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Mycobiont and Whole Thallus Cultures of *Roccella Montagnei* Bél. For the Biosynthesis of Secondary Compounds

S. Muthukumar, S. Karthik and G.N. Hariharan*

Abstract

The mycobiont and whole thallus cultures of *Roccella montagnei* Bel. were established using soredia as an inoculum.The mycobiont cultures showed optimum growth, biomass and biosynthesis of compounds in Lilly and Barnett medium with glucose as a carbon source, micronutrients and vitamins. After the incubation period of 180 days, the cultures were harvested, and their biomass and secondary compound profiles were analysed. The HPTLC chromatogram of the acetone extract of the NT and mycobiont cultures revealed erythrin as the major biosynthesized compound in both and identified as a key biosynthate by *R. montagnei.* Further, the NT biosynthesized 5 additional compounds and the mycobiont cultures biosynthesized 6 additional compounds. The molecular identity of the cultured mycobiont was confirmed using nuclear ribosomal Internal Transcribed Spacer (ITS) as well as the secondary chemistry. Lichen compound erythrin was identified as a key biosynthate by the cultures.

Key word:-*Roccella montagnei,* Mycobiont, Whole thallus culture, Soredia, Erythrin.

Abbreviation: - NT, Natural thallus; MYC, Mycobiont culture; WTC, Whole thallus culture; UV, Ultra violet wave length; HPTLC, High performance thin layer chromatography; ITS, Internal transcribed spacer.

Introduction

The natural thallus (NT) of the lichen *R. montagnei* (Roccellaceae) was used as a source of a natural dye for colouring the Royal fabrics during historical times and for the preparation of litmus paper until recently (Nash 2008; Muggia *et al,* 2009) and hence extensively collected. The orcinol group of secondary compounds in the NT of this lichen was found important as a dying agent. Further the natural thallus extracts of *R. montagnei* showed anti-pest activity against *Helicoverpa armigera* (Balaji *et al*, 2007) and antibacterial and antifungal activity against many of human pathogenic microorganisms (Balaji *et al*, 2006). Currently the interests on natural dyes from lichens are reviving and the natural thallus of *R. montagnei* is considered as a potential resource (Upreti *et al*, 2010 and Shukla and Upreti 2014). It is well known, that lichens grow extremely slow and produce low biomass in nature and large scale harvesting of the natural thallus of lichens will lead to loss of species as well as critically affect many its ecological functions.

Establishing lichen cultures for the biosynthesis of secondary compounds is an emerging and challenging area of research. There is no universal general culture recipe for the establishment of lichen cultures unlike common fungal and bacterial culture media. Lichen culture initiation is considered challenging since each lichen species as well as its constituent bionts show extreme preferences to culture media compositions specifically the carbon sources, inorganic salts, vitamins, amino acids and pH of the media (Karthik *et al*, 2016). Factors such as incubation temperature, RH, light and dark cycles also known to influence lichen culture initiation and its growth. Studies have established culture protocol for each species, based on the success of initiation, further growth leading to biomass (Molina *et al*, 2015) and synthesis of compounds in a specific medium after attempting various media compositions (Stocker Worgotter *et al*, 2009). These studies have used a range of media from low nutrient containing basal medium to complex and nutrient rich media (MY, LB, MS, BBM, PDA and SAB with modifications). Crittenden *et al* (1995), Sangvichien *et al,* (2011), and Mc Donald *et al,* (2012) has established culture of large number of mycobiont cultures (MYC) and

Lichen Ecology and Bioprospecting Laboratory, M.S. Swaminathan Research Foundation,
Taramani, Chennai 600 113, India.
*E-mail : gnhariharan@mssrf.res.in

"Valarmathi and Hariharan (2007)," "Fazio *et al*. (2012), Stocker Worgotter (2015) have demonstrated the initiation, growth and the synthesis of secondary compounds by select lichen species.

In general, the initiation of MYCs of fertile lichens can be achieved through ascospores derived cultures, but this method is not suitable for sterile lichens and for the initiation of WTCs. Hence thallus maceration cultures or asexual propagules were used as an inoculum for culturing whole thallus of sterile lichens. However thallus maceration protocols often result in heavy contamination by endolichenic fungi and other microbes present in the NT, which can rapidly establish in the culture plates prior to the growth of mycobiont (Mc Donald *et al*, 2012) effective protocols were also provided by Yamamoto et al. (1985), Fazio et al. (2009) to avoid contamination. Valarmathi and Hariharan (2007) has established WTC of *Dirinaria applanata* using soredia as an inoculum, and Stocker Worgotter and Turk R (1989) has established the MYC of *Peltigera didactyla* using soredia. In the above background, this paper reports the attempts made for culturing the mycobiont and whole thallus of *R. montagnei* using soredia as an inoculum under different media compositions. As a proof of identity of the mycobiont, the genetic similarity of the initiated MYCs were compared with that the NT using the conserved Internal transcribed spacer (ITS) region; and the secondary compound profiles of cultured and NT were compared for specific key biosynthates using High performance thin layer chromatography (HPTLC).ve

Material and Methods

Lichen sample collection

The specimens of *R. montagnei* was collected from Tamil Nadu, Kanchipuram District, Vadanemmeli village, lat.12°44'08.9"N, long.80°14' 04.4"E, alt. 6m msl on the bark of *Borassus flabellifer by* S. Muthukumar, S. Karthik and G.N. Hariharan, on 30.01.2014. One of the collected specimen has been preserved as a voucher and cited as MSSRF/Rm/Herb/15/2014. The species was identified based on morphological characters (D.D. Awasthi, 2007)" and secondary chemistry. Soon after collection, the thalli were wrapped in dry tissue paper. These samples were stored at 4°C in refrigerator in the laboratory.

Isolation and culturing mycobiont and whole thallus culture

The thalli were washed in running tap water for 1 hour and then treated with Tween 80 (2%) surfactant for 5 min, and the surfactant was removed by washing 20 times in double distilled water. Using a dissection zoom microscope, young soredia were dissected out with pointed needle and used as inoculum (Yoshimura, 2002) in to each 30 plates of Lilly Barnett medium (LB) (Lilly and Barnett, 1951), Malt Yeast Extract medium (MY) and Modified Murashige Skoog medium (MMS) (Valarmathi and Hariharan, 2007). The chemicals used in the media preparations were supplied by Hi Media laboratories Pvt. Ltd. Mumbai.

The inoculated plates (15 nos. of each medium) were incubated for 48 hrs in dark at 22-24°C under 70–80% relative humidity for mycobiont initiation. The remaining culture plates were incubated in 12/12 hr day and night cycle with similar temperature and RH regimen for the initiation of WTCs. All the inoculated plates were observed using an inverted microscope (Zeiss Primovert) between 20 days interval and initiation and growth stages were recorded. The initiated MYC and WTCs were subcultured into the same medium and plates that continued to grow in subculture were recorded as successfully established.

HPTLC of secondary metabolites of natural thallus and mycobiont culture of *R. montagnei*

The secondary compound profile of NT and the MYCs were analysed using HPTLC (Arup *et al,* 1993) (CAMAG, Switzerland). The acetone extract of the NT and MYCs (180 days old, harvested and dried) were analysed for its secondary compounds using standard lichen solvent system G (Toluene/Ethyl acetate/Formic acid 139/83/8) (Orange *et al,* 2001). The chromatogram prior as well as after its derivatization with 10% H_2SO_4 were viewed in visible light, UV 254 and 366 nm and scanned using UV 254 densitometry for secondary compound spots detection, its colour and Rf class. Compounds were identified based on Orange *et al*, (2001) using the Rf value, spot colour under UV 254 and derivatization with 10% aqueous sulphuric acid.

Isolation and characterization of Erythrin for using it as a standard

The NT of *R. montagnei* (30 gram) was air dried and soaked in acetone for 48 hours. The condensed acetone extract yielded 2.1 gram of dry weight. Column chromatography of this extract using silica gel (60-120 mesh) with the solvent gradient from *n*-hexane to EtOAc (100 to 10:20) were eluted, condensed and analysed using TLC, and the similar fractions were pooled and concentrated. The erythin containing fraction was purified and subjected to spectroscopic analysis (UV, FT-IR, ^{1}H and ^{13}C NMR with DEPT (Distortion less enhancement by polarization transfer), and the molecule was characterized as C_{20} $H_{22}O_{11}$ (438), [3-hydroxy-5-methyl-4-((2,3,4-

trihydroxybutoxy) carbonyl) phenyl 2,4-dihydroxy-6-methybenzoate] which is known as Erythrin. This molecule was used as a standard to detect the synthesis of erythrin by the MYC.

Molecular identity of the natural thallus and mycobiont culture

The identity of the mycobiont from culture was confirmed by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region. The genomic DNA was isolated from NT and MYC of *R. montagnei* using the CTAB method (Gargas and Taylor, 1992). The PCR amplification of the ITS region was performed in a reaction volume of 25 µL with 20 ng of genomic DNA, 1 µL of each of the primers viz. ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al. 1990) with 2.5 mM total dNTPs, 1X *Taq* polymerase buffer and 1U *Taq* polymerase. The PCR amplification conditions included an initial denaturation at 95° C (5min), 25 cycles each of denaturation at 95°C (1 min), annealing at 54°C (1 min), extension at 72°C (1 min) and a final extension step at 72°C (7 min). The obtained PCR product was cloned in a T/A vector (MBI Fermentas) and sequenced using M13 forward and reverse universal primers in an automated sequencer ABI Prism 3130 Applied Biosystems. Sequences were blasted against GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the methodology (Sayers et al. 2011) to approach the identity as *R. montagnei*.

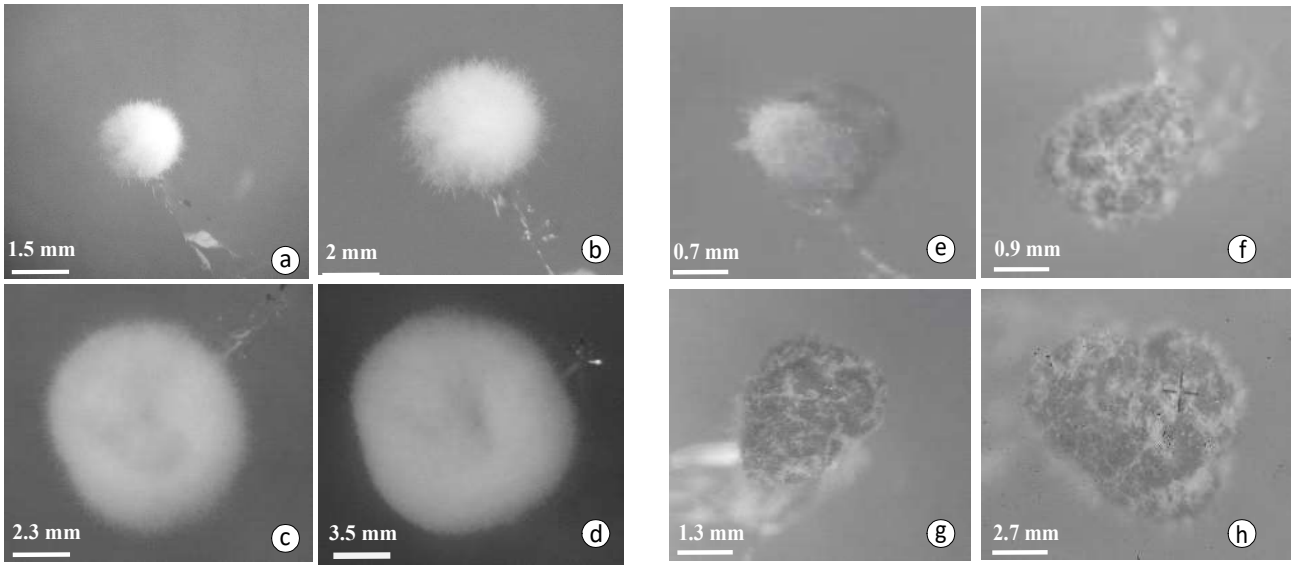
Results

Mycobiont and whole thallus culture initiation and its development

The MYC showed initiation in 8 out of 15 plates in LB medium and 2 out of 15 plates in MMS medium. The remaining plates were contaminated with endolichenic fungi and bacteria. There was no initiation of mycobiont in the plates with MY medium up to 60 days and these plates were also devoid of any contamination. The cultures initiated in MMS were subcultured on 20th day in the same medium and showed no growth thereafter. After 2-3 weeks the initiated MYCs in LB medium attained a circular growth with 1-2 mm in diameter on 20th day (Fig.1a) it was subcultured in 50 plates using the same medium. All the 50 plates showed growth of the mycobiont.

It was observed that the subcultured mycobiont on the 20thday attained a circular shape with a diameter of 2±0.3 mm and appeared as a white, cottony stage and continued to grow up to 50th day (Fig.1b). These cultures between 50th - 90th days turned pale white and appeared as compact mycelial colony, and measured 2.3±0.2 mm (Fig.1c) in diameter and after 180 days (Fig.1d) the culture were harvested for secondary compound analysis.

The initiated WTC on the 20th day attained a diameter of 0.7±0.1 mm and appeared as greenish gel drop with white mycelial growth (Fig. 1e). On the 50th day the cultures



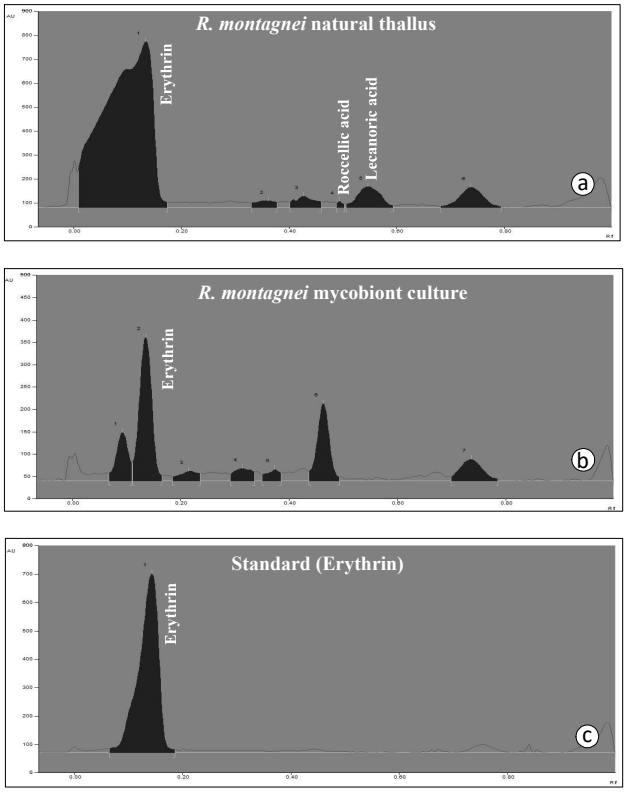
a. Initiation of mycobiont culture in LB medium on 20th day
b. Cottony growth stage of mycobiont culture on the 50th day
c. Compact mycelial stage of mycobiont culture at 90th day
d. Mycobiont culture at 180th day
e. Initiation of whole thallus culture in LB medium on 20thday
f. Cottony with photobiont combined stage of mycobiont on 50th day
g. Greenish white maculate clump stage at 90th day
h. Whole thallus culture on 150th day

Fig. 1. Growth stages of mycobiont and whole thallus cultures of *R. montagnei*

showed cottony growth with green and white streaks (Fig.1f). On 90th day, it appeared greenish white (maculate) clump and attained a diameter of 1.7±0.3 mm (Fig.1g) and on 150th day the cultures showed increment in diameter (2.5-2.7 mm). After this stage, there was no further growth and development observed in these cultures (Fig.1h). The photobiont was identified as *Trentipholia sp*.

HPTLC analysis of natural thallus and mycobiont culture

The secondary compound profiles of the acetone extract of the NT and MYC using HPTLC Densitograms (Fig.2, a,b,c) under UV 254 nm (Fig.3) indicated that the NT and MYC synthesized erythrin as major compound. Further, the NT biosynthesized 5 additional compounds and the MYC biosynthesized 6 additional compounds (180-days old in LB medium).



a. Densitogram of biosynthesized compounds by *R. montagnei* natural thallus
b. HPTLC densitogram of biosynthesized compounds by *R. montagnei* mycobiont culture
c. HPTLC densitogram of Standard (Erythrin)

Fig. 2. HPTLC densitogram of biosynthesized compounds by *R. montagnei* natural thallus, mycobiont culture and Standard (Erythrin)

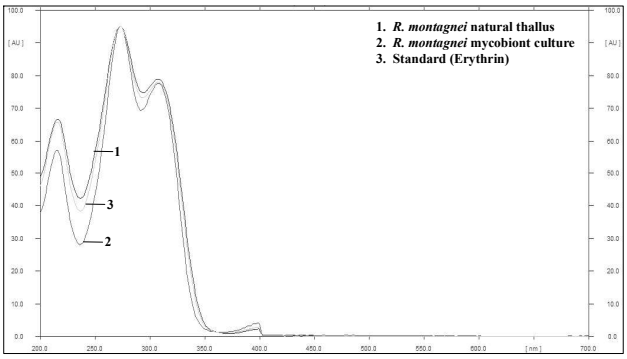


Fig.3. HPTLC spectral comparison of Erythrin between *R. montagnei* natural thallus, mycobiont culture and standard (Erythrin)

Molecular identity of natural thallus and mycobiont culture

The molecular identity of the MYC was confirmed by sequencing its ITS region with that of NT. The NCBI blast analysis showed 99% similarity between both the ITS sequences of *R. montagnei.* The sequences were submitted to NCBI (KU530114 & KU530115).

Discussion

The NT of *R. montagnei* due to its secondary compounds serve as a key source of dye, litmus paper and also exhibit antimicrobial and antifungal properties. It is extensively collected and hence it is essential to establish cultures protocols. Crittenden *et al,* (1995) attempted culturing of 7 species of *Roccella* in a defined medium using thallus fragments and spore germination method and indicated that both fragments and ascospores failed to grow. Stocker-Wörgötter (2015) has cultured *R. decipiens* in MS medium and shown the biosynthesis of erythrin, lecanoric and orsellinic acid. In this study, both MYC and WTC were established using soredia as inoculum. The MYC showed the biosynthesis of 7 secondary compounds among them erythrin as major substance.

The initiation of *R. montagnei* MYC and WTC cultures found challenging because of the overgrowth of contaminating fungi from thallus fragments and mature soredia. However, initiations of cultures from young soredia as inoculum were successful due to lesser contamination. This provides an option to establish WTC and MYCs in the absence of ascomata as well as in conditions where thallus fragments failed to establish (Valarmathi and Hariharan 2007). In this study, culture media viz MY, LB and MMS were used, the WTC and MYC were initiated in LB and MMS media, indicating that this fungus initiate in complex media with readily available

inorganic nutrients. In addition the growth of fungus in two media out of the three tried, iterate that lichen cultures can be achieved only through trial and error basis with different media compositions as indicated by many studies (Crittenden *et al*, 1995; Hamada *et al,* 1996; Mc Donald *et al*, 2012; Kinoshita *et al*, 2015).

The earlier lichen culture studies were focusing on the re-synthesis of lichen cultures from isolated bionts (Ahamadjian 1970). In late 1990s various researchers, had reported the detection of the biosynthesized known and novel secondary compounds by the symbiotic or aposymbiotically grown MYCs. These studies had indicated that the lichen cultures synthesize the secondary compounds under different carbon sources as well as under varying concentration of a specific carbon source (Hamada *et al*, 1996). Stocker-Wörgötter (2015) has indicated that the 5 month old cultures of *R. decipiens* in the MS medium produced erythrin alone and the 6 month old culturesin MS medium with 6% mannitol and 3% erythritol has quantified that 1gm of culture synthesized erythrin (40 mg), orsellinic acid (16 mg) and lecanoric acid (30 mg). However in the present study, we have used glucose as a carbon source and 6 month old MYC of *R. montagnei* biosynthesized erythrin as major substance along with 6 unidentified spots. It was observed that in many lichen culture experiments, glucose has been used as a preferred carbon source, since it is cheaper compared to other carbon sources and it is easily assimilated and metabolized - a necessity to initiate the pathways for secondary metabolite biosynthesis (Stocker-Wörgötter (2008).

Rao and Seshadri (1940) indicated that whenever the compound erythrin was found as a major substance, erythritol was obtained in traces and lecanoric and roccellic acids were absent or in traces in the NT of *R. montagnei*. Similar pattern was also observed in our study that we have isolated erythrin as a major substance and the remaining compounds were found in traces in NT. Further, in MYC erythrin was detected as a major distinct spot and the remaining spots were in traces.

In this study, the cultures were initiated in Lilly & Barnett medium (1951) without any modification, thus providing an option for the synthesis of secondary compounds of *R. montagnei* for suitable applications *in vitro* and hence there is a possibility to conserve the NT of this lichen.

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