Chemical and Structural Characterization of the Needle Epicuticular Wax of Two Clones of *Pinus strobus* Differing in Sensitivity to Ozone

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ABSTRACT

Trimble, J. L., Skelly, J. M., Tolin, S. A., and Orcutt, D. M. 1982. Chemical and structural characterization of the needle epicuticular wax of two clones of *Pinus strobus* differing in sensitivity to ozone. Phytopathology 72:652-656.

Two clones of eastern white pine (*Pinus strobus*), one sensitive and one tolerant to ozone (O_3), were exposed to 0.0 and 0.30 ppm O_3 for 6 hr/day for 7 consecutive days. Intact fascicles were excised from the terminal apex of each tree, and fine structure of the epicuticular wax was examined by scanning electron microscopy. All samples were characterized either by a fibrillar wax structure or an amorphous, platelike wax structure, regardless of O_3 exposure or previously determined O_3 sensitivity. Epicuticular wax removed from harvested fascicles was analyzed for alkane content by using gas-liquid chromatography. Hentriacontane (C_{31}) was the predominant alkane. Alkane concentration was significantly greater for the tolerant clone on both a fascicle surface area and fascicle weight basis, regardless of exposure to O_3.

The surfaces of plant leaves are characteristically covered by several layers of lipophilic material, the outermost being the epicuticular wax. It has been proposed that the functional and physiological roles of leaf epicuticular wax include the following: maintenance of water balance; modification of gas exchange; protection against mechanical and pest damage; protection against ultraviolet radiation; and protection against atmospheric pollutants such as O_3 (6). Rhine (16), in 1924, first noted that soot occluded stomatal chambers of Austrian pine (*Pinus nigra* Arnold). More recently, Percy and Riding (14) investigated the epicuticular wax structure of eastern white pines adjacent to and distant from anthropogenic sources of air pollutants. Needles of trees growing near a pulp mill were characterized by an amorphous epicuticular wax structure that completely occluded the

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epistomatal chamber. Conversely, needles of trees growing in a relatively pollution-free environment were characterized by a rodlike or fiberillar wax structure that only partially occluded the epistomatal chamber. Percy and Riding (14) also proposed that degradation of the fibrillar wax to amorphous wax may be attributed to exposure to atmospheric pollutants.

Epicuticular wax has been chemically defined as a complex and variable mixture of long-chain alkanes, alkenes, aromatic hydrocarbons, fatty acids, ketones, aldehydes, alcohols, and esters (2,12). The epicuticular waxes of conifers undergo changes in chemical composition as the result of plant maturation, needle age, or changes in environmental conditions (4,8,9). Hoffman et al (10) proposed that the fatty acid portion of the leaf wax may react with O_3 and with components of acidic precipitation.

The objectives of this study were: to characterize the fine structure of the epicuticular wax of eastern white pine; to compare the wax structure of two selected clones of eastern white pine, one sensitive and one tolerant to O_3, as previously determined by Nicholson (13); and to compare the alkane composition of the epicuticular wax of the two clones following exposure to O_3.

MATERIALS AND METHODS

Plant material. Two clones of 3-yr-old grafted eastern white pines with known sensitivity and tolerance to O_3 were maintained in 1.0-L plastic pots containing a medium composed of 1:2:2 (v/v) peat, vermiculite, and Weblite® (Weblite Corp., Webster, VA 24016). One gram of Osmocote® 14-14-14 (N-P-K) slow-release fertilizer (Sierra Chemical Company, Milpitas, CA 95035) was added to each pot in the early spring prior to budbreak. Trees were maintained in an outdoor cold frame during the winter months to induce dormancy. Eight weeks prior to fumigation, nine trees from each clone were moved into a greenhouse equipped with a charcoal air-filtration system to force budbreak. Trees were grown under ambient temperature and light while in the greenhouse, and were watered once daily and more frequently during the summer months.

Fumigation. Plants were divided into two treatments: 0.0 ppm O_3 and 0.30 ppm O_3 and were exposed for 6 hr (0800-1400) each day for seven consecutive days. Twelve trees, six from each clone, three per treatment, were exposed during each of two fumigations. Plants remained in the filtered-air greenhouse, except during fumigation in the continuous-stirred tank reactors (CSTR) as described by Heck et al (7).

Ozone was supplied by a Wellsbach Laboratory Ozonator Model T-408 (Wellsbach Ozone Systems Corporation, Philadelphia, PA 19129) and subsequently monitored by a Bendix Model 8002 Chemiluminescent Ozone Analyzer (Bendix Process Instruments Division, Lewisburg, WV 24901). The O_3 monitor was calibrated from a known source supplied by the Bendix Model 8852 Dynamic Calibration System (Bendix Process Instruments Division, Lewisburg, WV 24091). Light was supplied by high-pressure sodium lamps (Harvey Hubbell, Inc., Lighting Division, Christiansburg, VA 24073), which provided 28,000 lux and 415 μEinsteins/m^2/sec of photosynthetically active light. Humidity and temperature during fumigation were maintained at 65–86% RH and 27–35 C, respectively.

Scanning electron microscopy and gas-liquid chromatography. Three fascicles of 4- to 5-wk-old needles were excised from the terminal apex of each experimental tree at 6 hr and at 7 days after initiation of fumigation. Fascicles were stored in plastic bags that were folded, sealed loosely and refrigerated until examined by scanning electron microscopy (SEM). A 1.0 cm segment was removed from the midportion of each needle. The segments were affixed to aluminum stubs by using double-stick tape and aluminum paint. Segments were oriented so that the right-hand adaxial surface could be viewed. Specimens were coated with a gold-paladium alloy and viewed under an AMR Model 900 High-Resolution SEM (Advanced Metals Research Corp., Burlington, MA 01803) at 20 kV. Two predetermined locations were viewed on each segment, and the wax structure was designated fibrillar (rodlike projections of wax that covered more than 50% of a needle segment) or amorphous (solid platelike mass of wax that covered more than 50% of a needle segment).

Additional fascicles were harvested as before at 10 and 12 wk after fumigation for gas-liquid chromatographic (GLC) analysis of the wax composition. Each fascicle was cut 5.0 cm from the needle tips and weighed on a Mettler Balance. Fascicle leaf surface area was calculated as described by Wood (22). Wax was removed from the needle surface by dipping them for 30 seconds into 7.0 ml of redistilled chloroform contained in a preweighed glass test tube (11,19). The efficiency of wax removed was checked by SEM and all wax and particles were found to have been removed. The wax was filtered through Whatman No. 1 filter paper, evaporated to dryness under nitrogen, weighed, and redissolved in hexane. The net wax weight was determined. Three microliters of each sample were analyzed with a Bendix Model 2600 Gas-Liquid Chromatograph equipped with a flame ionization detector (Bendix Process Instruments Division, Lewisburg, WV 24901). Alkanes were separated on a 91 × 0.32 cm (OD) glass column packed with 3% SE-30 on Chromosorb Q (Applied Science Laboratories, Inc., State College, PA 16801). A temperature program of 150–250 C at 3 C/min was used. Alkane concentrations were expressed as equivalents of an external standard containing docosane (C_{22}). Peak areas were determined by a Spectra Physics Minigrator (Spectra Physics, Santa Clara, CA 95054). Alkane concentrations were expressed as follows: micrograms per sample; micrograms per fascicle weight in mg; micrograms per net weight of wax in mg; and micrograms per unit surface area (cm^2) of the fascicle. Data from each analysis of the wax content of fascicles harvested 10 wk and 12 wk after fumigation were analyzed independently.

RESULTS

Wax structure. In general, the morphology of the wax covering of all needles examined varied from fibrillar to amorphous. Wax structure was not correlated with O_3 sensitivity, since needles from the sensitive and tolerant clones were covered with both a fibrillar and amorphous wax structure. In addition, wax structure was not affected by O_3 exposure. The fibrillar and amorphous wax forms were characteristic of needles exposed to 0.0 ppm O_3 and 0.30 ppm O_3.The fibrillar wax form was characterized by tufts of rodlike tubules or fibrils of wax (Fig. 1A). The wax tufts sporadically covered the cuticular ridges of the needle except in regions adjacent to stomata where aggregates of wax tufts completely covered the needle surface. The amorphous wax form was characterized as an encrustation of solid, platelike layers of wax (Fig. 1B). The amorphous wax covering appeared to be solid, hard, and brittle, as evidenced by the cracking and disruption of the wax layer. The encrustation of amorphous wax prevalent around stomata and the stomatal pores of some specimens examined were completely occluded with wax. At high magnifications, the amorphous wax appeared to be formed by the "melting" of wax fibrils into a solid plate (Fig. 1C and D).

Alkane content. Long-chain alkanes were identified as the major components of epicuticular wax. Hentriacontane (C_{31}) and, to a lesser extent, tritriacontane (C_{33}) were the predominant alkanes. Alkane concentration was expressed as the sum of C_{31} and C_{33} (Table 1). Mean alkane concentrations per sample were significantly greater in the epicuticular wax of O_3-tolerant trees than in the wax of O_3-sensitive trees. In addition, wax of ozone-treated trees from both clones contained a higher alkane concentration per sample than did wax of control trees. This difference, however, was not statistically significant.

Alkane concentrations per fascicle weight varied, according to O_3 sensitivity and O_3 exposure. Only trees of the tolerant clone that had been treated with O_3 had significantly greater alkane concentration per fascicle weight. Results of the 12-wk harvest indicated a higher alkane concentration per fascicle weight for the tolerant clone, although this difference was not significantly greater than that of the sensitive clone.

In both the 10- and 12-wk harvest, the wax from the tolerant clone had a significantly higher alkane concentration per unit fascicle surface area than wax from the sensitive clone. Wax from

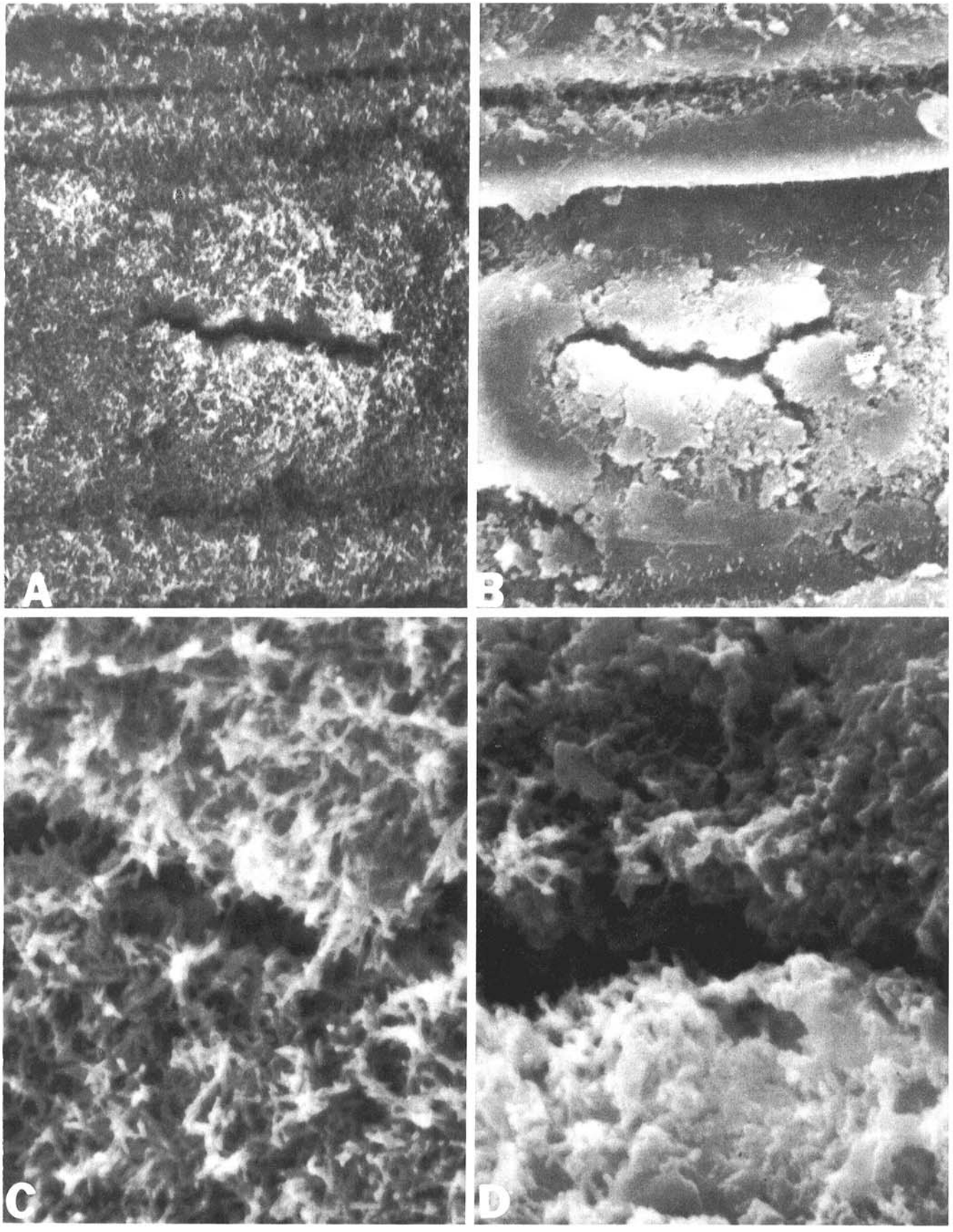

Fig. 1. The epicuticular wax structure on needles of *Pinus strobus* **A,** wax in tufts or rodlike tubules (×1,800); **B,** wax encrustation into platelike layers (×1,800); **C and D,** wax tubules and tufts coalescing into solid plates (×9,000 and ×8,500, respectively).

TABLE 1. Mean alkane concentration of the epicuticular wax from two clones of eastern white pine exposed to 0.0 and 0.30 ppm $)_3$ for 6 hr/day for 7 days

Clone treatment (O_3 conc. in ppm)	Fascicle weight (mg)	Fascicle surface area (cm^2)	Wax weight (mg)	Alkane conc. per sample (μg)	Alkane conc. per fascicle weight[y] ($\mu g/mg$)	Alkane conc. per unit surface area of fascicle[y] ($\mu g/cm^2$)
Exposure +10 wk:						
Sensitive (I-2)						
0.00	45.94 A[z]	4.53 A	0.110 A	60.01 A	1.31 A	13.27 A
0.30	49.73 AB	4.56 A	0.210 A	76.72 AB	1.55 A	16.75 AB
Tolerant (IV-2)						
0.00	63.05 C	4.91 A	0.572 AB	96.85 BC	1.54 A	19.83 BC
0.30	56.22 BC	5.05 A	0.920 B	124.14 C	2.28 B	24.66 C
Exposure +12 wk:						
Sensitive (I-2)						
0.00	59.55 A[z]	4.78 A	0.750 A	77.66 A	1.31 A	16.32 A
0.30	61.91 A	4.81 A	0.670 A	84.86 A	1.36 AB	17.54 A
Tolerant (IV-2)						
0.00	65.44 AB	4.75 A	0.850 A	120.94 B	1.87 B	25.66 B
0.30	73.71 B	5.07 A	1.700 B	129.34 B	1.75 AB	25.25 B

[y] Data from analysis of needles samples 10 and 12 wk following exposure.
[z] Within a column, means for each clone/treatment followed by the same letter are not significantly different, $P = 0.05$, according to Duncan's multiple range test.

ozone-treated trees within a clone had a higher alkane concentration than wax from trees not exposed to O_3. These increases were found in both experiments, but the differences were not statistically significant.

DISCUSSION

Variations in epicuticular wax structure have been previously reported by Wells and Franich (21) for *Pinus radiata* D. Don (Monterey pine) and by Hanover and Reicosky (6) for *Picea pungens* Engelm. (blue spruce). The varying wax structure was attributed to a number of factors, including environmental conditions and plant genetics (3,6). In this study of eastern white pine, the noted variations in wax structure could not be correlated to O_3 sensitivity, since the sensitive and tolerant clones displayed similar wax structure. Furthermore, exposure to O_3 did not appear to affect the wax structure of either clone. This is contrary to the findings of Percy and Riding (14) who reported that exposure to anthropogenic air pollutants appeared to modify the epicuticular wax of eastern white pine. Percy and Riding examined 30- to 50-yr-old trees grown in the field, whereas this study utilized 3-yr-old grafted trees grown under greenhouse conditions and exposed to O_3 only in controlled fumigation chambers.

The presence of two different wax forms on conifer needles, fibrillar and amorphous, as described in this study, has been previously reported by Bukovac and Widmoyer (1) and Grill (5). Franich et al (3) reported a "melting" phenomenon of wax fibrils into the amorphous wax form. This change in wax structure from fibrillar to amorphous may be indicative of mechanical injury to the plant surface, tissue maturation, tree age, physiological stress on the plant, or chemical changes in the wax constituents (1,3,14,15,21). Grill (5) reported that atmospheric conditions bring about changes in the amount of wax and in its structure on the needles of *Picea abies* L. Karsten (spruce).

Schütt and Schuck (18) ascertained that the chemical composition of *Pinus sylvestris* L. (Scotch pine) wax varied with season of the year and needle age. These findings are in accordance with those previously described by Herbin and Robins (8). Schuck (17) found quantitative differences in wax composition between provenances of Scotch pine. These differences were related to varying resistance to the needle blight fungus *Lophodermium pinastri* (Schrad.) Chev. The epicuticular needle waxes of the disease-resistant provenances contained a greater concentration of C_{29}-alcohol and C_{31}-paraffin than the more disease susceptible provenances (17). Similarly, Franich et al (4) proposed that wax composition of Monterey pine could be utilized in a genetic breeding program for resistance to *Dothistroma pini* Hulbarg. It has been suggested that resistance may be manipulated via regulation of the wax composition (17).

The chemical composition of eastern white pine epicuticular wax may provide a mechanism of tolerance to O_3. The correlation between the chemical composition of eastern white pine epicuticular wax and O_3 sensitivity has not been investigated prior to this study. The trend of increased alkane content in ozone-exposed epicuticular waxes is in agreement with the results of Trevathan et al (20), who found that tobacco leaf tissue contained a higher lipid content after exposure to 0.03 ppm O_3. The mechanism of this increase reported by Trevathan et al (20) and in this article can only be hypothesized at this time. It is unlikely that ozonation directly altered the alkane content of leaf surface wax. However, it is plausible that ozonation affects biosynthesis of alkanes in the epicuticular wax.

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