

Effects of *Cryptococcus laurentii* (Kufferath) Skinner on biocontrol of postharvest decay of arbutus berries

Xiaodong Zheng*, Hongyin Zhang, and Yufang Xi

College of Biosystem Engineering and Food Science, Zhejiang University, Hangzhou, 310029, P.R. China

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Abstract. *Cryptococcus laurentii* was tested as a biocontrol agent for reducing natural decay of arbutus berries caused by *Penicillium citrinum* and *Verticicladiella abielina* in semi-commercial postharvest trials. Three different preparations of *C. laurentii* were compared for antagonistic efficiency. Washed *C. laurentii* cells provided better protection against decay than yeast cultured in broth without washing while the culture supernatant free of yeast cells provided no protection. The protection provided by the washed yeast cells was dose-dependent. *Cryptococcus laurentii* was also effective in controlling decay at low temperature (4°C). The efficacy of *C. laurentii* was enhanced by the addition of 2% CaCl₂. Agar disks of *C. laurentii* NYDA cultures placed on PDA plates seeded with pathogens did not inhibit the growth of *P. citrinum* or *V. abielina*. Spore germination of the pathogens in potato dextrose broth was strongly inhibited in the presence of active cell suspensions.

Keywords: Arbutus berry; Biocontrol; CaCl₂; *Cryptococcus laurentii*; Postharvest decay.

Introduction

The shelf-life of the arbutus berry (*Mycira rubra* Sieb. et Zucc.) is very short because of its perishability and susceptibility to rot-causing pathogens. Postharvest pathogens cause major losses in arbutus berries in China. During storage and shipment of the arbutus berries, decay losses are mainly caused by *Penicillium citrinum* Thom and *Verticicladiella abielina* (Peck) Hughes. Synthetic chemical fungicides have been traditionally used to control these pathogens. Fungicide efficacy, however, is frequently decreased by the development of resistant strains of pathogens (Rosenberger and Meyer, 1981; Spotts and Cervantes, 1986). In addition, public concern and regulatory restrictions about the presence of fungicide residues on crops have emphasized the need to find alternative methods for disease control (Smilanick, 1994; Fan et al., 2000).

Biological control of fruit decay using a microbial antagonist has been considered a desirable alternative to synthetic fungicides. The control of major postharvest pathogens through application of biological agents was reported for stone fruits (Pusey and Wilson, 1984), pome (Janisiewicz, 1987, 1988; Janisiewicz et al., 1992), citrus (Chalutz and Wilson, 1990; Smilanick and Denis-Arrue, 1992), and other fruits (Lima et al., 1997).

Previous studies have shown that *Cryptococcus laurentii* (Kufferath) Skinner can be used as a bio-agent for postharvest control of gray and blue mold rot in apples (Roberts, 1990a), strawberries, kiwi fruits, and grapes (Lima et al., 1998), as well as of *Mucor* rot in pear (Roberts,

1990b). However, no literature is available on the postharvest biological control of mold rot in arbutus berries with a microbial antagonist. The objective of this study was to test whether *C. laurentii* can be used for postharvest biological control of mold rot in arbutus berries. We evaluated its effectiveness under semi-commercial conditions.

Materials and Methods

Fruits

The arbutus berries cultivars "Biji" were harvested at typical commercial maturity from Xianju of Zhejiang province. Fruits were used immediately after harvest.

Pathogen Inoculum

Penicillium citrinum and *Verticicladiella abielina* were isolated from infected arbutus berries and cultured on potato-dextrose agar medium (PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g; and deionized water, 800 ml). Spore suspensions were prepared by removing the spores from the sporulating edges of a 2-3-week-old culture with a bacteriological loop and suspending them in sterile distilled water. Spore concentration was determined with a hemacytometer and adjusted as required.

Antagonist

The culture of *C. laurentii* was obtained from Institute of Microbiology, Chinese Academy of Science (Beijing, P. R. China). Yeast cultures were maintained at 4°C on nutrient yeast dextrose agar (NYDA: 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar, in 1 L of distilled water).

*Corresponding author. Tel: 86-0571-86971167; Fax: 86-0571-86045315; E-mail: xdzheng@zju.edu.cn

Yeast were grown in 250-ml Erlenmeyer flasks containing 50 ml of NYD broth (NYDB) at 28°C on a rotary shaker (200 rpm) for 20 h. Cells were centrifuged at 6,000 rpm for 10 min and washed twice to remove the growth medium. Cell pellets were re-suspended in distilled sterilized water and brought to the initial concentration of 2×10^9 to 5×10^9 CFU/ml (CFU: colony-forming units). Cell concentration was then adjusted as needed for different experiments (Wisniewski et al., 1995).

Culture filtrates were prepared by filtering centrifuged culture of the antagonist through a 0.2 μ m polycarbonate membrane filter. The unwashed cells were grown with 20-h culture filtration and adjusted to 10^8 CFU/ml with additional culture filtration. The washed cell suspension was prepared as described above. Different treatments were performed as follows: A: culture filtrate, B: 1×10^8 CFU/ml unwashed cell culture mixture, C: 1×10^8 CFU/ml washed cell suspension, D: sterile distilled water as a control.

Efficacy of C. laurentii for Controlling Natural Infections

Fruits were treated by dipping them in suspensions A, B, C or D for 30 s, then air dried. The fruits were sealed in polyethylene-lined plastic boxes to retain high humidity and incubated at 20°C. Infection rate was determined 3 days after inoculation. There were three replicates of 16 fruits with a complete randomization in each test, and experiments were performed three times.

Effects of Concentrations of Yeast on Control Effectiveness

The suspensions of washed cells were adjusted to concentrations of 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 CFU/ml with sterile distilled water with the aid of a hemacytometer (Fan and Tian, 2000), with sterile distilled water as a control. Intact fruits were inoculated by dipping them in yeast suspensions for 30 s. Fruits were then air dried, sealed in polyethylene-lined plastic boxes to retain high humidity, and incubated at 20°C. Infection rate was determined 3 days after inoculation. There were three replicates of 16 fruits with a complete randomization in each test, and experiments were performed three times.

Efficacy of C. laurentii for Controlling Natural Infection under Cold Storage Conditions

The experiment conditions described above were repeated with the treated arbutus berries incubated at 4°C. The infection rate was determined 7 days after inoculation. There were three replicates of 16 fruits with a complete randomization in each test, and experiments were performed three times.

Effect of Calcium on Control Effectiveness

Suspensions of *C. laurentii* were prepared with washed cells and adjusted to 10^9 , 10^8 , 10^7 , 10^6 and 0 CFU/ml either in distilled water or in 2% calcium chloride. Fruits were treated with these suspensions as described above. Fruits

were incubated at 20°C and observed for percent infection at 3 days after challenge inoculation. There were three replicates of 16 fruits with a complete randomization in each test, and experiments were performed three times.

In Vitro Antagonism

To evaluate the interactions between the antagonist and the pathogens in culture, 15-mm-diameter disks from 5-day-old NYDA cultures of *C. laurentii* were cut and then placed on PDA plates seeded with 0.5 ml of a conidial suspension of each pathogen. The effect of the yeast antagonist on the growth of the pathogens was compared with that of *B. subtilis*, a known bacterial antagonist (Chalutz and Wilson, 1990).

The effect of *C. laurentii* on spore germination and germ tube elongation of pathogen was tested in potato dextrose broth (PDB). A 100- μ l quantity of 1×10^8 , 1×10^6 , 1×10^4 CFU/ml active yeast cell suspensions, culture filtrate or sterile distilled water (control) was added to a 10 ml glass tube containing 5 ml PDB. At the same time, aliquots (100 μ l) of spore suspensions (1×10^7 spores/ml) of *P. citrinum* or *V. abielina* were added to each tube. After 20 h of incubation at 25°C on a rotary shaker (50 rpm), at least 100 spores per replicate were observed microscopically to determine germination rate and germ tube length (Droby et al., 1997). All treatments consisted of three replicates, and experiments were performed three times.

Statistical Analysis of Data

All statistical analyses were performed using SAS (SAS Institute, Version 6.08, Cary, NC). To test for the effect of the treatments, the data were analyzed by one-way analysis of variance (ANOVA). Data for percentages of germinated spores (spore germination) were transformed into the arcsine square root values to normalize distribution before analysis of variance (Sukhvibul et al., 1999). Mean separations were performed using Duncan's multiple range test. The percentage of germinated spores shown are untransformed data.

Results

Efficacy of C. laurentii for Controlling Natural Infection

The highest level of control of mold rot was achieved with washed yeast cell suspension (Figure 1). The infection rate of the arbutus berries treated with 1×10^8 CFU/ml washed cell suspension was 39.6% after 3 days at 20°C, lower than that of the control and other treatments. The unwashed cell culture mixture provided significant control ($p < 0.05$), but less than the washed cell suspensions. Cell-free culture filtrate failed to provide protection.

Effects of Concentrations of Yeast on Control Effectiveness

Incidences of disease on fruits treated with all concentrations of *C. laurentii* were significantly lower than those

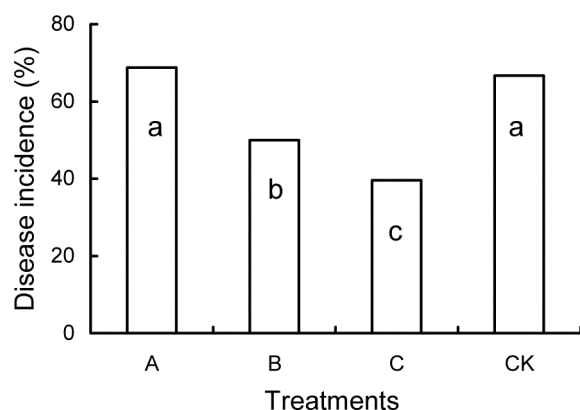


Figure 1. Inhibition natural infection of arbutus berries as affected by various treatments. A= culture filtrate; B= 1×10^8 CFU/ml unwashed cell culture mixture; C= 1×10^8 CFU/ml washed cell suspension, CK= Sterile distilled water. Each value is the mean of three experiments. Disease incidences were measured after 3 days at 20°C. Data in columns with different letters are significantly different according to Duncan's multiple range test at $p=0.05$.

treated with control except 1×10^6 CFU/ml ($p < 0.05$) (Figure 2). The results showed that the higher the concentrations of the antagonist, the lower the disease incidence. At the concentration of the washed cell suspension of *C. laurentii* of 1×10^9 CFU/ml, decay incidence was 41.7% while the control fruit incidence was 68.8%. At 20°C for 3 days, the relationship of the percentage of the arbutus berries that developed lesions (Y) to the concentration of *C. laurentii* applied to the fruits (X) was described by the equation: $Y = 64.0 - 1 \times 10^{-7}X + 1 \times 10^{-16}X^2$, $r^2 = 0.86$.

Efficacy of *C. laurentii* on Controlling of Natural Infection under Cold Storage Conditions

Cryptococcus laurentii significantly inhibited mold rot ($p < 0.05$) on arbutus berries stored at 4°C for 7 days at all

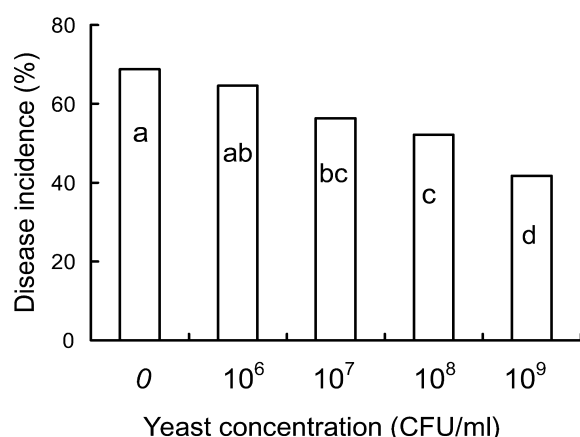


Figure 2. Effects of concentrations of yeast on control effectiveness. Each value is the mean of three experiments. Disease incidences were measured after 3 days at 20°C. Data in columns with different letters are significantly different according to Duncan's multiple range test at $p=0.05$.

concentrations (Figure 3). The concentrations of *C. laurentii* also significantly influenced control efficacy under cold storage condition. At 1×10^8 and 1×10^9 CFU/ml, the incidence of disease was reduced by 34.7% and 43.4% compared to the control, respectively. At 4°C for 7 days, the relationship of the percentage of the arbutus berries that developed lesions (Y) to the concentration of *C. laurentii* applied to the fruits (X) was described by the equation: $Y = 40.8 - 1 \times 10^{-7}X + 1 \times 10^{-16}X^2$, $r^2 = 0.68$.

Effect of CaCl_2 on Biocontrol Efficacy of *C. laurentii*

CaCl_2 significantly improved inhibition of decay by *C. laurentii* (Figure 4). Disease incidences of fruits treated with 2% CaCl_2 were significantly lower than those treated with water at all yeast concentrations ($p < 0.05$). The yeast concentrations either in water or in 2% CaCl_2 also significantly affected the biocontrol efficacy.

Biocontrol Activity of *C. laurentii* in Vitro

Agar disks of *C. laurentii* NYDA cultures placed on PDA plates seeded with pathogens did not inhibit the growth of *P. citrinum* or *V. abielina*. However, under similar conditions, *B. subtilis* inhibited the growth of *P. citrinum* and *V. abielina* by forming an inhibition zone (3- to 12-mm wide) around the disks.

Spore germination of pathogens in PDB was strongly inhibited in the presence of active cells of the antagonist (Table 1). The concentrations of *C. laurentii* significantly influenced control of spore germination of *P. citrinum* and *V. abielina*. The results showed that the higher the concentrations of the antagonist, the lower the spore germination rate and the smaller the germ tube length. When the concentration of the cell suspension of *C. laurentii* reached 1×10^8 CFU/ml, spore germination of *P. citrinum* and *V. abielina* was totally inhibited. Culture filtrate did not control spore germination or germ tube elongation ($p < 0.05$), and neither did autoclaved culture.

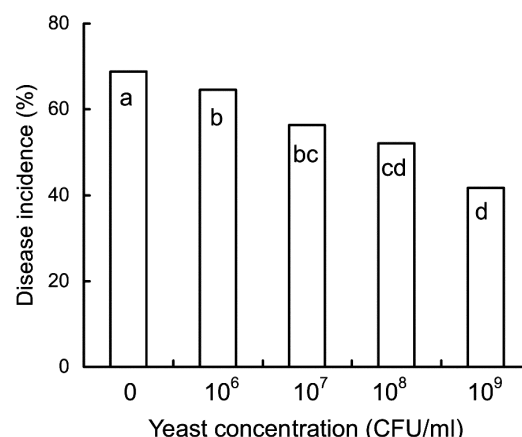


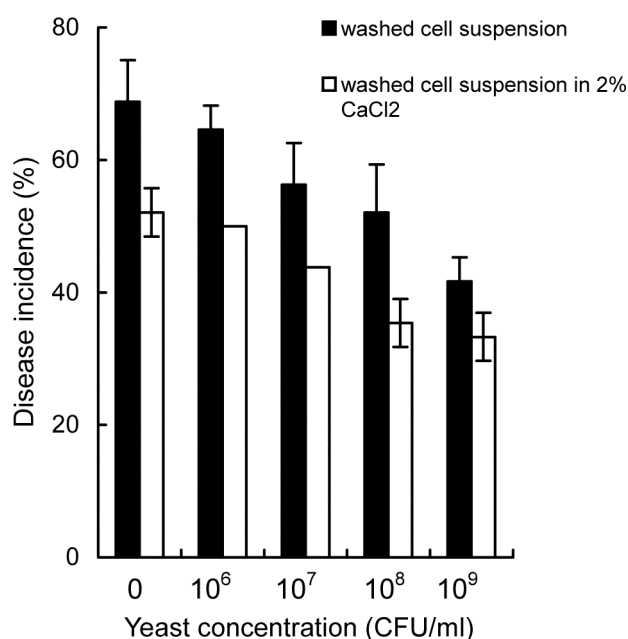
Figure 3. Efficacy of *C. laurentii* for controlling of natural mold rot under cold storage conditions. Each value is the mean of three experiments. Disease incidences were measured after 7 days at 4°C. Data in columns with different letters are significantly different according to Duncan's multiple range test at $p=0.05$.

Table 1. Effect of different concentration of washed cell suspensions, autoclaved cell and cell-free culture filtrates of *C. laurentii* on spore germination and germ tube elongation of *P. citrinum* and *V. abielina*.

| Treatments | Spore germination (%) ¹ | | Germ tube length (um) | |
|----------------------------|------------------------------------|--------------------|-----------------------|--------------------|
| | <i>P. citrinum</i> | <i>V. abielina</i> | <i>P. citrinum</i> | <i>V. abielina</i> |
| 1×10 ⁴ CFU/ml | 67.3b ² | 68.3b | 14.8b | 9.1b |
| 1×10 ⁶ CFU /ml | 36.8c | 16.1c | 8.2c | 5.4c |
| 1×10 ⁸ CFU /ml | 0 d | 0d | 0d | 0d |
| Autoclaved culture | 85.2a | 96.1a | 20.9a | 12.7a |
| Culture filtrate | 90.2a | 90.1a | 19.2a | 12.1a |
| Control (H ₂ O) | 89.4a | 97.8a | 20.1a | 14.4a |

¹Germination rate and germ tube length were measured after 20 h incubation at 25°C in PDB.

²Each value is the mean of three experiments. Values in each column followed by different letters are statistically different according to Duncan's multiple range test at p=0.05.

**Figure 4.** Effects of adding CaCl₂ to washed cell suspensions of yeast on control effectiveness. Each value is the mean of three experiments (±SD). Disease incidences were measured after 3 days at 20°C. Data in columns with different letters are significantly different according to Duncan's multiple range test at p=0.05.

Discussion

This study demonstrates that *C. laurentii* significantly reduced natural mold rot on the arbutus berries caused by *P. citrinum*, *V. abielina*. Culture filtrate did not control the disease. In addition, culture filtrate co-cultured with pathogen spores did not affect spore germination or germ tube elongation. In the test on PDA plates, *C. laurentii* had no effect on the growth of the pathogens in culture. These findings suggested that *C. laurentii* did not inhibit pathogens by producing antibiotics as *B. subtilis* did (Pusey and Wilson, 1984). Unwashed cell culture of *C. laurentii* showed decreased biocontrol effectiveness compared with the washed cell suspension. In addition, the antagonistic activity of *C. laurentii* was dependent on the concentra-

tion of the antagonist. The higher the concentration of *C. laurentii*, the better the biocontrol activity. These results suggest that competition for space and nutrition may play a major role in the biocontrol capability of *C. laurentii* against pathogens as previously demonstrated for other antagonistic yeasts (McLaughlin et al., 1992; Fan and Tian, 2001; Filonow, 1998). The demonstrated effect of initial concentration of *C. laurentii* on biocontrol efficacy provides presumptive evidence that biocontrol is achieved when actively multiplying populations of *C. laurentii* are present in wounds. Biocontrol by an antagonistic yeast involves several potential modes of action. Mycoparasitism, induced resistance, and the production of lytic enzymes such as β -1,3-glucanase and chitinase have been suggested (Wilson et al., 1991; Ippolito et al., 2000). The precise mode of action of *C. laurentii* should be investigated further.

An antagonist is very promising if it can be combined with routine postharvest treatments. Cold storage is a routine method of postharvest handling of fruits and vegetables. The demonstrated biocontrol effect of *C. laurentii* at low temperature (4°C) makes feasible an integrated control strategy under commercial conditions.

Postharvest CaCl₂ treatments represent safe and effective methods for improving the quality and extending the storage life of fresh fruit (Tsantili et al., 2002). The results of this study indicate the beneficial effect of calcium chloride on the biocontrol activity of *C. laurentii* against natural infection of the arbutus berries, which is in agreement with the result obtained by Fan and Tian (2001), who found that 20 g CaCl₂ per liter enhanced the control of rhizopus rot of nectarine fruits by *Pichia membranefaciens*. McLaughlin et al. (1990) reported that calcium salts improved the efficacy of yeast biocontrol agents against *Botrytis* and *Penicillium* rots of apple. They suggested that the effect of calcium on the biocontrol activity of the yeast antagonists was due to some interaction with the yeast or its metabolic products at the wound site rather than a direct effect of calcium on the pathogen or the fruit tissue. In contrast, Wisniewski et al. (1995) reported that calcium chloride reduced germination and germ tube elongation of *B. cinerea* and *P. expansum* in vitro. Droby et al. (1997) found that the effect of calcium in reducing infec-

tion of grapefruit wounds by *P. digitatum* could be due to direct effects on host tissue (making cell walls more resistant to enzymatic degradation) or the pathogen (interfering with spore germination, growth, and inhibition of fungal pectinolytic enzymes). The mechanism(s) by which calcium enhances the biocontrol effect of yeast antagonists on fruits is not yet fully understood. It may be a result of several different interactions taking place between calcium ions and the host, the pathogen, or the yeast.

In conclusion, the present results show that *C. laurentii* has potential as a biocontrol agent for the control of postharvest decay of the arbutus berries caused by *P. citrinum*, *V. abielina*, and other pathogens. The use of *C. laurentii* has been found to be compatible with several postharvest practices including cold storage and CaCl_2 treatment, thus making feasible an integrated control strategy under commercial conditions. Further studies are needed to determine the biological control efficacy of preharvest application of *C. laurentii* to control.

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羅倫隱球酵母對楊梅採後病害的生物防治效果

鄭曉冬 張紅印 席璵芳

浙江大學生物系統工程與食品科學學院

本文測定了羅倫隱球酵母在半商業儲藏條件下對楊梅由桔青黴、楊梅輪帶黴等致病黴菌引起的採後腐爛的抑制效果。比較了羅倫隱球酵母三種不同的處理液的拮抗效果：酵母細胞菌懸液的抑菌效果好於酵母培養液，而酵母濾液沒有抑菌作用。酵母細胞菌懸液的抑菌作用與細胞的濃度有關。羅倫隱球酵母在低溫條件下 (4°C) 對楊梅黴爛也有抑制作用。2% 的氯化鈣可以加強羅倫隱球酵母的抑菌效果。在馬鈴薯葡萄糖瓊脂固體培養基 (PDA) 體外抑菌實驗中，把生長有羅倫隱球酵母的營養酵母葡萄糖固體培養基 (NYDA) 切塊放置在塗布有桔青黴或楊梅輪帶黴孢子的馬鈴薯葡萄糖瓊脂固體培養基上一起培養，羅倫隱球酵母並不能抑制黴菌的生長；而在馬鈴薯葡萄糖液體培養基 (PDB) 體外抑菌實驗中，羅倫隱球酵母活細胞有效的抑制了黴菌孢子的萌發。

關鍵詞：羅倫隱球酵母；楊梅；採後腐爛；生物防治；氯化鈣。