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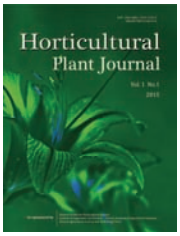

The Biocontrol Effects of the *Bacillus licheniformis* W10 Strain and Its Antifungal Protein Against Brown Rot in Peach

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Abstract

The biocontrol effects of *Bacillus licheniformis* W10 bacterial suspension and its antifungal protein on peach brown rot caused by *Monilinia fructicola* in storage peach fruits and the effects on fruit quality were investigated. The results showed that the fruit disease suppression of *B. licheniformis* W10 bacterial suspension and antifungal protein were significantly higher than that of the control. Inoculation of bacterial suspension and antifungal protein prior to *M. fructicola* gave a better biocontrol effect, and the higher concentrations of bacterial (1×10^{10} cfu · mL^{-1}) and antifungal protein (3.0 mg · mL^{-1}) performed better control effects. The environmental conditions, such as temperature and humidity, affected biocontrol effects of W10 bacterial suspension and antifungal protein. The influence of environment conditions on the activity of antifungal protein was less than that on bacterial suspension. Moreover, lower temperature (4 ℃) and relative humidity (RH 70%–75%) were favorable to prevent peach brown rot by W10 bacterial suspension and its antifungal protein. The W10 bacterial suspension and antifungal protein amended with calcium [0.1% $Ca(NO_3)_2$] could enhance the biocontrol effects, and obviously put off the occurrence of peach brown rot. In addition, the bacterial suspension and antifungal protein significantly reduced the natural decay rates of peach fruits during storage, and the effects were equal to carbendazim. Moreover, both W10 bacterial suspension and antifungal protein treatments did not have effects on external and internal fruit appearance, such as chromatic aberration parameter L^* of flesh, flesh firmness, soluble solids content and weight loss. Therefore, the *B. licheniformis* W10 is a potential biocontrol factor for peach brown rot.

Keywords: peach; *Bacillus licheniformis*; antifungal protein; *Monilinia fructicola*; biological control; fruit quality

1. Introduction

Brown rot, also known as *Sclerotinia* drop or fruit rot, was the first recorded postharvest fruit disease (Ogawa et al., 1995). Brown rot mainly occurs during the fruit late-growth and postharvest storage stage, resulting in loss of fruit value which causes significant economic losses (Ogawa et al., 1995; Li and Chen, 2009). In many parts of China, the primary pathogen causing peach brown rot is *Monilinia fructicola* (Zhu et al., 2005; Hu et al., 2011a, 2011b; Yin et al., 2013), which was first discovered in the eastern United States (Fan et al., 2010). At present, the major prevention and control method for peach brown rot is the use of chemical pesticides. However, pesticides will lead to antimicrobial resistance (Yin et al., 2010), and the

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pesticide residue on the peaches will be a threat to public health. In Europe, no chemical pesticides are allowed for the postharvest stone fruits at all (Casals et al., 2010). Biological control is an important approach for comprehensive management peach brown rot (Spadaro and Gullino, 2004) because it is non-toxic, leaves no residue, and causes no resistance. Biological control has become a "hot" research topic in the area of postharvest disease control and is expected to replace chemical pesticides (Sharma et al., 2009).

The *Bacillus licheniformis* W10 strain is a biocontrol strain of bacteria obtained from a plant rhizosphere in our laboratory. *B. licheniformis* W10 can produce an extracellular antifungal protein, which has a strong inhibitory effect against many plant pathogens and which has the same field control effect as chemical pesticides (Tong et al., 2000; Tang et al., 2005; Sun et al., 2007; Ji et al., 2008). Thus studying *B. licheniformis* W10 and its antifungal protein for the prevention of peach brown rot can improve peach storage by providing a basis for their application as a new biocontrol.

2. Materials and methods

2.1. Materials

The experiments were conducted in 2013 in the plant protection laboratory of Yangzhou University and Forestry and Fruit Research Institute, Shanghai Academy of Agricultural Sciences. In our laboratory, *B. licheniformis* W10 was isolated from tomato rhizosphere. *M. fructicola* was isolated from peach fruit that had brown rot disease at the storage stage in our laboratory. The stock and culture medium for the bacteria were nutrient broth agar (NA) and potato sucrose agar (PSA) medium, respectively. Fruit were obtained from the Institute of Fruit Trees, Shanghai Academy of Agricultural Sciences: nectarine cultivar 'Huyou 18' (hard) and peach cultivar 'Hujing Milu' (soft).

The *B. licheniformis* culture and its antifungal protein preparation were obtained as follows: activated *B. licheniformis* W10 was used to inoculate a 150 mL NA culture medium, which was maintained at 28 ℃ and 180 r · min^{-1} for 48 h. The culture concentration were measured by a plate count method and either adjusted to 1×10^{10} cfu · mL^{-1} or further diluted for the experiment. The antifungal protein was prepared according to the method of Ji et al. (2007). In brief, the *B. licheniformis* W10 culture was centrifuged at 4 ℃ and 8 000 r · min^{-1} for 15 min. The supernatant was passed through a 0.45 μm filter to obtain the culture filtrate. Ammonium sulfate was slowly added to the filtrate to achieve 30% saturation in order to precipitate the proteins at 4 ℃ overnight. The precipitate was collected by centrifugation. The protein precipitation was resuspended in 1/30 of the original volume which was 0.05 mol · L^{-1} Tris-HCl buffer (pH 6.8) and dialysed at 4 ℃ (the dialysis molecular weight cutoff was 8 000 D). The dialysis buffer was replaced every 8 h and was replaced three times. The dialysed protein solution was passed through a 0.22 μm filter to obtain the crude protein. The standard protein concentration curve of bovine serum albumin was measured and regressed as $y = 0.6751x$ ($r^2 = 0.9947$) where y is the absorbance OD_{280} value and x is the protein concentration (mg · mL^{-1}). The OD_{280} value of crude protein concentration was measured, and the protein concentration was calculated according to the standard curve equation. The crude protein was diluted to lower levels as stated in the experiment.

2.2. Testing parameters for the inhibition of B. licheniformis W10 and its antifungal protein on peach brown rot

Concentration: high, medium, and low concentrations of bacteria (1×10^{10}, 1×10^{8}, and 1×10^{6} cfu · mL^{-1}) and protein (3.0, 0.6, and 0.3 mg · mL^{-1}) were tested. The peach surface was first cleaned with 75% ethanol, then a 6 mm in diameter, 3 mm deep block was removed by a hole puncher. The hole was inoculated with either 50 μL bacteria or antifungal protein, and sterile water was used as the control. The fruit was inoculated with a 6 mm piece of brown rot mycelium 1 d later.

Inoculation time: 1. Samples were first inoculated with *B. licheniformis* W10 or its antifungal protein and were inoculated with *M. fructicola* 1 d later; 2. Samples were inoculated with both *B. licheniformis* W10 or its antifungal protein and *M. fructicola* at the same time; 3. Samples were first inoculated with *M. fructicola*, then inoculated with *B. licheniformis* W10 or its antifungal protein 1 d later. Sterile water was used as control for *B. licheniformis* W10 or antifungal protein. The concentration of *B. licheniformis* W10 was 1×10^{10} cfu · mL^{-1}, and the concentration of antifungal protein was 3.0 mg · mL^{-1}.

Temperature and humidity: both a low temperature of 4 ℃ and room temperature 25 ℃ were tested. A low humidity [relative humidity (RH) 70%−75%] and a high humidity (RH 95%−100%) were tested. The concentration of *B. licheniformis* W10 was 1×10^{10} cfu · mL^{-1}, and the concentration of antifungal protein was 3.0 mg · mL^{-1}.

Calcium solution spray: the fruits were sprayed with 0.1% $Ca(NO_3)_2$ solution first. After the surface was dried, samples were inoculated with *B. licheniformis* W10 (2×10^{9} cfu · mL^{-1}) or antifungal protein (3.0 mg · mL^{-1}), and with *M. fructicola* 1 d later.

In all the experiments, 10 peaches were tested for each treatment, and each treatment was repeated three times. The fruit were stored at 25 ℃ under RH 70%−75%. Different temperature and humidity tests were performed according to the specific temperature and humidity. The fruits were examined once a day for disease onset, and lesion diameter was measured.

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