Postharvest Biology and Technology 99 (2015) 93–98

Contents lists available at ScienceDirect

Postharvest Biology and Technology

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

Control of brown rot on jujube and peach fruits by trisodium phosphate

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ARTICLE INFO

Article history:
Received 26 March 2014
Accepted 1 August 2014
Available online 16 September 2014

Keywords:
Trisodium phosphate
Jujube fruit
Peach fruit
Monilinia fructicola
Alkaline pH
Membrane integrity

ABSTRACT

Trisodium phosphate (TSP) has been shown to be effective for control of food-borne bacteria, but little is known about its activity against phytopathogenic fungi that cause plant diseases. Here we show that TSP application reduced disease development of brown rot caused by *Monilinia fructicola* on jujube and peach fruits. The efficiency of TSP was positively correlated with its concentrations. Analysis of the influence of pH on efficiency of TSP indicated that the inhibitory effect of TSP against *M. fructicola* was only partially influenced by its alkaline pH. TSP directly inhibited spore germination, germ tube elongation, and mycelial growth of *M. fructicola* in the culture medium. To further investigate the mechanisms by which TSP inhibited fungal growth, we detected the integrity of the plasma membrane of *M. fructicola*. Our result show that TSP treatment resulted in the loss of plasma membrane integrity, leading to the release of intracellular contents such as soluble proteins, carbohydrates, and nucleic acids. Taken together, our data suggest that TSP was effective for controlling postharvest disease caused by *M. fructicola* on jujube and peach fruits and this antifungal activity was directly related to the disruption of cell membrane of the fungal pathogen.

1. Introduction

Postharvest diseases caused by various microbial pathogens result in the major losses in fruit and have been controlled mainly by synthetic fungicides. However, some fungicides are dangerous to human health and cause environmental pollution. Development of alternative methods has become urgent in recent years due to the increasing regulatory restrictions on the use of fungicides (Droby et al., 2009; Romanazzi et al., 2012). Promising approaches including biological control agents (Droby et al., 2009; Li et al., 2011; Liu et al., 2011, 2013; Hershkovitz et al., 2011; Ren et al., 2012; Sui et al., 2012), natural antimicrobials such as chitosan and plant extracts (Nigro et al., 2006; Martínez-Romero et al., 2007; Romanazzi et al., 2009; Qin et al., 2010), and substances generally regarded as safe (GRAS) (Lurie et al., 2006; Chervin et al., 2009; Guentzel et al., 2010) has been tested for their effectiveness to control of postharvest diseases in various fruits.

A great many of chemicals classified as GRAS have been applied to extend postharvest storage of fruit such as silicate (Qin and Tian, 2005), calcium (Ciccarese et al., 2013), carbonate and bicarbonate (Palou et al., 2002; Youssef et al., 2014), electrolyzed oxidizing water (Guentzel et al., 2010) and ethanol (Lurie et al., 2006; Chervin et al., 2009). Trisodium phosphate (TSP) is a GRAS substance defined by the US Food and Drug Administration (USDA-FSIS, 2012). As an inorganic compound, TSP is highly soluble in water producing an alkaline solution. A number of studies have shown that TSP is effective in controlling the growth of food-borne bacterial pathogens including *Salmonella* species, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Capita et al., 2003; del Rio et al., 2006, 2007). Most of these studies were carried out on meat products for bacterial decontamination (Dorsa et al., 1997). Recently, Su and D'Souza (2012) reported that TSP was effective in reducing *Salmonella* Typhimurium on plant produce such as lettuce and peppers. However, information about the effect of TSP against postharvest diseases of fruit is not available. Moreover, although the antimicrobial effects of TSP against bacterial pathogens have

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http://dx.doi.org/10.1016/j.postharvbio.2014.08.003
0925-5214/

been extensively investigated, little is known about the effects of TSP against fungal pathogens.

The objective of this study was to evaluate the effectiveness of TSP in controlling postharvest disease caused by fungal pathogen, *Monilinia fructicola*, on jujube and peach fruits, which contain particular nutritional qualities but are susceptible to fungal pathogens. We found that TSP treatment could reduce brown rot and inhibit the growth of *M. fructicola*. The modes of action of TSP on *M. fructicola* were investigated by analyzing the effect of TSP on the loss of membrane integrity and the leakage of cellular constituents in the fungal pathogen.

2. Materials and methods

2.1. Fruit

Jujube (*Zizyphus jujuba* cv. Dongzao) and peach (*Prunus persica* cv. Okubao) fruits were harvested from an orchard in Beijing at commercial maturity and immediately transported to the Institute of Botany, the Chinese Academy of Sciences. The firmness of jujube fruit was 48.6 N as determined by a penetrometer (FT-327; UC Fruit Firmness Tester, Milano, Italy), with total soluble solids of 18.6%. The firmness and total soluble solids of peach fruit were 55.8 N and 9.8%, respectively. Fruit without physical injuries or infections were disinfected with 2% (v/v) sodium hypochlorite for 2 min, washed with tap water, and air-dried prior to use.

2.2. Pathogen

M. fructicola was isolated from infected peach fruit and maintained on potato dextrose agar (PDA). Spores were obtained from the surface of the agar after culture for one week at 25 °C. The spores were suspended in 5 mL of sterile distilled water containing 0.05% (v/v) Tween 80. To remove any adhering mycelia, the spore suspensions were filtered through four layers of sterile cheesecloth. The concentration of the spore suspensions was determined using a hemacytometer.

2.3. Effect of TSP on brown rot on jujube and peach fruits

Jujube and peach fruits of were wounded (3 mm deep × 3 mm wide) with a sterile nail at the equator before inoculation. Then 10 μL of a spore suspension of *M. fructicola* at 1×10^5 spores/ml was added to the wounded sites. After the fruit were air-dried, 15 μL of TSP solution at different concentrations (0.5, 1, 1.5, and 2%, w/v) was added to the same wounded sites. Water-treated fruit were used as the control. Fruit were placed in plastic boxes (200 mm × 130 mm × 50 mm) with high humidity (about 95%) and stored at room temperature (25 °C). Decay incidence was measured daily after treatment. Three replicates were applied to each treatment (20 fruit per replicate) and the entire experiment was repeated twice.

To analyze the influence of pH value on the ability of TSP for control of *M. fructicola*, jujube and peach fruits were wounded and inoculated with the pathogen as described above. Fruit were then treated with 15 μL of TSP solution at 1% (w/v) with normal pH (about pH 12.0) or with pH 7.0 adjusted with HCl. Preliminary experiments showed that HCl at neutral pH had no effect against *M. fructicola* in both jujube and peach fruits (data not shown). Fruit treated with NaOH solution at pH 12.0 and deionized water were used as controls. Fruit were stored at 25 °C and disease incidence was measured after symptoms occurred. Each treatment was replicated three times with 20 fruit per replicate and the entire experiment was repeated twice.

2.4. Determination of spore germination and germ tube elongation

Inhibitory effects of TSP on spore germination and germ tube elongation of *M. fructicola* was examined microscopically as previously reported (Qin et al., 2003). Potato dextrose broth (PDB) media supplemented with TSP at different concentrations were inoculated with a spore suspension of *M. fructicola* to obtain a final concentration of 5×10^5 spores/mL. The inoculated PDB media were added to wells of a 24-well microtitration plate and cultured at 25 °C on a rotary shaker at 100 rpm. Spore germination was measured when the germ tube was equal to or greater than the diameter of the spore. Germination rate were expressed as the percentage of germinated spores out of the total number of evaluated spores. Germ tube length was detected with an ocular micrometer. About 200 spores were measured and the experiment was repeated twice with three replicates for each treatment.

2.5. Assay for mycelial growth

The effect of TSP on mycelial growth of *M. fructicola* was measured according to the method of Droby et al. (2003). In brief, a 5-mm diameter plug of mycelial agar obtained from the growing edge of 14-day-old cultures was placed in the center of a 9-cm-diameter Petri dish containing PDA medium with different concentrations of TSP solutions. Before being added to the PDA medium, the TSP solutions were sterilized by filtering through a 0.45 μm Millipore filter. Radial growth of *M. fructicola* was detected after incubated at 25 °C for 3, 5, 7 and 9 days. Mycelial growth was expressed as the diameter of the fungal colony (mycelium) in the Petri dish minus the diameter of the agar plugs (5 mm). Three replicates were applied to each treatment and the entire experiment was repeated twice.

2.6. Determination of plasma membrane integrity

For plasma membrane integrity observation, spores of *M. fructicola* were cultured in PDB medium containing TSP at 0 or 0.25% (w/v). After incubation for 2 h at room temperature (25 °C), spores were collected and stained for 5 min at 30 °C with 10 μg/mL propidium iodide (PI), which is commonly used for detecting membrane integrity of cells (Fish et al., 2000). The spores that had been stained were centrifuged and washed twice with phosphate buffered saline (PBS, pH 7.4) to remove residual dye (Li and Tian, 2006). A Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany) was used for fluorescence observation. Images were collected through an Axiocam MRc digital camera (Carl Zeiss). Three fields of view were chosen randomly from each cover slip, and the experiment was repeated twice.

2.7. Determination of cellular leakage

The leakage of cytoplasmic contents from the mycelia was detected following the method of Lewis and Papavizas (1987) with some modifications. *M. fructicola* was cultured in PDB medium on a rotary shaker at 100 rpm for 3 days at 25 °C. Then the mycelia were harvested and washed with sterile distilled water. The washed mycelia were resuspended in 100 mL of sterile distilled water containing TSP at 0, 0.03, 0.06, 0.12 and 0.25% (w/v), and incubated on a rotary shaker for 1, 2, 3, and 4 h. The mycelia were filtered and the filtrated solutions were used for the determination of leakage of soluble proteins, carbohydrates and nucleic acids. The Bradford assay (Bradford, 1976) was performed to quantify release of proteins. Soluble carbohydrate was detected with anthrone reaction, which uses glucose as the standard (Morris, 1948). The absorbance at 260 nm was applied to calculate the concentration of nucleic acids.