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Wound healing in citrus fruit is promoted by chitosan and *Pichia membranaefaciens* as a resistance mechanism against *Colletotrichum gloeosporioides*

Yijie Zhao[a,b], Lili Deng[a,b], Yahan Zhou[a], Jian Ming[a,b], Shixiang Yao[a,b], Kaifang Zeng[a,b,*]

[a] *College of Food Science, Southwest University, Chongqing 400715, PR China*
[b] *Chongqing Engineering Research Center of Regional Food, Chongqing 400715, PR China*

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ABSTRACT

Colletotrichum gloeosporioides is the most important pathogen of citrus fruit and is a serious concern in rainy, humid regions and seasons. In this study, the effects of chitosan and *Pichia membranaefaciens* on anthracnose control, wound healing, and the cell wall changes in citrus fruit and the relationship between wound healing and cell wall composition were evaluated. We found that chitosan and *P. membranaefaciens* increased the wound healing process via wound tissue appearance changes, lignin accumulation, increases in cell wall compounds (pectin, cellulose) and reduced activities of cell wall enzymes - polygalacturonase (PG); pectin methylesterase (PME); cellulase (CEL) - to control anthracnose. During wound healing, acid-soluble pectin (ASP) and cellulose had strong positive correlations with lignin.

1. Introduction

The pathogen that causes serious anthracnose disease in citrus fruit is *Colletotrichum gloeosporioides*, which was recently reported as the eighth most important group of plant pathogenic fungi that infects at least 1000 plant species worldwide (Aiello et al., 2015; Bill et al., 2016; Wu et al., 2016). *C. gloeosporioides* also causes preharvest symptoms such as withered twig tips, tear stain, stem-end rot of fruits and citrus post bloom fruit drop (Huang et al., 2013; Lima et al., 2011; Rhaiem and Taylor, 2016). Anthracnose particularly infects citrus that grows in rainy, humid regions and seasons and causes serious decreases in yield. In China, this pathogen is very common in Chongqing Province and is severe in some rainy months, such as May and June. Valencia orange is currently the major variety of orange (*Citrus sinensis*) grown in China. Wounding commonly occurs to fresh fruit from harvest to final use and induces a series of physiological and defensive responses to prevent further damage, which is the process called wound healing. Wound healing shows resistance to pathogen invasion (Ayon-Reyna et al., 2017; Leverentz et al., 2000; Shao et al., 2010; Spotts and Chen, 1987); thus, the aim of this project was to study anthracnose and the mechanism after wounding of Valencia orange.

Wound healing plays an important role in maintaining postharvest quality and shelf life. Plant responses to wounding have been described previously (Cheong et al., 2002; De Bruxelles and Roberts, 2001; Shanker and Venkateswarlu, 2011; Wu et al., 1997) and include (i) ROS burst, (ii) mechanical barriers to invading organisms, and (iii) defensive compounds accumulate from the wound, especially the lignin. Furthermore, PAL is an enzyme recognized as highly important in wound healing, particularly wound-induced suberization. Wounding in apple promotes the lignification process (Vilanova et al., 2014) and healing of minor injuries to Valencia orange is also associated with PAL increase and lignin accumulation (Ismail and Brown, 1979). One study found that potato tubers develop a wound barrier that is effective against pathogen infection (Kumar and Knowles, 2003). Robert showed that healing of wounds decreases the decay in pear caused by *B. cinerea*, *M. piriformis*, *P. expansum*, and *P. solitum* (Spotts et al., 1998), and Vilanova found that the wound response prevents infection by *P. expansum* at 20 °C (Vilanova et al., 2014). Earlier studies on wound healing suggest that ABA is involved in the regulation of wound healing in potato tuber (Espelie and Kolattukudy, 1985; Lulai et al., 2008; Soliday et al., 1978), *Arabidopsis* root (Efetova et al., 2007), tomato fruit (Tao et al., 2016), and kiwifruit (Han et al., 2018, 2017). Moreover, ethylene is relevant to wound healing in grapevine stems (Sun et al., 2007).

Many studies demonstrate that chitosan and *P. membranaefaciens*

Abbreviations: PAL, L-phenylalanine ammonia-lyase; RH, relative humidity; WSP, water-soluble pectin; ASP, acid-soluble pectin; PG, polygalacturonase; PME, pectin methylesterase; CEL, cellulase

* Corresponding author at: College of Food Science, Southwest University, Chongqing 400715, PR China.
E-mail address: zengkaifang@hotmail.com (K. Zeng).

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have antimicrobial activity against *Vaccinium corymbosum*, *Penicillium digitatum*, *P. expansum, Alternaria alternata*, *Botrytis cinerea*, *Rhizopus stolonifer*, and *M. fructicola* based on different mechanisms (Cao et al., 2015; Chan and Tian, 2005; Qin et al., 2004; Shao et al., 2015; Vieira et al., 2016; Wang et al., 2015; Hernandez-Montiel et al., 2018), which include competition for space and nutrients (Di Francesco et al., 2018, 2017); promotion of plant resistance (Parafati et al., 2016; Yan et al., 2018); decrease in firmness-related enzymatic activity (Jongsri et al., 2016; Luo et al., 2013); production of hydrolytic enzymes (Tian et al., 2018; Zhou et al., 2016); generation of volatile organic compounds (VOCs) (Hernandez-Montiel et al., 2018; Oro et al., 2018; Parafati et al., 2017); induction of oxygen radical scavenging enzymes and oxidative stress (Luo et al., 2013); and induction of lignin accumulation (Chan et al., 2007; Deng et al., 2015; Luo et al., 2012). Although PAL and lignin are relevant to wound healing (PAL, lignin), no direct study has been conducted on the effects of chitosan and *P. membranaefaciens* on wound healing. Moreover, wound healing is associated with appearance change, including cell wall changes (Cajuste and Lafuente, 2007; Carvajal et al., 2015; Vicente et al., 2013), which change during wound healing (Neubauer et al., 2012; Spotts et al., 1998). However, the link between wound healing and cell wall structure has not been studied. Thus, the objectives of this study were as follow: (1) to determine the effects of chitosan and *P. membranaefaciens* on wound healing and the cell wall changes in citrus fruit with and without *C. gloeosporioides* inoculation, and (2) to study the relationship between wound healing and cell wall components.

2. Materials and methods

2.1. Fruit and pathogen inoculum

Valencia orange [*Citrus sinensis* (L.) Osbeck] fruits were harvested at commercial maturity and transported to the laboratory. The fruits without wounds or rot were selected based on uniform size and color, disinfected in 2 g L^{-1} sodium hypochlorite solution for 2 min, and air-dried.

The fungal pathogen *C. gloeosporioides* (isolate Cg-7) was isolated from infected citrus fruit in the laboratory and maintained on a potato dextrose agar plate at 4 °C. The spore concentration of the suspensions was determined using a hemocytometer and adjusted to 10^5 spores mL^{-1} using sterile distilled water.

2.2. Treatment preparation

The *Pichia membranaefaciens* yeast strain was obtained from the Chinese General Microbiological Culture Collection Center, Beijing, China. *P. membranaefaciens* was prepared according to the method of Zhou (Zhou et al., 2016). According to the method of Luo and Zhou (Luo et al., 2012; Zhou et al., 2016), the yeast was re-suspended in sterile distilled water and adjusted to an initial concentration of 10^8 cells mL^{-1}.

Chitosan (Jinan Haidebei Marine Bioengineering Co., Ltd., Shangdong, China) with an intrinsic viscosity of 30 centipoise was obtained. According to the method of Zhou (Zhou et al., 2016), the chitosan was dissolved at 0.01 g of chitosan in 100 mL of sterilized distilled water containing 0.1 % acetic acid and subsequently adjusted to a pH value of 5.4.

2.3. Postharvest treatment and inoculation

Fruit was randomly divided into 2 treatment groups: group A and group B, with 300 fruits for each group. For group A, 10 µL of (1) distilled water was used as a control, (2) 0.01 % chitosan was added, or (3) 10^8 cells mL^{-1} *P. membranaefaciens* yeast were placed in a 1 mm wide, 2 mm deep wound made with a sterilized iron nail at 5 points in the equatorial region of each fruit. For group B, the same treatments were applied as those in group A. After 24 h at room temperature, 10 µL of 10^5 spores mL^{-1} pathogen was inoculated in the same wounds of group B. After air-drying, all fruit were separately packed in plastic bags and stored at 28 °C and 90 to 95 % room humidity (RH). The disease incidence and lesion diameter of each fruit were measured at 6, 9, 12, and 15 d during storage, with the lesion diameter measured without the width of the hole. Each treatment was performed in three replicates with ten fruits per replicate, and the experiment was conducted twice.

2.4. Sampling

The fruit pericarp was collected at 0, 3, 6, 9, 12, and 15 d and sampled at 5 mm from the edge of the wound after storage. Tissue samples were mixed and frozen immediately in liquid nitrogen and stored at −40 °C. Each treatment was replicated three times.

2.5. Water content determination

Tissue samples (5 mm wide × 2 mm deep) were excised, including the holes, with a sterilized hole puncher. Each fruit had 5 points in the equatorial region, and 5 fruits were used for each treatment. The samples were incubated at 65 °C for 12 h, and the results were calculated as the weight after drying divided by the fresh weight. Each treatment was replicated three times.

2.6. Determination of pectin, cellulose and lignin

Pectin was extracted according to the methods of Cheng and McComb and McCready (Cheng et al., 2008; McComb and McCready, 1952). Uronic acid concentrations in water-soluble pectin (WSP) and acid-soluble pectin (ASP) were measured as previously reported by Bu (Bu et al., 2013). The pectin concentration is expressed as mg GalA kg^{-1}. Cellulose was determined according to the method of Bu (Bu et al., 2013), and the cellulose concentration is expressed as g kg^{-1}. Lignin was determined according to the method of Deng (Deng et al., 2015), and the lignin concentration is expressed as ΔOD_{280} kg^{-1}.

2.7. Enzyme extraction and assay

The pectin methylesterase (PME) activity was measured according to the method of Carvajal (Carvajal et al., 2015) with some modifications. One gram of frozen tissue was ground in a mortar and extracted at 4 °C with 5 mL of 1 M NaCl containing 1 % PVPP. The homogenate was shaken for 4 h at 4 °C and then centrifuged at 4 °C and 8000 × g for 30 min. The supernatant was collected and adjusted to pH 7.5 with 1 mM NaOH. The reaction mixture contained 1 mL of enzymatic extract, 6 mL of 0.5 % (w v^{-1}) pectin pH 7.5, 1.5 mL of 0.01 % (w v^{-1}) bromothymol blue pH 7.5, and 1 mL of H_2O pH 7.5. The reduction in the absorbance at 620 nm was followed for 20 min. The PME activity is expressed as ΔOD_{620} s^{-1} kg^{-1} protein. The extraction method of polygalacturonase and cellulase was adapted from Zhang (Zhang et al., 2010). The polygalacturonase (PG) activity was assayed according to Zhang (Zhang et al., 2010), and PG activity is expressed as g GalA h^{-1} kg^{-1} protein. The cellulase (CEL) activity was assayed according to Carvajal (Carvajal et al., 2015). CEL activities were calculated using a glucose standard curve and are expressed as g Glc h^{-1} kg^{-1} protein. The PAL activity was measured according to the method of Gao (Gao et al., 2016) and is expressed as ΔOD_{290} s^{-1} kg^{-1} protein.

2.8. Statistical analyses

The experiments were performed using a completely randomized design. All statistical analyses for this experiment were performed using the SPSS statistical software package (SPSS Inc., Chicago, IL, USA). Data from repeated experiments were statistically analyzed using analysis of variance (ANOVA) applied to percentages previously subjected to arc-

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