

Research Article

Pathogenicity of *Colletotrichum* spp. Isolated from Shallot in Special Region of Yogyakarta and Central Java

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ABSTRACT

Anthracnose disease on shallots is an important diseases caused by *Colletotrichum* gleosporioides species complex. The purpose of the study was to determine the pathogenicity of 11 *Colletotrichum* spp. isolates, originating from several shallot production centers in Central Java and Special Region of Yogyakarta, as well as detect the ability of isolates to produce specific enzymes. The pathogenicity test was carried out by inoculating shallot varieties *Tajuk*, using a spore suspension. Inoculation was carried out by spraying 10 mL of spore suspension with a density of 10⁴/mL to each plant and were then covered using clear plastic, for 24 hours. Enzyme production tested in vitro included cellulase, amylase, laccase, and protease. Results showed that all isolates tested were pathogenic and the most infectious was the isolate UGM_CIM_P from Imogiri with disease incidence of 49%. All isolates were able to show cellulase activity, with the highest activity found in the UGM_CIM_P isolate. All isolates tested showed no amylase, laccase, and protease activities.

Keywords: Colletotrichum spp.; enzyme test; pathogenicity test; shallot

INTRODUCTION

Shallots (Allium cepa var. aggregatum) is one of Indonesia's top horticultural commodity after chili (Kementerian Pertanian Indonesia Republic of Indonesia's Ministry of Agriculturel, 2023). Shallot demand increases annually respective to population growth. According to the Badan Pusat Statistika [Republic Indonesia Bureau of Statistics] (2023), shallot demand in 2022 increased by 5.12% (40.51 thousand tons) compared to previous years. Unfortunately, increases of consumer's shallot demand and consumption are not followed by production. Shallot production in Indonesia in 2022 decreased by 22.23 thousand tons compared to 2021. Decrease of shallot production also occured in Yogyakarta and Central Java. Central Java and Yogyakarta shallot production in 2022 decreased by 7,745 (1.4%) and 7,502 tons (25.2%) compared to 2021 (Badan Pusat Statistika Republic of Indonesia Bureau of Statistics], 2023).

Decrease of shallot production is caused by various factors, including antrachnose (Perez & Alberto, 2020). Anthracnose is an important disease that infect shallots. In Indonesia, anthracnose infection on shallot is reported to be caused by *Colletotrichum gleosporioides* species complex (Amrullah *et al.*, 2023; Syafitri *et al.*, 2023). Anthracnose incidence have been reported in Special Region of Yogyakarta, specifically Bantul and Sleman Regencies (Amrullah *et al.*, 2023; Syafitri *et al.*, 2023; Budiarti *et al.*, 2022).

Pathogenicity test is done to detect the ability of pathogen to cause disease (Agrios, 2005). Pathogen host infection starts from conidia attachment to host and its germination to produce appressorium. Appressorium assist pathogen to penetrate plant tissues (Sutomo *et al.*, 2022). Appressorium will produce penetrating hyphae to penetrate host cuticle layer and plant cell walls (Perfect *et al.*, 1999). After entering plant tissue, pathogen will invade and colonize host plant cells and tissue. On the surface

of infected and symptonous plants, acervulus with conidia that function for secondary inoculum and further disease spread (Peres *et al.*, 2005; De Silva *et al.*, 2017).

During infection and colonization processes, pathogens will produce enzymes that determine Colletotrichum spp. pathogenicity (Ramos et al., 2010). Enzyme that degrade plant cell walls facilitate pathogen entrance and invasion to plant tissue (Liao et al., 2012). Armesto et al. (2019) stated that laccase directly involved in C. gleosporioides penetration in coffee plants. Amylase, cellulase, and protease can also accelerate penetration processes. Velho et al. (2018) stated that amylase was used to degrade starch in apples. De Silva et al. (2021) stated that cellulase also played roles in degradation of champak plant cell walls during early stages of infection. Meanwhile, protease have a role in plant invasion (Liao et al., 2012). Herath et al. (2021) demonstrated that C. gleosporioides species complex can infect shallot bulbs. This study aimed to test pathogenicity of various Colletotrichum spp. isolates from shallot originating from Special Region of Yogyakarta and Central Java.

MATERIALS AND METHODS

Study Location and Duration

Research was conducted from April – November 2023. The in vitro test were done in the Laboratory of Phytopathology; while pathogenicity test were done in screen houses of the Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta.

Colletotricum spp. Isolate Test

Collectivichum spp. isolates used in this study were collection from the Laboratory of Phytopathology, Faculty of Agriculture, Universitas Gadjah Mada that were isolated from different shallot production center in Yogyakarta and Central Java (Table 1). Colletotrichum spp. isolates were propagated on Potato Dextrose Agar (PDA). Isolate propagation was done using a complete randomized design with 10 replications for each isolate.

Pathogenicity Test

Shallot plant variety *Tajuk* were inoculated four weeks after planting (WAP) using 10 mL of spore

suspensions with density of 10⁴/mL. Innoculation were done in the afternoon by spraying shallot leaves. Shallot plants were then covered with transparent plastic for 24 hours. Pathogenicity tests were done using a complete randomized block design with 10 replicates. Observations were done for 7 days by scoring using classifications by Amallia *et al.* (2023) (Table 2). Disease intensity was caculated using the following formula by Suryadi *et al.* (2017):

$$IP = \frac{\sum (n \times v)}{(Z \times N)} \times 100\%$$

IP = disease intensity (%); n = number of infected leaves at each category; N = number of leaves observed; Z = highest categorical class used in this study; v = category number used at each pathogen infection.

Obtained disease intensity data were then used to calculate area under disease progress curve (AUDPC) to determine disease development rate using the formula used by Granada et al. (2020) as following:

$$AUDPC = \sum\nolimits_{i = 1}^n {\frac{{{Y_i} + {Y_{i + 1}}}}{2}} \; x\; (t_{i + 1} - t_i)$$

Yi = disease intensity at day-i; Yi+1= disease intensity at day-i+1; (ti+1-ti) = time differences between two observations; n = total observations.

Appressorium Formation

Observation of appressorium formation was done by following methods from Wang et al. (2020). Spore suspensions were prepared by adding 10 mL of sterilized water to ten day old fungal colonies and scraped using an L glass to obtain a spore suspension with density of 10⁶/mL. Spore suspensions were placed on object glass and covered using a cover glass. Covered object glass were then placed in a petri dish with a wetted filter paper on its bottom to maintain humidity. Spore suspensions were incubated in the dark at room temperature and observed every 6 hours to record spore germination and formation of appressorium. Five replicates were used in this study.

Enzyme Activity Test

Cellulase activity test. *Colletotrichum* spp. were grown on carboxymethyl cellulose with composition

Table 1. Colletotrichum spp. isolates used in this study

Isolate	Origin	Geographical Location	Provinces of Origin	Planting Pattern
UGM_CKPJ_P	Kajoran, Magelang	7°30'01"- 110°05'52"	Central Java	Polyculture
UGM_CIM_P	Imogiri, Bantul	7°54'55.6" – 7°55'28.1"	Yogyakarta	Polyculture
UGM_CKP_P	Panjatan, Kulonprogo	7.8596° - 110.1579°	Yogyakarta	Polyculture
UGM_CBT_P	Sanden, Bantul	7°58'05" — 110°15'57"	Yogyakarta	Polyculture
UGM_CIM_M	Imogiri, Bantul	7°54'55.6" - 7°55'28.1"	Yogyakarta	Monoculture
UGM_CSL_M	Seyegan, Sleman	7°43'16" - 110°18'31"	Yogyakarta	Monoculture
UGM_CSL_P	Moyudan, Sleman	7°46'22" - 110°15'14"	Yogyakarta	Polyculture
UGM_CBT_M	Sanden, Bantul	7°58'05" - 110°15'57"	Yogyakarta	Monoculture
UGM_CKP_M	Panjatan, Kulon Progo	7.8596° – 110.1579°	Yogyakarta	Monoculture
UGM_CGK_M	Pl.ayen, Gunungkidul	7°56'41" - 110°32'59"	Yogyakarta	Monoculture
UGM_CBB_M	Wanasari, Brebes	6°51'52" - 109°00'20"	Central Java	Monoculture

Table 2. Anthracnose scoring categories on shallot (Amallia et al., 2023)

Score	Description		
0	No symptoms		
1	Presences of white oval symptoms of leaves or chlorosis on leaves		
2	Acervulus appear on leaf surfaces with symptoms forming consentris lessions		
3	Appearence of concav necrotic spots with acervulus, dark lession with orange or salmon-colored conidia mass		
4	Lession unite and show symptoms of dead tips.		
5	Lession spreads, leaves die-off and dry-up.		

of 10g/L CMC and 20 g/L of agar. Fungi were incubated for 4 days at 28 °C. After four days, colonies were moved to temperature of 50 °C for 16 hours. After this period, 10 mL of congo red (0.05%) and 0.1 M buffer Tris-HCl pH 8 were added for 30 minutes at 25 °C. Transparent zone formed on media were observed and used to calculate cellulolytic index (De Silva et al., 2021). Calculation of cellulolytic index (CI) was done across five replicates using the formula from Onsori et al. (2005):

$$CI = \frac{[transparent\ zone\ diameter\ (cm) - colony\ diameter\ (cm)]}{colony\ diameter\ (cm)}$$

Amylase activity test. Amylase was tested using five replication on media containing 0.2% of starch in NA (pH 6). *Colletotrichum* spp. were grown on media containing 20g/L of NA + 2g/L of starch solution. Fungi were incubated for 5 days and 1 mL of iodine was added. Amylase activity were shown by the formation of transparent zones (Velho *et al.*, 2018).

Protease activity test. Protease activity tested on *Colletotrichum* spp. grown on media composition containing 37g/L of PDA + 4g/L of skim milk with five replicates. Fungal colony were incubated at room temperature for 5 days. Observations were done by measuring halo zones formed around colonies (Velho *et al.*, 2018).

Laccase activity test. Laccase activity test was done on *Colletotrichum* spp. grown on PDA added with 0.01% guaiacol. Five replicates were used for this study. Fungal colonies were incubated at room temperature for 5 days and observed whether brown halo zones were formed around fungal colony (De Silva *et al.*, 2021).

Data Analysis

AUDPC were analyzed using an ANOVA. If significant differences were detected, a post-hoc test was done using a Duncan's Multiple Range Test (DMRT) with confidence levels of 95%. All analysis were done using IBM SPSS Statistics version 25.

RESULTS AND DISCUSSION

Pathogenicity of Colletotrichum spp. Isolates

Pathogenicity test results showed that all tested isolates were infectious and caused anthracnose symptoms on shallots variety *Tajuk* (Figure 1, Figure 2, and Table 3). Results from disease intensity observation until the seventh observation day showed that UGM_CIM_P from Imogiri, Bantul showed the highest disease intensity of 49% (Figure 3) and AUDPC value of 173.3 while

UGM_CGK_M from Gunungkidul demonstrated the lowest disease intensity of 12% and AUDPC value of 32.15 (Figure 4). Meanwhile, ANOVA analysis showed no significant difference between isolates. Symptoms that occured during pathogenicity test were similar to ones found in the field (Figure 1). Symptoms start from white and wet lessions that later spread and cause chlorosis and necrosis with concentric rings that contain orange mass conidia. Symptoms will later develop into dried leaves and dead leaf tips if no intervention

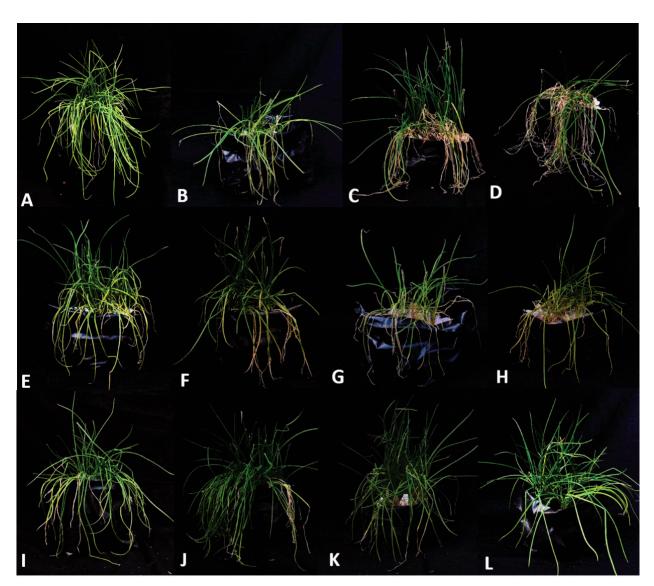


Figure 1. Anthracnose symptoms on shallot plants seven days after inoculated with *Colletotrichum* spp. A =UGM_CKPJ_P; B =UGM_CIM_P; C =UGM_CKP_P; D =UGM_CBT_P; E =UGM_CIM_M; F =UGM_CSL_M; G =UGM_CSL_P; H =UGM_CBT_M; I =UGM_CKP_M; J =UGM_CGK_M; K =UGM_CBB_M; L = Control

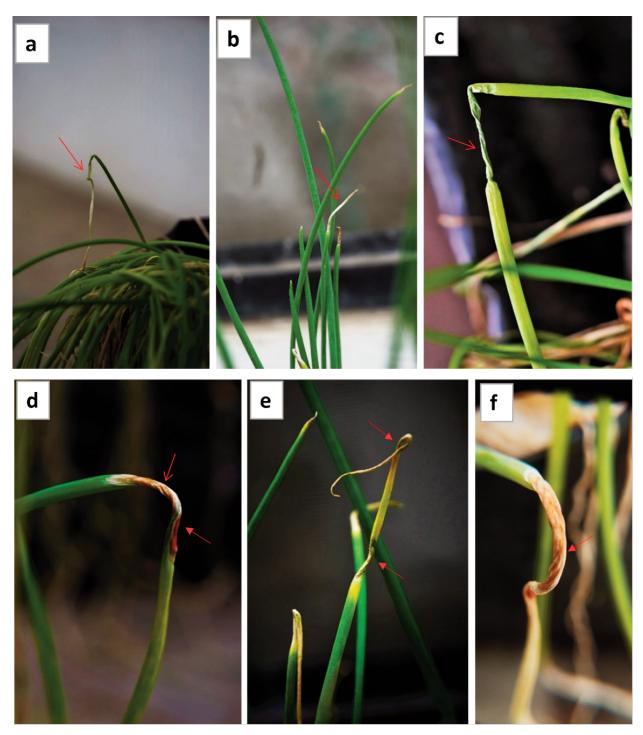


Figure 2. Anthracnose symptom variation on shallot plants seven days after inoculated with *Colletorichum* spp.: (a) dead tips, (b) tip necrosis, (c) spreaded leaf lessions that cause leaves to dry up and twirl, (d) orange conidia, (e) chlorosis and dried tips, (f) concentric rings on leaves and dead leaf tips.

are done (Figure 2e). These symptoms were consistent with ones stated by Budiarti *et al.* (2022) where anthracnose symptoms on leaves showed white oval to round lessions on shallot leaves. Untreated symptoms will develop broken and weak

leaves with necrotic lession with blackish orange conidia.

Appressorium formation tests showed that UGM_CIM_P had the ability to form appressorium 12 hours after innoculations (Figure 5 and Table 3).

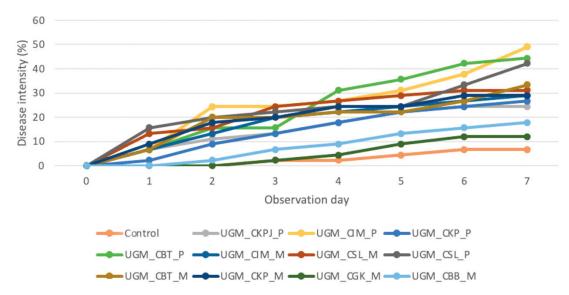


Figure 3. Anthracnose disease intensity development on shallot plant inoculated by *Colletotrichum* spp. UGM_CKPJ_P (Kajoran, Magelang; Polyculture), UGM_ CIM_P (Imogiri, Bantul; Polyculture), UGM_CKP_P (Panjatan, Kulon Progo; Polyculture), UGM_CBT_P (Sanden, Bantul; Polyculture), UGM_CIM_M (Imogiri, Bantul; Monoculture), UGM_CSL_M (Seyegan, Sleman; Monoculture), UGM_CSL_P (Moyudan, Sleman; Polyculture), UGM_CBT_M (Sanden, Bantul; Monoculture), UGM_CKP_M (Panjatan, Kulon Progo; Monoculture), UGM_CGK_M (Playen, Gunungkidul; Monoculture), UGM_CBB_M (Wanasari, Brebes; Monoculture).

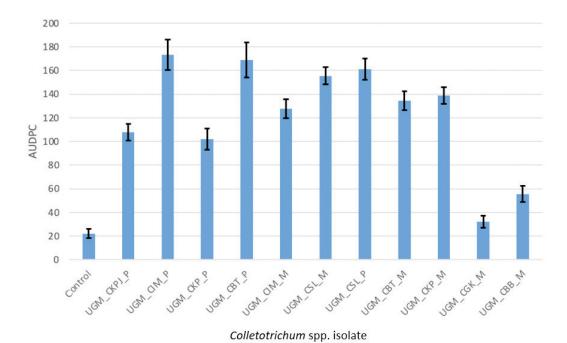


Figure 4. Area under disease progress curve (AUDPC) of anthracnose on shallot caused by *Colletotrichum* spp. UGM_CKPJ_P (Kajoran, Magelang, Polyculture), UGM_CIM_P (Imogiri, Bantul; Polyculture), UGM_CKP_P (Panjatan, Kulon Progo; Polyculture), UGM_CBT_P (Sanden, Bantul; Polyculture), UGM_CIM_M (Imogiri, Bantul; Monoculture), UGM_CSL_M (Seyegan, Sleman; Monoculture), UGM_CSL_P (Moyudan, Sleman; Polyculture), UGM_CBT_M (Sanden, Bantul; Monoculture), UGM_CKP_M (Panjatan, Kulon Progo; Monoculture), UGM_CGK_M (Playen, Gunungkidul; Monoculture), UGM_CBB_M (Wanasari, Brebes; Monoculture).

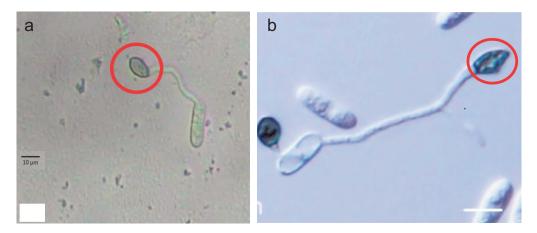


Figure 5. Appressorium formed by *Colletotrichum* conidia: (a) appressorium formed by UGM_CIM_P isolate on PDA media, 12 hours after inoculated; (b) appressorium form by *Colletotrichum gloeosporioides* according to Fu *et al.* (2019)

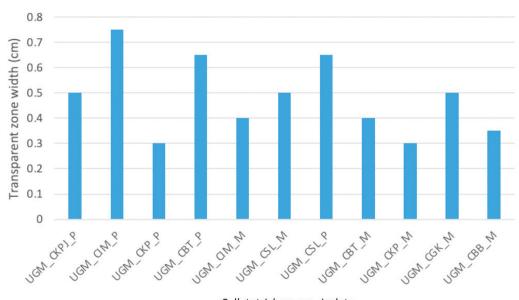
Table 3. Time required for *Colletotrichum* spp. isolates, pathogens of shallots, to form appressorium

pathogens of shallots, to form appressorium			
Isolate	Time required (hours)		
UGM_CKPJ_P	24		
UGM_CIM_P	12		
UGM_CKP_P	36		
UGM_CBT_P	12		
UGM_CIM_M	36		
UGM_CSL_M	12		
UGM_CSL_P	12		
UGM_CBT_M	24		
UGM_CKP_M	12		
UGM_CGK_M	48		
UGM_CBB_M	36		

Fukada et al. (2019) stated that Colletotrichum spp. appressorium required 6 hours. Kenny et al. (2012) reported that C. gloeosporioides and C. acutatum spore germination required 3–12 hours while appressorium forming required 6–48 hours. Appressorium formation rate affect the formation of penetrating hyphae and pathogens pathogenicity mechanisms. According to Alberto (2014), penetrating hyphae are used by pathogens to enter plant host tissue. Pathogen can enter through stomata and natural or artificial wounds or openings. After entering plant tissue, pathogen invade and colonize plant tissue and cause cell death to necrotic symptoms.

Colletotrichum spp. Isolate Enzymatic Activity

Enzyme activity test was done for cellulase, laccase, amylase, and protease. However, only cellulase demonstrated transparent zones in all isolates. UGM_CIM_P showed ability to form transparent zones of 0.75 cm (Figure 6) with cellulolytic index (0.25) that is higher than other isolates (Figure 7). This means that *Colletotrichum* spp. can degrade cellulose contained in media. This fungi has the ability to hydrolyze cellulose to simpler compounds for further use of this fungi (Akhir et al., 2022). Darwesh et al. (2020) stated that cellulose degradation in media by producing cellulase with three main process of exo-\betaglukanase, endo-β-glukanase and β-glukanase to degrade cellulose to glucose. Cellulase degrade plant cell walls during early stages of infection (De Silva et al., 2021). Results from this study demonstrated that Colletotrichum spp. were not able to degrade media tested using amylase, protease, and laccase due to shallot leaves not containing fat, starch, and protein. Sidhu et al. (2014) tested C. gloeosporioides from Piper betle where 9 from 14 tested isolates did not show laccase activity. Moraes et al. (2020) demonstrated that 29 Colletotrichum spp. isolates from soybean did not show amylase activity.



Colletotrichum spp. isolate

Figure 6. Cellulase activity of *Coletotrichum* spp. isolates from shallots.

UGM_CKPJ_P (Kajoran, Magelang; Polyculture), UGM_CIM_P (Imogiri, Bantul; Polyculture), UGM_CKP_P (Panjatan, Kulon Progo; Polyculture), UGM_CBT_P (Sanden, Bantul; Polyculture), UGM_CIM_M (Imogiri, Bantul; Monoculture), UGM_CSL_M (Seyegan, Sleman; Monoculture), UGM_CSL_P (Moyudan, Sleman; Polyculture), UGM_CBT_M (Sanden, Bantul; Monoculture), UGM_CKP_M (Panjatan, Kulon Progo; Monoculture), UGM_CGK_M (Playen, Gunungkidul; Monoculture), UGM_CBB_M (Wanasari, Brebes; Monoculture).

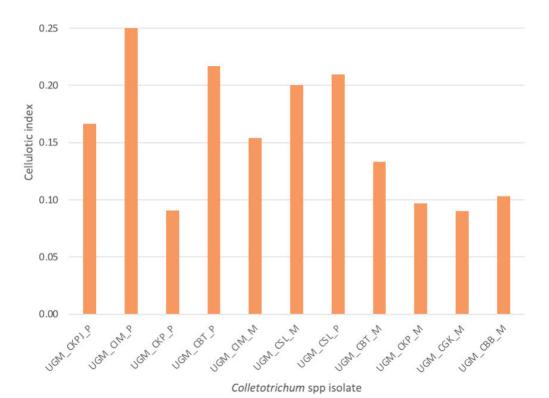


Figure 7. Cellulolytic index of *Coletotrichum* spp., cause of anthracnose on shallots.

UGM_CKPJ_P (Kajoran, Magelang; Polyculture), UGM_CIM_P (Imogiri, Bantul; Polyculture), UGM_CKP_P (Panjatan, Kulon Progo; Polyculture), UGM_CBT_P (Sanden, Bantul; Polyculture), UGM_CIM_M (Imogiri, Bantul; Monoculture), UGM_CSL_M (Seyegan, Sleman; Monoculture), UGM_CSL_P (Moyudan, Sleman; Polyculture), UGM_CBT_M (Sanden, Bantul; Monoculture), UGM_CKP_M (Panjatan, Kulon Progo; Monoculture), UGM_CGK_M (Playen, Gunungkidul; Monoculture), UGM_CBB_M (Wanasari, Brebes; Monoculture).

CONCLUSION

Pathogenicity test of 11 *Colletotrichum* spp. isolates from shallots showed that all isolates were pathogenic and UGM_CIM_P from Imogiri, Bantul was the most infectious. All *Colletotrichum* spp. isolates can form appressorium and produce cellulase, but not amylase, laccase, and protease.

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