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Research Article
Identification of *Colletotrichum* Species Associated with Chili Anthracnose in Indonesia by Morphological Characteristics and Species-Specific Primers

Widodo and Sri Hendrastuti Hidayat

Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University (Institut Pertanian Bogor), Jalan Kamper, Kampus IPB Darmaga-Bogor 16680, Indonesia

Abstract

Background and Objective: Anthracnose disease on chili pepper caused by *Colletotrichum* spp. is known as one of the devastated disease world-wide including Indonesia. This study was carried out to identify *Colletotrichum* species recovered from chili pepper fruits with anthracnose symptoms collected from some areas in Indonesia through morphological and molecular approaches. **Materials and Methods:** Infected fruit samples showed anthracnose symptom from several areas were isolated and purified using single spore methods. All of the isolates were then characterized based on conidial shape and colony color. The effect of temperature on the radial growth of six of the isolates represented three conidial morphotypes was determined. **Results:** Ninety seven single-spore isolates of *Colletotrichum* from chili pepper fruits were identified as *C. acutatum*, *C. gloeosporioides* and *C. capsici* based on morphological characters and/or polymerase chain reaction using species-specific primer. All isolates identified as *C. acutatum* produced tapering conidia and developed orange colony color. Meanwhile, conidia of isolates identified as *C. gloeosporioides* showed cylindrical shape with rounded on both ends and developed grey or olive gray colony colors. Among *Colletotrichum* species collected in this study, *C. capsici* was easily differentiated from two other species by its typical falcate conidial shape. Of the 6 tested isolates representing three species, *C. acutatum* showed the slowest growth rate in plate culture on potato dextrose agar. **Conclusion:** Out of 97 isolates collected in this study, *C. acutatum* was the most common species recovered from several chili pepper areas in Indonesia, then followed by *C. capsici* and *C. gloeosporioides*, respectively.

Key words: Anthracnose, *Colletotrichum*, chili pepper fruits , morphological characters, species-specific primer

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Corresponding Author: Widodo, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University (Institut Pertanian Bogor), Jalan Kamper, Kampus IPB Darmaga-Bogor 16680, Indonesia Tel: +62-251-8423048

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The genus of *Colletotrichum* is one of the common plant pathogenic fungal causing variety of plant diseases and serious problem for crop production in the tropics and subtropics world-wide[1]. Many kind of crops[2-8], either annual or perennial, have been reported as the target host of this fungus, including chili pepper[9]. Anthracnose of chili pepper caused by *Colletotrichum* spp. is considered as one of the major threats of the chili production in Indonesia and causing losses varied from approximately 10-80% and 2-35% in wet and dry season, respectively[9]. Up to now, the causal agents of chili anthracnose in Indonesia was identified as *Colletotrichum capsici* and *C. gloeosporioides*[10]. However, Wang *et al.*[11] found other species, i.e., *C. acutatum* from chili anthracnose in Indonesia based on Polymerase Chain Reaction (PCR) detection using species specific primers.

Differentiation between *Colletotrichum* species was commonly performed based on differences in morphological features such as size and shape of conidia, presence or absence of setae, colony color, optimal temperature, growth rate and existence of the teleomorph, *Glomerella*. This approach will be appropriate when the distinct traits were existed[12-15]. Due to the influences of environmental factors on the stability of morphological traits, sometimes these criteria were not adequate and reliable as differentiation characters among species of *Colletotrichum* and other fungi[16]. Among the most common *Colletotrichum* species reported on chili pepper, *C. capsici* is very easy to identify based on its falcate conidial shape which is very distinct with two other species, i.e., *C. gloeosporioides* and *C. acutatum.* However, conidial based identification for *C. acutatum* and *C. gloeosporioides* sometime is very difficult to differentiate between those two species.

In Indonesia, there is little information concerning the population structure of *Colletothrichum* involved in anthracnose disease in some growing areas of chili pepper. This information is necessary for designing the control measures strategies, e.g., breeding program and the strategy in application of fungicides. The research described here, was undertaken to identify and to find out the population structure of *Colletotrichum* species associated with anthracnose disease of chili pepper from some production areas in Indonesia based on some morphological characters and Polymerase Chain Reaction (PCR) using the species specific primers.

MATERIALS AND METHODS

Isolation and culture collection: Isolates of *Colletotrichum* collected from 23 districts in 8 provinces in Indonesia were examined during this study. Isolation was carried out by three methods, depending on the materials to be isolated, including diseased fruit with or without visible sporulation and seeds collected from diseased fruits. Isolates were obtained from fruits without visible sporulation and seeds from infected fruit using the procedure described by Photita *et al.*[17] with slight modification to the media used. Minor modification over Photita *et al.*[17] include: Addition of lactic acid 20% to the media and surface disinfection of diseased fruits by wiping with 70% alcohol wetted cotton before slicing into small pieces. Meanwhile, surface disinfection of infected seeds were performed by dipping in 5% sodium hypochlorite for 5 min and washing in 2 series of sterile water. All the disinfected materials were then placed on the surface of Potato Dextrose Agar (PDA) (Oxoid Ltd.) mixed with two drops of 20% lactic acid per Petri dish. Plates were incubated at room temperature (28-30°C) under continuous fluorescent light. The fungi were identified following sporulation and single-spore isolation on Water Agar (WA) (Oxoid Ltd.) was performed using the procedure described by Choi *et al.*[18]. Meanwhile, direct observation and single-spore isolation from sporulating diseased fruits was carried by touching a sterilized wire loop on the spore masses and streaking onto the surface of WA plates and were then incubated overnight (±16 h). All cultures were kept on PDA slant as stock culture for further experiments.

Conidial shape: Conidial shape characteristics were examined from the isolates grown on PDA for 8-9 days at room temperature under continuous fluorescent light. A conidial suspension was prepared from the culture by scrapping the colony surface and suspended in sterile distilled water. The shape of conidia of each isolate was then determined by examining 100 randomly chosen conidia and placing each in one of the following shape categories: (1) Fusiform, sides tapered to a point on both ends, (2) Cylindrical, sides straight with conidia pointed on one end and rounded on the other, (3) Cylindrical, sides straight with conidia rounded on both ends and (4) Conidia falcate, pointed on both ends.

Cultural characteristics and temperature response: Initial test of the characteristics of the culture and its response to

temperature was performed on six representative isolates suspected of belonging to the three species commonly found in chili pepper based on conidial shape. Single colonies of each isolates were initiated by inverting a 4 mm mycelial plug onto PDA in disposable plastic petri dishes at room temperature under continuous fluorescent light. Colony color was determined by examining the cultures after 7 days growth.

The effect of temperature on radial growth of the first six selected isolates was determined using the modified method of Smith and Black[14]. Four plates of six selected isolates were grown on PDA in the dark at 16, 18, 28, 32 and 36°C. A 4 mm mycelial plug from 7 days old culture of each selected isolates were initiated on PDA and incubated in room temperature for one day before being placed in incubators. The resultant radial growth of each isolate was measured 5 days after incubation.

Molecular identification using specific primer

Extraction of total DNA: Total DNA was extracted from *Colletotrichum* isolates following a procedure described by Doyle and Doyle[19] with minor modification, i.e., isopropanol and ammonium acetate was added in the same time during DNA extraction, overnight incubation for DNA precipitation and resuspension of DNA pellet on TE buffer without RNAse. Fresh 0.1 g mycelial tissue harvested from 7 days old culture on Potato Dextrose Broth (PDB) was ground in liquid Nitrogen to powder, 500 µL of CTAB buffer (10% Cetyl-trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 0.05 M EDTA, 0.5 M NaCl, 1% β-mercapto-ethanol) was added and the extract was transferred to 1.5 mL clean tube. The extract was incubated in water bath at 65°C for 1 h, then shaked every 10 min to separate lipid and protein. About 500 µL of chloroform/iso-amyl alcohol (24:1, v/v) was added to the liquid, then tube was vortexed for 5 min and centrifuged at 14000 rpm for 15 min. The supernatant was pipetted to 1.5 mL clean tube, 3 M ammonium acetate and isopropanol of 1/10 and 2/3 volume supernatant, was added, respectively. The liquid was mixed gently then incubated overnight at -20°C or 4 h at room temperature. After incubation, the liquid was centrifuged at 12000 rpm for 10 min to precipitate DNA and then discarded flow-through. The pellets were washed with 500 µL of 70% ethanol, centrifuged at 8000 rpm for 5 min and dried under room temperature after discarding the flow through. Dried pellets containing total DNA were dissolved in 50-100 µL of nuclease free water or TE buffer (pH 8) and the DNA was ready for amplification.

Amplification: Amplification of total DNA using specific primer pairs was conducted following method described by AVRDC[20] to confirm *Colletotrichum* species. The three specific primer pairs, i.e., CaInt2/ITS4, CcInt2/ITS4 and CgInt/ITS4, for *C. acutatum*, *C. capsici* and *C. gloeosporioides*, respectively (Table 1). The PCR reaction contains 10×PCR Buffer, 25 mMMgCl, 2.5 mMdNTPS, 10 µM each of primer, *Taq* polymerase (5 U μL^{-1}), 1 µL of DNA and the reaction was adjusted to 25 µL with nuclease free water. Amplifications was performed in GeneAmp PCR System 9700 machine with 5 min at 94.0°C for pre-heating, followed by 30 cycles of denaturation (1 min at 94.0°C), annealing (1 min at 46.0°C) and extension (2 min at 72.0°C). The last cycle was ended at 72.0°C for 10 min and cooled down to 4.0°C. Electrophoresis was done using 1% Agarose gel in 0.5×TBE (Tris-Boric acid-EDTA) buffer, run at 50 V for 50 min. Following electrophoresis, agarose gel then was soaked on to 0.1% EtBr for 5 min, washed with H_2O and visualized under UV transilluminator.

RESULTS

Colletotrichum isolates and morphological characteristics In this study, 97 isolates of *Colletotrichum* were recovered from chili pepper growing areas of 23 districts in 8 provinces (Table 2). Based on the conidial morphology, the isolates in this study were separated into three morphotypes. Morphotype 1 recovered the isolates that produced setae on PDA (Fig. 1a) and falcate-conidia (Fig. 1b); morphotype 2 represented by isolates produced cylindrical-conidia with rounded on both ends (Fig. 1c, d) and morphotype 3 isolates produced cylindrical-conidia with pointed end (Fig. 1e, f). The falcate conidial morphology is distinct characteristic of *C. capsici* which commonly occurred on chili pepper (Fig. 1b) and easily produced setae on media (Fig. 1a). All the isolates grouped into morphotype 2 and 3 failed to produce setae in culture. Most of the isolates forming falcate conidia (morphotype 1) formed grey cottony mycelium with olive grey to dark grey bottom color (Fig. 2a, b left). Colony color of morphotype 2 isolates showed olive gray cottony mycelium,

Table 1: Specific primers for amplification of *Colletotrichum* sp. using polymerase chain reaction[17]

Primer	Sequence	Target
Colletotrichum-F	5'-TCCTCCGCTTATTGATATGC-3'	
CaInt2 (R)	5'-GGCGCCGGCCCCGTCACGGGGG-3'	*C. acutatum*
CcInt2(R)	5'-TCTCCCCGTCCGCGGGTGG-3'	*C. capsici*
CgInt (R)	5'-GGCCTCCCGCCTCCGGGCGG-3'	*C. gloeosporioides*

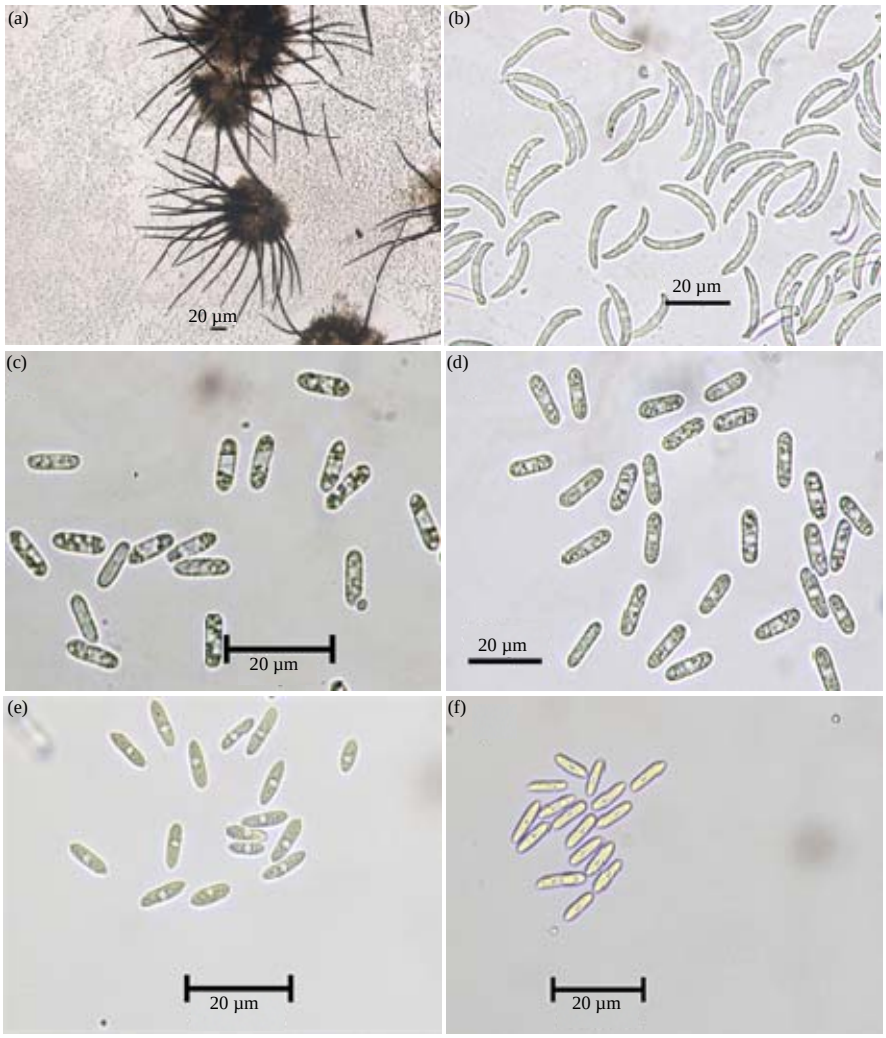

Fig. 1(a-f): Conidial characteristics of selected *Colletotrichum* isolates from chili pepper, (a) Setae formation of morphotype 1, (b) Morphotype 1 produced falcate conidia, (c, d) Morphotype 2 produced cylindrical with rounded on both ends conidia and (e, f) Morphotype 3 produced cylindrical with pointed end conidia

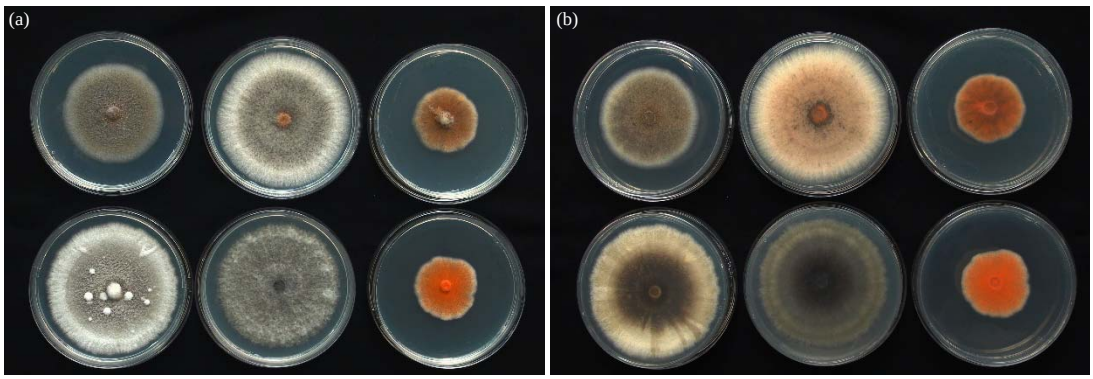

Fig. 2(a-b): Colony characteristics of 7 days old culture *Colletotrichum* isolates from chili pepper under continuous fluorescent light, (a) Upper colony surface and (b) Lower colony surface of *C. capsici* isolate BGR 1303 and BGR 11132 represented morphotype 1 (left), *C. gloeosporioides* isolate TGM 1105 and BGR 11133 represented morphotype 2 (middle), *C. acutatum* isolate BL 1303 and SMG 135 represented morphotype 3 (right)

Table 2: Molecular identification of *Colletotrichum* species collected from some chili pepper areas in Indonesia using species-specific primer and conidial shape

		Species		
Province	Number of isolates	*C. acutatum*	*C. capsici**	*C. gloeosporioides*
West Java	28	12	15	1
Central Java	31	27	3	1
Jogjakarta	4	3	0	1
East Java	9	5	1	3
West Sumatera	15	6	7	2
Lampung	6	2	0	4
Bangka- Belitung	2	0	2	0
Bali	2	2	0	0
Total	97	57 (58.76)**	28 (28.87)	12 (12.37)

*Some isolates identified based on its distinct falcate conidial shape, **Number in parentheses showed the percentage

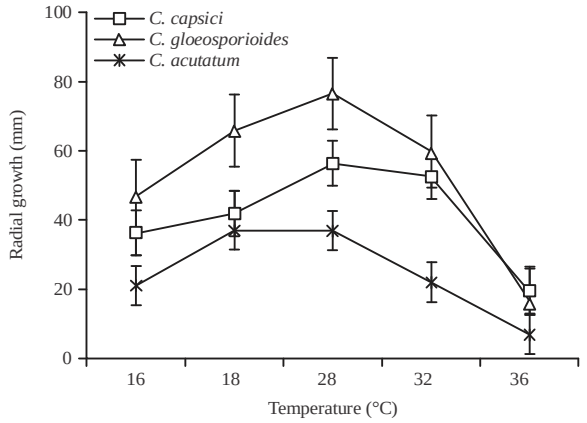

Fig. 3: Average colony diameter of 5 days old petri plate cultures of two *C. capsici* isolates (BGR 1303, BGR 11132), two *C. gloeosporioides* isolates (BGR 11133, TGM 1105) and two *C. acutatum* isolates (SMG 135, BL 1303) identified based on conidial shap

sometimes showing luxuriant orange conial masses with beige or gray to dark gray when cultures were viewed from the bottom (Fig. 2a, b middle). The colony color of morphotype 3 isolates showed flattened mycelium, colony color were orange or orange with grayish tinge when plate cultures viewed both from above or the reverse side (Fig. 2a, b right).

Temperature response: Two representative isolates of each morphotype were tested in the study of temperature effect on radial growth. As shown in Fig. 3, the mean growth of two isolates (TGM 1105 and BGR11133) represented morphotype 2 (*C. gloeosporioides*) were significantly fastest at the temperature range between 16 and 36°C; whereas two isolates (BL1303 and SMG135) of morphotype 3 (*C. acutatum*) showed the slowest radial growth on PDA in plate culture in all temperature ranges (Fig. 3). Two isolates represent morphotype 1 (*C. capsici*) showed the radial growth rate between the other two morphotypes at temperature range of 16 and 32°C.

Identification using specific primers: Molecular detection using species-specific primers was performed to confirm morphological- and temperature response-based identification of above six isolates. The species-specific primers CcInt2/ITS4 amplified a 460 bp fragment from two *Colletotrichum* isolates, i.e., BGR 1303 and BGR 11132, which was identified as *C. capsici* based on specific falcate conidial shape. There was no DNA amplification from four other tested isolates (Fig. 4a). The species-specific primers CgInt/ITS4 was successfully amplified 450 bp DNA fragments from two isolates, i.e., BGR 11133 and TGM 1105 (Fig. 4b); whereas a 490 bp DNA fragment were amplified from the remaining two selected isolates, i.e., SMG 135 and BL 1303, with species-specific primer CaInt2/ITS4 (Fig. 4c). Identification of isolates by PCR in present study was in agreement with identification of six isolates based on morphological characteristics, especially conidial shape and the response of isolates to temperature. These results showed that molecular tools such as PCR using species-specific primers are valuable in distinguishing species of *Colletotrichum* that can not be easily differentiated based on morphological characters.

***Colletotrichum* species of chili pepper in Indonesia based on molecular identification using species-specific primer and/or morphological characteristics:** Isolates of *Colletotrichum* collected from several places in Indonesia was identified using combination of morphological characteristics and molecular technique. Distinct falcate conidial character and formation of setae in fungal culture was used to differentiate *C. capsici* from the other two species; whereas identification of *C. acutatum* and *C. Gloeosporioides* was performed using of species-specific primers, i.e., CaInt2/ITS4

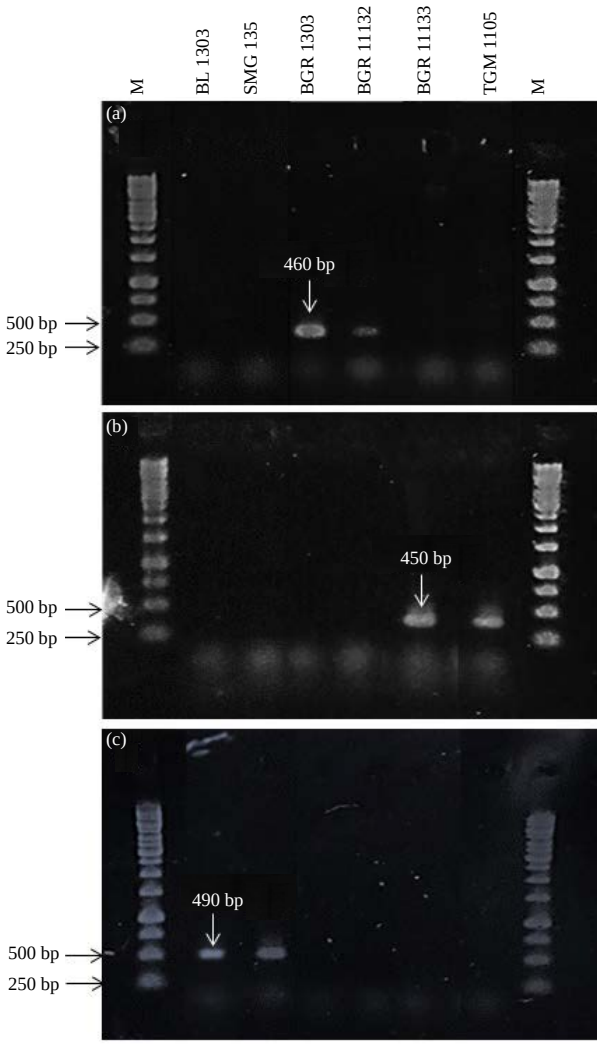

Fig. 4(a-c): Initial test using species-specific primers (a) CcInt2/ITS4, (b) CgInt/ITS4 and (c) CaInt2/ITS4 for identification of *Colletotrichum* isolates suspected as *C. capsici* (BGR 1303 and BGR 11132), *C. gloeosporioides* (BGR 11133 and TGM 1105) and *C. acutatum* (BL 1303 and SMG 135) based on morphological characters
M: 1 kb marker (Thermo Scientific, US)

and CgInt/ITS4, respectively. Out of 97 isolates of *Colletotrichum* species collected from 8 provinces and 23 districts in Indonesia, the majority isolates (58.7%) were identified as *C. acutatum* and the others as *C. capsici* (28.9%) and *C. gloeosporioides* (12.4%) (Table 2).

DISCUSSION

Identification of *Colletotrichum* isolates from chili pepper in this study was performed by combining morphological traits, including conidial morphology, colony color and presence or absence of setae; temperature response to radial growth rate and PCR methods using species-specific primer. On the basis of conidial morphology, we separated 97 *Colletotrichum* isolates into three species: *C. capsici*, *C. gloeosporioides* and *C. acutatum*. Among of three species, *C. capsici* was easily differentiated with two other species by its falcate conidia and the presence of setae. Two other isolates groups characterized by cylindrical conidia with rounded or pointed ends were fit well within the range of descriptor used for *C. gloeosporororioides*[14] and *C. acutatum*[14,21]. Conidial based identification sometimes is not consistent enough for discerning species with similar conidial shape, e.g., *C. gloeosporioides* and *C. acutatum*. In general conidia of *C. acutatum* are cylindrical and tapering with pointed in one or both ends; whereas

conidia of *C. gloeosporioides* are cylindrical with obtuse ends[22]. During this study, the presence of setae within acervuli of *C. gloeosporioides* and *C. acutatum* did not observe. However, this character might not be significantly important in distinguishing the species since the setae formation was varied on the strain and the media used as reported by previous authors[12,23-24]. Based on colony color, *C. acutatum* was easily differentiate with two other species by the domination of orange color on both upper and underside view of colony and rarely olive as previously reported by Smith and Black[14] on other host. However, other authors[21] divided *C. acutatum* into three subpopulation based on colony color, including pink, orange and gray.

Numerous papers reported that isolates of *C. acutatum* grew at a significantly slower than *C. gloeosporioides*[14,25] and other species, i.e., *C. fragariae*[14]. Than *et al.*[26] also found that among *Colletotrichum* species associated with chili pepper anthracnose in Thailand, *C. gloeosporiodes* showed the fastest growth followed by *C. capsici,* while *C. acutatum* was the slowest. In this study, similar results was also obtained and suggested the response of temperature might be worthy for differentiating the three species of *Colletotrichum* of chili pepper. However, according to Freeman *et al.*[22], differences in growth rate and optimal growth temperature is not always a reliable criterion for species identification. The authors stated that the use of species-specific primers for PCR amplification of unique rDNA fragments seems the most promising method[22].

Results in this study showed the most predominant species of *Colletotrichum* causing chili pepper anthracnose in several areas in Indonesia was *C. acutatum*. Based on morphological descriptions, chili anthracnose in Indonesia reported before 2007 was caused by *C. gloeosporioides* could have been caused by *C. cutatum.* According to Suryaningsih *et al.*[10], *C. acutatum* was not stated as the species causing anthracnose on chili pepper in Indonesia. Results of this study is not the first report on the existence of *C. acutatum* in Indonesia since it has been determined earlier by Wang *et al.*[11] with limited sample. However, there are no specific reports in Indonesia explaining the distribution and composition of the species in wider chili pepper areas. In current study here indicated that *C. acutatum* is an emerging species that may threaten the production of chili pepper and other crops where this species become established. Additional studies concerning the etiology and epidemiology of this species in Indonesia should be performed to describe management strategies of chili anthracnose in the future. Based on this study, plant breeders should aware of the potential of *C. acutatum* as a major plant pathogen causing chili anthracnose when developing the new cultivars. Since this species was first identified by Wang *et al.*[11] in Indonesia in 2007, some breeding researches to develop new chili pepper cultivars resistant to anthracnose have been performed[27-29]. However, the pathogenicity variation of *C. acutatum*[30] should be considered in the development of resistant cultivars to this pathogen.

The data from this study provide a basic understanding about the population structure of *Colletotrichum* species associated with chili pepper anthracnose of different areas in Indonesia. This information hopefully will help us to develop effective control measures, including the use of resistant cultivars and the application of fungicides. Species differentiation is important for control purposes using fungicides, since sensitivity of one species to a certain fungicides (e.g., benomyl) in mixed population of *C. acutatum* and *C. gloeosporioides* as opposed to the other. It might cause incomplete control and shift their population structure[22]. Recent study[31] by same authors showed that 9 isolates of three species (*C. capsici*, *C. acutatum* and *C. gloeosporioides*) from different areas indicated the presence of varying resistance to recommended concentration of chlorothalonil, mancozeb and propineb but not to benomyl which rarely used by farmers.

CONCLUSION

The study discovered the different isolates by analyzing their conidial formation by PCR related to *C. capsici*, *C. acutatum* and *C. gloeosporioides* and found that the *C. acutatum* is most prevalent species in majority of isolates.

SIGNIFICANCE STATEMENTS

Two species of *Colletotrichum*, i.e., *C. capsici* and *C. gloeosporioides* has been known as the main causal agent of anthracnose disease on chili pepper in Indonesia for a long time. Based on samples collected from 8 provinces in Indonesia, it was confirmed that another species of *Colletotrichum*, i.e., *C. acutatum* now became the most prevalent species causing the disease. Furthermore, this study shown that the response of each species of *Colletotrichum* to different temperature will affect their morphological characters, i.e., radial growth of the culture colony. The different response of radial growth of three *Colletotrichum* species to varied temperature is expected to be helpful in identification process. This new information is very important for development of disease management strategy.

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