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ORIGINAL ARTICLE

Control of gray mold of grape by *Hanseniaspora uvarum* and its effects on postharvest quality parameters

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Abstract The potential of antagonist P-2 for controlling postharvest gray mold decay of grape berries and their effects on postharvest quality of fruits was investigated. The results showed that antagonist P-2 at 1×10^8 CFU ml^{-1} completely inhibited the gray mold decay after 4 days, significantly inhibited the spore germination and lesion diameters of *Botrytis cinerea* in vitro, reduced the natural decay development of grape berries, and did not impair quality parameters, including TSS (total soluble solids), ascorbic acid, and titratable acidity. Moreover, the results of ITS (internal transcribed spacer) analysis indicated that antagonist P-2 belonged to *Hanseniaspora uvarum*.

Keywords Biocontrol · *Botrytis cinerea* · Grape berry · *Hanseniaspora uvarum* · Postharvest decay · Quality parameters

Introduction

Gray mold decay caused by *Botrytis cinerea* is one of the most destructive postharvest diseases of grapes (Cappellini et al. 1986; Latorre 2007). Currently, the disease is primarily controlled by sulphur dioxide fumigation (Smilanick et al. 1990) and SO_2 generator pads (Guzev et al. 2008). However, due to the public concern about the phytotoxicity and possible hypersensitivity in humans, researchers are trying their best to explore alternatives to synthetic fungicides for controlling postharvest diseases (Ragsdale and Sisler 1994; Cao et al. 2008).

Microbial biocontrol agents have shown great potential as alternatives to synthetic fungicides for the control of postharvest decay of fruits and vegetables (Wilson and Chalutz 1989; Smilanick 2004). More and more microbial antagonists, such as *Trichoderma harzianum* (Elad 1994), *Clonostachys rosea (*Peng and Sutton 1990), *Candida oleophila* and *C. guilliermondii* (Saligkarias et al. 2002), *Kloeckera apiculata* (Long et al. 2005), *Cryptococcus laurentii* (Yu et al. 2008), *Aureobasidium pullulans* (Adikaram et al. 2002), and *Bacillus subtilis* (Taguchi et al. 2003) have been reported to effectively control gray mold of fruits.

The main mechanism of the antagonists was considered as the competition for nutrients and space (Janisiewicz and Korsten 2002), production of cell wall-degrading enzymes (Droby et al. 2002), production of antifungal diffusible and volatile metabolites (Sujatha et al. 2005), and mycoparasitism (Bar-Shimon et al. 2004; Long et al. 2005).

The present study focused on the yeast strain P-2 isolated from the surface of the grape fruits. The objectives of the present work were (1) to evaluate the efficacy of P-2 for controlling gray mold decay, (2) to investigate the biocontrol activity of P-2 in vitro, (3) to assess the efficacy of P-2 in controlling natural decay development of grape clusters and its effects on quality parameters of fruits, and (4) to confirm the species of the yeast P-2.

Materials and methods

Pathogen inoculum Botrytis cinerea, purchased from China Centre for Type Culture Collection (CCTCC) in Wuhan University was maintained on Potato Dextrose agar (PDA)

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at 25°C. Spore suspensions were prepared by flooding 7-day-old PDA cultures with sterile distilled water. Spores were counted with a haemocytometer and then diluted to 1×10^5 or 1×10^6 spores ml^{-1} as required.

Antagonist The antagonist strain P-2 was isolated from the surface of grape berries using the method of Wilson and Chalutz (1989). The yeast was cultured in 250-ml Erlenmeyer flasks with 50 ml of Yeast Peptone Dextrose (YPD) on a gyratory shaker at 200*g* for 48 h at 25°C. Then the cells were harvested by centrifuging at 6,000*g* for 10 min and resuspended in sterile distilled water. The cell suspensions were adjusted to concentrations of 1×10^6 or 1×10^8 CFU ml^{-1} with a haemocytometer.

Fruits Grape berries (*Vitis vinifera* L. Kyoho) were harvested from the vineyard in Huazhong Agricultural University, and selected on size and the absence of physical injuries or infections. Prior to use, fruits were surface disinfected with 2% (v/v) sodium hypochlorite for 5 min, rinsed with tap water and dried in air.

Effect of antagonist strain P-2 on controlling gray mould decay of grapes with artificial inoculation The berries were wounded (about 3 mm deep and 3 mm diameters) on the equator by sterile dissecting needles. Each wound was added with 20 μl of cell suspensions of antagonistic yeast (1×10^6 and 1×10^8 CFU ml^{-1}) and distilled water (as control). Two hours later, 20 μl of suspension of *B. cinerea* (1×10^5 spores ml^{-1}) was inoculated into each wound. All treated fruits were placed in an incubator at 20°C and 90± 5% relative humidity. Disease incidence was determined by counting the number of infected wounds after 4 days. Each treatment contained three replications with 50 single berries per replication, and the entire experiment was repeated twice.

Biocontrol activity of antagonist P-2 in vitro To evaluate the interactions between the antagonist and the pathogen in culture, we cut 5-mm-diameter disks from PDA plates, then 20 μl of pathogen suspension (1×10^6 spores ml^{-1}) and antagonist P-2 (1×10^8 CFU ml^{-1}) were added to each wells. The plates were incubated at 25°C and lesion diameters were determined after 4 days. Each treatment was replicated three times and the experiment was repeated twice.

To assess the effect of antagonist P-2 on spore germination of *B. cinerea,* 100 μl of spore suspensions of *B. cinerea* (1×10^6 spores ml^{-1}) was transferred to glass tubes containing 5 ml of Potato Dextrose broth (PDB) with P-2 at 1×10^8 CFU ml^{-1} and no P-2 inoculum (as control). All flasks were put on a gyratory shaker (200*g*) at 25°C and incubated for 12 h. Approximately 100 spores per replicate were measured for germination and at least 5 microscope fields were observed. Conidia were considered germinated when germ tubes exceeded the conidium diameter. Each treatment was replicated three times and the experiment was repeated twice.

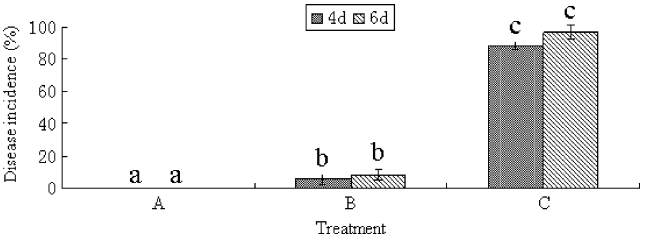

Fig. 1 Disease incidence of gray mold in grapes with artificial inoculation at 25°C for 4 and 6 days as affected by various treatments. **A** *Hanseniaspora uvarum* P-2 (1×10^8 CFU ml^{-1}), **B** *Hanseniaspora uvarum* P-2 (1×10^6 CFU ml^{-1}), **C** control (distilled water). *Vertical bars* represent standard deviations of the means. Data in columns with *different letters* are significantly different according to Duncan's multiple range test at $P\leq0.05$

Effect of antagonist strain P-2 on reducing natural decay development of grape clusters Grape berries were divided into clusters (approximately 500 g) and randomized, so that a portion of each cluster was represented in each replicate. Grape clusters were dipped in a suspension of antagonistic yeast P-2 (1×10^8 CFU ml^{-1}), carbendazim (500 mg l^{-1}, as positive control), and distilled water (as negative control), respectively. All the grape clusters were air dried for 1 h. About 1,000 g of grape clusters were placed in ventilated polyethylene bags and stored at 0°C. Infection rate was recorded after 50 days. There were three bags of grape berries in each treatment with three replicates. The experiment was repeated twice.

Identification of antagonist strain P-2 by Internal Transcribed Spacer (ITS) analysis The strain P-2 was identified by the rDNA-ITS molecular analysis. Genomic DNA was extracted using the method of Yang et al. (2006). The fungus-specific universal primers used were TCCGTAGGT GAACCTGCGG (ITS1) and TCCTCCGCTTATTGA TATGC (ITS4). Genomic DNA was used for the PCR reactions. The amplification reactions were performed in a total volume of 50 μl containing 2.0 μl of template DNA,

Table 1 Effect of *Hanseniaspora uvarum* P-2 on spore germination and lesion diameter of *Botrytis cinerea*

Treatments	Spore germination (%)	Lesion diameter (mm)
P-2	9.23±3.50 a	19.81±0.50 a
Control	74.25±2.78 b	67.65±1.92 b

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at $P\leq0.05$ according to Duncan's multiple range tests

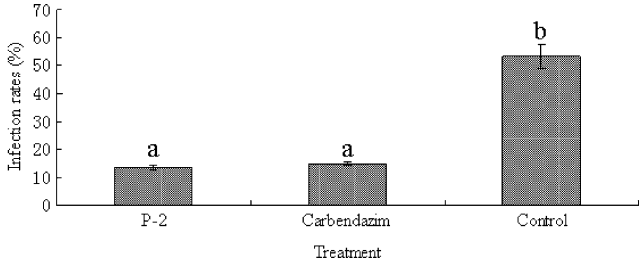

Fig. 2 Effect of *Hanseniaspora uvarum* P-2 on reducing natural decay development. *Vertical bars* represent standard deviations of the means. Data in columns with *different letters* are significantly different according to Duncan's multiple range tests at $P \leq 0.05$

1 μl of each primer (20 μmol ml^{-1}), 5.0 μl of 10X PCR buffer, 5.0 μl of $MgCl_2$ (25 mmol ml^{-1}), 2.0 μl of dNTPs (10 mmol ml^{-1}), 2.0 μl of BSA, and 0.4 μl of Taq DNA polymerase (5 U/μl). The PCR reactions were carried out under the following conditions: 4 min at 94°C followed by 36 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, and 10 min of final extension at 72°C. The PCR products were sequenced by Beijing Genomics Institute (Beijing, China). The ITS sequence was identified by searching databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nib.gov/BLAST).

Effects of antagonist strain P-2 on postharvest quality parameters of grapes To evaluate the effects of P-2 on postharvest quality of grapes, freshly harvested fruits were stored as described above. Quality parameters were measured after storage, on three replicates of five fruits each, and performed at 0°C. The testing methods are described below.

Total soluble solids Total soluble solids (TSS) 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) (Larrigaudiere et al. 2002).

Ascorbic acid 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 100 g sample (Zhang et al. 2005).

Titratable acidity Acidity was measured by titration with 0.1 mol l^{-1} 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 malic acid (Wright and Kader 1997).

Statistical analyses The data were analyzed by analysis of the variance (ANOVA). Mean separations were performed using Duncan's multiple range test. Differences at $P \leq 0.05$ were considered as significant.

Results

Effect of antagonistic yeast P-2 on controlling gray mould in grape with artificial inoculation

The disease incidence on all antagonist treated fruits was significantly lower than those on the control ($P < 0.05$). The concentrations of antagonist P-2 significantly influenced disease incidence of gray mould in grape berries (Fig. 1). Strain P-2 at 1×10^8 CFU ml^{-1} completely inhibited the gray mold decay after 4 days, while the control fruits had 88% disease incidence.

Inhibitory effect of antagonist P-2 on gray mould in vitro

As shown in Table 1, antagonist P-2 significantly inhibited the growth of *B. cinerea* in the test on the PDA plates. Moreover, spore germination of *B. cinerea* in PDB was greatly controlled by the living cell of P-2.

Efficacy of antagonist strain P-2 for reducing natural decay development

Our experiments evaluated the efficacy of antagonist P-2 in reducing the natural decay development at 0°C for 50 days in Fig. 2. The application of antagonist P-2 and the fungicide carbendazim resulted in low decay incidence, compared with water-treated control fruit. There was no significant difference of infection rates between antagonist P-2 and carbendazim.

Table 2 Effects of *Hanseniaspora uvarum* P-2 on postharvest quality parameters of grapes after 50 days storage at 0°C

Treatments	TSS[a] (%)	Titration acid (% malic acid)	Ascorbic acid (mg/100g)
P-2	13.9±1.02 a	0.30±0.06 a	3.39±0.13 a
CK	13.6±1.14 a	0.27±0.03 a	2.95±0.09 a

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple range tests

[a] Total soluble solids.

Identification of antagonistic yeast P-2

The 708-bp ITS region was successfully amplified from genomic DNA, by the fungus-specific universal primers pairs, ITS1–ITS4. The ITS sequence of P-2 showed 100% sequence identity with the strain of *Hanseniaspora uvarum* (GenBank accession no. DQ 640770.1), and 99% sequence identity with the strain of *Hanseniaspora uvarum* (GenBank accession no. DQ872854.1, AJ512432.1, AJ318411.1, AY046200.1, FJ515178.1). The results indicated that the strain P-2 belonged to *Hanseniaspora uvarum.*

Effects of antagonist strain P-2 on postharvest quality parameters of grapes

According to the data in Table 2, antagonistic yeast P-2 had no significant effect on titration acid, ascorbic acid, and soluble solids content during storage at 0°C.

Discussion

Antagonist yeasts are useful biocontrol agents for suppressing postharvest diseases in various fruits (Zhang et al. 2008a, b; Long and Gao 2009). However, there is little information concerning about the effect of *H. uvarum* on controlling the postharvest gray mold decay of grape berries and its effect on quality parameters of fruits. Thus, it is necessary to investigate the control efficacy of *H. uvarum* to postharvest gray mold decay and natural infections of grape berries and its effect on quality parameters of grapes.

On PDA plates, *H. uvarum* significantly inhibits the growth of *B. cinerea*. Under in vitro conditions, spore germination of *B. cinerea* was totally inhibited by the living cell of *H. uvarum*. Moreover, the application of *H. uvarum* resulted in low decay incidence of grapes with artificial inoculation and natural decay development. These results suggest that inhibitory effect on spore germination and lesion growth may be one of the major mechanisms of *H. uvarum* inhibiting postharvest gray mold decay. However, there are complex interactions between host, pathogen, antagonist, and microorganisms (Zhang et al. 2008a, b). These should be investigated more precisely concerning the mode of action.

Not impairing quality parameters of fruits is one of the characteristics of an ideal antagonist (Zhang et al. 2008a, b). In the present experiment, *H. uvarum* did not impair quality parameters of grapes during storage. The results suggested that *H. uvarum* has potential for commercialization.

Traditionally, the identification of yeast species has been based on assimilation and fermentation tests and morphological traits (Barnett et al. 2000). Recent progress in molecular biology has led to the development of new methods for yeast identification based on molecular techniques (Esteve-Zarzoso et al. 2001; Cadez et al. 2002). In this study, the strain P-2 was analyzed by ITS regions. Based on its morphological and physiological characteristics (data not shown), the strain P-2 was identified as *Hanseniaspora uvarum*.

Overall, the results reported here showed that *H. uvarum* strain P-2 has considerable potential as a biocontrol agent for controlling postharvest decay of grapes caused by *B. cinerea*. The application of *H. uvarum* strain P-2 significantly reduced postharvest natural infection of grape fruits. Future research will be aimed at developing the technology to be used under large-scale operations and investigating the precise mechanisms of *H. uvarum* for controlling postharvest gray mold decay of grapes.

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