Novel Microbial Sources of Tropane Alkaloids: First Report of Production by Endophytic Fungi Isolated from *Datura metel* L.

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Abstract Eighteen endophytic fungi were isolated from various tissues of *Datura metel* and genes encoding for putrescine *N*-methyltransferase (PMT), tropinone reductase 1 (TR1) and hyoscyamine 6 β -hydroxylase (H6H) were used as molecular markers for PCR-based screening approach for tropane alkaloids (TAs) producing endophytic fungi. These fungi were identified taxonomically by sequence analysis of the internal transcribed spacer region (ITS1–5.8S–ITS2) and also based on morphological characteristics of the fungal spore as *Colletotrichum boninense*, *Phomopsis* sp., *Fusarium solani*, *Colletotrichum incarnatum*, *Colletotrichum siamense* and *Colletotrichum gloeosporioides*. The production of TAs hyoscyamine and scopolamine by the fungi has been

ascertained using chromatography and spectroscopy methods by comparison with the standards. Among the fungi, the highest yields of hyoscyamine (3.9 mg/L) and scopolamine (4.1 mg/L) were found in *C. incarnatum* culture. This is the first report of endophytic fungi possess the *PMT*, *TR1* and *H6H* genes and produces TAs. These endophytic fungi have significant potential to be applied in fermentation technology to meet the demands for TAs economically.

Introduction

Tropane alkaloids (TAs), scopolamine and its precursor hyoscyamine, are produced by some genera of Solanaceae family namely *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* [7]. They are extensively used as anticholinergic agents [30], anaesthetics and in the treatment of motion sickness [15]. Scopolamine remains to be the preferred drug because of its lesser side effects than hyoscyamine. Scopolamine plays an important role in plant defence mechanism due to its implication in hampering the growth of insect larvae [27]. The roots are reported to be the site of scopolamine production from where it is translocated to other parts of the plant [19]. Large quantities of plant material are required for the production of these drugs as their alkaloid contents is low. Therefore, extensive efforts have been made in the recent years to facilitate the production of these valuable TAs from callus and hairy root cultures of TA-producing solanaceae plants by adapting different biotechnological approaches. But, they only accumulate in hairy root and are completely absent in callus and all suspension cultures [17]. Overexpression of *PMT* and *H6H* genes in transgenic hairy root culture of *Hyoscyamus niger* and *Atropa baetica* has been used for the production of TAs

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[29, 30]. Several approaches attempted in plant cell and organ culture systems failed to increase the accumulation of these alkaloids. The yields obtained so far are too low to allow commercial production. Efforts are on to look for alternate sources of these alkaloids.

Plant and endophytic fungi through mutualistic symbiosis produce secondary metabolites [8]. Ancient horizontal gene transfer is thought to have occurred between plants and microbes [20]. There is also a considerable amount of evidence showing that endophytes produce bioactive secondary metabolites associated with the host plant [5]. *Datura metel* L. (local name 'thorn apple'), is one of the medicinal plant and it is a rich source of the TAs hyoscyamine and scopolamine [22]. *D. metel* had been studied earlier for the composition, diversity and distribution of endophytic fungi within the plant [1, 21]. Some bioactive products have also been reported from endophytic fungi of *D. metel* from our lab [10], but not hyoscyamine and scopolamine or their structural analogues. We report here for the first time that the production of hyoscyamine and scopolamine by endophytic fungi isolated from *D. metel* plant and identified them based on molecular and morphological studies. The TAs production was confirmed by chromatographic and spectroscopic methods.

Materials and Methods

Plant Material and Isolation of Endophytic Fungi

Samples of *D. metel* fresh and healthy roots, stems and leaves were collected from the nursery, Indian Institute of Science, Bangalore, India. The plant parts were thoroughly washed with running tap water, rinsing with sterile distilled water (SDW) and then treated with 20% commercial bleach with 0.1% Tween-20 for 5 min and later again rinsed in SDW. They were then soaked in 100 mL solution containing fungicide bavistin (30 mg), tetracycline (0.6 mg), rifampicin (0.6 mg) and 0.1% Tween-20 for 20 min and rinsed in SDW. All samples were finally surface sterilized with 0.1% mercuric chloride for 10 min, followed by five to six washings with SDW. The surface sterilized root and stem segments were cut in to small pieces (1–2 mm), leaves were cut into 1 cm² pieces and placed on petri plates containing potato dextrose agar (PDA) supplemented with 100 mg/L ampicillin. The plates were labelled and incubated at 25 ± 2 °C with 12 h photoperiod or in darkness. The fungal mycelia that emerged from the cut surface of the segments after several days were transferred on to fresh PDA plates [18].

DNA Extraction and Genomic PCR-Based Screening for TAs-Producing Fungi

Eighteen different endophytic fungi were isolated and cultured in PDB medium. The genomic DNA was extracted from their mycelium and also from *D. metel* leaves by CTAB method [9]. The DNA quality was visualized under UV light, quantified by nanodrop and used for polymerase chain reaction (PCR) analysis. All endophytic fungi were screened for the presence of *N*-methyltransferase (*PMT*), tropinone reductase 1 (*TR1*) and hyoscyamine 6 β -hydroxylase (*H6H*) genes as molecular markers for TAs-producing fungi and *D. metel* gDNA was used as positive control. Based on the conserved sequences of *PMT*, *TR1* and *H6H*, the gene specific primers were designed and synthesized (Table S1). Each PCR reaction was performed in a total volume of 25 μ L reaction mixture containing 10 μ M of each forward and reverse primer, 200 μ M of each of the four dNTPs, 1 Unit Taq DNA polymerase and 100 ng template DNA. The PCR programme detail is given in Table S1. The amplified DNA fragments were analysed by agarose gel electrophoresis, purified using a GeneJETTM Gel Extraction kit, (Thermo Scientific, Waltham, MA, USA), sequenced and analysed with BLAST in the NCBI database.

Identification of Endophytic Fungi

The DNA of six PCR positive fungal isolates was used for the PCR amplification of ITS regions to identify the fungal isolates. The universal primers ITS1 and ITS4 (Table S1) were used for the amplification of the rDNA region of ITS1 and ITS2 and the 5.8SrRNA gene. The PCR reaction mixture contained all of the components as the same concentration described above and PCR programme details is given in Table S1. The PCR products were purified, sequenced and analysed as mentioned above. Sequence data were aligned and compared with sequences deposited in the GenBank data base NCBI BLASTIN programme. The fungi were assigned to a genus based in a 99–100% similarity index.

Mycelia pattern and conidial morphology of the six fungi were also determined using a light microscopy (Zeiss AX10 Imager A2, Zeiss, Germany). At least 30–50 conidia were studied for each isolate separately and their all characters determined and compared to that of the already reported [13, 16, 25] species.

Extraction of TAs from Endophytic Fungi

Six endophytic fungi were grown in 500 mL Erlenmeyer flask containing 100 mL modified nutrient growth medium (MNGB: Peptone 5 g/L, Yeast extract 3 g/L, Malt extract 3 g/L, Dextrose 10 g/L, pH 7.0 ± 0.2) and incubated at 28 ± 2 °C for 21 days. After 3 weeks of still culture, the

mycelia were harvested by filtration. Mycelia were thoroughly washed with sterile water, weighed and ground to fine powder using a mortar and pestle. The powdered mycelia and culture filtrate were processed independently for TAs extraction according to Gontier and Sangwan [6]. The culture filtrate and powdered mycelial material were extracted with the equal volume of methanol:chloroform:ammonia solution (50:50:1.4, v/v/v). The lower organic phase was collected and the solvent was then removed by rotary evaporator under reduced pressure at 40 °C. The residues were dissolved in 40 mL of 0.1 N HCl. After addition of 3 mL of ammonia solution, the alkaloids were extracted with 3 × 30 mL chloroform. After that the chloroform extracts were evaporated to dryness under reduced pressure at 40 °C. The dry residue was dissolved in 1 mL methanol for further use.

Detection of TAs from Fungal Extracts by Thin Layer Chromatography (TLC)

Extracted samples and standards hyoscyamine and scopolamine were separated by TLC on silica gel 60 GF259 (Merck, Kenilworth, USA) using a solvent system consisting of chloroform:methanol:acetone:ammonia (75:15:10:2, v/v/v/v). The alkaloids were detected by spraying the alkaloid specific Dragendorff's reagent on the plate. The spots showing R_f values corresponding to standard hyoscyamine and scopolamine (Sigma Aldrich, India) were scraped off from the non-sprayed TLC plate and eluted with methanol and purified fungal TAs were used for further analysis.

Quantification of TAs by High Performance Liquid Chromatography (HPLC)

The quantitative estimation of alkaloids from six fungal extracts was performed by HPLC. An Agilent 1120 HPLC system was used for separation. A photo iodide array detector measuring absorbance at 217 nm. A Phenomenex Luna C18 (5 µm, 250 × 4.6 mm) column was employed and fungal extracts were separated by using an isocratic elution of mobile phase consisting of 83% phosphate buffer saline (pH 3.3): 17% acetonitrile. The flow rate of mobile phase was set at 1.0 mL min⁻¹ using standards. The concentrations of hyoscyamine and scopolamine in the extracts were calculated by comparison with peak intensity obtained from standards.

Electrospray Ionization Mass Spectrometry/Mass Spectrometry (ESI-MS/MS) Analysis

The purified fungal samples were subjected to mass spectrometry (Thermo Electron Corporation, CA, USA) analysis. The conditions for MS/MS were as follows: the

determination mode was in scan mode, the ion–dipole was positive ion, the ionization mode was electro spray ionization (ESI), the nebulizer gas (helium) at a flow rate of 1 mL min⁻¹, the capillary temperature was 275 °C and the capillary voltage was 16 V. the data acquired over an range of 50–350 in positive ion mode and analysed using the xcalibur analysis software.

Results

Screening and Identification of Endophytic Fungi for Producing TAs

A total of 18 endophytic fungi were isolated from plant tissues, viz, leaf, root, stem and their fungal genome screened for the presence of TA biosynthetic genes. Out of 18 fungi, 6 fungi were found to be PCR positive for the conserved sequences of the *PMT* (196 and 256 bp), *TR1* (208 bp) and *H6H* (378 bp) genes (Fig. 1a–d). The sequence analysis of the *PMT*, *TR1* and *H6H* showed 99, 93 and 100% homology, respectively, with the host plant gene sequence (Table S2). The sequences of *PMT*, *TR1* and *H6H* DNA fragments have been deposited at the NCBI GenBank database with the accession numbers KM897684, KT358856, KT358857, KR024334, KT366043, KT366042, KT358853, KT358854, KT358855, KT358850, KT358851, KT358852 and KT358844, KT358845, KT358846, KT358847, KT358848, KT358849, respectively. These results confirmed the presence of *PMT*, *TR1* and *H6H* genes involved in TAs biosynthesis pathway in the six fungal isolates thus indicating their potential for production of hyoscyamine and scopolamine.

The six fungi amplified the 600 bp amplicon of ITS region and their sequences were blast analysed, which showed maximum homology with the earlier NCBI database. This molecular information was supporting with the morphological data, based on both results the endophytic fungi were identified as *Colletotrichum boninense* (Dm 9.3), *Phomopsis* sp. (Dm 10.2), *Fusarium solani* (Dm 13.4), *Colletotrichum incarnatum* (Dm 16.3), *Colletotrichum siamense* (Dm 17.3) and *Colletotrichum gloeosporioides* (Dm 18.2). The ITS sequences of six fungi were deposited to NCBI Genbank with the accession numbers (Fig S2). The detailed information for the identification is given in Supplementary results, Table S3, Figs. S1 and S2.

Structural Elucidation and Quantitation of Hyoscyamine and Scopolamine

The fungal extracts were assessed for the presence of hyoscyamine and scopolamine. The TLC analysis of fungal extracts showed the brown spots corresponding to standard hyoscyamine and scopolamine with the R_f value 0.21 and

Fig. 1 Representative genomic PCR analysis for the presence of genes encoding *PMT* (a, b), *TRI* (c) and *H6H* (d) in endophytic fungi. Lane 1: DNA markers (100 bp ladder), Lane 2: positive control (PCR product of gDNA of *D. metel*), Lanes 3–8 PCR products of Dm 9.3, Dm 10.2, Dm 13.4, Dm 16.3, Dm 17.3 and Dm 18.2, respectively, Lane 9: negative control (-template). Arrows show the amplified products

0.56, respectively (Fig. 2). This confirmed the presence of TAs in the fungal extracts.

The MS/MS spectra of fungal hyoscyamine showed the protonated ion at m/z 290.16 with a minor fragment ion at m/z 124.1 similar with the standard (Fig. 3a, b). The protonated ion of fungal scopolamine showed a very characteristic peak at m/z 304.15 and fragmented ions of m/z 156 and 138 similar to standard (Fig. 3c, d) demonstrating that these fungal endophytes can produce TAs.

The mycelial and broth extracts were analysed by HPLC in order to quantify the amounts of hyoscyamine and scopolamine produced by these fungi. These compounds were detected in the broth, but not in mycelial extracts suggesting that they were released into the medium in liquid cultures. The standard scopolamine eluted with the Rt 4.08 min and hyoscyamine observed with the Rt 6.31 min (Fig. 4a, b) similar peaks corresponding to standards were observed in all the six fungal extracts (Fig. 4c). The maximum amounts of hyoscyamine and scopolamine obtained in the

Fig. 2 TLC analysis of six fungal extracts with standard hyoscyamine and scopolamine. Lane 1: hyoscyamine, Lane 2: scopolamine, Lane 3–8: fungal extracts of Dm 9.3, Dm 10.2, Dm 13.4, Dm 16.3, Dm 17.3 and Dm 18.2, respectively

Fig. 3 ESI-MS/MS of standard hyoscyamine (a), fungal hyoscyamine (b), standard scopolamine (c), fungal scopolamine (d) (*C. incarnatum*)

Fig. 4 HPLC separation and quantification of TAs from fungal extract: **a** standard scopolamine, **b** standard hyoscyamine, **c** fungal hyoscyamine and scopolamine (*C. incarnatum*)

C. incarnatum were 3.9 and 4.1 mg/L, respectively, followed by other five fungi (Table 1).

Discussion

Plants continue to be the sole source for TAs drugs in view of its commercially unviable complex chemical synthesis [12]. *D. metal* is one of the major sources of the hyoscyamine and scopolamine [22]. It is believed that the horizontal transfer of some biosynthetic genes of secondary metabolites from the plant to the fungus occurs during their symbiotic association, resulting in the ability of the fungus to produce host plant-specific compounds. The aim of the present investigation was to search for an alternative and viable source of TAs. This is the first report for the isolation, characterization and identification of TAs-producing endophytic fungi from *D. metal*. The study confirms the presence of *PMT*, *TRI* and *H6H* genes in six endophytic fungi out of 18 fungi by gPCR approaches. Earlier searches reported the production of TAs from endophytic actinomycetes of *D. stramonium* not from

Table 1 Hyoscyamine and scopolamine production by the six fungal isolates by HPLC quantification

S. no.	Endophytic fungal code	Name of the endophytic fungi	Hyoscyamine (mg/L)	Scopolamine (mg/L)
1	Dm 9.3	<i>C. boninense</i>	3.4 ± 0.9 ^c	1.6 ± 0.2 ^b
2	Dm 10.2	<i>Phomopsis</i> sp.	3.0 ± 0.3 ^d	0.5 ± 0.1 ^f
3	Dm 13.4	<i>F. solani</i>	2.6 ± 0.4 ^e	1.0 ± 0.2 ^c
4	Dm 16.3	<i>C. incarnatum</i>	3.9 ± 0.3 ^a	4.1 ± 0.8 ^a
5	Dm 17.2	<i>C. siamense</i>	3.6 ± 1.0 ^b	0.8 ± 0.5 ^d
6	Dm 18.2	<i>C. gloeosporioides</i>	2.2 ± 0.4 ^f	0.7 ± 0.5 ^e

Values sharing a common letter within a column are not significant at $p < 0.05$; values are mean ± SD ($n = 3$)

endophytic fungi [14]. However, the molecular marker based screening of endophytic fungi for taxol production was studied by Xiong et al. [28] supports our findings. Moreover, the high homology of *PMT*, *TRI* and *H6H* genes between fungal and plant origin suggested that the genetic recombination of the endophytes with its host in evolutionary time could have led to the incorporation of the pathway genes into endophytes from its host [26]. In this study, based on molecular and morphological data, the gene positive fungi were identified as *C. boninense* (Dm 9.3), *Phomopsis* sp. (Dm 10.2), *F. solani* (Dm 13.4), *C. incarnatum* (Dm 16.3), *C. siamense* (Dm 17.3) and *C. gloeosporioides* (Dm 18.2), which were determined first time in *D. metel* plant. MNGB media was a very good base for the production of antimicrobial compounds and also for the production of TAs [14].

In the present work, the crude extracts extracted from 3 weeks fungal broth was examined by chromatography and spectroscopy analysis (TLC, HPLC and MS/MS) to confirm the TAs production. The TLC analysis of the methanol extracts showed the presence of TAs and they displayed the chromatographic properties and R_f value similar to standards. The fungal hyoscyamine and scopolamine ions mass fragmentation behaviour from MS/MS exhibited high similarities to standards and this mass ions fragmentation behaviour found to be similar to earlier report [3]. The HPLC analysis confirmed the presence of hyoscyamine and scopolamine with a peak retention time of 4.08 and 6.31, respectively, similar to standards, which indicates these six endophytic fungi are potential for the production of TAs. However, the scopolamine content and scopolamine/hyoscyamine ratio was found to be high in *C. incarnatum*. In contrast, the hyoscyamine levels are higher than the scopolamine in the case of other five fungi, indicating that the *Colletotrichum* species can be potential candidate for TAs commercial production. In recent years, reports mentioning fungal endophytes capable of producing natural products with therapeutic potential similar to their host plant compounds [2, 8, 24]. Some examples include endophytes producing paclitaxel [23], Podophyllotoxin [4] and camptothecin [11]. To meet the commercial need for scopolamine and

hyoscyamine, further work will focus on biotechnological approaches such as strain improvement, genetic manipulation and fermentation engineering.

Conclusion

In conclusion, 18 endophytic fungi were isolated from *D. metel* and 6 were identified as TAs-producing endophytic fungi by screening the 3 key genes of TAs biosynthetic pathway using gPCR. Moreover, the biosynthetic genes of fungal sequences showed the high homology with plant origin. This is the first report of TAs-producing endophytic fungi from *D. metel*. *C. incarnatum* showed the highest yields of hyoscyamine and scopolamine. These endophytic fungi could be the potential candidates to be applied in fermentation technology to meet the demands for TAs economically.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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