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ORIGINAL ARTICLE

Tryptophan over-producing cell suspensions of *Catharanthus roseus* (L) G. Don and their up-scaling in stirred tank bioreactor: detection of a phenolic compound with antioxidant potential

Priyanka Verma · Ajay K. Mathur · Nusrat Masood · Suaib Luqman · Karuna Shanker

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Abstract Five cell suspension lines of *Catharanthus roseus* resistant to 5-methyl tryptophan (5-MT; an analogue of tryptophan) were selected and characterized for growth, free tryptophan content and terpenoid indole alkaloid accumulation. These lines showed differential tolerance to analogue-induced growth inhibition by 30 to 70 mg/l 5-MT supplementation (LD_{50}=7–15 mg/l). Lines P40, D40, N30, D50 and P70 recorded growth indices (i.e. percent increment over the initial inoculum weight) of 840.9, 765.0, 643.9, 585.7 and 356.5 in the absence and, 656.7, 573.9, 705.8, 489.0 and 236.0 in the presence of 5-MT after 40 days of culture, respectively. A corresponding increment in the free tryptophan level ranging from 46.7 to 160.0 μg/g dry weight in the absence and 168.0 to 468.0 μg/g dry weight in the presence was noted in the variant lines. Higher tryptophan accumulation of 368.0 and 468.0 g/g dry weight in lines N30 and P40 in 5-MT presence also resulted in higher alkaloid accumulation (0.65 to 0.90 % dry weight) in them. High-performance liquid chromatography (HPLC) analysis of the crude alkaloid extracts of the selected lines did not show the presence of any pharmaceutically important monomeric or dimeric alkaloids except catharanthine in traces in the N30 line that was also unique in terms of a chlorophyllous green phenotype. The N30 line under optimized up-scaling conditions in a 7-l stirred tank bioreactor using Murashige and Skoog medium containing 2 mg/l α-naphthalene acetic acid and 0.2 mg/l kinetin attained 18-folds biomass accumulation within 8 weeks. Interestingly, the cell biomass yield was enhanced to 30-folds if 30 mg/l 5-MT was added in the bioreactor vessel one week prior to harvest. Crude alkaloid extract of the cells grown in shake flask and this bioreactor batch also showed the formation of yellow-coloured crystals which upon ^{1}HNMR and ESI-MS analysis indicated a phenolic identity. This crude alkaloid extract of bioreactor-harvested cells containing this compound at 50 μg/ml concentration registered 65.21, 17.75, 97.0, 100 % more total antioxidant capacity, reducing power, total phenolic content, and ferric-reducing antioxidant power, respectively, when compared with that of extracts of cells grown in shake flask cultures. The latter, however, showed 57.47 % better radical scavenging activity (DPPH) than the bioreactor-harvested cells.

Keywords *Catharanthus roseus* · 5-Methyl tryptophan tolerance · Bioreactor up-scaling · Antioxidant · Phenolic

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

A. K. Mathur
e-mail: ak.mathur@cimap.res.in

N. Masood · S. Luqman
Molecular Bio-prospection Department, Central Institute of Medicinal and Aromatic Plants (CIMAP), Council of Scientific and Industrial Research, PO CIMAP,
Lucknow 226015, India

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

Introduction

Catharanthus roseus (L.) G. Don is an important medicinal herb, primarily valued for its two anti-neoplastic bisindole alkaloids—vincristine and vinblastine and two anti-hypertensive monomeric alkaloids ajmalicine and serpentine. Agri-based production of these drug molecules for pharma industry is highly expensive because of their extremely low *in planta* accumulation (Verma et al. 2012). Biotechnological interventions to improve their production in plant or in cell/tissue cultures of *C. roseus* are, therefore, being rigorously explored (Pietrosuik et al. 2007; Zarate and Verpoorte 2007; Zhao and Verpoorte 2007; Verma et al. 2011, 2012). Commercial success in this direction has still been elusive because of several technological gaps such as non-availability of cell/tissue types with ideal physiological, biochemical and genetic backgrounds to push the metabolic flux towards the synthesis of target molecules and limited efforts to optimize the design and performance of existing bioreactors to suit industrial up-scaling of *C. roseus* cells/tissues (ten Hoopen et al. 1994; Zhao and Verpoorte 2007; Facchini and De Luca 2008). Current experimental strategies to engineer terpenoid indole alkaloids (TIAs) pathway in *C. roseus* are, therefore, revolving around the generation of cell and tissue types characterized by sufficient precursor availability, over-expression of limiting pathway enzymes, activation of regulatory transcription factors, silencing of competitive pathways to flux the pathway intermediates in desired direction and optimization of growth parameters for bioreactor based cultivation (Canel et al. 1998; Zhao et al. 2001; Hughes et al. 2004; Seth and Mathur 2005; Zarate and Verpoorte 2007; Zhao and Verpoorte 2007; Murata et al. 2008; van der Fits and Memelink 2000). Based on the knowledge so far gathered on spatial and temporal complexities associated with TIAs pathway in *C. roseus*, the involvement of at least 30 coordinately regulated enzymatic steps occurring in four different cell types (epidermis, internal phloem-associated parenchyma, laticifers and idioblast) and five intracellular compartments (chloroplast, vacuole, nucleus, endoplasmic reticulum and cytosol) has been implicated (van der Heijden et al. 2004; Facchini and De Luca 2008; Guirimand et al. 2010; Verma et al. 2012). Since biosynthesis of all TIAs proceeds from a common precursor molecule—strictosidine—which is a condensation product of tryptophan-derived indole ring donor tryptamine and a terpenoid moiety donor secologanin, limited availability of these precursor molecules from the primary shikimate and secoiridoid pools has often been documented as the most serious problem associated with low TIAs productivity in *C. roseus* (Goddijn et al. 1995; Canel et al. 1998; Whitmer et al. 2002; Seth and Mathur 2005). Since tryptamine is a decarboxylated product of amino acid tryptophan bulk of which is also required for auxin synthesis, efforts in these studies were made to over-express the enzyme tryptophan decarboxylase coded by tryptophan decarboxylase (*TDC*) gene to push its flux towards TIAs synthesis. Tryptophan otherwise is the least abundant amino acid in a plant cell and its concentration is strongly regulated by feedback inhibition of the enzyme anthranilate synthase (Radwanski and Last 1995). To enhance the level of free tryptophan in the cellular metabolic pool of the cells, the strategy to select 5-methyltryptophan (5-MT)-resistant mutants has been followed because such mutants are known to acquire resistance to this anti-metabolite by over-producing tryptophan via coding for a new form of anthranilate synthase that is less sensitive to feedback inhibition (Li and Last 1996; Cho et al. 2000; Tozawa et al. 2001; Hughes et al. 2004; Seth and Mathur 2005). Recent studies in *C. roseus* have also suggested that besides TIAs the plant is also a rich source of a wide spectrum of phenolic compounds such as 2, 3-dihydroxybenzoic acid, flavonoids, anthocyanins and cinnamic acid derivatives (Mustafa and Verpoorte 2007). These phenolics are derived from the amino acid phenylalanine and hence also compete with the indole alkaloid biosynthesis for common precursor chorismate. Majority of these phenolics are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups and are known for their antioxidant activity due to their redox properties (Zheng and Wang 2001; Ndhlala et al. 2010). We report here the selection and characterization of 5-methyl tryptophan resistant cell lines of *C. roseus* and recovery of a phenolic compound with strong antioxidant potential from them under shake flask and bioreactor based cultivation.

Material and methods

Establishment of cell suspension in shake flask cultures

5-Methyltryptophan (5-MT)-resistant callus lines were selected in three cultivars of *C. roseus* (*cv.* Nirmal, Prabal and Dhawal; CIMAP National Gene Bank Accession nos. 0865, 0862 and 0859, respectively) according to the procedure described earlier from our laboratory (Seth and Mathur 2005). The selected lines were stably maintained and multiplied in the presence of 5-MT for 20 subculture cycles on a Murashige and Skoog (1962) medium supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l 6-benzylaminopurine (BAP). Cell suspensions of five selected lines (Table 1) were initiated by transferring 5 g of callus into 50 ml of a MS liquid medium consisting of 2 mg/l α- naphthalene acetic acid (NAA) and 0.2 mg/l kinetin (Kn). Cultures were incubated on a rotary shaker (120 rpm) at 24±2 °C under 1,000-lux intensity illumination. Repeated sieving and frequent sub culturing every 15th day resulted in establishment of fine suspensions within 65–

Table 1 Characteristics of five selected 5-MT-tolerant cell lines used in the present study

Line no.	Parentage	Morphology of suspension	Threshold 5-MT tolerance limit (mg/l)[a] (approximately)	
			Callus	Cell suspension
NW	Nirmal (wild type)	Pale white, fine	18 (LD_{50}=10–12)	12 (LD_{50}=8–10)
N30	Nirmal	Chlorophyllous, light green, fine	35	30
DW	Dhawal (wild type)	Creamish, fine	22(LD_{50}=12–15)	18(LD_{50}=12–15)
D40	Dhawal	Creamish, fine	40	40
D50	Dhawal	Creamish, fine	50	50
PW	Prabal (wild type)	Pale white, coarse	12 (LD_{50}=7–10)	10(LD_{50}=7–12)
P40	Prabal	Pale white, coarse	40	40
P70	Prabal	Pale white, clumpy	70	65

[a] Threshold tolerance limit is defined as 5-MT concentration in which the survival and growth of the selected line is either comparable, better or at least >50 % of the growth of the corresponding wild-type line grown on 5-MT free control medium

70 days. The growth kinetics of these lines in shake flask cultures was studied at 20th and 40th day both in the presence and the absence of the analogue. Growth index (GI) of cultured cells was measured as percent increment over the initial inoculum weight. All growth assays were made with three replicates per treatment and experiment was repeated four times.

Bioreactor up scaling

The suspension culture of N30 line was up-scaled in a 7-l stirred tank bioreactor (Model ADI-1010; Applikon Biotechnology Holland). Seven independent batches were run under different sets of conditions. Variables examined and optimized for up-scaling included the type of impeller (marine vs. rushton blade), batch cycle duration (6–20 weeks), inoculum density (30–40 g) and medium volume (2–3 l). The cultures were aerated through a sintered steel sparger at an air flow rate of 2 l per min equivalent to 20 % dissolved oxygen (DO) level. Rotation speed was maintained at 100–120 rpm and the incubation was carried out at 25±3 °C under high illumination (3,000 lux) provided by a specially fabricated LCD light source kept around the glass vessel (Fig. 1a). Biomass accumulation was monitored by measuring cell pack volume, medium consumed and fresh and dry weight determinations. Effect of 5-MT on the growth and TIA yield in bioreactor was studied by adding 30 mg/l of analogue 1 week prior to harvesting. All process parameters during bioreactor cultivation were monitored and recorded using Bioexpert Ferm Software.

Chemical analysis

Tryptophan estimation in the free amino acid pool was done by using a modified procedure of Bieleski and Turner (1966) for sample preparation and Dalby and Tsai (1975) for colorimetric estimation. For estimating the total TIAs content in cultured cells, 1 g lyophilized dried cells were extracted with HPLC grade methanol (3×30 ml; 12 h each) at room temperature. The MeOH extracts were pooled and dried in vacuum to 10 ml, mixed with 10 ml dH_2O acidified using 10 ml of 3 % HCl and washed thrice with *n*-hexane (3×30 ml). The aqueous portion was extracted with chloroform (3×30 ml), washed with dH_2O, dried over anhydrous sodium sulphate, concentrated in vacuum and weighed. HPLC analysis of the crude alkaloid extract was performed using a modular HPLC apparatus (Waters Corp., Milford, MA, USA) equipped with a 600E multi-solvent delivery system and a 2,996 photodiode array detector. An RP-18e reversed-phase chromolith Performance HPLC column (100×4.6 mm i.d.) was employed in all the analysis. An optimum mobile phase (flow rate of 1.2 ml/ min) composition was achieved by using 21:79 (*v*/*v*) acetonitrile: 0.1 M phosphate buffer containing 0.5 % glacial acetic acid (Gupta et al. 2005). Data obtained were processed using Empower Pro (Waters Corp.) chromatographic software.

For TLC analysis of the total alkaloid extract, silica plates (MERCK, $60F_{254}$ aluminium/ glass sheets; 20×20 cm) were activated at 55 °C for 30 min prior to use. Solvent system (10 % methanol in chloroform with two to three drops of ammonia) was freshly prepared. The prominent spot of compound other than that of known reference standards was isolated by means of preparative TLC. The tentative authentication of the compound was performed through NMR spectroscopy (^{1}HNMR) and mass analysis (ESI-MS). For NMR analysis, 20 mg of compound was dissolved and evaporated two to three times in CCl_4 to remove the solvent protons and finally dissolved in $CDCl_3$. Similarly, 1–2 mg of isolated compound (dried) was used in the mass

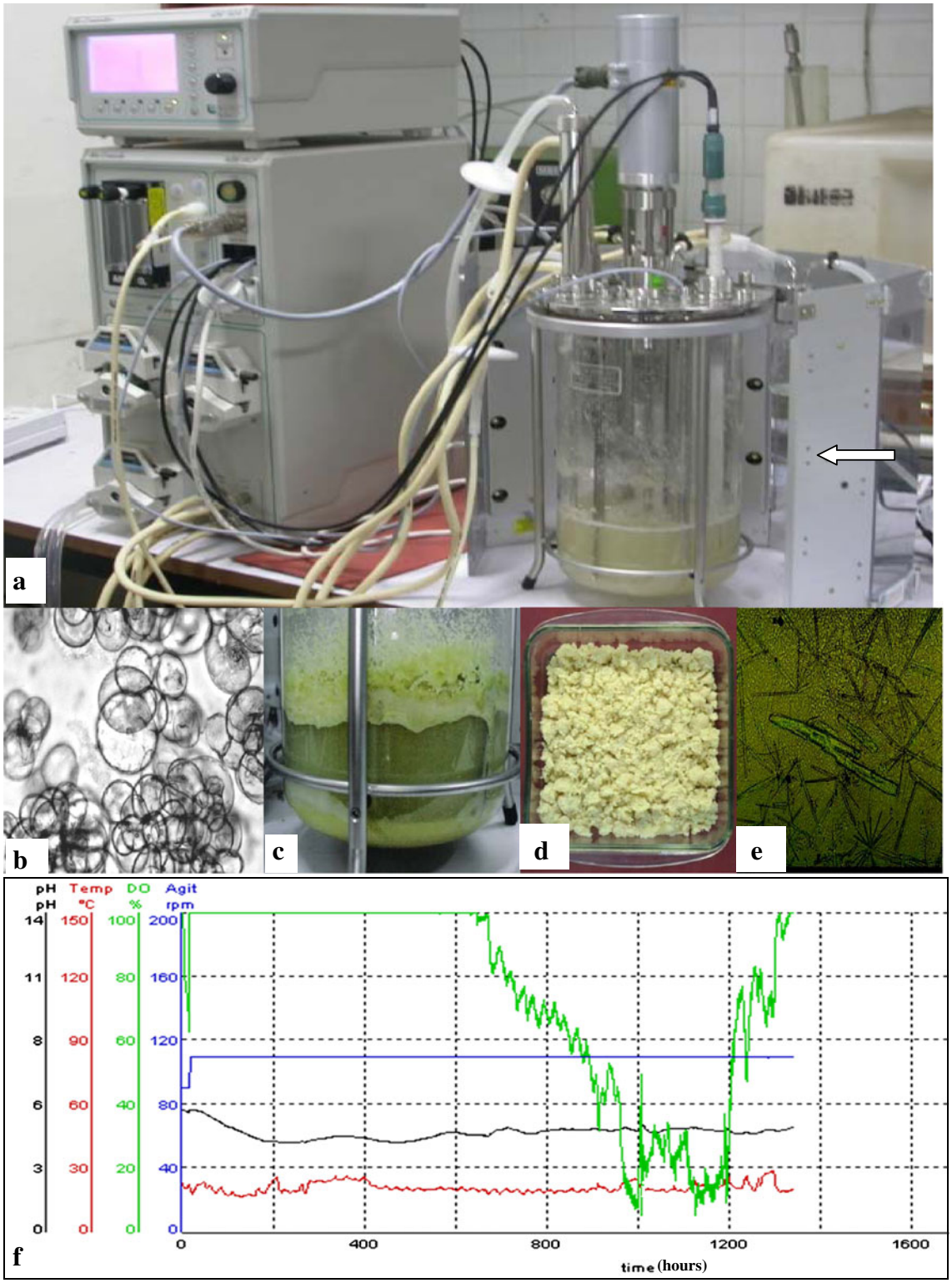

Fig. 1 Up-scaling of N30 cell line of *C roseus* in bioreactor. **a** Bioreactor set up with high illumination (*arrow* shows the LCD light source). **b** Cells after 20 days of growth. **c** Bioreactor vessel with full grown biomass after 8 weeks of growth. **d** Freshly harvested cell biomass from batch 6 of bioreactor run. **e** Photo-micrograph of the crystals obtained in the crude alkaloid extract. **f** Various process parameters recorded during bioreactor cultivation in batch 6 with rushton-type impellers and 8-week cycle

analysis. NMR analyses were done on a Bruker avance 300 MHz instrument with TMS as an internal standard. ESI mass spectra were recorded on Applied Biosystem's API-300.

In vitro antioxidant assays

Crude alkaloid extracts of bioreactor harvested tissue and shake flask grown suspension were dissolved in dimethy sulphoxide and with final concentration of 0.01 %. Ferric-reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was carried out by the method described by Pulido et al. (2000) and Chung et al. (2002), respectively. Total phenolic content of extracts was determined in terms of gallic acid equivalence (GAE) by Folin-Ciocalteu reagent method (Singleton and Rossi 1965). The total antioxidant capacity of extracts was measured using ascorbic acid as standards (Prieto et al. 1999).The reducing power of the extracts was determined by the procedure of Yen and Chen (1995). The results are given as mean ± SD of three independent experiments in replicate. Correlation among the antioxidant parameters was done by determining Pearson's coefficient using software SYSTAT11 (Luqman et al. 2009).

Results and discussion

Five variant cell lines showing differential threshold tolerance to growth inhibition by 30–70 mg/l 5-methyl-tryptophan were isolated from callus cultures of three genotypes of *C. roseus* (Table 1). The sensitivity of the wild-type callus lines of the three genotypes towards analogue (LD_{50}=7–15 mg/l) was found to be genotype-specific (*cv.* Dhawal>Nirmal>Prabal). The direct selection scheme developed by our group earlier (Seth and Mathur 2005) allowed the recovery of lethal-to-supra-lethal-to gradually-add on variants. The selected lines grew well in the presence of 5-MT whereas the survival or growth of all the corresponding wild type lines was completely checked at 25 mg/l level of 5-MT supplementation. The acquired trait was stably maintained through sustained selection pressure for over 20 biomass doubling cycles and was retained even when the variants were grown for four continuous passages in the absence of the analogue. For initiating cell suspensions, the callus lines were initially transferred to a liquid MS medium containing 1.0 mg/l 2, 4-D and 0.5 mg/l BAP but this medium did not support the formation of a fine suspension even after four to five subcultures. Fine cell suspensions were however obtained when a MS liquid medium supplemented with 2 mg/l NAA and 0.2 mg/l Kn was employed (Fig. 1b). Morphologically, all cell suspension lines grew as yellow to creamish cell mass except N30 line which had a chlorophyllous appearance. The selected callus and cell suspension lines showed a steady and comparable biomass accumulation both in the presence and absence of the analogue (Table 2). Growth measurement data obtained after 20 and 40 days of incubation indicated that wild-type callus and cell suspension lines of Nirmal, Prabal and Dhawal genotype attained a growth index (GI; percent biomass increase over the initial inoculum) of 575.8 and 109.1, 732.0 and 105.7 and 651.8 and 321.2 after 40 days of incubation, respectively. However, when these cultures were grown in the presence of 5-MT, none of them survived. Amongst the five selected analogue-tolerant cell suspension lines, highest GI on 5-MT free control medium was achieved by P40 (840.9) followed by D40 (765.0), N30 (643.9), D50 (585.7) and P70 (356.5) after 40 days of growth cycle. In the presence of corresponding threshold 5-MT, N30 and P40 with a GI of 705.8 and 656.7, respectively, grew better than the rest of the selected lines. P70 line with a clumpy tendency remained slow growing both in the presence or absence of 5-MT. All the 5-MT-tolerant cell lines in suspension showed more initial growth depression in presence of analogue till 20th day of cultures in comparison to corresponding callus phase that might be a consequence of more direct and uniform exposure of cells to the analogue in the liquid phase. When wild-type line and selected 5-MT-tolerant lines were analysed for their free tryptophan content it was observed that three wild type callus and cell suspension lines had either traces or very low level of tryptophan in them when maintained in the 5-MT free control medium (i.e. 34.0–122.0 μg/g dry weight in callus and >50.0 μg/g dry weight in suspension phase). In comparison, the tryptophan accumulation in the selected cell lines varied from 46.7 to 160.0 μg/g dry weight under analogue-free and, 168.0 to 468.0 μg/g dry weight under analogue supplemented conditions. Higher tryptophan accumulation of 368.0 and 468.0 μg/g dry weight in lines N30 and P40 under 5-MT also resulted in higher total TIAs accumulation (0.65 to 0.90 % dry weight) in them. In general, the alkaloid productivity level in cell suspensions was found lower than in corresponding callus due to lack of requisite cellular differentiation and sink capacity of the latter as has been suggested in earlier reports (Verma et al. 2012). HPLC analysis of the crude alkaloid extract of these lines did not show the presence of major TIAs except a faint peak of catharanthine in case of N30. This line because of its consistent growth behaviour, higher tryptophan content and better alkaloid profile was subsequently used for optimizing the cultural parameters for its bioreactor up-scaling.

Seven individual batches of up scaling of N30 line were run in a 7-l stirred tank bioreactor fitted with a steel sparger (Fig. 1a). Parameters namely impeller type, medium to inoculum ratio, volume of the medium consumed and cell pack volume (CPV) measurement were studied (Table 3). All runs were initiated using 30–40 g of cells from 30-day-old early stationary-phased shake flask cultures. In batch 1 wherein no impeller was used, the cells did not grow well, consumed only 240 ml out of the 2 l medium and yielded just 6-fold more biomass than the initial inoculum weight

Table 2 Growth, tryptophan and alkaloid profile of selected 5-MT tolerant lines of *C. roseus* in static and shake flask cultures under presence and absence of 5-MT

Line number	5-MT in medium (mg/l)	Culture age (days)	Parameters studied at static /shake flask level					
			Growth index (% increment over the initial inoculum weight)		Tryptophan content in free amino acid pool (μg/g.dry weight)		Crude alkaloid content (% dry weight)	
			Callus	Suspension	Callus	Suspension	Callus	Suspension
NW[a]	0	20	120.7±22.3	75.6±11.4	tr	tr	tr	tr
		40	575.8±45.3	109.1±15.8	34.0±6.6	tr	0.34±0.03	0.016±0.001
	30	20	28.1±5.6	ns	tr	–	nd	–
		40	16.1±9.7		tr		tr	
N30	0	20	280.4±34.3	446.8±75.6	81.0±8.7	32.0±7.4	nd	nd
		40	620.7±83.6	643.9±78.9	285.0±17.6	159.0±34.5	0.31±0.02	0.09±0.002
	30	20	310.6±45.6	324.7±45.6	485.0±23.5	247.0±25.4	nd	nd
		40	632.6±32.5	705.8±54.3	700.0±45.7	368.0±24.6	0.50±0.03	0.65±0.02
PW[a]	0	20	427.0±67.4	65.8±20.2	nd	nd	nd	nd
		40	732.0±59.4	105.7±19.7	122.0±23.4	46.0±14.6	0.04±0.01	tr
	40	20	65.5±9.5	ns	nd	–	nd	–
		40	10.6±2.4		tr		tr	
P40	0	20	464.5±45.8	450.5±67.8	39.0±8.9	40.9±8.6	tr	0.07±0.003
		40	1,058.5±89.7	840.9±88.6	291.1±17.8	160.0±15.6	0.14±0.05	0.17±0.05
	40	20	874.3±56.7	245.7±45.6	298.0±20.3	79.0±11.2	0.18±0.06	tr
		40	1,219.7±98.6	656.7±67.8	946.0±68.7	468.0±19.6	1.49±0.09	0.9±0.004
P70	0	20	437.6±56.6	211.8±44.5	tr	18.9±3.5	0.10±0.01	tr
		40	735.5±87.9	356.5±65.4	90.1±7.8	57.6±9.2	0.91±0.07	tr
	70	20	250.3±36.5	105.1±30.5	30.8±7.8	40.3±8.2	0.19±0.01	0.08±0.002
		40	637.5±79.8	236.0±35.6	247.2±16.8	168.0±17.9	1.24±0.09	0.23±0.04
DW[a]	0	20	328.4±66.7	276.8±24.8	29.8±9.8	tr	nd	nd
		40	651.8±54.8	321.2±54.5	80.5±11.4	tr	0.05±0.01	nd
	40	20	98.5±16.7	ns	tr	–	nd	–
		40	17.6±5.4		nd		tr	
D40	0	20	218.0±28.2	450.1±45.6	21.0±6.5	nd	0.09±0.01	tr
		40	948.3±89.5	765.0±36.5	71.1±13.2	98.9±8.7	0.35±0.04	0.11±0.01
	40	20	164.2±47.8	214.7±33.5	36.7±5.9	24.5±3.9	tr	tr
		40	350.0±67.5	573.9±64.5	323.3±48.5	215.6±20.4	0.48±0.03	0.25±0.09
D50	0	20	370.1±36.7	356.0±55.5	28.6±9.5	tr	nd	nd
		40	654.9±66.4	585.7±90.7	84.1±8.9	46.7±12.1	0.46±0.02	nd
	50	20	365.2±46.2	256.9±35.6	30.1±7.8	19.0±2.9	Nd	tr
		40	854.9±87.5	489.0±59.8	372.0±18.9	198.7±19.3	1.65±0.08	tr

ns no survival, *tr* traces, *nd* not detected

[a] Parent wild-type line

after 20 weeks with a CPV of 10.0 ml/100 ml suspension. A three-blade marine impeller also could not support cell growth due to excessive damage of the cells. The cell biomass obtained after 10 weeks in batch 2 run was >2.0-folds of the initial inoculum weight. The growth and biomass accumulation was significantly improved when six-blade rushton-type impellers were employed (batch 3) and the best results were obtained when two such impellers were placed at a height of 10 and 18 cm from the base of a 30-cm-long central shaft. The culture grew by over 16 times from its initial 2.0 % inoculum density within 18 weeks after consuming almost half of the 2.0 l medium. The biomass accumulation was further improved (batch 4) when volume of the initial medium was increased from 2.0 to 3.0 l and the harvesting was done after 6 weeks of bioreactor cultivation. Subsequently an inoculum weight of 40 g/3 l of medium and a culture cycle of 8 weeks (batch 5) were optimized for growth and alkaloid production. Maximum biomass

Table 3 Performance of N30 line of *C. roseus* under different sets of processing conditions in a stirred tank bioreactor

Batch no.	Impeller type[a]	Medium volume (1)	Inoculum weight (g)	Culture duration (weeks)	Final weight (g)	Medium consumed (ml)	Cell pack volume (ml/100 ml suspension)	Crude alkaloid content (% dry weight)
1	No impeller	2.0	30.0	20	180.5	240	10±0.5	0.09±0.008
2	Marine blade	2.0	30.0	10	56.6	200	8±0.3	0.10±0.007
3	Rushton	2.0	30.0	18	500.5	900	24±0.5	0.12±0.011
4	Rushton	3.0	30.0	6	550.3	1,000	25±0.7	0.12±0.009
5	Rushton	3.0	40.0	8	700.5	1,120	50±1.2	0.13±0.012
6	Rushton	3.0	40.0	8	1,200.3[b]	2,300	87±1.8	0.26±0.044
7	Rushton	3.0	40.0	8	1,150.8[b]	2,330	82±2.1	0.27±0.035

[a] Two in number

[b] 5-MT (30 mg/l) was added in the culture vessel 1 week prior to harvesting

accumulation attained under these standardized operational conditions was 18-folds more than the initial inoculum weight with a concurrent increase in the CPV value of 50.0 ml/100 ml cell suspension. The rapid biomass increment was also evident through a steep fall in DO level between 30 to 55 days under such culture conditions (Fig. 1c–f). Influence of appropriate impeller types and inoculum density in bioreactor-based cultivation of *C. roseus* cells has been frequently cited in published literature (Fulzele and Heble 1994; Huang and Mc Donald 2009). These studies have indicated that reducing shear force while increasing the mechanical mixing efficacy for optimal mass transfer needs to be fine-tuned when cultured cells are scaled up from shake flask to bioreactor. Use of an improper impeller type can exert undesirable hydrodynamic forces leading to poor cell viability and metabolite production due to shear mechanical shocks (Huang and Mc Donald 2009). The rushton-type impellers used in the present study are often preferred for plant cells because they follow a radial flow pattern for dispersion of cells in the medium with sufficient oxygen mass transfer and minimum shear stress (Zhao and Verpoorte 2007). Batches 6 and 7 were repeat runs wherein the influence of 5-MT was studied by adding 30 mg/l of the tryptophan analogue in the bioreactor vessel one week prior to harvesting (i.e. after 7 weeks of growth under analogue-free environment). N30 cells not only tolerate this inhibitory level of 5-MT but also had more than 1.5-fold biomass yield (i.e. 30 times more than the initial inoculum weight) than the non-5MT-treated cultures when harvested after 8 weeks. The total alkaloid content (0.26–0.27 % dry weight) was also doubled in these 5-MT-treated cells (Table 2). The free tryptophan level in cells up-scaled in bioreactor batches 4, 5 and 6 was found to be 142.45, 164.0 and 388.3 μg/g dry weight, respectively, and corroborated well with the tryptophan content of N30 cells grown in shake flask cultures. Like shake flask suspension cultures, the HPLC analysis of the total alkaloids extract of cells up-scaled in bioreactor also did not show the presence of any known pharmaceutically alkaloid except a faint peak of catharanthine highlighting again the necessity of requisite tissue differentiation for advancement of complete TIAs pathway in *C. roseus*. Interestingly, it was also observed that crude alkaloid extract of bioreactor up-scaled suspension showed the formation of yellow needle-like crystals in it (Fig. 1e) in comparison to transparent non crystalline extract of shake flask cultures. These crystals up on TLC analysis showed one non-alkaloidal spot of unknown compound under UV (Rf=0.44 at 254 nm; silica gel, solvent 10 % MeOH in $CHCl_3$). ^{1}HNMR spectra of this compound showed the presence of a –OH group directly attached to an aromatic ring (δ=4.2). The aromatic proton signals appeared at δ=7.51–7.69 (J=3.3, 5.7 Hz). The ESI-MS (positive mode) spectra showed major molecular peak at m/z=471. These initial characterizations suggested a phenolic identity of this compound that was co-eluted in the aqueous phase along with alkaloids during extraction. Keeping in view the possible phenolic nature of this compound, the crude extracts of the cells obtained from bioreactor and shake flask cultures were subjected to concentration- dependent ferric-reducing, free radical scavenging and total antioxidant assays (Fig. 2). Total antioxidant capacity (TAC) increased with increasing concentration of the extract and at 50 μg/ml concentration it was 65.21 % more in bioreactor harvested tissue than the cells grown in shake flask. Reducing power (RP) of both types of the extracts, however, showed almost equal absorbance at higher concentration (100 μg/ml) with a linear trend. FRAP at 50 μg/ml concentration was 100 % more in up-scaled cultures but at 100 and 1,000 μg/ml concentration the activity was better in cells of shake flask cultures (Fig. 2e). The free radical scavenging (DPPH) activity in the extract of shake flask suspensions on the other hand was consistently better (57.47 %) at all assayed concentrations when compared with that of bioreactor grown cells. In terms of total phenolic content (TPC)

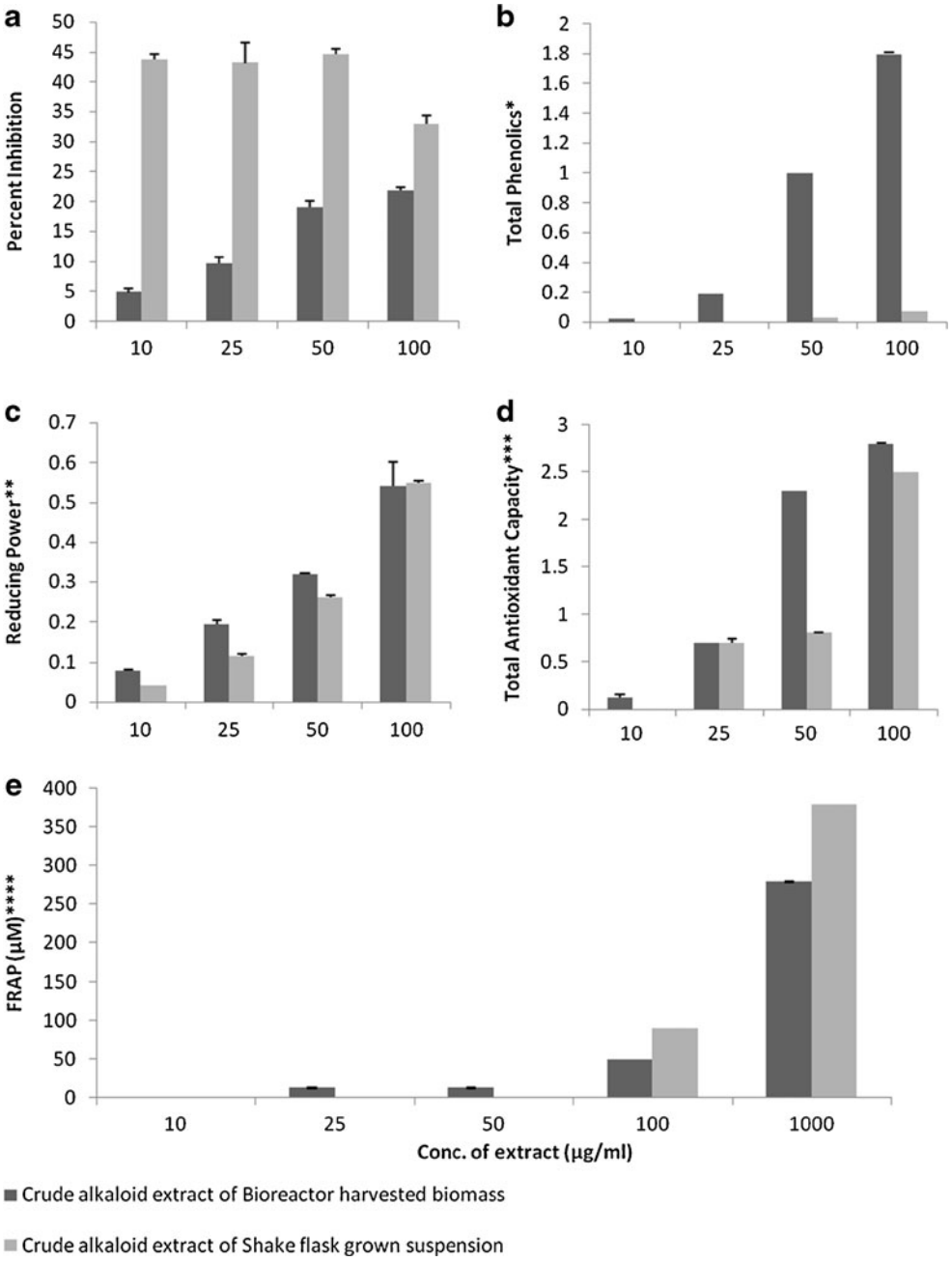

Fig. 2 Concentration dependent free radical scavenging activity (**a**); total phenolic content (**b**); reducing power (**c**); total antioxidant activity (**d**) and ferric-reducing antioxidant power (**e**) of crude alkaloid extracts of shake flask grown suspension and bioreactor harvested cell of N30 cell line of *C. roseus*. *Single asterisk* (*) expressed as gallic acid equivalence; *two asterisks* (**) determined in terms of absorbance at 700 nm; *three asterisks* (***) expressed as ascorbic acid equivalence; *four asterisks* (****) estimated in terms of ferrous sulphate equivalence

measured as GAE, the extract of bioreactor harvested tissue was found to be 97 % better (micrograms GAE=1.8) than the extract of shake flask suspension (micrograms GAE= 0.07). Table 4 summarizes the correlations between different performed assays based on their Pearson's coefficient. The data clearly suggested that crude alkaloid extract of bioreactor harvested cell was having better antioxidant potential than crude extract of shake flask grown suspension (Pearson's coefficient=0.8–0.998). The negative correlations between DPPH and FRAP, TPC, RP or TAC observed in the shake flask cell extract with Pearson's coefficient value of −0.856, −0.927, −0.856 and −0.994, respectively, can be ascribed to its higher (57.47 %) free radical scavenging power.

The results of the present investigation highlight and further substantiate three major issues in *Catharanthus* biotechnology, i.e. the constrain of the limited availability of indole precursor molecule tryptophan for TIAs biosynthesis can be overcome by selecting cell types resistant to growth inhibition imposed by its analogue 5-methyltrypthan; the over-synthesised tryptophan can not only push the pathway flux towards TIAs biosynthesis but can also enhance growth and biomass accumulation, possibly through its larger availability for auxin synthesis; the 5-MT-tolerant cell lines can

Table 4 Pearson coefficients based correlation analysis of the different in vitro assays of the crude alkaloid extracts of shake flask-grown suspension and bioreactor harvested biomass of N30 cell line of *C. roseus*

Correlation between the in vitro assays	Crude alkaloid extract of	
	Shake flask grown N30 suspension	Bioreactor grown N30 suspension
FRAP[a] and TPC[b]	0.905	0.910
DPPH[c] and TPC	−0.856	0.953
DPPH and FRAP	−0.994	0.795
TAC[d] and FRAP	0.942	0.800
TAC and DPPH	−0.927	0.998
TAC and TPC	0.937	0.965
RP[e] and FRAP	0.905	0.949
RP and DPPH	−0.856	0.945
RP and TPC	0.990	0.985
RP and TAC	0.937	0.948

[a] Ferric-reducing antioxidant power

[b] Total phenolic content

[c] 1,1-Diphenyl-2-picrylhydrazyl radical scavenging

[d] Total antioxidant capacity

[e] Reducing power

be conveniently scaled up in stirred tank bioreactor fitted with rushton-type impellers and; bioreactor-based cultivation under improved aeration can enrich the cells with phenolics having better antioxidant potential. The ability of plant cells to resist 5-MT induced toxicity has often been traced to their ability to over synthesize tryptophan via a relaxed feedback inhibition of the enzyme anthranilate synthase involved in its synthesis from chorismate (Li and Last 1996; Meijer et al. 1993; Scott et al. 1979; Radwanski and Last 1995; Seth and Mathur 2005). Selection of such 5-MT-tolerant variants is important because exogenous supplementation of tryptophan normally results in cell death due to the down regulation of several other metabolic pathways via activation of enzyme chorismate mutase that strongly pulls chorismate towards prephenate metabolism (Radwanski and Last 1995; Galili and Hofgen 2002; Whitmer et al. 2002). Hughes et al. (2004) have also shown that TIA accumulation in a transgenic hairy root line of *C. roseus* was favoured by the larger tryptophan availability achieved via over-expression of an *Arabidopsis* feedback-resistant anthranilate synthase gene in them. Though not yet experimentally validated, our results also suggested that 5-MT-tolerant variants, like N30 line used in the present study, can prove better starting material for ongoing genetic modification efforts in *C. roseus* revolving around the over-expression of pathway genes like *TDC*, *strictosidine synthase*, *geraniol-10-hydroxylase*, *LAMT* and *secologanin synthase*. Our finding that higher accumulation of free tryptophan in the 5-MT-tolerant cells can also influence production of phenolic in them would find notice for their further value addition.

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Conflict of interest The authors declare that they have no conflict of interest.

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