Symbiosis, 33 (2002) 215–226
Balaban, Philadelphia/Rehovot

Pestalotiopsis maculans: A Dominant Parasymbiont in North American Lichens

HENRY J. SUN[1,4*], PAULA T. DEPRIEST[2], ANDREA GARGAS[2,5], AMY Y. ROSSMAN[3], and E. IMRE FRIEDMANN[1,6]

[1]*Polar Desert Research Center and Department of Biological Science, Florida State University, Tallahassee, FL 32306-1100, USA;*
[2]*Botany, MRC-166, United States National Herbarium, National Museum of Natural History, Smithsonian Institution, P.O.Box 37012, Washington, DC 20013-7012, USA, Tel. +1-202-357-2545, Fax. +1-202-786-2563, Email. depriest.paula@nmnh.si.edu;*
[3]*Systematic Botany and Mycology Laboratory, USDA-ARS, Beltsville, MD 20705, USA, Tel. +1-301-504-5364, Fax. +1-301-504-5810, Email. amy@nt.ars-grin.gov;*
[4]*Present address: Jet Propulsion Laboratory, Mail Stop 183-301, California Institute of Technology, Pasadena, CA 91109-8099, USA, Tel. +1-818-354-0600, Fax. +1-818-393-4445, Email. henry.j.sun@jpl.nasa.gov;*
[5]*Present address: Department of Botany, University of Wisconsin, Madison, WI 53706, USA, Tel. +1-608-262-8644, Fax. +1-608-262-7509, Email. agargas@facstaff.wisc.edu;*
[6]*Present address: Space Science Division, 245-3, NASA Ames Research Center, Moffett Field, CA 94035, USA, Tel. +1-605-604-1136, Fax. +1-605-614-6779, Email. ifriedmann@mail.arc.nasa.gov*

Received April 25, 2002; Accepted November 12, 2002

Abstract

By culturing small thallus portions in nutrient medium, we showed that *Pestalotiopisis maculans* (Corda) Nag Raj is a dominant parasymbiont (secondary fungus) in North American lichens. *P. maculans* was present in all twelve lichen specimens (10 *Cladonia*, 1 *Usnea*, and 1 *Parmetroma*) studied in the eastern North

*The author to whom correspondence should be sent.

0334-5114/2002/$05.50

America between Ontario, Canada and Oaxaca, Mexico. In each lichen *P. maculans* was present throughout the length of the thallus. Cultures of excised tissue samples revealed that in the *Cladonia* thallus *P. maculans* is confined to the medulla, but not in direct contact with the photobiont cells contained therein. When growing in pure culture, *P. maculans* and the mycobiont *Cladonia subtenuis* show different hyphal morphologies in the environmental scanning electron microscope, but these characteristics are not present within the lichen thallus. Twenty-one lichens collected in Germany, the Canary Islands, New Zealand, and Israel contained other secondary fungi (but not *Pestalotiopsis*) with variable abundance and relatively narrow geographic distribution.

Keywords: Lichenicolous fungi, lichen symbiosis, *Pestalotiopsis*

1. Introduction

According to the classical definition of Schwendener (1868), lichens are symbiotic associations between a fungus, the mycobiont, and an alga or a cyanobacterium, the photobiont. Subsequent studies revealed that while most lichens contain only one fungus, i.e., the mycobiont, some have additional (secondary) fungi, recognizable by their pigmentation or morphology under the light microscope. Thus, the dark-pigmented dematiaceous secondary fungi contrast with the hyaline hyphae of their host (e.g. Lindsay, 1869; Clauzade and Roux, 1976; Hawksworth, 1979, 1981, 1982, 1988). While many lichenicolous fungi are parasitic, i.e. induce gall-like malformations or discolorations of the lichen thallus, some cause no apparent symptoms in the host and have been termed parasymbionts by Zopf (1897). Hawksworth (1988) suggested that parasymbiont-containing lichens are, in reality, three-member symbioses, with two mycobionts and one photobiont.

Recent studies indicate that lichens contain more secondary fungi than is evident from microscopic examination. Seemingly "pure" lichen thalli, cleaved to small pieces (tens of microns to 0.25 cm^2) and incubated on solid media, yielded a large variety of micro-fungi. For example, Petrini et al. (1990) obtained 506 secondary fungi from seventeen lichen specimens collected on a forest floor in Germany. Girlanda et al. (1997) isolated 117 fungal strains from two foliose lichens (*Parmelia taractica* and *Peltigera praetextata*) collected from a coniferous forest in Italy. Möller and Dreyfuss (1996) cultured 59 fungi from sixteen lichen species collected on King George Island, Antarctica. Many, if not all, of these isolates should be considered true secondary fungi in lichens.

The present study originated from an unexpected observation: while isolating mycobionts of some North American *Cladonia* species, we found that their thalli contained the secondary fungus *Pestalotiopsis maculans* (Corda) Nag Raj. Further investigation of non-*Cladonia* lichens as well as lichens from other continents suggests that *P. maculans* is associated only with North American lichens. The finding of secondary fungi in lichens, as discussed above, is not new. What is new is that the same secondary fungus was present in all parts of the lichen thallus and in specimens from a wide range of geographic localities. We suggest that *P. maculans* be considered a dominant parasymbiont, a secondary fungus present in many or most lichens within a certain geographic area.

2. Materials and Methods

Lichens were collected in North America (from Ontario, Canada, to Oaxaca, Mexico), Germany (Schleswig-Holstein), Israel, the Canary Islands, and New Zealand between 1993 and 1997. The lichens and localities are listed in Table 1.

Isolation and culturing

Lichen thalli were surface-disinfected by immersion in 0.5% phenol solution for one minute, followed by washings in 0.5% Tween detergent solution and three changes of sterile water. Under aseptic conditions, thalli were then cut into 3–5 mm^2 pieces. Fifty pieces were randomly selected and incubated on Malt Yeast Extract (MYE) agar plates at 20°C. Pure cultures were obtained by transferring emerging colonies to fresh medium. This method differs from those used by Petrini et al. (1990), Girlanda et al. (1997) and Möller and Dreyfuss (1997). Attempting to isolate all secondary fungi present in the thallus, these authors cut specimens into segments small enough to contain only a single fungal species. In contrast, we used large thallus segments to ensure that each contained at least some portion of *P. maculans* which, having a faster growth rate, outgrew other fungi.

The distribution of *P. maculans* within the thallus of the lichen *Cladonia subtenuis* (Abbayes) Mattick was studied in some detail. *C. subtenuis* has a tubular thallus consisting of two concentric layers: medulla and stereome, but no cortex. Only the medulla contains photobiont cells, in clusters (Figs. 1C and D). To assess the distribution of *P. maculans* along the length of the thallus three complete thalli were cleaved into segments from tip to base and all segments cultured. To localize *P. maculans* within the thallus cross section, we excised and incubated: (1) tissue portions containing only stereome, (2) portions of the

Table 1. Secondary fungi isolated from lichens of various geographic regions

Secondary fungus	Locality	Lichen	Abundance*
Pestalotiopsis maculans (Corda) Nag Raj	Toronto, Ont., Canada	*Cladonia rangiferina* (L.) F. H. Wigg.	100%
	Mattapoisett, MA, USA	*Cladonia subtenuis* (Abbayes) Mattick	100%
	Gambrill's State Park, MD, USA	*Cladonia rangiferina*	100%
	Owings Mills, MD, USA	*Cladonia subtenuis*	100%
	Zebulon, NC, USA	*Cladonia mitis* Sandst.	100%
	Franklin Co., NC, USA	*Cladonia subtenuis*	100%
	Stone Mountain, GA, USA	*Cladonia subtenuis*	100%
	Tallahassee, FL, USA	*Cladonia subtenuis*	100%
	Tallahassee, FL, USA	*Cladonia leporina* Fr.	100%
	Tallahassee, FL, USA	*Parmotrema perforatum* (Jacq.) A. Massal.	100%
	Tallahassee, FL, USA	*Usnea strigosa* (Ach.) Eaton	100%
	Oaxaca, Mexico, USA	*Cladonia rangiferina*	100%
Diplodia mutila Fr.	Golan Heights, Israel	*Tornabaena* sp.	100%
Alternaria sp.	Judean Mountains, Israel	*Caloplaca aurantia* (Pers.) J. Steiner	100%
Alternaria sp.	Judean Mountains, Israel	*Squamarina gypsacea* (Sm.) Poelt	100%
Non-sporulating fungus	Judean Mountains, Israel	*Xanthoria parietina* (L.) Th. Fr.	88%
Penicillium sp.	Judean Mountains, Israel	*Ramalina* cf. *duriaei* (De Not.) Bagl.	50%
Trichoderma polysporum (Link:Pers.) Rifai	Southern Island, New Zealand	*Leifidium tenerum* (Laurer) Wedin.	100%
Trichoderma sp.	Christchurch, New Zealand	*Usnea* sp.	60%
Penicillium sp.	Christchurch, New Zealand	*Pseudocyphellaria colensoi* (Bab. ex Hook.f.) Vain.	50%
Non-sporulating fungus	Christchurch, New Zealand	*Pseudocyphellaria coronata* (Müll. Arg.) Malme	45%
Non-sporulating fungus	Christchurch, New Zealand	*Pseudocyphellaria glabra* (Hook.f. & Taylor) C.W.Dodge	30%

Table 1. Continued

Secondary fungus	Locality	Lichen	Abundance*
Mucor sp.	Santiago del Teide, Canary Islands	*Heterodermia* sp.	88%
Myrothecium sp.	La Caldera, Canary Islands	*Ramalina* sp.	50%
Penicillium sp.	La Palma, Canary Islands	*Lobaria pulmonaria* (L.) Hoffm.	46%
Myrothecium sp.	Santiago del Teide, Canary Islands	*Parmotrema* sp.	34%
Penicillium sp.	Santiago del Teide, Canary Islands	*Stereocaulon* sp.	26%
Non-sporulating fungus	La Caldera, Canary Islands	*Usnea* sp.	16%
Non-sporulating fungus	Santiago del Teide, Canary Islands	*Usnea* sp.	6%
Penicillium sp.	Kiel, Germany	*Cladonia coniocraea* (Flörke) Spreng.	76%
Penicillium sp.	Kiel, Germany	*Evernia prunastri* (L.) Ach.	66%
Trichoderma sp.	Kiel, Germany	*Cladonia pyxidata* (L.) Hoffm.	64%
Mortierella ramanniana (Möll.) Linnem.	Kiel, Germany	*Cladonia pyxidata*	6%

*Percent thallus portions (out of 50) yielding the secondary fungus.

medulla large enough to contain several clusters of algae, and (3) small portions of the medulla each with a single algal cell cluster, isolated with a micropipette as described by Ahmadjian (1967).

Microscopy

Lichen thalli and fungal cultures were studied with the environmental scanning electron microscope, or ESEM (E-3, Electro Scan Co.) at the National High Magnetic Field Laboratory in Tallahassee, Florida. ESEM is a relatively new microscopy technique, which, unlike conventional scanning electron microscopy, requires no pretreatment or carbon coating of samples and allows the viewing of living biological specimens in a hydrated state. Because of their different growth rates, parasymbiont and mycobiont cultures were observed after one week and one month of growth on agar medium respectively. Longitudinal sections of lichen thalli were mounted on agar blocks for observation. All observations were made at 5.8 torr vapor pressure, 15 Kev filament voltage, and 20°C.

3. Results

Diversity of isolated fungi

For each of the fifty segments from each of the twelve North American lichen specimens (*Cladonia mitis* Sandst., *C. rangiferina* (L.) F.H. Wigg., *C. subtenuis* (Abbayes) Mattick, *C. leporina* Fr., *Parmotrema perforatum* (Jacq.) A. Massal., and *Usnea strigosa* (Ach.) Eaton), *P. maculans* appeared within three days as a uniform colony surrounding the segment. This indicates that *P. maculans* outcompeted the mycobionts and other secondary fungi in the lichens under the growth conditions provided. Furthermore, *P. maculans* was present throughout the length of the thallus, a conclusion verified in *C. subtenuis* by cultures of segments cleaved from apex to base of three complete thalli. Names of host lichens, their localities, the isolated fast-growing secondary fungi, and the percentages of thallus segments in which they appeared are listed in Table 1.

Lichens from Germany, the Canary Islands, Israel, and New Zealand showed a different pattern (Table 1). Of the 21 lichen specimens studied, only four yielded a fast growing parasymbiont in all cultured thallus segments: *Alternaria* sp. and *Diplodia mutila* Fr. in three lichens from Israel, and *Trichoderma polysporum* (Link:Pers.) Rifai in one lichen from New Zealand. Unlike *P. maculans*, these parasymbionts appear in only some, but not all, specimens of lichens in their area. Cultures of the other 17 lichen specimens yielded less abundant fast-growing fungi present in 6–88% of the incubated thallus segments. Also, it is of interest to note that the lichen *Cladonia pyxidata* (L.) Hoffm. from Kiel, Germany, yielded two different parasymbionts: *Trichoderma* sp. in one specimen, and *Mortierella ramanniana* (Möll.) Linnem. in the second. In segments where these fast growing secondary fungi were absent, further incubation sometimes gave rise to additional 1–3 fungi before confluent growth overwhelmed the plate. Because the occurrence of these slower growing fungi was inconsistent, they were not included in our study.

Localization of P. maculans in Cladonia thallus

The thallus of *Cladonia* species is a hollow tube consisting of medulla and stereome (Figs. 1C and D). Cultures of pieces of medulla, large (about 0.25 mm^2) enough to contain several photobiont cell clusters, yielded the secondary fungus *P. maculans*. However, smaller pieces containing a single cluster yielded, after 4–6 weeks of incubation, either the mycobiont or the photobiont, but no *P. maculans*. This observation indicates that *P. maculans* is present in the medulla excluding the immediate vicinity of the photobiont cells. Thallus portions containing only stereome (excised from the middle region of the thallus where it is wide enough to permit such operations) showed no signs of growth even after long periods of incubation. This observation suggests that the stereome hyphae are not viable or do not grow on the nutrient medium provided, and *Pestalotiopsis* is absent.

Morphology of hyphae in culture and in the lichen thallus

Light microscopic observations of thin sections of *C. subtenuis* showed that hyphae in the medulla are thicker than those in the stereome. However, this difference does not seem to be related to the presence of *P. maculans*, as within each layer hyphae appear morphologically uniform.

In the ESEM, the mycobiont *C. subtenuis* and the dominant parasymbiont *P. maculans*, grown on agar plates, exhibited different hyphal morphologies. The mycobiont formed a dense network of "knobby" hyphae (white arrows) with frequent branchings, anastomoses, and rounded corners at the junctions (Fig. 1A). In contrast, hyphae of *P. maculans* formed a relatively loose network of smooth hyphae with less frequent branchings and anastomoses, and acute angles at the junctions (Fig. 1B). Hyphae in the mature lichen thallus do not resemble those of either the mycobiont or *Pestalotiopsis* grown in culture. Nor could we detect, despite a very thorough search, any morphological evidence of two different fungi either in the medulla or in the stereome. Hyphae in the lichen, oriented primarily along the longitudinal axis of the thallus, show much fewer branchings and anastomoses than does mycobiont or *Pestalotiopsis* hyphae in culture (Figs. 1C and D). Morphological differences between hyphae of the medulla (om) and stereome (im) are not well defined. Knobby hyphae were frequent in both medulla and stereome and the balloon-like inflated "knobs" are much larger than those in the cultured mycobiont (Figs. 1A and D, white arrows). Hyphae of the medulla are more loosely spaced and have a rough surface (Fig. 1C), particularly around photobiont cells (algae, Fig. 1C, p), perhaps due to deposition of secondary metabolites. The smaller hyphae between and around photobiont cells are probably haustoria (Fig. 1C, p). Hyphae of the stereome are more compacted and covered by an apparently mucilaginous material, which obscures their morphology.

4. Discussion

In this paper, we define "dominant parasymbiont" as a secondary fungus which: 1) is present in practically all parts of the lichen thallus; 2) is growing in intimate association with the primary symbionts without causing them any apparent harm ("parasymbiont" according to Hawksworth's 1982 definition); and 3) is present in most or all lichens in a geographic area.

Based on a limited number of specimens studied, we consider *Pestalotiopsis maculans* a dominant parasymbiont in North American lichens. *P. maculans* was found in all twelve lichens (three genera and six species) collected from nine localities in the eastern North American continent, ranging from Ontario, Canada to Oaxaca, Mexico. Detailed culture studies of *C. subtenuis* confirmed that *P. maculans* was indeed present in all portions of the lichen thallus. As we

Figure 1. ESEM micrographs. A: Surface of a colony of mycobiont *Cladonia subtenuis* grown on nutrient agar showing curved hyphae with knob-like swellings (white arrows), frequent branchings and anastomoses (black arrow: liquid water between filaments); B: Surface of a colony of dominant parasymbiont *P. maculans* grown on nutrient agar showing straight hyphae with no swellings and relatively fewer branchings and anastomoses; C: Longitudinal section of *C. subtenuis* thallus showing stereome (im) and medulla (om) with photobiont cells (p). In the sterreome hyphae are covered by a seemingly mucilaginous material. In the medulla hyphae have a rough surface (deposits of secondary metabolites?) and fewer branchings and anastomoses. D: Longitudinal section similar to C showing knob-like enlargements of hyphae in both medulla and stereome (arrows). Bar = 10 µm.

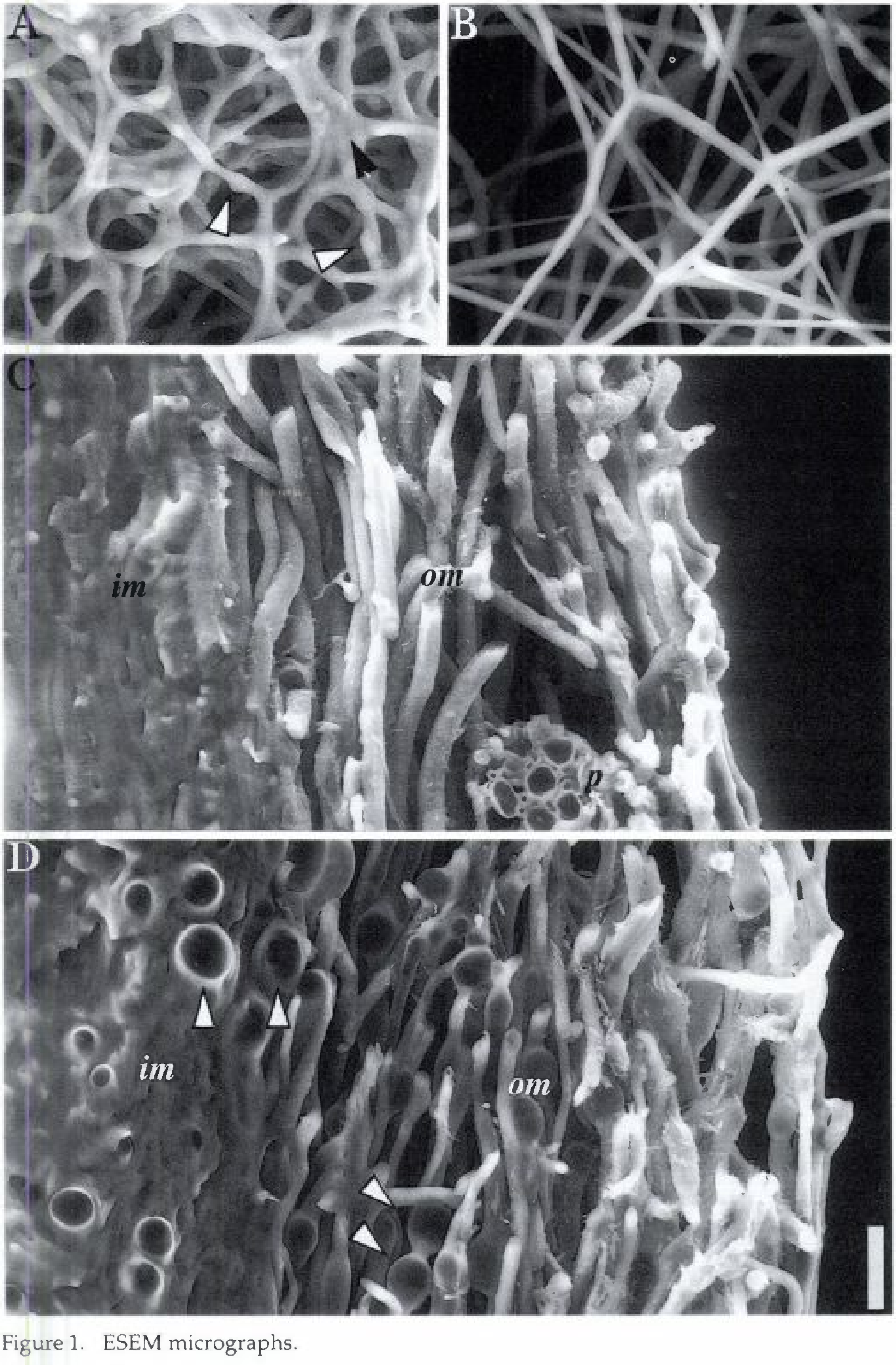

Figure 1. ESEM micrographs.

do not have data from the central and western North America, the exact geographic limit of *P. maculans* as a dominant parasymbiont remains undefined. Although we cannot provide a direct measure of the integration of *P. maculans* in the lichens, the frequency of their co-occurrence suggests a stable association. In the present study, the fast growth rate of *P. maculans* makes it easy to detect in lichens by cultures, but fast growth is not necessarily a characteristic of all dominant parasymbionts. It is possible that slow growing dominant parasymbionts also exist in lichens.

P. maculans of the ascomycete order Xylariales is a weak plant pathogen of worldwide distribution usually associated with lesions or dead, discolored leaves (Mordue and Holliday, 1971 as *P. guepini*; Nag Raj, 1993). Interestingly, while this organism appears to be common in North American lichens, it is not among the over 500 species of secondary fungi isolated by Petrini et al. (1990), Möller and Dreyfuss (1996) and Girlanda et al. (1997) from European and Antarctic lichens. Perhaps, the reason for this geographic pattern is that *P. maculans* is more virulent and infects more plants in North America than in other geographic regions. Because *P. maculans* can produce conidia when growing in plants, infected plants could effectively become *P. maculans* dispersers, inoculating lichens around them. Once the fungus establishes itself in a lichen as dominant parasymbiont, the association is easily maintained through asexual reproduction (thallus fragmentation) of the lichen.

For the purpose of comparison, we studied lichens from four other continents using the same isolation and culture method as applied to North American lichens. The results were varied, but none of the lichen groups yielded any dominant parasymbiont like *P. maculans*. Because of the limited number of samples studied and because our method reveals only fast growing dominant parasymbionts, it would be premature to conclude that no dominant parasymbionts were present in the lichens. Clearly, more systematic studies are needed to determine whether dominant parasymbionts, perhaps slow growing ones, exist in other geographic regions. But our results demonstrate the notable fact that secondary fungi, even fast growing ones, are not necessarily present in all parts of the lichen thallus. Thus the consistent presence of *P. maculans* in all parts of North American lichens is a valid characteristic of dominant parasymbionts.

Despite our extensive efforts, we were unable to locate and identify hyphae of *P. maculans* in the thallus of *Cladonia* species, either by light microscopy or by ESEM. In the ESEM, the mycobiont and *P. maculans* grown in pure culture are morphologically different from each other (Figs. 1A and B), but the differences are absent in the mature lichen thallus. It appears that the morphogenetic process of the lichen overrides the expression of the genetically controlled morphology of both the mycobiont and perhaps the dominant parasymbiont as well. This agrees with the observation that both mycobionts and photobionts in general exhibit different morphologies in pure culture as opposed to the organized lichen thallus. For example, in the lichen *Heppia echinulata* the cyanobacterial photobiont shows a unicellular, *Gloeocapsa*-like morphology. In pure culture, however, it produces filaments similar to those of the genus *Scytonema* (Marton and Galun, 1976).

The distribution of *P. maculans* within the *Cladonia* thallus is consistent with the morphological and functional differentiation of the lichen thallus. The stereome, composed of non-viable hyphae bounded by a mucilaginous material, may serve only as structural elements imparting mechanical strength to the thallus. The outer medulla harbors the photobiont, contains pore spaces between hyphae, and is metabolically more active. Thus, it stands to reason that the medullary hyphae immediately adjacent to the photobiont cells belong only to the mycobiont. The non-lichen-forming *P. maculans* probably fills the available spaces within the "scaffolding" formed by the mycobiont. In the thallus, the slower growing mycobiont is able to out-compete the more aggressive parasymbiont apparently because it is able to extract nutrition directly from the photobiont, perhaps through haustoria formation (Fig. 1C, p). The dominant parasymbiont may play the role of a "scavenger", living off organic substances passively leaked from the photobiont and the mycobiont during wet-dry and freeze-thaw cycles (Farrar, 1976). In ecological parlance, one can speculate that in the lichen thallus there is a niche for the dominant parasymbiont both in the spatial and nutritional sense.

Acknowledgements

We thank Drs. J. Garty (Tel-Aviv, Israel), L. Greenfield (Christchurch, New Zealand), J.C. Krug (Toronto, Canada), and L. Kappen (Kiel, Germany) for providing fresh lichen specimens. E.I.F. was funded by NASA grants NAGW-4044 and NAG5-4921. P.T.D. and A.G. were funded by grants from the Smithsonian Institution NMNH Research Initiative Programs, the Smithsonian Institution Scholarly Studies Program and the NSF PEET program (DEB-9712484) to P.T.D., and a Postdoctoral Fellowship to A.G. P.T.D. thanks Sue Lutz for editorial assistance. We thank two anonymous reviewers for their constructive comments that helped improve the paper.

REFRENCES

Ahmadjian, V. 1967. *The Lichen Symbiosis*. Blaisdell Publishing, Waltham, MA.

Clauzade, G. and Roux, C. 1976. Les champignons lichénicoles non lichénisés. Université des Sciences et Techniques du Languedoc, Montepellier.

Farrar, J.F. 1976. The lichen as an ecosystem: observation and experiment. In: *Lichenology: Progress and Problems*. D.H. Brown, D.L. Hawksworth, and R.H. Bailey, eds. Academic Press, London and New York, pp. 385–406.

Girlanda, M., Isocrono, D., Bianco, C., and Luppi-Mosca, A.M. 1997. Two foliose lichens as microfungal ecological niches. *Mycologia* **89**: 531–536.

Hawksworth, D.L. 1979. The lichenicolous Hyphomycetes. *Bulletin of British Museum of Natural History* (Botany Series) **6**: 183–300.

Hawksworth, D.L. 1981. The lichenicolous Coelomycetes. *Bulletin of British Museum of Natural History* (Botany Series) **9**: 1–98.

Hawksworth, D.L. 1982. Secondary fungi in lichen symbioses: parasites, saprophytes and parasymbionts. *Journal of the Hattori Botanical Laboratory* **52**: 357–366.

Hawksworth, D.L. 1988. The variety of fungal-algal symbioses, their evolutionary significance, and the nature of lichens. *Botanical Journal of the Linnean Society* **96**: 3–20.

Lindsay, W.L. 1869. Observations on new lichenicolous micro-fungi. *Transactions of the Royal Society of Edinburgh* **25**: 513–555.

Marton, K. and Galun, M. 1976. *In vitro* dissociation and reassociation of the symbionts of the lichen *Heppia echinulata*. *Protoplasma* **87**: 135–143.

Möller, C. and Dreyfuss, M.M. 1996. Microfungi from Antarctic lichens, mosses and vascular plants. *Mycologia* **88**: 922–933.

Mordue, J.E.M. and Holliday, P. 1971. *Pestaloptiopsis guepini*. *CMI Descriptions of Pathogenic Fungi and Bacteria* No. 320. Commonwealth Mycological Institute, Kew, pp. 2.

Nag Raj, T.R. 1993. Coelomycetous anamorphs with appendage-bearing conidia. *Mycologue Publications*. Waterloo, Ontario, pp. 1101.

Petrini, O., Hake, U., and Dreyfuss, M.M. 1990. An analysis of fungal communities isolated from fruticose lichens. *Mycologia* **82**: 444–451.

Schwendener, S. 1868. Ueber die Beziehungen zwischen Algen und Flechtengonidien. *Botanische Zeitung* **26**: 289–292.

Zopf, W. 1897. Ueber Nebensymbiose (Parasymbiose). *Berichte der Deutschen Botanischen Gesellschaft* **15**: 90–92.