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Autoclaved yeast enhances the resistance against *Penicillium expansum* in postharvest pear fruit and its possible mechanisms of action

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ABSTRACT

The study investigated the effect of autoclaved yeast on the control of blue mold in pear fruit and the possible mechanisms involved. The results demonstrated that autoclaved yeast *Rhodosporidium paludigenum* could stimulate remarkable resistance to the blue mold caused by *Penicillium expansum* in pear fruit. Autoclaved yeast had no direct antifungal activity against *P. expansum in vitro* and *in vivo* while it reduced germination of *P. expansum* in fruit wounds after 24 h of treatment. Moreover, the activities of four defense-related enzymes (including superoxide dismutase, catalase, peroxidase and phenylalanine ammonia-lyase) and the four pathogenesis-related protein genes (including *PR1-like*, *endoglucanase9*, *endochitinase-like* and *PR4*) were significantly enhanced and the lipid peroxidation was highly inhibited in the treatment with autoclaved yeast, which was closely related to the mechanism by which autoclaved yeast reduce the blue mold rot in pear fruit. The results from this study provides the basis for further research on the antagonistic mechanism of biocontrol yeasts in induced resistance of harvested fruit.

1. Introduction

Blue mold rot, caused by *Penicillium expansum* Link, is a major and wide-spread postharvest disease of pears, resulting in considerable economic losses during a long-term storage of harvested pear fruit (Wicks, 1977). Although the application of synthetic fungicide is still the most widely used method in controlling postharvest disease of fruit, the regulations on the use of new and existing fungicides are becoming more and more stringent because of the chemical residue, development of resistance biotypes, and the potential risk on environmental and human health (Calvo et al., 2017; Wisniewski et al., 2016). Therefore, there is a real imperative to develop the safe and effective alternative technologies for postharvest disease management of fruit.

Biological control agents as an alternative to synthetic fungicides for reducing postharvest losses of harvested commodities has been the focus of considerable research over the last 30 years (Wisniewski et al., 2016). Numerous microbial antagonists have been identified in laboratory, semi-commercial, and commercial studies (Droby et al., 2016). *Rhodosporidium paludigenum* Fell & Tallman was an effective antagonistic yeast, which could inhibit the black rot and gray mold in cherry tomato (Wang et al., 2008, 2010b), sour rot in citrus (Liu et al., 2010) and black mold in Chinese winter jujube (Wang et al., 2009). Moreover, it has been reported that the biocontrol efficacy of *R. paludigenum* could be improved by salt-adaptation, acid-adaptation, and chitin-adaptation in reducing infections of *P. expansum* in pears, *Alternaria alternata* in Chinese winter jujube, *P. expansum* in apples (Lu et al., 2014a; Wang et al., 2014, 2010a). Recently, Lu et al. (Lu et al., 2013a,b, 2014b) confirmed that applications of *R. paludigenum* effectively induced disease resistance of mandarin oranges by pre-harvested spraying or by post-harvested treatment, which is associated with the activation of ethylene-dependent signaling pathway and defense-related genes expression. However, the mechanisms by which *R. paludigenum* could induce resistance of harvest fruit is still unclear. And it was well known that various modes of action may simultaneously involve in the interaction between antagonisms and pathogens, therefore it is difficult to evaluate the contribution of each action mode possessed by the antagonistic yeast (Li et al., 2016).

In order to explore the mechanisms of induced resistance of *R. paludigenum*, this study was designed to investigate the effect of autoclaved yeast prepared from *R. paludigenum* on postharvest diseases and resistance response in pear fruit. The specific aims are to determine: (1) the effect of autoclaved yeast on inhibition of the blue mold rot in pear fruit wounds; (2) the antifungal activity of autoclaved yeast *in vitro* and *in vivo*; (3) the influence of autoclaved yeast on the activity of defense-

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related enzymes and the level of MDA in pear fruit wounds; (4) the changes in transcript expression of pathogenesis-related (PR) genes including *PR1-like*, *endoglucanase9*, *endochitinase-like* and *PR4* following autoclaved yeast treatment in pear fruit.

2. Materials and methods

2.1. Fruit

Pear fruits (*Pyrus pyrifolia* Nakai cv. Shuijing) were harvested at commercial maturity. Fruits without physical injuries or infections were selected based on uniformity of size, color and ripeness. The selected fruits were surface-disinfected by immersion for 2 min in 2% (v/v) sodium hypochlorite, rinsed with tap water, and allowed to air dried at room temperature.

2.2. Yeast

The yeast strain *Rhodosporidium paludigenum* Fell & Tallman (IMI 394084) was originally isolated from the East China Sea and identified by CABI Bioscience Identification Services (Egham, UK) (Wang et al., 2008). *Saccharomyces cerevisiae* (strain No. 2.3854) was obtained from the Institute of Microbiology, Chinese Academy of Sciences, China and used as a yeast control. The yeast cell was maintained on nutrient yeast dextrose agar (NYDA) medium (containing 8 g of nutrient broth, 5 g of yeast extract, and 10 g of glucose and 20 g of agar in 1 L of distilled water) at 4 °C. Before use, it was activated on NYDA plate at 28 °C for 3 days. Liquid cultures of the yeast were inoculated in 250 mL flask containing 50 mL of nutrient yeast dextrose broth (NYDB) medium at 28 °C for 24 h on a gyratory shaker at 200 rpm. After incubation, yeast cells were harvested by centrifuging at 3000*g* for 10 min and washed twice with sterile-distilled water to remove the medium. Cell pellets were then resuspended in sterile distilled water and adjusted to an appropriate concentration with a hemocytometer.

2.3. Pathogen

The pathogen strain *Penicillium expansum* (Pers.:Fr.) Sacc. was isolated from a decayed pear fruit and maintained on potato dextrose agar (PDA) medium (containing the extract from 200 g of boiled potato, 20 g of glucose and 20 g of agar in 1 L of distilled water) at 25 °C. Spore suspensions of the pathogen were prepared by removing the spores from a 7-day-old culture of *P. expansum* incubated and then suspending them in sterile distilled water. The spore concentrations were determined using a hemocytometer and adjusted with sterile distilled water as required.

2.4. Autoclaved yeast preparation

The yeast suspension was conducted by thermal treatment, achieved by holding the yeast at 121 °C for 20 min in automatic autoclave sterilizer (XLS-3750, Sannyo, Japan). This enabled the autoclaved yeast to be obtained.

2.5. Efficacy of autoclaved yeast in inhibiting the blue mold rot caused by P. expansum in pear fruit

2.5.1. Effect of autoclaved yeast at different concentrations on inhibition of blue mold rot caused by P. expansum in pear fruit

Pear fruit were wounded using a sterile nail (3 mm deep × 5 mm dia.) and the tissue plugs were removed. All fruit were wounded at the same time with one wound per fruit at the mid-point between calyx and stem end. A 30 μL aqueous of autoclaved *S. cerevisiae* or *R. paludigenum* suspension, at 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 cells mL^{-1} respectively, was applied to each wound. Sterile distilled water was used as the control. After incubation of 24 h, 30 μL of *P. expansum* suspensions at 1×10^4 spores mL^{-1} were inoculated into each wound. The fruits were then air dried and stored in enclosed plastic trays to maintain 90–95% relative humidity (RH) at 25 °C. Disease incidence and lesion diameter were observed. Each treatment included three replicates and each replicate consisted of nine fruits. Each test was performed twice.

2.5.2. Effect of the time between autoclaved yeast treatment and pathogen inoculation on inhibition of blue mold rot in pear fruit

Pear fruit were wounded as described above and each wound was treated with 30 μL of autoclaved yeast at 1×10^7 cells mL^{-1} or sterile distilled water as the control. At various time intervals (0, 6, 12, 24, and 36 h) after treatment, 30 μL of *P. expansum* suspensions at 1×10^4 spores mL^{-1} were inoculated into each wound. The fruits were then air dried and stored as above. Each treatment included three replicates and each replicate consisted of nine fruits. Each test was performed twice.

2.5.3. Effect of autoclaved yeast on different spore concentrations of P. expansum in pear fruit

The fruits were wounded on each pear fruit as described above and each wound was treated with 30 μL of autoclaved yeast (1×10^7 cells mL^{-1}) or sterile distilled water (as the control). After 24 h, 30 μL aqueous spore suspension of *P. expansum* containing 1×10^2, 1×10^4 or 1×10^6 spores mL^{-1} were inoculated into each wound. Each treatment included three replicates and each replicate consisted of nine fruits. Each test was performed twice.

2.6. Effect of autoclaved yeast on spore germination and survival of P. expansum in vitro

The effect of autoclaved yeast on spore germination of *P. expansum* was assayed in potato dextrose broth (PDB) amended with a suspension of autoclaved yeast in final concentrations at 0, 1×10^5, 1×10^6, 1×10^7, 1×10^8 cells mL^{-1}. Aliquots of 100 μL of pathogen suspensions were put into 10 mL glass tubes containing 2 mL of PDB to obtain a final concentration of 1×10^6 spores mL^{-1}. All test tubes were incubated on a rotary shaker at 200 rpm at 25 °C for 12 h. Above 150 spores of *P. expansum* were observed randomly using a hemocytometer to determine the germination rate. Each treatment was repeated three times with three replicates and the whole experiment was conducted more than twice.

Equal amount of the spore suspensions of *P. expansum* were mixed with a suspension of autoclaved yeast in final concentrations at 0, 1×10^5, 1×10^6, 1×10^7, 1×10^8 cells mL^{-1}, respectively. After 60 s, a volume of 100 μL of the suspensions were taken and plated on PDA. The fungal colonies forming units per plate were counted after 72 h of incubation at 25 °C. Each treatment was repeated three times with three replicates and the whole experiment was conducted more than twice.

2.7. Effect of autoclaved yeast on spore germination of P. expansum in pear fruit wounds

Four wounds were made on each pear fruit as described above. The wounds were treated with 30 μL cell suspensions of autoclaved yeast at 1×10^7 cells mL^{-1}, or sterile distilled water as the control. After treatment for 2 or 24 h, 30 μL of *P. expansum* spore suspensions at 1×10^7 spores mL^{-1} were inoculated into each wound. The fruits were then air dried and stored at 25 °C as above. After incubation for 12 h, spores were collected from the wound and above 150 spores of *P. expansum* were observed randomly using a hemocytometer to determine the germination rate. Each treatment was replicated three times and each replicate included nine fruits.