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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.

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 phytocompound 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 μmol m^{-2} 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 $CaCl_2 \cdot 2H_2O$ 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 Darshanie 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 × 10 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 × 10 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 μg 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 × *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) mg^{-1} 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 EU mg^{-1} protein min^{-1}. One unit of enzyme determines the amount necessary to decompose 1 μmol of H_2O_2 per min. CAT activity was calculated by using the co-efficient of absorbance 0.036 mM^{-1} 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) polyvinylpyrollidone (PVP), 1.0% (v/v) Triton X-100, and centrifuged at 8608.6 × *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 EU mg^{-1} 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 mM^{-1} cm^{-1}. The activity of APX was expressed in EU mg^{-1} protein min^{-1}. One unit of enzyme is the amount necessary to decompose 1 μm 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 mM^{-1} cm^{-1}. The GR activity was determined through glutathione-dependent oxidation of NADPH at 340 nm. The activity of GR was expressed in EU mg^{-1} protein min^{-1}. One unit of enzyme determines the amount necessary to decompose 1 μmol 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 ± 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 (μg 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 ($Rf = 0.28$) and VC ($Rf = 0.46$) 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 μg g^{-1} dry weight) in germinating embryos and the yield was improved (11.44 μg 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 μg 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 μg g^{-1} dry weight) and VC (4.14 μg 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 EU mg^{-1} protein min^{-1} and 2.03 EU mg^{-1} protein min^{-1}

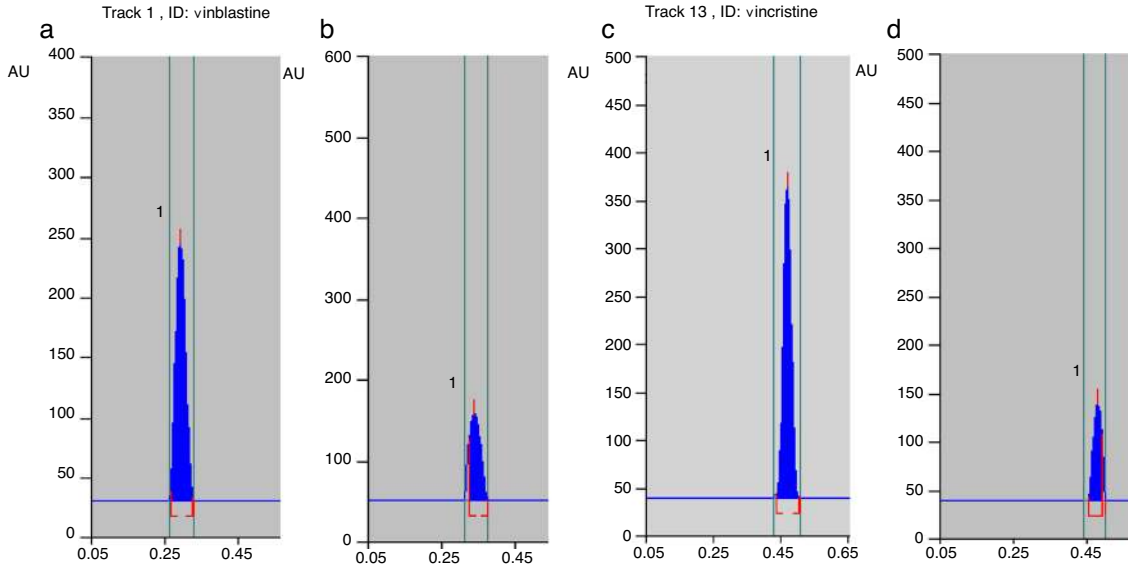

Fig. 1. (a) Standard peak of vinblastine, (b) peak showing the maximum yield of vinblastine in YE treated leaves at T3, (c) standard curve of vincristine and (d) peak showing the maximum yield of vincristine in YE treated leaves at T3 (*x*-axis presents peak area and *y*-axis presents R*f*).

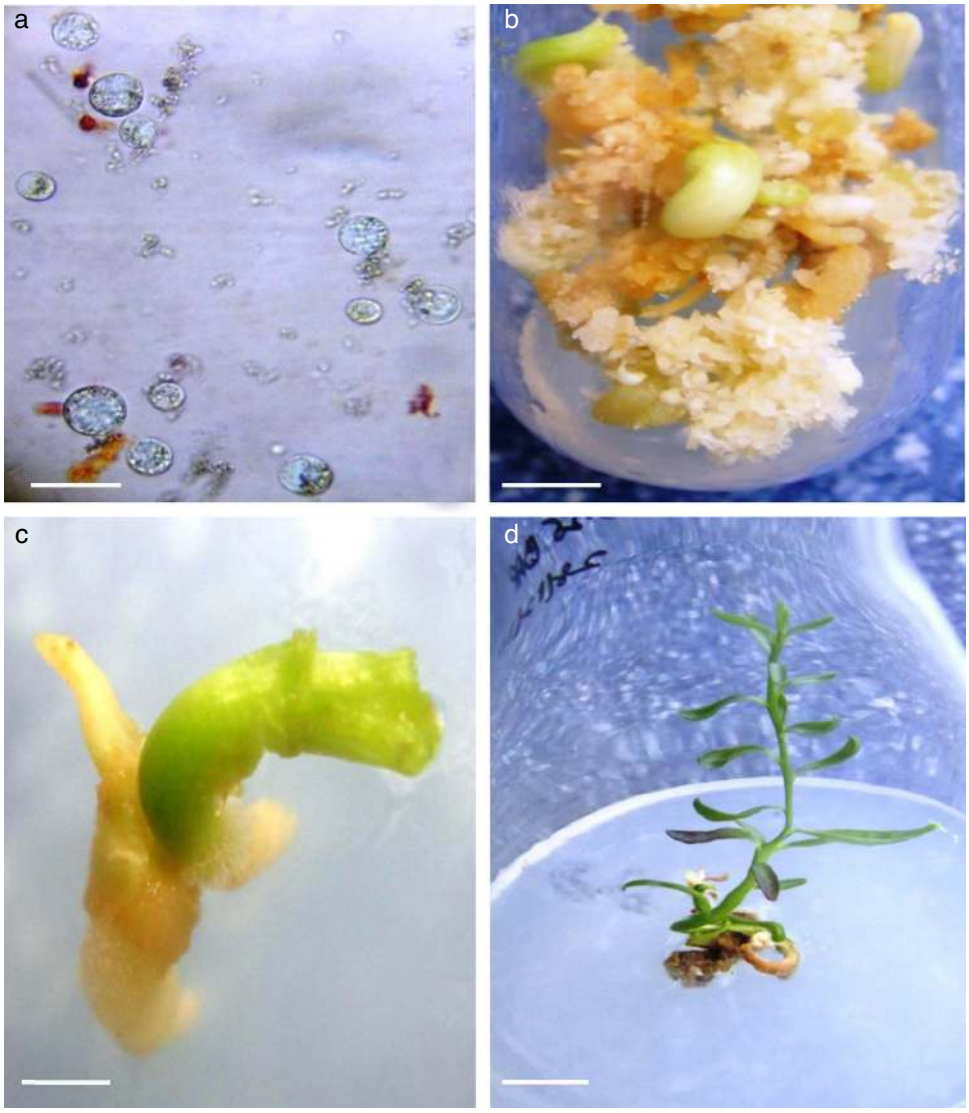

Fig. 2. (a) Isolated protoplasts; (b) development of embryogenic callus from protoplasts; (c) individual embryo developed from PDEC treated with T3 treatment of YE and (d) regenerated plantlet from protoplast derived embryo, grown in MS medium containing YE (T3). (a and b) bar = 200 μm, (c) bar = 2 mm,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

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

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

Values are expressed as means ± 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 (μg g^{-1} dry weight) in protoplast derived embryos/tissues in *Catharanthus roseus*. Tissues were added with different treatments of YE.

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

Values are expressed as means ± 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 ± 0.025^{d}	0.55 ± 0.018^{d}	2.87 ± 0.018^{d}	0.21 ± 0.014^{d}
C	1.46 ± 0.022^{c}	0.74 ± 0.011^{c}	4.27 ± 0.033^{c}	0.31 ± 0.011^{c}
T3	1.77 ± 0.015^{b}	1.01 ± 0.018^{b}	6.42 ± 0.048^{b}	0.67 ± 0.011^{b}
T4	1.85 ± 0.018^{a}	1.14 ± 0.022^{a}	7.05 ± 0.016^{a}	0.73 ± 0.019^{a}
LSD at 5%	0.041	0.033	0.080	0.031

Values are expressed as means ± 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 ± 0.018^{c}	0.65 ± 0.015^{d}	3.20 ± 0.011^{d}	0.29 ± 0.018^{d}
C	1.99 ± 0.022^{a}	0.86 ± 0.021^{c}	5.05 ± 0.018^{c}	0.39 ± 0.011^{c}
T3	1.89 ± 0.018^{b}	1.18 ± 0.011^{b}	7.24 ± 0.029^{b}	0.68 ± 0.029^{b}
T4	2.03 ± 0.014^{a}	1.26 ± 0.022^{a}	7.47 ± 0.021^{a}	0.85 ± 0.014^{a}
LSD at 5%	0.052	0.045	0.057	0.035

Values are expressed as means ± 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 ± 0.029^{d}	1.04 ± 0.036^{d}	2.10 ± 0.029^{d}	0.19 ± 0.014^{d}
C	3.27 ± 0.018^{c}	1.57 ± 0.018^{c}	3.15 ± 0.018^{c}	0.29 ± 0.002^{c}
T3	6.02 ± 0.032^{b}	1.96 ± 0.015^{b}	6.06 ± 0.022^{b}	0.52 ± 0.001^{b}
T4	7.52 ± 0.835^{a}	2.09 ± 0.011^{a}	7.06 ± 0.011^{a}	0.62 ± 0.002^{a}
LSD at 5%	0.063	0.057	0.039	0.039

Values are expressed as means ± 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 maturating- (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 rosmaric 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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References

Abraham, F., Bhatt, A., Keng, C.L., Indrayanto, G., Sulaiman, S.F., 2011. Effect of yeast extract and chitosan on shoot proliferation, morphology and antioxidant activity of *Curcuma mangga in vitro* plantlets. Afr. J. Biotechnol. 10, 7787–7795.

Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol. 105, 121–126.

Amakura, Y., Umino, Y., Tsuji, S., Tongai, Y., 2000. Influence of jam processing on the radical scavenging activity and phenolic content in berries. J. Agric. Food Chem. 48, 6292–6297.

Boller, T., 1995. Chemoperception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 189–214.

Cai, Z., Kastell, A., Mewis, I., Knorr, D., Smetanska, I., 2012. Polysaccharide elicitors enhance anthocyanin and phenolic acid accumulation in cell suspension cultures of *Vitis vinifera*. Plant Cell Tissue Org. Cult. 108, 401–409.

Darshanie, P., Rajapakse, Mendis, M.H., Jayawardene, S.D.G., 1991. Establishment of callus culture in Bg-94-1: a commercially cultivated rice in Sri Lanka. J. Nat. Sci. Coun. Sri Lanka 19, 163–170.

De Luca, V., St Pierre, B., 2000. The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci. 5, 168–173.

Dhindsa, R.H., Plumb-Dhindsa, P., Thorpe, T.A., 1981. Leaf senescence correlated with increased level of membrane permeability, lipid peroxidation and decreased level of SOD and CAT. J. Exp. Bot. 32, 93–101.

Dipti, T., Mujib, A., Maqsood, M., Ali, M., Zafar, N., 2016. *Aspergillus flavus* fungus elicitation improves vincristine and vinblastine yield by augmenting callus biomass growth in *Catharanthus roseus*. Plant Cell Tissue Org. Cult. 126, 291–303.

Elmaghrabi, A.M., Ochatt, S., Rogers, H.J., Francis, D., 2013. Enhanced tolerance to salinity following cellular acclimation to increasing NaCl levels in *Medicago truncatula*. Plant Cell Tissue Org. Cult. 114, 61–70.

Feher, A., 2015. Somatic embryogenesis – stress-induced remodeling of plant cell fate. Biochim. Biophys. Acta 1849, 385–402.

Foyer, C., Noctor, G., 2005. Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28, 1056–1071.

Foyer, C.H., Halliwell, B., 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133, 21–25.

Gao, F., Ying-hui, Y., Chuan-chao, D., 2011. Effects of endophytic fungal elicitor on two kinds of terpenoids production and physiological indexes in *Euphorbia pekinensis* suspension cells. J. Med. Plants Res. 5, 4418–4425.

Goodbody, A.E., Endo, T., Vukovic, J., Kutney, J.P., Choi, L.S.L., Misawa, M., 1988. Enzymic coupling of catharanthine and vindoline to form 3α,4α-anhydrovinblastine by horseradish peroxidase. Planta Med. 54, 136–140.

Hassanein, A.A., 2000. Physiological responses induced by shock and gradual salinization in rice (*Oryza sativa* L.) seedlings and the possible roles played by glutathione treatment. Acta. Bot. Hung. 42, 139–159.

Ibrahim, I.A., Abd El Kawi, M., Nower, A., Abdel Motaal, A., Abd El Aal, A., 2009. Alkaloid production and organogenesis from callus of *Hyoscyamus muticus* L. in vitro. J. App. Sci. Res. 5, 82–92.

Junaid, A., Bhat, M.A., Mujib, A., Sharma, M.P., 2006. Somatic embryo proliferation, maturation and germination in *Catharanthus roseus*. Plant Cell Tissue Org. Cult. 84, 325–332.

Kim, O.T., Bang, K.H., Shin, Y.S., Lee, M.J., Jung, S.J., Hyun, D.Y., Kim, Y.C., Seong, N.S., Cha, S.W., Hwang, B., 2007. Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. Plant Cell Rep. 26, 1941–1949.

Korsangruang, S., Soonthornchareonnon, N., Chintapakorn, Y., Saralamp, P., Prathanturarug, S., 2010. Effects of abiotic and biotic elicitors on growth and isoflavonoid accumulation in *Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica* cell suspension cultures. Plant Cell Tissue Org. Cult. 103, 333–342.

Kuboyama, T., Yokoshima, S., Tokuyama, H., Fukuyama, T., 2004. Stereo controlled total synthesis of (+) vincristine. Proc. Natl. Acad. Sci. U.S.A. 101, 11966–11970.

Kutney, J.P., 1990. Biosynthesis and synthesis of indole alkaloids in plant cell cultures: a personal overview. Nat. Prod. Rep. 7, 85–103.

Kutney, J.P., Choi, L.S.L., Nakano, J., Tsukamoto, H., Boulet, C.A., McHugh, M., 1991. Process of synthesis of vinblastine and vincristine. US Patent 5047528.

Lu, M.B., Wong, H.L., Teng, W.L., 2001. Effects of elicitation on the production of saponin in cell culture of *Panax ginseng*. Plant Cell Rep. 20, 674–677.

Ma, Q., Ding, Y., Chang, J., Sun, X., Zhang, L., Wei, Q., Cheng, Y., Chen, L., Xu, J., Deng, X., 2014. Comprehensive insights on how 2,4-dichlorophenoxyacetic acid retards senescence in post-harvest citrus fruits using transcriptomic and proteomic approaches. J. Exp. Bot. 65, 61–74.

Mahalingam, R., Fedoroff, N., 2003. Stress response, cell death and signalling: the many faces of reactive oxygen species. Physiol. Plant 119, 56–68.

Menke, F.L.H., Parchmann, S., Mueller, M.J., Kijne, J.W., Memelink, J., 1999. Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol. 119, 1289–1296.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.

Mizukam, H.T., Ogawa, H., Ohashi, B., Ellis, E., 1992. Induction of rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures by yeast extract. Plant Cell Rep. 11, 480–483.

Montesano, M., Brander, G., Tapio, P.E., 2003. Pathogen derived elicitors: searching for receptors in plants. Mol. Plant Pathol. 4, 73–79.

Moreno, P.R.H., Van der Heijden, R., Verpoorte, R., 1995. Cell and tissue cultures of *Catharanthus roseus*: a literature survey II. Updating from 1998 to 1993. Plant Cell Tissue Org. Cult. 42, 1–25.

Moreno, P.R.H., (Ph.D thesis) 1995. Influence of stress factors on the secondary metabolism in suspension cultured *Catharanthus roseus* cells. Leiden University, Leiden.

Mujib, A., Ilah, A., Aslam, J., Fatima, S., Siddiqui, Z.H., Maqsood, M., 2012. *Catharanthus roseus* alkaloids: application of biotechnology for improving yield. Plant Growth Reg. 68, 111–127.

Mukherjee, A.K., Basu, S., Sarkar, N., Ghosh, A.C., 2001. Advances in cancer therapy with plant based natural products. Curr. Med. Chem. 8, 1467–1486.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant 15, 473–497.

Murthy, H.N., Lee, E.J., Paek, K.Y., 2014. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell Tissue Org. Cult. 118, 1–16.

Mustafa, N.R., Kim, H.K., Choi, Y.H., Erkelens, C., Lefeber, A.W., Spijksma, G., Van der Heijden, R., Verpoorte, R., 2009. Biosynthesis of salicylic acid in fungus elicited *Catharanthus roseus* cells. Phytochemistry 70, 532–539.

Naz, R., Anis, M., Aref, M.I., 2015. Management of cytokinin-auxin interactions for *in vitro* shoot proliferation of *Althaea officinalis* L.: a valuable medicinal plant. Rend. Fis. Acc. Lincei. 26, 323–334.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.

Neurnberger, T., 1999. Signal perception in plant pathogen defense. Cell. Mol. Life Sci. 55, 167–182.

Ogata, A., Tsuruga, A., Matsunob, M., Mizukami, H., 2004. Elicitor-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures: activities of rosmarinic acid synthase and the final two cytochrome P450-catalyzed hydroxylations. Plant Biotechnol. 2, 393–396.

Ozgur, C., Sule, A., 2009. Defensive and secondary metabolism in *Astragalus chrysochlorus* cell cultures in response to yeast extract stressor. J. Environ. Biol. 30, 51–55.

Pauw, B., Duijn, B., Kijne, J.W., Memelink, J., 2004. Activation of the oxidative burst by yeast elicitor in *Catharanthus roseus* cells occurs independently of the activation of genes involved in alkaloid biosynthesis. Plant Mol. Biol. 55, 797–805.

Pazmino, D.M., Romero-Puertas, M.C., Sandalio, L.M., 2012. Insights into the differential response of wound and leaves to herbicides 2,4-dichlorophenooxyacetic acid in pea plants: role of reactive oxygen species. Plant Signal. Behav. 7, 425–427.

Prakash, G., Srivastava, A.K., 2008. Statistical elicitor optimization studies for the enhancement of azadirachtin production in bioreactor *Azadirachta indica* cell cultivation. Biochem. Eng. J. 40, 218–226.

Putalun, W., Luealon, W., De-Eknamkul, W., Tanaka, H., Shoyama, Y., 2007. Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua*. Biotechnol. Lett. 29, 1143–1146.

Qiong, Y., Ming, S., Janet, N., Jian, Y.W., 2006. Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Salvia miltiorrhiza* hairy roots. Plant Sci. 170, 853–858.

Radman, R., Saez, T., Bucke, C., Keshavarz, T., 2003. Elicitation of plants and microbial cell systems. Biotechnol. Appl. Biochem. 37, 91–102.

Ren, A.Z., Gao, Y.B., Liu, S., 2002. Response of some protective enzymes in *Brassica chinensis* seedling to Pb^{++}, Cd^{+} and Cr^{+++} stress. Chin. J. Appl. Ecol. 13, 510–512.

Rhee, H.S., Cho, H.Y., Son, S.Y., Yoon, S.Y.H., Park, J.M., 2010. Enhanced accumulation of decursin and decursinol angelate in root cultures and intact roots of *Angelica gigas* Nakai following elicitation. Plant Cell Tissue Org. Cult. 101, 295–302.

Samar, F., Mujib, A., Dipti, T., 2015. NaCl amendment improves vinblastine and vincristine synthesis in *Catharanthus roseus*: a case of stress signalling as evidenced by antioxidant enzymes activities. Plant Cell Tissue Org. Cult. 121, 445–458.

Sanchez-Sampedro, M.A., Fernandez-Tarrago, J., Corchete, P., 2005. Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn. J. Biotechnol. 119, 60–69.

Schmeller, T., Wink, M., 1998. Utilization of alkaloids in modern medicine. In: Roberts, M.F., Wink, M. (Eds.), Alkaloids, Biochemistry, Ecology and Medicinal Applications. Plenum Press, New York, pp. 435–459.

Smith, J.I., Amouzou, E., Yamaguchi, A., 2003. Peroxidase from bioreactor cultivated *Catharanthus roseus* cell cultures mediates biosynthesis of α-3′,4′-anhydrovinblastine. Biotechnol. Appl. Biochem. 10, 568–575.

Szabo, E., Thelen, A., Petersen, M., 1999. Fungal elicitor preparations and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of *Coleus blumei*. Plant Cell Rep. 18, 485–489.

Tanabe, S., Hayashi, N., Nishizawa, Y., Yamane, H., Shibuya, N., Minami, E., 2008. Elicitor and calatalse activity of conidia suspensions of various strains of *Magnaporthe grisea* in suspension-cultured cells of rice. Biosci. Biotechnol. Biochem. 72, 889–892.

Teixeira da Silva, J.A., Dobranszki, J., 2016. Tissue culture of *Muscari* species: present achievements and future perspectives. Rend. Fis. Acc. Lincei. 27, 427–441.

Valluri, J.V., 2009. Bioreactor production of secondary metabolites from cell cultures of periwinkle and sandalwood. Methods Mol. Biol. 54, 325–335.
Van Der Heijden, R., Jacobs, D.I., Snoeijer, W., Hallared, D., Verpoorte, R., 2004. The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr. Med. Chem. 11, 607–628.
Veerashree, V., Anuradha, C.M., Kumar, V., 2012. Elicitor-enhanced production of gymnemic acid in cell suspension cultures of *Gymnema sylvestre* R. Br. Plant Cell Tissue Org. Cult. 108, 27–35.
Verpoorte, R., Contin, A., Memelink, J., 2002. Biotechnology for the production of plant secondary metabolites. Phytochem. Rev. 1, 13–25.
Yoon, J.H., Kim, K.H., Ma, J.C., Huh, H., 2000. Induced accumulation of triterpenoids in *Scutellaria baicalensis* suspension cultures using a yeast elicitor. Biotechnol. Lett. 22, 1071–1075.
Zarate, R., Verpoorte, R., 2007. Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Don. Phytochem. Rev. 6, 475–491.
Zhao, J., Zhou, L., Wu, J., 2010. Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. Appl. Microbiol. Biotechnol. 87, 137–144.
Zhao, J., Zheng, B., Li, Y., Shan, T., Mou, Y., Lu, S., Li, P., Zhou, L., 2011. Enhancement of Diepoxin ζ production by yeast extract and its fractions in liquid culture of Berkleasmium-like endophytic fungus Dzf12 from *Dioscorea zingiberensis*. Molecules 16, 847–856.
Zubek, S., Mielcarek, S., Turnau, K., 2012. Hypericin and pseudohypericin concentrations of a valuable medicinal plant *Hypericum perforatum* L. are enhanced by arbuscular mycorrhizal fungi. Mycorrhiza 22, 149–156.