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Research article

Induction of defense-related enzymes in patchouli inoculated with virulent *Ralstonia solanacearum*

Jian-Hui Xie [a,b], Ting-Ting Chai [a], Ran Xu [a], Dan Liu [a], Yu-Xiu Yang [a], Zhi-Cheng Deng [a], Hua Jin [a], Hong He [a,*]

[a] School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, 232 WaiHuan East Road, Guangzhou Higher Education Mega Center, Guangzhou 510006, PR China
[b] Guangdong Provincial Key Laboratory of Clinical Research on Traditional Chinese Medicine Syndrome, The Second Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou 510120, PR China

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ABSTRACT

Background: Defense-related anti-oxidative response is a vital defense mechanism of plants against pathogen invasion. *Ralstonia solanacearum* is an important phytopathogen. Bacterial wilt caused by *R. solanacearum* is the most destructive disease and causes severe losses in patchouli, an important aromatic and medicinal plant in Southeast Asia. The present study evaluated the defense response of patchouli inoculated with virulent *R. solanacearum*.
Results: Results showed that the basic enzymatic activities differed not only between the leaves and stems but also between the upper and lower parts of the same organ. POD, SOD, PPO, and PAL enzymatic activities were significantly elevated in leaves and stems from patchouli inoculated with *R. solanacearum* compared to those in control. The variation magnitude and rate of POD, PPO, and PAL activities were more obvious than those of SOD were in patchouli inoculated with *R. solanacearum*. PAGE isoenzymatic analysis showed that there were one new POD band and two new SOD bands elicited, and at least two isoformic POD bands and two SOD bands were observably intensified compared to the corresponding control.
Conclusion: Our results suggest that not only defense-related enzymatic activities were elevated but also the new isoenzymatic isoforms were induced in patchouli inoculated with *R. solanacearum*.

1. Introduction

Pogostemon cablin (patchouli) is an important aromatic plant belonging to the family Lamiaceae, native to southeast Asia, and is now extensively cultivated in many tropical and subtropical countries, especially China, Indonesia, India, Brazil, Vietnam, Malaysia, Mauritius, Philippines, and Thailand. Patchouli oil, which is extracted from its leaves, is widely used in the modern perfume, cosmetic, and food industries. Modern research has repeatedly demonstrated the various pharmacological activities of this oil including anti-inflammatory, antiseptic, antibacterial, antifungal, antidepressant, and insect repellent properties [1]. In China, patchouli is also a famous herbal remedy that has long been used to treat common cold, nausea, pain, headaches, infections, and digestive problems.

Today, growing interest in its fragrance and medicinal applications has led to patchouli's widespread cultivation in southern China. However, patchouli is very sensitive to bacterial wilt, a serious disease caused by *Ralstonia solanacearum*. The genetic variability within patchouli is relatively limited; therefore, breeding potential for resistance to biotic stresses is also limited. Patchouli is propagated by stem cuttings, which further limits its available genetic pool. Furthermore, stem cuttings can harbor the pathogens, thus allowing the disease to be perpetuated through vegetative propagation. Despite various attempts in the past decades, effective prevention and control of patchouli bacterial wilt disease to date has hardly been obtained. Therefore, it is necessary to find a potential new strategy for protecting patchouli against attacks by *R. solanacearum*.

Defense-related enzymes constitute an important protective system for plants against pathogen invasion. In recent years, intensive efforts have been devoted to the elucidation of defensive responses to pathogen invasion [2,3,4,5,6,7,8]. Current research has determined superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) as vital defense-related enzymes of plants, and therefore, these enzymes are extensively studied in the research of plant defense against phytopathogens [9,10,11,12]. SOD, POD, and PPO are representative antioxidant enzymes and are important components of defense against membrane lipid peroxidation and oxidative stress during pathogen invasion [13]. SOD is the first enzyme involved in the antioxidant defense. The ability of plants to overcome oxidative stress partly relies on the induction of

* Corresponding author.
E-mail address: hehong67@hotmail.com (H. He).
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SOD activity and subsequently on the upregulation of other downstream antioxidant enzymes in the defense [14]. POD is an oxidoreductive enzyme that participates in cell wall polysaccharide processes such as oxidation of phenols, suberization, and lignification of host plant cells during defense reaction against pathogenic agents [15]. PPO is involved in the oxidation of polyphenols into quinones and lignification of plant cells during microbial invasion [16]. PAL is the primary enzyme in phenylpropanoid metabolism and plays an important role in the synthesis of several defense-related secondary compounds such as phenols and lignin [17].

R. solanacearum is an important phytopathogen and a causal agent of devastating plant wilt disease. Bacterial wilt caused by *R. solanacearum* is a serious soil-borne disease of many economically important crops, such as tomato, potato, tobacco, eggplant, and banana. Defense responses to *R. solanacearum* involving antioxidant enzymes were reported in some crops, especially plants of the *Solanaceae* family, such as tomato, potato, and eggplant. However, the study of defense response to *R. solanacearum* on its new host, especially medicinal plants such as patchouli has rarely been reported to date. *R. solanacearum* is a "species complex" with high heterogeneity on various hosts [18,19]. Currently, increasingly more number of *R. solanacearum* strains are being isolated from different plants. In addition, some new hosts of *R. solanacearum*, such as patchouli, were reported [20]. However, evidence for the induction of defense enzymes that protect plants from infection by the bacterium in new hosts is still preliminary and inconsistent. In the present work, attempt has been made to investigate the defense response of patchouli inoculated with *R. solanacearum*, which was isolated from a naturally wilted patchouli plant grown in Guangdong, China.

2. Materials and methods

2.1. Plant materials and culture

Leaves procured from elite patchouli mother plants (grown in Guangzhou, China, E 112°57′13″, N 222°6′35″) were washed 3 to 4 times in tap water and surface sterilized for 1 min in 75% ethanol, followed by immersion in 0.1% (*w*/*v*) $HgCl_2$ solution for 5 min, and finally rinsed thoroughly with sterile distilled water. *In vitro* regeneration of plantlets was achieved through a series of culturing steps under the following specified conditions: (1) for shoot initiation and multiplication, surface-disinfected explants were inoculated on full strength solid MT basal medium supplemented with 0.2 mg/L 6-benzyladenine and 3% (*w*/*v*) sucrose for 4 weeks.

Rapid and prolific shoot regeneration through direct somatic embryogenesis was initiated from leaf explants of patchouli after initial culturing; (2) for root development and true-to-type plantlets, profuse rooting from regenerated shoots was induced by transferring uniform proliferated shoots to MT medium devoid of phytohormones after 2 months. Established cultures were subcultured in the same medium at 4-week intervals. All the cultures were maintained at 25 ± 2°C under a 12-h photoperiod with a light intensity of 3000 lx provided by cool white fluorescent tubes. Seven to eight-week-old plants exhibiting 12–14 leaves were used for the experiments. Leaves were numbered according to their time of appearance. Leaves located at the base of the stem were numbered from 1 to 6, designated as lower parts, and those above were numbered from 7 to 12. Patchouli plantlets were sampled thrice for each treatment for the examination of enzymes. All plantlets were initially of identical length and similar size.

2.2. R. solanacearum and culture

In our previous investigation, a strain of *R. solanacearum* was isolated from the vascular bundles of patchouli affected by bacterial wilt in Guangdong Province, China [20]. The strain was routinely grown or maintained at 28°C on NA plate (beef extract 3 g/L, yeast extract 1 g/L, bacterial peptone 10 g/L, sucrose 10 g/L, pH 7.2). Stock cultures of *R. solanacearum* were preserved at −20°C in NA liquid medium containing 1% dimethyl sulfoxide prior to use. The isolate was routinely checked for any contamination during this investigation.

2.3. Inoculation of patchouli

A single colony of *R. solanacearum* was inoculated in nutrient broth and incubated in a shaking incubator at 200 rpm overnight and 28°C for 12 h (logarithmic phase). The density of the bacterial suspension for inoculation was prepared and adjusted to 1.0×10^8 CFU/mL. The suspensions were immediately used for the inoculation of patchouli.

Roots of patchouli plantlets were inoculated to evaluate its association with the phytopathogenic bacteria *R. solanacearum*. Bacterial suspensions of 1 mL volume were inoculated into the roots of patchouli by the root wounding method. Plants infiltrated with aliquots of sterile distilled water served as controls. At the end of each treatment period, plantlets inoculated with *R. solanacearum* were studied for the determination of defense enzymes of patchouli in the following days.

2.4. Enzyme extraction and assay

Each patchouli plantlet was separated into two parts, the upper half (top, leaves numbered from 7 to 12) and the lower part (bottom, leaves numbered from 1 to 6). Each part was further segregated into two tissues: leaf and stem. Leaves and stems (upper/lower, 0.2 g) were well-homogenized in a homogenizer in 2 mL ice-cold 50 mM sodium phosphate buffer (pH 6.0) for POD, PPO, and SOD assay and in 50 mM borate buffer (pH 8.8, containing 5 mM β-mercaptoethanol) for PAL assessment, individually. The homogenate was centrifuged at 12,000 × g for 10 min, and then the resultant supernatant was used for subsequent enzymatic activity assays. All operations were performed at 0°C to 4°C.

2.5. Peroxidase assay

POD (E.C. 1.11.1.7) activity was quantified by taking 0.1 mL homogenate in 3 mL of 0.1 M sodium phosphate buffer, pH 6.0 (containing 19 μL of 30% hydrogen peroxide (H_2O_2) as the oxidant and 38 μL guaiacol as the hydrogen donor per 50 mL buffer). The oxidation of guaiacol was measured by spectrophotometry at 470 nm and <25°C. The enzymatic activity was determined by measuring the maximum slope of the reaction line.

2.6. Superoxide dismutase assay

SOD (EC 1.15.1.1) activity was analyzed according to the method of Gay and Tuzun et al. [21], with the following modifications. The reaction mixture (3.0 mL) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 10 μM ethylenediaminetetra acetic acid disodium (EDTA-Na_2), 2.0 μM riboflavin (added last), and 0.1 mL homogenate in a final volume of 3 mL. The mixtures were illuminated by a fluorescent lamp (3000 lx) for 15 min, and the absorbance was measured immediately at 560 nm. Identical solutions held in the dark served as blanks. The reaction mixture without adding specific enzymes developed maximum color due to the highest reduction of NBT. One unit of SOD was defined as the amount of enzyme that caused a 50% decrease in the SOD-inhibitable NBT reduction. The specific activity was expressed as units per gram fresh weight (FW) per min (units g^{-1} FW min^{-1}).

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