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REGULAR ARTICLE

Effect of thymol on antifungal ability of chitosan coating against *Penicillium expansum* in Yali pear

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ABSTRACT

Chitosan can form a semipermeable film on fruit to reduce postharvest fruit losses, but using it in fruit storage still demonstrates some defects such as limited ability of inhibiting microorganisms. To improve antifungal ability of chitosan coating used in fruit storage, inhibitive ability of thymol to *Penicillium expansum in vitro* and effect of thymol on antifungal ability of chitosan coating against *P. expansum* in Yali pear (*Pyrus bretschneideri* Rehd.) were tested. Results showed that mycelial growth and conidial germination of *P. expansum* could be completely inhibited *in vitro* when thymol reached 0.2 g/L in culture media, antifungal ability of chitosan coating against *P. expansum* in Yali pear could be improved effectively by adding thymol (0.4 g/L, 0.8 g/L and 1.6 g/L) into, the resistance-related enzymes of POD, PPO and CHT in pear could be enhanced by thymol (0.8 g/L) and positive effects of chitosan coating on the weight loss, firmness and total soluble solids (TSS) in pear during storage should not be decreased when thymol (0.5 g/L) was added into.

Keywords: Thymol; Chitosan coating; Pear; *Penicillium expansum*; Antifungal

INTRODUCTION

Pear is a very popular fruit in the world, but the fruit deterioration caused by *Penicillium expansum* often appears during cold storage. Edible coating is one of promising methods to reduce postharvest losses of fruit. Chitosan, as a safe, biodegradable natural alkaline polysaccharide, has been used in a variety of postharvest fruits, such as grape (Romanazzi et al., 2002), papaya (Chien et al., 2013) and tomato (García et al., 2014). Though chitosan coating has many advantages to the preservation of postharvest fruit and vegetables, chitosan coating still demonstrates some defects, which include limited ability of inhibiting some microorganisms that lead fruit to decay. It was confirmed that antimicrobial activity of chitosan enriched with some agents could be improved (Sivakumar et al., 2005; Zivanovic et al., 2005) effectively.

Synthetic fungicides have excellent capability of inhibiting microorganisms, but in recent years, the using of them has caused consumers' concern and is becoming more and more restrictive due to carcinogenic effects, residual toxicity problems, environmental pollution, occurrence of microbial resistance and high inputs (Diánez et al., 2002; Marín et al., 2003; Rial-Otero et al., 2005). Essential oils (EOs) have long been known to provide effective control over fungal phytopathogens, but different components in EOs show complicated relationships in inhibiting microorganisms, such as synergistic and antagonistic effects, which result in some uncertainties in research work. Thymol is one component of EOs mainly derived from *Origanum Linn* and *Thymus Linn* plants and confers antimicrobial properties to these oils (Mahmoud et al., 1994; Shelaf et al., 1984; Sivropoulou et al., 1996; Pino et al., 1997; Evans and Martin 2000). Over the last two decades several studies *in vitro* have shown that thymol possesses antibacterial and antifungal properties (Marchese et al., 2016).

Accordingly, the present work was undertaken to evaluate the effect of thymol on antifungal ability of chitosan coating against *Penicillium expansum* in Yali pear and the possible mechanism of thymol boosting the antifungal ability of chitosan.

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MATERIALS AND METHODS

Thymol, pathogen and pear

Thymol (> 99%), obtained from Sigma Aldrich, was dissolved in dimethyl sulfoxide (≥ 99.7%, Sigma) to 100 g/L and stored in cold. *Penicillium expansum* was isolated from infected pear, then preserved in our lab and activated on potato dextrose agar (PDA) before using. Yali pear (*Pyrus bretschneideri* Rehd.) was purchased from one commercial orchard in Beijing.

Conidial suspension (1×10^{10} conidia/L)

Conidial suspension of *P. expansum* was prepared by flooding the 1-week-old culture dishes incubated at 27°C with sterile-distilled water containing 0.01% Tween-80 and adjusted to 1×10^{10} conidia/L with microscope (amplification factor: 10 × 40 times).

Chitosan solution (5 g/L)

Chitosan (deacetylation degree ≥ 90, molecular weight: 100 KD) was obtained from Haidebei Inc. (Shandong, China). To prepare 1 L of chitosan solution (5 g/L), 5.0 g of chitosan was dissolved in about 0.91 L of distilled water containing 1.0% (v/v) acetic acid, then the pH of the solution was adjusted to pH 5.0 with 2 mol/L NaOH and made up to 1 L.

Assessment antifungal activity of thymol against *P. expansum in vitro*

The assessment of antifungal activity of thymol against *P. expansum in vitro* was conducted according to methods used by Rios et al. (1998), Wu and Zheng (2007) and Droby et al. (1997) with slight modifications.

The thymol solution was dissolved in sterilized PDA at 45°C (final thymol containing: 0, 0.0125, 0.025, 0.05, 0.1 and 0.2 g/L) respectively, then the intermixture was poured into 9 cm of diameter glass Petri dishes (20 mL per dish). One 6 mm disc was excavated from the PDA in center of every dish (one hole per dish) and 50 µL of the conidial suspension was filled into the hole and then incubated in dark at 27 ± 2°C for 72 h, then the diameter of each colony was recorded. Three Petri dishes per treatment, three replicates.

Effect of thymol on conidial germination (%) of *P. expansum* was tested in potato dextrose broth (PDB). Thymol solution was added into a 10 mL of test tube containing 5 mL of PDB (final thymol containing: 0.05, 0.1, 0.15 or 0.2 g/L), 100 µL of the conidial suspension was added into each test tube. After 20 h of incubation at 27 ± 2°C on a rotary shaker (about 20.94 rad/s), 100 conidia per replicate were observed to determine germination percentage. Experiments were performed three times. The concentration of thymol for mycelial growth and conidial germination being inhibited completely in PDA or PDB was considered as the minimum inhibitory concentration (MIC) of thymol against *P. expansum in vitro* in this experiment.

Estimating effects of thymol on resistance-related enzymes in pears

In the experiment, both the thymol treated and control pears (30 mature pears per treatment, three replicates) were surface-sterilized with 70% ethanol and wounded with a sterilized nail at 3 points (3 mm deep, 3 mm wide) on the equator of each pear. 20µl of thymol solution (diluted to 0.8 g/L, which was 4 times of MIC) or sterile-distilled water (control) was pipetted into each wound site and then 15µL of the conidial suspension was injected into each of the wounded sites. Thereafter, the pears were kept in plastic containers and incubated at 25 ± 2°C, 95 - 100% RH. The activities of resistance-related enzymes of peroxidase (POD), polyphenol oxidase (PPO) and chitinase (CHT) were assayed at the indicated times according to methods described by Cao et al. (2007) and Reissig et al. (1995). Healthy tissue samples were dug below the peel of pear within 2 mm from lesion edge respectively.

For peroxidase (POD) assay, sampled tissue (5.0 g) was homogenized on ice with 5 mL of 0.1 mol/L sodium acetate buffer, pH 5.5, containing 40 g/L polyvinyl polypyrrolidone (PVPP), 1 mmol/L polyethylene glycol (PEG-4000) and 10 ml/L Triton X-100. The homogenate was centrifuged at 12 000 × g at 4°C for 30 min and the supernatant was collected for the enzyme assay.

For peroxidase (PPO) assay, sampled tissue (3.0 g) was homogenized on ice with 5 mL of 0.1 mol/L sodium acetate buffer, pH 5.5, containing 40 g/L PVPP, 1 mmol/L PEG-4000 and 10 ml/L Triton X-100. The homogenate was centrifuged at 12 000 × g at 4°C for 30 min and the supernatant was collected for the enzyme assay.

For chitinase (CHT) assay, sampled tissue (10.0 g) was homogenized with 10.0 mL of 0.1 mol/L sodium acetate buffer, pH 5.2, containing 5 mmol/L β-mercaptoethanol, 40 g/L PVPP and 1mmol/L ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10 000× g at 4°C for 30 min and the supernatant was collected for the enzyme assay.

In this experiment, activities of POD or PAL was expressed as units per gram fresh weight and activity of CHT was expressed as units per milligram fresh weight.

Inoculation and antifungal evaluation in pears

The effect of thymol on antifungal ability of chitosan coating against *P. expansum* was assessed in pear according to

methods used by Liu et al. (2005), Cao et al. (2006) and Zhu et al. (2008) with slight modifications. The pears (10 pears per treatment, three replicates) were disinfected with 70% ethanol, then rinsed in sterilized water for 2 min and left at room temperature until they dried completely. Subsequently, two round wounds (2 mm deep, 5 mm wide) were made on the equator of each pear with a sterilized nail, then the pears were dipped in sterile-distilled water (control) or chitosan solution (5 g/L) added with thymol solution (final thymol containing: 0, 0.4, 0.8 or 1.6 g/L) respectively. After 3 min, the pears were taken out from the solution, air-dried at room temperature for 30 min and 15 µL of conidial suspension was injected into each of the wounded sites. Thereafter, the pears were stored in plastic containers and incubated at 25 ± 2°C, 95 - 100% RH. Lesion diameter and disease incidence on each pear were recorded after 72, 96, 120, and 144 h.

Measuring quality of coated pears

Pear firmness, total soluble solids (TSS) and weight loss were assessed according to methods used by Zhu et al. (2008) with slight modifications. The pears (10 pears per treatment, three replicates) were dipped in sterile-distilled water or chitosan solution (5 g/L) added with thymol solution (final thymol containing: 0 or 0.5 g/L) respectively. After 3 min, the pears were taken out from the solution, air-dried at room temperature for 30 min and stored in plastic containers (25 ± 2°C, 95 - 100% RH). Pear firmness, total soluble solids and weight loss were recorded at the indicated time.

Fruit firmness was measured using a firmness tester (Model GY-B, Xingke Manufactory, Siping City, Jilin, China) equipped with a flat probe (6 mm diameter). Sections of skin were removed at the equator of the pears to allow four separate readings of each pear. Total soluble solids (TSS) content of the pears was determined using an Abbe refractometer (Model WYA, Shanghai Precision Scientific Instrument Co., Ltd, China). Weight loss was determined by weighing pears at intervals during storage and data were expressed as percentage of the initial weight.

Statistical analysis

Data were collected and analyzed by one-way analysis of variance using the statistical software of SPSS 12.0 (SPSS Inc., Chicago, IL, USA) for windows. Mean separations were performed using the least significant difference method (LSD test). P values of < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Antifungal ability of thymol against *P. expansum in vitro*

Effects of thymol on *P. expansum in vitro* were determined through testing percentage of conidial germination and diameter of each mycelial colony at different concentrations of thymol. It was revealed from the results (Fig. 1) that conidial germination percentage and mycelial colony diameters both were gradually decreased as concentration of thymol increased in the culture media. When thymol reached 0.1 g/L in PDA, mycelial colony diameter was 77.6% lower than that in the control. When thymol reached 0.15 g/L in PDB, the conidial germination was 98% lower than that in control. When concentration of thymol reached 0.2 g/L in culture media, both colonial growth and conidial germination of *P. expansum* could be completely inhibited, thus the MIC of thymol to *P. expansum* was 0.2 g/L.

Resistance-related enzymes of POD, PPO and CHT in the treated pears

The activities of POD (Fig. 2a) and PPO (Fig. 2b) in treated pears were significantly ($P < 0.05$) higher than those in control pears at 3 or 4 measurement points. In the treatment, activity of POD could reach 73.9% higher than that in the control on the 8th day, the activity of PPO in treated pears was higher than that in control pears at most of the measurement points and the activity of PPO in treated pear could reach 34.2% higher than that in control pear on the 6th day. The activity of defense enzyme of CHT (Fig. 2c) in treated pear also showed higher than that in control pear on the 4th, 6th and 8th day.

Induction of resistance to pathogen infection has been indicated as a promising approach for controlling postharvest diseases of fruit (Porat et al., 2003; Liu et al., 2005). It was reported that disease resistance in plants could be induced by some EOs (Li et al., 2012; Itako et al., 2013). Our data show that the disease resistance

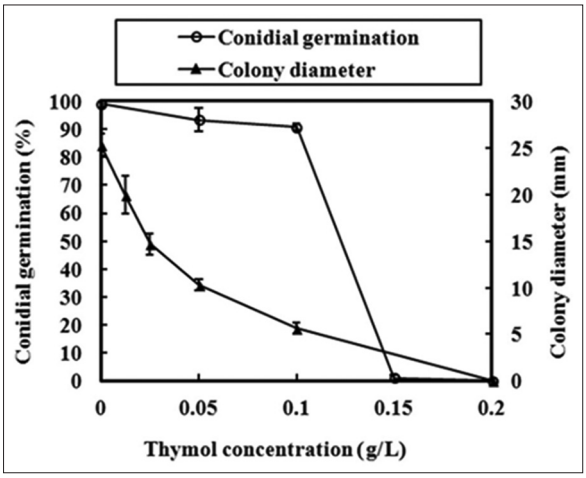

Fig 1. Antifungal ability of thymol against *P. expansum in vitro*. Conidial suspension of *P. expansum* was cultured at 27 ± 2°C in PDB (measuring conidial germination) or in PDA (measuring colony diameter) containing different concentrations of thymol for 20h (measuring conidial germination) or 72 h (measuring colony diameter). Bars represent standard errors for the means of three replications.

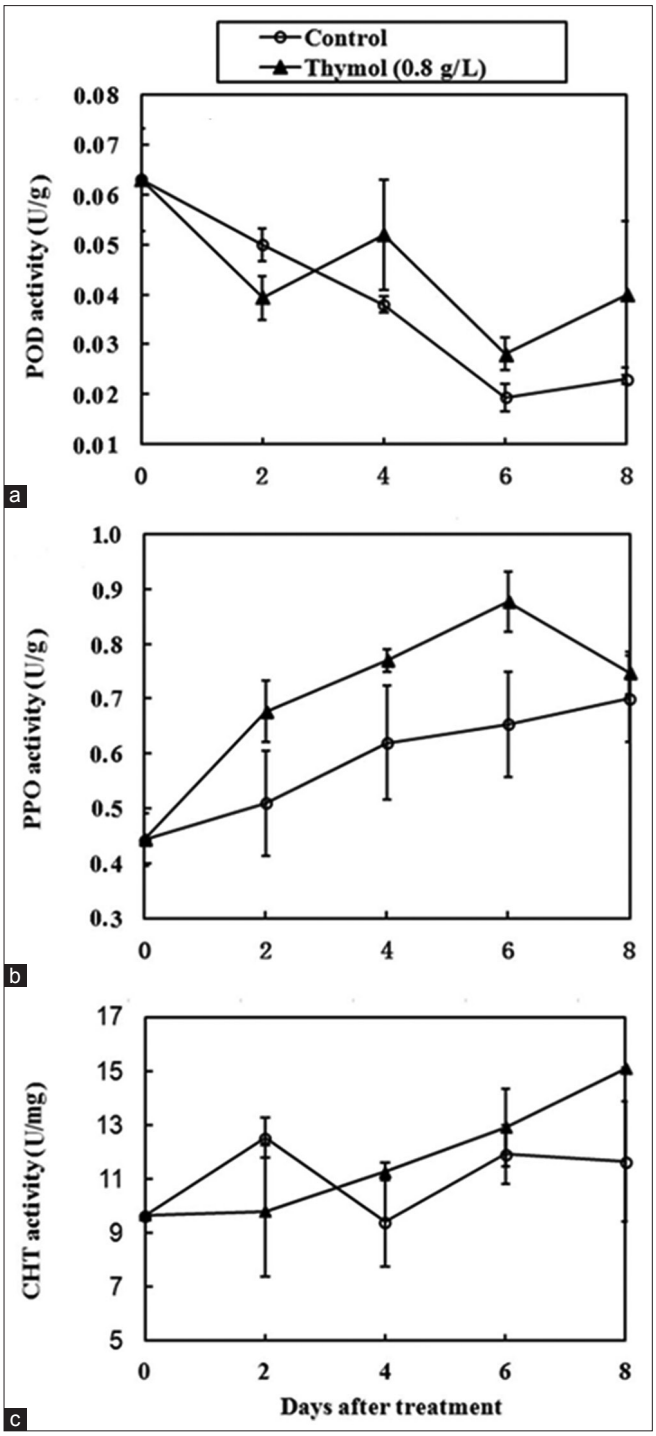

Fig 2. Effects of thymol treatment on the activity of POD, PPO and CHT. The coated pears were stored at 25 ± 2°C, 95 - 100% RH. Bars represent standard errors for the means of three replications.

of pear against *P. expansum* could be induced by thymol. In our most work, we focused on using essential oils or components of essential oils to improve antifungal ability of edible film and studying why EOs or components of EOs could improve antifungal ability of edible film and help fruit to resist pathogens. Thus we did the experiment of evaluating resistance-related enzymes induced by thymol *in vivo*. Nevertheless, in this paper, it's better if we concerned the complex coatings in.

Effect of thymol on antifungal ability of chitosan against *P. expansum* in pears

The disease development in pears inoculated with *P. expansum* was reduced by chitosan coating, but the antifungal ability of chitosan coating against *P. expansum* could be further improved by thymol. The average lesion diameter on pears coated complex coating containing 1.6 g/L of thymol was only 9.7 mm, which was 46.6% lower than that on pears coated with only chitosan after inoculation for 144 h (Fig. 3a). Until the end of the experiment, The average lesion diameter on pears coated with complex coating containing 0.8 g/L or 1.6 g/L of thymol was significantly ($P < 0.05$) less than coated with chitosan only.

As shown in Fig. 3b, the disease incidence in pears inoculated with *P. expansum* was significantly ($P < 0.05$) reduced by chitosan coating or complex coating at 72 h. As time went on, the disease incidence of pears gradually reached 100% if only chitosan coating was used, but the disease incidence was reduced significantly ($P < 0.05$) by adding thymol (final concentration: 0.8 or 1.6 g/L) into the coating. The disease incidence of pears treated with chitosan containing 1.6 g/L of thymol only reached 32.7% after inoculation for 144 h, when the disease incidence of pears treated with only chitosan reached 100%.

Many studies showed the potential application of chitosan in fruit storage, also some studies tried to use EOs to improve the antimicrobial ability of chitosan coating. Because any of EOs usually contains various components, those studies seldom demonstrated the antifungal mechanism of each component of Eos in fruit. This study proved that thymol (one native compound from some EOs) could improve the antifungal ability of chitosan against *P. expansum* effectively in pear. Thymol not only inhibited *P. expansum* in vitro, but also acted on resistance-related enzymes of POD, PPO and CHT in pear.

Weight loss, firmness and TSS of coated pears

The weight loss, firmness and TSS of pears were assessed during the storage. The reasons that cause weight loss of harvested fruit and vegetables mainly include transpiration and respiration. It was reported that after coating with chitosan on the surface of fruit and vegetables, more water can be reserved (Zhong and Xia 2007; Gao et al., 2013). In our experiment, chitosan coating (whether added with thymol or not) also retarded the weight loss of pears during the experiment, but the weight loss did not vary significantly ($P > 0.05$) between the control and the treatments during the experiment (Fig. 4a), the reason perhaps came from that the concentration of chitosan we used was somewhat low.

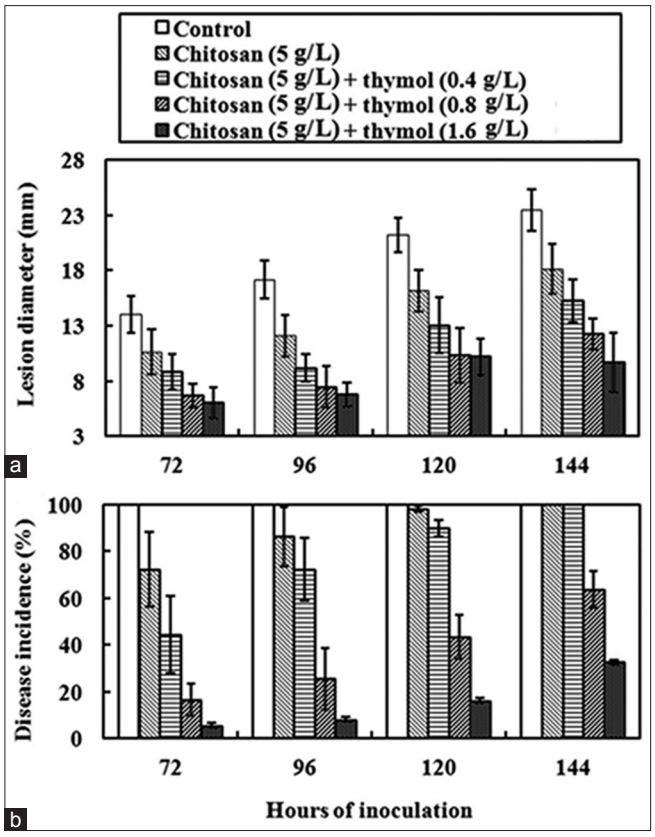

Fig 3. Effect of thymol on antifungual ability of chitosan coating to *P. expansum* in pear. The coated pears were incubated at 25 ± 2°C, 95 - 100% RH. Bars represent standard errors for the means of three replications.

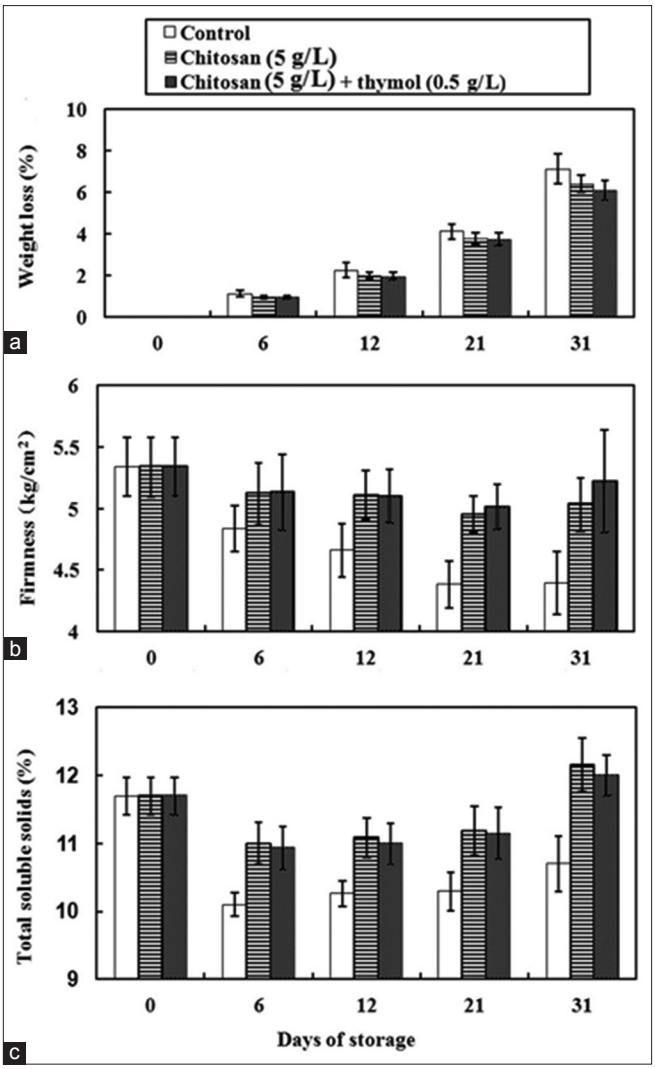

Fig 4. Effects of the complex coating on weight loss, firmness and total soluble solids. The coated pears were stored at 25°C, 95 - 100% RH. Bars represent standard errors for the means of three replications.

The decreases of firmness in pears were slowed down by chitosan coating or complex coating. As shown in Fig. 4b, the firmness of treated pears was significantly ($P < 0.05$) higher than that of control samples on the 12th, 21th and 31th day during storage, the firmness of pears treated with chitosan coating or complex coating was 14.6% or 18.8% higher than that of control samples after 31 days of storage, respectively.

As shown in Fig. 4c, the concentration of TSS in pear coated with chitosan or complex (chitosan and thymol) was higher than that in control pear at most of measurement points and kept varying little during the experiment. However, the concentration of TSS in control pear exhibited significantly ($P < 0.05$) differences among the day of experiment beginning, 6th and 31st during storage.

The results were in agreement with those previous studies which showed that chitosan has positive effects on fruit quality during storage (Qi et al., 2011; Velickova et al., 2013; Xiao et al., 2011) and previous studies also confirmed that some EOs can prevent fruit softening and decreasing weight losses (Xiao et al., 2011; Shirzadeh and Kazemi 2012). In our experiment, some data (such as the data of weight loss and firmness on the 21th and 31th day) also showed that when thymol was added into chitosan coating, the complex coating had better ability than only chitosan coating on maintaining pear quality. Though the positive effects of complex coating (by adding thymol into) did not reach significant difference level ($P > 0.05$) from those of chitosan coating (without adding thymol into) on maintaining pear quality in this experiment, those data were at least able to demonstrate that the positive effects of chitosan coating on weight loss, firmness and TSS in pears during storage should not be decreased when thymol was added into.

In order to prove the latent capacity of thymol on improving antifungal ability of chitosan, we used thymol of 0.4 g/L, 0.8 g/L and 1.6 g/L in the disease evaluation *in vivo*, but our previous studies showed that 0.4 g/L ~ 0.8 g/L thymol pipetted into wounded pears could reach the approximately

inhibitive effect as 0.2 g/L thymol tested *in vitro* on *P. expansum*, so we selected 0.8 g/L thymol for evaluation of resistance-related enzymes induced and 0.5 g/L thymol in complex coating for measurement of quality changing of coated pears. Nevertheless, in this section, perhaps selecting uniform concentration (both are 0.8 g/L or 0.5 g/L) should be better than selecting 0.8 g/L and 0.5 g/L respectively.

CONCLUSIONS

Thymol, the native compound from some EOs, can improve antifungal ability of chitosan coating against *P. expansum*. It not only inhibited conidial germination and mycelial growth of *P. expansum*, but also acted on resistance-related enzymes of POD, PPO and CHT in pears. Thymol is one of viable compounds from EOs to enhance antifungal effect of eatable coatings used in fruit storage.

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Authors contributions

J. Wang was involved in conducting experiments, sample analysis and manuscript preparation, B. Yang was involved in literature collection, S. Zhang involved in revising the language of this manuscript, J. Cao was involved in designing the study, W. Jiang was the project director and guided the overall experimental.

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