Pyricularia zingiberi, a causal agent of diamond shape leaf spot disease of ginger in Indonesia

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Abstract. Ginger is an economically important commodity of Indonesia. A diamond shape leaf spot disease has been reported in ginger cultivation areas. The aim of this study was to determine the causal agent of the disease. Two isolates were obtained by a single conidia isolation method on Pyricularia typical conidia, followed by propagation on a PDA medium. The conidial suspensions were sprayed onto leaf of white and red ginger types for pathogenicity test. The inoculated plants were placed at room temperature with 80%-90% air humidity for 48 hr, then put back in the greenhouse. The fungal identity was determined based on its morphological characteristic and confirmed by molecular characterization through DNA extraction and amplification using ITS5 and ITS4 primers. The optimal growth temperature of the fungus was assayed by planting each isolate onto PDA medium, then incubated at 4 level temperatures. The same temperature levels was also tested on conidial germination, on WA medium. The artificial inoculation showed both isolates infected all types of ginger, but red ginger showed lower in disease severity. Morphologically, both isolates are corresponding to Pyricularia zingiberi. The fungus grows optimally between 25 °C and 31 °C. PCR analysis supported the morphological observation results.

1. Introduction

Many diseases on ginger cultivation caused by various pathogens occurred in Indonesia. Wilt disease caused by Ralstonia solanacearum and leaf spot diseases caused by Phyllosticta sp. are still being a serious problem in Indonesia [1]. Phakopsora elettariae and Phyllosticta zingiberi were also reported infecting ginger leaves [2].

Recently, Pyricularia sp. had been considered as the causal agent of a serious leaves disease. Typical symptom of the disease found on ginger cultivations in Indonesia involved diamond shape spot on the leaves. Disease incidence caused by this fungus in Central Java, West Java and Bengkulu Provinces was varied between 1.4% and 92.0% [3]. This fungus was prevalent on a big ginger type, whereas data regarding the infection on the red type was not available [3]. Pyricularia species has been reported as a pathogenic fungus of ginger in Japan [4-5]. Meanwhile, other Pyricularia species have been reported as endophytic fungi on leaves of zingiberaceae, and the fungi do not cause any typical disease symptom [6]. Pyricularia species have been studied intensively based on their molecular characters worldwide, included species infecting ginger leaf [7].
Pyricularia is an airborne fungus with a polycyclic disease type. The occurrence of disease development is influenced by the environmental condition. The disease incidence in upper land of Bengkulu Province (700 m asl) was higher than the one of below 500 m asl in West Java [3]. A combination of environmental factors, i.e. wet leaf period, air temperature, and air humidity were important for the epidemic of Pyricularia grisea of rice in California [8].

The objectives of the present study were (1) to identify fungus associated with diamond leaf spot disease of ginger both morphologically and molecularly; (2) to determine optimal temperature for fungus growth; (3) to assay its pathogenicity against 2 gingers types.

2. Methods

2.1. Isolation
Leaves of white ginger (giant ginger) showing diamond shape spots were collected from 2 areas of ginger plantations in West Java, i.e. Bogor in Sept 2016 and Sukabumi in Oct 2017. Leaf samples were washed in tap water for 1 min then placed in petri dishes with wet tissue to maintain the air humidity. The dishes were placed under white tube light (Phillips 20 W; 400 lux) with 40 cm distance in room temperature for 48 to 72 hr for inducing sporulation [9].

Single conidia isolation was carried out by first scrapping the conidia from the surface of the infected leaf tissue. The conidial suspension was spread onto water agar (WA) medium amended with 1% lactic acid (5 mL: 1 L medium) in a petri dish [10]. The dishes were placed in an incubator with temperature adjusted to 28 °C for 24 hr. Microscopic observation of the conidial morphological characteristics was also carried out for confirmation.

Germinating conidia were observed, selected and transferred onto PDA medium for further study. Confirmation on the obtained fungal isolate identity was conducted by subculturing the colonies onto PDA medium with pieces (1 cm²) of sterilized ginger leaf inside, and incubated for 72 hr in a room with specific light condition as mentioned above. The colony was considered as Pyricularia if it produces lemon shape conidia. Two isolates were selected and recognized as Pyricularia from each respective cultures, namely Pyr-J 1 from Bogor and Pyr-J 2 from Sukabumi. The isolates were preserved in PDA medium slant in tubes for further study. The isolates were also deposited in the culture collection of ISMCRI, IAARD in Bogor.

2.2. Identification

2.2.1. Morphology. Fungal species were identified based on morphological characteristics, mostly the characters of conidiom and conidiophore. The fungal reproductive structures of each isolate were mounted in a drop of lactic acid on a slide glass, then placed under a light microscope for observation [11]. Images capture software with Meiij camera system was performed for documentation and measurement of the size of conidia and conidiophore. The obtained data were compared with those described previously as the characters of Pyricularia species [7, 12, 13].

2.2.2. Molecular characterization. DNA extraction of Pyr-J1 and Pyr-J2 isolates were conducted according to the protocol described [14]. Moreover, DNA amplification was performed by polymerase chain reaction (PCR) method in a 25 μL reaction mixture consisting of 2 μL of DNA template, 1 μL of each primer as a final concentration of 1 μM, 8.5 μL of nuclease free water, and 12.5 μL of 2xGo Taq Green Master Mix (Promega, Madison, WI). The fungus-specific universal primers ITS5 (5’–TCCTCGGTTATGTAGTC–3’) and ITS4 (reverse) (5’–TCCGTAGGTAACTTTCGC–3”) were used for PCR amplification [15]. These reaction were subjected to an initial denaturation of 5 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C for denaturation, 30 sec at 52 °C for annealing process, 30 sec at 72 °C for extension and 7 min at 72 °C for final extension. The PCR product was analysed by electrophoresis in 1.5% (wt/vol) agarose (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8]), and stained with 1% gel red. The electrophoresis condition was set at 110V for 30 min. A 1 μL of DNA
marker (1000 bp) was used in the gel. DNA visualisation was conducted by Gel Doc™ XR (BioRad, USA). The PCR products were sent to First Base (Malaysia) for sequencing.

Phylogenetic analysis was performed using Maximum Parsimony (MP) method in the PAUP 4.0.b10 program [16]. The heuristic method using tree bisection reconstruction (TBR) with the addition of 1000 random sequence algorithms was performed to obtain the optimal tree. Internal branch strength of the phylogenetic tree in MP analysis was tested by Bootstrap (BS) analysis using 1000 replications. Bootstrap (BS) value > 50% was displayed on the phylogenetic tree.

2.3. Pathogenicity
Pathogenicity test was performed by means of artificial inoculation. Two mo-old ginger plants were sprayed with conidial suspension adjusted at 10^5 conidia m/L with 0.01% tween 20 as spreading agent [17]. The conidial suspension of each respective isolates were prepared as mentioned above. The inoculated plants were incubated in a dark room with temperature about 25-28 °C and air humidity ranged 75%-80% for 72 hr, then put the plants back to greenhouse. Ginger (Zingiber officinale), white ginger var. Cimanggu and red ginger var. Halina 1 were used in this test. Symptom appearance was observed daily, disease incidence and severity were measured at 14 d after inoculation. Disease severity was measured by scoring as described by scoring system for P. oryzae of rice [18]. The plant was considered resistant or incompatible based on infection type categories [5].

2.4. Optimal temperature
The effect of ambient temperature on the fungus’ colony growth was tested by planting the fungal isolates onto PDA medium in petri dishes and incubated in incubators set at 25, 28, 31 and 34 °C. The colony diameters was measured after 7 d of incubation.

The effect of the tested temperature on percent germination and germ tube length of conidia as well as appressoria formation of each fungal isolates were carried out by dropping a conidial suspension onto a sterilized slide glass, then put them into petri dishes with wet filter paper inside. The dishes were placed in incubators with temperature adjusted to 25, 28, 31 and 34 °C for 24 hr. Each treatment was repeated 5 times.

Sporulation ability of the fungal isolates at different tested temperature levels were carried out by growing the isolates in medium and incubated at various levels of temperature [19], with modification of growing medium. A PDA medium was used instead of extract dextrose oat meal agar. The petri dishes were incubated in room temperature for 72 hr. Five pieces of autoclaved ginger leaves ca. 1 cm² width were put at the edge of an active growing colony of respective fungal isolates. Then, the dishes with the leaves inside were put into incubators with adjusted temperatures as mentioned above for 7 d. Number of conidia from each treatment was measured by dislodging each dish with water, and counted the conidial density with a haemocytometer.

3. Results and discussion
In natural conditions, the specific symptom is commonly appeared on the lower leaves which is fully opened on the productive phase of the plants (Fig. 1a and 1b). The conidiophores and the conidia were consistently found from infected leaves incubated in room temperature under humid condition and exposed by 12 photoperiods of light for 48 hr. This treatment is a suitable condition for the fungus to sporulate.

Solitary, long and cylindrical shape conidiophores developed from the typical infected leaf symptom; it is brown to hyaline, 170 – 280 µm long and 2.5-4.5 µm wide. The conidiophore mostly consists of 3-5 septate or more (Fig. 1c and 1d). The conidiogenous cell on the tip of conidiophore consists of 4 or more obviously conidial scars (Fig. 1e). Conidia is hyaline, lemon shaped with 2 septate, 17.5-21.3 µm long, 5.0-8.8 µm wide; apical cell 5.0-8.6 µm long, and 5.0-7.5 µm for basal cell, hilum as thickening structure at the basal end (Fig. 1f). Germination occurs mostly below 24 hr, with germ-tube raises from the upper cell with appressorium (Fig. 1g and 1h). Color of the colony is
light green at the center and white at the edge. Resting structure often seen from the old colony (Fig. 1i and 1j).

**Figure 1.** Symptom and the causal agent (*Pyricularia zingiberi*) of diamond shape spot disease of ginger. (A) and (B) Typical diamond shaped leaf spot; (C) Conidiophores; (D) Basal conidiophore; (E) Conidiogenous cell; (F) Conidia; (G) Germinating conidia; (H) Germinating conidia with appresorial structures (→); (I) Colony on PDA at 28 °C; (J) Colonies with resting structures. Note, Fig 1C to 1J are morphological characteristics of the Pyr-J2 isolate.
The MP tree obtained by sequence analysis of ITS rDNA of Pyr-J1 and Pyr-J2 and the sequences of 27 related sequences of Pyricularia obtained from sequence databank is represented in Fig. 2. Bootstrap analysis with 1000 bootstrap replications demonstrated 3 major branches. It showed that isolates Pyr-J1 and Pyr-J2 are in the same clade (Fig. 2). The 2 isolates are in the same clade with Pyricularia zingiberi from Japan by 99.7% Bootstraps (BS) value. Based on both morphology and molecular description, the Pyricularia isolates were identified as P. zingiberi. No sexual state was observed neither from the culture nor on infected leaf samples.

Pyricularia is well known as a wide host range plant pathogenic fungus, mostly on monocotyledon. The current isolates have morphological characteristics similar to P. zingiberi [13], and based on sequence of ITS5 and IST4, the current isolates were clustered in the same clade with the isolates of ginger from Japan that showed low pathogenicity to other plant species [5]. Therefore, Pyricularia isolates of ginger in Indonesia are identified as P. zingiberi. This present fungus was different from Pyricularia distorta of Alpinia and Catymbium (Zingiberaceae). The hilum of the present isolates is obvious; and no hilum for the P. distorta. This is the first reports confirming that P. zingiberis is existing in Indonesia.

The artificial inoculation results showed that both tested isolates infected 2 types of ginger, although the white ginger type was more susceptible than the red ginger. Yellow spot symptom was seen at 7 to 8 d after inoculation and the typical diamond spot symptom with grey center mostly distinct about 14 d after inoculation. The symptom was rarely seen at young but predominantly at well-developed leaves of both ginger types. There was no symptom at sheath leaf nor stem. If the spot developed at the leaf basal end, the infected leaves are sensitive to wind and fall prematurely. According to the resistance category [5], both 2 ginger types were categorized into infection types 3 or 4, which are considered as compatible or susceptible.

Pyricularia zingiberi have been reported infecting ginger plant in Japan [13, 5], India [20], and Malaysia [21]. Some species of Pyricularia are also reported living as endophytic in leaves of zingiberaceous crops in Thailand, namely P. costina, P. kookicola, P. longispora, and P. variabilii [6]. Pyricularia zingiberi seems narrow in host ranges [13, 5]. Therefore, pathogenicity test is an important step to determine this fungus identity.

Both fungal isolates showed similar growth patterns in regard of optimal temperature, conidial germination and germ-tube length characters. The fastest growth of both Pyricularia isolates was between 25 and 31 °C, the fungus grew more slowly outside the temperature range (Fig. 4a).

Temperature range 28 and 31 °C were also optimum for the conidia to germinate, with up to 70% of the conidia of both isolates germinated within 24 hr (Fig. 4b). The longest conidial germ-tube was observed at 28 and 31 °C (Fig. 4c). Germinating conidia also formed appressoria and 80% of the conidia formed appressoria if incubation was at 28 and 31 °C, higher than other tested temperatures. Temperatures between 28 and 31 °C was also suitable for the isolates to sporulate (data not shown).

The optimal temperature for P. zingiberi to grow seems between 25 and 30 °C, and 48 hr of high relative humidity is a suitable condition for the fungus to germinate and infects ginger leaf. The typical symptom is obviously recognized about 7 d after inoculation, with sporulation occurred within 48 hr. This finding are corresponding with Pyricularia grisea, a pathogenic species of rice blast. Growth speed and sporulation of P. grisea (M. grisea) are higher at 27 °C than the one incubated at 22 or 32 °C [19].

Pyricularia fungus of ginger may have existed a long time ago in Indonesia, because ginger has been cultivated for yr. Lacking of sporulation ability of this fungus on PDA medium may cause the fungus undetected along a leaf tissue isolation procedure.

Ginger plants are mostly planted in the early of rainy seasons. It is a favourable condition for Pyricularia to grow, develop and spread. In order to manage the diamond shaped leaf spot disease occurrence in the future, epidemiological study of the fungus on various ginger growing phase and its potent host ranges among other Zingiberaceae should be considered, as well as find an effective fungicide.
Figure 2. Maximum Parsimony (MP) tree generated from the ITS rDNA sequences of the Pyr-J1, Pyr-J2 and related sequences. The tree was rooted with *Rhizopus oryzae* CBS 112 07, CBS 330 53, and CBS 127 08. Bootstrap value >50% (1000 replicates) are shown at the branches.

Figure 3. Disease incidence (a) and severity (b) of 2 isolates of *Pyricularia zingiberi* on white and red gingers. Vertical line on each bar represents ranges of values. □ Pyr-J1 and ■ Pyr-J2 isolate (white ginger is var. Cimanggu; red ginger is var. Halina 1).
Figure 4. Effect of temperature on growth of *P. zingiberi* isolates. (a) Colony diameter, (b) Germination and (c) Germ-tube length. Vertical line on each bar represents ranges of values. □ Pyr-J1 and ■ Pyr-J2 isolate.

4. Conclusions

*Pyricularia zingiberi* is the causal agent of diamond shape leaf spot disease of ginger in Indonesia. White and red ginger can be the host of *P. zingiberi*.

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