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Use of Antagonistic Yeast in controlling anthracnose caused by *Colletotrichum gloeosporioides* in papaya in Sri Lanka

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Abstract:
Anthracnose, which is caused by *Colletotrichum gloeosporioides*, is one of the most prevalent papaya diseases in Sri Lanka. Although it is controlled by a variety of chemical fungicides, the possibility of the pathogen developing a chemical resistance and consumer demands made the need for a biocontrol technique to manage this disease. Additionally, using biocontrol techniques that have been developed in other countries may have negative effects on nontargeted organisms. Therefore, this study intended to assess the antagonistic yeasts' potential to inhibit the growth of *C. gloeosporioides* in papaya in Sir Lankan context. Four types of yeasts were isolated from *Carica papaya* L. (Papaya) leaf surfaces, *Psidium guajava* L. (Guava) leaf surfaces, Cocos nucifera (Coconut) water and Baker's Yeast solution (*Sacchsromyces cerevisiae*)(Y162, Y234, Y342, and Y467 respectively) were isolated. *C. gloeosporioides* was isolated from diseased papaya fruits and morphologically identified based on their colony characteristics and spore characteristics. The dual culture assay was used to examine the antagonistic activity. Commercial antibiotic, Fluconazole was used as the positive control. All four yeast isolates had shown significant antagonistic activity against *C. gloeosporioides* (One way ANOVA, $P < 0.05$), which was even higher than the positive control. The highest The Percent Inhibition of Radial Growth (PIRG %) was observed with Y162 yeast variety (59.3±2.0). In Vivo, the variety Y162 caused 66.2% reductions in disease incidence. According to these findings antagonistic yeast Y162 could be utilized as a possible biological control agent against the anthracnose disease caused by *C. gloeosporioides* in papaya in Sri Lanka.

Keywords: Anthracnose, papaya, biocontrol, *Colletotrichum gloeosporioides,* Anagonistic yeast

1. Introduction

Papaya (*Carica papaya* L.) belonging to Caricaceae family, is one of the highly used, nutrient dense tropical fruit. However, due to its high perishability post-harvest loss of papaya are comparatively higher compared to many other fruits. Particularly, it is estimated that the postharvest losses of papaya from fungal infections account for more than 50% of productivity, and Anthracnose caused by *Colletotrichum gloeosporioides*, being one of the major causes [1]. Even while the initial infection always happens before to harvest, symptoms usually start to show up afterward as a result of favorable storage conditions, which allow for continued fungal proliferation [2].Due to the availability of fungus in the surroundings, postharvest infections may occur. The situation is made worse when the fruit sustains significant injury after harvest [3]. To control anthracnose, fungicide dips or drenches are used during the packing process [3]. The search for alternate control strategies has gained significance currently due to the possible negative effects of fungicide poisoning on humans and the environment. Further development of the resistance to commonly used fungicides is also a major reason for finding alternative methods to control anthracnose [2].

Many of the alternative methods such as UV irradiation are expensive and could even affect physical and physiological conditions of the fruit [4]. Therefore, several initiatives with better alternatives had been studied to reduce anthracnose, including the use of antagonistic

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organisms. Due to their potent antagonistic activity against pathogens, yeasts are among the antagonistic organisms that have been described as postharvest biocontrol agents most frequently [5]. This is because yeasts are known to produce extracellular polysaccharides, which enhance their viability and inhibit the growth of other organisms [6]. Further, unlike other antagonistic fungi and bacteria, yeast just needs simple nutrients for growth and does not create any allergic spores or metabolites that could harm consumers [2]. However, it has been found that the efficiency of these antagonistic organisms depends on stability and the adaptation of the organism [7]. Furthermore, some bio controlling agents could affect negatively on other nontargeted organisms. Therefore, it is important to discover native yeast species to control anthracnose locally. Thus, the present study was conducted to isolate yeast verities from the local environment and study their effectiveness in controlling anthracnose in papaya.

2. Methodology

2.1. Isolation of *Colletotrichum gloeosporioides*

Papaya and Banana fruits having Anthracnose infected tissues were collected from Badulla area (geographical coordinates, 6° 59' 36.2436" N, Longitude: 81° 3' 17.9316" E) and placed in a moist chamber for 24 hours to induce the conidia of the pathogens. Diseased Papaya and Banana fruit tissues (1 cm^2) were cut under aseptic conditions. The tissues were surface sterilized using 70% alcohol. After three serial washings in sterile distilled water, Papaya and Banana tissues (5 mm^2) were sub-sectioned carefully with a sterile scalpel and then it was transferred aseptically onto solidified antibiotic rich (Ciprofloxacin 10 mg/L) Potato Dextrose Agar (PDA) (3 sub sections per plate) and incubated 30 °C for 7 days. Small fungal plugs were transferred from the pure culture into 5 new PDA Petri plates. The Petri plates were sealed and incubated at 30 °C until close to sporulation.

2.2 Identification of the *C. gloeosporioides* by slide culture method

A filter paper was placed in the petri dish. Two slides were stacked on the filter paper and the petri dish was covered by the lid and sterilized. Two thin PDA pieces were cut and placed on either edge of the upper slide in the petri dish. The fungus was inoculated to the PDA piece using a sterilized inoculation loop and the PDA pieces were covered with sterilized coverslips. Then sterilized distilled water was added to the filter paper and the petri dish was sealed and incubated [8]. After about 7 days the coverslips were observed under a microscope. Lacto phenol cotton blue stain was used for the microscopic analysis of the fungi and *C. gloeosporioides* was identified using a fungal identification key [9].

2.3 Isolation of Antagonistic Yeast

Healthy plant leaves of Guava (*P. guajava*) and Papaya (*C. papaya*) were collected from Badulla area. Leaves were cut into small pieces using a sterilized scalpel and 1g of leaf samples from each were placed in 10 mL of sterilized distilled water separately. They were kept in a shaker for 20 – 30 minutes.

Coconut water was obtained and allowed to ferment for four days. A 1 mL of fermented Coconut water was added to 9 mL of sterilized distilled water and shaken well. Serial dilutions were prepared as 10^{-2}, 10^{-3} and 10^{-4} separately for each sample. Spread plates were prepared from each dilution using antibiotic-rich GPYA (Glucose Peptone Yeast extract Agar) as the growth media. Plates were incubated at 30°C for 1-2 days. Isolated colonies that differed in colony characteristics and were streaked separately to obtain pure cultures.

2.4 Assay for antagonism using dual culture method

In PDA medium, the yeast isolates were cultured alongside the pathogen to evaluate their antagonistic capabilities. Seven days old mycelial plugs (5 mm diameter) of *C. gloeosporioides* were placed at the center of the plate. Two days old yeast culture was then streaked on the same plate 3 cm from each other. The dual culture plates were incubated at 30 ºC for four days.
Plates inoculated only with *C. gloeosporioides* served as negative controls and plates inoculated with *C. gloeosporioides* and Fluconazole fungicide were used as a positive control. The experiment was repeated with three replications of each treatment. The Percent Inhibition of Radial Growth (PIRG %) was calculated using the formula.

$$\textbf{PIRG}\ (\%) = \frac{\boldsymbol{C - T}}{\boldsymbol{C}} \times \mathbf{100}$$

C- Represents the distance (measured in mm) from the point of inoculation to the colony margin on the control plates
T- The distance of fungal growth from the point of inoculation to the colony margin on the treated plates in the direction of the antagonist
Efficiency of selected antagonistic yeast in controlling Anthracnose disease in papaya
Healthy twelve papaya fruits at color index two (green with a trace of yellow) within the weight range of 0.5 – 0.75 kg, were chosen and washed beforehand being soaked in sodium hypochlorite 0.5% for 5 minutes. They were then soaked in sterile distilled water for 1 minute. The fruits were surface-sterilized with 70% (v/v) ethanol after being air dried. They were then subjected to dipping treatment with the selected yeast solution (Y162) and sterile distilled water as the control. The fruits were then dehydrated, individually wrapped in white paper and placed in corrugated paper boxes (30 cm x 30 cm), where they were kept for eight days at room temperature. Six replications of each treatment were used in this experiment. After eight days of storage, the development of anthracnose disease symptoms on the fruit surfaces was observed and recorded using the method described by Illeperuma & Jayasuriya [8].
Following equation was used to determine disease incidence.

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

3.0 Results

3.1 Isolation of and identification of *C. gloeosporioides*

A total of 30 fungal isolates from *C. papaya* fruits with anthracnose symptoms were isolated. Based on culture morphological traits on PDA and spore characteristics were seen under a microscope, and the isolates were identified [9] [10]and [11]. The cultural and microscopic characteristics of the fungal isolate are presented in Table 01 and Figure 01. The isolates were subsequently verified using Koch's postulate, which involved inoculating healthy, ripe papaya fruits with ten-day-old pure cultures of the isolates grown on PDA. The papaya fruits developed a distinctive black mark after two days. Identical morphological, cultural, and spore properties were observed in the reisolated fungus from the damaged fruit as the original isolate.

Table 01: Colony characteristics of fungal isolates growing on potato dextrose agar and incubated at 25 ℃ for 6 days

Feature		Observation
Colony Colour	Upper Side	whitish, greyish, or creamish colour
	Reverse Side	greyish cream with circular orange-pinkish colour
Colony Texture		cottony, velvety
Colony Margin		regular
Colony Elevation		raised

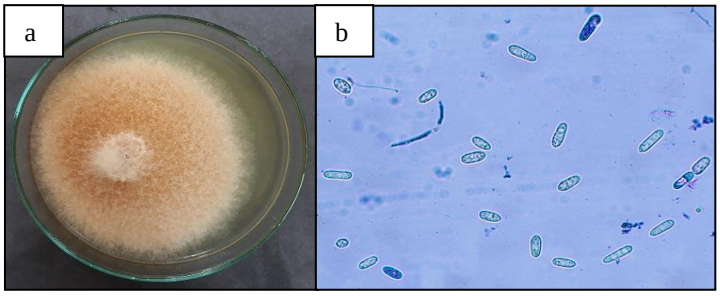
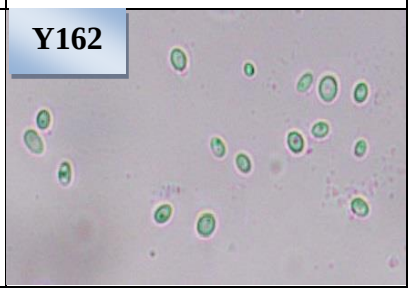


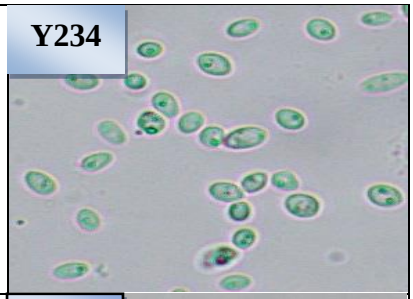
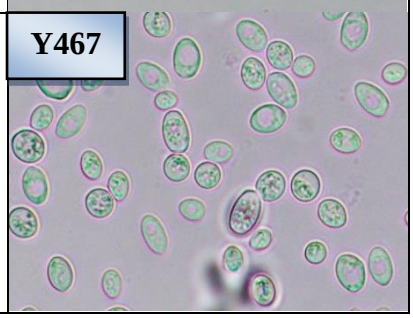
Figure 01: a) Isolated fungal cultures on PDA after six days incubation b) Conidia of isolated fungal culture (magnification 4 x 40).

3.2 Isolation and identification of yeast

Four types of yeasts were isolated from *C. papaya* L. (Papaya) leaf surfaces, *P. guajava* L. (Guava) leaf surfaces, *C. nucifera* (Coconut) water and Baker's Yeast solution (*S. cerevisiae*). The yeast types were confirmed by the colony characteristics, cell size and budding structure (Table 02).

Table 02: Colony and microscopic characteristics of Yeast isolates

Source	**Colony characteristics**	**Microscopic characteristics**	**Microscopic view (magnification 4x 40)**
Psidium guajava L. (Guava) leaf surface (Dilution -10^{-2})	Pale yellow colour, opaque, rough surfaced	Round shape small cells	

Cocos nucifera (Coconut) water (Dilution -10^{-4})	White colour, opaque, rugose surfaced	Oval shape middle sized cells	
Cocos nucifera (Coconut) water (Dilution -10^{-4})	White colour, opaque, rough surfaced	Short rod shape middle sized cells	
Carica papaya (Papaya) leaf surface (Dilution -10^{-4})	White colour, opaque, shiny surfaced	Oval shape large cells	

3.3 Screening of antagonistic yeast against *C. gloeosporioides*

All four yeast verities had shown positive antagonistic effects in different levels against *C. gloeosporioides*, after being co-cultivated in the same agar plate for seven days (One way ANOVA, P <0.05). Out of the four yeast strains selected, only one isolate (Y162), had more than 55% inhibitory effects compared to the control (0%) (Table 03)(Figure 02). Interestingly all four yeast species showed higher PIRG % compared to the positive control.

Table 3. Antagonistic activity of yeast isolates on growth of *C. gloeosporioides* in dual culture assay after seven days incubation at 28 ℃.

Yeast Isolate	PIRG %
Y162	59.3±2.0
Y234	30.3±1.5
Y342	55.3±1.5
Y467	45.0±1.7
Positive control	24.7±1.5

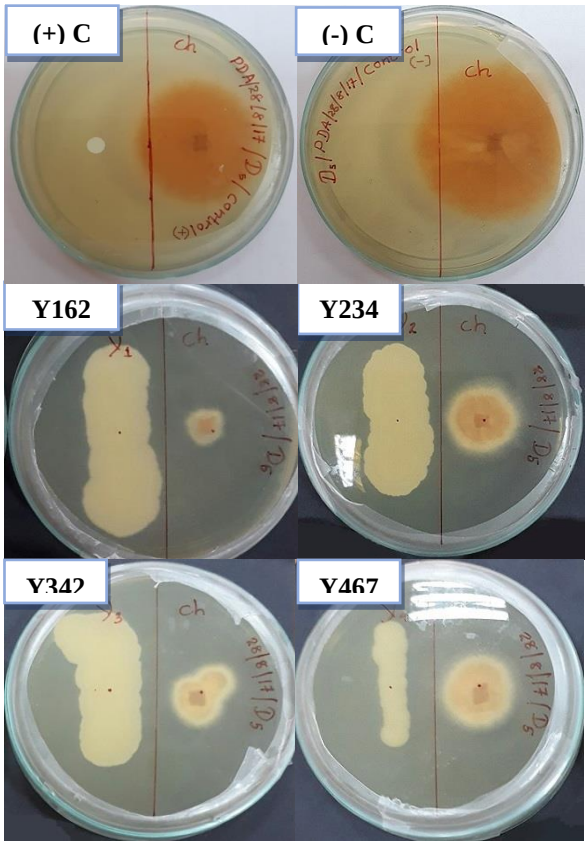


Figure 02: Growth of *C. gloeosporioides* in duel culture assay with antagonist yeast after seven days at 28 ℃ with positive and negative controls.((+C) : positive control; (-)C : negative control) (left side of the petri dish yeast variety ; right side of the petri dish *C. gloeosporioides.*)

3.4 Papaya disease incidence after antagonist yeast treatment

After eight days of storage, there was no statistically significant difference between the Y162 treatments and the control in terms of the disease incidence on the naturally infected fruits (Disease incidence of the control (%), 100, Disease incidence of the Y162 treated sample (%), was 66.2 % (One way ANOVA, $P > 0.05$)(Figure 03). However, the fruits treated with Y162 had a much lower observable severity than the control fruit. According to the results, Y162 was able to lower the severity of the disease incidence on the papaya even if it was unable to considerably prevent disease occurrence.

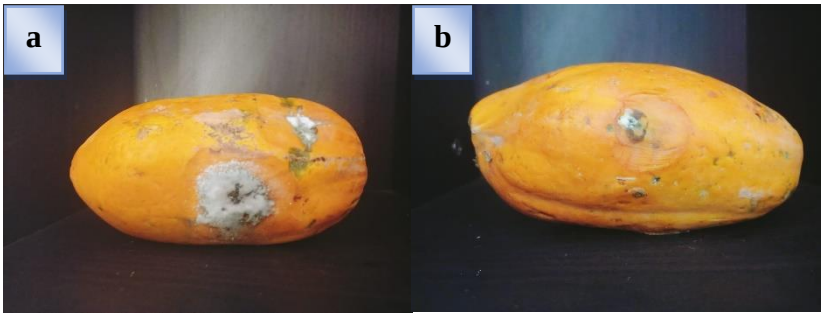


Figure 03: Disease incidence observed in a) the control papaya sample treated with sterilized distilled water and b) papaya sample treated with Y162 yeast solution.

4.0 Discussion

Biocontrol methods are becoming more popular due to their many advantages[12]. As a result, many antagonistic organisms have been identified and successfully patented as postharvest biocontrol agents[2][13]. However, these biocontrol agents could be dangerous to non-targeted organisms [14] and this could be minimized by selecting antagonistic agents from the same environments [15]. Therefore, the present study targeted, isolating an effective antagonistic yeast species from the local environment to control anthracnose in papaya, in Sri Lanka.
Although various sources from the local environment were used to isolate yeast species, yeast species that were isolated from *Psidium guajava L.* (Guava) leaf surface gave the best results for the dual culture assay where the PIRG % value was 59.3±2.0. The yeast isolated from *Cocos nucifera* (Coconut) water also gave more 50%, PIRG value. The yeast that was isolated from papaya leaves gave comparatively lower results. However, better results were received for yeast species isolated from papaya leaves, petioles, and fruit surfaces of papaya plants in a study conducted by Hassan et al. in Malaysia [2].

The antagonistic yeasts are anticipated to work through a variety of mechanisms, such as host resistance induction, competition for resources, mycoparasitism, and formation of secondary metabolites as toxins [16][17]. On the other hand, pathogens also contend with antagonistic yeasts for resources and habitat, which affects their colonization and development[16]. Additionally, a variety of environmental factors also affect the effectiveness of antagonistic yeasts [18][19]. All these factors must have more or less affected on the final PIRG% values of the studied yeast species. The most interesting finding was, all the yeast varieties isolated gave higher results than the positive control, Fluconazole. Fluconazole destroys pathogens by preventing the formation of ergosterol, which is essential for the fungal cell membrane and raises cellular permeability [20]. The action of yeast could be faster and more effective than this method of action of Fluconazole, on C. *gloeosporioides*.

Due to its efficiency in *in vitro* experiments Y162 was selected for further study as a possible antagonist agent against *C. gloeosporioides.* Again, Y162 confirmed its efficiency, by showing only 66.2% disease incidence, compared to 100% disease incidence in the control. Many studies revealed that after being applied to fruit surfaces, antagonist yeast interacts with host tissue to trigger the development of protective enzymes [21] [22]. Another essential component for the effective biocontrol activity of yeast is the attachment of it to the pathogen hyphae. This feature allows antagonistic agents to hinder the pathogen from starting an infection [23]. Due to direct attachment, yeast can absorb nutrients more rapidly than the target pathogens, reducing their spore germination and development. Arras et al., [24] reported this for the activity of *Pichia guilliermondii* (antagonistic yeast) on *Penicillium italicum*, a fungal pathogen in citrus fruit. Therefore, the presence of yeast should be there simultaneously with the infection or immediately after the infection. Consequently, the application of the yeast should be done directly after the harvest because *C. gloeosporioides* usually infect the papaya even before the harvest while it is on the tree.
As the future work of the study, molecular identification of the yeast Y162 should be carried out and it is required to acquire a better knowledge of the biocontrol activity of Y162 to prevent any negative impacts of it on people and the environment when used as a biocontrol agent.

5.0 Conclusions

Yeast isolated from *Psidium guajava* L. (Guava) leaf surface, showed the highest inhibitory action. In vitro and in vivo experiments with this antagonistic yeast revealed that *C. gloeosporioides* development was inhibited by its actions. Therefore, we can conclude that this antagonistic yeast variety has a strong potential for application as a biological agent to combat

the anthracnose caused by *C. gloeosporioides* in papaya in Sri Lanka.

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