**Biology Research in Thailand**

# Combining Yeasts and Chitosan Treatment to Reduce Anthracnose Fruit Rot in Mangoes

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**Abstract**

'Choke Anan' and 'Nam Doc Mai' mangoes were wounded and treated with one of two yeast antagonists (*Candida* sp. isolate ns 5 and ns 9) for 12 h before soaking with chitosan (0.25% and 0.5%) and followed by inoculation with the anthracnose pathogen *Colletotrichum gloeosporioides*. Treated fruits were stored at 25°C for 7 days. The results revealed that anthracnose lesions decreased on fruit in whose wounds antagonistic yeasts had been allowed to colonize before inoculation with the pathogen. The combination of antagonistic yeast with chitosan was more effective on the reduction of anthracnose incidence than yeast or chitosan alone. *Candida* sp. ns 9 in combination with 0.5% chitosan was the most effective in controlling anthracnose fruit rot in 'Choke Anan' and 'Nam Doc Mai' mangoes in which the average percentages of disease incidences were 6.7% and 13.3%, respectively, compared with 93.3% and 100% infection in the control fruits. As for fruits treated with hot water (55°C for 5 min), the disease incidences in 'Choke Anan' and 'Nam Doc Mai' were 73.3% and 86.7%, respectively.

*Key words:* Antagonistic yeasts, anthracnose, *Candida* sp., chitosan, *Colletotrichum gloeosporioides*, mangoes

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## Introduction

The most serious postharvest disease developing worldwide in mango fruits is anthracnose caused by *Colletotrichum gloeosporioides*. Mango fruits suffer significant losses from anthracnose disease after harvest in Thailand. Postharvest application of synthetic fungicides has been the main method used for controlling the disease. However, fungicide-treated product has become unacceptable to consumers and importing countries because of the risk of chemical residues (Sangchote 1998). In view of public concern on health risks and the possibility of pathogens developing resistance to fungicides, it is important to explore alternative

control procedures. Biological control of storage rot with antagonistic microorganisms holds the promise of providing an alternative to synthetic fungicides for the control of rot diseases in fruits and vegetables. Effective antagonists have been found in the epiphytic microflora (associated with the surfaces) of plants. We were particularly interested in finding antagonists that did not produce antibiotics as part of their mode of action. These antagonists would be more acceptable to the public as no potentially harmful antibiotics would be introduced into the fruits. The treatment of fruits with antagonistic yeast is a promising alternative control of wound infection caused by rot pathogens during storage (Janisiewicz 1991). Antagonistic yeast, *Candida oleophila* was shown to colonize in wounds and reduce the rot development in apple fruits (Wilson *et al*. 1993, Mercier and Wilson 1994). In addition, this yeast cannot grow at human body temperature (37°C) and does not produce antibiotics so it is safe to use it to treat the fruits. In our research work, we found two isolates of epiphytic yeast derived from the surface of mango leaves collected from an organic orchard in Chiang Mai province, Thailand. These two yeast isolates were further tested on mango fruits for controlling anthracnose fruit rot. Both isolates resulted in significant reduction of the rot.

Recently, several additives have been shown to augment the biocontrol activity of selected antagonists. A combination of antagonist with $CaCl_2$, nitrogenous compounds or a sugar analog, 2-deoxy-D-glucose, was shown to increase the effectiveness of the antagonists and reduce the microbial population to the degree required to give effective control (McLaughlin *et al*. 1990, Janisiewicz 1994, Wisniewski *et al*. 1995). Among the potential additives, chitosan (β-1,4-glucosamine polymer) could be a useful additive to antagonistic microorganisms. Chitosan and its derivatives such as glycolchitosan and carboxymethylchitosan are known to form a semipermeable film and have an inhibitory effect on a number of pathogenic fungi and also induce host defense responses (Allan and Hadwiger 1979, El Ghaouth *et al*. 1994). Combining antagonistic yeasts with chitosan will make it possible to exploit the antifungal and eliciting properties of chitosan and the biological activity of the antagonists (El Ghaouth *et al*. 2000). In the present paper we report on the effect of the combination of two isolates of epiphytic yeasts with chitosan on anthracnose fruit rot in mango fruits.

## Materials and Methods

*Fruit material and microorganisms*

Mango fruits cv. 'Choke Anan' and 'Nam Doc Mai' were surface sterilized and individually wounded at 3 sites with sterilized needles (3 mm wide X 5 mm deep). The pathogen was isolated from infected mango fruits and checked for pathogenesis ability by re-inoculation on mango fruits. It was identified as *Colletotrichum gloeosporioides***.** Conidia suspension was prepared from 7-day-old cultures on potato dextrose agar (PDA) plate and adjusted to $10^6$ conidia $ml^{-1}$. The number of conidia was determined with a haemocytometer. The biocontrol agents used in this study were two yeast strains (ns 5 and ns 9) isolated from mango leaves collected from an organic orchard in Chiang Mai province. These two yeasts were tentatively identified as being from the genera *Candida* by conventional morphological methods. The inocula of two yeasts were prepared from 48 h-old nutrient yeast dextrose agar (NYDA) grown cultures, suspended in 50 ml of 0.05 M phosphate buffer and mixed by a Vortex mixer for 1 min. The yeast suspension was centrifuged at 10,000 rpm for 15 min, and the supernatant was discarded to collect precipitate. The concentration of yeast cells was adjusted to 1.5 - 2.0 X $10^6$ colony forming units per ml (CFU $ml^{-1}$) using a spectrophotometer (absorbance of 0.155 at 550 nm).

*Population dynamics of yeasts in wounds*

The effect of chitosan on the survival of two

epiphytic yeast strains in mango wounds was determined. Mango fruits were wounded as described above, and each wound was inoculated with 40 µl of yeast cell suspension (ns 5 or ns 9) containing 0.5% chitosan and allowed to dry. Fruits were incubated in plastic trays at 25°C under humid conditions (95%). Individual fruit containing three wounds represented a single replicate in a randomized complete block design, and three replicates were sampled at 0, 2, 4, 6, 8 and 16 days after inoculation. Tissue samples containing the wounds were removed with a cork borer (10 mm in diameter), individually homogenized in 5 ml of sterilized distilled water, mixed well by a Vortex mixer, serially diluted and plated in triplicate on NYDA medium. The plates were incubated at 25°C for 48 h. Colonies were then counted and results were expressed as $\log_{10}$CFU per wound.

*Wound colonization by antagonistic yeasts and fungal pathogen*

Wounds on mangoes were inoculated with each yeast isolate ns 5 or ns 9 for 12 h and followed by inoculation with the fungal pathogen. Another treatment was interchanged in which wounds were inoculated with the pathogen for 12 h followed by the yeast. The fruits were incubated at 25°C for 24 h. Then, wounded tissues (2-3 $mm^2$) were excised from the treated fruits, immediately placed in 2.5% glutaraldehyde for 2 h, washed with 0.1 M phosphate buffer 2 times for 15-20 min, fixed with 1% Osmium for 2 h, washed with 0.1 M phosphate buffer 2 times, and dehydrated with an acetone series (for 20 min each). The samples were further dried by sublimation under high vacuum in a Critical Point Dryer. Dried tissue was mounted on an aluminium stud, coated with gold-palladium (Karabulat *et al*. 2002), and observed under a JEOL® JSM-6335F scanning electron microscope.

*Biocontrol assay*

The experiment consisted of the following treatments: (1) The wounded fruits were inoculated with 20 µl of a conidia suspension of fungal pathogen at $10^6$ conidia $ml^{-1}$as the control. (2) The wounded fruits were dipped in sterilized distilled water at 55°C for 5 min, and challenge-inoculated with the pathogen. (3) The wounded fruits were soaked in 0.25% or 0.5% chitosan, and allowed to air dry before challenge-inoculation with the pathogen. (4) The wounded fruits were treated with 40 µl of a cell suspension of yeast ns 5 at 1.5 X $10^6$ CFU $ml^{-1}$or yeast ns 9 at 2.0 X $10^6$ CFU $ml^{-1}$ for 12 h, and challenge-inoculated with a conidia suspension of the pathogen. (5) The wounded fruits were treated with yeast ns 5 or ns 9 as described above, followed by being dipped in 0.25% and 0.5% chitosan, and allowed to air dry before challenge-inoculation with the pathogen. Fruits were stored at 25°C under high humidity (95%) in enclosed plastic trays. Each treatment was applied to five replicates of five fruits each in each experiment. The entire experiment was repeated twice. The percentages of disease incidence and severity were determined at 7 and 10 days after inoculation.

**Results**

The growth rate of yeasts ns 5 and ns 9 were not influenced by chitosan. A similar growth pattern of yeasts ns 5 and ns 9 was observed in mango wounds in a preliminary experiment (data not shown). The population sizes of yeasts ns 5 and ns 9 increased continuously. The results showed that both antagonists could colonize and grow in mango wounds for a period of 8 days but thereafter decreased within 2 days (Fig. 1).

Electron micrographs revealed that both isolates of antagonistic yeasts effectively colonized wounds of mango fruit. Furthermore, the yeasts could attach to the conidia and mycelia of anthracnose pathogen when the yeasts were pre-inoculated in the wound sites for 12 h and followed by the pathogen (Figs. 2 and 3).

The addition of 0.25% and 0.5% chitosan in mango wounds enhanced significantly the efficacy

of the two epiphytic yeasts in controlling anthracnose fruit rot in 'Nam Doc Mai' and 'Choke Anan' mangoes. The combination of antagonistic yeast with chitosan was more effective in the reduction of anthracnose incidence than yeast or chitosan alone. In addition, the combination of yeast ns 9 with 0.5% chitosan was more effective in controlling anthracnose symptoms than the other treatments. *Candida* sp. ns 9 in combination with 0.5% chitosan was most effective in controlling anthracnose fruit rot in 'Choke Anan' and 'Nam Doc Mai' mangoes in which the average percentages of disease incidences were only 6.7% and 13.3%, respectively, when compared with 93.3% and 100% infection in the controls. As for fruits treated with hot water (55°C for 5 min), the disease incidences

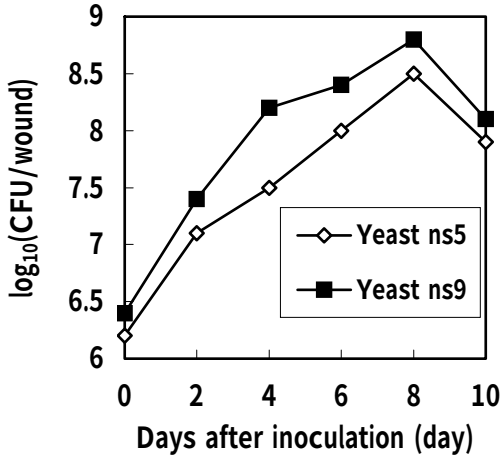


**Figure 1 Population dynamics of two isolates of antagonistic yeast ns 5 and ns 9 in wounds on mango fruits. CFU: colony forming units.**

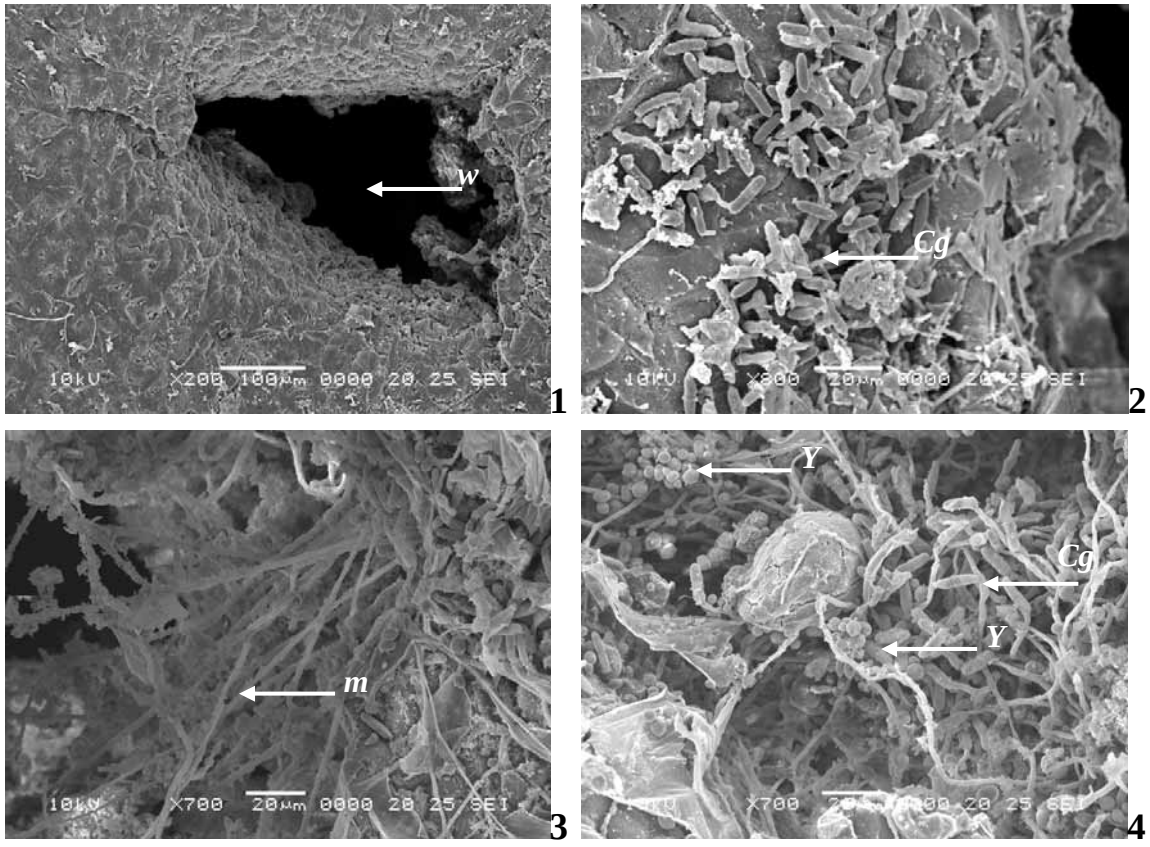


**Figure 2 Scanning electron micrographs showing the colonization by yeast cells and the pathogen in mango wounds. 1: A wounded site (*w*), 2: Colonization by *C. gloeosporioides* (*Cg*), 3: The mycelia of *C. gloeosporioides* (*m*) colonized in the wound which was pre-inoculated with *C. gloeosporioides* 12 h before the yeast inoculation, 4: The wound pre-inoculated with yeast (*Y*) 12 h before the *C. gloeosporioides* (*Cg*) inoculation. The tissue samples were taken from the treated fruits 24 h after the treatment.**

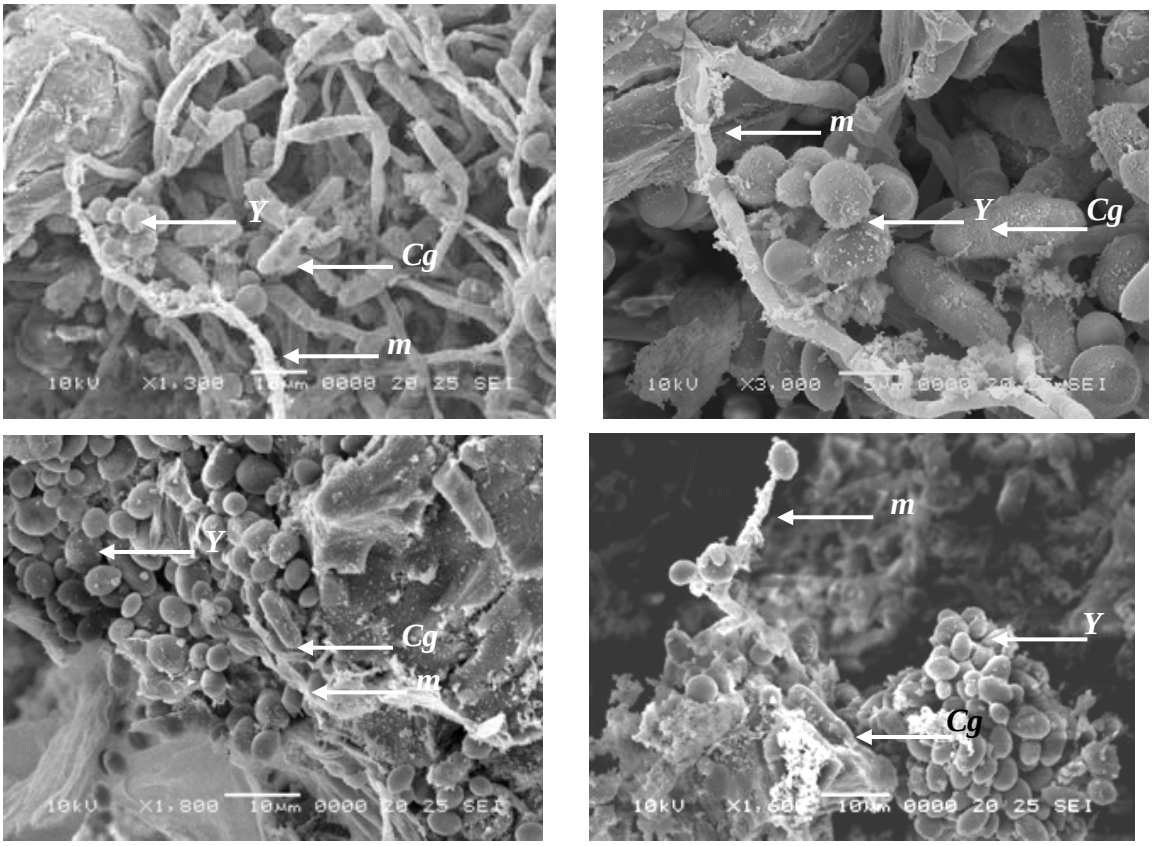


**Figure 3 Scanning electron micrographs showing the yeast ns 9 (*Y*) cells attached to the conidia (*Cg*) and mycelia (*m*) of *C. gloeosporioides* which was inoculated 12 h after the inoculation with yeast ns 9. The tissue samples were taken from the treated fruits 24 h after the treatment.**

were 73.3% and 86.7% in 'Choke Anan' and 'Nam Doc Mai' mangoes, respectively, and these fruits showed visible symptoms of infection by the second day of storage similar to the control fruits (Tables 1 and 2).

**Discussion**

The mechanism for biocontrol of the antagonistic yeasts (ns 5 and ns 9) has not been fully determined. However, the competition mechanism of these antagonists against anthracnose pathogen appeared to be the principle mode of action whereas the production of antibiotic substances was not detected. In the recent study carried out by El Ghaouth *et al.* (2000), the antagonistic yeast *Candida saitoana* was compatible with chitosan or chemically-modified chitosan. Chitosan was inhibitory to spore germination of major postharvest pathogens but showed no effect on the growth of *C. saitoana in vitro* and *in planta*. This makes it possible to exploit both the antifungal and eliciting properties of chitosan and the biological activity of the antagonists. The results from the present study confirm that combining antagonistic yeasts with chitosan can provide better control of anthracnose fruit rot than the use of biocontrol agent alone. The data showed that *Candida* sp. ns 9 combined with 0.5% chitosan treatment gave the best result to control anthracnose lesions on the fruits when compared with the other treatments.

The performance of the combination of antagonistic yeasts with 0.5% chitosan against anthracnose pathogen may be due to both the antifungal effect and film-forming property of chitosan (El Ghaouth *et al*. 2000). Because of its filmogenic property, chitosan may act as a barrier to the outflow of nutrients and, consequently, may

**Table 1 Efficacy of yeast antagonists, chitosan or their combination for controlling the severity of anthracnose lesions in 'Choke Anan' and 'Nam Doc Mai' mangoes**

| Treatment | Severity (%)* | |
|---|---|---|
| | Choke Anan** | Nam Doc Mai** |
| Control | 100 [d] | 100 [d] |
| Hot water 55°C, 5 min | 86.33 [c] | 80.80 [c] |
| 0.25% chitosan | 59.67 [b] | 51.69 [ab] |
| 0.5% chitosan | 56.59 [b] | 52.53 [b] |
| Yeast ns 5 | 54.26 [b] | 62.66 [b] |
| Yeast ns 9 | 51.69 [ab] | 59.92 [b] |
| Yeast ns 5 + 0.25% chitosan | 50.13 [ab] | 54.22 [b] |
| Yeast ns 5 + 0.5% chitosan | 47.29 [a] | 45.15 [a] |
| Yeast ns 9 + 0.25% chitosan | 50.90 [ab] | 51.69 [ab] |
| Yeast ns 9 + 0.5% chitosan | 42.12 [a] | 43.88 [a] |

Fruits were treated with hot water, 0.25% or 0.5% chitosan, yeast ns 5 or ns 9 cell suspension or the combination of yeast and chitosan 12 h before challenge-inoculation with conidia suspension of *C. gloeosporioides*.

* Severity (%) = LdA/LdC X 100, where LdC is the average lesion diameter in control wounds inoculated with the pathogen and LdA is the average lesion diameter in wounds treated with antagonist prior to inoculation with a pathogen.

**Within a column, values with the same letter are not significantly different at the P=0.01 levels according to Duncan's multiple range test.

**Table 2 Effectiveness of yeast antagonists, chitosan or their combination on anthracnose lesions in 'Choke Anan' and 'Nam Doc Mai' mangoes caused by *C. gloeosporioides***

| Treatment | Disease Incidence (%)* | |
|---|---|---|
| | Choke Anan** | Nam Doc Mai** |
| Control | 93.3 [d] | 100.0 [e] |
| Hot water 55°C, 5 min | 73.3 [d] | 86.7 [d] |
| 0.25% chitosan | 53.3 [c] | 53.3 [c] |
| 0.5% chitosan | 40.0 [b] | 40.0 [b] |
| Yeast ns 5 | 53.3 [c] | 60.0 [c] |
| Yeast ns 9 | 46.7 [c] | 40.0 [b] |
| Yeast ns 5 + 0.25% chitosan | 33.3 [b] | 33.3 [b] |
| Yeast ns 5 + 0.5% chitosan | 26.7 [b] | 26.7 [b] |
| Yeast ns 9 + 0.25% chitosan | 20.0 [b] | 33.3 [b] |
| Yeast ns 9 + 0.5% chitosan | 6.7 [a] | 13.3 [a] |

Fruits were treated with hot water, 0.25% or 0.5% chitosan, yeast ns 5 or ns 9 cell suspension or the combination of yeast and chitosan 12 h before challenge-inoculation with conidia suspension of *C. gloeosporioides*.

* Incidence (%) = number of rotten wounds / number of total wounds X 100.

**Within a column, values with the same letter are not significantly different at the P=0.05 level according to Duncan's multiple range test.

reduce the availability of nutrient to a level that will not sustain the growth of the pathogen. This contention is supported by the fact that a fungal pathogen exposed to chitosan often displays signs of nutrient deprivation (Vero *et al*. 2002).

In conclusion, this study revealed that the biocontrol activity of these two isolates of epiphytic yeasts against anthracnose fruit rot in

mango caused by *Colletotrichum gloeosporioides* was enhanced significantly by the addition of chitosan. The level of control conferred by the combination of *Candida* sp. ns 9 and 0.5% chitosan was superior to that conferred by *Candida* sp. ns 5 and 0.5% chitosan and appears to be due to additive interactions between the yeast and chitosan. Combining antagonistic yeasts with chitosan can be expected to provide better control of anthracnose fruit rot than the use of biocontrol agent alone. Future research will explore the possibility of biocontrol enhancement using mixtures of antagonists or some additives and try to formulate them into commercial products.

**Acknowledgement**

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