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ORIGINAL PAPER

Fungal elicitor-mediated enhancement in growth and asiaticoside content of *Centella asiatica* L. shoot cultures

Archana Prasad · Archana Mathur · Alok Kalra · Madan M. Gupta · Raj K. Lal · Ajay K. Mathur

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Abstract Effect of fungal elicitors on biomass and asiaticoside production in multiple shoot cultures of *Centella asiatica* was studied in a dose- and culture age- dependent manner. Addition of 3 % v/v *Trichoderma harzianum* culture filtrate (CF) in the growth medium on 10th day of 35 days culture cycle resulted in 9.63 mg g^{-1}dry weight content and 1.15 mg dry weight $culture^{-1}$ yield of asiaticoside that was 2.53 and 2.35 times higher than in the unelicited control shoots, respectively. The elicited cultures also registered 1.24 folds more biomass over the control with a growth index (GI) of 7.67, calculated as fresh weight increment over the initial inoculum weight. Elicitation of shoots with mycelial extract (ME) of the fungus *Colletotrichum lindemuthianum* (1.5 % v/v, added on 0 day) on the other hand, though favored highest biomass accumulation amongst all the elicitation treatments (GI = 16.10) resulted in a decreased asiaticoside content of 1.10 mg g^{-1} dry weight that was nearly 3.5 and 8.7 times lower than in the control or *T. harzianum* CF-treated shoots, respectively. Treatments with mycelial extract of *Fusarium oxysporum* (0.5–1.5 % v/v), in general, proved inhibitory for shoot growth if added on 0 day of the culture cycle with GI = 4.85–8.45 in comparison to 11.11 in the control with a poor asiaticoside yield of only 0.18–0.42 mg dry weight $culture^{-1}$. Though the shoot biomass accumulation was marginally improved (GI = 5.68–11.94) over the untreated control when *F. oxysporum* ME (0.5–1.5 % v/v) was added in the medium on the 30th day of culture but the asiaticoside yield (0.18–0.94 mg dry weight $culture^{-1}$) remained low. The results presented here demonstrate for the first time the potential application of *T. harzianum* CF in up-regulating the asiaticoside biogenetic pathway in *C. asiatica.*

Keywords *Centella asiatica* · Asiaticoside · Multiple shoot cultures · *Trichoderma harzianum* · Fungal elicitation

A. Prasad · A. Mathur (✉) · A. K. Mathur
Division of Plant Biotechnology, Central Institute of Medicinal and Aromatic Plants, Council of Scientific and Industrial Research (CSIR), PO CIMAP, Lucknow 226015, India
e-mail: archnacimap@yahoo.co.in; a.mathur@cimap.res.in

A. Kalra
Division of Microbiology and Entomology, Central Institute of Medicinal and Aromatic Plants, Council of Scientific and Industrial Research (CSIR), PO CIMAP, Lucknow 226015, India

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

R. K. Lal
Division of Genetics and Plant Breeding, Central Institute of Medicinal and Aromatic Plants, Council of Scientific and Industrial Research (CSIR), PO CIMAP, Lucknow 226015, India

Introduction

Centella asiatica (L.) Urban (Family-Apiaceae) is a herbaceous plant with great medicinal value. Triterpenoid saponins and sapogenins, particularly asiaticoside, madecassoside, asiatic acid and madecassic acid, are the chief bioactive molecules present in this plant (Mathur et al. 2007; Randriamampionona et al. 2007). Asiaticoside extracted from the aerial parts of the plant is known to exert strong diuretic, antispasmodic, circulation stimulatory and wound healing actions and is frequently used in the treatment of leprosy, ulcer, asthma, bronchitis, elephantiasis, eczemas, anxiety, mental disorders and urethritis (Mathur et al. 2007; Gohil et al. 2010). Asiaticoside

derivatives are also widely used in the treatment of Alzheimer's disease as they can protect cells against β-amyloid induced cell death (Mook-Jung et al. 1999). Recently, studying the possible mode of action of asiaticoside in wound healing and suppression of proliferation of keloid-derived fibroblasts and collagen production, Tang et al. (2011) have demonstrated that asiaticoside suppresses collagen expression and TGF-β/Smad signalling by inducing Smad7 and inhibiting TGF-β receptors RI and RII in keloid fibroblasts to prevent hypertrophic scars and keloid formation. The pharmaceutical companies depend on the supply of this herb from its wild populations growing near the swampy ditches that are often contaminated with heavy metal pollutants and other harmful chemical residues that adversely affect the medicinal efficacy of its herbal formulations (McCaleb et al. 2000). In vitro tissue culture- or hydroponics-based cultivation of *C. asiatica*, therefore, offers an interesting and sustainable alternative option to produce uniform, clean herb of consistent quality for pharmaceutical companies. Multiple shoot cultures of *C. asiatica* have been shown to produce asiaticoside in an age dependent manner under in vitro and hydroponics environment (Kim et al. 2004; Mangas et al. 2006; Hernandez-Vazquez et al. 2010; Prasad et al. 2012a, b). Callus, cell suspensions, transformed hairy roots or adventitious roots regenerated from leaf-derived callus have also been found to synthesize asiaticoside, but to a much lesser extent than the multiple shoots (Kiong et al. 2005; Aziz et al. 2007; Kim et al. 2007; Mangas et al. 2008; Bonfill et al. 2011; Mercy et al. 2012). In many of these studies, the asiaticoside biogenesis was also found to be up-regulated by abiotic elicitors like methyl jasmonate or by feeding pathway precursors like squalene and β-amyrin. Reports on the use of biotic elicitors to enhance triterpenoid saponins production in *C. asiatica* are, however, scanty. Kim et al. (2004) have found that whole plant cultures of *C. asiatica* produced 1.41 folds more asiaticoside when treated with 0.1 g l^{-1} yeast extract. Recently, Satheesan et al. (2012) have also reported that colonization of a growth-promoting endophytic fungus *Piriformospora indica* in roots of in vitro grown rooted plants of *C. asiatica* resulted in rapid shoot and root biomass accumulation along with two fold increase in asiaticoside content (0.53 % w/w) in the leaves. Based on Real-time PCR analysis, the authors have corroborated that such mutualistic association in *P. indica*-challenged plants led to enhanced asiaticoside synthesis due to higher expression of two pathway genes squalene synthase and β-amyrin synthase. Reports on the positive influence of biotic elicitation on triterpenoid saponins/glycosides pathway in cell and tissue cultures of many other plant systems like *Perilla frutescens, Calendula officinalis* and *Uncaria tomentosa* also exist in literature (Wang et al. 2004; Feria-Romero et al. 2005; Wiktorowska et al. 2010; Mathur and Mathur 2010).

In this communication we describe the elicitation effects of culture filtrate (CF) of *Trichoderma harzianum* and mycelial extract (ME) of *Colletotrichum lindemuthianum* and *Fusarium oxysporum* on biomass and asiaticoside accumulation in multiple shoot cultures of *C. asiatica.* To the best of our knowledge this constitutes the first report on dose- and age-dependent biotic elicitation of asiaticoside synthesis involving ME or CF of these fungal organisms.

Materials and methods

Raising of shoot cultures, media composition and incubation condition

Axillary shoot cultures of *C. asiatica* were established by culturing nodal explants on agar-gelled MS (Murashige and Skoog's 1962) medium supplemented with 2.5 mg l^{-1} kinetin (Kn) and 30 g l^{-1} sucrose. The axillary shoots thus generated were shifted to MS liquid medium of similar composition (10 ml/flask of 100 ml capacity) to form multiple shoots as described earlier (Prasad et al. 2012a). Multiple shoot cultures were maintained in vitro by regular sub-culturing every 6–8 weeks. For elicitation experiments, uniform nodal shoot explants (0.2–0.3 g fresh weight) were excised from 2 month old stock cultures and inoculated in 10 ml liquid medium in 100 ml Erlenmeyer flasks. The elicitors were individually added in the culture medium in defined doses at different stages of growth as described in the succeeding section. The pH of the culture medium was adjusted to 5.8 ± 0.04 with 1 N NaOH/HCl before autoclaving at 121 °C and 15 ψ for 15 min. All shoot cultures were incubated at 25 ± 3 °C under 16 h light (54 μmol m^{-2} s^{-1}) provided by cool white fluorescent tube lights.

Preparation of fungal elicitors

Fusarium oxysporum and *Colletotrichum lindemuthianum* were grown in potato dextrose broth. For this, 10.0 mm **(Ø)** disc of the actively growing fungal isolates on potato dextrose agar medium was inoculated into 250 ml of sterile potato dextrose broth in 500 ml Erlenmeyer flask and grown for 7–10 days at 30–34 °C. After incubation the mycelium was harvested through filtration and washed 4 times with sterile distilled water. The mycelium was then dried first at room temperature and then at 70 °C in a hot air oven. The dried mycelium was powdered and washed with chloroform: methanol (1:1) mixture followed by washing with acetone. The mycelium powder was again dried at room temperature and 1 g of it was mixed with

10 ml distilled water and autoclaved at 120 °C for 1 h. The autoclaved mixture was centrifuged at 5,000 rpm for 10 min and supernatant was collected. The pellet was re-suspended in 10 ml distilled water and autoclaved again and the procedure was repeated 3–4 times. The supernatants were pooled and concentrated in a Rotavapour to 5 ml and dialysed with distilled water at 4 °C for 24 h. The dialyzed extract was finally filter-sterilized through 0.22 µm membrane and used in elicitation experiments. *T. harzianum* (ATCC No.PTA 3701) culture filtrate was prepared by growing the fungus in 250 ml potato dextrose broth under static condition at 30 °C for 2 weeks. After 14 days, the mycelium was separated by filtration through Whatman No.1 filter paper. The resultant culture broth was finally filter-sterilized (0.22 µm) and used for elicitation.

Elicitation treatments

The mycelial extract of *F. oxysporum* and *C. lindemuthianum* were individually added to the *C. asiatica* shoot culture medium at 0.5, 1.0 and 1.5 % (v/v) concentration on 0 or 30th day of the culture cycle whereas the culture filtrate of *T. harzianum* was tested at 1.0, 2.0, 3.0 and 4.0 % (v/v) levels added on 0, 10th and 20th of the culture growth. The elicitation treatments tested in the present study were deduced on the basis of several trail experiments conducted with ME and CF of the tested fungal isolates on soil-grown potted plants of *C. asiatica* (data not presented here). All elicited or non-elicited cultures were harvested at the end of 35 days of culture cycle when maximum growth and asiaticoside productivity is normally attained in such cultures (Prasad et al. 2012a). The biomass accumulation was measured in terms of fresh weight increment over the initial inoculum weight and expressed as Growth Index (GI). The harvested shoots were lyophilized to constant dry weight and used for asiaticoside extraction. The asiaticoside content and yield was calculated on mg g^{-1}dry weight and mg dry weight $culture^{-1}$ basis, respectively.

Extraction and quantification of asiaticoside

For asiaticoside extraction 100 mg of lyophilized powdered shoot tissue was extracted thrice with 5 ml of methanol: water (80:20) for 24 h each. The pooled methanol extract was concentrated in Rotavapor to 5.0 ml and defatted twice with 20 ml n-hexane for 4 h. The n-hexane layer was discarded and the defatted methanol extract was collected, dried under vacuum and dissolved in 1.0 ml HPLC grade methanol for further analysis. The HPLC quantification of asiaticoside was carried out using the procedure described earlier (Verma et al. 1999). A gradient liquid chromatographic system (model LC-10A series, Shimadzu, Japan) fitted with two LC-10AD pumps controlled by CMB-10A interface module equipped with multi-dimesional UV–Vis detector (SPD-10A), 7725i manual injector (Rheodyne), 20 µl sample loop and a CLC-ODS (M) column (250 × 4.6 mm id; Shimadzu) was used. The elution was performed using the solvent system composed of 1 % trifluoroacetic acid (TFA): methanol (30:70 v/v) at a flow rate of 1 ml min^{-1} (column temperature of 26 °C). Detection was done at 220 nm wavelength. Reference compound of asiaticoside was purchased from Fluka Analytical, France.

Statistical analysis

The data on various growth and productivity parameters were collected from 3 independently performed experiments with a minimum of 3 replicated cultures per treatment. The data is expressed as mean performance of all replicates along with their standard deviation. The values were subjected to ANOVA analysis using random block design (Tables 1, 2, 3).

Results

Effect of fungal elicitors on growth of multiple shoot cultures

The multiple shoot cultures of *C. asiatica* on control medium (i.e., without any elicitor supplementation) attained a GI of 6.21 at the time of harvesting on 35th day of the culture cycle (Fig. 1a). Addition of 1, 2, 3 or 4 % (v/v) CF of *T. harzianum* in the medium on 0, 10th and 20th day of the culture cycle did not affect the shoot growth significantly except a 1.24 fold increase in the treatment where the shoots were exposed to 3 % CF after 10 days of growth (GI = 7.67). In fact, *T. harzianum* CF at all doses when applied at the beginning of the culture cycle on 0 day proved rather inhibitory for the culture growth (GI = 4.9–5.38) in comparison to untreated control shoots. Elicitor treatments given to 20 days old shoots again did not alter the growth indices (GI = 5.28–6.50) that remained more or less at par with that of untreated shoots (Fig. 1a).

Figures 2 and 3 summarise the influence of mycelial extracts of *C. lindemuthianum* and *F.oxysporum* on shoot growth. Lower doses of *C. lindemuthianum* ME (0.5 and 1 % v/v) when applied at the beginning of the culture cycle proved significantly inhibitory to shoot growth with GI = 5.09 and 8.12, respectively, when compared with the control cultures in this set of experiment (GI = 11.11). In contrast, the shoot growth improved by 1.45 folds with a GI of 16.11 in cultures grown on medium containing 1.5 %

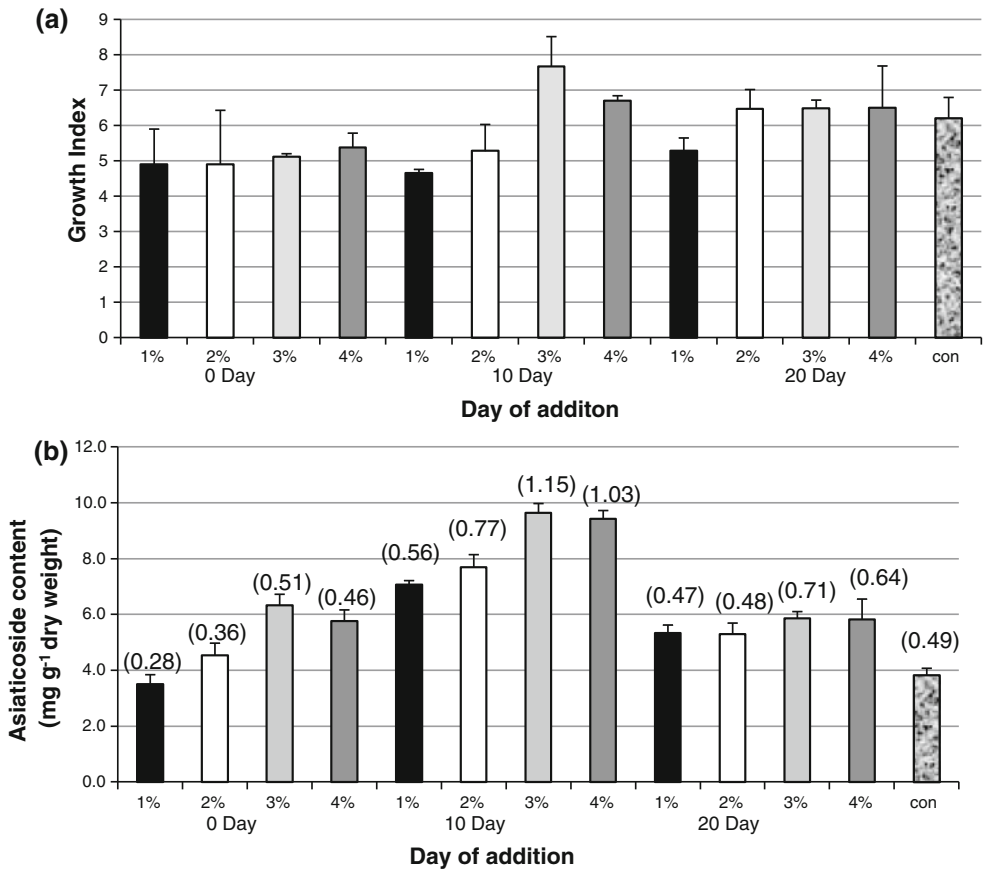

Fig. 1 Effect of *T.harzianum* CF at 1, 2, 3, and 4 % (v/v) doses on growth index (**a**) and asiaticoside content (**b**) of multiple shoot cultures of *C. asiatica*. Values in *parenthesis* represents total asiaticoside yield on mg dry weight culture^{-1} basis. con-Untreated control culture. *Error bars* represent standard deviation (N = 3)

Table 1 Analysis of variance using RBD for *T. harzianum* elicited cultures (**$P < 0.01$)

Source of variation	Degree of freedom	Mean sum of squares	
		Growth index (GI)	Asiaticoside content (GI) (mg g^{-1} dry weight)
Treatments	12	2.35**	0.87**
Error	26	0.56	0.01
Total	38		

ME of this fungus added on 0 day. The elicitor at this dose, however, was not found effective in influencing the shoot growth when applied on 30th day of the culture cycle (GI = 12.12; Fig. 2a). Treatments of *F.oxysporum* ME at all concentrations tested showed more pronounced growth inhibition ($P < 0.01$) except at 1.5 % v/v dose applied on 30th day of culturing where a marginal improvement in shoot growth (GI = 11.94) was noticed in comparison to control (GI = 11.11; Fig. 3a).

Effect of elicitors on asiaticoside content and yield

The effect of different fungal elicitors on asiaticoside content and yield was also monitored in a dose- and culture age-dependent manner (Fig. 1b, 2b, 3b). Out of the three biotic elicitors tested in this study, the highest asiaticoside content of 9.63 mg g^{-1} dry weight was recorded in shoots treated with 3 % v/v CF of *T. harzianum* added on 10th day of shoot growth (Fig. 1b). This was 2.53 fold more than that of untreated cultures (3.83 mg g^{-1} dry weight) at the time of harvesting on 35th day. Comparable enhancement in asiaticoside content (9.43 mg g^{-1} dry weight) was also noticed in shoots treated with 4 % CF of *T. harzianum* on 10th day. These two elicitation treatments were also found significantly superior in terms of asiaticoside yield of 1.15 and 1.03 mg dry weight culture^{-1} over the control (0.49 mg dry weight culture^{-1}) or rest of the elicitation treatments involving *T. harzianum* CF (0.28–0.77 mg dry

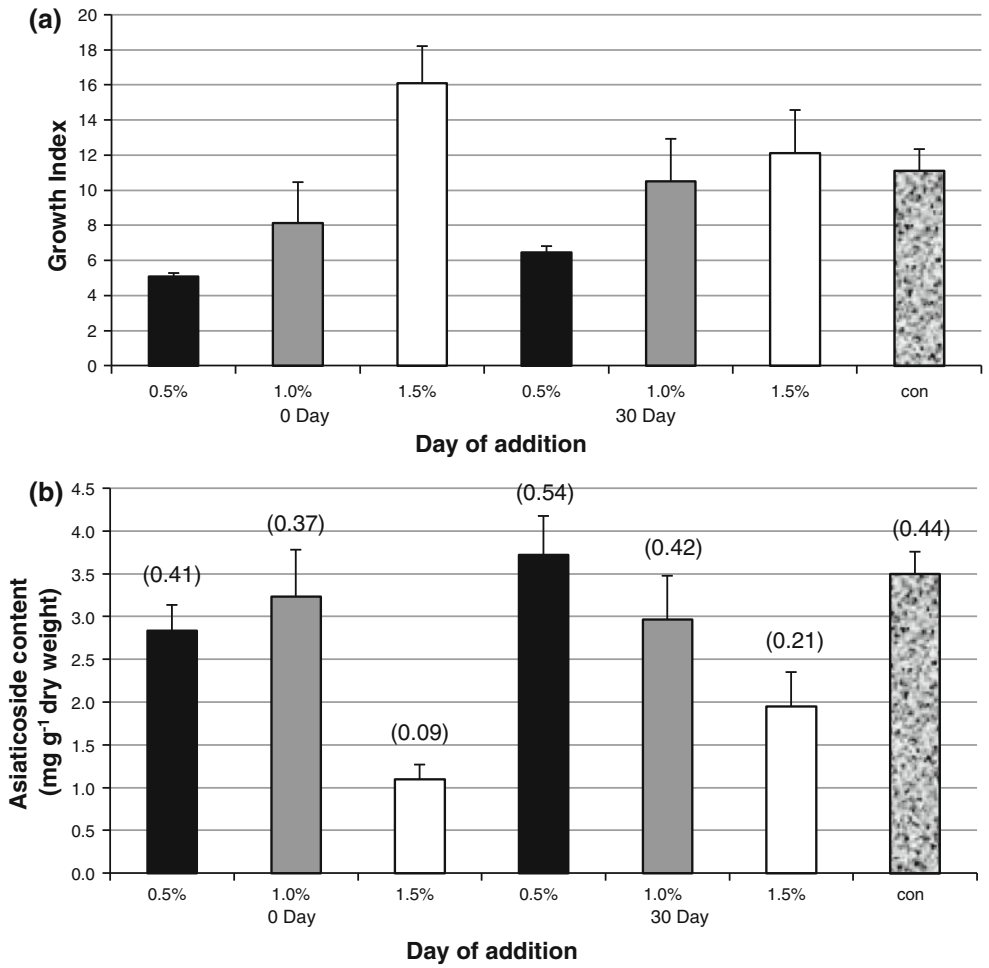

Fig. 2 Effect of *C. lindemuthianum* ME at 0.5, 1.0 and 1.5 % (v/v) doses on growth index (**a**) and asiaticoside content (**b**) of multiple shoot cultures of *C. asiatica*. Values in *parenthesis* represents total asiaticoside yield on mg dry weight culture^{-1} basis. Con-Untreated control culture. *Error bars* represent the standard deviation (N = 3)

Table 2 Analysis of variance using RBD for *C. lindemuthianum* elicited cultures (*$P < 0.05$, **$P < 0.01$)

Source of variation	Degree of freedom	Mean sum of squares	
		Growth index (GI)	Asiaticoside content (mg g^{-1} dry weight)
Treatments	6	41.73*	2.69**
Error	14	9.80	0.16
Total	20		

weight culture^{-1}). In contrast, addition of *F. oxysporum* ME on 0 day of the culture cycle showed reduction in asiaticoside content as well as yield at all the concentrations tested (Fig. 3b). Though, treatment of shoots with 0.5 % v/v of this ME after 30 days of growth resulted in 1.66 folds increase in asiaticoside content (5.84 mg g^{-1} dry weight) over the non-treated controls, but because of its negative influence on shoot growth the net asiaticoside yield per culture remained low when compared with shoots treated with 3 % CF of *T. harzianum* on 10th day (Fig. 1b).

Elicitation efforts made with ME of *C. lindemuthianum,* in general, proved more inhibitory for asiaticoside content and yield in a dose-dependent manner (Fig. 2b). Highest dose of this elicitor (1.5 % v/v) reduced the asiaticoside content to 1.1 and 1.95 mg g^{-1} dry weight when tested on 0 and 30th day of the culture cycle in comparison to 3.5 mg g^{-1} dry weight in the un-treated shoots. Lowering of elicitor dose to 0.5 % added after 30 days of growth though improved the asiaticoside content yield to 3.72 mg g^{-1} dry weight, did not enhance the metabolite

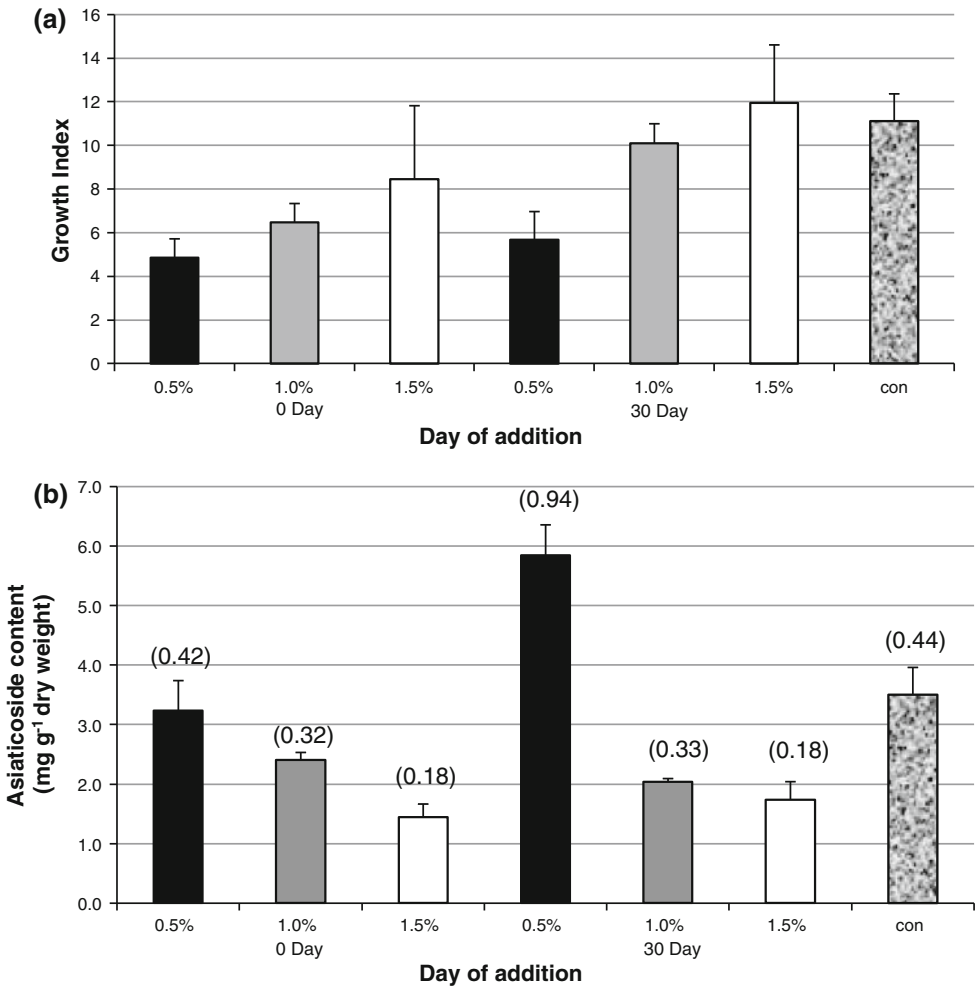

Fig. 3 Effect of *F.oxysporum* ME at 0.5, 1.0 and 1.5 % (v/v) doses on growth index (**a**) and asiaticoside content (**b**) of multiple shoot cultures of *C. asiatica*. Values in *parenthesis* represents total asiaticoside yield on mg dry weight culture^{-1} basis. Con-Untreated control culture. *Error bars* represent standard deviation (N = 3)

Table 3 Analysis of variance using RBD for *F. oxysporum* elicited cultures (**$P < 0.01$)

Source of variation	Degree of freedom	Mean sum of squares	
		Growth index (GI)	Asiaticoside content (mg g^{-1} dry weight)
Treatments	6	24.28**	6.80**
Error	14	3.45	0.12
Total	20		

yield significantly because of poor biomass accumulation in such cultures.

Discussion

Plant cell, tissue and organ cultures are frequently explored as efficient renewable resource for producing a variety of phytochemicals that are either difficult to be synthesised chemically or are produced in extremely low concentration in field-grown plants. The progress in this subject has been periodically reviewed by many workers (Dicosmo and Misawa 1995; Collins 2001; Verpoorte and Memelink 2002; Mulabagal and Tsay 2004; Martin et al. 2008; Karuppusamy 2009; Siahsar et al. 2011). Though use of callus and cell suspension cultures for this purpose is more preferred because of the ease with which they can be up-scaled in bioreactors, but in most cases the biosynthesis of these compounds has been found to be linked with higher level of cellular/tissue differentiation in the form of organised shoots or roots or both (Kornfeld et al. 2007; Vinterhalter et al. 2008; Shalaka and Sandhya 2009; Karuppusamy 2009). This situation is more demanding when desired metabolites are synthesised or accumulated in specialized cells or tissues of the plants (Facchini and DeLuca 2008; Verma et al. 2012). In vitro production of triterpene

glycosides particularly asiaticoside in *C. asiatica* studied in the present report falls in this category of secondary metabolites where leaves of the organized shoot cultures have been shown to be better metabolite yielder than callus or cell suspension (Kim et al. 2004; Mangas et al. 2006; Hernandez-Vazquez et al. 2010; Prasad et al. 2012a). From amongst the various experimental strategies for improving the productivity of plant secondary metabolites in cultured tissues, the use of biotic and abiotic elicitors to initiate a signal transduction mechanism for global activation of several inter-related pathway genes is being rigorously followed these days (Dörnenburg and Knorr 1995; Zhao et al. 2005; Namdeo 2007). Elicitors are generally defined as chemicals or bio-factors of abiotic or biotic origin that can trigger a signal transduction pathway for initiating or altering a physiological and/or morphological response of a plant cell to attain ecological fitness under a stressed environment (Zhao et al. 2001, 2005). While abiotic elicitors used for this purpose normally include metal ions and inorganic signalling molecules like Cd^{++}, Ca^{++},Hg^{++}, Al^{+++}, Cu^{++}, salicylic acid, methyl jasmonate etc., the biotic elicitors are generally derived from pathogenic or non-pathogenic micro-organisms, plant cell wall components, as well as chemicals released by plants at point of pathogen or herbivore attack (Wang et al. 2001, 2004; Namdeo et al. 2002; Fuss 2003; Jeong and Park 2007; Liu et al. 2007; Ajungla et al. 2009; Wiktorowska et al. 2010). Elicitors generally impart their influence by inducing rearrangement of metabolic fluxes between constitutively expressed pathways leading to the hyper accumulation of defence related secondary metabolites (Namdeo 2007). In the present study three biotic elicitors derived from fungi namely *C. lindemuthianum, F.oxysporum* and *T. harzianum* were tested for influencing the in vitro asiaticoside production in multiple shoot cultures of *C. asiatica*. Our results have clearly indicated, and are in agreement with earlier findings in *Centella* and many other plant systems, that parameters such as elicitor specificity, growth stage of the treated tissue, concentration and duration of exposure to elicitor have considerable influence on the elicitation process and outcome (Namdeo et al. 2002; Kim et al. 2004, 2007; Mangas et al. 2006; Liu et al. 2007; Vasconsuelo and Boland 2007; Wiktorowska et al. 2010; Bonfill et al. 2011). *C. asiatica* shoots in our study were found more responsive to elicitation for growth and asiaticoside accumulation by culture filtrate of *T. harzianum* than the mycelial extract of *F. oxysporum* or *C. lindemuthianum*. Also the efficacy of *T. harzianum* CF treatment was maximum in terms of highest asiaticoside content of 9.63 mg g^{-1} dry weight when it was applied at a dose of 3–4 % v/v on 10 days old cultures. While longer exposure of the shoots to this elicitor had a negative effect on growth and asiaticoside productivity, its supplementation at later growth stages did not have a marked influence. The improvement in asiaticoside production in our study was found to be much higher than reported levels in earlier abiotic elicitation efforts made in this plant (Kim et al. 2004; Mangas et al. 2006; Aziz et al. 2007). In comparison, addition of *F. oxysporum* mycelial extract at all doses during early stages of culture cycle caused inhibition of both growth and asiaticoside accumulation as was also reported for hyoscyamine and scopolamine synthesis in callus cultures of *Datura metel* (Ajungla et al. 2009). Members of the genus *Trichoderma* are well known for their effect on plant growth promotion and are frequently used as bio-protectant (Harman et al. 2004; Cordo et al. 2007; Vinale et al. 2008). Studies dealing with the mechanism of interaction between *Trichoderma* and plants have indicated that culture filtrate of this fungus contains macromolecules and low molecular weight compounds that induce strong changes in cytosolic Ca^{2+} level in plant cells and activates defence responses including the accumulation of plant secondary metabolites (Fuss 2003; Navazio et al. 2007). Present and earlier findings of our group on growth and asiaticoside production kinetics in in vitro and hydroponically-grown shoot cultures of *C. asiatica* (Prasad et al. 2012a, b) have also revealed an age-dependent nature of metabolite accumulation in this herb and a 35 days old growth period is optimal for maximum recovery of this compound. This phenomenon has also been observed for podophyllotoxin production by cell suspension cultures of *Linum album* (Shams-Ardakani et al. 2005) and triterpene production in *Tabernaemontana catherinensis* (Pereira et al. 2007).

Conclusion

The present study has conclusively demonstrated a strong positive influence of the culture filtrate of *Trichoderma harzianum* on growth and asiaticoside production in multiple shoot cultures of *C. asiatica*. This fungal elicitor at a defined dose and culture age stimulated the asiaticoside production by 2.35 folds (1.15 mg dry weight $culture^{-1}$) in the shoots. It is hoped that method developed in the present study will not only provide an alternate production platform for asiaticoside but would also be useful for the isolation and expression of genes associated with the biosynthesis of this bioactive compound. We are presently engaged in comparative transcript profiling of elicited versus non-elicited multiple shoots to understand the regulatory mechanisms of *in planta* synthesis of centellosides in *C. asiatica* herb.

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References

Ajungla L, Patil PP, Barmukh RB, Nikam TD (2009) Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L. Indian J Biotechnol 8:317–322

Aziz ZA, Davey MR, Power JB, Anthony P, Smith RM, Lowe KC (2007) Production of asiaticoside and madecassoside in *Centella asiatica* in vitro and in vivo. Biol Plant 51:34–42

Bonfill M, Mangas S, Moyano E, Clusido RM, Javier P (2011) Production of centellosides and phytosterols in cell suspension cultures of *Centella asiatica*. Plant Cell Tiss Organ Cult 104: 61–67

Collin HA (2001) Secondary product formation in plant tissue cultures. Plant Growth Regul 34:119–134

Cordo CA, Monaco CL, Segarra CI, Simon MR, Mansilla AY, Perello AE, Kripelz NI, Bayo D, Conde RD (2007) *Trichoderma* spp.as elicitors of wheat plant defense responses against *Septoria tritici*. Biocontrol Sci Technol 17:687–698

Dicosmo F, Misawa M (1995) Plant cell and tissue culture: alternatives for metabolite production. Biotechnol Adv 13: 425–453

Dörnenburg H, Knorr D (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzyme Microb Technol 17:674–684

Facchini PJ, DeLuca VD (2008) *Opium poppy* and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. Plant J 54:763–784

Feria-Romero I, Lazo E, Ponce-Noyola T, Cerda-Garcia-Rojas CM, Ramos-Valdivia AC (2005) Induced accumulation of oleanolic acid and ursolic acid in cell suspension cultures of *Uncaria tomentosa*. Biotechnol Lett 27:839–843

Fuss E (2003) Lignans in plant cell and organ cultures: an overview. Phytochem Rev 2:307–320

Gohil KJ, Patil JA, Gajjar AK (2010) Pharmacological review on *Centella asiatica*: a potential herbal cure-all. Indian J Pharm Sci 72:546–556

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2:43–56

Hernandez-Vazquez L, Bonfill M, Moyano E, Cusido RM, Palazon J, Novarro-Ocna A (2010) Conversion of α-amyrin into centellosides by plant cell cultures of *Centella asiatica*. Biotechnol Lett 32:315–319

Jeong GA, Park DH (2007) Enhanced secondary metabolite biosynthesis by elicitation in transformed plant root systems. Appl Biochem Biotechnol 130:436–446

Karuppusamy S (2009) A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. J Med Plant Res 3:1222–1239

Kim OT, Kim MY, Hong MH, Ahnn JC (2004) Stimulation of asiaticoside accumulation in the whole plant cultures of *Centella asiatica* (L.) Urban by elicitors. Plant Cell Rep 23:339–344

Kim OT, Bang KH, Shin YS, Lee MJ, Jung SJ, Hyun DY, Kim YC, Seong NS, Cha SW, Hwang B (2007) Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. Plant Cell Rep 26: 1941–1949

Kiong ALP, Mahmood M, Fadzillah M, Daud SK (2005) Effect of precursor supplementation on the production of triterpenes by *Centella asiatica* callus cultures. Pak J Biol Sci 8:1160–1169

Kornfeld A, Kaufman PB, Lu CR, Gibson DM, Bolling SF, Warber SL, Chang SC, Kirakosyan A (2007) The production of hypericins in two selected *Hypericum perforatum* shoot cultures is related to differences in black gland culture. Plant Physiol Biochem 45:24–32

Liu XN, Zhang XQ, Sun JS (2007) Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hypericum perforatum*. Plant Growth Regul 53:207–214

Mangas S, Bonfill M, Osuna L, Moyano E, Tortoriello J, Cusido RM, Piñol MT, Palazo′n J (2006) The effect of methyl jasmonate on triterpene and sterol metabolisms of *Centella asiatica*, *Ruscus aculeatus* and *Galphimia glauca* cultured plants. Phytochemistry 67:2041–2049

Mangas S, Moyano E, Osuna L, Cusido RM, Bonfill M, Palazon J (2008) Triterpenoid saponin content and the expression level of some related genes in calli of *Centella asiatica*. Biotechnol Lett 30:1853–1859

Martin EK, Vishal G, Susan CR (2008) Pharmaceutically active natural product synthesis via plant cell culture technology. Mol Pharm 5:243–256

Mathur A, Mathur AK (2010) In vitro saponin production in plant cell and tissue cultures. In: Arora R (ed) Medicinal Plant Biotechnology. CAB Internationals, UK, pp 115–137

Mathur A, Mathur AK, Yadav S, Verma P (2007) *Centella asiatica* (L.) Urban-Status and scope for commercial cultivation. J Med Arom Plant Sci 129:151–162

McCaleb R, Morien K, Schott T (2000) Market report on herbs and spices. Agribusinesss Sustainable Natural African Plant Products (A-SNAPP Online); http://www.herbs.org/africa/marketreport0400.html

Mercy S, Sangeetha N, Ganesh D (2012) *In vitro* production of adventitious roots containing asiaticoside from leaf tissues of *Centella asiatica* L. In Vitro Cell Dev Biol-Plant 48:200–207

Mook-Jung I, Shin JE, Yun SH, Huh K, Koh JY, Park HK, Jew SS, Jung MW (1999) Protective effects of asiaticoside derivatives against beta-amyloid neurotoxicity. J Neurosci Res 58:417–425

Mulabagal V, Tsay HS (2004) Plant cell cultures-An alternative and efficient source for the production of biologically important secondary metabolites. Int J Appl Sci Eng 2:29–48

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

Namdeo AG (2007) Plant cell elicitation for production of secondary metabolites: a review. Pharmacogn Rev 1:69–79

Namdeo A, Patil S, Fulzele DP (2002) Influence of fungal elicitors on production of ajmalicine by cell cultures of *Catharanthus roseus*. Biotechnol Prog 18:159–162

Navazio L, Baldan B, Moscatiello R, Zuppini A, Woo SL, Mariani P, Lorito M (2007) Calcium-mediated perception and defense responses activated in plant cells by metabolite mixtures secreted by the biocontrol fungus *Trichoderma atroviride*. BMC Plant Biol 7:41

Pereira PS, Ticli FK, França SC, Breves CMS, Lourenço MV (2007) Enhanced triterpene production in *Tabernaemontana catharinensis* cell suspension cultures in response to biotic elicitors. Quim Nova 30:1849–1852

Prasad A, Mathur A, Singh M, Gupta MM, Uniyal GC, Lal RK, Mathur AK (2012a) Growth and asiaticoside production in multiple shoot cultures of a medicinal herb *Centella asiatica* L. under the influence of nutrient manipulations. J Nat Med 66:383–387

Prasad A, Pragadheesh VS, Mathur A, Srivastava NK, Singh M, Mathur AK (2012b) Growth and centelloside production in hydroponically established medicinal plant-*Centella asiatica* (L.). Ind Crops Prod 35:309–312

Randriamampionona D, Diallo B, Rakotoniriana F, Rabemanantsoa C, Cheuk K, Corbisier AM, Mahillion J, Ratsimamanga S, Jaziri MEJ

(2007) Comparative analysis of active constituents in *Centella asiatica* samples from Madagascar: application for *ex situ* conservation and clonal propagation. Fitoterapia 78:482–489

Satheesan J, Narayanan AK, Sakunthala M (2012) Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in *Centella asiatica*. Mycorrhiza 22:195–202

Shalaka DK, Sandhya P (2009) Micropropagation and organogenesis in *Adhatoda vasica* for estimation of vasine. Pharmacogn Mag 5:359–363

Shams-Ardakani M, Hemmati S, Mohagheghzadeh A (2005) Effect of elicitors on the enhancement of podophyllotoxin biosynthesis in suspension cultures of *Linum album*. Daru J Pharm Sci 13:56–60

Siahsar B, Rahimi M, Tavasssoli A, Raissi AS (2011) Application of biotechnology in production of medicinal plants. Am-Euras J Agric Environ Sci 11:439–444

Tang B, Zhu B, Liang Y, Bi L, Hu Z, Chen B, Zhang K, Zhu J (2011) Asiaticoside suppresses collagen expression and TGF-b/Smad signaling through inducing Smad7 and inhibiting TGF-bRI and TGF-bRII in keloid fibroblasts. Arch Dermatol Res 303:563–572

Vasconsuelo A, Boland R (2007) Molecular aspects of the early stages of elicitation of secondary metabolites in plants. Plant Sci 172:861–875

Verma RK, Bhartariya KG, Gupta MM, Kumar S (1999) Reverse-phase high performance liquid chromatography of asiaticoside in *Centella asiatica*. Phytochem Anal 10:191–193

Verma P, Mathur AK, Srivastava A, Mathur A (2012) Emerging trends in research on spatial and temporal organization of terpenoid indol alkaloid pathway in *Catharanthus roseus*. Protoplasma 249:255–268

Verpoorte R, Memelink J (2002) Engineering secondary metabolite production in plants. Curr Opin Biotechnol 13:181–187

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Barbetti MJ, Li H, Woo SL, Lorito MA (2008) A novel role for *Trichoderma* secondary metabolites in the interactions with plants. Physiol Mol Plant Pathol 72:80–86

Vinterhalter B, Jankovic T, Sovikin L, Nikolic R, Vinterhalter D (2008) Propagation and xanthone content of *Gentianella austiaca* shoot cultures. Plant Cell Tiss Organ Cult 94:329–335

Wang C, Wu J, Mei X (2001) Enhanced taxol production and release in *Taxus chinensis* cell suspension cultures with selected organic solvents and sucrose feeding. Biotechnol Prog 17:89–94

Wang JW, Xia ZH, Chu JH, Tan RX (2004) Simultaneous production of anthocyanin and triterpenoids in suspension cultures of *Perilla frutescens*. Enzyme Microb Technol 34:651–656

Wiktorowska E, Dlugosz M, Janiszowska W (2010) Significant enhancement of oleanolic acid accumulation by biotic elicitors in cell suspension cultures of *Calendula officinalis* L. Enzyme Microb Technol 46:14–20

Zhao J, Zhu WH, Hu Q (2001) Selection of fungal elicitors to increase indole alkaloid accumulation in *Catharanthus roseus* suspension cell culture. Enzyme Microb Technol 28:666–672

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333