Fluorescence microscopy of Douglas fir foliage epiflora[1]

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Douglas fir needles stained with the fluorescent dye, primulin, were observed microscopically using epiillumination with a mercury arc light source. Microorganisms fell into three categories: algae, which exhibited primary fluorescence; actinomycetes and fungi, which exhibited the secondary fluorescence of the adsorbed dye; and darkly pigmented fungi, which appeared black on the background of the fluorescing Douglas fir needle. A scanning electron micrograph is included for the purposes of comparison.

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Des aiguilles de pin Douglas colorées à la primuline, une teinture fluorescente, ont été observées au microscope photonique avec épi-illumination et en se servant d'un arc à mercure comme source de lumière. Les microorganismes observés appartiennent à trois catégories: des algues qui révèlent la fluorescence primaire, des actinomycètes et champignons qui révèlent la fluorescence secondaire de la teinture adsorbée, enfin, des champignons à pigments foncés qui apparaissent noirs sur le fond fluorescent de l'aiguille de pin Douglas. Une micrographie prise au microscope électronique à balayage est incluse, et les avantages des techniques d'observations sont discutés. [Traduit par le journal]

Introduction

When observing a leaf surface microscopically, the researcher may find difficult the detection of hyaline fungi, actinomycetes, and green algae, which blend in with the green-leaf background. For work involving observation of microorganisms in place on the leaf, the use of fluorescent staining provides straightforward and inexpensive access to the microhabitat. Primulin has been used before to observe fungal development (Streiblova and Pokorny 1964). This report describes a method for primulin staining of hyaline fungi on the leaf surface. Scanning electron microscopy, previously suggested as a method for direct observation of leaf-inhabiting microorganisms (Barnes *et al.* 1968; Beech and Davenport 1971), has been carried out for the purposes of comparison.

Methods

Fluorescence Technique

Douglas fir needles were stripped from twigs and glued onto nonfluorescing Plexiglas slides with Testor's cement for plastic models (Testor Corporation, Rockford, Illinois 61101). The slides were stained for 5 min in a 1% solution of primulin (Eastman Organic Chemicals, Eastman Kodak Company, Rochester, New York), rinsed for 3 min in running water to remove excess dye, and stored in humid chambers until examination.

Microscope pictures were taken on 35-mm Kodak EHB (high-speed tungsten) color film and Ilford HP4 black and white film (Ilford Inc., West 70 Century Rd., Paramus, New Jersey 07650) with a Zeiss universal microscope (Zeiss, Obertochen, W. Germany) using a mercury arc source (HBO 200 W/4) (Osram, Berlin, W. Germany). This is an incident light system using a Zeiss BG-12 exciter filter which has transmission peaks at 404 nm and 435 nm when used in conjunction with the mercury arc source. Zeiss barrier filters 53 or 50 + 44 were used between the subject and the film or the eye to cut out the exciter wavelengths. A Nikon AFM photomicrographic unit (Nikon Inc., New York, U.S.A.) was used to record the image. The ASA (American Standards Association) of the EHB film was increased to 400 (equal to European Din 27) and the ASA on the Ilford film was increased to 1600 (approximately European Din 33). The EHB film was developed with Kodak E-4 processing. Ilford film was developed in Acufine (Acufine, Inc., 439-44 E. Illinois St., Chicago, Ill. 60611) for 4¼ min at 70°F.

Needles were glued to Plexiglas slides to prevent curling during observation. Moistening of the needles reduced the problem of microbial cell shrinkage and did not interfere with the optics of the fluorescence system.

A high intensity fluorescence is needed to expose the film. We used a Zeiss 16 × epiplan objective in preference to a Zeiss 6.3 × plan objective most of the time. Exposure times varied from ½ min to 4½ min; in general they were long.

Scanning Electron Microscopy

Needles were stripped from twigs and fixed in formalin–acetic acid–alcohol (FAA) with 5% (v/v) glycerol (Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.) for 2 days. Needles were then stored in glycerol until examination. Scanning electron microscopy was done by the A.H. Soeldner Electron Micro-tech Service (P.O. Box 528, Philomath, Oregon 97370). Mr. Soeldner used a Cambridge stereoscan mark II-A scanning electron microscope (Engis Equipment Company, 8035 Austin Ave., Morton Grove, Illinois 60053) located at Materials Analysis Cost

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Center, Department of Mining, Metallurgical, and Ceramic Engineering, University of Washington, Seattle, Washington. A 110-μA beam at 20 kV accelerating potential was used. Needles were drained on paper towels and were mounted directly on specimen stubs with conductive silver paint. They were examined without metal coating. Pictures were taken with a Polaroid land camera, which is part of the microscope assembly.

Results and Discussion

Figures 1 through 6 are representative of the epiflora seen on Douglas fir needles using the fluorescence technique. The needles themselves fluoresce red because of the primary fluorescence of the chlorophyll; epiphytic algae show a similar red fluorescence. Hyaline fungi, actinomycetes, and the cell walls of algae fluoresce yellow-green because of the secondary fluorescence of the primulin. Nonfluorescing epiphytic cells appear dark.

The alga shown in Fig. 1 is common on Douglas fir, especially on older needles. This figure shows the alga, *Protococcus viridis* Ag., on the leaf itself; often it is found epiphytic on *Atichia*, a fungus which appears as a dark, amorphous mass. In contrast with the mass of algal cells pictured, epiphytic lichens show a more organized structure of fluorescing algal cells interspersed with dark, nonfluorescing fungal cells. In color, lichens are quite distinct when observed with this technique.

Actinomycetes, like the one shown in Fig. 2, appear as brightly fluorescing masses of thin strands. Hyaline fungi have a similar appearance, although more structure is usually evident in the surface features.

Figure 3 shows a darkly pigmented fungus. It appears black against the fluorescing background of the needle. Several dark hyphal strands are also apparent in the upper right-hand corner. Such hyphae show up much better with the fluorescence technique than they do with white light epiillumination. The fluorescence technique seems to provide more contrast.

Figures 4 through 6 show rows of stomates on the lower surface of the needles as observed with the fluorescence technique. In Fig. 4, one can see a row of stomates with the stomatal pores plugged by fungi. There are strands of fungi radiating out from the stomatal pores. They tend to parallel the lines of the stomates. Figures 5 and 6 show the lower surface of another needle. In this case small algal cells are as common as fungal cells. One can take a series of pictures like Fig. 5 to construct a montage which shows general epiphytic patterns on the whole needle. Figure 6 is a higher magnification view of the same needle shown in Fig. 5. In color, the chloroplasts in the guard cells are quite obvious; they show less well in a black and white print. Figures 5 and 6 show the same pattern of microbes in stomatal pores and in lines parallel to the stomates as does Fig. 4. It seems that often the chloroplasts in the guard cells are not covered by microorganisms. Depressions in the needle surface around guard cells and in lines parallel to the stomates might provide protection and (or) a reservoir for nutrients and could account for the patterns of microorganism distribution described. Figure 7 shows a scanning electron micrograph of the lower surface of a Douglas fir needle similar to those shown in Figs. 4 through 6. Here again, stomatal pores are plugged with microorganisms. Depressions in the needle topography are evident, as are the microorganisms resident in them. Scanning electron microscopy provides more information about needle surface topography than the fluorescence technique, but the fluorescence technique is superior when the object is the identification of surface microbes or the quick and inexpensive estimate of microbial cover on the leaf.

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PLATE I

FIG. 1. *Protococcus viridis* on upper surface of 3-year-old needle. FIG. 2. Actinomycete on upper surface of 3-year needle. FIG. 3. Fungus, probably capnodiaceous, on lower surface of 11-year needle. FIG. 4. Lower surface of 6-year needle. Note mycelial strands and plugged stomatal pores. Figs. 1–4. The bar represents 20 microns (μ).

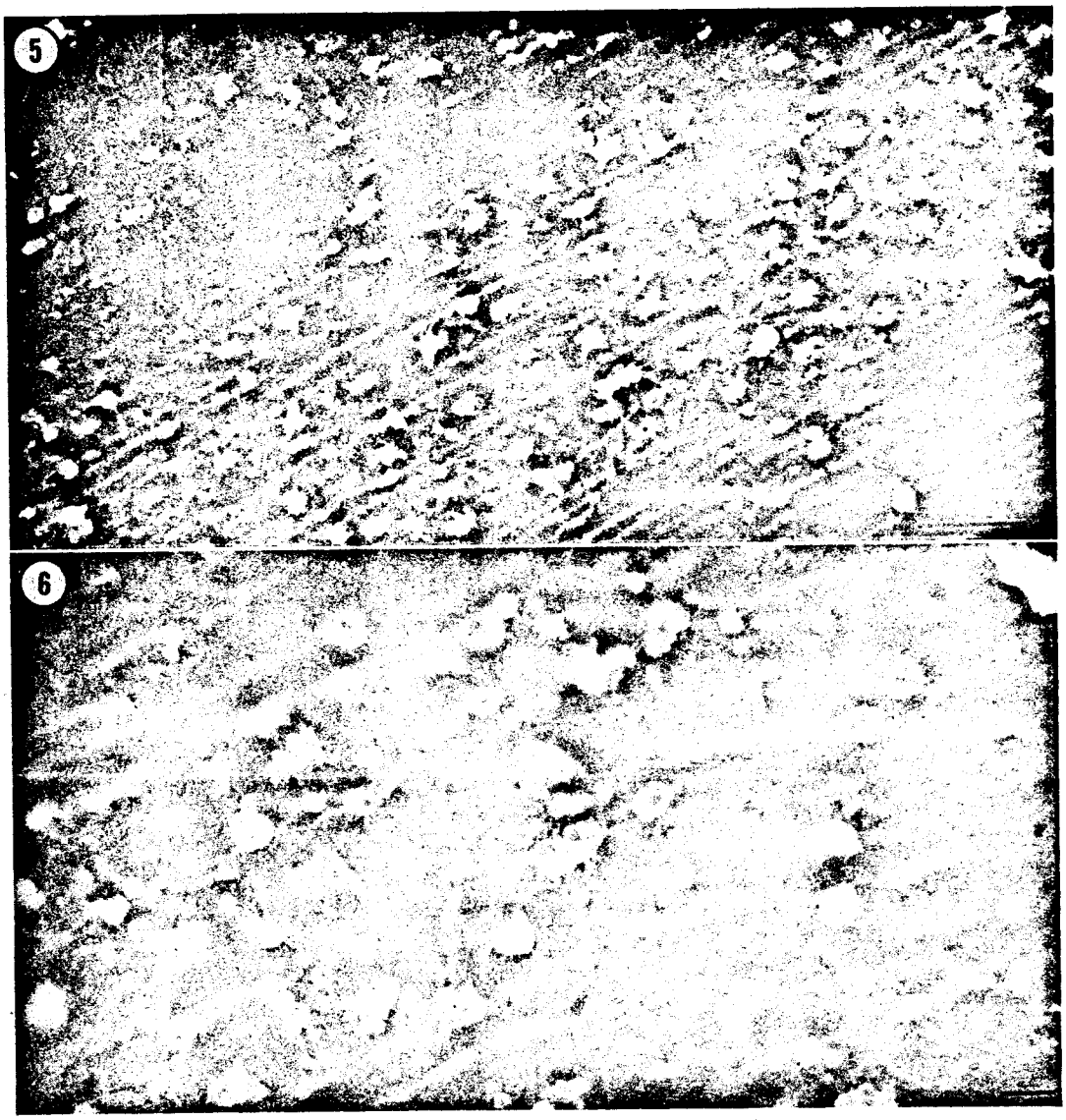

FIG. 5. Lower surface of 5-year needle. Note rows of stomates and dense microbial cover. Bar represents 100 μ. FIG. 6. Lower surface of 5-year needle. Bar represents 20 μ.

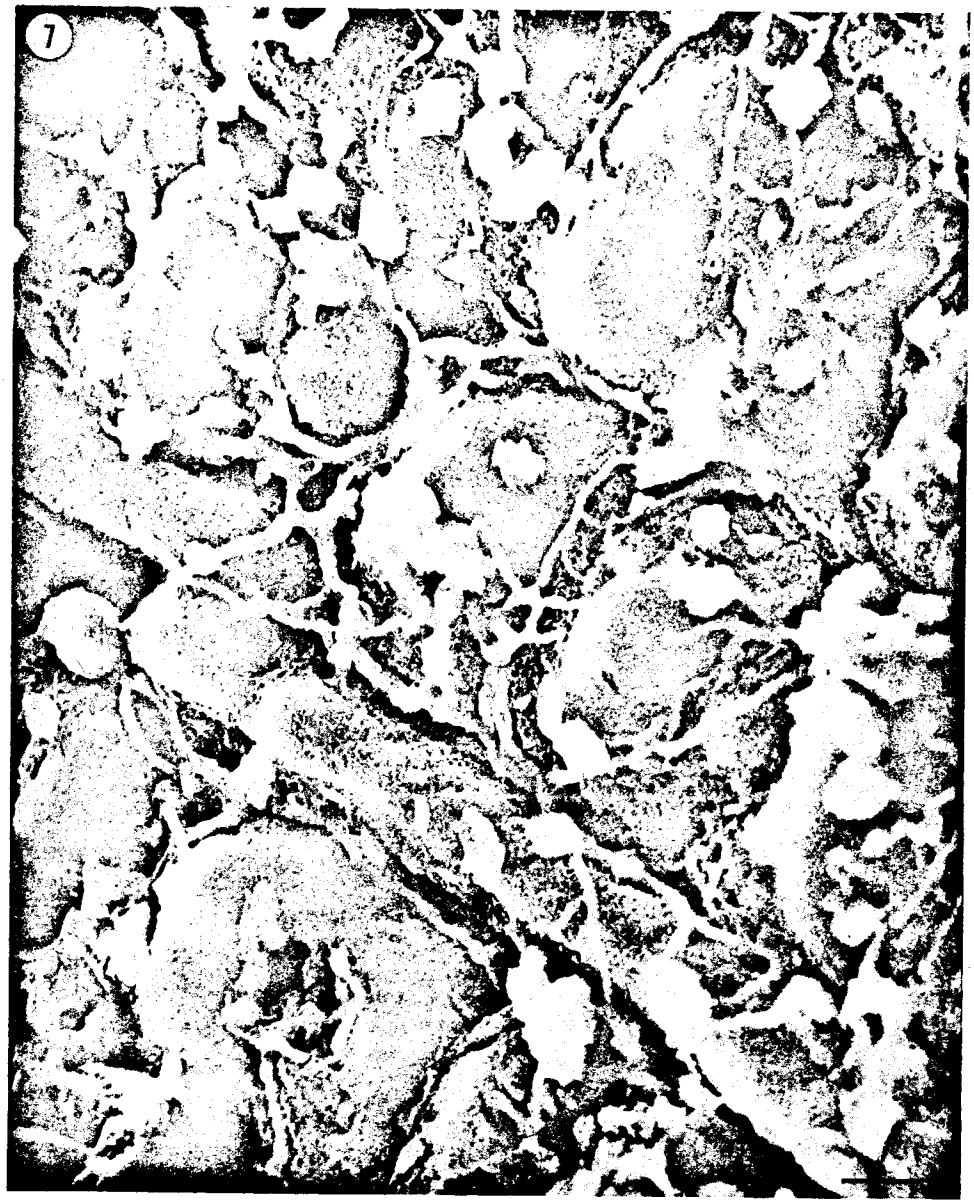

FIG. 7. Lower surface of 8-year needle. Bar represents 10 μ.