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Effect of Ribitol and Plant Hormones on Aposymbiotical Growth of the Lichen-forming Fungi of *Ramalina farinacea* and *Ramalina fastigiata*

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This study was aimed at evaluating the growth promoting effect of symbiotic algal polyol (ribitol) and plant hormones on the lichen-forming fungi (LFF), *Ramalina farinacea* (CH050010 and 40403) and *Ramalina fastigiata*. The addition of ribitol to basal (malt-yeast extract) medium enhanced the relative growth rates of all three LFF. *R. farinacea* (CH050010), *R. farinacea* (40403) and *R. fastigiata* (H06127) showed 35.3%, 29.0% and 29.3% higher growth rates, respectively, compared to the control. IBA (indole-3-butyric acid) and TIBA (2,3,5-tridobenzoic acid) also increased growth rates of the LFF by 34 to 64% and 7 to 28%, respectively, compared to the control. The combination of ribitol with IBA or TIBA synergistically increased the growth of all LFF. For example, ribitol and IBA treatments increased growth rates of *R. farinacea* (CH050010), *R. farinacea* (40403) and *R. fastigiata* (H06127) by 79.4%, 40.3% and 72.8% in, respectively, compared to those grown on the basal medium. The stimulating effect of ribitol and IBA on the LFF growth induced vertical development of the fungal mass in culture. We suggest that lichen-forming fungal growth of *Ramalina* lichens can be stimulated aposymbiotically by supplementing polyols and plant hormones to the basal medium in the mass production of lichen secondary metabolites under large scale culture conditions.

KEYWORDS : Fungal growth, Indole-3-butyric acid, Lichen-forming fungi, 2,3,5-Tridobenzoic acid, Ribitol

Lichens are a symbiotic relationship between a fungal and an algal partner, and they have evolved several biosynthetic pathways which produce an amazing diversity of secondary metabolites (Oksanen, 2006; Stocker-Wörgötter, 2008). Many lichen secondary metabolites have various biological activities and are potential sources of compounds for pharmaceutical and industrial applications (Boustie and Grube, 2007). But lichens have been overlooked by the pharmaceutical industry because of their slow growth in nature and difficulties in their artificial cultivation. Hence, the large-scale industrial production of lichen metabolites has never been accomplished. However, use of lichen-forming fungi (LFF) can overcome the disadvantage of natural lichen extracts for industrialization of their metabolites because of their faster growth rates and the greater production of metabolites in culture (Behera *et al*., 2005). However, LFF growth rates are still slow, even under optimum culture conditions, compared to other filamentous fungi. Therefore, there is strong demand to discover growth promoting or stimulating substances for LFF in culture.

In our previous study, LFF isolated from *Ramalina* species grew fast and produced antifungal substances against several plant pathogenic fungi (Oh *et al*., 2006). Green algae partners release a polyhydric sugar alcohol (polyol), such as ribitol, erythritol and sorbitol, to the fungal partner (Parmqvist *et al*., 2008). Ribitol is the carbohydrate supplied by symbiotic algae to lichen-forming fungi in *Ramalina* lichens. Plant hormones are initial resources for promoting plant growth and have a stimulatory effect on microbial growth (Mukhopadhyay *et al*., 2005). For growth promotion in large scale culture of *Ramalina* LFF, we evaluated the effect of ribitol as an additional carbon source, and some plant hormones as inducers of growth of *Ramalina* LFF.

Material and Methods

Two LFF strains of *Ramalina farinacea* (CH050010: Chinese lichen, 40403: Korean lichen) and one LFF strain of *Ramalina fastigiata* (H06127: Hungarian lichen) were obtained from Korean Lichen and Allied Bioresource Bank. Isolation by tissue culture method and confirmation by ITS sequence analysis of the LFF were previously described (Wei *et al*., 2008). Fungi were cultured on MY (malt-yeast extract) medium at 15°C for 1 month before the test.

In our preliminary test for screening 14 plant hormones, IBA (indole-3-butyric acid) and TIBA (2,3,5-tridobenzoic acid) showed the most significant effect on lichen-forming fungal growth of *Nephromopsis ornate*, *Myelochroa irrugans* and *Usnea longissima* at 2 μM/*l*. These two hormones were employed for the evaluation of growth promoting effect on *Ramalina* LFF in this study. All plant hormones were purchased from Sigma-Aldrich Company (UAS).

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The fungal mass of each LFF (approximate 500 mg DW) was aseptically ground and suspended in the liquid MY medium. The suspension (0.1 *ml*) was dropped on a pre-weighed cellulose-acetate filter placed on the surface of MY solid medium. Six different media were used for evaluation: 1) MY as a control, 2) MYR (MY amended with 1% ribitol), 3) MYI (MY amended with 2 μM/*l* IBA), 4) MYRI (MY amended with 1% ribitol and 2 μM/*l* IBA), 5) MYT (MY amended with 2 μM/*l* TBA) and 6) MYRT (MY amended with 1% ribitol and 2 μM/*l* TBA). Relative growth rates were calculated by measuring dry weight of the fungal biomass after a 50 day incubation period at 15°C. Five replicates were performed for each treatment.

Results and Discussion

Addition of ribitol to MY basal medium enhanced relative growth rates of all three LFF (Table 1). *R. farinacea* (CH050010), *R. farinacea* (40403) and *R. fastigiata* (H06127) showed 35.3%, 29.0% and 29.3% higher growth rates, respectively, than those grown on MY without ribitol. The growth promoting effect of ribitol on *Ramalina* LFF agreed well with a previous report that demonstrated that ribitol accelerated the LFF growth of *Usnea rubescens* (Yamamoto *et al*., 1985). Factors stimulating lichen-forming fungal growth, such as temperature, pH, and different carbon and nitrogen sources, have been discussed (Yamamoto *et al*., 1987, 1993). Different lichen-forming fungi have different carbon source preferences (Yamamoto *et al*., 1987). Translocation patterns of metabolites between mycobionts and photobionts vary among species, and are partly dependent on the chemical composition of the biont cell walls (Honegger, 1991). Green algae release a polyhydric sugar alcohol (polyol), such as ribitol, to the mycobiont. Once taken up by the mycobiont, the carbohydrate is rapidly and irreversibly metabolized into mannitol via the pentose phosphate pathway (Lines *et al*., 1989) making it unavailable to the photobiont (Galun, 1988). Therefore, it can be speculated that an additional supply of ribitol to the separated LFF of *Ramalina* species will trigger the translocation of ribitol and pentose pathway as it does when in symbiotic status. Thus, ribitol supplyment may be preferable to single supply of complex carbohydrates of MY for the fungal growth in culture. Ribitol also has a positive effect on secondary metabolite production in lichens. Solhaug and Gauslaa (2004) showed that physcion biosynthesis is more strongly activated by supplementation of ribitol than by mannitol in *Xanthoria parietina*.

Some plant hormones accelerated lichen-forming fungal growth of *Ramalina* species in our study (Table 1). Compared to the LFF grown on MY without hormones, IBA (indole-3-butyric acid) increased growth rates of the LFF by 34 to 64% and TIBA (2,3,5-tridobenzoic acid) increased growth rates by 7 to 28%. The stimulating effect of the hormones was most significant in the Chinese strains of *R. farinacea* (CH050010). Interestingly, there was differential sensitivity to the hormones between the Korean strain (40403) and Chinese strain of *R. farinacea.* Supplementation of ribitol and the hormones synergistically increased the growth of all the LFF. In particular, when LFF were cultured on the medium with IBA and ribitol, they grew much faster than on other media. Compared to the control, the Chinese strain of *R. farinacea* (CH050010), Korean strain of *R. farinacea* (40403) and *R. fastigiata* (H06127) showed the increased growth rates of 79.4%, 40.3% and 72.8% on the medium, respectively. The stimulating effect of ribitol and IBA on the LFF growth induced vertical development of the fungal mass in culture (Fig. 1). Yamamoto *et al*. (1993) reported that the plant hormones abscisic acid (ABA), indole-3-acetic acid (IAA), 2-(p-chlrophenoxy)isobutyric acid (CIBA), cinnamic acid (CA) and 1-Naphthaleneacetic acid (NAA) had no stimulating effect on lichen-forming fungal growth of *Usnea rubescens* in culture. This corresponds with our preliminary screening results in the present study (data not shown). In some cases, IAA and jasmonic acid inhibited the growth of several LFF. These findings imply that different plant hormones, even those belonging to same class,

Table 1. Relative growth rates of the lichen-forming fungi of *Ramalina* species on media supplied with ribitol and/or plant hormones

Species / Treatment	*R. farinacea* (CH050010)	*R. farinacea* (40403)	*R. fastigiata* (H06127)
Control (MY)	8.80 ± 0.20 a	4.49 ± 0.07 a	5.80 ± 1.27 a
MYI	14.44 ± 0.12 e	6.02 ± 0.10 e	9.04 ± 0.18 c
MYT	11.22 ± 0.22 b	4.82 ± 0.15 b	7.27 ± 0.21 b
MYR	11.91 ± 0.12 c	5.79 ± 0.05 d	7.50 ± 0.16 b
MYIR	15.79 ± 0.21 f	6.30 ± 0.08 f	10.02 ± 0.23 c
MYTR	12.33 ± 0.21 d	5.21 ± 0.11 c	7.70 ± 0.23 b

LFF were cultured at 15°C in the dark for 50 days. Data represent the mean ± SD of five replicates and were calculated by the following equation (Relative growth rate = Final dry weight/Initial dry weight). Means with the same letter are not significantly different at $P = 0.05$ (LSD). Control: malt-yeast extract medium (MY); MYI: MY + IBA (indole-3-butyric acid, 2 μM/*l*); MYT: MY + TBA (2,3,5-tridobenzoic acid, 2 μM/*l*); MYR: MY + ribitol (1%); MYIR: MY+IBA (2 μM/*l*) + ribitol (1%); MYTR: MY + TBA (2 μM/*l*) + ribitol (1%)

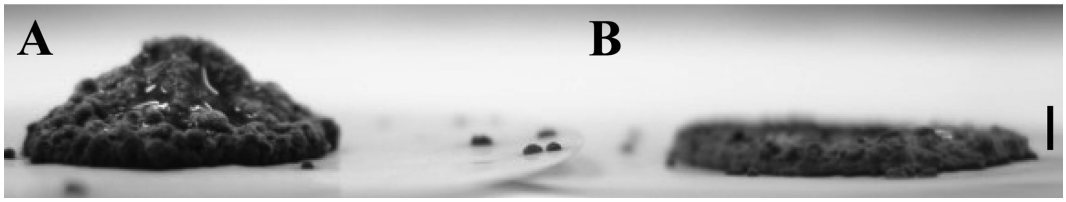

Fig. 1. Stimulating effects of ribitol and plant hormones on the lichen-forming fungal growth of *Ramalina faricacea* (CH050010). The fungi grown on the MY medium with 1% ribitol and 2 μM/*l* IBA showed vertical development of fungal mass (A), compared to those grown on the medium without ribitol and IBA (B) after a 50 day-incubation period at 15°C. Scale bar = 50 mm.

can influence LFF growth in different manners. Auxins play a critical role in numerous plant growth and developmental processes, including cell elongation, differentiation, tropism, apical dominance and root initiation. IBA is a well known plant rooting hormone. However, the effects of these auxins on fungal growth and developmental process is not known.

We conclude that the polyol (ribitol) excreted by symbiotic algae and some plant hormones (mainly auxins) can stimulate the fungal growth of the separated mycobionts of *Ramalina* lichens in a synergic manner. We are now analyzing the chemical profiles of the LFF in culture to optimize large scale cultural conditions of the LFF for mass production of lichen substances.

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