Biocontrol of Citrus green mould and postharvest quality parameters

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Abstract

The potential for using *Pichia guilliermondii* BCC 5389 or *Bacillus subtilis* ABS-S14 by themselves or in combination for the control *Penicillium digitatum* in citrus, and their effects on postharvest quality of fruit was investigated. The percentage of disease with the combined antagonists was completely inhibited. Rapid colonization of *P. guilliermondii* was observed in the wounds during the first day to 6 days at 25°C, whereas *B. subtilis*, increased marginally over 3 days. The populations then stabilized for the remaining incubation period. The percentage of spore germination of *P. digitatum* incubated with all treatments was inhibited by 100%. At concentrations of the combined antagonists of 1×10^8 CFU/mL, the incidence of green mould was reduced to 0% compared with the pathogen control itself (92.93%) after 5 days of incubation at 25°C. The combination did not impair any of the quality parameters of fruit following incubation at 25°C for 7 days.

Introduction

Postharvest diseases cause considerable losses to harvested fruits and vegetables during transportation and storage (Sharma *et al*., 2009). Green mould caused by *P. digitatum* is the major postharvest disease of citrus, wherever it is grown and causes serious losses annually (Eckert and Brown, 1986). Synthetic fungicides are the primary means to control postharvest diseases (Eckert, 1990), but there is public concern over the accumulation of chemical residues in the food chain and environmental safety (Arul, 1994). Biological control using microbial antagonists has emerged as one of the most promising alternatives to postharvest applications of chemical fungicides (Janisiewicz, 1998). A number of yeasts and bacteria have been reported to effectively inhibit postharvest decay of fruit (Janisiewicz and Korsten, 2002). Some formulations have been developed and commercialized, such as the registered biological control formulations SHEMER WDG (Metschnikowia fructicola; Agro Green, Israel) and Bio-Save 10 LP (*Pseudomonas syringae* Strain ESC-10; JET Harvest Solutions, Orlando, FL, USA) (Palou *et al.*, 2002), Aspire, containing *C. oleophila* Montrocher I-182, and Yield Plus containing *Cryptococcus albidus* (Saito) C.E. Skinner (Droby *et al.*, 2001). Moreover, many reports have investigated some natural antagonists

to control citrus fruits. Previously studies, it was shown that *Pichia guilliermondii* strain R13, showed antagonistic activity against *Colletotrichum capsici* in chilli (Chanchaichaovivat *et al.*, 2008) and was also effective in suppressing *P. digitatum* on citrus fruit (Abraha *et al.*, 2010).

Recent studies have shown that a cell-free culture supernatant from *B. subtilis* 155 containing secondary metabolites (SMs) produced the best inhibitory effect on mycelial growth and spore germination of the fungus *P. digitatum.* Using a *B. subtilis* endospore suspension, 24 h prior to fungal spore inoculation disease incidences decreased by 86.7%, while disease symptoms were delayed at 6 days and decay symptoms until day 9 (Leelasuphakul *et al.*, 2008). However, biological control by a single antagonist is not a broad-spectrum phenomenon and is not as effective as chemical fungicides (Chad-Goyal and Spotts, 1996). So to achieve a broader spectrum control of disease ways to enhance the activities of antagonistic microorganisms should be investigated (Janisiewicz and Korsten, 2002). A mixed antagonistic culture is perhaps one method to try for enhancement. The aims of this study were to evaluate the use of antagonistic microbes as a stand-alone treatment and as a combination of antagonistic microbes for the control of green mould, and to investigate their effect on the quality parameters of fruits, including weight loss, fruit firmness, total

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soluble solids, titratable acidity

Materials and Methods

Fruit preparation

Shogun mandarin oranges (*C. reticulata* Blanco cv. Shogun) with similar color, uniformity, size and maturity without wounds were used in this study. Fruits were washed with tap water and sterilized with 2% sodium hypochlorite and air dried prior to wounding.

Antagonists

A suspension of the yeast cells, *P. guilliermondii* BCC 5389 was prepared from a pure culture obtained from the Postharvest and Seed Pathology Laboratory, Department of Plant Pathology of Kasetsart University and screened for its antagonistic effect against *P. digitatum*. The yeast was grown in 250 mL Erlenmeyer flasks with 50 mL nutrient yeast dextrose broth (NYDB) on a rotary shaker at 200 rpm and 28°C for 24 h (stationary phase). Cell suspensions were prepared by centrifugation at 6000 rpm for 10 min (at 4°C) and washed twice with 0.85% NaCl in order to remove any growth medium. Cell pellets were resuspended in sterilized distilled water, counted using a haemacytometer and cell suspensions were adjusted to a concentration of 10^8 cells/mL.

The *B. subtilis* ABS-S14 isolate used in this investigation was originally obtained from soil samples, collected from citrus orchards around the south of Thailand, and it had earlier been screened for the antagonistic properties in vitro. Preparation of antagonist suspension: each isolate was grown on nutrient agar (NA) at 30°C for 24 h. A loopful of each culture was then transferred to a 250 mL conical flask containing 50 mL of nutrient broth (NB) and incubated on a rotary shaker (200 rpm) for 48 h at 30°C. Cultures were centrifuged for 15 min at 5000 rpm and pellets were resuspended in sterile distilled water and centrifuged for a second time. Bacterial cell suspensions were adjusted to 10^8 cells/mL.

Fungal pathogen inoculums

Spore suspensions of the fungus *P. digitatum* were prepared by removing spores from the sporulating edges of a week old culture with a sterile loop and suspension in sterile distilled water. The spore concentration was adjusted to 10^4 spores/mL and counted with a heamacytometer.

Biocontrol assay on citrus fruits

Wounds (3 mm deep and 3 mm wide) were made uniformly at the equator of fruits by puncturing with a sterile scalpel. Aliquots of 20 µL of each the

suspensions of 2% sodium bicarbonate, 500 ppm of imazalil, *P. guilliermondii* BCC 5389, *B. subtilis* ABS-S14, mixtures of BCC 5389 and ABS-S14 at concentration of 1×10^8 cells/mL were dropped into the wounds. Two hours later, a 20 µL suspension of *P. digitatum* (1×10^4 spores/mL) was inoculated into each wound. The treated citrus fruits were placed in 400 x 300 x100 mm plastic boxes with high moisture (about 95% relative humidity) and incubated at 25°C. Disease incidence caused by *P. digitatum* were determined daily for 5 days. Each treatment was composed 4 replicate trials of 20 fruits per treatment. The percentage of disease incidence is the percentage of the number of fruits with lesions over the total number of inoculated fruits. The lesions were observed under a compound light microscope. The experiment was repeated twice.

Population studies of antagonists in fruit wounds

The ability of antagonist cells to survive and multiply in the wound sites of citrus fruit was determined as follows. Mature citrus fruits were disinfected with 2% sodium hypochlorite for 2 min, and then rinsed in fresh sterile water. After wounding, inoculation with 20 µL of the cell suspension of antagonists at 1×10^8 cells/mL was performed, and fruits were placed in plastic boxes with high humidity and incubated at 25°C. The antagonistic population was monitored at day 0 (3 h after inoculation) 3, 6 and 9 days after treatment and the entire wound was then excised from the fruit with a cork borer (1 cm in diameter and 1 cm deep), placed in 10 mL of sterile 0.05 M phosphate buffer at pH 7.0, and ground with an autoclaved mortar and pestle in 50 mL of sterile 0.85% sodium chloride solution. Ten-fold serial dilution of the washings was made, and 0.1mL of each dilution was spread on NYDA medium. The plates were incubated at 25°C for 2 days before counting of colonies (Li *et al.*, 2008). The population densities of antagonists were expressed as log CFU/wound. There were two replicate trials of 10 fruits per treatment. The experiment was repeated twice.

Effects of antagonists on spore germination of the pathogen

Wounds (3 mm deep and 3 mm wide) are made uniformly at the equator of fruits by puncturing with a sterile scalpel. Treatments consisting of 20 µL aliquots of each suspension of a control (no antagonist), *B. subtilis* ABS-14, *P. guilliermondii* BCC 5389 and ABS-S14+ BCC 5389 dropped into the wounds. Then, aliquots (20 µL) of spore suspensions (10^4 spores/ mL of *P. digitatum* were added into the wound. The treated citrus fruits were placed in 400 x 300 x100 mm plastic boxes with high

moisture content and incubated at 25°C. There were two replicate trials of 10 fruits per treatment observed microscopically to determine the germination rate of fungal spores at 0, 24, 48 and 72 h.

Effects of antagonists and postharvest quality

To evaluate the effect of a combination of the antagonistic yeast *P. guilliermondii* BCC 5389, and *B. subtilis* ABS-S14 or both combined in NaHCO₃ solution on the development of the natural decay of intact fruit after inoculation with *P. digitatum* the pathogen inoculated fruit were dipped in the suspension of antagonistic microorganism at 8 cells/mL in 2% NaHCO₃ and sterile distilled water was used as the control, for 30 sec, and air-dried. The treated fruit were kept in polyethylene plastic boxes to retain a high humidity at 25°C for 7 days. Disease incidences were measured after incubation for 7 days. There were two replicate trials of 10 fruits per treatment with complete randomization design. The experiment was repeated twice.

Weight loss

The mass was measured using a balance (0.001 g) (Metter toledo AB204-S) before treatment (A) and after storage (B). The mass loss was calculated as (A-B)/A.

Fruit firmness

Firmness values of each individual fruit were measured at two points of the equatorial region by using the TA-XT2i Texture Analyser (stable Microsystems) with an 8 mm diameter flat probe. The probe descended toward the sample at 10 m/m and the maximum force (N) required was defined as the firmness. The firmness of each citrus fruit was measured twice on different sides.

Total soluble solids

Total soluble solids (TSS) were determined by measuring the refractive index of the juice diluted 1:1 using a hand refractometer "ATAGO N1" and the results were expressed as % Brix.

Titratable acidity

Acidity was measured by titration of 5 mL of juice with 0.1 N NaOH and phenolphthalein as an indicator. Titratable acidity was calculated as a percentage citric acid by the formula %TA = [(mL NaOH) (N NaOH) (meq.wt.acid)/mL sample] x 100.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by the least square difference test using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Efficacy of antagonists for control green mould

The major limitations with biocontrol compared to using synthetic fungicides are a lack of biocidal activity with the pathogen and a narrower spectrum of activity (Conway *et al*., 2004). Results from our experiments showed that the highest level of control of green mold rot was achieved with two combined antagonists that were as good as the synthetic fungicide imazilil. The highest level of control of *Penicillium* induced green mould was achieved with mixtures of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 (Figure 1). The disease incidence of citrus fruits after 5 days at 25°C, treatment with the mixed antagonists was 0 same as seen in the treatment with imazilil, compared to 92.93% with pathogen alone and 49.47% for NaHCO₃ alone. This finding confirms a previous observation that *P. guilliermondii* as a promising biocontrol agent of blue and green mould of citrus fruit (Droby *et al*., 1993; Kinay and Yildiz, 2008). Moreover, it has been widely reported as an effective BCA against fungal pathogens, *Botrytis cinerea*, *Alternaria alternata*, *Rhizopus stolonifer* in cherry tomato (Zhao *et al*., 2010). *B. subtilis* has been previously reported to control green and blue molds caused by *P. digitatum* and *P. italicum* (Obagwu and Korsten, 2003). However, mixed cultures of the microbial antagonists provide better control of postharvest diseases over individual cultures or strains (Sharma *et al*., 2009). Conway *et al.* (2007) showed that *Metschnikowia pulcherrima*, *Cryptococcus laurentii* in combination with sodium bicarbonate were more effective than used alone.

Population studies of antagonists in fruit wounds

The populations of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 were determined after incubation at 25°C for 9 days. The population of *P. guilliermondii* BCC 5389 in the wounds observed during the first day: recovery of log CFU/wound was 8.0 then rapid colonization occurred after 3, 6 and 9 days: recovery of log CFU/wound was 8.3, 10.13 and 11.58, respectively. The difference between populations in *B. subtilis* ABS-S14, were minor, log CFU/wound was 7.99 in the first day and then slightly higher on day 3, log CFU/wound was 8.92, but the populations slightly declined on days 6 and 9 days, log CFU/ wound was 7.97 and 7.95, respectively (Figure 2). The previously report showed that the combined *B. subtilis* ABS-S14 and *P. guilliermondii* BCC5389 with 2% sodium bicarbonate inhibited mycelium
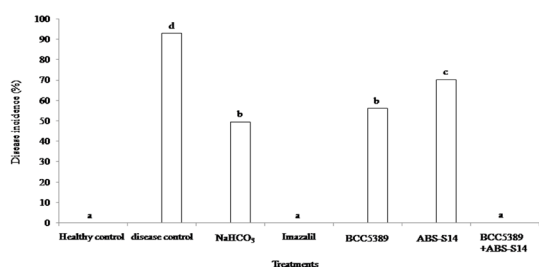
Figure 1. Effects of *P. guilliermodii* BCC5389, *B. subtilis* ABS-S14 alone and combination for control green mould of citrus fruit, disease incidence was recorded 5 days after inoculation with *P. digitatum* and incubation at 25°C. Means with the different letters are significantly different according to the LSD test at P = 0.05.
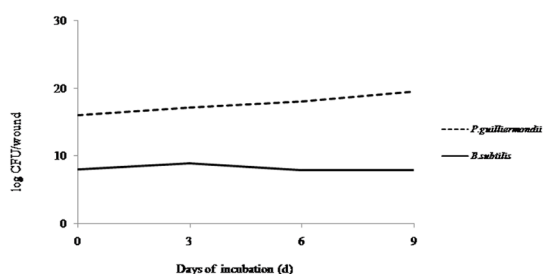

Figure 2. *P. guilliermondii* BCC5389 and *B. subtilis* ABS-S14 in wounds of citrus fruits incubated at 25°C. Data were represented the mean colony counts from three replicate fruits, each plated in triplicate at each sampling time for each temperature.
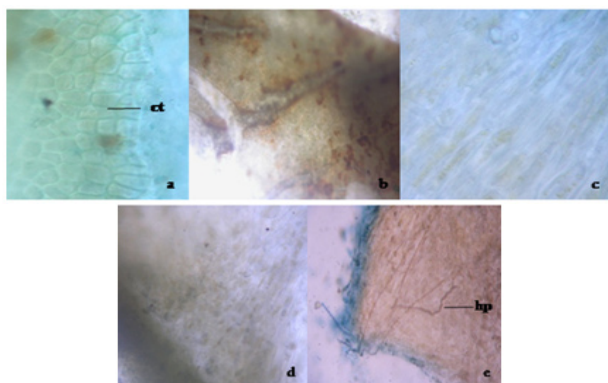

Figure 3. Compound light microscope images of *P. digitatum* hyphae at 72 h. A = healthy tissue, B-D = *P. digitatum* infected citrus tissue treated with *P. guilliermondii* BCC5389, *B. subtilis* ABS-S14, BCC5389 + ABS-S14, respectively., E = disease tissue; magnification x 400. ct = citrus tissue; hp = hypha.

and spore germination of *P. digitatum* (100%) and sodium bicarbonate solution has no effect on growth of both antagonistic microorganisms (Sangwanich *et al.*, 2010). `Obagwu *et al.* (2003) who reported *B. subtilis* combined with sodium bicarbonate was as effective as the fungicide (quazatine plus imazalil) treatment. In this situation *P. guilliermondii* present as mixed antagonists rapidly colonized citrus wounds at 25°C. This result was like Lahlali *et al.* (2011)

who reported *P. guilliermodii* was able to grow at the wounded sites in citrus fruit. Such rapid growth in the wounds indicates that it is well adapted to the wound environment of the fruit and has considerable potential as a biocontrol agent whereas *B. subtilis* slightly declined. Bacteria are early colonizers when nutrient levels on the surface are low. However, as nutrients increase due to cell leakage, the numbers of yeasts increase markedly. Furthermore, it has been known that bacteria showed a marked decline in populations even after short periods of dry weather, whereas yeast continued to colonize under prolonged periods of high temperature and dry conditions (Teixido *et al.*, 1998).

Effects of antagonists on spore germination of pathogen

Germination of *P. digitatum* incubated on citrus was observed with a compound light microscope after 24, 48 and 72 h of incubation (Figure 3) Observations made at 24, and 48 h and of fruit treated with the mixed antagonistic microorganisms revealed complete inhibition of *P. digitatum* spore germination when compared to the healthy citrus tissues (Figure 3A). At 72 h cells treated with *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 alone showed slight hyphal germination of fungus in citrus tissues (Figure 3B-D) but much less than the disease control (Figure 3E). This indicates that competition for nutrients may play a role using antagonistic yeast and bacteria. When orange peel extract was used as a nutrient source, the bacterium Pantoea agglomerans CPS-2 used as an antagonist prevented germination at a low nutrient concentrations but not at higher nutrient concentrations (Pope *et al.*, 2003). However, there are several different modes of action included in biocontrol by antagonistic yeast and bacteria, including mycoparasitism, induced resistance and the production of fungal lytic enzymes, e.g. β-1,3-glucanase, chitinase (Wilson *et al.*, 1991; Leelasuphakul *et al.*, 2006). However, the modes of action of combined yeast and bacteria to control postharvest disease of fruits are still not clear because of the difficulties encountered during the study of these complex interactions. The modes of action should be further investigated to determine their precise action.

Effects of antagonists on postharvest quality

Experiments to evaluate the efficacy of combined antagonists and with $NaHCO_3$ showed no significant effect on the quality of fruit parameters: weight loss, fruit firmness, total soluble solids, titratable acidity did decrease slightly after incubation for were shown

Table 1. Effects of antagonistic microorganisms in combination with NaHCO$_3$ on postharvest quality parameters of citrus fruit

Treatment	Weight loss[a] (%)	Firmness (N)	Soluble solids (%)	Titratable acidity (%)
Control	0.039±7.82a	8.20 ± 0.18a	5.2 ± 0.88a	0.549 ± 0.33a
BCC5389+ABS-S14	0.038 ± 5.79a	7.05 ± 0.13a	5.62 ± 0.91a	0.465 ± 0.14a
BCC5389+ABS-S14 +NaHCO$_3$	0.030 ± 5.01a	7.05 ± 0.15a	5.42 ± 0.56a	0.419 ± 0.11a

[a]Quality parameters of citrus fruit were obtained after 7 days at 25°C. Mean are for two trials. Values within columns followed by the same letter are not significantly different at P = 0.05 according to the LSD test.

not to be greatly affected by the treatments after storage at 25°C for 7 days. However, the values for the control treatment were slightly higher than the others. The results are presented in Table 1 (P < 0.05). In addition, it was shown that the combination did not impair the fruit quality parameters under commercial conditions and indicated that they had a potential for commercialization. In conclusion, our result highlights show that the combination of yeast and bacterium can be used as a non-chemical alternative treatment against postharvest diseases of Shogun mandarins. Future research will be aimed at investigating about the mode of action of the combination to control postharvest diseases of fruits.

Acknowledgement

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