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Antagonistic Endophytic Fungi from Papaya Fruit Against Anthracnose Causing Pathogens (*Colletotrichum gloeosporioides*) on Papaya Fruit

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Received May 26, 2023; revised July 09, 2023; accepted October 27, 2023

ABSTRACT

Anthracnose disease caused by *Colletotrichum gloeosporioides* considered a major disease on papaya fruit. One way to control plant diseases is to use antagonistic fungi as biocontrol agents. Several antagonistic fungi can be found in plant tissues (endophytic fungi). This study aims to get endophytic fungi from papaya fruit antagonistic to the fungus *C. gloeosporioides*. The research used a Completely Randomized Design (CRD) consisting of four treatments and five replications on *in-vitro* and *in-vivo* tests. The results showed that three isolates of endophytic fungi found from papaya fruit were *Fusarium* sp., *Aureobasidium* sp., and *Acremonium* sp., which had an inhibition of 63.5%, 67.86%, and 7.52%, respectively. *Fusarium* sp. and *Aureobasidium* sp. are potentially considered antagonist fungi in controlling the fungus *C. gloeosporioides* in *in-vitro* testing based on the inhibition results were more than 60%. *Aureobasidium* sp. is considered potential antagonist fungi according to the colonization or growth of *C. gloeosporioides* mycelium inhibition that emerges on papaya up to 97%.

Keywords: *Acremonium sp*, biocontrol agent, *Aureobasidium sp., Fusarium sp.*

Jamur Endofit Antagonis pada Buah Pepaya terhadap Patogen Penyebab Antraknosa (Colletotrichum gloeosporioides) pada Buah Pepaya

ABSTRAK

Penyakit antraknosa yang disebabkan oleh jamur *Colletotrichum gloeosporioides* merupakan penyakit utama pada buah pepaya. Salah satu cara pengendalian penyakit tanaman adalah dengan menggunakan jamur antagonis sebagai agen biokontrol. Beberapa jamur antagonis dapat ditemukan di dalam jaringan tanaman (jamur endofit). Penelitian ini bertujuan untuk mendapatkan jamur endofit dari buah pepaya yang bersifat antagonistik terhadap jamur *C. gloeosporioides*. Penelitian ini menggunakan Rancangan Acak Lengkap, yang terdiri atas empat perlakuan dan lima ulangan pada pengujian secara *in-vitro* dan *in-vivo*. Hasil dari penelitian menunjukkan adanya tiga isolat jamur endofit dari buah pepaya, yaitu *Fusarium* sp. *Aureobasidium* sp., dan *Acremonium* sp. dengan daya hambat berturut-turut 63,5%, 67,86%, dan 7,52%. *Fusarium* sp. dan *Aureobasidium* sp. merupakan jamur antagonis yang potensial dalam mengendalikan jamur *C. gloeosporioides* pada pengujian secara *in-vitro* berdasarkan hasil daya hambat yang lebih besar dari 60%. *Aureobasidium* sp. merupakan isolat jamur antagonis potensial berdasarkan kemampuannya dalam menghambat kolonisasi atau pertumbuhan miselium *C. gloeosporioides* pada buah pepaya dengan penghambatan mencapai 97%.

Kata Kunci: *Acremonium sp*, agen biokontrol, *Aureobasidium sp., Fusarium sp.*

INTRODUCTION

Indonesia is one of the main producers of the tropical fruit papaya (Vieira *et al.*, 2022). Indonesia harvests papaya up to 2 tons per hectare monthly (Demak Regency Agriculture and Food Service, 2021). On the other hand, papaya fruit production in Indonesia decreased by 3.23% in 2017 (Indonesia Central Bureau of Statistics, 2021). The number of papaya fruit decreased because it has a short shelf life and is attacked by anthracnose disease (Wiyono and Manuwoto, 2008). Pathogenic fungi from the genus *Colletotrichum* cause anthracnose disease. In general, the Colletotrichum fungus has six main species *Colletotrichum gloeosporioides, C. capsici, C. dematium, C. coccodes, C. acutatum,* and *Glomerella cingulate* (Kim *et al*., 1999).

Colletotrichum gloeosprorioides is a pre and post-harvest disease which causes 40-100% economic losses in developing countries (Ademe et al., 2013). The symptoms can be seen from sunken areas and

water-soaked spots, rapidly expanding, and the infected tissue becomes soft and necrotic (Ayón-Reyna *et al*., 2017). Therefore, controlling *C. gloeosprorioides* on papaya fruit is necessary, considering the vital effects and losses. Despite using fungicides that affect health and the environment, biological control can be a good option.

Disease management control using biological agents is the most carried out on post-harvest products. Endophytic fungi are microorganisms that live in plant tissues such as leaves, fruits, twigs, and plant roots, in more than one isolate, different endophytic fungi can be produced from one host plant (Istifadah & Suganda, 2010). Siddiqui & Shaukat (2003) stated that endophytic microorganisms have many benefits as biological agents because some are easily cultured *in vitro*, can increase plant resistance to parasites, do not attack or produce toxins in plants, and produce growth-stimulating hormones.

Some endophytic fungi can be used as biological agents against the cause of anthracnose disease in some fruits and vegetables. According to Siregar *et al.* (2007), *Bacillus polymyxa bacteria* and *Trichoderma harzianum* as and endofit fungi controlled the pathogen that causes anthracnose in chilli plants. The statement is reinforced by the statement of Zivkovic *et al*. (2010) that *T. harzianum* and *Gliocladium roseum* are endophytic microbes that can be used as biological agents against *C. acutatum* and *C. gloeosporioides* that cause anthracnose in fruits.

Study evidence on endophytic fungi *Trichoderma* sp. successfully suppresses *C. gloesoprorioides* fungi in chilli peppers was shown by Herwidyanti *et al*. (2013). Another study using *Aspergillus* fungus on chilli fruit also slowed the incubation period of anthracnose disease caused by *C. gloeosporioides* (Amaike & Keller, 2011). There has been no study on endophytic fungi from within papaya fruit tissue, which triggered the author to research the antagonistic potential of endophytic fungi against pathogens that cause anthracnose (*C. gloesoprorioides*) in post-harvest papaya fruit in vitro and in-vivo.

MATERIALS AND METHODS

The study was conducted at the Laboratory of Biotechnology Plant Protection, Department of Pests and Plant Diseases, Faculty of Agriculture, Universitas Padjadjaran, Sumedang Regency, West Java, Indonesia. Research was conducted from April to June 2022**.**

Colletotrichum gloeosporioides preparation

Papaya peel was cut 2x2 cm between symptomatic and healthy parts. Furthermore, the cut results are sterilized by soaking in 2% Clorox for a minute and rinsed using sterile *aquades*. It was then soaked again using 70% alcohol for 30 seconds. After that, the fruit pieces are drained using sterile filter paper. After dryed, the papaya skin is cut back into 1x1 cm in size.

The papaya fruit pieces are placed in prepared Potato Dextrose Agar (PDA) media, added with 0.5% Chloramphenicol antibiotic, and incubated at room temperature for several days. The pure cultures of *C. gloeosporioides* isolates were identified macroscopically and microscopically based on morphological and pigmentation characteristics on PDA media. The morphological and pigmentation characterization of the resulting *C. gloeosporioides* (Figure 1) draws on literacy studies from Barnett and Hunter (1998), Semangun (2008), and Sudirga & Ketut (2016). The identification results then purified in new PDA media.

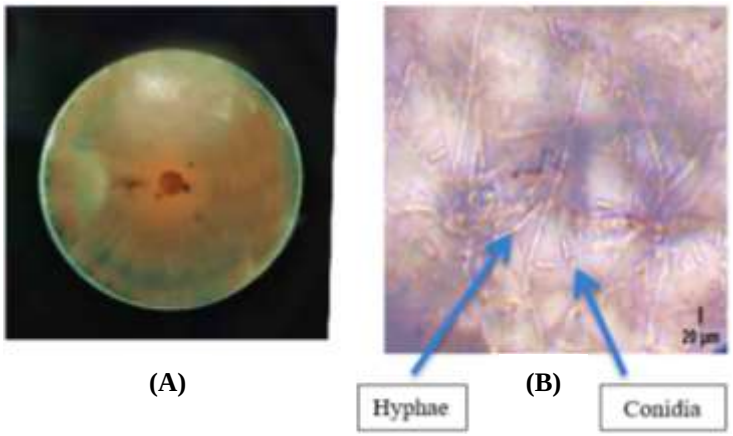


Figure 1. Colletotrichum gloeosporioides fungus. (A) Colonies on PDA media; (B) Conidia and hyphae with a size of 10.36 µm.

Isolation and identification of endophytic fungi from papaya fruit

Endophytic fungi are isolated by cutting the skin of a healthy papaya fruit with a size of 1x1x0.5 cm. The pieces are sterilized by soaking with 2% Clorox for a minute and rinsing using sterile *aquades*. After that, the pieces were dipped using 70% alcohol for 30 seconds and drained using filter paper. The dried pieces were then placed in the prepared PDA media, added

with 0.5% chloramphenicol antibiotics, and incubated at room temperature for several days.

The grown endophytic fungal isolates were then purified in the new PDA medium and identified, which referred to Barnett and Hunter (1998). The results of isolation, purification, and identification of endophytic fungi from papaya fruit obtained three isolates of endophytic fungi, namely *Fusarium* sp. (Figure 2), *Aureobasidium* sp. (Figure 3), and *Acremonium* sp. (Figure 4).

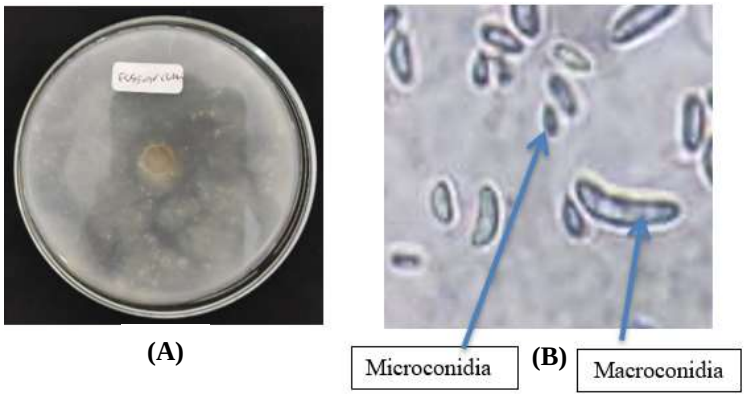


Figure 2. *Fusarium* sp. (A) Colonies on PDA media; (B) Conidia.

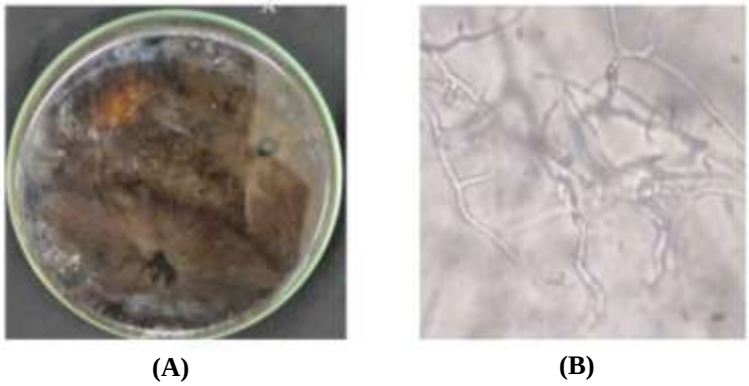


Figure 3. *Aureobasidium* sp. (A) Colony on PDA media; (B) Hyphae.

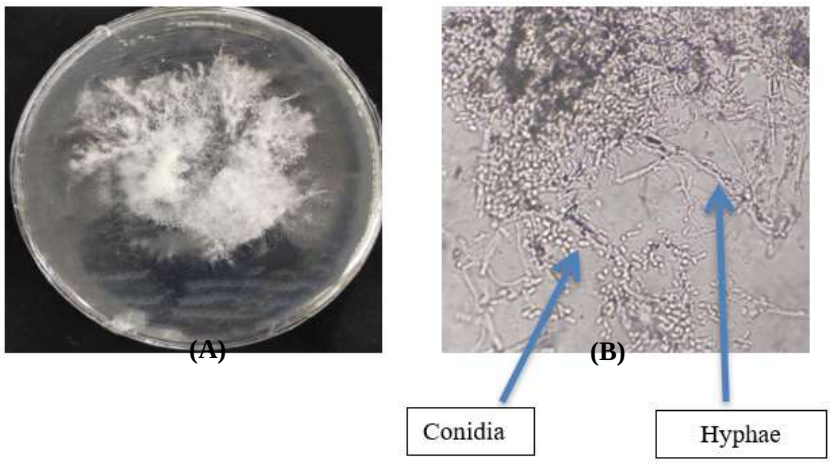


Figure 4. *Acremonium* sp. (A) Colony on PDA media; (B) Conidia.

***In-vitro* test**

Antagonistic fungi were tested in vitro using the dual culture method. The *C. gloeosporioides* and endophytic fungal isolate side by side with 3 cm on a 9 cm diameter petri dish PDA media (Figure 5). The isolates were then incubated for ten days at room temperature. The process aims to see the inhibition and magnitude of inhibition of endophytic fungal isolates against the growth of *C. gloeosporioides* on PDA media.

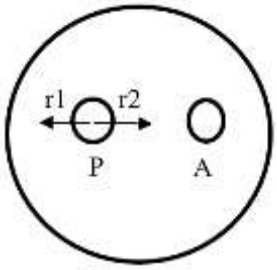


Figure 5. In-vitro antagonism test of endophytic fungi and *C. gloeosporioides* fungi.

Notes: (A) Isolate of *C. gloeosporioides* fungi; (P) Isolate of endophytic fungi; (r1) Radius of an isolate of pathogenic fungi that stay away from antagonistic fungi; (r2) Radius of isolates of pathogenic fungi that approach the antagonistic fungi.

The number of *in-vitro* antagonism test treatments was three treatments of endophytic fungi (*Fusarium* sp., *Aureobasidium* sp., and *Acremonium* sp.) + *C. gloeosporioides* and a control treatment, namely pathogenic fungal isolates without endophytic fungal isolates repeated five times. Furthermore, the calculation of inhibition of endophytic fungi against *C. gloeosporioides* fungi was carried out using the following equation:

$$I\ (\%) = \frac{r1-r2}{r1} \times 100\% \quad \ldots (1)$$

Notes:
I : The percentage of inhibition of antagonistic fungi to pathogens (%)
r1 : Radius isolates of pathogenic fungi that stay away from antagonistic fungi
r2 : Radius isolates of pathogenic fungi approaching antagonistic fungi

Ratnasari *et al.* (2014) stated that if the percentage of inhibitory power is less than 60%, then antagonistic fungi only have a minimal inhibitory effect on the growth of pathogenic fungi to attack. Nevertheless, antagonistic fungi are said to be able to inhibit the growth of pathogenic fungi to the maximum if the percentage of inhibition is more than 60%. Only endophytic fungi with more than 60% inhibition will be tested for antagonism *in-vivo*.

***In-vivo* test**

The papaya fruit used for the *in-vivo* antagonism test is a whole Calina variety papaya fruit aged one day after harvest. The fruit colour was 25% orange on the fruit's skin in healthy conditions with medium size with a fruit weight of 0.8-1 kg. This *in-vivo* test starts by sterilizing the surface of papaya fruit by spraying 70% alcohol and drying. The papaya fruit pierced 20 punctures on the surface with a depth of 2 cm (Figure 6). Then a suspension of endophytic fungi (spores/mL) was injected into the punctured wound of 5 µL each. After it incubated for a night, 5 µL suspension of *C. gloeosporioides* with 10^5 spore/mL density injected into each punctured wound, then stored at room temperature and observed.

Previous *in-vitro* test results showed that *Fusarium* sp. and *Aurediobasidium* sp. have an inhibition of more than 60%, so it is used for in-vivo tests. The *in-vivo* antagonism test consists of 4 treatments: *C. gloeosporioides* + *Fusarium* sp.; *C. gloeosporioides* + Aureobasidium *sp.*; positive control (*C. gloeosporioides* + fungicide Antracol-Propineb 70%); negative control treatment (pathogenic fungal isolate without endophytic fungal isolate or pesticides). The results of observing disease symptoms from *in-vivo* antagonism tests are then used to calculate disease intensity and inhibition of endophytic fungi. The formula calculates the intensity of the disease (%): (Number of affected punctures: number of all punctures) x 100%
The inhibition of endophytic fungi *in-vivo* was calculated based on the formula:

$$Pv\ (\%) = [(1-a) : b] \times 100\% \quad \ldots (2)$$

Notes:
Pv : Percentage of inhibition of antagonistic fungi *in-vivo* (%)
a : The number of damage to the treatment
b : The number of damage to the controls
*Endophytic fungi with an inhibitory power of more than 60% are declared capable of inhibiting *C. gloeosporioides* fungi.

Figure 6. *In-vivo* antagonism test of endophytic fungi and *C. gloeosporioides.*

Data analysis

The experiment used Complete Randomized Design (RAL) with five repetitions. The data obtained were then analyzed using the ANOVA test (variety analysis) in SPSS program version 25.0. The Duncan test at a level of 5% will be used if there is a discernible difference.

RESULT AND DISCUSSIONS

In-vitro* antagonism test of endophytic fungi and *Colletotrichum gloeosporioides

Endophytic fungi obtained from papaya fruit *Fusarium* sp. and *Aureobasidum* sp. were able to inhibit the growth of colonies of *C. gloeosporioides*, while *Acremonium* sp. did not significantly different to inhibit from controls. The difference in the results of the inhibition of endophytic fungi against the pathogen *C. gloeosporioides* was based on the antagonistic

ability of each different fungus. Artursson et al. (2006) showed that secondary metabolites produced by antagonistic fungi can cause different fungal responses to some pathogens.

Fusarium sp. could suppress the growth of *C. gloeosporioides in-vitro* by 63.5%. The antibiosis mechanism of antagonism of the fungus *Fusarium* sp. can be seen from the formation of an empty zone between pathogenic and antagonistic fungi; there was also thickening in the hyphae of *C. gloeosporioides* (Figure 7*)*. The antibiotic mechanism utilizes compounds produced by biocontrol agents to inhibit pathogenic hyphae that become abnormal/malformed.

Istifadah *et al.* (2006) found melanization and abnormalities in pathogenic hyphae due to secondary metabolites from endophytic fungi. *Fusarium* sp. produces toxic secondary metabolites called mycotoxins that diffuse from hyphae to their surroundings (Burgess *et al*., 2006). Endophytic fungi could produce antibiotic secondary metabolites (Radji, 2005). Nitao *et al*. (2001) also stated that *Fusarium equiseti* produces antagonistic toxins to fungal pathogens and parasitic nematodes in soybean plants. Moreover, *F. equiseti* can inhibit pathogenic fungi in the roots of barley plants (Maciá-Vicente et al., 2008).

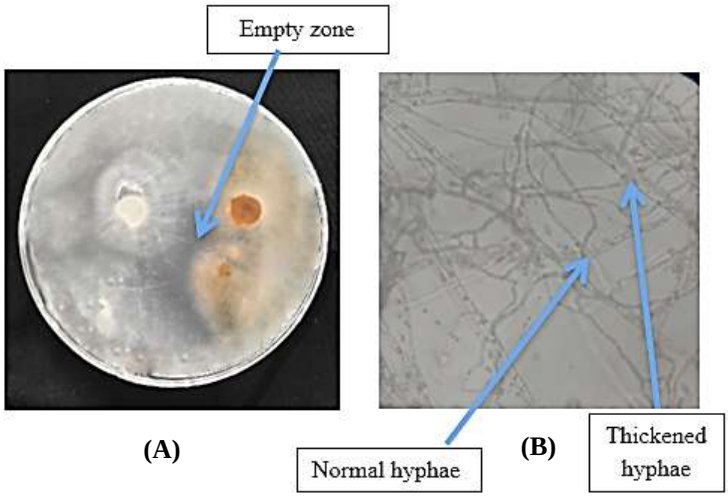
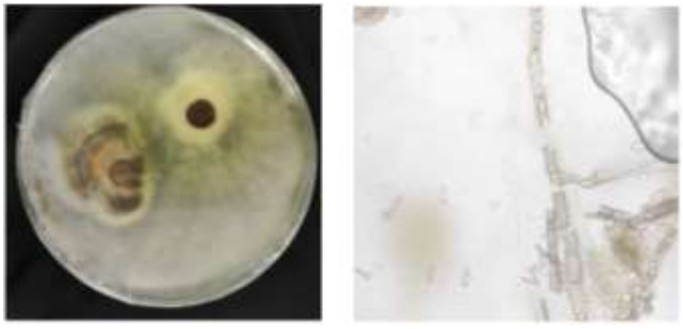


Figure 7. *Fusarium* sp. antagonism test. (A) Colonies on PDA media; (B) *C. gloeosporioides* thickened hyphae.

Based on Table 1, *Aureobasidium* sp. could suppress *C. gloeosporioides* colonies with the highest percentage (67.86%) of inhibition compared to other treatments. The antagonism mechanism of *Aureobasidium* sp. is considered a competition, characterized by the fungus covering colonies of *C. gloeosporioides*. In addition, *Aureobasidium sp.* grew faster and filled the 9 cm petri dish (Figure 8A), whereas *C. gloeosporioides* hyphae undergoes lysis (Figure 8B). *A. pullulans* is reported to be able to compete with other microbes for space and nutrients by excreting extracellular polysaccharides, enzymes, and other molecules (Bozoudi *et al*., 2018).

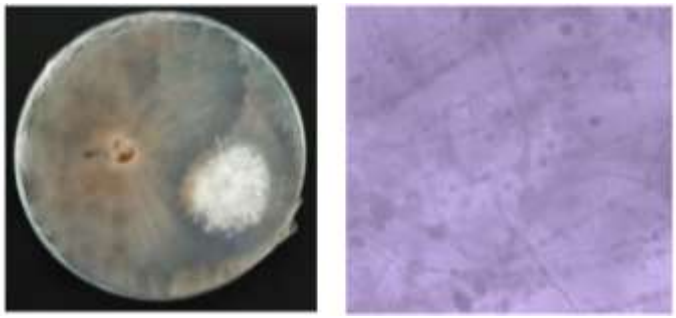


(A) **(B)**

Figure 8. Antagonism test of *Aureobasidium* sp. (A) Colonies on PDA media; (B) Lysis of *C. gloeosporioides* hyphae.

Acremonium sp. showed a low percentage of inhibition compared to other treatments at 7.52%, and the colony diameter of *C. gloeosporioides* almost covered the petri dish by 7.56 cm (Table 1). *Acremonium* sp. was also found to slightly inhibit the growth of *C. gloeosporioides* which can be seen from the empty zone between the fungi (Figure 9). The phenomenon happened because *Acremonium* sp. produces secondary metabolites as an antibiotic mechanism. However, these secondary metabolites do not inhibit the growth of *C. gloeosporioides.* The secondary metabolites found in some Acremonium types, Cephalosporins, belong to the antibiotic and anti-inflammatory class (Zhang *et al.,* 2009).

Grunewaldt-Stocker (2003) reports that *Acremonium* sp. can be an antifungal in tomato and hemp plants. *Acremonium* sp. could also reduce wilt symptoms due to *F. oxysporum* infection (Grunewaldt-Stocker, 2003). Wicklow et al. (2005) also showed that *Acremonium* sp. is effectively used as an antifungal of *A. flavus* and *F. verticillioides* that produce mycotoxins.



(A) **(B)**

Figure 9. Antagonism test of *Acremonium* sp. (A) Colonies on PDA media (B) *C. gloeosporioides* hyphae.

Table 1. *Colletotrichum gloeosporioides* colony diameter and *in-vitro* inhibition of endophytic fungi.

	Treatment	Diameter colony of *C. gloeosporioides* (cm)	Inhibition (%)
A	Control	8.76a	0.00
B	*Fusarium* sp. x *C. gloeosporioides*	5.90b	63.50
C	*Aureobasidum* sp. *x C. gloeosporioides*	6.00b	67.86
D	*Acremonium* sp. x *C.gloeosporioides*	7.56a	7.52

*Numbers followed by different letters in column 3 show significantly different data using the Duncan Test at a real level of 5%

In-vivo antagonism test of endophytic fungi and *C. gloeosporioides* fungi

Based on the *in-vivo* results of the antagonism test, it can be seen that the Antracol fungicide (control negative) treatment inhibited the growth of *C. gloeosporioides* by at most 100% with 0% disease intensity (Table 2; Figure 10 A). The result was in line with Sila and Sopialena (2016), which stated that Antracol fungicide has a strong disease-killing spectrum. The Antracol fungicide also slowly decomposed by the wind to more effectively suppress the development of brown spot disease on leaves and anthracnose in chilies (Sila & Sopialena, 2016).

The most effective endophytic fungal treatment in inhibiting *C. gloeosporioides* was the fungus *Aureobasidium* sp. with 97% inhibition and 3% disease intensity (Table 2; Figure 10B). The fungus *Aureobasidium* sp. is reported to control pathogenic fungi from several plants. Dimakopoulou et al. (2008) state that isolation of *Aureobasidium pullulans* was equally effective in controlling the causes of bunch rot disease in oil palm plants. Renouf et al. (2005) also report that *A. pullulans* can reduce the growth of *Botrytis cinerea* on the surface of fresh grapes.

Fusarium sp. fungal treatment inhibits 47% with a disease intensity of 53% (Table 2). *Fusarium* sp. fungus obtained from papaya fruit tissue is considered ineffective in controlling the pathogen *C. gloeosporioides* that causes anthracnose disease in papaya fruit *in-vivo* which can be seen from the severe effect of the symptoms produced in Figure 10C.

Figure 10. Positive control treatment results on papaya fruit (*C. gloeosporioides* with Antracol Fungicide) (A). *C. gloeosporioides* and *Aureobasidium* sp. treatment results on papaya fruit (B). *Treatment of C. gloeosporioides with Fusarium sp. on papaya fruit.* (C)

Table 2. Growth inhibition average of *Colletotrichum gloeosporioides* colonies on various treatments.

	Treatment	Disease Intensity (%)	Inhibition (%)
A	Negative control	100c	0
B	Positive control (Fungicide)	0a	100
C	*Fusarium* sp. x C. *gloeosporioides*	53b	47
D	*Aureobasidum* sp. x *C. gloeosporioides*	3a	97

*Numbers followed by different letters in column 3 show significantly different data using the Duncan Test at a real level of 5%

The effect of endophytic fungi on papaya fruit can be observed by the absence of growing colonies of *C. Gloeosporioides* in each hole treated. The occurrence happened because endophytic fungi could inhibit fungal growth through the antibiotic mechanisms, competition, and hyperparasitism. In line with the research of Pal and Paul (2013) that endophytic fungi can produce secondary metabolites that function as antimicrobial substances, antioxidants, cytotoxic compounds, growth hormones, and hydrolytic enzymes.

According to Burge (1988), fungi can produce various kinds of toxic compounds to fight other microorganisms. Most microorganisms produce and secrete one or more antibiotic compounds. In several studies, antibiotics produced by microorganisms are effective in suppressing pathogens that cause plant diseases (Pal & Gardener, 2006). Competition mechanisms can occur when two or more organisms have the same needs as space, nutrients, oxygen, and even light (Campbell, 1989). The mechanism of hyperparasitism occurs when antagonistic agents attack pathogens by coiling around pathogenic hyphae (Campbell, 1989).

CONCLUSIONS

Three isolates of endophytic fungi were obtained from the Calina papaya fruit tissue *Fusarium* sp., *Aureobasidium* sp., and *Acremonium* sp. The *Fusarium* sp. and *Aureobasidium* sp. are potential antagonistic fungal to control *C. gloeosporioides* pathogens, showing an average inhibition of more than 63%. *Aureobasidium* sp. is a potential antagonistic fungus to inhibit the colonization or growth of *C. gloeosporioides* mycelium on papaya fruit, with inhibition reaching 97%.

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