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ORIGINAL PAPER

Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in *Centella asiatica*

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Abstract *Centella asiatica* (Indian pennywort) has wide application in Indian and Chinese traditional medicines with documented evidence for wound healing and neuroprotective and anti-aging potential. Asiaticoside, a trisaccharide triterpene, is the most medicinally active compound in the plant. β-Amyrin synthase and squalene synthase have been identified as the two key genes in the triterpenoid pathway which regulate the production of asiaticoside in *C. asiatica*. The paper reports salient findings of our study utilizing the growth-promoting endophytic fungus *Piriformospora indica* to successfully colonize roots of *C. asiatica* in vitro cultures for investigating the effect of the mutualistic association on asiaticoside production. Co-cultivation of *P. indica* resulted in the rapid enhancement of root and shoot biomass of host plant, which was visible after 7 days of culture and continued up to 45 days. *P. indica* co-cultivation also favored the synthesis of asiaticosides, as evidenced by HPLC analysis which indicated about twofold increase (0.53% (*w*/*w*) in leaves and 0.23% (*w*/*w*) in whole plant) over control (0.33% (*w*/*w*) in leaves and 0.14% (*w*/*w*) in whole plant). Real-time PCR results confirmed the strong upregulation of squalene synthase and β-amyrin synthase transcripts in *P. indica*-challenged plants compared with the control. Our data demonstrate the potential use of *P. indica* as a means to enhance plant secondary metabolite production in planta with scope for further field evaluation.

Keywords Asiaticoside · *Centella asiatica* · Endosymbiosis · *Piriformospora indica*

Introduction

Centella asiatica (Indian pennywort) is a small herbaceous annual plant of the family Apiaceae native to India, Sri Lanka, Northern Australia, and some other parts of Asia. It is used as a medicinal herb in traditional Indian, African, and Chinese medicine. Several reports have documented the ability of *C. asiatica* to aid wound healing through stimulation of the production of type I collagen and effecting a marked decrease in inflammatory reaction and myofibroblast production (Widgerow and Chait 2000). The isolated steroids from the plant have been used to treat leprosy (Hausen 1993), and there are reports of potential applications of the plant in re-vitalization of the brain and nervous system (Brinkhause and Lindner 2000), neuroprotection by directly or indirectly modulating the activities of ATPases (Visweswari et al. 2010), and in combating aging (Bradwejn and Zhou 2000). Asiaticoside, a trisaccharide triterpene, has been identified as the most active compound in the plant, which also possesses other major

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bioactive triterpenoid glycosides (asiatic acid, madecassic acid), saponin glycosides (Brahmoside, Brahminoside), and flavonoids (Maquart et al. 1999). Growing evidence for the medicinal potential of these compounds has motivated researchers to utilize *C. asiatica* in vitro culture systems to enhance secondary metabolite production. Efforts to elicit the biosynthesis of centellosides mainly focus on using methyl jasmonate (MJ; Kim et al. 2004; Mangas et al. 2006), thidiazuron (Kim et al. 2004), and a permeabilization and feeding strategy by treatment with dimethyl sulfoxide alone or in combination with β-amyrin (Hernandez-Vazquez et al. 2010) in cell cultures, roots, and whole plants of *C. asiatica*. Efforts to improve the triterpenoid content of *C. asiatica* in in vitro shoot cultures by elicitation with exogenously supplied MJ resulted in a significant enhancement of the triterpenoid content at the expense of plant growth and decreased free sterol content (Mangas et al. 2006). However, eliciting transformed hairy root cultures of *C. asiatica* with MJ enhanced asiaticoside production as well as root biomass (Kim et al. 2007). It is evident in most of the reports that asiaticoside production is inhibited in undifferentiated cultures and is more favorably enhanced by whole plant cultures in a growth-correlated manner (Mangas et al. 2006; Aziz et al. 2007).

Piriformospora indica, discovered in the Indian Thar desert in 1997 (Verma et al. 1998), is a member of the *Sebacinales* (from genus *Agarimycotina*, *Basidiomycota*; Weiss et al. 2004). In contrast to arbuscular mycorrhizal fungi, this fungus can be easily cultivated in axenic cultures, where it asexually forms chlamydospores containing 8–25 nuclei (Verma et al. 1998). *P. indica* is able to transfer growth-promoting activity to its host plants and possesses a broad host range among mono- and dicotyledonous plants (Varma et al. 1999; Peskan-Berghöfer et al. 2004; Barazani et al. 2005; Serfling et al. 2007). In spring barley, *P. indica* colonization enhanced plant biomass, which was accompanied by grain yield increases of up to 11% (Waller et al. 2005). *P. indica* stimulates adventitious root formation in ornamental cuttings (Pham et al. 2004), while enhanced salt tolerance has been observed in barley (Waller et al. 2005). The growth promotion effect of *P. indica* on the medicinal plant *Adhatoda vasica* has been reported (Rai and Varma 2005), and recently, Baldi et al. (2009) demonstrated an enhanced production of podophyllotoxin in cell suspension cultures of *Linum album* by elicitation with culture filtrate and cell extract of *P. indica*. To date, there are no reports on the potential application of *P. indica* root symbiosis on the enhancement of secondary metabolites at the whole plant level. This paper reports for the first time the successful root colonization of *C. asiatica* by *P. indica*, which resulted in a significant enhancement of plant biomass and asiaticoside content.

Materials and methods

Plant material and initiation of in vitro cultures

C. asiatica plants were maintained at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India, under uniform conditions of growth. For initiating in vitro cultures, the plants were washed under running tap water for 1 h to remove dust and dirt, and soaked with mild detergent solution for 10 min followed by washing three times with distilled water. The nodal portions were used as explants for the experiments. The excised nodal portions (2–3 cm long) were soaked in 1% detergent solution for about 1 h and washed thoroughly under running tap water. Surface sterilization was done with 0.01% $HgCl_2$ (Himedia, Mumbai, India) for 5 min followed by a final rinse (three to four times) with sterile double distilled water. The explants were inoculated in 6-benzyl amino purine (2 mg l^{-1}) and naphthalene acetic acid (0.1 mg l^{-1}) containing half-strength MS (Murashige and Skoog 1962) medium fortified with 3% sucrose (Sisco Research Laboratories Pvt. Ltd, Mumbai, India) and 0.8% (*w*/*v*) agar (Sigma, St. Louis, MO, USA), adjusted to pH 5.75, and autoclaved at 121°C for 15 min. The cultures were maintained in sterile glass bottles at 23±2°C with a 16/8-h photoperiod and a light intensity of 25 μmol m^{-2} s^{-1}, provided by white fluorescent tubes and 55–65% relative humidity. After 2 weeks, rooted plantlets were transferred to half-strength MS basal liquid medium fortified with 3% sucrose maintained at pH 5.8 in sterile culture tubes.

Initiation and maintenance of *P. indica* culture

P. indica cultures for the present work were provided by Dr Anith, KN, Department of Microbiology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram, originally gifted by Prof. Ajit Kumar Varma; School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. The cultures were maintained in potato dextrose agar (PDA) medium (Sigma, St. Louis, USA) maintained at pH 7.0 and incubated in the dark at 28° C for a period of 10 days. Fungal hyphae (100 mg) were transferred to potato dextrose liquid culture and maintained in the dark at 28°C for 5 days.

Co-cultivation

Two-week-old *C. asiatica* plants maintained in MS liquid culture were transferred to the medium containing MS and potato dextrose broth (PDB; containing about 100 mg ml^{-1} fresh weight of *P. indica*) in a 1:1 ratio and incubated under identical conditions of light intensity and

temperature as described above for the maintenance of plant cultures, for a period of 45 days in the Plant Tissue Culture Laboratory at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. Plants maintained under identical conditions in MS/PDA (1:1) liquid medium without *P. indica* co-cultivation provided as control.

Staining of colonized root tissue

Trypan blue in lactophenol staining (Phillip and Hayman 1970)

Roots were collected, rinsed well in distilled water, and boiled with 10% KOH (Qualigens, Mumbai, India) in order to soften the root tissue, followed by neutralization with 2% HCl (Qualigens, Mumbai, India). Roots were then cut into 1-cm-long pieces and stained with 0.5% Trypan blue (Lobachemie, Mumbai, India) in lactophenol containing lactic acid (20%, *w*/*v*; Nice Chemicals Pvt. Ltd, Kerala, India), phenol (20%, *w*/*v*; Sisco Research Laboratories Pvt. Ltd., Mumbai, India), glycerol (40%, *w*/*v*; Sisco Research Laboratories Pvt. Ltd.), and water (20%, *v*/*v*) for a period of 10 min. The tissues were then washed with lactophenol solution for 15 min to remove excess stain. Slides were prepared from these samples and mounted in DPX mountant (Sisco Research Laboratories Pvt. Ltd.). Bright field images were taken in Image Proplus software using a fluorescent microscope (Nikon Instruments Inc., New York, USA).

WGA-AF 488 (wheat germ agglutinin-Alexa Flour 488) staining and confocal imaging

WGA-AF 488 staining was carried out with slight modifications of the procedure of Wright 1984. Both the control and the co-cultivated plant roots were collected and the roots fixed in trichloroacetic acid (TCA) fixation solution containing 0.15% (*w*/*v*) TCA in 4:1 (*v*/*v*) ethanol/chloroform followed by washing 5 min in 1X phosphate buffer saline (PBS, pH 7.4). The roots were then boiled for 1 min with 10% KOH and neutralized in 1X PBS. Thereafter, the root tissues were transferred to staining solution containing 100 μg ml^{-1} WGA-AF 488 (Invitrogen, Oregon, USA) dissolved in 1X PBS (pH 7.4). During incubation, root segments were vacuum-infiltrated three times for 1 min at 50 mmHg. After overnight incubation, the roots were destained by incubating overnight in PBS. The samples were viewed by confocal laser imaging on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany). The conjugated WGA-AF 488 was excited at 488-nm wavelength and detected at 500–600 nm. For viewing the *P. indica* mycelium and spores, the fungus was cultured by inverted agar block culture method and treated with conjugated WGA as described above.

Analysis of root colonization by *P. indica*

After 45 days of co-culture, root colonization was assessed as percentage colonization.

Percentage of colonization

$$= \frac{\text{Number of root segments colonized}}{\text{Total number of root segments observed}} \times 100$$

SEM analysis

Control and *P. indica*-colonized root tissues after 45 days of co-culture were fixed with 3% glutaraldehyde in Sorenson phosphate buffer (pH 7.4) and dehydrated by passage for 15 min each in 30%, 50% and 70% ethanol twice, followed by 30-min incubation in 90% and 100% ethanol. Critical point drying was carried out using HCP-2 critical point dryer (Hitachi, Japan) at 20°C. Samples were viewed in S-2400 scanning electron microscope (Hitachi, Europe).

Fungal and plant genomic DNA isolation and PCR analysis

Genomic DNA was isolated from 100 mg *P. indica* as well as the leaf and root tissue of control and *P. indica*-colonized (after 45 days of co-cultivation) *C. asiatica* by cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1994). For genomic DNA isolation from *P. indica*, initially, the fungi were inoculated on PDA for 3 weeks and then transferred into PDB at room temperature for 1 week. Mycelia were filtered and washed with sterilized water. Fungal biomass was frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. Genomic DNA was extracted from all samples using the CTAB method with slight modifications. To the samples, 1 ml of preheated (65°C) 2% (*w*/*v*) CTAB (Sigma, Germany) buffer was added and cooled to room temperature. Equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed well followed by centrifugation at 10,000 rpm for 10 min at 4°C. To the upper phase, 5 μl of RNase (stock concentration 10 mg ml^{-1}) was added and incubated at 65°C for 15 min. Chloroform/isoamyl alcohol extraction was repeated twice and DNA was precipitated from the upper aqueous phase by adding an equal volume of isopropanol. DNA was pelleted by centrifugation at 12,000 rpm for 5 min in a centrifuge (Jouan Inc., Winchester, USA) at room temperature. The pellet was air-dried and dissolved in nuclease-free water for PCR analysis.

PCR was performed using *PITEF* (*P. indica* transcription elongation factor) primers (Table 1) based on the sequence available in GenBank (accession no. AJ249911; Buetehorn et al. 2000). The reaction mixtures included 10 pM of forward and reverse primers, 2 μl of 2 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 1.5 μl of 25 mM $MgCl_2$, 2.5 μl of 10X PCR buffer, and 1 U Taq DNA polymerase (Promega, Madison, WI, USA). PCR conditions consisted of an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 2 min, 50°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 8 min.

Real-time PCR

Real-time PCR analysis was carried out to assess the effect of *P. indica* colonization on transcript accumulation of *C. asiatica SQS* (squalene synthase) and *BAS* (β-amyrin synthase), the two asiaticoside synthesis pathway genes. Gene-specific primers were designed based on sequence information provided by Mangas et al. 2008. Total RNA was isolated from leaf tissues of control (non-colonized) and *P. indica*-colonized plants after 15, 30, and 45 days of co-culture by Trizol method (Invitrogen, California, USA). RNA quantity was determined on NanoDrop™ 8000 Spectrophotometer (Thermo Scientific, USA). Approximately 1 μg of DNase (Sigma, St. Louis, USA) treated RNA was used to prepare cDNA using MMLV-RT following the manufacturer's protocol (Promega). Gene-specific primers of *C. asiatica SQS* and *BAS* (Table 1) were used for quantitative real-time PCR. The reaction was set up in a final volume of 20 μl containing 10 μl SYBR green PCR reagent (Applied Biosystems, California, USA), 1.5 μl of diluted cDNA (1:10 dilution), and 300 nM each of the designed primers; the conditions were: 50°C for 2 min initially followed by 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min in a real-time PCR machine (ABI 7500, Applied Biosystems). The plant-specific 5.8S rRNA gene served as control for constitutive gene expression in leaves. Primer sequences of 5.8S rRNA genes earlier reported by Mangas et al. 2008 are provided in Table 1.

Table 1 Primers for real-time PCR analysis

Name	Sequence 5′ to 3′
BAS	Forward: TCCCTCAGCAGGAAACAAC
	Reverse: CGGTACTCTCCAAGTGCCCATA
PITEF	Forward: TCGTCGCTGTCAACAAGATG
	Reverse: GAGGGCTCGAGCATGTTGT
SQS	Forward: CAAATTTTCCGTGGC
	Reverse: CAATGGGTTTATTTCTCCAGAAGAC
5.8S rRNA	Forward: CGGCAACGGATATCTGGCT
	Reverse: TCCGCCCCGACCCCTTTC

Comparative expression levels ($2^{-\Delta Ct}$) were calculated using SDS software (Applied Biosystems). Expression levels are relative to the level of 5.8S rRNA expression, which was constant in all RNA samples used and was set to 1. Values are the means of three replicates of two independent experiments assayed by quantitative PCR. The negative controls and the melting curve analyses carried out with each PCR reaction confirmed the absence of nonspecific PCR products and primer dimers.

Determination of plant growth

Control plants as well as plants colonized with *P. indica* were analyzed at 15-day intervals up to 45 days (0, 15, 30, and 45 days) to assess growth parameters including root and shoot numbers and lengths. Shoots and roots were separated and washed with distilled water. The length and the total number of roots as well as petioles and leaves were analyzed separately. For the determination of the dry weight, the materials were dried overnight in an oven at 40°C.

HPLC analysis

Control and *P. indica*-treated plants were oven-dried at 40° C and pulverized using a blender. Methanolic extracts were prepared from equal weights of the plants by the conventional Soxhlet extraction method. The extracts were incubated for 24 h at 4°C, filtered, and evaporated under vacuum to dryness. They were stored at 4°C for HPLC analysis. This was performed using a high-performance liquid chromatographic system equipped with LC8A pump, SPD-M 10A*vp* photo array detector in combination with Class LC 10A software. Chromatographic separation was performed using an Octyl silane C8 column (5-μm size, 250 × 4.6 mm in length; Merck, Darmstadt, Germany) with water–acetonitrile (HPLC grade, Merck) as the mobile phase. Separation was carried out at a flow rate of 1.5 ml min^{-1}. The sample injection volume was 20 μl at 25°C.

Ten milligrams of the asiaticoside standard was weighed and added to a 10-ml volumetric flask, dissolved with 5 ml of methanol, and finally made up to 10 ml with methanol (HPLC grade, Merck). *C. asiatica* extract equivalent to 100 mg of asiaticoside weighed and added to a 100-ml volumetric flask was dissolved in 50 ml of methanol, made up to 100 ml, and filtered through a Millex syringe-driven filter unit (Millipore Corporation, Bedford, USA) before injection.

The chromatogram was recorded at 210 nm and the injection was repeated four times; finally, the relative standard deviation of the area was calculated. The sample was injected in a similar way. The contents of asiaticoside and madecasso-

side in plant extracts were determined by comparing peak areas of plant samples with those of the standard.

Statistical analysis

The experiments were performed in vitro and plants were arranged in a completely randomized design with three replicates for each growth parameter studied. For each experiment, 15 *P. indica* co-cultured plants were analyzed and the experiments were repeated twice. Thus, a total of 30 treated plants were studied in comparison to ten control (untreated) plants. One plant each was maintained as a control for each growth parameter studied, i.e., five controls per experiment (5×2=10 control plants in total). The mean values were compared using Student's *t* test ($p<0.05$), and analysis of data was carried out using the SAS version 8.1 (SAS Institute Inc., USA).

Results

Colonization of *P. indica* in roots of *C. asiatica*

Our experiments provide evidence for the successful colonization of *P. indica* in roots of the host plant. Staining with a general dye like Trypan blue indicated the presence of chlamydospores inside the root cells of *C. asiatica* ("ESM 1"). Chitin-specific WGA-AF 488 provided evidence that the fungus colonizes the root surface, with hyphae running parallel to the root axis (Fig. 1c), and produces coiled hyphae intracellularly. The more detailed scanning electron microscopy (SEM) image indicates the penetration of fungal hyphae into the root epidermis ("ESM 2b"), apart from forming a mesh of hyphal networks and profuse chlamydospores on the surface. PCR analysis using *P. indica*-specific gene *PITEF* (GenBank accession no. AJ249911) produced an amplicon of about 250 bp from genomic DNA of the colonized root ("ESM 3a"), whereas a similar amplicon was absent in the leaf of colonized *C. asiatica* ("ESM 3b") and the control samples of root and leaf. This result confirms the effective root colonization of *P. indica* in *C. asiatica* roots.

Promotory effect of *P. indica* colonization on host plant biomass

P. indica colonization resulted in a rapid increase in root and shoot biomass of the host plant. The enhancement was visible after 7 days of co-culture, which progressed even after 45 days of culture. There was a significant increase ($p<0.05$) in the number of leaves of colonized plants (12 per node) compared with the control plants (eight per node; Table 2).

P. indica colonization favorably influences asiaticoside content

The HPLC profile indicated a twofold increase in asiaticoside content (percent, *w*/*w*) both in whole plants and in leaves after co-cultivation compared with non-colonized control plants and control leaves. Within the plant, asiaticoside content was maximum in leaves (0.33%, *w*/*w*, in control and 0.53%, *w*/*w*, in leaves of colonized *C. asiatica*), and the whole plant analysis showed an increase in asiaticosides in the colonized sample (0.23%, *w*/*w*) over the control (0.14%, *w*/*w*; Fig. 2).

P. indica improves asiaticoside production in *C. asiatica* through inducing the expression of *SQS* and *BAS*

SQS and *BAS* are two key genes in the asiaticoside biosynthesis pathway ("ESM 4"). It was evident by real-time PCR analysis that *SQS* and *BAS* transcripts are strongly upregulated (5 and 79 times, respectively) in the leaves of the colonized plants compared with the levels in control (Figs. 3 and 4). Root tissues recorded a very low transcript accumulation of *SQS* and *BAS*. The transcript levels showed a progressive increase in accumulation from 15 to 45 days of co-culture. The transcript level upregulation of the key genes in the triterpenoid pathway follows the pattern of corresponding asiaticoside levels as evidenced by HPLC, suggesting that the regulation occurs at the level of transcription.

Discussion

Our results indicate that *P. indica* effectively colonizes the roots of *C. asiatica.* Hyphae and spores were detected around the roots, in the extracellular space, and within the root cells. The co-cultivation of both organisms resulted in a rapid increase in shoot and root biomass. Endophytic fungi form mutualistic interactions with their host, which frequently leads to enhanced growth of the host (Khan et al. 2008). It is well known that AM fungi enhance nutrient uptake as this fungal symbiosis increases the abilities of the host plants to explore a larger volume of soil than roots alone and to take up phosphate from a greater surface area (Jakobsen et al. 1992). The high efficiency of colonization and host biomass enhancement in *C. asiatica* is in agreement with similar reports from Chinese cabbage, wherein *P. indica* colonization resulted in host growth enhancement and drought tolerance (Sun et al. 2010). Another important consequence of endophyte colonization in *C. asiatica* was the significant enhancement of asiaticosides in the colonized plants. Asiaticoside predominated in the leaves compared with the roots which showed very low level of *BAS*

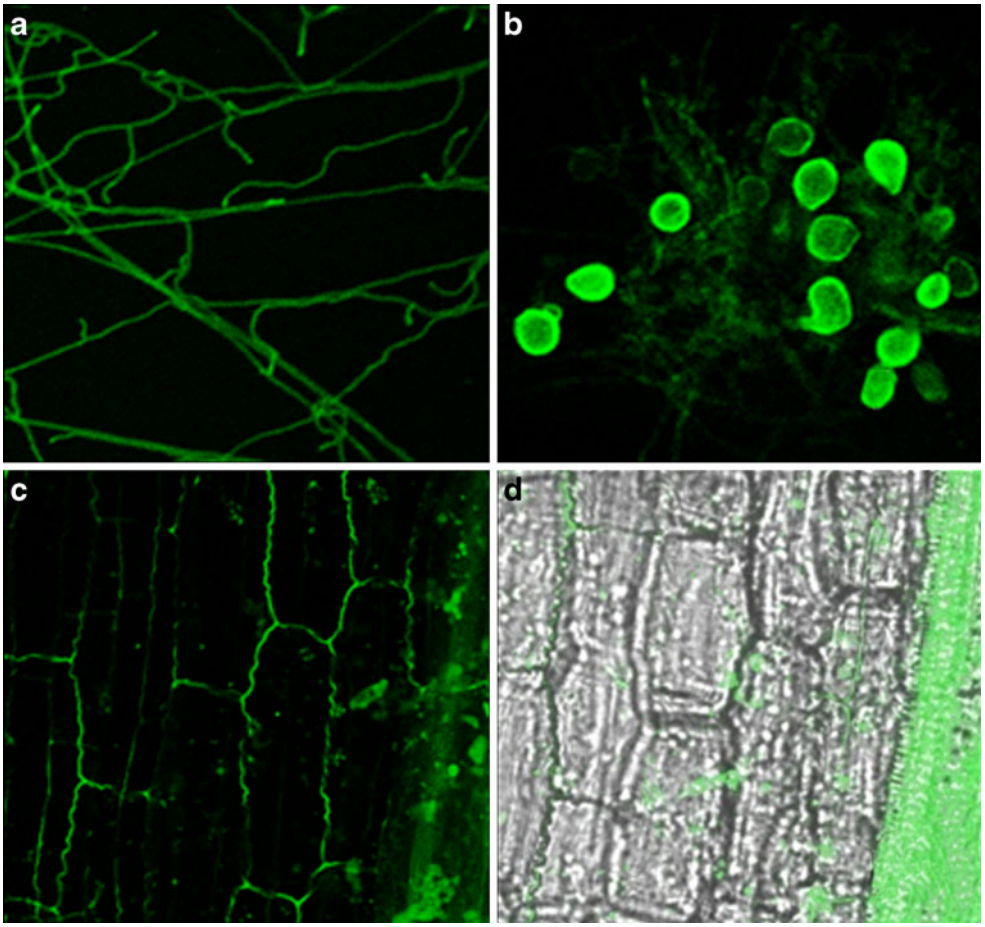

Fig. 1 Confocal laser image of WGA-AF 488 staining for *P. indica* analysis. **a** *P. indica* hyphae stained with WGA-AF 488. **b** WGA-AF 488-stained spores of *P. indica*. **c** *P. indica* hyphae on colonized root surface after 10 h of co-cultivation. **d** Overlay with bright field image. The image was excited at 488-nm wavelength and detected at 500–600 nm. The intercellular hyphae are seen closely aligned to the rhizodermal cell walls

transcripts. It is likely that the precursors are synthesized in the root and translocated to the leaf for bioconversion. This hypothesis is strengthened by the observation that asiaticoside and madecassoside were undetectable in root cultures of *C. asiatica* (Aziz et al. 2007), unless the culture medium is supplied with the elicitor MJ (Kim et al. 2007). It is evident in most of the earlier reports that asiaticoside production is inhibited in undifferentiated cultures and is more favorably enhanced by differentiation (Mangas et al. 2006; Aziz et al. 2007). Consistent with these findings, our results indicate that whole plant cultures are efficient targets for improved asiaticoside production in *C. asiatica*.

Table 2 *t* test: Two-sample analysis for assessing the effect of *P. indica* colonization on plant growth

	Control		Treatment	
	Initial	Final[a]	Initial	Final[a]
Plant biomass (g)				
Fresh weight	12.1	21.4	12.3	29.4*
Dry weight	0.77	1.194	0.77	2.098*
Leaf number	4.8	8	5	11.8*
Root number	10.2	14.6	9.8	19.8*
Shoot length (cm)	4.8	7.8	5.1	8.2
Root length (cm)	3.4	8.1	3.5	8.3

[a] Observations taken after 45 days of co-culture. Values are the means of three replications (n=3) per experiment; experiments were repeated twice

*p<0.05

The increase in secondary metabolite production effected by endosymbiosis could be due to elicitation of plant defense in response to fungal elicitors like lipopolysaccharides and glycoproteins formed by the action of plant-derived hydrolases secreted in response to endophyte colonization (Gao et al. 2010). Improved plant defense responses correlated with endophytic colonization are associated with the increased demands for energy-reducing equivalents and carbon skeletons provided by primary metabolic pathways (Bolton 2009; Gao et al. 2010).

Triterpene saponins are glycosylated plant secondary metabolites generated through the isoprenoid pathway. Squalene synthase represents a putative branch point in the isoprenoid biosynthetic pathway capable of diverting carbon flow specifically to the biosynthesis of sterols and, hence, is considered a potential regulatory point for sterol metabolism. Cyclization through dammarenyl, baccharenyl,

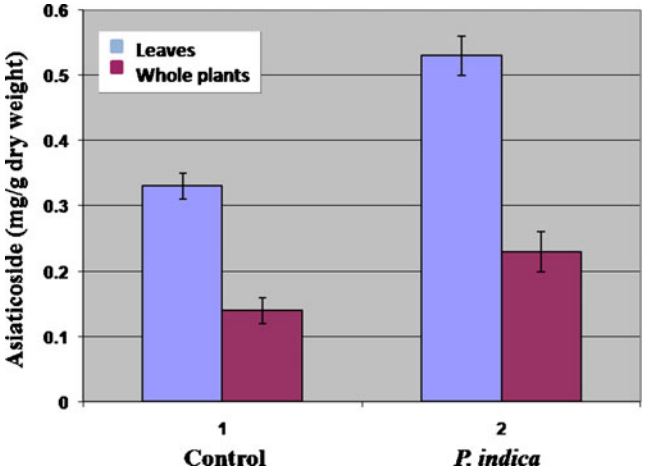

Fig. 2 Comparison of HPLC quantitation of asiaticoside. The amount of asiaticoside produced by control (*1*) and *P. indica*-colonized plants (*2*)

and lupenyl cation intermediates generates lupeol and α/β-amyrin (Jenner et al. 2005), the precursors of the *Centella* pentacyclic triterpenoid saponins. The α/β-amyrin synthase enzymes cyclize oxidosqualene via the dammarenyl cation and allow further ring expansion and some rearrangement before deprotonation to α-amyrin and β-amyrin, respectively (Haralampidis et al. 2002). Thus, β-amyrin synthase is the immediate precursor for the production of asiaticoside. In recent reports, the expression of *C. asiatica–BAS* (*C.a BAS*) gene, together with that encoding for squalene synthase (*C.a SQS*), was compared with the production of centellosides (asiaticoside, madecassoside, and their respective aglycones) in plants and callus cultures, and *BAS* and *SQS* have been identified as target genes to modify the production of triterpenoid saponins in *C. asiatica* (Mangas et al. 2008; Yendo et al. 2010).

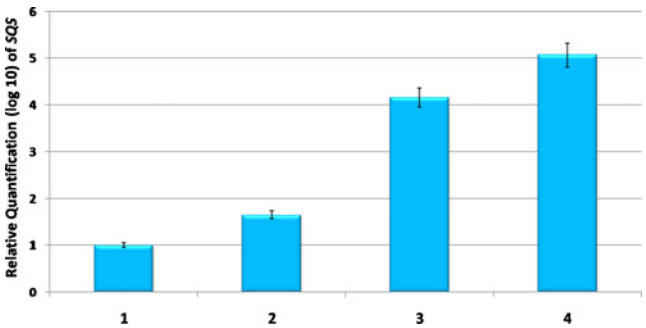

Fig. 3 Real-time PCR analysis of *SQS* expressed as fold expression in leaves of *Centella asiatia*. Analysis was performed in leaves of control (*1*) and *P. indica*-colonized in vitro plants of *C.asiatica* (*2–4*). Samples were isolated at 15, 30, and 45 days and analyzed in comparison to tissues of control plants maintained for 45 days in culture. Relative quantification represents the fold expression levels of *SQS* relative to the level of 5.8S rRNA gene expression, which was constant in all RNA samples. Number of replications (*n*)=3

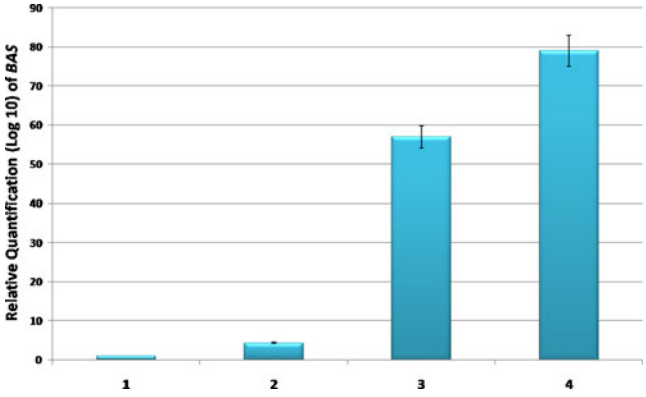

Fig. 4 Real-time PCR analysis of *BAS* expressed as fold expression in leaves of *Centella asiatia*. Analysis was performed in leaves of control (*1*) and *P. indica*-colonized in vitro plants of *C. asiatica* (*2–4*). Samples were isolated at 15, 30, and 45 days and analyzed in comparison to tissues of control plants maintained for 45 days in culture. Relative quantification represents the fold expression levels of *BAS* relative to the level of 5.8S rRNA gene expression, which was constant in all RNA samples. Number of replications (*n*)=3

Real-time PCR results suggest that the sterol synthesis pathway is preferentially favored, indicated by the significant accumulation of *SQS* transcripts in *P. indica*-colonized samples compared with the control samples, which results in the upregulation of *BAS*, eventually leading to the enhanced biosynthesis of asiaticoside, as evidenced by HPLC analysis. Correlated changes in *SQS* enzyme activity with changes in the level of the corresponding protein and mRNA have been reported before (Devarenne et al. 2002). Upregulation of the mRNA levels of *BAS* by MJ, along with increased activity and accumulation of soyasaponin, has been observed in licorice suspension cultures (Hayashi et al. 2003). Kim et al. (2004, 2005) reported that asiaticoside content was much higher in leaves treated with MJ than in untreated leaves, which correlated with an increase in transcript accumulation of *C. a BAS*.

To the best of our knowledge, this is the first report on the induction of a symbiotic association with a growth-promoting endophytic fungus for the enhanced production of *C. asiatica* triterpenoid. The use of whole fungal cultures and host plants obviates the need for infrastructure sophistications and labor involved in maintaining plant cell cultures. Further studies are currently in progress to extend this work to field conditions, and the preliminary results have been promising.

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