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Diagnostics of Epistomatal Wax of Californian Pine Needles, and Their Association with Ozone-Caused Chlorotic Mottle

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Abstract

Tropospheric ozone is a worldwide phenomenon causing injuries to forest trees. Californian ponderosa and Jeffrey pines are well known for their sensitivity to ozone, while other pine species have varied in their susceptibility. Sensitive pine species are known for their chlorotic mottle and tip burn symptoms, caused by ozone air pollution. Epistomatal wax plugs and filamentous waxes around stomata are typical for pine needle surfaces. In this study, we investigated epistomatal and epicuticular needle waxes in eight species of field-grown pines in 1985, 1986 and 2006. The epistomatal wax plugs were present in asymptomatic needles without chlorotic mottle (23% of needles). A lack of wax plugs in needles with chlorotic mottle and tip burn symptoms was common (76% of needles). More abundant existence of mottling associated with stomata without wax plugs in two-year-old needles, compared with one-year-old needles (43% and 33%, respectively), indicated chronic injury development over time.

Keywords

***Pinus coulteri*, *P. jeffreyi*, *P. ponderosa*, Epistomatal Wax Plugs, Scanning Microscopy (SEM)**

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1. Introduction

Ozone (O_3) injuries in montane coniferous forests in California have been recorded since the1950's [1] [2]. Diagnostic injuries of oxidant and winter fleck on Western pine needles were first recognized in the 1970's [3]. The most O_3 sensitive Western pines are *P. coulteri* D. Don (Coulter pine), *P. jeffreyi* Balf (Jeffrey pine), *P. lambertiana* Douglas (sugar pine), and *P. ponderosa* (ponderosa pine) [4]. Experimental results during different seasons revealed O_3 sensitivity to vary with exposure time, with the order of species sensitivity being: *P. ponderosa*, *P. jeffreyi*, *P. coulteri*, then *P. lambertiana*. Ozone injuries of the most sensitive pine species were associated with epidermal and hypodermal thickness, while in more tolerant species the injuries were found especially in substomatal mesophyll cells [5]-[7].

Stomata regulate the flow of gases in and out of leaves [8]. The stomata of many gymnosperms [9] and a few angiosperms appear to be partially blocked by filamentous waxes [10]. In the genus *Pinus*, the stomatal Florin ring and the stomata lantechamber [11] are covered with tubular epicuticular waxes, and this structural wax distribution reaches surrounding areas in the stomatal rows. These waxy plugs occlude the entrance of stomata, particularly in conifers [12] [13]. Stomatal waxes may inhibit the ingress of liquid water into the intercellular air spaces. On the other hand, the presence of large amounts of wax covering stoma affects the rate of leaf gas exchange by decreasing the area available for diffusion. Stomatal wax plugs and imbricacy have been shown to reduce maximum leaf conductance in conifers [13] [14]. However, the role of the stomatal antechamber wax plugs is not clear when air pollution is considered.

Changes in conifer needle epicuticular waxes, and especially epistomatal waxes, have been used as an early diagnostic tool in needle injuries [15] [16]. Experimental responses with different pollutants and case studies around mixed pollution sources indicate changes in epicuticular waxes especially at the top of and around the stomata. The observations vary due to species, environment, needle ontogeny, age and longevity [15] [17] [18].

In conifers, drought resistant features such as a general reduction in physiological activity in periods of drought, sunken stomata and thick dense cuticles are typical. Chronic O_3 injury in Californian pine species is related to increased O_3 uptake due to sluggishness of stomata and their partial opening at night [19] [20]. However, functional injury has not been directly connected with diagnostics e.g. chlorotic mottle symptoms, condition of epicuticular waxes, and existence of wax plugs. Chlorotic mottle is a visible needle injury symptom developing in Californian pines in the field.

The specific type of chlorotic mottling in ponderosa pine and O_3 needle mottle in seen in other pine species are one and the same disease [21]. Seasonal advancement of mottling, or the rate at which needles are injured by ozone, has been found to vary both between and within the different pine species. While some O_3-exposed trees may retain only current-year needles, others may show retention of affected needles for two, three or four seasons [21].

We studied epistomatal wax plugs and injury of epicuticular waxes of needles in several California pine species *in situ*. Our aim was to develop O_3 impact diagnostics using the condition of epicuticular wax and occurrence of wax plugs in needles of these selected pine species. Our hypothesis was that a lack of stomatal antechamber wax plugs and visible chlorotic mottle symptoms are connected, and that diagnostic differences between needle-age classes can be found. One aim was to find a species-specific early diagnostic tool that would combine microscopic injuries with visible chronic O_3 symptoms *in situ*. Repeated monitoring was used to assess annual variation in the condition of trees.

2. Material and Methods

2.1. Sites

The individual test trees were selected during the first sampling year in the San Bernardino Mountains and in the Riverside Botanic Gardens of southern California. Field samples were collected three times from the same trees, in 1985, 1986, and 2006. Needle sampling sites were located at forest sites with altitudes varying between 335 - 2800 m asl (**Table 1**).

Needle samples were taken from the bottom, westerly side of adult native trees (>2 m above ground). We used small or large open stands and dominant or co-dominant single trees for needle collection. Sampling took place in March 1985, 1986, and again in April 2006. Marked *Pinus* species were *Pinus albicaulis* Engelm., alpine white bark pine (not found in 2006); *Pinus attenuata* Lemmon, knob cone pine (not found in 2006); *Pinus coulteri*,

Table 1. Needle samples at field sites in Riverside and the San Bernardino Mountains, altitude and pine species collected in 1985, 1986 and 2006.

Site and sample number	Altitude (m asl) Latitude N, W	Species
0) Botanic Gardens Riverside (1 - 6), single tree	335 - 442 m 33˚58'10.71"N, 117˚19'9.77"W	*Pinus attenuata**, *P. coulteri*, *P. muricata**
1) Thurman Flats (7, 8), small open stand, dominant tree	1200 m 34˚5'51.02"N, 116˚57'44.56"W	*Pinus coulteri*
2) Highway 38 (9, 10), small open stand, dominant tree	1250 m 34˚5'55.27"N, 116˚58'25.21"W	*Pinus ponderosa*
3) Highway 38 (11,12), small open stand, dominant tree	1800 m 37˚7'28.84"N, 116˚59'10.23"W	*Pinus coulteri*
4) Highway 38 (13, 14 14a, 14b), small open stand, co-dominant tree	1900 m 34˚8'7.15"N, 116˚59'12.11"W	*Pinus coulteri*, *P. ponderosa*
5) Highway 38 (15 - 20) small open stand, co-dominant tree 15, 16; individual trees mixed with more numerous ponderosas 17, 18; individual tree near highway 19, 20	2000 m 34˚9'22.21"N, 116˚57'45.55"W	*Pinus albicaulis***, *P. coulteri*, *P. ponderosa*
6) Highway 38 (21, 22), large open stand, co-dominant tree	2000 m 34˚10'4.00"N, 116˚54'18.40"W	*Pinus ponderosa*
7) Barton Flats, (23, 24), large open stand, co-dominant tree	2000 m 34˚10'33.42"N, 116˚52'4.60"W	*Pinus ponderosa*
8) Onyx Summit (25, 26), large open stand, co-dominant tree	2800 m 34˚11'18.90"N, 116˚42'41.88"W	*Pinus jeffreyi*
9) Highway 38 (27, 28), sparesely located trees	2650 m 34˚12'55.06"N, 116˚44'25.15"W	*Pinus monophylla*
10) Highway 18 (29, 30), sparsely located trees	2000 m 34˚18'57.99"N, 116˚49'32.84"W	*Pinus monophylla*

*Tree has died, no samples in 2006; ***P. lambertiana* samples in 2006.

D. Don, Coulter pine; *Pinus jeffreyi* Balf, Jeffrey pine; *Pinus lambertiana* Douglas, sugar pine (only 2006); *Pinus monophylla* Torr. & Frem., single-leaf pinyon; *Pinus muricata* D. Don, bishop pine (not found in 2006); and *Pinus ponderosa* Douglas ex Lawson & C. Lawson var. ponderosa, ponderosa pine [22] [23]. Typical morphological characteristics of these pines are presented in **Table 2**. The one- and two-year-old needles were separated and studied independently. In some samples, three-year-old needles were also available. The needles were gently air dried at room temperature and placed in protective parcels, then air mailed to the University of Oulu where visible needle injuries (mottling and tip burn) were detected by MBC-stereomicroscope.

2.2. Scanning Electron Microscopy (SEM)

The air dried or/and freeze-dried portions of needles were cut from their middle part (pieces about 0.5 cm), placed on brass stubs and sputtered with 25 - 30 nm gold-palladium (POLARON 5100). The samples were studied with JEOL JSM-35 Scanning electron microscope (SEM) with 15 kV. In 2006, samples were sputtered for 30 sec with 30 nm palladium and studied with JEOL JSM-6400. The samples were micrographed in a standardized series under the microscope, where the field of view of selected mid-needle surface area (0.061 mm^2) was scored with 100× - 200×, and 400× magnifications, and epistomatal wax plugs with 1000× or 2000× magnifications. In some cases, wax fibrils were further micrographed at magnification of 10,000×. The condition of the waxes in 2 - 4 stomatal rows in the middle of the adaxial side of each needle were assessed in random order. Ten stomata (wax plugs and filamentous waxes) were classified under the SEM and notes of special features were listed as follows: (+++ very abundant, ++ moderately abundant, + exist to some extent, (+) exist to a minor extent, − not observed) for the following characteristics:

Table 2. Typical morphological features of the pine species studied.

Pine species	Number of needles in a fascicle and number of stomatal rows, *mean of* 20 *needles in this study*	Typical length of needles and their longevity; *mean length of* 20 *needles in this study*
P. albicaulis White bark pine	5 9.3	3 - 7 cm, 5 - 8 yrs 4.78 cm
P. attenuata Knob cone pine	3 24.4	9 - 15 cm, 4 - 5 yrs 12.21 cm
P. coulteri Coulter pine	3 22.5	16 - 30 cm, 3 - 4 yrs 19.55 cm
P. jeffreyi Jeffrey pine	3 16.00	12 - 15 cm, 4 - 6 yrs 9.76 cm
P. monophylla Single leaf pinyon	1 25.8	3 - 6 cm, 4 - 12 yrs 3.75 cm
P. muricata Bishop pine	2 12.4	7 - 15 cm, 2 - 3 yrs 9.33 cm
P. ponderosa Ponderosa pine	3 18.7	17 -25 cm, 4 - 6 yrs 15.30 cm
P. lambertiana Sugar pine	5 ?	6 - 11 cm, 2 - 4 yrs ?

Resource: Richardson, D.M. (1998) Ecology and Biogeography of *Pinus*. Cambridge University Press, 527 p [40].

1) Erosion of epistomatal tubular wax.
2) Crumbly epistomatal wax.
3) Stomatal wax breaks.
4) Stomatal blockage with small particles.
5) Stomata with wax plugs.
6) Stomata without wax plugs.

2.3. Diagnostics

Observations were carried out of visible and epicuticular injury symptoms by three people, and scores of their observations averaged. In order to keep the microscopic work feasible, one individual adult indicator tree of each species per site was selected for study. Needle morphology and number of stomata varies greatly between pine species, which is indicated by the number of stomata rows (**Table 2**). The specific aim of this SEM study was to evaluate specific O_3 symptoms of adult pine species. Work with adult trees is essential, since the limited time of the experimental work on O_3 effects on seedlings is often insufficient to induce symptoms with. Developing field diagnostics of species-specific O_3 symptoms is important, as the traditional biomonitoring tools for other air pollutants, such as their accumulation in plant tissues, cannot be used. The images of typical injury symptoms obtained with SEM were saved as paper micrographs. The total numbers of diagnostic-type figures saved, is 147 for samples collected in 1985-1986, and 83 in 2006.

3. Results and Discussion

Our study included pine individuals at typical sites with chronic O_3 pollution. Due to abundance of chlorotic mottling in trees sampled in 1985 and 1986, there were only 13 needle samples without mottling symptoms (**Table 3**).

3.1. Riverside Botanic Garden

We evaluated three sample trees in the Botanic Garden (**Figures 1(A)-(D)**, **Table 4**).

For *Pinus attenuata*, Whang *et al*. [24] characterize epicuticular needle waxes to be well developed. In 1985 and 1986 the needles collected had 8 mm tip burn and chlorotic mottle symptoms. The cuticular surface was classified to have eroded waxes but otherwise was healthy. By the 2006 sampling, the *P. attenuata* tree had died.

Table 3. The visible mottling symptoms under stereomicroscope in needle samples (three samples were omitted due to fungal infection/and or mold problems).

Samples	Mottling + tip burn two-year-old	Mottling + tip burn one year old	No symptoms
1985 + 1986, *n* = 60(57)	*n* = 25/57	*n* = 19/57	*n* = 13/57
	43.1%	33.3%	22.8%
2006, *n* = 26	*n* = 12/26	*n* = 11/26	*n* = 3/26
	46.2%	42.3%	11.5%

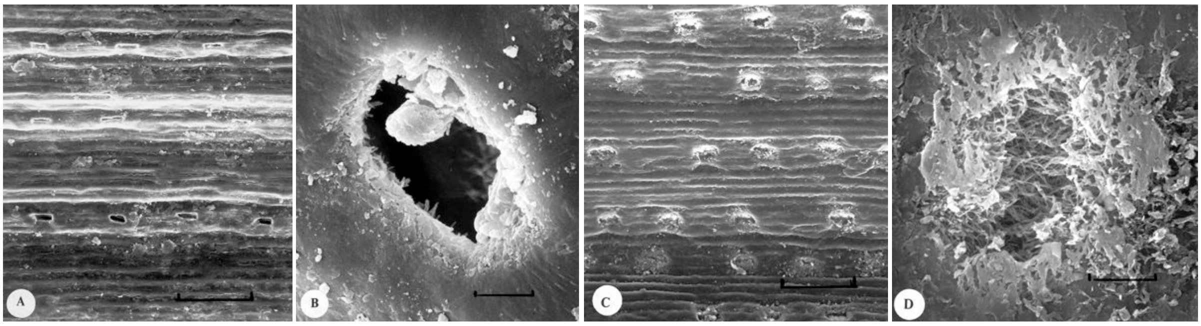

Figure 1. *Pinus muricata* ((A), (B)) epicuticular waxes and stomata without wax plug and *P. attenuata* ((C), (D)) eroded waxes and stomatal plugs. Riverside Botanic Gardens 1985, 1986. Bars (A) and (C) = 10 µm, (B) and (D) =1 µm.

For *P. muricata*, Whang *et al*. [24] characterize features of the outer cuticular surface to be weakly developed. The young needles of *P. muricata* tree in the first two samplings were classified as visibly healthy, but needle epicuticular waxes were in poor condition, and the whole epistomatal wax structure was destroyed (**Figure 1(A)**, **Figure 1(B)**). All micrographed stomata were empty, and stomatal plugs were lacking. By 2006, the tree had died.

For *P*. *coulter*, Whang *et al*. [24] consider the epicuticular wax surface to be well developed and stomatal wax plugs present. In the sample from the present study, *P. coulter* had visibly healthy young needles, however slight mottling was observed under light microscope in the two-year-old needles. In 2006 only the *P. coulteri* from all the marked test trees was still alive at the Botanical Garden, it exhibited slight microscopic injuries, but did not show visible injuries (**Figure 2**, **Table 4**).

3.2. San Bernardino Mountains

3.2.1. 1985 and 1986

Coulter pines in the San Bernardino Mountains had chlorotic mottling and tip burn symptoms in all needle age classes (1-year-old, 2-year-old, 3-year-old), and the epistomatal cuticular waxes were damaged. These results were similar to those found in 1985 and 1986 (**Figure 2**, **Table 4**).

Pinus albicaulis (white bark pine). Whang *et al*. [24] consider its cuticular wax surface to be well developed and stomatal plugs to be absent. In our sample tree, the needles were extremely xeromorphic with thick, ridged epicuticular waxes. Stomata were located in deep grooves. In many needle samples, the thick wax surface was broken into wax plates and cuticular injury diagnostics and comparison with other species was not possible. However, the stomatal wax plugs were present in young needles (**Figure 3(A)**, **Figure 3(B)**, **Table 4**).

P. *jeffreyi*. Most needles indicated epicuticular wax changes. The tube-like waxes were observable only in stomatal plugs of current year needles. The sample tree was growing at an altitude of 2800 m (**Figure 4**, **Table 4**).

P. *ponderosa*. The needles revealed chlorotic mottling and tip burn symptoms with intensive erosion of epicuticular waxes, and additionally some fungal infection. In samples with lower fungal infection, the differences in the wax quality between the needle years were observable. The waxes were classified to be in better condition in the eastern parts of the San Bernardino Mountains than in sites closer to the Los Angeles Basin and in Riverside. In young needles, stomatal wax plugs were abundantly present, but in older mottled needles they were absent in 52% of the documented samples. In many, cases some stomata poseessed plugs while some were without, depending on the stage of chlorotic mottling and tip burn of needles (**Figure 5**, **Table 4**).

Table 4. Species-specific diagnostic epicuticular wax symptoms and wax plug coverage of one/two-year-old needles. Abundance +++, ++, + (+); not observed −, n.d = not detected, combination of observations in 1985 and 1986.

Pinus albicaulis symptoms of one/two-year-old needles, combination of observations in 1985 and 1986. Visible symptoms: Mottling, no clear mottling in young needles 1985.						
Site number 5	Erosion of epistomatal wax	Crumbly epistomatal wax	Breaks of epistomatal wax	Particle blockage of stomata	With wax plugs	Without wax plugs
	++/n.d	−/n.d	−/n.d.	+/n.d.	+/n.d.	−/n.d.
Pinus attenuata wax symptoms of one/two-year-old needles: Visible tip burn, mottling in 1985 two-year-old, no visible symptoms in 1986 needles. Figure 1(C), Figure 1(D)						
Riverside botanic gardens	Erosion of epistomatal wax	Crumbly epistomatal wax	Breaks of epistomatal wax	Particle blockage of stomata	With wax plugs	Without wax plugs
	++/++(+)M	+/++M	−/+M	+(+)/++M	−/?*M	+++/?*M
Pinus coulteri symptoms of one/two-year-old needles combination of observations in 1985 and 1986. Visible symptoms: Mottling, tip burn.						
Site number	Erosion of epistomatal waxes	Crumbly epistomatal wax	Breaks of epistomatal wax	Particle blockage of stomata	With wax plugs	Without wax plugs
Botanic gardens	+/++	−/++	(+)/−	(+)/+(+)	+++/−	−/(+)
1	++/++	++ (fungi)/++	++/+++	(+)/(+)	−/−	−/−
2	?/++	(+)/(+)	(+)/−	?/++	?/−	?/−
3	++/++	+/++	+/+++	++/−	?/+++	?/+
4	+/++	+/+++	+++/+++	++/++	+/+	?/?
5	+++/n.d	n.d.	n.d.	n.d	(+)/(+)	+/+++
Pinus jeffreyi symptoms of one/two-year-old needles combination of observations in 1985 and 1986. Visible symptoms: Mottling, chlorosis, tip burn.						
Onys summit	Erosion of epistomatal wax	Crumbly epistomatal wax	Breaks of epistomatal wax	Particle blockage of stomata	With wax plugs	Without wax plugs
	++(+)/+++	−/(+)	(+)/(+)	−/−	+(+)	−/+++
Pinus monophylla symptoms of one/two-year-old needles combination of observations in 1985 and 1986. *No visible symptoms*.						
Site	Erosion of epistomatal wax	Crumbly epistomatal wax	Breaks of epistomatal wax	Particle blockage of stomata	With wax plugs	Without wax plugs
Site 9	+++/+++	−/++	−/+	+/++	+/+	+/−
10	+(+)/no sample	−/	−/	−/	+/	+/
Pinus muricata symptoms of one/two-year-old needles combination of observations in 1985 and 1986. Visible symptoms: Slight mottling in two-year-old, tip burn.						
Riverside botanic gardens	Erosion of epistomatal wax	Crumbly epistomatal wax	Breaks of epistomatal wax	Particle blockage of stomata	With wax plugs	Without wax plugs
	+++/+++	++/++	−/−	(+)/−	−/−	+++/+++
Pinus ponderosa symptoms of one/two-year-old needles (abundance +++, ++, + (+); not found −) combination of observations in 1985 and 1986. Mottling. Tip burn, except site 5 young needles in 1985.						
Site number	Erosion of epistomatal Wax	Crumbly epistomatal Waxes	Breaks of epistomatal waxes	Particle blockage of stomata	With wax plugs	Without wax plugs
2	n.d.	n.d	n.d.	n.d	n.d.	n.d

Continued

4	++/+	−/(+)	−/+	−/(+)	(+)/−	−/−
5	+++/+++	−/−	++/++	−/−	+/−	++/(+)
6	++/+++	−/−	+/+	−/+	−/−	(+)/+
7	+/+++	−/−	(+)/+	−/−	−/−	−/++

?* observations not confirmed, M = mottling.

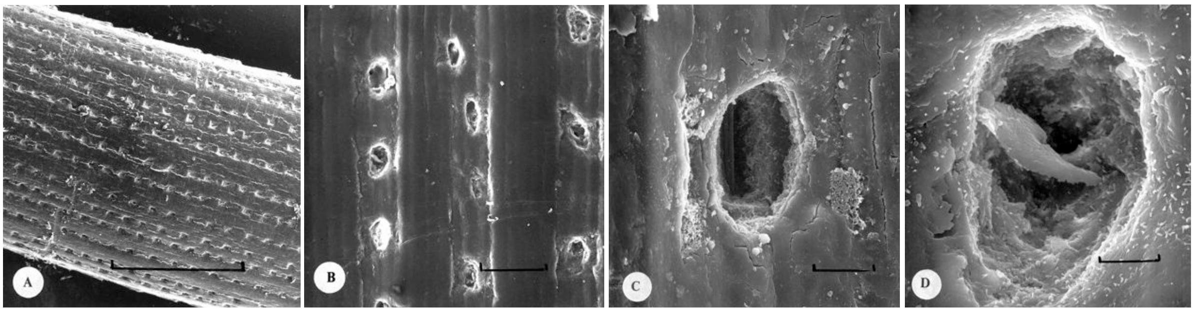

Figure 2. *Pinus coulteri* overview of the needle (A); The stomatal rows with and without wax plugs (B); Stomata without wax plugs ((C) and (D)). Riverside botanic gardens and San Bernardino Mountains, 1985, 1986. Bars (A) and (B) = 10 µm, (C) and (D) = 1 µm.

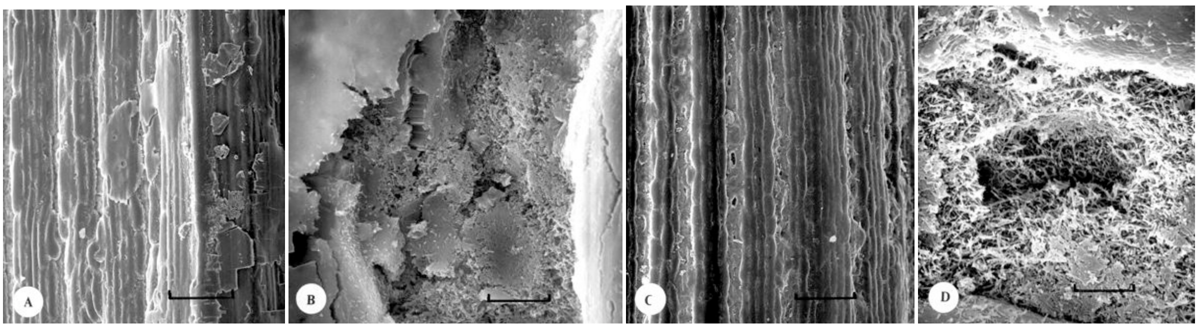

Figure 3. *Pinus albicaulis* (site 5) overview of the young needle (A); and stomata with wax plug (B); *P. monophylla* (site 10) overview of the young needle (C); and stomata with eroded epistomatal waxes of two-year-old needle (D). Bar (A) and (B) = 10 µm, (C) and (D) = 1 µm.

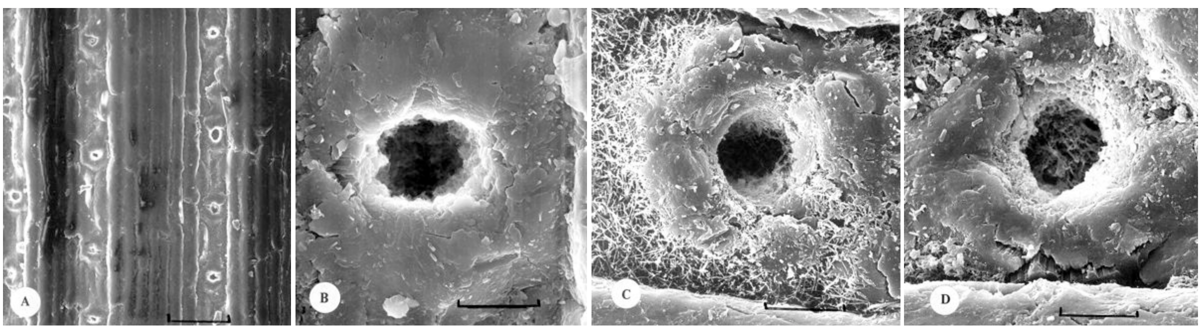

Figure 4. *Pinus jeffreyi* (site 8) overview of epicuticular waxes and stomata: needles of 1985 in March 1986 (A); Same needle stoma without wax plug. Needles with open stomata of 1985 in March 1986 (B); Needles of 1984 in March 1986 (C); Needles of 1983 in March 1986 (D). Bars (A) = 10 µm, (B) = 1 µm.

P. monophylla trees indicated chlorotic mottling at one site but not at the other. Samples typically featured erosion of epistomatal waxes, which was more abundant at the higher elevation site (2650 m) (**Figure 3(C)**, **Figure 3(D)**, **Table 4**).

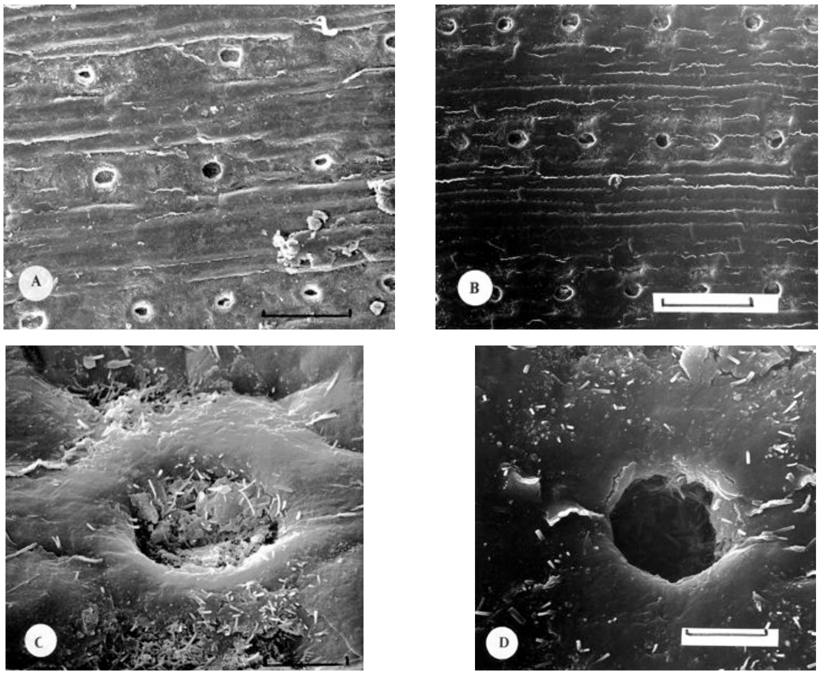

Figure 5. *Pinus ponderosa* ((A), (B)). An overview of stomatal rows, needles of 1984 in March 1985 ((A), site 7; (B), site 5). Young needles of *Pinus ponderosa* (site 5) in 1986 (C). Needle year 1984 in 1986 (D). Bar (A) and (B) = 10 µm, (C) and (D) = 1 µm.

3.2.2. 2006

In 2006, the same trees were sampled (no samples *P. attenuata* and *P. muricata*). Only three of the needle samples collected were found to be without mottling symptoms; these were *P. coulteri* one year old needles from the Botanic Garden and *P. monophylla* both one and two-year-old needles (from the same site as earlier with healthy needles). We micrographed needle epistomatal and epicuticular waxes with special emphasis on stomatal wax plugs. In four species *P. coulteri*, *P. jeffreyi*, *P. monophylla* and *P. ponderosa*, stomata with or without wax plugs were found both on adaxial and abaxial surfaces of needles. The lack of epistomatal wax plugs was evident in all species with mottling symptoms (**Figure 6**).

Pinuslambertiana Dougl. (sugar pine) was an additional species studied in 2006. Whang *et al.* [24] consider the wax surface well-developed and stomatal wax plugs to be present in this species. In our study, the stomatal waxes were well-developed and plugs were present. The high ridges of needle surface affected the wax diagnostics (results not shown).

The overall condition of the cuticular surfaces of the four species studied previously had improved when compared with samples collected in 1985 and 1986. This may be as a result of the improved air quality in the area [25] (**Figure 7**). However, visible mottling symptoms were common in all species (**Table 3**), and differences between needle age classes revealed a characteristic erosion of epicuticular waxes. Stomatal wax plugs were abundant, but stomata without wax plugs were found in all species.

Controlled exposure studies and field studies of O_3 impacts on Western conifer species have demonstrated chlorotic mottle as typical visible O_3 injury, which begins as a degradation of mesophyll cells below the epidermis [26]. This mesophyll degradation is mainly caused by acute O_3 responses [5] [6], and the lack of epistomatal wax plugs seem to be associated with chronic effects, e.g. changes in water holding capacity and gas exchange of needles.

In our study, we measured several epicuticular wax parameters in order to be able to connect observations with existing mottling and tip burn injuries. The variation of cuticle micromorphology in different pine species makes inter-species comparisons difficult, but response comparisons between sites in the same species have an indicative and diagnostic value.

The microscopic diagnosis for specified O_3-induced symptoms in the foliage of pine and spruce species is

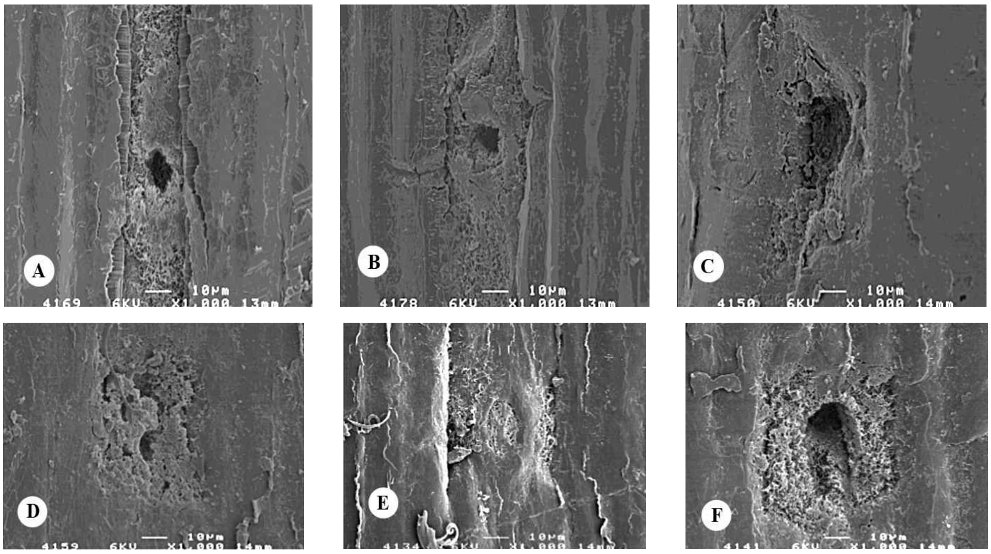

Figure 6. Stomata with or without wax plugs in *P. jeffreyi* ((A), (B)), *P. coulteri* ((C), (D)) and *P. ponderosa* ((E), (F)) in San Bernardino Mountains in 2006. Overall appearance of epicuticular and epistomatal waxes is clean (compared with 1985 and 1986 samples) without special injuries or particles on surfaces. Note: the micrographs have been taken with different scanning electron microscope.

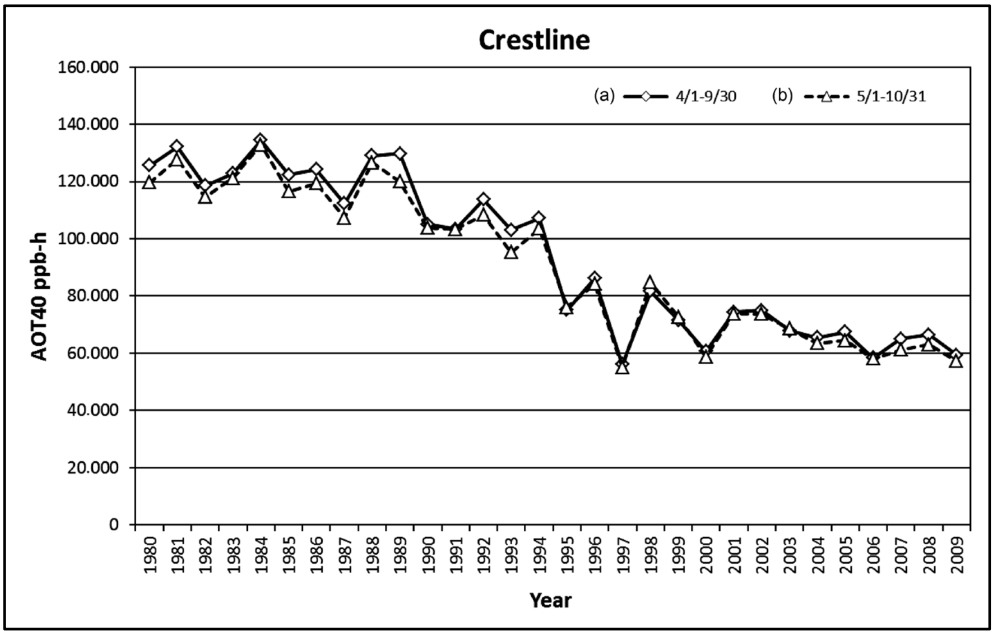

Figure 7. Ozone AOT 40 indices (ppb h) measured at the Crestline site, high exposed to air pollution location in the San Bernardino Mountains 1980-2010. These indices were calculated for two 6-month periods of the growing season: (a) April 1-September 30, and (b) May 1-October 31. A critical level of O_3 (a dose above which O_3-type damage on trees may develop) has been proposed as 10,000 ppb h [41].

mainly attributed to changes in needle mesophyll cells and their chloroplasts [27]-[29]. In experimental studies, the O_3 damage to epicuticular waxes and stomatal responses have either been related to number of stomata per needle, wax synthesis in young developing needles, needle ontogeny or wax amount [30] [31]. Smoothening (mild erosion) of cuticular wax due to O_3 exposure has been observed on *P. coulteri* [32]. Bytnerowicz and Turunen [18] found occluded stomata in young needles of the O_3-fumigated *P. ponderosa*, and O_3-induced erosion of *Piceaabies* (Norway spruce) needle wax has also been documented [33]. Due to variability in assessment methods, and seasonal versus environmental variability, cuticular and stomatal responses have been considered only of limited importance as environmental diagnostic tools [34]. However, plant-atmosphere interactions are of ultimate importance in understanding long-term air pollution and environmental phenomena effects. The cuticle micromorphology is even useful in indicating past environmental conditions [35]. The role of stomatal wax plugs in conifer-atmosphere interactions, in particular, should also be better understood. In pine needles, an assumption of seasonal existence of winter-time wax plugs, has long been held [36]. In cold climates, the erosion of epicuticular wax and epistomatal wax plugs is evident *in situ* [37].

Seedlings are used in fumigation experiments, due to seasonal and tree size limitations, and thus needle responses are measured mostly in current-year young needles. In these studies, the observations of stomatal wax plugs and epicuticular waxes have shown only minor injuries [32]. However, under field conditions, O_3 injuries in pines are detected in older needles [31] and are chronic. The observed changes related to chronic injuries and aging of needles as well as the role of wax plugs in stomatal conductance and gas exchange during whole needle retention time should be better understood. Currently in California, the physiological basis of O_3 injury assessment in conifers uses chlorotic mottle and needle retention [34], but characteristics of needle early injury development are not included.

Evans and Miller [5] [6] emphasized that there was no association between the O_3 injury observed, and changes in substomatal needle tissues of the non-fumigated and O_3-fumigated needles of *P. ponderosa*, *P. jeffreyi*, *P. coulteri* and *P. lambertiana*. However, their micrographs indicate a lack of wax plugs in *P. ponderosa* and *P. coulteri*. According to Whang *et al*. [24] stomatal wax plugs should be present in *P. ponderosa*, *P. coulteri*, and *P. lambertiana*, but not in *P. jeffreyi*. In our study, wax plugs were present in all these species. Wax plugs were abundant in one-year-old needles, but lacking in the stomata of many needles with chlorotic mottling. Stomatal wax plugs were less abundant in two-year-old needles, which may indicate more advanced mottling, or some aging phenomena [16].

In our study, the high elevation (2800 m) *P. jeffreyi* had stomata without wax plugs in older needles, but wax plugs were present in younger needles as previously noted [32]. In our study, the individual of *P. jeffreyi* was sampled three times and the observations of symptoms remained the same in all three sampling years. In 2006, chlorotic mottle and stomata without wax plugs were still observed in young needles in April (see **Figure 6**).

4. Conclusions

The lack of stomatal antechamber wax plugs and visible chlorotic mottle symptoms seem to be connected. Recently, Vollenweider *et al*. [29] showed that presence of cells of mottles is a hypersensitive response of *Pinus ponderosa* to ambient O_3. Whether the lack of wax plugs is a consequence of disturbances in early stomatal development, as found in acid rain-affected needles [37] or a later symptom of dysfunctions, remains unanswered. However, more abundant lack of wax plugs in older needles indicates their late development. The stomata without protective waxes are non-functional. This is also a factor that can predispose pines to specialized fungal diseases [38]), or to a water deficit.

The diagnostic knowledge of stomatal microstructures with or without injuries of different pine species helps to understand the O_3 sensitivity of tree species. However, of the main parameters determining whole-tree O_3 sensitivity, is stomatal conductance controlling effective O_3 uptake [39].

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