists of community enterprises in Pong Nam Ron district, Chanthaburi province, to be used in their chili plantation. It was found to have 2.64×10^5 CFU/g of total fungi and 32 fungal colonies with different morphologies were isolated. The number of total fungi in this study was not different from the previous study that explored the fungal communities in compost and vermicompost. They revealed that total fungi in compost and vermicompost were up to 8.2×10^5 CFU/g and 4.0×10^5 CFU/g, respectively [5].

Additionally, fungal species in vermicompost consisted of zygomycetes, ascomycetes, basidiomycetes, mitosporic fungi, and sterile mycelia that revealed their relative load to be 6, 4, 2, 76, and 12%, respectively. Based on the present findings, the mitosporic fungi (76%) dominated the vermicompost. The most abundant species were *Scedosporium* and *Pseudallescheria boydii,* and the genera with the highest load and number of species was *Penicillium* and *Aspergillus*. Interestingly, members of the genera *Trichoderma* were found in this study, including *Trichoderma hamatum* and *Trichoderma harzianum* [5]. *Trichoderma* spp. have previously been reported for their antifungal activity against plant pathogens including against the causative agents of anthracnose [2, 17, 18].

3.3 Vermicompost contained fungi inhibiting the fungal pathogen of chili

Thirty-two fungal colonies isolated from vermicompost were tested for antifungal activity against fungal pathogen of chili. Among these fungal isolates, 13 (40.63%) revealed antifungal activity against *Colletotrichum siamense,* a fungal pathogen of chili, and showed an inhibition percentage in the range of 46.85-72.64%. The isolates E10, E8, and F5 displayed the highest percent inhibition, with 72.75±4.81, 71.70±5.45, and 70.65±4.80%, respectively (Table 2 and Fig. 3).

In previous studies, the percentage of inhibition between *Trichoderma* spp. and *Colletotrichum capsica* was 30.87% [19]. In this study, the fungal colonies isolated from vermicompost (E10, E8, and F5) were classified in the genera of *Trichoderma* and displayed a higher percentage of inhibition than those in some other studies.

Table 2. Percent inhibition of fungal isolates from vermicompost against *Colletotrichum siamense*.

Isolates	Percent inhibition (%)
E5	66.18±4.83
E6	61.21±11.91
E7	65.41±3.15
E8	**71.70±5.45**
E9	58.13±2.79
E10	**72.75±4.81**
E19	46.85±0
F3	56.52±0
F4	66.18±4.83
F5	**70.65±4.80**
F6	54.91±2.79
F7	59.12±8.32
F12	69.6±1.82

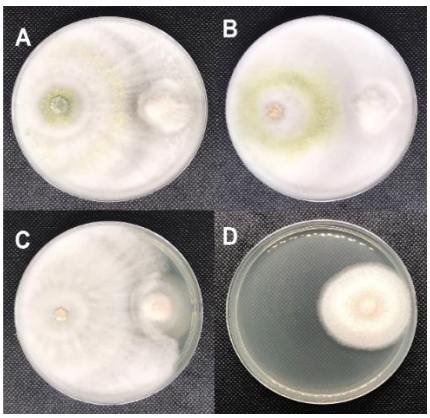

Fig. 3. Antifungal activity of some fungal isolates from vermicompost against *Colletotrichum siamense*. A: fungal isolate E10, B: fungal isolate E8, C: fungal isolate F5, D: *C. siamense* as a control.

3.4 Identification of antagonistic fungi

The fungal isolates from vermicompost with the highest antifungal properties were identified by morphological study and sequence analysis of ITS, RPB2,

and TEF1. The results found that these isolates were identified as *Trichoderma breve* (E10) and *T. zerobreve* (E8 and F5, respectively) (Figs. 4-5).

Trichoderma breve and *T. zerobreve* are found in soil [20] and have previously been reported to exhibit antagonistic properties against plant pathogens [2, 17, 18]. *T. breve* was associated with enhanced plant growth by height, leaf number, and chlorophyll index of Cocoa (*Theobroma cacao* L.), and it could colonize the root hairs of 66.67% [21]. Moreover, the efficacy of *Trichoderma* spp. against *Colletotrichum capsici*, a causative agent of chili anthracnose, in fruit infection was investigated. The results showed that all *Trichoderma* strains reduced the fruit infection rate more than the diseased control. *T. harzianum* IMI-392433 was found to be much more effective against *C. capsici* [22].

The biocontrol properties of *Trichoderma* spp. have previously been described to be based on 3 main mechanisms. Mycoparasitism, the hyphae of *Trichoderma* coil around and penetrate into the hyphae of the pathogen by producing a hook or knob-like structure (appressorium). Antibiosis, *T. harzianum* produced antibiotics like trichodermin, suzukacillin, and alamethicin that influence morphological or physiological sequences leading to its successful penetration. Finally, competition for nutrients or space to suppress plant pathogens [17, 18].

4. Conclusion

In conclusion, the fungal isolates from vermicompost (*Trichoderma breve* and *T. zerobreve*) with inhibitory activity against the chili pathogen *Colletotrichum siamense* could support the use of vermicompost on chili plantations, not only for promoting plant growth but also for use as a biological control agent against the fungal pathogens that cause chili anthracnose.

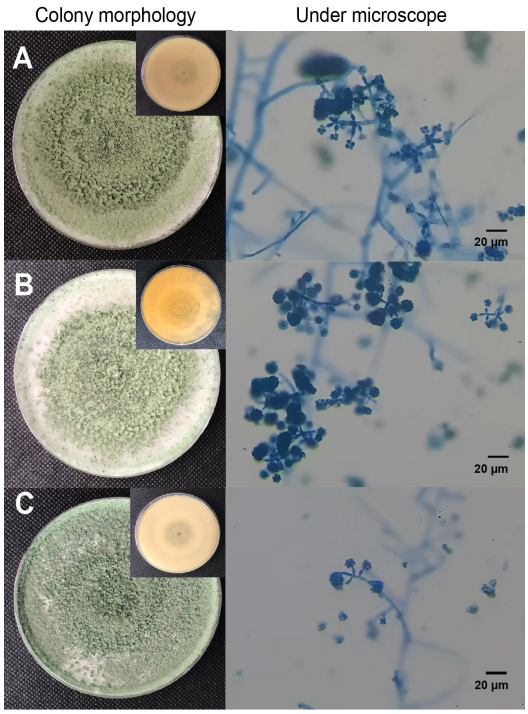

Fig. 4. Colony morphology and structure under microscope of fungi isolated from vermicompost that revealed antifungal activity. A: fungal isolate E10, B: fungal isolate E8, C: fungal isolate F5.

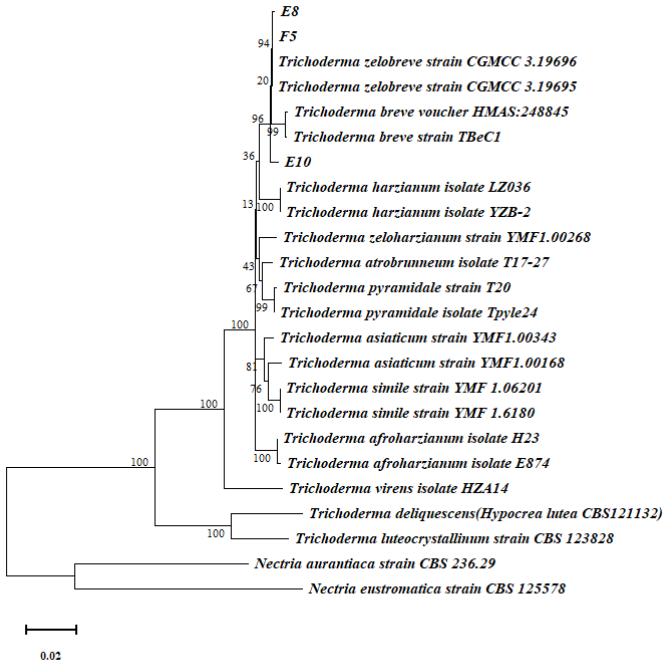

Fig. 5. Phylogenetic tree of nucleotides from fungal colonies isolated of vermicompost (isolates E10, E8, and F5) based on ITS, RPB2, TEF1.

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References

[1] Pakdeevaraporn P, Wasee S, Taylor PWJ, Mongkolporn O. Inheritance of resistance to anthracnose caused by *Colletotrichum capsici* in *Capsicum*. Plant Breeding, 2005;124(2):206-8.

[2] Saxena A, Raghuwanshi R, Gupta VK, Singh HB. Chilli Anthracnose: The Epidemiology and Management. Frontiers in Microbiology, 2016;7.

[3] Than PP, Jeewon R, Hyde KD, Pongsupasamit S, Mongkolporn O, Taylor PWJ. Characterization and pathogenicity of *Colletotrichum* species associated with anthracnose on chilli (*Capsicum* spp.) in Thailand. Plant Pathology, 2008;57(3):562-72.

[4] Pathma J, Sakthivel N. Microbial diversity of vermicompost bacteria that exhibit useful agricultural traits and waste management potential. SpringerPlus. 2012;1:26.

[5] Anastasi A, Varese GC, Marchisio VF. Isolation and identification of fungal communities in compost and vermicompost. Mycologia. 2005;97(1):33-44.

[6] AOAC. Offical Methods of Analysis of AOAC International. 18 ed. Maryland. USA2005.

[7] Yan Z, Anh V. Effect of *Trichoderma* sp. on Anthracnose Disease of Stored Chilli. Borneo Journal of Resource Science and Technology, 2018;8:90-102.

[8] Raja HA, Miller AN, Pearce CJ, Oberlies NH. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community.

Journal of Natural Products, 2017;80(3):756-70.

[9] Khodadadi F, González JB, Martin PL, Giroux E, Bilodeau GJ, Peter KA, et al. Identification and characterization of *Colletotrichum* species causing apple bitter rot in New York and description of *C. noveboracense* sp. nov. Scientific Reports, 2020;10(1):11043.

[10] Xing J-H, Sun Y-F, Han Y-L, Cui B-K, Dai Y-C. Morphological and molecular identification of two new *Ganoderma* species on *Casuarina equisetifolia* from China. MycoKeys, 2018;34:93-108.

[11] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 1987;4(4):406-25.

[12] Felsenstein J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution, 1985;39(4):783-91.

[13] Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences of the United States of America, 2004;101(30):11030-5.

[14] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution. 2018;35(6):1547-9.

[15] Suwannarat S, Steinkellner S, Songkumarn P, Sangchote S. Diversity of *Colletotrichum* spp. isolated from chili pepper fruit exhibiting symptoms of anthracnose in Thailand. Mycological Progress, 2017;16(7):677-86.

[16] de Silva DD, Groenewald JZ, Crous PW, Ades PK, Nasruddin A, Mongkolporn O, et al. Identification, prevalence and pathogenicity of *Colletotrichum* species causing anthracnose of *Capsicum annuum* in Asia. IMA Fungus, 2019;10(1):8.

[17] Mukhopadhyay R, Kumar D. *Trichoderma*: a beneficial antifungal agent and insights into its mechanism of biocontrol potential. Egyptian Journal of Biological Pest Control. 2020;30.

[18] Tyśkiewicz R, Nowak A, Ozimek E, Jaroszuk-Ściseł J. *Trichoderma*: The Current Status of Its Application in Agriculture for the Biocontrol of Fungal Phytopathogens and Stimulation of Plant Growth. International Journal of Molecular Sciences, 2022;23(4):2329.

[19] Zee Kar Y, Vu Thanh Tu A. Effect of *Trichoderma* sp. on Anthracnose Disease of Stored Chilli. Borneo Journal of Resource Science and Technology, 2018;8(2).

[20] Gu X, Wang R, Sun Q, Wu B, Sun J-Z. Four new species of *Trichoderma* in the Harzianum clade from northern China. MycoKeys, 2020;73:109-32.

[21] Chavez-Jalk A, Leiva S, Bobadilla LG, Vigo CN, Arce M, Oliva-Cruz M. Effect of Endophytic *Trichoderma* sp. Strains on the Agronomic Characteristics of Ecotypes of *Theobroma cacao* L. under Nursery Conditions Agronomy. 2022;2022:5297706.

[22] Rahman MA, Ansari TH, Alam MF, Moni JR, Ahmed M. Efficacy of *Trichoderma* against *Colletotrichum capsici* Causing Fruit Rot Due to Anthracnose of Chili (*Capsicum annum* L.). The Agriculturists, 2018;16(02):75-87.