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Attenuation of camptothecin production and negative relation between hyphal biomass and camptothecin content in endophytic fungal strains isolated from *Nothapodytes nimmoniana* Grahm (Icacinaceae)

Endophytic fungi, a group of fungi living inside the host plant tissues without causing visible symptoms of disease, are known to occur ubiquitously in plants[1]. Existing in a mutualistic association with their host plants, they have been shown to enhance the plant's ability to tolerate abiotic[2] and biotic stresses[3]. In culture, many endophytic species have been shown to produce a number of important secondary metabolites including anticancer, antidiabetic, antifungal and immunosuppressant compounds[4,5]. Many of these compounds closely mimic those produced by the respective host plants, suggesting that the fungi could in fact potentially serve as an alternative source of plant secondary metabolites[4,6]. Notwithstanding these findings, to date, there has been no major breakthrough in commercially exploiting the endophytic fungi as a source of important secondary metabolites[7,8]. Among the reasons attributed is the severe attenuation of production of the secondary metabolite by the fungi in culture. Li *et al.*[9] showed that successive cultures of an endophytic fungi *Pericornia* sp. isolated from *Torreya grandifolia*, resulted in the attenuation of taxol production, though the fungal growth itself was unaffected. Although the reasons for such attenuation are not extensively studied, it is conjectured that it could be due to lack of host stimulus in the culture media[9].

In this paper, we report the isolation of endophytic fungal strains producing an anticancer alkaloid, camptothecin (CPT), from *Nothapodytes nimmoniana* (Icacinaceae), a tree known to produce the highest ever reported levels of CPT[10,11]. CPT is a potent inhibitor of DNA topoisomerase-I[12]. CPT derivatives are currently being used to treat several cancers including colon cancer[13], uterine cervical cancer and ovarian cancer[14], and also AIDS[15]. Two clinically used antitumour compounds, topotecan and irinotecan, are semi-synthesized using natural CPT from plant sources[11]. Here, we demonstrate the attenuation of CPT production by the endophytic fungal strains across serial subculture generations. Further, we also report an intriguing negative relation between the hyphal biomass in culture and its respective CPT content. We discuss these results in the light of using endophytic fungi as an alternative source for secondary metabolite production.

Endophytic fungi were isolated from inner bark of 15 trees of *N. nimmoniana*, Grahm (Icacinaceae) from the Western Ghats, a megadiversity hotspot in south India, following Arnold *et al.*[16]. Bark explants sampled from the trees were treated with 95% ethanol for 2 min and then the alcohol was allowed to evaporate. The outer bark was then removed with a sterile blade. After thoroughly washing the tissue with sterile water, small pieces of the inner bark (approximately 1.5 cm^2) were placed on aqueous agar amended with streptomycin (100 mg/l) to eliminate bacterial growth and incubated at 28 ± 2°C until fungal growth started. Once the endophytic fungi emerged from the cut ends of the tissues, they were transferred to sterile Sabouraud agar (dextrose: 40 g/l; peptone: 10 g/l; agar: 20 g/l) and incubated. All pure fungal isolates were routinely maintained on Sabouraud agar and were stored in their vegetative form as slants and in 50% (v/v) glycerol at –80°C.

All the fungal strains were identified using rDNA homology. ITS1 and ITS4 primers were used to amplify ITS1, 5.8S and ITS2 regions of rDNA[17]. Genomic DNA was isolated from the mycelial mass using the method described by Vainio *et al.*[18].

The isolated fungi were fingerprinted using ITS rDNA region and the sequences submitted to GenBank. Amplification was performed in a reaction volume of 25 µl with approximately 150 ng of DNA template, 1 mM each of dNTP, 1X PCR reaction buffer (Bangalore Genei, India, containing 1.5 mM $MgCl_2$) and 1 U *Taq* polymerase (Bangalore Genie, India) together with 5 pmole of primers ITS1 and ITS4. All the components in the reaction except DNA were mixed together. The PCR amplification cycle consisted of 3 min at 95°C, followed by 40 cycles of 1 min at 92°C, 1 min at 50°C and 2 min at 72°C and with a final extension of 10 min at 72°C in an Eppendorf thermal cycler. PCR products were analysed by electrophoresis at 70 V for 3 h in a 1.8% agarose gel in 1X tris buffer and inspected under UV light in a transilluminator following ethidium bromide staining. The PCR products were eluted using an Eppendorf Perfect Prep Gel Cleanup Kit, and confirmed by fractionating on 1.8% agarose gel and visualized by ethidium bromide staining. The sequencing of the eluted fragments was done at MWG, Bangalore.

BLAST analysis was performed with full length ITS sequences as queries to reveal relationships to GenBank database of fungal nucleotide sequences in NCBI database. Based on highest homology and total score, the strains were identified using the following criterion that was described in Higgins *et al.*[19] and Marquez *et al.*[20]. Genus and species of the database match were accepted whenever the identity between our sequence and that of the database was greater than 97%; only the genus was accepted when identity to a database match was from 95% to 96.9%, and when the similarity was less than 95% the isolates were considered unidentified. The sequences have been deposited in the GenBank and the accession numbers (FJ158119–FJ158141) are provided in Table 1.

Fungal isolates were grown in liquid media following the method described by Amna *et al.*[21]. For each pure isolate, single hyphal tips were incubated in presterilized Sabouraud broth with dextrose as sole carbon source (4% w/v) and peptone (1% w/v) as nitrogen source. Though it is well known that secondary metabolite production depends on several culture conditions (type of medium, carbon and nitrogen sources, temperature, pH, cultivation time, etc.), for the present study, we restricted the experiments to Sabouraud broth as previous studies had demonstrated the production of CPT in this medium[21]. Shake flask experiments were carried out in 250 ml conical flasks containing 50 ml of medium, agitated at 200 rpm on a rotary shaker at 28 ± 2°C for four days.

Table 1. Endophytic fungal strains isolated from *N. nimmoniana* along with their GenBank accession numbers

Isolate code	GenBank accession number	Percentage similarity with GenBank database match	Species name
UAS001	FJ158119	93	Unidentified
UAS002	FJ158120	98	*Diaporthe conorum*
UAS003	FJ158121	99	*Fusarium solani*
UAS004	FJ158122	99	*Fusarium* sp.
UAS005	FJ158123	99	*F. verticillioides*
UAS007	FJ158124	99	*F. oxysporum*
UAS008	FJ158125	93	Unidentified
UAS009	FJ158126	100	*Irpex lacteus*
UAS010	FJ158127	95	Unidentified
UAS011	FJ158128	99	*Fusarium* sp.
UAS013	FJ158129	99	*F. sacchari*
UAS014	FJ158130	100	*Phomopsis* sp.
UAS015	FJ158131	100	*Botryosphaeria parva*
UAS017	FJ158133	99	*F. subglutinans*
UAS018	FJ158134	98	*F. oxysporum*
UAS019	FJ158135	100	Unidentified
UAS020	FJ158136	99	*F. oxysporum*
UAS021	FJ158137	100	Unidentified
UAS022	FJ158138	99	*F. oxysporum*
UAS024	FJ158139	98	*Galactomyces* sp.
UAS025	FJ158140	100	*F. oxysporum*
UAS026	FJ158141	97	*F. solani*

Sequence quality of UAS006, UAS012, UAS016 and UAS023 was poor and hence these isolates were not identified. BLAST score of isolates UAS019 and UAS021 was very low and hence they were not considered further for their identification.

Each of the fungal strains was passed through four subculture generations. From each of the mother cultures (regarded as the first generation culture), hyphal tips were inoculated to obtain the second-generation culture. From these, further hyphal tip inoculations were made to obtain respectively the third and fourth subculture generations. Each of these subculture generations was then put through shake-flask fermentation as described here.

CPT was extracted from the fungal mycelia and broth as described by Wall *et al.*[22] for all isolates at each subculture generations. Mycelia and broth were separated by filtration. Mycelia were thoroughly washed with sterile distilled water, weighed and homogenized in a cell disintegrator. Both cell homogenate and cell-free broths were extracted four times with equal volumes of chloroform : methanol (4 : 1 v/v). The solvent was evaporated using a rotary evaporator leaving behind the organic residue, which was dissolved in DMSO : methanol (1 : 3 v/v) and analysed by using high performance liquid chromatography (HPLC) and LC-MS/MS.

For a few samples, the presence of CPT was validated by LC-MS/MS (Figure 1). Analysis was done by a Waters Quattro Premier micromass LC-MS system consisting of a reversed phase C18 column (150 × 2.1 mm, 5 µ). Mobile phase consisting of acetonitrile/water (60 : 40) containing 0.1% formic acid was pumped at a flow rate of 0.3 ml/min. High quality argon gas was used for collision and nitrogen gas for desolvation. The mass spectrometer was operated in the positive ion mode using electrospray ionization. Conditions for mass spectrum analysis were set at a capillary voltage of 2.99 kV, cone at 18 V, source temperature at 100°C, desolvation temperature at 300°C and collision temperature at 25°C.

The molecular ion peak at *m*/*z* 349 $[M + H]^+$ was taken for MS/MS studies. This parent ion upon ionization and fragmentation exhibited different daughter ions. Both parent and the daughter ions were monitored simultaneously in a MRM (multireaction monitoring) mode. Among the daughter ions monitored, the one with best intensity transition was used for quantification and the rest for confirmation. The daughter ion peak at *m*/*z* 305 was taken up for quantification. Quantification of CPT in the fungal extracts from different isolates was done on the basis of the calibration curve established by injecting six concentrations of the standard CPT in the concentration range from 5 to 5000 ppm. Linear calibration curve of 305 ion of CPT indicated a curve coefficient of $R^2 = 0.982$.

Once the chemical identity of CPT was demonstrated by the LC-MS/MS analysis, quantification of CPT in the subcultured generations of the various isolates was done by performing a reverse phase HPLC analysis.

The amount of CPT in each sample was determined by HPLC performed on reverse phase (HPLC model, Shimadzu, Japan) on a C18 column (250 × 4.6 mm, 5 µm). The HPLC conditions were: 254 nm as the detector wavelength, 1.6 ml/min flow rate and 10 µl sample loop. The mobile phase was adjusted as follows: 40% acetonitrile and 60% water + 0.1% trifluroacetic acid (TFA) in an isocratic mode. A CPT (Sigma, 95% HPLC purified) standard was procured from Sigma Chemicals. A standard graph was developed by injecting different concentrations of standard CPT (0.1–1.0 mg/ml). The CPT in the extract was identified by comparing its retention time with that of standard CPT (3.5 min). Quantity of CPT in the extract was calculated by using the standard graph and expressed as CPT in µg per 100 mg dry weight of the mycelia. CPT was estimated for all the fungal isolates at the second, third and fourth subculture generations.

Twenty-six fungal strains were isolated from the inner bark tissue of 15 *N. nimmoniana* trees and genotyped using *ITS rDNA* homology (Table 1). Seventeen out of 25 isolates were identified based on ITS sequence homology with the GenBank sequences. Twelve out of 17 (70% of the isolates) identified isolates belonged to the genus *Fusarium* and the remaining five isolates belonged to four other genera namely, *Diaporthe*, *Irpex*, *Botryosphaeria* and *Galactomyces*. All isolates belonged to Ascomycetes group of fungi, except isolate *Irpex*; which belonged to the Basidiomycetes group (Table 1).

All the 25 fungal strains produced CPT in culture. Most of the CPT was recovered from the mycelia with little or none from the broth. For a few of the strains, the CPT signals were validated by an LC-MS/MS analysis (Figure 1). The presence of CPT was confirmed by the appearance of the characteristic *m*/*z* 305 ion $[M–CO_2]$ after fragmentation of

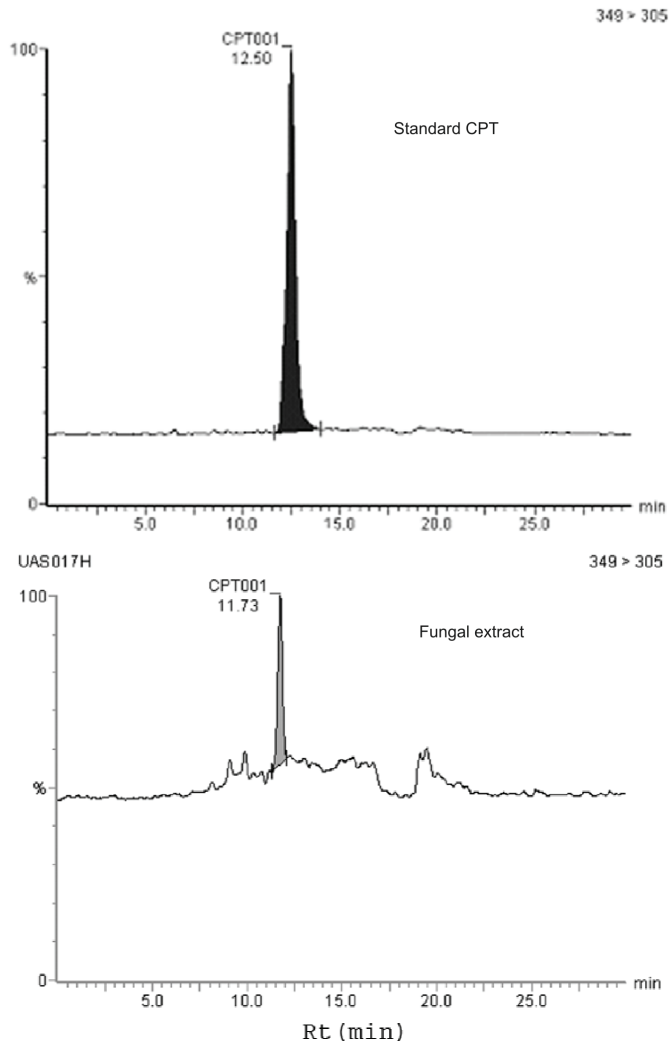

Figure 1. LC-MS/MS chromatogram of standard CPT and fungal extract (UAS017).

Table 2. Mean CPT (μg/100 mg) content of 25 endophytic fungal isolates at second, third and fourth subculture generations. Mean followed with dissimilar superscripts are significant $P < 0.05$ (Student's *t*-test for independent samples)

Generation	*N*	Range	Mean	± SE
Second	25	0.22–8.80	2.17[a]	0.52
Third	25	0.01–9.82	1.14[b]	0.44
Fourth	25	0.004–7.72	0.66[b]	0.30

parent molecule *m*/*z* 349 [M + H$^+$] in LC-MS/MS (Figure 1). The LC-MS/MS spectrum of fungal CPT was identical to the standard CPT spectrum.

Figure 2 presents the attenuation of CPT production for four strains across the subculture generations. Over the three-subculture generations, for all the fungal isolates, the mean CPT content decreased significantly from 2.17 μg/100 mg in the second subculture generation to 0.66 μg/100 mg in the fourth subculture generation ($P < 0.05$, Student's *t*-test for independent samples; Table 2). We also observed a significant negative relation between the hyphal biomass and its respective CPT content at the end of the second subculture generation (Figure 3).

Our study has demonstrated (a) a clear attenuation of CPT production by the endophytic fungal strains isolated from *N. nimmoniana* across their respective subculture generations and (b) a clear negative relation between hyphal biomass and CPT content across the fungal strains. Both these findings have important implications in as far as realizing the potential of endophytic fungi as alternative sources of plant secondary metabolites.

Attenuation or the loss of virulence is well known in microbiological literature[23–25]. More recently, Kusari *et al.*[26] reported the attenuation of CPT production in an endophytic fungus isolated from *Camptotheca acuminata*. One of the possible reasons attributed for the attenuation is the lack of host stimulus in the axenic culture medium. However, efforts to reverse attenuation in endophytic fungal cultures by host stimuli have been far too few and where attempted, have not been highly successful. Li *et al.*[9] reported the reversal of attenuation of taxol production by endophytic fungi on supplementing the fungal culture with host tissue extract. We failed to reverse the attenuation of CPT production by the endophytic fungi despite supplementing the fungal growth medium with host tissue extracts. The lack of success in reversing the attenuation of endophytic fungi, we believe, rests on our current limited understanding of the evolutionary significance of these organisms and their dynamic interaction with their respective hosts[27].

The observed negative relation between the hyphal biomass and CPT content is intriguing and might be related to the trade-off between the cost of production of CPT on one hand, and energy required for growth of the fungus on the other. Accordingly, fungal isolates that allocate a larger proportion of their carbon pool to CPT production (reflected in a higher CPT content) would tend to have lesser hyphal biomass and vice versa. While direct evidence for this hypothesis is lacking, previous studies have shown that accumulation of defence compounds (secondary metabolites) in plants may often incur a cost in the form of reduced growth or biomass production[28–30]. Yet another explanation for the observed negative relation could be based on the assumption that CPT produced by the fungus actually inhibits further hyphal growth and multiplication. Thus isolates that produced a higher level of CPT in shake flasks would tend to be inhibited in their growth compared

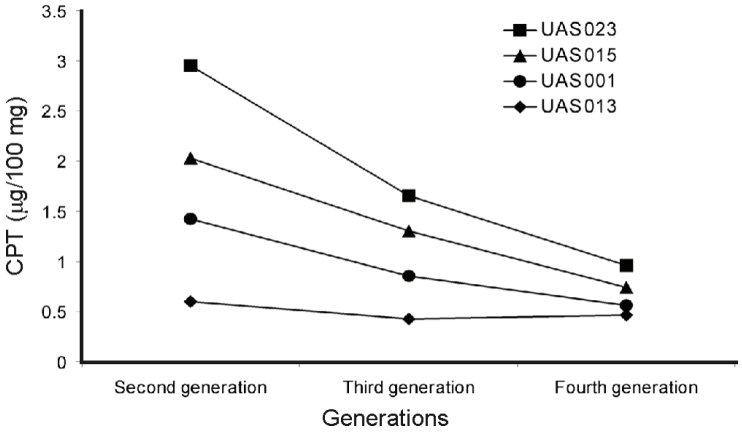

Figure 2. CPT yield of isolates, UAS023, UAS015, UAS001 and UAS013 at second, third and fourth subculture generations.

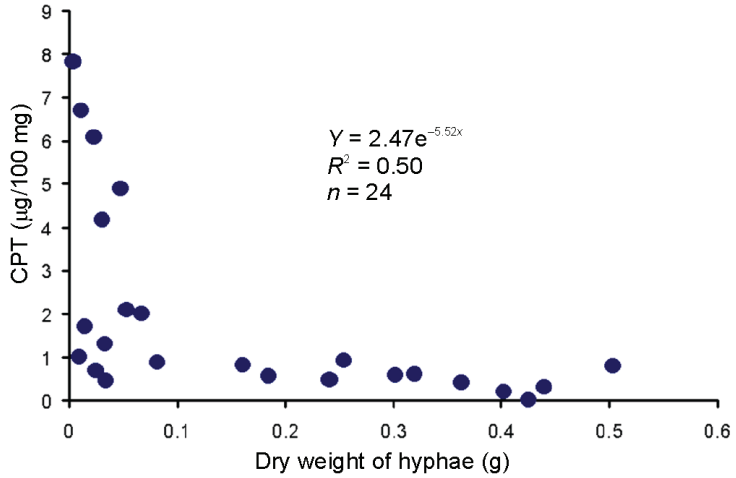

Figure 3. Negative relation between hyphal dry weight and CPT content of endophytic fungal isolates from *N. nimmoniana* ($y = 2.47e^{-5.52x}$, $R^2 = 0.50$, $n = 24$).

to isolates that produced less or no CPT at all. However studies at our laboratory showed that exogenous application of CPT in culture flasks did not inhibit the growth of the endophytic fungi. In fact Liu and Reinscheid[31] reported that endophytic fungi isolated from *Camptotheca acuminata*, a tree producing CPT, were insensitive to exogenously applied CPT.

Both our findings, namely, the attenuation of production of CPT and the negative relation between hyphal biomass and CPT production, offer important challenges that need to be addressed before one could realize the potential of endophytic fungi as alternative sources of secondary metabolites. In particular, if these challenges have to be addressed successfully, more research would be required to understand the biology of the endophytic fungi and their intricate relationship with the host.

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Changes in the essential oil content and composition of *Origanum vulgare* L. during annual growth from Kumaon Himalaya

The members of the genus *Origanum* L. are usually perennial herbs belonging to the mint family (Lamiaceae). The genus *Origanum* comprises 49 taxa belonging to 10 different sections characterized by a large morphological and chemical diversity. Most of them are found exclusively in the eastern Mediterranean region[1]. In India, it is represented by a single species, *Origanum vulgare*, widely distributed in subtemperate/temperate Himalaya[2]. *Origanum* species is an important culinary herb in world trade. It is reported to be widely used as a traditional remedy to treat various ailments such as whooping and convulsive coughs, digestive disorders and menstrual problems[3]. The essential oil of this plant has proven to be antimicrobial, fungicidal and antioxidant[4–7].

Essential oil composition of *Origanum* spp has been extensively investigated[8–14]. However, considering its huge genetic diversity, the information on this important plant from India is scanty[15–17]. The present study was carried out to study the variability of essential oil content and composition of carvacrol chemotype of *O. vulgare* during annual growth from Kumaon region of western Himalayas.

The planting of *O. vulgare* was done in October and plants were raised following normal agricultural practices in the experimental field of CIMAP Research Centre, Purara, Uttarakhand. Climatologically, the site falls in the temperate region (1250 m) of western Himalaya. The fresh herb of *O. vulgare* was collected every month (March 2007 to February 2008) and hydrodistilled in Clevenger apparatus for 3 h to extract the essential oil. The oil was dried over anhydrous sodium sulphate, measured and stored in refrigerator prior to analysis.

The gas spectrograph (GC) analysis of the oil samples was carried out on a Nucon Gas Chromatograph Model 5765 and Perkin-Elmer Auto XL GC equipped with FID using two different stationary phases, BP-20 (30 m × 0.25 mm × 0.25 μm film thickness) and PE-5 (60 m × 0.32 mm; 0.25 μm film thickness) fused silica columns respectively. Hydrogen was the carrier gas at 1.0 ml/min. Temperature programming was done from 70°C to 230°C at 4°C/min with initial and final hold time of 2 min (for BP-20) and from 70 to 250°C at 3°C/min (for PE-5). The injector and detector temperatures were 200°C and 230°C on BP-20 and 220°C and 300°C on PE-5 column respectively. The injection volume was 0.02 μl neat and Split ratio was 1 : 30.

The GC–mass spectrometer (MS) analysis of the oils was carried out on a Perkin-Elmer Turbomass Quadrupole Mass Spectrometer fitted with Equity-5 (Perkin-Elmer) fused silica capillary column (60 m × 0.32 mm; 0.25 μm film thickness). The column temperature was programmed 70°C, initial hold time of 2 min, to 250°C at 3°C/min with final hold time of 3 min using helium as carrier gas at a flow rate of 1.0 ml/min. The injector temperature was 250°C, injec-

Table 1. Essential oil content of *Origanum vulgare* L. during annual growth

Month	Phenological stage	Oil content (%)*
February	Vegetative	0.25 (0.01)**
March	Vegetative	0.50 (0.02)
April	Vegetative	0.70 (0.01)
May	Vegetative	0.70 (0.02)
June	Flowering initiated	0.71 (0.02)
July	Full flowering stage	1.30 (0.04)
August	Green seed stage	1.00 (0.02)
September	Brown seed stage	0.80 (0.02)
October	Seed shattering	0.60 (0.01)
November	Seed shattering	0.52 (0.02)
December	Hibernation	0.35 (0.02)
January	Hibernation	0.20 (0.01)

*Calculated on fresh weight basis. **Values in parenthesis are standard deviation.