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LEAF SPOT CHARACTERISTICS OF *PHOMOPSIS DURIONIS* ON DURIAN (*DURIO ZIBETHINUS* MURRAY) AND LATENT INFECTION OF THE PATHOGEN

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Abstract

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A survey of leaf spot disease on durian caused by *Phomopsis durionis* was conducted in durian growing areas in eastern Thailand, Chanthaburi and Trat provinces. It was found that lesions with yellow halos on both mature and young leaves are variable in sizes (1–10 mm in diameter). In this study, nine morphologically distinct isolates of *Phomopsis* were obtained from durian leaf spots. Some of them produced small number of pycnidia on potato dextrose agar after incubation for 30 days. Artificial inoculation on wounded leaves of durian seedlings, resulted in the production of browning areas with yellow halos and pycnidium formation at 13 days and 20 days after inoculation, respectively. Furthermore, red-brown spots with yellow halos on leaf tissues were observed at 32 days after inoculation. High density of *Phomopsis* was observed in durian symptomless leaves and flowers indicated the latent infection of the pathogen in the fields. Interestingly, crude extract of durian leaf with preformed substances demonstrated inhibition of spore germination and germ tube growth of the pathogen, *Phomopsis* sp., on water agar. In addition, bioassay on TLC plate displayed inhibition zone of growth of the fungus, *Cladosporium oxysporum* at retention factor (R_f) of 0.29–0.88. This indicates that preformed substances in leaf tissues might act as compounds affecting latent period of pathogen.

Keywords: *Durio* spp., quiescent infection, latent period, preformed substance, TLC bioassay

INTRODUCTION

The genus *Phomopsis* (telemorph *Diaporthe*) belongs to the Phylum Ascomycota, class Sordariomycetes, order Diaporthales, and family Diaporthaceae (Webster and Weber, 2007). Several species of the *Phomopsis* are plant pathogenic fungi causing diverse symptoms, i.e., spot, canker, dieback, root rot, fruit rot, blight, decay and wilt on a vast variety of hosts (Uecker, 1988; Uecker and Johnson, 1991; Uceker and Kuo, 1992; Santos and Phillips, 2009). Among them, *P. durionis* has been reported as the causal agent of durian leaf spot disease which was found in several durian growing areas of Thailand (Lim and Sangchote, 2003). The disease affects both seedlings and mature plants, particularly in susceptible cultivars such as 'Mon Thong', the most commercially grown cultivar of Thailand. The leaf spot symptom is characterized by dark brown necrotic spots, approximately 1 mm in diameter, with yellow halos. These spot lesions are more prevalent on durian leaves during the mature stage, in particular when durian growing fields are poorly managed. Additionally, shabby or unhealthy plants can be noticed due to reduction of photosynthesis. Although Phomopsis leaf spot is not a major cause of loss in durian production, the fruit rot after harvesting caused by several fungal pathogens including *P. durionis* is considered to be more significant in losses of durian yield (Sangchote *et al.*, 2012). Various important pathogens, i.e., *Colletotrichum gloeosporioides*, *Lasiodiplodia theobromae*, *Phytophthora palmivora* and *P. durionis* were found to be causal agents of durian fruit rot; however, *P. durionis* was the most prevalent pathogen among members

of the same population (Sangchote *et al.*, 2012). *P. durionis* has also been found to be a new causal agent of leaf spot disease on *Pachira macrocarpa* in China. It causes severe symptoms and is widespread throughout the areas growing this plant (PingGen *et al.*, 2000).

The appropriate climate for the development of durian leaf spot disease was reported to be favored by high humid condition (Lim and Sangchote, 2003). In Thailand, indeed in eastern region, *P. durionis* spread over durian growing areas throughout the years. The observation in 2013 in durian growing areas of Chanthaburi, one of the provinces in the east of Thailand, indicated that relative humidity at 80% or above lasted for 7 months of the year. Moreover, rainfall amounts between 400–1000 mm were observed for half of the year (unpublished data). Similar observation was found in *P. viticola* on grape. Necrotic spots on cane and leaf as well as fruit rot of grape were more produced when the grape plants were grown during the period with high rainfall amount (more than 100 mm) (Pscheidt and Pearson, 1991).

In several studies, various species of *Phomopsis* have been reported to undergo a phase of latent infection in which pathogens infect plant organs and colonizes in tissues without visible symptoms. When the condition is suitable such as older foliage stage of plants or plant senescence, the symptoms can be expressed. Latent period of *P. viticola* has been reported for 3–4 weeks after pathogen infection on grape leaves before necrotic spots occur (Pscheidt and Pearson, 1991; Hilton, 2012). Similarly, asymptomatic fruits of grape have been noticed after infection of *P. viticola* when they are almost ready to be harvested, fruit rots then are visible (Anco *et al.*, 2011). Furthermore, it has been reported that narrowed-leaf lupine infected by *P. leptostromiformis* has latent and symptomless period of 20 days after infection (Williamson *et al.*, 1991). In case of strawberry plants infected by *P. obscurans,* invisible symptoms can be investigated in early growing season, whereas the symptoms of leaf blight develop in the late season (Ellis and Nita, 2008). Plant organs infected with latent pathogens are the main sources of inoculum for disease spread under appropriate climate conditions. Rawnsley (2012) reported that high humid condition was required for pathogens to release their spores from their fruiting bodies. Likewise, spores of *P. viticola* can be dispersed to any plant parts by rain splash. The factors involving with latent infection of pathogens have been reviewed by Prusky (1996), in which preformed substances in plant tissues are able to suppress the growth of pathogens. Everett (1997) and Wang *et al.* (2006) discovered that an antifungal compound, l-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene, obtained from the peel of unripe avocado fruits was shown to suppress the growth of the *Colletotrichum gloeosporioides*. Similarly, a mixture of antifungal substances, 5-(12-cis-heptadecenyl)- and 5-pentadecyl-resorcinol, which was extracted from the peels of immature mango fruit displayed the possibility to inhibit *Alternaria alternata* (Droby and Prusky, 1986).

Plants without symptom during latent stage become one of obstacles to identify cause of disease problem. This brings about a failure to establish an effective disease management program in time. Elucidation on factors relating to latent infection such as preformed substances involving in pathogen growth suppression may open up a new avenue for disease management in the future.

Although several latent pathogens have been studied in detail, information on *Phomopsis durionis* infected on durian leaf is still elusive. Therefore, the present study aimed to:

1) characterize durian leaf spot symptom and its causal pathogen, *P. durionis*,
2) study on latent infection of *P. durionis*, and
3) examine the factor affecting latent infection of pathogen in durian leaves.

MATERIALS AND METHODS

Disease Observation and Pathogen Isolation

The survey of Phomopsis leaf spot of durian cultivar Mon Thong was conducted at two locations of durian growing areas in eastern Thailand, Chanthaburi and Trat provinces. Diseased samples were randomly collected from twenty plants of each orchard, and then were characterized. The pathogens were isolated by tissue transplanting method and were maintained on potato dextrose agar (PDA) at room temperature (25–32 °C). Pure cultures were kept in PDA slants for further study.

Latent Infection of Leaf Spot Disease on Durian Seedlings

Inoculum Preparation

A highly virulent isolate of *P. durionis* obtained from the previous study of Tongsri (2013) was cultured on PDA. Sterile durian leaf tissues, 1 cm^2 in size, were placed on the PDA surface for spore induction at room temperature for 10 days. To prepare conidial suspension, leaf tissues containing large amount of conidial mass were immersed in distilled water. The sample suspension was then filtered through three layers of sheet clothes.

Pathogen Inoculation

A sterile needle was used to make wound on the leaf surface of durian seedlings cultivar Mon thong. 20 µl of the conidial suspension at the concentration of 1×10^6 conidia/ml was dropped on the wound sites. Inoculated seedlings were incubated in a 95% relative humidity in plastic bag at 28–33 °C for 48 hours, and subsequently the moisture was released by unsealing the bag. Seedlings then were still maintained in unseal bag for 14 days. The infected seedlings were removed from plastic

bag into greenhouse under normal condition until symptom was developed. The time period of symptom expression was then recorded.

Latent Infection of Leaf Spot Disease in Durian Orchards

Symptomless durian leaves and flowers (cultivar Mon Thong) were collected from two durian orchards in different growth stage. Pathogen detection on the symptomless leaves and flowers was performed by tissue transplanting technique on PDA. Ten pieces (2 mm^2 in size) of leaf and flower tissues were placed on each PDA Petri dish, and were incubated at room temperature for 7 days. The experiment was repeated twice with three replications (10 Petri dishes per replication). Percentage of pathogen population was determined. Latent infection of *Phomopsis* was confirmed by freezing technique with modified method of Luo and Michailides (2001). Durian leaves without symptoms were placed overnight at –20 °C, and then were transferred to moist chamber. The leaf samples later on were incubated at room temperature for 7 days, and then pycnidial production was observed.

Antifungal Substances in Durian Leaf Tissues as a Factor Affecting Latent Infection

Leaf Extract Preparation

Durian leaves (cultivar Mon Thong) without symptoms were chosen from the orchards. They were blended to powder (300 g) for soaking in 500 ml 95% methanol for 7 days. The mixture was filtrated through Whatman No. 1 filter paper, and the filtrate was collected. The filtrate was dried with a rotary evaporator (Buchi, Rotavapor R110) at 40 °C, subsequently the residues were resuspended in 5 ml of absolute methanol. Crude extract was stored in the dark vials and kept at –20 °C until used.

Antifungal Activity on Water Agar (WA)

500 µl of crude extract was dried in air for 2 hours to dismiss toxic solvent, and then the rest of crude extract was pipetted onto 0.2% WA Petri dish. Twenty microliter of *P. durionis* conidial suspension was also pipetted to Petri dish. The mixture was spread using sterile glass rod. The Petri dish was incubated at room temperature for 10 hours. Percentage of conidial germination and germ tube growth were determined, and then were compared with the controls of evaporated methanol and distilled water. Five hundred conidia were counted in total to examine conidial germination and germ tube growth with three replications.

Antifungal Activity on Thin Layer Chromatography (TLC) Plate

100 µl of crude extract were load onto TLC plate (aluminium plate coated with 0.2 mm-thick silica gel 60 F254, MERK) using micro haematocrit tubes (Vitrex Medical AIS, Denmark). The loaded spot was completely dried, and then the plate was developed in running solvent, dichloromethane:methanol (98:2 v/v, modified from the method of Peret-Almeida *et al.*, 2005) for 1 hour. The plate was removed from running solvent and air dried for 2 hours for removing of toxic solvents. The TLC plate was sprayed with conidial suspension of either *P. durionis* or *Cladosporium oxysporum* as indicator fungus in potato dextrose broth using an airbrush (BADGER AIR-BRUSH™, U.S.A.). Since *C. oxysporuma* produced dark conidia, the evaluation of inhibition zone can be easily achieved on TLC plates. The plate was then incubated in a moist plastic chamber at room temperature for 3 days. Inhibition zones of fungal growth were observed and the retention factors (R_f) values of the active compounds were calculated as following equation (modified from Zainuri, 2006).

$$R_f = \frac{\text{Distance traveled by the active compounds}}{\text{Distance traveled by the running solvent}}.$$

RESULTS AND DISCUSSION

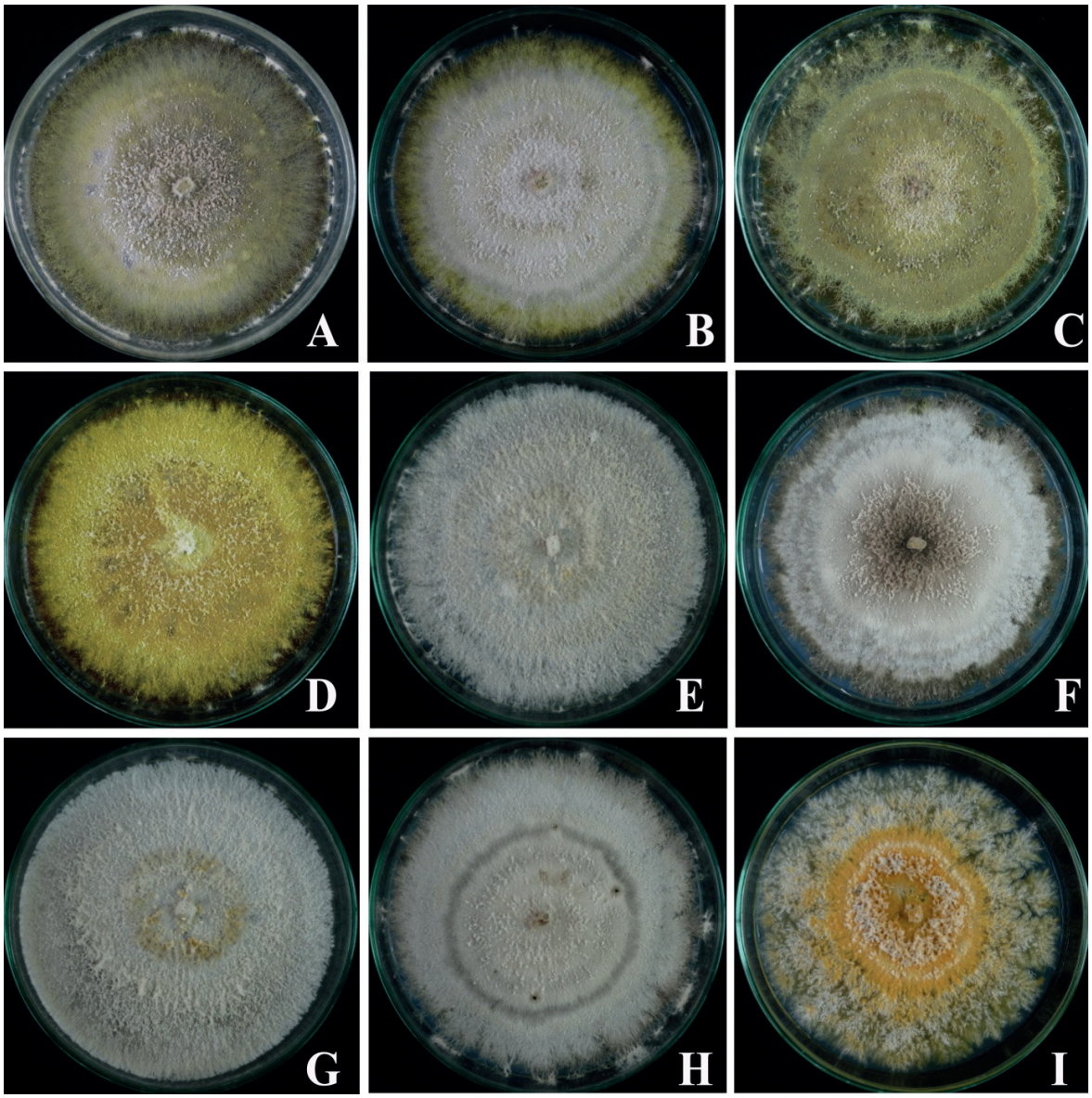
Disease Characteristics and Pathogen Isolation

Leaf spot disease of durian cultivar Mon Thong caused by *P. durionis* is variable in size, ranging from 1 10 mm in diameter, with dark red-brown margins and yellow halos surrounding the lesions. The disease expresses on both mature and young leaves, but numerous spots are prevalent on maturity stage of leaf (Fig. 1). Lim and Sangchote (2003) have been reported that the leaf spot symptom on durian usually presents the small lesions, approximately 1 mm in sizes. However, we initially report distinct observation on lesion size which is larger than 1 mm in diameter with yellow halo in this study. Morphological study of *Phomopsis* isolated from leaf spots with different sizes revealed the nine morphologically distinct isolates of *Phomopsis* on PDA culture (Fig. 2). In addition, small numbers of pycnidia from some isolates were observed on PDA cultures after 30 days of incubation. Indeed, two distinct types of conidia, α- and β-conidia, were obtained. However, the latter ones were rare (Fig. 3). Similar result was also reported in the study of Brayford (1990) in which two groups of *Phomopsis* isolates from elm were obtained due to morphological differences and ability to produce pycnidia. Moreover, a group of *Phomopsis* only produced pycnidia on sterile elm tissues.

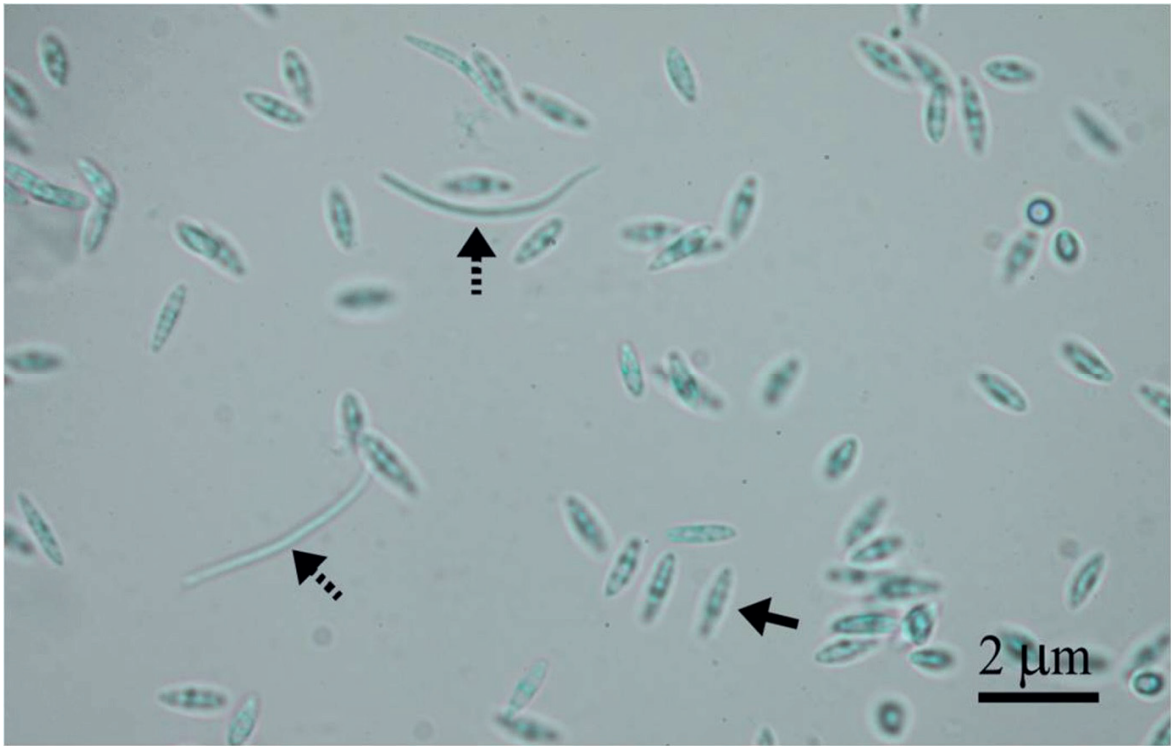
Latent Infection of Leaf Spot Disease

Artificial inoculation by wounding method on seedlings demonstrated browning areas on the wound sites of durian leaves after 13-day inoculation. Later on, lesion expansion and necrotic area with yellow halos were observed. Pycnidial production, tiny black spots seen by naked eyes, was investigated on the surface of necrotic tissues

1: *Leaf spot disease on durian cultivar Mon Thong caused by Phomopsis durionis. (A) Small spots (~ 1 mm in diameter) with yellow halos on mature leaves. (B) Large necrotic lesions (~ 2–10 mm in diameter) with yellow halos on mature leaves*



2: *Morphologically distinct isolates of Phomopsis isolated from leaf spots on durian cultivar Mon Thong. (A–C) The isolates of C04, C10 and C08 from Chanthaburi province. (D–I) The isolates of TP01-5, TP02-1, TP02-3, TP02-4, TP03-1 and TP03-2 from Trat province*

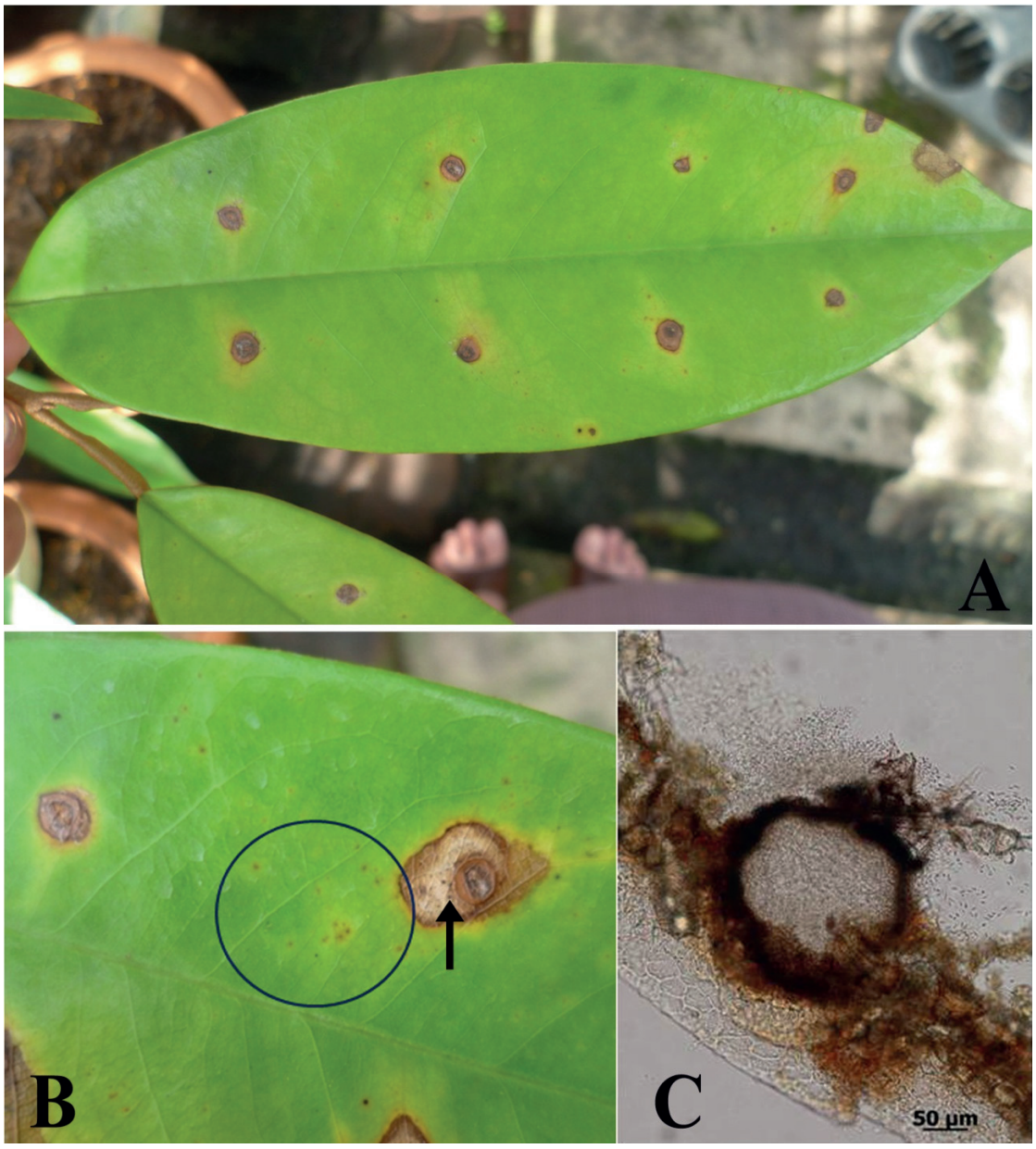


3: *Two types of conidia of Phomopsis durionis isolate C10. Numerous α-conidia (continuous arrow) and rare β-conidia (dash arrow) released from pycnidium*

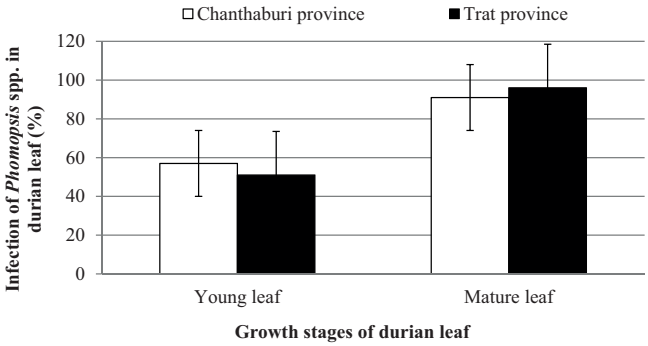
after 20 days of infection. Free-hand cross section of pycnidia exhibited large amounts of α-conidia inside. Subsequently, small red-brown spots about 1 mm in sizes were observed after 32 days of inoculation (Fig. 4). The spot lesions were then re-isolated, and the same pathogen was detected after investigation. Rawnsley (2008) reported that *P. viticola* caused brown spot on grapevine leaves in the period of 21 days after infection, while the spots on canes appeared at 28 days or more. In addition, Pscheidt and Pearson (1991) reported that grapevine leaves infected with *P. viticola* still showed no symptoms at the earlier period of infection. Until the leaf tissues became senescent, the symptoms then became visible. Similar observation was observed on the grapevine fruits in which the spots on them were shown about 7–21 days before harvest. Williamson *et al.* (1991) found that *P. leptostromiformis*, the causal agent of stem blight on lupine, produced symptomless plant tissues after infection for 20 days. Altogether, these evidences confirm the existence of a latent period developed by the pathogens.

Pathogen investigation in symptomless durian leaves and flowers (cultivar Mon Thong) from two durian orchards indicated higher density of *Phomopsis* in the mature leaves than that of the young leaves (Fig. 5). It might be possible that the old leaves are suitable for pathogen colonization. Sinclair (1991) found that soybean plants infected with *P. phaseoli* always occurred in vascular system of the mid and late stage of vegetative growth, although there were no visible symptoms on plant tissues. Furthermore, the symptoms expressed on the stem and senescent cotyledons in the period of 30 days after infection were detected in the study. Besides symptomless durian leaf tissues, the pathogen was also able to produce invisible symptom on durian flowers. Indeed, the outer layer of floral parts – epicalx – was found the highest infection particularly in 8-week-old stage of flowers, the initial period of blooming (Fig. 6). An overnight-deep freezing at –20 °C following by seven-day incubating of natural infection of *Phomopsis* in durian leaves exhibited brown and water-soaked tissues with numerous black pycnidia. Hence, this observation also supports an existence of latent infection of the pathogen in this study. Latent period phenomenon was observed in extensive detail in the study of Williamson *et al.* (1991), in which asymptomatic plant tissue displayed conidium germination as well as pathogen penetration into the plant cells. Furthermore, subcuticular coralloid hyphae between cuticle and epidermis layers were also observed. When plants developed into mature stage or became senescent, the visible symptoms were then observed.

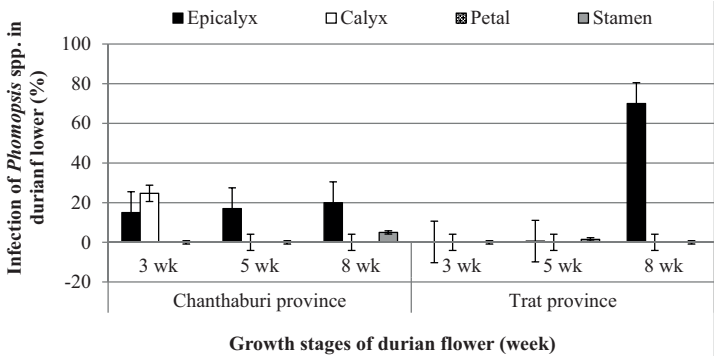
It is speculated that plant tissues containing latent pathogens may act as sources of inoculum, when condition is suitable for development of pathogen-reproductive structure. As shown by the study of Rawnsley (2008), fruiting body structure of a latent pathogen, *P. viticola*, was produced under high humid condition. Later on these spores were dispersed by rain splash to new plants. Therefore, *Phomopsis*



4: *Artificial inoculation of Phomopsis durionis on wounded leaves of durian seedlings. (A) Necrotic spots surrounded by yellow halos. (B) Small red-brown spots with yellow halos (circled), 32 days after inoculation, and tiny black pycnidia of pathogen on necrotic tissue (arrow). (C) Freehand cross section of Phomopsis pycnidium with numerous α-conidia obtained from necrotic tissue*



5: *Latent infection of Phomopsis spp. on young and mature stages of durian leaves obtained from Chanthaburi and Trat provinces. Bars represent the standard error of means in three replications*

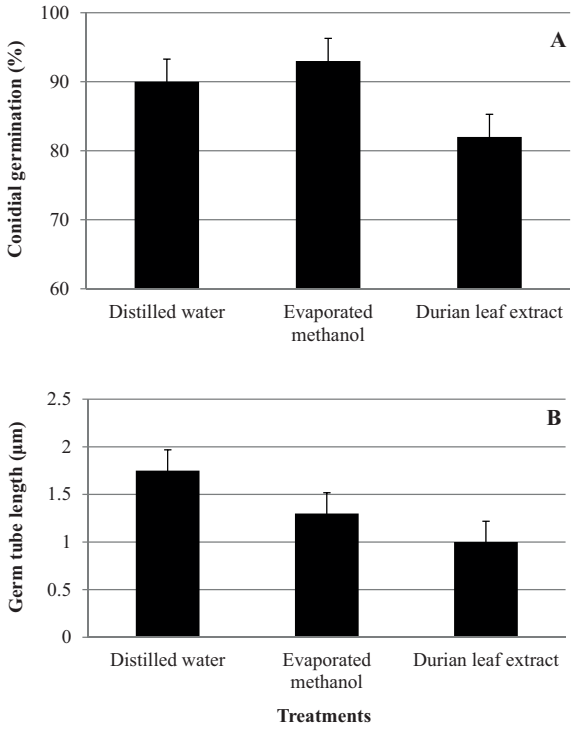


6: *Latent infection of Phomopsis spp. on different parts of durian flowers obtained from Chanthaburi and Trat provinces. Bars represent the standard error of means in three replications*

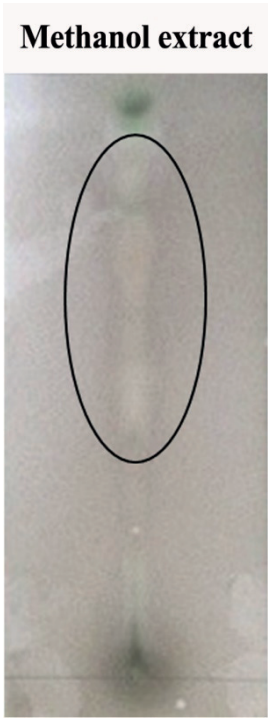
population that presented on durian leaves and flowers may act as important source of inoculum for pathogen dispersal and disease spread.

Antifungal Substances in Durian Leaf Tissues as a Factor Affecting Latent Infection

Crude extract from durian leaves significantly inhibited conidial germination of *P. durionis* on water agar. Germinating conidia treated by the crude extract exhibited the value of 82%, while the controls of evaporated methanol and distilled water showed the values of 93% and 90%, respectively. Furthermore, the leaf extract was able to reduce germ tube length compared with one of the controls, an evaporated methanol, as indicated by the average values of 1 µm and 1.3 µm in length, respectively (Fig. 7). Moreover, bioassay on TLC plate exhibited a wide range of inhibition zones at R_f 0.29–0.88 against *P. durionis* and *C. oxysporum* (Fig. 8). This may indicate the action of preformed-antifungal substances containing in durian leaf tissues on pathogen growth inhibition. Chemical components in durian leaves including hydroxytryptamines, mustard oils, saponin, fats, and formic acid were reported by Brown (1997). Some of these chemicals displayed the potential of antimicrobial activity. Similarly, Lauren *et al.* (2011) demonstrated that



7: *Effects of durian leaf crude extract on conidial germination (A) and germ tube length (B) of Phomopsis durionis on water agar, compared to the controls, distilled water and evaporated methanol. The result was observed after 10 hour incubation at room temperature (25–32°C). Bars represent the standard error of means in three replications*



8: *TLC plate showing inhibition zones (circled) at R_f 0.29–0.88 where the growth of Cladosporium oxysporum (indicator fungus) was inhibited by preformed-antifungal compounds obtained from durian leaf extract*

saponin, one of preformed chemicals, from cereal exhibited the ability of microbial suppression. Furthermore, Prusky (1996) reviewed that preformed substances in plant tissues such as diene extracted from pericarp of avocado fruit can affect latent infection by suppressing pathogen growth. Although, antifungal substances in plant tissues reduced spore germination and germ tube growth of the pathogen, *Monilinia fruticola*, appressoria were not affected by the same substance (Janisiewicz *et al.*, 2011). Thus, fractionations of crude extract with appropriate solvents need to be performed for analysis of pure compound in future studies.

CONCLUSION

In this present study, we report the distinct characteristics of *P. durionis*, a causal agent of durian leaf spot disease, which were obtained in eastern Thailand. The durian leaf spot lesions were variable in sizes with distinct morphological appearances of the pathogen. Latent stage of the pathogen was studied in details in which browning areas with yellow halos on inoculated wound-leaves of seedling appeared at 13 days after inoculation with the virulent isolate of *Phomopsis*. Pycnidial formation on necrotic tissues subsequently expressed at 20 days of inoculation. In addition, the new symptom of red-brown spots (~ 1 mm in size) with yellow halos exhibited at 32 days of inoculation. Furthermore, natural infection of *Phomopsis* on the leaves and flowers without symptoms were abundantly observed in the actual durian orchards. Interestingly, preformed substances in durian leaves displayed the ability to inhibit spore germination and germ tube length of the pathogen. Therefore, the phenomenon of pathogen infecting in plant tissue with no visible symptoms may be related to some factors available in plants that affect latent period of the pathogen.

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