Effects of fungal extracts on vinblastine and vincristine production and their biosynthesis pathway genes in *Catharanthus roseus*

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**Abstract**

*Catharanthus roseus* is an important medicinal plant due to the production of valuable anticancer secondary metabolites including vinblastine and vincristine. Due to the importance of these alkaloids and their small amounts in *C. roseus*, one of the strategies to increase their production is using of elicitors. Fungal extracts recently showed that they could act as an elicitor and alter the expression levels of secondary metabolite biosynthesis genes. This study aimed to investigate the effects of fungal extracts on vinblastine and vincristine production in *C. roseus*. The cell suspension of *C. roseus* optimized through multiple hormonal treatments and concise analysis of the type of calluses. Fungal extracts of *Prformospora* and *Trichoderma* applied in multiple concentrations and in different times to the cell suspensions of *C. roseus*. The HPLC analysis results showed that the amount of vinblastine was increased; in contrast, it was found that vincristine amount decreased after treatments compared to the controls. In the Real-Time PCR assay, the results showed that the effect of these extracts on the expression levels of *DHT* and *D4H* genes was significant and their expression levels was increased in mRNA level. The present study revealed that the fungal extracts have the potential to increase the vinblastine and vincristine accumulation in *C. roseus* plant.

**1. Introduction**

*Catharanthus roseus* is one of the most important medicinal plants of the *Apocynaceae* family, which produces various secondary metabolites (Sain and Sharma 2013). This plant contains more than 130 types of alkaloids (Almagro et al. 2015) and has been used due to its various therapeutic and their expression levels was increased in recent years (Sato et al. 2001). The micropropagation of the *C. roseus* in vitro is very important and may be a suitable way for the production of its valuable alkaloids (Pietrosiuk et al. 2007). One of the most important methods for increasing the production of secondary metabolites is the use of elicitors (Esmaeilzade and Sharifi 2013). Elicitors are biological or chemical compounds that can cause an increase in biosynthesis and accumulation of secondary metabolites through the induction of defensive responses, physiological changes and accumulation of phytoalexins. Biological elicitors include polysaccharides, proteins, glycoproteins, and cell wall fragments of fungi, plants (cellulose and pectin), and microorganisms (chitin and glucan) (Shilpa et al. 2010). Biological elicitors may include a certain compound such as chitin and chitosan, or a combination of biological compounds such as homogenous fungi and yeast extracts (Esmaeilzade and Sharifi 2013). Biological elicitors have been efficiently used to increase the production of secondary metabolites in cell culture of many medicinal plants (Jonkova, 2007). The modification of cell culture medium using biological elicitors is one of the important strategies for inducing secondary metabolism and increasing the production of valuable secondary metabolites (Esmaeilzade and Sharifi 2013). Various investigation have shown the effect of biological elicitors in increasing of secondary metabolites (Gadzovska Simic et al. 2014;
The results of variance analysis of callusing and mean of callus weight

Table 3

| Type of variation | Degree of freedom | Mean of callus weight | Callusing |
|-------------------|-------------------|-----------------------|-----------|
| Treatment         | 8                 | 0.223**               | 1157.404**|
| Explant           | 1                 | 0.049**               | 3344.907**|
| Treatment × Explant | 8              | 0.010**               | 844.907** |
| Error             | 36                | 0.005                 | 347.222   |
| CV                |                   | 26.8                  | 24.6      |

*Significance in 5%, **Significance in 1% and ns None-significant

Table 1

Different hormonal combination used in callus induction experiment

| Medium name | Hormones |
|-------------|----------|
| MS1         | 1 mg/l 2,4-D + 0 mg/l KN |
| MS2         | 1 mg/l 2,4-D + 0.5 mg/l KN |
| MS3         | 1 mg/l 2,4-D + 1 mg/l KN |
| MS4         | 1/5 mg/l 2,4-D + 0 mg/l KN |
| MS5         | 1/5 mg/l 2,4-D + 0.5 mg/l KN |
| MS6         | 1/5 mg/l 2,4-D + 1 mg/l KN |
| MS7         | 2 mg/l 2,4-D + 0 mg/l KN |
| MS8         | 2 mg/l 2,4-D + 0.5 mg/l KN |
| MS9         | 2 mg/l 2,4-D + 1 mg/l KN |

Table 2

Sequence of primers used for real time PCR

| Gene | Sequence (5′-3′) | Amplicon size (%) | GC |
|------|------------------|-------------------|----|
| DAT  | GCTGATCCGTCGAGTTATCA 50 | 18S rRNA | 148 55 |
| DAT  | GCAACAAACCCCGACTTCTG 148 | 18S rRNA | 125 55 |
| D4H  | TTGCACCCGGACTTCTTG 118 | 18S rRNA | 115 55 |
| D4H  | TTGGGACAAGCAAGCACTCA 118 | 18S rRNA | 125 55 |
| D4H  | GCTGATCCGTCGAGTTATCA 50 | 18S rRNA | 148 55 |
| D4H  | GCTGATCCGTCGAGTTATCA 50 | 18S rRNA | 125 55 |
| D4H  | GCTGATCCGTCGAGTTATCA 50 | 18S rRNA | 115 55 |

2. Materials and methods

2.1. Preparation of plant materials

Seeds of *Catharanthus roseus* plant were purchased from Pakan-Bazar co, Isfahan, Iran. Seeds were sterilized using 70% ethanol for 30 seconds, 1% sodium hypochlorite for 20 minutes, and thereafter washed 3 times for 5 seconds with sterile distilled water. Sterilized seeds were cultured on hormone free MS medium and placed in a growth room at 25°C under dark conditions. After germination, the seeds were transferred to a growth room with 16 hour light/8-hour darkness.

2.2. Callus induction and Cell Suspension

In order to study the effects of hormone and explant on callus formation, a completely randomized, factorial experiment was conducted in triplicate. Root and shoot pieces (about 1 cm in length) were placed in the MS medium with hormonal treatment (Table 1) containing kin (0, 0.5 and 1 mg/l) and 2,4-D (1, 1.5 and 2 mg/l).

![Figure 1](Image)

Fig. 1. Callusing rate of different treatments in root and shoot explants
2.3. Preparation and application of fungal extract

The P. indica endophyte fungus was cultured in the Kafer solid medium (Käfer 1977) and stored for 10 days at 28°C. The fungus was transferred to Kafer liquid medium, and placed in shaker incubator (120 rpm at 28°C) for two weeks. T. tomentosum was first cultured in PDA medium and then in PD8 medium in similar conditions to P. indica. After sufficient growth of fungi, the fungus-rich biomass, which included mycelium and fungal spores, was filtered by Buchner funnel. After filtration, the fungal cells were dried for 24 hours at 65°C. Finally, the filtered extracts were used as an elicitor (Namdeo et al. 2002). For treatment, 250 ppm stokes were prepared from fungal extracts. The extract of the fungus prepared by P. indica 1%, T. tomentosum 1% and the interaction of both fungi 1% v/v. This extracts were applied to cell suspension in three repeats (Tashackori et al. 2016b). Sampling from cells was performed at 0, 24, 48, 72 hours after treatment (Hasanloo et al. 2015). For cell sampling, cell suspensions were filtered by Buchner funnel and treated for 24 hours at 50°C.

2.4. Extract preparation, chromatography and data analysis

Dried samples completely powdered and 100 mg of powder was solved in 1 ml of methanol in a 2 ml tube. The mixture was shaken for 10 minutes and then placed in an ultrasonic apparatus at 30°C for 1 hour. After ultra-sonication, centrifugation was performed at 14000 rpm for 15 minutes, the supernatant removed and poured into the new tubes (Pan et al. 2010). To improve the final extract, the centrifuge was repeated twice. The vinblastine (code V03000 Sigma Aldrich) and vincristine (code V7988 Sigma) standards were purchased. 0.5 mg of vincristine and 0.5mg of vinblastine were solved in 0.5 ml of HPLC methanol and 0.5 ml of acetonitrile respectively. In order to plot the standard curves, different concentrations of vincristine: 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8 and 10 mg/l (ppm) and vinblastine: 100, 150, 200, 300, 400 and 500 mg/l were prepared and 20 μl injected into HPLC machine. Optical absorptions were recorded at 210 nm and the standard curve plotted. Chromatography was carried out using an HPLC manufactured by KNauer Germany and a UV detector with a C-eighteen column (4.6 x 250 μm TSKgel-ODS-looV 5) manufactured by Japan’s TOSOH BIOSCIENCE. HPLC water and acetonitrile with a 50:50 ratio were used for the mobile phase. Flow rate was set to 1 ml per minute and light absorption measured at 210 nm. The amount of vinblastine and vincristine in different treatments calculated and compared (Saravanan and Karthi 2014). In this experiment, a factorial experiment used in a completely randomized design with three repeats. SPSS software was used to analyze the data. The Means were also compared by Duncan’s multiple range test at a probability level of 1%.

2.5. Expression Analysis

This experiment was also carried out in a completely randomized design. Total RNA was extracted with RNxPlus kit (Sinaclo co, Iran) and cDNA was synthesized with MMLV reverse transcriptase (Fermentase). Primers were designed for D4H and DAT genes (Table 2) and synthesized by Macrogene Company. Real time PCR reaction carried out with SIBRGREEN dye through 10 min at 95°C, 15 s at 95°C, 20s at 60°C and 20s in 72°C. The 18SrRNA gene was used as internal control and the results were calculated as relative changes compared to the control sample. SPSS software was used to analyze the data.

3. Results and discussion

3.1. Callus induction

After onemonth of induction, calluses were sampled and the traits (callusing and callus weight) were measured. The results of analysis of variance for callusing showed that there was a significant difference between hormonal treatments and explants in terms of callusing. It was also observed that the interaction of explants and hormonal treatments on callusing percentage at 5% probability level is significant. Analysis of variance of mean of callus weight showed that there was a significant difference between hormonal treatments and between explants in terms of mean of callus weight, but the effect of hormonal and explant treatment on mean of callus weight was not significant (Table 3). Comparison of mean of interactions between explants and hormonal treatments on callus formation also showed that the highest percentage of callus was related to root explants in MS7, MS8 and MS9 and stem

![Fig. 2. Callus weight mean in different hormone treatments](image)

![Fig. 3. Callus weight mean in shoot and root explants](image)

Table 4

| Variation        | Degree of freedom | Cell count | Settled cells volume |
|------------------|-------------------|------------|----------------------|
| Hormone          | 8                 | 510.10**   | 537.04**             |
| Explant          | 1                 | 1066.67**  | 1075.57**            |
| Explant×Treatment| 8                 | 223.00**   | 221.28**             |
| Error            | 36                | 2.83       | 4.2                  |
| CV               |                   | 23.6       | 26                   |

* Significance in 5%, ** Significance in 1% and ns None-significant
Fig. 4. Settled cell volume mean of hormone treatments in shoot and root explants.

Fig. 5. Cell count mean in ml ($\times 10^4$) of hormone treatment in shoot and root explants.

Fig. 6. Standard curves of A: Vincristine and B: Vinblastine.
Table 5
Variance analysis of vinblastine levels under the treatment of fungal extracts and time

| Variation          | Sum of square | Degree of freedom | Mean of square |
|--------------------|---------------|-------------------|----------------|
| Fungal extract     | 1.168         | 3                 | 0.389**        |
| Time               | 2.729         | 3                 | 0.910**        |
| Fungal extract × Time | 3.65         | 9                 | 0.406**        |
| Error              | 3.42          | 32                | 0.107          |
| Total              | 553.088       | 48                |                |

*Cv = 9.7
* Significance in 5%, ** Significance in 1% and ns None-significant

Table 6
Variance analysis of vincristine levels under the treatment of fungal extracts and time

| Variation          | Sum of square | Degree of freedom | Mean of square |
|--------------------|---------------|-------------------|----------------|
| Fungal extract     | 1.236         | 3                 | 0.997**        |
| Time               | 2.99          | 3                 | 0.412**        |
| Fungal extract × Time | 3.635       | 9                 | 0.404**        |
| Error              | 2.635         | 32                | 0.085          |
| Total              | 17.109        | 48                |                |

*Cv = 17.7
* Significance in 5%, ** Significance in 1% and ns None-significant

3.2. Cell Suspension

The results of analysis of variance of settled cells and cell count showed that the effect of hormonal treatments, explants and the interaction of hormonal and explants on the volume of cells and cell count was significant at 1% level (Table 4). Comparison of the mean of interactions between explants and hormonal treatments on the volume of settled cells showed that the highest mean of settled cell volume was related to root explants in MS9 medium. The smallest volume of settled cells was related to the stem explants in MS7 and MS6 (Fig. 4). Comparison of mean of interactions between explants and hormonal treatments on number of cells showed that the highest number of cells was related to root explants in MS9 and stem explants in MS4. The smallest number of cells belonged to the stem explants in MS6 and MS7 (Fig. 5).

3.3. Effect of fungal extract and time on vinblastine and vincristine production

According to the results of the cell growth curve based on SCV and cell count, it was found that the best time to apply treatment was related to 7 days after cellular uniformity. According to standard curves of vinblastine and vincristine (Fig. 6), results of analysis of variance of vinblastine in fungal extract treatment and time showed that the effect of fungal extracts and time and the interaction of extract and time on the production of vinblastine (Table 5) and vincristine (Table 6) was significant at 1%. The comparison of the mean of interactions of time and fungal extract also showed that the highest amount of vinblastine production was related to 48 hours after treatment for the fungal extracts of Priformospora and Trichoderma (Fig. 7). Comparison of the interactions of fungal extract and time treatments showed that the highest amount of vincristine production in 72 hours after treatment was related to Priformospora fungi extract. In contrast, the lowest amount of vincristine obtained in 48 hours after treatments was related to Priformospora and Trichoderma fungi extracts (Fig. 8). The vinblastine (Fig. 7B) and vincristine (Fig. 9A) standard curve revealed that their absorption peaks appear around 3:30 and 4 minute respectively. The comparison of the peaks of vinblastine and vincristine in the control samples (Fig. 10B) and samples treated by the fungal extracts (Fig. 10A) showed vinblastine was increased and vincristine was decreased in compared to the control sample.

3.4. Gene expression analysis

Analysis of variance of fungal extract and time on D4H and DAT genes expression level showed that the effect of extract, time and interaction of extract and time on expression of both genes was significant at
1% level (Table 7). Analysis of the interaction between time and extract showed that the highest expression of D4H (Fig. 11) and DAT (Fig. 12) genes was around 48 hours after treatment with *P. indica* and *T. tomentosum* fungi extracts. The expression began to increase after 24 hours and reached its maximum level in 48 hours. Finally after 72 hours it was decreased to the primary level. The study of the mix of fungal extracts (p + t) effect showed that there was no effect on the expression of these genes compared to the control.

Vinblastine and vincristine are two valuable drugs against cancers. Because of the plant origin of these drugs and small yield from plants, attempts to increase the production of these drugs in *C. roseus* have been continuously carried out for decades. The same compounds, like efficient anticancer drug Taxol is under extensive research and bioinformatics analysis to increase the yield in *Taxus baccata* plant (Jafari et al., 2016; Jafari and Dehghan 2018. Nowadays, tissue culture, Cell suspensions and hairy roots are the strategies for production of these valuable drugs without cutting down the whole of old plants that living multiple decades. It is shown that the use of elicitors can increase the production of some secondary metabolites in plants (Bahabadi et al.2011). In our study, the results of treatments of cell suspension of *C. roseus* by *Penicillium citrium* and *P. spimulorum* fungi extracts showed that the production rate of vinblastine increased and vincristine decreased. It is known that the effect of the elicitors on the increase or decrease of secondary metabolite production depends on several factors. One of these factors is the type of the elicitor. Different fungi extracts have a different effect on different plants and metabolites. For example, *Penicillium citrium* and *P. spimulorum* fungi could increase the production of Ajmalicine in the *C. roseus* cells and *Absidia cristata* extract increases serpentin production, while the *Mucor* extract has a poor effect (Tang et al. 2011). Another factor is the type of secondary metabolite. A specific type of fungal extract may have a different effect on the two types of secondary metabolites. For example, research on the effect of *Pythium aphanidermatum* fungi showed that *Pythium* extract increases the amounts of ajmalicine, but reduces the amounts of phenol and terpenoide and decreases the activity of the terpenoide pathway enzymes (Moreno et al. 1996). One of the other factors contributing to the effect of the stimulus on the secondary metabolite is the concentration of the elicitor (Khosroushahi et al. 2006). Different concentrations of elicitors have different effects on the production of secondary metabolites. For example, high concentrations of *Rhizopus stolonifera*...
(50 mg / l) fungi extract in cultured taxus cells have produce the highest cell death and the lowest levels of Taxol, while its low concentration (25 mg / l) produces the highest levels of Taxol and the lowest mortality rate of cells (Khosroushahi et al. 2006). The time period that the cells exposed to the elicitors also plays an important role in the production of secondary metabolites (Bahabadi et al. 2011). In flaxseed culturing, the presence of fungal extract at various times has a different effect on the lignan content (Bahabadi et al. 2011). The highest amount of podophyllotoxin and larysic resinol in flaxen cells treated with Fusarium graminearum, Rhizoctonia solani, Rhizopus stolonifera and T richodema viride elicitors was observed on the fifth day after treatment and with Sclerotinia sclerotiorum elicitor on the third day after treatment (Bahabadi et al. 2012). Increasing the amount of vinblastine in the present study may be due to the presence of unknown compounds such as indole acetic acid, flavonoids, and cell wall enzymes such as cellulase and xylanase, and compounds such as oligo saccharides, hormones, enzymes and peptides that can act as a stimulus factor (Kumar et al. 2016). In addition, the cause of increasing the amount of vinblastine by the fungal extract may be due to the activation of the defense paths of the plant by the fungal cell wall compounds. The activation of the plant's defense system results in the secretion of hydrolytic enzymes thought to have

![Fig. 10. Vinblastine and vincristine absorption in A: samples treated with fungal extract and B: in control plant.](image_url)

![Fig. 11. Interaction of Fungal extracts and time treatment to DAT gene expression.](image_url)

| Variation        | Degree of freedom | Mean of square D4H | Mean of square DAT |
|------------------|-------------------|--------------------|--------------------|
| Fungal Extract   | 3                 | 1.06**             | 1.14**             |
| Time             | 3                 | 2.7**              | 3.37**             |
| Fungal extract × | 9                 | 0.501**            | 0.57**             |
| Time             | 32                | 0.009              | 0.01               |
| Error            | 32                |                    |                    |
| Total            | 48                | CV = 13%           | CV = 11%           |

* Significance in 5%, ** Significance in 1% and ns None-significant.
an important role in the plant’s defense activity (Ahlawat et al. 2016). Another possibility of the mechanism of the effect of the fungal extract in increasing of secondary metabolite production is that the compounds of the fungus extract may have an effect on the expression of the biosynthetic pathway of vinblastine and the increased activity of the biosynthetic pathway enzymes has led to an increase in the production of vinblastine. Identification of active compounds of fungal extracts will help to understand the mechanism of interaction between plant cells and fungal elicitors and defense responses. In the biosynthesis pathway of vinblastine and vincristine, vincristine synthesis occurs with the demethylation and formylation of vinblastine (Ishikawa et al. 2009). Among these modifications, acid anhydride (HC2O) and formic Acid (HCOOH) also play a role in this reaction. The fungal extract may have an affect on demethylation and formylation of vinblastine and reduce the production of vincristine. The results of our experiments showed that the fungal extracts increased the expression of the DAT and D4H genes. Wang et al. studied the effect of ethephon on the production of derivatives of vindolin and vinblastine and on the expression of the D4H, DAT, T16H genes. They showed that treatment with 60 to 100-μm ethephon increased catarantin and Vinodolin and high concentrations of ethophon increased the expression of D4H and T16H genes by three-fold, and low concentrations increased the DAT gene expression by three times. However, at high concentrations of ethephon Vinblastine level increased and in low concentrations it was decreased (Wang et al. 2016). Pauw et al. found that stimulation with yeast extract in C. roseus increased the expression of ZCT (inhibitor) and ORCA (activator) transcription factors (Pauw et al. 2004). Also, the results of another experiment showed that the increased levels of expression of the CRWRRK2 transcription factor (which was expressed in response to methyl jasmonate) in the C. roseus hairy roots resulted in increased level of ORCA3 activator transcription factor and ZCT inhibitor transcription factor expression (Suttpanta 2012). Synchronous expression of activator and inhibitor factors may be necessary to switch on and off the expression of genes in response to stimuli. Regarding to these results, it is possible that the treatment with fungal extract induces simultaneously activator and inhibitor transcription factors and, in an unspecified way, initially activators increases expression of both genes in 24 hours, then inhibitors decreases the expression of them and biosynthesis pathway returns to the basal levels in 48 hours.

4. Conclusions

Fungal extracts have different compounds that they stimulate the production of secondary metabolites. The present study focused to investigate the production of C. roseus alkaloids and the expression of their biosynthetic pathway gene by using fungal extract. Considering the results, fungal extracts have increased the amount of vinblastine and the expression of pathway genes; we suggest investigating the effect of different concentrations of priformospora and Trichoderma extracts and the effect of other fungi extracts on the production of these alkaloids as well as the expression of other genes. In addition, given that the increase in vincristine was not accompanied by the simultaneous increase of vinblastine. The factors that affect methylation, demethylation and formylation of vinblastine and the effective enzymes in the conversion of Vienna-vinblastine to vincristine is need to further study.

Contributions:

R. A carried out the most experimental procedures, H. R provided the necessary documentation and knowledge, S. B provided additional knowledge about plant cell culture and J. A contributed in article writing formatting and documentation of the research.

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The authors declare that they have no conflict of interest.

Declarations of interest:

none
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