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Unusual patterns in the distribution of calcium oxalate in spruce needles and their possible relationships to the impact of pollutants

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SUMMARY

The patterns of distribution of calcium oxalate crystals in spruce needles have been investigated in healthy needles and those subjected to either acid precipitation (ph 2·5) or various ozone fumigations. In the acid treatment, deposits of Ca oxalate were lacking in the outer walls of the epidermal cells. Under the impact of ozone, unusual complexes of Ca oxalate crystals embedded in a matrix of callose and cellulose projected from the walls inwards into the cells, filling the lumina of epidermal and hypodermal cells completely. In mesophyll cells, the complexes projected more or less from the walls into the vacuoles. In other cases, crystals appeared within the vacuoles of mesophyll cells. In dead cells, precipitates of Ca oxalate or phosphate apparently indicate destruction of the cytoplasm after a major influx of Ca^{2+}. It is speculated, that the physiological significance of enhanced calcium leaching has been overestimated. With regard to ozone impact, it seems that the major problem of thereby enhanced membrane permeabilities may not be an increased efflux of mineral elements, but rather an increased influx of Ca into the symplast and subsequent disturbances in metabolism.

Key words: Acid precipitation, ozone, spruce needles, calcium metabolism, compartmentation.

INTRODUCTION

In current research on 'New-type Forest Decline' there has been increasing evidence accumulated in the past years that many of the decline symptoms are associated with disturbances in mineral nutrition, especially with regard to Mg, Ca, K, and Zn. Many of these nutrient deficiencies can be explained by changes in the soil caused by enhanced input of acidity and nitrogen from atmospheric pollution (Roberts, Skeffington & Blank, 1989). Consequently, it was shown that addition of the lacking mineral elements as fertilizers can restore the health of the trees, though they are still growing in the same polluted air (Hüttl & Fink, 1988).

On the other hand, there is only limited evidence that the direct impact of gaseous air pollutants, especially ozone, is a major cause of 'New-type Forest Decline' (Roberts *et al.*, 1989). Direct injury by ozone, observed, for example as visible injury to pine trees in southern California, has been produced in Norway spruce in controlled fumigation experiments but similar symptoms have not yet been found in this species in the field (Fink, 1988). However, there are still a number of indications that gaseous pollutants and the direct impact of acidic deposition upon the foliage are linked to the development of decline symptoms. Consequently, the possible interactions between gaseous pollutants, acidic deposition upon needles/leaves, and mineral nutrition have been of major interest in the past years. The models concerning such interactions have mainly dealt with the phenomenon of enhanced leaching of mineral nutrients from the foliage by the impact of acidic precipitation, possibly further enhanced by an increase in internal membrane permeability of cells which could facilitate the outward diffusion of ions (Scherbatskoy & Klein, 1983; Hutchinson & Adams 1987; Mengel, Lutz & Breininger, 1987; Klumpp & Guderian, 1990).

From field studies it has long been known that cation exchange processes at the forest canopy surface can neutralize a major part of the acidity in acid depositions, and that substantial amounts of mineral elements can be translocated within an ecosystem by these processes. The amounts depend on the forest type, the predominant leaf structure, and the acidity of the precipitation (Bosch, *et al.*, 1983; Ulrich, 1983; Lowett *et al.*., 1985; Joslin, McDuffie & Brewer, 1988; Waldman & Hoffmann,

1988; Yoshida & Ichikuni, 1989). In general, Ca is by far the most important cation in this process, though some of it may also originate from external depositions.

Little information, however, is available about the exact micromorphological location of such exchange processes at the needle/leaf surfaces, and about changes occurring in the internal compartmentalization of mineral elements under the impact of pollutants. With regard to Ca, first approaches have been made recently to show the general microscopical pattern of Ca distribution in healthy conifer needles (Fink, 1991 *a*, *b*). In the present paper some deviations of Ca distribution from this normal pattern will be presented and their possible connection to the impact of pollutants discussed.

MATERIALS AND METHODS

Needles of Norway spruce (*Picea abies*) were sampled from a number of controlled experiments. (1) Seedlings sprayed for one vegetation period with artificial acid rain either of pH 5·5 or 2·5 (courtesy Dr N. Cape, ITE Edinburgh). (2) Seedlings subjected for 2 months to ozone concentrations of 100, 300, or 600 $\mu g\ m^{-3}$ (courtesy Dr Prinz, Essen, FRG); (3) Seedlings subjected for 4 months to ozone concentrations of 200 $\mu g\ m^{-3}$ (courtesy Dr Landolt, Birmensdorf, Switzerland). (4) Seedlings subjected for 1 yr to fluctuating ozone concentrations from 100 to 300 $\mu g\ m^{-3}$ (courtesy Dr Scholz, Reinbek, FRG). Furthermore, needles were collected from various sites of declining Norway spruce in Germany, on acidic and calcareous soils.

For the microscopical investigations, about 2–3 mm long segments of needles were immediately fixed in cold formaldehyde/glutaraldehyde in 0·1 M phosphate buffer, pH 7·2, according to Karnovsky (1965). After 12 h, the samples were postfixed for 4 h in cold 2% osmium tetroxide in the same buffer. The samples were then dehydrated in an ascending series of acetone and embedded in epoxy resin according to Spurr (1969). Semithin sections were stained with 1% toluidine blue in 1% sodium tetraborate and evaluated with a Zeiss Axiomat light microscope. For the histochemical localization of calcium the reaction with glyoxal-bis-(2-hydroxyanil) was used (Pearse, 1985); the sections were then counterstained with malachite green. Polarizing light microscopy was also applied. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM 9 transmission electron microscope.

RESULTS

The distribution of Ca-oxalate crystals in normal, healthy needles has been described by Fink (1991 *a*) and should be considered for comparison in the following. The changes in the pathological material will be described here according to the individual tissues.

Epidermis

In undisturbed spruce needles grown with a good supply of Ca, numerous minute crystals of Ca oxalate normally tend to accumulate within the epidermal walls. This accumulation usually starts in the outer epidermal walls and cuticular layers and then proceeds to the inner epidermal walls, so that frequently the epidermal cells are totally encircled by rings of Ca oxalate in their cell walls (Figs 1, 2, 13). When spruce seedlings were sprayed experimentally for one vegetation period with artificial rain of either pH 5·5 or 2·5, this pattern of Ca distribution was essentially unchanged by the higher pH treatment. With the low pH, however, Ca oxalate was confined to the inner epidermal walls, whereas the outer epidermal walls stayed almost free of crystals (Figs 3, 14). The deposits in the inner walls tended to be heavier than in the controls. This absence of crystals from the outer epidermal walls was restricted to this one experiment, however, and was not found in any material collected from damaged trees in the field, where the calcium was always concentrated within the outer epidermal walls.

Another unusual distribution of Ca oxalate in the epidermis was found in several needles from fumigation trials with ozone. Normally and, as described above, even under the impact of acidic deposition where only the relative patterns change, the crystals are restricted to the apoplast of the epidermal walls. Following ozone fumigation, by contrast, massive deposits of Ca oxalate were found *within* several epidermal cells (Figs 4, 15, 16). In these cases, massive deposits of Ca oxalate were growing into the vacuole, thus nearly filling the cells completely, whereas the surrounding cell walls were completely free of any crystals. This phenomenon occurred in a number of normal epidermal cells, but in some cases from the ozone fumigations also in subsidiary cells of the stomata (Fig. 5) and even in guard cells of stomata of some older needles, where crystals filled the vacuole (Fig. 6). Other guard cells from the fumigation experiments showed an unusual accumulation of tiny crystals in their walls directly at the edges of the stomatal pores (Fig. 30), though this area is normally nearly free of Ca oxalate (Fig. 29).

Hypodermis

Very similar patterns of intracellular appearance of Ca oxalate as in the epidermal cells could also be observed in some sclerenchyma cells of the hypodermis in fumigated needles. Whereas normally the crystals are restricted to the area between the thin primary and the thick secondary cell wall (Fig 17), in

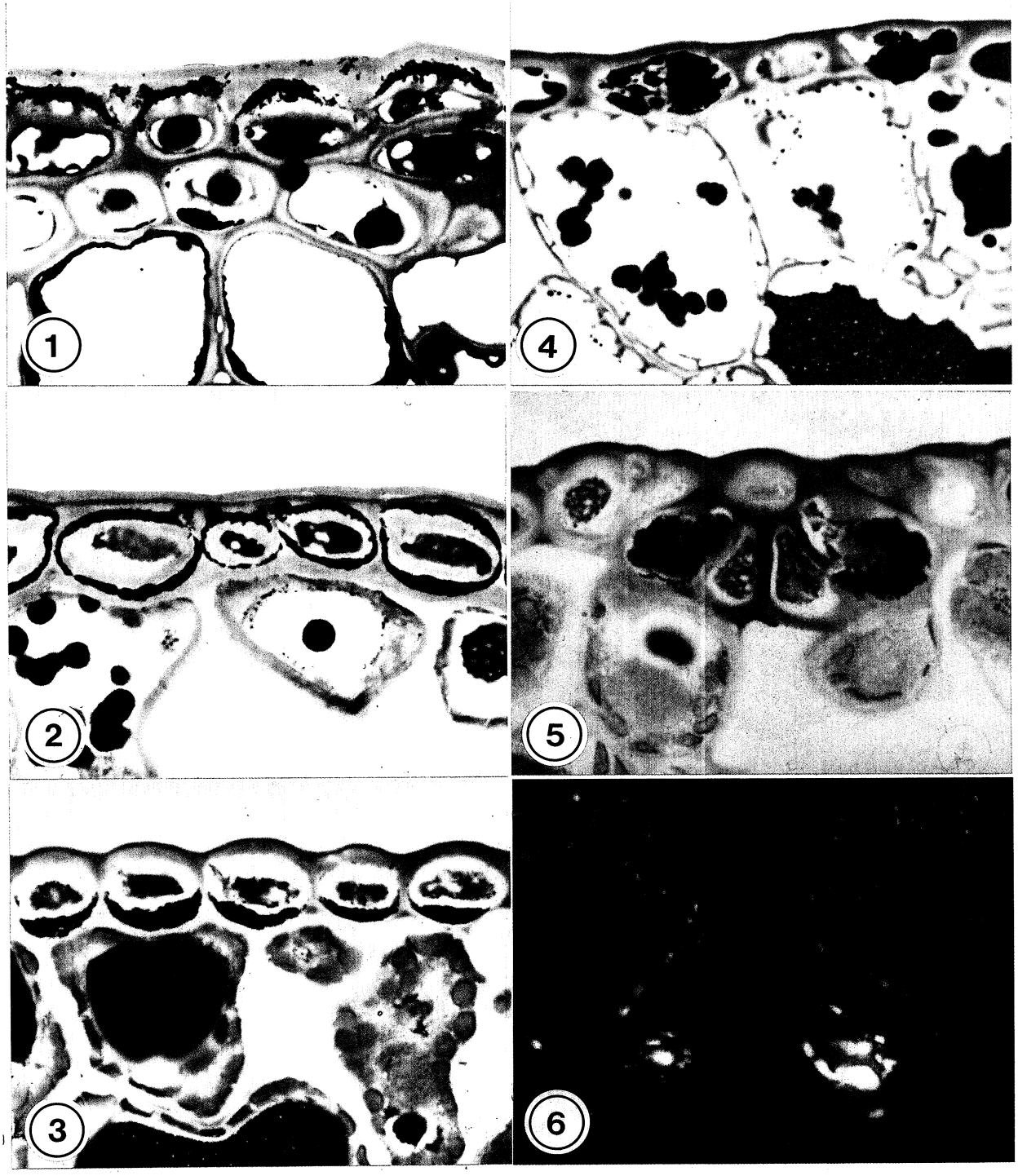


Figure 1. Calcium oxalate crystals (red) accumulating in the outer epidermal walls of a 1-yr-old healthy spruce needle from the field. ×1080. **Figure 2**. Calcium oxalate crystals (red) completely surrounding epidermal cells in 2-yr-old spruce needle grown with high Ca supply. ×980. **Figure 3**. Calcium oxalate crystals (red) restricted to the inner epidermal walls in 1-yr-old spruce needle after treatment with acid rain (pH 2·5) for one vegetation period. ×1100. **Figure 4**. One-yr-old spruce needle fumigated for 2 months with 600 $\mu g\ m^{-3}\ O_3$; Ca oxalate crystals accumulating within epidermal cells. ×980. **Figure 5**. The same sample, crystals accumulating within subsidiary cells of a stoma. ×1100. **Figure 6**. Calcium oxalate crystals within the vacuoles of guard cells in 4-yr-old spruce needle from the field. Polarized light. ×1200.

these cases the walls stayed free of crystals which, instead, filled the lumina of these cells (Fig. 18).

Mesophyll

Similar phenomena could also be observed in the mesophyll cells of needles from these ozone fumigations as well as in some needles from damaged trees in the field. Whereas normally Ca oxalate is precipitated outside the mesophyll cells along the walls in the intercellular spaces (Fig. 7), in these cases heavy deposits of Ca-containing crystals could

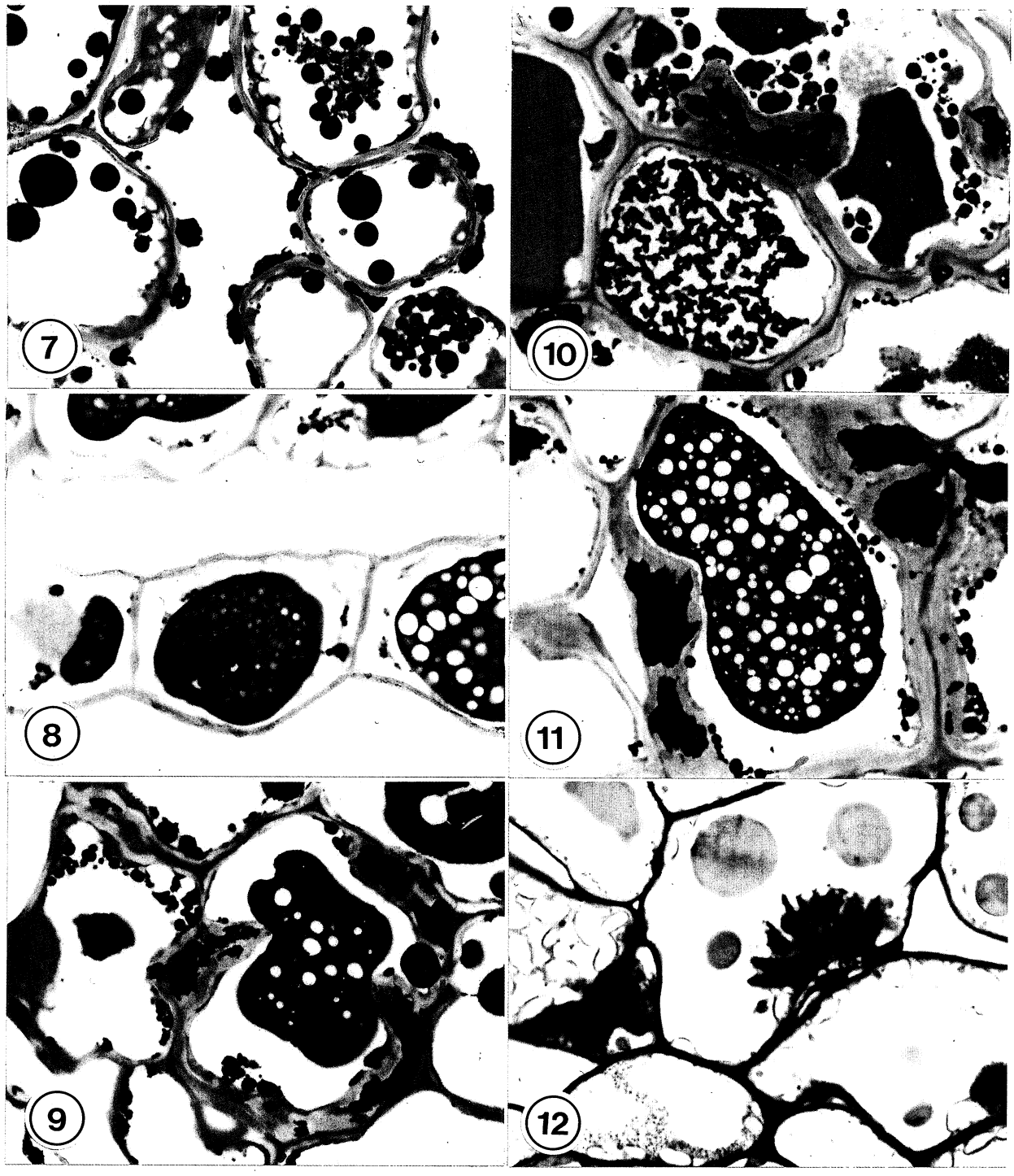


Figure 7. Calcium oxalate crystal accumulated within the intercellular spaces outside mesophyll cells in 2-yr-old healthy spruce needle. ×870. **Figure 8**. Heavy Ca deposit within the tannin-rich vacuole of a mesophyll cell in 3-yr-old spruce needle from a decline area in the Black Forest. ×920. **Figure 9**. Aggregates of Ca oxalate crystals growing from the walls inside mesophyll cells in 1-yr-old spruce needle fumigated for 2 months with 600 $\mu g\ m^{-3}\ O_3$. ×940. **Figures 10, 11**. Aggregates of crystals growing into the lumina of 2-yr-old spruce needles from a damaged stand on calcareous soil (Schwäbische Alb). ×945. **Figure 12**. Wall outgrowths containing Ca oxalate crystals from same material as Figures 10 and 11; staining with toluidine blue to show callose-and cellulose-containing wall outgrowths. ×920.

be observed within the mesophyll cells. Three different types of deposits could be distinguished:

(1) Small outgrowths from the inner wall into the cell lumina are initiated by deposition of callose along some points of the wall (Fig. 19). These outgrowths subsequently enlarge and, besides callose, also cellulose is deposited; within these outgrowths masses of small crystals of Ca oxalate

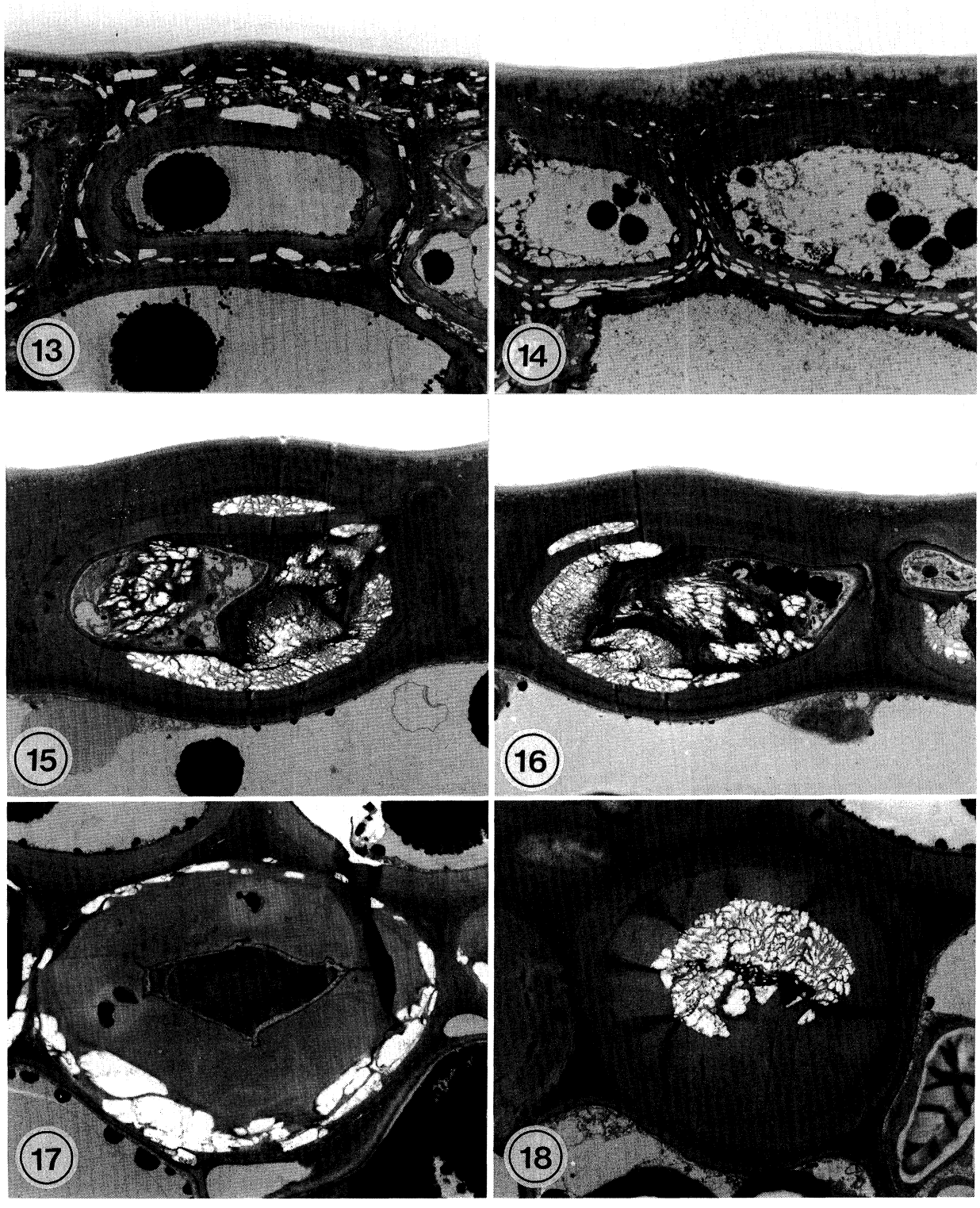


Figure 13. Electron micrograph of the normal distribution of minute crystals of Ca oxalate around the epidermal cell of a 1-yr-old spruce needle well supplied with Ca (control). ×3300. **Figure 14**. The corresponding pattern in the needle from the acid-rain experiment (pH 2·5); only in the inner epidermal walls deposits of crystals occur, whereas the outer part remains nearly free. ×3100. **Figures 15, 16**. Massive aggregates of Ca oxalate crystals embedded in wall material projecting into the cell lumen of epidermal cells of 1-yr-old spruce needles after fumigation for 2 months with 600 μg m^{-3} ozone. ×3100. **Figure 17**. Regular appearance of Ca oxalate crystals in the thick walls of a sclerenchymatic cell of the hypodermis in a healthy control needle. ×3400. **Figure 18**. The same as in Figure 17, but in an ozone-fumigated needle, exhibiting massive aggregations of crystals in the small cell lumen. ×3300.

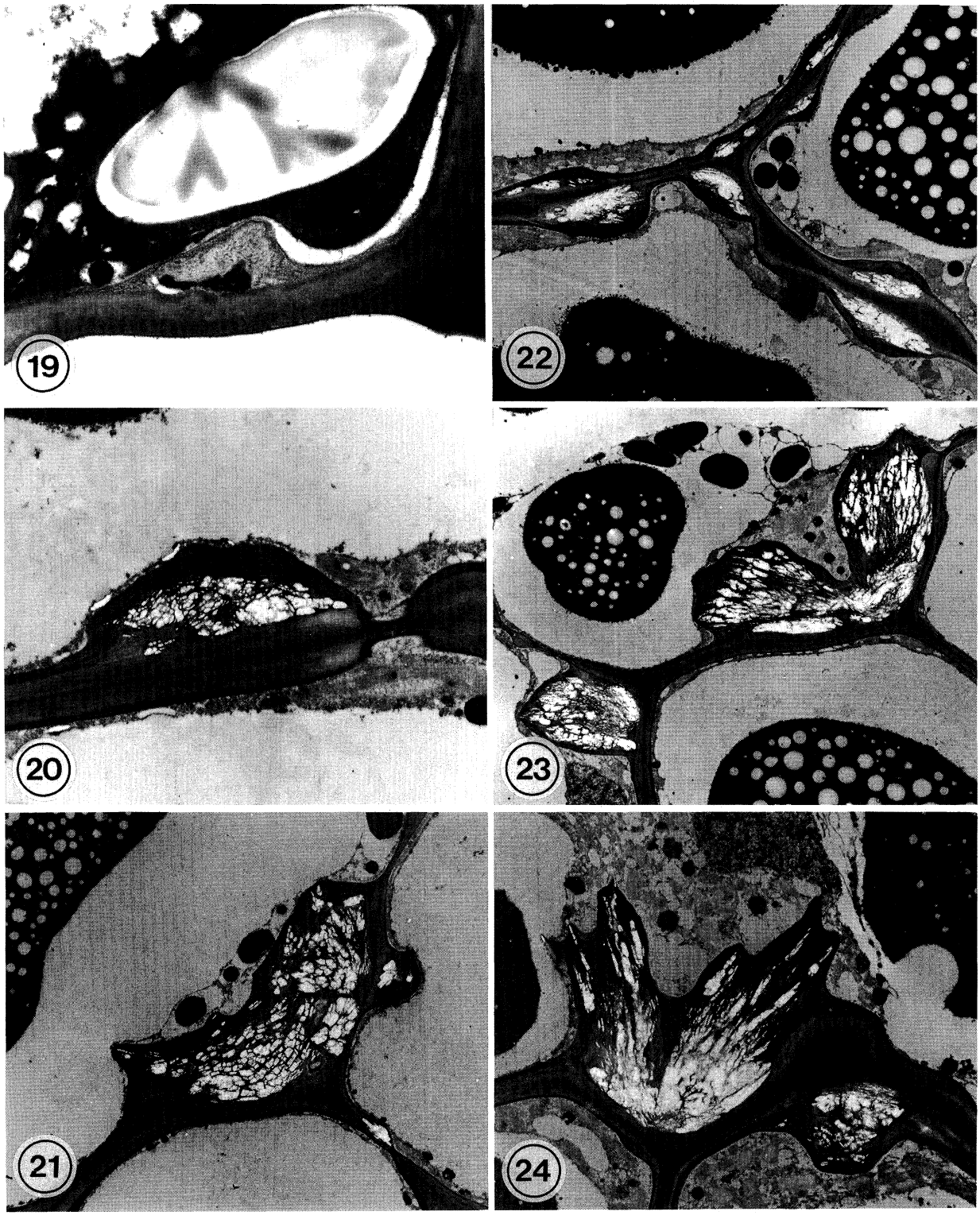
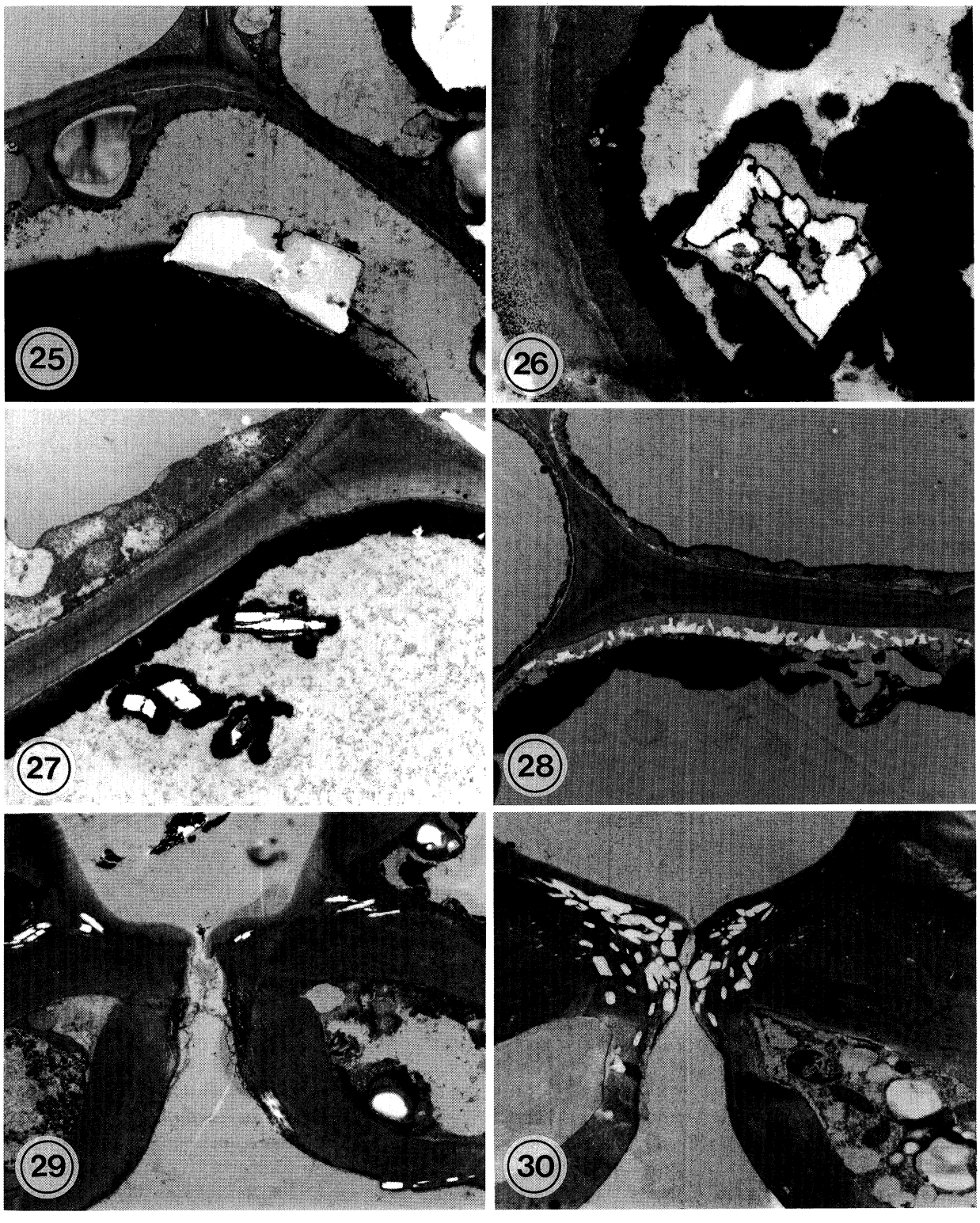


Figure 19. Local accumulation of callose along the inner wall of the mesophyll cell of a 2-yr-old spruce needle exposed for 2 months to 600 μg m^{-3} ozone. × 5200. **Figures 20, 21**. Later stages of wall outgrowths with internal deposits of Ca oxalate crystals in the same material. × 5800 (Fig. 20); × 4100 (Fig. 21). **Figures 22–24**. Massive internal crystal aggregates in 2-yr-old spruce needles from a damaged stand on calcareous soil (Schwäbische Alb). × 4200 (Fig. 22); × 4500 (Fig. 23); × 4300 (Fig. 24).

precipitate (Figs 9, 20, 21). Along the cell walls, these crystalliferous outgrowths can either line up as a series of relatively flat wall thickenings (Fig. 22), or as massive complexes protruding into the cytoplasm and the vacuole (Figs 10–12, 23, 24). In no case could any infection by fungal hyphae, which could theoretically have provoked such wall alterations, be detected microscopically.



Figures 25–27. Vacuolar inclusions of Ca oxalate, surrounded by heavy tannin deposits, in 1-yr-old ozone-fumigated needles. ×5300 (Fig. 25); ×6300 (Fig. 26); ×4900 (Fig. 27). **Figure 28**. Precipitation of calcium oxalate or phosphate within the cytoplasm of a dead mesophyll cell in 2-yr-old spruce needle after ozone fumigation. ×5300. **Figure 29**. Occurrence of guard cells in a 2-yr-old control needle; hardly any crystals in the walls. ×4500. **Figure 30**. Calcium oxalate crystals accumulating around the stomatal pore in the walls of guard cells from a 2-yr-old needle fumigated for 4 months with 200 $\mu g\ m^{-3}$ ozone. ×6300.

(2) Calcium oxalate suddenly appears within the vacuoles in the form of single large crystals or as numerous smaller crystals (Figs 8, 25–27). Frequently, these crystals are surrounded by heavy agglomerations of dark-staining tannins. In some cases, such crystals appear to be eroding (Fig. 27), pointing to the fact that they may become dissolved again with time.

(3) In some apparently dead mesophyll cells from ozone-fumigated needles an irregular white line was observed between the cell wall and the tonoplast (Fig. 28). At first glance it seemed to be an artifact caused by tearing of the section or incomplete infiltration by the embedding resin, but complementary investigations with some histochemical tests at the level of the light microscope proved their nature as crystalline Ca compounds. It remained open, however, whether they consisted of Ca oxalate or Ca phosphate.

DISCUSSION

With regard to the altered patterns in crystal distribution under the experimental impact of acid precipitation there are several possible explanations. (*a*) Ca is leached from the outer epidermal walls by the acid rain before it is precipitated as oxalate. (*b*) Oxalic acid is leached from the walls so that no precipitation can take place. (*c*) The pattern of secretion of oxalic acid is altered, so that it is primarily secreted towards the inner epidermal walls and here already scavenges any Ca^{2+} moving towards the cuticle.

At the moment, no conclusive interpretation can be given, but this seems to be the first direct visualization of the leaching effect. This would mean that the leaching process itself is restricted mainly to the outer epidermal cell walls, whereas the inner walls seem to be unaffected and even show an enhanced deposition of Ca oxalate. It would also mean that the fraction of Ca which had been leached additionally due to the acid would otherwise have become bound as oxalate in the outer cell wall, i.e. it would have become physiologically inactivated anyhow. In the light of this assumption the data on mineral leaching probably need to be reevaluated, since the physiological effects of leaching losses would depend upon the chemical fraction of the leached element.

It has been shown before that leaching is primarily an exchange process from the apoplast of the epidermis (Mecklenburg, Tukey & Morgan, 1966; Tukey, 1970; Adams & Hutchinson, 1984; Hutchinson & Adams, 1987; Foster, 1990). It is a normal process to get rid of some of the excess minerals that are transported with the transpiration stream to the epidermis but cannot move further when the water evaporates through the stomata. It is interesting that the first publications on leaching interpreted this process more as an excretion of excess minerals rather than an unwanted loss of physiologically essential nutrients (Arens, 1934). This is especially true for calcium which would be toxic for the cells if it were to accumulate in the symplast. Thus the precipitation as Ca oxalate is only an alternative to leaching, since in the first case it is inactivated *in situ* whereas in the latter it is effectively lost; in both cases it physiologically 'disappears'.

Crystals of Ca sulphate have been repeatedly observed on the surface of conifer needles under the impact of acidic deposition, both in the field and in controlled experiments (Bosch *et al.*, 1983; Nebe, Schierhorn & Ilgen, 1988; Huttunen, Turunen & Reinikainen, 1990). There is evidence that this represents a fraction of the calcium leached from the epidermis, which can recrystallize upon the surface under certain weather conditions when the calcium is not immediately washed away by precipitation. There is still some question of whether the sulphate comes from sulphuric acid in the rain or originates, at least partially, from within the leaf, since such crystals have also been observed under the impact of nitric acid depositions (Adams, Caporn & Hutchinson, 1990). It is uncertain whether the Ca oxalate crystals themselves in the outer epidermal walls may become dissolved again under the action of wet acid depositions on the cuticle, even if the acidity of the deposits is increased by evaporation of water. In order to dissolve Ca oxalate, pH values below 1 are needed. It is thus more probable that the Ca is leached before it is precipitated as oxalate. This would be supported by the experiments of Mecklenburg *et al.* (1966) with ^{45}Ca in bean plants, where leaching was primarily restricted to the mobile fraction which had recently been transported into the leaf, whereas the major portion of the previously absorbed Ca was already incorporated in relatively unleachable forms in the leaf. It is interesting, however, that Huttunen *et al.* (1990) observed more intensive gypsum crystal formation on spruce needles (with the Ca concentrated in the epidermal cell walls) than on pine needles (with the Ca concentrated within the vacuoles of the epidermal cells, thus being more protected against leaching; cf. Fink, 1991*b*).

If little Ca exists in the outer epidermal wall, however, this may enhance the permeability of the cuticle for other substances as well, since Ca^{2+} can form bridges between molecules and thus partially 'close' the ionic channels in the cuticle for other ions (Berg *et al.*, 1990). Consequently, Tukey & Morgan (1963) observed that in leaves of squash, with Ca and P deficiency, much more nutrient leaching occurred than in well-supplied plants. This effect of higher leaching rates in Ca deficient plants was also partially confirmed for spruce needles (Klumpp & Guderian, 1990). Among the elements, potassium is frequently less influenced by the acidity of deposition, since this cation concentrates more in the symplast and is thus more under control of the plant and protected against leaching, while Ca and Mg occur more in the apoplast (Thomas & Runge, 1988).

In contrast to these effects of acid precipitation, ozone seems to alter the Ca metabolism in quite a different way. Since conifers in general seem to keep the Ca in the apoplast outside the cells (in contrast to

angiosperms which precipitate Ca within their cells), the described observations of the intracellular occurrence of Ca precipitates in epidermal, hypodermal, and mesophyll cells would point to an uncontrolled penetration of Ca into the cells, followed by some sort of defence reaction in order to encapsulate the Ca deposits. Since such reactions were also found in needles from damaged trees in the field, it is not yet possible to say if this is really a sign for ozone impact, or if other factors could also lead to such phenomena.

However, at the present time a hypothesis may be put forward at least as a first approach to these changes. It is known that ozone and other air pollutants enhance the permeability of membranes (cf. Jäger & Klein, 1980; Keitel & Arndt, 1983; Castillo & Heath, 1990). This could lead to an enhanced influx of Ca (which is normally kept within the apoplast) into the cytoplasm; as a consequence, callose deposition starts. It is known meanwhile from phytopathological studies, that callose formation is triggered by such an influx of Ca, normally induced by enhanced membrane permeability as a result of fungal attack (Kauss, 1987, 1990). Experimentally, enforced influx of Ca^{2+} into the cytoplasm always activates 1,3-β-glucan synthase and callose synthesis starts. In the present case, a continuing imbalance between Ca^{2+} influx and the proceeding synthesis of callose and other cell wall substances could finally lead to the development of the strange crystalliferous wall outgrowths described in the present paper. In this respect it is interesting to notice that Ca oxalate crystals have been found in various other cases to be enclosed by callose sheaths (Eschrich, 1956; Thaler & Weber, 1957).

There would, however, also exist the possibility of an unimpeded influx of Ca through the plasma membrane and the tonoplast into the vacuole, where Ca oxalate finally crystallizes. Whether this interferes with the normal metabolism of the mesophyll cells, cannot be decided at the moment. The unusual agglomeration of heavy tannin deposits around such crystals would perhaps point to such a disturbance. On the other hand, however, such crystals can apparently be redissolved, which would point to a marked regulation mechanism. Dissolution of intracellular Ca oxalate crystals has been described in angiosperms before (e.g. during seed germination), though the underlying biochemical mechanisms cannot yet be explained (Kohl, 1889; Alexandrow & Timofeev, 1926; Franceschi & Horner, 1980).

Finally, the appearance of calcium deposits in dead mesophyll cells (Fig. 28) would lead to the assumption that in the worst case an enhanced influx of Ca into the cytoplasm could lead to the precipitation of cytoplasmic phosphate or oxalate and consequently to the death of the cells. Uncontrolled influx of calcium into cells is also a general phenomenon of senescence, triggering the catabolic process by binding to calmodulin and activating phospholipase and lipoxygenase, which then cause a more rapid recycling of membranes and a further enhancement of their permeability (Ferguson, 1984; Leshem, 1987). In summary, high apoplastic and low symplastic Ca^{2+} delay senescence, whereas lower apoplastic and higher symplastic Ca^{2+} promote senescence.

Increased influx of Ca into the subsidiary and guard cells of the stomata, for which some evidence is presented here, could cause specific disturbances to stomatal functions. Apoplastic Ca^{2+} which enters the cytosol of guard cells normally reduces stomatal aperture, and scavenging of calcium by oxalic acid in the epidermis may be a mechanism to keep the Ca^{2+} concentration low close to stomata (Atkinson, Mansfield & Davies, 1990). Changes in this complex mechanism by affecting apoplastic Ca through acid rain, or enhancing symplastic Ca through ozone, may seriously upset the system.

Consequently, if in conifer needles Ca is normally restricted to the apoplast and prevented from entering the symplast, enhanced membrane permeabilities could lead to an increased influx of Ca into the symplast, disturbing metabolism. On the other hand, efflux of Mg into the apoplast could occur where it would, under certain conditions, also be precipitated by oxalic acid as Mg oxalate and thus become physiologically inactive (which would not show up in total nutrient analyses of the samples). Whether this really happens in conifer needles remains to be shown, but Rao & Tewari (1989) found on surface lesions of coffee leaves infected by *Mycena citricolor*, crystals of both Ca oxalate, and Mg oxalate. It is postulated, that leaf magnesium could diffuse out of the cells because of pathogen-induced permeability changes and was subsequently sequestered by fungal oxalic acid.

In any case, the presented observations inevitably provoke some question about our current ideas on the interaction between mineral nutrients and atmospheric pollutants. Possibly the phenomenon of *leaching*, which has been predominant in this discussion, is less important than supposed; in contrast, questions of the disturbance of the *internal compartmentalization* of minerals within the cells could be of much more importance. Such shifts in the location and chemical binding form would not show up in any analysis of the total nutrient content as usually performed. Since the microscopical localizations of Ca oxalate shown here represent only a very drastic shift in mineral localization, more subtle techniques are needed to detect earlier changes. Also the impact of ozone in connection with mineral nutrition has been mainly seen as enhancing permeability of membranes with subsequent *efflux* of minerals which could then be more easily leached by acid rain (Klumpp & Guderian, 1990). The present observations, however, suggest that the main prob-

lem may be the increased *influx* of Ca into cells as promoted by ozone, finally causing accelerated senescence reactions.

Unfortunately, no biochemical investigations exist upon the biosynthesis of oxalic acid in conifers. In angiosperms, synthesis of oxalic acid is frequently linked to nitrate reduction in leaves. In conifers, however, the nitrate is already reduced in the roots and no reduction takes place in the needles. Furthermore, preliminary observations have shown no indications of differences in oxalate content from spruces growing on sites with much or with no nitrate in the soil solution. Another possible major metabolic pathway is the synthesis of oxalic acid from L-ascorbic acid as a precursor (Franceschi & Horner, 1980). The spruce needle is known for its generally high content of ascorbic acid which could be a source for further synthesis of oxalic acid (Esterbauer & Grill, 1980). The synthesis of the antioxidant ascorbic acid is known to increase under exposure to oxidative stress as a protective reaction (Elstner, 1982); high amounts have also been reported in the needles of damaged trees in the field (Osswald, Senger & Elstner, 1987). Thus, one could speculate that under oxidative stress higher amounts of ascorbic acid could finally be converted into higher amounts of oxalic acid. This could then lead to an impoverishment of free calcium if uncontrolled precipitation takes place.

In earlier studies, increased synthesis of Ca oxalate has been reported in spruce needles under the impact of SO_2 (Hartig, 1896), in fir needles under the impact of fluoride (Garrec *et al.*, 1978), and in *Gingko* leaves subjected to high levels of air pollution (Umemoto & Hozumi, 1972). This has been interpreted as an effect of enhanced respiratory activity under these conditions and interference with normal enzymatic processes, which finally lead to premature aging. In needles of declining spruces and firs with low Mg and Ca contents in the Black Forest, however, lower amount of Ca oxalate have been reported as a consequence of the low amounts of available calcium (Parameswaran, Fink & Liese, 1985).

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