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Yeast application for controlling apple postharvest diseases associated with *Penicillium expansum*[1]

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Abstract. *Penicillium expansum* is one of the main pathogens causing decay in fruits and vegetables. In recent years, researchers have discovered that some yeasts have antagonism against *Penicillium expansum*. In this study, two species of yeast that can biocontrol apple diseases caused by *Penicillium expansum* were discovered. At the same time, the antagonistic conditions—such as yeast concentration, concentration of Fe^{2+} in the yeast culture medium, and inoculation time—were preliminarily studied, and the best conditions for yeast antagonism were determined.

Keywords: Apple; Biocontrol; Yeast.

Introduction

Apple is one of the most important fruits produced in China. To provide fruit throughout the year, fresh apples are stored after harvest. Postharvest losses caused by fungal diseases are the major factor limiting the storage life of apples. Postharvest fungal diseases of apple are mainly caused by *Penicillium expansum* (Romano et al., 1983). Traditionally, this disease is controlled by the application of synthetic fungicides (Eckert and Ogawa, 1988). However, the potential impact on environment as well as human health largely limits their application (Eckert et al., 1994). It is reported that some microbes become fungicide-resistant (Spotts and Cervantes, 1986; Holmes and Eckert, 1999), and thus a fungicide's effect on controlling fungal growth may be greatly reduced. Considering the human health and pollution risks, some fungicides are prohibited from use in many developed countries (e.g. America, England).

Recently, biological control has been developed as an alternative to synthetic fungicides (Wilson et al., 1993), and considerable success has been achieved by utilizing antagonistic microorganisms for controlling postharvest diseases. Ever since Guter reported that *Bacillus subtilis* was antagonistic toward fruit pathogens, many studies involving antagonistic microorganisms to control postharvest diseases of fruits and vegetables have been done (Wisniewski et al., 1991; Smilanick, 1992; Caztoria et al., 1997; Chand-Goyal and Spotts, 1997; Leibinger et al., 1997; Droby et al., 1998; Filnow, 1998; Benbow and Sugar, 1999; Sugar and Spotts, 1999; Ippolito and Franco, 2000). Roberts (1990) discovered that *Cryptococcus laurentii* has antagonistic activity against many postharvest pathogens. The competition for nutrients may play a role in the antagonism of *Cryptococcus laurentii*. Decay caused by *Rhizopus* sp. is reduced 70% when strawberries are treated with *Aureobasiduim pullulans* before storage (Lima et al., 1997). Calvente (Calvente et al., 1999) found that *Rhodotorula glutinis* produced rhodotorulic acid, which enhanced biocontrol activity of *Rhodotorula glutinis* against *Penicillium expansum* in postharvest apples. When applied to wounds 12 h after inoculation, *Pseudomondia corragata* significantly reduced brown rot in nectarines and peaches caused by *Monilinia fructicola* (Smilanick et al., 1993). Studies on postharvest biocontrol of fruits and vegetables have become an important new area of research.

Advantages of utilizing antagonistic microorganisms include reducing environmental pollutation, effectively controlling postharvest diseases, and producing high quality and safe food. Unfortunately, only a few studies in this field were reported in China (Fan and Tian, 2000a,b; Fan and Tian, 2001; Tian and Fan, 2001). There is a new opportunity to study biological control of postharvest diseases of fruits and vegetables in our country.

In this paper the capability of some kinds of yeast to control apple postharvest diseases caused by *Penicillium expansum* was studied, and the one with the best effect was identified. The antagonistic conditions of that yeast and the methods of antagonism were also discussed.

Materials and Methods

Materials

Fruit: Red Fushi Apple.

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Microorganisms: Antagonists—*Saccharomyces* sp., *Candida* sp., *Cryptococcus* sp., and *Rhodotorula* sp. Pathogen --- *Penicillium expansum*, isolated from microorganisms of rotten fruits.

Reagents: pH 7.0, 0.05 M phosphate buffer; 70% alcohol; 0.5% NaCl; $FeSO_4$.

Instruments: haemocytometer; microscope; incubator; rotary shaker.

Antagonist Isolation

5 g soil under a pear tree was added to 50 mL sterile water and mixed well. The solution and its diluents were streaked onto wort agar plates. The plates were incubated at 28°C for a few days. Red yeast and other yeast single-cell colonies were re-cultured on wort agar plates and stored in test tubes.

Pathogen Isolation

5 g rotten apple tissue was dissolved in 50 mL sterile water. The solution and the diluents were streaked on to PDA plates containing 1.6 μg/mL penicillin and incubated at 28°C.

In Vitro Test

Four yeasts, *Saccharomyces* sp., *Candida* sp., *Cryptococcus* sp. and *Rhodotorula* sp. were cultured in wort liquid culture medium on a rotary shaker (100 mL culture medium volume in a 500 mL Erlenmeyer flask, 200 rpm) for 72 h at 28°C. *Penicillium expansum* spores and washed yeast cells were counted with a haemocytometer and adjusted to a concentration of 4.4×10^6 spores/mL and 6×10^6 cells/mL, respectively. Aliquots of 0.2 mL *Penicillium expansum* spore suspension were added to PDA plates and then stored in the refrigerator for 2 h. Washed yeast cell suspension was injected into holes (diameter 6.5 mm; four holes per plate) on PDA plates and cultured at 28°C. Plates were checked once or twice a day.

In Vivo Test

Washed *Cryptococcus* sp. cells were cultured in wort liquid culture medium amended with Fe^{2+} (0 μmol/L, 2.5 μmol/L, 5 μmol/L or 20 μmol/L) on a rotary shaker (25 mL culture volume in a 100 mL Erlenmeyer flask, 200 rpm) for 72 h at 28°C. The yeast cells were harvested by centrifugation at 2,000 rpm for 10 min, washed twice with sterile phosphate buffer (pH=7.0), and suspended in phosphate buffer. The suspension was adjusted to concentrations of 3.5×10^8 cells/mL, 3.5×10^7 cells/mL, or 7×10^6 cells/mL.

Apple fruits (no wound or scar in the surface, fresh) were selected for experiment. They were surface-sterilized with 0.5% NaCl for 5 min and then washed with tap water. After air-drying, apples were treated with 70% ethanol. Each fruit was wounded four times at its equator with the sterile head of a finishing nail (6 mm diameter×4 mm deep). To obtain the best biocontrol efficacy of the yeast, apple fruits were divided into four groups to study four factors (the concentration of the yeasts, inoculatation time, concentration of Fe^{2+} in culture medium, and storage temperature) that influence the antagonism. 10 fruits were arranged in a randomized complete block for each group, and the experiment was repeated three times. All data presented in the figure are averages.

The antagonistic effect of the different yeast concentrations. Wounds were inoculated with 20 μL yeast (grown in culture medium that contained 2.5 μmol/L Fe^{2+}) cell suspension at the concentrations of 7.0×10^6, 3.5×10^7, and 3.5×10^8 cells/mL. After 12 h, a 20 μL *Pencillium expansum* suspension at a concentration of 1×10^4 spores/mL was added to each wound. The apples were then stored at 15°C.

The antagonistic effect of different inoculation times. Wounds were inoculated with 20 μL yeast (cultured in culture medium that contained 2.5 μmol/L Fe^{2+}) cell suspension at a concentration of 3.5×10^7 cells/mL. After 1 h, 12 h, 36 h, a 20 μL *Pencillium expansum* suspension in a concentration of 1×10^4 spores/mL was added to each wound, and the apples were stored at 15°C.

Antagonistic effect of Fe^{2+} concentration in culture medium. Wounds were inoculated with a 20 μL yeast (grown in culture medium that contained 0 μmol/L, 2.5 μmol/L, 5 μmol/L, and 20 μmol/L Fe^{2+}, respectively) cell suspension at concentration of 3.5×10^7 cells/mL. After 12 h, a 20 μL *Pencillium expansum* suspension in a concentration of 1×10^4 spores/mL was added to each wound, and the apples were stored at 15°C.

Antagonistic effect of different storage temperatures. Wounds were inoculated with a 20 μL yeast (grown in culture medium that contained 2.5 μmol/L Fe^{2+}) cell suspension at 3.5×10^7 cells/mL. After 12 h, a 20 μL *Pencillium expansum* suspension in 1×10^4 spores/mL was added to each wound. The apples were stored at 10°C, 15°C, and 20°C, respectively.

Controls. CK1 control means nothing was added to wounds at 15°C, and CK2 control means only *Pencillium expansum* was added to wounds at 15°C.

Statistical analysis. The incidence and severity of decay were analyzed by an analysis of variance (ANOVA) with SAS Software (SAS Institute, version 6.08, Cary, NC). Statistical significance was judged at the level $p<0.01$. When the analysis was statistically significant, Duncan's Multiple-Range Test (SSR Test) was used to test mean separations among mean values of each treatment.

Results

Effects of the Antagonist

An antagonistic effect was observed on PDA plates which had *Pencillium expansum* for 2 h followed by *Saccharomyces* sp., *Candida* sp., *Cryptococcus* sp., or *Rhodotorula* sp. *Candida* sp. and *Cryptococcus* sp. had greater antagonistic efficacy than *Rhodotorula* sp. and *Saccharomyces* sp. Since *Cryptococcus* sp. had the greatest effects, it was selected for further study.

Effects of Yeast Concentration on Decay

High, middle and low concentrations of yeast (LC=7.0 $\times 10^6$ cells/mL; MC=3.5$\times 10^7$ cells/mL; HC=3.5$\times 10^8$ cells/mL) were inoculated into wounds of apple. The apple fruits were kept at 15°C. Lesion diameters were recorded every 24 h after the treatment (Figure 1).

It was obvious from Figure 1 that the antagonism was related to the concentration of antagonist. When concentration of the yeast reached 3.5$\times 10^7$ cells/mL and spore suspension of the pathogens was 1$\times 10^4$ spores/mL, distinct antagonism was observed. The postharvest biocontrol capability of the apple fruits was enhanced with the increase of the antagonist concentration.

Effects of Different Inoculation Times on Decay

The pathogens were added 1 h, 12 h, 36 h or 60 h after the inoculation of the antagonist. Then the fruits were stored at 15°C, and the disease severity was observed every 24 h after inoculation (Figure 2).

From Figure 2, it can be seen that the later the pathogens were introduced after the inoculation of the yeast, the better the antagonism was. The reason may be that with enough time the yeast had reproduced and used the nutrient (we call it competitive antagonism), or the yeast had secreted enough antagonistic substances. So we should apply the antagonist early after the harvest to prevent the pathogens from infecting.

Effects of Concentrations of Fe^{2+} in Culture Medium on Decay

Others have reported that the Fe^{2+} concentration in the cells has a great effect on the antagonism (Calvente et al., 1999). We added different concentrations of Fe^{2+} into the culture medium to make the yeast cells contain different concentration Fe^{2+} and then inoculated the wounds with these yeasts. We stored the fruits at 15°C and observed the results 35d after the inoculation (Figure 3).

The experimental results showed (Figure 3) that the yeast had its greatest antagonism when cultured in a medium containing 2.5 μmol/L Fe^{2+}, but when too much Fe^{2+} was in the culture medium, the yeast accelerated the decay. The antagonism appears to be related to the competition for Fe^{2+}. Yeast cells became enriched in the wounds by chelated Fe^{2+} to put off the germination of pathogens and then strengthened biocontrol efficacy. Charlang et al. (1981) reported that spores could not germinate without taking up large quantities of Fe^{2+}.

Effect of Different Storage Temperatures on Decay

Apple fruits were stored at 10°C, 15°C, 20°C after the inoculation of the antagonist and the pathogen. Lesion diameters were recorded every 24 h after challenge inoculation (Figure 4).

We can see from Figure 4, the higher the storage temperature, the faster the apple decay. The antagonistic effects were inversely related to the storage temperature; the antagonism was better when the storage temperature was lower. It is important to keep apple fruit at a low temperature.

Summary of the Analysis of Variance for the Effects of Each Treatment

The effects of *Cryptococcus laurentii*'s concentration, inoculation time of *Pencillium expansum*, concentration of Fe^{2+} in culture medium, and storage temperature on decay

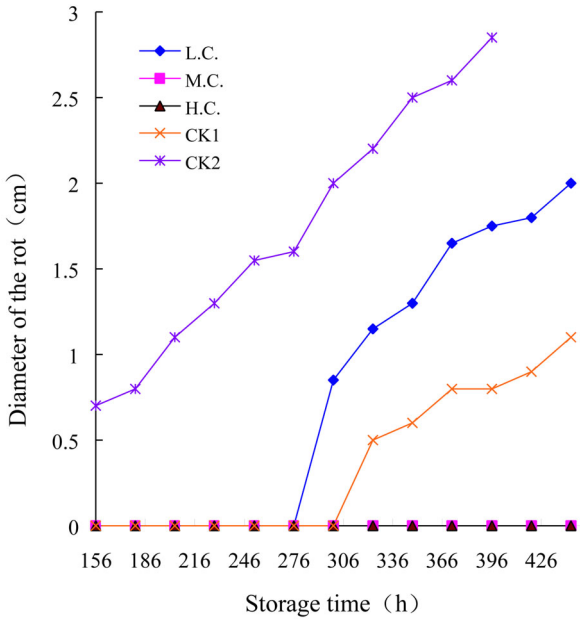

Figure 1. Effects of *Cryptococcus laurentii* concentration on apple decay.

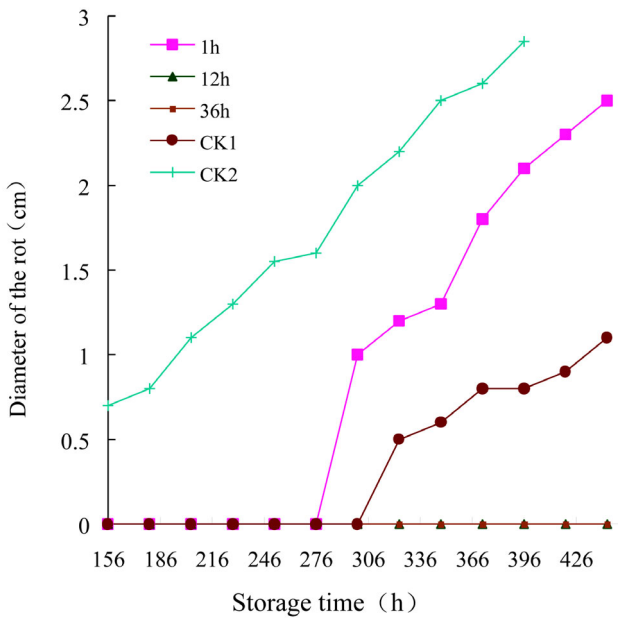

Figure 2. Effect of inoculation time of *Pencillium expansum* on apple decay.

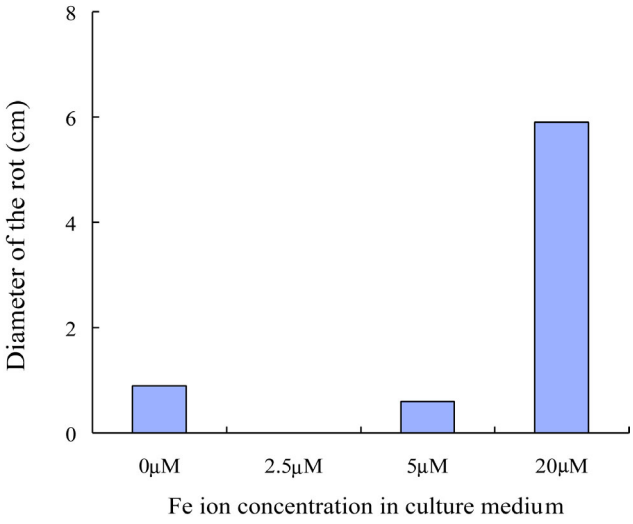

Figure 3. Effects of concentration of Fe^{2+} in culture medium on apple decay.

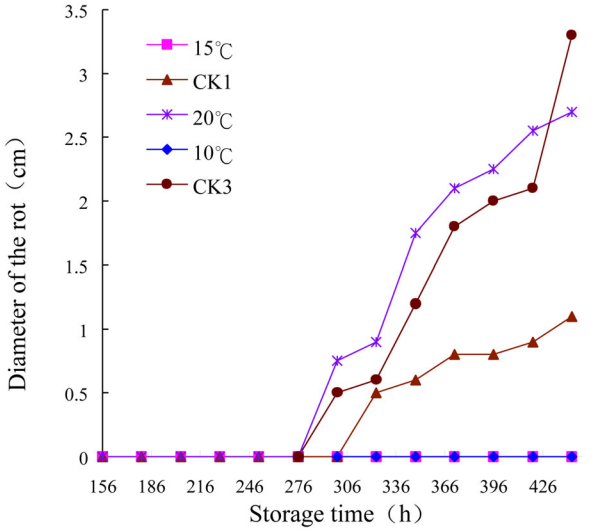

Figure 4. Effect of storage temperature on apple decay. CK3 control means nothing was added to wounds at 20°C.

were analyzed by analysis of variance (ANOVA). At hour 828, diameter of rot to Fe^{2+} treatment were used for statistical analysis, and other treatments' diameters of rot were measured at hour 396 for statistical analysis. It's evident that all treatments significantly reduced the decay (Table 1).

As the effect of every treatment was significant (Table 1), Duncan's Multiple-Range Test was adopted to compare mean separations among mean values of every treatment (Table 2). In this analysis, diameter of rot was indicated as mean value±standard deviation. The letters behind the standard deviation of the mean indicate significant differences where the same letters are not significantly different at $P<0.05$. We can see from Table 2 that increasing the concentration of yeast, delaying *Penicillium*'s inoculation time, and reducing the storage temperature can significantly enhance antagonism. But Fe^{2+} in culture medium should be adjusted to an appropriate concentration to achieve the best antagonism.

Discussion

The results of this study indicate that *Pencillium expansum* causes postharvest decay of apple and that *Cryptococcus* sp. and *Candida* sp. have antagonistic biocontrol efficacy. The biocontrol efficacy of *Cryptococcus* sp. was superior. Biocontrol of postharvest decay caused by different epiphytes may need different antagonists.

Yeast was the most common biological control agent. This study showed a valuable method to control epiphyte pathogen caused postharvest decay by using antagonistic yeasts. However, the methods of yeast biocontrol include many aspects and need further study. Better methods

Table 1. Summary of the analysis of variance for the effects of all treatment on the diameter of the rot.

Treatment	Source	df	SS	s^2	F	$F_{0.01}$
Cryptococcus laurentii's Concentration[a]	Treatment	4	17.03	4.26	68.24**	5.99
	Error	10	0.62	0.062		
	Total	14	17.65			
Inoculation time of *Pencillium expansum*[a]	Treatment	4	18.34	4.585	86.64**	5.99
	Error	10	0.54	0.054		
	Total	14	18.88			
Concentration of Fe^{2+} in culture medium[b]	Treatment	3	68.06	22.69	615.24**	7.59
	Error	8	0.30	0.037		
	Total	11	68.36			
Storage temperature[a]	Treatment	4	13.21	3.30	52.89**	5.99
	Error	10	0.62	0.062		
	Total	14	13.83			

[a]Data were measured 396 hours after inoculation with yeast.
[b]Data were measured 828 hours after inoculation with yeast.
The significance is indicated by the F ratio: **P<0.01.

Table 2. Effects of each treatment expressed as mean (±standard deviation) diameter of rot. Comparison between mean values of treatment were made using Dancun's Multiple Range Test. Letters behind the standard evidation indicate significant differences where the same letters are not significantly different at $P<0.05$.

Yeast's concentration (cells/mL)[a]	Diameter of rot (cm)[a]	Inoculation time *Penicillium* (h)[b]	Diameter of rot (cm)[a]
CK1	0.8±0.30bc[c]	CK1	0.85±0.82bc
CK2	2.85±0.30a	CK2	2.8±1.06a
7×10^6	1.75±0.28ab	1	2.1±0.3ab
3.5×10^7	0.07±0.11c	12	0.08±1.09c
3.5×10^8	0.08±0.07c	36	0.03±0.96c
Concentration of Fe2+ (μM)	Diameter of rot (cm)	Storage temperature (°C)	Diameter of rot (cm)
0	1.2±0.17b	CK1	0.8±0.90bc
2.5	0.05±0.63c	CK3	2.0±0.93ab
5	0.8±0.52bc	10	0.06±0.04c
20	6.1±2.33a	15	0.04±0.04c
		20	2.25±1.04a

[a]Data were measured 396 hours after inoculation with yeast.
[b]Data were measured 828 hours after inoculation with yeast.
[c]Values are means of three repetitions±standard deviation of the mean. Means are averaged values of three trials±the standard error.

are needed to assist decay control and to combine microorganisms with storage of farm produce.

Currently, very few studies are done in our country on the ability of antagonistic microorganisms to control fresh fruit and vegetable pathogens causing postharvest decay. This area has great potential and deserves more focus.

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酵母在蘋果採後青黴病害生物學防治上應用的初探

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青黴是引起果蔬採後腐爛的主要病原體。近些年來的研究發現，一些酵母能與青黴產生拮抗作用，從而抑制青黴的生長。本文通過體外平板實驗篩選出兩株對蘋果青黴病害具生物防治作用的酵母，並就酵母的濃度、酵母培養基中鐵離子濃度、接種時間等對蘋果採後病害的生物防治的影響進行了研究，確定了酵母的最佳拮抗作用條件。

關鍵詞：酵母；蘋果；生物防治。