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CONTROL OF *PENICILLIUM EXPANSUM* BY COMBINING *BACILLUS SUBTILIS* AND SODIUM BICARBONATE

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Abstract

In the recent years, biological control has been explored as an alternative to the use of synthetic fungicides for managing postharvest decay. Some exogenous substances, such as chitosan, amino acids, carbohydrates, carbonate and bicarbonate salts have been studied to enhance biocontrol capability of antagonists against fungal pathogens. Simultaneous application of chemicals and biocontrol agents could provide more effective means of control and consistent results than that of one approach alone. The objective of the present study was to evaluate and compare the biocontrol efficacy of *Bacillus subtilis* (CFBP 4228) with and without sodium bicarbonate (SBC) against *Penicillium expansum* on apple fruits. The addition of 3% (w/v) SBC in the suspension of *B. subtilis* completely inhibited spore germination of *P. expansum* in potato dextrose broth medium. In combination with *B. subtilis*, SBC exhibited a consistent ability to enhance the biocontrol performance of antagonist against *P. expansum*. Lesion diameter of apple fruits treated with mixture of *B. subtilis* and SBC was significantly reduced, in contrast to inoculation with *B. subtilis* alone. The results of this study show that combination of *B. subtilis* and SCB provided a more effective control on *P. expansum* than applying the antagonist or SBC alone, and can be used as a non-chemical alternative treatment against blue mold on apple fruits.

Key words: Penicillium expansum, postharvest decay, Bacillus subtilis, sodium bicarbonate

Introduction

Blue mold caused by *Penicillium expansum* Link is economically important postharvest disease of apple fruits. Conidia of *P. expansum* are present in the orchards, fruit storage rooms, dump-tank and flotation-tank water, and packing facilities. Wounds such as punctures, and bruises on the fruit created at harvest and during postharvest handling are the primary avenue for infection of fruit by this fungus (Rosenberger, 1990). Patulin, a mycotoxin produced by *Penicillium* spp. during fruit spoilage, is a major concern, since exposure can result in severe acute and chronic toxicity, including carcinogenic, mutagenic, and teratogenic effects (McCallum et al., 2002).

Synthetic chemical fungicides, have been traditionally used to control and reducing postharvest diseases (Eckert and Ogawa, 1988). However, the development of fungicide resistance by postharvest pathogens and an increasing environmental concern over fungicide residues in food, have stimulated to find alternative means for controlling postharvest decay (Holmes and Eckert, 1999). Antagonistic biocontrol involves the use of naturally occurring nonpathogenic microorganisms that are able to reduce the activity of plant pathogens and thereby suppress diseases. Antagonistic microorganisms can complete with pathogens for nutrients, inhibit pathogen multiplication by secreting antibiotics or toxins, or reduce pathogen population through hyperparasitism (Mahadtanapuk et al., 2007). Several strains of the genus *Bacillus* produce spores that are resistant to various physical and chemical treatments, such as desiccation, heat, UV

irradiation, and organic solvents, and serve as excellent biological control agents (BCAs) against a wide range of plant pathogens by their production of antibiotics (iturin, surfactin and fengycin), cell wall-degrading enzymes (chitinase and β -1, 3 glucanase), and antifungal volatiles (Fiddaman and Rossall, 1993; Knox et al., 2000; Leelasuphakul et al., 2008). *Bacillus subtilis* isolated from citrus fruit surface was successfully evaluated for control of citrus green and blue molds caused by *P. digitatum* and *P. italicum*, respectively (Obagwu and Korsten, 2003).
However, BCAs do not have as wide a spectrum of activity under various conditions as fungicides, and most of them cannot achieve the effectiveness of fungicides even under optimal conditions (Conway et al., 2007). Therefore, simultaneous application of several physical and chemical methods could provide more effective means of control and consistent results than that of one approach alone (Zamani et al., 2009). Some exogenous substances, such as chitosan, amino acids, carbohydrates, carbonate and bicarbonate salts have been studied to enhance biocontrol capability of antagonists against fungal pathogens (Depasquale and Montville, 1990; El-Ghaouth et al., 1992; Tian et al., 2002). Among them, sodium bicarbonate (SBC) is effective in controlling postharvest decay of fruits (Smilanick et al., 1999; Karabulut et al., 2001; Yao et al., 2004).
The objective of the present study was to evaluate and compare the biocontrol efficacy of *Bacillus subtilis* (CFBP 4228) and SBC (alone and in combination) against *Penicillium expansum* on apple fruits.

Material and methods

Pathogen and Antagonist

P. expansum was isolated from decayed apple fruits. The pathogen was maintained on potato dextrose agar (PDA) at 4°C. Conidial suspension of *P. expansum* was prepared as follows: the pathogen was grown on PDA under constant fluorescent light. After 2 weeks incubation at 25°C, spores were harvested by flooding the plates with 10 ml of sterile distilled water containing 0.05% (v/v) Tween 80, scraping with a rubber spatula, and then filtering the suspension through double layers of cheesecloth. The spores were counted with a haemocytometer, and adjusted with sterile distilled water to $1x10^6$ conidia/ml.
The antagonistic bacterium, *B. subtilis* (CFBP 4228), was obtained from the French Collection of Plant associated bacteria. The bacterial strain was maintained on nutrient agar (NA).

Effect of *B. subtilis* and SBC on *P. expansum* spore germination

Aliquots of 5 ml potato dextrose broth (PDB) were placed in glass tubes; 100 µl of conidial suspension of *P. expansum* ($1x10^6$ conidia/ml) and 100 µl of bacterial suspension of *B. subtilis* ($1x10^8$ CFU/ml), with or without 1, 2 or 3% (w/v) SBC were added into the glass tubes. All treated tubes were placed in a rotary shaker at 110 rpm at 25°C. After 18 h incubation, 100 spores of fungal pathogen were measured for germination rate and germ tube elongation. Spores were considered germinated when germ tube length was equal to or greater than spore length.

Biocontrol test

Apple fruits (cv. Golden Delicious) were dipped in ethanol (70%) for 2 min, rinsed with sterile distilled water, air-dried, and punctured with a sterile micropipette tip at the equatorial region (3-4 mm depth). Aliquots of 25 µl of bacterial suspension of *B. subtilis* ($1x10^8$ CFU/ml), and 3% solution of SBC, either alone or in combination, were pipetted into each wound. After 1 h, wound was inoculated with 25 µl of conidial suspension of *P. expansum* ($1x10^6$ conidia/ml). The positive control fruits were inoculated only with fungal conidial suspension, and the negative control with

sterile distillate water. The all apples were placed in moist chamber and incubated at 25°C. After 7 days treatments were compared based on lesion diameters. There were three replicates for each treatment, and the experiment was repeated twice.

Data analysis

All data were analyzed by analysis of variance (ANOVA). Mean values were compared using Duncan's multiple range test, and significance was evaluated at $P<0.05$. Statistical analysis was performed using STATISTIKA v.6 (StatSoft, Inc.).

Results and discussion

Control of *Penicillium* decay of apple fruit is known as very difficult to achieve by biological means, due to the high competitiveness of the pathogen in the wound niche (Wilson and Wisniewski, 1989; Janisiewicz and Korsten, 2002). The effectiveness of antagonist to control diseases caused by *Penicillium* spp. on various fruit improved when they were used with different organic and inorganic additives. Among these additives, bicarbonate salts have showed broad spectrum antimicrobial properties and potential for controlling postharvest decay on various fruits (Smilanick et al., 1999; Karabulut et al., 2001; Yao et al., 2004; Convay et al., 2007). The inhibitory effect of bicarbonate salts on fungal pathogens is probably due to the reduction of cell turgor pressure that results in collapse and shrinkage of hyphae and spores, and consequent inability of fungi to sporulate (Fallik et al., 1997).

The results of this study indicated that conidial germination and germ tube growth of *P. expansum* in PDB was completely inhibited when *B. subtilis* combined with 3% solution of SBC. Zamani et al. (2008) indicated that the presence of SBC in antagonist suspension enhanced the inhibition of spore germination and germ tube elongation of *P. digitatum.* Significant differences were observed among the other treatments and positive control (Table 1).

The result of biocontrol test indicated that 3% SBC was not significant effective in controlling decay incidence of apple fruits caused by *P. expansum* (Table 2). This result was consistent with the observation of Yao et al. (2004), who found that treatment with 2% solution of SBC was not effective in reducing blue mold decay on pear fruits. Biocontrol efficacy of *B. subtilis* was better as compared to SBC, and the disease incidence on antagonist treated fruits was significantly lower than those on the control. However, treatment of fruits with mixture of *B. subtilis* and 3% solution of SBC significantly reduced lesion diameter of apples infected by fungal pathogen. The addition of SBC markedly enhanced the biocontrol activity of *B. subtilis* and showed significantly better disease control in comparison with the antagonistic bacterium alone.

Similar results were observed by Palou et al. (2001), who reported that significant control of *P. italicum* resulted when fruit were treated with 2, 3, and 4% solutions of SBC. Treatment with 1% solution of SBC was ineffective. The SBC treatment was mainly fungistatic, and not very persistent since the fungus survived the treatment. The presence of bicarbonate residues in the wounds was thought to be the cause of the fungistatic effect. The SBC presence delayed spore germination in the treated wounds. Porat et al. (2003) observed that 2% solution of SBC inactivated spore germination of *P. digitatum* in citrus fruit wounds. Also, antagonist *B. subtilis* with SBC improved decay control of *P. digitatum* and *P. italicum* (Obagwu and Korsten, 2003). It was concluded that the space created by the disruption of the pathogen development at the wound site by SBC may have given the antagonist a competitive advantage.

Table 1. Effects of *B. subtilis* and SBC on spore germination and germ tube length of *P. expansum*.

Treatment	Spore germination (%)	Germ tube length (µm)
Control of *P. expansum*	95.2 ± 2.7* a**	112.3 ± 0.8 a
B. subtilis	46.5 ± 2.1 e	55.5 ± 0.9 e
1% SBC	89.2 ± 1.5 b	90.4 ± 1.3 b
2% SBC	68.9 ± 2.3 c	72.2 ± 1.1 c
3% SBC	58.2 ± 0.8 d	62.3 ± 0.6 d
B. subtilis + 1% SBC	31.3 ± 0.9 f	40.5 ± 1.6 f
B. subtilis + 2% SBC	19.4 ± 1.1 g	27.9 ± 1.4 g
B. subtilis + 3% SBC	0 h	0 h

*Data represented standard deviations of the means.
**Values in each column followed by a same letter are not statistically different by Duncan's multiple range test ($P < 0.05$).

Table 2. Biocontrol effects of *B. subtilis* and SBC against *P. expansum* decay on apple fruits.

Treatment	Lesion diameter (mm)
Positive control	34.6 ± 1.5* a**
Negative control	0 e
B. subtilis	18.3 ± 1.1 c
3% SBC	26.3 ± 0.5 b
B. subtilis + 3% SBC	10.0 ± 1.7 d

*Data represented standard deviations of the means.
**Values in column followed by a same letter are not statistically different by Duncan's multiple range test ($P < 0.05$).

Conclusion

Under *in vitro* conditions, SBC had significantly potential to enhance the antifungal effect of *B. subtilis*. Suppressive effects include reduced sporulation and germ tube growth of *P. expansum.* The addition of 3% (w/v) SBC in the suspension of *B. subtilis* completely inhibited spore germination of fungal pathogen in PDB medium. However, the inhibitory effects *in situ* were not as strong as those *in vitro*. The results of biocontrol test showed that combination of antagonistic *B. subtilis* with 3% (w/v) SBC provided a more effective control than the application of antagonist or SBC alone, which should be considered to be a useful and promising measure for controlling postharvest decay of *P. expansum* on apple fruits.

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