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Soredial culture of *Dirinaria applanata* (Fée) Awasthi: Observations on developmental stages and compound production

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Abstract

We established soredial cultures of the lichen *Dirinaria applanata* under controlled laboratory conditions using soredia and made observations on development, biomass and compound production. The successive developmental stages in culture, observed using light microscopy and macro photography, were the initial contact stage on the 4th–6th day, arachnoidal shape on 15th day, white cottony on the 20th day, radial thallus on the 27th day, lobe formation on the 30th–35th day and soredia like formations on the 73rd day. The cultured thalli resembled the natural thalli (foliose-like sub orbicular growth form) by the 27th day of culture with a biomass of 12.9 mg. Thin layer chromatography analyses on the 55th day of culture showed the presence of atranorin and divaricatic acid, and on the 73rd day, in addition to the above compounds, sekikaic acid and two unknown compounds were also observed.

Keywords: Lichen culture, soredia, developmental stages, atranorin

1. Introduction

The interest on lichen secondary compounds is increasing since they exert a wide array of biological activity (Oksanen, 2006). The slow growth rate and adpressed growth forms of lichen in nature make it difficult to harvest large-scale biomass to extract these compounds, and such extractions are likely to conflict with conservation interests. Hence the establishment of lichen cultures is considered basic for the production of secondary compounds (Crittenden et al., 1995).

The antimicrobial potentialities of the secondary compounds of the sorediate lichen *Dirinaria applanata* (Fée) Awasthi (Awasthi, 1975) against human bacterial pathogens, were established using TLC Bioautographic overlay assay (Gibbons and Gray, 1998) by the authors (unpublished results). Hence there is need to culture *D. applanata* for compound production. Crittenden et al. (1995) attempted to culture five species of *Dirinaria* through ascospore and thallus fragments and isolated the mycobionts of *D. picta* and *D. applanata* with limited success. Behera et al. (2002) demonstrated secondary compound production using whole thallus cultures of *Bulbothrix setschwanensis*, and the developmental stages and thallus formation of several lichen taxa have been successfully achieved using soredia (Stocker-Wörgötter and Turk, 1988; Schuster et al., 1985). In the present study, soredia were used as inoculum to culture *D. applanata*, and observations were made on the developmental stages, biomass and secondary compound production.

2. Materials and Methods

The thalli of *D. applanata* with well-formed soredia and producing atranorin (a β orcinol para-depside) and divaricatic acid (a orcinol para-depside) (Awasthi, 1975; Orange et al., 2001) growing under natural conditions were used for this study. The lichen was collected at Tamil Nadu, Kanchipuram District, Vadanemmeli (latitude: N 12°44' 08.9", longitude: E 80°14' 04.4") alt. 6 m msl, on bark of *Borassus flabellifer*. A part of the material has been preserved as voucher specimen and cited as 15.08.2005 G.N. Hariharan, P. Balaji and R. Valarmathi (MSSRF/Herb/DIRa/902/05).

Soredial culture

Lichen cultures were started within 7 days of collection.

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Table 1. The composition of culture media used to culture lichen soredia. Amounts are in g/l of medium which was solidified with 1.8% w/v agar.

Composition	Medium for soredial culture initiation	Medium for soredial culture establishment, rapid growth and secondary compound production	
	Malt Yeast Agar medium (MY)	Modified Bolds Basal medium (4% MBBM)	Murashige and Skoogs medium (modified from Stenroos et al., 2003) (4% MMS)
Malt extract	10	Nil	0.5%
Yeast extract	4	Nil	0.5%
Sucrose	Nil or 40 (4%)	40 (4%)	40 (4%)
Micronutrients	Nil	$NaNO_3$ - 3.0 $MgSO_4.7H_2O$ - 1.5 KH_2PO_4 - 2.0 $CaCl_2$ - 0.1 K_2HPO_4 - 3.0 NaCl - 0.1	NH_4NO_3 - 165.00 KNO_3 - 190.00 $MgSO_4.7H_2O$ - 37.0 $MnSO_4.H_2O$ - 1.69 $ZnSO_4.7H_2O$ - 0.86 $CuSO_4.5H_2O$ - 0.0025 $CaCl_2.2H_2O$ - 44.0
Trace elements	Nil	H_3BO_3 - 11.42 $FeSO_4.7H_2O$ - 0.82 $ZnSO_4.7H_2O$ - 0.40 $MnCl_2.4H_2O$ - 0.43 $(NH_4)_6MoO_{24}.4H_2O$ - 0.20 $CuSO_4.5H_2O$ - 1.57 $Co(NO_3)_3.6H_2O$ - 0.98 EDTA - 15.0 KOH - 3.10	KI - 0.083 $CoCl_2\ 2H_2O$ - 0.0025 KH_2PO_4 - 17.0 H_3BO_3 - 0.62 $Na_2\ MoO_4\ 2H_2O$ - 0.025 $FeSO_4\ 7H_2O$ - 2.784 Na_2 EDTA - 3.724
Vitamins and aminoacids	Nil	Nil	Thiamine HCL - 0.10 Nicotinic acid - 0.05 Pyridoxine HCL - 0.05 Glycine - 0.2

Healthy sorediate thalli were selected under dissection zoom microscope. The thalli were washed in running tap water for 2 hours and then treated with a surfactant Tween 80 (2%) for 5 min, and the surfactant was removed by washing 30 times in double distilled water. Using a dissection zoom microscope, young soredia still attached to the soralium were scooped out, with inoculation needles and used as inoculum. Replicates consisting of 150 soredial inocula were initiated on MY medium (Table 1). These cultures were incubated for 48 hrs in the dark for reduced contamination and rapid initiation of the mycobiont of *D. applanata*. Subsequently, these cultures were incubated in light dark cycle consisting of a 12 hr day (with a light intensity of 50–100 µmol m^{-2} s^{-1} PPFD) and a 12 hr night at 22–28°C under 70–80% RH. The *D. applanata* cultures attained the arachnoidal shape in 10–15 days. The cultures were sub-cultured onto MY medium supplemented with 4% w/v sucrose and MBBM and MMS media also with this level of sugar (Table 1). The aim was to enhance the rapid and synchronized growth of the bionts and secondary compound production. The pH of all media was adjusted to 6.6 with 1 N HCl and 1 N NaOH and 1.8% agar was employed to solidify the media. Mycobiont cultures were established by incubating soredia in MY medium and keeping these continuously in the dark for up to four months at 22–28°C and 70–80% RH.

Lichen symbionts (mycobiont and photobiont) are usually difficult to initiate on media containing 4% w/v sucrose as they attract heavy contamination (Yoshimura et al., 2002). For this reason, initially MY without sugar was used, however it often results in weak and superficial growth of the bionts as time progresses. So transfer of cultures to a medium containing carbon nitrogen and micronutrients was made to support the rapid and synchronized growth of bionts (Molina and Crespo, 2000).

The initial stages of lichenization were observed under an inverted microscope, and the cell density was measured using a haemocytometer. The radial increment of culture and biomass production was measured at 7 day intervals. The developmental stages of the cultures were recorded under dissection microscope and photographed. Transverse sections of the cultured and natural thallus were prepared using a Leica cryomicrotome and photographed.

Secondary compound extraction and identification

The secondary compounds produced by the cultures were determined by Thin Layer Chromatography (TLC),

using standard solvent systems and micro crystallization (Orange et al., 2001). Samples of atranorin and divaricatic acid were provided by Prof. Veranja Karunaratne, Department of Chemistry, University of Peradeniya, Sri Lanka, and used as standards in the TLC analysis.

The atranorin content of the cultures were quantified from the 55th day onwards, using 2 mg of the harvested and dried culture mass by 2,4-dinitrophenylhydrazone method (Jordan and Veatch, 1964). The atranorin content of this mass was separated using preparative TLC (Gibbons and Gray, 1998), and 1.0 ml of a KOH-EtOH solution (KOH : H_2O : EtOH 2.8 g : 150 ml : 100 ml) and 2 ml of 2,4-dinitrophenylhydrazine were added to produce a wine-red solution. After an incubation time of 30 min at 25±3°C, the absorption was measured with a spectrophotometer at 470 nm, and the atranorin content was quantified.

3. Results

Initiation, growth and structure of cultured lichens

Smear preparations of the soredia, prior to inoculation, showed a dense aggregation of photobiont cells and mycobiont mycelium in a completely lichenized state (Fig. 1). The inoculum appeared greenish due to the greater number of variously sized photobiont cells (61.5×10^6 cells of photobiont) and smaller amount of mycelium. After an initial 48 hrs of incubation of all the cultures in MY in dark, 22–28°C and 70–80% RH, the association between the bionts seemed to disintegrate (Fig. 2). The cells of photobiont (*Trebouxia* sp.) became free and appeared light greenish and there was a reduction in the number of photobiont cells (40×10^6 cells of photobiont) observed. The initiation of new fungal hyphae was noted after a lag phase of 2 days (48 hrs) and the fungal hyphae established contacts with photobiont. In some of the hyphae, originating from the soredial inoculum, the terminal cell appeared obpyriform to ampulliform in shape. Between 72–96 hrs, photobiont cells were found in a mycelial mesh, and a few photobiont cells were found in contact with the hyphae. Light microscopic images revealed that the hyphae penetrate the photobiont's cell wall and reach the cytoplasm (Fig. 3).

The cultures acquired an arachnoidal shape at 10 days (Fig. 4), after which time they were transferred to 4% MMS secondary medium. After this transfer, on the 15th day, a rapid growth of mycobiont occurred, and the mycelial growth completely covered the photobiont cells, appeared cottony, and acquired a ≅ radial shape after 20 days (Fig. 5). At this stage, the mycobiont established haustorial connections with a few cells of photobiont, and the developing thallus showed radiations at the margin. The ridges between two furrows appeared similar to the lacinae of the natural thallus of *D. applanata* on 30th–35th day (Fig. 6).

The upper cortex of the cultures on the 73rd day showed several minute openings at the centre of the thallus, exposing the algal layer (Fig. 7), which resembled primordia of soredia as minute verucae and burst open to produce white farinose soredia similar to natural thallus. A transverse section (T.S.) of this region indicated an accumulation of photobiont cells compared to the fewer photobiont cells in other portions of the thallus. The cultures of *D. applanata* on 4% (sucrose) MMS produced 230.5 mg biomass and showed a radial increment of 16.35 cm on the 153rd day. The transverse sections of the thallus on the 73-day-old culture showed differentiation of cortex, photobiont layer, medulla and lower cortex (Fig. 8) and resembled the transverse section of the natural thallus of *D. applanata* (Fig. 9).

Secondary metabolite production by cultured thalli

The production of secondary compounds was observed after the 55th day of culture. The thin layer chromatography analyses on the 55th day using harvested cultured biomass showed the presence of atranorin and divaricatic acid. On the 73rd day in addition to the above compounds, sekikaic acid and two unknown compounds (Rf class 4 and 5, yellowish orange) were also observed (Fig. 10). This confirmed by the microcrystallization method using GE and GAW. These compounds are apparently not produced by the natural lichen growing in our collecting localities, at least at the concentrations detected in the cultures. The mean concentration of atranorin on the 55th day was 0.18 µg/2 mg dw of the harvested cultured thalli and on the 111th day the mean atranorin concentration was measured as 3.61 µg/2 mg dw.

Mycobiont culture

The mycobiont cultures incubated in continuous darkness showed initial stages of development similar to those of soredial cultures incubated in a light and dark cycle. After 35 days, the cultures showed the formation of a few ridges; the ridges radiated towards the center, and even after four months of dark incubation, the ridges were not differentiated into the lobes that were observed in soredial cultures. Thin layer chromatographic analyses of extracts from the mycobiont cultures did not show any secondary compounds.

4. Discussion

The successive developmental stages in cultures of vegetatively reproducing *D. applanata* with green

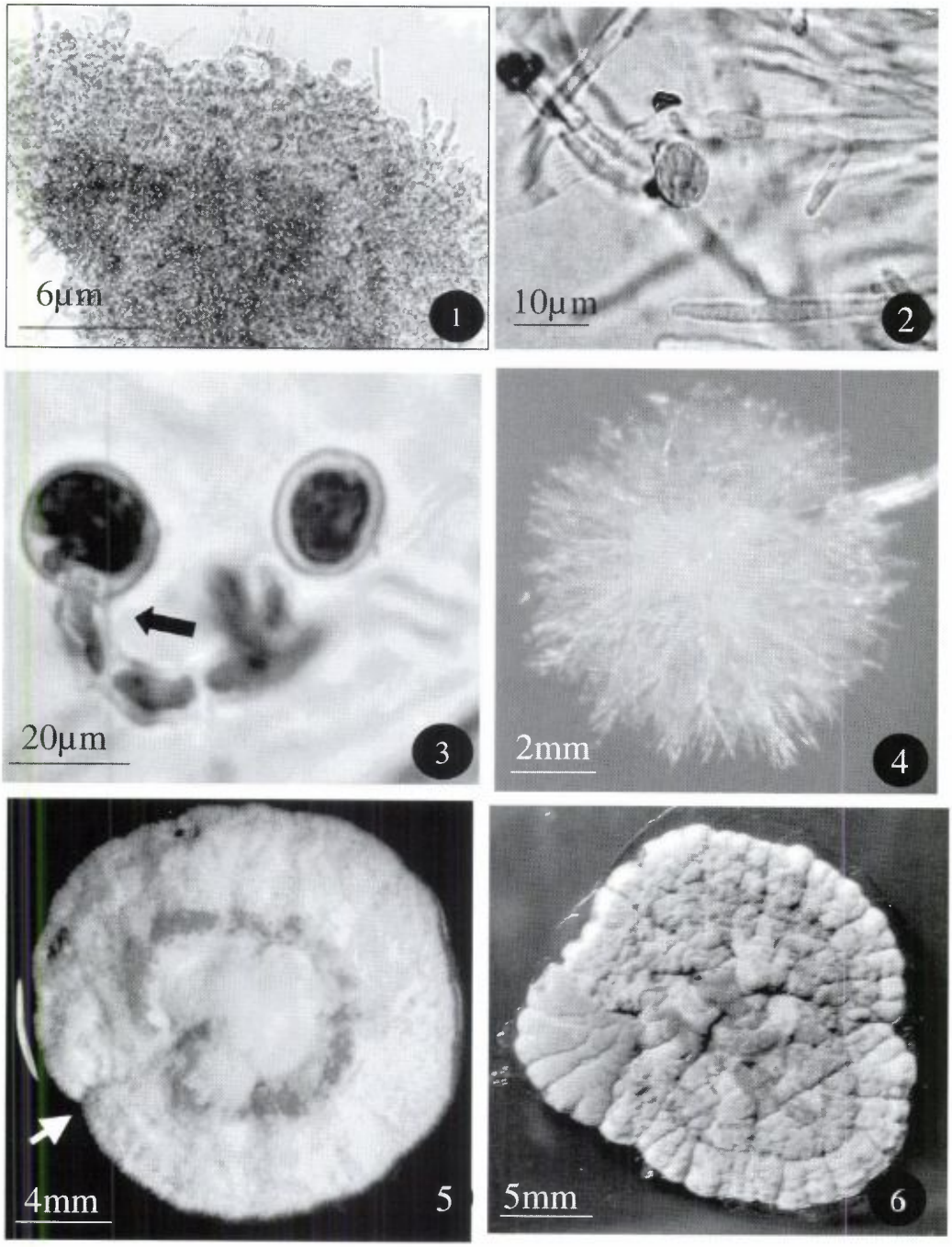
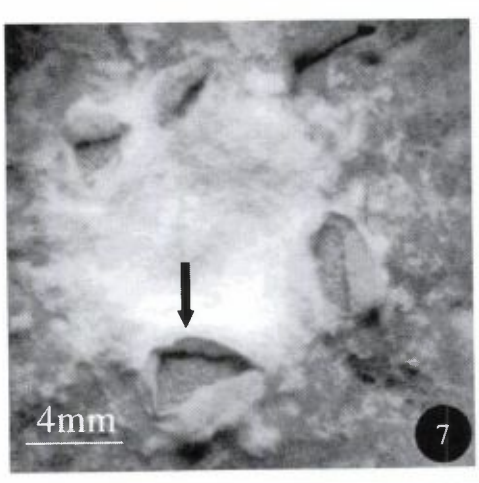
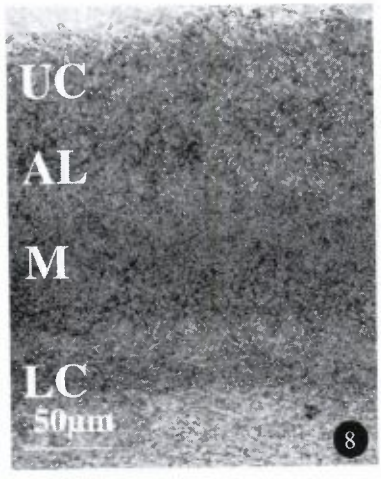
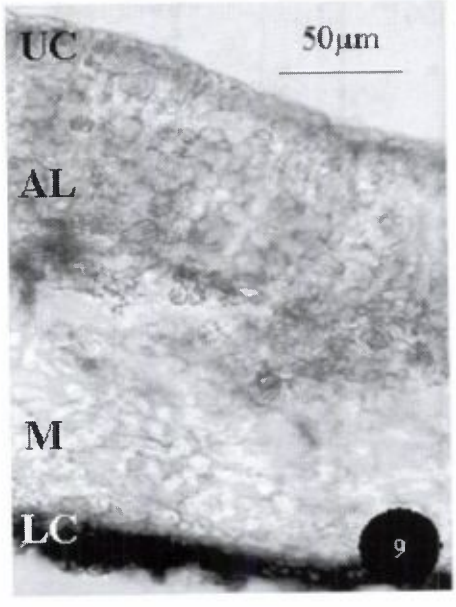


Figure 1. Appearance of the soredial inoculum.
Figure 2. Disintegrated bionts after 48 hrs of dark incubation.
Figure 3. Smear preparations of culture showing haustorial connections
Figure 4. Culture with arachnoidal shape after 10 days of inoculation.
Figure 5. Radial shape after 7 days of transfer in 4% MMS showing indentations in the margins (arrow).
Figure 6. Well-developed heteromerous thallus after 73 days of culture.

photobiont in MMS with 4% sucrose were similar to those in soredial cultures of *Peltigera didactyla,* on sterilized soil substratum under controlled laboratory conditions (Stocker-Wörgötter and Turk, 1988). The cultures of *D. applanata* showed lichenization between the 21st and 27th day, and initiation of cortex and lobe differentiation after the 55th day of inoculation. Cultures of *P. didactyla* showed lobe differentiation and cortex formation after four







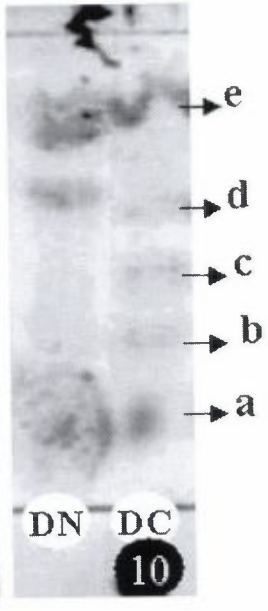


Figure 7. Minute verucae at the center of the thallus exposing the algal layer.
Figure 8. Transverse section of cultured thallus showing different thallus layers, LC-Lower cortex, M-Medulla, AL-Algal layer, UC-Upper cortex.
Figure 9. Transverse section of natural thallus showing different thallus layers, LC-Lower cortex, M-Medulla, AL-Algal layer, UC-Upper cortex.
Figure 10. TLC of both natural and cultured thallus (DN-Natural thallus, DC-Cultured thallus) showing compounds: a. Divaricatic acid; b&c. Unknown compounds in culture (Rf class-4 and 5); d. Unknown compound in natural and cultured thallus (Rf class-6); e. Atranorin.

months. In further studies, lobes of *Parmelia sulcata* developed after 12 months in culture. Thus, as noted by Stocker-Wörgötter and Turk (1988), even though the different developmental stages occur in a similar sequence, the time period for development and vary for different lichen species.

Lobe differentiation and compound production appeared only in the soredial culture incubated in a light and dark cycle and were not observed in mycobiont cultures even after four months of dark incubation. The *D. applanata* cultures produced atranorin, divaricatic acid and sekikiac acid as well as two compounds unknown in the natural thallus. The production of extra compounds, other than the known compounds of natural thalli, was also observed by Ahmadjian and Jacobs (1985) in re-established synthetic cultures of *Usnea strigosa*. They attributed this to the different growing conditions of the lichen culture or the genetic variations that occur in the inoculum.

In this study, the cultures grown on MY and MBBM media with 4% sucrose as a carbon source showed slow growth compared to growth on MMS medium with 4% sucrose. 4% MMS medium in combination with additional carbon and nitrogen source, showed rapid growth, thallus differentiation and compound production, indicating possible specific nutrient requirements of *D. applanata*. It also indicates that some culture media are better than others, but this may not reflect specific nutrient requirements in the media. More experiments on this aspect are clearly needed.

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