World J Microbiol Biotechnol
DOI 10.1007/s11274-013-1432-z

ORIGINAL PAPER

# Enhancement of vindoline and vinblastine production in suspension-cultured cells of *Catharanthus roseus* by artemisinic acid elicitation

**Jinwei Liu · Jianhua Zhu · Le Tang · Wei Wen · Shuangshuang Lv · Rongmin Yu**

Received: 6 October 2012 / Accepted: 10 July 2013


**Abstract** Elicitation is an important strategy to improve production of secondary metabolites in vitro. Artemisinic acid was studied as a novel elicitor to enhance the yield of terpenoid indole alkaloids in the present paper. Our results demonstrated that the concentrations of vindoline and vinblastine were increased by sixfold and twofold, respectively, compared to those of the control group after treatment with artemisinic acid. To elucidate the underlying mechanism, we investigated the gene expression of four enzymes involved in the biosynthetic pathway of vinblastine in the suspension-cultured cells of *Catharanthu sroseus*. RT-PCR experiment showed that artemisinic acid was able to up-regulate the transcriptions of tryptophan decarboxylase, geraniol 10-hydroxylase, tabersonine 16-hydroxylase and deacetoxyvindoline 4-hydroxylase.

**Keywords** *Catharanthus roseus* · Suspension-cultured cells · Vindoline · Vinblastine · Artemisinic acid · Elicitation

## Introduction

*Catharanthus roseus* is an important source of terpenoid-indole alkaloids (TIAs). Vinblastine and vincristine isolated from *C. roseus* exhibit significant cytotoxic activity and are used as antineoplastic agents in the clinical treatment (Keglevich et al. 2012). As global demand for these compounds increases, new methods to enhance their production are needed because plant extract naturally has very low yield but is currently the only commercial source (Van der Heijden et al. 2004). Plant cell cultures are considered to be an alternative for producing indole alkaloids. Suspension-cultured cells of *C. roseus* have been studied for many years as a potential way to produce therapeutically important indole alkaloids such as vinblastine, catharanthine and ajmalicine (Osman et al. 2007). However, few successful industrial applications can be cited due to many biological and technological limitations, the biggest of which is the low yield of those compounds in cell cultures (Zhao et al. 2005).

The use of elicitors is one of the most effective strategies employed to increase the production of important alkaloids in cells and organ cultures (Fabricant and Farnsworth 2001; Pandey et al. 2010). There are two types of elicitors: biogenic and abiogenic. Biogenic elicitors can be further divided into exogenous and endogenous elicitors. Abiogenic elicitors include heavy metal ions, inhibitors of some metabolic steps, some antibiotics and fungicides, and UV radiation. Alkaloids in cultures of *C. roseus* were enhanced by both biogenic and abiogenic elicitors (Zhao et al. 2005). Although elicitors such as methyl jasmonate and salicylic acid could accelerate the biosynthesis of TIAs in the cultures of *C. roseus*, the production of TIAs could not be increased significantly (Gautam et al. 2011). Therefore, searching for more efficient elicitor is urgently needed and actively being investigated.

Artemisinic acid, a cadinane-type sesquiterpene isolated from *A. annua*, was supposed to be an intermediate in biosynthesis pathway leading to artemisinin (Ferreira and Luthria 2010). During a series of investigation about the biosynthesis pathway of artemisinin, we unexpectedly

Jinwei Liu and Le Tang equally contributed to this work

J. Liu · L. Tang · W. Wen · S. Lv · R. Yu
Biotechnological Institute of Chinese Materia Medica, Jinan University, Guangzhou 510632, China

J. Zhu (✉) · R. Yu (✉)
Department of Natural Medicinal Chemistry, Jinan University, Guangzhou 510632, China
e-mail: tzhujh@jnu.edu.cn

R. Yu
e-mail: rongminyu99@hotmail.com

Published online: 18 July 2013

discovered that suspension-cultured cells of *C. roseus* could produce octadecanoic acid and its analogs upon addition of artemisinic acid. The biosynthetic pathway of octadecanoids has been reported to be involved in elicitor-induced alkaloid production (Peebles et al. 2009). Jasmonate and its octadecanoid precursors have been implicated as intermediate signaling molecules in elicitor-induced secondary metabolite accumulation (Gundlach et al. 1992; Mueller et al. 1993). The biosynthetic pathway of jasmonate is an integral part of the elicitor-triggered signal transduction pathway that resulted in the coordinate expression of the tryptophan decarboxylase (TDC) and strictosidine synthase (STR) genes (Menke et al. 1999). Thus, we were very interested to find out whether artemisinic acid could stimulate the biosynthesis of indole alkaloids in suspension cultured cells of *C. rosues*. In our previous study, we found that artemisinic acid could increase the yield of catharanthine (Wen et al. 2012). In order to have a comprehensive understanding of artemisinic acid's role on the production of TIAs in cultured-cells of *C. roseus*, especially for vindoline and vinblastine, the inducing effects of artemisinic acid as an elicitor were investigated in this paper.

The biosynthesis of TIAs is strictly regulated. Many enzymes involved have been purified and characterized. The first committed steps towards TIAs biosynthesis are the conversion of tryptophan to tryptamine by TDC and the conversion of geraniol to 10-hydroxygeraniol by geraniol 10-hydroxylase (G10H). The pathway leading to the formation of vindoline from tabersonine has been studied in *Catharanthus* (De Luca et al. 1988). The regulation of vindoline synthesis is achieved mainly through three enzymes: tabersonine 16-hydroxylase (T16H), deacetoxyvindoline 4-hydroxylase (D4H) and deacetylvindoline acetyl CoA acetyltransferase (DAT) (De Luca and Laflamme 2001). Expression of genes upstream of T16H and D4H is needed for vindoline production (He et al. 2011). The TIA pathways are not only regulated developmentally and tissue-specifically, but also affected by external biotic and abiotic factors (Geerings et al. 2000). Therefore, external regulatory strategies activating D4H and T16H transcription may contribute to the accumulation of vindoline. Vindoline is condensed with catharanthine to form vinblastine and vincristine. Many studies on plant regulation and metabolic engineering are based on gene expression profiling, which requires quantification of specific mRNA sequences. Currently, one of the most widely used methods for conducting these analyses is to perform reverse transcription PCR (RT-PCR). To elucidate the underlying mechanism of artemisinic acid as a novel elicitor, transcriptional levels of four key enzymes (TDC, G10H, T16H and D4H) involved in the biosynthetic pathway of vinblastine were measured by RT-PCR in this paper.

## Materials and methods

### Plant materials

*C. roseus* suspension-cultured cells were directly induced from leaves in our laboratory (Yan et al. 2008). Suspension-cultured cells of *C. roseus* were transplanted to 500 ml conical flask containing 200 ml of MS medium, supplemented with indole-3-acetic acid (IAA) 1 $mgL^{-1}$, 6-benzylaminopuriple (6-BA) 0.5 $mgL^{-1}$, sucrose 40 g $L^{-1}$. The suspension-cultured cells were incubated under light at 25 °C and shaken at 110 rpm.

### Treatment with artemisinic acid

The solutions of artemisinic acid were prepared in absolute ethanol. Different concentrations of artemisinic acid were added to 12-day-old suspension-cultured cells of *C. roseus*, and the final concentrations of artemisinic acid were 10, 30 and 50 mg $L^{-1}$, respectively. For the control treatment absolute ethanol alone was used. The suspension-cultured cells and culture media were collected at 24, 48 and 72 h after elicitation.

### Dry weight determination

After treatment at 24, 48 and 72 h with 10, 30 and 50 mg $L^{-1}$ of artemisinic acid, cells and media were separated by filtration with suction. The cells were dried at 55 °C. After total elimination of water was achieved, the dried cells were weighed. Each sample was done in triplicate. Absolute ethanol was used as the control group.

### Alkaloid extraction and determination

The extraction and determination of alkaloids were carried out as references (Zhao et al. 1999, 2000). Briefly, dry cell power was extracted sonically with methanol three times. After concentration of combined extract solutions under vacuum, the residue was dissolved in 2.5 % sulfuric acid (v/v) and washed twice with ethyl acetate. The pH value of the aqueous solution was adjusted to 10 and then the solutions were partitioned for three times. The ethyl acetate layer was concentrated under vacuum, and then the residue was re-suspended with 1 ml of methanol and stored at 4 °C for alkaloid analysis. Alkaloids in the medium were directly partitioned into ethyl acetate after the pH value was adjusted to 10. The extracts of ethyl acetate were combined and concentrated into dryness. The residue was dissolved in methanol and then analyzed by HPLC.

Separation of TIAs was performed using a reversed phase $C_{18}$ column (Hydro-RP, 250 × 4.6 mm, particle size of 4 μm, Phenomenex). The temperature was maintained at 35 °C, and UV detection at 220 nm. Alkaloids were eluted with a mobile phase of methanol/acetonitrile/0.025 mol $L^{-1}$ ammonium acetate/triethylamine (15:40:45:0.1) at a flow rate of 1 mL $min^{-1}$. The sample injection volume was 10 μL. Alkaloids were identified and quantified by comparison with their commercial standard.

### RNA extraction and RT-PCR

The total RNA was extracted from different cell samples using Trizol Reagent. Semi-quantitative RT-PCR was used to measure the transcript levels of TDC, G10H, T16H and D4H. β-actin was used to calibrate the cDNA samples and as an internal reference. The forward and reverse primers for TDC, G10H (Wei 2010), T16H, D4H and β-actin (He et al. 2011) were listed in Table 1. The PCR reaction cycle numbers were optimized for each gene, so that the accumulation of products was in the exponential range. The cycles of PCR reaction varied as following: 30 cycles for β-actin, 35 cycles for TDC and T16H, and 40 cycles for G10H and D4H. The conditions for PCR reaction were as follows: denaturing at 94 °C for 5 min, followed by various cycles with different genes of denaturing at 94 °C for 30S, annealing at 60 °C for 30S, extension at 72 °C for 1 min, and final extension for 5 min at 72 °C. The products of PCR reaction were loaded onto 1.5 % agarose gel. RT-PCR images were obtained and band intensities were quantified by Image J software.

**Table 1** Sequence for forward and reverse primers of the TIA biosynthetic pathway genes monitored (TDC, G10H, T16H and D4H)

| Gene | Primer sequence (5′ → 3′) |
|---|---|
| G10H-Forward primer | TGAATGCTTGGGCAATTGGA |
| G10H-Reverse primer | GCAAATTCTTCGGCCAGCAC |
| TDC-Forward primer | TCCGAAAACAAGCCCATCGT |
| TDC-Reverse primer | AAGGAGCGGTTTCGGGGATA |
| T16H-Forward primer | ATGACTTTGAAGCCCCACTG |
| T16H-Reverse primer | CCACCAAATGGTAGATACTCG |
| D4H-Forward primer | TAGGATCCGAGCAAGAAAGAAAAATG |
| D4H-Reverse primer | ATCTGCAGGACGCTTATTTAGTCCAAC |
| β-actin-Forward primer | GGCGGATGCTGAGGATATTC |
| β-actin-Reverse primer | TCCAGAGTCCAGAACAATACCA |

## Results

### Effect of artemisinic acid on the growth of suspension-cultured cells

The average dry weight of the cells was decreased after they were treated with artemisinic acid at concentrations of 30 and 50 mg $L^{-1}$. This information indicated that artemisinic acid at high concentrations might have a negative effect on cell growth. However, there were no significant differences between the artemisinic acid-treated groups and the control group for each time point (Table 2).

### Effect of artemisinic acid on the production of vindoline

Significant differences in the concentrations of vindoline between the artemisinic acid-treated groups and the control group were observed at 24, 48 and 72 h after elicitation. The highest concentration of vindoline was found at 24 h after elicitation. As shown in Fig. 1, the concentration of vindoline in the artemisinic acid-treated groups was significantly higher than that of control group. The maximal concentration of vindoline obtained in the artemisinic acid-treated groups was 142 ± 9.8 μg $L^{-1}$, six-fold increase to that of control group. More importantly, most (75–95 %) vindoline was excreted into medium, which would greatly facilitate the recovery of this compound.

### Effect of artemisinic acid on the production of vinblastine

In elicited experimental groups, artemisinic acid was added on the induction day (day 12) at 10, 30 and 50 mg $L^{-1}$. As the results showed in Fig. 2, rapid increase of vinblastine occurred after 24 h, which continued up to 72 h at 10 mg $L^{-1}$ for artemisinic acid group. The production of vinblastine in the artemisinic acid-treated groups maximized at 160 ± 23.4 ng $L^{-1}$, which was 2.23-fold higher than that of the control group.

**Table 2** Dry weights of suspension-cultured cells of *C. roseus* treated with artemisinic acid in different concentrations (10, 30 and 50 mg $L^{-1}$)

| Time (h) | Dry cells weight (g $L^{-1}$) | | | |
|---|---|---|---|---|
| | 0 | 10 | 30 | 50 |
| 24 | 23.51 ± 0.22 | 22.48 ± 1.15 | 20.66 ± 0.21 | 18.41 ± 0.90 |
| 48 | 19.65 ± 2.34 | 19.40 ± 1.22 | 18.55 ± 1.21 | 15.75 ± 0.32 |
| 72 | 18.81 ± 2.32 | 19.65 ± 3.80 | 19.01 ± 0.66 | 15.05 ± 0.32 |

Values represent mean ± SD from triplicate sample

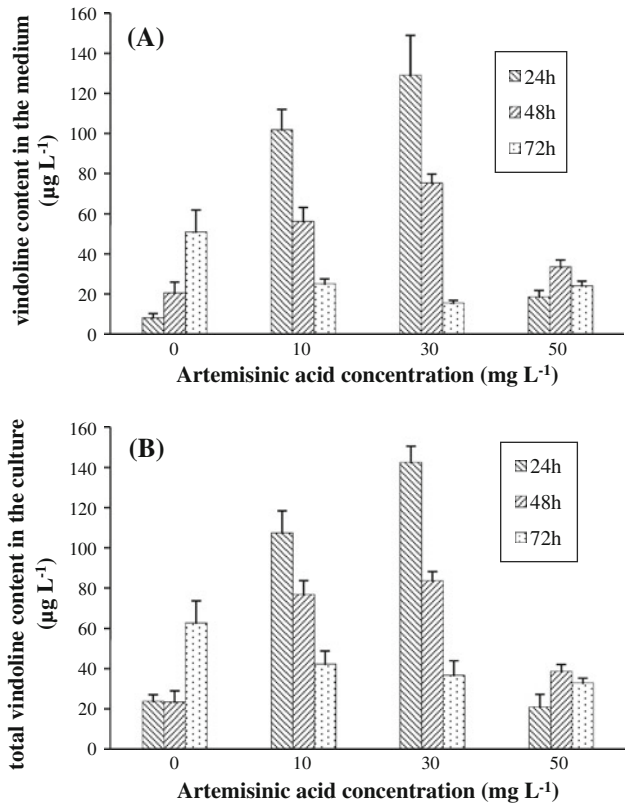


**Fig. 1** Effect of Artemisinic acid in different concentrations (10, 30 and 50 mg $L^{-1}$) on extracellular vindoline production (**A**) and total vindoline production (**B**); values represent mean ± SD from triplicate sample

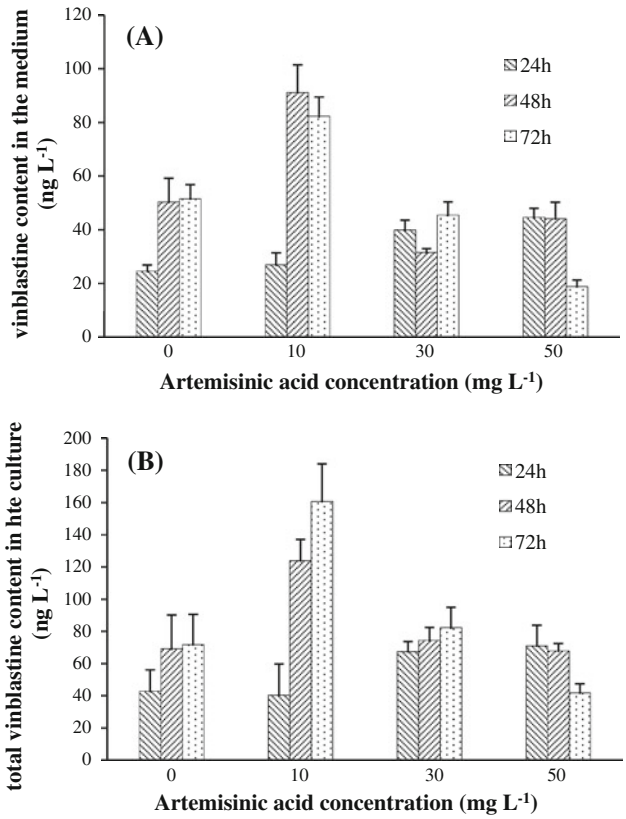


**Fig. 2** Effect of artemisinic acid in different concentrations (10, 30 and 50 mg $L^{-1}$) on extracellular vinblastine production (**A**) and total vinbalstine production (**B**); values represent mean ± SD from triplicate sample

### Effect of artemisinic acid on TDC, G10H, T16H and D4H transcript abundance in suspension-cultured cells of *C. roseus*

Transcript levels of β-actin (internal reference), TDC, G10H, T16H and D4H were determined at 48 h after treatment with artemisinic acid, and the results were shown in Fig. 3. The results demonstrated that the transcript level of TDC increased obviously after elicitation. In the group treated with artemisinic acid at concentration of 30 mg $L^{-1}$, the highest transcript level of TDC was eightfold higher than that of the control group. The transcript level of G10H was also increased in response to the treatment with artemisinic acid. The highest transcript level of G10H was approximately fivefold higher than that of the control group after elicitation. The above information indicated that artemisinic acid could induce the transcription of TDC and G10H in suspension-cultured cells of *C. roseus*. Similarly, the artemisinic acid-treated groups showed a notable increase in the transcript level of T16H. In the group treated with artemisinic acid at concentration of 30 mg $L^{-1}$, the highest transcript level of T16H was approximately tenfold higher than that of the control group. The transcript level of D4H was transiently responsive and reached its peak when the concentration of artemisinic acid was 10 mg $L^{-1}$, followed by a return to the level of control group. The highest transcript level of D4H was only approximately twofold higher than that of the control group.

## Discussion

Regulation of total indole alkaloids can be controlled either by developmental or exogenous signals. Light is thought to have an effect on enzyme induction and activation. Zhao et al. (2001) found that light significantly influenced the biosynthesis of vindoline. Light also plays a critical role for vindoline biosynthesis and these effects are exerted through the last two enzymes in the process, D4H and DAT, which require the involvement of phytochrome (Aerts and De Luca 1992; Vazquez-Flota and De Luca 1998). Therefore, the cells of *C. roseus* were cultured under light in this study.

After the treatment of artemisinic acid, the yield of vindoline increased significantly (23.8 ± 2.2 → 142 ± 9.8 μg $L^{-1}$).

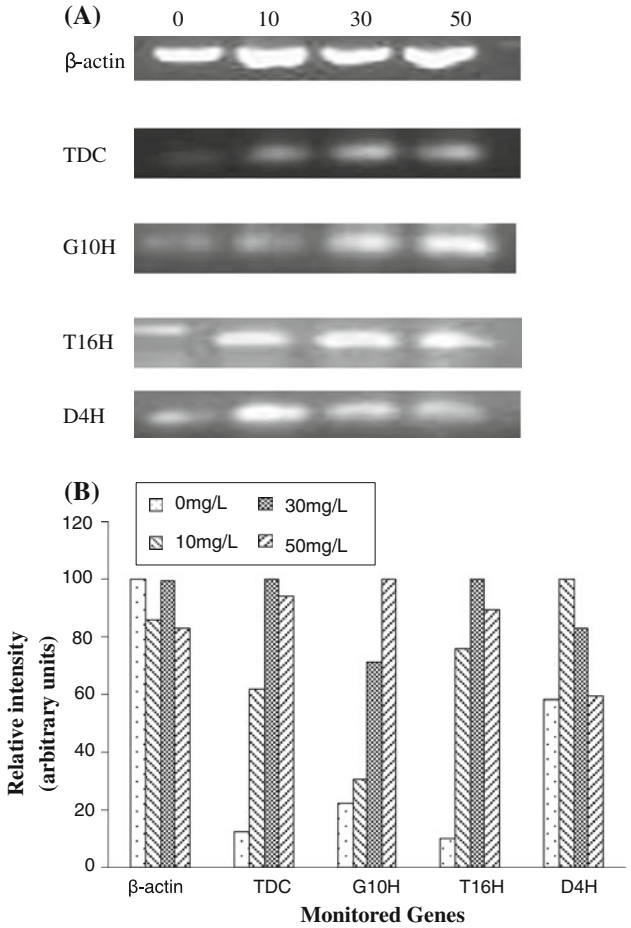


**Fig. 3** Effect of artemisinic acid elicitation at various concentrations (10, 30 and 50 mg $L^{-1}$) at 48 h on the gene expression of TDC, G10H, T16H and D4H in suspension cultures of *C. roseus*, **A** Representative gel images; **B** Relative intensities of the transcripts of TDC, G10H, T16H and D4H. β-actin was used as internal standard

The optimal condition for vindoline production was as follows: cells of *C. roseus* were pre-cultured for 12 days under light and then treated with artemisinic acid (10 mg $L^{-1}$) for 24 h. It was reported that the treatment of jasmonate generated 75.3 ng $g^{-1}$ vindoline of dry weight (He et al. 2011). Our investigation showed that under the optimal condition, the production of vindoline was significantly higher than what had been previously reported (He et al. 2011).

Although substantial progress has been made in the metabolic engineering of TIAs in *C. roseus*, the bisindole alkaloids vinblastine and vincristine still could not be successfully produced on an industrial scale, owing to various biological and technological limitations. The bisindole alkaloids occurred in extremely low quantities in plant due to their highly cytotoxic nature, which posed the problem of cellular containment (De Luca and Cutler 1987). The TIA's biosynthetic pathway of *C. roseus* has attracted a lot of attention not only because they could produce diverse alkaloid compounds of significant pharmaceutical importance, but also because there is a complex secondary metabolic pathway in the plants. Vindoline and catharanthine are the precursors of bisindole alkaloids in *C. roseus*. As shown in Fig. 1, the yield of vindoline reached its maximal level at 24 h after elicitation. The yield of vinblastine in the culture increased from 24 h after elicitation and maximized at 72 h, which may have occurred because vindoline was the precursor of vinblastine (Fig. 2).

It had been assumed that the transcriptional block of genes leading to vindoline biosynthesis resulted in a lack of vindoline in cultured cells of *C.roseus* (St-Pierre and De Luca 1995; Vazquez-Flota et al. 2002). Therefore, variations at gene transcription levels should reflect early regulations in a biosynthetic pathway. TDC catalyses the formation of tryptamine from tryptophan, which is the first step in the synthesis of the indole moiety of the *Catharanthus* alkaloids. TDC activity could be increased after treatment with methyl jasmonate and ethylene in suspension cultures (Vazquez-Flota et al. 2009). In the present study, we report that TDC activity could be transiently enhanced after elicitation with artemisinic acid. The expression level of the terpenoid pathway gene for the elicited condition was shown in Fig. 3. These results suggested that artemisinic acid could stimulate the expression of G10H on monoterpenoid indole alkaloid biosynthesis in the TIA biosynthetic pathway. Overexpression of G10H in transgenic hairy-root cell lines significantly enhanced the accumulation of ajmalicine, vincristine and vinblastine (Montiel et al. 2007). In this study, overexpression of G10H in *C. roseus* suspension-cultured cells increased the accumulation of vindoline and vinblastine. Hernandez-Dominguez et al. (2004) found that maximal vindoline accumulation in *C. roseus* in vitro shoot cultures coincided with maximum activities of DAT, D4H and TDC. In our study, although vindoline could be hardly detected in unelicited in suspension culture cells, it was found vindoline increased after the elicitation procedure treated with artemisinic acid. This was corroborated by the observed increase in T16H and D4H transcript levels upon elicitation.

In summary, we conclude that the addition of artemisinic acid could enhance the accumulation of alkaloids as a result of increasing the activities of TDC, G10H, T16H and D4H in the TIA biosynthetic pathway. The result of this experiment indicated that artemisinic acid could be used as a promising elicitor, especially for the production of TIAs.

**Acknowledgments** This research work was financially supported by National Natural Science Foundation of China (No. 81073004 and No. 81102771) and Guangdong Natural Science Foundation (No. S2011040003028).

## References

Aerts RJ, De Luca V (1992) Phytochrome is involved in the light-regulation of vindoline biosynthesis in *Catharanthus*. Plant Physiol 100:1029–1032

De Luca V, Cutler AJ (1987) Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthusroseus*. Plant Physiol 85:1099–1102

De Luca V, Laflamme P (2001) The expanding universe of alkaloid biosynthesis. Curr Opin Plant Biol 4:225–233

De Luca V, Fernandez JA, Campbell D, Kurz W (1988) Developmental regulation of enzymes of indole alkaloid biosynthesis of *Catharanthusroseus*. Plant Physiol 86:447–450

Fabricant DS, Farnsworth NR (2001) The value of plants used in traditional medicine for drug discovery. Environ Health Persp Suppl 109:69–75

Ferreira JFS, Luthria DL (2010) Drying affects artemisinin, dihydroartemisinic acid, artemisinic acid, and the antioxidant capacity of *Artemisia annua* L. Leaves J Agr Food Chem 58:1691–1698

Gautam S, Mishra A, Tiwari A (2011) *Catharanthus* alkaloids and their enhanced production using elicitors: a review. Int J Pharm Technol 3:713–724

Geerings A, Ibanez MML, Memelink J, van der Heijden R, Verpoorte R (2000) Molecular cloning and analysis of strictosidine β-D-glucosidase, an enzyme in terpenoidindole alkaloid biosynthesis in *Ctharanthusroseus*. J Biol Chem 275:3051–3056

Gundlach H, Muller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA 89:2389–2393

He LH, Yang L, Tan RH, Zhao SJ, Hu ZB (2011) Enhancement of vindoline production in suspension culture of the *Catharanthusroseus* cell line C20hi by light and methyl jasmonate elicitation. Anal Sci 27:1243–1248

Hernandez-Dominguez E, Campos-Tamayo F, Vazquez-Flota F (2004) Vindoline synthesis in vitro shoot cultures of *Catharanthusroseus*. Biotechnol Lett 26:671–674

Keglevich P, Hazai L, Kalaus G, Szántany C (2012) Modifications on the basic skeletons of vinblastine and vincristine. Molecules 17:5893–5914

Menke FLH, Parchmann S, Mueller MJ, Kijne JW, Memelink J (1999) Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoidindole alkaloid biosynthetic genes in *Catharanthusroseus*. Plant Physiol 119:1289–1296

Montiel G, Breton C, Thiersault M, Burlat V, Jay-Allemand C, Gantet P (2007) Transcription factor Agamous-like 12 from Arabidopsis promotes tissue-like organization and alkaloid biosynthesis in *Catharanthusroseus* suspension cells. Metab Eng 9:125–132

Mueller MJ, Brodschelm W, Spannagl E, Zenk MH (1993) Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc Natl Acad Sci USA 90:7490–7494

Osman MEH, Elfeky SS, Abo El-Soud K, Hasan AM (2007) Response of *Catharanthusroseus* shoots to salinity and drought in relation to vincristine alkaloid content. Asian J Plant Sci 6:1223–1228

Pandey VP, Cherian E, Patani G (2010) Effect of growth regulators and culture conditions on direct root induction of *Rauwolfiaserpentina* L. (Apocynaceae) Benth by leaf explants. Trop J Pharm Res 9:27–34

Peebles CA, Shanks JV, San KY (2009) The role of the octadecanoid pathway in the production of terpenoidindole alkaloids in *Catharanthusroseus* hairy roots under normal and UV-B stress conditions. Biotechnol Bioeng 103:1248–1254

St-Pierre B, De Luca V (1995) A cytochrome P-450 monooxygenase catalyzes the first step in the conversion of tabersonine to vindoline in *Catharanthusroseus*. Plant Physiol 109:131–139

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11:607–628

Vazquez-Flota FA, De Luca V (1998) Developmental and light regulation of desacetoxyvindoline 4-hydroxylase in *Catharanthusroseus* (L.) G. Don. Evidence of a multilevel regulatory mechanism. Plant Physiol 117:1351–1361

Vazquez-Flota F, De Luca V, Carrillo-Pech M, Canto-Flick A, De L, Miranda-Ham M (2002) Vindoline biosynthesis is transcriptionally blocked in *Catharanthusroseus* cell suspension cultures. Mol Biotechnol 22:1–8

Vazquez-Flota F, Hernandez-Dominguez E, Lourdes MM, Monforte-Gonzalez M (2009) A differential response to chemical elicitors in *Catharanthusroseus* in vitro cultures. Biotechnol Lett 31:591–595

Wei S (2010) Methyl jasmonic acid induced expression pattern of terpenoidindole alkaloid pathway genes in *Catharanthusroseus* seedlings. Plant Growth Regul 61:243–251

Wen W, Zhu JH, Liu JW, Yu RM (2012) Impact of artemisinic acid on the growth and catharanthine production in *Catharanthusroseus* cultured cells. J Med Plants Res 6:2019–2028

Yan CY, Ma WL, Yu RM (2008) Induction of *Catharanthusroseus* callus and optimization on its culture conditions. J Chin Med Master 44:350–356

Zhao J, Zhu WH, Wu YQ, Hu Q (1999) Determination of indole alkaloids in *Catharanthusroseus* tissue and cell cultures by RP-HPLC. Acta Pharm Sinica 34:539–542

Zhao J, Zhu WH, Hu Q (2000) Enhanced ajmalicine production in *Catharanthusroseus* cell cultures by combined elicitor treatment: from shake-flask to 20-l airlift bioreactor. Biotechnol Lett 22:509–514

Zhao J, Zhu WH, Hu Q (2001) Selection of fungal elicitors to increase indole alkaloid accumulation in *Catharanthusroseus* suspension cell culture. Enzyme Microb Tech 28:666–672

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333