Appl Biochem Biotechnol (2014) 173:663–672
DOI 10.1007/s12010-014-0883-5

Regulation of vincamine biosynthesis and associated growth promoting effects through abiotic elicitation, cyclooxygenase inhibition, and precursor feeding of bioreactor grown *Vinca minor* hairy roots

Priyanka Verma · Shamshad Ahmad Khan · Ajay Kumar Mathur · Karuna Shanker · Raj Kishori Lal

Received: 18 December 2013 / Accepted: 24 March 2014 /
Published online: 11 April 2014

Abstract Hydroxylase/acetyltransferase elicitors and cyclooxygenase inhibitor along with various precursors from primary shikimate and secoiridoid pools have been fortified to vincamine less hairy root clone of *Vinca minor* to determine the regulatory factors associated with vincamine biosynthesis. Growth kinetic studies revealed that acetyltransferase elicitor acetic anhydride and terpenoid precursor loganin significantly reduce the growth either supplemented alone or in combination (GI=140.6±18.5 to 246.7±24.3), while shikimate and tryptophan trigger biomass accumulation (GI=440.2±31.5 to 540.5±40.3). Loganin also downregulates total alkaloid biosynthesis. Maximum flux towards vincamine production (0.017±0.001 % dry wt.) was obtained when 20-day-old hairy roots were fortified with secologanin (10 mg/l) along with tryptophan (100 mg/l), naproxen (8.4 mg/l), hydrogen peroxide (20 μg/l), and acetic anhydride (32.4 mg/l). This was supported by RT PCR (qPCR) analysis where 2- and 3-fold increase in tryptophan decarboxylase (TDC; RQ=2.0± 0.09) and strictosidine synthase (STR; RQ=3.3±0.36) activity, respectively, was recorded. The analysis of variance (ANOVA) for growth kinetics, total alkaloid content, and gene expression studies favored highly significant data ($P<0.05$–0.01). Above treated hairy roots were also up-scaled in a 5-l stirred-tank bioreactor where a 40-day cycle yielded 8-fold increase in fresh root mass.

P. Verma (✉) · S. A. Khan · A. K. Mathur
Department of Plant Biotechnology, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Council of Scientific and Industrial Research, PO CIMAP, Kukrail Picnic Spot Road, Lucknow 226015, India
e-mail: priyankaurbest@gmail.com

K. Shanker
Department of Analytical Chemistry, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Council of Scientific and Industrial Research, PO CIMAP, Lucknow 226015, India

R. K. Lal
Department of Plant Breeding and Genetics, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Council of Scientific and Industrial Research, PO CIMAP, Lucknow 226015, India

Keywords *Vinca minor* · Vincamine · Hairy roots · Bioreactor up-scaling · Indole alkaloid regulation

Introduction

Vinca minor is commonly known as myrtle or lesser periwinkle. It is a member of family Apocyanaceae and considered as evergreen perennial herbaceous plant. More than 50 indole alkaloids have been isolated from this plant. *V. minor* contains monomeric eburnamine-type indole alkaloids including vincamine which has modulatory effects on brain circulation and neuronal homeostasis as well as antihypoxic and neuroprotective potencies [1, 2]. Vincamine is used for the prevention and treatment of cerebrovascular insufficiencies and disorders. It increases cerebral blood flow, oxygen consumption, and glucose utilization [3, 4]. It is therefore included in numerous medical preparations and serves as a basis for several semi-synthetic medicines with an enhanced or modified physiological effect [5]. The limited yield of bioactive molecules from plants, however, presents a significant challenge for their large-scale industrial usage in drug development programs. Recent efforts made in the area of metabolic engineering of some medicinal crops have led to the development of plant cell and tissue systems as viable alternative production platforms for some of the important phytoceuticals that can be scaled up in a controlled environment [6–11]. The great pharmacological values, low *in planta* occurrence, unavailability of synthetic substitutes and exorbitant market cost of these alkaloids have prompted to understand the basic architecture and regulation of biosynthesis of indole alkaloids in *V. minor* plant and its cultured tissues. As *V. minor* is a poorly investigated plant, very less information regarding details of pathway have been elucidated. It starts with the strictosidine—which is a condensation product of an indole ring donor tryptamine and a terpenoid moiety donor secologanin (Fig. 1). Strictosidine that heralds the first indication of a switchover of carbon flux from primary to secondary metabolism reacted upon by the enzyme strictosidine β-glucosidase (SGD) to yield a highly reactive ring-opened unstable aglycon to yield various types of alkaloids [12]. Aglycon leads to another branch point, i.e., tabersonine. From tabersonine, in the root tissue serpentine/reserpine/minovincine/minovincinine/minovine, etc., types of alkaloids are formed through the action of cyclooxygenases [13]. Under these conditions, there is only a limited flux of intermediary metabolites flowing to the vincamine pathway. If that can be blocked through the incorporation of cyclooxygenase inhibitors, there are chances to increase flux towards vincamine biosynthesis. Another approach is to elicit the secondary metabolite pathway through the introduction of hydroxylase elicitor along with precursor feeding that can also regulate the factors associated with alkaloid synthesis. In the present study, the regulatory effects of different elicitors and various flux inhibitors have been investigated to draw maximum flux towards vincamine biosynthesis. The most effective factors have been screened, and the best treated roots were up-scaled in a 5-l stirred-tank bioreactor.

Material and Methods

Hairy Root Induction and Establishment

Leaf explants from multiple shoot cultures of *V. minor* were used for hairy root induction. The mother stock shoot cultures were maintained on a MS [14] medium supplemented with 1.0 mg/l 6-benzyladenine (BA), 0.1 mg/l α-naphthaleneacetic acid (NAA), 0.4 mg/l thiamine hydrochloride, and 4.0 g/l Phytagel. For co-cultivation, the bacterial suspension of

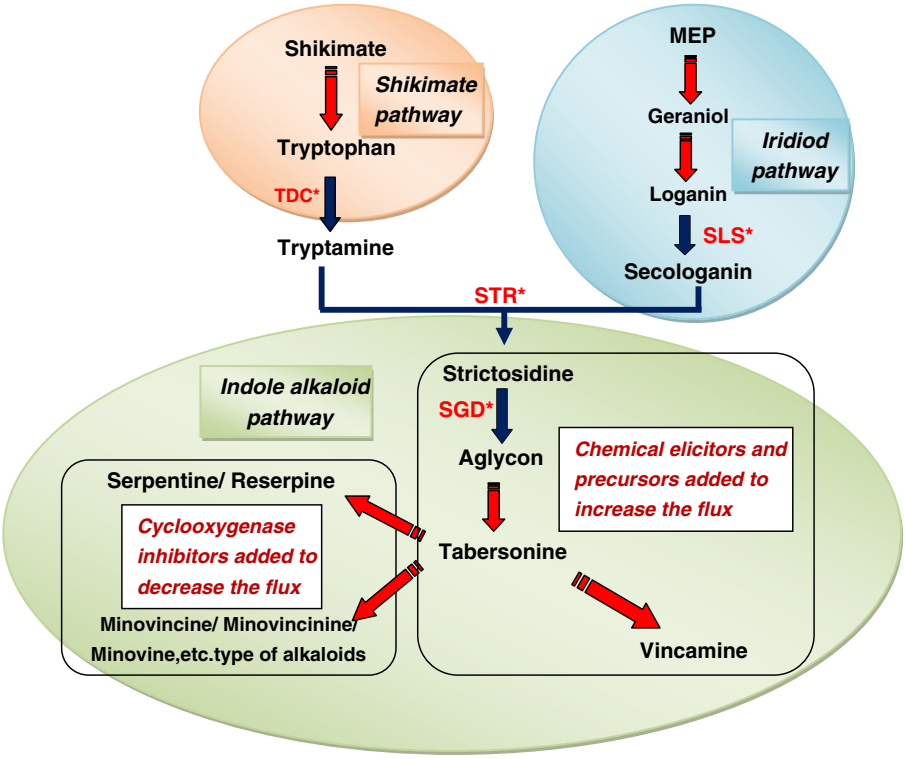

Fig. 1 Terpenoid indole alkaloid pathway operating in *V. minor* and the regulation strategy applied to enhance flux towards vincamine biosynthesis in the present study. *Red broken arrows* represent multi-step or uncharacterized reactions. **TDC* tryptophan decarboxylase, *SLS* secologanin synthase, *STR* strictosidine synthase, *SGD* strictosidine β-glucosidase

Agrobacterium rhizogenes strain A4 was raised in liquid YMB medium [15] supplemented with 50 mg/l kanamycin. Twenty-four-hour-old bacterial culture (OD 0.5 at 600 nm) was used for infecting the explants by wounding them with sterile needles dipped in the bacterial suspension. Untreated explants were plated to serve as controls. The treated explants were placed on a hormone-free MS medium for 5 days. After co-cultivation, the explants were shifted to a MS basal medium supplemented with cephalexin and ampicillin (500 mg/l each) for 15–20 days for bacterial elimination and root induction. The resultant roots from independent transformation events were carefully excised and transferred to one-fourth strength of Gamborg [16] B5 liquid medium for growth and multiplication via regular sub-culturing through a 5-week culture cycle. Growth index (GI) of hairy roots was measured as percent increment over the initial inoculum weight [GI= {(final wt.–initial wt.)/initial wt.}×100]. All growth assays were made with three replicates per treatment, and experiment was repeated three times. The root cultures were incubated on a rotary shaker (80–100 rpm) under a 16-h light and 8-h dark photoperiod.

Elicitor Treatments, Inhibitor, and Precursor Feeding

Based on the previous studies conducted by Giddings et al. [17] and Guo et al. [18] on a very closely related plant *Catharanthus roseus*, following experimental strategy was designed to

enhance the flux towards vincamine synthesis in *V. minor*. Cyclooxygenase inhibitors, hydroxylase, and peroxidase elicitors were added to the 20-day-old hairy roots growing in liquid medium. Naproxen (cyclooxygenase inhibitor) was added to inhibit serpentine/type of alkaloid biosynthesis at the concentration of 8.4 mg/l. To enhance the activities of hydroxylase and peroxidase, hydrogen peroxide (20 μg/l) was added, while for increased acetyltransferases activity, acetic anhydride (32.4 mg/l) was used. Another cyclooxygenase inhibitor benzotriazole was also tested at the concentration of 0.5 μM/l. Five precursor molecules of terpenoid indole alkaloid pathway, i.e., shikimate (100 mg/l), tryptophan (100 mg/l), tryptamine (100 mg/l), loganin (10 mg/l), and secologanin (10 mg/l) have been tested in various combinations with cyclooxygenase inhibitors, hydroxylase, and peroxidase elicitors to screen out the best treatment favoring the vincamine biosynthesis (Table 1). The concentrations of all the cyclooxygenase inhibitor, hydroxylase/peroxidase elicitor, and precursor were taken according to the previous studies conducted on *C. roseus* [17, 18]. Before addition, all the elicitors and inhibitors were filter-sterilized with membrane filter (0.22 μ). Roots were harvested 7 days after addition, and growth index was calculated. All experiments were carried out in triplicate, and experiments were repeated thrice.

HPLC Analysis

One gram oven-dried tissue was ground to fine powder and extracted with methanol (3×30 ml). Pooled methanolic extract was filtered, concentrated in vacuum at 40 °C to 10 ml, diluted with distilled water (10 ml), acidified with 3 % HCl (10 ml), and extracted with hexane (3×30 ml). The aqueous extract was cooled (10 °C), basified with NH_4OH (pH 8.5), and extracted with chloroform (3×30 ml). Combined chloroform extract was dried over Na_2SO_4 and air-dried to a constant weight. Vincamine estimation was performed by using high-performance liquid chromatography–photodiode array detector (HPLC–PDA-MS) system—Shimadzu Kyoto, Japan. Ten microliters of the extract was loaded onto a RP-C18 (4.6×100mm) HPLC column. The mobile phase used consisted of A (45 % acetonitrile) and B (55 % sodium phosphate buffer pH-6). A constant flow rate of 1.5 ml/min was used for all the analysis. Run time for each cycle was 14 min. Detection was done at 254 nm. The data represents the mean value of three independent experiments each performed in triplicate.

Real-Time PCR (qPCR)

Real-time PCR was performed on the StepOne Real-Time PCR System (Applied Biosystems; Foster City, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems). Total RNA was isolated from best treated hairy roots following LiCl precipitation method. Random primers were used to generate complementary deoxyribonucleic acid (cDNA) from the RNA from each tissue, using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA of untreated roots serves as controls. All real-time PCR quantification was performed with a non-template control and the endogenous control Actin. The gene expression levels were interpolated from standard curves for relative expression and normalized to Actin in the same tissue. Real-time transcript analysis was performed with two candidate genes tryptophan decarboxylase (TDC) and strictosidine synthase (STR). Respective primers used for the amplification were F-5′-TGTTGCAATGGCGAAGATGT-3′, R-5′CACGTCTGAACCTGGTAAAGGTT-3′ (TDC); F-5′-AGGTGGACAGCATGGGAA AG -3′, R-5′-TTCTTGGATTTGTTCGAAGTGTTC-3′ (STR), and F-5′ACGTGGATATCA GGAAGGATCTG-3′, R-5′-TTCTATCTGCAATACCTGGGAACA-3′ (Actin). The cycling program consisted of an initial hold at 50 °C for 2 min and 10 min incubation at 95 °C

Table 1 Effect of different chemical elicitor, inhibitor, and precursor feeding on growth and alkaloid production of *Vinca minor* hairy roots

S. no.	Treatment	Growth index*	Total alkaloid (% dry wt.)	Vincamine (% dry wt.)
1	Control	315.0±14.3	2.6±0.2	0.0
2	Naproxen[a]	320.6±32.7	2.5±0.1	0.0
3	Benzotriazole[b]	285.0±17.0	2.3±0.1	0.0
4	Acetic anhydride[c]	240.5±26.1	2.9±0.3	0.0
5	Hydrogen peroxide[d]	322.4±22.1	2.9±0.2	0.0
6	Shikimate[e]	540.5±40.3	2.7±0.1	0.0
7	Tryptophan[f]	440.2±31.5	2.5±0.1	0.0
8	Tryptamine[g]	345.6±20.3	2.5±0.1	0.0
9	Loganin[h]	205.0±15.6	2.1±0.1	0.0
10	Secologanin[i]	300.0±12.6	2.6±0.3	0.0
11	Naproxen+hydrogen peroxide	330.4±38.5	3.1±0.2	0.0
12	Naproxen+hydrogen peroxide+benzotriazole	346.5±35.0	2.5±0.2	0.0
13	Naproxen+hydrogen peroxide+acetic anhydride	246.7±24.3	3.0±0.1	0.0
14	Naproxen+hydrogen peroxide+acetic anhydride+shikimate	343.4±10.5	3.2±0.4	0.0
15	Naproxen+hydrogen peroxide+acetic anhydride+tryptophan	350.5±21.4	3.1±0.2	0.0
16	Naproxen+hydrogen peroxide+acetic anhydride+tryptamine	285.5±22.8	3.0±0.2	0.0
17	Naproxen+hydrogen peroxide+acetic anhydride+loganin	160.4±30.3	2.3±0.1	0.0
18	Naproxen+hydrogen peroxide+acetic anhydride+secologanin	290.8±29.4	3.5±0.3	0.015±0.001
19	Naproxen+hydrogen peroxide+acetic anhydride+shikimate+loganin	175.8±27.8	2.2±0.2	0.0
20	Naproxen+hydrogen peroxide+acetic anhydride+shikimate+secolaganin	280.9±30.6	3.2±0.3	0.011±0.001
21	Naproxen+hydrogen peroxide+acetic anhydride+tryptophan+loganin	200.0±19.4	2.1±0.1	0.0
22	Naproxen+hydrogen peroxide+acetic anhydride+tryptophan+secologanin	340.5±20.4	3.7±0.3	0.017±0.001
23	Naproxen+hydrogen peroxide+acetic anhydride+tryptamine+loganin	140.6±18.5	2.0±0.1	0.0
24	Naproxen+hydrogen peroxide+acetic anhydride+tryptamine+secologanin	300.8±34.3	3.2±0.2	0.011±0.001

*Calculated on the 27th day

[a] 8.4 mg/l

[b] 0.5 μM/l

[c] 32.4 mg/l

[d] 20μg/l

[e–g] 100mg/l

[h–i] 10mg/l

followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fold increase/RQ was calculated using formula $RQ=2^{-\Delta\Delta Ct}$, where ΔCt=average Ct (gene of interest)—Average Ct (Actin) and ΔΔCt=ΔCt—Δ Ct (control of the experiment; cDNA of untreated hairy roots).

Bioreactor Up-Scaling

The hairy root clone PV8 with optimized elicitation and precursor treatments was up-scaled in a 5-l stirred-tank bioreactor (Model ADI-1010; Applikon Biotechnology, Holland). The cultures were aerated through a sintered steel sparger at an air flow rate of 2 l per min equivalent to 20 % dissolved oxygen (DO) level. Three-liter medium with 30 g of fresh hairy roots as inoculum was used. Marine type impeller has been used with a rotation speed of 70–100 rpm. A nylon mesh support was tagged to the central shaft of the glass culture vessel of the reactor in such a way that the roots remained in constant contact with the medium without any damage caused by intermingling with the sparger rod. The incubation was carried out at 25± 3 °C under high illumination (3,000 lx) provided by a specially fabricated LCD light source kept around the glass vessel. Biomass accumulation was monitored by fresh and dry weight determinations. All process parameters during bioreactor cultivation were monitored and recorded using BioExpert Ferm software.

Statistical Analysis

All the values expressed are mean±standard deviation of mean (SD). The data was statistically analyzed for significance by analysis of variance (ANOVA). The differences were considered significant at $P<0.05$.

Results and Discussion

Hairy roots were induced from leaf explants after 3 weeks of co-cultivation in the presence of the antibiotics. No hairy root formation was observed in control explants. Transformation frequency of A4 strain was 40 % for leaf explants (Fig. 2 a). A total of 35 independent clones were observed, out of that four were finally established in shake flask culture in one-fourth Gamborg (B5) liquid medium. On HPLC analysis, one of the clones PV8 was found to be vincamine less and also fast proliferating and was used as starting material for studying the regulatory factors (Fig. 2b, c). This clone showed total alkaloid production of 2.6±0.2 % dry wt. and maximum biomass production on the 20th day (GI=320.0±24.4). The other clones were PVG, PV3, and PV11. PVG was found to be vincamine positive while PV3 and PV11 were slow growing. Differences in the growth capacity that were observed among the transformed roots might be due to variations in the expression of *rol* genes, which could alter the ratio of endogenous growth regulators [19]. Hairy roots are considered to be a better candidate for in vitro production because of their higher level of cellular differentiation and improved genetic or biochemical stability in culture. Fast growth, hormone autotrophy, and efficient metabolite production kinetics in shake flask as well as bioreactors were some of the other attributes of transformed roots that attracted scientific attention towards them as improved alternate production platform for plant secondary metabolites [20]. Clone PV8 has complete machinery to biosynthesize alkaloids (total alkaloid), but no vincamine was detected due to flux diversions in sub-pathways operating in indole alkaloid pathway, making it ideal material for studying regulatory factors. As vincamine pathway is poorly investigated, and very less steps are known till date (Fig. 1), it is difficult to design a strategy for enhanced vincamine production and regulation. Various combinations of treatments are shown in Table 1. Growth kinetic studies revealed that acetyltransferase elicitor acetic anhydride and terpenoid precursor loganin significantly reduce the growth index either alone or in combination (Table 1—S. no. 4, 9, 13, 17, 19, 21, and 23; GI=140.6±18.5 to 246.7±24.3). Roots

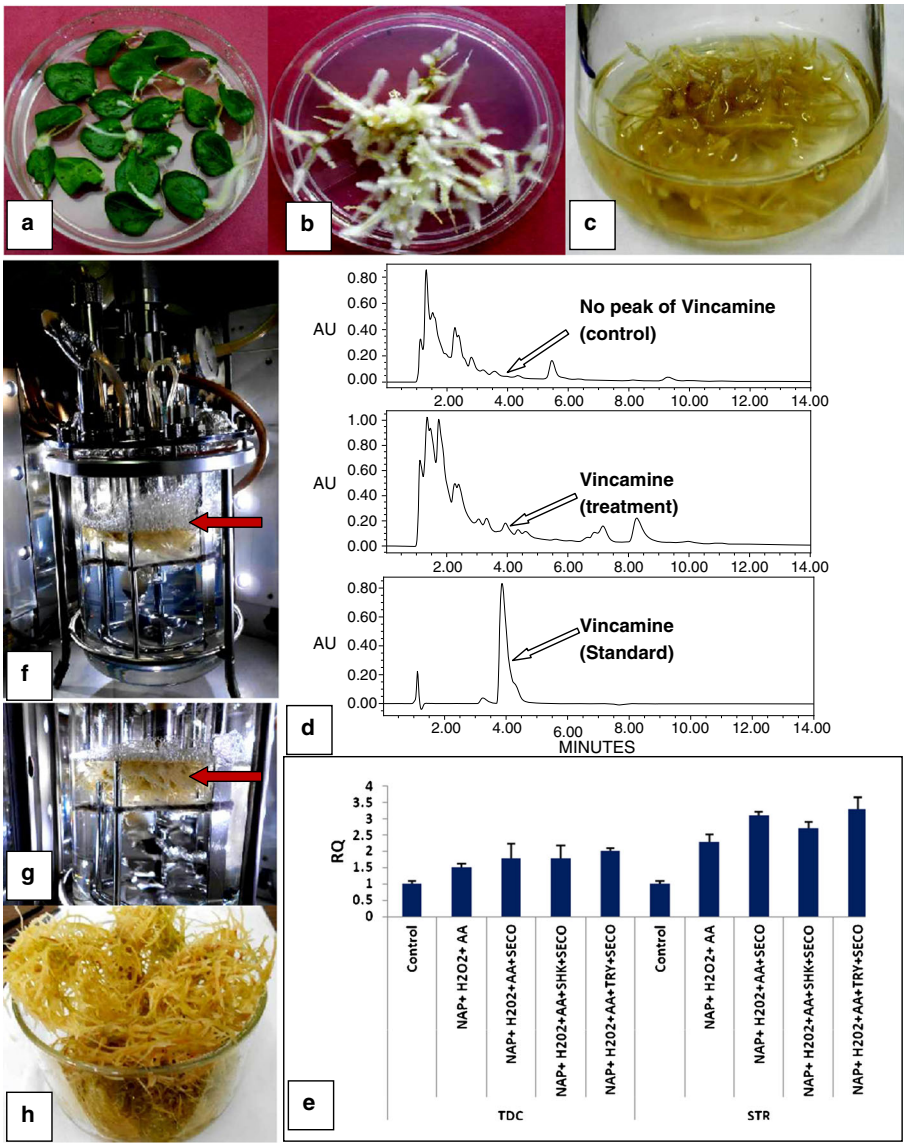

Fig. 2 Hairy root induction, establishment, bioreactor up-scaling, HPLC, and RT (qPCR) analysis in *V. minor*. **a** Hairy root induction from leaf explants after co-cultivation with *A. rhizogenes* strain A4. **b**–**c** Clone PV8 on semisolid MS medium and in one-fourth B5 liquid medium. **d** Chromatogram showing detection of vincamine (in comparison to standard and control) in hairy roots treated with naproxen, tryptophan, secologanin, hydrogen peroxide, and acetic anhydride. **e** RT (qPCR) analysis of TDC and STR genes in the treatments showed the presence of vincamine [data are means±SD (n=3 biological replicates), and *Y*-axis represents relative quantity (RQ)]. **f**–**g** PV8 roots growing in bioreactor on a nylon mesh support (*arrow*). **h** Harvested root biomass of PV8 after 40 days of bioreactor cultivation run. *NAP* naproxen, H_2O_2 hydrogen peroxide, *AA* acetic anhydride, *SECO* secologanin, *SHK* shikimate, *TRY* tryptophan

fortified with the indole donor shikimate and tryptophan showed enhanced biomass production (Table 1—S. no. 6, 7; GI=540.5±40.3 and 440.2±31.5, respectively) when applied alone, but

Table 2 ANOVA for total alkaloid production and growth index shown in Table 1

Source of variability	D.F.	Mean of sum of squares	
		Total alkaloid	Growth index
Treatments	23	0.634*	22,977.44*
Error	48	0.044	681.771
Total	71		

D.F. degree of freedom
*$P<0.01$

in combination, no significant increase in biomass was observed (Table 1—S. no. 14, 15, 20, 22; GI=280.9±30.6 to 350.5±21.4). Supplementation of another terpenoid moiety secologanin did not register any significant effect on growth index (Table 1—S. no 10, 18, 20, 22; GI=280.9±30.6 to 340.5±20.4). Tryptamine supplementation also did not effected the biomass accumulation much (Table 1—S. no.8, 16, 24; GI=285.5±22.8 to 345.6±20.3) except in the presence of loganin where significant reduction in growth index was observed (Table 1—S. no. 23; GI=140.6±18.5). Benzotriazole is a P-450 monooxygenase inhibitor. In *C. roseus* cell culture, the addition of benzotriazole influences strictosidine synthesis and blocks the lochnericine biosynthetic pathway [18]. As a result, more precursors enter the tabersonine metabolic pathways. Here, the addition of benzotriazole alone or in combination with elicitors resulted neither into increased total alkaloid content nor to vincamine biosynthesis; therefore, it was omitted from the further combinations. Cyclooxygenase is a key enzyme in serpentine biosynthesis, which competitively inhibits other monomeric alkaloid biosynthesis in the *C. roseus*. The use of cyclooxygenase inhibitor naproxen specifically blocks the serpentine flux [13]. In the present study, naproxen introduction along with hydroxylase and acetyltransferase elicitor in the presence of various terpenoid indole alkaloid precursors is an effective step towards total alkaloid and vincamine synthesis (Table 1). Hydrogen peroxide is a strong oxidizing agent, which can increase NADPH oxidase activity, cell oxidase, and hydroxylase activity. Hydrogen peroxide can act as a signal molecule to stimulate the defense mechanisms of plant cells by enhancing gene expression of plant secondary metabolites [18, 21]. In *C. roseus*, its addition (20 μg/l) resulted into maximum tabersonine production. Although final steps involved in vincamine biosynthesis are still unknown, acetylating elicitor is introduced just to check whether any acetylation step is being involved or not. Supplementation of both hydrogen peroxide and acetic anhydride in combination with naproxen was able to induce 15.38–42.30 % increase in total alkaloids in the presence of various precursors. The limited availability of precursor molecules from the primary shikimate and secoiridoid pools has often been documented as the most serious problem associated with low terpenoid indole alkaloids productivity [2, 22–29]. Therefore, various precursor molecules viz shikimate, tryptophan, tryptamine, loganin, and secologanin were supplemented along with naproxen, hydrogen peroxide, and acetic anhydride to draw maximum flux towards vincamine synthesis. It was observed that loganin presence alone or in

Table 3 ANOVA for TDC and STR expression through real-time PCR shown in Fig. 2e

Source of variability	D.F.	Mean of sum of squares	
		TDC expression	STR expression
Treatments	4	0.482*	2.500**
Error	10	0.075	0.048
Total	14		

D.F. degree of freedom
*$P<0.05$; **$P<0.01$

combination significantly downregulate the total alkaloid biosynthesis (2.0±0.1–2.3±0.1 % dry wt. in comparison to control 2.6±0.2 % dry wt.), while the best results were obtained when secologanin was introduced along with tryptophan, naproxen, hydrogen peroxide, and acetic anhydride. This treatment successfully drawn flux towards vincamine biosynthesis (Table 1). Maximum vincamine (0.017±0.001 % dry wt.) was registered in this treatment which was followed by treatment having only secologanin (without tryptophan supplementation) (0.015± 0.001 % dry wt.) and lastly when tryptophan was replaced by shikimate (0.011±0.001 % dry wt.). The ANOVA showed high level of significance ($P<0.01$) in both the cases of growth index and total alkaloid production (Table 2). HPLC showed vincamine peak at retention time of 4.0 min (Fig. 2d). In the above vincamine producing elicited hairy root cultures, gene expressions of the two genes, tryptophan decarboxylase (TDC) and strictosidine synthase (STR) related to indole alkaloid biosynthesis, were analyzed by conducting real-time PCR (qPCR). TDC activity was enhanced only to 1.5–2 fold, while the maximum of 3-fold (RQ= 3.3±0.36) increase in the STR activity was registered in cultures fortified with secologanin, tryptophan, naproxen, hydrogen peroxide, and acetic anhydride (Fig. 2e). The ANOVA revealed significant ($P<0.05$) and highly significant ($P<0.01$) data of TDC and STR activity, respectively (Table 3). Hairy roots treated with above optimized elicited conditions were also up-scaled in a 5-l stirred-tank bioreactor. Three individual batches of up-scaling were run in a 5-l stirred tank bioreactor fitted with a steel sparger (Fig. 2f–g). All runs were initiated using 30 g of roots from 20-day-old shake flask cultures. A 40-day-long bioreactor batch run of PV8 line yielded 8-fold increase in fresh root mass (Fig. 2h). The alkaloids analysis data of the harvested roots was comparable to that of respective shake flask cultures except a slight increase in total alkaloid production (4.1±0.2 % dry wt.) in comparison to shake flask cultures (3.7±0.3 dry wt.). Vincamine production in bioreactor grown hairy roots was found to be similar to hairy roots grown in shake flask cultures. The strategy of regulating the vincamine biosynthesis in *V. minor* hairy root cultures succeeded in delivering the desired metabolic product. This was achieved by blocking the serpentine/type of alkaloids (through the addition of naproxen), promoting the transformation of tabersonine towards vincamine through various elicitor and precursor feeding treatments. Present investigation is the first report of vincamine regulation in hairy root cultures of *V. minor*. In spite of the fact that very less information regarding vincamine biosynthesis has been known till date, the present study highlights the role of hydroxylase and acetyltransferase elicitors and precursor availability along with the cyclooxygenase inhibitors in the vincamine biosynthesis.

Acknowledgments The work presented here has been supported by DST-FAST TRACK SERC/LS-261/2012. The help rendered by Jyotsana Priya during HPLC analysis has been highly acknowledged.

References

1. Bruneton, J. (1995). *Pharmacognosy, Phytochemistry, Medicinal Plants* (p. 915). Paris: Lavoisier Publ.
2. Verma, P., Mathur, A. K., & Shankar, K. (2012). *Plant Cell, Tissue and Organ Culture, 111*, 239–245.
3. Belal, T. S., Barary, M. H., Ibrahim, M. E. A. L., & Sabry, S. M. (2009). *Journal of Food and Drug Analysis, 17*, 415–423.
4. Molchan, O., Romashko, S., & Yurin, V. (2012). *Plant Cell, Tissue and Organ Culture, 108*, 535–539.
5. Sabry, S. M., Belal, T. S., Barary, M. H., & Ibrahim, M. E. A. L. (2010). *Journal of Food and Drug Analysis, 18*, 447–457.
6. Kutchan, T. M. (1995). *Plant Cell, 7*, 1059–1070.

7. Morgan, J. A., & Shanks, J. V. (2000). *Journal of Biotechnology, 79*, 137–145.
8. Verpoorte, R., & Alfermann, A. W. (2000). *Metabolic Engineering of Plant Secondary Metabolism*. Dordrecht, The Netherlands: Kluwer Academic Publishers.
9. Sato, F., Hashimoto, T., Hachiya, A., Tamura, K. I., Choi, K. B., & Murashige, T. (2001). *Proceedings of the National Academy of Sciences of the United States of America, 98*, 367–372.
10. Facchini, P. J., & De Luca, V. (2008). *Plant Journal, 54*, 763–84.
11. Ziegler, J., & Facchini, P. J. (2008). *Annual Review of Plant Biology, 59*, 735–769.
12. Geerlings, A., Martinez-Lozano Ibanez, M., Memelink, J., van der Heijden, R., & Verpoorte, R. (1999). *Journal of Biological Chemistry, 275*, 3051–3056.
13. Guo, Z.-G., Liu, Y., & Xing, X.-H. (2011). *Process Biochemistry, 46*, 783–787.
14. Murashige, T., & Skoog, F. (1962). *Physiologia Plantarum, 15*, 473–497.
15. Hooykaas, P. J. J., Klapwijk, P. M., Nuti, M. P., Schilperoort, R. A., & Rorsch, A. (1977). *Journal of General Microbiology, 98*, 477–482.
16. Gamborg, O. L., Miller, R. A., & Ojima, K. (1968). *Experimental Cell Research, 50*, 151–158.
17. Giddings, L.-A., Liscombe, D. K., Hamilton, J. P., Childs, K. L., Della Penna, D., Buell, C. R., & O'Conner, S. E. (2011). *Journal of Biological Chemistry, 286*, 16751–16757.
18. Guo, Z.-G., Liu, Y., Gong, M.-Z., Chen, W., & Li, W.-Y. (2013). *Plant Cell, Tissue and Organ Culture, 112*, 43–54.
19. Bulgakov, V. P. (2008). *Biotechnology Advances, 26*, 318–324.
20. Christey, M. C. (2001). *In Vitro Cellular & Developmental Biology. Plant, 37*, 687–700.
21. Wang, Y., Dai, C. C., & Zhao, Y. W. (2011). *Process Biochemistry, 46*, 730–735.
22. Goddijn, O. J. M., Pennings, E. J. M., Van der Helm, P., Schilperoort, R. A., et al. (1995). *Transgenic Research, 4*, 315–323.
23. Canel, C., Lopes-Cardoso, M. I., Whitmer, S., Van der Fits, L., et al. (1998). *Planta, 205*, 414–419.
24. Whitmer, S., Canel, C., Hallard, D., Goncalves, C., & Verpoorte, R. (1998). *Plant Physiology, 116*, 853–857.
25. Whitmer, S., Van der Heijden, R., & Verpoorte, R. (2002). *Plant Cell, Tissue and Organ Culture, 69*, 85–93.
26. Whitmer, S., Van der Heijden, R., & Verpoorte, R. (2002). *Journal of Biotechnology, 96*, 193–203.
27. Seth, R., & Mathur, A. K. (2005). *Current Science, 89*, 554–547.
28. Verma, P., Mathur, A. K., Massod, N., Luqman, s., & Shankar, K. (2012). *Protoplasma, 250*, 371–380.
29. Verma, P., Mathur, A. K., & Shankar, K. (2012). *Applied Biochemistry and Biotechnology, 168*, 568–579.