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REVIEW ARTICLE

Ozone effects on plants in natural ecosystems

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ABSTRACT

Tropospheric ozone (O_3) is an important stressor in natural ecosystems, with well-documented impacts on soils, biota and ecological processes. The effects of O_3 on individual plants and processes scale up through the ecosystem through effects on carbon, nutrient and hydrologic dynamics. Ozone effects on individual species and their associated microflora and fauna cascade through the ecosystem to the landscape level. Systematic injury surveys demonstrate that foliar injury occurs on sensitive species throughout the globe. However, deleterious impacts on plant carbon, water and nutrient balance can also occur without visible injury. Because sensitivity to O_3 may follow coarse physiognomic plant classes (in general, herbaceous crops are more sensitive than deciduous woody plants, grasses and conifers), the task still remains to use stomatal O_3 uptake to assess class and species' sensitivity. Investigations of the radial growth of mature trees, in combination with data from many controlled studies with seedlings, suggest that ambient O_3 reduces growth of mature trees in some locations. Models based on tree physiology and forest stand dynamics suggest that modest effects of O_3 on growth may accumulate over time, other stresses (prolonged drought, excess nitrogen deposition) may exacerbate the direct effects of O_3 on tree growth, and competitive interactions among species may be altered. Ozone exposure over decades may be altering the species composition of forests currently, and as fossil fuel combustion products generate more O_3 than deteriorates in the atmosphere, into the future as well.

INTRODUCTION

Although ozone (O_3) is generated by ultraviolet radiation in sunlight, its overall tropospheric concentration is relatively low due to rapid degradation, surface deposition and atmospheric mixing. Judging from the amount present in one of the cleanest atmospheres of the globe (Antarctica; Oltmans *et al.* 2006), plants likely evolved under O_3 concentrations of less than 20 nl $O_3 \cdot l^{-1}$. This concentration has not been found to be harmful to any extant plants. In the early 20th century, fossil fuel use by both industry and transportation sectors significantly increased O_3 concentrations due to the release upon combustion of a wide range of organic molecules (Atkinson *et al.* 1996). Organic molecules and NO_x exposed to as little as half full sunlight generate oxidants, of which O_3 is the major component. Current global concentrations are now much higher because although unstable, the degradation of O_3 is slower than its generation (Fowler *et al.* 1999). In some areas O_3 concentrations are so high that injury to plants is visible and deleterious to production.

Oxidant-induced injury to plants was first noted and identified in Southern California (Haagen-Smit 1952) as the population and industry rapidly increased in Los Angeles after World War II. Southern California has a Mediterranean-type climate, with high radiant energy and moderate to high temperatures much of the year, which accelerates oxidant production. The Los Angeles Air Basin is surrounded by 3500-m mountain ranges, with a narrow pass limiting air flow to the desert to the east. Onshore winds from the cooler Pacific Ocean move from west to east and concentrate pollutants in the inland valleys. Both primary (CO_2, NO_x) and secondary (O_3, NO_x) pollutants are produced, and O_3 concentrations of 300–400 nl $O_3 \cdot l^{-1}$ were common in the 1970s in these inland valleys (Atkinson 2000). The deleterious effects of pollutants on humans and plants drove state and federal agencies to set protective air quality standards, which were met only by the mid-1990s (Tingey *et al.* 2004).

Understanding the atmospheric chemistry of fossil fuel combustion and the secondary pollutants were critical steps to understanding their effects on biological systems in the late 1950s through the 1960s. However, generating the organic oxidants and maintaining their concentrations in experimental exposure chambers was difficult. In particular, peroxy-actyl nitrate was studied (Stephens 1969), but other more complex organics were too difficult to make, and so few such experiments were performed. Many of these organic compounds are believed to be formed in concentrations too low to cause damage.

AMOUNTS AND EXPOSURE DURATION OF OZONE

Agreement among scientists for biologically effective, comparable experimental exposures has been elusive. The fundamental discourse has revolved around what concentration, for how long and how often. Should both nocturnal and diurnal

exposure be accounted for? How does pre-experiment exposure affect response, and should it be applied before the experiment? How closely can and should the natural exposure be mimicked in experimental exposures? Throughout, 'O_3 exposure' is used on a qualitative level, both in terms of range and experimental exposure relative to ambient concentrations in the agricultural or unmanaged ecosystem of the investigated species. The terms 'low' (roughly 20–70 nl $O_3 \cdot l^{-1}$), 'moderate' (70–150 nl $O_3 \cdot l^{-1}$) and 'high' exposure (>150 nl $O_3 \cdot l^{-1}$) is assumed unless otherwise noted. Much of the referenced literature used exposure, but stomatal conductance (g_s) was not consistently included, so O_3 flux cannot be back-calculated uniformly across references.

It is critical to measure O_3 concentrations in the air near the leaf, plant, stand or ecosystem of interest. For several decades, daytime hourly O_3 concentrations were accumulated by the hour (exposure), summing all hourly O_3 concentrations (SUM00), or only values above a threshold and specified duration such as a defined season length and daylight hours (reviewed in Musselman *et al.* 2006). AOT40 was used for decades in Europe until the background O_3 levels regularly exceeded 40 nl $O_3 \cdot l^{-1}$. At the time of writing, the U.S. criteria for acceptable O_3 concentration in ambient air was an hourly O_3 concentration not exceeding 120 nl $O_3 \cdot l^{-1}$ more than once per year, and the 3-year average of the annual fourth-highest daily (8-h daytime) maximum was ≤70 nl $O_3 \cdot l^{-1}$ (Federal Register 2017). However, specialists have argued for a weighted standard (higher hourly O_3 concentrations mathematically weighted; Musselman *et al.* 2006). Weighted exposure metrics can be correlated with visible foliar injury; however, not all species have visible symptoms even when the exposure reduces biomass (*e.g.* white fir, *Abies concolor* (Gord. & Glend) Lindl. ex Hildebr.; Retzlaff *et al.* 2000). Also, indices heavily biased on high O_3 concentrations can fail to provide quantitative relationships to biological responses over long exposures (Massman *et al.* 2000; Musselman *et al.* 2006). Over the last 15 years, there has been strong scientific support for calculating dose (year O_3 uptake per leaf area or stoma, PODy, year·nmol·m^{-2} PLA·s^{-1}; Matyssek *et al.* 2007b; UNECE 2010). This approach is biologically appropriate as it accommodates both concentration and uptake when the plant is biological active. An additional step that has been proposed is to take into consideration the antioxidant defence capacity of the plant (Di Baccio *et al.* 2008), such that uptake that exceeds the defence capacity would be considered deleterious. The endpoints of experimental exposure include production of seed or fruit, vegetative growth, plant health (variety of approaches), lack of susceptibility to insects and disease, and in conjunction with other stressors, survival.

We first present case studies, as many of the early studies were conducted in unmanaged ('natural') settings and continue to frame our questions on plant response to O_3. Methodological approaches to environmental and plant attributes, and experimentally manipulated O_3 exposures are then briefly described. The emphasis of this review is the description of known and supported physiological responses to O_3, with explanations of underlying biochemical and molecular processes involved. Overall, responses from about 60 vascular plant species in unmanaged systems have been described. Much of our knowledge of biochemical and genetic pathways of plant response to O_3 was developed from agricultural species, and we cite responses experimentally derived from a dozen species as well as from the global experimental plant, thale cress (*Arabidopsis thaliana* L. Heynh). The role of genetic and population diversity in shifting composition and structure in communities, and in altering ecosystem processes, is discussed. Lastly, we summarise next-steps in diverse aspects of O_3 effects research.

CASE STUDIES

Ecosystem function is dependent on the constituent species and their genetic variation, and the dynamics of the interactions among species and their respective response to the biotic and abiotic environment (Agrawal & Agrawal 2000). We present a series of case studies of relatively few species that have shaped our thinking of and approach to research on O_3 effects on plants, and how our knowledge to date can be used to help understand processes at the ecosystem and sub-landscape level.

Plantago major

One of the first field-based, long-term studies of O_3 impacts on plants was conducted in the United Kingdom where ambient O_3 levels were sufficient to influence the composition of native plant communities (Reiling & Davison 1992; Davison & Reiling 1995; Lyons & Barnes 1998). With elevated O_3, sensitive populations of English plantain (*P. major* L.) had both decreased growth (Reiling & Davison 1992; Pearson *et al.* 1996; Whitfield *et al.* 1997) and lower reproductive success (Reiling & Davison 1992; Pearson *et al.* 1996). While spatial comparisons of population responses to O_3 are complicated by other confounding factors, such as other pollutants present or different environmental conditions, Davison & Reiling (1995) demonstrated that rapid changes in population resistance of English plantain occurred in response to different ambient O_3 exposures. The research team concluded that the observed change in population O_3 tolerance was due to differential selection of genotypes already present, rather than through an influx of new English plantain germplasm (Wolff *et al.* 2000).

San Bernardino Mountains, California

Often cited as the classic case linking tropospheric O_3 (and accompanying nitrogen (N) deposition) to whole ecosystem impairment (Fenn *et al.* 1996; Bytnerowicz 2002; Garner *et al.* 2006), the mixed conifer forest of the San Bernardino Mountains (with elevations to 3500 m) has been described and studied since the late 1950s (Table 1). Miller *et al.* (1963) first correlated visible injury (chlorotic mottle on needles) with O_3 exposure in ponderosa pine (*Pinus ponderosa* Lawson & C. Lawson). Average concentrations of 100–120 nl $O_3 \cdot l^{-1}$ over 24 h with 1-h peaks well into the 200 nl $O_3 \cdot l^{-1}$ range were common across the area in the 1960s and 1970s (Bytnerowicz 2002). Political action pressured regulators to set limits on pollution to reduce peak daytime O_3 concentrations (Lee *et al.* 2003), but nighttime concentrations have remained the same or increased (Grulke *et al.* 2004).

High ambient O_3 exposure has been linked to moderately high N deposition: the effects of the two pollutants cannot be separated in field studies (Fenn *et al.* 1996; Grulke *et al.* 1998; Bytnerowicz 2002). High O_3 and accompanying N deposition was associated with foliar chlorotic mottle and reduced needle

Table 1. Growth and productivity.

growth and productivity	O_3	O_3 +N	O_3 $-H_2O$
foliar biomass	n.d.	↑	↓
height growth	n.d.	↑	↓
bole diameter growth	↓	↑	↓
fine root biomass	↓	↓	↑
leaf surfaces			
stomatal occlusion	↑	n.d.	n.d.
trophic interactions			
bark beetle	n.s.	↑	↑
fungal infection	↑	n.d.	n.d.
ecosystem level			
Competitive indices	n.d.	↓	↑
Mycorrhizal biomass	↑	n.s.	n.s.
gas exchange	**O_3**	**O_3 +N**	**O_3 $-H_2O$**
A_{max} lower canopy	n.d.	↑	↓
A_{max} whole canopy	↓	n.d.	↓
A_{max} seedlings	↓↑	n.d.	n.d.
stomatal limitation	α to ps	n.d.	↑
stomatal conductance	↓	↓↑	↓
foliar respiration	n.s.	↑	↓
soil respiration	↓	n.d.	n.d.
O_3 flux	↓	n.s.	↓

foliar biochemistry and tissue chemistry	O_3	O_3 +N	O_3+ $-H_2O$
total ascorbate	↓	↓	↑
dehydroascorbate	↑	n.d.	↓
total glutathione	↓	↑	↓
oxidised glutathione	↑	↑	↓
α Carotenoids	↑	n.d.	↓
foliar nitrogen	↓	↑	↓
C:N ratio of foliage	↑	n.d.	↓
starch	n.d.	↓	↑
chlorophyll content	↓	↑↓	↓

Effects of ozone (O_3), O_3 and nitrogen (+N) deposition, and O_3 and drought stress ($-H_2O$) on western yellow pine in the Sierra Nevada and the San Bernardino Mountains, California, USA.

retention (Grulke & Lee 1997; Arbaugh *et al.* 1998). Net assimilation was modified by both high N deposition and syncopated drought (Grulke *et al.* 2002, Grulke *et al., 2003b*), altered carbohydrate allocation within all tree age classes (Grulke *et al.* 2001) and reduced root biomass (Grulke *et al.* 1998). The consequence of reduced root biomass is increased tree susceptibility to drought, wind throw, greater susceptibility to successful insect attack and the frequency of root diseases (Grulke *et al.* 2009). High O_3 exposure alters forest successional pathways (Miller *et al.* 1989), favouring more O_3-tolerant species (incense cedar, *Calocedrus decurrens* (Torr.) Florin and white fir; Arbaugh *et al.* 2003), shifting species dominance in the understorey (Temple 1999) and influencing insect and disease incidence (Pronos *et al.* 1999). Confounding factors such as drought and fire suppression add to the complexity of the San Bernardino Mountain story (Minnich *et al.* 1995; Grulke *et al.* 2009).

Sierra Nevada

The western slope of the Sierra Nevada in California has been adversely affected by O_3 since the early 1980s (Carroll *et al.* 2003). Daytime means of 60–80 nl $O_3 \cdot l^{-1}$ are common (Böhm *et al.* 1995; Takemoto *et al.* 1997; Bauer *et al.* 2000; Bytnerowicz *et al.* 2002; Panek *et al.* 2002). Similar to forests in the Transverse Range, the western Sierra Nevada mixed conifer forests are also exposed to a wide range of additional gaseous and particulate pollutants including various sulphur (S) and nitrogenous compounds (Bytnerowicz *et al.* 1992; Takemoto *et al.* 2001; Fenn *et al.* 2003), pesticides and herbicides transported from agricultural lands located to the west (Davison & Knapp 2007). Similar to other areas, elevated O_3 effects must be evaluated within the context of other pollutants and environmental stressors.

Seedlings of the iconic giant sequoia (*Sequoiadendron giganteum* (Lindl.) J. Buchholz) were symptomatic at elevated O_3 levels in chamber studies (Temple 1988) and were symptomatic in the mid- to late 1980s at ambient O_3 levels in Sequoia National Park, *in situ* (P. Miller, personal communication). Symptomatic seedlings required more light (higher intensity and longer duration) to maintain a positive annual carbon budget (Grulke *et al.* 1989). Pole-size and larger trees of giant sequoia are asymptomatic under past and current levels, suggesting that as a species, it may be relatively tolerant to O_3 (Grulke & Miller 1994). However, O_3 exposure of branches *in situ* showed that each (of three) trees differed in their response: one tree had reduced chlorophyll concentration, another had higher endogenous respiration rates that reduced net assimilation (Grulke *et al.* 1996).

While the overall effects of oxidant pollution on conifers in the Sierra Nevada are comparable to 30 years earlier in the Transverse Range of southern California (Staszak *et al.* 2007), symptoms of O_3 injury have been found on pines in all of the Sierra Nevada National Forests and Parks (Carroll *et al.* 2003). Typical O_3-induced visible foliar symptoms, including chlorophyll degradation, chlorotic mottle and premature senescence, are observed in ponderosa pine (Peterson *et al.* 1991; Arbaugh *et al.* 1998), Jeffrey pine (*Pinus jeffreyi* Balf.; Peterson *et al.* 1987; Patterson & Rundel 1993; Grulke *et al.* 2003a,b; and both species: Staszak *et al.* 2007). Ozone exposure symptoms have been recreated in chamber exposure experiments (Andersen *et al.* 1997; Momen *et al.* 2002).

Visible symptoms have been linked to decreased bole radial growth in both ponderosa (Peterson *et al.* 1991) and Jeffrey (Peterson *et al.* 1987; Patterson & Rundel 1993) pine. Ozone uptake and expression of O_3 effects in mature Jeffrey pine canopies were greater in perennially mesic *versus* xeric microsites (Grulke *et al.* 2003a,b). Isozyme frequency was more homozygous in pole-sized ponderosa pine (more sensitive to O_3) that experienced increasingly higher O_3 concentrations over their lifetime relative to mature and old-growth trees (Staszak *et al.* 2007). In the same study, isozyme frequency of pole-sized trees of Jeffrey pine (more tolerant) was more heterozygous. These genetic shifts are expected to drive changes in forest stand composition and community structure (Patterson & Rundel 1993; Takemoto *et al.* 2001).

In the Sierra Nevada, drought is common (Preisler *et al.* 2017), and may occur in half of all years[1] (Grulke *et al.* 2009). Although xeric microsites decreased O_3 uptake in Jeffrey pine (Grulke *et al.* 2003a), chronic and severe drought stress reduces

[1]Defined as <60% of the long-term average precipitation in an area based on physiologically-defined drought stress.

plant carbon balance (McDowell *et al.* 2008) *via* restricted carbohydrate gain and/or retention, *via* overall loss of hydraulic conductivity and carbon starvation, and can lead to mortality from drought alone or from increased susceptibility to insect attack. Over the last 5 years, the Sierra Nevada has experienced prolonged, exceptionally extreme drought, accompanied by loss of millions of trees to drought, bark beetle and wood borer (Preisler *et al.* 2017). Tree mortality was concentrated in the Transverse Range and the western Sierra Nevada, the areas of greatest O_3 exposure. Outbreaks of insect infestation are believed to be indicators, not a cause, of existing stress in the forest (Wickman 1992).

Appalachian mountains

The southern Appalachian Mountain region experiences some of the highest O_3 concentrations of any natural area in the eastern United States (Mueller 1994; Hildebrand *et al.* 1996; Chappelka & Samuelson 1998). Since the region is the home of the Shenandoah and Great Smoky National Parks,[2] there has been considerable investigation of the region's dominant forest species to determine how O_3 is impacting these ecosystems. Other air pollutants are found in this ecosystem, but not at the high deposition values found in the California studies.

Under ambient O_3 concentrations, visible foliar symptoms of O_3 were found on common sassafras (*Sassafras albidum* (Nutt.) Nees; Chappelka *et al.* 1999), black cherry (*Prunus serotina* Ehrh.; Samuelson 1994; Neufeld *et al.* 1995; Hildebrand *et al.* 1996; Chappelka *et al.* 1997; Samuelson & Kelly 1997), tulip poplar (*Liriodendron tulipifera* L.) and white ash (*Fraxinus americana* L.; Hildebrand *et al.* 1996). Three species of conifer, Table Mountain pine, Virginia pine and eastern hemlock (*Pinus pungens* Lamb., *Pinus virginiana* Mill., *Tsuga canadensis* (L.) Carrière, respectively) were asymptomatic (Neufeld *et al.* 2000). Visible foliar symptoms in the same species have been induced in exposure chambers (Duchelle *et al.* 1982; Neufeld *et al.* 1992; Samuelson 1994; Fredericksen *et al.* 1995).

Somers *et al.* (1998) correlated visible injury symptoms of tulip poplar and black cherry to decreased radial growth. Ambient O_3 also affected understorey species and vegetation (Duchelle & Skelly 1981; Duchelle *et al.* 1982, Duchelle *et al., 1983*; Chappelka *et al.* 1997, Chappelka *et al., 2003*; Chappelka 2002; Davison *et al.* 2003; Grulke *et al.* 2007) and affected community composition (Barbo *et al.* 1998) through effects on growth and reproduction (Chappelka 2002). Kim *et al.* (1998) have also shown effects of O_3 on litter decomposition rates, as was found in the San Bernardino Mountains with long-term elevated O_3 exposure.

Aspen FACE project

The Aspen FACE (Free Air CO_2 Exposure) project utilised an open-air exposure system (Karnosky *et al.* 1999, Karnosky *et al., 2001*) that allowed for replicated exposure of 30-m diameter plots with elevated O_3, alone or in combination with elevated CO_2 without the confounding factors associated with chamber exposures. Each experimental ring was subdivided into halves, with one half containing five clones of trembling aspen (*Populus tremuloides* Michx.) and the other half containing combinations of trembling aspen and paper birch (*Betula papyrifera* Marshall) or trembling aspen and sugar maple (*Acer saccharum* Marshall) trees. The O_3 exposures were ambient (~36 nl $O_3 \cdot l^{-1}$, 12-h·day^{-1} over the growing season) and 1.5× ambient. The Aspen FACE project contributed new findings from the molecular and biochemical levels to the ecosystem level (summarised in Karnosky *et al.* 2001; King *et al.* 2013). The most striking finding of the project was that elevated O_3 offset CO_2-stimulated growth of both trembling aspen (Isebrands *et al.* 2001) and paper birch (Percy *et al.* 2002). The effects on the above- and belowground growth and physiological processes have cascaded through the ecosystem, even affecting microbial communities (Larson *et al.* 2002; Phillips *et al.* 2002). This project illustrated how the co-effects of elevated CO_2 and O_3 had complex effects on tri-trophic interactions involving key tree species, important insect pests and their natural enemies (table 4; Percy *et al.* 2002; Holton *et al.* 2003). The phenologies of host growth (trembling aspen), amount and constituent chemistry of foliar wax, incidence of foliar rust (*Melanpsora medusa* Thuem) and abundance of aphids and their natural enemies were affected by the co-effects of elevated CO_2 and O_3 (Percy *et al.* 2002). The growth enhancing aspects of elevated CO_2 exposure sometimes, but not always, or did not wholly mitigate the deleterious impact of O_3 exposure.

Kranzberger forest free air ozone exposure

A mature stand of European beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) Karst.) was intensively studied in southern Germany from 2000 to 2007 (Matyssek *et al.* 2007b). A portion of the stand was exposed to twice ambient O_3 concentrations (but limited to <150 nl $O_3 \cdot l^{-1}$). This study is comparable to that of the Aspen FACE study in that the experimental O_3 exposure was in free-air, and plant competition as well as multi-trophic-level effects could be examined. In the Aspen FACE experiment, very young trees were planted into and investigated within a 'model' ecosystem. The Kranzberger Forest study was unique in that trees grown under relatively low ambient O_3 concentrations were then exposed to elevated O_3, imposing a stressor on otherwise healthy mature trees with perhaps a growth 'legacy'. One discovery was a change in bole allometry in Norway spruce in elevated O_3, which was not observed in European beech (Pretzsch *et al.* 2010). Diameter growth of Norway spruce at breast height was slightly less than trees in ambient O_3, but diameter was slightly greater at greater bole heights. Elevated O_3 decreased bole volume in European beech by 44%, but was slightly increased in Norway spruce, a result that would not have been reported had diameter been measured only at one bole height. Nunn *et al.* (2006) calculated whole tree O_3 flux for the two species. European beech had higher O_3 flux per leaf surface area and retained only the upper 4 m of foliage on the tree. Norway spruce had lower O_3 flux per needle surface area, but up to eight needle age classes and green foliage retained on much of the bole length. No differences in O_3 flux per tree were found, negating classifications of O_3 flux based on leaf physiognomy (Reich 1987). Although fine and medium root mass was reduced by O_3 exposure of Norway spruce in the litter, A_1 and B_1 horizons, the same size roots in European beech had greater mass in the A_1, B_1, B_2 and B_3 soil horizons. This is an

[2]National Parks have Class I air quality designations by the 1977 US Clean Air Act.

unexpected effect attributed to O_3-induced degradation of cytokinins (CK) in foliage, lower CK translocation to roots and less CK suppression of fine root growth in European beech (Winwood *et al.* 2007). An extreme drought was experienced in 2003, which reduced stomatal conductance and O_3 uptake. Much knowledge of both abiotic (O_3, and fortuitously drought) and biotic (intra- and interspecies competition) drivers was gained in this medium-term experiment.

EXPOSURE TO OXIDANTS IN CONTROLLED CONDITIONS

Much of the connection between what is observed at the whole plant level is the product of biochemical and physiological responses to oxidant exposure and uptake. Exposure conditions in controlled conditions are matched to those of specific natural surroundings to the extent possible. Most agricultural experiments have been carried out with a single cultivar planting in a field, with the focus on individual plants and population-level statistics. In 'natural' ecological settings such as free-air enrichment experiments, a similar approach has been taken to match *in situ* exposure conditions, also with individuals and populations as the statistical unit.

Once O_3 concentration and the pattern of exposure are determined, other environmental conditions must be fixed for exposure chambers, or carefully quantified for free-air exposures. The environmental conditions that will most influence plant growth and response are soil characteristics; intensity, duration and quality of light; ranges of temperature (above- and belowground); humidity; amounts of CO_2 and oxidants; plant to plant competition and plant–microbe symbiosis. In addition to the variables above, wind speed and direction, the spatial arrangement and species mix of trees and the surrounding landforms will influence responses of interest in free-air exposures. In many experimental exposures, plant growth conditions are constructed to optimise plant growth, but plants with higher growth rates (and stomatal uptake and O_3 dose) are often more deleteriously affected at a given O_3 exposure than plants growing under poorer conditions, such as in the field.

There are four main approaches to investigating O_3 effects on plants: (i) controlled laboratory, growth boxes or greenhouse experiments, in which all parameters of exposure are tightly controlled and only one parameter is varied; (ii) field chamber experiments, in which varied cultivars or populations are grown in a uniform, open expanse and the exposure is varied; (iii) free-air exposure experiments, where many environmental attributes vary but are measured and whose effects are measured and statistically differentiated; and (iv) natural ecosystems across environmental gradients including pollutant exposures, where if locations are carefully selected and environmental variables are quantified, evaluation can yield effective answers to co-varying effects. Taken together, these experimental settings yield insightful conclusions, especially if the species is long-lived and cumulative exposures result in a measurable effect.

MEASUREMENTS OF OZONE-INDUCED CHANGES

Visible injury to leaf surfaces

The first standard developed for O_3 injury was visible discoloration of foliage ('visible injury;' Jacobson & Hill 1970; Miller *et al.* 1996; Grulke & Lee 1997; Brace *et al.* 1999). The level of injury (chlorosis, chlorotic mottle, bronzing, necrosis), the proportion of leaf surface area affected, where it occurred (young or older leaves, shaded or exposed surfaces) and what plant age was most responsive are recorded after a quantified exposure (leaf from a plant with little *versus* known, higher O_3 exposure; Fig. 1). Visible symptoms of O_3 vary genetically and among species (Heath 1980). The U.S. Forest Service Inventory and Analysis Program (US FIA) observes visible foliar O_3 assessments at forested sample plots across the United States as one measure of forest health (Smith *et al.* 2003). For example, 12% black cherry, 15% loblolly pine (*Pinus taeda* L.) and 24% sweetgum (*Liquidambar styraciflua* L.) trees expressed foliar injury in sampled plots in the northeast and mid-Atlantic states (Coulston *et al.* 2003).

Productivity measurements

Specific visible symptoms are definitive for symptomatic species and can be replicated with controlled O_3 exposures (*e.g.* Brace *et al.* 1999). Visible symptoms have been correlated to losses in both seed (sand blackberry, *Rubus cuneifolius* Pursh; Chappelka 2002) and biomass (ponderosa pine; Arbaugh *et al.* 1998) production. However, some species have losses in production without visible symptoms (white fir; Retzlaff *et al.* 2000). In crops, 'loss' is related to the plant part that is economically important: seeds (soybean, *Glycine max* (L.) Merr.; Zhang *et al.* 2014; common bean, *Phaseolus fava,* now *Phaseolus vulgaris* L.; Burkey *et al.* 2012), fruits (common grape vine, *Vitis vinifera* L.), leafy foliage (common lettuce, *Lactuca sativa* L.; Temple *et al.* 1986) and roots (common beet, *Beta vulgaris* L.; Munir *et al.* 2016; see economic assessment in Booker *et al.* 2009). For species growing in unmanaged ecosystems, a much larger range of symptoms have been reported. These include reduced photosynthesis (Patterson & Rundel 1993; Darrall & Jager 1984; Heck *et al.* 1988), basal area increment or bole diameter in trees (Peterson *et al.* 1987; Karnosky *et al.* 1996; Somers *et al.* 1998; McLaughlin *et al.* 2007a), shifts in allocation between above- and belowground tissues or carbohydrate reserves or forms (Grulke *et al.* 2001), foliar loss of macro- or micronutrients, reduced reproduction (Luck 1980; Black *et al.* 2000; Chappelka 2002) and competitive status (blue wild rye, *Elymus glaucus* Buckley, ponderosa pine; Andersen *et al.* 2001; McDonald *et al.* 2002) or an increase in mortality (Karnosky 1981; Karnosky *et al.* 2001; McDonald *et al.* 2002). Competition alters plant condition, O_3 uptake, allocation of most within-plant resources and the capacity to detoxify O_3 effects. Unfortunately, the majority of O_3 studies have been conducted on open-grown plants, often grown in pots where competition is absent both above- and belowground.

It is presumptive to predict mature tree responses to O_3 from seedling response studies (Karnosky *et al.* 2001) as: (i) the capacity for resource acquisition varies; (ii) patterns of allocation among root, stem and leaf differs; (iii) the competitive pressure differs by life stage, species composition, tree density, leaf area distribution in space and time, and these factors may change in response to O_3 enrichment (all of these factors influence gas exchange in the canopy); and (iv) post-Reich (1987), attempts to correlate plant functional type to O_3 responses have not always been successful (Nunn *et al.* 2006).

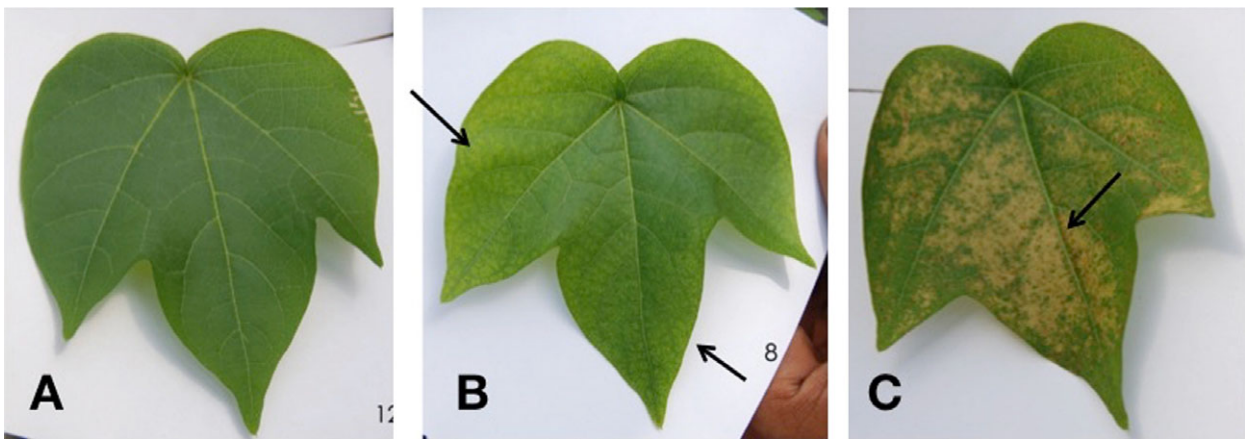

Fig. 1. Examples of no to extreme O_3 exposure effects in primary cotton leaves. (A) No exposure; (B) 15-min exposure to 1200 nl $O_3 \cdot l^{-1}$; (C) 15-min exposure to 2400 nl $O_3 \cdot l^{-1}$. Arrows in (B) indicate leaf margin 'burn' while arrow in (C) indicates lack of necrosis near the vein. Note the irregularity of the chlorosis in (B) and necrosis in (C) (photograph by D.A. Grantz).

Up until the mid-1990s, experimentation was focused on tree seedlings in exposure chambers (Chappelka & Samuelson 1998). In the late 1990s to the present, a considerable effort was made to conduct research on O_3 effects on mature trees, especially within the context of FACE systems. Examples of the focus on mature trees include pines, fir and oaks in California forests, deciduous trees in the Appalachian Mountains, pines, spruce, beech, birch and oak trees across Europe (Hoshika *et al.* 2012). Studies comparing response to O_3 of different black cherry tree age classes suggest that some responses are similar (Fredericksen *et al.* 1995). In most other cases, however, organismal age affects physiology, resource storage capacity and so the response to O_3. In conifers, O_3 exposure was more deleterious to needle function in young *versus* mature trees (ponderosa pine: Dugger & Ting 1970b; Tingey *et al.* 1975; Momen *et al.* 1996; Samuelson & Kelly 1997; Kolb & Matyssek 2001; white fir: Grulke & Retzlaff 2001; red spruce, *Picea rubens* Sarg.: Rebbeck *et al.* 1993; Norway spruce: Wieser *et al.* 2002). Even though photosynthesis was unchanged, Momen *et al.* 1996 found increased leaf respiration in juvenile but not mature ponderosa pine, suggesting a respiratory cost of repair of O_3 injury. In deciduous hardwoods, mature trees had greater O_3 sensitivity than seedlings (English oak, *Quercus robur* L.: Kelly *et al.* 1995; red oak, *Quercus rubra* L.: Samuelson & Kelly 1996; European beech, Braun *et al.* 1999; silver birch, *Betula pendula* Roth: Oksanen 2003). Questions of tree age-dependent O_3 responsiveness remain important research investigations in attempting to scale to ecosystem-level responses. Modelling ecosystem response is limited to mono-specific plantations or assigning average responses to a mix of species in the same physiognomic plant class (Weinstein *et al.* 2005).

PHYSIOLOGICAL EFFECTS

Plant strategies to survive O_3 stress include exclusion of O_3 (through stomatal limitation) or tolerance of it or its products (through production and activity of antioxidants; Levitt 1980). Specific details of how these events occur, and in what order, are few. Air pollutants can be physically excluded from the tissues or cells *via* stomatal closure or by membrane non-permeability. However, stomatal closure is not always complete and stomatal function may be compromised by O_3 exposure (Paoletti & Grulke 2005). Below, observations of O_3 injury are reviewed (Reddy *et al.* 1993; Harris & Bailey-Serres 1994; Heath 2007; Matyssek *et al.* 2014a) many of these events are likely to have been initiated at the level of gene expression.

Carbon assimilation

All green plants generate and transfer energy through the processes of photosynthesis and respiration. Whole-plant net carbon uptake is a function of carbon assimilation (A) and respiratory (R_s) losses, the gain and loss of leaf area, leaf phenology and carbon allocation to reproduction, growth, carbohydrate storage and structure. Ozone has been shown to depress assimilation in sensitive individuals and species. When sensitive species are dominant in unmanaged ecosystems, such as ponderosa pine (Miller *et al.* 1969; Weber *et al.* 1993; Takemoto *et al.* 1997; Grulke *et al.* 2002) and trembling aspen (Coleman *et al.* 1995a,b; Yun & Laurence 1999; Noormets *et al.* 2001a,b; Sharma *et al.* 2003), shifts in dominance, stand composition and sometimes loss of those species can occur. In a study of mature Jeffrey pine, trees in mesic microsites had higher O_3 uptake over the growing season relative to trees in xeric microsites (Grulke *et al.* 2003a). Higher O_3 uptake was correlated with low and mid-canopy needle retention (representing 65% of the foliar biomass, in Grulke & Balduman 1999), lower branchlet diameters and basal area increments, and lower foliar N content. Ozone exposure in chamber studies also negatively affected leaf area in black cherry seedlings (Neufeld *et al.* 1995), European ash (*Fraxinus excelsior* L.; Wiltshire *et al.* 1994) and canopy structure in poplar (*Populus* spp.; Dickson *et al.* 2001). Ozone exposure reduces foliar retention in both chamber and field studies (Miller *et al.* 1963; Miller 1973; Karnosky *et al.* 1996; Grulke & Lee 1997; Pell *et al.* 1999; Topa *et al.* 2001).

Fava bean (*Vicia faba* L.) grown in clean air, then subjected to short-term acute O_3 levels (defined as 150 nl $O_3 \cdot l^{-1}$ for 8 h) developed significant visible injury (20% necrotic; Turcsányi

et al. 2000), yet plants grown in 'chronic' O_3 (low or moderate levels over a longer time with the same overall exposure) developed no visible injury. Within an hour of acute O_3 exposure, the stomatal conductance was lower by 40%, and assimilation was reduced 18% relative to plants in clean air. With chronic O_3 exposure, stomatal conductance was lowered only by 21%, while assimilation was similar between chronic and acute exposures. The similarity of assimilation between O_3 exposure levels indicates that there was no stomatal limitation to assimilation in either exposure. Plants subjected to repeated exposures, at low levels, will become more resistant to later exposures (McCool *et al.* 1988), another indication that changes in gene expression may play a role in the response. However, the cost to plant growth is not known. It is also not known how rapidly a plant will recover after a single exposure (Grantz & Farrar 2000), but there is evidence for both short- term (Grulke *et al.* 2007) and long-term 'memory' of exposure from up-regulated antioxidant content from exposure prior to the experimental exposure (Langebartels *et al.* 1998). It is suspected that the longer the time period between high peaks, the more able the plant is to maintain normal growth and development. However, a long-term shift in energy allocation under continuous O_3 exposure may be less detrimental than long periods between temporary acute exposures.

Energy flow in plant communities can be altered by O_3 through changes in carbon allocation. Elevated O_3 affects carbon allocation to roots by decreasing or inhibiting phloem loading of carbohydrates. This leads to depressed root growth and the potential for affected species to have an increased susceptibility to drought. Ozone and accompanying N deposition alters element ratios in foliage, litter, soil (Boerner & Rebbeck 1995; Andersen *et al.* 2001; Lindroth *et al.* 2001) and global ecosystems (Peñuelas *et al.* 2013). Concentrations of tannins, lignin and phenolics (Findlay *et al.* 1996; Kim *et al.* 1998; Baumgarten *et al.* 2000; Saleem *et al.* 2001) are also affected by O_3 exposure. Ozone can alter carbon cycling in the ecosystem by affecting the chemical composition and rate of decomposition of leaf litter (Fenn & Dunn 1989), litter build-up in the ecosystem and can also negatively affect belowground food webs.

Respiration

In contrast to relatively consistent findings with assimilation, O_3 effects on respiration vary. In silver birch, Matyssek *et al.* (2002) found no O_3 effect on stem respiration. The lack of effect suggested that construction and or maintenance costs of stems were not impacted. However, the bole represents a large storage pool of carbohydrates in mature trees, and the timing of phenological events between individual trees may confound the ability to detect statistically significant differences in carbon storage across pollutant exposures (Grulke *et al.* 2001). Ozone induced a change in bole allometry in European beech and Norway spruce (Pretzsch *et al.* 2010) that at the forest level, could result in increased respiratory losses. Using a traditional measurement of bole diameter, Braun *et al.* (2014) estimated an 11% loss of biomass in Switzerland attributable to ambient O_3 exposure. A simulation of the effect of O_3 exposure on bole growth of ponderosa pine showed a 15% reduction in biomass (Weber & Grulke 1995), largely influenced by differences in carbohydrate allocation as well as repair costs elsewhere in the tree. Foliar respiration is generally increased under elevated O_3 as maintenance costs of leaves damaged by O_3 are higher than normal (Grulke 1999; Noormets *et al.* 2001a). Although foliar respiration is more variable than that of the bole, 120 measures were necessary to show significant differences among treatments (unpublished data, referenced in Grulke 1999). Thus, increased respiration is often found, but not reported as a significant effect of O_3 exposure.

Soil respiration was depressed with O_3 exposure to aboveground tissues (Coleman *et al.* 1996; Andersen & Scagel 1997; Scagel & Andersen 1997; King *et al.* 2001). Decreased soil respiration is thought to be due to reduced root growth under O_3, but it could also be explained by decreased microbial respiration (Andersen 2000). Increased soil respiration has also been found in ponderosa pine seedlings exposed to O_3 (Scagel & Andersen 1997), likely because of a stimulation of soil microbial respiration. The sensitivity of whole soil pedon respiration to O_3 illustrates the role that O_3 plays in altering ecosystem carbon balances (Andersen 2000). Foliar carbohydrate studies also suggest more carbon is used for repair processes with O_3 exposure (Einig *et al.* 1997; Grulke *et al.* 2001; Topa *et al.* 2001), a result supported by increased respiration.

The net effect of O_3 impacts on assimilation and respiration for sensitive components of natural ecosystems is that height and radial growth (Isebrands *et al.* 2001; Oksanen 2003) can be negatively affected by O_3. This has been extrapolated to depressed net primary production (NPP) of whole ecosystems (Hogsett *et al.* 1997; Laurence & Andersen 2003; Laurence *et al.* 2003).

Stomatal conductance

Stomata respond to external (light, humidity, oxides) and internal (CO_2, C_i, cellular osmoticum) conditions across a broad range of time scales, and are governed by the developmental, metabolic state (Turcsányi *et al.* 2000) and genetic capacity of each plant. Stomata control water and carbon cycles, as well as O_3 uptake (Hetherington & Woodward 2003). Ozone exposure induces a decline in assimilation (Reich 1987; Saxe 1991; Heath 1996; Bortier *et al.* 1999; Tingey *et al.* 2001; Grulke *et al.* 2002), attributable to a decline in carboxylation efficiency, the electron transport system and/or indirect effects on stomata (Kull *et al.* 1996; Kellomäki & Wang 1997; Reichenauer *et al.* 1997). The associated decline in stomatal conductance is a secondary response to declining assimilation.

Stomatal responses to air pollutants have been extensively reviewed and are complex, varying among species, leaf and tree age, and are modified by other environmental stressors (Darrall 1989; Saxe 1991; Mansfield 1998). Sensitive trees may have intrinsically higher stomatal conductance, thus have higher O_3 uptake and more symptoms. Sensitive trees may have similar stomatal conductance but have lower defence capacities. Sensitive trees may also differ in their endogenous respiration rate: carbon balance is lower with O_3 exposure, C_i is lower, stomatal conductance increases, and O_3 uptake is increased. Many combinations of subtle variations in the carbon, water or nutrition can lead to O_3 sensitivity (Heath & Taylor 1997; Grulke 1999).

Differences in stomatal density may also lead to O_3 exposure sensitivity. In clones of silver birch and downy birch (*Betula pubescens* Ehrh.), O_3 tolerance was related to higher stomatal density, but the opposite was true for black cherry, with

sensitive genotypes having higher stomatal density (Ferdinand *et al.* 2000). Changes in stomatal density developed after O_3 exposure in birch spp. (Matyssek *et al.* 1991; Günthardt-Goerg *et al.* 1993; Pääkkönen *et al.* 1995a,b; 2003; Frey *et al.* 1996; Maurer & Matyssek 1997), European ash (Wiltshire *et al.* 1996) and Canadian poplar (*Populus* × *euramericana*, currently referenced as *Populus* × *canadensis*, Günthardt-Goerg *et al.* 1996). High stomatal density permits optimisation of stomatal regulation and water use efficiency (Hetherington & Woodward 2003). Small stomata can open and close more rapidly (Aasamaa *et al.* 2001). Smaller apertures should also translate to lower O_3 uptake per stoma, reducing the need for antioxidant capacity in a given volume of apoplastic water. Nunn *et al.* (2006) provide one example of scaling these attributes up to the whole tree level.

Groups of stomata may have localised, coordinated responses (*e.g.* stomata in some areas may have larger apertures than stomata in other areas of the same leaf). Ozone exposure exacerbates patchy stomatal responses (Omasa *et al.* 1981; Ellenson & Amundson 1982; Beyschlag & Eckstein 1998). Localised independent stomatal behaviour supports opportunistic capture of resources, such as sun flecks in a broad lamina (Mott & Buckley 2000).

Moderate (~70 nl $O_3 \cdot l^{-1}$ (hourly average)) levels of O_3 exposure or short-term exposure (a few hours) generally stimulate a rapid reduction in stomatal aperture. At higher levels of O_3 with longer-term exposure, or in highly sensitive species, stomatal responses can become sluggish (Reich & Lassoie 1984; McAinsh *et al.* 2002; Grulke *et al.* 2007), and the relationship between assimilation and stomatal conductance is uncoupled (Reich & Lassoie 1984; Matyssek *et al.* 1991; Flagler *et al.* 1994; Tjoelker *et al.* 1995; Clark *et al.* 1996; Paoletti & Grulke 2005, 2010). Sluggish stomatal responses are also observed when O_3 exposure is combined with drought stress (Youngglove *et al.* 1988; Patterson & Rundel 1989; Pearson & Mansfield 1993; Grulke 1999), fluctuating light (Keller & Häsler 1984; Reich & Lassoie 1984; Barnes *et al.* 1990; Grulke *et al.* 2003a; Paoletti & Grulke 2010) or rapid changes in humidity (coneflower, Grulke *et al.* 2007). Ozone exposure is known to accelerate senescence processes (Heath & Taylor 1997) and sluggish stomatal responses are also typical of ageing leaves (Reich 1984). Sluggish stomatal response translates to poor control of water loss: stomata are more open when environmental conditions are unfavourable (*e.g.* high leaf to air vapour pressure deficit, VPD) and partially closed when conditions are favourable for assimilation (Patterson & Rundel 1993).

After O_3 exposure during the daytime, the sluggish stomatal response may persist (Barnes *et al.* 1990; Grulke *et al.* 2007) leading to incomplete stomatal closure at night. California black oak (*Quercus kelloggii* Newberry) and blue oak (*Q. douglasii* Hook. & Arn.) were exposed to no ('naïve') or chronic O_3 exposure for 1 or 2 months (respectively, in these deciduous and evergreen/wintergreen oaks) in open-top chambers, and a small portion of a leaf was additionally exposed to no or acute O_3 exposure for 1 h during the day. Following daytime exposure, nighttime transpiration (T_r) was measured. Both species exposed to chronic daytime exposure had elevated nighttime transpiration relative to controls (1.9× *versus* 1.6× higher, respectively). In California black oak, a 1-h acute exposure had little effect on nighttime transpiration in naïve seedlings (1.2× higher), but both medium-term chronic and a 1-h acute exposure increased nighttime transpiration (2.4× higher). In contrast, nighttime transpiration was much higher in 'naïve' blue oak seedlings than in higher exposed previously to chronic O_3 (1.9× and 1.7×, respectively). The effect lasted for 3 days before full recovery of stomatal closure at night in both species (Grulke *et al.* 2007). Possible mechanisms through which O_3 induces both sluggish opening and closing have been discussed by Kaiser & Paoletti (2014).

Ozone absorption by leaves: Multiple levels

There are three sequential processes from the external concentration of O_3 into the leaf, to the detoxification of O_3 in the apoplastic water, to the movement of detoxification products into the cell, which combine to stimulate genetic up-regulation of defences (Heath 1980).

Entry of the gaseous pollutant into the leaf

This includes gaseous diffusion through the leaf boundary layer, the stomata and into the substomatal cavity. The movement of pollutants through the leaf surface boundary layer into the stomata is rate-limiting, especially in the case of low wind velocity. Once through the boundary layer, the gas must pass through the stomata and into the leaf. The entry of gases into a leaf (flux) is dependent on physical (linearly dependent on the difference in concentrations of gases inside and outside of the leaf and stomatal size) and chemical processes of the gas phase and cell surfaces. The proportionality coefficient is the conductance (g) to O_3. For both water and CO_2, this formulation has been used for years (Farquhar & Sharkey 1982; Ball 1987). For O_3, internal concentration seems to be very close to zero (Laisk *et al.* 1989) because it is extremely reactive with cellular chemicals. Thus, the effective delivery rate is: stomatal conductance × ambient O_3 concentration, with stomatal conductance being the primary regulatory control of flux (Taylor *et al.* 1982; Amiro *et al.* 1984). However, the level of O_3 within the plant is not zero, as otherwise no reaction with any biochemical species could take place. In fact, O_3 most probably reacts with various biochemicals as it enters the guard cell pairs and beyond (see Heath 1994a). The level of O_3 probably drops at each point along its pathway up to the apoplasm. To date, no experiments have elucidated these processes on a microscopic scale.

Reactions of gases in the apoplasm, and reaction of these products with biochemicals within the cell wall

Ozone reacts with organic molecules at double bonds to form carbonyl groups and peroxides (H_2O_2; see Fig. 2), as well as with sulphydryls, with the formation of disulphide bridges or sulphones (Mudd 1973). These chemical reactions are poorly understood, although there are some fundamentals (Heath 1987, Heath, 1988, Heath Heath 1996). In water, the reactions become more confusing, but some products have been described by Heath (1987). In the apoplasm, an equilibrium between the gas and aqueous phase occurs at the interface where the gaseous species dissolves into the water according to Henry's Law (Heath 1980, Heath, 1987; Hewitt *et al.* 1990). Effective detoxification reactions can occur here *via* antioxidant metabolites and enzymes, such as ascorbate, glutathione and superoxide dismutase (Matters & Scandalios 1987). Chemical modification of wall-specific biochemicals is also possible (Castillo *et al.* 1987). In many studies, the *in vivo* activity of plant enzymes was shown

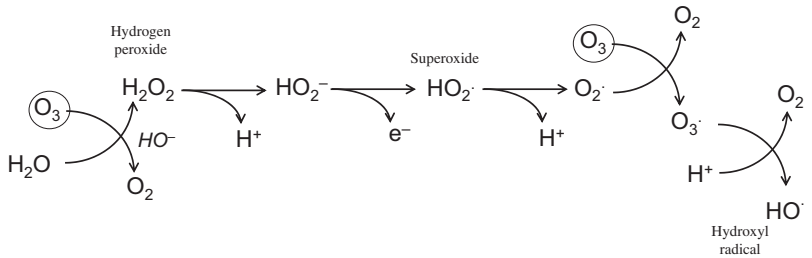

Fig. 2. Ozone reactions and electron structure of some products of O_3 and water. Adapted from Jans & Hoigné (2000) and Walcek *et al.* (1997). Many of the chemical species have unpaired electrons associated with the compound, which makes them highly reactive. In addition, some species can easily take up a proton from water, depending upon the pH of the medium, which alters its characteristics and reactivity.

to be altered by O_3 by testing the rate of the specific enzyme activity after their extraction from exposed tissues. Some enzymes from whole tissues were thought to be critical for metabolism, such as glucan synthase (Ordin *et al.* 1969), or for intracellular movement of ions, such as Ca^{2+} (Castillo *et al.* 1987). Other experiments were focused on enzymes within extracellular fluid (such as diamine oxidase; Peters *et al.* 1988) after the tissue containing those fluids was exposed to atmospheric O_3.

Movement of reaction product(s) into the cell and enzymatic or chemical transformations within the cell

It is believed that the initial site of O_3 damage is the plasma membrane. Membrane functions, such as membrane fluidity (Pauls & Thompson 1980), permeability (Elkiey & Ormrod 1979), K^+ exchange *via* ATPase reactions (Dominy & Heath 1985) and Ca^{2+} exclusion (Castillo & Heath 1990) are rapidly lost. If O_3 reacts with components of the cell wall, those alterations may connect to the cytoplasm through the wall and membrane by membrane-specific proteins not directly linked to transport. The similarity of wounding responses (Langebartels *et al.* 1991) and O_3-induced membrane disruption suggests the induction of wound-regulated genes (Mehlhorn *et al.* 1991).

After the normal physiological homeostasis is disturbed, attempts of re-normalisation occur within cells and between tissues, with activation of genetic responses. Most of these studies are recent due to the great strides in genomics and centre upon pathogen response (PR) activity and senescence (discussed later). The production of defence compounds (antioxidants) is an energetically costly response, and they increase under elevated O_3 (Sheng *et al.* 1997; Tausz *et al.* 1999, 2002). In mature Jeffrey pine, stomatal uptake of O_3 elicited one complex of antioxidant defences in mesic microsites, while endogenously-generated free radicals in the chloroplast elicited a second complex of antioxidant defences in xeric microsites (Grulke *et al.* 2003b).

Mesophyll cells of leaves receive the products of O_3 with water and wall- or membrane-linked biochemicals. Arguments have been made about the types of chemicals which can be formed (Heath 2007), yet none have been well proven to exist within the leaf except for hydrogen peroxide (H_2O_2), which is also made by the leaf cell. In alkaline media, O_3 reacts and forms H_2O_2 and many other compounds (Fig. 2). In acid media, O_3 is relatively stable in the absence of metal ions. The pH of the cell walls within the substomatal cavity is more acid than alkaline, but the wall does have diverse charges and surfaces, which may alter these simplified conclusions.

From the reaction coefficients given by Atkinson (1990), the rates of reaction of O_3 with double-bond compounds includes: the Creigee mechanism (equation 1), ring breakage *e.g.* ascorbate (equation 2) and sulphydryl oxidation (equation 3; see Heath 1987).

$$\text{R-CH}_2\text{-CH=CH-CH}_2\text{-R}' \rightarrow \text{R-CH}_2\text{-CH(OH)} + \text{O=CH-CH}_2\text{-R}' \quad (1)$$

$$\text{C-Ring with [double bond]} + \text{O}_3 \rightarrow \text{Broken C-ring at [double bond]} \quad (2)$$

$$\text{G-SH} + \text{G-SH} + \text{O}_3 \rightarrow \text{G-S-S-G} + \text{H}_2\text{O} + \text{O}_2 \quad (3)$$

Reactions with double bonds and with sulphydryl groups have been seen to alter protein structures and thereby inactivate them.

While many reactions could occur and alter some metabolites, most pathways are well regulated, and for any small disruption, the pathway would tend to return to near its former stability. Thus, many measurements of metabolites or even enzymes may result in undetectable changes due to the smallness of the change and experimental variability. The most accurate measurement of changes is the flow of metabolites through a pathway, and those measurements are difficult at best.

Hydrogen peroxide

In the past, H_2O_2 was thought to be a toxic compound for cells as there are so many antioxidants and enzyme systems that eliminate it. However, it is clear that cells generate it for specific purposes, notably to attack invading pathogens in defence (Mehdy 1994; Simon-Plas *et al.* 1997). In fact, it is believed that the chemical species generated is superoxide through a one-electron reduction of molecular oxygen (reactive oxidative species, ROS). In the acid region of the cell wall, superoxide is immediately converted to H_2O_2 by a protonation and dismutation. That conversion is carried out by NAD(P)H oxidase located on the cell membrane, facilitated by cytochrome b6 (Auh & Murphy 1995). Furthermore, H_2O_2 is used for oxidation in lignification (Schopfer 1994; Schopfer *et al.* 2001). The induced oxidative burst by ROS likely plays a role in stimulating Cl^- and K^+ efflux, and in generating alkalinisation of the extracellular space, since these processes are inhibited by preventing the burst (Cazale *et al.* 1998).

In the wall region, H_2O_2 is not that toxic as no necrosis is reported when 500 mM peroxide is infiltrated into leaf tissue.

However, the production of salicylic acid and benzoic 2-hyroxylase are induced with 30 and 0.3 mM H_2O_2, respectively, indicating some metabolic signalling (Leon *et al.* 1995). A 1 M H_2O_2 solution infiltrated into soybean will generate lipid peroxidation after 1 h (Degousee *et al.* 1994). The cells can react to the total system and generate peroxide scavenging compounds within a few hours, which eliminate excess H_2O_2 (Baker *et al.* 1995).

Hydrogen peroxide has been found in the wall after O_3 exposure in silver birch (Pellinen *et al.* 1999, 2002). By using $CeCl_2$ as a cellular stain for H_2O_2 (as a cerium perhydroxide precipitate, see Liu *et al.* 1995), a gradual development of stain was found after 8 h of an acute O_3 exposure. Ozone itself may induce some formation of H_2O_2, and so an additional 25 min passed after exposure for it to be dissipated with the expectation that any O_3-induced H_2O_2 would be eliminated by reactions with apoplastic antioxidants. After 2 h of exposure, H_2O_2 staining was visible on the surfaces of both sets of mesophyll cells. Accumulation of H_2O_2 stain continued for 16 h after the exposure, suggesting a triggered reaction rather than O_3 decomposition itself. There was H_2O_2 stain present in the mitochondria, peroxisomes and cytoplasm, but not in the chloroplast. These experiments indicate that O_3 *per se* does not generate the H_2O_2 but rather triggers a stress-related H_2O_2 formation similar to pathogen attack (ROS). Ozone breakdown can also add more H_2O_2. The presence of higher than normal levels of H_2O_2 within the apoplastic space implies a stimulation of the normal pathogen defence pathway. Thus, all the events and activation of pathways/genes caused by pathogen defence should be observed upon O_3 exposure of plants.

Hydrogen peroxide has been linked to abscisic acid (ABA, a hormone)-induced stomatal closure by activating Ca^{+2} influx in guard cells (Pel *et al.* 2000). The addition of H_2O_2 to a guard cell preparation at a level of only 5 mM will cause a dramatic increase (ca. 9×) in membrane current (due to ion flow) at the hyperpolarising potential of −200 mV. Amounts as low 50 μM H_2O_2 will also cause a sizable current increase. While the membrane stability was unaffected by H_2O_2, the activation of the channel required only about 23 min. Abscisic acid also induced the production of H_2O_2 through ROS accumulation (see Shapiro & Zhang 2001). A recessive ABA signalling mutant (gca2) showed insensitivity of stomatal closure to the hormone, and a lack of stimulation of the Ca^{2+} influx. On the other hand, H_2O_2 inhibits ABA-induced stomatal closure. Thus, a model of interactions that links H_2O_2 with ion flux, and ABA production with an inhibiting signal molecule of ABA-insensitive gene 2 (ABI2, equations 4, 5):

$$H_2O_2 \rightarrow [Ca^{2+}\text{influx and ABA production}] \quad (4)$$

$$\text{ABA} \rightarrow \text{stomatal closure [but inhibited by ABI2]} \quad (5)$$

In the past it was not understood why, in some cases, O_3 would not always decrease stomatal conductance (see Heath 1994b). The phospho-tyrosine-specific protein phosphatase (ABA-insensitive gene 2) is an inhibitor of stomatal closure induced by ABA but is inhibited by H_2O_2 (Meinhard *et al.* 2002). The sensitivity of stomatal closure to ABA is modified by H_2O_2 to make the stomatal complex more sensitive to the hormone. Thus, for a given level of ABA present in the guard cell complex due to environmental factors such as low humidity, soil water potential or high air temperature, any generation of H_2O_2 would inhibit ABA-insensitive gene 2 and increase stomatal closure *via* increased sensitivity to the hormone.

Photosynthesis inhibition: Alteration of mRNA coding of Rubisco by O_3

There is a large body of literature that shows that O_3 can induce declines in cellular concentrations of Rubisco (Pell *et al.* 1994). Treatment of a variety of plants with a moderate level of O_3 induces a loss of Rubisco due to reduced mRNA coding for both subunits of Rubisco. With the important role of Rubisco in the production of carbohydrates (see Fig. 3), any loss could have severe consequences for plant productivity.

The sequence of the formation and functional activation of Rubisco is a useful example of genomics, and serves as a model for linking genomics, plant physiology and ecology. While it may seem that assaying the Rubisco mRNA would predict O_3 injury, there are many events that will change the production of Rubisco mRNA.

For example, the level of carbohydrate influences the amount of mRNA for Rubisco (*rbc*S). As stated in Krapp *et al.* (1993), there are four indications of this: (i) removal of a sink organ leads to an inhibition of assimilation at the source; (ii) given an increased level of CO_2, after a transitory increase in assimilation, the rate returns to that measured previously; (iii) using transformed cells to prevent sucrose export (the addition of invertase to the cell wall) leads to an increase in sugar content and a decline in assimilation; (iv) sugars fed through the transpiration stream lead to a decline in assimilation. This may partially explain why total productivity of a leaf may not reflect functioning area of the leaf.

Williams *et al.* (1994) developed a correlation between ABA levels after drought stress in thale cress leaves and the loss of Rubisco mRNA. The ABA level had a half-life rise time of about 1–2 h, and the Rubisco mRNA level had a half-life decline of about 2–4 h. This suggests that drought stress may alter the relationships much more than merely closing the stomata. If ABA lowers Rubisco mRNA by whatever mechanism, it may be a poor marker of O_3 exposure except under very controlled conditions.

Carbohydrate transformations and allocation

Many of the experiments with O_3 have shown that exposure reduces the net assimilation (the balance of carbon gain and loss through assimilation and respiration) and accumulated dry mass of the plant. If the stomata close partially, photosynthetic activity will decline which should lower carbon gain, which in turn will reduce growth. However, fixed carbon alone does not account for the complete equation. Repair or prevention of injury (antioxidant response) requires resources and energy, which additively reduces growth, but little work has been done on the amount of energy so diverted and its ramifications.

Adams *et al.* (1990) found that there was 'no statistically significant effects of elevated O_3 (up to 95 nl $O_3 \cdot l^{-1}$ for 7 h) on either carbon gain or photosynthate at any specific time during the growing season'. In the same study, he reported that

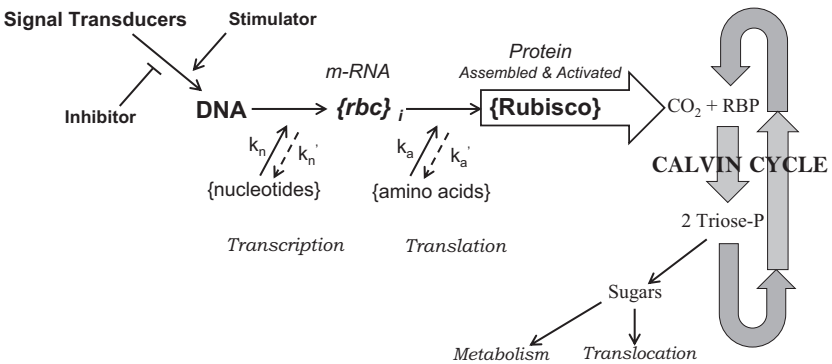

Fig. 3. The formation and use of Rubisco following the Central Dogma Sequence. DNA produces mRNA, which is the code for the protein structure(s). With Rubisco, two distinct subunits must be produced and assembled to make a working protein. The stimulation of the DNA–RNA sequence is controlled by a number of signal transduction agents, which alters the ability of DNA to be transcribed. Rubisco is the key enzyme in the Calvin Cycle of photosynthesis, which fixes a CO_2 molecule into sugars (ribulose 1,5-bisphosphate carboxylase/oxygenase). The CO_2 enters the leaf through the stomata, which has a conductance.

loblolly pine seedlings grown at twice ambient O_3 levels consistently exhibited lower assimilation, higher respiration and a reduction of allocation to the fine roots. It is likely that high plant-to-plant differences in response belied statistical discovery. On the other hand, Volin *et al.* (1998) reported lower leaf area, leaf weight, specific leaf area and root weight in trembling aspen and two C_3 grasses (western wheatgrass. *Agropyron smithii,* now *Pascopyrum smithii* (*Rydb.*) *À. Löve* and prairie Junegrass, *Koeleria cristata,* now *Koeleria macrantha* (Ledeb.) Schult.), but not in red oak or C_4 grasses (side oats grama, *Bouteloua curtipendula* (Michx.) Torr. and little bluestem, *Schizachyrium scoparium* (Michx.) Nash). There was no statistically significant change in any species in leaf stomatal conductance nor in assimilation. They described a correlation over all species between growth decline and increased stomatal conductance, which may be an example of loss of stomatal control at high O_3 exposures (see above).

Maximum concentrations of carbohydrates in 1-year-old ponderosa pine needles declined with an increase in pollution (O_3 and N deposition) along an anthropogenic pollution gradient east of Los Angeles, CA (Grulke *et al.* 2001). Monosaccharide concentrations in fine roots were depressed, along with starch, suggesting that needle sugars were limiting and that led to root sugar limitations as well. No attempt was made at balancing the total productivity since the data were taken over a full growing season of mature trees with many source–sink interactions. In a shorter-term exposure (9 days; Smeulders *et al.* 1995), low O_3 exposure increased the retention of labelled photosynthates within the needle (see Grantz & Farrar 2000), but at higher exposures (400 *versus* 200 or 0 nl $O_3 \cdot l^{-1}$), total starch within the needle declined, suggesting that less carbohydrate was produced within the cells.

There is a pronounced reduction in the allocation of carbohydrate from shoot to root, with the shoot maintaining a larger proportion of carbon recently fixed. In cotton, Grantz & Yang (2000) found that the O_3-mediated allocation differed from that driven by foliar loss (pruning). Ozone triggers a plant-wide response, possibly regulated by long-distance signals that modify the delivery of resources to these sinks in parallel. Stitt (1996) suggested that '. . .allocation is regulated by long-distance signals that act to influence growth of selected sinks and to modify the delivery of resources to these sinks in parallel'. Cooley & Manning (1987) further suggested that there were three possible ways that O_3 exposure might alter translocation: (i) malfunction of the phloem loading process; (ii) increased allocation to repair damage within the source; and (iii) altered balance between source and sink.

Ozone exposure can induce a shift in the carbon transfer between roots and shoots, which is amplified by mild drought (Gerant *et al.* 1996). Further, wounding the leaves of red goosefoot (*Chenopodium rubrum* L.) induced defence responses, which also regulated source–sink relations (Roitsch 1999). Ethylene (C_2H_2, discussed below) can repress the expression of extracellular invertase, which is critical for control and downloading of sucrose derived from the translocation stream.

Although inhibition of phloem loading into the stem has been reported with O_3 exposure (Grantz & Farrar 2000), it is unclear whether or not sugars are translocated out of the leaf. Dugger & Ting (1970a) suggested this nearly 40 years ago, but few have returned to this question. Some observations suggest that assimilation within the leaf declines, translocation is inhibited even more so, such that fewer growing points of the plant are stimulated to grow and root/shoot ratios are altered (Tjoelker *et al.* 1995; Gerant *et al.* 1996).

Ethylene interaction with injury and conductance

Increased ethylene (C_2H_2) production is a known response to wounding and has been also identified as a common plant response to O_3 exposure. For a variety of plants, the production of C_2H_2 (induced by up to 750 nl $O_3 \cdot l^{-1}$ for 4 h) was exponentially correlated with external O_3 exposure and linearly related to foliar injury (Tingey *et al.* 1976).

Tingey (1989) demonstrated that C_2H_2 release was stimulated by O_3 exposure. The amount of C_2H_2 produced after 24 h was species- and dose-dependent. The observation that an inhibitor of C_2H_2 formation, aminoethoxyvinyl glycine (an inhibitor of acetyl-CoA carboxylase synthase) blocks C_2H_2 formation and inhibits visible injury production further implicates the wounding response (Mehlhorn *et al.* 1991). While it was possible that the effect was not specific for C_2H_2, elimination of its formation prevented visible injury. This response could be due to elimination of visible injury or through chemical induction of stomatal closure. However, Taylor & Gunderson (1988) and

Taylor *et al.* (1988) found that aminoethoxyvinyl glycine did not close stomata nor inhibit assimilation *per se* in soybean. Ormrod & Beckerson (1986) injected polyamine into the transpiration stream and prevented injury, suggesting a close involvement of the pathway linking C_2H_2 to polyamines with the production of visible injury. The lack of C_2H_2 production or an increased level of polyamines slowed or prevented visible injury. The amount of O_3-induced C_2H_2 release declined with repeated exposure, indicating an acclimatisation to O_3, as was also found by Stan & Schicker (1982) and Stan *et al.* (1981). Stress-induced C_2H_2 production correlated better with concentration rather than duration of O_3 exposure.

The correlation of ethylene release with O_3-induced visible injury was also shown in common pea (*Pisum sativum* L.) cultivars by Dijak & Ormrod (1982). With O_3 exposure (6 h at 300 nl $O_3 \cdot l^{-1}$), stomates closed by half in 3 h. Both sensitive and insensitive cultivars with visible injury released ethylene, with greater visible injury and ethylene release for a given exposure in sensitive cultivars. This precedes the observation that ethylene release is required for visible injury (or vice versa), shown by the work of Mehlhorn & Wellburn (1987, summarized in Wang *et al.* 1990). Soybean exposed to exogenous ethylene (Taylor & Gunderson 1988) decreased both stomatal conductance and assimilation, but not simultaneously. Exogenous ethylene slightly increased a difference between internal leaf CO_2 concentration and ambient CO_2 concentration (by changes in the former) in contrast with that observed by Farage *et al.* (1991) with O_3 exposure. These differing observations may have confounded earlier studies.

Ethylene (with a double bond) released within the substomatal cavity reacts with internal O_3, generating relatively noxious chemicals (see equation 1). Using the data of Taylor & Gunderson (1988), the internal concentration of C_2H_2 is calculated to be about 2000 $nl \cdot l^{-1}$. While this seems small, it can react with O_3 to form a variety of noxious low molecular weight compounds, which can penetrate cell membranes.

Antioxidants (principally ascorbate and glutathione) within the mesophyll play a role in preventing O_3-induced injury. The apoplastic space surrounding the substomatal cavity would be the 'first line of defence' in this prevention of injury from O_3. Once that line is overrun by excess oxidants and antioxidants that are unable to sufficiently regenerate, then other parts of the cell become 'at risk', the next being the plasma membrane. Although there are a number of biochemicals in the apoplasm that react with O_3, the predominant species is ascorbate.

There are a number of antioxidants within the cell, and measurement of one compound can give misleading results. For example, ascorbate is present within the wall (Padu *et al.* 2005; Pignocchi *et al.* 2006), cytoplasm (Davletova *et al.* 2005) and chloroplasts (Smirnoff *et al.* 2001). Once tissue is ground up and assayed for ascorbate, all sources have been combined into one measurement. If the wall ascorbate drops by 50% due to O_3 exposure but all others remain the same, the measurement of the total drop is dependent upon the amount of ascorbate within the wall. Through measurements, Turcsányi *et al.* (2000) estimated that a 50% loss of apoplastic ascorbate would be converted into only a 2–3% loss of total ascorbate, which is difficult to measure let alone show statistical significance. These types of problem are at the heart of how difficult it is to measure biochemicals as markers of O_3 injury.

Wounding and pathogen attack

The production of hydrogen peroxide after O_3 exposure seems to be a typical wounding response. In retrospect, the paucity of studies demonstrating a correlation between O_3-induced wounding and pathogen attack is surprising. The linkage between the pathogen wound responses and visible injury are well established (see Conklin & Barth 2004). Langebartels *et al.* (2000) used an O_3 exposure which injured a sensitive tobacco (*Nicotiana tabacum* L.) cultivar but not a tolerant cultivar (Bel B and W3). They also showed that the biochemical response to O_3 occurred in the same order as that for a pathogen attack.

The hypersensitive response of plants is a mechanism used to prevent the spread of infection by microbial pathogens. It is characterised by rapid cell death in the local region surrounding an infection, which restricts the growth and spread of pathogens. It precedes a slower systemic response, which leads to systemic acquired resistance. The hypersensitive response is triggered by cellular recognition of gene products, secreted by a pathogen, that interact with the product of a plant resistance gene. There are a wide variety of plant resistance genes enabling plants to recognise virulence products produced by different pathogens. Reactive oxygen species also trigger the deposition of lignin and callose, as well as cross-linking of some compounds in the cell wall, which serve to form a barrier to spread of the infection.

The activation of plant resistance genes triggers an ionic flux: efflux of hydroxide and potassium from the cells, and influx of calcium and hydrogen ions into the cell. Concurrently, the cells involved in hypersensitive responses generate an oxidative burst by producing reactive oxidant species, superoxide anions, hydrogen peroxide, hydroxyl radicals and nitrous oxide. These compounds impair the ability of the cellular membrane to function by inducing lipid peroxidation (Zheng *et al.* 2011), through lipid oxidase or a membrane lipase to produce jasmonic acid or inositol triphosphate. Jasmonic acid and inositol triphosphate act at the systematic acquired resistance level to change the production of defence gene products.

Mehdy (1994) described a model of how an elicitor produced by the pathogen attack also activated a G-protein, which opened the inward flowing Ca^{2+} channel. The flow of Ca^{2+} into the cytoplasm raises the internal concentration at the μM level, and so activates a protein kinase, that increases the activity of the plasma membrane NAD(P)H oxidase, generating more superoxide (O_2^-). Superoxide dismutase converts this into hydrogen peroxide.

In essence, the defence activation which leads to localised cellular death is caused by a pathogen recognition sequence. The defence activation is localised by proteins within the apoplasm and by membrane transport changes, leading to ROS production. Once that begins, it can lead to a global response or resistance caused by 2-hydroxylase activation and the production of benzoic acid, leading in turn to production of salicylic acid.

Local pathogenic responses seem to require the involvement of cysteine proteases. The induction of cell death and the clearance of pathogens also require active protein synthesis, an intact actin cytoskeleton and the presence of salicylic acid. Copper amine oxidase, which catalyses the oxidative deamination of polyamines (especially putrescine), is involved in generation of ROS and the release of hydrogen peroxide and

ammonia. Other enzymes thought to play a role in ROS production include xanthine oxidase, NADPH oxidase, oxalate oxidase, peroxidases and flavin-containing amine oxidases. In some cases, the cells surrounding the lesion synthesise antimicrobial compounds, such as phenolics, phytoalexins and pathogenesis-related proteins, including β-glucanases and chitinases. These compounds act by puncturing bacterial cell walls, by delaying maturation or by preventing reproduction of the attacking pathogen.

Jasmonic acid and salicylic acid

Salicylic acid (SA) and jasmonic acid (JA) are regulators of plant defence response but tend to respond more slowly than ethylene, with broader effects within the plant (Fig. 4). They are both involved in plant responses to O_3, once again linking pathogen/wounding defence to O_3-induced injury. However, their exact roles are far from clear. Methyl jasmonate is involved in resin biosynthesis, which accumulates within the ducts and xylem (Martin *et al.* 2002) by stimulating the isopentenyl pathway, through the mevalonate pathway. Ozone induces a similar stimulation, linked to JA.

One of the lipoxidase isoforms is activated by pathogen infection within 6 h and accumulated for a week (Kolomiets *et al.* 2000). The first stage of this pathway leads to 13-hydroperoxide linolenic acid, which then is converted either to allene oxide through allene oxide synthase or to C6 aldehydes through hydroperoxide lyase. These aldehydes act as signalling agents *via* systemin (Sivasankar *et al.* 2000) or to volatile odiferous compounds (oxylipins), which are antimicrobial toxins (Froehlich *et al.* 2001). These compounds are associated the chloroplast envelope where they affect its metabolism. As 13-hydroperoxide linolenic acid and allene oxide are both implicated in plant defence and are activated by O_3, they may be related to why chloroplast enzymes and mRNAs (specifically for Rubisco) are involved in O_3-induced injury.

The activation of passive Ca^{2+} influx would serve the same purpose as activation of the G-protein. The level of cytoplasmic Ca^{2+} would rise, and all else would follow. Plant exposure to O_3 activates passive Ca^{2+} influx, but Castillo & Heath (1990) demonstrated that *in vivo* exposure of common bean both inhibits the outward-directed ATP-requiring Ca^{2+} pump and increases the passive permeability of Ca^{2+}, which may be an alteration of the channel control. It was thought that the Ca^{2+} transporter system had a sensitive sulphydryl group, which, if oxidised, alters normal movements. Dominy & Heath (1985) showed that K^+-activated ATPase (involved in K^+ transport) was inactivated by *in vivo* exposure to O_3 and that too was traced to a sensitivity to sulphydryl. O_3-induced change in Ca^{2+} permeability is believed to be the trigger to most, if not all, wounding responses.

Stress-induced alterations in gene expression

A number of abiotic stresses have been described and investigated using molecular biology techniques. In most cases, stress causes alterations in protein synthesis: either a class of proteins has appeared or disappeared. However, when the levels of certain proteins change only slightly, it is likely that mRNA indicates an alteration of gene expression (Sachs & Ho 1986). In a number of stress responses, changes in protein synthesis can be traced back to regulation of DNA transcription. In some cases, control of gene expression under a number of stress conditions is post-transcriptional.

In comparison to other abiotic stresses, relatively few studies have addressed the qualitative and quantitative effects of air pollution on protein metabolism (see above and summarised in Harris & Bailey-Serres 1994). Those few studies suggest that the physiological and metabolic consequences of pollutant exposure may be mediated by altered gene expression. Air pollution-induced alterations in electrophoretically separated proteins have been reported for bilberry (*Vaccinium myrtillus* L.) chronically exposed to nitrogenous air pollutants (*e.g.* NO_2, SO_2, NH_3, urea and fertiliser dust, Pietila *et al.* 1990), corn (*Zea mays* L.) acutely exposed to SO_2 (Ranieri *et al.* 1990), Norway spruce chronically exposed to O_3 and acid mist (Schmitt & Sandermann 1990), common pea acutely exposed to O_3 (Beckett *et al.* 1989, Beckett *et al., 1990*) and wheat (*Triticum aestivum* L.) exposed to 200–400 nl $O_3 \cdot l^{-1}$ (Farage *et al.* 1991; Nie *et al.* 1993). Altered gene expression due to plant habituation to O_3 stress may be of adaptive significance. Habituated plants exhibit reduced signs of tissue injury with previous O_3 treatment (McCool *et al.* 1988).

Ecologists have likened leaf response to O_3 as 'early senescence' (Barth *et al.* 2004; Gielen *et al.* 2007). Early abscission and reduced Rubisco activity induced by O_3 is also common in leaf senescence. However, few investigators have done the necessary enzymatic measurements for long enough duration to show that these (and other) metabolic changes induced by O_3 are simply accelerated aging (He 2002).

Genetic diversity/population structure

Elimination of sensitive individuals is the first stage of natural selection (Bradshaw & McNeilly 1991). While the idea of natural selection induced by O_3 and related changes in natural plant communities was first proposed and demonstrated by Dunn (1959), the evolution of O_3 tolerance is not widely accepted. Heagle *et al.* (1991) were able to show adaptation of a white clover (*Trifolium repens* L.) population to elevated O_3 in just two growing seasons. Davison & Reiling (1995) were able to compare the O_3 resistance of common plantain populations grown from seed collected from the same sites over a period with increasing O_3. The two (independent) populations studied both increased O_3 resistance, consistent with selection for tolerance. Using random amplified polymorphic DNA primers, the latter populations were subsets of the earlier ones, supporting *in situ* evolution, rather than catastrophic loss and replacement of the populations (Wolff *et al.* 2000).

An example of change in the genetic make-up of natural ponderosa and Jeffrey pine populations was investigated in Sequoia National Park, CA (Staszak *et al.* 2007). Ozone concentrations were low initially, continuously measured from 1982 to present, and equalled those east of Los Angeles, with a lag of 30 years. In unmanaged natural populations, isozymes known to be relevant to O_3 tolerance were selected, and their level of heterogeneity was assessed in two species differing in sensitivity in three size/age classes: pole-sized (<35 years old), mature (80–120 years old) and old-growth (>200 years old). Genetic variation differed between pole-sized trees, with an increase in homozygosity relative to older tree size classes in the more sensitive species, ponderosa pine (Miller 1973), *versus*

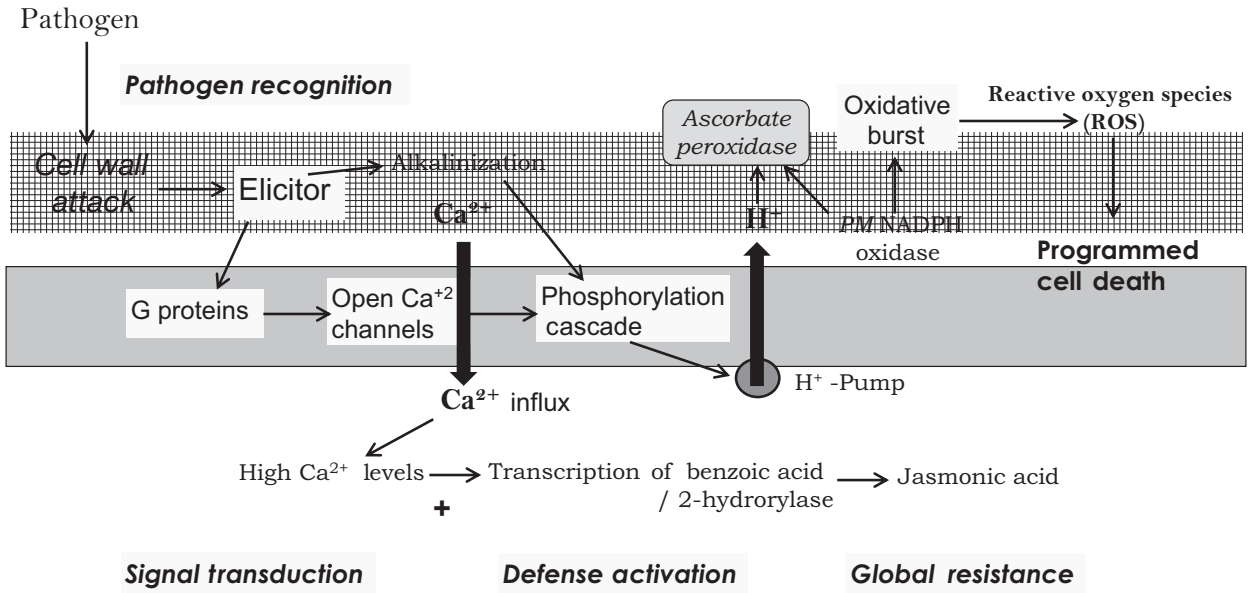

Fig. 4. Schematic diagram of the Pathogenic Response Sequence. The initial attack from the pathogen begins at the top left *via* a recognition of the proteins and wall fragments released by the attack. The initial response is due to a series of signal transduction events that alters the membrane and so alters the ionic balance across the membrane. This in turn generates activation of the events through protein cascades that generate reactive oxygen species (ROS) and lead to the global response of signalling molecules such as jasmonic acid and salicylic acid. Generation of ROS leads to localised cell death appearing as a sequence in senescence and activation of events far removed from the initial site (modified from Leon *et al.* 1995.).

an increase in heterozygosity (relative to older trees) in Jeffrey pine. Loss of genetic diversity in ponderosa pine implied loss of diversity in mature and old-growth trees contributing to the youngest generation. In atmospherically clean areas, the genetic diversity of ponderosa pine is greater than that of Jeffrey pine (Potter *et al.* 2013). Across anthropogenic gradients of O_3 concentrations in Wisconsin, foliar injury of trembling aspen was correlated to O_3 concentrations, but no change in gene frequency among populations was observed (Berrang *et al.* 1986, Berrang *et al., 1989*, Berrang *et al.* Berrang *et al.* 1991; Bell *et al.* 1991; Reiling & Davison 1992). Paludan-Müller *et al.* (1999) showed that Northwest European provenances of European beech were more sensitive to O_3 than were Southeast European provenances, which had experienced higher O_3 levels. However, if the southeastern provenance experienced more drought, these differences could be attributable to differences in O_3 uptake, not genetic change.

Changes in genetic diversity induced by O_3 is an area deserving additional research. Repeat assessment of genetic diversity over time from wild populations experiencing high O_3 concentrations, sampling genetic diversity along known O_3 gradients and the use of modern biotechnological approaches to characterise and quantify genetic diversity should all be useful approaches to test for O_3-induced selection for tolerance in natural ecosystems. Another important question to address is what plant traits were lost as a result of intolerance to O_3 exposure.

Natural selection for O_3 tolerance can also be facilitated by reductions in fitness, where fitness is defined by population size, reproductive proportion of the population, number of flowers and number of viable seeds produced (Harper 1977). The impacts of O_3 on reproductive development (reviewed by Black *et al.* 2000) can occur by influencing: (i) age of flowering, particularly in long-lived trees that often have long juvenile periods of early growth without flower and seed production; (ii) flower bud initiation and development; (iii) pollen germination and pollen tube growth; and (iv) seed, fruit or cone yields and seed quality.

Elevated O_3 decreased numbers of flower spikes and seed capsules per plant in common plantain (Reiling & Davison 1992; Pearson *et al.* 1996; Lyons & Barnes 1998). Similar responses were seen for mustard (*Brassica campestris* L.) plants exposed to a single dose of 100 nl $O_3 \cdot l^{-1}$ for 6 h (Stewart *et al.* 1996). Rapeseed (*Brassica napus* L.) exposed to a short duration of elevated O_3 increased flower bud abortion (Bosac *et al.* 1998). Floral initiation was delayed in O_3 sensitive dogbane (*Apocynum androsaemifolium* L.) grown under ambient O_3 in the eastern USA. In one of the few comparisons of whole plant O_3 sensitivities with that of male gametophytes, Hormaza *et al.* (1996) found a high correlation between O_3 sensitivity and pollen tube elongation, net photosynthesis and relative growth rates for six species of fruit tree. Together these studies suggest that O_3 can affect fitness of plants either by affecting the sporophyte or the gametophyte.

ORGANISMAL EFFECTS CASCADE TO COMMUNITY AND ECOLOGICAL PROCESSES

The well-known effects of O_3 exposure cascade into community- and ecosystem-level perturbations. Ozone exposure at moderate levels disrupts and alters community and ecosystem carbon, nutrient and water balance and dynamics. Ozone exposure reduces carbon acquisition, inhibits phloem loading (Grantz & Farrar 2000), lowers carbon allocation to roots (Andersen *et al.* 1991; Grulke *et al.* 1998; Andersen 2003), increases susceptibility to drought (Grulke *et al.* 2009),

increases successful bark beetle and wood borer attack (Preisler *et al.* 2017), weakens trees, increases probability of mortality and may increase susceptibility of air pollution-affected forests to wildfires (Grulke *et al.* 2009). Ozone-induced reduction in carbon acquisition also results in early leaf senescence, with fewer nutrients extracted before abscission, elemental imbalances in foliage, higher leaf turnover rates and loss of shaded foliage and branches, greater litter build-up and lower litter decomposability (Fenn & Dunn 1989).

Carbon dynamics

Ozone-induced disturbances in aboveground carbon and nutrient pools and dynamics cascade to belowground dynamics. Shifts in within-plant allocation affect carbon loss from the ecosystem in terms of respired carbon and leached aqueous dissolved organic and inorganic carbon. A biogeochemical model (BGC) was parameterised to capture long-term effects of O_3 exposure, N deposition and climate in a ponderosa pine-dominated, mixed conifer site in the western San Bernardino Mountains. The effect of simulated O_3 exposure resulted in faster production and turnover of foliage, and a shift in carbon from the canopy (15% reduction) to the forest floor (increase of 50–60%; Arbaugh *et al.* 1998). In this model, when O_3 exposure was combined with N deposition (*e.g.* in the western *versus* eastern San Bernardino Mountains), litter mass exponentially increased. Elevated O_3 exposure and N deposition over decades alters ecosystem macro- and micronutrient balances due to soil acidification and growth stimulation by excess N (Poth & Fenn 1998). Lower root mass and root carbohydrates (Grulke *et al.* 2001) can disrupt belowground community dynamics (Allen & Allen 2017).

Water dynamics

Ozone exposures large enough to affect assimilation also affect plant water balance. At moderate O_3 exposures, decreases in assimilation reduce stomatal conductance concomitantly following Farquhar & Sharkey (1982). At high O_3 exposures, decreases in assimilation are not necessarily followed by a timely reduction in stomatal conductance. Sluggish stomatal behaviour (Paoletti & Grulke 2005) has been demonstrated in a number of species (field study: California black oak, Grulke *et al.* 2005; chamber exposure studies: Handley & Grulke 2008; blue oak, Grulke *et al.* 2007; sensitive and tolerant common bean, Paoletti & Grulke 2010; Palmer amaranth, *Amaranthus palmeri* S. Watson, Paudel *et al.* 2016). The repercussion for sluggish stomatal behaviour is that under conditions favourable for assimilation, stomatal conductance may be limiting; under unfavourable conditions (such as high vapour pressure deficit), stomatal conductance is not decreased with assimilation (Patterson & Rundel 1993), more transpirational water is lost, and plant drought stress is more likely, especially in xeric microsites or during severe and/or prolonged drought conditions. In droughted Pima cotton (*Gossypium barbadense* L.) exposed to elevated O_3, the relative reduction in stomatal conductance was half that of well-watered plants with elevated O_3 and 20% that of well-watered plants grown in low O_3 in early to mid-morning (Grantz *et al.* (2015). Production in Pima cotton was correlated with early morning, not midday assimilation. Also, with short- or long-term acute O_3 exposure, stomatal closure is impaired at the end of the day, and stomata may remain partially open for up to 3 days (field study: ponderosa pine, Grulke *et al.* 2004; greenhouse study: California black oak, blue oak; Grulke *et al.* 2008). These demonstrations provide the mechanistic basis for increased transpiration, statistically linked to reduced stream flow in oak-dominated forests (McLaughlin *et al.* 2007a,b). However, stomatal sluggishness was not associated with increased transpiration at the whole tree level in an O_3-sensitive poplar due to stomatal closure and reduction of transpirational leaf surface area by premature leaf abscission (Hoshika *et al.* 2012).

Nutrient dynamics

Much of the research on the effects of pollutants on nutrient dynamics in ecosystems has focused on acidic deposition effects. In areas with moderate and high pollutant deposition, N enrichment cannot be separated from O_3 exposure, and reviews of the effect of acidic (and N) deposition on ecosystem nutrient dynamics are important to consider within this context (Binkley 1992; Fenn *et al.* 1998, Fenn *et al., 2003*). For example, at moderately high pollution sites, foliar N content is higher than that at a lower pollution exposure site, but so is potassium, magnesium and iron (Poth & Fenn 1998). At low and moderate O_3 exposure (<200 nl $O_3 \cdot l^{-1}$ cumulative over the active growing season), N deposition is low and its effect may be negligible as a direct contributing factor to ecosystem nutrient dynamics.

Ozone exposure imposes a stress on the nutritional status of plants. The largest annual nutrient input to the ecosystem is from foliar and root turnover. Birch grown in highly fertilised conditions exhibited higher leaf turnover with O_3 exposure. Leaves were formed faster but were abscised prematurely, while those in low nutrient conditions had longer leaf retention but higher respiration (Maurer & Matyssek 1997). Ozone injury may not itself induce leaf abscission, but rather abscission is coordinated according to nutrient status.

Excision of plant parts and whole plant mortality are potentially much larger, but syncopated, ecosystem inputs. Ozone exposure reduces nutritional content of foliage because of *in situ* degradation of chlorophyll. Reconstruction of chlorophyll may be limited by nutritionally poor soils or low soil moisture, or reduced root mass from the O_3 exposure itself. Foliar exposure to O_3 may also increase leaching of nutrients (Kerstiens & Lendzian 1989). Ozone exposure promotes early senescence of foliage (Miller & Ederman 1977; Heath & Taylor 1997) with higher nutrient content than if excised later in the growing season (Poth & Fenn 1998). Leaf litter in high O_3 exposure sites has lower decomposability (Fenn & Dunn 1989) and so builds up. The accumulation of soil organic matter from increased leaf litter alone, even without N deposition, can lower soil pH (Binkley 1992). Lower soil pH can promote loss of nutrients from the system, further reducing nutrient availability to the plant. Complex organic compounds in decomposing litter may also tie up nutrients and render them less available to plants. A biogeochemical model was used to understand the potential role of prescribed fire in influencing ecosystem N residence time (Gimeno *et al.* 2009). Even if N deposition ceased and prescribed fire were applied every 7 years, it would take over 100 years to reduce soil N to pre-pollution levels (Gimeno *et al.* 2009).

Although trees dominate nutrient cycling in forest ecosystems, understorey plants also contribute to nutrient cycling in the ecosystem through litter decomposition and mycorrhizal associations (Kozlowski *et al.* 1991). The direct effect of O_3 exposure on belowground nutrient dynamics is poorly understood. In the forest stand most impacted by pollution in the San Bernardino Mountains, CA, fine root biomass was significantly reduced in ponderosa pine due to the combined effects of elevated O_3 and N deposition (Grulke *et al.* 1998). In moderately high O_3 exposure sites, mycorrhizal colonisation of roots was lower in Polish forests (Kieliszewska-Rokicka *et al.* 1998). Reduction in fine root systems (fine roots + mycorrhizal tips + extramatrical hyphae) could alter ecosystem nutrient dynamics in fundamental ways (Allen & Allen 2017). Liu *et al.* (2015) demonstrated deleterious legacy effects on plant production due to long term O_3 exposure effects on soil microbiota.

Community composition

A few of the case studies above describe shifts in community composition (Davison & Barbo 1998; Miller *et al.* 1989; Arbaugh *et al.* 1998) from prolonged, elevated O_3 exposure. In an experimental exposure of an early successional forest community in the southeastern US, Barbo *et al.* (1998) found changes in species performance, canopy structure, species richness and diversity consistent with the view that O_3 can shift species dominance. Genetic selection against sensitive species (*e.g.* favouring Jeffrey over ponderosa pine in the Sierra Nevada; Patterson & Rundel 1993; Staszak *et al.* 2007; trembling aspen *versus* paper birch; Karnoski *et al.* 2003) will likely lead to shifts in community or stand composition. McBride *et al.* (1999) applied a life table projection to tree demographic data taken across the anthropogenic pollution gradient in the Transverse Range, southern California to forecast species composition. By 2074, ponderosa pine nearly disappears in all tree age classes, and the community is dominated by California black oak, followed by incense cedar and sugar pine (*Pinus lambertiana* Douglas). However, in a complex calculation of O_3 flux of whole trees, Norway spruce was no less sensitive than European beech (Nunn *et al.* 2006). No other flux analysis has been conducted to properly assess among-species O_3 sensitivity in these terms, considering differences in stomatal conductance, foliar longevity and effects of lower canopy shading. Based on sensitivity determined in exposure chambers, the analysis of Nunn *et al.* (2006) and the flux approach in general (Matyssek *et al.* 2007b), O_3 is likely to change our forecasts for species survival and future community composition. Process models aid in interpreting O_3 exposure effects at the ecosystem level (Büker *et al.* 2015; DO_3SE).

Species diversity in the understorey can be quite high, making studies of O_3 effects on understorey community dynamics challenging. However, some attempts to quantify understorey responses have been made, ranging from simple ranking of relative sensitivity of component species using visible symptoms (Treshow & Steward 1973; Temple 1999) to complex measures of community structure and composition (*e.g.* McBride & Miller 1999; Karnosky *et al.* 2001). Ozone-induced changes in canopy species (ponderosa pine, Andersen *et al.* 2001; loblolly pine, Barbo *et al.* 2002) can also influence understorey species assemblages. In coastal sage scrub, Westman (1979) attributed and correlated low cover and species diversity with high O_3 exposures. In a Mediterranean pasture, plants in the *Fabaceae* family were more symptomatic than those of *Poaceae*, with some species responsive to current and elevated O_3 levels, and others only at elevated O_3 concentrations (Bermejo *et al.* 2003).

Trophic interactions/food webs

Although insects and diseases are dynamic components of forest ecosystems, trees can be more susceptible to outbreaks due to the presence of multiple stressors, such as drought and pollutant exposure (summarised in Grulke *et al.* 2009). Ozone can have direct effects on diseases. Oxidant exposure predisposed ponderosa pine to the root pathogen *Fomes annosus* (James *et al.* 1980). One of the first descriptions of inter-trophic level interactions in natural communities was the O_3-induced predisposition of ponderosa pine to attack by bark beetles (Cobb *et al.* 1958; Stark *et al.* 1968; Stark & Cobb 1969). Trees exposed to oxidant injury had lower resin flow and exudation pressure. Also, several attributes associated with tree defence against beetle attack were compromised by oxidant exposure, including sapwood, phloem moisture content and phloem thickness (Pronos *et al.* 1999).

Bark beetle predators and parasitoids had higher densities in healthy ponderosa pine trees than did trees with foliar O_3 symptoms (Dahlsten *et al.* 1997). These data suggest that O_3 exposure influenced host suitability for natural enemies of the bark beetles. Similar findings were presented by Percy *et al.* (2002) for aphids, whose abundance increased in young trembling aspen stands exposed to elevated O_3: the levels of predators of aphids (ladybirds, lacewings, spiders and parasitoids) significantly decreased under elevated O_3. Other biochemical changes relevant to insect infestation in O_3-exposed plants are reviewed in Riemer & Whittaker (1989). The timing and nature of leaf quality changes associated with O_3 exposure and other environmental stressors, and the timing of attack and subsequent use of the tree by insects, may preclude identification of cause and effect in field studies. The complex nature of the effects of O_3 on trophic interactions and food webs calls for additional basic research and modelling.

As yet, there have been no comprehensive studies on the effects of O_3 on structural or functional components of soil food webs (Andersen 2003). However, Phillips *et al.* (2002) found evidence for changes in the bacterial and fungal biomass belowground in trembling aspen and trembling aspen/paper birch stands exposed to elevated O_3. A subsequent study showed that O_3 decreased cellobiohydrolase activity in the soil microorganisms, driving the change in the microbial community (Larson *et al.* 2002).

No publications were discovered on O_3 as a limitation to ecosystem extent. Effects at the landscape level may be significant if O_3-sensitive species such as western yellow pine (ponderosa and Jeffrey pine) are lost in great numbers during the last decade in the Sierra Nevada and the Transverse Range of California (Preisler *et al.* 2017). Multi-trophic impacts of elevated O_3, and elevated O_3 and CO_2 (Karnosky *et al.* 2001) in simplified stands have been shown to have cascading effects on forest composition and are likely to play out in future, unmanaged ecosystems.

Natural disturbances

There has been little research on the susceptibility of O_3-impacted plants or communities to abiotic natural disturbances. High O_3 exposure and N deposition significantly reduced root mass in the western San Bernardino Mountains, east of Los Angeles, California (Grulke *et al.* 1998), which could make trees more susceptible to windthrow. However, because this highly impacted forest had an already excised lower canopy, shaded primary branches due to air pollution exposure and the lowest live branch was high, and the Old Fire (October 2003) converted from a whole-crown chaparral fire to a ground fire when it burned into one of the most pollution-impacted forests.

There have been enough ecophysiological studies to state that O_3 predisposes plants and communities to drought stress. Under moderate drought stress, Norway spruce trees grown under elevated O_3 had higher stomatal conductances and transpiration (Karlsson *et al.* 1995). Pearson & Mansfield (1993) showed that successive O_3 episodes disrupted stomatal function, making European beech seedlings more susceptible to drought. Similarly, there have been enough studies to state that O_3 predisposes plants and communities to low winter temperature events. Trees living near the limits of their freezing tolerance range may be particularly susceptible to predisposition of freezing injury by O_3 exposure (Sheppard *et al.* 1989; Prozherina *et al.* 2003). The influence of elevated O_3 on freezing tolerance was carried over from summer to winter for Sitka spruce (*Picea sitchensis* (Bong.) Carrière; Lucas *et al.* 1988), Aleppo pine (*Pinus halepensis* Mill.; Wellburn & Wellburn 1994) and red spruce (Waite *et al.* 1994). However, isolating the role of elevated O_3 from elevated CO_2, N deposition or other phenomena that may affect senescence and winter hardiness is difficult at best (Skärby *et al.* 1998).

CONCLUSIONS

Tropospheric O_3 is an important stressor in natural ecosystems, with well-documented impacts on soils, biota and ecological processes. In turn, the effects of O_3 on individual plants and processes are scaled up through the ecosystem through effects on carbon and nutrient dynamics, and hydrology. Ozone can impact the occurrence and impact of landscape-level natural disturbance.

There are two separate paths of investigation into O_3 effects: controlled experimental studies and field-level empirical studies. The former are carried out under carefully controlled conditions and serve to lead to understanding of the fundamental or basic processes which occur. Here the plants could be grown under optimal conditions, except for the addition of atmospheric oxidants. Primary research may be able to suggest the most pressing field investigations to be conducted. In field investigations, the focus should be on species that dominate ecological processes in unmanaged ecosystems, accompanied by environmental monitoring. The more completely the environmental conditions are described (soil characterisation, water regimes, radiation loads, temperature and humidity, wind dynamics, air chemistry), the better the understanding and modelling of the biological response will be.

In a sense, the model for O_3 injury to a single plant is simple: (i) O_3 enters the stomata, governed by normal gas flow; (ii) O_3 reacts with water at the cell membrane level and forms hydrogen peroxide; (iii) hydrogen peroxide reacts with apoplastic antioxidants and decomposes, resulting in no change, except for the need for more reducing power (energetically costly); (iv) if excess hydrogen peroxide is not eliminated, the cells within a small region of tissue react as if a pathogen has attacked (hypersensitive response) and activate protection reactions resulting in necrosis. Step (iii) is an integrative response of all antioxidants, with reducing power coming from within the cell. Step (iv) is also an integrative response involving many of the cell's components. Loss of leaf area through this process, and of reductive power, results in a loss of plant productivity. Enough loss, and the plant weakens and either eventually succumbs (slowly) or becomes susceptible to attack by other biological or deficiency agents (quickly).

There is some evidence that O_3 may also directly affect guard and subsidiary cells (Kaiser & Paoletti 2014), reducing stomatal responsivity to changes in environmental conditions. No complete model has been developed for all of the processes and repercussions of O_3 exposure to plants. The collection of responses is complex: once a signal is detected (*e.g.* membrane protein), other signals are generated through a signal transduction pathways (Singh *et al.* 2002). This is a biological shotgun approach: some of the generated signals are limited by other signals from still other environmental and developmental cascades. Some signals are not limited but rather modified – weakened or strengthened. Gradually another process is turned on. It is impossible to predict since that depends upon all the signals and how they are integrated throughout the cascade. At this point we do not know enough about these integrative processes. Ultimately, we may be able to understand them since progress in underlying genetic controls is improving rapidly.

Of particular note, the pathogen response spreads in at least two ways: as an agent (hydrogen peroxide) and as a signal to antioxidants, such as ascorbate and glutathione. There are indications that hydrogen peroxide can be weakened by lowering its concentration (reactions with superoxide dismutase, ascorbate or glutathione) or eliminated by decomposition (peroxidases or catalase) (Evans *et al.* 2005; Willems *et al.* 2016). Any change in its composition then affects what other signals are important or used 'downstream' from this reaction. It is not understood if the pathogen response events generated by O_3 exposure can influence real pathogen attack responses that occur later: is the response to the real pathogen lessened to the detriment of the plant? The detected changes from research to date suggest how these integrated systems work.

Systematic injury surveys demonstrate that foliar injury occurs on sensitive species throughout the globe. However, deleterious impacts on plant carbon, water and nutrient balance can occur without visible injury. Because sensitivity to O_3 may follow coarse physiognomic plant classes, species' sensitivity is perhaps better assessed by cumulative O_3 uptake and defence capacity.

Investigations of the radial growth of mature trees, in combination with data from many controlled studies with seedlings, suggest that ambient O_3 is currently reducing the growth of mature trees in some locations. Modelling efforts demonstrate that modest effects of O_3 on growth may accumulate over time, may interact with other stresses and alter species composition. Based on ASPEN-FACE with experimentally elevated O_3, and DUKE-FACE conducted concurrently with moderate ambient elevated O_3 and modelling efforts (Büker *et al.* 2015), the growth

stimulatory, elevated CO_2 and growth suppressing, elevated O_3 interactions suggest further complexities of response that may only be resolved by re-calculating or estimating doses of prior experiments, and using physiological process-based models to suggest the possible balance of their effects. Further, results of process-based models need to be incorporated into dynamic vegetation models (MC2, ED2) in order to inform carbon, nutrient and hydrologic dynamics and balance at the landscape and global level into the future. Due largely to increases in temperature, O_3 concentrations are projected to increase over a broad portion of the U.S. by the end of the century (USGCRP 2018).

Knowledge for assessing the range of ecological effects of O_3 on natural ecosystems is growing, but there remain significant uncertainties regarding O_3 effects at the ecosystem level. There is a need for the following ecosystem-level responses:

- *Ecosystem processes*: Very little is known about the effects of O_3 on water, carbon and nutrient cycling at the plant community, watershed and landscape level. Effects on belowground ecosystem processes in response to O_3 exposure alone and in combination with other stressors and growth enhancers are critical to projections at larger scales. Although O_3 effects on belowground biomass have been described, little is known about the effects of O_3 on soil food webs and how this affects species diversity (Andersen 2003).
- *Biodiversity and genetic diversity*: O_3 impacts on genetic diversity in natural ecosystems have been largely correlational (Pitelka 1988; Winner *et al.* 1991; Davison & Barbo 1998; western yellow pine, Staszak *et al.* 2007), although they have been assessed in some experimental exposures (aspen clones, Karnosky *et al.* 2001). These studies could be strengthened by using modern molecular methods to quantify impacts on changes in underlying diversity. Studies of competitive interactions under elevated O_3 are needed (Miller *et al.* 1989; Laurence *et al.* 2003), using both temporal and spatial variability to detect change. The translation of O_3 exposure into dose to better evaluate plant and system response to O_3 is also needed.
- *Natural ecosystem interactions with the atmosphere*: Little is known about feedbacks between O_3 and climate change on volatile organic compound (VOC) production, which in turn, could affect O_3 production (Fuentes *et al.* 2001). Ozone-induced elevated VOC production may also play a role in increased insect herbivory (Dicke & Baldwin 2010; Holopainen & Gershenzon 2010). At moderate to high O_3 exposure, uncoupled A and g_s response and sluggish stomatal behaviour may affect tree water balance, and if the sensitive tree species are dominant, hydrologic balance at the watershed and landscape level could be affected (*e.g.* McLaughlin *et al.* 2007b). This has not been addressed in any model to date because if O_3 exposure effects are included in the effort, they have been assumed to have a linear relationship.
- *Other interactive environmental components*: Interaction with other environmental components (*i.e.* increased temperature, increased atmospheric CO_2, N deposition, drought, etc.) or with various biotic stressors are needed to better predict complex interactions likely in the future (Laurence *et al.* 2003). Whether O_3 will negate the positive effects of increasing CO_2 on plant carbon and water balance is not yet known for sufficient species in managed or unmanaged ecosystems, nor is it known if these effects will scale up to the landscape level. Moderately high O_3 concentrations may increase tree susceptibility to drought, increasing successful insect attack (Skärby *et al.* 1998; Grulke *et al.* 2009).
- *Fitness*: The impacts of O_3 on reproductive processes and reproductive development under realistic field or forest conditions are needed (Black *et al.* 2000).
- *Conduct studies on mature trees*: The vast majority of O_3 studies of trees have been conducted with young, immature trees and in trees that have not yet formed a closed canopy. Questions remain as to the comparability of O_3 effects on juvenile *versus* mature trees, and on trees grown in the open *versus* those in a closed forest canopy in a competitive environment (Chappelka & Samuelson 1998; Kolb & Matyssek 2001; Samuelson & Kelly 2001).
- *Scaling up*: Scaling the effects of O_3 from the responses of single or a few plants to effects on communities, ecosystems and landscapes is complex and will require more data in unmanaged ecosystems as well as extensive modelling efforts. Although atmospheric O_3 concentrations can be detected with some satellite-based imagers, it is unlikely that foliar O_3 injury or reductions in NDVI (Normalised Difference Vegetation Index) could be distinguished from other environmental stressors (such as oxidative stress from drought or root disease) using remote sensing.
- *Ecosystem services*: Assessments of O_3 impacts on ecosystem services are needed. An approach for this has been developed by Srivastava and Nowak (*in progress*).
- *Leaf to landscape linkage*: Linking research of basic physiological processes with scaled, landscape-level observations is critical and needed. Understanding the biochemical reactions affected by O_3 and their repercussions on downstream plant processes and understanding the level and uptake of O_3 over a given time period which causes injury that affects higher-scale processes are essential. Biochemical research can be undertaken in laboratory settings and incorporated into our understanding of plant processes. Exposure indices and uptake should be conducted in FACE experiments designed to investigate O_3 interactions with other growth stressor enhancers, and along 'natural' gradients in environmental stressors, including elevated O_3. The technology is well developed for both delivery of gases and monitoring of physiological processes.

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