Effects of yeast antagonists in combination with hot water treatment on postharvest diseases of tomato fruit

Yuanyuan Zong, Jia Liu, Boqiang Li, Guozheng Qin, Shiping Tian

Abstract

Hot water treatment (HWT) and two yeast antagonists, Candida guilliermondii and Pichia membranaefaciens were investigated separately and together for controlling Botrytis cinerea, and natural infection in tomato fruit stored at 20 °C. Applied separately, both HWT and antagonists inhibited decay caused by B. cinerea, and natural infection. The combination of antagonists and HWT showed better control efficacy. Application of HWT did not affect the growth of C. guilliermondii and P. membranaefaciens in tomato wounds, while HWT induced significant increase in the activities of phenylalanine ammonia-lyase (PAL), chitinase (CHI) and β-1,3-glucanase in fruit. The mechanism by which HWT enhanced the biocontrol efficacy of the antagonistic yeasts may be related to the elicitation of biochemical defense responses in tomato fruit. The combination of antagonistic yeasts and HWT could be a promising method for the control of postharvest diseases of tomato fruit.

1. Introduction

Postharvest pathogens, including Botrytis cinerea Pers.:Fr., cause major losses in tomato production (Badawy and Rabea, 2009). Although the use of synthetic fungicide is still the primary means for controlling postharvest diseases, the development of resistance to many fungicides by pathogens (Holmes and Eckert, 1999) and public concern over the potential impact of fungicides on human health and the environment (Spadaro and Gullino, 2004; Droby et al., 2009), have created interest in alternative methods of disease control.

Biological control using microbial antagonists has shown great potential for control of postharvest disease of fruit and vegetables (Tian, 2006; Sharma et al., 2009). Among these antagonists, yeasts have been pursued actively, as production of toxic secondary metabolites is not involved in their activities against pathogens (Qin et al., 2006) and considerable information is available with respect to techniques for their genetic manipulation, production and storage (Reeveder, 2004). Candida guilliermondii (Castellani) Langeron & Guerra and Pichia membranaefaciens Hansen, as two antagonistic yeast strains, have been studied for biological control of postharvest fungal pathogens, such as B. cinerea, Penicillium expansum Link, Rhizopus stolonifer (Ehrenb.:Fr.) Vuill, Monilinia fructicola (Wint.) Honey and Colletotrichum acutatum J.H. Simmonds (Zahavi et al., 2000; Scherm et al., 2003; Qin et al., 2004; Xu et al., 2008; Cao et al., 2009).

Hot water treatment (HWT) is also used as one of the most promising non-pesticide technologies for postharvest control of decay (Fallik, 2004). Hot water rinsing and brushing were shown to enhance resistance against B. cinerea in fresh harvested tomato fruit (Fallik et al., 2002). Nafussi et al. (2001) observed that a hot-water dip for 2 min at 52–53 °C prevented decay for at least 1 week in lemon fruit inoculated with Penicillium digitatum Sacc., and Dimitris et al. (2005) found that hot water brushing at 60 °C for 30 s or 65 °C for 20 s significantly reduced the decay incidence of natural infection in cactus pear. The resistance mechanisms elicited in hot water treated fruit included production of lignin-like materials (Nafussi et al., 2001), stabilization of membranes and induction of pathogenesis-related proteins (Schierr, 2000).

Although HWT and biological control have been shown to be effective in reducing postharvest decay of fruit, each exhibits limitations that can affect its commercial applicability (Zhang et al., 2007). The preferred alternative to chemical treatments in the future is likely to be a combination of different methods (Teixidó et al., 2001). Therefore, the overall objective of the present study was to evaluate the effects of HWT and two yeast antagonists C. guilliermondii and P. membranaefaciens, used separately or in combination, on controlling postharvest diseases of tomato fruit. The effects of HWT on the population dynamics of the two biocontrol agents, and on the induction of phenylalanine-ammonia-lyase (PAL), chitinase (CHI) and β-1,3-glucanase in fruit were also determined.
2. Materials and methods

2.1. Fruit

Tomato fruit (*Lycopersicon esculentum* Mill. cv. Fenhong) were harvested at the mature green stage, and sorted based on size and the absence of physical injuries or disease infection. The color of the fruit was green, and the average quality values of brix, acidity, and firmness were 5.85 °Bx, 2.1 mmol/100 g fruit weight and 120 N/cm², respectively. Before treatments, fruit were surfaced-disinfected with 2% sodium hypochlorite for 3 min, then rinsed with tap water, and air-dried according to Liu et al. (2007).

2.2. Pathogen inoculum

*B. cinerea* was isolated from infected tomato fruit and maintained on potato dextrose agar (PDA) (Oxoid, UK) at 4 °C. The pathogen was inoculated into apple fruit and re-isolated onto PDA before the experiment. Spore suspension was obtained from 2-week-old cultures. The number of spores was calculated with a hemocytometer, and intact fruit were inoculated by dipping the antagonist treatment as described above, then air-dried; (4) fruit did not receive any hot water or yeast treatment and served as the control.

After 24 h, fruit in the four groups were inoculated with 5 µL of a conidial suspension of *B. cinerea* at 5 × 10^4 spores mL⁻¹. Treated fruit were stored at 20 °C for 4 days, and disease incidence and lesion diameter of tomato fruit caused by *B. cinerea* were determined. Each treatment contained three replicates with 10 fruit per replicate and the experiment was repeated twice.

2.3. Yeast antagonists

*Candida guilliermondii* (CGMCC 2.63) was obtained from the Institute of Microbiology, the Chinese Academy of Sciences, Beijing. *Pichia membranaefaciens* was isolated in our previous experiment (Fan and Tian, 2000) and identified by CABI Bioscience Identification Services (International Mycological Institute, UK). Fifty-milliliter of nutrient yeast dextrose broth (NYDB: 8 g of nutrient broth (Oxoid, UK), 5 g of yeast extract (Oxoid, UK) and 10 g of dextrose (Beihua Fine Chemicals Co., Ltd., China) in 1000 mL water) was prepared in 250-mL conical flasks, inoculated with *C. guilliermondii* or *P. membranaefaciens* to an initial concentration of 10^5 cells mL⁻¹, and incubated for 48 h at 25 °C on a shaker at 200 rpm.

2.4. Efficacy of hot water treatment on control of gray mold development in tomato fruit

As it is known, improper heat treatment would damage the tissue of the fruit. Therefore, in the present study, 42 °C within 1 h was chosen according to the previous study (McDonald et al., 1999) and our preliminary trials, which guaranteed no occurrence of heat injury on tomato fruit.

Fruit were randomly grouped into four lots. Three lots of fruit were subjected to hot water immersion treatment at 42 °C for 20, 40 or 60 min. The bath temperature was constantly monitored by thermometers and maintained less than 1 °C below or above the established value during each treatment. After the treatment, the fruit were forced-air cooled immediately to 20 °C, and then air-dried. The fourth lot of non-heat treated fruit at 20 °C served as control, for preliminary study showed that there was no significant difference in disease resistance between non-heat treated fruit and those immersed in room temperature tap water (about 20 °C) within 60 min (data not shown). After 24 h, two wounds (3 mm deep and 3 mm wide) were made with a sterile nail on the equator of each fruit. A 5-µL conidial suspension of *B. cinerea* (5 × 10^5 spores mL⁻¹) was inoculated to each wound. Treated fruit were stored at 20 °C for 4 days, and disease incidence and lesion diameter of tomato fruit caused by *B. cinerea* were determined. Each treatment contained three replicates with 10 fruit per replicate and the experiment was repeated twice.

2.5. Effects of yeasts in combination with hot water treatment on gray mold development in tomato fruit

Tomato fruit were divided into four groups: (1) fruit were immersed in a circulating water bath at 42 °C for 40 min, then forced-air cooled immediately to 20 °C, and air-dried; (2) fruit did not receive HWT, aliquots (5 µL) of washed cell suspension of *C. guilliermondii* or *P. membranaefaciens* (5 × 10^7 cells mL⁻¹) were pipetted into wounds; (3) fruit first received HWT as described in Group 1, then after 20 min of forced-air cooling to 20 °C, they were treated with yeasts as described in Group 2; (4) fruit did not receive any hot water or yeast treatment, and served as the control.

After 24 h, fruit in the four groups were inoculated with 5 µL of a conidial suspension of *B. cinerea* at 5 × 10^4 spores mL⁻¹. Treated fruit were stored at 20 °C for 4 days, and disease incidence and lesion diameter of tomato fruit caused by *B. cinerea* were determined. Each treatment contained three replicates with 10 fruit per replicate and the experiment was repeated twice.

2.6. Effects of yeasts in combination with hot water treatment on natural infection of intact fruit

The effects of HWT with or without *C. guilliermondii* or *P. membranaefaciens* on development of natural decay were evaluated according to Zhang et al. (2008), with some modification. Intact fruit was divided into four groups: (1) fruit were immersed in a circulating water bath at 42 °C for 40 min, then forced-air cooled immediately to 20 °C, and air-dried; (2) the suspensions of washed cells of *C. guilliermondii* or *P. membranaefaciens* were adjusted to concentrations of 5 × 10^5 cells mL⁻¹ with sterile distilled water by a hemocytometer, and intact fruit were inoculated by dipping them in the suspension of washed cells for 30 s, and air-dried; (3) fruit first received the HWT as described above, then after 20 min of forced-air cooling to 20 °C, the fruit continued to receive the antagonist treatment as described above, then air-dried; (4) fruit did not receive any of the two treatments and served as the control. Treated fruit were stored at 20 °C for 20 days, and the percentage of infected fruit was recorded. Each treatment contained three replicates with 10 fruit per replicate and the experiment was repeated twice.

2.7. Determination of population dynamics of the antagonists in fruit wounds

Tomato fruit were immersed in a circulating water bath at 42 °C for 40 min, and forced-air cooled immediately to 20 °C. Fruit without HWT served as control. Then, fruit were treated with aliquots (5 µL) of washed cell suspension of *C. guilliermondii* or *P. membranaefaciens* at 5 × 10^7 cell mL⁻¹. Samples were prepared at different time points after treatment according to the previous study (Fan and Tian, 2001). The yeasts were recovered by removing 10 wound tissues with a cork borer (1 cm diameter × 1 cm deep), ground with a mortar and pestle in 10 mL sterile distilled water. Then 50 µL of serial 10-fold dilutions were spread on NYDA plates. Samples taken at 1 h after treatment for population measurement served as time 0. Fruits stored at 20 °C were assessed every 1 day for 5 days. Colonies were counted after incubation at 20 °C for 5 days and expressed as the Log_{10} CFU per wound. There were three replicates in each treatment, and the experiment was repeated twice.

2.8. Assay of defense-related enzyme activities

Tomato fruit were immersed in a circulating water bath at 42 °C for 40 min, and forced-air cooled immediately to 20 °C. Fruit with-
out HWT served as control. For the enzyme assay, fruit samples were obtained from 10 fruits containing the pericarp and flesh at 0, 1, 2, 3, 4 and 5 days after treatment (DAT). Each treatment contained three replicates and the experiment was repeated twice.

PAL was extracted by the method of Zhao et al. (2008), with some modification. Tissue sample (10 g) was mixed with 4 mL of ice-cold sodium borate buffer (100 mM, pH 8.7) and ground thoroughly at 4 °C. The homogenate was centrifuged at 17,000g for 30 min at 4 °C, and the resulting supernatant was collected for the enzyme assay. PAL activity was analyzed using the method of Assis et al. (2001), with some modification. One milliliter of enzyme extract was incubated with 2 mL of borate buffer and 0.5 mL of L-phenylalanine (20 mM) for 60 min at 37 °C. The reaction was stopped with 0.1 mL 6 mol L⁻¹ HCl. PAL activity was determined by the production of cinnamate, measured by the absorbance change at 290 nm. The blank was the crude enzyme preparation mixed with L-phenylalanine with zero time incubation. The specific enzyme activity was defined as nmol cinnamic acid h⁻¹ mg⁻¹ of protein.

For chitinase (CHI) and β-1,3-glucanase, enzymes were extracted according to Yao and Tian (2005). Tissue samples (10 g) with 0.3 g polyvinyl polypyrrolidone (PVPP) were ground with 30 mL sodium acetate buffer (50 mmol L⁻¹, pH 5.0) at 4 °C. The homogenate was centrifuged at 17,000g for 30 min at 4 °C, and the resulting supernatant was collected for the enzyme assay. CHI activity was determined using the method of Wirth and Wolf (1990), with slight modification. CHI activity was measured by mixing 1 mL of crude enzyme solution with 2 mL of 2% dye-labeled carboxymethyl chitin in 50 mmol L⁻¹ sodium acetate buffer (pH 5.0). After 1 h of incubation at 37 °C, the reaction was stopped by adding 1 mL of 1 mol L⁻¹ HCl, the reaction mixture was cooled and centrifuged. The absorbance of the supernatant was measured at 550 nm. The specific enzyme activity was expressed as μmol product h⁻¹ mg⁻¹ protein.

β-1,3-Glucanase activity was assayed by measuring the amount of reducing sugar released from the substrate by the dinitrosalicylate method (Ippolito et al., 2000), with some modification. A total volume of 250 μL of enzyme preparation was incubated with 250 μL of 0.5% laminarin (w/v) for 1 h at 37 °C. Two hundred microliters of sterile distilled water was added to 50 μL of the reaction mixture. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The reaction was stopped by adding 250 μL of 3,5-dinitrosalicylate and boiling for 5 min in a water bath. The solution was diluted with 4 mL of distilled water and the amount of reducing sugars was measured at 500 nm. The specific activity of β-1,3-glucanase was expressed as the formation of 1 μmol glucose equivalents h⁻¹ mg⁻¹ protein.

Protein content was determined according to Bradford (1976) with bovine serum albumin (Sigma Chemicals Co., St. Louis, USA) as standard.

2.9. Statistical analysis

The results from three independent experiments were recorded, and data from one representative experiment are presented in this paper. All statistical analyses were performed with SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Data from assays of population dynamics and enzyme activities were compared in a Student’s t-test. Others were analyzed by one-way ANOVA. Mean separations were performed by Duncan’s multiple range tests. Differences at P < 0.05 were considered significant.

3. Results

3.1. Efficacy of hot water treatment on control of gray mold in tomato fruit

Disease incidence of gray mold in hot water treated fruit was significantly lower than that of the control (P < 0.05), except for the fruit treated for the longest time (60 min) (Fig. 1A). Moreover, HWT at all treatment time significantly reduced lesion diameter (P < 0.05) (Fig. 1B). The best inhibition of this disease was achieved when the fruit were treated with hot water at 42 °C for 40 min. Therefore, HWT for 40 min was chosen for further study to evaluate the effect on disease control of the combination with antagonistic yeasts, the effect on population dynamics of the yeasts and on induction of defense-related enzyme activities of fruit.

3.2. Effects of yeasts in combination with hot water treatment on control of gray mold in tomato fruit

Disease incidence and lesion diameter in all treated fruit were significantly lower than those of the control fruit (P < 0.05) (Fig. 2), HWT (40 min), C. guilliermondii and P. membranaefaciens, as stand-alone treatments, reduced the disease incidence from 81.7% (control) to 61.7%, 40.0% and 45.0%, respectively. The combination of the two yeasts and HWT exhibited a synergistic effect, which decreased the disease incidence to a lower level, 21.7% for C. guilliermondii and 26.7% for P. membranaefaciens, respectively.

---

**Fig. 1.** Efficacy of hot water treatment for control of gray mold caused by B. cinerei in tomato fruit. All hot water treatments were at 42 °C. Disease incidence (A) and lesion diameter (B) were determined 4 days after inoculation at 20 °C. Bars represented standard deviations of the means. Values followed by different letters are significantly different according to Duncan’s multiple range test at P < 0.05.
Values followed by different letters are significantly different according to Duncan’s multiple range test at P < 0.05.

3.3. Effects of yeasts in combination with hot water treatment on natural infection of intact fruit

The pathogens causing natural infection on tomatoes using in our experiment were mainly B. cinerea, Alternaria arborescens and R. stolonifer, with B. cinerea being predominant. Applications of HWT (40 min), C. guilliermondii and P. membranaeefaciens resulted in decay incidence of natural infection on fruit after storage at 20 °C for 20 days at 33.3%, 38.3% and 45.0%, compared with 58.3% in the control fruit (Fig. 3). In fruit treated with the combination of HWT and C. guilliermondii or P. membranaeefaciens, the percentages of decayed fruit were 21.7% and 23.3%, resulting the lowest ones among the treatments tested.

3.4. Effects of hot water treatment on population dynamics of the antagonists in fruit wounds

There were no significant differences between population dynamics of C. guilliermondii and P. membranaeefaciens in wounds of control and heat treated fruit at 20 °C (P > 0.05) (Fig. 4). Both antagonistic yeasts multiplied rapidly in tomato fruit wounds, and C. guilliermondii grew a little more rapidly than P. membranaeefaciens. After 1 day, the number of cells increased over 10 times for both yeasts, while the number of cells became gradually stable after 3 days.

3.5. Effects of hot water treatment on induction of defense enzymes

HWT induced the activities of PAL, CHI and β-1,3-glucanase in tomato fruit stored at 20 °C (Fig. 5). The activity of PAL in control fruit increased at 1 DAT, then declined and maintained a relatively low level during storage (Fig. 5A). The tendency of the enzyme change both in hot water treated fruit and the control fruit were similar. PAL activity in hot water treated fruit also reached its highest level at 1 DAT, and the level was almost 1.5 times higher than control fruit at the same time. Throughout the whole storage period, hot water treated fruit showed significantly higher enzyme activity than the control (P < 0.05).

The change of CHI activity was shown in Fig. 5B. Both hot water treated fruit and the control demonstrated similar change trend. With prolonged storage period, the hot water treated fruit showed continuously higher activity than the control. Especially at 1 and 2 DAT, the differences were more obvious. The peak value of CHI activity appeared at 3 DAT in both hot water treated fruit and the control. As shown in Fig. 5C, β-1,3-glucanase activity could be stimulated by HWT. β-1,3-Glucanase activity reached its peak value at 2 DAT, and then decreased rapidly. Comparatively, the peak value in the control was detected at 3 DAT. It suggested that HWT induced the appearance of the peak value of β-1,3-glucanase activity 1 day before the control. Furthermore, significantly higher β-1,3-glucanase activity was observed in hot water treated fruit as compared with the control during the first 3 days of storage (P < 0.05).

4. Discussion

Hot water treatment was reported to be effective in controlling postharvest diseases in fruit (Schirra et al., 2000; Fallik, 2004). In the present study, it was found that 20- and 40-min HWT significantly reduced disease incidence and lesion diameter of tomato fruit caused by B. cinerea, and the 60-min treatment significantly reduced just the lesion diameter (Fig. 1). This indicated that the time of HWT correlated closely with control efficiency. A similar result was also obtained by Zhang et al. (2008), who reported that within 20 min of treatment, HWT at 46 °C for 15 min showed better efficiency on P. expansum in pear fruit than those for 5, 10 or 20 min.
Due to the best efficacy of 40-min HWT, it was chosen to control postharvest diseases of tomato fruit combined with antagonistic yeasts. When the HWT was combined with C. guilliermondii or P. membranaefaciens for control of gray mold in artificially inoculated fruit, the efficacy was higher than either HWT or antagonistic yeast treatment alone (Fig. 2). Similar results were obtained in the experiment with natural infection of intact fruit, the combination of antagonistic yeasts and HWT also showed the best control efficacy (Fig. 3). This suggested that HWT could enhance the biocontrol efficacy of antagonistic yeasts on tomato fruit. Such results confirmed other findings that the combination of Metschnikowia pulcherrima (Pitt.) M.W. Miller and hot water treatment was more effective to control P. expansum and B. cinerea on apple fruit than individual treatment (Spadaro et al., 2004), and the biocontrol efficacy of Cryptococcus laurentii (Kufferath) Skinner against R. stolonifer and natural infection on strawberries was enhanced by hot water immersion (Zhang et al., 2007). The synergistic effect may be a result of several different interactions taking place among HWT, the yeast and the fruit.

Population density of antagonistic yeasts plays a major role in competing for nutrients and space, which is an important mechanism of biological control (Janisiewicz and Korsten, 2002). The results of our study suggested that C. guilliermondii and P. membranaefaciens multiplied quickly in wounds of tomato fruit stored at 20°C. This indicated that the two yeasts were well-adapted to the wound environment. Moreover, HWT did not significantly influence the population of C. guilliermondii or P. membranaefaciens. Each of them showed almost the same population dynamics in wounds of control and hot water treated fruit (Fig. 4).

One of the possible action mechanisms of HWT is induction of antifungal-like substances that inhibit fungal development in fruit tissue and defense-related enzymes (Schirra et al., 2000). PAL, CHI and β-1,3-glucanase have been suggested to be the crucial enzymes against fungal infection (Yao and Tian, 2005; Zhao et al., 2008). PAL is the first enzyme of phenylpropanoid pathway and involved in the biosynthesis of phenolics, phytoalexins and lignins (Qin et al., 2003). Chitin, as an essential component of the cell wall of many fungal pathogens, can be degraded by CHI. Additionally, β-1,3-glucanase is one of the most fully characterized pathogenesis-related (PR) proteins, and it can act directly by degrading cell walls of pathogens or indirectly by releasing oligosaccharide and eliciting defense reactions (Rose et al., 2002). Both of these processes are potential defense mechanism against fungal infection (Tian et al., 2007). The results of the present study showed the markedly higher activities of PAL, CHI and β-1,3-glucanase in hot water treated fruit compared to control (Fig. 5).

**Fig. 4.** Effects of hot water treatment (42°C, 40 min) on population dynamics of C. guilliermondii (A) or P. membranaefaciens (B) in wounds of tomato fruit stored at 20°C. Bars represented standard deviations of the means.

**Fig. 5.** Changes of PAL (A), CHI (B) and β-1,3-glucanase (C) in control and hot water treated fruit stored at 20°C. Bars represented standard deviations of the means.
water treated tomato fruit (Fig. 5), which may correlate with the lower infection incidence. Similar results were obtained by Benitez et al. (2006), who reported that hot water dipping treatment at 55 °C for 5 min reduced resistance in mango fruit at mature green stage against Colletotrichum gloeosporioides (Penz) Penz & Sac. by enhancing activities of PAL and β-1,3-glucanase. Moreover, Pavoncello et al. (2001) observed that hot water brushing treatment at 62 °C for 20 s promoted the accumulation of CHI and β-1,3-glucanase proteins of grapefruit, resulting in an increase in resistance against P. digitatum.

In conclusion, the combination of HWT and C. guilliermondii or P. membranaefaciens is more effective to control postharvest diseases of tomato fruit than the single treatments. It suggests that the combined strategy of biological and physical control may partially substitute the utilization of synthetic fungicides in tomato fruit. However, future research on development of the technology at large scale is needed.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 30671473) and by the Knowledge Innovation Program of the Chinese Academy of Sciences (Grant No. 69714G1001).

References


