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Full Length Article

Effect of NO-Mediated Endophytic Fungal Elicitors on Essential Oil Accumulation in Suspension Cells of *Cinnamomum longepaniculatum*

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Abstract

Nitric oxide (NO) 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 and *Neurospora crassa* 3J1) for promoting essential oil accumulation in *Cinnamomum longepaniculatum*, changes in the contents of NO 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 NO donor sodium nitroprusside (SNP) into the culture. The release of NO was suppressed and the essential oil contents decreased after the NO-specific scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazol ine-1-oxyl-3-oxide (cPTIO) was added into the cell cultures. cPTIO 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 stimulate NO release and essential oil synthesis in *C. longepaniculatum* cells. Reliable evidence that NO 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. © 2019 Friends Science Publishers

Keywords: Plant secondary metabolism; Nitric oxide (NO); Signaling;

Introduction

Cinnamomum longepaniculatum (Gamble) N. Chao is a commercial crop that contains volatile terpenoids, such as γ-terpinene, α-terpilenol, and 1,8-cineole (Li *et al*., 2014; Xu *et al*., 2014). The benefits of essential oil obtained from *C. longepaniculatum* can be attributed to their anti-inflammatory-, antioxidant-, and antimicrobial properties (Bakkali *et al*., 2008). The demand for these natural essential oils from *C. longepaniculatum* has increased exponentially along with continuous improvements in socioeconomic status and standards of living of people (Wei *et al*., 2016). However, the yield and quality of *C. longepaniculatum* essential oils vary due to multiple factors, such as instability of secondary metabolites and environmental constraints (Yan *et al*., 2017). Therefore, sustainable retrieval of essential oils from *C. longepaniculatum* is an important research focus.

Recent studies demonstrated that endophytic fungal elicitors can effectively increase secondary metabolite production (Xie *et al*., 2011; Jia *et al*., 2016; Clifton *et al*., 2018; Rao *et al*., 2018). Many efforts have been made to elucidate the signal transduction mechanisms involved in the accumulation of secondary metabolites induced by endophytic fungal elicitors (Gao *et al*., 2012; Cui *et al*., 2017; Bastías *et al*., 2018); however, the signal transduction mechanism through which endophytic fungal elicitors promote the accumulation of volatile terpenoids in aromatic plant cells remains unknown (Cui *et al*., 2018; Leylaie and Zafari, 2018; Silva *et al*., 2018). Therefore, it is necessary to study this mechanism further. Many studies report that NO-mediated fungal elicitors can promote the accumulation of plant secondary metabolites (Zhang *et al*., 2011; Girija *et al*., 2016; Shi *et al*., 2017; Ali *et al*., 2018). Understanding the signaling effects of NO involved in the synthesis and regulation of *C. longepaniculatum* essential oil will be useful in promoting its accumulation in *C. longepaniculatum* cells. In this study, the role of NO in the accumulation of essential oils in *C. longepaniculatum* suspension cells induced by fungal elicitors was investigated, and signal transduction pathways of the fungal elicitor mediated promotion of essential oil accumulation were also analyzed.

Materials and Methods

Experimental Materials

Samples of *C. longepaniculatum* leaves were collected

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from the Hongyan Mountain in Yibin (located at 27°50' N; 105°20' E) and were identified by Professor Qin Wei from Yibin University. Two strains of endophytic fungi (*Penicillium commune*, 2J1, and *Neurospora crassa*, 3J1) were isolated from *C. longepaniculatum* plants (Yan *et al.*, 2017). These fungi were cultured and maintained on potato dextrose agar medium (PDA).

Suspension Culture for *C. longepaniculatum* Cells

Young leaves of *C. longepaniculatum* were used for callus induction. The callus was subcultured two to three times. About 1.0 g callus with vigorous growth and loose texture was inoculated into 150 mL conical flasks with 50 mL B_5 culture medium. Cultures were cultured on a shaker at 120 rpm at 25°C and subcultured every 7 days. Fresh cells were subjected to decompression and suction filtration to determine the essential oil content in suspension cells of *C. longepaniculatum*.

Preparation of Endophytic Fungal Elicitors

2J1 and 3J1 were cultured on the potato dextrose agar medium (PDA) and incubated at 28°C. After subculture, the mycelia were transferred to a flask containing 80 mL PDA and cultured in the medium at 28°C, 150 rpm. After the mycelia were harvested, they were filtered and ground with a mortar and a pestle. The homogenate was autoclaved at 121°C for 20 min. The fungal suspension after autoclaving was used as the elicitor. Then, anthrone–sulfuric acid method standardized elicitor concentration (Tao *et al*., 2011).

NO Test

The amount of NO in different treated suspension cells was evaluated by Hu *et al*. (2003). Suspension cells were filtered with a microporous membrane at 4°C. One milliliter filtrate and 1 mL Griess reagent were mixed and incubated at 25°C for 30 min. Absorbance was measured at 550 nm using a spectrophotometer. The NO content was calculated by querying a standard curve of $NaNO_2$.

Effect of Endophytic Fungal Elicitors on NO Release and Essential Oil Accumulation

To explore the role of fungal elicitors in NO burst and essential oil accumulation in *C. longepaniculatum* suspension cells, endophytic fungal elicitors (40 mg/L) prepared from *Penicillium* sp. 2J1 and *Neurospora* spp. 3J1 were used. 15 g of fresh dried cells were extracted at 7, 14, 21, 28 and 35 d after the addition of elicitors. The NO contents were determined at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h. The cells were subjected to decompression and suction filtration to determine the essential oil content in the suspension cells.

To investigate the effects of elicitors on NO release and essential oil contents, the concentrations of elicitors was set to gradients of 0, 20, 40, 60, and 80 mg/L in the 7-day-old cell cultures. The NO contents were determined at 18 h and essential oil accumulation of *C. longepaniculatum* cells was determined at 14 d.

Isolation and Analysis of Essential Oils

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

These are the following chromatographic conditions: chromatographic column, DB-FFAP; capillary column (30 m × 0.25 mm × 0.25 μL); carrier gas, He; carrier gas flow, 0.7 mL/min; and injection port temperature, 250°C. In the heating program, the temperature increased from 50°C to 100°C at a rate of 10°C/min, held at 100°C for 1 min, then increase to 220°C at 20°C/min, and held at 220°C for 13 min; the injected volume was 1.0 μL and the split ratio was 10:1. Mass spectrometer conditions were the following: interface temperature, 280°C; ion source temperature, 230°C; ionization mode, EI; ionization energy, 70 eV; and mass scan range, 29.0–500.0 amu. Meanwhile, appropriate amounts of 1,8-cineole and α-terpilenol were weighed as the standards for use in an external standards method for quantitative analysis (Wang *et al*., 2014).

Treatment of *C. longepaniculatum* Cells by SNP and cPTIO

C. longepaniculatum cells of 7-day-old culture were chosen, and NO donor SNP solution that had been filtered by 0.22 μm Millipore filter was added. The mixture was cultured for 18 h, and SNP was eliminated with fresh medium. Then, cells were suspended again in fresh culture medium. cPTIO was added 20 min. The NO content was evaluated for 18 h, and essential oil content was tested at the day 14. The control group was mixed with an equal volume of sterile water. All the treatments were done in triplicate.

To study relationship of NO signaling molecules and endophytic fungal elicitors in *C. longepaniculatum* cells, seven groups were created, including a control group A without elicitors and experimental groups B (2J1, 40 mg/L), C (3J1, 40 mg/L), D (2J1, 40 mg/L and cPTIO, 2.5 mmol/L), E (3J1, 40 mg/L and cPTIO, 2.5 mmol/L), F (SNP, 7.5 mmol/L), and G (SNP, 7.5 mmol/L and cPTIO, 2.5 mmol/L) and tested them in the experimental setting.

Statistical Analysis

The yield of essential oil and the activity of enzymes from each treatment were measured using three replications. The

data were described as mean values ± standard deviation and analyzed by one-way analysis of variance (ANOVA). Duncan multiple comparison test was used to detect differences between the means of all varieties. Differences were considered significant at a $p \leq 0.05$. All correlation and path coefficient analyses were performed with SPSS Statistics 19.0 and Excel 2007.

Results

Effect of Elicitors on NO Release and Essential Oil Accumulation

As shown in Fig. 1, the NO contents increased following elicitor addition and peaked after 3 h and 18 h. The trends seen for different groups upon addition of elicitors were basically consistent. As can be seen, the NO contents peaked at 5.6 μ mol/L (2J1) and 6.19 μmol/L (3J1) after 3 h and were 2.02- and 1.95-fold higher than that of the control, respectively. On the contrary, the NO contents of the control group without an elicitor remained at a low level. Essential oil contents of cells treated with the fungal elicitor changed with time and were significantly higher than those of the control group (Fig. 2), which indicated that elicitors significantly stimulated the biosynthesis of essential oils in *C. longepaniculatum* cells. Moreover, the contents of essential oils peaked at 14 d after the treatment. After that, the essential oil contents of the treated cells gradually decreased, indicating that a reasonable treatment time is very important for increasing the yield of essential oil.

Effect of different Concentration Elicitors on No Release and Essential Oil Accumulation

The NO contents of cells treated with 2J1 and 3J1 continuously increased with peaked at 40 mg/L (Fig. 3). At the same time, the essential oil contents were also significantly improved with the increase in elicitor concentration (Fig. 4). The changes in essential oil contents in the 2J1 and 3J1 groups were found to correspond with the fluctuation in the NO contents. Furthermore, the control group without elicitors did not exhibit significant changes of both NO and essential oil contents, which indicates that NO is an important signal molecule involved in the promotion of essential oil accumulation by fungal elicitors.

Effect of NO-mediated Elicitors on Essential Oil Accumulation

As shown in Fig. 5 and 6, the cPTIO accompanying elicitor treatments could reduce NO release and inhibit essential oil accumulation. Adding exogenous SNP could increase NO release in cells to a level that is slightly higher than that in the experimental groups in which only elicitors were added. Meanwhile, adding SNP could promote essential oil accumulation, but the essential oil accumulation capacity

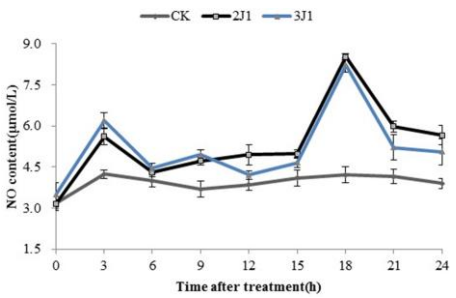

Fig. 1: Time effect of elicitor on NO accumulation in *C. longepaniculatum* cells

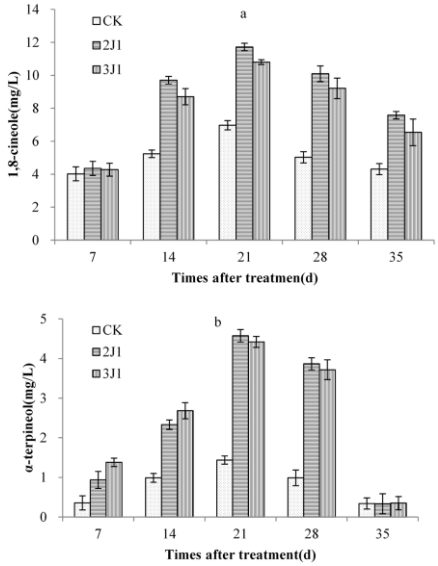

Fig. 2: Time effect of elicitors on essential oil (**a**: 1,8-cineole, **b**: α-terpineol) accumulation in suspension cell of *C. longepaniculatum*

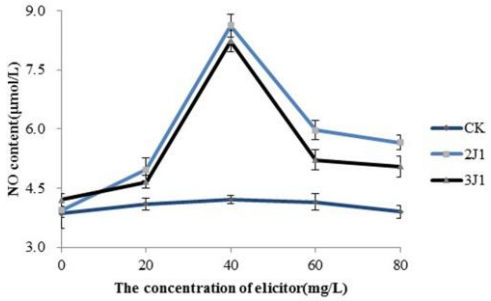

Fig. 3: The effect of different concentration of elicitors on NO release in *C. longepaniculatum* cells

was poorer than that in the experimental groups with elicitors. Compared with the control group, the experimental groups with SNP and cPTIO addition showed similar NO and essential oil contents, indicating that SNP mediated promotion of essential oil synthesis can be suppressed by cPTIO. Adding exogenous SNP can facilitate essential oil synthesis in *C. longepaniculatum* cells, whereas cPTIO can inhibit NO release in cells and reduce essential oil contents.

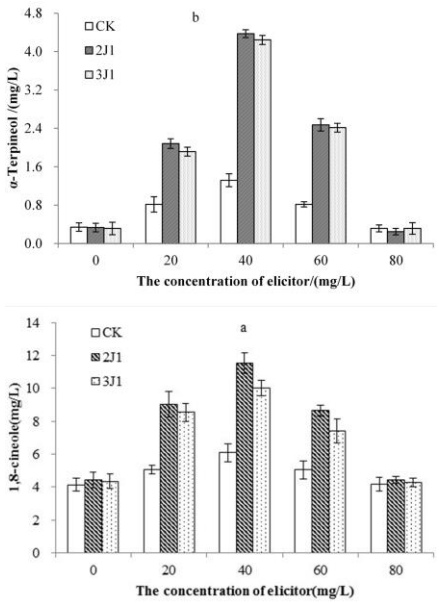

Fig. 4: The effect of different concentrations of endophytic fungal elicitors on essential oil (**a**: 1,8-cineole, **b**: α-terpineol) accumulation in suspension cell of *C. longepaniculatum*

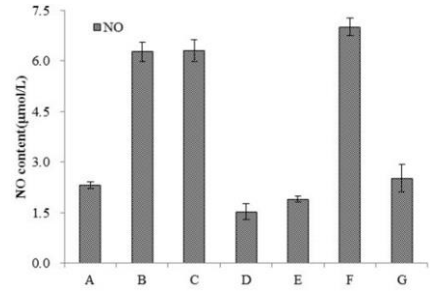

Fig. 5: The NO contents of different experimental groups

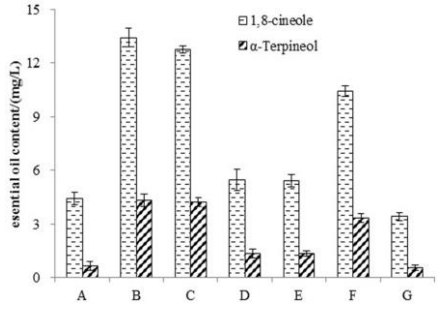

Fig. 6: The essential oil contents of different experimental groups

Discussion

Studies have shown that the production of plant secondary metabolites involves a complex series of biochemical reactions and is controlled by related genes in the cell (Simic *et al*., 2015). Stimulation signals of endophytic fungal elicitors are responsive and transmitted by the corresponding signal transduction mechanisms, thereby controlling the synthesis of plant secondary metabolites (Pusztahelyi *et al*., 2015). The results showed that the release of NO can promote the synthesis of essential oils in *C. longepaniculatum* cells under the action of endophytic fungal elicitors. SNP can significantly promote the accumulation of essential oils, whereas cPTIO can significantly inhibit NO release in *C. longepaniculatum* cells, thereby reducing the content of essential oils. This indicates that there is a pathway involved in the regulation of essential oil synthesis from NO in the signal transduction pathway. NO is a major intracellular signaling molecule that activates plant cell responses by fungal elicitors (Rath *et al*., 2014; Huang *et al*., 2015), and NO release is an early response of many plant cells under fungal elicitor treatment (Rasul *et al*., 2012; Samalova *et al*., 2012). In this study, the burst of NO occurs earlier than the synthesis of essential oil, which may be due to its function as an intracellular messenger; this might regulate the expression of related synthetic genes in the cell, increasing the content of essential oil.

The effects of various signaling pathways on the synthesis of plant secondary metabolites are not independent. Instead, these signaling molecules and their signal transduction pathways form a complex network that mediates external factors and promotes the biosynthesis of secondary metabolites (Gandhi *et al*., 2014; Rahimi *et al*., 2014). The results were also confirmed that the synthesis of essential oil from *C. longepaniculatum* cells could not be completely inhibited by cPTIO. The results further indicated that endophytic fungal elicitors can promote the synthesis of essential oil through other signal transduction pathways, and that the NO pathway is only one of the signaling pathways in *C. longepaniculatum* cells. Therefore, this result can form a basis for the effective regulation of essential oil synthesis from *C. longepaniculatum* and comprehensively reveal the signal transduction mechanism underlying essential oil synthesis in *C. longepaniculatum*.

Conclusion

Through this study, reliable evidence was provided regarding the important role played by NO in the synthesis of essential oil in *C. longepaniculatum* cells induced by endophytic fungal elicitors. Moreover, the addition of SNP can facilitate essential oil synthesis, whereas cPTIO can inhibit NO release and reduce essential oil synthesis in *C. longepaniculatum* cells. However, cPTIO could not completely inhibit essential oil accumulation in *C. longepaniculatum* cells induced by endophytic fungal elicitors, and this indicates that endophytic fungal elicitors can also promote essential oil synthesis in *C. longepaniculatum* cells through other signal transduction pathways.

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