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ORIGINAL ARTICLE

Inhibitory activity of tea polyphenol and *Hanseniaspora uvarum* against *Botrytis cinerea* infections

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Abstract

Aims: To investigate the effect of tea polyphenol (TP) and *Hanseniaspora uvarum* alone or in combination against *Botrytis cinerea* in grapes and to evaluate the possible mechanisms involved.

Methods and Results: TP alone was effective in controlling grey mould in grape at all concentrations. TP at 0·5 and 1·0% in combination with *H. uvarum* (1×10^6 CFU ml^{-1}) showed a lower infection rate of grey mould. TP at 0·01% or above significantly inhibited the spore germination of *B. cinerea*. TP at 0·1% showed inhibition ability on mycelium growth of *B. cinerea*. The addition of TP did not affect the growth of *H. uvarum in vitro* and significantly increased the population of *H. uvarum in vivo*.

Conclusions: TP exhibited an inhibitory effect against *B. cinerea* and improved the biocontrol efficacy of *H. uvarum*. The inhibitory effects of spore germination and mycelial growth of *B. cinerea* and the increased populations of *H. uvarum in vivo* may be some of the important mechanisms of TP.

Significance and Impact of the Study: The results suggested that TP alone or in combination with biocontrol agents has great potential in the commercial management of postharvest diseases of fruits.

Introduction

Grey mould caused by *Botrytis cinerea* is one of the most destructive postharvest diseases of grapes (Cappellini *et al.* 1986; La Guerche *et al.* 2007; Latorre 2007). Antagonist yeast has shown potential as an alternative measure to synthetic fungicides for disease control (Ippolito *et al.* 2000; Smilanick 2004). However, their biocontrol efficacy under semi-commercial conditions is often lower than synthetic fungicides (Droby *et al.* 1998; Spadaro and Gullino 2004). To substitute synthetic fungicides, more environment-friendly compounds should be developed as alternative control methods for postharvest diseases.

Tea polyphenol (TP), a safe natural product, has been shown to have antimicrobial activity against human and animal disease-related bacteria, food-borne bacteria (Diker *et al.* 1991; Linke and LeGeros 2003; Bandyopadhyay *et al.* 2005; Si *et al.* 2006), pathogenic viruses (Cheng *et al.* 2002; Liu *et al.* 2005; Xu *et al.* 2008), phytopathogenic bacteria (Fukai *et al.* 1991) and pathogenic fungi (Toyoshima *et al.* 1993; Hirasawa and Takada 2004; Liu *et al.* 2010b). However, little information is available regarding effect of TP on grey mould of grape and improvement of the biocontrol effect of antagonist *Hanseniaspora uvarum*. We found that *H. uvarum* was an effective antagonist against grey mould of grape (Liu *et al.* 2010a,c). The objectives of the present work were as follows: (i) to assess the potential of TP to enhance the efficacy of *H. uvarum* against grey mould *in vivo*; (ii) to determine the effect of TP on the spore germination and mycelial growth of *B. cinerea*; (iii) to evaluate the effect of TP on the growth of *H. uvarum in vitro* and *in vivo*.

Materials and methods

Plant material

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, the fruit was surface disinfected with 2% (v/v) sodium hypochlorite for 5 min, rinsed with tap water and dried in air.

Yeast antagonist and pathogen

H. uvarum was isolated from the surface of grape with the method of Wilson and Chalutz (1989) and identified by morphological, physiological experiments and ITS analysis (GQ480362). The yeast was cultured in yeast peptone dextrose (YPD) for 48 h at 25°C. Then the cells were collected by centrifugation at 6000 ***g*** for 10 min. The cell suspensions were adjusted to concentrations of 1×10^6 or 1×10^8 CFU ml^{-1}, respectively, with sterile distilled water.

B. cinerea was purchased from the China Center for Type Culture Collection (CCTCC) in Wuhan University and maintained on potato dextrose agar (PDA) at 25°C. Spore suspensions were prepared by flooding 7-day-old PDA cultures with sterile distilled water. Spore concentrations were determined with a haemocytometer and adjusted to the required concentrations.

Chemicals product

The green TP, with a total TP content of more than 99·3% (analysed by HPLC), was purchased from Nanjing Qingze Medical Technological Development Co. Ltd (China). It was supplied as a fine powder (through 80 mesh) and prepared 1 week before we bought it.

Effect of TP and *H. uvarum* on controlling grey mould in grapes

Grapes were wounded (about 3 mm deep × 3 mm diameter) on the equator by sterile dissecting needles (3 mm). Each wound was added with 20 μl of the treatment suspensions as follows: TP (0·01, 0·05, 0·1, 0·5 and 1·0%, respectively), TP (0·01, 0·05, 0·1, 0·5 and 1·0%) in combination with *H. uvarum* (1×10^6 CFU ml^{-1}), *H. uvarum* (1×10^6 CFU ml^{-1}) and *H. uvarum* (1×10^8 CFU ml^{-1}). Fruit treated with sterile distilled water acted as controls. After 2 h, 20 μl of *B. cinerea* at 1×10^6 conidia ml^{-1} was inoculated into each wound. Treated fruits were put in an incubator at 28°C and 90 ± 5% relative humidity (r.h.). Disease incidence was determined by counting the number of infected wounds after 4 days. There were 100 berries in each replication and three replicates in each treatment. The experiment was repeated twice.

Effect of TP on conidia germination of *B. cinerea in vitro*

The inhibition of conidia germination of *B. cinerea* by TP was assayed in potato dextrose broth (PDB). Hundred microlitres of pathogen suspensions (1×10^6 conidia ml^{-1}) was transferred to 50 ml of PDB, which contained different concentrations of TP (0, 0·01, 0·05, 0·1, 0·5 and 1·0%). All flasks were put on a rotary shaker at 200 rev min^{-1} at 28°C and incubated for 12 h. Conidia germination rates were determined microscopically. Approximately 100 conidia per replicate were measured for germination, and at least five 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.

Effect of TP on mycelium growth of *B. cinerea in vitro*

To assess the effect of TP on mycelium growth of *B. cinerea,* PDA containing different concentrations of TP (0, 0·01, 0·05, 0·1, 0·5 and 1·0%) was divided equally (20 ml) into each Petri dish (90 mm in diameter). A Petri dish was inoculated in the centre with a single 5-mm-diameter agar disc from the margin of a 7-day-old colony of *B. cinerea*. The plates were incubated at 28°C, and colony diameter was determined after 4 days. Each treatment was replicated three times, and the experiment was repeated twice.

Effect of TP on growth of *H. uvarum in vitro and in vivo*

YPD were autoclaved (120°C, 15 min) prior to adding 0, 0·01, 0·05, 0·1, 0·5 and 1·0% TP and cell suspensions of *H. uvarum* (1 ml) to reach an initial concentration of 1×10^6 CFU ml^{-1}, and then incubated at 28°C for 48 h. The number of CFU of the yeast was determined by dilution plating 48 h after incubation at 28°C and expressed as Log_{10} CFU ml^{-1}. There were three replicates per treatment, and the experiment was conducted twice.

For the *in vivo* population dynamics study, the fruit were wounded as described earlier. Twenty microlitres of *H. uvarum* (1×10^6 CFU ml^{-1}) alone or in combination with TP (0, 0·01, 0·05, 0·1, 0·5 and 1·0%) was pipetted on to the wounds. Treated fruits were incubated at 28°C and 90 ± 5% RH. Tissue samples were removed from five single fruit with a sterile cork borer (approximately 5 mm deep × 5 mm diameter) at 48 h and homogenized in 5 ml of sterile distilled water, and then plated 100 μl of a 10-fold dilution on YPD. Colonies were counted after incubation at 28°C as described earlier. There were three replicates per treatment, and the experiment was conducted twice.

Statistical analysis

The data were analysed by analysis of the variance (ANOVA). Mean separations were performed using Duncan's

multiple range test. Differences at $P \leq 0{\cdot}05$ were considered as significant.

Results

Effect of TP and *H. uvarum* on controlling grey mould in grape

As shown in Fig. 1, treatment with TP alone resulted in significantly lower disease incidence of grey mould on grape compared with the controls. The addition of TP to *H. uvarum* markedly enhanced the biocontrol efficacy against grey mould. The combined treatment of *H. uvarum* (1×10^6 CFU ml^{-1}) with TP (0·5 and 1·0%) was effective as 1×10^8 CFU ml^{-1} *H. uvarum* alone, and better than *H. uvarum* (1×10^6 CFU ml^{-1}) alone.

Inhibitory effect of TP on spore germination of *B. cinerea in vitro*

As shown in Fig. 2, treatment with TP showed inhibitory effects on the spore germination of *B. cinerea in vitro*. The spore germination rate of *B. cinerea* was significantly lower in the TP-treated (all concentrations) samples than the control. The highest inhibitory rate on spore germination was recorded at 1·0% TP ($P < 0{\cdot}05$).

Effect of TP on mycelial growth of *B. cinerea in vitro*

The effect of TP on mycelial growth of *B. cinerea in vitro* is shown in Fig. 3. Mycelium growth was significantly inhibited by TP at 0·1, 0·5 and 1%, but not by TP at 0·01 and 0·05%. The highest inhibitory rate on mycelium growth was recorded at 1·0% TP ($P < 0{\cdot}05$).

Effect of TP on population dynamics of *H. uvarum in vitro* and *in vivo*

There was different effect on the growth of *H. uvarum* between in YPD medium and wounds of grapes (Table 1). Adding TP did not significantly influence the growth of *H. uvarum* in YPD medium. While in the presence of TP at 0·5% or above, the populations of *H. uvarum* increased in wounds.

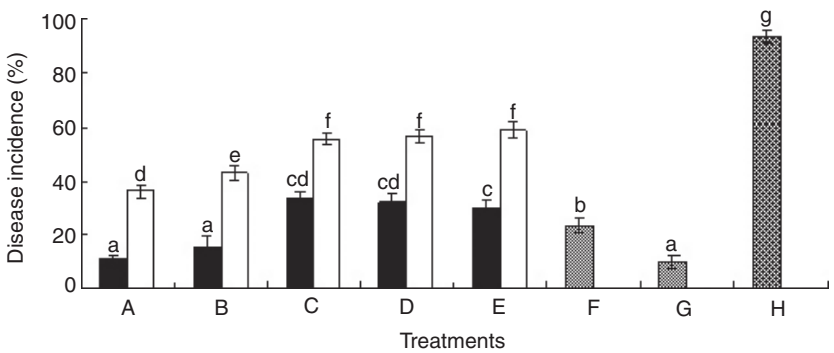

Figure 1 Disease incidence of gray mold in grape fruits at 28°C for 4 d as affected by various treatments: (a–e) Tea polyphenol (0·01, 0·05, 0·1, 0·5 and 1·0%) alone (□); or combined with *Hanseniaspora uvarum* (1×10^6 CFU ml^{-1}) (■); (f) *H. uvarum* (1×10^6 CFU ml^{-1}); (g) *H. uvarum* (1×10^8 CFU ml^{-1}); (h) 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 $P \leq 0{\cdot}05$.

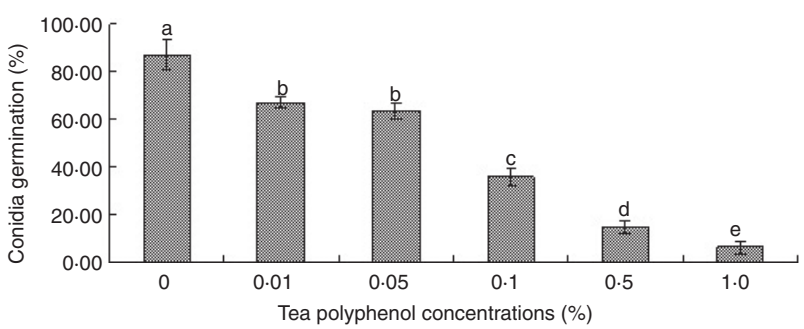

Figure 2 Effect of tea polyphenol at various concentrations on spore germination of *Botrytis cinerea in vitro* at 28°C. Bar represent standard deviations of the means. Data in columns with different letters are significantly different according to Duncan's multiple range test at $P \leq 0{\cdot}05$.

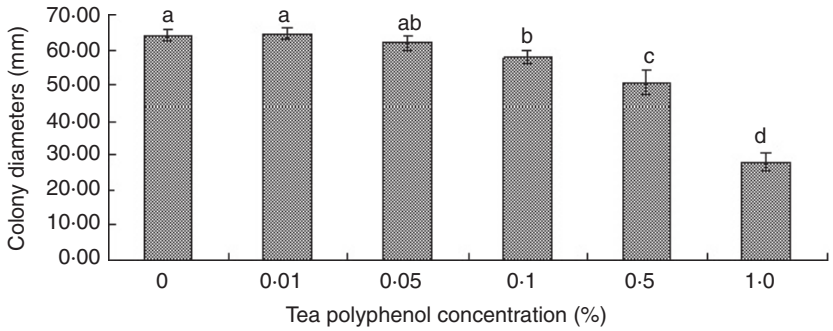

Figure 3 Effect of tea polyphenol at various concentrations on mycelium growth of *Botrytis cinerea in vitro* at 28°C. Bar represent standard deviations of the means. Data in columns with different letters are significantly different according to Duncan's multiple range test at $P \leq 0{\cdot}05$.

Table 1 Effect of tea polyphenol at various concentrations on growth of *Hanseniaspora uvarum in vitro* and *in vivo*

	Population growth	
Tea polyphenol concentration (%)	*In vitro* (Log $_{10}$ CFU ml^{-1})	*In vivo* (Log $_{10}$ CFU ml^{-1})
0	7·65 ± 0·19 a	6·51 ± 0·15 a
0·01	7·61 ± 0·12 a	6·62 ± 0·13 a
0·05	7·60 ± 0·14 a	6·93 ± 0·17 a
0·1	7·47 ± 0·16 a	7·25 ± 0·21 a
0·5	7·41 ± 0·22 a	8·19 ± 0·09 b
1	7·22 ± 0·17 a	8·82 ± 0·11 c

Data are treatment means of pooled data ± standard errors.
Values of each column followed by different letters are significantly different at $P \leq 0{\cdot}05$ according to Duncan's multiple range tests.

Discussion

Results from our experiments showed that the significant control effects against grey mould were achieved with TP (Fig. 1). Similar inhibitory effects were reported on several human and animal disease-related bacteria, phytopathogenic bacteria and pathogenic fungal. Friedman (2007) reported that TP exhibited inhibitory effects against *Bacillus cereus*. Jeong *et al.* (2009) showed that catechol significantly inhibited the growth of *Clostridium perfringens*, *Clostridium difficile* and *Escherichia coli*. Liu *et al.* (2010b) observed that TP had an obvious inhibitory effect against stem-end rot caused by *D. natalensis*. Moreover, we found that the combined treatment of *H. uvarum* (1×10^6 CFU ml^{-1}) with TP (0·5 and 1·0%) could significantly reduce grey mould on grape in comparison with application of *H. uvarum* (1×10^6 CFU ml^{-1}) or TP alone. This result suggests that TP enhanced the biocontrol activity of *H. uvarum* against grey mould caused by *B. cinerea*. This result was in accordance with previous reports of Liu *et al.* (2010b), who found that the addition of TP greatly enhanced the biocontrol efficacy of *C. ernobii* against stem-end rot of citrus.

In our experiment, the spore germination of *B. cinerea* was significantly inhibited by TP at all concentrations (Fig. 2). Furthermore, mycelium growth was significantly inhibited by TP at 0·1% and above (Fig. 3). The beneficial effect of TP on inhibiting spore germination and mycelium growth was also observed by Park *et al.* (2006) and Wang *et al.* (2008), who reported that the spore germination and mycelium growth of *Bipolaris maydis*, *Colletotrichum musae*, *Fusarium oxysporum* f.sp. and *C. albicans* were significantly inhibited by TP, and by Hara-Kudo *et al.* (2005), who reported that green tea catechins (the major compound of green TP) showed antibacterial action on pathogenic bacteria spore, such as *Clostridium botulinum* and *Clostridium butyricum*. Moreover, we observed that TP significantly inhibited the spore germination and mycelium growth of *D. natalensis* in the previous study. These results suggest that the inhibitory effect on spore germination and mycelium growth may be one of the major mechanisms of TP inhibiting postharvest fungal disease. However, the precise interaction between TP and the spore or hypha of postharvest fungal pathogens is still unknown and needs to be further studied.

In this study, the addition of TP did not affect the growth of *H. uvarum in vitro* and significantly increased the population of *H. uvarum in vivo* (Table. 1). Our results showed that the increased population of *H. uvarum* may be related to the enhanced biocontrol efficacy of *H. uvarum*. Moreover, the environment of the wounds was more complex than that of YPD. The differences *in vitro* and *in vivo* showed that specific salt–host tissue interactions may involve biochemical reactions. It was reported that TP promoted the scavenging of ROS and exhibited the antioxygenic property (Friedman 2007). The differences could be correlated with the mechanisms of TP (Liu *et al.* 2010b).

Our results indicated that TP significantly inhibited the infection of postharvest pathogen and greatly enhanced the growth of antagonist. The results were similar to those of Ishihara *et al.* (2001), who observed that green TP decreased the population of harmful bacteria such as *Fusobacterium* and increased the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria*. Ahn *et al.*

(2005) reported that TP not only helped to kill the intestinal pathogens but also promoted the growth of beneficial flora in the intestine such as *Bifidobacteria*. Moreover, we observed that TP had an obvious inhibitory effect against stem-end rot and enhanced the growth of *C. ernobii* in the previous study. It is therefore inferred that the antimicrobial activity of TP has selectivity, which could inhibit the growth of pathogens and keep a balance of beneficial flora.

TP is a natural extract of tea and an environmentally safe and inexpensive reagent. The present work showed that TP exhibited inhibitory effect against postharvest fungal disease. Therefore, TP could be used to improve the efficacy of *H. uvarum*. TP has great potential in commercial development. The inhibitory effects of TP on spore germination and mycelial growth of *B. cinerea* and the increased populations of *H. uvarum in vivo* may be some of the important mechanisms of TP. Further research will focus on the mechanisms of TP against fungal pathogens at the molecular level.

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