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ORIGINAL PAPER

Restoration of camptothecine production in attenuated endophytic fungus on re-inoculation into host plant and treatment with DNA methyltransferase inhibitor

M. M. Vasanthakumari[1] · S. S. Jadhav[1,2] · Naik Sachin[1] · G. Vinod[1,2] · Singh Shweta[1] · B. L. Manjunatha[1,2] · P. Mohana Kumara[4] · G. Ravikanth[1,3] · Karaba N. Nataraja[2] · R. Uma Shaanker[1,2,3]

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Abstract Fungal endophytes inhabit living tissues of plants without any apparent symptoms and in many cases are known to produce secondary metabolites similar to those produced by their respective host plants. However on sub-culture, the endophytic fungi gradually attenuate their ability to produce the metabolites. Attenuation has been a major constraint in realizing the potential of endophytic fungi as an alternative source of plant secondary metabolites. In this study, we report attempts to restore camptothecine (CPT) production in attenuated endophytic fungi isolated from CPT producing plants, *Nothapodytes nimmoniana* and *Miquelia dentata* when they are passed through their host plant or plants that produce CPT and when treated with a DNA methyl transferase inhibitor. Attenuated endophytic fungi that traversed through their host tissue or plants capable of synthesizing CPT, produced significantly higher CPT compared to the attenuated fungi. Attenuated fungus cultured in the presence of 5-azacytidine, a DNA methyltransferase inhibitor, had an enhanced CPT content compared to untreated attenuated fungus. These results indicate that the attenuation of CPT production in endophytic fungi could in principle be reversed by eliciting some signals from plant tissue, most likely that which prevents the methylation or silencing of the genes responsible for CPT biosynthesis.

Keywords Endophytic fungi · Attenuation · In vitro regeneration · *Nothapodytes nimmoniana* · Epigenetic modifiers · GFP

Introduction

Endophytic fungi are an important group of fungi that reside asymptomatically within plant tissues. Often the endophytic fungi have been shown to impart tolerance to their host plants against herbivory, heat, salt, disease and drought stress (Azevedo et al. 2000; Rahman and Saiga 2005) and thus improve the fitness of plants. In in vitro culture, endophytic fungi have been shown to produce a number of compounds that may have potential applications in agriculture, medicine and food industry (Murray et al. 1992; Li et al. 2000; Azevedo et al. 2000; Strobel 2002; Berg et al. 2004). Specifically, the endophytic fungi have been shown to produce a number of secondary metabolites, often similar to those produced by their respective host plants (Stierle et al. 1993; Stierle and Strobel 1995). Examples of these compounds include taxol, camptothecine, podophyllotoxin, vinblastine, hypericin, diosgenin, azadirachtin, rohitukine, capsaicin among others (Wang et al. 2000; Yang et al. 2004; Zhou et al. 2004; Kusari et al. 2008, 2009; Mohana Kumara et al. 2011; Shweta et al. 2010, 2013; Kumara et al. 2014; Devari et al. 2014). These studies have fueled the expectation that the endophytes could serve as an alternative

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✉ R. Uma Shaanker
umashaanker@gmail.com

[1] School of Ecology and Conservation, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka 560065, India

[2] Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka 560065, India

[3] Ashoka Trust for Research in Ecology and the Environment, Royal Enclave, Srirampura, Jakkur, Bengaluru, Karnataka 560064, India

[4] Department of Chemistry, Indian Institute of Technology Madras, Chennai, Tamilnadu 600036, India

source of important plant secondary metabolites and thus relieve dependence on their respective host plants for the metabolites. However till date, this expectation remains unfulfilled primarily due to the low yields as well as the attenuation of production of the metabolites on sub-culture of the fungi (Li et al. 1998; Gurudatt et al. 2010; Kusari et al. 2011; Xiang et al. 2013; Kumara et al. 2014; El-Elimat et al. 2014). Successive sub-culture of the endophytic fungi, *Periconia* sp. isolated from *Torreya grandifolia*, resulted in the attenuation of taxol production (Li et al. 1998). Similarly, endophytic fungi producing the anti-cancer alkaloid, camptothecine (CPT) have been shown to attenuate over sub-culture generation (Gurudatt et al. 2010; Kusari et al. 2011). Although the exact mechanism leading to the attenuation is not clear, it is attributed to either the absence of host stimulus in the culture media and or silencing of genes in axenic cultures (Priti et al. 2009; Sachin et al. 2013). Attempts to reverse the attenuation by supplementing axenic cultures with respective host tissue extracts have not been successful (Gurudatt et al. 2010; Kusari et al. 2011).

Recently, a few studies have demonstrated that fungi may possess gene clusters encoding the production of previously unobserved secondary metabolites (Beau et al. 2012). Activation of these attenuated or silenced gene clusters to obtain either improved titers of known compounds or new ones altogether has been a subject of considerable interest (Shwab et al. 2007; Wang et al. 2010). In fact, in an effort to obtain new drug leads from silent biosynthetic pathways, epigenetic modifying substances have been used as a promising tool for manipulating the secondary metabolism of fungi (Shwab et al. 2007; Williams et al. 2008; Henrikson et al. 2009; Fisch et al. 2009).

In this study we report the isolation of endophytic fungi from *Nothapodytes nimmoniana* (Graham) Mabb. (Icacinaceae) a tree known to produce the anti-cancer alkaloid, camptothecine (CPT). The fungi produce CPT when cultured in potato dextrose broth, independent of the host tissue. However on sub-culture, the fungi attenuate and lose their ability to produce CPT. Here, we have attempted to revive the production of CPT in the attenuated fungus following three approaches. In the first, the attenuated fungus was inoculated into in vitro regenerated *N. nimmoniana* plant devoid of endophytic fungus or other plants that produce CPT and then re-isolated and examined for the restoration of CPT production. The second approach was similar to the first, except that here an attenuated endophytic fungus that was labeled with green fluorescent protein (GFP) was used to track the fungus through the process of inoculation and its re-isolation. In both the cases, attenuated endophytic fungi that were passed through their host tissue or plants that produced CPT, had significantly higher CPT compared to attenuated fungus not treated thus. In the third approach, the attenuated fungus was cultured in the presence of 5-azacytidine, a DNA methyltransferase inhibitor. Fungus, thus treated showed an enhanced CPT content compared to untreated attenuated fungus. These results indicate that the attenuation of production of CPT by the endophytic fungi could in principle be reversed by eliciting some signals from plant tissue, most likely that which prevents the methylation or silencing of the genes responsible for CPT biosynthesis. We discuss the implications of the study with reference to realizing the potential of endophytic fungi as alternative sources of plant secondary metabolites.

Materials and methods

Plant material

Nothapodytes nimmoniana (Graham) Mabb. (synonyms, *Nothapodytes foetida* and *Mappia foetida*) (family Icacinaceae) is a small tree occurring in the Western Ghats, a mega diversity hot spot, in southern India. The tree is an important source of the anti-cancer alkaloid, CPT (0.3 % by dry weight of stem bark) (Ramesha et al. 2008).

Collection of plant material and shoot regeneration

Fruits of *N. nimmoniana* were collected from trees growing in the evergreen forests in Nelliyampathy (10.23′52.95″N–76.36′30.27″E), Western Ghats of Kerala, India. The pericarp of the fruits was cracked open and seeds were separated and were soaked in sterilized distilled water for 10 h. They were treated with 0.005 % Tween-20 for 15 min and washed thrice with sterile distilled water followed by treatment with Bavistin [0.1 % (w/v)] for 30 min. Further, seeds were disinfected with 4 % (v/v) NaOCl for 10 min and finally rinsed five times with sterile distilled water to remove traces of disinfectant. Embryos were isolated from the surface disinfected seeds and were used as explants. Embryonal explants were placed on Murashige and Skoog (1962) basal medium with 0.3 % (w/v) sucrose and 0.8 % (w/v) agar (Hi-Media, India). The pH of the medium was adjusted to 5.8. Following the inoculation, all the cultures were incubated at 23 ± 2 °C under 16 h photoperiod at 80 % relative humidity. Emerged hypocotyl segments were aseptically excised and were cut into segments (0.5–1.0 cm). These hypocotyl segments were placed on MS medium supplemented with growth regulator TDZ (Thidiazuron) (0.5 mg l^{-1}) and were incubated as described earlier for 8 weeks. The induced adventitious multiple shoots were sub-cultured on the same media at 4 week intervals.

Screening of in vitro regenerated *N. nimmoniana* for endophytic fungal occurrence

The shoots of in vitro regenerated plants were cut into 1.0 cm long segments and placed on petridish containing potato dextrose agar media (PDA, Himedia, Mumbai) amended with streptomycin (0.1 g l^{-1}). Each petridish contained five segments. The petridishes were incubated in a light chamber under 12/12 h cycles of light and darkness at 23 ± 2 °C for 2 weeks.

Experiment 1: inoculation of attenuated endophytic fungi to in vitro regenerated *N. nimmoniana* plants and its effect on reversal of attenuation

The endophytic fungus, *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx. (L-6) was obtained from the culture collections maintained at the School of Ecology and Conservation, University of Agricultural Sciences, GKVK, Bangalore. The fungus was isolated from stem segments of *N. nimmoniana*. In culture, the fungus produced CPT; however on sub-culture the fungus lost its ability to produce CPT. The fungus was characterized based on the cultural characteristics (Arx 1981) and by sequencing the internal transcribed spacer (ITS) fragments (ITS1-5.8S-ITS2) using the universal primers ITS1 and ITS4. The sequence has been deposited in NCBI GenBank (Accession Number KF152941).

Inoculation of attenuated endophytic fungi to in vitro regenerated *N. nimmoniana* plants

Single hyphal tip from the colony cultures of fourth sub-culture generation of *B. rhodina* that had attenuated was cultured aseptically on PDA. At the fourth sub-culture the fungus had not yet completely lost its ability to produce CPT but had significantly attenuated. These cultures therefore seem to retain their genetic machinery to produce CPT. Such cultures were selected to examine the possibility of revival of the production of CPT. Five-day-old colony was used for preparing the mycelial suspension (Dhingra and Sinclair 1993). Mycelial suspension (2×10^6 cfu ml^{-1}; 20 µl) was prick-inoculated to leaves of in vitro regenerated *N. nimmoniana* and these inoculated plants were incubated at 23 ± 2 °C, in light/dark for 7 days. The plants inoculated with 20 µl of sterile distilled water served as control. The colonization ability of *B. rhodina* was determined by isolating the fungus from the point of inoculation and from sites away from the place of inoculation. Inoculated plants were cut into 1-cm long segments and placed on PDA (amended with streptomycin 0.1 g l^{-1}) and incubated as described earlier.

Effect of inoculation on reversal of attenuation

The fungal colonies emerging from the incubated plant segments (from both the site of inoculation and sites away) were purified. The purified fungal isolates were fingerprinted by sequencing the internal transcribed spacer (ITS) fragments (ITS1-5.8S-ITS2) using the universal primers ITS1 and ITS4 and compared with that of the mother culture. The purified isolates were also identified morphologically (Arx 1981). Further the cultures from both sites (from the site of inoculation and away) were cultured in potato dextrose broth (PDB) and CPT quantified.

Experiment 2: inoculation of EGFP labeled endophytic fungus, *Phomopsis* sp. to in vitro regenerated *N. nimmoniana* and *Ophiorrhiza mungos* plants and its effects on reversal of attenuation

Endophytic fungus, *Phomopsis* sp. (GenBank Accession Number JX139522) isolated from stem segments of *Miquelia dentata* (Icacinaceae), a plant producing CPT, was used in this experiment (Ramesha et al. 2013; Shweta et al. 2013). The fungus produces CPT in culture but rapidly attenuates on subculture. The culture loses its ability to produce CPT by the sixth sub-culture.

Transformation of *Phomopsis* sp. using enhanced green fluorescence protein (EGFP) gene

Agrobacterium strain EHA105 (pSK1019 binary vector) containing the hygromycin phosphotransferase B resistance gene (*hph*) under the *Aspergillus nidulans* trp C promoter and the enhanced green fluorescence protein (EGFP) gene under a ChGPD promoter was grown on Luria–Bertani medium (LB) supplemented with 50 mg l^{-1} kanamycin at 28 °C. The strain was kindly provided by Dr. David Ezra, Department of Plant Pathology and Weed Research, ARO, Israel (Ezra et al. 2010).

Transformation was done following Michielse et al. (2008) with minor modifications. *Agrobacterium tumefaciens* strain EHA105 bearing the pSK1019 vector was taken from the glycerol stock solution and 100 µl of this glycerol stock solution was inoculated into 3 ml LB broth with 50 mg l^{-1} of kanamycin and 100 mg l^{-1} of rifamycin. The culture was incubated overnight on a rotary shaker (225 rpm at 28 °C). One ml of the above culture was transferred into 9 ml LB broth with 50 mg l^{-1} kanamycin and 100 mg l^{-1} rifamycin. The remaining culture was saved into glycerol stock. The culture was incubated for 8 h on a rotary shaker (100 rpm) at 28 °C. The culture (1.5 ml) was centrifuged at 2400 g for 10 min at room

temperature (RT) to remove the supernatant and the cells were washed by resuspending the pellet in 250 µl of induction medium (IM) and centrifuged (2400 g for 5 min at RT) and the pellet was re-suspended in 10 ml of liquid IM with 5 µl of 0.2 m acetosyringone (AS), 50 mg l^{-1} kanamycin and 100 mg l^{-1} rifamycin. The culture was incubated for 4–6 h at 28 °C at 100 rpm until the culture reached an optical density (OD) of 0.3–0.4. The OD was measured in a spectrophotometer at 600 nm and adjusted with IM to obtain a final OD of 0.3. Simultaneously, the fungal mycelia was harvested by adding 5 ml of physiological salt and scraping the surface using a sterile scalpel. The harvested mycelia were aliquoted in the eppendorf tubes. *Agrobacterium* culture (100 µl) and 100 µl of fungal culture were mixed in an eppendorf tube at different ratios (1:1, 1:2, 2:1). With the help of sterile tweezers, a sterile Hybond N+ filter was placed on the petriplates contacting solid IM + AS. Mycelia infected with *Agrobacterium* (200 µl) was spread on a solidified IM and incubated at 27.5 °C for 3 days in an incubator. The nylon membrane was later transferred into selection medium with 30 mg l^{-1} of hygromycin and 200 mg l^{-1} of cefatoxamine. The plate was observed for the emergence of putative transformants. The putative transfomants were picked and pure cultured on 50 mg l^{-1} of hygromycin.

Identification of transformants

Putative transformants were grown in PDB containing 50 mg l^{-1} of hygromycin and 100 mg l^{-1} of cefatoxamine for 5 days at 28 °C. Mycelia was harvested and ground to fine powder in presence of liquid nitrogen in a mortar and pestle (Vainio et al. 1998). The *hph* and EGFP gene in putative transformants was detected by PCR using the primers *hph*F (5′GCTGGGGCGTCGGTTTCCAC 3′), *hph*R (5′CGGGTTCGGCCCATTCGGAC 3′), and EGFPF (5′GACGTAAACGGCCACAAGTT 3′), EGFPR (5′GAACTCCAGCAGGACCATGT 3′) respectively. Each 25 µl of master mix contained buffer (2.5 µl), Taq polymerase (1 U), dNTPs (2.5 µl), primers (3 pmol) and 1 µl of DNA. The PCR was programmed for 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 55 °C for 50 s and 72 °C for 3 min with a final extension of 72 °C for 10 min. All PCR products were sequenced to confirm the identity of the product (Chromous Biotech, Bengaluru).

Mitotic stability of transformants

Randomly selected putative transformants were cultured on PDA media without hygromycin at 28 °C for 5 days. The putative transformants was sub-cultured. After 5 sub-cultures, the putative transformants were inoculated onto PDA media with 50 mg l^{-1} hygromycin.

Confocal microscopy

Colonies were examined for EGFP fluorescence using confocal microscopy (FV 1000 Confocal Microscope-Olympus at the NCBS-C-CAMP Confocal and TEM imaging facility, http://www.ccamp.res.in/imaging). For the green fluorescence detection, mycelial samples were excited under 488 nm image laser wavelengths and 509 nm emission max with the excitation and emission filters at 485/20 and 530/25 respectively. EGFP fluorescence inside the plant cells was examined by taking thin cross sections of leaf and stem.

Inoculation of EGFP labeled endophytic fungus, *Phomopsis* sp. to in vitro regenerated *N. nimmoniana* and *Ophiorrhiza mungos* plants

In this experiment, in vitro regenerated plants of *N. nimmoniana* and *Ophiorrhiza mungos* (Rubiaceae) were used for re-inoculation of the attenuated endophytic fungi, *Phomopsis* sp., obtained from *Miquelia dentata*. *Ophiorrhiza mungos* is a small herbaceous plant occurring in the Western Ghats of India. The plant has been reported to produce CPT and 10-methoxy camptothecine (Tafur et al. 1976). All plants were prick inoculated with the EGFP transformed *Phomopsis* sp. in a sterile environment and allowed to colonize for 3 weeks. Later the fungus was re-isolated from the plants at sites other than where they were inoculated. As a control, a non-CPT producing plant, in vitro generated *Nicotiana tabacum* plants were also prick inoculated with *Phomopsis* sp. In all the cases, the fungus was re-isolated, cultured in PDB and examined for the revival of attenuation.

Experiment 3: effect of DNA methyl transferase inhibitor on reversal of attenuation

Fifteen agar plugs (5 mm dia) containing mycelia from third subculture generation of *B. rhodina* were placed into conical flask containing 50 ml of sterile PDB. After incubating for 72 h (25 °C on a rotary shaker at 170 rpm), cultures were treated with 5-azacytidine (1, 5, 7.5 or 10 µM), a DNA methyltransferase (DNMT) inhibitor and allowed to grow for an additional 5 days. Control (untreated) and treated fungi were harvested and CPT was analyzed.

Extraction and analysis of CPT

Camptothecine was extracted from the fungal mycelia following the protocol described by Shweta et al. (2010). The mycelial mat was separated from the PDB through filtration, and dried at 60 °C for 96 h in a hot air oven.

The dried mycelial mat was thoroughly crushed in sterile pestle and mortar to obtain fine powder. One hundred milligrams of fine tissue powder of the sample was taken in 15 ml vial and extracted in 10 ml of 61 % ethanol at 60 °C for 3 h in a shaking water bath. After cooling to room temperature, the extract was centrifuged (10,000 rpm for 10 min at 4 °C). The supernatant was passed through a 0.2 μ filter (Tarsons, India) and analyzed for the presence of camptothecine.

HPLC analysis

The extracted samples were analyzed by reverse-phase HPLC (LC-20AD, Shimadzu, Japan) and the analysis was done under following chromatographic conditions: column: Luna 5u C18(2) (250 × 4.6 mm, 5 μ, Phenomenex, USA), Detector: SPD-M20A photodiode array detector, Wavelength: 254 nm for CPT and 266 nm for 9-methoxy camptothecine (9-MCPT). The flow rate was adjusted to 1.5 ml min^{-1} with an injection volume of 20 μl. The mobile phase consisted of 25 % acetonitrile (Pump A) and 75 % water + 0.1 % trifluro-aceticacid (Pump B) in an isocratic mode. The CPT standard (stock solution) was prepared using DMSO and methanol in 1:3 (v/v) ratio at the concentration of 1 mg ml^{-1}, 10-hydroxycamptothecine (10-HCPT) was prepared using 100 % methanol (1 mg ml^{-1}) and 9-methoxycamptothecine (9-MCPT) was prepared using chloroform and methanol in 1:3 (v/v) ratio at a concentration of 1 mg ml^{-1}. The working solution was prepared using the above stock solution at the concentration range of 100–500 μg ml^{-1} and the standard curve was prepared by injecting (20 μl) the different concentrations of the working solution to the HPLC system (Ramesha et al. 2008). The total run time was 30 min with a retention time of CPT at 14 min, and 9-MCPT at 25 min. The amount of CPT and its derivatives in the samples was calculated based on the regression curve of respective standards ($R^2 = 0.99$). Limits of detection (LOD) and limits of quantification (LOQ) for CPT was found to be 0.065 and 0.197 μg ml^{-1}, respectively, providing further evidence for the sensitivity of the system.

HPLC–MS analysis

HPLC–MS analysis was done to examine the presence of CPT and related compounds in the extract using LC–ESI–MS (LCMS-2020, Shimadzu, Japan). The LC analysis was coupled to an ion trap mass analyzer. The mass analyzer was equipped with atmospheric pressure ionization source, electrospray ionization (ESI). High purity nitrogen from a nitrogen generator was employed as both the drying and nebulizer gas. The LC conditions for the analysis were set as follows: Column:Luna 5u C18(2) (250 × 4.6 mm, 5 μ, Phenomenex, USA), Detector: UV–visible, wave length: 254 nm for CPT and 266 nm for 9-MCPT. For the analysis the flow rate was adjusted at 1.5 ml min^{-1} with an injection volume of 20 μl. The mobile phase for the analysis consisted of 25 % acetonitrile (Pump A) and 75 % water (Pump B) in an isocratic mode. Following conditions were set for mass spectrum analysis: dry gas flow rate 10.0 l min^{-1}, nebulizer pressure 35 psi, nebulizing gas flow 1.5 l min^{-1}, DL Temperature: 250 °C and the mass range was adjusted to 100–700 m/z (Ramesha et al. 2013).

ESI–MS/MS

The presence of CPT in the extract was further confirmed by an ion trap LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA). Spectra were obtained under following conditions: solvent flow rate at 1.0 ml min^{-1}, nebulizer pressure 110-psi (N2), damping gas: Helium at 10.0 ml min^{-1} and 5 kV spray voltage on an ion trap LTQ XL mass spectrometer. Spray solvent: methanol:water (8:2 v/v) and mass spectra were acquired in full scan, Positive ion mode, over the mass range from m/z 100 to 1000. By applying these conditions, the data acquisition was accomplished by Xcalibur for MS^2/MS^3 fragmented ions of CPT.

Statistical analysis

All experiments were replicated at least thrice. Paired *t* test was used to analyze the statistical significance of the different treatment effects.

Results and discussion

Multiple shoot regeneration

Shoot regeneration was achieved with hypocotyl segments cultured on MS medium amended with TDZ (0.5 mg l^{-1}). Shoot buds began to appear 8 weeks after culture initiation. The adventitious shoot buds started from the proximal end of the hypocotyl segments. Shoot regeneration frequency ranged from 50 to 60 %. There was no endophytic fungal emergence from stem and leaf segments of in vitro regenerated *N. nimmoniana* plated on PDA. As a matter of abundant caution, the plates were observed for over a month and yet there was no emergence from any of the plates. The in vitro regenerated shoots devoid of endophyte were found to produce CPT; the retention time of CPT from the plant tissue samples were identical to those obtained for authenticated standard (Fig. 1).

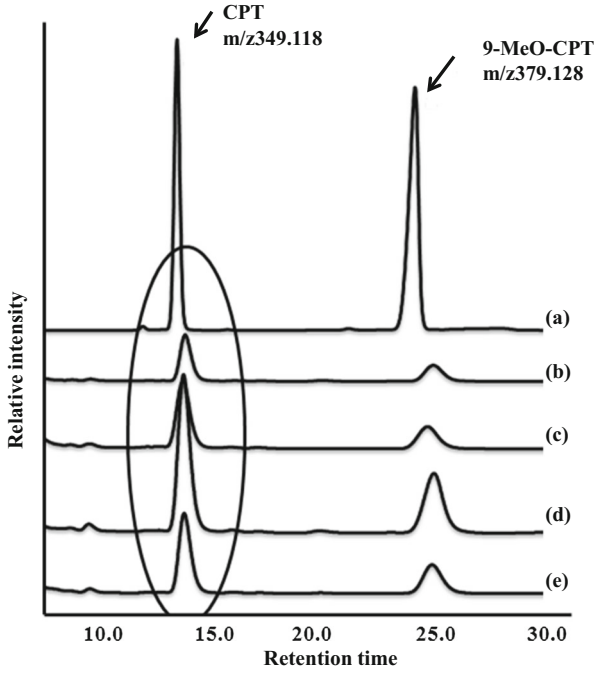

Fig. 1 HPLC spectra of camptothecine (CPT) and 9-methoxy-camptothecine (9-MeO-CPT) from (*a*) standard reference compound, (*b*) endophyte free in vitro regenerated plants of *N. nimmoniana,* (*c*) attenuated endophytic fungus, (*d*) attenuated endophytic fungus reisolated from other than place of inoculation and (*e*) attenuated endophytic fungus re-isolated from the place of inoculation. In all cases the fungi used was *Botryosphaeria rhodina*, isolated from its host plant, *N. nimmoniana*

Inoculation of endophytic fungi to in vitro regenerated *N. nimmoniana* plant and its effects on reversal of attenuation

The endophytic fungi, *B. rhodina* isolated from *N. nimmoniana* readily colonized the in vitro regenerated shoots of *N. nimmoniana* as evident by the isolation of the fungus from not only the site at which they were inoculated but also from sites distant to the place of inoculation (Fig. 2). The genetic identity of the re-isolated fungus, determined by sequencing the inter-genic spacer regions of rDNA, was similar to that of the mother culture indicating that the inoculated fungi had successfully invaded the host tissue. The re-isolated fungus was also characterized based on morphological characteristics (Arx 1981) and was found to be similar to the one inoculated.

The re-isolated fungus, both from the site at which they were inoculated and from sites distant to the place of inoculation yielded significantly ($P = 0.0001$) higher amount of CPT compared to fungi that had not passed through the host plant. Thus, compared to an average yield of 71.83 μg 100 mg^{-1} of CPT in the attenuated fungus, the yield from the fungus at the place of inoculation and away were respectively 197.71 and 170.18 μg 100 mg^{-1} (Table 1; Fig. 1). The presence of CPT was confirmed by the distinct m/z of 349 corresponding to standard CPT in an LC–MS and ESI–MS/MS analysis showing characteristic fragmentation of CPT (m/z 305, 277 and 249) (Fig. S1).

Inoculation of EGFP labeled endophytic fungus, *Phomopsis* sp. to in vitro regenerated *N. nimmoniana* and *O. mungos* plants and its effects on reversal of attenuation

Agrobacterium tumefaciens strain Eha105 successfully transformed the endophytic fungus *Phomopsis* sp. The resultant transformant was resistant to hygromycin at 75 g l^{-1} and expressed the EGFP fluorescence (Fig. 3). The transformant was mitotically stable as evident by its resistance to 75 g l^{-1} of hygromycin and EGFP fluorescence even after 5 consecutive sub-culture in a non-selection media. The genomic DNA amplification of *hph* gene and *egfp* gene were observed at 601 and 600 bp respectively (Fig. 3). Wild type *Phomopsis* sp. (MD 86) did not show any fluorescence and served as the control.

The EGFP transformed *Phomopsis* sp. successfully colonized the in vitro regenerated plants of *N. nimmoniana* and *O. mungos* 3 weeks after incubation at room temperature. Tissue sections of *N. nimmoniana* and *O. mungos* exhibited fungal emergence after 5 days of incubation in the selection media (Fig. 4). Colonization was not successful in in vitro regenerated plants of tobacco. The re-isolated fungi from *N. nimmoniana* and *O. mungos* were resistant to hygromycin (75 g l^{-1}) as well as exhibited the green fluorescence (Fig. 4). However, attempts to detect the EGFP transformed fungus in vivo (in the plant) were not successful due to high background auto-fluorescence.

The HPLC analysis of the re-isolated endophytic fungus along with the wild type attenuated and transformed fungus showed a peak eluting at retention time corresponding to standard CPT (12 min); there was a distinct spike in the CPT peak in the re-isolated endophytes compared to the respective wild type and transformed fungi that was not passed through the host (Figs. 5, 6). The presence of CPT, was further confirmed by ESI–MS/MS analysis which showed fragmented MS^3 ion of CPT at 277.17 m/z (Fig. S2).

Effect of DNA methyltransferase inhibitor on reversal of attenuation

Treatment with 5-azacytidine increased the CPT yield in the attenuated fungus. Compared to 36.20 μg 100 mg^{-1} CPT in the untreated attenuated fungus, treatment with 0.01 g l^{-1} of azacytidine increased the CPT content to 88.14 μg 100 mg^{-1} (Table 2). However, concentrations beyond 1.0 μM 0.01 g l^{-1} were inhibitory. Finally the

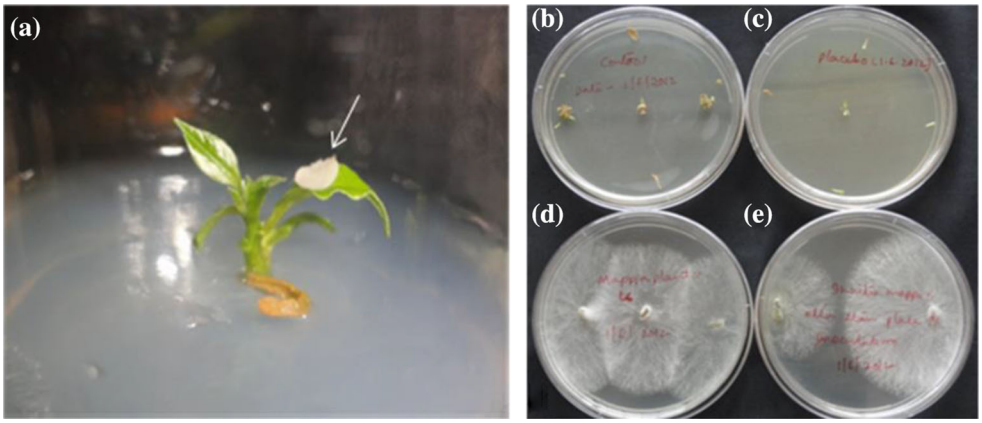

Fig. 2 **a** Inoculation of spore suspension of attenuated endophytic fungi *Botryosphaeria rhodina* for colonization in in vitro regenerated *N. nimmoniana* plants, **b** in vitro regenerated *N. nimmoniana* segments devoid of endophytic fungal assemblages in control treatment, **c** in vitro regenerated *N. nimmoniana* segments devoid of endophytic fungal assemblages upon treatment with sterile distilled water (placebo), **d** emergence of endophytic fungi, *B. rhodina* from the place of inoculation and **e** emergence of endophytic fungi, *B. rhodina* from site away from the place of inoculation

Table 1 Reversal of attenuation of CPT (μg 100 mg^{-1}) production by the endophyte, *Botryosphaeria rhodina*, upon inoculation into its host plant, *N. nimmoniana*

S. no.	Region of isolation	Mean ± SD*
1	Attenuated fungi	71.83 ± 7.36^{a}
2	Re-isolated (from site of inoculation)	197.71 ± 10.05^{b}
3	Re-isolated (from site away)	170.18 ± 9.29^{c}

* Dissimilar superscripts indicate significant difference (student *t* test, $P < 0.001$)

identity of the CPT in the endophytic fungus was confirmed by LC–MS (m/z 349) (Fig. 7) and ESI–MS/MS analysis (m/z 305, 249 and 277) with reference to standard CPT (Fig. S3).

One of the major constraints in realizing the potential of endophytes in the production of plant secondary metabolites is the observed attenuation of production of the metabolite on subculture of the fungi (Li et al. 1998; Kusari et al. 2009; Gurudatt et al. 2010; Xiang et al. 2013; Kumara et al. 2014). Several studies have attributed the attenuation to lack of

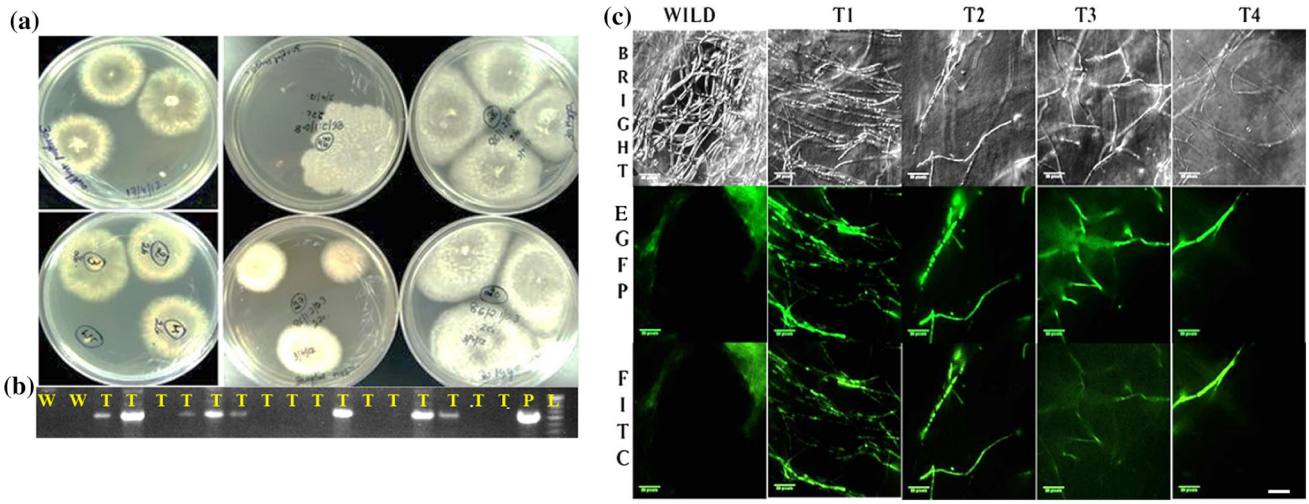

Fig. 3 **a** Putative *Phomopsis* sp. transformants growing on 75 μg ml^{-1} of hygromycin, **b** gel picture of the amplification of *hph* gene (610 bp) and EGFP gene (609 bp), (*W*—wild type, *T*—transformant, *P*—plasmid, *L*—ladder 1 kb), **c** flurosence imaging of *Phomopsis* sp. MD 86 hyphae. The first column is the wild type (control) and *T1*, *T2*, *T3* and *T4* are transformants. *Scale* 10 micrometer

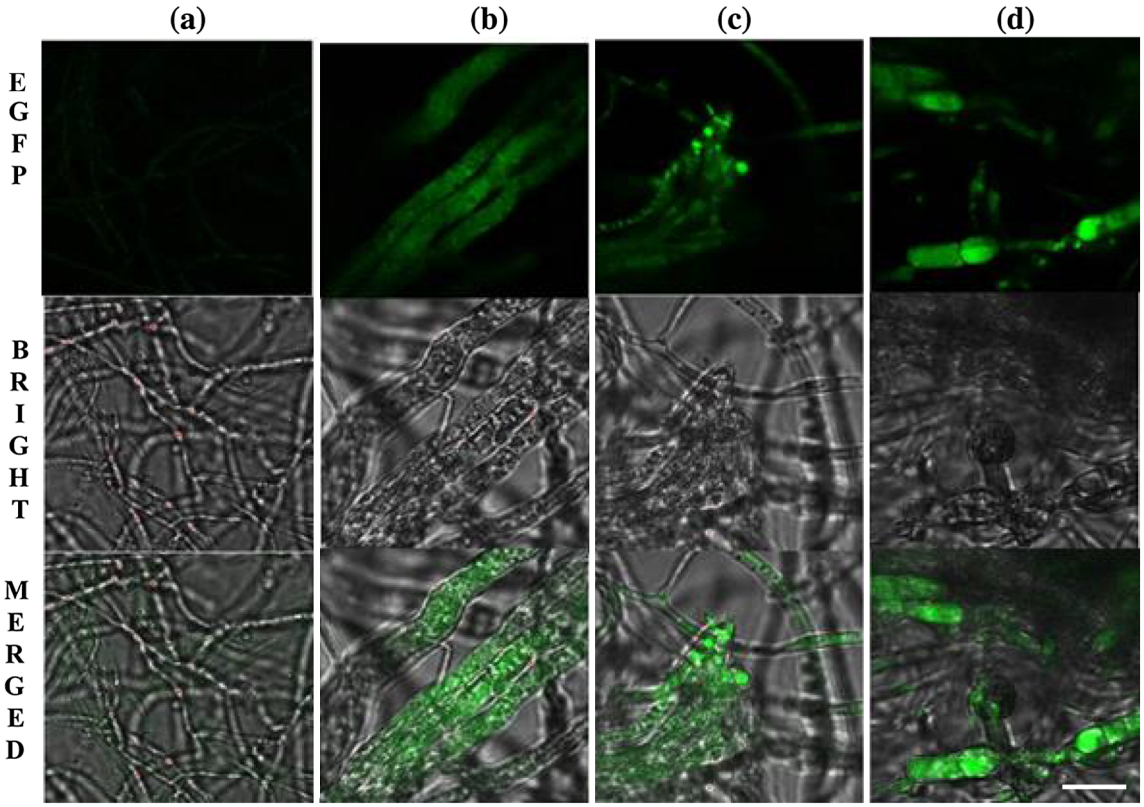

Fig. 4 Confocal fluorescence image of *Phomopsis* sp. re-isolated after 3 weeks of inoculation into in vitro regenerated *N. nimmoniana* (**c**) and *O. mungos* (**d**). The images of control (**a**) and transformed fungus (**b**) are also shown. The hyphal cells were fluorescing *green* under the blue light. *Scale* 50 µm. (Color figure online)

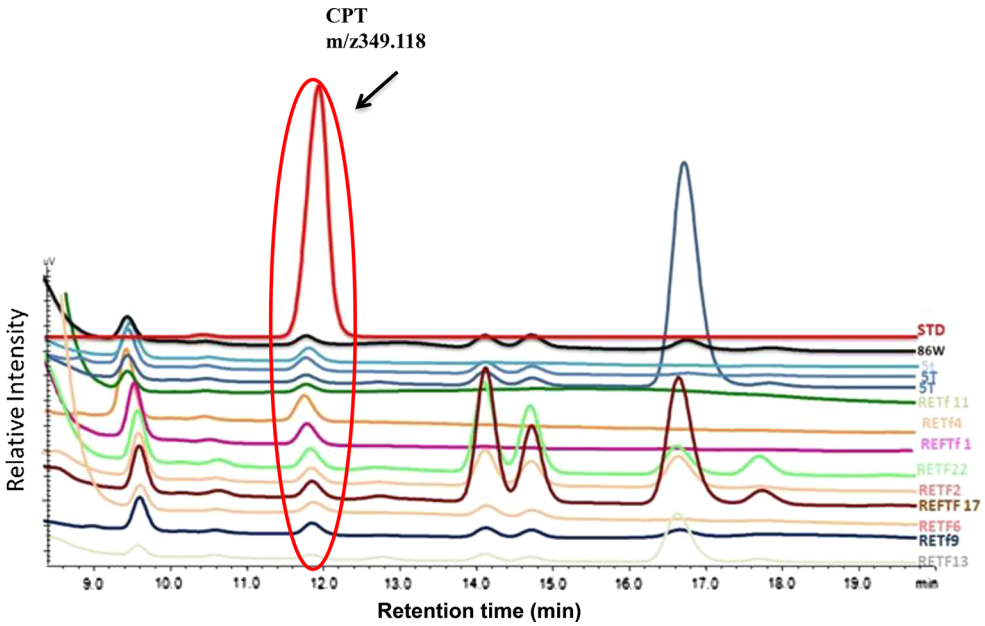

Fig. 5 Extracted HPLC chromatograms of endophytic fungus *Phomopsis* sp. MD 86 (86 W), GFP transformed (5t,5T) and re-isolated GFP transformed fungus from *N. nimmoniana* stem (RETFf 4), *N. nimmoniana* leaf (RETf 11, RETF 13), *O. mungos* stem (RETF 2,22,17, RETf 1,9), *O. mungos* leaf (RETF 6)

contact of the fungus with the host in axenic culture, due to which certain presumed signaling by the host are lost and hence leading to attenuation. In an effort to address this, few studies have in the past attempted to recycle or pass the attenuated fungus through the host and examine if attenuation could be reversed (Li et al. 1998; Gurudatt et al. 2010; Kusari et al. 2011). But these studies had not yielded conclusive results. A major drawback in these studies is the fact

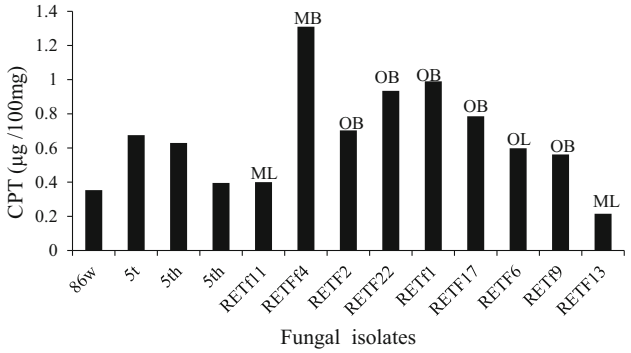

Fig. 6 Concentration of CPT in endophytic fungus *Phomopsis* sp.MD 86 wild type (86 W), EGFP transformed (5t, 5T) and isolates of reisolated EGFP transformants (RETF11,4,2,22,1,17,6,9,13) from CPT producing plants. *MB—N. nimmoniana* stem portion, *ML—N. nimmoniana* leaf portion, *OB—O. mungos* stem portion, *OL—O. mungos* leaf portion

Table 2 Reversal of attenuation of CPT (µg 100 mg^{-1}) production by the endophyte, *Botryosphaeria rhodina*, upon treatment with DNA methyltransferase inhibitor, 5-azacytidine

Sl. no.	Treatment (µM)	Mean ± SD*
1	Control	36.20 ± 7.84^{a}
2	1.00	49.75 ± 13.62^{ab}
3	5.00	78.07 ± 12.32^{c}
4	7.50	75.28 ± 17.97^{abc}
5	10.0	88.14 ± 13.6^{c}

* Dissimilar superscripts indicate significant difference (student *t* test, $P < 0.001$)

that none of them had used endophyte free plants and thus the results could have been confounded by interactions with endogenous flora. In the current study, we used endophyte free in vitro regenerated plants of *N. nimmoniana, O. mungos* as well as *N. tabacum*. Furthermore and in an additional experiment, we used EGFP transformed attenuated fungus that allowed the continuous monitoring of the fungus through its passage in the host. In both the re-inoculation experiments, we found a significant increase in the CPT content of attenuated fungus when they were passed through their host plants compared to attenuated fungus that were not passed through the host. Additionally our study also showed that reversal of attenuated fungus could occur when passaged through non-host plants also, as long as the plants have the ability to produce CPT. Thus, the attenuation of the endophyte, *Phomopsis* sp. from *Miquelia dentata* was able to be reverted when passed through non-host plants that produced CPT such as *N. nimmoniana* and *O. mungos.* These results seem to suggest that certain critical signaling may be necessary for the fungus to maintain its endogenous production, in the absence of which the gene(s) responsible for the biosynthesis could be switched off or rendered silent. Preliminary results, using DNA methyltransferase (DNMT) inhibitor suggest that this could be a possibility. Fungi on their path to attenuation (in the second sub culture) when treated with 5-azacytidine partially reversed their attenuation indicating that during the process of sub-culture certain genes may be silenced. In summary, these results indicate the possibility that attenuation of endophytic fungus in axenic culture could be due to the absence of some critical signaling

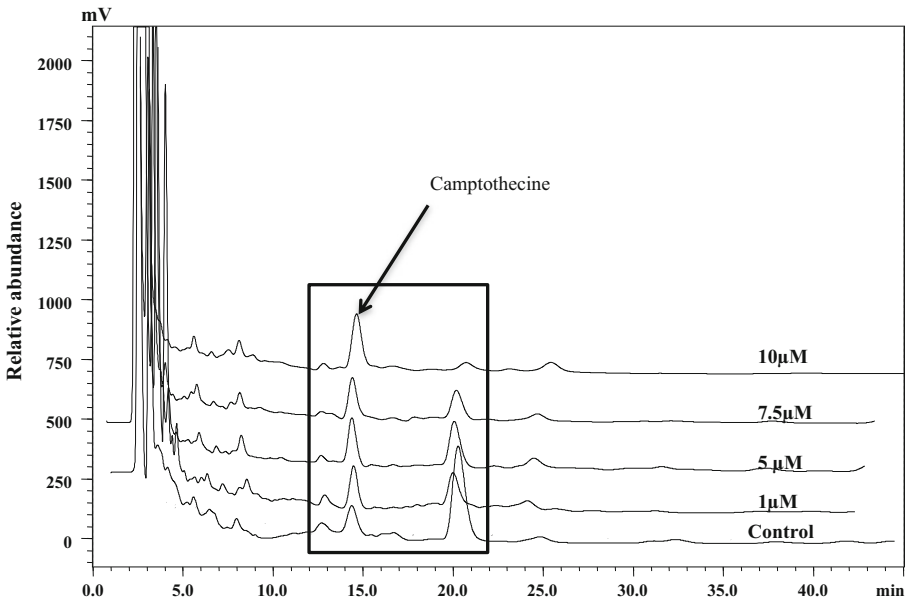

Fig. 7 Overlay of LC/MS chromatograms of extracts of attenuated fungi: control, 1, 5, 7.5 and 10 µM 5-azacytidine treated fungi. Region highlighted indicates varying levels of the peaks of interest

from the plants that could lead to methylation or silencing of the genes. These conclusions are strengthened by some very recent findings that methylation events could be crucial in regulating secondary metabolite production in endophytic fungi. For example, Chen et al. (2013) showed that treating endophytic fungi isolated from the medicinal plant *Datura stramonium* with histone deacetylase (HDAC) inhibitors could successfully activate secondary metabolite biosynthetic pathways that are dormant under normal conditions. In a more conclusive manner, Chujo and Scott (2014) demonstrated the role of epigenetic modifications to be responsible for silencing of alkaloid biosynthetic genes in the endophyte, *Epichloe festucae* associated with *Lolium perenne*. The endophyte produces bioprotective lolitrems and ergot alkaloids in association with its host plant. However in axenic cultures, the endophyte fails to produce the compounds. Chujo and Scott (2014) showed that histone H3 lysine 9 and lysine 27 trimethylation (H3K9me3/H3K27me3) at the alkaloid gene loci were critical determinants of transcriptional activity. Deletion of *E. festucae* genes encoding the H3K9-(ClrD) or H3K27-(EzhB) methyl transferases led to de-repression of *ltm* and *eas* gene expression under non-symbiotic culture conditions and a further enhancement of expression in the double deletion mutant. We are currently investigating the underlying molecular mechanisms that might be responsible in revoking the attenuation of production of camptothecine by endophytic fungi when they are passed through their host plants.

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