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Yeast antagonist and microwave treatment control blue mold rots of harvested jujube fruits

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Abstract

In this study, the functions of antagonistic yeast alone or in combination with microwave treatment in controlling blue mold rot of jujube fruit were investigated. Meanwhile, their effects on the quality of the harvested fruits were also detected. Therefore, *in vitro* and *vivo* experiments were both adopted for the study. For *in vitro* test, the growth of *Penicillium citrinum* was almost completely inhibited (98%) by 2 and 3 min 2450 MHz microwave treatments, respectively. In addition, the population density of the pathogenic bacteria in the surface wounds of fruit was also significantly lower than that of the control *in vivo* test. The results showed that the microwave treatment reduced disease incidence and lesion diameter of infected wounds from 100% to 36.0% and 1.92 cm to 1.38 cm, respectively. Similarly, the *Metschnikowia pulcherrima* were capable of reducing incidence from 100% to 45.0% and lesion diameter from 1.92 cm to 1.50 cm, respectively. The disease incidence and lesion diameter were only 21.0% and 1.00 cm, respectively, when treated with the combination of microwave and antagonist yeast. Meanwhile, the incidence of the natural decay on treated fruits was similar to that of the inoculated fruits. Therefore, the results also demonstrated that all the treatments did not influence the quality parameters of Jujube fruit. In short, our research suggested that yeast antagonist in combination with microwave treatment could be an alternative to synthetic fungicides for controlling the postharvest blue mold rot of jujube fruits.

Keywords: blue mold rot; Jujube; microwave treatment; *M. pulcherrima*; quality parameters.
Abbreviations: NYDB_nutrient-yeast-dextrose broth; PDA_potato dextrose agar; TSS_Total soluble solids.

Introduction

As a traditional food with abundant nutrients and good flavor, Jujube (*Zizyphus jujuba* cv. Dongzao) fruit has increasing popularity (Li et al., 2007; Zhang and Li 2014). However, acting as an important food stuff and medicine, Jujube fruit is susceptible to postharvest diseases caused by various pathogenic fungi (Cao et al., 2013). *P. citrinum* is one of the most important postharvest infection pathogens and causes serious postharvest losses annually (Pérez et al., 2011). Meanwhile, Jujube fruit can also be infected by other pathogens such as *Alternaria alternana* (Fr.:Fr.) Keissl., *Monilinia fructicola* (G.Wint) Honey, *Penicillium expansum* Link and *Rhizopus stolonifer* (Ehrenb.:Fr.) Vill. The pathogenic bacteria always invade through wounds that occur during harvest or packing processes (Horwitz 2000; Asghari and Aghdam 2010; Cao et al., 2013; Guo et al., 2014). Although the postharvest blue mold rot of jujube fruit could be inhibited by synthetic fungicides, the application of fungicides is increasingly limited (Droby et al., 2009; Wu et al., 2010). One reason is the development of the fungicide resistance from the pathogens and the other reason is the public concern regarding the potential harmful effects of fungicide residues on the environment and human health (Wu et al., 2010). With the development of the innovative research techniques, biocontrol agents, which are attracting more and more attentions can serve as important alternatives to synthetic fungicides (Xu et al., 2008; Sharma et al., 2009). Recently, some isolated bacteria and yeast from fruit surfaces have already been demonstrated to be efficient in the postharvest biocontrol (Sharma et al., 2009; Zong et al., 2010). Among all these microbial agents, researches have specially focused on yeasts (Roberts 1994; Porat et al., 2002; Lima et al., 2007). That is due to that the production of toxic secondary metabolites is not involved in their activities against pathogens and more importantly there is considerable available information about the techniques for their genetic manipulation, production and storage (Reeleder 2004; Csutak et al., 2007; Csutak et al., 2013). Studies using yeasts as biocontrol agents are aiming to isolate and test the antimicrobial and antifungal properties of various species belonging to *Candida*, *Debaryomyces*, *Metschnikowia*, *Pichia* and *Rhodotorula* genera (Sharma et al., 2009). The yeast antagonist *M. pulcherrima* has been demonstrated to be effective against postharvest diseases of several fruits (Csutak et al., 2013).

Microwave heating has been widely used in food processing for drying, pasteurization, sterilization, thawing, tempering and baking for several decades (Gupta and Leong 2008; Chandrasekaran et al., 2013). More importantly, microwave heating has gained popularity in food processing due to its ability to achieve high heating rates, significant reduction in cooking time, more uniform heating, safe handling, ease of operation and low maintenance (Zhang et

al., 2006; Salazar-González et al., 2012). Recently, the use of microwave power for the control of postharvest diseases of fruits and vegetables has been developed (Palou 2009; Janisiewicz and Conway 2010; Sisquella et al., 2013).
So far, there are several non-fungicidal methods which have already entered the commercial stage to control several diseases in different crops, including the use of yeast antagonists and microwave treatment (Wilson et al., 1993; Smilanick et al., 1999; Tian 2000; Teixidó et al., 2001; Wisniewski et al., 2007). However, to be commercially successful, any product/technology to be used in the postharvest phase has to be able to control diseases at a rate of more than 95%. Therefore, the use of these methods as an alternative of the synthetic fungicides is an integrated strategy and also the use of these methods is supposed to be able to take full advantages of the additive or synergistic effects of different treatments in order to improve the efficacy of a single one.

The primary objective of this study was to evaluate the effectiveness of a yeast antagonist in the control of blue mold rot in jujubes as a single treatment or in combination with microwave treatment. Furthermore, the effects of these treatments on the quality of jujubes after storage such as firmness, total soluble solids, ascorbic acid and titratable acidity were investigated.

Results

The effect of microwave on pathogen in vitro

The effect of microwave treatment on the viability of *P. citrinum* spores was tested *in vitro*. Spore germination of *P. citrinum* was completely inhibited by 2450 MHz microwave treatment for 2 or 3 min, whereas that of *P. citrinum* was only partly inhibited by the treatment for 1 min (Fig. 1).

Effect of microwave treatment on viability of P. citrinum in surface wounds

The results shown in Fig. 2 indicated that the population density of *P. citrinum* in surface wounds of microwave treatment fruits were significantly lower than that of the control ($P \leq 0.05$). However, the population density of *P. citrinum* in surface wounds of jujube fruit treated with microwave was no significant difference between 2 min and 3 min.

Effects of microwave treatment and yeast antagonist on decay development in inoculated fruits

The results presented in Fig. 3 indicated that in fruit inoculated with *P. citrinum*, the disease incidence and lesion diameter in all treated jujube fruits were significantly lower than those of the control fruits ($P \leq 0.05$). Microwave treatment and yeast antagonist, as stand-alone treatments, were capable of reducing the disease incidence of rotten fruits from 100% to 45% and 36%, and lesion diameter from 1.92 cm to 1.50 cm and 1.38 cm, respectively. However, in fruits treated with the combination of microwave treatment and antagonist yeast, the disease incidence of rotten jujube fruits and lesion diameter was only 21% and 1.00 cm, respectively.

Effects of microwave treatment and yeast antagonist on natural infections and postharvest quality of jujube fruit

Our experiments evaluated the efficacy of microwave and yeast antagonist, as stand-alone treatments or in combination, in reducing the natural development of decay after storage at 2±1 ºC for 45 days and at 22 ºC for 7 days. The results presented in Table 1 indicated that the application of microwave power and *M. pulcherrima* resulted in low average decay incidence on jujube fruit after storage at 2±1 ºC for 45 days and 22 ºC for 7 days ranging from 11.6% to 12.3%, compared with 28.6% in the water-treated control jujube fruit. The combination of microwave and *M. pulcherrima* was the most effective treatment and the percentage of decayed fruits was only 6.2.

Microwave treatment alone or in combination with yeast antagonist had no significant effect on jujube fruit firmness, titratable acidity or TSS contents compared to the control fruits, after storage at 2±1 ºC for 45 days followed by 22 ºC for 7 days (Table 1). The Ascorbic acid contents of jujube fruits treated with microwave, antagonist and both of them were also similar to those of the control jujube fruits.

Discussion

Microwave treatments with a 2450 MHz household oven (between 0.4 and 0.45 kW) have been previously studied to control postharvest diseases such as *B. cinerea*, *P. expansum* and *R. stolonifer* in peaches (Karabulut and Baykal 2002; Zhang et al., 2004). Although microwave heating has been demonstrated to control different diseases in stone fruit, no information is available about the use of microwave heating to control *P. citrinum* in jujube fruit.

The effects of the microwave treatment on P. citrinum

The results of the study have demonstrated that microwave treatment has the potential to control *P. citrinum* in jujube fruits. This is in agreement with the results obtained by Zhang et al. (Zhang et al., 2006), who found that microwave power could control the postharvest blue mold rot of pear. Under *in vitro* conditions, the growth of *P. citrinum* was completely inhibited by a 2450 MHz microwave heating for 2 or 3 min. Moreover, in *P. citrinum*, the survival rate in surface wounds and the population densities were significantly lower after 2 or 3 min of microwave treatment than control fruit ($p \leq 0.05$). In the studies designed to determine the effects of microwave treatment and yeast antagonist on the decay development in artificially inoculated and wounded fruits or naturally infected un-wounded fruits, the percentage of infected wounds or fruits treated by microwave treatment were significantly lower than that of the control.

The effects of the microwave treatment combined with M. pulcherrima on P. citrinum

The results also showed that the several combined alternative control methods may be used to control postharvest blue mold rot of jujube fruits. Our experiments revealed that the effectiveness of microwave treatment combined with *M. pulcherrima* in controlling artificially inoculated *P. citrinum* as well as natural infections on intact fruit was higher than that of either microwave power treatment or *M. pulcherrima* treatment. Control of pathogens after inoculation is important because most infections occur through wounds inflicted during or just after harvest (Roberts 1994; Teixidó et al., 2001). The microbial antagonists have a poor ability to eradicate pre-existing infections, while microwave treatment can compensate for this weakness to control the recently

Table 1. Effects of microwave treatment and yeast antagonist on natural infections and postharvest quality of unwounded fruit.

Treatment	Disease incidence (%)	Firmness (N)	Ascorbic acid (mg/100g)	TSS (%)	Titratable acidity (% malic acid)
Control	28.6±3.15a	15.38±1.60a	249.45±1.52ab	21.60±0.52a	0.43±0.01a
Microwave	12.3±2.12b	15.60±0.52a	253.83±2.02b	21.82±0.62a	0.44±0.15a
Antagonist	11.6±1.23b	14.94±1.32a	251.59±1.30a	22.62±0.51a	0.43±0.05a
MW+AT	6.2±1.12c	15.35±1.12a	254.87±0.58b	22.58±0.64a	0.42±0.20a

Disease incidence was recorded after 45 days storage at 2±1℃ and additional 7days storage at 22℃. Means are averaged values of three trials ± the standard error. Values followed by the same letter are not significantly different at p≤ 0.05 according to analysis by Duncan's multiple range test.

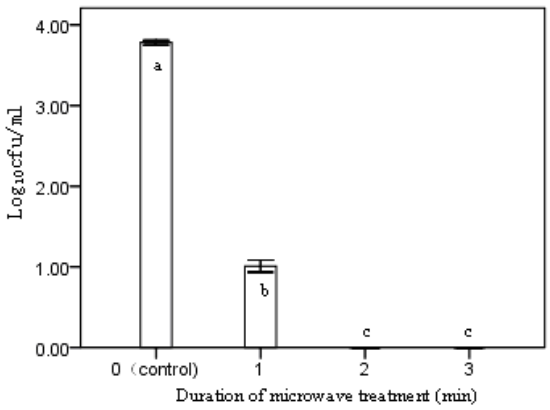

Fig 1. *In vitro* effect of microwave treatment on the survival of *P. citrinum* spores. Bars represent the standard errors of the means. Means with the different letters are significantly different according to Duncan's multiple range test at p ≤0.05.

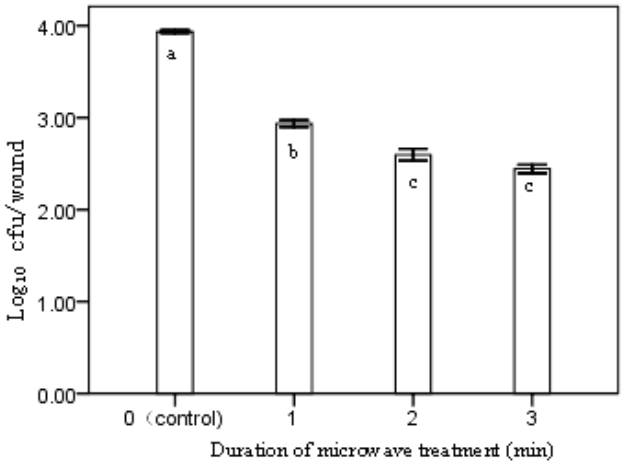

Fig 2. Effect of microwave treatment on the survival of *P. citrinum* spores *in vivo*. Bars represent the standard errors of the means. The population densities were significantly lower than that of control. However, there was no significant difference between 2 min and 3 min treatments.

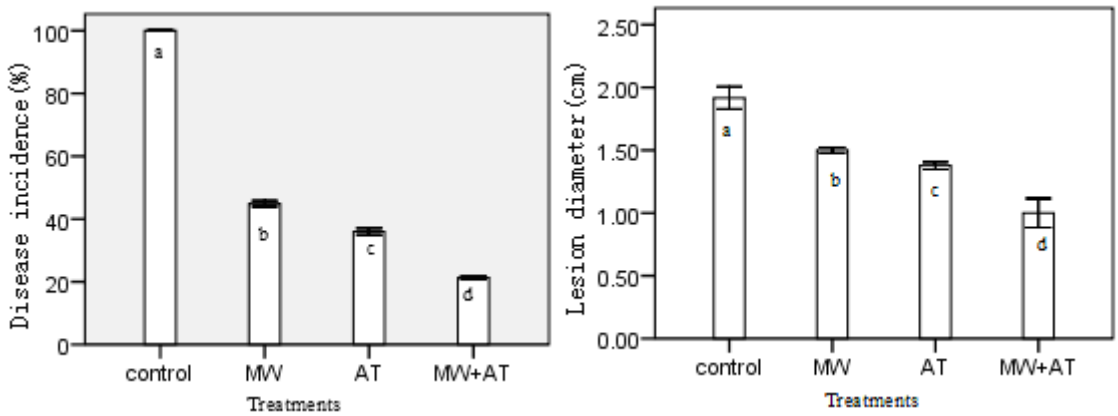

Fig 3. Effects of microwave treatment and yeast antagonist on decay development in artificially inoculated and wounded fruits. *MW*, microwave treatment; *AT*, antagonist yeast treatments; *MW + AT*, the combination of microwave and antagonist yeast treatment. Bars represent the standard errors of the means. Means with the different letters are significantly different according to Duncan's multiple range test at p≤ 0.05.

established (within 24 h) infections. Similarly, microwave treatment does not exhibit persistent protection of the fruits from re-infection, while the protection of microbial antagonists can persist for long time. Therefore, we suggest that their combined application can protect the fruits from re-infection effectively (Smilanick et al., 1999).

Clearly, many more studies are needed to screen out the best strategy to utilize these combinations (UV-C, hot water, temperature, method of application, etc.) in the future (Zhang et al., 2006; Janisiewicz and Conway 2010; Zong et al., 2010).

Materials and Methods

Fruit material, fungal cultures and microwave oven

'Dongzao' Jujube fruits were harvested at commercial maturity stage (with red surface and be going to soften) from Alar of Xinjiang province, China and sorted based on the size and the absence of physical injuries or infections. Fruits were either used immediately or stored at 2±1 ºC until use. Before treatment, fruits were disinfected with 2% (v/v) sodium hypochlorite for 2 min, washed in tap water and dried in air.

The P. citrinum was obtained from infected 'Dongzao' jujube fruits and cultured on potato dextrose agar (PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g; agar powder, 20 g and distilled water, 800 ml) at 4 ºC. Spore suspensions were prepared by flooding a petri plate containing a 2-week-old sporulating culture with sterile distilled water. The spore concentration of *P. citrinum* was determined with the aid of a haemocytometer and adjusted to 1×10^5 spores/ml with sterile distilled water.

The yeast antagonist *M. pulcherrima* was isolated from the surface of 'Dongzao' jujube fruit according to the method of Wilson and Chalutz (Wilson and Chalutz 1989). *M. pulcherrima* was identified by Sangon Biotech Co., Ltd (Shanghai). Yeast cells were cultured in nutrient-yeast-dextrose broth (NYDB: 8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose in 1000 ml of distilled water) for 24 h at 28 ºC. Following incubation, the cells were centrifuged at 5,000×g for 10 min and washed twice with sterile distilled water in order to remove the growth medium. The concentration of the yeast was determined with a haemocytometer and adjusted to 1×10^8 cells/ml.

A domestic (kitchen type) 2,450 MHz microwave oven (Galanz, China) was used for the experiments, providing 0.45 KW microwave power. The uniformity of the microwaves in the cavity was enhanced by a turntable.

In vitro tests

Sterile screw-capped glass tubes containing 1.8 ml sterile distilled water were prepared in advance. 0.2 ml of concentrated *P. citrinum* spore suspension was added to the tubes to achieve a final concentration of 1×10^5 spores/ml. The glass tubes containing spore suspension were then placed in the microwave oven and heated for 1, 2 or 3 min, respectively and the conidial suspension without treatment was used as a control. After treatment, tubes were kept in the laboratory for 25 min to allow the heat to redistribute and to equilibrate with room temperature. Aliquots of 100 µl of each suspension were plated onto PDA agar plates. After 72 h incubation at 25 ºC, the colonies were counted and the results were represented as the mean number of colony-forming units (cfu)/ml (Zhang et al., 2006).

Survival of P. citrinum in surface wounds

The effect of microwave treatment on the survival of *P. citrinum* was determined in wounds on 'Dongzao' jujube fruits which were wounded with two wounds of each jujube fruit. The wounds were approximately 3 mm in diameter and 3-4 mm in depth. The wounds were allowed to dry for 2 h and then 25 µl of spore suspension (1×10^5 spores/ml) was pipetted into each wound. The wound-inoculated fruits were divided into two groups: one were placed in the microwave oven and heated for 1, 2 or 3 min. The other did not receive the microwave treatment and was regarded as the control. Tissue samples borer, individually homogenized in 50 ml of sterile water, vortexed, serially diluted and plated in triplicate on PDA medium. The plates were incubated at 25 ºC for 48h. Colonies were then counted and results were expressed as $\log_{10}$ cfu/wound. There were three replicates for each treatment and the experiment was repeated three times.

Effects of microwave treatment and yeast antagonist on decay development in artificially inoculated and wounded fruit

In this experiment, jujube fruits were wounded (3 mm deep×3 mm wide) with a sterile nail at the equator of each fruit. Each wound was added with 20µl of spore suspension of *P. citrinum* (1×10^5 spores/ml). Inoculated fruit were left to air dry for 2 h. All treated jujube fruits were then divided into four groups: (1) Jujube fruits were placed in the microwave oven and heated for 2 min. After treatment, fruits were kept in the laboratory for 30 min to allow the heat to redistribute and to equilibrate with room temperature. (2) Aliquots (25 µl) of fresh (shake culture) cell suspension of *M. pulcherrima* (1×10^8 cfu/ml) were pipetted onto wounds. (3) Jujube fruits were first received microwave treatment for 2 min, and then aliquots (25 µl) of fresh cell suspension of *M. pulcherrima* (1×10^8 cfu/ml) were pipetted onto wounds after 30 min of heat redistribution. (4) Jujube fruits did not receive any of these two treatments serving as the control. All treated jujube fruits were stored at 25 ºC for 5d and the percentage of infected wounds and lesion diameters were recorded afterwards. There were three replicate trials of 400 jujube fruits per treatment with complete randomization. The experiment was repeated three times.

Effects of microwave treatment and yeast antagonist on natural infections and postharvest quality of jujube fruit

To evaluate the effect of microwave treatment with or without the yeast antagonist on development of natural decay, intact jujube fruits were divided into four groups. In the first group, fruits were placed in the microwave oven and heated for 2 min. Then, the treated fruits were kept in the laboratory for 30 min to allow the heat to redistribute and to equilibrate with room temperature. In the second group, jujube fruits were dipped into cell suspension of *M. pulcherrima* (1×10^8 cfu/ml) for 1 min at room temperature and then air dried. In the third group, jujube fruits were first received microwave treatment as described above. After 30 min of heat redistribution, jujube fruits were then received antagonistic yeast treatment as described above. In the fourth group, jujube fruits were dipped into tap water for 1 min at room temperature and air dried and served as control. Treated jujube fruits were stored at 2±1 ºC for 45 days followed by 22 ºC for 7 days in order to determine disease development under shelf-life conditions.

The percentage of infected fruits was recorded afterwards. There were three replicate trials of 400 fruits per treatment with complete randomization. The experiment was repeated three times.

To evaluate the effect of microwave power, alone or in combination with antagonist yeast on postharvest quality of jujube fruits, the harvested fruits were treated and stored under the storage conditions as described above. Quality parameters were measured after storage. Quality measurements were made on three replicates of 400 fruits each, and performed at ambient temperature (about 22 ºC).

The testing methods

Fruit firmness: Firmness values of each individual jujube was measured at three points of the equatorial region by using the TA-XT2i Texture Analyser (Microstable Instruments, UK) with a 5 mm diameter flat probe. The probe descended toward the sample at 5.0 mm s^{-1} and the maximum force recorded (N) was defined as firmness. The firmness of each jujube was measured three times on different sides (Zhang et al., 2007).

Total soluble solids: Total soluble solids (TSS) were determined by measuring the refractive index of the same juice with a hand refractometer (WYT-II; Qingyang Optical Instrument Co., Ltd., Chendu, China) and the results were expressed as percentages (g per 100 g fruit weight) (Palou et al., 2001).

Ascorbic acid: The 2, 6-dichloroindophenol titrimetric method (Horwitz 2000) was used to determine the ascorbic acid content of the pressed fruit juice. Results were expressed as milligrams of ascorbic acid per 100 g sample.

Titratable acidity: Acidity was measured by titration with 0.1 M NaOH to pH 8.1. 4 g juice diluted with 20 ml distilled water was evaluated for each replicate. Titratable acidity was calculated as percent malic acid (Wright and Kader 1997).

Statistical analyses

All treatments were arranged in a randomized complete block design and were conducted at least three times. The data were from one individual experiment and representative of three independent experiments with similar results. The data were analyzed by one-way analysis of variance (ANOVA) in the statistical program SPSS version 17.0. When the analysis was statistically significant, Duncan's multiple range test was applied to separate means. Differences among parallel tests at $P \leq 0.05$ were considered as significant.

Conclusion

The combination of microwave treatment with the biological control agent overcomes the significant limitations of one of these treatments alone. Besides, this treatment did not impair the quality parameters of jujube fruit. The combination among the antagonists and microwave treatments could be a reliable solution to control postharvest diseases in jujube fruit. Therefore, a combined strategy of biological and physical measures may partially substitute synthetic fungicides.

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