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Needle wax surface structure of *Pinus sylvestris* as affected by ammonia

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SUMMARY

Wax surfaces of current, first and second year needles of *Pinus sylvestris L.* from Open Top Chambers, both fumigated with ammonia and unfumigated, were examined with the scanning electron microscope. In the course of time the wax rodlets on peristomatal rims of treated and untreated plants became fused and agglomerated to various extents into morphologically different types of crusts. Compact peristomatal crusts were most frequent. Porous, particulate and 'melted' peristomatal crusts as well as epistomatal crusts were of rare occurrence and presumably represented a random phenomenon. No correlation was found between appearance of wax crusts and ammonia fumigation. Ammonia treatment did not affect fungal growth either. Quantitative estimates indicated a very weak, statistically insignificant, tendency of ammonia to increase the rate of the normal, age dependent wax degradation. Observations on young needles developing after prolonged fumigation are suggestive of an indirect effect of ammonia on the rate of crystalline wax degradation.

Key-words: air pollution, ammonia, epicuticular wax, *Pinus sylvestris*, stomata.

INTRODUCTION

Forests in The Netherlands, composed for a large part of *Pinus sylvestris* plantations and growing on acid and poor sandy soils have been declining during the past few decades. One of the factors likely to accelerate the natural acidification of the soils is air pollution (Krause *et al.* 1984; Nihlgard 1985). In this respect, ammonia is probably a major pollutant, locally deposited in high concentrations (Buijsman *et al.* 1984; Erisman *et al.* 1987) as a consequence of intensive livestock farming. Excess of nitrogen is thought to be one of the most serious causes of forest decline, not only in The Netherlands but in major parts of Europe (Nihlgard 1985).

Recent studies carried out within the Dutch Priority Programme on Acidification have confirmed that forest decline is correlated with an increase in ammonia concentration. Several negative effects of ammonia have been observed: bud injuries, a nutrient imbalance, decreases in mycorrhiza infection and increases in sensitivity to frost and drought stresses or to fungal pathogens (Van der Eerden *et al.* 1990).

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This research has also revealed the negative effect of ammonia on *Pinus sylvestris* needle surfaces resulting in degradation of the wax layer. In a later study, it was suggested that artificial rain treatment with $(NH_4)_2SO_4$ (2500 µl^{-1}) may affect the needle surface of *Pseudotsuga menziesii* by increasing wax fusion. In an experiment in growth chambers, the effect of NH_3 (180 µg m^{-3}) combined with clean artificial rain was rather similar to the effect of $(NH_4)_2SO_4$, although the fusion was more irregular (Van der Eerden *et al.* 1991). On the other hand, Thijsse & Baas (1990) found an ambiguous effect of NH_3 on needle wax in Douglas fir, when fumigated in Open Top Chambers.

In the present study, variations in epicuticular needle wax morphology in treelets of *Pinus sylvestris* as related to needle age and fumigation with ammonia are described and quantified.

MATERIALS AND METHODS

Sampling data are summarized in Table 1. Most of the material studied came from a long-term fumigation experiment using 5-year old trees of *Pinus sylvestris* L. in Open Top Chambers (OTC) of the Institute for Plant Protection at Wageningen. For a description of the OTC's see Thijsse & Baas (1990). Controls were fumigated with ambient air with an average level of *c.* 10 µg NH_3 m^{-3}. In two other OTC's trees were subjected to 60 µg NH_3 m^{-3} and 100 µg NH_3 m^{-3}, respectively from 6 August 1990 to June 1991. Needles of different age-classes were collected at regular intervals during the 1990 season, and in April and May 1991. Some very young current-year needles (still flushing and before completion of length growth) were sampled in May 1991 in order to study the early stages of crystalline wax appearance and its degradation. In addition, field samples from 7-year-old trees growing in monitoring sites in Vreedepeel, the Netherlands, subjected to a mean of 20 µg NH_3 m^{-3} ± daily or annual fluctuation from nearby intensive farming, and in Wageningen (*c.* 10 µg NH_3 m^{-3}) were studied for comparison. Ambient NH_3 levels may fluctuate considerably, and the averages of 20 and 10 µg m^{-3} are based on a limited number of measurements only. Needles from unpolluted areas (background less than 2 µg NH_3 m^{-3}) were not available for study.

Segments of *c.* 6 mm from the middle region of the needles, previously air dried for at least 24 h and stored in a refrigerator to prevent fungal contamination, were cut and fixed to stubs using double-sided adhesive tape, subsequently sputter-coated with gold in a Polaron E5100 apparatus, and finally examined in a Jeol JSM-35 scanning electron microscope at × 600, × 2000 and × 18 000 magnification.

In the first series of observations, stomata were assessed from SEM-micrographs (*c.* 500 photographs at × 2000 or × 600), following classification of various types of epicuticular/peristomatal wax morphology (see results). In subsequent observations, stomata were directly assessed from the SEM-screen.

Quantification of structural changes

To quantify wax changes, the relative amount of amorphous wax on peristomatal rims was used as a criterion for the level of crystalline wax degradation. Five stages were recognized in the extent to which peristomatal rims of stomata were covered with wax crusts (Fig.2):

stage 1: free of amorphous wax,
stage 2: 1–20% covered by amorphous wax,

Table 1. Summarized data on needles sampled and stomata assessed

OTC Treatment (code)		Sampling dates	Number of trees	Number of needles per tree: Current-year needles	First-year needles	Second-year needles	Number of stomata (abaxial surface)
Open Top Chamber samples							
6	Ambient	12-06-1990	5	5	5		810
	air	26-07-1990	5	5	5		810
	(*c*. 10 μg	11-09-1990	5	5	5		810
	NH_3 m^{-3})	15-10-1990	5	5	5		810
		23-04-1991	3		3	3	360
		07-05-1991	1	5			100
		28-05-1991	1	5			100
3	60 μg	11-09-1990	5	5	5		810
	NH_3 m^{-3}	15-10-1990	5	5	5		810
	from	28-11-1990	5	5	5		810
	August 1990	23-04-1991	3		3	3	360
	to	07-05-1991	1	5			100
	May 1991	28-05-1991	1	5			100
1	100 μg	28-11-1990	5	5	5		810
	NH_3 m^{-3}	23-04-1991	3		3	3	360
	from	07-05-1991	1	5			100
	August 1990	28-05-1991	1	5			100
	to May 1991						
Field samples							
Vreedepeel		30-10-1990	3	5	5		486
Wageningen		23-05-1991	3		3	3	360

stage 3: 21–50% covered by amorphous wax,
stage 4: 51–80% covered by amorphous wax,
stage 5: 81–100% covered by amorphous wax.

Twenty abaxial stomata from the middle part and from the middle row of each needle (four needles each from five trees) and one stoma of each needle (one needle from one tree) from a preliminary pilot study, sampled in the period June–November 1990, as well as twenty abaxial stomata of each needle sampled in April and May 1991, were attributed to one of the above mentioned stages.

Statistical analysis

Twenty stomata per needle of current-year age and from different treatments, collected in the period June–November 1990, were assigned to the stage number of peristomatal crust formation. The number of observations per needle in each amorphous wax stage was multiplied by its rank and then divided by 20 (stomata), resulting in a mean wax-stage index per needle. The mean index per tree was then calculated and treatment differences per sampling date were analysed with Student's *t*-test (Sokal & Rohlf 1981).

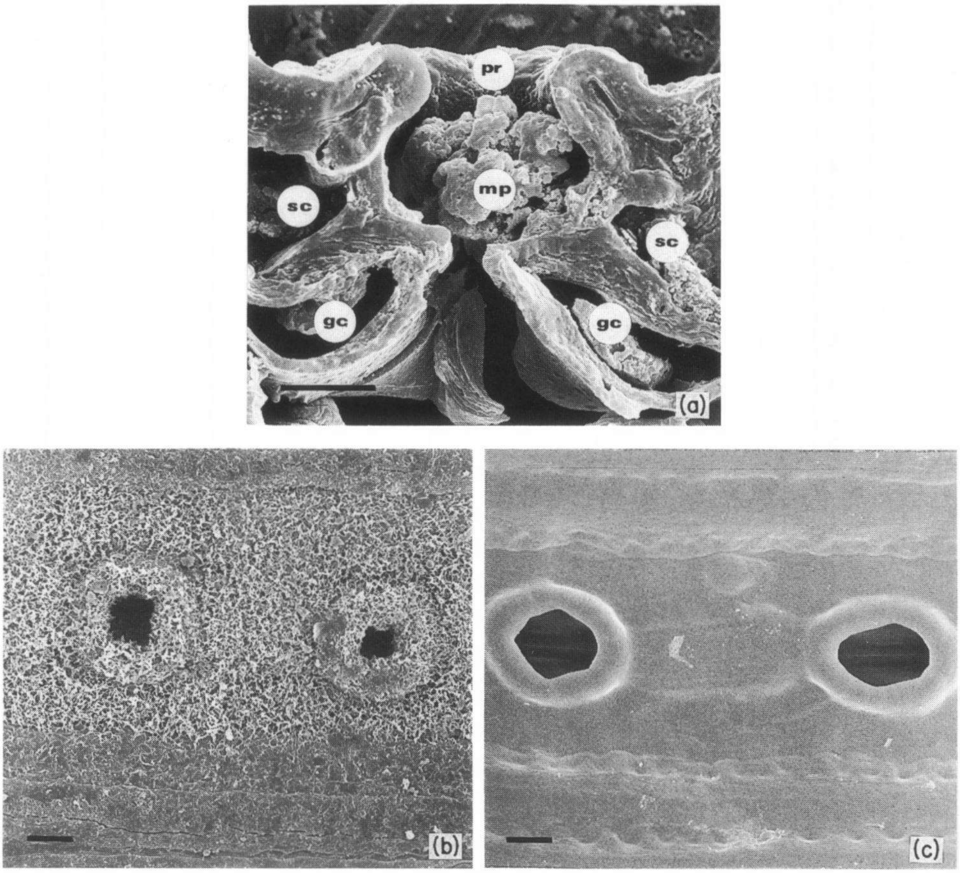

Fig. 1. (a) Stomatal complex of *Pinus sylvestris* needles in cross section showing peristomatal rim with incorporated subsidiary cell wall (pr), subsidiary cells (sc), sunken guard cells (gc) and stomatal antechamber filled with massive wax plug (mp). Scale bar = 10 μm. (b) Current-year needle showing two peristomatal rims covered with crystalline and amorphous wax between stomatal rows predominantly free of crystalloids. Scale bar = 10 μm. (c) Current-year needle, wax dissolved by chloroform, showing smooth cuticle. Scale bar = 10 μm.

In total, 582 needles, 326 unfumigated and 256 fumigated, were investigated, over 9800 stomata were assessed, over 700 SEM-photomicrographs were analysed, and many more SEM images observed and recorded.

RESULTS

General observations

Like in other coniferous species, guard cells of stomata in *Pinus sylvestris* are deeply sunken and overtopped by subsidiary cells forming a conspicuous peristomatal rim which is the only part of the stomatal complex always visible in surface view (Fig. 1a and b).

The needle surface, both adaxial and abaxial, is primarily covered with a thin continuous layer of amorphous wax, on top of which the crystalloids of so-called structural wax arise. The crystalloids appear as simple rodlets and are arranged either as individual crystals or in the form of tufts and are oriented at any angle between 0 and 90°

perpendicular to the needle surface. Although the rodlets are known to be tubular, their tips are nearly always occluded. Only in a few photomicrographs, the hollow nature was clearly visible, possibly representing broken, 'closed' tubules.

When the wax is dissolved by chloroform, the cuticle appears smooth with no striations (Fig. 1c).

With time, the structure of wax rodlets is altered to various extent, mostly resulting in an amorphous and almost flat wax crust (Fig. 2).

This process apparently has a different start and intensity depending on needle region and side. Thus, on the regions situated closer to the needle margin or on the adaxial side, the degradation is slower and wax tends to retain the rodlet form for a longer time than in the central region of the abaxial side.

On peristomatal rims, where gradual changes of rodlets started to appear first and where they were best visible, three principal stages of wax degradation could be observed:

(a) fusion and agglomeration of rodlets,
(b) advanced stage of agglomeration,
(c) crust formation.

Our screening showed that peristomatal wax crusts most often started to develop on one side of the rim and then spread gradually, in an arc, around the epistomatal pore, covering the whole rim (Fig. 2). Only a few stomata analysed showed two incipient parts of amorphous wax crust, each concentrated on one side of the peristomatal rims.

As judged from photomicrographs at high magnification ($\times$ 2000), peristomatal rim wax crusts on *P. sylvestris* needles were not identical: three different types, compact (Figs 2e and d), porous (Fig. 3a) and particulate (Figs 3b and c), could be distinguished.

Compact crusts occurred in all needles, from all sampling dates, including all unfumigated and fumigated samples as well as field samples, and presumably formed gradually. Porous crusts, which may be considered as incomplete crusts, although covering the whole rim, were present in needles collected from June to November 1990 only. For porous crusts there is no evidence that they arose gradually. Particulate crusts, probably a stage of crust degradation, consist either of coarsely flattened particles like those in Figure 3b, or of minor granulated ones like those in Figure 3c. Particulate crusts were detected in all needles except very young current-year needles collected in May 1991.

A number of compact crusts appeared to consist of aggregated outer-most parts of rodlets only, as the crystalloid structure underneath the crusts remained preserved (Fig. 3d).

Some peristomatal rim wax crusts, either entirely or locally, near the epistomatal pore, looked as if they were 'melted' or partly dissolved (Fig. 4a). A 'melted' appearance has also been noted in earlier stages where individual rodlets inside the epistomatal chambers and on peristomatal rims appeared to be affected (Fig. 4b).

Moreover, fused rodlets inside the stomatal antechambers forming a flat and solid wax plug or a crust above the pore, completely or partially occluding the stomata, were also found (Fig. 4c).

'Melted' wax as well as occluded stomata occurred in low frequency at all sampling dates except in the very young current-year needles collected in May 1991.

The emergence of rodlets could be detected on stomatal areas in very young current-year needles which flushed in the first half of May 1991 and were still surrounded by the needle fascicle. The most extensive crystalline cover occurred on and around the peristomatal rims or inside the stomatal antechambers. On non-stomatal areas the rodlets

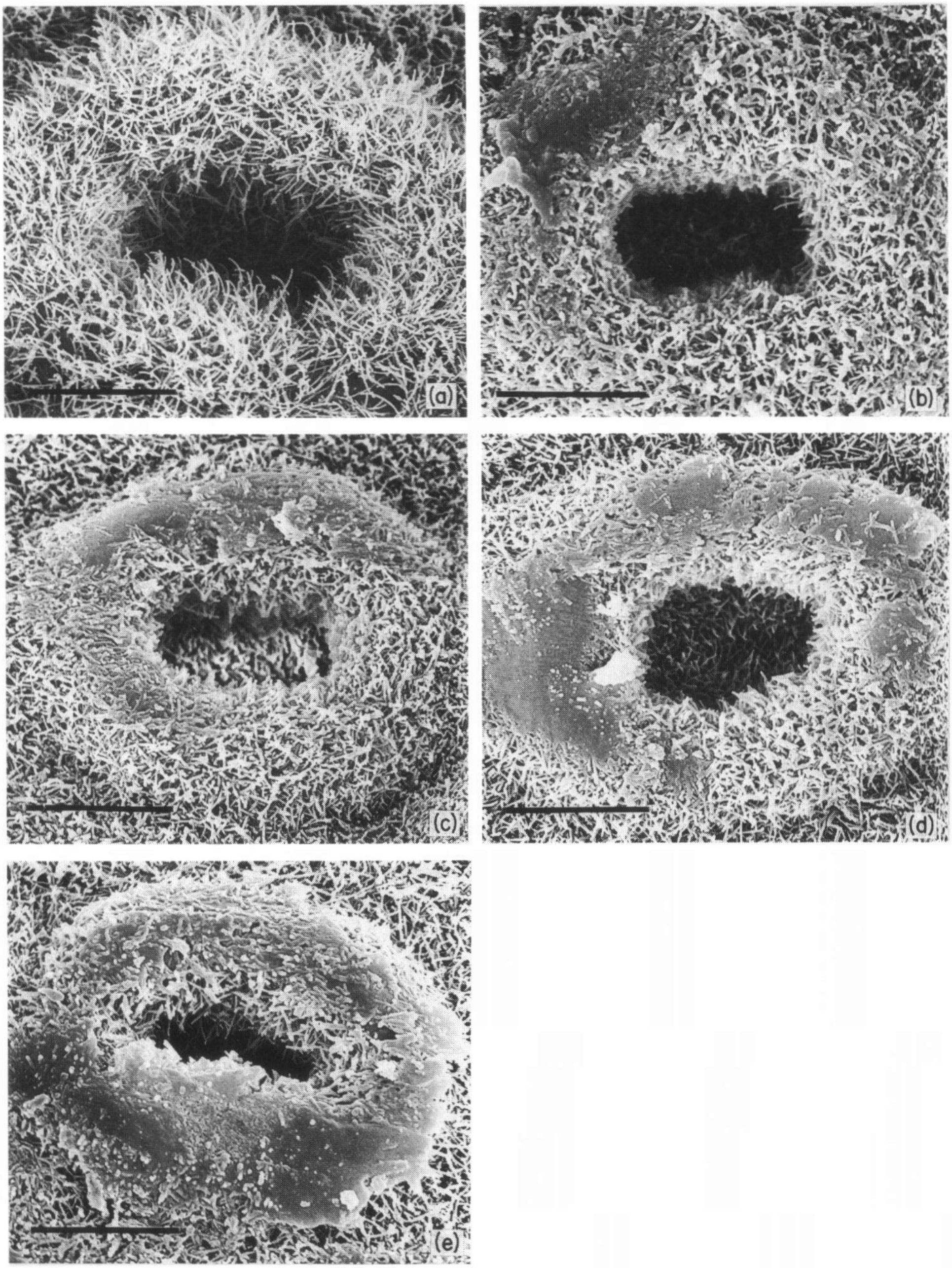

Fig. 2. Representative images of stomata showing structural and amorphous wax stages on peristomatal rims. Scale bar = 10 µm. (a) Stage 1. Amorphous wax absent. (b) Stage 2. 1%–20% of peristomatal rim covered with amorphous wax. (c) Stage 3. 21%–50% covered with amorphous wax. (d) Stage 4. 51%–80% covered with amorphous wax. (e) Stage 5. 81%–100% covered with amorphous wax.

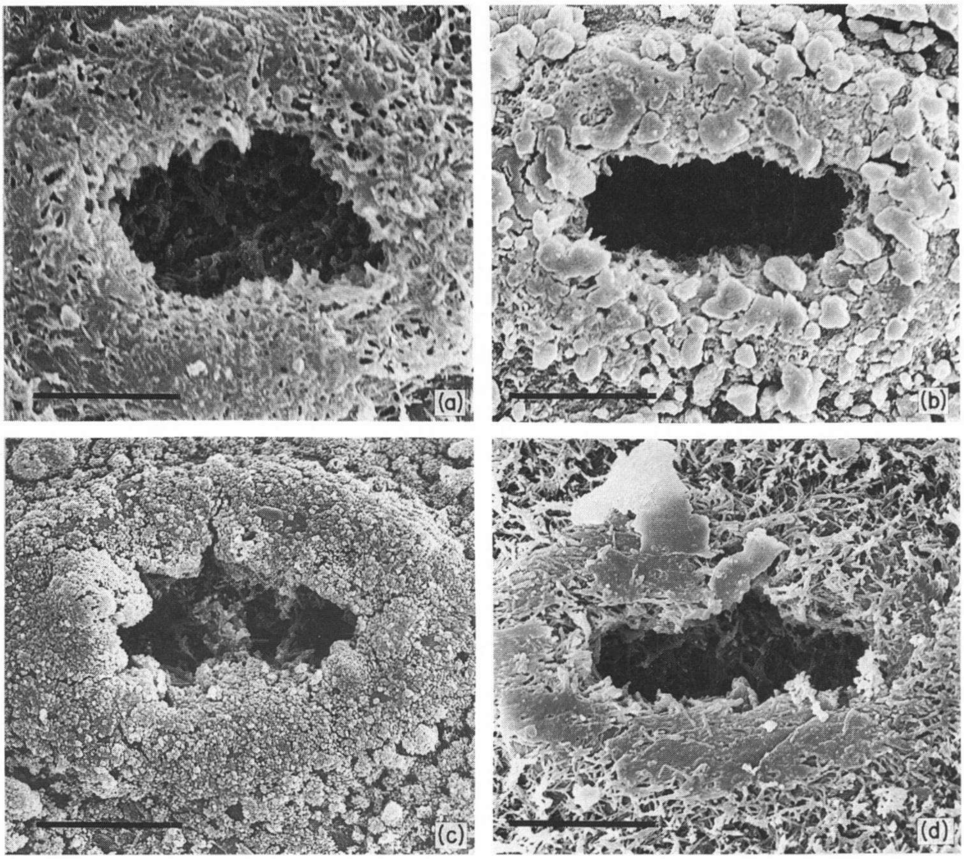

Fig. 3. Peristomatal rims covered with different types of wax crusts (see also text). Scale bar = 10 µm. (a) A porous wax crust. (b) A particulate wax crust on a current-year needle consisting of irregular and flat particles. (c) A particulate wax crust on a first-year needle consisting of granular particles. (d) A crust with underneath parts of unaffected rodlets on a current-year needle.

started to emerge at the same time, but were very sparse. On the same very young needles, however, the degradation of rodlets into amorphous crusts could already be observed.

Three different types of fungal mycelium, all probably belonging to *Hyphomycetes* (*Deuteromycotina*), were found on needle surfaces. The first type of narrow thread-like hyphae, mainly growing among stomata (Fig. 4d), was most frequent. The other two types consisting of thick hyphae similar to each other, one of septate, another of unseptate hyphae, covering the areas around peristomatal rims and the rims themselves (Figs 4e and f), were of rare occurrence.

Fungi first appeared in approximately 2-month-old needles and were present at all later stages. The per cent of current-year needles from the first growing season contaminated with fungi was appreciably lower than that of first-year needles, whereas fumigated needles as well as field samples were similarly or slightly less infected than unfumigated ones. In the young current-year needles collected in April 1991, fungal presence was noted only sporadically, whereas in young current-year needles collected in May 1991 they have not been found at all (Fig. 5).

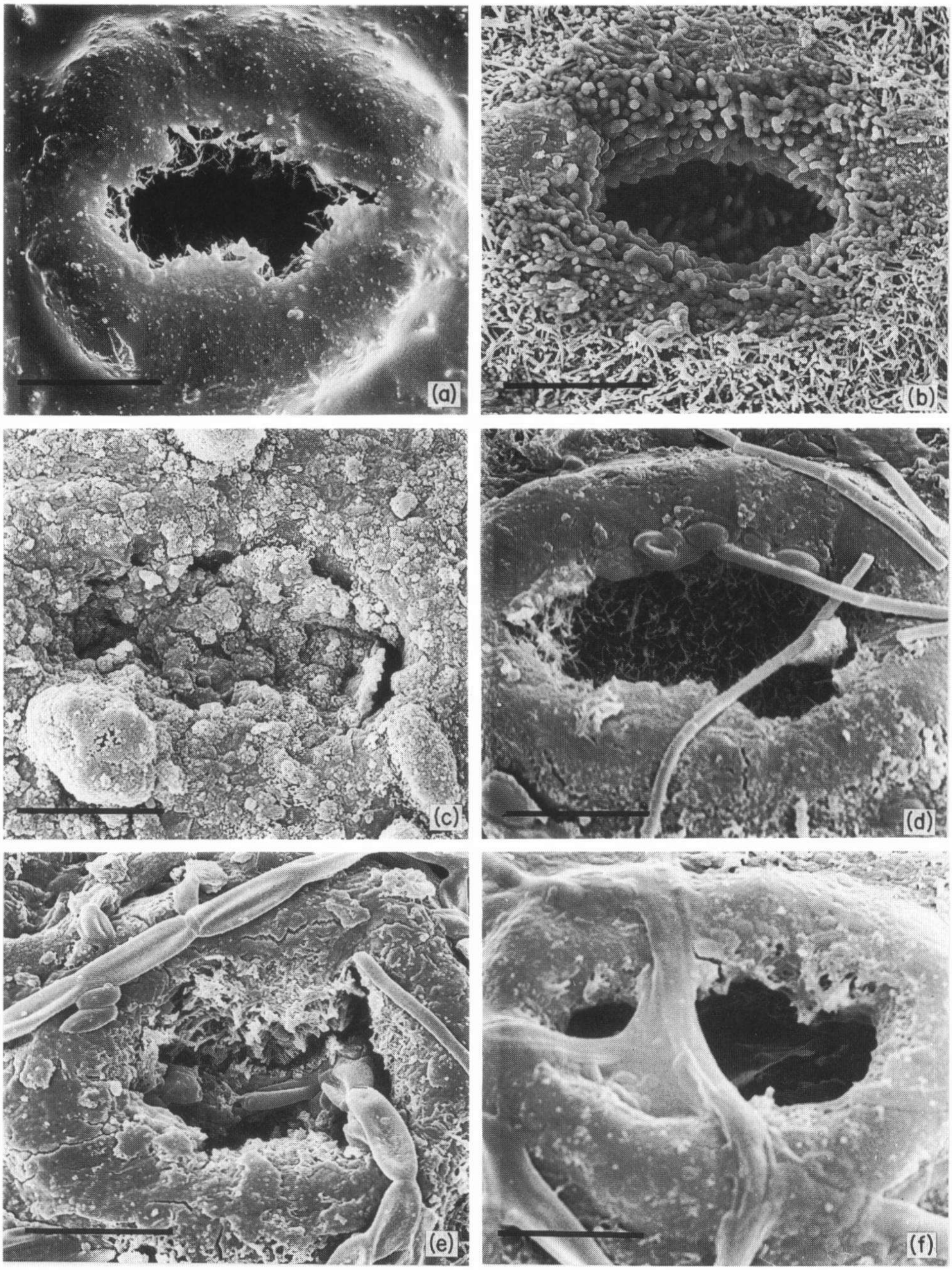

Fig. 4. (a) 'Melted' wax crust on and around peristomatal rim on a current-year needle. Scale bar = 10 μm. (b) 'Melted' rodlets close to and inside the pore on a current-year needle. Scale bar = 10 μm. (c) Wax crust above the stomatal antechamber on a current-year needle. Scale bar = 10 μm. (d–f) Fungal mycelia belonging to Hyphomycetes. Scale bar = 10 μm. (d) Narrow hyphae on a 1-year-old needle. (e) Thick septate hyphae on a current-year needle from field sample (Vreedepeel). (f) Thick unseptate hyphae on a 1-year-old needle.

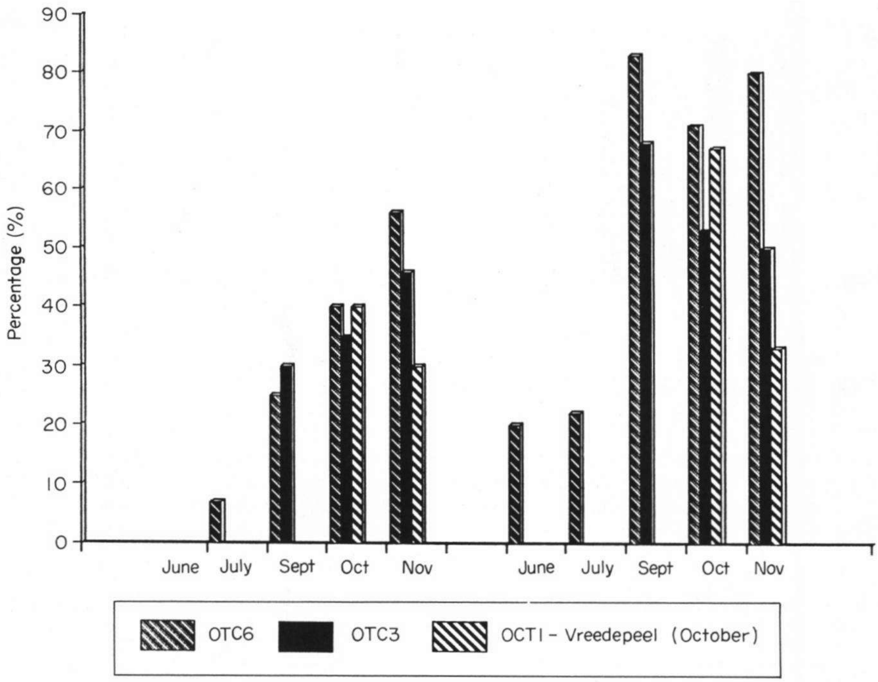

Fig. 5. Percentage of stomata on current (left) and first (right) year needles contaminated with fungi from OTC and field samples collected in the period June–November 1990. OTC6: control; OTC3: 60 µg NH_3 m^{-3}; OTC1: 100 µg NH_3 m^{-3}.

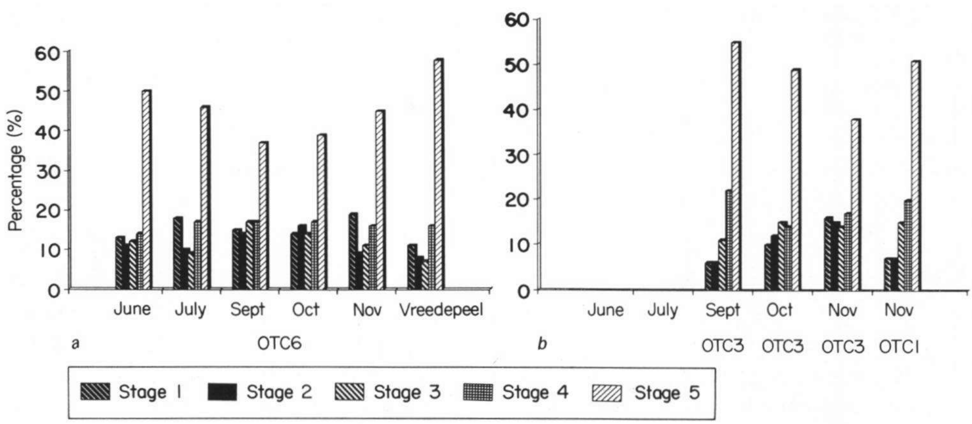

Fig. 6. (a) (left) Frequency distribution of amorphous wax stages on peristomatal rims for unfumigated current-year needles from OTC and field samples (Vreedepeel) collected in the period June–November 1990. (b) (right) Frequency distribution of amorphous wax stages on peristomatal rims for fumigated current-year needles from OTC collected in the period September–November 1990 (fumigation started on 6 August 1990).

Quantification of changes in wax morphology on peristomatal rims

Frequency distributions of structural and amorphous wax stages for peristomatal rims of needles collected during the period June–November 1990, show distinct differences between current- and first-year needles (Figs 6 and 7). Although stage 5 is already

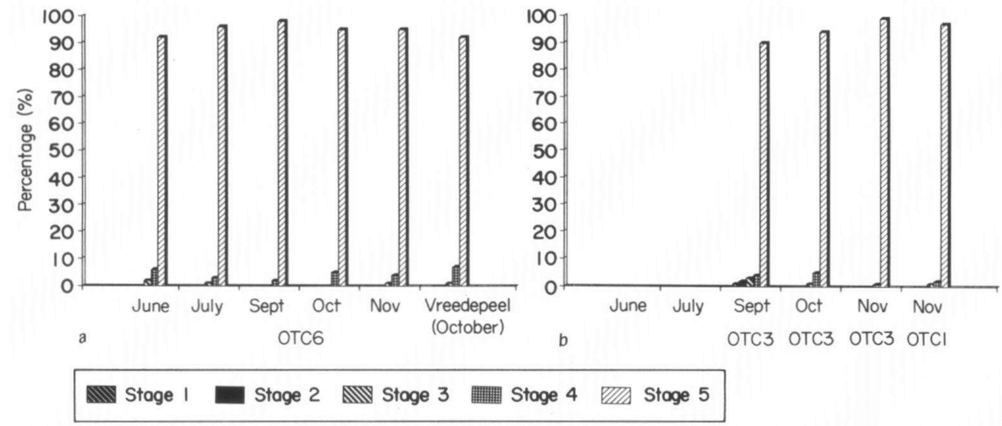

Fig. 7. (a) (left) Frequency distribution of amorphous wax stages on peristomatal rims for unfumigated first-year needles from OTC experiments and field samples (Vreedepeel) collected in the period June–November 1990. (b) (right) Frequency distribution of amorphous wax stages on peristomatal rims for fumigated first-year needles from OTC collected in the period September–November 1990 (fumigation started on 6 August 1990). OTC3: 60 µg NH_3 m^{-3}; OTC1: 100 µg NH_3 m^{-3}.

Table 2. Statistical analysis of differences between current-year needles subjected to different treatments

Sampling date	NH_3 concentration (µg m^{-3})	Wax stage classification $\bar{x}$	SD
September	10	3·42	0·76
September	60	4·06	0·36
October	10	3·50	0·47
October	60	3·84	0·89
November	10	3·56	0·58
November	60	3·47*	0·47
November	100	4·00*	0·27

**t*-test: difference is significant at $P = 0{\cdot}06$; other differences are not significant ($P > 0{\cdot}10$). The fumigation started on 6 August 1990.

predominant in current-year needles at all sampling dates, the first four stages still occur relatively frequently; in first-year needles, stage 5 is twice as frequent and stages 1 and 2 are completely absent. Contrary to our expectations, within current-year needles, stage 1 increased in frequency and stage 5 decreased during the period June–July 1990. There are only very slight and inconclusive differences between fumigated and unfumigated needles. Fumigation (from September 11 onwards) with 60 µg m^{-3} NH_3 resulted in a slightly higher frequency of stomata with a high proportion of peristomatal amorphous wax in the September and October samples, but the reverse was true for the November samples. Treelets fumigated with 100 µg m^{-3} NH_3 showed only a very slightly higher level of amorphous crust formation than the controls and were very similar in needle wax morphology to the field samples collected in Vreedepeel.

As seen in Table 2, the effect of the NH_3 treatment on current-year needles was very low. Differences between treatments are insignificant except between the November needles fumigated with 60 or 100 µg NH_3 m^{-3}.

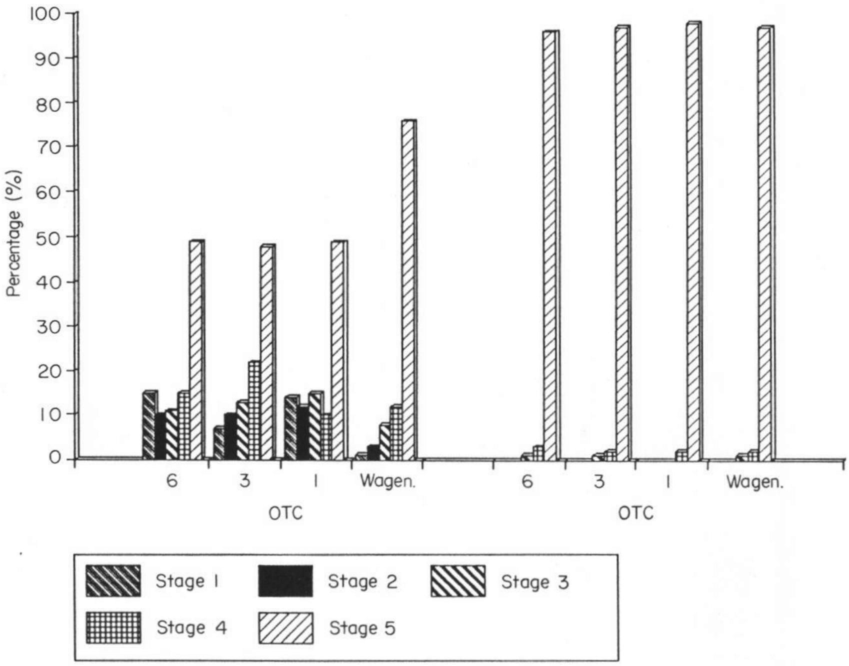

Fig. 8. Frequency distribution of amorphous wax stages on peristomatal rims for 1- (left) and 2-year-old (right) needles from OTC and field samples (Wageningen) collected in April 1991. OTC6: control; OTC3: 60 µg NH_3 m^{-3}; OTC1: 100 µg NH_3 m^{-3}.

The samples from April 1991 showed almost the same wax pattern in the two age-classes as the samples collected in November 1990. A slight further increase in the advanced amorphous wax stages, particularly in stage 5, continued in the needles subjected to ambient air or 60 µg NH_3 m^{-3} and field samples, while the reverse was true in the needles subjected to 100 µg NH_3 m^{-3}. The level of amorphous wax stages in 1-year-old needles was slightly lower than of first-year needles from June 1990, and differences between treatments had decreased (Fig. 8).

The frequency distributions of amorphous wax stages for peristomatal rims of very young current-year needles showed that the wax degradation had increased from early to late May 1991. At the end of this month its level, expressed as per cent of stomata completely covered with wax crusts, in the needles subjected to ambient air or 60 µg NH_3 m^{-3} had almost reached the level found in the needles from June and September 1990, respectively. In the needles subjected to 100 µg NH_3 m^{-3} the wax degradation was even markedly higher than in corresponding needles from November 1990 (Fig. 9).

The percentage of stomata with porous, particulate and 'melted' peristomatal wax crusts, as well as of those with epistomatal wax crusts, on needles sampled between June and November 1990 and on those sampled in April 1991, was low and fluctuated irrespective of needle age, sampling date and treatment. In addition, these kinds of crusts were usually only found in at most one to two or three stomata out of the investigated 20 per needle.

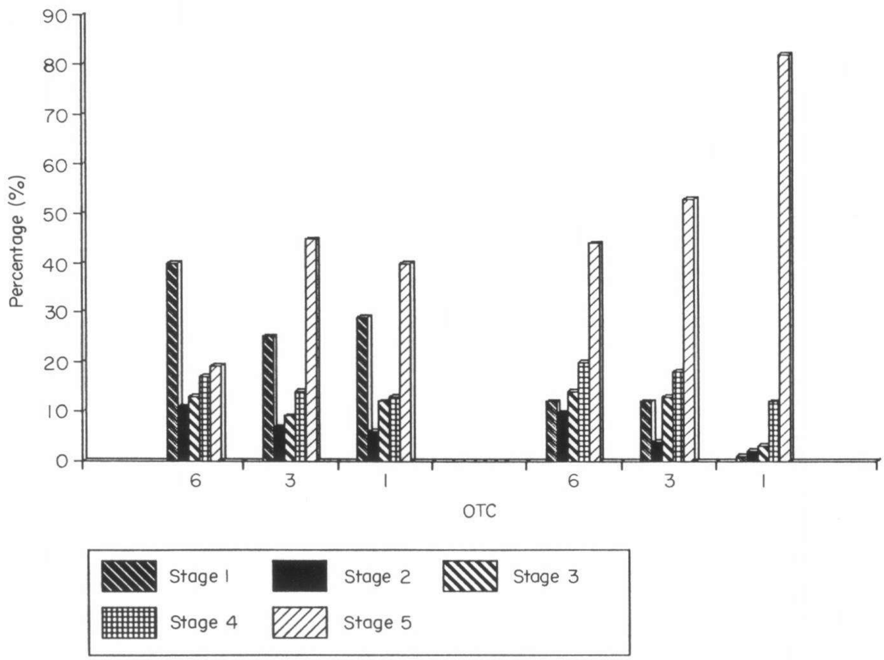

Fig. 9. Frequency distribution of amorphous wax stages on peristomatal rims for unfumigated and fumigated young current-year needles collected on 7 May (left) and 25 May 1991 (right).

DISCUSSION

The epicuticular waxes on conifer needles, particularly crystalloids situated in the stomatal areas, are thought to have several functions and physiological roles. Wettability, gas exchange, water balance, protection against ultraviolet radiation, fungal growth, and wet or dry deposition may be influenced (Goss 1973; Franich *et al.* 1977; Yoshie & Sakai 1985; Berg 1989; Schulze *et al.* 1989).

For this reason an increased rate of rodlet degradation probably affects unfavourably needle or tree physiology. According to many studies (Grill 1973; Cape & Fowler 1981; Cape 1983; Huttunen & Laine 1983; Riding & Percy 1985; Berg 1989; Turunen & Huttunen 1990, 1991) it appears that air pollution, especially with SO_2 and O_3, increases the rate of wax alteration under both natural and controlled conditions. This structural degradation is considered an irreversible process (Huttunen & Laine 1983).

Despite these effects of air pollution, it seems that wax rodlet degradation is first of all a natural phenomenon, resulting from the physical and chemical properties of the rodlets (Schulze *et al.* 1989). Wax tubules on the surface of needles are by themselves rather labile structures which are naturally prone to degradation (Fox 1958). Their progressive fusion and agglomeration into crusts with time is, therefore, a normal phenomenon (Reicosky & Hanover 1976; Franich *et al.* 1977; Crossley & Fowler 1986; Thijsse & Baas 1990).

As the main changes in epicuticular wax morphology in *Pinus sylvestris* affect the peristomatal rims and not the wax over the stomatal pores, any major effect of these structural changes on gas exchange and protection against water stress is highly unlikely.

Markedly pronounced differences in frequency of structural and amorphous wax stages found between current- and first-year needles collected in the first growing season

(June–November 1990) and between 1- and 2-year-old needles collected in the second growing season (April 1991), clearly show that crust formation is primarily a process depending on needle age. An early beginning of wax rodlet degradation of amorphous wax stages, taking place independently of ammonia treatment, seems likely to be also a time-dependent phenomenon. Similar observations have been reported for *P. sylvestris* needles by Huttunen & Laine (1983).

In addition, the different forms of the wax degradation found in this study do not suggest an effect of ammonia. Special forms like porous and particulate peristomatal wax crusts and the rare incidence of epistomatal ones (i.e. directly above the pore) occurred independently of the fumigation treatments. Their incidence was, moreover, very low and fluctuated irrespective of needle age, sampling date and treatment. For this reason, these two crust forms seem to be a random phenomenon.

The 'melted' kind of degradation is thought to be caused either by air pollutants or by high temperature. Schmitt *et al.* (1977) found heavily 'melted' wax after SO_2 fumigation and acid rain treatment. Unusually increased temperatures, however, had been ruled out as a cause of 'melted' wax as no structural changes could be observed when needles were subjected to temperatures of 50°C (Sauter & Vox 1986). As 'melted' wax in the needles studied was of rare occurrence and the per cent of stomata with it varied independently of needle age, sampling date and treatment, its appearance cannot be attributed to ammonia impact.

The appearance of a kind of compact peristomatal wax crust, with non-fused parts of rodlets beneath its surface, suggests an inconstant rate of changes through the wax layer: faster at the tips than at the bases of the tubules. According to Crossley & Fowler (1986), these crusts imply that the effect was mediated from the outside. Barnes *et al.* (1988) also suggested that the pollutant they studied (O_3) had a direct influence rather than an indirect one through an effect on metabolism and wax production. In our study these crusts were found in the needles from all treatments thus making the impact of ammonia an unlikely cause.

As shown in Figure 6 and Table 2, the effect of the NH_3 treatment was not pronounced and mainly statistically insignificant, and any accelerating effects on the rate of wax degradation must be very limited.

The higher levels of advanced amorphous wax stages in very young current-year needles (sampled in May 1991, Fig. 9) treated with ammonia, particularly in those subjected to higher concentration, seem to indicate a stronger effect on wax degradation. These young current-year needles on trees were already fumigated for nearly 8 months and by themselves could not fully have been subjected to direct effects of NH_3 because of their young age and protected micro-environment in the fascicles. This is suggestive of an indirect effect of NH_3 on the stability of crystalline wax.

The wax on the first-year needles collected in 1991 does not show any further progress in wax degradation compared with current-year needles of the November 1990 samples. Many SEM images suggested the presence of recrystallization. Moreover, the number of peristomatal rims free of crusts (stage 1) had increased while stomata in stage 5 showed a decrease in number over the period June–July 1990. These observations lend support to Barnes *et al.* (1988) and Günthardt (1985) who suggested that young needles can replace structurally degraded waxes through continued production of crystalline rods while waxes in older needles probably represent a final metabolic product.

Our results have given no indication of a correlation between fungal presence and atmospheric ammonia concentration. Their growth appeared to be mainly needle-age dependent and apparently they had no dissolving effects on the wax layer as the fungi on *Pseudotsuga menziesii* needles (Thijsse & Baas 1990).

From our observations it appears that in *Pinus sylvestris* the needle age has an overriding effect on epicuticular wax morphology. Ammonia has only a very minor influence after prolonged fumigation, possibly via indirect effects.

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