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PLANT CRUDE EXTRACTS AND YEAST AS ALTERNATIVE TO SYNTHETIC FUNGICIDE FOR CONTROLLING POSTHARVEST GREEN MOULD ON CITRUS FRUIT

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Abstract

SUKORINI HENIK, SANGCHOTE SOMSIRI, KHEWKHOM NETNAPIS: *Plant crude extracts and yeast as alternative to synthetic fungicide for controlling postharvest green mould on citrus fruit.* Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 2013, LXI, No. 3, pp. 795–801

The objectives of this research was to evaluate the effectiveness of the combination of plant crude extracts and yeasts as an alternative to replace the synthetic fungicide to control green mould rot caused by *Penicillium digitatum* on citrus fruit. This pathogen caused 90% of citrus losses during the storage. Control of this pathogen mainly with chemicals, but concerned with environmental contamination, human health, and pathogen resistance, chemical treatment is frequently decreased. *Eugenia caryophylata* crude extracts and *Candica utilis* showed to be the best combination to attain a reduction in green mould incidence by 90.3% and disease severity by 96.26%. Furthermore, the combination of *E. caryophylata* crude extracts and *C. utilis* had a more potent antifungal activity than imazalil. The effectiveness of the combination of plant crude extractss and yeasts can be an alternative treatment to replace the synthetic fungicide to control *P.digitatum* on citrus fruit, but the application in the packaging line needs further investigation.

citrus fruit, green mould, plant crude extracts, yeast, *Penicillium digitatum*

Harvested citrus fruits are very susceptible to wound infection by *Penicillium digitatum* (Pers.:Fr.) Sacc, which is commonly known as green mould rot. It causes 90% of losses during the storage and also serious damages in commerce (Canamas *et al*., 2008). Minimizing wounds on fruit, proper temperature management, and postharvest fungicide treatments are the main methods of reducing losses by this pathogen (Eckert and Eaks, 1989).

Applications of synthetic fungicides increase an environmental contamination and human health problems. In contrast, control of postharvest pathogens has been accomplished mainly with the use of chemicals. *P. digitatum* developed resistance to the fungicide thiabendazole (TBZ) in the California citrus industry in 1981 (Holmes and Eckert, 1999), sodium ortho-phenylphenate (SOPP) and imazalil 1-[2-(2,4-dichlorophenyl)-2-(2-prophenyloxy)ethyl]-1H-imidazole, and caused reduction in chemical efficacy (Holmes and Eckert, 1999). Therefore, alternative strategies to control postharvest disease of citrus fruits are needed.

Recently, integrated control is often used to enhance the effectiveness of a control measure. Biological control has emerged as one of the most promising alternative, antagonist organism has been used either alone or as part of an integrated control strategy to reduce synthetic fungicide (Fan and Tian, 2000). Combination of *Rhodotorula glutinis* with 1 $mmolL^{-1}$ and NH_4Mo provided an effective control on *P. expansum* and *Alternaria alternata* than applying the yeast or NH_4Mo alone. *P. expansum* completely eliminated by combination of *Metschnikowia pulcherrima, Cryptococcus laurentii,* and sodium bicarbonate on fruit stored under CA conditions (Conway *et al*., 2007). Mixture of *Pantoea agglomerans* CPA-2, 3% sodium carbonate or sodium bicarbonate (SBC) aqueous solutions heated to

40 °C effectively protected pre-existing rind wounds *P. digitatum* during seven days of storage at 10 °C (Usalla *et al*., 2008). A treatment comprising *Bacillus amyloliquefaciens* HF-01 combined with 50 µg mL^{-1} tea saponin was revealed to be as effective as the fungicide treatment with excess of 90% control of green, blue moulds, and sour rot (Hao *et al*., 2011).

MATERIALS AND METHODS

Plant crude extracts preparation

Preparation of the plant crude extracts for the antifungal screening against pathogenic fungi was conducted as previously described by Win (2007). Seven plants including, fresh *Cymbopogon citratus* stem, *Zingiber officinale* rhizome, *Momordica charantia* fruit, *Curcuma longa* rhizome, dried *Eugenia caryophyllata* flower bud, *Cinnamomum cassia* bark, and *Tinospora crispa*. The fresh plants were dried under shade and blended to powder. Powdered plant with a mass of 300 g was soaked in 400 mL of 96% ethanol for 3 days with frequent agitation. The mixture was filtrated through Whatman filter paper no 1 and the crude extract was collected. The crude extract was evaporated at 40 °C with a rotavapor at 200 mbar. An extract was collected and mixed in ratio 1:3 CH_2Cl_2, and left for 30 minutes prior to filtration. The filtrate was dried with a rotary evaporator, and subsequently added with 20% ethanol and kept in –20 °C until used.

Antagonist

Seven antagonist which used in this research were *Candida tropicalis* TISTR 5010/ATCC 13803, *Pichia membranefaciens* TISTR 5093, *Candida utilis* TSITR 5001, *Aureobasidium pullulans* TISTR 3384, *C. guillermondii* BCC 5389, *C. sake* TISTR 5143, and *Debaryomyces hansenii* TISTR 5155. The yeast cells were cultured on yeast malt extract agar (YMA) medium and incubated at 28 °C for 48 hours. The cell suspension was then prepared by adding with 10 mL of sterile distilled water and counted with a haemocytometer. The cell suspension was adjusted to 1×10^8 cells mL^{-1}.

Pathogen inoculum

A highly virulent strain of *P. digitatum* was obtained from decayed citrus "Sai Num Pung" tangerine fruit. The pathogen was grown on potato dextrose agar (PDA) at 25 °C for seven days. The conidial suspension was prepared by adding 10 mL of sterile distilled water with 0.01% Tween 80 to the cultures and counted with a haemocytometer. The cell suspension was adjusted to 1×10^5 conidia mL^{-1}.

Fruit preparation

"Sai Num Pung" tangerine fruits (*Citrus reticulata* Blanco) were obtained from a commercial orchard in Fang, Chiang Mai province, Thailand, and fruits free of defect were chosen. The fruits surfaces were disinfected with immersed in 1% sodium hypochlorite for three minutes, rinsed with sterile water, and dried in a sterile chamber.

Effect of antagonist and plant crude extracts to control *P. digitatum* on citrus fruit

Seven plant crude extracts and seven yeasts were screened, after that two promising plant crude extracts and two yeasts were tested on citrus fruit. A sterile needle was used to make wound on 3 mm depth. Ten micro liter of conidial suspension (1×10^5 conidia mL^{-1}) of *P. digitatum* were added with a sterile pipette on the citrus wounds, immediately after pricking and allowed to dry under aseptic conditions, 10 µL of yeast at 1×10^8 cells mL^{-1} were also applied on the same wound. After drying, 10 µL of the plant crude extracts were added on the wound site. The inoculated fruits were incubated in a 100% RH chamber at 25 °C for 7 days. Each treatment contained 45 fruits in 3 replications and the experiment was arranged as randomized complete block design. Disease incidence and disease severity were observed after 7 days of incubation. The disease incidence calculated using this formula: Disease incidence = (number of infected fruits/total number of fruit assessed) × 100. The disease severity on citrus was determined according to the portion of the infected area of citrus fruit. The disease severity calculated using this formula: Disease severity = (infected tissue area/total tissue area) × 100%.

Comparison plant crude extracts and yeast with commercial synthetic fungicide

This study used promising plant and yeast as a result from previous experiment. A sterile needle was used to make wound on 3 mm depth. Ten micro liter of conidial suspension (1×10^5 conidia mL^{-1}) of *P. digitatum* was added with a sterile pipette on the citrus wound, after pricking and allowed to dry under aseptic conditions. Ten micro liter of yeast at 1×10^8 cells mL^{-1} were also applied on the same wound. After drying, 10 µL of the plant crude extracts were applied on the wound site then incubated in a 100% RH at 25 °C. In comparison with synthetic fungicide, the fruits which were inoculated with *P. digitatum* (1×10^5 conidia mL^{-1}, 10 µL), treated with imazalil 10 µL to the wounds at 50,100, or 150 mgL^{-1}. Each treatment contained 45 fruits in 3 replications and the experiment was arranged as randomized complete design. Disease incidence and disease severity were observed after 7 days of incubation. The disease incidence calculated using this formula: Disease incidence = (number of infected fruits/total number of fruit assessed) × 100. The disease severity on citrus was determined according to the portion of the infected area of citrus fruit. The disease severity calculated using this formula: Disease severity = (infected tissue area/total tissue area) × 100%.

Effect plant crude extracts to yeast conidia and pathogen spore growth

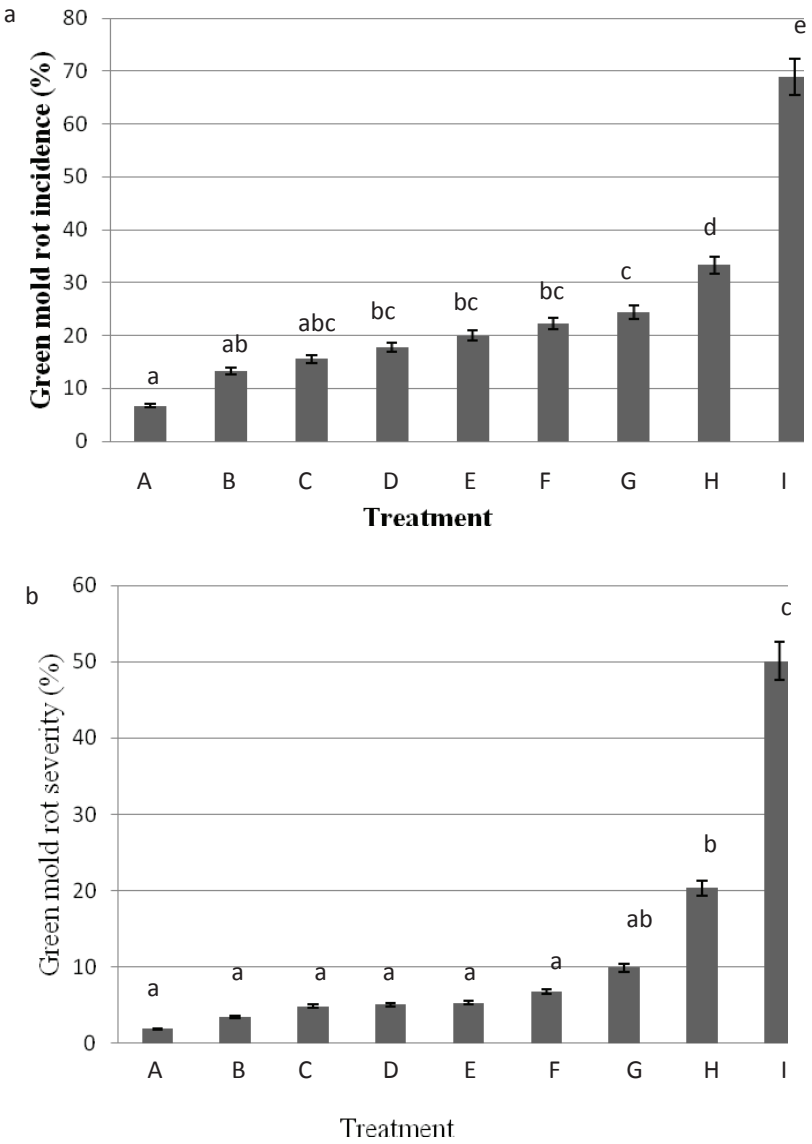
To know the effect of yeast and plant crude extracts to *P. digitatum* and plant crude extracts on *C. utilis* growth were investigated. Both microorganisms were grown on the PDA, PDA plus 5,000 mgL^{-1} of *E. caryophyllata*, and PDA plus 5,000 mgL^{-1} of *E. caryophyllata* and citrus juice 2% as origin substrate. Effect of *E. caryophyllata* and *C. utilis* to *P. digitatum* was observed under fluorescent microscope, Carl Zeis GmbH, Germany. Isolates were transferred from petri dish then smeared on a glass slide and stained with one drop of Nile red solution (1mg mL^{-1} of 100% ethanol), waited for 3 minutes, after ward observed under the microscopes with normal and fluorescent light source.

Statistical analysis

All data were analyzed by Statistical analysis of variance (ANOVA) procedure and regression analysis using Statistical analysis system (SAS) software. Statistical significant was assessed at $p < 0.05$ and Tukey'S HSD Multiple range test was used to separate means.

RESULT

All of the combinations of plant crude extracts and yeast could reduce the disease incidence in excess of 70%. The combination of *E. caryophylata* at 15,000 mgL^{-1} and *C. utilis* at 1×10^8 cells mL^{-1} was the best combination. It reduces the disease incidence by 90.3%, while the combinations of *C. longa* at



1: *Effect of plant extract and yeast to control of green mould rot incidence (a) and disease severity (b). (A) E. caryophylata and C. utilis; (B) C. longa and C. utilis; (C) E. caryophylata and C. tropicalis; (D) C. utilis; (E) C. longa and C.tropicalis (F) F. C.tropicalis; G. E. caryophylata; H. C. longa; I. control. Bars represent the standard deviations of the mean.*

30,000 mgL^{-1} and *C. utilis* at 1×10^8 cells mL^{-1}; *E. caryophylata* at 15,000 mgL^{-1} and *C. tropicalis* at 1×10^8 cells mL^{-1}; *C. longa* at 30,000 mgL^{-1} and *C. tropicalis* at 1×10^8 cells mL^{-1}, the reduction was 80.7%, 77.4%, and 71%, respectively (Fig. 1a). Likewise, for the disease severity, the combination of *E. caryophylata* at 15,000 mgL^{-1} and *C. utilis* at 1×10^8 cells mL^{-1} was found to be the best combination to attain a reduction in disease severity of 96.26%, while the combinations of *C. longa* at 30,000 mgL^{-1} and *C. utilis* at 1×10^8 cells mL^{-1}; *E. caryophylata* at 15,000 mgL^{-1} and *C. tropicalis* at 1×10^8 cells mL^{-1}; *C. longa* at 30,000 mgL^{-1} and *C. tropicalis* at 1×10^8 cells mL^{-1}, the reduction was 93.2%, 90.3%, and 89.4%, respectively (Fig. 1b).

Since combination of *E. caryophylata* crude extracts at 15,000 mgL^{-1} and *C. utilis* at 1×10^8 cells mL^{-1} was the best, it was compared with imazalil at several concentrations. It showed that the combination of *E. caryophylata* crude extracts at 15,000mgL^{-1} and *C. utilis* at 1×10^8 cells mL^{-1}, had a higher antifungal activity than imazalil. The disease incidence was 31.1% whereas imazalil 150 mgL^{-1} was 57.8% and the disease severity was 14.47% and 21.1% respectively (Tab. I).

The possibility of mode of action of *E. caryophyllata* and *C. utilis* to inhibit *P. digitatum* growth had been tested on PDA by poisonous food technique. The result showed that *P. digitatum* which was cultured on PDA plus *E. caryophyllata* and 2% citrus juice (Fig. 4A) compared with control (4B), grew but subsequent died (Fig. 4C and D). It was indicated by cytoplasmic coagulation and vesiculation of hyphal content (Fig. 4E and F). However, this result was better than *P. digitatum* growth on PDA plus 5,000 mgL^{-1} *E. caryophyllata* without citrus juice (Fig. 2A). *P. digitatum* did not germinate (Fig. 2B) and the number of alive conidia also reduced (Fig. 2C). On the other hand, *C. utilis* was still alive as shown by staining method (Fig. 3).

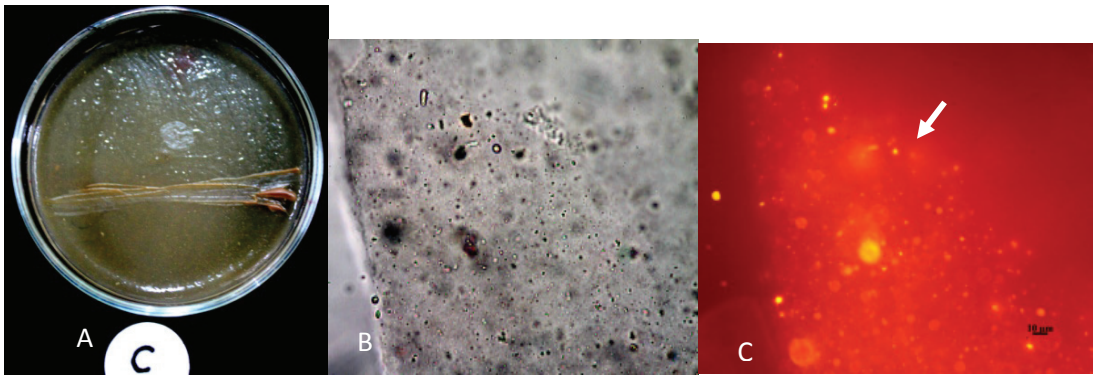
DISCUSSION

This study clearly showed that integrated treatment of *E. caryophyllata* crude extracts and *C. utilis* significantly reduced green mould rot of *P. digitatum* on the citrus fruits. It was indicated that *E. caryophyllata* crude extracts and *C. utilis* have fungicidal properties to control green mould rot disease on citrus fruits. Bardin *et al.* (2003), reported that combined applications of a biocontrol agent and synthetic chemicals or plant materials often provide better plant protection than individual treatments. The combined application of *Paeonia suffruticosa* (medicinal plant) and *T. harzianum* was more effective than each treatment to control *Rhizoctonia* damping-off (Lee *et al.*, 2008). The result agree with Lee *et al.*

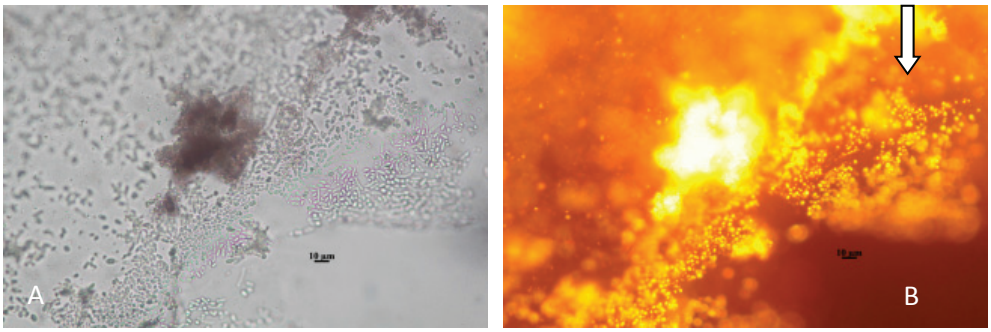
I: *Green mold rot incidence (%) and severity (%) on citrus fruits after treated with E. caryophyllata, C. Utilis, or imazalil at different concentration.*

Treatment	Disease Incidence (%)	Disease Severity (%)
Imazalil 50 mg/L	95.57 e	44 d
Imazalil100 mg/L	71.10 d	25.87 b
Imazalil 150 mg/L	57.80 b	21.10 ab
Eugenia caryophyllata	53.30 b	26.43 b
Eugenia caryophyllata + *C.utilis*	31.10 a	14.47 a
C. utilis	64.43 c	28.43 bc
Water (control)	100.00 e	55.43 e
ETOH 20%	100.00 e	34.77 c

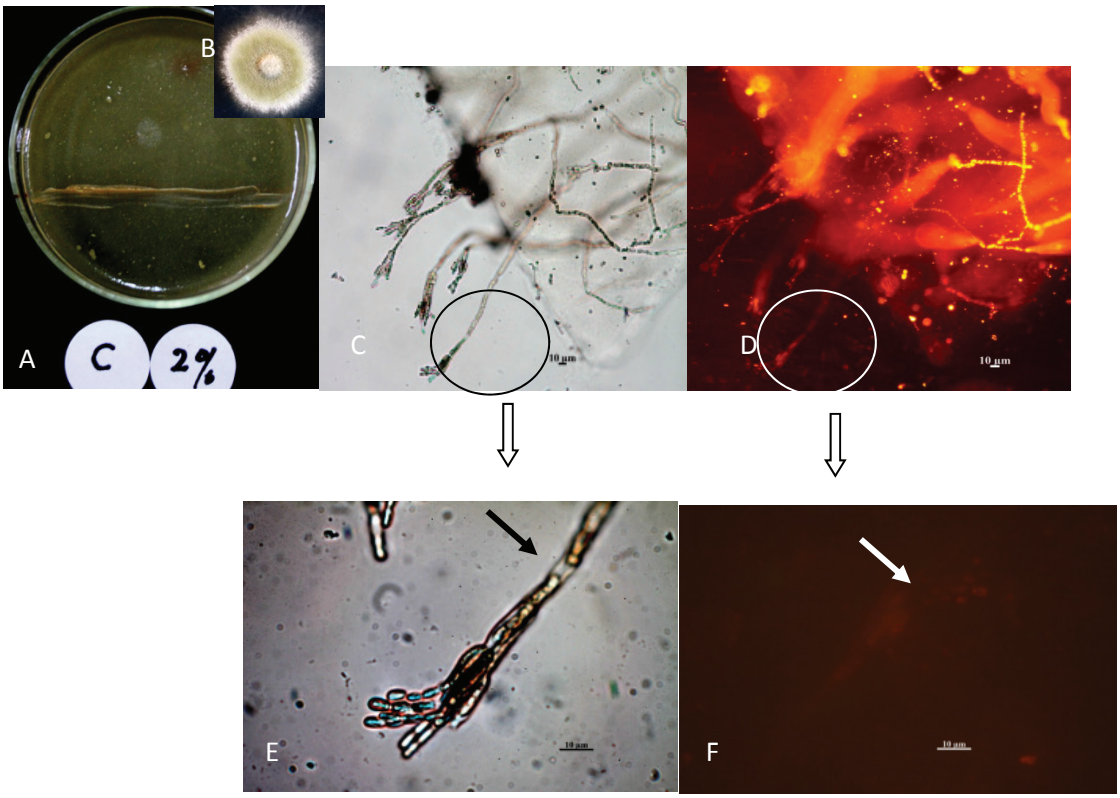
Values with the same letters in the same coloum were not significant different from each other based on the Tukey'S HSD Multiple range test (p = 0.05).



2: *(A) Dual culture P. digitatum and Candida utilis on PDA + 5,000 mgL^{-1} of E.caryophyllata. (B) The microscopic view of Candida utilis (400X), under normal and (C) fluorescent light source. The fluorescent of P. digitatum conidia indicated that the conidia were alive (showed by an arrow). Bar indicated 10 µm.*



3: *The Candida utilis were cultured on PDA + E.caryophyllata. (A) The microscopic view of Candida utilis (400X), under normal and (B) fluorescent light source. The fluorescent of C. utilis cells indicated that the conidia were alive (showed by an arrow). Bar indicated 10 µm.*



4: *(A) Dual culture P. digitatum and Candida utilis on PDA +5000 mgL^{-1} E.caryophyllata + 2% citrus juice. (B) Control. (C) The microscopic view of P. digitatum under normal light source (200X) and (E) (1000X)) and (D) Fluorescent light source (200x) and (F, 1000x). Cytoplasmic coagulation and vesiculation on hyphae were indicated by circle. The gold fluorescent absent in the hypha of P. digitatum indicated that the pathogen was dead (showed by a white arrow). Bar indicated 10 µm.*

(2011) who tested fifty five species of medicinal plant materials for their antifungal activity *in vitro* against *R. solani* AG 2-1 to improve the biocontrol efficacy of *T. harzianum*. Six species were found to be effective against *R. solani* AG 2-1. Among these six medicinal plant materials, *E. caryophyllata* flower bud and *C. loureirii* stem bark have high efficacy against *R. solani* AG 2-1 mycelial growth which inhibited by 73.7% and 71.1%, respectively.

The *P. digitatum* showed no growth on PDA plus 5 000 mg/L *E. caryophyllata* crude extracts for 7 days, approved that crude extracts was effective to control green mould rot of citrus fruits a short period (days), nonetheless, its efficacy should be improved when protection was required for long periods. Moreover, *C. utilis* cells grew on PDA plus 5 000 mg/L *E. caryophyllata* crude extracts, this result acclaimed that *E. caryophyllata* crude extracts had no effect to the *C. utilis* growth then it could control *P. digitatum* for long period. Cell death because its loss of cell contents and the initiation of autolysis. Besides this, the permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Armstrong, 2006). The damage was irreversible and the changes showed general disorganization of the cytoplasm

as well as cytoplasm leakage, probably caused by loss of conidial membrane integrity which could eventually lead to fungal cell death (Bakkali, 2008). In this regard, Denyer (1990) affirms that leakage of intracellular material was a general phenomenon provoked by many antimicrobial substances. Perhaps eugenol an active ingredient in *E. caryophyllata* induces either hyperpolarization of the inner mitochondrial membrane or mitochondrial swelling or both. Usually, cells in which mitochondria are destabilized and finally broken down will decrease in the coupling efficiency of the electron-transport chain and therefore can generate ROS intermediates which can lead to oxidative stress (Martindale and Holbrook, 2002). In addition, Park *et al*. (2007) studied effect of clove oil on *Trichophyton mentagrophytes* under transmission electron microscope, the inner mitochondrial membranes were partially destroyed, with complete destruction of the cell wall.

The mode of action of antagonist yeast on postharvest pathogen was complex. It might involve nutrient competition (Droby *et al*., 1989) site exclusion and direct parasitism (Ippolito *et al*., 2000), production of hypha enzyme (El-Ghaouth *et al*., 1998) and induce resistance (Arras, 1996). Wisniewski *et al*.,1991, found the tenacious attachment of *P. gyilermondii* to pathogen hypha and the production of β -1,3 glucanase by the yeast. However, many researchers supported competition for nutrient was the main mode of action of antagonistic yeast against pathogen (Droby *et al*., 1989). The yeast cell colonize rapidly and compete effectively with the pathogen for nutrient in wound, resulting in the nutritional starvation of the pathogen (Mc Laughin *et al*., 1990).

CONCLUSIONS

All of the combinations of plant crude extracts and yeast can reduce the disease incidence in excess of 70%. The combination of *E. caryophylata* and *C. utilis* was the best combination to attain a reduction in disease incidence by 90.3% and disease severity by 96.26%. The combination of *E. caryophylata* crude extracts and *C. utilis* had a more potent antifungal activity than imazalil at 150 mgL^{-1}. *E. caryophyllata* crude extracts was effective to control green mould rot of citrus fruits for short period and *C. utilis* can control *P. digitatum* for long period. This combination might be used as a drench treatment in packaging line, but still need more detail research especially how to apply the combination.

SUMMARY

The objectives of this research was to evaluate the effectiveness of the combination of plant crude extractss and yeasts as an alternative to replace the synthetic fungicide to control *P. digitatum* on citrus fruit. Seven plants including, fresh *C. citratus* stem, *Z. officinale* rhizome, *M. charantia* fruit, *C. longa* rhizome, dried *E. caryophyllata* flower bud, *C. cassia* bark, and *T. crispa*. The other seven antagonists which used in this research were *C. utilis* TISTR 5100, *C. tropicalis*, *P. membranefaciens, A. pullulans* TISTR 3384, *C. guillermondii* BCC 5389, *C. sake* TISTR 5143, and *D. hansenii*. Both plant crude extracts and yeasts were screened to get the promising combination through *in vitro* and *in vivo*. The combination of *E. caryophylata* and *C. utilis* was the best combination to attain a reduction in disease incidence by 90.3% and disease severity by 96.26%. The combination of *E. caryophylata* crude extracts and *C. utilis* had a more potent antifungal activity than imazalil.

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