CERTAIN PHYSICAL CHARACTERISTICS OF THE WAX OF THE LEAVES OF DOUGLAS-FIR
PSEUDOTSUGA MENZIESII (MIRB.) FRANCO

by

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Typed by Dorothy Louise Ehrichs
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Certain Physical Characteristics of the Wax of the Leaves of Douglas-Fir
Pseudotsuga Menziesii (Mirb.) Franco.

Introduction

Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco.) was discovered by Archibald Menzies at Nootka Sound, Vancouver Island, Canada in 1792. Later, when the Rocky Mountain form was discovered by Dr. Parry in Colorado in 1861 (6), trees from the Pacific coast region became known as the coast form. Frothingham (6) stated that the two forms differ in physiological and morphological characteristics such as rate of growth, sensitivity to frost, form of crown, size and shape of cones, grain of wood and character of foliage. He further stated that in German plantations, foliage of the coast form suffered from severe winter temperatures but the Rocky Mountain form showed no injury and that the waxy cuticle of the mountain form protects it from excessive winter transpiration.

The taxonomic separation into two forms within the species rests on many criteria, but Douglas-fir appears to be polymorphic with respect to color of foliage which is exemplified by variability of leaf coloration within a local population.
Harlow and Harrar (8) state "... sometimes trees with blue-green foliage and others with yellow-green leaves are found standing together." Buesgen (2) considered the "bluish color" to be due to wax on the leaves. Examination of certain physical characteristics of wax of leaves of Douglas-fir to determine the nature of such color differences in the range from green to blue-green is the purpose of this thesis. Two Douglas-fir trees growing in McDonald Forest near Corvallis, Oregon, were selected for the study.
LITERATURE REVIEW

Literature relating to the wax of the leaves of Douglas-fir is rather scarce. However, references to wax of other conifers and many herbaceous plants are available (1, 3, 4, 10, 12, 13, 14, 15, 20, 22, 25, 26, 27, 32).

Following the terminology of Esau (5), the "epidermis" includes epidermal cells, stomatal guard cells, trichomes and other specialized cells. The epidermis is covered by a continuous, tough, varnish-like skin, the cuticle, which develops during maturation of the shoot. According to Roelofsen (20), this is the true cuticle; it is separable from the cells at all stages of growth (5). Later, the cellulose matrix of the outer walls of epidermal cells, especially of leaves, may become heavily impregnated with cutin, wax, pectic compounds and other substances. Internal cutin fuses with the true cuticle to form a thick structure that is more firmly attached to individual cells; this structure is the cuticular membrane (20). Wax may occur on the surface of the true cuticle, inside the cuticular membrane, or in mixture with cutin in the cellulose matrix of the outer walls of epidermal cells (2, 5, 12, 13, 17,
It does not occur alone in the cellulose matrix or free in the cell contents (4).

Wax may assume various forms on the surface of leaves. In 1884, De Bary (4) divided the types of wax coverings into four chief groups: strata or crusts, rods, simple granules and aggregates. The first type is found on Thuja orientalis L., T. occidentalis L., and Taxus baccata L. among others. The second type is especially prevalent on grasses and grass-like plants. The third type is characterized by small granules or rods about 1 micron long that are spaced on the cuticle. Closely arranged granules are responsible for the white or blue bloom typical of glaucous plants. The aggregate type is variable, being composed of simple layers interspersed with rods or multiple layers. Examples of the last type are found in the white eucalypti, Eucalyptus globus Labill., E. pulverulenta Sims., and the acacias, Acacia huegelii Benth., A. cultriformis Cunn. Other forms of deposit do occur; wax on the leaves of Agave americana L. is intermediate between the third and fourth types according to De Bary (4).
The development of wax layers on the surface of leaves has not been explained. One of the earliest hypotheses was that wax is extruded and does not owe its origin to metamorphosis of the cuticle or cell-membrane (4). Several investigators (12, 15, 22, 23, 24, 25, 26) have attempted to determine the validity of this theory in the past eighty years.

Extrusion of wax would require some type of opening or "pore" in the cuticular membrane. The shape and size of such an opening would affect the form of the wax deposit and the distribution of such openings would control the distribution of wax on the surface. Plasmodesmatal-type structures have been reported in outer walls of epidermal cells of Citrus L. leaves and fruits (23, 24) and in the leaves of Primula L. (15). Juniper (12) found minute projections extending from the surface of the cuticle in eight species of angiosperms and "assumed (these) to be of wax extruded through the cuticle". However, Scott et al (25) did not find any plasmodesmata in the epidermis of Allium cepa L. when the tissue was studied with the electron microscope. Schieferstein and Loomis (22) also used the electron microscope to study epidermal structure of 60 species of plants, about half of which have waxy
leaves and they did not find any evidence of passage-ways through the primary cell wall or cuticle.

The pattern of wax distribution that occurs in the center of the surface of a rapidly growing cell wall does not change as the growth continues (21). Wax may be deposited only on the developing surface and is not regenerated if removed from older surfaces (3, 21). However, wax production does not cease with maturation since in conifers, cutin and wax are deposited in the outer wall of epidermal cells of mature leaves for 13 years (27). It is common in many plants to find a thickening of the cuticular membrane after elongation of cells is completed (5). Schieferstein and Loomis (22) have stated: "... surface wax in maize is extruded only through the very young cell wall and cuticle, and that new wall, cuticle and surface wax deposits are formed at the margins of the outer walls of growing epidermal cells. This growth pattern would account for the unchanging wax distribution on growing leaves and for the unimpeded growth of epidermal cells partially covered with a non-plastic cuticular layer."
Both wax and cutin contain the same elements: carbon, hydrogen and oxygen. Warth (29, p. 92) reported that the essential processes involved in the natural formation of wax by plants are: "carboxylation, reduction, hydrolysis, oxidation, decarboxylation, and a photochemical reaction." Juniper (12) verified the necessity for the photochemical reaction when he grew *Pisum sativum* L. in the dark. He found that no wax was formed until the plant was exposed to light; then wax was formed quickly on immature leaves. The annular pattern of wax on the surface of the immature leaves conformed to the phasic growth pattern of the cells underlying the cuticle. Other reports concur with both the occurrence of a photochemical reaction and the annular pattern of wax produced after exposure to light (21, 22). Kreger (13) found that plants in widely divergent families have cuticle waxes of almost the same chemical composition and concluded that long-chain carbon compounds, precursors of wax, are formed in the protoplasm.

No reference to the processes in the production of cutin was found. De Bary (4, p. 81) cites the percentage composition of the elements in cutin as: C, 73.66;
H, 11.37; and O, 14.97 per cent. However, in contrast to wax, cutin is deposited in the absence of a light stimulus. A true cuticle will form on shoots of plants grown in the dark (10, 12, 21, 22, 26). Cutin has also been identified on walls of cells bordering the air space of the mesophyll (5).

Barber (1) reports that four species of Eucalyptus L'Herit have waxes with melting points between 45 and 48°C and that, after a single hot day, "leaves fully exposed to the sun may have faded while leaves in the shade may still be fully glaucous." He concludes that the wax melts and forms a smooth layer which does not scatter light.

High light intensity causes an increase in wax production (2, 26, 27). Sun leaves of Hedera helix L. produced heavier cuticles with greater wax content than shade leaves (26). Under greenhouse lighting conditions, Cunze (3) found that high atmospheric moisture would reduce this effect of light. He grew 8 species of conifers and angiosperms under similar light intensity, but with different moisture conditions. All seedlings produced some wax, but, in the moist environments, the amount was reduced to half of that in the normal environment. In Eucalyptus L., under extremely moist conditions,
the dermal tissue degenerated, mesophyll cells broke through the epidermis as intumescences and expanded about 100 times. The transpiration then increased about 70-fold.

Drought has been associated with increased wax production (2, 3, 26). Pines growing in xeric habitats produced thicker cuticles with more wax than the same species growing in more favorable habitats (27). An increase in air temperature will cause an increase in wax produced (26); but, excessively high temperatures will decrease the amount according to results with Prosopsis juliflora var. velutina DC. (10).

Other conditions such as position in crown (7), sun or shade (26), season (7), and age of leaves (17, 27) influence wax production. Barber (1) found that amount of wax in Tasmanian eucalypts varied directly and clinally with amount of frost along a transect from low to high elevation. He concluded that species which could not produce sufficient wax were restricted by frost activity to a given elevational zone.

Many investigators, who studied wax in relation to environmental factors, have offered hypotheses to explain its function. Buesgen (2, p. 406) reported that conifers growing
in southerly or elevated continental regions with low atmospheric humidity are "regularly provided with a bluish wax deposit ... or if this is absent with a thick hypodermal layer." He further states: "By such means, they protect themselves both against strong cuticular evaporation and against excessive light; two climatic factors which regularly go together. . .".

Cunze (3) stated:

"Auch die Nadeln der Coniferen erniedrigen durch das Wachs ihre Verdunstung bedeutend. (Picea pungens [Engelm.] um 400%). Bie ihnen besteht eine Beziehung zwischen Wachs und Hypoderm derart, dass das Wachs die Streifen gegen Verdunstung schützt, deren Hypoderm nicht gleichmaessig ausgebildet werden kann, weil es von Spaltoeffnungen durchbrochen wird."

Sutherland expressed a similar opinion after studying the anatomy of pine leaves (28). Barber (1) proposed that wax acts as insulation against frost. Wax also may act as a filter, absorbing the ultraviolet rays, to control quality of light entering the leaf (2). Leaves of Picea pungens var. glauca Regel turn from blue-grey to grey-green with age as the originally thick wax covering weathered (17). The heavy concentrations of wax in the stomatal region of the leaves of Douglas-fir,
recognized in the species description (18, 19), are considered
to be effective in reducing evaporation from the leaves (2).
Most other hypotheses explain the function of wax in terms of
reducing evaporation through all portions of the cuticular
membrane (5, 6, 16, 26, 27, 32). Skoss (26) has shown that
isolated, de-waxed cuticle of Hedera helix L. is permeable
to water in vitro. He found permeability inversely correlated
with amount of impregnated waxes.

Sutherland (28) reported a "water layer" (an extra row
of thin-walled cells without cell contents, interposed between
the epidermis and the first thickened hypodermal layer) in two
of three varieties of Pinus ponderosa Laws growing in New
Zealand. The varieties with this "water layer" appeared to be
darker than those without such a layer. Eucalyptus gigantea Dehnh.
displays a bluish hue on its leaves even after wax is removed.
Barber (1) called this "structural glaucousness" and explained
it on the basis of the configuration of the epidermal cell's outer
wall. Similar structures in Douglas-fir have not been reported.

Only one reference was found on the genetics of wax
production. Wellensiek (30, 31, 32) studied glaucousness in
Pisum (Tourn.) L. and found segregation in the F-2 and F-3 generations. All of the emerald varieties (non-waxy) differ from the glaucous ones by a factor, $B_1$, but the degree of glaucousness, when $B_1$ is present, varies according to the allelic status of another factor, $W$. 
MATERIALS AND METHODS

The two trees selected for this study are growing on the north edge of a large opening in McDonald Forest about eight miles north of Corvallis, Oregon, at an elevation of approximately 1750 feet. The blue tree is 105 feet high and 28 inches in diameter 4 1/2 feet above ground. Respective values for the green tree are 120 feet and 37 inches. Both trees are exposed to direct sunlight from the south and southeast (Figure 1).

The difference in foliar color was easily seen in the natural environment. When branches were brought into the laboratory, it was found difficult to detect this difference. Under fluorescent or weak incandescent light, branches from the two trees could not be separated on the basis of foliar color. However, the difference in color appeared under high intensity incandescent light, sunlight through window glass or full sunlight. Unfiltered sunlight or an electronic strobe light was markedly superior to any other lighting for black and white photography (Figure 2). Color film was sensitive to the difference in all conditions, but the most satisfactory results were obtained with full sunlight or strobe light.
Figure 1. The two trees used in this study as they stand in McDonald Forest, about 8 miles north of Corvallis, Oregon. A - the east side of the blue tree; B - the west side of the green tree.
Early in July, 1958, branches of the blue and green trees were taken from the south sides of the lower crowns. Immediately after collection the branches were cut into three segments representing the growth during the years 1958, 1957, and 1956. These segments, with leaves attached, were placed in closed glass jars containing moist cotton and stored in a refrigerated room.

Leaves were selected from the branch segments for a study of leaf morphology. Forceps were used for handling in this and all subsequent procedures. Observations with reflected light were made with a binocular dissecting microscope at magnifications of 7 to 25 diameters and at 590 diameters with a compound microscope equipped with an ultra pack vertical illuminator. Certain morphological characteristics considered pertinent to the presence of wax were checked. The number of stomatal rows and the presence of "bloom" was compared between leaf samples. To compare the area of leaves of the same length from different samples, a study was made of the tip, center, and base of leaves from each tree. Photographs were taken of the adaxial and abaxial surfaces of leaves from each age class of both trees.
Distribution of wax was noted with the aid of a Fisher-Johns Meltingpoint Apparatus. The copper stage of the melting-point apparatus was covered with an 18 mm circular cover glass of #1 thickness and preheated to various temperatures. A fresh leaf was placed, for 15 seconds, in one of four positions; with the adaxial surface, abaxial surface or one of the sides touching the glass. When the leaf was removed, the presence or absence of a wax deposit was recorded. The leaf was observed through the binocular dissecting microscope to insure that unheated surfaces remained unaffected during this treatment. Leaves from each age class of both trees were tested. Lowest temperature at which a deposit of wax could be seen was recorded.

Samples for wax analyses and anatomical study were selected to eliminate the variation in length of leaves that existed between the two trees. Individual leaves were removed from the branch segments and each leaf examined for defects. In a typical selection, the first 100 acceptable leaves of a given age from one tree constituted the first sample. The lengths of these leaves were recorded in millimeters. Acceptable leaves of the same age from the second tree were paired by length with those of the first sample. This permitted a total of six paired observations.
When pairing of the leaves was completed, the samples were wrapped separately in aluminum foil envelopes containing moist cotton and returned to a refrigerated room for 48 hours. The moisture content of the leaves thereby reached equilibrium, after desiccation that may have occurred during the sampling procedure.

Special aluminum foil dishes were prepared for weighing (Figure 3). The dishes were made by molding an aluminum foil sheet between the top and bottom halves of a petri dish. The shiny surface of the foil was on the inside of the finished dish. A section of foil approximately 1 inch wide and 2 inches long was left on one side to serve as a handle, as trim allowance for adjusting weight and as a place to number each dish. The dishes were dried in an electric oven at 34°C for 24 hours and subsequently weighed. Then, by trimming the handle, weight was adjusted to fall within the range of 0.9300 to 0.9500 grams. The dishes were returned to the oven, weighed after 8 hours and again after an additional 16 hours. The tares were recorded.

After the 48-hour rehydration period, the leaves were spread on paper towels in the laboratory for 15 minutes to evaporate excess moisture. The fresh weight of each sample was determined and the samples placed in numbered aluminum
Figure 2. General appearance of branches taken from the south side of the blue and green trees. The illumination was sunlight through window glass.

Figure 3. Aluminum foil dishes used in removal of wax from leaves.
foil dishes.

The removal of wax was accomplished by introducing 50 ml of reagent grade benzene into each aluminum foil dish. Controls, i.e., empty dishes and dishes with benzene only, were processed concurrently with the samples. The sample was constantly agitated for two minutes and the leaves were removed. This time interval was selected from previous tests which showed that wax from the surface was removed but subcuticular waxes and resins were unaffected by two minutes in benzene. All foil dishes containing benzene and dissolved wax were placed under a hood until evaporation of benzene was complete. The dishes were placed in the electric oven at 34°C until weight remained constant. Oven-dry weight for each sample of wax was calculated.

Melting point of each sample of wax was determined on the Fisher-Johns apparatus while the sample was observed under the binocular microscope. Temperature at first sign of melting and at completion was recorded.

Immediately following wax removal, each sample of leaves was sub-divided into two groups. Group 1 contained 2 leaves from each of the 23, 24, 25, 26 and 27 mm length
classes. Group 2 contained the remaining 90 leaves in the sample.

Leaves of Group 1 were placed in small vials containing a saturated aqueous solution of phenol for protection against fungal attack and were set aside. Later, these leaves were sectioned on the sliding microtome at 80 microns, stained with Sudan IV and mounted on a slide in Karo after the methods given by Johansen (11, p. 24, 191). Untreated leaves from the same length classes were prepared in like manner. The anatomical features were studied under a compound microscope with transmitted light. The outline of each cross section was traced after projection through a compound microscope onto a glass surface. The width, thickness and perimeter of each leaf was measured.

Leaves of Group 2 were placed in a numbered aluminum foil dish. The dishes were placed in loosely covered, plastic laboratory trays. After evaporation of absorbed benzene, the dishes were placed in an electric oven at 34° C until weight remained constant. Oven-dry weight of the 90 leaves was recorded and converted to an estimate of oven-dry weight for 100 de-waxed leaves and for 100 untreated leaves.

Certain portions of this study were not considered subject to statistical analyses. These include the effect of light quality
on color of foliage and basic coloration after removal of wax from the surface of leaves. Measurements of perimeter, width and thickness at the middle of leaves from the two trees were analyzed by t-tests. The factors of replication and age class were recorded in such a manner that the data on melting point of wax, fresh weight and oven-dry weight of leaves, and weight of wax removed from the leaves could be analyzed by t-tests, analyses of variance, and analyses of covariance.
RESULTS

Observations of leaves of Douglas-fir show that the adaxial surfaces of leaves from the blue tree have a broader wax deposit in the groove than leaves of the green tree. Abaxial surfaces are very similar in both trees (Figure 4). Number of stomatal rows are randomly distributed between 6 and 11 and "stomatal bloom" is present on all leaves. When wax on the leaves is removed with drops of benzene, the color difference disappears. Leaves from both trees are dark green when wet with benzene and glossy green when dried after treatment. Later, a brown tone appears as cell contents decompose in the treated leaves.

Cross sections of leaves show that anatomical features are normal for the species (Figure 5). The cuticular membrane, stained with Sudan IV, appears as one layer, approximately equal in thickness to the epidermal wall below it. This structure measures 3 to 5 microns in leaves of both trees, before or after removal of wax from the surface of leaves. Variation in thickness of this layer appears to be random. Under polarized light, the cuticular membrane is birefringent.
Figure 4. Surface view of leaves of Douglas-fir. A - adaxial surface, B - abaxial surface at 3.3 X. From left to right, the leaves are: Blue - 1958, 1957, 1956 and Green - 1958, 1957, 1956 in both A and B. Scale in millimeters.

Figure 5. Cross sections (80 microns) from the middle of two-year-old leaves stained with Sudan IV and mounted in Karo. A - from blue leaf and B - from a green leaf. (Note the sclerid in the blue leaf and the holes in the mesophyll of both leaves where these sclerids were ripped out by the microtome knife.) Magnification is 48 X.
Wax was found on the leaves by tests conducted with the preheated coverslip and melting point apparatus. At temperatures above 57°C, wax was deposited from adaxial surfaces while sides and abaxial surfaces required temperatures of 59°C to form a wax deposit. Distribution of wax was observed on all portions of the leaves by vertical illumination with a compound microscope at 590X magnification. The configuration of the wax resembled the simple granules in De Bary's (4) system. The density of wax granules was greatest on leaves of the blue tree.

Preliminary studies of the central portion of leaves indicated that margins were essentially parallel and that thickness was relatively constant within any one leaf from 5 mm above the base to within 3 mm of the tip. This suggested that the greatest variant affecting area of a leaf was total length. This fact was considered in the selection of samples for further study (Table 1). However, cross sections taken from the center of 100 individual leaves indicated that three factors affecting area were not controlled by the sampling technique. The relative perimeter, width and thickness of the projected images of the 100 cross sections showed that variation within samples was uniform (Table 2).
Table 1. Distribution of the 100 leaves in each half of a paired sample by Age Class and Replicate Number within the millimeter length classes.

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Table 2. Relative size of projected images of cross sections of fresh leaves and leaves in Group 1.

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<td>12 11 12 10 14 10 11 12 14 10</td>
</tr>
<tr>
<td>11 11 12 11 13 11 11 11 13 10</td>
</tr>
<tr>
<td>13 11 11 11 14 11 11 11 13 11</td>
</tr>
<tr>
<td>12 11 10 10 14 12 11 11 12 11</td>
</tr>
<tr>
<td>13 11 11 12 14 12 11 11 12 10</td>
</tr>
<tr>
<td>12 10 11 10 14 12 12 11 12 11</td>
</tr>
<tr>
<td>13 11 12 11 14 12 9 11 12 10</td>
</tr>
<tr>
<td>11 11 12 11 14 12 12 11 13 12</td>
</tr>
<tr>
<td>11 11 12 12 14 11 11 11 12 10</td>
</tr>
<tr>
<td>120 109 115 108 137 115 111 111 125 108</td>
</tr>
</tbody>
</table>
When these data were converted to average perimeter, width and thickness measurements in millimeters (Table 3), it was found that all measurements were greater for the blue tree than for the green tree.

Table 3. Means of perimeter, width and thickness measurements for fresh leaves and leaves in Group I derived from the relative values in Table 2.

| Type of Measurement | FRESH LEAVES | | | TREATED LEAVES | | |
|---------------------|--------------|----------------|----------------|----------------|----------------|
|                     | 1958 | 1957 | 1956 | 1958 | 1957 |
| **Perimeter**       |      |      |      |      |      |
| Blue                | 4.02 | 3.99 | 4.30 | 3.90 | 4.25 |
| Green               | 3.45 | 3.38 | 3.57 | 3.50 | 3.47 |
| Difference          | .57  | .61  | .73  | .40  | .78  |
| **Width**           |      |      |      |      |      |
| Blue                | 1.72 | 1.70 | 1.82 | 1.67 | 1.81 |
| Green               | 1.45 | 1.39 | 1.46 | 1.44 | 1.43 |
| Difference          | .27  | .31  | .36  | .23  | .38  |
| **Thickness**       |      |      |      |      |      |
| Blue                | .72  | .69  | .82  | .67  | .75  |
| Green               | .65  | .65  | .69  | .67  | .65  |
| Difference          | .07  | .04  | .13  | .00  | .10  |

Data for width and thickness (Table 3) were used to compare actual perimeter with estimated perimeter (Table 4), calculated by the equation for approximate circumference of an ellipse.
Table 4. Comparison of actual and estimated perimeter at the middle of Douglas-fir leaves. The equation used to estimate perimeter was for approximate circumference of an ellipse:

\[ 2\pi \sqrt{\frac{a^2 + b^2}{2}} \]

where:
- \( a \) = width, and
- \( b \) = thickness of leaf from Table 3.

<table>
<thead>
<tr>
<th>Source</th>
<th>FRESH LEAVES</th>
<th>TREATED LEAVES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1958</td>
<td>1957</td>
</tr>
<tr>
<td>Blue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual</td>
<td>4.02</td>
<td>3.99</td>
</tr>
<tr>
<td>Estimated</td>
<td>4.14</td>
<td>4.08</td>
</tr>
<tr>
<td>Diff. (E-A)</td>
<td>.12</td>
<td>.09</td>
</tr>
<tr>
<td>Green</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual</td>
<td>3.45</td>
<td>3.38</td>
</tr>
<tr>
<td>Estimated</td>
<td>3.53</td>
<td>3.41</td>
</tr>
<tr>
<td>Diff. (E-A)</td>
<td>.08</td>
<td>.03</td>
</tr>
</tbody>
</table>

Perimeter of green leaves varied only slightly from the estimate, but data for blue leaves were substantially different from the elliptic estimate. In both cases, however, measured perimeter was smaller than estimated perimeter. The lack of fit obtained with the equation precluded mathematical calculation of surface area from available data. An equation that would provide an
estimate of total area would require multiple measurements of perimeter at intervals along the length of individual leaves. However, an estimate of total area is not essential since photographs show that wax occurs as discrete granules on the surface of leaves (Figure 6). Thus, variation among trees in spacial arrangement of wax granules may be observed directly and estimates may be made of differences in amount of wax on small areas.

Melting point of wax removed from the leaves (Table 5) varies from 59 to 62° C in all age classes for both trees.

Table 5. Range of melting point for wax removed from the 100 leaves in each half of each paired sample by Age Class and Replicate Number.

<table>
<thead>
<tr>
<th>Tree</th>
<th>1958 Replicate No.</th>
<th>1957 Replicate No.</th>
<th>1956 Replicate No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C 1</td>
<td>°C 2</td>
<td>°C 1</td>
</tr>
<tr>
<td>Green</td>
<td>59-60</td>
<td>60-61</td>
<td>60-61</td>
</tr>
</tbody>
</table>

* First sign of melting - melting completed.
Figure 6. Granules of wax on three different portions of the surface of leaves of Douglas-fir. A-D, adaxial surface; E-H, stomatal region; K-N, abaxial surface without stomata. A, E, K are one-year-old and B, F, L are three-year-old leaves from the blue tree; C, G, M are one-year-old and D, H, N are three-year-old leaves from the green tree. Note the differences in number and distribution of wax granules between leaves from the two trees, especially on the adaxial surface; also the mode of clustering of granules and the discreteness of granules even after three years of weathering.
Differences between the samples of wax are not significant when the sign test is applied to the means from each range. This physical characteristic and the crystallization pattern that was observed indicate that waxes from the two trees are chemically similar, if not identical.

The data from the paired observations indicated that blue leaves yielded more wax than did the green leaves (Table 6). The same relationship is indicated by the results for fresh weight; i.e., blue leaves are heavier than green leaves (Table 7).

Table 6. Weight of wax removed by treatment with benzene of the 100 leaves in each half of each paired sample by Age Class and Replicate Number.

<table>
<thead>
<tr>
<th>Tree</th>
<th>1958 Replicate No.</th>
<th>1957 Replicate No.</th>
<th>1956 Replicate No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Blue</td>
<td>14.7</td>
<td>16.3</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>Green</td>
<td>10.9</td>
<td>14.5</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.9</td>
</tr>
<tr>
<td>Difference</td>
<td>3.8</td>
<td>1.8</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.6</td>
</tr>
</tbody>
</table>
Table 7. Fresh weight of the 100 leaves in each half of each paired sample by Age Class and Replicate Number.

<table>
<thead>
<tr>
<th>Tree</th>
<th>1958 Replicate No.</th>
<th>1957 Replicate No.</th>
<th>1956 Replicate No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>Blue</td>
<td>1637.0</td>
<td>2071.8</td>
<td>2071.8</td>
</tr>
<tr>
<td></td>
<td>2032.5</td>
<td>2097.0</td>
<td>1568.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1638.5</td>
</tr>
<tr>
<td>Green</td>
<td>1518.0</td>
<td>1605.0</td>
<td>1348.0</td>
</tr>
<tr>
<td></td>
<td>1608.0</td>
<td>1650.0</td>
<td>1342.0</td>
</tr>
<tr>
<td>Difference</td>
<td>119.0</td>
<td>466.8</td>
<td>220.0</td>
</tr>
<tr>
<td></td>
<td>424.5</td>
<td>447.0</td>
<td>296.5</td>
</tr>
</tbody>
</table>

In fresh weight, three-year-old leaves were lighter than two-year-old and heavier than one-year-old leaves.

Oven-dry weight of the 90 de-waxed leaves in group 2 (Table 8) were converted to an estimate of oven-dry weight of 100 de-waxed leaves (Table 9). Total length of the 100 leaves in each sample was used as a correction factor in the following formula:

Oven-dry weight of 100 de-waxed leaves equals

\[
\frac{\text{oven-dry weight of 90 de-waxed leaves}}{\text{(length of 100 leaves in mm minus 250 mm for leaves in Group 1)}} \times \text{(length of 100 leaves in mm)}
\]

Weight of wax removed was added to values calculated to obtain an estimate of oven-dry weight for 100 untreated leaves for each sample (Table 10). In all cases, oven-dry weight of blue leaves is greater than that of green leaves. Three-year-old leaves are
Table 8. Oven-dry weight of the 90 de-waxed leaves in Group 2 from each half of each paired sample by Age Class and Replicate Number.

<table>
<thead>
<tr>
<th>AGE CLASS</th>
<th>1958</th>
<th>1957</th>
<th>1956</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate No.</td>
<td>Replicate No.</td>
<td>Replicate No.</td>
</tr>
<tr>
<td>Tree</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>Blue</td>
<td>418.2</td>
<td>493.8</td>
<td>628.4</td>
</tr>
<tr>
<td>Green</td>
<td>410.2</td>
<td>438.2</td>
<td>524.2</td>
</tr>
<tr>
<td>Difference</td>
<td>8.0</td>
<td>55.6</td>
<td>104.2</td>
</tr>
</tbody>
</table>

Table 9. Oven-dry weight of the 100 de-waxed leaves from each half of each paired sample by Age Class and Replicate Number. Calculated according to the formula:

\[
\text{O.D. Wt. 100 de-waxed leaves} = \frac{(\text{O.D. Wt. 90 de-waxed leaves})(\text{Length of 100 leaves in mm})}{(\text{Length of 100 leaves in mm minus 250 mm for leaves in Group 1})}
\]

<table>
<thead>
<tr>
<th>AGE CLASS</th>
<th>1958</th>
<th>1957</th>
<th>1956</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate No.</td>
<td>Replicate No.</td>
<td>Replicate No.</td>
</tr>
<tr>
<td>Tree</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>Blue</td>
<td>463.6</td>
<td>544.8</td>
<td>694.4</td>
</tr>
<tr>
<td>Green</td>
<td>454.7</td>
<td>483.5</td>
<td>579.2</td>
</tr>
<tr>
<td>Difference</td>
<td>8.9</td>
<td>61.3</td>
<td>115.2</td>
</tr>
</tbody>
</table>
lighter than two-year-old leaves, but approximately equal to one-year-old leaves in oven-dry weight.

Table 10. Oven-dry weight of the 100 de-waxed leaves plus wax, from each half of each paired sample by Age Class and Replicate Number.

<table>
<thead>
<tr>
<th>Tree</th>
<th>1958 Replicate No.</th>
<th>1957 Replicate No.</th>
<th>1956 Replicate No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>Blue</td>
<td>478.3</td>
<td>561.1</td>
<td>706.2</td>
</tr>
<tr>
<td>Green</td>
<td>465.6</td>
<td>498.0</td>
<td>587.7</td>
</tr>
<tr>
<td>Difference</td>
<td>12.7</td>
<td>63.1</td>
<td>118.5</td>
</tr>
</tbody>
</table>

The t-tests on paired observations show that differences in perimeter, width and thickness at the middle of leaves, in fresh weight and oven-dry weight of leaves and in amount of wax removed are all significant at the 5 per cent level of significance (Table 11). Analyses of variance show that age class of leaves and tree from which they were collected influence fresh weight and oven-dry weight of leaves and weight of wax removed.
Table 11. Results of t-tests for paired observations on Douglas-fir leaves.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of Freedom</th>
<th>t-value</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perimeter</td>
<td>4</td>
<td>9.3</td>
<td>1</td>
</tr>
<tr>
<td>Width</td>
<td>4</td>
<td>11.2</td>
<td>1</td>
</tr>
<tr>
<td>Thickness</td>
<td>4</td>
<td>3.0</td>
<td>5</td>
</tr>
<tr>
<td>Table 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight of wax</td>
<td>5</td>
<td>10.2</td>
<td>1</td>
</tr>
<tr>
<td>Table 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh weight</td>
<td>5</td>
<td>5.7</td>
<td>1</td>
</tr>
<tr>
<td>Table 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oven-dry weight</td>
<td>5</td>
<td>3.3</td>
<td>5</td>
</tr>
</tbody>
</table>

There was no significance in differences between replications and interaction between factors was absent (Table 12).

A rearrangement of the data was used in the analyses of covariance in order to pool most of the non-significant variation with experimental error. Variation due to trees, age class and trees x age class was substracted from the total variation between samples. The regression coefficient, $b$, and correlation coefficient, $r$, were calculated from the remaining variation.
Table 12. Summary of analyses of variance for observations on Douglas-fir leaves.

<table>
<thead>
<tr>
<th>Source and Factors</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 6. (wt. of extracted wax)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Replications</td>
<td>1</td>
<td>1.76</td>
<td>NS*</td>
</tr>
<tr>
<td>Years</td>
<td>2</td>
<td>60.46</td>
<td>5</td>
</tr>
<tr>
<td>RxY (a)</td>
<td>2</td>
<td>2.53</td>
<td>NS</td>
</tr>
<tr>
<td>Trees</td>
<td>1</td>
<td>25.23</td>
<td>1</td>
</tr>
<tr>
<td>TxY</td>
<td>2</td>
<td>.98</td>
<td>NS</td>
</tr>
<tr>
<td>TxR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TxYxR (b)</td>
<td>3</td>
<td>.34</td>
<td></td>
</tr>
</tbody>
</table>

| Table 7. (fresh weight of leaves) | | | |
| Replications | 1 | 32,054.01 | NS |
| Years | 2 | 147,316.97 | NS |
| RxY (a) | 2 | 14,572.81 | NS |
| Trees | 1 | 324,657.21 | 1 |
| TxY | 2 | 12,320.76 | NS |
| TxR | | | |
| TxYxR (b) | 3 | 8,297.88 | |

| Table 10. (oven-dry wt. of leaves) | | | |
| Replications | 1 | 1,476.30 | NS |
| Years | 2 | 22,044.88 | 5 |
| RxY (a) | 2 | 940.98 | NS |
| Trees | 1 | 13,500.52 | 1 |
| TxY | 2 | 2,621.23 | NS |
| TxR | | | |
| TxYxR (b) | 3 | 337.65 | |

* NS = non significant F-value obtained.
Table 13. Summary of analyses of covariance for observations on Douglas-fir leaves.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares (xx)</th>
<th>Sum of Products (xy)</th>
<th>Sum of Squares (yy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>fresh wt.</td>
<td>oven-dry wt.</td>
<td>wt. of wax</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>730,025.94</td>
<td>6,829.34</td>
<td>154.18</td>
</tr>
<tr>
<td>Trees</td>
<td>1</td>
<td>324,657.21</td>
<td>2,862.01</td>
<td>25.23</td>
</tr>
<tr>
<td>Years</td>
<td>2</td>
<td>294,633.94</td>
<td>3,431.99</td>
<td>120.92</td>
</tr>
<tr>
<td>Txy</td>
<td>2</td>
<td>24,641.52</td>
<td>68.18</td>
<td>.20</td>
</tr>
<tr>
<td>Remainder</td>
<td>6</td>
<td>86,093.27</td>
<td>467.16</td>
<td>7.83</td>
</tr>
</tbody>
</table>

b (remainder) = SP_{xy}/SS_{xx} = + 0.0054
r (remainder) = (SP_{xy})^2/SS_{xx}SS_{yy} = 0.324

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares (xx)</th>
<th>Sum of Products (xy)</th>
<th>Sum of Squares (yy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>oven-dry wt.</td>
<td></td>
<td>wt. of wax</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>67,203.95</td>
<td>-320.86</td>
<td>154.18</td>
</tr>
<tr>
<td>Trees</td>
<td>1</td>
<td>13,500.52</td>
<td>583.63</td>
<td>25.23</td>
</tr>
<tr>
<td>Years</td>
<td>2</td>
<td>44,089.76</td>
<td>-1,057.42</td>
<td>120.92</td>
</tr>
<tr>
<td>Txy</td>
<td>2</td>
<td>5,242.46</td>
<td>31.09</td>
<td>.20</td>
</tr>
<tr>
<td>Remainder</td>
<td>6</td>
<td>4,371.21</td>
<td>121.84</td>
<td>7.83</td>
</tr>
</tbody>
</table>

b (remainder) = SP_{xy}/SS_{xx} = + 0.0279
r (remainder) = (SP_{xy})^2/SS_{xx}SS_{yy} = 0.434
existing for weight of wax on fresh weight and weight of wax on oven-dry weight of leaves (Table 13).

The $b$ values show that, regardless of tree or year, the amount of wax removed increases 0.0054 units for every unit increase in fresh weight and it increases 0.0279 for every unit increase in oven-dry weight of leaves. However, the $r$ values of 0.324 for wax on fresh weight and 0.434 for wax on oven-dry weight indicate that weight of leaf can not account for more than 10 to 19 per cent of the variation in weight of removable wax.
DISCUSSION

Since the two trees selected for this study are growing under apparently similar conditions, the differences found between the phenotypes may to some extent be attributed to genetic differences.

Color of foliage was found to be a reliable indicator of the presence of wax in that more wax could be removed from the leaves of the blue tree than from leaves of the green tree. Wax occurs on the leaves in the form of discrete granules about 0.25 microns in diameter. These granules are distributed on the surface of the true cuticle singly or in small clusters, however the granules were never fused to form any type of continuous layer on any of the leaves observed. Visual observation of the difference in color between the two trees depends on the intensity and spectrum of the light used for illumination. Sunlight, sunlight through window glass, electronic strobe light and high intensity incandescent light proved to be the best illumination. When the wax is disturbed or removed, either physically or chemically, the glaucousness disappears regardless of the illumination used. This agrees with De Bary's observation that
physical structure of the wax causes the phenomenon of glaucousness (4).

Wax is also identifiable in the cuticular membrane of leaves. The portion of the epidermis that was stained with Sudan IV contained both cutin and wax according to the birefringence observed with polarized light when the cross sections of leaves were examined. However, wax from inside of the true cuticle was not removed by the processes used for removal of wax on the surface of leaves. Therefore, findings reported here do not involve internal wax.

When the study was started, it was thought necessary to determine total area of leaves in order to estimate differences in amount of wax per unit of surface. However, the mathematical equation for approximate circumference of an ellipse yielded estimates which did not agree with actual measurements of perimeter. Within a single tree, the estimate varies from the actual by a relatively constant amount, but, between the two trees, this comparison was disproportionate. Since perimeter is not related to length of leaves in a constant way, this approach was abandoned, even though repeated measurement of perimeter
at measured intervals along the length of individual leaves may provide a realistic estimate of area. In addition, the need for an estimate of total area disappeared when it was found that wax occurs as discrete granules and not as a continuous or broken layer as was believed in the beginning. Thus, with suitable equipment, a compound microscope equipped with a vertical illuminator, the amount of wax per unit area (in terms of the number of granules) may be estimated directly without involving the entire area of the leaf.

Two physical properties of waxes were used to evaluate the possibility of chemical differences in the wax from these two trees. Firstly, melting point of wax, in situ, was found to be slightly lower than melting point of wax removed with benzene. However, the two trees did not differ in this respect. Secondly, observations indicate that shape and size of the wax granules are the same in both trees. Even in the stomatal region, where the granules often appear to be massed to form waxy plugs in the stomatal openings, no indication was found of fusion between granules. Yet, wax granules were observed singly and in small clusters. The grouping could well result from surface tension in unoriented wax as it shrinks during
drying upon exposure to the atmosphere before crystallization occurs. This hypothesis agrees with the conclusions of Kreger (13). On the basis of melting point determinations and observed crystallization pattern, chemical differences do not exist between waxes from these two trees.

The method of sampling, pairing of leaves by length, was time consuming and would be costly if large samples were required. However, this design permits a rather close evaluation of the possibility of using other criteria as a basis for comparing the amount of wax on different leaves. Determinations of fresh weight or oven-dry weight of leaves would be rapid and would probably result in less disturbance of the wax granules than would length comparisons. A correlation was found between these weights and weight of wax removed from the 100 leaves in each sample, but the correlation coefficients were not very large. Ten per cent of the variation in amount of wax removed can be accounted for by variation in fresh weight of leaves and 19 per cent by variation in oven-dry weight of leaves. This emphasizes the fact that leaves of the same length (and comparable area) from two trees do not have the same weight. These differences between the
phenotypes would invalidate the use of weight of leaf, either fresh or oven-dry, as a basis for sampling in determinations of wax on the surface of the leaf.

Many authors cited environmental factors associated with the presence of wax on leaves of other species. In these reports, such factors as high light intensity (especially that rich in ultra-violet rays), severe frost, drought and low atmospheric humidity are indicated as favoring individuals capable of producing wax on their leaves. It is interesting to note that characteristics of foliage, associated with amount of wax in this study, also have been associated with these environmental conditions (2).

All colors of foliage have been observed in the Pacific Coast Region where environment is optimum for growth of Douglas-fir. This would indicate that the genetic base for color is multifactorial. The assumption seems reasonable that individual variation in color of leaves will be useful in future studies of genetic diversity.

Further investigation based on the results of this work has been initiated. Pairs of trees, within the green to blue-green range of foliar color, have been selected in several
environments. Scions from these trees have been collected and grafted onto root stocks. When these grafts are old enough to produce flowers, reciprocal pollinations will be made between the respective pairs of trees. Progeny obtained will be placed under various environmental conditions, such as drought, etc., to study their response to such conditions. Future results will indicate the heritability of color and may show whether blueness of leaves is a suitable criterion for selection of drought tolerant individuals.
SUMMARY

Wax occurs on leaves of Douglas-fir as discrete granules, about 0.25 micron in diameter, which are distributed singly or in small clusters on the surface of leaves. Wax is also identifiable in the cuticular membrane by the use of Sudan IV and polarized light. Melting point determinations indicate that wax is chemically the same in both trees.

The granular crystallization pattern of the wax causes the phenomenon of glaucousness. When the structure of wax is disturbed or wax is removed, the blueness disappears and leaves from both trees appear to be of the same dark green color.

Since blue leaves have more wax than green leaves of the same length, amount of wax controls the intensity of the blue color observed. Though amount and distribution of wax on the abaxial surface, especially in the stomatal region, appears to be similar on leaves from both trees, quantitative differences are visible on the adaxial surface.

Other differences existing between leaves of these trees were measured and their association with wax evaluated.
Although leaves of the blue tree are slightly larger at the middle and heavier in both fresh and oven-dry weight than leaves of the same length from the green tree, the correlation coefficient for the amount of wax on either of these weights was small. Thus, weight of leaf is not a suitable basis for determination of the amount of wax on the surface of the leaf.

Determination of total area of a leaf, earlier considered to be the most suitable basis for comparing amount of wax, is found to be impractical and unnecessary. Wax granules per unit area may be estimated directly with suitable optical equipment.

The analyses also indicate that significant variation occurs between different age classes of leaves for many of the characteristics measured. Future studies of wax should consider this effect by comparing leaves of the same age from the phenotypes being investigated.
BIBLIOGRAPHY


