HYPOVIRULENT ISOLATES OF *FUSARIUM* COLLECTED FROM CHILI CROPS IN BOYOLALI REGENCY, CENTRAL JAVA, INDONESIA

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ABSTRACT

Fusarium, a genus of filamentous fungi, has many species which serving as important pathogens to many diseases in crops. Till today, there have not been effective and efficient control methods for such fungi. Recently, scientists agree that application of biological agents is a tactful choice. Development of hypovirulent strains of fungus as biocontrol agents is very limited. This research was aimed to find hypovirulent isolates of *Fusarium* from field as biological agents. A hundred isolates of *Fusarium* from chili were collected in Boyolali, Central Java. Morphological characterization revealed that isolates performed varied colony phenotypes. Based on colony phenotype pattern, isolates were classified into five groups. From each group, one hypovirulent isolate was selected based on colony growth rate on potato dextrose agar media. The selected hypovirulent isolates were used for virulence assay in apple. The result showed that there were four hypovirulent isolates i.e.: B6, C15, D19, and E20 isolates. Total RNA extraction of the identified hypovirulent isolates revealed the existence of viral RNA in C15 isolate. Based on the existence of viral RNA in C15 isolate, the hypovirulent traits were due to mycoviral infection, whereas the hypovirulent traits performed by the other three were due to genetic factors.

Keywords: biological control agent, *Fusarium*, hypovirulent isolate, virulence assay

INTRODUCTION

A filamentous fungus, *Fusarium* is the causal agent of many diseases in crops such as vascular wilt, root rot, corm rot, damping-off, yellows, and others. The fungus has a broad host range including rice, horticultural crops, ornamental plants, and others. Overall, the distribution of *Fusarium* is known to be cosmopolitan. In Indonesia, *Fusarium* causes diseases in almost all agricultural commodities. The fungus is found almost in all agriculture land. In general to date, there have not been effective and environmental friendliness control methods for the fungus. Therefore, utilizing a biological control method is a tactful choice (Farr *et al.*, 1989; Agrios, 1997).

In solid media culture, depending on the species, *Fusarium* colonies can have varying appearances. In general, the aerial mycelium first appears white, and then may change to a variety of colors ranging from white to dark purple. Furthermore, colony phenotypes may also vary according to strain within a species (Alexopoulos *et al.*, 1996).

In the field, there are many strains within a species of fungi. Some of them are virulent whereas the others are hypovirulent. The hypovirulent isolates have 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).

In the biological control practice, the hypovirulent strains of *Fusarium* has been utilized for controlling the virulence strains which infect fanilla in the field. Here, the hypovirulent strains control the virulent strains via antagonism mechanism (Ogoshi, 1987; Agrios, 1997). On the other hand, to control *F. graminearum* on wheat, people have developed *F. graminearum* Virus1-DK21 (FgV1-DK21) (potex-like virus) (Chu *et al.*, 2002; Chu *et al.*, 2004). The other mycovirus, *F. graminearum* virus from China, 9 isolate (FgV-ch9) has been also developed for controlling *F. graminearum* on wheat and maize (Darissa *et al.*, 2011). In this case, the mechanism of the hypovirulent strains in controlling the virulent strains is incorporated with viral infection. The hypovirulent

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strains are viral infected, and during application, the viruses spread to and infect the virulent strains, and change them into hypovirulent (Lakshman *et al.*, 1998; Ghabrial, 2001; Milgroom and Cortesi, 2004; Nuss, 2005).

In Indonesia, the development of hypovirulent strains of fungus as biocontrol agents is limited. Related to this condition, a preliminary study was needed to find hypovirulent isolates of Fusarium from field as biological control agents. Morphological and virulence traits were used to identify hypovirulent isolates.

MATERIALS AND METHODS

Collection of *Fusarium* Isolates from Fields

Fusarium isolates were collected from endemic areas of the fungus in the center of chili-producing regions in Boyolali regency, Central Java, Indonesia. A hundred pieces of infected trunks were collected from 10 fields (10 samples per 1 hectare of field). Each sample was put into a plastic, and then labelled and kept in an icebox. When samples were arrived in the laboratory, then the samples were transferred to refrigerator prior to fungi isolation.

Isolation and Culture of *Fusarium* Isolates on Artificial Media

Isolation was done according to the procedure of Streets (1972) with modification. Each infected sample was soaked in 2% NaOCl for 2 min and rinsed in sterile distilled water. Small pieces of the infected samples were transferred to 90 x 15 mm Petri dishes containing 20 ml potato dextrose agar (PDA) (Oxoid Laboratories, Detroit, Mich.), and incubated for 10 days at 25°C. *Fusarium* isolates, were transferred to new 35 x 10 mm diameter Petri dishes containing 8 ml PDA and incubated at room temperature for 7 days. The isolates were identified to the genera level by the procedures of Nelson *et al.* (1983). The selected isolates were maintained on regeneration medium at 4°C in a refrigerator as stock cultures.

Morphological Characterization of *Fusarium* Isolates

Morphological characterization was done as described by Hillman *et al.* (1990). Experimental cultures were initiated by inoculating 3 x 3 x 3 mm agar cubes, excised from the margins of 7- to 10-day stock cultures, at the center of 90 x 15 mm Petri dishes 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 growth rate), colony nature, colony color, amount and color of aerial mycelium.

Virulence Assay of *Fusarium* Isolates

Virulence assay was done in laboratory as described by Elliston (1985). Mature apples (Rainbow, imported from China) were washed with 5% PURELOX, then five 5-mm diameter x 3-mm-deep plugs of tissue were removed with a burned cork borer and spatula from points equally spaced around each apple. Each apple was inoculated with five isolates of *Fusarium sp*. 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 35 x 25 x 7 cm plastic boxes under bench top conditions. Lesion diameters were measured at 3, 5 and 7 day after inoculation.

Total RNA extraction of *Fusarium* Isolates

Total RNA Extraction of *Fusarium* isolates was performed using TRIzol®RNA Extraction Kit supplied by Invitrogen™, following protocol from the manufacturer. A 10-day-old culture of *Fusarium* in 20 ml potato dextrose broth (PDB) was harvested by straining through sterile Miracloth (Calbiochem). Fungal tissue (≈75 mg) was pulverized in liquid nitrogen with a mortar and pestle. One ml of Trizol reagent was added to the mycelial powder to form a slurry, then was transferred to a 1.5 ml micro-centrifuge tubes followed by 5 min incubation at room temperature. The undesired materials were pelleted by centrifugation at 12,000 x g for 10 min. The supernatant was phase separated by adding 0.2 ml chloroform, followed by fortexing for 15 seconds and centrifugation at 12,000 x g for 15 minutes. RNA was precipitated by adding 0.5 ml isopropyl alcohol to the supernatant followed by incubation at room temperature for 10 minutes. The precipitated RNA was pelleted by centrifugation at 12,000 x g for 15 minutes. 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 in TBE buffer. RNA in the gel was stained with ethidium bromide and photographed.

RESULTS AND DISCUSSION

Collection of *Fusarium* Isolates from Fields and Culture in Artificial Media

Fusarium isolates were collected from the center of chili production field in Boyolali regency, Central Java. From this field were collected 100 specimens of infected trunks, and from the specimens were isolated a hundred *Fusarium* isolates. A hundred *Fusarium* isolates were cultured on 8 ml PDA plates in 35 x 10 mm diameter Petri dishes at room temperature. On this medium, the cultures of *Fusarium* isolates showed varied colony morphology from smooth, thin, and cream colored colonies to thick, pink colored colonies with white aerial mycelium. All *Fusarium* isolates were then used for colony morphology characterization.

Colony Morphology Characterization of *Fusarium* Isolates

All *Fusarium* isolates were morphologically characterized. Each isolate was cultured on a 20 ml PDA plate in 90 x 15 mm Petri dish and incubated on the bench top at room temperature for a week (Figure 1). Five days after inoculation (DAI), when the *Fusarium* colonies performed their best appearance, they were grouped into 5 groups based on their colony morphology pattern (Figure 2). Group A consisted of smooth and thin colonies, was creamy colored, with a few of white aerial myselium; group B consisted of smooth colonies, was purple colored, with much of white aerial mycelium; group C consisted of thick colonies, was pink colored, with white aerial mycelium; group D consisted of rather thick colonies, was white dirty colored, with much of aerial mycelium like cotton; and group E consisted of rather rugged colonies, was white dirty colored, forming a ring pattern, with white aerial mycelium. Then, all the groups of *Fusarium* isolates were used for colony growth rate assay by culturing them on 20 ml PDA plate in 90 x 15 mm Petri dishes at room temperature, and observed at 5 DAI. The result showed that colony growth rate of isolates within each group was varied. From this assay, one selected isolate showed the fastest colony growth rate from group A, and one another showed the slowest colony growth rate from group B, C, D and E. As a result, there were five selected isolates i.e.: A2, B6, C15, D19 and E20 isolates with colony sizes 88 mm, 60 mm, 31 mm, 50 mm and 45 mm in diameters respectively (Figure 3). Based on these data, from the five selected isolates, we coined that isolate A2 was virulent, and the other four isolates were hypovirulent.

From mycovirology perspective, phenotype deviations on fungal colonies are the important sign indicating viral infection on them. The deviation could happen on colony profile, colony color, colony growth rate, aerial mycelium quantity, and also sporulation (Ghabrial, 2001). *Fusarium graminearum* virus-DK21 (FgV-DK21), which causes hipovirulent on its host *Fusarium graminearum*, changes morphological traits of the fungus including reduction in mycelial growth, increased pigmentation, and decreased (60-fold) production of trichothecene mycotoxins (Chu *et al.*, 2002). In the case of *Cryphonectria hypovirus-1* (CHV1), which infects *C. parasitica*, the virus changed the colony phenotype of the fungus from smooth white yellowish color to rough and white color. Whereas infection of *Mycovirus-1* (MyRV1) on the same fungus changed the colony phenotype to brown with colony growth rate restricted (Hillman *et al.*, 2004; Suzuki *et al.*, 2004; Supyani *et al.*, 2007).

In this research, the variation on phenotype colony of *Fusarium* isolates indicated the possibility of mycoviral infection. The change of phenotype colony of fungus usually correlates to its virulence. Based on the obtained data of colony profile and colony growth rate, the selected five *Fusarium* isolates were subjected to virulence assay.

Figure 1. Culture of *Fusarium* isolates on artificial medium. *Fusarium* isolates were cultured on 20 ml potato dextrose agar (PDA) plates in 90 x 15 mm Petri dishes and incubated on the bench top at room temperature for a week
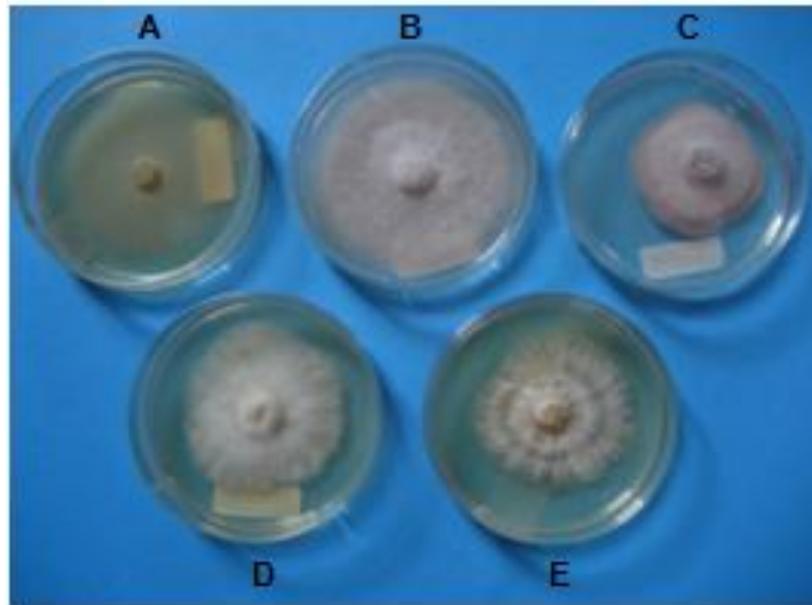
Figure 2. Five groups of *Fusarium* isolates based on colony morphology. Group A: Smooth and thin colonies, creamy colored, with a few of white aerial mycelium; Group B: Smooth colonies, purple colored, with much of white aerial mycelium; Group C: Thick colonies, pink colored, with white aerial mycelium; Group D: Rather thick colonies, white dirty colored, with much of aerial mycelium like cotton; Group E: Rather rugged colonies, white dirty colored, form a ring pattern, with white aerial mycelium. *Fusarium* isolates were cultured on 20 ml PDA plates in 90 x 15 mm Petri dishes and incubated on the bench top at room temperature for a week
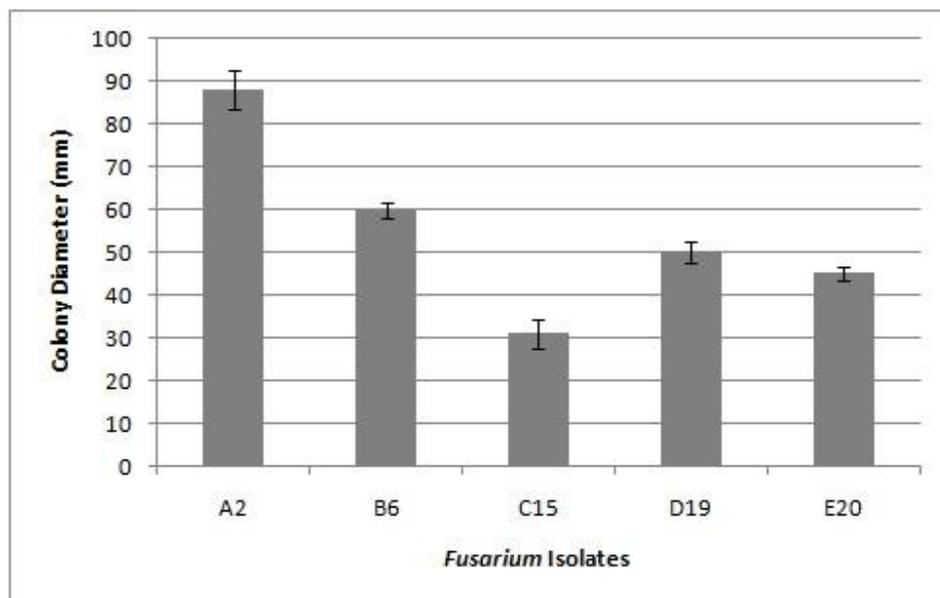

Figure 3. Quantified results of colony growth rate assay. Five selected isolates of *Fusarium* were cultured on 20 ml PDA plates in 90 x 15 mm Petri dishes and incubated on the bench top at room temperature for 1 week. Data were collected at 5 day post inoculation (DPI) in five replicates. Each bar represents the average from five replicates with standard deviation

Virulence Assay

Virulence assay was performed by inoculating the selected 5 *Fusarium* isolates onto apples in five replicates. The result showed that the five *Fusarium* isolates showed varied virulence level. At 5 DAI, the highest virulence level was shown on isolate A2, in which lesion 60 mm in diameter could be produced, and whereas the lowest virulence level was shown by isolate C15 which could only produce lesion 10 mm in diameter. Furthermore, B6, D19 and E20 isolates produced lesion 40 mm, 24 mm and 15 mm in diameter respectively (Figure 4 and Figure 5).

The other most important indication of viral infection on fungus is the decrease on fungal virulence level (Ghabrial, 2001). Infection of *Cryphonectria hypovirus-1* (CHV1) on *Cryphonectria*

parasitica decreases its virulence level by 25%, whereas the infection of *Mycovirus-1* (MyRV1) on the same fungus decreases its virulence level by 80% (Enebak *et al.*, 1994; Hillman *et al.*, 2004; Supyani *et al.*, 2007). In the case of mycovirus, *F. graminearum* virus-DK21 (FgV-DK21) reduces pathogenicity of the fungus which was proven by reducing its virulence towards wheat (Chu *et al.*, 2002). The other mycovirus, *F. graminearum* virus from China 9 isolate (FgV-ch9) also reduces pathogenicity of the fungus, by reducing its virulence towards wheat and maize (Darissa *et al.*, 2011).

In this research, based on the phenotype colony traits and virulence, the *Fusarium* isolate A2 represents virulent isolate, whereas the other 4 selected isolates represent hypovirulent isolates.

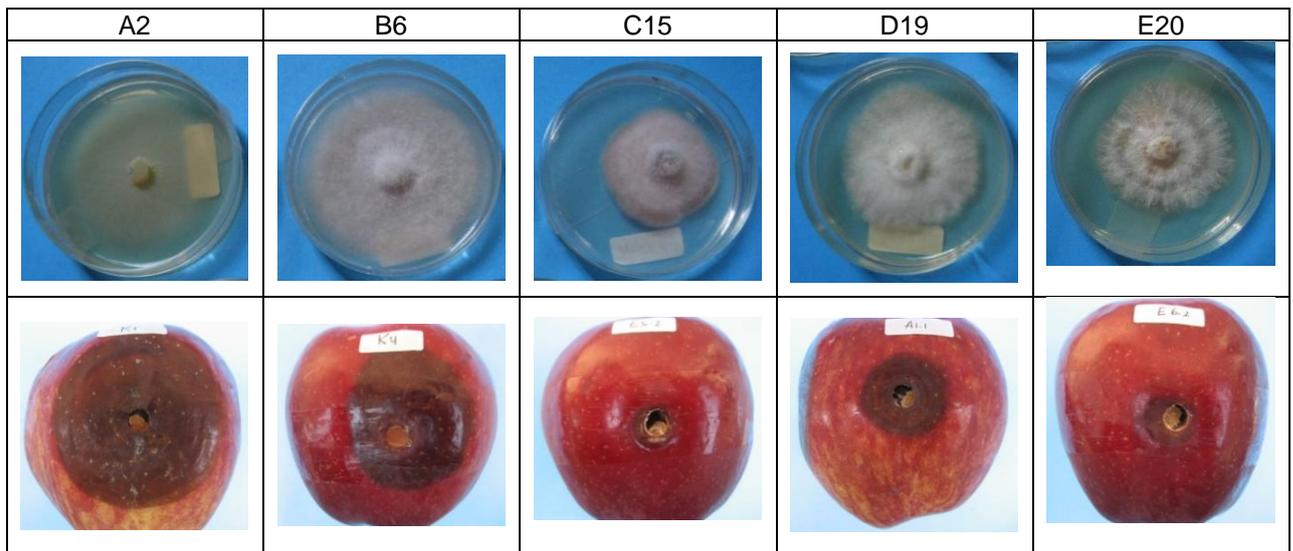| A2	B6	C15	D19	E20

Figure 4. Biological characters of five selected isolates of *Fusarium*: virulent (A2), hypovirulent (B6, C15, D19 and E20). **Upper**: colony profile on PDA plate. Selected *Fusarium* isolates were cultured on 20 ml PDA plates in 90 x 15 mm Petri dishes and incubated on the bench top at room temperature for a week. **Lower**: virulence assay on apple fruit. Selected *Fusarium* isolates were inoculated on apple fruit and incubated under room temperature for five days
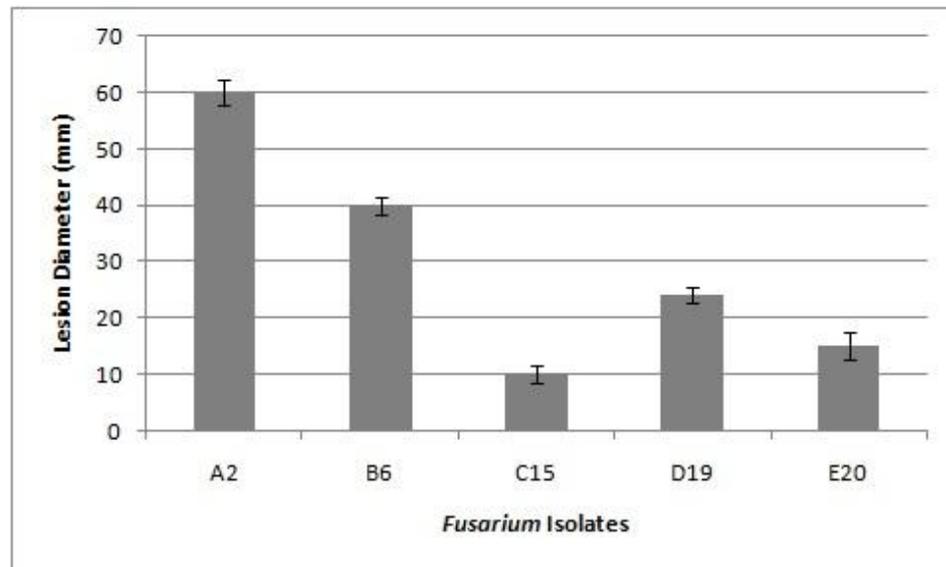
Figure 5. Quantified results of virulence assay. Each selected *Fusarium* isolate was inoculated on apple fruit and incubated under room temperature. Data were collected at 5 day post inoculation (DPI). Each bar represents the average from five replicates with standard deviation

Total RNA Extraction

To prove whether the hypovirulent isolates were infected by mycoviruses, total RNA from mycelia of the five selected isolates was extracted. The result of total RNA electrophoresis was shown in Figure 6. From the Figure 6, only C15 isolate showed specific RNA bands. C15 isolate clearly shown had two specific RNA bands sized around 1.0 kbp and 0.8 kbp (pointed by arrows). Here, as a consideration, the ribosomal RNA of the fungus was 28s and 18s ribosomal RNA with size 2.5 kbp and 2.0 kbp respectively (marked by their molecular size). It indicated the existence of mycoviral in C15 isolate. King *et al.* (2012) reported that to date there are more than 200 mycoviruses identified, and almost all of their genomes were dsRNA. The size of their RNA genomes ranged between 1 and 12.7 kbp. Especially on *Fusarium* sp, genome size of mycoviral which has been reported to date is 0.8-11.5 kbp dsRNA on *F. poae* (Compel *et al.*, 1999), 1.4-1.6 kbp dsRNA on *F. solani* (Nogawa *et al.*, 1996), 2.2-4.0 kbp dSRNA on *F. Oxysporum* (Kilic and Griffin, 1998) and 2.4 to 7.5 kbp dsRNA on *F. Graminearum* (Chu *et al.*, 2002; Chu *et al.*, 2004; Darissa *et al.*, 2011).

From this result, it is suggested that the hypovirulent traits performed by C15 isolates of *Fusarium* in this research were due to mycoviral infection**.** The hypovirulent traits performed by isolates B6, D19 and E20 of *Fusarium* in this

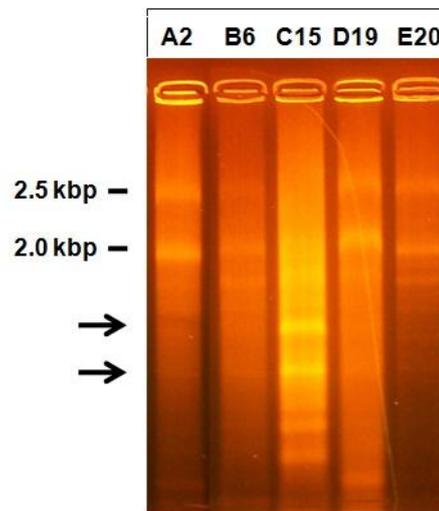research was probably manifestation of fungal genetic variability.

Figure 6. Electrophoresis of total RNA extracted from mycelia of selected *Fusarium* isolates on 1% agarose gel in TBE buffer. Lane A2 was virulent isolate, whereas lanes B6 to E20 were hypovirulent isolates. Marks indicate host fungal ribosomal RNA. Upper mark, 28s ribosomal RNA (2.5 kbp); lower mark, 18s ribosomal RNA (2.0 kbp). Arrows indicate putative mycoviral RNA

CONCLUSIONS

Fusarium isolates infecting chili crops in Boyolali, Central Java showed varied colony phenotype. Based on virulence assay, some of the isolates showed hypovirulency. Total RNA extraction of the hypovirulent isolates revealed the existence of viral RNA in isolate C15. It is suggested that the hypovirulent traits performed by isolates C15 of *Fusarium* in this research was due to mycoviral infection, whereas the hypovirulent traits performed by the other isolates were due to fungal genetics factor.

ACKNOWLEDGEMENTS

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