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Does the Lichen Alga *Trebouxia* Occur Free-Living in Nature: Further Immunological Evidence

A. MUKHTAR, J. GARTY* and M. GALUN
Department of Botany and *Institute for Nature Conservation Research, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel*
Tel. 972 3 6409163, Fax 972 3 6409380

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Abstract

Morphological and immunological methods have revealed free-living *Trebouxia* cells among the first settlers in an area that has been completely sterilized by a forest fire. These cells could be detected three years after the fire, and before any lichen colony had been established. We provide evidence that these free-living *Trebouxia* cells are identical by morphological and immunological criteria with the photobiont of *Xanthoria parietina* and of *Buellia* sp. that developed in another area of the same region which was exposed to a forest fire four years earlier. It appeared that the *X. parietina* colony examined contained two (or more) different *Trebouxias*.

Keywords: lichen, *Trebouxia*, photobiont, *Buellia* sp., immunoassay

1. Introduction

De Bary (1887) claimed "that lichen algae have become adapted to symbiosis with fungi and cannot exist in the free-living state." A consequence of this statement would be, that lichens reproduce only vegetatively, by diaspores which contain both components – the fungus and the alga. There are indeed many lichen species with vegetative reproduction units, such as soredia and isidia (Jahns, 1988). However, a large number of foliose species and the

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majority of crustose species are not equipped with vegetative diaspores and depend therefore on resynthesis between germinated spores and the appropriate photobiont to give rise to new lichenized thalli.

Considering the distribution of such lichens, resynthesis between germinated ascospores and free-living photobionts has been assumed to be a frequent phenomenon (Bowler and Rundel, 1975; Smith, 1981; Tehler, 1982).

However, the existence of *Trebouxia*, the most common lichen photobiont, as a free-living organism in nature, has been a subject of debate. Ahmadjian (1967, 1970, 1988, 1993) "feels" that the *Trebouxia* cells on bark and wood observed by Tschermak-Woess (1978) and the free-living *Trebouxia* cells observed by Bubrick et al. (1984) being identical with *Trebouxia* of *Xanthoria parietina*, were in fact"... *Trebouxia* cells that escaped from asexual propagules"; yet, *X. parietina* does not have asexual propagules. Here we had the opportunity to demonstrate free-living *Trebouxia* cells from a site where "escapees" are most unlikely.

2. Materials and Methods

Site

Two areas were selected on Mt. Carmel (Israel) that were subjected to forest fires in 1983 and 1989, respectively. In 1992 *Trebouxia* isolations were performed from *Xanthoria parietina* and from *Buellia* sp. that grew close to each other, on a rock exposed to the fire in 1983. Free-living *Trebouxia* was also isolated from a rock exposed to fire in 1989.

Isolation of symbiotic photobionts

Fresh thallus of *X. parietina* was washed for 30 min under running tap water, blotted dry, soaked in tap water for a few minutes and blotted again. The thallus was then cut into tiny pieces (less than 1 mm^2) and soaked for 10–15 min in BBM-soil (according to Nichols and Bold, 1965 with 1% soil extract). Then 50 mg of the thallus fragments were transferred for about 2 weeks to 10 ml vials containing 2–3 ml BBM-soil medium. These fragments were then mounted on TA(Amp-Tet) (*Trebouxia* medium, Ahmadjian, 1993, diluted 2-fold and agarized with 1% agar, containing 0.3 mg/ml ampicillin and 2.5 μg/ml tetracyclin) in sterile Petri plates, ca 1 cm apart from each other. Within 2–3 weeks, algal cells emerged from the thallus fragments. Algal microcolonies that developed from single cells after several transfers on the same medium were used. Further growth was achieved in 50 ml BBM-soil medium containing ampicillin (0.3 mg/ml) in 100 or 125 ml erlenmeyer flasks.

All cultures on agarized medium were maintained at 18°C, under 10^5 erg cm^{-2} sec^{-1} light (16 hr light/8 hr dark) and the algal cultures in liquid medium were shaken (100 rpm) at 22–24°C under the same light/dark conditions. These procedures yielded five isolates: XpT1–XpT5.

The isolation of *Trebouxia* from *Buellia* sp. was performed by Prof. Dr. E. Tschermak-Woess. Tiny pieces were scrapped off the thallus surface and distributed in a drop of water on BBA3N (agarized BBM3N, Ahmadjian, 1993). Large algal cells were then picked out from the loose assemblage under a dissecting microscope by a platinum hook and transferred on small agar blocks. After several transfers, two isolates were obtained: BT1 and BT2.

Isolation of free-living photobionts

Rock surfaces (the rock exposed to fire in 1989) were scrapped and ground into powder, 0.2 g of which were incubated in ~3 ml BBM-soil (Amp) at 22–24°C under light conditions as above. A few weeks later several types of green algae developed on the growth medium. Further algal proliferation was achieved in 50 ml BBM-soil(Amp) in erlenmeyer flasks as above.

Preparation of antisera and immunization schedule

Antisera against one isolate of *Trebouxia* and from *X. parietina* (XpT1) and one *Trebouxia* isolate from *Buellia* sp. (BT1) were generated as previously described by Frensdorff et al. (1979).

Antisera assays

Algal cells obtained from BBM-soil(Amp) or from TA(Amp-Tet) and suspended in BBM-soil(Amp) were used. Aggregates were separated by homogenizing and algal cells were then pelleted twice by low speed centrifugation and suspended in PBS (phosphate buffered saline) (KH_2PO_4, 1.5 mM Na_2HPO_4, 3 mM, KCl, 2.7 mM, $MgSO_4$, 0.4 mM; NaCl, 137.9 mM; pH 7.4). About 10^6 algal cells were pelleted again in 1.5 ml microtubes for 30 min in 50 μl of blocking solution (10% BSA in PBS). Blocking was performed by gentle vortexing. Cells were then incubated for 2 hr in a 1:200 diluted (in blocking solution) antiserum. Cells were pelleted, the supernatant discarded and the cells washed with 200 μl PBS by gentle vortexing for 10 min. Cells were pelleted again, the supernatant discarded and the cells blocked as before for 15 min, incubated with diluted (1:160) secondary IgG (Goat anti-rabbit IgG-FITC conjugate,

Sigma) in blocking solution and then washed as before in 200 μl PBS. Algal cells were then pelleted, mounted on slides in borate-buffered glycerol and examined with a Zeiss epifluorescence microscope (BG-12 exiter filter, no. 50 barrier filter). A Fujicolor 400 ASA slide film was used for photography. Controls were cells incubated with preimmune serum, with antiserum alone and with secondary IgG alone, respectively.

3. Results

αXpT1 was screened for its ability to react selectively with the homologous isolate (XpT1) and with the isolated XpT2, XpT3, XpT4 and XpT5. α BT1 was screened for its ability to react selectively with the homologous isolate (BT1) and the isolate BT2. Cross-reactivity of both antisera was examined, i.e. αXpT1 against BT1 and BT2, and αBT1 against all five *X. parietina–Trebouxia* isolates: XpT1–XpT5.

As shown in Table 1, αXpT1 reacted with XpT1 (the homologous cells) and with isolate XpT2 (Fig. 1a), but did not recognize the three other isolates: XpT3, XpT4 or XpT5. αXpT1 also gave a positive reaction with BT1 (Fig. 1b) and with BT2 (Fig. 1c). αBT1 reacted positively with both its homolog BT1 (Fig. 1d) and with isolate BT2 (Fig. 1e). αBT1 cross reacted with isolates XpT1 and XpT2 (Fig. 1f), whereas no reaction was detected with isolates XpT3, XpT4 or XpT5. From the "rock powder" (of the rock exposed to the fire of 1989), a myriad of algal cells developed in culture. Several algal cells in this mixture cross-reacted with αXpT1 and with αBT1 (Fig. 1g and h). This rock was at the time of collection completely devoid of lichen colonies, whereas the rock exposed to fire in 1983 was already settled with *X. parietina*

Table 1. Immunofluorescence assay of antisera with homologous and heterologous algae

Algal isolate	Antiserum			
	αXpT1		αBT1	
XpT1	+	(hm)	+	
XpT2	+		+	
XpT3; XpT4; XpT5	−		−	
BT1	+		+	(hm)
BT2	+		+	

XpT1–XpT5: isolates of *Trebouxia* sp. from *Xanthoria parietina*. BT1 and BT2 isolates of *Trebouxia* sp. from *Buellia* sp. αXpT1: antiserum against XpT1. αBT1: antiserum against BT1. +: positive reaction; −: negative reaction; hm: homologous alga.

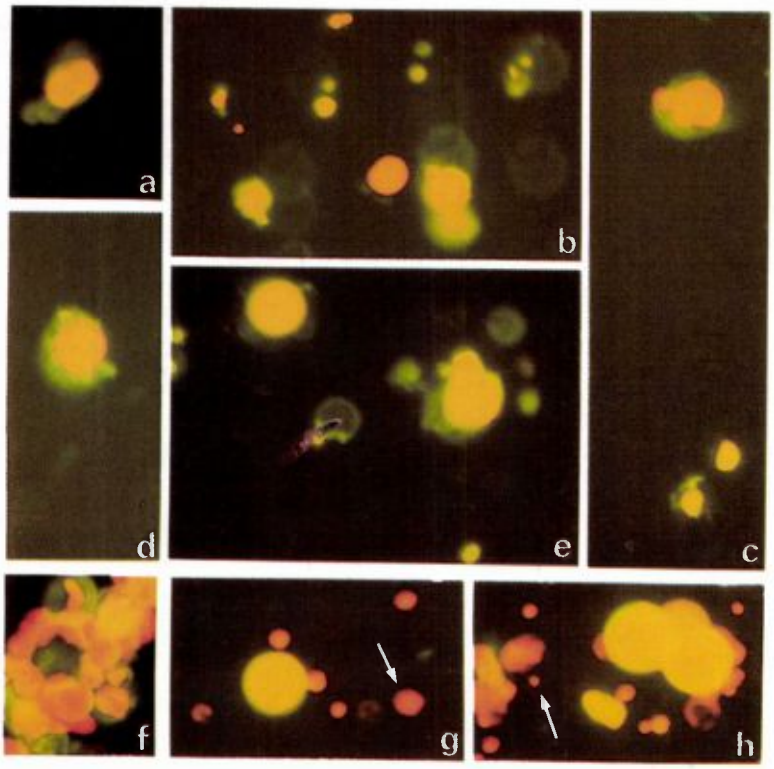

Figure 1. Indirect immunofluorescent staining of *Trebouxia* cells.
(a) αXpT1 reacted with XpT2; (b) αXpT1 reacted with BT1; (c) αXpT1 reacted with BT2; (d) αBT1 reacted with BT1; (e) αBT1 reacted with BT2; (f) αBT1 reacted with XpT2; (g) αXpT1 reacted with free-living *Trebouxia*; (h) αBT1 reacted with free-living *Trebouxia*; arrows indicate negative reaction of free-living algae. XpT = *Trebouxia* isolated from *Xanthoria parietina*; BT = *Trebouxia* isolated from *Buellia* sp..

and *Buellia* sp., from which the *Trebouxia* cells used were isolated. There was no reaction with any of the controls.

4. Discussion

The two fires that occurred in 1983 and in 1989 at two localities on Mt. Carmel (Israel), which completely destroyed the entire vegetation, gave us the opportunity to prove that *Trebouxia*, the most common lichen photobiont, belongs to the free-living algal community. By employing highly sensitive immunological methods, we show that among the many different algae that

developed in surface crevices of a rock, about three years after the fire, still devoid of lichens or any other higher vegetation, there were *Trebouxia* cells with surface molecules similar to those of intact photobionts grown in culture.

The antiserum against one isolate from *X. parietina* (XpT1) recognized only two isolates from this lichen (i.e. its homologue XpT1 and XpT2), but did not react with the three additional isolates (XpT3, XpT4 or XpT5), suggesting that *X. parietina* can contain two (or more) different photobionts. This suggestion was strongly supported by the similar cross-reactivity with αBT1. αBT1 also recognized the two isolated XpT1 and XpT2, but gave no reaction with the three other *X. parietina* isolates (XpT3–XpT5). These results lead to the conclusion that the *Buellia* sp. contains the same photobiont as the *X. parietina* specimen.

Bubrick et al. (1984) documented the presence of free-living *Trebouxia* and *Pseudotrebouxia* and showed, with the aid of morphological and immunological methods (the same as applied in this study), that both the free-living photobiont as well as the mycobiont spores detected were of *X. parietina*. Surprisingly, this evidence, in addition to the observations by Tschermak-Woess (1978) on free-living *Trebouxia* colonies, did not end the argument on the existence of *Trebouxia* as free-living in nature. According to Ahmadjian (1967, 1970, 1988, 1993), there are no free-living populations of *Trebouxia* and "... such cells ... probably originated from thallus fragments or propagules whose associated hyphae died." The presence of *Trebouxia* cells on a completely bare rock "sterilized by a fire," before the establishment of the "host" lichen, provides unequivocal evidence to conclude this long-lasting dispute.

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