Annals of Microbiology, **59 (1)** 77-81 (2009)

Kloeckera apiculata strain (34-9) to control *Botrytis cinerea* during the pre- and postharvest handling of strawberries

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Received 21 November 2008 / Accepted 26 January 2009

Abstract - The efficacy of *Kloeckera apiculata* strain (34-9) in controlling gray mould (*Botrytis cinerea*) of strawberry fruit was evaluated in pre- and postharvest handling. Dynamic growth of *K. apiculata* strain (34-9) was tested in the field on strawberry. Antagonist population was 2.2 x 10^6 CFU/ml in strawberry fruit after it was treated 2 h in the field, then decreased slightly, and then the population stabilized at the concentration 10^5 CFU/ml during the period of strawberry growth. The effect of *K. apiculata* strain (34-9) (1.0 x 10^8 CFU/ml) on *B. cinerea* was evaluated. Preharvest (34-9) treatment was the most effective, while postharvest (34-9) and Sumilex treatment equally reduced the incidence of decay, caused by gray moulds (*B. cinerea*). Light microscopy revealed attachment of the yeast cells to the pathogen hyphae. *Kloeckera apiculata* strain (34-9) did not alter any quality parameters of fruit when assessed at the end of storage.

Key words: biological control; *Kloeckera apiculata* strain (34-9); *Botrytis cinerea*; strawberry; pre- and postharvest handling.

INTRODUCTION

Gray mould, caused by *Botrytis cinerea*, is the primary cause of decay on strawberry. Fungicides are often used to control decay; however, postharvest fungicides are not available for strawberry. With increasing consumer concerns and the loss of fungicide registrations, alternatives are necessary to provide decay control. Biological control agents have potential to be fungicide alternative (Swadling and Jeffries, 1996; Wilson, 1997). Swadling and Jeffries (1996) screened 559 microorganisms isolated from strawberry; 27 of these inhibited *B. cinerea* growth in several *in vitro* tests and were further tested *in vivo* on strawberry. Lima *et al*. (1997) tested the effectiveness of yeast *Aureobasidium pullulans* and *Candida oleophila* on strawberry to control postharvest rots caused by *B. cinerea* and *Rhizopus stolonifer*. Guinebretiere *et al*. (2000) isolated one strain of *Candida reukaufii*, one strain of *Candida pulcherrima*, and two *Enterobacteriaceae*, which effectively colonized strawberry fruit wounds and strongly inhibited *B. cinerea* spore germination in vitro. Wszelaki (2001) tested heat treatment, biological control and controlled atmospheres (CA) as alternatives to pesticides in control of *B. cinerea* during postharvest handling of strawberries. She found high oxygen atmospheres could be used as a substitute for traditional CA, that *B. cinerea* germination was hindered with hot water treatments of 45 and 50 °C, and that three yeasts (*Pichia guilliermondii*, *C. oleophia* and *A. pullulans*) had biocontrol capabilities against gray mould. *Pichia gulliermondii* provided the most effective control, without causing fruit injury.

Biological control agents are believed to have several modes of action, including competing for space and nutrients on the commodity, antibiosis, restricting the action of pathogen hydrolytic enzymes, production of enzymes to degrade pathogen cell walls, and direct parasitism of the pathogen (Elad, 1996). Long *et al*. (2006) found antagonist yeast *K. apiculata* strain (34-9) cells surrounding the germinating spores of blue mould and attachment of the yeast cell was observed. Guinebretiere *et al*. (2000) tested antagonist bacterial cells encircling the germinating spores of *B. cinerea* and attachment of the antagonist cell, indicating direct parasitism.

The paper reports pre- and postharvest experiments and pilot tests using the yeast antagonist 34-9 which was applied to strawberry. The purpose of these experiments was to evaluate the commercial potential of this yeast for the control of postharvest decay of strawberry fruit, with the goal of obtaining effective, non-chemical decay prevention against *B. cinerea* using strawberries as a model system.

MATERIALS AND METHODS

Yeast and pathogen cultures. *Kloeckera apiculata* strain (34-9) was isolated from the epiphytes of strawberry roots. Cultures of 34-9 used for fermentation were grown on Yeast Peptone

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Dextrose (YPD) containing 20 g peptone, 10 g yeast extract, and 20 g of D-glucose in 1 litre 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. The concentration of *K. apiculata* strain (34-9) in the suspension was counted with a hemocytometer and adjusted to 1.0 x 10^8 colony-forming unit (CFU)/ml with sterile distilled water.

Cultures of *B. cinerea* were obtained from decayed strawberry, grown on potato Dextrose agar (PDA) , and kept on PDA slants at 4 °C for later use.

The strawberry fruit. Strawberries were hand-picked in the field in the local strawberry orchard, preferably in the morning before outside temperatures became too high, which raised the fruit temperature and accelerated deterioration. Fruits were kept at 0 °C for later use.

Population dynamics. *Kloeckera apiculata* strain (34-9) suspension (1.0 x 10^8 CFU/ml) applied to strawberry plants during the period of growth. The dynamic growth of *K. apiculata* strain (34-9) was first tested 2 h after when it was applied in the field. For the treatment three replicates of four fruits were homogenized in 10 ml of sterile physiological solution (0.9% NaCl). The suspension was diluted 10 to 10^6 fold according to treatment and 100 µl of each dilution was spread on bean sprouts medium containing 30 mg/l chloramphenicol. The plates were incubated at 25 °C and after 2-3 days the number of colonies of isolate 34-9 was recorded. The yeast population was monitored every two weeks after treatment.

Efficacy of *Kloeckera apiculata* on natural decay development and quality parameters of strawberry. The ability of *K. apiculata* to reduce the development of postharvest decay on strawberry fruit was evaluated. In the experiments conducted with strawberry fruit, the variety of "Fengxiang" and "Hongjia" was used after harvest. Fruit was dipped momentarily in a cell suspension of *K. apiculata* strain (34-9) (1.0 x 10^8 CFU/ml), allowed to air-dry for 1 h, and then packed in commercial cartons and stored at 0 °C. Fruit dipped in plain water was used as control. There were three boxes for each treatment and each box served as a single replicate (50 fruits per box). During the storage, the development of natural decay, including moulds and other rots, was determined. The results were expressed as percentage of decay.

To evaluate the effect of 34-9 on postharvest quality of strawberry, freshly harvested fruit were treated, and stored as described above to evaluate the effect of *K. apiculata* on reducing natural decay development. Quality parameters were measured after storage, on three replicates of five fruit each, and performed at 0 °C. The testing methods are described below.

Soluble solids. Total soluble solids were determined by measuring the refractive index of the same juice with a hand refractometer and the results expressed as percentages (g per 100 g fruit weight) (Larrigaudière *et al*., 2002).

Ascorbic acid. The 2,6-dichloroindophenol titrimetric method was used to determined the ascorbic acid content of pressed fruit juice. Results were expressed as milligrams of ascorbic acid per 100 g sample (Zhang *et al*., 2005).

Titratable acidity. Acidity was measured by titration with 0.1 N NaOH to pH 8.0; 4 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).

***In vitro* interaction of strain 34-9 with pathogen hyphae.** The possible interaction of strain 34-9 with pathogen hyphae was assessed *in vitro*. A uniform wound (3 mm deep by 2 mm wide) was made at the equator of strawberry fruits using a sterile nail. First, wounds were inoculated with a 20 µl of *K. apiculata* strain (34-9) suspension. After 4 h, a 20 µl of *B. cinerea* suspension was added to each wound. The fruits were put into a 400 mm by 300 mm by 100 mm plastic tray wrapped with a high density polyethylene sleeve in order to maintain about 95% relative humidity, then stored at 25 °C for 24 h. Light microscopy was used for interaction observation.

Statistical analysis. The results were submitted to analysis of variance and the mean values were compared using Duncan's multiple range test.

RESULTS

Colonization of wounds by *Kloeckera apiculata* strain (34-9)

The populations of strain *K. apiculata* strain (34-9) decreased slowly in fruits in the field. At the beginning of the experiment (time 0 h), the yeast population was 2.2 x 10^6 CFU/ml per strawberry fruit, and reduced to 2.0 x 10^5 CFU/ml in strawberry during the period of growth (Fig. 1), then stabilized thereafter (6.0 x 10^5 CFU/ml to 7.0 x 10^5 CFU/ml per strawberry).

Efficacy of *Kloeckera apiculata* strain (34-9) for controlling of *Botrytis cinerea*

The highest level of control of *B. cinerea* was achieved with preharvest treatment of *K. apiculata* strain (34-9), which inhibited lesion development of *B. cinerea* in strawberry fruit at 0 °C after 14 days cold storage (Fig. 2 and Fig. 3). The infection incidence of strawberry fruit treated by preharvest and postharvest of *K. apiculata* strain (34-9) were 16.7 and 23.3% after 14 days at 0 °C, respectively, which were significantly lower than control (53.3%). Disease incidence of strawberry fruit with Sumilex was 23.3%, which equalled that after postharvest treatment with *K. apiculata* strain (34-9).

Efficacy of *Kloeckera apiculata* strain (34-9) on quality parameters of strawberry

Kloeckera apiculata strain (34-9) had no significant effect on total soluble solids, ascorbic acid or titratable acidity during storage at 0 °C (Table 1).

Light microscopy

Light microscopy observations were made on *K. apiculata* strain (34-9) and the co-inoculated gray mould (*B. cinerea*). Pictures shown in Fig. 4 exhibit the presence of large numbers of yeast cells in wounded tissues and the attachment of yeast cells to the hyphae of the pathogen.

DISCUSSION

Botrytis cinerea of strawberry was effectively controlled by the application of *K. apiculata* strain (34-9) (Fig. 2 and Fig. 3). Preharvest treatment was the most effective, followed by post-

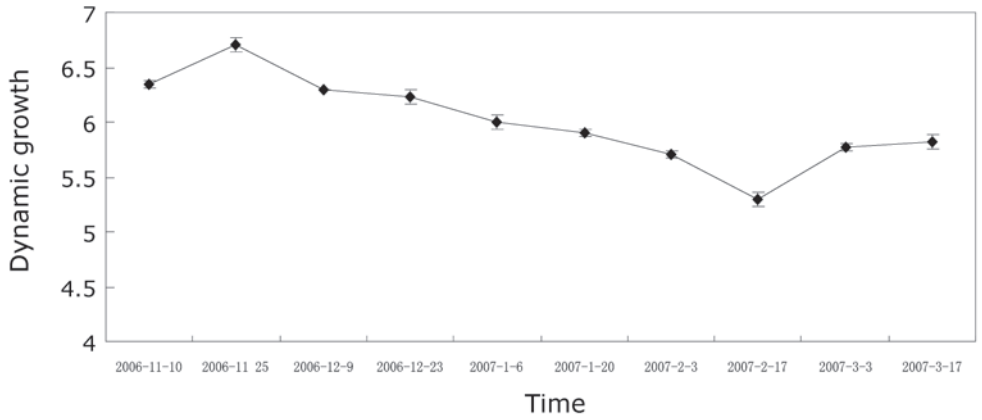

FIG. 1 - Dynamic growth of *Kloeckera apiculata* strain (34-9) when it was used field on strawberry.

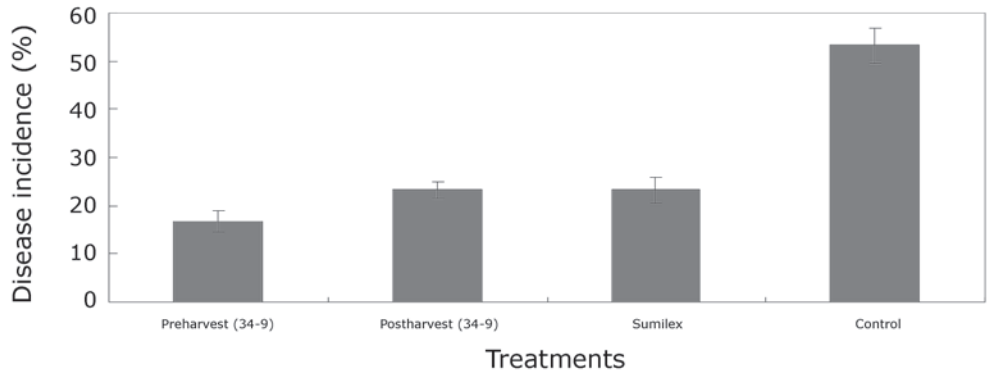

FIG. 2 - Effect of different treatments in strawberry after 14 d cold storage.

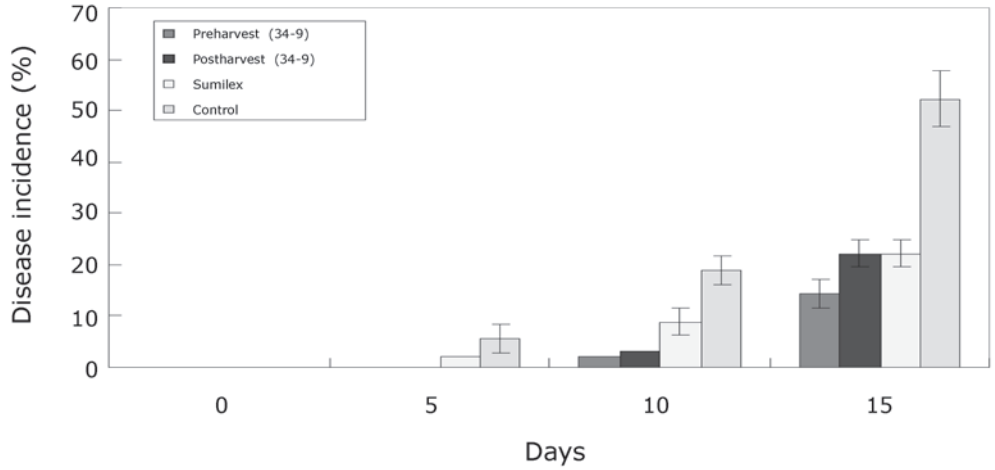

FIG. 3 - Disease incidence of strawberry with different treatments during storage.

TABLE 1 - Effects of different treatments on the nutrition content of strawberry during storage

Treatments	TSS	Titration acid	Vc (mg/100g·fw)
Preharvest	6.0a	1.01a	68.9a
Postharvest	5.4a	1.23a	58.7a
Sumilex	6.4a	1.24a	60.3a
Control	6.3a	1.38a	62.0a

Means with the same letter are not significantly different (p = 0.05) as determined by LSD.

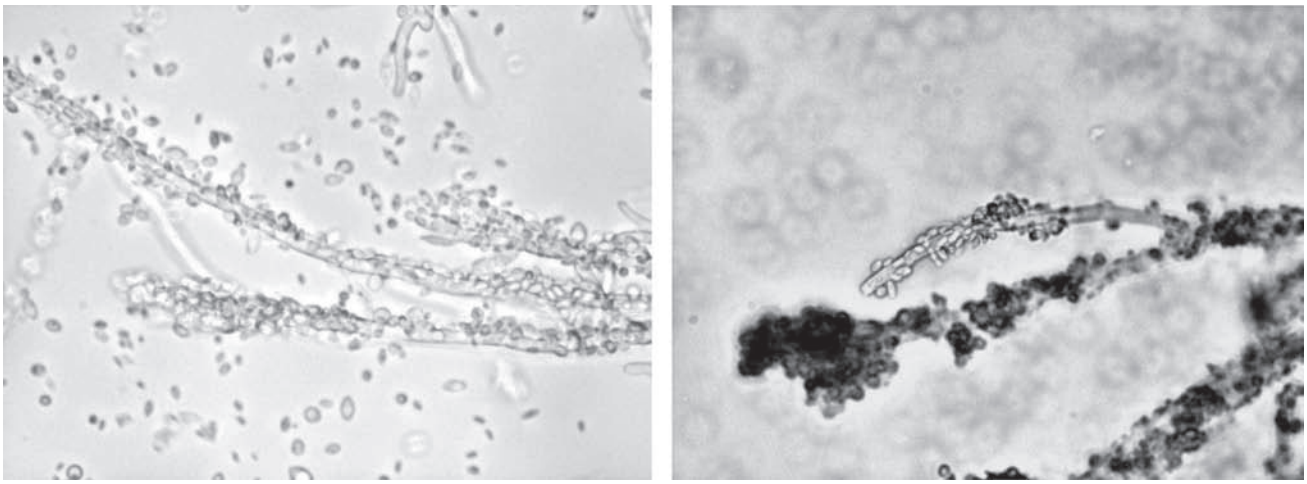

FIG. 4 - Antagonistic effect on *Kloeckera apiculata* strain (34-9) against *Botrytis cinerea* (magnification 400X).

harvest treatment and Sumilex application. The difference in application of antagonists could explain the difference in decay control. Huang *et al*. (1995) found that effective biocontrol was dependent upon the successful establishment of a bacterial antagonist (*Pseudomonas glathei*) as the wound site prior to challenge by the pathogen. McLaughlin *et al*. (1990) applied *P. guilliermondii* twenty-four hours before challenging with *B. cinerea*, while we applied *K. apiculata* strain (34-9) before harvest, to simulate field or latent biocontrol. Furthermore, the lack of significant decay control by the antagonist treatments could be related to maturity and ripened stage of the fruit at the time of the experiment. Janisiewicz *et al*. (1998) found that greater concentrations of biocontrol antagonists were needed to control decay on mature apple fruit than on less mature fruit. Although strawberries are picked near fully ripe, unlike many climacteric fruits, such as peach, which can be harvested "mature green" and ripened later, there is still considerable variation in maturity between pickers and fields. A biological control agent that could grow or maintain high levels to control decay during ripening and senescence would be optimal.

Rapid colonization of *K. apiculata* strain (34-9) in strawberry was observed during the period of growth, at an early stage *K. apiculata* strain (34-9) populations declined slowly, and then stabilized for the remaining time, respectively. The initial recovery after 1 h was 2.2 x 10^6 CFU/ml per strawberry fruit. Then, the yeast population decreased slowly after application. The dynamic growth of the yeast in strawberry plant and its ability to control *B. cinerea* indicated that *K. apiculata* strain (34-9) was well adapted to the field environment in fruit and had considerable potential as a biocontrol agent.

The observed restriction of fungal ingress and preservation of host wall integrity strongly indicate that *K. apiculata* strain (34-9) may have affected the ability of *B. cinerea* to degrade host tissue and establish a nutritional relationship. A similar attachment pattern was also observed in vitro with other yeast species (Douglas, 1987; Wisniewski *et al*., 1988; El-Ghaouth *et al*., 1998) including the antagonistic yeast *P. guilliermondii*, when co-cultured with *B. cinerea*, and was attributed to a lectin-type recognition (Wisniewski *et al*., 1991). As to the role played by the attachment capability of antagonistic yeasts in the observed control of lesion development, it has been suggested that attachment may enhance nutrient competition as well as interfere with the ability of the pathogen to initiate infection. Analysis in plant of the effect of treatments that negate attachment may provide further insight regarding the extent of the role played by attachment in the biocontrol activity of antagonistic yeasts.

Kloeckera apiculata strain (34-9), which is present in soil, fruit, leaf, wine, and grape must, is generally considered to be non-pathogenic (Identification Report-CNAL/No. L0726/09014, Hubei Institute of Health Surveillance, Analysis and Protection) and wound be safe to use for biological control proposes. Moreover, we found that the use *K. apiculata* strain (34-9) for biological control is compatible with several common postharvest practices including Carbendazim use and cold storage (Long *et al*., 2006), thus permitting an integrated control approach under commercial conditions.

Acknowledgements

This research was financially supported by a grant from the National Natural Science Foundation of China to B.D. (NSFC No. 30700555).

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