การใช้ยีสต์ *Issatchenkia orientalis* เพื่อป้องกันกำจัดโรคแอนแทรคโนสของผลมะม่วงหลังการเก็บเกี่ยว

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บทคัดย่อ

งานวิจัยนี้เป็นการศึกษาประสิทธิภาพของยีสต์ *Issatchenkia orientalis* ในการป้องกันกำจัดโรคแอนแทรคโนสของผลมะม่วงหลังการเก็บเกี่ยว โดยจากผลการทดสอบการเลี้ยงเชื้อร่วมกันบนอาหารเลี้ยงเชื้อในห้องปฏิบัติการพบว่า ยีสต์สามารถยับยั้งการเจริญของเส้นใยและการงอกของสปอร์ของเชื้อรา *Colletotrichum gloeosporioides* ซึ่งเป็นสาเหตุของโรคแอนแทรคโนสได้ ในส่วนของการทดสอบบนผลมะม่วงได้ผลเช่นเดียวกัน คือสปอร์ของเชื้อราไม่สามารถงอกได้ และพบว่าส่วนผิวของสปอร์ที่มีของยีสต์เกาะติดอยู่มีลักษณะยุบตัวลง การศึกษาแสดงให้เห็นว่าสารระเหยบางชนิดที่ยีสต์สร้างและซึมแผ่ไปในอาหารเลี้ยงเชื้อมีผลในทางลบต่อการเจริญของเส้นใยของเชื้อราอันเป็นสาเหตุของโรคแอนแทรคโนส โดยผลการทดสอบประสิทธิภาพการป้องกันกำจัดโรคโดยแช่ผลมะม่วงในยีสต์แขวนลอยในน้ำกลั่นเป็นเวลา 40 นาที พบว่าสามารถลดการเกิดโรคแอนแทรคโนสบนผลมะม่วงหลังบ่มสุกได้ดี ประสิทธิภาพการป้องกันกำจัดโรคเพิ่มขึ้นเมื่อใช้ยีสต์ร่วมกับการใช้น้ำร้อน คือ แช่ผลมะม่วงในน้ำร้อนอุณหภูมิ 52 องศาเซลเซียส นาน 5 นาที แล้วตามด้วยการแช่ในยีสต์แขวนลอยในน้ำกลั่นนาน 30 นาที

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Postharvest Application of *Issatchenkia orientalis* for the Control of Anthracnose of Mango

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Abstract

The effectiveness of the yeast *Issatchenkia orientalis* in the control of anthracnose of postharvest mango was evaluated. In vitro test showed that hyphal growth and spore germination of *Colletotrichum gloeosporioides* were inhibited in the presence of the yeast. Spores inoculated on mango fruits were also unable to germinate. Some spores were damaged due to the attachment of the yeast cells showing a sunken surface. Diffusible and volatile substances were produced by the yeast and had adverse effect on hyphal growth of the fungal pathogen. Application of the yeast by immersing the mango fruits in ayeast suspension (40 min) could reduce the postharvest development of anthracnose lesions. By integrating the use of the yeast and a hot water treatment (52°C, 5 min) the effectiveness in controlling mango anthracnose was increased.

Keywords : Anthracnose / Biocontrol / Mango / Postharvest / Yeast

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1. Introduction

Colletotrichum gloeosporioides (Penz.) Penz. and Sacc. cause anthracnose on mango, reducing the quality and marketability of the fruit. Immature, green mangoes show no symptoms from quiescent infection of the pathogen, but upon ripening, the disease develops and symptoms appear as dark brown or black spots. Fungicides are used as the main practice to prevent losses. Fungicide is sprayed for 8-10 times (7-day-interval) before wrapping the mango fruits in paper bags. Fungicide is also used in postharvest treatment by dipping the mango fruits in a fungicide solution before packing, storage and/or transportation to the markets. However, the use of chemical fungicides is becoming more restricted by the increasing concerns of toxic residues either in fruits or the environment. Moreover, several countries have launched new regulations, and as a result, using fungicides has now become more restricted. Therefore, alternatives counter this serious disease is crucially needed. Some of the most promising new methods use chitosan [1] and microorganisms.

Biocontrol agents, used against several postharvest fruit diseases, include fungi, bacteria and yeasts. Yeasts deserve particular attention as their mode of action tends to be competition for space and nutrients, rather than production of antibiotics or other toxic secondary metabolites [2]. Several yeast isolates showed distinct control of postharvest fruit diseases. Metschnikowia pulcherrima has been proved to be highly effective in the control of blue and grey mold of apple [3] as well as *Cryptococcus laurentii* for gray mold rot of pear [4] and *Pichia guilliermondii* for black rot of pineapple [5]. *C. oleophila* is another promising antagonistic yeast [6], which is currently registered by the Environmental Protection Agency in the United States for controlling postharvest diseases of apple, pear and citrus fruit.

For evident control results of postharvest diseases in tropical fruits,few microorganisms, including yeasts, have been reported. Among those limited isolates, *C. oleophila* effectively controlled anthracnose of papaya [7]. In Thailand, *C. tropicalis* showed high potential for the control of fruit rot, caused by *Lasiodiplodia theobromae*, on mango [8]. However, other yeast isolates, especially for the control of anthracnose of mango, and their effectiveness has not been reported.

Among 54 isolates of yeast isolated from fruits and vegetables, *Pichia guilliemondii*, *Candia musae*, *C. quercitrusa* and *Issatchenkia orientalis*, were effective in growth inhibition of *Colletotrichum capsici*, the causal agent of *chili anthracnose*, and could reduce disease incidence in chili fruits [9]. These yeasts showed potential as bio control agents of anthracnose and *I. orientalis* was of interest in this study.

This study evaluates the antagonistic effect of the yeast *Issatchenkia orientalis* against *C. gloeosporioides* and its effectiveness in preventing the development of anthracnose lesions on mango. Integrated use of the yeast with a hot water treatment was also evaluated.

2. Materials and methods

2.1 Source of Mango

Mangoes, cv. Nam Dok Mai, were used throughout. The mature mango fruits were harvested from the orchard in Agricultural Technology Research Institute, Rajamangala University of Technology Lanna, Muang district, Lampang. The fruits were washed with distilled water and air-dried. Those fruit showing no sign of lesion or abnormal appearance were selected

for the experiments.

2.2 Fungal and yeast cultures

C. gloeosporioides, the fungal pathogen of anthracnose, was isolated from mango, cultivar Nam Dok Mai, showing anthracnose symptom. A pure fungal culture was grown on potato-dextrose agar (PDA) and stored at 5°C until use. The yeast *Issatchenkia orientalis* was isolated from the ripe sapodilla (named in Thai as La Mud, scientific name: *Manilka razapota* (L.) P. Royen. Peel and flesh fruit (10 g) cut into small pieces, were shaken in 100 mL of sterile distilled water for 40 min at 150 rpm on a rotary shaker (New Brunswick, Model Imnova 2050, U.S.A.). Aliquots of serial dilutions of the wash were then plated onto PDA. The Petri dishes were incubated at room temperature (30+2°C) for 5 days and the creamy white colonies of the yeast were transferred to fresh PDA plates. The most effective yeast isolate was screened by a dual culture test. Pure culture of the selected yeast isolate was grown on PDA and stored at 5°C until use. Identification of this yeast isolate was carried out by BIOTEC, the National Science and Technology Development Agency and the result revealed it as *Issatchenkia orientalis*.

2.3 Inhibition of hyphal growth and spore germination in vitro

The hyphal growth of *C. gloeosporioides* in the presence of the yeast *I. orientalis* was observed. Three 7-mm-diameter agar discs taken from a 10-day-old PDA culture of *C. gloeosporioides* were transferred to 20 mL of potato and dextrose broth (PDB) in Petri dishes. Yeast cell suspension at a concentration of 2.8 10^8 cells/mL, prepared from 4-day-old PDA culture, was then added into the Petri dishes at different volumes (0.1, 0.5 and 1.0 mL). Sterile distilled water was used in the control treatment. There were ten replicated Petri dishes in each treatment. The Petri dishes were incubated at room temperature for 15 days. Dried weight of hyphal mass was collected from each treatment and compared to the control treatment.

Inhibition of spore germination of *C. gloeosporioides* by the yeast *I. orientalis* was evaluated. Spore suspension of *C. gloeosporioides* was prepared from 10-day-old PDA culture at concentration of 6.4 10^8 spores/mL. A cell suspension of the yeast *I. orientalis* was prepared from 4-day-old PDA culture at a concentration of 3.3 10^8 cells/mL. These two suspensions were then added, 1 mL spores and 0.2 mL yeast, into a flask containing 10 mL of PDB. There were 3 replications for each treatment. After 12 h of incubation at room temperature, spore germination of *C. gloeosporioides* was counted using a haemacytometer (BOECO, Germany) under light microscope (National, model 173MS, China)

2.4 Inhibition of spore germination on mango fruit

Spore suspension of *C. gloeosporioides* was prepared from 10-day-old PDA culture at concentration of 5.4 10^8 spores/mL. Cell suspension of the yeast *I. orientalis* was prepared from 4-day-old PDA culture at concentration of 4.3 10^8 cells/mL. For surface sterilization mangoes, cv. Nam Dok Mai, were immersed in 1% sodium hypochlorite, for 5 min. and then rinsed with sterile distilled water and left to dry. Suspension of the fungal pathogen and yeast was inoculated on the mango fruit in the same marked area, 1 mL each. For the control treatment, only a suspension of the fungal pathogen was inoculated. The mangoes were incubated in a plastic box for 5 days at room

temperature. Pieces of mango peel were then processed for scanning electron microscope observation. Spore germination in the presence of the yeast was investigated.

2.5 Production of inhibiting substances

Production of diffusible and volatile substances, which have adverse effect on growth of *C. gloeosporioides*, were evaluated in separate trials. For diffusible substances, a dual culture was carried out on PDA plates incubating for 15 days at room temperature. Colony appearance of the pathogen, both on the test and control plates, was then observed and compared. For volatile substances, a 7-mm-diameter agar disc taken from a 10-day-old PDA culture of *C. gloeosporioides* was placed in the center of PDA plate. The yeast was inoculated by streaking on another PDA plate. The plate containing the pathogen was inverted over the plate of the yeast. Both plates were then sealed together with polyethylene film. For the control treatment, each plate of the pathogen was inverted over fresh PDA plate. There were ten replicated plates for each treatment. The test plates were incubated at room temperature. The diameter of *C. gloeosporioides* was measured at 10 days after incubation and compared to the control treatment.

2.6 Reduction of anthracnose lesion development

Mangoes, cv. Nam Dok Mai, were washed in distilled water and left to dry. Cell suspension of *I. orientalis* was prepared from 4-day-old PDA culture at concentration of 10^8 cells/mL. The mangoes were then immersed in the yeast cell suspension for 40 min. For the control treatment, mangoes were immersed in distilled water for the same period of time. There were three replicates each with twenty fruits arranged in RCBD. Since this study paid attention on postharvest development of anthracnose after quescent infection broke out, there was no artificial inoculation. Hence, the disease that developed on mangoes was caused by natural infections. The mangoes were placed in plastic baskets and covered with newspapers. After fifteen days of incubation at room temperature, lesion development was assessed using the following scores: *0* = no disease; *1* = tiny lesion (pin-headed size) developed, 2-3 lesions per fruit; *2* = 3-4 lesions of 3 to 4–mm diameter size were found and the damaged area was less than 5 % on the fruit; *3* = 5-12% of damaged area found on the fruit; *4* = 13-25% of damaged area found on the fruit; *5* = 26-50% of damaged area found on the fruit; *6* = more than 50% of damaged area found on the fruit.

2.7 Integrated use of the yeast and hot water treatment

Mangoes, cv. Nam Dok Mai, were washed in distilled water and left to dry. Cell suspension of *I. orientalis* was prepared from 4-day-old PDA culture at concentration of 10^8 cells/mL. The mangoes were immersed in hot water (52°C) for 5 min then immersed in yeast cell suspension for 30 min. The mangoes in the control treatment, after hot water treatment, were immersed in distilled water instead. There were three replicates each with fifteen fruits arranged in RCBD. The mangoes were placed in plastic baskets and covered with newspapers. After fifteen days of incubation at room temperature, lesion development was assessed using the same scores previously mentioned.

3. Results and discussion

3.1 Inhibition of hyphal growth and spore germination in vitro

The hyphal growth of *C. gloeosporioides* in the presence of *I.orientalis* apparently decreased. Therefore, the dried weight of hyphal mass of *C. gloeosporioides*, grown in the presence of yeast, was less than that from the control plate where *C. gloeosporioides* was grown singly in a PDB plate. Used as an inoculum, the number of initial yeast cells affected the quantity of hyphal mass production as shown by the different dried weights of hyphal mass produced by the different initial yeast cells. The dried weights of hyphal mass of *C. gloeosporioides* were found to be 0.04, 0.03, 0.01 mg/plate with initial yeast cell inocula of 0.01 10^8, 0.07 10^8, 0.14 10^8 cells/plate, respectively (Table 1).

Spore germination of *C. gloeosporioides* was completely inhibited as indicated by 0.00% germination in a PDB flask containing yeast. By comparison in the control flask (no yeast), the percentage of spore germination was 85.78%.

Table 1 Dried weight of hyphal mass of *C. gloeosporioides* grown in the presence of *I. orientalis* and singly, at 15 days after incubation at room temperature.

Initial inoculum (mL/plate)	Concentration of yeast cells at initial (cells/plate)	Dried weight of hyphal mass of *C. gloeosporioides* (mg/plate)
0.1	0.01×10^8	0.04 b[1]
0.5	0.07×10^8	0.03 c
1.0	0.14×10^8	0.01 d
0.0 (control)	0.00	0.12 a

[1] Means followed by different letters were significantly different using Duncan's Multiple Range Test ($p<0.01$), C.V. = 28.92%

3.2 Inhibition of spore germination on mango fruit

Spores of *C. gloeosporioides* inoculated on mango fruit did not germinate. Normally, the spores germinate within 12 h after incubation. But, in the presence of the yeast, they did not. The surface of the spore appeared sunken (Fig. 1b). Some yeast cells attached on the spores of the fungal pathogen (Fig. 1c) Anthracnose lesion was not found on the yeast-treated mango fruits but found on the untreated ones. Abundant yeast cells were found on the surface of the treated mango fruits (Fig. 1a).

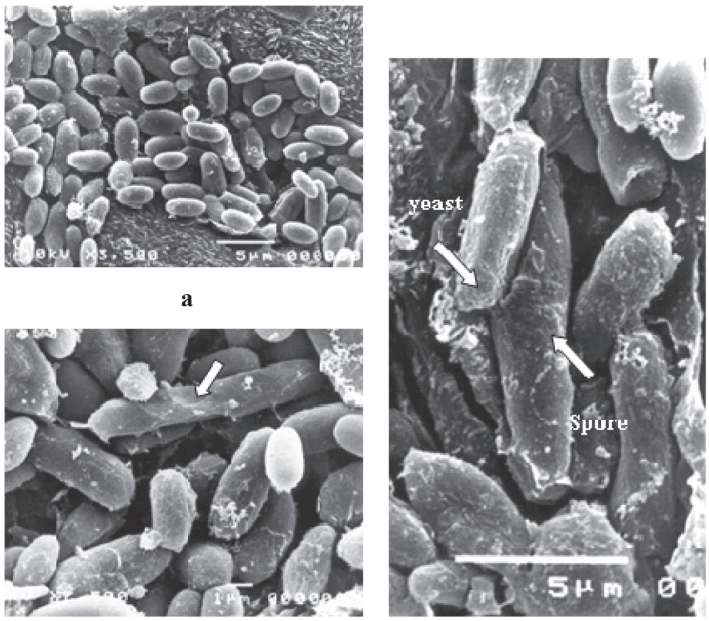

Fig. 1 Monographs of SEM showing (a) Abundant yeast cells on surface of the mango fruit, after 5 days of incubation, (b) Spore surface appeared sunken and (c) A yeast cell attached on surface of *C. gloeosporioides* spore.

The inhibition of hyphal growth and spore germination of *C. gloeosporioides* and the reduction of anthracnose lesion development on mangoes cv. Nam Dok Mai was demonstrated in this study. A sunken surface of the spores indicated that the yeast may secrete certain substances that damaged the spores. Competition for nutrient and space was not clearly demonstrated in the present study. However, rapid growth of the yeast in the culture medium indicated that this mechanism could be involved. Modes of action of the yeasts tend to be competition for space and nutrients, rather than production of antibiotics or other toxic secondary metabolites. The substances secreted by yeasts mostly are enzymes, e.g. polygalacturonase, β-glucosidase [2]. The yeast *Metschnikowia pulcherrimahas* shown to produce polygalacturonase [10].This yeast was proven to be highly effective in the control of blue and grey mold of apple [3]. The yeast *I. orientalis* is also used for winemaking and therefore, may have the ability to produce certain enzymes. A further study should clarify this assumption.

3.3 Production of inhibiting substances

I. orientalis produced diffusible and volatile substances that inhibited the growth of *C. gloeosporioides*. Dual culture on PDA revealed that certain substances were produced and diffused around the yeast colony. These substances retarded growth of the pathogen since the radius of the pathogen's colony in the test plate was shorter than that in the control plate. In addition, mycelia at the edge of the colony of *C. gloeosporioides* growing nearby the yeast's colony were scarce.

The production of volatile substances was also demonstrated in the present study. *C. gloeosporioides* challenged by *I. orientalis* in sealed plate and grew poorly. The average diameter measured at 10 days after incubation was 1.0 cm, while that of *C. gloeosporioides* grown singly in the control plate measured 7.01 cm.

3.4 Reduction of anthracnose lesion development

The yeast *I. orientalis* evidently showed effectiveness in reducing anthracnose lesion development on mangoes cv. Nam Dok Mai. The average disease score on mango treated with the yeast was found to be 1.88 while the un-treated one (control treatment) was 2.63.

3.5 Integrated use of the yeast and hot water treatment

Hot water treatment followed by immersion in yeast cell suspension gave the best control result in reducing anthracnose lesion development when compared to a single application, whereas a mango of the control treatment showed severe disease symptoms (Fig. 2). Disease scores of the control treatment, hot water treatment (HWT), yeast+HWT and yeast treatment were 5.23, 1.92, 0.27 and 1.86, respectively. The advantage of yeast+HWT is clear (0.27).

Integrated use of the yeast and other treatments, e.g. hot water treatment, chemicals, food preservatives, wax, etc. are mentioned in several reports [11-12] and proved to enhance the effectiveness of controlling the postharvest diseases in fruits. The finding in the present study confirmed that integrated use of yeast and hot water treatment provided the best treatment for mango anthracnose.

4. Conclusion

The yeast *I. orientalis* effectively reduced lesion development of anthracnose on mango. Its ability to inhibit hyphal growth and spore germination of *C. gloeosporioides* in vitro and on mango fruits was demonstrated. Integrated use of the yeast and hot water treatment controlled postharvest development of anthracnose lesions on mango fruits. The yeast *I. orientalis*, therefore, has a high potential to be used as bio control agent for mango anthracnose.

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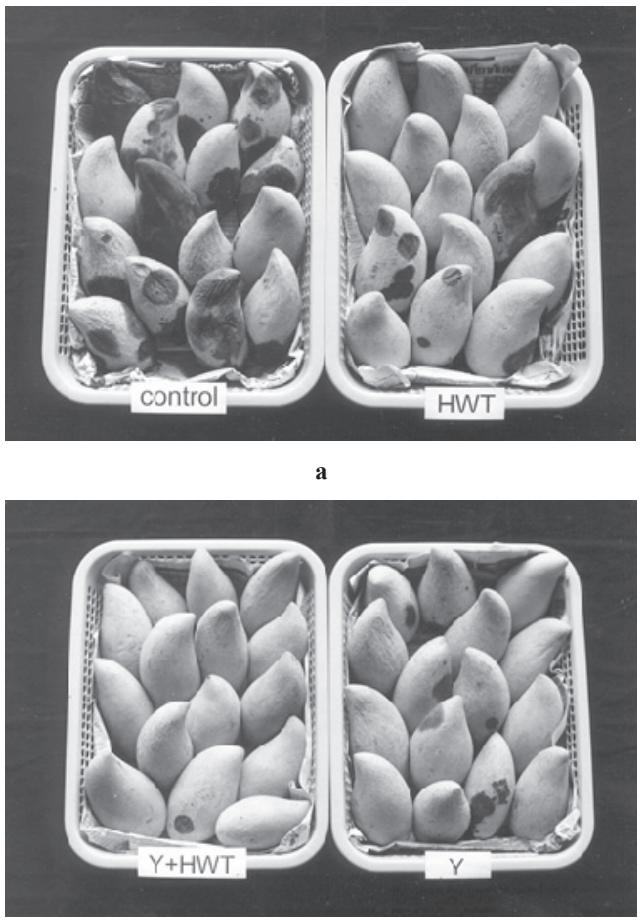

Fig. 2 Anthracnose lesions on mangoes of different treatments: control (distilled water), hot water treatment (HWT), yeast *I. orientalis* (Y)+HWT, and yeast alone (Y) where (a) Immersed mangoes in distilled water (left) and hot water (right) and (b) Immersed mango in yeast suspension followed by hot water (left) and in yeast suspension alone (right).

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