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PAPER • **OPEN ACCESS**

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To cite this article: Suparman *et al* 2018 *IOP Conf. Ser.: Earth Environ. Sci.* **102** 012080

Cross inoculation of anthracnose pathogens infecting various tropical fruits

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Abstract. Anthracnose disease is very important disease of tropical fruits causing significant yield losses. The disease is caused by *Colletotrichum* spp. and infects almost all tropical fruit species, especially the succulent ones. Various species of Colletotrichum infect various tropical fruits and there are possibilities for cross inoculation to occur among tropical fruits which might cause severe infection. An experimental research was conducted to examine the effect of cross inoculation of anthracnose pathogen among papaya, eggplant, chili and common bean on the infection development and severity of the disease on each inoculated fruit species. *Colletotrichum* spp. were isolated from naturally infected papaya, eggplant, chili and common bean. Each fungal isolate was purified and identified to determine the species name. The spores of each isolate were then used to separately inoculate healthy and sterilized papaya, eggplant, chili and common bean. The results showed that cross infection developed on chili, eggplant and papaya but not on bean. Chili showed the highest susceptibility to all Colletotrichum isolates and significantly different from eggplant and papaya. The anthracnose pathogen isolated from common bean showed no pathogenicity to other hosts and might be used as cross protection inoculant to the disease in the other hosts.

Keywords*: anthracnose, cross inoculation, tropical fruits.*

1. Introduction

Papaya (*Carica papaya* L.), eggplant (*Solanum melongena* L.), chili pepper (*Capsicum annum* L.), and common bean (*Phaseolus vulgaris* L.) are highly demanded fruits in Indonesia [1]. All of the fruits are continuously available because, in the tropics, the crops are cultivated in such order that the fruits can be harvested regularly for daily needs. Like other succulent fruits, papaya, eggplant, chili pepper and common bean are subject to infection by *Colletotrichum* spp. causing anthracnose disease. The disease is very important during pre- and post-harvest phases. According to Dean *et al* [2], anthracnose disease has been considered as very important plant diseases based on its economic and scientific significance.

Colletotrichum spp. infect various fruit crops in tropical and subtropical regions, causing serious damage and significant yield losses, both quantitative and qualitatively in all growth phases of the crops. The pathogen is more threatening in humid tropics and contributes significant decrease to pre and post-harvest products [3]. Instead of causing anthracnose, the pathogens also inciting other diseases such as dieback, leaf spot, flower rot, fruit rot, stem end rot, and root rot [4, 5]. The fungus infects almost all of the above-ground parts of the host plants of all growth stages. Infection symptoms are obvious on leaves and fruit and sometimes appear on stems and flowers. Symptom on leaves is irregular or circular brown spot which eventually dries. Infection on stem causes irregular, sunken, brown spots with orange conidial masses eventually appear on the spots [6]. Symptoms appear on fruits are typical of anthracnose disease, a black round and sunken spots with black acervuli are often visible in the middle of the sot. Under favorable environmental conditions and susceptible hosts, lesions may coalesce [7]. Under natural ecosystem conditions, Colletotrichum infects several closely related host species but some species are able to infect a wider taxonomic range of hosts. It is also common to find multiple hosts infected by a single species of Colletotrichum, and single host infected by multiple species of the pathogen [8].

As a pathogen, Colletotrichum is a polyphagous fungal genus and is widely spread in both hemispheres, from tropical to subtropical countries on all continents [9]. The fungus is a hemi-biotrophic pathogen exploiting biotrophic and necrotrophic strategies to infect the host, producing appressoria, penetration peg, intracellular hyphae, and necrotrophic mycelia [10, 11]. Infection is started with the attachment of conidia to the surface of the host plant. Under humid conditions, the conidia germinate to form melanised appressoria to facilitate mechanical penetration. The penetration peg develops at the base of the appressorium to pierce the host cell. After penetration, infection vesicle and primary hyphae are formed in the epidermal cell throughout biotrophic phase and causes no harm to the host [12]. The fungus then switches to necrotrophic phase, produces secondary hyphae which then ramify inter-cellularly and kill the host cells by using hydrolytic enzymes [13].

Papaya is very susceptible to *C. gloeosporioides*, the causal pathogen of anthracnose, and losses to the disease ranges from 1 to 93% with average of 25%, depending on the postharvest handling applied [14]. In the case of eggplant, anthracnose is the most important and prevalent diseases which may cause reduction of 10 to 80% of crop production, occurring both in the field and during postharvest period [15]. Various species under genus Colletotrichum have been identified as the causal agent of anthracnose disease on eggplant, including *C.gloeosporoides* f.sp. *melongenae*, *C. nigrum*, *C.capsici*, *C. dematium* and *C. lindemuthianum* [16].

Chili pepper is another solanaceous crop severely infected by Colletotrichum causing the main problem on mature fruit. In the Colletotrichum-chili pepper pathosystem, different *Colletotrichum* species have been identified to be in association with the same host, including *C. acutatum*, *C. capsici*, and *C. gloeosporioides* [17] and cause an average yield losses up to 50% [18]. *C. capsici* is more frequently found infecting mature chili, whereas the other two Colletotrichum species infect both green and ripening chili fruits [19]. Anthracnose disease on common bean caused by *C. lindemuthianum* was reported to cause yield losses ranged from 40 to 90%, particularly in the high altitude and low temperature areas [20], since the anthracnose of common bean is favored by temperatures of 13-26°C and relative humidity above 92% [21]. *C. lindemuthianum* is the only species known as anthracnose pathogen on common bean, of which more than 25 races have been isolated and identified [22].

Colletotrichum intensely produces conidia bearing acervuli on the surface of infected host tissues. The conidia are released to become primary inoculum of anthracnose disease. The inoculum is disseminated by wind and water splashes to surrounding areas resulting in the development of secondary infections [23]. The inoculum causing secondary infection on previously healthy and younger fruit is produced by mature anthracnose spots on leaves or older fruit. Under high humidity conditions, conidia spread to immature fruit, causing quiescent infection on the fruit occurring during pre-harvest fruit development stage. The disease itself will appear and develop more apparently on harvested fruit and well known as post-harvest disease. The latent infection of anthracnose occurs due to the unsuitable conditions of the fruit to support the disease initiation. The initiation and development of anthracnose disease is also favored by the ethylene produced by the host fruit during ripening process [8]. The optimal conditions for the initiation and development of anthracnose disease in most hosts are at 25°C, even though the infection may occurs under wider range of temperature (18–33°C) and higher moisture (95–100%) [24].

Considering the continuous availability of papaya, eggplant, chili pepper and common bean under similar pre- and post-harvest conditions, and the fact that all of the four fruits species are subjected to Colletotrichum infection, it was assumed that there might be natural cross inoculations of

Colletotrichum spp. among them, as indicated by Stankova *et al* [9] that, due to the wide variability of Colletotrichum causing anthracnose, the conidia from infected host might be dangerous not only to the same host, but also to other crops. The outcomes of pathogen-host interaction vary among hosts [25] indicating host preference or host specialization [26]. The pathogen aggressiveness might be related to host susceptibility which can be determined by measuring disease or infection parameters such as incubation period, infection frequency, lesion size, and inoculum production. However, there has been not enough information of the aggressiveness of *Colletotrichum* spp. on papaya, eggplant, chili pepper and common bean on other hosts naturally grown under mixed cropping system or closed each other, and of the susceptibility of the fruits species to Colletotrichum from other hosts.

In this research, we evaluated the host specificity of the multi host pathogen Colletotrichum isolated from papaya, eggplant, chili pepper and common bean, by making cross-inoculation of the isolated pathogens. The research emphasized on the differentiation of each isolate of Colletotrichum on each inoculated host.

2. Materials and Methods

The cross-inoculation experiment was conducted in the Laboratory of Phytopathology, Department of Plant Protection, Sriwijaya University in 2016. The experiment was made using local fruits collected directly from farmers' fields in Ogan Ilir Regency and Palembang vegetable market in South Sumatra. Fruits used in this cross inoculation tests were papaya (*Carica papaya* L.), eggplant (*Solanum melongena* L.), chili pepper (*Capsicum annum* L.), and common bean (*Phaseolus vulgaris* L.). *Colletotrichum* spp. isolates used in the experiment were originated from diseased fruits obtained from the same field and market from where the healthy fruits used for cross inoculation experiment were collected.

2.1. Fungal Isolation:

Colletotrichum spp. were isolated from naturally infected papaya, eggplant, chili pepper and common bean. The pathogens were isolated from fruit without visible sporulation, by cutting fragments of 5x5 mm2 from the boundaries between anthracnose lesion and symptomless surrounding tissues. The fragments were then dipped into 1% sodium hypochlorite for 5 minutes, rinsed with sterile water, and dried with sterile tissue paper [27].

The sterilized tissue fragments were placed on potato dextrose agar in a Petri dish and incubated under room temperature until the hyphae of Colletotrichum grew and long enough to be transferred for further isolation and purification. Growing fungi showing typical colonies of *Colletotrichum* were transferred to fresh Potato Dextrose Agar medium to make pure cultures of the fungi. New colonies of *Colletotrichum* were made by single spore isolation technique. Conidia masses were streaked onto the surface of water agar 1.5% using sterilized loop and then incubated overnight under room temperature. A single germinating spore was transferred onto PDA using sterilized needle to make a pure culture [28]. Pure cultures were incubated for 14 days under daylight with temperature of 25 $\pm$ 2°C suitable for the fungal sporulation.

2.2. Pathogenicity Test

The spores used in pathogenicity test were harvested from 14 days old pure culture of *Colletotrichum* spp. isolated from the four fruit species. The spores were collected by pouring 10 mL of sterile water containing 0.1% of surfactant Tween 20. The agar surface was scrapped with paint brush to quicken the release of the spores. To make a pure spore suspension, the water containing scrapped fungal culture was filtered using double layers of cheese cloth. The spore density was adjusted to 1 x 10^6 spore mL^{-1} using haemocytometer [27, 28].

Fresh physiologically mature papaya, eggplant, chili, and common bean were graded based on size and conditions of the fruit species for further use. The selected fruits were cleaned with tap water before being sterilized by dipping into 70% ethanol for 3 minutes followed by immersing into 1% sodium hypochlorite solution for 5 minutes. The fruits were then rinsed 3 times in sterile water for 2 minutes and dried with sterile tissue paper. The sterilized fruits were placed on plastic box in which chicken wire mesh was laid two cm above the box bottom. Sterile tissue moistened with sterile water was laid on the box floor to maintain high relative humidity inside the box [29]. Each fruit species was inoculated with *Colletotrichum* spp. Isolated from all fruit species in an experiment arranged in a completely randomized design (CRD) with 4 colletorichum isolates as treatment and 5 replications. The CRD was used in the experiment because it was conducted in the laboratory under homogenous environmental conditions. Each set of treatment involving 5 fruit in each plastic box. .

Sampled fruits arranged in plastic boxes were inoculated with prepared spore suspension using micropipette. Ten inoculation points were marked on each fruit to be inoculated. Wounds were then made on the marked inoculation sites by puncturing the fruit surface using sterilized needle point to a 1 mm depth [30]. 10 µl spore suspension was dropped on each wound using micropipette. Mock inoculation was made to control fruits by dropping 10µl sterile water on wounds made on the fruits. Each set of inoculated fruits in the plastic box was incubated separately. Every plastic box containing a set of inoculated fruits was wrapped individually with transparent plastic bag to retain high humidity required for disease initiation. The boxes containing inoculated fruits were incubated under temperature of 25 ± 2°C with 12 hour light/dark cycle. The bags were removed 2 days after inoculation [27].

Inoculated fruits were visually evaluated daily to record data of infection parameters caused by *Colletotrichum* spp. cross-inoculated to papaya, eggplant, chili pepper and common bean. The parameters measures included incubation period, infection frequency, anthracnose size, number of acervuli and number of conidia per acervuli. Incubation period was measured as period (in day) when 50% of inoculation points show the first symptom of anthracnose; infection frequency was measured as the percentage of infection points developed anthracnose symptom; anthracnose was measured as the longest diameter; number of acervuli was measured is the average numbers of acervuli appeared on anthracnose symptom; and number of conidia per acervulus is the average number of conidia released from acervuli appeared on anthracnose symptom.

3. Results and Discussion

C. gloeosporioides, C. capsici and *C. lindemuthianum* were isolated from four different tropical fruits. The identification of the pathogens was based on the disease symptoms, culture characteristics, and morphological characteristics [23, 31, 32]. All Colletotrichum isolates have ability to infect other fruit species, except the one isolated from common bean. *C. gloeosporioides* isolated from papaya and eggplant and *C. capsici* isolated from chili pepper could infect all tested fruits but common bean. Inversely, *C. lindemuthianum* originated from common bean could infect only original host and failed to infect other inoculated hosts. This is in accordance with the reports by Thomazella *et al* [22] that common bean could only be infected by *C. lindmeuthianum* which is consisted of more than 25 races. The failure of *C. lindemuthianum* to infect other hosts might also be caused by environmental condition, especially the temperature of the room used to incubate the inoculated fruits which was set to 25± 2°C which might be too high for *C. lindemuthianum* which needs optimum temperature of 13-26°C with an optimum of 17°C, and relative humidity above 92% [21]. The cross inoculation experiments demonstrated the variations in *Colletotrichum* spp. pathogenicity on their original host and on other hosts. The level of host preference among *Colletotrichum* spp. isolated from four different fruits and the susceptibility of the hosts varied significantly.

3.1. Pathogenicity of Colletotrichum spp.

All isolates of *Colltotrichum* spp. were proved as the pathogens causing anthracnose on all fruits producing typical anthracnose symptoms after being inoculated with the pathogens. This was verified by the fact that mock inoculation using sterile water had no effect on inoculated fruits. *C. gloeosporioides, C. capsici* and *C. lindemuthianum* isolated from different hosts were able to establish anthracnose not only on their original hosts but also on other hosts through cross inoculation. This demonstrate that most *Colletotrichum* spp. have wide range of host species, and many host species are subject to more than one species of Colletotrichum as reported by Barrett *et al* [33] that, under natural ecosystem, most pathogens may have wide host range, and most hosts may suffer from various pathogen infections.

3.1.1. Cross inoculation to papaya. Papaya inoculated with *Colletotrichum* spp. Isolated from papaya, eggplant and chili produced typical symptoms of anthracnose. The results of this cross inoculation revealed that Colletotrichum isolated from the four fruit species naturally infected by the fungus could initiate anthracnose on papaya at similar rate and severity (Table 1). The surprising result was the fact that the anthracnose disease developed on original hosts was not different from those developed on non-original hosts. The short incubation period (< 3 days) on all infected fruits did not automatically indicated the susceptibility of the host since other infection parameters showed the opposite. Means of incubation period (2.06 days), infection frequency (14.06%), anthracnose spot size (1.84 mm), number of acervuli per spot (0.60) and number of conidia per acervulus (1.31) showed lower values than those of natural infections, and no significant difference among hosts, except number of conidia per acervulus, in which papaya was significantly different from other three infected hosts. This significant difference is quite creditable since the conidia are very important factor in the disease epidemic. Under favorable condition, conidia produce in high rate under temperature of 25°C and relative humidity of 95-100% [24]. The size of lesson developed on papaya as original hosts when inoculated with Colletotrichum isolated from naturally infected papaya was not larger than those developed on papaya inoculated with Colletotrichum from other fruits. This result was inconsistent with those found by Lakshmi *et al* [27] that *C. gloeosporioides* isolated from a fruit species could cross-infect fruits of other species, and the fungus isolate was most aggressive when inoculated to its original host.

Table 1. Anthracnose disease produced by cross-inoculation of Colletotrichum isolated from different fruits on papaya.

Isolate	Incubation period[a]	Infection frequency[a]	Anthracnose size[a]	Number of acervuli[a]	Number of conidia $(x\ 10^4)^a$
Papaya	1.75 a	13.43 a	1.32 a	1.00 a	2.22 b
Eggplant	1.81 a	15.89 a	1.49 a	0.35 a	0.77 a
Chili pepper	2.36 a	14.67 a	2.39 a	0.33 a	0.70 a
Common bean	2.33 a	12.23 a	2.15 a	0.71 a	1.55 a
Mean	2.06	14.06	1.84	0.60	1.31

[a]Means with same letters in the same column were not significantly different according to HSD 5%.

The low values of anthracnose disease parameters on papaya also indicated the low virulence of the pathogens used in the inoculation or the unfavorable environmental conditions during disease development, even though room temperature and humidity had been adjusted to suit the needs of anthracnose development. Furthermore, there has been no report of other species of Colletotrichum causing anthracnose disease on papaya except *C. gloeosporioides.* Therefore, the low values of infection parameters of anthracnose on papaya caused by Coleltorichum isolated from other fruits might not be caused by low aggressiveness of the pathogens but more likely to be caused by host specificity.

3.1.2. Cross inoculation to eggplant. Eggplant inoculated with *Colletotrichum* spp. isolated from papaya, eggplant and chili also developed anthracnose on all inoculated tested fruits with some differences in some infection parameters. As can be seen in Table 2, the results of this cross inoculation revealed that anthracnose developed almost equally in all infected tested fruits (Table 2). The surprising result was the fact that the anthracnose disease developed better on eggplant compared to those developed on papaya. All infection parameters of anthracnose developed on eggplant were higher than those on papaya, except average number of ecervuli per anthracnose spot. Means of incubation period (2.27 days), infection frequency (26.30%), anthracnose spot size (2.78) are higher than mean values of the same parameters measured on papaya. These phenomena revealed that eggplant is more susceptible to anthracnose pathogen than papaya, or the anthracnose pathogens Colltotrichum spp. are more virulent when inoculated to eggplant. Virulence of anthracnose pathogens is affected by host conditions such as availability of nutrients and enzymes required by the pathogens, or the presence of antifungal compounds [27].

The significantly higher infection frequency on papaya than that on eggplant is not quite surprising since both fruits are natural host of *C. gloeosporioides*. However, number of acervuli and number of conidia per acervulus were significantly higher on eggplant than on papaya indicating that eggplant is more suitable host for *C. gloeosporioides* isolated from eggplant. This is in accordance with previous finding that anthracnose infection was better on its natural host than on other hosts. As occurred in cross infection to papaya, cross infection to eggplant also resulted in higher number of conidia produced on natural host rather than on non-natural host and this might make difference in the next step of disease spread and development. More abundant conidia will make more effective disease spread and development. The more prolific anthracnose developed on eggplant is consistent with the report of Mathur and Kongsdal [35] that anthracnose on eggplant might be caused by *C. gloeosporoides* f. sp. *melongenae*, *C. nigrum*,*C. capsici*, *C. dematium*, *C. lindemuthianum* and *Gloeosporium melongenae.*

Table 2. Anthracnose disease produced by cross-inoculation of Colletotrichum isolated from different fruits on eggplant.

Isolate	Incubation period[a]	Infection frequency[a]	Anthracnose size[a]	Number of acervuli[a]	Number of conidia (x 10^4)[a]
Papaya	2.40 a	31.54 b	2.91a	0.41 b	0.80 b
Eggplant	2.21 a	20.82 a	2.30a	1.09b	2.35 c
Chili pepper	2.10 a	24,34 a	2.26a	0.00a	0.00 a
Common bean	2.37 a	28.51 b	3.64a	0.91b	2.29 b
Mean	2.27	26.30	2.78	0.60	1.36

[a]Means with same letters in the same column were not significantly different according to HSD 5%.

3.1.3.Cross inoculation to chili pepper. Cross inoculation of *C. capsici* isolated from chili papaya, eggplant, common bean and chili pepper itself showed that the pathogen could infect other host with significant different in infection frequency, anthracnose size, number of acervuli and number of conidia per acervulus (Table 3). The three parameters are very important infection parameters in determining the aggressiveness of the pathogen and the susceptibility of the hosts. In this case, the difference of the parameters is more likely to determine the difference of pathogens aggressiveness since the parameters were observe on the same host but of different pathogen isolates. Means of incubation period (2.13 days), infection frequency (53.63%), anthracnose spot size (3.69), number of acervuli (1.16) and number of conidia per acervulus (5.35 x 10^4) are higher than those measured on papaya and eggplant. This clearly indicates that there are different levels of host susceptibility among the tested fruit species, and chili pepper is the one showing the most susceptible to *Colletotrichum* spp. than papaya, eggplant and common bean. In general, chili pepper naturally infected by several species of Colletotrichum, including *C. acutatum*, *C. gloeosporioides*, *C. coccodes* and *C. dematium* [23] and in Indonesia, chili pepper frequently infected by *C. acutatum*, *C. capsici*, and *C. gloeosporioides* [21].

Table 3. Anthracnose disease produced by cross-inoculation of Colletotrichum isolated from different fruits on chili pepper.

Isolate	Incubation period[a]	Infection frequency[a]	Anthracnose size[a]	Number of acervuli[a]	Number of conidia (x 10^4)[a]
Papaya	2.00 a	51.16 b	3.89 b	0.91 a	6.45 a
Eggplant	1.96 a	47.02 b	3.59 b	0.53 a	3.18 a
Chili pepper	2.17 a	62.41 c	4.41 c	1.78 b	4.06 a
Common bean	2.37 a	53.91 b	2.89 a	1.40 a	7.71 a
Mean	2.13	53.63	3.69	1.16	5.35

[a]Means with same letters in the same column were not significantly different according to HSD 5%.

3.1.4.Cross inoculation to common bean. All Colletotrichum isolated from papaya, eggplant and chili pepper failed to develop anthracnose disease when inoculated to common bean. The fruit could only be infected by Colletotrichum isolated from the same fruit species which mean that the host factor is very influential to the initiation and development of the disease. Furthermore, anthracnose on common bean is favored by cool and wet weather with temperature ranges from 13 to 26°C and an optimum of 17°C [24], which could not be met during the experiment.

4. Conclusions

Cross inoculation of *Colletotrichum* spp. isolated from naturally infected papaya, eggplants and chili pepper could produce anthracnose symptoms not only on original but also on non-original hosts. Since the fruits are continuously available in the field and market and mostly cultivated under mixed cropping system, the cross inoculation potential make the vulnerability of the disease even greater and needs serious management. Colletotrichum causing anthracnose on common bean could only produce anthracnose symptoms on original host might be used to biologically control anthracnose disease of other fruits through cross protection inoculation.

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