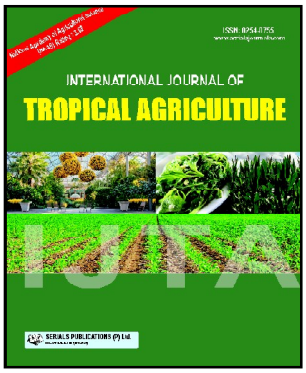

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Isolation and Identification of Phylloplane Microorganisms on the Stems of Asparagus for Biological Control of Anthracnose and Stem Blight Diseases

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Abstract: The objectives of this research project were to isolate antagonistic microorganisms from stem surface of asparagus (*Asparagus officinalis* L.) for *Colletotrichum gloeosporioides* and *Phomopsis asparagi* diseases and identify these microorganisms and to test if these microorganisms can cause diseases.Nine isolates were obtained from the research project. *Rhizopus arrhizus* (D1F5), D1F4, D1F3, D1F6 and D1F9 caused reduction in growth of *Colletotrichum gloeosporioides* for80.20, 75.10, 65.08, 56.28 and 55.03%, respectively. D1F5 also had the highest reduction in growth of *Phomopsisasparagi* for 64.31%, whereas other eight isolates caused reduction on growth of *Phomosis asparagi* for lower than 50%. These isolates were inoculated on asparagus stems for five days to observe pathogenicity of the isolates. The stems showed normal symptoms 3 DAI. However, at 5 DAI, D1F4 and D1F5 had the highest change in the wounds of the stems. The inoculated stems were yellow and the wounds developed mold colonies with dark green and brown colors, respectively. These isolates may cause the disease on asparagus. The isolates D1F1, D1F2 and D1F8 had the lowest change on asparagus stem as the stemswere still green.

Key words: Asparagus; disease; phylloplane; microorganisms; Thailand.

I. INTRODUCTION

Asparagus is an important vegetable crop in Thailand, and the demand for asparagus is increasing both fresh vegetable for export and processed products. The production area is also increasing especially in Kanchanaburi. Most farmers grow asparagus for export and Japan is the major import country. The area planted to asparagus in 2015 in

Thailand was estimated at 1,908.2 ha with the total production of 13,271 tons[1]. However, asparagus production in Thailand has several problems including unsuitable land, high labor cost, insect pests, diseases, residual contamination of harmful chemicals and the lack of local market as asparagus depends solely on export. The asparagus growers need four areas of extension programs including suitable production technology, the extension program from government sector, the support for production factors and marketing. Suitable technology for asparagus production in Thailand is being developed. Many production areas in Kanchanaburi are not able to produce asparagus to meet the quality required by the exporters. Insect pests, diseases and the contamination of chemicals in the products are major important problems of asparagus production in this province. Contamination of chemicals can cause health problems to consumers and export problem. Contamination is not only a problem for asparagus growers but also a problem for exporters and consumers. Root rot, anthracnose and stem blight are economically important diseases in asparagus production in Thailand. Asparagus growers use chemicals to control the diseases to sustain yield and maintain product quality.

Several methods are available for disease control in asparagus. High hydrostatic pressure treatment (HHP) is used to control diseases of asparagus under storage [2], and the most extensively used method is the application of chemicals [3]. Integrated management control by using several methods is an effective option to control the diseases of asparagus [4]. For biological control, the promising antagonistic organisms include *Pseudomonas putida* (MGY2), *Trichoderma* sp., *Streptomyces hygroscopicus*, *S. noursei, S. natalensis*, *Bacillus subtilis, Bacillus thuringiensis*[5] and *Streptomyces globisporus*[6].

The use of antagonistic organisms to control the diseases is currently well accepted by asparagus growers as a safe and economy method. It does not cause contamination to the products and frequent use of antagonistic organisms is not necessary as these organisms can persist in the soil. However, the organisms are specific to the growing areas and indigenous organisms are required for more effective control of the disease. *Streptomyces* spp. (ME2-27-19A) isolated from the asparagus field could suppress the growth of *F. oxysporum* f. sp. *asparagi* and *F. moniliforme*, which cause root rot in asparagus [7]. *F. redolens* is another pathogen of root rot in asparagus and it is similar to *F. oxysporum* for morphologyand symptoms [8]. *Aspergillus niger* isolated from the root zone of asparagus had antagonistic effect on *F. oxysporum* growth[9]. The phylloplane microorganisms or normal flora on the surface of host plants have not been well researched in asparagus, and screening of these organisms is necessary to discover the effective organisms for use as biological control of the diseases. The research on screening of antagonistic organisms to control the diseases on the aerial parts of asparagus was the continuation of our previous project, which focused on root rot diseases by screening antagonistic organisms in the root zones of asparagus [9].

In this study, indigenous antagonistic organisms on the aerial parts of asparagus were screened. The assumption underlying the research project is that indigenous antagonistic organisms should be advantageous because they adapt to growing areas. The objectives of this study were to isolate and identify the antagonistic organisms from stem surface for controlling *Colletotrichum gloeosporioides* and *Phomopsis asparagi* causing anthracnose disease and stem blight disease in asparagus and to test pathogenicity of these antagonistic organisms. The information obtained in this study is important for development of biological control of anthracnose disease and stem blight disease in asparagus.

II. MATERIALS AND METHODS

A. Isolation of Microorganisms on Stem Surface of Asparagus

Stem samples of asparagus were taken from two farmer's fields in Dan Makham Tia district, Kanchanaburi province and one farmer's field in Kamphaeng Saen district, Nakhon Pathom province. The samples were cut into small pieces and washed in flasks containing autoclaved distilled water at the ratio of 10 grams of samples per 90 grams of water. The samples were shaken for better contact of sample surface and water. The samples were further diluted at the dilution factors of 10^{-1} to 10^{-3}. The samples of 0.1 ml were spread on hard potato dextrose agar (PDA) medium in the petri dishes and incubated in an incubator at temperature of 8-10°C for 24-72 hours. The colonies on the petri dishes were isolated on the same medium for further efficacy test.

B. Efficacy Test

The isolated fungi were cultured with the target pathogens using dual culture technique in 2015. The colonies of the isolated fungi were cultured using spot inoculation technique. The fungi with three replicates including three control petri dishes were cultured on PDA medium for 5 days. The fungi and the target pathogens were further transferred to new PDA medium, put on the same petri dishes at the distance of 3 cm and incubated at 25-30 °C for 7 days. The ability of the isolated fungi to suppress the target pathogens were evaluated using the following equation;

$$\% \text{ growth reduction} = (Dc - Ds) \times 100/Dc$$

Where Dc is the diameter of the isolated fungi on the control plate and Ds is the diameter of the isolated fungi growing with the target pathogen. Some isolated fungi that could be cultured in laboratory for long period were identified of species.

The experiment was repeated in 2016 using the same procedure except that the evaluation was carried out at three days after culture.

C. Pathogenicity Test

The isolated fungi were evaluated by stem attachment method for the causal agents of plant diseases to ascertain that they will not cause diseases in asparagus. The isolated fungi were cultured on PDA medium for 3-4 days. The colonies of the isolated fungi were cut into pieces of 0.5 × 0.5 cm. The cut pieces were attached on the stems of asparagus, which were wounded at 10 and 20 cm above soil surface. The inoculated stems were placed on moist paper with 2 cm in thickness in trays, and the trays were wrapped with transparent shrink film to maintain high moisture and prevent the spreading of the fungal spores. The samples were incubated at room temperature for 3-7 days. Pathogenicity was evaluated by observation of the lesions on inoculated stems.

Evaluation of pathogenicity was carried out at 5 and 7 days after inoculation (DAI) using the disease severity rating scales of 1 to 5, one is very slightly severe and 5 is most severe.

III. RESULTS AND DISCUSSION

A. Isolation of Microorganisms on Stem Surface of Asparagus

Twelve fungi that could grow and develop mycelium and spores on PDA medium were isolated from stem surface of asparagus. The 12 isolates would be further tested for efficacy in controlling anthracnose and stem blight in asparagus.

Low number of the isolates obtained in this study would be mainly to poor environmental conditions as the time for collecting the samples was in the dry season when soil and air moistures were low and the temperature was high. Another reason for low number of isolates was the low number of samples collected from only three fields.

B. Efficacy Test

From 12 isolates, 9 isolates showed antagonistic effects on anthracnose caused by *Colletotrichum gloeosporioides* and stem blight caused by *Phomopsis asparagi* (Table 1). These isolates were designated as D1F1 to D1F9. Percentages of growth reduction of *C. gloeosporioides* were from 7.45% in D1F8 (unidentified species) to 80.20% in D1F5 (identified as *Rhizopus arrhizus), and* Percentages of growth reduction of *P. asparagi* ranged between 1.67% in D1F4 and 64.31% in D1F5. D1F5 had the highest percentages of reduction of anthracnose (80.20%) and stem blight (64.31%). D1F6 (identified as *Aspergillus* sp.) also had high percentages of growth reduction of *C. gloeosporioides* (56.28%) and *P. asparagi* (42.87%), andD1F9 also showed similar results for percentages of reduction of *C. gloeosporioides* (55.03%) and *P. asparagi* (42.49%). D1F3 (unidentified) had high percentages of growth reduction of *C. gloeosporioides* (65.08%) and *P. asparagi* (42.71%). Other isolates such as D1F2 and D1F4 (unidentified) had high percentages of growth reduction of *C. gloeosporioides* (45.17 and 75.10%, respectively), but they did not showed high percentages of growth reduction of *P. asparagi*. These isolates may be useful as a source of biological control of these diseases in asparagus.

The data in 2016 showed that D1F5 had the highest growth reduction of *C. gloeosporioides* (50.04%) and the other isolated fungi showed similar growth reduction of *C. gloeosporioides*, ranging from 4.81 to 23.41%. D1F5 also had the highest growth reduction of *Phomopsis asparagi* (58.45%) followed by D1F6 (24.81%), and the other isolated fungi had growth reduction of *Phomopsis asparagi* lower than 15%.

Dual culture of some isolated fungi against *Colletotrichum gloeosporioides*and *Phomopsis asparagi,* the causal agents of anthracnose and stem blight, were shown in Figure 1 and 2 respectively.

In previous study, *Trichoderma virens* effectively suppressed the growth of *C. gloeosporioides* which is the causal pathogen of stem rot in papaya for more 60% [5]. In this study, the isolated fungus D1F5 identified as *Rhizopus arrhizus*, D1F4 (unidentified) and D1F3 (unidentified) were also effective in controlling the growth of *C. gloeosporioides* under dual culturecondition.

Table 1
The ability of the isolated fungi from stem surface of asparagus to suppress the growth of *Colletotrichum gloeosporioides* and *Phomopsis asparagi*.

	Growth reduction (%)				*Fungal identification*[/1]	
	C. gloeosporioides		*P. asparagi*			
Isolated fungi	*2015*	*2016*	*2015*	*2016*	*Identified as*	*Similarity (%)*
D1F1	22.49^{g}	13.57^{b}	28.45^{d}	5.31^{d}	*Schizophyllum commune*	99%
D1F2	45.17^{e}	23.41^{b}	21.59^{e}	14.97^{c}	Unidentified	
D1F3	65.08^{c}	21.95^{b}	42.71^{b}	9.66^{cd}	Unidentified	
D1F4	75.10^{b}	22.32$_{b}$	1.67^{f}	8.21^{d}	Unidentified	
D1F5	80.20^{a}	50.04^{a}	64.31^{a}	58.45^{a}	*Rhizopus arrhizus*	100%
D1F6	56.28^{d}	14.66^{b}	42.87^{b}	24.81^{b}	*Aspergillus* sp.	100%
D1F7	32.50^{f}	13.93^{b}	35.64^{c}	7.25^{d}	Unidentified	
D1F8	7.45^{h}	4.81^{b}	28.55^{d}	10.14^{cd}	Unidentified	
D1F9	55.03^{d}	18.30^{b}	42.49^{b}	5.80^{d}	*Aspergillus* sp.	100%

Means in the same column followed by the same letter are not statistically different at 0.05 probability level by DMRT.
[/1] Identified by BIOTEC, Thailand

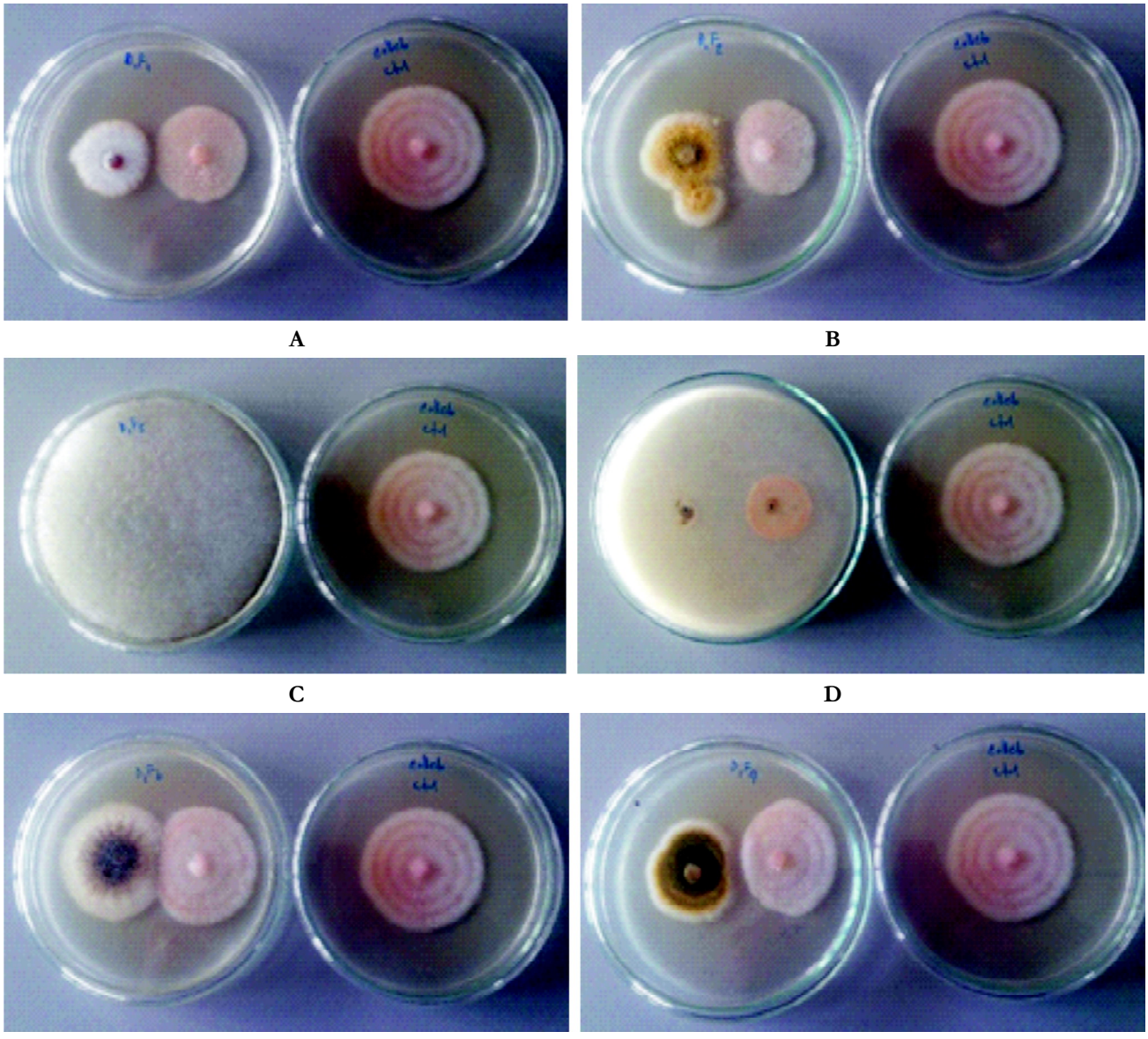

Figure 1: Dual culture of some antagonistic fungal isolates *Colletotrichum gloeosporioides* on left petri dish and control on the right petri dish; A. D1F1, B. D1F2, C. D1F5 (front), D. D1F5 (back), E. D1F6 and F. D1F9.

For controlling of stem blight caused by *Phomopsis asparagi*, the isolated fungus D1F5 is also effective in suppressing the growth of *P. asparagi* for more than 50% under dual culture condition. However, other isolated fungi did not show good suppression of the pathogen as they could reduce the growth of *P. asparagi* for lower than 50%. The results in this study were comparable to those reported previously. In previous study, *Streptomyces globisporus* JK-1 could reduce the growth of *P. asparagi* for 46.3% [6]. *Rhizopus arrhizus* (D1F5) in this study was somewhat better than *Streptomyces globisporus* JK-1 in previous study in controlling *P. asparagi*. However, the difference in experiment conditions should be considered when compared the results from different studies.

This study could also indentify two isolated fungi having ability to control the growth of

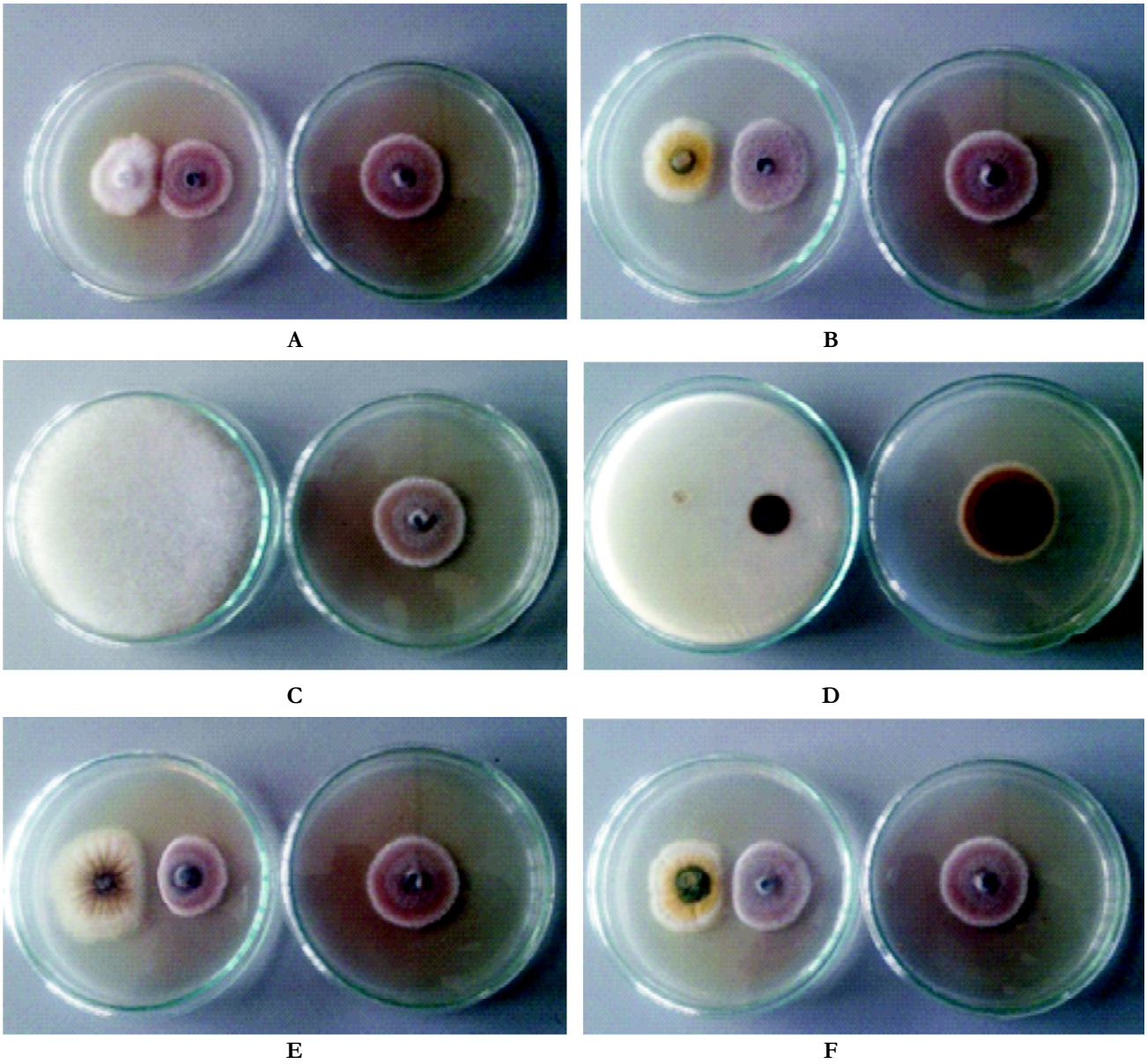

Figure 2: Dual culture of some antagonistic fungal isolates against *Phomopsis asparagi* on the left petri dish and control on the right petri dish; A. D1F1, B. D1F2, C. D1F5 (front), D. D1F5 (back), E. D1F6 and F. D1F9.

anthracnose and stem blight. The isolated fungus D1F1 was identified as *Schizophyllum commune,* whereas the isolated fungi D1F6 and D1F9 were identified as *Aspergillus* sp. Other isolated fungi in this study could not be identified because they were not able to persist in long term culture in laboratory.

C. Pathogenicity Test

At 3 DAI, the plant showed normal symptom, the evaluation of pathogenicity, therefore, was carried out at 5 and 7 DAI. After 5 DAI, the inoculated stems showed symptoms of 1-3 severity levels, and, after 7 DAI, the inoculated stems showed symptoms of 2-5 severity levels (Table 2).

At 5 DAI, the isolated fungi D1F4 and D1F5 had the highest disease severity levels of 3 (Figure 3), and D1F3, D1F6. D1F7 and D1F9 had disease severity levels of 2, whereas D1F1, D1F2 and D1F8 had disease severity level of 1. After 7 DAI, D1F8

Table 2
Pathogenicity test of 9 isolated fungi evaluated at 5 and 7 days after inoculation.

	Severity levels of symptom		
Isolate	*5 DAI*	*7 DAI*	*Symptom at 5 DAI*
D1F1	1[/1]	2	Stem was green and yellow at the tip. The wound had pale brown and short mycelium.
D1F2	1	2	Stem was green and yellow at the tip. The wound had white and short fungal mycelium.
D1F3	2	4	Stem was green and yellow at the tip. The wound had white and short mycelium.
D1F4	3	4	Stem was green and yellow. The wound had black mycelium.
D1F5	3	4	Stem was green and yellow. The wound had brown and long mycelium.
D1F6	2	4	Stem was green and pale yellow. The wound had short mycelium with black spores.
D1F7	2	4	Stem was green and yellow. The wound had white and short mycelium.
D1F8	1	5	Stem was green and yellow at the tip. Edge of the wound was pale yellow, and the wound had long and white mycelium with rapid growth, leading to the most severe symptom at 7 DAI. At 7 DAI, whole stem was brown.
D1F9	2	4	Stem was green and yellow. The wound had white mycelium with green spores.

[/1]Severity rating of 1-5, 1 = very slightly severe to 5 = most severe.

Control **D1F1** **D1F5** **D1F6**

Figure 3: Pathogenicity test of some isolated fungal isolates at 5 days after inoculation; normal stems of control and D1F1 inoculation; green andyellowstems of D1F5 and D1F6 inoculation.

had the highest disease severity level (5) followed by D1F3, D1F4, D1F5, D1F6, D1F7 and D1F9 (level 4), where as D1F1 and D1F2 had the lowest disease severity level (2).

When antagonistic and pathogenetic properties of the isolated fungi were considered at 5 DAI, D1F5 (*Rhizopus arrhizus*) was the best isolated fungi with good ability to control anthracnose caused by *C. Gloeosporioides*and stem blight caused by *P. asparagi*but it had high risk for causing disease in wounded asparagus stem.

Other isolated fungi had low risk for causing disease in asparagus except for D1F4 which had severity at the same level as D1F5.

Rhizopus arrhizus was reported to cause Rhizopus rot disease in root stocks of mulberry in Japan [10] and soft rot disease in water melon (*Citrullus vulgaris*) in Korea [11]. *Rhizopus arrhizus* has ability toproduce some phytochemicals such as siderophore that inhibits the growth of other microorganisms [12]. *Rhizopus arrhizus* also produces lactic acid, fumaric acid and ethanol at considerable amount for industrial use [13]. In order to develop biological control of anthracnose and stem blight disease in asparagus by using *R. arrhizus*, the farmers should ascertain that the fungus is safe for use. The use of *R. arrhizus* strains that are not harmful to the crops through genetically modified methods might be possible.

The use of D1F1 (*Schizophyllum commune*) and D1F2 for controlling anthracnose and stem blight in asparagus would be an acceptable option. Although these isolated fungi are not as effective as D1F5, they have low risk to cause diseases in asparagus. The isolated fungi can be used as a component of the integrated management control to control the diseases [4]. The use of chitosan derivatives with broad spectrum effect on plant diseases as a biological polymer wrapper for fruits and vegetables under storage might be a good option for disease control [3].

IV. CONCLUSION

In this study, the author tested the assumption that indigenous antagonistic organisms should be advantageous because they adapt to growing areas, identified the antagonistic organisms from stem surface for controlling anthracnose disease and stem blight disease in asparagus and verified that the antagonistic fungi did not cause diseases in healthy asparagus at 3 DAI. The author found that the isolated fungus D1F5 identified as *Rhizopus arrhizus* showed the best growth reduction of anthracnose disease cause by *C. gloeosporioides* and stem blight disease cause by *Phomopsis asparagi* under laboratory condition.

The results are important as an initial step to develop biological control of the diseases and further investigations are still required. The isolated fungi should be tested for pathogenicity under greenhouse and field conditions. Collection of the samples should be undertaken in more growing areas of asparagus and other crops both in soil and stem surface to increase the chances to find the best antagonistic fungi. The information on the persistence of the fungal colonization on stem surface of asparagus is important for development of biological control.

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