

Antagonistic Activity of Fungal Endophytes Isolated from *Garcinia atroviridis* against *Colletotrichum gloeosporioides*

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ABSTRACT

The extensive use of synthetic fungicides in controlling plant disease generates detrimental impacts on the environment and human health. In response to this problem, an alternative method was developed, known as biological control using antagonistic microorganisms. Since investigation on fungal endophytes of *Garcinia atroviridis* is still unclear, it was chosen for the study. The aim of the present work was to evaluate biocontrol potential of endophytic fungi against *Colletotrichum gloeosporioides*, a phytopathogen that caused anthracnose disease. A total of 92 endophytic fungi were isolated from different tissue parts of *Garcinia atroviridis* including leaves, petioles, branches, and fruits. Results demonstrated that, most of endophytic fungal isolates showed some inhibitory action over the growth of *C. gloeosporioides* during dual culture growth. Endophyte isolate F14 showed the highest antagonistic activity against *Colletotrichum gloeosporioides* with 67.38% percentage inhibition radial growth (PIRG). However, 7 out of 92 isolates showed no inhibitory effect against *Colletotrichum gloeosporioides*. In conclusion, endophytic fungi isolated from *G. atroviridis* indicate the potential as biocontrol agents. It is hoped that the finding of isolated endophytic fungi in this study with antagonistic activity against anthracnose pathogen may be used in biocontrol programmes of plant disease in the region.

1. Introduction

Garcinia atroviridis is locally called as Asam gelugur. Belongs to the family of Clusiaceae, it can be found in Malaysia, Myanmar, India and is widely cultivated in Sumatera Island as one of export commodity purposes (MyBIS, n.d; Bayu *et al.* 2018). Other than being used in cooking as flavouring agent to replace tamarind, *G. atroviridis* is also being used traditionally in many ways to promote human health (Sultana *et al.* 2014). The plant parts has been used by the old folks as an abdominal reliever during pregnancy, cough and throat irritation remedy, to combat dandruff problem and can enhance better blood circulation in body (Taher *et al.* 2017). Based on Sultana *et al.* (2014); Taher *et al.* (2017); Bayu *et al.* (2018), the fruit of this plant contains many organic compounds but the most interesting is the

hydroxycitric acid. This acid has been commercialized under weight management product. Besides that, the extracts from different plant parts have antibacterial, antifungal, antioxidants, and antitumor potential. However, investigations based on endophytic microorganism associated with *G. atroviridis* is still limited and needs to be explored (Sim *et al.* 2010).

Anthracnose is one of the most problematic and economically harmful plant diseases occur on a variety of crops. This disease infected most of the plant parts such as fruits, leaves, stems, and flowers (Kimaru *et al.* 2018). Anthracnose is favoured by humid, wet, warm condition and transmitted by rain splash, moist wind and infected seeds (Sharma and Kulshrestha 2015). There are several causal agents of this disease that have been documented, but the most commonly known is species of the genus *Colletotrichum* including *C. gloeosporioides*, *C. acutatum*, *C. capsici*, and *G. musae* (Than *et al.* 2008; Abd-Elsalam *et al.* 2010; Sarkar 2016; Waghunde *et*

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al. 2017). These species causes considerably damage to large number of crops and tropical fruits such as coffee, chilli, strawberry, mango, banana, and avocado (Freitas *et al.* 2013; Oo and Oh 2016; Uddin *et al.* 2018). It is characterised by the development of dark sunken necrotic lesions on affected plant part (Crump 2009). The critical phases for disease control are during flowering and fruit set, and after harvest which requires both pre and post treatments.

Commercial synthetic fungicides such as propineb, difenoconazole, chlorothalonil, propiconazole, and mancozeb are used to overcome anthracnose caused by *Colletotrichum* sp. on various fruits and vegetables (Crump 2009). These chemicals are undoubtedly effective in controlling the disease, however, many of them have been banned in some countries due to their debits such as causing acute poisoning, birth defects, and endocrine disruption (PAN 2017). Hence, interest has been developed to create an alternative way that is safe and effective to be used in plant disease management. Replacing fungicides with biological control agents (BCAs) has been acknowledge for years as it could offer better results and cause no effect towards environment (Talapatra *et al.* 2017). It is primarily relies on the use of antagonistic organism that universally or specifically target the pathogen.

Endophytes are microorganisms that colonize within the plant cells without causing any harm and eventually establish mutualistic relationship with its plant host (Padhi *et al.* 2013). The host supplying nutrients and protection to endophytes while in return, endophytes producing novel bioactive metabolites which help in improving the host's ability to tolerate with biotic and abiotic stresses, promoting the plant growth and enhancing the resistance of its host to various types of pathogens (Tan and Zou 2001; Patra *et al.* 2016). Studies done by the researches demonstrated that endophytic microorganisms could be recognized successful biopesticides in controlling plant damage caused by the phytopathogens (Phongpaichit *et al.* 2006; Bivi *et al.* 2010; Landum *et al.* 2016; Marcellano *et al.* 2017). To date, all plants that have been studied from various habitats, harbour one or more endophytic microorganisms (Jia *et al.* 2016). Therefore, the focus of this study was to test antagonistic activity of endophytic fungi isolated from *Garcinia*

atroviridis against *Colletotrichum gloeosporioides* (*C. gloeosporioides*).

2. Materials and Methods

2.1. Samples Collection

Sampling activity was carried out by following method by (Martin and Dombrowski 2015). Different parts (leaf, petiole, branch, fruit) of healthy host plants, *Garcinia atroviridis* were collected from Nasuha Herbal Farm, Muar, Johor, Malaysia. Samples were stored in an aseptic zipper bags separately during collection.

2.2. Isolation of Fungal Endophyte

All the plant tissues were thoroughly washed with running water and cut into 0.5 cm pieces. The method described by Zheng *et al.* (2017) were slightly modified and used for surface sterilization. The samples were surface sterilized by following series of immersion sequential steps: 70% Ethanol for 2 minutes, 2% Sodium Hypochlorite for 3 minutes, sterile distilled water for three times and allowed to dry on sterilized filter paper. After drying, the small pieces (3-5) of each plant parts were placed on Potato Dextrose Agar (PDA) supplemented by chloramphenicol. The plates were invertedly incubated for 2 weeks at 28°C for fungal development. Each colony that appeared from the edge of tissues was continuously sub-cultured in order to obtain pure culture. Colonization rate was calculated by dividing the total number of plant segments infected by fungal endophyte over total number of segments incubated. Isolation rate was determined as the number isolates obtained from plant segments divided by the total number of segments incubated (Sun *et al.* 2008; Pal *et al.* 2012; Wu *et al.* 2019).

2.3. Preparation of Pathogenic Fungus

Antagonistic activity of all endophytic fungi was evaluated against *Colletotrichum gloeosporioides* (FRIM 1319), which was obtained from Laboratory of Mycology and Pathology, Forest Research Institute Malaysia, Kepong. The fungal pathogen was sub-cultured on PDA plate and incubated at 28°C. The mycelial plugs of fungal pathogen were stored in 30% glycerol at -80°C for long term preservation.

2.4. *In vitro* Evaluation of Endophyte Fungi against *Colletotrichum gloeosporioides*

The antagonistic potential of endophytic fungal isolates was assessed through direct confrontation method in accordance to Katoch and Pull (2017). The experiment was performed in triplicate and the mean values were recorded. A 5 mm agar plug of 7 day old endophyte fungus and pathogen were co-cultured at opposite site in PDA plate. The pathogen alone (without endophyte) was served as control. All the plates were invertedly incubated at 28°C for two weeks. The radial growth of pathogen cultured with/without endophyte fungal was measured daily. Then, the data was transformed into percentage inhibition of radial growth (PIRG) using the formula:

$$\text{PIRG (\%)} = \frac{\text{CDC} - \text{CDT}}{\text{CDC}} \times 100$$

Where,

CDC :radial growth of pathogen colony in the absence of endophyte (measured in cm)
CDT :radial growth of pathogen colony in the presence of endophyte (measured in cm)

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

3. Results

3.1. Isolation of Fungal Endophytes

A total of ninety two endophytic fungi were successfully isolated from different plant parts (branches, fruits, leaves, and petioles) of *Garcinia atroviridis*. Figure 1 shows the colonization frequency of endophytic fungi in branch (96.3%) was higher

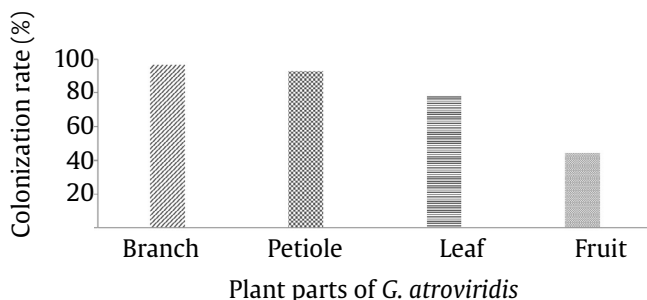


Figure 1. Colonization rate (%) of endophytic fungi isolated from *G. atroviridis*

as compared to petiole (92.6%), leaf (77.85%), and fruit (44.4%). Similarly, endophytic fungi in branch recorded highest isolation rate (1.14), followed by petiole (1.11), leaf (0.67), and fruit (0.48) (Figure 2).

3.2. Screening of Endophytic Fungi against *Colletotrichum gloeosporioides* in Dual Culture Assay

The potential antagonistic activities of all endophytic fungi against *C. gloeosporioides* pathogen were investigated in dual culture assay. In dual culture plate, the percentage of inhibition radial growth (PIRG) of endophytes towards *C. gloeosporioides* was observed on 7th days and 14th day of incubation. All the endophyte isolates showed different degrees of inhibition toward the mycelial growth of pathogen, ranging from 0.00% and 67.38% (Figure 3). Generally, all endophyte isolates showed inhibitory activity on the pathogen except for the seven isolates which were F7, F50, F61, F68, F76, F78, and F85. From all the tested endophyte isolates, only 14 endophytic fungi showed >50% of PIRG and the others were categorised <50% of PIRG (Table 1). The highest PIRG value was observed in F14 (67.38%) and F65 isolates was recorded as the lowest PIRG value (5.08%).

4. Discussion

Medicinal plants have been targeted for their endophyte microorganisms as the endophytes have capability of producing similar secondary metabolites as their plant host (Zhao *et al.* 2010; Nazir and Rahman 2018). These fungal endophytes are now great used by practitioners in biological control practices against plant pathogens and pests. They have been investigated as potential biocontrol agents and proved to be successful antagonists towards

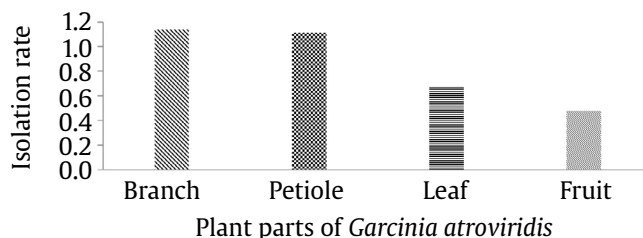


Figure 2. Isolation rate of endophytic fungi isolated from *G. atroviridis*

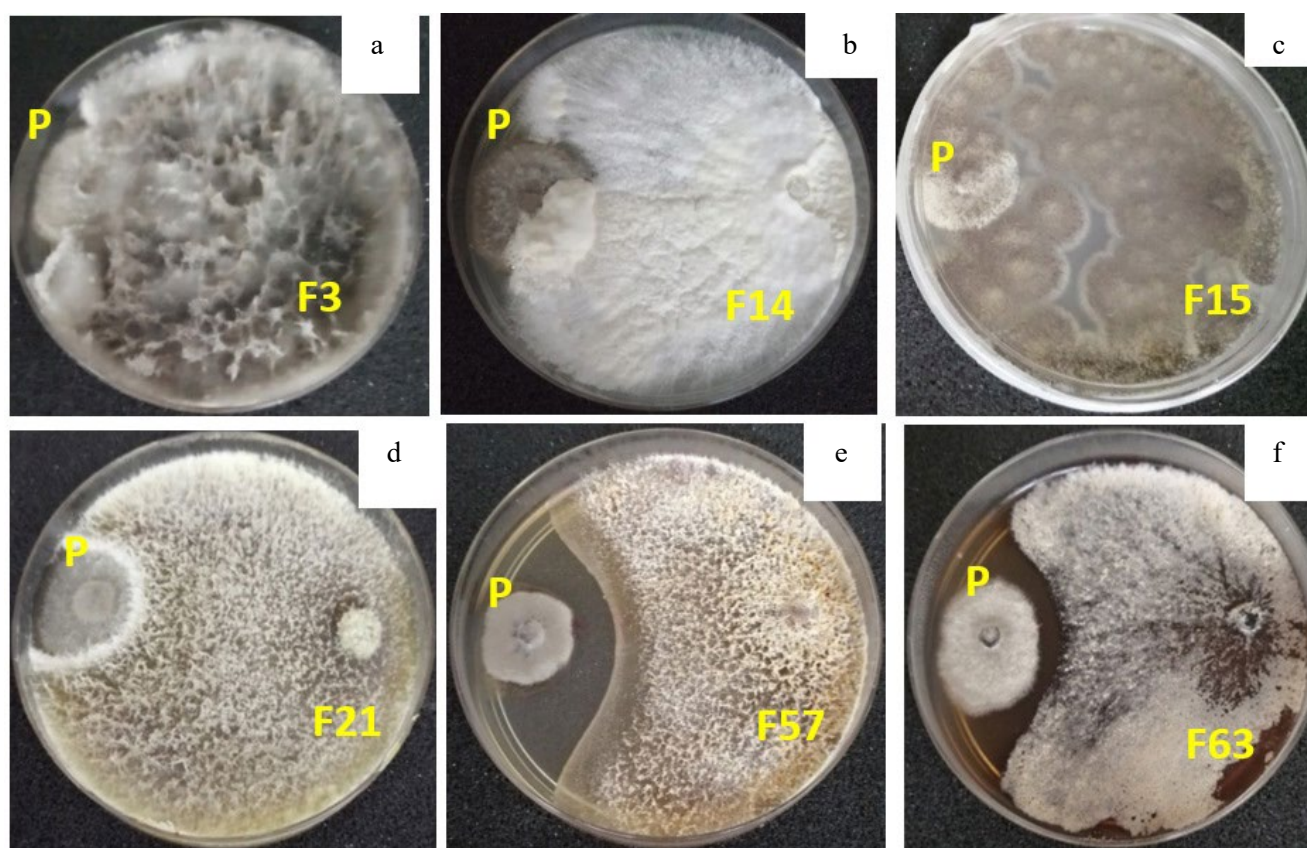


Figure 3. Antagonistic effect of endophytic fungi on *C. gloeosporioides* (P) after 14 days incubation in dual culture plate. (a) F3, (b) F14, (c) F15, (d) F21, (e) F57, and (f) F63

Table 1. Mean PIRG (%) of antagonistic potential of endophytic fungi against *C. gloeosporioides* on dual culture test

Endophytic fungi	Day 7 (%)	Day 14 (%)
F3	30.95 ^a ±3.37	58.36 ^b ±0.66
F4	30.95 ^a ±3.37	53.95 ^b ±5.58
F13	38.69 ^a ±4.21	51.32 ^b ±1.86
F14	47.62 ^a ±4.12	67.39 ^b ±4.22
F15	52.78 ^a ±3.37	64.39 ^b ±1.67
F19	38.10 ^a ±6.73	56.47 ^b ±4.99
F20	36.91 ^a ±4.30	53.95 ^b ±5.58
F21	44.44 ^a ±4.81	62.33 ^b ±3.17
F47	20.63 ^a ±5.62	51.86 ^b ±1.62
F53	28.57 ^a ±6.19	51.56 ^b ±4.08
F57	42.26 ^a ±0.84	59.41 ^b ±0.83
F63	21.03 ^a ±4.18	51.25 ^b ±9.48
F70	41.67 ^a ±6.87	61.45 ^b ±5.03
F95	50.00 ^a ±0.84	64.71 ^b ±2.41

Values of PIRG (%) are means from 3 replications. Means followed by the same letter in each row are not significantly different at $p < 0.05$ according to Tukey's test

pathogens and phytophagous insects through many studies.

In this study, about 92 endophytic fungi were cultivated from *G. atroviridis*. The colonization rate was calculated on the purpose of comparing the degrees of infected by fungal endophytes between different plant parts while the isolation rate could demonstrated the fungal richness in a given sample of plant parts (Sun *et al.* 2008). Based on the results, branches demonstrated the highest isolation and colonization of endophyte isolates followed by petioles, leaves and fruits. The result was align with the study made by Sun *et al.* (2008) which reported that the colonization of endophytic fungi in branches is higher than the leaves. The same results were pointed out by Phongpaichit *et al.* (2006). In contrast to these studies, Yu *et al.* (2018) reported that endophytic fungi isolated from *Camellia oleifera* was highest in leaves compared to other parts of

the plant. It can be said that the potential of fungal endophytes to colonize inter- or intracellular of plant is depends on their capability to utilize different substrates synthesize in different parts of the plant host (Sun *et al.* 2008; Pandey *et al.* 2014). This specific distribution has been looked as a strategy in order to reduce intense rivalry between the endosymbionts and preventing the plant host from an excessive population of endophytic microorganisms (Gimenez *et al.* 2007).

Dual culture assay is the basic method in order to assess antagonistic the pathogen tested (Rahman *et al.* 2010). This technique has been used in the studies done by (Devi and Singh 2015; Naidu *et al.* 2016; Yasmin *et al.* 2017). Results of direct inhibition test showed that 85 out of 92 endophytic fungi isolated from *Garcinia atroviridis* have the ability to inhibit the growth of *C. gloeosporioides*. In this study, fourteen endophytic fungi (Figure 1) were classified as strong antagonist that might have potential to be used in biocontrol management plan to combat anthracnose disease caused by the pathogen. Similarly, Ting *et al.* (2009) and Naidu *et al.* (2016) reported in their studies that endophyte isolates with percent of inhibition radial growth (PIRG) above 50% against the pathogens were grouped as great antagonist. Although at first both endophytes and the pathogen were co-cultured in opposite direction, most of the endophyte mycelium grows towards the pathogen as the incubation period started and showed different patterns of interaction after two weeks as reported by Begum *et al.* (2008) and Naidu *et al.* (2016). Endophytic fungi have several mechanisms in order to reduce or suppress the growth of fungal pathogens such as competing nutrient or space, mycoparasitism and producing extracellular metabolites (Scott 2016; Hamzah *et al.* 2018). The hyperparasitism action of F14 isolate can be seen on the fifth day as its mycelium begun to in contact with the mycelial of the pathogen. The endophyte then started to overgrow the pathogen on day 8 and giving the highest PIRG (67.39%) on day 10. The same mechanism reported by Lahlali and Hijri (2010) as the *R. solani* colony was entirely invaded by fungal endophyte of potato plant, *T. atroviride*. On the other hand, confrontational activity of *Xylaria* sp. and *Phoma* sp. on *F. solani* were demonstrated by secreting secondary metabolite into the agar which have adverse effect on the pathogen's growth (Hamzah *et al.* 2018).

5. Conclusion

Overall, there were ninety-two endophytic fungi were successfully isolated from the branches, petioles, leaves and fruits of *Garcinia atroviridis*. A total of 85 fungal endophytes demonstrated different degrees of antagonistic properties against anthracnose pathogen, *Colletotrichum gloeosporioides*, where the F14 isolates could be the potential biocontrol agent for *C. gloeosporioides* as it has the highest PIRG (67.39%). Finding in this study suggested the initial step in utilizing the isolates for inhibitory application in agriculture and forestry in the region.

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