



Effects of SA and H₂O₂ Mediated Endophytic Fungal Elicitors on Essential Oil in Suspension Cells of *Cinnamomum longepaniculatum*

Kuan Yan, Linman He*, Kangjia Yang

College of Sichuan Tea, Yibin University, Yibin, China

Email: *1398421407@qq.com

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Abstract

Salicylic acid (SA) and hydrogen peroxide (H₂O₂) are signal molecule that plays crucial roles in plant secondary metabolism. In order to explore their roles in mediating the effect of endophytic fungal elicitors on essential oils in the suspension cells of *Cinnamomum longepaniculatum* fungus, an elicitor made of *Penicillium* sp. was used as material in this experiment. Cell suspension culture was used to study the effects of SA and hydrogen peroxide on the essential oils in the suspension cells of *Cinnamomum longepaniculatum* fungus. The results showed that there were signal pathways regulating essential oil synthesis by SA and H₂O₂ in *Cinnamomum longepaniculatum* fungus suspension cells, but the two pathways had no obvious succession. Adding endophytic fungal elicitors, AOPP and CAT at the same time could reduce the essential oil synthesis induced by elicitors in *Cinnamomum longepaniculatum* fungus suspension cells, but not completely inhibit it. It indicated that endophytic fungal elicitors could also promote the synthesis of essential oil in oil suspension cells through other signal transduction pathways.

Subject Areas

Biochemistry, Biotechnology

Keywords

Cinnamomum longepaniculatum, Endophytic Fungal Elicitor, Salicylic Acid, Hydrogen Peroxide, Suspension Cell

1. Introduction

Cinnamomum longepaniculatum (Gamble) N. Chao is an evergreen tree of the family *Cinnamomum*, and it is one of the second-class national key protected

plants. It is mainly distributed in Sichuan and is rich in natural aromatic oils. The whole body is treasure [1]. Further research found that essential oil from camphor oil has analgesic, anti-cancer and antibacterial effects [2] [3]. Endophyte of a plant is a type of fungus that grows in a plant and does not cause any pathological characteristics of the plant itself during the entire growth cycle or part of a healthy plant's growth cycle [4]. The first endophytic fungus was discovered in 1898. Until 1993, Sierle *et al.* isolated an endophyte capable of producing paclitaxel from *Taxusb revifolia* bark. Fungi (*Taxomyces andreanae*) [5]. Although the number of researchers has increased, the main research directions are still focused on the secondary metabolites of endophytic fungi and the diversity of biological activity and the relationship between endophytic fungi and the host [6] [7] [8]. In recent years, researchers have isolated endophytic fungi from a variety of plants, and some can produce the same substances or components as the host [9]. Plant endophytic fungi can promote the growth of host plants, enhance host resistance and reversibility, promote the synthesis and accumulation of effective substances or ingredients in plants, participate in the formation of essential substances in plants, etc. [10] [11].

At present, research finds that endophytic fungi of *C. longepaniculatum* can help the synthesis and accumulation of essential oil in *C. longepaniculatum* cells, and can increase the activity of protective enzymes of free radical scavenging system and can further promote the up-regulation of gene expression levels of key enzymes during the monoterpene synthesis [12] [13]. But so far, the research on the effect mechanism of endophytic fungi and essential oils of *C. longepaniculatum* is not enough, so it limits the endogeneity to a certain extent understanding of fungi affecting essential oil quality. Salicylic acid (SA) is hydroxybenzoic acid, a phenolic compound that is commonly found in plants [14]. Its biosynthetic pathway is mainly shikimic acid pathway in plants and is related to phenylalanine ammonia lyase (PAL). When plants induce necrosis symptoms through biotic or abiotic stress factors, PAL and other enzymes are induced in this pathway. The combined effect of these enzymes resulted in the accumulation of SA [15]. Today, as a signal molecule, H_2O_2 is receiving more and more attention [16]. At the same time, H_2O_2 considered being one of the main intracellular messenger substances in the process of endogenous fungal elicitors inducing plant cell defense responses. Recent studies have also begun to focus on H_2O_2 for various plant by-products accumulated effects, such as betulin [17], atractylodesin [18], etc.

In order to better understand the signal transduction mechanism of endophytic fungal elicitors through salicylic acid (SA) and hydrogen peroxide (H_2O_2) to mediate the essential oil synthesis of *C. longepaniculatum* suspension cells to promote *C. longepaniculatum* oil, in this study, the suspension cells of *C. longepaniculatum* were used as the research object to study the synthesis of essential oil from *C. longepaniculatum* cells (this study mainly examined 1,8-eucalyptus) and the SA and H_2O_2 mediate the relationship between endophytic fungal elicitors, further revealing that SA and H_2O_2 mediate endophytic fungal elicitors (2)1) as signal molecules that influence essential oil production in

C. longepaniculatum suspension cells, and it will provide a reference for the subsequent research on the signal transduction mechanism of endophytic fungal elicitors to mediate the synthesis of monoterpenoids through other signal molecules.

2. Materials and Methods

2.1. Materials

The *C. longepaniculatum* was collected from the *C. longepaniculatum* base of Hongyan Mountain in Yibin, and an endophytic fungus 2J1 (*Penicillium commune*) was isolated from the *C. longepaniculatum* plant and identified in the early stage. Frozen storage using glycerol tube storage method.

2.2. Method

2.2.1. Establishment of Suspension System of *C. longepaniculatum*

Select the fresh and tender *C. longepaniculatum* leaves soaked in washing powder water for 5 minutes, then place them under the faucet, rinse them with running water, soak in 75% alcohol for 15 s, rinse three times with sterile water; disinfect with mercury for 8 minutes, and finally rinse at least 5 times with sterile water. The inoculated explants were light cultured at about 25°C. And then the callus was subcultured twice after the callus induction was completed. The well-grown and loosely-brown callus was inoculated into a conical flask containing 50 mL B5 liquid culture medium, at about 25°C, cultivate with shading and shaking at 120 r/min speed. Subsequent once every 2 weeks, at least twice.

2.2.2. Preparation of Endophytic Fungal Elicitors

The 2J1 was inoculated on the culture PDA medium using a plate streak method. It was sealed and stored in a plastic wrap and cultured at 28°C for seven days. Activated endophytic fungi were inoculated into the PDA liquid culture medium, set the temperature at 28°C, the rotation speed was 130 r/min, and cultured for 7 days in suspension and shaking. The gauze was used to filter and isolate the bacteria from the fermentation broth, and the bacteria were ultrasonically broken. Then, it is mixed with the fermentation broth, filtered under reduced pressure, and finally the filtrate is put into a high-temperature autoclave, and autoclaved at 121°C for 20 minutes to prepare an endophytic fungal elicitor. The total sugar was used to calibrate the elicitor concentration, and the content of endophytic fungal elicitor sugar at a concentration of 40 mg/L was determined by the fluorenone-sulfuric acid method. (Glucose standard curve: $y = 0.1436x + 0.0119$, $R^2 = 0.9919$).

2.2.3. Data Measurement Method

Accurately weigh 0.3 g of dried *C. longepaniculatum* suspension cells, add cyclohexane to cold soak overnight (the ratio of cyclohexane to cells is 4:1), and then perform ultrasonic extraction for 30 min. Centrifuge on the centrifuge for 4 min at a speed of 5000 r/min, extract the supernatant, make up to 5 mL. And determine the content of essential oil. Analyze by GC-MS. The chemiluminescence method was used to determine the H₂O₂ concentration [18]. Determined

the SA concentration by high performance liquid chromatography (HPLC) [19].

2.2.4. Exogenous Substance Addition Method

C. longepaniculatum suspension cells were cultured to the 7th day with endophytic fungal elicitor 2J1 and substances filtered through a 0.22 μm microporous membrane, namely SA and SA synthesis inhibitor AOPP

(L- α -aminooxy- β -phenylpropionic acid), H_2O_2 , catalase (CAT); of which the addition concentration of exogenous SA, H_2O_2 is 5 mmol/L, the addition concentration of AOPP and CAT is 20 mmol/L, the addition time 20 minutes before the endogenous fungal elicitor or exogenous was added. The suspension cells of each control group were added with an equal volume of PDA liquid medium.

3. Result

3.1. Effect of Endophytic Fungal Elicitors on SA, H_2O_2 and Essential Oil in Suspension Cells of *C. longepaniculatum*

Different concentrations of inducers were added to the suspension cells of *C. longepaniculatum* cultured for 7 days, with a concentration gradient of 0, 20, 40, 60, 80 mg/L. The endophytic fungi 2J1 induction of different concentrations was detected the effect of *C. longepaniculatum* suspension cells on the release amount of SA and H_2O_2 after 21 h of cultivation of *C. longepaniculatum* suspension cells. The amount of essential oil synthesis was measured at 14 days after treatment. The results are shown in **Figures 1-3**.

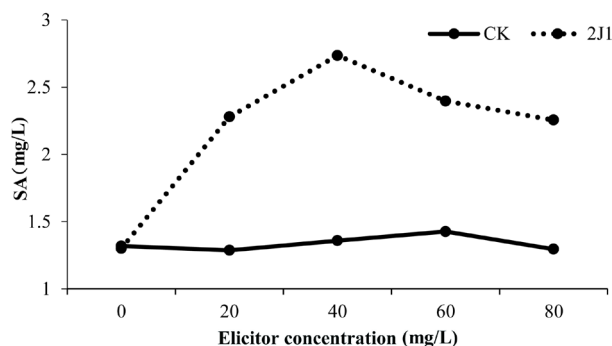


Figure 1. Effect of different inducer concentrations on SA accumulation.

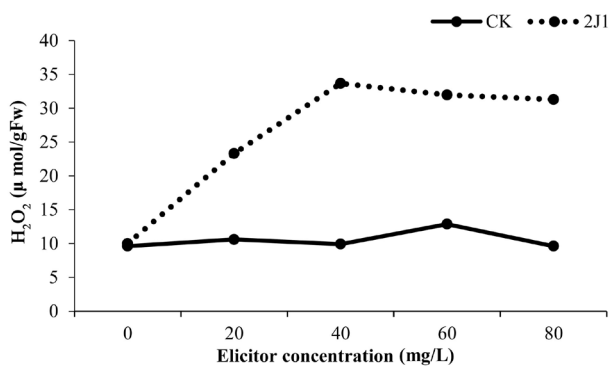


Figure 2. Effect of different inducer concentrations on H_2O_2 accumulation.

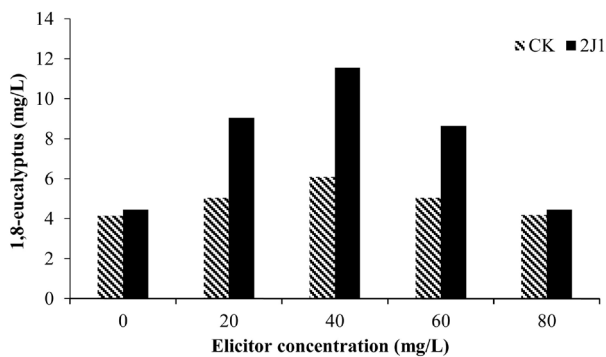


Figure 3. Effect of different elicitor concentrations on 1, 8-eucalyptus oil content.

The comparison shows that when the concentration of the inducer is less than 40 mg/L, the accumulation of SA and H₂O₂ gradually increases, and when the concentration of the inducer is 40 mg/L, the accumulation amount of SA and H₂O₂ reached a peak, and when the concentration was greater than 40 mg/L, the accumulation amount of SA gradually decreased. This phenomenon showed that the accumulation of essential oil from suspension cells of *C. longepaniculatum* was similar to the dependence of the endogenous fungal 2J1 elicitor concentration. Detect the effect of endophytic fungus 2J1 elicitor on SA, H₂O₂ release and essential oil synthesis in *C. longepaniculatum* suspension cells for 24 h. The results are shown in **Figure 2**, **Figure 4-6**.

In summary, it can be seen that the suspension cell of *C. longepaniculatum* treated with an appropriate amount of endogenous fungal 2J1 elicitor, the best induction effect was achieved on the 21st day, and the essential oil produced was significantly increased; but the induction time was too long, which was not conducive to *C. longepaniculatum* suspension accumulation of essential oil in cells.

3.2. The Role of SA and H₂O₂ in Promoting the Synthesis of Essential Oil from *C. longepaniculatum* Suspension Cells by Endogenous Fungal Elicitors

Add endogenous fungal 2J1 elicitors, exogenous SA, 2J1 and AOPP, 2J1 and CAT, SA and AOPP, H₂O₂ and CAT, to detect the accumulation of SA (**Figure 7**), H₂O₂ (**Figure 8**) and the synthesis of essential oil (**Figure 8**) were compared with the blank group without any addition. The endophytic fungus 2J1 inducer was added at a concentration of 40 mg/L. The source SA, H₂O₂ was 5 mmol/L, AOPP, CAT was 20 mmol/L. The detection time of SA and H₂O₂ was 21 h after treatment, the detection time of the synthetic amount of and the essential oil (1,8-eucalyptus) was 14 days after treatment.

It can be seen from the comparison that the addition of the endophytic fungus 2J1 elicitor can promote the accumulation of SA and the accumulation of essential oil. The addition of exogenous SA can promote the increase of SA concentration in the suspension cells of camphor, and the addition of exogenous H₂O₂ can promote the increase of H₂O₂ concentration and promote the accumulation of essential oil. Adding endophytic fungus 2J1 inducer and AOPP will inhibit the

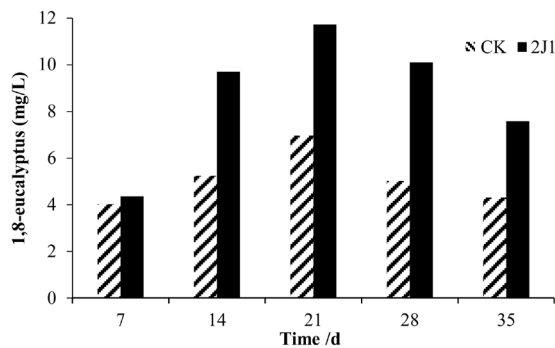


Figure 4. Effect of 2J1 on 1, 8-eucalyptus oil synthesis.

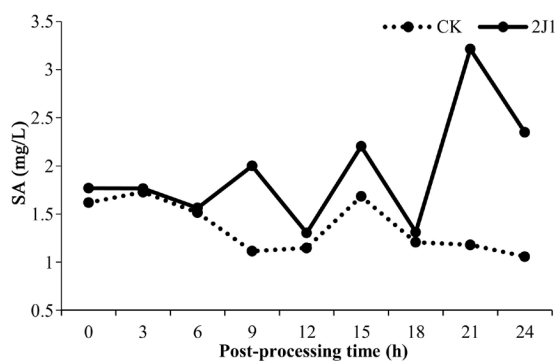


Figure 5. The effect of 2J1 on the amount of SA.

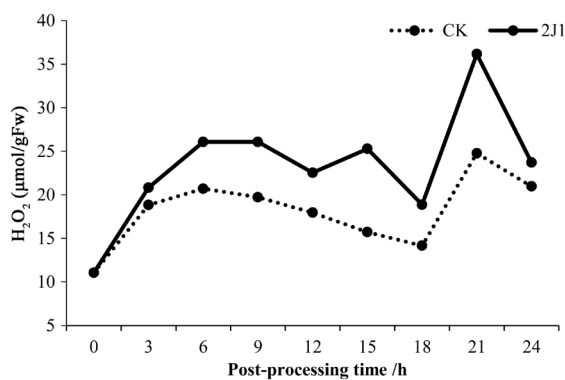


Figure 6. Effect of 2J1 on the amount of H₂O₂.

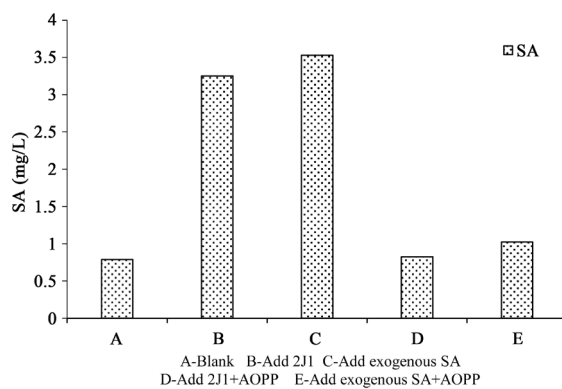


Figure 7. Effect of SA on the amount of SA in suspension cells of *C. longepaniculatum*.

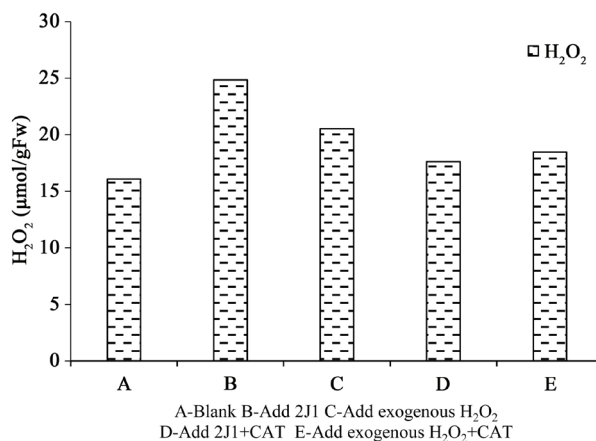


Figure 8. Effect of H₂O₂ on the amount of H₂O₂ in *C. longepaniculatum* suspension cells.

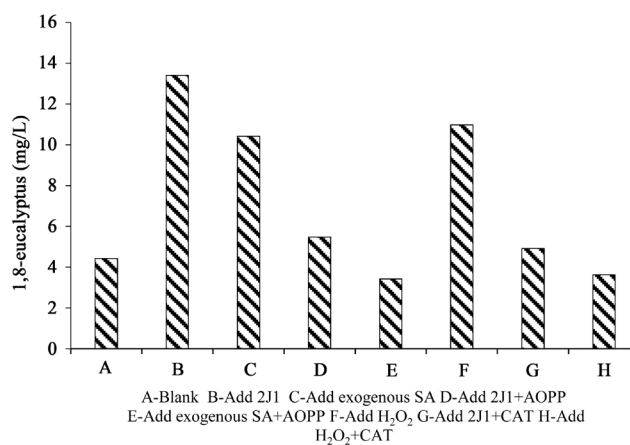


Figure 9. Effect of SA and H₂O₂ on the amount of essential oil synthesis in suspension cells of *C. longepaniculatum*.

accumulation of SA, The addition of endophytic fungus 2J1 crude elicitor and CAT can inhibit the accumulation of H₂O₂ in *C. longepaniculatum* suspension cells, and at the same time, it will partially inhibit the synthesis of essential oil, but it cannot completely inhibit it. Adding exogenous SA and AOPP, the accumulation of SA in suspension cells increased slightly compared to the control group. The accumulation of H₂O₂ in suspension cells of exogenous H₂O₂ and CAT slightly increased compared with the control group, the content of essential oil decreased slightly. This shows that AOPP can inhibit the promotion of essential oil synthesis by endogenous fungal elicitors and exogenous SA, and CAT can inhibit the promotion of essential oil synthesis by endogenous fungal elicitors and exogenous H₂O₂.

3.3. Interactions of SA and H₂O₂ in Endophytic Fungal Elicitors to Promote the Synthesis of Essential Oil from *C. longepaniculatum* Suspension Cells

Although the above experimental results can confirm that SA and H₂O₂ can be used as signal molecules to mediate the endophytic fungal elicitor to promote

the synthesis of essential oil from *C. longepaniculatum* suspension cells, the relationship between the two during the mediation process is not clear. In this study, the suspension cells of *C. longepaniculatum* were treated for 7 days, and a blank group, a control group (with the addition of the endophytic fungus 2J1) and 3 experimental groups (with the addition of the endophytic fungus 2J1 inducer and AOPP; 2J1 elicitor and CAT; 2J1 elicitor, AOPP, and CAT were added at the same time) were established to detect the accumulation of SA and H₂O₂ and the Synthetic situation of essential oil (1,8-eucalyptus) in the *C. longepaniculatum* suspension cells, respectively. The concentration of the inducer is 40 mg/L, and the concentration of AOPP and CAT is 20 mmol/L. The time for detecting the release amount of SA and H₂O₂ from *C. longepaniculatum* suspension cells was 21 h after treatment. The time for detecting the synthesis time of essential oil from *C. longepaniculatum* cells was 14 days after treatment. The experimental results are shown in Figures 10-12.

According to the experimental results, it can be seen that in the experimental group added SA inhibitor AOPP, the accumulation of SA and the synthesis of

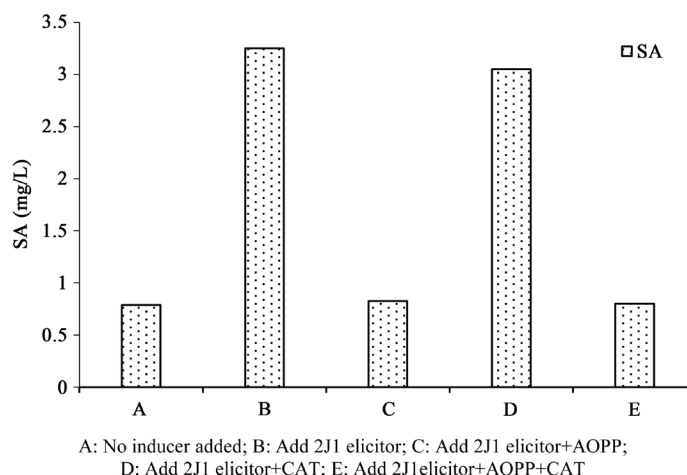


Figure 10. The accumulation of SA in the suspension cells of *C. longepaniculatum*.

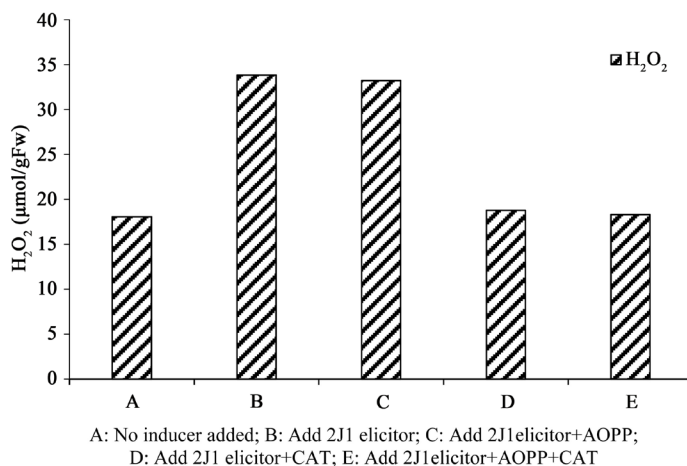


Figure 11. Accumulation of H₂O₂ in suspension cells of *C. longepaniculatum*.

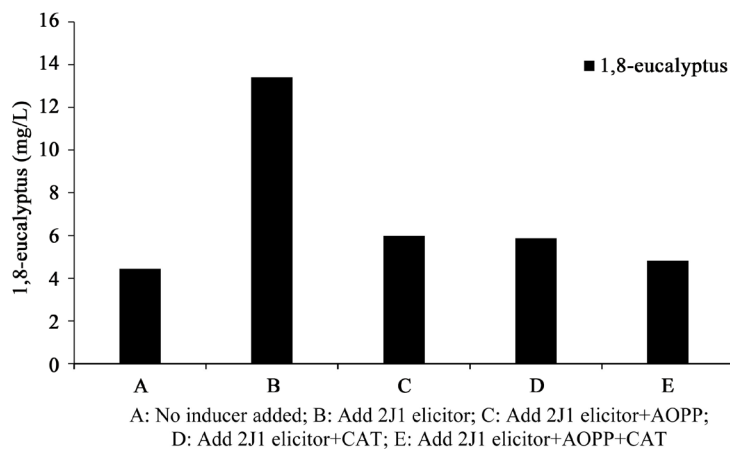


Figure 12. Synthesis of essential oil in *C. longepaniculatum* suspension cells.

essential oil caused by the endogenous fungal 2J1 elicitor were inhibited, but the accumulation of the H_2O_2 caused by 2J1 has no significant effect; the addition of CAT can simultaneously inhibit the accumulation of H_2O_2 and essential oil caused by the 2J1 elicitor, but has no significant effect on the accumulation of SA. At the same time, the addition of 2J1, AOPP, and CAT inhibits the release of SA and H_2O_2 caused by the addition of 2J1, AOPP, and CAT, which is more significant than the inhibitory effect of 2J1 and a single inhibitor. Even so, the addition of 2J1, AOPP, and CAT at the same time also cannot completely inhibit the accumulation of essential oil in *C. longepaniculatum* suspension cells caused by the 2J1 elicitor.

4. Discussion

Since endophytic fungal elicitors belong to extracellular materials and cannot directly enter the cell to play a role, the process of endophytic fungal elicitors to influence the secondary metabolism of plant cells through signal pathways will first identify and bind to the plant specific receptors on the cell membrane, change the structure of the cell to promote the production of specialized intracellular messenger substances. These messenger substances can regulate the expression of related genes in the nucleus through a series of signal transduction pathways. Finally, the defensive secondary metabolic system is activated, and the synthesis of secondary metabolites [20]. Based on this theory, we can speculate that after treatment of *C. longepaniculatum* by the endogenous fungal 2J1 elicitor, the accumulation of SA and H_2O_2 may promote the early reaction in the synthesis of essential oil from *C. longepaniculatum*, and SA and H_2O_2 act as intracellular messenger substances and undergo a series of reactions, which promotes synthesis of essential oil in *C. longepaniculatum*.

Studies have found that plant cells can produce a variety of signal molecules in the cell after being stressed by stressors such as inducers, and resist the external stress signal through corresponding signal pathways [21]. With more and more intensive research of the signal molecule and signal transduction mechanism

was conducted, SA and H₂O₂ were found to play important signaling molecules in many plants. This study also shows that the SA pathway and the H₂O₂ pathway exist two signal pathways in *C. longepaniculatum* cells that simultaneously affect the essential oil synthesis of *C. longepaniculatum* suspension cells. In the study of these two pathways mediating the role of endogenous fungal elicitors in promoting the essential oil synthesis of *C. longepaniculatum* suspension cells, it was found that the addition of SA inhibited agent AOPP had no significant effect on the release of H₂O₂ caused by the 2J1 inducer, and the addition of the quencher CAT with H₂O₂ did not significantly affect the accumulation of SA. There are no obvious upstream and downstream relationships in this approach.

Although this study further studied the role of SA and H₂O₂ mediated endogenous fungal elicitors on the synthesis of essential oil from *C. longepaniculatum*, there are three aspects that can be further studied: first, the crude fungal elicitor is the filtrate inactivator of homogenized hyphae, and the endophytic fungal elicitor is divided into 4 categories: oligosaccharides, glycoproteins, proteins, unsaturated fatty acids, and the components are more complex. In this experiment, it is speculated that oligosaccharides are one of the more common elicitors [22]. In order to fully understand the induction of elicitors, it is necessary to further isolate and purify the crude endophytic fungus 2J1 and to detect the structure, explore a better preparation method [23]. Second, in this study, only 1,8-eucalyptol in essential oil was detected, which represented the synthesis amount of essential oil, and the detection of the products was not comprehensive, so it is possible to further study the role of the two signal regulation pathways of SA and H₂O₂ in the synthesis of different essential oil components. Finally, add AOPP and CAT, and after double inhibitor treatment, the synthesis and accumulation of essential oils induced by the endophytic fungal crude elicitor in suspension cells was reduced to zero and not completely inhibited, indicating that there are also pathways other than SA and H₂O₂ in the endogenous fungal 2J1 elicitor to promote the accumulation and accumulation of essential oil in *C. longepaniculatum* suspension cells. The synthetic pathway is very complicated and requires further research.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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