

## Review

# Ozone effects on forests

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Accepted 31 December, 2012

**Tropospheric ozone concentration has been rising in the last decades, due to industrial and other human activities. For plants, ozone constitutes one of the most damaging air pollutants. Main effects of ozone on forest species are reviewed: visible symptoms caused by acute exposure at the anatomical, structural and metabolic level, and the long run effects on growth and development derived from chronic exposure. Particular attention is given to photosynthesis and the effects on stomatal functioning, as major ozone injuries are inflicted to the plant after entering through the stomata. Plant detoxification capacity, carbohydrate allocation, growth and development are also revised, as well as the effects at the ecosystem level, defence mechanisms of plants against ozone, and their sensitivity and tolerance. The rising problem of tropospheric ozone contamination should awaken the international awareness and measures should be taken to control ozone atmospheric levels considering their transnational implications.**

**Key words:** Abiotic stress, atmospheric pollution, climatic change, environmental pollution, forest decay, forest ecosystems, forest species, ozone, reactive oxygen species, tropospheric ozone.

## INTRODUCTION

The ozone (O<sub>3</sub>) layer that is formed in the stratosphere by ionisation of oxygen absorbs most ultraviolet radiation coming from the sun, thus protecting life from an excess of high energy radiation harmful to living organisms. Thinning of this stratospheric layer (between 20 and 30 km altitude) is the cause of more ultraviolet radiation passing through and producing tropospheric ozone. Most atmospheric ozone (ca. 90%) is stratospheric, while the tropospheric ozone concentration is usually small, in the order of a few ppb (Seinfeld and Pandis, 2006).

The increment of tropospheric ozone concentration, dispersion and effects have been related the industrial activities (Borell et al., 1997; Martin et al., 1991; Millán et al., 2000). The reaction of nitrogen oxides (NO<sub>x</sub>) with ultraviolet light, oxygen and exhaust gases generates O<sub>3</sub>, which adds to that brought down from the stratosphere by

vertical winds produced during electrical storms. Ozone concentration is minimal by night but builds up to phytotoxic levels in the atmosphere during calm, warm, sunny weather when pollutants accumulate in stagnant air. This situation is typical of spring and summer days. Accumulation also occurs during atmospheric inversions in valleys and basins. The combination of these processes contributes to maintain a basal concentration of about 20 to 45 ppb (nmol/mol) in intermediate latitudes of the northern hemisphere. The concentration of O<sub>3</sub> has been registered since the end of the XIX century, indicating an increment at a rate between 1 and 2.5% annually (Jonson et al., 2006).

In Japan, concentrations above 100 ppb have been recorded in both urban and mountainous areas (Watanabe et al., 2012). In a polluted atmosphere in which emissions of NO<sub>x</sub> and volatile organic compounds feed the photochemical reactions, O<sub>3</sub> concentration may reach levels as high as 200 to 400 ppb (Emberson et al., 2003; Fiala et al., 2003). It has been stated that ozone is the most damaging air pollutant to plants (Gimeno et al., 1995; Peñuelas et al., 1999), cultivated crops as well as

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forest vegetation (Treshow and Stewart, 1973) and numerous shrub species (Findley et al., 1997a). Nowadays, susceptibility to ozone is a criterion to choose a plant crop, especially for ornamental woody plants (Sacramento Tree Foundation, 2010).

The aim of this review was to emphasize the importance of tropospheric ozone contamination, underlining its effects on forests and natural ecosystems. Until recently, most research on ozone damage was focused on commercial crops, and the effects on forests were not considered as important as those caused by pests and diseases. We try to give a global scope of ozone damage on plant growth and development, from the physiological to the ecosystem level. In a context of global climate change, the interaction of ozone with increasing CO<sub>2</sub> concentrations and with the water balance of plants should increase public awareness of the problem.

## **SYMPTOMS OF OZONE-DERIVED TOXICITY IN WOODY PLANTS**

Symptoms of ozone damage may be visible symptoms such as brown or red-brown punctures or chlorotic bands in the leaves, caused by acute, high ozone concentrations during short periods, or premature leaf senescence and reduced growth and productivity, usually caused by moderate, chronic concentrations during long periods. Morpho-anatomical symptoms include tissue collapse, interveinal necrosis, and markings on the upper surface of leaves known as stipple (numerous tiny spots of different pigmentations), flecking (silver or bleached straw white spots), mottling (irregular blotches of green, light green, and yellow), yellowing, bronzing, or bleaching. Ozone-affected leaves may show the most severe injuries within the palisade tissue (Paoletti et al., 2009). The main way of O<sub>3</sub> entrance into the plant is the stomata. Certain species are sensitive to very low levels (0.05 ppm). Furthermore, ozone produces other toxic compounds, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>), atomic oxygen (O), and hydroxyl radical (·OH). Some visible symptoms frequently attributed to O<sub>3</sub> are black, reddish or brownish spots in the limb (Fumagalli et al., 2001; Hayes et al., 2007). In a more advanced stage, old leaves appear brilliant white mottled. Conifers frequently show a yellow to brown mottling and tipburn, pink spots or a yellow to brown or orange-red flecking and banding of the needles (Anttonen and Kärenlampi, 1996; Baker and Allen, 1996; Vollenweider et al., 2003).

On the other hand, many toxic effects caused by ozone to some woody plants, including premature aging, may occur in the absence of visible symptoms (Günthardt-Goerg, 1996; Günthardt-Goerg et al., 1996; 1997; Vollenweider et al., 2003). Foliar damage attributed to tropospheric ozone was first observed to be phytotoxic to

*Vitis vinifera* in southern California in the 1950s (Richards et al., 1958) and in the 1960s, "X" disease of ponderosa pines within the San Bernardino Mountains was likewise determined to be due to O<sub>3</sub> (Karnosky et al., 2007). Foliar O<sub>3</sub> symptoms have been verified for seedlings under controlled chamber conditions, but due to complex interactions within forest stands, evidence of similar losses within mature tree canopies remains difficult to find. Investigations on tree growth, O<sub>3</sub> flux, and stand productivity are being conducted along natural O<sub>3</sub> gradients and in open-air exposure systems to better understand O<sub>3</sub> effects on forest ecosystems (Karnosky et al., 2007).

In ozone affected plants, stomata lose turgor and close. Toxic radicals alter the membrane permeability, chloroplasts disaggregate and the whole metabolism is affected. Some species, e.g. poplars, are especially susceptible, and in ash, although no visual symptoms are obvious, the epidermis may collapse and chloroplasts may start to degenerate. Ozone toxicity is determined by its absorption process through the stomata and the plant mechanisms of detoxification and repair (Massman, 2004; Wieser and Matyssek, 2007). In addition to the external O<sub>3</sub> concentration, the uptake of O<sub>3</sub> by plants is primarily influenced by stomatal conductance ( $g_s$ ), which is strongly dependent on climatic conditions, varying between species and site characteristics (Manzanera and Martinez-Chacon, 2007), the position of leaves within the canopy as well as leaf and plant age (Matyssek et al., 2004). However, ozone concentration in the intercellular space is very low, probably because it is decomposed after uptake (Laisk et al., 1989).

## **OZONE EFFECTS ON PHOTOSYNTHESIS**

Ozone has a marked effect on photosynthesis, limiting the net assimilation rate and the chlorophyll content. However, the major alterations take place in the electron transport chain and in the carbon fixation role of ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco). In ponderosa pine needles following exposure to ozone, an observed broadening of the chlorophyll absorption band has been interpreted as a consequence of chloroplast disorder and granulation of the thylakoid membranes (Ustin et al., 2009). Indirectly, stomatal guard cell function is impaired by ozone. Photosynthesis as a whole is variably affected by limiting the net assimilation rate, the chlorophyll content, the electron transport chain, the carbon fixation role of Rubisco and the stomatal guard cell function. These effects also depend on the genotype and the stage of development. Furthermore, defence mechanisms are weakened and ozone-killed tissues are readily infected by certain fungi, decreasing the detoxification capacity of the plant and consequently, increasing damage (that is, Massman, 2004). Greitner and Winner (1989) demonstrated that ozone reduced the

photosynthetic rate in *Alnus frangula* and observed that the plant cells of the root nodules were damaged, but not those of the *Frankia* symbiont. Alterations of photosynthesis vary depending on the species, clone or ecotype and developmental stage.

Normally, woody perennials are more tolerant than deciduous broadleaved species (Calatayud et al., 2010). Another general rule is that more tolerant species to other types of stress, for example hydric stress, are also more tolerant to ozone. This occurs in mediterranean sclerophyllous species, such as *Quercus ilex* (Calatayud et al., 2010) and *Pinus halepensis* (Alonso et al., 2001). Other cases of intraspecific diversity in the capacity of response to ozone absorption have been tested in long run Open Top Chamber (OTC) experiments with two ecotypes of *Quercus coccifera* (Elvira et al., 2004), where one-year old plants of ecotype *garriga* show a decrease in net assimilation and  $g_s$  for two years, as compared to plants of other ecotype. Also, younger leaves show less damage and less chlorophyll destruction than mature leaves (Broadmeadow and Jackson, 2000) and a greater capacity of detoxification (Alonso et al., 2001; Massman, 2004).

## EFFECTS ON GROWTH AND DEVELOPMENT

Plant growth is often stunted (Leisner and Ainsworth, 2012). Findley et al. (1997b) demonstrated that ozone concentrations below the sensitivity threshold to cause visible foliar symptoms nevertheless cause growth and flowering drop in *Buddleia davidii*. Diameter growth and size-growth relationships are also affected in spruce and beech (Pretzsch and Dieler, 2011). Root development is inhibited by ozone (Bassirirad, 2000) and it has been postulated that ozone may cause greater and earlier disruption of below-ground growth with long-term consequences for productivity. As a result the hydraulic capacity to provide the transpiring shoots with water is reduced. This reduction in root capacity could reduce photosynthesis and plant water use. Total root biomass is reduced, the fraction of plant biomass in root tissues declines, and the number and branching patterns of roots is altered by ozone. Root dry weight show a tendency towards reduction in *Q. pyrenaica* plants fumigated with 30 ppb ozone ( $-45\%$ ,  $p < 0.1$ ), and biomass partitioning was significantly altered in this species: reduction in root growth was stronger than reduction in above-ground biomass (13%); thus, above-ground biomass/below-ground biomass ratio increased significantly in this species (50%), but other oak species were not so sensitive (Calatayud et al., 2011).

Ozone effects on roots or on the above-mentioned ratio have been reported as a consequence of reduction in  $CO_2$  assimilation but also of photo-assimilate allocation from source tissues of the leaves to sink tissues in the roots (Andersen, 2003). Under natural conditions, root re-

duction and changes in biomass partitioning in tree species may reduce resistance to wind throw (Broadmeadow and Jackson, 2000). In many species, it is expected that ozone-driven severe symptoms and strong effects on photosynthesis are parallel to the highest biomass reductions. However, effects at leaf-level, and especially visible injury, are frequently uncoupled with growth or biomass reductions: the latter effects can be limited if, for example, leaves become affected toward the end of the growing season, when growth has already stopped or is reduced (Novak et al., 2007), or by compensatory responses of younger or non-affected leaves.

Present tropospheric ozone concentrations and those projected for later this century are toxic to trees and have the potential to reduce the carbon sink strength of these forests (Wittig et al., 2009). Current ambient  $O_3$  (40 ppb on average) significantly reduced the total biomass of trees by 7% compared with trees grown in charcoal-filtered controls, which approximate preindustrial ozone concentration in the atmosphere. Above and belowground productivity were equally affected by ambient levels in these studies. Elevated concentrations of 64 ppb reduced total biomass by 11% compared with trees grown at ambient levels, while an elevated  $O_3$  concentration of 97 ppb reduced total biomass of trees by 17% compared with charcoal-filtered controls. The root-to-shoot ratio was significantly reduced by elevated  $O_3$  amounts, indicating a greater sensitivity of roots to this contaminant. At elevated  $O_3$  concentrations, trees had significant reductions in leaf area, Rubisco content and chlorophyll content, which may underlie significant reductions in photosynthetic capacity. Trees also had lower transpiration rates, and were shorter in height and had reduced diameter when grown at elevated concentrations.

Further, at elevated tropospheric  $O_3$ , gymnosperms were significantly less sensitive than angiosperms. Taken together, these results demonstrate that the carbon-sink strength of northern hemisphere forests is likely reduced by current  $O_3$  and will be further reduced in future if  $O_3$  amount rises. This implies that a key carbon sink currently offsetting a significant portion of global fossil fuel  $CO_2$  emissions could be diminished or lost in the future (Wittig et al., 2009). Radial growth and structure of five 5-year-old trembling aspen (*Populus tremuloides*) clones and the wood characteristics of paper birch (*Betula papyrifera*) were affected by the interaction of ozone in an atmosphere with elevated concentrations of  $CO_2$  (Kostiainen et al., 2008). Material for the study was collected from the Aspen FACE (Free-Air  $CO_2$  Enrichment) experiment in Rhinelander, WI, where the samples had been exposed to four treatments: control, elevated  $CO_2$  concentration (560 ppm), elevated  $O_3$  concentration (1.5 times ambient) and their combination for five growing seasons. Wood properties of both species were altered in response to exposure to the treat-

ments. Ozone also may cause changes in flowering timing and less flower and fruit production (Hayes et al., 2012; Leisner and Ainsworth, 2012). Seed germination rate may be reduced, as well as pollen germination and growth, as it has been observed in *Pinus strobus* (Benoit et al., 1983), *Prunus*, *Malus* and *Pyrus* (Black et al., 2000; Hormaza et al., 1996).

## EFFECTS AT THE ECOSYSTEM LEVEL

Ozone weakens forest plants, which become more susceptible to drought and diseases. In a climatic change context of CO<sub>2</sub>-rich atmosphere, it is expected that stomatal regulation should be difficult for ozone absorption. However, experiments combining both gases show a high variability in response (Fiscus et al., 2005). After four years of experiments using an open-air exposure system, trying to assess the impact of elevated atmospheric CO<sub>2</sub> and O<sub>3</sub> on the O<sub>3</sub>-sensitive species trembling aspen (*Populus tremuloides*) and paper birch (*Betula papyrifera*), as compared to the O<sub>3</sub>-tolerant species sugar maple (*Acer saccharum*), the responses to these interacting greenhouse gases have been remarkably consistent in pure aspen stands and in mixed aspen/birch and aspen/maple stands, from leaf to ecosystem level, for O<sub>3</sub>-tolerant as well as O<sub>3</sub>-sensitive genotypes and across various trophic levels. These two gases act in opposing ways, and even at low concentrations (1.5 times ambient, with ambient averaging 34-36 ppb during the summer daylight hours), ozone offsets or moderates the responses induced by elevated CO<sub>2</sub>. After three years of exposure to 560 µmol/mol CO<sub>2</sub>, the above-ground volume of aspen stands was 40% above those grown at ambient CO<sub>2</sub>, and there was no indication of a diminishing growth trend. In contrast, O<sub>3</sub> at 1.5 times ambient completely offset the growth enhancement by CO<sub>2</sub>, both for O<sub>3</sub>-sensitive and O<sub>3</sub>-tolerant clones (Karnosky et al., 2003).

More recently, leaf biomass production was monitored in the same aspen, birch and maple stands for seven years, concluding that the overall effect of elevated ozone was to decrease leaf mass by 13%. Interactions with CO<sub>2</sub> concentration, forest community composition and stand development process were observed. Ozone also retarded nitrogen cycling (Talhelm et al., 2012). Another good example of ozone effects at ecosystem level is the San Bernardino Mountains forest, California, formerly covered by *Pinus ponderosa* and *P. jeffreyi* (Arbaugh et al., 1998). Those pines lose foliage and vigour, and were attacked by bark beetles, due to drought and ozone-driven weakening. Other ozone-tolerant species outcompeted both pines and the species composition changed. Mediterranean plants are often adapted to different oxidative stress factors (e.g., high temperature, strong sun-light and drought) that can make them more tolerant to ozone stress, as molecular responses to all

these stresses may be convergent (Bussotti, 2008; Calatayud et al., 2010; Pell et al., 1997). Overall, the results recorded until now are consistent with previous studies comparing related evergreen and deciduous species, which showed a much higher tolerance in evergreen species (Calatayud et al., 2010).

Ozone may influence chemical processes of litter and organic matter decomposition with impact on soil microorganisms and roots, as observed in ponderosa pine (Olszyk et al., 2001). Furthermore, some species show natural selection responses in sensitivity to ozone (Berrang et al., 1991). Interspecific competence may also be altered, as leguminous plants in general are more sensitive to ozone. This has a potentially negative effect on nitrogen fixation (Andersen, 2003).

## DEFENCE MECHANISMS OF PLANTS AGAINST OZONE

Reactive oxygen species formation takes place in normal plant metabolism by partial reduction of molecular oxygen. When O<sub>2</sub> accepts one or two electrons, produces superoxide (O<sub>2</sub><sup>-</sup>) or peroxide (O<sub>2</sub><sup>2-</sup>) anions, or the hydroxyl radical (·OH), which are dangerously reactive, oxidizing proteins and causing DNA mutations. Also, singlet oxygen can be produced from the photosynthetic light-harvesting chlorophyll molecules and is highly reactive. To avoid those dangerous free radicals and reactive oxygen species, plants possess different protection systems. In fact, there is formation/destruction equilibrium of those toxic radicals but this equilibrium may be affected by several factors, such as drought, solar radiation, high temperature or ozone contamination, among others. Several enzymes delete those radicals. Ascorbate peroxidase plays this protective role in chloroplasts, cytoplasm and mitochondria, using ascorbate as a substrate to reduce peroxide. In fact, ascorbic acid and apoplastic ascorbate are antioxidants that react with free radicals in plant cells. Other antioxidant cell protectants, such as glutathione, react with singlet oxygen. For instance in spruce (*Picea abies*), superoxide dismutase activity declined under ozone stress, while the redox states of the ascorbate and the glutathione pools were not affected by any treatment, suggesting that spruce needles seem to be able to acclimate to ozone stress by increasing their ascorbate pools and protecting pigments (Kronfuss et al., 1998). Alonso et al. (2001) exposed two year old Aleppo pine (*Pinus halepensis*) plants to high ozone concentrations. These authors observed that the pines activated protection mechanisms against oxidative stress in the newest needles, as opposed to older needles, which were damaged. Younger needles show higher detoxification capacity thanks to the induction of antioxidant enzyme activity. Those detoxification mechanisms decrease ozone impact but at a high photo-

assimilate expense, thus depending on environmental and ecophysiological conditions, such as hydric stress. Plant responses to ozone are therefore expressed as phenologic and physiological changes, indicating that plants activate their protection systems in increasing ozone atmospheres (Alonso et al., 2001).

## SENSITIVITY AND TOLERANCE

Identification and classification of ozone-sensitive species has been difficult and controversial. For instance, holm oak (*Quercus ilex ssp. ilex*) show stippling symptoms and was more sensitive to ozone than *Q. ilex ssp. ballota*, *Olea europaea cv. vulgaris* and *Ceratonia siliqua*, in a two year long OTC experiment of exposition to ozone (Ribas et al., 2005). However, other authors have not recently included holm oak as a sensitive species (Calatayud et al., 2010; 2011). Differences in O<sub>3</sub> sensitivity between pioneer and climax forest tree species has been reviewed by Matyssek et al. (2010), based on recent evidence from novel phytotron and free-air ozone fumigation experiments in Europe and America. As previously mentioned, O<sub>3</sub> counteracts some effects of elevated CO<sub>2</sub> on plant growth, the response being governed by genotype, competitors, and ontogeny rather than by the species. Complexity in O<sub>3</sub> responsiveness increased under the influence of pathogens and herbivores (Matyssek et al., 2010). Gerosa and Ballarin-Dente (2003) identified risk areas for trees and shrubs in Lombardy, Northern Italy, by combining distribution maps of sensitive species and ozone concentration maps with critic levels. In the particular case of shrub species, a methodology of damage monitoring based on symptomatology has been developed both in Europe and USA (Bussotti et al., 2006; 2009; Ferretti et al., 2008). Mills et al. (2011) collected a database with 644 cases of ozone pollution and their effects in 18 European countries. 22.9 % of the cases referred to shrubs, 39% were for crops (27 species), and 38.1% were for (semi-) natural vegetation (95 species). The effects of ozone could be generalized, fitting better with the modelled accumulated stomatal flux over a threshold 3 nmol/m<sup>2</sup>/s than with the Accumulated Ozone concentrations over a Threshold of 40 µg/m<sup>3</sup> h (AOT40) index. In the mid 90's, the AOT40 index was adopted for defining O<sub>3</sub> exposure instead of one, 10, 12 or 24 h average concentrations, recognizing the importance of cumulative exposure approaches (Kärenlampi and Skärby, 1996). However, a consensus has recently evolved that O<sub>3</sub> phytotoxic effects are more closely related to the amount of pollutant entering the plant through the stomatal pores and reaching the sites of damage within the leaves (Musselman et al., 2006).

Ozone sensitivity has shown to be a genetic trait in black cherry, where families differed significantly in their response to ozone treatments in severity of adaxial stipple,

but not in leaf senescence or growth. Family heritability estimates for foliar injury, calculated by treatment and week of measurement, were generally above 0.5 under 90 and 120 ppb ozone treatments. The relative ozone sensitivity of these cherry families in Continuously Stirred Tank Reactor (CSTR) chambers corresponded well with susceptibility rankings of their 27-year-old parents, replicated in a clonal seed orchard, and growing under ambient ozone exposures. The existence of localized, heritable variation in ozone sensitivity in wild populations has obvious implications for the use of bioindicators in forest health monitoring (Lee et al., 2002). The ozone sensitivity in oak species, namely *Quercus ilex*, *Q. faginea*, *Q. pyrenaica* and *Q. robur* is the result of the interaction between ozone uptake and species-specific leaf characteristics, e.g., leaf habit, thickness, stomatal density (Calatayud et al., 2011). Leaf Mass per Area (LMA), has been used to distinguish between ozone-tolerant and ozone-sensitive species. This index is also considered an index of sclerophylly, with the threshold at 7.5 mg/cm<sup>2</sup> (Bussotti, 2008), reaching 9.7 mg/cm<sup>2</sup> in *Q. faginea* and 15.2 in *Q. ilex*. Sclerophyllous adaptations include the development of cells with thick walls and more supportive tissue that can affect gas diffusion inside the leaves.

Thick leaves are considered to be more ozone-tolerant than thinner leaves (Bennet et al. 1992; Karlsson et al. 2004; Lyons et al. 2000; Pääkkönen et al., 1995a; 1995b), in part because of differences in the gas-phase diffusion pathways (Chappelka and Samuelson, 1998). The presence of cells with thick walls strongly influences the length of the diffusion pathway for ozone and modifies the interaction with oxidative constituents of the apoplast. The density of the cell wall (degree of cross-linking, suberification or lignification) would also be expected to influence the tortuosity of the diffusion pathways for ozone (Lyons et al., 2000). The dense trichoma layer presents in the lower leaf surface of *Q. ilex* increases boundary layer resistance, and eventually may increase the surface of reaction with ozone, contributing to its depletion. In addition to these processes, leaves with higher LMA values have been correlated with higher antioxidant capacity levels (Matyssek et al., 2007) and a high tissue density is considered to be able to better feed detoxification processes (Bussotti, 2008).

## CONCLUSION

Public opinion is increasingly aware of the risks of ozone to human health, the environment and forests in particular. Efforts to limit tropospheric ozone have been undertaken by industrialised countries of the northern hemisphere, and expectedly will extent all around the world. We have tried to underline the damage on forests and natural ecosystems. We have revised ozone damage on photosynthesis, plant growth and development, from

the individual to the ecosystem level. Implications of tropospheric ozone contamination should be considered at three levels: coordinated efforts among countries to reduce ozone pollution; adoption of measures of environmental management to mitigate ozone effects; and search for species or ecotypes with greater tolerance to ozone and more capacity of detoxification, taking into account the genetic diversity and phenotypic plasticity of plant species.

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