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Tissue-culture of selected species of the *Graphis* lichen and their biological activities

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Abstract

The results on the biological activities of the in vitro culture of *Graphis guimarana*, *G. nakanishiana* and *G. schizograpta* lichen are reported. The methanolic extracts of natural thalli and their cultures were found to inhibit tyrosinase, xanthine oxidase and to scavenge superoxide.

Keywords: Lichen; *Graphis*; Tyrosinase; Xanthine oxidase; Superoxide scavenging activity

1. Introduction

Metabolites produced by microorganisms and fungi are a resource of recognized therapeutic potential. Nevertheless, the potential of fungal spp. obligate symbionts in lichen remains largely unexplored for their slow growing in nature and for the difficulties in their artificial cultivation [1–3]. Furthermore, the lichen biomass in the spontaneous flora, with some exceptions, is not sufficiently voluminous to allow its economic exploitation, and in the future it will continuously diminish and alter due to the predictable increase of the pollution degree. In general, lichen tissue cultures grow much faster than natural thalli; as a consequence in vitro culture of lichens have been studied in order to produce high quantities of biomass [1,4–8].

In our previous studies a foliose lichen *Bulbothrix setschwanensis* was cultured in vitro. These cultures and the natural thalli of 77 species of the lichen Graphidaceae have been screened for their potential superoxide scavenging activity, inhibition of xanthine oxidase and tyrosinase [9–12].

2. Experimental

2.1. Plants

Fresh natural thalli of *Graphis guimarana* Vainio, *G. nakanishiana* Kulk. et Patw. and *G. schizograpta* Müll. Arg. (Graphidaceae) were collected from Khandala and Dongarwadi, Maharashtra State. A part of the material of each species has been preserved as specimen in the Ajrekar Mycological Herbarium (AMH), Pune, India.

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2.2. Thalli induction and cultures

Lichen thalli (5 mg) were cut into pieces of 1 cm^2, washed with tap water for overnight and homogenized with 5 ml of distilled water under sterile conditions. The suspensions were passed through a sterilized 500 μm mesh. Further the filtrates were passed through a nylon filter with a 150 μm mesh. Small segments from the second filtration were picked up with sterilized stainless steel loop and were inoculated onto slant media in the petriplates size of 9 cm. The inocula were grown at 18 °C and with alternating photoperiod of 8 h light (400 lx) / 16 h dark. The culture media were selected to be poor nutrient so as to ensure a proportionate growth of both the photobiont and the mycobiont and to enable the formation of cell aggregates. The following culture media adjusted to various pH ranging from 5.0 to 9.5 were used: malt-yeast extract (MY) [13], Lilly-Barnett (LB) [14], Murashige and Skoog [15], Bold's basal medium (BBM) [16], Bischoff and Bold's [17] and modified Bold's basal medium (Table 1) [9].

Lichen thalli were cultured successfully in vitro at 18 °C with an 8 h period of light (400 lx) alternated with a period of 16 h dark and 50–80% relative humidity in culture room. After 3 months, cultures were collected from the petriplates. Out of 366 inoculations of three species ca. 240 cultures were obtained without contamination. These cultures were dried at 40 °C for 72 h and then weighed. Before drying these cultures were compared with their natural counterpart for morphological confirmation by stereo-binocular and transmission light microscopes for the development of photobiont and mycobiont in culture and recorded by macro and microphotography. Lactophenol was used as the stain medium. The chemical data have been obtained by the standardized method of TLC [18] by using the standard solvent system BDA (benzene : dioxane : acetic acid, 180 : 45 : 5, 230 ml) and HEF (hexane : ethylether : formic acid, 30 : 80 : 20, 230 ml). Norstictic acid is present in *G. guimarana* and stictic acid in *G. nakanishiana* in the natural thalli.

Lichen cultures composed of mycobiont and photobiont were maintained for more than 1 year by subculturing them in the fresh modified Bold's basal medium, at an interval of 3 months, under the conditions as mentioned above. The pH of the medium used has been adjusted to 6.6 with 1 N NaOH.

2.3. Cultured lichens and natural thalli biological activities

In order to screen the cultured lichen and natural thalli for their potential activitites, inhibition of tyrosinase and xanthine oxidase and superoxide scavenging activity, 5 mg of *G. guimarana*, *G. nakanishiana* and *G. schizograpta*

Table 1
Chemical composition of the modified Bold's basal medium

Chemicals	Gram per litre in distilled water
Part 1[a]	
NH_4NO_3	1.5
$MgSO_4 \cdot 7H_2O$	1.0
KH_2PO_4	2.0
$CaCl_2$	0.1
K_2HPO_4	3.0
Na_2SO_4	0.1
Part 2[b]	
i. H_3BO_3	4.02
ii. $FeSO_4 \cdot 7H_2O$	0.82
$ZnSO_4 \cdot 7H_2O$	0.40
$MnCl_2 \cdot 4H_2O$	0.43
iii. $(NH_4)_6MoO_{24} \cdot 4H_2O$	0.20
$CuSO_4 \cdot 5H_2O$	0.57
$CO(NO_3)_3 \cdot 6H_2O$	0.98
iv. Na_2-EDTA	15.0
KOH	3.10
Agar	20.0
Sucrose	10.0

[a] Add 10 ml of each stock solution to 940 ml of distilled water.
[b] Add 1 ml of each stock solution to the Part 1 solution).