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Review

The challenge of making ozone risk assessment for forest trees more mechanistic

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Clarifying and linking mechanisms of O_3 uptake and effective dose are research challenges highlighted in view of recent progress and perspectives towards cause–effect based risk assessment.

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ABSTRACT

Upcoming decades will experience increasing atmospheric CO_2 and likely enhanced O_3 exposure which represents a risk for the carbon sink strength of forests, so that the need for cause–effect related O_3 risk assessment increases. Although assessment will gain in reliability on an O_3 uptake basis, risk is co-determined by the effective dose, i.e. the plant's sensitivity per O_3 uptake. Recent progress in research on the molecular and metabolic control of the effective O_3 dose is reported along with advances in empirically assessing O_3 uptake at the whole-tree and stand level. Knowledge on both O_3 uptake and effective dose (measures of stress avoidance and tolerance, respectively) needs to be understood mechanistically and linked as a pre-requisite before practical use of process-based O_3 risk assessment can be implemented. To this end, perspectives are derived for validating and promoting new O_3 flux-based modelling tools.

1. Introduction

The methodology for assessing risks imposed by enhanced ground-level O_3 regimes on trees and forests has been debated for quite some time (Kärenlampi and Skärby, 1996; Stockwell et al., 1997; Fuhrer et al., 1997; Matyssek and Innes, 1999; Ashmore et al., 2004; Krause et al., 2005; Percy et al., 2007; Matyssek et al., 2007b). This debate is fed by prognoses that chronic exposure of vegetation to ground-level ozone will likely increase during upcoming decades (Fowler et al., 1999; Vingarzan, 2004; Ashmore, 2005), although declines may occur regionally to varying extents (McKendry and Lundgren, 2000; Oltmans et al., 2006). Still, O_3 exposure will stay at levels higher than during pre-industrial times, constituting a risk for vegetation, including forests (Skärby et al., 1998; Matyssek and Sandermann, 2003). As the latter are determinants of global carbon cycling (Schlesinger, 1997; Körner, 2006), ground-level ozone being a factor of climate change (IPCC, 2001, 2007; Giles, 2005; Ashmore, 2005) tends to mitigate carbon sink strength and modify metabolic response under increasing atmospheric CO_2 concentration (Grams et al., 1999; Fiscus et al., 2005; Kozovits et al., 2005; King et al., 2005; Karnosky et al., 2003, 2005; Kubiske et al., 2006, 2007; Valkama et al., 2007). This is relevant for agroforestry and energy farming, renewable resource management and post-Kyoto policies, as the productivity of fast-growing pioneer rather than climax tree species appears to be at risk under O_3 impact (Karnosky et al., 2003; Matyssek et al., 2007a). Given the ecological and economic significance, cause–effect related O_3 risk assessment is needed.

Two different concepts of O_3 risk assessment currently exist, i.e. methodologies based on (1) exposure to O_3 concentration and (2) O_3 flux and uptake into plants (e.g. Fuhrer et al., 1997; Musselman et al., 2006; Percy et al., 2007; Percy and Karnosky, 2007; Paoletti and Manning, 2007; Matyssek et al., 2007b). It was initially

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believed that O_3 exposure would tightly be linked with O_3 uptake of plants, as suggested mainly by studies on juvenile trees in exposure chambers (Kolb and Matyssek, 2001). This link has turned out to be poor in the field, however, varying with altitude or with light or water limitation (Matyssek et al., 2004, 2006, 2007b). In particular, exposure-related risk assessment becomes unreliable during dry periods of the Mediterranean climate (Paoletti, 2006; Paoletti et al., 2006; Wieser et al., 2006a) or summers as experienced in 2003 in Central Europe (Ciais et al., 2005), although scientifically elaborated advances strive to improve exposure concepts because of their practicability of use (Percy et al., 2007; Paoletti and Manning, 2007). In fact, North America is continuing to use exposure-based methodologies to support air quality standards. Nevertheless, O_3 uptake under high O_3 exposure can be lower during dry than humid summers with low O_3 exposure (Löw et al., 2006), such a decoupling being caused by stomatal regulation in response to site factors (Schulze and Hall, 1982). Most distinctively, however, the O_3 flux is phytomedically relevant and mechanistic (Wieser and Matyssek, 2007) as it yields the O_3 dose taken up through stomata into the leaves (cf. Medical Dictionary, 2003; Wieser and Havranek, 1993; Musselman et al., 2006), where the derivatives of ozone may incite stress reactions within the confines of the plant's metabolic sensitivity (Sandermann et al., 1997; Musselman et al., 2006; Tausz et al., 2007). In general toxicology, risk assessment needs to employ mechanistic (i.e. process-based, cause–effect related) models as part of the database (Klaassen, 1986). This latter requirement is intrinsically represented by the O_3 flux rather than exposure approach. Still, it is not the aim of this paper to evaluate advantages and shortcomings of exposure versus flux concepts, which has comprehensively been done elsewhere (Paoletti and Manning, 2007). Rather, mechanistic approaches will be proposed, which are to comprise basic processes in O_3 uptake and defence-related plant metabolism. Recent progress and research challenges will be outlined towards mechanistically founded O_3 risk assessment.

Cause–effect related O_3 flux has not yet been commonly adopted in risk assessment because (1) mechanistic clarification is still required before simplification can be elaborated for routine use in practice – and (2) the flux approach is felt to be too complicated, although recent advances have allowed routine computation of O_3 fluxes (Matyssek et al., 2007b) and, in particular, its initiation in risk assessment at the pan-European landscape level (Wieser and Tausz, 2006). Since O_3 flux-related risk assessment is based on modelling (Emberson et al., 2000; Tuovinen et al., 2001), a major shortcoming presently is the lack of empirical data sets for validation. It is important, therefore, to examine novel validation approaches as the ultimate pre-requisite for new flux-based modelling tools in O_3 risk assessment of trees and forests (cf. Klaassen, 1986).

It is tempting to claim risk to be understood when based solely on O_3 uptake. However, uptake is just one component of O_3 risk (Matyssek et al., 2004, 2007b), which is co-determined by the plant's sensitivity per unit of O_3 uptake, i.e. the effective O_3 dose (Wieser et al., 2002; Musselman et al., 2006; Ashmore, 2005). Hence, two components require attention, according to

$$O_3 \text{ risk} = f(O_3 \text{ uptake},\ \text{sensitivity per unit of } O_3 \text{ uptake}) \quad (1)$$

Care must be taken, therefore, not to confound conceptual differences between expressions like O_3 exposure, O_3 dose and effective O_3 dose (Musselman et al., 2006; Wieser and Tausz 2006). O_3 uptake inherently reflects traits of stress avoidance through the extent of O_3 exclusion by stomatal regulation, whereas upon uptake, sensitivity is an expression of metabolic O_3 stress tolerance. Hence, both O_3 uptake and effective dose are process-based, which renders the O_3 flux approach inherently mechanistic relative to exposure concepts. Understanding the effective O_3 dose (*sensitivity per unit of O_3 uptake* in Eq. (1)) is complex, as the plant may delay its metabolic response relative to the instant of O_3 impact and even "actively" amplify the oxidative stress (Sandermann, 2004). The extent to which the plant (or genotype) is able to handle oxidative stress depends on phenology, onotogeny and site conditions (Löw et al., 2007), being influenced by O_3-mediated, phytohormonal effects on plant sensitivity (Mahalingam et al., 2006; Winwood et al., 2007), and is linked with the capacity of the antioxidant, detoxification and repair systems as intrinsic features of plant metabolism. A major genetic component is involved as evidenced by heritable traits that influence relative O_3 sensitivity among closely related plant lines. Given this complexity, findings highlighted from herbaceous plants will set the stage for clarification in trees.

According to Eq. (1), this paper will elucidate (1) recent advances in assessing O_3 uptake at the whole-tree and stand level through empirical approaches and (2) the state of knowledge on the metabolic control of the effective O_3 dose. Beyond the scope of previous accounts (e.g. Musselman et al., 2006; Matyssek et al., 2007b; Percy et al., 2007; Tausz et al., 2007), the mechanistic grounds and evidential advances of both components of risk assessment, i.e. O_3 uptake and effective dose, will be reviewed from a common, integrative perspective. This includes extending understanding of O_3 detoxification towards the role of stress amplification and secondarily formed toxicants (e.g. singlet oxygen, peroxides of lipids and ascorbate, SH-groups of proteins) in stress tolerance. In view of such challenges, the present focus needs to be on current research efforts rather than on elaborating protocols of O_3 risk assessment for practical use. Nevertheless, policy-relevant strategies will be outlined towards practicability, as only mechanistic understanding of O_3 risk will meet the increasing demand for reliable assessment methodologies.

2. Scaling of O_3 flux and uptake from leaf to stand

Mechanisms of relevance for O_3 risk assessment are defined by the spatio-temporal resolution that is intrinsically associated each with O_3 uptake and effective O_3 dose (cf. Eq. (1), Sandermann and Matyssek, 2004). The mechanistic dimension of O_3 uptake is represented by the process of diffusive O_3 influx into leaves as determined by boundary layers and stomatal regulation, which are influenced by multi-factorial, plant-related and environmental impacts. Such process-based bridging of O_3 regimes with plant response to yield the effective O_3 dose as the outcome of cause–effect relationships renders the flux approach of Eq. (1) ultimately mechanistic. In the absence of such functional settings, exposure concepts fail, therefore, to be mechanistic. In the following, the functional basis of O_3 flux is highlighted to strengthen mechanistic understanding in scaling O_3 uptake to the tree and stand level.

2.1. Scope of O_3 flux assessment at the leaf level

The empirical derivation of the O_3 influx into plants has typically been based on the gas exchange measurement of CO_2 and water vapour of single leaves, employing the *water vapour surrogate method* (Matyssek et al., 1995). This approach makes use of the stomatal conductance for water vapour (g_{s-H_2O}), which is derived from the leaf transpiration rate (J_{H_2O}), and the gradient of high vapour pressure inside the intercellular spaces (e_i, approximating saturation pressure at leaf temperature) to the typically lower e_a (absolute humidity) in the ambient air (Nobel, 1983; von Willert et al., 1995). Expressing the gradient as the difference of the mole fractions of water vapour in air (Δw; cf. Cowan, 1977) yields

$$g_{s-H_2O} = J_{H_2O}/\Delta w\left(\text{mmol m}^{-2}\text{ s}^{-1}\right). \quad (2)$$

Δw represents $e_i - e_a$ as divided by air pressure P, and g_{s-H_2O} can be converted into the stomatal conductance for ozone by exchanging diffusion coefficients accordingly (Laisk et al., 1989)

$$g_{s-O_3} = 0.613 g_{s-H_2O} \quad (3)$$

The influx of ozone, J_{O_3} (alike J_{H_2O}), is a diffusion process, although the "upper end" of the O_3 gradient lies in the ambient air. Sound evidence was provided by Laisk et al. (1989) that the O_3 concentration at the "lower end" approaches nil[1] in the intercellular leaf space, as ozone finds its sink in the apoplast, decomposing into oxidative derivatives (Heath, 1994; Sandermann, 1996). Hence, the ambient O_3 level outside the leaf (p_{O_3}) virtually represents the O_3 gradient across the stomata. The stomatal pathway determines the O_3 influx (i.e. stomatal O_3 deposition), as O_3 uptake through the leaf cuticle can be excluded (Hoigne, 1988; Kerstiens and Lendzian, 1989) so that J_{O_3} is calculated as

$$J_{O_3} = g_{s-O_3} p_{O_3}\left(\text{nmol m}^{-2}\text{ s}^{-1}\right) \quad (4)$$

The unit of J_{O_3} reflects the trace gas feature of ozone in air. The temporal integral of J_{O_3} yields the cumulative O_3 uptake (COU, as "μmol mol^{-1} h"; Musselman et al., 2006; Wieser and Tausz 2006), which is the phytomedically relevant O_3 dose (Matyssek et al., 2007b; Wieser and Matyssek, 2007).

The strength of this leaf level approach is that stomatal regulation becomes apparent in J_{O_3} and COU (see Eq. (4)). However, the assessment of g_{s-O_3} inevitably suffers during gas exchange measurement from the destruction of the leaf boundary layer (Nobel, 1983; von Willert et al., 1995), representing a diffusion resistance that counteracts O_3 uptake. Overestimation of J_{O_3} may be the result relative to leaf exposure under field conditions. Obviously, stomatal regulation and O_3 interaction with air boundaries and transpired water molecules (not accounted for, so far) constitute the mechanistic dimension of O_3 uptake. Another shortcoming relates to the representativeness of measured leaves within the foliage, given the wide range of leaf differentiation, age classes in evergreen trees and exposure conditions within crowns. Uncertainty governs scaling, therefore, of J_{O_3} and COU from the leaf to the tree and stand level.

Nevertheless, the leaf level approach has provided the basis for modelling O_3 flux and uptake as currently used in risk assessment (e.g. Emberson et al., 2000; Tuovinen et al., 2001), taking the O_3 concentration of the free atmosphere as the starting point for stand level flux calculation (Fig. 1A). Hence, g_{s-O_3} is modelled through a top-down approach, to the extent species and site-specific parameterizations are available (Emberson et al., 2000; Nunn et al., 2005). Since g_{s-O_3} does not embody boundary layer conductances, the latter need to be modelled for estimating the O_3 concentration within boundary layers (p_{O_3-b}) at the stand and leaf levels, as p_{O_3-b} is relevant for J_{O_3} calculation; p_{O_3-b} being drained by O_3 sinks in plant and soil surfaces, the non-stomatal O_3 deposition needs to be modelled in addition. Such calculations depend on approximations so that variation in non-stomatal O_3 deposition, apparently depending on moisture conditions, has remained controversial (Fredericksen et al., 1996; Fowler et al., 2001; Altimir et al., 2006). This controversy, mirroring the uncertainty about the mechanistic basis of O_3 flux, can only be met with empirical data sets. These hardly exist; however, for validation empirical methods are available for partitioning stomatal and non-stomatal O_3 deposition (Massman, 1993; Nikolov and Zeller, 2003).

2.2. Sapflow approach of whole-tree O_3 flux assessment

The above outlined mechanistic shortcomings can be overcome through the sapflow approach, as recently suggested and demonstrated by Wieser et al. (2003), Matyssek et al. (2004), Nunn et al. (2007) and Köstner et al. (2007). The pursued bottom-up perspective (Fig. 1B) is based on the sapflow measurement through tree trunks (Q_w, e.g. Cermak et al., 1973; von Willert et al., 1995), as Q_w feeds the crown transpiration (E_c), according to the proportionality:

$$Q_w \sim P_e/(c_w \Delta T), \left(\text{l h}^{-1}\right) \quad (5)$$

with the specific heat of water (c_w) relating vertically induced temperature differences (ΔT) at given electrical energy input (P_e) to mass flow of water. Upon correction for heat dissipation through non-moving water, trunk structure and air, Q_w becomes the mass flow rate of xylem water (being positively correlated with P_e – or negatively with ΔT, if P_e is kept constant). Eq. (5) forms the basis of several variants of Q_w assessment (Cermak et al., 1973; Sakuratani, 1981; Granier, 1985) and E_c estimation, the latter being approximated by Q_w on a daily and seasonal basis (Schulze et al., 1985; Köstner et al., 1998). E_c is converted into the O_3 influx of the foliage (J_{O_3-f}) by (1) determining the crown conductance for water vapour (G_c) as

$$G_c = E_c/\Delta w_c. \quad (6)$$

Δw_c is the mole fraction difference of water vapour between the intercellular space of foliage and the ambient air at canopy height (cf. Cowan, 1977; see above). As an approximation, air and foliage temperature may be regarded as similar, as is the case under closed-canopy conditions, with the major part of the stand foliage being shaded. Hence, e_i becomes the saturation vapour pressure at air temperature within the canopy for calculating Δw_c. (2) G_c is converted into the crown conductance for ozone (G_{O_3}) in analogy to Eq. (3). If Δw_c is assessed at canopy height, boundary layer and aerodynamic conductances are accounted for by G_c and G_{O_3}, the latter being scaled to the foliage area of the study tree (cf. Section 2.3). (3) J_{O_3-f} is then calculated as (cf. Nunn et al., 2007):

$$J_{O_3-f} = p_{O_3-b} G_{O_3}\left(\text{nmol m}^{-2}\text{ s}^{-1}\right) \quad (7)$$

p_{O_3-b} as nl l^{-1} (=ppb) represents the O_3 concentration within the boundary layer of the foliage, being the "upper end" of the O_3 diffusion gradient. The O_3 concentration at the "lower end" is again regarded as nil in the intercellular airspace of the foliage (see above; cf. Laisk et al., 1989). Hence, p_{O_3-b} as the reference concentration for F_{O_3-f} is located in the vicinity of the leaves (contrasting with current modelling approaches, see above), although gradients in O_3 concentration still may exist within the canopy (Baumgarten et al., 2000). J_{O_3-f} represents the approximated stomatal O_3 deposition at the crown level under the influence of within-canopy boundary layers. In view of policy-related risk assessment requirements (UNECE, 2003; Matyssek et al., 2007b), sapflow-derived J_{O_3-f} offers a mechanistic basis for calculating mean O_3 uptake across the tree foliage on a daily basis. Hence, daily O_3 uptake (OU_{day}) is the integral of J_{O_3-f} during the diurnal course. The seasonal sum of OU_{day} yields the "cumulative O_3 uptake" (COU) as the phytomedically relevant whole-tree O_3 dose (Musselman et al., 2006; Matyssek et al., 2007b).

Using the sapflow approach at within-day time resolution of J_{O_3-f}, correction is needed of the temporal offset between onset of

[1] Research has shown that intercellular O_3 may be greater than zero (Moldau and Bichele, 2002), although those findings were favoured experimentally by high O_3 levels. Under ambient O_3 concentrations, it should be expected that the ultimate sink for O_3 in the apoplast would result in negligible amount of intercellular O_3 and serves as a basis for the approximation of the intercellular zero-level used in this analysis.

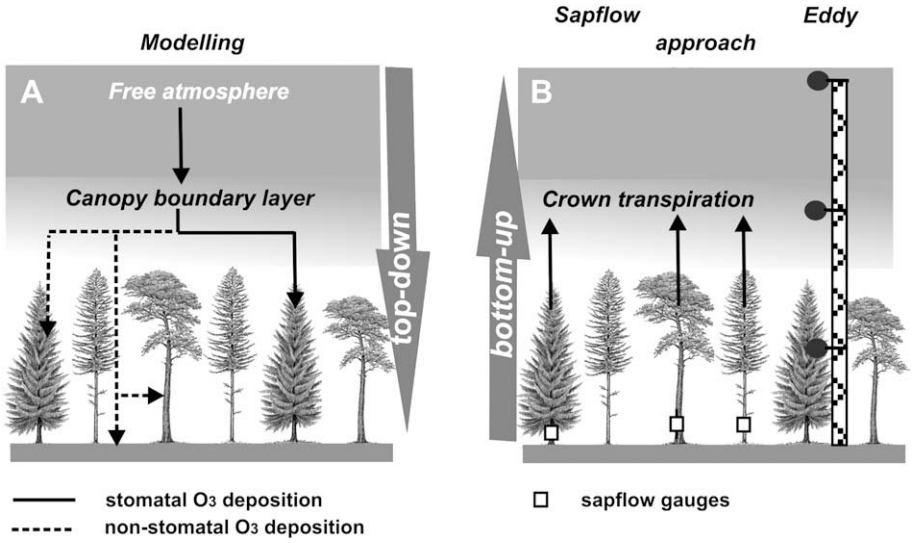

Fig. 1. Schematic view, illustrating the contrasting approaches in flux-based O_3 risk assessment: (A) "top-down" through modelling, calculating stomatal and non-stomatal O_3 deposition with the O_3 concentration of the free atmosphere as the starting point; (B) "bottom-up", empirically assessing via sapflow measurement, stomatal O_3 deposition of whole trees, with subsequent upscaling by means of silvicultural data to the stand level; may be combined with eddy covariance assessment of stand O_3 flux (i.e. sum of stomatal and non-stomatal O_3 deposition) to yield non-stomatal O_3 deposition (needed for validation of current risk modelling concepts) empirically as the difference of the outcomes of both approaches (see text for details; tree silhouettes adapted from U. Hecker, 1995. Bäume und Sträucher, BLV, München, Germany, 479 pp.).

foliage transpiration at sunrise (when stomata open) and sapflow at trunk breast height (typical position of sapflow gauges) a few hours later; accordingly, continuing sapflow past sunset requires attention. The offset reflects use of stored water in trunk and branches for transpiration (Tyree and Zimmermann, 2002), as the storage capacitance (varying with tree species and morphology) is emptied during the morning hours and refilled at night (Schulze et al., 1985; Köstner et al., 1998). Offset correction is achieved by regarding, during data processing, both sapflow at breast height and E_c to be triggered by sunrise (as demonstrated in Matyssek et al., 2004; Fig. 2), given the similar shapes of both time courses. This synchronization relates sapflow-derived E_c at within-day time resolution to the diurnal courses of Δw_c and p_{O_3-b} between sunrise and sunset for J_{O_3-f} calculation. Correction refinement at within-day time resolution must be subjected to further research.

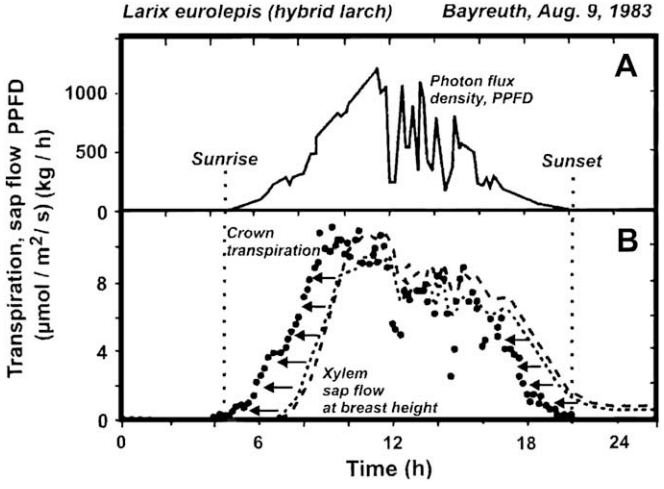

Fig. 2. Time course of (A) PPFD and (B) crown transpiration rate (dots) and xylem sapflow through the trunk at breast height (lines) of hybrid larch during one summer day. Arrows indicate the time correction in order to estimate crown transpiration from sap flow at the due time during the daily course (see text); adapted from Matyssek et al. (2004).

Fig. 3 exemplifies high J_{O_3-f} not necessarily to be the consequence of high p_{O_3-b}, but to result from moderate O_3 exposure, if G_c is high, for example, as under humid weather conditions (cf. Nunn et al., 2007).

The practicability of the sapflow approach for whole-tree and COU-related O_3 risk assessment will depend on model applications, which must not become too complex, although being mechanistic (Matyssek et al., 2007b; Emberson et al., 2000). One step towards simplification is the finding that the dependence of G_c on Δw obeys a similar relationship across a wide range of contrasting tree species and site conditions (cf. Schulze and Hall, 1982). Normalizing this relationship as demonstrated in Fig. 4 (Nunn et al., 2007; Köstner et al., 2007) allows us to derive one underlying, exponential function. Although generalizations require caution, they will foster the development of process-based modelling in O_3 risk assessment for practical use.

2.3. Scaling O_3 flux to the stand level

Temporal scaling of J_{O_3-f} towards whole-tree COU needs to be linked with spatial scaling to arrive at the stand level. This is achievable by linking COU of the foliage with allometric relationships, valid for respective stands, between foliage area and tree-structural parameters such as trunk diameter or cross-sectional sapwood area at breast height. Stand level COU can then be expressed per unit of ground area (Matyssek et al., 2007b). The sapflow approach pursues bottom-up tree-to-stand scaling (Wieser et al., 2003, 2006a,b; Matyssek et al., 2007b), as exemplified for O_3, G_{O_3}, F_{O_3} and COU of a *Pinus canariensis* (Tenerife Mediterranean climate) and a *Pinus cembra* forest (Austria, temperate climate; Fig. 5). Stand level COU during the growing season was 54 and 140 mmol m^{-2} ground surface area in the two species, respectively, and corresponded to COU at the needle level of 15.9 (*P. canariensis*) and 13.6 mmol m^{-2} (*P. cembra*) per unit of total needle surface area each. The scaling step between leaf and stand is

$$COU_{stand} = COU_{leaf} LAI. \quad (8)$$

Empirical data and scaling as shown in Fig. 5 enable validation of models as currently used in O_3 risk assessment.

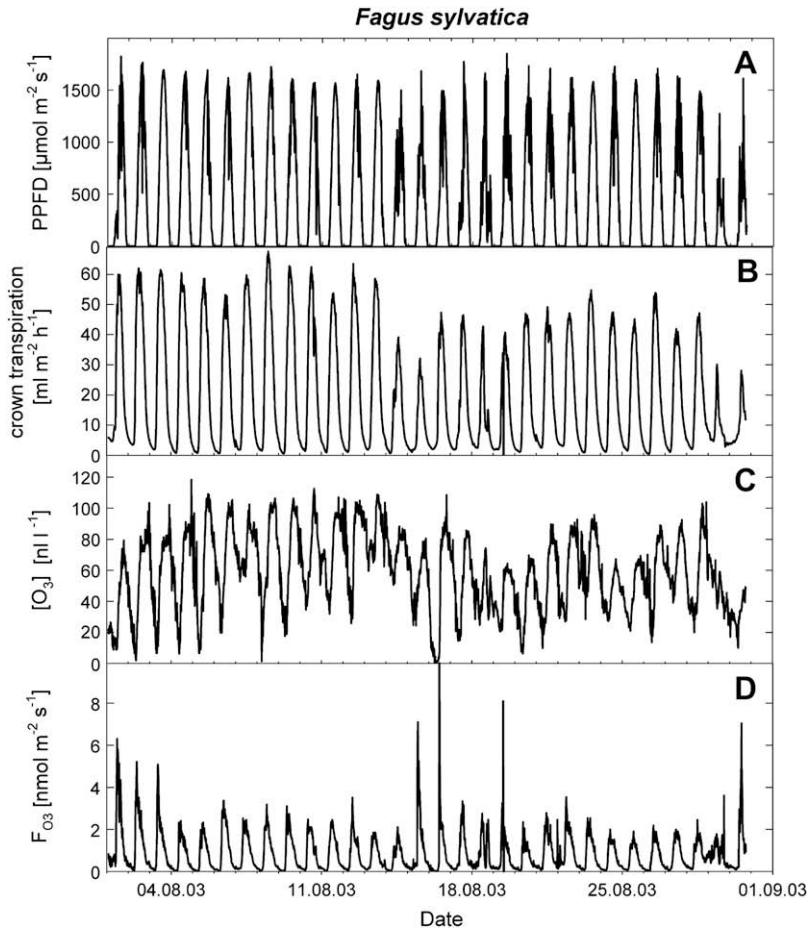

Fig. 3. Seasonal course of (A) PPFD; (B) crown transpiration; (C) O_3 concentration; and (D) whole-tree O_3 flux of adult beech (*Fagus sylvatica*) at Kranzberg Forest/Germany (adapted from Nunn et al., 2007).

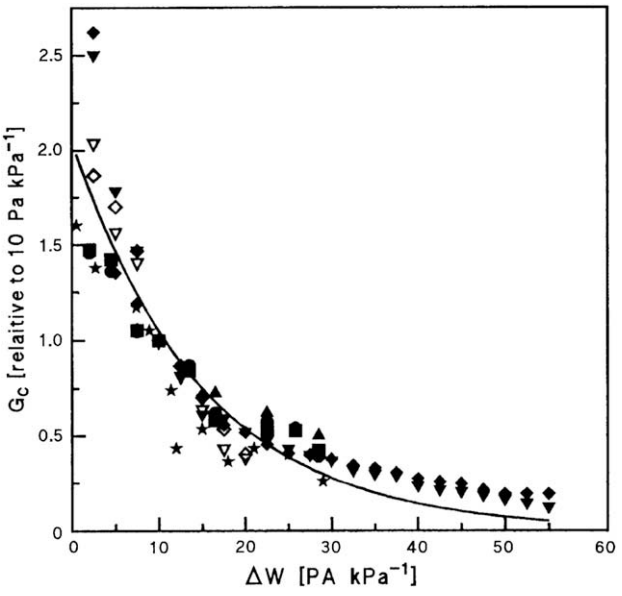

Fig. 4. Relative canopy conductance of *Picea abies* (●), *Pinus cembra* (■), and *Larix decidua* (▲) at Mt. Patscherkofel (1950 m a.s.l.); of *Fagus sylvatica* during a dry (▼) and a wet year (▽), and *Picea abies* during a dry (▼) and a wet year (◇) at Kranzberg forest (485 m a.s.l.); as well as of *Pinus canariensis* (★) in the Mountains of La Victoria, Tenerife (1650 m a.s.l.). Points were fit by the mono-exponential equation $y = 2.046 \exp(-0.0669x)$; $r^2 = 0.87$.

Average G_{O_3} at the leaf level can be obtained by relating sapflow-derived G_{O_3} at the stand or tree level to foliage area, which circumvents effects of boundary layer destruction as encountered during leaf level gas exchange assessments (see Section 2.1). The comparison of Table 1 between mean G_{O_3}, derived at the alpine timberline in the same tree species, either from leaf gas exchange or sapflow assessment, did not render significant differences across leaf, tree and stand levels. Such consistencies may be favoured by the open-canopy structure of timberline forests in the absence of large boundary layers. Still, clarification is needed for closed-canopy forest conditions.

2.4. Stand level O_3 flux assessment through eddy covariance approach

Other than the sapflow-based O_3 flux assessment which yields, upon upscaling, the stomatal O_3 deposition of stands, the eddy covariance approach can determine the stand level O_3 flux as the sum of stomatal and non-stomatal O_3 depositions (Mikkelsen et al., 2000; Vitale et al., 2005; Gerosa et al., 2005; Altimir et al., 2006; Cieslik, 1998, 2004). The latter approach, based on the turbulence theory, allows direct measurement of the total fluxes of O_3 (atmosphere to stand surface) and water vapour (from stands). Vapour efflux is equivalent to evapotranspiration, if the latter is assumed to be the only source of water vapour. Since the O_3 flux through stomata as one component in total flux is neither accessible through the eddy covariance nor the alternative vertical gradient methodology, a both empirical and theory-based algorithm need to be applied for

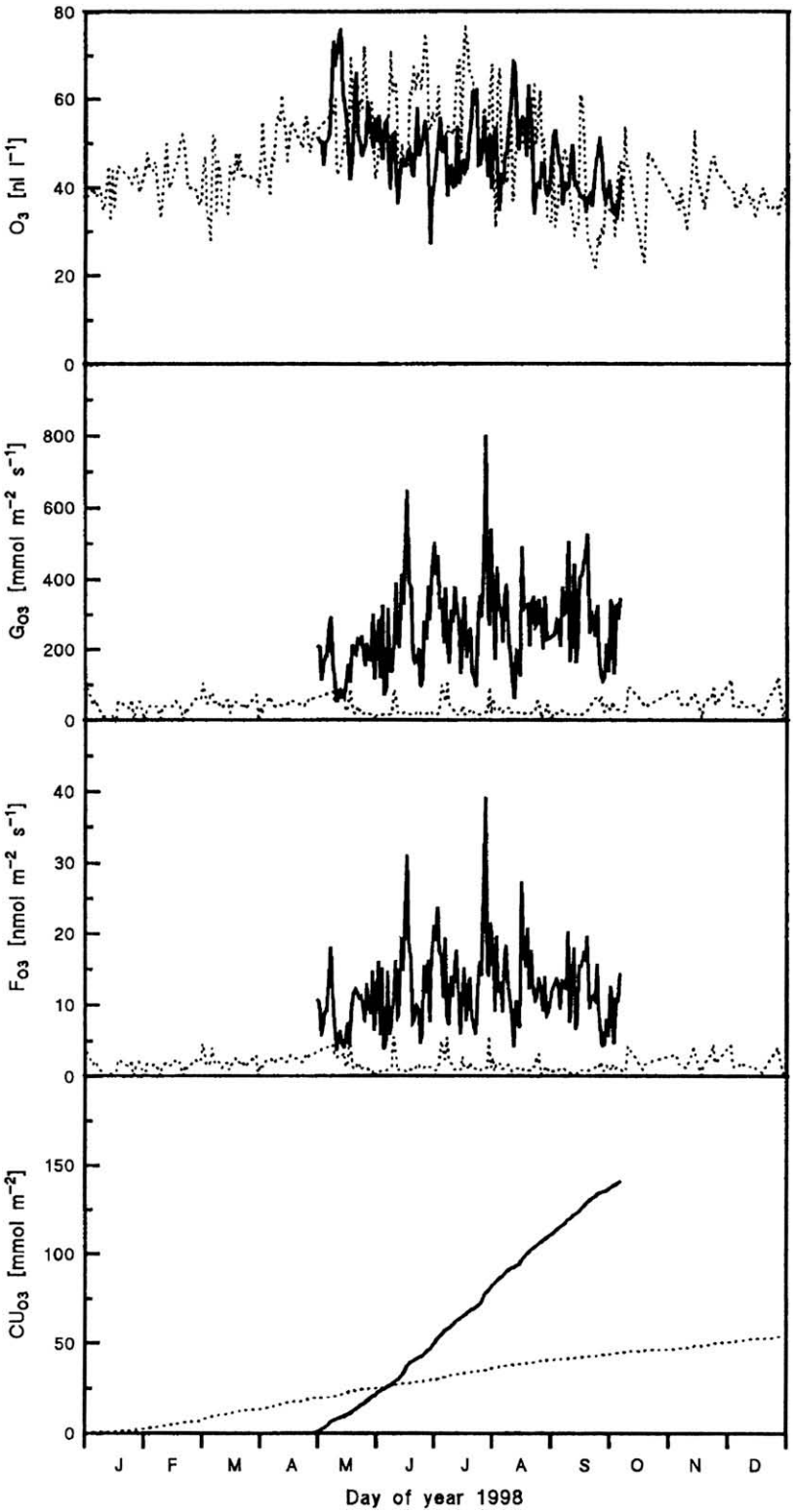

Fig. 5. Seasonal course of daily mean ambient ozone concentration [O_3], ground area scaled daily mean canopy conductance for ozone [G_{O_3}], ground area scaled daily O_3 uptake rate [F_{O_3}]; and ground area scaled cumulative O_3 uptake [CU_{O_3}] in *Pinus cembra* forest at 1950 m a.s.l. in the Central Austrian Alps (solid lines), and in a *Pinus canariensis* forest at 1650 m a.s.l. Tenerife, Canary Islands (dotted lines) (modified after Wieser, 2004, unpublished data, in Matyssek et al., 2007b and Wieser et al., 2006a,b). Stand characteristics of the two sites.

Parameter	*P. canariensis* forest Tenerife	*P. cembra* forest Austria
Stand age [years]	50	80–100
Stand density [trees ha^{-1}]	825	1038
Basal area [m^2 ha^{-1}]	53.8	47.6
LAI [m^2 m^{-2}] total surface area	3.4	10.4

Table 1
Average stomatal conductance for O_3 [mmol m^{-2} s^{-1}] expressed on a total needle surface area obtained from gas exchange measurements at the leaf level, sapflow measurements at the tree level and up-scaled to the whole stand in a *Pinus cembra* forest near the Klimahaus research Station on Mt. Patscherkofel south of Innsbruck, Austria 1950 m altitude

Tree species	Gas exchange measurements	Tree level sapflow	Whole stand
Picea abies	22.5	24.3	
Pinus cembra	19.8	26.1	25.1
Larix decidua	31	31.9	

The subalpine study site is located at the alpine timberline at 1950 m a.s.l. near the Klimahaus research Station on Mt. Patscherkofel (47°12′N, 11°27′E) south of Innsbruck, Austria and represents an open subalpine mixed forest (*Larici–Pinetum cembrae*) of cembran pine (*Pinus cembra* L.), European larch (*Larix decidua* Mill.), and Norway spruce (*Picea abies* [L.] Karst.). The trees were 70–90 years old and their height ranged between 9 and 14 m. The stand density was 1038 trees ha^{-1}, with a basal area of 47.6 m^2 ha^{-1} (84% *P. cembra*, 7%, *P. abies*, 9% *L. decidua*). The leaf area index was 10.4 m^2 m^{-2} on a total needle surface area, respectively.

calculating stomatal O_3 deposition from micro-meteorological approaches. The algorithm both considers the stomatal and non-stomatal O_3 flux (e.g. to soil, water, dead or live organic matter and influenced by VOCs):

$$F_T = F_{ST} + F_{NS} \quad (9)$$

where F_T is the total O_3 flux, F_{ST} is the stomatal component and F_{NS} is the sum of all non-stomatal pathways. The calculation of F_{ST} assumes the air motion in the stomatal cavity to be laminar so that molecules of a trace gas like ozone strictly obey molecular diffusion. This assumption also underlies empirical assessments of stomatal O_3 flux at the leaf and whole-tree level (see above). As pointed out for leaf gas exchange and sapflow approaches, the conversion of water vapour flux through stomata into flux of ozone is based on replacing the molecular diffusion coefficients of the gases accordingly. In relation to the eddy covariance method, stomatal conductance for water vapour is assessed through the Penman–Monteith approach (Monteith, 1981), if other relevant parameters are known. These are radiative and ground heat fluxes, temperature and humidity (accessible through measurements) along with aerodynamic and laminar resistances (accessible through parameterizations, cf. Hicks et al., 1987). An alternative assessment is provided by the Evaporation–Resistance approach (Thom, 1975), which derives leaf from air temperature and the sensible heat flux between leaf and air. In the literature, both the Penman–Monteith (Lamaud et al., 2002; Gerosa et al., 2003, 2004; Cieslik, 1998, 2004) and the Evaporation–Resistance approach (Fowler et al., 2001; Coyle et al. (2005) have been employed. Gerosa et al. (2007) did recently show the equivalence of both approaches. Fig. 6 gives hourly averages of F_T and F_{ST} for two campaigns conducted in a "pseudosteppe" near Rome (Italy), as based on the eddy covariance in combination with the Penman–Monteith approach. The first campaign (Fig. 6A) fell into a dry period (June 1993) with dead vegetation upon drought so that evaporation was low. Hence, the F_{ST}/F_T ratio was low, whereas this ratio was high in the second campaign (Fig. 6B) during a humid period (May 1994) with physiologically active vegetation.

F_{ST} of Fig. 6 is still calculated through the Penman–Monteith based algorithm (see above), so that non-stomatal O_3 flux is obtained by difference between (measured) total and (calculated) stomatal O_3 flux (given the lack of measured data sets of both parameters from same experiments at the present stage). By combining the sapflow with eddy covariance approach, non-stomatal O_3 deposition, as one mechanistic component of the atmosphere-to-plant O_3 flux, is obtained, however, as the difference between the outcomes of two independent empirical assessments. Hence, F_{NS} becomes empirically accessible as well – indirectly at least, via F_T (through eddy covariance) and F_{ST} (through sapflow). Such an achievement is appreciable compared to present estimations of non-stomatal O_3 deposition based on calculations or modelling.

In the long term, the need for combining the two methodologies may become obsolete, as the empirical sapflow approach intrinsically yields F_{ST}, which is crucial in flux-based risk assessment. Hence, new modelling approaches will not require knowledge of F_{NS} anymore (unlike currently used models). In summary, the sapflow approach closes a significant mechanistic gap in scaling O_3 flux and uptake from leaves to stands (cf. Fig. 1B).

3. The role of plant responsiveness in defining an effective O_3 dose

This section highlights the functional basis of the effective O_3 dose as the second component in O_3 risk assessment (cf. Eq. (1)). This component is commonly regarded as the mechanistic foundation of risk assessment, as being controlled by molecular and biochemical processes. However, the effective O_3 dose can only be functionally understood in relation to the specific spatio-temporal scales of O_3 uptake which were pointed out in Section 2, as biophysical and physiological mechanisms associated with the O_3 diffusion into leaves do exist beyond the molecular level (Sandermann and Matyssek, 2004). In the following, challenges encountered upon O_3 uptake are addressed towards fostering the mechanistic basis comprehensively, sensu Eq. (1), of O_3 risk assessment.

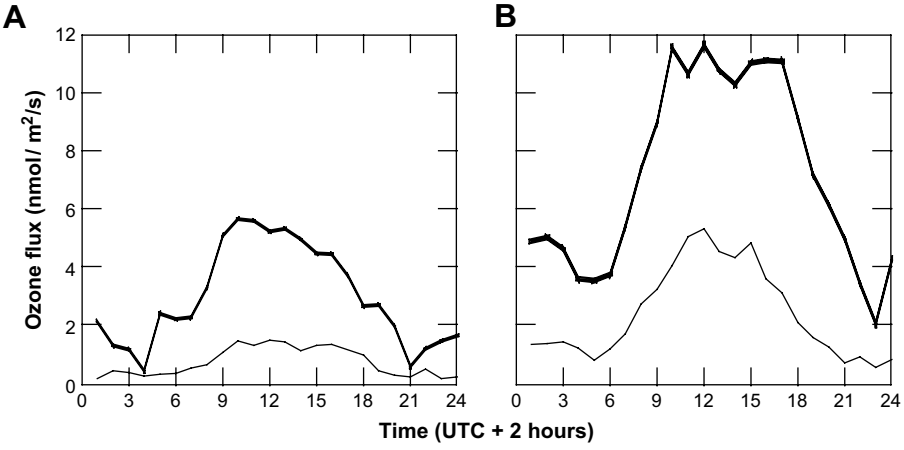

Fig. 6. Hourly averaged total (bold line) and stomatal (thin line) O_3 fluxes over the Mediterranean pseudosteppe located at Castelporziano. (A) refers to a campaign made in June 1993, whereas (B) describes the situation in May 1994.

3.1. Plant response to acute versus chronic O_3 exposure

Section 2 has shown how cumulative O_3 uptake, i.e. the O_3 dose, can be derived from measurements of stomatal conductance or sapflow. The question then is: how is the O_3 dose perceived by the plant and how does the plant respond? Past consideration of these questions (Krupa et al., 2001) has distinguished acute effects caused by short episodes of high O_3 versus chronic effects by extended exposure to near-ambient O_3 that in most cases is enhanced above pre-industrial background levels. Acute or chronic O_3 effects not only depend on the exposure regime, but also on the responsiveness of plants. Similar O_3 regimes can lead to injury symptoms or induced resistance in different plant species or genotypes (Langebartels et al., 2000). It is not yet widely recognized that under special circumstances O_3 can increase plant resistance to oxidative stress and pathogens (Langebartels et al., 2000; Sandermann, 2004; Luedemann et al., 2005). Such observations have so far only been made in controlled laboratory studies and are difficult to detect under field conditions.

The wide range of O_3 effects was first demonstrated for tobacco where exposure to acute O_3 led to a burst of ethylene, a biphasic oxidative burst, and subsequent acute visible symptoms in variety Bel W3 but not in variety Bel B (Schraudner et al., 1998). The important second burst peak and visible injury developed long after the end of O_3 exposure, thus revealing a central role of biochemical plant responsiveness (Schraudner et al., 1998). Stomatal O_3 uptake and ascorbate contents were comparable, but tobacco Bel B responded to O_3 by induction of antioxidative phenolic conjugates. Both tobacco lines formed new transcripts that were in part identical (e.g. for glutathione peroxidase) although tobacco Bel W3 expressed additional transcripts related to hypersensitive cell death such as genes of the shikimate pathway (Janzik et al., 2005). The O_3-treated tobacco variety Xanthi-nc developed resistance towards tobacco mosaic virus (Yalpani et al., 1994).

In summary, the tobacco case studies indicated that a given O_3 dose, dependent on plant genotype, can lead to greatly divergent responses that range from acute visible symptoms to strengthened antioxidative protection, and from growth inhibition to induced resistance against pathogens.

3.2. The oxidative burst response to acute O_3 exposure

An acute O_3 pulse (150 ppb, 5 h) can lead to a biphasic oxidative burst in the O_3-sensitive tobacco variety Bel W3 (Schraudner et al., 1998). The first oxidative peak occurred during O_3 uptake like in the O_3-tolerant variety Bel B, whereas the second and much more pronounced peak appeared subsequent to O_3 exposure only in Bel W3 (Fig. 7). Histochemically, the induced ROS (H_2O_2, superoxide anion, potentially singlet oxygen) could be stained as spots at the later sites of visible symptom development near the leaf veins (Schraudner et al., 1998). By many criteria, including induced signalling substances, transcripts and stress molecules, the acute reaction to O_3 resembled the well-known hypersensitive response that occurs in incompatible plant–pathogen interactions (Sandermann et al., 1998). Ozone was, therefore, termed as an abiotic elicitor of plant defence reactions (Sandermann, 1996; Sandermann et al., 1998). In analogy to biotic elicitors, O_3 was postulated to act via an initial O_3 receptor (Sandermann, 1996). In analogy to other redox-sensitive processes (Buchanan and Balmer, 2005) this could be a regulatory protein with sensitive SH-groups, but the putative ozone receptor has not been identified. The stimulatory roles of ethylene and salicylic acid and the lesion containment by jasmonic acid have been well characterized by use of defined mutants of *Arabidopsis thaliana* (reviewed: Kangasjärvi et al., 2005). The original research on tobacco (Schraudner et al., 1998) has been step-by-step reproduced, confirmed and considerably extended in an independent laboratory (Pasqualini et al., 2003). The decisive second oxidative peak could be attributed to a plasma membrane NADPH oxidase whose transcript was itself stimulated by O_3 (Langebartels et al., 2002). It is also plausible that cell-wall peroxidases are involved in the oxidative burst induced by O_3. Bolwell and Wojtascek (1997) concluded that cell-wall peroxidases were responsible for much of the H_2O_2 produced in the oxidative burst in response to incompatible pathogens. If so, the extracellular peroxidases would require a reductant such as cysteine or GSH plus Fe^{2+} in the apoplastic fluid as well as an increase in pH to neutral or basic conditions to be effective. Some candidate reductants induced by O_3 in the apoplastic space, such as certain phenolic compounds, have already been identified (Langebartels et al., 2002). Interestingly, the burst phenomenon was confined to leaves of intermediate age so that ontogeny had a strong influence on O_3 responsiveness (Schraudner et al., 1998). Initiation of the first and second oxidative burst in response to an acute O_3 exposure may be mediated by G-proteins (Joo et al., 2005). Null mutants for the α subunit of the heterotrimeric G-protein complex (gpa1–4) failed to show an increase in an indicator of H_2O_2 after exposure to 350 nl O_3 l^{-1} for 6 h. Joo et al. (2005) also found that O_3-induced lesion formation and ion leakage were less in null mutants for NADPH oxidases.

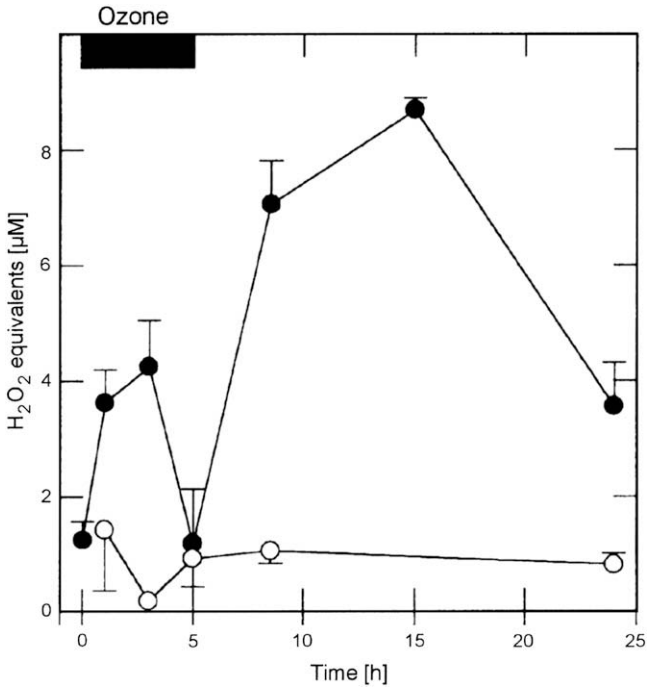

Fig. 7. Biphasic oxidative burst (measured as H_2O_2 equivalents) upon exposure of tobacco Bel W3 to a pulse of O_3 (150 nmol mol^{-1}, 5 h) (●) or control air (○). The O_3-tolerant tobacco line Bel B (not shown) displayed only the small initial burst peak (Schraudner et al., 1998).

Conversely, mitochondria appeared to be involved in O_3 responses. For example, the antioxidative alternative oxidase (AOX) pathway was induced by O_3, with nitric oxide (NO) acting as an indispensable messenger molecule (Ederli et al., 2006). The O_3 sensitivity of tobacco depended strongly on mitogen-activated protein (MAP) kinases (Samuel and Ellis, 2002). The oxidative burst has been characterized as a general response to acute O_3 exposure of tobacco, tomato, birch and *Arabidopsis* (Kangasjärvi et al., 2005), as well as several additional sensitive wild plant species (Wohlgemuth et al., 2002) and sensitive accessions of the model legume, *Medicago trunculata* (Puckette et al., 2007).

In summary, O_3 effects from acute exposure may proceed via the second peak of a biphasic oxidative burst. In contrast to earlier

views, these effects are not directly caused by the reactive O_3 molecule, but by an amplifying biochemical system resembling that of the hypersensitive response in incompatible plant–pathogen interactions. This system may have multiple entry points for various stressors so that the definition of O_3 limit values may become difficult for field sites. It is recognized that most of the addressed laboratory experiments, examining mechanistic processes in response to acute ozone, used O_3 concentrations far above those that occur in ambient air; and that response might be different with exposure at O_3 concentrations nearer ambient levels. Nevertheless, these experiments have identified mechanistic processes that might be expected to occur, especially under those situations where plants are exposed to high ambient O_3 episodes.

3.3. Unforeseen link between acute O_3 and salt stress

The selection of O_3-sensitive mutants of *Arabidopsis* led to the discovery of the *RCD1* gene for radical-induced cell death (Overmyer et al., 2000). This gene encodes an ADP-ribosyl transferase domain containing protein with a largely nuclear location (Ahlfors et al., 2004). Recent studies have shown that *RCD1* mutations lead to decreased salt tolerance due to interaction of the plasma membrane Na^+/K^+-antiporter SOS1 with *RCD1* (Katiyar-Agarwal et al., 2006). The *vtc-1* mutant of *Arabidopsis* which is O_3-sensitive due to distinctly lowered ascorbate contents (see below) had increased salt sensitivity (Huang et al., 2005). These recent studies further illustrate the phenomenon of cross-protection and cross-sensitivity that is often observed in plant ecotoxicology (Sandermann, 2004).

3.4. Chronic O_3 as part of the plant ROS network

Plants generate ROS during photosynthesis, respiration and growth processes. In addition, for most abiotic and biotic environmental stress factors plants generate ROS as part of their toxic action. Plants have evolved a complex ROS defence system, operating through antioxidative metabolites (e.g. vitamins C and E, glutathione, some phenolics, especially flavonoids and conjugated polyamines) and enzymes (e.g. superoxide dismutase, catalases, ascorbate peroxidases). The corresponding genes are usually inducible and lend themselves to knockout and overexpression experiments. In recent years, it has been recognized that the ROS defence system is not only for defence but also for multiple signalling purposes. Excellent reviews are available (Mittler et al., 2004; Foyer and Noctor, 2005; Buchanan and Balmer, 2005). Cellular organelles usually have their own ROS scavenging system, such as the highly active ascorbate/glutathione cycle of chloroplasts. Conversely, the cellular compartments are connected and have common output points. This has been demonstrated for O_3 acting primarily on the apoplast, for H_2O_2 generated in peroxisomes and for singlet oxygen generated in plastids (Sandermann, 2004). In all these cases, the signalling substance salicylic acid and total cellular H_2O_2 increased as plants became resistant to certain pathogens (Sandermann, 2004). The *vtc-1* mutant of *Arabidopsis* mentioned above had lowered ascorbate content. This led not only to O_3 sensitivity, but also to increased resistance against a bacterial pathogen and premature senescence (Conklin and Barth, 2004). Although selected originally by acute O_3 treatment, the *vtc-1* mutant may also represent a model for chronic O_3 phytotoxicity for which premature senescence is highly typical (Krupa et al., 2001). As a case study of chronic O_3 toxicity in herbaceous plants, the suppression of biomass accumulation in soybean and eventual decline in net photosynthesis caused by 1.5× ambient O_3 (71 nmol mol^{-1}, 12 h daily average) is depicted in Fig. 8. The inhibitory effect (cf. Fiscus et al., 2005) of O_3 on growth is evident approximately 30 d before effects on net photosynthesis and visible injury appear in upper canopy leaves of this determinate growth cultivar. Foliar O_3 injury is apparent in the lower canopy before it develops in the upper canopy. Chronic O_3 is clearly inhibitory to growth from early in the life cycle and results in premature (or accelerated) senescence.

The *vtc-1* mutant of *Arabidopsis* has drawn attention to the apoplastic ascorbate pool as a first line of biochemical defence against O_3. Overexpression of a dehydroascorbate reductase (Eltayeb et al., 2006; Chen and Gallie, 2005) or a monodehydroascorbate reductase (Eltayeb et al., 2007) led to O_3 tolerance. Overexpression of a dehydroascorbate reductase also led to salt tolerance (Ushimaru et al., 2006). Changing the apoplast redox state by overexpression of ascorbate peroxidase led to transcript and defence changes reminiscent of O_3 treatment (Pignocchi et al., 2006).

The ROS defence networks of plants also contain signalling components that are not at first sight related to ROS defence. This includes the signalling molecules ethylene, salicylic acid, jasmonic acid and NO as well as MAP kinases (see above). Heterotrimeric G-proteins are also involved in O_3 sensitivity or tolerance (Booker et al., 2004; Joo et al., 2005). The differential O_3 sensitivity of two clones of *Trifolium pratense* was due to parts of the ROS defence network other than apoplastic ascorbate (D'Haese et al., 2005). Most recently, the isoprene emission of certain plant species has been discussed as an O_3 protectant (Loreto and Fares, 2007) although this effect had been negated in earlier studies (Chameides, 1989). The relative efficacy and reduced synthesis rate in response

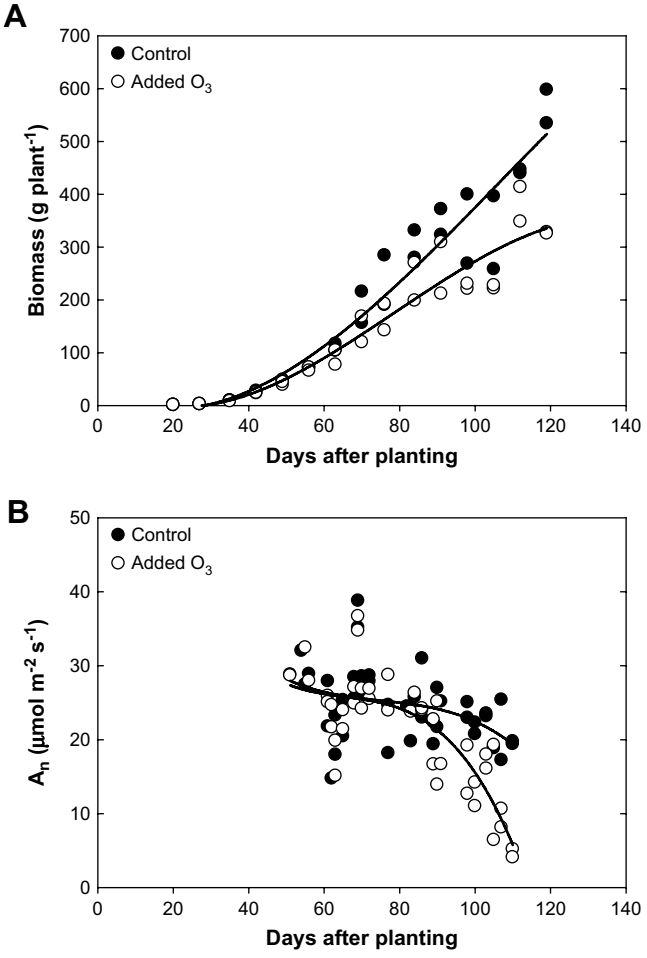

Fig. 8. Effect of charcoal-filtered air (control, 20 nmol O_3 mol^{-1}, 12 h average) and 1.5× ambient ozone (75 nmol mol^{-1}) on Essex soybean (A) biomass accumulation and (B) net photosynthesis (A_n) in open-top field chambers. A_n was measured on leaves at node 14, counting acropetally (upper canopy leaves). For further details, see Fiscus et al. (2005).

to O_3 (Calfapietra et al., 2007) leave open many questions about the role of isoprene in plant O_3 responses.

In summary, plants possess highly complex ROS protective systems. Chronic O_3 may feed into this system at one or more entry points and lead to multiple changes including growth and cell death events and interaction with other abiotic and biotic stress factors. Typical symptoms of chronic O_3 exposure are chlorotic mottling and premature senescence (Krupa et al., 2001). It should be remembered that O_3 itself likely occurs only in the apoplast and is quenched there whereas the intracellular defence systems are confronted with a multitude of secondary toxicants. The presence of O_3 in the apoplast results from the continual influx, and once arrived at the apoplast, O_3 concentrations approach nil (see above), as defences rapidly react, unless overwhelmed.

3.5. Generation of toxicants during O_3 detoxification

Early studies in experiments with acute O_3 fumigations had shown that the rapid decomposition of O_3 by reaction with relatively high concentrations of phenolic acids was accompanied by formation of highly reactive hydroxyl radicals (Grimes et al., 1983). Soluble and bound phenolics are typical apoplastic components although the concentration of soluble phenolics may be low (Chameides, 1989). The consumption of O_3 by oxidation of protein –SH groups may inactivate functional proteins if the oxidizable amino acid is at the active site of the enzyme (Mudd, 1996). Ozone addition products and other reactive products with fatty acids of plasma membrane lipids, or with isoprene (see above), may have toxic potential. Even in the case of the protectant ascorbate there is the possibility of forming reactive ozonides and peroxy or aldehydic products if O_3 attacks the double bond. Early studies have emphasized the toxic potential of such O_3 reaction products (Mudd, 1996) but more recently the attention has shifted to less reactive second messenger molecules induced by O_3 (ethylene, salicylic acid, jasmonic acid, nitric oxide; see above). It seems that the old concept of toxic O_3 second messenger molecules is still of value (Fuhrer and Booker, 2003). Some amino acid residues are carbonylated by O_3 which contributes to the pool of damaged enzymes and is likely implicated in aging processes in animals (Stadtman, 1992). Carbonyl concentrations of the Rubisco small subunit increased in bean leaves visibly injured by O_3 and correlated with exposure level (Kanoun et al., 2002). Obviously carbonylation of residues at the active sites of the enzyme would be most critical in impairing function.

It is apparently not widely recognized that the reaction with L-ascorbate is not only an important quenching and detoxification mechanism for O_3 (Chameides, 1989; Conklin and Barth, 2004; Musselman et al., 2006) but may also be a source for toxicants. The high reaction velocity and the double bond of ascorbate as an attack site were recognized in early studies (Chameides, 1989; Runeckles and Chevone, 1992). However, it appears that the principles of O_3 reactions with organic compounds and in particular the classical Criegee mechanism (Bailey, 1954) have so far not been applied to the reaction between ascorbate and O_3. Fig. 9 depicts one of the expected two zwitter ions that leads to peroxy-L-threonic acid and oxalic acid. The second expected zwitter ion (not depicted) leads to peroxy-oxalic acid and L-threonic acid. The peroxy-acids are highly reactive and likely phytotoxic (Sandermann, 2008). It is furthermore known that O_3 is transformed to singlet oxygen (Kanofsky and Sima, 2000) whose reaction with L-ascorbate again produces peroxy-compounds (Kwon and Foote, 1988). The detoxification of O_3 by apoplastic ascorbate that is emphasized in literature (Chameides, 1989; Conklin and Barth, 2004; Musselman et al., 2006) thus appears to apply only when the toxic by-products of O_3 quenching can also be detoxified. The concept of defining an effective O_3 dose as a result of flux and detoxification (Wieser et al., 2002; Musselman et al., 2006; Tausz et al., 2007; Wieser and Matyssek, 2007) should, therefore, be extended by including the bioactivation aspect illustrated here by induced oxidative burst reactions, and by the formation of toxic by-products.

3.6. Ozone responses: transcript level

Induced biochemical plant responses are expected to take place in the sequence signalling – transcripts – proteins – metabolites. It has been established that O_3 is a powerful inducer of antioxidative, detoxification and defensive genes and a repressor of growth-related genes, such as those for photosynthesis (Sandermann, 1996; Sandermann et al., 1998; Kangasjärvi et al., 2005). The use of high-density array transcript analyses and real-time PCR is quite recent and it can now be determined if acute and chronic O_3 exposures lead to distinguishable typical transcript signatures.

3.6.1. Acute exposure

The isolation of O_3 response EST (expressed sequence tag) clones resulted in the development of macro/microarrays for diagnosis of various stresses in plants. Acute O_3 exposure (200 nmol mol^{-1}, 6–12 h) resulted in the induction of well-known O_3-responsive transcripts for PR proteins, ascorbate peroxidase, cytochrome P450 and glutathione *S*-transferase, as well as numerous ESTs (Tamaoki et al., 2004), so far shown to be induced by O_3. Expression profiles revealed that O_3 stress could be distinguished from other stress factors like UV-B irradiation, heavy metal exposure, drought or wounding, although overlaps were also present (Matsuyama et al., 2002; Tamaoki et al., 2004; Heidenreich et al., 2005). Analysis of about 12,000 ESTs from *Arabidopsis* resulted in the isolation of 205 O_3-responsive ESTs, with 157 induced and 48 repressed transcripts upon acute O_3 exposure

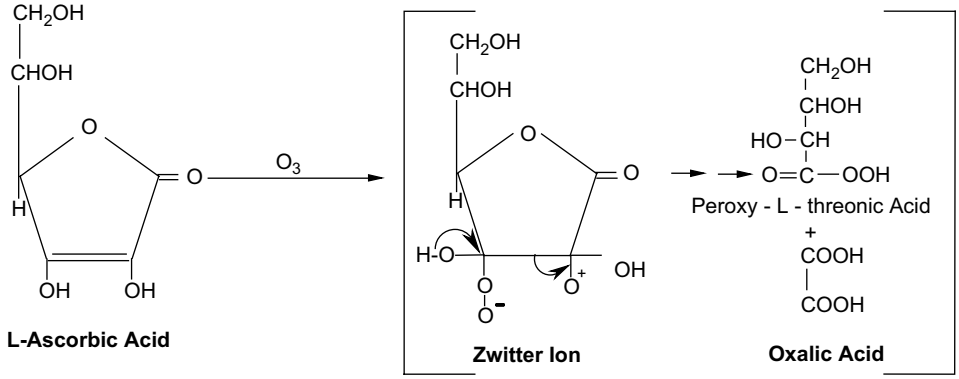

Fig. 9. Ozone attack on L-ascorbic acid according to the Criegee mechanisms (Bailey, 1954).

(Tamaoki et al., 2003). However, functional classification showed that up to 47% were unknown ESTs. Hierarchical clustering revealed jasmonate-, ethylene- and salicylic acid-induced genes, as well as overlapping hormone clusters (Tamaoki et al., 2003). Similarly, new genes and pathways, other than those already reported on as an O_3 response, were recently published by Tosti et al. (2006). They concluded that induction of WRKY transcription factor genes provided strong evidence for their participation in stress perception and signal transduction in O_3-treated plants, possibly related to redox-regulated processes. The temporal profile of O_3-induced oxidative burst in *Arabidopsis* ecotype Col-0 resulted in early and late up-regulated transcripts, as well as down-regulated genes (Mahalingam et al., 2005). Whole genome expression profiling in the O_3-sensitive *Arabidopsis* ecotype Ws-0 showed early induction of hormone-responsive and proteolysis genes and a down-regulation of genes involved in energy metabolism, carbon utilisation and signalling, indicating an activation of an oxidative cell death pathway and inefficient defence responses (Mahalingam et al., 2006).

Most microarray analysis was carried out with *Arabidopsis* using high O_3 concentrations and little is known for other plants. In European beech saplings that were exposed to O_3 for up to 30 d (300 nmol mol^{-1}, 8 h d^{-1}) microarray analysis showed O_3-responsive genes that were already known in herbaceous plants like glutathione *S*-transferase, catalase and PR genes (Olbrich et al., 2005). The shikimate pathway, important for aromatic metabolites and all aromatic amino acids, plays a central role in mediating between primary and secondary metabolism in plants. In the O_3-sensitive tobacco cultivar Bel W3 an acute O_3 pulse resulted in transiently elevated levels of several shikimate pathway transcripts (Janzik et al., 2005). This could also be demonstrated in European beech by quantitative real-time RT-PCR (qRT-PCR), indicating transcriptional similarities between herbaceous and woody plants upon acute O_3 exposure (Betz et al., in press).

3.6.2. Chronic exposure

Chronic O_3 exposure (1.2× ambient) of different *Arabidopsis* ecotypes in a field experiment within SoyFACE (http://www.soyface.uiuc.edu/) for several weeks until the rosette stage or flowering resulted in 58 up- and 577 down-regulated transcripts. About 70% of elevated transcripts were unknown or hypothetical and 12% involved redox control and cell signalling (Miyazaki et al., 2004). Surprisingly when comparing transcription profiles under field conditions with those obtained under growth chamber conditions, the numbers of up-regulated transcripts increased dramatically to about 400, and down-regulated to about 800 (Miyazaki et al., 2004). This indicates that additional stress factors in the field like daily temperature change and local weather conditions may lead to substantial differences in gene expression patterns. In another FACE experiment with an O_3 exposure of 25% over ambient *Arabidopsis* ecotype WS showed the greatest number of changes in gene expression (2.926) compared to ecotype Col-0 (630) and Cvi-0 (1303) and a relative *Thellungiella halophila* (Li et al., 2006). Moreover a cross-ecotype comparison showed a different gene expression pattern (Li et al., 2006). Stress-induced changes were observed for core metabolism between WS and Cvi-0, whereas Col-0, Cvi-0 and Th were little affected by O_3. Cvi-0 showed elevated transcript levels for transcription factors, ethylene-responsive genes and PR genes, whereas in WS photosynthetic genes, ascorbate glutathione cycling, cell-wall metabolism and marker gene for programmed cell death were down-regulated. Reversals of relative sensitivities of gene expression under chronic compared to acute O_3 stress were observed by Li et al. (2006). Novel ozone-resistance pathways under chronic O_3 exposure may be activated that are different from the established hormone-related pathway under acute high O_3 exposure.

At the Kranzberg Forest free-air O_3 fumigation site (ambient and 2× ambient O_3; Matyssek et al., 2007a) qRT-PCR of RNA isolated from sun leaves of 60 year-old European beech showed only a weak induction of shikimate pathway genes in contrast to controlled greenhouse conditions with beech saplings (30 d, 300 nmol mol^{-1}, 8 h d^{-1}; Betz et al., in press). Similarly genes connected with the antioxidative system, shikimate pathway and lignin formation were not significantly affected within a 2-year observation, although an ACC synthase transcript was up-regulated in adult trees at the Kranzberg field site (Jehnes et al., 2007). These differences between the field and greenhouse are likely caused by the O_3 regimes used, tree ontogeny, local weather conditions or additional abiotic/biotic stress.

In the Aspen FACE experiment (Karnosky et al., 2007) an O_3-tolerant poplar clone was exposed for a period of 5 years to an O_3 concentration of 1.5× ambient. Out of 4600 analyzed ESTs, 185 were significantly O_3-regulated within two replication years (Gupta et al., 2005). Higher expression was observed for signalling, defence-related genes, senescence-associated and flavonoid biosynthesis genes, whereas photosynthesis and energy-related genes were repressed. In addition interacting effects of elevated CO_2 and O_3 on the gene expression profile were observed that were not seen with single stress treatments (Gupta et al., 2005).

In summary, transcript patterns have been successfully analyzed as early plant responses to O_3. Comparing chronic versus acute O_3 exposure, some genes are similarly up- or down-regulated. However, transcription profiles under chronic O_3 in the field currently represent snap-shots and more experiments are necessary to define transcript specificities. In addition, different O_3-sensitivities for different ecotypes or clones have to be considered. The systematic analysis of O_3 responses at the levels of proteins (proteomics) and metabolites (metabolomics) is under way and is not covered in this short review.

4. Towards mechanistic O_3 risk assessment in practice

Sections 2 and 3 outlined the mechanistic basis of what has been widely accepted, that plant response to O_3 depends on the amount of O_3 entering the leaves via stomata and the plant's sensitivity in relation to O_3 uptake, termed "effective O_3 dose" and determined by the metabolic redox control and resulting defence capacity (see Eq. (1); Runeckles and Vaartnou, 1997; Matyssek et al., 2004; Panek et al., 2002; Musselman and Massman, 1999; Massman et al., 2000; Musselman et al., 2006; Wieser and Matyssek, 2007). Hence, progress towards obtaining more mechanistic O_3 risk assessment needs to pursue functional and integrated unraveling of O_3 stress tolerance (redox regulation under internal and external impacts) and avoidance (stomatal regulation in concert with O_3 diffusion). This paper strives to present the concept of the required integration of O_3 stress tolerance and avoidance along with recent progress towards mechanistic risk assessment. Given this rationale and complexity as outlined above, detailed methods of how mechanistic risk assessment can be achieved in practice are not possible at present. Simple but meaningful practical applications have not yet been developed, and this is not the purpose of this paper. Rather, promising augmentation in functional evidence is reported herein as a suggested concept towards mechanistically based assessment methods for practical use. To this end, a unique new approach to the required integrative concept is introduced here.

Aiming at mechanistic O_3 risk assessment, the primary challenge at present is the need for approximation and simplification for practical application. However, protocols for routine use can only be developed after clarification of the mechanistic processes. In the case of O_3 stress avoidance, stomatal conductance is the metric needed to assess O_3 uptake at prevailing ambient O_3 levels (Tausz et al., 2007). Both at the leaf and canopy level, conductance depends on environmental factors which drive internal processes and stomatal regulation (Schulze and Hall, 1982; Schulze, 1994).

Such prominent factors are light, air humidity and soil water availability. Concerning the latter, knowledge is scarce about effects on O_3 uptake, at least with respect to reliable risk modelling (Nunn et al., 2005; Emberson et al., 2000; Wieser and Tausz, 2006, and further references therein). Clearly soil moisture effects on O_3 uptake, e.g. in relation to rooting depth and soil characteristics, although being highly important, have been neglected in the mechanistic clarification of O_3 stress avoidance. Also neglected have been interactions with VOC emissions from leaves, as well as molecular collision effects between transpired water vapour and stomatal O_3 influx. In addition, impact of O_3 on stomata (Reich, 1987; Nunn et al., 2006), either directly on the performance and differentiation of the stomatal apparatus (Maier-Maercker, 1989) or indirectly via changed leaf metabolism (Taylor et al., 1988; Matyssek and Sandermann, 2003; Warren et al., 2007), requires mechanistic assessment prior to arriving at simplification for new modelling tools.

Although models are available that describe O_3 flux into the leaves (Wieser and Tausz, 2006 and further references therein), it is currently not possible to model the component of O_3 stress tolerance (i.e. effective dose, Eq. (1); Musselman et al., 2000; Tausz et al., 2007). As outlined in Section 3, amplification events are important in addition to detoxification, and at present it cannot be excluded that some secondary toxicant rather than ozone itself is responsible for toxicity. The introduced integrative concept also requires the "effective O_3 dose" to be translated into practical application (i.e. modelling) without becoming too complicated for routine use (Matyssek et al., 2007b). Again, simplification is needed upon mechanistic clarification. One perspective towards simplification is the rationale that increasing demands for O_3 detoxification and repair along with increasing O_3 uptake may progressively curtail that portion of carbon gain required for other metabolic demands. Therefore, we postulate O_3-induced injury to occur when metabolic demand for repair and defence exceeds the assimilate supply through net photosynthesis; and that beyond a critical range of O_3 uptake stress tolerance collapses because of assimilate shortage (cf. also Wieser and Matyssek, 2007). Such collapse is in analogy to what is known about defence against metabolic poisoning in the liver of humans (McKillop and Schrum, 2005).

Based on the conceptual model of Musselman et al. (2006) to include a defence component which counteracts O_3 flux into the leaves, Kolb and Matyssek (2001) suggested the ratio between photosynthesis (as an estimate of resource availability for repair and detoxification) and O_3 uptake to be related to ozone sensitivity, i.e. mirroring the effective O_3 dose. A decreasing ratio would, hence, indicate increasing O_3 sensitivity. Musselman and Massman (1999) have suggested the plant's O_3 defence to be related to photosynthesis, and Massman (2004) derived a model to use photosynthesis as a surrogate for defence.

Wieser et al. (2002, 2003) linked O_3 uptake to the leaf area-based antioxidant level. In this concept, the specific leaf area (SLA, leaf area per unit dry mass) is used as an estimate of the depth of biochemical defence lines, given by the number of cell rows behind the leaf surface area (as reflected by SLA), through which (via the stomata) the area-based O_3 influx intrudes into leaves (Matyssek et al., 2007b). Indeed, the carbon gain/O_3 uptake ratio appears to be plausible for estimating O_3 stress tolerance, because detoxification and repair require energy (Smirnoff, 1996; Noctor and Foyer, 1998). Wieser et al. (2002) were able to interpret, based on this ratio, differences in O_3 susceptibility in relation to canopy position (sun versus shade) and tree age. Matyssek et al. (2007b) used SLA as a proxy of O_3 susceptibility, as leaf area-based antioxidant levels tended to decline with increasing SLA (Tegischer et al., 2002; Wieser et al., 2002, 2003). Consistently, O_3 susceptibility was shown to increase as SLA increased, along with decreasing photosynthetic capacity, while decreasing C gain/O_3 uptake indicated increasing O_3 sensitivity (Kolb and Matyssek, 2001). Such simplifications still require rigorous examination before becoming valid for use in modelling. Nevertheless, they provide a basis for respective hypothesis evaluation and a perspective to arrive at mechanistically derived proxies for practical use. Of course, the addressed simplifications may not be the only way for approaching the ultimate aim of mechanistic O_3 risk assessment on a routine basis. As the effective O_3 dose relates to the plant's intrinsic redox system (as pointed out above), also proxies like the NAD(P)H pool or the ratio between carboxylases (cf. Saurer et al., 1995) as well as photosynthetic water-use efficiency (relating C gain to O_3 uptake via stomatal opening, cf. Kolb and Matyssek, 2001) have been proposed (Dizengremel et al., 2008).

Although the flux-based O_3 risk assessment presently appears to be more complicated in use than the exposure-based approach, it is not so complicated that progress cannot be achieved and perspectives should not be examined. After the present stage of basic clarifications and parameterizations, including the necessary components described above, fluxed-based modelling tools will guide users in ways similar to current exposure-based tools. Such latter tools are still being used because of the challenge of implementing flux-based methodology, not because exposure-based methods are intrinsically better. The major deficit of the exposure approach is the lack of a mechanistic basis (being intrinsic, however, to the flux-based approach). Still, policy issues are beyond the scope of this paper and are discussed elsewhere (Percy et al., 2007; Paoletti and Manning, 2007).

5. Conclusions

Consensus has increased within the scientific community to derive O_3 risk assessment of vegetation from a sound mechanistic basis. Apart from recent considerations about exposure-based approaches (Percy et al., 2007), the O_3 flux concept of risk assessment, representing a dose-related (i.e. phytomedically relevant), cause–effect based and, hence, intrinsically mechanistic approach is to be promoted (Matyssek et al., 2007b). This latter approach integrates two components: (1) the O_3 influx (yielding the "physical" uptake and, hence, O_3 dose) and (2) the "effective O_3 dose" (i.e. the plant's sensitivity per unit of O_3 uptake). Both components require unveiling of their specific spatio-temporal mechanisms each, which is the pre-requisite of approximations for novel modelling tools to be used in O_3 risk assessment on a routine basis. This account summarized the current state of mechanistic understanding and perspectives towards approximations prior to practical use.

Regarding (1), the sapflow approach of empirically deriving whole-tree O_3 uptake has opened a new perspective in using the flux concept in risk assessment. In combination with the eddy covariance approach of assessing O_3 flux at the stand level, the non-stomatal O_3 deposition can be derived empirically so that new data sets become available for validating such flux-based models which are currently employed in O_3 risk assessment. To the extent that sapflow-based data sets on different sites and tree species will be elaborated, new risk assessment models will become available to overcome present uncertainties about boundary layer effects on O_3 flux and about non-stomatal O_3 deposition. Knowledge about the latter parameter could become obsolete, as new modelling tools derived from the sapflow approach will inherently cover the stomatal O_3 flux, which is of primary interest in risk assessment. The mechanistic basis (1) relates to stomatal regulation as a means of O_3 stress avoidance under multi-factorial impact. Soil moisture effects require paramount attention in this context. Mechanistic coverage of molecule collisions between O_3 and H_2O on their pathway through stomata and interactions with VOCs released from leaves are crucial, in addition, in view of required evidence.

This relates to a process-based assessment of canopy-internal O_3 gradients which must not be overlooked in flux calculations, as knowledge of O_3 regimes within boundary layers will become crucial.

In view of (2), concepts become relevant as known from medical sciences, as plants undergo detoxification or bioactivation of enzymes similar to animals upon oxidative stress so that the "green liver" concept has been developed (Sandermann, 2004; Wieser and Matyssek, 2007). The above outlined methodology of stomatal O_3 flux assessment, yielding the phytomedically relevant O_3 dose, can now be combined with evidence on the plant's dose-related sensitivity to derive limit ranges of O_3 risk in plant response. Many of the signalling, stress amplification, detoxification and toxification events rendering the dose-related sensitivity ("effective O_3 dose") have been elucidated in recent years as summarized above but still need to be included (upon simplification) in novel flux models of O_3 risk assessment. The challenge is that single metabolic master reactions in control of plant response to O_3 have not been identified. Basic questions, such as the role of O_3 peak episodes or interactions with other abiotic and biotic stressors, are still in need of additional study. This could, for example, solve the long-standing question of which biotic plant diseases are enhanced by elevated O_3, and if O_3 can induce resistance under field conditions.

The forest level approach, based on sapflow methodology in flux-based O_3 risk assessment, needs to be conceptually cross-linked with controlled free-air O_3 fumigation experiments in plantations of fast-growing pioneer tree species. Such pioneers are likely to be sensitive to an extent that allows unravelling mechanisms in control of the effective O_3 dose. In pursuing such a perspective, O_3 risk analysis must not overlook interaction with other pollutants (in particular, nitrogen deposition and increasing atmospheric CO_2 concentration) and altered site factors (temperature and moisture regimes) along topographic gradients under the conditions of climate change. Risk analysis must be extended beyond vegetation to human health as part of "environmental health" in order to enhance political awareness and increase socio-economic appreciation of intact "ecosystem services" for well-being. This requires attention also to effects of vegetation on tropospheric O_3 regimes (e.g. through VOC emissions, land-use changes, species associations in forests). Interfaces must be developed that accommodate risk assessment across "forest", "crop" and "semi-natural vegetation" systems (Wieser et al., 2008), as at the landscape level O_3 risk of each of these vegetation types needs to be integrated into one common assessment concept.

O_3 risk assessment must extend beyond estimations of yield losses. Rather, probability-based evaluations of O_3-induced injury and damage as well as environmental/ecological changes in a wider context need to be developed (e.g. effects on population genetics and biodiversity). This is a pre-requisite for coping with an increasing demand for liability aspects in a legal context. Such latter demands, but also tropospheric O_3 as a component of "Global Change", with consequences for "carbon sink strength" of woody plant systems, productivity of renewable resources through energy farming and post-Kyoto policies require enhanced precision in quantitative O_3 risk assessment. Policy-relevant deliverables of potential new research projects need to be the replacement of exposure-based concepts with a mechanistic but still practicable alternative. O_3 flux-based risk assessment sensu Eq. (1) is such an alternative, as it will offer reliable evaluations of the likeliness of injuries, damages or other alterations ascribed to O_3 impact in plant systems.

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