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HYPOVIRULENT ISOLATES OF *Rhizoctonia solani* COLLECTED FROM RICE IN KARANGANYAR REGENCY, CENTRAL JAVA, INDONESIA

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ABSTRACT

A filamentous fungus *Rhizoctonia solani* Kuhn is the important pathogen of many diseases on crops. The fungus has a broad host range including rice, horticulture crops, ornamental plants, and others. To date, there is no effective control method to the disease. So, the biological control method is a tactful choice. In the field, there are some hypovirulent strains within a species of a fungus. The hypovirulent isolates has two possibilities, they are genetically hypovirulent or infected by mycovirus. From phytopathologycal perspective, both of them are interesting as they could be developed as biocontrol agents. Binucleate *Rhizoctoia* is an example in which hypovirulent isolate is used as biocontrol agent. To control the same fungus, recently was developed *Rhizoctonia solani virus 717*, a virus that was isolated from a hypovirulent *R. solani*. In Indonesia, the development of hypovirulent strains of fungus as biocontrol agents is still very limited. This research was aimed to isolate hypovirulent isolates of *R. solani* from the field, as preliminary study toward development of the strains as biocontrol agents. We have collected 400 isolates of *R. solani* from the field. Morfological characterization and virulence tests revealed that the collected isolates perform varied colony phenotype and virulence. Finally, we identified 5 hypovirulent isolates i.e., isolate 11, 13, 15, 19, and 12 which perform relatively slower in colony growth and weaker in virulence compared to the virulent isolate. Total RNA isolation of the hypovirulent isolates revealed that there is no mycovirus infection on them. It means that the hypovirulent traits performed by *R. solani* isolates in this research were not caused by mycovirus infection. They were suggested caused by genetics factor.

Keywords: *Rhizoctonia solani*, isolate, hypovirulence.

INTRODUCTION

A filamentous fungus *Rhizoctonia solani* Kuhn is the causal agent of many diseases on crops such as vascular wilt, yellows, corm rot, root rot, damping-off, and others. The fungus has a broad host range including rice, horticulture crops, ornamental plants, and others. Overall, the distribution of *R. Solani* is known to be cosmopolitan. In Indonesia, the fungus is found almost in all agriculture land. In general to date, there is no effective and environmental friendliness control method to the fungi. Therefore, utilizing a biological control method is a tactful choice (Agrios, 1997; Semangun, 1996).

In solid media culture, such as potato dextrose agar (PDA), *R. Solani* colonies can have varying appearances. In general, the mycelium first appears white, and then may change to a variety of colors - ranging from bright to dark brown - according to the strain. After several days in media culture, the colonies produce scterotia in varied size and formation (Alexopoulos, 1996).

In the field, there are many strains within a species of fungi. Some of them are virulent whereas the others are hypovirulent. In the case of the hypovirulent isolates, there are at least two possibilities, they are genetically hypovirulent or are infected by mycovirus. From phytopathological perspective, both of them are interesting as they could be developed as biocontrol agents (Ogoshi, 1987; Ghabrial, 2001).

Within a species of fungus, there are some strains that show varied in virulence. Binucleate *Rhizoctoia* for example, was naturally isolated from field and shows hypovirulent traits. In the plant protection practices, people use this hypovirulent strain for biocontrol agents against the virulence *R. solani* strains. In this case, the hypovirulent strain controls the virulent one via antagonism mechanism (Ogoshi, 1987; Sneh *et al*., 1996).

On the other hand, there is mechanism of biocontrol among fungi instead of antagonism. To control the same fungus, recently was developed *R. solani* isolate Rhs 717, a hypovirulent *R. Solani* strain containing *Rhizoctonia solani virus 717* (Partitivirus). In this case, the mechanism of the hypovirulent strain in controlling the virulent one is incorporated with viral infection. The hypovirulent strain is viral infected, and during application, the viruses spread to and infect the virulent one, and change it to be hypovirulent (Nuss, 2005; Milgroom and Cortesi, 2004; Ghabrial, 2001; Lakshman *et al*., 1998).

In Indonesia, development of hypovirulent strains of fungus as biocontrol agents is still very limited. This research was aimed to collect hypovirulent isolates of *R. solani* from the field, as a preliminary study toward development of the strains as biocontrol agents.

In this research, we have collected 400 isolates of *R. solani* from the field. Based on morfological and virulence traits, we identified 5 hypovirulent isolates. Based on Total RNA analysis it was revealed that the hypovirulent traits performed by *R. solani* isolates in this research were not caused by mycovirus infection. They were suggested caused by genetics factor.

MATERIALS AND METHODS

Collection of *R. solani* isolates from fields

R. Solani isolates were collected from endemic areas of the fungus in the center of rice-producing regions in Karanganyar regency, Central Java, Indonesia. Rice

crops showing typical symptom of *R. Solani* infection were marked and selected. Stems and midribs showing the symptom were cut off, inserted in plastic clip, labeled, and kept in ice box. After getting in laboratory, the samples were transferred to refrigerator prior fungi isolation.

Isolation and culture of *R. solani* isolates on artificial medium

Isolation was done according to the procedure of Streets (1972) with modification. Each sample (diseased stems and midribs) were soaked in 2% NaOCl for 2 min and rinsed in sterile distilled water. Small pieces of the diseased stems and midribs were transferred to 90 mm petridishes containing 20 ml potato dextrose agar (PDA) (Oxoid Laboratories, Detroit, Mich.), and incubated for 10 days at 25°C. All isolates were labeled or marked using similar number given when collecting from field. Cultures were maintained on regeneration media at 4^{o}C in a refrigerator.

Morphological characterization of *R. Solani* isolates

Morphological characterization was done as described by Hillman, *et al*. (1990). Experimental cultures were initiated by inoculating 3x3x3 mm agar cubes, excised from the margins of 7- to 10-day stock cultures, at the center of 90-mm-diameter petridishes containing 20 ml of PDA. Plates were incubated under standard bench top conditions at room temperature. Cultures were observed at 3, 5, and 7 day after inoculation (DAI). The observed and recorded traits were: colony diameter, colony color, amount of aerial mycelium.

Virulence assay

Virulence assay was performed in laboratory and greenhouse. Virulence assay in laboratory was done as described by Elliston (1985). Mature apples (Rainbow, imported from China) were washed with 5% PURELOX, then four 7-mm diameter x 3-4-mm-deep plugs of tissue were removed with a burned cork borer and spatula from points equally spaced around each fruit. Each apple was inoculated with four isolates of *R. Solani*. One inoculum plug was inserted into each wound, with mycelium facing inward, and pressed with a sterile spatula into complete contact with the tissue. Sites were covered with small pieces of parafilm to retard drying, then the apples were incubated in 35x25x7-cm plastic boxes under bench top conditions. Lesion diameters were measured at days 3, 5, and 7.

Virulence assay in greenhouse was conducted by the method of Kanematsu *et al*. (2004) with modifications. Seeds of rice (Membramo variety) were sown in a plastic tray containing 1:1 of autoclaved soil and compost, and grown in a greenhouse for about two weeks. Seedlings then were transplanted to a single plastic container (20 cm in diameter) containing autoclaved latosol soil. *R. solani* isolates were cultured on PDA at 25°C for 2 weeks. At 36 days after transplanting (DAT), the rice plantlets were inoculated with a single sclerotium of *R. solani* isolates by inserting the sclerotium in the midrib of rice plantlets. Inoculated rice plantlets were grown in greenhouse for 60 days.

Total RNA extraction

Total RNA Extraction was performed using Trizol Reagent (Total RNA Isolation Reagent) supplied by Invitrogen followed manual from the manufacturer. A 10-day-old culture of *R. solani* in 20 ml potato dextrose broth was harvested by straining through sterile Miracloth (Calbiochem). Fungal tissue (75 mg) was pulverized in liquid nitrogen with a mortar and pestle. Homogenation was done by adding 1 ml of Trizol Reagent to the mycelial powder to form a slurry, then was transferred to 1.5 ml microcentrifuge tubes followed by 5 min incubation at room temperature and centrifugation at 12, 000 x g for 10 min. Phase separation was done by adding 0.2 ml chloroform to the supernatant followed by fortexing for 15 sec and centrifugation at 12, 000 x g for 15 min. RNA precipitation was done by adding 0.5 ml isopropyl alcohol to the supernatant followed by incubation at room temperature for 10 min and centrifugation at 12, 000 x g for 15 min for getting pellet. After being washed using 75% ethanol, the pellet was dissolved in 30 µl DEPC treated water. Total RNA was analyzed by electrophoresis through 1% agarose gel cast in TBE buffer. Gels were stained with ethidium bromide and photographed.

RESULTS AND DISCUSSIONS

Collection of *R. solani* isolates from fields and culture in artificial medium

R. Solani isolates were collected from endemic areas of the fungus in the center of rice-producing regions in Karanganyar regency, Central Java. From this field were collected 300 specimens of diseased plants. From these specimens we isolated 600 *R. solani* isolates. All the 600 *R. solani* isolates were cultured on 90 mm diameter of potato dextrose agar (PDA) medium plates under standard condition. On this medium, the cultures of *R. solani* isolates showed varied phenotype morphology from smooth and white color until rough cottony and white yellowish. Based on these phenotype, we roughly screened and got 400 selected isolates.

Colony phenotype characterization of *R. solani* isolates

All of the 400 *R. solani* isolates were phenotype characterized. Each of the isolate was cultured on PDA in 90 x 15 mm petridish and incubated on the bench top under standard condition for 1 week. After 1 week culture, the 400 isolates of *R. solani* colonies showed varied colony morphology. Based on the colony morphology, we screened the 400 isolates and finally we got 31 selected isolates showing unique traits such as colony growth was very fast or slow, colony color was darker or bolder, aerial mycelium was much or few, and sclerotia was amass or spread.

To the 31 selected isolates were then subjected to colony growth rate assay by culturing them on PDA plate. Colony growth was observed at 3, 5, and 7 day after inoculation (DAI). The result showed that colony growth

rate among the isolates was varied. At 5 DAI observations, the longest colony diameter was demonstrated by isolate 7 that were 83 mm, whereas the shorthest colony diameter was demonstrated by isolate 11 that was 5 mm. Furthermore, we also identified 4 other isolates showing relatively low colony diameter i.e., isolate 13, 15, 19, and 12 with colony diameter was 32, 45, 65, and 74 mm, respectively (Figures 1 and 2). Based on these data, we coined that isolate 7 is virulent, whereas isolate 11 and other 4 isolates are hypovirulent.

To the 31 selected isolates, we also observed colony profiles. Colony profiles of virulent isolate and the 5 selected hypovirulent isolates are shown in Figure-1. The virulent isolate (isolate 7) showed relatively fast in colony growth rate, colony color was white yellowish, formed a ring, and sclerotia was formed before 4 DAI. Contrary, the hypovirulent isolates showed varied colony phenotipe. Colony growth rate of isolate 11 was very low, mycelium was cottony smooth and white yellowish color, and sclerotia was formed after 14 DAI. Colony of isolate 13 grew slowly, white yellowish color, did not form a ring, not yet formed sclerotia at 4 DAI, a lot of aerial mycelium, and center area of the colony was dark color. Isolate 15 resembled to isolate 13, but grew faster and formed a ring. Colony of isolate 19 was grew fastly, white transparent color, did not form a ring, not yet formed sclerotia at 4 DAI, center area of the colony was white yellowish color and based on microscopic observation, the micelia was corpulent. Furthermore, colony of isolate 12 grew swiftly, white transparent color, did not form a ring, and not yet formed sclerotia at 4 DAI (Figures 1 and 5).

From mycovirology perspective, one of the important sign indicating virus infection on fungi is phenotype colony deviation. The deviation can be happen on colony growth rate, colony color, colony profile, aerial mycelium quantity, and also sporulation. Take some examples, Infection of *Cryphonectria hypovirus-1* (CHV1) on *Cryphonectria parasitica* change colony phenotype from smooth white yellowing color to rough and white color. Whereas infection of *Mycovirus-1* (MyRV1) on the same fungus changes the colony phenotype to brown with colony growth rate is restricted (Hillman *et al*., 2004; Suzuki *et al*., 2004; Supyani *et al*., 2007). The other case, hypovirulent *R. solani* isolated from diseased potato in North America, recently was proved to be mycovirus infected. In this case, mycovirus infection restricts colony growth rate and delayed sclerotia formation (Lakshman *et al*., 1998).

In this research, the variation on phenotype colony of *R. solani* isolates indicating the possibility of mycovirus infection. The phenotype colony changing on fungus usually correlates to its virulence. So this trait could be used as guidance for hypovirulence screening. Based on the obtained data of colony profile and colony growth rate, the selected 31 *R. solani* isolates were subjected to virulence assay.

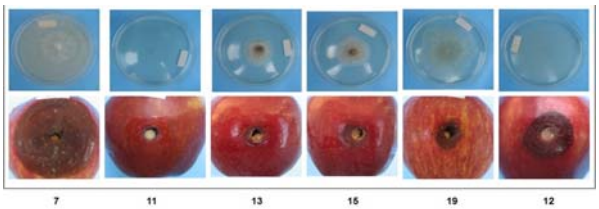

Figure-1. Biological character of *R. solani* isolates: virulent (7), hypovirulent (11, 13, 15, 19, and 12). Upper: colony profile on PDA plate, Lower: virulence assay on apple fruit.

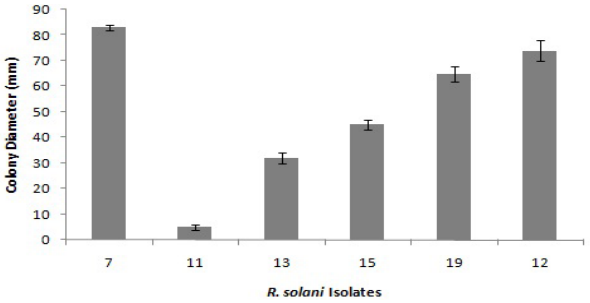

Figure-2. Quantified results of colony growth rate assay. Each *R. solani* isolate was cultured on PDA plate under room temperature in triplicates. Data were collected at 5 dpi (day post inoculation). Each bar represents the average from triplicates with standard deviation.

Virulence assay

In the laboratory, virulence assay was performed by inoculating the selected 31 *R. solani* isolates onto apples in triplicates. The result showed that the 31 *R. solani* isolates were shown varied virulence level. At 5 DAI, the highest virulence level was shown on isolate 7, in which could produce lesion 40 mm in diameter, whereas the lowest virulence level was shown by isolate 11 which could only produce lesion 5 mm in diameter. Furthermore, we also coined 4 other isolates showing relatively low in virulence level i.e. isolate 13, 15, 19, and 12 with lesion diameter was 8, 10, 12, and 24 mm respectively (Figures 1 and 3).

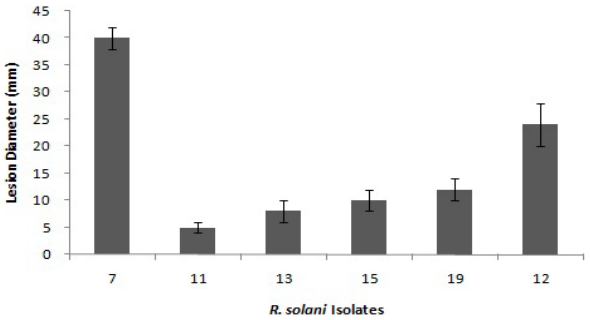

Figure-3. Quantified results of virulence assay. Each *R. solani* isolate was inoculated on apple fruit and incubated under room temperature. Data were collected at 5 dpi (day post inoculation). Each bar represents the average from triplicates with standard deviation.

In the greenhouse, virulence assay was performed by inoculating the selected 31 *R. solani* isolates onto rice crops in triplicates. The result showed that the 31 *R. solani* isolates were shown varied virulence level. At 30 DAI, the highest virulence level was shown on isolate 7, in which could cause 73% disease intensity, whereas the lowest virulence level was shown by isolate 11 which could only cause 48% disease intensity. Furthermore, we also coined 4 other isolates showing relatively low in virulence level i.e., isolate 13, 15, 19, and 12 with disease intensity was 53, 58, 60, and 64 % respectively (Figure-4).

One of the most important indications of viral infection on fungus is the decrease on its virulence level (Ghabrial, 2001). Infection of *Cryphonectria hypovirus-1* (CHV1) on *Cryphonectria parasitica* decreases its virulence level 25%, whereas infection of *Mycovirus-1* (MyRV1) on the same fungus decreases its virulence level 80% (Hillman *et al*., 2004; Supyani *et al*., 2007; Enebak *et al*., 1994). The other case, *Rhizoctonia solani* isolated from diseased potato in North America which recently was proved to be mycovirus infected was also indicated decrease in its virulence level (Lakshman *et al*., 1998).

In this research, based on the phenotype colony traits and virulence, the *R. solani* isolate 7 represents virulent isolate, whereas the other isolates especially isolate 13, 15, 19, and 12 represent hypovirulent isolates.

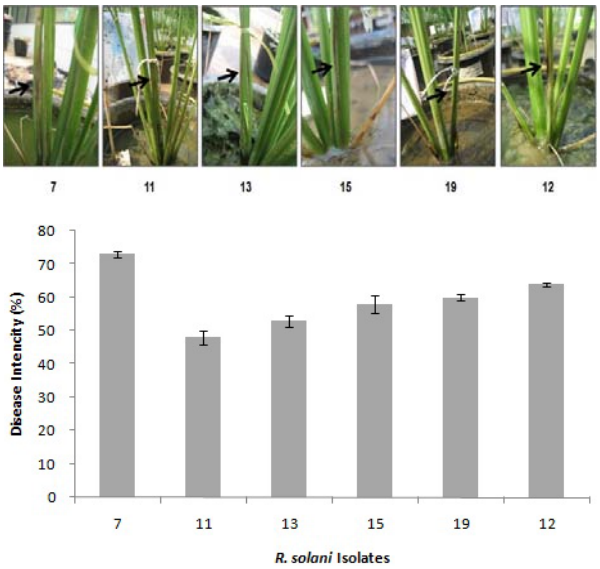

Figure-4. Virulence assay in greenhouse. Rice clumps (*Oryza sativa* var Membramo) were planted in plastic containers (one clump per container), cultured for 36 days and subsequently inoculated with single sclerotium of *R. solani* isolates according to the number. Each clump was inoculated with each isolate in triplicates. Phenotypes of rice plants 4 weeks following inoculation are shown. Arrows indicate sites of inoculation and symptom development (A). (B) Quantified results of virulence assay showing mean disease intensity and standard deviations from triplicates. Data were collected at 30 dpi (day post inoculation).

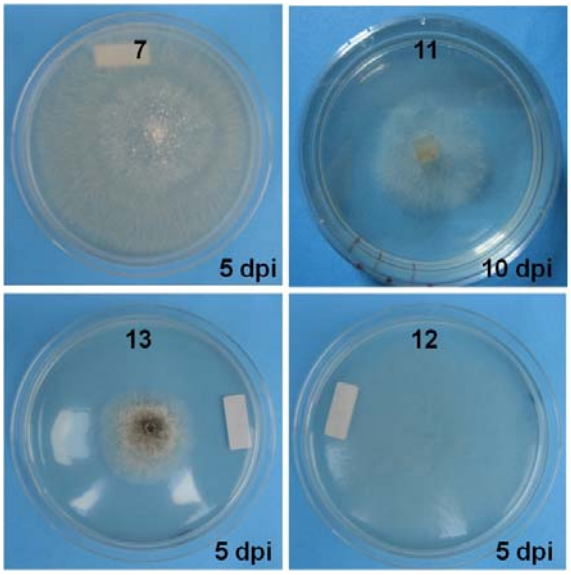

Figure-5. Colony morphology on potato dextrose agar of selected *R. Solani* isolates. Numbers represent isolates of the fungus. Pictures were taken at 5 days post inoculation (dpi) for isolates 7, 13, 12 and 10 dpi for isolate 11.

Total RNA extraction

To prove whether the hypovirulent isolates were infected by mycoviruses, we extracted total RNA from mycelia of the five selected isolates. The result of total RNA electrophoresis is shown in Figure-6. From the figure we can see that there are no specific bands in each isolates positioned at upper and lower their ribosomal RNA, 28s and 18s ribosomal RNA with size 2500 bp and 2000 bp respectively. It indicates that there are no typical bands of mycoviral genomes. King *et al*. (2012) reported that to date there are more than 200 mycoviruses were identified, and almost all of their genome were RNA. The size of their RNA genomes were ranged between 1 and 12.7 kb.

From this result we can suggest that the hypovirulent traits performed by isolate 11, 13, 15, 19, and 12 are not caused by mycoviral infection. They probably are manifestation of fungal genetics variability.

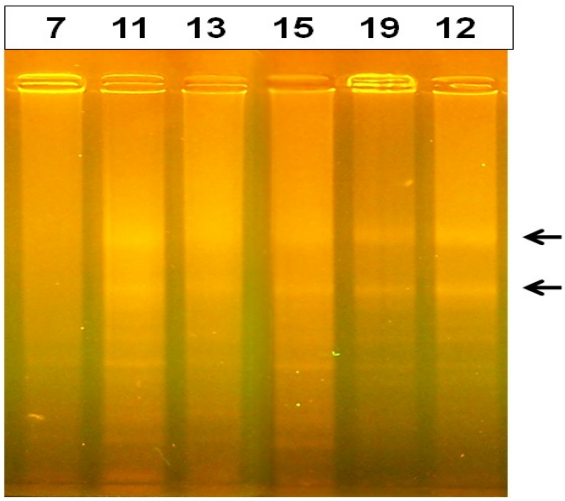

Figure-6. Electrophoresis of total RNA extracted from mycelia of *R. solani* isolates on 1% agarose gel in TBE buffer. Lane 7 is virulent isolate, whereas lanes 11 to 12 are hypovirulent isolates. Arrows indicate host fungal ribosomal RNA. Upper arrow, 28s ribosomal RNA (2500 bp); lower arrow, 18s ribosomal RNA (2000 bp).

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