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RESEARCH ARTICLE

In vitro growth and content of vincristine and vinblastine of *Catharanthus roseus* L. hairy roots in response to precursors and elicitors

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Abstract

Catharanthus roseus L. is a medicinal plant that produces numerous indole terpenoid alkaloids, including vincristine and vinblastine, which are used for cancer treatment. The effect of specified precursors (L-phenylalanine, L-tyrosine) and elicitors (chitosan, methyl jasmonate) on *C. roseus* hairy roots (CHR) growth has been examined in order to increase the content of vincristine and vinblastine. Our results showed that CHR generated by an *Agrobacterium rhizogenes* strain isolated in Vietnam was capable of producing both vincristine and vinblastine when subjected to precursors, but only vinblastine when exposed to elicitors. However, both precursors and elicitors were evaluated to have an effect on increasing the accumulation of TIAs in CHR. In particular, the use of elicitors required more time to find the appropriate induction conditions, while the use of precursors gave outstanding efficiency in the treatment with 1 µM phenylalanine. The greatest yields of vincristine (51.99 µg g^{-1} DW) and vinblastine (699.92 µg g^{-1} DW) were obtained in the 7th week (with 0.306 g DW biomass). This result is the first time we might boost the levels of vincristine and vinblastine in our CHR clone generated by the Vietnam strain of *A. rhizogenes*.

Keywords

Catharanthus roseus, elicitors, hairy root, precursors, vinblastine, vincristine.

Introduction

Catharanthus roseus L. is a tropical perennial medicinal plant in the family Apocynaceae. *C. roseus,* native and also endemic to Madagascar, is a herb, 30–100 cm tall. It grows wild in the tropical and subtropical areas of the world and is widely cultivated (1). There are two common cultivars of *C. roseus* named based on their flower color that is the pink-flowered 'Rosea' and the white-flowered 'Alba' (1, 2). *C. roseus* is propagated by seeds or cuttings *(3)*. The flowers bloom year-round and are white to dark pink with a dark red center, with a base tube about 2.5-3 cm and a corolla about 2-5 cm diameter with five-lobed petals (4). The flowers of the wild plant are naturally pale pink with purple in the center, but gardeners have developed varieties (more than 100) with colors ranging from white, pink to purple (3).

Catharanthus roseus is used as a folk remedy in many countries around the world and has been time-tested and validated by people's beliefs (2). *C. roseus* is used in the treatment of many diseases such as diabetes, blood pressure, asthma, constipation, cancer, menorrhagia, rheumatism, indigestion, skin diseases, bloody diarrhea and depression (3, 4). All parts of *C. roseus,* including roots, leaves, flowers and stems are used as

herbal medicine. Because of its therapeutic usefulness, it has attracted a lot of attention from scientific researchers. Published studies show that *C. roseus* is a medicinal plant with many pharmacological effects such as antimicrobial, antioxidant, anthelmintic, antifeedant, antisterility, antidiarrheal, antidiabetic (1). Therefore, the research on *C. roseus* will be further invested in to exploit the more herbal value of this plant.

Catharanthus roseus has received much pharmaceutical attention because of its chemical composition, which contains more than 130 different indole terpenoid alkaloids (TIAs), some of which are pharmacologically important and potent, such as vinblastine and vincristine. These were demonstrated with the high effectiveness of antioxidant, hypertension, diabetes and cancer treatments (5, 6). Vinblastine and vincristine are two popular *C.* roseus TIAs regarded as the first natural drugs used in the prevention and cure of malignant tumor diseases (7). Both vincristine and vinblastine are very expensive because the accumulated concentrations from the whole plant are very low (approximately 0.0005% w/w) (6), it is estimated that 500 kg of dried *C. roseus* leaves produces only 1 g of vinblastine, this makes the extraction of *C. roseus* very expensive. Low levels of vincristine and vinblastine are mostly connected with the geographical separation of biosynthetic sites in the plant. It mostly refers to the TIAs biosynthesis pathway of great degree specialization in leaf cells (8). Because of the poor recovery of vinblastine and vincristine from the costly extraction approach, scientists are particularly interested in increasing their production using various biological strategies.

Biotechnology has succeeded in producing many species of medicinal plants with high activity by *in vitro* culture. The most promising biotechnological tool for high production of these compounds is hairy root culture (9). Hairy root is formed by a mediate interaction between soil bacterium *Agrobacterium rhizogenes* and plant. This transformation process has delivered a precious by-product, “hairy root” that can develop unlimitedly in culture media (10). Hairy root might regard as plant factory that generated similar secondary metabolites and even higher than parents in a short period of time (10). Hairy root culture may completely expand to industrial scale due to independent growth in liquid medium without exogenous hormones, stable genetic and biochemical expression.

In order to enhance content and reduce harvest time of valuable secondary metabolites in hairy root, precursors and elicitors have been proposed as promising strategies (11). Many previous studies have demonstrated that precursors (phenylalanine, tyrosine) and elicitors (methyl jasmonate, chitosan, fungal elicitation) may accelerate the TIAs contents in CHR such as ajmalicine, serpentine, ajmalicine, catharanthine, tabersonine and lochnericine (12-14). Furthermore, vinblastine and vincristine accelerations were less studied, maybe because these compounds located in almost final positions of TIAs biosynthesis pathways (5).

In the current work, the effects of precursors (types and concentrations) and elicitors (types, dosages, and time exposure) on CHR growth and alkaloids (vinblastine and vincristine) accumulation were evaluated.

Materials and Methods

Agrobacterium rhizogenes preparation

Agrobacterium rhizogenes C18 strain was isolated in the Laboratory of Plant Biotechnology, Faculty of Biology - Biotechnology, Ho Chi Minh University of Sciences, Vietnam National University (15). *A. rhizogenes C18* strain was cultured and stored on Yeast Mannitol Broth (YMB) agar for 48 hrs at 25 °C and shaken at 180 rpm. Bacterial broth after culture with an OD_{600} nm of about 1.7-1.8 was diluted to an OD_{600} nm 1.0 to be used as a source of bacteria for infection.

CHR preparation

CHR was induced by *A. rhizogenes C18* according to the procedure of Nhut et al. (16).

Preparation of seedlings in vitro

Catharanthus roseus VIN077 seeds were provided by Vietnam Flower Seed Company FVN (Ho Chi Minh City). Seeds were washed with diluted soapy water for 10 min and then rinsed with clean water. Next, the seeds were washed with 80% ethanol for 1 min and soaked in 5% NaOCl solution for 15 min, and then the seeds were washed with sterile distilled water. Seeds after sterilization were placed on 1/2MS medium (Murashige & Skoog) supplemented with 3% (w/v) sucrose and 0.8% (w/v) phytoagar (pH 5.8) at 25°C with dark conditions. After 5 days, transfer the seedlings to 1500 lux white light (16 hrs/day). After 8 weeks of culture, the plants are about 5-7 cm tall and have 4-5 pairs of developed true leaves that will be used as infectious materials to induce hairy roots.

The procedure for hairy root induction

The true leaves *in vitro* were wound in the main veins of the leaves. Then, the sample was soaked in *A. rhizogenes* suspension for 5 min. Next, the samples were co-cultured on 1/2MS medium in the dark for 7 days at 25 °C. Remove *A. rhizogenes* by transferring samples to *1/2* MS medium supplemented with cefotaxime 500 mg L^{-1} and cultured in the dark for 7 days at 25 °C. After that, the samples were transferred to 1/2MS medium and continued to grow in the dark at 25 °C. Hairy roots are formed after 2-3 weeks of infection.

Hairy roots culture in liquid medium

CHR was cultured in liquid ½ B5 medium (Gamborg's B5) (17) containing sucrose 2% (w/v), pH = 5.7 ± 0.1. The initial culture density was 0.2 g fresh weight with 50 ml liquid medium in a 250 ml erlen, shaken in an 80 rpm rotary shaker at 25 °C in a fully dark setting. Precursors and elicitors will be added to the culture medium to investigate the growth and content of vincristine and vinblastine of hairy roots. The control treatment was hairy roots grown in the medium without adding precursors and elicitors.

Investigating the effect of precursors

L-phenylalanine (HiMedia, India) and L-tyrosine (Merk, Germany) were provided at the beginning with different concentrations: 0, 1, 10 and 100 µM. CHR dry weight (DW) and contents of vincristine and vinblastine were measured every week for 8 weeks.

Investigating the effect of elicitors

In the late exponential phase of culture (6th week): chitosan (from shrimp shells, >= 75% (deacetylated), China): 0, 50, 100 and 150 mg L^{-1} and methyl jasmonate (Sigma, American): 0, 50, 100 and 150 µM were added to the culture media. The exposure times for each elicitor dosages were 2, 4 and 6 days. CHR dry weight (DW) and contents of vincristine and vinblastine were measured after 2, 4 and 6 treated days.

Analysis of vincristine and vinblastine contents by HPLC method

For vincristine and vinblastine (µg/g DW) measurement, 0.5 g dried CHR was extracted from harvested samples and then evaluated by HPLC using a previously published method (18). HPLC analysis used a chromatographic system consisting of a G1312 BinPump, a 7725-type injector valve with a 20 µl loop, a G1314A variable wavelength probe, a PT100 column oven, and an Agilent ChemStation. Reversed phase column (150 mm × 4.6 mm; 5 µm) operated at 25 °C and eluted with 0.1% triethylamine (A) and methanol (B) according to the following procedure: 0–40 min, linear gradient from 50:50 (A: B) to 30:70; 40–50 min, elution class with 10:90; and 50-60 min, elution class with 50:50 to equilibrate the column. All chromatographic experiments had a flow rate of 0.8 ml/min, an injection volume of 10 µl and the effluent was monitored at 280 nm (18).

Statistical analysis

All experiments in this study were repeated 3 times and each treatment in these experiments was repeated 10 times with the follow-up parameters being dry weight (g DW) and vincristine and vinblastine content (µg g^{-1} DW). All data were analyzed using the SPSS 16.0 (Copyright SPSS Inc.). Experimental results were represented as mean ± standard deviation (SD). Differences between means were evaluated by Duncan's multiple range tests. Statistical significance was accepted at a level of $p < 0.05$.

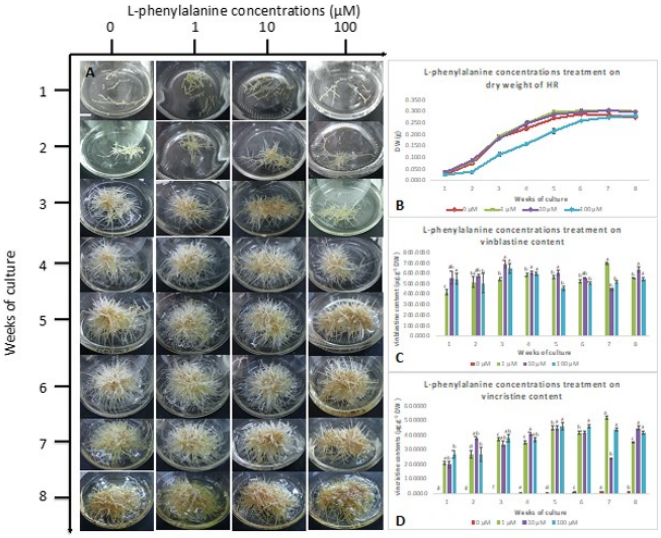

Fig. 1. Effect of various L-phenylalanine concentrations on CHR. **(A)** CHR phenotypes, **(B)** dry weight, **(C)** vinblastine contents and **(D)** vincristine contents. Error bars represent standard deviations. The value in line marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test). Scale bar = 1 cm.

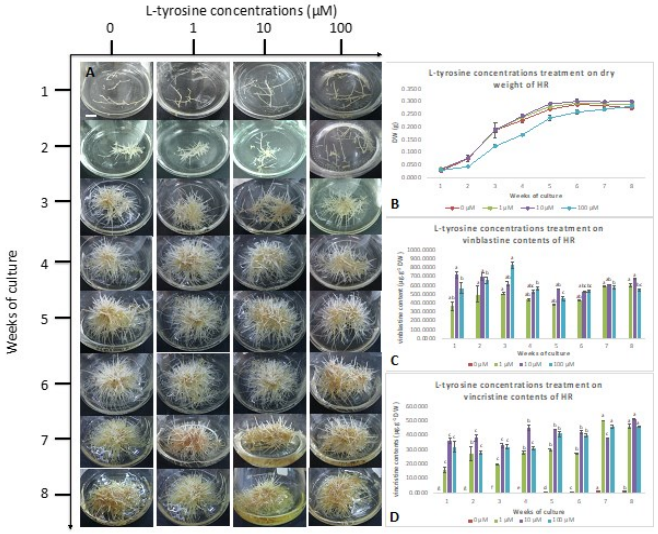

Fig. 2. Effect of various L-tyrosine concentrations on CHR. (A) CHR phenotypes, (B) dry weight, (C) vinblastine contents and (D) vincristine contents. Error bars represent standard deviations. The value in line marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test). Scale bar = 1 cm.

Results and Discussion

The effect of precursors

Fig. 1 and 2, Table 1 and 2 show that phenylalanine and tyrosine introduction increased both vincristine and vinblastine accumulation, whereas the control sample was noted in traces.

Effects of L-phenylalanine

The DW, VBL, and VCR were evaluated after adding various PHE concentrations during 8 weeks of culture.

As shown in Fig. 1B, by feeding 0, 1, and 10 µM PHE, the lag phase of CHR (1 week) was shortened in comparison with the 100 µM PHE addition (2 weeks). At week 4 of culture, the presence of 1 and 10 µM PHE could enhance the growth of CHR comparable with the root control. By only adding 1 µM PHE, the CHR may develop its growth similar to the 10 µM feeding. The growth curve may be clearly divided into [1] the lag (1 – 2 weeks), [2] the exponential (2 – 5 weeks), and [3] the stationary (5 – 8 weeks) growth stage. The CHR accelerated and reached a peak of DW at week 5^{th} (0.2987 g).

Regarding TIA contents (Fig. 1C), there were various distributions of VBL and VCR contents in different dosages of PHE addition. The VBL did not observe in the CHR control but strongly accumulated when using precursor. By feeding 10 and 100 µM PHE, CHRs early achieved their highest VBL contents in week 3^{rd} (690.00 µg g^{-1} DW) and 1^{st} (550.00 µg g^{-1} DW) respectively. However, their VBL contents remained or even reduced gradually in further weeks. The addition of 1 µM PHE could reveal an outstanding effect, CHR achieved the highest VBL content at week 7^{th} (699.92 µg g^{-1} DW). A

Table 1. Effect of various L-phenylalanine concentrations on CHR

Week of culture		1	2	3	4	5	6	7	8
DW (g)	0 µM	0.0266^{f}	0.0746^{e}	0.1870^{d}	0.2268^{c}	0.2699^{b}	0.2879^{a}	0.2844^{a}	0.2732^{a}
	1 µM	0.0367^{e}	0.0824^{d}	0.1927^{c}	0.2491^{b}	0.2987^{a}	0.3024^{a}	0.3064^{a}	0.3006^{a}
	10 µM	0.0366^{e}	0.0884^{d}	0.1841^{c}	0.2481^{b}	0.2877^{a}	0.2986^{a}	0.3039^{a}	0.2977^{a}
	100 µM	0.0254^{e}	0.0387^{e}	0.1133^{d}	0.1605^{c}	0.2153^{b}	0.2600^{ab}	0.2737^{a}	0.2818^{a}
VBL (µg g^{-1} DW)	0 µM	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1 µM	419.6185^{c}	520.2104^{bc}	549.9049^{b}	589.9210^{b}	569.9364^{b}	530.0000^{b}	699.9238^{a}	560.0000^{b}
	10 µM	560.0000^{ab}	580.0000^{ab}	690.0000^{a}	610.0819^{a}	610.0000^{a}	559.9375^{ab}	449.9507^{b}	639.9283^{a}
	100 µM	550.0000^{a}	510.4393^{b}	650.1912^{a}	599.8754^{a}	459.9288^{b}	510.0654^{b}	520.0000^{b}	550.0000^{a}
VCR (µg g^{-1} DW)	0 µM	0.0500^{g}	0.0800^{g}	0.1200^{f}	0.3700e	0.7000^{d}	1.1101^{c}	1.6302^{a}	1.3300^{b}
	1 µM	20.9809^{e}	27.0109^{d}	36.9936^{c}	34.9953^{c}	44.9950^{b}	42.0000^{b}	51.9943^{a}	35.0000^{c}
	10 µM	20.0000^{b}	38.0000^{ab}	34.0000^{ab}	41.0055^{a}	45.0000^{a}	41.9953^{a}	23.9974^{b}	44.9950^{a}
	100 µM	27.0000^{b}	27.0233^{b}	38.0112^{ab}	36.9923^{ab}	45.9929^{a}	46.0059^{a}	44.0000^{a}	42.0000^{a}

The value marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test)

Table 2. Effect of various L-tyrosine concentrations on CHR

Week of culture		1	2	3	4	5	6	7	8
DW (g)	0 µM	0.0266^{f}	0.0746^{e}	0.1870^{d}	0.2268^{c}	0.2699^{b}	0.2879^{a}	0.2844^{a}	0.2732^{a}
	1 µM	0.0356^{e}	0.0760^{d}	0.1880^{c}	0.2388^{b}	0.2800^{a}	0.2934^{a}	0.2913^{a}	0.2900^{a}
	10 µM	0.0318^{e}	0.0770^{d}	0.1890^{c}	0.2429^{b}	0.2913^{a}	0.3005^{a}	0.2992^{a}	0.3006^{a}
	100 µM	0.0298^{e}	0.0436^{e}	0.1252^{d}	0.1697^{c}	0.2360^{b}	0.2590^{a}	0.2692^{a}	0.2820^{a}
VBL (µg g^{-1} DW)	0 µM	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1 µM	370.0000^{ab}	499.7810^{a}	509.9096^{a}	440.0000^{ab}	380.0452^{ab}	430.0000^{ab}	589.9325^{a}	600.0000^{a}
	10 µM	720.7547^{a}	700.3641^{a}	619.8906^{ab}	530.0000^{abc}	560.0000^{ab}	529.9412^{abc}	610.0680^{ab}	680.0000^{a}
	100 µM	570.0000^{b}	659.4954^{b}	830.0000^{a}	570.1120^{b}	450.0000^{c}	540.0000^{bc}	580.0000^{b}	550.0650^{bc}
VCR (µg g^{-1} DW)	0 µM	0.0500^{g}	0.0800^{g}	0.1200^{f}	0.3700^{e}	0.7000^{d}	1.1101^{c}	1.6302^{a}	1.3300^{b}
	1 µM	16.0000^{c}	26.9882^{b}	19.9964^{c}	28.0000^{b}	30.0036^{b}	27.0000^{b}	49.9943^{a}	46.0000^{a}
	10 µM	36.0377^{c}	38.0198^{c}	32.9942^{c}	45.0000^{b}	44.0000^{b}	41.9953^{b}	38.0042^{c}	51.0000^{a}
	100 µM	32.0000^{c}	27.9786^{c}	32.0000^{c}	31.0061^{c}	41.0000^{b}	40.0000^{b}	46.0000^{a}	46.0054^{a}

The value marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test).

description of VCR contents by adding different PA concentrations was revealed in Fig. 1D. The VCR was presented in all cultures but was remarkably increased when only adding PHE. In 1, 10 and 100 µM PHE, CHR accumulated the greatest VCR contents at week 7th (51.99 µg g^{-1}DW), 4th (41.01 µg g^{-1}DW) and 5th (45.99 µg g^{-1}DW) respectively. The HPLC analysis of VCR accumulated in CHR cultured in 1 µM PHE and the control was given in Fig. 6A and 6B respectively.

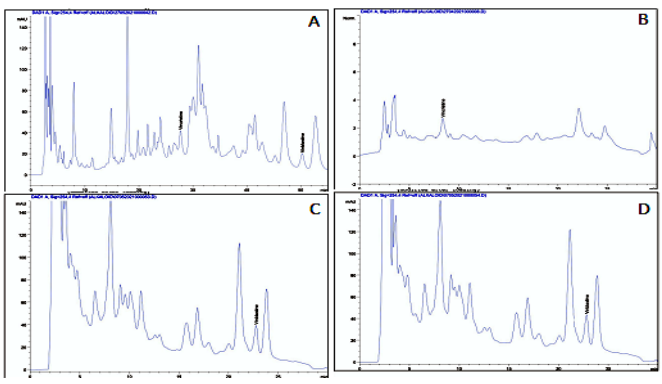

Fig. 6. HPLC analysis of the CHR extract with addition of (A) 1 µM PHEL, (B) the control hairy root after 7 weeks of culture, (C) 150 mg L^{-1} chitosan after 6 days of elicitation, (D) 50 µM methyljasmonate after 2 days of elicitation.

Effects of L-tyrosine

CHR was cultured in liquid medium contained numerous TYR concentrations. The DW, VBL and VCR were evaluated periodically in a duration of eight weeks.

The effect of TYR concentrations on DW was described in Fig. 2B. When adding 0, 1 and 10 µM TYR, the lag phase of CHR (1 week) was shortened in comparison with the 100 µM PHE addition (2 weeks). At week 5 of culture, the appearance of 1 and 10 µM TYR could elevate the growth of CHR comparable with the root control. Furthermore, with only providing 1 mM TYR, the CHR may generate growth similar to that of a 10 mM feeding. The growth curve can be clearly divided into three stages: [1] the lag (1 – 2 weeks), [2] the exponential (2 – 5 weeks), and [3] the stationary (5 – 8 weeks) growth stage. The CHR developed and then achieved a peak of DW at week 5th (0.2800 g).

The effects of different dosages of TYR on VBL and VCR contents were revealed in Fig. 1C and 1D. The VBL in CHR has just started to accumulate when only feeding TYR

into cultural medium. In the treatment of TYR, the VBL achieved the greatest content quite early in lag phase (from the 1st to 3rd week) and then nearly maintained during 8 weeks. The significant production of VCR happened in the temporary growth stage. The additions of 1, 100 (in the 7th week) and 10 µM (in the 8th week) reached the highest content: 49.99, 46.00 and 51.00 µg g^{-1} DW respectively.

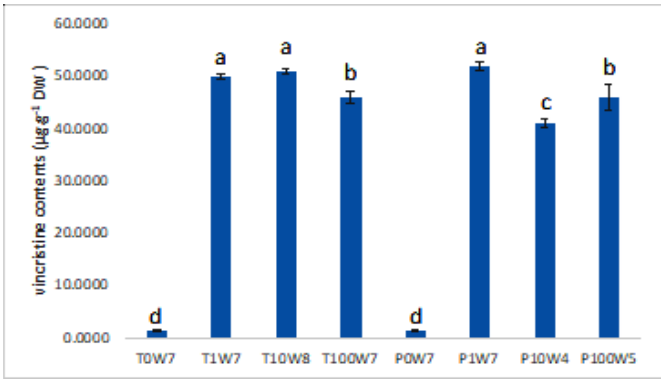

Fig. 3. The VCR (B) contents among the most effective PA and TYR concentrations. Error bars represent standard deviations. The value in line marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test). (T: tyrosine concentration, P: phenylalanine concentration, W: week).

A comparison between PHEL and TYR concentrations affected on VCR content was illustrated in Fig. 3. The VCR content parameter was priority chosen due to its economic value. Although VBL and VCR could help to specifically treat many cancer diseases. The cost for using VCR ($ 15 million/kg) is much more than VBL ($ 2 million/kg) . The T1W7, T10W8 and P1W7 had created a remarkable effect on increasing VCR content. The VBL contents accumulated in them were then compared. As a result, the T10W8 and P1W7 could produce significantly higher amounts of VBL than the T1W7. Regarding economic efficiency, P1W7 is not only using less precursor dosage but also shortening the harvest time in comparison with T10W8. Therefore, in this study, using 0.1 µM PHEL may consider as a reasonable strategy for effectively obtaining VBL and VCR in our CHR culture.

At the appropriate concentration, both PHEL and TYR could increase the growth of CHR in comparison with the control. This root growth encouragement may be due to the extra supply of nitrogen nutrients as amino acids characteristics of PHEL and TYR However, precursors at high concentrations may hamper the absorbent of other nutrients lead to reduce biomass production .

There was a noted observation of the VBL and VCR contents. While the VBL often accumulated in early stage of culture (week 1 – week 3), the VCR presented in later phase (week 7 – week 8). This issue could be explained by understanding the TIA biosynthesis pathway. Firstly, vinblastine (a dimeric indole alkaloid) was made by coupling vindoline and catharanthine under catalysis of horseradish peroxidase (21). Afterwards, vinblastine is then converted into vincristine by the oxidation of its methyl group

However, the interesting discovery was by adding 1 µM PHEL, the CHR could produce both the greatest VBL and VCR contents after 7 weeks of culture. It could be considered that PHEL is more appropriate than TYR for TIA biosynthesis pathway. For the increase of TIA content in CHR, there were several studies using terpenoid precursor such as loganin that could enhance the catharanthine by 26%, ajmalicine by 84%, lochnericine by 119% and tabersonine by 225% in the induced samples (23). A study, it was reported that with loganin feeding, ajmalicine content (3.27 mg L^{-1}) was improved by 2.3-fold and there was 1.8-fold increase in the yield of serpentine (2.51 mg L^{-1}) when compared with control cultures (1.42 and 1.39 mg L^{-1} respectively) (24). Our study was firstly using PHEL and TYR for elevating VBL and VCR in CHR. In addition, the VCR was increased 31-fold comparable with the control CHR. The CHR in our study required a significant primary growth stage before stepping to the secondary metabolite accumulation. PHEL may be applicable for both cultural phases as a supportive nutrition in the first stage and then become an effective precursor in the second stage.

The effect of elicitor

Fig. 4 and 5 Table 3 revealed only vinblastine was produced in CHR when using chitosan and methyl jasmonate elicitation.

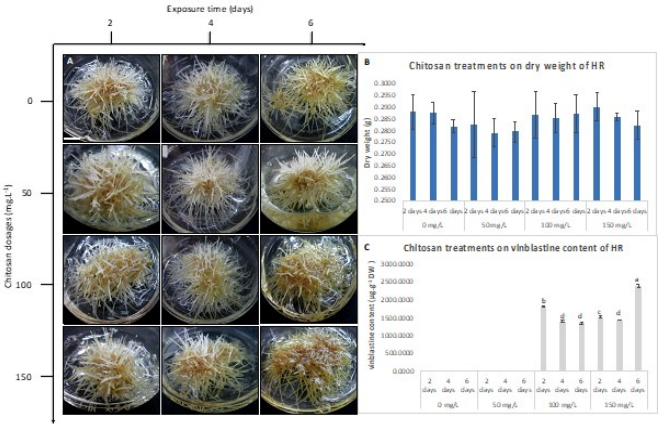

Fig. 4. Effect of the chitosan dosages and exposure time on CHR. (A) CHR phenotypes, (B) dry weight and (C) vinblastine contents. Error bars represent standard deviations. The value in line marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test). Scale bar = 1 cm.

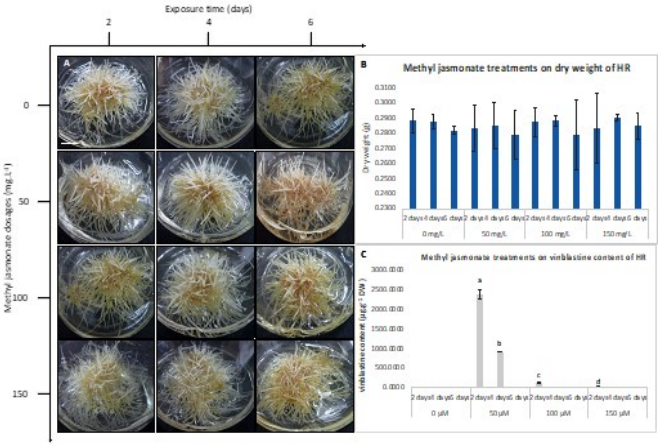

Fig. 5. Effect of the methy jasmonate dosages and exposure time on CHR. (A) CHR phenotypes, (B) dry weight and (C) vinblastine contents. Error bars represent standard deviations. The value in line marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test). Scale bar = 1 cm

Table 3. Effect of chitosan and methyl jasmonate dosages and time exposure on CHR

Chitosan dosages	Time exposure	DW (g)	VBL (µg g^{-1} DW)	Methyl jasmonate	Time exposure	DW (g)	VBL (µg g^{-1} DW)
	2 days	0.2879	0.0000		2 days	0.2879	0.0000
0 mg/L	4 days	0.2877	0.0000	0 µM	4 days	0.2877	0.0000
	6 days	0.2818	0.0000		6 days	0.2818	0.0000
	2 days	0.2825	0.0000		2 days	0.2830	2370.2790[a]
50 mg/L	4 days	0.2792	0.0000	50 µM	4 days	0.2847	911.4933[b]
	6 days	0.2796	0.0000		6 days	0.2788	0.0000
	2 days	0.2869	1833.5870[b]		2 days	0.2871	125.9854[c]
100 mg/L	4 days	0.2855	1400.7873[d]	100 µM	4 days	0.2880	0.0000
	6 days	0.2872	1363.0420[d]		6 days	0.2789	0.0000
	2 days	0.2901	1526.5750[c]		2 days	0.2832	48.4800[d]
150 mg/L	4 days	0.2859	1437.6680[d]	150 µM	4 days	0.2903	0.0000
	6 days	0.2824	2401.0166[a]		6 days	0.2846	0.0000

The value marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test)

Effects of chitosan

As shown in Fig. 4, CTS dosages or time exposure did not reduce the growth of CHR comparable with the control ($p > 0.05$). However, eliciting with CTS at high concentrations (100 and 150 mg L^{-1}), the CHR revealed a significant increase of VBL content. In addition, increasing time exposure could help to enhance the production of VBL. After 6 days of elicitation, adding 150 mg L^{-1} CTS could reveal a remarkable result (2401.02 µg g^{-1} DW). The color of CHR and medium turned into yellow only exposing with 150 mg L^{-1} CTS. The HPLC analysis of VCR accumulated in CHR cultured in150 mg L^{-1} CTS after 6 days of elicitation was described in Fig. 6C.

Effects of methyl jasmonate

As shown in Fig. 5, MJ with any dosages or time exposure could not make detrimental effect on the growth ability of CHR in comparison with the control. The greatest content of VBL was produced in using 50 µM and 2 days of MJ elicitation (2370.30 µg g^{-1} DW). The HPLC analysis of VCR accumulated in CHR cultured in 50 µM MJ after 2 days of elicitation was revealed in Fig. 6D.

In the comparison between two elicitors, using methyl jasmonate with lower dosage as well as shorter exposure time of elicitation than chitosan, it could produce the same amount of vinblastine. Methyl jasmonate works as an exogenous signaling molecule; it increases the transcription of genes encoding enzymes involved in TIAs production (25). The methyl jasmonate effect in TIAs elevation was reported in many previous studies. In *C. roseus,* methyl jasmonate increased ajmalicine accumulation in cell suspension cultures (26), vindoline, catharanthine, and ajmalicine accumulation in meristem cell cultures in bioreactor systems (25), and ajmalicine and catharanthine accumulation in hairy root culture (27). Furthermore, it had a significant effect on the expression levels of TIAs biosynthesis genes in *C. roseus* prior blooming *(28)*. In this study, methyl jasmonate promoted vinblastine accumulation (2.370 µg g^{-1} DW) in CHR, whereas the control sample did not. However, vincristine was not discovered in these treatments.

A comparison between using precursor and elicitor on VBL and VCR production in the CHR

There were just a few reports working on VBL and VCR content of CHR. Determine the VCR was 442.3 ng mg^{-1} FW but VBL was not detected. In comparison with our CHR, the VCR accumulated less content (51.99 µg g^{-1} DW) but significantly higher VBL content. It is noteworthy that VBL (which presented abundantly in our CHR) will transform into VCR during times of culture. After 7 weeks, CHR cultured in 1 µM PHEL could produce both VBL and VCR with higher content than any others. This issue may indicate that CHR will be able for a remarkable VCR increase in further weeks of culture.

On the other hand, there was not any appearance of VCR when exposed to CTS or MJ. This issue could be elaborated by the transformation process from VBL to VCR. [1] The effect of elicitor (CTS or MJ) may release inhibited signals which inactivated of the enzyme transformed VBL to VCR and [2] VBL play a key role in responding to CTS or MJ and therefore VCR did not require. In this study, our CHR had sufficient ability to respond to the elicitor at the appropriate dosage, which led to an increase in VBL content, but not a reduction in growth by the time of elicitation. However, the less efficient in VCR production may due to a limitation in the experimental design of elicitor. It could be seen clearly in the use of 150 mg L^{-1} CTS with 6 days of elicitation which produced the highest of VBL content. Consequently, it is very likely that using a higher dosage or extending the time exposure will reveal a buildup of VCR content in our CHR.

Therefore, it is clear that in culturing hairy roots of *Catharanthus roseus*, the use of both precursor and elicitor have an impact on increasing the accumulation of TIAS if we use it appropriately. Among them, using precursor (phenylalanine 1 µM) is the most clearly demonstrated.

Conclusion

Precursors or elicitors treatment enhanced the production of TIAs in our CHR clones. In particular, the use of elicitors required more time to find the appropriate induction condi-

tions, while the use of precursors gave outstanding efficiency in the treatment with 1 M phenylalanine. The greatest yields of vincristine (51.99 µg g^{-1} DW) and vinblastine (699.92 µg g^{-1} DW) were obtained in the 7th week (with biomass 0.306 g DW). This highlights the high potential in using precursors to produce vincristine from CHR, especially CHR clones generated by *Agrobacterium rhizogenes* isolated in Vietnam.

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Authors contributions

PTBV carried out the precursors and elicitors effect investigation and drafted the manuscript. DMC participated in the precursors and elicitors effect investigation and drafted the manuscript. ALB participated in the design of elicitors and precursors effect investigation and performed the statistical analysis. NNN carried out the CHR preparation and CHR resource. LVB and PNDQ conceived of the study, participated in manuscript editing, its design, and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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