Postharvest Biology and Technology 99 (2015) 37–43

Contents lists available at ScienceDirect

Postharvest Biology and Technology

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

Effect of postharvest application of chitosan combined with clove oil against citrus green mold

Xingfeng Shao [a,*], Baoying Cao [b], Feng Xu [a], Shuhui Xie [a], Dandan Yu [a], Hongfei Wang [a,*]

[a] Department of Food Science and Engineering, Ningbo University, Ningbo 315211, Zhejiang, PR China
[b] Information School, Ningbo City College of Vocational Technology, Ningbo 315000, Zhejiang, PR China

ARTICLE INFO

Article history:
Received 14 April 2014
Accepted 24 July 2014
Available online 24 August 2014

Keywords:
Chitosan
Clove oil
Satsuma mandarin
P. digitatum
Green mold

ABSTRACT

The antifungal activity of chitosan combined with clove oil against *Penicillium digitatum*, the causal agent of citrus green mold, was tested *in vitro* and *in vivo*. Chitosan combined with clove oil inhibited mycelial growth more than individual treatments, which was related to the greater release of cellular material and the largest alterations in hyphal morphology of *P. digitatum*. However, compared to chitosan alone, 1% chitosan coatings combined with various amounts of clove oil (0.5, 1 or 2 mL/L) showed no greater ability in controlling decay development on artificially inoculated citrus fruit. 1% chitosan combined with 0.5 mL/L clove oil appeared to slightly reduce lesion diameter and enhanced the activities of defense enzymes, including chitinase and phenylalanine ammonia-lyase at the later stages of incubation. This study indicated that the synergistic antifungal activity of chitosan-clove oil observed in *in vitro* studies was not found in *in vivo* tests. Therefore, the data suggest that a coating of 1% chitosan alone, not combined with clove oil, can effectively contribute to the control of green mold on citrus fruit.

1. Introduction

Green mold, caused by *Penicillium digitatum*, is a disease that inflicts major postharvest damage on citrus fruit. Traditionally, synthetic fungicides, such as imazalil, are used to control this disease (Smilanick et al., 1997). However, due to the increasing awareness of potentially harmful chemical compounds on human health and the environment, current research aims at developing alternative strategies for reducing the use of chemical additives in the food industry. In this context, environmentally friendly plant extract agents, such as essential oils, have shown great potential as alternatives to synthetic fungicides in disease control and quality maintenance in tomatoes (Soylu et al., 2010), blueberries (Mehra et al., 2013), strawberries (Shao et al., 2013a), avocadoes (Sellamuthu et al., 2013) and citrus (Fan et al., 2014).

Clove oil, extracted from the dried flower buds of the clove *Eugenia caryophyllata* L. Merr. & Perry (Myrtaceae), is widely used and well known for its antimicrobial, antioxidant, antifungal and antiviral activity (Chaieb et al., 2007), and has agricultural and food protection applications (Bakkali et al., 2008). The main compounds of clove oil are phenylpropanoids such as eugenol (76.8%), β-caryophyllene (17.4%), α-humulene (2.1%), and eugenyl acetate (1.2%) (Chaieb et al., 2007). Clove oil has been proven to be very effective against *P. digitatum* (Hall and Fernandez, 2004; Yahyazadeh et al., 2008), *Penicillium italicum* (Anjum and Akhtar, 2012) and *Botrytis cinerea* (Vesaltalab et al., 2012). Eugenol, the principal chemical component of clove oil, is regarded as the most effective component against various pathogens (Chaieb et al., 2007).

Although essential oils prove to be good antifungal agents, their use for maintaining fruit quality and reducing decay development is often limited because of their application costs and various other disadvantages (*i.e.*, high volatility, strong flavor, and potential toxicity) (Bakkali et al., 2008). The incorporation of essential oils into an edible coating is an effective method for solving some of these problems, as well as in controlling fruit fungal disease by lowering the diffusion processes and maintaining high concentrations of active molecules on the surface of the fruit (Aloui et al., 2014). In this sense, some researchers have reported the effects of essential oils combined with hydroxypropylmethylcellulose, locust bean gum, *Aloe vera* gel, wax or chitosan to control the decay development and extend storage life of different fruit (Sánchez-González et al., 2011; Perdones et al., 2012; Aloui et al., 2014; Fan et al., 2014; Paladines et al., 2014).

Among these polysaccharides, chitosan is the most interesting edible coating combined with essential oils. It is non-toxic, high biodegradable, and has strong antimicrobial and antifungal

* Corresponding authors. Tel.: +86 574 87609573; fax: +86 574 87608347.
E-mail addresses: shaoxingfeng@nbu.edu.cn (X. Shao), wanghongfei@nbu.edu.cn (H. Wang).

http://dx.doi.org/10.1016/j.postharvbio.2014.07.014
0925-5214/

activities that may effectively control various causes of fruit decay (Kong et al., 2010; Zhang et al., 2011). Chitosan–lemon oil coatings show higher antifungal ability than individual treatment both in *in vitro* tests and in inoculated strawberries (Perdones et al., 2012). Xing et al. (2011) reported that a chitosan–cinnamon oil coating might be a promising candidate to maintain the quality of sweet peppers. Tea tree oil containing chitosan exhibited the highest effective control on the growth of *P. italicum*, but did not lead to any relevant changes in the quality of orange fruit (Cháfer et al., 2012). To the best of our knowledge, no data are available regarding chitosan coatings with clove oil and their role against *P. digitatum* in *in vitro* tests and in citrus fruit.

Control of postharvest diseases by chitosan or essential oil treatments seems to occur through two different mechanisms: a direct germicide effect on pathogens and an indirect effect by inducing defense mechanisms in fruit tissue (Liu et al., 2007; Zhang et al., 2011; Shao et al., 2013a). As an exogenous elicitor, chitosan can induce resistance in the host by increasing the activities of several defense-related enzymes, such as phenylalanine ammonia-lyase (PAL), chitinase (CHI) and β-1,3-glucanase (Zhang et al., 2011). Shao et al. (2013a) reported that tea tree oil can induce the disease defense ability of strawberry through higher activities of PAL and β-1,3-glucanase. There is no information about the effect of chitosan-essential oils on the disease resistance ability of fruit during postharvest handling and storage.

The objectives of this study were to (1) investigate the antifungal ability of chitosan combined with clove oil on mycelial growth, membrane permeability and ultrastructure of *P. digitatum*, (2) study the effects of chitosan coating with clove oil on green mold development and defense-related enzymes in artificially inoculated citrus fruit.

2. Materials and methods

2.1. Raw materials and pathogen

Mature satsuma mandarin (*Citrus unshiu* Marc. cv. Miyagawa wase) fruit were harvested from a commercial orchard in Ninghai, Zhejiang province, China. All fruit were uniform in size and color and free of physical injury or signs of infection.

A highly virulent *P. digitatum* strain was isolated from spoiled satsuma mandarin fruit and the strain identified and lodged in a fungal culture collection as described in our previous study (Xei et al., 2013). The strain was grown for 7 days on potato dextrose agar (PDA; 1 L of an infusion from potatoes containing 20 g/L glucose and 15 g/L agar) at 25 °C prior to experimentation. Spores from a 7-day-old culture were suspended in 0.05% Tween 20 and adjusted to 10^5 conidia/mL by using a hemacytometer.

Clove oil was purchased from Chengdu Kediya Reagent Co. Ltd. (Chengdu, China). Chitosan, with a deacetylation degree of 95% (CAS Number 9012-76-4) was purchased from Aladdin Industrial Corporation, Shanghai, China.

2.2. Assessment of the effects of chitosan combined with clove oil on mycelial growth in vitro by agar dilution method

According to our previous results (Xei et al., 2013), the minimum inhibitory concentration (MIC) value of chitosan and clove oil against *P. digitatum* is 0.25% (w/v) and 6 mL/L (v/v) in *in vitro* test, respectively. A 2.5 g aliquot of chitosan was dispersed in 100 mL of sterile water with 1% (w/w) glacial acetic acid, creating a mother solution of chitosan (2.5%, w/v). Chitosan mother solution, clove oil, or a combination was added to the sterile PDA medium at a temperature of 40–45 °C to reach a final concentration of 0 (Control), chitosan 1/8 MIC + clove oil 1/8 MIC, chitosan 1/4 MIC + clove oil 1/4 MIC. Then, these amended PDA media were immediately added into the glass Petri dishes (90 mm in diameter).

P. digitatum was inoculated immediately by plating in the center of each plate with a 5 mm diameter disc of the fungus, cut with a sterile cork borer from the edge of actively growing cultures on PDA plates. The Petri dishes were incubated at 25 °C for 5 days. The efficacy of treatment was determined by measuring the average of two perpendicular diameters of each colony. The percentage inhibition of the radial growth of the fungi by treatment, compared with the control, was calculated at day 5. Percentage mycelial inhibition = $[(dc - dt)/dc] \times 100$, where dc is the mean colony diameter for the control sets and dt is the mean colony diameter for the treatment sets. Five Petri dishes were used per replication, and all tests were repeated five times.

2.3. Effects of chitosan combined with clove oil on membrane permeability and ultrastructure of P. digitatum

P. digitatum was inoculated into potato dextrose broth (PDB) medium and incubated at 25 °C and 140 rpm for 2 days. Chitosan and/or clove oil were then added to the medium to reach a final concentration of 2 MIC. The suspensions were incubated at 25 °C and 140 rpm for 32 h. Samples from the suspensions were collected at 4 h intervals during incubation. The suspensions collected at different times were centrifuged at 4000 rpm for 10 min to obtain the supernatant and collect fungal cells. All experiments were repeated three times.

To find the effect of chitosan-clove oil on membrane permeability, the absorbance at 260 nm (A260) of the supernatants were determined according to the methods of Shao et al. (2013b), which reveals the release of cellular material by a UV/Vis spectrophotometer (UV-2000; UNICO Instrument Co., Ltd., Shanghai, China).

In order to observe the ultrastructure of *P. digitatum* by transmission electron microscopy (TEM), the fungal cells were collected by centrifugation at 4000 rpm for 10 min and washed twice in sterile saline at the end of incubation for 36 h. Mycelia were fixed with 2.5% glutaraldehyde for 2 h and then 1% osmium tetraoxide for 1 h, at room temperature. They were washed three times with 0.1 mol/L phosphate buffer (pH = 7.2), each time for 15 min. After fixation, the samples were dehydrated in a graded ethanol series (30, 50, 75, 85 and 100%) for a period of 15 min in each series. Finally, they were dipped into epoxy propane for 15 min. The specimens were then passed through the solution of epoxy propane and epoxy resin (1:1) for 1 h each and embedded in epoxy media. Using a diamond knife, blocks were sectioned into ultrathin sections of about 70 nm. The ultrathin sections were contrasted with uranyl acetate followed by lead citrate for 30 min each and examined on a TEM (Model JEM-1230; Hitachi). Five samples from each repeated treatment were examined by TEM.

2.4. Effects of chitosan combined with clove oil on green mold development in artificially inoculated citrus fruit

For the antifungal ability *in vivo* assays in 2011, fresh fruit were surface-sterilized by dipping in 2% sodium hypochlorite solution (v/v) for 2 min, and then washed with distilled water. Thereafter, fruit were wounded (3 mm deep and 3 mm wide) on opposing sides with a sterile needle, and inoculated with 20 μL of a spore suspension of *P. digitatum* (10^5 spores mL^{-1}), and left to air-dry. After inoculation, the fruit were randomly distributed into eight equal groups and dipped into the chitosan (1%), clove oil (0.5, 1 or 2 mL/L), chitosan-clove oil (1% chitosan combined with 0.5, 1.0 or 2.0 mL/L clove oil) and control (distilled water) for 2 min, and left to air-dry again. Then, these inoculated and treated fruit were kept at 5 °C to observe the decay development. Five fruit constituted a