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EFFECTS OF FERTILIZATION ON EPICUTICULAR WAX MORPHOLOGY OF NEEDLE LEAVES OF DOUGLAS FIR, PSEUDOTSUGA MENZIESII (PINACEAE)[1]

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Fertilized stands of *Pseudotsuga menziesii* were found to have glaucous needles. We investigated the morphological and quantitative characteristics of the epicuticular waxes of needles of fertilized and control trees. Glaucousness was caused by ornate tubular epicuticular wax. Dipping needles in chloroform, which dissolves waxes, eliminated the glaucous appearance. Based on cryostage scanning electron microscopic observations, the epicuticular waxes in the nonstomatal region were much more ornate on the needles of the fertilized trees (experimental needles) than in unfertilized trees (control needles). The stomatal region in both experimental and control needles showed similarly ornate waxes. Quantities of waxes were similar in experimental and control needles. The glaucousness was not the result of greater quantities of wax; rather, fertilization altered wax morphology in the nonstomatal regions.

The correlation of leaf glaucousness with the morphology of epicuticular wax has been documented in many taxa (Eglinton and Hamilton, 1967) including needles of conifers (Hanover and Reicosky, 1971; Clark and Lister, 1975). Changes in epicuticular wax morphology associated with aging (Hallam, 1970; Reicosky and Hanover, 1976; Thijsse and Baas, 1990), physical environmental effects (Hall and Jones, 1961; Hull, Morton, and Wharrie, 1975; Reed and Tukey, 1982), and air pollutants (Günthardt-Goerg and Keller, 1987; Thijsse and Baas, 1990) have been recorded. The morphologies of epicuticular waxes are categorized as either amorphous or crystalline. Crystalline waxes include rod, granular, tubular, and other types (Baker, 1982). Both amorphous and crystalline waxes have been reported to occur on needles of *Pseudotsuga menziesii* (Hanover and Reicosky, 1971; Thair and Lister, 1975; Thijsse and Baas, 1990).

In a large silvicultural experiment established throughout the intermountain portion of the western United States, field foresters noted that needles of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, fertilized with nitrogen and potassium were bluer (more glaucous) than those left unfertilized (Intermountain Tree Nutrition Cooperators, personal communication). The objective of this study was to investigate the effect of mineral nutrition on the morphology of epicuticular waxes in this species.

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MATERIALS AND METHODS

Experimental and control plots, each 405 m^2 in area and containing at least ten mature trees of *Pseudotsuga menziesii,* were established at each of four sites (installations in Table 1) in the fall of 1987. Experimental plots were fertilized with 228 kg N (urea) ha^{-1} and 228 kg K (KCl) ha^{-1}. At each installation, the unfertilized control plots were adjacent to the fertilized plots. During 1988, needles were collected from one experimental and one control tree at each installation. Current-year twigs were collected from the branches that were three whorls from the top of the tree. Twigs bearing needles were collected by climbing the individual trees, packaged to minimize abrasion to the needle surfaces, immediately placed on ice, and then air-expressed to Michigan State University. Needles were collected at 12 mo after fertilization of experimental plots (Table 1).

For convenience, needles from trees that had been fertilized are referred to hereafter as "experimental needles" and needles from trees growing in the control plots are called "control needles."

Microscopy—To examine the basic needle anatomy, free-hand sections of needles were prepared for light microscopy as described by Ewers (1982).

For cryostage scanning electron microscopy (cryo-SEM), the median 2 mm of experimental and control needles was excised with a razor blade, attached to specimen stubs with Tissue-Tek II OCT compound, cryo-fixed by plunging into liquid nitrogen under an atmosphere of argon in an EMscope SP2000 sputter-cryo system, and then transferred in an evacuated chamber to another chamber in which specimens were sputter-coated with gold at 0.05 Torr and 20 mA for 4 min. The frozen specimens shrouded under vacuum were transferred to the JEOL JSM-35C scanning electron microscope specimen stage for examination. On the abaxial surface of needles of *P. menziesii,* there are two parallel strips (regions) of stomates (Fig. 1).

TABLE 1. *Location and description of four Douglas fir fertilizer installations. Fertilized and control plots were at each installation*

Installation	202	204	237	243
Region	Central Idaho	Northern Idaho	Western Montana	Northeast Washington
Latitude	44°27′	46°35′	47°4′	48°32′
Longitude	115°55′	116°4′	133°8′	117°43′
Elevation (m)	1,677	1,006	1,341	1,006
Soil parent material	Granite	Volcanic ash	Glacial till	Glacial till
Average tree age (yr)	58	41	90	73
Basal area (m^2 ha^{-1})	16.3	23.3	35.8	23.2
Tree ha^{-1}	593	617	573	593
Fertilizer application date	2 Sept 87	27 Oct 87	30 Sept 87	16 Nov 87
Needle collection date	27 Sept 88	20 Oct 88	12 Sept 88	8 Nov 88

Photographs were taken of three needles per treatment both in stomatal and nonstomatal regions. Those of the nonstomatal region were all taken on the abaxial surface halfway between the two stomatal regions. Some experimental and control needles were treated with chloroform to dissolve the epicuticular wax before cryo-SEM preparation.

Wax quantification—One set of 134 to 319 experimental and control needles was used from each of the four installations. To extract the epicuticular wax, each needle was held by the petiole with a pair of forceps and immersed for 10 sec in successive 30-ml portions of chloroform. The chloroform extracts from each needle sample were filtered through Whatman No. 1 filter papers and then combined. Chloroform was removed from the sample by rotary evaporation under reduced pressure (water aspirator) at 40 C. The residues remaining after removal of the chloroform were dissolved in 6 ml chloroform, and then transferred to preweighed aluminum weighing boats (W_i). After drying to a constant weight in a desiccator, the final weight (W_f) was determined. The amount of epicuticular wax was the difference between W_f and W_i minus the weight of dry residue of the same amount of chloroform. Data were calculated and expressed as mean ± standard error.

The wax types, tubular or scalelike, were evaluated from four sets of cryo-SEM micrographs of experimental and control needles. The needle areas covered by tubular and scalelike wax were calculated by measuring cutouts of photographs with a Li-Cor 3000 portable area meter.

Needle surface area and dry weight—After extraction of epicuticular wax, each needle was cut with a razor blade through the plane that separates the needle as a distal cone and a proximal frustum (Fig. 1). The lengths of the distal cone (L_d) and the proximal frustum (L_p) were precisely measured under the dissecting microscope. The circumferences of the bottom of the distal cone (P_d) and the middle of the proximal frustum (P_p) were traced with a Nikon SMZ-10 dissecting microscope attached to the camera lucida, digitized by the DAP computer program, and calculated to obtain the perimeter by J. A. Elhai's DNALENGTH program (unpublished), which is a personal computer software routine to accumulate the length between two digitized points. The total needle surface area was the sum of the surface areas of the distal cone and the proximal frustum, as calculated by equations shown in Fig. 1. Needle dry weight was determined after drying to a constant weight in a 100 C oven.

RESULTS

The entire surface of experimental needles tended to be more glaucous than control needles. When experimental leaves were dipped in chloroform, they lost their glaucous appearance. Upon chloroform treatment, control leaves lost the glaucous strips that were macroscopically visible in the stomatal regions.

The epicuticular waxes of nonstomatal regions of experimental needles appeared with cryo-SEM to have an ornate, tubular structure. This was especially true in the valleys between the ridges (Figs. 2, 3). Examined by freehand sections, the valleys between the ridges corresponded to the radial anticlinal walls of epidermal cells. When the epicuticular waxes of experimental needles were dissolved by chloroform, a relatively smooth and clear surface topography (Fig. 4) corresponded to files of epidermal cells beneath the cuticle. Although pictures of nonstomatal

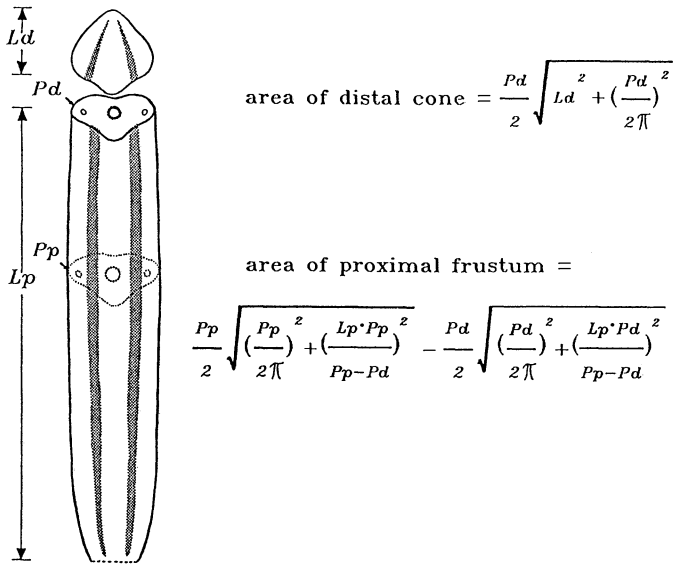
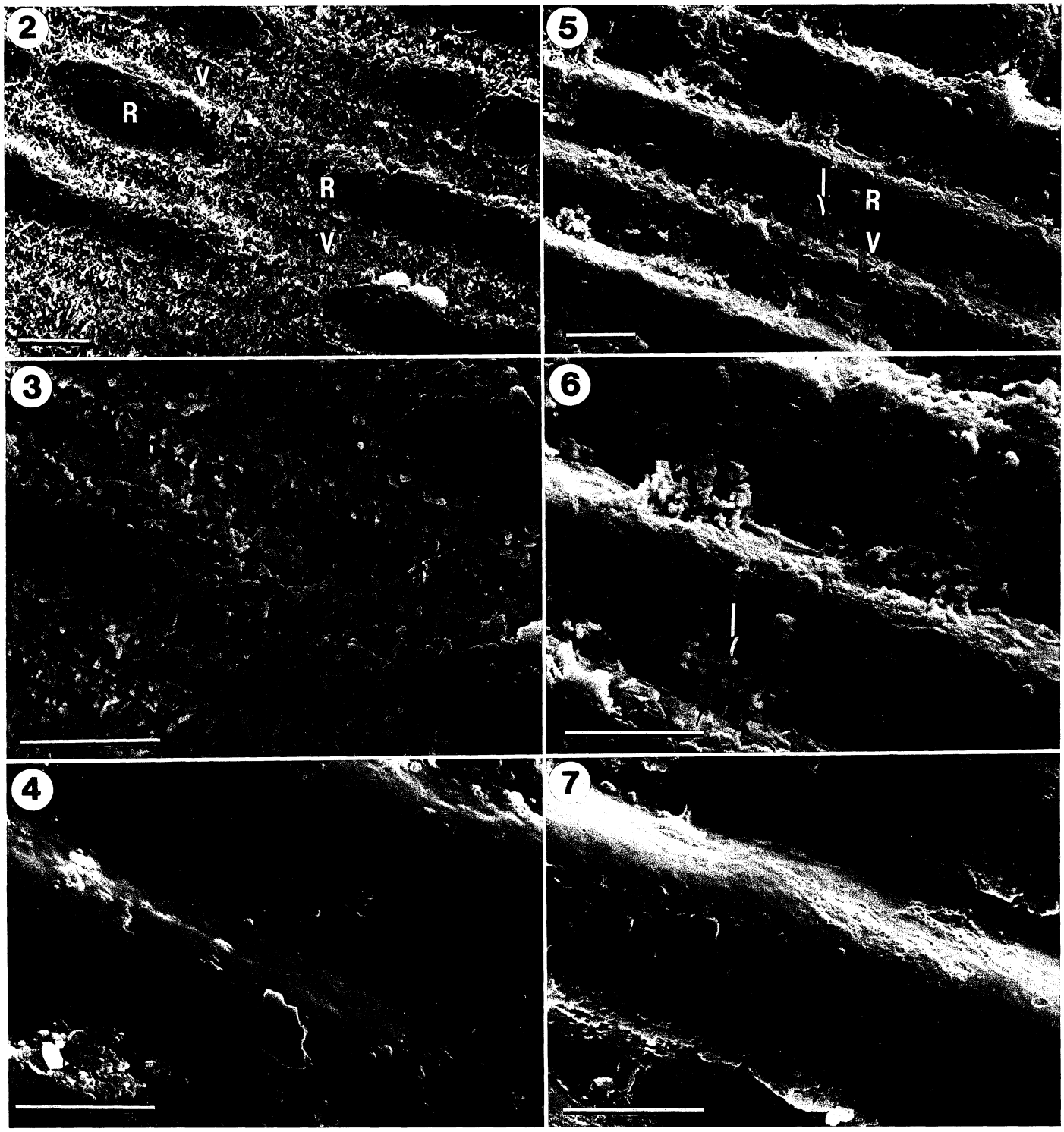


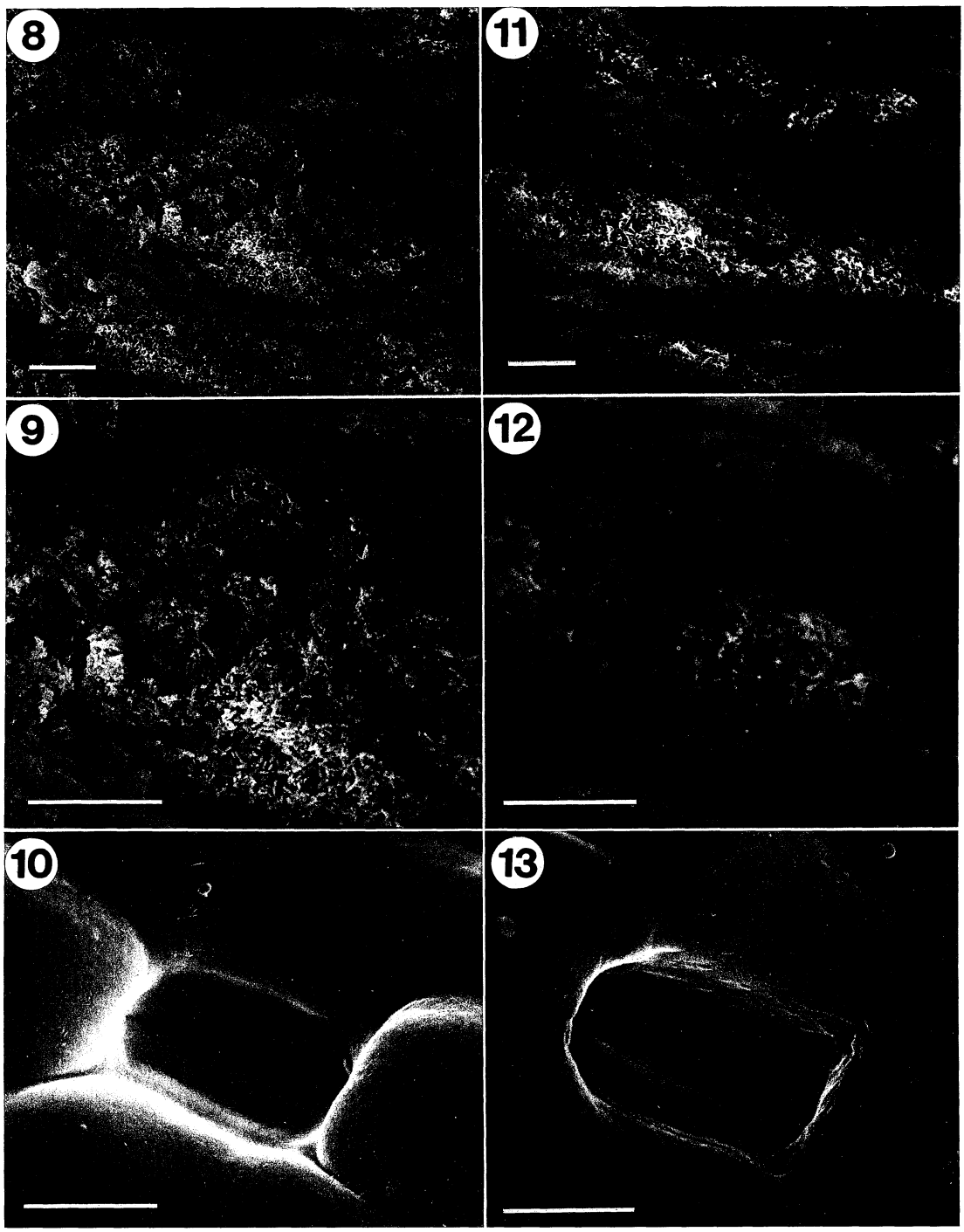
Fig. 1. The geometrical calculation of needle area including a distal cone and a proximal frustum; where L_d and L_p are the lengths of the distal cone and the proximal frustum, and P_d and P_p are the perimeters of the bottom of the distal cone and the middle of the proximal frustum. The shadowed areas represent the stomatal regions on the abaxial surface of needles.



Figs. 2–7. Cryo-SEM micrographs of the nonstomatal regions of *Pseudotsuga menziesii* needles. **2, 3.** Experimental needle (K + N fertilization) showing tubular epicuticular wax. **4.** Experimental needle that was treated with chloroform to dissolve the wax. **5, 6.** Control needle showing scalelike epicuticular wax. Arrows indicate the sparsely distributed tubular wax. **7.** Control needle that was treated with chloroform to dissolve the wax. R = ridge; V = valley. Bars = 10 μm.

regions were all taken on the abaxial surface halfway between the stomatal regions, the other nonstomatal regions were similarly ornate.

In contrast to experimental needles, the control needles in the nonstomatal region appeared to be relatively smooth with scalelike wax (Figs. 5, 6). No tubular wax was found on the ridges, and tubular wax did not occur, or, on some micrographs (Figs. 5, 6), occurred only sparsely in the valleys. The tubular wax covered 52.2% (±5.1%) of the nonstomatal area in the experimental needles in contrast



Figs. 8–13. Cryo-SEM micrographs of the stomatal regions of *Pseudotsuga menziesii* needles. **8, 9.** Experimental needle showing tubular epicuticular wax that obscures the stomates. **10.** Experimental needle that was treated with chloroform to dissolve the wax, making visible the sunken stomate. **11, 12.** Control needle showing tubular epicuticular wax that obscures the stomates. **13.** Control needle that was treated with chloroform to dissolve the wax, making visible the sunken stomate. Bars = 10 μm.

to 4.4% (±2.8%) in the controls. The chloroform treatment revealed the same pattern of surface topography on the control needles (Fig. 7) and the experimental ones (Fig. 4).

In the stomatal region, both experimental (Figs. 8, 9) and control (Figs. 11, 12) needles had ornate, tubular epicuticular wax deposits that almost completely occluded the epistomatal chambers. Although stomates were visible in surface view with a dissecting microscope as white (glaucous) specks, the tubular wax obscured the stomates when they were viewed with cryo-SEM (Figs. 8, 9, 11, 12). The fine structure of tubular wax on stomatal regions of experimental and control needles was similar (Figs. 9, 12). After removing the wax with chloroform, stomatal complexes were readily identified in experimental (Fig. 10) and control needles (Fig. 13). The surfaces of the

experimental and control needles appeared quite similar after chloroform treatment both in nonstomatal (Figs. 4, 7) and stomatal regions (Figs. 10, 13).

The mean quantity of surface wax per unit needle area was 3.66 ± 1.22 $\mu g/mm^2$ for the experimental needles and 4.39 ± 1.13 $\mu g/mm^2$ for the controls. The amount of surface wax per needle was 0.158 ± 0.019 mg in the experimentals and 0.160 ± 0.038 mg in the controls. The experimental needles had about 16.6% less wax per surface area and about 1.3% less total wax per needle than the controls. However, based on the analysis of variance (ANOVA), the mean weight of the wax, whether expressed per surface area or per needle, was not significantly different between the experimental and control needles. The experimental needles (dry weight per needle = 3.93 ± 0.72 mg) were about 35.8% heavier than the controls (=2.89 ± 0.27 mg), except those from installation 237 located in western Montana. The F value of ANOVA for the mean needle dry weight is 1.825 ($P = 0.225$). Long-term growth records (8 yr) showed that unlike the other sites, stem wood growth of trees from installation 237 had not responded to fertilization.

DISCUSSION

Our results demonstrated that glaucousness is caused by an ornate, crystalline wax morphology. In the nonstomatal regions glaucous (experimental) needles had much more tubular wax than did nonglaucous (control) needles. Experimental and control needles were similarly glaucous in the stomatal regions, where the wax was tubular. Removing wax with chloroform eliminated the glaucous appearance in each case. Fertilization did not result in the production of greater quantities of epicuticular wax, but rather in a change in wax morphology.

Glaucousness appears to be due to the production of greater quantities of tubular wax and lesser quantities of scalelike wax. The chemical nature and metabolic pathways for the production of these two wax types need to be determined. In *Pinus radiata* D. Don there is evidence that chemically different waxes were produced in stomatal and nonstomatal regions (Franich and Wells, 1980). It is possible that the different regions produce wax from different monomers or by changes in concentration and crystallization during growth. In vitro recrystallizations of epicuticular wax of *P. menziesii* suggested that the final dimensions and morphology of the wax crystals were functions of physical properties of the component molecules, rather than an enzyme-dependent polymerization (Lister and Thair, 1981). The method of epicuticular wax deposition remains unclear, but possible hypotheses include 1) an extrusion process through pores in the cuticle; 2) diffusion of wax components in a solvent system through cuticle, followed by crystallization on the leaf surface; or 3) transport of the wax components by a carrier (McWhorter and Paul, 1989).

In *Picea pungens* Engelm. (Reicosky and Hanover, 1976) and *Pinus strobus* L. (Johnson and Riding, 1891), tubular wax of the stomatal region is developed before needles emerge from the bud; in contrast, the epicuticular wax of the nonstomatal region develops later. However, Thijsse and Baas (1990) reported that the epicuticular wax on both stomatal and nonstomatal regions develops before bud break in *P. menziesii.*

Barthlott (1981) claimed that epidermal characters were only slightly affected by environmental conditions, and that great structural diversity of epidermal cells provided valuable systematic criteria. Our results revealed that most epidermal characters on both treatment and control leaves were similar. However, glaucousness may not always be a good taxonomic character because, at least in *P. menziesii,* it is strongly influenced by mineral nutrition.

Whether the production of glaucous needles by *P. menziesii* in response to fertilization is an adaptative response is unknown at this time. The effect of enhanced glaucousness of leaves is to reduce the intensity of the UV waveband of light. This may be advantageous especially for high elevation species where UV radiation can cause "sunscald" on nonglaucous leaves (Clark and Lister, 1975). In addition to reducing excess absorption of radiation, the projections of tubular waxes could also result in a greater boundary layer for transpiration and energy exchange with the surrounding cooler air (Barthlott and Wollenweber, 1981). In addition, the surface "roughness" may cause turbulence in laminar air flow and therefore increase thermodynamic exchange. Barthlott (1981) reported that woody species in moist tropical or subtropical environments often have little or no surface sculpturing. In contrast, plants growing under dry conditions normally have a greater surface sculpturing than those plants not living under water or temperature stress. Lastly, the sculpturing of epicuticular waxes increases the repellency of water and hydrophilic liquids. Wettable leaf surfaces, owing to the existence of the surface water film, tend to contain pollutants and are often highly colonized by microorganisms. In contrast, in water-repellent leaves it is usually quite hard to find any trace of contamination (Barthlott and Wollenweber, 1981).

In conclusion, our study shows that fertilization with potassium and nitrogen results in enhanced glaucousness in the nonstomatal areas of needles. Glaucousness is caused by a highly ornate tubular wax morphology and is not related to the total quantity of surface wax. The chemistry and metabolic pathways resulting in the different wax morphologies remain unclear.

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