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

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Abstract. Sumartiniputut. 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 Cl₂) 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 (ɸ = 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 10⁵/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, multisepaote (3-5 septeate), and hyaline. A colony of *Fusarium* sp.2 is brown with a white surface, smooth, microconidia ellipsoid (0-2-sepate), macroconidia fusoid (3-5-sepate), and hyaline (Figure 3). A colony of *Fusarium* sp.3 is white in color, smooth, circular, conidia fusoid with tapering towards both ends, multisepate (3-5 septeate), and hyaline. A colony of *Fusarium* sp.4 is white in color, smooth, conidia fusoid with tapering towards both ends, multisepate (3-5 septeate), and hyaline. A colony of *Fusarium* sp.5 is white in color, smooth, conidia fusoid with tapering towards both ends, multisepate (3-5 septeate), 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*         |
| Brondong    |              | Leaf spot (+)     | *C. canescens*         |
| Pasuruan    | Kejapananan  | Leaf spot (+)     | *C. canescens*         |
| Banyuwangi  | Genteng      | Leaf spot (+)     | *C. canescens*         |
| Malang      | Kepanjen     | Leaf spot (+)     | *C. canescens*         |
|             | Pakisaji     | Leaf spot (+++)   | *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)

Table 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)   | *C. canescens* (cm)   | *C. canescens* (cm)    |
| **Fusarium sp.1 (KH KJP 2A)** | 0.7                  | 2.1                   | 2.2                    |
| **Fusarium sp.2 (KH KJP1 B)** | 0.7                  | 1.96                  | 2.5                    |
| **Fusarium sp.3 (KH 3.7)** | 0.6                  | 2.14                  | 2.5                    |
| **Fusarium sp.4 (KH 8.16)** | 0.6                  | 2.12                  | 3.0                    |
| **Curvularia sp.1 (KH JBG B)** | 0.6                  | 2                     | 2.2                    |
| **Curvularia sp.2 (KH 6.13)** | 0.6                  | 1.96                  | 2.5                    |
| **Curvularia sp.3 (KH 5.10)** | 1                    | 1.86                  | 2.5                    |
| **Aspergillus flavus (KH 1.2)** | 0.6                  | 0.8                   | 1.0                    |
| **Cercospora canescens** | 0.8                  | 2.08                  | 4.0                    |

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.
## Table 1: Isolates, Colonies, and Conidia

| Isolates       | Colonies | Conidia |
|---------------|----------|---------|
| *Fusarium* sp.1 (KH-KJP-2A) | ![Image](image1.png) | ![Image](image2.png) |
| *Fusarium* sp.2 (KH-KJP-1B) | ![Image](image3.png) | ![Image](image4.png) |
| *Fusarium* sp.3 (KH 3.7) | ![Image](image5.png) | ![Image](image6.png) |
| *Fusarium* sp.4 (KH 8.16) | ![Image](image7.png) | ![Image](image8.png) |
| *Curvularia* sp.1 (KH JBG B) | ![Image](image9.png) | ![Image](image10.png) |
| *Curvularia* sp.2 (KH 6.13) | ![Image](image11.png) | ![Image](image12.png) |
| *Curvularia* sp.3 (KH 5.10) | ![Image](image13.png) | ![Image](image14.png) |

**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   | 67                        |                   |
| (KH KJP 2A)       |                           |                   |
| *Fusarium* sp.2   | 61                        |                   |
| (KH KJP1 B)       |                           |                   |
| *Fusarium* sp.3   | 61                        |                   |
| (KH 3.7)          |                           |                   |
| *Fusarium* sp.4   | 45                        |                   |
| (KH 8.16)         |                           |                   |
| *Curvularia* sp.1 | 66                        |                   |
| (KH JBG B)        |                           |                   |
| *Aspergillus* flavus (KH 1.2) | 85  |                   |

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  | 1       |
| *Fusarium* sp.1 (KH KJP-2A)       | 34.67  | 36.00  | 49.33  | 6.34    |
| *Fusarium* sp.2 (KH-KJP-1B)       | 29.67  | 29.00  | 42.33  | 19.63   |
| *Fusarium* sp.3 (KH 3.7)          | 34.33  | 44.33  | 49.00  | 6.96    |
| *Fusarium* sp.4 (KH-816)          | 37.33  | 40.00  | 48.33  | 8.23    |
| *Curvularia* sp.1 (KH-JBG-B)      | 24.67  | 31.66  | 44.00  | 16.46   |
| *Curvularia* sp.2 (KH-6.13)       | 26.33  | 40.00  | 53.67  | 1.89    |
| *Curvularia* sp.3 (KH 5.10)       | 30.67  | 39.67  | 55.33  | 5.05    |
| *Aspergillus* flavus (KH-1.2)     | 30.33  | 35.00  | 51.00  | 3.17    |
| *C. canescens*                    | 39.00  | 44.33  | 52.67  | -       |

Note: WAA = weeks after application, I= inhibition at 4 weeks after sowing
### Figure 4. Biocontrol activity in vivo in the greenhouse

| Treatment | 3 weeks after application | 4 weeks after application |
|-----------|---------------------------|---------------------------|
| *Fusarium* sp.2 (KH1B-KJP) | ![Image](image1) | ![Image](image2) |
| *Fusarium* sp.3 (KH 3.7) | ![Image](image3) | ![Image](image4) |
| *Fusarium* sp.1 (KJP-KH2A) | ![Image](image5) | ![Image](image6) |
| *Fusarium* sp.4 (KH-816) | ![Image](image7) | ![Image](image8) |
| *Curvularia* sp.2 (KH-6.13) | ![Image](image9) | ![Image](image10) |
| *Curvularia* sp.1 (KH-JBG-B) | ![Image](image11) | ![Image](image12) |
| *Aspergillus flavus* (KH-1.2) | ![Image](image13) | ![Image](image14) |
| Control (C. canescens) | ![Image](image15) | ![Image](image16) |

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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**REFERENCES**

Amaike S, Keller NP. 2011. *Aspergillus flavus*. Ann Rev Phytopathol 49: 107-133.

Agrios NG. 2005. Plant Pathology. 5th ed. Department of Plant Pathology, University of Florida, USA.

Baker KF. 1987. Evolving concepts of biological control of plant pathogens. Ann Rev Phytopathol 24: 67-85

Barrett IL, Hunter BB. 1977. Illustrated genera of imperfect fungi 4th ed. Burgess, Edina, MN.

Bhat EA, Mohiddin EA, Hilal AB. 2014. Reaction of green gram (*Vigna radiata*) to *Cercospora canescens* (Ellis & Martin). Indian J Agric Res 48 (2): 140-144.

Blakeman JP. 1982. Potential for biological control of plant diseases on the phylloplane. Ann Rev Phytopathol 20: 167-192.

Burgdorff RJ, Laing MD, Morris CD, Jamal-Ally SF. 2014. A procedure to evaluate the efficiency of surface sterilization methods in culture-independent fungal endophyte studies. Brazilian J Microbiol 45: 977-983.

Chen Y, Yang X, Zhang AF, Zang HY, Gu CY. 2016. First report of leaf spot caused by *Nigrospora sphaerica* on kiwifruit in China. Plant Dis 100: 2326.

Coskuntuna A, Ozer N. 2008. Biological control of onion basal rot disease using *Trichoderma harzianum* and induction of antifungal compound in onion set following seed treatment. Crop Protect 27: 330-336.

Crous PW, Braun U. 2003. *Mycophaerella* and its anamorphs. 1. Names published in *Cercospora* and *Passalora*. CBS Biodiversity Series 1: 1-571.

Dutta J, Gupta S, Thakur D. 2015. First report of *Nigrospora* leaf blight on tea caused by *Nigrospora sphaerica* in India. Plant Dis 99: 417.

Ginting E, Ratnaningsih, Iswanto R. 2008. The physical and chemical characteristics of mungbean seeds derived from 17 genotypes. In: Harsono A, Taufiq A, Rahmiana AA, Suharsono, Adie MM, Rozi F, Wijanarko A, Wijono A, Soehendi R (eds). Legumes and Tuber Crops Technology Innovation, Supported Inhouse Foods Production and Energy Supply. Indonesian Legumes and Tuber Crops Research, Malang.

Grewal JS, Machendra P, Kulsheethra DP. 1980. Control of *Cercospora* leaf spot of green gram by spraying Bavistin. Indian J Agric Sci 50: 707-711.

Haider A, Ahmed S. 2014. Study on seed quality and performance of some mungbean varieties in Pakistan. J Biol Agric Healthc 4 (23): 161-165.

Ichikawa K, Aoki T. 2000. New leaf spot disease of *Cymbidium* species caused by *Fusarium subglutinans* and *Fusarium proliferatum*. J Gen Plant Pathol 66: 213-218.

Iqbal SM, Ghafoor A, Bashir M, Malik BA. 1995. Estimation of losses in yield components of mungbean due to *Cercospora* leaf spot. Pakistan J Phytopathol 7: 80-81.

Nelson PE, Toussoun TA, Marasas WFO. 1983. *Fusarium* species. An Illustrated Manual for Identification. The Pennsylvania State University Press. University Park, London.

Nelson PE, Dignani MC, Anaissie EJ. 1994. Taxonomy, Biology, and Clinical Aspect of *Fusarium* Species. Clin Microb Rev 7: 479-504.

Pawar NV, Patil VB, Kamble SS, Dixit GB. 2008. First report of *Aspergillus niger* as a plant pathogen on *Zingiber officinale* from India. Plant Dis 92: 1368.

Rahayu M, Sumartini. 2016. A survey of groundnut and mungbean diseases in Central and East Java. Report of Legumes and Tuber Crops Research in the Year 2016. Indonesian Legumes and Tuber Crops Research, Malang.

Sarita, Buts AK, Singh R. 2014. Study of seed borne mycoflora of mungbean (*Phaseolus aureus*) treated with potassium nitrate during storage. Adv Appl Sci Res 5 (6): 11-13.

Semangu H. 2004. Diseases of Food Crops in Indonesia. Gajah Mada University Press, Yogyakarta.

Sharma R, Joshi A, Ramesh CD. 2012. A brief review on mechanism of *Trichoderma* fungus use as biocontrol agents. Internat. J Innov Bio-Sci. 2: 200-210.

Pande A, Rao VG. 1998. A Compendium Fungi on Legumes from India. Scientific Publishers (India), Jodhpur.

Thakur S, Harsh NSK. 2014. Phylloplane fungi as biocontrol agent against *Alternaria* leaf spot disease *Splanthes oleracea*. Biosci Discover 5: 139-144.

Vorholt JA. 2012. Microbial life in the phyllosphere. Nat Rev Microbiol 10: 828-840.