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Mechanisms of glycine betaine enhancing oxidative stress tolerance and biocontrol efficacy of *Pichia caribbica* against blue mold on apples

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HIGHLIGHTS

- GB enhanced biocontrol efficacy of *P. caribbica* to blue mold decay of apples.
- *P. caribbica* treated with GB exhibited faster growth in wounds of apples.
- GB increased oxidative stress tolerance of *P. caribbica*.
- GB reduced ROS accumulation, protein carbonylation, lipid oxidation of *P. caribbica*.
- GB induced differential expression of some proteins of *P. caribbica*.

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ABSTRACT

The effects of glycine betaine (GB) treatment on biocontrol efficacy of *Pichia caribbica* against blue mold decay of apples and oxidative stress tolerance were investigated. To reveal the mechanism of GB enhancing oxidative stress tolerance and biological control efficacy of *P. caribbica*, the accumulation of intracellular reactive oxygen species (ROS), protein carbonylation, lipid oxidation and differentially expressed proteins were analyzed. Compared with *P. caribbica* treated without GB, *P. caribbica* treated with GB exhibited enhanced biocontrol activity (disease incidence decreased from 48.81% to 32.14%) and oxidative stress tolerance when exposed to oxidative stress (survival ability increased from 60.1% to 77.7%), as well as improved growth in wounds of apples. GB treatment reduced accumulation of ROS, levels of oxidative damage to cellular proteins and lipids in *P. caribbica*. The proteomic analysis showed that 51 proteins were differentially expressed in *P. caribbica* after GB treatment, 33 proteins of which were successfully identified by MALDI-TOF-MS and database Queries. The up-regulation of some identified proteins related to carbohydrate transport and metabolism (enolase, pyruvate kinase and isocitrate lyase), stress response and regulation (peroxisomal catalase and Serine/threonine protein kinase) were involved in the enhancement of oxidative stress tolerance and biocontrol efficacy of *P. caribbica*.

1. Introduction

Postharvest losses of fruits and vegetables caused by fungal pathogens are very serious throughout the world. Furthermore, some fungi could produce mycotoxins that is a major health hazard for consumers (Mahunu et al., 2016; Liu et al., 2017). Blue mold decay caused by *Penicillium expansum* is one of the most important postharvest rots of apples, which affects fruit quality and causes significant economic losses (Fan and Tian, 2000; Zhang et al., 2007; Calvo et al., 2007). *P. expansum* is also regarded as a major producer of patulin (PAT), a mycotoxin which has cytotoxic, genotoxic and immunosuppressive activity (Wouters and Speijers, 1996). Traditionally, the control of fungal diseases is mainly based on chemical treatments (Salomao et al., 2008; Sansone et al., 2005). However, the use of chemical fungicides is restricted due to the concerns on the resistance of spoilage fungi to fungicides, toxicity of residual fungicides and the environmental pollution (Ragsdale and Sisler, 1994; Sharma et al., 2009; Förster et al., 2007).

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Biological control with microbial antagonists alone or integrated with other measures is considered as a promising alternative strategy, which could reduce synthetic fungicide usage (Wilson and Wisniewski, 1994; Wisniewski et al., 2007; Sui et al., 2015). A number of antagonistic yeasts have been used to control postharvest decay of fruits and vegetables. The antagonistic yeasts have showed significant biocontrol activity against pathogenic fungi strains, such as *P. expansum,* which lead to the decay of apples fruits(Janisiewicz and Korsten, 2002; Fan and Tian, 2001; Droby et al., 2003; Droby, 2006; Ippolito et al., 2000; Zhang et al., 2009; Sharma et al., 2009). Recent reports have concerned the important role of antagonistic yeasts not only in the control of fungal contamination but also mycotoxin produced by fungi in fruits, such as patulin by *P. expansum* in apple fruits (Castoria et al., 2002, 2005). However, some studies reported that antagonistic yeasts used alone cannot provide as satisfactory biocontrol efficacy against decay of fruits as chemical fungicide. Therefore, some compounds, such as indole-3-acetic acid (IAA), chitosan, glycine betaine or antioxidant compounds, were used to enhance the biocontrol activity of antagonistic yeasts (Yu et al., 2007, 2009; Sharma et al., 2009; Liu et al., 2011; Zhao et al., 2012).

Oxidative stress, a potential threat for most aerobic organisms, has a pivotal role in biocontrol systems (Castoria et al., 2003; Macarisin et al., 2010). The presence of oxidative stress (i.e. hydrogen peroxide) in the environment of wounds in fruits represents part of the plant defense response to microbial attack. The previous study demonstrated that antagonistic yeasts could rapidly colonize wounds of fruits and then limit the growth of pathogenic fungi even in this oxidative stressful environment (Castoria et al., 2003). The tolerate ability of antagonistic yeasts to oxidative stress effected their biocontrol ability against fungal pathogens greatly. Therefore, increasing survival ability of antagonistic yeasts under oxidative stress conditions would be a useful strategy for enhancing biocontrol efficacy (Liu et al., 2011; Sui et al., 2015).

Glycine betaine (GB, *N, N, N*-trimethylglycine), one of the most common compatible solutes, plays a vital role in osmotic regulation of bacteria, fungi, and plants (Ashraf and Harris, 2004; De Zwart et al., 2003; Rhodes and Hanson, 1993). GB can protect enzymes by stabilizing the protein structure and maintains the integrity of membranes against osmotic stress (Ashraf and Foolad, 2007; Boncompagni et al., 1999; Wang et al., 2007). GB induces antioxidant defense responses in plant species such as tea (Kumar and Yadav, 2009), wheat (Raza et al., 2007), and rice (Farooq et al., 2008). However, there are few reports about the effect of GB on antioxidant systems and biocontrol efficacy of antagonistic yeast, not to mention the mechanism involved. Liu et al. (2011) report that GB-treated *Cystofilobasidium infirmominiatum* exhibited greater biocontrol activity against *P. expansum*. The accumulation of reactive oxygen species (ROS) and protein oxidation in GB-treated cells decreased. While the activities of antioxidant enzymes, including catalase, superoxide dismutase and glutathione peroxidase of GB-treated cells increased. The results by Sui et al. (2012) also showed that GB-treated *Candida oleophila* exhibited greater biocontrol activity against *P. expansum* and *Botrytis cinerea*. GB-treated cells exhibited less accumulation of ROS and lower levels of oxidative damage to cellular proteins and lipids. Additionally, the expression of major antioxidant genes, including peroxisomal catalase, peroxiredoxin TSA1, and glutathione peroxidase was elevated in the yeast by GB treatment.

The objective of the present study was to determine the effect of GB on oxidative stress tolerance and biocontrol efficacy of antagonistic yeast *Pichia caribbica,* and explore the mechanism of GB enhancing oxidative stress tolerance and biocontrol efficacy of *P. caribbica.* We investigated (1) the effects of GB on biocontrol efficacy of *P. caribbica* against blue mold decay of apples caused by *P. Expansum*, population dynamics of *P. caribbica* in the wounds of apples, viability of *P. caribbica* under oxidative stress; (2) the effects of GB on reactive oxygen species (ROS) accumulation in *P. caribbica*, protein carbonylation and lipid oxidation; (3) the effects of GB on proteome of *P. caribbica*.

2. Material and methods

2.1. *Yeast*

P. caribbica preserved in the China General Microbiological Collection Center (CGMCC, No. 3616) was stored at 4 °C on nutrient yeast dextrose agar (NYDA) in our laboratory. NYDA medium contained (per liter): 8 g nutrient broth, 5 g yeast extract, 10 g glucose, and 20 g agar. Liquid cultures in nutrient yeast dextrose broth (NYDB) inoculated with a loop of the above culture were incubated at 28 °C for 24 h. And then, cultures were centrifuged at 7000*g* for 10 min and precipitates were washed three times with sterile distilled water to remove residual growth medium. The cells was resuspended and adjusted to the concentration of 5×10^8 cells/ml with sterile distilled water.

2.2. Pathogen

P. expansum isolated from infected apples was stored at 4 °C on potato dextrose agar (PDA). PDA medium contained (per liter): extract of boiled potato (200 g), 20 g dextrose, 20 g agar. Spore suspensions of *P. Expansum* were prepared by collecting spores from the sporulating edges of a 7-day-old culture into sterile distilled water and adjusted to the appropriate concentration.

2.3. Fruits

Apples (*Malus* × *domestica Borkh*, cv. Fuji) were harvested at commercial maturity from a orchard in Yantai, Shangdong Province. Fruits without apparent injury or infection were selected based on uniformity of size, ripeness and color, and disinfected with 1% (w/v) sodium hypochlorite for 1–2 min, then washed with tap water and air-dried. These fruits were used in subsequent biocontrol assays.

2.4. GB treatment of P. caribbica

P. caribbica at concentration of 5×10^8 cells/ml was prepared according to the method described as 2.1. Afterward, 1 ml of the above-mentioned *P. caribbica* suspension was cultivated in NYDB (not-GB-treated) or NYDB amended with GB (GB-treated) at different concentrations (0.5 mM, 1 mM, 2 mM and 3 mM) in a rotary shaker at 180 rpm at 28 °C for 24 h. Afterwards, cultures were centrifuged at 7000*g* for 10 min and washed three times with sterile distilled water to remove residual GB and medium. Cell pellets were re-suspended and adjusted to a concentration of 1×10^8 cells/ml with sterile distilled water for subsequent experiments.

2.5. Biocontrol assay of P. caribbica against P. expansum on apples

Three wounds were made on the equator of each apple using a sterile cork borer (approximately 5 mm in diameter, and 3 mm in depth). Each wound was inoculated with the following solutions: (1) suspension of not-GB-treated *P. caribbica* (1×10^8 cells/ml) prepared according to the method described as 2.4; (2) suspension of GB-treated *P. caribbica* (1×10^8 cells/ml) prepared according to the method described as 2.4; (3) sterile distilled water as the control. After three hours, 30 μl of *P. expansum* suspension (5×10^4 spores/ml) prepared according to the method described as 2.2