CHAPTER 12

Defense Strategies against Ozone in Trees: The Role of Nutrition

Andrea Polle, Rainer Matyssek, Madeleine S. Günthardt-Goerg, and Stefan Maurer

CONTENTS

12.1 INTRODUCTION

Tropospheric ozone is a major air pollutant in industrialized countries. It is formed by photochemical oxidation of primary pollutants released into the air by burning of fossil fuels. In the presence of high irradiance the generation of ozone (O_3) is initiated by nitrogen dioxide (NO_2) and driven by volatile hydrocarbons and other components present in exhaust from traffic, power plants, or industrial productions.[1] Ozone is also a natural component in air at low concentrations of 5 to 15 ppb.[2] However, during the last 100 years these background levels have approximately doubled. Under clear and sunny weather conditions, O_3 rises to peak levels and occasionally reaches concentrations above 120 ppb.[1]

Ozone is highly phytotoxic. In aqueous phases it degrades into reactive oxygen species such as $O_2^{\cdot-}$ or H_2O_2.[3,4] Ozone itself or its oxidative degradation products easily oxidize cellular targets such as unsaturated fatty acids in membranes, thiol groups in enzymes, etc.[5] Antioxidative defense systems which are found in all aerobic organisms may prevent damage, if present at sufficient activities to counterbalance oxidative injury.[6] Sudden peak values of O_3 may, however, overwhelm protective systems and cause acute injury in sensitive plant species. Such increases in O_3 to high, immediately toxic peak concentrations occur occasionally and are typically confined to a local scale, whereas chronic exposure to persistently enhanced mean O_3 concentrations during diurnal

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and seasonal courses is a general, regionally widespread phenomenon. It is questionable whether plants, in particular trees with their long reproduction cycles, have already adapted to the high stress levels imposed by O_3 or have sufficient metabolic flexibility to acclimate to such conditions. Protective measures against O_3 may be related to structural features limiting the access of O_3 to sensitive targets or physiological factors such as allocation of cellular resources to repair and detoxification processes.

There is now a large body of data showing that exposure to chronically elevated O_3 may cause changes in carbohydrate allocation patterns, decreases in photosynthesis, reductions in biomass, changes in growth patterns, premature senescence, etc.[5,7-10] These reactions are also accompanied by alterations in biochemical defense systems.[5,6,11] However, consistent O_3 responses have not always been found. A reason may be that the O_3 sensitivity is affected by the interaction of internal plant-specific factors on the one hand and external, environmental factors on the other hand. To date, little attention has been paid to the question of how defense mechanisms against O_3 may be affected by interaction with nutrition. The present chapter focuses on O_3-induced stress responses in tree species as affected by nutrition and developmental stage and aims at providing an integrative view from the cellular to the whole-plant level. Since there are only few and scattered data on this particular subject, the major body of this chapter will exemplify results of an experiment conducted on young birch trees (*Betula pendula*, Roth.) grown at high or low nutrient supply and under chronic O_3 exposure.

12.2 OZONE UPTAKE AS MODIFIED BY DEVELOPMENT, STRUCTURAL CHANGES, AND INJURY

Ozone is transported to the surface of plants by turbulent transport. In fully differentiated leaves, cuticles represent a nearly impermeable barrier[12] and O_3 is almost exclusively taken up via the open stomata. Therefore, stomatal conductance is an important factor determining the internal O_3 dose, i.e., the O_3 influx during exposure time.[13] The stomatal conductance, a feature given by the number of stomata per leaf area and their aperture, varies, however, largely during diurnal and seasonal courses and depends on water availability, nutrient supply, light regime, and developmental stage of a plant. In trees stomatal conductance is generally higher in deciduous than in evergreen species.[13] Thus, a given external O_3 concentration may lead to large differences in the internal O_3 dose between the two types of foliage, implying distinct differences in the need for defense systems between species, habitats, and under fluctuating environmental conditions.

With respect to the O_3 dose, it is important whether species with indeterminate shoot growth, i.e., species forming new leaves throughout the season (e.g., birch, poplar) or species with determinate growth, i.e., those forming only one or two flushes per season (e.g., conifers, beech) are considered, and during which ontogenic stage O_3 is present. During the initial stages of leaf formation, before the cuticle and outer epidermal cell wall have thickened and guard mother cells divide to form the stomata, the influence of O_3 is independent of stomatal regulation. At this early stage, the barrier properties of the cuticles may not be as efficient as in mature leaves. An example shows that in this ontogenic stage, the presence of chronic O_3 levels (40 ppb during the night, 90 ppb during the day) caused a reduction in leaf expansion in birch (Figure 12.1A) and resulted in smaller leaves (Figure 12.1B). These leaves displayed relatively higher densities of stomata, scales, small hairs, and veins than those from trees grown in filtered air.[14] Higher stomatal densities have also been found in various other birch clones after O_3 exposure.[15] The O_3-mediated reduction in leaf size was highly significant in high- but not in low-fertilized cuttings, which per se formed small leaves (Figure 12.1B). Still, in O_3-exposed leaves from low-fertilized birch, decreased guard cell sizes and increased stomatal density demonstrated that the influence of O_3 on differentiation was not necessarily bound to individual leaf expansion (Figure 12.1C vs 12.1B). In young, expanding leaves injury to subcellular structures was not yet apparent.

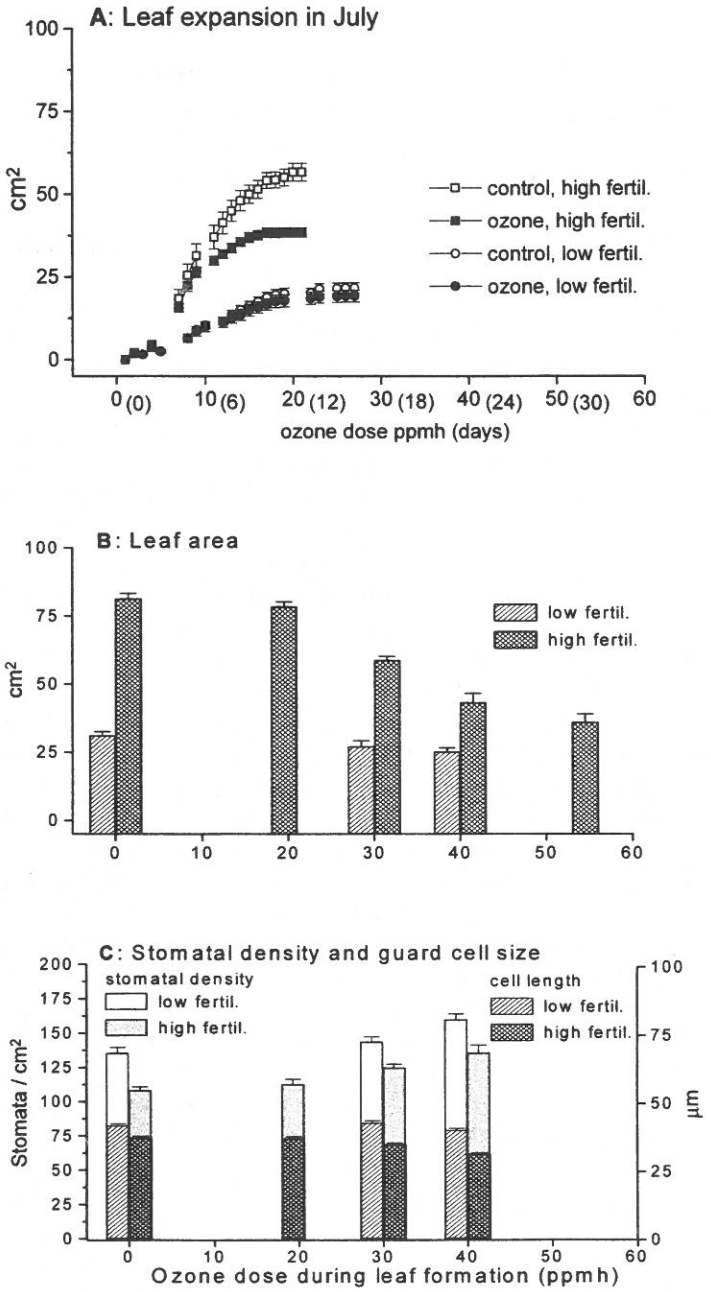

Figure 12.1 Leaf expansion (A), leaf area (B), stomatal density and size of guard cells (C) in high- and low-fertilized birch (*Betula pendula* Roth) trees as dependent on the external O_3 dose (concentration x time) during leaf formation. Young trees were grown from cuttings and fertilized regularly with a commercial fertilizer solution containing macro- and micronutrients either at a dilution of 0.005% (low fertilization) or 0.05% (high fertilization). The plants were exposed to filtered air (< 3 ppb, control) or to filtered air with added O_3 from before leaf flush to autumnal leaf loss in 20 field fumigation chambers in 1993. Ozone was generated from pure oxygen. Details of the experiment have been described elsewhere.[14,16,25] Leaves of the same age were investigated on individual trees. Data represent means ± standard error of $n = 10$ (A) or $n = 20$ trees (B and C). The number of determinations for stomatal density and guard cell size were 20 and 50, respectively, per leaf. The effects of O_3 and fertilization were significant ($P < 0.001$). Leaf expansion and leaf area were affected by significant interactions between O_3 and nutrition ($P < 0.005$).

In fully differentiated birch leaves, incipient subcellular structural changes appear in spongy parenchyma cells situated in intercostal fields adjacent to the free air space in the vicinity of stomatal openings.[16] Because of its high reactivity, O_3 is probably decomposed in the cell wall matrix and does not enter the intracellular space.[17] The diffusive pathway of O_3 within the leaf is likely to be small. It has been reported that in spruce the lignin content of guard cells is lower in O_3-exposed needles, whereas the mesophyll, which also contains lignin, did not show this reduction, pointing to O_3-induced disturbances at distinct locations.[18] Ozone-induced cell wall responses have been studied by cytochemistry in birch and several other species.[16,19] Under chronic O_3 exposure of birch and irrespective of the nutritional state of the plants, the outermost pectinaceous layer (calcium pectate) of spongy parenchyma cells was found to swell and protrude into the free air spaces, sometimes forming bubbles and at later stages wartlike structures (Figure 12.2B as compared with control in filtered air Figure 12.2A and D, vs. control 12.2C). The cytosol, the nucleus, and the mitochondria became relatively dark, whereas the chloroplasts appeared more translucent (Figure 12.2B vs. 12.2A). It is not yet known whether such initial ultrastructural changes are reversible; but after continued exposure the delimitation of the membranes became less distinct and the ultrastructural changes also spread to the palisade parenchyma. Only at this point did initial O_3 symptoms become macroscopically visible as light green dots on the adaxial leaf side in transmitted light.

With the appearance of initial O_3 symptoms and their development into stipplings and collapse of individual cells (Figure 12.3A), it was observed histochemically that starch granules remained accumulated along small leaf veins; this may indicate inhibited phloem loading (Figure 12.2F vs. 12.2E)[14,20-23] simultaneously with a reduction in CO_2 assimilation (see below). Autoradiography after $^{14}CO_2$ feeding showed the irregular CO_2 uptake in leaves with visible O_3 symptoms as compared with leaves from trees exposed to filtered air (Figure 12.2H vs 12.2G). In contrast to starch accumulation along small veins, the O_3-induced decline in the mesophyll cells was characterized by lowered starch content. At this stage the nucleus was condensed and the cell walls thickened by cellulose and pectin deposition (Figure 12.2D vs. 12.2C). These reactions that precede cell collapse led to an increase in dry mass at the expense of intercellular air spaces (Figure 12.3B). As injury proceeded, the cell-to-cell contact was interrupted and finally phenolic polymers appeared as a result of oxidative processes in cytosol and the vacuoles. The latter processes were partly caused by membrane disintegration irrespective of the species and the preceding stress.[24]

The sensitivity and appearance of initial leaf symptoms and their further development to cell injury and stippling varies inter-[21,24] and intraspecifically.[19] In birch, the stomatal density is increased by both low nutrition and exposure to O_3.[14,15,25] However, the role of O_3 in leaf differentiation cannot be generalized since in poplar reduced stomatal densities have been found.[20,26] Conflicting results have also been reported on the impact of O_3 on gas exchange: net CO_2 uptake rate, stomatal conductance, and water-use efficiency decreased, increased, or even remained unaffected.[5,8] One may ask whether nutrition can explain some of the variability found in the gas exchange behavior under O_3 stress. High nutrition typically stimulates leaf metabolism including CO_2 uptake rates.[27] This may increase the capacity for repair and detoxification processes[28] and, thereby, enhance the O_3 tolerance of photosynthesis. However, stomatal conductance may be enhanced as well[29] so that an increase in O_3 uptake into the leaves may counteract the benefits of high nutrition. By contrast, low nutrition may reduce the influx of O_3 and, thereby, the risk of injury. Moreover, the sensitivity of stomatal regulation is known to be mediated by nutrition.[30]

In spite of increased stomatal densities in birch leaves grown with low nutrient supply, under the influence of O_3 stomatal conductance decreased to the level of trees at high nutrition.[31] Thus, under O_3 impact partial stomatal closure in low-fertilized plants compensated for the increase in stomatal density.[25,32] Apparently, this effect was less pronounced in leaves of birch grown with high nutrient supply than in those from plants with low nutrient supply, thereby resulting in similar O_3 uptake rates in both nutrient regimes (Figure 12.4A and B).

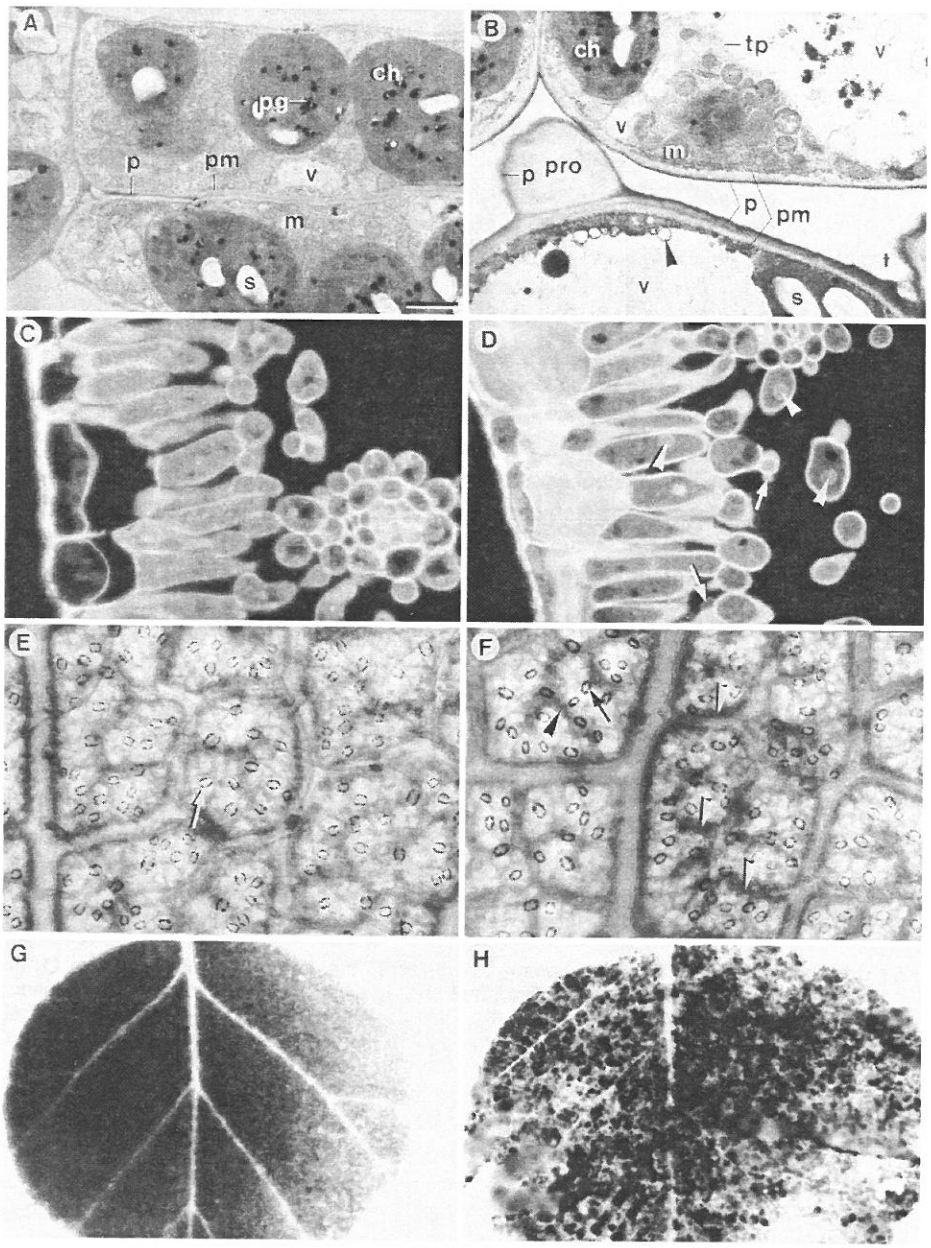

Figure 12.2 Birch (*Betula pendula*) leaf structures and ultrastructures after growth in filtered air (A, C, E, G) or in filtered air with 90 ppb O_3 added during daylight hours and 40 ppb during night (B, D, F, H). (A and B) TEM micrographs of palisade parenchyma cells in 26-day-old birch leaves from the high fertilization regime (bar = 1.5 µm); (A) control with distinct mitochondria (m) and chloroplasts (ch), small plastoglobuli (pg) and vacuole (v), and thin pectin layer (p). (B) With initial visible O_3 symptoms (O_3 dose = 43 ppmh): large vacuoles with tannin deposit (upper cell) and lipid droplet (lower cell). In the lower cell, plasmamembrane (pm) confining a darkened cytoplasm, tonoplast (tp) with proliferations (arrow head), thickened pectin layer with bubblelike projections (pro). (C and D) Cross sections stained with coriphosphine for pectinaceous substances (white), which in (D) are increased in the cell walls (sometimes forming bubbles or warts as indicated by arrows) and in the nuclei (arrow heads), bar = 22 µm. For technical details see Reference 16. (E and F) Surface view of lower leaf surface, stained for starch with I/KI, stomata with black amylopectin granules (arrows), and starch accumulated along small leaf veins (arrow heads), bar = 0.15 mm. (G and H) Autoradiographies 12 h after feeding with $^{14}CO_2$; (H) irregular CO_2-uptake.

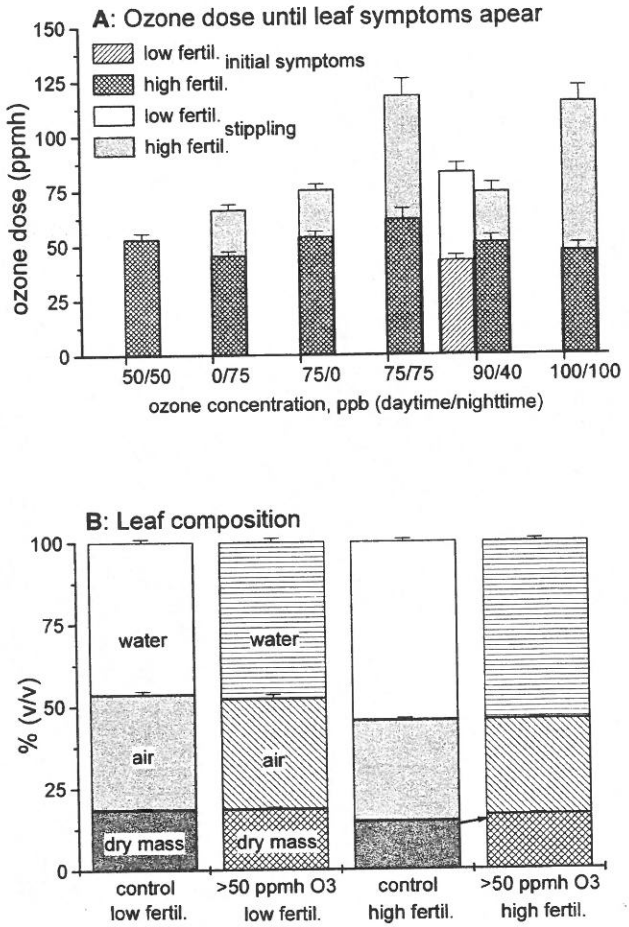

Figure 12.3 Development of visible leaf symptoms as related to the external O_3 dose and the O_3 exposure regimes (A) and effects on the relative distribution of water, air, and dry matter within foliar volume elements in leaves of *Betula pendula* (B). Data are means of $n = 10$ (A) and 40 (B). The effects of O_3 on the development of leaf symptoms and dry matter were significant ($P < 0.002$). The effects of fertilization were significant ($P < 0.03$), except on the appearance of stipplings.

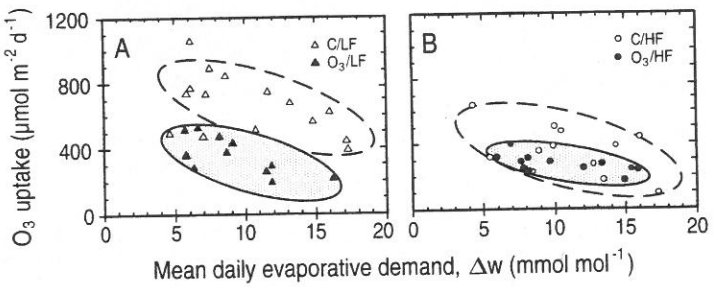

Figure 12.4 Daily O_3 uptake (i.e., O_3 influx density) into birch leaves as dependent on the mean daily evaporative demand of the ambient air (expressed as the difference in the leaf/air mole fraction of water vapor): (A) Low-fertilized plants; (B) high-fertilized plants. For the control in filtered air at each nutrient supply (open symbols), the potential O_3 uptake was calculated assuming the same O_3 regime as applied to the O_3-exposed plants (closed symbols): Abbreviations: C = control plants in filtered air; O_3 = plants exposed to O_3 (90 ppb during daylight hours, 40 ppb at night); LF = low-fertilized plants; HF = high-fertilized plants. (Modified from Maurer et al.[31])

Under O_3 stress, stomatal narrowing may be initiated by an increasing CO_2 concentration in the intercellular space of the mesophyll (*ci*) as frequently observed in O_3-exposed leaves.[33-35] In O_3-exposed birch, ci increased regardless of nutrition or the extent of stomatal closure.[31] Elevated ci at lowered stomatal conductance can only be explained by a decline in the enzyme-driven CO_2 consumption. In fact, O_3 made the rate of CO_2 uptake decrease relative to the stomatal conductance.[32] Thus, CO_2 uptake was limited by a diminished CO_2 demand in the mesophyll rather than by lowered stomatal conductance. Therefore, the increase in $\delta^{13}C$ in the plant biomass observed under O_3 stress[36] cannot be caused by stomatal limitation of photosynthesis[37] but was probably a result of stimulated phosphoenol pyruvate carboxylase (PEPC) activity (see below). The observation that stomatal narrowing is preceded by a decline of the adjacent mesophyll cells cells and parallelled by raised *ci* suggests that the decreased aperture is a consequence of injury. It is likely that the supply of guard cells with ions, hormones, etc., necessary for stomatal regulation is disturbed when neighboring cells have been damaged. But more research is needed on the osmotic and hormonal control of the stomatal width under O_3 stress.[38] Furthermore, the accumulation of immobile amylopectin in narrowed guard cells (Figure 12.2F vs. 12.2E) may also be interpreted as a defense strategy to reduce high apoplastic sucrose levels when mesophyll sucrose efflux exceeds translocation.[22,39] In this manner gas exchange for further assimilate production is restricted. It should be emphasized that the reduction in stomatal conductance found in low-fertilized birch resulted in a 50% decrease in the daily O_3 uptake relative to the potential O_3 influx in the absence of stomatal effects (see Figure 12.4).

The interaction between nutrition and O_3 effects on stomatal conductance is, however, species dependent. In contrast to birch, in alder O_3 caused increases in stomatal conductance and, thereby, in O_3 uptake, irrespective of the nitrogen supply.[40] If water supply is high, stomatal conductance may increase even in the absence of O_3 by low nutrition (see Figure 12.4).[41] It has typically been reported for conditions of low nutrient availability that CO_2 uptake rates remain low relative to stomatal conductance.[31,42,43]

12.3 PHOTOSYNTHESIS AND CARBON METABOLISM UNDER THE INFLUENCE OF O_3 AND NUTRITION

It has frequently been observed that O_3 leads to a decrease in CO_2 uptake rates[8,13,44] although the primary impact of O_3 is not in the chloroplasts but in cell walls and adjacent plasmalemma.[10,19] Functional and structural breakdown of the chloroplasts has been reported, e.g., photoinhibition, loss of ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) protein and activity.[5,45] In birch, the variable fluorescence (F_v/F_m) remained stable, until CO_2 uptake had almost reached compensation.[31] This observation indicates the dependence of CO_2 uptake rates on the collapse of entire mesophyll cells,[32] whereas photosystem II apparently stayed intact in the persisting clusters of green cells in the O_3-injured leaves. Inhibition of nitrate reductase by O_3 may be significant for plant nutrition,[46] as the balance between nitrate reductase and Rubisco activities may exert the ultimate control on the carbon and nutrient allocation of the whole plant.[47] Remarkably, O_3-exposed birch plants of low nutrition displayed increases in CO_2 uptake rates and Rubisco activity in young leaves relative to the corresponding control in filtered air.[22,31] Such effects were absent at high nutrition. Thus, nutrition determines the extent to which photosynthesis of newly formed leaves may compensate for the decline in older O_3-injured foliage.[8] By contrast, photosynthesis of unnodulated alder was more sensitive to O_3 at low nitrogen supply.[40] However, it is not known whether the limitation in a specific nutritional element such as nitrogen may have an effect similar to that caused by overall reduction in nutrient supply.

High nutrition could not prevent leaf injury in birch, but low nutrition delayed the impairment of photosynthesis and maintained the life span of O_3-exposed leaves almost throughout the entire growing season.[31] Leaves of high-fertilized trees displayed O_3 symptoms earlier (see Figure 12.3A),

along with more pronounced declines in photosynthetic capacity, water-use efficiency, apparent CO_2 uptake efficiency, and quantum yield than did leaves from low-fertilized birch. Given the similar O_3 uptake under both nutrient regimes (see Figure 12.4B), leaf maintenance at low nutrition was apparently more efficient relative to leaves of high nutrition.

The influence of O_3–nutrient interactions on photosynthesis of birch was reflected in distinct changes in the carbohydrate metabolism of leaves. The levels of glucose, fructose, and sucrose were significantly enhanced by O_3 in young birch plants at low but not so at high nutrient supply.[22] Consistently, low nutrition was associated with an inhibited synthesis of sucrose as indicated by the reduced sucrose phosphate synthase activity and increased levels of fructose-2,6-bisphosphate[50] — a metabolite known to regulate sucrose synthesis.[47] In parallel to sucrose accumulation, enzymes of sucrose degradation like sucrose synthase and alkaline invertase were stimulated, probably contributing to the enhanced levels of glucose and fructose. The flux of assimilates was apparently redirected from sucrose synthesis to starch formation, as sustained by reduced starch phosphorylase and ADP-glucose-pyrophosphorylase activities, and to the glycolytic pathway. In relation to glycolysis, the induced high-PEPC activity was striking[22] as this enzyme initiates the anaplerotic synthesis of oxalacetate and malate through nonphotosynthetic CO_2 incorporation and, thereby, promotes the citrate cycle. The raised supply of C_4 acids may fuel the respiratory ATP production and provide substrate for repair and detoxification processes.[48,49] These interrelationships are consistent with the observed increase in $\delta^{13}C$ in the plant biomass, which can be explained by the stimulated PEPC activity and its low ^{13}C discrimination. Furthermore, increases in malate, respiration, and ATP-to-ADP ratio were found under O_3 stress.[22,36] Raised PEPC activity has been reported previously for O_3-exposed pine and poplar plants.[51-53]

Elevated carbohydrate levels as found in low-fertilized birch under O_3 stress may also contribute via end product inhibition to a decline in photosynthetic capacity.[54] However, in O_3-exposed birch of high nutrition sugar levels were not raised.[22] The decrease in photosynthetic capacity in these plants appeared to be determined by cell collapse rather than by diminished Rubisco activity.[22,31,32] Inositol levels were decreased by O_3 at both nutrient regimes, the cyclitols being regarded as scavengers of O_3-induced hydroxyl radicals,[55] although findings conflict about cyclitol responses to O_3.[52,56,57] Overall, the extent of nutrition did not prevent the decline in the leaf structure and photosynthesis of birch.

It is difficult to decide on the basis of carbohydrate responses alone whether high nutrition is advantageous for O_3 tolerance. One can state, however, that the carbohydrate metabolism responded to O_3 more sensitively in leaves of low nutrition, and that the life span of such leaves was longer than at high nutrition.[31] This latter finding is important with respect to the indeterminate shoot growth in birch: leaf formation and longitudinal growth of shoots continue throughout the growing season when nutrition is high, but cease early at low nutrition. Hence, it would appear advantageous to maintain the relatively fewer leaves formed at low nutrition even if they are injured. By contrast, at high nutrition the premature loss of the aging leaves on the lower shoot sections after the O_3 dose has become injurious might be balanced by new uninjured leaves formed at the elongating shoot tips.

Remarkably, protein levels, Rubisco activity, and photosynthetic capacity were higher in young O_3-exposed leaves on upper shoot parts relative to individuals in filtered air when nutrition was low, whereas such effects were absent at high nutrition.[31,50] At low nutrition, perhaps plant-internal nitrogen retranslocation to the young leaves compensated to some extent for the O_3-caused photosynthetic decline of the aging leaves. By contrast, as long as the nitrogen availability is high, the "opportunity costs"[47] seem to be lower in forming new leaves and abandoning the aging and injured ones instead of maintaining them. As a consequence of new leaf and side branch formation, the proportion of O_3-injured leaves in the whole-plant foliage area was decreased at high relative to low nutrition.[58,59] Regardless of nutrition, the proportion of nitrogen allocated to the whole-plant foliage was significantly increased under O_3 stress, in particular when taking into account the content and low retranslocation of nitrogen in the prematurely shed leaves. Contrasting with birch,

plants (Figure 12.6A and C). When such plants were grown in the presence of chronic O_3 levels, the pigment content decreased more rapidly than under filtered air (Figure 12.6 A and C). This is in accordance with the development of leaf injury at the macroscopic and microscopic scale (see above). The question was whether SOD activity or antioxidant levels were associated with mediating O_3 protection. Interestingly, in young leaves of O_3-exposed, high-fertilized plants the activity of SOD was diminished as compared with controls, even though these leaves did not show visible injury (Figure 12.5A). In high-fertilized plants, elevated SOD activities appeared with development of foliar symptoms of injury (Figure 12.5A). These data show that O_3 exposure may result in any kind of response: increases, decreases, and no effects. Such conflicting results have frequently been reported in the literature.[11] One of the regular functions of SOD is the detoxification of superoxide radicals formed during photosynthesis when electrons are transferred to oxygen instead of $NADP^+$. In O_3-exposed leaves, low SOD activity might indicate that initially when symptoms of injury are not yet apparent, the turnover of NADPH/$NADP^+$ is accelerated because more reductant is needed to compensate oxidative stress. Under such conditions, there may be a higher availability of $NADP^+$, thereby outcompeting O_2 as an alternative electron acceptor. At beginning of injury, the demand for NADPH by the Calvin cycle may decline, requiring an increased detoxification of $O_2^{\cdot-}$ radicals since there was no evidence for a general decrease in PSII-mediated electron flux.[31] The highest SOD activity was found in injured leaves from high-fertilized birch, perhaps in response to unspecific oxidative degradation of cellular components. Taken together, these data suggest that in foliage of high-fertilized plants, SOD activities do not respond actively to protect plants but follow cellular events occurring as a result of O_3 stress.

In contrast to high-fertilized plants, in leaves of low-fertilized plants the age-dependent decline in SOD activity was not found reflecting a trend similar to that found for photosynthesis in filtered air.[31] High SOD activities and elevated ascorbate contents were maintained under low nutrient supply for all the leaf age classes (Figure 12.5B and D). In low-fertilized leaves the glutathione content was lower than in leaves from high-fertilized plants but O_3 induced a partial increase in this component, especially in older leaves (Figure 12.5F). Obviously, the response of antioxidants to O_3 is strongly affected by the interaction between nutrition and leaf age. The data support the hypothesis that high antioxidative capacity protects from O_3 injury.[11] Such elevated capacities were present in low-fertilized and to a lesser extent in leaves from high-fertilized plants. The differences in antioxidant contents and their modification by O_3 are striking, since the analyzed leaves of high-fertilized birch with lower antioxidative protection were shed earlier than the leaves of low-fertilized plants with high antioxidant capacities. It will be necessary to address these O_3–nutrient interactions in other species, especially in such ones with determinate growth. This may be important because young expanding foliage from spruce or beech contains lower antioxidative capacities than the analyzed birch leaves.[76,77] It has been proposed that this ontogenic stage may be the "Achilles' heel" with respect to oxidative injury.[77]

Since O_3 is probably degraded in the cell wall, apoplastic defense mechanisms have attracted considerable attention in the last few years. Ozone-acclimated spruce trees grown at high altitude contain up to 5 m*M* apoplastic ascorbate and show increases in response to O_3 exposure.[78] In beech, O_3 induced a delayed response in ascorbate.[79] In an herbaceous species (tobacco), the ascorbate pool was rapidly depleted in presence of O_3.[80] In contrast to relatively O_3-resistant species such as beech or spruce, the O_3-sensitive birch clone did not contain significant concentrations of apoplastic ascorbate (Figure 12.7A). The apoplast contained predominately dehydroascorbate, which is not active in O_3 detoxification (Figure 12.7A). Little, if any apoplastic ascorbate has been found in poplar, a species which is also O_3 sensitive (Polle, unpublished results). In O_3-exposed birch leaves with visible injury (stipplings) the apoplastic matrix contained significant approximately threefold higher total ascorbate (= ascorbate + dehydroascorbate) concentrations than healthy leaves, an important fraction of this pool being in the reduced stage (Figure 12.7A). In low-fertilized leaves the O_3-induced increases in apoplastic ascorbate and dehydroascorbate were less pronounced than in high-fertilized leaves (Figure 12.7A). The concentrations of apoplastic glutathione were similar

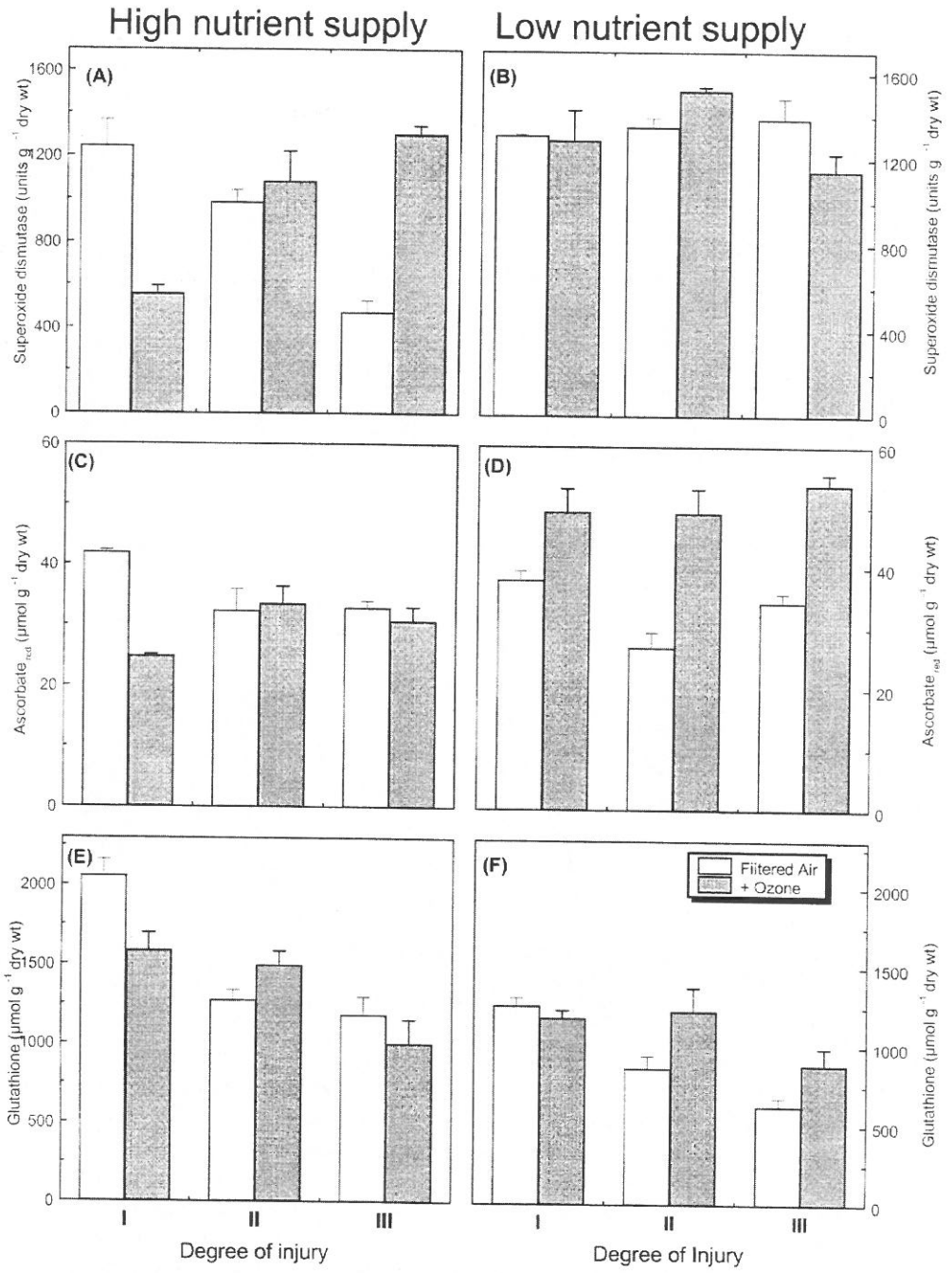

Figure 12.5 Response of SOD (A and B), ascorbate (C and D), glutathione (E and F) to O_3 in birch (*Betula pendula*) in dependence on leaf age and nutrient supply. The foliage was collected along the main axes of birch seedlings grown for about 4 months in the presence of O_3 (90/40 ppb, day/night) or filtered air (control). I, II, and III indicate leaf ages of about 4, 6, and 8 weeks, respectively, and correspond to increasing degree of injury for O_3-exposed plants. Limited nutrients were supplied by watering the plants with a 10-fold diluted fertilizer solution during the whole growth phase (for detailed growth conditions see Refernce 31). Data are means of six individual trees measured in three replicates (± SD).

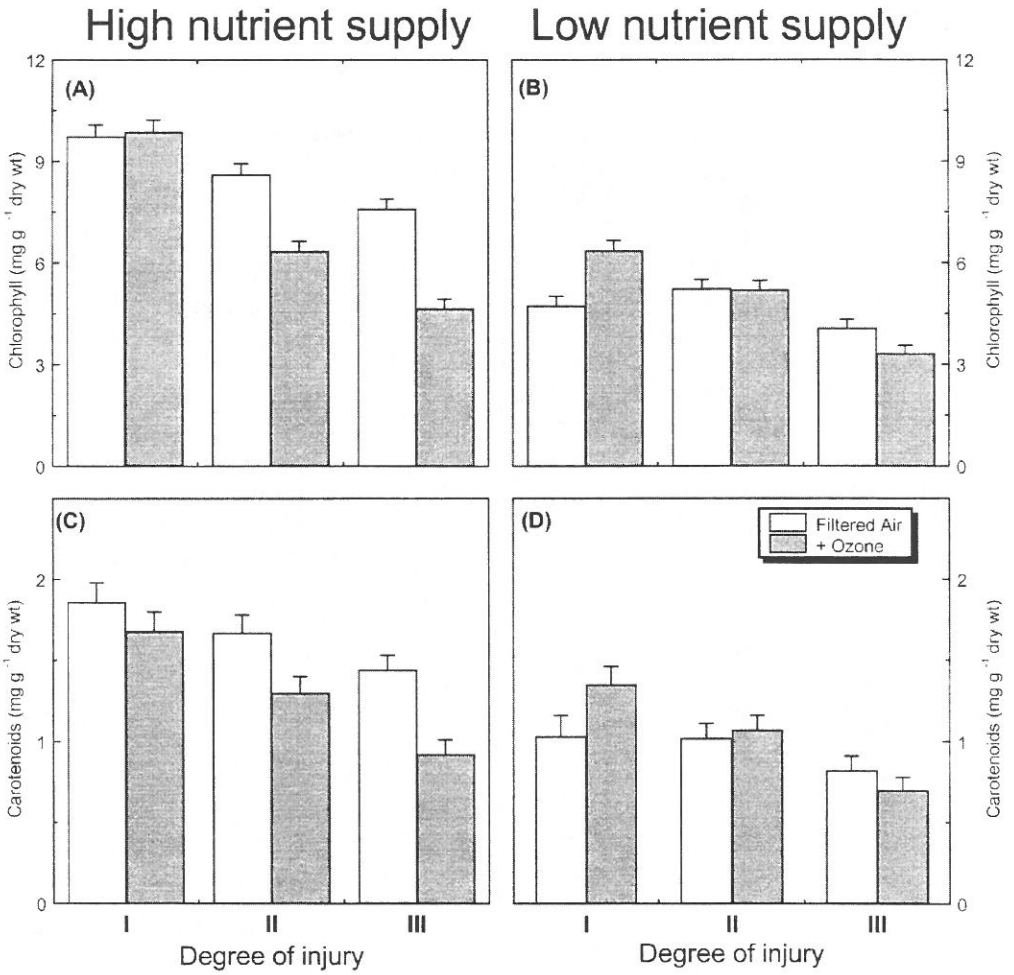

Figure 12.6 Response of chlorophyll (A and B) and carotenoids (C and D) to O_3 in birch (*Betula pendula*) in relation to leaf age and nutrient supply. For details see Figure 12.5.

in low- and high-fertilized plants (Figure 12.7C). In O_3-exposed foliage, the relative contents of antioxidants in the apoplast amounted to about 5 to 9% of the total foliar contents of these components (Figure 12.7B and D), which is relatively high compared with other studies.[80,81] The relative activities of apoplastic peroxidases — natural constituents in the apoplast — were in the same range as those of apoplastic antioxidants (Figure 12.7E), whereas glutathione reductase activity — a marker for symplastic components — was not significantly increased in O_3-exposed as compared with unstressed foliage (Figure 12.7F). This observation may indicate that O_3 caused a specific induction of apoplastic defenses. Unfortunately, it is not clear whether the relative increase in apoplastic antioxidants was caused by "easier" leakage of small solutes through slightly injured membranes than that of large proteins. However, regardless the causes of an increased presence of antioxidants in the apoplast, these will mediate some protection against unspecific oxidation.

The apoplastic phase also contains other solutes which may contribute to O_3 detoxification. For example, in spruce needles components like picein, p-hydroxyacetophenon, catechin, *p*-hydroxybenzoic acid glucoside, ferulic acid, kaempherol-3-glucoside, as well as unknown phenolic components have been found in significant concentrations.[11] Furthermore, the apoplastic defense systems can only operate if reductant is delivered at sufficiently high rates. The capacity and identity

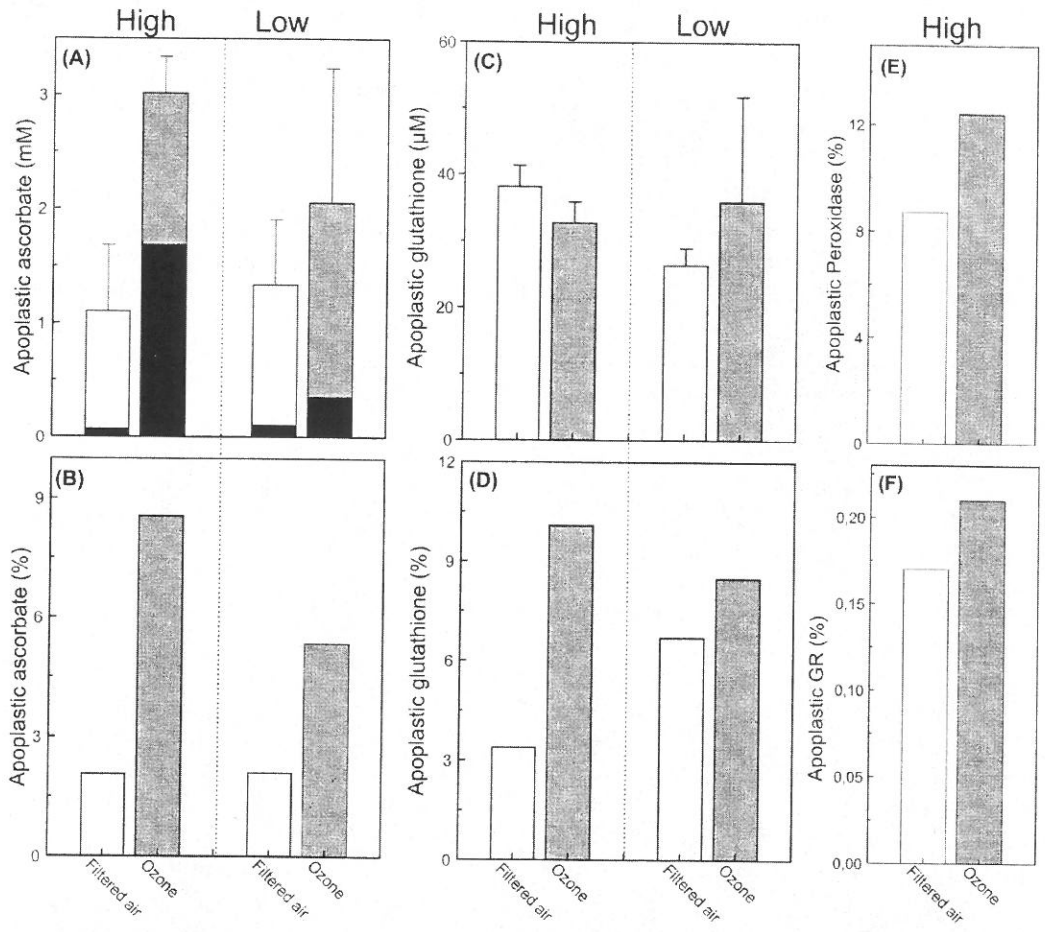

Figure 12.7 The effects of O_3 and nutrition on apoplastic ascorbate (A and B) and glutathione (C and D) in relation to apoplastic and symplastic enzymes in *Betula pendula*. The foliage was collected along the main axes of birch seedlings grown in the presence of O_3 (90/40 ppb, day/night) or filtered air (control). Leaves with visible injury symtoms were used for the analysis of apoplastic and symplastic components. The activities of peroxidase and glutathione reductase and the content of ascorbate and glutathione in foliar extracts were set as 100%. Ascorbate [(A) apoplastic concentration of ascorbate (black bars) and dehydroascorbate (white, gray bars), (B) relative occurrence in the apoplastic space], glutathione [(C) apoplastic concentration, (D) relative occurrence in the apoplastic space], peroxidase (E) and glutathione reductase activities (F), relative occurrence in the apoplastic space. Data indicate means of $n = 6$ (± SD).

of this system is still unclear. There is preliminary evidence that O_3 causes a rapid flux of electrons across the plasma membrane from the inner to the outer side (U. Heber, personal communication). Such a transmitting pathway might connect intracellular, symplastic with extracellular, apoplastic antioxidative systems. A better characterization of these systems is urgently needed in order to understand fully their role in O_3 detoxification. Furthermore, it will be necessary to resolve the reactions of the defense systems on a microspatial scale within the leaf because most O_3 that enters a leaf seems to react in the vicinity of the stomatal apertures. Since under both nutrient regimes the daily fluxes of O_3 into the leaves were similar to each other (see Figure 12.4), higher concentrations of apoplastic antioxidants can be expected to afford higher protection. The most important conclusion from the birch study is that low-fertilized plants allocate elevated amounts of substrate

and energetic resources to detoxification systems probably in order to provide their leaves *a priori* with enhanced defense measures important for longer leaf persistence in a stressful environment.

12.5 OZONE–NUTRIENT INTERACTION IN WHOLE-PLANT CARBON ALLOCATION AND BIOMASS PRODUCTION

Both the short- and the long-term nutrient availability determines the fate of assimilated carbon for plant survival. During ontogenic development, carbon allocation is modified by chronic O_3 concentrations. Low nutrient availability and drought result in favored root growth,[82-84] whereas O_3 generally leads to an opposite effect resulting in decreased root–shoot ratios.[8,85] The O_3-induced changes in plant performance appear to be associated with an impeded assimilate translocation from the leaves to the roots.[9] The disturbance of the carbohydrate metabolism is also apparent from starch accumulation which occurs in O_3-injured leaves along the small veins (see Figure 12.2F).[22,23] Starch accumulation was observed under both nutrient regimes. Because of its specific localization along the veins, this phenomenon was not comparable with an overall accumulation of starch recorded in spruce mesophyll cells after an early necrosis of phloem cells by Mg or K deficiency.[86] A direct influence of O_3 and its reactive products on the small leaf veins cannot be ruled out since birch leaves have a high proportion of air space (see Figure 12.3B). Such an influence may also be relevant for stomatal regulation under O_3 stress as inhibited by abscisic (ABA) retranslocation from the leaves due to phloem dysfunction which may favor stomatal narrowing.[87] In leaves displaying visible O_3 injury, the cell walls in small leaf veins were similarly thickened as those in mesophyll cells, the cytoplasm was darkened, vacuoles were filled with dark or coarse tannin precipitation (Figure 12.8B vs. 12.8A, E) in contrast to the sandy tannin appearance in the control.

With respect to whole-plant carbon allocation, it has to be considered that birch grown at relatively low nutrition maintains a higher proportion of O_3-injured foliage area than that grown at high nutrition. Therefore, it would be expected that carbon supply to roots may be more limited at low as compared with high nutrient supply. In fact, the root–shoot biomass ratio (R/S), which was often reduced under O_3 stress,[8] was only slightly lowered by O_3 at high nutrition, whereas O_3 reversed R/S at low nutrition from values > 1[54] to < 1, i.e., to about the level of the high-nutritional plants.[58,88,89] Although R/S was altered dramatically by O_3 at low nutrition, the proportions of fine and coarse roots remained unchanged in the total root biomass as did the specific root length in each root class. However, the coarse roots displayed overall smaller cell sizes and fewer starch granules (Figure 12.8H vs. 12.8G). The fine roots appeared darker and contained phenolic substances (stained black in Figure 12.8F) in conducting tissues, endodermal cells, and the pericycle. Such symptoms were not found in roots from unstressed plants.

Furthermore, an important factor modifying carbon allocation is mycorrhizal infection. In birch, interactions among nutrition, O_3, and mycorrhizae have not been studied. In *Acer saccharum* seedlings, O_3 exposure can cause morphological changes of mycorrhizae and stimulate the fungal, presumably nonmycorrhizal biomass in the rhizosphere, perhaps reflecting increased risk of pathogenic infection.[90] In O_3-exposed *Picea abies*, mycorrhizae were reduced in calcareous rather than acidic soils[91]; moreover, decreases in root and soil respiration have been found.[92] Probably, low carbon allocation to the root of O_3-exposed trees inhibits mycorrhizal and rhizospheric activities.[93] It has been shown, however, recently that in O_3-exposed seedlings of *Pinus ponderosa* O_3 stimulated (as long as O_3 levels were not too high) the fungal and bacterial biomass in the rhizosphere and increased root respiration, the latter effect being driven by low rather than high nutrition.[94] In parallel, the respiratory CO_2/O_2 quotient was increased by O_3, indicating changing substrate in the belowground respiration. In mature trees rather than seedlings of *Quercus rubra*, O_3 increased the root respiration while decreased the production and turnover of the fine roots.[95] It was concluded that high root respiration favored nutrient uptake to meet the enhanced demand of the O_3-exposed foliage. It is unclear, however, whether mycorrhizae become a "burden" in trees under O_3 stress,

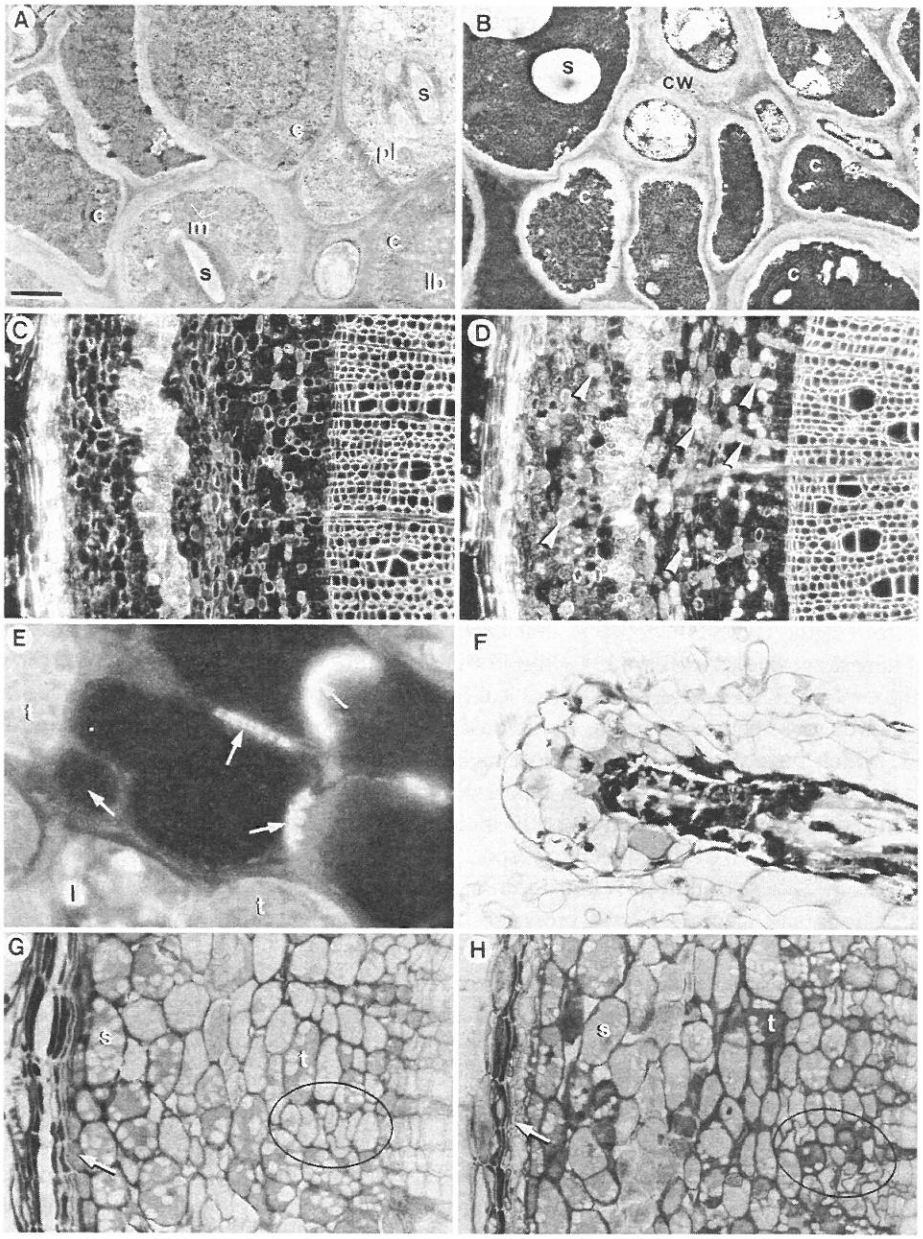

Figure 12.8 Transverse section of birch (*Betula pendula* Roth) leaves grown in filtered air (A, C, G) or filtered air with added O_3 (B, D, E, F, H) with low or high fertilization. Ozone was added during daylight hours (90 ppb) and night (40 ppb). The transverse section were prepared as described elsewhere.[16] (A and B) Bar = 1.3 μm; TEM micrographs of small leaf veins in 35-day-old birch leaves from the low-fertilization regime; (B) leaf with visible O_3 symptoms (stippling, O_3 dose at harvest = 58 ppm x h). In B (vs. control A) the cell walls (cw) are thickened, plasmodesmata (pl) and cytoplasmic lipid bodies (lb) are rare, starch grains (s) are large, the cytoplasm is electron dense and as dark as the mitochondria (m). (C and D) High fertilization, bar (see A) = 89 μm, stem section (age 150 days) viewed by light microscopy under dark field + phase contrast (unstained). Arrow heads in D (vs. control C) with an O_3 dose at harvest of 250 ppm x h denote phenolic (luminescent) cell content. (E) Detail from (D), stem phloem, bar = 7 μm. Arrows denote sieve pores narrowed by callose (white, stained by Aniline, viewed under UV fluorescent light) and an empty (black) companion cell; t = coarse tannin depositions, l = vacuolar lipid droplet. (F) Detail from fine root, lateral section, bar = 36 μm, phenolic compounds stained black by Os/KI. (G and H) Bar = 36 μm, coarse roots. H (vs. control G) shows declined phellogen cells (arrow), smaller storage starch grains (s), darkened vacuols (particularly tannin, t), and disordered phloem cell rows (circled).

taking into account the strong carbon sink represented by the fungus, the limited carbon allocation to the root, potential increases in root respiration, and, overall, the lowered photosynthetic capacity. Findings of unchanged R/S of mycorrhizal relative to nonmycorrhizal plants with reduced R/S under O_3 impact may support this view[96] and perhaps reflect the competition for assimilates required in the maintenance of the O_3-exposed foliage. As an issue also for herbaceous plants,[97] the nutritional impact on the cost–benefit relations of mycorrhizae under O_3 stress has not been clarified yet.

Stem growth can also be limited by O_3, although often radial rather than longitudinal increment appears to be affected.[8] One hypothesis is that O_3 and its reactive products may reach the phellogen from outside through the lenticells by diffusion through the intercellular space or the apoplast, where they may affect cellular differentiation or induce decline in stem tissues. Another possibility is that the supply of carbohydrates to the stem is limited via inhibited phloem loading. Microscopically, it can be observed that O_3 leads to a deterioration of the phellogen and phellem cells near the stem surface in young birch trees.[14] In the cortical parenchyma of the shoot more cells have their "disposal bags," the vacuoles, filled with phenolic substances (mostly tannin) at low rather than at high nutrient supply. Ozone-induced decline of cells becomes visible, when tannin precipitations become coarser and parenchyma cells become plasmolyzed and brownish,[14] which can be detected by their luminescence using the microscopical dark field technique (Figure 12.8D, arrow heads vs. control 12.8C). Similar cell decline occurs in the stem phloem parenchyma, and the rows of sieve elements (Figure 12.8, controls 12.8C and G) become disordered (12.8D and H). Irrespective of nutrition, the cambial activity is shut down earlier in the season and the xylem cells near the cambium show thicker cell walls (white in Figure 12.8D vs. 12.8C) than the younger xylem cells of the control. Before onset of autumnal leaf fall in early November, the stems from the control treatment still show open sieve pores. In contrast, the sieve pores in stems of the trees in the O_3 treatment, which have already partially lost their foliage, are narrowed by callose, have fewer protein bodies, and their companion cells appear dead without luminescent cytoplasm. The changed luminescence of the adjacent parenchyma cells indicates altered storage substances (Figure 12.8E).[19] Ozone-induced shutdown of the phloem transport forces the trees to suspend growth until the next growing season. This also inhibits retranslocation of assimilates. Cell decline was not observed at low fertilization, but xylem tissue was proportionally more decreased by low fertilization together with O_3 than the bark tissue.[19]

Ozone had minor impact on stem growth at low nutrition, and, therefore, the amount of respired CO_2 throughout the growing season per unit of volume increment was similar to the control in filtered air (Maurer and Matyssek, unpublished results). This ratio was enhanced by O_3; however, at high nutrition stem growth was significantly reduced and ceased earlier in the season than in the absence of O_3. Apparently, the proportion of growth respiration declined under O_3 stress relative to the respiratory maintenance costs which remained unchanged throughout the growing season (Maurer and Matyssek, unpublished results). The O_3-caused premature loss of the leaves at the lower stem probably was responsible, at high nutrition, for the lowered radial growth of the stem and, to some extent, for the limitation of root growth.[47,98] Overall, the actual respiratory costs related to wood formation did not seem to depend on nutrition or O_3 regime, which is consistent with findings about other factorial influences.[99,100]

Ozone has the potential of inhibiting the branching of the crown,[23] and so does low nutrition; consequently, suppressed branch formation by O_3 was most evident in birch of high nutrition.[58] Also, leaf expansion is inhibited by O_3 and low nutrition (Figure 12.1A and B), so again O_3 impact was significant only at high nutrition. In the latter nutrient regime, the lowered number of branches, apart from premature leaf loss and reduced leaf size, was mostly responsible for the smaller foliage area relative to plants in O_3-free air. At low nutrition, the foliage area was not reduced by O_3; however, the proportion of O_3-injured foliage was high. Thus, the carbon gain strongly depended on the photosynthetic performance of the injured leaves and on their maintenance.[58] The water loss was reduced per unit of whole-plant foliage area similar to the observation at the single leaf level, whereas no such effects were found at high nutrition. By modifying the crown architecture and the

cost–benefit balance of water loss vs. carbon gain as related to the allometry, O_3 affects the mechanistic basis of plant competitiveness — mediated through nutrition.[101,102]

The concentrations of N, P, and K of whole birch plants were enhanced by O_3 at low nutrition, but differences diminished as high nutrition raised the whole-plant biomass and nutrient contents, with the greatest stimulations occurring in the absence of O_3.[58] Enhanced nutrient contents under O_3 have been reported previously,[103] although the overall findings conflict, due to changes in nutrient uptake vs. demand or plant-internal retranslocation.[8] Applying the concept of nutritional analysis by Timmer and Morrow[104] to birch with its indeterminate shoot growth, the response to nutrition would indicate nutrient demand at low nutrition. However, it should be noted that birch leaves did not develop visual symptoms of deficiency apart from decreased pigment contents (see Figure 12.6). The concentrations of Ca and Mg were not affected by O_3 at high and low nutrition. However, they declined as high nutrition increased the biomass and the nutrient contents of the plants, these changes being greatest in the absence of O_3. Thus, Ca and Mg seem to have been nonlimiting at low nutrient supply. Ozone did not fundamentally change the whole-plant interrelationships between levels and contents of nutrients and biomass production, but only decreased the extent of interaction between biomass production and nutrient relations.[58] Overall, the biomass production was driven by nutrition, whereas O_3 appeared to play a secondary role. Remarkably, the proportional limitation of annual growth by O_3 was similar in both nutrient regimes and tended to be smaller even at low nutrition. The whole-plant carbon balance of the second half of the growing season (after the O_3 dose had become a constraint on production) revealed that at low nutrition only a small proportion of the carbon gain was used for growth under O_3 stress, indicating high respiratory costs and explaining the low "water-use efficiency" of biomass production at the whole-plant level (Figure 12.9).

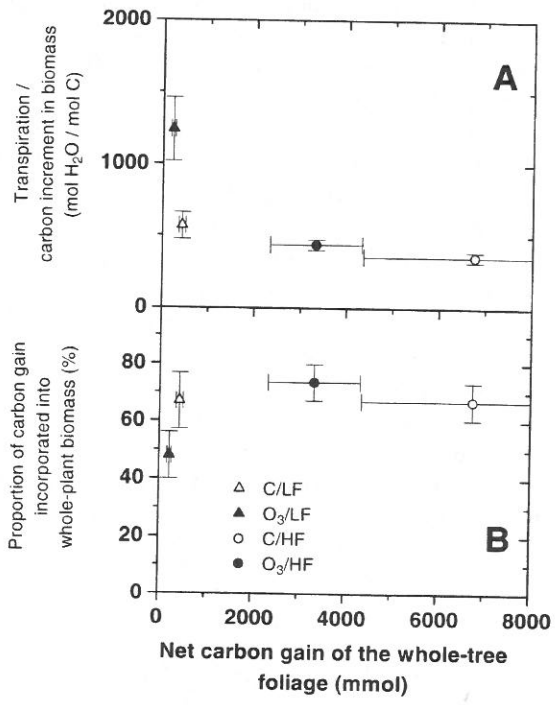

Figure 12.9 Transpiration as based on the carbon increment in the whole-plant biomass (A) and proportion of the carbon gain incorporated into the whole-plant biomass in relation to the net carbon gain of the foliage (B) in *Betula pendula* plants during the second half of the growing season (August 3 to October 3, 1993). (Calculated from data in Reference 58.) For abbreviations, see Figure 12.4.

It seems that birch can "choose" between two different "strategies" for assuring biomass production under O_3 stress, depending on the nutritional status and, as a consequence, on the rate of leaf formation. One has to be aware, however, that the extent of leaf longevity varies between years because of the additional variable impact of the accompanying environmental scenarios.[21] In indeterminate-growing trees, low nutrition can result in changes in whole-plant carbon allocation, and high respiratory costs and leaf maintenance; high nutrition allows for high leaf turnover[59] with minor impact on the whole-plant carbon allocation. Both nutritional strategies apparently allow for similar efficiencies in biomass production under O_3 stress,[105] and in these terms, high nutrition is not a prerequisite for high O_3 tolerance. Low nutrition did not override or balance the effects of O_3 stress on whole-plant carbon allocation, as suggested by Greitner and Winner,[40] Weinstein et al.,[106] or Mooney and Winner.[85] Considering the "bias" by nutrition on the carbon, water, and nutrient relations and on the allometric differentiation under O_3 impact, it is certainly imaginable that conflicting reports on responses to O_3 may relate to variable plant nutrition.[8] As well, conditions requiring the maintenance rather than the replacement of the O_3-injured foliage may render trees more susceptible to changes in the plant-internal resource allocation which, via altered root and crown architecture and related consequences for the "resource gathering capacity," can affect competitiveness.[107]

12.6 CONCLUSIONS

Since O_3 is taken up into the mesophyll via the open stomata, plants respond to O_3 fluxes rather than to ambient O_3 concentrations. The intrinsic O_3 exposure is determined by structural features of the leaf such as stomatal conductance, intracellular air spaces, thickness of cell walls, etc. A case study on birch shows that these features are strongly affected by nutrition on the one hand and are changed under the impact of O_3 on the other hand. Exposure to chronic O_3 levels frequently results in reduced O_3 fluxes into the leaf. Anatomical studies suggest that this influx is a consequence of injury rather than of regulated acclimation to O_3. Several lines of evidence suggest that O_3 injures pathways for assimilate transport, thereby affecting cellular carbohydrate metabolism. Low nutrient supply shifted assimilate resources to antioxidative defenses, thereby enabling a maintained life period of leaves. However, there was no evidence that O_3 as a single factor caused an induction of antioxidative defenses. In contrast, leaves from high-fertilized trees were shed earlier under O_3 stress so that the carbon gain overall rather depended on the uninjured foliage as compared with the low-fertilized plants, in which the carbon gain relied on the O_3-injured foliage. Ozone exposure also affected stem and roots growth. One reason was probably the diminished supply of carbohydrates. In the stem, effects on anatomical structures were also apparent. These O_3 effects were modified by the extent of nutrient supply. Since the case study was performend with birch, a species with indeterminate shoot growth and with young plants, further studies with older trees, preferably grown and exposed to experimentally elevated O_3 regimes under stand conditions and especially characterized by determinate growth patterns, are required. In addition to the variation in nutrients, other relevant factors such as light or water supply will have to be varied. Such factorial studies are important for identification of the most sensitive response mechanisms and for a scaling of O_3 effects from the cellular to the whole plant and up to the stand level.

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