Cell Wall Extract from *Piriformospora indica* Enhances Valerenic Acid Content in Valerian Roots via Modulating Sesquiterpene Synthase Genes

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Abstract

Valerenic acid (VA) as a sesquiterpene constituent responsible for medicinal properties is derived from valerian (*Valeriana officinalis*). It is well documented that the mutualistic basidiomycete *Piriformospora indica* substantially promotes secondary metabolites production. Nevertheless, our knowledge about the molecular mechanism involved is rudimentary. In this study, we aimed to find the effect of *P. indica* cell wall extract (CWE) in the modulation of the transcriptional rate of sesquiterpene synthase (Sesqui-TPS) genes involved in VA biosynthesis in the host plant, *V. officinalis*. For this purpose, relative expression of putative terpene syntheses (*VoTPS1*, *VoTPS3* and *VoTPS7*) genes was quantified by quantitative real-time PCR in the roots of valerian plants inoculated by two concentrations of the extract (2 and 4 % v/v) prepared from the cell wall of *P. indica* for various time intervals. As well as, VA content of roots of *V. officinalis* was measured. According to the results, The 4% fungal CWE application produced more VA than 2% CWE treated plants at the exposure time of 72 h. Such enhancement was correlated with increased transcript expression of two sesquiterpene synthases (sesqui-TPSs) including *VoTPS1* and *VoTPS7* which provided intermediates probably serving in the VA-biosynthetic pathway, while different expression pattern of *VoTPS3* showed that it probably has no influence on the biosynthesis of VA. Since the accumulation of VA is a dose (and time) dependent response at roots of *V. officinalis*, the present study suggests that the mechanism(s) responsible for the enhancement of VA could be related to the fungus-plant association.

Keywords: *Piriformospora indica*, Sesquiterpenes Synthase, *Valeriana officinalis*, Valerenic acid

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Introduction

Valerenic acid (VA) as biologically active sesquiterpene compound responsible for sedative and hypnotic properties is extracted from *Valeriana officinalis* (valerian) [1]. Sesquiterpenes, as a class of terpenes, are originated from the condensation of the 3 isomeric isoprene C5 units namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [2]. Cytosolic pool of IPP/DMAPP is converted to farnesyl diphosphate (FPP, C15), which serves as a precursor for the production of numerous structurally diverse sesquiterpenes (e.g. germacrene B/C/D and valerenadiene) [3]. It is thought that valerenic acid may be derived from valerenadiene. Among characterized sesquiterpene synthases (sesqui-TPSs), it is found that *V. officinalis* terpene synthase 1 (VoTPS1) encodes valerenadiene synthase and VoTPS7 functionally acts as a germacrene synthase [4]. It is also proposed that germacrene intermediate may contribute to valerenadiene biosynthesis [3]. However, biochemical mechanism of its biosynthesis has been remained enigmatic. Unlike VoTPS1 and VoTPS7, which are expressed more in root tissue and utilize significant amount of FPP rather than geranyl diphosphate (GPP) as substrate, *VoTPS3* mRNA is present in all tissue types examined (leaves, stems and roots) and uses GPP rather than FPP as substrate [4].

Piriformospora indica, as a plant growth-promoting mycorrizal fungus (PGPF), has the ability to colonize the root cortex of a broad variety of monocot and dicot plant species [5, 6]. *P. indica* promotes the host plant growth and productivity via adaptation to biotic and abiotic stresses [7]. There are many reports demonstrating the stimulation of bioactive compounds production in a wide variety of medicinal plants by their interaction with *P. indica*, but not much is known about the mechanism involved [8-10]. In this study, in order to investigate the effect of *P. indica* cell wall extract (CWE) in modulation of transcriptional rate of sesquiterpene synthese (Sesqui-TPS) genes, which may influence the yield of valerenic acid (VA), relative expression of putative terpene syntheses (*VoTPS1*, *VoTPS3* and *VoTPS7*) genes was quantified by quantitative real-time PCR (qRT-PCR) in the roots of valerian plants inoculated by two concentrations of the extract (2 and 4 % v/v) prepared from the cell wall of *P. indica* for various time intervals. As well as, VA content of roots of *V. officinalis* was measured.

Materials and Methods
Plant materials and growth conditions
Seeds of *V. officinalis* were obtained from Pakanbazr Company, Esfahan, Iran. The seeds were surface sterilized

with 70 % (v/v) ethanol for a min and rinsed with sterile distilled water. Seeds were then treated with 2.5 % sodium hypochlorite (NaClO) for 10 min, followed by 3 times washes with sterile water to remove the trace of bleach. Surface-sterilized *V. officinalis* seeds were germinated on half-strength Murashing and Skoog (MS) medium [11], supplemented with 30 g/L sucrose and solidified with 8 g/L agar. The pH of medium was adjusted to 5.8. The cultures were maintained under controlled conditions in tissue culture room (16/8 h light/dark cycle at 25 ± 2°C).

Culture conditions and CWE preparation

The fungus was first cultivated on solid complex medium (CM) [12] containing 20X salt solution (50 ml/L), glucose (20 g/L), peptone (2 g/L), yeast extract (1 g/L), casamino acid (1 g/L), microelements solution (1 g/L), agar (15 mg/L) and then a piece of grown fungus was transferred to MYPG medium containing malt and yeast extracts (3 g/L), peptone (5 g/L) and glucose (10 g/L) at pH 6.2, temperature 25 ± 2°C on a shaker incubator. The cell wall extract was prepared using the protocol of Anderson-Prouty and Albershem [13] with minor modification as reported by Vadassery *et al.*, [14]. The cultures were harvested in the late log phase (14th day). The fungal biomass was collected by passing through Whatman filter paper (No.1). The residue on the paper surface was washed three times with distilled water. This preparation was dried at 30°C. Dried biomass was then homogenized using mortar and pestle. Elicitor fractions were prepared from the mycelia cell wall by suspending 10 g of cell wall in 100 mLwater and autoclaving for 15 min at 120°C. Autoclaving releases the active fractions of the cell wall. The suspension was centrifuged at 500 rpm for 10 min. The resulting supernatant was designated as cell wall extract and stored at 4°C for further use.

Treatment with extracted P. indica cell wall

Two months after growth of seedlings on half-strength MS media, plants were subcultured on the media containing different concentrations of cell wall extract from *P. indica* (2 and 4 % v/v). Control plants were subcultured on cell wall extract free- half-strength MS media. The root samples were collected at 12, 24, 48, 72 and 144 h after inoculation to evaluate dynamic responses of terpene synthase genes and VA content.

VA Quantification

The roots of *V. officinalis* were dried and made in to a coarse powder. The root powder (approx.200 mg) was extracted three times with 5 ml of 70 % MeOH, sonicated for 10 min and then diluted to a final volume of 15 ml with MeOH.

All samples were filtered through a 0.45 µm micro-filter (Merck, Germany) and then subjected to reverse-phase high-performance liquid chromatography (HPLC) analysis as described in previous studies [15].

HPLC analysis was carried out using a Knauer HPLC system (Germany). VA was separated by reverse-phase isocratic C18 column (250×3 mm) with a pre-column (25×4.6 mm, particle size 5 µm) and detection by UV was conducted at 225 nm. The mobile-phase consisted of 0.5 % (v/v) phosphoric acid (A) and 0.5 % phosphoric acid:methanol mixture (27:73) (B) delivered at a flow rate of 1 ml/min and an injection volume of 20 µl. VA was provided by Sigma-Aldrich Co. (USA). Standard solution was prepared by dissolving VA in methanol to obtain solutions with 5 different concentrations in the range of 5 to 25 ppm. The calibration curves were constructed based on measuring their respective peak areas. This experiment was carried out with three replicates. The results were expressed as mean values ± SD. The statistical significance of VA contents was analyzed using a t test with Excel software for significant differences between cell wall extract of the endophyte-treated and control plants.

RNA extraction, cDNA synthesis, primers design and qRT-PCR reaction

Total RNAs were extracted from the roots using GeneAll[®] RiboEx ™ kit (BioFrontier, Korea) based on manufacturer's protocol. First strand cDNA synthesis was done from 500 ng of total RNA using Fermentas kit (Revert Aid ™ First Strand cDNA Synthesis Kit) according to the manufacturer's instructions. Primer pairs of the terpene synthase genes including *VoTPS1*, *VoTPS3* and *VoTPS7* were designed using online IDT Primer Quest™ Software of Integrated DNA according to the predicted CDS sequences. The primers sequences designed for qRT-PCR amplification of target genes are listed in Table 1.

The qRT-PCR was performed using HIFI SYBR[®] Green kit (Iran) Master Mix and Step One Plus[®] (ABI, USA) machine.

**Table 1.** Primer sequences designed for qRT-PCR.

Target gene	Accession number	Primer sequence (5'→3')	Product size (bp)
VoTPS1	JX494699	F: GTAGGCATGGGAGTAACTACAG R:TCGTGTCCAACCTTATCATCC	119
VoTPS3	JX494701	F: CGTGCGTAAAGTCTTACG R: CTACTCACTCCGTGTTCTTTC	114
VoTPS7	JX494705	F: GCTAGGCTCGACAATGATAAAG R: TGAACTCTACAACCACTTCTTC	124
18S rRNA	AJ236003	F: AGGATGGCAAGCGTATTGG R: TGCATTTCCCAGAGTGTCTTC	118

PCR conditions were an initial denaturation of 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 20 s and extension at 72°C for 20 s. Relative fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ comparative method [16]. The threshold cycle (CT) for each gene was normalized against the Ct for *18S rRNA* gene (AJ236003) of *V. officinalis*, which was used as the internal reference. Relative expression software tool (REST)® software [17] was used to analyses the obtained data. This experiment was carried out with three biological and two technical repeats.

Results

Effect of different concentrations of fungal CWE on valerenic acid production in V. officinalis elicitor-treated cultures
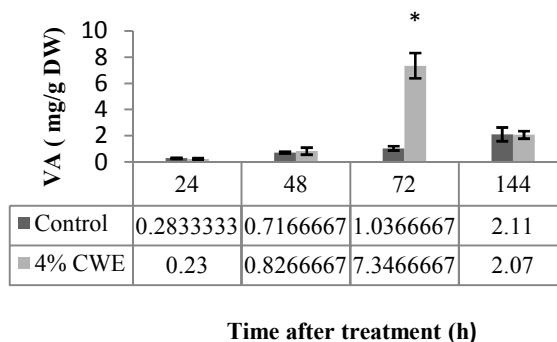
To evaluate the effects of fungal cell wall on stimulating VA production, HPLC analysis was performed. Elicitors derived from the fungal CWE were screened for dose concentrations (2 and 4% v/v) at the exposure time of the elicitor 24 to 144 h. As shown in Fig. 1, the maximum increments of 2.75 times (0.77 mg/g DW) in VA content, in comparison to control culture (0.28 mg/g DW) were obtained, when 2% (v/v) CWE of *P. indica* was added for 24 h. Furthermore, about 1.8 fold enhancement in VA production by *V. officinalis* elicited with CWE of *P. indica* was observed with an exposure time of 48 h. However, further increase of exposure time (after 48 h) resulted in a steep decline in quantity of VA. It is clear that maximum accumulation of VA (7.3 mg/g DW) was recorded at 4% (v/v) dose with a 7 fold increase compared to control culture (1.03 mg/g DW) at 72 h and it decreased thereafter (Fig. 2).
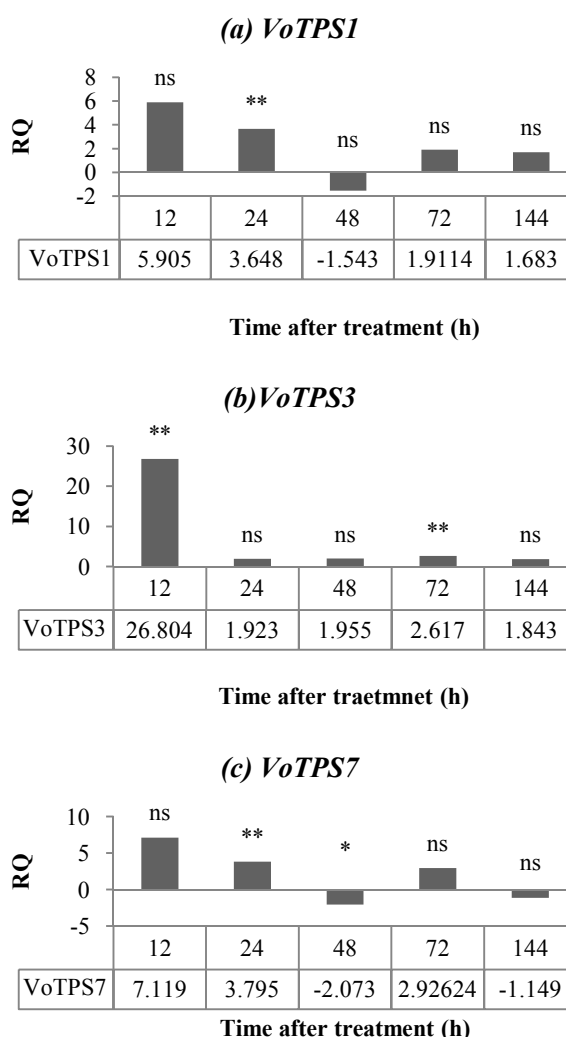

Time after treatment (h)	24	48	72	144
■ Control	0.28	0.72	1.03	2.11
▨ 2% CWE	0.77	1.34	0.97	0.36
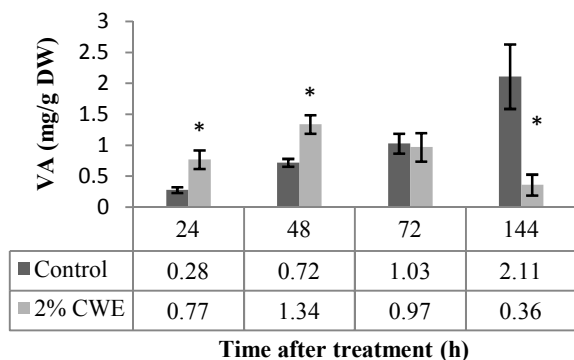
Figure 1. Time-course effects of fungal CWE (2% v/v) on valerenic acid production in the root of *V. officinalis* plants. The data were averaged from three replicates and analyzed using a *t*-test for significant differences between cell wall extract of the endophyte-treated and control plants (P< 0.05).

Effect of different concentrations of fungal CWE on expression pattern of Sesqui-TPS genes in V. officinalis roots

To explore the effects of fungal cell wall in modulation of the sesquiterpenes biosynthesis in the host plant, transcript abundance of *VoTPS1*, *VoTPS3* and *VoTPS7* genes was quantified by qRT-PCR in the root of *V. officinalis* plants (Fig. 3 & Fig. 4). As shown in Fig. 3a-c, the fungal CWE

Time after treatment (h)	24	48	72	144
■ Control	0.2833333	0.7166667	1.0366667	2.11
▨ 4% CWE	0.23	0.8266667	7.3466667	2.07

Figure 2. Time-course effects of fungal CWE (4% v/v) on valerenic acid production in the root of *V. officinalis* plants. The data were averaged from three replicates and analyzed using a *t*-test for significant differences between cell wall extract of the endophyte-treated and control plants (P< 0.05).

(a) VoTPS1

Time after treatment (h)	12	24	48	72	144
VoTPS1	5.905	3.648	-1.543	1.9114	1.683

(b) VoTPS3

Time after traetmnet (h)	12	24	48	72	144
VoTPS3	26.804	1.923	1.955	2.617	1.843

(c) VoTPS7

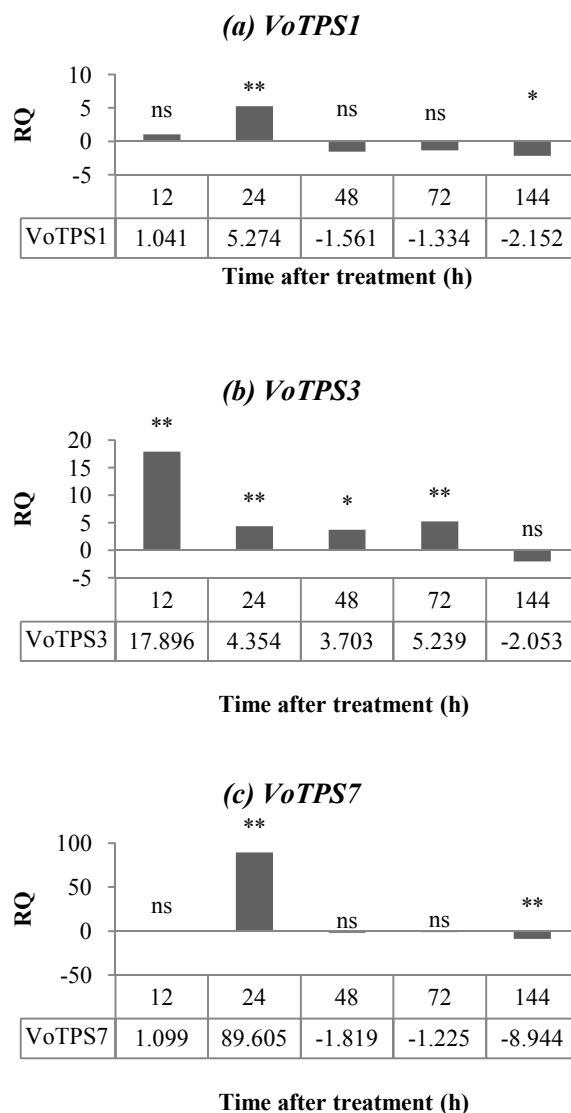| Time after treatment (h)	12	24	48	72	144
VoTPS7	7.119	3.795	-2.073	2.92624	-1.149

Fig. 3. Effect of fungal CWE (2% v/v) on expression of genes involved in sesquiterpenes biosynthesis. Transcript abundance of (a) *VoTPS1*, (b) *VoTPS3*, (c) *VoTPS7* genes were analyzed at exposure times of 12, 24, 48, 72 and 144 h in the root of *V. officinalis* plants inoculated with *P. indica* CWE. For normalization, *18S rRNA* gene was used as the endogenous gene. The Y-axis represents relative quantity. **, * and "ns" indicate significant differences respectively at (P<0.01), (P<0.05) and non-significant differences as compared to the endophyte CWE-free control plants.

at a concentration of 2% did not affect the expression of *VoTPS1* and *VoTPS7* genes at the time exposure of 12 h, while *VoTPS3* expression was significantly (~27 fold) higher than that in CWE-free control plants. This dose of the elicitor increased expression of *VoTPS1* and *VoTPS7* genes 3.6 and 3.8 times more compared to the non-elicited control, respectively at the time exposure of 24 h, but did not induce the expression *VoTPS3* significantly compared to control. As shown in Fig. 4a-c, the fungal CWE at a concentration of 4% did not induce the expression of *VoTPS1* and *VoTPS7* genes significantly compared to control plants at the time exposure of 12 h, while transcript expression of these genes reached the peak at 24 h (~5 and 90 times more compared to the endophyte CWE-free control plants, respectively) and then went back to the same levels with the control at 48 and 72 h. Transcript abundance of these genes reached their lowest level at the time exposure of 144 h. The *VoTPS3* gene showed higher expression level when the plants treated with 4% CWE level from 12 to 72 h and had the highest expression level at 12 h of exposure time; the expression level of *VoTPS3* returned to the same level with the control at the time exposure of 144 h.

Discussion

The *P. indica* CWE contains many known compounds such as conjugated protein, peptide, glycoprotein, lipid and oligosaccharide, which can mimic the presence of the fungus and acts as elicitors [18]. To evaluate the possible mechanism involved in *P. indica* CWE-mediated enhancement of VA biosynthesis, we have monitored the transcription rate of three possible terpene synthase gene candidate including *VoTPS1*, *VoTPS3* and *VoTPS7*. The qRT-PCR data presented here clearly demonstrated that a maximum increase of 5 fold in the expression level of *VoTPS1* gene was achieved upon addition of *P. indica* CWE at a level of 4% (v/v) for an exposure time of 24 h. An increase of 90 fold in the expression level of *VoTPS7* was obtained upon addition of *P. indica* CWE under similar condition. Expression of *VoTPS1* and *VoTPS7* genes is coordinately induced in response to fungal elicitor treatment. This consistence of gene expression suggested that these genes might be regulated by the same mechanism. Coordinated induction of terpenoid indole alkaloids (TIA) biosynthetic genes by fungal elicitor treatment was recently reported [19]. Sequence analysis of several *Catharanthus* TIA pathway promoters revealed the presence of several important *Cis*-regulatory elements including fungal elicitor-responsive elements. Two key families of transcription factors from *Catharanthus* bound to elicitor responsive element regions of the multiple *Catharanthus* TIA pathway promoters and their expression was rapidly induced by elicitor treatment [20]. These findings indicate that increased expression of sesquiterpene synthase genes is possibly modulated by an elicitor-responsive signal transduction pathway in *V. officinalis*. Addition of *P. indica* CWE for a shorter period, i.e., 12 h, resulted in comparatively lesser increment in *VoTPS1* and *VoTPS7* transcript levels. This might be due to insufficient time

available for signaling compounds in *P. indica* CWE to execute their effects.

(a) VoTPS1

	12	24	48	72	144
VoTPS1	1.041	5.274	-1.561	-1.334	-2.152

Time after treatment (h)

(b) VoTPS3

	12	24	48	72	144
VoTPS3	17.896	4.354	3.703	5.239	-2.053

Time after treatment (h)

(c) VoTPS7

	12	24	48	72	144
VoTPS7	1.099	89.605	-1.819	-1.225	-8.944

Time after treatment (h)

Figure 4. Effect of fungal CWE (4% v/v) on expression of genes involved in sesquiterpenes biosynthesis. Transcript abundance of (a) *VoTPS1*, (b) *VoTPS3*, (c) *VoTPS7* genes were analyzed at exposure times of 12, 24, 48, 72 and 144 h in the root of *V. officinalis* plants inoculated with *P. indica* CWE. For normalization, 18S rRNA gene was used as the endogenous gene. Y-axis represents relative quantity. **, * and "ns" indicate significant differences respectively at ($p<0.01$), ($p<0.05$) and non-significant differences as compared to the endophyte CWE-free control plants.

While, further increase of exposure time (after 24 h) resulted in a steep decline in *VoTPS1* and *VoTPS7* transcript levels probably due to reduced signal transduction after 24 h. However, the exact mechanism underlying the influence of the *P. indica* CWE-derived molecules in triggering signal transduction pathways leading to enhanced transcription rates of these putative terpene synthases needs to be further studied.

In present study, *P. indica* CWE- induced sesqui-TPS genes (*VoTPS1* and *VoTPS7*) was accompanied by substantial enhancement of the VA content in the *V. officinalis* roots. Our results support the hypothesis that valerenadiene and germacrene B/C/D synthase (respectively encoded by *VoTPS1* and *VoTPS7*) might provide intermediates in the VA-specific biosynthetic pathway [4]. In contrast, expression pattern of *VoTPS3* is quite different from *VoTPS1* and *VoTPS7*. A maximum of increase in the expression level of *VoTPS3* was achieved upon addition of *P. indica* CWE at both levels of 2 and 4% (v/v) for an exposure time of 12 h. Since *VoTPS3* mRNA exhibited to have low level activity with FPP, Yeo *et al*., [4] suggested this encodes an enzyme with a monoterpene synthase activity, It may be concluded that *VoTPS3* does not appear to have any significant influence on the biosynthesis of VA.

Our study provides evidence that different concentrations of *P. indica* CWE (2 and 4% v/v) were able to substantially enhance the VA content in the root of *V. officinalis*. The 4% fungal CWE application compared to 2 % produced more VA in treated plants.

Thus, the accumulation of VA is a dose elicitor-dependent response of roots of *V. officinalis*. Similar phenomenon of dose-dependent elicitation of ursolic acid (UA), oleanolic acid (OA) and betulinic acid (BA) was observed when the suspension culture of *Lantana camara* were treated with 2.5% v/v filter-sterilized culture filtrate of *P. indica* [10]. Furthermore, the response to elicitation is dependent on the duration time of incubation with elicitor. The optimum incubation time for VA induction with 4% CWE was found to be 72 h. However, further increase of exposure time led to decrease in VA content. Such incubation time-dependent results have been supported by the finding of Namdeo *et al*., [21], and Baldi *et al*., [8]. Therefore, in addition to the concentration of an elicitor, the exposure time of elicitor to plant cultures is also an important parameter for synthesizing the maximum concentration of desired bioactive molecules [22].

An elicitor requires minimum dosage to execute its effect. An elicitor, in a low dose, causes the elicitor binding sites in cells are not fully occupied for triggering the elicitation whereas high dosage of elicitor may have harmful effects on the production of desired metabolites due to induction hypersensivity response leading to cell death. Similarly, a longer exposure time may cause a deleterious effect on the formation of desired phytochemicals either due to their degradation or elicitation of other secondary metabolites [8, 23].

Conclusion

This is one of the first studies to show that CWE of *P. indica* may play an important role in promoting the VA content by modulating the expression of genes involved in sesquiterpenes biosynthesis. These results suggest that the mechanism(s) responsible for the enhancement of metabolites could be related to the fungus-plant interaction. Further studies are required to shed more light on molecular mechanisms of signaling cascade triggered by active fractions of CWE for activation of the transcriptional rate observed in this study.

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