

Morphology and Accumulation of Epicuticular Wax on Needles of Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*)

Abstract

Past studies have documented differences in epicuticular wax among several tree species but little attention has been paid to changes in accumulation of foliar wax that can occur during the year. We sampled current-year needles from the terminal shoots of Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) in late June/early July, late August and early November. Needles were sampled from two sites that differed in their climate and shoot phenology. Adaxial (upper), abaxial (lower) and cross-sectional surfaces were examined on scanning electron micrographs. Wax thickness increased significantly ($P < 0.01$) during the year (from $2.9 \pm 0.26 \mu\text{m}$ in late June/early July to $4.4 \pm 0.13 \mu\text{m}$ in early November). Mean wax thickness was slightly thicker on adaxial ($4.0 \pm 0.16 \mu\text{m}$) than on abaxial ($3.5 \pm 0.22 \mu\text{m}$) surfaces ($P = 0.03$). There were no significant differences in wax thickness between needles sampled at the base of the terminal shoot or near the tip of the shoot. Tubular or rod-shaped epicuticular wax crystals were sparsely developed on adaxial surfaces, completely covered abaxial surfaces (including filling all stomatal cavities), and had the same general structure and appearance across sites and sampling dates. Some erosion of epicuticular wax crystals on adaxial surfaces and presence of amorphous wax on abaxial surfaces was observed late in the year when epicuticular wax thickness was the thickest. Fungal hyphae were observed on top of epicuticular wax crystals and emerging from stomatal pores.

Keywords: Epicuticular wax, stomatal wax, morphology, Douglas-fir, *Pseudotsuga menziesii* var. *menziesii*, foliar fungi

Introduction

In land plants, cuticular and epicuticular waxes form a barrier between the above-ground portion of a plant and its environment and these waxes serve many functions (Eglington and Hamilton 1967, Juniper 1995, Shepherd and Griffiths 2006). The wax barrier is primarily thought to reduce water loss, but it could also enhance conditions for gas exchange and photosynthesis (Smith and McClean 1989, Ishibashi and Terashima 1995), protect against insect or disease attacks (Blakeman 1973), shield plants from pollutants and foliar sprays (Post-Beittenmiller 1996) and serve as a repository for lipid-soluble materials used in signaling (Juniper 1995). Although past studies have documented epicuticular wax crystals on tree foliage (c.f., Hanover and Reicosky 1971, Clark and Lister 1975, Jeffree et al. 1971, Johnson and Riding 1981, Chiu et al. 1992, Thijssse and Baas 1990, Apple et al. 2000), there has been little attention paid to changes in the thickness of the

wax layer or the crystals over the growing season. Exceptions would be studies of changes during the growing season in crystalline and amorphous wax in and around stomatal cavities exposed to NH_3 (Thijssse and Baas 1990) and responses of needle waxes to pollution (Turunen et al. 1997). Foliar wax, however, is a critical component of how plants interact with their environment and warrants additional study, particularly as predicted warmer temperatures (and associated lower relative humidities) in the future (IPCC 2013) could trigger changes in wax production and composition (Sheppard and Griffiths 2006).

Many needle characteristics in conifers have been determined by the time of full needle expansion and cannot be altered; however, since needle waxes are deposited exterior to the cells, it is possible that they could change after needles are fully formed (Apple et al. 2000). Thus, changes in wax characteristics after needle formation could represent an important way for conifers to modify leaf traits in order to acclimate to changes in their environment. This potential plasticity in responding to environmental conditions could be especially important to conifers which retain

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their needles for several years. Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco) is the most common and commercially important tree species in the Pacific Northwest. Predicted warmer and drier conditions under climate change could impact its ability to reduce water loss, but the impacts may be greater or less than predicted without examination of possible mechanisms. For example, we recently reported that Douglas-fir families growing at a warm, dry site had 40% lower rates of water loss compared to the same families growing at cooler, moister sites (Bansal et al. 2015); however, the mechanisms associated with that reduced water loss were not examined. Due to the lack of information about foliar wax development in conifers in general and Douglas-fir in particular, we initiated a preliminary trial to investigate two questions related to needles on young Douglas-fir. We asked: does the (1) thickness of the epicuticular wax layer or the (2) morphology of the epicuticular wax crystals differ based on date of sampling during the year, needle surface (abaxial versus adaxial), position of the needle on the shoot or local environment?

Methods

The Douglas-fir Seed Source Movement Trial is a set of 9 common garden studies in Washington and Oregon planted in the fall of 2008. We selected two seed sources from two common gardens that we would expect to have the greatest difference in the timing of needle formation based on their shoot phenology (date of terminal budburst and budset). This was a pilot study; we planned to sample more sites and genotypes per site if the initial results indicated significant differences between the needles sampled at the two locations. The seed source/common garden locations we sampled were: a high elevation source from the Washington Cascades (seed source location: 1431 m amsl, 46.94°, -121.47°) planted in southern Oregon (415 m amsl, 42.35°, -122.94°; site name Stone Nursery), hereafter “warm, dry” and a California coastal source (seed source location: 188 m amsl, 39.18°, -123.74°) planted at in the central Washington Cascades (860 m amsl, 46.95°, -122.01°, site name Doorstop), hereafter “cool, moist.” Based on on-site weather stations, mean

maximum air temperature in August was 32 °C at the warm, dry site and 21 °C at the cool, moist site; mean minimum relative humidity in August was 26% at the warm, dry site and 59% at the cool, moist site; total warm season (May–September) precipitation was 90 mm at the warm, dry site and 363 mm at the cool, moist site.

We sampled two to four healthy trees at each site on three dates in 2011. Trees were 2 to 3 m tall during the sampling period; we used a ladder when sampling the taller trees to avoid damaging the shoots. Trees at the warm, dry site were sampled June 28, August 22, and November 7; corresponding collections were made at the cool, moist site July 5, August 29, and November 2. Samples were taken from fully expanded needles at the base of the 2011 terminal shoot and from just below the tip of the shoot, thus, sampling the first- and last-formed needles on the shoot at each date. Four needles were collected per tree at each site on each date. The sampled shoots had set bud by the first sampling date at the warm, dry site and remained in budset for the rest of the year. One of the trees at the cool, moist site was in continuous growth (i.e., terminal shoot had not set bud) at the first sampling date, all trees were in continuous growth at the second sampling date (i.e., trees had re-flushed), and all trees had set bud by the final sampling date. As part of methods development for this study, we also sampled 1-year-old needles in the spring on several saplings from local seed sources near Olympia, WA.

Needles were clipped from the stem just above the needle base using surgical scissors to avoid damaging the stem. Samples were handled using forceps or gloved hands to avoid contamination and placed in plastic sampling bags in a cooler for transport to the laboratory. Samples were kept refrigerated for 2–4 days until they were processed for viewing with a scanning electron microscope. Healthy sections of the needles were selected (abaxial, adaxial, and cross sections), cut from the rest of the needle with surgical carbon steel razor blades (GEM®), freeze dried, mounted on sampling blocks, and stored in desiccators until ready for viewing. One day prior to viewing, samples were gold coated. Samples were viewed

with a scanning electron microscope at several magnification levels and micrographs taken. Images of the abaxial surface were made with special attention to the stomata. Measurements of wax thickness were made on the cross-sectional micrographs (using Image J software). Thickness was measured from the exterior edge of the cell wall to the top of the solid wax layer and did not include surface wax crystals. Number of leaves for each sample date and leaf surface ranged from 5 to 15. Generally 15 to 30 measurements of wax thickness were taken per needle surface to provide a good estimate of the mean.

We fit generalized linear mixed-effects models of wax thickness, with sampling date (date of sampling, normalized by subtracting the mean and dividing by the standard deviation), side of leaf (adaxial or abaxial) and position of the leaf along the stem (near the base or near the tip of the shoot) included as fixed effects. The models included site identity, tree identity (nested within site) and leaf identity (nested within tree) as random effects to account for the non-independence of samples taken from the same site, tree or leaf. Each model had a gamma error distribution. We used likelihood ratio tests to assess the significance of the fixed effects.

Results

Epicuticular wax thickness increased significantly over the study period from summer through fall ($P < 0.001$) (Figure 1). Wax was also slightly thicker on the adaxial side of the leaf ($4.0 \pm 0.16 \mu\text{m}$) than the abaxial side ($3.5 \pm 0.22 \mu\text{m}$) ($P = 0.03$) and

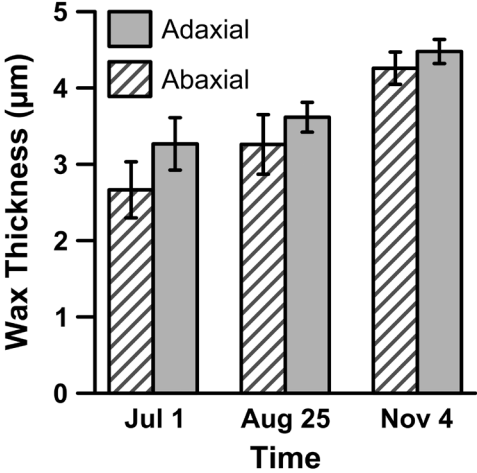


Figure 1. Mean and standard error bars for wax thickness of epicuticular wax on Douglas-fir needles by sampling date and needle surface.

this effect was consistent at each sampling date (Figure 1), i.e., the interaction between sampling date and leaf side was non-significant ($P = 0.20$). Leaf position along the terminal shoot did not affect wax thickness ($P = 0.88$); that is, wax thickness did not differ between the first-formed and last-formed needles on a shoot. Mean wax thickness was greater at the warm, dry site ($4.2 \pm 0.22 \mu\text{m}$) than at the cool, moist site ($3.3 \pm 0.18 \mu\text{m}$) but our experimental design did not permit testing for differences between the sites/seed sources.

Rod-like or tubular wax crystals were present on both leaf surfaces, however, the epicuticular wax crystals were much more sparsely developed on the adaxial compared to the abaxial surfaces (Figure 2). Crystals completely covered the abaxial

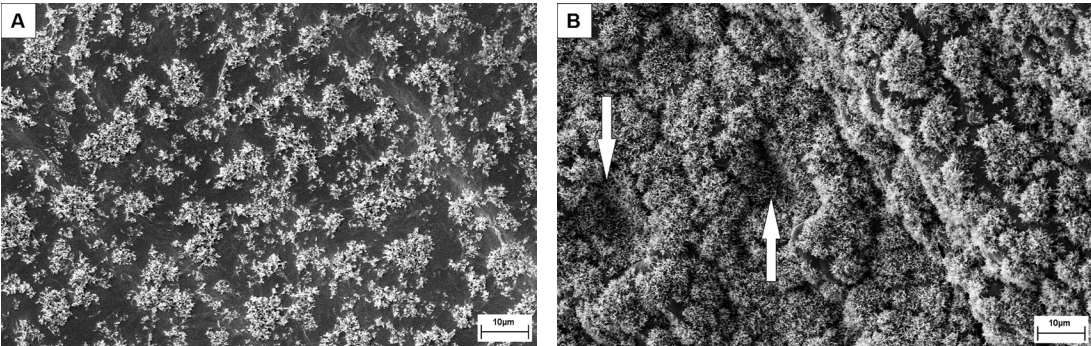


Figure 2. Epicuticular wax crystals on current-year Douglas-fir needles on the terminal shoot: (A) adaxial (upper) surface and (B) abaxial (lower) surface (stomatal cavities visible in the center and lower left of the image, see arrows).

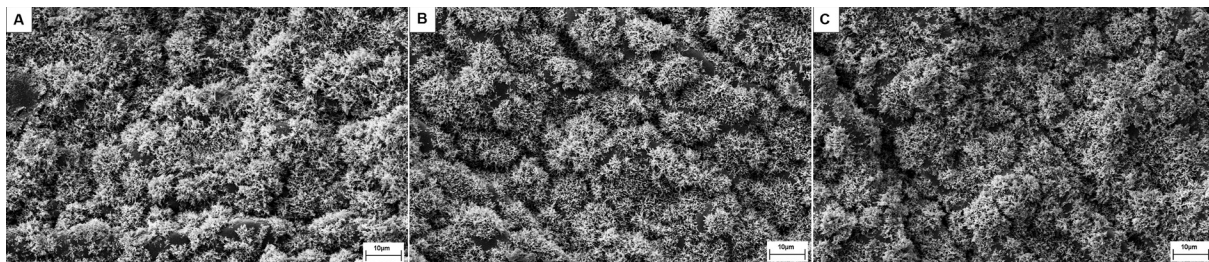


Figure 3. Abaxial surface of late-formed needles at the warm, dry site. Sampled: (A) June 28, (B) August 22, (C) November 7.

surface of most samples. All stomatal cavities (stomata on the abaxial surface) were completely filled with epicuticular wax crystals. There was no obvious difference in this coverage of the abaxial surface (including the stomatal cavities) between sites, by sampling date, or between needles formed early or late in the growing season. Epicuticular wax crystals had the same general structure and appearance on the needles at both sites at all sampling dates (Figure 3). Wax crystals had the same appearance on needles sampled from the most recently formed fully expanded needles as on the first-formed needles of the season.

Erosion of epicuticular wax crystals was observed on some samples; this was most common along the ridges of epidermal cells on adaxial surfaces (Figure 4a). In addition, there was an increase in amorphous rather than crystalline wax on guard cells and other areas on abaxial surfaces (Figure 4b). Amorphous wax did not extend into the stomatal cavities. The presence of amorphous wax was most common on samples taken at the end of the season (and also observed on samples

from previous-year needles evaluated as part of methods development).

Although we selected needles in the field that appeared healthy, some samples collected in November had fungal hyphae that crossed the abaxial surface (Figure 5a). In addition, samples of previous-year needles taken in the spring as part of methods development, exhibited substantial proliferation of epiphytic hyphae of *Phaeocryptopus gaeumannii* (Figure 5b) which radiated from pseudothecia that developed in stomatal cavities (Stone et al. 2008).

Discussion

Current season foliage of Douglas-fir can adapt to changes in its environment by increasing wax thickness. These changes likely have adaptive value as foliar wax is an important characteristic in preventing water loss from the non-stomatal portions of the needles (Shepherd and Griffiths 2006). The climate throughout most of the range of coastal Douglas-fir becomes droughty several months after budburst and needle expansion.

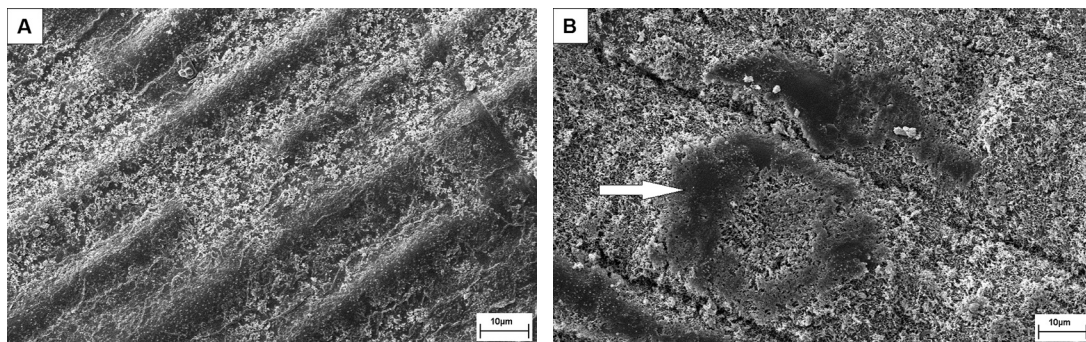


Figure 4. Within-season changes in appearance of epicuticular wax on Douglas-fir needles. (A) Wax erosion on adaxial surface of early-formed needles at the warm, dry site. (B) Amorphous wax on guard cells on late-formed needles at warm, dry site.

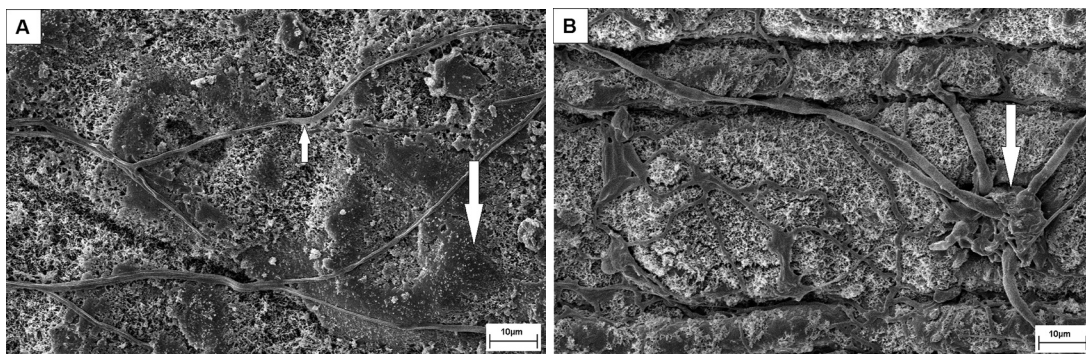


Figure 5. Fungal hyphae on abaxial surfaces of Douglas-fir needles. (A) November sample on early-formed current-year needle from cool, moist site. Wide arrow points toward amorphous wax, narrow arrow points to fungal hyphae. (B) May 2011 sample from previous-year needles collected near Olympia, WA. Arrow points to stomatal pore with epiphytic hyphae of *Phaeocryptopus gaeumannii* emerging from pseudothecia (terminology from Stone et al. 2008).

Thus, increases in wax thickness after leaf expansion should help reduce rates of cuticular water loss (i.e., loss after stomatal closure) later in the growing season. In a related study, Bansal et al. 2015 found that cuticular water loss of Douglas-fir shoots was less from trees growing on a site with hot and dry conditions than from trees of the same genotypes growing on sites with cooler and more humid conditions. Based on the results from this study, we suspect the hotter, drier site conditions would have induced greater wax thickness, and thus, played an important role in reducing the rate of cuticular water loss. The ability of Douglas-fir to increase wax thickness after needle expansion is likely to be an important trait for acclimating to the higher levels or longer time periods of summer moisture stress predicted under future climate change.

Results from this pilot study indicate epicuticular wax thickness of Douglas-fir increases substantially during the growing season. Previous reports have indicated loss of epicuticular wax with rainfall (or simulated rainfall) on goldenrod (Mayeux and Jordan 1987), loss due to physical abrasion by other plants or wind-blown soil (Martin and Juniper 1970), or to physical damage (Englinton and Hamilton 1967), and no regeneration of epicuticular wax was observed after full leaf expansion of cabbage (Flore and Bukovac 1974). In fact, wax is reported to decline with leaf age (Juniper 1995). Leaf wax content was reported to increase during drought treatments

with maize (Premachandra et al 1991) but the study design did not permit separation of leaf age with response to drought. In this study, however, wax thickness increased substantially during the season at both sites. It is possible that wax synthesis and transport could function differently in a species such as Douglas-fir where the foliage persists for several years as opposed to foliage of annual plants or perennial plants with annual leaves. Future timing of sampling for needle waxes for Douglas-fir, and presumably for other conifers, needs to take into account the increase in wax thickness which occurs from summer to fall in first-year needles.

Plants grown at low (20–30%) relative humidities have been shown to have higher mass of leaf wax than those grown at high (98%) relative humidity (Koch et al. 2006) and other environmental factors such as temperature or light intensity can also influence wax development (Baker 1974). Since this pilot study only sampled field sites where environmental conditions were not controlled, we cannot determine which factors could have been involved in triggering the development of wax during the season. Wax thickness was greater early in the season on the warm, dry site than on the cool, moist site but our experimental design did not permit testing the effects of site or seed source. Previous reviews have suggested that factors such as light intensity, relative humidity, heat or cold temperatures are important factors in inducing wax development (Post-Beittenmiller

1996, Shepherd and Griffiths 2006). As summer temperature is predicted to increase (and relative humidity will decrease) under global warming (IPCC 2013), we anticipate that wax production will increase and wax composition could be altered for many species. Since wax synthesis and transport are carbon and energy intensive processes, it would be useful to conduct studies under controlled environments to understand the degree to which various environmental factors control these processes and the variation associated with genotype.

The appearance of the rod-like wax crystals was similar to those seen on Douglas-fir needles in previous reports (Clark and Lister 1975, Hanover and Reicosky 1971, Lister and Thair 1981, Thijssse and Baas 1990). Past researchers have indicated that the appearance of epicuticular wax crystals in Douglas-fir is related to the chemistry of the wax rather than biologically-dependent processes (Lister and Thair 1981) and that wax morphology is generally determined by the composition of the wax (Baker 1982). The appearance of a glaucous bloom on Douglas-fir needles following nitrogen and phosphorus fertilization in field plots was reported to be associated with increased wax crystals in the non-stomatal regions but the appearance of the crystals did not differ (Chiu et al. 1992). In this study, the general appearance of the wax crystals on Douglas-fir foliage was very similar at different sampling dates, age of needle formation, and at two sites/seed sources. In addition, the appearance was similar to that observed in sampling at other locations in western Washington (data not shown) and for Douglas-fir growing in the Netherlands (Thijssse and Baas 1990), Vancouver Island, Canada (Thair and Lister 1975), Idaho, Montana, and northeast Washington, USA (Chiu et al. 1992) or in the samples taken by Hanover and Reicosky 1971 (location not specified). The appearance of the surface wax can be altered by cultural or environmental factors (more amorphous wax reported by Thijssse and Baas 1990, more wax crystals by Chiu et al. 1990). However, there is no evidence that there are major differences in the general morphology, or by implication in the chemistry, of cuticular needle wax for coastal Douglas-fir. The design of this trial did not per-

mit separating the effects of genotype from local environment. Thus, it cannot be ruled out that quantitative analysis of wax chemistry with more genotypes or sites could reveal differences in wax chemistry or morphology associated with genotype or environment. Within-species differences in wax composition have been reported for species in the Cupressaceae and these differences could confer greater plant fitness under hot summer or cold winter temperatures (Dodd and Afzal-Rafii 2000, Dodd and Poveda 2003).

The observed occlusion of the stomatal chambers with wax crystals was consistent with previous reports for Douglas-fir (Hanover and Reicosky 1971, Thair and Lister 1975, Chiu et al. 1992, Thijssse and Baas 1990) and other conifers (Reicosky and Hanover 1976). Some have suggested that this occlusion reduces water loss by increasing the tortuosity of the path of gas exchange (c.f., Jeffree et al. 1971, Reicosky and Hanover 1976). It has also been suggested that while occlusion of stomatal chambers does serve to reduce transpiration, the wax plugs could have evolved as an adaptation to wet rather than dry conditions as the wax crystals help keep the chambers free of water (Brodribb and Hill 1997). Leaf wetting can inhibit photosynthesis (Ishibashi and Terashima 1995) and reducing wettability can increase conductance and photosynthesis under wet conditions (Smith and McClean 1989). Thus, keeping stomatal chambers dry could be advantageous to species which experience long wet periods with temperatures warm enough to permit photosynthesis. We observed occlusion by wax crystals at all sampling dates. Occlusion of the stomatal chambers has been observed on needles still in the bud or as the needles emerged from the buds of Douglas-fir (Thijssse and Baas 1990) and for several other North American conifers (Reicosky and Hanover 1976, Johnson and Riding 1981); this likely indicates an important physiological role for wax in stomatal chambers as this phenomenon has evolved in many coniferous species (Brodribb and Hill 1997). The presence of epicuticular wax crystals could also serve to partially protect the stomatal chambers from fungal invasion (Brodribb and Hill 1997) but fungi have co-evolved to develop mechanisms to reproduce (Stone et al. 2008).

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