International Journal of Food Microbiology 164 (2013) 141–147

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Investigating the efficacy of *Bacillus subtilis* SM21 on controlling *Rhizopus* rot in peach fruit

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ARTICLE INFO

Article history:
Received 2 January 2013
Received in revised form 28 March 2013
Accepted 15 April 2013
Available online 22 April 2013

Keywords:
Bacillus subtilis SM21
Peach fruit
Postharvest disease
Biological control
Antagonism
Induced disease resistance

ABSTRACT

The efficacy of *Bacillus subtilis* SM21 on controlling *Rhizopus* rot caused by *Rhizopus stolonifer* in postharvest peach fruit and the possible mechanisms were investigated. The results indicated *B. subtilis* SM21 treatment reduced lesion diameter and disease incidence by 37.2% and 26.7% on the 2nd day of inoculation compared with the control. The *in vitro* test showed significant inhibitory effect *of B. subtilis* SM21 on mycelial growth of *R. stolonifer* with an inhibition rate of 48.9%. *B. subtilis* SM21 treatment significantly enhanced activities of chitinase and β-1,3-glucanase, and promoted accumulation of H_2O_2. Total phenolic content and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity were also increased by this treatment. Transcription of seven defense related genes was much stronger in fruit treated with *B. subtilis* SM21 or those both treated with *B. subtilis* SM21 and inoculated with *R. stolonifer* compared with fruit inoculated with *R. stolonifer* alone. These results suggest that *B. subtilis* SM21 can effectively inhibit *Rhizopus* rot caused by *R. stolonifer* in postharvest peach fruit, possibly by directly inhibiting growth of the pathogen, and indirectly inducing disease resistance in the fruit.

1. Introduction

Peach fruit usually have a very short shelf life at room temperature, mainly due to their high susceptibility to pathogens. *Rhizopus* rot caused by *Rhizopus stolonifer* is one of the most common postharvest diseases of peach fruit (Northover and Zhou, 2002). Traditionally, control of postharvest diseases in fruit is dependent on fungicides, and many commercial fungicides are available for postharvest treatment to reduce decay and extend the shelf-life of peach fruit (Fan and Tian, 2000). However, public concern about chemical residues and the fungicide resistance in pathogens have promoted the development of alternative approaches to control postharvest diseases (Russell, 2006).

Biological control is usually regarded as safe for humans and the environment, thus it has emerged as an alternative strategy to combat major postharvest diseases of fruit (Janisiewicz and Korsten, 2002; Sharma et al., 2009). *Pichia membranaefaciens* (Fan and Tian, 2000; Tian et al., 2002), *Debaryomyces hansenii* (Singh, 2004; Mandal et al., 2007) and *Cryptococcus laurentii* (Zhang et al., 2007) have been reported as effective biocontrol agents against *Rhizopus* rot in peach fruit. Recently, some strains of *Bacillus* spp. have been shown effective in controlling postharvest pathogens of peaches (Arrebola et al., 2010; Zhou et al., 2011). *Bacillus subtilis* has also been reported to control green mold disease caused by *Penicillium digitatum* in citrus fruit (Leelasuphakul et al., 2008), anthracnose rot caused by *Colletotrichum lagenarium* in watermelon (Kim and Chung, 2004), anthracnose rot caused by *Colletotrichum gloeosporioides* in mangoes and avocadoes, and postharvest diseases caused by *Alternaria alternata* in melon (Wang et al., 2010). Although the mechanisms by which microbial antagonists suppress postharvest diseases have not been clearly elucidated, competition for nutrients and space is considered as the major mode of their action. In addition, several other biocontrol mechanisms including induction of resistance in host tissue, production of antibiotics, and direct parasitism have been suggested to be effective against postharvest diseases in fruits (Sharma et al., 2009).

In our preliminary experiments, we found that a *B. subtilis* strain SM21 could effectively inhibit postharvest decay of peaches caused by *R. stolonifer*. However, the mode of actions of *B. subtilis* SM21 on inhibiting peach fruit decay was not clear. The objective of this work was to investigate the efficacy of *B. subtilis* SM21 for the control of *Rhizopus* rot caused by *R. stolonifer* in postharvest peach fruit and to explore the possible mechanisms involved.

2. Materials and methods

2.1. Fruit material

Peach (*Prunus persica* Batsch) fruit of cv. Baifeng, a major cultivar widely cultivated in southern China, was hand-harvested at firm-mature stage from a commercial orchard in Nanjing, Jiangsu province, and transported to the laboratory on the day of collection. In the laboratory, the fruit was selected for uniform size and maturity and absence of visual

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.04.010

defects. Fruit was surface-sterilized with 75% ethanol, and air dried prior to wounding.

2.2. Biocontrol agent and pathogen

The biocontrol agent, *B. subtilis* SM21, was kindly supplied by Prof. Jianhua Guo of College of Plant Protection, Nanjing Agricultural University, China. *B. subtilis* SM21 was originally isolated from the forest soil of Zhenjiang City, Jiangsu Province, China, and the CGMCC number is 2058 (Wang et al., 2012). The bacterial strains were all cultured with LB medium in a 1 L conical flask at 30 °C and 200 rpm. A working volume of 500 mL of LB medium was used as a growth medium after inoculation with 1% (v/v) of an inoculum. Bacterial cells were harvested at the beginning of the stationary phase (24 h) by centrifugation at 5000 g for 5 min at 20 °C in an Avanti-TMJ-25I centrifuge (Beckman, Palo Alto, CA, USA). The cell paste was resuspended in sterile distilled water and the cell concentration was adjusted to 1×10^8 CFU/mL.

The pathogen *R. stolonifer* was isolated from the surfaces of infected peach fruit and cultured on potato dextrose agar (PDA) medium (containing the extract of 200 g boiled potatoes, 20 g dextrose and 20 g agar in 1000 mL of distilled water). A sporangiospore suspension was prepared from 2-week-old cultures incubated at 26 °C. Spores were removed from the surface of each Petri dish culture and suspended in 5 mL of sterile distilled water. The number of spores was calculated with a hemocytometer counting chamber, and then the spore concentration was adjusted to 1×10^5 spores per mL with sterile distilled water.

2.3. Effect of biocontrol agent on controlling of Rhizopus rot

Peaches were wounded with the tip of a sterile dissecting needle to make two uniform wounds 4 mm deep and 2 mm wide on two sides of each fruit around the fruit equator. 20 μL of washed-cell suspension of *B. subtilis* SM21 at 1×10^8 CFU/mL or distilled water (as control) was pipetted onto each wound. The fruit were then air dried and put into 400 × 300 × 100 mm plastic trays wrapped with high density polyethylene sleeve to maintain high humidity for the growth of *B. subtilis* SM21(Wang et al., 2013). After keeping at 20 °C for 12 h, peaches were inoculated with 15 μL of a suspension of 1×10^5 spores per ml *R. stolonifer* in each wound. The fruit was incubated at 20 °C with high humidity (about 95%) for 3 days. Disease incidence and lesion diameter on each fruit wound were observed at 1, 2 and 3 days post inoculation. Meanwhile, fruit samples were taken for enzyme assays and measurements of protein, total phenolic and H_2O_2 content, DPPH radical-scavenging activity and quality parameters. There were three replicates of 10 fruit each per treatment, and the experiment was conducted three times.

2.4. Effect of B. subtilis SM21 on mycelial growth of R. stolonifer in vitro

The interactions between *B. subtilis* SM21 and *R. stolonifer* in culture were evaluated on PDA plates. A 5-mm *R. stolonifer* agar plug from actively growing mycelium of *R. stolonifer* was placed centrally on the agar test plates. Four 10 μL quantity of 1×10^8 CFU/mL *B. subtilis* SM21 cells or sterile distilled water as a control were inoculated on 4 sites of PDA plate at equal distance from each other 3 cm distance from the colony of *R. stolonifer*. All the plates were incubated for 2 days at 28 °C and then the antagonistic effect of the *B. subtilis* SM21 on mycelial growth *of R. stolonifer* was calculated according to the method of Zhou et al. (2011).

2.5. Assay of enzyme activity

Chitinase (EC 3.2.1.14) was extracted from 1 g of frozen tissue sample with 5 mL of 50 mM sodium acetate buffer (pH 5.0). Chitinase activity was measured by the release of *N*-acetyl-D-glucosamine (NAG) from colloidal chitin according to the method of Abeles et al. (1971). A unit of chitinase activity is defined as the amount of enzyme required to catalyze the production of 1 μg NAG per hour at 37 °C.

β-1,3-Glucanase (EC 3.2.1.58) activity was determined using the colorimetric assay based on the hydrolysis of laminarin according to the previous method (Abeles et al., 1971). 1 g of frozen tissue sample was ground with 5 mL of 50 mM sodium acetate buffer (pH 5.0). 1 mL of enzyme preparation was incubated for 1 h at 37 °C with 1 mL of 4% laminarin (Aldrich, Chemical Co., Milwaukee, WI, USA). The reaction was terminated by heating the sample in boiling water for 5 min and the amount of reducing sugar was measured spectrophotometrically at 540 nm after reaction with 250 μL 3,5-dinitrosalicyclic reagent. One unit is defined as the amount of enzyme catalyzing the formation of 1 μmol glucose equivalents in 1 h.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by the method of Rao et al. (1996). 1 g of frozen tissue was ground with 5 mL of 50 mM sodium phosphate buffer (pH 7.8). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 14 mM methionine, 3 μM EDTA, 1 μM nitro blue tetrazolium (NBT), 60 μM riboflavin and 0.1 mL crude enzyme extract. One unit of SOD activity is defined as the amount of enzyme causing 50% inhibition of NBT.

Catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Chance and Maehly (1955). Frozen tissue (1 g) was ground with 5 mL of 50 mM sodium phosphate buffer (pH 7.0). The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 12.5 mM H_2O_2 and 20 μL of enzyme extract. One unit of CAT activity is defined as the amount of enzyme that decomposed 1 μmol H_2O_2 per min at 30 °C.

Ascorbate peroxidase (APX, EC 1.11.1.11) measurement was adapted from the method of Vicente et al. (2006). 1 g frozen tissue was ground with 5 mL of 50 mM sodium phosphate buffer (pH 7.0), containing 0.1 mM EDTA, 1 mM ascorbic acid and 1% polyvinyl-pyrrolidone (PVP). The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C and the supernatant was used to determine APX activity. One unit of APX activity is defined as the amount of enzyme that oxidized 1 μmol ascorbate per minute at 30 °C.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was extracted with 0.2 M sodium borate buffer at pH 8.7 that contained 20 mM of β-mercaptoethanol. The assay medium contained 0.1 mL of enzyme extract and 1 mL of L-phenylalanine. After incubation at 40 °C for 1 h, the reaction was stopped by adding 0.2 mL of 6 M HCl. PAL activity was assayed according to the method of Assis et al. (2001) with some modification. One unit of PAL activity is defined as the amount of enzyme that caused an increase in absorbance of 0.01 at 290 nm in 1 h under the assay conditions.

Peroxidase (POD, EC 1.11.1.7) was extracted from 1 g of frozen tissue with 5 ml of 50 mM sodium phosphate buffer (pH 8.7). The extracts were then homogenized and centrifuged at 10,000 *g* for 20 min at 4 °C. POD activity was assayed according to the method of Kochba et al. (1977) using guaiacol as donor and H_2O_2 as substrate. One unit of POD activity is defined as the amount of enzyme required to cause an increase in absorbance of 0.01 at 470 nm per minute.

Polyphenol oxidase (PPO, EC 1.10.3.1) was determined by adopting the method described by González et al. (1999). The frozen tissue (1 g) was ground with 5 mL of 0.2 M sodium phosphate buffer, pH 6.5, together with 1% of polyvinylpolypyrrolidone (PVP). The crude PPO extraction was centrifuged at 10,000 *g* for 20 min. Each 3 mL of assay medium contained 0.1 M catechol, 0.1 M sodium phosphate buffer, pH 6.5, and 0.1 mL enzyme extract. The increase in absorbance at 420 nm at 25 °C was recorded. One unit of PPO activity is defined as the amount of enzyme that caused an increase of 0.01 at 420 nm per minute.

Protein content in the enzyme extracts was determined by the Bradford (1976) method, using bovine serum albumin as a standard. Specific activity of all of the enzymes was expressed as units per milligram of protein.