



Original Article

Yeast extract elicitation increases vinblastine and vincristine yield in protoplast derived tissues and plantlets in *Catharanthus roseus*



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ABSTRACT

Catharanthus roseus (L.) G. Don, Apocynaceae, is an immensely important medicinal plant, produces a variety of anticancerous compounds. The yield of two most investigated alkaloids vinblastine and vincristine is unfortunately very low. A vast array of technologies including elicitation have recently been used to enrich *Catharanthus* alkaloid in culture. Yeast extract is a biotic elicitor, the polysaccharide and the peptide moiety have been recognized as a signalling element in enriching secondary metabolites. In this study, the yeast extract elicitation on vinblastine and vincristine was studied in various protoplast derived tissues and plantlets. Four different yeast extract treatments (T1 = 0.5 g/l, T2 = 1.0 g/l, T3 = 1.5 g/l and T4 = 2.0 g/l) were prepared and used. The alkaloid was quantified and a comparative account of yield were presented by the use of High performance thin layer chromatography. The yeast extract amendment in medium improved vinblastine and vincristine yield in cultivating tissues, maximum being in germinating embryos and in *in vitro* raised leaf. The highest yield was in T3 (1.5 mg/l) in which 22.74% vinblastine and 48.49% vincristine enrichment was noted in germinating embryos; the enhancement was however, treatment-specific. Antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase activities were investigated as addition of yeast extract caused cellular stress and had enriched level of alkaloids.

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Introduction

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) belongs to the family Apocynaceae. It is an important medicinal plant and it contains a wide variety of anti-cancerous alkaloids (Van Der Heijden et al., 2004; Mujib et al., 2012). Among those, vinblastine (VB) and vincristine (VC) are the most important drugs (Moreno, 1995) and these two alkaloids are used as a therapeutic agent to treat against a number of cancers (Mukherjee et al., 2001). The low yield of alkaloids in plant has encouraged researchers to explore other alternative production methods by using *in vitro* cultures (Verpoorte et al., 2002; Naz et al., 2015), metabolic engineering (De Luca and St Pierre, 2000), semi-synthesis (Kutney et al., 1991) or even total chemical synthesis (Kuboyama et al., 2004). In *C. roseus*, various semi-synthetic procedures were developed by using chemicals and enzymatic coupling of commercially available catharanthine and vindoline (Goodbody et al., 1988; Moreno et al., 1995). In recent years, various cultural practices such as optimization of culture media, selection of high alkaloid producing cell lines,

precursor feeding, bioconversion and metabolic engineering are successfully utilized for enriching alkaloids in cultivated cells (Van Der Heijden et al., 2004; Zarate and Verpoorte, 2007; Mujib et al., 2012; Teixeira da Silva and Dobranszki, 2016). It has earlier been noted that an exogenous amendment of specific groups of compounds to medium drastically increased the yield of secondary metabolites (Ibrahim et al., 2009; Murthy et al., 2014). These external triggers are biotic and abiotic in nature; in the biotic types the microbial extracts such as *Pythium*, *Botrytis*, *Phytophthora*, *Pseudomonas* and *Aspergillus* are observed to be very effective in a variety of cell cultures including *C. roseus* (Valluri, 2009; Mustafa et al., 2009; Dipti et al., 2016).

Yeast extract (YE) is an important elicitor and is found to be rich in vitamin B-complex. It also contains essential components like chitin, *N*-acetyl-glucosamine oligomers, β -glucan, glycopeptides and ergosterol (Boller, 1995); these compounds elicit plant defense responses by triggering metabolite synthesis (Putalun et al., 2007; Cai et al., 2012). In other cultural experiments, complex biological preparations have also been used as elicitors where the molecular structure of active compound is not fully known (Radman et al., 2003; Zubek et al., 2012). The addition of YE has successfully been used in culture and overproduction of important phytochemical was observed in several studied plant genera (Prakash and

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Srivastava, 2008; Zhao et al., 2010; Cai et al., 2012). The addition of external compounds causes stress in cultivating tissues and improves alkaloid biosynthesis (Elmaghrabi et al., 2013; Samar et al., 2015; Dipti et al., 2016). Induced stress has been measured by studying antioxidant enzymes activity and other stress markers in cultured tissues that overproduce important phyto-compounds (Feher, 2015). In this present article of *C. roseus*, we therefore examined the role of YE on VB and VC yield on cultivated tissues/plants developed from protoplast.

Material and methods

Plant material

Catharanthus roseus (L.) G. Don, Apocynaceae, seeds were collected from herbal garden, Hamdard University, New Delhi, and was identified by taxonomist (Dr. M.P. Sharma), Department of Botany; the specimen was deposited in Departmental archive for future reference. The seeds were surface-sterilized with 0.1% (w/v) HgCl_2 for 2 min, rinsed four-times with sterilized double-distilled water and were placed in a conical flask containing 50 ml of MS medium (Murashige and Skoog, 1962) without organic compounds and plant growth regulators (PGR). The seeds were germinated and the seedlings were grown for 3 weeks. Various explants (e.g., hypocotyls, leaves and nodal segments) were excised and cultured on same MS, amended with various PGR for callus/embryogenic induction. The detailed procedure was described previously (Junaid et al., 2006) and was followed. The medium pH was kept at 5.7 before sterilization at 121 °C temperature. The cultures were kept in an incubation room with 25 ± 2 °C and 16-h photo period regime provided by cool white fluorescent tubes ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Protoplast isolation and culture

Protoplasts were isolated from 4-day-old embryogenic suspension. Suspended cells (0.5 g fresh weight) were incubated with 10 ml of enzyme solution containing 2% cellulase, 1% pectinase, 0.02% (w/v) macerozyme (Sigma Aldrich) and 0.5% (w/v) driselase (Sigma Aldrich) along with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and sorbitol. The mixture was later incubated on a rotary shaker (50 rpm) for 12 h under constant light conditions at 25 °C for liberating protoplasts. Isolated protoplasts were cultured in liquid medium at a density of 5×10^5 protoplasts/ml. Protoplasts were poured into a conical flask containing 25 ml MS liquid medium amended with PGR, which facilitated protoplast division.

Micro-colony, callus, embryo formation from protoplasts

Suspended protoplasts were cultured on medium supplemented with appropriate PGR concentrations, i.e. 0.25 mg/l L-naphthaleneacetic acid (NAA), 0.50 mg/l NAA, 0.25 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.50 mg/l 2,4-D, and combination of 0.25 mg/l NAA + 0.25 mg/l 2,4-D, 0.50 mg/l NAA + 0.50 mg/l 2,4-D for developing micro colonies. The induced colonies transformed into callus, which later converted into embryogenic callus. The embryogenic calli were placed on MS, added with 1 mg/l NAA + 1.5 mg/l 6-benzylaminopurine (BA) for differentiation of embryos. Later, protoplasts-derived embryos were cultured in optimized gibberellic acid (GA_3) amended medium (1.5 mg/l) for embryo maturation and subsequent germination/plant recovery. The cultured tissues were sub cultured regularly at an interval of 4 weeks, unless mentioned otherwise.

Elicitor treatment

Various *in vitro* grown tissues were subject to YE treatments. Four different concentrations of YE (Merck, India) were prepared and were designated as: T1 = 0.5 g/l, T2 = 1 g/l, T3 = 1.5 g/l and T4 = 2 g/l. The above designated doses of YE were amended to MS following Darshan et al. method (1991). After YE addition, the pH was maintained at 5.7 and the medium was sterilized at 15 lb (121 °C). The yield of alkaloid was measured at different tissues/embryos, and plantlets originated from protoplasts.

Quantification of VB and VC using high performance thin layer chromatography

Extraction

The VB and VC contents were measured in different tissues using standard VB and VC, procured from Sigma-Aldrich (St. Louis, MO, USA). Select tissues (25 weeks old) were cultured in optimized media and were harvested when the tissues were at their best growth. Tissue sample (one gram [g], dry weight) was refluxed in 30 ml of methanol for about 5–6 h and the recovered supernatant was heated at 60 °C until the volume reduced to 1–2 ml.

Preparation of stock solution and calibration curve

One mg of VB and VC was dissolved in 1 ml of methanol to make a stock solution of concentration 1 mg ml^{-1} . From stock solution, different dilutions were made (minimum five) to obtain 200, 400, 600, 800 and 1000 μg per band of standard and analyzed independently by HPTLC. Standard curve was plotted between peak area (y-axis) and concentration (x-axis).

HPTLC and quantification in different samples

Pre-coated Thin Layer Chromatography (TLC) Aluminium sheets ($20 \times 10 \text{ cm}$) with Silica gel (60 F 254, Merck) were used as stationary phase. The mobile phase was freshly prepared, consisting of toluene, carbinol, acetone and ammonia in the ratio of 40:20:80:2. Samples were applied by the help of 100 μl microsyringe (CAMAG) using Linomat 3 (CAMAG) applicator. After the application of samples, the silica plates were air dried for 10–15 min and placed in a developing chamber (Twin through Chamber CAMAG $20 \times 10 \text{ cm}$) containing mobile phase. Solvent system was allowed to move upwards up to 85 mm. The plate was removed from the chamber and air dried again for 10–20 min. Silica gel plates were documented by using the Reprostar (CAMAG) in the presence of UV-light. Stationary phase along with the VB and VC was scanned by using the Scanner 3 (CAMAG). VB was scanned at 280 nm and the VC was scanned at 300 nm. Alkaloid content was measured as $\mu\text{g g}^{-1}$ dry weight basis. The peak of VB and VC was fixed and the detection of the same alkaloids in the samples was compared with the peaks of standard.

Antioxidant enzymatic activity

Fresh plant material was homogenized in 2 ml of 0.1 M common extraction buffer (pH 7.5, 0.1 M K-phosphate, 0.5 mM EDTA, 1.0 mM ascorbic acid) and centrifuged at $8608.6 \times g$ for 20 min at 4 °C. Supernatant (enzyme extract) was taken for enzyme analysis and the activity of enzymes was expressed in Enzyme Unit (EU) $\text{mg}^{-1} \text{protein min}^{-1}$.

Catalase

Catalase (CAT) activity was determined by the method of Aebi (1984). CAT activity was determined by monitoring the decomposition in H_2O_2 , measuring a decrease in the absorbance at 240 nm of reaction mixture containing 1 ml of 0.5 M reaction phosphate buffer (Na-phosphates, pH 7.5), 0.1 ml EDTA, 0.2 ml enzyme extract and

0.1 ml H_2O_2 . The reaction was run for 3 min. The enzyme activity was expressed in $\text{EU mg}^{-1} \text{protein min}^{-1}$. One unit of enzyme determines the amount necessary to decompose $1 \mu\text{mol}$ of H_2O_2 per min. CAT activity was calculated by using the co-efficient of absorbance $0.036 \text{ mM}^{-1} \text{ cm}^{-1}$.

Superoxide dismutase

The method of [Dhindsa et al. \(1981\)](#) was followed with slight modification to estimate superoxide dismutase (SOD) activities. Embryos (0.1 g) at different developmental stages were homogenized in 2 ml of extraction mixture containing 0.5 M sodium phosphate buffer (pH 7.3), 3 mM EDTA, 1.0% (w/v) polyvinylpyrrolidone (PVP), 1.0% (v/v) Triton X-100, and centrifuged at $8608.6 \times g$ at 4°C . The SOD activity in the supernatant was assayed by its ability to inhibit the photo-chemical reduction. The assay mixture consisting of 1.5 ml reaction buffer, 0.2 ml methionine, 0.1 ml of enzyme extract with equal volume of 1 M NaCO_3 and 2.25 mM Nitro Blue Tetrazolium (NBT) solution, 3 mM EDTA, riboflavin, 1 ml of Millipore H_2O was taken in test tubes and incubated under light for 10 min at 25°C . A 50% reduction in colour is one unit and the enzyme activity was expressed in $\text{EU mg}^{-1} \text{protein min}^{-1}$.

Ascorbate peroxidase

The method developed by [Nakano and Asada \(1981\)](#) was used to determine the activity of ascorbate peroxidase (APX). The reaction mixture contained 1 ml of 0.1 M sodium buffer (pH 7.2), 0.1 ml of EDTA and 0.1 ml of tissue extract. Ascorbate was added and the reaction was run for 3 min at 25°C . Ascorbate oxidation was monitored spectrophotometrically at 290 nm by a reduction of absorbance and using the absorption coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. The activity of APX was expressed in $\text{EU mg}^{-1} \text{protein min}^{-1}$. One unit of enzyme is the amount necessary to decompose $1 \mu\text{mol}$ of ascorbate per min.

Glutathione reductase

The glutathione reductase (GR) activity was determined by the method of [Foyer and Halliwell \(1976\)](#). The enzyme extract was rapidly assayed for the GR activity by taking 0.1 ml of enzyme extract and 1 ml of reaction buffer (Tris 0.1 M/pH 7.0, to 100 ml of Tris 0.2 mM NADPH and 0.5 mM GSSG was added). The reaction was run for 3 min at 25°C . The GR activity was calculated by using the co-efficient of absorbance $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. The GR activity was determined through glutathione-dependent oxidation of NADPH at 340 nm. The activity of GR was expressed in $\text{EU mg}^{-1} \text{protein min}^{-1}$. One unit of enzyme determines the amount necessary to decompose $1 \mu\text{mol}$ of NADPH per min.

Statistical analysis

The data on the effects of YE on protoplast callus, embryogenesis, alkaloid yield and on enzyme activity were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. Values are expressed as means $\pm$ standard errors of three replicates of two experiments. The presented mean values were separated using LSD at $p \leq 0.05$ level.

Results

Protoplast isolation, callus formation, embryo formation and plantlet regeneration

The hypocotyl induced embryogenic callus was used for protoplast isolation study; this embryogenic callus henceforth is called as normal embryogenic callus (NEC). Protoplast isolation was carried from NEC suspension by using various wall degrading enzymes. Significant numbers of protoplasts (data not shown

here) were obtained on treatment with cellulase (2%), pectinase (1%), driselase (0.5%), macerozyme (0.02%) and sorbitol (0.6 mM) ([Fig. 1a](#)). Repeated divisions of protoplasts formed micro-colonies; and the maximum numbers of micro colonies/calli were induced on 0.50 mg/l NAA + 0.50 mg/l 2,4-D added medium within 12 weeks of incubation. Protoplast originated micro-calli gradually transformed into embryogenic calli, called as protoplast derived embryogenic calli (PDEC). The micro-calli had slow growth initially, but with time the tissues showed normal fast growth as those cultivated cells overcame stress gradually, caused by enzyme mixtures and osmoticum treatment. The micro-calli of PDEC origin started to induce somatic embryos within 8 weeks of culture (induction stage). In MS embryo proliferation medium, a heterogeneous mixture of embryos was formed (proliferation stage). Globular and heart-stage embryos were more in numbers compared to torpedo and cotyledonary embryos, which differentiated in good numbers later. The embryo maturation was very high on 1.5 mg/l GA_3 added medium and with time the maturation percentage improved. The embryos started to regenerate plants in the same maturation medium. The entire process from 'protoplast to plantlet' recovery was achieved through somatic embryogenesis following embryo induction, proliferation, maturation and embryo germination methods ([Fig. 2a and d](#)). The whole process took about 40 weeks to obtain a plant from protoplast.

VB and VC content ($\mu\text{g g}^{-1}$ dry weight)

The yield of VB and VC was measured in different tissues. The mobile phase of standards produced a sharp and compact VB ($R_f = 0.28$) and VC ($R_f = 0.46$) peak ([Fig. 1a and c](#), respectively). Scanning of VB and VC was made at 280 and 300 nm, respectively. The regression analysis showed a good linear relationship with VB $r = 0.999$ ($r^2 = 0.998$) and VC $r = 0.994$ ($r^2 = 0.989$) with respect to peak area and concentration range of 200–1000 μg per spot. The regression equation of VB was $Y = 943.7X + 180.0$ and of VC was $Y = 813.5X + 1684$.

From [Tables 1 and 2](#), it is clear that VB was maximum ($9.32 \mu\text{g g}^{-1}$ dry weight) in germinating embryos and the yield was improved ($11.44 \mu\text{g g}^{-1}$ dry weight) with YE elicitation, especially in T3 treatments. Compared to VB, the yield of VC was low and in germinating embryos, the yield was high. With YE elicitation, VC yield was further improved, maximum being in T3 ($3.46 \mu\text{g g}^{-1}$ dry weight). We also quantified VB and VC yield in protoplast regenerated leaves. *In vitro* leaf tissue had maximum amount of VB compared to other tissues/embryos. T3 (1.5 mg/l YE) harvested leaves had highest yield of VB ($15.47 \mu\text{g g}^{-1}$ dry weight) and VC ($4.14 \mu\text{g g}^{-1}$ dry weight), followed by T4 treatment ([Fig. 1b and d](#)). The VB and VC level increased 22.74% and 48.49%, respectively, with YE elicitation in germinating embryos over non-treated control tissue.

CAT, APX, SOD and GR activities

The addition of elicitors often cause stress in cultivating tissue we therefore analyzed and compared antioxidant enzyme activities (CAT, APX, SOD and GR) as an indicator of stress. Protoplast derived embryogenic tissue showed higher antioxidant enzyme activities compared to normal embryogenic tissue as these tissues derived from digestion with wall degrading enzymes, which caused moderate to severe stress to cells/tissues. With YE elicitation, the anti-oxidant enzyme activities were further up suggesting extra stress on tissues. Compared to embryo induction and proliferation stages, the alkaloid yield was high on maturing- and germinating embryos, so was the higher level of enzyme activities. The CAT activity was $1.85 \text{ EU mg}^{-1} \text{protein min}^{-1}$ and $2.03 \text{ EU mg}^{-1} \text{protein min}^{-1}$

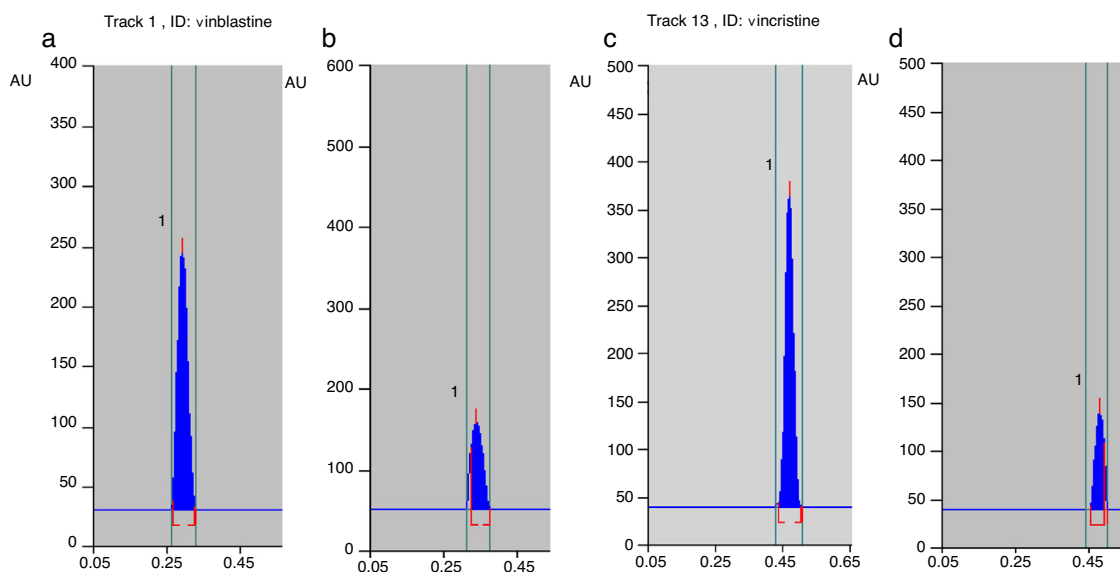


Fig. 1. (a) Standard peak of vinblastine, (b) peak showing the maximum yield of vinblastine in YE treated leaves at T3, (c) standard curve of vincristine and (d) peak showing the maximum yield of vincristine in YE treated leaves at T3 (x-axis presents peak area and y-axis presents Rf).

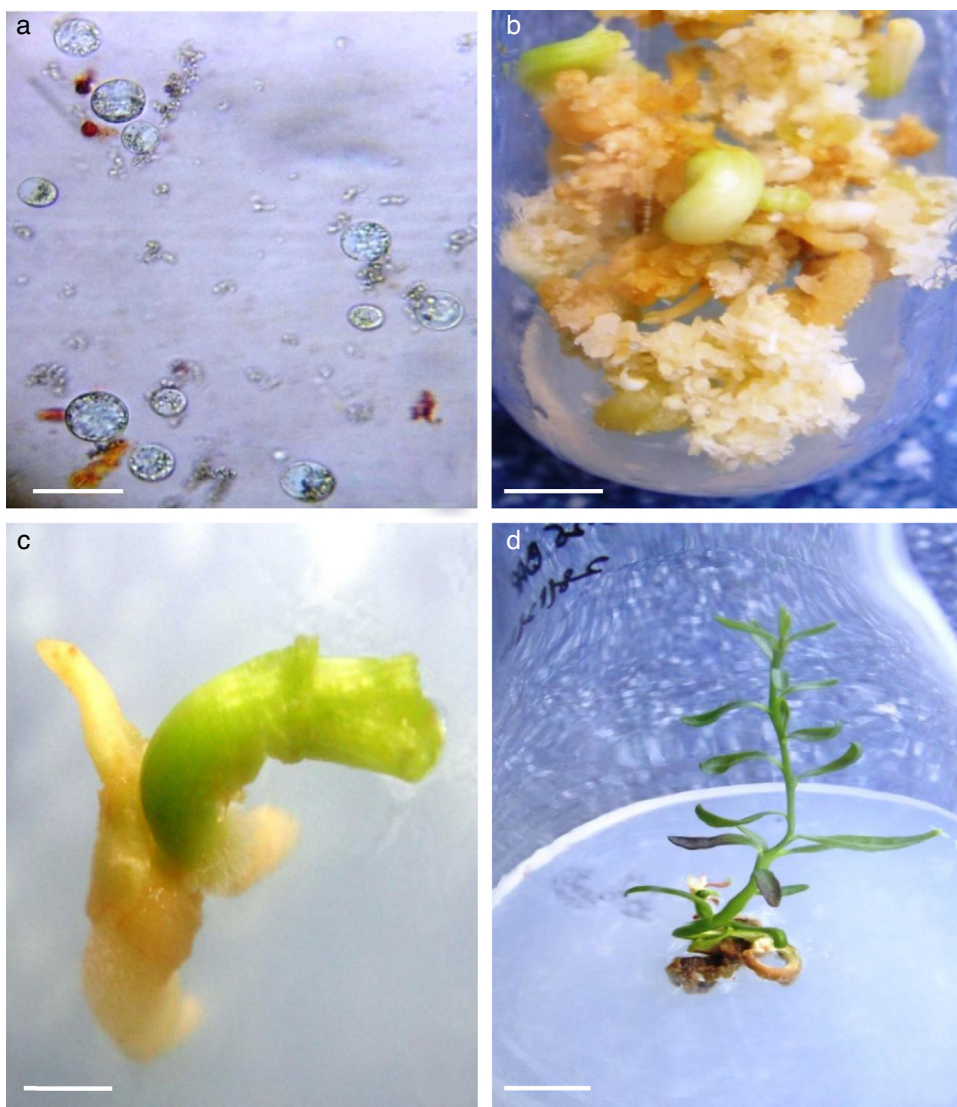


Fig. 2. (a) Isolated protoplasts; (b) development of embryogenic callus from protoplasts; (c) individual embryo developed from PDEC treated with T3 treatment of YE and (d) regenerated plantlet from protoplast derived embryo, grown in MS medium containing YE (T3). (a and b) bar = 200 μ m, (c) bar = 2 mm, (d) bar = 4 mm.

Table 1

Vinblastine (VB) content ($\mu\text{g g}^{-1}$ dry weight) in protoplast derived embryos/tissues in *Catharanthus roseus*. Tissues were added with different treatments of YE.

Sample	Vinblastine ($\mu\text{g g}^{-1}$ dry weight)				
	Induction	Proliferation	Maturation	Germination	Leaves (<i>in vitro</i>)
C	1.82 $\pm$ 0.015 ^d	4.44 $\pm$ 0.022 ^e	7.45 $\pm$ 0.025 ^e	9.32 $\pm$ 0.028 ^e	13.10 $\pm$ 0.007 ^d
T1	1.93 $\pm$ 0.018 ^c	4.81 $\pm$ 0.043 ^d	7.65 $\pm$ 0.025 ^d	9.64 $\pm$ 0.029 ^d	13.25 $\pm$ 0.031 ^c
T2	2.04 $\pm$ 0.022 ^b	5.25 $\pm$ 0.015 ^c	7.87 $\pm$ 0.033 ^c	10.34 $\pm$ 0.015 ^c	13.24 $\pm$ 0.094 ^c
T3	2.13 $\pm$ 0.019 ^a	6.03 $\pm$ 0.015 ^a	8.06 $\pm$ 0.036 ^a	11.44 $\pm$ 0.014 ^a	15.47 $\pm$ 0.018 ^a
T4	1.94 $\pm$ 0.023 ^c	5.86 $\pm$ 0.044 ^b	7.97 $\pm$ 0.014 ^b	11.07 $\pm$ 0.019 ^b	14.21 $\pm$ 0.007 ^b
LSD at 5%	0.048	0.426	0.071	0.051	0.108

Values are expressed as means $\pm$ standard errors of three replicates of two experiments. Data were taken after 6th of culture. Different superscript letters (a–f) indicate significance at $p \leq 0.05$ level according to LSD. T1 = 0.5 mg/l; T2 = 1.0 mg/l; T3 = 1.5 mg/l; and T4 = 2.0 mg/l. C, control, i.e. protoplast derived embryogenic tissue.

Table 2

Vincristine (VC) content ($\mu\text{g g}^{-1}$ dry weight) in protoplast derived embryos/tissues in *Catharanthus roseus*. Tissues were added with different treatments of YE.

Sample	Vincristine ($\mu\text{g g}^{-1}$ dry weight)				
	Induction	Proliferation	Maturation	Germination	Leaves (<i>in vitro</i>)
C	0.79 $\pm$ 0.058 ^{de}	1.79 $\pm$ 0.011 ^d	2.21 $\pm$ 0.047 ^e	2.33 $\pm$ 0.014 ^d	3.65 $\pm$ 0.022 ^e
T1	0.95 $\pm$ 0.018 ^c	2.08 $\pm$ 0.022 ^c	2.54 $\pm$ 0.018 ^d	2.94 $\pm$ 0.025 ^c	3.82 $\pm$ 0.011 ^d
T2	1.09 $\pm$ 0.027 ^b	2.46 $\pm$ 0.029 ^b	2.99 $\pm$ 0.018 ^c	3.27 $\pm$ 0.011 ^b	3.96 $\pm$ 0.022 ^c
T3	1.18 $\pm$ 0.011 ^a	2.68 $\pm$ 0.018 ^a	3.35 $\pm$ 0.016 ^a	3.46 $\pm$ 0.016 ^a	4.14 $\pm$ 0.015 ^a
T4	0.83 $\pm$ 0.025 ^d	2.33 $\pm$ 0.011 ^b	3.13 $\pm$ 0.015 ^b	3.35 $\pm$ 0.015 ^b	4.07 $\pm$ 0.011 ^b
LSD at 5%	0.068	0.048	0.058	0.281	0.040

Values are expressed as means $\pm$ standard errors of three replicates of two experiments. Data were taken after 6th of culture. Different superscript letters (a–f) indicate significance at $p \leq 0.05$ level according to LSD. T1 = 0.5 mg/l; T2 = 1.0 mg/l; T3 = 1.5 mg/l; and T4 = 2.0 mg/l. C, control, i.e. protoplast derived embryogenic tissue.

Table 3

Enzyme activities (EU mg^{-1} protein min^{-1}) at maturation stages of embryo; MS medium was added with 0.5 mg/l GA_3 .

Sample	CAT	APX	SOD	GR
NEC	1.02 $\pm$ 0.025 ^d	0.55 $\pm$ 0.018 ^d	2.87 $\pm$ 0.018 ^d	0.21 $\pm$ 0.014 ^d
C	1.46 $\pm$ 0.022 ^c	0.74 $\pm$ 0.011 ^c	4.27 $\pm$ 0.033 ^c	0.31 $\pm$ 0.011 ^c
T3	1.77 $\pm$ 0.015 ^b	1.01 $\pm$ 0.018 ^b	6.42 $\pm$ 0.048 ^b	0.67 $\pm$ 0.011 ^b
T4	1.85 $\pm$ 0.018 ^a	1.14 $\pm$ 0.022 ^a	7.05 $\pm$ 0.016 ^a	0.73 $\pm$ 0.019 ^a
LSD at 5%	0.041	0.033	0.080	0.031

Values are expressed as means $\pm$ standard errors of three replicates of two experiments. Data were taken after 6th of culture. Different superscript letters indicate significance at $p \leq 0.05$ level according to LSD. T3 = 1.5 mg/l and T4 = 2.0 mg/l. C, control, i.e. protoplast derived embryogenic tissue; NEC, normal embryogenic tissue.

Table 4

Enzyme activities (EU mg^{-1} protein min^{-1}) at germination stages of embryos; MS medium was added with 0.5 mg/l BA + 0.5 mg/l NAA.

Sample	CAT	APX	SOD	GR
NEC	1.67 $\pm$ 0.018 ^c	0.65 $\pm$ 0.015 ^d	3.20 $\pm$ 0.011 ^d	0.29 $\pm$ 0.018 ^d
C	1.99 $\pm$ 0.022 ^a	0.86 $\pm$ 0.021 ^c	5.05 $\pm$ 0.018 ^c	0.39 $\pm$ 0.011 ^c
T3	1.89 $\pm$ 0.018 ^b	1.18 $\pm$ 0.011 ^b	7.24 $\pm$ 0.029 ^b	0.68 $\pm$ 0.029 ^b
T4	2.03 $\pm$ 0.014 ^a	1.26 $\pm$ 0.022 ^a	7.47 $\pm$ 0.021 ^a	0.85 $\pm$ 0.014 ^a
LSD at 5%	0.052	0.045	0.057	0.035

Values are expressed as means $\pm$ standard errors of three replicates of two experiments. Data were taken after 6th of culture. Different superscript letters indicate significance at $p \leq 0.05$ level according to LSD. T3 = 1.5 mg/l and T4 = 2.0 mg/l. C, control, i.e. protoplast derived embryogenic tissue; NEC, normal embryogenic tissue.

Table 5

Enzyme activities (EU mg^{-1} protein min^{-1}) of *in vitro* harvested leaf tissues, cultivated on MS + 0.50 mg/BA + 0.50 mg/l NAA.

Sample	CAT	APX	SOD	GR
NEC	1.05 $\pm$ 0.029 ^d	1.04 $\pm$ 0.036 ^d	2.10 $\pm$ 0.029 ^d	0.19 $\pm$ 0.014 ^d
C	3.27 $\pm$ 0.018 ^c	1.57 $\pm$ 0.018 ^c	3.15 $\pm$ 0.018 ^c	0.29 $\pm$ 0.002 ^c
T3	6.02 $\pm$ 0.032 ^b	1.96 $\pm$ 0.015 ^b	6.06 $\pm$ 0.022 ^b	0.52 $\pm$ 0.001 ^b
T4	7.52 $\pm$ 0.835 ^a	2.09 $\pm$ 0.011 ^a	7.06 $\pm$ 0.011 ^a	0.62 $\pm$ 0.002 ^a
LSD at 5%	0.063	0.057	0.039	0.039

Values are expressed as means $\pm$ standard errors of three replicates of two experiments. Data were taken after 6th of culture. Different superscript letters indicate significance at $p \leq 0.05$ level according to LSD. T3 = 1.5 mg/l and T4 = 2.0 mg/l. C, control, i.e. protoplast derived embryogenic tissue; NEC, normal embryogenic tissue.

in maturing- and germinating embryos, respectively, at high YE (T4) treatment (Tables 3 and 4). The APX activity was also similarly more in maturing- (1.14 EU mg^{-1} protein min^{-1}) and germinating embryos (1.26 EU mg^{-1} protein min^{-1}). The SOD

activity followed the same trend (7.05 EU mg^{-1} protein min^{-1} and 7.47 EU mg^{-1} protein min^{-1} , respectively) and so was the GR activity (0.73 EU mg^{-1} protein min^{-1} and 0.85 EU mg^{-1} protein min^{-1} , respectively). The T4 harvested *in vitro* leaf tissue also had enriched

CAT, APX, SOD and GR activities, which yielded highest level of VB and VC (Table 5).

Discussion

The present study was carried out to investigate the effect of YE on VB and VC in protoplast derived tissues/plantlets in *C. roseus*. We noted enhanced yield of VB and VC in response to YE elicitation. There are several similar reports on YE-induced synthesis of triterpenoids and other metabolites in cultivated plant tissues such as in *Scutellaria baicalensis* (Yoon et al., 2000), *Panax ginseng* (Lu et al., 2001), *Centella asiatica* (Kim et al., 2007), *Angelica gigas* (Rhee et al., 2010) and *Pueraria candollei* (Korsangruang et al., 2010). It is known that the plants' response to pathogen is based on its ability to recognize signal molecules of foreign agent and these molecules or elicitors are primarily proteins and/or cell wall-derived oligosaccharides (Neurnberger, 1999; Mahalingam and Fedoroff, 2003). Elicitors are capable of evoking a variety of plant defense responses including the synthesis of reactive oxygen species (ROS), the hypersensitive response, the synthesis of phytoalexin, antimicrobial secondary compounds and other manifestations of defensive nature (Montesano et al., 2003). Several valuable bioactive compounds like azadirachtin, artemisinin and tanshinones accumulation were reported to be stimulated by YE elicitor (Putalun et al., 2007; Prakash and Srivastava, 2008; Zhao et al., 2010). The mechanism of elicitation was, however, varied in different plant groups and in most cases 'elicitor-receptor' complex was formed and a vast array of biochemical responses were manifested (Radman et al., 2003; Cai et al., 2012).

The activities of antioxidant enzymes such as APX, SOD and CAT are often used as marker in evaluating physiological, biochemical alterations of plants in response to stresses (biotic and abiotic) and in studies with plants' systemic acquired resistance system (Tanabe et al., 2008); and these enzyme activity increase at the onset and increasing levels of stress (Ren et al., 2002). In plant systems, the major ROS scavenging mechanisms include the activity of SOD, APX and CAT (Mittler, 2002). In *Euphorbia pekinensis*, increased CAT activity and synthesis of H_2O_2 were noted post elicitor treatment in cultivated tissues (Gao et al., 2011). The protoplast derived tissues had high enzymatic activity and the activity was even more on YE added conditions; this increase may be due to overproduction of ROS. In *Oryza sativa* cultivar, increased antioxidative enzyme activity scavenged cellular ROS successfully and prevented membrane injury by improving oxidative stress tolerance (Hassanein, 2000). Foyer and Noctor (2005) noted that the enzyme glutathione reductase (GR) – a key ascorbate-glutathione cycle enzyme, helped maintain a balance of GSH/GSSG ratio and protected cells from oxidative damage. Current proteomic and molecular analyses suggest that Salt Overly Sensitive 1 (SOS1) gene, genes encoding transcription factors, stress proteins, enzymes catalyzing secondary compounds genes are noted to be upregulated by stress or stress inducers (Pazmino et al., 2012; Ma et al., 2014).

Protoplast derived embryogenic tissue showed an enriched level of alkaloid, the yield of VB and VC was even more in YE amended conditions. The action of YE was earlier reported to be associated with activation of terpenoid indole alkaloids (TIA) biosynthetic genes *STR* and *TDC* encoding strictosidine synthase and tryptophan decarboxylase in biosynthesis of alkaloids (Pauw et al., 2004). A semi-synthetic method of chemical or enzymatic coupling of monomeric indole alkaloids vindoline and catharanthine in forming vinblastine was successfully made in previous studies (Goodbody et al., 1988; Kutney, 1990) in which peroxidase catalyzed coupling reaction step of synthesis (Smith et al., 2003). In this present study, we observed increased enzyme activity on application of YE that might trigger enhanced level of VB and VC

synthesis in culture. Abraham et al. (2011) studied YE effect in *Curcuma mangga* and higher antioxidant enzyme activity was noted to be due to enriched level of phenolic accumulation in tissues. The correlation between accumulated phenolic levels with radical scavenging was earlier noted in different berry species and thus, the antioxidant enzyme activities and the synthesis of secondary metabolite are considered to be invariably related (Amakura et al., 2000). In *Catharanthus*, there is enrichment of alkaloids, so the study is important as it is the only source of expensive, important chemotherapeutic drugs with anti-cancerous activity, which are present in a very trace (0.0005% DW) amount (Schmeller and Wink, 1998). The synthesis of rosmarinic acid (RA) and phenolic compounds in *Salvia miltiorrhiza* hairy roots was similarly stimulated by YE and Ag^+ , an abiotic elicitor (Qiong et al., 2006). The biotic and abiotic elicitor induced (YE and MJ) RA accumulation was observed in other investigated plants such as *Orthosiphon aristatus* (Mizukam et al., 1992) and *Coleus blumei* (Szabo et al., 1999). In *L. erythrorhizon*, the addition of YE in suspension rapidly and transiently enhanced RA accumulation (Ogata et al., 2004). Sanchez-Sampedro et al. (2005) reported about three-fold production of silymarin following YE treatment in *Silybum marianum*. The amendment of YE at 0.5 g/l was found to be an optimum treatment for elicitation in *Gymnema sylvestre* in which over 5.25 folds of gymnemic acid production was noted within 20 days of culture (Veerashree et al., 2012). Over 113.3% increase in total phenolic content over control was reported in YE treated cells in *Astragalus chrysochlorus* (Ozgur and Sule, 2009). In our study, enriched VB and VC alkaloid yield was noticed on YE treatments and 1.5 g/l was observed to be the optimum dose. YE contains several components like chitin, N-acetylglucosamine oligomers, β -glucan, glycopeptides and ergosterol (Boller, 1995) but the specific fraction triggers the synthesis of alkaloids is still not elucidated clearly. The peptide and polysaccharide moieties present in YE have been suggested to be the molecules of stimulating nature (Menke et al., 1999; Zhao et al., 2011). The protoplasts and the protoplast derived tissues showed stress as this tissue underwent adverse cellular conditions, caused by wall degrading enzymes, PGRs and influx/efflux of required ions. The addition of YE in medium may raise stress level further (as was evidenced from increasing levels of stress marker enzymes) that may help in synthesizing enhanced level of VB and VC in cultured tissues. This is the first ever study of systematic monitoring of VB and VC in protoplast regenerated tissues in *Catharanthus* under YE influence. This study may complement to other efforts aiming to enrich VB and VC yield in culture.

Conclusion

This is the first report on the effect of YE on VB and VC yield in protoplast regenerated plants in *C. roseus*. The observation suggests that YE efficiently enhanced alkaloid yield however, the accumulation was treatment-specific. The identification of signalling component of YE in biosynthesis could be a very effective approach for large scale augmentation of alkaloid yield of pharmaceutical importance.

Authorship

The first author conceived the idea and performed most of the experiments and the second author edited the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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