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# ZOOSPOROGENESIS IN *TREBOUXIA GELATINOSA*: ULTRASTRUCTURE POTENTIAL FOR ZOOSPORE RELEASE AND IMPLICATIONS FOR THE LICHEN ASSOCIATION

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**Abstract:** Zoosporogenesis was observed in the algal symbiont *(Trebouxia gelatinosa)* of *Parmelia caperata*. This is the first detailed report of this phenomenon in a *Trebouxia* species and, more importantly, in lichens, where zoosporogenesis in this alga is usually suppressed and the arrested zoospores form aplanospores. Observations of zoospore formation by algal cells within the natural thallus and in thallus fragments incubated on a mineral medium suggest that zoospores are released within thalli and may escape from the thallus to form free-living microcolonies. These colonies potentially could unite with hyphae derived from spores of either the same, or genetically different mycobionts (fungal symbionts) to establish new lichen associations, as has been shown in laboratory studies. Such natural resyntheses could be one cause for the observed heterogeneity characterizing fungi in many lichen populations. Other implications of free-living *Trebouxia* populations are discussed.

## Introduction

During the course of an investigation of the ultrastructural effects of sulphur dioxide on *Parmelia caperata* (Slocum 1977), various stages of zoosporogenesis in the phycobiont, *Trebouxia gelatinosa* Ahm., were observed in hydrated thallus sections. This unusual observation prompted us to further examine the conditions under which zoosporogenesis might be initiated in the lichen thallus as little is known regarding this developmental process *in situ*.

Zoosporogenesis has been commonly reported in *Trebouxia* and *Pseudotrebouxia* species growing in liquid culture media or on agarized media (see Ahmadjian 1967b; Archibald 1975), but it is usually suppressed in the lichen association, with aplanospores forming from arrested zoospores. It has been suggested that this is perhaps due to a lack of sustained hydration under natural conditions in the thallus. Frequent wetting and drying cycles characterize most *Trebouxia*-containing lichen habitats and the thick-walled aplanospores are probably better adapted to withstand periods of desiccation. Indeed, the initiation of zoosporogenesis in lichenized algae has been previously documented only in hydrated thalli. Ahmadjian (1970) observed late stages of zoosporogenesis in the phycobiont (*Pseudotrebouxia potteri* (Ahm.) Archibald) of *Rhizoplaca chrysoleuca* (Sm.) Zopf collected during a wet spring season and reported seeing a free-swimming zoospore in a crushed thallus preparation, thus demonstrating the potential motility of these cells within the lichen. Santesson (1952) observed zoosporangia in several trentepohlialean phycobionts of

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foliicolous lichens. Motile zoospores would provide a means whereby a phycobiont could disperse itself outside of a thallus and establish free-living colonies. The mycobiont similarly has the capability to establish a separate existence by means of sexual and asexual spores.

There are scattered reports of free-living colonies of *Trebouxia* [some species of which are now placed in the genus *Pseudotrebouxia* (Archibald 1975); see discussion in Ahmadjian (1967b); Nakano (1971a, 1971b); Tschermak-Woess (1978)], but these algae occur almost exclusively as phycobionts in a wide range of associations with lichen fungi. Because the 'free-living' nature of some reported *Trebouxia* isolates was questioned, and also due to the infrequency of such reports, Ahmadjian (1970) hypothesized that these phycobionts might not have recognizable free-living counterparts, rather they had been morphologically modified through their symbiotic existence with the fungus. This hypothesis was recently supported by a suggestion that unicellular *Trebouxia* phycobionts represent a morphologically reduced stage of a free-living, *Pleurastrum*-like, filamentous alga (Molnar *et al.* 1975). Inasmuch as many filamentous blue-green phycobionts, such as *Scytonema* species, are broken up into individual cells as a result of hyphal constriction within the thallus (Ahmadjian 1967a; Marton & Galun 1976), this idea does not seem unreasonable.

Our paper addresses the contradiction that exists between the reports of free-living strains of *Trebouxia* and *Pseudotrebouxia* and the hypothesis that these algae no longer exist apart from the lichen symbiosis. We also examined factors which might promote zoospore formation in *Trebouxia gelatinosa* in nature and the means by which zoospores might escape from a thallus and establish free-living colonies.

## Materials and Methods

### 1. Thalli from natural populations and laboratory incubation studies

Peripheral lobes (several mm²) of *Parmelia caperata* (L.) Ach. were collected from the northeast side of a large *Quercus* sp. located at the junction of South Rd and Reservoir Rd, Holden, Massachusetts, U.S.A. Collections were generally made at weekly or biweekly intervals for *c.* 1 yr from 13 September 1977 to 20 October 1978. Only small thalli with apparently healthy lobes were chosen, and careful records made as to the condition of the thallus at the time of collection, particularly following prolonged periods of persistent rainfall. Specimens were immediately returned to the laboratory and light microscopic observations of algal zoosporogenesis were carried out utilizing squash preparations of thalli. Thalli were also washed under running tap water for 10–15 min and then pieces of the whole thallus, algal layer and soredia were first examined for the presence of zoosporogenesis in the algal cells and then incubated in distilled water, soil extract agar (Ahmadjian 1967a) and Bold's Basal Medium (Deason & Bold 1960) at 15°C, 17°C or 23°C and under either a 12 : 12 or 16 : 8 LD cycle for periods of up to 11 days. In some of the studies, thallus pieces were preincubated under the same conditions for 3–5 days in plastic petri dishes on filter paper moistened with distilled water.

### 2. Light and Electron Microscopy

Specimens of *Parmelia caperata*, also containing *Trebouxia gelatinosa* as a phycobiont, were collected on the bark of *Quercus alba* in March on the campus of Dennison University, Granville, Ohio. Excised sections (*c.* 1–2 mm²) of healthy peripheral lobes of a single thallus were hydrated in 0·2 M (pH 7·0) phosphate buffer for one hr and returned to the laboratory where they were processed for electron microscopic observation following incubation on control or sulphur dioxide fumigation solutions (see Slocum 1977).

Specimens were rinsed in 0·2 M sodium cacodylate buffer (pH 7·2) and fixed at 23°C using standard fixation techniques employing primary fixation in Karnovsky's solution (Karnovsky 1965) in cacodylate buffer for 2 h followed by post fixation in 1% osmium tetroxide in the same buffer for 2 h. Samples were

dehydrated in a graded acetone series at 30 min intervals followed by two 15 min changes in propylene oxide, all steps at 4°C, and embedded in Spurr's epoxy resin (Spurr 1969). Sections were cut on a diamond knife using a Porter Blum MT-1 ultramicrotome, mounted on uncoated 200-mesh grids, and post-stained with uranyl acetate and lead citrate. Specimens were examined and photographed using a Phillips EM 300 transmission electron microscope.

Light microscopic observations of zoosporangia in the thallus were made using 1% methylene blue-stained thick plastic sections (0·5–1·0 $\mu$m) of embedded materials examined with electron microscopy, and recorded on an Olympus Model AH photomicroscope. Living algal cells were examined in squash preparations of the thallus and photographed using a Zeiss Universal photomicroscope equipped with Nomarski optics.

## Results

Various stages of zoosporogenesis were observed among algal cells in squashed preparations of thallus fragments and soredia that were examined from natural populations throughout the year of study, but most of the cells were in the vegetative condition. During the periods October–early December and April–June, more cells were involved in the process of zoosporogenesis than in the remaining months.

Up to eight zoospores were present in mature zoosporangia. Representative data for these periods are seen in our observations of thallus samples collected on 19 May 1978, which revealed among a total of 369 randomly selected cells the following frequencies: zoosporangia (four or more cell segments) 22 (6·0%); aplanosporangia 26 (7·0%); vegetative cells 321 (87·0%). In the periods July–September and January–March, very few division stages were evident in the *Trebouxia* cells, and those seen were primarily the beginning stages of division, i.e., first and second cleavages. Of 184 randomly selected cells observed on 26 September 1977, only one aplanosporangium was recorded, the remaining cells being vegetative.

What appeared to be a seasonal influence on zoosporogenesis could not be correlated with long-term climatological data for the collection site (see Table 1). More importantly, however, we did not find any correlation between the degree of zoosporogenesis and short-term hydration patterns, as was expected. We were particularly sensitive to the possibility that more algal cells would be dividing in the thallus following periods of sustained rainfall and generally made collections every two days during these periods. The 26 September 1977 observations reported above followed eleven days of continuous rain, and as seen during other rainy periods in July–September and January–March, nearly all cells were in the vegetative condition.

Thallus fragments and soredia that were incubated on mineral medium in the laboratory contained algal cells that commonly formed zoosporangia within 3–11 days and free-swimming zoospores could be seen in crushed thallus preparations. The number of zoospores produced in a sporangium varied up to a maximum of 64 (Figs 13–14). Similar thallus fragments that were incubated in distilled water and on soil-extract agar exhibited algal cells in which zoosporogenesis was initiated but not completed during the period of incubation (11 days). A comparison between thallus fragments that were pre-incubated and fragments placed directly into mineral media did not reveal any significant differences in the frequency of algal zoosporogenesis. Division of the algal cells in the incubated material was seen first in the soredia and algal layer fragments and later in pieces of the whole thallus. There was no

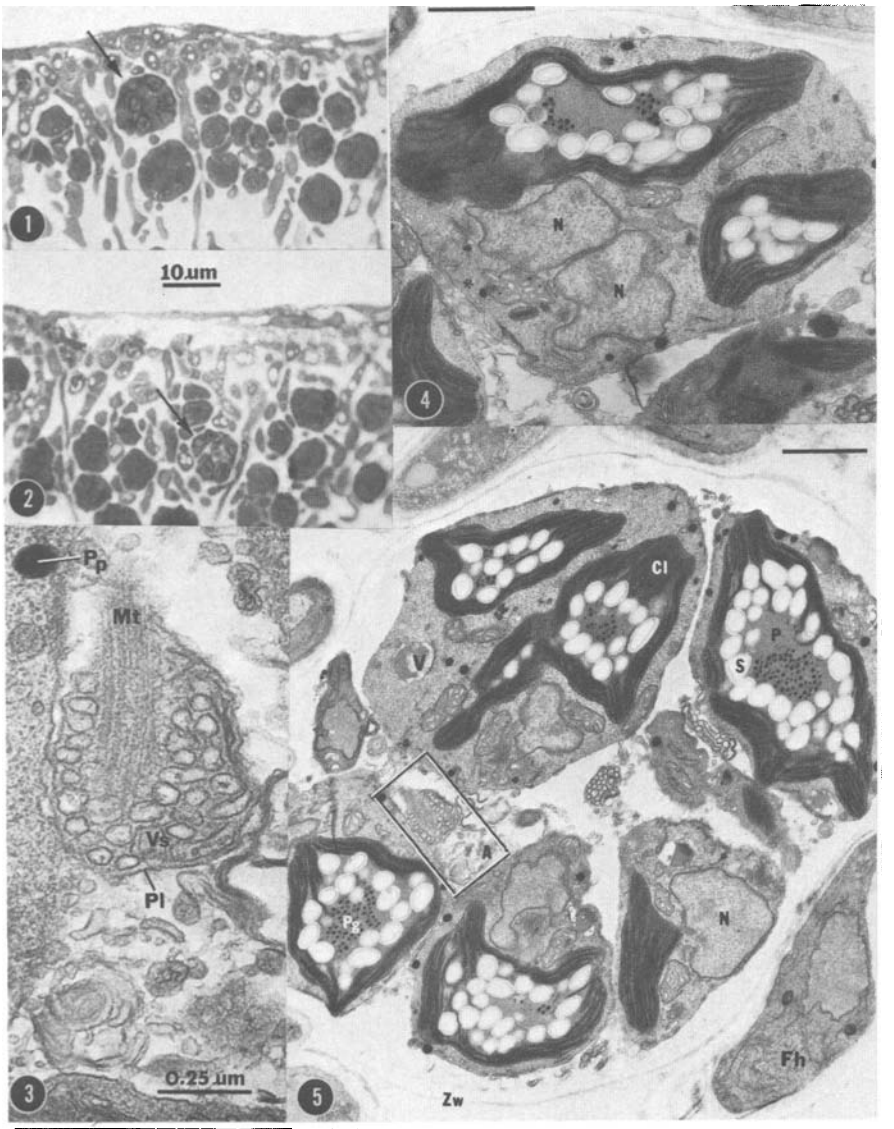
TABLE 1. *Climatological data for Worcester, Massachusetts during 1977–1978 Study Period (U.S. National Weather Service, Worcester, Mass.).*

| Month | Average temperature (°C) | Average precipitation (cm) | Relative Humidity (hr 7/Hr 13) (%) | Cloud cover (Days) Clear/Partly Cloudy/Cloudy |
|---|---|---|---|---|
| 1977 | | | | |
| O | 9·6 | 16·1 | 75/59 | 7/8/16 |
| N | 5·1 | 9·4 | 77/64 | 4/5/21 |
| D | −3·3 | 12·7 | 71/60 | 9/5/17 |
| Average | 3·8 | 12·7 | 74·3/61·0 | 6·7/6·0/14·7 |
| 1978 | | | | |
| J | −6·1 | 25·2 | 70/61 | 6/8/17 |
| F | −6·7 | 5·3 | 62/46 | 11/7/10 |
| M | −0·5 | 8·2 | 62/47 | 6/13/12 |
| Average | −4·4 | 12·9 | 64·7/51·3 | 7·7/6·0/13·0 |
| A | 5·8 | 5·7 | 60/43 | 8/7/15 |
| M | 13·6 | 9·4 | 75/53 | 4/13/14 |
| J | 17·9 | 4·0 | 70/55 | 9/12/9 |
| Average | 12·4 | 6·4 | 68·3/50·3 | 7·0/10·7/12·7 |
| J | 20·2 | 9·1 | 73/57 | 7/10/14 |
| A | 20·1 | 12·7 | 81/70 | 3/8/20 |
| S | 14·2 | 2·6 | 79/56 | 11/10/9 |
| Average | 18·2 | 8·1 | 77·7/61·0 | 7·0/9·3/14·3 |

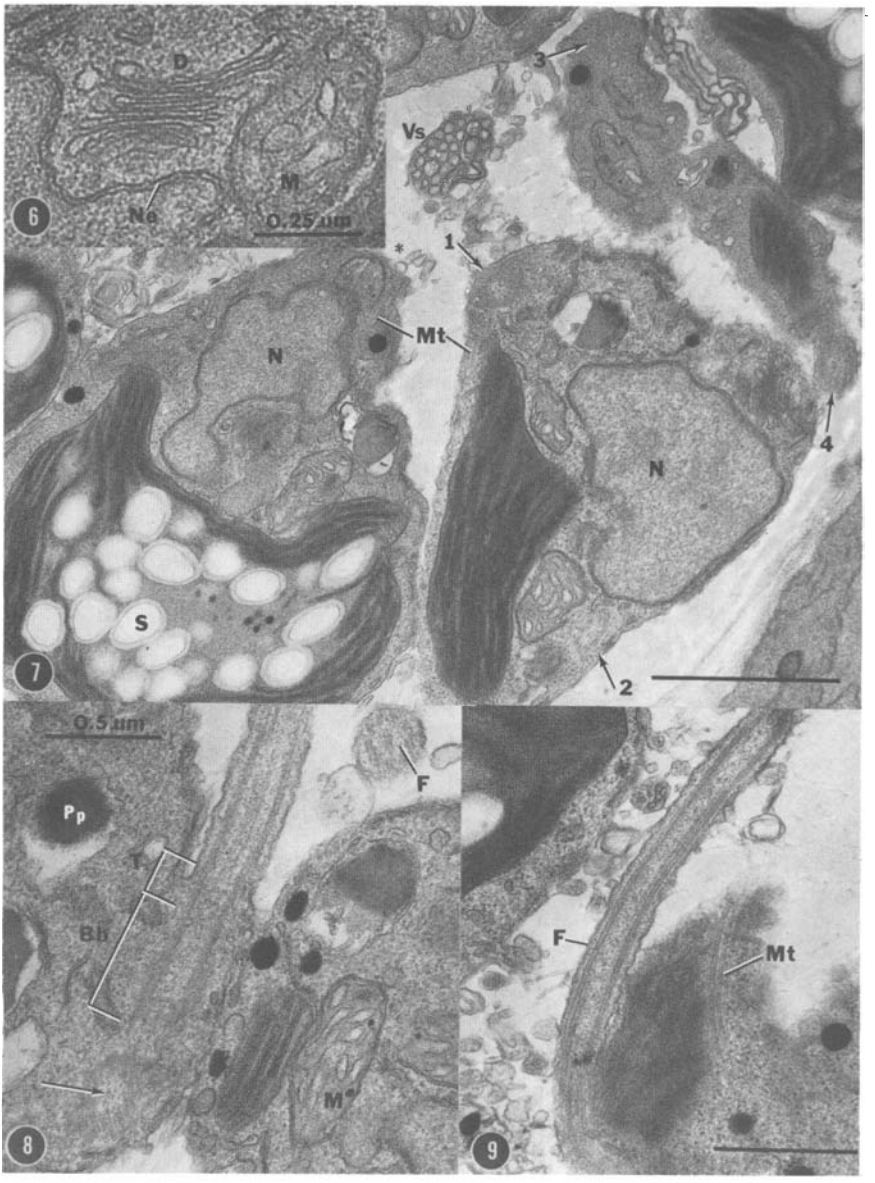
demonstrable relationship between zoosporogenesis and either temperature or photoperiod regimes employed in this study.

In transverse sections of the lichen thallus, zoosporangia were located in the uppermost portions of the algal layer, surrounded by closely-appressed fungal hyphae (Figs 1–2). Zoosporangial cells measured 8–10 $\mu$m diam and typically contained eight zoospores per cell, each zoospore approximately 3–4 × 5–6 $\mu$m in size and exhibiting a subellipsoidal morphology. The multicellular zoosporangia were readily differentiated from the vegetative cells at the light microscope level and zoospores were further distinguishable from aplanospores as a result of an intense staining of their plastids and lack of a cell wall. Zoosporogenesis apparently is not synchronized to any extent within a given region of the thallus, since vegetative cells surrounding a zoosporangium showed no indication of zoospore formation.

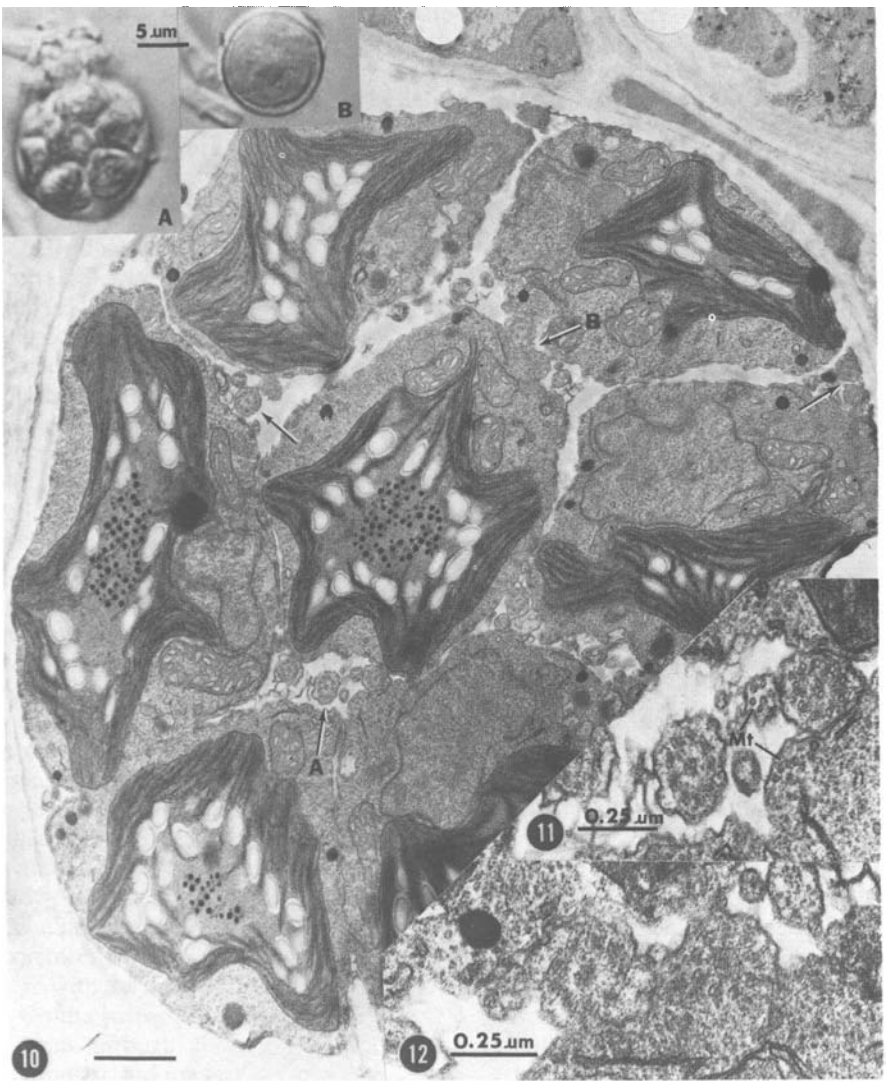
At the ultrastructural level, the intimate nature of fungus-alga contact is evident (Fig. 5) and contact is generally of an appressorial nature, with haustorial penetration of the zoosporangium or adjacent vegetative cells being observed only infrequently. Zoospores initially appear to be at different stages of development within a



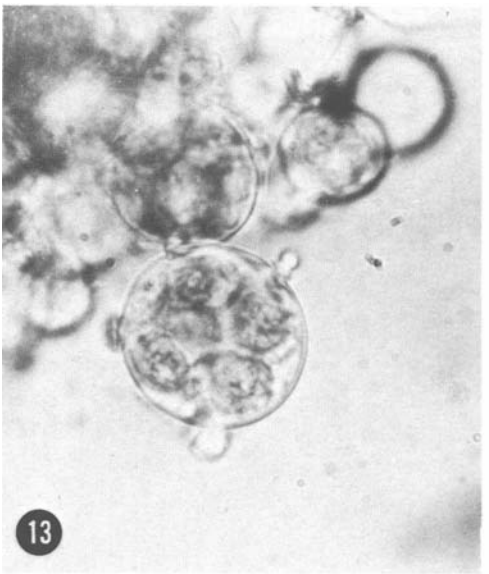

FIGS 1–5. FIGS 1–2. Light micrographs; thick plastic sections of *Parmelia caperata* thallus. Algal zoosporangia 'arrows' are located in the medullary region just below the upper cortex. FIGS 3–12. TEM micrographs of *Trebouxia gelatinosa* zoospores. FIG. 3. Enlargement of bordered area A in Fig. 5 showing distal peripheral flagellar fibrils terminating in the vesiculate structure enclosed within the flagellar membrane; note extra-cellular membraneous debris. FIG. 4. Serial section through zoospore initial seen at upper left in Fig. 5, showing adjacent nuclear profiles proximal to basal body region indicated by asterisk, but not seen in this plane of sectioning which passes through the two flagella. FIG. 5. Zoosporangium surrounded by tightly-appressed fungal hyphae; zoospores, in various stages of development, exhibit typical lobate plastids with large, central pyrenoids, starch granules and pyrenoglobuli; prominent dictyosomes are associated with the nuclei; naked zoospores are pulled away from the parent cell wall; the large cell in upper left has completed chloroplastic and nuclear division prior to cytokinesis. Enlargement of bordered region A is shown in Fig. 3.

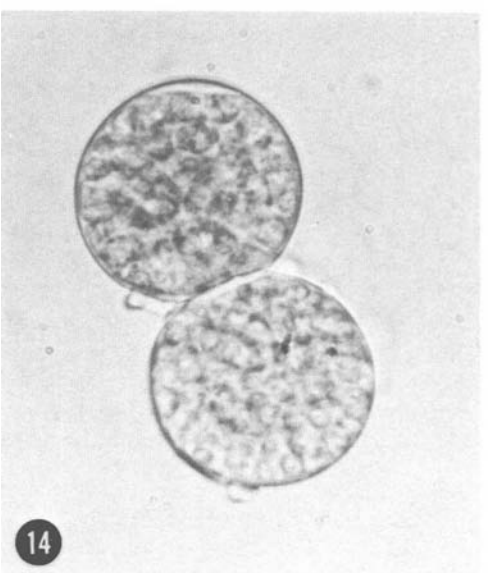



FIGS 6–9. FIG. 6. Typical dictyosome occurring in a depression on the surface of the nucleus. FIG. 7. Enlargement of part of Fig. 5 showing basal body profiles (arrows) in two zoospore daughter cells; in serial sections, an additional basal body was observed at the position indicated by the asterisk; the associated microtubular rootlet complex is seen in these cells. FIG. 8. Longitudinal section through the transition-basal body region of a zoospore flagellum; basal body region of second flagellum is seen in oblique transverse section (arrow). FIG. 9. Near median longitudinal section through the flagellum proper, and adjacent cytoskeletal microtubules.




FIGS 10–12. FIG. 10. Algal zoosporangium with appressed fungal hyphae; numerous flagellar profiles are seen in transverse section (arrows); arrows A and B enlarged in Figs 11–12 respectively. Insets: Nomarski interference light micrographs of an aplanosporangium (Fig. 10A) and typical vegetative cell showing large central pyrenoid (Fig. 10B) and illustrating usual sequence for lichenized *Trebouxia*, from zoospore arrest to aplanospore release. FIG. 11. Transverse section through a flagellum shown at arrow A in Fig. 10; note typical 9 + 2 fibril arrangement and cytoplasmic microtubules. FIG. 12. Transverse section through transition-basal body region of a pair of flagella shown at arrow B in Fig. 10.




FIGS 13–14. FIG. 13. Squash preparation of natural thallus, incubated in mineral medium, showing eight-celled zoosporangium. Note appressorial hyphae. FIG. 14. Sixty-four-celled zoosporangium from similarly incubated natural thallus.

**Abbreviations used in the figures:** Bb, basal body; Cl, chloroplast; D, dictyosome; ER, endoplasmic reticulum; F, flagellum; Fh, fungal hypha; M, mitochondrion; Mt, microtubule; N, nucleus; Ne, nuclear envelope; P, pyrenoid; Pg, pyrenoglobuli; Pl, plasmalemma (or flagellar membrane); Pp, polyphosphate granules; S, starch; T, transition region; V, vacuole; Vs, vesiculate structure; Zw, zoosporangium wall. Scale = 1 $\mu$m unless otherwise indicated.

given sporangium (Fig. 5), with cell division occurring via a successive bipartitioning of the zoospore parent cell. Zoospores are, at first, loosely surrounded by the parent cell wall and the lumen of the sporangium is filled with membraneous cytoplasmic debris. In Fig. 10, showing a later stage of development, zoospores are slightly larger and closely appressed to one another, occupying the entire volume of the zoosporangium. It is at this stage that zoospores presumably retract their flagella and secrete a wall, entering the aplanosporic condition illustrated in Fig. 10A. Upon release from the aplanosporangium, these cells eventually obtain the typical 5–10 $\mu$m vegetative cell diameter (Fig. 10B). No evidence of a zoospore exit pore or other discharge structure was observed in the parent cell wall.

Individual zoospores contain a prominent axial plastid which exhibits a large, centrally-located pyrenoid traversed by thylakoids that are characteristically bordered by pyrenoglobuli. In no instance was an eyespot observed in the zoospore chloroplasts. Large starch granules are embedded within the pyrenoid matrix and starch accumulation appears to a much greater extent in these cells as compared to adjacent vegetative cells. The zoospore has a single lobate nucleus positioned laterally or anteriorly to the chloroplast. Invariably, one to several dictyosomes per cell are located within depressions on the surface of the nucleus (Fig. 6), and endoplasmic reticulum elements (Fig. 4) typically border the plasmalemma, which delimits the wall-less protoplasts. Each cell contains numerous mitochondrial profiles exhibiting somewhat dilated, saccate cristae. Numerous electron-dense polyphosphate granules are observed in these cells (Figs 3, 8) and vegetative cells, undoubtedly a result of their incubation in phosphate-buffered solutions prior to fixation (see Fisher 1971). Of particular interest is the 'vesiculate structure' (Fig. 3) in which the flagella terminate at their distal ends. In this structure, the peripheral and central flagellar fibrils distend into a mass of membrane-bounded vesicles enclosed within the flagellar membrane. Each zoosporangium exhibits numerous profiles of these structures, measuring 0·5–0·8 $\mu$m wide by as much as 2·0 $\mu$m long, giving the flagellar tips a swollen, bulbous appearance. The two equal length flagella of the zoospore (Figs 8–9) were otherwise similar in organization to those of other chlorophycean algae. The axoneme exhibits a typical 9 + 2 arrangement of the central and peripheral fibrils in transverse section (Fig. 12). In the transition-basal body region of the flagellum, seen in transverse section in Fig. 11 (see also Fig. 8), the nine peripheral doublets are interconnected by a nine-pointed star-shaped structure (Fig. 11). Broad microtubular rootlets are frequently observed in association with the basal bodies, as seen in Fig. 7.

On the basis of light microscope observations and also a limited number of serial sections through zoospores in various stages of development, it appears that chloroplast and nuclear division occur somewhat synchronously (Fig. 4), followed by cytokinesis. Flagella apparently are not retracted prior to the onset of cell division. The chloroplast first divides into two halves which remain juxtaposed at the longitudinal axis of the cell. Following nuclear division, a pair of daughter nuclei are also located one on either side of this axis just posterior to the two basal bodies (see Fig. 4). We have been unable to observe actual mitotic figures during zoosporogenesis to date, thus it is not yet clear whether this alga has a closed mitotic spindle or if the spindle is centric or acentric. There is little doubt, however, that cytokinesis is characterized by the development of a phycoplast initiated between the basal bodies at the anterior ends of the dividing daughter zoospores, the perpen-

dicular cleavage furrow partitioning a single nucleus and plastid segment in each zoospore. Evidence for this event is seen in Fig. 7 in which cytokinesis has been recently initiated (between asterisk and arrow 1), with one basal body going to each cell. Prior to cytokinesis, the daughter nuclei lie close to each other (Fig. 4), suggestive of a collapsing spindle.

## Discussion

One of the characteristics of the genus *Trebouxia* (Chlorococcales), as contrasted with *Pseudotrebouxia* (Chlorosarcinales), is the absence of desmoschisis, or true vegetative cell division (Archibald 1975). The only form of cell division that exists in *Trebouxia* is zoosporogenesis. Under natural conditions, naked zoospores are produced in the zoosporangium, but the vast majority of these cells eventually become aplanosporic (i.e. they round up and secrete a cell wall). Aplanospores are subsequently released from the sporangium where they enlarge as vegetative cells. In view of the large number of algal cells in a given lichen thallus (estimated 5000–7500 cells $mm^{-3}$ of medullary agal layer in this study), all of which are presumably derived from aborted zoospores, it seems clear that the process must be of common occurrence in this lichen alga in nature. Inasmuch as zoosporogenesis is of central importance to the reproductive success of this alga, it is surprising that this phenomenon has not been more extensively investigated. The present study of zoosporogenesis in *T. gelatinosa* has revealed several interesting features of this process which may be more broadly applied to the study of other *Trebouxia*-containing lichens.

### 1. Observations of zoosporogenesis

While zoospores and zoosporangia of cultured *Trebouxia* isolates have been previously seen in electron micrographs (Ahmadjian 1967a: 23; Jacobs & Ahmadjian 1971), details of zoosporogenesis in this genus have not been published. Furthermore, the present study is the first *in situ* ultrastructural documentation of zoosporogenesis for any lichenized alga.

The general morphology of the biflagellate zoospores and the major features of zoosporogenesis in this alga, as seen with electron microscopy, were in agreement with light microscopic observations of Ahmadjian (1967b). Zoospores were completely devoid of a cell wall. The absence of an eyespot in zoospore plastids, at the ultrastructural level, supports Archibald's (1975) observations for this algal species, although she reported the presence of this structure in several other *Trebouxia* species, in contrast with Starr's (1955) finding that zoospores of this genus lacked eyespots. It was noted, however, that the very small size of these structures may render them difficult to observe in many species using light microscopy. In addition, Jacobs & Ahmadjian (1971) demonstrated an eyespot in electron micrographs of *T. erici* zoospores, but only when this alga was cultured in an inorganic medium.

The dilated cristae of mitochondria observed both in zoospores and vegetative cells may be explained as a result of hydration of the thallus. This mitochondrial morphology is not seen in these cells under normal conditions, and can be induced by hydration of desiccated thalli. A similar phenomenon has been observed under

anaerobic conditions following prolonged hydration of *Triticale* root cells (Oliveira 1977).

Dictyosomes were much more prevalent in zoospores than in the surrounding vegetative algal cells. Profiles of this organelle appeared to be restricted to regions of the nucleus with the forming face promimal to the nuclear envelope. Interestingly, Brown & Bold (1964) reported an increased number of dictyosomes during the morphogenesis of *Chlorococcum* zoospores, which, unlike the naked zoospores of *Trebouxia*, produce a cell wall. Zoospores of *Trebouxia* also produce a cell wall upon encystment, shortly after release from the sporangium, and the golgi apparatus may play a role in this process at that time.

We are unaware of previous reports of the 'vesiculate structure' for flagella of other algae, although a morphologically similar structure has been reported at the distal ends of the so-called 'club-foot' cilia of various representatives of the sponges (Bergquist *et al.* 1977). At the light microscopic level, we have observed bulbous swellings at the distal ends of the flagella of a number of green algae prior to the shedding or resorption of these organelles; it is entirely possible, however, that these swellings are not structural analogs of the vesiculate structure seen in electron micrographs of *Trebouxia* zoospores, but rather represent regions of localized pressure build-up in the flagellum, as has been suggested for the palmelloid-forming mutants of *Chlamydomonas eugametos* (Nakamura *et al.* 1978). In his excellent discussion of the resorption of flagella in various motile cell types, Bloodgood (1974) indicated that flagellum retraction often involved the retraction of the axoneme with the concomitant incorporation of the flagellar membrane into the plasmalemma. Evidence for the latter phenomenon, with few exceptions, came from observations that flagellar mastigonemes were dispersed over surfaces of certain cells upon encystment, and also that membrane-like structures were seldom seen in the cell cytoplasm following retraction. In contrast, we propose that the vesiculate structure of *Trebouxia* zoospores would provide a means by which excess flagellar membrane could readily be taken up into the cytoplasm of an encysting cell. Alternatively, this structure could serve to distribute new membrane to a rapidly elongating flagellum during zoosporogenesis.

Cell division events during zoosporogenesis could not be elucidated with certainty from the limited number of sections examined, a problem which stemmed from our inability to produce *in situ* synchronization of zoosporogenesis in this alga. A number of micrographs, however, suggest that cell division during zoosporogenesis in *Trebouxia* shares certain similarities, and at the same time a few differences, with events recorded for other chlorococcalean algae (e.g. Pickett-Heaps 1975). Actual mitotic events, as was previously mentioned, are not yet established, though it appears that the spindle does not persist at teleophase. A phycoplast forms during cytokinesis and the cleavage furrow is initiated between the anterior basal body pair. Zoosporogenesis in *Trebouxia* and *Pseudotrebouxia* is currently being investigated in culture and it is hoped that these studies will provide a better understanding of cell division events in these algae.

Molnar *et al.* (1975) suggested that *Trebouxia* may be a morphologically reduced form of a *Pleurastrum*-like filamentous alga, primarily on the basis of ultrastructural similarities in the pyrenoids of the two algae, but also because of the *Trebouxia*-like appearance of single-celled individuals in polymorphic *Pleurastrum* cultures and preliminary data indicating that certain cell division events were shared by each. Our

study of zoosporogenesis in *Trebouxia*, though incomplete, indicates that there may indeed be similarities in the cell division events of these algae. Each exhibits both a collapsing spindle and a phycoplast formed during cytokinesis, with the cleavage furrow initiated between the two basal bodies (see Molnar *et al.* 1975). It should be noted, however, that in the above study, cell division events were examined in *Trebouxia impressa* Ahm. (Mattox, *pers. comm.*), a species subsequently transferred to *Pseudotrebouxia* (*P. impressa* (Ahm.) Archibald), as it exhibits true vegetative cell division (Archibald 1975). While an interesting comparison can be made between *Pleurastrum* and *Pseudotrebouxia*, both of which exhibit desmoschisis and similar ultrastructural features, we think that *Trebouxia* is probably not closely related to these genera as its representatives do not exhibit desmoschisis. Certainly, additional representatives of the three genera need to be examined before more definitive ideas can be set forth regarding the evolution of these large groups of lichen algae.

## 2. Factors inducing zoosporogenesis

We observed mature zoosporangia among the algal population of a thallus during certain times of the year and thallus fragments incubated in a mineral medium formed zoospores within a few days. What appeared to be a seasonal pattern in zoosporogenesis of the natural phycobiont could be due to several factors, one of which is the moisture content of a thallus and also the duration of hydration. Although this is not apparent from either short or long-term climatological data for the collecting area during the study period, we nonetheless feel that hydration is the single most important factor in the initiation of zoosporogenesis. We have found that prolonged hydration of the thallus is prerequisite to the formation of zoospores in the *Trebouxia* cells and even distilled water is sufficient to elicit this response, though zoospore maturation appears to be dependent upon other factors (see discussion below). The large number of starch grains observed in developing zoospores and vegetative cells (Figs 4–5,10) are evidence for a sustained high moisture content in the thallus prior to zoosporogenesis (Jacobs & Ahmadjian 1971).

Aside from hydration effects *per se*, another factor that might stimulate zoosporogenesis or be important in zoospore maturation is mineral availability. We found that the algae in thallus fragments incubated in mineral solution readily formed free-swimming zoospores within several days after incubation, while *Trebouxia* cells in fragments incubated in distilled water initiated zoosporogenesis but did not release zoospores during the study period. Experiments have shown that lichens can absorb ammonium and nitrate from simulated rain water (Lang *et al.* 1976), and uptake of these compounds might be important in the process of zoosporogenesis. We need additional data on the availability of nutrients to the lichen at various times of the year. In addition, the asynchronization of zoosporogenesis in the thallus (Figs 1–2), where all algal cells would be expected to experience similar photoperiod and water availability, might reflect microenvironmental differences in nutrient flux. Support for this theory may be found in the observation that zoosporogenesis was induced in *Trebouxia* cells in soredia and pieces of medulla well before this process was seen in intact thallus fragments. The intact fungal cortex undoubtedly presents a barrier to or removes some nutrients from solution before they reach the algal cells, at least during the initial stages of hydration.

There appears to be no correlation between zoosporogenesis and the temperature

and light regimes used in this study. Furthermore, zoosporogenesis was observed either directly or indirectly (i.e. aplanospores) in all months of the year in the natural *Trebouxia* population, suggesting that these parameters may not be instrumental in the initiation of zoosporogenesis.

We still know virtually nothing about the various factors influencing zoosporogenesis in *Trebouxia* in nature, however, it would seem that a higher degree of synchronization of this process, which ultimately is responsible for the reproduction of this alga in thallus populations, would be necessary to accomodate the rapid growth conditions reported for *Trebouxia*-containing lichens in the late spring and again in the fall months in temperate environments (e.g. Hale 1970; Showman 1976; Lawrey & Hale 1977). Our observations on the frequency of zoosporogenesis in a natural population during these periods support this hypothesis.

### 3. Potential for zoospore release

Our findings suggest that under certain, as yet undefined, conditions, some algal cells in a natural lichen thallus do complete the process of zoosporogenesis and release zoospores into the surrounding microenvironment. This would seem to be supported by reports of free-living *Trebouxia* (Tschermak-Woess 1978). Once freed, *Trebouxia* zoospores, which have a maximum width of 4–5 $\mu$m, could be readily accommodated by the large cracks in the cortex, measuring tens of microns or more. Such cracks are common features of lichen thalli, which are generally very brittle when dry. Observations of zoosporangia proximal to the upper cortex in *Parmelia caperata* further suggest that released zoospores would be required to swim only relatively short distances to the thallus surface.

The actual escape mechanism of zoospores from the sporangium in a thallus is not known, but would appear to involve the simple rupture of the zoosporangial wall. There is no ultrastructural evidence of a discharge apparatus or similar modification of the parent cell wall as was reported for sporangia of *Trebouxia erici* (Jacobs & Ahmadjian 1971).

Despite the potential avenues of zoospore escape from the thallus, it would seem that the intimate nature of fungus-alga contact in the lichen would represent a considerable barrier to zoospore release. There did not, however, appear to be any fungal restriction on the ability of an algal cell to form zoospores. Tschermak-Woess (1978) noted that *Trebouxia* cells associated with tightly-appressed fungal hyphae, either in free-living colonies or in the lichen thallus, produced only aplanospores, whereas algal cells entirely free of fungal contact formed abundant zoosporangia and released free-swimming zoospores. This evidence appears to indicate that the degree of fungal contact and perhaps physical constraint imposed on the sporangium by closely-appressed hyphae may be factors in determining the actual release of zoospores, even under optimal conditions for the initiation of zoosporogenesis. As few algal cells in a thallus would be expected to be completely free of contact with the fungus, this observation might explain the relatively large number of algal cells that eventually become aplanosporic and, concomitantly, a smaller incidence of free-living *Trebouxia* populations.

### 4. *Trebouxia* as a free-living alga

The few zoospores released from a thallus could potentially form free-living

microcolonies of *Trebouxia* which then could re-enter into lichenized associations with mycelia derived from various types of reproductive propagules of either the same or, perhaps, genetically unrelated fungi. Tschermak-Woess (1978) observed free-living colonies of *Myrmecia* and *Trebouxia* near lichen thalli which contained these algae as phycobionts. She suggested that such algae could unite with the germinated propagules of the mycobiont to establish new lichens. In addition, sexual reproduction by motile gametes of this alga (Ahmadjian 1967b) would be facilitated in the free-living condition, although the union of gametes within the thallus cannot be precluded. New strains of *Trebouxia* might thus be established outside the lichen thallus. Sexuality in these algae has been reported from cultural studies but its occurrence was not common (Ahmadjian 1959).

The evolution of the lichen symbiosis is a subject of considerable speculation both because of a poor fossil record and our ignorance of the basic genetic processes which govern these associations. Studies on the chemical variation in morphologically very similar taxa strongly suggest that some type of hybridization must take place between lichenized fungi, although the mechanism for such hybridization is not known. In the case of the *Ramalina siliquosa* species complex, algae isolated from different chemical races grown *in vitro* did not exhibit cultural variations that could be associated with the chemical variations in donor lichens (Culberson 1969). Brodo (1978) has suggested that hybridization between lichenized fungi might occur as a result of diploidization through hyphal fusions in the mycelia of adjacent thalli.

Certainly, the establishment of free-living forms of *Trebouxia* and *Pseudotrebouxia*, phycobionts that may be encountered in as many as half of all known lichen associations (Ahmadjian 1970), would suggest a second possibility. Since the ascospores of lichen mycobionts appear to be products of genetic recombination (Ahmadjian 1964), such spores could combine with free-living colonies of a given algal strain to produce the genetic heterogeneity that characterizes the fungi of many lichen populations. The implications of a free-living *Trebouxia* go further, in that a given fungal strain could also associate with many different algal strains independently. Recently, it has been shown, in lichen associations involving a single mycobiont and discrete green and blue-green algal populations, that each alga is capable of influencing not only thallus morphology but possibly the biosynthesis of certain lichen substances (James & Henssen 1976). Consequently, the observed morphological and biochemical variations in *Trebouxia*-containing lichens and lichen populations, in general, may reflect differences in fungal genotypes as well as possible algal modification of gene expression in the mycobiont. Laboratory studies of lichen re-synthesis on natural substrates (Ahmadjian 1980) have demonstrated that algal and fungal symbionts which have been artificially separated and grown in culture can recombine to form lichen thalli with the same morphological and chemical attributes of the naturally-occurring morph. These studies have also shown that one mycobiont can form thalli with different species of *Trebouxia* or *Pseudotrebouxia* phycobionts.

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