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Postharvest Application of Antagonistic Yeast, *Candida metapsilosis* to Control *Colletotrichum gloeosporioides* Caused Anthracnose Disease on Mango Fruits and Possible Mechanisms

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ABSTRACT:

The research was tested efficacy of antagonistic yeast Candida metapsilosis to control the pathogen Colletotrichum gloeosporioides on mangoes. This research found, antagonistic yeast C. metapsilosis showed high inhibition to C. gloeosporioides of spore germination at 33.23%. The result of disease incidence on mango that treated with yeast and spore of pathogen showed disease symptom at level 2 (4.15%). Control of anthracnose disease control at 14 days storage time, the mango that treated with antagonistic yeast and sodium bicarbonate demonstrated great result, because these not found the disease incidence. However, mango in the control group showed highest of disease at 69.00%. From all results, yeast C. metapsilosis was play role to control pathogen by hydrolytic enzyme, nutrients, and space competition. The nutrients mechanism found that, mango treated with yeast and spore of pathogen could be presented good of disease incidence (DI) at 71.17%. Addition possible of mechanism, space competition showed the mango tested with yeast and simultaneous drip with pathogenic spore of pathogen had highest of inhibition rate (IR) at 37.38%. The important of both mechanisms, yeast can produce and secreted chitinase enzyme to damage pathogen. This enzyme activity found the average activity at 156.40 U/ml. The mango that treated with antagonistic yeast C. metapsilosis showed great resulting to control spore germination of pathogen, high capacity to reduce anthracnose lesion development and good action to control pathogen for 14 days storage time. Additional reason of yeast, that play role by an important mechanism; nutrient, space, and enzyme activity to damaged and control the pathogen C. gloeosporioides of anthracnose disease on mango fruits.

Keywords: Antagonistic yeasts *Candida metapsilosis*, Mango, Anthracnose disease, *Colletotrichum gloeosporioides*

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INTRODUCTION

Postharvest diseases are the main factors causing damage to postharvest fruits and vegetables during transportation and distribution. Anthracnose is a major disease-causing damage to many fruits such as apples, avocados, bananas, mangoes, etc. [1]. The disease causes up to 20-30% of the damage and more than 50% of the fruit production industry [2]. *Colletotrichum gloeosporioides* (Penz.) Penz and Sacc, is the main pathogen in mango and resulting in a decrease in quality and productivity. In the past, chemicals were used to control diseases in mangoes. Mango (*Mangifera indica* L.) is a popular fruit consumed worldwide. It is grown in many countries and sold worldwide [3]. It is a good quality mango of Thailand. It looks like a mango that has a distinctive feature: large fruit, long shape, thick skin, delicious taste, suitable for export. However, crops were destroyed by anthracnose in post-harvest and transportation stages. More than 50% of the destruction by anthracnose disease was found in crop plant [3]. For control of anthracnose, benzimidzole has been used to kill pathogenic fungi in the past. These chemicals are harmful to humans and pathogenic fungi have been found to be more resistant to these chemicals [4]. Pathogen control instead of this chemical, yeast and bacteria have many advantages in biological control of plant pathogens. There is a research report on the use of yeast species, including *Pichia membranifaciens* to control and inhibit the growth of *Rhizopus* fungi that cause fruit rot disease and antagonist yeast *P. guilliermondii* was used to controlling pathogenic fungi of type *C. capsici* and *C. gloeosporioides* that causes anthracnose disease in chilli fruit and *Candida membranifaciens* is also used same role. It can also be used to control anthracnose disease in mangoes fruits [5], which is consistent with research by [6] used yeast antagonists combined with NH_4MO to control and inhibit mycelium growth and germination of spores of *C. gloeosporides* in mangoes. This study found that yeast isolate VCU24 combined with 0.5% NH_4MO was the most effective in inhibiting pathogenic mycelium by 57.1% and spore germination by 78.6% compared to the control group. The combined application of yeast cultures with these agents can promote the inhibition of pathogenic fungi. In addition, there have been reports of the use of this agent in conjunction with yeast *Hanseniaspora uvarum* to inhibited mycelium growth well in grapes [7], including *C. membranifaciens* yeast. Several yeast isolates from fruits and vegetables have been reported, including *P. guilliermondii*, *Candida musae*, *C. quercitrusa* and *Issatchenkia orientalis* effective to control the mycelium of *Colletotrichum capsici*, causative agent of anthracnose in chilli fruit [5]. In addition, the use of yeast antagonist *C. metapsilosis* has been reported to be effective in controlling pathogenic mycelium of *C. capsici* were also found to have 95.16% survival of chilli fruit from anthracnose and this yeast was able to generate and secrete chitinase, which is an important mechanism for disease control by chitinase activity was 5,071.11 mlU/mg protein [8]. The yeast strain *Candida haemulonii* had good effect and positive effect on chilli fruit with survival rate 66.25% at 20 days after postharvest storing time [9]. *Candida* yeast antagonists, especially *C. metapsilosis*, is an interesting because of their good results and does not cause disease in humans. In Thailand, a yeast antagonist *C. tropicalis* has been reported to control fruit rot disease in mango caused by pathogenic

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fungi, *Lasiodiplolodia theobromae* [10]. However, usage of antagonistic yeasts for the control of anthracnose in mangoes has not been reported. The management and techniques for biocontrol still have many limitations because the specificity of biocontrol agents for each pathogen is not established [11]. The application of yeast as a biocontrol agent is a promising approach for biocontrol and good control of postharvest diseases in fruit [12]. Yeast strains are also capable use to control wide range of pathogenic fungi [13]. In this study, *C. metapsilosis* was used to control postharvest anthracnose in mango. In Kalasin it's also province that produces a lot of mangoes for sale, but anthracnose causes a drop in yield. Therefore, the objective of this research is to use yeast species *C. metapsilosis* to control anthracnose caused by pathogenic *C. gloeosporioides* of mango fruit after harvest.

METHODS

2.1. Experimental; fruit, antagonist yeast and pathogen fungi

This research used mango (*M. indica* L.) from community market, Muang district, Kalasin province, Thailand. We used the healthy mangoes, no wounds, no disease and fruits are same size. Then, washed with sterile distilled water and dried. The purified antagonistic yeast *C. metapsilosis* was collected from chili farmers' plots, Bueng Wichai Subdistrict, Mueang District, Kalasin Province, Thailand. This yeast was cultured on yeast malt extract agar (YMA) medium and stored at 4°C until testing. For pathogen fungi, *C. gloeosporioides* was separated from diseased mango fruit by cutting the diseased mango skin into small pieces. Next, placed on potato dextrose agar (PDA) agar medium and incubated at 30 °C for 14 days or until the fungi was grew in culture plate. After that, used cork borer No. 5 to puncture the tip of the mycelium and culture it on PDA medium and incubate at 30 °C for 7 days until pure fungi are obtained and stored the fungi at 4°C until testing.

2.2 Efficacy of yeast antagonist *C. metapsilosis* to control pathogenic fungi *C. gloeosporioides*

2.2.1 Inhibition of spore germination on mango fruits

Cultivation of *C. gloeosporioide*s on PDA medium for 14 days until spores were obtained. Spores were counting by hemacytometer concentration at $5x10^8$ spores/ml. Yeast antagonist *C. metapsilosis* was cultured on YMA medium for 5 days and counted by hemacytometers to obtain concentration at $4x10^8$ cells/ml. Spore and yeast cell solutions were mixed 1 ml in 20 ml of potato dextrose broth (PDB), mixed well, and incubated at 30 °C for 24 h. Mangoes were cleaned with 1% sodium hypochlorite for 5 min followed by sterile distilled water and desiccate in the air drop. Then drop spore solution 1 ml and yeast cell 1 ml onto the marked area of the mango fruit. For control group, sterile distilled water was used instead of yeast cells. Next, placed mangoes in a plastic box and incubated at 25 °C for 5 days. For the control group, sterile distilled water was used instead of yeast cells. The percentage inhibition was calculated from the formula.

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Percent inhibition (%) = (AB)/A x100

Where A = spore germination of test group
B = spore germination of control group

2.2.2 Disease incidence of anthracnose disease on storage quality of mango fruits

Mangoes were cleaned with 1% sodium hyperchloride for 5 min followed by sterile distilled water and desiccate in the air. The yeast antagonist *C. metapsilosis* and spore of *C. gloeosporioide*s were used the same concentration with 2.2.1. The mangoes were immersed in 300 ml of yeast cell solution for 40 minutes. Sterile distilled water was used for the control group. Mango fruits were placed in plastic boxes and incubated at 25°C for 14 days. Natural lesions were collected, and lesion was scored as follows: 0=no lesion, 1=2-3 wounds/fruit, 2=3-4 lesions/fruit or lesions occur less than 5% of the fruit, 3=wounds occur 5-12% of the fruit, 4=wounds occur 13-25% of the fruit, 5=wounds occur 26-50% of the results and 6=more than 50% of the lesions occurred.

2.2.3 Control of anthracnose disease on mango fruits after harvest

This tested used concentration of yeast cell and pathogen spore at $4x10^8$ cells/ml and $5x10^8$ spores/ml, respectively. The test was performed using antagonistic yeast compared with sodium bicarbonate 2%, mancozeb and control group. All mangoes were incubated at 25°C for 7 days and 14 days. The lesions of disease were collected by scoring of anthracnose disease and experiments were divided as follows: T1=mango soaked in yeast cell solution for 5 minutes, T2=mango soaked in yeast cell solution for 10 minutes, T3=mango soaked in 2% sodium bicarbonate solution for 5 minutes, T4=mango soaked in 2% sodium bicarbonate solution for 10 minutes, T5=Mango soaked in mancozeb solution (1 g/1 liter) for 5 minutes, T6=mango soaked in mancozeb solution (1 g/1 liter) for 10 min and T7=Control group

2.3 Possible mechanism of yeast antagonist

C. metapsilosis to control *C. gloeosporioides* on mango fruits.

2.3.1 Assessment of nutrients competition

Mangoes were cleaned and make an incision on the skin 1 ml deep with cork borer No. 5, drip nutrients into the wound. The carbon source was used 2% of glucose/l (G), nitrogen source was used 3% of potassium nitrate (N) at 50 µl for each source, after which 25 µl of yeast cell solution was added at the concentration $4x10^8$ cells/ml. (A), and 25 µl of pathogenic spores' solution at a concentration $5x10^8$ spores/ml (P), for the control group used 50 µl of phosphate solution (pH 6.5) instead of the above nutrients. Mangoes were stored in plastic boxes at 25 °C for 6 days. The treatments were divided to: T1=Control T2=A+P, T3=A+P+G and T4=A+P+N. The increase in development of disease was calculated with following:

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$$\text{Disease incidence (\%DI)} = LD_R/LD_C \times 100\%$$

Where, LD_R = wound diameter of the test treatment
LD_C = 2nd treatment wound diameter (A+P)

2.3.2 Assessment of space competition

Mangoes were cleaned make a 1 ml deep incision on the mango skin with cork borer No. 5. Yeast cultures and fungal spores were used at $4x10^8$ cells/ml and $5x10^8$ spores/ml, respectively. The test was divided into 6 treatments, each treatment was dripped with 25 µl of solution. All mangoes were placed in a plastic box at 25 °C. for 6 days. The treatment divided in to: T1 = control group (25 µl sterile distilled water + 25 µl pathogenic spores simultaneously dripped), T2 = Yeast infusion 12 h before, followed by pathogenic mold spores, T3 = Yeast infusion 24 h before, followed by pathogenic mold spores, T4 = Drops of pathogenic mold spores 12 h first, followed by yeast, T5 = Drops of pathogenic mold spores 24 h first, followed by yeast and T6 = Simultaneous drip of pathogenic mold spores and yeast. The inhibition rate (IR) was collected according to the following formula:

$$\text{Inhibitory rate, \%IR)} = (LDC - LDR)/LDC \times 100\%$$

Where, LD_C = wound diameter of control group
LD_R = wound diameter of test group

2.3.3 Assessment of chitinase activity of yeast antagonist *C. metapsilosis*

Chitinase activity was measured according to the method of [14], using colloidal chitin azure (remazol brilliant violet 5R, Sigma-aldrich, C3020) as a substrate. The absorbance was measured at 550 nm by the chitinase unit, measured from the chitinase standard curve from *S. griseus*, C6137. By 1 unit/ml of chitinase enzyme activity is 1 µmol of N-acetyl-D-glucosamine. released from chitin in 1 h was calculated from the formula:

$$\text{Enzyme units} = 4xnxA_{280}/(0.01x10)$$

Where n = dilution rate
4 = final volume in reaction (4 ml)
10 = Reaction curing time (min)

2.4 Data analysis

Percent inhibition of spore germination, score of disease severity, activity of the enzyme chitinase were tested by one-way ANOVA using SPSS 19.0 for Windows program. The mean difference was determined by Duncan multiple range test at the confidence level of $P<0.05$.

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3. RESULTS AND DISSCUSSION

3.1 Characteristics of *Candida metapsilosis* and *Colletotrichum gloeosporioides*

Characteristics of yeast *C. metapsilosis* when cultured on YM agar medium. The colonies presented mucoid, glistering, and raised when yeast cells are examined under a microscope. It was found that yeast cells are solitary cells and there are sprouts for the average cell size. This species was identified by nucleotide sequences, the D1/D2 region, 600 bases long, lies on the 5´ side of the 26s rDNA with D1/D2 being the fast-evolving and divergent regions of bases [15]. Comparing the nucleotide sequences of the yeasts on the Genbank database, found this strain was showed *C. metapsilosis* as in the clade Metschikowia. [16]. Characteristic of *C. gloeosporioides* after inoculation 72 h was found it can cause disease starting to see the surface of the fruit collapse into a small point. When the fungi were isolated by growing them on PDA media, it was found that the colonies first formed white mycelium, later changed to pink and gray. It produces spores called conidia that are crescent-shaped. This type of mycelium grows relatively slowly. The colonies will fully develop on the surface of the feed when incubated at 30 °C for 14 days (Figure 1). This morphological feature is consistent with stating that fungi *Colletotrichum* sp. The conidia spores are crescent-shaped, including *C. capsici*, *C. acudatum*, *C. falcatum*, etc., with long, black-brown spines setae and [17] isolated *Colletotrichum* sp. from chili plants and indicates that when growing on the fruit, it produces black ulcers, clustered with acervulus (85-245 µm long), brown to black spines setae (70-135 µm long); crescent (17-26 µm long). Growing on PDA, mycelium is white and by age 10 it is 85 mm in diameter. When sequencing ITS, it was *C. capsici* and this species grew relatively slower on PDA medium.

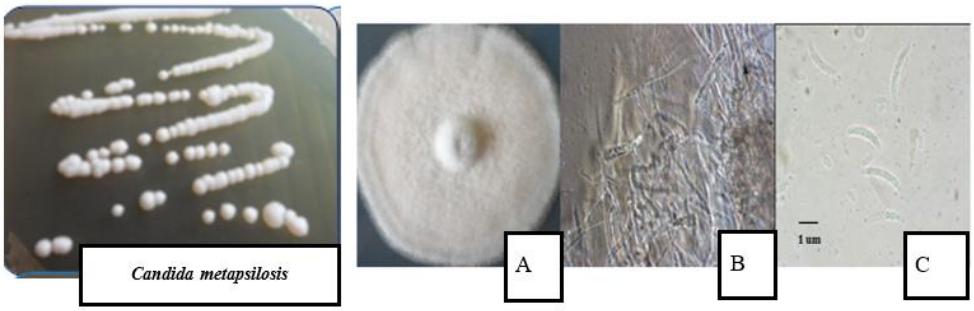

Figure 1. Characteristic of yeast *C. metapsilosis* on YM agar and characteristic of *C. gloeosporioides* (A) on PDA medium (B) conidiophore (C) conidia spore

3.2 Efficacy of yeast antagonist *C. metapsilosis* in controlling *C. gloeosporioides*

Inhibition of spore germination on mango fruit that incubated at 25 °C for 5 days and inhibition percentage was collected. The result found inhibition at 33.23%. For disease incidence of anthracnose disease on storage quality of mango fruit at 14 days was found at 4.15% and conclude into level 2. However, in control group showed 16.99% of disease incidence at level 4 (see table 1). Anthracnose disease control on mango fruit after harvest, the result found that

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after incubating for 7 days, most of the mangoes had no lesions. There was pathogenicity at level 0, except mangoes in the T6 test group (10 min mancozeb infusion) had 1 score of disease (1.20%) and the diseased group in the T7 control group had 2 score of disease (3.75%). For disease incidence at 14 days on mangoes found that in groups T1, T3 and T4 were not detected. However, T2 with yeast infusion for 10 min had a slightly disease incidence at level 1 (0.89%), While disease incidences were found in mango that treated with mancozeb chemical at 46.70% and 56.00% (see table 2 and figure 2). All results indicated that the antagonist yeast was effective in controlling pathogenic fungi on mango fruit up to 14 days after harvest.

Table 1. Disease incidence of anthracnose disease on storage quality of mango fruit at 14 days

Rep	Treatment			
	Control		Test	
	Disease score	Disease incidence)%)	Disease score	Disease incidence)%)
1	4	24.72	0	0.00
2	2	5.00	3	8.27
3	4	21.25	2	4.20
Average	**4**	**16.99**	**2**	**4.15**

Table 2. Control of anthracnose disease on mango fruit after harvest at 7 days and 14 days

Treatments	7 days		14 days	
	score	Disease incidence)%)	score	Disease incidence)%)
T1)yeast infusion, 5 min(	0	0.00	0	0.00
T2)yeast infusion, 10 min(	0	0.00	1	0.89
T3)sodium bicarbonate 2%, 5 min(	0	0.00	0	0.00
T4)sodium bicarbonate 2%, 10 min(	0	0.00	0	0.00
T5)mancozeb, 5 min(	0	0.00	5	46.70
T6)mancozeb, 10 min(	1	1.20	6	56.00
T7 control	2	3.75	6	69.00

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Figure 2. Control of anthracnose disease on mango fruit after harvest at 7 days and 14 days, T1: yeast infusion 10 min, T2: yeast infusion 10 min, T3: sodium bicarbonate 2% 5 min, T4: sodium bicarbonate 2% 10 min, T5: mancozeb 5 min, T6: mancozeb 10 min, T7: control

Efficacy of yeast *C. metapsilosis* to controlling *C. gloeosporioides*, found that yeast can inhibited spore germination of pathogen at 33.23%. After 5 days of mango harvest, the antagonist yeast infection was still able to colonize on the mango fruit and, good control of pathogenic mold spores because if the antagonist yeast is not used in the control, it will be found it on mango fruits and will be spoiled quickly. Because the anthracnose fungus *C. gloeosporioides* able to grow quickly was up to 30% if it was attached to the mango fruit for 4-8 days [18]. Disease incidence of anthracnose on mango fruit, it was found that the mangoes in the control group had highest of disease incidence at 16.99%. The mangoes in the test group with pathogenic spores and yeast spores had the incidence of 4.15%, which was low and clearly more than control group. The results were similar with [19] who tested the disease incidence in Nam Dok Mai mango. The yeast strain *Issatchenkia orientalis* controls the anthracnose pathogen caused by *C. gloeosporioides*. Disease incidence was 1.88% in the yeast-treated group and 2.63% in the control group. However, the antagonistic yeast strains were effective in controlling the fungal pathogens on mango fruits up to 14 days after harvest. Anthracnose control test on mango fruits after harvesting period showed that after incubation for 7 days, most of the mangoes were not lesions or diseased. But when the mangoes were incubated for up to 14 days, it was found that the mangoes in the antagonistic yeast-infected group that received sodium bicarbonate gave the best results. No occurrence of the disease was found. The high disease incidences were found on mangoes that treated with mancozeb solution and in control. The pathogenicity was at level 6 (69.00%), followed by mangoes soaked in mancozeb for 10 minutes, pathogenic at level 6 (56.00%), and mangoes soaked in mancozeb for 5 minutes had pathogenicity at level 5 (46.00%). 70%), respectively. The treatments that using yeast cultures still gave a good control resulting although not equivalent to sodium bicarbonate. However, the mangoes in the mancozeb-treated group showed poor results because the incidence of mangoes was like the highest disease incidence of the control treatment. The disease was occurred first on the fruit poles and then spreads to other parts of the mango fruit. In previous study, mancozeb was showed great in control to pathogen and was found that the inhibition was up to 60%, but it might be due to testing in different stages of pathogenic fungi.

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They are also different types of fruits and the amount of intensity of substances used is different. From all reasons that showed relationship of yeast antagonist *C. metapsilosis* in controlling pathogen *C. gloeosporioides* on mango in secretion hydrolytic enzyme and nutrient and space competition.

3.3 Possible mechanism of yeast *C. metapsilosis* to control *C. gloeosporioides* on mango fruits.

Assessment of nutrient competition between antagonist yeast *C. metapsilosis* and the pathogenic fungus *C. gloeosporioides*, it is an important mechanism for control of anthracnose disease on mango fruits. The result found group 3 (A+P+G) that received yeast cell solution 2% with pathogen spores and activated carbon had highest disease incidence at 89.52%, followed by group 4 (A+P+G+N) received yeast and pathogen spore and 3% of potassium nitrate at 81.21% and group 2 (A+P) received yeast and fungal spore showed disease incidence at 71.17% (see figure 3). Mechanisms of space competition is another mechanism to study the association of yeast antagonist *C. metapsilosis* to control pathogen *C. gloeosporioides* on mango fruits in the different time at 6 days. The inhibition found that treatment 6 had highest inhibition rate at 37.38%, followed by treatment 3 (24.35%) and treatment 5 (22%) respectively. The inhibition of all groups was significantly different ($p<0.05$) (figure 4). Addition mechanism is chitinase activity of yeast *C. metapsilosis*. The result found this strain had an average enzyme activity at 156.40 U/ml, total protein content at 96.36 mg protein, and a specific chitinase activity at 1.62 U/mg protein (see table 3).

The mechanism of yeast antagonist *C. metapsilosis* to control *C. gloeosporioides* on mango fruits. The mango that received yeast cell solution with spores of pathogenic fungi and 2% carbon had highest of disease incidence at 89.52%, followed by group treated with yeast cell solution plus pathogenic fungal spores and 3% potassium nitrate (81.21%). From the experimental results, it was found that mangoes fed with only the antagonist yeast infested gave the best results. Maybe because of the treatments were added carbon and nitrogen, caused the pathogenic fungi can used this carbon source as an energy source for cell division and growth as well as using nitrogen for protein synthesis. In addition, carbon is also a factor that affects to production and secretion of chitinase enzymes of antagonist yeast fungi. Addition mechanism of yeast antagonist *C. metapsilosis* to inhibit the pathogen *C. gloeosporioides* by regarding the chitinase activity, the yeast antagonist *C. metapsilosis* had an average enzyme activity at 156.40 U/ml, an average total protein content of 96.36 mg protein, and a specific chitinase activity of 1.62 U/mg protein. In addition, *P. guillermondii* yeast produced more chitinase when cultured in medium containing with CWP, this enzyme will promote yeast cells to adhere the pathogenic fungal hyphae more effectively and good chitin digestion of the pathogenic fungal wall. This result is depending on the proteins responsible for memorization between yeast cells and mycelium, as a single peptide protein, the C-terminal side has a base that signals the transport of enzymes to the chitin of the cell wall of pathogenic fungal hyphae [20]. The secretion of this enzyme is also one mechanisms of yeast induces plant resistance to

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pathogenic fungi. The pathogenesis of fungi is a good stimulus for the yeast to recognize that it has invaded thereby rapidly producing enzymes to digest pathogenic fungal hyphae [21]. This enzyme mechanism is efficacy controlling anthracnose disease caused by pathogen *C. gloeosporioides*. Another mechanism to compete for living space of yeast antagonist *C. metapsilosis* and pathogen *C. gloeosporioides*. The mangoes that inoculated with pathogenic spores and yeast had the highest inhibition rate (37.38%), while the mangoes that were inoculated with yeast infestation before or after had less inhibition. This may be because if the pathogenic fungi are dropped first, the fungi can enter and colonize on the mango skin and grow faster. When the yeast infection was dripped down, it can lessen to inhibited of mycelium of pathogen. But, when it drops at the same time, both pathogenic fungi and yeasts compete for clinging to the surface of the mango. The yeast may cling well because it is a cell whereas the pathogenic fungi are spore-forming. As a result, the antagonist yeast bacteria compete for more living space [22].

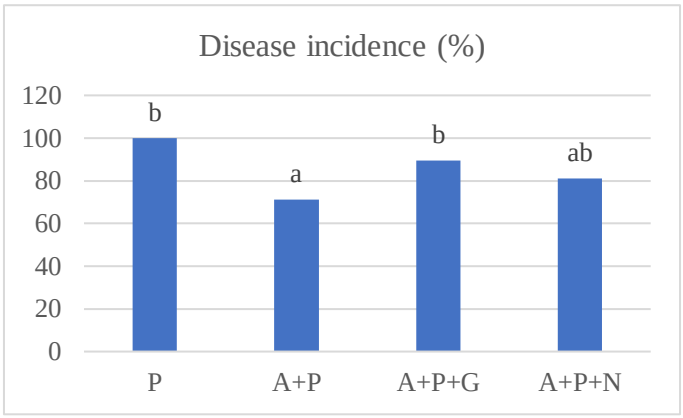

Figure 3. Disease incidence from nutrient competition of yeast antagonists *C. metapsilosis* and *C. gloeosporioides* on mango fruits. The different letters a, b, c in figure were statistically difference at the 95% confidence.

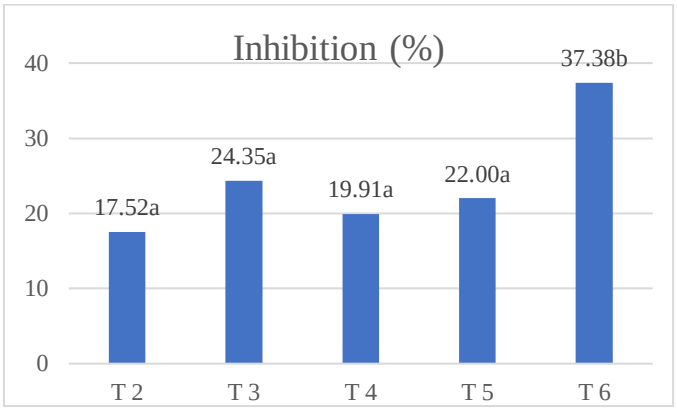

Figure 4. Inhibition rate from space competition of yeast antagonist *C. metapsilosis* and *C. gloeosporioides* on mango fruit at 6 days. The different letters a, b, c in figure were statistically difference at the 95% confidence.

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Table 3. Chitinase activity and protein content of yeast *C. metapsilosis*

Rep	Chitinase (U/ml)	Protein (mg)
1	136.70	67.65
2	145.00	113.70
3	155.87	100.33
4	175.20	96.41
5	169.25	103.72
average	**156.40**	**96.36**

CONCLUSIONS

The mangoes in group treated with a yeast antagonist, *C. metapsilosis*, had an inhibitory effect to germination of pathogenic fungal spores, reduce the development of anthracnose or disease incidence on mango fruit, control of anthracnose disease on mango fruits after harvesting up to 14 days better than mancozeb chemical. The mechanisms by yeast to control anthracnose diseases include competing for nutrients, space and chitinase enzymes activity.

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