Nitrogen fixation in lichens is important for improved rock weathering

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It is known that cyanobacteria in cyanolichens fix nitrogen for their nutrition. However, specific uses of the fixed nitrogen have not been examined. The present study shows experimentally that a mutualistic interaction between a heterotrophic N_2 fixer and lichen fungi in the presence of a carbon source can contribute to enhanced release of organic acids, leading to improved solubilization of the mineral substrate. Three lichen fungi were isolated from *Xanthoparmelia mexicana*, a foliose lichen, and they were cultured separately or with a heterotrophic N_2 fixer in nutrient broth media in the presence of a mineral substrate. Cells of the N_2-fixing bacteria attached to the mycelial mats of all fungi, forming biofilms. All biofilms showed higher solubilizations of the substrate than cultures of their fungi alone. This finding has bearing on the significance of the origin and existence of N_2-fixing activity in the evolution of lichen symbiosis. Further, our results may explain why there are N_2-fixing photobionts even in the presence of non-fixing photobionts (green algae) in some remarkable lichens such as *Placopsis gelida*. Our study sheds doubt on the idea that the establishment of terrestrial eukaryotes was possible only through the association between a fungus and a phototroph.

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1. Introduction

Upon colonization of the land surface, microorganisms would face, among other problems, low concentrations of many nutrients in available water. They are known to utilize a variety of methods to extract nutrients from mineral surfaces, among which the most prevalent may be the secretion of organic acids (Robert and Berthelin 1986; Jones 1998). Fungi within lichens are known to secrete a great number of organic compounds, which are essential for supplying lichen with minerals from the substrate (Huneck and Yoshimura 1996; Purvis 2000; Neaman *et al* 2005). It has been reported that there are great qualitative and quantitative differences in the spectrum of organic acids between intact lichens and cultured lichen fungi (Huneck and Yoshimura 1996), suggesting that fungal acid production is altered in the lichen.

Weathering of stones and rocks is caused by physical, chemical (e.g. air pollution and acid rain) and biological processes (Goudie and Parker 1999). Organic acids produced by microorganisms as by-products of their metabolism are responsible for biological weathering of rocks. With the emergence of fungi and the evolution of microbial consortia such as lichens, organic acids such as phenolic acids became important in solubilizing nutrients from inorganic substrates (Neaman *et al* 2005).

Biofilm formation is a prominent feature of microbial growth in nature. Biofilms have been observed in a number of environments, but little is known about their effects on the release of minerals from substrates. A recent study experimentally showed that a heterotrophic N_2 fixer colonized mycelia of common soil fungi forming biofilms (Seneviratne and Jayasinghearachchi 2003). Nitrogenase activity and nitrogen accumulation were detected in them (Jayasinghearachchi and Seneviratne 2004). A comparable observation has been made in *Chiodecton sanguineum*, a lichen, where its hyphae were surrounded by purple bacteria (Uphof 1925). Nitrogen fixation in cyanolichens has also been reported (McCune 1993). The biofilms produced promising results in the P solubilization of rock phosphate

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(RP) (Jayasinghearachchi and Seneviratne 2006). Further, the biofilms enhanced N and P availabilities when inoculated in the soil (Seneviratne and Jayasinghearachchi 2005). Thus, the present study developed such microbial biofilms using lichen fungi in order to investigate the possible role played by fixed N_2 in cyanolichens. It is hypothesized here that fixed N_2 is important in rock weathering by lichens. The implications of this study are also discussed in this paper.

2. Materials and methods

2.1 *Isolation of lichen fungi and co-culturing for biofilm formation*

A foliose lichen, "salted rock-shield" (*Xanthoparmelia mexicana* Gyeln. Hale) (figure 1a) was used for the study. Lichen-forming fungi were isolated using pieces of the lichen thallus. They were surface sterilized by immersing in a 0.2% Hg_2Cl_2 solution for 1 min, washed with six changes of sterile distilled water, crushed into small pieces and placed on Sabouraud dextrose agar (SDA) medium. Fungi grown on the plates were isolated and identified as (i) *X. mexicana*, the mycobiont of the lichen, (ii) *Botrydiplodia theobromae* (a plant pathogen), and (iii) *Syncephalastrum racemosum* (a rare causative agent of human zygomycosis, a serious infection resulting from an invasion of the blood vessels). These fungi were co-cultured for biofilm formation with a heterotrophic N_2 fixer, *Bradyrhizobium elkanii* SEMIA 5019, a soybean nodulating strain. The use of a heterotrophic N_2 fixer is crucial in this study, because it only induces the N_2 fixing activity in the co-cultures of lichen fungi. If a cyanobiont was used for this, then the lichen fungi would get the benefit of photosynthetic activity, in addition to the N_2 fixing activity. This would not reflect the effect of the N_2 fixing activity alone on the function of the lichen fungi.

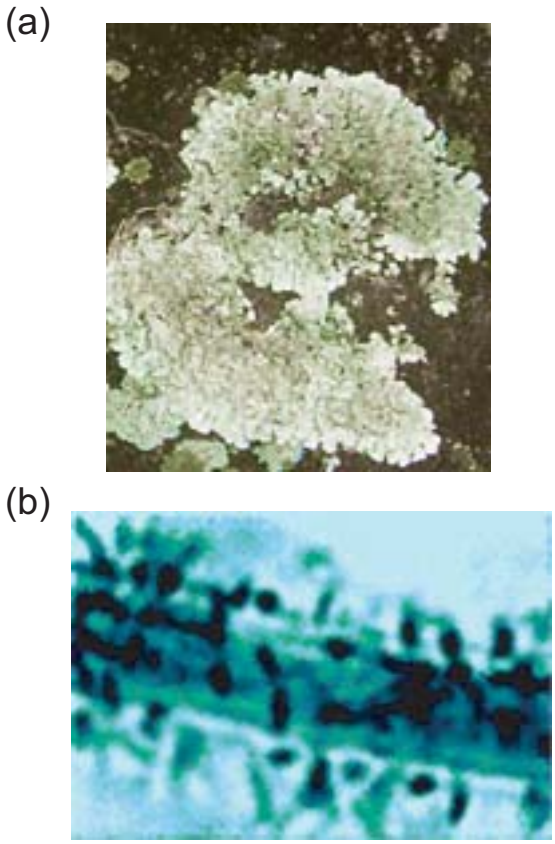

Figure 1. **(a)** Thallus of "salted rock-shield" (*Xanthoparmelia mexicana*), a foliose lichen growing on a rock. **(b)** A mycelial filament of its mycobiont, *X. mexicana,* colonized by *Bradyrhizobium elkanii* SEMIA 5019 (magnification: x 2000).

Eppawala rock phosphate (ERP, total P concentration 22.5%), an RP from a deposit in Sri Lanka, was used as the test material for rock weathering. It was tested with the microbial cultures for P solubilization for 15 days. All these microorganisms are deposited in the culture collection of the Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka. Bradyrhizobial cultures were maintained in yeast manitol broth (YMB) (Somasegaran and Hoben 1994). They were incubated on a rotary shaker at 28°C for 6 days. Pure cultures of the fungi were maintained on potato glucose agar (PGA) and incubated at 28°C for 3–4 days depending on their growth. The ERP was ground and sieved (< 0.5 mm). Plate cultures of the fungi and the bradyrhizobial strain were inoculated into a series of 100 ml Erlenmeyer flasks containing 50 ml of a modified Pikovskaya broth, as mentioned below. The original medium of glucose 10 g, $Ca_3(PO_4)_2$ 5 g, NaCl 0.2 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, $MnSO_4.7H_2O$ 0.0025 g, $FeSO_4.7H_2O$ 0.0025 g, $(NH_4)_2SO_4$ 0.5 g and yeast extract 0.5 g in a litre of aquadest (Narsian *et al* 1995) was modified by replacing $Ca_3(PO_4)_2$-P with the ERP P. The microbial treatments were (i) *X. mexicana* alone, (ii) *B. theobromae* alone, (iii) *S. racemosum* alone, (iv) *B. elkanii* SEMIA 5019 alone, (v) *X. mexicana* + *B. elkanii* SEMIA 5019 biofilm, (vi) *B. theobromae* + *B. elkanii* SEMIA 5019 biofilm, (vii) *S. recemosum* + *B. elkanii* SEMIA 5019 biofilm, and (viii) the control (i.e. nutrient medium + ERP particles). The experiment was arranged in a completely randomised design. Six replicates were maintained for each treatment and the control. The cultures were incubated on a shaker at 4 rpm and room temperature (28°C) for 15 days, since in a preliminary study mycelial growth was found to reach a maximum biomass at 15 days.

2.2 *Microbial observations and sample analyses*

At day 7, a loop of the broth culture was removed from each flask using a sterilized inoculating loop. It was observed using a light microscope with an oil immersion lens. Lactophenol cotton blue was used to visualize the mycelia

and biofilms. At day 15, the supernatant of the flasks was collected by centrifugation at 2147×*g* for 20 min (Thomas *et al* 1985). The pH of the supernatant was measured using a pH meter. The $NaHCO_3$-extractable P in the supernatant was extracted. Phosphorous was analysed spectrophotometrically at 880 nm using the molybdenum blue method (Anderson and Ingram 1993). Thereby, the ERP P solubilized by the microbial treatments was calculated. The efficiency of P release was calculated using the percentage of P release from the ERP.

2.3 *Data analyses*

All data were analysed using SAS (1998) software. Means of pH and ERP P solubilized of the fungal cultures and their corresponding biofilms were compared using two-tailed student's *t*-tests. The relationship between pH and percentage of ERP P solubilized was derived using non-linear regression analysis.

3. Results and discussion

Bradyrhizobial cells attached to the mycelial mats of all fungi forming biofilms were observed under the light microscope at 7 days of incubation. The fungal mycelium of *X. mexicana* was profusely colonized by *B. elkanii* SEMIA 5019 (figure 1b). The differences in P solubilization by the different microbial treatments used in this study varied in their significance ($P = 0.003 – 0.332$; table 1). The *X. mexicana* + *B. elkanii* SEMIA 5019 biofilm released the highest amount of P (i.e. 2.1% of ERP P) when compared with the other microbial treatments. In general, all biofilms showed higher P releases than the cultures of their fungi alone, although the differences were not statistically significant at 5% probability level. This could be due to community level gene expression for organic acid production, which may be unique to the biofilm (Vilain and Brözel 2006), and different from gene expressions of original microbes that formed the biofilm. To our knowledge, this is the first study in which lichen-forming fungi have been employed for rock phosphate solubilization. During the biofilm formation, attachment to biotic or abiotic surfaces stimulates exopolysaccharide synthesis by some bacteria (Vandevivere and Kirchman 1993). Further, the presence of a N_2 fixer in the biofilm also aids acid production, because it has been shown that N is limiting in the production of acids by microbes especially in P solubilizing systems (Singh and Amberger 1998). This may have helped a higher production of the organic acids in the biofilms. The H^+ concentrations calculated from the pH

Table 1. Eppawala rock phosphate P (ERP P) solubilized and pH of culture media containing different microbial treatments of lichen fungi after 15 days of incubation.

			ERP P solubilized		
Treatment	pH	Difference†	(mg g^{-1} ERP P)	Difference†	(%)
X. mexicana alone	3.8 ± 0.05		16.1 ± 1.40		1.6
		0.1 (0.228)		4.7 (0.118)	
X. mexicana + SEMIA 5019	3.7 ± 0.01		20.8 ± 0.91		2.1
B. theobromae alone	5.6 ± 0.37		6.7 ± 1.10		0.7
		1.1 (0.102)		1.8 (0.332)	
B. theobromae +SEMIA 5019	4.5 ± 0.02		8.5 ± 0.36		0.8
S. racemosum alone	5.1 ± 0.40		9.3 ± 1.29		0.9
		0.9 (0.187)		3.7 (0.193)	
S. racemosum +SEMIA 5019	4.2 ± 0.02		13.3 ± 1.06		1.3
SEMIA 5019 alone	4.3 ± 0.01		9.7 ± 0.39		1.0
		1.1 (< 0.001)		6.0 (0.003)	
Control	5.4 ± 0.02		3.7 ± 0.56		0.4

Mean ± SE, $n = 6$. †Differences of the parameters between the fungus alone and its biofilm with SEMIA 5019 were tested for significance using the two-tailed *t*-test. Probability levels are within parentheses.

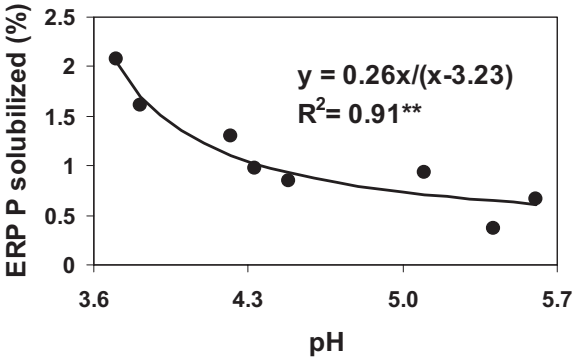

Figure 2. Relationship between pH of microbial culture media and Eppawala rock phosphate P (ERP P) solubilized by the cultures used in the study.

of the culture media of the three biofilms of *X. mexicana*, *B. theobromae* and *S. racemosum*, were respectively 1.3-, 13- and 8-fold higher, compared to the cultures of their respective fungi. The results established a clear negative relationship between pH in the culture media and the ERP P solubilized (figure 2); the more acidic the media, the higher the amount of P solubilized. The culture with *B. elkanii* SEMIA 5019 alone, attached to the ERP particles, also showed high P release relative to the control possibly due to its inherent ability of organic acid production (Halder *et al* 1990). The variability of the organic acid production may be due to varying specificities for the attachment of bacteria to fungi (Seneviratne and Jayasinghearachchi 2003), which in turn may govern their interactions, and the quality and quantity of the acids secreted into the medium (Reddy *et al* 2002).

The prevalence of heterotrophic N_2 fixers has been reported from diverse environments. Molecular evidence indicates their presence in deep-sea and hydrothermal vent environments (Mehta *et al* 2003), and marine intertidal microbial mat consortia (Olson *et al* 1999). Heterotrophic N_2 fixers have been characterized even in the presence of phototrophic N_2 fixers in microbial consortia associated with the ice cover of soil habitats of Lake Bonney, Antarctica (Olson *et al* 1998). Moreover, phototrophic N_2 fixers have been observed even in the presence of non-fixing phototrophs in lichens such as *Placopsis gelida* (Lamb 1947). In such lichens, *Nostoc* – the phototrophic N_2 fixer, often dwells within gall-like structures called cephalodia. Heterocyst differentiation within cephalodia in such cases is greater than when *Nostoc* are the primary symbionts in lichens (Rai 1990). This demonstrates the specialization of *Nostoc* for N_2 fixation in the presence of non-fixing phototrophs. Thus, although the prevalence of N_2 fixation and the high demand for it in microbial biofilms have been earlier reported. The significance of N_2 fixation in the biofilms has not been adequately explained. Our study provides evidence that N_2 fixation in such microbial consortia is important in enhanced weathering of their mineral substrate.

Although the importance of phototrophy in the establishment of land flora during terrestrialization has been emphasized (Selosse and Le Tacon 1998), our study provides evidence for a possible terrestrialization of heterotrophs, which could have occurred in the presence of Fischer-Tropsch type synthetic reactions for the formation of organic compounds.

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