Food Chemistry 254 (2018) 208–216

Contents lists available at ScienceDirect

Food Chemistry

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

Effect of *Cryptococcus laurentii* on inducing disease resistance in cherry tomato fruit with focus on the expression of defense-related genes

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ARTICLE INFO

Keywords:
Cryptococcus laurentii
Induced disease resistance
Defense-related proteins
Gene expression
Hormone signaling pathway
Fruit quality

ABSTRACT

The objective of this study was to prove and explain the disease resistance-inducing ability of *Cryptococcus laurentii* on cherry tomato, as well as assay its effect on fruit quality. Apart from disease incidence, activities of defense-related enzymes and expression of critical genes were studied. With pre-treatment of *C. laurentii*, disease incidences of *Botrytis cinerea* and *Alternaria alternate* infected fruits were both significantly reduced. Corresponding mechanism could be explained as *C. laurentii* can induce resistance in cherry tomato by activating the expression of important defense-related genes, such as genes involved in salicylic acid (SA) and jasmonic acid (JA) signaling pathways and genes encoding pathogenesis related proteins, thus activating comprehensive defense reaction against pathogen invasion. Coupled with the results that fruit color was improved and other physicochemical parameters remained uninfluenced, our study suggests that pre-treatment with *C. laurentii* can be a promising method to preserve cherry tomato fruits.

1. Introduction

Induced resistance is considered to be a promising strategy to replace synthetic fungicides to control postharvest decay of fruits and vegetables, which provides long-term systemic resistance to a broad spectrum of pathogens (Walling, 2001) and can be achieved by physical, chemical and biological means (Romanazzi et al., 2016). As explained, induction of plant resistance is one of the most important mechanisms of the biocontrol activity of antagonistic yeasts (Sharma, Singh, & Singh, 2009). *C. laurentii* is a widely studied biocontrol yeast which is capable of managing postharvest fungal diseases of fruits and vegetables such as peach, pear, apple and tomato (Wei et al., 2016). Previous studies indicated that *C. laurentii* can noticeably induce resistance in pears (Tian, Wan, Qin, & Xu, 2006), jujube fruits (Tian et al., 2007), peaches (Xu, Qin, & Tian, 2008), and table grapes (Meng & Tian, 2009).

However, little knowledge has been known about the mechanism of antagonistic yeasts' resistance-inducing effect on fruits. Information limits in the accumulation of phytoalexin scoparone (Spadaro & Droby, 2015) and some defense-related enzymes like β-1,3-glucanase, chitinase and peroxidase (Ippolito, El Ghaouth, Wilson, & Wisniewski, 2000; Tian et al., 2006). (jiang, Zheng, & Chen, 2009) gave an overall view on transcript modification in cherry tomato after treated with *C. laurentii*, but detailed and interrelated information are missing. More importantly, no study has been conducted to assay the resistance-inducing effect of antagonistic yeast separately from other effects on fruits. As is known to all, the biocontrol activity of antagonistic yeast on postharvest pathogen is the combined outcome of four modes of action which are competition for nutrients and space, production of antibiotics, direct parasitism and induced resistance (Sharma et al., 2009). Therefore, new method of antagonistic yeast treatment should be taken to focus on its resistance inducing effect.

This study aims to (1) evaluate the effect of *C. laurentii* on inducing resistance against *B. cinerea* and *A. alternata* of cherry tomato; (2) explain the mechanism of this induction effect at two levels: activities of defense-related enzymes and expression of defense-related genes; (3) evaluate the effect of *C. laurentii* on fruit quality during storage.

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https://doi.org/10.1016/j.foodchem.2018.01.100
Received 25 September 2017; Received in revised form 7 January 2018; Accepted 12 January 2018
Available online 31 January 2018
0308-8146/

2. Materials and methods

2.1. Fruit and pretreatment

Mature red cherry tomato fruits (*Solanum lycopersicum* var. *cerasiforme*) of the cultivar 'Qian Xi' were hand-harvested without injuries or infections from Liang Yi Agricultural Development Co., Ltd. in Yuhang County, Zhejiang Province, China, and immediately transported to the laboratory in Zhejiang University. Fruits with uniform shape and size were selected and then surface-sterilized in 0.1% (v/v) sodium hypochlorite aqueous solution for 2 min, rinsed thoroughly with tap water, and dried in the air prior to the experiment.

2.2. Yeast and pathogen

Yeast *C. laurentii* (Kufferath) Skinner (Strain zju 10) was originally isolated from pear fruit. It was first cultured on nutrient yeast dextrose agar (NYDA) at 25 °C, then inoculated to 50 mL of nutrient yeast dextrose broth (NYDB) in 250 mL conical flasks and incubated at 28 °C for 24 h on a rotary shaker at 3.3 s^{-1}. Yeast cells were pelleted at 4000 g for 10 min. After being washed twice, they were re-suspended with sterile-distilled water. Then, the number of cells was counted on a hemocytometer and the concentration was adjusted to 1×10^8 cells mL^{-1}.

The pathogen *B. cinerea* and *A. alternata* were individually isolated from infected cherry tomato fruit and cultured on potato dextrose agar (PDA) at 25 °C for 7 d and 14 d respectively prior to use. Then, spores were obtained by flooding the culture with sterile-distilled water, and the concentration of the suspension was adjusted to 1×10^4 cells mL^{-1} by a hemocytometer.

NYDA: 8 g of nutrient broth, 5 g of yeast extract, 10 g of glucose and 20 g of agar in 1 L of distilled water. NYDB: NYDA without agar. PDA: 200 g of potato extract, 20 g of glucose and 20 g of agar in 1 L of distilled water. All the media were sterilized at 121 °C for 20 min.

2.3. Assay of disease incidence

Cherry tomato fruits were dipped in (1) sterile distilled water as control, or (2) 1×10^8 cells mL^{-1} suspension of *C. laurentii* for 10 min. Then, the fruits were stored in enclosed plastic trays at 25 °C with high Relative Humidity (90–95%). After 48 h (optimized by earlier experiment, data not shown), one wound was made (5 mm diameter and 2 mm deep) at the equator of the fruit by a sterile borer. Each fruit was inoculated with 20 μL of a spore suspension of *B. cinerea* (1×10^4 cells mL^{-1}). The fruits were then stored at the same condition mentioned above. Disease incidence was recorded after 48, 72 and 96 h. Fruits were determined to be infected when mycelium was observed on wounds. Each treatment consisted of three replicates with 20 fruits per replicate, and the experiment was conducted twice.

The effect on *A. alternata* was tested exactly the same as above.

2.4. Assay of defense-related enzyme activity

2.4.1. Treatment

Cherry tomato fruits were treated with sterile distilled water or *C. laurentii*, and stored in the same way as Section 2.3, but no pathogen was inoculated. At the time of 0, 24, 48, 72 and 96 h after treatment, samples of the tissue (without peel) were taken, immediately frozen in liquid nitrogen and stored at −80 °C. Each sample consisted of tissues from nine fruits.

2.4.2. Extraction

Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) share the same extraction to analyze their activities. 1 g of frozen sample was ground on ice in a mortar and pestle with 3 mL of cold 50 mM sodium phosphate buffer (pH 7.8) containing 1.33 mM EDTA and 1% (w/v) PVP. For the extraction of phenylalanine ammonia-lyase (PAL), 0.5 g of frozen sample was mixed with 2 mL of 100 mM sodium borate buffer (pH 8.8) containing 5 mM β-Mercaptoethanol, 1 mM EDTA and 1% (w/v) PVP at 4 °C. For β-1,3-glucanase (GLU) and chitinase (CHI) extraction, method was the same as PAL except that the buffer was sodium acetate buffer (pH 5.0).

The homogenates were centrifuged at 12,000*g* for 10 min. Then the supernatants were collected to assay the enzyme activities and protein contents. The protein contents were determined according to the Bradford method. The values of enzyme activities were expressed as units per milligram protein.

2.4.3. SOD and CHI activity assay

SOD and CHI activity were determined by assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). According to manufacturer's instruction, one unit of SOD and CHI activity was defined as the amount of enzyme resulting in 50% inhibition rate in 1 mL of reaction solution and producing 1 mg N-acetylglucosamine respectively.

2.4.4. POD activity assay

POD activity was measured using guaiacol as substrate (Lurie, Fallik, Handros, & Shapira, 1997). 100 μL of crude enzyme extract, 140 μL of 0.3% (v/v) guaiacol and 60 μL of 0.3% (v/v) H_2O_2 were added together. The reaction was detected immediately at 470 nm every 30 s for 5 min. One unit of POD activity was defined as the amount of enzyme that produced an increase of A_{470} by 0.01 per min.

2.4.5. CAT activity assay

CAT activity was assayed based on the method of (Aebi, 1984). The reaction mixture consisted of 250 μL of 30 mM H_2O_2 and 50 μL of crude enzyme extract. The reaction was detected immediately at 240 nm every 30 s for 5 min. One unit of CAT activity was defined as the amount of enzyme that produced a decrease of A_{240} by 0.01 per min.

2.4.6. PAL activity assay

According to the method of (Zucker, 1968), a reaction mixture of 250 μL of crude enzyme extract, 500 μL of 100 mM sodium borate buffer (pH 8.8) and 250 μL of 20 mM l-Phenylalanine was incubated at 37 °C for 6 h. The reaction was terminated by adding 50 μL of 6 M HCl and the absorbance of the mixture was detected at 290 nm. The initial absorbance (0h), which should be deducted from the absorbance data at 6 h, was measured by adding HCl immediately to the reaction mixture without incubation. One unit of PAL activity was defined as the amount of enzyme that caused an increase of A_{290} by 0.01 per hour.

2.4.7. GLU activity assay

GLU activity was determined according to the method of (Ippolito et al., 2000) with some modifications. 250 μL of crude enzyme extract and 250 μL of 4 g L^{-1} laminarin (Sigma) were mixed and incubated at 37 °C for 8 h. Then, 500 μL of DNS was added to the mixture and the enzymatic reaction was terminated by heating the mixture in boiling water for 5 min. After being cooled to room temperature, 4 mL of water was added to the mixture and the absorbance was measured at 500 nm. The initial absorbance (incubated for 0 h) was detected and deducted. The linear relationship between A_{500} and glucose content was determined by developing a standard curve.

2.5. Assay of expression of defense-related genes

2.5.1. Treatment

The treatment was similar as 2.4.1, only the time interval is 24 and 48 h.

2.5.2. RNA extraction

Frozen tissue was fully ground and total RNA was extracted using