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Elicitors' influenced differential ginsenoside production and exudation into medium with concurrent Rg3/Rh2 panaxadiol induction in *Panax quinquefolius* cell suspensions

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Abstract Cobalt nitrate, nickel sulphate, hydrogen peroxide, sodium nitroprusside, and culture filtrates of *Pseudomonas monteili*, *Bacillus circularans*, *Trichoderma atroviridae*, and *Trichoderma harzianum* were tested to elicit ginsenoside production in a cell suspension line of *Panax quinquefolius*. Abiotic elicitors preferentially increased panaxadiols whereas biotic elicitors upregulated the panaxatriol synthesis. Cobalt nitrate (50 μM) increased total ginsenosides content by two-fold (54.3 mg/L) within 5 days. It also induced the Rc synthesis that was absent in the control cultures. Elicitation with *P. monteili* (2.5 % *v*/*v*, 5 days) also supported 2.4-fold enhancement in saponin yield. Elicitation by *T. atroviridae* or hydrogen peroxide induced the synthesis of Rg3 and Rh2 that are absent in ginseng roots. The highest ginsenosides productivity (3.2-fold of control) was noticed in cells exposed to 1.25 % *v*/*v* dose of *T. atroviridae* for 5 days. Treating cells with *T. harzianum* for 15 days afforded maximum synthesis and leaching (8.1 mg/L) of ginsenoside Rh1.

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Introduction

Plant secondary metabolites are low molecular weight products that play a pivotal role in imparting ecological fitness to a plant, particularly during defense responses. These metabolites are often induced by stress signals, such as pathogen attack, environmental stresses, and nutrient deficits. Several such phytomolecules are also pharmaceutically important to mankind as disease preventive/curative drugs (Rao and Ravishankar 2002). Since these plant secondary metabolites are invariably synthesized using the precursors drawn from primary metabolic pool, they are produced in a need-based manner and in quantities that are just sufficient for a plant to tide over a stressful situation. The low in planta productivity levels of desired phytomolecules often mismatch with their industrial demands and have necessitated two lines of scientific investigation to boost their production. First, the modern -omics tools of biotechnology are being frequently applied to hyper express the desired biosynthetic route(s) through various pathway engineering strategies in homo-to heterologous systems and, second, cell-culture-based alternate production platforms are being tested for their in vitro production (Akula and Ravishankar 2011). Besides easing out the immense pressure of unsustainable harvesting of natural plant populations, these alternate production pedestals are becoming viable options for those high-value phyto-molecules that are exclusively obtained from a plant source and defy chemical synthesis due to their complex chirality.

Ginseng (*Panax ginseng* and *Panax quinquefolius*) is an important oriental herb well known for its revitalizing, longevity-promoting, and aphrodisiac virtues. The bioactives

attributed to its neuroprotective, cardioprotective, immunomodulatory, and cerebral health maintenance belong to a group of pentacyclic triterpenoid saponins, ginsenosides, predominantly found in the roots (Wu and Zhong 1999). Based on the structural differences, these ginsenosides are broadly classified into two groups, i.e., 20(S) protopanaxadiol group (Rb1, Rb2, Rc, Rd, Rg3, Rh2) and 20(S) protopanaxatriol group (Re, Rg1, Rg2, Rh1). The biggest obstacle for pharma industries to procure ginsenosides via organized ginseng cultivation is the long gestation gap of 5–6 years prior to crop harvesting for roots. Consequently, ginseng biotechnologists have long been investigating the in vitro ginsenoside production in cell culture platforms (Wu and Zhong 1999; Mathur et al. 1994, 2010). Inspite of efforts spanning decades, commercial-level success is still limited. Low cellular stability, lower ginsenosides content, their poor recovery from cultured tissues, and a sketchy knowledge of the biosynthetic pathway are some of the major constraints that require a closer scrutiny (Wu and Zhong 1999).

Elicitation of ginsenosides biogenetic pathway in cultured cells and tissues of *Panax* spp. has been frequently attempted in many past studies (Thanh et al. 2005; Wang et al. 2005, 2011; Huang and Zhong 2013; Huang et al. 2013). To recall, elicitors are defined as substances that can trigger a cascade of signaling events that in turn can activate the expression of defense-related genes and synthesis of secondary metabolites (Chandra and Chandra 2011). Different elicitors may act at different levels depending upon their differential elicitor–membrane receptor binding, G protein activation, cytoplasmic acidification by activation of membrane H^+-ATPase pumps, and ROS generation via second messengers such as IP3 and cyclic AMP, followed by early/late defense gene expression leading to secondary metabolite synthesis (Zhao et al. 2005). Elicitors so far tested to overexpress ginsenoside synthesis in cell and tissue cultures of *Panax* species were primarily abiotic in nature such as methyl jasmonate, hydrogen peroxide, sodium nitroprusside, vanadate, and Ni^{++}. (Hu et al. 2003a; Yu et al. 2005; Jeong and Park 2006; Tewari et al. 2008; Tewari and Paek 2011; Huang and Zhong 2013; Huang et al. 2013; Rahimi et al. 2015a). Majority of these studies were carried out using cell and tissue cultures of Korean or Chinese species of ginseng (*P. ginseng* and *Panax notoginseng*). Related information in case of American ginseng (*P. quinquefolius*) is scanty. This literature scan has also revealed that >80 % of such studies in *Panax* were carried out using methyl jasmonate as the eliciting agent that depicted a wide spectrum of variable responses ranging from positive to negative to no influence on ginsenoside productivity. Use of biotic elicitors for upregulating ginsenosides production in cell and tissue cultures of *Panax*, on the other hand, is confined to just few reports. Xu and co-workers (2005) employed a fungal elicitor obtained from *Colletotrichum* which was found to enhance the production of saponins via singlet oxygen release. In another report, suspension cultures of *P. ginseng* were treated with *Botrytis cinerea* and yeast preparations to produce a novel anti-bacterial compound in cultures (Kim et al. 2001).

In the present investigation, we have tested the efficacy of four abiotic (cobalt nitrate, hydrogen peroxide, sodium nitroprusside, and nickel sulphate) and four biotic (culture filtrate of *Pseudomonas monteili*, *Bacillus circularans*, *Trichoderma atroviridae*, *Trichoderma harzianum*) elicitors on the pattern of ginsenosides production over a 50-day growth cycle of a cell suspension culture line of *P. quinquefolius* (US patent 632602B1). This selected cell line is being stably maintained in our laboratory for more than 20 years now (Mathur et al. 1994; Mathur et al. 2010). The choice of elicitors used in the present study was based on their well-documented influences on signaling cascade involved in plant secondary metabolism. For example, hydrogen peroxide is known to be a signal transducer of stress response that can modulate gene expression by its ability to generate reactive oxygen species (Hu et al. 2003a; Zhao et al. 2005). Similarly, sodium nitroprusside, a NO donor, has been shown to be associated with upregulation of several secondary metabolic pathways in different plant systems including *P. ginseng* (Hu et al. 2003b; Zhang et al. 2012; Rahimi et al. 2015a). The two heavy metal elicitors cobalt and nickel used in the present study in the form of their nitrate and sulphate salts, respectively, are well-known upregulators of plant secondary metabolite synthesis (Zhao et al. 2005). Nickel and vanadate (a compound containing an oxoanion of vanadium in its highest oxidation state of +5) have previously been shown to hyper-express ginsenosides synthesis in hairy root and cell suspension cultures of *P. ginseng* (Jeong and Park 2006; Huang and Zhong 2013). Biotic elicitors of microbial origin are also gaining importance these days because of their ability to mimic a disease response that, in turn, switches on a defense signal cascade and hence the synthesis of secondary metabolite in a plant system. Such microbial elicitors are normally applied in the form of mycelia extracts, culture filtrate, or cell-free extracts (Mañero et al. 2012; Chodisetti et al. 2013; Qianliang et al. 2013; Prasad et al. 2013; Awad et al. 2014; Verma et al. 2014).

It is also pertinent to mention here that so far only six major ginsenosides constituents (Rb1, Rb2, Rc, Rd, Re, and Rg1, present in the roots of field grown *Panax* plants) are in primary focus of elicitation studies carried out with ginseng cell and tissue cultures. The present investigation also highlights the influence of elicitation treatments on the accumulation of four de-glycosylated minor ginsenoside components (Rg2, Rh1, Rg3, and Rh2) that are presently gaining immense importance as potent anti-cancer pharmacophores (Park et al. 2010). We have also monitored for the first time in this study the

efficiency of elicitation treatments that could afford the exudation of biosynthesized ginsenosides into the culture medium that can have great relevance in reducing the cost of extraction and isolation during downstream processing.

Materials and methods

In vitro maintenance of *P. quinquefolius* cell suspensions

The high ginsenoside producing cell suspension was originally developed from the root callus of *P. quinquefolius* (Mathur et al. 2001 US patent 6326202B1, voucher specimen designated as CIMAP/Pq-1 being maintained in the ex situ repository of Gene Bank, CIMAP, Lucknow) and was stably maintained in vitro on a modified MS (Murashige and Skoog 1962) media supplemented with 3.0 % sucrose, 0.01 % myoinositol, 0.33 μM thiamine hydrochloride, 2.5 μM pyridoxine hydrochloride, 4.0 μM nicotinic acid, 5.4 μM α-naphthaleneacetic acid (NAA), and 1.2 μM Kinetin. Cell suspension cultures were regularly subcultured after every 6 weeks. The pH of all the media was adjusted to 5.8 ± 0.03 before autoclaving at 1.04 kg/cm^2 pressure (121 °C) for 15–20 min. The cultures were incubated under a 16:8-h light/dark photoperiod provided by cool white fluorescent tubes with 15 μE m^{-2} s^{-1} intensity. The temperature and relative humidity in the culture room varied from 25 to 28 °C and 60 to 70 %, respectively.

Growth kinetic studies

For growth and metabolite productivity measurements of the cell suspensions, 3 g (FW) of cell inoculum was aseptically transferred to 40 mL medium in 250-mL Erlenmeyer flasks and kept on a rotator shaker at 100 rpm. Cells were harvested every 10 days of growth until the completion of a 50-day-long cell cycle. Biomass increments were monitored by measuring the fresh weight (FW) of harvested cells. The harvested cells were lyophilized (Labconco, Free zone 2.5, USA) to constant weight for dry matter determination. The growth rate was measured as percent biomass increment [(= *Weight of cells at harvest*/*Weight of initial inoculums*) × 100]. A minimum of five replicates were run for all the treatments, and the experiments were repeated thrice.

Ginsenoside quantification using HPLC-UV

Ginsenoside extraction and quantification were done following an optimized methodology previously reported by us (Biswas et al. 2015). Briefly, 1 g of dried lyophilized cell sample was sonicated (120 W; Rivotek, Ultrasonic cleaner, STD 2025) for 30 min in 50 mL methanol (four times). The methanolic extracts were pooled and sequentially partitioned using diethyl ether and water-saturated n-butanol, and the resultant butanolic extract was vacuum-concentrated to dryness using a Rotavapor (BUCHI, Vacuum controller V-850, Switzerland). The dried extract was re-dissolved in HPLC-grade methanol for HPLC analysis. For spent media analysis, 50 mL of the media was lyophilized to a powder form and extracted as mentioned above for cells. HPLC-UV analysis was carried out using a HPLC modular system (Waters Millford, USA) equipped with a Waters symmetry C_{18} 4.6 × 150 mm (3.5 μm) column, 600 E Waters pump, 2996 photodiode detector, and 717 auto-sampler. A gradient elution system comprising of water (A) and gradient-grade acetonitrile (B) was developed. The flow conditions employed were as follows: 0 min, % A = 80, % B = 20; 30 min, % A = 66, % B = 34; 48 min, % A = 48, % B = 52; 55 min, % A = 55, % B = 45; 60 min, % A = 15, % B = 85; and 70 min, % A = 80, % B = 20. Ten ginsenosides, namely, Rb1, Rb2, Rc, Rd, Re, Rg1, Rg2 Rg3, Rh1, and Rh2, were eluted in a total run time of 70 min. The order of elution was Rg1, Re, Rh1, Rb1, Rc, Rb2, Rg2, Rd, Rg3, and Rh2 with retention time of 41.8, 42.1, 51.6, 53.4, 53.9, 54.2, 54.7, 55.9, 63.1, and 64.9 min, respectively. The ginsenosides were identified in the samples by matching corresponding UV spectra and retention times of authentic reference compounds. Peak identity was further confirmed by spiking the sample with the respective authentic. For quantitative analysis, calibration curves were plotted. Linearity was performed for each ginsenoside standard. Standard solutions containing 2–20 μg of each ginsenoside were analyzed in triplicate of each concentration. Calibration curves were constructed by plotting peak areas against analyte concentration. The linearity was assessed by calculating the slope, *y* intercept, and determination coefficient (R^2) using least squares regression (Rb1, 0.9971; Rb2, 0.9904; Rc, 0.9991; Rd, 0.9842; Re, 0.9902; Rg1, 0.9921; Rg2, 0.9906; Rg3, 0.9924; Rh1, 0.9961; Rh2, 0.9926). Reference samples of ginsenosides were purchased from ChromaDex Ltd. and Sigma-Aldrich, USA. All samples were analyzed at 203 nm using the Empower 2 software.

Preparation and application of elicitors

Preparation of abiotic elicitors

Stock solutions (1 mg/mL) of cobalt nitrate, sodium nitroprusside, and nickel sulphate were prepared in double distilled water. Hydrogen peroxide (30 %) solution was also prepared in double distilled water. The solutions were filter-sterilized using 0.22 μ PVDF filter unit (Millipore, USA). The elicitors were added to the cell suspensions after 25 days of cell growth at two concentrations: cobalt nitrate at 50 (CN 50) and 100 μM (CN 100), hydrogen peroxide at 50 (HP 50) and 100 μM (HP 100), sodium nitroprusside at 75 (SNP 75) and 100 μM (SNP 100), and

nickel sulphate at 15 (Ni 15) and 20 μM (Ni 20). The cell suspensions were harvested after 5 and 15 days of exposure to the elicitor treatments.

Preparation of biotic elicitors

P. monteili strain Cb8 (MTCC No. HQ995498) and *B. circularans* strain Pf6 (MTCC No. 10012) were maintained on the nutrient agar medium (15 g/L peptone, 3 g/L beef extract, 5 g/L NaCl, 0.3 g/L KH_2PO_4, and 15 g/L agar). Single bacterial colonies were inoculated in 10-mL nutrient broth medium and maintained on a rotary shaker (160 rpm) at 28 °C for 24 h. One milliliter of the suspension was further inoculated into 9-mL fresh nutrient broth (NB) under similar conditions and allowed to grow for 12 h ($OD_{660} = 1$). The cultures were centrifuged at 10, 000 rpm for 10 min to pellet out the cells. The supernatant was filter-sterilized using a 0.22-μm PVDF filter unit (Milllipore, USA). *T. atroviridae* TA (sequence deposited to NCBI: ACCESION NUMBER JX002658) and *T. harzianum* TH (ATCC No. PTA 3701) fungal cultures were maintained on potato dextrose agar (PDA). One-square-centimeter disc of the fungal isolate was inoculated onto 250 mL of potato dextrose medium and incubated on a rotary shaker (180 rpm) for 20 days. The mycelia were filtered off through a Whatman filter paper and the filtrate was sterilized using Millipore (0.22 μ) PVDF filter unit for further use.

The elicitors were added to the *P. quinquefolius* cell suspensions after 25 days of growth at two concentrations as follows: culture filtrate of *P. monteili* at 1.25 % *v*/*v* (Cb8 1.25 %) and 2.5 % *v*/*v* (Cb8 2.5 %), culture filtrate of *B. circularans* at 1.25 % *v*/*v* (Pf6 1.25 %) and 2.5 % *v*/*v* (Pf6 2.5 %), culture filtrate of *T. atroviridae* at 1.25 % *v*/*v* (TA 1.25 %) and 2.5 % *v*/*v* (TA 2.5 %), and culture filtrate of *T. harzianum* at 1.25 % *v*/*v* (TH 1.25 %) and 2.5 % *v*/*v* (TH 2.5 %). The plant cells were harvested after 5 and 15 days of elicitor treatments. The respective controls were set using addition of equal amounts of nutrient broth (Ct NB for bacterial elicitors) and potato dextrose broth (Ct PDB) for fungal elicitor treatments.

Statistical analysis

All experiments were carried out with five replicates (duplicated thrice). The ginsenoside yields were calculated on per liter medium basis and expressed as milligrams per liter. The growth and metabolite production kinetics of *P. quinquefolius* cell suspensions were subjected to ANOVA using complete randomized design (CRD; Singh and Chaudhary 1979). All the data was further subjected to Pearson's linear co-relation coefficient analysis using SPSS software version 13.

Results

Growth and metabolite production kinetics of unelicited *P. quinquefolius* cell suspensions

The unelicited cell suspension cultures on the control medium followed a bi-phasic growth pattern during a 50-day-long cultivation cycle (Tables 1 and 2). After registering a marginal fall in biomass increment between the 10th and 20th day of culturing, the cells grew exponentially and attained their highest growth rate of 172.3 on the 40th day of the culture cycle. The cell growth was significantly retarded during next 10 days attaining a growth rate of only 136.94 on 50th day. Further prolongation of the culture cycle caused extensive cell death and hence not deemed suitable. The cultured cells also showed a differential age-dependent in vitro accumulation pattern of ginsenosides in them (Tables 1 and 2). Only eight out of ten analyzed ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rg1, Rg2 ,and Rh1) were detected in these cultures on the control medium, out of which Re and Rg1 ginsenosides also showed their partial leaching into the spent medium at all harvest. Rg3 and Rh2 ginsenosides were not detected in any cell or medium samples. The highest cumulative yield of the analyzed ginsenosides in the cells + spent medium (27.3 mg/L medium) was obtained after 30 days of growth. This was nearly 2.8-fold more than in samples harvested on the 10th and 20th days of incubation and 4.5-fold more than in 50th-day samples. Protopanaxatriol ginsenosides Re and Rg1 were the major (>90 %) constituents of the pentacyclic triterpenoid saponin fractions in all the analyzed samples. The protopanaxadiol fraction of the ginsensosides, on the other hand, was dominated by Rb2 (0.03–0.1 mg/L) during initial 30 days of culture after which it was not detected. In later stages of growth between the 30th and 50th day, this decline in Rb2 concentration was compensated with a con-committal rise in Rb1 fraction (0.14–0.09 mg/L). The protopanaxadiol Rc showed its presence (0.04 mg/L) in only the cell samples harvested on the 20th day of growth, whereas ginsenoside Rd accumulation that was also peaked (0.13 mg/L) in 30-day-old cells was not detected in the 50th-day harvest. The two other analyzed panaxatriol ginsenosides Rg2 and Rh1 were produced in minor amounts (0.06–0.12 mg/L) in the cultured cells between the 20th and 40th days of growth cycle (Tables 1 and 2).

Effect of elicitation treatments on cell biomass and ginsenosides production

As mentioned earlier, biomass accumulation and ginsenosides productivity patterns were monitored as a function of dose and duration of exposure of four individually added abiotic and four biotic elicitors in the medium. All elicitors were added in the cell medium on the 25th day of the culture in two doses, and the cells were exposed to these elicitation treatments for

Table 1 Growth and metabolite production kinetics in *Panax quinquefolius* cell suspensions

Age (days)	% Biomass increase (Mean ± SD)	Ginsenoside yield calculated as mg/L suspension																				Total
		Rb1		Rb2		Rc		Rd		Re		Rg1		Rg2		Rg3		Rh1		Rh2		
		C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	
10	118.61 ± 6.5	nd	nd	0.037	nd	nd	nd	nd	nd	8.57	0.06	2.51	0.07	nd	nd	nd	nd	nd	nd	nd	nd	11.25
20	109.14 ± 10.5	nd	nd	0.08	nd	0.04	nd	0.06	nd	7.2	0.07	2.73	0.38	0.06	nd	nd	nd	nd	nd	nd	nd	10.62
30	150.0 ± 8.94	0.14	nd	0.10	nd	nd	nd	0.13	nd	19.9	1.53	3.77	1.54	0.12	nd	nd	nd	0.07	nd	nd	nd	27.3
40	172.30 ± 0.77	0.09	nd	nd	nd	nd	nd	0.06	nd	10.5	2.2	2.3	0.9	nd	nd	nd	nd	0.07	nd	nd	nd	16.12
50	136.96 ± 4.1	0.09	nd	nd	nd	nd	nd	nd	nd	3.9	0.5	1.4	0.5	nd	nd	nd	nd	nd	nd	nd	nd	6.4
CD (5 %)	12.92	0.03	0	0.02	0	0.01	0	0.02	0	1.12	0.43	0.76	0.50	0.03	0	0	0	0.01	0	0	0	1.4
CD (1 %)	18.36	0.04	0	0.03	0	0.01	0	0.04	0	1.6	0.61	1.09	0.72	0.04	0	0	0	0.02	0	0	0	2.06

C content in cells, *M* content in leftover medium, *nd* not detected

either 5 or 15 days before harvesting on 30th and 40th days of culturing, respectively. The efficacy of different elicitation treatments was quantified in terms of their influence on total and individual yield of panaxadiol and panaxatriol constituents in the cell and medium samples as compared to non-treated controls. The obtained data is summarized in Tables 3, 4, 5, and 6, and Fig. 1.

Effect of abiotic elicitors

The biomass accumulation patterns in cultures exposed to abiotic elicitor treatments for 5 days was more or less comparable to that of 30-day-old non-treated controls except in case of 100 μM cobalt nitrate and 100 μM hydrogen peroxide doses that caused nearly 30 % decrease in the biomass yield. Sodium nitroprusside (100 μM) and nickel sulphate (15 μM), on the other hand, enhanced the cell biomass gain by 30 % when compared with non-treated control cultures (Table 3). When duration of exposure to elicitors was increased to 15 days, the cell mass gains in all the treatments were comparable to 40-day-old controls.

The proportion of triol ginsenosides was always higher than that of the protopanaxadiols which constituted a small fraction of the total yield under all abiotic elicitation regimes. Among the four abiotic elicitors used in this study, the most significant enhancements in total ginsenoside productivity were observed when 25-day-old cell cultures were separately exposed to 50 μM cobalt nitrate, 15 μM nickel sulphate, or 75–100 μM sodium nitroprusside treatments for 5 days (Table 3). All these elicitation treatments nearly doubled the total ginsenoside recovery from the cultures (54.3–46.5 mg/L) when compared with the 30-day-old non-treated controls (26.8 mg/L). Interestingly, barring cobalt nitrate that favored a larger triol ginsenosides recovery from the harvested cells (39.1 mg/L), all other abiotic elicitors also afforded greater leaching of the biosynthesized saponins into the spent medium that was maximum in case of 75 μM sodium nitroprusside and 15 μM nickel sulphate treatments (31.2 and 31.6 mg/L, respectively). Hydrogen peroxide at lower dose of 50 μM reduced the total ginsenosides recovery to almost half (15.2 mg/L), but at higher dose of 100 μM, it also elicited a greater production and leaching of panaxatriol ginsenosides (21.5 mg/L) into the medium. At 50 and 100 μM doses, hydrogen peroxide elicited the highest accumulation of panaxadiol ginsenosides in the cells (1.2 mg/L) as well as the spent medium (5.7 mg/L), respectively. The yield of total ginsenosides in the cell and medium of 40 days harvested samples, on the other hand, was significantly reduced when

Table 2 Analysis of variance (ANOVA) using CRD

Sources of variation	DF	% BI	Mean sum of squares																			
			Rb1		Rb2		Rc		Rd		Re		Rg1		Rg2		Rh1		Total			
			C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M				
Treatment	4	1896.2**	0.012**	0	0.007**	0	0.001**	0	0.009**	0	110.3**	2.6**	2.1**	0.97**	0.009**	0	0.003**	0	193.5**			
Error	10	50.3	0	0	0	0	0	0	0	0	0.384	0.05	0.1	0.07	0	0	0	0	0.63			
Total	14																					

C content in cells, *M* content in leftover medium

**$P < 0.01$

Table 3 Effect of abiotic and biotic elicitors on the diol and triol ginsenoside production in cultures of *Panax quinquefolius*

Elicitor Treatment	Ginsenoside content after 5-day treatment						Ginsenoside content after 15-day treatment					
	% Biomass increase	Diols (mg/L)		Triols (mg/L)		C + M	% Biomass increase	Diols (mg/L suspension)		Triols (mg/L suspension)		C + M
Abiotic treaments		C	M	C	M			C	M	C	M	
Control	157.55 ± 1.4	0.2 ± 0.06	Tr[a]	23.5 ± 01.3	3.1 ± 0.8	26.8	176.34 ± 2.43	0.11 ± 0.06	Tr	8.8 ± 1.02	3.7 ± 0.99	12.6
CN 50	159.56 ± 2.6	0.4 ± 0.03	5.2 ± 0.04	39.1 ± 1.54	9.6 ± 0.9	54.3	132.40 ± 1.9	0.1 ± 0.02	1.3 ± 0.98	18.1 ± 1.23	4.6 ± 0.84	24.1
CN 100	117.62 ± 1.01	0.3 ± 0.05	Tr	26.0 ± 1.04	5.0 ± 0.13	31.3	95.70 ± 1.54	0.2 ± 0.07	Tr	11.0 ± 1.92	1.4 ± 0.07	12.6
HP 50	152.40 ± 1.92	1.2 ± 0.09	Tr	9.3 ± 1.05	4.7 ± 0.62	15.2	88.06 ± 1.21	1.0 ± 0.05	Tr	6.2 ± 1.03	2.3 ± 0.09	9.5
HP100	118.98 ± 2.7	Tr	5.70 ± 0.07	12.3 ± 1.23	21.5 ± 1.71	39.6	87.68 ± 1.1	Tr	2.9 ± 0.09	10.1 ± 1.72	10.8 ± 0.14	23.8
SNP 75	181.31 ± 1.02	0.41 ± 0.04	Tr	14.9 ± 1.76	31.2 ± 1.21	46.5	163.08 ± 2.65	0.2 ± 0.05	Tr	10.2 ± 1.66	27.1 ± 0.99	37.5
SNP 100	205.01 ± 0.93	Tr	0.1 ± 0.04	22.0 ± 2.13	25.3 ± 1.25	47.4	151.65 ± 2.03	Tr	Tr	14.1 ± 2.03	12.3 ± 1.03	26.4
NI 15	205.74 ± 1.2	Tr	0.2 ± 0.03	19.5 ± 1.65	31.6 ± 1.63	51.3	192.5 ± 1.02	Tr	0.2 ± 0.002	9.4 ± 0.95	22.1 ± 2.93	31.7
NI 20	150.10 ± 2.3	Tr	Tr	16.5 ± 2.1	16.4 ± 1.05	33.0	136.16 ± 1.98	Tr	Tr	7.8 ± 1.01	7.3 ± 0.09	15.1
Biotic (bacterial)												
Ct NB	154.34 ± 1.5	0.26 ± 0.04	Tr	22.6 ± 1.2	3.0 ± 0.95	25.9	181.23 ± 2.03	0.1 ± 0.07	Tr	8.3 ± 1.06	3.6 ± 0.11	12.0
CB8 1.25 % *v/v*	130.64 ± 2.13	0.3 ± 0.03	0.2 ± 0.03	29.2 ± 1.03	12.8 ± 0.87	42.5	137.25 ± 1.31	0.3 ± 0.02	Tr	33.3 ± 2.09	15.7 ± 1.22	49.3
CB8 2.5 % *v/v*	143.01 ± 0.43	0.6 ± 0.03	0.1 ± 0.01	42.5 ± 1.5	22.9 ± 1.04	66.3	134.74 ± 1.21	0.2 ± 0.06	Tr	27.0 ± 1.59	8.0 ± 0.32	35.2
PF6 1.25 % *v/v*	163.91 ± 1.02	1.0 ± 0.05	1.0 ± 0.03	34.1 ± 1.32	5.1 ± 1.1	41.3	119.03 ± 0.99	0.5 ± 0.01	Tr	27.6 ± 1.26	23.0 ± 1.93	51.1
PF6 2.5 % *v/v*	138.75 ± 1.8	0.6 ± 0.01	Tr	18.0 ± 1.08	6.0 ± 0.97	24.6	131.86 ± 1.21	0.9 ± 0.05	0.3 ± 0.03	34.4 ± 1.09	21.3 ± 0.39	56.9
Biotic (fungal)												
Ct PDB	150.12 ± 1.75	0.2 ± 0.04	Tr	23.7 ± 1.3	3.5 ± 0.65	27.4	175.75 ± 1.2	0.1 ± 0.002	Tr	9.1 ± 1.03	3.8 ± 0.09	13.0
TA 1.25 % *v/v*	153.60 ± 2.3	1.0 ± 0.05	1.3 ± 0.04	77.8 ± 1.54	7.5 ± 1.01	87.6	142.85 ± 2.38	1.6 ± 0.09	1.2 ± 0.93	30.0 ± 2.31	27.4 ± 1.76	60.2
TA 2.5 % *v/v*	112.16 ± 0.96	2.6 ± 0.05	0.5 ± 0.05	44.7 ± 1.62	7.2 ± 0.92	55.0	109.05 ± 2.61	0.6 ± 0.05	0.2 ± 0.04	36.3 ± 1.25	21.6 ± 1.97	58.7
TH 1.25 % *v/v*	121.16 ± 1.04	0.64 ± 0.04	0.5 ± 0.02	45.1 ± 1.03	1.9 ± 0.65	47.14	133.72 ± 1.52	0.1 ± 0.04	2.0 ± 0.09	47.2 ± 2.84	33.9 ± 2.31	83.2
TH 2.5 % *v/v*	103.38 ± 0.86	1.3 ± 0.11	1.2 ± 0.05	42.1 ± 1.65	8.2 ± 1.01	52.8	123.48 ± 1.04	0.6 ± 0.01	2.0 ± 0.07	40.0 ± 1.21	20.1 ± 1.55	62.7

Values represented as mean ± SD (five replicates). All elicitors were added in 25-day-old cultures. Initial inoculums at start of culture cycle: 3.0 g/flask in 40-mL medium

C content in cells, *M* content in spent media

[a] Traces

Table 4 Differential efficacy of various treatments on individual ginsenoside profiles within 5 days of elicitation in *P. quinquefolius* cultures

Elicitor treatments	Ginsenoside content (mg/L)										
	Panaxadiols							Panaxatriols			
		Rb1	Rb2	Rc	Rd	Rg3	Rh2	Re	Rg1	Rg2	Rh1
Control	C	0.2 ± 0.05[a]	Tr	nd	Tr	nd	nd	20.3 ± 0.1	2.8 ± 0.6	0.2 ± 0.05	0.2 ± 0.04
	M	nd	nd	nd	Tr	nd	nd	1.2 ± 0.2	1.9 ± 0.3	nd	nd
CN50	C	nd	0.2 ± 0.01	nd	Tr	0.2 ± 0.01	nd	25.0 ± 0.5	13.1 ± 0.1	0.5 ± 0.04	0.5 ± 0.06
	M	nd	nd	5.0 ± 0.08	Tr	0.2 ± 0.01	nd	2.1 ± 0.3	4.0 ± 0.5	nd	3.5 ± 0.05
CN100	C	nd	0.1 ± 0.003	nd	nd	0.2 ± 0.02	nd	18.9 ± 0.2	6.2 ± 0.3	0.9 ± 0.03	Tr
	M	nd	nd	nd	nd	nd	nd	2.4 ± 0.2	2.2 ± 0.3	nd	0.3 ± 0.07
HP50	C	0.8 ± 0.04	nd	0.4 ± 0.08	Tr	nd	nd	7.0 ± 0.6	2.3 ± 0.5	nd	Tr
	M	nd	nd	nd	Tr	nd	nd	2.2 ± 0.1	2.5 ± 0.1	nd	nd
HP100	C	nd	nd	nd	Tr	nd	nd	8.7 ± 0.1	3.6 ± 0.6	nd	nd
	M	nd	nd	1.6 ± 0.07	Tr	nd	4.1 ± 0.04	20.4 ± 0.7	1.1 ± 0.5	nd	nd
SNP75	C	0.4 ± 0.03	nd	nd	nd	nd	nd	9.8 ± 0.5	4.5 ± 0.8	0.1 ± 0.04	0.5 ± 0.04
	M	nd	nd	nd	nd	nd	nd	13.5 ± 0.4	12.1 ± 0.6	0.4 ± 0.03	5.2 ± 0.07
SNP100	C	nd	0.1 ± 0.003	nd	Tr	nd	nd	16.5 ± 0.1	4.8 ± 0.2	0.7 ± 0.05	nd
	M	nd	nd	nd	Tr	nd	nd	12.6 ± 0.1	7.0 ± 0.2	nd	5.7 ± 0.08
NI15	C	nd	nd	nd	nd	nd	nd	10.7 ± 0.8	8.1 ± 0.4	0.4 ± 0.04	0.3 ± 0.05
	M	nd	0.2 ± 0.06	nd	nd	nd	nd	13.4 ± 0.4	16.4 ± 0.2	0.2 ± 0.03	1.6 ± 0.04
NI20	C	nd	nd	nd	nd	nd	nd	8.7 ± 0.1	7.3 ± 0.6	0.4 ± 0.06	0.1 ± 0.04
	M	nd	nd	nd	nd	nd	nd	4.0 ± 0.1	6.0 ± 0.1	nd	6.4 ± 0.08
Control NB	C	0.2 ± 0.04	0.06 ± 0.01	nd	Tr	nd	nd	19.6 ± 0.6	2.5 ± 0.3	0.3 ± 0.09	0.2 ± 0.05
	M	nd	nd	nd	Tr	nd	nd	1.5 ± 0.5	1.5 ± 0.4	nd	nd
Cb8 1.25 %	C	Tr	0.1 ± 0.03	0.2 ± 0.04	Tr	nd	nd	17.2 ± 0.3	11.0 ± 0.1	0.5 ± 0.03	0.5 ± 0.02
	M	nd	nd	nd	Tr	nd	0.2 ± 0.01	6.1 ± 0.5	5.0 ± 0.1	nd	1.7 ± 0.07
Cb8 2.5 %	C	0.3 ± 0.03	0.2 ± 0.01	0.1 ± 0.01	Tr	nd	nd	26.2 ± 0.1	15.7 ± 0.5	0.2 ± 0.05	0.4 ± 0.03
	M	nd	nd	nd	Tr	nd	0.1 ± 0.03	14.7 ± 0.7	8.2 ± 0.2	nd	nd
Pf6 1.25 %	C	nd	0.1 ± 0.003	0.2 ± 0.02	Tr	0.6 ± 0.05	0.1 ± 0.01	20.3 ± 0.5	12.5 ± 0.2	0.9 ± 0.08	0.4 ± 0.05
	M	0.9 ± 0.05	nd	nd	Tr	nd	0.1 ± 0.02	2.3 ± 0.4	2.1 ± 0.1	nd	0.7 ± 0.06
Pf6 2.5 %	C	nd	Tr	nd	Tr	0.6 ± 0.01	Tr	10.5 ± 0.1	6.5 ± 0.4	0.3 ± 0.03	0.7 ± 0.05
	M	nd	nd	nd	Tr	nd	Tr	3.5 ± 0.1	2.4 ± 0.7	nd	nd
Control PDB	C	0.2 ± 0.02	Tr	nd	Tr	nd	nd	20.4 ± 0.3	2.8 ± 0.4	0.3 ± 0.09	0.2 ± 0.06
	M	nd	nd	nd	Tr	nd	nd	1.8 ± 0.4	1.7 ± 0.1	nd	nd
TA 1.25 %	C	0.3 ± 0.04	nd	0.3 ± 0.1	Tr	0.4 ± 0.01	nd	48.8 ± 0.7	27.7 ± 0.2	0.9 ± 0.01	0.4 ± 0.01
	M	0.8 ± 0.07	nd	nd	Tr	0.3 ± 0.05	0.2 ± 0.01	3.6 ± 0.1	2.4 ± 0.1	0.6 ± 0.01	0.9 ± 0.08
TA 2.5 %	C	0.6 ± 0.02	nd	nd	Tr	0.2 ± 0.01	1.8 ± 0.05	22.2 ± 0.3	21.0 ± 0.2	0.8 ± .04	0.7 ± 0.04
	M	Tr	nd	nd	Tr	0.4 ± 0.06	0.1 ± 0.01	3.7 ± 0.5	2.6 ± 0.6	0.6 ± .03	0.3 ± 0.05
TH 1.25 %	C	0.14 ± 0.02	0.1 ± 0.01	0.1 ± 0.03	Tr	0.3 ± 0.01	nd	22.7 ± 0.2	22.2 ± 0.4	Tr	0.2 ± 0.05
	M	nd	Tr	0.2 ± 0.02	Tr	0.3 ± 0.05	nd	0.7 ± 0.1	0.9 ± 0.2	Tr	0.3 ± 0.06
TH 2.5 %	C	Tr	Tr	0.1 ± 0.03	Tr	0.4 ± 0.02	0.8 ± 0.01	26.2 ± 0.5	15.4 ± 0.3	0.3 ± 0.04	0.2 ± 0.04
	M	0.9 ± 0.07	nd	nd	Tr	nd	0.3 ± 0.02	4.5 ± 0.2	3.0 ± 0.4	0.2 ± 0.05	0.5 ± 0.09

Key to treatment code as mentioned in the "Materials and methods" section

nd not detected, *Tr* traces, *C* Cellular content, *M* medium content

[a] Mean ± S.D

compared with samples analyzed after 5-day exposure, except in case of 75 μM sodium nitroprusside treatment that continued to support high level of ginsenosides recovery (37.5 mg/L) from the cells and the spent medium.

Besides total saponin production, the different elicitor treatments also showed a profound effect on the synthesis and exudation of the individual major or minor ginsenoside constituents (Tables 4 and 5). For example,

Table 5 Differential efficacy of various treatments on individual ginsenosides profiles within 15 days of elicitation in *P. quinquefolius* cultures

Elicitor treatments	Ginsenoside content (mg/L)										
	Panaxadiols							Panaxatriols			
		Rb1	Rb2	Rc	Rd	Rg3	Rh2	Re	Rg1	Rg2	Rh1
Control	C	0.1 ± 0.01[a]	nd	nd	Tr	nd	nd	6.3 ± 0.4	2.4 ± 0.1	0.1 ± 0.05	Tr
	M	nd	nd	nd	Tr	nd	nd	2.2 ± 0.5	1.5 ± 0.4	nd	nd
CN50	C	nd	nd	nd	Tr	0.1 ± 0.01	nd	11.5 ± 0.7	6.3 ± 0.1	0.1 ± 0.02	0.2 ± 0.01
	M	nd	nd	1.1 ± 0.01	Tr	0.2 ± 0.01	nd	1.1 ± 0.7	1.2 ± 0.1	nd	2.3 ± 0.05
CN100	C	nd	nd	nd	Tr	0.2 ± 0.02	nd	8.4 ± 0.2	2.1 ± 0.2	0.5 ± 0.03	Tr
	M	nd	nd	nd	Tr	nd	nd	0.6 ± 0.2	0.6 ± 0.1	nd	0.2 ± 0.02
HP50	C	0.9 ± 0.02	nd	0.1 ± 0.02	nd	nd	nd	2.4 ± 0.6	3.8 ± 0.1	nd	Tr
	M	nd	nd	nd	nd	nd	nd	1.0 ± 0.4	1.3 ± 0.4	nd	nd
HP100	C	nd	nd	nd	Tr	nd	nd	4.6 ± 0.7	5.5 ± 0.1	nd	nd
	M	nd	nd	0.5 ± 0.09	Tr	nd	2.4 ± 0.03	10.3 ± 0.8	0.5 ± 0.1	nd	nd
SNP75	C	0.2 ± 0.03	nd	nd	nd	nd	nd	7.2 ± 0.5	2.8 ± 0.4	nd	0.2 ± 0.03
	M	nd	nd	nd	nd	nd	nd	16.0 ± 0.4	8.4 ± 0.5	0.4 ± 0.04	2.3 ± 0.07
SNP100	C	nd	nd	nd	Tr	nd	nd	11.7 ± 0.5	2.1 ± 0.1	0.3 ± 0.05	nd
	M	nd	nd	nd	Tr	nd	nd	4.6 ± 0.1	3.1 ± 0.1	nd	4.6 ± 0.06
NI15	C	nd	nd	nd	Tr	nd	nd	4.8 ± 0.1	4.3 ± 0.3	0.2 ± 0.07	0.1 ± 0.03
	M	nd	0.2 ± 0.06	nd	Tr	nd	nd	7.5 ± 0.3	12.1 ± 0.6	0.1 ± 0.06	2.4 ± 0.03
NI20	C	nd	nd	nd	Tr	nd	nd	2.6 ± 0.2	5.1 ± 0.3	0.1 ± 0.03	Tr
	M	nd	nd	nd	Tr	nd	nd	1.0 ± 0.1	2.6 ± 0.4	nd	3.7 ± 0.06
Control NB	C	0.1 ± 0.01	nd	nd	Tr	nd	nd	5.9 ± 0.1	2.3 ± 0.1	0.1 ± 0.07	Tr
	M	nd	Tr	nd	Tr	nd	nd	2.4 ± 0.1	1.2 ± 0.4	nd	nd
Cb8 1.25 %	C	0.3 ± 0.02	nd	Tr	Tr	nd	nd	17.9 ± 0.4	15.0 ± 0.4	0.2 ± 0.04	0.2 ± 0.07
	M	nd	nd	nd	Tr	nd	Tr	7.4 ± 0.7	7.2 ± 0.5	nd	1.1 ± 0.02
Cb8 2.5 %	C	0.2 ± 0.06	nd	Tr	Tr	nd	nd	12.8 ± 0.1	13.6 ± 0.1	0.2 ± 0.05	0.4 ± 0.03
	M	nd	nd	nd	Tr	nd	nd	3.2 ± 0.1	4.8 ± 0.3	nd	nd
Pf6 1.25 %	C	nd	nd	Tr	Tr	0.5 ± 0.01	Tr	16.3 ± 0.3	11.2 ± 0.1	0.1 ± 0.04	Tr
	M	Tr	nd	nd	Tr	nd	Tr	12.4 ± 0.3	10.4 ± 0.3	0.2 ± 0.04	nd
Pf6 2.5 %	C	0.2 ± 0.02	Tr	nd	Tr	0.7 ± 0.02	Tr	20.3 ± 0.5	13.4 ± 0.6	0.2 ± 0.06	0.5 ± 0.08
	M	nd	nd	nd	Tr	nd	0.3 ± 0.1	12.4 ± 0.3	8.9 ± 0.1	nd	nd
Control PDB	C	0.1 ± 0.01	Tr	nd	Tr	nd	nd	6.5 ± 0.1	2.5 ± 0.1	0.1 ± 0.02	Tr
	M	nd	nd	nd	Tr	nd	nd	2.7 ± 0.1	1.1 ± 0.2	nd	nd
TA 1.25 %	C	0.9 ± 0.04	0.3 ± 0.01	Tr	Tr	0.4 ± 0.01	nd	13.0 ± 0.4	15.2 ± 0.1	0.4 ± 0.07	1.3 ± 0.02
	M	0.1 ± 0.07	0.2 ± 0.01	0.7 ± 0.06	Tr	Tr	0.2 ± 0.01	4.6 ± 0.4	20.3 ± 0.5	Tr	2.5 ± 0.08
TA 2.5 %	C	0.4 ± 0.02	Tr	nd	Tr	Tr	0.2 ± 0.01	18.4 ± 0.4	17.4 ± 0.5	0.5 ± .04	Tr
	M	Tr	0.2 ± 0.03	nd	Tr	Tr	nd	4.7 ± 0.2	16.7 ± 0.1	Tr	0.2 ± 0.03
TH 1.25 %	C	nd	0.1 ± 0.01	Tr	Tr	nd	nd	26.7 ± 0.2	18.3 ± 0.3	0.4 ± 0.04	1.8 ± 0.01
	M	1.3 ± 0.04	nd	Tr	Tr	nd	0.6 ± 0.04	16.0 ± 0.2	11.4 ± 0.1	Tr	6.5 ± 0.08
TH 2.5 %	C	nd	nd	Tr	Tr	nd	0.6 ± 0.01	26.2 ± 0.8	13.2 ± 0.3	0.3 ± 0.02	0.2 ± 0.04
	M	nd	nd	1.9 ± 0.05	Tr	nd	0.1 ± 0.02	12.4 ± 0.7	6.9 ± 0.6	nd	0.8 ± 0.06

Key to treatments code as mentioned in the "Materials and methods" section

nd not detected, *Tr* traces, *C* cellular content, *M* medium content

[a] Mean ± SD

among the panaxadiol constituents, ginsenoside Rb1, detected only in the cellular extracts, was best synthesized in cultures that were treated with 50 μM hydrogen peroxide (0.8 mg/L). Ginsenoside Rb2, in general, was produced in very less amount in all the treatments. The panaxadiol constituent Rc that was not detected in the control

Table 6 Intercorrelations (Pearson's coefficient) between the biomass and ten analyzed ginsenosides in *P. quinquefolius* cell suspensions after 5 and 15 days of elicitor treatment

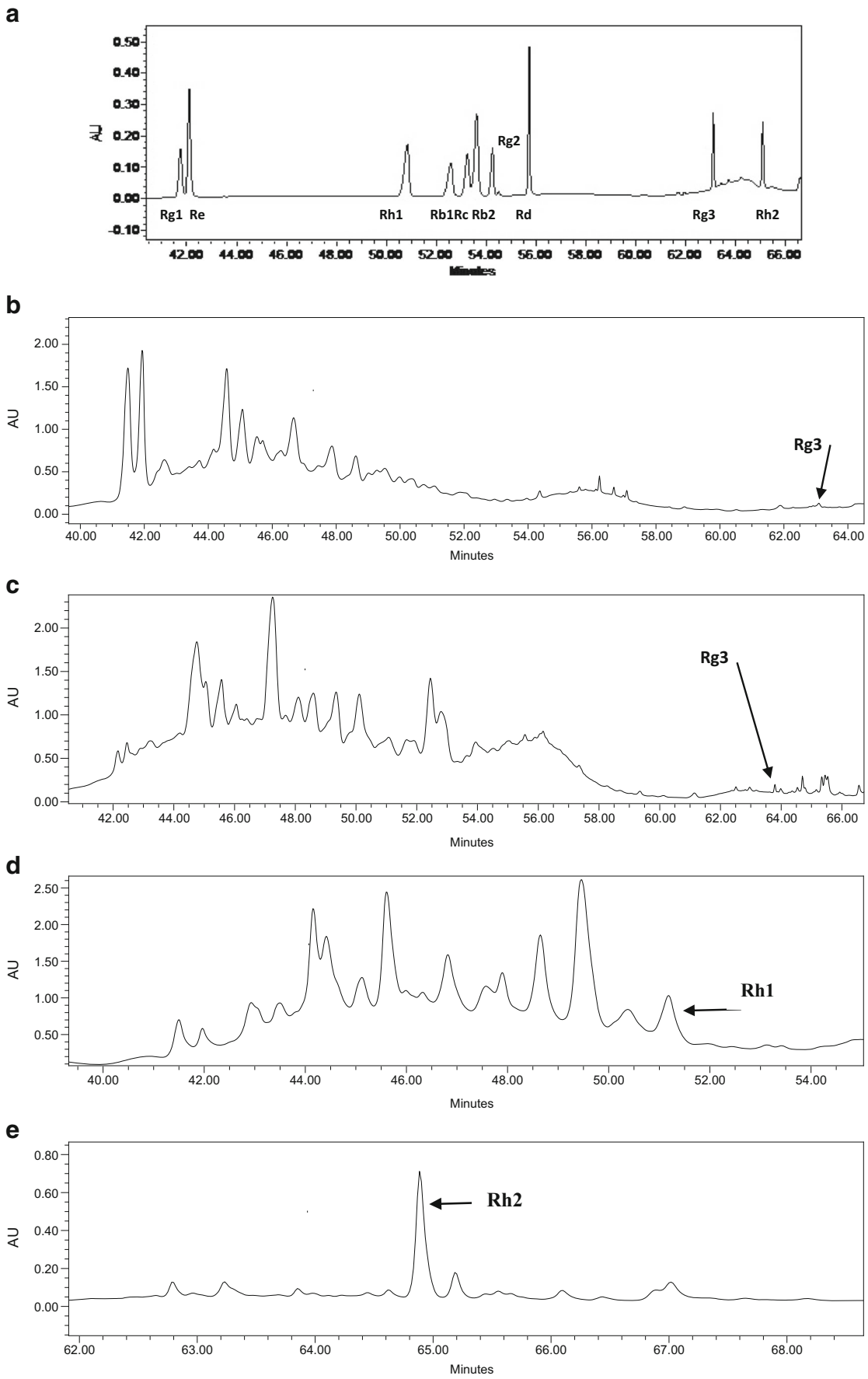
	Rb1	Rb2	Rc	Rd	Re	Rg1	Rg2	Rg3	Rh1	Rh2	CGC	(BY)
After 5 days of elicitor treatment												
Rb1	1.000	−0.121	−0.232	−0.023	0.352	0.305	0.411	0.518*	−0.330	−0.002	0.331	−0.174
Rb2		1.000	0.362	0.306	0.144	0.446	0.049	0.294	−0.145	−0.317	0.344	0.231
Rc			1.000	−0.035	0.062	−0.035	−0.091	0.176	0.143	0.140	0.135	0.005
Rd				1.000	0.316	0.290	−0.044	−0.065	−0.015	−0.159	0.359	−0.022
Re					1.000	0.646**	0.380	0.349	−0.004	0.117	0.901**	0.067
Rg1						1.000	0.522*	0.592*	0.058	−0.176	0.871**	0.052
Rg2							1.000	0.719**	0.042	−0.065	0.521*	−0.033
Rg3								1.000	−0.257	0.075	0.552*	−0.336
Rh1									1.000	−0.295	0.048	0.547*
Rh2										1.000	0.044	−0.462
CGC											1.000	0.042
BY												1.000
After 15 days of treatment												
Rb1	1.000	0.635**	−0.039	0.262	0.463	0.714**	0.447	0.534*	0.631**	0.097	0.700**	0.021
Rb2		1.000	0.108	0.528*	0.068	0.687**	0.454	0.752**	0.247	−0.048	0.432	0.089
Rc			1.000	0.026	0.285	0.115	−0.056	0.267	−0.010	0.074	0.236	−0.187
Rd				1.000	0.175	0.460	0.083	0.276	−0.134	−0.184	0.315	0.238
Re					1.000	0.653**	0.371	0.030	0.270	0.097	0.909**	−0.045
Rg1						1.000	0.488*	0.460	0.229	−0.045	0.897**	−0.025
Rg2							1.000	0.460	0.378	−0.328	0.492*	0.095
Rg3								1.000	0.271	−0.098	0.302	−0.126
Rh1									1.000	−0.041	0.381	0.267
Rh2										1.000	0.051	−0.392
CGC											1.000	−0.022
BY												1.000

BY biomass yield, *CGC* total ginsenoside content

*$P < 0.05$; **$P < 0.01$

cultures was most strongly elicited when 50 μM Cobalt nitrate treatment was applied (5.0 mg/L), followed by treatment with 100 μM hydrogen peroxide (1.6 mg/L). In addition to Rc, the cobalt nitrate treatment also stimulated the synthesis of Rg3 to limited extent (0.4 mg/L). Rh2 diol that was not present in any of the treatments was produced in a significant amount in cultures treated with hydrogen peroxide at 100 μM dose within 5 days of exposure (Table 4 and Fig. 1e). Interestingly, ginsenosides Rc and Rh2 synthesized in above two treatments also completely leached out into the spent medium. It is pertinent to high light here that Rg3 and Rh2 are minor panaxadiols that are generally absent in field grown ginseng roots. Ginsenoside Rd, on the other hand, was detected only in trace amounts (<0.05 mg). An interesting variation was observed with cobalt nitrate at 50-μM dose that led to a concurrent detection of both Rg3 and Rd (synthesized from Rg3 via UDP-glycosyl transfer) in extracts obtained from either cells or spent media. Higher dose of this elicitor selectively synthesized only Rg3 in the cells (0.2 mg/L).

Among the individual panaxatriol, Re and Rg1 were the major ginsenoside fractions (>90 % of total) in *P. quinquefolius* cell suspensions under all elicitation treatments. All elicitors also facilitated their simultaneous exudation into the media but to varying degrees. The total Re yield in cells and medium in cultures treated with 50 μM cobalt nitrate, 100 μM hydrogen peroxide, 100 μM sodium nitropruside, and 15 μM nickel sulphate was comparable (23–28 mg/L) but the latter three elicitation doses proved better in terms of Re release into the spent medium (Table 4). Similar pattern of production and leaching was observed for panaxatriol ginsenoside Rg1. Maximum Rg1 elicitation was observed within 5 days of


a
AU
Rg1 Re
Rh1
Rb1Rc Rb2
Rg2
Rd
Rg3
Rh2
Minutes
b
AU
Rg3
Minutes
c
AU
Rg3
Minutes
d
AU
Rh1
Minutes
e
AU
Rh2
Minutes

◀ **Fig. 1** **a** HPLC chromatogram of ten authentic ginsenoside; **b**, **c** Rg3 peak identification in cells and medium samples following 5-day elicitation with 1.25 % culture filtrate of *T. atroviridae*; **d** Rh1 peak identification in spent medium sample after 5-day elicitation with 20-μM dose of nickel sulphate; **e** HPLC peak identification of Rh2 in spent media after 5 days of 100 μM hydrogen peroxide treatment

treatment with 15 μM nickel sulphate (24.5 mg/L in cells and medium combined) that was slightly more than in 50 μM cobalt nitrate–treated cultures (17.1 mg/L). However, the former treatment afforded four times more leaching of Rg1 into the media. The ginsenoside Rg2 yield was also highest in cultures elicited with cobalt nitrate but at a higher dose (100 μM) exposure for 5 days. The synthesized Rg2 thus produced in cells was released into the medium upon prolongation of culture cycle to 40 days. The synthesis of triol fraction Rh1 was highest (6.5 mg/L) in 20 μM nickel sulphate (Fig. 1d) treatment that was exclusively released into the medium. Similar trends were also observed with 75 and 100 μM sodium nitroprusside. Prolongation of the elicitor exposure duration to 15 days led to a general decrease in all individual panaxadiol as well as panaxatriol ginsenosides content and hence was not found suitable.

Effect of biotic elicitors

Exposure of cultures to biotic elicitors for 5 days did not significantly alter the pattern of biomass gains, but in 15-day treatments, they caused 25–30 % of decrease in biomass when compared with the respective nutrient broth controls (Table 3). Among the four biotic elicitors used, the most spectacular enhancement in ginsenoside productivity within 5 days of treatment was visible when culture filtrate of *T. atroviridae* at 1.25 % (*v*/*v*) dose was used. The total ginsenosides recovery in this treatment was 87.6 mg/L that was followed by 66.3 mg/L yield in cultures exposed to CF of *P. monteili* (2.5 % *v*/*v*). The ginsenoside production in rest of the biotic elicitor treatments for 5 days consistently ranged between 41.3 and 55.0 mg/L in comparison to 25.9 and 27.4 mg/L in the nutrient broth control media. Culture filtrate of *B. circularans* (2.5 % *v*/*v*) was the only biotic elicitor treatment that caused a minor decline in total ginsenoside recovery (24.6 mg/L). Like abiotic elicitor treatments, the bulk of the total ginsenoside content (>90 %) under biotic elicitations was accounted for by the synthesis of panaxatriols than their diol counterparts. The productivity levels of total diols in biotic elicitor treatments ranged between 0.2 and 2.6, and 0.5 and 1.3 mg/L in cells and spent medium, respectively. However, unlike abiotic elicitors, the biotic elicitation treatments for 5 days tend to favor more retention of biosynthesized panaxatriol and panaxadiol ginsenosides (>85 %) in the cells than their release into the left over medium. Prolongation of the elicitation duration to 15 days indicated that culture filtrate of *T. harzianum* at 1.25 % *v*/*v* dose treatment that was least effective after 5 days treatment afforded the highest accumulation of total ginsenosides (83.2 mg/L) that was comparable to the treatment of 1.25 % *v*/*v* *T. atroviridae* after 5 days. However, this *T. harzianum* treatment also significantly improved the leaching of both diol and triol ginsenosides into the medium (Table 3).

Analogous to abiotic treatments, the biotic elicitation treatments presented a very different profile of individual ginsenoside components (Tables 4 and 5). The yield of the three panaxadiol ginsenosides (Rc, Rg3, and Rh2) was significantly less than what was achieved with the use of abiotic elicitors. While Rc was detected at 0.1 to 0.2 mg/L level in only 5-day exposure treatments, Rg3 production was favored by treatment of culture filtrates of both *Trichoderma* species (0.4–0.7 mg/L). Rg3 was almost equally distributed in cell and medium extracts (Fig. 1a–c). Rh2 accumulation was induced by all the biotic elicitors, maximum being when 2.5 % *v*/*v* of *T. atroviridae* culture filtrate was employed for 5 days (Table 4). As opposed to preferential production of either Rb1 or Rb2 with abiotic elicitors, biotic elicitation led to simultaneous production of both these diol ginsenosides. Rb1 production was highest (1.335 mg/L) when 1.25 % *v*/*v* culture filtrate of *T. harzianum* was applied for 15 days (Table 5). However, increasing the dose to 2.5 % *v*/*v* led to a comparable recovery of this diol within 5 days. Extensive Rb2 production was observed when cell suspensions were exposed to culture filtrate of *T. atroviridae* (1.25 % *v*/*v*) for 15 days. Ginsenoside Rd production was observed only in traces.

Among the panaxatriols, total Re elicitation was most pronounced (52.4 mg/L) when 1.25 % *v*/*v* dose of *T. atroviridae* culture filtrate was used (Table 4). It was 2.5-fold higher than in the non-treated control cultures (21.5 mg/L). Only minimum amount of Re showed its release into the medium in both these sets of cultures. When, in place of *T. atroviridae*, the culture filtrate of *T. harzianum* was employed as a biotic elicitor, more synthesis and leaching of Re was noticed after 15 days of treatment. Bacterial elicitation treatments with *B. circularans* and *P. monteili* culture filtrate also favored Re production with similar trends of leaching upon delayed harvesting. In comparison to the control cultures, the productivity of triol component Rg1 was 7–8-fold higher (30–35 mg/L) under both the elicitation doses of *T. atroviridae* after 15 days of exposure (Table 5). Treatment with *T. harzianum* culture filtrate, on the other hand, was effective (29.7 mg/L) only at lower elicitation dose of 1.25 % *v*/*v* after 15 days. Similar trend in Rg1 synthesis was also evident with bacterial elicitors. The synthesis of third analyzed panaxatriol component Rg2 was upregulated by *T. atroviridae* elicitor doses, but its release into the spent medium was more (40–50 % of total) in only 30-day-old cultures. In vitro production of Rh1 was preferentially

favored by prolonged exposure to lower dose of *T. harzianum* culture filtrate and >80 % of the biosynthesized Rh1 leached out into the medium (6.5 mg/L). The bacterial elicitors were not found effective in enhancing the Rh1 yield *in vitro*.

Correlation studies between biomass, total, and individual ginsenosides yields

The linear correlations calculated between biomass and ten analyzed individual ginsenosides after 5 and 15 days of all elicitation treatments are summarized in Table 6. Some interesting inferences could be made from this statistical analysis that largely corroborated with the data obtained under different elicitation treatments as described above. The panaxatriols Re, Rg1, and Rg2 that formed the bulk of the total saponin fraction showed a strong positive correlation with the total ginsenoside productivity after both 5 ($r=0.901$, 0.871, and 0.521) and 15 days ($r=0.909$, 0.897, and 0.492) exposure of cells to various elicitation regimes, respectively. Ginsenosides Re and Rg1 also indicated a positive correlation among themselves at both stages of harvest ($r=0.646$ and 0.653). Among the panaxadiols fractions, Rb1 and Rg3 were positively correlated ($r=0.518$, 0.534) in 5- and 15-day treatments. Such positive correlations among Re and Rg1, and Rg3 and Rb1 was expected based on their back-to-back synthesis in the biogenetic route via UDP-glycosylation of OH^- groups at different carbon positions in the dammaranediol skeleton. A positive correlation between diol moieties Rb1 and Rb2 with the triol component Rg1 ($r=0.714$ and 0.687, respectively) after 15-day elicitation treatments also suggested that an increased flux toward protopanaxadiol pathway may have supported their subsequent conversion toward protopanaxatriol skeletons. Alternately, since conversion of Rh1 to Rg1 and Rd to Rb1 or Rb2 involves a glycosyl transfer at the same C-20 position, it is likely that effective elicitor treatments were upregulating the common UDP-glycosyl transferases system to channelize the flux toward both diol and triol classes of ginsenosides in our cultures. No analyzed ginsenoside except Rh1 ($r=0.547$) showed any significant correlation with biomass accumulation in this study.

Discussion

The wealth of information accumulated over last two decades has suggested that *Panax* species are fairly amenable systems for developing cell-culture-based alternate production platforms for their high-value bioactive molecules—ginsenosides (Huang and Zhong 2013; Zhang et al. 2012; Huang et al. 2013). The biosynthetic pathway leading to the formation of different types of ginsenosides can not only be expressed in vitro but can also be manipulated by various abiotic and biotic treatments. The present study on in vitro production of ginsenosides in abiotically and biotically elicited cell suspensions of *P. quinquefolius* largely conforms to the research trends available in other *Panax* species (Rahimi et al. 2015b). Besides generating excellent base material for upscaling the customized production and recovery of different types of ginsenosides under specific elicitation regimes and harvesting schedules, our study has also highlighted the efficacy of certain treatments comprising of *T. atroviridae* culture filtrate or hydrogen peroxide that could also induce the biosynthesis of two anti-neoplastic ginsenoside constituents Rg3 and Rh2 that have so far been not found in plant roots of any *Panax* species. It is likely that these specific elicitation treatments were able to restrict the biosynthetic pathway branch to Rd, Rb1, and Rb2 that operates in the plant roots as indicated earlier by Wang et al. (2015) and Park et al. (2010) who resolved to microbial biotransformation options to obtain Rg3 and Rh2 from authentic standards of Rd and Rb1.

Culture filtrate and extract of several *Pseudomonas*, *Bacillus*, and *Trichoderma* species have been extensively used to elicit secondary metabolism in several plant systems (Algar et al. 2012; Chodisetti et al. 2013; Prasad et al. 2013; Qianliang et al. 2013; Awad et al. 2014;Verma et al. 2014). It is presumed that these microbial preparations are rich in chitosans and glucans and hence mimic a disease response that in turn triggers the plant defense machinery via NO-signaling and secondary metabolism. Our study also suggested that in case of *P. quinquefolius* cell suspensions, biotic elicitors of fungal and bacterial origin at different doses were effective in enhancing the accumulation of panaxatriol group of ginsenosides (Re and Rg1, Rg2 and Rh1) whereas the synthesis and leaching of panaxadiol constituents (Rg3, Rh2, and Rc) in the medium was preferentially favored by abiotic elicitors, particularly nickel sulphate and hydrogen peroxide. The abiotic elicitors used in our study were found effective within 5 days of exposure for all the ginsenosides. Biotic elicitors, however, exhibited a variable scenario. The best elicitation treatment in terms of total ginsenoside yield (87.3 mg/L with 52.5 mg/L Re and 30.4 mg/L Rg1) was afforded within 5 days by elicitor treatment comprising of 1.25 % *v*/*v* *T. atroviridae* culture filtrate. However, 15-day prolonged exposure to *T. harzianum* culture filtrate was more effective for production as well as leaching of these two triols along with Rh1. As indicated earlier, auto-leaching of a desired metabolite into the spent medium has an important industrial consideration as it not only offers an economically more viable non-destructive mode of metabolite recovery but also promotes the development of open flowing cultivation system that further adds to the reduction of production cost (Cai et al. 2012). In the absence of auto-leaching tendency of the cultured cells, different workers have used other strategies to induce cell lysis for this purpose. Reports on increasing the osmolarity of the medium by addition of excess sucrose or inducing membrane

fluidity by pH shocks to the cells are available to leach camptothecin in *Nothapodytes nimmoniana* cell suspensions (Karwasara and Dixit 2013) and azadirachtin from *Azadirachta indica* hairy roots (Satdive et al. 2007). Since, in addition to the involvement of membrane transporters, the passive metabolite exudation from cell also depends upon the molecular size of the compound (Cai et al. 2012), it seems likely that leaching of Rg1, Rg2, Rh2, and Rh1 from *P. quinquefolius* cells in this study was favored because of their smaller size with less glycosylated sugar chains as compared to Rb1 and Rb2 and allowed them to diffuse past the lipid membrane across a concentration gradient. The elicitor regimes used by us might have supplemented this leaching tendency by increasing the membrane fluidity and upregulation of ABC-type transporters or H^+ gradient mechanisms in the cultured cells as was proposed for resveratrol and viniferin exudation in cell cultures of *Vitis vinifera* (Santamaria et al. 2011).

The two heavy metal abiotic elicitors, namely, cobalt nitrate and nickel sulphate, used in the present investigation have not been used in any earlier cell culture study in *Panax* species. Only one report on use of nickel salts to influence ginsenoside accumulation in hairy root cultures of *P. ginseng* exists in literature wherein a 1.4-fold increment was observed (Jeong and Park 2006). Among the two heavy metal salts used in the present investigation, 50 μM of cobalt nitrate and 15-μM doses of nickel suphate showed maximum elicitation of total ginsenoside recovery. A dose of 50 μM of cobalt nitrate was particularly effective in enhancing the yield of panaxadiol Rc. In comparison to this, the third abiotic elicitor hydrogen peroxide used by us did not lead to any substantial elicitation of ginsenoside pathway except in inducing the leaching of ginsenoside Rh2 by a possible repression of downstream glycosyl transfer to ginsenoside Rd and other diols. Heavy metals and other abiotic elicitors are known to influence the plant secondary metabolic pathways as signaling precursors through the action of NADPH oxidase under the influence of oligalacturonic-acid-like modulators that have been implicated in enhanced ginsenoside production in cell cultures of *P. ginseng* (Hu et al. 2003b; Yu et al. 2005; Huang and Zhong 2013).

It may be concluded from the above discussion that abiotic or biotic elicitation of plant secondary metabolism in cell cultures largely follows a non-specific pattern. This may be because the cellular mechanism(s) operative at the cell surface for recognizing an elicitor may vary in different sets of condition (Zhao et al. 2005). This may result in non-exclusivity/specificity of a particular elicitor to influence the synthesis of even similar types of molecules in all plant systems. Our present understanding level about the mode of cellular actions of these elicitors is still meager. Some of the more likely factors associated with the manifestation of elicitor influence may include trans-membrane activation of G-protein kinases leading to ROS generation and cytoplasmic acidification (Yoshikawa et al. 1983; Schmidt and Ebel 1987; Vasconsuelo and Boland 2007). Other important cellular event linked with elicitor binding may be the ion fluxes such as K^+/H^+ exchange and Ca^{2+} influx that act as second messenger in signal transduction mechanism that in turn switches on the pathway specific transcription factor(s) for the global expression of a multi-step secondary metabolic route (Zhao et al. 2005). Ca^{2+} influx can also manifest its influence through ROS generation such as superoxides and H_2O_2. Since most of the secondary metabolites in plants are produced in response to various microbial threats and environmental stresses, involvement of NO in the signal transduction machinery has also been implicated in many elicitation studies with plant cell cultures. Further studies related to the molecular aspect behind the observed phenomena in this particular *P. quinquefolius* cell suspension line are underway.

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Compliance with ethical standards

Conflict of interest Tanya Biswas declares that she has no conflict of interest. Alok Kalra declares that he has no conflict of interest. A K Mathur declares that he has no conflict of interest. R K Lal declares that he has no conflict of interest. Manju Singh declares that she has no conflict of interest. Archana Mathur declares that she has no conflict of interest.

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Ethical compliance This article does not contain any studies with human participants or animals performed by any of the authors.

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