




Article

Selecting Antagonistic Yeast for Postharvest Biocontrol of *Colletotrichum gloeosporioides* in Papaya Fruit and Possible Mechanisms Involved

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Abstract: *Colletotrichum gloeosporioides* causes anthracnose disease in papaya fruit resulting in tremendous economic loss due to its latent infection. This study aimed to evaluate the biocontrol activity of antagonistic yeasts against *C. gloeosporioides* in papaya and determine the possible mechanism involved. One hundred and ten yeast strains were isolated from different parts of the papaya plant. Among them, only five strains, namely F001, F006, L003, FL013 and LP010, showed more than 55% radial growth inhibition of *C. gloeosporioides*. These five potent yeast strains were further evaluated in vitro and in vivo. The results indicated that strain F001 had the strongest biocontrol activity based on spore germination and fungal growth inhibition. In Vivo, the strain F001 caused 66.7% and 25% reductions in disease incidence and severity, respectively. Based on molecular identification, the strain F001 was confirmed as *Trichosporon asahii*. Despite there was no significant induction of defense enzyme activities found on the treated fruits, SEM observation showed direct attachment of *T. asahii* with the fungal hyphae and interfere in their establishment to the fruit surface. Based on these findings, the antagonistic yeast *T. asahii* strain F001 may be used as a potential natural biological control agent against anthracnose disease in papaya fruit.

Keywords: biocontrol; *Colletotrichum gloeosporioides*; anthracnose; papaya; *Trichosporon asahii*



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1. Introduction

Anthracnose in papaya fruit is a devastating postharvest disease caused by *Colletotrichum gloeosporioides* where losses can be up to 62%, with disease incidence ranging from 90–98% in Malaysia [1]. The fungus infection generally starts during the flowering stage and remains dormant until postharvest when it becomes favorable for colonization on the fruit tissue. Even though initial infection always occurs before harvest, symptoms typically become visible after harvest, as fungal development continues due to suitable storage conditions. Postharvest infections may occur due to inoculum availability in the processing environment, and situation becomes worse when the fruit is subjected to considerable amount of wounding after harvest [2]. Synthetic fungicides are commonly used to protect perishable fruit and vegetable from postharvest fungal diseases due to their efficacy and convenience [3,4]. However, prolonged use of registered chemicals fungicides result in decline in efficacy because of increasing pathogen-resistance [5,6].

Many efforts have been made to control postharvest diseases using safer alternatives [7]. The manipulation of naturally occurring antagonistic organisms in controlling

postharvest diseases such as anthracnose disease has attracted the interest of many researchers [8]. Yeasts are one of the antagonistic organisms most reported as postharvest biocontrol agents and widely developed into registered products [9]. According to previous reports, natural yeasts are potent as biological control agents due to their strong antagonistic activities against pathogen diseases [9,10]. Yeast possesses some unique features, including production of extracellular polysaccharides, which promote their viability and limit the pathogens' propagules growth [11–13]. Yeast only requires simple nutrition for propagation, has flexibility in colony establishment even on dried-surfaces, and high tolerance to chemical treatments [14,15]. Furthermore, yeasts do not produce any allergenic spores, mycotoxins and antibiotic metabolites like other antagonist fungi and bacteria [16]. Thus, they leave no harmful residue for consumers [17].

In previous studies, it was shown that the origin of the antagonist microbe may affect the efficacy of its biocontrol activities since it involves the agent's adaptation and stability under different environments [18]. Thus, native yeast isolated from specific host plants is the most preferred to obtain reliable antagonist yeast agents. To date, there has been no reports on successful biocontrol yeast agents against *C. gloeosporioides* that causes anthracnose disease in papaya fruit cv. Solo planted in the tropical region. Therefore, this study was conducted to: (a) isolate potential antagonist yeasts from papaya plants; (b) evaluate their in vitro and in vivo antagonist activities against *C. gloeosporioides* in papaya; (c) identify antagonist yeast by molecular identification; and (d) determine the possible yeast mechanisms of action, including induction of defense enzyme activities and attachment on *C. gloeosporioides*, in controlling anthracnose disease in papaya fruit.

2. Materials and Methods

2.1. Isolation of Antagonistic Yeast

Antagonistic yeasts were randomly isolated from the leaves, petioles, and fruit surfaces of papaya plants grown in Lanchang, Pahang (geographical coordinates: 3°24'0" N, 103°26'0" E, Malaysia). Tissue samples were cut into 6 mm in diameter using a sterile cork borer. Ten pieces of tissues from each sample were then soaked in test tubes containing 10 mL of sterile distilled water. The tubes were shaken on a shaker for 5 min and the mixtures serially diluted to reduce the cell concentration. Subsequently 0.1 mL of each dilution was plated on nutrient yeast dextrose agar (NYDA) medium using a sterile glass spreader. All the cultured plates were then incubated at 28 ± 2 °C for 48 h. The isolated yeasts were re-cultured on new NYDA media until pure cultures were obtained. Colonies of yeasts were chosen based on different morphological distinctions.

2.2. Screening of Antagonist Yeast

2.2.1. Dual Culture Assay

Preliminary in vitro screening was conducted for all of the isolated yeasts to evaluate their antagonistic capabilities against *C. gloeosporioides* according to the method described by Hasan [15], with minor modifications. The *C. gloeosporioides* used for the experiment was sourced from the Plant Pathology Laboratory, Universiti Putra Malaysia. For antagonistic capability assessment, the yeast isolates were cultured together with the pathogen on potato dextrose agar (PDA) medium. A 6 mm mycelial plug was taken from a seven-day-old *C. gloeosporioides* culture and placed at the center of the plate. Two-day-old yeast culture was then streaked on the same media at 1.5 cm from the plate edge. Fungus placed on a culture plate without the yeast served as the control. The plates were then incubated at room temperature (28 ± 2 °C) for seven days. Percent inhibition of radial growth (PIRG) was recorded based on the following formula [16]:

$$PIRG = \frac{R1 - R2}{R1} \times 100 \quad (1)$$

where, R1 = Radial growth of *C. gloeosporioides* in the control plate; and R2 = Radial growth of *C. gloeosporioides* cultivated with potential antagonistic yeast.

Only isolates with *PIRG* > 55% were selected to proceed with the next experiment. This study was done in triplicates for each treatment.

2.2.2. Agar Well Test

Potential yeast strains with *PIRG* more than 55% were selected for further testing using the agar well test method described by Chanchaichaovivat et al. [17] with some modifications. A 0.02 mL yeast cell suspension (5×10^6 cells mL⁻¹) was placed into a well at the middle of PDA mixed with 15% juice of papaya. The plate was kept for 1–2 h to enable diffusion of the yeast suspension. Subsequently, 0.02 mL spore suspension of *C. gloeosporioides* (5×10^4 spores mL⁻¹) was injected into the well. Sterile distilled water was added, instead of the yeast cell suspension, to the control plate. After incubation at 28 ± 2 °C for seven days, the fungal growth in each plate was observed and the diameters of the fungal growth measured. This experiment was done using five replicates for each treatment.

2.2.3. In Vivo Assay

Healthy papaya fruits cv. Solo, within the weight range of 0.3–0.45 kg and at color index two (green with a trace of yellow), were washed before being soaked in sodium hypochlorite 0.5% for 5 min, followed by soaking in sterile distilled water for 1 min. After being air dried, the fruits were surface-sterilized with 70% (*v/v*) ethanol. Two wounds were then inflicted on each fruit with a sterile cork borer and 0.02 mL antagonistic yeast suspension (5×10^6 cells mL⁻¹) applied to the wounds. After 2 h, 0.02 mL of *C. gloeosporioides* spore suspension (5×10^4 spores mL⁻¹) was placed on the wounds. For the control treatment, only *C. gloeosporioides* was inoculated onto the wounds. The fruits were stored at room temperature (28 ± 2 °C) in plastic containers covered with polyethylene plastic. Disease development was recorded after six days by measuring the diameter of the lesions formed. The average lesion expansion and percentage of disease reduction over the control was then calculated using the following formulae [18,19]:

$$\text{Lesion expansion average (cm day}^{-1}\text{)} = \frac{\text{Lesion diameter day 6 (cm)} - \text{Lesion diameter on day 0 (cm)}}{\sum \text{storage day (6 days)}} \quad (2)$$

$$\text{Disease reduction over control percentage (\%)} = \frac{\text{Lesion diameter of control fruit (cm)} - \text{Lesion diameter of treated fruit (cm)}}{\text{Lesion diameter of control}} \times 100 \quad (3)$$

This experiment was conducted using six replicates for each treatment.

2.2.4. Inhibition of *C. gloeosporioides* Spore Germination

The effects of the different yeast cell concentrations on pathogen spore germination was tested using the method described by Droby et al. [20], with minor modifications. Five milliliters of potato dextrose broth (PDB) was poured into sterile glass tubes. Then, 0.1 mL of the treatment and pathogen spore suspension (5×10^4 spores mL⁻¹) were added into each tube simultaneously. Treatments used in this study were 5×10^4 , 5×10^6 , and 5×10^8 cells mL⁻¹ for isolates F001 and FL013, while Benocide 50WP® (BEN, active ingredient: benomyl 50% WP, at the concentration of 0.33 g L⁻¹) and sterile distilled water (SDW) served as the positive and negative controls respectively. All the treated tubes were placed on a rotary shaker at $3 \times g$ at 28 °C for 20 h. After 20 h of incubation, 100 random pathogen spores were examined under a light microscope (Standard 25, Carl Zeiss, Oberkochen, Germany) and the germination rate calculated. This experiment was carried out using three replicates for each treatment.

2.2.5. Production of Diffusible Antagonist Substance

The diffusible antagonistic substance produced by the yeast was determined according to methods the described by Rahman et al. [21], with minor modifications. Sandwich plates

were prepared using two types of media. Ten milliliters of PDA was first placed into each petri dish as a bottom layer. A filter paper (Whatman filter paper no. 1) was then placed on the top of the PDA layer and 10 mL of molten NYDA poured on top of it. After the top layer had solidified, 0.1 mL of F001 and FL013 suspension at 5×10^8 cells mL⁻¹ was poured and spread over the surface of the NYDA in the sandwich plate. After three days of incubation, the NYDA medium inoculated with the yeast and the filter paper layers were removed leaving only the PDA layer on the plates. Then, the PDA layer was inoculated with a mycelial disk of a seven days old culture of *C. gloeosporioides*. The plate was then incubated at 28 ± 2 °C. After seven days, the radial growth of the fungus was measured. A control treatment was also carried out by substituting sterile distilled water for the yeast suspension. The results were expressed using the average diameter of the *C. gloeosporioides* growth for all treatments. This experiment was conducted using five replicates for each treatment.

2.3. Identification of Antagonistic Yeast

Genomic DNA extraction of the most effective yeast was performed using the CTAB methods described by Edwards et al. [22], with minor modifications. Amplification of ribosomal DNA (rDNA) of yeast strain F001 was performed according to the method described by White et al. [23], using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCCG-3') as the forward primer and ITS4 (5'-TCCTCCGCTTAATTGATATGC-3') as the reverse primer, synthesized at First Base Laboratories Sdn. Bhd., Malaysia. PCR reaction was performed according to the protocol described by Hata et al. [24]. The amplification was performed in a BioRad iCycler (Bio-Rad) thermocycler as described by Nghia et al. [25].

The PCR product was purified using Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified PCR product was then sent to NextGene Sdn. Bhd., Malaysia for sequencing. The resulting sequences were aligned and identified using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Multiple sequence alignment was performed using Clustal W, and phylogenetic analysis constructed by Neighbour Joining method using MEGA software version 6. Bootstrap values illustrated on the phylogenetic dendrogram were generated with 1000 replicates [26,27]. The isolate was deposited in the Microbial Collection Unit (UNiCC) of the Institute of Biosciences (IBS), Universiti Putra Malaysia. Identification of yeast was also done by referring to morphology characteristics.

2.4. In Vivo Efficacy of Selected Antagonistic Yeast in Controlling Anthracnose Disease in Papaya Fruit

The papaya fruit, 'Solo' variety, used for this study was chosen and sanitized as described in Section 2.2.3. After being air dried, the fruit was subjected to dipping treatment with: (i) sterile distilled water and (ii) *T. asahii*. The treated fruits were then dried, and wrapped individually with clean white paper and packed into corrugated paper boxes before being stored at 28 ± 2 °C for eight days. This experiment was conducted with four replications for each treatment. After eight days of storage, the development of anthracnose disease symptoms and its severity on the fruit surfaces were observed and recorded. Disease incidence and severity were calculated using the following formula and scale.

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected fruit}}{\text{Total number of fruit}} \times 100 \quad (4)$$

Data on disease severity (DS) was indexed on a 0–4 scale, where, 0 = no disease symptoms or infection on the fruit surface area, 1 = 1–10% area of disease or infection, 2 = 11–20% area of disease or infection, 3 = 21–30% area of disease or infection and 4 = 31% and above area of disease or infection [28]. Disease severity was calculated using the following formula, as described by Singh [29]:

$$\text{Disease severity, DS (\%)} = \frac{\Sigma (\text{Severity rating} \times \text{number of fruit with that rating})}{\text{Total number of fruits assessed} \times \text{highest scale}} \times 100 \quad (5)$$

The fruit pulp from this study was used for enzyme activity determination. The fruits were individually chopped, ground, and freeze-dried using liquid nitrogen. The samples were stored in sealed polyethylene bags at $-80\text{ }^{\circ}\text{C}$ until ready for extraction.

2.5. Effects of Yeast Application on Selected Defensive Enzyme Activities

2.5.1. Tissue Extraction and Determination of Polyphenol Oxidase (PPO) Enzyme Activities

All enzyme extraction procedures were carried out at $4\text{ }^{\circ}\text{C}$. The method of extraction was adapted from Venkatachalam and Meenune [30] with minor modifications. Two grams of frozen papaya pulp tissues were ground with 10 mL of cold 0.2 M sodium phosphate buffer (pH 6.4) and homogenized using a mortar and pestle at $4\text{ }^{\circ}\text{C}$. The homogenate was filtered through a layer of cheesecloth and the filtrate centrifuged at $3360 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$. The supernatant was collected for PPO activity assay, according to the method by Tian et al. [31]. The reaction mixture contained 1.5 mL of 0.5 M 4-methylcatechol in 0.2 M sodium phosphate buffer (pH 6.4) and 0.05 mL of the crude enzyme extract. The absorbance was measured at 398 nm at $25\text{ }^{\circ}\text{C}$ for 1 min using UV/VIS spectrophotometer (Thermo Scientific Multiskan Go, Vantaa, Finland equipped with Thermo Scientific SkanIt Software version 3.2, Vantaa, Finland).

One unit of PPO activity is defined as the amount of enzyme extracts producing an increase in absorbance for 1 min. The enzyme activity is expressed as changes in the absorbance unit g^{-1} tissue according to the formula described by Kokkinakis and Brooks [32] as follows:

$$\text{Unit g}^{-1} \text{ tissue} = \frac{\text{optical density} \times \text{dilution factor}}{\text{g of tissue used in the assay}} \times 100 \quad (6)$$

2.5.2. Tissue Extraction and Determination of Phenylalanine Ammonia-Lyase (PAL) Enzyme Activities

For the PAL assay, 2 g of pulp tissue was homogenized in 4 mL of 0.1 M sodium borate buffer (pH 8.0) solution containing 0.2 g of insoluble polyvinylpyrrolidone (PVP), 5 mM mercaptoethanol and 2 mM ethylenediaminetetraacetic acid (EDTA). Then, the homogenate was centrifuged for 20 min at $4\text{ }^{\circ}\text{C}$ and $17,500 \times g$ [30]. PAL activity was determined according to the method of Jiang and Joyce [33], with slight alterations. An aliquot (0.1 mL) of the enzyme extract was incubated together with 2.9 mL of 0.1 M sodium borate buffer (pH 8.0) solution containing 3 mM L-phenylalanine for 1 h at $37\text{ }^{\circ}\text{C}$. An increase in PAL activity at 290 nm, due to the formation of trans-cinnamate, was measured using UV/VIS spectrophotometer (Thermo Scientific Multiskan Go, Vantaa, Finland, Finland equipped with Thermo Scientific SkanIt Software version 3.2, Vantaa, Finland). One unit of enzyme activity is defined as the amount of enzyme causing a decrease in absorbance of 0.01 per min. The results were expressed as unit g^{-1} tissue.

2.5.3. Tissue Extraction and Determination of Catalase (CAT) Enzyme Activities

Crude extract for CAT activity was obtained using the method of Wang et al. [34]. Two grams of pericarp tissues were homogenized in 4 mL cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 g of polyvinyl polypyrrolidone (PVPP). The homogenate was then centrifuged ($4\text{ }^{\circ}\text{C}$, $17,500 \times g$, 20 min) following which the supernatant was immediately analyzed. Determination of CAT activity was performed according to the method described by Beers and Sizer [35], with slight modifications. The reaction mixture consisted of 0.5 mL enzyme extract, 2 mL of 50 mM sodium phosphate buffer (pH 7.0) and 0.5 mL of 40 mM hydrogen peroxide (H_2O_2) with a total volume of 3.0 mL. The decomposition of H_2O_2 was measured using UV/VIS spectrophotometer (Thermo Scientific Multiskan Go, Vantaa,

Finland equipped with Thermo Scientific SkanIt Software version 3.2, Vantaa, Finland) at 240 nm absorbance.

One unit of enzyme activity was defined as micromoles of hydrogen peroxide, oxidized per milliliter per minute at 25 °C. The results were expressed as enzyme unit per gram fresh weight (U g⁻¹ FW). The extinction coefficient of 39.4 mM cm⁻¹ was used to calculate the CAT activity.

2.6. Yeast Interaction and Attachment to the Pathogen and Papaya Peel

Attachment of the antagonistic yeast to the pathogen and papaya peel was studied using a scanning electron microscope (SEM), with reference to the methods used by Hasan [15], with minor adjustments. A matured green papaya was washed with tap water and its surface sterilized with 75% ethanol. After being air dried, the center of the fruit was wounded with a sterile needle and it was then dipped in F001 suspension (5 × 10⁸ cells mL⁻¹) for 3 min. Then, 100 µL of pathogen spore suspension at 5 × 10⁴ spores mL⁻¹ was inoculated into the wounded area and the fruit stored at 28 ± 2 °C for three days in a covered plastic tray. For the control fruit, there was no pathogen inoculated into the wound. After three days, the fruit's peel at the wounded area was removed and cut into 2 mm³ pieces and then fixed using 2.5% buffered glutaraldehyde for 24 h at 4 °C. The sample was then washed with 0.1 M sodium cacodylate buffer (pH 7.7) and post-fixed in 1% sodium tetroxide for 2 h at 4 °C. Subsequently, the sample was washed again with 0.1 M sodium cacodylate buffer, for three times at 10 min each. A series of dehydration processes were performed with eight different concentrations of ethanol (30, 40, 50, 60, 70, 80, 90 and 100%) for 30 min each. The samples were dried in a Baltec 030 Critical Point Drying apparatus and affixed onto an aluminium stub and coated with gold in a Polaron Sputter Coater and viewed under SEM (JSM 6400, JOEL, Akishima, Japan).

2.7. Experimental Design and Statistical Analysis

The experiment was conducted using a completely randomized design. The results were analyzed using analysis of variance, SAS version 9.4 (SAS Institute, Cary, NC, USA) and the means were compared using Duncan's multiple range test (DMRT) at $p \leq 0.05$. Data for disease incidence and severity percentage were log-transformed before analysis.

3. Results

3.1. Isolation of Antagonistic Yeast from the Papaya Plant

A total of 110 yeasts strains were isolated from the leaves, petioles, and fruit surfaces of randomly selected papaya plants based on different colony colors, sizes and shapes. All the isolates were preserved and maintained for further assessment of their antagonistic activities against *C. gloeosporioides*.

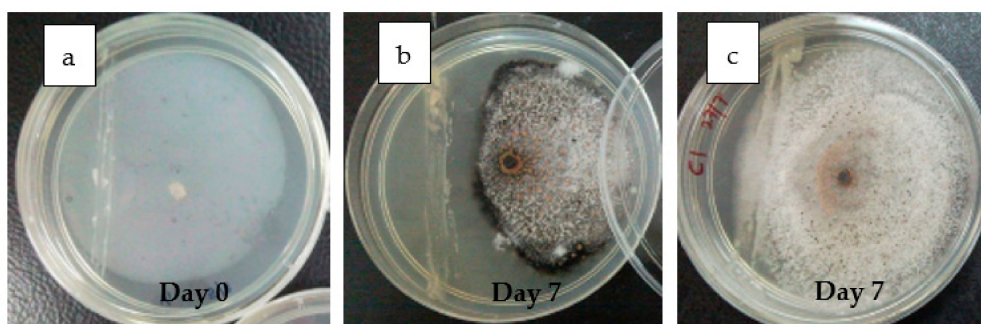
3.2. Screening of Antagonist Yeast Against *C. gloeosporioides*

Using dual culture screening, the antagonistic activities of the yeast strains were measured by their abilities to resist fungal invasion or inhibit fungal growth before yeast streaking. Out of the 110 yeast isolates, only 25 were found to have positive antagonistic effects against *C. gloeosporioides*, after being co-cultivated in the same agar plate. All 25 yeast strains showed different degrees of antagonistic activity as illustrated by the PIRG values after seven days of incubation. Out of the 25 yeast strains selected, only five isolates, namely, F001, F006, L003, FL013 and LP010, had more than 55% inhibitory effects compared to the control (0%). The PIRG of strains F001, F006 and FL013 were 59.5, 60.8 and 70.3, respectively, and 59.5% for L003 and LP010, after seven days of incubation (Table 1 and Figure 1).

Table 1. Effects of antagonist yeast isolates on radial growth of *C. gloeosporioides* in dual culture assay after seven days incubation at 28 ± 2 °C.

Yeast Isolate	Percentage Inhibition of Radial Growth (PIRG) (%)
F001	59.46 ± 5.41 abc ^z
F002	50.00 ± 4.05 bcde
F006	60.81 ± 1.35 ab
F010	28.38 ± 1.35 hi
F015	45.95 ± 2.70 cdef
F022	32.43 ± 2.70 ghi
F026	54.05 ± 0.00 bcde
L003	59.46 ± 8.11 abc
FL002	31.08 ± 1.35 ghi
FL006	35.14 ± 2.70 fgh
FL013	70.27 ± 0.00 a
FL015	36.49 ± 1.35 fgh
FL016	54.05 ± 0.00 bcde
FP001	40.54 ± 2.70 efgh
FP002	54.05 ± 0.00 bcde
FP006	54.05 ± 2.70 bcde
FP013	36.49 ± 1.35 fgh
FP014	51.35 ± 2.70 bcde
LP005	41.89 ± 1.35 defg
LP010	59.46 ± 8.11 abc
FLP004	33.78 ± 4.05 fghi
FLP007	54.05 ± 0.00 bcde
FLP009	52.03 ± 2.03 bcde
FLP011	21.62 ± 2.70 i
FLP016	50.00 ± 1.35 bcde

^z Means values within each column followed by different letter indicate significant differences between treatments according to DMRT ($p \leq 0.05$). Values after \pm represent the standard error (SE).

**Figure 1.** Radial growth of *C. gloeosporioides* co-cultured with antagonist yeast after seven days at 28 ± 2 °C; (a,b) *C. gloeosporioides* co-cultured with LP010 and (c) *C. gloeosporioides* co-cultured with FLP011.

3.3. Effect of Yeast Suspension on *C. gloeosporioides* Growth in Agar Well Test

Five potential yeast strains selected from the previous screening were further evaluated in agar well tests. Among these selected yeast strains, LP010 showed the lowest ability for spore inhibition (Table 2), whereas yeast suspension of F001, F006, and L003 did not show significant differences in the inhibition of *C. gloeosporioides* spores. Meanwhile, FL013 yeast suspension showed remarkable ability to totally inhibit the growth of *C. gloeosporioides*. This experiment showed that FL013 had the most robust antagonistic activity, followed by strains F006, F001, L003, and LP010, in that order.

Table 2. Antagonistic effects of yeast suspension on mycelial growth of *C. gloeosporioides* in agar well tests after seven days incubation at 28 ± 2 °C.

Treatment	Diameter of Mycelial Growth (cm)
Control	6.82 ± 0.20 a ^z
F001	1.96 ± 0.88 c
F006	0.80 ± 0.35 cd
L003	2.25 ± 0.33 c
FL013	0.00 ± 0.00 d
LP010	4.73 ± 0.37 b

^z Means values within each column followed by different letters indicate significant differences between treatments according to DMRT ($p \leq 0.05$). Values after \pm represent SE.

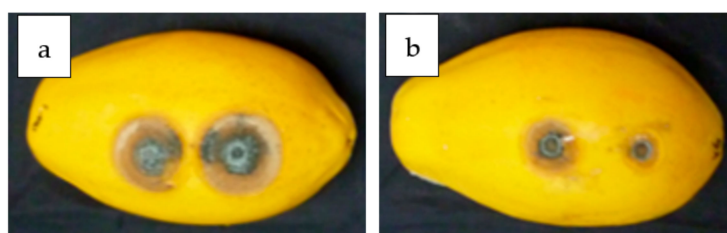
3.4. Effect of Antagonist Yeast on In Vivo Anthracnose Disease Control

Many researchers prefer Agar-based screening of potential biocontrol agents to select the most probable candidates due to cost and time factors [21,36,37]. It is also an excellent approach to validate the results obtained from other in vitro screening tests. However, in vivo studies are still needed to verify the results obtained, even after several in vitro screening studies have been done. This study (Table 3) showed that all wounds on the papayas treated with the yeasts had significantly smaller anthracnose lesion diameters than the control, except for isolates L003 and LP010. The results for isolate F001 showed the highest biocontrol efficacy with only 1.99 cm lesion diameter, 0.33 cm day⁻¹ lesion expansion rate, and 51.19% disease reduction as compared to the control (Figure 2).

Table 3. Effects of antagonist yeast treatments in papaya inoculated with *C. gloeosporioides* after storage at 28 ± 2 °C for six days.

Treatment	Lesion Diameter (cm)	Lesion Expansion (cm day ⁻¹)	Disease Reduction over Control (%)
Control	4.08 ± 0.06 a ^z	0.68 ± 0.01 a	–
F001	1.99 ± 0.17 d	0.33 ± 0.03 d	51.19 ± 4.17 a
F006	3.45 ± 0.22 bc	0.58 ± 0.04 bc	15.44 ± 5.48 bc
L003	3.68 ± 0.08 ab	0.61 ± 0.01 ab	9.93 ± 1.94 c
FL013	3.13 ± 0.19 c	0.52 ± 0.03 c	23.20 ± 4.77 b
LP010	3.62 ± 0.14 ab	0.60 ± 0.02 ab	11.36 ± 3.37 bc

^z Mean values within each column followed by different letters indicate significant differences between treatments according to DMRT ($p \leq 0.05$). Values after \pm represent SE.

**Figure 2.** Radial growth of *C. gloeosporioides* in papaya wounds treated with antagonist yeast after six days of inoculation; (a) papaya wound without yeast treatment, (b) papaya wound treated with F001 yeast.

3.5. Effects of Antagonist Yeast Concentrations on *C. gloeosporioides* Spore Germination

The ability of the biocontrol treatments at different concentrations in suppressing the germination of fungal spore was investigated. Only two isolates were chosen for this study, F001 and FL013, based on their effectiveness as biological agents in previous tests. Treatments with these two isolated yeasts significantly reduced *C. gloeosporioides* spore germination percentages compared to distilled water (Table 4). The spore germi-

nation percentages were shown to decrease significantly, from 90% to 30% for the F001 isolate and from 76% to 64% for the FL013 isolate, with increasing yeast cell concentrations. However, the increment in concentration from 5×10^6 to 5×10^8 cells mL⁻¹ for yeast isolate FL013 did not demonstrate significant spore germination inhibition. Isolate F001 demonstrated better overall control with the lowest spore germination of 30% at the 5×10^8 cells mL⁻¹ yeast concentration. Compared to the commercial fungicide, Benocide 50WP[®], both yeast isolates showed significantly higher inhibition of spore germination at only 5×10^6 cells mL⁻¹. Due to the better antagonistic results shown for yeast suspension at concentration 5×10^8 cells mL⁻¹, the same concentration was used for the next assessment.

Table 4. Effects of different solutions on spore germination percentage of *C. gloeosporioides* after 20 h incubation at 28 °C in PDB.

Solution	Spore Germination (%)
Sterile distilled water	100.0 ± 0.00 a ^z
Benocide 50WP [®]	78.6 ± 3.62 c
F001 5×10^4 cells mL ⁻¹	89.5 ± 0.25 b
F001 5×10^6 cells mL ⁻¹	48.9 ± 1.41 e
F001 5×10^8 cells mL ⁻¹	30.3 ± 1.77 f
FL013 5×10^4 cells mL ⁻¹	76.2 ± 1.16 c
FL013 5×10^6 cells mL ⁻¹	67.0 ± 4.15 d
FL013 5×10^8 cells mL ⁻¹	64.3 ± 2.99 d

^z Mean values with different letters within each column are significantly different at $p \leq 0.05$ using DMRT. Values after ± represent SE.

3.6. Diffusible Compound Production by Antagonist Yeast

The ability to produce antifungal metabolites is one of the biocontrol mechanisms of antagonist organisms. This study showed that both yeast strains significantly inhibited fungal mycelial growth compared to the control (Table 5). Mycelial growth was completely inhibited by diffusible antifungal substances produced by isolate F001 at the concentration of 5×10^8 cells mL⁻¹. However, *C. gloeosporioides* mycelia could still survive on PDA containing diffusible antifungal substances produced by isolate FL013. It was observed that the diffusible compounds produced by isolate F001 were more potent than FL013 as demonstrated by its better control of fungal growth.

Table 5. Effects of diffusible substances generated by antagonist yeasts on *C. gloeosporioides* mycelia growth diameter after seven days incubation at 28 °C.

Treatment	Mycelia Growth Diameter (cm)
Sterile distilled water (SDW)	6.91 ± 0.09 a ^z
F001 5×10^8 cells mL ⁻¹	0.00 ± 0.00 c
FL013 5×10^8 cells mL ⁻¹	5.69 ± 0.50 b

^z Mean values with the different letters within each column are significantly different at $p \leq 0.05$ using DMRT. Values after ± represent SE.

3.7. Antagonist Yeast Identification

Following in vitro and in vivo screenings, isolate F001 was found to be stable and effective in both the controlled environment (in vitro) and natural environment (in vivo). Thus, only this isolate was identified and further tested for its biocontrol ability against *C. gloeosporioides*. The DNA of antagonist yeast F001 was successfully extracted using the CTAB method. The ITS region was amplified using a pair of universal primers, ITS1 and ITS4. After amplification, a product of 527 bp was obtained from the isolate. The sequence obtained was deposited in the GenBank and used to search for similar sequences in various databanks using the BLAST program. The phylogenetic tree generated using the Neighbour Joining (NJ) method showed that isolate F001 has high homology (99%) to

Trichosporon asahii (accession number: KX580068) and was clustered together with other sequences of *T. asahii* as evidence of high homology (99%). However, there are minor variations between *T. asahii* and *T. inkin*, as visualized by the cluster separation illustrated in Figure 3.

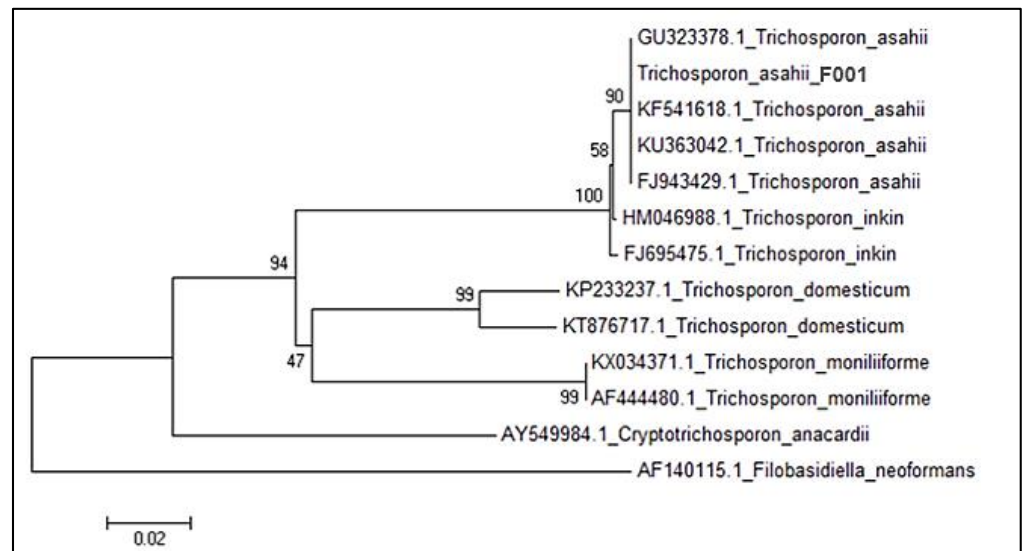


Figure 3. Phylogenetic tree based on ITS rDNA sequence of *Trichosporon asahii* F001 and reference sequences of *Trichosporon* with outgroup genus *Cryptotrichosporon* and *Filobasidiella*. Tree generated using Neighbour Joining analysis with 1000 bootstrap simulations. Numbers on the branches represent bootstrap values.

BLAST comparison of the ribosomal DNA sequences from the selected yeast strain showed that isolate F001 had 99% similarity to the sequences of *Trichosporon asahii*. Observation of the morphological (colony morphology) and microscopic (cell shape and size) characteristics of *T. asahii* supports the rDNA sequencing results through referring Kurtzman and Fell [38]. The yeast colony of the F001 strain, identified as *T. asahii*, was white, with a wide, dry, fine zonate margin and farinose at the center (Figure 4a). Culture odor was lacking or faintly cheese-like and the colony diameter was around 16–24 mm. Meanwhile, the yeast cells in Figure 4b showed chains of spores in different shapes which were round, rectangular or irregular. All of the morphological and microscopic characteristics shown by the selected isolate cell matched with *T. asahii* characteristics.

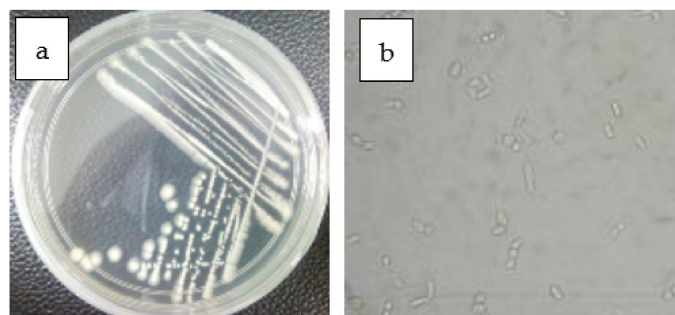


Figure 4. Morphological characterization of *T. asahii*; (a) *T. asahii* on PDA plate, (b) *T. asahii* cell shape.

3.8. Disease Incidence and Severity in Papaya Treated with Antagonist Yeast

After eight days of storage, the disease incidence on the naturally infected fruits treated with *T. asahii* showed no significant difference as compared with the control treatments (Table 6). However, in terms of disease severity, the fruit treated with *T. asahii* showed significantly lower severity percentage than that in the control fruit, which was 50% reduction. With reference to the results, although *T. asahii* was unable to avoid disease incidence significantly, it still managed to reduce the severity of the disease on the papaya.

Table 6. Effects of treatments (sterile distilled water and *Trichosporon asahii*) on disease incidence and severity percentage on Solo papaya (*Carica papaya* L.) stored at 28 °C for eight days.

Treatment	Disease Incidence (%)	Disease Severity (%)
Sterile distilled water	100 ± 0.00 a ^z	75 ± 13.7 a
<i>Trichosporon asahii</i>	66.7 ± 14.43 a	25 ± 9.1 b

^z Mean values within each column followed by different letters indicate significant differences between treatments according to DMRT ($p \leq 0.05$). Values after ± represent SE.

3.9. Polyphenol Oxidase (PPO), Phenylalanine Ammonia-Lyase (PAL), Catalase (CAT) Enzyme Activity

Defense-related enzymes measured for this study were PPO, PAL and CAT. After eight days of storage, the PPO enzyme activities in Solo papaya treated with *T. asahii* showed no significant difference with the control treatments (Table 7). There was also no significant difference shown for the other two enzymes measured: PAL and CAT.

Table 7. Effects of treatments (sterile distilled water and *Trichosporon asahii*) on polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and catalase (CAT) on Solo papaya (*Carica papaya* L.) during storage at 28 °C for eight days.

Treatment	PPO (Unit g ⁻¹ Tissue)	PAL (Unit g ⁻¹ Tissue)	CAT (U g ⁻¹ FW)
Sterile distilled water	274.6 ± 4.8 a ^z	2012.7 ± 11.4 a	2.34 ± 0.3 a
<i>Trichosporon asahii</i>	274.3 ± 5.9 a	1986.0 ± 13.6 a	2.27 ± 0.4 a

^z Means values within each column and factor followed by different letters indicate significant differences between treatments according to DMRT ($p \leq 0.05$). Values after ± represent SE.

3.10. Interaction of Antagonist Yeast and *C. gloeosporioides* in Papaya

An ultrastructure study was done to investigate the interaction between *T. asahii*, the pathogen and the host. As shown in Figure 5b,c, *T. asahii* and the fungal pathogen exhibited successful colonization on the wounded papaya peel three days after inoculation. Meanwhile, Figure 5d illustrates that the *T. asahii* cells attached tenaciously to the hyphae of *C. gloeosporioides* growing on the wounded papaya peel.

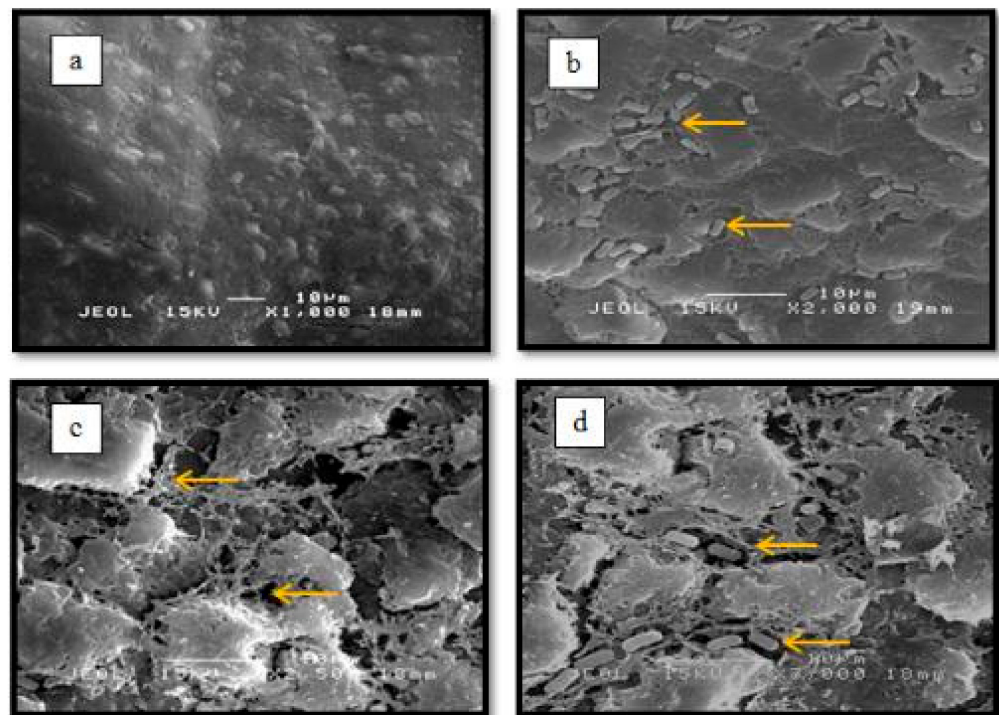


Figure 5. Attachment and interaction of *C. gloeosporioides*, *T. asahii* and papaya peel after three days of inoculation, viewed using scanning electron microscope. (a) papaya peel without any inoculation, (b) papaya peel inoculated with *T. asahii* (arrows show yeast cells), (c) papaya peel inoculated with *C. gloeosporioides* spores (arrows show fungal hyphae) and (d) papaya peel inoculated with *C. gloeosporioides* and *T. asahii* (arrows show yeast cells attached to fungal hyphae).

4. Discussion

The typical strategy for biocontrol is to use living organisms to suppress or inhibit the growth, infection, or reproduction of another organism [39]. Disease control by exploiting antagonist organisms has become a viable disease management strategy, and several microorganisms have been successfully patented as postharvest biocontrol agents [40–43]. According to Alvinda and Natsuaki [44], the most viable way of using antagonist microorganisms as biocontrol agents is by supporting and maintaining existing antagonists which already reside and are established on the target plant environments. Consequently, epiphytic yeast isolated from the papaya plant may be an optimum choice for the isolation of antagonist agents because of their ability to colonize the papaya fruit naturally. Introducing new antagonist microorganisms to a host may result in undesirable effects such as complexities arising from biocontrol agent and host compatibility. Such biocontrol agents cannot multiply or inhabit the host naturally and may require several applications to maintain effectiveness. Furthermore, study on any possible side effects from introducing new microorganisms to a host needs to be conducted to avoid any new disease outbreaks or adverse impacts on the host and consumers. Thus, exploiting the native antagonist microorganisms as the source biocontrol agents is safer and more reliable than the introduction of new biocontrol agents to the produce.

The establishment of biocontrol products involves screening large numbers of possible antagonists. The selection of appropriate screening methods is essential in determining effective biocontrol agents against pathogenic diseases. Optimally, biocontrol organism candidates should be screened on plants or *in vivo* rather than on plate or *in vitro* cultures. However, huge-scale screening study on whole plants is usually time-consuming and labor-intensive, thus the conventional screening approach remains prevalent among researchers [45].

In the dual culture assay, out of the 110 yeast strains isolated, only five strains, namely, F001, F006, L003, FL013, and LP010, displayed more than 55% inhibitory effects compared to the control (0%). These five yeast strains were further screened using an agar well test. The results showed that isolates F001 and F006 worked better when immediately in contact with the pathogen but this was not so for LP010. The weaker antagonistic effect of LP010 in the agar well test may be due to it having a different mode of action in controlling pathogens as compared to the other isolates, as suggested by Alvindia and Natsuaki [44], where the antagonist's mode of action is said to influence its capability in preventing the activity of the pathogen tested. For example, the yeast's ability to colonize rapidly and maintain an adequate population level is vital in nutrients competition with pathogens and to maintain its effectiveness in disease suppression [46]. However, in the current study, the actual reason for the outcome was not thoroughly examined since the main focus of this experiment was to screen for potential candidates as antagonist yeast strains against *C. gloeosporioides*.

Meanwhile, F001 showed better antagonist activity than the other isolates in in vivo screening tests as compared to the in vitro tests. Capdeville et al. [2] also discovered a similar finding where some yeast strains successfully managed the pathogen in vitro but not in vivo. Some successfully managed the disease in vivo but were unable to effect high suppression against the fungus in vitro. In contrast, others managed to control the fungus and the disease in vitro and in vivo. This situation may be due to differences in adaptation of the yeast to the environment, where the in vitro environment was more stable than the in vivo assay. In Vivo the pathogen inoculum amount is difficult to be defined since it is under exposed conditions. Further, postharvest disease infection can be influenced by inoculum in the processing environment and the extent of wounding on fruits after harvest and handling [2].

Biocontrol efficacy of microbial antagonists can be enhanced by the additional density of the antagonists. Quantitative interaction between the concentration of antagonists applied and the resulting effectiveness of the biocontrol agents was also observed in the present study. Zhang et al. [47] also demonstrated that in vitro co-culturing of *Pseudozyma fusiformata*, *Metschnikowia* sp., and *Aureobasidium pullulans* at higher concentrations had a higher significant effect in suppressing spore germination or germ tube elongation of *Monilinia laxa*, a postharvest pathogen in peach. These scenarios indicate that antagonists and pathogens compete for the same nutrients to survive [48]. However, the additional concentration of FL013 from 5×10^6 to 5×10^8 cells mL⁻¹ unable to significantly reduce spore germination, as shown in the results obtained. According to Sharma et al. [49], this qualitative response is strongly influenced by the antagonist's ability to multiply and spread at the wound site. Studies done by Droby et al. [50] also showed that mutant *Pichia guilliermondii* lost its antagonist activity against *Penicillium digitatum* on grapefruit and against *Botrytis cinerea* on apples even at concentrations of up to 10¹⁰ CFU/mL. This situation was due to the static cell population of this mutant yeast at the wound sites, while the wild type multiplied 10–20 fold within 24 h. In general, antagonist microbes are most effective as biocontrol agents when applied at 10⁷ to 10⁸ CFU mL⁻¹ [17,47,51,52], and rarely, higher concentrations are needed [49].

Yeast's antagonistic mode of action also includes the ability to generate antagonistic substances, for example, killer toxins, peptides, and antibiotic metabolites [9,53,54]. Some yeasts display killing activity through secretion of proteins or glycoproteins, killing toxins commonly lethal to other yeast species, pathogenic filamentous fungi, and bacteria through different mechanisms, including hydrolysis of the primary cell wall component β -1,3-glucans [55–58]. Yeasts with the ability to secrete killer toxins such as *Saccharomyces cerevisiae* and *Pichia membranifaciens* are generally immune to killer toxins of their class and this gives the yeast cells an advantage over their competitors [59–61].

The metabolites from both the yeast strains tested, namely F001 and FL013, were found to have significant inhibitory effects on the pathogen's growth compared to the control. However, diffusible antifungal metabolites produced by isolate F001 had a better effect in

inhibiting the growth of *C. gloeosporioides* mycelia compared to FL013. After reference to all the in vitro and in vivo screening tests, only F001, identified as *Trichosporon asahii*, was chosen for further study as a potential antagonist agent against *C. gloeosporioides*, due to its effectiveness shown in several assessments.

Several studies showed that antagonist yeast could interact with the host tissue to induce production of defensive enzymes after application of the yeast on the fruit surfaces [7]. Chitinase, β -1,3-glucanase, phenylalanine ammonia-lyase (PAL), catalase (CAT), peroxidase and polyphenol oxidase (PPO) are considered important enzymes in disease resistance mechanisms against pathogens. However, in the present study, the induction of PPO, PAL and CAT were not measurable after the application of *T. asahii* on the papaya fruit. The results showed that the induction of PPO, PAL and CAT responses are not *T. asahii* antagonist's mode of action even though the fruit treated with this yeast treatment showed lower disease severity compared to the sterile distilled water treated fruit. Since the disease defensive response of fruit also involves many other types of mechanisms, this situation might be due to the generation of microenvironments that did not favor the growth of pathogens on the treated fruit, such as competition for nutrients and space or direct parasitism of biocontrol agents against the pathogen [62].

Attachment of antagonist microorganisms to the pathogen hyphae is also an essential factor for successful biocontrol activity. With this ability, the biocontrol agent is able to disrupt the pathogen's action to begin infection, thus, controlling lesion development on the produce. The importance of this attribute can be seen in the relationships of *Enterobacter cloacae* and *Rhizopus stolonifer* [63], and *Pichia guilliermondii* and *Penicillium italicum* [64]. In Vitro studies performed on these relationships showed that antagonistic yeasts and bacteria absorb nutrients faster than target pathogens due to direct attachment, thus preventing spore germination and pathogen growth [63,65,66].

Meanwhile, according to in vitro studies done by Wisniewski et al. [67], yeast attachment on fungal hyphae occurs within 12 to 16 h of incubation, while for in vivo studies, attachment of yeast to pathogen hyphae, *Botrytis* spp. was discovered within 48 to 72 h after inoculation. Considering that most fungal pathogens begin invading the fruit tissue within 6 to 10 h, attachment of antagonist microbes might have limitations in preventing decay if applied after or simultaneously with disease infection [68]. The micrograph obtained in the present study after 48 h of simultaneous inoculation, shown in Figure 5d, shows that the pathogen hyphae had already covered the papaya fruit peel, and the yeast was unable to inhibit spore germination. Observation of the attachment effect should be improved by viewing the interaction activity 72 h after inoculation or by applying the biocontrol earlier, before the pathogen spores are introduced to the papaya peel.

Trichosporon species are widely distributed in nature. Other than being commonly isolated from soil, fruit, and other environmental sources, it is also present in the human gastrointestinal and respiratory tracts [69,70]. From the results obtained, *T. asahii* isolated from the papaya fruit has proven its antagonistic ability against *C. gloeosporioides* and can thus be used to effectively control postharvest anthracnose disease in papaya fruit. The possible mechanisms of action include: (1) space and nutrition competition with *C. gloeosporioides*; (2) inhibition of pathogen growth by production of diffusible antifungal substances; and (3) direct parasitism against *C. gloeosporioides* by attachment to the pathogen hyphae to reduce disease severity on the wounded papaya. However, the application of *T. asahii* on produce needs to be fully elucidated to gain better understanding in manipulating the strain as a biocontrol agent and to avoid any side effects on humans and the environment.

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