Free Rad. Res. Comms., Vol. 3, No. 1–5, pp. 185–192
Photocopying permitted by license only

Printed in Great Britain

INVESTIGATIONS ON SPRUCE DECLINE IN THE BAVARIAN FOREST

W.F. OSSWALD and E.F. ELSTNER

Institut für Botanik und Mikrobiologie Technische Universität München, Arcisstrasse 21, 8000 München 2, Bundesrepublik Deutschland

(Received September 10th 1986)

KEY WORDS: "Waldsterben", photodestruction, host-parasite relationships, p-hydroxyacetophenon.

INTRODUCTION

Phytomedical "diagnostic data" concerning recent spruce decline in the Bavarian Forest indicate that parallel to photodynamic bleaching reactions, oxidative damage may cause the loss of resistance towards different fungal invaders. The loss of resistance is manifested by the destruction of the wax plugs covering the stomatal cavities while increased fungal infection is measured from the increase of ergosterol in damaged needles. Thus, needle loss appears to be a two step process involving predamage by environmental factors such as ozone followed by necrotic processes induced by secondary fungal infection.

RESULTS AND DISCUSSION

The results of some experiments concerning the reasons for spruce deline in Bavaria are reported in this investigation. The symptoms we found on spruce are complex. In most cases the oldest needles are bleached, with the exception of the current year's growth. The oldest bleached needles eventually become necrotic and abscise. A very important observation is that the needles of the upper side of a branch are frequently more strongly bleached than needles of the opposite side.[1] This can be taken as an indication of light dependent processes being involved in the bleaching reaction. The symptoms described can be found at higher altitudes in the Bavarian Forest (about 800 meters above sea level). In the Bavarian Forest very low sulphurdioxide concentrations are measured in the air. Therefore, these areas are called "clean air regions." In these areas photooxidants (with ozone as the main component) are assumed to play a certain role in spruce decline.

Pigment bleaching is well-known in the plant kingdom. Bleaching reactions are often caused or accelerated by light. Reactive oxygen species such as superoxide, hydrogen peroxide or hydroxyl radicals are considered to be initiators of pigment bleaching. These reactive oxygen species are formed in the chloroplast under conditions of "overreduction" in the electron transport chain.[2] A detoxification cycle is localized in the stroma of the chloroplast which converts superoxide radicals and

TABLE I
Content of ascorbic acid and glutathione of differently damaged spruce needles

Ascorbic acid/Glutathion										
year symptom	84 green	84 green/yellow	83 green	83 yellow	82 green	82 yellow	81 green	81 yellow	80 green	80 yellow
Chlorophyll (mg/g dw)	2.2	2.0	2.7	1.7	3.2	0.76	3.3	0.7	2.9	1.4
Ascorbic acid (mg/g dw)	1.97	4.4	3.9	5.1	5.5	7.6	4.6	6.2	4.4	7.8
DH Asc acid (mg/g dw)	0.39	1.0	0.3	0.8	0.06	0.3	0.6	0.6	0.2	2.0
GSH (μg/g dw)	51	84	46	104	84	203	57	120	41	202

hydrogen peroxide to water at the expense of ascorbic acid, glutathione and NADPH[3]. Therefore, we first looked for the metabolites of this cycle and determined the content of oxidized and reduced ascorbic acid and glutathione, (GSH; GSSG) of green and bleached needles of different ages.

In Table I the amount of ascorbic acid and glutathione in bleached and green needles are compared. All bleached needles exhibit a higher amount of both ascorbic acid and glutathione as compared to green needles. The increased amount of ascorbic acid and glutathione in all bleached needles may be taken as indicative of the fact that chloroplasts may be overflooded with reactive oxygen species during bleaching, thus enhancing these counteracting metabolites. The increased detoxification capacity will not prevent pigment oxidation which is mainly caused by singlet oxygen within the thylakoids. However, ascorbic acid which is localized in the stroma will detoxify superoxide radicals and hydrogen peroxide. Therefore further oxidative processes e.g. lipid peroxidation which will lead to membrane leakage will be hindered.

Before we performed experiments concerning internal metabolites we took a great number of scanning-electron-microscopic pictures, comparing the surfaces of green needles with those of bleached and necrotic ones. Figure 1 shows the surface of a healthy green needle, with the typical wax plugs covering the stomata. Figure 2 shows the surface of a bleached needle. The main differences are the partially or completely destroyed wax plugs. Sometimes gaps are formed which may allow pathogens to penetrate the needles. On necrotic needle surfaces, we often see typical reproductive structures of several forest pathogens. In Fig. 3 we see the pycnidia of the forest pathogen *Rhizosphaera kalkhoffii* growing through the stomata carrying wax plugs on top of the pycnidia. It is very hard, however, to quantify these visible symptoms. In our case we had to quantify fungal infection. Therefore we looked for a specific fungal metabolite which indicates a biochemical indicator for fungal infection. We chose ergosterol, since it is a specific membrane component of fungi and cannot be found in higher plants. This method was introduced by Seitz *et al.* in 1977.[4] Seitz *et al.* used ergosterol for quantifying fungal infections of differently infected cereal crops. We transferred this method to spruce needles.[5]

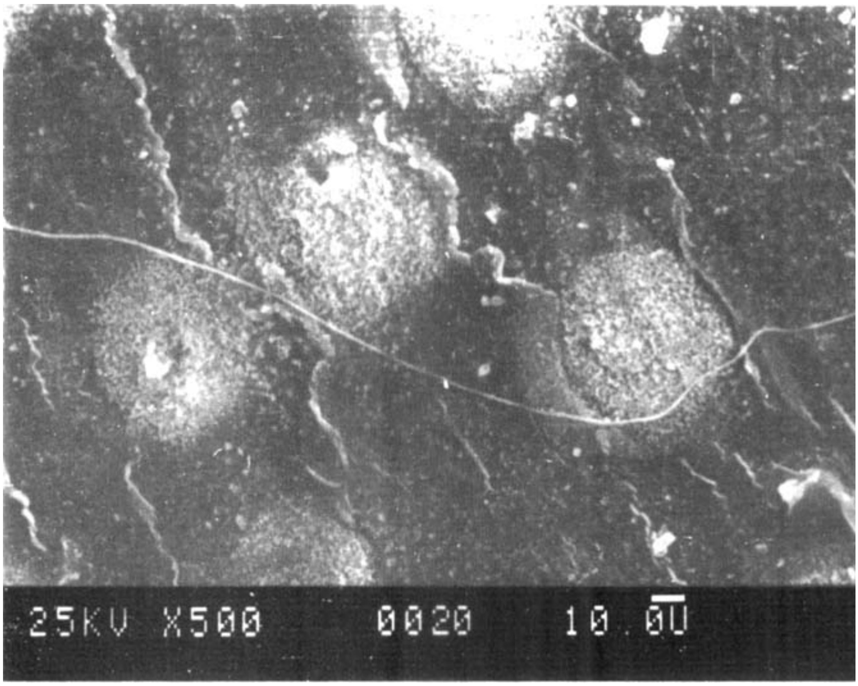

FIGURE 1 Surface of a green needle (magnification × 500).

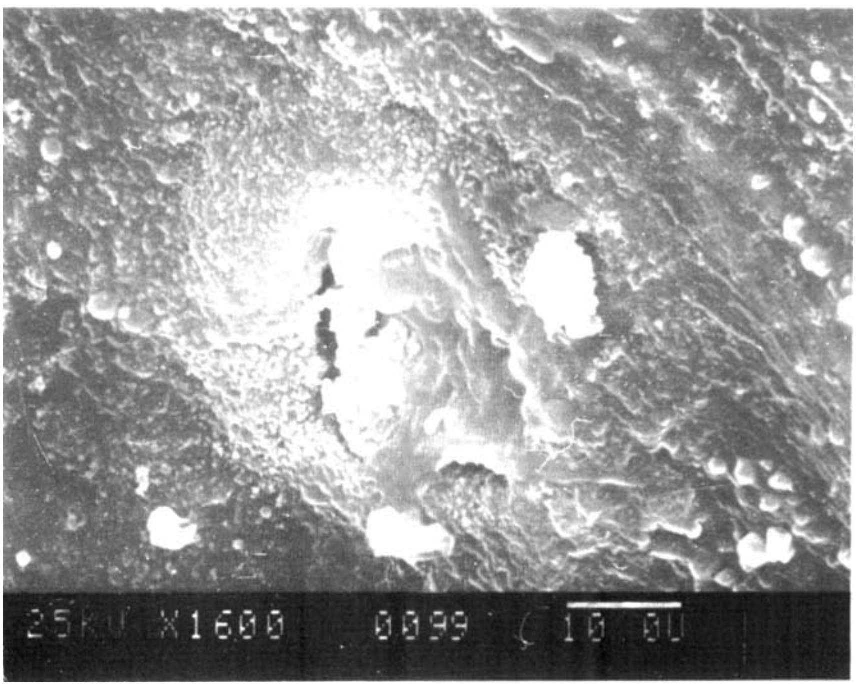

FIGURE 2 A wax plug of a bleached needle (magnification × 1600).

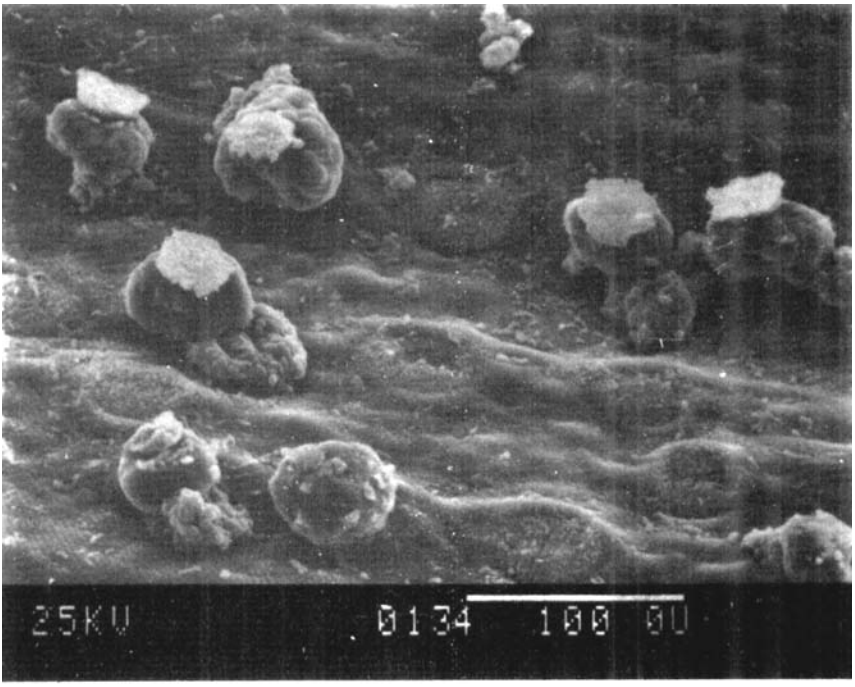

FIGURE 3 Surface of a necrotic spruce needle with pycnidia of *Rhizosphaera kalkhoffii* breaking through the stomata (magnification × 500).

In Table II the ergosterol contents of different important forest pathogens are shown; (obtained from Prof. Butin, Braunschweig). We compared the ergosterol content of green, yellow and necrotic needles of different age. Most green and bleached needles exhibited small ergosterol contents. Often the ergosterol level of bleached needles was greater compared with green needles. However, all necrotic needles showed the highest ergosterol contents. The ergosterol concentration correlated with the amount of necrotic parts of the needles (Tables III and IV). We believe that green needles are already infected to a certain degree. The fungi inside bleached needles may grow faster, and fungal infection may be finally responsible for the necrotic reaction. Necrotic needles are dead, whereas bleached needles are still alive.

Host-parasite interactions are well known in animals as well as in the plant world. Most higher plants contain fungitoxic compounds which are stored as inactive glycosides and which are activated during the growth of the fungi inside the host.

TABLE II
Ergosterol content of different forest pathogens

	Ergosterol content (μg/g dw)
Rhizosphaera kalkoffii	255
Sirococcus Typ II	119
Lophodermium piceae	95

TABLE III
Ergosterol content of green and bleached spruce needles

year symptom	84 green	84 green/yellow	83 green	83 yellow/green	82 green	82 yellow	81 green	81 yellow
Chlorophyll (mg/g dw)	2.2	2.0	2.7	1.7	3.2	0.76	3.3	0.7
Ergosterol (ng/g dw)	200	387	227	533	334	551	540	479

TABLE IV
Ergosterol content of green, bleached and necrotic spruce needles

year symptom	84 green	84 green/yellow	83 green	83 green/yellow	82 green	82 yellow	green/yellow + necrotic green/yellow	more than 50% necrotic tips
Chlorophyll (mg g dw)	3.5	2.8	2.6	2.0	3.2	1.4	—	—
Ergosterol (ng g dw)	—	22	36	47	37	32	489	3295

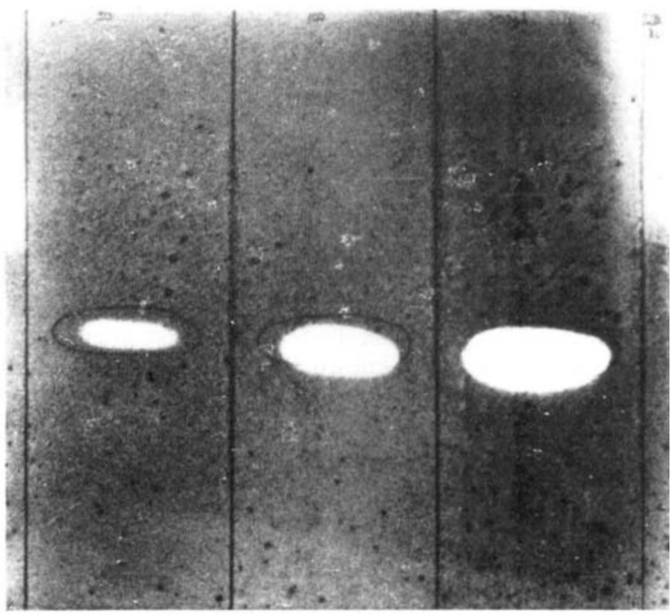

FIGURE 4 TLC-plate with white inhibition zones of the growth of *Rhizosphaera kalkhoffii* brought about by chromatography of needle extracts.

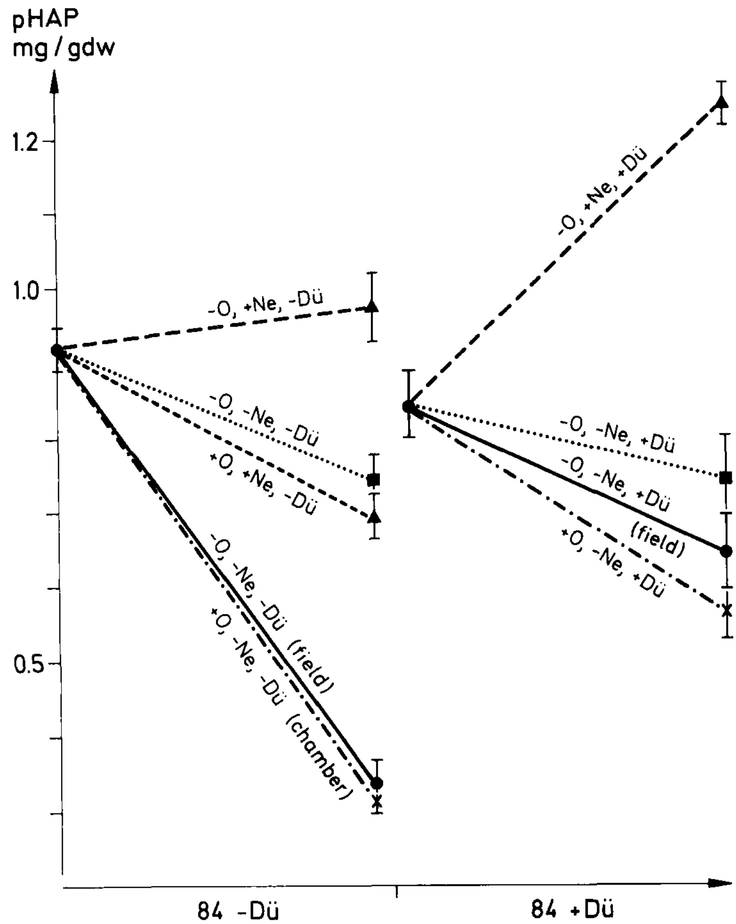

FIGURE 5 Content of p-hydroxyacetophenon of one year old spruce needles after different treatments. +/−O = with and without ozone (90 $\mu g/m^3$), +/−Ne = with and without acid fog (pH = 3.0, pH = 5.6), +/−Dü = with and without fertilizing.

Other fungitoxic compounds are synthesized by the host after contact with the pathogen. These substances are well-known in herbaceous plants and are called "phytoalexins". However, little is known about conifers with the exception of the work of Franich *et al.*[7] They found derivatives of the dehydroabietic acid in the wax layer of *Pinus radiata* needles which showed fungistatic activity towards the pathogen *Dothistroma pini*. They further demonstrated that these substances may play an important role in the defence reaction towards this pathogen. We performed similar experiments with spruce needles. We extracted a compound which showed fungitoxic activity towards *Cladosporium cucumerinum* and *Rhizospaera kalkhoffii* in the plate test according to Homans and Fuchs.[8] Its structure was deduced as p-hydroxy-acetophenol (p-HAP) which has been known for a long time as a component of spruce needles.[9] For the first time we could demonstrate that this compound exhibits fungitoxic activity (Fig. 4). Further experiments are currently performed to show whether

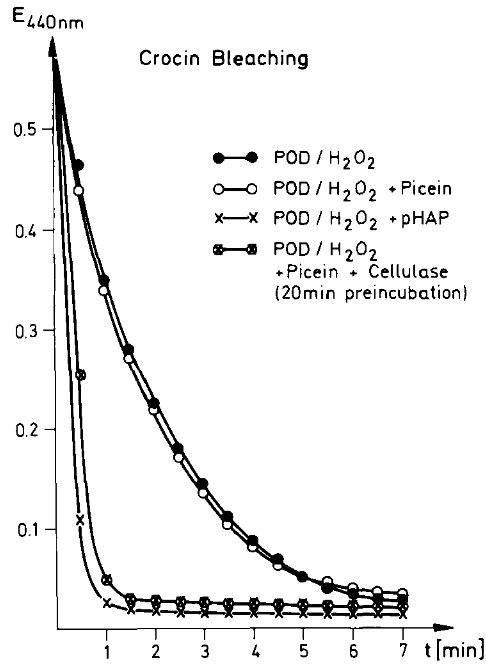

FIGURE 6 Bleaching of "crocin" with Peroxidase (POD), H_2O_2, p-hydroxyacetophenon and Picein.

p-hydroxyacetophenon plays a role in the defence reaction of *Picea abies* towards different forest pathogens.

In the first experiments which were carried out in collaboration with the "GSF" in Munich/Neuherberg we could demonstrate that the content of p-HAP is reduced in the needles after exposure to ambient ozone concentrations (Fig. 5).[10] We could further demonstrate that p-HAP may be involved in the bleaching reaction as shown in Fig. 6. Peroxides in the presence of hydrogen peroxide bleaches the model carotenoid crocin to some extent. This reaction is strongly stimulated by p-HAP. If p-HAP is substituted by the native picein no stimulation is observed. After a preincubation of picein with cellulase (introduced by the pathogen), which converts picein to p-HAP, we observe a stimulation of the bleaching reaction comparable to that in the presence of p-HAP.

SUMMARY

The primary damaging reactions in spruce needles may operate as follows:

1) Trees under "stress" produce the plant hormone ethylene.

2) Ethylene and ozone react extremely fast forming hydrogen peroxide and formaldehyde, compounds which may damage the wax layer.

3) Ozone as a very agressive oxidant will inactivate membrane bound enzymes through oxidation of their thiol groups. Thus the translocation of sugars from the chloroplast into the phloem may be inhibited or blocked. The result will be an

"overreduction" of the electron transport chain resulting in the formation of reactive oxygen species in the light. These reactive oxygen species will induce lipid peroxidation and pigment cooxidation.

4) The visible effects are bleached needles and an impairment of structural resistance against fungal infections.

5) In addition ozone will directly reduce the content of antifungal compounds such as p-HAP.

6) Furthermore p-HAP may be involved in the bleaching reaction after its release from picein.

7) Finally, fungi may penetrate the needles and eventually grow faster in bleached needles. Infected needles will become necrotic and abscise.

Acknowledgments

We wish to thank Prof. Butin (Insititut für Pflanzenschutz im Forst, Braunschweig) for the gift of different forest pathogens. We also thank H. Senger, W. Schütz, S. Zieboll and S. Lachner for performing different experiments. This work was financially supported by the Bayer. Staatsministerium für Landesentwicklung und Umweltschutz.

References

1. Oßwald, W.F. und Elstner, E.F. *Forstwiss. Cbl.*, (in press) (1986).
2. Elstner, E.F., Oßwald, W.F. and Youngman, R.J. *Experientia*, **41**, 591–597, (1986).
3. Foyer, C.H. and Halliwell, B. *Planta*, **133**, 21–25, (1976).
4. Seitz, L.M., Mohr, H.E., Burroughs, R. and Sauer, D.B. *Cereal Chem.*, **54**, 1203–1207, (1977).
5. Oßwald, W.F., Höll, W. and Elstner, E.F. *Z. Naturforsch.*, **410**, 542–546, (1986).
6. Bailey, J.A. and Mansfield, J.W. Phytoalexins. Blackie, Glasgow, (1982).
7. Franich, J.A., Gadgil, P.D. and Shain, L. *Physiol. Plant Pathol.*, **23**, 183–195, (1983).
8. Homans, A.L. and Fuchs, S. *J. Chromatogr.*, **51**, 327–329, (1970).
9. Esterbauer, H.D., Grill, D. und Beck, G. *Phyton (Austria)*, **17**, 87–99, (1975).
10. Oßwald, W.F., Heinisch, H. und Elstner, E.F. *Forstwiss. Cbl.*, **105**, 261–265, (1986).

Accepted by Prof. H. Sies