

Full Length Research Paper

Effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus*

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Accepted 9 March, 2011

This study examined the effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus*. Endophytic fungus and its elicitors were inoculated in cell suspension cultures of *C. roseus* respectively. The biochemical change of suspension cells was then tested. The results showed when the pH rises, the concentration of MDA increases as the activities of antioxidative enzymes (peroxidase and catalase) rises. The critical enzymes of alkaloid synthesis include phenylalanine ammonialyase and tryptophan decarboxylase in suspension cells were also found to increase, along with the alkaloid yield rises. The alkaloid yields reached 770.36 693.76 and 693.76 µg/gFW in the co-culture and elicitor-induced groups, which are 48 and 32% higher than the control group, respectively.

Key words: *Catharanthus roseus*, suspension cell, endophytic fungus, alkaloid.

INTRODUCTION

Catharanthus roseus (L.) G. Don, or periwinkle, is a tropical plant that belongs to the pocynaceae family. The plant is of interest because it is capable of synthesizing useful secondary metabolites, including vindoline, vincristine and vinblastine, which were prevalently used in the treatment of cancer (Lounasmaa et al., 1989). Available for harvest all year round, the plant itself is poisonous. The secondary metabolites found in *C. roseus* extracts and the biosynthetic pathways leading to the compounds have been described in the literature (Vander et al., 1989, 2004; Facchini, 2001; De Luca et al., 2000,

2001).

It is noteworthy that some plants generating bioactive natural products have associated endophytes that can produce the same compounds (Strobel, 2003). In the past decades, plenty of secondary metabolites from endophytic fungi have been tested and found to have applications as medicinal and agrochemical candidates (Strobel, 2003; Huang et al., 2008). Fungal elicitor can induce cell synthesis and accumulation of secondary metabolites in some hypersensitive plants, such as phytoalexin, flavonol, alkaloid and others. As a kind of special chemical signal in the interaction between plants and microbes, they can induce the specific gene in plants metabolism to express quickly, specificity and selectively (Eiler, 1987). The reason might be related to a genetic recombination of the endophytic fungi with their host that occurs in evolutionary time (Tan and Zou, 2001). Endophytic fungi, the latent phytopathogens or mutualistic symbionts presenting inter- and/or intracellularly in the normal tissue of host plants, have proved

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Abbreviations: *C. roseus*, *Catharanthus roseus*; **MDA**, malonic dialdehyde; **POD**, peroxidase; **CAT**, catalase; **PAL**, phenylalanine ammonialyase; **TDC**, tryptophan decarboxylase.

to be a promising source of secondary metabolites with novel structures and/or strong bioactivities (Tan and Zou, 2001; Schulz et al., 2002).

In recent years, in the study of secondary metabolite production in plant cell cultures, a new method has received widespread attention: the application of elicitor treatment on plants which can promote culture cells synthesized a large number of useful secondary metabolites rapidly (Wu et al., 2002). At the same time, a large number of studies shown that endophytic fungus participated in the synthesis of active ingredients or the transformation of plant secondary metabolites (Eilert et al., 2004; Boller, 2005). The enzymes of tryptophan decarboxylase (TDC) and phenylalanine ammonialyase (PAL) were used as the rate-limiting enzymes in the synthesis of *C. roseus* alkaloid. To explore the effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis of *C. roseus*, endophytic fungus and its elicitors were inoculated in cell suspension cultures of *C. roseus*, the biochemical change of suspension cells was then tested.

MATERIALS AND METHODS

C. roseus callus and suspension cell culture

Calli were initiated from the explants that were taken from the second leaf under apical bud in *C. roseus*, and was cultured in our laboratory for a year. Two grams of lax callus was selected and crushed carefully with forceps, and then loaded into a 100 ml flask with 30 ml MS liquid culture medium (contains 0.5 mg/L 2, 4-D and 0.1 mg/L BA). Then it was cultured with a shaker for 120 r/min at 25°C in a dark room. During the subculture, 5 ml suspension cell liquid was added into 30 ml fresh liquid culture medium after the *C. roseus* cell suspension had been cultured for 10 days.

Endophytic fungi culture

Endophytic fungi was isolated from *C. roseus* in our laboratory and named F9, it was identified belonging to *Fusarium oxysporum* Schlecht. F9 was taken and inoculated into a potato glucose liquid medium, cultured with a shaker for 100 r/min at 28°C and harvested after 7 days.

Preparation of the endophytic fungal elicitor

After mycelium was harvested, washed it with methanol: chloroform (V/V=1:1) and phosphate buffer (0.1 mol/L, pH 7.0) solution respectively. After being grinded and filtered, the fungi mycelium was sterilized at 121°C for 20 min. The sugar content was measured using the Anthrone-sulfuric acid colorimetric method, and glucose was used to calibrate the standard concentration (Sornani et al., 1987).

Mensuration of the suspension cell growth curve

After vaccination, samples were taken every 2 days, vacuum-filtrated until they were no longer soaked in water and then weighed. Culture time was used as abscissa, and the fresh weight of the suspension cells was used as ordinate to draw a growth

curve of *C. roseus* suspension cell.

Treatment of the suspension cells of *C. roseus*

After 10 days, the suspension cell culture was divided into three groups and treated as follows:

Group I: Inoculation of the endophytic fungi in suspension cells, cultured and labelled "Co-culture group".

Group II: Inoculation of the endogenous fungal elicitor in suspension cells, cultured and labelled "elicitor-induced group", with an elicitor final concentration of 1 µg/ml.

Group III: Added the same volume of sterile water solution in suspension cells as elicitor, cultured and labeled "control group".

Within the first 36 h after inoculation, sampling was done on the three groups with 6 h intervals.

Sampling was followed by vacuum filtration, after which the samples were quickly frozen with liquid nitrogen and kept at a low temperature in a refrigerator.

Determination of the medium pH was done using Precise Delta320 pH Meter (Mettler-Toledo Co. Ltd), peroxidase (POD) activity using $\Delta A_{470}/\text{min/gFW}$ was used to express the enzyme activity of POD (Braker et al., 1980), catalase (CAT) activity using $\Delta A_{240}/\text{min/gFW}$ was used to express the enzyme activity of CAT (Braker et al., 1980) and phenylalanine ammonialyase (PAL) activity using $\Delta A_{290}/\text{h/gFW}$ was used to express the enzyme activity of PAL (Ouyang et al., 1985).

Determination of TDC activity required 1 ml reaction liquid (containing 0.05 mol/L phosphate buffer, 1 mmol/L tryptophan, 1 mmol/L phosphopyridoxal and 3 mmol LDTT/ pH7.6) mixed with 1 ml sample solution, to which 3 ml methanol was added after 30°C and was kept warm for 30 min.

The test solution was then measured at 595 nm, and $\Delta A_{595}/\text{h/gFW}$ was used to express the enzyme activity of TDC (Pennings et al., 1989).

Protein content was determined using Coomassie brilliant blue G-250 (Bradford et al., 1976). Malondialdehyde (MDA) content was identified by using ($\Delta A=A_{532}-A_{600}$) (Liu et al., 1994).

Total content of alkaloids was determined in accordance with the method of Yuan (1991).

RESULTS

The growth of *C. roseus* suspended cells

After 5 ml of *C. roseus* suspended cells have been inoculated into a 30 ml fresh liquid medium for 10d, subjecting it to subculture five times, a steady growth of suspension cells was obtained. During the sixth subculture, samples were taken every 2 days, and the suspended cell growth curve (Figure 1) was measured. From the curve, *C. roseus* suspended cells showed growth delay but a relatively stable cell biomass on the first 4 days of the culture. On the fourth day, the cells entered the logarithmic growth phase, with their biomass increasing until the 10th day which got the highest biomass of 109.593 g/L, and then they progressed into a stable growth period, with the biomass remaining high and approximately unchanged.

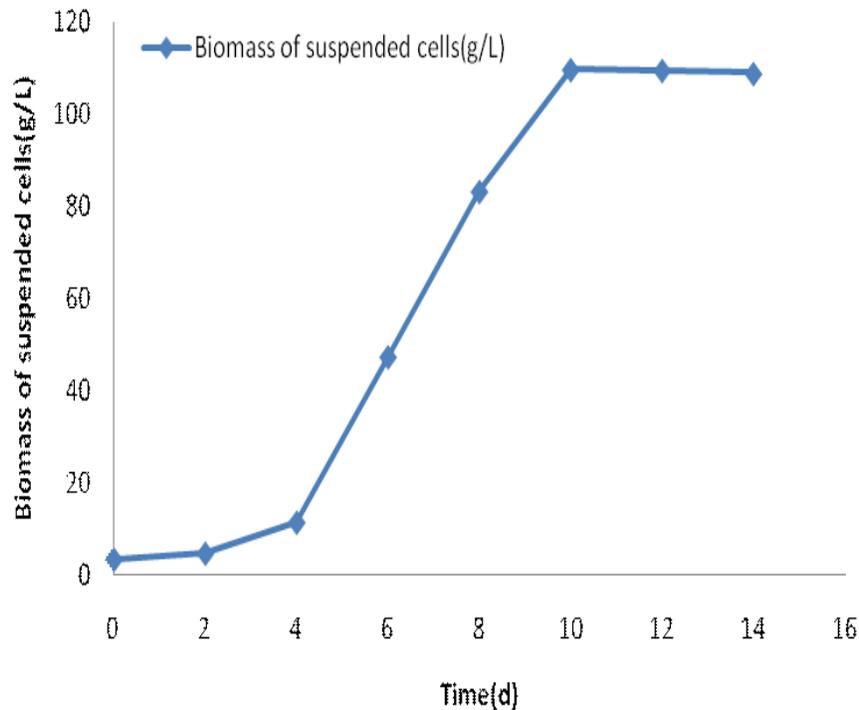


Figure 1. Growth curve of *C. roseus*.

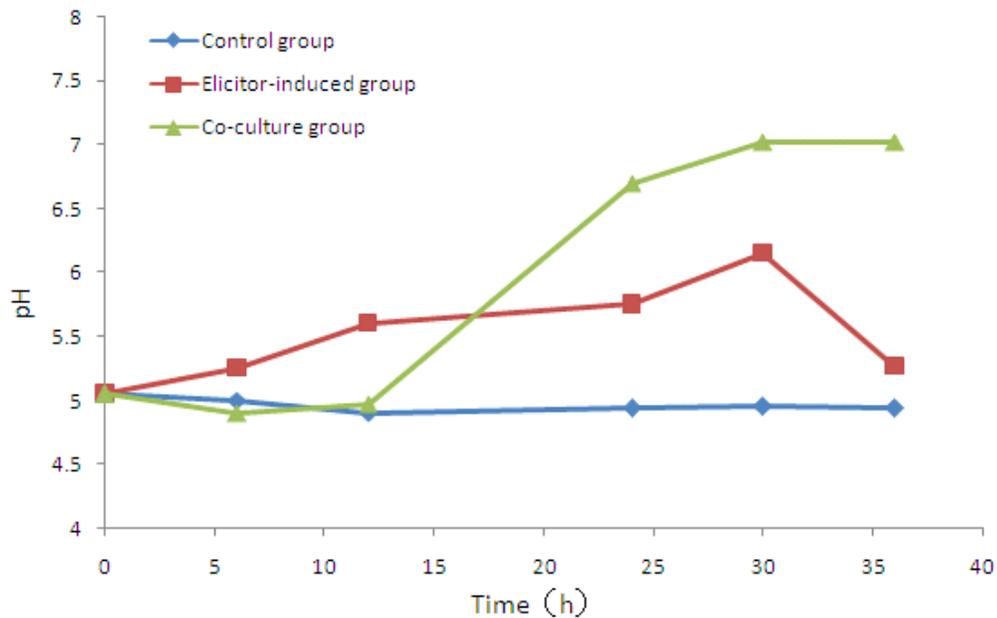


Figure 2. Effects of endophytic fungi and elicitors on the pH of *C. roseus* suspension cells.

Effects of endophytic fungi and elicitors on the pH of *C. roseus* suspension cells

As seen in Figure 2, in the Elicitor-induced group, the culture medium pH level increased and reached the

maximum pH of 6.15 at 30 h, and then declined. The pH level in the co-culture group was consistent with that of the control group before 12 h, and then the pH level increased until it reached the maximum pH 7.02 at 30 h. This may be due to the endophytic fungi still being in the

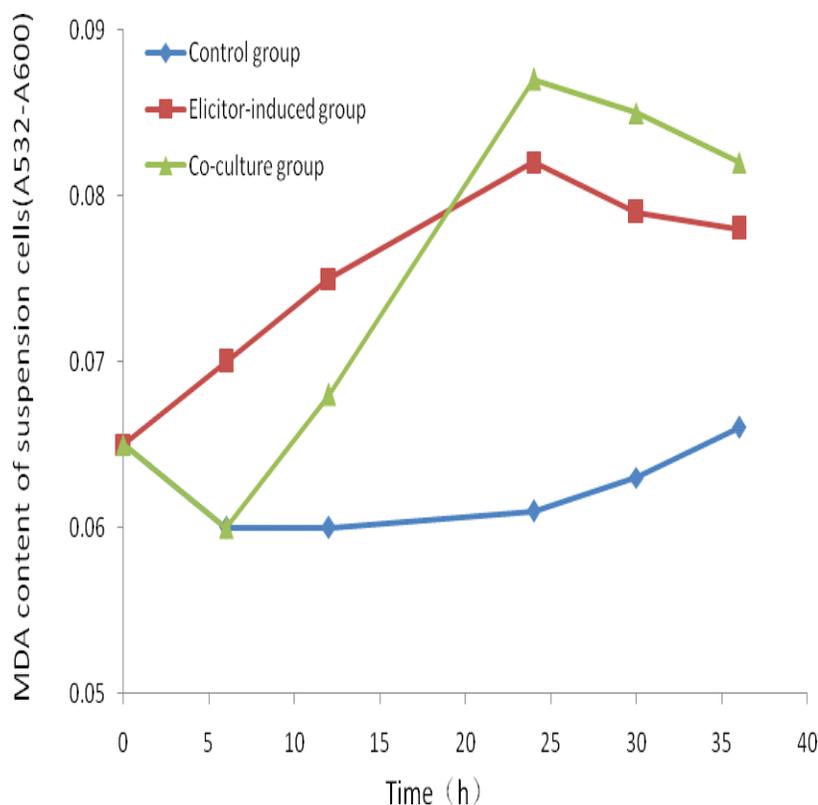


Figure 3. Effects of endophytical fungi and elicitors on the content of MDA in *C. roseus* suspension cells.

lag phase, where the biomass growth is less, corresponding signal molecules do not generate, or very little or no stimulation fails to trigger the physiological response in co-culture suspended cells. The addition of F9 and elicitor led to the alkalization of the co-culture and elicitor-induced groups. The increase in pH level meant the proton concentration decreased in the cell culture medium. To maintain the ion concentration balance inside or outside of the cells, cations in the cells must flow outside, triggering a series of biological effects.

Effects of endophytic fungi and elicitors on the MDA content in the cell suspension

After inoculating the endophytic fungi or their elicitors in the suspension cells, the MDA content of cell suspension (A532—A600) increased substantially, which is consistently higher than the control group. In the elicitor-induced group, the MDA content kept increasing until it reached its maximum value of 0.082 at 24 h, and then declined. In the co-culture group, the MDA content increased after 6 h and obtained the highest 0.087 at 24 h, then subsequently followed a downward trend which is slower than the decline in the elicitor-induced group. As shown in Figure 3, the MDA content of the suspension

cells increased after the endophytic fungi and their elicitors were added, which indicates that the *C. roseus* suspension cells increase lipid overoxidation.

Effects of endophytic fungi and elicitors on the total soluble protein content of *C. roseus* suspension cells

After adding the elicitors or the endophytic fungi was inoculated in *C. roseus* suspension cells, there was an obvious change in the amount of intracellular soluble protein.

As shown in Figure 4, the total soluble protein content in the co-culture group was higher than that in the control group except at 6 h. Compared with the control group, the total soluble protein content in the elicitor-induced group was lower in the first 6 h, then increased and exceeded that of the control group, until it got 7620 $\mu\text{g/gFW}$ at 12 h. It subsequently followed a downward trend before 24 h which it got 6262 $\mu\text{g/gFW}$, and then it increased until it got 8280 $\mu\text{g/gFW}$ at 30 h. This may be attributed to the elicitor temporarily suppressing cell anabolism, keeping the protein content low at first. Later, with the start and strengthening of the secondary metabolism of a specific metabolic pathway, the protein content increased.

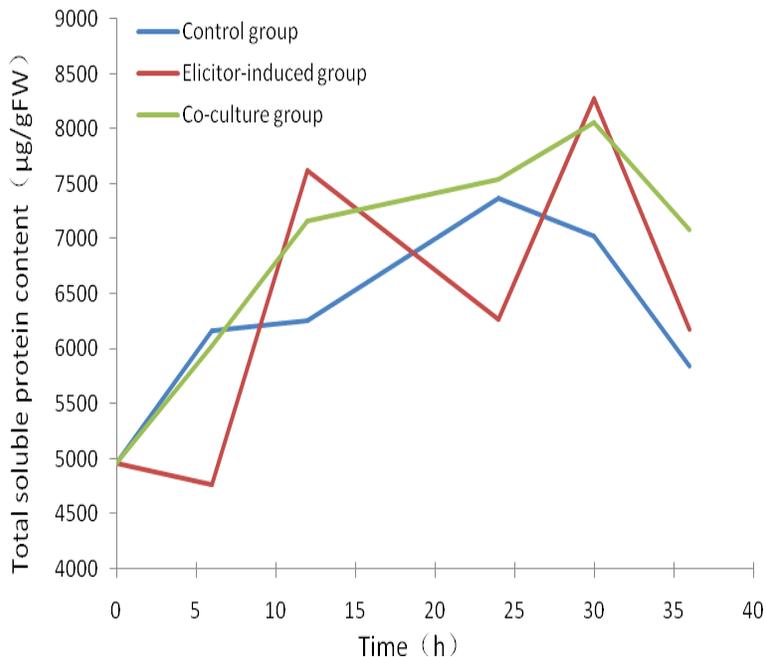


Figure 4. Effects of endophytic fungi and elicitors on the total soluble protein content of *C. roseus* suspension cells.

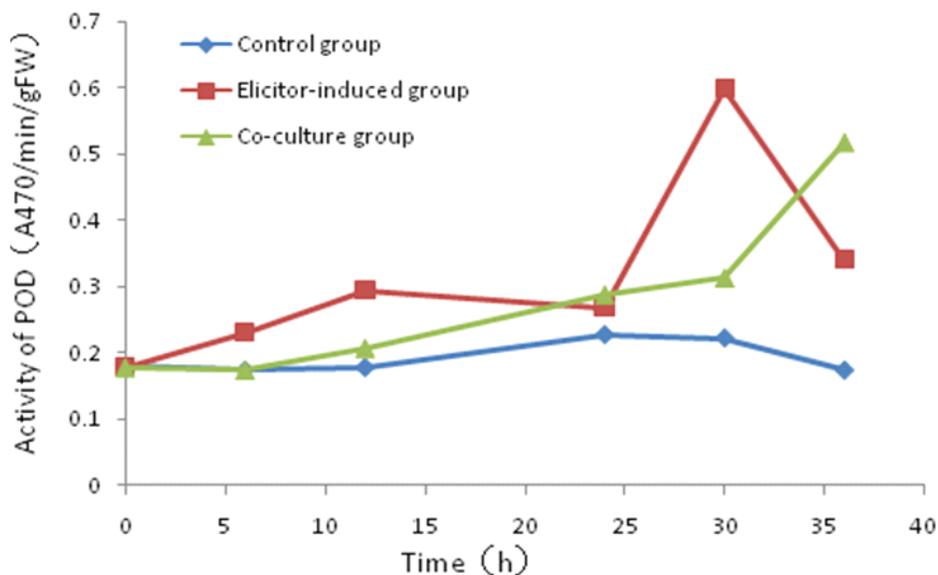


Figure 5. Effects of endophytic fungi and elicitors on the activity of POD in *C. roseus* suspension cells.

Effects of endophytic fungi and elicitors on the free radical scavenging activity of *C. roseus* suspension cells

As shown in Figure 5, after inoculation in the suspension cells, POD and CAT activity increased as seen in the

time sequence, the activity of POD rose and reached its peak value of 0.598 A470/min/gFW at 30 h in the elicitor-induced groups and of 0.516 A470/min/gFW at 36 h in the co-culture groups. As shown in Figure 6, the activity of CAT was consistent in both co-culture and elicitor-induced groups, with H₂O₂ accumulated in high

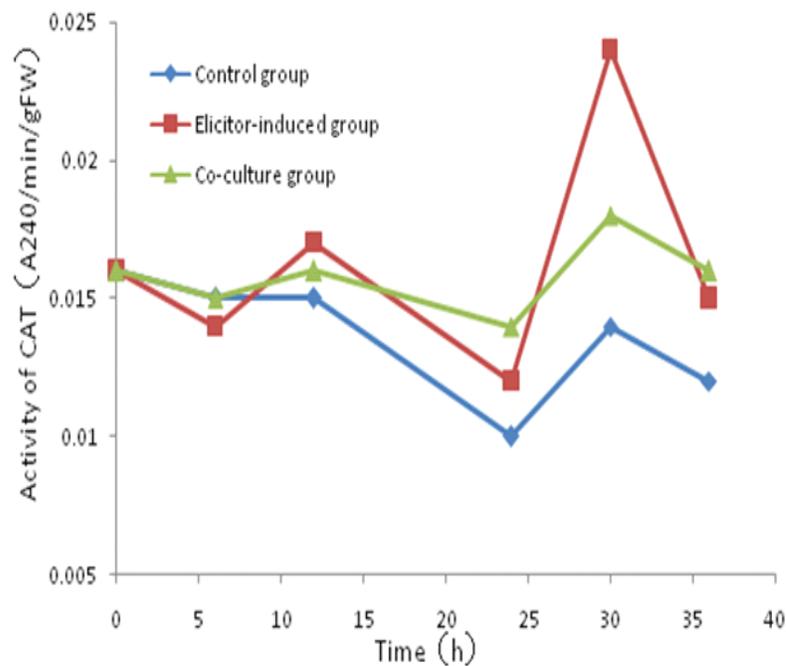


Figure 6. Effects of endophytic fungi and elicitors on the activity of CAT in *C. roseus* suspension cells.

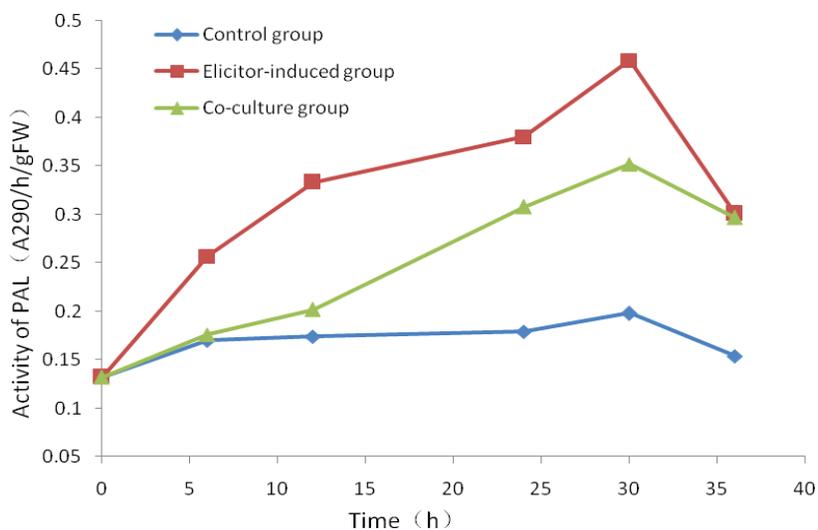


Figure 7. Effects of endophytic fungi and elicitors on the activity of PAL in *C. roseus* suspension cells.

concentrations early in the inoculation, then CAT eliminated the excess H_2O_2 and reduced its toxic effects to the cells. The CAT activity in the elicitor-induced group increased higher than that in the control group after 12 h, then declined at 24 h, and it increased again until it reached its peak of 0.024 A240/min/gFW at 30 h after 24 h.

Effects of endophytic fungi and elicitors on the PAL activity of *C. roseus* suspension cells

The activity of PAL increased continuously and reached the peak value of 0.459 A290/h/gFW and 0.352 A290/h/gFW at 30 h in the elicitor-induced and co-culture group, respectively (Figure 7), after which it decreased.

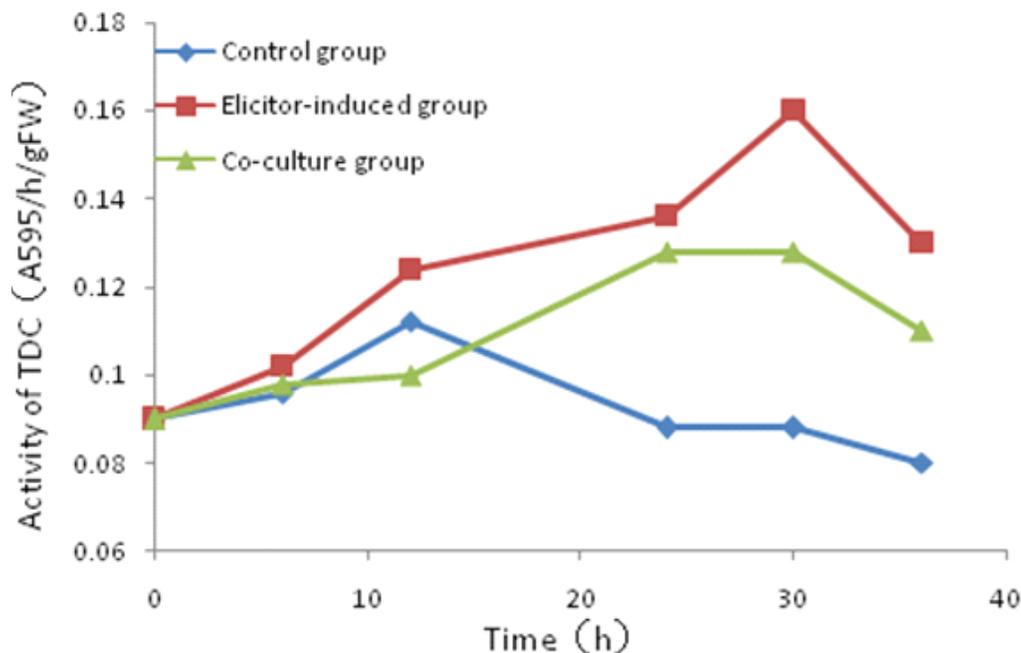


Figure 8. Effects of endophytic fungi and elicitors on the activity of TDC in *C. roseus* suspension cells.

The PAL activity rose significantly within a short time, early after inoculation in the elicitor-induced group, which may be due to the rapid response in *C. roseus* suspension cells toward elicitors. *C. roseus* suspension cells induced genes to express immediately. In the later stage of inoculation, the PAL activity decreased because the secondary metabolites involved in defense were synthesized, and other enzymes produced byproducts.

Effects of endophytic fungi and elicitors on the tryptophan activity of *C. roseus* suspension cells

As shown in Figure 8, endophytic fungi and their elicitors strongly stimulated the activity of the key enzyme of TDC in *C. roseus* suspension cells. The change in TDC activity in the elicitor-induced and co-culture groups is similar to that of PAL, its increased continuously and reached the peak value of 0.160 A595/h/gFW and 0.128 A595/h/gFW at 30 h in the elicitor-induced and co-culture group respectively. But the TDC activity in the control group increased slowly at the very start and decreased slowly after 12 h.

Effects of endophytic fungi and elicitors on the alkaloid synthesis of *C. roseus* suspension cells

As shown in Figure 9, the alkaloid content in the co-culture group began to increase after 30 h and in the elicitor-induced group after 24 h. Both got the highest

value of 693.76 and 770.36 $\mu\text{g/gFW}$ after 36 h, compared with the control group, these increased rates were 48 and 32%, respectively. After the treatment of suspended cells, the synthesis of alkaloids did not increase immediately compared with that in PAL and TDC. This was because PAL and TDC catalyzed alkaloid biosynthesis in the early and middle phases of the reactions. In comparison, the synthesis of alkaloids needed to follow a series of catalytic reactions, so that the accumulation of alkaloids lagged behind. Although PAL and TDC activity decreased in the later stage, they were still higher than that in the control group, with alkaloid accumulation increasing further.

DISCUSSION

The experiment trying to establish the co-culture of *C. roseus* suspension cell and endophytic fungi, and studied the relationship between them on the synthesis of alkaloid.

In the establishment of co-culture system, because the isolated filamentous fungi were not easy to count, in order to facilitate the experimental operation, endophytic fungi were inoculated by the number of rings that was used for vaccination. In the experiments, suspension cells are basically the same alkaloid production with the number of fungal inoculation loops or less the case. Therefore, in co-culture, all were inoculated with endophytic fungi of a ring, and the final co-culture system used MS medium, in the first 10d suspension cell cultures

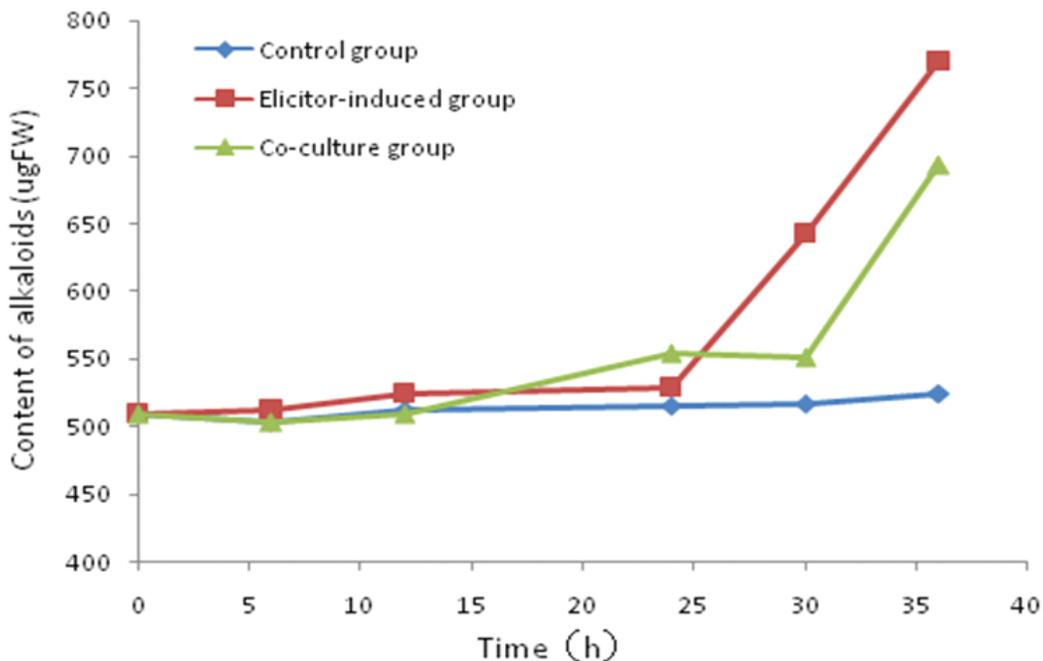


Figure 9. Effects of endophytic fungi and elicitors on the content of alkaloids in *C. roseus* suspension cells.

inoculated with endophytic fungi were cultivated for 36 h.

Effects of endophytic fungal on the synthesis of secondary metabolism led by the type of fungi species, the type of elicitors, their density and other factors

Different types of endophytic fungal has different type of induced information, the speed and the intensity (Jungui, 2000). The results had proved the point in the former experiment, where 11 endophytic fungal elicitors had different effects on the synthesis of *C. roseus* alkaloid, and different effects when different volumes of the same elicitors were added. Therefore, the endophytic fungal F9, in which fungi and suspension cells was cultured on the *C. roseus* alkaloids was promoted stronger than that of other fungi, and its elicitor treatment effect was better than that of the other endophytic fungi. The elicitor of F9 endophytic fungal treatment was better than F9 with the best effect when the concentration is 1 $\mu\text{g/ml}$.

The fungi elicitors induce metabolic changes, which not only start or enhance a particular secondary metabolic pathway but also actively synthesize a secondary metabolism of plant defense, such as phytoalexin, lignin and so on. These processes were mainly achieved with the regulation of enzymes (Yuan et al., 1991). In addition, the accumulation of plant secondary metabolites cannot be limited; too many secondary metabolites had toxic reactions toward the cells themselves. In the cells, the decrease in PAL activity was facilitated by the

appropriate channels closing or reducing the synthesis of key enzymes in order to limit the amount of secondary product accumulation. The enzyme of PAL is the first and key enzyme of the metabolic pathway of phenylpropanes, and an increase in its activity would result in the secondary metabolism product such as alkaloid increase.

Plant cells can strongly control the external pH in normal circumstances; endophytic fungi or elicitors can cause proton influx. We presumed that the change in pH was caused by endogenous fungi or their elicitors' unique induction mechanism on plant cells in the liquid medium. In plant secondary metabolism, the metabolic pathway of phenylpropanes played an important role. A change in the cell metabolism structure is caused by a change in the cell situation, which increases the flow of logistics to the metabolic synthesis of alkaloids. For the suspension culture of *C. roseus* cells, secondary metabolites such as alkaloid accounted for the major part of the secondary metabolic pool. In general, there is a positive correlation between the secondary metabolism of key enzymes in the branch pathway and the enzyme activity in primary and secondary metabolism, and between the specific metabolic pathway activation and the accumulation of products (Zhang et al., 2002). In the biosynthesis of the metabolic pathway of *C. roseus* alkaloid, strictosidine glucoside synthetase (SSS) and TDC were the two major key enzymes, but the regulation of TDC was stronger (Kutchan, 1993).

This was because increased PAL and TDC activity led to the increase in the alkaloid synthesis of *C. roseus* suspension cell culture. Further experiments, we plan to

inoculate both endogenous fungal elicitor and precursor substances to the combination of suspension cells were inoculated, and then detected alkaloids.

Conclusion

In this study, endophytic fungi and elicitors caused rapid response and action in cultured *C. roseus* suspension cells. The cell membrane became damaged, and the redox state changed at the cellular level. The specific signal transduction pathways were activated in the process of releasing and clearing free radicals. Some specific secondary metabolic pathways, such as phenylpropanes metabolic pathways, were opened or strengthened.

Additionally, enzyme expression in the path strengthened. After treatment with endophytic fungi and elicitor, PAL and TDC activity saw a notable increase in *C. roseus*. Meanwhile, the alkaloid content of the cells improved significantly.

ACKNOWLEDGMENTS

This work is financially supported by the International Cooperation Project on Traditional Chinese Medicines of Ministry of Science and Technology of China (No. 2007DFA40680), the Technological Item of Hunan Province, P.R.C (06SK3061) and the Youth Grant of Hunan Agriculture University (10QN20).

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