

An endophytic fungus, *Gibberella moniliformis* from *Lawsonia inermis* L. produces lawsone, an orange-red pigment

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Abstract Lawsone (2-hydroxy-1, 4-naphthoquinone), also known as hennotannic acid, is an orange red dye used as a popular skin and hair colorant. The dye is produced in the leaves of *Lawsonia inermis* L, often referred to as the “henna” tree. In this study, we report the production of lawsone by an endophytic fungus, *Gibberella moniliformis* isolated from the leaf tissues of *Lawsonia inermis*. The fungus produced the orange-red dye in potato dextrose agar and broth, independent of the host tissue. Presence of lawsone was confirmed spectrometrically using HPLC and ESI–MS/MS analysis. The fragmentation pattern of lawsone was identical to both standard lawsone and that extracted from plant

tissue. This is a first report of lawsone being produced by an endophytic fungus, independent of the host tissue. The study opens up interesting questions on the possible biosynthetic pathway through which lawsone is produced by the fungus.

Keywords Endophytic fungus · *Lawsonia inermis* · Lawsone · *Gibberella moniliformis* · Orange red dye

Introduction

Endophytes, both fungi and bacteria, inhabit living tissues of plants without causing any apparent symptoms (Bandara et al. 2006; Khanam and Chandra 2015). They are ubiquitous and have been recovered from almost all plants examined (Nair and Padmavathi 2014; De Carvalho et al. 2016). A number of studies have documented their ability to confer fitness benefits to their respective host plants, including tolerance to abiotic and biotic stresses (Yang et al. 1994; Yuan et al. 2016). In culture, outside their host tissue, endophytic fungi have been shown to be an important and novel source of natural bioactive products with potential application in agriculture, medicine and food industry (Shweta et al. 2010; Kumara et al. 2012; El-Hawary et al. 2016). They have been reported to produce a number of important secondary metabolites including anticancer, antifungal, antidiabetic and immunosuppressant compounds (Kumara et al. 2012;

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Shweta et al. 2013a, b; Gouda et al. 2016). Interestingly, many of these compounds (such as campothecin, podophyllotoxin, vinblastine, hypericin, diosgenin, azadiracthin, rohitukine) are similar to those produced by their respective host plants (Kumara et al. 2014; Shweta et al. 2014). These latter findings have led to the belief that endophyte could substitute plants as sources of important secondary metabolites (Tan and Zou 2001; Priti et al. 2009). Attempts have been made to enhance production of several secondary metabolites by optimizing the fermentation techniques (Visalakchi and Muthumary 2010; Kushwaha et al. 2014; Venugopalan and Srivastava 2015) that could make the production of the secondary metabolites cost effective (Jia et al. 2014).

Among a range of secondary metabolites, endophytes are also known to produce a variety of pigments. Several endophytic fungi isolated from *Ginkgo biloba* (Cui et al. 2012), *Coptis deltoidea* (Huang et al. 2012) and *Camellia sinensis* (Dongsheng et al. 2009) have been reported to produce pigments in culture. Considering the importance of natural dyes compared to synthetic dyes (Siva 2007; Siva et al. 2012), synthesis of dyes by fungi offers an opportunity to rapidly produce dye throughout the year by optimizing fermentation processes (Jiang et al. 2005).

In this paper, we report the production of lawsone (2-hydroxy-1, 4-naphthoquinone), an orange-red dye, by an endophytic fungi, *Gibberella moniliformis*, isolated from *Lawsonia inermis* L. Lawsone also known as hennotannic acid, is a well known pigment produced naturally in the leaves of *L. inermis* (Krishnamurthy et al. 2002; Siva 2003). The shrub is distributed widely and is native to tropical and subtropical regions of Africa, northern Australia and southern Asia (Sharma et al. 2011). The dye has numerous industrial applications (Heo et al. 2011). The halo oximes of lawsone and other derivatives have also been shown to have anticancer, antibacterial, antiparasitic as well as molluscicidal activity (Zaware et al. 2011; Mahal et al. 2017). Besides, the dye is reported to have antioxidant, antiviral, antidermatophytic, tuberculostatic, cytotoxic, enzyme inhibitory, nematocidal, anti-coagulant and wound healing properties (Brahmeshwari et al. 2012). The fungus produced the orange-red dye in potato dextrose agar and broth, independent of the host tissue. Presence of lawsone was confirmed spectrometrically using HPLC

and ESI-MS/MS analysis. The fragmentation pattern of lawsone was identical to both standard lawsone and that extracted from plant tissue. This is a first report of lawsone being produced by an endophytic fungus, independent of the host tissue. We discuss the results in the light of the wider interest of using endophytes as an alternative source of secondary metabolite. We also discuss the possible biosynthetic pathway that may be adopted by the endophyte to produce the dye.

Materials and methods

Isolation of endophytic fungus from *Lawsonia inermis*

Plant samples of *L. inermis* L. were collected from the Botanical Garden, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India. The tissue samples were washed thoroughly in running tap water to remove surface dust and other contamination and then subsequently dried on blotter disk. The endophytic fungi were isolated from stem, fruit and leaf samples following Shweta et al. (2010).

Leaf, fruit and stem segments (including mid-vein of the lamina) of approximately 0.5 cm² were excised using a sterile blade. The segments were thoroughly washed with distilled water, surface disinfected by immersion in ethanol (70%, v/v) for 5 s, followed by immersion in sodium hypochlorite (4% available chlorine) for 90 s and then rinsed three times for 60 s each with sterile demineralised water. The surface disinfected tissue segments were incubated on petri plates containing potato dextrose agar (PDA) amended with streptomycin (0.1 g/ml) (Taylor et al. 1999; Parthasarathy and Sathiyabama 2014) at 28 ± 2 °C. When the hyphae grew to a length of about 2 cm, single hyphal tips were isolated and sub-cultured on potato dextrose agar (PDA) (HiMedia, Bangalore) and incubated at 28 ± 2 °C. A serial sub-culturing was performed to obtain a pure culture. Fungal isolates were stored in their vegetative form as slants and in 50% (v/v) glycerol at 8 °C.

Based on the emergence of fungi, the per cent colonization frequency was computed as the ratio of the number of tissue segments showing emergence to the total number of tissue segments incubated (Fisher and Petrini 1987). To check for the effectiveness of sterilization, surface impressions of the surface

disinfected tissue segments were made on PDA plates and incubated at 28 ± 2 °C for 96 h. The plates were monitored for fungal growth. To evaluate the pigment production, the pure culture of the isolates was cultured in 250 ml flask containing 150 ml of PDB at 28 ± 2 °C for 15 days.

Morphological and molecular identification of endophytic fungus

Of the 11 isolates, only one isolate was found to produce the orange-red pigment. The identity of this isolate was ascertained based on the culture characteristics, morphology of the fruiting body and characteristics of the conidia/spores following standard mycological manuals (Sutton 1980; Arx 1981; Ellis and Ellis 1985).

The identity of the isolate was further confirmed by sequencing the internal transcribed spacer (ITS) of the nuclear DNA region of the genome. The fungal isolate was cultured in pre-sterilized potato dextrose broth (PDB) at 28 ± 2 °C in a flask and agitated at 250 rpm for 7 days in a rotary shaker. The fungal mycelia were freeze-dried and the genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Vainio et al. 1998). The internal transcribed spacer region was amplified with ITS1 and ITS4 primers following protocols as described previously (White et al. 1990). The PCR conditions used were initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 15 s, 50 °C for 1 min, 72 °C for 45 s, and a final extension at 72 °C for 7 min. The 50 µl reaction mixture contained 1× PCR buffer, dNTP's (200 µM each), 1.5 mM MgCl₂, and 10 pmol of each primer (Sigma Life Sciences), 1–5 ng of DNA and 2.0 units of *Taq* DNA polymerase. The amplified products were sequenced (Chromous Biotech, India) and the sequences were analysed using the Basic Local Alignment Search Tool (BLAST) (Koetschan et al. 2010). The Blast analysis was performed with full length ITS sequence as queries to reveal relationships with published sequences. The sequence with the highest homology, maximum query coverage and maximum score was used as a reference to assign the identity of the endophytic fungus (Higgins et al. 2007). A UPGMA phylogenetic tree was constructed using sequences of the related species obtained from GENBANK in MEGA 6.0.

Extraction of pigment from endophytic fungal mycelia, broth and plant

The endophytic fungal isolate was cultured in liquid media. Single hyphal tip was inoculated into 150 ml of pre-sterilized PDB medium in a 250 ml conical flask. Flasks were incubated in a rotary shaker at 250 rpm at 28 ± 2 °C for 15 days. After 15 days of incubation, the mycelia mass was separated from the broth, washed in sterile water and dried (5% moisture content) in a hot-air oven at 50 °C for 4 days.

The dried mycelial mat was crushed to fine powder in sterile mortar–pestle. About 0.5 g of fine tissue powder was taken in 15 ml vial and extracted with 5 ml volume of methanol (99.7%). The suspension was incubated in hot water bath for 3 h at 50 °C. The extract was then subjected to centrifugation at 10,000 rpm for 10 min and supernatant was collected and concentrated using rotary evaporator at 45 °C for 4 h to get crude extract and stored at 4 °C for further analysis (Dass and Mariappan 2014).

Pigment was extracted from the broth by adding equal amount of chloroform (1:1 v/v) after mixing for 30 min using a separating funnel and then kept for separation. Lower solvent layer was collected and concentrated under vacuum using rotary evaporator at 48 °C and stored at 4 °C for further use (Goswami et al. 2011).

The leaves of the plant were washed in running tap water and kept for drying in an oven at 50 °C for 48 h. The dried leaves were then powdered mechanically in mortar and pestle and subjected to extraction in 100% methanol and kept in water bath at 60 °C for 3 h (Anju et al. 2012; Dass and Mariappan 2014). The extract was centrifuged and the upper solvent layer was collected, filtered with Whatman number 1 filter paper and solvent was evaporated to get crude leaf extract. These were stored at 4 °C until further use (Patil et al. 2010; Suman et al. 2012).

HPLC analysis

The presence of lawsone in the broth, mycelia and plant extract was analysed by reverse phase-HPLC (LC-20AT, Shimadzu, Japan). The conditions used for chromatographic analysis were as follows: Column: Luna C18 (250 mm × 4.6 mm), 5 µm pore size (Phenomenex, USA), Detector: SPD-M 20A photodiode array, oven temperature 35 °C, wave length was

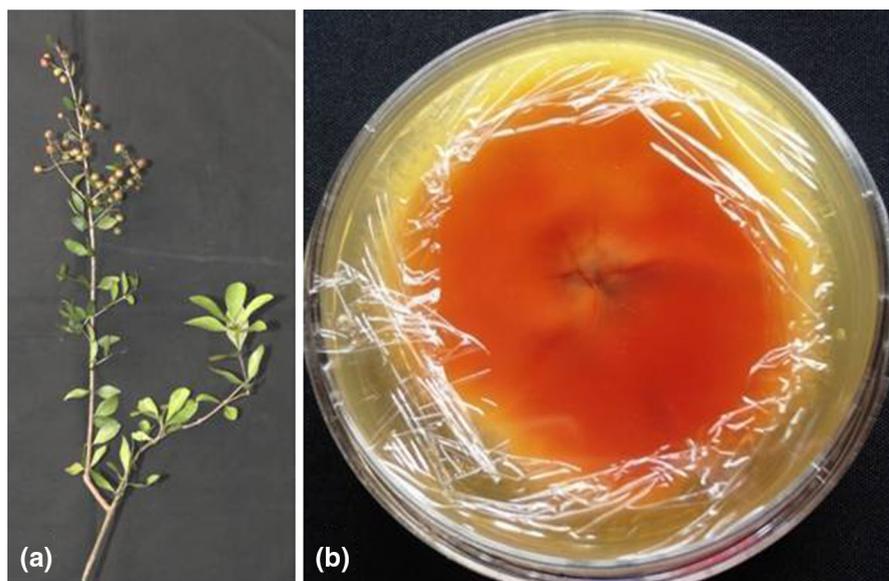


Fig. 1 **a** Twig of *Lawsonia inermis* L. **b** *Gibberella moniliformis* (KX579923) isolated from *Lawsonia inermis* L. showing orange red pigmentation on potato dextrose agar

set at 280 nm. The flow rate was adjusted to 1.5 ml/min with an injection volume of 20 μ l. The mobile phase consisted of 0.1% TFA (Pump A) and HPLC gradient methanol (Pump B) in binary gradient mode with the following ratios of solvents (A:B) 100:0,75:25,68:32,45:55,80:20,100:0 with run times of 0–1,1–5,5–10,10–20,20–35,35–36 min respectively. After 100% B, the system returned to the initial condition within 1 min. The lawsone standard (Sigma-Aldrich, Bangalore, India) was prepared using 100% methanol at a concentration of 1 mg/ml and 20 μ l of the solution was injected into the HPLC system with a total run time of 35 min. The presence of lawsone in the sample was analysed by comparing the retention time with standard lawsone and based on the regression of the standard curve, the amount of lawsone in the samples was calculated (Ei-Shaer et al. 2007).

ESI-MS/MS analysis

The presence of lawsone in the crude extract was further confirmed by ESI-MS/MS analysis. Mass spectra of the samples were obtained under following conditions: Spray solvent used for the analysis was dichloromethanol (HPLC grade). Solvent flow rate at 1 ml/min, nebulizer gas (N₂) pressure 110- psi, damping gas: helium at 10.0 ml/min and 5 kV spray voltage was maintained. ESI-MS/MS measurements

were performed using 3200 Q TRAP LC/MS/MS (Applied Biosystems). Spectra were collected in the range of m/z 50–1500 and data were averaged for 100 scans. MS²/MS³ fragmentation was performed at 25 V collision energy. The fragmentations were compared with those reported in literature and Metlin database (Gan et al. 2015).

Results and discussion

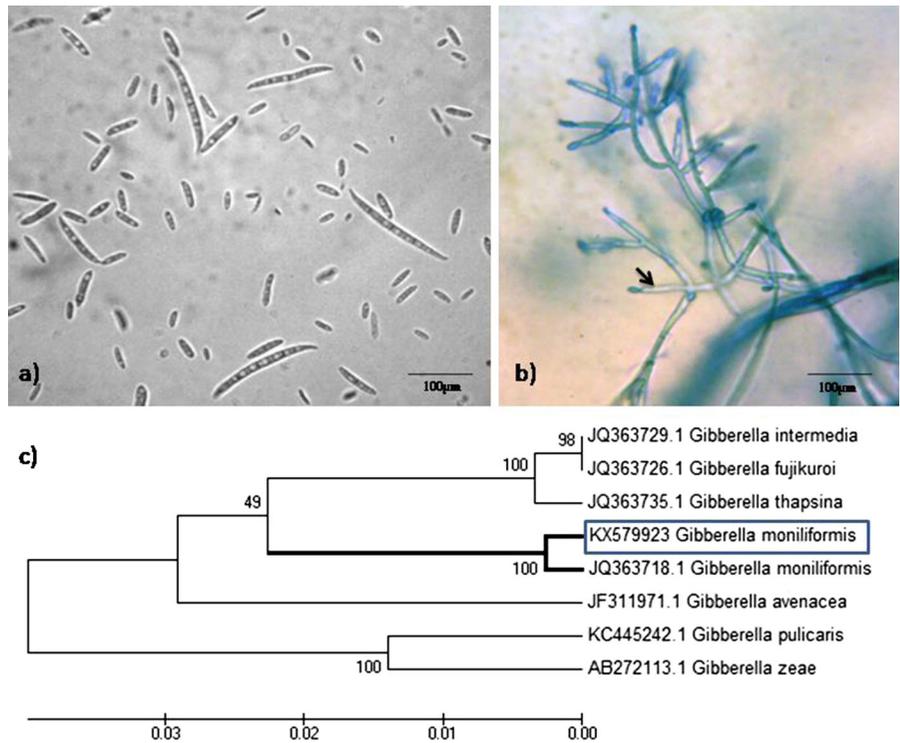
Isolation of endophytic fungi from *Lawsonia inermis*

The fungal mycelium was isolated from cut ends of leaves, fruits and stem. A total of 11 OTU's were isolated and brought to broth culture. The colonization frequency of the endophytic fungi was 28% (from fruits), 60% (from stem) and 77% (from leaves). Among the 11 OTU's, only one, from stem showed pigmentation (orange-red) which was selected for further analysis (Fig. 1).

Morphological and molecular characterisation of the isolated endophyte

Based on the colony and spore characteristics, the fungus was identified as *Gibberella moniliformis*. The

Fig. 2 **a** Microscopic image of micro and macro conidia of *Gibberella moniliformis* (KX579923). **b** Conidiophores with phialides shown in *arrows* (imaging was done using OLYMPUS IX 81 live cell imaging system). **c** UPGMA phylogenetic tree based on ITS rDNA sequence of *Gibberella moniliformis* (KX579923, in *box*) and its closest ITS rDNA matches from Gen Bank. *Numbers* at the nodes indicate bootstrap values. The phylogenetic tree was constructed with bootstrap value of 1000 replicates



fungal colonies were found to grow 5 cm in 5 days on potato dextrose agar. The aerial mycelium of the fungus was dense, collapsing with age, white at first and then turning into orange red. The orange sporodochia was formed as the colony aged. Conidiophores initially arose as single, lateral phialides, sometimes bearing polyphialides producing shorter conidia of irregular shape. Macroconidia were sickle shaped, narrow at both ends. Predominantly 3–7 septate, microconidia were thin walled, one celled ovate, scattered in orange red aerial mycelium. (Fig. 2a, b) (Domsch et al. 1980). The ITS rDNA sequence of the fungus showed 99% similarity in the NCBI database entry for *G. moniliformis* (Fig. 2c). The ITS rDNA sequence was deposited in the NCBI database (KX 579923).

HPLC analysis and ESI–MS/MS analysis

The presence of lawsone in the broth and fungal mycelial culture was confirmed and quantified by HPLC and ESI–MS/MS analysis. The retention time of standard lawsone was 25.48 min with total run time of 35 min. The plant extract, mycelial extract and the broth yielded a peak at 25.48 min comparable to that

of standard lawsone. The HPLC profiles indicated the presence of lawsone in fungal mycelium (0.395%; from 69.2 mg of residue weight), broth filtrate (0.032%; from 17.3 mg of residue weight) and plant extract (0.576%; from 70.5 mg of residue weight) (Fig. 3).

Further, spectroscopic evidence for the identity of the lawsone in the samples was obtained by electron spray mass spectrometry. The ESI–MS/MS analysis of the crude extract of mycelia, broth and plant showed molecular ion signatures in the range of m/z 50–1500 under negative ion mode analysis. The reference lawsone yielded an m/z 173 and fragmented into m/z 145[M+H]⁺. The plant extract, fungal extract and broth also yielded a mass spectrum with the major ion being m/z 173 fragmented into m/z 145. These results clearly indicated the presence of lawsone in the fungal culture grown under in vitro conditions independent of the host. The identity of lawsone in the fungal samples was further confirmed by MS²/MS³ fragmentation. The fragmentation of the ion of standard lawsone m/z 173 yielded m/z 145. Further fragmentation resulted in m/z 101 and m/z 77. All the samples exhibited a molecular adduct in negative ion mode similar to the standard. These fragmentations are exactly similar to

Fig. 3 HPLC profile of lawsone from standard, plant, *G. moniliformis* mycelia and broth

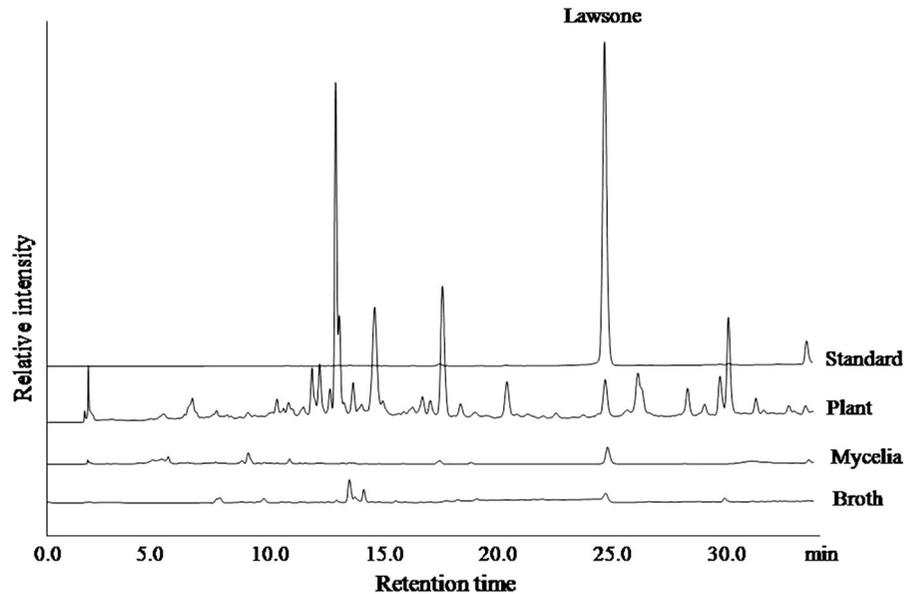
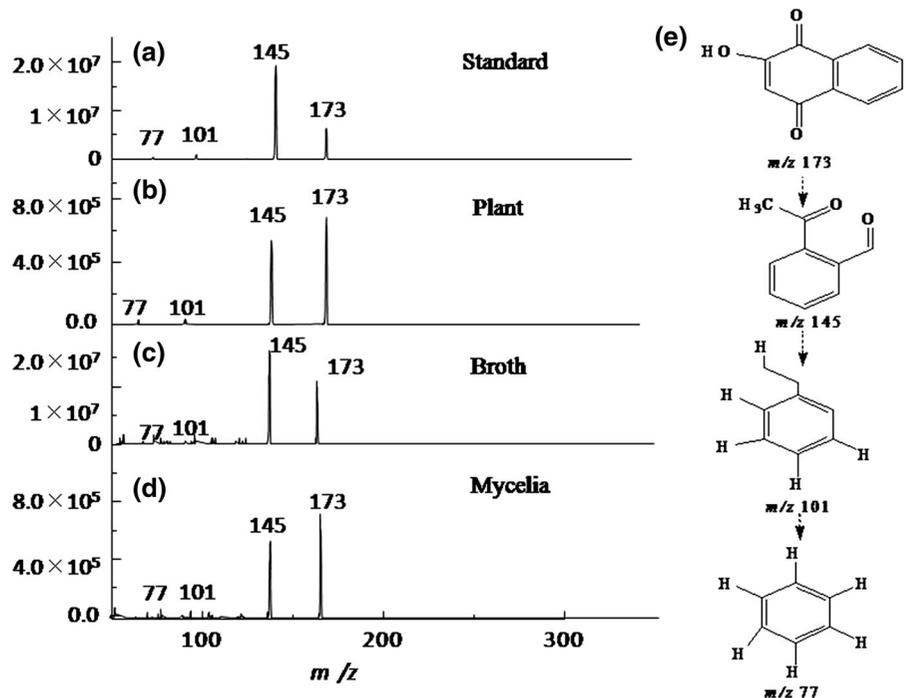


Fig. 4 ESI-MS/MS spectra of **a** Standard, **b** *L. inermis* plant, **c** *G. moniliformis* broth, **d** mycelia, **e** MS/MS fragmentation pattern of lawsone in negative ion mode

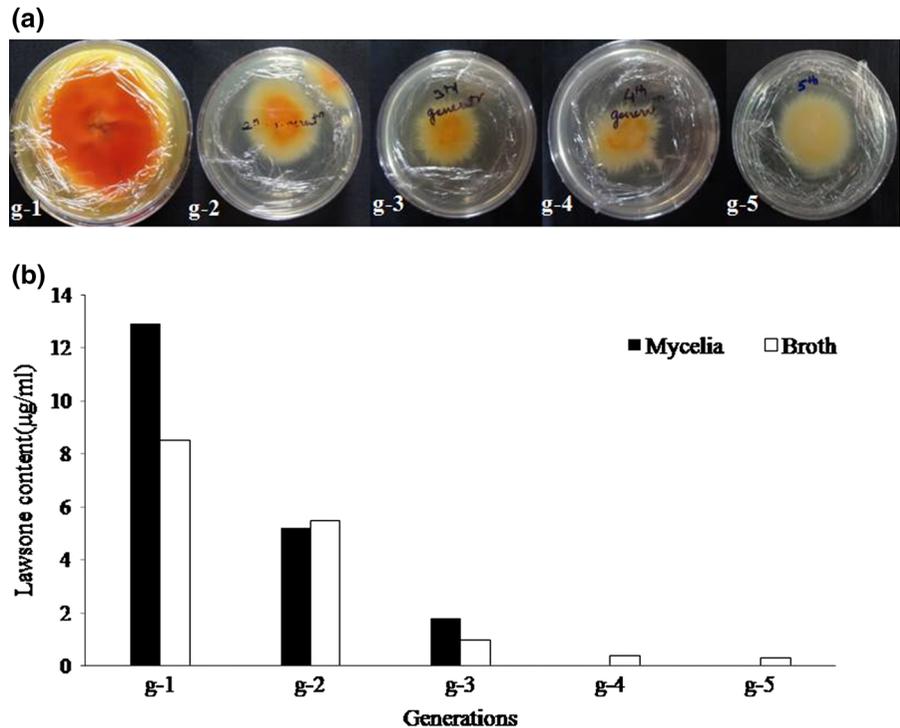


those reported for lawsone in the Metlin metabolite database. (Fig. 4) (Folk et al. 1973; Gan et al. 2015).

This is perhaps the first study to report the production of lawsone by an endophyte isolated from the plant *L. inermis*. Several earlier reports have demonstrated the production of dyes by endophytes in culture. For example, Khanam and Chandra (2015)

isolated an endophytic bacteria which produced bright red pigment from the dye yielding plant *Beta vulgaris*, while the orange coloured compound quercetin glycoside was isolated from fungal endophytes of *Ginkgo biloba* L. (Wang and Liu 2004). However in this study we show that the endophytes associated with the leaves of *L. inermis* were able to independently

Fig. 5 a Attenuation of production of lawsone over subculture generation by *G. moniliformis* on PDA
b Lawsone ($\mu\text{g/ml}$) in fungal mycelial and broth over subculture generation (g1–g5)



produce lawsone. Independent production of lawsone by the fungus raises interesting question of whether the endophytes possess an independent biosynthetic machinery to produce the dye.

Lawsone (2-hydroxy-1,4-naphthoquinone) is a derivative of 1–4 naphthoquinone, produced via the shikimate pathway (Maeda and Dudareva 2012; Widhalm and Rhodes 2016). C^{14} labelled experiments have shown that the quinone portion of the molecule is derived directly from shikimic acid in plants. However in the case of microorganisms, *p*-hydroxybenzoic aldehyde is the precursor for formation of quinone rings (Bohm 1967; Muller and Leistner 1976). A number of naphthoquinone compounds are produced by plants, cyanobacteria, bacteria, filamentous fungi and diatoms (Yoshida et al. 2003; Ikeda et al. 2008; Babula et al. 2009; Shintani et al. 2009). Several of these compounds in plants are produced as a response to plant–microbe interactions as well as plant–plant interaction, perhaps serving as a defense or allelopathic molecule (Fernandez et al. 2016; Zhu et al. 2016). Similarly, in microbes too, the naphthoquinone compounds are produced during microbe–microbe interactions as well as under various stress conditions (Rohrbacher and St-Arnaud 2016). In filamentous fungi, naphthoquinones molecules are found

in the mycelial and sexual fruiting bodies presumably to protect against ultraviolet and desiccation stress and also against insects (Studt et al. 2012).

The production of lawsone by the endophyte was limited to only the first few generations. The production of the dye by *G. moniliformis* attenuated with subsequent sub-culture generations (Fig. 5a, b). Attenuation of host metabolite produced by the fungus or bacteria is well known in microbial literature (Vasanthakumari et al. 2015). More recently, El-Hawary et al. (2016) also reported the attenuation of solamargine production in fungal isolate isolated from *Solanum nigrum*. The attenuation might occur both due to absence of host signals in the axenic culture as well in silencing of the genes responsible for the biosynthesis of the compound (Sachin et al. 2013; Vasanthakumari et al. 2015).

The identification of a new organic source of the dye from the endophyte offers promise in exploiting the fungus as alternative source for lawsone. Further studies are however required to optimise the growth as well as other culture conditions that would not only predispose the fungus in producing higher quantities of the dye but also without any perceptible attenuation in the production of the dye over sub-culture generations.

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