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Full Length Article

Effect of Endophytic Fungal Elicitors on Essential Oil Accumulation in Cell Suspension Cultures of *Cinnamomum longepaniculatum*

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Abstract

A *Cinnamomum longepaniculatum* (Gamble) N. Chao cell suspension culture system was established using four endophytic fungal elicitor strains, *Penicillium commune* (2J1), *Neurospora crassa* (3J1), *Aspergillus fumigatus* (5J2) and *Penicillium paneum* (YB) to explore the mechanisms underlying the effects of endophytic fungal elicitors on essential oil (1,8-cineole and α-terpineol) accumulation in suspension cells. Essential oil content reached a peak at 21d after treating cells with 40 mg/L 2J1 and 3J1. The contents of 1,8-cineole increased by 68.21 and 54.96% and those of α-terpineol increased by 218.51 and 207.65%, respectively. Synthesis of the essential oil of *C. longepaniculatum* was significantly enhanced within the elicitor concentration range of 20–60 mg/L and reached a maximum at 40 mg/L. Furthermore, the peroxidase (POD) content, catalase (CAT) activity, and malondialdehyde (MDA) content of suspension cells increased significantly, and these increases were to an extent positively correlated with essential oil accumulation. No significant differences were detected between *C. longepaniculatum* cells treated with 5J2 and YB elicitors and the control group in terms of essential oil accumulation, CAT activity, and POD and MDA contents. The results showed that treatment with 2J1 and 3J1 elicitors could significantly increase the accumulation of essential oil in *C. longepaniculatum* cells. © 2019 Friends Science Publishers

Keywords: *Cinnamomum longepaniculatum*; Endophytic fungal elicitors; Essential oil; Suspension cells

Introduction

Cinnamomum longepaniculatum (Gamble) N. Chao is an important industrial crop. Essential oils can be extracted from its roots, stem, leaves and seeds; the main constituents of leaf essential oil are terpenoids (>85%) (Tao *et al*., 2002; Wang *et al*., 2004; Xu *et al*., 2014) including 1,8-cineole, α-terpilenol and γ-terpinene (Li *et al*., 2014; Xu *et al*., 2014). These essential oils have antioxidant, anti-inflammatory, and antimicrobial properties (Wang *et al*., 2007; Wei *et al*., 2016). However, their yield is low due to the variable content of secondary metabolites and the effects of other factors such as environmental conditions. Substantial efforts have been made to improve the yield of *C. longepaniculatum* essential oil using new technologies.

Previous plant studies have mainly focused on external environmental factors such as illumination, temperature and humidity, but have neglected the influence of internal environmental factors (*e.g*., microorganisms), particularly endophytic fungi, which can improve stress resistance and promote the accumulation of active components (Phongpaichit *et al*., 2007; Gao *et al*., 2010; Xie *et al*., 2011; Jia *et al*., 2016; Clifton *et al*., 2018; Rao *et al*., 2018). In recent years, although many studies concerning the effects of endophytic fungi on the accumulation of secondary metabolites in medicinal plants have been reported (Jiang *et al*., 2010; Bhagat *et al*., 2012; Mishra *et al*., 2012; Nath *et al*., 2015; Santos *et al*., 2015), there have been few reports related to the effect of endophytic fungi on the synthesis of plant essential oils and the underlying mechanisms. In this regard, cell culture can significantly promote the accumulation of secondary metabolites, and cell-based production of essential oils is the best way to solve the current imbalance between supply and demand.

In this study, we established a suspension culture system to investigate the effects of endophytic fungal elicitors on essential oil (1,8-cineole and α-terpineol) accumulation in suspension cells of *C. longepaniculatum*. The results provide references for elucidating the mechanisms underlying the influence of endophytic fungi on secondary metabolite synthesis in host plants, and also for the anticipated cloning of related regulatory genes and artificial regulation of secondary metabolite synthesis in *C. longepaniculatum*.

Materials and Methods

Experimental Material

Cinnamomum longepaniculatum (Gamble) N. Chao were

collected from Hongyan Mountain, Yibin, China (located at 27°50' N;105°20' E). Four strains of endophytic fungi (*Penicillium commune*, 2J1), (*Neurospora crassa*, 3J1), (*Aspergillus fumigatus*, 5J2), and (*Penicillium paneum*, YB) were isolated from *C. longepaniculatum* plants (Yan *et al*., 2017). These fungi were cultured and maintained on potato dextrose agar medium (PDA).

Methods

Separation and purification of endophytic fungi: Leaves of fresh *C. longepaniculatum* plants were collected and repeatedly rinsed with tap water. The leaves were then cut into segments and surfaced sterilized: 5–8 min sterilization with 0.1% mercury bichloride, three washes with sterile water, 20 s sterilization with 75% ethyl alcohol and a further three washes with sterile water. Subsequently, the sterilized explants were cut into 0.3–0.5 cm sheets and inoculated onto a PDA plate. Thereafter, the explants were cultured for 8–10 d at 28°C. When hyphae had grown on sample edges, portions of the hyphae at the tissue block edges were cut for further culture and inoculated and separated continuously until purified.

Suspension Culture for *C. longepaniculatum* Cells

Small tender leaves of *C. longepaniculatum* were used for the induction of calli. These calli were subsequently subcultured two to three times. Approximately 1.0 g of callus, characterized by vigorous growth and loose texture, was selected and placed in a 150 mL conical flask containing 50 mL B5 culture medium, which was incubated in the dark at 25°C, 120 rpm and pH 7.4. An initial subculture was performed after 7 d, followed by a further two to three subcultures at 7-d intervals. The culture was filtered and the filtrate was used for further culture. This preparation was used as the initial suspension culture.

Preparation of Endophytic Fungal Elicitors

The endophytic fungi were cultured on PDA, and incubated at 28°C. A PDA with no fungal inoculation was used as a control. From 7 day-old cultures, 1 cm^2 of mycelia was transferred to a 250 mL Erlenmeyer flask containing 80 mL potato dextrose medium, and the mycelia were maintained in the medium at 150 rpm at 28°C until harvest. Mycelia were harvested by filtering, and ground with a mortar and pestle. The homogenate was diluted in water and autoclaved for 20 min at 121°C. The autoclaved fungal suspension was used as the elicitor. Elicitor concentration was standardized using the anthranone–sulfuric acid method (Tao *et al*., 2011).

Effect of Endophytic Fungal Elicitors on Essential Oil Accumulation in *C. longepaniculatum* Suspension Cells

The four endophytic fungal elicitors (40 mg/L) were added to the suspension cell cultures of *C. longepaniculatum*. A control group was by prepared (adding blank fungal cultures). 15 g of fresh dried cells were extracted at 0, 7, 14, 21 and 30 h after the addition of elicitors. The cells were subjected to decompression and suction filtration to determine the essential oil content in the suspension cells.

We also examined the effects of different concentrations (20, 40, 60 and 80 mg/L) of endophytic fungal elicitors on essential oil accumulation in the suspension cells of *C. longepaniculatum*. A control group was established by adding the corresponding volume of distilled water. After 21 d of culture, 15 g of fresh cells were subjected to decompression and suction filtration to determine the essential oil content in suspension cells of *C. longepaniculatum*. All experiments were done in triplicate.

Isolation and Analysis of Essential Oils

Essential oil was obtained by hydrodistillation for 5 h using a Clevenger-type apparatus for 5 h. The volatile distilled oils were dried over anhydrous sodium sulfate and weighed using an electronic balance (accuracy to 0.0001 g) to calculate the yield of essential oil. All samples were stored at 4°C until analysis. The oils were yellow in color and had a distinct sharp odor.

Gas chromatography-mass spectrometry (GC-MS) analysis was performed using an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass spectrometer using an HP-5MS fused silica capillary column (30 m×0.25 mm internal diameter, 0.25 μm film thickness). The temperature program used was follows: 50°C for 2 min, increased to 120°C at a rate of 3°C/min, held at 120°C for 2 min, then increased to 250°C at 15°C/min, and held at 250°C for 2 min. The carrier gas was helium, used at a flow rate of 0.7 mL/min; the injected volume was 1.0 μL (1:10 in Et_2O) and the injector temperature was 220°C. Mass spectrometer conditions were as follows: GC–MS interface temperature, 250°C; ion source temperature, 230°C; quadrupole temperature, 150°C; ionization mode, EI; and ionization energy, 70 eV.

Compounds were identified by comparing their mass spectra with the mass spectra obtained from an MS database (NIST 08), and by comparing the retention indices (RI) of the samples with those of reference compounds run under identical conditions. Laboratory library and literature data for the main compounds identified were confirmed by comparing the GC retention times of the analyzed samples with those of pure standards (Hu *et al*., 2011). Pure standard compounds of 1,8-cineole and α-terpineol were provided by Prof. Qin Wei (China). Then, the external standard method for quantitative for both terpenes.

Preparation of Crude Enzyme Solutions from Suspension Cells of *C. longepaniculatum*

To 0.05 g of collected suspension cells, we added 0.01 g polyvinyl pyrrolidone (PVP) and 5 mL 0.1 mol/L phosphate

buffer [pH 7.8 containing 0.2 mmol/L ethylene diamine tetra-acetic acid (EDTA) and 0.4 mmol/L β-mercaptoethanol] for ice-bath grinding. The mixture was then centrifuged for 10 min at 4℃ and 12,000 rpm. The supernatant was used as a test solution.

Determination of CAT Activity

Test solution (100 μL) was added into 3 mL with 0.1% H_2O_2 100 mmol/L phosphate buffer (pH7.0). Absorbance was measured at 30-s intervals at 240nm and CAT activity was expressed as ΔA/min/g.

Determination of POD Activity

Guaiacol (28 μL) was added to 50 mL100 mmol/L phosphate buffer (pH7.0). Thereafter, the mixture was cooled, followed by the addition of 19 μL 30% H_2O_2 (POD reaction solution). During the test, 100 μL test solution, 2 mL POD reaction solution, and 1 mL 0.2 mol/L monopotassium phosphate solution were mixed together. Absorbance was measured at 30-s intervals and POD activity was expressed as ΔA/min/g.

Determination of MDA Content

Test solution (1.0 mL) and 5.0 mL 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) were mixed evenly and then sealed using a plug. The mixture was boiled in a water bath for 30 min and then rapidly cooled. The solution was then centrifuged for 10 min at 4000 rpm. The control used was 20% TCA. Absorbance of the supernatant was measured three times at 532 nm and 600 nm and the mean values were taken as the final values. MDA content was expressed as ΔA (ΔA =A532-A600).

Statistical Analysis

The yield of essential oil and the activity of enzymes from each treatment was measured using three replications. The data were described as mean values ± standard deviation and analysed by one-way analysis of variance (ANOVA). A Duncan multiple-comparison test was used to detect differences between the means of all samples. Difference were considered significant at a p value < 0.05. All correlation and path coefficient analyses were performed with SPSS Statistics 19.0 and Excel 2007.

Results

Effect of Endophytic Fungal Elicitors on Essential Oil Accumulation in *C. longepaniculatum* Suspension Cells

Endophytic fungi can facilitate the production and accumulation of secondary metabolites in host plant under certain conditions (Sachin *et al*., 2013) In the present study, we investigated the effects of four endophytic fungal elicitors (2J1, 3J1, 5J2 and YB) on essential oil (1,8-cineole and α-terpineol) accumulation in *C. longepaniculatum* suspension cells.

The suspension cells of *C. longepaniculatum* treated with the four endophytic fungal elicitors had significantly different accumulations of 1,8-cineole and α-terpineol (Table 1 and 2). At 0d, no significant difference was observed among the different groups in terms of the accumulations of 1,8-cineole and α-terpineol. Subsequently, essential oil accumulation in all groups increased to different extents. 2J1 and 3J1 elicited the highest increases, showing significantly higher essential oil accumulations after 7 d. Although the essential oil accumulations elicited by 5J2 and YB increased to a certain extent, they were not significantly different from that of the control groups. Essential oil contents of cells treated with the fungal elicitor changed with time and were significantly higher than control group, which indicated that elicitors significantly stimulates the essential oil biosynthesis of *C. longepaniculatum* cells. Moreover, the contents of essential oils peaked at 21 d after the treatment. After that, essential oil contents of the treated cells gradually decreased, but those of the 2J1 and 3J1 groups were still at significantly higher levels than those of the other groups. On the basis of these results, we were able to identify the dominant strain and the best oil extraction time, which will be conducive to increasing practical production output.

Effect of Concentrations of Endophytic Fungal Elicitors on Essential Oil Accumulation in Suspension Cells of *C. longepaniculatum*

Four different concentrations of the four endophytic fungal elicitors were added to cell suspension cultures of *C. longepaniculatum* and significant differences of essential oil accumulations were observed among the cultures after 21 d. The essential oil contents were also significantly improved with the increase of elicitors concentration. Compared to the control group, the 20 mg/L 2J1 and 3J1 groups showed significant increases in essential oil accumulation. The 1,8-cineole contents were 1.79- and 1.69-fold higher than that of the control group (Fig. 1), respectively, whereas the α-terpineol contents were 2.56- and 2.35-fold higher than that of the control group (Fig. 2). When the elicitor concentration was increased to 40 mg/L, the 2J1 and 3J1 groups showed peak essential oil accumulations. The 1,8-cineole contents were 5.461 mg/L and 4.951 mg/L higher than that of the control group (Fig. 1), respectively, whereas the α-terpineol contents were 3.504 mg/L and 2.924 mg/L higher (Fig. 2). At 60 mg/L, although the essential oil accumulations of the 2J1 and 3J1 groups were still considerably higher than that of the control group, they were significantly lower than those at 40 mg/L. At 80 mg/L, no significant difference was observed among the different test groups in terms of essential oil contents. Thus, 2J1 and 3J1

Table 1: Effect of endophytic fungal elicitors on 1,8-cineole accumulation in suspension cells of *C. longepaniculatum.* Effect of different elicitors of endophytic fungal (2J1, *P. commune*; 3J1, *N. crassa*; 5J2, *A. fumigatus*; YB, *P. paneum*) elicitors on 1,8-cineole accumulation in suspension cells of *C. longepaniculatum*

Endophytic fungi	1,8-cineole (mg/L)				
	0d	7d	14d	21d	28d
CK	4.022a	4.310d	5.243c	6.971c	5.027c
2J1	4.355a	7.584a	9.705a	11.725a	10.099a
3J1	4.275a	6.540b	8.704b	10.802b	9.210b
5J2	4.182a	4.469d	5.417c	7.244c	5.077c
YB	4.196a	5.149c	5.839c	6.849c	5.466c

Values followed by the same lowercase letters in the same column are not significantly different at the 5% level

Table 2: Effect of endophytic fungal elicitors on α-terpineol accumulation in suspension cells of *C. longepaniculatum.* Effect of different elicitors of endophytic fungal (2J1, *P. commune*; 3J1, *N. crassa*; 5J2, *A. fumigatus*; YB, *P. paneum*) elicitors on α-terpineol accumulation in suspension cells of *C. longepaniculatum*

Endophytic fungi	α-terpineol (mg/L)				
	0d	7d	14d	21d	28d
CK	0.341a	0.356c	0.988d	1.437d	0.989d
2J1	0.335a	0.938b	2.330b	4.577a	3.868a
3J1	0.350a	1.379a	2.684a	4.421b	3.719b
5J2	0.339a	0.366c	1.451c	2.138c	1.500c
YB	0.309a	0.354c	0.936d	1.289d	0.908d

Values followed by the same lowercase letters in the same column are not significantly different at the 5% level

at concentrations of 20-60 mg/L can facilitate significant synthesis of essential oil in the suspension cells of *C. longepaniculatum* and the contents of essential oils reached a maximum at 40 mg/L. However, the essential oil contents of the 5J2 and YB groups changed only slightly with an increase in elicitor concentration. On the basis of these observations, the dominant fungi and the optimal oil extraction time and elicitor concentration could be identified, and this information will be conducive to increasing practical production output.

Effect of Endophytic Fungal Elicitors on Protective Enzymes of the Radical Scavenging System and Membrane Lipid Peroxidation of Suspension Cells

After endophytic fungal elicitors were added to cell suspension cultures, we found that POD and CAT activities in cells changed accordingly. Although POD activity changed slightly after treating cells with 5J2 and YB, the variation was, the same as that observed for the control group (CK). The POD activity of the YB group reached a peak at 12h, but was not significantly different compared to CK. In contrast, the maximum POD activities of cells treated with 2J1 and 3J1 were significantly higher (473.23% and 389.51%, respectively) than that of CK. The POD activity of cells treated with 5J2 reached a maximum at 6 h, and then declined gradually and was

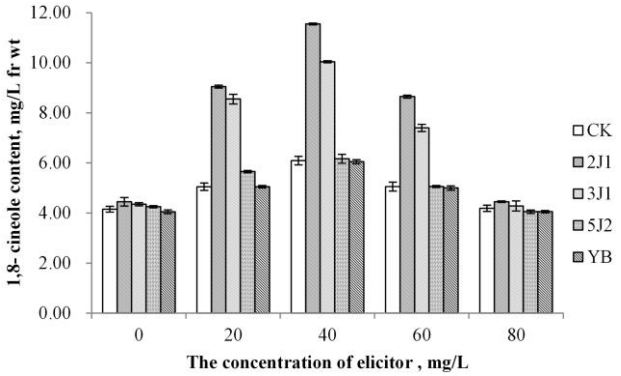

Fig. 1: Effect of different concentrations of endophytic fungal (2J1, *P. commune*; 3J1, *N. crassa*; 5J2, *A. fumigatus*; YB, *P. paneum*) elicitors on 1,8-cineole accumulation in suspension cells of *C. longepaniculatum*

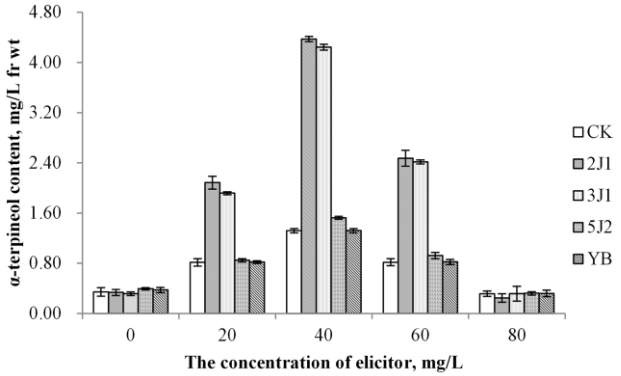

Fig. 2: Effect of different concentrations of endophytic fungal (2J1, *P. commune*; 3J1, *N. crassa*; 5J2, *A. fumigatus*; YB, *P. paneum*) elicitor on α-terpineol accumulation in suspension cells of *C. longepaniculatum*

similar to that of CK (Fig. 3). The CAT activities of the 2J1, 3J1, 5J2 and YB groups reached peaks at 18 h, 18 h, 6 h and 12 h, respectively. At 18h, the CAT activities of cells treated with 2J1 and 3J1 were significantly higher than that of CK, by 1.88- and 1.49-fold, respectively. Although the CAT activities of cells treated with 5J2 and YB showed peaks, they were maintained for a short time and did not show a sharp increase (Fig. 4). These observations demonstrate that the POD activities of cells treated with 2J1 and 3J1 can be maintained at a relatively high level for a certain period of time. CAT activity increased slowly during the early period of culture and thus H_2O_2 was maintained at a certain concentration. H_2O_2 is an important signal molecule in cells and can activate or enhance specific metabolic pathways. However, excessive H_2O_2 will have toxic effects on cells. CAT activity increased sharply during the late period of culture and remained significantly higher than that of CK, thus eliminating the toxic effects of excessive H_2O_2 on cells. Therefore, active oxygen in *C. longepaniculatum* cells increased the activity of defensive enzymes that

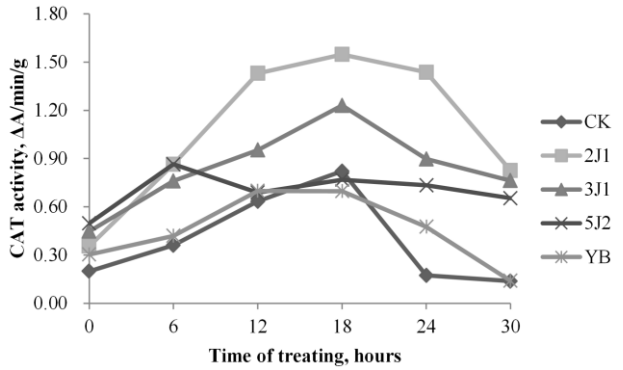

Fig. 3: Activity of catalase (CAT) in cell suspension cultures of *C. longepaniculatum* induced by endophytic fungal (2J1, *P. commune*; 3J1, *N. crassa*; 5J2, *A. fumigatus*; YB, *P. paneum*) elicitors

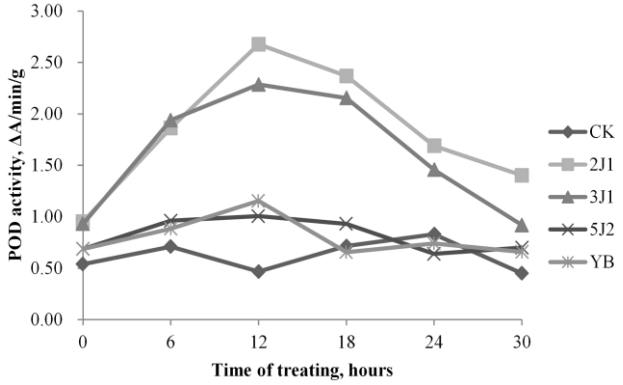

Fig. 4: Activity of peroxidase (POD) in cell suspension cultures of *C. longepaniculatum* induced by endophytic fungal (2J1, *P. commune*; 3J1, *N. crassa*; 5J2, *A. fumigatus*; YB, *P. paneum*) elicitors

were enhanced after treating cells with 2J1 and 3J1. Dynamic changes in the oxidation-reduction enzymes that function in radical scavenging systems may change the redox state of *C. longepaniculatum* cells and thereby stimulate changes in physiological status and metabolic activity, thus influencing the accumulation of secondary metabolites.

Organ aging or injury to plants is often accompanied by membrane lipid peroxidation. MDA is the final decomposition product of membrane lipid peroxidation and MDA content can reflect the degree of injury to plants. The MDA contents of the four test groups increased to different extents. The MDA contents of groups 5J2 and YB changed relatively slightly and were essentially comparable to that of CK. The MDA contents of groups 2J1 and 3J1 reached a peak at 18 h and were 5.11- and 4.53-fold higher, respectively, than that of CK (Fig. 5), indicating that 2J1 and 3J1 can increase MDA content significantly, and hence intensify membrane lipid peroxidation significantly.

Discussion

As a type of unique chemical signal produced by fungal elicitors can rapidly and exclusively induce the expression of specific genes in plant cells, activate specific secondary metabolic pathways, and promote the accumulation of specific secondary metabolites (Simic *et al*., 2015). Use of endophytic fungal elicitors has become an important technique for inducing secondary metabolite accumulation in plant cells. Hence, in many recent studies, they have been widely used to process plant cells, thereby enabling the rapid acquisition of abundant related secondary metabolites. For example, found that fungal elicitor can increase phenylpropanoid and naphtodianthrone contents of *Hypericum perforatum* L. cell cultures (Zhai *et al*., 2017). used endophytic fungal elicitors to enhance rutine production in *Fagopyrum tataricum* hairy root (Zhao *et al*., 2014). The promotion effect of endophytic fungal elicitors on secondary metabolite synthesis of suspension cells is influenced by various factors, including type of endophytic fungi, and type and concentration of elicitors. The activities of different types of elicitors have different speeds and intensities (Zhao *et al*., 2015). Although elicitors can promote the synthesis of secondary metabolites in cells, they can also induce cells to produce substances that are cytotoxic. Therefore, it is important to select appropriate concentrations of elicitors (Tao *et al*., 2011).

Treatment of plant cells with endophytic fungal elicitors not only increases the synthesis of secondary metabolites in cells but can also induce physiological and biochemical changes in plants, and thereby alter related enzyme activities. It is widely accepted that the induced activity of characteristic enzymes is directly correlated with secondary metabolite accumulation (Tao *et al*., 2011; Chen *et al*., 2016). Our research results also confirmed that variations in the enzyme activities in *C. longepaniculatum* cells are positively correlated with the synthesis of essential oil.

Conclusion

Although this study revealed the type and optimal concentration of endophytic fungal elicitors and optimal incubation times favoring essential oil accumulation in *C. longepaniculatum*, further studies are needed to address how elicitors enhance the activities of related key enzymes in metabolic pathways, how signal transduction is realized, why the capability of endophytic fungal elicitors to promote essential oil synthesis begins to “degrade” when under culture for a certain period and what is the relationship between such “degradation” and the host. In future studies, we will continue to examine the mechanisms underlying the effect of endophytic fungal elicitors on essential oil synthesis in *C. longepaniculatum* cells, with the aim of providing references for the subsequent cloning of related regulatory

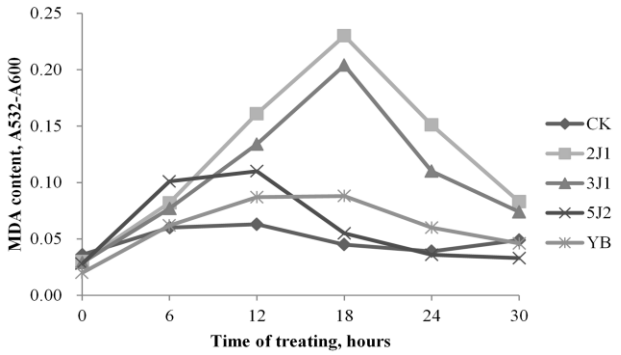

Fig. 5: Content of malondialdehyde (MDA) in cell suspension cultures of *C. longepaniculatum* induced by endophytic fungal (2J1, *P. commune*; 3J1, *N. crassa*; 5J2, *A. fumigatus*; YB, *P. paneum*) elicitors

genes and artificial control of essential oil synthesis in *C. longepaniculatum.*

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