CONIFER EPICUTICULAR WAX AS A BIOMARKER OF AIR POLLUTION: AN OVERVIEW

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ABSTRACT

Epicuticular wax covering the conifer tree species surface has been used, mainly in conifers, as a biomarker of air pollution damage. Using Scanning Electron Microscopy (SEM) various alterations in wax structure and chemistry caused by natural and anthropogenic factors have been noticed. SEM enables to evaluate wax deterioration at a very early stage, before visible symptoms occur. Symptoms of wax injury are, in general, not specific to the air pollutant type. Most common alterations in wax were the following: an undeveloped structure, various type of wax tubes fusion or erosion (deformed and disfunctioned stomatal complexes, a decrease in wax tube distribution, increased enrichment of completely amorphous stage), shifted annual wax erosion rate, chemical and needle wettability changes. To use SEM as an accurate tool for evaluating wax alteration, it is essential to distinguish air pollution and natural factors from artefacts caused by inappropriate usage of technique.

KEY WORDS: Epicuticular wax, structure and chemical alterations, scanning electron microscopy (SEM), biomarker, anthropogenic and natural factors, air pollution, artefacts, conifers.

INTRODUCTION

Cuticle covering the conifer needle surface acts as a barrier between needle and its atmospheric environment. It is the most exposed part of the needle to a variety of effects. Cuticle plays various important functions, protecting forest trees from: loss of water (due to transpiration), nutrient and organic substances (in leaching processes), as well as, effects of natural (climatic and weather conditions, epiphylic micro-organisms, physical abrasion), and anthropogenic factors (air pollution) (Hanisch and Kilz 1990; Turunnen and Huttunen 1990; Wolfenden and Mansfield 1991; Zalewska-Gorzelska 1991; Bermadinger-Stabentheiner 1995; Bermadinger-Stabentheiner and Grill 1995; Kerstiens 1996; Rieder et al. 1996; Schreiber 1996; Turunen 1996a; Schreiber et al. 1997; Werner 1998). The cuticle's protective role is especially crucial for species exposed to extreme natural and anthropogenic conditions (Turunen and Huttunen 1996; Turunen and Huttunen 1997).

The cuticle, 1-5 μm thick, consists of two elements: the cutin (an insoluble polyester built up by dihydroxy – and trihydroxy fatty acids) and cuticular waxes – a complex mixture of soluble lipids (long chain – 16-36 carbon atoms – aliphatic compounds). Waxes are deposited within the inner cutin layers (intracuticular waxes) and onto the outer surface of the cuticle (epicuticular wax) (Kolattukudy 1996; Rieder et al. 1996; Neinhuis and Barthlott 1997; Schreiber et al. 1997; Turunen and Huttunen 1997). Epicuticular wax occurs as a wax coating particularly in and around the stomatal entrances (Huttunen 1994).

Epicuticular wax morphology varies between the species, in conifers it is well developed and rather uniform within the whole systematic group. Conifer wax occurs in form of tubes, rods, plates or filaments built up by the secondary alcohol nonacosan-10-ol (Hanisch and Kilz 1990; Zalewska-Gorzelska 1991; Bermadinger-Stabentheiner and Grill 1995; Neinhuis and Barthlott 1997; Huttunen 1997). Appearance of wax structures is determined by its chemical composition, primary alcohols crystallise in form of plates, β-diketones produce thin tubes and aldehydes crystallise as rods (Bermadinger-Stabentheiner 1995; Bermadinger-Stabentheiner and Grill 1995). The epicuticular wax quantity and chemistry are determined genetically by many natural factors, and vary between the species. Higher amount of epicuticular wax on the leaf surface was observed in subarctic pines (Turunen 1996a) and species growing at high altitudes (Grodzińska-Jurczak 1994, 1996; Turunen 1996a). Its structure however is in most cases poorly developed with short wax tubes (Turunen 1996a).

Epicuticular wax and cuticle change over a needle's life stage, during foliage development needles are almost completely surfaced with epicuticular waxes whereas the cuticle is rather thin. Since needle growth is completed, parts of wax erode and cuticle thickness increase (Neinhuis and Barthlott 1997; Turunen and Huttunen 1997). The crystalline wax structure of healthy needles is completely developed after a vegetation period, natural and anthropogenic factors may lead to various alterations (Hanisch and Kilz 1990). Wax quantity changes also with age, and amounts from 0.5% to over 2% dry weight in young pine needles (Huttunen 1994). After the growing process is completed, epicuticular wax of needles cannot be resynthesized. However while eroded, it may become recrystallised within ca. 6 weeks (Huttunen 1994;

Neinhuis and Barthlott 1997; Schreiber et al. 1997). In mechanically injured spruce needles, a regrowth of wax tubes was noticed (Bermadinger-Stabentheiner and Grill 1996).

Since the early 70-ties, changes in the epicuticular wax structures, above all in conifers, have been successfully used as a biomarker of pollution damage (Turunen and Huttunen 1990; Woźny 1991; Hellqvist et al. 1992; Bermadinger-Stabentheiner and Grill 1995; Werner 1998; Cape and Percy 1998). Bioindication is based on direct observations of alterations into wax structure or indirect methods such as evaluating needle wettability (Turunen and Huttunen 1990). Estimation of epicuticular wax degradation of conifer needles has been carried out mainly in the Scanning (SEM) and Transmission Electron Microscopy (TEM) (Hanisch and Kilz 1990; Bermadinger-Stabentheiner 1995; Trimbacher et al. 1996). SEM has been considerably helpful in examination of needles from highly polluted areas or exposed to various chemical substances under experimental conditions (Godzik 1982; Turunen 1990). SEM enables to evaluate wax morphology changes at a very early stage, before visible symptoms have occurred (Godzik 1982; Bermadinger-Stabentheiner 1995; Turunen and Huttunen 1996). A variety of alterations into epicuticular wax morphology were found. Using a SEM with attached energy disspersive X-ray analyser allows to characterise particulars (their size, shape, structure, chemical composition and distribution) deposited on the needle surface (Godzik 1982). SEM as a method is rather easy to perform, valuable information about the needle surface may be obtained without an intricate or laborious preparation (Bermadinger-Stabentheiner 1994).

In this paper the role of conifer epicuticular wax as a biomarker of air pollution damage as well as the growing literature on field and experimental studies focused on factors affecting wax structure and chemistry are reviewed. The purpose was: to summarise the variety of epicuticular wax alterations that may appear in response to certain natural and anthropogenic (main pollutants) agents in conifer trees of various species, age and conditions they grow in and to provide appropriate tools for evaluating wax changes and their accurate usage.

EPICUTICULAR WAX ALTERATIONS

NATURAL FACTORS EFFECTS

Climatic and mechanical factors

A great number of natural environmental factors (rough climate, strong winter with long periods of low temperature, large amounts of rain-, and snowfall, wind etc.) may result in various types of epicuticular wax structure alterations: an undeveloped structure, a decrease in wax tube distribution (Bermadinger-Stabentheiner and Grill 1995; Turunen 1996; Turunen and Huttunen 1996; Back et al. 1997; Turunen and Huttunen 1997), faster enrichment of completely amorphous wax stage (Turunen and Huttunen 1991, 1997; Ylimatimo et al. 1993) deformed and/or dysfunctioned stomata (Huttunen 1994; Turunen 1996a), increased annual wax erosion rate and needle wettability change (Huttunen 1994; Turunen 1996a). Large amount of fog, increased radiation and low temperature alter in wax chemical changes: shifted amounts of secondary alcohols, alkanes and alkylesters (Turunen et al. 1993; Turunen and Huttunen 1996). Short, cold growing seasons, long winters may disturb nutrient availability for wax development (Ylimatimo et al. 1993; Turunen 1996a) leading to occurrence of lower amounts of stomata with well-developed and well-preserved epicuticular wax structures of the needles (Turunen and Huttunen 1991).

Epicuticular wax deterioration rate (an undeveloped and/or eroded wax structure, thinner cuticle, higher permeability and wettability of the needles) are, in most cases, positively correlated with the altitude, the higher elevation, the more severe damage to the needle (Grodzińska-Jurczak 1994, 1996; Turunen and Huttunen 1996). Altitude coincides naturally with climatic conditions (higher rain-, and snowfall, strong, wind, lower temperature, prolonged winter) (Grodzińska-Jurczak 1994; Turunen and Huttunen 1996).

Wax surface is very susceptible to mechanical damages (forces), many injuries to wax are caused e.g. by: (a) wind-blown particles (dust, sand, snow, ice), (b) high temperature (melting point of spruce waxes: 45-60°C), (c) abrasion. Most frequent damages (smeared epicuticular wax layer and squashed tubes; melting wax structure; loss of wax) occurred in wax at stomatal chambers and on the surrounding surface. As a consequence, the crystalline wax structure is destroyed, and such mechanically-induced alteration estimated as an effect of air pollution (Bermadinger-Stabentheiner 1994; Werner 1998).

Age

Structure of epicuticular wax in and around the stomata changes with age of the needles from not completely crystalline, thin and flat layer at the first phase of formation into fully crystalloid formations after a vegetation period (Hanisch and Kilz 1990; Donnelly and Dowding 1994; Huttunen 1994; Werner 1998). Since it had been fully developed, may be altered only by external factors (Hanisch and Kilz, 1990; Bermadinger-Stabentheiner and Grill 1995). The natural ageing of the needles results in changes in the epicuticular wax structure and chemistry manifested by: (1) a reduced crystalloid wax tubes quantity (tubes in and outside stomatal rows in young needles; cracked, eroded wax forms to amorphous wax with empty cavities in the stomatal chambers in older needles) (Hanisch and Kilz 1990; Hellqvist et al. 1992; Turunen et al. 1993; Huttunen 1994; Bermadinger-Stabentheiner 1995; Turunen 1996a; Turunen and Huttunen 1996, 1997), (2) declined wax tube distribution (WTD) (more evident after the first overwintering) (Hellqvist et al. 1992; Turunen et al. 1993; Huttunen 1994; Turunen and Huttunen 1996; Turunen 1996a; Turunen et al. 1997; Turunen and Huttunen 1997), (3) a higher cuticular permeability and increased wettability (reduced water droplet contact angle (DCA) (Staszewski et al. 1994; Turunen 1996a) as well as (4) a reduced proportion of dehydroabietic acid, hydroxy fatty acids and secondary alcohol nonacosan-10-ol and increases in alkyl esters, di-esters and estolides amount (Hanisch and Kilz 1990; Turunen 1996a; Turunen et al. 1997; Turunen and Huttunen 1997).

Ageing processes may be accelerated by a variety of natural (light intensity, wind, frost, drought) and anthropogenic factors (air pollution e.g.: acid rain, heavy metals, ammonium, nutrient deficiency) (Hanisch and Kilz 1990; Turunen et al. 1993, 1997; Huttunen 1994; Turunen and Huttunen 1996; Back et al. 1997).

ANTHROPOGENIC FACTORS

Air pollution effect on wax structure and chemistry

Effects of various air pollutants (e.g. SO_2, NO_2, acid rain, heavy metals and ammonia) on needle surface (epicuticular wax included) have been extensively studied both in the field and laboratory experiments (open-top and climate controlled chambers). Results of those investigations varied, some researchers observed no pollutant-induced alterations to epicuticular wax. However most of them, noticed that epicuticular wax changes, while exposed to the external chemicals. Alterations in wax are often similar to those caused by natural factors, but occurring at accelerated rates (Cape 1988; Hanisch and Kilz 1990; Huttunen 1994; Turunen and Huttunen 1996, 1997; Turunen 1996a; Turunen et al. 1997; Bermadinger-Stabentheiner and Grill 1995; Wolfenden and Mansfield 1991).

Air pollution may result in changes into epicuticular wax structure, chemistry, wettability and transport properties. The rate of foliar injury depends on pollution type and dosage, genetics (clone, species), development stage and age of the needle, as well as other environmental factors. In general, symptoms of wax injury are not specific to the kind of pollutant (Godzik 1982; Bytnerowicz and Turunen 1993; Huttunen 1994; Turunen and Huttunen 1997; Werner 1998). A direct cause of the needle injury may be established if there is only one source of pollution in the immediate vicinity of the trees and, chemical composition of the emitted wastes is known (Hanisch and Kilz 1990; Bytnerowicz and Turunen 1993). Structure and chemical composition of epicuticular wax may be altered by pollutants indirectly (affecting cuticle synthesis through the altered foliage metabolism and decreased tree health) and/or directly (chemical reactions between wax and air pollution components, e.g. acid rain or mist compounds) (Turunen and Huttunen 1990, 1997; Turunen et al. 1993; 1997; Huttunen 1994; Jetter 1996; Trimbacher et al. 1996; Turunen 1996a).

In clean air areas changes in wax structure appear more frequently as tubular wax type while those grown in polluted regions present eroded forms (Tuomisto and Neuvonen 1993). The most frequently observed wax structure injuries were: delayed development of cuticles, various type of wax tubes fusion or erosion (deformed stomatal complexes, breakdown and aggregations of wax structures on stomatal chambers), increased wettability and conductivity of the needles (Godzik 1982; Turunen and Huttunen 1990; Turunen et al. 1993, 1997; Huttunen 1994). Changes the wax always start, as in case of ageing processes, from a wax-tubes fusion leading to a complete degradation of the crystalline structure (Bermadinger-Stabentheiner and Grill 1995; Neinhuis and Berthlott 1997).

Changes in epicuticular wax chemistry (altered wax synthesis and its composition) was attributed only to the air pollution effects (Turunen et al. 1993, 1997) and lead to its structure alterations afterwards. Acid rain treatment lead to decreases in production of diols and hydroxyacids and a lower production of secondary alcohols (Turunen 1996a). Concentration of diterpene acids (particularly dehydroabietic acid) in pine needles increased towards the emitter and might have been a metabolic response to sulphur dioxide and heavy metals and/or direct reactions of SO_2-derived sulphuric acid on the needle surface (Schmitt et al. 1998; Turunen, 1996a; Turunen et al. 1997). As a consequence of chemical changes in wax, a decreased tollerance of plants to insect attacks was observed (Turunen and Huttunen 1990).

Higher degradation of wax structure of one- and two-year-old conifer needles exposed to various industrial pollution (SO_2, acid rain, heavy metals) compared to the current ones was found by many authors (Hanisch and Kilz 1990; Turunen et al. 1993; Turunen et al. 1997; Turunen and Huttunen 1996; Grodzińska-Jurczak 1994, 1996; Grodzińska-Jurczak and Szarek-Łukaszewska 1997) (Fig. 1). Sulphur dioxide, dust and ammonium emission resulted in WTD increase, ca. from 31-70% (1-yr.-old) to 71-100% (a current-yr. needle) (Turunen et al. 1993, 1997). The older the needle the higher the occurrence of deposited particles and epyphyllic micro-organisms (algae and fungi) on its surface (Turunen et al. 1993, 1997; Ylimatimo et al. 1993; Turunen 1996a; Turunen and Huttunen 1996; Back et al. 1997; Grodzińska-Jurczak 1994, 1995). Ageing processes affect needle physiology (photosynthetic ability, re-crystal-

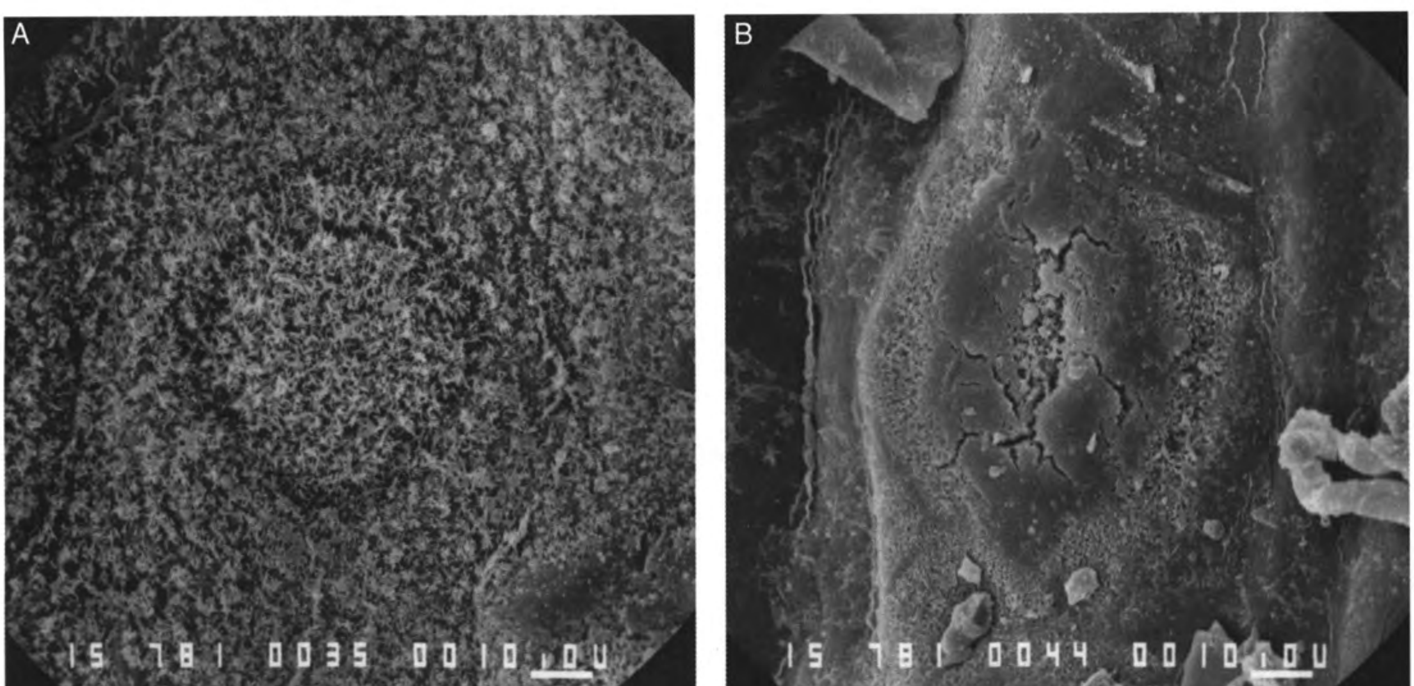

Fig. 1. SEM – micrographs of alterations in epicuticular wax morphology of *Picea abies* caused by various air pollution (SO_2, acid rain, heavy metals) (x 2000).
A – a current-yr-old needle, no visible degradation or aggregation of wax rodlets;
B – a 2-yr-old needle, most severe aggregation and degradation of the formerly structural wax and formation of a continuous wax layer on 9/10 of the epistomatal chamber.

lisation of the wax), the older the needle the more visible consequences are seen (Turunen and Huttunen 1990).

Many authors found a correlation between the rate of epicuticular wax erosion and the distance from the industrial source. The closer to the emitter the more wax alteration occurred (Turunen et al. 1993, 1997; Manninen and Huttunen 1995; Turunen and Huttunen 1996). Wax changes were manifested in a decrease of the stomata density (Turunen 1996a; Turunen and Huttunen 1996, 1997), reduced wax tube distribution with characteristic fusions (Huttunen 1994; Turunen 1996a) and increased needle surface wettability (Turunen 1996b; Turunen and Huttunen 1996, 1997). In case of trees growing in further location from the pollution source, their response to low concentrations of chemicals may be masked by many natural and anthropogenic factors (soil chemistry, climatic conditions, fungal and insect diseases) (Turunen et al. 1993; Kupcinskiene 1996; Turunen and Huttunen 1997).

The rate of air-pollutant-induced wax damage is estimated by various classifications, most of which estimate the overall condition of the wax of a needle or only a stomatal area (Huttunen and Laine 1983; Crossley and Fowler 1986; Bacic et al. 1990; Turunen and Huttunen 1991). Wax is classified into quality classes distinguishing: (1) distribution of crystalline waxes in epistomatal areas, (2) occurrence of particulate deposition, (3) occurrence of fungi hyphae, insect damage and blue-green algae (Turunen et al. 1992, 1993). Ylimatimo et al. (1993) formulated a classification which enables establishing wax coverage and morphology disturbances. Trimbacher et al. (1995) used a five degree classification estimating the degree of melted wax of the stomatal area and different wax forms appeared during degradation processes (Trimbacher et al. 1996). Bacic et al. (1990) quantified wax changes on the basis of the relative amount (%) of amorphous wax on peristomatal rims of stomata.

Alterations in epicuticular wax structure and chemistry induced by air pollution may lead to a decreased tree viability mainly through the following processes: (1) nutrient leaching after increased needle wettability, rain retention and needle permeability, (2) desiccation (due to increased cuticular and stomatal transpiration), (3) fungal and insects diseases cused by increased wax permeability and wettability (Turunen and Huttunen 1990).

Many plant species (especially those growing in smelter vicinity) evolved tolerance to air pollutants through evolution (e.g. enzymes tolerant to metals) or physiological plasticity (needles of lower stomatal density and wettability which absorb lower amounts of the gaseous pollutant) (Turunen and Huttunen 1997). Turunen and Huttunen (1996) observed that Scots pine needles (especially of olderage classes) exposed to air pollution since 1950 did not show any wax damage, fungal or insects diseases. Only most tolerant trees survived the pollutants' effects in a natural selection process.

Acid rain

Most investigations of air pollution effects on conifer needles damage were orientated on acid rain. Experiments were performed on various conifer species, both in the field and under controlled conditions. Acidic precipitation comprising sulphur (sulphate) results in more severe wax injuries than that of nitrogen (nitrate or ammonium) content or a mixture of both (nitrate and sulphate) (Cape 1988; Turunen and Huttunen 1990, 1991, 1996; Cape 1994; Turunen et al. 1995, 1997). Acid rain may affect directly by chemical reaction between its components (H^+, NH_4^+, SO_4^{--}, NO_3^-) and the waxes or their precursors, or indirectly via the soil and root systems (Turunen et al. 1995, 1997)

In general, precipitation of pH 3-4 may induce alterations in epicuticular wax. Injury rates and symptoms however vary between the species and depend on occurrence of natural, as well as anthropogenic factors (Turunen and Huttunen 1991; Turunen et al. 1993; Grodzińska-Jurczak 1994). An artificial acid rain precipitation (pH 3,5) resulted in increased cuticular and stomatal conductivity of Norway spruce needles in comparison to rain of pH 5,6 (Huttunen 1994). Pine seedlings treated with rain of pH 3 and pH 4 resulted in wax synthesis delay and decrease in its quantity. Needles of adult trees had a poorly (changed forms of crystalloids) or undeveloped (short and sparsely distributed) wax structure and deformed stomatal complex (narrow, half-complexes, occluded and double sized) (Turunen and Huttunen 1991; Wolfenden and Mansfield 1991; Bytnerowicz and Turunen 1993; Turunen et al. 1993; Huttunen 1994, Turunen 1996a).

Acid rain irrigation may lead to nutrient leaching from the tree foliage and changes in needle chemistry, their rate depends on rain acidity, its duration, nutritional soil status and tree species. Increased concentrations of macroelements (K, Ca, Mg and Na) and heavy metals (Cd, Pb, Zn) in throughfalls under acidic precipitation exposition was reported by Mengel et al. (1987); Schier (1987), Cape and Lightowlers (1988); Grodzińska-Jurczak (1994, 1995) and Zimka and Stachurski (1996). In all cases leaching was paired with severe wax damage of the growing spruce. Back et al., (1995); Turunen, (1996) treated Norway spruce and Scots pine seedlings with water mixture of nitric and sulphuric acids acidified to pH 3 or pH 4. In case of spruce a significant decrease in foliage Ca concentration was observed while Scots pine reacted in shifting of the foliar Ca content. Different conifer species fumigated with SO_2 and NO_2 together with a Ca deficient solution resulted in a decreased concentration of Ca, K and P concentration in needles (Rantanen et al. 1994).

Acid rain induced changes to the wax surface may be accelerated or multifold by extreme climatic conditions. Rough climate with low temperature, prolonged winter seasons, poor summers, high frequency of precipitation, exposure to snow, ice crystals and fog affected to a greater extent the epicuticular wax structure of pine needles (erosions and a delayed cuticle development) (Turunen and Huttunen 1991, 1997; Huttunen 1994). Interactions between acidic pollutants' effect and low-temperature stress was suggested by Wolfenden and Mansfield (1991). Tolerance of Sitka spruce to low temperature increased after exposition to SO_2 and NO_2 alone or in combination (Wolfenden and Mansfield 1991). Damages of spruce and dwarf-pine wax increased with the altitude, the higher the elevation the more significant erosions of wax (Grodzińska-Jurczak 1994; Grodzińska-Jurczak and Szarek-Łukaszewska 1997).

Sensitivity of needle to acid rain depends on the age and phenology stage of the needle. In most cases, current-year needles were more affected by acid rain than 1- or 2-year-old ones (Grodzińska-Jurczak 1994; Rantanen et al. 1994; Grodzińska-Jurczak and Szarek-Łukaszewska 1997). Least tollerant are needles with a low quantity of wax: 1) young, elongated with undeveloped cuticle and 2) old, eroded needles containing less wax than well-developed trees (Turunen and Huttunen 1991).

Heavy metals

Heavy metal effects on the epicuticular wax have not been studied in details. Metals are principally taken up from the soil (an indirect mechanism) or in minor cases directly via stomata and cuticle (Turunen 1996a; Turunen et al. 1997).

Heavy metals in insoluble form are not toxic. In acidic or humid environment however (an increased acidity of water film or rain droplets on the needle surface) may dissolve and change to ion form. Their toxicity increases significantly (Turunen 1996a). Mostly damaged wax (changed wax chemistry, altered wax structure and epistomatal chambers occluded with heavy metals particles) was observed in the trees growing close to the smelter where concentrations of heavy metals were the highest. High concentration of Ni, Cu and Fe resulted also in inhibition of stomatal closing and alterations in needle wettability characteristics (Turunen et al. 1993, 1997; Huttunen 1994; Turunen 1996a). In general, heavy metal effect on the needle surface is less severe than that caused by acid rain (Turunen et al. 1993; Turunen and Huttunen 1997).

Ammonia

Tree exposition to ammonia did not result in any specific alterations in the wax structure and chemistry. Only slight changes in needles' microstructure (e.g. increase in thylackoids thickness, enlarging of the intrathylakoid areas, curling of granal membrans) (Back et al. 1997) and wax alterations (a very weak, insignificant icreased rate of the normal, age dependent wax degradation) were observed (Bacic et al. 1990). Surface of pine needles growing in immediate vicinity to farms were colonised by a higher amount of microflora (algae and fungi) than those from the control area. It was, however, most likely due to the ammonium compounds deposition than wax structure (Back et al. 1997). In contrary, Bacic et al. (1990) did not find any correlation between fungal presence on the Scots pine needle surface and atmospheric ammonia concentration. Their growth was rather needle-age dependent, having no dissolving effects on the wax layer.

Ozone

The results of studies on ozone effect upon epicuticular wax structure vary. Most authors do not proof any morphological changes caused by ozone (Huttunen 1994; Bermadinger-Stabentheiner 1995; Cape et al. 1995; Wolfenden and Mansfield 1991; Werner 1998). Lutz et al. (1990) (after Werner, 1998) did not find any wax structure alterations of spruce treated with a mixture of acidic fog and ozone at 100-360 μg m^{-3}. Fumigation with 200 μg m^{-3} of ozone resulted in slight alterations in spruce (*Picea abies*) wax structure (Ojanpera and Huttunen 1989) while the same dose made changes only in wax quantity without any structure alterations at Norway spruce wax (Gunthardt-Goerg and Keller 1987). Barnes et al. (1988) found ozone to speed a wax degradation manifested in stomatal occlusion. Four species of pine growing under ambient level of photochemical smog showed foliar injury symptoms (e.g. cracks on needle surface, deep stomatal cavities) (Bytnerowicz 1989). Wax alterations (wax tubes fusion, stomatal occlusion) were also observed in Ponderosa pine exposed to ozone in Sierra Nevada (CA, USA). Changes in wax structure may be explained by: (1) disturbances in wax synthesis, expressed as an aberrant wax tubes' fusion or (2) chemical changes of wax (a decrease in secondary alcohols, diols, alkylesters, fatty acids) caused by ozone (Barnes 1996).

UV-B radiation

Field and laboratory studies on UV-B radiation effect on the needle showed significant damaging effects depending on the species and a radiation dose. Needle response to the radiation is dictated mainly by the optical properties of the outer tissue layer (Barnes et al. 1994). The mechanism by which UV-B alters wax chemical composition and physical properties are still unknown. One of the explanations is, that radiation affects directly enzymes activity resulting in disturbing a natural balance of wax biosynthesis (Gordon et al. 1998). Gordon et al. (1998) studies showed that UV-B affects the epicuticular wax biosynthesis in various conifer tree species (Norway, red, black and white spruces) at doses found in natural forest ecosystems. Response to increasing radiation was species specific, least obvious symptoms in epicuticular wax composition were found in white spruce, wax of other three species was significantly changed. Barnes (1996) and Kinnunen and Huttunen (1996) found that most tolerant to UV-B radiation are species growing in high ultraviolet irridiance environments. Kinnunen and Huttunen (1996) observed highest changes in wax structure (a slight increase in tubular waxes distribution) of Scots pine growing naturally in low UV-B conditions. A higher amount of wax might be produced as a reaction against severe irridiance (Huttunen 1994; Kinnunen and Huttunen 1996; Krauss 1996; Rieder et al. 1996).

Changes in epicuticular wax chemistry and structure caused by UV-B radiation are of big concern because they may result in increased seedling sensitivity to the changing atmospheric environment, mainly in the regions under effect of co-occurring pollutants (O_3, CO_2, acid rain) (Gordon et al. 1998).

Nutrient supply of trees

Influence of nutrient supply of trees on epicuticular waxes was in most cases studied in laboratory. An unbalanced nutrient status, induced mainly by air pollution, may alter the epicuticular wax structure, however its symptoms are different, depending on the nutrient type and dosage (Ylimatimo et al. 1993). Fir needles of fertilised trees with nitrogen and potassium had enriched tubular waxes whereas needle wax from the control sites were smooth (Chiu et al. 1992). An increased concentration of nitrogen in needles resulted also in higher tolerance of spruce seedlings to freezing (Back and Huttunen, 1992). Schwab et al. (1994) did not find any effects of calcium and magnesium deficiency on epicuticular wax appearance in spruce. On the contrary, Back and Huttunen (1992); Ylimatimo et al. (1993) and Turunen and Huttunen (1996) observed in spruce and pine seedlings, treated with nutrient deficient solution (Mg, K and Ca deficiency), a significant decrease in wax amount in stomatal and epistomatal needle chambers, changes in its structure from tubelike to more fused and netlike. Additionally, spruce showed alterations in needles micromorphology (a collapsed floem, large starch grains, decreased number of thylakoids) (Back and Huttunen 1992). In most cases, wax deterioration appeared a year after treatment. Nutrient imbalance induced changes in wax morphology might be caused by changes in the synthesis of wax or a belated synthesis (Ylimatimo et al. 1993).

Particles occurrence

The occurrence of anthropogenic origin particles on the needle surface correlates in most cases with the range of air pollution in a particular area and distance from the smelter and households. The shorter the proximity the more surface of the needle covered with particles (Durasovic 1997; Godzik and Sassen 1978; Turunen et al. 1993, 1997; Grodzińska-Jurczak 1994; Manninen and Huttunen 1995; Turunen and Huttunen 1996; Grodzińska-Jurczak and Szarek-Łukaszewska 1997, Schmitt et al. 1998) (Fig. 2). Particles were scattered over the whole needle surface (especially close to the smelter), but their highest deposition was observed mainly in the stomatal regions of the needles. The highest occurrence of the

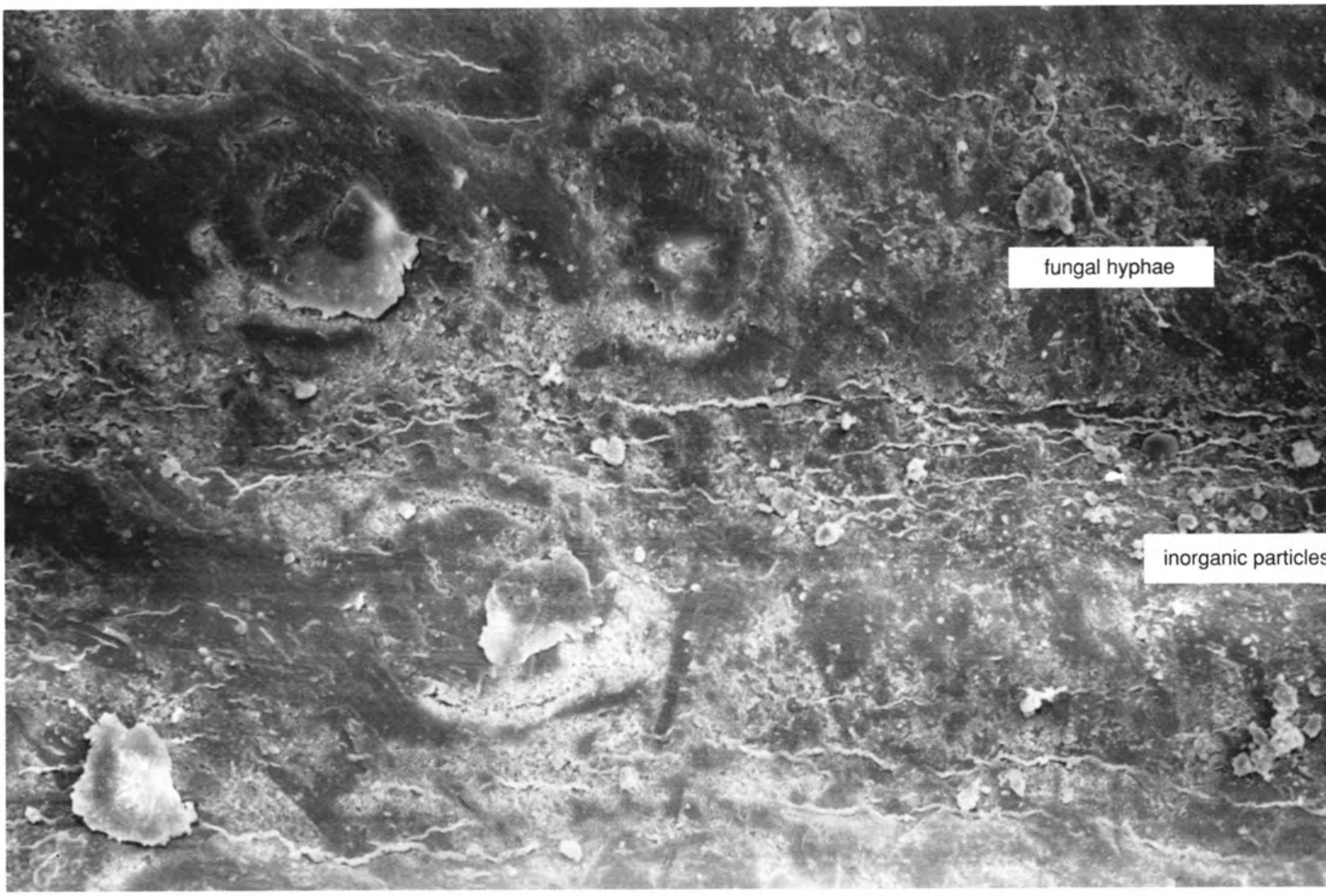

Fig. 2. Strongly eroded epistomatal wax of a 2-yr-old *Picea abies* needle, (x 350). A seriously damaged structure with no wax tubes left, occurrence of inorganic particles and fungal hyphae.

epicuticular wax there made stomatal area rough and though, easy to absorb particles (Burkhardt and Peters 1996; Turunen et al. 1997). The toxicity of particles to the needles depends on their size and chemistry. Most harmful are particles of a diameter less than 1 µm (aerosols). They may easily penetrate into needles via stomata and in many cases dissolve in its surrounding leading to phytotoxic effects in needle tissue (Turunen et al. 1992; Burkhardt and Peters 1996). Needle wax structure and chemistry may be altered due to the reactions between certain components of the cuticle with dust particles or surface tension effects (Turunen and Huttunen 1990). Dust particles may also disturb water economy of the plants by increasing the water loss. Some particles may be removed from wax surface naturally by rain, snow, fog, if the wax is intact (Neinhuis and Berthlott 1997).

Microorganisms

Leaf surface of various plant species is naturally colonised by epiphyllic micro-organisms (mainly bacteria, yeast, fungi and algae). In non-polluted areas micro-organisms may fully absorb polar chemicals avoiding their penetration through stomata. Epiphyllic organisms lead also to improvement of leaf surface condition, mainly by increasing its wettability (Schreiber 1996). Fungal penetration of the needle surface is a multistep, complicated process depending on several factors such as free moisture of the needle surface, a proper adhesion surface, humidity, low defence of host plant (Turunen and Huttunen 1990; Ylimatimo et al. 1993; Mendengen 1996). Air pollution, by altering the acidity and/or chemistry of the leaf surface, may promote or inhibit the fungal hyphae growth (Turunen and Huttunen 1990). In general, acid rains decrease the number of microofungi on the needle surface (Huttunen 1996). Trees already damaged by natural or anthropogenic factors are more vulnerable to the fungi infections leading in most cases to necrosis and needle abscission (Evans 1982; Hanks and Wright 1986; Godzik and Sienkiewicz 1990; Turunen and Huttunen 1990; Grodzińska-Jurczak 1994, 1996). The occurrence of fungi hyphae on the needle surface is positively correlated with the vicinity to the emitter, the closer to the emitter the higher number of fungi (Turunen and Huttunen 1996)

ESTIMATION OF NEEDLE WETTABILITY

Besides estimation of epicuticular wax erosion, physical and chemical plant surface alterations may be distinguished by a needle wettability assessment. Both methods give information on the leaf surface changes and should be used simultaneously or exchangeably depending on the research nature (Cape et al. 1994; Jagels 1994). Evaluation of needle wettability may be achieved by measuring the water droplet contact angles (DCA) or the water-holding capacity on needle surface (Cape 1983; Turunen and Huttunen 1990; Turunen et al. 1993; Neinhuis and Berthlott 1997). While analysing contact angles must be measured very fast after leaves sampling (within 1-3 hrs), practically often in the field conditions (Cape et al. 1994). Wettability is of great importance to the trees, since it determines ion and water exchange through the cuticle and pollutant absorption (Cape 1996; Turunen and Huttunen 1997). Needle wettability is induced mainly by structure (amounts of tubular and amorphous deposits of wax) and chemical composition (type and distribution of functional groups) of epicuticular wax. Depending on the hydrophobicity rate, particles deposited on the needle surface may also influence its wettability (Burkhardt and Eiden 1996; Schreiber 1996; Turunen and Huttunen 1997; Turunen et al. 1997).

Wettability changes may be explained by natural and anthropogenic factors. Their ratio alters with needle age, the older the tree the higher its wettability (Turunen and Huttunen 1991, 1996; Turunen et al. 1993, 1995, 1997; Donnelly and Dowding 1994; Cape et al. 1995, Kupcinskiene 1996, Schreiber 1996). Needles of trees growing in extreme climatic conditions (far north, low temperature sum) show a lower wettability than those from the southern, mild areas (Turunen and Huttunen 1996, 1997). It is essential to understand the role of the mentioned natural factors to assess properly a pollution damage to the needle surface. A mixture of anthropogenic pollutants and natural compounds (sea salts, dust particles) often found in the field can confound the interpretation of DCA and wettability measurement (Jagels 1994; Staszewski et al. 1994). Air pollutants such as ozone, sulphur dioxide and acidic fog result in increased wettability of the needle surface (Cape and Fowler 1981; Huttunen and Lane 1983; Turunen et al. 1993; Jagels 1994; Staszewski et al. 1994; Barthlott and Neinhuis 1996; Cape 1996; Turunen and Huttunen 1996; Wolfenden and Mansfield 1991). Cape et al. (1995); Turunen and Huttunen (1996) and Turunen (1996a) found changes in wettability caused by an increased SO_2 concentration in the air and lower long-term temperature sum. One of the first symptoms of needle surface to acid rain is its increased wettability. Two to three growing seasons treatment with acidic precipitation (pH 3) containing sulphuric acid or a mixture of sulphuric and nitric acids lead to their increased wettability (mostly in the oldest needles) (Turunen, 1996a, 1996b; Turunen and Huttunen 1996). Needles exposition to sulphuric acid (pH 3.1) did not result in changes to wettability while the increased contact angles of water droplets were significant after combined treatment of sulphuric acid and heavy metals (copper and nickel sulphate) (Turunen and Huttunen 1991; Turunen 1996aa; Turunen et al. 1997). Increased acidity caused shifted wettability depending on the tree species (Turunen and Huttunen 1997). In case of spruce higher wettability was observed in needles treated with acid rain of pH 3 than with water and acid rain of pH 4. The order of wettability for pine was as follows: water pH 3 > pH > 4. No effect of ozone on wettability was found (Turunen and Huttunen 1991; Cape et al. 1995). A pronounced pH dependence of wetting is mainly due to the presence and activity of epiphyllic microorganisms, while chemical composition of epicuticular wax of conifer needles does not refer to this phenomenon (Schreiber 1996). He reported that alterations in leaves' wettability property may be caused either by changes in chemistry and/or the fine structure of the wax or by increased amounts of epiphyllic microorganisms on the leafsurface. Greater extent of needle surface wettability was observed closer to the pollution sources (Kupcinskiene 1996, Turunen and Huttunen 1997). Wettability change may be a more accurate biomarker of early wax alterations at the regional level than in immediate vicinity of the smelter, where the effect may be masked by e.g. deposition of particles or fungi hyphae on the needle surface (Turunen 1996a; Turunen and Huttunen 1996; Turunen et al. 1997).

ARTEFACTS IN SEM EVALUATION OF EPICUTICULAR WAX ALTERATIONS

To use Scanning Electron Microscopy (SEM) as a successful tool for evaluating epicuticular wax structure alterations, it is important to distinguish air pollution and effects of natural factors from artefacts caused by inappropriate usage of technique (Huttunen 1994; Bermadinger-Stabentheiner 1994; 1995; Bermadinger-Stabentheiner and Grill 1995). Due to a high sensitivity of epicuticular-wax structure, a proper sampling, storage and preparation itself are of high importance (Godzik 1982; Turunen et al. 1997). Although a considerably small amount of samples is needed for SEM analysis, to make evaluation appropriate and statistically secured a large number of leaves or needles must be sampled (Godzik 1982; Hanisch and Kilz 1990; Turunen and Huttunen, 1990). While sampling the following remarks must be taken into account: time, day and season of collection, height of the tree the samples are taken from, exposition to the polluting substance, age of the needle, characteristics of natural factors (temperature, wind), conducting a time series (frequent sampling e.g. in monthly intervals) (Hanisch and Kilz 1990). If sampling time is not standardized a detailed description of season, month, and time of the day of collection must be included into the protocol. Wax synthesis or erosion degree may be affected by the time factor (Cape et al. 1994). A careful sample handling is needed, while transporting a mechanical abrasion and overheating must be avoided, since it may result in superficially squashed wax structures in the antechamber and on the surrounding surface. Similar symptoms of severe damage (a smeared epicuticular wax layer and squashed tubes) might be caused also by a number of natural factors (e.g. wind-borne particles, snow, ice) (Bermadinger-Stabentheiner 1994; 1995). The SEM method should not be used if the plant surface is covered by many particles or biotic infection because samples are analysed together with the wax tubes (Manninen et al. 1996). For a safe storage leaves should be either cryostored or kept in a turgid state protecting from eventual biological deterioration (e.g. microbial growth) (Cape et al. 1994). It is recommended to use paper bags instead of air-tight glass vials or air-tight boxes for storage. Epicuticular wax structure of needles kept in glass vials and air-tight boxes were deteriorated (a melted antechamber wax with no crystalline structures) comparing to those from the paper bags. It has been supposed that water or volatile substances may accumulate within the needle durring storage, then are emitted resulting in wax structure destroy (melting or dissolving them). Usage of air-permeable paper bags may significantly decrease the wax alteration rate (Bermadinger-Stabentheiner 1994). An appropriate material storage enables the use of needles at least for two years after sampling (Bermadinger-Stabentheiner 1995). After collection fresh wax material may be fixed in 70% alcohol, FAA fixative or 2% (W/v) osmium vapour (Godzik 1982; Huttunen 1994). Using fresh leaves, they should be kept in non-humid environment; high humidity may result in fungal development identified in SEM analysis as natural epiflora (Cape et al. 1994). Using not fresh material, a proper needle fixation is needed. The most preferable method is freeze-drying. Air-dried leaves are to show more artifacts (wax plugs extrusion, wax cracking) than fresh, cryostored ones (Cape et al. 1994). If using it, gentle air-drying in a mild room temperature should be introduced, as least injuries to wax structure in that condition were noticed (Godzik 1982; Huttunen 1994). Although recently freeze-drying processes have been favoured, using them one ought to interpret SEM micrographs carefully. Freeze-drying (e.g. liquid nitrogen) may lead to remarkable alterations in ultrastructure and appearance of epicuticular wax (Godzik 1982; Huttunen 1994; Bermadinger-Stabentheiner 1995; Bermadinger-Stabentheiner andd Grill 1995). Particularly sensitive to freeze-drying are young needles. Shrinkage processes caused by freezing result in appearance of fissures in antechamber wax. These symptoms do not arise in well developed needles. The use of LTSEM may eliminate such artefacts (Hanisch and Kilz 1990; Bermadinger-Stabentheiner 1995; Bermadinger-Stabentheiner andd Grill 1995).

CONCLUSIONS

Structure and chemistry of epicuticular wax covering the plant surface may be changed directly or indirectly by a variety of air pollutants. The rate of damage depends on the pollutant type, dosage, tree age, species and growth conditions. Using scanning microscopy (SEM), it is possible to distinguish forest damage at a very early stage, before visible symptoms occur. However, only a proper sampling, storage, preparation and methods enable a high repeatability and can make wax a sensitive and accurate biomarker of air pollution damage. Future research should be focused on detecting and better understanding of physiological and biochemical changes in leaves, as well as considering the possible effects of epiphytic microorganisms' wetting, which may result in epicuticular wax deterioration. If possible, epicuticular wax as an indicating method should be included into the national forest monitoring system and used parallely with conventional methods of tree damage evaluation (defoliation, disscoloration of foliage).

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WOSKI EPIKUTYKULARNE DRZEW IGLASTYCH JAKO BIOMARKER ZANIECZYSZCZEŃ POWIETRZA – PRZEGLĄD BADAŃ

STRESZCZENIE

Woski epikutykularne okrywające powierzchnię roślin są używane jako biomarkery oceny zanieczyszczeń powietrza. Opisanie wielu zmian w budowie i składzie chemicznym wosków na skutek czynników antropogenicznych i naturalnych było możliwe dzięki zastowaniu skaningowej mikroskopii elektronowej (SEM). SEM pozwala na rozpoznanie zniszczeń wosków we wczesnym etapie, jeszcze przed wystąpieniem widocznych symptomów. Symptomy uszkodzeń wosków nie są na ogół specyficzne dla poszczególnych rodzajów zanieczyszczeń. Do najczęściej opisywanych zmian w strukturze i chemiźmie wosków należą: nierozwinięta struktura krystaliczna wosków, różne rodzaje zlewania się pałeczek woskowych lub ich erozja (zdeformowanie i dysfunkcja kompleksów aparatów szparkowych, obniżenie nagromadzenia woskowych pałeczek, przeobrażanie się wosków o budowie krystalicznej w formę amorficzną), zwiększone roczne tempo erozji wosków, zmiany w składzie chemicznym i zwilżalności igieł. W celu poprawnej oceny degradacji wosków, konieczne jest rozróżnienie ich uszkodzeń spowodowanych czynnikami naturalnymi lub zanieczyszczeniami powietrza od artefaktów wystepujących na skutek nieprawidłowości przy stosowaniu metody SEM.

SŁOWA KLUCZOWE: woski epikutykularne, zmiany w strukturze i składzie chemicznym, mikroskopia skaningowa (SEM), biomarker, czynniki antropogeniczne i naturalne, zanieczyszczenia powietrza, artefakty, drzewa iglaste.