Crystal occurrence and wax disruption on leaf surfaces of cabbage treated with simulated acid rain

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Summary

Epicuticular waxes on leaves of Brassica oleracea L. (cabbage) were studied using scanning electron microscopy after a single treatment with simulated rain of pH 5.6, 3.0 or 2.5 which was either sprayed on to plants in an exposure chamber or applied as droplets with a micropipette. Treatments with acidified rain caused serious structural degradation of the wax crystals. The alteration of crystalline wax structures was similar for leaves treated with nitric acid solutions, but less severe, than for leaves treated with sulphuric acid solutions. With both H₂SO₄ and HNO₃-derived rain solutions numerous gypsum (CaSO₄) crystals were found in and near lesions on the leaves treated with rain of pH 3.0 and 2.5. The crystals probably resulted from damage of cuticular membranes by acidic rain which significantly altered their permeability to ions in the area of lesions. Crystalline leaf waxes may be an important target for acidic pollutants, and the physiological consequences of their degradation are discussed.

Key words: Brassica oleracea, epicuticular wax, acid rain, degradation, gypsum crystals.

Introduction

Epicuticular waxes on leaf surfaces of higher plants are an important barrier to ion and water movement across the cuticle. These crystalline deposits which overlay the cuticle occur in a variety of forms, such as plates, ribbons, tubes and rods (Baker, 1982; Juniper & Jeffree, 1982). Differences in wax morphologies may be caused by differences in the chemical composition of the material, or they may be due to a different arrangement of the same compounds (Jeffree et al., 1973; Baker, 1982). Epicuticular waxes are important in determining the surface properties of leaves. In general, leaves with more epicuticular wax are more hydrophobic, resulting in less contact between the surface of the cuticle and water droplets. This has implications for nutrient leaching, the retention of water drops, pesticide spray retention, sensitivity of leaves to acid rain, and infection by pathogens.

Wax development may be influenced by environmental factors, such as light, temperature and humidity, (Whitecross & Armstrong, 1972; Baker, 1974; Reed & Tukey, 1982) and in addition to influencing wax production, the environment may cause erosion of epicuticular waxes from leaf surfaces by direct abrasion of crystalline structures, for example by wind and rain (Hall & Jones, 1961; Hallam, 1970; Baker & Hunt, 1986). Studies have also shown alterations in the crystalline structure of epicuticular waxes as a result of air pollution. Observations on conifer species growing in polluted areas have detected damage to the wax cover; the rodlets of epicuticular wax become fused and in the most severe cases of degradation a continuous wax layer covers the entire stomatal chamber. This type of damage to the needle wax may be a result of exposure to various pollutants, including SO₂ (Fowler et al., 1980; Cape & Fowler, 1981), ozone (Braun & Sauter, 1983; Magel & Ziegler, 1986; Schmitt et al., 1987), salt spray (Krause, 1982), vehicle emissions (Riding & Percy, 1985; Sauter et al., 1987), and alkaline dusts (Grill & Golob, 1983; Grill et al., 1987).

Damage to cuticular wax of plants in the field (e.g. Crossley & Fowler, 1986) can rarely be attributed to
a specific pollutant, but several studies using trees suggest a role of acidic precipitation. The erosion of epicuticular waxes by acid rain was first reported by Shriner (1974), in which he observed, using scanning electron microscopy (SEM), increased weathering of epicuticular waxes on *Quercus prinus* and *Liriodendron tulipifera* when exposed to stimulated rain of pH 3-2 in comparison with that of pH 5-6. In contrast, others have asserted that waxes on the leaf or needle surface would not be susceptible to modification by acids because of the inert nature of the wax components (primarily hydrocarbons, primary and secondary alcohols, β-diketones and fatty acids) (see reviews by Evans, 1984; Seymour Berg, 1985).

It is apparent that exposure to acidic precipitation at high elevation can be severe. Lengthy periods of very high acidities in cloudwater (pH 1-5-2-8) have been measured on Mount Mitchell in the southern Appalachians (R. Bruck, pers. comm.) and also in cloudwater at other montaine, coastal and urban locations (Munger et al., 1983; Weathers et al., 1986; Sigg et al., 1987; Fowler et al., 1988). There is increasing evidence linking acidic deposition to the forest declines now widespread in North America and in Central Europe.

Examination of needle surfaces using SEM of individuals in decline has shown that for red spruce in North America (Bruck, pers. comm.) and Norway spruce and silver fir in Central Europe (Sauter & Voss, 1986; Grill et al., 1987), at high elevation sites, the needle wax is severely eroded, especially in the epistomatal chambers. Recently, in four separate studies, Norway spruce and silver fir seedlings were exposed to acid rain treatments over 2-30 months and in all cases damage to the waxes similar to the symptoms observed on declining trees in the field was found, rodlets having fused in response to the acidic sprays (Magel & Ziegler, 1986; Rinallo et al., 1986; Schmitt et al., 1987; Mengel et al., 1989).

Wax production may also be altered by acid rain as reported by Percy & Baker (1987) for dwarf bean, field bean, pea and rape. In their studies, this was often accompanied by changes in wax composition and alteration of the crystalline wax structure. Thickness of the cuticular membrane was decreased in leaves of dwarf bean, field bean and pea, and membrane ultrastructure was also altered in dwarf bean at pH 2-6.

In an SEM study initiated in our laboratory to examine whether differences in surface features of cotyledons and leaves of cabbage (*Brassica oleracea*) could account for the much greater sensitivity of the cotyledons of this plant to injury by acid rain (Caporn & Hutchinson, 1986), we observed significant changes in the epicuticular waxes on both cotyledons and leaves after a single, short (30 min) exposure to simulated rain of pH 3-0. Based on this unexpected observation of damage to epicuticular waxes we decided to use cabbage as a model system to test the effect of acidic rain solutions on crystalline waxes. An advantage of using cabbage for this work is that the structure, chemical composition and development of its crystalline waxes has been studied more extensively (e.g. Baker, 1974; Sutter, 1983) than the epicuticular waxes of tree species. Our initial observation was thus followed by repeated experiments with simulated rain of three pH values (5-6, 3-0 and 2-5) and varying chemical compositions, either sprayed on plants or applied as droplets with a micropipette. In the present paper we report the morphological changes we observed in the crystalline wax structure on leaves exposed to acid rain. We also describe calcium and sulphur-rich crystals found on the surface of leaves which received the acidic rain treatments.

**MATERIALS AND METHODS**

**Growth conditions**

Cabbage plants (*Brassica oleracea* L.) cv. Viking Golden Acre were grown in a greenhouse on four different dates throughout the year in experiments to determine the effect of simulated acid rain on surface wax. In each of the experiments, cabbage seedlings were grown in 10 cm diameter plastic pots containing equal parts of loam, peat and sand. Growth conditions (e.g. light, temperature and relative humidity) varied for the four sets of plants. Supplementary lighting, using either cool fluorescent tubes or high pressure sodium vapour lamps, depending in which of two greenhouses the plants were grown, extended the photoperiod to 14 h light. Plants were grown at a minimum temperature of 18-20 °C and the maximum temperature varied with the time of year for the four sets of plants; approximately 25 °C in the winter months and 35 °C in the summer. Plants were watered from saucers below each pot in order not to wet the leaves and possibly disrupt the surface waxes. Similarly a liquid fertilizer solution of equal parts of nitrogen, phosphorus and potassium, was supplied from below each pot every other week.

**Simulated rain spraying experiments**

Two experiments were carried out in which plants were exposed to a single simulated rain treatment 4 weeks after planting. In the first experiment 10 replicate plants were exposed to either rain of pH 3-0, acidified with 2-1 equivalent sulphuric/nitric acid, or a non-acid rain of pH 5-6. The simulated rain solution used in the first experiment contained cations and anions similar to the concentrations occurring in rain in southern Ontario (described by Hutchinson and Adams, 1987). In the second experiment, five replicate plants were exposed to pH 5-6 or 3-0 simulated rain, with and without calcium (Ca) in the rain solution. The pH 3-0 rain was
acidified with either 2:1 equivalent sulphuric to nitric acid or nitric acid only. This gave a total of two treatments of pH 5-6 and four of pH 3-0.

Simulated rain was applied in an enclosed exposure chamber in the greenhouse. Rain was delivered from a stainless steel rain nozzle (Bete Fog), 1.75 m above the plants. Rain droplets ranged in diameter from 200 to 1100 μm, with a mean of 480 μm. Plants were exposed to one simulated rain event lasting 30 min and delivering 0.9 cm rain.

The 4-week-old plants in both experiments generally had 7-9 leaves. For SEM we sampled the fourth leaf below the apex at a stage where leaf expansion was still occurring. Leaves were sampled for SEM 1 day after the rain treatments. Since cabbage leaves are highly water repellent, tissue was generally cut in areas where there was obvious visible damage (lesions) for the pH 3-0 treatment. However, some pH 3-0 tissue with no visible damage was also used.

**Droplet experiments**

In another experiment small drops of water of various pH values were gently pipetted onto the adaxial surface of cabbage leaves. This technique was developed in order to assess the effect of rain acidity alone on epicuticular waxes without the complication of some physical abrasion by the impact of simulated rain, as reported by Baker and Hunt (1986). It also had the added benefit that all of the pH treatments could be applied to a single leaf for comparison, eliminating variability in the surface waxes of different leaves due to leaf age or position.

Three pHs (5-6, 3-0 and 2-5) of simulated rain were used, as well as an unwetted area of the leaf surface. Distilled water was either acidified with 2:1 equivalent sulphuric/nitric acid to produce the acidic pHs of 3-0 and 2-5, or adjusted to pH 5-6 with sodium hydroxide.

The highly water repellent nature of the cabbage leaf surface made it impossible to get droplets to remain on the spot where they were applied. Therefore, lanolin was squirted from a syringe in a thin line (c. 2 mm wide, 2 mm high) to delineate squares (1 cm × 1 cm) on the adaxial surface of leaves into which 300 μl of rain solution was then pipetted. Care was taken not to abrade the leaf surface. The squares were set out in a grid fashion on either side of the leaf mid-vein, approximately 1 cm away from the vein. Placement of the three pH treatments, as well as an unwet treatment, in the grid was random, with at least one empty square separating the treatments to prevent drops from running together (Fig. 1). The treatments were applied to 10 replicate plants, on one leaf, which was 7th-9th below the apex. Plants were 63-d-old when the first 5 replicates were treated and 70-d-old when the second five replicates were treated at which time they generally had 12-15 leaves. Leaves were sampled for SEM on the day after the drops were applied. Tissue was cut from the centre area of the treated squares, to avoid the possibility that the lanolin itself might have an effect on the leaf surface. Comparison of unwet areas adjacent to the grids but well away from any lanolin, and unwetted areas in squares marked out with lanolin, using SEM, showed lanolin had no effect on the leaf surface except right alongside the grid. In preliminary tests with the lanolin on cabbage leaves we found that it spread out over the leaf surface at temperatures above 22 °C obliterating the crystalline wax structure, and consequently the plants were kept at 18 °C during the period that droplets were on the leaves.

**Epicuticular wax examination**

Leaf pieces (c. 4 × 4 mm), were mounted on aluminium stubs with double-sided adhesive taking care to avoid wax abrasion. Material from the rain-spraying experiments was rapidly frozen in liquid N₂ after mounting and then freeze-dried overnight. In contrast, we examined fresh leaf tissue for the droplet experiments. In this case, all of the treatments for a single leaf were represented on one stub. Freeze-dried and fresh specimens were gold-coated and adaxial surfaces examined with a Cambridge S-180 scanning electron microscope (SEM) operated at 25 kV. One to several areas of the surface of each piece of tissue were selected for the scanning micrographs to illustrate the typical appearance of
epicuticular waxes. Photographs were taken at three standard magnifications (320 x; 1020 x; 3200 x) using Polaroid P55 film. The amount of alteration of the wax structure was evaluated on the basis of the micrographs and general observations by comparing each pH 3-0 or 2-5 tissue with the corresponding pH 5-6 control tissue in each experiment. In the droplet experiments this meant that corresponding samples for the different pH treatments were all from the same individual leaf, while for the rain spraying experiments the samples compared were taken from different plants using the same approximate areas of leaves in the same position from the shoot apex.

Crystal morphology and chemistry

Crystals with distinctive forms were observed using SEM on acid-treated leaves. Their chemical composition was determined by X-ray microanalysis (EDAX) of carbon-coated specimens using a JEOL JSM-840 SEM operated at 15 kV. Crystal refractive indices were determined with an optical microscope using oil immersion and an index oil of 1:550. Another technique which was used to study the crystals was to place pH 5-6, 3-0 and 2-5 droplets (with no additional salts) carefully on to cabbage leaves, then to remove them with a micropipette after an hour and allow the droplet to dry on a glass coverslip. This allowed crystals to be examined for their morphology, refractive characteristics and chemical composition without interference from the leaf surface itself. Rain droplets from the same initial batch were also placed directly on a glass coverslip.

Some observations of the entire surface of leaves from the simulated rain experiment were also made using a Zeiss stereo SVB dissecting microscope.

RESULTS

Wax morphology

The adaxial surfaces of cabbage leaves are covered with dense, crystalline wax fibrils projecting from an underlying layer of amorphous wax and small platelets that covers the cuticle surface. Both the density and fine structure of the crystalline waxes varied with the growth conditions for the various experiments, which were run at different times of the year in two different greenhouses. The normal appearance of epicuticular wax, both on plants from the pH 5-6 treatment and on unsprayed plants, will be described first, followed by a description of the alteration of wax structure and coverage that occurred in the acidic treatments.

In the two experiments in which rain was sprayed from a nozzle onto the plants, wax was present as tubes projecting away from the leaf, mainly perpendicular to the cuticle surface. The tubes were interspersed with some flat irregular plates (Figs 2, 3). The waxes on the leaf surfaces of plants used in the rain droplet experiment had a somewhat different appearance. In this case the tubular wax structures were also present, but overlying about half of the tubes were cup-like wax structures known as dendrites (Figs 6, 7). The area of leaf surface covered by epicuticular wax was greater on the plants from the rain droplet experiment, both due to the presence of the dendrites and a greater density of tubes. Both of these wax forms are characteristic for cabbage; the formation of dendrites in preference to upright tubes is favoured at higher temperatures and with high radiant energy rates (Baker, 1974; Baker & Hunt, 1986). Waxes on cabbage are known to respond very rapidly to fluctuations in environmental conditions. For example, Baker (1974) found that plants grown in conditions that favoured the formation of tubes produced dendrites on top of the tubes within 48 h after the temperature and radiant energy were increased.

Alteration of crystalline waxes in response to acidic rain

The relatively large deposits of crystalline waxes on cabbage make the leaves extremely water repellent. Raindrops both bounce and roll off the leaves when plants are sprayed with simulated rain. Also, the droplets pipetted onto leaves generally would not stay in place without the lanolin grid. After a rain treatment only a small number of water droplets were left on the leaves which probably minimized the damage to the leaf surface; less than 2% of the leaf area had visible injury.

Regardless of whether cabbage leaves had tubular waxes, or a composite arrangement of tubes and dendrites, a similar alteration of the crystalline wax structure was observed after a single treatment with pH 3-0 rain (Figs 4, 5, 8-11). The damage was also similar whether the acidic rain was sprayed or applied as drops with a micropipette. Typically the tube waxes and dendrites, characteristic of the undamaged (pH 5-6 and unsprayed) leaf, were replaced by large scattered amorphous clumps of wax. The damage to the pH 3-0 treated leaves was less than with pH 2-5 rain; areas with degraded wax as well as areas with normal crystalline structure were observed (Figs 4, 8). This gave a patchy appearance to the wax coverage with considerably more smooth surface visible between the wax structures. On leaves treated with pH 2-5, however, the degradation was so much more severe that, in very large areas, there was almost no fibrillar wax structure left, but rather the leaf surface had an amorphous appearance (Figs 10, 11). In some cases the surface was quite smooth (Fig. 10), but in others the wax formed a kind of crust (arrowed) on the surface (Fig. 12) and even occasionally the wax plugged the stomata (Fig. 13). In Figure 13 there are smooth areas, an area with a solid thick crust and also areas where the wax appears as
Crystals and wax on leaves of cabbage exposed to acid rain

Figures 2-5. Scanning electron micrographs showing the epicuticular wax structure, mostly consisting of tubes (T) and some irregular shaped plates (P), on the adaxial surface of pH 5.6-treated leaves of cabbage (Figs 2, 3) and the degradation of wax crystalloids to amorphous clumps (C) on pH 3.0-treated leaves (Figs 4, 5). Plants received a 30-min spraying of simulated rain, which contained added ions to approximate ambient rain in southern Ontario. pH 3.0 was acidified with 2:1 H₂SO₄:HNO₃. S, stomatal pore. Scale bars for Figures 2 and 4, 10 μm; for Figures 3 and 5, 30 μm.

spherical globules. The structural alteration of epicuticular waxes was similar whether induced by nitric acid-rain alone or by rain acidified with a sulphuric acid/nitric acid mixture. However, less damage to the leaves occurred in those treated with the nitric acid rain. Leaf tissue from the pH 3.0 treatment which showed no macroscopic injury also showed damage to the crystalline waxes, although it was less common than on tissue taken from the area of lesions. Thus, structural alteration of crystalline waxes occurred outside regions of cell collapse and obvious visible injury.

Presence of gypsum crystals

SEM studies also showed crystals of various sizes and forms on acid-injured cabbage leaves (Figs 14-20). Scanning of a number of whole cabbage leaves using a dissecting microscope (x 60 mag.) showed that these crystals generally occurred in the immediate (up to 1 mm away) vicinity of lesions. Very few were observed away from the lesions. The abundance of crystals of various shapes and sizes in one of the lesions, with a diameter of approximately 500 μm, is shown in Figure 14. Epidermal cells in the lesion, which have collapsed to form the depression, also show marked erosion of their crystalline waxes. The wax covering in another leaf surface, shown in Figure 15, not only appears amorphous and degraded, but wax has also formed a crust over the numerous crystals that precipitated following the acid rain treatment. The crystals occurred as an elongate blade, parallel to the crystallographic axis. Crystals varied from single prismatic or tubular rods, with monoclinic morphology, approximately 6 μm thick and 20-80 μm long, to complex clusters with many side arms, approximately 90 μm in diameter, to dense clump-like crystals with no side arms but rather upward-pointing arms. Twinning of the blades is common, especially swallow tail twins (these are illustrated in Fig. 20). Similar crystals were observed on acid-injured leaves, whether the source acid in the pH 3.0 rain treatment was a mixture of sulphuric and nitric.
acids or only nitric acid, and when calcium and other ions that could perhaps precipitate out of the rain solution were deliberately not added to the simulated rain (i.e. no ions other than the acids were added to the rain). Crystals occurred only on leaves that had been treated with the acid rain (pH 3.0 and 2.5) and not on control (pH 5.6 and unwet) leaves. Identical crystals were also formed on a glass slide by removing pH 3.0 rain drops from cabbage leaves within an hour of applying the drops with a micropipette and letting them dry on the slide (Figs 18-20). In this experiment pH 3.0 rain acidified with either H$_2$SO$_4$, HNO$_3$, or a mixture of these acids all resulted in similar crystal forms. pH 3.0 rain solution that had
Crystals and wax on leaves of cabbage exposed to acid rain

Figures 12, 13. Scanning electron micrographs showing degradation of the crystalline wax by pH 2.5 where it forms a crust (arrowed) over the surface (Fig. 12) or deposits over stomata (arrowed) (Fig. 13). S, stomatal pore; G, globules; Cr, crust. Scale bars, 10 μm.

no contact with cabbage leaves and also pH 5.6 rain solution removed from cabbage leaves did not form the crystals on the glass slides.

Fifty-five crystals, including some crystals of each of the described morphologies (i.e. single rods, clumps, clusters with sidearms) were characterized by means of electron probe X-ray microanalysis (EDAX). The crystals were all comprised principally of calcium (Ca) and sulphur (S). We also found the same elemental composition (Ca and S) for the different types of acids that were used to acidify rain (H₂SO₄, HNO₃ or a mixture of these two acids). For about 70% of the crystals, Ca and S were the only peaks measured with EDAX, the remaining 30% of the crystals having very small peaks of K, Mg or Na, as well as the distinctive Ca and S peaks. The ratio of S:Ca varied from high to low, with some showing equal amounts of both elements. This variation would be expected even on a single crystal, however, simply due to the positioning of the electron probe. The variation was the same for the different acids that were used to acidify the rain. One means of differentiating between the hydrated (gypsum) and anhydrite forms of CaSO₄ is the characteristic refractive indices (n) of these two forms. When this was determined for the crystals it was found to be consistent with gypsum (n = 1.53).

DISCUSSION

These experiments demonstrate a severe alteration in the appearance of crystalline leaf waxes on cabbage caused by exposure of the leaf surface to acidic rain. While the erosion of epicuticular wax layers on several conifer species by gaseous air pollutants has been demonstrated (e.g. Huttunen & Laine, 1983; Riding & Percy, 1985; Crossley & Fowler, 1986), until recently there has been scepticism that epicuticular wax layers would be susceptible to morphological changes by rain acidity (see reviews by Evans, 1984; Seymour Berg, 1985). In the present study, therefore, the extent of the structural degradation from tubular wax crystalloids to a fairly amorphous and melted-looking wax was an unexpected finding. This occurred after exposure to only one short period of rain, or even with single droplets, and at pH values presently encountered under ambient conditions. By applying multiple droplets having different pH values to a single leaf we were certain that degradation from simulated rain of pH ≤ 3.0 was caused by the acidity and not by physical abrasion from simulated rain, as has been shown by Baker & Hunt (1986). This suggests that acidic solutions, whether applied as rain or mist can cause comparable degradation of the epicuticular wax of the leaf. Although most of the samples were taken from lesions, the wax structure was also degraded, though to a smaller extent, outside regions of visible injury.

Percy & Baker (1987) described reductions in quantity of wax on leaves of Phaseolus vulgaris (dwarf bean) due to acidic rain, with no apparent effects of acidity on the morphology of amorphous wax for leaves of this species and also of Vicia faba (field bean). However, there were changes due to the pH of simulated rain in the morphology of wax for Pisum sativum (pea) and Brassica napus (rape), which were also studied and which have crystalline wax. In this study plants were repeatedly exposed to simulated rain, from the time of leaf emergence to that of full leaf expansion, at pH values between 5.6 and 2.6. Percy and Baker concluded that changes in wax morphology, as well as decreases in the amounts of epicuticular wax per unit area of leaf, were due to a specific effect of simulated acid rain on wax production. The chemical composition of epicuticular wax of dwarf bean and pea leaves, but not field bean or rape, was also altered by the pH of the rain. One of their most significant findings was that in addition to affecting the epicuticular waxes, changes in the
Figures 14-20. Scanning electron micrographs of crystals of various morphologies on the adaxial surface of cabbage leaves (Figs 14-17) and on glass slides after droplets were removed from cabbage leaves and then allowed to dry on the slides (Figs 18-20). All micrographs are of pH 3.0 material; crystals did not occur with pH 5.6 rain. Figure 14. Crystals (arrowed) in the region of a lesion in which epicuticular wax is also severely degraded. Rain acidified with HNO₃, no ions added. Figure 15. Area in a lesion showing single prismatic rods (R) as well as more complex clusters (C) with sidearms. Crystals are coated in wax from the severely degraded leaf surface. Rain acidified with 2:1 H₂SO₄: HNO₃, with ions added. Figure 16. Dense clump-type crystal with upward-pointing arms. Rain acidified with HNO₃, with added ions. Figure 17. Several crystals of the cluster type with side arms. Wax structure is crystalline on the left of the micrograph and amorphous on the right. Rain acidified with 2:1 H₂SO₄: HNO₃, with no ions added. Figure 18. Crystals which formed in a circle at the periphery of a pH 3.0 droplet that was removed from a cabbage leaf and allowed to dry on a glass slide. Figures 19, 20. Higher magnifications of the crystals in Figure 18. Crystal in Figure 20 shows distinctive swallow tail twin (arrowed) characteristic of gypsum. Rain acidified with 2:1 H₂SO₄: HNO₃, with no ions added. Scale bars for Figures 16, 19 and 20, 10 μm; for Figures 15 and 17, 30 μm; for Figures 14 and 18, 100 μm.
cuticular membrane ultrastructure were brought about by the acidic treatments. In contrast to our findings with cabbage concerning structural degradation of wax crystalloids, where tubes and dendrites were degraded to large clumps of wax or, in severe cases, either thick wax crusts or very large areas of continuous smooth wax, Percy and Baker found subtle changes in wax morphology for their species with the crystalline wax. Acidic treatment caused a greater number of smaller tubes of wax in rape and a larger number of plates in pea. One major difference between these two studies, besides the different species that were used, appears to be the way in which tissue was sampled for SEM. We generally examined leaf segments selectively taken from an area containing a lesion, although some undamaged tissue was also included, whereas in Percy and Baker’s work leaf segments (only three per pH and species) were not selectively chosen. The small amount of visible foliar injury from the pH 3.0 treatment (1.3 and 4.4% of the leaf area damaged in pea and rape respectively), combined with the hydrophobic nature of leaves that have crystalline waxes probably indicates that the tissue pieces they examined randomly did not have droplets drying on them, since these would be rare. The alteration in numbers of tubes and plates and changes in chemical composition, are probably, as they suggest, an effect of the general stress produced by acid treatment of leaves, altering physiological pathways such as wax synthesis, rather than a direct structural breakdown of wax crystalloids as we have found.

Although we have found serious structural damage of the epicuticular waxes near lesions, leaves with crystalline waxes, including cabbage, tend to be very hydrophobic, and the leaf remains essentially unwetted after a rain event, in comparison with less waxy leaves on which numerous droplets remain after rains (Adams & Hutchinson, 1987). An understanding of the significance to the plant of the damage we observed requires more extensive study of the areas of the leaf showing no macroscopically visible injury. Here the droplets may not have dried on the surface but there will have been short periods of contact as droplets rolled over the surface. From the few leaf samples exposed to pH 3.0 which had no obvious visible damage we have indications of cuticular injury but it appears to be less frequent and of lesser severity. Since the duration of contact between acidic solutions and leaves is likely to determine the extent of injury, substantial damage to plant surfaces may be great for those species which are enveloped for lengthy periods in acidic mists.

Our results with cabbage indicate that crystalline waxes are sensitive to acidic precipitation of ≤ pH 3.0. The frequency of occurrence of precipitation (rain and cloudwater) of at least this level of acidity (Bruck, pers. comm.; Weathers et al., 1986) indicates that foliar epicuticular waxes are an important target for acidic precipitation. Four recent studies in which Norway spruce was exposed to simulated acid rain under controlled conditions (Magel & Ziegler, 1986; Rinallo et al., 1986; Schmitt et al., 1987; Mengel et al., 1989) have all observed similar wax degradation to that found for cabbage. Symptoms like these are also now being reported for needles of conifers at high elevation sites in the USA (Bruck, pers. comm.) and in Germany (Sauter & Voss, 1986). These authors report that on declining trees the waxes in the epistomal chamber degrade to the point that a fairly solid wax cover virtually occludes the stomata. Sauter and Voss found a good relationship between the number of open stomata and the shedding of needles, such that the shedding of needles begins when the number of viable stomata becomes lower than about 15%. Based on these findings, Sauter and Voss proposed a new hypothesis for the present decline of spruce and fir throughout Central Europe, in which the observed structural occlusion of stomata caused by acidic rain and/or fog degrading the crystalline wax, could be responsible for the premature shedding of needles. They suggest also that the symptoms of nutrient deficiency (e.g. Mg, Ca and K), typical of damaged trees, could result from reduced transpiration associated with the damage to epicuticular waxes by acidic deposition.

Structural degradation of waxes may have many consequences. Epicuticular waxes are important in the control of gaseous exchange and their modification or loss has been reported to increase transpiration (Grncaric & Radler, 1967; Denna, 1970) and influence gaseous exchange (Jeffere et al., 1971). Effects on the regulation of leaf temperature and frost tolerance may result (Martin & Juniper, 1970). Since the crystalline structure of the wax determines leaf wettability, wax degradation of the sort observed on the acid-treated leaves would undoubtedly reduce the hydrophobicity. There is indirect evidence to show that the sensitivity of leaves to acid rain and the extent of injury are related to the wettability of the leaf surface and leaf water-holding capacity (Keever & Jacobson, 1983; Haines et al., 1985; Caporn & Hutchinson, 1986). Leaf surfaces that are wetted more easily are also more susceptible to leaching (Tukey, 1971) and are more permeable to pesticides and other chemicals used on plants (Hunt & Baker, 1982; Whitehouse et al., 1982). Percy & Baker (1988) reported changes in retention of fluorescein, and in foliar penetration of some inorganic ions. They were able to relate this to reductions in droplet contact angles by acidic rain exposures. Another consequence of decreased hydrophobicity, which is more subtle and difficult to measure, is that fungal pathogens may establish more readily in the humid environment on the leaf surface. Davies (1961) reported different patterns of deposition of water-borne spores on plant surfaces, depending on the ease of wetting of the surfaces. Air
pollutants might be absorbed more easily by leaves if the waxes are damaged (Swiecki, Endress & Taylor, 1982). These numerous physiological consequences and altered leaf surface interactions, as a result of morphological alteration of epicuticular waxes, require investigation.

Although we observed similar degradation of waxes in leaves exposed to H_2SO_4 as in those exposed to HNO_3, suggesting that the damage was caused by H^+ reacting with substances, such as alkanes, secondary alcohols and ketones which are the major components of cabbage leaf waxes (Baker, 1982; Sutter, 1984), other pollutants including sulphur dioxide, nitrogen oxides and ozone appear to produce quite similar symptoms in a range of plant species. Thus two important questions remain: (1) which factor(s) in the acidic rain is primarily involved in the structural breakdown of wax crystalloids and (2) which components of the wax structure are susceptible and react with the pollutants.

In addition to altering epicuticular wax structure, a second consistent effect of the acidic rain treatments was the appearance of crystals on the adaxial surface, in the vicinity of lesions. The crystal morphology and composition were similar when induced by sulphuric acid or nitric acid rain or a mixture of the two acids; S and Ca were the two peaks identified in every crystal using X-ray microanalysis, suggesting the crystals could be gypsum (CaSO_4·2H_2O). The limitations of X-ray microanalysis do not allow definitive identification of the crystals as gypsum and complementary techniques such as X-ray diffraction are required for absolute identification. Other lines of evidence, besides the consistency of Ca and S peaks, suggest that the crystals are primarily gypsum are: (1) the morphological characteristics of the crystals (elongate blades, typically twinned and prismatic parallel to the c axis) fit with descriptions of the structure of gypsum crystals (Palache et al., 1951). Anhydrite CaSO_4 usually does not occur as euhedral crystals but typically are thick and tabular or rarely prismatic parallel to the c axis, (2) the refractive indices determined with an optical microscope using oil immersion and an index oil of 1.550 were consistent with gypsum and not in keeping with anhydrite CaSO_4 and (3) Mg, K and Na, which were sometimes present as small peaks in the crystal scans, are known to substitute for Ca in the structure of gypsum. Our results indicate that the source of these cations is the leaf, since removal of cations from the simulated rain solution did not have any effect on crystal formation. Similarly, we must conclude that S is also coming from the leaf in large enough quantities to precipitate with available Ca without the S contribution from sulphuric acid in the acidified rain, because the crystals produced by the nitric acid rain treatment had the same distinct composition of S and Ca. The presence of CaSO_4 crystals on pH 5.6 treated leaves, but not on leaves from the pH 5.6 treatment, suggests increased foliar leaching by acidic rain. Dry deposits on the surface of leaves may also be solubilized by acidic rain. However, since crystals did not occur other than in the vicinity of lesions, as determined by scans of whole leaves with a dissecting microscope, the Ca and S are probably both derived from significant tissue damage which increases membrane permeability to both cations and anions. This is supported by a previous study, with cabbage and other crop species, in which we showed that rain droplet pH was considerably increased when droplets were placed on the leaf surface above lesions in comparison with the smaller pH increase over uninjured leaf tissue due to H^+ exchange and solubilization of leaf surface deposits (Adams & Hutchinson, 1987). Other studies have shown that leaching of cations and anions can be significantly altered by acidic rain (Fairfax & Lepp, 1975; Evans, Curry & Lewin, 1981; Gaber & Hutchinson, 1988). However, our results indicate that in the area of lesions this can be much more substantial.

Gypsum crystals have been reported on beech leaves and needles of Norway spruce examined by SEM in studies related to forest decline (Coe & Lindberg, unpub.; Bosch et al., 1983; Hafner, 1986). However, there was no means of relating their presence to acidic deposition and increased leaching in these field studies. This study has demonstrated first, that acid rain applied under controlled conditions causes structural modifications of epicuticular waxes on cabbage leaves, and second, that CaSO_4 crystals are present in the area of lesion, suggesting significant alterations in membrane permeability to ions. In view of these results and other recent evidence that epicuticular waxes are damaged by acidic rain, it is important that the physiological consequences of such damage be assessed in future research.

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References


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