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Time and dosage effects of an endophytic fungal elicitor on the volatile oil production and physiology of *Atractylodes lancea* suspension cells

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The effects of an endophytic fungal elicitor from *Cunninghamella* sp. (AL4) on the volatile oil production and defence responses of host plant *Atractylodes lancea* were investigated. The aim was to improve the capability of endophytic fungi resources in promoting medicinal plant secondary metabolites. The results showed that the endophytic fungal elicitor significantly stimulated volatile oil accumulation. The best inducing concentration of the elicitor for volatile oil production was 30 mgL⁻¹. The highest amount of volatile oil was harvested 9 days (d) after elicitor treatment. The activity of 3-hydroxy-3-methylglutaryl CoA reductase increased by 2.79-folds compared with the control of 7 d after treatment. The elicitor also enhanced the activities of phenylalanine ammonia-lyase, peroxidase superoxide dismutase, polyphenol oxidase, and catalase. These enhanced activities led to improved plant defence-related secondary metabolite synthesis ability. In addition, the elicitor-treated cells were consistently healthy, and had slightly increased biomass. The results demonstrated for the first time the optimal induction time and concentration of the endophytic fungal elicitor, as well as the changes in physiological indices after endophyte-host interaction. The information provided in the present study may be applied in large-scale medicinal plant suspension cultures.

Key words: Endophytic fungus, fungal elicitor, *Atractylodes lancea*, volatile oil, *Cunninghamella* sp.

INTRODUCTION

Secondary metabolites from plants are used as drugs or drug components (Bérdy, 2005), such as paclitaxel for cancer (Wang et al., 2001) and shikonin for HIV (Wu et al., 2009). *Atractylodes lancea*, a member of the Compositae family, is a traditional medicinal plant widely distributed in China (Juan et al., 2002; Yuan et al., 2009; Chandran et al., 2011). Volatile or essential oils from *A. lancea* show antimicrobial activities as well. These oils are a mixture of active secondary metabolites (Wang et al., 2009), including atractylone, hinesol, β -eudesmol, and atractylodin. However, the supply of such medicinal

materials does not usually meet the demand because of environmental contaminations. Plant cell suspension culturing is one of the methods for producing plant secondary metabolites (Cheng et al., 2008). This method has the advantages of stable quality, insensitivity to climate, and easy controllability. Nevertheless, commercial production by plant cell cultures is still limited by low productivity. This limitation is addressed by the use of biotic and abiotic elicitors, which improve the productivity of plant cell cultures (Roberts and Shuler, 1997). Fungal elicitors from pathogenic microorganisms, when added to a plant cell suspension, stimulate the production of target substances. For example, *Candida albicans* and *Staphylococcus aureus* act as elicitors to increase the production of bilobalide and ginkgolides in *Ginkgo biloba* cell suspension cultures (Kang et al.,

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2009). However, there is a tradeoff between the secondary metabolites yield and the plant growth. The growth of plant cells is significantly inhibited after incubation with a pathogenic fungal elicitor (Yuan et al., 2002; Kang et al., 2009). This tradeoff may be minimised using endophytic microbes. These microbes are an intriguing group of organisms associated with various tissues and organs of terrestrial and some aquatic plants. The infection processes of these microbes are inconspicuous, and the infected host tissues are at least transiently symptomless (Stone et al., 2000). Fungal endophytes are synergistically symbiotic with plants. These endophytes can promote plant growth and enhance resistance against phytopathogens (Glenn et al., 1996; Wilhelm et al., 1998). Nonetheless, little research has been done on the effects of endophytic fungal elicitors on host plant suspension cultures.

Plants naturally have a variety of relationships with different fungi. Such relationships include mutualistic associations with mycorrhizal fungi, or antagonistic associations with different pathogenic fungi (Groppe et al., 1999). A series of defensive mechanisms and different physiological responses protects plants against external stresses. As part of these defence responses, the levels of plant secondary metabolites are often mediated by defence-related enzymes. Such enzymes include phenylalanine ammonia-lyase and polyphenol oxidase (Han et al., 2009).

The positive effects of endophytic fungal elicitors on cell cultures of *A. lancea* have been confirmed in previous studies (Fang et al., 2009a, b). In the present work, the optimal elicitor amount and induction time that yields the highest amount of target metabolites were determined. The mechanism of regulating the accumulation of host active secondary metabolites was also investigated. The results may provide theoretical bases for the effective utilization of endophyte resources.

MATERIALS AND METHODS

Cell suspension culture

The *A. lancea* suspended cell line was obtained using the procedures described in our previous report (Fang et al., 2009b). The culture medium was a Murashige and Skoog (1962) medium supplemented with 0.5 mgL⁻¹ naphthaleneacetic acid, 1.0 mgL⁻¹ 6-benzyladenine, and 30 gL⁻¹ sucrose. The medium pH was adjusted to 6.0 before autoclaving. The 25 ml cultures were shaken at 120 rpm in darkness at 23°C in 100 ml Erlenmeyer flasks, and were subcultured every 2 weeks.

Endophytic fungal elicitor preparation and treatment

The fungal endophyte AL4 (*Cunninghamella* sp.) was isolated from *A. lancea* cultured on potato dextrose agar, and then incubated at 28°C (Chen et al., 2008). From 7 day-old cultures, mycelia (1 cm²) were transferred to a 250 ml Erlenmeyer flask containing 80 ml of potato dextrose medium. The flask was rotated at 150 rpm and 28°C for 5 days (d). The mycelia were harvested by filtering and

grinding with a mortar and pestle. The mixture was then diluted 10 times with distilled water (w/v), and autoclaved for 20 min at 121°C. The autoclaved fungal suspension was used as an elicitor (Yuan et al., 2009). Fungal extract was quantified by a phenol-sulphuric acid method using glucose as the standard (Dubois et al., 1956).

For dosage effect investigation, 14 d-old *A. lancea* cultures were treated by 5, 10, 20, 30, and 40 mgL⁻¹ carbohydrate equivalents elicitor; the cells used for experiments were harvested 7 d after treatment. Equal volume of sterile double distilled water was used as control. For inducing time investigation, 14 d-old *A. lancea* cultures were treated by 30 mgL⁻¹ carbohydrate equivalents elicitor; the cells were harvested at 1, 3, 5, 7, 9 and 11 d after treatment individually.

Extraction of volatile oil and gas chromatography (GC) analysis

The dried cell sample (500 mg) was powdered, sonicated for 15 min in 30 ml of hexamethylene, and centrifuged at 5000 g for 5 min. The supernatant was filtered through a 0.45 µm membrane and evaporated. The residue was dissolved in 1 ml of hexamethylene, and then stored in a dark glass bottle at 4°C for GC analysis.

GC was carried out using a Hewlett-Packard 1890 series GC column equipped with a flame ionization detector at 240°C. A polyethylene glycol column with 0.3 µm film thickness was used with special temperature procedures prescribed by Fang et al. (Wang et al., 2011). Nitrogen was the carrier gas, and the flow rate was 4 ml/min. The 4 main components of the volatile oil (atractylone, hinesol, β-eudesmol, and atractylodin) were quantitatively analysed according to our previously published work (Wang et al., 2011).

Enzyme assays

The activities of superoxide dismutase (SOD) were assayed by measuring its capacity in inhibiting the photochemical reduction of nitro-blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 10 mM methionine, 1.17 mM riboflavin, 56 mM NBT, and 30 µl of enzyme extract. The absorbance of the solution was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the photochemical reduction of NBT.

The activity of peroxidase (POD) was analysed according to Seema and Upendra, (2001) with some modifications. Fresh cells (500 mg) were homogenised with 10 ml of 0.1M phosphate buffer (pH 7.0), 1 mM EDTA, and 1.5% w/w polyvinylpyrrolidone. The homogenate was centrifuged at 15000 g for 20 min at 4°C. The supernatant was used as the crude extract for the enzyme activity assays. POD activity was measured following the change in absorbance at 470 nm due to guaiacol oxidation. The activity was assayed for 1 min in a reaction solution (final volume = 3 ml) composed of 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H₂O₂, and 50 µl of crude extract. One unit of POD activity was defined as an absorbance change in 0.01 unitmin⁻¹.

Catalase (CAT) activity was determined by the consumption of H₂O₂ at 240 nm for 30 s (Aebi 1974). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H₂O₂, and 50 µl of cell extract in 3 ml total volume. CAT activity was expressed as the absorbance change in cell optical density at 240 nm/min/g (OD₂₄₀) fresh weight.

The activity of phenylalanine ammonia-lyase (PAL) was analysed following the method of Cheng and Breen (1991) with some modifications. About 500 mg of cells were homogenised for 2 min in 5 ml of 0.1 M borate buffer (pH 8.8) containing 600 mg of polyvinylpyrrolidone, 5 mM β-mercaptoethanol, and 2 mM EDTA.

Table 1. Effect of elicitor concentration on the growth of suspension cells and volatile oil content of *Atractylodes lancea*.

Elicitor (mgL ⁻¹)	Dry weight of cells (g·L ⁻¹)	Atractylone (μg·g ⁻¹)	Hinesol (μg·g ⁻¹)	β-eudesmol (μg·g ⁻¹)	Atractylodin (μg·g ⁻¹)	Total (μg·g ⁻¹)
0	6.98 ± 0.47 ^a	0 ^a	0 ^a	18.08 ± 0.29 ^a	0 ^a	18.08 ± 0.29 ^a
5	8.22 ± 0.54 ^b	5.26 ± 0.65 ^b	15.44 ± 0.56 ^b	15.11 ± 0.40 ^b	4.49 ± 0.66 ^b	40.3 ± 2.26 ^b
10	7.41 ± 0.49 ^a	8.21 ± 0.50 ^c	18.06 ± 0.17 ^c	22.48 ± 0.37 ^c	7.99 ± 2.26 ^c	56.75 ± 3.29 ^c
20	7.18 ± 0.48 ^a	15.13 ± 1.15 ^e	33.33 ± 2.31 ^e	41.64 ± 2.61 ^e	7.95 ± 1.18 ^c	98.04 ± 7.26 ^e
30	7.06 ± 0.45 ^a	15.53 ± 0.45 ^e	35.71 ± 1.96 ^e	45.19 ± 0.75 ^f	9.87 ± 0.83 ^c	106.29 ± 3.98 ^f
40	6.98 ± 0.44 ^a	12.65 ± 0.56 ^d	29.17 ± 1.72 ^d	35.32 ± 0.94 ^d	8.03 ± 0.08 ^c	85.15 ± 3.3 ^d

The results are represented by their mean ± standard deviation (SD) of triplicate samples. Values followed by different letters differ significantly at $P = 0.05$.

The homogenate was centrifuged for 15 min at 14000 g , and the supernatant was collected for enzyme activity determination. PAL activity was measured by incubating 0.5 ml of supernatant with 2 ml of 0.1 M borate buffer (pH 8.0) containing 3 mM L-phenylalanine for 1 h at 30°C. The increase in absorbance at 290 nm due to the formation of trans-cinnamate was spectrophotometrically measured. PAL activity was expressed as the absorbance change in 240 nm/min/g (OD₂₉₀) fresh weight.

The activity of 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase (HMGR) was determined by the method of Toroser and Huber (1998). The enzyme extract was added (75 mg·protein·ml⁻¹) to 50 mM Tris-HCl assay buffer (pH7.0) containing 0.3 mM HMG-CoA (Sigma, Cat.H6132), 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), and 4 mM dithiothreitol. NADPH oxidation in the reaction solution was monitored at 25°C by the decreasing absorbance at 340 nm, with the solution free of HMG-CoA as a blank. One HMGR enzyme unit was equivalent to the oxidation of 1 mM NADPH per minute.

Polyphenol oxidase (PPO) activity was determined according to the method of Wang et al. (1991) using 30 μg of total protein, 25 mM citrate-phosphate buffer at pH 6.4, and 5 mM L-proline in a reaction mixture volume of 1 ml. The sample was aerated, and pyrocatechol (1, 2-dihydroxybenzene) at a final concentration of 20 mM was used as the substrate. Absorbance readings were performed at 515 nm, 25°C, and 10 s intervals for 1 min. Using 0.01 absorbance as a unit of enzyme activity (U), enzyme activity was expressed as U g⁻¹FW. The soluble protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Statistical analysis

Data were analyzed using Microsoft Excel. The values were represented as means of three replicates (mean ± SD) for each treatment. One-way ANOVA was performed with SPSS version 13.0 (IBM Corporation, Somers, NY, USA). Duncan's Multiple Range Test was used to measure the difference among the means at a level of $P = 0.05$.

RESULTS

Effects of the elicitor on the regeneration and volatile oil production of *A. lancea* suspension cells

Effects of applied elicitor concentration on the volatile oil production

Cell growth promotion was 117.73% of the control when

the elicitor concentration was 5 mgL⁻¹ (Table 1). With increased elicitor concentration, the promotion of biomass regeneration was greater but weaker. When 40 mgL⁻¹ elicitor was added, the dry weight (DW) of the suspension cells of the treatment group was equal to that of the control group.

Only β-eudesmol was detected in the suspension cells without an elicitor (Table 1). In contrast, all four main medical components (attractylone, hinesol, β-eudesmol, and atractylodin) were detected with different concentrations of elicitor. Volatile oil production showed obvious concentration dependence to the AL4 fungal elicitor; volatile oil accumulation peaked at 30 mgL⁻¹ elicitor concentration.

Effects of elicitor induction time on the volatile oil production

To further determine the optimal fungal elicitor induction time on volatile oil production, 30 mgL⁻¹ elicitor as previously proved was added to the *A. lancea* suspension cells at different times (Table 2). β-eudesmol reached the highest amount of 53.02 μg g⁻¹ at 9 days after elicitor application (Table 2). One day after elicitor application, the β-eudesmol content was 41.68% higher than that of the control group, and all 3 other volatile oils components of *A. lancea* began to accumulate in the suspension cells treated by 30 mg/l elicitor for 3 days (Table 2). As seen in Table 2, 9 days was the best induction time, atractylone, hinesol, β-eudesmol, and atractylodin reached their highest amount, 12.85, 31.46, 53.02 and 18.35 μg g⁻¹, respectively.

Effects of the fungus elicitor on plant defence related enzyme activity

To understand the primary defence responses of the host treated with the endophytic fungal elicitor, the activities of POD, SOD, and CAT were assayed. The activities of the 3 antioxidant enzymes significantly increased in chronological order (Figure 1). First, SOD activity induced

Table 2. Time course of the growth of suspension cell and volatile oil content of *A. lancea*.

Components	Treatment	0 days	1 days	3 days	5 days	7 days	9 days	11 days
Atractylone ($\mu\text{g}\cdot\text{g}^{-1}$)	Control	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	Elicitor	0 ^a	0 ^a	3.96 $\pm$ 0.65 ^b	8.35 $\pm$ 0.09 ^c	13.44 $\pm$ 0.76 ^d	12.85 $\pm$ 1.82 ^d	4.85 $\pm$ 0.65 ^b
Hinesol ($\mu\text{g}\cdot\text{g}^{-1}$)	Control	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	Elicitor	0 ^a	0 ^a	9.29 $\pm$ 0.10 ^b	19.21 $\pm$ 1.43 ^d	30.50 $\pm$ 0.62 ^e	31.46 $\pm$ 0.39 ^e	13.18 $\pm$ 0.16 ^c
β -eudesmol ($\mu\text{g}\cdot\text{g}^{-1}$)	Control	0 ^a	5.11 $\pm$ 0.22 ^b	11.35 $\pm$ 0.21 ^c	15.29 $\pm$ 0.60 ^e	19.62 $\pm$ 0.13 ^f	14.97 $\pm$ 0.84 ^g	13.55 $\pm$ 0.39 ^d
	Elicitor	0 ^a	7.24 $\pm$ 0.62 ^b	16.34 $\pm$ 0.20 ^c	27.26 $\pm$ 1.02 ^e	48.68 $\pm$ 1.48 ^f	53.02 $\pm$ 0.63 ^e	23.75 $\pm$ 1.38 ^c
Atractylodin ($\mu\text{g}\cdot\text{g}^{-1}$)	Control	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	Elicitor	0 ^a	0 ^a	3.64 $\pm$ 0.24 ^b	8.64 $\pm$ 0.87 ^c	7.67 $\pm$ 1.22 ^c	18.35 $\pm$ 0.49 ^e	10.64 $\pm$ 0.63 ^d
Total ($\mu\text{g}\cdot\text{g}^{-1}$)	Control	0 ^a	5.11 $\pm$ 0.22 ^b	11.35 $\pm$ 0.21 ^c	15.29 $\pm$ 0.60 ^e	19.62 $\pm$ 0.13 ^f	14.97 $\pm$ 0.84 ^g	13.55 $\pm$ 0.39 ^d
	Elicitor	0 ^a	12.35 $\pm$ 0.83 ^b	44.58 $\pm$ 1.38 ^c	78.75 $\pm$ 4 ^e	119.91 $\pm$ 4.19 ^f	130.66 $\pm$ 4.16 ^g	65.95 $\pm$ 3.2 ^d

The results are represented by their mean $\pm$ standard deviation (SD) of triplicate samples. Values followed by different letters differ significantly at $P = 0.05$.

by the fungal elicitor rapidly increased within a short time. The maximum level was reached at 2.5 and 7.5 h, and the level decreased to that of the control at 15 h (Figure 1a). Subsequently, POD activity increased at 5 h, reached the highest point at 10 h, and equalled the control at 17.5 h (Figure 1b). CAT activity was low in the early period compared with the control (Figure 1c). In contrast, minimal variations were observed in the control groups.

The PAL activity induced by the fungal elicitor immediately increased and peaked early (Figure 2). The highest level was 3.3-folds higher than that in the control groups (Figure 2a). On the other hand, the highest levels of PPO activities in the elicitor-treated groups were found at day 5, and the values were 1.7 times those of the control (Figure 2b).

Effects of the fungus elicitor on HMGR

As sesquiterpenes synthesis rate-limiting enzyme in higher plants (Chappell et al., 1995), HMGR may control volatile oil accumulation in *A. lancea*; hence, HMGR activity was investigated. When the fungal elicitor was applied on day 1, HMGR activity began to increase, and reached the highest point (2.79-folds) 7 days after induction (Figure 3).

DISCUSSION

The induced effects of elicitor concentration may be classified into two types. One is the saturation-reaction type. In this type, the production of secondary metabolites increases with increased elicitor concentration until the

maximum amount is reached. The other is the optimum-concentration type. In this type, the production of secondary metabolites reaches the maximum amount when the optimum concentration of the elicitor is applied. In the present study, the induction effect of the fungal elicitor was of the optimum-concentration type. The optimum concentration was determined as 30 mg L⁻¹ (Table 1). The induction time also significantly affects the accumulation of secondary metabolites. In the current work, 1 day after elicitor application, the *A. lancea* cells began to produce volatile oils, and the synthesis was increasing till 9th day (Table 2).

The synthesis of many secondary metabolites in plants is widely accepted to be part of the defence responses of plants. Endophytes belonging to the Clavicipitaceae family can accumulate several classes of fungal metabolites. These metabolites serve as relief mechanisms to grasses resisting biotic and abiotic stresses, including fungal diseases (Kuldau and Bacon, 2008). The activities of POD, SOD, and CAT are usually used to evaluate the physiological and biochemical responses of plants to biotic and abiotic stresses, as well as the systemic acquired resistance of the plant (Peltonen et al., 1997; Gechev et al., 2003). POD participates in a variety of plant defence mechanisms, and is involved in plant resistance against certain diseases (Silva et al., 2008; Dutsadee and Nunta, 2008). SOD, POD and CAT, which play important roles in the metabolism of reactive oxygen species, could be induced by environmental stresses, including fungal elicitors (Pachten and Barz, 1999; Tanabe et al., 2008). Our results showed that POD activity was up-regulated to eliminate the H₂O₂ produced by SOD in chronological order (Figure 1). Interestingly, CAT activity was low in the early period which may have enabled the culture to maintain the H₂O₂ concentration,

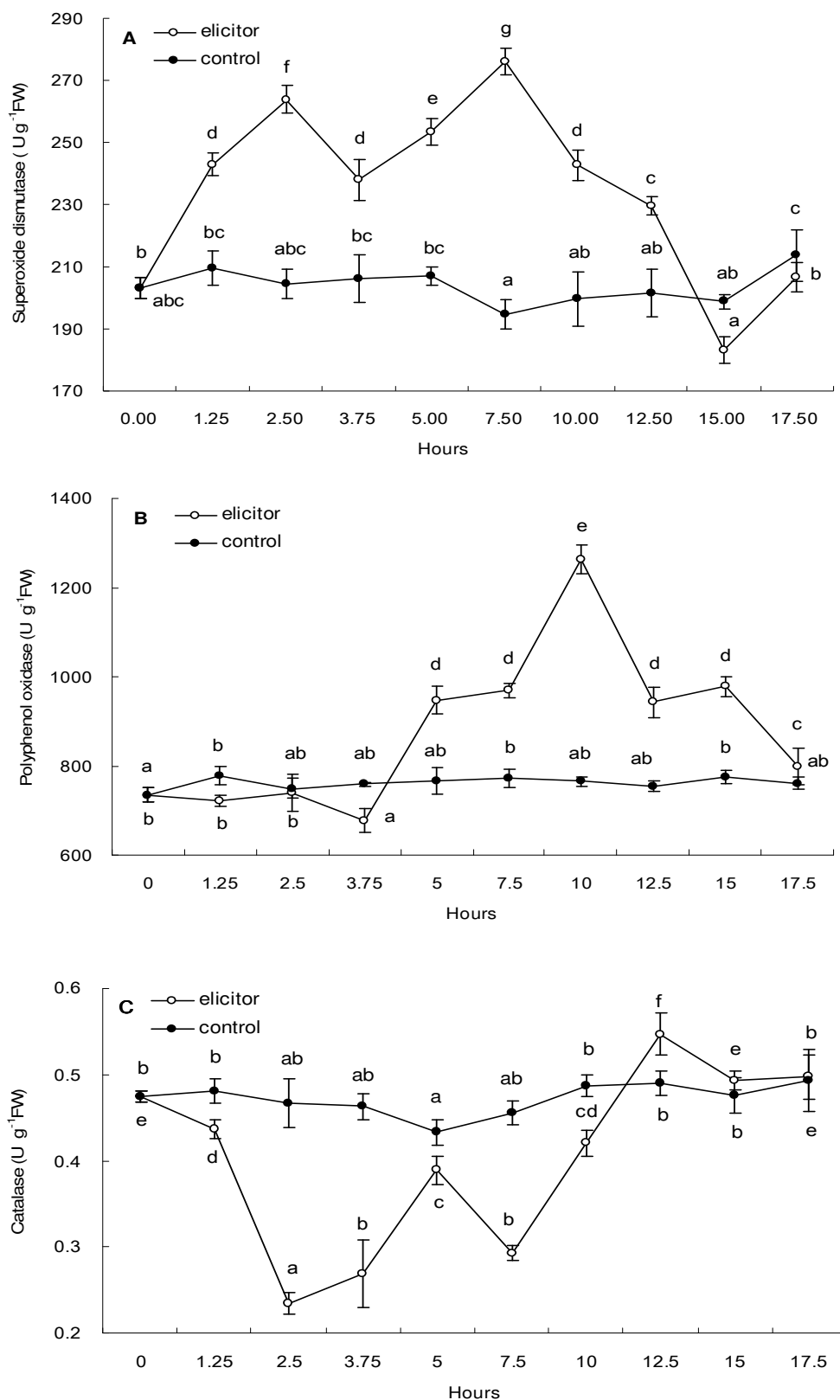


Figure 1. Effects of the AL4 crude elicitor on the primary defence responses of the suspension culture. Error bars represent standard deviation of three replicates. Values followed by different letters differ significantly at $P = 0.05$. FW= fresh weight of *Atractylodes lancea* cells. (A) Effects of the AL4 crude elicitor on the activity of SOD; (B) effects of the AL4 crude elicitor on POD activity; (C) effects of the AL4 crude elicitor on CAT activity.

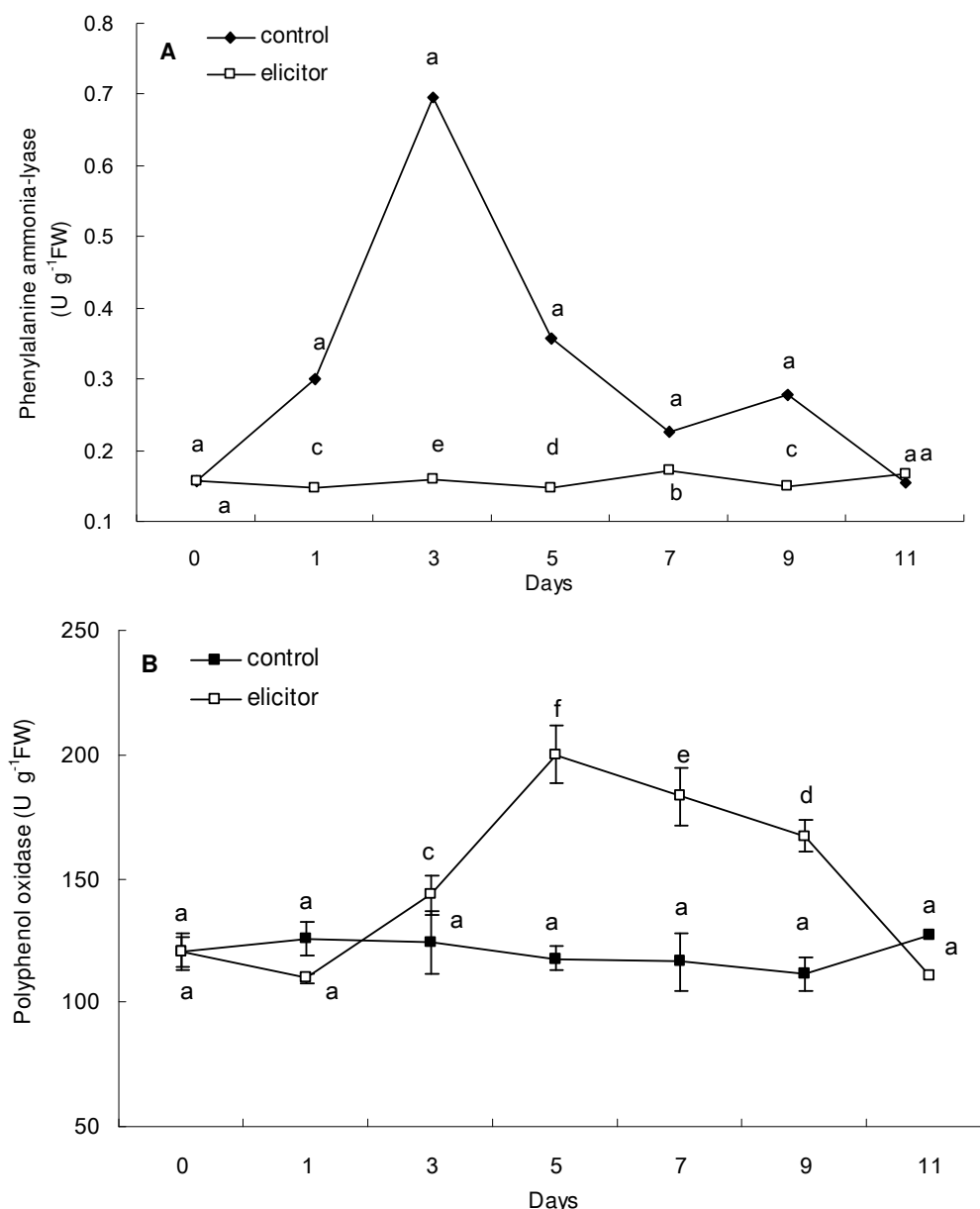


Figure 2. Effects of the AL4 crude elicitor on the activities of the resistance-related enzymes of the host. Error bars represent standard deviation of three replicates. Values followed by different letters differ significantly at $P = 0.05$. FW=fresh weight of *A. lancea* cells. (A) Effects of the AL4 crude elicitor on PAL activity; (B) effects of the AL4 crude elicitor on PPO activity.

and then H_2O_2 was used to stimulate the accumulation of secondary metabolites in plant cells. CAT activity eventually increased to eliminate H_2O_2 , which would have harmed the cells at certain accumulated amount (Figure 1c). PAL is the key enzyme of phenols in plants, and PPO can oxidise various phenols into quinones. Both are involved in the resistance-related reactions of plants (Xu and Dong, 2005). PAL has been reported to be up-regulated in *Catharanthus roseus* cell cultures induced by *Aspergillus niger* elicitor (Chen et al., 2009; Juan et al., 2002). Our result showed that the activities of both PAL

and PPO sequentially reached their peaks, indicating that the fungal elicitor treatment initiated the resistance-related reaction. It implies that volatile oil accumulation in the *A. lancea* suspension cells were closely linked with plant defence.

In higher plants, mevalonate (MVA) pathway that mainly exists in the cytosolic and endoplasmic reticulum (ER) compartments is responsible for biosynthesis of sesquiterpenes, triterpenes and polyterpenes (Ha et al., 2003). HMGR which catalyses the NADP-dependent synthesis of mevalonate from HMG-CoA, is considered

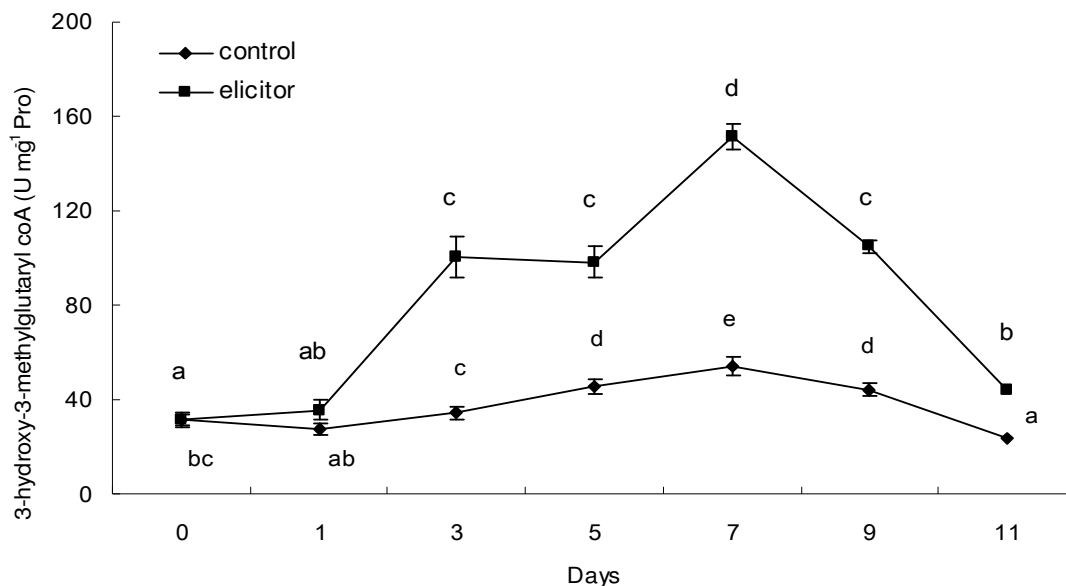


Figure 3. Effects of the AL4 crude elicitor on HMGR activity. Error bars represent standard deviation of three replicates. Values followed by different letters differ significantly at $P = 0.05$. FW= fresh weight of *A. lancea* cells.

the first step in the MVA pathway (Chappell et al, 1995). Overexpression of HMGR gene in *Salvia miltiorrhiza* significantly improves tanshinone production, indicating that HMGR is important in isoprene metabolism (Kai et al., 2006). Our results showed that HMGR was enhanced greatly (Figure 3) and followed by volatile oil accumulation in *A. lancea* (Table 2).

Conclusion

In conclusion, endophyte elicitor from *Atractylodes lancea* enhances the activities of SOD, POD, CAT, PAL and PPO suggests that the endophyte AL4 elicitor can improve the resistance of *A. lancea* cells, do not suppress plant cells growth significantly. Subsequently, the content of secondary metabolites is increased.

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