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PRELIMINARY SCREENING OF ENDOPHYTIC FUNGI FROM *Capsicum annuum* L. FOR BIOCONTROL ACTIVITY AGAINST *Colletotrichum gloeosporoioides*

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Abstract

Anthracnose is a plant disease caused by the *Colletotrichum sp*., which responsible for declining the production of chilli production worldwide. Farmers have been using various agronomic practices in managing plant diseases, such as planting resistant crop varieties, applying chemical fungicides and introducing biological control agents. However, due to the drawbacks of chemical fungicide usage, biological control has become an alternative option. This study was conducted to determine the potential endophytic fungi isolated from chilli as biocontrol agents against phytopathogen, *Colletotrichum gloeosporoioides.* In the present work, we isolated, identified and characterized a total of 85 fungal isolates harboring inside the leaves, fruits and stems of *Capsicum annuum* L. The endophytic fungi isolates were most frequently found in the leaves of *C. annuum* (1.17) compared to stems (0.24) and fruits (0.08). The cultural-morphological identification was grouping these isolates into 14 groups. Based on the rDNA ITS gene sequence, 14 fungal isolates belonged to Phylum Ascomycota. Most of the dominating fungal endophytes were from the genus *Trichoderma*. For antagonistic test, *Hypoxylon sp*. (F2) and *Trichoderma reesei* (F4) isolates recorded a promising potential against *C. gloeosporoioides* phytopathogens with 87.21% and 76.26% of radial growth inhibition, respectively. The finding in this study suggested the initial step in utilizing the isolates for further development of these endophytic fungal as antagonists and antibacterial agents for future biotechnological applications in chilli growing fields.

INTRODUCTION

Chili or botanically known as *Capsicum annuum* L. is one of the most widely domesticated crops in the world due to its economic significance. Belongs to the Solanaceae family, this plant has been commonly used in culinary as flavouring agents and has its own nutritional and medicinal interests. It is rich in vitamin A, B and C and is a good source of minerals such as potassium, calcium and iron [1]. The main bioactive compound in chili, namely capsaicin, gives the spicy sensation that helps in lowering blood sugar levels and improving cardiovascular health and has analgesic properties for arthritis, diabetes and headaches [2]. According to the Food and Agriculture Organization of the United Nations (FAO), one-third of the world's food produced was estimated lost or wasted and directly impacted both consumers and national economies. Improper management during pre-harvest and post-harvest could lead to this problem. Diseases caused by pathogens such as bacteria,

fungi and viruses are the major restrictions to fruit production, including chilli.

Anthracnose disease has been acknowledged as a major cause of decreasing chilli production in tropical and subtropical countries due to environmental factors [3]. Globally, several species of fungal belong to the genus *Colletotrichum* were the etiological agents for anthracnose disease, such as *Colletotrichum capsici*, *C. acutatum*, *C. truncatum* and *C. gloeosporioides* [4]. Infected chilli can be seen with symptoms such as dark, sunken necrotic tissues, with orange to pink spores on fruit surfaces [5]. Even small lesions on fruits could reduce their marketable value. Various management strategies have been applied in order to control the disease, including the usage of fungicide by farmers. However, the long-run implications of this practice can cause pathogen resistance and negative impacts on humans and the environment. Hence, a safe and environmentally friendly control is warranted to replace or decrease the usage of chemical fungicides. The application of biological control agents in managing anthracnose disease has been mentioned by [6, 7].

Endophytes, mostly fungi and bacteria, are microorganisms that reside in the tissues of living plants without causing any harm to the host [8, 9]. Almost all plants that have been studied consist of one or more types of endophytes and can be isolated from the stem, roots, fruits, seeds, and leaf segments [10, 11, 12]. They are endosymbionts, which help increase plant growth, act as biocontrol agents (BCAs) against pathogen and enhance the ability of the host to withstand various types of biotic and abiotic stresses [13]. In recent years, research into the beneficial use of fungal endophytes has increased worldwide as they could produce many secondary metabolites that essential to biotechnology interest. Thus, the objective of this study was to investigate the presence, distribution, and antagonistic activity of endophytic fungi in fruiting stages of chili pepper (*Capsicum annuum* L.) to suppress the growth of *Colletotrichum gloeosporoioides.*

MATERIALS AND METHODS

Materials

Fungal pathogen, *Colletotrichum gloeosporoioides* was obtained from Laboratory of Mycology and Pathology, Forest Research Institute Malaysia (FRIM). Potato Dextrose Agar was purchased from Oxoid, UK.

Methods

Samples Collection and Procedure

Healthy leaves, stems, and fruits of chili were collected from the greenhouse at field 16, Faculty of Agriculture, Universiti Putra Malaysia. It is located on 3°02′ N latitude and 101°42′ East longitude and altitude is 31 m above sea level. Plants were chosen during the fruiting stage of the chili pepper growth. In our research, the fruiting stage was described as the point at which chili pepper fruits began to appear after 40 days of planting. A total of fifteen healthy plant samples from each field were randomly chosen at the study site. One branch with leaves was randomly selected and excised from each plant. All the samples were then brought to the laboratory in a separated and labelled sterile zipper bag. The method described by [14] was slightly modified for the isolation of endophytic fungi. All the plant tissues were thoroughly cleaned with running water and air-dried before being cut into 0.5x0.5cm pieces. These fragments were surface sterilized with 70% ethanol for 2 minutes, 2% sodium hypochlorite for 3 minutes and rinsed with sterile distilled water three times. The excess moisture was blotted by sterile filter papers.

Isolation of Fungal Endophytes

The tissue fragments of each plant part were placed on Potato Dextrose Agar supplemented with chloramphenicol. The plates were incubated for 2 weeks at 28°C for fungal development. Pure fungal endophyte cultures were obtained by picking the hyphal tips of the developing colonies. Each pure culture was then grouped according to colony morphology. The isolation rate was calculated as the number of endophytic fungi isolated from plant segments were divided by the total number of segments incubated, while the colonization frequency was calculated by dividing the total number of plant segments colonized by fungal endophytes with the total number of segments observed [15, 16].

Morphological Identification of Endophytic Fungi

Each pure culture was first examined both macroscopically and microscopically based on colony texture, colour and reproductive characteristics. The identification of all the fungal isolates was carried out with the help of a microscope (Olympus Bx51, Tokyo, Japan) and all the procedures were adopted as previously described by [17].

Molecular Characterization of Endophytic Fungi

Genomic DNA was extracted from fresh mycelia grown in PDB by using CTAB method proposed by [18] with slight modification. The fresh mycelia were taken from broth and transferred to 1.5mL centrifuge tube containing 500µL of 2X CTAB extraction buffer. The mixture was homogenized completely using cell homogenizer. Then, the samples were incubated in a water bath for 30 minutes at 65°C with intermittent mixing. The tubes were centrifuged at 10 000rpm for 10 minutes. The supernatant was collected and transferred into a new tube. Next, an equal volume of chloroform:isoamyl alcohol (24:1) was added into the tubes and the solution was mixed by shaking vigorously forming an emulsion. The tubes were then centrifuged at 13,000rpm

for 12 minutes, which was long enough to produce a clear supernatant. The upper aqueous layer was collected, and re-extraction process was repeated twice. Two volume of ice cold isopropanol was added in the tubes and stored overnight in freezer (-20°C) for DNA precipitation. The precipitate was collected by centrifugation at 10 000rpm and was washed with 1ml of 70% ethanol followed by air drying. The dried pellets were resuspended in 50µL of TE buffer. PCR amplification of the endophytic fungal genomic was performed by using the following universal primer, ITS 1 (5'TCCGTAGGTGAACCTGCGG3') and ITS 4 (5'TCCTCCGCTTATTGATATGC3'). The PCR mixture was set up in 25µL comprised of 12.5µL of Go Taq ® Green Master Mix, 1.0µL of each primer, 2.0 µL of each DNA template and 8.5µL of sterile distilled water. The amplification process was conducted using the same cycling program as mentioned by [17]. The PCR products were sequenced by the Bio Basic Asia Pacific Private Limited using the same primers. The sequence of ITS regions was matched with those in the nucleotide database of National Centre for Biotechnology Information (NCBI) by using BLAST Tool.

Fungus Pathogen

Colletotrichum gloeosporoioides (FRIM 1352) obtained from Laboratory of Mycology and Pathology, Forest Research Institute Malaysia, Kepong was used as the pathogenic fungus in antagonistic study. The pathogen was sub-cultured on PDA plate and incubated at 28°C.

Antagonistic Activity of Fungal Endophyte Isolates against *Colletotrichum* sp.

The abilities of endophytic fungi to act as antagonists were determined in dual plate confrontation assays against the phytopathogen, *C. gloeosporoioides* in accordance with protocol mentioned by [19]. Based on the groupings, one isolate from each group was selected for antagonistic assay purposes. A 5mm fungal plug of 7 days old pathogen was placed 1cm from the margin of the PDA plate. A similar size plug of the endophytic fungus isolate was placed in the opposite direction from pathogen plug. *C. gloeosporoioides* alone was served as control. Three replicates were used for each endophytic fungus tested and all the plates were incubated at 28°C for two weeks. The percentage inhibition of radial growth (PIRG) was calculated as follows:

$$\text{PIRG}\ (\%) = \frac{\text{R1}-\text{R2}}{\text{R1}} \times 100$$

Where,
R1 = radial growth of pathogen in control plate
R2 = radial growth of pathogen in test plate

The data obtained from the observation on the fungal colony radial were subjected to analysis of variance (2-way ANOVA). The means were separated by Tukey's test at $p<0.05$ with SPSS statistical software.

RESULTS AND DISCUSSION

Exploration and identification of numerous endophytic fungi from various plants over the years show the effectiveness of using them against phytopathogens with different degrees of success. Recently, this practice has gained increased attention due to its ability to provide a sustainable agricultural approach to controlling the disease. The present study was conducted to isolate and evaluate their potential as biocontrol agents against *Colletotrichum gloeosporoioides.*

A total of eighty-five endophytic fungi were isolated from leaves, fruits and stems of chilli plant. Isolated endophytic fungi were confirmed with the absence of epiphytic fungal colonies on control plates, as stated by [20]. The endophytic fungi frequency was varied according to the respective organ (leaves, fruits and stems). The highest isolation rate of endophytic fungi was observed in the leaves of *C. annuum* with 1.17, whereas the fruits of *C. annuum* showed the lowest isolation rate with approximately 0.08 (Figure 1). Thus, it is indicated that the endophytic fungi were abundant and showed species richness in leaves parts. The present study is in line with [21] who found the highest diversity of the endophytic population from leaves samples compared to fruit samples in similar plants. The study of *Melastoma malabraticum* by [22] also supported the same hypothesis whereby the highest number of endophyte species were found to be harboured in leaves compared to the root and stems. According to [23], the leaf segment has a greater diversity of endophytic fungi due to the surface area exposed to the atmosphere and the presence of leaf stomata providing passage to the fungal mycelia entrance. Compared to previous investigations by [14] the harvested endophytic fungi from chilli plant were found to be the most abundant in roots compared to the leaves and stems. The difference distribution of endophytic fungi in various plant parts was common and influenced by the variables such as nutrient availability, ecological factors, experimental parameters and plant physiology [24]. Present study demonstrated the low number of isolated fungus in chili plants as compared to previous study. As stated by [14], the sampling range of the plant age is strongly associated with the number of endophytic fungal species, so the likelihood of colonisation by endophytic fungi varies depending on the climate, resulting in infinite species diversity and quantities of endophytic fungi. Therefore, it is suggested to extend the sample collection by expanding the sampling range and collecting the different age of plant samples to allow the isolation of additional endophytic fungi [14].

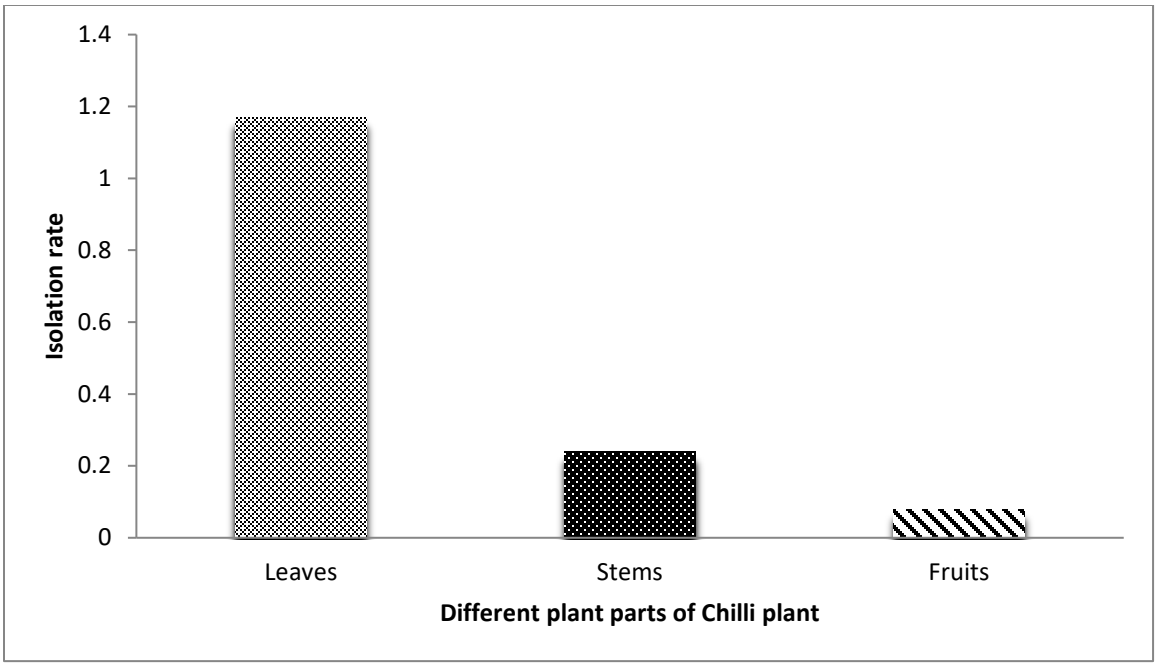

Figure 1. Fungal endophytes richness isolated from different plant parts of *Capsicum annuum* L.

The morphological identification of isolates endophytic fungi was performed on PDA medium to determine the macroscopic appearances of cultural characteristics of the endophytic fungi collection based on the features and colour of the fungal colonies (Table 1). Those isolates were divided into 14 groups based on their macromorphological characteristics. Most of these isolated endophytes were rapidly grown as they were fully filling the plates after 2 weeks. From the result obtained in Figure 2, both endophytic fungi isolated from the leaf shows circular colony form and filiform colony margin except for isolate F3 with irregular colony form and margin. Isolate F1 expresses a white colony with thin aerial mycelia with a mass of orange conidia. Isolate F8 in this study had brown with white conidia mass.

Meanwhile, isolates F5, F7, and F9 exhibited similar culture characters of white mycelium. However, isolate L showed a brown colour mycelium with a brown vein. Among endophytic fungi isolated from fruits (Figure 2), isolate F6 showed different morphologically when it was the only one with irregular colony form, undulate colony margin, and umbonate colony elevation with light brown colour with black conidia. Isolated endophyte, F10 had the smallest growth diameter size with rigid and rough texture black colour mycelia. Endophytic fungi isolated from stems demonstrated irregular colony form, undulate colony margin, and umbonate colony elevation. Isolates F11 showed the black colour mycelia, however, with an average smooth texture with the orange colour colonizing the agar after one-week growth. Next, isolate F12 performed a white thick texture green mycelium in the middle and isolate F14 showed a thick yellow with white conidia mass. Isolate F13 and F4 showed a white mycelium. However, isolate F13 showed a greyish colour of mycelia in the middle.

Table 1. Description of cultural characteristics and conidia morphology of endophytes isolated obtained from *Capsicum annuum* L.

Fungi Species (isolates code)	Morphological characteristics		
	Colonies texture upper surface	**Colonies colour upper surface**	**Colonies colour below surface**
F1	White thick cottony mycelia with orange conidia	White and orange	Orange conidia mass
F2	Thick white- brownish surface with white crusty texture at centre	White and brown	Black and white
F3	White greyish thick cottony mycelia with orange conidia	White and grey	White and grey
F4	Smooth white mycelium	White	White
F5	Rough white mycelium	White	White
F6	Rough texture with black conidia	Light brown with black conidia	Light brown with black conidia
F7	Smooth white mycelium	White	White
F8	Brown with white conidia mass	Brown and white conidia	Dark brown
F9	Thick white cottony mycelia	White	White and brown vein
F10	Black of rough rigid texture	Black	Black
F11	Average smooth texture	Shinning black	Black
F12	White thick texture with green mycelium	Green	Red
F13	White smooth texture with greyish mycelium	White and grey	White
F14	Thick yellow with white conidia mass	Yellow and white	Yellow

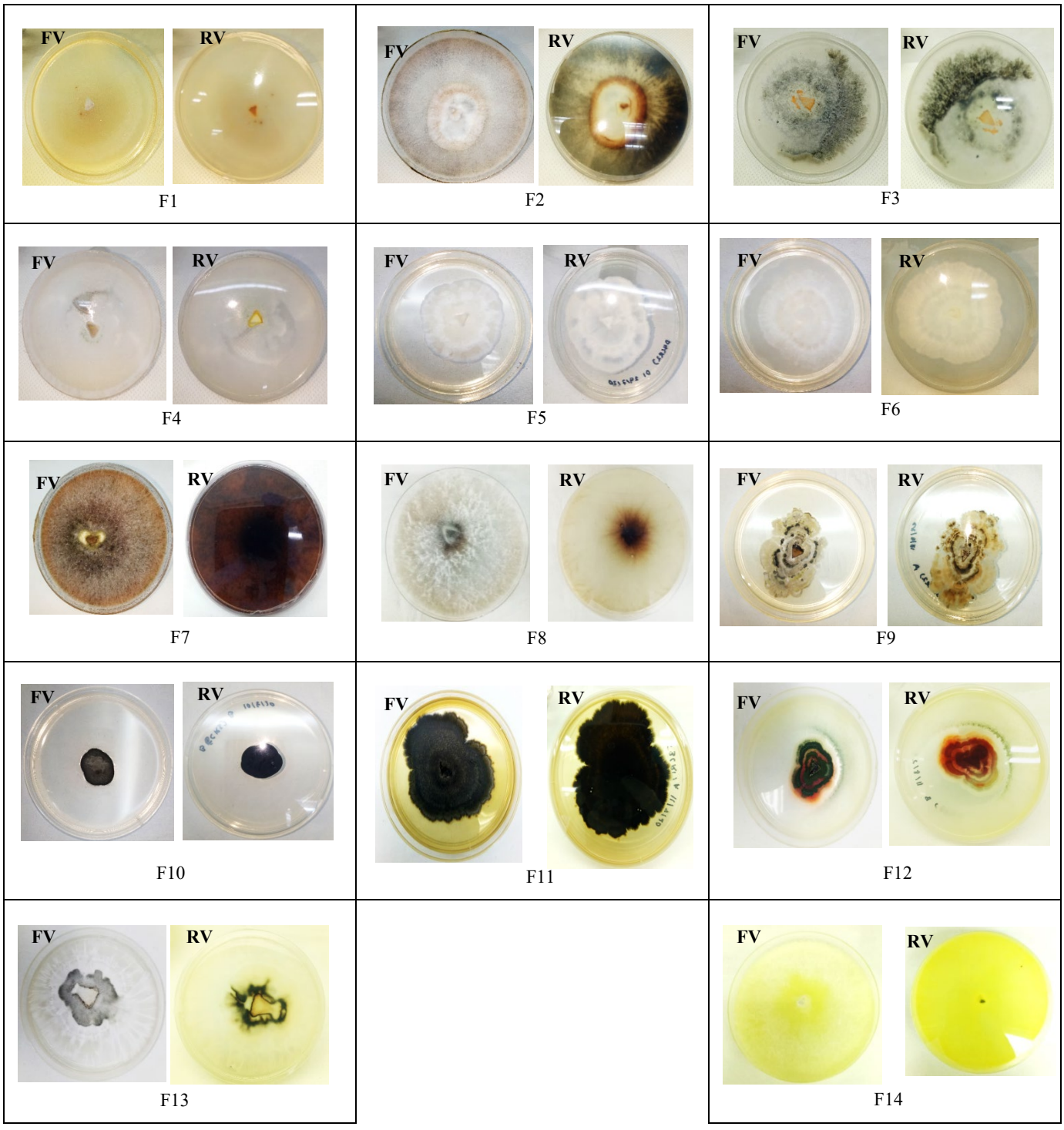

Figure 2. Macromorphological on the basis of colony colour on PDA (FV) and reverse colour (RV) of each isolate

Based on the 14 groupings, each group of endophytic fungal isolates was chosen for molecular identification, of which only 11 were successfully amplified using primer ITS1 and ITS4. BLAST searches revealed their identities as members of 7 different genera (Table 2), with all isolates belonging to phylum Ascomycota. Two genera, *Aspergillus novofumigatus* IBT 16806 and *Bipolaris sorokiniana* ND90Pr found in this study have been reported as plant pathogenic [25]. The dominating genus in this study was *Trichoderma reseei.* Other identified genera were *Aspergillus awamori, Aulographum hederae, Hypoxylon sp, Macroventuria anomochaeta,* and *Trichoderma aureviride.* These endophytes were found in different hosts as reported in several previous studies [26, 27, 28]. Species of Trichoderma has been acknowledged as an environmentally friendly and efficient BCA for various plants as they were able to produce various types of defensive secondary metabolites [29]. According to [27], *A. awamori* is an endophyte that has the capability to produce IAA, enhancing the growth of plant host. Meanwhile, volatile organic compounds (VOCs) of *Hypoxylon sp.* could inhibit the development of certain phytopathogen as reported by [30].

Table 2. Closest relatives of *Capsicum annuum* L. endophytic fungal isolates based on BLAST search analyses

Phylum	Isolate no.	Sequence based identification	Similarity %	Accession number
Ascomycota	F1	*Aspergillus awamori*	100	WP_2062078661/ GCB24478.1
	F2	*Hypoxylon sp.* EC38	100	OTA50905.1 / EGR48663.1
	F3	*Trichoderma reesei* QM6a	97.92	EGR48663.1
	F4	*Trichoderma reesei* QM6a	98.11	EGR48663.1
	F5	*Hypoxylon sp*. EC38	100	OTA50905.1
	F6	No similarity found	-	-
	F7	*Aspergillus novofumigatus* IBT 16806	97.56	PKX88236.1
	F8	No similarity found	-	-
	F9	*Aspergillus novofumigatus* IBT 16806	97.56	PKX88236.1
	F10	*Aulographum hederae* CBS 113979	100	KAF1980620.1
	F11	*Bipolaris sorokiniana* ND90Pr	100	EMD58209.1
	F12	*Patellaria atrata* CBS 10160	97.78	KAF2834113.1
	F13	*Trichoderma reesei* QM6a	100	EGR48663.1
	F14	No similarity found	-	-

Dual plate inhibition assays were carried out to screen the potential antagonistic activity of endophytic fungi against anthracnose fungal pathogen, *Colletotrichum gloeosporoioides* on PDA plates (Figure 2). *Colletotrichum sp*. is a major cause of anthracnose disease, mainly in chili cultivation in Malaysia. Anthracnose causes yield losses of up to 50% [30] and the typical anthracnose symptoms on chilli fruit include sunken necrotic tissues, with concentric rings of acervuli. This assay was carried out for two weeks to ensure the consistency of antagonism capability of each endophytic fungus. Antagonism is generally explained as any activity of an organism, which in some way adversely affects another growth associated with it [32]. Fungal antagonists are divided into three types including mycoparasitism, competition, and the production of extracellular metabolites (antibiosis) [33]. The present study showed that the isolated endophytic fungi express competition antagonism against these phytopathogens.

The antagonistic activity of each isolates was significantly different and ranging from 0 % to 87.21%. Dual culture test revealed that among 14 strains tested, only 4 isolates, namely F2, F4, F12 and F14 demonstrated the highest percentage of inhibition (87.21%, 76.26%, 51% and 65%, respectively). In contrast, the lowest were F6 and F7 isolates with similar percentage of inhibition, 9.59% as shown in Table 3. F2, F4, F12 and F14 isolates were classified as strong antagonists and might have the potential to be used in biocontrol management to combat anthracnose disease caused by the pathogen. Similarly, [34] and [35] reported in their studies that endophyte isolates were grouped as a broad antagonist with a percentage of radial growth inhibition (PIRG) above 50 percent against the pathogens.

Table 3. Inhibition radial growth (%) of *Colletotrichum sp.* by endophytic fungi.

Endophytic fungus	Day 7 (%)	Day 14 (%)
F1	$22.76^{a} \pm 1.41$	$36.53^{a} \pm 3.95$
F2	$77.24^{b} \pm 1.41$	$87.21^{b} \pm 0.79$
F3	$39.27^{c} \pm 2.09$	$47.15^{c} \pm 3.73$
F4	$57.73^{d} \pm 2.82$	$76.26^{d} \pm 1.58$
F5	$8.95^{e} \pm 3.73$	$21.46^{e} \pm 11.81$
F6	$7.32^{f} \pm 2.44$	$9.59^{f} \pm 0.00$
F7	$0.83^{g} \pm 1.44$	$9.59^{f} \pm 0.00$
F8	$9.59^{h} \pm 0.00$	$27.50^{h} \pm 2.50$
F9	$9.59^{h} \pm 0.00$	$25.83^{i} \pm 1.44$
F10	$6.39^{i} \pm 2.09$	$19.17^{j} \pm 2.89$
F11	$9.59^{h} \pm 0.00$	$25.83^{k} \pm 1.44$
F12	$6.39^{i} \pm 2.09$	$51.67^{l} \pm 1.44$
F13	$9.59^{h} \pm 0.00$	$20.83^{m} \pm 3.82$
F14	$9.59^{h} \pm 0.00$	$65.00^{n} \pm 2.50$

**Mean of three replications. Means in a column followed by different superscript letters are significantly different according to Tukey's test*

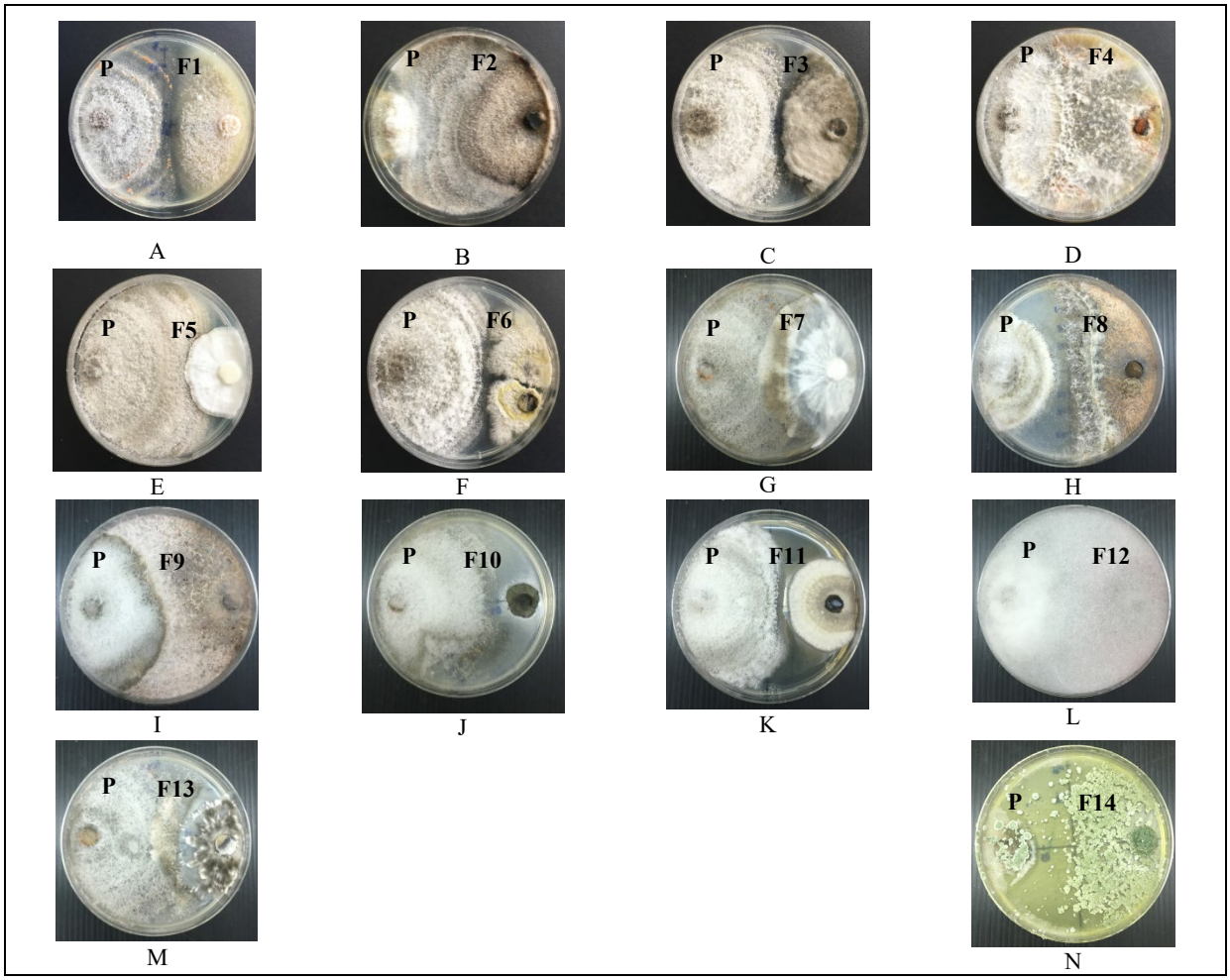

Figure 3. Varying degrees of pathogen (*Colletotrichum sp.*) growth inhibition by fungal endophyte isolates in dual culture plate assay.

CONCLUSION

A total of 85 fungal endophytes demonstrated different degrees of antagonistic properties against anthracnose pathogen, *C. gloeosporoioides* where the *Hypoxylon sp*. (F2) and *T. reesei* (F4) isolates could be the potential biocontrol agent for *C. gloeosporioides* as it has the highest PIRG (87.21%) and (76.26%), respectively. The finding in this study indicated the initial phase in the use of isolates for inhibitory application in agriculture.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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