



Effect of Ca^{2+} -Mediated Endophytic Fungal Elicitors on Essential Oil Accumulation in Suspension Cells of *Cinnamomum longepaniculatum*

Kuan Yan¹, Rui Li^{2*}, Qin Wei²

¹College of Sichuan Tea, Yibin University, Yibin, China

²College of Life Science and Food Engineering, Yibin University, Yibin, China

Email: *1156560931@qq.com

How to cite this paper: Yan, K., Li, R. and Wei, Q. (2019) Effect of Ca^{2+} -Mediated Endophytic Fungal Elicitors on Essential Oil Accumulation in Suspension Cells of *Cinnamomum longepaniculatum*. *Open Access Library Journal*, 6: e5764. <https://doi.org/10.4236/oalib.1105764>

Received: September 5, 2019

Accepted: September 14, 2019

Published: September 17, 2019

Copyright © 2019 by author(s) and Open Access Library Inc.

This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

Abstract

Calcium ion (Ca^{2+}) is a signal molecule that plays crucial roles in plant secondary metabolism. In order to explore the signaling mechanism of endophytic fungal elicitors (*Penicillium commune* 2J1) for promoting essential oil accumulation in *Cinnamomum longepaniculatum*, changes in the contents of Ca^{2+} and essential oil were investigated after the addition of elicitors to the *C. longepaniculatum* cultures. The essential oil contents in *C. longepaniculatum* cells were increased upon addition of the CaCl_2 into the culture. The concentration of Ca^{2+} was suppressed and the essential oil contents decreased after the Ca^{2+} channel blocker (LaCl_3) was added into the cell cultures. LaCl_3 did not completely inhibit essential oil accumulation in *C. longepaniculatum* cells induced by endophytic fungal elicitors. Based on these experimental results, adding elicitors were found to significantly increase Ca^{2+} concentration and essential oil synthesis in *C. longepaniculatum* cells. Reliable evidence that Ca^{2+} can effectively mediate endophytic fungal elicitors to promote essential oil synthesis in *C. longepaniculatum* cells is presented in this study. It is further demonstrated that endophytic fungal elicitors may facilitate essential oil synthesis in *C. longepaniculatum* suspension cells through other signal transduction pathways.

Subject Areas

Biochemistry, Biotechnology

Keywords

Endophytic Fungal Elicitor, Calcium Ion (Ca^{2+}), Suspension Cells, Signal Transduction, Essential Oil

1. Introduction

Essential oil is a kind of natural spice oil. The essential oil extracted from Yibin's eucalyptus is mainly 1,8 eucalyptus oil [1], which is widely used in many industries [2]. How to improve the production of essential oil, more effective, low cost and sustainable access to essential oil, has become an important content and new development direction of *C. longepaniculatum* resources and plant aromatic oil research. Endophytic fungus has a variety of species and roles [3] [4] [5] [6], which play an important role in the synthesis of volatile substances [7] [8] [9] [10] [11]. Calcium is a universal second messenger for plants in response to biotic and abiotic stresses. Studies have shown that calcium signaling pathways play an important role in the synthesis of volatile substances [12] [13] [14] [15].

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-eucalyptol, α -terpineol) and amount of Ca^{2+} by adding endophytic fungi elicitors, to reveal the signal transduction mechanism of endophytic fungal elicitors through Ca^{2+} -mediated synthesis of volatile oil in *C. longepaniculatum* suspension cells, and is a signal for subsequent study of endophytic fungal elicitors to mediate monoterpenoid synthesis via calcium signaling pathway. The transduction mechanism provides a reference and also has a certain reference significance for the signal transduction mechanism of endophytic fungi through the synthesis of monoterpenoids by other signaling 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. It was preserved in PDA medium [16].

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 was 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 strain 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 homogenizing, 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. Extraction and Determination of Essential Oil from Endophytic Fungi of *C. longepaniculatum*

The endophytic fungus of the *C. longepaniculatum* was removed from the culture flask under sterile conditions every 7 d and dried at 55 °C. Accurately weigh 0.3 g of endophytic fungus, 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 essential oil, which was analyzed by GC-MS. Chromatographic conditions: column temperature 60 °C; HP-5MS column, 30 m × 0.250 mm; injection volume 1 µL; temperature programming: starting from 60 °C, rising to 190 °C at a heating rate of 10 °C/min and maintaining 2 min, then, it is raised to 210 °C for 2 min at a heating rate of 5 °C/min, and then raised to 220 °C for 8 min at a heating rate of 10 °C/min. After injection into GC-MS, the standard curve is obtained, that is, the essential oil content is obtained. The response value was used to calculate the essential oil content (1,8-eucalyptol: $Y = 73,900X - 299,200$, $R^2 = 0.999$, α -terpineol $Y = 51,620X + 162,200$, $R^2 = 0.9992$).

2.2.4. Determination of Ca²⁺ Concentration in Suspension Cells of *C. longepaniculatum*

The *C. longepaniculatum* cells were dried at 70 °C, ground into a powder, and weigh 0.2 g into a Nitrifying tube. 10 ml of concentrated sulfuric acid-perchloric acid (5:1) was added to the mixture. Set overnight, digested in the fume hood the next day. The standard solution of Ca²⁺ was set, and then 2 mL of 5% LaCl₃ solution was added separately, and the volume was made up with ion-free water. Take 2 - 10 ml of the solution to be tested and blank into a 100 ml volumetric flask, add 2 mL of 5% LaCl₃ solution, and make up to volume with ion-free water. The concentration of Ca²⁺ was measured using a flame photometer [17] [18].

2.2.5. Method for Adding Exogenous Ca²⁺

The *C. longepaniculatum* suspension cells cultured for 14 days were added to a Ca²⁺ solution with a concentration of 10 mmol/l after sterilization.

2.2.6. Addition Method of Ca²⁺ Channel Blocker LaCl₃

The *C. longepaniculatum* suspension cells cultured for 14 days were used, and the Ca²⁺ channel blocker LaCl₃ was added 20 min before the endogenous fungal inducer or exogenous Ca²⁺ was added, and the concentration was 20 mmol/L [19].

3. Result

3.1. Endophytic Fungal Elicitors Induce Accumulation of Ca²⁺ and Essential Oil in Suspension Cells of *C. longepaniculatum*

40 mg/L of endophytic fungus 2J1 crude elicitor was added to the *C. longepaniculatum* suspension cell culture until the 7th day, and an equal amount of PDA medium was added as a control. The effects of endophytic fungus 2J1 crude elicitor on Ca²⁺ concentration and essential oil content in *C. longepaniculatum* suspension cells within 24 h were shown in **Figures 1-3**, respectively. The results shown in the figure are the average of 3 independent experiments.

It can be seen from **Figure 1** that the *C. longepaniculatum* suspension cells treated by the 2J1 crude elicitor can produce a change in Ca²⁺ concentration. The maximum intracellular Ca²⁺ concentration is 21 h after the elicitor treatment, and the Ca²⁺ concentration in the treated group at this time is about 1.66 times that of the control group. As shown in **Figure 2** and **Figure 3**, the amount of essential oil produced by the *C. longepaniculatum* cell suspension cells after the inducer treatment is significantly different from that of the control group, and the content of the cells grows with the growth of the cells, and the cells grow for 21 days, (14 d after treatment), the yield peaked. At this time, the cells treated with the inducer 2J1 produced 1,8-eucalyptus oil in an amount of 11.725 mg/L, and the produced α -terpineol was 4.577 Mg/L. The amount of volatile oil that is subsequently produced gradually decreases.

3.2. Effects of Different Concentrations of Endophytic Fungi Elicitors on Ca²⁺ Concentration Changes and Essential Oil Accumulation in Suspension Culture Cells

After 7 days of culture of suspension culture of *C. longepaniculatum*, 2J1 crude elicitors of endophytic fungi with different concentrations (0, 20, 40, 60, 80 mg/L) were added to detect the change of Ca²⁺ concentration and accumulation of volatile oil in suspension cells of *C. longepaniculatum*. The amount is shown in **Figures 4-6**. The results shown in the figure are the average of 3 experiments.

Figure 4 shows that the change in Ca²⁺ concentration in the suspension cells of *C. longepaniculatum* is dependent on the concentration of endophytic 2J1 elicitor. When the concentration of elicitor was 0 - 40 mg/L, the concentration of Ca²⁺ in the suspension cells of *C. longepaniculatum* increased gradually. When the concentration of inducer was 40 mg/L, the concentration of Ca²⁺ in the suspension cells of *C. longepaniculatum* peaked. When the concentration is greater than 40 mg/L, the intracellular Ca²⁺ concentration gradually decreases. This phenomenon is similar to the dependence of the accumulation of volatile oil in the *C. longepaniculatum* suspension cells on the endophytic fungal 2J1 elicitor concentration as shown in **Figure 5** and **Figure 6**. This suggests that the Ca²⁺ signaling pathway may be one of the signaling pathways that mediate endophytic fungal elicitors to promote the accumulation of volatile oil in *C. longepaniculatum* suspension cells.

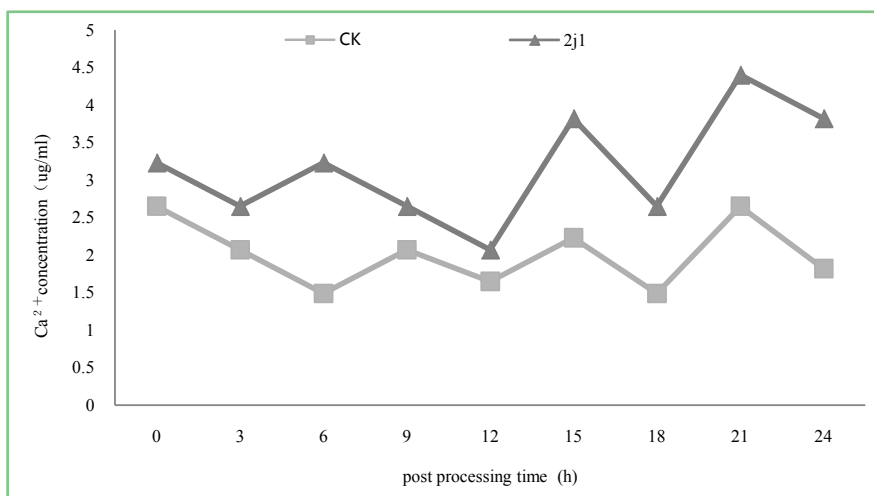


Figure 1. Endophytic fungal elicitors induce changes in Ca²⁺ concentration in suspension cells of *C. longepaniculatum*.

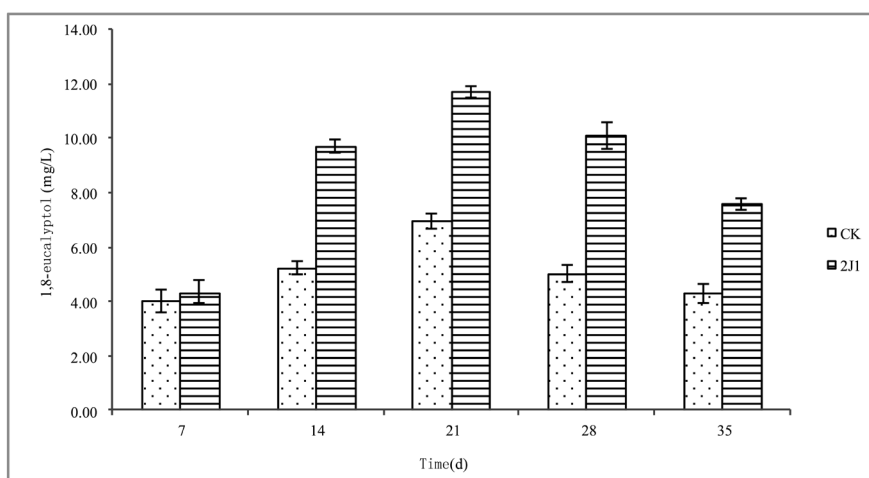


Figure 2. Effects of endophytic fungal elicitors on the production of 1,8-eucalyptol in suspension cells of *C. longepaniculatum*.

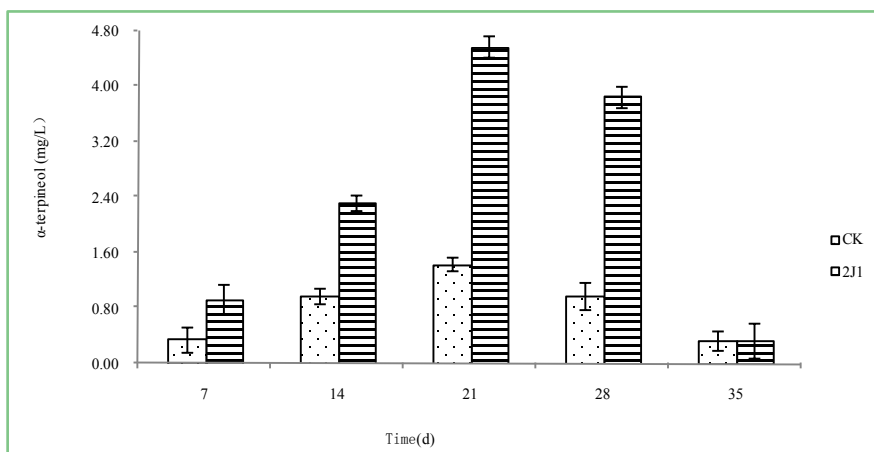


Figure 3. Effects of endophytic fungal elicitors on the production of α-terpineol in suspension cells of *C. longepaniculatum*.

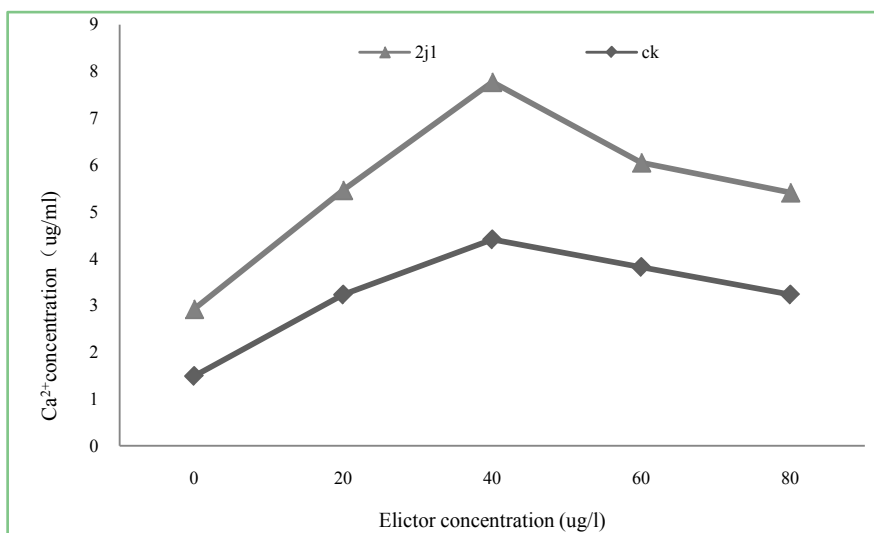


Figure 4. Effects of different elicitor concentrations on Ca²⁺ in suspension cells of *C. longepaniculatum*.

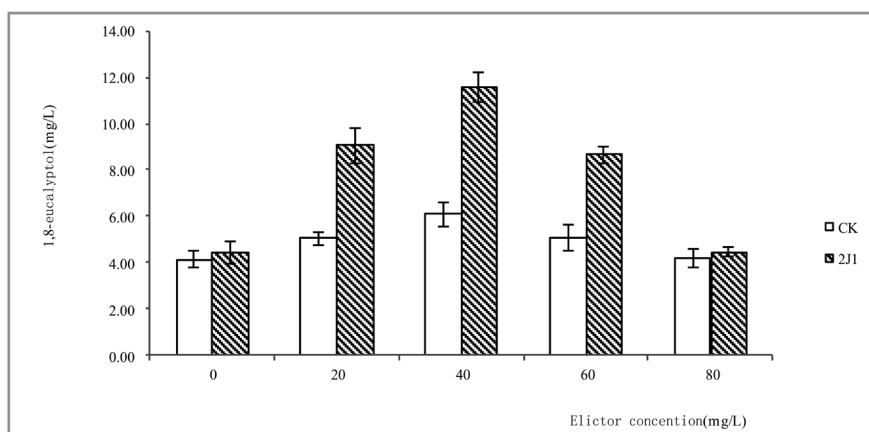


Figure 5. Effects of different elicitor concentrations on the production of 1,8-eucalyptus in suspension cells of *C. longepaniculatum*.

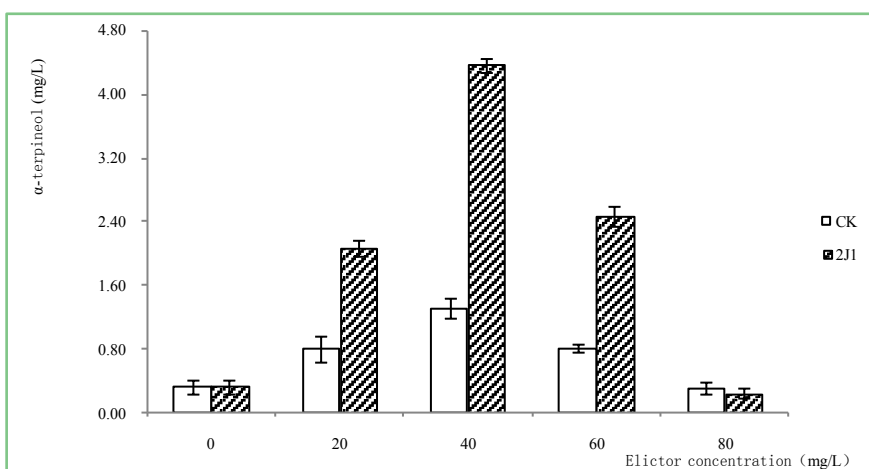


Figure 6. Effects of different elicitor concentrations on the production of alpha-terpineol in suspension cells of *C. longepaniculatum*.

3.3. Role of Ca²⁺ Endophytic Fungal Elicitor in Promoting Volatile Oil Synthesis from *C. longepaniculatum* Suspension Cells

In order to further study the role of Ca²⁺ in the endophytic fungus 2J1 crude elicitor in the synthesis of volatile oil from the suspension of *C. longepaniculatum*, the concentration of Ca²⁺ in the suspension of *C. longepaniculatum* in 5 groups was detected after 7 days of culture. The situation (Figure 7) and the synthesis of volatile oil (Figure 8), wherein the endophytic fungi 2J1 crude elicitor, exogenous Ca²⁺, LaCl₃ were added at a concentration of 40 mg/L, 10 mmol/L, 20 mmol/L.

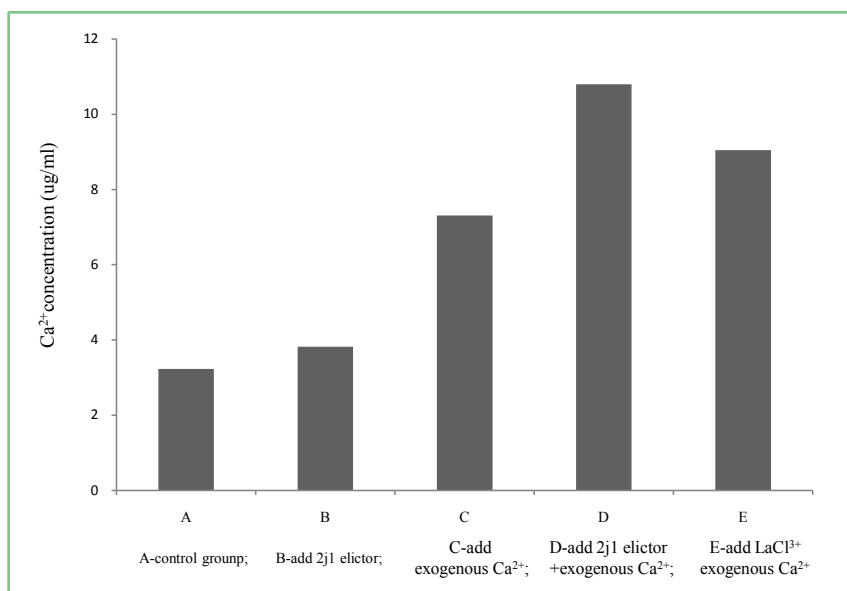


Figure 7. The changes of Ca²⁺ concentration in 5 groups.

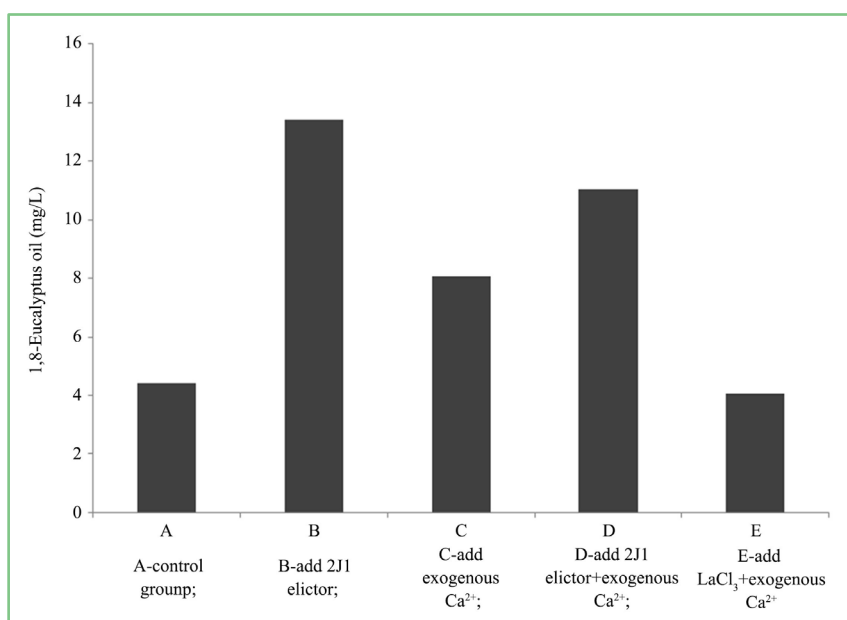


Figure 8. Synthesis of volatile oil in 5 groups.

As can be seen from **Figure 7** and **Figure 8**, in the control group without the inducer added, the concentration of Ca^{2+} in the suspension cells of the *C. longepaniculatum* was low, and the amount of 1,8-eucalyptus oil that can be detected was only 4.43 mg/L. Adding the endophytic fungus 2J1 crude elicitor can increase the concentration of Ca^{2+} in the suspension cells of *C. longepaniculatum* (compared with A and B in **Figure 7**); it can also promote the accumulation of volatile oil, and the 1,8-eucalyptol under the action of 2J1 elicitor, the amount of synthetic oil was 13.41 mg/L, which was 3.03 times that of the control group (compared with A and B in **Figure 8**). The addition of exogenous Ca^{2+} promoted the increase of Ca^{2+} concentration in the suspension of *C. longepaniculatum* (compared with A and C in **Figure 7**); it also promoted the accumulation of volatile oil (compared with A and C in **Figure 8**), but The promotion effect was inferior to the addition of the endophytic fungus 2J1 crude elicitor experimental group (comparison of A, B and C in **Figure 8**). Adding endophytic fungus 2J1 crude elicitor and exogenous Ca^{2+} promoted the increase of Ca^{2+} concentration in the suspension of *C. longepaniculatum*, and the Ca^{2+} concentration reached the maximum (compared with A and D in **Figure 7**), and also promoted the synthesis of volatile oil (compared with A and D in **Figure 8**). The concentration of Ca^{2+} in the suspension cells was increased after add exogenous Ca^{2+} and LaCl_3 compared with the control group, but the content of volatile oil decreased slightly (compared with A and E in **Figure 7**, A and in **Figure 8**). This indicates that LaCl_3 can inhibit the promotion of volatile oil synthesis by endophytic fungal elicitors and exogenous Ca^{2+} .

4. Discussion

It is generally believed that the signal transduction process of endophytic fungal inducer synthesis of secondary metabolites in plant cells is: endophytic fungal elicitor as an extracellular stimulator first recognizes and binds to specific receptors on plant cell membranes, thereby promoting the cells produce specific intracellular messenger substances and regulate the expression of related genes in the nucleus through corresponding signal transduction pathways [19], ultimately activating the defensive secondary metabolic system in the cells to promote secondary metabolite synthesis. Therefore, according to this theory, it can be speculated that the change of Ca^{2+} concentration in cells after 2J1 crude elicitor treatment may be the pre-reaction, and calcium as a common second messenger in plant cells, after a series of process reactions, eventually leading to the accumulation of volatile oil. Under stress conditions such as elicitors, plant cells can sense and transduce external stress signals through a variety of signaling molecules and signaling pathways [19]. Studies have shown that the calcium signaling pathway is located upstream of the NO signaling molecule [13]. The calcium signaling pathway is thought to be an important pathway for fungal elicitors to induce plant cell defense responses [12]. The results of this study indicated that the concentration of Ca^{2+} reached the peak at 21 h after culture of the

endophytic fungus 2J1 crude elicitor. At the same time, the endophytic fungus 2J1 crude elicitor was used to treat the suspension of *C. longepaniculatum*. After data analysis, it was found that the concentration of Ca^{2+} and the accumulation of volatile oil in the suspension of *C. longepaniculatum* showed a similar trend with the change of concentration; further study the role of Ca^{2+} on the synthesis of volatile oil in the suspension of *C. longepaniculatum* by 2J1 crude elicitor. The *C. longepaniculatum* suspension cells may exist a signal pathway regulating the synthesis of volatile oil: calcium signaling pathway. And this pathway has different effects in regulating the different components of synthetic volatile oil. Adding endophytic fungus 2J1 crude elicitor can promote the synthesis of α -terpineol to 3.31 times of the control group, while 1,8-eucalyptol can only increase by 1.90 times; addition of exogenous Ca^{2+} can also promote the increase of volatile oil, but the effect is not as good as after the addition of inducer. Moreover, the three experimental groups added with LaCl_3 showed that the synthesis of volatile oil was significantly inhibited.

Although this study has further studied the regulation mechanism of Ca^{2+} -mediated endophytic fungal elicitors on the synthesis of oil volatile oil, there are two aspects that can be further studied: the endophytic fungus crude inducer is used in this study. The filtrate of the mycelium is inactivated, and the endophytic fungal inducers are divided into four categories: oligosaccharides, glycoproteins, proteins, and unsaturated fatty acids. The components are complex, and it is presumed to be oligosaccharides in this experiment. Oligosaccharides are one of the more common inducers [20]. However, in order to fully exert the inducer effect, it is necessary to further isolate and purify the endophytic fungus 2J1 crude elicitor, and explore a better method for preparing elicitors [21]. The addition of LaCl_3 could not completely inhibit the accumulation of volatile oil from the endophytic fungus 2J1 crude elicitor on the suspension of *C. longepaniculatum*, indicating that endophytic fungal elicitors can also cause accumulation of volatile oil by other means, or it may be calcium signal. With multiple calcium signaling pathways, LaCl_3 may only inhibit one of the signaling pathways. Therefore, the synthetic pathway of plant secondary metabolites is very complicated, especially the involvement of calcium signaling pathways in secondary metabolic regulation is particularly complicated, and further research is needed.

5. Conclusion

In this study, the induction time, induced concentration of endophytic fungal elicitors and the addition of exogenous Ca^{2+} and LaCl_3 were investigated. The endophytic fungal elicitors were induced by Ca^{2+} to mediate the accumulation of volatile oil in the suspension cells. The results indicated that there may be a signal pathway for the regulation of volatile oil synthesis by Ca^{2+} as a signaling molecule in *C. longepaniculatum* suspension cells. The addition of exogenous Ca^{2+} promotes the synthesis of volatile oils in *C. longepaniculatum* cells. LaCl_3 inhi-

bits the accumulation of some Ca^{2+} in the cells and leads to a decrease in the yield of volatile oil. However, the addition of LaCl_3 did not completely inhibit the accumulation of volatile oil in *C. longepaniculatum* cells caused by endophytic fungal elicitors, suggesting that endophytic fungal elicitors may not completely inhibit all pathways of calcium signaling, and may also through other signal transduction pathways to promote the synthesis of volatile oil in *C. longepaniculatum* suspension cells, so this aspect needs further study.

Conflicts of Interest

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

References

- [1] Yin, L., Yue, L., Du, Y., *et al.* (2014) Principal Component Analysis of Essential Oils in Yibin Oilseed Vegetative Organs. *Jiangsu Agricultural Sciences*, **42**, 348-350+355.
- [2] Xu, S., Yin, Z.Q., Wei, Q., *et al.* (2014) Anti-Hepatoma Effect of Safrole from *Cinnamomum longepaniculatum* Leaf Essential Oil *in Vitro*. *International Journal of Clinical and Experimental Pathology*, **7**, 2265-2272.
- [3] Ye, K.C. (2012) *In Vitro* Anti-Hepatocarcinoma Activity and Its Mechanism of Action of *Cinnamomum longepaniculatum* Leaf Extract. Sichuan Agricultural University, Ya'an.
- [4] Su, Z., Mao, L., Li, N., *et al.* (2013) Evidence for Biotrophic Life Style and Biocontrol Potential of Dark Septate Endophyte *Harpophora oryzae* to Rice Blast Disease. *PLoS ONE*, **8**, e61332. <https://doi.org/10.1371/journal.pone.0061332>
- [5] Jiang, D.H. (2015) Endophytic Fungi and Their Prospects. *Chinese Journal of Biological Control*, **31**, 742-749.
- [6] Xia, C., Zhang, X., Christensen, M.J., Nan, Z. and Li, C.J. (2015) *Epichloe* Endophyte Affects the Ability of Powdery Mildew (*Blumeria graminis*) to Colonise Drunkenhorse Grass (*Achnatherum inebrians*). *Fungal Ecology*, **16**, 26-33. <https://doi.org/10.1016/j.funeco.2015.02.003>
- [7] Zhai, Z.D., Shan, L., Jin, Z.Y., *et al.* (2018) A Brief Analysis of the Relevant Research Progress of Paclitaxel. *Science Bulletin*, **34**, 15-20.
- [8] Ludwig-Muller, J. (2015) Plants and Endophytes: Equal Partners in Secondary Metabolite Production. *Biotechnology Letter*, **37**, 1325-1334. <https://doi.org/10.1007/s10529-015-1814-4>
- [9] Lin, Y. and Wei, H. (2012) Research and Application Prospects of Endophytic Fungi in Plants. *Fujian Forestry Science and Technology*, **39**, 186-193.
- [10] Ren, A., Zhang, L. and Meng, X.G. (2010) Research Progress and Existing Problems of Endophytic Fungi in Plants. *Guangdong Agricultural Sciences*, **37**, 103-106.
- [11] Zhou, L.M. and Wei, F. (2018) Study on the Influence of Calcium Ion on Pollen Tube Growth. *Henan Agriculture*, No. 11, 87+96.
- [12] Yu, X.C., Ren, N. and Zhang, S. (2012) Research Progress on the Role of Calcium Signaling in Plant Disease Resistance. *Chinese Agricultural Science Bulletin*, **28**, 12-16.
- [13] Zhe, Z., Song, S., Bian, Z., *et al.* (2011) Research Progress of Calcium Ion in Plant Cells. *Journal of Anhui Agricultural Sciences*, **39**, 14525-14527.

-
- [14] Ren, C.G. (2014) Study on the Signal Regulation Mechanism of Endophytic Bacteria Promoting the Accumulation of Volatile Oil in Medicinal Plant *Atractylodes lancea*. Nanjing Normal University, Nanjing.
- [15] Galon, Y., Finkler, A. and Fromm, H. (2010) Calcium-Regulated Transcription in Plants. *Molecular Plant*, **4**, 653-669. <https://doi.org/10.1093/mp/ssq019>
- [16] Kuan, Y., Fang, C. and Qin, W. (2017) Effects of Endophytic Fungi on the Accumulation and Physiological and Biochemical Characteristics of Essential Oil from *C. longepaniculatum*. *Biotechnology Bulletin*, **33**, 138-143.
- [17] Ma, Y., Li, Z., Dong, D.D., Dai, J. and Niu, J. (2018) Rapid Determination of Calcium in Health Products by Flame Atomic Absorption Spectrometry. *Food Industry*, **39**, 299-301.
- [18] Wei, L., Yang, W., Kong, L., *et al.* (2018) Determination of Potassium, Sodium, Calcium and Magnesium in Fuel Ethanol Production Process by Flame Atomic Absorption Spectrometry. *Brewing Technology*, No. 11, 110-112+129.
- [19] Yang, L. (2014) Characteristics and Role of Calcium Signal in Bamboo Seedlings under Drought Stress. Zhejiang Agriculture and Forestry University, Hangzhou.
- [20] Tao, J.H., Wang, D.G., Qi, X.L., *et al.* (2014) Nitric Oxide and Salicylic Acid Mediate Endophytic Fungal Elicitors to Promote Signal Transduction of Atractylodesin Biosynthesis in *Atractylodes lancea* L. *Chinese Herbal Medicine*, No. 5, 701-708.
- [21] Jiao, J. (2016) Regulation and Signal Transduction of Endophytic Fungi on Secondary Metabolite Accumulation of *Rhodiola*. Shanxi University, Taiyuan.