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Effect of H_2O_2-Mediated Endophytic Fungal Elicitors on Essential Oil Accumulation in Suspension Cells of *Cinnamomum longepaniculatum*

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Abstract

Hydrogen peroxide (H_2O_2) is a signal molecule that plays a crucial role in plant secondary metabolism. In order to explore the signaling mechanism of endophytic fungal elicitors (*Penicillium commune* 2J1) for promoting 1,8-eucalyptus accumulation in *C. longepaniculatum*, changes in the contents of H_2O_2 and 1,8-eucalyptus were investigated after the addition of elicitors to the *C. longepaniculatum* cultures. The experimental results showed that the 1,8-eucalyptus contents in *C. longepaniculatum* cells were increased upon addition of the endophytic fungal elicitors into the culture. It's maybe through Hydrogen peroxide (H_2O_2) signal molecule. When different concentrations of elicitors were added to *C. longepaniculatum* suspension cells, the changes in the content of H_2O_2 and 1,8-eucalyptus showed the same trend, and both reached the highest value at 40 mg/L of elicitor concentration, indicating that the endogenous fungal elicitors affect the accumulation of 1,8-eucalyptus through H_2O_2 signaling molecular pathway. When CAT was added, the 1,8-eucalyptus decreased but was not completely inhibited, indicating that the elicitors also have other signaling pathways.

Subject Areas

Biochemistry, Biotechnology

Keywords

Endophytic Fungal Elicitor, H_2O_2, 1,8-Eucalyptus, Signal Transduction, CAT

1. Introduction

Essential oil is a kind of natural spice oil and Yibin government attaches great importance to the development of *C. longepaniculatum* resources [1] [2]. The essential oil extracted from Yibin's eucalyptus is mainly 1,8-eucalyptus [3], which is widely used in many industries. Endophytic fungus has a variety of species and roles [4]-[10], which play an important role in the synthesis of volatile substances [11] [12]. H_2O_2 is a universal second messenger for plants in response to biotic and abiotic stresses. Studies have shown that H_2O_2 signaling pathways play an important role in the synthesis of volatile substances [13]-[18].

In this study, the research object is endophytic fungi of the *C. longepaniculatum*, studies the relationship between the synthesis of the main volatile oils (1,8-eucalyptus) and amount of H_2O_2 by adding endophytic fungi elicitors (*Penicillium commune* 2J1) [19] [20] [21], to reveal whether the endogenous fungi could regulate the accumulation of volatile oil (1,8-eucalyptus) in *C. longepaniculatum* through the molecular pathway of H_2O_2. The transduction mechanism provides a foundational theory for the further development and utilization of endophytic fungal resources.

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. It was preserved in PDA medium.

2.2. Method

2.2.1. Establishing the *C. longepaniculatum* Suspension Cell System

Collect fresh *C. longepaniculatum* leaves, and then disinfect them with washing powder water, running tap water, 75% alcohol, sterile water, mercury, and sterile water. The inoculated explants were light cultured at about 23˚C. After the callus induction was completed, subcultured twice. The well-grown and loosely-brown callus were inoculated into a 150 mL Erlenmeyer flask containing 50 mL of B5 medium at 25˚C, 120 r/min rotation speed, shading and shaking culture. 14 d subcultured once, followed by 2 times.

2.2.2. Preparation of Endophytic Fungus Elicitor

The 2J1 strain stored in the test tube was inoculated on potato medium and cultured at 28˚C for seven days. The activated endophytic fungi were inoculated into liquid PDA medium, and cultured at 28˚C, 130 r/min for 7 d. After the fermentation, the cells were separated from the fermentation broth by gauze. After crushing and homoge-nizing, it was mixed with the fermentation broth, suction filtered, and the filtrate was autoclaved at 121˚C for 20 min to prepare an endophytic fungus inducer. The content of the elicitor sugar was then determined by

the fluorenone-sulfuric acid method.

2.2.3. Determination of H_2O_2 Concentration in Suspension Cells of *C. longepaniculatum*

First draw the calibration curve of the H_2O_2 standard solution, then take 1 g of the cultivated *C. longepaniculatum* suspension cells, add 1 ml of acetone treated with low temperature in advance. A small amount of quartz sand was added to slurry, load it into a centrifuge tube, and centrifuge in a high-speed refrigerated centrifuge at 3000 r/min and 10˚C for 10 min to extract the supernatant which is the sample extract. Take 1 ml of sample extraction solution, 0.1 ml of 5% titanium sulfate was added to it. 0.2 ml of concentrated ammonia water was added to the mixture and wait for precipitation to form, then centrifuge at 5000 rpm/min and 10˚C for 10 min, discard the supernatant. The precipitate was washed with acetone to remove the cytochrome. After removing the pigment, 5 ml of 2 mol/L sulfuric acid was added to the precipitate, and the precipitate was completely dissolved, and the absorbance was measured at a wavelength of 415.

2.2.4. Extraction and Determination of Volatile Oil from Suspended Cells of *C. longepaniculatum*

Accurately weigh 0.3 g of suspended cells, add 4 times (1:4) of cyclohexane overnight cold soak, then ultrasonic extraction for 30 min, centrifugation at 5000 r/min at 25˚C for 4 min, then take the supernatant, the volume was adjusted to 5 mL with cyclohexane. The liquid was extracted with a syringe, and the filter was filtered into a sample bottle to determine the content of the volatile oil, which was analyzed by GC-MS.

2.2.5. Method for Adding Exogenous H_2O_2

Under sterile conditions, 25 μmol/g hydrogen peroxide solution was added to the suspended cells through a 0.22 μm microporous filter.

2.2.6. Addition Method of Catalase CAT

Under sterile conditions, suspended cells were passed through a 0.22 μm microporous filter, and the Catalase CAT was added 20 min before the endogenous fungal inducer or exogenous H_2O_2 was added.

3. Results

3.1. Effect of Endophytic Fungal Elicitors on H_2O_2 and Volatile Oil Accumulation

The experimental group adds 40 mg/L endophytic fungus 2J1 elicitor to the cultivated *C. longepaniculatum* cells, and the control group was added to an equal amount of PDA medium. As for the date measured, the concentration of H_2O_2 was measured by every 3 h and the content of volatile oil was measured by every 7d. The results shown in the figure are the average of 3 independent experiments.

From **Figure 1**, it can be seen that H_2O_2 can be produced by the elicitor, the

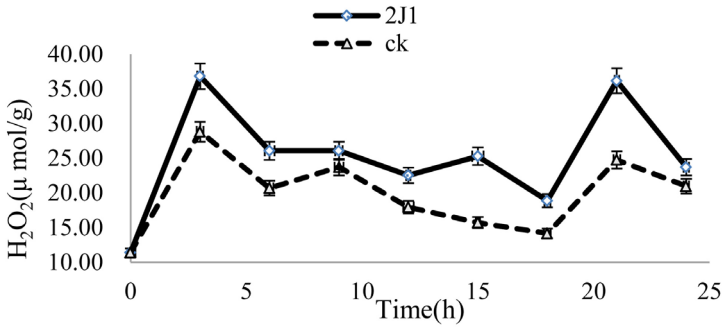

Figure 1. Endophytic fungal elicitors induce H_2O_2 bursts of *C. longepaniculatum* suspension cells.

control group on the suspension cell of *C. longepaniculatum* has the same function. The content of H_2O_2 in the experimental group is always higher than that in the control group, and the experimental group completed a rapid increase at 18 h. It is indicating that the elicitor has a certain regulation effect on H_2O_2 production. From **Figure 2**, the accumulation of 1,8-eucalyptus in the control group and the experimental group both increased, it shows that has a substance in the control group takes a slight effect. And the content of 1,8-eucalyptus in the experimental group was higher than that in the control group, indicating that the endophytic fungus 2J1 elicitor played a significant role in accumulating *C. longepaniculatum*. On the 21st day, the maximum amount of volatile oil accumulation approximately was 2 times of the control Group.

In summary, it can be seen that the treatment of *C. longepaniculatum* suspension cells by the endogenous fungal elicitor of 2J1 produce H_2O_2 in the cells, and promotes the accumulation of volatile oil from *C. longepaniculatum*. This study indicated that the effect of endogenous fungi to volatile oil accumulation was related to the H_2O_2 signaling molecular pathway.

3.2. Effects of Different Concentrations of Endophytic Fungal Elicitors on H_2O_2 and Volatile Oil Accumulation

Different concentrations (0, 20, 40, 80 mg/L) of endophytic fungus 2J1 elicitors were added to the cultivated *C. longepaniculatum* suspension cells. The control group was added with an equal amount of PDA medium. According to the experimental data above-mentioned, the concentration of H_2O_2 in the suspension cells treated with endophytic fungal elicitor at different concentrations was measured at 21 h, and the accumulation of volatile oil in the suspension cells of *C. longepaniculatum* treated with different concentrations of endophytic fungal elicitors was measured on the 14th day. The measurement results are shown in **Figure 3** and **Figure 4**.

As shown in **Figure 3** and **Figure 4**, the suspension cells of *C. longepaniculatum* were treated with endophytic fungal elicitors of different concentrations, and it was found that the H_2O_2 bursts when the elicitor concentration was 0 - 40 mg/L. The accumulation of volatile oil from *C. longepaniculatum* was positively correlated with the concentration of elicitor. The accumulations of volatile oil in

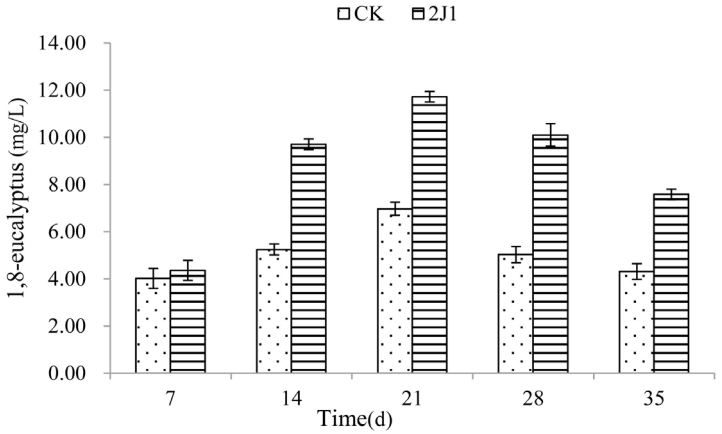

Figure 2. Effect of endogenous fungal elicitors on 1,8-eucalyptus content in suspension cells of *C. longepaniculatum.*

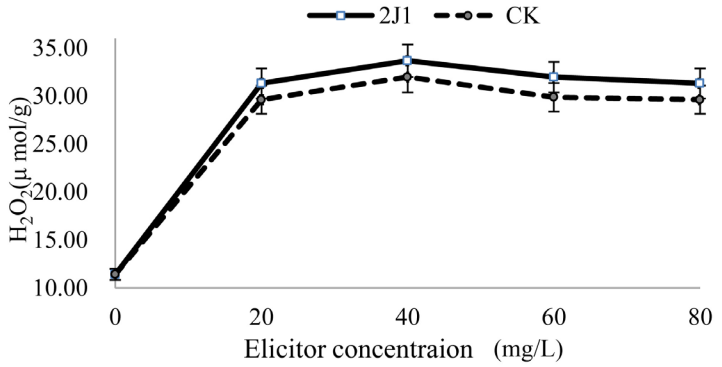

Figure 3. Effect of different elicitor concentrations on H_2O_2 bursts of *C. longepaniculatum* suspension cells.

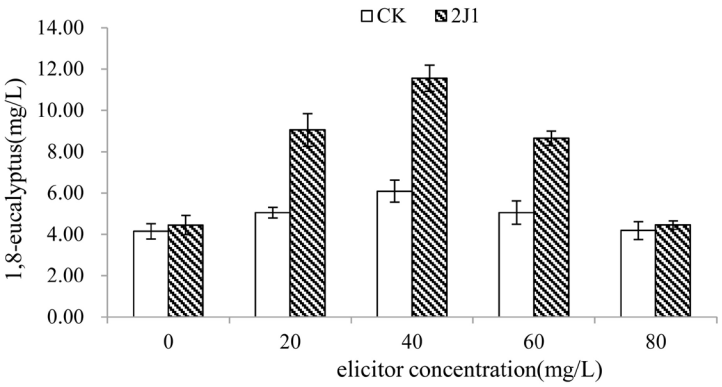

Figure 4. Effects of different elicitor concentrations on 1,8-eucalyptus production in suspension cells of *C. longepaniculatum.*

C. longepaniculatum were negatively correlated with the concentration of elicitor when the concentration of elicitor was 40 - 80 mg/L. The correlation between the concentration of H_2O_2 and the endogenous fungal 2J1 elicitor is consistent with the correlation between the accumulations of volatile oil in suspension cells of *C. longepaniculatum* and the concentration of the 2J1 elicitor in endophytic

fungi. The elicitor concentration is 40 mg/L had the best effect. It is indicated that H_2O_2 was involved in the inducer in accelerating the accumulation of volatile oil from *C. longepaniculatum*. It is suggested that the endogenous fungal 2J1 elicitor was responsible for the accumulation of volatile oil from *C. longepaniculatum*, but whether the H_2O_2 signaling molecular pathway is the only pathway needs further study.

3.3. The Effect of H_2O_2 in Endogenous Fungal Elicitors Promoting the Suspension Cells of *C. longepaniculatum* Volatile Oil Synthesis

In order to further investigate whether the H_2O_2 signaling molecular pathway is an endogenous fungal influence on the accumulation of volatile oil in *C. longepaniculatum*, and to explore whether the pathway is the only one. The conditions under which H_2O_2 affects volatile oil in *C. longepaniculatum* cells need to be further studied. The cultured the suspension cells of *C. longepaniculatum* were used as materials, 2 control groups and 4 experimental groups were set up. The concentration of H_2O_2 in the suspension cells of *C. longepaniculatum* (**Figure 5**) and the synthesis of volatile oil (**Figure 6**) were measured in 6 groups. The endophytic fungus 2J1 elicitor has a concentration of 40 mg/L and the PDA culture solution is concentrated to prepare the elicitor. The concentrations of exogenous H_2O_2 and CAT were 25 umol/ml and 100 mmol/ml. The time to detect the H_2O_2 concentration of the *C. longepaniculatum* suspension cells was 21 h after treatment, and the time to detect the synthesis of volatile oil from the *C. longepaniculatum* suspension cells was 14 d.

As can be seen from **Figure 5** and **Figure 6**: group E and group F is the lowest H_2O_2 accumulation of the suspension cells of *C. longepaniculatum*; in group E and group F 1,8-eucalyptus is lower than group C and D. It can be seen that CAT decomposes H_2O_2 to make 1,8-eucalyptus lower than the E group with the same conditions without CAT, Group F. It is indicated that the accumulation of volatile oil from *C. longepaniculatum* was affected to the endogenous fungal 2J1 elicitor through the H_2O_2 signaling molecular pathway. The inhibition of the

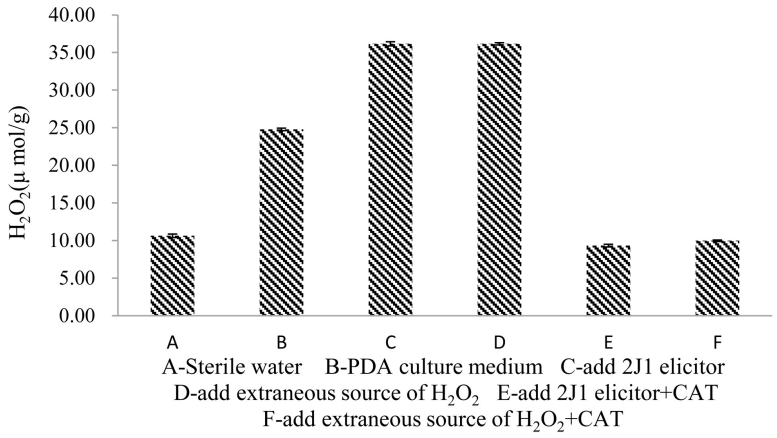

Figure 5. The burst of H_2O_2 in 6 groups.

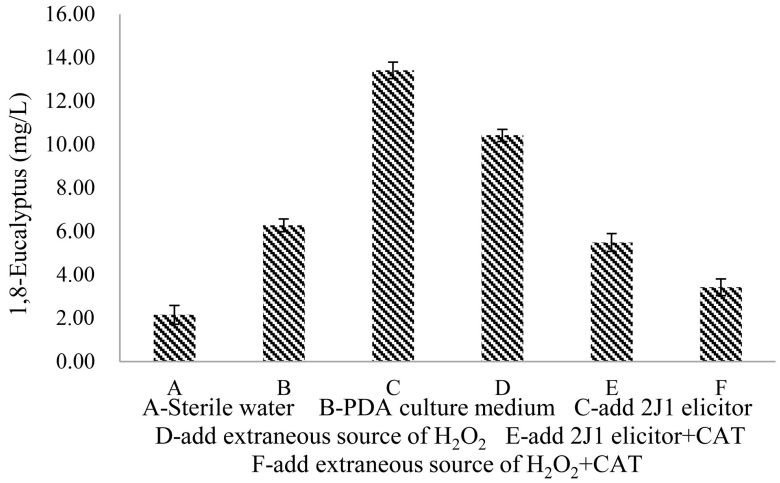

Figure 6. Synthesis of volatile oil in 6 groups.

H_2O_2 signaling molecular pathway reduces the accumulation of 1,8-eucalyptus. But 1,8-eucalyptus in group E is higher than those in groups A and F, indicating that there are other pathways affecting the accumulation of volatile oil in addition to the H_2O_2 signaling molecular pathway.

In summary, it can be seen that the elicitor affects the synthesis of volatile oil from *C. longepaniculatum* through multiple signaling molecular pathways. The signaling of H_2O_2 molecular pathway is one of the pathways. The addition of H_2O_2 enzyme CAT through this pathway inhibits the accumulation of a certain amount of volatile oil. But the accumulation of volatile oil cannot completely be inhibited. The presence of some other substances in the PDA culture medium can also promote the burst of H_2O_2 and the accumulation of essential oil.

4. Discussion

This experiment is based on the effect of the endophytic fungus 2J1 of *C. longepaniculatum* on the accumulation of 1,8-eucalyptus. The study found that the accumulation of volatile oil was promoted to add the 2J1 endophytic fungal elicitor to the *C. longepaniculatum* suspension cells. And H_2O_2 was also produced in the cells, indicating that the effect of endophytic fungi on the accumulation of volatile oil may be related to H_2O_2. It provided the possibility for the experiment to continue, and also made basic assumptions based on the results of the first step experiment, and the H_2O_2 signal molecular pathway may be a pathway for the effect of endophytic fungi on the accumulation of volatile oil in *C. longepaniculatum*.

In this study, different concentrations of endophytic fungal elicitors were used to treat *C. longepaniculatum* suspension cells, and it was found that the correlation between the concentration of H_2O_2 produced by suspension cells. And endophytic fungi 2J1 inducers were consistent with the correlation between the accumulation of 1,8-eucalyptus and the concentration of 2J1 elicitors of endophytic fungi. It was further confirmed that the effect of endophytic fungi on the

accumulation of volatile oil through H_2O_2 pathway, showed that the effect of endophytic fungi on the accumulation of volatile oil in *C. longepaniculatum* had H_2O_2 signal molecular pathway.

In order to further explore whether this pathway was the only pathway and how H_2O_2 participated in the process of endogenous fungal elicitors to promote the accumulation of volatile oil in suspension cells of *C. longepaniculatum*, the study added exogenous H_2O_2 and the experimental results showed that the addition of exogenous H_2O_2 could also increase the intracellular H_2O_2 concentration and the accumulation of volatile oil, indicating that the inducer acts on the upstream of H_2O_2. Besides, the accumulation of volatile oil is not completely inhibited after the addition of H_2O_2 enzyme CAT, indicating that there are other pathways besides the H_2O_2 signaling molecular pathway [22] [23].

During the course of this study, it was found that compared to the sterile water control group, PDA culture medium still exists substance that affects H_2O_2 burst and 1,8-eucalyptus accumulation, but the specific substances need to be further studied. This study proves that H_2O_2 is a signal molecular pathway that promotes the synthesis of 1,8-eucalyptus, but it remains to be explored which genes and enzymes work in the cell. At present, a lot of research on endophytic fungi in other plants is needed to reveal the mechanism of internal action and to study the gene level.

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Conflicts of Interest

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

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