

Microbial Populations on Douglas Fir Needle Surfaces

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Abstract. The surface microflora on Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco.) foliage from old-growth trees in western Oregon has been examined by epifluorescence and scanning electron microscopy. Colonies of microorganisms on both upper and lower surfaces of 1-, 3-, 5-, and 8-year-old needles from three heights in the canopy of a single tree have been counted in belt transects, and the relative abundance of various categories of microorganisms has been computed. Aggregations of microbial cells are prevalent in the midrib depression along the upper surface and in stomatal cavities and gutters between rows of epidermal cells on the lower surface. Darkly pigmented hyphae and clumps of cells occur, in general, more frequently on the upper needle surface, a habitat more subject to desiccation and UV exposure. *Protococcus* colonies become abundant on both upper and lower surfaces of older needles. Microbial cover was found to be significantly higher on the bottom of the needle than on the top. The factors involved in this effect are considered and discussed.

Introduction

Coniferous foliage comprises a major portion of the total photosynthetic surface within the coniferous forest biome. Insofar as the net primary productivity of the biome depends on the efficient functioning of this surface, microorganisms living on or within the needles may exert a disproportionately large influence on the overall activities of the forest. Obvious effects may include the direct utilization of carbohydrates produced by the needles and hastened senescence of the needles through infection. More subtle effects may involve leaching and/or uptake of carbohydrates and nutrients from the phyllosphere. Surface yeasts have been implicated in the erosion of leaf cuticles (7,10), a process presumed to make leaves more vulnerable to leaching. Witkamp (15) has shown the surface microflora of tropical broad-leaved evergreens to be highly active in the uptake of radionuclides.

Preliminary studies in this laboratory (11) revealed a significant microflora associated with the surface of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco.) needles from an old-growth stand. The present work was undertaken as a first step in assessing the importance of this canopy microflora. It has involved

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identification of the categories of microorganisms present, description of variation in patterns of microbial distribution as a function of location on the needle surface and of needle age, and estimation of microbial cover.

Materials and Methods

Description of Trees, Treatment of Branches, and Needle Selection

Most of the foliage examined in this study was taken from a single old-growth Douglas fir in Watershed 10 of the Andrews Experimental Forest (44°13' north latitude, 122°15' west longitude) in the Willamette National Forest near Blue River, Oregon. This watershed has been designated an intensive study site for the Northern Coniferous Biome of the International Biological Program (IBP). The study tree (tree 286 on the IBP stem map) was approximately 450 years of age and was located on a south-facing slope at approximately 460 m elevation. Branches were taken from the tree during September 1972 by climbers using the modified rock climbing technique described by Denison et al. (3). The height for each branch system was recorded in meters or was estimated simply as the upper, middle, or lower third of the tree canopy.

In developing the fluorescence technique and for scanning electron microscopy of the leaf surface, we also used foliage from trees approximately 100 years old located in the Pioneer Memorial Cemetery near the University of Oregon and in Hendricks Park, Eugene, Oregon (44°02' north latitude, 123°08' west longitude). Foliage from the Eugene trees came from the lower canopy, 1.5 m to 2.5 m above the ground.

Branches from tree 286 were placed in plastic bags and brought back to the laboratory where they were cut into segments by age class, sorted into paper bags, and stored in a freezer at -15°C for times varying between 2 and 10 months before examination. Prior to staining, a randomly chosen collection of 102 needles was taken from an arbitrarily selected group of segments for a given age class. From these 102 needles 10 were picked haphazardly for examination under the fluorescence microscope.

In estimating the proportion of the needle surface covered by microbial cells on foliage, we sampled five needles with five transects per needle for each combination of the following variables: needle surface (top and bottom), age (1, 3, 5, and 8 years), and canopy height (lower, middle, and upper).

It may be argued that storage at -15°C for prolonged and varying periods prior to observation has caused changes in the surface microbial populations and introduced artifacts into our data. Such changes might involve either microbial growth or death and disappearance of microbial cells. We feel both to be improbable. Temperatures as low as -15°C seldom occur in the low-elevation forests where our samples were taken, and the microorganisms resident there are unlikely to be extreme psychrophiles. The fluorescence stain used in these studies binds to microbial cell walls (13) and does not distinguish between living or dead microorganisms. Although prolonged exposure to subzero temperatures may kill microbial cells, complete lysis and disappearance of their walls must occur only rarely. Further, additional studies now nearing completion by one of us (G.C.C.) based on freshly collected needles confirm the patterns of microbial distribution reported here.

Fluorescence Technique

Douglas fir needles were stripped from twigs and glued onto nonfluorescing Plexiglas slides. The slides were stained for 5 min in a 1% solution of primulin, 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 with a Zeiss universal microscope using a mercury arc source (HBO 200 W/4). 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.

Sampling for Microbial Cover and Colony Frequency

The length and width at the widest point of each needle were measured in millimeters with a stage micrometer. Because of difficulties in observing the twisted needle petiole with epifluorescence microscopy, needle petioles were excluded from this study.

To estimate the microbial cover, we took belt transects running at right angles to the long axis at random positions along the length of the needle. They consisted of a series of contiguous quadrats 68.8 μ on a side, delimited by a grid in the microscope eyepiece. A Zeiss 16 \times epiplan objective and an 8 \times eyepiece were used giving a magnification of 128 \times . The microbial cover in each quadrat was estimated to the nearest 5%.

For the determination of the distribution of needle surface microorganisms among various categories, we used the left half of the quadrats chosen for the cover estimates. These transects were 34.4 μ wide and as long as the width of the needle. The first 200 microorganisms observed in each age-surface class were scored as belonging to one of the several categories listed in Table I. When additional transects were needed to reach 200 microorganisms, they were located using a random numbers table. In order to avoid the bias introduced by sampling only one needle or by sampling the area near the edge of the needle more often than the area near the midrib region, we looked at as many complete transects on a needle as were needed to observe a minimum of 40 microorganisms. The last transect observed for each set of five needles was generally not completed, since the sampling stopped when 200 microorganisms had been observed. Each distinguishable microbial cell clump was counted as one microorganism. A hypha was counted as a single microbial colony each time it made distinctive lines or branches in the transect.

Scanning Electron Microscopy

Needles were stripped from twigs and fixed in formalin-acetic acid-alcohol (FAA) with 5% (v/v) glycerol for 2 days. Needles were then stored in glycerol until examination with a Cambridge stereoscan mark II-A scanning electron microscope.

Data Analysis

All estimates of microbial cover were arcsin transformed, and statistical tests were applied to the transformed numbers. D'Agostino's test (16) and the computation of the g_1 statistic as a measure of skewness (12) were used to estimate deviations from normality of the arcsin-transformed numbers. Inequality of variances for the arcsin-transformed values was checked with Bartlett's test.

Ninety-five percent confidence intervals of the means were calculated by adding $t s_x$ to the means and subtracting $t s_x$ from the means (where t is Student's t and s_x is the standard error of the mean). An approximate t -test was used to evaluate the differences of means of microbial cover between two samples (12). A Pearson product-moment correlation coefficient was calculated to relate microbial cover to needle size.

Results and Discussion

Distribution of Microbial Cell Types

Prior to discussion of microbial distribution on needle surfaces, the most conspicuous features of the topography of the needle itself should be mentioned. These include a deep midrib groove on the upper surface and a midrib flanked by two rows of stomata on the lower surface. Each needle becomes more terete and twists at the petiole end; toward the tip the upper midrib groove becomes progressively shallower and ultimately disappears as the needle abruptly tapers

Table 1. Relative frequency of categories of microorganisms on Douglas fir needle surfaces from upper canopy

Microorganism Category	Bottom of Needle Age (years)				Top of Needle Age (years)			
	1	3	5	8	1	3	5	8
Darkly pigmented fungal hyphae	1.5	23	32.5	24.5	27	30	43	27
Hyaline fungal hyphae	20	34.5	22.5	22.4	21	3.5	3	6.5
<i>Protococcus viridis</i>	0	6	16	28	0	15.5	17	16.5
Darkly pigmented colonies of fungi (black dots)	41	9.5	12	11	20.5	26	26.5	33.5
Hyaline microbial colonies	34	21	11.5	13	31.5	25	10.5	15
Other	3.5 ^a	6 ^a	5.5 ^a	1 ^a	0	0	0	1.5 ^b

^a Appeared as bright clumps of cells usually in stomata; probably *Protococcus*. See text for further explanation.

^b *Trentepohlia*.

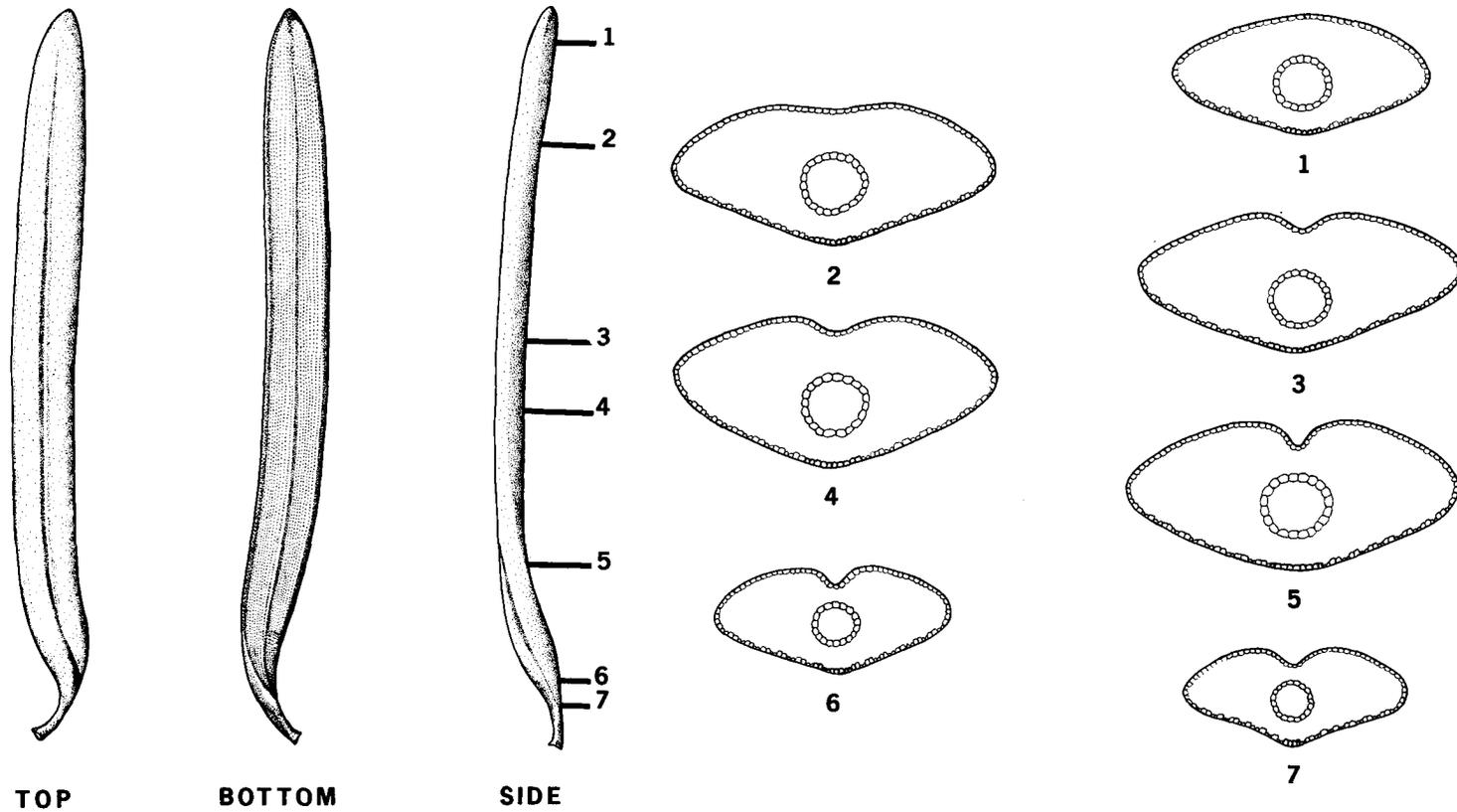
to a point. Internally, a central vascular bundle and two lower, symmetrically placed resin ducts lateral to the stomatal rows are apparent (Fig. 1). At a microscopic level, gutters between individual epidermal cells (Fig. 3) and deep substomatal chambers provide important habitats for microepiphytes.

Although epifluorescence microscopy may provide inexpensive access to the needle microhabitat, the low resolution inherent in the optical system and poor depth of focus give rise to ambiguities in identifying specific microbial taxa. Consequently, microorganisms have been grouped by cell types into the categories listed in Table 1 in considering relative abundance. General comments on the taxa in each category are included with the presentation of the quantitative data below.

Protococcus viridis Ag., a nonmotile green alga in the Protococcaceae, is a common epiphyte of the Douglas fir needle and twig surface. On the top of the needles, *Protococcus* is most often found in or near the midrib depression, where it frequently occurs epiphytically on colonies of *Atichia*, a black, yeast-like fungus. On the bottom of the needles, *Protococcus* colonies are most often associated with the stomatal openings. Clumps of yellow fluorescing cells also seen in the stomata probably represent shriveled *Protococcus* cells in which the primary fluorescence has faded due to prolonged exposure to UV light under the microscope; such colonies are included in the "other" category for the bottom of the needle in Table 1.

In terms of frequency, this alga becomes established on 1- or 2-year-old needles. Thereafter, its relative abundance increases steadily on the lower surface but remains relatively constant after 3 years on the upper surface.

The distribution of epiphyllous algae on leaf surfaces has been described previously for a number of tropical plants. Palm (8) noted *Stomatochroon* growing in the substomatal chambers of tropical plants from 20 families. Allen (1) performed a pattern analysis for *Phycopeltis expansa* Jennings on leaves of a tropical orchid. He observed a positive correlation between the occurrence of algae and shallow "gutters" between the epidermal cells, suggesting that algal zoospores might be stranded there on the drying leaf and that such colonies might have free water available to them for longer periods. *Protococcus* does not have



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Fig. 1. Top, bottom, and side views of a typical Douglas fir needle. Position of cross-sectional profiles is indicated by numbered lines. Note disappearance of the midrib groove toward the needle apex. Drawing by Jill Suttles.

any specialized cells for penetrating substomatal openings. Further, since *Protococcus* produces no zoospores, Allen's explanation for the colonization of intercellular gutters by *Phycopeltis* cannot apply. However, *Protococcus* does appear to be a true leaf epiphyte, exploiting surface depressions for protection and moisture. Cells from existing colonies of *Protococcus* may be carried in the winter rains to young needles, where they become lodged in the midrib depressions between parallel rows of epidermal cells, and in the stomata.

Darkly pigmented fungal hyphae often appear in lines running parallel to the long axis of the needle with short bridges between. As a group they include several taxonomic categories. Dark hyphae are an important component of the microbial flora on the top, but not on the bottom of 1-year needles; at 5 years they become the most abundant category of microorganisms on both sides of the needles; on older needles they decline in relative frequency as other microorganisms become more abundant.

The category "darkly pigmented colonies," referred to as "black dots," consist of: areas of infection by leaf parasites, spores, unidentified nonfilamentous clumps of black or brown cells, and *Atichia*, the sterile state of a Loculoascomycete and the predominant black fungus observed here. *Atichia* appears as a nonmycelial, yeast-like cluster of cells in a gelatinous matrix; it occurs more abundantly on the top of the needle than on the bottom and is particularly common in the midrib depression.

The top and bottom of the needles show different patterns of relative frequency of black dot colonies. On the top of the needles black dots accounted for 20% of the counts on 1-year needles, increasing to 35% at 8 years. On the bottom of 1-year needles black dots were the most common category of microorganisms (41% of the counts); their relative frequency decreased on 3- to 8-year needles. We suggest that these colonies may be disease organisms and that the diseased needles fall off the tree and are not seen as 3-, 5-, and 8-year needles. A similar distribution pattern was reported for *Schizothyrium* sp. on Douglas fir needles by Sherwood and Carroll (11) and can be inferred from the data of Gourbière (4,5) on the total fungal epiphyte populations on *Abies alba* Mill.

Black dots and darkly pigmented fungi occur, in general, more frequently on the top of the needle than on the bottom. These data are consistent with previously reported observations suggesting that pigments may protect fungal cells in the phyllosphere from desiccation and UV light (6,9).

"Hyaline hyphae" include both actinomycetes and fungi. Actinomycetes can be separated from fungi on the basis of filament width; actinomycete filaments are 1 μ or less in diameter (14). Although they were originally designated as a separate category, they have been lumped with the hyaline fungal filaments for purposes of discussion. Actinomycetes seem rare on Douglas fir needles, although they are often isolated in plating out throughfall samples.

Hyaline fungal hyphae often appear between clumps or colonies of other microbial cells (Fig. 2). They seem to grow in lines parallel to rows of stomata and into the stomata themselves. On the top surface hyaline hyphae are common on 1-year needles, but less abundant on 3-, 5-, and 8-year needles. On the bottom surface they make up $\geq 20\%$ of the counts in all age classes observed. Such hyphae may darken with age and be scored as "dark hyphae" on older needles, thus creating a spurious impression of decreasing abundance with needle age.



Fig. 2. Fluorescence microscopy. Top of 3-year needle from tree 286. Hyaline hyphae connect hyaline microbial colonies. Many of these hyphae are growing in lines parallel to the long axis of the needle. Bar equals 20 μ .

“Hyaline colonies” comprise a category of clumps of nonfilamentous microorganisms, all of which fluoresce yellow (suspected colonies of *Protococcus* excluded). Occasionally, individual yeast-like cells composing a colony can be seen. Hyaline colonies occur most frequently on 1-year needles; their relative frequency drops by 3 years on the bottom of the needles and by 5 years on the top of the needles. Again, increasing pigmentation of cells with age may be involved.

Because of the limitations of epifluorescence microscopy noted above, we performed a qualitative check on patterns of microbial distribution with the scanning electron microscope. Such observations at higher magnification and resolution have confirmed the buildup of microbial cell mass in the midrib groove and in stomatal cavities on older needles. Growth of fungal hyphae in gutters between rows of epidermal cells is also evident (Fig. 3).

In general, the distribution of microorganisms seems to be related to the topography of the needle surface. Surface depressions may provide protection from physical elements such as sunlight and desiccation and from biological hazards such as grazing insects; they may further serve as reservoirs for moisture and nutrients leached from the canopy by rain.

Microbial Cover on the Douglas Fir Needle

The transects on which cover estimates are based were randomly positioned across the needles perpendicular to the long axis. A random sampling scheme was chosen both for its simplicity and because we felt such transects would allow for sampling various microhabitats on the needles in a manner proportional to their occurrence.

Several biases may have been introduced by this sampling method. It was

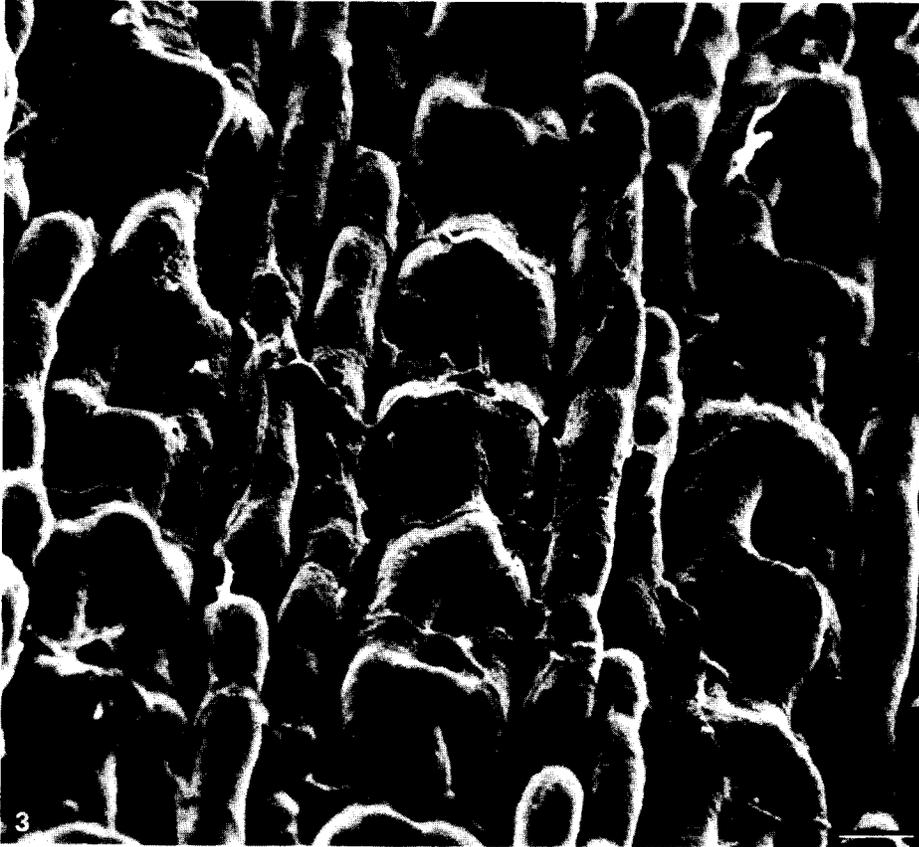


Fig. 3. Scanning electron micrograph of the bottom of a 1-year needle from tree 286. The needle was fixed in 5% aqueous glutaraldehyde and critical point dried. Bar represents 10 μ .

assumed that variation in the size of the needle (and therefore the length of the transects) would not influence the results. An analysis of the data for ages 1, 3, 5, and 8 years lumping all three canopy heights for the bottom of the needle does not show significant correlation at the 0.05 level of transect length with microbial cover (Pearson product-moment correlation coefficient = 0.028, DF = 298). However, the same calculations done on lumped data for the top of the needle show a significant correlation of transect length with microbial cover (Pearson product-moment correlation coefficient = 0.131, $p < 0.05$, DF = 298). Although this correlation coefficient is low, the sample size is large, and we feel the effect to be real. Since the final selection of needles was not carried out in the random fashion, a slight bias may have resulted.

Our study has further assumed that a single harvest of persistent 1- to 9-year-old needles will reveal patterns of increasing microbial cover similar to those seen if a single year's crop of needles were to be examined for 9 years in succession. Although we feel this to be a reasonable assumption, we have not tested it, and thus it should be explicitly stated.

In analyzing the data on cover for the purposes of two sample comparisons, we have encountered several difficulties. Although all data were arcsin transformed, about a third of the data sets for individual groups of transects proved to be still non-normally distributed; the normally distributed data sets were subjected to Bartlett's test for equality of variances, and the data were found to be heteroscedastic. Consequently, an approximate *t*-test described in Sokal and Rohlf (12) was used in two sample comparisons of means instead of the more commonly encountered Student's *t*-test.

Figures 4 through 6 show the average microbial cover for the age classes sampled at three canopy heights. Samples from the lower, middle, and upper canopy were taken at 30 m, 42 m, and 52 m from the ground, respectively. Vertical lines represent 95% confidence intervals of the mean; if none is obvious, the confidence interval was smaller than the symbol representing the point. Since the data were arcsin transformed, the 95% confidence intervals are not necessarily symmetrical around the mean. Data from both top and bottom of the needle are graphed in the same figure for each canopy height; when two points for the same age class overlap, one of them has been slightly offset to make it more easily seen.

Differences in microbial cover between upper and lower needle surfaces (Figs. 4–6) are significant at the 0.05 level for all age classes and heights in the canopy (approximate *t*-test, $DF = 24$). Microbial cover is not as great on the upper needle surface as on the lower surface. There may be at least two possible explanations for this observation: aside from the groove along the midrib, the top surface of the needle offers only shallow depressions on which microorganisms can become established; the top and bottom of the needle may be exposed to different microclimates due to their orientation on the tree or due to features of their surface topography. The side of the needle oriented toward the sun would be expected to have a lower amount of moisture at the needle surface because of the drying effects of sunlight. The surface area within depressions and the type of depression (midrib, stoma, or space between rows of cells) may also influence the amount of surface moisture and sunlight available to microbial cells.

Just as the relative abundance of microorganisms changes with needle age, an increase in microbial cover also occurs with increasing age (Table 2). This increase is apparent on the tops of the needles for all three canopy heights. In the lower and middle canopies cover increases steadily from 1 to 8 years. In the upper canopy cover also increases from 1 through 8 years, although the difference between cover at 5 and 8 years is not significant. The decrease in cover at 9 years in the lower canopy represents a significant decrease.

The increase in microbial cover with age appears even more marked for the bottom surfaces of needles. Exceptions to a steady and significant increase in microbial cover with age were seen only in the upper canopy, where a significant decrease in cover occurs between 3 and 5 years, and in the middle canopy, where cover does not increase between 5 and 8 years.

In order to determine the effect of needle age and height in canopy on microbial cover, we performed a two-way analysis of variance. However, in view of the non-normality of some of the data sets and the heteroscedasticity of the data, the results must be considered ambiguous. Beyond this, a significant interaction factor between needle age and height in the canopy was discovered.

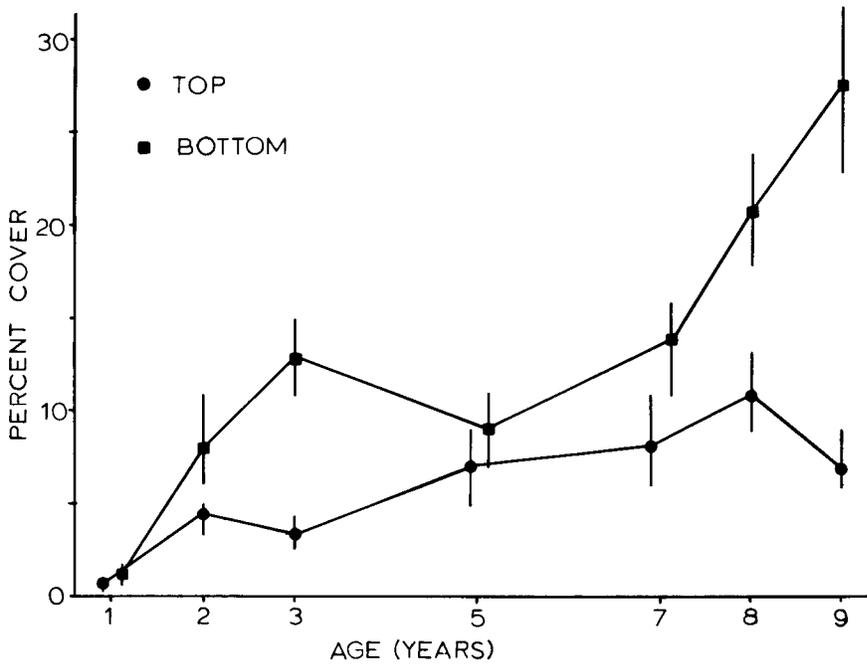


Fig. 4. Microbial cover on the needle surfaces. Lower canopy of tree 286,30 m above the ground. Data were arcsin transformed. Vertical lines indicate 95% confidence intervals of the mean; $n = 25$.

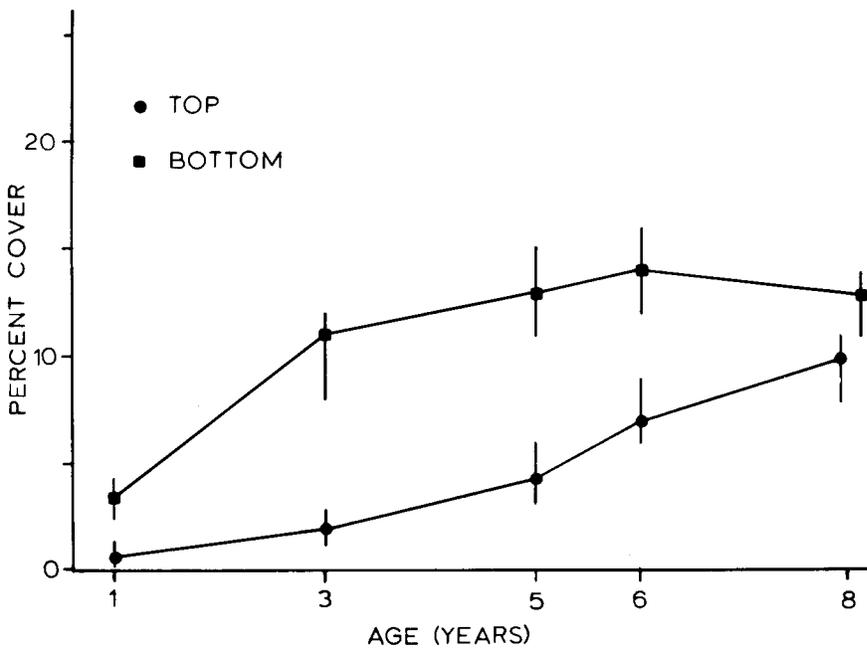


Fig. 5. Microbial cover on the needle surfaces. Middle canopy of tree 286,42 m above the ground. Data were arcsin transformed. Vertical lines indicate 95% confidence intervals of the mean; $n = 25$.

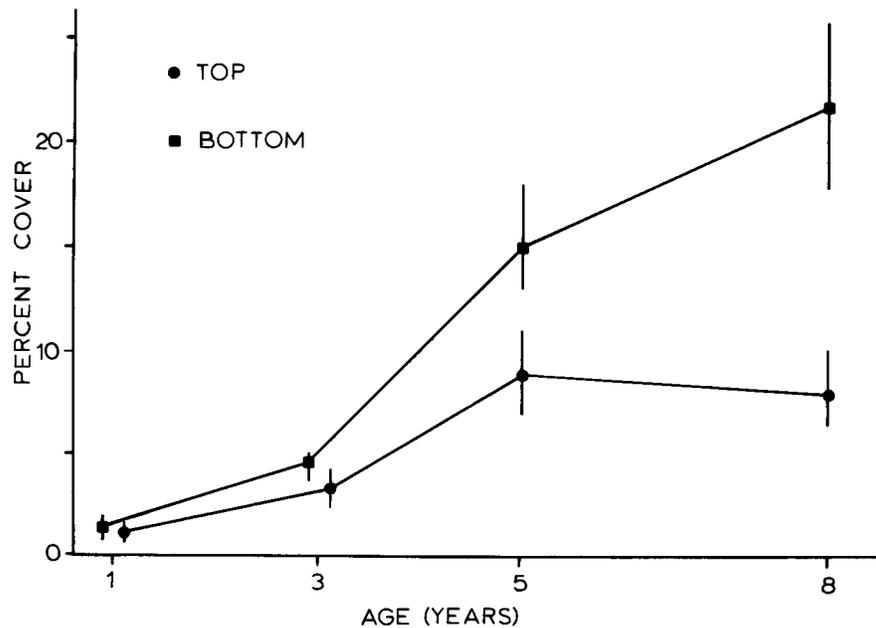


Fig. 6. Microbial cover on the needle surfaces. Upper canopy of tree 286, 52 m above the ground. Data were arcsin transformed. Vertical lines indicate 95% confidence intervals of the mean; $n = 25$.

For these reasons no conclusions on variations in microbial cover with location of the needles in the canopy are presented here.

In general, one might expect microbial populations to respond to variations in temperature, humidity, and light, to the relative availability of dissolved substances in canopy wash, and to differing grazing pressures. Studies currently in progress at the University of Oregon have revealed marked differences in all of these parameters from one location in the canopy to the next. Our failure to demonstrate clear systematic variations in microbial populations in response to these differences can perhaps be attributed to the use of cover as an estimator of

Table 2. Mean microbial cover on the bottom and top of 1-, 3-, 5-, and 8-year Douglas fir needles at three heights in the canopy of tree 286

Needle Age (years)	Side of Needle	Mean microbial cover		
		Lower Canopy	Middle Canopy	Upper Canopy
1	Bottom	1.1	3.4	1.4
3	Bottom	13	11	4.6
5	Bottom	9	13	15
8	Bottom	21	13	22
1	Top	0.6	0.6	1.2
3	Top	3.4	2	3.3
5	Top	7	4.3	9
8	Top	11	10	8

standing crop. In fact, the thickness of a cell or aggregate of cells here measured as cover can vary from 1 to 25 μ . One of us (G.C.C.) is now measuring microbial cross-sectional areas in transverse sections at varying positions along the length of the needle. It is expected that such measurements will permit more accurate estimation of microbial cell volumes and that the data will prove more amenable to statistical analysis. With such estimates in hand, systematic variation in microbial standing crops with needle age and height in canopy should emerge.

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