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Commercial testing of *Kloeckera apiculata*, isolate 34-9, for biological control of postharvest diseases of citrus fruit

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Abstract - The efficacy of the yeast *Kloeckera apiculata* strain 34-9 to control the natural incidence of postharvest decay of citrus fruit under laboratory and commercial conditions was evaluated. Small-scale experiments with citrus fruit dipped into the yeast cell suspension were carried out to test its inhibitory effect, and the development of decay in citrus was effectively inhibited. The yeast was compatible with a low concentration of a commonly used fungicide. In packinghouse tests, combining the yeast with 40 mg/L MBC (Carbendazim) resulted in a reduction in the incidence of decay, caused by the green and blue moulds (*Penicillium digitatum* and *Penicillium italicum*, respectively), equal to a conventional fungicide treatment of 200 mg/L MBC. In commercial packinghouse tests, the efficacy of *K. apiculata* strain 34-9 could be maintained to be effective in controlling the decay of several cultivars under packinghouse conditions at a cell concentration of the yeast antagonist 3 x 10^8 cells/mL. In all experiments, after storage at 5 °C for 90 days, *K. apiculata* strain 34-9 did not alter any quality parameters of fruit.

Key words: postharvest disease, commercial testing, green mould, blue mould.

INTRODUCTION

Postharvest losses of fresh fruit and vegetables can be very costly depending on species, harvest methods, length of storage, and marketing conditions. Postharvest diseases often account for a major part of the losses (Bull *et al.*, 1997; Droby *et al.*, 1998; Pailly *et al.*, 2004) and their control requires use of a large amount of fungicides (Wilson *et al.*, 1993; Holmes and Eckert, 1999). Concerns regarding human health and environmental risks associated with chemical residues in foods have been the main driving force for the search for new and safe postharvest disease control methods. In addition, after prolonged use, the effectiveness of many of the fungicides declines because of the selection and proliferation of fungicide-resistant biotypes of pathogens (Holmes and Eckert, 1999). Losses have increased in most citrus-producing areas of the world because of this problem (Eckert *et al*., 1990).

Biological control of postharvest diseases by antagonistic microorganisms has been a promising alternative to fungicides (McGuire, 1994; Wilson *et al.*, 1996; Zhang *et al.*, 2005). Substantial progress in advancing this technology from the laboratory to practical commercial application has been realized (Droby *et al*., 1998; Hofstein *et al.*, 1994; Long *et al.*, 2006). Some of this technology has been patented and commercial products such as Aspire (Ecogen Corporation, Langhorne, PA) and Biosave 110 (EcoScience Inc., Worcester, MA) have been registered for commercial use to control postharvest decay of horticultural products.

Green and blue moulds of citrus fruit, caused by *Penicillium digitatum* (Pers.: Fr) Sacc. and *Penicillium italicum* Wehme respectively, are among the most economically important postharvest diseases of citrus worldwide. It is estimated that losses of citrus due to blue mould infection is 30 to 50% in China (Deng *et al.*, 2002), the world's largest producer of citrus. One major research focus in this area has been selection and development of effective and environment-friendly agents for biological control of this disease (Fan *et al*., 2000; Long *et al*., 2005).

The paper reports results of pilot tests using *Kloechera apiculata* strain 34-9 which was conducted during three successive seasons (2003, 2004, and 2005) in a commercial citrus packinghouse in HZAU (Huazhong Agricultural University). The purpose of these experiments was to evaluate the commercial potential of this yeast to control of postharvest decay of citrus, particularly the efficacy of *K. apiculata* strain 34-9 applied to Guoqing 1, Owari, Ponkan and Newhall Navel Orange.

MATERIALS AND METHODS

Yeast preparations and fruit material. *Kloechera apiculata* strain 34-9 was isolated from among the microbial the epiphytes of citrus roots (Long *et al*., 2005). Cultures of *K. apiculata* strain 34-9 used for fermentation were

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grown on yeast peptone dextrose (YPD) medium containing 20 g peptone, 10 g yeast extract, and 20 g of D-glucose in 1 L of distilled water. Erlenmeyer flask containing 5 mL of YPD were inoculated with 100 µL of a 24-h old starting culture of *K. apiculata* strain 34-9 and incubated on a rotary shaker at 25 °C for 48 h. Cultures were then used as inocula applied in a commercial citrus packinghouse. The cells were harvested by centrifugation at 8000 rpm (about 4700 *x g*) for 10 min, washed twice with 0.9% NaCl and resuspended in distilled water. The concentration of cells in the suspension was counted with a haemocytometer and adjusted to $3x10^8$ CFU(colony-forming unit)/mL with sterile distilled water.

The cultivars Guoqing 1 (*Citrus unshiu* Marc cv. Guoqin No. 1), Owari (*Citrus unshiu Marc* cv. Owari), Ponkan (*Citrus reticulate* Blanco cv. Ponkan) and Newhall Navel Orange (*Citrus sinensis* Osbeck cv. Newhall navel orange) were commercially harvested in a nearby orchard (Yichang Hubei province, Shimen Hunan province) and used within 24 to 48 h after harvest, and stored in the ventilated warehouse at 5 °C.

Efficacy of *Kloechera apiculata* strain 34-9 in small-scale experiments and quality of treated citrus fruit. The ability of *K. apiculata* strain 34-9 to reduce the development of postharvest decay on citrus was evaluated on the variety 'Guoqing 1' mandarin. Fruit were dipped in 30 s in a cell suspension of *K. apiculata* strain 34-9 containing 3 x 10^8 CFU/mL, and allowed to dry for in air for 72 h, and then packed into commercial cartons and stored at 5 °C. Non-treated fruit were used as controls. There were three boxes for each treatment and each box served as a single replicate (50 fruit per box). Each treatment consisted of three replicate of 50 fruits in each experiment, and the experiment was conducted three times. Following three month of storage, the development of natural decay, including moulds and other rots, was determined. The results were expressed as percentage of fruits that were decayed.

To evaluate the effect of *K. apiculata* strain 34-9 on the postharvest quality of citrus, freshly harvested fruit were treated, and stored as described above to evaluate the effect of *K. apiculata* strain 34-9 on reducing natural decay development. Quality parameters were measured after storage at 5 °C on three replicates of five fruit each, and performed at 5 °C. The testing methods are described below.

Total soluble solids were determined by measurement of the refractive index of the pressed fruit juice collected from the five fruit of each replicate with a hand refractometer and the results expressed as percentages (g per 100 g fruit weight) (Larrigaudière *et al.*, 2002).

The 2,6-dichloroindophenol titrimetric method was used to determine the ascorbic acid content of pressed fruit juice. Results were expressed as milligrams of ascorbic acid per 100g sample (Ozden and Bayindiril, 2002).

Acidity was measured by titration with 0.1 N NaOH, pH 8.0; 1 g of juice diluted with 20 mL of distilled water was evaluated for each replicate. Titratable acidity was calculated as percent citric acid (Wright and Kader, 1997)

Commercial packinghouse tests. All experiments were conducted in a standard packinghouse with commercial processing equipment located at HZAU. The standard layout of the packinghouse processing line is shown in Fig. 1. At the entering station the fruit received an extensive sorting and washing with plain water, then they are dried in air. The fruit was then passed through a drencher station, where fungicides were applied. The fruit were then air-dried naturally and packed. Our experiments were integrated on the daily routine of the packinghouse operation.

The tests were performed during three successive seasons (2002/2003, 2003/2004, and 2004/2005) with a total of 12 tests. Three treatments were applied: 1) control, unbagged fruit (packed directly from the fruit bins just before entering the packing line); 2) *K. apiculata* strain 34-9 applied at a cell concentration of the yeast antagonist at 3 x 10^8 cells/mL plus 40 mg/L MBC; and 3) a commercial treatment [200 mg/L MBC plus 200 mg/L 2,4-D(2,4-dichlorophenol)]. In these tests, the commercial treatment served as a positive control for *K. apiculata* strain 34-9. *Kloechera apiculata* strain 34-9 and MBC were compatible with each other (data not shown). In addition, a series of three tests was conducted, separately with four different citrus cultivars, at different times during the season of 2003/2004, to evaluate the efficacy of *K. apiculata* strain 34-9 alone and the combination of *K. apiculata* strain 34-9 + 40 mg/L MBC or 200 mg/L MBC alone. Fruits treated with water and run on the packing line served as one treatment (Guoqing 1: 50 fruit/carton; Owari: 50-60 fruit/carton; Ponkan: 50-60 fruit/carton and Newhall Navel Orange: 45-55 fruit/ carton). Unless otherwise stated, fruit in all treatments were stored at 5 °C (85% relative humidity), and the development of decay was determined after 3 months. Each treatment consisted of three replicate, and the experiment was conducted three times.

Statistical analysis. Data were subjected to analysis of variance (ANOVA) using SAS Software. Statistical significance was assessed at $p < 0.05$ and Duncan's Multiple Range Test was used to separate means. The percentages of decayed fruit and germinated spores were transformed into arcsine square root values to normalize and improve homogeneity of variety of the data before analysis of variance was applied (Sukhvibul *et al.*, 1999). The percentages shown are untransformed data.

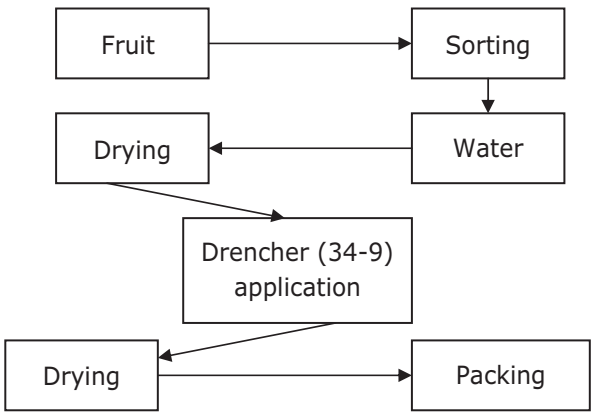

FIG. 1 - Schematic diagram of the packinghouse line steps in HZAU, China.

TABLE 1 - Efficacy of *Kloechera apiculata* strain 34-9 on natural decay development and quality parameters of citrus

Treatment	Disease incidence (%)	Soluble solids (%)	Ascorbic acid (mg/100 g)	Titratable acidity (% citric acid)
Control	25.2[a]*	10.6[a]	34.6[a]	0.50[a]
Antagonist	5.3[b]	10.8[a]	32.4[a]	0.52[a]

Means are for 3 trials. Values followed by the same letter are not significantly different at $p < 0.05$ (Duncan's Multiple Range Test).
* Decay and parameters of citrus quality were determined after 90 days storage at 5 °C.

RESULTS

Efficacy of *Kloechera apiculata* strain 34-9 in small-scale experiments and the quality of treated citrus fruit

Our experiments evaluated the efficacy of yeast antagonist in reducing the natural development of decay following storage at 5 °C for 90 days. The application of *K. apiculata* strain 34-9 resulted in average decay incidence among the fruit of 5.3% compared with 25.2% among the unbagged control fruits (Table 1). The yeast antagonist had no significant influence on firmness, total soluble solids, ascorbic acid or titratable acidity after three months of storage at 5 °C (Table 1).

Commercial packinghouse tests

Efficacy of Kloechera apiculata *strain 34-9 against "Guoqing 1" fruit rot*

A low concentration of MBC (200 mg/L, 1/5 to 1/10 of the commercially recommended concentration) was added to *K. apiculata* strain 34-9 preparations in most subsequent packinghouse tests. The combination of the antagonist cell suspension and MBC resulted in the lowest incidence of fruit decay (Fig. 2). Weight loss rates among the treatments (34-9+MBC and MBC) differed significantly from that of the control.

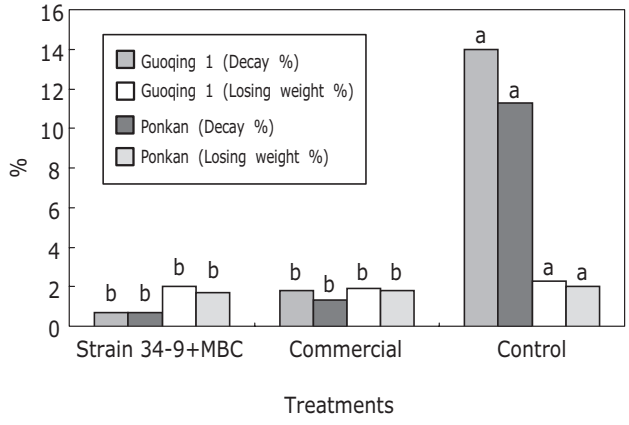

FIG. 2 - Effect of the combination of *Kloechera apiculata* strain 34-9 with a low concentration of MBC and its effect on the development of natural *Penicillium* rot infection and the losing weight rate in citrus after 90 days of storage at 5 °C. Columns with unlike letters are significantly different at $p < 0.05$ according to Duncan's multiple range test.

Efficacy of Kloechera apiculata *strain 34-9 against Owari fruit rot*

After 45 days storage, there was notable difference on both decay (including bagged fruit and unbagged fruit) and losing weight rate (including bagged fruit and unbagged fruit) among the treatments and the control. The decay of bagged fruit that had been treated with 34-9+MBC or MBC was 1.22% and 1.76%, respectively, compared to 7.68% among the control fruit. The decay of unbagged fruit that had been treated with 34-9+MBC or MBC was 2.83% and 2.29%, respectively, compared to 17.89% among the control fruit. Weight loss was more rapid among the control fruit than those treated with 34-9+MBC or MBC (Table 2).

After 90 days, both decay and weight loss were higher than after 45 days of storage. The treatments had significantly reduced the decay of bagged fruit, decay of 34-9+MBC, MBC and control were 3.03, 4.04, and 10.02%, respectively (Table 3). The decay and losing weight rate of unbagged fruit were higher than that of bagged fruit. The magnitude of control of decay and trends of weight loss associated with the 34-9+MBC and MBC treatments were similar in these tests with cultivar Owari to those with Guoqing 1. Therefore, the yeast was compatible with a mixture of commonly low concentration of a chemical fungicide.

Efficacy of Kloechera apiculata *strain 34-9 against "Ponkan" fruit rot*

The efficacy of 34-9+MBC was evaluated in three separate tests conducted with Ponkan fruit (Fig. 2). The combination of *K. apiculata* strain 34-9 and MBC (40 mg/L) had the lowest number of decayed fruit compared to the controls or those treated with 200 mg/L MBC alone (Fig. 2).

Efficacy of Kloechera apiculata *strain 34-9 against "Newhall Navel Orange" fruit rot*

The 34-9+MBC treatment effectively reduced the development of decay in Newhall Navel Orange (Table 4). The efficacy and weight loss rate among fruit after 34-9+MBC or commercial MBC treatment were similar.

Efficacy of Kloechera apiculata *strain 34-9 against rots of various citrus fruit cultivars during three seasons of experiments*

Results obtained from the packinghouse tests performed with various citrus fruits over three seasons showed the efficacy of the combination of *K. apiculata* strain 34-9 and

TABLE 2 - Effect of each treatment on the Owari after 45 days storage

Treatment	Decay (%)		Losing weight rate (%)	
	Bagged fruit	Unbagged fruit	Bagged fruit	Unbagged fruit
34-9+MBC	1.22[b]	2.83[b]	1.07[b]	4.13[c]
MBC	1.76[b]	2.29[b]	1.05[b]	5.27[b]
Control	7.68[a]	17.89[a]	1.42[a]	7.91[a]

Means are for 3 trials. Values followed by the same letter are not significantly different at $p < 0.05$ (Duncan's Multiple Range Test).

TABLE 3 - Effect of each treatment on the Owari after 90 days storage

Treatment	Decay (%)		Losing weight rate (%)	
	Bagged fruit	Unbagged fruit	Bagged fruit	Unbagged fruit
34-9+MBC	3.03[c]	4.12[c]	2.42[b]	5.44[b]
MBC	4.04[b]	6.84[bc]	2.58[b]	5.95[b]
Control	10.02[a]	21.51[a]	3.43[a]	8.38[a]

Means are for 3 trials. Values followed by the same letter are not significantly different at $p < 0.05$ (Duncan's Multiple Range Test).

a reduced concentration of MBC (Table 3, Table 4, Fig. 2). The addition of 40 mg/L of MBC to the yeast preparation resulted in a marked reduction in the development of postharvest rots in Guoqing 1 , Owari, Ponkan, and Newhall Navel Orange. The incidence of decay in the 34-9+MBC was reduced to a level of 4.2% lower while in the unbagged fruit, the decay incidence was 10.02% to 14%, depending on the cultivar used (Fig. 3).

DISCUSSION

Kloechera apiculata strain 34-9 was isolated from soil of a citrus grove, and our results indicated that *K. apiculata* strain 34-9 could significantly reduce citrus postharvest rots (Long *et al*., 2005). The small-scale experiments were conducted to compare the efficacy of the yeast cell suspensions with a common commercial treatment in protecting against the development of natural decay on 'Guoqing 1' mandarin (*Citrus unshiu*). Generally, reduction of decay by *K. apiculata* strain 34-9 compared with control was 10 to 40% , this variability was expected and could be a result of several factors related to the initial quality of the fruit used in each test, the susceptibility of the fruit (early or late season), and the time elapsed between picking and treatment.

TABLE 4 - Effect of each treatment on the "Newhall Navel Orange"

Treatment	Decay (%)	Losing weight rate (%)
34-9+MBC	4.2[b]	2.82[b]
MBC	4.4[b]	3.04[b]
Control	12.6[a]	3.41[a]

Means are for 3 trials. Values followed by the same letter are not significantly different at $p < 0.05$ (Duncan's Multiple Range Test).

Results of the packinghouse tests conducted with various citrus cultivars have indicated that the feasibility of large-scale production and application of *K. apiculata* strain 34-9 as a biological control agent of postharvest diseases of citrus is promising. *K. apiculata* strain 34-9 as a standalone treatment is not sufficient to reduce the decay to commercially acceptable levels. Insufficient and inconsistent performance is a common problem with commercial treatment; it is still a feasible method of postharvest decay control since decay levels did not exceed the commercially acceptable levels (above 4%). In order to achieve high efficacy comparable with the standard commercial treatment, *K. apiculata* strain 34-9 should be used in conjunction with a low concentration of MBC (40 mg/L). Although the combination of 34-9+MBC did not always result in a significantly lower incidence of decay compared with MBC, the 34-9+MBC is preferable because it provided a much lower

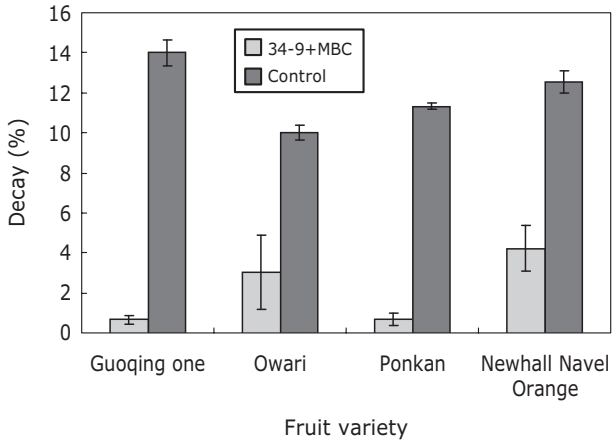

FIG. 3 - Effect of initial pH values on the oxidation of Fe^{2+} by strain YSK.

level of chemical fungicides. In addition to lower residues of MBC in the fruit, the costs of MBC would also be reduced since much less of it is needed. Also, compared with the MBC treatment alone, the 34-9+MBC resulted in a more consistent control, possibly because isolates resistant to MBC were controlled by this treatment. In order to eliminate the requirement to add MBC, other efficacy-enhancing methods must be developed. Research aimed at enhancing the efficacy of the yeast with physical control measures (hot water, ultraviolet light, controlled atmospheres, and cold storage) is currently being evaluated.

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