Asian Australas. J. Biosci. Biotechnol. 2023, 8 (2), 23-29; https://doi.org/10.3329/aajbb.v8i2.66927

Asian-Australasian Journal of Bioscience and Biotechnology
ISSN 2414-1283 (Print) 2414-6293 (Online)
https://www.ebupress.com/journal/aajbb/

Article

Colletotrichum truncatum, an endophytic fungus derived from *Musa acuminata* (AAA group): antifungal activity against *Aspergillus* isolated from COVID-19 patients and indole-3-acetic acid (IAA) production

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Received: 02 May 2023/Accepted: 22 June 2023/Published: 26 June 2023

Abstract: Fungal endophyte is a fungal that lives in plant organism as mutualism association. The role of fungal endophyte is a growth promoter or/and microbial pathogen inhibitor. This study investigated antifungal activity of *Colletotrichum truncatum* E10, an endophytic fungus derived from *Musa acuminata* (AAA group), against 7 isolates of *Aspergillus* obtained from lower respiratory samples of COVID-19 patients. In addition, IAA production of this strain was also observed. All isolates of *Aspergillus* were identified using MALDI-TOF MS. The fungal endophyte, *C. truncatum* E10, was screened for IAA induction with and without 0.1, 2 and 8 mg/mL of L-tryptophan based on colorimetric method using Salkowski reagent which produced pinkish to reddish solution indicating the presence of IAA. Antagonist activity was based on dual culture assay measured in colony growth inhibition (CGI). *C. truncatum* E10 produced the highest IAA concentration of 112.81±0.12 µg/mL when 8 mg/mL of L-tryptophan added. The strong antagonist activities were shown by *C. truncatum* E10 against 5 *Aspergillus* isolates including 2 *A. fumigatus*: sp442/6 (CGI=57.83±5.11%) and sp269/11 (CGI=53.01±8.52%), 1 *A. niger* sp26/7 (CGI=57.83±15.33%) and 2 *A. flavus*: sp26/7 (CGI=56.63±13.63%) and sp36/7 (CGI=57.23±0.85%), whereas the colony growth inhibition (CGI) of other 2 isolates including *A. fumigatus* sp567/6 and *A. flavus* sp269/11 were less than 50%. In this study, *C. truncatum* E10 produced substances that inhibited human fungal pathogen including *A. fumigatus*, *A. flavus* and *A. niger*. Moreover, it can produce IAA activity. Further investigations are being conducted to expand the plant growth promotion effects and determine IAA biosynthesis pathway. For antifungal activity, the bioactive metabolites produced by this endophytic fungal isolate should be characterized to specify the effective compounds.

Keywords: fungal endophyte; *Colletotrichum truncatum*; *Musa acuminata*; IAA; *Aspergillus* spp.

1. Introduction

Fungal endophyte is a fungus which resides in healthy plant tissues and lives as mutualism association with various types of plants. By the way, endophytes will produce secondary metabolite compounds which protect host from pathogens or/and being growth promoters (Fadiji and Babalola, 2020; Baron and Rigobelo, 2022). Fungal endophytes can be found in various plant such as rice, banana and soursop (Potshangbam *et al*., 2017; Henao *et al*., 2019; Silva *et al*., 2022). There were many fungal endophyte species such as *Fusarium* spp., *Penicillium* spp., *Curvularia* spp., *Aspergillus* spp. and *Colletotrichum* spp. (Hamzah *et al*., 2018; Rashmi *et al*., 2019). The well-known secondary metabolites produced from fungal endophytes is often described in many studies in laboratory scale are auxins or Indole-3-acetic acid (IAA) as plant growth promoting compound. Each fungal endophytic strain produces IAA in a different concentration. L-tryptophan is the substrate used to induce better IAA synthesis (Numponsak *et al*., 2018; Khan *et al*., 2021; Jahn *et al*., 2021). In addition, other properties which often do the research are antimicrobials. The study of Souza *et al*. (2014) revealed that endophytes from banana enhanced tolerance to diseases via growth enhancement (Souza *et al*., 2014). Endophytic fungi could produce effective antifungal metabolites which can resolve growing invasive fungal infections (Deshmukh *et al*., 2018). At present, there is the respiratory epidemics caused by a virus including Coronavirus 2019 (COVID-19) and influenza, which is often coinfected with *Aspergillus*, complicating patient's condition. Reizine *et al*. (2021) reported that 22.5% of ICU patients admitted for severe viral infection developed influenza-associated pulmonary aspergillosis (IAPA) (23.9%) and COVID-19-associated pulmonary aspergillosis (CAPA) (20.4%) (Reizine *et al*., 2021). The objective of this study was to investigate antifungal activity of *Colletotrichum truncatum,* an endophytic fungus derived from *Musa acuminata* (AAA group) against 7 isolates of *Aspergillus* obtained from COVID-19 patients. In addition, IAA production of this strain was also observed.

2. Materials and Methods

2.1. Ethical approval

The experiment was carried out in accordance with the guidelines of Human Research Ethics Committee of Thammasat University (Science), Thailand (No. 043/2561).

2.2. The strains of fungi used in this study

C. truncatum E10, an endophytic fungus**,** was isolated from *M. acuminata* (AAA group) in Pathum Thani province, Thailand. which have no symptoms and fungal pathogens. Briefly, *C. truncatum* E10 was carried out from banana components by using sterilization technique. Small pieces of banana component were soaked in 70% ethanol for 60 seconds, 1% Sodium hyperchloride (NaOCl) for 3 minutes and rinsed for 2 times in sterile water for 30 seconds then dried on sterile paper (Photita *et al*., 2001). All components were put on potato dextrose agar (PDA) medium and observed for endophytes growth. *C. truncatum* E10 was one of endophytic fungal strains that could be isolated and identified into species level ITS gene sequencing by ITS1 primer: 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 primer: 5' TCCTCCGCTTATTGATATGC 3' to amplify ITS region and sequencing (Núñez-Trujillo *et al*., 2013). The phylogenetic tree of ITS sequence of *Colletotrichum* was presented in Figure 1.

The stocked culture of seven strains of *Aspergillus* spp., including 3 blue green colonies of *Aspergillus* sp442/6, sp567/6 and sp269/11, 1 black colony of *Aspergillus* fg26/7 and 3 light green colonies of *Aspergillus* fg26/7, fg36/7, sp269/11, were used in this study. These strains were isolated from unidentified COVID-19 patients and stocked in Microbiology Laboratory, Thammasat University Hospital, Thailand.

2.3. MALDI-TOF mass spectrometry analysis

Aspergillus were cultured on potato dextrose agar (PDA) medium for 3 days at room temperature. Hyphae and/or conidia were collected with a sterile cotton swab from 1 cm in diameter of growth and inoculated into sabouraud dextrose broth (SDB) medium on rotator at room temperature for 16-18 hours. Mycelia sediment was transferred into 1.5 mL microtube and centrifuged at 13000 rpm for 2 min. The supernatant was carefully removed and then 1 mL of HPLC-grade water was added to the pellet, mixed by vortex and centrifuged at 13,000 rpm for 2 min. After that the supernatant was carefully removed again. This washing process was done twice.

Mycelia sediment was added with 300 µL of HPLC-grade water and washed with 900 µL of 70% ethanol. The pellet was suspended by equal volume of 70% formic acid and acetonitrile, then centrifuged at 13,000 rpm for 2 min. One microliter of sample supernatant was dropped onto a MALDI target plate and dried at room temperature. One microliter of alpha-Cyano-4-hydroxycinnamic acid (HCCA) was applied and allowed to dry prior to analysis. The spectrum pattern was analysed by MALDI-TOF MS (Bruker Daltonics, Inc) with MT

filamentous fungi library 3.0 and MBT compass library revision L. MALDI-TOF MS was performed on an autoflex maX™ TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with smartbeam-II laser with FlexControl™ software 3.4 (Bruker Daltonics) for automatic acquisition of mass spectra in the linear positive mode within a range of 2 to 20 kDa. Each spectrum was acquired with 2,000 laser impulses at frequency of 200 Hz. Sample was triplicate collecting the spectra. The mass spectrometer was periodically calibrated using a Bacterial Test Standard (BTS) (*Escherichia coli* ATCC 25922). All isolates were confirmed species by MBT compass explorer, library version 4.1 (9,999 entries; Bruker Daltonics) and analyzed as score value. The score >2.0 was highly propable species identification, whereas scores 1.7-1.9 were propable genus identification and score <1.7 were not reliable identification.

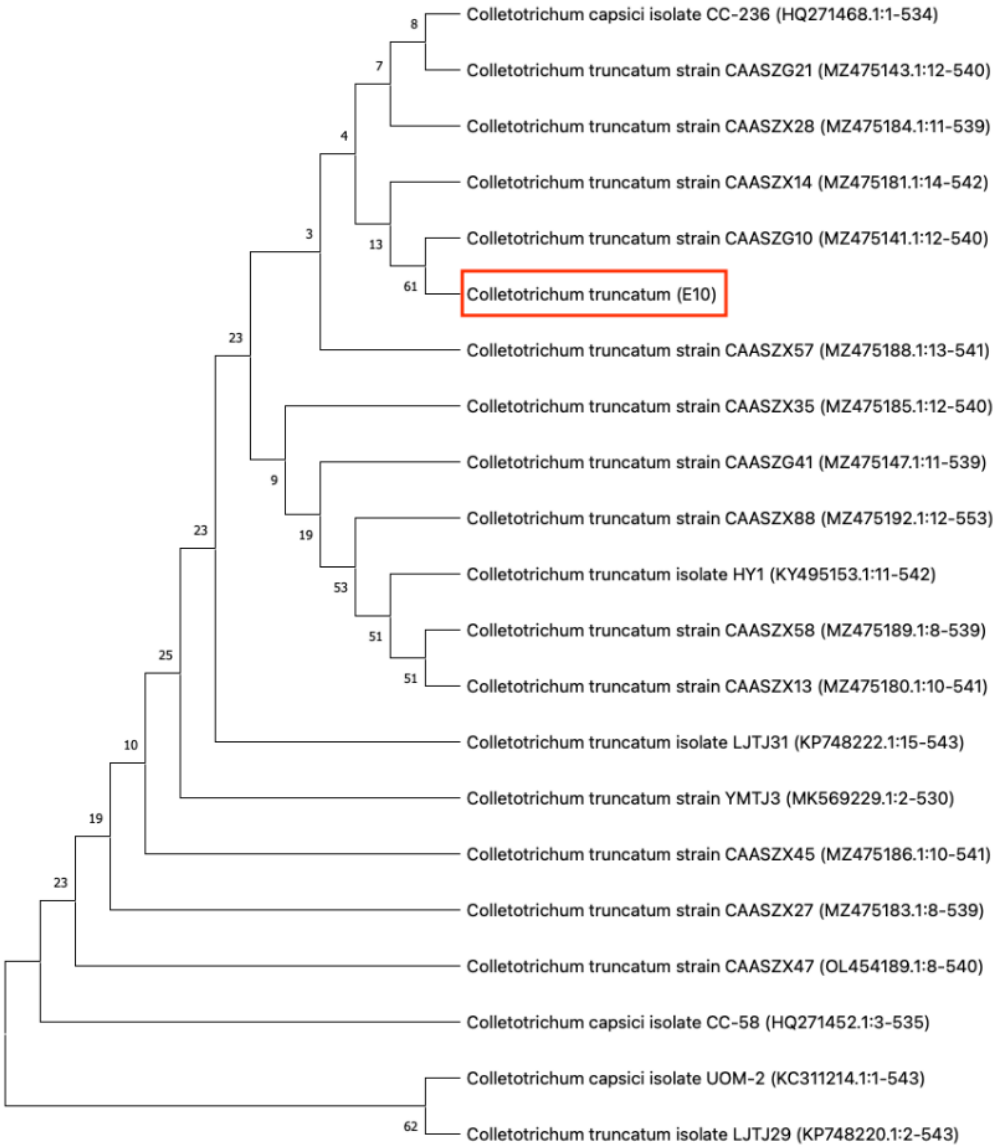

Figure 1. The phylogenetic tree showing relationship of *Colletotrichum truncatum* (E10), indicated by a red rectangle, based on the ITS region (ITS1 primer). Phylogenetic tree generated in MEGA 11 software and inferred using the neighbor-joining analysis. Numbers at branch points indicate bootstrap values.

2.4. Detection of IAA hormone

C. truncatum E10 was cultured on potato dextrose agar (PDA) medium for 7 days at room temperature. A disc (5 mm in diameter) of fungal endophyte colony was applied into potato dextrose broth (PDB) medium with 0.1, 2 and 8 mg/mL of L-tryptophan and incubated 7 days at 25ºC with dark condition in shaker with 150 rpm (Syamsia *et al*., 2017).

After 7 days, 1 mL of fungal endophyte supernatant was collected and mixed with 1 mL Salkowski reagent (12 g/L $FeCl_3$ in 429 mL/L H_2SO_4), then incubated for 24 hours in the dark condition. The color change was observed and measured an absorbance by using spectrophotometer with a wavelength of 530 nm. IAA concentrations were compared with IAA standard curve. The experiments were carried out in triplicate. Values are expressed in µg/mL.

2.5. Antifungal testing: dual culture plate

C. truncatum E10 was cultured on PDA medium for 7 days at 25°C. Then mycelial plug (5 mm diameter) of each fungus were prepared. Each plug of *C. truncatum* and *Aspergillus* was placed 5 cm apart in the opposite site and incubated at 25°C for 5-7 days. The antagonism was described as colony growth inhibition (CGI) (Ibrahim *et al*., 2017). Colony growth inhibition (CGI) or antifungal activity from endophytic fungi was calculated using following formula: CGI (%) = (R1−R2)/R1 x 100%. Where, R1 represents the diameter of the colony of *Aspergillus* (human clinical pathogenic fungi) without *C. truncatum* E10 (endophytic fungi) and R2 represents the diameter of the colony of *Aspergillus* towards the growth of *C. truncatum* E10. Each colony growth inhibition (CGI) percentage was scored as follows: CGI > 75% or Very Strong activity (++++), 75 ≥ CGI > 50% or Strong activity (+++), 50 ≥ CGI > 25% or Mild activity (++), 25 ≥ CGI > 0% or Weak activity (+), and CGI = 0% or No activity (–) (Lutfia *et al*., 2021). All experiments were performed in duplicate.

3. Results

3.1. The identification of *Aspergillus* isolates using MALDI-TOF MS

Seven isolates of *Aspergillus* were correctly identified by MALDI-TOF MS at the species level with a score >2.0 (highly propable species identification). Three blue green colonies of *Aspergillus* sp442/6, sp567/6 and sp269/11, 1 black colony of *Aspergillus* fg26/7 and light green colonies of *Aspergillus* fg26/7, fg36/7 were *A. fumigatus* (n=3), *A. niger* (n=1) and *A. flavus* (n=3), respectively. The different species of *Aspergillus* presented the different protein mass spectra as shown in Figure 2.

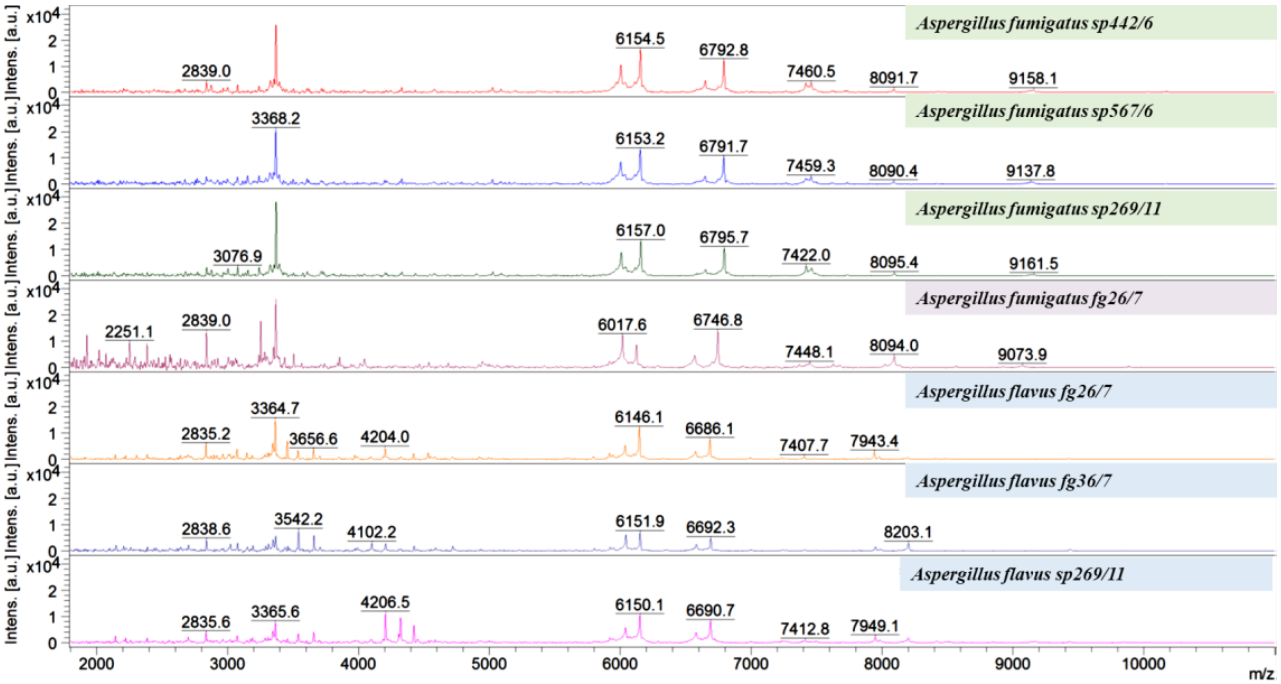

Figure 2. Protein mass spectra of *Aspergillus* spp. X-axis; m/z, Y-axis; intensity.

3.2. Detection of IAA hormone

After *C. truncatum* E10 was cultured in PDB medium with 0.1, 2 and 8 mg/mL of L-tryptophan for 7 days with dark condition, the isolates that produced IAA hormone was changed in pink color after added Salkowski reagent and incubated for 24 hours. Compared with IAA standard curve, *C. truncatum* produced the highest IAA concentration of 112.81±0.12 µg/mL in 8 mg/mL of L-tryptophan followed by IAA concentration of 107.81±0.08 µg/mL in 2 mg/mL of L-tryptophan and 23.21±0.51 µg/mL in 0.1 mg/mL of L-tryptophan.

3.3. Antagonist activity by dual culture plate assay

Antagonist activity was based on dual culture assay measured in colony growth inhibition (%). The strong antagonist activities were shown by *C. truncatum* E10 against 5 isolates of *Aspergillus* including 2 isolates of *A. fumigatus*: sp442/6 (CGI=57.83±5.11%) and sp269/11 (CGI=53.01±8.52%), 1 isolate of *A. niger* fg26/7 (CGI=57.83±15.33%) and 2 isolates of *A. flavus*: fg26/7 (CGI=56.63±13.63%) and fg36/7 (CGI=57.23±0.85%). The colony growth inhibition (CGI) of 2 isolates including *A. fumigatus* sp567/6 and *A. flavus* sp269/11 were 48.19±1.70% and 48.19±5.11%, respectively. Dual culture plate between each isolate of *Aspergillus* against fungal endophytes were shown in Figure 3.

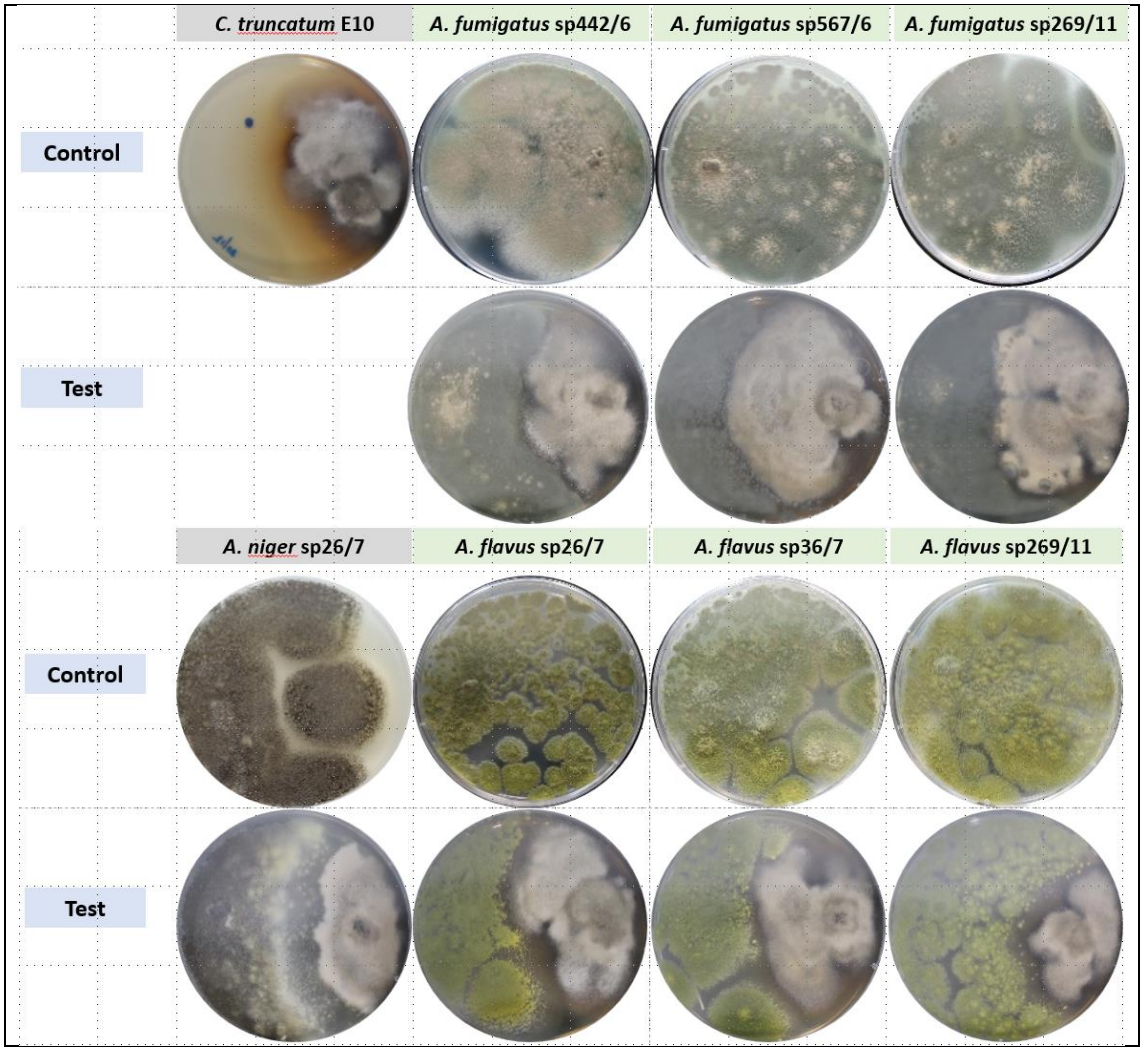

Figure 3. Dual culture plate assay between each *Aspergillus* isolates (left), and *Colletotrichum truncatum*, a fungal endophyte (right). Dual culture plate assay between each *Aspergillus* isolates (left), and *Colletotrichum truncatum* E10, a fungal endophyte (right). Control: *Colletotrichum truncatum* E10 and each *Aspergillus* isolate was cultured on PDA for 7 days, Test: the colony of *Aspergillus* was inhibited by the growth of *Colletotrichum truncatum* E10.

4. Discussion

The IAA produced from various fungal endophytes including *Colletotrichum* spp. was often described in many studies as plant growth promoting compound (Numponsak *et al*., 2018). Each fungal endophytic strain produces IAA in different concentration range from 23.21±0.51 µg/mL to 112.81±0.12 µg/mL which is lower than previous study that isolating endophytic fungi from *Lilium davidii* found that *Acremonium* sp. Ld-03 showed IAA production ranged from 53.12±3.20 µg/mL to 167.71±7.12 µg/mL under different tryptophan concentrations from 0 mg/mL to 4 mg/mL exogenous tryptophan (Khan *et al*., 2021). Our study found that L-tryptophan, an efficient precursor for IAA biosynthesis was the substrate used to induce better IAA synthesis like previous studies (Numponsak *et al*., 2018, Jahn *et al*., 2021). The IAA concentration increased from

862.26±28.03 µg/mL to 1205.58±151.89 µg/mL after extending the incubation time from 0 to 26 days with 8 mg/mL tryptophan (Numponsak *et al*., 2018). Other than L-tryptophan concentration, IAA production could be influenced by many factors, including incubation temperature and pH (Lebrazi *et al*., 2020). Together with plant growth promoting compound produced from *C. truncatum* E10 in this study, it also produces substances that could inhibit human fungal pathogen including *A. fumigatus*, *A. flavus* and *A. niger*. The respiratory epidemics caused by a virus including COVID-19 and influenza, which is often coinfected with *Aspergillus*, complicating patient's condition. The emergence of azole-resistant *A. fumigatus* COVID-19-associated pulmonary aspergillosis was reported in an immunocompromised patient admitted in ICU patient (Meijer *et al*., 2020). Other endophytic fungal isolates were strong antagonist activity against human pathogenic bacteria such as *S. aureus* and *E. coli*, especially, the isolate of Gp07, *Colletotrichum siamense*, presented the wide range of antibacterial activities (Lutfia *et al*., 2021). The bioactive compounds from *Aspergillus niger*, yanuthone D, K, L, M and X2, displayed antifungal activity toward *Candida albicans* with yanuthon D was the most active property (IC50 = 3.3 µM) (Petersen *et al*., 2014, Holm *et al*., 2014). In addition, the Omomowo *et al.* (2023) reviewed the different bioactivities of several compounds synthesized from several fungal including anti-microbial activity such as phomoenamide and phominitroester from *Phomopsis* spp. with antitubercular activity, 7-amino-4-methylcoumarin from *Xylaria* spp. with wide antifungal properties and so on (Omomowo *et al*., 2023).

5. Conclusions

In our study, *C. truncatum* E10 produced substances that inhibited human fungal pathogen including *A. fumigatus*, *A. flavus* and *A. niger*. Moreover, it can produce IAA activity. Further investigations are being conducted to expand the plant growth promotion effects and determine IAA biosynthesis pathway. For antifungal activity, the bioactive metabolites produced by this endophytic fungal isolate should be characterized to specify the effective compounds.

Acknowledgements

The authors gratefully acknowledge the financial support provided by Thammasat University Research Fund under the TU Research Scholar, Contract No. 81/2561.

Data availability

Data are contained within the article.

Conflicts of interest

None to declare.

Authors' contribution

Panarat Hematulin and Thirawatthana Pharamat: assisted in data collection and gathering information; Jiraporn Yansombat and Chollanant Khattiyawech: designed the experiment, analyzed the data, and wrote the draft of this manuscript; Worada Samosornsuk, Seksun Samosornsuk and S.M. Lutful Kabir: supervised and revised the final manuscript. All authors have read and approved the final manuscript.

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