Combination of the biocontrol yeast *Cryptococcus laurentii* with UV-C treatment for control of postharvest diseases of tomato fruit

Changfeng Zhang · Kunsong Chen · Guoli Wang

Abstract Integrated management that combines different methods is being actively pursued in the control of postharvest disease. In this study, the biocontrol yeast *Cryptococcus laurentii* and ultraviolet-C (UV-C) treatment were evaluated for controlling infection following artificial inoculation with *Botrytis cinerea* or *Alternaria alternata*, and natural infection in tomato fruit. Applied separately, *C. laurentii* and UV-C (4 kJ m^{-2}) effectively inhibited decay caused by *B. cinerea* or *A. alternata*, and natural infection. The combination of *C. laurentii* and UV-C showed better control efficiency. UV-C treatment did not affect yeast growth in fruit wounds, while the treatment induced the transcript expression of *β-1,3-glucanase*, *phenylalanine ammonia-lyase*, *peroxidase* and *superoxide dismutase* based on real-time PCR analysis, as well as increased the activity of these enzymes in tomato fruit. Results indicate that the mechanism by which UV-C enhanced the biocontrol efficacy of *C. laurentii* may be associated with the elicitation of defense response in tomato fruit.

Keywords Biocontrol yeast · Defense response · Postharvest disease · Tomato fruit · UV-C

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C. Zhang (✉) · G. Wang
Shandong Key Laboratory of Storage and Transportation Technology of Agricultural Products, Shandong Institute of Commerce and Technology, 4516 Travel Road, Caishi, Licheng District, Jinan 250103, Shandong, China
e-mail: zcf202@163.com

C. Zhang · K. Chen
Laboratory of Fruit Quality Biology/The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Huajiachi Campus, Hangzhou 310029, China

C. Zhang · G. Wang
National Engineering Research Center for Agricultural Products Logistics, Jinan 250103, China

Introduction

Fungal pathogens, including *Botrytis cinerea* and *Alternaria alternata* which respectively cause gray mold and black mold, result in major losses in tomato production (Mari et al. 1996; Polevaya et al. 2002; Cota et al. 2007; Su and Gubler 2012). Although current management of postharvest diseases of fruits and vegetables is mainly relying on the use of synthetic chemical fungicides, the development of pathogen's resistance to fungicide and public concern over the potential impact on environment and human health have created interest in new strategies for the disease management (Janisiewicz and Korsten 2002; Spadaro and Gullino 2004).

In recent years, biological control using microbial antagonists has shown great potential for postharvest

disease management (Droby et al. 2009; Sharma et al. 2009; Jamalizadeh et al. 2011). Among these microbial agents, the utilization of yeasts had been especially emphasized (Mclaughlin et al. 1990; Reeleder 2004; Wisniewski et al. 2007) and the yeast antagonist *Cryptococcus laurentii* has been demonstrated to be effective against postharvest diseases of apple (Lima et al. 1998; Janisiewicz et al. 2008), tomato (Chand-Goyal and Spotts 1997), peach (Zhang et al. 2007), orange (Zhang et al. 2005), grape (Meng et al. 2010) and jujube (Qin and Tian 2004). In addition to biological control, physical control like UV-C irradiation has been also applied as another promising technology for postharvest disease management (Shama and Alderson 2005; Stevens et al. 2005; Cia et al. 2010). Preharvest UV-C treatment (3 or 8 kJ m^{-2}) of mature green tomato fruit delayed ripening and increased resistance to *Penicillium digitatum* (Obande et al. 2011), and treatment of postharvest tomato fruit with UV-C at the dosage of 3.7 kJ m^{-2} could induce disease resistance to *B. cinerea* (Charles et al. 2008c). Likewise, UV-C illumination (5 kJ m^{-2}) effectively induced resistance against *Monilinia fructicola* in harvested tomato fruits (Li et al. 2010), UV-C irradiation (7.5 kJ m^{-2}) markedly reduced *Penicillium expansum* development in apple fruit (de Capdeville et al. 2002), and the three dosages of UV-C irradiation (0.43, 2.15 and 4.30 kJ m^{-2}) significantly reduced natural infection in strawberry fruits (Erkan et al. 2008). Moreover, Stevens et al. (2005) found that appropriate UV-C treatment on apple, peach and tangerine induced their resistance to postharvest pathogens, respectively. The resistance mechanisms involved in UV-C treated fruits included induction of pathogenesis-related (PR) proteins (Charles et al. 2009), antioxidant enzymes (Erkan et al. 2008), phenolic compounds (Charles et al. 2008a), and cell structure reinforcement (Charles et al. 2008b).

Even though either biocontrol using yeast or UV-C irradiation has been shown to be effective in reducing postharvest decay, a preferred alternative to fungicide treatment is integrated management, such as a combination of different methods (Teixidó et al. 2001; Korsten 2006; Janisiewicz and Conway 2010; Smilanick 2011). Recently, Xu and Du (2012) reported that UV-C treatment enhanced the biocontrol efficacy of *Candida guilliermondii* on control of blue mold and green mold in pear fruit. Integration of UV-C with the biocontrol yeast *Debaryomyces hansenii* could be as effective as the postharvest fungicide benomyl treatment in reducing storage rots in peach, tangerine and sweetpotato (Stevens et al. 1997). However, so far limited information is available on the mode of action by which UV-C treatment enhanced biocontrol efficacy of antagonistic yeast against postharvest diseases, especially in terms of the induction of defensive response of tomato fruit at both transcript and enzyme activity levels.

The overall objective of the present study was to evaluate the effects of the yeast antagonist *C. laurentii* and UV-C treatment, used separately or in combination, on control of postharvest diseases of tomato fruit. More specifically, we investigated the effects of (i) *C. laurentii* and/or UV-C on postharvest diseases of tomato fruit caused by *Botrytis cinerea* or *Alternaria alternata*, or natural infection; (ii) UV-C on the population dynamics of *C. laurentii* in fruit wounds; (iii) UV-C on the transcript expression of β-1,3-glucanase (GNS), phenylalanine ammonia-lyase (PAL), peroxidase (POD) and superoxide dismutase (SOD) using real-time PCR, and the activity of these enzymes in tomato fruit.

Materials and methods

Yeast

The yeast antagonist *C. laurentii* was isolated from apple fruit surface and identified by the CABI Bioscience Identification Services (International Mycological Institute, Egham, UK). Fifty milliliters 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, China) in 1,000 ml water) was prepared in 250-ml conical flasks and inoculated with *C. laurentii* to an initial concentration of 10^5 cells ml^{-1}, as determined by a hemocytometer. Yeast cultures were incubated on a rotary shaker at 200 rpm for 48 h at 25 °C. Before use for biocontrol test, yeast cells were pelleted at $8,000\times g$ for 3 min and washed three times with sterile-distilled water in order to remove residual medium.

Pathogens

Botrytis cinerea or A. alternata were isolated from infected tomato fruits and maintained on potato

dextrose agar (PDA) (Oxoid, UK) for 14 days at 25 °C. The spore suspensions of both pathogens were obtained from surface of the PDA cultures with 5 ml of sterile distilled water containing 0.05 % (v/v) Tween 80 by glass spreading rod, and then filtered through four layers of sterile cheesecloth to remove any adhering mycelia (Qin and Tian 2004). The number of spores was calculated using a hemocytometer, and then the spore concentration was adjusted to 5×10^4 spores ml^{-1} with sterile distilled water.

Fruit

Tomato (*Solanum lycopersicum* L. cv. Jinguan) fruits were harvested at mature green maturity. The average quality values of firmness, soluble solids content and titratable acidity were 110.5 N, 6.2 and 2.4 %, respectively. Fruits without wounds or rot were selected based on uniform size and absence of physical injury or disease infection. Before treatment, fruits were surface-disinfected with 2 % (v/v) sodium hypochlorite for 2 min, rinsed with tap water and air-dried.

Efficacy of UV-C treatment on control of *B. cinerea* and *A. alternata* in tomato fruits

It is known that improper UV-C treatment would damage fruit tissue. Therefore, in the present study, a maximum duration of UV-C treatment of 18 min (the dosage of 6 kJ m^{-2}), which guaranteed no occurrence of UV injury to tomato fruits, was chosen based on previous studies (Liu et al. 2011; Obande et al. 2011) and our preliminary trials.

Fruits were randomly grouped into four lots. Three lots of fruits were treated by UV-C according to Li et al. (2010). Specifically, the fruits were placed on trays under two UV-C lighting tubes (peak emission at 254 nm) at a distance of 20 cm. The radiation intensity of the lamps was measured by a UV digital radiometer (BrightStars, China). Each lot of fruits was exposed to UV-C radiation for 6 (2 kJ m^{-2}), 12 (4 kJ m^{-2}) or 18 min (6 kJ m^{-2}). To provide uniform irradiation, fruits were rotated to the opposite side at the half-time of each treatment. The fourth lot of fruits without UV-C treatment served as the control. After 24 h, two (3 mm deep × 3 mm wide) wounds were made with a sterile nail on the opposite sides at the equator of each fruit. A 5 μl spore suspension of

B. cinerea or *A. alternata* (5×10^4 spores ml^{-1}) was inoculated to each wound. Treated fruits were stored at 20 °C for four days. Disease incidence and lesion diameter on each tomato fruit were recorded. Each treatment consisted of three replicates with 20 fruits per replicate and the experiment was repeated three times.

Effects of *C. laurentii* in combination with UV-C treatment on disease development in tomato fruits

Tomato fruits were divided into the following four groups:

1. Fruits which were firstly treated by UV-C for 12 min (4 kJ m^{-2}), then wounded as described above (two wounds on the opposite sides at the equator of each fruit);
2. Fruits which were not treated with UV-C, but wounded and inoculated by pipetting 5 μl of washed cell suspension of *C. laurentii* (5×10^7 cells ml^{-1}) into each wound;
3. Fruits which were firstly treated with UV-C as described in Group 1, then inoculated with *C. laurentii* as described in Group 2;
4. Fruits which were wounded as described above but without UV-C or yeast treatment as the control.

After 24 h, all fruits in the four groups were inoculated with 5 μl of spore suspension of *B. cinerea* or *A. alternata* at 5×10^4 spores ml^{-1}. All fruits were stored at 20 °C for four days. Disease incidence and lesion diameter on each tomato fruit were recorded. Each treatment consisted of three replicates with 20 fruits per replicate and the experiment was repeated three times.

Effects of *C. laurentii* in combination with UV-C on natural infection of intact fruits

The effects of UV-C with or without *C. laurentii* on development of natural decay of tomatoes were evaluated. Intact fruits were divided into the following four groups:

1. Fruits which were illuminated by UV-C for 12 min (4 kJ m^{-2});
2. Fruits which were inoculated by dipping for 30 s into a washed cell suspension of *C. laurentii* (5×10^7 cells ml^{-1}), and then air-dried;

3. Fruits which firstly treated with the UV-C treatment as described in Group 1, and then inoculated with the yeast as described in Group 2;

4. Fruits which did not receive any treatment and served as the control.

All the fruits were stored at 20 °C for 20 days, and the percentage of infected fruits was recorded. Each treatment consisted of three replicates with 20 fruits per replicate and the experiment was repeated three times.

Determination of population dynamics of *C. laurentii* in fruit wounds

Tomato fruits were illuminated by UV-C for 12 min (4 kJ m^{-2}) as described above. Fruits without UV-C treatment served as the control. Afterwards, two wounds (3 mm deep × 3 mm wide) were made on the opposite sides at the equator of each tomato fruit with a sterile nail. All fruits were treated by pipetting 5 μl of a washed cell suspension of *C. laurentii* (5 × 10^7 cell ml^{-1}) into each fruit wound, and stored at 20 °C. Fruit samples were collected every day over a period of five days after treatment and yeast populations were measured as described by Xu and Du (2012). The yeast was recovered by removing 20 samples of wound tissues with a cork borer (1 cm diameter × 1 cm deep). Samples were then ground with a mortar and pestle in 10 ml of sterile distilled water. Then, 50 μl of serial ten-fold dilutions were spread on NYDA (NYDA: as for NYDB with addition of 20 g of agar) plates. Samples taken at 1 h after treatment for population measurement served as time 0. Colonies were counted after incubation for five days and expressed as the log$_{10}$ colony-forming unit (CFU) per wound. There were three replicates in each treatment, and the experiment was repeated three times.

RNA isolation and quantitative real-time PCR analysis of defense gene expression

Tomato fruits were illuminated by UV-C for 12 min (4 kJ m^{-2}) as described above, and the fruits without UV-C treatment served as the control. Fruit samples were obtained from 20 fruits stored at 20 °C containing the pericarp and flesh at 0, 1, 2, 3, 4 and 5 days after treatment (DAT). Each treatment consisted of

three replicates and the experiment was repeated three times. Total RNA from tomato samples at each time point was isolated using Concert^{TM} Plant RNA Reagent (Invitrogen, USA) according to the manufacturer's instructions (Wisniewski et al. 2011). Extracted RNA was treated with TURBO^{TM} DNase (Ambion, USA) and purified with RNeasy Mini Kit (Qiagen, China). Real-time PCR analysis was performed using 40 ng of total RNA, SuperScript III Platinum SYBR Green One-Step RT-qPCR Kit with ROX (Invitrogen, USA), and 20 pmol of each primer per reaction. The ABI 7900 (Applied Biosystems, USA) was set to cycle as follows: cDNA synthesis at 48.0 °C for 30 min, 95.0 °C denaturation for 5 min, 40 cycles of 95.0 °C for 15 s followed by 60.0 °C for 1 min, 40.0 °C for 1 min, dissociation step. The primers of the target genes of GNS, PAL, POD and SOD (Table 1) were designed according to the previous studies (Wang et al. 2009; Song et al. 2011). The standard curve method was used to calculate transcript abundance relative to *β-actin* as a reference gene (Wang et al. 2009). There were three replicates in each treatment, and the experiment was repeated three times.

Assay of enzyme activities in tomato fruits

Tomato fruits were illuminated by UV-C for 12 min (4 kJ m^{-2}) as described above, and the fruits without UV-C treatment served as the control. For the enzyme assay, fruit samples were obtained from 20 fruits stored at 20 °C containing the pericarp and flesh at 0, 1, 2, 3, 4 and 5 DAT. Each treatment consisted of three replicates and the experiment was repeated three times.

β-1,3-glucanase (GNS) enzyme was extracted according to Cao and Jiang (2006). Fruit tissue samples (10 g) were homogenized in 20 ml of ice-cold sodium acetate buffer (100 mM, pH 5.0) containing 5 mM *β*-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid (EDTA) with a tissue grinder. The homogenate was centrifuged at 13,000×*g* for 20 min at 4 °C, and the resulting supernatant was collected for the enzyme assay. GNS activity was assayed, with laminarin as the substrate, following the method described by Ippolito et al. (2000). Reaction production was measured spectrophotometrically at 500 nm. The specific activity of GNS was expressed as U mg^{-1} protein, where one unit was defined as the production of 1 μmol glucose equivalent h^{-1}.

Table 1 Primers used in real-time PCR analysis for defense gene expression

Gene name	NCBI accession No.	Primer sequence
GNS	M80608	F: AGGAACGATGTTAGATGGTTTACT
		R: ATCCATCGCAGCATAAACAG
PAL	M90692	F: CTTTGATGCAGAAGCTGAGACA
		R: TCGTCCTCGAAAGCTACAATCT
POD	L13654	F: GAATGTGCCAACTGGTAGAAGA
		R: GAGCAACGAGACACTCCAATAG
SOD	M37151	F: GCGGGTGACCTGGGAAACAT
		R: CCACAAGTGCTCGTCCAACAAC
β-actin	U60480	F: ACATTGTGCTCAGTGGTGGTACT
		R: CCACCTTAATCTTCATGCTGCT

GNS β-1,3-glucanase, PAL phenylalanine ammonia lyase, POD peroxidase, SOD superoxide dismutase
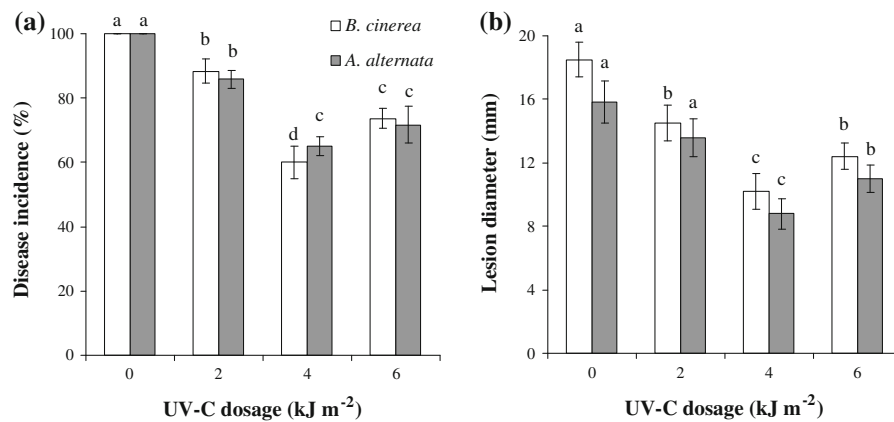

Fig. 1 Efficacy of UV-C treatment for control of gray mold and black mold caused by *B. cinerea* and *A. alternata* in tomato fruits, respectively. Disease incidence (**a**) and lesion diameter (**b**) were determined four days after inoculation at 20 °C. The data presented are the means of nine replicates (20 fruits each) pooled from three experiments. Each error bar represents SE. Columns with different letters in each disease are significantly different according to Duncan's multiple range test at $P < 0.05$

Phenylalanine ammonia lyase (PAL) enzyme was extracted by the method of Jin et al. (2009). Tissue sample (10 g) was homogenized with 20 ml of ice-cold sodium borate buffer (100 mM, pH 8.7) containing 5 mM β-mercaptoethanol and 4 % (w/v) polyvinylpyrrolidone (PVP) with a tissue grinder. The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4 °C, and the resulting supernatant was collected for the enzyme assay. Enzyme extract (0.3 ml) was incubated with 0.6 ml of sodium borate buffer and 0.3 ml of L-phenylalanine (20 mM) for 60 min at 37 °C. The reaction was stopped with 0.3 ml of 1 M HCl. PAL activity was determined by the production of cinnamate, with the absorbance change at 290 nm

(Assis et al. 2001). The blank was the crude enzyme preparation mixed with L-phenylalanine with zero time incubation. The specific enzyme activity was defined U mg^{-1} protein, where one unit was defined as the production of 1 nmol cinnamic acid h^{-1}.

For peroxidase (POD) and superoxide dismutase (SOD), enzymes were extracted according to Chan and Tian (2006). Tissue samples (10 g) with 0.3 g polyvinyl polypyrrolidone (PVPP) were homogenized in 20 ml of ice-cold sodium phosphate buffer (50 mM, pH 7.8) with a tissue grinder (Kriens-LU, Switzerland). The homogenate was centrifuged at $13,000 \times g$ for 20 min at 4 °C, and the resulting supernatant was collected for the enzyme assay. POD activity was analyzed using

Table 2 Statistical analysis (one-way ANOVA) of the effects of UV-C dosage and different treatments on disease index caused by *B. cinerea*, *A. alternata* or natural infection in tomato fruits

	Disease incidence				Lesion diameter			
	F-value	df (factor)	df (error)	*P* value	*F*-value	df (factor)	df (error)	*P* value
B. cinerea								
UV-C dosage	196.15	3	32	<0.001	73.96	3	32	<0.001
Treatments	1145.23	3	32	<0.001	255.41	3	32	<0.001
A. alternata								
UV-C dosage	124.72	3	32	<0.001	62.37	3	32	<0.001
Treatments	887.40	3	32	<0.001	294.33	3	32	<0.001
Natural infection								
Treatments	214.78	3	32	<0.001				

UV-C dosage represents 0, 2, 4 and 6 kJ m^{-2} of UV-C treatment on tomato fruits in Fig. 1, while treatments represent control, UV-C (4 kJ m^{-2}), yeast (*C. laurentii*), and the combination of UV-C (4 kJ m^{-2}) and yeast (*C. laurentii*) in Figs. 2 and 3
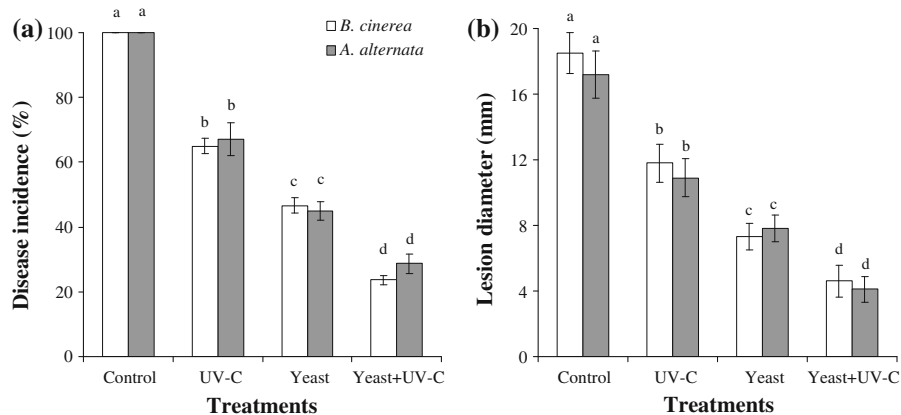

Fig. 2 Effect of UV-C irradiation (4 kJ m^{-2}) and the antagonistic yeast, *C. laurentii* (5 × 10^7 cells ml^{-1}) applied alone or in combination on disease incidence (**a**) and lesion diameter (**b**) caused by *B. cinerea* and *A. alternata* in tomato fruits. The data presented are the means of nine replicates (20 fruits each) pooled from three experiments. Each error bar represents SE. Columns with different letters in each disease are significantly different according to Duncan's multiple range test at *P* < 0.05

guaiacol as a substrate (Yao and Tian 2005). The increase in absorbance at 460 nm was spectrophotometrically assayed after H$_2$O$_2$ was added. The specific activity was defined as U mg^{-1} protein, where one unit was defined as the increase in the rate of absorbance per minute. SOD activity was determined with nitro blue tetrazolium (NBT) as a substrate (Wang et al., 2004). The absorbance was determined at 560 nm after activation of 10-min illumination with a fluorescent lamp (60 μmol m^{-2} s^{-1}). The specific activity was defined as U mg^{-1} protein, where one unit of SOD activity was defined as the amount of enzyme causing 50 % inhibition in the NBT reduction. Protein content was determined according to the Bradford assay (Bradford 1976). Bovine serum albumin (Sigma-Aldrich, China) was used as a standard.

Statistical analysis

All statistical analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Data from assays of population dynamics, gene expression and enzyme activity were compared in a Student's *t* test. Others were analyzed by one-way ANOVA, and mean separations were performed by Duncan's new
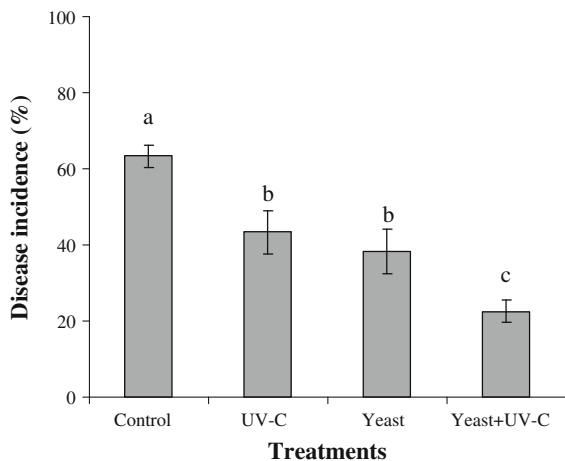
Fig. 3 Effect of UV-C irradiation (4 kJ m^{-2}) and the antagonistic yeast, *C. laurentii* (5 × 10^7 cells ml^{-1}) applied alone or in combination on natural infection of tomato fruits. Disease incidence was determined after 20 days of storage at 20 °C. The data presented are the means of nine replicates (20 fruits each) pooled from three experiments. Each error bar represents SE. Columns with different letters are significantly different according to Duncan's multiple range test at $P < 0.05$
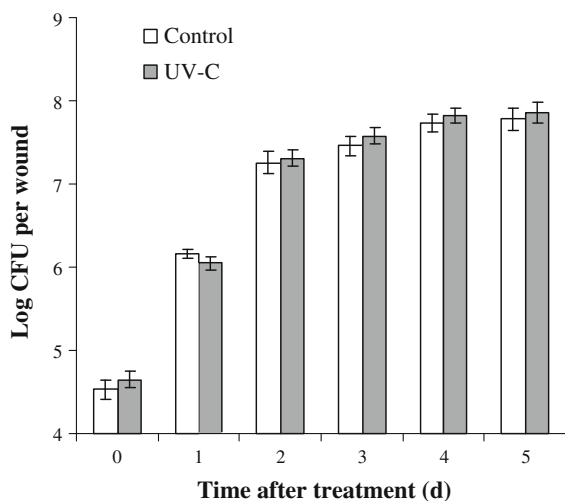

Fig. 4 Population dynamics of *C. laurentii* in wounds of UV-C treated (4 kJ m^{-2}) and untreated control tomato fruits stored at 20 °C for five days. The data presented are the means of nine replicates (20 wounds each) pooled from three experiments. Each error bar represents SE

multiple range test. Differences at $P < 0.05$ were considered significant. Data presented in this paper were pooled across three independent repeated experiments, as the interaction between treatment and experimental replication was not significant.

Results

Efficacy of UV-C treatment on control of *B. cinerea* and *A. alternata* on tomato fruits

UV-C at all dosages used (2, 4 and 6 kJ m^{-2}) significantly reduced disease incidence of gray mold and black mold, caused by *B. cinerea* and *A. alternata* in tomato fruits, respectively (Fig. 1a; Table 2). Moreover, lesion diameter caused by the two pathogens was significantly lower in all UV-C-treated (except 2 kJ m^{-2}) tomato fruits compared to untreated control fruits (Fig. 1b; Table 2). Generally, the best inhibitory effect on the two diseases was achieved when the fruits were treated with UV-C at the dosage of 4 kJ m^{-2}. Therefore, such UV-C at 4 kJ m^{-2} was chosen for further studies of the effects on disease control when combined with the antagonistic yeast *C. laurentii*, as well as on the population dynamics of the yeast and induction of defensive and antioxidant gene expression and enzyme activity in fruits.

Effects of *C. laurentii* in combination with UV-C treatment on disease development in tomato fruits

All treatments significantly reduced the disease incidence and lesion diameter caused by *B. cinerea* and *A. alternata* (Fig. 2; Table 2). UV-C treatment (4 kJ m^{-2}) and *C. laurentii* (5 × 10^7 cells ml^{-1}), as stand-alone treatment, reduced disease incidence of gray mold from 100 % of control fruits to 65 % and 67 %, respectively. The combination of *C. laurentii* and UV-C treatment exhibited a synergistic effect, with the lowest disease incidence of 24 % (Fig. 2a). When lesion diameter of gray mold reached 18.5 mm in control fruits, UV-C treatment, *C. laurentii* and the combination treatment reduced lesion diameter to 11.8, 7.3 and 4.6 mm, respectively (Fig. 2b). Likewise, the combination of *C. laurentii* and UV-C treatment also showed best control effect on black mold according to disease incidence and lesion diameter. Collectively, the combination of UV-C (4 kJ m^{-2}) and *C. laurentii* (5 × 10^7 cells ml^{-1}) treatment increased the control efficacy of each single treatment against both mold diseases in tomato fruits.

Table 3 Statistical analysis (Student's *t* test) of the effects of UV-C treatment (4 kJ m^{-2}) on population dynamics of *C. laurentii* in tomato fruit wounds, gene expression and enzyme activity of fruits for each day over a period of five days

		Time (day)					
		0	1	2	3	4	5
Log$_{10}$ CFU per wound	*t*	−1.40	1.85	−0.84	−1.04	−1.18	−0.91
	P value	0.18	0.08	0.41	0.31	0.26	0.35
Gene expression							
GNS	*t*		−3.35	−3.19	−9.93	−5.15	−12.03
	P value		0.004	0.006	<0.001	<0.001	<0.001
PAL	*t*		−16.24	−12.43	−10.56	−9.73	−11.83
	P value		<0.001	<0.001	<0.001	<0.001	<0.001
POD	*t*		−7.42	−7.81	−8.37	−10.56	−11.25
	P value		<0.001	<0.001	<0.001	<0.001	<0.001
SOD	*t*		−8.50	−6.34	−10.33	−9.22	−4.83
	P value		<0.001	<0.001	<0.001	<0.001	<0.001
Enzyme activity							
GNS	*t*		−3.28	−3.72	−7.66	−4.38	−2.06
	P value		0.005	0.002	<0.001	<0.001	0.06
PAL	*t*		−11.63	−10.23	−9.53	−12.46	−7.86
	P value		<0.001	<0.001	<0.001	<0.001	<0.001
POD	*t*		−13.41	−9.26	−14.76	−7.52	−10.23
	P value		<0.001	<0.001	<0.001	<0.001	<0.001
SOD	*t*		−3.11	−3.69	−4.63	−5.24	−2.69
	P value		0.007	0.002	<0.001	<0.001	0.016

The degrees of freedom (df) of all the following *t* tests is 16

GNS *β*-1,3-glucanase, PAL phenylalanine ammonia lyase, POD peroxidase, SOD superoxide dismutase

Effects of *C. laurentii* in combination with UV-C on natural infection of intact fruits

The pathogens causing natural infection on tomatoes used in this experiment were mainly *B. cinerea*, *A. alternata* and *P. expansum*, with *B. cinerea* being predominant. The combination of *C. laurentii* and UV-C treatment showed the best control effect on natural infection in tomato fruits after storage at 20 °C for 20 days. As compared to control fruits with natural decay incidence of 63 %, UV-C treatment, *C. laurentii* and the combination treatment significantly decreased the incidence to 43, 38 and 23 %, respectively (Fig. 3; Table 2).

Effects of UV-C treatment on population dynamics of *C. laurentii* in fruit wounds

The biocontrol yeast, *C. laurentii*, multiplied rapidly in tomato fruit wounds (Fig. 4). The number of the yeast increased more than ten-fold after one day, and became gradually stable after three days when the cells reached the stationary phase. Moreover, the difference of *C. laurentii* population dynamics in wounds between untreated control and UV-C-treated (4 kJ m^{-2}) fruits was non-significant (Table 3).

Effect of UV-C treatment on induction of defensive and antioxidant gene expression in tomato fruit

The transcript level of the four genes, GNS, PAL, POD and SOD, was increased significantly by UV-C treatment (4 kJ m^{-2}) (Fig. 5; Table 3). Among the four tested genes, GNS expression was the least affected by UV-C but was significantly induced during the five-day period (Fig. 5a). PAL expression in UV-C treated fruit increased dramatically at one day after treatment (DAT), while it peaked at 3 DAT in control fruit (Fig. 5b). POD expression in control fruit showed
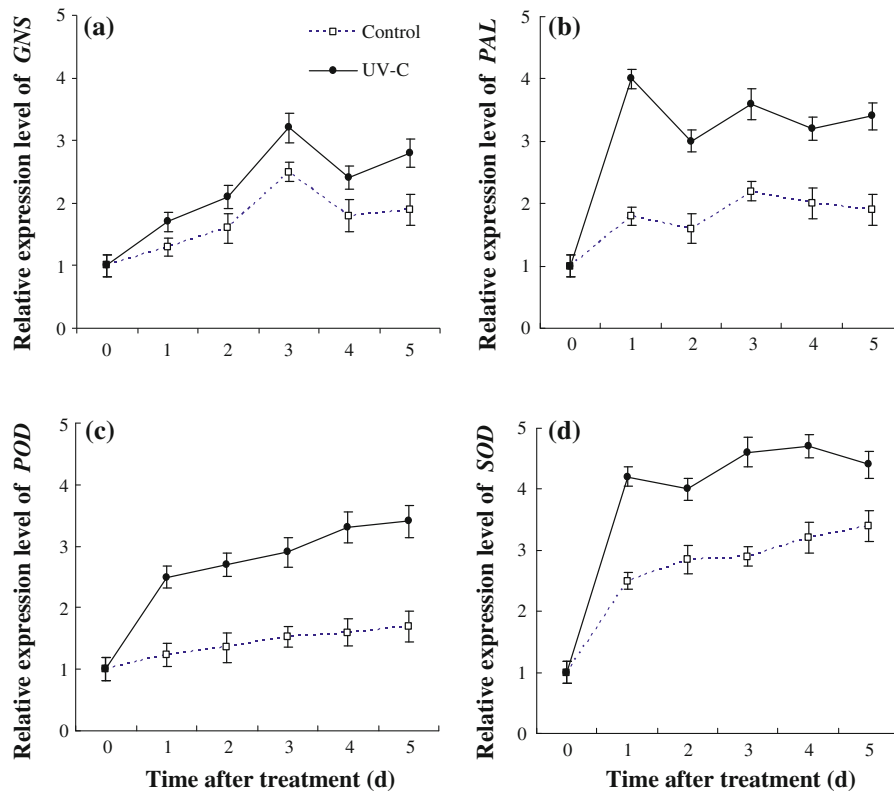
Fig. 5 Relative transcript expression of β-1,3-glucanase (GNS, **a**), phenylalanine ammonia lyase (PAL, **b**), peroxidase (POD, **c**) and superoxide dismutase (SOD, **d**) in UV-C treated (4 kJ m^{-2}) and untreated control tomato fruits stored at 20 °C. Values were normalized to that at time 0, arbitrarily set to unity. The data presented are the means of nine replicates (20 fruits each) pooled from three experiments. Each error bar represents SE

relatively stable change pattern over the five-day period. In contrast, the expression was consistently higher expression in UV-C treated fruit (Fig. 5c). SOD expression increased markedly at 1 DAT and then kept relatively stable in control fruit, while the gene expression showed similar pattern but was induced in UV-C treated fruit. For instance, SOD expression in treated fruit was 1.7-fold higher than that in control at 1 DAT (Fig. 5d).

Effects of UV-C treatment on induction of defensive and antioxidant enzyme activity

Corresponding to the pattern of gene expression observed for the four genes examined, UV-C treatment (4 kJ m^{-2}) also significantly induced the activity of GNS, PAL, POD and SOD enzymes in tomato fruits stored at 20 °C (Fig. 6; Table 3). The activity of GNS in the control fruits increased gradually and peaked at 3 DAT, and declined afterwards. The pattern of enzyme activity both in UV-C treated and the untreated control fruits were similar (Fig. 6a). With a prolonged storage period, the UV-C treated fruit showed continuously higher PAL activity than the control. The peak value of PAL activity in both control and treated fruits appeared at 1 DAT (Fig. 6b). POD activity in the control fruits was relatively stable, while UV-C treatment increased the activity, especially during the first three days of storage (Fig. 6c). As shown in Fig. 6d, SOD activity was stimulated by UV-C treatment, increasing rapidly and reaching a maximum at 3 DAT.

Discussion

Among postharvest physical treatments, UV-C has been reported to be an effective method of managing fungal diseases in fruits (González-Aguilar et al. 2001; Romanazzi et al. 2006; Cia et al. 2010). In the present
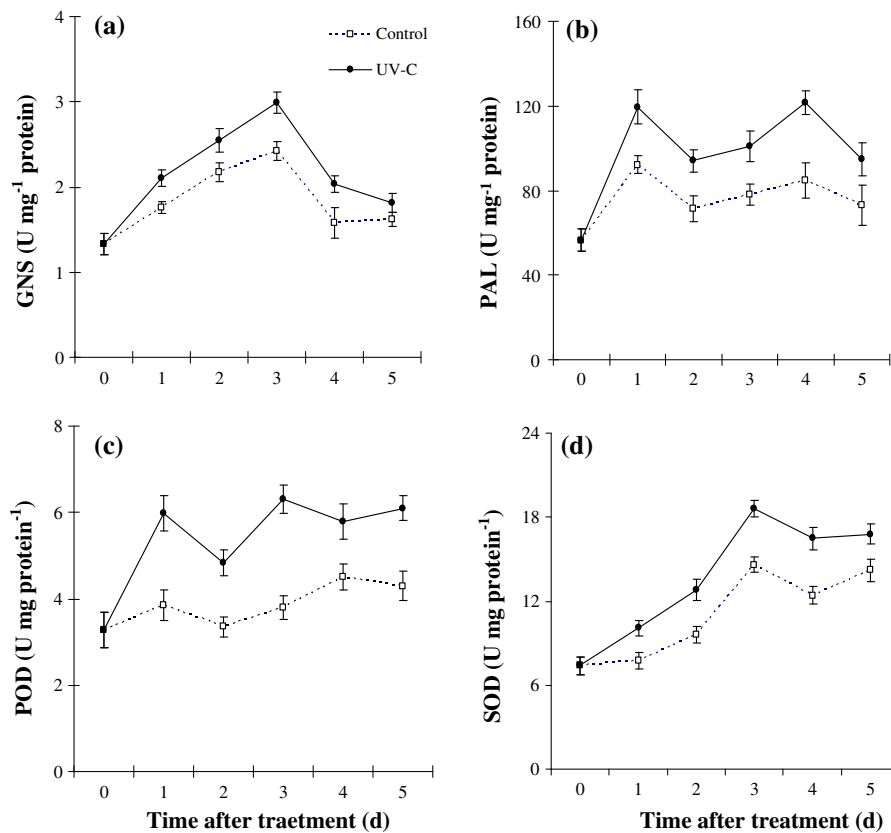
Fig. 6 Enzyme activity of β-1,3-glucanase (GNS, **a**), phenyl-alanine ammonia lyase (PAL, **b**), peroxidase (POD, **c**) and superoxide dismutase (SOD, **d**) in UV-C treated (4 kJ m^{-2}) and untreated control tomato fruits stored at 20 °C. The data presented are the means of nine replicates (20 fruits each) pooled from three experiments. Each error bar represents SE

study, we found that UV-C treatment alone at all the dosages (2, 4 and 6 kJ m^{-2}) effectively controlled postharvest diseases of tomato fruits caused by *B. cinerea* and *A. alternata*. Among them, the treatment at 4 kJ m^{-2} showed the best control, indicating that the dosage of UV-C correlated closely with inducible host resistance against pathogens. A similar result was recently obtained by Xu and Du (2012), who reported that UV-C treatment at 5 kJ m^{-2} had better inducible defense effects on pear fruit against *B. cinerea* and *P. expansum* than UV-C at 3.33 and 6.67 kJ m^{-2}.

Due to the result that UV-C treatment at 4 kJ m^{-2} had the best efficacy, it was selected to study the control of postharvest diseases of tomato fruits combined with the biocontrol yeast *C. laurentii*. When the UV-C treatment was combined with *C. laurentii* for control of gray mold and black mold in artificially inoculated fruits, the efficacy was higher than any individual treatment. Similar effects were obtained in

the control of natural infection in intact fruits, that is, the combination of *C. laurentii* and UV-C also showed the best control efficacy, suggesting that UV-C could enhance the biocontrol efficacy of *C. laurentii* on tomato fruits. These results confirmed the previous reports about the combined application of biocontrol yeast and UV-C treatment on control of postharvest diseases in other fruits. The combination of the biocontrol yeast *D. hansenii* and UV-C treatment was more effective for control of *M. fructicola* on peach and *P. digitatum* on tangerine than the individual treatments (Stevens et al. 1997). Similarly, the combination of the yeast *Candida guilliermondii* with UV-C irradiation exhibited a synergistic effect in reducing artificially inoculated *P. expansum* and *B. cinerea* diseases, as well as natural infection on pear fruit, being better than either yeast or UV-C treatment alone (Xu and Du 2012). The synergistic effect may be a result of triangle interactions among UV-C

treatment, the yeast antagonist and the fruit host. High population density of microbial antagonists is an advantage in competing for nutrients and space, both of which play a major role in biocontrol efficacy (Wisniewski et al. 2007; Sharma et al. 2009). The results of our study suggested that *C. laurentii* multiplied quickly in wounds of tomato fruits stored at 20 °C. This indicated that the yeast was well-adapted to the wound microenvironment. Moreover, UV-C treatment did not markedly affect the in vivo growth of *C. laurentii*, according to the assay of population dynamics in wounds of control and UV-C-treated fruits.

The ability of UV-C to induce fruit host defense mechanisms has been previously postulated (Li et al. 2010; Charles et al. 2011). GNS, PAL, POD and SOD have been suggested to play a crucial role in defense against fungal infection. GNS is one of the most fully characterized pathogenesis-related (PR) proteins, and can act directly by degrading cell walls of pathogens or indirectly by releasing oligosaccharides that elicit defense reactions (Lee et al. 2006; Tian et al. 2007). PAL is the first enzyme in the phenylpropanoid pathway, which involves in the biosynthesis of phenolics, phytoalexins and lignins (Dixon et al. 2002). All these physiological processes are potential defense mechanisms in tomato fruit against pathogen infection (Wang et al. 2011). In addition to defense-related enzymes, antioxidant enzymes like POD and SOD are closely related to the defense of plant host against fungal pathogens, because they play a role in detoxifying reactive oxygen species and alleviating oxidative damage caused by pathogen invasion (Xu and Tian 2008; Zeng et al. 2010). In the present study, UV-C treatment markedly induced gene expression of GNS, PAL, POD and SOD, and their enzyme activity as well, which corresponded well to the control effect of gray mold and black mold. Similar results were obtained by El Ghaouth et al. (2003), who reported that UV-C treatment at the dosage of 7.6 kJ m^{-2} induced disease resistance in peach fruits by enhancing transcript expression of GNS, PAL and *chitinase*. Li et al. (2010) reported that the induction of enzyme activity of PAL, GNS, SOD, catalase, and glutathione reductase in pear fruit by UV-C treatment contributed to reduction of brown rot caused by *M. fructicola*. Moreover, Lamikanra et al. (2005) found that UV-C treatment elicited POD activity of fresh-cut cantaloupe melon, leading to increase of resistance against

microbial infection and thus extension of shelf life. The present report, however, is the first report demonstrating the inductive effect of UV-C on defensive and antioxidant enzymes at both the transcript and enzyme activity levels in tomato fruit.

In conclusion, the combination of the antagonistic yeast *C. laurentii* and UV-C treatment was more effective in controlling postharvest diseases of tomato fruits than the single treatment. Therefore, a combined strategy of biological and physical measures may represent a viable approach used to manage postharvest diseases under the variable conditions found in packinghouses and storage facilities.

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Author Biographies

Changfeng Zhang is an associate professor of developmental biology in Shandong Key Laboratory of Storage and Transportation Technology of Agricultural Products (China). He is studying postharvest pathology of tomato fruit and writing the manuscript.

Kunsong Chen is a professor in Zhejiang University, China. He is involved in material preparation and investigation of fruit in response to UV-C.

Guoli Wang is a professor in National Engineering Research Center for Agricultural Products Logistics. He is studying biocontrol efficacy of *Cryptococcus laurentii*. This work is a team effort to shed light on the combined effect of *C. laurentii* and UV-C on postharvest disease management.