



# Enhanced expression of ginsenoside biosynthetic genes and in vitro ginsenoside production in elicited *Panax sikkimensis* (Ban) cell suspensions

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## Abstract

Dual metabolite, i.e., ginsenoside and anthocyanin, co-accumulating cell suspensions of *Panax sikkimensis* were subjected to elicitation with culture filtrates of *Serratia marcescens* (SD 21), *Bacillus subtilis* (FL11), *Trichoderma atroviridae* (TA), and *T. harzianum* (TH) at 1.25% and 2.5% v/v for 1- and 3-week duration. The fungal-derived elicitors (TA and TH) did not significantly affect biomass accumulation; however, bacterial elicitors (SD 21 and FL11), especially SD 21, led to comparable loss in biomass growth. In terms of ginsenoside content, differential responses were observed. A maximum of 3.2-fold increase (222.2 mg/L) in total ginsenoside content was observed with the use of 2.5% v/v TH culture filtrate for 1 week. Similar ginsenoside accumulation was observed with the use of 1-week treatment with 2.5% v/v SD 21 culture filtrate (189.3 mg/L) with a 10-fold increase in intracellular Rg2 biosynthesis (31 mg/L). Real-time PCR analysis of key ginsenoside biosynthesis genes, i.e., *FPS*, *SQS*, *DDS*, *PPDS*, and *PPTS*, revealed prominent upregulation of particularly *PPTS* expression (20–23-fold), accounting for the observed enhancement in protopanaxatriol ginsenosides. However, none of the elicitors led to successful enhancement in in vitro anthocyanin accumulation as compared to control values.

**Keywords** *Panax sikkimensis* · Cell suspension · Ginsenoside · *Trichoderma* · Elicitation

## Introduction

Ginseng has been revered as a valued oriental herb in traditional systems of Chinese healing, most often used to restore the opposing Yin and Yang energy balance of the human body.

Also known as “Man root,” ginseng was an important constituent of ancient medicinal potions as a revitalizer, aphrodisiac, and to restore youth and vitality (Court 2000). Even today, ginseng, particularly roots, is a crucial component of pharmaceutical preparations for its immunomodulatory, anti-aging, anti-neoplastic, anti-stress, and anti-diabetic activities (Christensen 2009). The world ginseng market was estimated at 2040 million US dollars in 2013, indicating the extensive global demand of this herb (Baeg and So 2013).

The supply of the roots of this “elixir” herb is mostly dependent on traditional agricultural output of *Panax ginseng* (Korean ginseng) and *P. quinquefolius* (American ginseng) from China, Korea, Vietnam, and the USA. Currently, the best quality ginseng is obtained from Ontario, Canada. The extensive range of bioactivities exhibited by ginseng is attributed to triterpenoid dammaranes, i.e., ginsenosides, found predominantly in roots and to some extent in the leaves and berries (Christensen 2009). Synthesized via the mevalonic acid (MVA) pathway in the cytosol, ginsenosides are broadly classified as 20(S) protopanaxadiols (Rb1, Rb2, Rc, Rd, Rg3,

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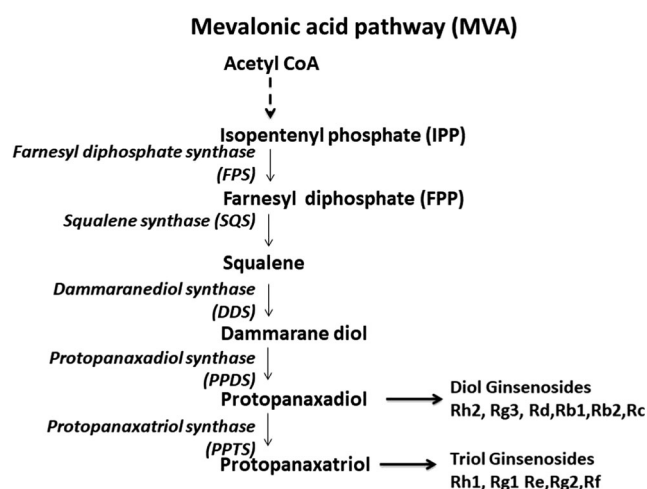
Rh2) and 20(S) protopanaxatriols (Re, Rg1, Rg2, Rh1), based on the differences in their aglycone chemical structures. The overall biosynthetic pathway leading to production of the protopanaxadiol and protopanaxatriol backbone is described in Fig. 1. In brief, acetyl CoA is converted to IPP via a series of enzymatic reactions following which farnesyl pyrophosphate synthase (*FPS*) catalyzes the synthesis of IPP to FPP. Squalene synthase (*SQS*) catalyzes the conversion of farnesyl pyrophosphate (FPP) to squalene. Specifically, *SQS* is generally considered as a crucial branch-point enzyme and a potential regulatory point controlling the carbon flux (Lee et al. 2004; Robinson et al. 1993). Squalene undergoes cyclization to form 2,3-oxidosqualene, and it is diverted to the dammarane-type ginsenoside biosynthesis pathway, initiated by formation of dammarenediol skeleton by dammarenediol-II synthase (*DDS*). *DDS* catalyzes the cyclization of oxidosqualene to dammarenediol-II and is regarded as a rate-limiting enzyme (Tansakul et al. 2006) for ginsenoside biosynthesis. Protopanaxadiol synthase (*PPDS*) and protopanaxatriol synthase (*PPTS*), belonging to a family of cytochrome P450-dependent monooxygenases, catalyze the conversion of the dammarane skeleton to protopanaxadiol (PPD) and protopanaxatriol (PPT) aglycone respectively. The different ginsenosides are then synthesized from these PPD and PPT aglycones via subsequent glycosylations at C3/C20 positions for the protopanaxadiols and C6/C20 positions for the protopanaxatriols.

Traditional cultivation of ginseng has long been riddled with complications such as stringent soil and climate pre-requisites. Ginseng cultivations are extremely susceptible to pest and pathogen attacks. The biggest obstacle for pharma industries to procure ginsenosides via organized ginseng cultivation is the long gestation gap of 5–6 years prior to economic harvesting. Consequently, ginseng biotechnologists have been investigating the potential of in vitro ginsenoside production

via cell cultures such as cell suspensions, hairy roots, and adventitious roots (Wu and Zhong 1999), as alternate ginsenoside production pedestals for sustained metabolite procurement. However, in spite of efforts spanning decades, commercial success is limited, probably due to low cell stability, low in vitro ginsenoside production, and a sketchy knowledge of their biosynthesis routes. The complications associated with cell cultures in terms of inherently low secondary metabolite production may be reasonably solved through elicitation strategies (Dörmenburg and Knorr 1995). Exogenous application of abiotic (heavy metal salts, signaling messengers such as salicylates and jasmonates, etc.) or biotic (derived from plant/animal origin, chitosan, oligogalacturonic acids, etc.) stress-inducing factors often trigger a cascade of signaling events that lead to downstream activation of defense-related gene expression and ultimately improved production of these secondary metabolites (Rao and Ravishankar 2002).

Ginseng cell cultures, particularly those of *P. ginseng*, have been extensively exploited for elicitation-based enhancement in ginsenoside biosynthesis. Methyl jasmonate is the most widely used elicitor, which has been reported to exhibit a range of responses in terms of upregulating ginsenoside biosynthesis (Kim et al. 2004; Thanh et al. 2005; Paek et al. 2009; Wang et al. 2013) in a host of ginseng tissues such as cell suspensions, adventitious roots, and hairy roots. Exogenous addition of heavy metals such as vanadate (Huang and Zhong 2013) and organic germanium (Yu et al. 2005) and compounds such as salicylates, hydrogen peroxide (Hu et al. 2003), sodium nitroprusside (SNP), and DCCD (Huang et al. 2013) have also been reported to enhance ginsenoside accumulation within cell cultures. Xu et al. (2005) reported enhanced saponin biosynthesis via a fungal elicitor from *Colletotrichum* in *P. ginseng*. Literature scans also reveal that majority of elicitation studies have been attempted in *P. ginseng*. Elicitation of *P. quinquefolius* system is comparatively less investigated with a few reports on ginsenoside enhancement using lactalbumin hydrolysate (Wang et al. 2011), cobalt nitrate, and SNP elicitation (Biswas et al. 2016) among abiotic elicitors. Recently, ginsenoside enhancements in *P. quinquefolius* cultures were observed using elicitors from *Trichoderma* (Biswas et al. 2016), *Aspergillus* (Li et al. 2016), and pathogenic fungus (Yu et al. 2016).

In the past decade, in vitro cultures of *Panax notoginseng* (Wang et al. 2005; Hu and Zhong 2008), *Panax vietnamensis* (Thanh et al. 2007), and *Panax japonicus* (Smolenskaya et al. 2007) have also been exploited for their ginsenoside production potential. The North Eastern Himalayan region of India is a rich biodiversity hotspot and is home to many ginseng species such as *P. sikkimensis*, *P. assamicus*, *P. bipinnatifidus*, *P. pseudoginseng*, and *P. sokpayensis* (Mehta and Haridasan 1992). Very few reports regarding study of these Indian ginseng species exist which mostly focus on cytological and taxonomic aspects (Sharma et al. 2010; Sharma and Pandit 2011). Literature dealing with tissue culture studies and



**Fig. 1** Biosynthetic pathway operative for production of the protopanaxadiol and protopanaxatriol aglycones for ginsenoside biosynthesis

in vitro ginsenoside production potential of these species is sparse (Mathur et al. 2010; Biswas et al. 2015a, 2015b; Gurung et al. 2018). *P. sikkimensis* is a perennial rhizomatous herb that grows in the conifer-rhododendron forests of Eastern Himalayas and is naturally distributed between elevations of 2600 and 4000 m. Recognizing the need for alternate production of ginsenosides via cell cultures of these Indian ginseng congeners, the host laboratory at CSIR-CIMAP had earlier reported and patented a purple-pigmented anthocyanin-producing callus line by cell aggregate selection method in this species (Mathur et al. 2002:US Patent No. 6368860; Mathur et al. 2010). The laboratory has also reported the dual metabolite, i.e., ginsenoside and co-accumulation of anthocyanins in cell suspensions of *P. sikkimensis* (Biswas et al. 2015b). The present study was undertaken to investigate the effect of exogenous addition of culture filtrates of *Serratia marcescens* (SD 21), *Bacillus subtilis* (FL11), *Trichoderma atroviridae* (TA), and *Trichoderma harzianum* (TH) on ginsenoside and anthocyanin biosynthesis in the cell suspensions of *P. sikkimensis* as a means to enhance metabolite productivity.

## Materials and methods

Growth and metabolite production kinetics of the *Panax sikkimensis* cell suspension cultures have been earlier reported by us (Biswas et al. 2015b). Based on the data published in the previous report, the experiment was initiated with 3.0 g of suspended cells inoculated in a 40-mL medium/flask and grown for 2 weeks. The elicitor preparation was decided to be added at this stage of the culture cycle. Cells were harvested for their growth and metabolite content after 1 and 3 weeks of elicitor treatment. Biomass accumulation was recorded through fresh weight determination of the harvested cell mass. For dry matter determination, the cells were dried to constant weight in a lyophilizer (Labconco, FreeZone 2.5, USA). The growth rate was measured as percent biomass increment (= weight of cells at harvest/weight of initial inoculum  $\times$  100). A minimum of three replicates were run for all the treatments, and the experiments were repeated thrice.

## Elicitor preparation and addition

### Preparation of biotic elicitors of bacterial origin

*Serratia marcescens* strain SD 21 (MTCC No. 9233) and *Bacillus subtilis* strain FL11 (MTCC No. 10010) were maintained on nutrient agar medium (15 g/L peptone, 3 g/L beef extract, 5 g/L NaCl, 0.3 g/L  $\text{KH}_2\text{PO}_4$ , and 15 g/L agar) in the Microbiology and Entomology Division in CSIR-CIMAP. Single bacterial colonies were inoculated in 10 mL nutrient broth medium and were maintained on a shaker incubator (120 rpm) at 28 °C for 24 h. One milliliter of the culture was

further inoculated into 9 mL fresh nutrient broth (NB) and maintained in similar conditions and allowed to grow for 12 h ( $\text{OD}_{660} = 1$ ). The microbial cultures were centrifuged at 10,000 rpm for 10 min to pellet out the cells. The supernatant was filter sterilized using 0.22  $\mu$  PVDF filters (Millipore, USA). 0.5 mL (1.25% v/v coded as FL11 1.25 and SD 21 1.25) and 1.0 mL (2.5% v/v coded as FL11 2.5 and SD 21 2.5) of the elicitor preparation were added to *P. sikkimensis* cell suspensions. The control was set by the addition of equal amounts of plain nutrient broth.

### Preparation of the biotic elicitors of fungal origin

*Trichoderma atroviridae* (TA; sequence deposited to NCBI: accession number JX002658) and *Trichoderma harzianum* (TH; ATCC No. PTA 3701) fungal cultures were maintained on potato dextrose agar (PDA) in the Microbiology and Entomology Division of CSIR-CIMAP. One square centimeter disc of active fungal isolate was inoculated onto 250 mL of potato dextrose medium and incubated on a shaker incubator (120 rpm) for 20 days. The mycelia were filtered off through Whatman filter paper and were filter sterilized using Millipore (0.22  $\mu$ m) PVDF filter unit for further use in the different elicitation treatments. 0.5 mL (1.25% v/v coded as TA 1.25 and TH 1.25) and 1.0 mL (2.5% v/v coded as TA 2.5 and TH 2.5) of the prepared elicitor were added aseptically to *P. sikkimensis* cell suspensions. The corresponding control comprised of addition of equal amounts of plain potato dextrose broth.

### Ginsenoside quantification using HPLC-UV

Ginsenoside extraction and quantification was done by an optimized methodology developed for ginseng cell suspensions, previously reported by us (Biswas et al. 2015a). Briefly, the samples were sonicated (120 W; Rivotek, Ultrasonic cleaner, STD 2025) for 30 min in 50 mL methanol, repeated four times. The methanolic extract was then 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 sample was redissolved in HPLC grade methanol for HPLC analysis. For leftover medium 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 Milford, USA) equipped with a Waters symmetry C<sub>18</sub> 4.6  $\times$  150 mm (3.5  $\mu$ ) 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; 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, 51.1, 52.8, 53.4, 53.8, 54.5, 55.9, 63.1, and 64.9 min, respectively. Linearity was performed for each ginsenoside standard as in our previous report (Biswas et al. 2016). 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).

The ginsenosides were identified in samples by matching corresponding UV spectra and retention times. In cases where a minor shift in retention times of ginsenosides was observed in the crude samples, particularly Rb2, peak identity was confirmed by spiking the sample with the respective authentic. Reference samples of ginsenosides Rb1, Rb2, Rc, and Rd, and Re, Rg1, Rg2, Rg3, Rh1, and Rh2 were purchased from ChromaDex Ltd. and Sigma-Aldrich, USA, respectively. All the samples were analyzed at 203 nm using the Empower 2 software.

## RNA isolation and quantitative real-time expression analysis

Total RNA from elicited and non-elicited cells was isolated using TRI reagent (Sigma-Aldrich Chemicals Pvt. Ltd., India) as per the manufacturer's instructions.

The first-strand complementary DNA was synthesized using Thermoscript™ RT-PCR kit (Life Technology, Invitrogen™ BioServices Pvt. Ltd., India) according to the manufacturer's instructions. In brief, 2.5 µg of total RNA was mixed with random hexamer primer and dNTPs (10 mM) and incubated at 65 °C for 5 min for sample denaturation prior to primer hybridization. The samples were incubated on ice for 1 min followed by addition of 8 µL of cDNA synthesis reaction mix [consisting of 4 µL of 5× cDNA synthesis buffer, 1 µL DTT (0.1 M), 1 µL RNase OUT™ (40 U/µL), and 1 µL Thermoscript™ RT (15 U/µL), 1 µL nuclease-free water]. This reaction mixture was incubated at 25 °C for 10 min followed by 60 min at 50 °C. The reaction was terminated by incubating at 85 °C for 5 min. One microliter of RNase H (2 U/µL) was added to remove RNA followed by incubating this mixture at 37 °C for 20 min. The cDNA was stored at –20 °C and was used for quantitative real-time PCR analysis.

The expression level of the respective genes in selected elicited vs non-elicited cultures was measured by Real Time PCR with SYBR green I-chemistry (Takara, Japan). Primers were designed with the Primer Express Software v.2.0 (Applied Biosystems, USA) and tested to ensure amplification

of single discrete fragment size with no primer-dimers. The primers were custom synthesized by Europhins Scientific, USA, and the sequences and necessary information are presented in Table 1. The reactions comprising of 15 ng cDNA, 0.4 µM of each forward and reverse primer, and 5 µL of SYBR Green PCR master mix (2×) (Applied Biosystems, USA) with the final volume adjusted to 10 µL with Milli-Q water were assembled. The reactions were carried out in three replicates in a 7900HT Fast Real Time PCR System (Applied Biosystems, USA), and the specificity of the reactions was verified by melting curve analysis with the thermal cycling parameters: initial denaturation (95 °C for 30 s), and 40 amplification cycles (95 °C for 5 s; 55 °C for 30 s; and 72 °C for 1.5 min).

Relative mRNA levels were quantified with respect to the selected internal/endogenous control housekeeping gene of *P. sikkimensis*, i.e., GAPDH. Sequence Detection System (SDS) software v2.2.1 (Applied Biosystems) was used for relative quantification of gene transcript using  $\Delta\Delta C_T$  method. The relative quantity was determined as  $2^{-\Delta\Delta C_T}$ .

## Statistical analysis

All experiments were repeated thrice and the values represent the average of the triplicates. The ginsenoside content is reported as milligrams per gram DW and the ginsenoside yield is reported as amount in milligrams, extrapolated for unit liter of cell suspension culture. Wherever found suitable, the means were compared using Duncan's multiple range test (DMRT), using Assistant Software Version 7.7 (Silva and Azevedo 2016). All the gene transcript fold enhancements were subjected to "Student's *T* test" to determine level of significance (Singh and Chaudhary 1979).

## Results

Cell suspensions were found to be differentially affected by the various biotic elicitors examined. Almost all the elicitors led to an increase in total ginsenoside contents within 1 week of elicitor exposure within the range of 1.1–3.2-fold enhancements with the exception of FL11 1.25 treatment. Prolonging the elicitor exposure for 3 weeks led to a consistent decline in ginsenoside content, as compared to the contents after 1 week of treatment. Maximum ginsenoside content was achieved with *Trichoderma harzianum* culture filtrate (TH) treatment for 1 week at a concentration of 2.5% v/v, which led to a ginsenoside yield of 222.2 mg/L (3.2-fold more than the control; Table 2).

None of the biotic elicitors tested led to an appreciable increase in anthocyanin accumulation of the *P. sikkimensis* cell suspensions. The amounts generated were either significantly less or only marginally more than control values, at either of



**Table 1** Real time primers used for the expression analysis of the selected genes

| Genes                                                   | Sequences                                                                                    | GenBank ID of the corresponding sequence from <i>P. quinquefolius</i> /melting temp/product size |
|---------------------------------------------------------|----------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| <i>GAPDH</i> (glyceraldehyde 3 phosphate dehydrogenase) | Forward primer 5'-GTCACCGTCTTTGGTATTAGGAATC-3'<br>Reverse primer 5'-TTAGCACCACCCTTCAAATGG-3' | GU075684/55 °C/130 bp                                                                            |
| <i>FPS</i> (farnesyl phosphate synthase)                | Forward primer 5'-CTCATACGCGCAGAGGTCAA-3'<br>Reverse primer 5'-GTAAGGCTTTGTCTGGAAATGC-3'     | KC524468/55 °C/130 bp                                                                            |
| <i>SQS</i> (squalene synthase)                          | Forward primer 5'-GCCGGAGAGTTCATGTAATCG-3'<br>Reverse primer 5'-TGCATCAATTTCATTAAACAATCC-3'  | KC524469/57 °C/130 bp                                                                            |
| <i>DDS</i> (dammarenediol synthase)                     | Forward primer 5'-GGTGCACCTACATGCCAATG-3'<br>Reverse primer 5'-GCGCTGTTGATTCCACTTTATCT-3'    | KC316048/56 °C/130 bp                                                                            |
| <i>PPDS</i> (protopanaxadiol synthase)                  | Forward primer 5'-GAACCGATGGCAATCTTGTGT-3'<br>Reverse primer 5'-GCGTTGGATTCTCCATGAGATC-3'    | JX569336/55 °C/130 bp                                                                            |
| <i>PPTS</i> (protopanaxatriol synthase)                 | Forward primer 5'-GGACAACGAGGCAGCACTTT-3'<br>Reverse primer 5'-CACCTGCACCGGATCATTTA-3'       | KC190491/56 °C/130 bp                                                                            |

the concentrations of the elicitor used and at either of the treatment durations (Table 2).

### Effect of bacterial culture filtrates on growth and metabolite content

#### Effect on cell biomass accumulation

In terms of cell biomass production, application of culture filtrate of SD 21 at both the concentrations for 1 week was not observed to have a detrimental effect (% BI comparable to control values). However, with increase in culture duration, cell biomass

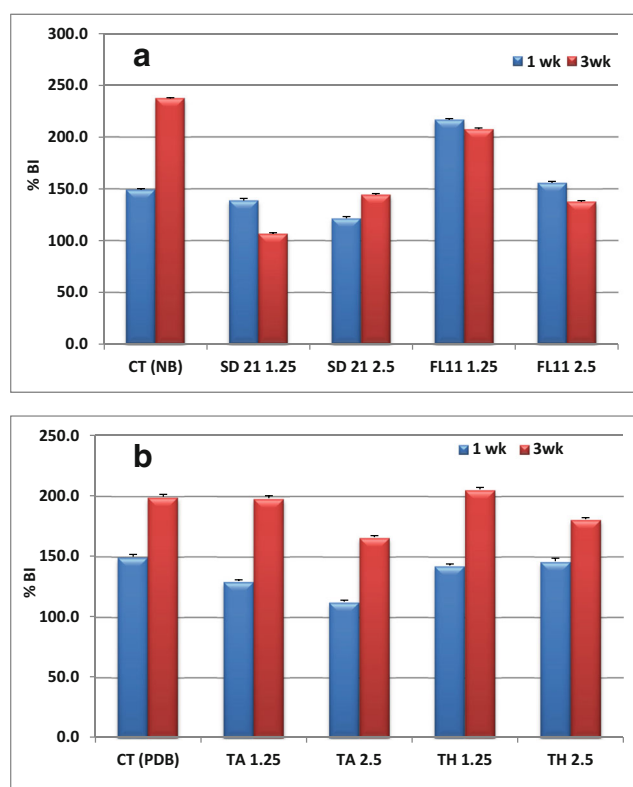
accumulation was not observed to increase exponentially, as was observed with the control (% BI = 237.5) cultures (Fig. 2a), rather a decrease in % BI was observed with the use of SD 21 1.25 treatment (% BI = 106.5). Application of culture filtrate of FL11 at 1.25% v/v for 1 week was observed to facilitate an increase in cell biomass accumulation (% BI = 216.78) as compared to control values (% BI = 149.1). Cell biomass accumulation was maintained at a % BI of 207.5, even after 3 weeks of elicitor exposure. Use of FL11 2.5 treatment for 1 week led to a biomass accumulation (% BI = 155.7) comparable to control (% BI = 149.1), which was not observed to increase with increase in elicitor exposure to 3 weeks.

**Table 2** Effect of the different biotic elicitations on ginsenoside and anthocyanin productivity of the *P. sikkimensis* cell suspensions after 1 and 3 weeks of treatment (see the “Materials and methods” section for treatment code). Averages followed by the same letter are not significantly different between themselves ( $P < 0.05$ ) as determined by Duncan's test

| Treatment                         | After 1 week of treatment |               |               |               | After 3 weeks of treatment |               |               |               |
|-----------------------------------|---------------------------|---------------|---------------|---------------|----------------------------|---------------|---------------|---------------|
|                                   | GC                        | GY            | AC            | AY            | GC                         | GY            | AC            | AY            |
| <b>Bacterial culture filtrate</b> |                           |               |               |               |                            |               |               |               |
| CT (NB)                           | 7.23 ± 0.1c               | 73.1 ± 1.3d   | 12.9 ± 0.8b   | 120.9 ± 1.8b  | 1.96 ± 0.04c               | 33.1 ± 0.4c   | 12.3 ± 1.7a   | 193.3 ± 0.7a  |
| FL11 1.25                         | 4.16 ± 1.2d               | 59.4 ± 1.1e   | 6.02 ± 0.6d   | 86.26 ± 0.9d  | 0.85 ± 0.05d               | 11.28 ± 1.1d  | 9.97 ± 0.8b   | 129.46 ± 1.5b |
| FL11 2.5                          | 13.32 ± 1.6b              | 144.8 ± 1.2b  | 7.74 ± 0.6c   | 79.68 ± 1.0e  | 2.28 ± 0.04b               | 37.9 ± 0.6b   | 8.90 ± 0.7b   | 126.02 ± 1.1c |
| SD 21 1.25                        | 12.80 ± 1.01b             | 121.7 ± 0.98c | 14.68 ± 0.35a | 134.54 ± 1.4a | 0.71 ± 0.09e               | 4.98 ± 0.11e  | 9.71 ± 0.3b   | 68.39 ± 1.1e  |
| SD 21 2.5                         | 23.13 ± 0.9a              | 189.3 ± 1.2a  | 13.67 ± 0.23b | 109.72 ± 1.1c | 3.79 ± 0.11a               | 41.06 ± 0.13a | 8.90 ± 0.5b   | 84.88 ± 1.2d  |
| <b>Fungal culture filtrate</b>    |                           |               |               |               |                            |               |               |               |
| CT (PDB)                          | 6.91 ± 0.99d              | 70.0 ± 1.1e   | 13.12 ± 0.32a | 107.06 ± 1.0a | 1.60 ± 0.04e               | 21.7 ± 0.8e   | 13.86 ± 0.42a | 213.66 ± 1.7b |
| TA 1.25                           | 10.55 ± 0.32bc            | 91.4 ± 1.2c   | 7.82 ± 0.87c  | 55.19 ± 0.53e | 4.42 ± 0.07c               | 60.5 ± 0.13c  | 13.49 ± 0.4a  | 220.45 ± 2.2a |
| TA 2.5                            | 10.2 ± 0.12c              | 80.01 ± 1.1d  | 11.28 ± 0.99b | 69.12 ± 0.77c | 2.37 ± 0.03d               | 33.4 ± 0.11d  | 9.39 ± 0.2c   | 128.47 ± 1.1e |
| TH 1.25                           | 11.37 ± 0.15b             | 110.6 ± 1.1b  | 10.24 ± 0.33b | 79.49 ± 0.21b | 6.56 ± 0.32b               | 89.8 ± 0.89b  | 10.41 ± 0.44b | 176.42 ± 1.1d |
| TH 2.5                            | 22.37 ± 0.54a             | 222.2 ± 2.1a  | 7.96 ± 0.65c  | 63.34 ± 0.62d | 11.81 ± 0.11a              | 148.6 ± 1.1a  | 13.81 ± 0.45a | 205.97 ± 2.3c |

Values are represented as mean ± SD (mean of three replicates).

GC ginsenoside content (mg/g DW), GY ginsenoside yield (mg/L), AC anthocyanin content (mg/g DW), AY anthocyanin yield (mg/L)



**Fig. 2** Effect of the different bacterial culture filtrate (a) and fungal culture filtrate (b) elicitation on cell biomass accumulation in *Panax sikkimensis* cell suspensions after 1 and 3 weeks of treatment (three replicates employed per treatment; initial inoculum 3.0 g FW/40 mL medium)

### Effect on metabolite, i.e., ginsenoside and anthocyanin content

In terms of total ginsenoside content and yield after elicitor treatment for 1 week, 1.25% v/v dose of FL11 culture filtrate led to a decrease in ginsenoside yield (59.4 mg/L) as compared to control cultures (73.1 mg/L). This was the only treatment in the entire study that led to a decline in the ginsenoside contents of the cell suspensions. Use of higher dose of this culture filtrate, i.e., 2.5% v/v led to a 1.8-fold increase in ginsenoside content and a 1.9-fold increase in total ginsenoside yield (Table 2). Ginsenoside contents were observed to decrease when the elicitor exposure was increased to 3 weeks.

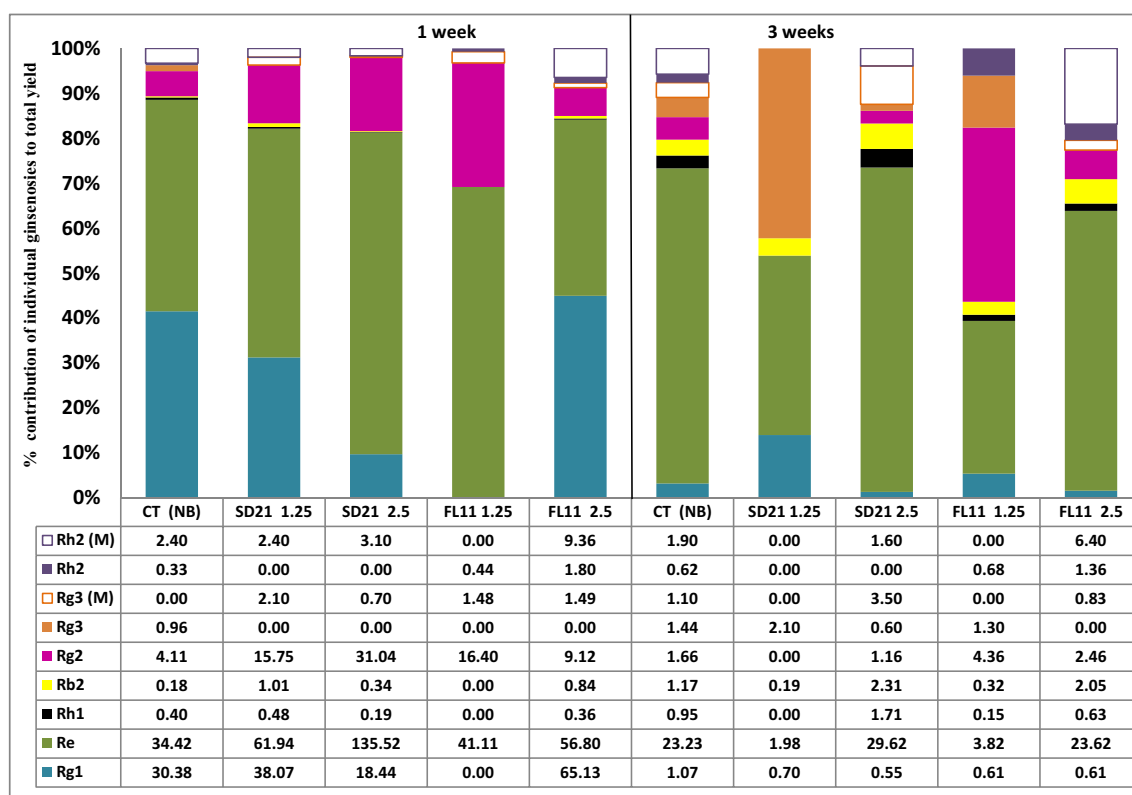
On the other hand, SD 21 culture filtrate, when applied for 1 week, proved stimulatory for ginsenoside biosynthesis, with 1.6- and 2.58-fold enhancements in ginsenoside yield with use of 1.25 and 2.5% v/v concentrations respectively. However, on extending the exposure duration to 3 weeks, a sharp decline in ginsenoside content and corresponding ginsenoside yield of the cell suspensions was observed, especially with the SD 21 1.25 treatment. This could possibly be due to corresponding decline in cell biomass accumulation with the use of this treatment for 3 weeks (Fig. 2a). None of the elicitation treatments

when applied for 1 week could fruitfully enhance anthocyanin accumulation beyond control values (120.9 mg/L; Table 2), with the exception of SD 21 1.25. It led to a marginal increase in anthocyanin yields to the tune of 134 mg/L. Upon increasing the elicitor exposure to 3 weeks, interestingly anthocyanin content was observed to increase with use of FL11 culture filtrates and decrease with use of SD 21 culture filtrates. But none of the treatments could exceed the corresponding control values of anthocyanin content, and hence, these bacterial elicitors were deemed unfit for anthocyanin elicitation (Table 2).

The effect of application of these culture filtrates on the individual ginsenoside biosynthesis of the cell suspensions is outlined in Fig. 3. The major ginsenosides accountable for the enhancement in ginsenoside yield with both the doses of SD 21 (1.25 and 2.5% v/v) when applied for 1 week is primarily due to extensive accumulation of protopanaxatriols Re and Rg2 within the cells, especially with use of 2.5% dose. An intracellular ginsenoside Re accumulation was observed to the tune of 138 mg/L (3.5-fold more than control; Fig. 3) and Rg2 accumulation to the extent of 31 mg/L (10-fold more than control; Fig. 3). Elicitation of ginsenoside Rg1 was not observed to be as pronounced, rather a decrease in Rg1 was observed with the use of SD 21 2.5 treatment (8.4 mg/L) when compared with control values (30.4 mg/L). Upon increasing elicitor exposure to 3 weeks, a sharp drop in the levels of these ginsenosides was observed, especially with SD 21 1.25 treatment. As opposed to elicitation for 1 week, ginsenoside Rg2 was not detected in cultures treated with SD 21 1.25, rather a marginal increase in Rg3 levels was observed (Fig. 3). Levels of Rg1 and Re were also reduced to extremely low amounts. With the use of SD 21 2.5 for 3 weeks, Re and Rg1 biosynthesis was observed to drop; however, this treatment was significantly improving biosynthesis of ginsenoside Rh1 (1.7 mg/L) and biosynthesis and exudation of Rg3 into the medium (3.5 mg/L) as compared to control [Rh1 = 0.95 mg/L; Rg3 (M) = 1.1 mg/L]. The biosynthesis of the other protopanaxadiols such as Rb2 and Rh2 was not significantly enhanced.

With the use of FL11 culture filtrates for 1 week, ginsenoside Re was observed to be increased with both the concentrations. However, presence of Rg1 was absent in cultures treated with FL11 1.25, which could be one of the prime reasons for decline in total ginsenoside content in comparison to control with the use of this treatment. Interestingly, use of FL11 2.5 led to a 2-fold increase in Rg1 (Fig. 3) and 2.2-fold increase in Rg2 accumulation. Among exuded ginsenosides, Rg3 (1.4 mg/L) and Rh2 (9.3 mg/L) were most prominently detected with the use of this treatment. Increase in the elicitor exposure duration to 3 weeks led to sharp decline in the levels of these ginsenosides at both the concentrations used.

None of the bacterial culture filtrates were able to induce production of Rb1, Rc, and Rd ginsenosides. They were



**Fig. 3** Percentage distribution (bar chart representation) and amount in milligrams per liter (tabular data) of the individual ginsenosides produced in cells and exuded into medium under the influence of 1.25 and 2.5% v/v application of the respective bacterial culture filtrate after 1 and 3 weeks of elicitation duration. Values in table are averages of three replicates.

(M): amount of the respective ginsenoside exuded into the leftover media; colored bars: respective ginsenoside content analyzed from cells; blank bars with colored borders: respective ginsenoside content analyzed from leftover media

consistently absent from unchallenged as well as elicited cell suspension cultures under the parameters of the present study.

## Effect of fungal culture filtrates on growth and metabolite content

### Effect on cell biomass accumulation

Application of culture filtrate of *T. harzianum* (TH) at 1.25% v/v (% BI = 142.1) and 2.5% v/v (% BI = 145.7) for 1 week was observed to have no profound effect as compared to corresponding control (% BI = 149.5) on cell biomass accumulation pattern of *P. sikkimensis* cell suspensions. Figure 2b clearly demonstrates that the registered % BI of cells were comparable to control values even when the duration of elicitor exposure was increased to 3 weeks. However, application of culture filtrate of *T. atroviridae* (TA) at either of the concentrations for 1 week led to a slight decrease in cell biomass accumulation (% BI = 129.2 and 112.2 with 1.25 and 2.5% v/v). Interestingly, with increase in elicitor exposure for a total of 3 weeks, biomass accumulation was recovered to control values with the use of 1.25% v/v concentration of the culture filtrate (Fig. 2b).

### Effect on metabolite, i.e., ginsenoside and anthocyanin content

In terms of ginsenoside content and ginsenoside yield of the cell suspensions, culture filtrates of both the *Trichoderma* spp. led to an enhanced accumulation of ginsenosides. Use of TA culture filtrate at 1.25% v/v dose for 1 week led to 1.3-fold enhancements and 2.5% v/v dose was observed to elicit a 1.14-fold enhancement in total ginsenoside yield respectively. Use of TH culture filtrates at both the doses for 1 week led to a 1.6–3.2-fold increase in total ginsenoside yield (Table 2). In the present study, maximum ginsenoside accumulation was observed with the use of TH culture filtrate at 2.5% v/v dose for 1 week which led to a ginsenoside content of 22.3 mg/g DW and a corresponding ginsenoside yield of 222.2 mg/L (3.2-fold more than control).

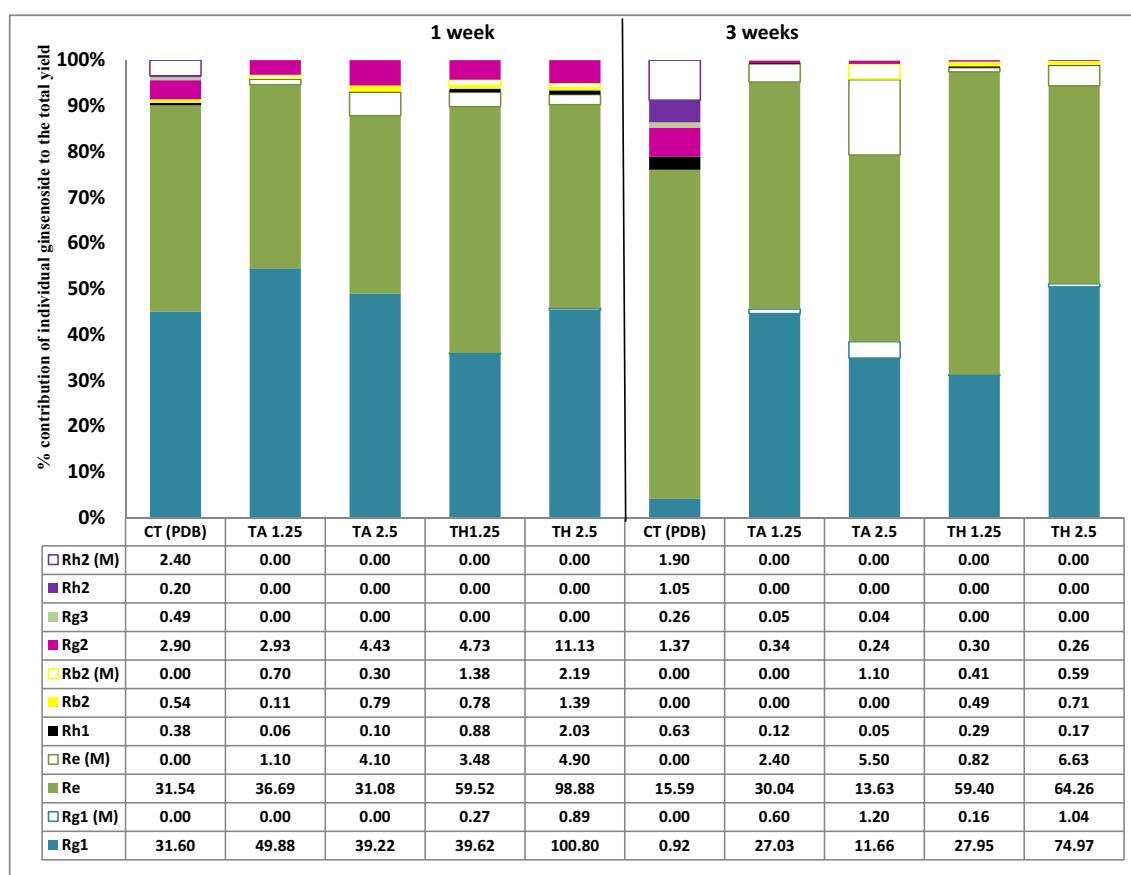
On the other hand, anthocyanin accumulation was initially observed to be depressed with 1 week of treatment with the fungal culture filtrates (Table 2). However, with increase in treatment duration to 3 weeks, an accelerative increase in anthocyanin biosynthesis was observed, particularly with the use of TA 1.25 treatment. A 2–4-fold enhancement in anthocyanin accumulation (as compared to yield after 1 week of elicitor exposure) was recorded with

the use of the fungal culture filtrates. However, in spite of such increase in anthocyanin biosynthesis potential, none of the treatments could facilitate in vitro accumulation significantly beyond control values (Table 2).

Figure 4 provides detailed information about the effect of fungal elicitation on the individual ginsenosides. With the use of *T. atroviridae* culture filtrates, it was found that treatment with either of the dosages for 1 week resulted in marginally increased Re/Rg1 synthesis; however comparatively, the extent of enhancement was more with TA 1.25% treatment (1.1–1.6-fold more than control; Fig. 4). Prolonging the exposure to 3 weeks was observed to have a depreciating effect on the individual yields. However, marginal exudation of these ginsenosides was observed (Fig. 4). In terms of the other ginsenosides, biosynthesis of ginsenoside Rg2 was enhanced in cells treated with TA 2.5% for 1 week (1.5-fold more than control). Interestingly, this treatment also led to marginal enhancement in the accumulation and partial exudation of ginsenoside Rb2. Ginsenosides Rg3 and Rh2 were not detected, in either cells or spent medium. Prolonging treatment duration led to a decrease in the overall content of these ginsenosides (Fig. 4).

With the use of *T. harzianum* culture filtrates at a concentration of 2.5% v/v for 1 week, a 3.2-fold enhancement in total ginsenoside yield (222.2 mg/L; Figs. 4 and 5a–c) was observed. This treatment led to an accelerated protopanaxatriol biosynthesis especially Re, Rg1, and Rg2 (3.3-, 3.1-, and 3.6-folds more than control respectively; Fig. 4). Increased accumulation of ginsenoside Rh1 (2.03 mg/L) and enhanced Rb2 biosynthesis [Rb2 = 1.3 mg/L; Rb2 (M) = 2.1 mg/L] was also observed. Use of 1.25% v/v dose for 1 week was also observed to have an accelerative effect on protopanaxatriol synthesis; however, the level of accumulation was significantly less than with the use of 2.5% v/v dose (Fig. 4). Prolonging the treatment duration for 3 weeks led to a rapid decline in the levels of all these ginsenosides, with the use of either of the concentrations, with the exception of level of ginsenoside Re with TH 1.25 treatment. It was comparable to ginsenoside Re yield after 1 week of exposure (Fig. 4).

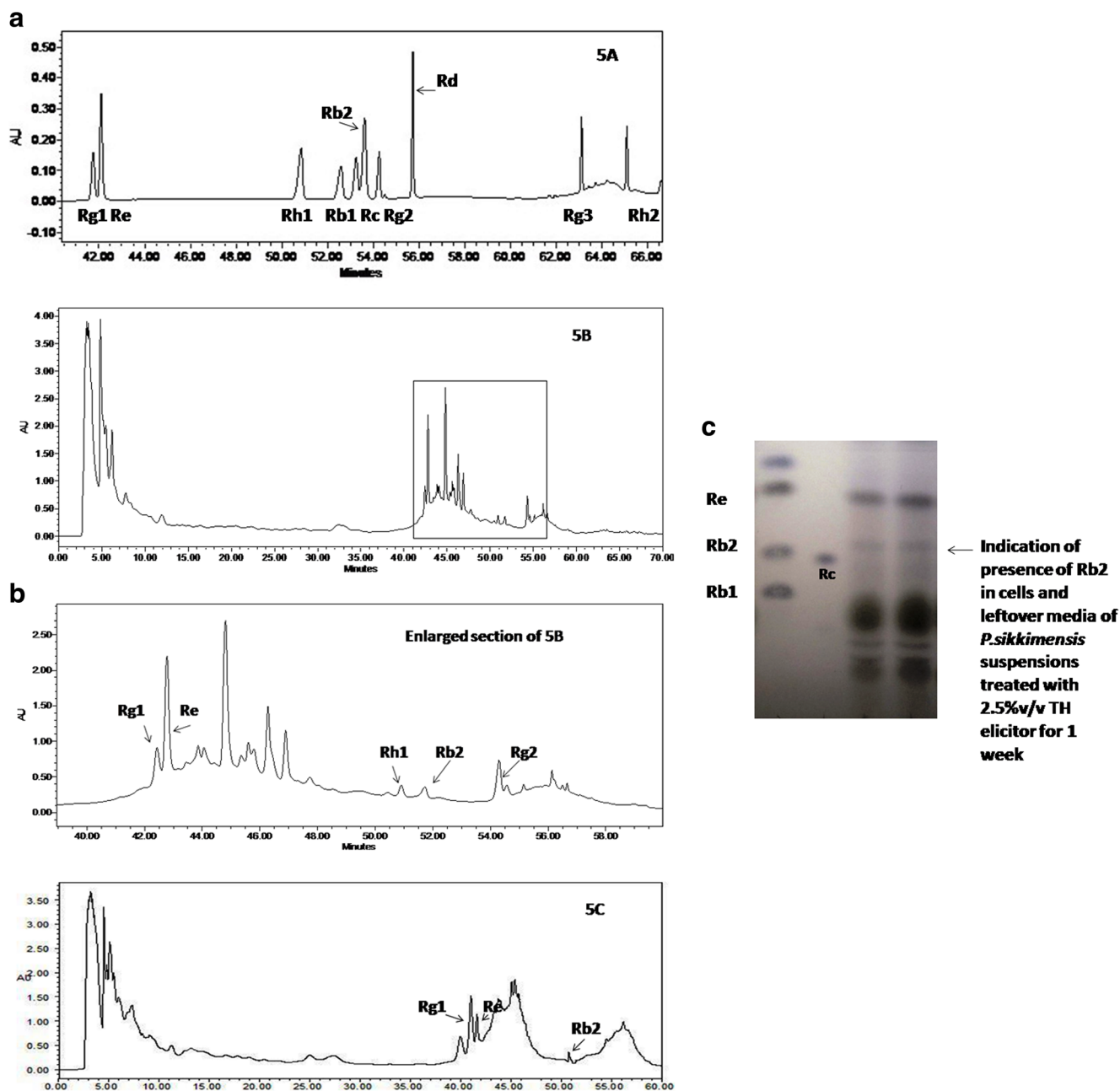
Elicitation of the desired ginsenosides, i.e., Rg3 and Rh2, was not observed. Their production under the treated conditions was comparatively less than the untreated control cultures.



**Fig. 4** Percentage distribution (bar chart representation) and amount in milligrams per liter (tabular data) of the individual ginsenosides produced in cells and exuded into medium under the influence of 1.25 and 2.5% v/v application of the respective fungal culture filtrate after 1 and 3 weeks of elicitation duration. Values in table are averages of three replicates. (M):

amount of the respective ginsenoside exuded into the leftover media; colored bars: respective ginsenoside content analyzed from cells; blank bars with colored borders: respective ginsenoside content analyzed from leftover media





**Fig. 5** HPLC chromatogram of ten ginsenoside authentic standards (a), *Panax sikkimensis* cell suspensions treated with 2.5% v/v culture filtrate of *T. harzianum* for 1 week (b), and leftover media of *Panax sikkimensis* cell suspensions treated with 2.5% v/v culture filtrate of *T. harzianum* for 1 week (c)

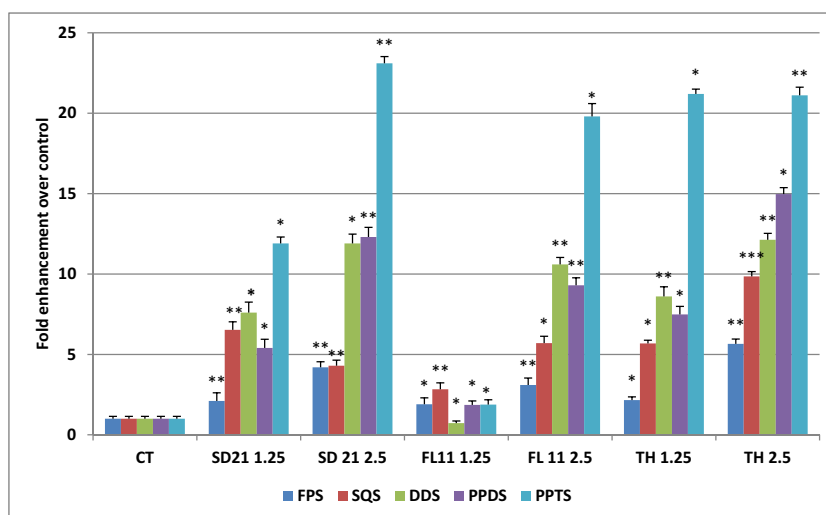
As was observed with the use of bacterial culture filtrates, none of the fungal culture filtrates were able to induce production of Rb1, Rc, and Rd ginsenosides, within the conditions and parameters of the present study.

#### qRT-PCR analysis of five ginsenoside biosynthesis genes in selected treatments

*Serratia marcescens* (SD 21), *B. subtilis* (FL11), and *Trichoderma harzianum* (TH) treatments were selected for real-time PCR analysis of the five key ginsenoside

biosynthesis genes, i.e., squalene synthase (*SQS*), farnesyl diphosphate synthase (*FPS*), dammarediol synthase (*DDS*), protopanaxadiol synthase (*PPDS*), and protopanaxatriol synthase (*PPTS*). The positive (SD 21 1.25, SD 21 2.5, TH 1.25, TH 2.5, and FL11 2.5) treatments were selected based on their potential for maximum elicitation of ginsenoside biosynthesis in *P. sikkimensis* cell suspensions. In order to eliminate favorable bias, and to build a hypothesis to determine crucial genes involved in ginsenoside biosynthesis, a treatment corresponding to a decline in total ginsenoside yields, i.e., FL11 1.25 (the only treatment where the total ginsenoside yield is less than

**Fig. 6** qRT-PCR analysis of five ginsenoside biosynthesis genes in selected treatments in *P. sikkimensis*. Vertical bars represent standard deviation from mean ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$



corresponding control), was also selected for qRT-PCR studies.

Figure 6 shows the relative fold enhancement in the transcript levels of these five genes in the control vs treated conditions. It can be clearly observed that with the exception of FL11 1.25, expression of all the genes was upregulated in the treated cells manifold, as compared to control treatment. Among the five positive treatments (Fig. 6), maximum fold enhancement in all the studied genes was observed in cells treated with 2.5% *v/v* *T. harzianum* culture filtrate (5–21-fold more than control). This was expected as this treatment correspondingly registered the maximum ginsenoside accumulation, among all the elicitation treatments (222.2 mg/L). Although expression of *PPTS* gene in 2.5% *v/v* TH-treated cells was comparable to its expression in 1.25% *v/v* of the same treatment, but expression of *PPDS*, *SQS*, and *FPS* was comparatively less, rather almost halved (Fig. 6) with the use of 1.25% *v/v* concentration. Interestingly, use of 2.5% *v/v* culture filtrate of *S. marcescens* led to a significantly enhanced *PPTS*, *PPDS*, and *DDS* expression (comparable to 2.5% TH treated; Fig. 6); however, expression of the upstream genes, i.e., *FPS* and *SQS*, was comparatively less than 2.5% TH-treated cells. This probably explains the decrease in total ginsenoside content achieved with this treatment (189.3 mg/L) when compared to content elicited by 2.5% TH treatment. Levels of all five genes were also significantly elevated in cells treated with FL11 2.5 treatment (Fig. 6). However, levels of *PPDS* and *PPTS* gene expression were comparatively less than SD 21 2.5 and TH 2.5 treatments, which probably explains a comparatively lower ginsenoside yield (144 mg/L) with use of FL11 2.5 treatment.

Interestingly, levels of gene transcripts for *SQS*, *FPS*, *PPDS*, and *PPTS* genes in FL11 1.25 treatment were marginally more than control cultures (1.9-, 2.8-, 1.8-, and 1.88-fold more than control respectively), but levels of *DDS* gene (0.73-fold of control) were observed to be decreased when compared to control by a factor of 0.27. This gene codes for an important protein

(dammarenediol synthase enzyme) that generates the dammarane diol skeleton for biosynthesis of the ginsenosides and decrease in its transcript levels may be the reason as to the observed decline in the total ginsenoside yields (59.4 mg/L) as compared to control values (73.1 mg/L) with the use of FL11 1.25. This observation points towards the possibility of *DDS* being one of the crucial genes that influence ginsenoside biosynthesis. Elicitation with culture filtrate of FL11 1.25 was down-regulating accumulation of *DDS* gene transcripts that may have led to a decline in the observed ginsenoside yields.

## Discussion

In the present study, cell suspensions of *Panax sikkimensis*, an Indian congener of ginseng, were subjected to biotic elicitation with the aim to augment biosynthesis of ginsenosides. Additionally, this line also co-accumulates anthocyanins, well known as “heart friendly,” dietary antioxidants (Matkowski 2008). Keeping in mind the commercial importance of this line (owing to its dual metabolite synthesizing tendency), efforts were undertaken to investigate the effect of biotic elicitor preparation from culture filtrates of *S. marcescens*, *B. subtilis*, *T. harzianum*, and *T. atroviridae* on the ginsenoside and anthocyanin biosynthesis in the cell suspensions.

Reports of ginsenoside elicitation using biotic elicitors are quite scanty. Xu et al. (2005) have reported a *Colletotrichum*-derived elicitor which was found to enhance saponins via singlet oxygen release in ginseng cells. *P. ginseng* suspension cultures were also reported to produce novel antibacterial compounds using *Botrytis cinerea* and yeast preparations (Kim et al. 2001). Recently, ginsenoside enhancements in *P. quinquefolius* cultures were observed using elicitors from *Trichoderma* (Biswas et al. 2016), *Aspergillus* (Li et al. 2016), and pathogenic fungus (Yu et al. 2016). Biotic elicitors are applied in many forms such as elicitor preparation from

mycelium, cell-free extracts, or fermentation broth. Culture filtrates or extracts of several *Pseudomonas*, *Bacillus*, and *Trichoderma* species have been reported to elicit secondary metabolism in many plant systems (Mañero et al. 2012; Chodisetti et al. 2013; Qianliang et al. 2013; Awad et al. 2014; Verma et al. 2014). It is hypothesized that these preparations are rich in chitosans, oligosaccharides, lipids, or small peptides, known as elicitors which mimic a disease response and elicit plant defense via NO signaling, SA production, JA production, etc. (Radman et al. 2003; Vasconsuelo and Boland 2007). Some of these elicitors interact with cell surface receptors whereas some are reported to directly enter the cells and affect gene expression (Radman et al. 2003). In the present study, a maximum of 3.2-fold enhancement in total ginsenoside content was observed with the use of culture filtrate of *Trichoderma harzianum*. Indeed, harzianolide, a plant growth regulator, was reported to be produced by a particular strain of *Trichoderma harzianum*, which led to increase in tomato cell biomass with harzianolide treatment. It was also observed to induce systemic resistance in plants, indirectly activating plant defense (Cai et al. 2013). Ginsenoside elicitation was also prominent with the use of bacterial culture filtrate, especially with the use of higher concentrations of *S. marcescens* culture filtrate. These particular plant growth-promoting rhizobacteria (PGPR) have been previously reported to induce systemic resistance in tobacco plants (Ryu et al. 2013). They are known to manifest the induction of systemic resistance via quorum sensing which is sensitive to bacterial cell density. It is probable that during the growth phase of bacterial strain, due to extensive quorum communication, they might exude certain “elicitors” into the culture filtrate, which might have triggered secondary metabolite biosynthesis.

Interestingly, culture filtrate of *S. marcescens* (1.25% v/v) was also observed to lead to a 2-fold increase in total ginsenoside accumulation in *Panax quinquefolius* cell suspensions (Biswas 2016). Analogous to *P. sikkimensis* cell suspensions, this elicitor also led to protopanaxatriol enhancement, particularly of ginsenoside Rg1 (5-fold increase than control) in *P. quinquefolius*. However, almost half of biosynthesized ginsenoside Rg1 was exuded into the medium, as opposed to *P. sikkimensis*, where exudation of the protopanaxatriols was not observed. This may be possibly due to differences in the cell membrane permeability of the two different *Panax* cell lines. Elicitation induced modulations in the membrane fluidity, physiological state, and protein chemistry at the cellular biomembrane might be responsible for such an observed variation in ginsenoside exudation within the two cell lines. Another probable mechanism of exudation may be due to presence of ABC transporters (ATP-binding cassette) which may be expressed by the plant cell under influence of external stimulus. They can actively transport chemically and structurally unrelated compounds from the cells (Martinoia et al. 2002), which may be responsible for exudation of even

heavily glycosylated ginsenosides such as Rb2 which is observed in *Trichoderma*-elicited cell suspensions of *P. sikkimensis*.

The host laboratory has earlier reported the effect of fungal culture filtrates on *Panax quinquefolius* cell suspensions (Biswas et al. 2016). Application of *Trichoderma atroviridae* and *T. harzianum* culture filtrates to *P. quinquefolius* cell suspensions (Biswas et al. 2016) was observed to have opposing effects on the ginsenoside biosynthesis, when compared to *P. sikkimensis* cell suspensions. In case of *P. quinquefolius*, application of 1.25% culture filtrate of *T. atroviridae* led to a 3.1-fold enhancement in total ginsenoside yield after 5 days of treatment, whereas application of *T. harzianum* culture filtrate at the same concentration led to only a marginal increase in ginsenoside accumulation, whereas in the case of *P. sikkimensis*, *T. harzianum* treatment for 1 week was better than *T. atroviridae* treatment for ginsenoside enhancement. Also, increasing duration of TH culture filtrate exposure led to an increase in ginsenoside yield for *P. quinquefolius* (3-fold more than control; Biswas et al. 2016) and a decrease in ginsenoside yield for *P. sikkimensis*. These observations may be accountable due to many reasons. Elicitors are most effective at optimum concentrations and at right stage of culture (Cai et al. 2013), which may vary from plant system to plant system. What may be an elicitor for one system may fail to invoke a suitable response in another plant system. Several previous works have demonstrated the importance of elicitor addition at the correct stage of cell growth. Some elicitors are required to be added at the exponential phase, while some require addition at the lag phase. Condori et al. (2010) have reported the dependence of cellular developmental stage on acetate-mediated production and exudation of resveratrol in peanut hairy root cultures. Increased hypericin production was observed with ozone elicitation during exponential phase (Xu et al. 2011) but enhanced peruvoside production and exudation in *Thevetia*, with MeJA elicitation in the lag phase (Zabala et al. 2010). It is a possibility that with progression in culture cycle, dynamic changes may occur in the cellular biomembranes such as altered membrane fluidity or expression of certain receptors at later stages of cell culture, which may then interact with the elicitor optimally. Hence, the effects of elicitation may be perceived at later stages of cell growth, which probably explains why TH culture filtrates on prolonged exposure were able to stimulate ginsenoside biosynthesis in *P. quinquefolius* and not in *P. sikkimensis*. Alternatively, elicitation signal may be transmitted in form of secondary messengers which might remain sequestered within the cells and may only amplify the signal after an intermittent gap.

In a nutshell, TA 1.25 treatment led to a 3.1-fold increase in ginsenoside yield in *P. quinquefolius* (Biswas et al. 2016) and TH 2.5 treatment led to 3.2-fold increase in *P. sikkimensis*. This phenomenon of a substance to act as an elicitor for one system, while not demonstrating elicitor activity under

identical conditions in another system, is one of the biggest hurdles in the field of elicitation science (Radman et al. 2003). However, we cannot rule out the possibility that a non-elicitor may act as an elicitor at a different concentration for a particular cell culture system. It might be due to the differences in the physiological state and the differential elicitor receptor chemistry at the biomembranes of the two cell suspension lines which may be the reason for the non-uniformity in response to a particular elicitor. Alternatively, the cell signaling pathways stimulated by a particular elicitor are never a one-way traffic of secondary messengers. It involves complex crosstalk among different messengers and simultaneous signals (Zhao et al. 2005). It might be possible that the differences in the gene expression pattern of the two cell suspension lines (challenged by a particular elicitor) may arise at this level, which has numerous different potential targets at the cellular level and leads to differential results. Characterization and identification of elicitors have been exhaustive in the past sesquidecade; however, it is essential to clearly understand the molecular events which lead to the observed effect, and a robust strategy to unravel the process is still lacking (Radman et al. 2003).

It would be interesting to study whether elicitation affects transcription levels of the genes in the ginsenoside biosynthetic pathway. Different studies have established the upregulated expression of these genes under the influence of various elicitors (Huang and Zhong 2013; Huang et al. 2013; Rahimi et al. 2016). Real-time PCR analysis of the genes involved in the ginsenoside biosynthetic pathway in the treated cells vs non-treated cells provided an insight into the differential transcript status among the two. In the present study, among the SD 21 and TH treatments, *PPTS* transcripts were consistently most abundantly present (Fig. 6). *DDS* and *PPDS* gene transcripts, although present in significantly high amounts than control, varied among themselves within these two treatments. Application of the lower dose was observed to facilitate *DDS* > *PPDS* transcript ratio whereas the reverse was observed on application of higher dose. Apparently, increase in elicitor concentration was observed to upregulate *PPDS* expression comparatively more than *DDS* expression particularly for TH treatments (Fig. 6). Levels of upstream gene transcripts, i.e., *FPS* and *SQS*, were consistently less than *DDS*, *PPDS*, and *PPTS* transcripts. *FPS* was consistently the least abundant among all five genes studied for the positive treatments. This indicates that probably these biotic elicitor preparations were targeting the downstream gene expression comparatively more rigorously than the upstream gene expression, which facilitated the increased flux flow through the committed step of ginsenoside biosynthesis (downstream of *DDS*), which resulted in enhanced ginsenoside accumulation within the cells. Another interesting observation was that elicitation mostly led to maximum enhancement in the protopanaxatriols such as Re and Rg1. This may be explained by the fact that as

the *DDS* and *PPDS* gene expression was elevated in the treated cells, it ensures the synthesis of the protopanaxadiol aglycone backbone. However, due to the corresponding abundance of *PPTS* transcripts in the treated cells, it is probable that bulk of the protopanaxadiol backbone is diverted downstream for synthesis of protopanaxatriols such as Re and Rg1, rather than their glycosylation via UDP-glycosyltransferases to protopanaxadiols such as Rh2, Rg3, and Rb2.

In the case of FL11 1.25, it was the only treatment in the present study that was observed to result in a decline in the total ginsenoside yield. Hence, it was expected that the relative gene transcript population of FL11 1.25 should be significantly less than the positive treatments. Interestingly, significant difference in levels of *FPS*, *SQS*, *PPDS*, and *PPTS* transcripts was not observed in comparison to control (1.8–2.8-fold of control). However, *DDS* transcripts were present only by a 0.73 factor of the corresponding level of *DDS* transcripts in control. This decline in *DDS* levels may be the causative factor of the observed decrease in ginsenoside yield in spite of the marginally elevated levels of the other four genes. However, gene expression levels of these five genes might not provide conclusive evidence when explaining pattern of biosynthesis of individual ginsenosides. *PPDS* and *PPTS* gene expression merely ensure availability of protopanaxadiol and protopanaxatriol backbones, but conversion of these backbones to the individual ginsenosides is via the action of UDP-glycosyltransferases, which actually catalyze formation of the different ginsenosides via glycosylation of these backbones at specific positions. This is an important family of enzymes which lead to stepwise addition of different monosaccharides at either C3/C6 (diol/triol) and C20 positions of the corresponding protopanaxadiol or protopanaxatriol aglycone, resulting in the synthesis of the different ginsenosides.

In the case of FL11 1.25 treatment, it can be seen that the decrease in total ginsenoside contents as compared to control is primarily due to absence of ginsenoside Rg1 (Fig. 3). However, there is an increase in Re and Rg2 biosynthesis as compared to control. Most probably, due to decline in *DDS* transcripts, flux through this branch-point may already be limited as compared to control. But since *PPDS* and *PPTS* transcripts are present at 1.8-fold of control level, they ensure a steady flux flow for formation of protopanaxadiol and protopanaxatriol backbone. However, differences in the transcript levels of different UDP-glycosyltransferases that catalyze formation of Rh2 to Rg3 from protopanaxadiol and Rh1 to Rg1 to Re to Rg2 (via different pathways not necessarily in that order) from protopanaxatriol may be accounting for the absence of Rg1 and a concurrent increase in accumulation of Re and Rg2. Hence, for conclusive evidence and to pinpoint exactly how an elicitor modulates expression of biosynthetic genes to result in the production of a particular ginsenoside, knowledge about these UDP-glycosyltransferases is critical. Recent research has been targeted towards these UDP-



glycosyltransferases, but they are still largely an unknown group. Only a few well-characterized UDP-glycosyltransferase systems are reported (as reviewed by Kim et al. 2015). Expression patterns of this gene family under challenged conditions may provide further clarity in terms of the molecular events triggered by the different elicitors.

The present study provides interesting leads in terms of commercial production of ginsenosides from this Indian ginseng cell line of *P. sikkimensis*. This cell line is of immense industrial importance for its potential bioreactor level upscaling. However, since the elicitors tested in the present study were unable to simultaneously enhance anthocyanin, it would be interesting to discover elicitors that can co-elicite ginsenosides as well as anthocyanins to maximize the commercial utility from this important ginseng cell line.

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## Compliance with ethical standards

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

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

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