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Effect of Different Inducers on the Essential Oil of Suspension Culture Cells from *Cinnamomum longepaniculatum*

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Abstract

In this study, the effects of different inducers on the accumulation of essential oil (1,8-eucalyptus) in suspension cells of *Cinnamomum longepaniculatum* were studied by adding different inducers, and the differences in the effects of different inducers on the yield and quality of essential oil in suspension cells of *C. longepaniculatum* were revealed. The results showed that under the same conditions, the addition of $CaSO_4$ 1.5 mg/L, Li_2SO_4 1.0 mg/L, SA15 mg/L and H_2O_2 9 mmol/L in B5 medium could significantly promote the accumulation of essential oil, which was 60.94%, 54.69%, 36.72% and 35.16% higher than that of CK, reaching 0.0824 mg/L, 0.0792 mg/L, 0.0700 mg/L and 0.0692 mg/L, respectively. Through orthogonal test, it was found that 2.0 mg/L $CaSO_4$ combined with, H_2O_2 9 mmol/L, SA 15 mg/L and Li_2SO_4 0.5 mg /L presented significant differences compared with other combinations ($P < 0.05$), and the yield reached 0.1768 mg/L, which increased by 245.31%. The experiment showed that the addition of inducer was an effective way to promote the accumulation of essential oil in suspension cells, and provided a reference for the manual regulation and strengthening of metabolism synthesis in this lifetime of suspension cells.

Subject Areas

Biochemistry, Biotechnology

Keywords

Cinnamomum longepaniculatum, Suspension Cells, Single Factor Orthogonal Test

1. Introduction

Cinnamomum longepaniculatum (Gamble) N. Chao is an evergreen tree of the

genus *Cinnamomum* of the family *Lauraceae*. *C. longepaniculatum* is a precious tree species in the genus *Aphididae*. The distribution is mainly concentrated in Taiwan, China and Yibin, Sichuan. The oil of *C. longepaniculatum* can be extracted from the leaves, stems and roots of *C. longepaniculatum* by steam distillation. At this stage, the oil extracted from *C. longepaniculatum* in Yibin is mainly essential oil [1].

The essential oil of *C. longepaniculatum* is mainly composed of dozens of different chemicals [2], and the main component 1,8-eucalyptus [3] is widely used in many industries, such as spices, food, medicine, chemical and defense. With the increase of the world's population and the improvement of people's material living standards, the demand for medicines, foods, spices, etc. has increased, and the demand for 1,8-eucalyptus has increased rapidly [4] [5] [6] [7].

Suspension cell culture refers to establish callus or excised plant cells and transfers to a liquid medium for sterile shaking culture [8]. The relatively uniform cells obtained by this culture method not only provide a unique experimental system for studying cell proliferation and differentiation, but also have a rapid cell proliferation rate and are suitable for large-scale culture. Therefore, there is a huge application potential in the industrial production of plant products [9] [10].

With the rapid development of society and the continuous improvement of people's quality of life, the demand for natural flavors such as essential oil has doubled, leading to a growing contradiction between supply and demand. Coupled with the plant's secondary metabolite instability and environmental constraints, the yield and quality of the essential oil cannot meet the demand. Therefore, the study of the influencing factors of essential oil is of great significance for significantly increasing the yield of essential oil, and has become an important content and a new development direction of *C. longepaniculatum* resources and plant aromatic oil research.

In the process of suspension culture of plant cells, the activity of enzymes in cell metabolism can be activated by adding exogenous inducers [11], thus increasing the production of secondary metabolites in oil suspension cells, and sometimes even inducing the new production of secondary metabolites in oil suspension cells [12] [13], with the continuous development of plant tissue and cell culture technology, cell suspension culture system is becoming more and more perfect. It has always been a hot spot for researchers to study how to use plant cell suspension culture technology to produce useful secondary metabolites and increase metabolite production [14] [15]. This technique of using elicitors to increase the production of secondary metabolites in oil suspension cells in the short term is currently a more mature method [16]. Therefore, finding suitable inducers can effectively increase the secondary metabolites of suspended cells and improve the oil production rate, which has high research value and application prospects.

2. Materials and Methods

Materials

All young leaves were sourced from the *C. longepaniculatum* base of Hongyan Mountain in Yibin. Cut the leaves blade into the appropriate size (should be placed in a sterile container) and rinse with running water for a few minutes to 30 minutes. Then soaked in 70% alcohol for 10 to 30 seconds and washed it 3 to 10 times with sterile water. Inoculated the treated aseptic explants in a 100 ml solid triangular medium.

Main Drugs and Medium

Inducers: $CuSO_4$, $FeSO_4$, Li_2SO_4, $MnSO_4$, $CaSO_4$, H_2O_2, tryptophan, SA (salicylic acid), sodium benzoate B5 medium: A large number of elements 1: KNO_3 50 g, 1 L (take 50 ml); A large number of elements 2: $CaCl_2$-$2H_2O$ 3 g, $MgSO_4$-$7H_2O$ 5 g, $(NH_4)_2SO_4$ 2.68 g, NaH_2PO_4-$2H_2O$ 3 g, 1L (take 50 ml); Trace element 1: KI 75 mg, H_3BO_3 300 mg, $MnSO_4$-H_2O 1000 mg, $ZnSO_4$-$7H_2O$ 200 mg, Na_2MoO_4-$2H_2O$ 25 mg, 500 ml (take 5 ml); Trace element 2: $CuSO_4$-$5H_2O$ 25 mg, CoCl-$6H_2O$ 25 mg, 500 ml (take 0.5 ml); Iron salt: $FeSO_4$-$7H_2O$ 2780 mg, Na_2-EDTA-$2H_2O$ 3730 mg, 500 ml (take 5 ml); Organic matter: Inositol 10,000 mg, niacin 100 mg, Pyridyl hydrochloride 100 mg, Thiamine hydrochloride 1000 mg, 500 ml (take 5 ml).

Test Design

B5 medium was used as the basic medium, and the single factor test of different levels of the same inorganic inducer was set up (Table 1), the single factor test of different levels of the same organic inducer (Table 2) and different inducers. Orthogonal test (Table 5), the medium was dispensed into a 150 mL Erlenmeyer flask, repeated for 3 bottles treatment, and sterilized at 115˚C for 20 min in an autoclave. After cooling, callus solid cells were transferred to liquid medium. The callus solid cells were cultured for 28 days in 25˚C in a constant temperature incubator, and its essential oil production was measured every 7 days during its cultivation.

Table 1. Single factor test of inorganic inducer.

Inorganic inducer	Level			
$CuSO_4$ (mmol/L)	0.01	0.02	0.03	0.04
$FeSO_4$ (mmol/L)	0.02	0.035	0.05	0.075
Li_2SO_4 (mg/L)	0.5	1	1.5	2
$MnSO_4$ (mmol/L)	1	3	5	7
$CaSO_4$ (mg/L)	0.05	0.1	0.15	0.2
H_2O_2 (mmol/L)	3	6	9	12

Table 2. Single factor test of organic inducer.

Inducer	Level			
Tryptophan (g/L)	0.14	0.144	0.148	0.152
SA (mg/L)	5	15	25	35
Sodium benzoate (mg/L)	5	10	15	20

Methods

Cultivation of suspension cells and establishment of suspension system

The inoculated explants were placed in a culture chamber, and the temperature was controlled at about 23˚C. After the callus induction was completed, it was subcultured twice. The transfer was performed twice, and about 2.0 g of the well-grown and loose pink callus was inoculated into a 150 mL Erlenmeyer flask containing 50 mL of B5 medium at a speed of 110 r/min at 25˚C. Shake culture under shading conditions. 14 days were subcultured once, followed by 2 times.

Inducer Single Factor Test

B5 medium was prepared, and the inorganic inducer was added to the same amount of inducer after each component, and sterilized at 115˚C for 20 min in an autoclave with an inoculation needle; organic inducer for sterilization in a high-pressure steam sterilization pot at 115˚C for 20 min, after cooling, an equal amount of each organic concentration inducer was removed by filtration under a sterile condition using a 0.22 μm water filter, as shown in Table 1 and Table 2. Then, it was placed in a suspension culture at 28˚C, 120 r/min for 28 days, and each concentration was repeated 3 times, and 3 controls were set at the same time.

Different Inducer Orthogonal Test

According to the results of single factor test, after the significance test of spss data processing system, the inducers with significant differences were selected for orthogonal test, and the yield of essential oil was used as the index to carry out the orthogonal test of $L_{16}(4^5)$ (Tables 3-6), each set of experiments was repeated 3 times.

Extraction and Determination of Essential Oil from Suspension Cells of *C. lngepaniculatum*

The suspension cells were removed from the culture flask under sterile conditions every 7 days and dried at 55˚C. Accurately weigh 0.2000 g - 0.6000 g of *C. longepaniculatum* suspension cells, add 4 times (1:4) of cyclohexane or petroleum ether for cold soak overnight, then treat in ultrasonic cleaner at 70˚C for 30 min, and finally centrifuge at 5000 r/min for 4 min. The supernatant was taken and made up 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 is 1 μL; programmed temperature: from 60˚C after the temperature increase rate of 10˚C/min to 190˚C and maintained for 2 min, Then, it was raised to 210˚C for 2 min at a temperature increase rate of 5˚C/min, and then increased to 220˚C for 8 min at a temperature increase rate of 10˚C/min, and then injected into a GC-MS to obtain a standard curve, that is, essential oil content response value was obtained. Further, the essential oil content was calculated [17] (1,8-eucalyptol: $Y = 73900X - 299200$, $R^2 = 0.9993$).

Table 3. Effects of different inorganic inducers on the accumulation of essential oil in suspension culture cells.

Time (d)	1,8-eucalyptus oil (mg/L)			
	7 d	14 d	21 d	28 d
$CuSO_4$ (0.010 mmol/L)	0.0370	0.0396	0.0423	0.0399
$CuSO_4$ (0.020)	0.0410	0.0530*	0.0492	0.0460
$CuSO_4$ (0.030)	0.0400	0.0456	0.0461	0.0428
$CuSO_4$ (0.040)	0.0397	0.04351	0.0432	0.0395
$FeSO_4$ (0.020 mmol/L)	0.0351	0.0401	0.0389	0.0380
$FeSO_4$ (0.035)	0.0384	0.0425	0.0489	0.0465
$FeSO_4$ (0.050)	0.0435	0.0533	0.0635*	0.0619
$FeSO_4$ (0.075)	0.0385	0.0486	0.0598	0.0541
Li_2SO_4 (0.5 mg/L)	0.0489	0.0569	0.0652	0.0650
Li_2SO_4 (1.0)	0.0590	0.0653	0.0792**	0.0790
Li_2SO_4 (1.5)	0.0543	0.0658	0.0732	0.0721
Li_2SO_4 (2.0)	0.0511	0.0600	0.0632	0.0620
$MnSO_4$ (1 mmol/L)	0.0452	0.0496	0.0495	0.0487
$MnSO_4$ (3)	0.0536	0.0557	0.0632*	0.0624
$MnSO_4$ (5)	0.0480	0.0525	0.0590	0.0586
$MnSO_4$ (7)	0.0431	0.0543	0.0598	0.0591
$CaSO_4$ (0.5 mg/L)	0.0650	0.0680	0.0692	0.0670
$CaSO_4$ (1.0)	0.0689	0.0742	0.0798	0.0765
$CaSO_4$ (1.5)	0.0732	0.0768	0.0824**	0.0804
$CaSO_4$ (2.0)	0.0651	0.0684	0.0675	0.0659
H_2O_2 (3 mmol/L)	0.0521	0.0556	0.0562	0.0552
H_2O (6)	0.0601	0.0621	0.0634	0.0620
H_2O (9)	0.0601	0.0642	0.0692**	0.0675
H_2O (12)	0.0561	0.0614	0.0634	0.0624
Control	0.0351	0.0467	0.0512	0.0436

Note: * There is a significant difference in inorganic inducers.

Table 4. Effect of different organic inducers on the accumulation of essential oil in suspension cells.

Time (d)	1,8-eucalyptus (mg/L)			
	7 d	14 d	21 d	28 d
Tryptophan (0.140 g/L)	0.0442	0.0505	0.0563	0.0553
Tryptophan (0.144)	0.0451	0.0513	0.0576	0.0566
Tryptophan (0.148)	0.0482	0.0549	0.0597*	0.0589
Tryptophan (0.152)	0.0472	0.0542	0.0536	0.0531
SA(5 mg/L)	0.0499	0.0596	0.0699	0.0676

Continued

SA (15)	0.0513	0.0656	0.0700**	0.0630
SA (25)	0.0495	0.0584	0.0675	0.0634
SA (35)	0.0503	0.0607	0.0653	0.0622
Sodium benzoate (5 mg/L)	0.0486	0.0546	0.0561	0.0551
Sodium benzoate (10)	0.0487	0.0593	0.0674*	0.0658
Sodium benzoate (15)	0.0488	0.0577	0.0649	0.0599
Sodium benzoate (20)	0.0469	0.0489	0.0485	0.0451
Control	0.0351	0.0467	0.0512	0.0436

Note: *There is a significant difference in inorganic inducers.

Table 5. Orthogonal test of different inducers.

Test number	Factor Factor					1,8-eucalyptus (mg/L)
	A	B	C	D	E	
1	1 (0.5)	1 (3)	1 (5)	1 (0.5)		0.0988
2	2 (1)	2 (6)	1	2 (1.0)		0.1121
3	3 (1.5)	3 (9)	1	3 (1.5		0.1342
4	4 (2)	4 (12)	1	4 (2.0)		0.1656
5	2	4	3 (25)	1		0.1321
6	4	3	2 (15)	1		0.1768
7	3	2	4 (35)	1		0.1321
8	1	4	4	3		0.1154
9	4	1	4	2		0.1541
10	1	3	3	2		0.0879
11	2	3	4	4		0.1103
12	2	1	2	3		0.0854
13	3	1	3	4		0.1103
14	3	4	2	2		0.1384
15	4	2	3	3		0.1569
16	1	2	2	4		0.0843
k1	0.3864	0.4486	0.5107	0.5398		
k2	0.4399	0.4854	0.4849	0.4925		
k3	0.5150	0.5092	0.4872	0.4919		
k4	0.6534	0.5515	0.5119	0.4705		
k1	0.0966	0.1122	0.1278	0.1350		
k2	0.1100	0.1214	0.1212	0.1231		
k3	0.1288	0.1273	0.1218	0.1230		
k4	0.1634	0.1379	0.1280	0.1176		
R	0.0668	0.0257	0.0068	0.0174		

Note: A: $CaSO_4$ (mg/L); B: H_2O_2 (mmol/L); C: SA (mg/L); D: Li_2SO_4 (mg/L).

Table 6. Analysis of variance of essential oil accumulation in orthogonal experiment of different inducers.

Variance source sv	Square sum ss	Degree of freedom df	Mean square ms	F	P
A	0.010	3	0.003	36.990	0.007*
B	0.001	3	0.000	5.130	0.106
C	0.000	3	5.341E−005	0.589	0.663
D	0.001	3	0.000	2.360	0.250
Error	0.000	4	9.072E−005		
Total variation	0.261	16			

Note: *$p < 0.01$.

Significance Test of Experimental Data

The experimental data was entered into the SPSS data processing system table and statistical analysis of the single factor test was performed on a completely random design of the test statistics tab. Perform a significant test on the experimental data and create a line graph of the experimental results in an Excel spreadsheet.

3. Results

The Effect of Single Factor of Inorganic Inducer on the Yield of Essential Oil from Oil Cell Suspension Cells

B5 medium was prepared, and the essential oil production was measured on the 7th, 14th, 21st and 28th day of suspension cell culture. The effect of single factor of each inorganic inducer on the accumulation of essential oil in the suspension cells of *C. longepaniculatum* was examined. The results are shown in Table 3. The results shown in the table are the average of 3 trials.

Table 3 shows that the oily suspension cells treated with each inorganic inducer showed an increase in the yield of most of the essential oil in the range of 21 d, and reached the maximum at 21 d. When the culture time was greater than 21 d, the essential oil content in the suspension cells gradually decreased; as the concentration of the inorganic inducer increased, the essential oil production increased first and then decreased, but the cells may be aging, and some cells reached the maximum in the second cycle. The overall trend of the increase was higher than that of the no-inducer group (CK). The effects of essential oil accumulation on the *C. longepaniculatum* cell suspension cells treated with inorganic inducers were basically consistent. Among them, at the 21st day, the induced concentrations of $CuSO_4$, $FeSO_4$, Li_2SO_4, $MnSO_4$, $CaSO_4$, H_2O_2 were 0.02 mmol/L, 0.05 mmol/L, 1.0 g/L, 3 mmol/L, 1.5 mg/L, 9 mmol/L, respectively. The essential oil yield was the largest under the treatment of this inorganic inducer, 0.0530 mg/L, 0.0635 mg/L, 0.0792 mg/L, 0.0632 mg/L, 0.0824 mg/L, 0.0692 mmol /L, respectively. The yield increased by 3.52%, 24.02%, 54.69%, 23.44%, 60.94%, 35.16%, respectively. According to the significance test, the induced concentrations of $CuSO_4$, $FeSO_4$, Li_2SO_4, $MnSO_4$, $CaSO_4$ and H_2O_2 were respec-

tively 0.02 mmol/L, 0.05 mmol/L, 1.0 mg/L, 3 mmol/L, 1.5 mg/L, 9 mmol/L at 21 d, there was a significant difference between the yield of 1,8-eucalyptus oil and other concentrations under the inducer at this time ($P < 0.05$). According to the SPSS significance test, $CaSO_4$ had the most significant difference compared with other inorganic inducers on the 21st day.

Effects of Organic Inducer Single Factor on the Yield of Essential Oil from Suspension Cells

B5 medium was prepared, and the same concentration of organic inducer was added after sterilization and cooling. The essential oil production was measured on the 7th, 14th, 21st and 28th day of suspension culture, and the single factor of each organic inducer was detected. The results are shown in Table 4, the results shown in the table are the average of 3 trials.

Table 4 shows that the essential oil production increased in the range of 21 d when the cultured cells were treated with organic inducers, and reached the maximum at 21 d. When the culture time was greater than 21 d, the content of essential oil in suspended cells decreased gradually. With the increase of organic inducer concentration, the yield of essential oil increased first and then decreased. The yield was higher than that of the no-inducer group (CK). The effects of essential oil accumulation on the suspension cells of *C. longepaniculatum* treated by organic inducers were basically consistent. Among them, on the 21st day, when the concentrations of tryptophan, SA and sodium benzoate were 0.148 g/L, 15 mg/L and 10 mg/L, respectively, the essential oil yield was the highest under the treatment of organic inducer, which was 0.0597 mg/L, 0.0700 mmol/L, 0.0674 mmol/L, the yield increased by 16.60%, 36.72%, 31.64% compared with CK; according to significant test analysis, on the 21st, tryptophan, SA, sodium benzoate induced concentration of 0.148 g/L, 15 mg/L and 10 mg/L, the yield of 1,8-eucalyptus oil was significantly different from that of other concentrations under the inducer at this time ($P < 0.05$).

Different inducer orthogonal test

Four factors including $CaSO_4$, H_2O_2, Li_2SO_4 and SA were selected by orthogonal test, and $L_{16}(4^5)$ was used as the index of essential oil production of orthogonal test (Table 5). The results of the variance analysis of the essential oil production in the inducer induction test are shown in Table 6. The variance analysis of essential oil production by orthogonal test of different inducers showed that the inducer for the significant difference in essential oil production was $CaSO_4$, and the other three inducers did not reach a significant level for the essential oil production. Through the orthogonal test, the influence of the R value of the four factors in Table 5 on the essential oil yield is: $CaSO_4 > Li_2SO_4 > SA > H_2O_2$.

The yields of essential oil and the K value of each group in different orthogonal test tables were analyzed. The optimal combination of 4 groups was selected, namely $A_4B_4C_1D_4$, $A_4B_4C_4D_4$, $A_4B_4C_1D_1$, $A_4B_4C_4D_1$ 4 groups conduct verification tests. The results showed that when $CaSO_4$ 2.0 mg/L, H_2O_2 9 mmol/L, SA 15 mg/L, Li_2SO_4 0.5 mg/L were added to the suspension medium, the yield was the

largest. It was 0.1768 mg/L, and compared with the yield of CK which have no inducer was increased by 245.31%.

4. Discussion

Studies have found that the addition of inducers can alter the pathway of secondary metabolite synthesis; it can also promote or inhibit the activity of key rate-limiting enzymes in the bio-metabolite synthesis pathway [18]; this experiment shows that there are differences between different inducers against *C. longepaniculatum* suspension cells and the effects of essential oil production, indicating that different inducers can affect the accumulation of essential oil in suspension cells to varying degrees.

In the single factor experiment, the suspension cells were treated with inorganic inducers $CaSO_4$, H_2O_2, Li_2SO_4, and the essential oil yield was compared with other inducers. There are significant difference ($P < 0.05$), and $CaSO_4$ was more significant than the other two, the yield was larger, reaching 0.0824 mg/L, which was 60.94% higher than CK. This is related to calcium ion Activator-related, it activates certain secondary metabolic pathways to increase metabolite production; H_2O_2 regulates gene expression and signaling as a signaling molecule to regulate secondary metabolic pathways [19]. The yield of essential oil treated by $CuSO_4$, $FeSO_4$ and $MnSO_4$ is higher than that of CK, but the difference is not significant, which promotes the accumulation of essential oil, but it is not significant, and the specific reasons need to be further carried out and explored. In summary, the promotion of the inorganic inducer on the accumulation of essential oil in the suspension cells of *C. longepaniculatum* is $CaSO_4$ > Li_2SO_4 > H_2O_2 > $FeSO_4$ > $MnSO_4$ > $CuSO_4$.

Treatment of *C. longepaniculatum* suspension cells with organic inducer SA can significantly promote the yield of essential oil from *C. longepaniculatum* suspension cells, reaching 0.0700 mmol/L, which is 36.72% higher than CK. It is found that SA acts as a signal molecule involved in signal transduction, activation and inhibition. The corresponding transcription factors affect the expression of genes and regulate the synthesis of secondary metabolites [20]. In summary, the promoting effect of organic inducers on the accumulation of essential oil in oil suspension cells was: SA > sodium benzoate > tryptophan. Therefore, based on the difference in the accumulation of essential oil, the dominant inducer can be found, thereby optimizing the way to induce the accumulation of essential oil in the suspension of cells.

In the orthogonal experiment, $CaSO_4$ had significant differences in the yield of essential oil from the *C. longepaniculatum* cell suspension compared with H_2O_2, Li_2SO_4, SA. The three inducers had no yield for essential oil. To achieve the level of significance, the effect of promoting essential oil production in orthogonal test was: $CaSO_4$ > Li_2SO_4 > SA > H_2O_2. Orthogonal test showed that when the callus suspension cells were added $CaSO_4$ 2.0 mg/L, H_2O_2 9.0 mmol/L, SA 15 mg/L, Li_2 SO_4 0.5 mg/l. The yield of essential oil was the highest at 0.1768 mg/L,

which was 245.31% higher than that without inducer. This indicated that the orthogonal combination of different inducers had the best effect on the yield of essential oil in suspension cells and the inducers in several different pathways added simultaneously have synergistic effects [21].

In this experiment, the effects of nine inducers and their partial combinations on the accumulation of essential oil in the suspension cells of *C. longepaniculatum* were studied, which promoted the accumulation of essential oil to some extent, but also encountered some problems in the experiment: such as the freshness of the cells and the effect of the length of the suspension cell culture cycle on the accumulation of essential oil, which needs further study. In the process of suspension culture of callus in the future, we can also optimize the culture conditions of its growth stage and metabolite synthesis stage, which lays a foundation for artificial regulation and optimization of the accumulation of secondary metabolites in induced callus.

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Conflicts of Interest

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

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