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Variation in symptoms and morphology of *Fusarium* spp. on shallot associated with basal plate rot disease in Brebes District, Central Java Province, Indonesia

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Abstract. *Marianah L, Nawangsih AA, Munif A, Giyanto, Tondok ET. 2024. Variation in symptoms and morphology of* Fusarium *spp. on shallot associated with basal plate rot disease in Brebes District, Central Java Province, Indonesia. Biodiversitas 25: 2198-2208.* Basal plate rot disease is an important disease of shallots that causes losses in the field and storage. The disease is caused by *Fusarium* complex species, with different levels of virulence and host susceptibility. The six species of *Fusarium* spp. reported to cause basal plate rot disease of onion were *F. oxysporum* f.sp. *cepae, F. proliferatum, F. redolens, F. solani, F. acutatum* and *F. tricinctum*. Information about the variation in symptoms and morphology of fungi that cause basal plate rot disease on shallots, especially in Indonesia, is still very limited. Besides, the species that cause basal plate rot disease on shallots has not been molecularly confirmed using specific primers. The objective of this study was to isolate, identify, and study the variation in symptoms of the fungus associated with basal plate rot disease. The fungi were isolated from shallot plants symptomatic of basal plate rot disease collected from Brebes, Central Java Province. Eight isolates of *Fusarium* with different morphologies were successfully isolated and identified. Five isolates were identified as *F. solani* (isolate BC1, BC2, BC3, BBS1, and BBS4), two isolates as *F. oxysporum* f.sp. *cepae* (isolate BC4 and BBS6) and one isolate *F. proliferatum* (isolate BBS5). All eight isolates were pathogenic on shallots with different levels of virulence and symptom variations. BC4 isolate had the highest level of virulence, resulting in plant death, and was identified as *F. oxysporum* f.sp. *cepae.*

Keywords: Basal plate rot disease, *Fusarium oxysporum,* molecular identification, pathogenicity, shallots, virulence

INTRODUCTION

Basal plate rot disease is an important disease on shallots reported in Indonesia as well as several onion-producing countries such as Mexico, India, Cina, and Vietnam (Nguyen et al. 2017; Cahyaningrum et al. 2019; Shamyuktha et al. 2020; Le et al. 2021b; Tirado-Ramirez 2021). It has also been reported that disease incidence reaches 100% and yield losses reach 78%, even causing severe damage in storage (Sintayehu et al. 2014; Dinata et al. 2021; Mariani et al. 2022; Maulidha 2023). In recent years, there has even been a trend of increasing cases, and the spread of basal plate rot disease in onion production centers in several countries, including Indonesia (Wiyatiningsih et al. 2009; Safitri et al. 2019; Kalman et al. 2020; Supyani et al. 2021).

Brebes is the center of shallot production in Central Java and the largest shallot-producing province in Indonesia. Basal plate rot disease in Brebes was reported to increase disease severity on shallots from 2018 to 2020 by 9.43%, and increase in disease severity occurred especially during the rainy season of epidemic nature (Wiyatiningsih et al. 2009; Supyani et al. 2021). According to BPS Brebes District (2023), the temperature in Brebes in 2022 ranges from 24 to 32°C, and the humidity ranges from 67-94%. These conditions are very favorable for the development and infection of the fungus *Fusarium* spp. The optimum temperature for the development of *F. oxysporum* f.sp. *cepae* ranges from 24 to 30°C, and the optimum temperature to infect in the field is 25-27°C (Abawi and Lorbeer 1972; Lee and Magan 2010; Safitri et al. 2019). This disease not only attacks shallot plants but can also attack other types of onions, such as shallots (*Allium cepa*), garlic *(A. sativum),* and leeks (*A. fistulosum),* and has a wide host range (Le et al. 2021a; Kalman et al. 2020; Tirado-Ramirez 2021).

The complexity of the disease is due to different *Fusarium* species with differences in virulence and the host's degree of susceptibility (Le et al. 2021a). The study by Haapalainen et al. (2016) showed that basal plate rot disease in shallots in Finland is caused by five species of *Fusarium*, namely *F. oxysporum, F. proliferatum, F. redolens, F. Solani,* and *F. tricinctum,* and found two or more species in one of infected tuber of a diseased plant. The three *Fusarium* species, *F. oxysporum, F. Proliferatum,* and *F. redolens,* are virulent pathogens and cause many deaths in onion plants. However, *F. oxysporum* f.sp. *cepae* is the most pathogenic species on shallot plants compared to other species (Taylor et al. 2013). Basal plate rot disease on shallots in Indonesia was also reported to be caused by *F. oxysporum* f.sp. *cepae.*

Moreover, several reported a shift in the cause of basal plate rot disease on shallot bulbs in some areas. In East Java, Yogyakarta, Central Java, and South Sulawesi, basal plate rot disease is caused by two species of *Fusarium*, i.e., *F. solani* and *F. acutatum* (Lestiyani et al. 2016; Cahyaningrum et al. 2019). Herlina et al. (2021) reported that *F. solani, F. proliferatum, F. verticillioides,* and *F. pallidoroseum* cause basal plate rot disease on Bogor, Demak and Probolinggo, Java Island. Information about the causes and symptoms of basal plate rot disease in shallots in Indonesia, especially in the Brebes District, and the morphology of the *F. oxysporum* f.sp. *cepae* fungus is still very limited. Therefore, a study is still needed to investigate the cause of the disease based on molecular identification so that the information becomes more reliable. The aim of this study was to evaluate the morphology of pathogenic fungus *Fusarium* spp. caused basal plate rot disease on shallots.

MATERIALS AND METHODS

Study area

This study was conducted in the mycology laboratory and greenhouse of the plant protection department at IPB University, Bogor, Indonesia. Samples of diseased plants were taken in three sub-districts of shallot production in Brebes, namely Wanasari, Larangan, and Bulakamba.

Procedures

Isolation of pathogenic fungi

Samples of shallot were washed under running water and sterilized using a 1.5% NaOCl solution and 70% alcohol, then rinsed with sterile water three times and then dried. The sample was cut into pieces of 0.5 cm, then placed in a petri dish containing PDA media and incubated at room temperature for 3-5 days. For each growing fungus, the tip of hyphae was removed and regrown on PDA medium until a pure culture of a single spore was obtained. All acquired fungi were characterized morphologically, macroscopically by observing the growth and color of their colonies and microscopically. The observed conidia were compared with references (Bektast and Kusek 2019; Kalman et al. 2020).

In vitro pathogenicity test

The pathogenicity tests were carried out using the modified method of Haapalainen et al. (2016). Conidial suspensions of *F. oxysporum* f.sp. *cepae* were prepared from 5-day-old cultures on PDA media, and five plugs of *F. oxysporum* f.sp. *cepae* inoculum were grown in 100 mL of 50% PDB and incubated in incubator shakers at 120 rpm for 7 days. The fungus was filtered, washed using sterile water, and refiltered. The fungus hyphae mixed in 50 mL of sterile water and chopped into small pieces using an ultra-turrax shredding machine. Shallot bulbs were removed from the outer skin and sterilized on the surface by soaking in a 3% NaOCl solution for 3 minutes, 70% alcohol for 1 minute, and rinsing using sterile water three times. The bulbs were soaked in a systemic fungicide solution with active 80% mancozeb for 1 hour, then left to dry in sterile conditions for 1 week. The bulb was split vertically; one part dripped 50 µL of hyphal suspension of *F. oxysporum* f.sp. *cepae* at the base of the bulb, and another part dripped sterile water for control with the same volume. The treatment was repeated five times. The treated shallot bulbs were put in sterile boxes with moistened sterile tissue paper and incubated for 7 days at room temperature. The test result was positive if symptoms of decay occurred in the inoculated part and the control part was asymptomatic.

Pathogenicity test in greenhouse

Shallot bulbs of Bima Brebes variety ready for planting are cleaned of the outer skin, washed, and sterilized surfaces using 3% NaOCl for 5 minutes, 70% alcohol for 1 minute, and rinsed with sterile water three times. Tubers were soaked using a synthetic chemical fungicide with 80% mancozeb active ingredients at a concentration of 3 g/L for 1 hour, then drained and left to dry in a sterile incubator for 7 days. Two plugs of *F. oxysporum* f.sp. *cepae* inoculum was grown in 100 mL of 50% PDB, incubated for 7 days in a shaker at 120 rpm, then centrifuged at 5,000 rpm for 20 minutes at room temperatures. Next, the pellets were taken, added to 100 mL of sterile water, and chopped using ultra-turrax. The inoculum fungus of *Fusarium* spp., as many as 25 mL/polybag (20 x 20 cm), was poured on sterile planting media (1 kg) with a composition of soil, sand, and organic fertilizer, which is a 1:1:1 ratio. Shallot bulbs dried for 1 week and planted in polybags after 1 week of inoculation with pathogenic fungi were selected for further analyses. Two shallot bulbs were planted in each polybagand and plants kept in greenhouses and watered daily. The experiment was repeated five times. Observations were carried out daily until disease incidence reached 100%.

DNA genomic extraction

Fusarium oxysporum f.sp. *cepae* grown on PDA media aged 7 days were taken in 2 borers (diameter 15 mm), grown in 10 ml of 50% PDB media, then incubated for 5 days on a shaker at 120 rpm at room temperature. The fungus mycelia was harvested by centrifugation at 10,000 rpm for 5 minutes, and the supernatant discarded. The mycelium pellets were extracted for DNA extraction using Zymo Research Corp.'s Quick-DNATM Fungal/Bacterial Miniprep Kit based on the public protocol.

DNA genomic amplification

The identification of *F. oxysporum* f.sp. *cepae* isolates was confirmed by PCR using ITS1 and ITS4 universal primers and three specific primers, namely SIX3, C5, and CRX1 (Table 1). Amplification was carried out on a PCR machine with a reaction mixture of 25 µL containing a mixture of Bioline *My Taq Red* 2x 12.5 µL, *nucleus-free water* 9.5 µL, primary pairs of 1 µL each (20 pmol), and DNA template 1 µL. PCR amplification for universal primers was performed based on Singha et al. (2016) standard thermocycling conditions as follows: one 2-minute cycle at 94°C; 30 cycles of 45 seconds at 94°C; 1-

minute annealing at 56°C; and 1 minute at 72°C, followed by one 5-minute cycle at 72°C. PCR amplification using specific primers was carried out based on Taylor et al. (2016), which was modified at the annealing temperature. PCR amplification for all specific primers was performed under standard thermocycling conditions as follows: one 2-minute cycle at 94°C; 30 cycles of 45 seconds at 94°C, annealing 30 seconds (Table 1 for temperature); and 1 minute at 72°C, followed by one 5-minute cycle at 72°C. The PCR products were visualized using electrophoresis on 1.5% agarose gel and migration at 50 volts for 50 min, then observed under a UV transilluminator. The PCR results of universal primers were sequenced via sequencing services Genetika Science Indonesia, and ITS gene sequences were compared to fungal gene sequences taken from the NCBI database using BLAST nucleotides in GenBank. PCR amplification using specific primers was carried out on isolates whose sequence results were stated as *F. oxysporum* to obtain the *F. oxysporum* f.sp. *cepae* isolates further.

Data analysis

The observation and sequencing data were analyzed descriptively using Bioedit and MEGA7 software.

RESULTS AND DISCUSSION

Symptoms of basal plate rot diseases on shallots in the field

Symptoms of basal plate rot on shallot bulbs found in the field were divided into three symptoms. The first symptom observed was yellowish-green plant with twisted leaves and tubers did not form when pulled (Figure 1.A). The second symptom was that the leaves turn yellow-green and become flat (Figure 1.B), and the last symptom was that the diseased plant had dry leaves with rotten tubers compared to the healthy plants (Figure 1.C).

Morphological characteristics of *Fusarium* spp.

Result of isolation showed that a total of eight isolates with different macroscopic and microscopic characteristics were isolated from samples (Table 2). Isolates of BC1, BC2, and BC3 colonies were white and produced non-pigment (Figures 2.A, B, and C), while BC4 colonies were white to purple, and the colony surface was slightly wet (Figure 2.D). The BBS1 isolate colony differed greatly from the other 5 isolates due to thick, aerial mycelium growth (Figure 2.E). BBS4 colonies were similar to BC1, BC2, and BC3 colonies, but BBS4 colonies sometimes formed a purple pigment at the colony's center (Figure 2.F). BBS5 colonies were similar to BBS6 colonies as they were purple-white with thick and fin hyphal growth, but the BBS6 colonies were darker purple than BBS5 colonies, and the pigment produced was more concentrated (Figures 2.G and 2.H). Microscopic characteristics of eight isolates of *Fusarium* spp. that cause basal plate disease on shallots in Brebes have differences in both shape and size of conidia. The conidia of BC1 and BC2 isolates had one-insulated conidia with conidia length of 10.33-16.54 µm and width of 2.35-3.94 µm (Figures 3.A and 3.B). The shape of BC3 and BC4 isolates conidia was crescents and pointed, but conidia of BC4 isolate were more pointed and smaller. BC3 conidia size with a length of 9.08-15.11 µm and a width of 2.23-4.06 µm, while conidia BC4 with a length of 6.92-10.06 µm and a width of 1.13-2.82 µm. Conidia of isolate BBS1 and BBS4 were oval to crescent shaped and wide (Figures 3.E and 3.F), but the size of conidia isolate BBS1 was larger than isolate BBS4 conidia isolate. The length of conidia BBS1 was 11.48-14-42 µm and width 3.33-4.50 µm, while the length of conidia BBS4 was 7.27-12.37 µm with a width of 1.50-2.43 µm. The conidia of BBS5 and BBS6 isolates had a long and pointed shape, where the BBS5 conidia had a length of 7.61-16.51 µm with a width of 1.59-3.04 µm, and the conidia of BBS6 isolate had a length of 8.31-12.01 µm with a width of 1.94-2.78 µm (Figures 3.G and 3.H).

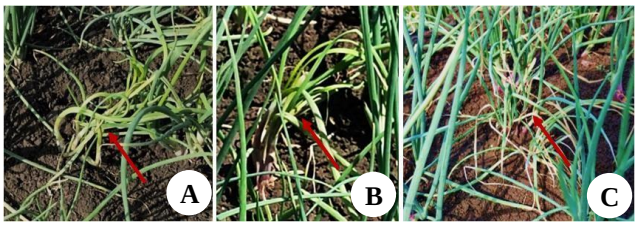

Figure 1. Symptoms of basal plate rot diseases on shallots in the field: A. Symptoms of leaf twisting; B. Stunted growth, flat and wilted leaves; C. Dried leaf tips

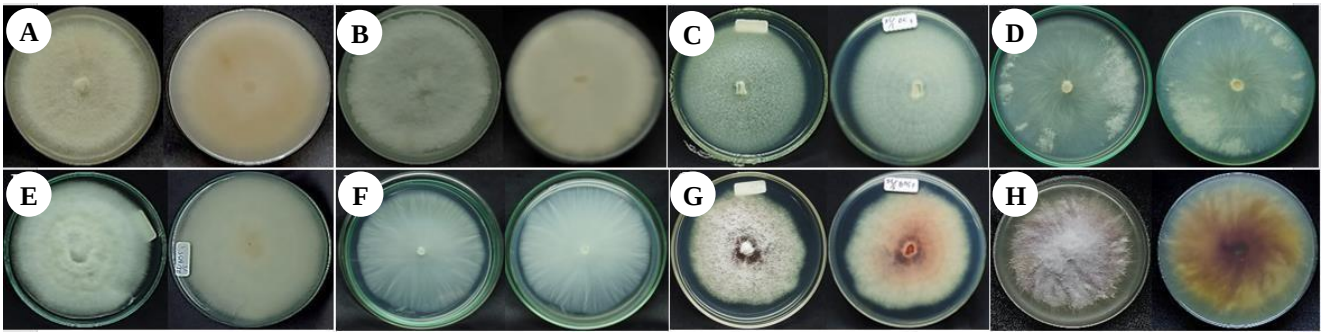

Figure 2. Macroscopic characteristics of *Fusarium* spp. isolates (Front and reverse view): A. Colony of BC1 isolate; B. BC2; C. BC3; D. BC4; E. BBS1; F. BBS4; G. BBS5 and H. BBS6

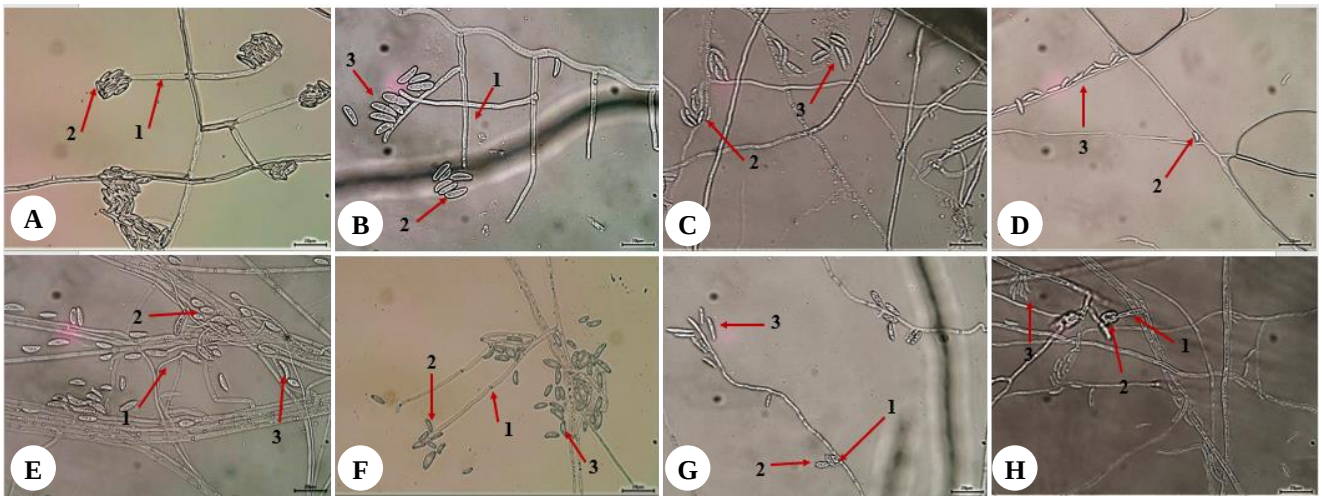

Figure 3. Microscopic characteristics of *Fusarium* spp.: 1. Conidiophore; 2. Micro conidia; 3. Macro conidia; A. BC1 isolate; B. BC2; C. BC3; D. BC4; E. BBS1; F. BBS4; G. BBS5 and H. BBS6

Table 1. Primers and PCR amplification conditions

Targets	Primers	Base sequence (5'- 3')	Annealing temperaturs	PCR products
ITS	ITS1	5'-TCC GTA GGT GAA CCT GCG G-3'	56.0°C, 1 min	550-570 bp
	ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'		
SIX3	SIX3 forward	5'-CCA GCC AGA AGG CCA GTTT-3'	52.9°C, 1 min	608 bp
	SIX3 Revers	5'-GGC AAT TAA CCA CTC TGCC-3'		
C5	C5 forward	5'-AGA GTG TGA AGT GAG GAC GAG GGA-3'	63.8°C, 1 min	1,064 bp
	C5 Revers	5'-CTA CGT TCG CCT CAC TCA TTG CCT-3'		
CRX1	CRX1 forward	5'-CAC CAT CTG TCT ACA TAA GGC CGC CC-3'	62.2°C, 1 min	856 bp
	CRX1 Revers	5'-AAA GTT CAA GGA CCG GAC CGC CG-3'		

Table 2. Morphological characteristics of *Fusarium* spp. isolates

Isolates	Species	Characteristics of fungus colonies in PDA media cultures	Characteristics of macro conidia	Shape of micro conidia
BC1	*F. solani*	Colonies were white and not pigmented, thin mycelium and unaerial.	The shape of macroconidia was widest at the middle with length of 10.33-11.94 µm and a width of 2.35-2.93 µm	Fusiform to reniform
BC2	*F. solani*	Colonies were white, and form a gradation of radius-like lines, thin mycelium without pigmentation.	The shape was widest at the middle with length of 10.37-16.54 µm and a width of 2.49-2.94 µm	Reniform
BC3	*F. solani*	The colony was brownish-white, thin mycelium, unpigmented, reverse surface of the colony had a gradation of circular lines.	The shape was widest at the middle with length of 09.08-15.11 µm and a width of 2.23-4.06 µm	Reniform to allantoid
BC4	*F. oxysporum*	The colonies were broken white to light orange, thin mycelium, surface of the colony was slightly wet with orange pigment.	The shape of macroconidia was curved with parallel walls with length of 08.01-10.06 µm and a width of 1.13-2.82 µm	Reniform
BBS1	*F. solani*	Colony was yellowish-white, unpigmented, cotton like mycelium, thick and aerial.	The shape was widest at the middle with length of 11.48-14.42 µm and a width of 3.33-4.50 µm	Fusiform to oval
BBS4	*F. solani*	The colony was brownish-white and the center of the colony was purple. Sometimes formed a purple pigment at the center of the colony.	The shape was widest at the middle with length of 07.27-12.37 µm and a width of 1.50-2.43 µm	Ovoid to reniform
BBS5	*F. proliferatum*	Colonies were white to pink-purple, cotton-like mycelial, slightly aerial and produced purplish-pink pigment.	The shape was widest at the basal portion with length of 07.61-16.51 µm and a width of 1.59-3.04 µm	Fusiform to ovoid
BBS6	*F. oxysporum*	The colony was purple, mycelium was smooth and the surface of the colony was slightly wet with dark purple pigmention.	The shapewas curved with parallel walls with length of 08.31-12.01 µm and a width of 1.94-2.78 µm	Allantoid

Table 3. Alignment of *Fusarium* spp. isolates sequencing results with data in GenBank

Isolates	Base length	Species	Query cover (%)	Percent identity (%)	Identity of isolate resource
BC1	567 bp	*F. solani*	100	99.82	MN602620, 574bp, XXTF8 of Chinese sugarcane soil.
BC2	568 bp	*F. solani*	100	99.82	MN856274, 568 bp, a rubia endophytic fungus from China.
BC3	569 bp	*F. solani*	100	99.65	MN856274, 568 bp, a rubia endophytic fungus from China.
BC4	1,074 bp	*F. oxysporum*	100	99.82	OK560835, 553 bp, fruit peels of Malaysia.
BBS1	567 bp	*F. solani*	100	99.82	KX583231, 567 bp, watermelon root rot of Mexico.
BBS4	621 bp	*F. solani*	91	99.82	KX583231, 567 bp, watermelon root rot of Mexico.
BBS5	562 bp	*F. proliferatum*	99	99.46	MW793553, 556 bp, onion basal rot of China.
BBS6	849 bp	*F. oxysporum*	100	99.82	MG846495, 545 bp, soil samples from Egypt.

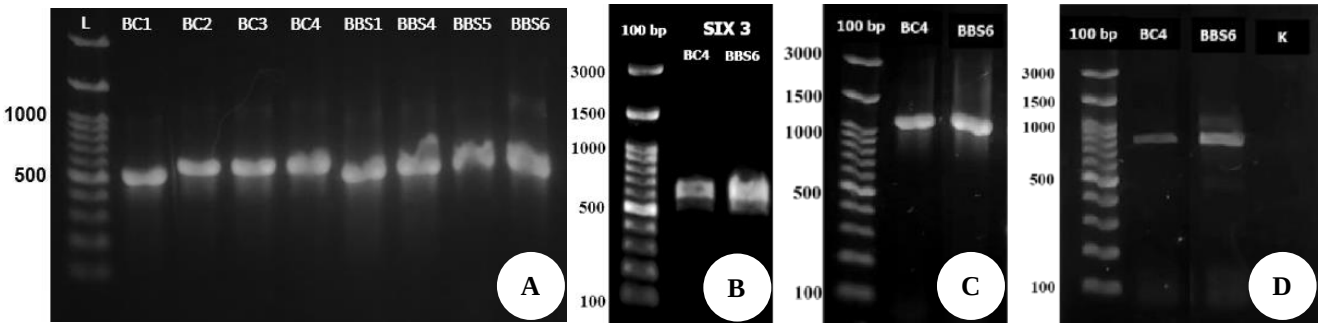

Figure 4. PCR amplification results of *Fusarium* spp.: A. ITS1 and ITS4 universal primer; B. SIX3 specific primer; C. C5 specific primers, and D. CRX1 specific primer

Molecular identification of *Fusarium* spp., causal agents of basal plate rot disease on shallots

Eight isolates of *Fusarium* spp. isolated from shallot plants symptomatic of basal plate rot disease were identified molecularly using ITS1 and ITS4 universal primers and sequenced (Figure 4.A). The results, which were aligned with the data in GenBank using Blast showed that five isolates, BC1, BC2, BC3, BBS1, and BBS4, were belonged to *F. solani*, while two isolates, namely BC4 and BBS6 were of *F. oxysporum*, and one isolate BBS5 was of *F. proliferatum* (Table 3). BC4 and BBS6 isolates had a homology of 99.82% with *F. oxysporum*, so these two isolates were further PCR amplified using specific primers PCR amplification results showed that both isolates were detected in SIX3-specific primers (Figure 4.B), C5-specific primers (Figure 4.C), and also in CRX1-specific primers (Figure 4.D).

The base sequence of the sequencing results was analyzed phylogenetically using MEGA 7, and phylogenetic trees were made using the neighbor-joining method with bootstrapping 1,000 times to see the kinship relationships between isolates. The analysis showed that eight isolates were divided into two groups (Figure 5). The first group consisted of five isolates: BC1, BC4, BBS1, BBS4, and BBS5. The second group consisted three isolates, namely BC2, BC3, and BBS6 from the same group. The results also showed that two of those eight isolates belonged to the same species: BC2 and BC3.

In vitro pathogenicity test

Results of in vitro pathogenicity test showed that hyphae of *Fusarium* spp. from the eight isolates began to grow on the bulb, and they were able to colonize the bulbs five days after inoculation. However, hyphal growth was visible in some isolates three days after inoculation, such as in isolate BC4 (Figure 6.A). All the eight pathogenic fungal isolates were virulent and can infect shallots. The virulence levels of the eight isolates appeared to be different, as seen from the frequency of infected bulbs 7 days later, where bulbs inoculated with isolates BC4, BBS6, BBS5, BC1, BC3, and BBS1 were 100% infected. The other 2 isolates (BC2 and BBS4) were only 60 and 80% infected, respectively. The bulb samples inoculated by isolate BC4 had the most severe infection compared to the seven isolates, where 60% of the test samples were infected on day 3 post-inoculation (dpi). In comparison, on day 5 dpi of all samples of shallot bulbs had rotted and could not grow back (Figure 6.D). Some isolates can cause disease with a high severity level even though the frequency of disease occurrence was low, such as isolate BBS1 (Figure 6.C). In contrast, the frequency of infected tuber samples was high, with a low level of disease severity, such as isolates BC2 and BBS4 (Figure 6.B). This shows the eight fungal isolates had different levels of virulence, although several isolates had the same level of virulence. In general, the eight isolates showed that six isolates had high virulence, namely BC4, BBS6, BBS5, BC1, BC3, and BBS1, while the other two isolates, namely BC2 and BBS4, were in the moderate virulence category.

Pathogenicity test in greenhouse

Pathogenicity tests in greenhouses showed that symptoms of basal plate rot disease on shallots began to appear in BC4 isolate at nine dpi, followed by BBS6 and BC1 isolates at day 10. The frequency of plants infected due to BC4 isolate infection reached 100% at 15 dpi, while

BBS6 isolate infection at the same time was only 80%, BC1, BBS1, and BBS4 reached 50%, BC2 and BBS5 reached 40%, and the BC3 isolates reached 30%. The frequency of infected plants at the end of the observations showed that the degree of virulence of each isolate was different. The highest virulence level was shown by BC4 isolate with a frequency of plants infected as much as 100%, followed by BBS6 isolates with as much as 90%, BBS4 with as much as 80%, BC1, BC2, BC3, BBS1, and BBS5 as much as 50%. The highest severity of the disease occurs due to infection with BC4 isolate, in which infected plants wither and die. While some plants were infected with the other seven isolates.

Results of pathogenicity tests in the greenhouses showed the symptoms caused by infection from each of the eight species of *Fusarium* spp. fungus isolates were different (Figure 7). Symptoms caused by BC1 isolate infection were stunted plant growth, yellowing of leaves, and drying of leaves (Figure 7.B). BC2 isolate caused symptoms in the form of stunted plant growth; wrinkled leaves, stiff green color like attacked by small leaf growth virus and dried leaf tips (Figures 7.C and 7.D). BC3 isolate caused stunted plant growth, chlorosis and drying of leaf tips (Figures 7.B and 7.D); the next symptom was broken leaves and dried leaf tips (Figure 7.E). Symptoms caused by BC4 isolate infection early in plant growth resulted in stunted plant growth, infection after the plant growth caused yellowing and twisting of leaves, and eventually the plant withered and died (Figures 7.F and 7.G). In comparison, mild symptoms appear as leaf tips dry up and the plant's leaf growth becomes smaller. In case of BBS1 isolate infection, the leaves of the plant broke easily and the leaf tips dried up, these symptoms were similar to those of BC2 infection (Figure 7.D). The BBS4 isolate causes mild infection symptoms, with leaves breaking easily and leaf tips drying up, while severe symptoms included yellowing, flattening of leaves and plant death over time (Figures 7.D and 7.E). Symptoms due to BBS5 isolate infection were yellowing of leaves, slight twisting accompanied by drying of leaf tips, and over time the plant died (Figure 7.E). Symptoms due to BBS6 isolate infection were similar to symptoms in BC4 infection, such as yellowing plant leaves, twisting, and over time the plant withered and died (Figures 7.E and 7.G).

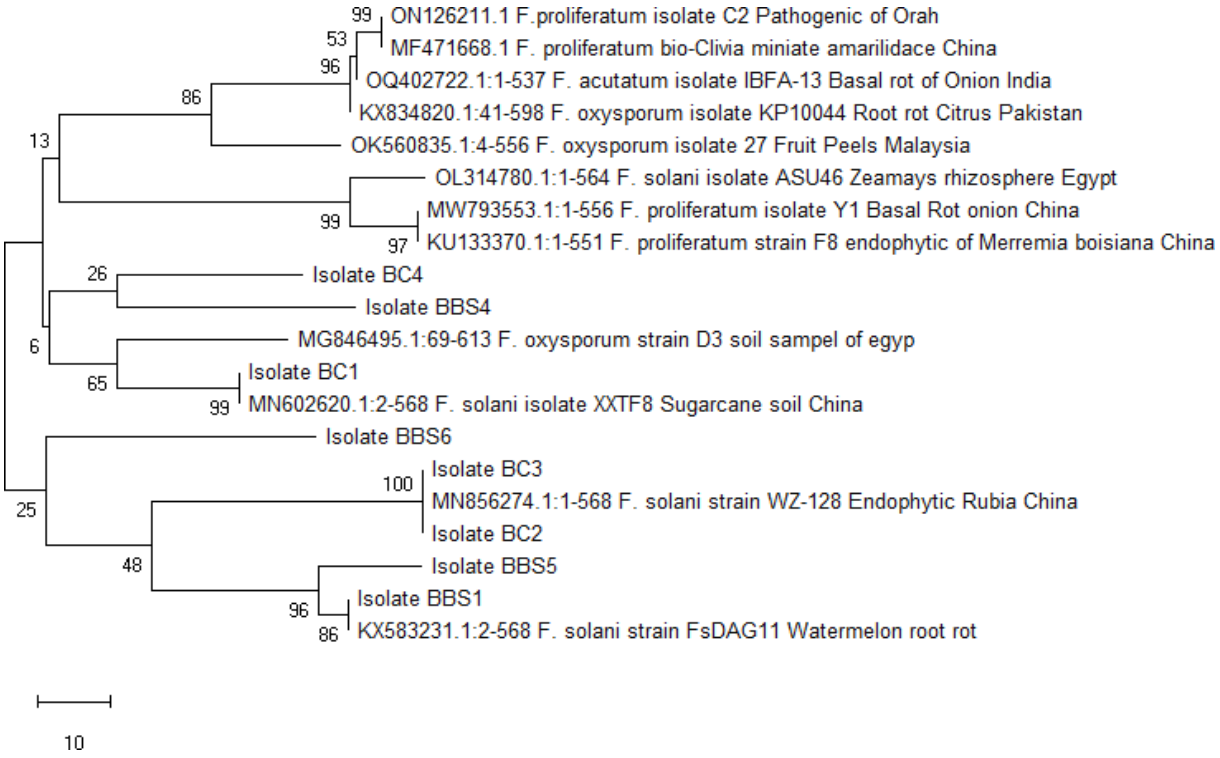

Figure 5. Phylogenetic tree of *Fusarium* spp. isolate based on the Neighbor-Joining method used MEGA 7 with 1000 bootstrap replications

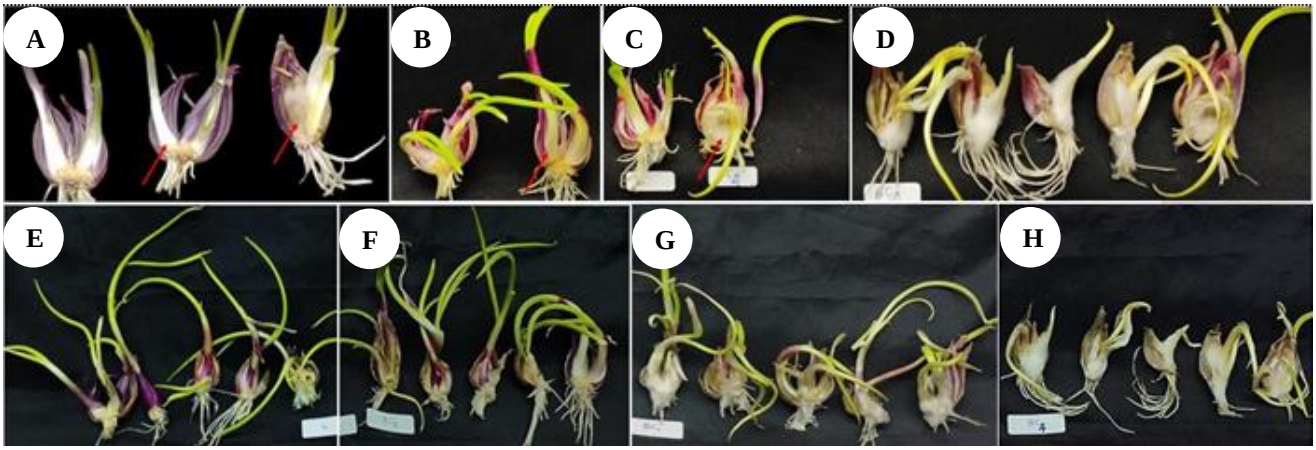

Figure 6. Pathogenicity test of *Fusarium* spp. isolate on shallots of Bima Brebes variety: A. Condition of bulb at 3 dpi (on the left control, uninfected bulb in the middle, and infected bulb in the right); B. The uninfected bulb at 5 dpi; C. Hypa colonization on infected bulb at 5 dpi; D. Condition of bulb infected by BC4 isolates at 5 dpi; E. Bulbs in control at 7 dpi; B. Bulbs at low virulence; C. Bulbs at moderate virulence; D. Bulbs at high virulence

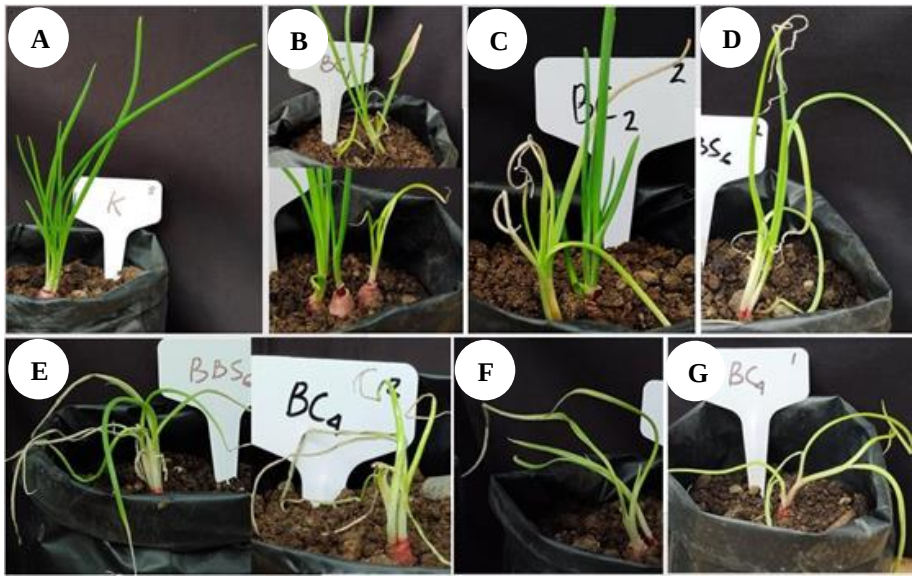

Figure 7. Variations in symptoms of basal plate rot disease on shallots due to infection by the pathogenic fungus *Fusarium* spp. in the greenhouse: A. control; B. stunted growth; C. dried leaf tips ; D. yellowing of leaves accompanied by dried tips; E. wilting; F. leaves twisted and flat; G. leaf twisting

Discussion

Basal plate rot disease on shallots is a systemic disease caused by the *Fusarium* species complex with varying symptoms. Typical symptoms commonly known by people in the field are yellowing and twisting of the leaves; however, based on our investigation in the field, other symptoms were also found, such as wilted plants with flattened leaves and other symptoms in the form of dry leaf tips. Symptoms on tubers also vary, from tuber growth not developing to tubers rotting at the base entirely and some tubers not rotting at all. That means we often need to correct things when identifying diseases directly in the field. The variation in basal plate rot disease symptoms in the field is caused by the timing of infection by fungal pathogens at different plant growth phases. According to Kintega et al. (2020), *Fusarium* sp. can infect onion plants in the seedling phase; however, typical symptoms will be seen if the infection occurs in mature plants or after harvest, namely in the form of yellowing leaves followed by wilting and curling. It is essential to understand and study the symptoms of basal plate rot disease in the field, both on shallot plants and other types of onion plants, because identifying the cause of the disease is one of the critical factors in controlling the disease. Variations in symptoms can also occur due to infection from different *Fusarium* species.

Isolation of pathogenic fungi from the three variations of shallot basal plate rot disease symptoms encountered in the field showed that these symptoms were caused by infection with the fungus *Fusarium* spp. The eight isolates

obtained were morphologically different. Morphological characteristics are one of the keys to identifying fungal pathogens of a plant. However, more is needed to identify complex species of *Fusarium* fungi, such as those that cause basal plate rot disease on shallots. The difficulty of observing morphological differences in *Fusarium* spp. is an obstacle, so DNA base sequence analysis is needed for more accurate identification results (Kalman et al. 2020).

The PCR results for the universal primers ITS1 and ITS4 showed that the eight fungal isolates responsible for basal plate rot disease in shallot bulbs in Brebes were all different *Fusarium* spp. Five isolates were of species *F. solani*, two were *F. oxysporum* species, and one was of *F. proliferatum* species. The results of PCR amplification showed that BC4 and BBS6 belonged to *F. oxysporum* f.sp. *cepae*. Taylor et al. (2016) reported that C5 and CRX1 genes were only found in *F. oxysporum* f.sp. *cepae*. On the other hand, the SIX3 gene was found in both *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *cepae*, but with a different base sequence. The SIX3 gene can be used to identify *F. oxysporum* f.sp. *cepae* (Taylor et al. 2016; Herlina and Istiaji 2020). The present study's findings demonstrated that the three species of *Fusarium* spp. fungi, namely *F. oxysporum* f.sp. *cepae*, *F. solani*, and *F. proliferatum* were responsible for the basal plate rot disease on shallots in Brebes. These findings differ from those of previous studies by Lestiyani et al. (2016) and Herlina et al. (2021), who reported that *F. solani*, *F. acutatum*, and *F. oxysporum* are the main causes of shallot basal plate rot disease in Java and *F. proliferatum*, *F. verticillioides*, and *F. pallidioroseum* are the main causes of shallot basal plate rot disease in Indonesia. According to Haapalainen et al. (2016), *F. oxysporum* and *F. proliferatum* are virulent and aggressive pathogens on shallots, causing death in shallot plants. In the present study, it observed that *F. oxysporum* f.sp. *cepae* caused basal plate rot disease in shallots in Indonesia. This fungus was not found in the previous study, even though specific primers were used. The pathogen also causes basal plate rot disease in shallots in Israel (Kalman et al. 2020). The results of phylogenetic analysis showed that the eight fungal isolates consisted of two groups. Group one consists of BBS6, BC2, and BC3 isolates, while group two consists of BC1, BC4, BBS1, BBS4, and BBS5 isolates.

The eight isolates had different macroscopic and microscopic characteristics, but molecular identification showed that isolates in the same species have different morphological characteristics. These suggests that *Fusarium* is a complex species that is not enough to be identifed by morphological characteristics only; it must be confirmed using molecular identifications. Five isolates of *F. solani* had white colonies with thin to thick hyphal growth. One isolate of *F. proliferatum* (BBS5) had pink-purple colonies with smooth hyphal growth. Two isolates of *F. oxysporum* f.sp. *cepae* had a variety of white to orange colonies with orange pigment and purple-colored colonies with dark purple pigments at the colony's centre. Shamyuktha et al. (2020) also suggested differences in *F. oxysporum* f.sp. *cepae* colony characteristics include thin to thick hyphal growth and white to cream mycelium, forming pink to purple pigment in the colony's center. The pigment is only produced in the colony's centre and has a radial pattern with pigment colors varying from purple to ocher and bright orange; some isolates do not produce pigment (Haapalainen et al. 2016). The two isolates also had different microscopic characteristics. Microconidia were oval to ovoid, and macroconidia were crescent-shaped with 3-4 septa. It also produces 1-2-celled chlamydospores with thick cell walls (Shamyuktha et al. 2020). *Fusarium solani* fungi have macroconidia that are usually sickle-shaped, widening in the middle of their length, and microconidia that are oval, reniform, elongated, or sometimes ovoid with a truncated base, mostly aseptate (Chehri et al. 2015). Microconidia of *F. proliferatum* are club-shaped with flat, aseptate bases and form chains or false heads in mono- and polyphialids, and macroconidia are straight or slightly curved with underdeveloped basal cells and 3-5 septa. Meanwhile, *F. oxysporum* microconidia are oval, with one septum but usually aseptate, and are produced on monophyalides; macroconidia are slightly curved, with foot-shaped or grooved basal cells and 3-5 septa (Shin et al. 2023).

The results of pathogenicity test showed that BC4 and BBS6 (*F. oxysporum* f.sp. *cepae*) were more virulent than the other six isolates (*F. solani* and *F. proliferatum*). However, the virulence of BC4 isolate was higher than the virulence of BBS6 isolate. The results of the pathogenicity test using shallot bulbs were in line with the results of the pathogenicity test in the greenhouse. Shallot plants in the greenhouse that were inoculated with the BC4 isolate give rise to symptoms faster (9 dpi) than plants inoculated with the other seven isolates (12 to 14). Isolates BC4 and BBS6 botsh are *F. oxysporum* f.sp. *cepae* could infect plants at the beginning of growth, but the BC4 isolate was infected more quickly with an incubation period of 9 dpi, while the BBS6 isolate began infected at 10 dpi. This incubation period is faster than the results of Wiyatiningsih et al. (2009), namely 14 dpi. Plants infected with isolate BC4 showed symptoms of severe infection, and within seven days, the plants wilted and died. Likewise, shallot plants that were inoculated with BBS6 isolate. Both isolates had high virulence, with disease incidence reaching 100% (BC4) and 80% (BBS6) at 15 dpi. These results indicate that the two isolates had higher virulence and caused more severe damage than the other six isolates (*F. solani* and *F. proliferatum*). According to Taylor et al. (2013), the species *F. oxysporum* f.sp. *cepae* is a highly virulent pathogen in shallot plants. PCR amplification also showed that the two isolates belonged to *F. oxysporum* f.sp. *cepae* with high pathogenicity. The three target genes detected, SIX3, C5, and CRX1, are closely related to the pathogenicity of *F. oxysporum* f.sp. *cepae*. Pathogens with high pathogenicity are only found in plants infected with this fungus in agricultural fields (Taylor et al. 2016). The statement by Taylor et al. (2016) was strengthened the results of our research, where the source of an isolate of *Fusarium* was from diseased plants taken directly from shallot planting fields in Brebes.

The results of the pathogenicity tests in greenhouses showed that infection by the fungus *Fusarium* spp. at the

beginning of shallot growth, plants growth is stunted, and the plant even fails to grow. Meanwhile, infection after the plants are more than two weeks old causes the plant to be unable to form tubers at all and causes the plant to wilt. In contrast, if the infection of the *Fusarium* occurs after the plant is the tuber formation phase, the formation of tubers will be hampered so that the size becomes small. Inhibition of plant growth and symptoms of plant wilt was likely due to the effects of mycotoxins produced by the fungus *Fusarium* spp. during plant infection. According to Munkvold (2017), *F. oxysporum* and *F. solani* complex species fungi produce trichothecene mycotoxin, which has an impact on cellular oxidative stress, cell cycle termination, apoptosis, and cell membrane dysfunction, thus causing reduced seed germination, stunting of coleoptiles, roots, and shoots, chlorosis, wilting, and necrosis. The results of this study also show that the *Fusarium* spp. can infect shallot plants in the all growth phases. According to Le et al. (2021a), the pathogenic fungus *Fusarium* spp. can attack at all stages of shallots plant growth, from seed to harvest, and infect bulbs in storage. Le et al. (2021b) stated that basal plate rot disease attacks that occur both before and after planting could cause yield losses, and the critical period of shallot plants against basal plate rot disease is 1-2 weeks at the star of plant growth.

The symptoms of basal plate rot disease in shallots caused by infection with the *Fusarium* spp. fungus shows several different symptoms. Shallot plants infected with *F. solani* cause symptoms in the form of stunted growth, chlorosis and dry leaf tips, wilted plants and rotting bulbs. The fungus *F. solani* produces trichothecene mycotoxins, which cause reduced seed germination, stunting of coleoptiles, roots, shoots, chlorosis, and wilting (Munkvold 2017). *Fusarium proliferatum* fungus infection on shallots causes symptoms of stunted growth, chlorotic leaves, and drying starting from the tips. These symptoms are likely due to the effects of the fumonisin mycotoxin produced by the *F. proliferatum* fungus during infection. According to Munkvold (2017), fumonisin mycotoxin can inhibit the growth of shoots and roots, causing wilting, chlorosis, and necrosis, and *F. proliferatum* is one of the *Fusarium* species that produces this mycotoxin besides *F. fujikuroi* and *F. verticillioides*. Meanwhile, the symptoms caused by the *F. oxysporum* f.sp. *cepae* infection means the leaves are chlorotic and twisted; sometimes the leaves become flat; the tubers do not develop; the following symptom is that the leaves start to dry out and wither, and the plant dies and the tubers rot. The fungus *F. oxysporum* produces the fusaric acid mycotoxin, which significantly affects its pathogenicity and induces wilting symptoms (Munkvold 2017). The rotting of the bulbs in shallots is caused by the pectic enzyme produced by the fungus *F. oxysporum* f.sp. *ceape*, namely exo- and endo polygalacturonase, where this enzyme can break down the pectin in the cell walls of shallots, so causing rot in the bulbs (Holz and Knox-Davies 1985). This variation in symptoms shows the uniqueness of the symptoms caused by the infection of each species of *Fusarium* spp. in shallot plants.

The symptoms that appear in the results of pathogenicity tests in greenhouses were also the same as those of observing symptoms in the field. According to Lestiyani et al. (2016), the symptoms of basal plate rot disease in shallots are wilting, tuber rot, and twisting. The fungus *F. solani* causes wilt in shallots; the combination of *F. solani* and *F. acutatum* can cause twisting symptoms, and rot symptoms are caused by *F. oxysporum.* That suggests that one plant infected with basal plate rot disease on shallots could be found in one or more species of *Fusarium* spp. According to Wiyatiningsih et al. (2009), shallot plants attacked by basal plate rot disease show symptoms in the form of pseudo-stems and leaves that grow longer and curling, pale green leaf color but are not wilted, tubers are smaller and do not show decay, and further attacks cause plants to dry and die. According to Cahyaningrum et al. (2019), in addition to pale yellow and curly leaves, shallot bulbs also rot. Decay starts from the roots and quickly spreads throughout the tuber; damaged root tissue is slightly pink and rots during the early phases of disease development (Haapalainen et al. 2016).

Two isolates (BC4 and BBS6) were expressed as *F. oxysporum* f.sp. *cepae*, the same species, have different macroscopic and microscopic characteristics and virulence levels. This suggests that it is likely that *F. oxysporum* f.sp. *cepae* have races with varying levels of virulence, as mentioned in Le et al. 2021a, so the symptoms caused are also different. The fungus *F. oxysporum* f.sp. *cepae* has a high level of virulence, which increase the incidence and severity of basal plate rot disease. The fact that this pathogen can attack shallot plants at all stages of growth means that this pathogen must be taken into account and receive more attention. The high level of virulence in *F.oxysporum* f.sp. *cepae* requires effective and environmentally safe treatment. Effective and sustainable control alternatives will be needed to suppress the growth of the fungus, including the application of biocontrol agents that can not only inhibit the growth of the fungus but also induce host plant resistance. As biocontrol agents, endophytic actinomycetes could be helpful because they make bioactive compounds that kill fungi and can make host plants more resistant, less stressed, and better able to grow (Subhashini and Singh 2014; Singh et al. 2017; Vurukonda et al. 2018; Vijayabharathi et al. 2018).

This disease has long been found in Indonesia. It was previously a secondary disease that was not considered an important disease in shallot plants, so it was not a concern for farmers and academics (Herlina et al. 2021). That causes the spread of disease in almost all shallot-production centres in Indonesia (Supyani et al. 2021; Safitri et al. 2019; Mariani et al. 2022). As the number of diseases keeps increasing, we need to use a combination of methods to control them (Integrated Pest Management). These include using resistant varieties like Batu ijo (Maulidha 2023), endophyte actinomycete biocontrol agents like the one we were studying, using fungicides wisely, and handling crops properly after harvesting. Based on the results of the present study, it can be concluded that basal plate rot disease on shallots in the Brebes area was caused by *F. oxysporum* f.sp. *cepae*, *F. solani*, and *F. proliferatum. F.*

oxysporum f.sp. *cepae* was more virulent than *F. solani* and *F. proliferatum.* One shallot plant that showed symptoms of basal plate rot disease can be infected with one or more species of *Fusarium* spp. Therefore, the possibility of interaction of *F. oxysporum* f.sp. *cepae* along with other species, is a secondary pathogen that requires further study.

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