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# EPISTOMATAL WAX INJURY TO RED SPRUCE NEEDLES (*PICEA RUBENS* SARG.) GROWN IN ELEVATED LEVELS OF OZONE AND ACIDIFIED RAIN

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## Abstract

Red spruce seedlings (*Picea rubens* Sarg.) were exposed to charcoal-filtered air, at 0.07 ppm or 0.15 ppm ozone ($O_3$), alone or in combination with pH 4.2 or pH 3.0 acidified rain, and examined with scanning electron microscopy (SEM) to determine if epistomatal wax fine structure was affected. Acidified rain in combination with 0.15 ppm $O_3$ produced changes in wax tubule morphology. Changes were moderate at pH 4.2 and severe at pH 3.0. Needles collected from Whiteface Mountain, New York, displayed injured epistomatal wax structure similar to that observed on needles exposed in the laboratory to 0.15 ppm $O_3$ plus pH 3.0 acidified rain.

**Key Words:** Red Spruce, forest decline, acid deposition, stomatal wax plugs, acid rain, ozone, sulfur dioxide.

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## Introduction

During the last decade, increased research has described decreased growth and vigor of spruce/fir stands in the eastern United States (4). Although the northeastern mountains are remote from sources of industrial pollutants, they receive high rates of acid deposition in the form of rain, snow, and cloud moisture (4, 12). This situation has led to speculation that acid deposition has initiated spruce decline or that it has aggravated conditions created by drought and non-biological stress (16). These forest ecosystems are highly complex and may be impacted by acid deposition and gaseous pollutants through various biological and chemical pathways. Forest responses to multiple stresses may require many years before subtle changes become obvious in themselves (4) or the ingress of secondary pests into weakened trees.

Atmospheric deposition can be viewed as stress to the ecosystem, with response occurring in a series of stages influenced by the severity of exposure. The interaction of gaseous pollutants, such as ozone ($O_3$) and sulfur dioxide, may produce different or more severe response than either pollutant alone. This may be intensified in the presence of acid deposition. Cuticular wax production, the process of wax regeneration, and stomatal function (4, 14, 15, 19) could be altered in conjunction with other physiological (10) and cellular changes (20). Scanning electron microscopy (SEM) can be used to observe such surface structure changes (1, 2, 4-9, 11, 17-19).

The purpose of the current study was to determine if the wax morphology of red spruce needles was affected by laboratory exposure to $O_3$ and/or acidic rain and by ambient atmospheric deposition levels experienced in northeastern forests of the United States.

## Materials and Methods

### Cultural practices

Two-year-old red spruce (*Picea rubens* Sarg.) seedlings, grown from a common seed source at the U.S.

Department of Agriculture, Forest Service, Northeastern Forest Experiment Station, Durham, New Hampshire were transplanted in September into 10.2 x 10.2 x 35.6 cm plastic pots containing equal parts (v/v) of peat moss and vermiculite and placed outdoors in Ohio. Each $m^3$ of media was amended with 3 kg lime, 1.2 kg 0-20-0 superphosphate, 4.5 kg 18-12-6 Osmocote slow release fertilizer (Sierra Chemical Co., Milpitas, CA), 0.9 kg Aqua-Gro non-ionic soil wetting agent (Marshall Thomas Co., Inc., Lexington, KY), and 0.04 kg Peters trace elements (W.R. Grace & Co., Fogelsville, PA).

In early December, seedlings were placed in a cold room at 5 °C. At the end of January, just prior to budbreak, 50 seedlings of uniform size were transferred to 1.07-meter-diameter continuous stirred tank reactor (CSTR) chambers (3) in a controlled temperature room. Chamber temperature was maintained at 24 ± 1 °C day and 20 ± 1 °C night. Relative humidity was 39 ± 2%.

A 15 hour photoperiod (0400-1900 h Eastern Standard Time, EST) was provided to each chamber by a metal halide lamp (MS1000/BU, Phillip's Lighting Corp., Bloomfield, NJ) which emitted a light intensity of 290 W m at top of plant canopy. Seedlings were watered twice weekly with 250 ml tap water applied to the soil.

### Pollutant treatments and monitoring

Ten seedlings each were exposed to one of the following treatments: charcoal-filtered air (CFA), CFA plus 0.7 ppm $O_3$, CFA plus 0.15 ppm $O_3$, CFA plus 0.15 ppm $O_3$ plus pH 4.2 acidified rain, CFA plus 0.15 ppm $O_3$ plus pH 3.0 acidified rain. A treatment with acidified rain only was not included in this study because similar doses failed to produce foliar injury in correlative study (16). Space limitations also necessitated only five treatments and one CSTR chamber per treatment.

Plants were exposed to $O_3$ for 6 hours (1000 to 1600 EST) seven days each week. $O_3$ was generated by a corona discharge generator (Model 03V10-0, Ozone Research and Equipment Co., Phoenix, AZ). Concentrations of $O_3$ were monitored (Model 49 Ozone Analyzer, Thermal Electron Corp., Hopkinton, MA) just above the foliage in the center of each chamber using an automated time sharing system. The monitor was calibrated with a Monitor Labs Model 8500 Permacal $O_3$ calibrator (Monitor Labs, San Diego, CA).

Rain treatments were applied at 0700 hour three times weekly (Monday, Wednesday, Friday) by dispensing 1.25 cm rain (approx. 10 min.) through a nozzle (Bete Nozzle P80, Greenfield, MA) positioned in the center of the chamber just below the impeller. Impeller action and nozzle spray pattern combined to give uniform rain distribution. Rain droplet size, measured using water sensitive paper methods (CIBA-Geigy Ltd.), was: 82-165 $\mu$m = 58%; 166-260 $\mu$m = 30%; 261-345 $\mu$m = 12%. Rain solutions were prepared for each precipitation event by adjusting deionized water to pH 4.2 ± 0.11 or pH 3.0 ± 0.09 with a mixture of two parts 1 M $H_2SO_4$ and 1 part 1 M $HNO_3$.

The $O_3$ and rain treatments were continued eight weeks from late January until early April. At the end of the treatment period, current year needles were removed with fine forceps from the distal 3 cm of terminal branches sampled in the cardinal axes of seedlings from each treatment. Needle samples were immediately sealed in plastic bags, labeled, and refrigerated at 5 °C.

### Field collections

For comparison with needles grown under controlled laboratory conditions, red spruce needles were collected from the same individual trees in fall 1985, spring 1986, and fall 1986 on Whiteface Mountain, New York, a spruce decline area, where atmospheric deposition monitoring documented high levels of ambient acidic deposition (12). Branches were cut with a pole pruner from unshaded canopy in the cardinal axes of trees from each of the 3 locations. Needle samples were placed on ice in a cooler for transport to the Delaware, Ohio laboratory. Similar red spruce needle samples were collected at Dawes Arboretum near Heath, Ohio to represent needles grown in an area of low ambient air pollution (13).

### Scanning electron microscopy (SEM)

Needle samples were prepared using a modification of the $OsO_4$ vapor-fixation technique developed by Krause and Houston (9). To quickly kill and immobilize epidermal cells as well as preserve the integrity of the epicuticular wax, needles were affixed to microscope slides with double-sided tape and suspended over $OsO_4$ crystals for 30 minutes in a glass desiccator. Vapor-fixation with $OsO_4$ of coniferous foliage is preferred over conventional aqueous preparative procedures because the ethanol used during dehydration could alter epicuticular wax (10). Current year needles were selected at random, mounted and sputter-coated with gold or carbon (Anatech, Springfield, VA). Tissue was examined with an Hitachi Model S-500 SEM set at 20 kV at 5 mm working distance.

A total of ten needles, 1 needle/tree, were observed for each experimental treatment and field location. Three SEM fields of four stoma each were selected at random for observation at 500 X for each needle processed from the field locations. Observations of experimentally treated needles were limited to one field per needle based on the consistency of needle condition observed in field-collected needles. The ratio of stoma with agglomerated epistomatal wax to stoma with regular epistomatal wax was recorded for each SEM field. The

**Table 1.** Frequency of numbers of *Picea rubens* stoma per a 4-stomatal SEM field[a] showing epistomatal wax injury exhibited as agglomerated wax tubules.

| | Ratio of stoma with agglomerated epistomatal wax: stoma with regular epistomatal wax | | | | |
|---|---|---|---|---|---|
| | 0:4 | 1:3 | 2:2 | 3:1 | 4:0 |
| Experimental Treatments[b] | | | | | |
| Carbon filtered air | 10 | 0 | 0 | 0 | 0 |
| 0.7 ppm $O_3$ | 10 | 0 | 0 | 0 | 0 |
| 0.15 ppm $O_3$ | 10 | 0 | 0 | 0 | 0 |
| 0.15 ppm $O_3$ + pH 4.2 rain | 5 | 4 | 1 | 0 | 0 |
| 0.15 ppm $O_3$ + pH 3.0 rain | 0 | 0 | 2 | 3 | 5 |
| Field Collections[c] | | | | | |
| Dawes Arboretum, Newark, Ohio | 24 | 6 | 0 | 0 | 0 |
| Whiteface Mountain, New York | 0 | 4 | 9 | 6 | 11 |

[a] Samples based on a field of 4 stomata selected at random and observed at x500.

[b] Samples based on 1 field/needle/seedling.

[c] Samples based on 10 needles, 3 fields/needle.

frequencies of these ratios were compared for the laboratory treatments and field locations using the chi-square test.

## Results and Discussion

Macroscopic symptoms (i.e., chlorosis, necrosis, etc.) were not detected on needles of *P. rubens* from any of the experimental treatments. Figure 1 represents a typical field of red spruce stomata on the abaxial needle surface observed with SEM in the current study. No injury was observed at x200 magnification on any needle at any treatment. Figure 1 is from field grown needles sampled at Dawes Arboretum, Ohio in relatively clean ambient air. Neither Adaxial nor abaxial surface of needles from plants grown in CFA revealed microscopic symptoms when examined with SEM. Abaxial surfaces of needles from plants grown in CFA had turgid convex epistomatal cells with epistomatal wax tubules (Figures 2 and 3). No injury was observed with SEM on needles from plants grown only in $O_3$ at 0.7 ppm or 0.15 ppm (Figure 4). Figure 4 is representative of the appearance of wax tubules observed in previous studies of *P. rubens* exposed to acidified rain only (14). Epistomatal wax tubules appeared to be regular in shape and structure. By contrast, light injury to needles from seedlings grown in 0.15 ppm $O_3$ plus pH 4.2 acidified rain was expressed as agglomerated wax tubules (Figure 5). Severe epistomatal injury observed on needles grown in 0.15 ppm $O_3$ plus pH 3.0 acidified rain (Figure 6) was expressed as agglomerated wax tubules and sheets of wax. The later is similar in appearance to *Picea abies* needle-wax damage, induced by pH 3.0 acidic fog, that was described by Mengel *et al.* (11) as "a melted appearance of the small wax threads."

Wax erosion was not observed in the current study. Our results in the laboratory indicate that epicuticular and epistomatal wax was not eroded after it was produced by the plant cells. Rather, it was modified as it was produced and deposited onto the needle surfaces during exposure to the treatment.

No differences in wax structures were detected between the $O_3$ treatments and the control (Table 1). The ratio of agglomerated to regular stomatal waxes observed from different treatments was significantly altered by pH 4.2 and pH 3.0 rainfall with $O_3$ (chi-square $P < 0.01$). In one-half of the needles sampled, the observed stoma on needles treated with pH 4.2 rainfall plus 0.15 ppm $O_3$ showed no injury, whereas all of the observed stoma on half of the samples on needles that received pH 3.0 rainfall plus 0.15 ppm $O_3$ were injured.

Comparison of field-collected needles with those from the experimental treatments revealed that needles from spruce at Dawes Arboretum, Ohio showed injury similar in extent to that found on needles in the pH 4.2 rainfall plus 0.15 ppm $O_3$ treatment (Figure 7). Injury

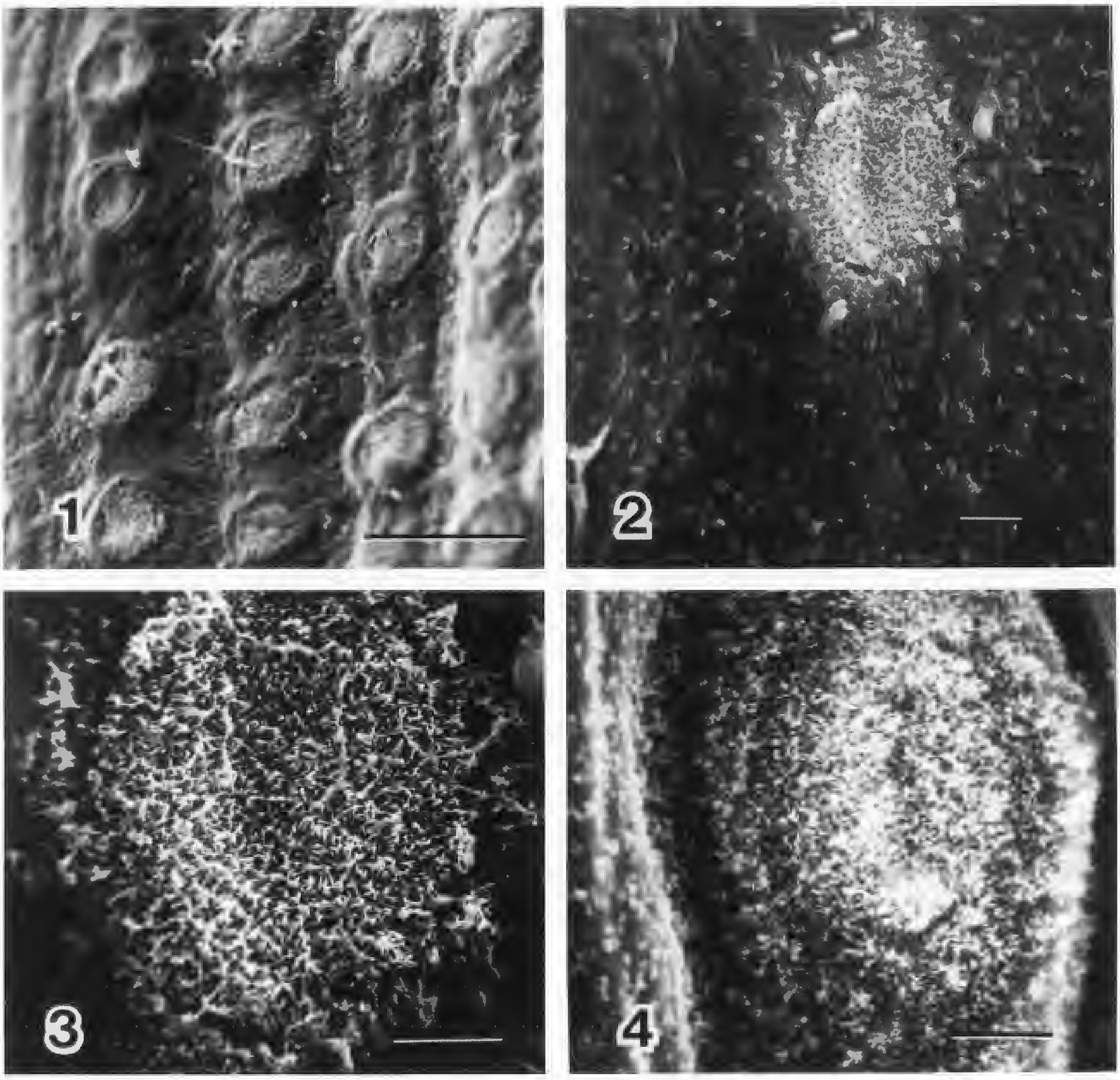


**Figure 1.** A typical field of red spruce stomata on the abaxial needle surface with SEM. Needle sample was taken in relatively clean ambient air from field samples grown at Dawes Arboretum, Ohio. Bar = 50 $\mu$m.

**Figures 2 and 3.** Epistomatal wax tubules from a red spruce needle grown in clean air. Bar = 5 $\mu$m.

**Figure 4.** Representative micrograph of epistomatal wax on needles grown in 0.07 ppm $O_3$ or 0.15 ppm $O_3$ showing regular wax tubules. Bar = 5 $\mu$m.

to epistomatal wax of needles collected from Whiteface Mountain, NY (Figure 8) was similar in extent to that on needles in the pH 3.0 rainfall plus 0.15 ppm $O_3$ treatment (Table 1). Acid rain at either pH apparently induced some degree of injury when applied with $O_3$. Variation in epistomatal wax injury due to site or time of collection was not detected. Wax erosion was not detected. This could have been a reflection of the relatively short time span of this study (14). Wax aberrations probably are due to developmental wax deposition controlled by the physiological state of each cell type (14). Longer exposure periods at chronic pollution levels

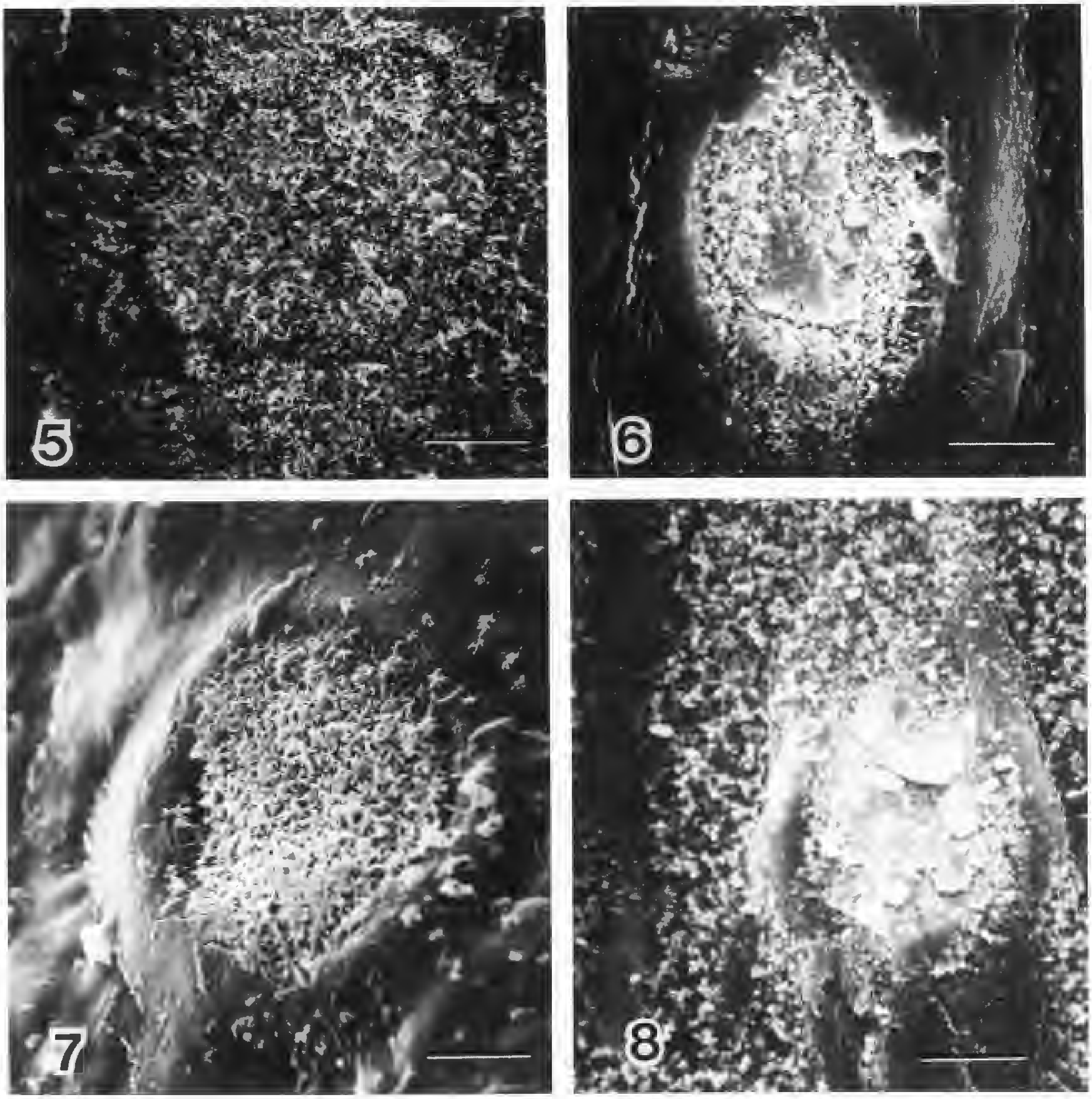


**Figure 5.** Light epistomatal wax injury to needles exposed in the laboratory to pH 4.2 acidified rain + 0.15 ppm $O_3$ expressed as agglomerated wax tubules. Bar = 5 $\mu$m.

**Figure 6.** Severe injury to epistomatal wax on needles exposed to pH 3.0 acidified rain + 0.15 ppm $O_3$ expressed as agglomerated wax tubules fused together in sheets. Bar = 5 $\mu$m.

**Figure 7.** Needle grown at Dawes Arboretum, Ohio exhibiting light epistomatal wax injury. Bar = 5 $\mu$m.

**Figure 8.** Needle grown at Whiteface Mountain, New York displaying severe injury to epistomatal wax; agglomerated wax tubules fused together in sheets. Bar = 5 $\mu$m.

could yield a clearer picture of epistomatal wax injury and needle survival.

The morphology of epistomatal needle wax is a reliable indicator of plant health and the plant's inherent resistance to air pollution stress (14). Survival of a particular tree species on a specific site could be altered due to degradation of the waxy cuticle. The first line of defense against ambient extremes is the waxy cuticle of a plant (14). Changes in leaf wettability, changes in rates of transpiration, and loss of solutes from leaf cells are some of the effects that result from disruption of epicuticular/epistomatal wax. In the current study, $O_3$ alone did not change the configuration of wax tubules under the experimental conditions tested. This is not to imply that $O_3$ would not cause injury if greater dosages or alternate exposure patterns were utilized.

Air pollutant stress cause-and-effect relationships are difficult to establish between laboratory results and field observations. The natural environmental stresses at Whiteface Mountain and Dawes Arboretum were not quantified in this study and may have influenced the extent of epistomatal injury detected. However, the similarity of epistomatal wax injury to needles from naturally occurring red spruce at Whiteface Mountain to that produced by pH 3.0 rainfall plus 0.15 ppm $O_3$ laboratory treatment, and the injury to needles from planted red spruce at Dawes Arboretum to pH 4.2 rainfall plus 0.15 ppm $O_3$ treatment suggests a pattern of response of red spruce to these air pollutants.

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## Discussion with Reviewers

**S.R. Shafer** : Are the seasonal mean ozone concentrations and median pH levels for the field sites available?
**H. Tuomisto**: What were the concentrations of the relevant air pollutants and the pH of rain water in the field sites? What kind of forest was there, and how were the collected needles selected?
**Authors**: No, seasonal mean ozone concentrations and median pH levels are not available. The concentrations of total oxidants, air pollutants and pH rain water at both field sites during the current experiment (i.e., Spring, 1985 through Fall, 1986) were as follows:

*Dawes Arboretum, OH*: Total oxidants/24 hour period = 0.02 to 0.04 ppm (39 - 78 $\mu g/m^3$); rain water = pH 5.4 ($\pm$ 0.3).

*Whiteface Mountain, NY*: Total oxidants/24 hour period = 0.05 to 0.10 ppm (98 to 196 $\mu g/m^3$); rain water = pH 4.5 ($\pm$ 1.2).

Seasonal means did not best characterize the physiological impact of pollutants to plant function. High concentrations of ozone exposure (episodes) in nature appear to be more important (Lejohn AS, Lucier M, 1991; Spatial and temporal variability of ozone exposure in forested areas of the United States and Canada: 1978-1988. J. Air Waste Management Assoc. **41**, 694-701). Spruce-Fir forest was the dominant forest type on Whiteface Mt., NY, with red spruce the primary species. Needles were sampled at random.

**H. Tuomisto**: Air pollution studies often suffer from subjectivity. This can be reduced by using a "blind" survey of the samples, i.e., by not revealing the origin of the samples to the observer until afterwards. Did you apply this method?
**Authors**: Yes, original SEM observations were made without knowledge of treatments to reduce biased conclusions. Once patterns of injury were suspected, replications of known treatments were used to verify the occurrences of various symptomologies.

**H. Tuomisto**: How did you define the limit between the wax classes, and how were the stomata showing intermediate wax treated in Table 1?
**Authors**: The limits between wax injury classes were not defined other than ratios of regular and agglomerated epistomatal wax. Intermediate injury to wax was not addressed in Table 1.

**S. Flegler**: Do you think cryo-preparation of the needles would produce significantly different results from that obtained with your osmium vapor technique?
**Authors**: Cryo-preparation would have produced similar results to those presented using $OsO_4$ in this study based on previous research completed and published from this laboratory (see text references 9 and 10).

**H. Tuomisto**: How long was refrigeration time after sampling?
**Authors**: The samples were refrigerated two days after sampling until $OsO_4$ vapor fixation.

**Reviewer I**: How have the intermediate degrees of injury been treated in the numerical analysis in Table 1?
**Authors**: Intermediate degrees of injury were not observed in this study.

**H. Tuomisto**: The influence of air pollution is not always clear-cut, and there are basically two approaches by which it can be unraveled. One can either use a large set of samples, or do a detailed job with a limited number of samples. In your study only two classes of epicuticular wax are recognized: agglomerated and regular. Do you think that the number of samples is large enough for so coarse a measure?
**Authors**: Yes, we feel that we used a large enough number of samples to measure agglomerated or regular epistomatal wax injury. To use more classes of injury would have been to seek details that were not present in the current study. Future studies could be designed to observe greater number of samples.

**H. Tuomisto**: It has been shown that the epicuticular wax structure may change rather rapidly in young needles. You mention three sampling events for the field sites, but no exact dates. How old were the needles collected each time, when were the ones contributing to Table 1 collected, and how were these selected?
**Authors**: The age of the needles collected in the field were as follows: Fall, 1985 - 6 months old; Spring, 1986 - current years growth; Fall, 1986 - 6 month old. Needles collected in the field and measured and recorded in Table 1 were from Fall, 1986. All were selected at

random.

**H. Tuomisto**: You suggest that the differences in wax are not due to erosion but to differences in wax formation. Have you got any data from needles of different ages to support this conclusion?
**Authors**: At Whiteface Mt., New York, needles were sampled in Fall, 1985. The following Spring (1986), the same ranking of needles (from the 1985 growing season) were sampled after over-wintering in the field following exposure to winter conditions, relatively high ambient air pollution, and acid precipitation exhibited no changes in epistomatal wax structures. However, epistomatal wax injury appeared in immature needles sampled during periods of rapid growth in Spring, 1986. In Fall, 1986, epistomatal changes persisted with SEM observations similar to those reported by Percy *et al.* (14).

**H. Tuomisto**: The wax around the stoma in Figure 8 is in surprisingly good condition for the stoma to be included in the severely injured class. Was the epicuticular wax outside the stomatal area observed in your study?
**Authors**: Observations of epicuticular wax outside the stomatal area did not provide any significant patterns of change, injury, or developmental morphology.

**A.F. Vogelmann**: What is the physiological significance of the agglomerated wax? How much agglomeration (Figure 5 versus Figure 6) can a tree/needle sustain without serious repercussions?
**Authors**: While we did not examine injured red spruce needle cells in cross-section, the amount of stomal agglomerated wax indicates the relative health or injury to particular trees due to problems of air quality. Without photosynthetic, respiratory, and growth/response data correlated with the occurrence of agglomerated wax long term survival or the vigor of particular tree cannot be predicted.

**A.F. Vogelmann**: Do you have any idea of the distribution of agglomeration over a tree? Does it increase with increasing needle age? Is it less on needle protected by overhanging branches?
**Authors**: We do not have any data on the spatial distribution of needle wax agglomeration over a tree. It was present on current years needles as well as previous years needles from Whiteface Mountain, New York. We have no information related to its presence on overhanging branches.

**S.L. Flegler**: You postulate that wax aberrations are probably due to developmental wax deposition controlled by the physiological state of each cell type. Do you then believe that the wax aberrations are a cause or a symptom of spruce decline?
**Authors**: Wax aberrations are indicators of spruce decline.

**S.R. Shafer**: Would you have seen any "rain" effect if you had simply rained with deionized water onto spruce needles?
**Authors**: Since we did not use deionized water, we cannot predict what the effect would have been. The rain acidity of pH 4.2 that we used was to approximate the acidity of ambient rain in central Ohio.