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Naturally produced citral can significantly inhibit normal physiology and induce cytotoxicity on *Magnaporthe grisea*

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ABSTRACT

Given the importance of finding alternatives to synthetic fungicides, the antifungal effects of natural product citral on six plant pathogenic fungi (*Magnaporthe grisea, Gibberella zeae, Fusarium oxysporum, Valsa mali, Botrytis cinerea*, and *Rhizoctonia solani*) were determined. Mycelial growth rate results showed that citral possessed high antifungal activities on those test fungi with EC_{50} values ranging from 39.52 to 193.00 μg/mL, which had the highest inhibition rates against *M. grisea*. Further action mechanism of citral on *M. grisea* was carried out. Citral treatment was found to alter the morphology of *M. grisea* hyphae by causing a loss of cytoplasm and distortion of mycelia. Moreover, citral was able to induce an increase in chitinase activity in *M. grisea*, indicating disruption of the cell wall. These results indicate that citral may act by disrupting cell wall integrity and membrane permeability, thus resulting in physiology changes and causing cytotoxicity. Importantly, the inhibitory effect of citral on *M. grisea* appears to be associated with its effects on mycelia reducing sugar, soluble protein, chitinase activity, pyruvate content, and malondialdehyde content.

1. Introduction

One of the major classes of organisms that cause plant disease are phytopathogenic fungi. Over the past decades, the actions of these organisms worldwide have caused millions of dollars of damage to the agriculture industry [1–4]. Combating this destruction usually relies on the frequent use of synthetic fungicide. Although effective, this frequent use of synthetic fungicide results in serious complications, including disrupted biological control of natural enemies and outbreaks in some plant diseases, resistance to other fungicides, toxic effects on organisms besides the targeted plants, and general environmental consequences [5–7].

As a result of these adverse consequences, alternative measures have been developed for better crop protection. This has necessitated the use of eco-friendly biopesticides and other plant-derived substances in the development of botanical fungicides. When compared to synthetic fungicides, these new, eco-friendly derivatives exhibit low to non-existent toxicity, further underscoring the potential for plant-derived fungicides [8–11]. Therefore, botanical fungicides are expected to remain important tools for the management of plant diseases. In turn, their use will help to increase future crop yields and quality as well as decrease environmental pollution in agricultural ecosystems [12–14].

Litsea cubeba is a member of the Lauraceae family and is found mainly in the tropical and subtropical regions of Australia, New Zealand, North America, South America, and Asia [15,16]. Like other plants of the genus *Litsea, L. cubeba* produces an essential oil, deriving from two parts of the plant (its leaves and fruit). The essential oil from its leaves contains a greater percentage of 1,8-cineole than citral. This oil is frequently found in perfumes. It is also a common ingredient in Southeast Asian cuisine as well as various chemical and medicinal enterprises [17,18].

Comparatively, the fruit-derived essential oil contains some 75% citral. Citral (3,7-dimethyl-2,6-octadienal) is thus one of the major compounds extracted from *L. cubeba*. As with its leaf-derived oil, this citral-heavy oil is used in various culinary dishes as well as a fragrance additive in cosmetics. In addition, citral is used as a raw ingredient in the manufacturing of various vitamins and supplements (e.g. citral, vitamins A, E, and K, ionine) as well as in perfumes. Because of its prevalence in many industries, citral has long been accepted by Western regulatory bodies in the US and Europe. As a result, it has been afforded the status of 'generally recognized as safe' (GRAS) [16,19].

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Although citral has many industrial and medical applications, recent work has focused on elucidating its use in human applications. Such work has led to insight into a multi-faceted role for citral, to include antifungal, antibacterial, and antioxidant properties. Interestingly, these results also point to its ability to act as an anti-insecticide [20–23]. Despite this recent research, there is currently little known about citral's use in agriculture, what is responsible for the bioactivities of citral, and the exact mechanism of its antimicrobial action. Our recent antifungal agent discovery screen study provides some insight, as we found that an acyclic monoterpene aldehyde citral showed considerable antifungal activities against the tested fungi [24].

The following study sought to further investigate the antifungal mechanisms of citral and its derivatives. Past work has shown that hyphal cell wall composition, cell wall-degrading enzymes, energy metabolism, and additional proteins have been suggested to be primary determinants of medicament control [25–31]. We examined the effects of citral on hyphal morphology, membrane permeability, and levels of various biochemical substrates (e.g. pyruvate, chitinase, reducing sugar, soluble protein and malondialdehyde (MDA)) in *Magnaporthe grisea*. The ultimate goal of understanding such mechanisms is to allow the better design and synthesis of new compounds with antifungal capabilities against pathogenic fungi.

2. Materials and methods

2.1. Fungal species and chemicals

The fungicidal activity of citral were evaluated against six candidate fungi (*M. grisea, G. zeae, F. oxysporum, V. mali, B. cinerea*, and *R. solani*) using the mycelium growth rate test. All of the candidate fungal species were provided by the Institute of Crop Protection (Guizhou University, Guiyang, China). Citral (purity ≥ 95%) was isolated from the essential oils derived from *L. cubeba*, dissolved in acetone to make a 1 mg/mL stock solution, and stored at 4 °C. Potato dextrose agar (PDA) was obtained from Xiya Reagent Co. Ltd. (Chengdu, China). Tris was supplied by Beijing Solarbio Science and Technology Co. Ltd. (Beijing, China). Tween 80, glutaraldehyde, 3,5-dinitro salicylic acid (DNS), *p*-dimethylaminobenzaldehyde (DMAB), 2,4-dinitrophenylhydrazine, and Coomassie brilliant blue G-250 were purchased from Aladdin Co. Ltd. (Shanghai, China). Thiophanate methyl (97% W/W) was obtained from Wuhan Chuangsheng Chemicals Co. Ltd. (Wuhan, China). Kasugamycin (76.5% W/W) was purchased from Shanxi Lvdun biological products Co. Ltd. (Pucheng, China). Zhongshengmycin (97% w/w) was obtained from Hubei Shengtian Hengchuang biotechnology Co. Ltd. (Wuhan, China).

2.2. Antimicrobial activity of citral on mycelial growth

Water and Tween 80 (0.5% v/v) were used to create a citral emulsion. This emulsion was then added to PDA before being plated on 90 mm dishes. The temperature was kept within a range of 45–50 °C. Controls received the same quantity of Tween 80 and PDA, minus the added citral. After plating, a 6 mm diameter disc cut from the actively growing front of an old colony of the desired pathogenic fungus was then placed with the inoculum side down in the center of each treatment plate, aseptically. Plates were then incubated in the dark at a constant temperature of 28 °C. All procedures utilized proper aseptic technique. All experiments were performed in triplicate before statistical analyses. Citral was tested against three pathogenic fungi, namely, *G. zeae, F. oxysporum*, and *R. solani* at different concentrations of 50, 100, 150, 200, and 250 μg/mL. For both *M. grisea* and *B. cinerea* mycelial, citral concentrations were set up as 12.5, 25, 50, 100, and 200 μg/mL. Meanwhile, citral was tested against *V. mali* at different concentrations of 25, 50, 100, 200, and 400 μg/mL. Mean growth values were obtained and then converted into an inhibition percentage of mycelial growth in relation to control treatment by using the following formula:

$$\text{Inhibition rate}\,(\%) = [(M_c - M_t)/(M_c - 0.6)] \times 100$$

where M_c and M_t represent the mycelial growth diameter in control and citral-treated conditions, respectively. The EC_{50} (effective dose for 50% inhibition) values were estimated statistically by probit analysis with the probit package of SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) [32].

2.3. Scanning electron microscopy examination of the effects of citral on hyphal morphology of M. grisea

For morphological studies, treatments were prepared by adding different concentrations of citral in PDA to each petri dish. *M. grisea* was then grown on these petri dishes at 28 °C for 10 days. Scanning electron microscopy (SEM) imaging and sample preparation were conducted as previously described, with the following modifications [32–34]. After citral incubation, mycelia were fixed overnight with 4% (v/v) glutaraldehyde at 4 °C. Mycelia samples were then washed twice for 10 min each in 0.1 mol/L phosphate buffered saline (PBS, pH = 7.2) and then dehydrated by subjecting them to a gradient of t-butanol washes (30%, 50%, 70%, and 90%) for ten minutes per concentration. The final 100% t-butanol wash was performed in triplicate for 20 minutes per wash. After dehydration, the specimens were dried in a freeze drier (LGJ-10D; Beijing Fourth Ring Scientific Instrument Co., Ltd., Beijing, China), and sputter-coated with gold. Microscopy was performed using a SEM (S-3400N; Hitachi, Tokyo, Japan) operated at an accelerating voltage of 20 kV. Controls consisted of untreated mycelia, which were prepared in parallel with experimental samples.

2.4. Effect of citral on the relative permeability rate of mycelia cell membrane

Citral-incubated *M. grisea* mycelia were gathered and washed twice for 2 min with sterilized water. One gram of the mycelia was placed in 15 mL centrifuge tubes containing one of three concentrations of citral (0, 100, and 200 μg/mL). Conductivity was measured at the following time points: 0 (J_0), 5, 10, 15, 20, 25, 30, 60, 90, 120, 180, 240, 300, 420, 540, and 720 min (J_1). Samples were boiled and cooled, after which their respective conductivities (J_2) were measured. Permeabilities (P%) were calculated by the following formula:

$$P\% = [(J_1 - J_0)/(J_2 - J_0)] \times 100\%$$

2.5. Preparation of crude extract of M. grisea mycelium

A total of six mycelial samples (6 mm in diameter) were harvested from a colony grown on a PDA-containing plate. Samples were placed in an Erlenmeyer flask containing 100 mL of sterilized Czapek medium (0.2% $NaNO_3$, 0.131% $K_2HPO_4{\cdot}3H_2O$, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.00183% $FeSO_4{\cdot}7H_2O$, 3% sucrose, pH = 6.8) [30] and incubated in a whirly shaker 125 rpm at 28 °C. The incubation period was 15 days, after which citral was dripped into the culture media at one of three different concentrations (0, 100, and 200 μg/mL). Mycelia were filtered, collected, and washed orderly at 0, 0.5, 1, 3, 6, 9, 12, 24, 48, and 72 hours. After soaking the water with filter paper, the dried mycelia were weighed and stored at –20 °C. One gram of the dried mycelia in liquid nitrogen was mixed with approximately 3 mL of Tris-HCl buffer (0.05 mol/L, pH = 7.5) and reduced with a mortar and pestle into a slurry. The slurry was centrifuged at 12,000 rpm for 20 min at 4 °C. The clear upper layer liquid (CULL) was isolated and stored at –20 °C. It should be noted that all experimental treatments were done in triplicate prior to analyses.