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

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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 H2O2. 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.

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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 gloeosporioides in mangoes and avocados, 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...
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 × 10⁶ 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 × 10⁴ 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 × 10⁶ 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 × 10⁵ 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 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 H₂O₂ 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 × 10⁶ 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-dinitrosalicylic 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 H₂O₂, and 20 μL of enzyme extract. One unit of CAT activity is defined as the amount of enzyme that decomposed 1 μmol H₂O₂ 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% polyvinylpyrrolidone (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 l-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 H₂O₂ 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 milli-gram of protein.
2.6. Measurement of total phenol, H_2O_2 content and DPPH radical-scavenging activity

Total phenolic content was determined using the modified Folin-Ciocalteu procedure described by Slinkard and Singleton (1977). Samples (1 g) were homogenized in 5 ml of 80% cold acetone and centrifuged at 10,000 g for 20 min; the supernatant was used for analysis. The result was expressed as milligrams of gallic acid equivalent (GAE) per 100 g of fresh weight.

H_2O_2 content was determined using a method based on titanium oxidation described by Patterson et al. (1984). Frozen tissue (2 g) was ground and homogenized with 5 ml of chilled 100% acetone and then centrifuged at 10,000 g for 20 min at 4 °C. Absorbance of the supernatant was measured at 412 nm. Absorbance values were calibrated against a standard curve (generated using known concentrations of H_2O_2) and expressed as μmol g^{-1} FW.

DPPH radical-scavenging activity was estimated using the method of Larrauri et al. (1998). Half a gram of frozen sample was extracted with 50% ethanol and centrifuged at 10,000 g for 20 min at 4 °C. An ethanolic solution of DPPH served as control. The DPPH radical-scavenging activity was calculated according to the following formula:

\[
\text{DPPH radical scavenging activity (\%)} = 1 - \left( \frac{\text{absorbance of sample}}{\text{absorbance of control}} \right) \times 100\%.
\]

2.7. Analysis of defense-related gene expression by RT-PCR

RT-PCR was used to analyze the expression patterns of the defense-related gene nonexperser of PR gene (NPR1-like), pathogenesis-related (PR-like), chitinase (CHI), β-1,3-glucanase (GNS), Phenylalanine ammonia lyase (PAL), lipoxygenase (LOX1) and catalase (CAT1) in peach fruit inoculated only with distilled water (Mock), R. stolonifer or B. subtilis SM21, and in those both treated with B. subtilis SM21 and inoculated with R. stolonifer. Total RNA was extracted from peach fruit according to the method described by Chang et al. (1993) with some modifications. RT-PCR was performed using the PrimeScriptTM 16 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). Short and conserved segments of PpNPR1-like (GenBank ID: DQ149935), PpPR-like (GenBank ID: AF362989), PpCHI (GenBank ID: AF206635), PpGNS (GenBank ID: U49454), PpPAL (GenBank ID: AF206634), PpLOX1 (GenBank ID: EU883638) and PpCAT1 (GenBank ID: AJ496418) were cloned by degenerate primers. Independent PCR with 25 cycles was performed using aliquots (1 μl) of cDNA samples, and a constitutively expressed gene 18S-rRNA (GenBank ID: L28749.1) was used as a quantitative control in the RT-PCR analysis. The sequences of primers used for RT-PCR analysis were the following: PpNPR1-like forward: 5'-GACCCAAACATGCC AGCAGTG-3', PpNPR1-like Reverse: 5'-ATCTTCTGCCGTCTAACC-3'; PpPR1-like Reverse: 5'-TTCCAGCCCTTACCA-3'; PpPR1-like forward: 5'-ATCAACTGGGACTTGCGTACT-3'; PpCHI forward: 5'-GTGGACGGGACTCAGATTACCA-3'; PpCHI reverse: 5'-TTTGGGGTTGTACTCATTCA-3'; PpGNS forward: 5'-ATTTCTCTTGCTGGTCTTG-3'; PpGNS reverse: 5'-CTCTGGGTCTTTCTATTCT-3'; PpPAL forward: 5'-TGAC CGCGTACGTTTT-3'; PpPAL reverse: 5'-CTGGTTGGGTTGCTGAT T-3'; PpLOX1 forward: 5'-GGACGGGACTAGATTACA-3'; PpLOX1 reverse: 5'-GTGCCGACTTGTGGACTA-3'; PpCAT1 forward: TCACTGATATTTCTCACGCTTACA-3'; PpCAT1 reverse: 5'-CTCCAACCTTAA
GCTTCAT-3′; 18S-rRNA_forward: 5′-ATGGCCGTTCTTAGTTGGTG-3′,
18S-rRNA_reverse: 5′-GTACAAAGGGCAGGGACGTA-3′.

Relative mRNA levels of genes were analyzed based on densitometry values obtained using the Quality One software of Bio-Rad.

2.8. Statistical analysis

All the experiments were conducted twice using completely randomized design and each treatment was replicated three times. All statistical analyses were performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA) for this experiment. Analysis of variance (ANOVA) was used to compare the means. Mean separations were performed using Duncan’s multiple range tests. Differences at \( P < 0.05 \) were considered as significant.

3. Results

3.1. Effect of B. subtilis SM21 on Rhizopus rot of peach fruit

The lesion diameter and disease incidence of Rhizopus rot in fruit treated with B. subtilis SM21 were significantly \( (P < 0.05) \) lower than those in the control at the first 2 days of incubation at 20 °C (Fig. 1). Compared with the control, B. subtilis SM21 treatment reduced lesion diameter and disease incidence by 37.2% and 26.7%, respectively, on the 2nd day of inoculation (Fig. 1A and B). Although all the inoculated wounds in both B. subtilis SM21 treated and control fruit developed decay symptoms after 3 days of the inoculation, the lesion diameter in fruit treated with B. subtilis SM21 was still significantly \( (P < 0.05) \) lower than that in control fruit (Fig. 1A).

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

In dual culture assay on PDA plate, the R. stolonifer hyphae in the control plates grew actively and spread over the entire PDA plate when B. subtilis SM21 was absent (Fig. 2A). B. subtilis SM21 showed strong antifungal activities against mycelial growth of R. stolonifer with an inhibition rate of 48.9% (Fig. 2B).

3.3. Effect of B. subtilis SM21 on chitinase and \( \beta\)-1,3-glucanase activities in peach fruit

The activities of chitinase (CHI) and \( \beta\)-1,3-glucanase (GNS) increased gradually during storage and were maintained at significantly \( (P < 0.05) \) higher levels in B. subtilis SM21 treated fruit than in control fruit (Fig. 3).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3.png}
\caption{Effect of B. subtilis SM21 treatment on chitinase (A) and \( \beta\)-1,3-glucanase (B) activities in peach fruit inoculated with R. stolonifer and incubated at 20 °C for 3 days. Data are expressed as the mean of triplicate samples. Vertical bars represent the standard errors of the means.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Effect of B. subtilis SM21 treatment on SOD (A), CAT (B) and APX (C) activities and \( \text{H}_2\text{O}_2 \) content (D) in peach fruit inoculated with R. stolonifer and incubated at 20 °C for 3 days. Data are expressed as the mean of triplicate assays. Vertical bars represent the standard errors of the means.}
\end{figure}

The activities of chitinase (CHI) and \( \beta\)-1,3-glucanase (GNS) increased gradually during storage and were maintained at significantly \( (P < 0.05) \) higher levels in B. subtilis SM21 treated fruit than in control fruit.
higher levels in fruit treated with *B. subtilis* SM21 and inoculated with *R. stolonifer* compared with the controls. The fruit that were both treated with *B. subtilis* SM21 and inoculated with *R. stolonifer* showed 89.8% higher activity of CHI and 38.0% higher activity of GNS after 3 days of inoculation (Fig. 3A and B).

3.4. Effect of *B. subtilis* SM21 on activities of SOD, CAT, APX and H$_2$O$_2$ in peach fruit

*B. subtilis* SM21 treatment significantly (*P* < 0.05) promoted the increases and delayed the decreases in activities of SOD and CAT, the activities of both enzymes were higher in *B. subtilis* SM21 treated and *R. stolonifer* inoculated fruit than those in control fruit during the whole storage time (Fig. 4A and B). *B. subtilis* SM21 treatment induced significantly (*P* < 0.05) higher SOD activity on the 1st and 2nd days compared with the control (Fig. 4A). Fruit treated with *B. subtilis* SM21 maintained significantly (*P* < 0.05) higher CAT activity on the 2nd and 3rd days of incubation (Fig. 4B). APX activity in peach fruit decreased during the incubation, *B. subtilis* SM21 treatment significantly (*P* < 0.05) inhibited the activity of APX on the 2nd and 3rd days of incubation (Fig. 4C). The level of H$_2$O$_2$ in both control and *B. subtilis* SM21 treated fruit increased during the inoculation. *B. subtilis* SM21 treatment promoted the accumulation of H$_2$O$_2$, and significantly (*P* < 0.05) higher H$_2$O$_2$ content was observed in *B. subtilis* SM21 treated fruit throughout the storage time (Fig. 4D).

3.5. Effect of *B. subtilis* SM21 treatment on activities of PAL, PPO and POD in peach fruit

PAL activity in control fruit increased slightly during storage. *B. subtilis* SM21 treatment promoted the increase and maintained significantly (*P* < 0.05) higher PAL activity during the whole storage period compared with control fruit (Fig. 5A). POD and PPO activities increased with storage, *B. subtilis* SM21 treatment significantly (*P* < 0.05) increased their activities. The fruit that was treated with both *B. subtilis* SM21 and inoculated with *R. stolonifer* showed 65.6% higher activity of POD after 2 days of inoculation (Fig. 5B) and 51.3% higher activity of PPO after 3 days of inoculation (Fig. 5C) than the control fruit.

3.6. Effect of *B. subtilis* SM21 treatment on total phenolic content and DPPH radical-scavenging activity in peach fruit

The level of total phenolic compounds in control fruit decreased gradually during storage. *B. subtilis* SM21 treatment induced the accumulation of total phenolic content, which was significantly (*P* < 0.05) higher than that in control fruit during the whole storage period (Fig. 6A). DPPH radical-scavenging activity in peach fruit was significantly (*P* < 0.05) enhanced by treatment with *B. subtilis* SM21. Although the activity showed a little decrease after it got a peak value on the 1st day of storage, DPPH radical-scavenging activity in *B. subtilis* SM21 treated fruit always remained at a significant (*P* < 0.05) higher value than in control fruit throughout storage time (Fig. 6B).

3.7. Effect of *B. subtilis* SM21 treatment and *Rhizopus* inoculation on defense-related genes expression in peach fruit

As shown in Fig. 7, transcripts of the seven defense related genes were retained at very low level in the fruit treated only with *R. stolonifer* or distilled water, however, their transcripts were significantly enhanced in the fruit treated with *B. subtilis* SM21. In the fruit treated with SM21 and inoculated with *R. stolonifer*, transcripts of all the seven defense related genes were significantly enhanced and attained at higher level compared with those treated with *B. subtilis* SM21 alone, suggesting that *B. subtilis* SM21 treatment induced stronger expression of the defensive genes in peach fruit upon challenge by the pathogen of *R. stolonifer*.

4. Discussion

In this study, we found that *B. subtilis* SM21 treatment significantly reduced *Rhizopus* rot in peach fruit wounds inoculated with *R. stolonifer* (Fig. 1). The *in vitro* experiment showed that *B. subtilis* SM21 significantly inhibited mycelial growth of *R. stolonifer* (Fig. 2). These results suggest that *B. subtilis* SM21 possesses strong antagonistic activity against the pathogen.

Induced resistance has been inferred to be one of the major mechanisms of biocontrol agents in inhibiting postharvest diseases of horticultural crops (Terry and Joyce, 2004). CHI and GNS have been found to play a crucial role in plant defense against pathogen infection (Ferreira et al., 2007; Liu et al., 2012). CHI hydrolyzes the β-1→4-linkage in chitin which is an essential cell wall component of fungi, while GNS directly degrades cell walls of pathogens or indirectly releases oligosaccharide and elicits defense reactions (Lee et al., 2006). Induction of these two defensive enzymes by *P. membranaeformis* was observed in harvested peach, apple, loquat and Chinese bayberry fruit, and this was correlated to increased disease resistance and reduced disease severity (Fan and Tian, 2000; Iippolito et al., 2000; Chan and Tian, 2005; Gao et al., 2008; Wang et al., 2011). In addition, PAL is the first enzyme in the phenylpropanoid pathway leading to the biosynthesis of phenolics, phytoalexins, lignins...
and many other compounds associated with disease resistance in plants (Cheng et al., 2001). The results of this study indicated that \textit{B. subtilis} SM21 treatment markedly up-regulated the expression of \textit{CHI}, \textit{GNS} and \textit{PAL}, and also promoted their enzyme activities (Figs. 3, 5A and 7).

Meanwhile, levels of total phenolic compounds in peach fruit were enhanced by \textit{B. subtilis} SM21 treatment (Fig. 6A). These results indicate that the induced disease resistance is involved in the mechanisms by which \textit{B. subtilis} SM21 suppressed \textit{Rhizopus} rot in peach fruit.

NPR1 is commonly recognized as a key regulator in defense signaling pathways that leads to increased induction of pathogenesis-related (PR) genes and enhanced disease resistance (Kinkema et al., 2000). Lipoxygenases (LOXs) are also reported to be crucial for lipid peroxidation processes during plant defense responses to pathogen infection. The function of LOXs in defense against pathogens is likely to initiate the synthesis of fatty acid hydroperoxides and of volatile products leading to cell death (Rusterucci et al., 1999; Hwang and Hwang, 2010). The up-regulation of NPR1-like, PR-like and LOX1 (Fig. 7) in this study suggests that the defense signaling networks in peach fruit was induced by \textit{B. subtilis} SM21 treatment. This result further indicates that the induced disease resistance is involved in the biocontrol activity of \textit{B. subtilis} SM21 against \textit{Rhizopus} rot in peach fruit.

The accumulation of reactive oxygen species (ROS) has the potential to serve not only as protectants against invading pathogens, but as signals activating further plant defense reactions and can be induced by a variety of chemical elicitors or pathogens (Lamb and Dixon, 1997). Generally, the metabolism of ROS is controlled by an array of enzymes including SOD, CAT, and APX. Recently, increasing evidence has shown a close relationship between \textit{H}_{2}\textit{O}_{2} accumulation and decreased fruit susceptibility to decay after harvest. For example, higher levels of \textit{H}_{2}\textit{O}_{2} content were correlated with reduced anthracnose rot in methyl jasmonate-treated loquat fruit (Cao et al., 2008), and the lower \textit{Rhizopus} rot in peach fruit treated with the biocontrol agent \textit{B. cereus} AR156 was closely related to increased \textit{H}_{2}\textit{O}_{2} content (Wang et al., 2013). In this study, \textit{B. subtilis} SM21 treatment maintained higher activities of SOD and CAT (Fig. 4A and B), but lower APX activity (Fig. 4C), thus resulting in higher \textit{H}_{2}\textit{O}_{2} content (Fig. 4D) in peach fruit. These results suggest that enhanced \textit{H}_{2}\textit{O}_{2} generation may be one of the major factors that trigger the disease resistance in \textit{B. subtilis} SM21-treated peach fruit.

In conclusion, our results suggest that \textit{B. subtilis} SM21 can effectively inhibit \textit{Rhizopus} rot caused by \textit{R. stolonifer} in postharvest peach fruit, possibly by directly inhibiting growth of the pathogen, and indirectly inducing disease resistance in the fruit.

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Fig. 6. Effect of \textit{B. subtilis} SM21 treatment on total phenolic content (A) and DPPH radical-scavenging activity (B) in peach fruit inoculated with \textit{R. stolonifer} and incubated at 20 °C for 3 days. Each data point is the mean of three replicate samples. Vertical bars represent standard errors of the means.

Fig. 7. Expression of representative defense related genes. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using 18S-rRNA as a standard. Relative mRNA levels of genes were analyzed based on Quality One software of Bio-Rad.
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References


