Effects of ozone on organic acids in needles of Norway spruce and Scots pine

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Summary. The effect of ozone, needle age, and season on the pH of homogenate and acid contents of Scots pine and Norway spruce needles is presented. In addition enzyme activities of cytochrome C-oxidase (cyt. C-ox), phosphoenolpyruvate-carboxylase (PEPC), shikimic acid-dehydrogenase (SHDH) and malate-dehydrogenase (MDH) were measured in Scots pine needles. In freshly sprouted spruce needles the level of quinic acid is high and the pH of the needle homogenate is low. Shikimic acid starts at low levels, increases with increasing needle age and becomes dominant, whereas the quinic acid content decreases. Malic acid has a marked seasonal trend; no trend was found in citric acid. Ozone ($200 \mu\text{g}/\text{m}^3$) decreased shikimic acid and quinic acid, whereas pH, malic acid and citric acid increased. Ozone ($100 \mu\text{g}/\text{m}^3$) had a similar effect, except in the current-year spruce needles. In Scots pine needles ozone led to increased enzymatic activities of cyt. C-ox, PEPC and SHDH, and a decrease in the activity of MDH. This effect was more pronounced in summer than in autumn, but the visible damage was greater in autumn. These effects can be found with other stresses and are not specific for ozone.

Key words: Enzymes – Needle age – Norway spruce – Organic acids – Ozone

Introduction

Effects of ozone on the carbohydrate metabolism of conifers have been repeatedly shown in the past (cf. Guderian et al. 1985). Our fumigation experiments with Scots pine revealed a decreased inositol and an increased pinitol content in ozone treated needles (Landolt et al. 1989). At the same time, a change in pH of Scots pine needle homogenates was observed. This finding was the starting point for intensified analyses of organic acids in ozone treated conifer needles,

mainly those of the shikimic acid pathway and the citric acid cycle. Because fumigation of plants with pollutants can lead to an increase or decrease of enzyme activities, thus modifying metabolic pools or fluxes (Tingey et al. 1975, 1976a; Pierre and Queiroz 1981, 1982; Sarkar and Malhotra 1979), enzyme activities related to the acid metabolism [cytochrome C-oxidase (cyt. C-ox), PEPC, malate-dehydrogenase (MDH) and shikimic acid-dehydrogenase (SHDH)] were also measured.

In woody plants the shikimic acid pathway is of great importance. It leads to one of the main components of the cell wall, lignin, and to aromatic amino acids and phenols. Conifer needles contain large amounts of shikimic and quinic acids (Lindner and Grill 1978). There is a close link between carbohydrate and shikimic acid metabolism, especially in young leaves and needles, because considerable amounts of carbohydrates are metabolized through this pathway (Dittrich and Kandler 1971).

Until now, hardly any information was available on possible ozone effects on the shikimic acid pathway. The studies have been mainly restricted to buffer capacities and possible changes in pH in connection with acid gases (Skye 1968; Pfanz and Heber 1986). In the context of tree damage in Germany, a recent paper by Dittrich et al. (1989), based on labelling experiments with spruce needles, points to possible impairment of the shikimic acid metabolism in damaged Norway spruce trees. However, the cause of this damage could not be elucidated.

For the fumigation experiments Norway spruce was chosen as the most widespread forest tree in Switzerland and Scots pine because it is one of the more sensitive to ozone (Davis and Wood 1972).

Materials and methods

Cloned plants were used for all the experiments, either 4-year-old grafts of Scots pine (*Pinus sylvestris* L., clone Birmensdorf 90) or 6-year-old cuttings of Norway spruce [*Picea abies* (L.), Karst. clone Birmensdorf 4] in 12-l pots with standard soil mixture. They were exposed in open-air closed-top fumigation chambers at our Institute. The experimental conditions are described in detail elsewhere (Landolt et al. 1989). Three of four rows with five closed-top fumigation chambers received charcoal-fil-

Fig. 1. Changes in pH-value and organic acids in the homogenate of spruce needles during the first 2 years after bud-break. Ten samples per needle age from 6 trees at two different locations were taken once a month for 2 years. Thus each circle represents the mean of 20 single measurements

tered air, the fourth row received unfiltered ambient air. The ozone concentrations in these experiments were based on the levels found in Switzerland at higher altitudes (Bucher et al. 1986), where there are no peak values and not much difference between day and night. One of the rows with filtered air was continuously supplied with $100 \mu\text{g}/\text{m}^3$ ozone, another with $200 \mu\text{g}/\text{m}^3$ ozone. Ozone was produced by electrical discharge of pure oxygen by an ozone generator (Fischer Mod. 502) and recorded on a strip chart recorder. The monthly means of ozone concentrations in the ambient air during summer 1987 were around $50 \mu\text{g}/\text{m}^3$ and the maximum half-hour mean was $170 \mu\text{g}/\text{m}^3$. More information about the local air quality during the fumigation periods can be found in Bleuler and Landolt (1988, 1989).

The plants (five per treatment) were distributed among the five chambers of each treatment. The first fumigation experiment started in mid-May, the second at the end of August 1987, and they were repeated in 1988.

The first sampling took place before the beginning of the fumigation (= week 0) except for the current-year needles in summer, which were analysed as soon as they were fully expanded. The beginning of the fumigation is termed week 1. The samples were always taken on the same day of the week during the same time period (between 0700 hours and 1000 hours). As very little material was used, consecutive sampling did not harm the plants.

The samples from the big trees were taken from five spruces in the garden of the Institute with branches that could be reached from the ground and from two levels of the crown of a tree at the study site Lägern, where a scaffold had been built around the tree for other purposes. The two youngest needle ages were analysed once a month over 2 years.

For pH measurement and acid analysis 100 mg needle material was homogenized in 1 ml water (Braun Microdismembrator). After centrifugation for 10 min at $12000 \times g$, 500 μl of the supernatant was diluted with NaCl solution to obtain an ionic strength (200 mM NaCl) similar to the intracellular conditions, and the pH-value was measured with a glass electrode. Acid analysis was carried out on 250 μl of the supernatant.

The acids were determined based on the method of Lindner and Grill (1978). They were purified by absorption on an anionic resin (Serva, Sordolit blue micro) and released by 2 M formic acid. After drying the residue was dissolved in 400 μl dimethylformamide (DMF) containing the internal standard phthalic acid. An aliquot of this solution was silylated with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) at room temperature. Analysis by capillary gas chromatography followed.

For the assay of cyt. C-ox 50 mg of needles was homogenized (Braun Microdismembrator) in 1 ml buffer (50 mM Na/K-phosphate pH 7, 1% (w/v) Tween 80) and centrifuged for 10 min at $12000 \times g$. To 40 $\mu\text{l}/\text{ml}$ of the supernatant (50 mM Na/K-buffer pH 7.4, 1 mM EDTA, 2% PVP K 30), 0.5 mg/ml reduced cytochrome C was added and the oxidation recorded at 550 nm.

For the other enzymes the extraction method of Weimar and Rothe (1986) was adapted to a sample of 100 mg needles. For all three enzymes the activity was recorded following the change of absorbance at 340 nm.

The assay conditions were:

1. MDH: 0.2 M Na/K-phosphate pH 7.5, 2 mM MgCl_2 , 1 mM mercaptoethanol, 180 $\mu\text{g}/\text{ml}$ NADH, 0.3 mM oxalacetic acid, 5 $\mu\text{l}/\text{ml}$ purified needle extract.

2. PEP-C: 0.1 M Tris/HCl pH 7.5, 6.25 mM NaHCO_3 , 6.25 mM MgSO_4 , 0.12 mg/ml NADH, 0.2 mg/ml PEP, 100 $\mu\text{l}/\text{ml}$ needle extract. No addition of MDH was necessary.

3. SHDH: 0.2 M Tris/HCl pH 8, 2 mM shikimic acid, 0.2 mg/ml NADP, 50 $\mu\text{l}/\text{ml}$ needle extract.

Enzyme activities and metabolite concentrations are expressed on a fresh weight basis, the statistics calculated using the test by ranks of Kruskal-Wallis.

Results

Beside large amounts of shikimic and quinic acid, malic and citric acid are also found in measurable amounts in spruce and pine. Seasonal changes in organic acids of conifers have been described before (Oechssler 1968), the analysis was made by paper chromatography. The quantitatively more accurate gas chromatographical analysis has confirmed the general trend of quinic and shikimic acid and given more details on the behaviour of malic and citric acid (Fig. 1). In the newly appearing needles the level of quinic acid is very high and the pH of the needle homogenate is low. Quinic acid decreases first quickly and then more slowly in older needles while pH-values increase. Shikimic acid starts at low levels and increases during the first summer and later becomes dominant.

Malic acid has a marked seasonal trend. The concentration is low in summer and high in winter. Citric acid shows no trend either with needle age or season. Its level is generally low but varies from one sampling to the next and from one tree to another.

In needles more than 2 years old, quinic and shikimic acids decrease steadily over the years. Winter levels of malic acid decrease too, but very slowly, and citric acid changes only a little. Increase in pH is smaller than might be expected from the diminishing acid contents (Fig. 2).

In the control plants from the fumigation experiments, the acids and the pH showed the same trend as in the big trees (Figs. 3–7). The pH in the pine needles is higher than

Fig. 2. pH-value and content of organic acids in needle homogenates of increasing age. Means from samples of 5 trees (1 sample per tree) taken in February at the forest research station in Birnensdorf
● pH-value, □ quinic acid, ■ shikimic acid, △ malic acid, ○ citric acid

Fig. 3. The effect of ozone on pH-value in needle homogenates of two needle ages from Norway spruce and Scots pine during the fumigation experiment of summer 1987. Each value represents the mean of 5 plants. (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, $P \leq 0.05$ △ 200 $\mu\text{g}/\text{m}^3$ O₃, □ 100 $\mu\text{g}/\text{m}^3$ O₃, ● filtered air (control), ▲ ambient air

in spruce. In the sprouting needles the difference is small and probably due to a lower content of quinic acid. In the 1-year-old needles the difference is greater and the concentration of shikimic acid is clearly lower.

In week 6 of the summer experiment 1987, after 5 1/2 weeks of fumigation, a couple of days after the sampling for biochemical analysis, the pines treated with 200 $\mu\text{g}/\text{m}^3$ ozone developed reddish-brown spots on some 1-year-old needles of the main shoot. The needles showed more and more signs of damage until most of them were brown at the end of the fumigation. While we always tried to choose healthy-looking needles for the biochemical analysis, this was no longer possible in the last sampling. The current-year needles had no visible damage except

Fig. 4. The effect of ozone on quinic acid in needles of 2 needle ages from Norway spruce and Scots pine during the fumigation experiment of summer 1987. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$ △ 200 $\mu\text{g}/\text{m}^3$ O₃, □ 100 $\mu\text{g}/\text{m}^3$ O₃, ● filtered air (control), ▲ ambient air

some yellow needle-tips on one branch of a single plant at the end of the experiment. The plants treated with 100 $\mu\text{g}/\text{m}^3$ ozone or ambient air did not look different from the control group, at least not during the 12 weeks of the experiment. Spruce was not visibly damaged during the ozone fumigation.

In 200 $\mu\text{g}/\text{m}^3$ ozone the pH-value of the needle homogenate increased in both species (Fig. 3) and quinic acid decreased (Fig. 4) compared to the control. The increase of shikimic acid in the current-year needles was smaller in 200 $\mu\text{g}/\text{m}^3$ ozone than in the control. In the 1-year-old needles shikimic acid decreased with both ozone fumigations (Fig. 5), with 200 $\mu\text{g}/\text{m}^3$ ozone to approximately half of the concentration of the control. The effect of 100 $\mu\text{g}/\text{m}^3$ ozone on shikimic acid in the 1-year-old needles was visible earlier in spruce but at the end of the fumigation it was about the same in both species. Malic and citric acids increased during fumigation (Figs. 6, 7). In the current-year needles of spruce the difference between 200 $\mu\text{g}/\text{m}^3$ ozone and the control was statistically no more significant in the last sampling, whereas in pine it increased continuously. As with shikimic acid, malic and citric acids in the 1-year-old needles of spruce also reacted in 100 $\mu\text{g}/\text{m}^3$ ozone. In the 1-year-old needles of pine only citric acid at the end of the experiment was different in 100 $\mu\text{g}/\text{m}^3$ ozone from the control. Ambient air had no effect on the organic acids in either species.

The activity of cyt. C-ox, which was measured during the summer and autumn 1987 experiment, increased in 200 $\mu\text{g}/\text{m}^3$ ozone and in the current-year needles in autumn

Fig. 5. The effect of ozone on shikimic acid in needles of 2 needle ages from Norway spruce and Scots pine during the fumigation experiment of summer 1987. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$ Δ 200 $\mu\text{g}/\text{m}^3$ O_3 , $\square$ 100 $\mu\text{g}/\text{m}^3$ O_3 , $\bullet$ filtered air (control), $\blacktriangle$ ambient air

Fig. 7. The effect of ozone on citric acid in needles of 2 needle ages from Norway spruce and Scots pine during the fumigation experiment of summer 1987. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$ Δ 200 $\mu\text{g}/\text{m}^3$ O_3 , $\square$ 100 $\mu\text{g}/\text{m}^3$ O_3 , $\bullet$ filtered air (control), $\blacktriangle$ ambient air

Fig. 6. The effect of ozone on malic acid in needles of 2 needle ages from Norway spruce and Scots pine during the fumigation experiment of summer 1987. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$ Δ 200 $\mu\text{g}/\text{m}^3$ O_3 , $\square$ 100 $\mu\text{g}/\text{m}^3$ O_3 , $\bullet$ filtered air (control), $\blacktriangle$ ambient air

even in 100 $\mu\text{g}/\text{m}^3$ ozone (Fig. 8), but in the 1-year-old needles that were soon visibly damaged no change in activity was observed.

A fumigation experiment with pine during the following summer resulted in the same changes of pH-value and organic acids. In addition the effect of ozone on the activity of PEPC, MDH and SHDH was investigated.

The activity of PEPC in Scots pine increased in summer in both needle ages in 200 $\mu\text{g}/\text{m}^3$ ozone (Fig. 9). In autumn it increased only in the 1-year-old needles and this increase was smaller and came later but simultaneously in both ozone treatments. MDH decreased in the 1-year-old needles in summer (Fig. 10). In autumn MDH was lower in the ozone treatments in the 1-year-old needles than at the beginning of the fumigations but did not decrease further. SHDH increased in summer with 200 $\mu\text{g}/\text{m}^3$ ozone in both needle ages (Fig. 11), but in autumn it did not change.

Discussion

The acids of the shikimic acid pathway decrease in the fumigated plants and the change in the pH-value seems to be a direct consequence of this decrease. This effect is known from SO_2 fumigation in Norway spruce (Grill et al. 1980) and Jack pine (Sarkar and Malhotra 1979). A change in malic and citric acid was not described there.

An increase in acids from the citric acid cycle is a typical stress reaction which has been described in several plants subjected to different stresses (Lance and Rustin

Fig. 8. The effect of ozone on the activity of cytochrome C-oxidase in two needle ages of Scots pine during the fumigation experiments of summer and autumn 1987. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$
 Δ 200 $\mu\text{g}/\text{m}^3$ O_3 , $\square$ 100 $\mu\text{g}/\text{m}^3$ O_3 , $\bullet$ filtered air (control), $\blacktriangle$ ambient air

Fig. 10. The effect of ozone on the activity of malate dehydrogenase in 2 needle ages of Scots pine during the fumigation experiments of summer and autumn 1988. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$
 Δ 200 $\mu\text{g}/\text{m}^3$ O_3 , $\square$ 100 $\mu\text{g}/\text{m}^3$ O_3 , $\bullet$ filtered air (control), $\blacktriangle$ ambient air

Fig. 9. The effect of ozone on the activity of PEP carboxylase in 2 needle ages of Scots pine during the fumigation experiments of summer and autumn 1988. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$
 Δ 200 $\mu\text{g}/\text{m}^3$ O_3 , $\square$ 100 $\mu\text{g}/\text{m}^3$ O_3 , $\bullet$ filtered air (control), $\blacktriangle$ ambient air

Fig. 11. The effect of ozone on the activity of shikimic acid dehydrogenase in 2 needle ages of Scots pine during the fumigation experiments of summer and autumn 1988. Each value represents the mean of 5 plants (1 sample per plant). Significantly different from control at (a): $P \leq 0.01$, (b): $P \leq 0.05$
 Δ 200 $\mu\text{g}/\text{m}^3$ O_3 , $\square$ 100 $\mu\text{g}/\text{m}^3$ O_3 , $\bullet$ filtered air (control), $\blacktriangle$ ambient air

1984), such as water stress (Becker and Fock 1986; Timpa et al. 1986), heavy metals strain (Grünhage and Jäger 1982), insufficient mineral nutrition (Lipton et al. 1987), and SO₂ fumigation (Pierre and Queiroz 1981) and the increase of malic acid in autumn can be regarded as an adaptation to cold stress. It is generally assumed that this increase provides reduction equivalents (Lance and Rustin 1984) and is connected to an increase in metabolic activity (Pierre and Queiroz 1981).

Only limited conclusions about the state of the cell in vivo can be drawn from the pH of the needle homogenate. It can be expected that the changes only concern the vacuole. The pH of the cytoplasm is assumed to be around 7. Therefore the greater part of the acids must be stored in the vacuole though the citric acid cycle is located in the mitochondrion and the shikimic acid pathway in the plastid (Mousdale and Coggins 1985).

pH measurement illustrates the quantitative relations between the different organic acids very well, as can be clearly seen in pine. pH-values are always near the pK-value of the most abundant acid. In the young needles there are large amounts of quinic acid and the pH of the homogenate lies at 3.4 (pK of quinic acid = 3.45), later shikimic acid dominates and pH rises above 4 (pK of shikimic acid = 4.15). In fumigated plants pH-values increase above 5 and there is about the same amount of malic and citric acid (pK₂ of malic acid = 5.2, pK₂ of citric acid = 4.75, pK₃ = 5.41) as shikimic and quinic acid.

From visual and biochemical aspects it is obvious that older needles are more sensitive to ozone than the younger ones. In pine the first biochemical differences were noticed even before the first visual symptoms but not all parameters showed statistically significant differences at that time, and the time lapse between the appearance of the first biochemical and the first visual symptoms was very short.

The lower sensitivity of Norway spruce compared to Scots pine was confirmed by the occurrence of visible symptoms but not by the biochemical reaction. Therefore a question arises as to the significance of the changes found for the tree.

Comparing the acid concentrations in fumigated plants with the levels in big, healthy-looking trees, we can see that only the values of the 1-year-old needles were obviously outside the natural variation range. In the current-year needles malic and citric acid were only slightly higher and quinic and shikimic acid were in a period of rapid changes where differences are difficult to detect anyway.

The role these acids play when they are accumulated in such amounts is not entirely clear. They are important precursors for amino acids, phenols, and lignin. The pool size found in fumigated plants should still be sufficient for these needs.

Another aspect is defence against fungi and insects. The low pH hinders growth of fungi. The fungi increase the pH of the needle homogenate and clones of *Pinus sylvestris* with lower buffer capacity are more sensitive to attack by *Lophodermium pinastri* (Scholz and Stephan 1974). Schopf (1986) studied the influence of the different compounds in needles in relation to feeding, growth, and survival of insects. He defined a term where shikimic and quinic acid influence insect growth negatively.

It is possible that without a direct influence on plant health a decrease in quinic and shikimic acids by high doses of ozone may reduce resistance in additional stress situations such as drought, pathogen or insect attack.

Activities of enzymes in vitro, also called enzyme capacities, are much higher than the activities in vivo but mostly show the same changes (Queiroz 1988). Therefore it seems justified to conclude from higher capacities to higher activities in vivo.

Ozone fumigated plants have higher activities of cyt. C-ox. This is a sign of increased respiration. Higher dark respiration upon fumigation has been found before (Barnes 1972; Reich 1983; Dizengremel and Citerne 1988). It was related to higher metabolic needs (Amthor and Cumming 1988) or thought to be a consequence of cell damage (Pell and Brennan 1973). Our results support the first hypothesis, as the intensity of the increase was reciprocal to the observed visible damage.

The two biochemical pathways where the main organic acids are found, the citric acid cycle and the shikimic acid pathway, are linked by the common precursor phosphoenolpyruvate (PEP). If in the fumigated plants more PEP is used to increase the pool of malic and citric acids, there is probably less available to be withdrawn to the shikimic acid pathway.

However, the significance of an increased PEPC activity in the plant cell metabolism is not yet fully understood. It seems that the increased PEPC activity and the increased levels of malic and citric acid as well as the enhanced cyt. C-ox activity point to enhanced respiration. In addition there is the possibility that a higher PEPC activity (and malic enzyme) would allow the TCA cycle to function anaplerotically to provide carbon for biosynthesis as well as for oxidation thus producing ATP (Still and Steup 1985). Higher respiration might supply the energy for repair and PEPC compensate this respiration partly, at the same time getting substrates ready for further enhanced metabolic activities. The increase in the activity of SHDH might be consistent with the decrease in quinic acid but not with the decrease in shikimic acid. Therefore the possibility cannot be excluded that the shikimic acid pathway might supply the aromatic amino acids for protein or phenol synthesis that may be increased in ozone-treated plants (Tingey et al. 1976b).

There seems to be a discrepancy between the biochemical and the visible effects in summer and in autumn. While the biochemical effects were more pronounced in summer, the visible damage was much more severe in autumn. The reason for this behaviour of the plants is not clear. Possibly there are two thresholds, one between strain avoidance and tolerance (Queiroz 1988) and the other between tolerance and susceptibility. These thresholds lie at different ozone levels depending on additional environmental factors.

The increase in enzyme activity is often much stronger in younger needles, possibly because of a general higher metabolic activity. Accordingly they are much less damaged than the older needles.

In C₃ plants the activity of PEPC is much lower than that of RuBP-carboxylase (RuBPC). The relation is around 6 (DeGroote and Kennedy 1977) and 15 (Melzer and O'Leary 1987). This means that a 4-fold increase in PEPC

such as we found can compensate for a considerable decrease in photosynthesis or increase in dark respiration without any detectable effect on gas exchange measurements. A discrimination of RuBPC in favour of PEPC has been found in isolated chloroplasts treated with sulfite (Libera et al. 1975), and in osmotically stressed wheat (Thind and Malik 1988). Useful information could be obtained by analysis of the $\delta^{13}\text{C}$, especially in the fraction of the four carbon acids, which is expected to become less negative. Additional measurement of stomatal conductance would give valuable information as it is an important factor in discriminating between ^{12}C and ^{13}C . Martin et al. (1988) found a change in $\delta^{13}\text{C}$ in the wood of a temporarily polluted area in the corresponding tree rings and in plants fumigated with SO_2 and O_3 . He concluded that a changed stomatal resistance might be responsible for this effect. Based on these results there is some evidence that an altered PEPC activity might also be involved.

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