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### Determination of Endophytic Fungi as Induce Resistance Agent of Chilli Pepper Against Pepper Yellow Leaf Curl Disease

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#### ABSTRACT

Pepper yellow leaf curl disease caused by *Pepper yellow leaf curl virus* becomes a major problem on chilli pepper in Indonesia since 1999. Endophytic fungi has the potency to suppress plant diseases by acting as induced resistant agent. The objective of this research was to determine the effect of endophytic fungi application on incidence of yellow leaf curl disease of chilli pepper. Four isolates of endophytic fungi, i.e. *Cercospora nicotianae* isolate H5, *Curvularia* sp. isolate H12, *Fusarium* sp. isolate AC-2.7 and AC-4.5 were applied as seed treatment and leaf spray on 2 chilli varieties, 'Biola' and 'Luwes'. Observations of incubation period showed that delayed symptom occurred on application of *Fusarium* sp. isolate AC-2.7 and *Curvularia* sp. isolate H12. Disease incidence reached 100 % in all treatments. Less severe symptom was observed on application of *Curvularia* sp. isolate H12. Most plants infected by PYLCIV showed obvious symptoms of yellow mosaic, leaf curling and stunting. Plant productivity analysis showed that application of *Fusarium* sp. isolate AC-2.7 resulted higher fruit weight and this treatment may induce tolerant response of the plants to PYLCIV infection. Initial effort to evaluate endophytic fungi as biocontrol agents for pepper yellow leaf curl disease should be studied further.

#### INTRODUCTION

Epidemic of pepper yellow leaf curl disease in Indonesia occurred since 1999. The disease has been reported in West Java, Central Java, West Sumatra, and Bali. Disease incidence may reach 96.4 % in chilli pepper production area and severe infection of the disease may cause significant yield loss (20 – 100%). Pepper yellow leaf curl disease is caused by *Pepper yellow leaf curl Indonesia virus* (PYLCIV), a member of the family Geminiviridae, genus Begomovirus. Infection of PYLCIV is easily recognized by its specific symptoms of very bright yellow mosaic of the leaf, sometimes followed by leaf curling, and plant stunting. The virus is only transmitted by an insect vector, i.e. whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). PYLCIV has broad host range in the families of Solanaceae, Leguminosae, Compositae. In addition, several weed species can also become host range of PYLCIV (Sharma, Gaur, & Ikegami, 2010).

Disease control methods have been applied with little success. This efforts involved the use of healthy transplants, management culture practices, management of vector populations, resistance plant, and plant growth promoting rhizobacteria to induce the resistance of the host plant (Sharma, Gaur, & Ikegami, 2010). No report was found regarding to resistant or tolerant response on commercial varieties of chilli pepper. Breeding for resisting the disease has not been successful. Breeding for Begomovirus PYLCIV resistance is very challenging due to the complex genetics nature of its resistance and its transmission dependence on viruliferous whiteflies (Polston & Capobianco, 2013; Rodríguez-López et al., 2011). Therefore, exploration on alternative control method to manage this disease is important.

The use of endophytic fungi as biocontrol agent has been reported for many plant diseases. Endophytic fungi are able to inhibit the development of plant pathogenic fungi (Khastini, Ogawara, Sato,

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& Narisawa, 2014), bacteria (González-Teuber, Jiménez-Alemán, & Boland, 2014) and nematodes (Yan, Sikora, & Zheng, 2011). Endophytic fungi may produce antimicrobial compound (Jalgaonwala, Mohite, & Mahajan, 2011), including alkaloids, peptides, steroids, terpenoids, phenols, quinones, aliphatic compounds and flavonoids (Yu et al., 2010), isopestacin (Lugtenberg, Caradus, & Johnson, 2016), sordaricin (Pongcharoen et al., 2008), and jesterone (Zhou et al., 2009). In addition, endophytic fungi may also have antifeedant for insect (Crawford, Land, & Rudgers, 2010), or induce resistance of the plant (Hardoim et al., 2015). Therefore, this research was conducted to determine the potential of endophytic fungi as biological agents for chilli pepper yellow leaf curl disease.

## MATERIALS AND METHODS

Research activities were conducted in screen house at Cikabayan Screen house, Plant Clinic and Laboratory of Plant Virology, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University from February to August 2015.

### Fungal Isolates, Virus Isolate, Chilli Pepper Varieties and Insect Vectors

The four isolates of endophytic fungi, i.e. *Cercospora nicotianae* isolate H5, *Curvularia* sp. isolate H12, *Fusarium* sp. isolate AC-2.7 and *Fusarium* sp. isolate AC-4.7 used in the study was provided by Plant Clinic, Bogor Agricultural University. Isolate of PYLCIV was originally collected from Brebes, Central Java. The virus isolate was transmitted and propagated on chilli pepper using whitefly transmission method (Polston & Capobianco, 2013) with modification. Identity of virus isolate was confirmed using polymerase chain reaction (PCR) technique (Nurulita, Hidayat, Mutaqin, & Thomas, 2015) followed by direct sequencing of PCR product. Sequence data showed that virus isolate had the highest homology (98 %) with *Pepper yellow leaf curl Indonesia virus* (GenBank #AB267834.1).

Two commercial chilli pepper varieties, i.e. 'Biola' and 'Luwes', were selected for this study based on preliminary experiment. The varieties showed highly susceptible and susceptible response to PYLCV infection (data not shown).

*B. tabaci* was originally collected from eggplant (*Solanum melongena*) in Bogor, West Java and was reared on healthy cotton plants (*Gossypium hirsutum*) in insect cage and was warmed with 5

Watt light bulb. The insect cage then was kept in a greenhouse.

### Application of Endophytic Fungi

Mycelia of each isolate of endophytic fungi were removed (one circle of cork borer) from potato dextrose agar (PDA), and transferred into glass flasks containing 100 ml of potato dextrose broth (PDB). Mycelia were harvested from the media after shaking the PDB at 100 rpm for 5 days (d), then filtered, and washed with sterilized water. The mycelia were mixed with 100 ml sterilized water then grinded using homogenizer (IKA® T18 basic Ultra Turrax®) for 5 minutes (mins) to obtain propagule suspension. Mycelial density from each suspension of endophytic fungi isolates was assayed by plating method on PDA. Final concentration of propagule suspension for application was adjusted to  $10^5$  cfu  $ml^{-1}$ , based on dilution formula ( $C_1V_1 = C_2V_2$ ). Tween 0.001 % was added to propagule suspension as adhesive reagent before application.

The application of endophytic fungi was done twice, i.e. as soaking seeds and propagule spray. The seeds were soaked consecutively on 70 % alcohol for 1 min, 2 % sodium hypochlorite (NaOCl) for 1 min, then washed 3 times with aquades. The seeds was treated with endophytic fungi by soaking them on propagule suspension for 2 hours (h) before planting them on seedling growing media. The application of endophytic fungi was given again on seedlings 3 weeks after planting (WAP) by propagule spraying using hand sprayer with application volume of 5 ml per plant. The seedlings were then transferred into plastic pot (35 cm x 30 cm) containing growing media, consisted of mix of soil and manure (2:1 w/w). Fertilizer (NPK) was given to the plant on 7 WAP (1.5 g per plant).

### Inoculation of PYLCIV Using *Bemisia tabaci*

Seedlings were inoculated with PYLCIV using imago *B. tabaci* at 4 WAP (4 leaf stage) following the method described by Polston & Capobianco (2013) with modification. The white flies were given access to PYLCIV-infected chilli pepper plants for 24 h. After acquisition access period, the whiteflies were collected individually using an aspirator and transferred to healthy plants for 48 h - inoculation access period. Ten imago whiteflies per plant were used for inoculation access period. All whiteflies were removed from the plants by spraying insecticides after inoculation access period and the plants were held in screen house for symptom development.

### Experimental Design and Statistical Analysis

The experimental design was completely randomized factorial designs with 2 treatment factors i.e endophytic fungi consisting of 4 isolates and chilli pepper varieties consisting of 2 varieties. The experiment was consisted of 3 replications and each replication consisted of 15 test plants. Plants given endophytic fungi treatment without virus inoculation were used as check control.

Incubation period, disease incidence and severity, yield components (weight and number of fruits) were recorded. Disease incidence was recorded as the percentage of plant showed typical symptoms of PYLCIV over the total number of plants. Disease symptoms were scored based on a 5 point scale, i.e. 0 = no symptoms, 1 = yellowing, 2 = yellowing and leaf curling, 3 = yellowing, leaf cupping with leaf curling, upward or downward, 4 = yellowing, leaf cupping with leaf curling, upward and downward, and 5 = yellowing, leaf curling, stunting. All data were analyzed using the SAS 9.1 program. Means and analysis of variance (ANOVA) were computed and analyzed based on Least Significant

Difference (LSD) test. A significant test was obtained at  $P = 0.05$

### RESULTS AND DISCUSSION

Most plants infected by PYLCIV showed obvious symptoms of yellow mosaic, leaf curling and stunting (Fig. 1). Disease incidence reached 100 % in all treatments, although severity of the symptom varied among treatments. In general, the disease symptom on var. 'Biola' was more severe than those on var. 'Luwes'. However, the number of plants showing most severe symptoms (score 5) was found higher on var. 'Luwes' (Table 1). Disease symptom was developed faster on var. 'Luwes' than on var. 'Biola'. Delay symptom development was observed when *Fusarium* sp. isolate AC-2.7 and *Curvularia* sp. isolate H12 were applied (Table 2, Fig. 2). However, there were no significant differences on incubation period of the disease based on statistical analysis. It indicated that symptom development was affected either by plant varieties or endophytic fungi (Table 2).



Fig. 1. Symptoms of PYLCIV infection on var. Luwes (A) and var. Biola (B).



**Table 1.** Disease score measurement on application of four endophytic fungi isolates (AC-2.7, AC-4.7, H5, and H12) on two chilli pepper varieties ('Luwes' and 'Biola').

Score	Number of plants									
	AC-2.7		AC-4.7		H5		H12		Control	
	Luwes	Biola	Luwes	Biola	Luwes	Biola	Luwes	Biola	Luwes	Biola
0	0	0	0	0	0	0	0	0	0	0
1	1	0	1	0	1	0	3	0	1	1
2	8	12	8	8	8	9	11	11	10	6
3	19	15	22	13	25	17	25	19	26	24
4	12	17	9	18	8	18	4	15	1	6
5	5	1	5	6	3	1	2	0	7	8

**Table 2.** The effect of endophytic fungi application on incubation period and severity of the disease, fruit weight and number of fruits.

	Incubation period (dai)*	Disease severity (%)*	Fruits weight per plant (g)*	Number of fruits per plant*
Varieties				
'Luwes'	18.0 ± 3.42 <sup>a</sup>	61.7 ± 4.29 <sup>b</sup>	43.2 ± 8.12 <sup>a</sup>	5.5 ± 0.91 <sup>a</sup>
'Biola'	21.7 ± 3.79 <sup>b</sup>	65.2 ± 4.36 <sup>a</sup>	22.7 ± 10.00 <sup>b</sup>	4.6 ± 2.03 <sup>a</sup>
Isolates				
AC-2.7	21.2 ± 3.48 <sup>ab</sup>	64.2 ± 1.77 <sup>a</sup>	40.7 ± 13.93 <sup>a</sup>	6.4 ± 1.56 <sup>a</sup>
AC-4.7	17.7 ± 4.67 <sup>bc</sup>	66.9 ± 4.80 <sup>a</sup>	32.0 ± 9.07 <sup>a</sup>	4.6 ± 0.72 <sup>a</sup>
H5	19.5 ± 3.76 <sup>bc</sup>	63.3 ± 3.24 <sup>a</sup>	30.5 ± 15.02 <sup>a</sup>	4.5 ± 1.75 <sup>a</sup>
H12	23.5 ± 3.32 <sup>a</sup>	58.9 ± 3.90 <sup>b</sup>	29.8 ± 8.19 <sup>a</sup>	4.9 ± 1.51 <sup>a</sup>
Control	17.3 ± 1.96 <sup>c</sup>	63.8 ± 5.56 <sup>a</sup>	31.7 ± 20.73 <sup>a</sup>	4.6 ± 2.03 <sup>a</sup>
Interaction (Varieties x Isolates)				
'Luwes' x AC-2.7	18.8 ± 2.48 <sup>a</sup>	65.3 ± 0.00 <sup>a</sup>	49.9 ± 8.89 <sup>c</sup>	6.6 ± 0.47 <sup>a</sup>
'Luwes' x AC-4.7	14.7 ± 3.66 <sup>a</sup>	64.0 ± 4.00 <sup>a</sup>	38.4 ± 8.82 <sup>bc</sup>	4.3 ± 0.87 <sup>a</sup>
'Luwes' x H5	17.7 ± 4.87 <sup>a</sup>	61.8 ± 3.36 <sup>a</sup>	42.3 ± 3.82 <sup>c</sup>	5.4 ± 0.43 <sup>a</sup>
'Luwes' x H12	21.1 ± 0.52 <sup>a</sup>	56.0 ± 3.53 <sup>a</sup>	35.1 ± 2.19 <sup>b</sup>	4.8 ± 0.74 <sup>a</sup>
'Luwes' x Control	17.7 ± 2.69 <sup>a</sup>	61.3 ± 3.53 <sup>a</sup>	50.1 ± 3.25 <sup>c</sup>	6.2 ± 0.41 <sup>a</sup>
'Biola' x AC-2.7	23.6 ± 2.72 <sup>a</sup>	63.1 ± 2.04 <sup>a</sup>	31.5 ± 12.30 <sup>b</sup>	6.3 ± 2.41 <sup>a</sup>
'Biola' x AC-4.7	20.7 ± 3.80 <sup>a</sup>	69.8 ± 4.07 <sup>a</sup>	25.5 ± 2.00 <sup>ab</sup>	4.9 ± 0.41 <sup>a</sup>
'Biola' x H5	21.4 ± 1.01 <sup>a</sup>	64.9 ± 2.78 <sup>a</sup>	18.6 ± 11.38 <sup>a</sup>	3.6 ± 2.26 <sup>a</sup>
'Biola' x H12	25.9 ± 3.02 <sup>a</sup>	61.8 ± 0.77 <sup>a</sup>	24.6 ± 8.97 <sup>a</sup>	5.1 ± 2.27 <sup>a</sup>
'Biola' x Control	17.0 ± 1.43 <sup>a</sup>	66.2 ± 6.84 <sup>a</sup>	13.3 ± 6.97 <sup>a</sup>	3.1 ± 1.72 <sup>a</sup>

Remarks: \* = Means followed by the same letters within one column are not significantly difference at 5 % level using LSD analysis; dai = day after inoculation

Further detail observation showed that all endophytic fungi application on var. 'Luwes' tended to cause fast symptom development, i.e. 6–10 days after inoculation (dai) (Fig. 2). On the other hand, symptom development on var. 'Biola' varied from one treatment to the others. The highest frequency of incubation period on *Fusarium* sp. isolate AC-2.7 and *Curvularia* sp. isolate H12 treatments was in the range of 31 dai to 35 dai (Fig 2A and 2D), whereas *Fusarium* sp. isolate AC-4.7 treatment caused faster symptom development, i.e. 6 dai to 10 dai (Fig 2B). Interesting result was observed on *Cercospora nicotianae* isolate H5 treatment, which caused fluctuated range, i.e. 11 dai to 15 dai and 26

dai to 30 dai (Fig. 2C).

Application of endophytic fungi in this experiment could delay symptom development on chilli pepper plant, although it did not inhibit virus infection. Different result was reported by Jaber & Salem (2014) where application of endophytic fungi *Beauveria bassiana* had tendency to delay *Zucchini yellow mosaic virus* (ZYMV) symptom development on *Cucurbita pepo*, and it significantly reduced disease incidence and severity. According to Hipper, Brault, Ziegler-Graff, & Revers (2013) delayed symptom development might be caused by inhibition of viral movement.

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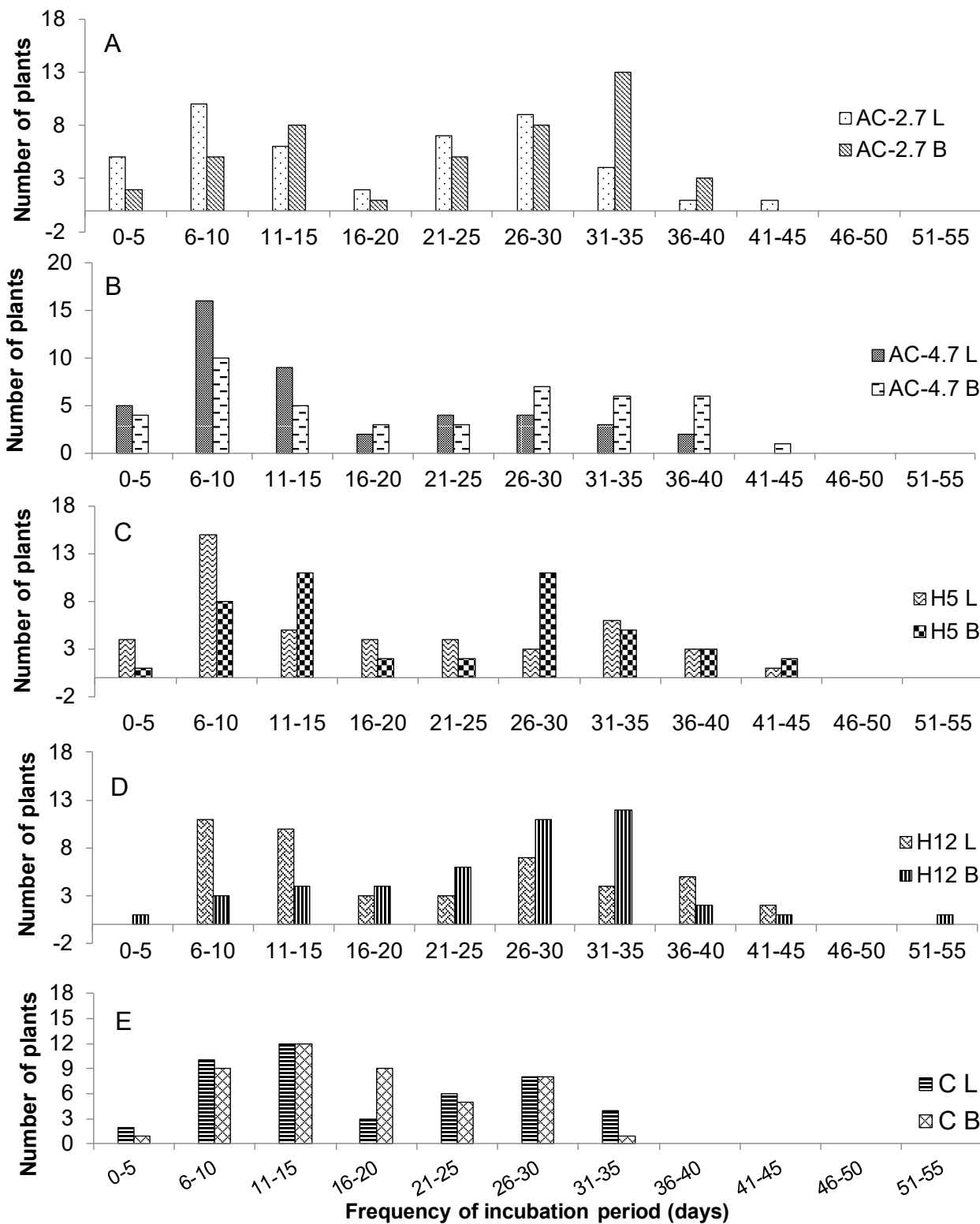


Fig. 2. The effect of endophytic fungi application on incubation period of pepper yellow leaf curl disease on var. 'Luwes' (L) and 'Biola' (B).

Analysis on yield components indicated that there was no difference on number of fruits per plant in all treatment. Var. 'Luwes' has higher fruit weight than var. 'Biola', and application of endophytic fungi did not affect fruit weight of var. 'Luwes'. Different result was observed for var. 'Biola', in which application of *Fusarium* sp. isolate AC-2.7 resulted higher fruit weight (Table 2). This treatment may induce tolerant response of the plants to PYLCIV infection.

Further data analysis revealed that productivity of plant did not always had a positive correlation with disease severity. The highest productivity was observed on plants showing high disease severity, and vice versa. High disease severity ( $65.3 \% \pm 0.00$ ) on var. 'Luwes' was observed on plants treated with *Fusarium* sp. isolate AC-2.7, but the productivity of this plants was the highest. On the other hand, the lowest disease severity ( $56.0 \% \pm 3.53$ ) was observed on plants treated with *Curvularia* sp. isolate H12, and the productivity of this plants was the lowest. Similar data was observed for var. 'Biola' (Table 2).

Previous research showed the role of endophytic fungi as a plant induced resistance agent. Achatz, Kogel, Franken, & Waller (2010) explained that the increasing plant yield occurred through accelerated growth of plants early in the development due to providing the basis for higher yield at harvesting time. Rúa, McCulley, & Mitchell (2013) showed that endophyte infection may increase negative effect of virus infection. In different cultivar, virus infection decreased yield in endophyte-infected individuals, but not endophyte-free individuals. Beneficial effects of endophytic fungi were influenced by host plant stages. The effectiveness of fungal endophyte occurred only in the early plant stage. It is possible that the consideration of mature plants and sexual reproduction would have different yield results regarding how the fungal endophyte contributes to niche partitioning (Kazenel et al., 2015).

Relationship between endophytes and host plant might turned/change from mutualistic to be negative when the environment has been changed (Moricca & Ragazzi, 2008). The presence of endophytes may cause disadvantages to the plant host. For instance, tall fescue grass (*F. arundinaceae*) containing endophyte showed enhanced crown and root rot disease caused by

*Pythium graminicola* compared to endophyte-free grass. According to Rodriguez, White, Arnold, & Redman (2009), susceptibility of grasses to *Pythium* will be higher when nutrient demands and stress on grass individuals increased due to high endophyte content in the grass. Busby et al. (2013) reported that *Penicillium* sp. endophyte infection prior inoculation with the necrotrophic leaf pathogen *Drepanopeziza populi* in *Populus angustifolia* genotypes showed greater symptom severity than plants inoculated with pathogen only. The different growth condition, especially the soil nutrient content might explained the different results. In this current research, the source of nutrient was relied only from the manure and once NPK application at 7 weeks seedlings. It might make chilli pepper more susceptible to PYLCIV infection, although according to Hull (2014) nutritional conditions that were most favorable for plant growth were also those giving greatest susceptibility to virus infection and there was no evidence that one particular element increased susceptibility to the plant.

Application of endophytic fungi should consider dosage/concentration and frequency of application for optimal effect. Although, the effectiveness of endophytic fungi was different among the host plant, fungi, virus, and their interaction. Suspension concentration of endophytic fungi applied in this presence research was  $10^5$  cfu ml<sup>-1</sup> by soaking seeds and leaf spraying in seedling stage. In comparison, Jaber & Salem (2014) used suspension concentration  $1 \times 10^8$  conidia ml<sup>-1</sup> with only once spraying application on the upper and lower surfaces of cucurbits crop and they could inhibit ZYMV infection until four weeks after inoculation. Meanwhile, Fakhro et al. (2010) reported that disease severity of *Verticillium dahliae* and concentration of *Pepino mosaic virus* was suppressed by application of fungal endophyte with suspension concentration  $10^5$  cfu ml<sup>-1</sup>.

Begomovirus replication inside its host cell is very rapid (Yadava, Suyal, & Mukherjee, 2010). On the other hand, endophytic fungi requires period of time to establish inside the host cell (Biswas, Dey, Satpathy, & Satya, 2012). Therefore, application of endophytic fungi should be done long before virus infection in order to give time for fungi colonization. Improvement on time and concentration of application should be proceeded to increase the efficiency of endophytic fungi as biocontrol agents.

### CONCLUSION

Potential role of endophytic fungi as beneficial microbes in controlling plant diseases has been reported. Application of endophytic fungi on chilli pepper did not suppress diseases caused by PYLCIV, although one isolate (*Curvularia* sp. isolate H12) had the potential to delay symptom development. The effect of endophytic fungi on plant tolerance varies between varieties or fungi isolates. Application of *Fusarium* sp. isolate AC-2.7 on var. 'Biola' could induce high plant yield despite of PYLCIV infection.

Initial effort to evaluate endophytic fungi as biocontrol agents for pepper yellow leaf curl disease should be studied further under field conditions since cultural practices and natural pests as well as disease condition might affect the efficiency of endophytic fungi to suppress disease development.

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