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Full Length Article

First Report of *Phomopsis* sp. PV-S G38 Caused Leaf Ring Spot Disease on Black Pepper (*Pipper nigrum* L.) in West Java, Indonesia

Dian Safitri[1*], Maisya Zahra Al Banna[2], Albertus Fajar Irawan[1], Septrial Arafat[1] and Muhammad Iqbal Fauzan[1]
[1]*Department of Agriculture, Faculty of Animal and Agricultural Sciences, Universitas Diponegoro 50275, Indonesia*
[2]*Faculty of Teacher Training and Education, Universitas Patompo, Makassar, Indonesia*
[*]For correspondence: diansafitrifpp@lecturer.undip.ac.id
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Abstract

Phomopsis sp. PV-S G38 have been frequently reported as plant pathogens, non-pathogenic endophytes or saprophytes, commonly infecting several agriculturally important crops. Infection of *Phomopsis* sp. PV-S G38 on black pepper in Indonesia has never been reported. The purpose of this study was to identify the cause of leaf ring spot disease on black pepper leaves. We used the most efficient method by combining morphological and molecular identification of the pathogen and analyzed the isolates that displayed the symptoms in the field. The primers of ITS 1 and ITS 4 were used. The GelDoc visualization ranged from 500–600 bp. This research discovered that *Phomopsis* sp. PV-S G38 was a pathogen in black pepper. The symptom of this disease was shown by the appearance of single heliocentric spot on the leaf. This evidence was supported by the sequence report. It revealed that the isolate was 100% identical to *Phomopsis* sp. PV-S G38.

Keywords: Black pepper; Indonesia; Leaf spot symptom; *Phomopsis* sp.

Introduction

Black pepper is one of the important commodities in Indonesia which support the country's gross domestic product (GDP) from the sector of agriculture (Bermawie *et al.* 2019; Azhari *et al.* 2021). It has generated income for many Indonesian farmers such as in the Provinces of Lampung, Bangka Belitung, Sulawesi and West Java, as cultivation of this crop is predominantly managed by smallholder plantations (Karmawati *et al.* 2020). The total production of this commodity in Indonesia on a total area of 119,200 ha in 2020 was 86 m tons (Manda *et al.* 2020). It is far below the annual production in Vietnam which attained 268.5 m tons in the same year (Oanh *et al.* 2021). A few years back, the black pepper production in Indonesia experienced a significant decline to 83.2 m tons (Manda *et al.* 2020).

A decline of black pepper production is attributed to various factors. One of them was the attacks of plant and disease. A stem rot disease caused by *Phytophthora capsici* in black pepper is the ultimate disease which can lead to the death of the plant (Suriany *et al.* 2021). In Indonesia, this disease results in a degradation of yield from 30-40% at some farms and others can even attain 100% loss (Nam 2012). In Malaysia, where 95% of the cultivated farms were infected by *P. capsici*, the production decreased by 5-10% (Kueh 1990). In India, yield loss reaches 30% (Anandaraj 2000). The attack of *P. capsici* caused a significant mortality rate. In the North Central Cost of Vietnam, it causes 80% plant mortality rate (Truong *et al.* 2008) and in some cases, 100% pepper vines were dead off (Ton *et al.* 2005).

A leaf spot disease with a heliocentric characteristic has been recently discovered in black pepper in the District of Bogor and Sukabumi, Indonesia. A pathogen causing the leaf spot disease is not identified yet, but the authors thought it to be caused by *Phomopsis* sp. PV-S G38 This disease also threatens black pepper farmers. However, the loss of the pepper yield caused by this disease is still not reported. *Phomopsis* sp. PV-S G38 have been reported in reducing the loss of yield in several crops. *Phomopsis vexans* (fruit blight) had been reported to cause around 15-20% losses in fruit yield of brinjal (Mahadevakumar *et al*. 2017). The presence of *P. phaseolorum* causing stem cancer and pod blight of soybean leads to 10% reduction in seed weight and 50% reduction in germination capacity (Sinclair 1993). *Phomopsis* sp. has been previously reported causing stem blight disease in asparagus plant (Yang *et al.* 2016; Thao and Dung 2019) and fruit blight on eggplant with heavy loss of yield (Saha *et al.* 2021).

Phomopsis sp. caused a leaf blight disease on several crops. *Phomopsis obscurans* is a common fungus infecting

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strawberry leaves and fruits (Howard and Albregets 1973). In 1984, the infection of these diseases expanded on other tissues, such as petiole, fruit trusses and stolon (Eshenaur 1989). The infection and development of this disease on leaves and runners significantly increased with the increased air temperature (Eshenaur 1989). *P. viticola* was also found causing the leaf spot on grape (Nita *et al.* 2008). *P. heveicola* was firstly reported causing leaf blight of coffee in China in 2019 (Gong *et al.* 2019). *Phomopsis* sp. has recently been reported infecting the leaf of *Macrotyloma uniflorum* in India (Pant *et al.* 2024). However, it has not been reported yet infecting the leaves of black pepper.

A recent study has reported that *Phomopsis* sp. was discovered attacking fruits of black pepper in Malaysia at the phase of asexual (Diaphorte) (Zakaria and Noor 2020). However, that study was still poorly documented because the symptoms were not explained in detail and the fungus morphologies were not studied yet. The study aimed at identification of the causative agent through morphological and molecular analysis. The implication of this study may provide important information that can be used to drive policy decisions focused at disease management in black pepper cultivation.

Materials and Methods

Site of study

This study was done during September 2020 to March 2021. Leaf samples affected by the disease were collected from smallholder plantations in Bogor and Sukabumi Districts, Indonesia. The cultivated area belonged to a community-owned black pepper plantation with an approximate size of 2.0 ha. The isolation process was carried out at the Mycology Laboratory, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University. This study used two stages of observation, a fungal isolation and fungal morphology and molecular identification.

Fungal isolation and field sampling

Sample collection: Direct observation on leaf samples was performed. Leaves exhibiting symptoms of brown spots with specific characteristics on the serrated leaf edges and dark brownish spots with specific heliocentric features were collected by cutting them off using a sterile knife. These samples were then placed in transparent plastic bags, stored in a cooler box, and transported to the laboratory. A total of 25 leaf samples were collected. The plant samples were three-month old seedlings.

Media sterilization: Petri dishes, measuring glasses, beakers, inoculation needles, and test tubes were washed with detergent, rinsed with clean water, and air-dried. They were wrapped in plastic and sterilized using an autoclave for 15 min at 121°C with a pressure of 2 atm following the method described by Fernandez and Chen (2005).

The Potato Dextrose Agar (PDA) media was prepared. A 200 g of potatoes were diced into small pieces resembling cubes. Thereafter, they were placed in a beaker glass and supplemented with 1 L of distilled water. The beaker glass was placed on a hot stir plate with setting temperature of 200°C and left for 1.5 h. The potato broth was then filtered and added with 20 g of dextrose, 17 g of pure microbiological agar, and 0.1 g of chloramphenicol.

Isolation of fungus from infected black pepper leaves: The isolation process was carried out based on the procedure described by Thilagam *et al.* (2018). Prior to isolation, the collected fungus-infected leaves were rinsed with running water for 5 min. They were, then, cut into approximately 1 cm^2 with half side of each piece carried healthy symptoms and the other carried diseased symptoms. Surface sterilization was done by dipping the samples on 1% NaOCl solution for about 2 min. Thereafter, they were rinsed with distilled water three times. A total of 5 sterilized samples were then planted onto a prepared PDA media in petri dish for fungal growth observation in a promptly purified environment.

Identification of fungal morphology: The fungal identification was conducted by macroscopic and microscopic observations. The macroscopic observation was carried out by examining colony color, shape, edge shape, elevation shape, and surface of the fungi. Microscopic observation was carried out under a microscope connected with the display (Olympus CX21) to obtain a detailed visual of the object. The process in preparing the object on semi-permanent slides was done following the standard method in the laboratory of microbiology. Mycelium was placed on a glass slide, added with 1 drop of Lacto Phenol Cotton Blue solution and spread evenly. It was left for a few minutes before covering up with a coverslip. The identification of *Phomopsis* sp. PV-S G38 morphology referred to several references. Those were soil and seed fungi by Watanabe (2002), introduction to common tropical molds by Gandjar *et al.* (1999) and illustrated genera of imperfect fungi. Structures of the hyphae whether they were septate or non-septate and hyaline or pigmented, as well as their asexual and sexual reproductive structures, and fruiting body structures were examined.

Fungal molecular identification

DNA from *Phomopsis* sp. PV-S G38 isolates were extracted and purified with DNAeasy Plant Mini Kit (Qiagen, Germany). DNA amplification of *Phomopsis* sp. PV-S G38 was carried out on a Thermo Cycler PCR machine using universal fungal primers, namely ITS 1 and ITS 4 following the PCR method described by White *et al*. (1990). The purified mycelia were submitted to Indonesian Center for Biodiversity and Biotechnology (ICBB) for molecular identification.

The amplified products were analyzed with a 1% agarose gel in 1x Tris-Borate EDTA (TBE) buffer.

Electrophoresis was set at 50 volts for 60 min. The agarose gel was immersed in ethidium bromide (EtBr) for 15 min and continued with sterile water for 5 min. The results of electrophoresis were visualized using a UV transilluminator and documented with a digital camera. DNA sequencing of the PCR products was performed by Frist-BASE Laboratories, Malaysia. The sequencing results were analyzed using multiple alignment program, Clustal W, with Bioedit 7.2.5 software and aligned with nucleotide sequences published on the GenBank site using BLAST-N (Basic Local Alignment Search Tool-Nucleotides) program on the NCBI (National Center for Biotechnology Information) website. A Clustal X 2.1 was employed to determine homology values on each sample. A phylogenetic tree was constructed using Mega 7.0 software with the neighbor-joining method and 1000 bootstrap repetitions following the method described by Anandaraj and Sarma (1995).

Results

Fungal isolation

Based on the isolation results, there were two prominent pathogen symptoms observed during field observations. The first symptom was necrosis without the presence of heliocentric and serrated edges at its tip, which was likely indicative of leaf ring spot caused by *Phomopsis* sp. PV-S G38. The second symptom was necrosis. A change of color was observed from green to black with appearance of heliocentric mark in the middle of the leaves. This symptom was suspected to be caused by *Phomopsis* sp. PV-S G38.

Fungal morphological identification

The hyphae of this fungus appeared as hyaline hyphae without septa, resembling cotton or wool on white-colored media as shown in Fig. 2. Authors did not find single spores during microscopic observation which complicated the process of morphological identification. Therefore, molecular identification was required to confirm the fungal species was *Phomopsis* sp. PV-S G38.

Fungal molecular identification

PCR and electrophoresis results indicated that the length of the amplified DNA band was approximately 542 bp (Fig. 3). The appearance of DNA bands might confirm that the obtained DNA sample more likely originated from *Phomopsis* sp. Based on the sequence alignment analysis in NCBI GenBank Nucleotide database, the sequences found in the ITS 1 and ITS 4 regions of the isolate sample exhibited a similarity level of 100% with *Phomopsis* sp. PV-S G38.

Pathogenicity test

One of the leaves from the round variety of Natar black pepper

Fig. 1: The symptoms of the disease on black pepper leaves (*Piper nigrum* L.) in Sukabumi district include leaf spot symptoms caused by *Phomopsis* sp. PV-S G38, characterized by the formation of heliosentris on black pepper leaves

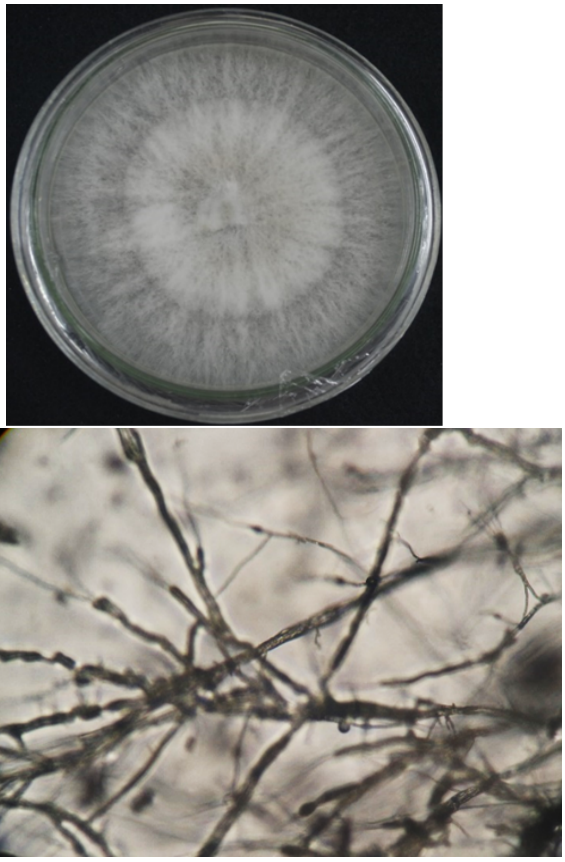

Fig. 2: Macroscopic and microscopic observation of *Phomopsis* sp. PV-S G38 hyphae

was washed using distilled water. Next, sample was immersed in 70% ethanol and rinsed it three times with distilled water. We placed the leaves onto a petri dish containing sterilized tissue culture medium after surface sterilization.

We cultured *Phomopsis* sp. PV-S G38 isolates in the center of potato dextrose agar (PDA) media and

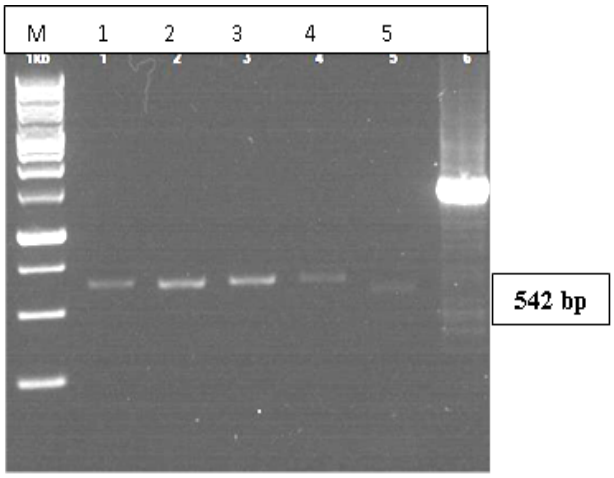

Fig. 3: Amplification result of gene 1TS1F rDNA (M) H Marker 1kb (ThermoScientific USA); Lane 5 is 542 bp for *Phomopsis* sp. PV-S G38

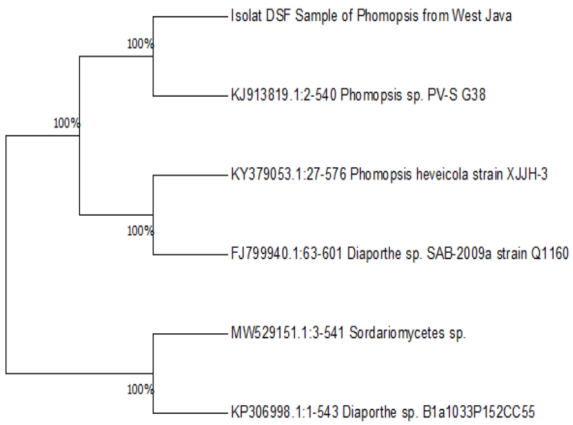

Fig. 4: Depicts the maximum likelihood phylogenetic tree from infected black pepper isolates using ITS sequence data, employing the maximum composite likelihood parameter model. The model and probability scores are provided at the nodes, and the scale bar indicates the number of substitutions per position. *Phomopsis* sp. PV-S G38 is considered the out-group

incubated them for 7 days in a temperature-controlled room. After the incubation period, we inoculated the central area of the black pepper leaf in a petri dish with approximately 1 cm of the *Phomopsis* sp. PV-S G38 isolate growing on PDA. We observed symptoms of infection after three days of incubation. The control group received only water agar medium inoculation without the pathogen (*Phomopsis* sp. PV-S G38).

Discussion

One of the diseases affecting black pepper plants originates from the Diaporthe group, representing the anamorphic phase of *Phomopsis* sp. PV-S G38 (Zakaria and Noor 2020). This phase is also responsible for diseases in dragon fruit in Malaysia (Huda-Shakirah *et al.* 2021). There have been no reports of *Phomopsis* sp. PV-S G38 as a pathogen in black pepper cultivation in Indonesia, until now. Huda-Shakirah *et al.* (2021) noted that necrosis characterized by a heliocentric shape and ring spots on the leaves were symptoms associated with *Phomopsis* sp. PV-S G38 infection. The infection of this pathogen resulted in a change in leaf color from green to brown, accompanied by irregular edges. These findings are consistent with those of Manda *et al.* (2020), who examined blight disease in eggplants caused by *Phomopsis* sp. PV-S G38. Based on our field observations, infected leaves turned dark brown and dried, displaying a heliocentric pattern. These symptoms varied in severity (Fig. 1) and were supported by the pathogenicity test, which elicited similar symptoms on leaves following inoculation with *Phomopsis* sp. PV-S G38 (Fig. 5) At the seedling stage, *Phomopsis* sp. PV-S G38 is frequently observed. *P. longicolla* was first reported to cause soybean stem blight in China during the seedling stage (Cui *et al.* 2009). Additionally, *P. vexans* has been reported to cause damping-off in eggplant seedlings (Manda *et al.* 2020).

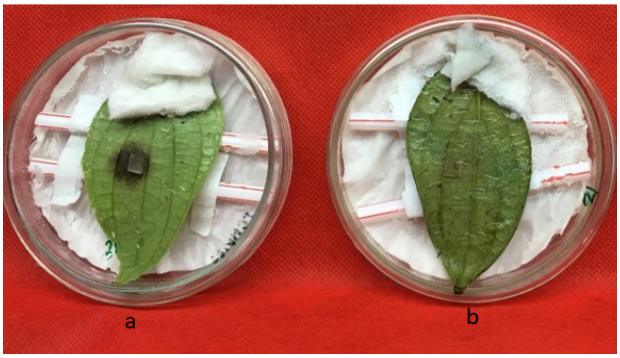

Fig. 5: Pathogenicity test: (**a**) leaves inoculated with *Phomopsis* sp. PV-S G38; (**b**) control treatment, in which only water agar was applied to the leaves without the inoculation of the pathogen

The difficulty in forming single spores may be due to factors of environment and genetic of the pathogen. *Phomopsis* sp. and its teleomorphic phase, Diaporthe, have hyphal sizes of 7-9 μm, with branched hyphae. Our findings were in line with the findings of Xu *et al.* (2021). Furthermore, Udayanga *et al.* (2011) identified morphological characteristics of hyphal structure of *Phomopsis* sp. PV-S G38 in the forms of hyaline, filiform, straight or curved, and aseptate. This fungus also had dark pycnidia and generally rounded. These findings were in line with the identification conducted by Watanabe (2002). He reported that *Phomopsis* sp. PV-S G38 produced pycnidia within the tissue of diseased plants, where one pycnidium can produce thousands of spores (conidia).

The appearance of DNA bands in the electrophoresis process was a significant finding because it was able to indicate that the obtained DNA sample most likely originates from *Phomopsis* sp. based on PCR results.

Nevertheless, they were still insufficient to definitively state that the isolate was *Phomopsis* sp. PV-S G38 because universal primers were still used in the PCR process. Our results were supported by a previous study which achieved a successful amplification using ITS 1 and ITS 4 primers with sizes ranging between 380–620 bp (Singha *et al.* 2016; Nishimitha *et al.* 2022), which finally gave more accurate information. Therefore, it could be concluded that the isolates showing necrosis symptoms found on leaves of black pepper in our study was confirmed as *Phomopsis* sp. PV-S G38. Our results obtained from the phylogenetic tree (Fig. 4) were in line with the findings of Udayanga *et al.* (2011) who identified the presence of *P. vexans* on leaves of eggplant, which showed leaf ring spot symptoms.

Mitigation of the diseases in black pepper by applying of organic matter had been reported. The presence of soil beneficial microorganisms would reduce the population of pathogenic agent such as nematodes and fungi (Nguyen *et al.* 2020). Besides their important roles in the metabolism and decomposition of complex organic compounds, their invasion on roots stimulates plant growth and increases a resistance to pathogens by producing antibiotics or enzymes that destroy cell wall. Furthermore, they are able to compete for nutrition and habitat with pathogens (Nielsen and Sorensen 1997; Tian and Riggs 2000; Khan *et al.* 2008). Further studies on the impact of *Phomopsis* sp. PV-S G38 on yield loss of black pepper and its mitigation method are required. This discovery provides the importance of molecular analysis in species identification and understanding the interaction between pathogens and plants, which, in turn, facilitate the effective plant disease management.

Conclusion

Morphological and molecular analysis revealed that a pathogen causing a leaf ring spot with heliocentric mark on leaves of black pepper was *Phomopsis* sp. PV-S G38. This is the first report of *Phomopsis* sp. PV-S G38 discovered on black pepper in Indonesia. The symptoms of the disease were characterized by the presence of circular, dark brown to black spots on leaves with a ring-like pattern. These findings highlight the need for further research to understand the epidemiology, management and control of this disease in the region.

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Authors Contribution

DSF was responsible for conceptualizing, conducting the study, collecting data, and writing the manuscript. MZA and AFI were responsible for advising and suggesting the title and contents of the manuscript. SAF and MIF were responsible for editing the manuscript.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the first author.

Ethics Approval

Not applicable to this paper.

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