Ascorbic Acid and Chlorophyll Content in Cell Cultures of Spruce *(Picea abies)*: Changes by Cell Culture Conditions and Air Pollutants

Burkhard Messner and Jürgen Berndt

Gesellschaft für Strahlen- und Umweltforschung München, Abteilung für Zellchemie,
D-8042 Neuherberg, Bundesrepublik Deutschland

Conifers contain ascorbic acid which is probably a protective against oxidative attacks. In yellow needles of damaged conifers the ascorbic acid content is increased, this suggests an induction by air pollutants and/or photooxidants of a protection mechanism in trees. To study this mechanism in more detail, a green cell suspension culture of spruce *(Picea abies)* has been established. These cells contain chlorophyll and have a photosynthetic capacity, but they need carbohydrates (sucrose) for growth and are thus photoheterotrophic. Their ascorbic acid content and its regulation depends on carbohydrates, the cells resemble healthy green needles in this respect; they appear to be qualified for studies on the induction of a defense mechanism.

Treatment of these cells with SO_2 (20 ppm, 4 days) ozone (1 ppm, 14 days) and vaporous H_2O_2 revealed a decrease of the ascorbic acid content and (to a lesser extent) also of chlorophyll. It is concluded that (photo-)oxidants obviously do not play such a dominant role in bleaching of needles as it is assumed. The increased ascorbic acid content found in needles of damaged conifers is probably not an induced protection mechanism against oxidants.

Introduction

During recent years in large forest areas in Germany and other countries a rapidly increasing decline of spruce and other conifers has been observed [1]. As a visible symptom, the oldest needles become yellow and finally necrotic before abscission. In most cases, the light exposed needles show stronger symptoms than shaded needles. Several hypotheses have been put foreward to explain this forest dieback. Besides mineral deficiencies and extreme climatic conditions, different pathogens [2] and gaseous air pollutants as SO_2, ozone [3] and other peroxidants [4, 5] are discussed to be involved in the spruce decline. These discussions still continue [6, 7]. To find out the factors causing this decline, a great number of investigations was started, and many biochemical parameters in the needles have been measured. Since it is generally assumed that oxidative pollution plays a dominant role in the damaging process, interest was thus focussed on protection mechanisms against oxidative attacks.

In yellow needles an elevated level of ascorbic acid (and of glutathion) was found and generally a decrease in the content of chlorophyll was observed. This increase in ascorbic acid content was about two-fold compared to green needles of the same tree and was found to be widespread within different damaged areas [8]. The higher ascorbic acid level was interpreted as a defense mechanism induced against oxidative attacks. This assumption is supported by the results of investigators who could demonstrate that the increase of the content of antioxidants in various conifers [9] and also herbaceous plants [10] occurs after fumigation with SO_2 or ozone. It seems to be a contradiction, however, that in spruce needles with an elevated ascorbic acid content and hence well protected against oxidative stress, the chlorophyll content is decreased as a consequence of bleaching by photooxidants.

Since SO_2, ozone and, more recently, also H_2O_2 [11, 12] are assumed to participate in lipid peroxidation and pigment destruction [5, 13], we have studied their effect on the content of ascorbic acid and chlorophyll. For this purpose we have established a cell culture of spruce *(Picea abies)*, since plant cell cultures are known to be a good tool to study plant defense mechanisms [14], they are much easier to handle than plants and, last but not least, the cells are available at any time.

Materials and Methods

Cell culture

Picea abies seedlings were washed thoroughly with sterile Tween 80 solution (0.01%, 5 min), desinfected in 15% H_2O_2 (1 min), rinsed twice with sterile distilled water (2 min) and immersed in 0.1% $HgCl_2$ for 2 min. The surface sterilized seeds germinated on humid tissue cloth (Kleenex) in the dark under sterile conditions after 14 days. These aseptically germinated cotyledons were maintained for three days in the light and then immersed in 70% ethanol (5 sec), 30% H_2O_2 (5 sec) and rinsed with sterile distilled water (15 sec). The sterile cotyledons were placed on Murashig and Skoog medium with 1% agar supplemented with 3% sucrose, the phytohormones benzylaminopurine (1 mg/l) and naphthylacetic acid (3 mg/l) (these amounts were found to be optimal), incubated at 27 °C and they were illuminated with Fluora fluorescent lamps (Osram L 40 W/77, 1000 lux). After 4−6 weeks, the cotyledons become covered with a green-brown callus, containing also many white cells; this callus was subcultured every two weeks. A green callus culture with a chlorophyll content of 200−300 µg/g fresh weight was obtained by the selection. However, these calli were not yet suited for the experiments designed, because their ascorbic acid and chlorophyll content varied from callus to callus to an intolerable extent.

For improvement of the homogeniety of these cells a cell suspension culture of spruce cells was established. Pieces of the above callus were transferred to a liquid culture medium of the same composition as used for the callus culture, but without the agar. The suspension cultures were grown at 27 °C in 1800 ml Fernbach flasks containing 300 ml of the medium and rotating at 60 rpm under continuous light as described above. After three days the growth medium was decanted and substituted by fresh growth medium, after additional four days 150 ml of the suspension culture was added to 150 ml fresh medium. Then again after three days the medium was decanted and fresh growth medium was added, after an additional four days again the suspension culture was divided and so on. Principally, the suspension culture was divided after seven days and the medium was changed during this growth period. This rather laborious procedure was found to be obligatory for the continuous suspension culture of green spruce cells, otherwise the cells became brownish, probably due to production and secretion of phenolic compounds (?) into the medium.

When necessary, in a final step homogenous callus probes were obtained by spreading suspension cells on a double layer of thick filter paper soaked with medium, and cultured in Petri dishes under light conditions as described above at 27 °C. After about seven days the filter paper strips are covered with newly grown cells, these cells were designated "secondary callus" and were used in most experiments.

Assay of CO_2-fixation

In the standard assay 100−300 mg of cells (secondary callus) were placed in a styrene tube (vol 13 ml) on a filter strip (2 × 6 cm) moistened with 0.6 ml of culture medium. At the neck of the lying tube, two separate droplets of each, 20 µl $NaH^{14}CO_3$ (5.78 µmol, 1 µCi) and 20 µl 1 N H_2SO_4, were placed; additionally, a small magnetic stirrer was placed beside the droplets. The tube was then closed with a screwcap and the $^{14}CO_2$ was released by mixing the droplets with the internal magnetic stirrer which was moved by an external magnetic stirrer. The final CO_2-concentration was 1% in the gaseous phase. The tubes with the cells were maintained at 25 °C under the illumination of 10,000 lux (halogen vapour lamp, Osram TL 40/25) for 30 min. Tubes serving as dark controls were covered with aluminium foil. After the fixation period, tubes were opened and CO_2-fixation was terminated by the addition of 2 ml of a mixture of ethanol and 1 N HCl (4 + 1). The filter strips were removed and were washed with the ethanol-HCl mixture, the cells were then homogenized in this ethanol-HCl mixture by use of a Potter homogenizer. The homogenate was brought to a final volume of 5 ml by the addition of the ethanol-HCl mixture. A sample of 1 ml was mixed with scintillation cocktail (Rotiscint 22) and CO_2-fixation was measured in a scintillation counter. In the fixation experiments with cotyledons, four small green bunches (38−51 mg total) were used instead of secondary callus cells.

Incubation of spruce cells with carbohydrates, SO₂, ozone and vaporous H₂O₂

When the effect of carbohydrates was studied, the culture medium of the secondary callus was sucked off by vacuum, the cells were washed with the desired medium containing carbohydrates and then placed in Petri dishes with the new medium and incubated.

In the experiments with SO_2 and ozone exposure, the strips with the secondary callus were placed in Petri dishes with caps slightly lifted (2−3 mm) to enable gas exchange. The treatment with SO_2 (20 ppm) was carried out in small styrene boxes (27 °C, Fluora Osram 1000 lux) combined with a gas dosing system (Dimikur from Wösthoff, Bochum). The exposure to ozone (1 ppm) was carried out in climate chambers containing air (control) or air plus ozone.

For the treatment with vaporous H_2O_2, the strips with the secondary callus were placed in small open dishes (50 mm in diameter) and these were then put into larger dishes (90 mm in diameter). The larger dishes contained 10 ml of H_2O_2-solution of the appropriate H_2O_2-concentration. The larger dishes were then closed and wrapped with parafilm so that the vaporous H_2O_2 could reach the strips with the secondary callus, but the H_2O_2-solution had no contact with the cells. The actual H_2O_2 concentration which was effective to the cells, was determined in separate, closed dishes. H_2O_2 solution as used in the cell experiments were incubated for 5, 20 and 70 h respectively. After this time periods, the H_2O_2 concentration was measured in the condensate formed at the cover of the dishes. 20 µl of condensate was diluted with 1 ml H_2O and measured at 240 nm. Equilibrium between H_2O_2 solution at the bottom and the condensed solution at the cover was reached after about 20 h. The effects of H_2O_2 on the ascorbic acid and chlorophyll content as shown in Fig. 5 are thus caused by actual H_2O_2 concentrations varying between zero and about 5 percent.

Extraction and determination of ascorbic acid and of chlorophyll

For ascorbic acid determination cells were homogenized in 1.5% metaphosphoric acid (100 mg cells in 1 ml acid, at 0 °C) by use of a Potter homogenizer, the homogenate was sonified (1 min)

and centrifuged at $15,000 \times g$ (5 min, 0 °C). The supernatant was used for ascorbic acid determination by HPLC analysis: an ion exchange HPLC column from Serva (DEAE Daltosil 100, 4 µ, 46 × 250 mm) in combination with ammonium phosphate buffer (100 mM, pH 4.5) as the mobile phase (flow rate 1 ml/min) was used. Ascorbic acid was monitored at 272 nm and was referred to an authentic standard (Sigma München). For chlorophyll determinations the cells were extracted with 80% acetone and the chlorophyll content in this extract was measured according to Arnon [15].

The results are referred to as g fresh weight. The cells (suspension cells were collected by filtration) were placed on Kleenex tissues, slightly pressed and after 2 min weighted (without the tissue). The values of fresh weight were well reproducible and the deviations were less than 10%. The cells were stored in liquid nitrogen.

Results

Cell growth, photosynthetic capacity, ascorbic acid content, effect of carbohydrates

The establishment of callus cultures of conifers has been described [16, 17], but the cultivation of spruce cells as a green cell suspension has not yet been reported. The difficulties to maintain such cell suspension cultures are probably due to latent infections of the calli which are used to initiate the suspension culture. In the procedure described here special attention has been paid therefore to the preparation of sterile cotyledons serving as source for the calli.

The green callus culture of spruce which was prepared from seedlings (see Materials and Methods) contains ascorbic acid, but the content varied from callus to callus and no reproducible results could be obtained for unknown reasons. Therefore we have established the suspension culture and from this the secondary callus was prepared.

The growth rates of cells in suspension and those of the secondary callus produced from the suspension culture are quite similar (Fig. 1). During the logarithmic growth phase the fresh weight of the cultures doubled within seven days. The chlorophyll content of both, callus and suspension cells, is about 100−150 µg/fresh weight, which is similar to that of other cells grown in culture (*Chenopodium rubrum*, 80 µg/g [18], *Cytisus sco-*

parius, 100 μg/f fresh weight [19]), but much lower than the chlorophyll content in healthy needles (1500 μg/g).

The cells (secondary callus) have a photosynthesizing capacity. Table I summarizes the results of the CO_2-fixation experiments, and it is evident that the cells make photosynthesis. Remarkably, the photosynthetic rate measured as μmol/mg chlorophyll × h^{-1} is the same in cultured cells and intact plants, i.e. in cotyledons. In cotyledons, however, the chlorophyll content is much higher and, as a consequence of this, CO_2-fixation measured as μmol/g fresh weight × h^{-1} is about six times higher in cotyledons than in the cells. Further, the dark-light difference in the CO_2-fixation rate is markedly higher in the cotyledons.

These CO_2-fixation experiments clearly show that the chlorophyll in the secondary callus cells does not make the cells merely green but is part of a functional photosynthetic apparatus, like in the intact plant. Due to the low chlorophyll content in the cells compared to that in the cotyledons, however, their photosynthesizing capacity is low. This and the fact that the cells need sucrose for growth suggest that the cells have to be classified as photomixotroph or even photoheterotroph, they are not photoautotrophic cells.

The ascorbic acid content was higher in the secondary callus as in the cells from the suspension and varied between 50 and 250 μg/g fresh weight at the beginning of the growth phase of the cells (see day zero in Fig. 1−5). During growth a maximum of the ascorbic acid content was found in the secondary callus in most cases, the reason for this increase is unknown. The ascorbic acid content in the cells is lower than that in green needles, which is between 1.3 and 2.7 mg/g dry weight [8], corre-
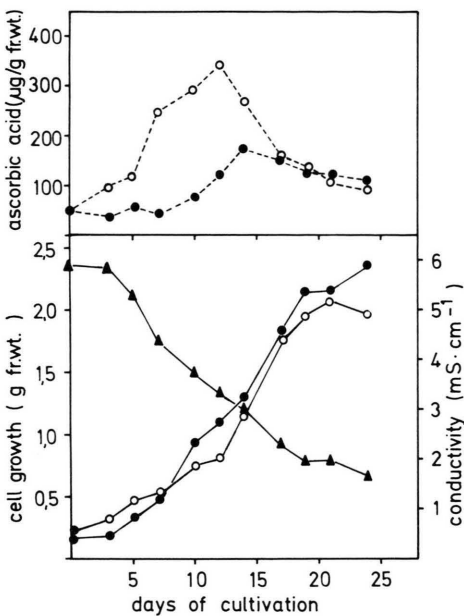

Fig. 1. Growth curves and ascorbic acid content of cells grown in suspension or as secondary callus. Cells were grown as described in Materials and Methods. ●——● cells in suspension, ○——○ secondary callus, ▲——▲ conductivity of growth medium; ascorbic acid content in suspension cells ●−−−● and in secondary callus ○−−−○. Mean values of two experiments.

sponding to about 650−1350 μg/g fresh weight. Since the secondary callus can easily be produced and can much better be handled than suspension cultures, it has been used in most experiments.

To simulate the variations of the ascorbic acid content which occur under physiological conditions in needles, the cells were cultured with different sugars. As can be seen from Fig. 2, the level of ascorbic acid in secondary calli grown in the pres-

Table I. CO_2-fixation of spruce cells (secondary callus) and of spruce cotyledons. Cells and cotyledons were incubated with $^{14}CO_2$ as described in Materials and Methods. Cotyledons were 14 days old. Data of CO_2-fixation are means of four determinations ± SEM, data of chlorophyll are means of two samples.

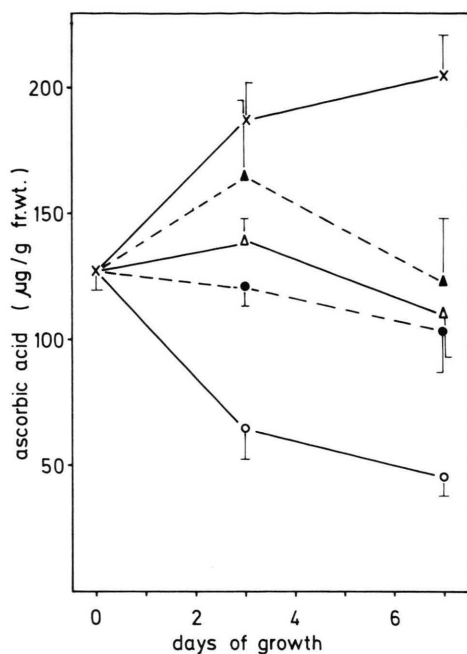|	Chlorophyll μg/g fr.wt.	CO_2-fixation			
		μmol/mg Chl. × h^{-1}		μmol/g fr.wt. × h^{-1}	
		light	dark	light	dark
Second. callus					
Expt. 1	290	55.9 ± 0.9	11.5 ± 0.7	16.2 ± 0.3	3.3 ± 0.2
Expt. 2	285	50.0 ± 1.2	10.5 ± 0.8	14.3 ± 0.4	3.0 ± 0.2
Cotyledons	1846	53.1 ± 1.8	0.76 ± 0.11	98.0 ± 3.3	1.4 ± 0.2

Fig. 2. Effect of carbohydrates on the ascorbic acid content in spruce cells. Secondary callus cells were cultured as described in Materials and Methods. After seven days of culture, the medium was changed and cell culture was continued in medium containing (90 mм each): sucrose ×——×, galactose ▲——▲, fructose △——△, glucose ●——●, without sugar ○——○. Mean values from four probes with SEM.
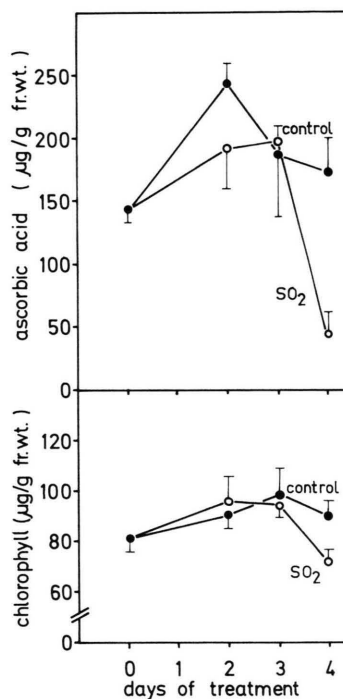

Fig. 3. Effect of SO_2 on the chlorophyll and ascorbic acid content in spruce cells. Secondary callus were cultured for seven days as described in Materials and Methods. The cells were then treated with 20 ppm gaseous SO_2. Mean value of four probes with SEM.

ence of sucrose is higher than in those grown with galactose, fructose or glucose. Without sugar the content of ascorbic acid declined to low values. The decrease from a maximum of the ascorbic acid content (Fig. 1), however, is not the consequence of sugar depletion in the medium (3% sucrose), since cell growth still continues (lower pannel in Fig. 1).

In trees also a close correlation exists between the content of carbohydrates and the ascorbic acid content, because both, sugar and ascorbic acid content, show the same annual rhythm: during winter the content of sugar and ascorbic acid is higher than in summer [20].

Effect of SO_2, ozone and vaporous H_2O_2 on the chlorophyll and ascorbic acid content

Fig. 3 shows the effect of 20 ppm SO_2 on the chlorophyll and ascorbic acid content. A decrease

of ascorbic acid content occurred, but not earlier than at prolonged treatment of four days, while chlorophyll was less affected. Ozone also has an effect after prolonged treatment and here also ascorbic acid was more sensitive than chlorophyll (Fig. 4). In the control cells the chlorophyll content slightly increased during cell growth in two weeks, while in ozone treated cells the chlorophyll content remained almost unchanged. Ozone thus probably affects rather the synthesis of chlorophyll but does not destroy chlorophyll itself.

Recently it was suggested [11, 12] that H_2O_2 may be effective as an oxidative agent involved in the destruction of conifer needles. We therefore studied the effect of vaporous H_2O_2 on the content of ascorbic acid and chlorophyll. In these experiments the cells were exposed to increasing concentrations of vaporous H_2O_2 for 70 h. As can be seen from Fig. 5, concentrations of up to 4% H_2O_2 (in the surrounding solution, which is less in the va-
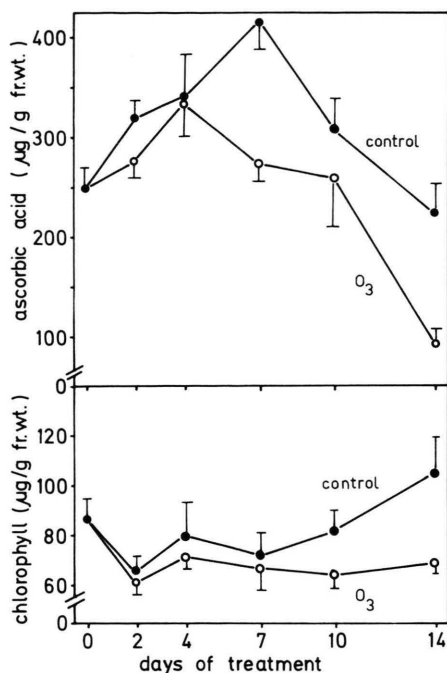
Fig. 4. Chlorophyll and ascorbic acid content in ozone treated cells. Secondary callus cells were precultured for seven days and then in a climate chamber treated with air (control) or air containing 1 ppm of ozone. Mean values of four to five probes.
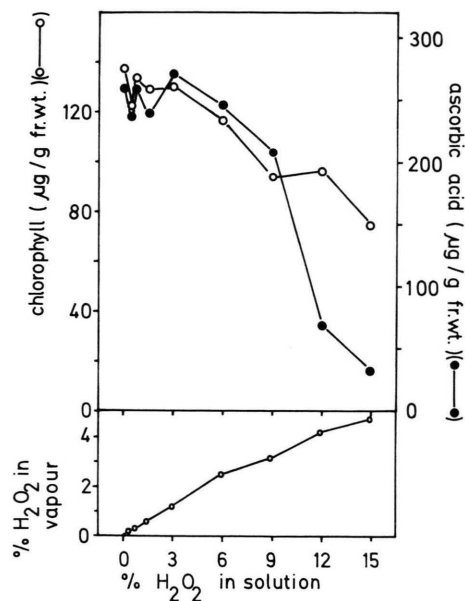

Fig. 5. Effect of vaporous H_2O_2 on ascorbic acid and chlorophyll content in cells. Secondary callus cells were precultured for seven days and then incubated with vaporous H_2O_2 as described in Materials and Methods. The incubation time was 70 h. ●——● ascorbic acid, ○——○ chlorophyll. Mean values of two experiments. In the lower pannel the actual H_2O_2 concentration being effective is shown. This is only about one third of the H_2O_2 concentration in the H_2O_2 solution (see Materials and Methods).

pour, see lower pannel in Fig. 5) had no significant effect, while with higher concentrations the ascorbid acid content declined rapidly. As with SO_2 and ozone, also with H_2O_2 the ascorbic acid content is more sensitive than chlorophyll to this oxidant.

Discussion

The spruce cells from the newly established suspension culture have an ascorbic acid content somewhat lower but comparable to that in green spruce needles. The amount of ascorbic acid can be influenced by the quality (Fig. 2) and the quantity (not shown) of carbohydrates; this indicates their involvement in the regulation of the ascorbic acid level. Additionally, phenylalanine ammonia lyase, a readily inducible enzyme in several plant cells for the synthesis of phenylpropanoids, can also be induced in the spruce cells by a fraction of the fungus *Rhizosphära kalkhoffii*, showing the ca-

pacity of the cells to induce a defense mechanism against biotic attack (not shown). With respect to these properties the cells resemble spruce needles. Since the growth of the cells depends on the presence of carbohydrates (sucrose) in the medium, the cells have to be classified as photoheterotrophic and in this respect they are different from green needles.

An increased ascorbic acid level as it is found in needles of damaged trees suggests an induction of a defense mechanism against oxidative stress. We have chosen SO_2, ozone and vaporous H_2O_2 as representatives of air pollutants to study whether they are able to induce in cells such a defense mechanism. In no case an elevation of ascorbic acid content with a concomitant fall in the chlorophyll content could be obtained. By contrast, the ascorbic acid declined drastically while the chlorophyll content was changed only slightly. A simple explanation for this discrepancy would be the fact

that the needles are photoautotrophic while the cells are photoheterotrophic. This includes of course differences in their metabolism.

The ascorbic acid level, however, in needles as well as in cultured cells, depends on the presence of sufficient carbohydrates. In the photoautotrophic needle this is achieved by photosynthesis, while in cultured cells sucrose added to the medium serves as source for the synthesis of a substantial amount of ascorbic acid in the cells (Fig. 2). This occurs in spite of a rather low chlorophyll content and a low photosynthetic capacity of the cells. To study the inducibility of a defense mechanism against oxidants, as tried here, the ability of the cells to synthesize ascorbic acid appears to be more important than their photosynthetic capacity. We conclude that, if there occurs an induction of such a defense mechanism, this should occur in photohetero-

trophic cells as well as in photoautotrophic needles.

Since this does not occur, in cells presumably ascorbic acid and, to a lesser extent, chlorophyll are destroyed directly by the air pollutants used. It is rather improbable that the increased ascorbic acid content found in needles of damaged conifers is a protection mechanism against external oxidants. Photooxidants probably do not play such a dominant role in the bleaching and destruction processes of needles as it is assumed. Changes in transport processes (for instance carbohydrates) or alterations in the activity of metabolically regulatory enzymes may rather be responsible for the bleaching of needles than a direct action of photooxidants. Otherwise the same symptoms should be found in treated cells and in damaged needles.

[1] L. W. Blank, Nature **314**, 311−314 (1985).
[2] O. Kandler, in: Waldschäden (G. v. Körtzfleisch, ed.), pp. 20−50, Oldenbourg Verlag, München 1985.
[3] U. Arndt, ibid., pp. 195−212.
[4] E. F. Elstner and W. F. Osswald, Naturwiss. Rdsch. **37/2**, 52−61 (1984).
[5] E. F. Elstner, W. F. Osswald, and R. J. Youngman, Experientia **41**, 591−597 (1985).
[6] O. Kandler, W. Miller, and R. Ostner, Allg. Forst Zeitschr. **27−29**, 715−723 (1987).
[7] M. Senser, K. A. Höpker, A. Peuker, and B. Glashagen, Allg. Forst Zeitschr. **27−29**, 709−714 (1987).
[8] W. F. Osswald, H. Senger, and E. F. Elstner, Z. Naturforsch. **42c**, 879−884 (1987).
[9] H. Mehlkorn, G. Seufert, A. Schmidt, and K. J. Kunert, Plant Physiol. **82**, 336−338 (1986).
[10] H. L. Edward, J. A. Jersey, C. G. Gifford, and J. Bennett, Environm. Exptl. Botany **24**, 331−341 (1984).
[11] J. Kandleck, S. McLaren, N. Camarota, V. Mohnen, and J. Wilson, in: Precipitation Scavenging, Dry Deposition and Resuspension, Proc. 4th Int. Conf. Santa Monica Calif., pp. 103−113, Elsevier Sci. publ. 1983.
[12] G. L. Kok, Atmos. Environ. **4**, 653−656 (1980).
[13] G. D. Peiser and F. Y. Shang, Plant Physiol. **60**, 277−281 (1977).
[14] J. Ebel, Annu. Rev. Phytopathol. **24**, 235−264 (1986).
[15] D. J. Arnon, Plant Physiol. **24**, 1−15 (1949).
[16] S. Arnold and T. Eriksson, in: Biotechnology in Agriculture and Forestry (Y. P. S. Bajaj, ed.), **Vol. 1**, pp. 291−320, Springer Verlag, Berlin 1986.
[17] C. H. Bornmann, in: Cell and Tissue Culture in Forestry (J. M. Bouga and D. J. Durzan, eds.), **Vol. 3**, pp. 2−29, Martinus Nijhoff Publ., Dordrecht, Boston, Lancester 1987
[18] W. Hüsemann and W. Barz, Physiol. Plant **40**, 77−81 (1977).
[19] F. Sato, N. Nakagawa, T. Tanio, and Y. Yamada, Agric. Biol. Chem. **45**, 2463−2467 (1981).
[20] W. Franke, Z. Pflanzenphysiol. **60**, 30−37 (1968).