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Short Communication: Biocontrol activity of Phyllosphere fungi on mungbean leaves against *Cercospora canescens*

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Abstract. *Sumartini. 2017. Short Communication: Biocontrol activity of Phyllosphere fungi on mungbean leaves against* Cercospora canescens*. Biodiversitas 18: 720-726.* Examination of biocontrol activity of phyllosphere fungi on mungbean leaves against *Cercospora canescens*, a causal agent of mungbean leaf spot disease, was conducted during February-July 2016. Samples of symptomatic mungbean leaves were collected from several production areas in East and Central Java. Symptomatic leaves with leaf spot were collected randomly by detaching the symptomatic leaves and kept in a plastic bag for laboratory studies. Fungi associated with leaf spot were isolated using surface sterilized technique on PDA medium. In vitro and in vivo antagonism assay was carried out in the laboratory and greenhouse, respectively. Fungi associated with leaf spot on mungbeans such as *Fusarium* spp.*, Curvularia* spp.*, Aspergillus flavus*, and *Nigrospora* sp. were tested against *C. canescens* in the antagonism assay. Highest inhibition activity against *C. canescens* was found on *Fusarium* sp. 2 (KH-KJP-1B) (in vitro = 61%, in vivo = 19.63%) and *Curvularia* sp.1 (KH-JBG-B) (in vitro = 66%, in vivo = 16.46%).

Keywords: biocontrol, *Cercospora* leaf spot, mungbean, phyllosphere fungi

INTRODUCTION

Mungbean [*Vigna radiata* (L.) R.Wilczek] is an important plant because contains 23-29% of protein (Ginting et al. 2008). This plant, therefore, provides high nutrition for the majority of Indonesian people that used to eat mungbean sprout. Several commercial snacks were also made from mungbean seeds. Mungbean is usually planted after the first and second of harvest season of rice harvest or after harvest season of rice and soybean, or after rice and corn harvest season. However, production of mungbean in Indonesia has been decreased due to several constraints, such as incursion of several plant diseases. One of the destructive plant diseases on mungbean leaves is *Cercospora* leaf spot.

Cercospora leaf spot is generally caused by *Cercospora canescens* (Semangun 2004). This fungus found in almost all parts of mungbean production areas. Losses of mungbean yield due to plant disease were reported up to 61% (Iqbal et al. 1995). The *Cercospora* leaf spot on mungbean leaves is easily recognized by its initial small brown spot and further developed to form larger spot (Semangun 2004). The disease usually appears about 30-40 days after planting, depending on temperature and humidity. *Cercospora canescens* spreads rapidly in susceptible varieties causing premature defoliation and reduction in the size of pods and grains (Grewal et al. 1980).

Management of leaf spot disease control generally involved several practices, such as planting resistant varieties and spraying fungicides. Integrated plant disease management, an effort to minimize yield losses by controlling pests and diseases using environmentally friendly approach, has become more prominent approach in managing plant diseases worldwide. It is due to the urgent need for reducing chemicals (fungicides) uses that unsafe to the environment and living beings. One of the environmentally friendly approaches is applying a natural enemy of the pathogen or bio-fungicide. Phyllosphere fungi such as *Trichoderma* spp. has been reported as a natural enemy for particular plant pathogen (Baker 1997; Thakur and Harsh 2014).

Phyllosphere comprises aerial parts of plants and it is dominated by the leaves. Most studies of the phyllosphere microorganisms have focused on bacteria and fungi (Vorholt 2012). Until recently, there have been no reports regarding biological control of *C. canescens* on mungbean leaves. Therefore, this study was aimed at determining and isolating fungi associated with leaf spot on mungbean leaves, and examining their potential in controlling *C. canescens*.

MATERIALS AND METHODS

Sampling

A sampling of symptomatic leaves of mungbean was conducted in several production areas of East Java and Central Java during February-July 2016. Six locations were randomly selected. On each location, 15 plants with leaf spot symptoms were selected. Three symptomatic leaves from each plant were collected, kept in plastic bags, and

labeled. All samples were kept in the cool ice box for further study in the laboratory.

Isolation of microfungi

Isolation of fungi from mungbean leaf spot was conducted using surface sterilized method (Burgdorf et al. 2014). Symptomatic part of leaves was cut into small pieces and disinfected according to the following procedure: part of symptomatic leaves immersed in a NaOCl solution (0.5% free Cl_2) for 1 min, 70% ethanol for 1 min, and washed with sterilized distilled water for three times. The samples were further dried on sterile filter paper (remove excess water), placed on Potato Dextrose Agar (PDA) medium, sealed, and then incubated at room temperature. Mycelium growing out from the samples were transferred and purified into a new medium. Identification of the fungal isolates was morphologically carried out using Barnett and Barry (1977), Nelson et al. (1983). All fungal isolates obtained in this study were kept on the PDA slant for further study.

In vitro antagonism assay

In vitro antagonism assay was conducted using dual culture technique on petri dishes (Coskuntuna and Ozer 2008). *Cercospora canescens* was placed at the periphery of the PDA plates (ϕ= 9 cm). Another agar dice of the same size of the fungal antagonist was placed at the periphery, but on the opposing site of the same Petridish. An isolate of *C. canescens* was placed in a similar manner on a fresh PDA, but without a fungal antagonist, was used as a control. All plates were incubated at 28°C. Antagonistic activity (I) was examined at 3, 7, and 17 days after incubation by measuring radius between the antagonist colony (R2) and *C. canescens* colony (R1). Antagonistic activity was calculated using the following formula:

$$I = \frac{R1 - R2}{R1} \times 100\%$$

In vivo antagonism assay

Vima-1 of mungbean variety were grown in plastic pots. Each treatment consisted of five pots, two plants/pot. Inoculation of the antagonist fungi was done at one-month-old plants by spraying a spore suspension isolates selected antagonist with spore density 10^5/ml. Artificial inoculation with leaf spot was done at two hours after to ensure a disease infected plants. Parameter observations were: the intensity of leaf spot disease on mungbean.

RESULTS AND DISCUSSION

Isolation and identification

Five fungal taxa were isolated from the phyllosphere of mungbean leaves, namely *C. canescens* Ellis & G. Martin (Table 1, Figure 1), *Fusarium* sp. (4 isolates), *Curvularia* sp. (4 isolates), *A. flavus*, and *Nigrospora* sp. (Figure 2.A-2.D, Figure 3). Among them, *C. canescens* has been known as plant pathogenic fungus causing leaf spot disease worldwide (Crous and Braun 2003). The intensity of leaf spot disease caused by *C. canescens* depended upon the weather and the resistance of variety. The intensity of leaf spot disease on mungbean can be reached 30% on moderately susceptible variety (Bhat et al. 2014). *Cercospora canescens* on mungbean leaf spot from this study is characterized by McKenzie (2013). The colony is gray, smooth, and secrete some substance purple-reddish (cercosporin), conidiophore is pale brown, long (20-200 x 3-6.5µm), with some septate, conidia is long (50-150 x 3-5.5 µm) with some septate, hyaline.

A colony of *Fusarium* sp.1 is white in color, smooth, circular and concentrate, conidia fusoid with tapering towards both ends, multiseptate (3-5 septate), and hyaline. A colony of *Fusarium* sp.2 is brown with a white surface, smooth, microconidia ellipsoid (0-2-septate), macroconidia fusoid (3-5-septate), and hyaline (Figure 3). A colony of *Fusarium* sp.3 is white in color, smooth, circular, conidia fusoid with tapering towards both ends, multiseptate (3-5 septate), and hyaline. A colony of *Fusarium sp*.4 is white in color, smooth, conidia fusoid with tapering towards both ends, multiseptate (3-5 septate), and hyaline.

Table 1. Leaf spot disease intensity on mungbean from several selected areas in this study.

District	Subdistrict	Disease intensity	Phyllosphere fungi
Lamongan	Sidomukti	Leaf spot (+)	*C. canescens* *Fusarium* sp.
	Brondong	Leaf spot (+)	*C. canescens* *Curvularia* sp. *A. flavus*
Pasuruan	Kejapananan	Leaf spot (+)	*C. canescens*
Banyuwangi	Genteng	Leaf spot (+)	*C. canescens*
Malang	Kepanjen	Leaf spot (+)	*C. canescens*
	Pakisaji	Leaf spot (++)	*C. canescens* *Nigrospora* sp.

Note: + = 5-10%, ++ = 10-20%

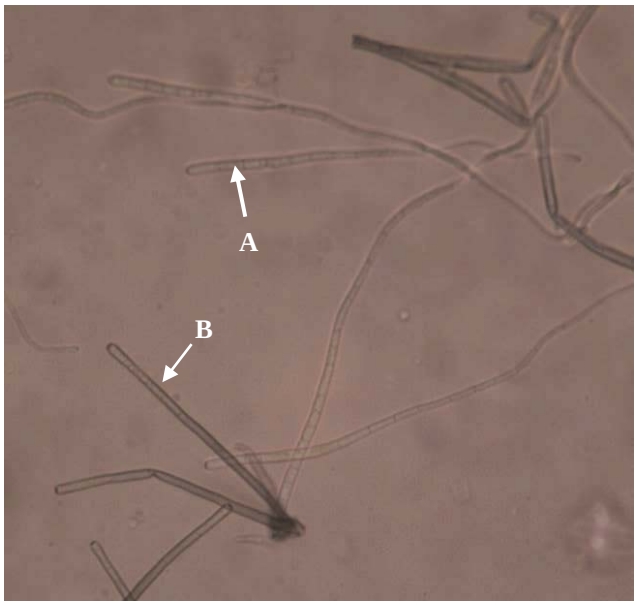


Figure 1. Conidia and conidiophores of *C. canescens* on mungbean leaf spot. A. Conidium, B. Conidiophore

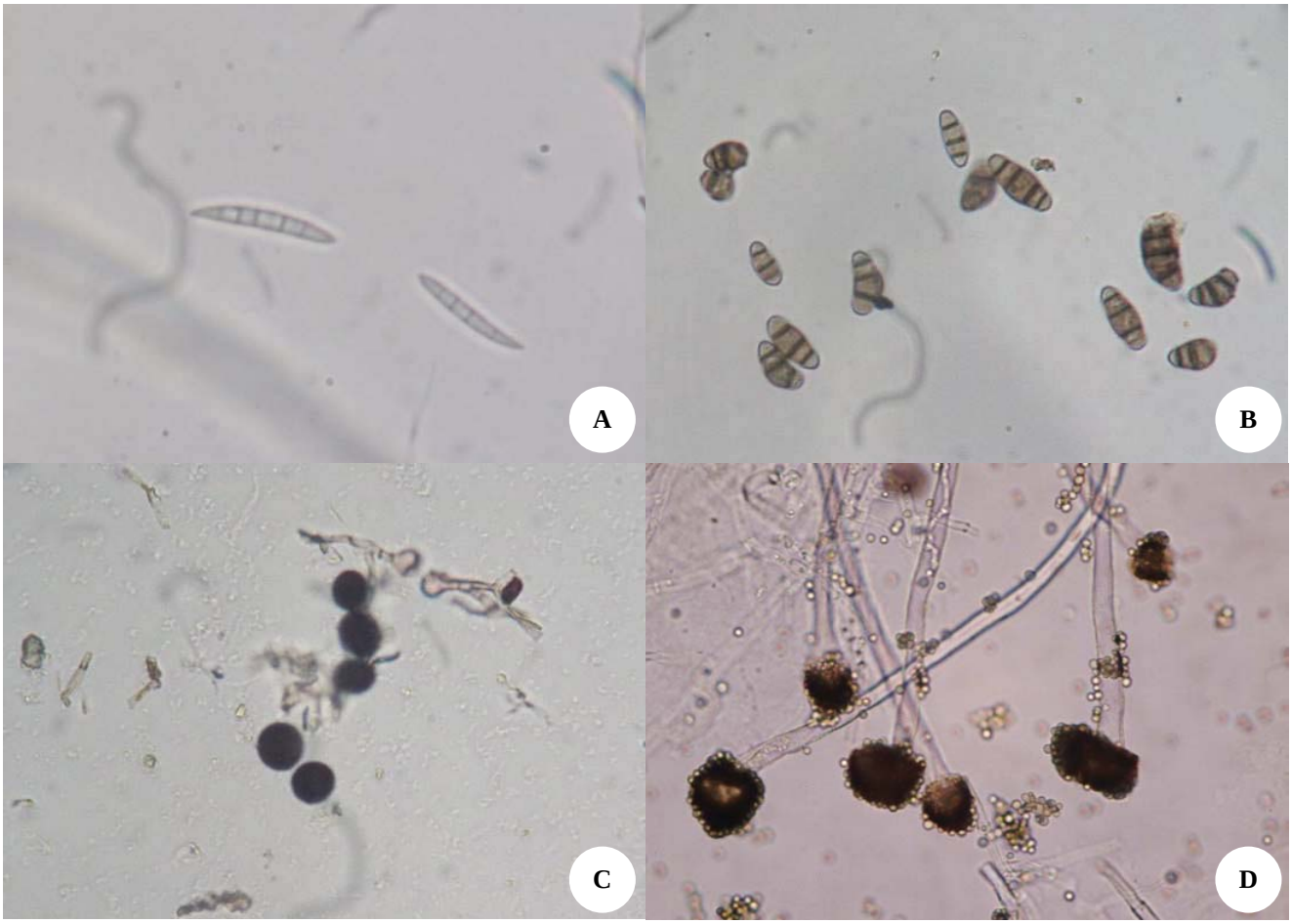


Figure 2. Fungi associated with leaf spot of mungbean leaves. A. *Fusarium* sp. (Isolate KH 2 A), B. *Curvularia* sp. (Isolate KH 1-3), C. *Nigrospora* sp. (Isolate KH JBG-A), D. *Aspergillus flavus* (Isolate KH-1.2)

Tabel 2. The growth of *C. canescens* during in vitro dual culture assay at 3, 7, 17 days

Isolates	Observation at 3 days		Observation at 7 days		Observation at 17 days	
	C. canescens (cm)	Isolates (cm)	*C. canescens* (cm)	Isolates (cm)	*C. canescens* (cm)	Isolates (cm)
Fusarium sp.1 (KH KJP 2A)	0.7	3	2.1	6.06	2.2	6.8
Fusarium sp.2 (KH KJP1 B)	0.7	2.9	1.96	6.2	2.5	6.5
Fusarium sp.3 (KH 3.7)	0.6	3	2.14	6.3	2.5	6.5
Fusarium sp.4 (KH 8.16)	0.6	2.5	2.12	3.8	3	5.5
Curvularia sp.1 (KH JBG B)	0.6	3	2	4.2	2.2	6.5
Curvularia sp.2 (KH 6.13)	0.6	3.2	1.96	5.76	2	7
Curvularia sp.3 (KH 5.10)	1	3.5	1.86	5.86	2.5	6.3
Aspergillus flavus (KH 1.2)	0.6	2.5	0.8	4.5	1	6.5
Cercospora canescens	0.8	-	2.08	-	4	-

A colony of *Curvularia sp* 1, 2, and 3 is dark green in color and will change to dark brown when mature. Conidiophore of *Curvularia sp*.1, 2, and 3 mostly is simple, bearing spores apically or on new sympodial growing points; conidia are yellow, more or less fusiform, four cells, end cell lighter, with one of the central cell enlarged and dark brown in color. A colony of *Aspergillus flavus* is green to yellowish in color, secrete aflatoxin yellow in PDA, conidia green in color. Conidiophore with phialide apically, one phialide bearing many conidia. A colony of *Nigrospora* sp.on PDA has gray wooly color with some black spot. Conidiophores simple, hyaline, globose, bearing single conidia apically. Conidia aleuriosporous, black in color, subglobose or disc-shaped, occasionally apiculate in the upper part.

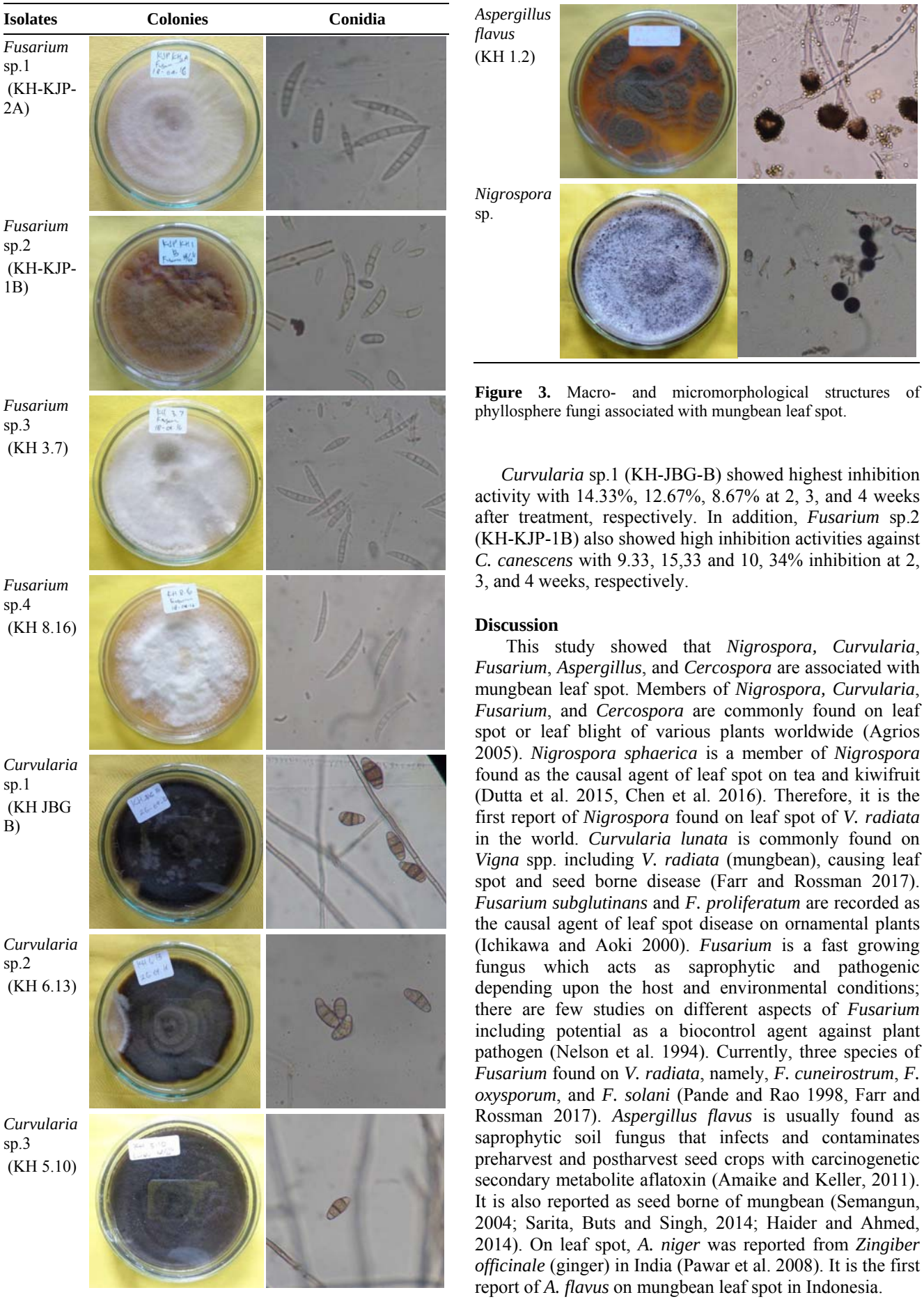


Figure 3. Macro- and micromorphological structures of phyllosphere fungi associated with mungbean leaf spot.

Curvularia sp.1 (KH-JBG-B) showed highest inhibition activity with 14.33%, 12.67%, 8.67% at 2, 3, and 4 weeks after treatment, respectively. In addition, *Fusarium* sp.2 (KH-KJP-1B) also showed high inhibition activities against *C. canescens* with 9.33, 15,33 and 10, 34% inhibition at 2, 3, and 4 weeks, respectively.

Discussion

This study showed that *Nigrospora, Curvularia*, *Fusarium*, *Aspergillus*, and *Cercospora* are associated with mungbean leaf spot. Members of *Nigrospora, Curvularia*, *Fusarium*, and *Cercospora* are commonly found on leaf spot or leaf blight of various plants worldwide (Agrios 2005). *Nigrospora sphaerica* is a member of *Nigrospora* found as the causal agent of leaf spot on tea and kiwifruit (Dutta et al. 2015, Chen et al. 2016). Therefore, it is the first report of *Nigrospora* found on leaf spot of *V. radiata* in the world. *Curvularia lunata* is commonly found on *Vigna* spp. including *V. radiata* (mungbean), causing leaf spot and seed borne disease (Farr and Rossman 2017). *Fusarium subglutinans* and *F. proliferatum* are recorded as the causal agent of leaf spot disease on ornamental plants (Ichikawa and Aoki 2000). *Fusarium* is a fast growing fungus which acts as saprophytic and pathogenic depending upon the host and environmental conditions; there are few studies on different aspects of *Fusarium* including potential as a biocontrol agent against plant pathogen (Nelson et al. 1994). Currently, three species of *Fusarium* found on *V. radiata*, namely, *F. cuneirostrum*, *F. oxysporum*, and *F. solani* (Pande and Rao 1998, Farr and Rossman 2017). *Aspergillus flavus* is usually found as saprophytic soil fungus that infects and contaminates preharvest and postharvest seed crops with carcinogenetic secondary metabolite aflatoxin (Amaike and Keller, 2011). It is also reported as seed borne of mungbean (Semangun, 2004; Sarita, Buts and Singh, 2014; Haider and Ahmed, 2014). On leaf spot, *A. niger* was reported from *Zingiber officinale* (ginger) in India (Pawar et al. 2008). It is the first report of *A. flavus* on mungbean leaf spot in Indonesia.

Table 3. Inhibition of several fungal isolates against *C. canescens* isolate

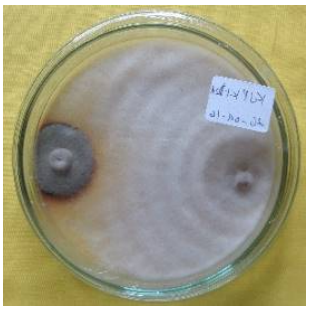
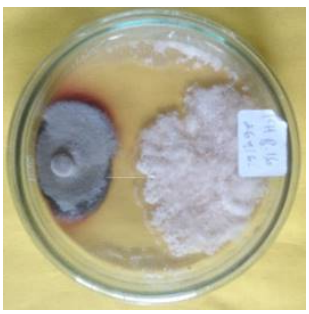
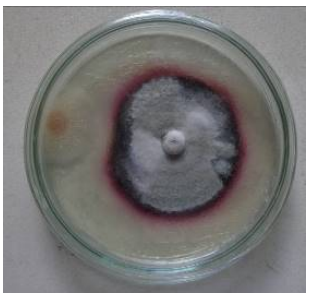
Isolates	Inhibition at 17 days (%)	Figures at 17 days
Fusarium sp.1 (KH KJP 2A)	67	
Fusarium sp.2 (KH KJP1 B)	61	
Fusarium sp.3 (KH 3.7)	61	
Fusarium sp.4 (KH 8.16)	45	
Curvularia sp.1 (KH JBG B)	66	
Curvularia sp.2 (KH 6.13)	71	
Curvularia sp.3 (KH 5.10)	60	
Aspergillus flavus (KH 1.2)	85	
C. canescens	-	

Table 4. The intensity of leaf spot disease (*Cercospora*) on mungbean in 2, 3, and 4 weeks after treatment.

Treatments	Leaf spot intensity (%)			
	2 WAA	3 WAA	4 WAA	I
Fusarium sp.1 (KH KJP-2A)	34.67 bc	36.60 bc	49.33 bc	6.34
Fusarium sp.2 (KH-KJP-1B)	**29.67 ef**	**29.00 e**	**42.33 d**	**19.63**
Fusarium sp.3 (KH 3.7)	34.33 bcd	44.33 a	49.00 bc	6.96
Fusarium sp.4 (KH-816)	37.33 ab	40.00 ab	48.33 bc	8.23
Curvularia sp.1 (KH-JBG-B)	**24.67 g**	**31.66 de**	**44.00 cd**	**16.46**
Curvularia sp.2 (KH-6.13)	26.33 fg	40.00 ab	53.67 ab	1.89
Curvularia sp.3 (KH 5.10)	30.67 cde	39.67 b	55.33 a	5.05
Aspergillus flavus (KH-1.2)	30.33 def	35.00 cd	51.00 ab	3.17
C. canescens	39.00 a	44.33 a	52.67 ab	-
cv (%)	10.22	9.08	9.13	
LSD 0.05	4.197	4.428	5.823	

Note: WAA = weeks after application, I= inhibition at 4 weeks after sowing

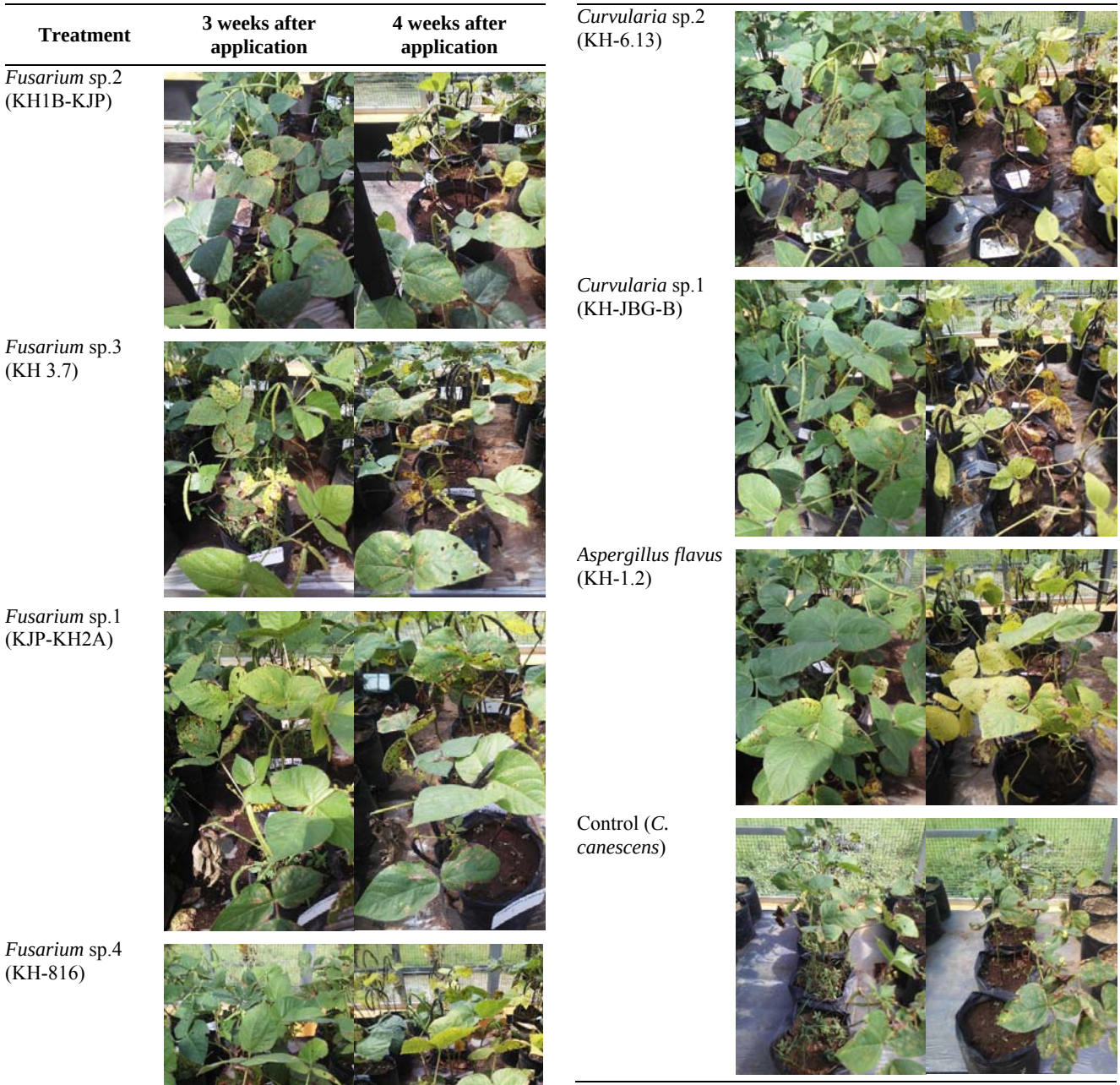


Figure 4. Biocontrol activity in vivo in the greenhouse

The most frequent fungus found on mungbean leaf spot was *Cercospora*, in particular, *C. canescens* (Farr and Rossman 2017). This fungus is a slow growing on the artificial medium. Therefore it was always inferior against others fungi in vitro (Table 3), but always superior against other fungi in vivo or the field (Table 4).

Figure 4 showed that some leaves have already dried and fallen on untreated plants compared to treated plants in two weeks after antagonist application. Untreated plants were also produced pods less than treated plants.

The current study showed that mechanism of antagonism against *C. canescens* possibly involving competition of space and nutrient, and antibiosis (Table 3). Competition of space and nutrient against *C. canescens* was clearly showed by *A. flavus* isolate, and antibiosis mechanism exhibited by *Fusarium* spp. Mechanism of biocontrol generally includes (i) mycoparasitism, biocontrol agents degrade cell walls of the fungi target by secretion of different lytic enzymes and coil around them; (ii) Antibiosis, the biocontrol agent secreted some substance which was lethal to the plant pathogen, such as gliotoxin in *Trichoderma*, (iii) Competition on space and nutrient, the biocontrol agent generally grow very fast and rapidly colonized the target fungus; and (iv) Induced resistance, in activation of the pathogens enzymes (Sharma et al. 2012).

This report is the first study of fungal diversity on mungbean leaf spot and their potential as biocontrol agents against *C. canescens* in Indonesia. Among them, *Fusarium* sp.2 (KH-KJP-B) and *Curvularia* sp.1 (KH-JBG-B) showed highest antagonistic activity with 20% and 16% inhibition of *C. canescens* growth, respectively. Both fungal isolates also exhibited potential biocontrol activity during greenhouse assay.

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