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Pathogenicity and Detection of Phytohormone (Gibberellic Acid and Indole Acetic Acid) Produced by *Fusarium* spp. that Causes Twisted Disease in Shallot

Pengujian Patogenisitas dan Fitohormon (Asam Giberelat dan Asam Indol Asetat) pada *Fusarium* spp. yang Menyebabkan Penyakit Moler pada Bawang Merah

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ABSTRACT

The twisted disease is one of the essential diseases in shallots caused by *Fusarium* spp. This study aimed to study pathogenicity and identify Fusarium species isolated from shallot plants with twisted symptoms in Nganjuk and Bantul areas. The Fusarium isolates were identified and then tested for pathogenicity levels and the effect of the hormones GA_3 and IAA on shallot symptoms. Molecular identification using NF2 and NF4 successfully identified one isolate of *Fusarium oxysporum*, three isolates of *F. acutatum*, and three isolates of *F. solani*. Each of these species produces different symptoms. Pathogenicity test showed that all isolates had disease incidence reaching 100%, except isolates of *F. solani*[1] causing wilt and *F. solani*[3] causing twisted have the lower disease incidence were 77.8% and 77.7%, respectively. The investigation caused twisted shallot related to different symptoms was tested using the Thin Layer Chromatography (TLC) method. The result indicates that all isolates did not find IAA hormone. In contrast, the hormone GA_3 was found in *F. solani*[2] and *F. solani*[3] isolates, caused bulb rot and twisted disease, respectively. Detection of IAA, GA_3, and other hormones in shallot plants showed different symptoms should be studied further.
Keywords: *F. acutatum, F. oxysporum*, *F. solani,* GA_3, IAA, Shallot, Thin Layer Chromatography, Twisted Disease

ABSTRAK

Penyakit moler merupakan salah satu penyakit penting pada pertanaman bawang merah yang disebabkan oleh *Fusarium* spp. Penelitian ini bertujuan mengidentifikasi spesies Fusarium yang diisolasi dari tanaman bawang merah yang bergejala moler di daerah Nganjuk dan Bantul. Isolat-isolat Fusarium yang diperoleh diidentifikasi selanjutnya diuji tingkat patogenisitasnya, serta mengetahui pengaruh hormon GA_3 dan IAA terhadap gejala yang timbul pada bawang merah. Hasil identifikasi molekuler menggunakan primer NF2 dan NF4 diperoleh berturut-turut 1 isolat *Fusarium oxysporum*, 3 isolat *F. acutatum* dan 3 isolat *F. solani*. Setiap spesies tersebut

menghasilkan gejala yang berbeda. Uji patogenisitas menunjukkan bahwa semua isolat memiliki patogenisitas yang tinggi mencapai 100%, kecuali isolat *F. solani*[1] penyebab penyakit layu dan *F. solani*[3] penyebab penyakit moler memiliki kejadian penyakit yang lebih rendah berturut-turut yaitu 77,8% dan 77,7%. Semua isolat tidak memproduksi hormon IAA. Sedangkan hormon GA_3 ditemukan pada isolat F. solani[2] yang menyebabkan busuk umbi dan F. solani[3] yang menyebabkan gejala moler. Deteksi lanjut pada hormon GA_3 dan IAA serta hormon-hormon lain yang kemungkinan menyebabkan gejala pada bawang merah, perlu dikaji lebih lanjut.

Kata kunci: Bawang Merah, *F. acutatum*, *F. oxysporum, F. solani,* GA_3, IAA, Kromatografi Lapis Tipis, Penyakit Moler

INTRODUCTION

Shallot (*Allium cepa* L. Var. *Agregatum* (L.)) is a type of Alliaceae bulbous plant widely cultivated globally, especially in Asia (Elizani and Sulistyaningsih, 2019). The twisted disease is one of the most critical diseases becoming a problem in shallots (Triwidodo et al., 1998). The leaf of shallot symptoms was pale green and curving (Lestiyani et al., 2016). According to Ebenebe (1980), the twisted disease was first reported near Zaria, northern Nigeria, in 1969. In 1980, this disease had been considered a severe problem in the north of Nigeria, with yield losses up to 50-100%. The same situation occurred in the Kalpitiya Peninsula, the north-western Province of Sri Lanka, with yield losses up to 20-30%. This disease originated in Indonesia and was initially classified as a minor disease in 1977. The incidence and severity of the disease have increased every year (Triwidodo et al., 1998). The twisted disease has become a primary disease in various shallot production areas in Indonesia (Wiyono, 2007; Wiyatiningsih et al., 2010).

Previous studies have explained that the twisted disease of shallot could be caused by *Fusarium oxysporum*, *F. solani*, and *F. acutatum*. However, each species has its role in causing twisted disease, i.e., *F. solani* and *F. acutatum* causes symptoms of shallot wilting; bulb rot is caused by *F. solani*, *F. acutatum* or *F. oxysporum*. In contrast, the twisted symptoms are caused by *F. solani* or *F. acutatum* (Lestiyani et al., 2016). Meanwhile, Patil et al., 2018 stated that inoculated onion seedlings with *Colletotrichum gloeosporioides*, *F. oxysporum,* and *Meloidogyne* sp. create twisted disease symptoms. It was supported by the application of Gibberellic Acid (GA_3) or Indole acetic acid (IAA), and a combination of onion seedling revealed the appearance of twisting symptoms.

At the same time, pathogens can stimulate growth hormones and form hormones or inhibit the plant production of growth regulators or growth inhibitors. Whatever the mechanism of action, pathogens frequently cause an imbalance in the plant hormonal system and cause abnormal growth responses incompatible with healthy development (Agrios, 2005). More IAA has been detected in diseased plants, such as in hyperplasia (Semangun, 2006). The increase in IAA levels was caused by reduced IAA breakdown due to inhibition of the IAA-oxidase enzyme activity. As in *Pseudomonas syringae*, which also produces auxin, it will change the biology of the host auxin during infection so that auxin levels in plants increase (Marois et al., 2002).

In comparison, gibberellins have pronounced growth-promoting properties. They promote flowering, stem and root elongation, and fruit growth in dwarf varieties and hasten their stretching to regular sizes. Gibberellins appear to activate previously "turned off" genes. No difference in gibberellin content has been reported between healthy and virus- or mollicute-infected plants. Spraying diseased plants with gibberellin alleviates some of the symptoms caused by these pathogens (Agrios, 2005). For example, After treatment with gibberellin, the stunting of corn plants infected with corn stunt spiroplasma was reversed (Nault, 1980).

Based on the data above, there is no clear information on the twists involved in the twisted disease of shallots. This study aims to determine whether *Fusarium* spp. resulted from pathogenicity tests that show different symptoms can produce hormones such as IAA and GA_3; these have wilt, bulb rot, and twisted symptom. This research using Thin Layer Chromatography (TLC), the easiest hormone testing method can be for quantitative growth hormone testing.

METHODOLOGY

This research was conducted at the Clinical Plant Disease Laboratory, Department of plant protection, Faculty of Agriculture, Universitas Gadjah Mada. This research had done in January-July 2018.

Fungal Identification and Pathogenicity test

Shallot plants with twisted disease symptoms from the field in Bantul (Yogyakarta) and Nganjuk (East Java) were pulled from the soil, placed in a plastic bag, and transported to the laboratory for fungal isolation. The bulb from twisted disease symptoms was washed, sterilized with 70% ethanol before cut into small pieces, and placed directly on Potato Dextrose Agar (PDA) medium. The CTAB (cetyltrimethyl-ammonium bromide) method was used to extract fungal DNA. IGS primer 5'-CTGAACGCCTCTAAGTCAGA-3' (NF2) and 5'-CCTGCGGACGCTCAAAAACTT-3' (NF4) (Chen et al., 2009) were used to amplify fungal DNA. PCR was carried out on 20-µl of the reaction mixture that contained 10-pmol of each primer, 10-µl of Quick taqTM HS dye Mix (Nippon gene), and 80 ng of template DNA. The thermocycler program began with initial denaturation for 2 minutes at 94°C, followed by 29 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 68°C. Electrophoresis on 1.2% agarose gel separated amplicons, which were stained with ethidium bromide (EtBr) (Lestiyani et al., 2014).

Fusarium spp. that identified pathogenicity testing is performed to determine the disease incidence and visible symptoms. Shallot bulbs in good condition were disinfected with 0.1% NaOCl for 30 minutes and rinsed with sterile water, and dried. The dried seeds were soaked in conidia suspension of 10^6 conidia/mL density for 1 hour (Dissanayake et al., 2009). Bulbs were planted in plastic pots filled (3 blubs/pot) with sterile soil that has been incubated in glasshouse condition at a temperature of 28-32°C for four weeks and observed every week. Disease incidence was calculated using the formula:

$$\mathrm{DI} = \frac{\mathrm{a}}{\mathrm{b}} \times 100\%$$

DI = disease incidence
A = number of disease plants
b = total number of plants

Hormone Analysis of IAA and GA_3

Each of the *Fusarium* spp. isolates were grown at 250 mL of Potato Dextrose Broth for seven days at room temperature. Filtrate

culture was filtered by Whatman paper no. 42. The pH of the filtrate was adjusted to 2.5-3.0, with the addition of 0.1 N HCl or KOH (Bhalla et al., 2009). Ethyl acetate was used for solvent extraction. Filtrate culture was extracted by adding ethyl acetate as much as the filtrate culture (250 mL) with a separating funnel. The upper organic phase was separated and dried over anhydrous sodium sulfate. The evaporation was then performed on a rotary vacuum evaporator at 40^{o}C, 30 rpm. The resulting residue was dissolved with acetonitrile and stored for TLC analysis (Rachev et al., 1993).

TLC plate type 60F 254 (Merck) was run with an eluent consisting of benzene: n-butanol: acetic acid (6: 3: 1) to the GA_3 test. The resulting spot can be visualized at UV (254 nm) after spraying with ethanol: concentrated sulfuric acid (95: 5) (Bhalla et al., 2009). IAA testing used eluents consisting of isopropanol: ammonia: water (10: 1: 1) and sprayed with 3% H_2SO_4 in methanol + 50 mg $FeCl_3$ and heated 80^{o}C for 10 minutes (Hasan, 2002).

Qualitative analysis was compared with standards that were dissolved using the same solvent as the sample compound. Samples with the same value of the retention factor (Rf) can be determined to be identical to the standard. Rf was calculated using the formula distance traveled by sample/ distance traveled by solvent (Fessenden et al., 2001).

RESULTS

Fungal Isolation and Identification

Shallots displayed typical symptoms of twisted disease with shallot leaf shown pale green and curving in the field survey (Figure 1). The isolation results from various sources obtained seven isolates with different *Fusarium* spp, then identified based on the molecular method, as shown in Table 1.

Figure 1. Symptom of twisted disease on shallot

Pathogenicity tests revealed that seven isolates of *Fusarium* spp. were capable of causing various disease symptoms on shallot. Specifically, wilting caused by *F. acutatum* or *F. solani*, twisted caused by *F. solani* or *F. acutatum*, and bulb rot caused by *F. acutatum* or *F. sonali* or *F. oxysporum*. Another result from the pathogenicity test showed that *F. solani* causing wilt and *F. solani* causing twisted have the lower disease incidence were 77.8% and 77.7%, respectively (Table 1).

Wilting on shallots causes the leaves to wilt, turn yellow, and then dry. Bulb rot symptoms were nearly identical to wilting symptoms; tubers were smaller and more rotten than wilting symptom, also turn yellow and wilt faster than shallots with the wilting symptom. Shallots with twisted symptoms have yellowed and twisted leaves (Figure 2).

Hormone analysis of IAA and GA_3

The result of TLC testing showed that from 7 isolates, only two isolates (*F. solani*^{2}, and *F. solani*^{3}) confirmed GA_3 with an Rf value of 0.73, wherein bands of light brown colour were observed on the TLC plates (Figure 2A). In contrast, none isolates confirmed IAA (Rf = 0.85) (Figure 2B).

Table 1. List of isolate and symptom that caused *Fusarium* spp.

Species	Location	Symptom	Disease incidence (%)
F. acutatum[1]	Bantul	Wilt	100
F. solani[1]	Nganjuk	Wilt	77.8
F. oxysporum[1]	Bantul	Bulb Rot	100
F. acutatum[2]	Bantul	Bulb Rot	100
F. solani[2]	Nganjuk	Bulb Rot	100
F. solani[3]	Bantul	Twisted	77.7
F. acutatum[3]	Nganjuk	Twisted	100

Note. [1],[2],[3] showed isolate number

Figure 2. Fusarium spp. caused disease symptoms on shallot: a) healthy shallot; b) wilting; c) bulb rot; d) twisted disease

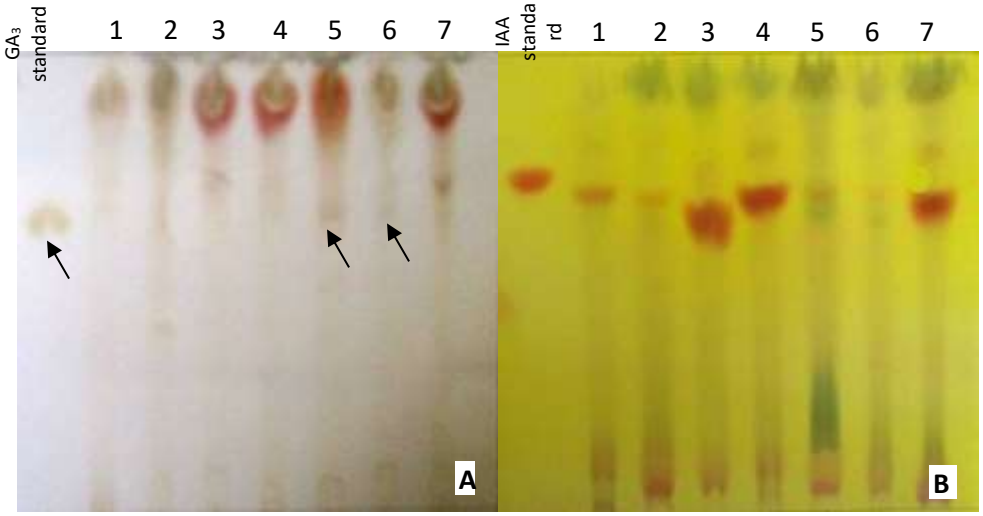

Figure 3. Hormone test in Fusarium spp. by TLC method: A) GA_3 testing; B) IAA testing; 1) *F. acutatum*[1]; 2) *F. solani*[1]; 3) *F. oxysporum*[1]; 4) *F. acutatum*[2]; 5) *F. solani*[2]; 6) *F. solani*[3]; 7) *F. acutatum*[3]

DISCUSSION

Fusarium spp. has various pathogenicities. Inoculation of *Fusarium* spp. to host plant is a simple method for determining the pathogenic or non-pathogenic. Strains that do not cause symptoms in plants are considered nonpathogenic for that plant (Tjamos et al., 1992).

Pathogenicity of *F. oxysporum* f. sp. *cepae* caused root rot and has become a disease in garlic, onions and leeks have done with much research. *F. oxysporum* f. sp. *cepae* invade plants through the roots. The development of this disease starts from necrosis on the basal plate, death of old leaves and rot on the inside of the tuber (Hitch et al., 2005). Two other species, *F. acutatum* and *F. solani*, are less well known for causing disease, but (Kalman et al., 2020) found that *F. acutatum* can cause bulb rot in red onions, and (Boehnke et al., 2015) said that *F. solani* is causing bulb rot in onions. The same species of these were also found in this study.

Seven Fusarium isolates from this study are known to infect plants and cause different symptoms. Wilting caused by *F. acutatum* or *F. solani*, twisted caused by *F. solani* or *F. acutatum*, and bulb rot caused by *F. acutatum* or *F. sonali* or *F. oxysporum*. The data needs investigating further. The statement was supported by Carrieri et al. (2013) studied that identified the causative agent of pink rot of onion roots and onion bulbs that primarily isolated three fungi: *Fusarium proliferatum*, *Fusarium trincticum*, and *Pyrenochaeta terresteris*. When combined, all Fusarium species will affect the high severity disease; for example, Fusarium Head Blight (FHB) disease affected mycotoxin production. Inoculation using one species, *F. graminearum,* or combining *F. graminearum* with other species produces the highest infection (Ghahderijani, 2008).

Phytohormone was suspected of playing a role in the symptom of the disease. The result of this study was two isolates confirmed GA_3 were *F. solani*[2], and *F. solani*[3] caused bulb rot and twisted disease, respectively. This result suggests that *F. solani* caused twisted disease is affected by GA_3. Previous research was done (Bhalla et al., 2009; Seo et al., 2012) considered that *F. solani* could produce GA_3. In the other study, the essential role of GA_3 in plant growth is an elongation cell of the stem, roots, flower, and fruit (Achard and Genschik, 2009; Davière and Achard, 2013; Hedden and Sponsel, 2015). However, GA_3 can induce the disease as though Bakanae (Matic et al., 2017) induced by *Giberella fujikuroi* (Desjardins et al., 2000; Hedden and Sponsel, 2015; Sulyanti, 2017; Salazar-Cerezo et al., 2018). *G. fujikuroi* secreting GA_3, which made symptoms in seedlings appear to be taller, more slender, and slightly chlorotic (Cen et al., 2020). That statement is similar to Wiyatiningsih et al. (2010) about the characteristic twisted disease of shallots showing a longer pseudostem and curved leaves dan chlorotic. A possibility that twisted disease is affected by GA_3 in that needed advanced technology to analyze it. However, GA_3 was also detected on rot symptom, it has not yet been found that GA_3 caused it, and further research is needed.

IAA may also act synergistically, such as elongation, similar to that caused by GA_3 (Agrios, 2005). Furthermore, IAA regulates plant growth and development of differentiation, tropism, senescence, and flowering (Li et al., 2015). IAA was detected more on plants diseased caused pathogen inducing IAA to produce gall (Fu and Wang, 2011; Denancé et al., 2013; Semangun, 2006), such as clubroot caused *Plasmo-*

diophora brassicae (Tian et al., 2019) and smut on maize caused *Ustilago maydis* (Aydo et al., 2015). Other examples are gall-caused *Agrobacterium tumefaciens* (Borges et al., 2019), late blight caused *Phytophthora infestans* (Saville et al., 2016), Fusarium wilt of bananas caused *F. oxysporum* f.sp. *cubense* (Di et al., 2016), and Fusarium head blight caused *F. Graminearum* (Yang et al., 2013). The other function, IAA, can reduce germination of *Fusarium* sp. at the tomato and barley (Sharaf and Farrag, 2004; Petti et al., 2012; Raffi et al., 2017).

In the other study, Ongoagwanit (1991) detected the increase of IAA on onion after *F. oxysporum* induce as a cause of twisted disease. Patil et al. (2018) showed abnormal elongation of the onion plant after IAA and GA_3 spraying but did not show any pathogen structure. The statement contrast with this research that IAA was not found in all isolates. Meanwhile, the mechanism of the IAA is not well understood in this study.

CONCLUSION

Three *Fusarium* spp. were identified, i.e., *F. solani*, *F. acutatum* or *F. oxysporum.* Wilting, bulb rot, and twisted disease are three classified symptoms of shallot. Wilt symptom on shallot caused by *F. solani* and *F. acutatum*, bulb rot by *F. solani*, *F. acutatum* or *F. oxysporum*, and twisted disease by *F. solani* or *F. acutatum*. *F. solani* produced the GA_3 hormone at *F. solani*[2] and *F. solani*[3] isolate, while all isolates testing did not produce IAA hormone. The role of the hormone in the symptoms is unclear. *Fusarium* spp. does not produce the hormone required to cause a more severe infection. However, these assays were successful in identifying the cause of twisted disease in shallot and *Fusarium* spp. identified as the disease causal agents in this country region. The pathogenicity assays were also helpful in determining the various symptoms caused by each *Fusarium* spp. The findings of the study are an essential step toward developing a disease-prevention program.

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