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Tropospheric ozone concentrations in the Catalan Pyrenees and their effects on Mountain pine (*Pinus uncinata* Ram.)

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SUMMARY

This thesis aimed to study the O_3 concentrations in the Pyrenees and their effects on Montain pine (Pinus uncinata Ram.), the dominant tree species of the subalpine forests of this mountainous range.

We first studied the O_3 concentrations trends during the last 16 years in the Catalan Pyrenees as well as the pattern of distribution of O_3 and its possible origin in an altitudinal gradient in central Catalan Pyrenees. We found that the O_3 concentrations in the overall Catalan Pyrenees have greatly exceeded the thresholds for protection of the vegetation set by the UNECE's CLRTAP and the European Directive 2008/50/EC from 1994 to 2009 and show a general increasing trend over time with a slight decrease during the last 3 years. In the altitudinal transects of the Central Catalan Pyrenees we found that O_3 concentrations significantly increased with altitude, with annual means at the highest site (2300 m a.s.l.) ranging from 38 to 67 ppb, showing values 35-38% greater in the warm period than in the cold period and with a characteristic pattern of minimum values early in the morning, a rise during the morning and a decline overnight. O_3 mainly originated from urban areas and was transported to high mountain sites. These results suggest thus that plant life in this mountainous range is at risk of damage by O_3, specifically at high-altitude sites, where exceedances of these thresholds are greater.

After characterizing the O_3 distribution in the Pyrenees, we surveyed the O_3-like visible injury, crown defoliation and tree mortality in the previous altitudinal gradients. Under conditions of relatively high summer water availability (summer precipitation/potential evapotranspiration above 0.96), increases in the severity of the O_3 visible injury were related with increases in [O_3]. Crown defoliation and tree mortality mean values of 20.4-66.4% and 0.6-29.6%, respectively, pointed to poorer tree vitality than reported in previous works and were positively correlated with the accumulated O_3 exposure and with variables referring to soil water availability, which again pointed to be result of a possible greater O_3 uptake. Although O_3 is believed to be part of the cause of the observed crown defoliation and tree mortality, further research is needed to determine the contribution of the multiple other acting stress factors.

The O_3-induced injury observed in *Pinus uncinata* stands from our study had the form of tiny and diffuse green-yellowish mottles located around the stomata lines on the light

exposed side of the needles and at the microscopic level, we observed chloroplasts reduction in size and an increased oxidation of cell content from the inner to the outer mesophyll cell layers, cell wall thickenings and wart-like protrusions, accumulation of antioxidants, and accelerated cell senescence processes. The observation of the same typology of mottles in a free-air O_3 fumigation experiment with *Pinus uncinata* saplings and the fact that most of the cytochemical markers observed below mottles in our symptomatic samples had already been observed to be induced by O_3 under controlled conditions in other tree species, confirm that the observed mottles in field mature individuals of *Pinus uncinata* are most probably caused by the effect of O_3.

Finally, we conducted a two-year experiment with O_3 fumigation, where it was confirmed that exposures to O_3 concentrations as those of the Pyrenees, led to high occurrence and intensity of visible injury and a 24-29% reduction of root biomass on saplings of *Pinus uncinata*, which may render trees more susceptible to other stresses and imply long-term effects on the nutrient, carbon, and water cycles of the ecosystem.

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INTRODUCTION AND AIMS

1. TROPOSPHERIC OZONE

1.1. What is tropospheric ozone and why is it important?

Ozone (O_3) is an allotropic form of oxygen that is present in the atmosphere as a natural constituent. It can be found in the stratosphere, at about 25 km of altitude, and in the troposphere, between the terrestrial surface and 10 km of altitude (Seinfield and Pandis, 2006). In the stratosphere, O_3 is produced by the photolysis of molecular oxygen and it constitutes the "O_3 layer", which protects us from the ultraviolet (UV) radiation from the sun. In the troposphere, O_3 is generated through the reaction of NO_x and VOC with the sunlight and it is considered a **secondary pollutant**.

There are several reasons that make O_3 an important pollutant at global and local scale (Fowler et al., 2008):

- It has an annual average lifetime of 22±2 days in the troposphere (Stevenson et al., 2006), although it can vary from 1-2 days in the boundary layer to several weeks in the upper troposphere.
- It can be produced from its precursors long after they have been emitted and far away, which makes very difficult to control the concentrations. Precursors can be transported long-range distances.
- It is a greenhouse gas and, hence, it has a warming effect on the climate system (Forster et al., 2007; Shindell et al., 2009).
- Concentrations are usually greater in suburban, rural and mountainous areas than in urban centres due to rapid chemical interactions with other pollutants.
- It has impacts on vegetation, usually at concentrations above 40 ppb although it depends on the species and the environment conditions. It reduces tree growth, carbon sequestration and the yield of staple crops, and it can affect the composition of natural plant communities (Ainsworth, 2008; Feng et al., 2008b; Fiscus et al., 2005; Fuhrer, 2009).
- Ambient concentrations (around 35 ppb) are though to have an impact on human health, although some individuals can be more sensitive than others. Impacts are

related to the respiratory system and include reduced lung function, lung irritation and in extreme cases, mortality (EEA, 2007).

1.2. Budget of tropospheric O_3: processes of formation and destruction

The concentration of tropospheric O_3 registered in a region is the result of the balance between the processes of formation, destruction and transportation. Therefore, the total amount of tropospheric O_3 in the atmosphere will be determined by the rates of these processes. The main sources of O_3 in the troposphere are the **photochemical formation,** which has been estimated to account for approximately 4500 Tg y^{-1}, and the **transportation of O_3 from the stratosphere**, that accounts for 540 Tg y^{-1}. The two loss processes are the **chemical destruction**, that accounts for approximately 4100 Tg y^{-1}, and the **dry deposition** to the Earth's surface, accounting for 1000 Tg y^{-1} (Denman et al., 2007; Gettelman et al., 1997; Olsen et al., 2004; Olsen et al., 2001; Prather et al., 2001; Stevenson et al., 2006; Wild, 2007; Wu et al., 2007)(Fig. 1). Ozone photochemical formation and chemical destruction rates are thus several times higher

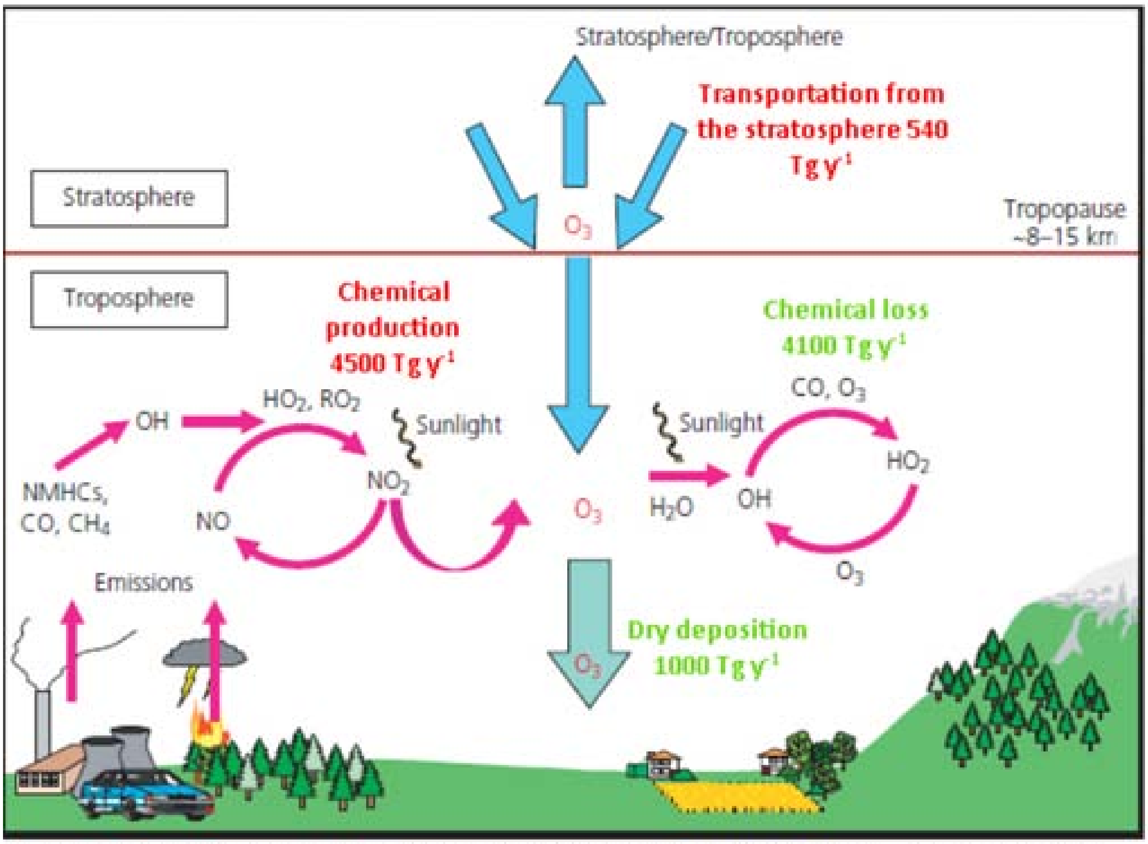


Fig. 1. Scheme of the sources and sinks of tropospheric O_3. Sources of O_3 include chemical production and transportation from the stratosphere, and O_3 sinks include chemical loss in the troposphere and deposition to terrestrial and marine surfaces. These global fluxes of O_3 are calculated using a global chemistry–transport model. Adaptation from Denman et al. (2007).

than the influx from the stratosphere and the dry deposition flux.

The **photochemical formation** of O_3 implies the presence of free-radical intermediates and other compounds as COV, CH_4, CO and NO_x. It also depends on meteorological conditions such as solar intensity, temperature and pressure and the concentration of water vapour in the atmosphere. The process starts when UV wavelengths shorter than about 320 nm, interacts with O_3 (1), giving place to hydroxyl (OH) radicals in the presence of water vapour (2) or reforming O_3, after interacting with an inert molecule (M), most commonly nitrogen (N_2) (3).

$O_3 + hv \rightarrow O + O_2$ (1)

$O + H_2O \rightarrow OH + OH$ (2)

$O + O_2 + M \rightarrow O_3 + M$ (3)

OH radicals play a central role in tropospheric O_3 chemistry. These OH react with CH_4, CO and VOC to initiate the reaction cycles that produce and remove O_3. The result of these cycles mainly depends on the concentration of NO_x and other organic compounds in the air. We can distinguish three different situations depending on the concentration of NO_x:

- *Low NO_x (<20 ppt):* it occurs in remote regions of the atmosphere such as the South Pacific Region, and is characterized by net O_3 removal. OH radicals formed in (1) react with CO and CH_4 to form peroxy radicals, CH_3O_2 and HO_2, which will be removed to form methyl hydroperoxide (CH_3OOH)(4), and hydrogen peroxide (H_2O_2)(5), which act as OH sinks and do not regenerate the O_3 destroyed in the production of OH. In this case, O_3 can also be removed when interacting with HO_2 radicals to regenerate the OH used in the CO oxidation (Fig. 2a).

$CH_3O_2 + HO_2 \rightarrow CH_3OOH + O_2$ (4)

$HO_2 + HO_2 \rightarrow H_2O_2 + O_2$ (5)

- *Intermediate NO_x (20-1000 ppt):* it occurs in rural areas of most industrialised countries and is characterised by net O_3 formation. O_3 formation in this regime is referred to as "NO_x-limited" because O_3 increases with increasing NO_x concentrations. The reactions of the proxy radicals intermediates with NO result in the conversion of NO to NO_2 (6) (7) with the following photolysis of NO_2 to generate O_3 (8)(3) (Fig. 2b).

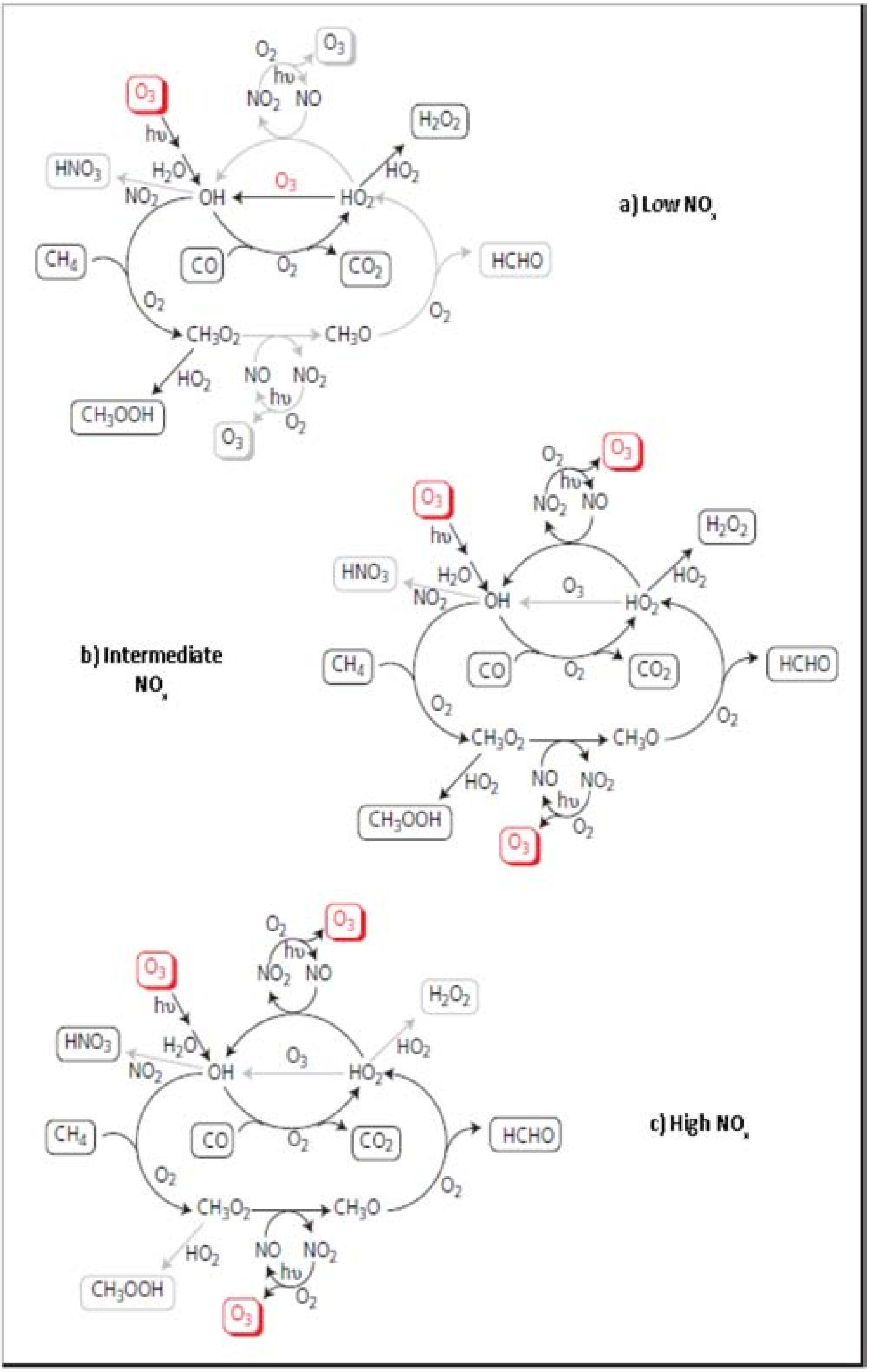


Fig. 2. Scheme of the processes of O_3 production and loss, under three different regimes as a function of NOx levels. Dominant processes are shown in black. Adapted from Fowler et al. (2008).

$CH_3O_2 + NO \rightarrow CH_3O + NO_2$ (6)

$HO_2 + NO \rightarrow OH + NO_2$ (7)

$NO_2 + hv \rightarrow NO + O$ (8)

- *High* NO_x *(>1000 ppt):* it occurs in locations close to pollution sources e.g. urban environments, and is characterised by the inhibition of O_3 formation. The reaction of NO to NO_2 are predominant and NO_2 reacts with OH to form HNO_3 (9). HNO_3 becomes the main sink of OH, stopping the entrance of OH into the cycle of O_3 formation. In urban environments, O_3 can highly decrease when NO emissions increase; however, O_3 peak episodes can occur when anthropogenic VOCs (e.g. from road transport or solvent evaporation) and/or biogenic VOCs (most notably isoprene) are injected in a NO_x rich environment. It is for this that the formation of O_3 in this case is referred to as "VOC-limited" (Fig. 2c).

$OH + NO_2 + M \rightarrow HNO_3 + M$ (9)

The main process of tropospheric **O_3 destruction** is the halogen-catalysed removal that takes places in marine areas, particularly at high latitudes during spring time. Both organic and inorganic bromine and iodine-containing species are released from surface reactions on sea-salt aerosols or ice surfaces or emitted by the ocean. Photolysis of these compounds will lead to Br and I atoms that react with O_3, giving place to net O_3 loss.

Dry deposition of O_3 to terrestrial and marine surfaces is another process of O_3 destruction. O_3 is a very reactive gas that is rapidly deposited to any surface as soil, vegetation, building materials and water surfaces. Dry deposition occurs as a result of reactions with the surfaces of soil and vegetation and uptake through plant stomata. The rate of deposition depends on meteorological variables, surface and substance properties and the interrelation between these factors. Turbulence, wind speed, the presence of water on vegetation and stomata opening (which respond to sunlight, temperature, humidity, soil moisture and CO_2 concentration), are the factors that regulate dry deposition on vegetation.

1.3. Temporal cycles of O_3

The reactions described above depend on the meteorological conditions and the O_3 precursors concentrations in the region. This makes O_3 concentration vary diurnally, seasonally and interannually.

At **day scale**, O_3 production is at its maximum during the central hours of the day, when solar radiation and temperature reach the highest values. Solar radiation is required for the photolysis of several species involved in O_3 formation, and the rate of some of the reactions increase with temperature. O_3 minimum concentrations are usually registered during night.

Seasonal variations are generally characterised by maximum concentrations during the warmer sunny weather of spring and summer, and minimum concentrations during winter and autumn. In areas with no influence of O_3 precursors emissions, O_3 maximum occurs at the end of winter or beginning of spring, as a result of the accumulation of precursors that do not react during winter due to low solar radiation and temperature. O_3 intrusions from the stratosphere during spring also contribute to the maximums observed in spring. In areas with influence of O_3 precursors emissions, O_3 maximum concentrations occur during spring and summer. Under hot, sunny conditions, if soils are dry, the soil water deficit leads to closure of stomata leading to lower rates of dry deposition and even larger O_3 concentrations.

Annual variability of O_3 concentrations are mainly linked to differences in weather conditions from year to year and different O_3 precursors concentrations. Warm sunny anticyclonic weather is related to peak O_3 episodes, for the reasons already explained above. Thus, yearly variations in the frequency and duration of weather suitable for O_3 episodes will influence the O_3 experienced during that year.

1.4. O_3 distribution at urban, rural and mountainous areas

In **urban environments**, elevated NO_x levels lead to O_3 depletion due to a direct reaction with emitted NO (known as "NO_x titration effect"). This phenomenon keeps moderate or low concentrations of O_3 in urban areas, but, of course, all processes take place simultaneously and dry deposition also contributes to the depletion of O_3 in these environments. On the contrary, rural, coastal and mountainous areas under the influence of O_3 precursors emissions are known to experience higher O_3 concentrations.

In **rural areas**, transportation of O_3 precursors and natural emissions of COVs lead to a situation of "NO_x-limited" net production of O_3, in which O_3 concentration increases with NO_x (Fowler et al., 2008). O_3 concentrations have also been observed to be higher in **mountainous areas** (Alonso and Bytnerowicz, 2003; Aneja et al., 1994b;

Ribas and Peñuelas, 2006). High O_3 concentrations in mountainous areas have been related to higher solar radiation, local air recirculation linked to orographic systems (e.g. valley-mountain systems or mixing boundary layers) (Sanz and Millán, 2000), ozone intrusions from the stratosphere (Viezee et al., 1983) and less chemical loss under low NO concentrations (Naja et al., 2003).

In rural areas, local air recirculation linked to orographic systems has important effects on **O_3 profiles**. In **sheltered valleys** during the night, thermal inversions often occur. The layer of air in contact with the earth's surface experiences O_3 depletion due to chemical loss and dry deposition. This often leads to a sharp decline in O_3 concentrations because O_3 depletion is faster than vertical transport from layers above the thermal inversion. **Hill tops** or areas above the thermal inversion experience larger O_3 concentrations because the supply of O_3 from higher levels in the boundary layer exceeds the rate of depletion by dry deposition and chemical reactions. These areas, either with no precursors emission or influenced by pollutant transportation, show O_3 daily profiles quite constant around a certain value. When the thermal inversion is broken in the morning due to surface warming, the higher O_3–enriched layer gets mixed with the lower "clean" layers and O_3 concentrations rapidly increase in the valleys during the first hours of the morning (Fowler et al., 2008).

1.5. O_3 distribution at global scale

O_3 distribution **at global scale** is determined by atmospheric transportation of O_3 and its precursors and by the O_3 formation and depletion reactions which are influenced by O_3 precursors concentrations and meteorological and topographical factors.

High O_3 levels have been observed over regions of high NO_2 and CO concentration in the northern and southern hemispheres such as USA, eastern China, north-north-eastern India (Indo-Gangetic Basin), southern America, Europe, Australia and central and southern Africa (Ghude et al., 2010) (Fig. 3a). In the southern hemisphere, seasonal distribution of tropospheric O_3 and its precursors shows a correlation with the seasonal variation of biomass burning in Africa and South America (Duncan et al., 2003). In the northern hemisphere, summer O_3 pollution is widespread throughout the middle latitudes, and high O_3 levels seem to be plumes downwind of Asia, North America and Europe (Ghude et al., 2010). In effect, O_3 levels in the Mediterranean area, the Middle

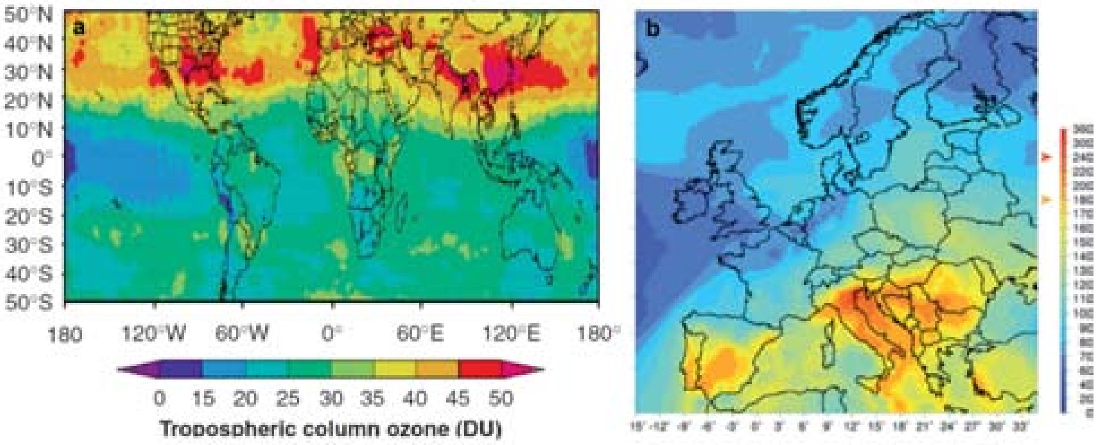


Fig. 3. **a)** Global distribution of three year averaged (2003–2005) tropospheric O_3 during May (in Dobson Units). Adapted from Ghude et al. (2010), **b)** tropospheric O_3 concentration ($\mu g \cdot m^{-3}$) during an episode of high pollution in June 2008. Adapted from Gabrielsen et al. (2009).

Est and North Africa are strongly affected by the southward outflow from North America and Europe (Ghude et al., 2010).

At European scale, the highest levels of O_3 are registered in the Mediterranean area. The high levels of solar radiation and temperature and the atmospheric stability in this area during summer and spring time contribute to the high O_3 levels found in this region. However, O_3 pollution can be very high in all over Europe under certain situations, as it happened during summer 2003 (Fiala et al., 2003). Figure 3b shows O_3 distribution in Europe during summer 2008 with the highest concentrations located in the Mediterranean region.

1.6. Trends in tropospheric O_3 and its relationship with climate change

Concentrations of ground-level O_3 have been increasing during the last century, from a mean level of 10 ppb (Anfossi and Sandroni, 1994b) at the end of the 19th century to a present annual average concentration of 35-40 ppb in the mid-latitudes of the Northern Hemisphere (Fowler et al., 2008). In Europe, background O_3 concentrations have increased by a factor of 5 since the beginning of the 20th century (Marenco et al., 1994) and by a factor of 2 during the last 30 years (Vingarzan, 2004). Moreover, the IPCC (Intergovernmental Panel on Climate Change) predicts an increase in concentrations of O_3 of 20-25% from 2015 to 2050 based on scenarios of high emissions (IPCC, 2007a). Nonetheless, in many places of Europe and North America O_3 peak episodes have decreased during the last 10-15 years (Jaffe and Ray, 2007; Jonson et al., 2006) due to the reduction of O_3 precursors emissions conducted since 1980 in these areas (Jonson et al., 2006). These contrasting trends of increasing background concentrations and decreasing O_3 peak episodes could be explained by less effective reductions of NOx emissions in summer than in winter (Jonson et al., 2006).

Future trends of O_3 concentrations need to be considered in the scenario of climate change, since photochemical production of O_3 depends on several factors that are influenced by climate change as temperature, solar radiation, air humidity or pollutants transportation (Fowler et al., 2008). Moreover, tropospheric O_3 is the third most important greenhouse gas after CO_2 and CH_4 that contributes to global warming (Fowler et al., 2008). Although the influence of climate change on O_3 is expected to be spatially and temporally variable, the general tendency is that O_3 increases over polluted land regions and decreases over the oceans (Fowler et al., 2008). Increases over land regions are expected to occur in areas with major emission sources from biomass burning or lightning and as result of increases in water vapour (which will lead to enhanced net O_3 production, (Isaksen et al., 2005)), temperature and changes in atmospheric circulation, which would affect long-range transportation of O_3 precursors and the frequency and duration of anticyclonic situations favourable for O_3 production. Decreases in rainfall would make O_3 photochemical production increase, although it would decrease the rate of dry deposition (Fowler et al., 2008). In any case, the scenarios of anthropogenic emissions are expected to have a stronger effect on global tropospheric O_3 concentrations than the scenario of climate change (Dentener et al., 2006).

2. OZONE EFFECTS ON VEGETATION

Ozone effects on vegetation depend on the **amount of O_3 entering the leaves** and **the plant's sensitivity to O_3** determined by the capacity of detoxification and repair processes within the leaf (Matyssek et al., 2008).

The amount of O_3 entering the leaves is directly proportional to the gradient of O_3 concentration between the interior and exterior of cells and indirectly proportional to several resistances against the movement of molecules (Fowler et al., 1999). A first aerodynamic resistance (R_a) conditions the movement from the atmosphere to the layer of air in contact with the vegetation surface and it depends on the structure and height of the vegetation cover and wind speed. A second resistance, of the boundary layer (R_b), determines the O_3 concentration at the layer of air in contact with the foliar surface and it varies with wind speed and leaf morphology (size, shape, orientation and texture). The final O_3 concentration that enters the leaves depends on the surface resistance (R_{sur}), which has two components: the cuticular resistance (R_{cut}), which determines the rate of reaction of O_3 with the cuticule, and the stomatal resistance (R_{sto}), which is the most important parameter that determines O_3 uptake by vegetation. R_{sto} mainly depends on the level of stomata opening which is regulated by environmental (temperature, air relative humidity, solar radiation, etc.) and physiological (phenology, phytohormones, age, etc.) factors (Emberson et al., 2000).

Plants have the capacity of activate several mechanisms of defence, protection and reparation against the damage caused by O_3. When O_3 concentration is higher than the defence capacity of plants, we can observe direct effects on physiology and growth. However, moderate O_3 concentrations can also have indirect effects due to the cost of activating and maintaining the detoxification and repair processes.

2.1. Effects at cell level

Once O_3 has entered the leaves through stomatas, it diffuses within the apoplast (diffusional space outside the plasma membrane) where it rapidly decomposes to free radicals and reactive oxygen species (ROS) as hydroxyl (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2)(Heath and Taylor, 1997). These may be detoxified by enzymes (superoxide dismutase, catalases and glutathione peroxidase), antioxidant molecules (ascorbate, vitamin C, glutathione) and other secondary metabolites (phenols, carotenoids). These radical scavengers constitute the main protection system of plants against the ROS and free radicals which are also formed during the vegetal metabolism, even under optimal growth conditions.

After reacting with the protection molecules, the free radicals and ROS react with the plasma membrane lipids, amino acids in plasma membrane proteins or apoplastic enzymes, and a variety of organic metabolites localized in the cell wall (Fiscus et al., 2005). The products of these reactions could serve as the initial signals leading to O_3 responses. These responses vary and include changes in ion concentrations as calcium, water loss, changes in enzymes concentration and activity, stimulation of production of antioxidants and ethylene, changes in gene expression, unregulated cell death, hypersensitive response leading to programmed cell death, and accelerated senescence (Black et al., 2000; Fiscus et al., 2005; Heath, 2008; Pell et al., 1997; Schraudner et al., 1997).

Many studies have also reported reductions in net assimilation which are related to reductions in the Rubisco activity and chlorophyll content, negative effects on photosynthetic electron transport, dysfunction on guard cells from the stomata and increases in respiration rates probably due to increases in the detoxification and repair processes (Andersen, 2003; Feng et al., 2008a; Fiscus et al., 2005; Heath, 2008; Peñuelas et al., 1994; Schraudner et al., 1997).

2.2. Effects at plant level

All the processes described above lead to physiological changes in leaves that eventually affect the amount of carbon available for allocation to meet growth and metabolic needs (Andersen, 2003). These changes include decreased carbon assimilation, increased metabolic costs, and possibly decreased phloem loading, as said before.

The effect of O_3 in **decreasing carbon assimilation** is due to the alteration of stomatal conductance, to decreases in the activity of Rubisco and to reductions in leaf longevity (Dann and Pell, 1989; Miller et al., 1999; Pell et al., 1994; Zheng et al., 2002). The **increase in metabolic costs** induced by O_3 is associated with processes of maintenance and reparation of the molecules affected by ROS (Dizengremel, 2001; Guderian et al., 1985; Landolt et al., 1997) and with synthesis of antioxidants that will avoid O_3 stress (Alscher and Amthor, 1988; Tingey, 1991). Ozone has been suggested to **impair phloem loading,** possibly by damaging plasmalemma or plasmodesmata in the mesophyll cells (Grantz and Farrar, 2000; Landolt et al., 1997; Rennenberg et al., 1996). Hence, decreased phloem loading and increased carbohydrate concentrations would finally give place to a feedback inhibition of photosynthesis (Koch, 1996).

Changes in the amount of carbon available for allocation to sink tissues – source strength- gives place to changes in the translocation patterns to, and hence the growth rate of, different plant organs. However, the translocation patterns also depend on sink activities, thus the effect of O_3 will finally depend on partitioning priorities of carbon at the time of exposure (Ashmore, 2005). In fact, the effect of O_3 on resource allocation is considerably varied between species; in some species O_3 has caused reductions in both vegetative and reproductive growth, in some others, resources have been found to be switched to reproductive growth, and in other cases, they have been switched to vegetative growth (Bergmann et al., 1995).

Among the changes in the translocation patterns, reduced allocation of carbon to **root growth** has been highlighted since it produces changes in the hydraulic functionality, negative effects on mychorrizal development, alterations in N fixation in legumes and actinorrhizal species, alterations in nutrients absorption, alterations in carbon flux to soil through altered rhizodeposition, including exudation, and root and hyphal turnover, reductions in plant vitality and changes in plant sensitivity to other stresses (Andersen, 2003; Ashmore, 2005; Fuhrer and Booker, 2003; Skarby et al., 1998).

Changes in the translocation patterns due to O_3 are also known to affect the **reproductive organs** (Ashmore, 2005). Nonetheless, O_3 may also have direct effects on reproductive structures by altering pollen germination and tube growth, fertilization, and the abscission or abortion of flowers, pods and individual ovules or seeds (Black et al., 2000; Bosac et al., 1994; Schoene et al., 2004; Stewart et al., 1996). **Changes in foliar chemistry** and surface characteristics induced by O_3 have also been found to yield important secondary effects on the incidence of viral and fungal diseases and the impact of insect pests (Fluckiger et al., 2002).

2.3. Effects at ecosystem level

Effects of O_3 on forest and semi-natural ecosystems need to be considered on a long-term basis (Ashmore, 2005). Ozone have been found to affect the **biogeochemical cycle**, although the effects are still poorly understood (Ashmore, 2005). Changes in litter chemistry, root chemistry, and root exudates due to the effect of O_3 seem to influence soil microbial diversity, biomass and activity, and consequently, carbon and nitrogen cycling (Islam et al., 2000; Kim et al., 1998; Olszyk et al., 2001). Moreover, the response of plant to O_3 stress is affected by nutrient availability (Utriainen and Holopainen, 2001; Whitfield et al., 1998), therefore, any change in nutrient cycling will lead to **feedback effects on plant sensitivity to O_3**.

Ozone has been also found to alter plant interspecific competition which can cause secondary **effects on community composition**. However, these effects cannot readily be predicted from the known sensitivity of individual species (Ashmore, 2005; Barbo et al., 1998). Moreover, theses changes in the community composition can include reductions in leguminous proportion, which seem to be more sensitive to O_3 (see Bassin et al., 2007a), giving place to negative effects on atmospheric N fixation in the ecosystem (Andersen, 2003).

Ozone can act as a **predisposing factor that weaken the trees**' resilience to a range of biotic and abiotic stresses such as drought, pathogen attacks and nutrients deficiency (Skarby et al., 1998). Interactions between O_3 and other stress factors such as soil moisture availability or increasing CO_2 atmospheric concentrations, are also very important at the ecosystem level (Ashmore, 2005). High levels of CO_2 as the ones predicted in the scenarios of global change, could function as protection against O_3 by reducing stomatal conductances (Fiscus et al., 1997) and increasing the availability of assimilated products for repair and detoxification processes in presence of O_3 (Heagle et al., 1999).

The impacts of O_3 stress and the interaction with other stresses have been thoroughly studied on the forest ecosystem composition in the San Bernadino mountains (Arbaugh et al., 2003; Arbaugh et al., 1998; Miller and McBride, 1999). Ponderosa pine (*Pinus ponderosa*) and Jeffrey pine (*Pinus jeffreyi*) are the historically dominant species of the mixed-conifer forests in San Bernadino mountains, due to their tolerance of the frequent forest fires. However, these species are also the most sensitive to O_3 showing severe foliar injury, reduced needle longevity and radial growth, and being more susceptible to bark beetle attacks. Regeneration in these forests is now greater for other species more resistant to O_3 and also more fire-sensitive, which are favored by current fire exclusion. Nonetheless, changes in the community composition are attributable not only to O_3 exposure but also to a mix of factors including nitrogen deposition, precipitation and management (Arbaugh et al., 2003).

Therefore, the long-term O_3 effects on species composition and ecosystem function still needs to be further studied together with their interaction with other factors such as nutrient availability and cycling and water stress, since they are important components of global change (Ashmore, 2005).

3. ASSESSMENT OF O_3 EFFECTS ON VEGETATION

3.1. The Convention on Long-range Transboundary Air Pollution (CLRTAP) and its protocols

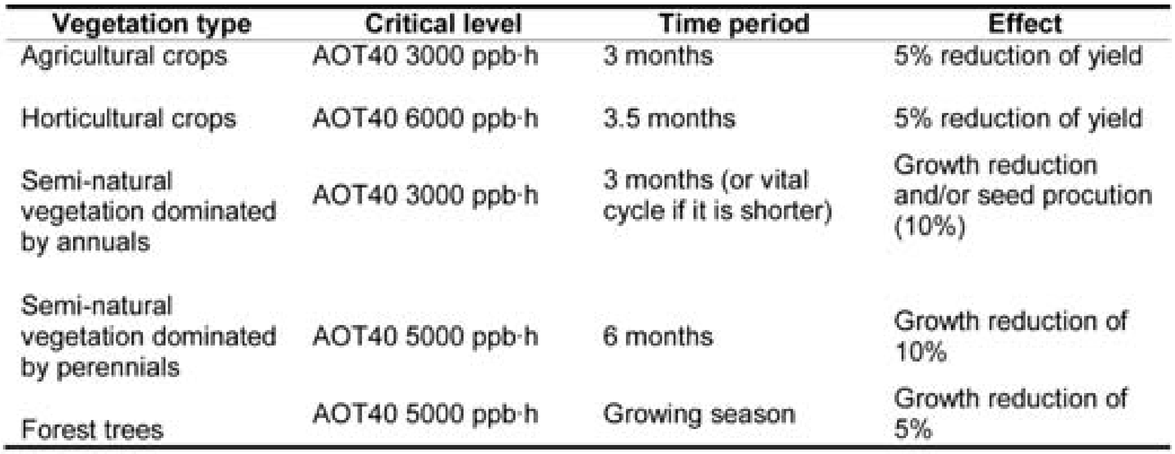
The first major action to combat transboundary air pollution in Europe took place in 1979 with the CLRTAP (Convention on Long-range Transboundary Air Pollution, in force in 1983) of the UNECE (United Nations Economic Commission for Europe) composed by 30 Western and Eastern European countries, the United States, Canada and the European Community (Schroeder and Yocum, 2006). With the aim of reducing the adverse effects of atmospheric pollution, they created the "multi-pollutant" **Gothenburg Protocol** in 1999 (UNECE/CLRTAP, 1999). In this protocol, **critical levels of O_3** that could damage plants were determined to protect vegetation in accordance with the convention's Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends (UNECE, 2010). CLRTAP and its protocols set a precedent for international environmental policy that the European Community adopted to a large extent (Schroeder and Yocum, 2006).

3.2. Establishment of the critical levels of O_3

Critical levels set up in the Gothenburg Protocol are defined as the highest load of pollutant that will not cause chemical changes leading to long-term harmful effects on the most sensitive ecological ecosystems in a designated area. The two approaches for defining critical levels of O_3 are based on either stomatal flux or O_3 concentration.

Critical levels based on stomatal flux provide an estimate of the amount of O_3 entering plants through the stomata and reaching the sites of damage within the leaf. These critical levels take into account the varying influences of air temperature, water vapor pressure deficit of the surrounding leaves, light, soil water potential, O_3 concentration and plant development on the stomatal flux of O_3. The phytotoxic O_3 dose (**POD**), defined as the accumulated flux above a flux threshold of Y nmol·m^2·s^{-1} accumulated over a stated time period during daylight hours, is the parameter used to establish the critical levels above which direct adverse effects may occur according to present knowledge. **Critical levels based on concentration** only consider the O_3 concentration at the top of the canopy but not the influence of climatic factors on stomatal conductance. Even though the determination of critical levels based on concentration has several important limitations and uncertainties, especially in the Mediterranean area where climatic factors strongly affect stomatal conductances, they are still used for estimating the risk of damage when climatic data or suitable flux models are not available (UNECE, 2010).

The **AOT40**, defined as the sum of the daylight hourly O_3 concentration, recalculated for canopy height, exceeding the threshold of 40 ppb_v over a certain period of time, is the most commonly used concentration-based critical level for forests and seminatural vegetation. The UNECE's CLRTAP determines different critical levels for O_3 for different accumulated periods and types of vegetation (e.g. three months for seminatural vegetation in communities dominated by annuals, six months for forest trees, etc.)(Table 1). An AOT40 of 5000 ppb_v·h over six months (April−September) has been set for the protection of forest trees and semi-natural vegetation dominated by perennials in mountainous areas of the Mediterranean region (UNECE, 2010). Above this critical level, forest trees and semi-natural vegetation begin to suffer a reduction in growth of at least 5 % and 10 %, respectively (UNECE, 2010).



Vegetation type	Critical level	Time period	Effect
Agricultural crops	AOT40 3000 ppb·h	3 months	5% reduction of yield
Horticultural crops	AOT40 6000 ppb·h	3.5 months	5% reduction of yield
Semi-natural vegetation dominated by annuals	AOT40 3000 ppb·h	3 months (or vital cycle if it is shorter)	Growth reduction and/or seed procution (10%)
Semi-natural vegetation dominated by perennials	AOT40 5000 ppb·h	6 months	Growth reduction of 10%
Forest trees	AOT40 5000 ppb·h	Growing season	Growth reduction of 5%

Table 1. Critical levels of O_3 for different types of vegetation set by the CLRTAP/UNECE. Exceedances of the critical levels indicate risk of damage (UNECE, 2010).

3.3. European Union air pollution policy

Parallel to the development of the Gothenburg Protocol, in 1996 the European Union launched the **Air Quality Framework Directive** on ambient air, which rather than establishing concrete air quality objectives, set up the basis for ambient air quality monitoring and management, from which more specific "daughter directives" could be adopted (Schroeder and Yocum, 2006). After some years of work, the final Directive had specific target values for emissions and, in 2001, a focus on ground level O_3 was added, constituting the National Emissions Ceilings Directive (NEC Directive), which would be a step toward the EU's long-term objectives of not exceeding critical loads of harmful pollutants (Schroeder and Yocum, 2006). In 2008, the **European Directive 2008/50/EC** (2008) established an O_3 target value and long-term objective for the protection of vegetation without distinguishing between types of vegetation. From 2010, the 5-year running average of the AOT40 calculated from May to July was set below the target value of 9000 ppb_v·h, while a longer-term objective was set below 3000 ppb_v·h (Table 2). The AOT40 for these targets was calculated by summing the differences between 40 ppb_v and the hourly O_3 concentrations above 40 ppb_v for the hours between 8.00 h and 20.00 h Central European Time (CET) each day.

Objective	Target value	Averaging period	Date by which it should be met
Protection of vegetation	AOT40 (calculated from 1 h values) 9000 ppb·h averaged over five years	May to July	1.1.2010
Protection of human health	61 ppb not to be exceeded on more than 25 days per calendar year averaged over three years	Maximum daily eight-hour mean	1.1.2010
Objective	**Long-term objective**	**Averaging period**	**Date by which it should be met**
Protection of vegetation	AOT40 (calculated from 1 h values) 3000 ppb·h	May to July	Not defined
Protection of human health	61 ppb	Maximum daily eight-hour mean within a calendar year	Not defined

Table 2. Target values and long-term objectives of O_3 for the protection of vegetation and human health established in the European Directive 2008/50/CE on ambient air quality (2008).

4. THE CASE OF THE PYRENEES

4.1. General description

The Pyrenees is a range of mountains situated in the southwest of Europe. It forms a natural border of 435 km long between France and Spain, from the Atlantic Ocean to the Mediterranean Sea. Hence, the Spanish Pyrenees are part of several provinces:

Guipuzcoa in the Basque Country, Navarra, Huesca in Aragon and Lleida, Barcelona and Girona in Catalonia.

Physiographically, the Pyrenees are divided into three different sectors: Western, Central and Eastern Pyrenees. The Western Pyrenees, from the Atlantic Ocean to Anie peak (which is the highest point, at 2504 m a.s.l), have moderately low average elevation with the typical Atlantic landscape. The Central Pyrenees, from Anie peak to Pimorent pass, are the highest part of the Pyrenees (with Aneto as the highest summit at 3404 m a.s.l) and they show significant differences in the climate and vegetation in the northern, with very humid and typical Atlantic vegetation, versus the southern sides, with the typical Mediterranean vegetation. The Eastern Pyrenees, from Cadí to Cap de Creus, maintain a relatively high elevation until very close to the sea (Canigó, at only 50 km far from the sea is 2785 m high). In this case, the most humid side is the southern one since it receives the influence of the Mediterranean Sea (Vigo, 2009).

The Catalan Pyrenees comprises the Eastern Pyrenees and part of the Central Pyrenees. However, they are often divided into three parts: the Western, from Val d'Aran to Pallars Sobirà counties, the Central, from Andorra to La Cerdanya, and the Eastern Catalan Pyrenees, from Ripollès to Alt Empordà counties, but including the Cadí range (Fig. 4).

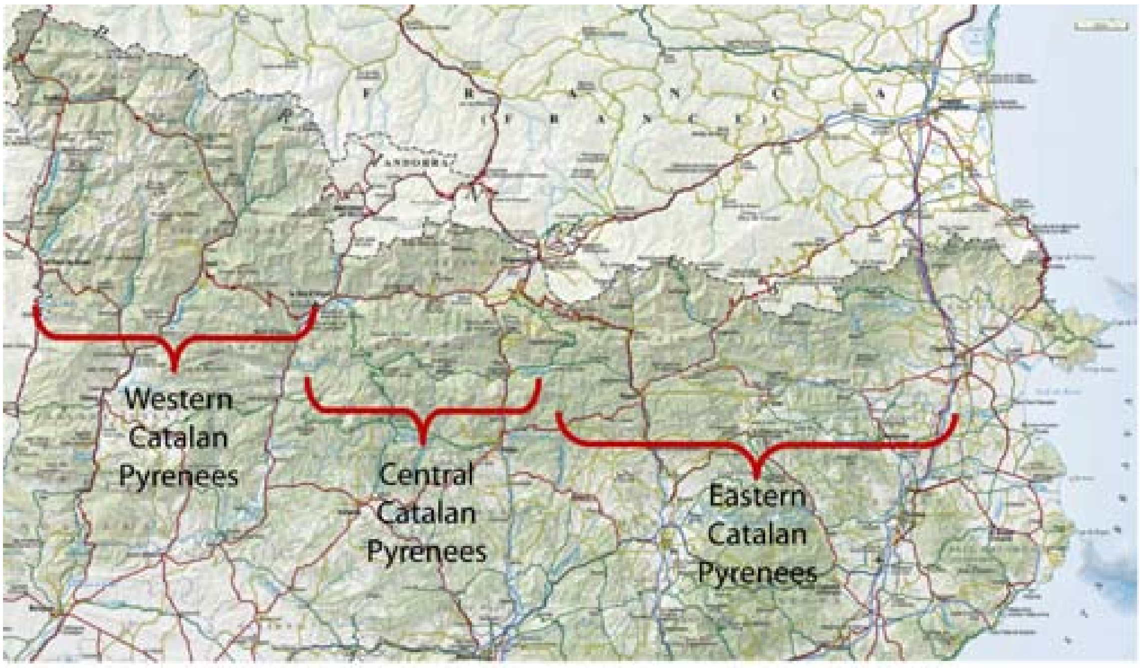
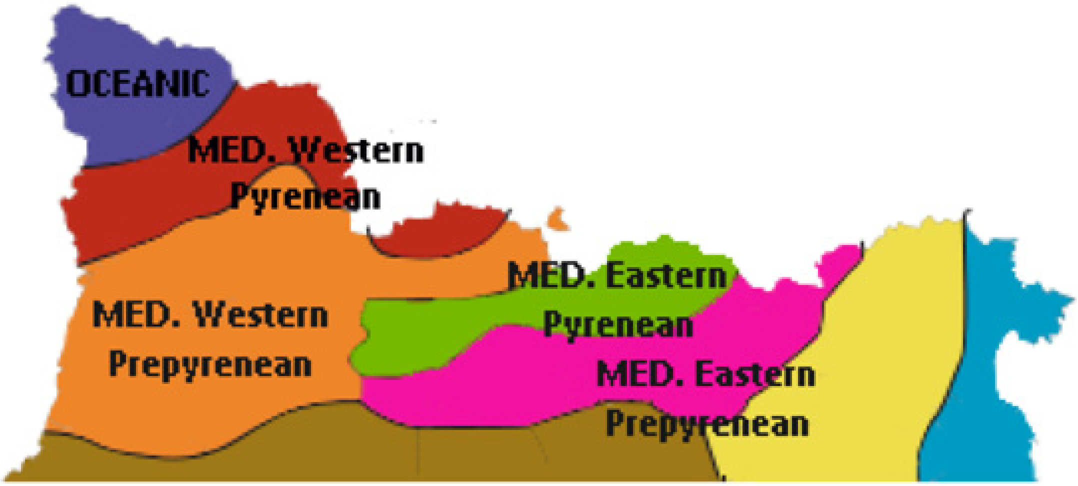


Fig. 4. Map of the Catalan Pyrenees. Adapted from the Institut Cartogràfic de Catalunya. www.icc.cat

4.2. Climate in the Catalan Pyrenees

The Catalan Pyrenees show a relatively high variety of climates (Fig. 5). We find the Oceanic climate and several Mediterranean climates: the Western Pyrenean, the Western Prepyrenean, the Eastern Pyrenean and the Eastern Prepyrenean. The Oceanic climate is the only one that has a balanced accumulated precipitation all year round whereas the other climates show the maximum precipitation in the summer or spring and the minimum in the winter. The annual accumulated precipitation is variable among the climates and temperatures are usually cold in winter and mild in summer, being hotter in the Prepyrenean climates than in the Pyrenean ones (Martín-Vide, 1992) (Fig. 5).



Type		Subtype	Annual precip. (mm)	Seasonal rainfall pattern	Mean annual temperature (ºC)
OCEANIC		Val d'Aran	900-1100	Balanced	6-10
MEDITERRANEAN	Pyrenean	Eastern	1000-1200	Maximum in summer and minimum in winter	3-9
		Western	1000-1300		2-9
	Prepyrenean	Eastern	850-1100	Maximum in summer o spring and minimum in winter	9-12
		Western	650-900		

Fig. 5. Distribution and characteristics of the Oceanic, Mediterranean Eastern and Western Pyrenean and Mediterranean Eastern and Western Prepyrenean climates in Catalonia. Adapted from Martin-Vide (1992).

Therefore, the rainfall pattern in the Pyrenees show a clear tendency of maximum rainfall during spring-summer (usually between May and June), so that the warm season is also humid, which will be favorable for plant development and growth (Vigo, 2009). Nonetheless, we need to take into account that year-to-year variations in the amount and distribution of precipitation are a feature very common in the Mediterranean climate. Thus, changes in the amount and distribution of precipitation can often occur in the mountainous areas with Mediterranean Prepyrenean and Pyrenean climates and dry summers can even take place in the high mountainous areas (Vigo, 2009).

4.3. Vegetation in the Catalan Pyrenees

Vegetation in the Pyrenees is characterized by a clear altitudinal zonation that forms contrasting vegetation belts (Ninot et al., 2007). A first **basal belt** spreads up to 600-900 m a.s.l., which in the Eastern and Iberian side of the Central and Western Catalan Pyrenees will be dominated by typical Mediterranean vegetation whereas in the northern side of the Central and Western Catalan Pyrenees will be dominated by the typical Atlantic vegetation (Vigo, 2009).

The **submontane belt**, whose limit is at 1100-1300(1500) m a.s.l. depending on areas and exposure, coincides mostly with the dominance of submediterranean formations such as forests of *Quercus pubescens* and *Quercus faginea*, and their related pinewoods of *Pinus nigra* subsp. *Salzmannii* or *P. sylvestris* (Ninot et al., 2007).

The **montane belt**, with its upper limit at 1600-1700 m a.s.l. on north-facing exposures and at 1700-1900 m a.s.l. on south-facing aspects, is characterized by mixed forests of *Fagus sylvatica* and *Abies alba* in the areas with Atlantic influence. *Abies alba* can form pure firwoods in north-facing slopes on acidic bedrocks with subcontinental bioclimate, as it occurs in the upper part of the belt and in the lower subalpine belt in Val d'Aran. In the areas where the climate is too continental or too dry for *Fagus* or *Abies* or where site conditions are more unfavorable (poor soils, dry shelves, rocky slopes, etc.), *Pinus sylvestris* dominate the forested landscapes (Ninot et al., 2007). The montane belt has suffered continous deforestations that gave place to a wide variety of scrubs, pastures and grasslands, which are in the present being used for livestock or agriculture.

The **subalpine belt** is the domain of *Pinus uncinata*, which forms dense and well-structured pinewoods of diverse type in the lower part of the belt (mainly to 2000 m a.s.l.), combining north- and south-facing and acidic and lime-rich soils. Above this elevation, the pinewoods become lighter and more irregular until the treeline, which occurs at varying altitudes, mostly between 2200 and 2450 m a.s.l., depending on continentality, exposure and landform (Carreras et al., 1996). However, in most of the Pyrenees the human action has strongly affected the primitive vegetation and the actual border between the subalpine forest and the alpine belt is not natural at all, but the result of the human influence for centuries (Vigo, 2009).

The **alpine belt** is characterized by particular plant communities in the most extreme habitats (rocks, scree, snow-beds), whereas various types of short grasslands cover more balanced soils (Illa et al., 2006). The limit of this belt runs up to 2800 m a.s.l., where it generally starts the subnival belt. The **subnival belt** is characterized by the strong dominance of rocks and scree, and the scarcity of surfaces bearing soil. It only occurs on some small surfaces corresponding to peaks, crests and cliffs and it presents sparse rupicolous vegetation and a few communities related to scree and other rocky surfaces (Ninot et al., 2007).

Most of the forest trees and herbaceous species found along the mentioned vegetation belts have been found to be sensitive to O_3. *Fagus sylvatica*, *Pinus sylvestris*, *Abies alba* and *Betula pendula* are some of the O_3-sensitive tree species (Karlsson et al., 2003; Larsen et al., 1990) distributed along the montane belt and subalpine belt clearings in the Pyrenees (Vigo, 2009). Regarding semi-natural vegetation, *Trifolium ssp., Phleum alpinum, Leontodon hispidus, Dianthus deltoides, Valeriana officinalis, Hieracium pilosella,* and *Silene acaulis* are some of the O_3-sensitive herbaceous species (Hayes et al., 2007) also present in the Pyrenees (Vigo, 2009). Nonetheless, O_3 sensitivity of *Pinus uncinata* has never been studied up till now, although it is the dominant species in the subalpine forests of the Pyrenees. In fact, significant increases in crown defoliation in stands of *P. uncinata* in the Pyrenees have already been detected by the Catalan forestry service and have been reported in a previous study (Carnicer et al., 2011). However, the effect of O_3 as a contributing factor in the observed forest decline has never been proved.

4.4. O_3 concentrations in the Catalan Pyrenees

Ozone concentrations have been found to increase with increasing altitude in mountainous areas (Aneja et al., 1994a; Aneja et al., 1994b; Cooper and Peterson, 2000; Ribas and Peñuelas, 2006; Sanz et al., 2007; Skelly et al., 1997). Higher solar radiation (Volz and Kley, 1988), local air recirculation linked to orographic systems (eg. valley-mountain systems or mixing boundary layers) (Lefhon, 1992; Sanz and Millán, 2000),

O_3 intrusions from the stratosphere (Viezee et al., 1983) and less chemical O_3 loss under low NO concentrations (Naja et al., 2003) are all factors which contribute to high O_3 mixing ratios at high altitude.

Although the distribution of O_3 has been studied in the Mediterranean area (Gimeno et al., 1995; Ribas and Peñuelas, 2000, 2003, 2004; Sanz et al., 2000; Ziomas, 1998) and in several mountain ranges from Europe (e.g. the Alps (Chevalier, 2007), the Carpathian Mountain Range (Manning et al., 2002)) and North America (eg. White Mountains, Adirondack Mountains (Aneja et al., 1994a; Aneja et al., 1994b), San Bernardino Mountains (Arbaugh et al., 2003; Arbaugh et al., 1998; Bytnerowicz et al., 2007b), Sierra Nevada (Arbaugh et al., 1998), Wasatch Mountains (Wager and Baker, 2003) and in the mountains around Mexico City (de Bauer and Hernandez-Tejeda, 2007)), prior the beginning of this work no thorough studies had been conducted in the mountain ranges of the Mediterranean region in Europe. In the Pyrenees, permanent O_3 monitoring has been carried out at only two observatories: in Navarra since 1999 (at the EMEP station of Irati, 1400 m a.s.l.) and in the Haut-Pyrenees department since 2001 (at the Pic du Midi, 2875 m a.s.l.), although former data series at Pic du Midi sparsely cover all the 20th century (see Chevalier, 2007; Marenco, 1986; Marenco et al., 1994). In the Catalan Pyrenees, to our knowledge there is only a preliminary study, which indicates high O_3 concentrations with strong interannual, seasonal and daily variations (Ribas and Peñuelas, 2006).

Although the Pyrenees are often directly exposed to oceanic air masses (in the case of north-westerly flux), these masses are usually unaffected by the European continental pollution (Gheusi et al., 2011). However, in the case of south to southwesterly advection, the influence of pollutants from the Iberian Peninsula may be detected on the Pyrenean summits (Gheusi et al., 2011). In summer, furthermore, the eastern and central parts of the Catalan Pyrenees receive sea breezes that carry pollutants from the highly populated and industrialised areas on the coast and central Catalonia (Jimenez-Guerrero et al., 2008) and from central Europe (background concentrations of 50–60 ppb), pollutants that have been previously transported by north and northwest winds and added to local emissions, all of which are re-circulated by the coastal breezes (Gangoiti et al., 2001).

5. AIMS OF THE THESIS

This thesis was designed based on the following points:

a) The *Pinus uncinata* subalpine forests of the Pyrenees have a high conservation interest as they shelter protected and endangered species and are functionally important for soil and water protection and scenic landscape values. Thus, impacts on these forests and on the livelihoods of forest-dependent communities could result in major ecological, economic, and social consequences.

b) Significant increases in crown defoliation in stands of *P. uncinata* in the Pyrenees are detected by the Catalan forestry service and have been reported in a previous study (Carnicer et al., 2011).

c) Ground-level O_3 is pointed as one of the possible factors contributing to worsen the health of *P.uncinata* stands. However, O_3 concentrations in the Catalan Pyrenees have not thoroughly been studied even though this area has the conditions to present high O_3 concentrations (see section 4.4.)

d) There is no information on the *Pinus uncinata* sensitivity to O_3.

The thesis aims to fill these gaps in our knowledge. In particular, the aims of this thesis, which are subsequently developed in each chapter, are:

1) To analyze the trends of O_3 concentrations in the overall Catalan Pyrenees during the last years, and the consequent risk of damage to vegetation (Chapter 1).

2) To thoroughly describe the pattern of distribution and possible origin of O_3 in a more concrete area of the Pyrenees, along an altitudinal gradient (Chapter 2).

3) To evaluate the severity of O_3-like visible injury in two *Pinus uncinata* stands along an altitudinal gradient in the Pyrenees and assess their crown defoliation and mortality as indicators of health status (Chapter 3).

4) To describe the visible and microscopic injury potentially caused by high natural O_3 concentrations on *Pinus uncinata* growing in the field and to visually and microscopically confirm that the O_3-like symptoms are caused by O_3 (Chapter 4).

5) To determine the specific effects of O_3 on the physiology of *Pinus uncinata*, by conducting a free-air fumigation experiment with O_3 (Chapter 5).

CHAPTER 1

Trends of the AOT40 at three sites in the Catalan Pyrenees over the last 16 years

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Abstract

Ozone mixing ratios were monitored at three stations at different altitudes along the Catalan Pyrenees from 1994 to 2009. The AOT40 greatly exceeded the critical level for the protection of forest and semi-natural vegetation set by the UNECE's CLRTAP and the target value and long-term objective for the protection of vegetation set by the European Directive 2008/50/EC. The AOT40 showed an overall increasing trend over time with a slight decrease during the last 3 years, although longer-term records of O_3 levels are required before affirming with certainty a declining or stabilising trend. These results indicate that plant life in the Pyrenean region can be at risk of O_3 damage due to the high O_3 mixing ratios detected. Nevertheless, more effort is warranted to determine the uptake of O_3 by vegetation in this mountainous range. An O_3 flux-based index that takes into account the local environmental conditions, plant phenology, and nocturnal uptake of O_3 would provide a more accurate assessment of the risk from O_3 for the particular vegetation in each area.

1.1. INTRODUCTION

Vegetation may be more sensitive to the phytotoxicity of O_3 at higher than at lower altitudes (Ribas and Peñuelas, 2006). A higher sensitivity could result from an increase of O_3 mixing ratios with increasing altitude in mountainous areas (Aneja et al., 1994b; Coyle et al., 2002; Díaz-de-Quijano et al., 2009) and the enhanced stomatal conductance found at high altitudes (Wieser et al., 2000), because trees rarely have the need for restricting the loss of water in these areas (Tranquillini, 1979).

With the aim of reducing the adverse effects of atmospheric pollution, the CLRTAP (Convention on Long-range Transboundary Air Pollution) of the UNECE (United Nations Economic Commission for Europe) created the “multi-pollutant” Gothenburg Protocol in 1999 (UNECE/CLRTAP, 1999). In this protocol, critical levels of O_3 that could damage plants were determined to protect vegetation in accordance with the convention’s Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends (UNECE, 2010). The two approaches for defining critical levels of O_3 are based on either stomatal flux or O_3 concentration. Critical levels based on stomatal flux provide an estimate of the amount of O_3 entering plants through the stomata and reaching the sites of damage within the leaf. Critical levels based on concentration only consider the O_3 concentration at the top of the canopy but not the influence of climatic factors on stomatal conductance. Even though the determination of critical levels based on concentration has several important limitations and uncertainties, especially in the Mediterranean area where climatic factors strongly affect stomatal conductances, they are still used for estimating the risk of damage when climatic data or suitable flux models are not available (UNECE, 2010).

The AOT40, defined as the sum of the daylight hourly O_3 concentration, recalculated for canopy height, exceeding the threshold of 40 ppb_v over a certain period of time, is the most commonly used concentration-based critical level for forests and semi-natural vegetation. The UNECE’s CLRTAP determines different critical levels for ozone for different accumulated periods and types of vegetation (e.g. three months for semi-natural vegetation in communities dominated by annuals, six months for forest trees, etc.). An AOT40 of 5000 $ppb_v \cdot h$ over six months (April–September) has been set for the protection of forest trees and semi-natural vegetation dominated by perennials in mountainous areas of the Mediterranean region (UNECE, 2010). Above this critical level, forest trees and semi-natural vegetation begin to suffer a reduction in growth of at least 5 % and 10 %, respectively (UNECE, 2010).

Parallel to the development of the Gothenburg Protocol, the European Union established the National Emissions Ceilings Directive in 2001 (NEC Directive) that would be a step toward the EU's long-term objectives of not exceeding critical loads of harmful pollutants (Schroeder and Yocum, 2006). In 2008, the European Directive 2008/50/EC (2008) established an O_3 target value and long-term objective for the protection of vegetation without distinguishing between types of vegetation. From 2010, the 5-year running average of the AOT40 calculated from May to July was set below the target value of 9000 $ppb_v \cdot h$, while a longer-term objective was set below 3000 $ppb_v \cdot h$. The AOT40 for these targets was calculated by summing the differences between 40 ppb_v and the hourly O_3 concentrations above 40 ppb_v for the hours between 8.00 h and 20.00 h Central European Time (CET) each day.

The Catalan Pyrenees offer an excellent opportunity to study the O_3 trends in high mountains and their effects on montane, subalpine, and alpine vegetation. Most of the urban centers in the area double their populations in the summer due to tourism that, in addition to the relatively high irradiance and temperatures, can favour the increase of O_3 mixing ratios in the area. In summer, furthermore, the eastern and central parts of the Pyrenees receive sea breezes that carry pollutants from the highly populated and industrialised areas on the coast and central Catalonia (Jimenez-Guerrero et al., 2008) and from central Europe (background concentrations of 50–60 ppb_v), pollutants that have been previously transported by north and northwest winds and added to local emissions, all of which are re-circulated by the coastal breezes (Gangoiti et al., 2001). On the other hand, the Catalan Pyrenees have a Mediterranean Pyrenean climate, with the maximum rainfall occurring in summer and increasing with altitude (SMC, 2010), which differs from the typical Mediterranean climate characterised by dry and warm summers. Vegetation at these sites is thus less likely to suffer restrictions of water, and its consequently stomatal closure during the daylight right when concentrations of O_3 are the highest. The overestimation of the risk to vegetation from O_3 when using concentration-based indexes in areas with a Mediterranean climate (Simpson et al., 2007) is therefore less likely to occur in our case, since the lower restriction of water will not cause stomatal closure when levels of O_3 are at their maximum.

In the Pyrenees, concentrations of O_3 are thought to have increased by a factor of five (1.6 %/year) from the end of the 19th century to the early 1990s (Marenco et al., 1994). In the Central Catalan Pyrenees, an increase of 5.1 $ppb_v \cdot year^{-1}$ was observed at an altitude of 2300 m from 2004 to 2008 (Díaz-de-Quijano et al., 2009). Increasing concentrations of O_3 with altitude; strong yearly, seasonal, and daily variations; and the exceedance of the AOT40 thresholds for the protection of humans and vegetation have been observed in the Central Catalan Pyrenees (Díaz-de-Quijano et al., 2009; Ribas and Peñuelas, 2006). The trends of the AOT40 and the exceedance of thresholds for the protection of vegetation, with its associated risk of damage to vegetation, however, have not been thoroughly studied for the entire extent of the Catalan Pyrenees. The aims of this study were: 1) to analyse the trends over the last 16 years of the AOT40 at the three available sites in the Catalan Pyrenees, and 2) to assess the risk of damage to vegetation from O_3 in this mountain range.

1.2. MATERIALS AND METHODS

Study area

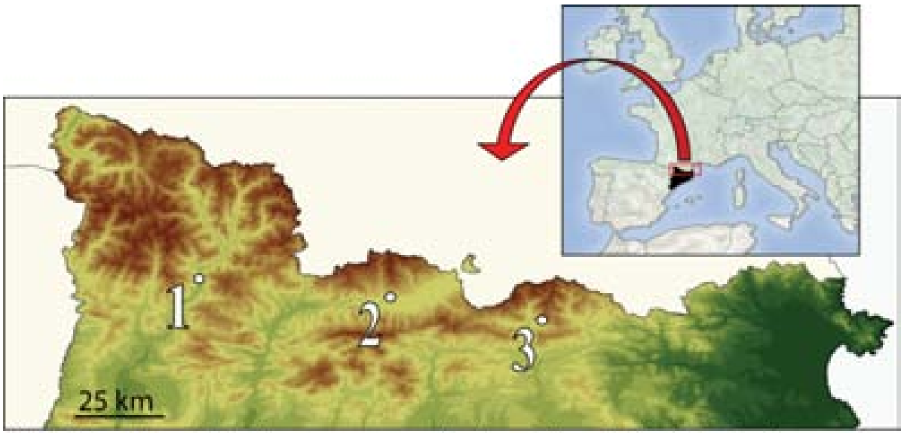


Fig. 1. Location of the three ozone-monitoring stations in the Catalan Pyrenees. 1: Sort, 692 m a.s.l.; 2: Bellver, 1040 m a.s.l.; 3: Pardines, 1224 m a.s.l.

Levels of O_3 were monitored at three permanent monitoring stations along the Catalan Pyrenees, and levels of NO_x were monitored only at the Bellver station. These stations are part of a regional network of rural monitoring stations operated by the Department of the Environment and Housing of the Government of Catalonia. All three ozone-monitoring stations are situated in villages. The village of Sort is located in the Western Catalan Pyrenees, Bellver in the Central Catalan Pyrenees, and Pardines in the Eastern Catalan Pyrenees (Fig. 1). All three villages have a population less than 2500. They are located in rural areas with abundant vegetative cover, experience the typical pattern of montane winds, and have almost no industrial activity. Bellver and Pardines can also receive sea breezes in the late afternoon during summer. Sort is not close to large urban areas, and Bellver and Pardines are 18 km and 20 km, respectively, from the nearest urban areas, which are considered small but have seasonally changing populations due to tourism (XVPCA, 2001)(Table 1).

Ozone monitoring and AOT40 calculation

Monitoring levels of ozone began in January 1994 in Sort and Bellver and one year later in Pardines. Levels of NO_x were monitored only in Bellver from 2004 to 2009. Measurements of ozone were made with Thermo 49i analysers (Thermo Scientific, Inc., Franklin, USA), and levels of NO_x were determined by using chemiluminescence with a Casella Monitor ML9841B (Casella Measurement Ltd., Bedford, UK). Data were collected at intervals of one minute, and then hourly averages were subsequently computed. The AOT40 was calculated following the methods described in the European Directive 2008/50/EC. For each given period, the AOT40 was calculated by summing the differences between 40 ppb_v and the hourly ozone concentrations above 40 ppb_v for

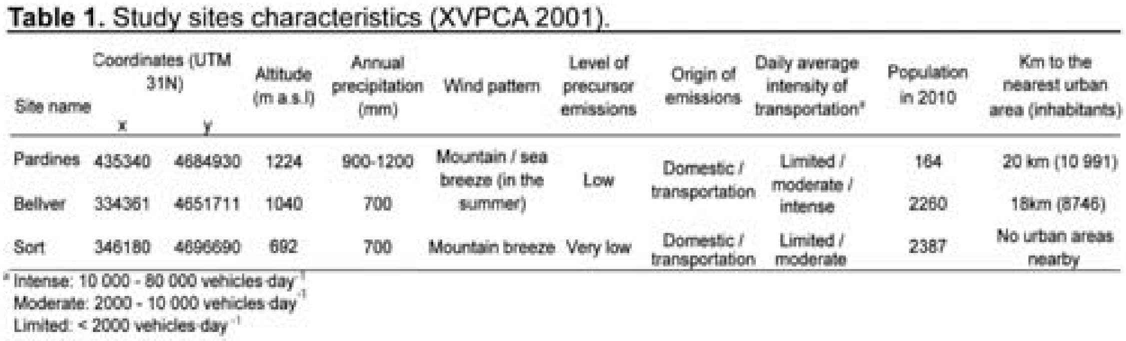


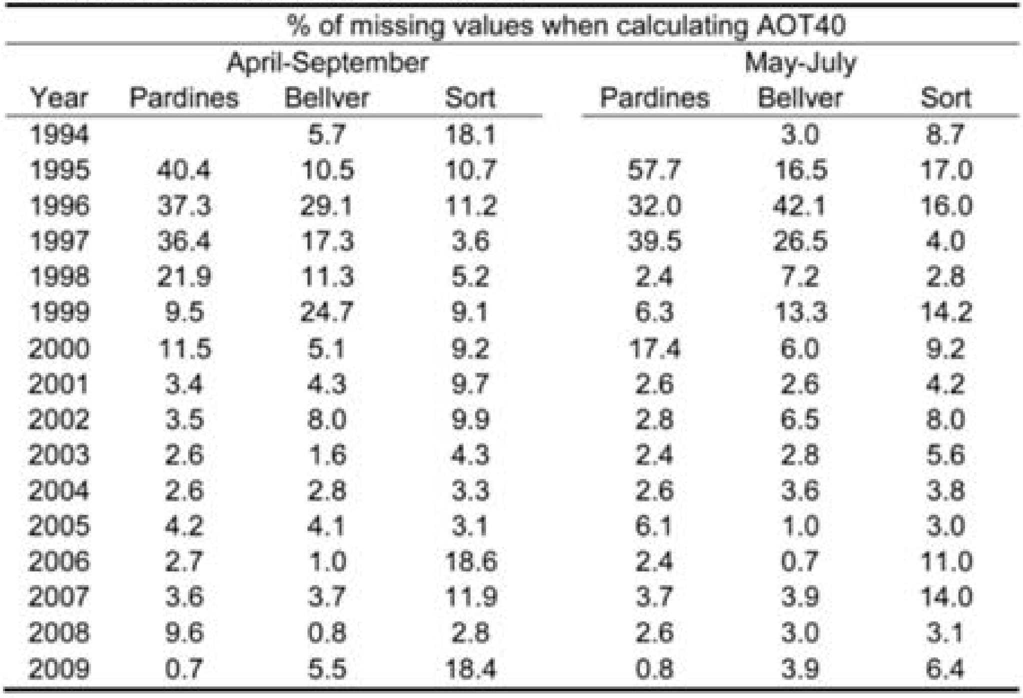
Table 1. Study sites characteristics (XVPCA 2001).

Site name	Coordinates (UTM 31N)		Altitude (m a.s.l)	Annual precipitation (mm)	Wind pattern	Level of precursor emissions	Origin of emissions	Daily average intensity of transportation[a]	Population in 2010	Km to the nearest urban area (inhabitants)
	x	y								
Pardines	435340	4684930	1224	900-1200	Mountain / sea breeze (in the summer)	Low	Domestic / transportation	Limited / moderate / intense	164	20 km (10 991)
Bellver	334361	4651711	1040	700					2260	18km (8746)
Sort	346180	4696690	692	700	Mountain breeze	Very low	Domestic / transportation	Limited / moderate	2387	No urban areas nearby

[a] Intense: 10 000 - 80 000 vehicles·day^{-1}
Moderate: 2000 - 10 000 vehicles·day^{-1}
Limited: < 2000 vehicles·day^{-1}

the hours between 8.00 and 20.00 Central European Time (CET) each day. When less than 90% of all possible measured data was available, the following factor was used to calculate AOT40 values: $AOT40_{estimate} = AOT40_{measured}$ * (total number of hours of the period) / (number of measured hourly values) (Directive, 2008). The percentage of missing values for the calculation of each annual AOT40 Apr-Sep and May-July is given in Table 2.

Table 2. Percentage of missing values when calculating AOT40 from April to September and from May to July for each site and year.



	% of missing values when calculating AOT40					
	April-September			May-July		
Year	Pardines	Bellver	Sort	Pardines	Bellver	Sort
1994		5.7	18.1		3.0	8.7
1995	40.4	10.5	10.7	57.7	16.5	17.0
1996	37.3	29.1	11.2	32.0	42.1	16.0
1997	36.4	17.3	3.6	39.5	26.5	4.0
1998	21.9	11.3	5.2	2.4	7.2	2.8
1999	9.5	24.7	9.1	6.3	13.3	14.2
2000	11.5	5.1	9.2	17.4	6.0	9.2
2001	3.4	4.3	9.7	2.6	2.6	4.2
2002	3.5	8.0	9.9	2.8	6.5	8.0
2003	2.6	1.6	4.3	2.4	2.8	5.6
2004	2.6	2.8	3.3	2.6	3.6	3.8
2005	4.2	4.1	3.1	6.1	1.0	3.0
2006	2.7	1.0	18.6	2.4	0.7	11.0
2007	3.6	3.7	11.9	3.7	3.9	14.0
2008	9.6	0.8	2.8	2.6	3.0	3.1
2009	0.7	5.5	18.4	0.8	3.9	6.4

Meteorological data

Meteorological data for each site was obtained from the Net of Automatic Meteorological Stations that belongs to the Meteorological Service of Catalonia (SMC). These stations provided us with hourly averages of wind speed and direction, temperature, and accumulated precipitation.

Statistical analyses

Correlation analyses were performed between the AOT40 values and the accumulated precipitation from April to September for AOT40 Apr-Sep and from May to July for AOT40 May-July, at each of the three sites. Daily mean and maximum ozone concentrations and temperature, and daily mean wind speed from April to September from 1994 to 2009 were also tested for correlation. Correlations between nocturnal ozone concentrations and nocturnal wind speed and temperature from April to September were performed using daily means calculated with the hourly averages from 20.00h to 7.00h (local time).

All correlations and the analyses of AOT40 trends over time at each site were performed with STATISTICA version 6 software.

1.3. RESULTS

Average daily patterns of ozone mixing ratios from April to September and from October to March for the years 1994-2009 are shown in Fig. 2. During spring and summer (April to September), the two valley-bottom sites (Sort and Bellver) showed the characteristic pattern of minimum values in the early morning (8.00h), a significant rise during the morning with increasing solar radiation and wind speed, peaking in the afternoon between 15.00h and 18.00h (CET), and then declining during the night (probably due to NO titration). Nevertheless, this daily pattern faded with altitude. Only a small daily variation was found at Pardines (1224 m a.s.l.), where ozone mixing ratios were also high during the night. During fall and winter, the daily pattern at Sort and

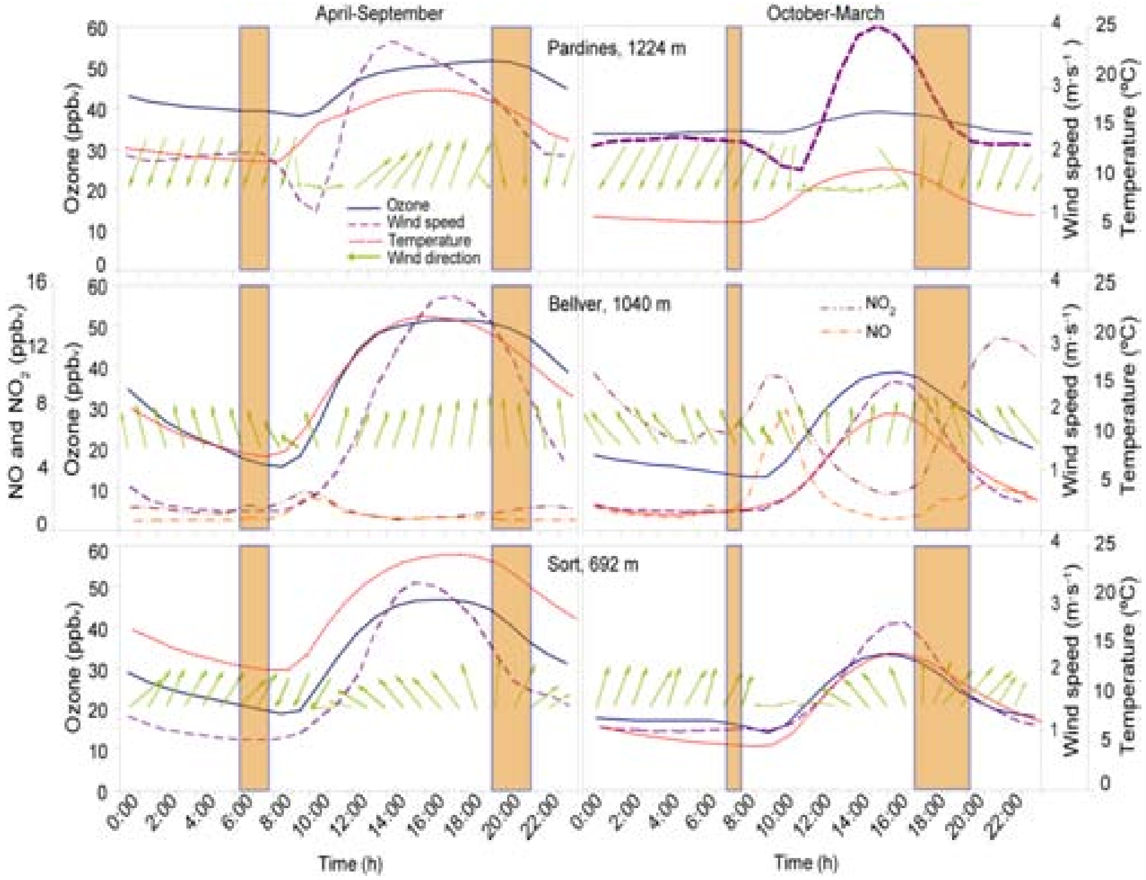
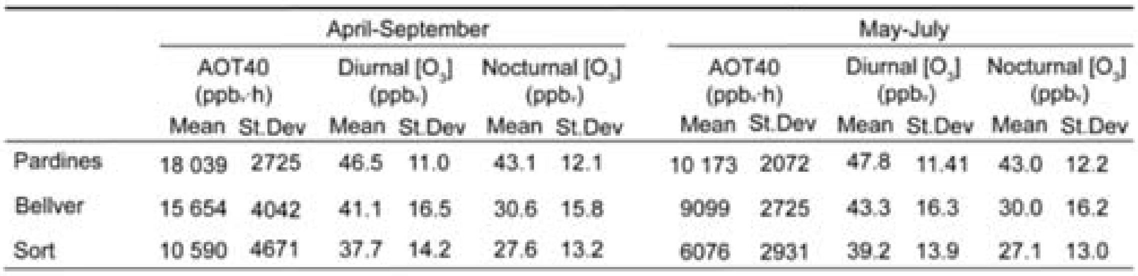


Fig. 2. Diurnal patterns of ozone, wind speed, wind direction (note that arrows point the direction where the wind is blowing to) and temperature from April to September (first column) and from October to March (second column) for years 1994-2009 in Pardines, Bellver and Sort (n = 2060-2777). NO_2 and NO data is only available for Bellver station from 2004 to 2009 (n = 1018-1053). Vertical bands indicate approximate time for sunrise and sunset.

Bellver was similar to the pattern observed for spring and summer. Minimum ozone values occurred one hour later in the morning (9.00h), also increased during the morning with increasing solar radiation and wind speed but peaked earlier than in spring and summer (between 14.00 and 17.00 (CET)), and then declined during the night. At Pardines, the daily variation of ozone was even smaller than during spring and summer. At all stations, the ozone maximum was between 30 and 40 ppb_v from October to March. Peak ozone concentrations did not occur at night at any of the sites for any of the stations, and nocturnal mean ozone concentrations were much lower than 40 ppb_v at Bellver and Sort and around 40 ppb_v at Pardines (Table 3). The critical levels for the protection of forest and semi-natural vegetation set by the UNECE's CLRTAP and the long-term objective for the protection of vegetation set by the European Directive 2008/50/EC have been greatly exceeded since 1994 at all stations (Fig. 3a and 3c, Table 3). Only the lowest location (Sort, 692 m a.s.l.) had a five-year running average of the AOT40 (May-July) below the target value (Fig. 3b).

Table 3. AOT40 and diurnal (8.00h-19.00h, local time) and nocturnal (20.00h-7.00h, local time) O_3 concentrations for the Apr-Sept and May-Jul periods from 1994 to 2009 in Bellver and Sort and from 1995 to 2009 in Pardines. Note that critical level for the AOT40 Apr-Sept is set at 5000 ppbv•h and long-term objective for the AOT40 May-Jul at 3000 ppb_v•h.



	April-September						May-July					
	AOT40 (ppb_v·h)		Diurnal $[O_3]$ (ppb_v)		Nocturnal $[O_3]$ (ppb_v)		AOT40 (ppb_v·h)		Diurnal $[O_3]$ (ppb_v)		Nocturnal $[O_3]$ (ppb_v)	
	Mean	St.Dev	Mean	St.Dev	Mean	St.Dev	Mean	St.Dev	Mean	St.Dev	Mean	St.Dev
Pardines	18 039	2725	46.5	11.0	43.1	12.1	10 173	2072	47.8	11.41	43.0	12.2
Bellver	15 654	4042	41.1	16.5	30.6	15.8	9099	2725	43.3	16.3	30.0	16.2
Sort	10 590	4671	37.7	14.2	27.6	13.2	6076	2931	39.2	13.9	27.1	13.0

The AOT40 Apr-Sep over time fit to a quadratic polynomial curve for Sort (R^2 = 0.78, $p < 0.001$; Fig. 3a). It increased at a rate of 1621 ppb_V·h·year^{-1} from 1994 to 2001 but later slowly became more stable. The five-year running average of AOT40 May-July showed a statistically significant increasing trend over time at Pardines, with a rate of increase of 267 ppb_V·h·year^{-1}, and at Bellver, with a rate of increase of 177 ppb_V·h·year^{-1} (Pardines: R^2 = 0.78, $p < 0.001$; Bellver: R^2 = 0.56, $p < 0.01$; Fig. 3b). At Sort, the five-year running average of AOT40 May-July fit better to a quadratic polynomial curve (R^2 = 0.96, $p < 0.001$; Fig. 3b) with a rate of increase of 703 ppb_V·h·year^{-1} until the 2002-2006 average. The AOT40 May-July did not show a

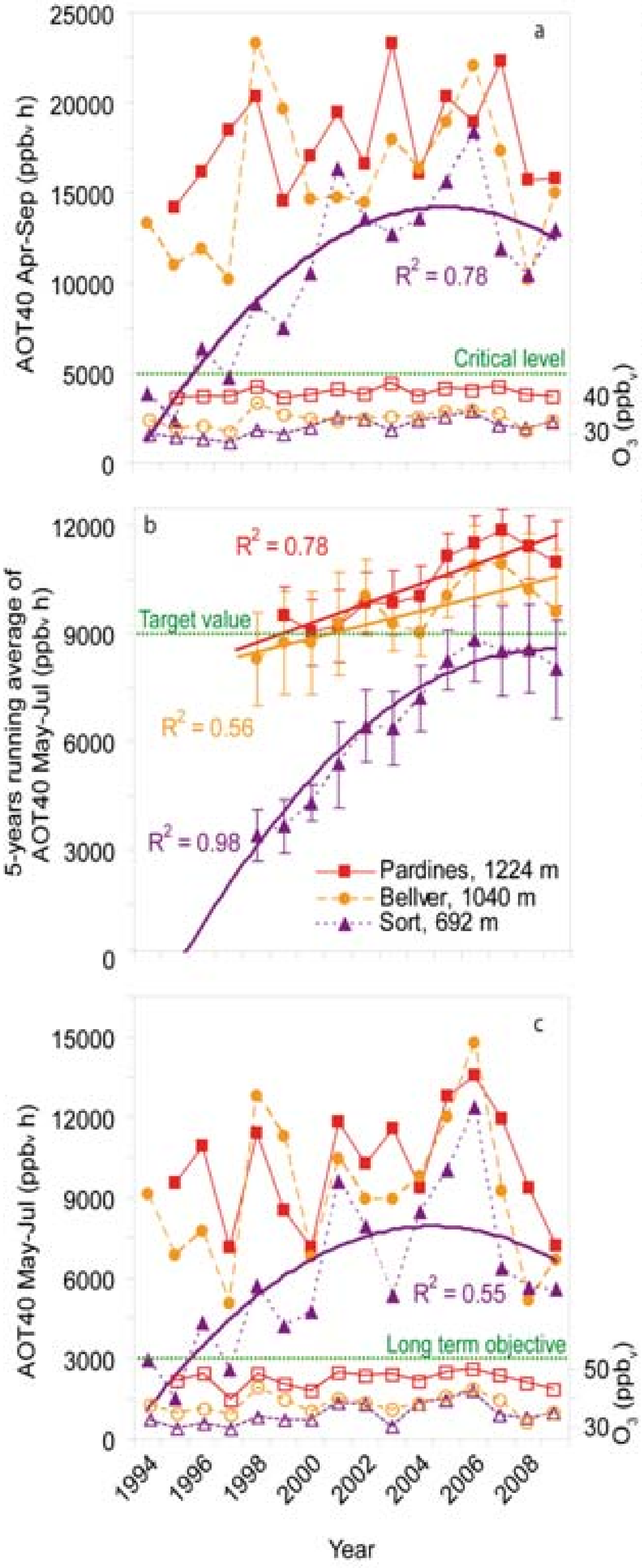


Fig. 3. Yearly AOT40 from 1994 until 2009 in Sort, Bellver and Pardines. a: AOT40 calculated over six months (April-September), critical level at 5000 ppb$_v$•h; b: 5-years running average of the AOT40 calculated over three months (May-July) ± SEM (Standard Error of the Mean, n = 5), target value at 9000 ppb$_v$•h. First value represented in the graph corresponds to the AOT40 average for years 1994-1998, the second one corresponds to 1995-1999, and so on; c: AOT40 calculated over three months (May-July), long-term objective at 3000 ppb$_v$•h. (Line fitting equations: a: Sort: $y = -107x^2 + 427897x - 4.29E+08$; b: Pardines: $y = 267x - 525561$, Bellver: $y = 177x - 344765$, Sort: $y = -49x^2 + 195789x - 1.97E+08$; c: Sort: $y = -63x^2 + 252291x - 2.52E+08$). Open figures in a) and c) show ozone means for each year from April to September (a) and from May to July (c).

statistically significant increasing trend at Pardines and Bellver, whereas at Sort, it increased at a rate of 735 ppb$_V$·h·year^{-1} until 2006 (best fit for a quadratic polynomial curve: $R^2 = 0.55$, $p < 0.01$; Fig. 3c). Nonetheless, seasonal mean ozone concentrations from April to September and from May to July did not show any significant increasing trend over time (Fig. 3a and 3c).

No significant correlations were found between AOT40 values and accumulated rainfall at any of the three sites. Daily mean and maximum ozone concentrations, however, showed a statistically significant relationship with daily mean and maximum temperatures and mean wind speed at the three sites (Table 4). Nocturnal ozone concentrations showed a positive statistically significant relationship with wind speed at the three sites, whereas nocturnal ozone concentrations and temperature were significantly correlated only at Pardines (Table 4).

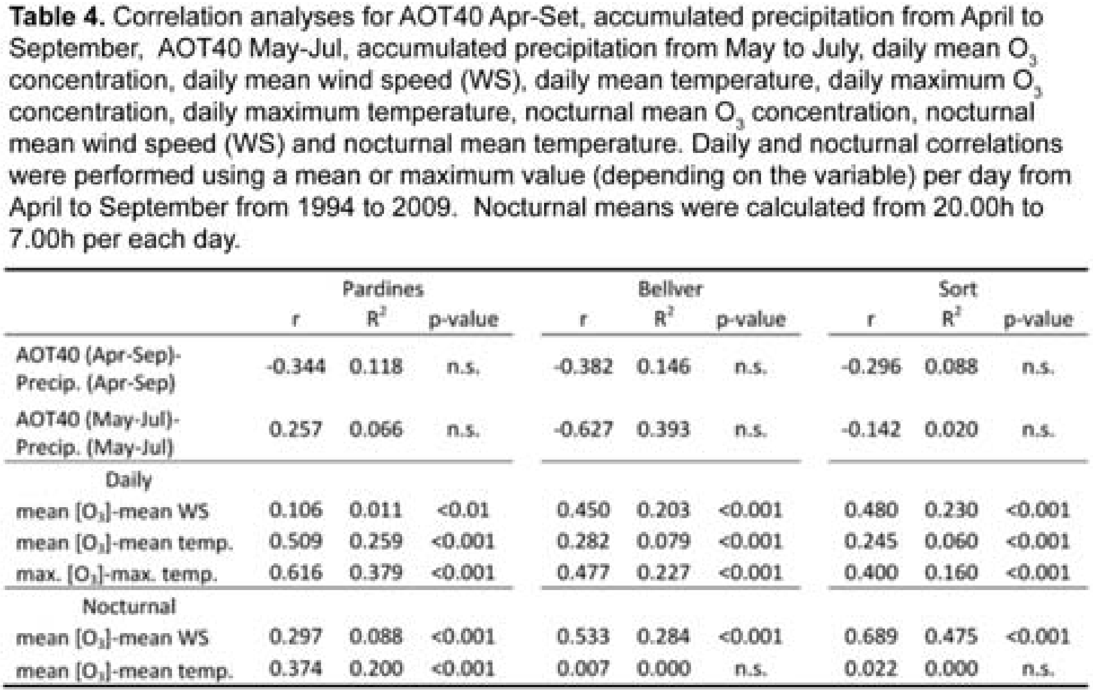


Table 4. Correlation analyses for AOT40 Apr-Set, accumulated precipitation from April to September, AOT40 May-Jul, accumulated precipitation from May to July, daily mean O_3 concentration, daily mean wind speed (WS), daily mean temperature, daily maximum O_3 concentration, daily maximum temperature, nocturnal mean O_3 concentration, nocturnal mean wind speed (WS) and nocturnal mean temperature. Daily and nocturnal correlations were performed using a mean or maximum value (depending on the variable) per day from April to September from 1994 to 2009. Nocturnal means were calculated from 20.00h to 7.00h per each day.

	Pardines			Bellver			Sort		
	r	R^2	p-value	r	R^2	p-value	r	R^2	p-value
AOT40 (Apr-Sep)-Precip. (Apr-Sep)	-0.344	0.118	n.s.	-0.382	0.146	n.s.	-0.296	0.088	n.s.
AOT40 (May-Jul)-Precip. (May-Jul)	0.257	0.066	n.s.	-0.627	0.393	n.s.	-0.142	0.020	n.s.
Daily									
mean $[O_3]$-mean WS	0.106	0.011	<0.01	0.450	0.203	<0.001	0.480	0.230	<0.001
mean $[O_3]$-mean temp.	0.509	0.259	<0.001	0.282	0.079	<0.001	0.245	0.060	<0.001
max. $[O_3]$-max. temp.	0.616	0.379	<0.001	0.477	0.227	<0.001	0.400	0.160	<0.001
Nocturnal									
mean $[O_3]$-mean WS	0.297	0.088	<0.001	0.533	0.284	<0.001	0.689	0.475	<0.001
mean $[O_3]$-mean temp.	0.374	0.200	<0.001	0.007	0.000	n.s.	0.022	0.000	n.s.

1.4. DISCUSSION

The AOT40 Apr-Sep at the three stations in the Pyrenees was 2-5 times higher (depending on the site and year) than the critical level for the protection of forest and semi-natural perennial vegetation set by the UNECE's CLRTAP. Regarding the ozone thresholds to be achieved over a longer time set by the European Directive 2008/50/EC, only one of the stations (Sort) showed a five-year running average of the AOT40 (May-July) below the target value to be achieved from 2010, whereas the long-term objective set at 3000 ppb_V·h was exceeded by factors of 1.4-5, depending on the year and site. Although ozone thresholds have been greatly exceeded in our study, other rural areas with similar altitude in the Mediterranean basin have higher values of AOT40 (Paoletti et al., 2007; Sicard et al., 2011). For example, Pino Torinese, at 600 m a.s.l. in northern Italy, had an average of 21560 ppb_V·h for the AOT40 May-July for 2000-2004, whereas Sort, at 692 m a.s.l., had an average of 7196 ppb_V·h for the same period (Paoletti et al., 2007). Air quality at Pino Torinese, though, is likely influenced by of the city of Turin, whereas Sort is a small village far from any metropolitan area. The AOT40 measured in Mercantour National Park (south-eastern France) is also higher than the values found in the Pyrenees. Cians, at 1484 m a.s.l. in Mercantour National Park, had a mean AOT40 May-July of 17818 ppb_V·h for the period 2001-2008 (Sicard et al., 2011), whereas Pardines had an average of 11340 ppb_V·h for the same period. Thus, although the Pyrenees show lower values of AOT40 than other sites at similar altitude in the Mediterranean area, sites at higher elevations, such as Cians and Pardines, have more similar AOT40 values than sites at lower elevations, such as Pino Torinese and Sort.

The exceedance of the critical level for ozone as it occurs in at our sites has been positively correlated with typical ozone-induced injuries in vegetation in several previous studies, under both controlled experiments (Gimeno et al., 2004; Manninen et al., 2003a; Marzuoli et al., 2009; Peñuelas et al., 1994; Ribas et al., 2005) and field conditions (Calatayud et al., 2007; Cvitas et al., 2006; Waldner et al., 2007). Furthermore, the AOT40 is higher at higher altitude, as has been seen in other mountainous areas where ozone mixing ratios rise with increasing altitude (Aneja et al., 1994a; Aneja et al., 1994b; Cooper and Peterson, 2000; Ribas and Peñuelas, 2006). Vegetation at these montane sites and also at higher altitudes can thus be affected by ozone, since exposures to ozone are already much above the critical level and tend to increase with altitude. In fact, most of the forest trees and herbaceous species found along the altitudinal gradient in the Pyrenees have been found to be sensitive to ozone. *Fagus sylvatica, Pinus sylvestris*, *Pinus uncinata* and *Betula pendula* are some of the ozone-sensitive tree species (Díaz-de-Quijano et al., 2012b; Karlsson et al., 2003) distributed along the montane and subalpine clearings in the Pyrenees (Vigo, 2009). *Trifolium* ssp., *Phleum alpinum, Leontodon hispidus, Dianthus deltoides, Valeriana officinalis, Hieracium pilosella*, and *Silene acaulis* are ozone-sensitive semi-natural herbaceous species (Hayes et al., 2007) also present in the Pyrenees (Vigo, 2009).

Even though nocturnal peaks in the levels of ozone have not been detected in the daily patterns, the stomatal uptake of ozone at night cannot be totally discarded. Some authors have reported significant nocturnal stomatal conductances (Caird et al., 2007; Daley and Phillips, 2006; Dawson et al., 2007), and the nocturnal uptake of ozone has been found to account for 24% of the total daily uptake even though the ozone concentrations were very low at night (Mereu et al., 2009). Whole-plant production by *Betula pendula* was more sensitive to nocturnal exposure to ozone compared to daytime exposure (Matyssek et al., 1995). Other tree species in the Pyrenees, such as *Abies alba, Larix decidua*, and *Pinus sylvestris*, also have significant nocturnal leaf conductances and/or incomplete stomatal closure at night (Caird et al., 2007). Thus, the AOT40 may be underestimating the real risk of O_3 to vegetation at these sites. In contrast, the AOT40 has been found to overestimate the risk of O_3 to vegetation in southern Europe compared to an O_3 flux-based index (Simpson et al., 2007).

Unlike the typical Mediterranean climate characterised by dry and warm summers, the Pyrenees present a Mediterranean Pyrenean climate with the maximum rainfall occurring in summer and increasing with altitude (SMC, 2010). Hence, in our study, the AOT40 might not overestimate the risk of O_3 to vegetation, because the vegetation at these sites is less likely to suffer restrictions of water, thereby preventing the typical stomatal closure that normally occurs in Mediterranean ecosystems during the day when drought and the highest concentrations of O_3 co-occur (Gerosa et al., 2009). The calculation of the AOT40 from April to September does not take into account the ozone concentrations during fall and winter that can affect the perennial montane vegetation. Even though the hourly mean ozone concentrations from October to March are usually below 40 ppb_v, as it can be seen in the daily patterns, hourly values may be higher in some cases. Further research is thus needed to determine if ozone concentrations in fall and winter should be considered when establishing critical levels for the risk of damage to perennial vegetation from ozone. In any case, a flux-based index that takes into account local environmental parameters, plant phenology, and the possible nocturnal uptake of ozone will be more appropriate than the exposure-based index when determining the real risk of O_3 to vegetation in the Catalan Pyrenees.

Even though the increasing trend of the AOT40 over time is clear for all AOT40 calculations only at Sort and for the AOT40 May-July five-year running average at

Bellver and Pardines, the AOT40 seems to have stabilised or even slowly decreased beginning in 2007. In fact, Sort has experienced an increase in emissions of ozone precursors in recent years due to the relatively recent development of an important tourism industry for winter and nature sports (Àvila et al., 2010). In the period 1994-2009, Sort's population increased 48% (INE 2010), and the number of vehicles increased 41.4% during the period 1997-2007 (IDESCAT, 2011). The populations of Bellver and Pardines increased 40% and 18% during the period 1994-2009 (INE 2010), with 29.5% and 32.3% increases in vehicles for the 1997-2007 period, respectively (IDESCAT, 2011). These changes may explain why Sort had lower ozone concentrations during the earlier years but a higher rate of increase of AOT40 than the other two sites.

Total emissions of NO_x in Spain increased 16.9% from 1990 to 2007 (EEA, 2009) but have decreased 24% in the last three years (1263.65 Gg in 2007 to 966.50 Gg in 2009) (EEA, 2011). The increase in emissions of NO_x is due to a 13.6% increase in the Spanish population, a 58% increase in gross domestic product, and a 61% increase in the consumption of primary energy during 1990-2005 (INE, 2011). Similarly, the AOT40 showed a general increasing trend from 1994 until 2006-2007 and a decreasing trend since 2007, which may be explained by the similar patterns observed in the total emissions of NO_x in Spain. Longer-term ozone records, however, would be necessary to confidently affirm the declining or stabilising trend of recent years. The increasing trends of AOT40 over time together with the absence of trends in the seasonal means suggest that ozone concentrations increase from year to year during daylight hours - when ozone concentrations account for the calculations of AOT40, whereas at night, ozone concentrations decrease from year to year, balancing the overall seasonal mean. In effect, increases in emissions of NO_x over time at these rural sites (Àvila et al., 2010) favour the photochemical production of ozone during the day and the depletion of ozone during the night due to NO_x titration.

Variations from year to year in the AOT40 values do not appear to be related to rainfall, whereas daily mean and maximum O_3 concentrations did show a positive significant relationship with daily mean and maximum temperatures and mean wind speed at the three sites. Nocturnal ozone concentrations were positively correlated with wind speed at all three sites and with temperature at Pardines. These correlations suggest that nocturnal temperature inversions and low wind speeds may lead to low ozone concentrations. High ozone concentrations are thus more likely to occur with moderate or strong winds that carry pollutants from highly polluted areas rather than from the accumulation of pollutants under stagnant atmospheric conditions caused by temperature inversions and very low winds. Hence, the yearly variations in AOT40 appear to be related more to differences in the long-range transport of pollutants or in the local production of ozone from varying emissions of precursors and from warming temperatures.

Even though the three monitoring stations do not provide a complete spatial coverage of ozone pollution in this mountainous range, the current data set provides an indication of what we may encounter in the Catalan Pyrenees as a whole. More O_3 monitoring stations are nevertheless required to obtain a more complete spatial coverage representing a greater range of altitudes. More extensive monitoring would enable us to determine if the increase of AOT40 found at the higher elevations is an effect of altitude or the nearby urban areas.

1.5. CONCLUSIONS

The AOT40 has greatly exceeded the critical levels for the protection of forests and semi-natural vegetation set by the UNECE's CLRTAP and the long-term objective for the protection of vegetation set by the European Directive 2008/50/EC at the three studied sites (Pardines (1224 m a.s.l.), Bellver (1040 m a.s.l.) and Sort (692 m a.s.l.)). However, the target value for the protection of vegetation set by the European Directive 2008/50/EC has only been exceeded at the two highest stations in the Catalan Pyrenees (Pardines and Bellver). The AOT40 also shows an increasing trend over time with a slight decrease during the last three years, although longer-term ozone records are required before affirming with certainty a declining or stabilising trend. These results indicate that plant life in the Catalan Pyrenees is at risk of damage by ozone. Exceedances of the critical levels, target values, and long-term objectives are greater the higher the altitude. Plant life is therefore expected to be under a higher risk of damage from ozone, specifically at high-altitude sites. Nevertheless, calculation of an ozone flux-index that takes into account the local environmental conditions, plant phenology, and possible nocturnal uptake of ozone is needed to more realistically assess the risk from ozone for vegetation in this mountainous area.

Acknowledgements

We are grateful to the Department of the Environment and Housing of the Government of Catalonia for providing the ozone data from the three stations. This research was funded by the Spanish Government projects CGL 2006-04025/1305, CGC 2010-17172 and Consolider Montes (CSD 2008-00040), and by the Catalan Government project SGR 2009-458.

CHAPTER 2

Increasing interannual and altitudinal ozone mixing ratios in the Catalan Pyrenees

Díaz-de-Quijano, M., Peñuelas, J., Ribas, À., 2009. Increasing interannual and altitudinal ozone mixing ratios in the Catalan Pyrenees. Atmospheric Environment 43, 6049-6057.

Abstract

Interannual, seasonal, daily and altitudinal patterns of tropospheric ozone mixing ratios, as well as ozone phytotoxicity and the relationship with NO_x precursors and meteorological variables were monitored in the Central Catalan Pyrenees (Meranges valley and Forest of Guils) over a period of 5 years (2004-2008). Biweekly measurements using Radiello passive samplers were taken along two altitudinal transects comprised of thirteen stations ranging from 1040 to 2300 meters a.s.l.. Visual symptoms of ozone damage in Bel-W3 tobacco cultivars were evaluated biweekly for the first three years (2004-2006). High ozone mixing ratios, always above forest and vegetation protection AOT40 thresholds, were monitored every year. In the last 14 years, the AOT40 (Apr-Sept.) has increased significantly by 1047 $\mu g \cdot m^{-3} \cdot h$ per year. Annual means of ozone mixing ratios ranged between 38 and 67 ppb_v (38 and 74 ppb_v during the warm period) at the highest site (2300 m) and increased at a rate of 5.1 $ppb_v \cdot year^{-1}$. The ozone mixing ratios were also on average 35 - 38 % greater during the warm period and had a characteristic daily pattern with minimum values in the early morning, a rise during the morning and a decline overnight, that was less marked the higher the altitude. Whereas ozone mixing ratios increased significantly with altitude from 35 ppb_v at 1040 m to 56 ppb_v at 2300 m (on average for 2004-2007 period), NO_2 mixing ratios decreased with altitude from 5.5 ppb_v at 1040 m to 1 ppb_v at 2300 m. The analysis of meteorological variables and NO_x values suggests that the ozone mainly originated from urban areas and was transported to high-mountain sites, remaining aloft in absence of NO. Ozone damage rates increased with altitude in response to increasing O_3 mixing ratios and a possible increase in O_3 uptake due to more favorable microclimatic conditions found at higher altitude, which confirms Bel-W3 as a suitable biomonitor for ozone concentrations during summer time. Compared to the valley bottom site the annual means of ozone mixing ratios are 37 % larger in the higher sites. Thus the AOT40 for the forest and vegetation protection threshold is greatly exceeded at higher sites. This could have substantial effects on plant life at high altitudes in the Pyrenees.

2.1. INTRODUCTION

During the pre-industrial period ozone concentrations were approximately 10 -15 ppb_v (Finlayson-Pitts and Pitts, 1997). However, these concentrations rapidly increased when NO_x emissions became more intense due to the switch to fossil fuels that took place in the industrial revolution (Finlayson-Pitts and Pitts, 1997). Nowadays, the annual average background ozone concentrations over the mid-latitudes of the Northern Hemisphere ranges approximately between 20 - 45 ppb_v, with variability depending on location, atmospheric elevation and distance to other pollutants' emission sources (Finlayson-Pitts and Pitts, 2000).

Overall, in mountainous areas ozone mixing ratios seem to rise with increasing altitude (Aneja et al., 1994a; Aneja et al., 1994b; Cooper and Peterson, 2000; Ribas and Peñuelas, 2006; Sanz et al., 2007; Skelly et al., 1997). Higher solar radiation (Volz and Kley, 1988), local air recirculation linked to orographic systems (eg. valley-mountain systems or mixing boundary layers) (Lefhon, 1992; Sanz and Millán, 2000), ozone intrusions from the stratosphere (Viezee et al., 1983) and less chemical ozone loss under low NO concentrations (Naja et al., 2003) are all factors which contribute to high ozone mixing ratios at high altitude. Moreover, stomatal conductance is found to increase with rising altitude (Wieser et al., 2000), since trees rarely have the need for restricting water loss at high altitudes (Tranquillini, 1979). Therefore, vegetation at higher altitudes can be more easily affected by ozone toxicity than at lower sites (Ribas and Peñuelas, 2006).

The distribution of ozone in mountainous regions has been studied in Europe in the Alps (Chevalier, 2007) and the Carpathian Mountain Range (Manning et al., 2002) and, in North America in the White Mountains, Adirondack Mountains (Aneja et al., 1994a; Aneja et al., 1994b), Appalachian Mountains (Skelly et al., 1997), San Bernardino Mountains (Arbaugh et al., 2003; Arbaugh et al., 1998; Bytnerowicz et al., 2007a), Sierra Nevada (Arbaugh et al., 1998), Wasatch Mountains (Wager and Baker, 2003) and in the mountains around Mexico City (De Bauer et al., 1985). Although ozone concentrations have been studied in the Mediterranean area (Gimeno et al., 1995; Ribas and Peñuelas, 2000, 2004; Sanz et al., 2000; Ziomas, 1998), no thorough studies have been conducted in the mountain ranges of the Mediterranean region in Europe. In this region, preliminary studies have indicated high ozone mixing ratios which are often above the human and vegetation protection threshold and strong interannual, seasonal and daily variations (Ribas and Peñuelas, 2006).

The aim of this study was to describe the interannual, seasonal and daily pattern of distribution of ozone in the mountains of the European Mediterranean area by monitoring ozone concentrations along two altitudinal gradients in the Pyrenees during a period of five years. We also studied ozone phytotoxicity in order to discern the suitability of Bel-W3 for biomonitoring ozone mixing ratios in long-term studies and the relationship between ozone mixing ratios and variations in NOx precursors, as well as meteorological variables with the aim of determining the possible source of ozone in this area.

2.2. MATERIAL AND METHODS

Field monitoring set up

From 2004 to 2007, during the whole year, and in 2008, during summer, thirteen sites were studied along two altitudinal gradients: one from 1040 meters a.s.l. to 2300 meters a.s.l. on the south-facing slopes of Puigpedrós (Meranges transect) and the other one from 1300 m a.s.l. to 2200 m a.s.l. facing north-east (Guils transect). Both were in the La Cerdanya county, located in the Central Catalan Pyrenees (Bellver 42º 22’N, 1º 46’E). The sampling sites were located approximately every 200 m in altitude (Fig. 1) in a forested area dominated by *Pinus uncinata*. Ozone mixing ratios and their phytotoxicity, as well as NO_2 mixing ratios were measured at all stations every two weeks in the warm period and once a month in the cold period. Phytotoxicity was

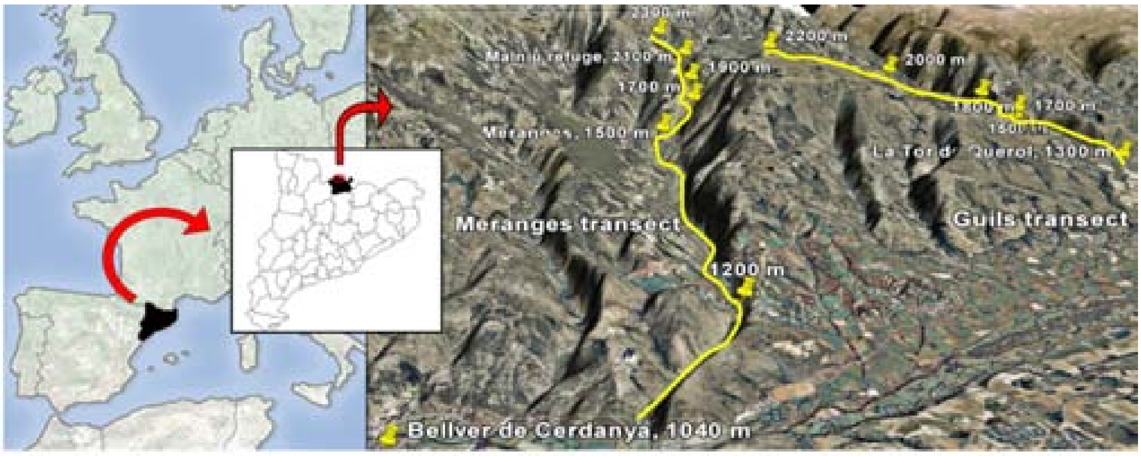


Fig. 1. Map of the altitudinal transect sites studied in the region of La Cerdanya in the central Catalan Pyrenees.

measured during the warm period from 2004 to 2006 at all sampling sites except the highest one at 2300 m since the irregularity of terrain hindered the installation of plants and the weather was excessively cold for the tobacco plants used as biomonitors.

Physico-Chemical measurements

Radiello radial symmetry passive samplers (Cocheo et al., 1996) were used to analyze ozone and nitrogen dioxide at all sampling sites. The use of these samplers is very common in studies conducted in remote areas (Cooper and Peterson, 2000; Krupa and Legge, 2000) where logistic limitations such as power supplies make it difficult to accurately quantify spatial patterns.

At 1040 m a.s.l., at Bellver, the lowest valley-bottom site of the south-facing transect, and at 1500 m a.s.l., in the village of Meranges, ozone measurements were also performed with a Thermo 49i analyzer (Thermo, Franklin, USA) in the first station and with a SIR S-5014 analyzer (SIR, Madrid, Spain) in the latter. NO_x levels were determined by using chemiluminescence with a Casella Monitor ML9841B (Casella Measurement Ltd., Bedford, UK) in Bellver and a SIR S-5012 (SIR, Madrid, Spain) in Meranges. These two sites are part of a regional network of rural monitoring stations run by the Department of the Environment and Housing of the Government of Catalonia (Generalitat de Catalunya) that consists of a network of ozone monitors and meteorological stations. The second station (Meranges, at 1500 m a.s.l.) was monitored with a mobile unit only during summer 2008.

Intercalibrations were made between passive samplers and monitors at these points. As in other previous works (Krupa and Legge, 2000; Skelly et al., 2001), close relationships were found between passive and active measurements (Ozone mixing $\text{ratio}_{\text{passive sampler}} = 0.9468\text{Mixing ratio}_{\text{monitor}}+7.4734$, $R^2=0.73$, $p<0.0001$). Nitrogen dioxide from passive samplers was also significantly correlated with NO_2 active measurements ($\text{Mixing ratio}_{\text{passive sampler}} = 0.5958\text{Mixing ratio}_{\text{monitor}}+1.8214$, $R^2=0.65$, $p<0.001$).

Temperature and humidity were measured at six of the sampling sites by means of a weatherproof Temperature/RH Datalogger (HOBO Pro, Sistemas y Instalaciones, Madrid, Spain), with sensors located at 1500, 2100 and 2300 m a.s.l. in the south facing transect, and at 1300, 1800 and 2200 m a.s.l. in the north-east transect. These parameters in addition to wind direction and speed were also available for the valley-bottom station (1040 m a.s.l.) and Meranges mobile unit (1500 m a.s.l.) operated by the Department of the Environment and Housing of the Government of Catalonia. Extrapolations from linear models for each biweekly period were used to estimate the temperature for the rest of sites.

Description of ozone phytotoxic levels: active bioindication

Phytotoxic effects were evaluated in terms of the ozone damage present in sensitive tobacco (*Nicotiana tabacum*) cultivars Bel-W3 and the resistant cultivar Bel-B that are commonly used in ozone bio-indicator programs (Heggestad, 1991; Klumpp et al., 2002). Tobacco seeds were germinated in ozone-free conditions to obtain homogeneous plants, thereby avoiding any possible early ozone contamination. When seedlings were at the fourth leaf stage, they were transplanted to 8 L pots filled with 25% peat, 25% vermiculite, 25% perlite and 25% sand. The soil pH of these pots was adjusted to 6.0 by adding $CaCO_3$. A NPK 15:11:13 slow-release fertilizer (Osmocote plus) was also added. A self-watering system was used in which each pot was placed above individual self-watering reservoirs connected by two wicks. Every fortnight, from May to September/early October, we used six new plants in each one of the stations (Fig. 1). Phytotoxic levels were defined according to the percentage of damaged leaf area in the Bel-W3 cultivar (Bytnerowicz et al., 1993; Ribas and Peñuelas, 2003; Ribas and Peñuelas, 2006). After each fortnight, the percentage of ozone-induced lesions on the oldest four leaves was visually recorded. These percentages were estimated in 5 % intervals.

2.3. RESULTS

Meteorological variables

The expected decrease in temperature with increasing altitude was observed along the transects. During the study period (2004-2008) biweekly average temperature ranged from 12 to 14ºC (at 1040 m and 1300 m a.s.l. in the Meranges and Guils transect respectively) and from 5.6 to 7.8ºC (at 2300 m and 2200 m a.s.l. in the Meranges and Guils transect respectively), that is, temperatures decreased by approximately 0.5 – 0.7 ºC per 100 m altitude. Average values of relative humidity for 2004 in the Meranges transect were as follows: 71.9 % at 1040 m, 63.4 % at 1500 m, 68.48% at 2200 m and 65.84 % at 2400 m; nevertheless, these differences were not statistically significant. The prevalent winds at the Meranges site during the period monitored (summer 2008) were from N (23 % frequency) and SE (21 %) (Fig. 2). Winds blowing from the south and west summed a total frequency of 50 %, and had the highest wind speed and ozone averages (2.39 – 3.55 m·s^{-1} and 44 – 48 ppb$_v$, respectively). These winds typically blew from 09:00 to 21:00 (local time) (Fig. 3).

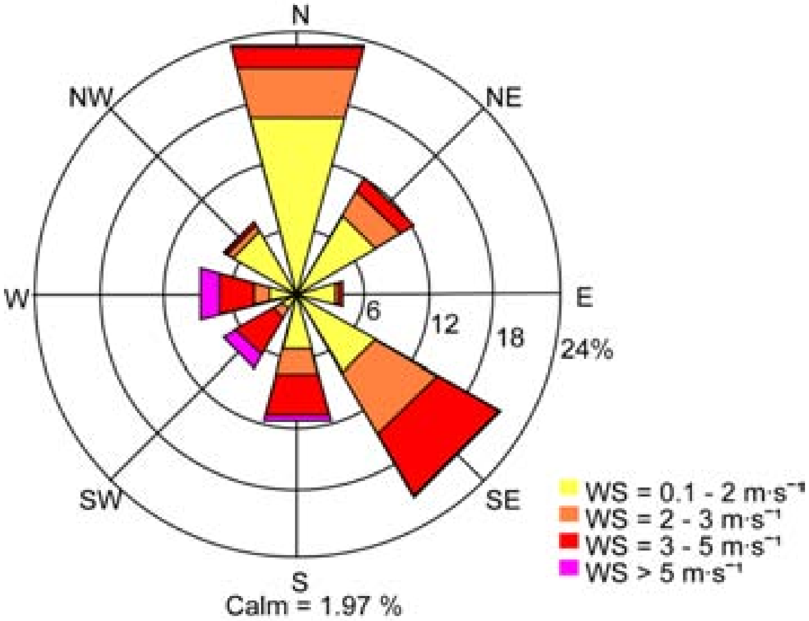


Fig. 2. Frequency of wind direction and speed recorded at Meranges mobile unit during summer 2008.

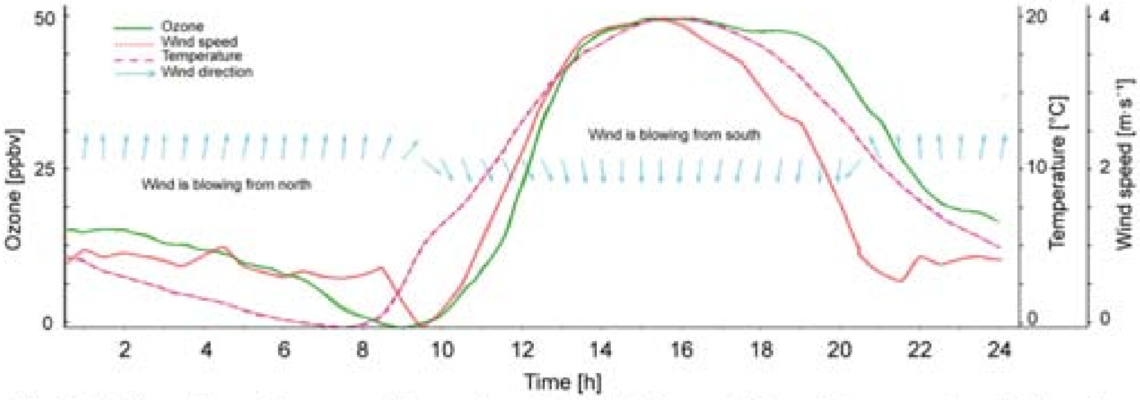


Fig. 3. Daily pattern of ozone mixing ratios (ppbv), wind speed (m•s^{-1}), temperature (°C) and wind direction (note that arrows point the direction where the wind comes from) during summer 2008 at the Meranges site.

O_3 threshold excess

The vegetation protection threshold has been set at AOT40 = 6000 μg·m^{-3}·h by the Directive 2002/3/EC of the European Parliament relating to ozone in ambient air, where (Directive, 2002) AOT40 is defined as the sum of the diurnal hourly ozone concentration exceeding the threshold of 40 ppb$_v$ over the period 1 May - 31 July. This threshold has been exceeded every year since 1994 in the valley-bottom site (data from monitor in Bellver station, Fig. 4). The highest AOT40 (May-July) value was reached in 2006 (6 times higher than the protection threshold). The forest protection threshold (AOT40 = 20000 μg·m^{-3}·h, calculated over the period 1 April - 30 September) has also been exceeded every year since 1994 (Fig. 4). The highest AOT40 (Apr-Sept.) value was reached in 1998 (2.3 times greater than the protection threshold for forests). There has been a statistically significant increasing trend over time in the AOT40 (Apr-Sept.) of 1047 μg·m^{-3}·h·year^{-1} ($p<0.05$, $R^2 = 0.30$).

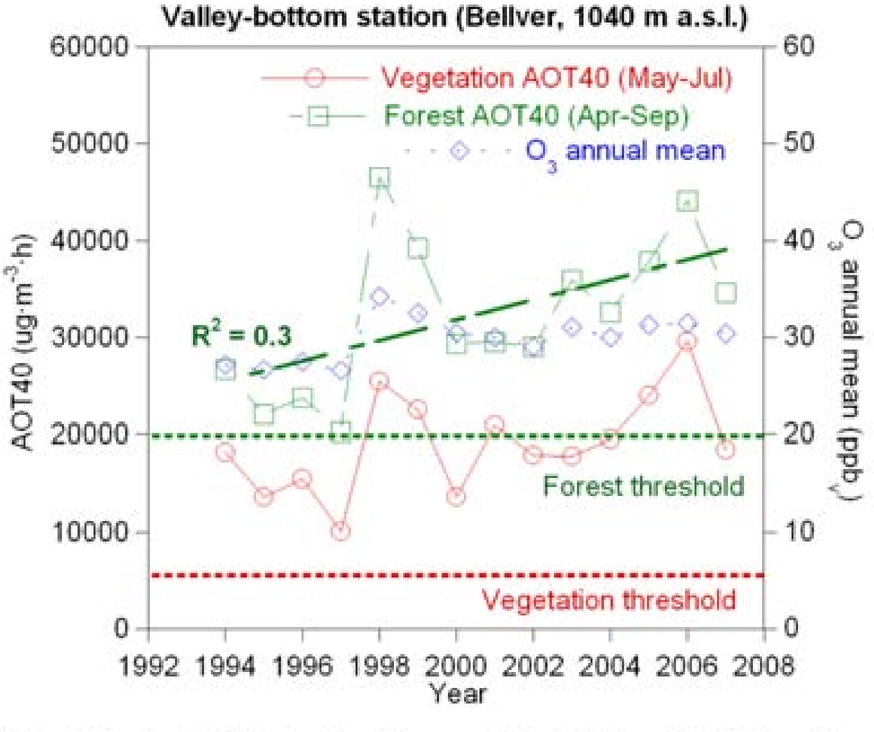


Fig. 4. Yearly AOT40 calculated for vegetation (1 May – 31 July) and for forest (1 April – 30 September) from 1994 to 2007 at the valley-bottom site (Bellver station, 1040 m a.s.l.).

O_3

Ozone mixing ratios increased with altitude in both transects: from an annual mean (for the 2004-2007 period) of 35 ppb$_v$, measured at the lowest site studied (1040 m a.s.l.), to 56 ppb$_v$ at the highest site (2300 m a.s.l.) in the Meranges transect (Fig. 5).

Thus, mean ozone mixing ratios for the whole period in the valley-bottom site (1040 m a.s.l.) were 37 % smaller than in the highest site (2300 m a.s.l.). The increase in ozone mixing ratios was 1.6 ppb_v per every 100 m of altitude. This increase was less clear along the intermediate altitudes of the Guils transect. There, ozone mixing ratios ranged from 46 ppb_v at 1300 m a.s.l. to 48 ppb_v at 2200 m a.s.l. (Fig. 5). Even though the difference between the ozone mixing ratios at the lowest and highest site in the Guils transect are smaller than those in the Meranges transect, there was a significant relationship between ozone mixing ratios and site altitude in the Guils transect for data gathered between 2005 and 2007 (O_3 mixing ratios=36.749+0.007altitude, R^2=0.77, $p<0.05$).

Yearly variations were found in both altitudinal gradients (Fig. 5). Ozone mixing ratios in 2004 were on average 31 % and 28 % smaller than the average values for 2004-2007 in the Meranges and Guils transects, respectively. The highest annual values were found in 2007 in the Meranges transect and in 2005 in the Guils transect (21 % and 12 % greater than the average value for 2004-2007 in Meranges and Guils transects, respectively). On average, the Guils transect had ozone mixing ratios 11% greater than the Meranges transect. Even though the AOT40 (April - September) has been increasing significantly since 1994, we have not detected a significant increasing trend in the annual means of ozone mixing ratios in the valley-bottom station (Bellver, 1040 m a.s.l.) (Fig. 4). At the highest site (2300 m), annual means of ozone mixing ratios ranged between 38 and 67 ppb_v (38 and 74 ppb_v during the warm season) and increased 5.1 ppb_v per year during the period sampled (2004-2007).

The seasonal pattern of ozone mixing ratios occurred as expected being on average 35 % and 38 % greater during the warm period (April - September) than during the cold period (October - March) in the Meranges and Guils transect, respectively (Fig. 6A and 6C).

Average daily patterns of ozone mixing ratios for years 2004 to 2007 in the valley-bottom site (Fig. 7) showed the characteristic pattern of minimum values in the early morning, a significant rise during the morning with increasing solar radiation (which coincided with the NO_2 minimum), peaking in the afternoon between 13:00 and 15:00 (local time) and then declining during the night (probably due to NO titration) (Fig. 7). Nevertheless, this daily pattern faded with altitude. Only a small daily

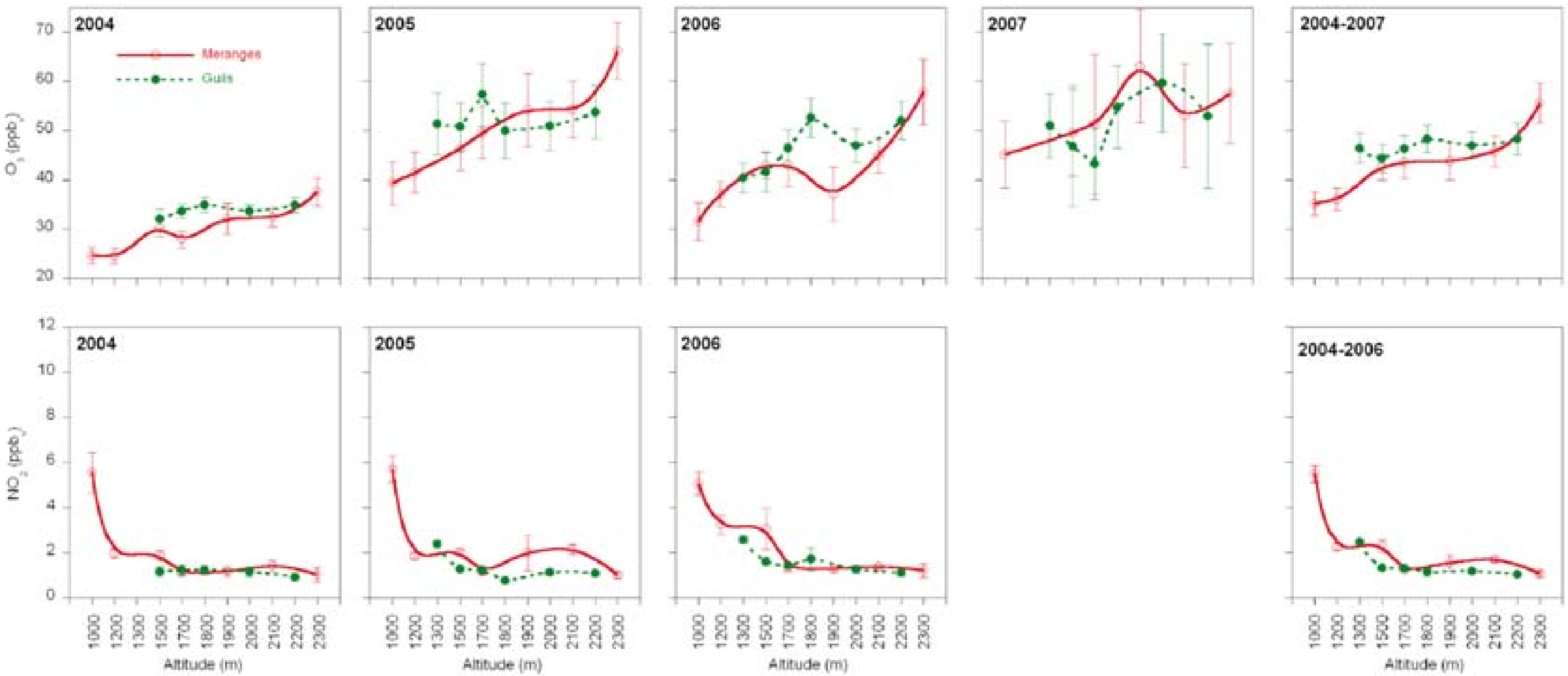


Fig. 5. Mean ozone mixing ratios (O_3) ± SEM (Standard Error of the Mean) for each year in the Meranges (n=4-24) and Guils altitudinal gradients (n=6-22) and the average for years 2004 to 2007 (Meranges: n=32-61; Guils:n=42-59), calculated for values obtained from passive samplers throughout the study period (first row). Mean NO_2 mixing ratios (NO_2) ± SEM for each year in the Meranges (n=7-20) and Guils altitudinal gradients (n=8-20) and the average for years 2004 to 2006 (Meranges: n=30-43; Guils: n=30-47))(second row).

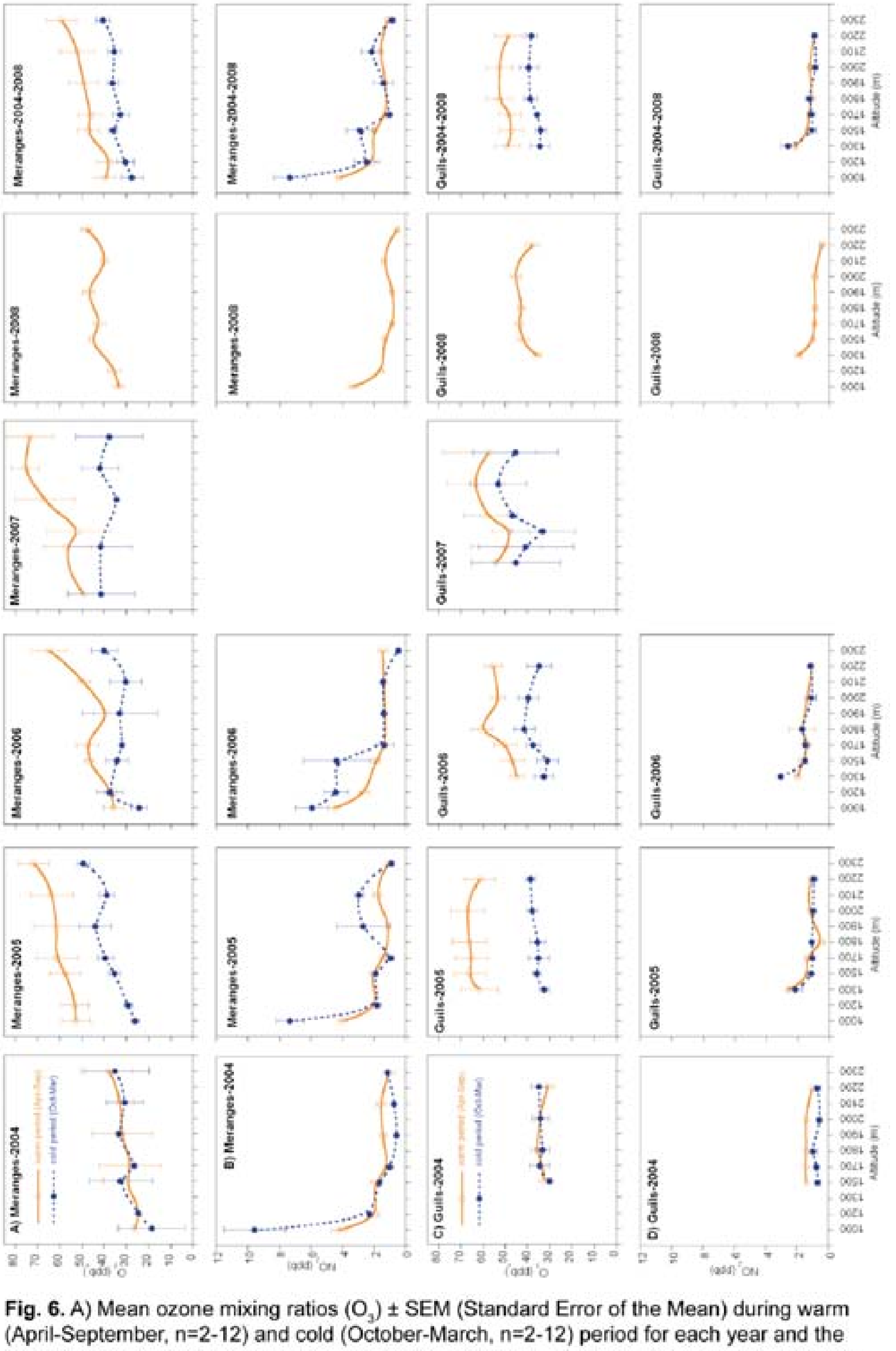


Fig. 6. A) Mean ozone mixing ratios (O_3) ± SEM (Standard Error of the Mean) during warm (April-September, n=2-12) and cold (October-March, n=2-12) period for each year and the 2004-2008 average (warm period: n=4-5; cold period: n=3-4) in the Meranges transect. B) Mean nitrogen dioxide mixing ratios (NO_2) ± SEM (Standard Error of the Mean) during warm (April-September, n=6-11) and cold (October-March, n=2-10) period for each year and the 2004-2006 average (warm period: n=4; cold period: n=3) in the Meranges transect. C) the same as the first row but for the Guils transect (O_3) ± SEM (Standard Error of the Mean) (for each year: warm period, n=4-12, cold period, n=2-11; 2004-2007 average: warm period, n=4-5, cold period, n=3-4). D) the same as the second row but for the Guils transect (NO_2) ± SEM (Standard Error of the mean) (for each year: warm period, n=7-11, cold period, n=2-9; 2004-2007 average: warm period, n=3-4, cold period, n=2-3).

variation was found at the Meranges station (1500 m a.s.l.), where ozone mixing ratios were also very high during the night-time (Fig. 8).

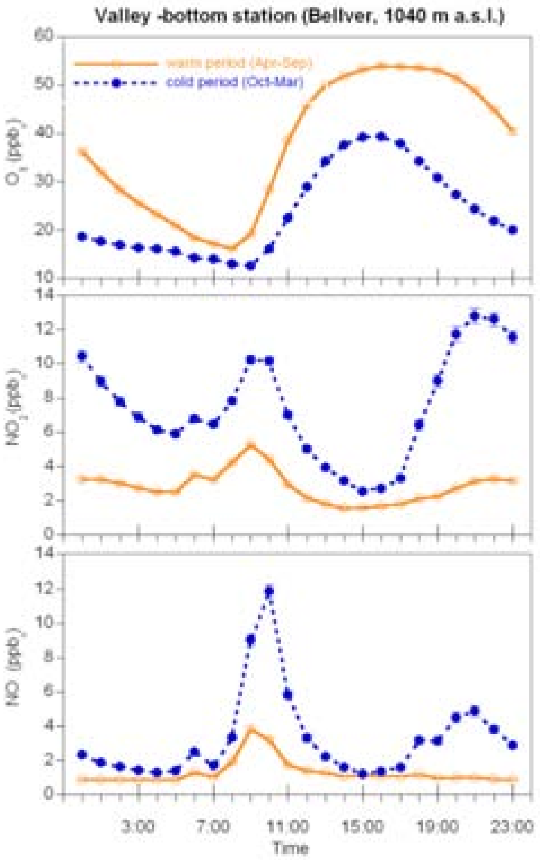


Fig. 7. Daily pattern of mean O_3, NO_2 and NO mixing ratios during 2004-2007 for the valley-bottom site (Bellver station at 1040 m ASL) ± SEM (Standard Error of the mean) in the cold (Oct-Mar. measurements, n=619-684) and warm periods (Apr-Sep. measurements, n=663-706).

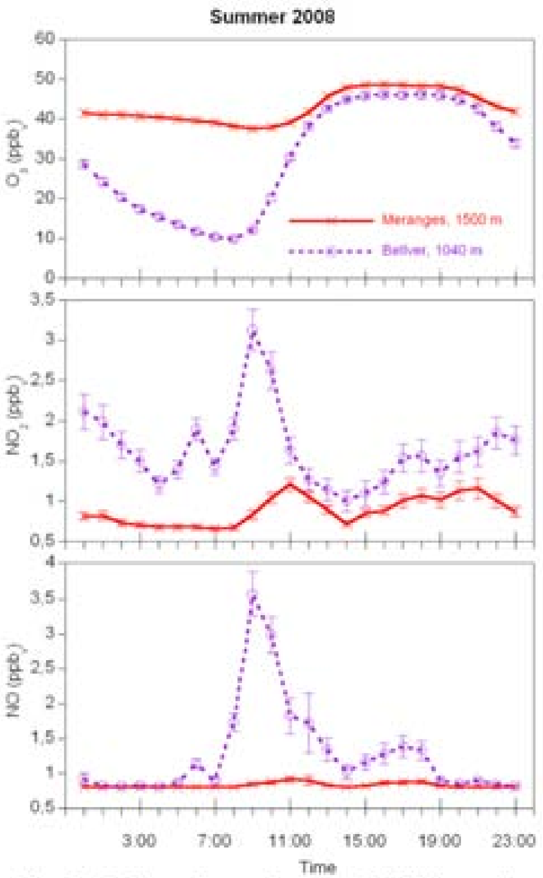


Fig. 8. Daily pattern of mean O_3, NO_2 and NO mixing ratios during summer 2008 (20 June to 30 September) for the valley-bottom site (Bellver station at 1040 m ASL) ± SEM (Standard Error of the mean, n=91-101) and mid-elevation site (Meranges at 1500 m ASL) + SEM (Standard error of the Mean, n=67-101).

NO_2 and NO

We found no significant increasing or decreasing trend over time for NO_2 and NO mixing ratios recorded since mid-February 2004 in the Bellver station (1040 m a.s.l.)(data not shown).

Yearly variations of NO_2 mixing ratios were minimal in both altitudinal gradients. Mean NO_2 mixing ratios over 2004-2006 remained at approximattely 1-2 ppb_v in all sites in both altitudinal transects. Only at the lowest sites, in the town of Bellver (5.5 ppb_v) and in the town of La Tor de Querol (2.5 ppb_v, Guils transect), did NO_2 exceed 2 ppb_v (Fig. 5).

NO_2 mixing ratios followed the expected seasonal pattern with higher values during the cold period than during the warm period, especially in those sites with nearby human activity (1000 m, 1500 m and 2100 m at Bellver town, Meranges village and Malniu mountain refugee, respectively) (Fig. 6B).

NO_2 and NO maximum mixing ratios were recorded in the morning before ozone mixing ratios rose and at the beginning of the night when human activity increases (Fig. 7 and 8), a pattern that became more accentuated during the cold period (Fig. 7). Mixing ratios in Meranges mobile station (1500 m a.s.l.) during summer 2008 were very low and close to the limit of instrument accuracy, showing no peak comparable to those found in Bellver.

Phytotoxic levels: spatial variation of leaf damage

Phytotoxicity increased with altitude and it was on average greater in the Meranges transect than the Guils transect (Fig. 9). Substantial ozone injury symptoms were found at all altitudes on the Bel-W3 tobacco plants exposed to the ambient air, although damage was more intense in the plants at greatest altitudes (Fig. 10). More than 30-40% of the surface area of Bel-W3 was affected at all sites in the Guils and Meranges transects, although at the highest sites (2200 m in the Guils transect and 2100 m a.s.l. in the Meranges transect) the mean damaged leaf area was 60-80% in the sensitive Bel-W3 tobacco plants and 16-40% in the Bel-B resistant cultivar. The resistant cultivar (Bel-B) was less affected overall and damaged leaf areas ranged from 5 to 15 % in the remaining sites (Fig. 10).

The percentage of ozone-induced damaged leaf area in the sensitive Bel-W3 cultivar had a significant quadratic relationship with ozone mixing ratios (Damaged leaf area Bel-W3 (%) = 213.29 – 8.03Ozone mixing ratio + 0.08(Ozone mixing ratio)^2, $R^2 = 0.68$, $p < 0.03$, Fig. 11).

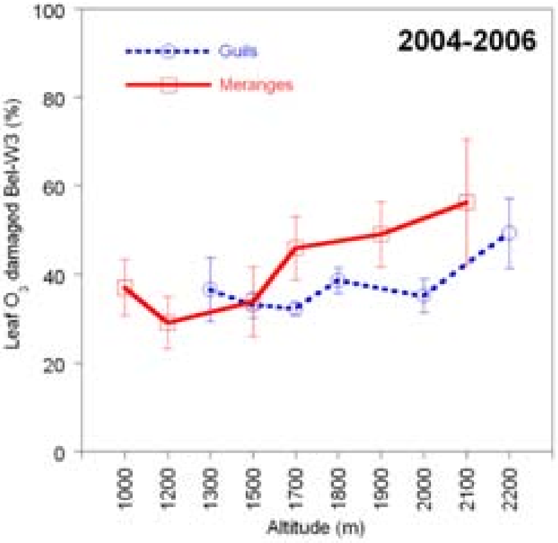


Fig. 9. Mean damaged leaf area (%) from 2004 to 2006 (calculated for May-September period) in *Nicotiana tabacum* Bel-W3 cultivar for each altitude ± SEM (Standard Error of the mean, n=2-3) in the Meranges and Guils transects.

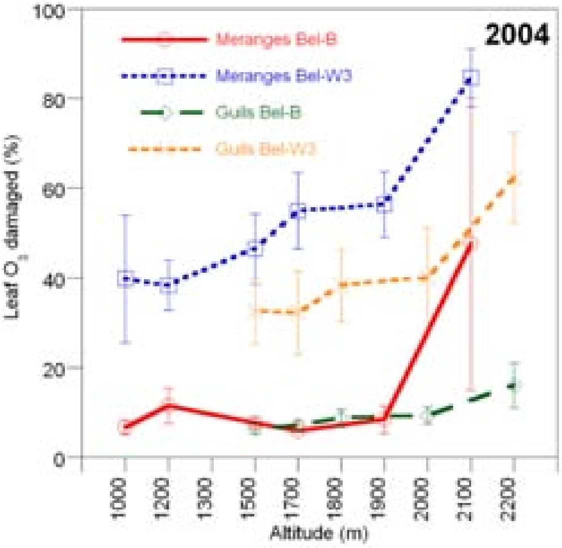


Fig. 10. May-September 2004 mean damaged leaf area (%) in *Nicotiana tabacum* Bel-W3 and Bel-B cultivars for each altitude ± SEM (Standard Error of the mean, n= 2-8) in the Meranges and Guils transects.

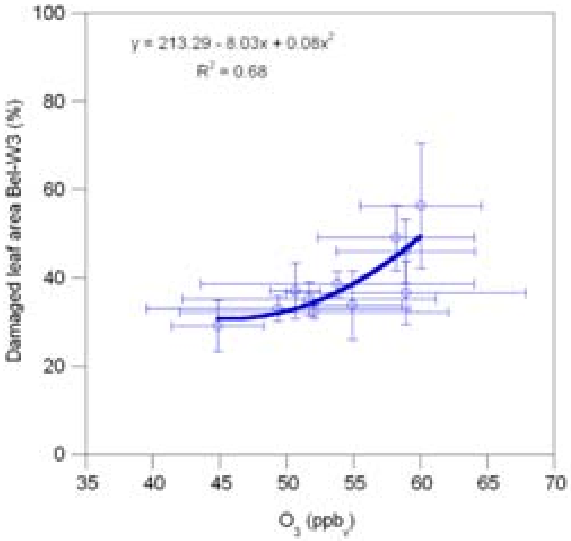


Fig. 11. Relationship between damaged leaf area (%) in *Nicotiana tabacum* Bel-W3 and mean ozone mixing ratios values (ppbv). Each dot represents the average values for 2004-2006 in each site along the altitudinal gradients.

2.4. DISCUSSION

Yearly exceedance of the AOT40 threshold for forest and vegetation protection has also been reported in other areas of the Mediterranean basin (Ferretti et al., 2007; Klumpp et al., 2006; Nali et al., 2002a; Paoletti et al., 2007; Saliba et al., 2008). Likewise, typical O_3-induced injuries in vegetation have been positively related to O_3 exceeding the critical level with a variable degree of intensity (Calatayud et al., 2007; Cvitas et al., 2006; Nali et al., 2002b; Waldner et al., 2007). Hence, our data provide evidence of an O_3 risk also for the vegetation of the high mountain areas of this Mediterranean region.

The ozone mixing ratios we measured fall into the highest range of the average ozone levels recorded in non-mountainous areas from the Mediterranean basin (Kalabokas et al., 2000; Lelieveld et al., 2002; Nali et al., 2002a, b) and they are similar to measurements reported by previous studies in valley sites (Ribas and Peñuelas, 2004). Mean growing season values of 31 - 41 ppb_v and maximum hourly values of 84 - 118 ppb_v monitored in our valley-bottom site since 1994 are also close to the O_3 mixing ratios reported in high mountain ecosystems in the European Alps and in the Carpathian mountains, where mean growing season values of 40-50 ppb_v (Bytnerowicz et al., 2004; Wieser et al., 2006) and O_3 maximum hourly values of 105-107 ppb_v (Bytnerowicz et al., 2004) have been reported. These data thus document the existence of persistent medium-to-high O_3 levels that contrast with the peak-type episodes found in central and northern Europe (Sanz and Millán, 2000).

Ozone mixing ratios increased with altitude along the transects studied (Fig. 5), a phenomenon which also has been reported in other mountainous regions (Cooper and Peterson, 2000; Peterson et al., 1999; Sanz et al., 2007). The average rate of ozone mixing ratio increase in the Meranges transect (1.6 ppb_v per 100 m of altitude, 0.3 in Guils transect) is quite close to the 1.3 ppb_v reported in the western Washington Mountains (USA) (Cooper and Peterson, 2000). A positive correlation has been found between ozone mixing ratios and solar radiation (Duenas et al., 2002), the latter tending to rise with increasing altitude. Nonetheless, in our study the altitudinal variations in ozone mixing ratios seem to be more related to air pollutant transport and diminished loss processes rather than to increased irradiance with altitude. Firstly, at mid-elevation sites (Meranges, 1500 m a.s.l.), solar radiation does not differ statistically from the values recorded in the valley-bottom site (Bellver de Cerdanya, 1040 m a.s.l.), where the average values from 08:00 to 19:00 are 457 and 464 W·m^{-2} in Meranges and Bellver, respectively. Secondly, 50 % of the wind blows from S and W, corresponding to the areas from which ozone precursors have most probably originated (main urban areas and roads). These winds blow during the daytime and they have the highest ozone concentrations. This suggests that they are probably carrying the ozone produced in the urban areas and roads where they come from. Furthermore, NO_2 and NO production is very low at the valley-bottom site in comparison with urban areas (mean annual concentrations of 2 ppb_v of NO and 5 ppb_v of NO_2 in Bellver versus 23 ppb_v and 22 ppb_v in several urban sites in Catalonia (data not published, courtesy of the Department of the Environment and Housing)) and they become even lower at Meranges site (1500 m a.s.l., Fig. 5). Thus, the low NO mixing ratios found at the Meranges site (and probably at higher altitudes as well) could explain the high night-time mixing ratios of ozone that contribute to enhancing the overall mean ozone mixing ratio. In effect, some of the highest ozone mixing ratios ever detected are at high-altitude rural sites (Brace and Peterson, 1998), where local production of NO is minimal and ozone night losses are low (Lefhon, 1992). Nonetheless, further research should be done in order to clarify how much stratospheric intrusions of O_3 and how much lower vertical mixing contributes to the increasing ozone mixing ratios found with rising altitude.

The AOT40 (Apr-Sept.) at the Bellver site (1040 m a.s.l.) has increased 1047 µg·m^{-3}·h per year since the measurements started in 1994 (Fig. 4). Nevertheless, the annual mean of ozone mixing ratios does not necessarily follow AOT40, as can be seen in 2007. In that year the AOT40 was relatively low in comparison with the previous years (Fig. 4), while the annual mean ozone mixing ratio reached its highest value in the whole period 2004-2007 (Fig. 5). Therefore, special attention should be paid to AOT40 measurements, which give a more realistic estimation of the potential risk for vegetation than annual means and, as we have seen, it can differ significantly from the annual means calculated for the same period.

In our case study, higher ozone mixing ratios in the warm period are probably a result of anabatic transport of photochemically produced ozone in nearby urban areas during the daytime. This process has been reported in other regions of the Mediterranean basin (Duenas et al., 2002; Gonçalves et al., 2009; Millán et al., 2000).

Even though anabatic and katabatic winds are formed all year long, the photochemical production of ozone is greater during the warm period due to higher temperatures, which explains the higher ozone mixing ratios found during this period. However, other factors like stratospheric ozone flux during early spring (Viezee et al., 1983) or excess hydrocarbons released during bud break (Aneja et al., 1991) could contribute to the observed seasonal variability.

Ambient conditions at Meranges station (1500 m a.s.l.) followed the typical daily pattern of high altitude sites. During the day, the valley floor warms up and the air rises up the slopes of the surrounding mountains (anabatic wind or valley breeze). These winds (blowing from S and W, Fig. 3) transport the ozone produced photochemically in urban areas to the reservoir layer, the highest ozone concentration being reached at mid-afternoon (15:00 local time, Fig. 8). After that, ozone mixing ratios start to decrease very slowly but still remain high over night (Fig. 8), as has been recorded in other high altitude sites (Millán et al., 2000). Indeed, the reservoir ozone layer created the day before can continue to cover high altitude sites the next day (Millán et al., 2000). Katabatic winds (also called mountain breeze and, in this case, blowing from N, Fig. 3), which are usually very gentle (<2 $m \cdot s^{-1}$ in Meranges, Fig. 3), keep the reservoir layer almost stagnant, thus maintaining elevated ozone concentrations during the night. Moreover, NO mixing ratios at Meranges station are so low or even inexistent (around 1 ppb_v, Fig. 8) that they could hardly contribute to ozone depletion. The diurnal minimum in Meranges takes place in the morning, probably when the onset of anabatic winds occurs, bringing O_3-depleted and/or NO enriched air from the valley or other more urbanized areas to the mountain tops, as has been reported in previous studies (Millán et al., 2000).

Similarly, Gonçalves et al. (2009) found that in the North-Eastern Iberian Peninsula the maximum contributors to ozone surface were net transport and vertical diffusion. High ozone mixing ratios were not produced in situ, but came from horizontally advected flows during the morning and gas-phase chemical contributions. During the afternoon and dusk, vertical advective flows injected pollutants in layers above the atmospheric boundary layer, which accumulated due to stagnant conditions and contributed positively to surface concentrations the following day (Gonçalves et al., 2009).

Annual means of NO_2 mixing ratios recorded by passive methods during 2004-2006 (Fig. 5) and by monitoring in Bellver station since 2004 (data not shown), did not present a statistically significant trend. This is despite the fact that NO_x emissions have apparently decreased since the mid-1980s in Western and Northern Europe and even in the Mediterranean region, although in the latter the decrease of NO_x emissions was smaller and it started later than in the rest of Europe (Brönnimann et al., 2002; De Leeuw, 2000).

The daily pattern of NO_2 mixing ratios at Bellver station suggests that local photochemical production of ozone takes place in the morning since NO_2 mixing ratios start to drop precisely when ozone mixing ratios start to increase, coinciding with increasing solar radiation (Fig.7). Conversely, the low or even inexistent levels of NO_2 and NO recorded at higher altitude, in Meranges (Fig. 8), could indicate again that the variations of ozone mixing ratios in this station are probably a result of net transport and vertical diffusion rather than local photochemical production and/or NO titration.

In the tobacco plants, the percentage of leaf area which was damaged increased with altitude (Fig. 9 and 10) in response to increasing O_3 mixing ratios and likely increases in O_3 uptake with greater altitude (Kaufmann, 1976; Wieser, 1999). In effect, there was a positive relationship between damaged leaf area of Bel-W3 and ozone mixing ratios (Fig. 11) which also has been reported in previous studies (Ribas and Peñuelas, 2006). The intense damage response found in these mountain sites is probably because the sensitivity threshold for the Bel-W3 cultivar (40 ppb_v) (Heggestad, 1991) was frequently exceeded. This was coupled to microclimatic conditions such as high radiation or humidity that enhance stomatal conductance and ozone uptake (Kaufmann, 1976; Penuelas and Inoue, 1999). These results confirm the value of tobacco plants as good biomonitor tools for ozone concentrations and phytotoxicity in the Catalan Pyrenees during summer time whereas the use of passive samplers is probably more appropriate for long-term monitoring of ozone mixing ratios in mountainous areas with hard winter conditions.

It should be highlighted that the AOT40 threshold for plants' and forests' protection has been greatly exceeded every year since 1994 in the valley-bottom site, where ozone mixing ratios are on average 37 % smaller than in the higher sites. Therefore, the AOT40 for vegetation and forest protection is even more exceeded at high altitudes which, in accordance with recent literature on critical levels (Pleijel, 2000; VanderHeyden et al., 2001), could have negative effects on plant life at high altitude sites in the Pyrenees.

Acknowledgements

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CHAPTER 3

Ozone-like visible injury, defoliation and mortality in two *Pinus uncinata* stands of the Catalan Pyrenees

Abstract

Ozone concentrations in the Pyrenees have exceeded the thresholds for forest protection during the last 16 years. We here surveyed the severity of O_3 visible injury, crown defoliation and tree mortality of *Pinus uncinata*, the dominant species in subalpine forests in this mountainous range, along two altitudinal and O_3 gradients in the Central Catalan Pyrenees and analyzed their relationship with site environmental conditions. O_3 visible injury severity showed higher values with increasing annual mean $[O_3]$ when summer water availability (assessed here as summer P/PET) was above a threshold of 0.96, whereas in drier conditions, higher $[O_3]$ did not yield to higher O_3 visible injury. Crown defoliation and tree mortality mean values ranged between 20.4-66.4% and 0.6-29.6%, respectively, depending on the sites. They were positively correlated with the accumulated O_3 exposure during the last 5 years and with variables related to soil water availability, which favours greater O_3 uptake through increased stomatal conductance. The results indicate that O_3 is involved in the observed crown defoliation and tree mortality. Further research is warranted, though, to determine the contribution of the multiple stress factors contributing to the observed crown defoliation and mortality in *Pinus uncinata* stands of the Catalan Pyrenees.

3.1. INTRODUCTION

Ozone concentrations in the Catalan Pyrenees have been found to exceed the critical level, the target value and the long-term objective for forest and semi-natural vegetation protection set by the CLRTAP/UNECE and the European Directive 2008/50/EC during the last 16 years (Díaz-de-Quijano et al., 2012a). It has also been observed in the Pyrenees an increase of the O_3 concentrations by a factor of five (1.6%/year) from the end of the 19th century to the early 1990s (Marenco et al., 1994). A study of the O_3 concentrations along an altitudinal gradient in the Central Catalan Pyrenees has also revealed a significant increase of O_3 concentration with altitude, from 35 ppb_v at 1040 m to 56 ppb_v at 2300 m on average for the 2004-2007 period, whereas the concentrations during the warm period (April-Septembre) reached 38 and 74 ppb_v at these same sites (Díaz-de-Quijano et al., 2009).

Ozone concentrations in the Pyrenees are, therefore, high enough to produce a negative effect on semi-natural vegetation and forests (Díaz-de-Quijano et al., 2012a). In fact, the exceedance of the critical level for O_3 as it occurs in Pyrenees has been positively correlated with typical O_3-induced injuries in vegetation in several studies, under both controlled experiments (Gimeno et al., 2004; Manninen et al., 2003b; Marzuoli et al., 2009; Peñuelas et al., 1994; Ribas et al., 2005) and field conditions (Calatayud et al., 2007; Cvitas et al., 2006; Waldner et al., 2007). Some of the forest trees and herbaceous species found along the altitudinal gradient in the Pyrenees have also been found to be sensitive to O_3 as it is the case of *Fagus sylvatica, Pinus sylvestris, Betula pendula* (Karlsson et al., 2003) and *Phleum alpinum, Leontodon hispidus, Valeriana officinalis, Silene acaulis* and *Hieracium pilosella* (Hayes et al., 2007).

The Mountain pine (*Pinus uncinata* Ram.) is an autochthonous European species which represents the dominant subalpine forest species in the Central and Eastern Pyrenees from 1600 to 1800 (depending on the area) to 2400 m a.s.l. (Burriel et al., 2004). A recent study with 7-years-old saplings of Mountain pine has revealed that O_3 concentrations similar to those registered in the Pyrenees decrease root biomass and produce O_3 visible injury in needles after two years of O_3 fumigation in a free-air facility in the Swiss Alps (Díaz-de-Quijano et al., 2012b). Moreover, mature individuals of *P. uncinata* under field conditions and exposed to elevated natural O_3 concentrations show O_3 visible injury, which has been thoroughly described and microscopically validated (unpublished Chapter 4). Possible impacts of O_3 on these subalpine forests of *Pinus uncinata* in the Pyrenees and on the livelihoods of forest-dependent communities could result in major ecological, economic, and social consequences. However, no thoroughly studies have been conducted yet in order to determine the extent of O_3 visible injury on *Pinus uncinata* stands in the Pyrenees and the general health status of this tree species.

The aims of this study were: 1) to evaluate the severity of O_3 visible injury in two *Pinus uncinata* stands along an altitudinal gradient in the Pyrenees, and 2) to assess crown defoliation and mortality as indicators of health status, in the mentioned *Pinus uncinata* stands, where O_3 concentrations had been previously monitored during several years.

3.2. MATERIALS AND METHODS

Study site

The study site is located in the area of La Cerdanya, in the Central Catalan Pyrenees (Fig. 1). This region has the typical Mediterranean Pyrenean climate, with the maximum rainfall occurring in summer and increasing with altitude (Martín-Vide, 1992), which differs from the typical Mediterranean climate characterised by dry and warm summers. We surveyed O_3-like visible injury, crown defoliation and tree mortality in two transects dominated by *Pinus uncinata* along two altitudinal gradients. One of the transects ranges from 1500 m a.s.l. to 2200 m a.s.l. facing north-east (Guils transect) and the other transect ranges from 1700 m a.s.l. to 2500 m a.s.l. on the south-facing slopes of Puigpedrós (Meranges transect). In total, we had twelve sites, situated approximately every 200 m in altitude (sometimes every 100 m) (Fig. 1). Nine of these sites were surveyed for O_3 visible injury and six of them for crown defoliation and tree mortality (Fig. 1). For the tree mortality assessment, we established ten 20x20 m square plots per site and three of these ten plots were also assessed for crown defoliation measurements.

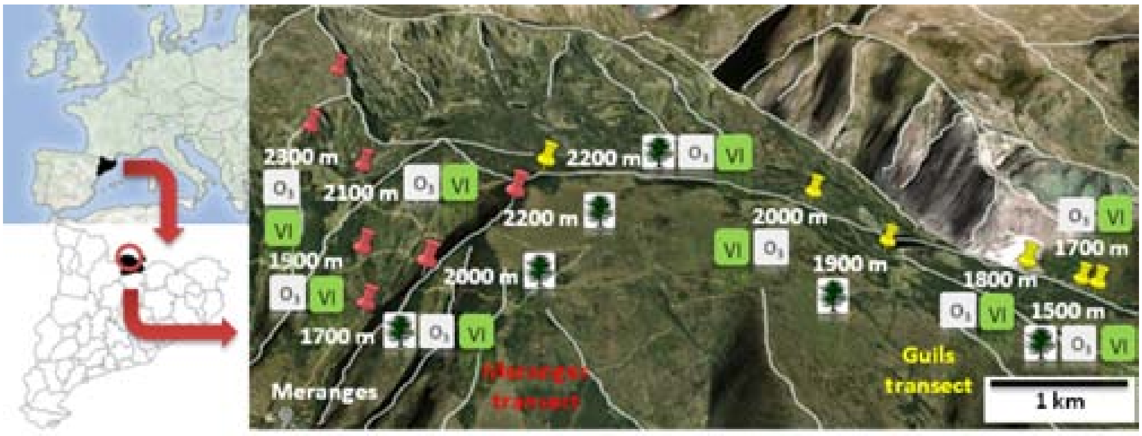


Fig.1. Map of the location of the sites in the area of La Cerdanya, Central Catalan Pyrenees. Ozone visible injury assessment was performed in all sites showing the "VI" icon. The tree icon indicates the sites where the percentage of defoliation and tree mortality was measured. The "O_3" icon indicates the sites were ozone measurements were performed in a previous work (Díaz-de-Quijano et al., 2009).

Variables defining site conditions

Soil depth was measured five times in each site and in the three plots where crown defoliation was measured by hammering a 1.3 m metal bar into the soil until reaching the rock bed. Soil water retention capacity was obtained by dividing the mass of water retained after 24 h by a soil core taken at 20 cm depth into the dry mass of the soil.

Topographic wetness indexes are used to characterize spatial soil moisture conditions at within-catchment scales (Beven and Kirkby, 1979; O'Loughlin, 1986). These indices assume that topography play a key role in controlling and modifying the hydrology at the hillslope scale (Grayson et al., 1999). We used the same topographic index as in Galiano et al. (2010), which accounts for the contributing area in the catchment that drains into a given point, and for the slope of the terrain. It ranged from 8.42 to 13.37, with higher values representing wetter conditions.

Summer water availability was assessed as summer P/PET, where P is the average (1951-1999) precipitation from July to September and PET is the average potential evapotranspiration for the same period.

O_3 concentrations had been monitored by passive sampling in nine sites during 2004-2008 (Díaz-de-Quijano et al., 2009) (Fig. 1). We used the average of the annual mean O_3 concentrations from 2005 to 2007 and from 2004 to 2008, and the sum of the fortnight mean O_3 concentrations from 2004 to 2008. Annual mean O_3 concentrations from 2005 to 2007 were used in the analyses of O_3 visible injury since they showed the best correlation with O_3 injury in a previous unpublished work (by Kefauver et al.). Annual mean O_3 concentration 2004-2008 and the sum of fortnight mean O_3 concentrations from 2004 to 2008 were used in the analyses of crown defoliation and tree mortality.

Assessment of the ozone visible injury severity

A total of 27 individuals of *Pinus uncinata* were examined for O_3 visible injury during May 2007. Three trees were chosen at each site along a mini-transect whose centre was as closest as possible to the O_3 passive sampler station established in Díaz-de-Quijano (2009). Trees were at least 2 m high, with a diameter at breast height (DBH) larger than 10 cm and with accessible branches (less than 2 meters height) and exposed to the sun. We used a tree pruning pole for sampling at mid-canopy height on exterior, non-terminal branches on the north, south and top of the tree which were old enough to have a full 5 years of needles present.

Ozone visible injury severity (VI-severity) is part of the Ozone Injury Index (OII) created with the aim of offering a reliable and quantitative visual-based tool of assessment of tree health (Arbaugh et al., 1998; Duriscoe et al., 1996 and see http://www.fs.fed.us/psw/publications/documents/gtr-155/ for further details). It corresponds to the % of the area of all symptomatic needles showing chlorotic mottling and it can be assigned to a score of from 1 to 5 (1:1-6%, 2:7-25%, 3:26-50%, 4:51-75%, 5:>75%).

Crown defoliation and tree mortality assessment

Crown defoliation was assessed in three plots per site following an adaptation of the manual from the International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests (UNECE/CLRTAP, 2006). In each plot, we chose 4 subplots oriented along the main compass directions at a distance of 8 m from the centre. On each subplot the 6 trees nearest to the subplot centre were selected as sample trees, resulting into 24 sample trees per plot. Defoliation was estimated in 5% classes relative to a reference tree as suggested in the manual (UNECE/CLRTAP, 2006).

A total of 60 20x20 m square plots were surveyed for tree mortality at the G1500, G1900, G2200, M1700, M2000, and M2200 sites (10 plots per site) in 2008. Plots were selected within homogenous parts of the stand, with no signs of recent disturbance or management, at least 30 m far from each other, and with a similar slope within each site.

Total number of live and dead trees and their DBH were measured in each plot. Tree density, percentage of dead trees and total basal area (BA) were then calculated for each plot.

Statistical analyses

General linear models were used to study the relationship between site characteristics and visible injury severity, defoliation and mortality. Parameters of the fitted models (β) were estimated using maximum likelihood methods. The selection of the model was based on a stepwise selection procedure using the Akaike information criterion (AIC). Data were transformed when needed to satisfy the assumption of normality (log(VI-sev), log(defoliation), log(mortality+1)). All analyses were performed with R, version 2.12.2 (2011, The R Foundation for Statistical Computing).

3.3. RESULTS

Assessment of ozone visible injury severity

Ozone visible injury severity (VI-sev) tended to increase with altitude in both transects ranging 1-2 at the Guils transect and 1.4-3.2 at the Meranges transect (Table 1). The average VI-sev was significantly lower at the Guils transect (Mean±SE: 1.26±0.2) than at the Meranges transect (Mean±SE: 2.08±0.4). The final model for VI-sev fitted using stepwise model selection is shown in Table 2. In this case, the interaction between the explanatory variables included in the model was significant. This means that, in conditions of summer P/PET above 0.96 (VI-sev=19.42-

18.76*summer P/PET+(-0.418+0.436*summer P/PET)*$[O_3]$), higher VI-sev was associated with higher $[O_3]$, whereas in conditions of summer P/PET below 0.96, higher VI-sev was not associated with higher $[O_3]$. Relationships of VI-sev with summer P/PET and annual mean $[O_3]$ for the 2005-2007 period are shown in Fig 2.

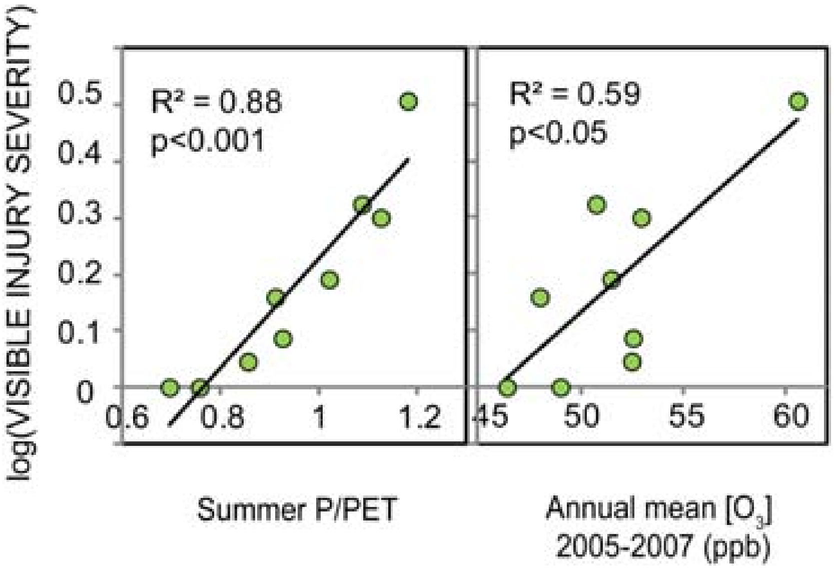


Fig. 2 Visible injury severity according to values of summer P/PET and annual mean $[O_3]$ for the 2005-2007 period. Data from both transects is represented. (Line-fitting for summer P/PET is y=0.9 x-0.7, and for the annual mean $[O_3]$ 2005-2007 is y=0.03x-1.5).

Defoliation and tree mortality

Defoliation and tree mortality values from the six studied sites are shown in Table 3. Defoliation showed mean values between 20 and 66%, being in general higher in the Guils transect than in Meranges transect. Defoliation also showed a clear increasing trend with altitude in Guils transect, which also coincided with an increasing trend of tree mortality (Table 3). This pattern was not so clear at Meranges transect, where the highest defoliation was found at the mid-altitude site. Tree mortality increased with altitude in both transects but it showed higher values at the Guils transect, ranging between 1-30% whereas in Meranges transect it ranged between 1-7.5%. On the whole, clear increasing trends with altitude and also higher values of defoliation and mortality were found in Guils transect, whereas in Meranges the values were lower and they did not show such as clear altitudinal pattern (Table 3). Guils transect also presented wetter conditions than Meranges, as it can be seen with the general higher values for the variables related to site water availability (e.g. soil depth, soil water retention capacity and the topographic index of wetness) (Table 3).

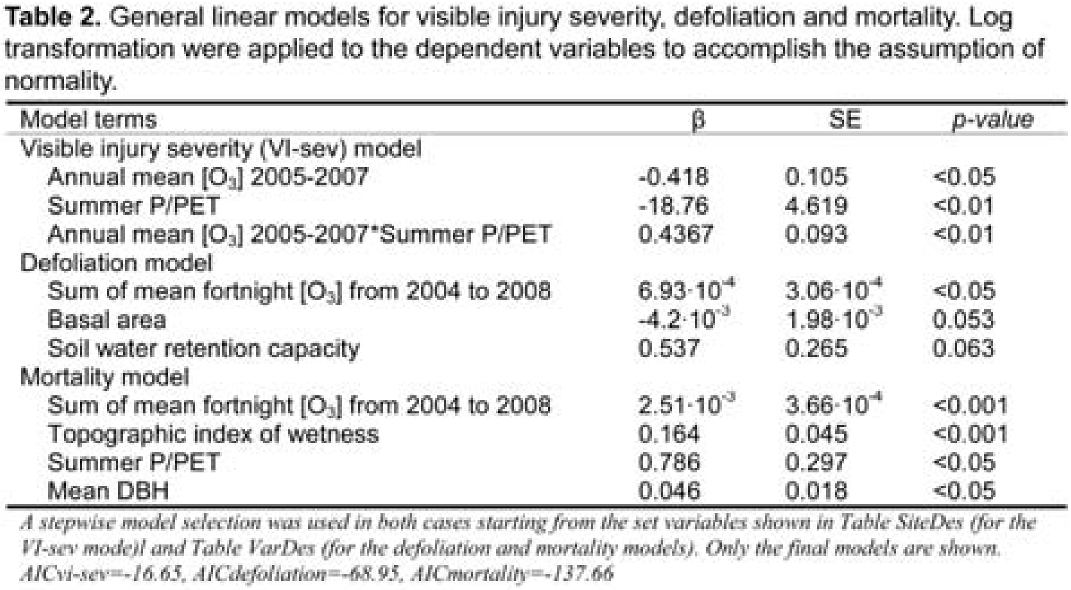


Table 2. General linear models for visible injury severity, defoliation and mortality. Log transformation were applied to the dependent variables to accomplish the assumption of normality.

Model terms	β	SE	*p-value*
Visible injury severity (VI-sev) model			
Annual mean $[O_3]$ 2005-2007	-0.418	0.105	<0.05
Summer P/PET	-18.76	4.619	<0.01
Annual mean $[O_3]$ 2005-2007*Summer P/PET	0.4367	0.093	<0.01
Defoliation model			
Sum of mean fortnight $[O_3]$ from 2004 to 2008	$6.93 \cdot 10^{-4}$	$3.06 \cdot 10^{-4}$	<0.05
Basal area	$-4.2 \cdot 10^{-3}$	$1.98 \cdot 10^{-3}$	0.053
Soil water retention capacity	0.537	0.265	0.063
Mortality model			
Sum of mean fortnight $[O_3]$ from 2004 to 2008	$2.51 \cdot 10^{-3}$	$3.66 \cdot 10^{-4}$	<0.001
Topographic index of wetness	0.164	0.045	<0.001
Summer P/PET	0.786	0.297	<0.05
Mean DBH	0.046	0.018	<0.05

A stepwise model selection was used in both cases starting from the set variables shown in Table SiteDes (for the VI-sev mode)l and Table VarDes (for the defoliation and mortality models). Only the final models are shown. AICvi-sev=-16.65, AICdefoliation=-68.95, AICmortality=-137.66

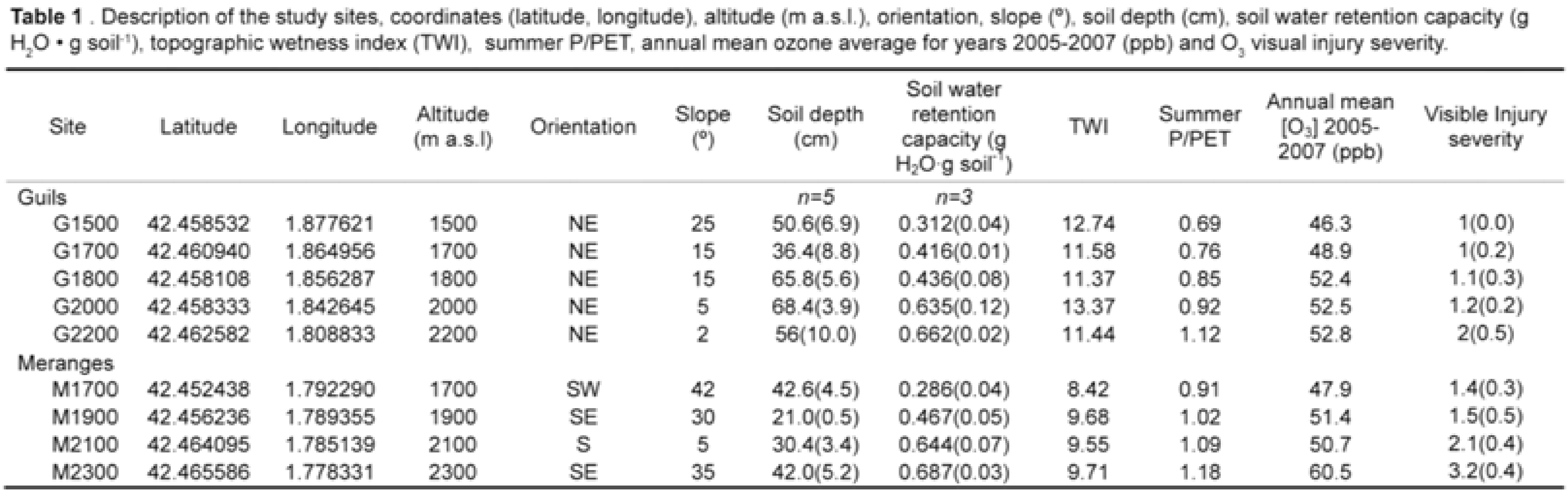


Table 1 . Description of the study sites, coordinates (latitude, longitude), altitude (m a.s.l.), orientation, slope (°), soil depth (cm), soil water retention capacity (g H_2O • g $soil^{-1}$), topographic wetness index (TWI), summer P/PET, annual mean ozone average for years 2005-2007 (ppb) and O_3 visual injury severity.

Site	Latitude	Longitude	Altitude (m a.s.l)	Orientation	Slope (°)	Soil depth (cm)	Soil water retention capacity (g H_2O·g $soil^{-1}$)	TWI	Summer P/PET	Annual mean [O_3] 2005-2007 (ppb)	Visible Injury severity
Guils						*n*=5	*n*=3				
G1500	42.458532	1.877621	1500	NE	25	50.6(6.9)	0.312(0.04)	12.74	0.69	46.3	1(0.0)
G1700	42.460940	1.864956	1700	NE	15	36.4(8.8)	0.416(0.01)	11.58	0.76	48.9	1(0.2)
G1800	42.458108	1.856287	1800	NE	15	65.8(5.6)	0.436(0.08)	11.37	0.85	52.4	1.1(0.3)
G2000	42.458333	1.842645	2000	NE	5	68.4(3.9)	0.635(0.12)	13.37	0.92	52.5	1.2(0.2)
G2200	42.462582	1.808833	2200	NE	2	56(10.0)	0.662(0.02)	11.44	1.12	52.8	2(0.5)
Meranges											
M1700	42.452438	1.792290	1700	SW	42	42.6(4.5)	0.286(0.04)	8.42	0.91	47.9	1.4(0.3)
M1900	42.456236	1.789355	1900	SE	30	21.0(0.5)	0.467(0.05)	9.68	1.02	51.4	1.5(0.5)
M2100	42.464095	1.785139	2100	S	5	30.4(3.4)	0.644(0.07)	9.55	1.09	50.7	2.1(0.4)
M2300	42.465586	1.778331	2300	SE	35	42.0(5.2)	0.687(0.03)	9.71	1.18	60.5	3.2(0.4)

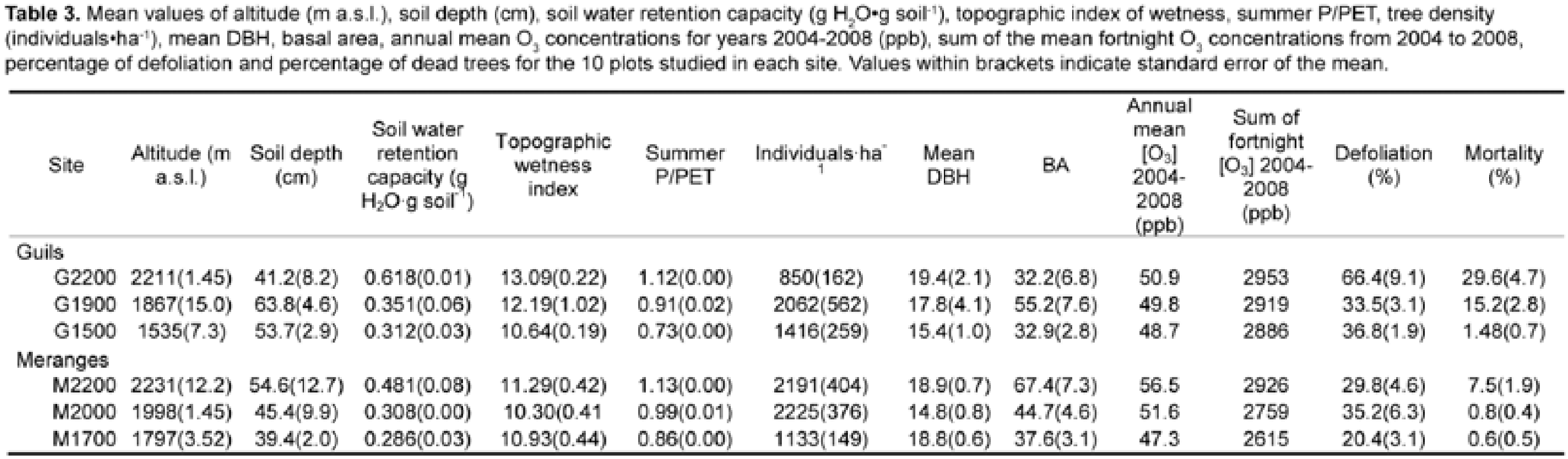


Table 3. Mean values of altitude (m a.s.l.), soil depth (cm), soil water retention capacity (g H_2O•g $soil^{-1}$), topographic index of wetness, summer P/PET, tree density (individuals•ha^{-1}), mean DBH, basal area, annual mean O_3 concentrations for years 2004-2008 (ppb), sum of the mean fortnight O_3 concentrations from 2004 to 2008, percentage of defoliation and percentage of dead trees for the 10 plots studied in each site. Values within brackets indicate standard error of the mean.

Site	Altitude (m a.s.l.)	Soil depth (cm)	Soil water retention capacity (g H_2O·g $soil^{-1}$)	Topographic wetness index	Summer P/PET	Individuals·ha^{-1}	Mean DBH	BA	Annual mean [O_3] 2004-2008 (ppb)	Sum of fortnight [O_3] 2004-2008 (ppb)	Defoliation (%)	Mortality (%)
Guils												
G2200	2211(1.45)	41.2(8.2)	0.618(0.01)	13.09(0.22)	1.12(0.00)	850(162)	19.4(2.1)	32.2(6.8)	50.9	2953	66.4(9.1)	29.6(4.7)
G1900	1867(15.0)	63.8(4.6)	0.351(0.06)	12.19(1.02)	0.91(0.02)	2062(562)	17.8(4.1)	55.2(7.6)	49.8	2919	33.5(3.1)	15.2(2.8)
G1500	1535(7.3)	53.7(2.9)	0.312(0.03)	10.64(0.19)	0.73(0.00)	1416(259)	15.4(1.0)	32.9(2.8)	48.7	2886	36.8(1.9)	1.48(0.7)
Meranges												
M2200	2231(12.2)	54.6(12.7)	0.481(0.08)	11.29(0.42)	1.13(0.00)	2191(404)	18.9(0.7)	67.4(7.3)	56.5	2926	29.8(4.6)	7.5(1.9)
M2000	1998(1.45)	45.4(9.9)	0.308(0.00)	10.30(0.41	0.99(0.01)	2225(376)	14.8(0.8)	44.7(4.6)	51.6	2759	35.2(6.3)	0.8(0.4)
M1700	1797(3.52)	39.4(2.0)	0.286(0.03)	10.93(0.44)	0.86(0.00)	1133(149)	18.8(0.6)	37.6(3.1)	47.3	2615	20.4(3.1)	0.6(0.5)

The final models for defoliation and mortality fitted using stepwise model selection from the initial set of explanatory variables presented in Table 3 are shown in Table 2. Both defoliation and mortality were mostly affected by the sum of the mean fortnight $[O_3]$ from 2004 to 2008, but were also associated with explanatory variables defining site water availability and stand characteristics (Table 2). Increases in defoliation and mortality were associated with higher accumulated exposures of O_3 and higher water availability, which in the case of defoliation was represented by soil water retention capacity and in the case of mortality by the topographic wetness index and summer P/PET (Fig. 3, Fig. 4). In the defoliation model, stand basal area was negatively correlated with defoliation, although the relationship was only marginally significant as it happened also with soil water retention capacity, whereas mean DBH was positively correlated with mortality.

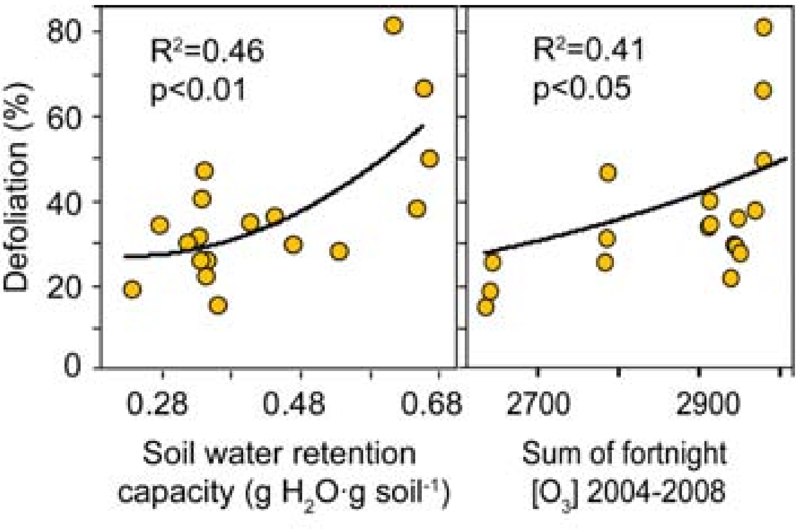


Fig. 3. Defoliation according to values of soil water retention capacity and the sum of fortnight mean $[O^3]$ from 2004 to 2008. Data from both transects is represented. (Line-fitting for soil water retention capacity is $y=169.3x^2-71.7x+35.4$, and for the sum of fortnight mean $[O^3]$ from 2004 to 2008 is $y=0.079e^{0.0021x}$)

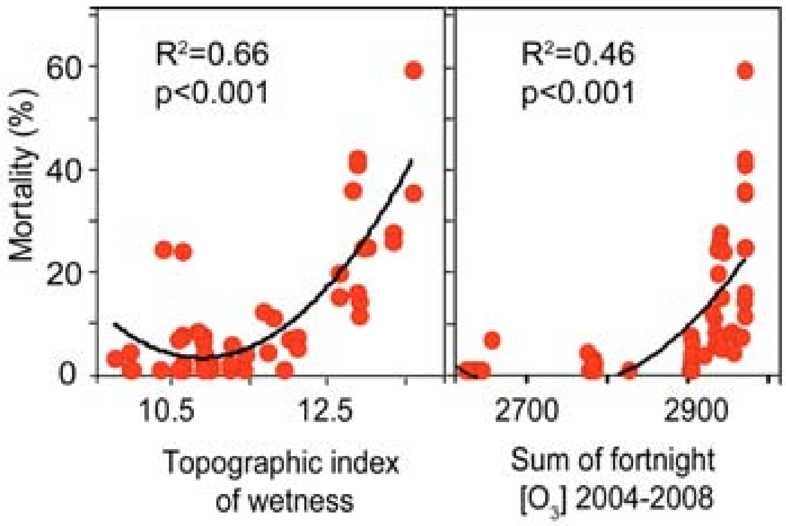


Fig. 4. Mortality according to values of topographic index of wetness and the sum of fortnight mean $[O_3]$ from 2004 to 2008. Data from both transects is represented. (Line-fitting for the topographic index of wetness is $y=5.5x^2-118.2x+640.8$, and for the sum of fortnight mean $[O_3]$ from 2004 to 2008 is $y=5 \cdot 10^{-4}x^2-2.8x+3804.8$).

3.4. DISCUSSION

Severity of O_3 visible injury depending on summer P/PET and annual mean $[O_3]$ for the 2005-2007 period

Ozone effects on vegetation are well-known to depend on the amount of O_3 entering the leaves and the plant's sensitivity to O_3 (Matyssek et al., 2008). O_3 uptake is highly influenced by the soil moisture availability since it directly affects stomatal conductance (Nunn et al., 2005a; Patterson et al., 2000; Schaub et al., 2007; Schaub et al., 2003). Soil water availability has also been considered as one of the most important site factors influencing the response of trees to O_3 stress (Lefohn et al., 1997; Ollinger et al., 1997). This is in agreement with our results showing that under situations of relatively high summer P/PET (>0.96), the severity of O_3 visible injury increased with increasing $[O_3]$. Stomatal conductance, and the consequent O_3 uptake, were very likely high under high summer P/PET. In the same way, lower VI-sev with increasing $[O_3]$ under conditions of low summer P/PET could be explained by the fact that under a certain level of water availability, stomatal conductances are expected to be highly decreased and even though [O_3] are high, the O_3 uptake will remain low resulting on lower injury.

O_3 visible injury could thus be much better predicted using a stomatal flux-based model that would take into account the factors influencing stomatal conductance and the specific [O_3] at each site. More effort should be thus put on characterizing the micro-environmental conditions that affect stomatal conductance, which are usually influenced by local topography and stand structure, with the aim of better analyzing the relationship between O_3 visible injury and the specific environmental conditions for each site. In any case, although the mean % of the area of all symptomatic needles showing chlorotic mottling in each site was not higher than 30% (VI-sev score of 3.22), visible injury could have appeared much after below-ground responses to O_3 and negative effects on a microscopic level may have started (Andersen, 2003; Laurence and Andersen, 2003).

Higher crown defoliation and tree mortality coinciding with higher accumulated O_3 exposures and higher water availability

Mean values of crown defoliation between 20 and 66% in our study sites were not surprising if taking into account that crown defoliation of *Pinus uncinata* in the Iberian Peninsula for the period 1996-2006 increased from 15 to 25% (Carnicer et al., 2011). The rate of mortality followed the same pattern as defoliation, being higher in those sites with higher defoliation. The average of mortality rate for all sites was 9.19%, which is not far from the overall 6% measured from 1997 to 2007 for the same species all over the Iberian Peninsula in the work of Carnicer et al. (2011). In fact, several studies have revealed significant relationships between crown condition decline and tree mortality (Dobbertin and Brang, 2001; Drobyshev et al., 2007; Eckmullner and Sterba, 2000). The high crown defoliation and tree mortality, with defoliation considerably above the defoliation figure of 25% loss of leaves or needles, considered to be indicative of bad-health of a tree (Innes, 1998), show that *Pinus uncinata* stands studied here presented overall poor vitality status.

Crown defoliation and tree mortality were mostly related to the accumulated O_3 exposure during the last five years and to variables characterizing soil water availability. Plant response to O_3 depends on the amount of O_3 entering the leaves and the plant's sensitivity to O_3 (Matyssek et al., 2008), and the amount of O_3 entering the leaves is mainly affected by the stomatal conductance and by the O_3 concentration in the air (Ro-Poulsen et al., 1998). Stomatal conductance is controlled by a range of environmental variables such as light intensity, temperature, vapour deficit pressure and soil water availability (Zierl, 2002). Soil water availability is one of the major environmental factors limiting stomata conductance and, subsequently, the O_3 uptake by the plant (Nunn et al., 2005a; Panek and Goldstein, 2001; Patterson et al., 2000; Schaub et al., 2007; Schaub et al., 2003). Therefore, it seems that the higher defoliation and mortality found in those sites with greater soil water availability and accumulated O_3 exposure could be due to higher uptake of O_3. In fact, the highest values of crown defoliation and tree mortality were observed in the Guils transect, which was found to be significantly wetter than the Meranges transect.

Nonetheless, the relationship between O_3 exposure and crown defoliation and subsequent tree mortality cannot be actually established. Crown assessment basically based in crown defoliation has been found to be one of the best indicators of tree vitality (Dobbertin, 2005). However, tree vitality is influenced by a multitude of different stress factors (i.e. meteorological (e.g. air temperature, frost events), hydrological (e.g.

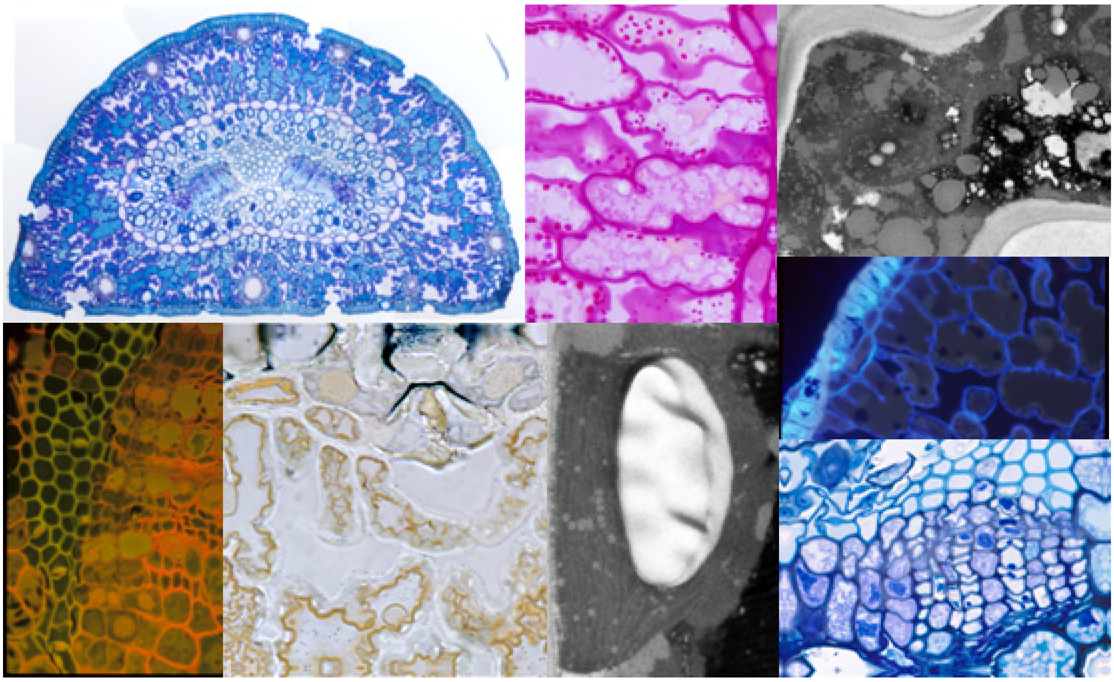
droughts, floods), biological (e.g. fungal disease, insects), chemical (e.g. air or soil pollution, soil nutrients), and physical (e.g. windbreak)) (Aamlid et al., 2000; De Vries et al., 2000; Landmann and Bonneau, 1995; Wellbum, 1994; Zierl, 2002), which makes that a meaningful relationship between crown defoliation and any of these environmental or anthropogenic stresses is hard to be detected (Zierl, 2002).

3.5. CONCLUSIONS

This study on severity of O_3 visible injury, crown defoliation and tree mortality along two altitudinal and O_3 gradients of *Pinus uncinata* in the Catalan Pyrenees indicates that O_3 contributes in part to the observed reduced tree vitality in this region. The severity of O_3 visible injury increased with increasing annual mean $[O_3]$ when summer P/PET was above a threshold of 0.96, whereas in drier conditions, higher $[O_3]$ did not yield higher O_3 visible injury. Crown defoliation and tree mortality were positively correlated with the accumulated O_3 exposure during the last 5 years and with variables referring to soil water availability, which also points to a likely greater O_3 uptake since soil water availability highly influences stomatal conductance. Nonetheless, the effect of O_3 cannot be established conclusively and definitively as the main cause factor of the crown defoliation and tree mortality observed in our study, since a multitude of different stress factors might be also contributing to the observed poor tree vitality. In any case, we can conclude that O_3 is one of the factors involved in the observed crown defoliation and tree mortality although further research is clearly warranted to determine the contribution of the multiple acting stress factors.

CHAPTER 4

Visible and microscopic ozone injury in foliage of *Pinus uncinata* from the Catalan Pyrenees



Abstract

The Catalan Pyrenees have been experiencing high levels of tropospheric O_3, exceeding the thresholds for forest protection during the last 16 years. Simultaneously, foresters expressed concerns about the vitality of *Pinus uncinata*, the dominant tree species at the subalpine forests of this region, whose sensitivity to O_3 has been proved in a free-air fumigation experiment with 7-years-old saplings. Aims in this study were to determine O_3 sensitivity of *Pinus uncinata* mature trees in the field by thoroughly describing the O_3-like visible injury and by validating the injury diagnosis with microscopy. Needles of 4 trees selected among 120 trees with O_3-like visible injury were collected to perform microscopical analyses in light, fluorescence and electron microscopy using serial sections centred on mottles identifying structural markers of the physiological responses induced by O_3 stress. We found the typical visible O_3 injury in the form of tiny and diffuse greenish mottles centred on stomata lines of light-exposed side of the needles. The symptoms were observed on 2 year or older needles and were similar to those induced by O_3 in the free-air fumigation experiment. Mottles were underlined by numerous changes in the cell wall structure and cell content - especially the chloroplast structure - peaking in the outer mesophyll layers and in cells below stomata. Overall, structural changes in mottles indicated increased oxidative stress and accelerated cell senescence processes, both typical of O_3 stress effects. Hence, there is evidence that *Pinus uncinata* mature trees in the field are sensitive to the ambient O_3 concentrations found in the Pyrenees and macroscopical and micrsocopical O_3 injury for this tree species remains now described in detail in the present work.

4.1. INTRODUCTION

Tropospheric ozone is considered one of the most important air pollutants affecting forest health (Matyssek et al., 2007). In the end of the 19th century, the mean ground-level O_3 concentrations were around 10 ppb (Anfossi and Sandroni, 1994b) but O_3 annual mean concentration have been increasing since then to a present level of 35-40 ppb in the mid-latitudes of the Northern Hemisphere (Fowler et al., 2008). Furthermore, these O_3 annual mean concentrations are expected to increase 20-25% from 2015 to 2050 according to the predictions of the Intergovernmental Panel on Climate Change based on the scenarios of high emissions (IPCC, 2007a). At present, O_3 concentrations in many areas at mid-latitudes of the Northern Hemisphere already exceed the threshold levels for vegetation protection and the risk for vegetation is expected to increase even more considering the projected concentrations for the future (Fowler et al., 2008).

Several studies have revealed that O_3 has negative effects on the health status of both broadleaved and conifer trees (Calatayud et al., 2011; Krupa et al., 1998). Ozone damage on some tree species such as *Fagus sylvatica, Betula pendula, Picea abies, Picea sitchensis, Pinus ponderosa, Pinus strobus* or *Pinus sylvestris* have been thoroughly studied due to their ozone-sensitiveness, abundance, distribution and economical value (Arbaugh et al., 2003; Bartholomay et al., 1997; Manninen et al., 2003b; Novak et al., 2008; Peñuelas et al., 1994; Pretzsch et al., 2010; Schaub et al., 2007; VanderHeyden et al., 2001). Nonetheless, the effect of O_3 on other tree species not so widely distributed but with a very important ecological value is starting to be studied. This is the case for *Pinus uncinata* in the Pyrenees mountains.

In the Catalan Pyrenees, the AOT40 has greatly exceeded the critical level, the target value and the long-term objective for forest and semi-natural vegetation protection set by the CLRTAP/UNECE and the European Directive 2008/50/EC during the last 16 years (Díaz-de-Quijano et al., 2012a). The Mountain pine (*Pinus uncinata* Ram.) is the dominant subalpine forest species in this mountain range from 1600-1800 (depending on the area) to 2400 m a.s.l. (Burriel et al., 2004) although the distribution of this European autochthonous species also comprises the Central and Western Alps and the Northern Appennines. At the end of the 90s, the Catalan Forestry Service started to report some processes of decline in the *Pinus uncinata* stands of the Central Catalan Pyrenees and significant increases in crown defoliation of this species in the Pyrenees have also been recently reported (Carnicer et al., 2011). In a recent study with free-air O_3 fumigation on *Pinus uncinata* saplings in the Swiss Alps it has been revealed that this species is sensitive to O_3 and that the ambient O_3 concentrations in the Pyrenees are likely to already be affecting *P.uncinata* stands (Díaz-de-Quijano et al., 2012b). However, these results were found on 7-years old saplings and even though the climatic conditions and O_3 exposure in the experiment were very similar to those in the Pyrenees, the grade of O_3 affectation on mature trees in field conditions still needs to be assessed.

Visible symptoms in the foliage of tree species are very important to detect and scale up the effects of different stress factors (Vollenweider and Günthardt-Georg, 2006). Nonetheless, the attribution of a given symptom to a specific stress factor is usually very difficult and, consequently, symptoms often require validation with microscopic analyses or experimental tests in controlled conditions (Evans et al., 1996; Günthardt-Goerg and Vollenweider, 2007; Novak et al., 2003). In the case of O_3 injury, the microscopic validation of visible symptoms are among the most efficient tools to validate O_3 effects in foliar material growing in the field (Günthardt-Goerg and Vollenweider, 2007). At microscopic level, there are several markers at characteristic tissues, cells and sub-cellular locations mostly in the mesophyll that are typical for plant responses induced by O_3 (Gravano et al., 2004; Günthardt-Goerg and Vollenweider, 2007; Kivimäenpää et al., 2003; Oksanen et al., 2004; Vollenweider et al., 2003). We here make use of these microscopic tools to validate the O_3-like symptoms in mature trees from the Pyrenees, in order to determine the effect of O_3 on this species under natural conditions. In this study we aimed 1) to thoroughly describe the visible and microscopic injury potentially caused by O_3 on this species in field conditions, and 2) to visually and microscopically confirm that the O_3-like symptoms found in individuals of *Pinus uncinata* growing in the field and exposed to high natural O_3 concentrations are caused by $O_{3.}$

4.2. MATERIAL AND METHODS

Study area

The study site is located in the area of La Cerdanya, in the Central Catalan Pyrenees. Two stands dominated by Pinus uncinata along two altitudinal gradients were chosen for the study. One of the transects ranges from 1500 m a.s.l. to 2200 m a.s.l. facing north-east (Guils transect) and the other transect ranges from 1700 m a.s.l. to 2500 m a.s.l. on the south-facing slopes of Puigpedrós (Meranges transect). In total, we had twelve sites, situated approximately every 200 m in altitude (sometimes every 100 m) (Fig. 1: A-C), where first crown defoliation, mortality and ozone-like visible injury in 120 trees (10 trees per site) had been assessed in a previous study (data not published). O_3 concentrations had been monitored by passive sampling in nine of these sites during 2004-2009 (Díaz-de-Quijano et al., 2009).

Sampling for morphological and microscopic analyses

For this study, we chose the two sites out of the twelve sites studied in the previous work (data not published), where symptoms were the most evident. These two sites corresponded to Guils 2200 m a.s.l. and Meranges 1700 m a.s.l. (Fig. 1: A,B). Four trees were sampled for ozone-like symptoms (chlorotic mottling) in these two sites. For each tree, 9 symptomatic and 3 asymptomatic (control) needle segments were photographed and fixed for morphological and microscopic analyses. Pictures of needle segments were used to both identify the exact location of the symptom and to describe the morphology of typical ozone-induced symptoms in our study species. Measurements on symptom morphology (area, circularity ($4\pi(\text{area}\cdot\text{perimeter}^{-2})$, a value of 1 indicates a perfect circle and as values approach 0, it indicates an increasingly elongated shape), number of symptoms per needle surface and area of needle covered by symptoms) were performed using Adobe Photoshop CS4 Extended (Adobe Systems Inc., San Jose/CA, USA).

As symptomatic material we always used the youngest needles showing symptoms to avoid as much as possible effects from other possible damaging factors, whereas for the controls we used year 2008 needles (1-year old needles, since the current year needles were still growing in May).

Microscopy

For microscopic analyses, 1/3 of needle segments were immediately fixed in methanol (material for the hand-cuttings), 1/3 in 2.5% glutaraldehyde and buffered at pH 7.0 with 0.067 M Soerensen phosphate buffer (material for the light microscopy) and 1/3 in 2.5% EM grade glutaraldehyde buffered at pH 7.0 with 0.067 M Soerensen

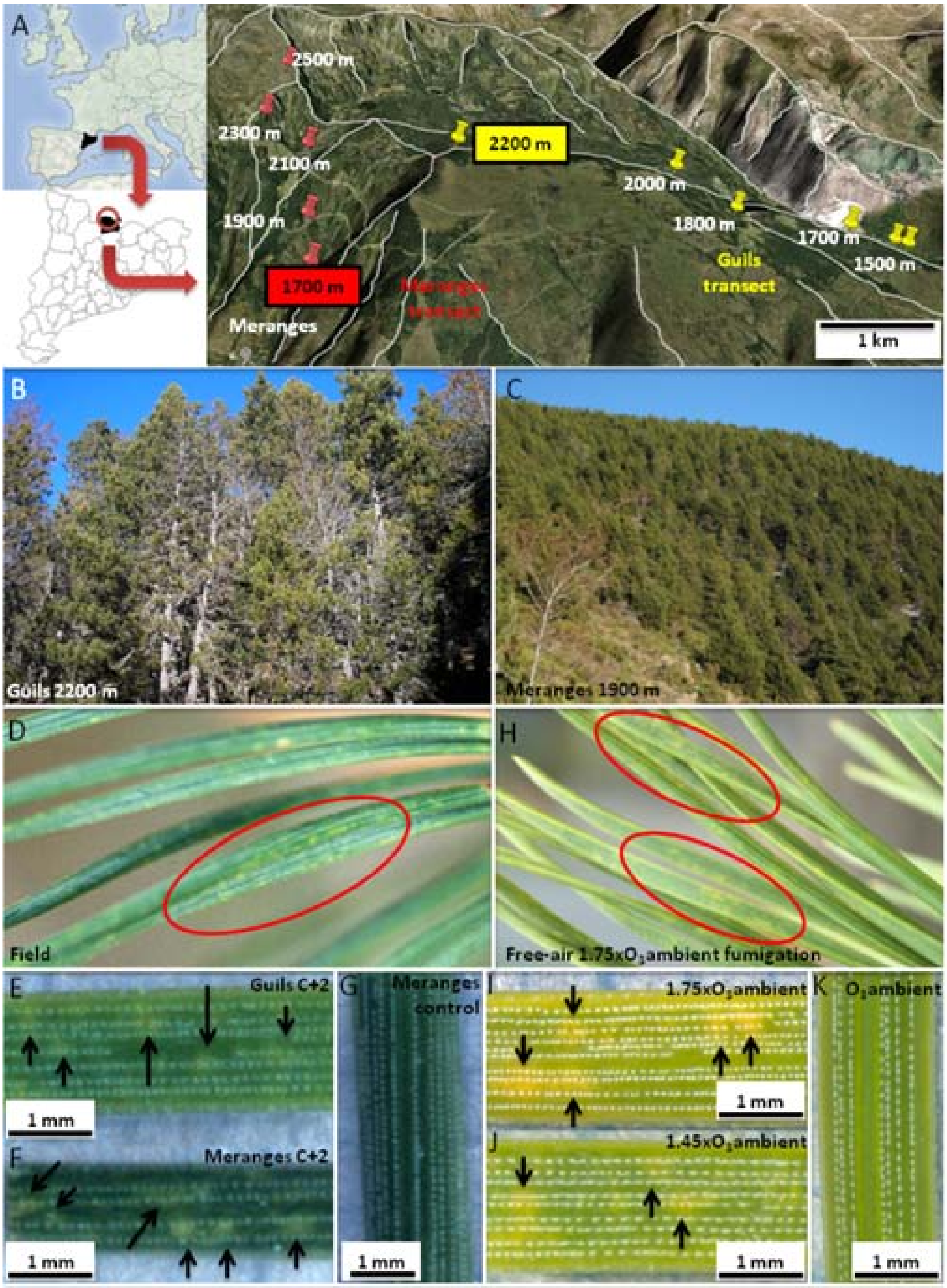


Fig. 1. A) Detailed map of the altitudinal transect sites studied in the area of La Cerdanya, in the Central Catalan Pyrenees. Sites highlighted with coloured squares are the ones where sampling for microscopy was performed; B) Guils site overview (G2200); C) Meranges site overview (M1900); D) detail of C+2 year whorl from Meranges showing the O_3-induced symptoms (red circle); needle segments of C+2 year from Guils (E) and Meranges (F) sites; G) needle segment of C+1 year from Meranges site (control); H) detail of a current year whorl from the free-air fumigation experiment in Alp Flix, Swiss Alps, fumigated with 1.75xO_3ambient; I) current year needle segment from a tree fumigated with 1.75xO_3ambient (mean O3 concentrations of 64.35 ppb during the growing season) in Alp Flix; J) current year needle segment from a tree fumigated with 1.45xO_3ambient (mean O_3 concentrations of 53.55 ppb during the growing season) in Alp Flix, and K) needle fumigated with O_3ambient (control, mean O_3 concentrations of 37.2 ppb during the growing season) in Alp Flix. Arrows show the O_3-induced mottles.

entirely infiltrated with the solution by evacuation before storing at 4 ºC until further processing. Histological, cytochemical and ultrastructural observations were performed using 30-µm hand-microtomed cuttings, 2.5-µm semi-thin and 70-nm thin cuttings.

Semi-thin cuttings were obtained after dehydrating the fixed material with 2-methoxyethanol (three changes), ethanol, n-propanol, n-butanol and embedding them in Technovit 7100 (Kulzer HistoTechnik). Thin cuttings were post fixed in 1:1 2% OsO_4 and phosphate buffer, dehydrated in ethanol at 25%, 50%, 70%, 90% and 100% and embedded in Epon (Sigma-Aldrich, Switzerland). Hand-cutting were obtained with a hand microtome and semi-thin and ultra-thin cuttings were obtained using a Reichert Ultracut S microtome. All material was stained (Table 1) and mounted either in the reagent, water, DePex or on Athene, 100 mesh copper grids (Plano GmbH) depending on the staining and observation technique. On average, we examined seven mottles per tree with a total of 130 hand cuttings, 190 semi-.thin cuttings and 4 ultra-thin-sections.

Table 1. Staining methods.

Stain	Reference	Solution	Staining time (min)	Color in transmitted light	Excitation (nm)	Application target	Fig.
Vanillin acid	Sarkar and Horwarth (1976)	10% vanillin in ethanol:37% HCl = 2:1	Observed in reagent	Red	-	Proanthocyanidins	LM: A-D
Fuchsin acid-toluidine blue	Adapted from Feder and O'Brien (1968)	1% fuchsin acid Aq.; 0.05% toluidine blue, acetate buffer pH 4.4; acetate buffer pH 4.4	20; 20; 1	Blue and red	-	Metachromatic stain	LM: F, H, J, K.
Coomassie blue	Adapted from Fisher (1968)	0.025% coomassie blue in ethanol 100%:acetic acid = 3:1; 7% acetic acid	35; 5	Blue	-	Proteins	LM: G, I, N, O.
Sudan black	Roth 0292.1	10 mg sudan black in 5 ml ethanol 94%:glycerine = 1:1	Observed in reagent	Black	-	Saturated lipids	LM: L, M.
Phloroglucinol	Fluka 79330	Saturated phloroglucinol in 18% HCl	Observed in reagent	Pink	-	Lignin	LM: E.
Coriphosphine	Weis et al. (1988)	0.03% coriphosphine Aq.	2	-	450-490	Pectins	LM: P-R.

Hand and semi-thin cuttings were observed using a Leica DM/RB 5× to 100× objectives and diascopic (with phase contrast) and episcopic (fluorescence) light illumination. Pictures were taken using either the digital Leica DC 500 camera interfaced by the Leica DC500 TWAIN software under control of the Image Access Enterprise 5 (Imagic, Glattbrugg, Switzerland) image management system (transmitted light microscopy), or the analogous micrograph system Wild MPS 548/52 using Kodak Ektachrome 400 Asa films (phase contrast and fluorescence microscopy). Pictures with the 40× objective or higher magnification were taken at different focal distances and stacked for a sharper image using Combine Z software (Hadley A.).

Ultrastructural observations were performed with the transmission electron microscope (TEM) Philips CM12 (Philips, Amsterdam, Holland) equipped with a side mounted Gatan digital camera (Gatan, Pleasanton, USA). For each sample observed at the TEM (one asymptomatic and one symptomatic for each of the two chosen plots), six cells were randomly chosen at different depths of the mesophyll and 4 or 5 chloroplasts were measured in each of the cells. Measurements of chloroplasts and starch grains size and shape and plastoglobuli density were performed using Adobe Photoshop CS4 Extended (Adobe Systems Inc., San Jose/CA, USA).

To assess the level of microscopic injury in our samples, we listed all the indications of possible ozone injury observed in each symptom (mottle) examined. Each indication was then assigned to a frequency category (0: not present, 1: present in one cell, 2: present in a few cells, but less than 50% of cells in the mottle, 3: present in more than 50 % of cells in the mottle or more than 50% of the area of the mottle). Frequency categories were averaged to obtain a score for each symptom, the so-called symptom score, which was the average of the category of frequency of all indications found in the studied mottle.

Statistical analyses

The analysed variables were the following: symptom area, symptom circularity, symptom density, percentage of sample area covered by symptoms, symptom score and number of indications per symptom. We tested these variables for differences between sites and trees using a nested design, with tree as the factor nested in site. Differences in the previously mentioned variables between needle side were tested by analysis of variance (ANOVA).

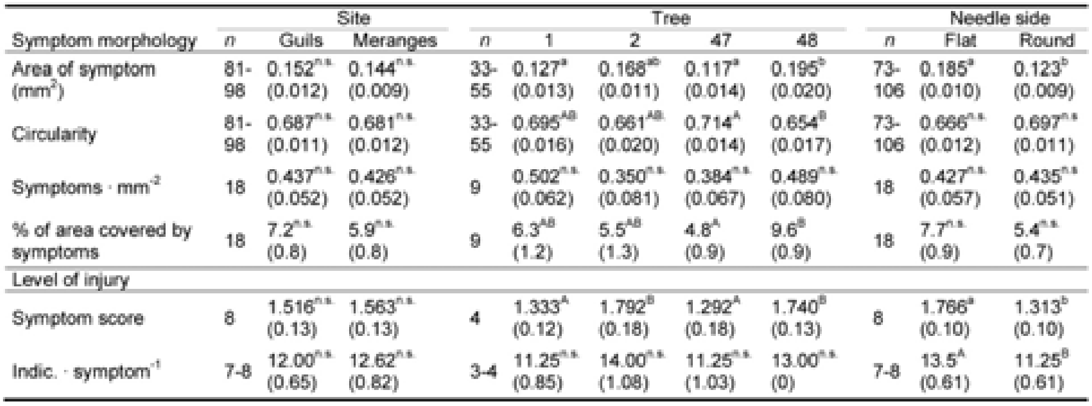
Changes in chloroplast and starch grains area and shape as well as plastoglobule (PG) density and number were tested by analysis of covariance (ANCOVA) using general linear models with categorical (symptomatic/asymptomatic and site) and continuous (chloroplast depth) predictor variables. The rate of change in chloroplast and starch grain area and number and density of plastoglobuli as a function of the chloroplast depth was modelled using linear regression after transforming the variables to accomplish the assumption of normality. All tests were conducted using STATISTICA 6 software (StatSoft Inc., Tulsa/OK, USA).

4.3. RESULTS

Symptom morphology

Ozone-induced visible injury had the form of tiny and diffuse green-yellowish mottles. These mottles were always located along stomata lines on the light-exposed side of the needles (Fig. 1: D-F). Morphology and location of the observed mottles were very similar to the morphology and location of mottles induced by ozone on the same tree species after being fumigated by O_3 during two growing seasons in a free-air O_3 fumigation facility in the Swiss Alps (Díaz-de-Quijano et al., 2012b) (Fig. 1: H-K). The symptoms were observed on 2-year or older needles and showed no morphological differences between plots (Table 2). Mottle size (mean area of symptoms), shape (circularity) and the percentage of sample area covered by symptoms were significantly different among trees, whereas symptom density did not significantly differ among trees (Table 2) Mottle area also differed among needle side, being bigger those mottles found

Table 2. Average of symptom area, circularity, number of symptoms per mm^2, percentage of sample area covered by symptoms, symptom score and number of indications per symptom (SE) for each site, tree and needle side. Symptom score is defined as the average of the category of frequency (0: not present, 1: present in one cell, 2: present in a few cells, but less than 50% of cells in the mottle, 3: present in more than 50 % of cells in the mottle or more than 50% of the area of the mottle) of all indications found in each mottle. Capital letters indicate statistical differences at $p < 0.05$ and lower case letters at $p < 0.001$.



	Site			Tree					Needle side		
Symptom morphology	*n*	Guils	Meranges	*n*	1	2	47	48	*n*	Flat	Round
Area of symptom (mm^2)	81-98	0.152$^{n.s.}$ (0.012)	0.144$^{n.s.}$ (0.009)	33-55	0.127^{a} (0.013)	0.168^{ab} (0.011)	0.117^{a} (0.014)	0.195^{b} (0.020)	73-106	0.185^{a} (0.010)	0.123^{b} (0.009)
Circularity	81-98	0.687$^{n.s.}$ (0.011)	0.681$^{n.s.}$ (0.012)	33-55	0.695^{AB} (0.016)	0.661^{AB} (0.020)	0.714^{A} (0.014)	0.654^{B} (0.017)	73-106	0.666$^{n.s.}$ (0.012)	0.697$^{n.s.}$ (0.011)
Symptoms · mm^{-2}	18	0.437$^{n.s.}$ (0.052)	0.426$^{n.s.}$ (0.052)	9	0.502$^{n.s.}$ (0.062)	0.350$^{n.s.}$ (0.081)	0.384$^{n.s.}$ (0.067)	0.489$^{n.s.}$ (0.080)	18	0.427$^{n.s.}$ (0.057)	0.435$^{n.s.}$ (0.051)
% of area covered by symptoms	18	7.2$^{n.s.}$ (0.8)	5.9$^{n.s.}$ (0.8)	9	6.3^{AB} (1.2)	5.5^{AB} (1.3)	4.8^{A} (0.9)	9.6^{B} (0.9)	18	7.7$^{n.s.}$ (0.9)	5.4$^{n.s.}$ (0.7)
Level of injury											
Symptom score	8	1.516$^{n.s.}$ (0.13)	1.563$^{n.s.}$ (0.13)	4	1.333^{A} (0.12)	1.792^{B} (0.18)	1.292^{A} (0.18)	1.740^{B} (0.13)	8	1.766^{a} (0.10)	1.313^{b} (0.10)
Indic. · $symptom^{-1}$	7-8	12.00$^{n.s.}$ (0.65)	12.62$^{n.s.}$ (0.82)	3-4	11.25$^{n.s.}$ (0.85)	14.00$^{n.s.}$ (1.08)	11.25$^{n.s.}$ (1.03)	13.00$^{n.s.}$ (0)	7-8	13.5^{A} (0.61)	11.25^{B} (0.61)

on the flat side of the needles (Table 2).

Microscopic analyses

Needle structure of asymptomatic samples from Guils and Meranges

Needle cross sections of asymptomatic needles from Guils and Meranges sites showed a rather vital structure of tissues. Meranges site sample showed the most vital structure of all examined samples, probably due to the favourable site conditions (lower elevation and milder temperature). On the whole, no traces of oxidation were found in cells from the mesophyll as it could be noticed by the homogenous staining of all mesophyll cells, usually being fucsia or even red, depending on the amount of olygomers of prothoancyanidins (OPC) accumulated (Fig. 2: A, C).

We found a normal epidermis and hypodermis cell structure. In the mesophyll cells, almost no cell wall protusions were found but a gradient from the inner to the outer mesophyll cells could be observed (i.e. decrease in the chloroplast and starch grain frequency and size and an increase in the stroma area and mitochondria frequency (Fig. 3: F, G)), although this gradient was not as sharp as in the symptomatic samples (Fig. 3: H, I). Cell structure showed several traits typical of rather healthy assimilative cells such as 1) thick and uncondensed cytoplasm (Fig. 3: A), 2) uncondensed nuclei (Fig. 3: A), 3) moderate membranes resolution (Fig. 3: A, F, G) 4) large chloroplasts showing thick grana with few transluscent PG, abundant no-transluscent PG and a low-moderate stroma condensation with nucleoid region (Fig. 3: F, G), 5) normal mitochondria structure (Fig. 3: A), and 6) a moderate lipid accumulation (Fig. 3: A) and homogenous condensed tannins deposition in the vacuoles. Cell structure in the Guils sample showed less vitality as expected from a site with more severe conditions. Cytoplasm was more

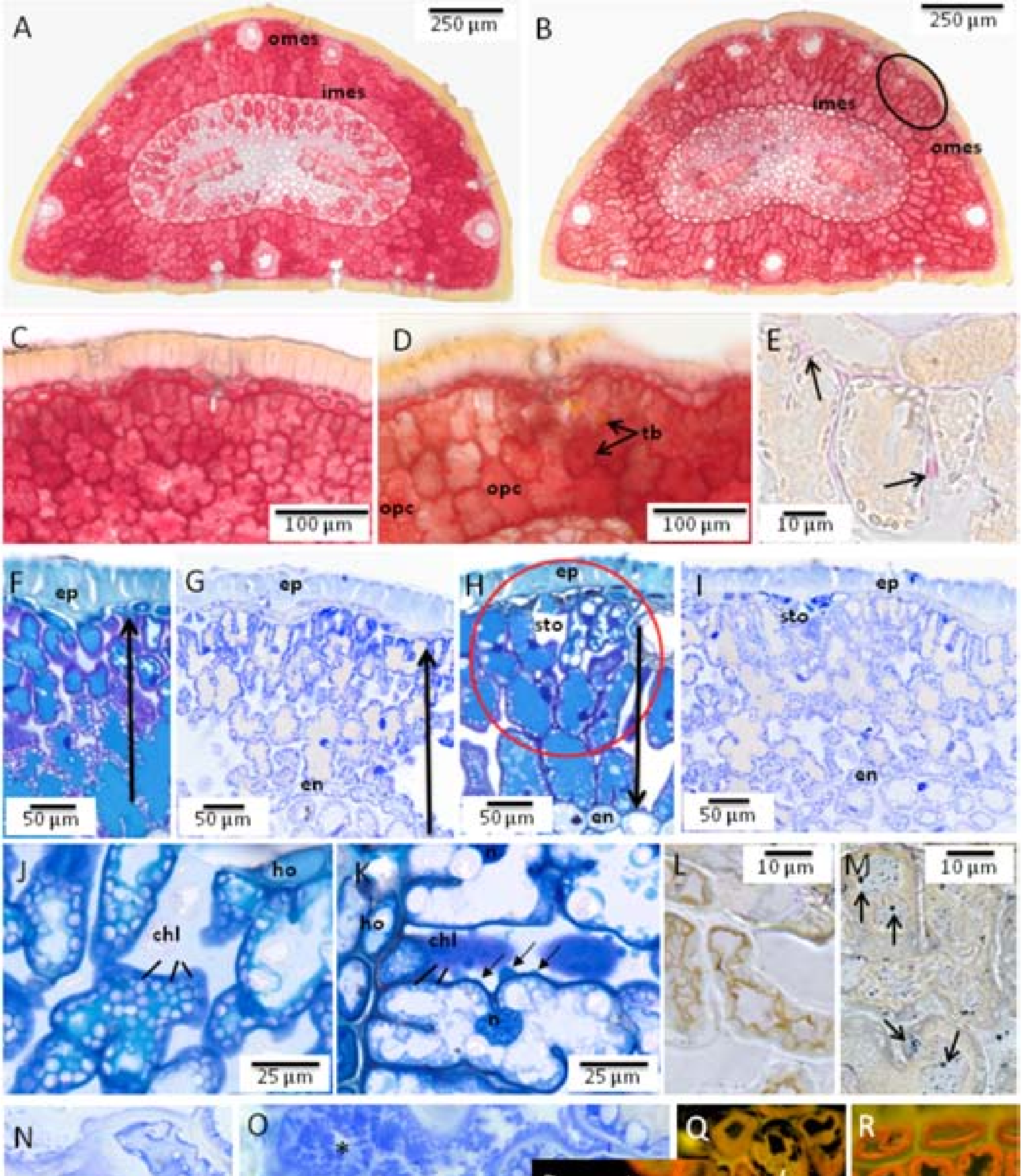


Fig. 2. Histological and cytological changes in symptomatic (B, D, E, H, I, K-Q) versus asymptomatic (A, C, F, G, J, L, R) samples. Overview of a symptomatic sample (B) with oxidation of cells in the outer mesophyll (omes) as can be seen with the brownish colour, especially right in the mottle (black circle), against the less oxidized cells from the inner mesophyll (imes) and from the asymptomatic sample (A), which are stained redder-fucsia. Detail of the mesophyll from a symptomatic sample (D) showing a greater oxidation of oligomers of proanthocyanidins (opc) around the stomata and an increased size and frequency of tannin bodies (tb) versus an asymptomatic sample (C). Lignin thickenings (arrows) in cell walls of mesophyll of symptomatic samples (E). Normal increasing gradient of protein content from the inner to the outer mesophyll (as shown by the violet staining in F and dark blue in G) in asymptomatic samples (F, G) against the inverted (H) or even lost (I) gradient in symptomatic samples. Red circle in H indicates where the mottle is located. Diminution in size and frequency of chloroplasts (chl) in cells underlying the mottle (K) versus chloroplasts from asymptomatic samples (J). Black arrows in K indicate wall-thickenings. Accumulation of oxidised lipids (arrows) in vacuols in cells from symptomatic samples (M) versus almost no-existent oxidised lipids in asymptomatic samples (L). Protein cell wall thickenings (arrows in N) and extracellular material (* in O) in a cell right in the mottle. White arrows (P, Q) show pectinic wart-like protusions on symptomatic cell walls in respect to asymptomatic cell walls (R). Ho: hypodermis, n: nucleus, ep: epidermis, en: endodermis, sto: substomatal chamber.

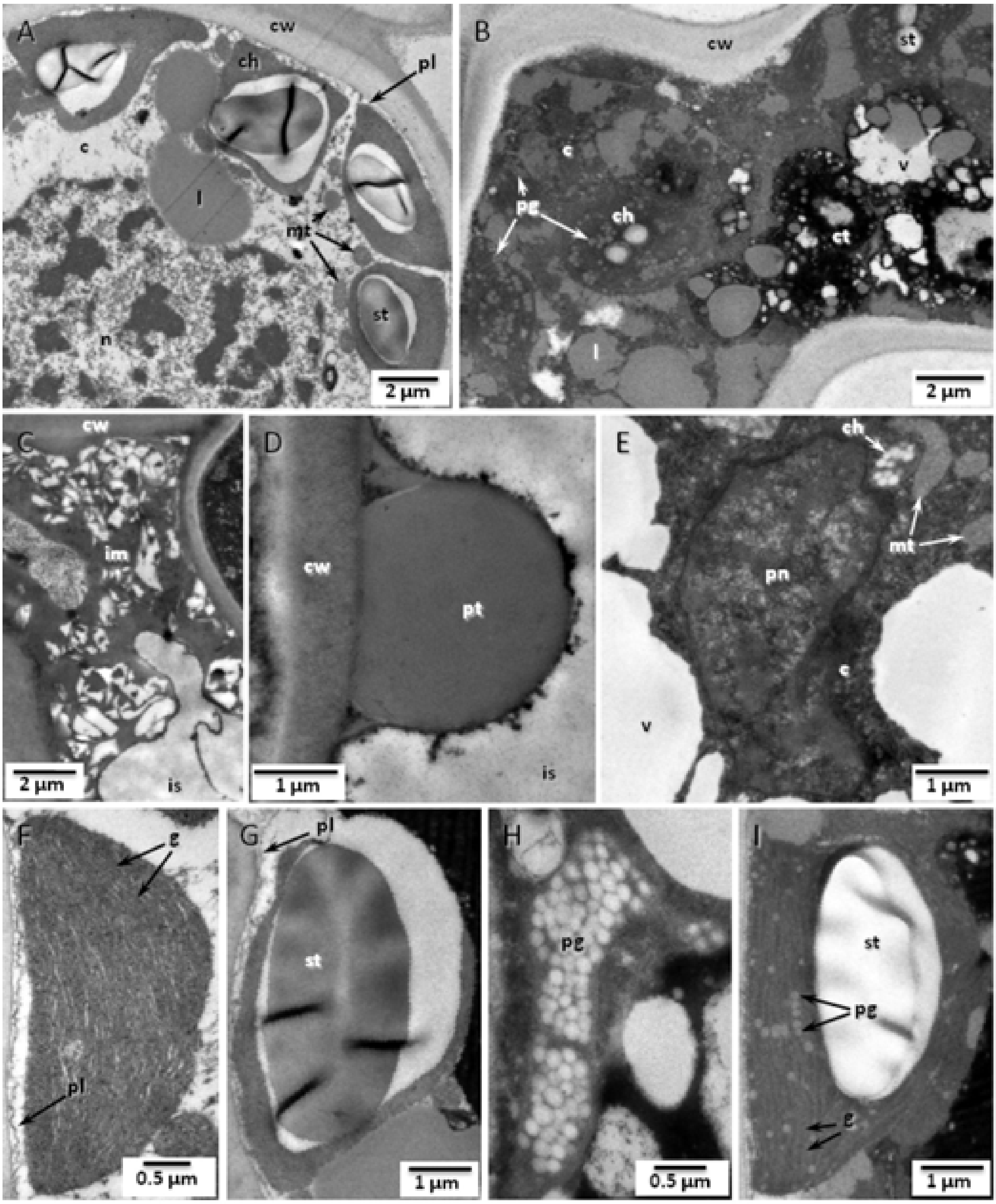


Fig. 3. Ultrastructural changes in symptomatic (B-E, H, I) versus asymptomatic samples (A, F, G). B) vs. A) within mottling symptoms, the mesophyll cell content showed increasing decompartmentation with cytoplasm interspersed with vacuolar regions, following tonoplast blebbing, and few structures/organelles still recognizable partly because of the fuzzy surrounding membranes. The cytoplasm (c) was condensed and showed many lipid inclusions (l). Chloroplasts (ch) showed condensation, poorly resolved grana and thylacoid membranes and increased plastoglobule (pg) frequency. C), D) Changes in apoplast included the accumulation of crystallites embedded within an amorphous substance in the intercellular space (is) and, on cell walls (cw), the occurrence of wart-like protrusions (pt) made of material similar to middle lamella. E) picnotic nucleus (pn) within an outer mesophyll cell from a mottling symptom. In comparison to normal nucleus (n) structure (A), nuclear material had shrunken to a small, deformed and highly condensed structure. F-I) structural differences between the chloroplasts from outer (F, H) vs. inner (G, I) mesophyll layers within symptomatic (H, I) vs. asymptomatic (F, G) tissue. The chloroplasts from inner layers were larger, contained more starch (st) but had their matrix and grana content proportionally reduced in comparison to the organelles from outer layers. Within mottling symptoms, the chloroplast size was noticeably reduced, especially within outer layers, but the platoglobule (pg) frequency and density was raised, especially within the chloroplasts just below hypodermis. The chloroplast envelope and inner membranes were no longer distinct. Other structures: pl: plasmallemma, mt: mitochondria, ct: condensed tannins, v: vacuole, im: intercellular material, g: grana.

asymptomatic samples.

Structural changes underlying mottling

The worst injury was found right in the mottles within the outer mesophyll cells adjacent to the substomatal chamber, in the exposed part of the needles (just where O_3 is expected to enter inside the needles). On the whole, a sharp gradient of degenerated cells could be observed from the outer mesophyll to the inner mesophyll layers, showing particularly degenerated cells in the mottle area. In the outer mesophyll or even in the hypodermis, cells were highly oxidated as it could be noticed by the brownish colour of the OPC against the redder-fucsia colour of the inner cells of the mesophyll (Fig. 2: B, D). This increase of oxidation in the outer part of the mesophyll was also clear when looking at the protein content. On the whole, protein content increased from the inner to the outer mesophyll due to chloroplasts proteins (Fig. 2: F, G) but this gradient was lost or even inverted right in the mottles, indicating an oxidative process starting around the stomata (Fig. 2: H, I).

In mottles, we found accumulation of crystallites (Fig. 3: C) and proteinic intercellular material (Fig. 2: O) in the intercellular space around the subestomatic chambers. The outer mesophyll cells showed wart-like protrusions in the cell wall (Fig. 3: D) made of material similar to middle lamella, pectins (Fig. 2: P, Q vs. R) and proteins (Fig. 2: N). Ligninic cell wall thickenings which are indicative of an increased oxidative stress were also found in mottles (Fig. 2: E). Both ligninic thickenings and pectinic wart-like protrusions were also found in other parts of the needles and even in some asymptomatic samples, but always in less abundance.

Membranes as plasmalema, tonoplast and nuclei membrane usually had very poor resolution, making the identification of organelles more difficult and leading to an appearance of cell content decompartimentation with condensed cytoplasm mixed together with vacuolar regions (Fig. 3: B).

Nuclei were highly condensed and deformed (picnotic nuclei, Fig. 3: E), mitochondria became smaller and contracted in the outer mesophyll layers (Fig. 3: E) and many lipid inclusions were found in the cytoplasm (Fig. 3: B). Tannin bodies, which are usually present in mesophyll cells, increased in size and frequency in the symptomatic samples, specially just below the mottle (Fig. 2: D vs. C). Oxidised lipids in vacuols (Fig. 2: M vs. L) as well as cytoplasm decompartimentation and nucleous condensation were found in almost all symptomatic samples but they affected only a few cells in the mottle.

Chloroplast and starch grain size and frequency decreased markedly from inner to outer mesophyll, grana thickness and frequency decreased, and stroma condensation and transluscent plastoglobuli density increased (Fig. 3: H, I). Contrary to asymptomatic samples, chloroplasts from cells in the mottle were drastically reduced in size and completely filled with plastoglobuli Fig. 3: H) and, even in some cases, they seemed to be completely destroyed (Fig. 2: K vs. J).

The level of microscopic injury did not vary between Guils and Meranges sites, as it can be seen with the symptom score obtained for each site (Table 2). Nonetheless, microscopic injury did significantly differ among trees and was significantly higher on the flat side of the needles (Table 2). That is, mottles located on the flat side of the needle showed a greater ozone injury, which can be seen with the higher symptom score and the significantly higher number of indications that they show in comparison with mottles located on the round side of the needle (Table 2).

Table 3. Chloroplast, starch grain and plastoglobuli traits and number at different locations in the mesophyll layers (inner, mid and outer mesophyll) for asymptomatic and symptomatic needles and summary of the main effects of symptom (symptomatic, asymptomatic), chloroplast depth (covariate), site (Guils, Meranges), the two-way interaction (site*symptom) and the three-way interaction (site*symptom*chloroplast depth). A separate slope design was used to model each factor's influence on chloroplast parameters, starch circularity and PG parameters because of the significant interaction between the covariate and categorical factors, whereas a traditional analysis of covariance design was computed for the starch area (n.s.).

				Chloroplast			Starch			PG	
Sample type	Mesophyll location	Depth (µm) (SE)	*n*	Area (µm²) (SE)	Circularity (SE)	*n*	Area (µm²) (SE)	Circularity (SE)	*n*	PG num. ·chloroplast^{-1} (SE)	PG number · µm^{-2} (SE)
Asymptomatic		157.81(9.77)	60	14.89(0.78)	0.65(0.01)	48	6.8(0.53)	0.69(0.01)	60	3.17(0.68)	0.21(0.05)
Symptomatic		163.21(10.47)	53	13.84(1.29)	0.62(0.02)	39	7.05(0.8)	0.72(0.02)	53	28.94(2.6)	3.9(0.7)
Asymptomatic	inner	251.49(14.15)	15	15.13(0.84)	0.64(0.03)	15	7.31(0.74)	0.7(0.02)	15	6.87(1.29)	0.48(0.09)
Asymptomatic	mid	163.71(6.76)	25	17.7(1.25)	0.72(0.01)	25	7.47(0.77)	0.73(0.01)	25	3.48(1.21)	0.22(0.08)
Asymptomatic	outer	80.18(6.36)	20	11.19(1.27)	0.58(0.02)	8	3.73(1.15)	0.58(0.04)	20	0	0
Symptomatic	inner	243.4(8.06)	17	22.51(1.66)	0.61(0.02)	16	10.05(1.39)	0.72(0.01)	17	25.35(3.54)	1.29(0.25)
Symptomatic	mid	174.81(9.8)	18	15.06(1.65)	0.65(0.03)	16	6.54(0.61)	0.73(0.03)	18	33.83(5.5)	2.24(0.29)
Symptomatic	outer	75.86(4.41)	18	4.44(0.56)	0.6(0.03)	7	1.37(0.41)	0.69(0.05)	18	27.44(4.15)	8.03(1.64)
ANCOVA											
			d.f.								
Symptom			1	<0.001	n.s.		n.s.	n.s.		<0.001	<0.01
Chloroplast depth			1	-	-		<0.001	-		-	-
Site			1	n.s.	n.s.		<0.001	<0.001		n.s.	n.s.
Site*Symptom			1	n.s.	<0.001		n.s.	<0.001		<0.05	n.s.
Site*Symptom*Chlp.depth			4	<0.001	n.s.		-	<0.001		<0.001	<0.001

Quantitative changes in chloroplast structure

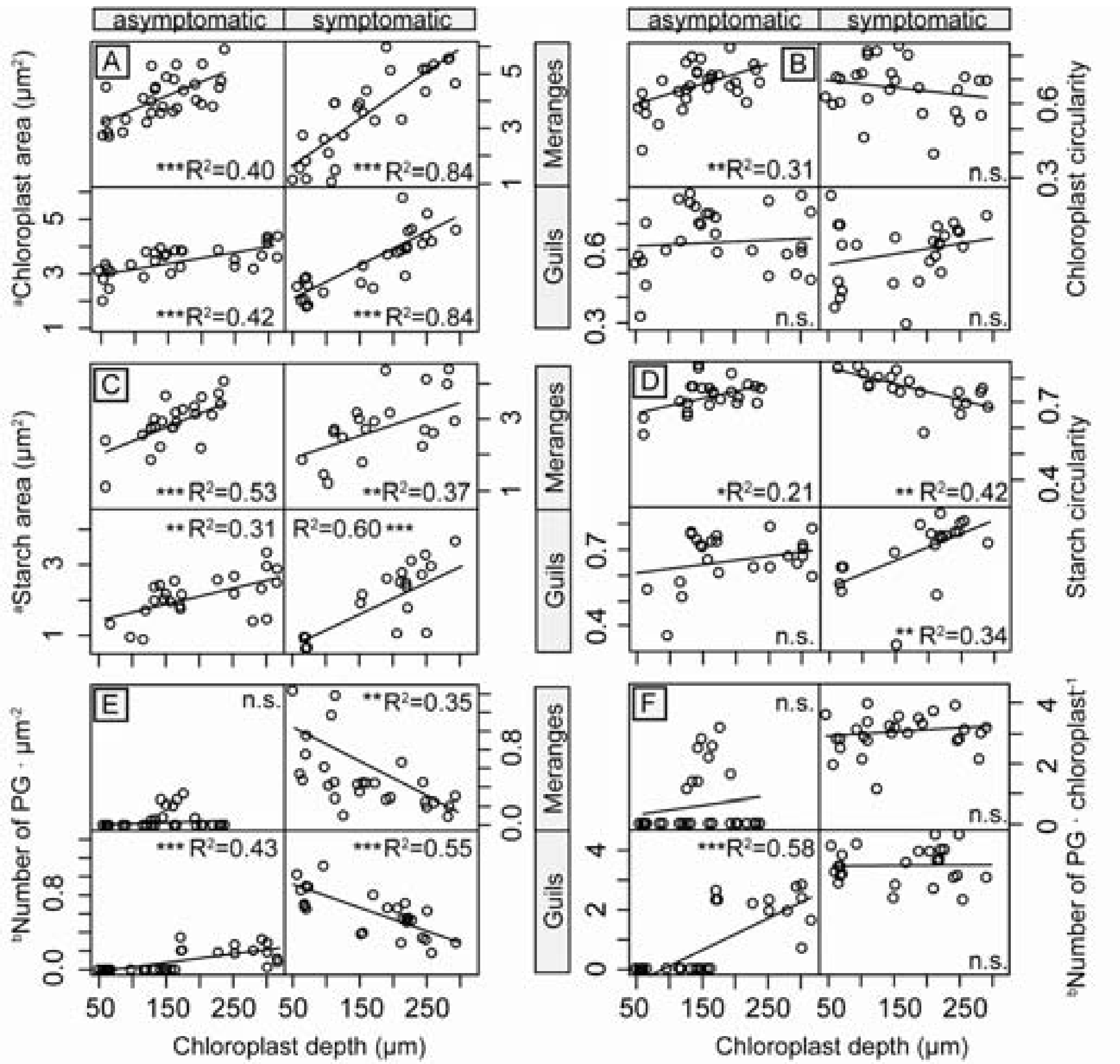
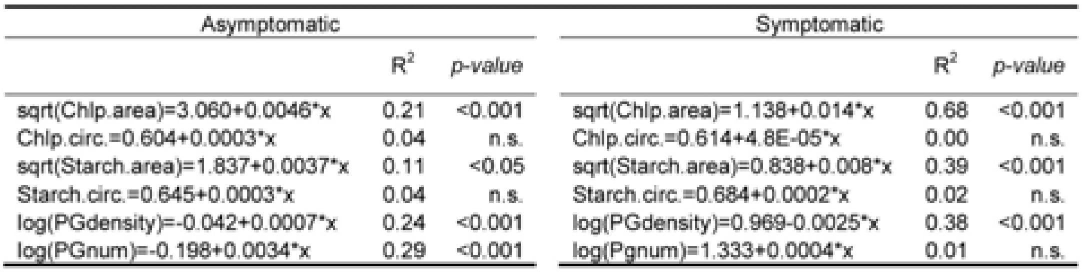


Fig. 4. Chloroplast area (A) and circularity (B), starch grain area (C) and circularity (D) and plastoglobuli density (E) and number (F) as function of the chloroplast depth inside mesophyll in asymptomatic and symptomatic needles, for each site. Chloroplast and starch area were transformed using a square root function (a) and PG density and number using a log function (b) prior to regression. R^2 is shown when there is statistically significance at * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. n.s. = not significant.

Chloroplast area, starch grain area and shape and plastoglobuli number and density showed significant differences according to the location of chloroplast in the mesophyll (Table 3, Fig. 4: A, C-F). Chloroplast area and plastoglobuli number and density also showed significant differences between asymptomatic and symptomatic samples (Fig. 4: A, E, F; Table 3).

Table 4. Regression equations of chloroplast area, starch grain area and plastoglobuli density as function of chloroplast depth in the mesophyll, for symptomatic and asymptomatic needles. Dependent variables were transformed using a square root function (for chloroplast and starch area) and a log function (for PG density and PG number) prior to regression.



Asymptomatic			Symptomatic		
	R^2	*p-value*		R^2	*p-value*
sqrt(Chlp.area)=3.060+0.0046*x	0.21	<0.001	sqrt(Chlp.area)=1.138+0.014*x	0.68	<0.001
Chlp.circ.=0.604+0.0003*x	0.04	n.s.	Chlp.circ.=0.614+4.8E-05*x	0.00	n.s.
sqrt(Starch.area)=1.837+0.0037*x	0.11	<0.05	sqrt(Starch.area)=0.838+0.008*x	0.39	<0.001
Starch.circ.=0.645+0.0003*x	0.04	n.s.	Starch.circ.=0.684+0.0002*x	0.02	n.s.
log(PGdensity)=-0.042+0.0007*x	0.24	<0.001	log(PGdensity)=0.969-0.0025*x	0.38	<0.001
log(PGnum)=-0.198+0.0034*x	0.29	<0.001	log(Pgnum)=1.333+0.0004*x	0.01	n.s.

Chloroplast in the outer mesophyll (depth = 78 µm) had an average surface 34% smaller than in the inner mesophyll (depth = 247 µm) (Table 3, 4) and this size gradient explained up to 21 % of the chloroplast area variance (Table 4). This difference was more pronounced in the symptomatic samples, where chloroplasts in the outer mesophyll were 76% smaller than in the inner mesophyll layers (Fig. 4: A, Table 3, Table 4) and the size gradient accounted for 68 % of the chloroplast area variance (Table 4). Whatever the factor, chloroplast shape hardly showed any change (Table 3; Fig. 4G: B).

Starch grain area also significantly increased with chloroplast depth but this increase did not significantly differ between symptomatic and asymptomatic samples (Fig. 4: C, Table 3). In the outer mesophyll (depth = 78 µm), starch grain area was 40% smaller than in the inner mesophyll (depth = 247 µm) for the asymptomatic samples and this difference was 1.82 times bigger for the symptomatic samples (Fig. 4: C, Table 3, Table 4). The shape of starch grains also significantly changed depending on chloroplast depth, becoming rounder towards the inner part of the mesophyll, except in the symptomatic samples of Meranges, where they behaved quite the opposite –starch grains were rounder in the outer mesophyll (Fig. 4: D, Table 3).

The number of plastoglobuli were primarily responsive to the presence of mottling, and both the number and density of plastoglobuli per chloroplast in symptomatic vs. asymptomatic material was significantly increased (by 9.1 and 18.6 times, for number and density, respectively; Table 3). Interestingly, the smaller chloroplasts within the outer layers of symptomatic needles showed high plastoglobule densities as shown by the negative correlation between the plastoglobule density and chloroplast size ($p<0.01$, $R^2=8.1\%$; Fig. 4: E, Table 3). However, chloroplast depth had little influence on the number of plastoglobuli per chloroplast in the symptomatic samples (Fig. 4: F). Only in the asymptomatic samples of Guils, larger chloroplasts, preferentially within the deeper layers of mesophyll, accumulated more plastoglobules, as shown by the significant positive correlation between the plastoglobule number and chloroplast size ($p<0.05$, $R^2=5.4\%$, Fig. 4: F).

4.4. DISCUSSION

Typical O_3-induced visible symptom morphology

Several previous studies show that mottling mostly in pine species and chlorophyll bleaching are the usual visible symptoms in response to O_3 stress in conifers (Arbaugh et al., 1998; Dalstein et al., 2002; Sanz et al., 2000). The diffuse green-yellowish mottles on the light-exposed side of the needles found in our trees coincide with the same symptoms reproduced during several controlled or semi-controlled exposures of the same and other pine species to O_3 (Anttonen et al., 1998; Díaz-de-Quijano et al., 2012b; Elvira et al., 1995; Flagler and Chappelka, 1995; Gimeno et al., 1992). The analogous mottles observed in *P.uncinata* from the free-air O_3 fumigation experiment in the Swiss Alps confirm, then, that mottles observed in our pines from la Cerdanya are most probably caused by O_3 (Díaz-de-Quijano et al., 2012b). In fact, both O_3 mean concentrations and values of AOT40 at the free-air fumigation experiment (Díaz-de-Quijano et al., 2012b) are comparable to the O_3 mean concentrations monitored at our study sites and to the AOT40 values monitored at other nearby sites in the Catalan Pyrenees (Díaz-de-Quijano et al., 2012a) (mean [O_3]: 62.1 ppb at M2300 vs. 63.5 ppb at O_3++; 53.9 ppb at G2000 vs. 53.5 ppb at O_3+; 46.1-46.9 at G1500-M1700 vs. 37.2 ppb at the control; AOT40 Apr-Sept: 17.4 ppm·h at 1224 m in the Catalan Pyrenees vs. 19.2 ppm·h at O_3+).

Nonetheless, we need to consider that results from the experiment in the Swiss Alps are based on 7-years old saplings whose stomatal conductances, and consequent O_3 uptake, are higher than those in field mature trees, as it has been seen in the author's unpublished data and also in other species (Nunn et al., 2005b). This can explain why mottles were observed in 2-years old or older needles in the field, whereas in the fumigation experiment mottles appeared in the current year needles. Consistently to our results, in other studies current year needles have been observed to be affected when trees were exposed to particularly high levels of ozone (see Barnes et al., 1999), which would be the case in the fumigation experiment due not to a higher O_3 concentrations but to a greater O_3 uptake as consequence of higher stomatal conductances.

Location of mottles at the light exposed tree, branch and needle side is a typical feature of O_3-induced injury (Günthardt-Goerg and Vollenweider, 2007). O_3 yields to an additional source of reactive oxygen species (ROS) which in addition to the light-dependant generation of ROS caused by high light (photooxidative stress), outbalances ROS detoxification systems giving place to an increased oxidative damage (Foyer et al., 1994). This synergetic action of O_3 and light seems to be the cause of finding mottles on the light exposed side of our needles.

Differences in symptom morphology variables among trees but not among sites suggest that expression of symptom morphology might depend on tree-intrinsic characteristics and/or different environmental conditions at small scale around each tree. Differences in mottle area between needle sides could be result of different stomatal conductances between both needle sides, due to different microclimatic conditions between both sides e.g. incidence of light only on one of the needle sides.

Microscopic analyses

Typical structure and location of markers for the oxidative stress caused by O_3

Nature and location of most of the cytochemical markers observed below mottles in our symptomatic samples have been already observed to be induced by O_3 under controlled conditions in other tree species (Vollenweider et al., 2003 and references within it). This further indicates that the observed mottles in *P. uncinata* from la Cerdanya are most probably caused by the effect of O_3.

Most conifers with O_3 symptoms show a clear increasing gradient of injured cells from the inner to the outer cell layers of the mesophyll (Anttonen and Karenlampi, 1996; Dalstein et al., 2002; Vollenweider et al., 2003). Photooxidative stress seems to interact with O_3 stress by enhancing visible and microscopical damage (Dalstein et al., 2002; Foyer et al., 1994; Günthardt-Goerg and Vollenweider, 2007; Vollenweider et al., 2003), which could explain why the injury was usually found in the outer mesophyll of sun-exposed parts of needles. In fact, visible O_3 injury either in conifers or broadleaved trees has been found especially after sunny periods and high O_3 concentrations mostly at those trees, branches, leaves or needles exposed to light (Maurer et al., 1997).

The enhanced oxidation of OPC as well as bigger and more frequent tannin bodies in the outer mesophyll of the sun-exposed part of the symptomatic needles has also been found in other studies (see Günthardt-Goerg and Vollenweider, 2007). This type of tannins, the proanthocyanidines, is especially sensible to oxidation (Amiot et al., 1992). They have, among others, an antioxidant and radical scavenging functions and they are usually present in gymnosperms (Kraus et al., 2003). However, their oxidation can be an indicator of a change in the cellular redox potential (Vollenweider et al., 2003), which can be modified by the effect of O_3 (Schraudner et al., 1997), as it seems to happen in our symptomatic samples below the mottles and around the substomatal chamber, right in the place where O_3 enters the needles.

Cell-wall thickenings and wart-like protrusions are common O_3 responses (Günthardt-Goerg et al., 1997; Vollenweider et al., 2003), although they can also be induced by other oxidative stressors, e.g. heavy metals (Hermle et al., 2007) or drought (Pääkkönen et al., 1998). The presence in much less abundance of these cell-wall markers in some asymptomatic samples have also occurred in asymptomatic material from field samples in other studies (Günthardt-Goerg, 1996; Günthardt-Goerg et al., 1997). It has been suggested that they might occur prior to the development of visible injury, as a first result of the cascade of oxidative reactions that take place after O_3 penetrates in the needle apoplast (Oksanen et al., 2004; Vollenweider et al., 2003).

Intercellular cell content remnants leaked from necrotic cells below mottles have also been observed in O_3-fumigated and field samples (Günthardt-Goerg and Vollenweider, 2007; Paoletti et al., 2010). This shows again that ROS target macro-molecules in cell walls being able to cause a final cell wall rupture (Paoletti et al., 2010). However, in our samples we did not observe cell wall rupture as it has also been observed in mesophyll cells in most conifer needles, where collapse do not occur with ambient O_3 dose because cells are thicker and sturdier than in leaves (Günthardt-Goerg and Vollenweider, 2007).

Ozone is known to adversely affect membrane lipids as the ROS liberated following the decomposition of O_3 oxidize the numerous double bonds present in membrane lipids (Mudd et al., 1971; Tomlinson and Rich, 1969). Therefore, poor membrane resolution with the subsequently cytoplasm decompartimentation are also expected from the effect of O_3, suggesting a decreased desaturation of fatty acids (Anttonen et al., 1995).

The observed condensation of cytoplasm, nucleus and chloroplasts together with the accumulation of secondary compounds (phenolics) in mesophyll cells are indicative of a process of accelerated cell senescence (ACS; Vollenweider et al., 2003). ACS occur in stressed foliage organs before autumn and/or in young needles and it comprises different physiological reactions (Pell et al., 1997) and structural changes (Vollenweider et al., 2003) that resemble natural senescence (Günthardt-Goerg and Vollenweider, 2007). Ozone has been observed to accelerate cell senescence (Bortier et al., 2000; Pell et al., 1997), and location of ACS symptoms in all still living mesophyll and epidermis cells has been used as criteria in differential diagnosis of O_3 symptoms (Vollenweider et al., 2003).

Ozone has also been found to reduce the size of mitochondria, probably due to high ADP concentration or to osmotic properties of the cells (summarized by Bereiterhahn and Voth, 1983). Lipid inclusions in the cytoplasm may arise from O_3-induced injuries to the lipids of the cellular membranes, which would also be manifested in ultrastructure as increased plastoglobuli (Anttonen and Karenlampi, 1996). Modifications in plastoglobuli number and chloroplast and starch grains size and frequency are further discussed in next section.

Microscopic injury in the same way as symptom morphology differed among trees rather than among sites, which again suggests that intrinsic characteristics of trees and/or environmental conditions at the tree micro-scale may have a bigger influence on symptom microscopical severity than the environment conditions found in each site. Higher number of indications and symptom scores found in greater mottles suggest that area of the mottles can be used as an indication of severity of microscopic injury.

Chloroplasts structure typical for the O_3 diagnosis

Ozone has been found to induce the same alterations in the structure of the chloroplasts of several coniferous species either in laboratory chambers, open-top chambers and open-field (Anttonen et al., 1995; Anttonen and Karenlampi, 1996; Holopainen et al., 1996; Sutinen, 1987, 1990). Choloroplasts with a reduction in size and the stroma electron dense, being both alterations more pronounced in the outer mesophyll cell layers and in the upper side of the needle compared to the inner layers and lower side are the three characteristics that being present all together can be used for O_3 diagnosis (Kivimäenpää et al., 2005). These three features were clearly present in our symptomatic samples, which gives evidence again of the affection of O_3 on our sampled trees. However, a reduction in the chloroplast size in the outer mesophyll cell layers was also observed in the asymptomatic samples, but not as pronouncedly as in the symptomatic material. Asymptomatic samples were 1-year old, so they had also been under ambient O_3 exposure for one year. Therefore, it seems normal to find a slight O_3 injury in these samples whereas symptomatic older needles are expected to show higher amount and more severe injury than younger ones due to longer exposure or greater susceptibility of the older needles (Anttonen et al., 1996; Wulff et al., 1996). In many conifer species, needles with no visible injury symptoms, as our asymptomatic samples, have shown a decrease in the size of chloroplasts as the typical response to O_3 stress (Kivimäenpää et al., 2005).

Other changes in chloroplasts like changes in starch accumulation or in the amount and color of plastoglobuli reported in needles fumigated with O_3 are not commonly used in O_3 diagnosis because the direction of the changes are variable (Holopainen et al., 1996; Sutinen, 1987; Sutinen et al., 1990; Utriainen et al., 2000; Wellburn and Wellburn, 1994) and they have also been reported with other stress factors (Holopainen et al., 1992; Sutinen and Koivisto, 1995). Actually, we observed a decrease in the size of starch grains in the outer mesophyll cell layers as it has been often reported in the mesophyll of conifers needles exposed to moderately O_3 concentrations (Anttonen and Karenlampi, 1996; Sutinen et al., 1990; Utriainen and Holopainen, 2000) but in our case, no significant differences in starch grain size were found between symptomatic and asymptomatic samples.

Although changes in the amount and color of plastoglobuli are not usually used for O_3 diagnosis, accumulation of plastoglobuli in the chloroplasts have been reported in several conifer species after O_3 exposure (Anttonen and Karenlampi, 1996; Sutinen and Koivisto, 1995; Sutinen et al., 1990; Wellburn and Wellburn, 1994). Plastoglobules are plastid-localized lipoproteins that can synthesize, store and traffic tocopherols and other molecules that function as antioxidants in the chloroplast (Bréhélin et al., 2007). Increases in the number and size of plastoglobuli after O_3 exposure might be related to membrane decomposition (Anttonen and Karenlampi, 1996). In our symptomatic samples, the amount of non-osmiophilic (light or transluscent) plastoglobuli was increased. Nonetheless, it is not clear if this increase is related to processes of senescence and ageing as it has been previously reported in other studies (Bréhélin et al., 2007) or if, on the contrary, these changes are not related to the plastoglobuli changes typical of ageing or senescence that experience many angiosperms (Kivimäenpää et al., 2010).

4.5. CONCLUSIONS

With the present study and the evidence provided by the mentioned free-air O_3 fumigation experiment we can conclude that *Pinus uncinata* is a tree species sensible to O_3 and that the effect of O_3 is noticeable not only on fumigated saplings, which are expected to show greater O_3 uptake, but also on mature trees in the field.

Pinus uncinata stands from the Catalan Pyrenees have been found to present the typical visible injury caused by O_3. At the macroscopic level, the O_3-induced visible injury had the form of tiny and diffuse green-yellowish mottles located around the stomata lines on the light exposed side of the needles. The observation of the same typology of mottles in the mentioned free-air O_3 fumigation experiment confirms that the observed mottles in field mature trees are most probably caused by the effect of O_3. At the microscopic level, most of the cytochemical markers observed below mottles in our symptomatic samples had already been observed to be induced by O_3 under controlled conditions in other tree species. The most typical indications for O_3-induced injury found in this study were chloroplasts reduction in size with stroma electron dense -being more pronounced in the outer mesophyll cell layers-, increased gradient of oxidation of cell content from the inner to the outer mesophyll, cell wall thickenings and wart-like protrusions, accumulation of antioxidants, and accelerated cell senescence processes.

Therefore, we can confirm that the O_3 uptake by *Pinus uncinata* mature trees in the Catalan Pyrenees are high enough to produce the typical O_3-induced visible injury, which has now been macroscopically and microscopically described in detail for this species in the present work.

Acknowledgement

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CHAPTER 5

Ozone visible symptoms and reduced root biomass in the subalpine species *Pinus uncinata* after two-years of free-air ozone fumigation

Díaz-de-Quijano, M., Schaub, M., Bassin, S., Volk, M., Peñuelas, J., 2012. Ozone visible symptoms and reduced root biomass in the subalpine species *Pinus uncinata* after two years of free-air ozone fumigation. Environmental Pollution, doi:10.1016/j.envpol.2012.1002.1011.

Abstract

Concentrations of ozone often exceed the thresholds of forest protection in the Pyrenees, but the effect of ozone on *Pinus uncinata,* the dominant species in subalpine forests in this mountainous range, has not yet been studied. We conducted an experiment of free-air ozone fumigation with saplings of *P. uncinata* fumigated with ambient O_3 (AOT40 May-Oct: 9.2 ppm h), 1.5xO_{3amb} (AOT40 May-Oct: 19.2 ppm h), and 1.8xO_{3amb} (AOT40 May-Oct: 32.5 ppm h) during two growing seasons. We measured chlorophyll content and fluorescence, visible injury, gas exchange, and above- and below-ground biomass. Increased exposures to ozone led to a higher occurrence and intensity of visible injury from O_3 and a 24-29% reduction of root biomass, which may render trees more susceptible to other stresses such as drought. *P. uncinata* is thus a species sensitive to O_3, concentrations of which in the Pyrenees are already likely affecting this species.

5.1. INTRODUCTION

Tropospheric ozone is considered the most perturbing air pollutant for forests (Matyssek et al., 2007). Concentrations of ground-level O_3 have been increasing during the last century, from a mean level of 10 ppb (Anfossi and Sandroni, 1994a) at the end of the 19th century to a present annual average concentration of 35-40 ppb in the mid-latitudes of the Northern Hemisphere (Fowler et al., 2008). Moreover, the IPCC (Intergovernmental Panel on Climate Change) predicts an increase in concentrations of O_3 of 20-25% from 2015 to 2050 based on scenarios of high emissions (IPCC, 2007b). At present, concentrations of O_3 at mid-latitudes of the Northern Hemisphere already exceed the threshold levels for the protection of vegetation, and the risk to vegetation is expected to increase as the projected concentrations increase (Fowler et al., 2008).

Several studies have revealed that O_3 has negative effects on the health of both broadleaved and coniferous trees (Calatayud et al., 2011; Krupa et al., 1998). Damage from ozone has been extensively studied for, among other species, *Fagus sylvatica, Betula pendula, Picea abies, Picea sitchensis, Pinus ponderosa, Pinus strobus* and *Pinus sylvestris* due to their sensitivity to ozone, abundance, distribution, and economic value (Arbaugh et al., 2003; Bartholomay et al., 1997; Manninen et al., 2003b; Novak et al., 2008; Peñuelas et al., 1994; Pretzsch et al., 2010; Schaub et al., 2007; VanderHeyden et al., 2001). However, we need to further study the possible effect of O_3 on other species of trees that, like *Pinus uncinata,* are very important in particular regions where concentrations of O_3 highly exceed the thresholds for the protection of vegetation, as occurs in the Pyrenees.

The Mountain pine (*Pinus uncinata* Ram.) is an autochthonous European species found in the Central and Western Alps, the Central and Eastern Pyrenees, and the Northern Appennines. In the Pyrenees, the lower altitudinal limit of *P. uncinata* is at 1200 m a.s.l. and it represents the dominant subalpine forest species from 1600-1800 (depending on the area) to 2400 m a.s.l. (Burriel et al., 2004). Possible impacts of O_3 on forests of *Pinus uncinata* and on the livelihoods of forest-dependent communities could result in major ecological, economic, and social consequences. In fact, significant increases in crown defoliation in stands of *P. uncinata* in the Pyrenees have already been detected by the Catalan forestry service and have been reported in a previous study (Carnicer et al., 2011). The study of the sensitivity to O_3 of this tree species common in subalpine forests is thus necessary. The valley bottoms in the central region of the Catalan Pyrenees have been experiencing high levels of tropospheric ozone, with AOT40 (Apr-Sept) ranging from 10.1 to 23.2 ppm h and increasing by more than 0.5 ppm h per year over the last two decades (Díaz-de-Quijano et al., 2009). Furthermore, AOT40 exposures are expected to be much greater at higher elevations, since O_3 mixing ratios have been reported to be 58% higher at 2300 m a.s.l than in the valleys (1040 m a.s.l.) (Díaz-de-Quijano et al., 2009).

The aims of this study are: 1) to determine if the Mountain pine is sensitive to O_3, and 2) to clarify if ambient concentrations of O_3 in the Pyrenees have an effect on pigments, gas exchange, the development of visible injury, and growth of this species.

5.2. MATERIALS AND METHODS

Free-air O3 fumigation system

Our study was conducted in 2009 and 2010 in a free-air O_3 fumigation system that was in operation from the spring of 2004 until the autumn of 2010 at Alp Flix (Sur), Canton Grisons, Switzerland (2000 m a.s.l., 9º39'N/46º32'E, (Bassin et al., 2007b)). Alp Flix is a grassland plateau situated approximately 200 m below the natural treeline near Julier Pass in the Central Alps. Climatic conditions are similar to those of the Pyrenees and are characterized by winters with snow cover lasting from December until April. Precipitation peaks in summer (long-term average of 120-140 mm monthly from May to September and 750 mm accumulated from May to October) with an annual total of 1200 mm. Annual mean temperature at Alp Flix is 1.1ºC. During the growing season (May-October), the average temperature for 2004-2010 was 7ºC, and monthly average temperatures were highest in July and August (10-9ºC).

The free-air O_3 fumigation system consisted of nine rings 7 m in diameter arranged in three linear blocks on a small ridge 150 m in length (for details of the system and its performance, see Volk et al. (2003)). Three fumigation treatments (ambient (control), 1.4-1.5 × O_3 ambient concentration (O_3+), and 1.7-1.8 × O_3 ambient concentration (O_3++)) were randomly assigned to the three rings in each block. The target concentrations of ozone were set for 24h/day, while the performance of the fumigation system depended on wind conditions. Increased concentrations of O_3 were maintained from 30 April to 30 October in 2009 and from 17 April to 13 October in 2010.

Micrometeorological measurements

Global radiation (W m^{-2}), wind speed (m s^{-1}), air temperature (ºC), relative humidity (%), and precipitation (mm h^{-1}) were recorded at a reference height of 2 m outside the fumigation rings. For details about the equipment used for these measurements, see Volk et al. (2003).

Plant material

A total of 72 seven-year-old saplings of *P. uncinata* from the Eastern Catalan Pyrenees were arranged into the 9 rings (8 saplings per ring) during the first week of May 2009. The saplings had been grown in the nursery of the Forestal Catalana, in Sant Feliu de Buixalleu (Girona), in 70% black peat and 30% white peat. Each sapling was transplanted into a 12-litre pot and placed in pits flush with the surrounding surface. The final soil composition was 72% black peat, 27% sand, and 1% white peat. Saplings were watered when needed during the first two weeks and later received only water from rain. At the beginning of the second growing season, they were redistributed among the rings within each O_3 treatment to minimize confounding effects of microclimatic differences between rings.

Chlorophyll fluorescence

The maximum photosystem II (PSII) quantum yield of primary photochemistry was measured on three twigs of six saplings per ring (3 x 6 x 3 = 54 twigs per treatment) from 12.00 to 16.00 h (local time) using a PAM-2000 fluorometer (Walz, Effeltrich, Germany). Measurements were taken 3-4 times during each growing season. Needles were kept in the dark for 30 min (dark adaptation) prior to the measurements, and then initial fluorescence (F_0, when all PSII reaction centres were open) was measured. The maximum fluorescence (F_m, when all PSII reaction centres were closed) was next measured applying a saturating light pulse. Maximum quantum yield of primary photochemistry was calculated as $F_v/F_m = (F_m-F_0)/F_m$ (Repo et al., 2004).

Measurements of chlorophyll content

Chlorophyll content was determined non-destructively using a SPAD-502 meter (Minolta Co, LTD, Osaka, Japan). Measurements were conducted on all saplings (measurements for each sapling were a mean of three twigs per plant) 3-4 times each growing season. Relative units of concentration of leaf chlorophyll are given by this instrument, which uses measurements of transmitted radiation at red and near-infrared wavelengths. Close linear correlations between SPAD values and concentrations of extractable chlorophyll have been reported for a wide range of species, including plants exposed to elevated levels of O_3 (Ribas et al., 2005).

Measurements of gas exchange

Rates of steady-state net photosynthesis (A) and stomatal conductances (g_s) were measured with a portable gas-exchange system, LiCor 6400, that was equipped with the conifer chamber EB PMK-5 (LI-COR Biosciences GmbH, Bad Homburg, Germany). Three saplings per ring were measured on each occasion (3-4 times each growing season) from 12.00 to 16.00h (local time), and values were expressed on the basis of a projected needle area. Measurements were always made at a temperature of 21-25ºC and with a photosynthetically active radiation (PAR) of 1600-1900 µmol m^{-2} s^{-1}. Needles were scanned, and the projected needle area was measured using Photoshop CS4 Extended.

Visible injury

All saplings were examined for visible injury - ozone, biotic, and frost injury - at the end of the second growing season. Ozone injury (OI), as typical chlorotic mottling, was assessed in all needles of the current year according to the ICP-Forests Manual on the Assessment of Ozone Injury, part VIII (Schaub et al., 2010). Ozone injury was scored in percentage of total surface affected and assigned to injury classes (score 0 = no injury present; 1 = low: 1-5% of the surface affected; 2 = moderate: 6-50 % of the surface affected; 3 = high: 51-100 % of the surface affected) (Schaub et al., 2010). Biotic injury (BI) was identified by directly detecting the remnants or the parasite itself, once the absence of an injury gradient had been confirmed. BI was assessed using the same scale as for ozone injury but only in current (C) and one-year-old needles (C+1), because older needles were usually too damaged to determine the origin of injury. Frost injury, as the typical weather fleck, was measured in one- and two-year-old needles (C+1, C+2). The frost-injury index (FII) was calculated by multiplying the percentage of affected needles by the percentage of affected area. FII thus ranged between 0 (no frost injury) and 10 000 (100% of affected needles × 100% of affected area). Different types of injury were determined following the procedures described by Vollenweider and Günthardt-Georg (2006) and Günthardt-Georg and Vollenweider (2007).

Measurements of growth

The height of each sapling was measured from a black ink mark approximately 2 cm above the soil to the tip of the terminal bud 3-4 times during each growing season. Two readings of stem diameter were recorded at the black mark using a vernier calliper. Stem log-size ($y = \log10(d^2h)$) was used for the analysis of growth (Lucas and Diggle, 1997).

At the end of the second growing season, individuals were harvested and dried at 60ºC to constant weight. Total above-ground (separated into needles and stem plus branches) and below-ground biomass were measured. Stem-weight, needle-weight, and root-weight ratios were calculated by dividing the dry weights of stem plus branches, needles, and roots, respectively, into the dry weight of the whole plant.

Statistical analyses

The effects of ozone treatments and time of exposure on fluorescence, chlorophyll content, measurements of gas exchange, and stem log-size were tested using linear mixed effects models with O_3, time, and block as fixed factors. Random factors (tree nested in ring) were considered by applying the AIC (Akaike Information Criterion). The method of generalised least squares was used when data did not fulfil the criteria for homogeneity of variances. In this case, data were analysed independently for each year.

The effects of ozone treatment on dry biomass, visible ozone injury, biotic injury, and frost injury at the end of the two years of exposure were tested as explained above but without including time as a factor. The model for biotic and frost injury also included needle year as a fixed factor. The method of generalised least squares was used when data did not fulfil the criteria for homogeneity of variances. Data were transformed when needed to satisfy the assumption of normality (log(OI + 1), sqrt(FII)).

All analyses were performed with R, version 2.12.2 (2011, The R Foundation for Statistical Computing).

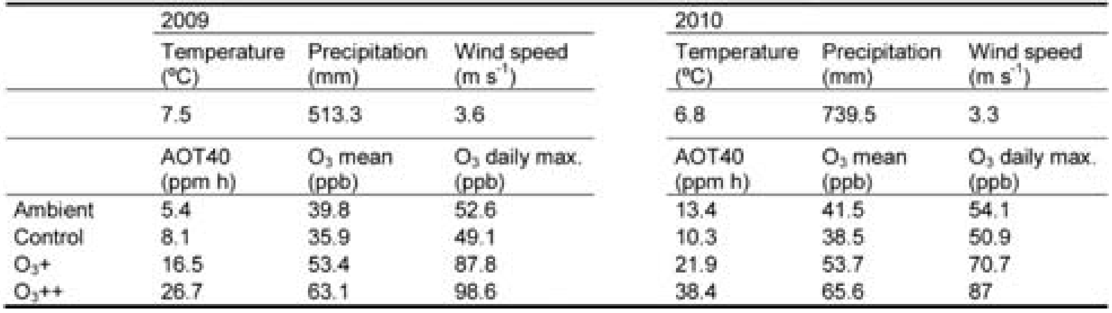
5.3. RESULTS

Climatic conditions and exposures to ozone

Weather conditions were slightly different between the two years (Table 1); the growing season in 2009 was warmer and drier than 2010 and the averages for the 2004-2010 period in Alp Flix. Mean accumulated precipitation (626.4 mm) and mean temperature (7.15ºC) at Alp Flix during our experiment were similar to those at similar altitudes in the eastern part of the Pyrenees where the experimental plants originated (Malniu meteorological station at 2230 m a.s.l.: 609.1 mm and 9.0ºC from May to October (data from Digital Climatic Atlas of Catalonia, http://www.opengis.uab.cat/acdc/en_index.htm)).

The average accumulated exposures for the two years over a threshold of 40 ppb (AOT40, from April to October) were 9.2 ppm h in the control plots and 19.2 ppm h and 32.5 ppm h in the O_3+ and O_3++ treatments, respectively (Table 1). Variations in AOT40 in the O_3+ and O_3++ treatments were associated with variations in meteorological conditions. The daily average concentration of ambient O_3 during the growing season was 40.7 ppb, and the average of the daily maximal concentration of O_3 was 53.3 ppb (for 2009 and 2010, Table 1).

Table 1. Mean temperature, precipitation, wind speed, AOT40, mean O_3 concentrations and maximum daily O_3 concentration measured in the O_3 fumigation system at Alp Flix during the growing seasons of 2009 (April 30th – October 30th) and 2010 (April 17th –October 13th). Ambient measurements refer to the measurements outside of the rings.



	2009			2010		
	Temperature (ºC)	Precipitation (mm)	Wind speed (m s^{-1})	Temperature (ºC)	Precipitation (mm)	Wind speed (m s^{-1})
	7.5	513.3	3.6	6.8	739.5	3.3
	AOT40 (ppm h)	O_3 mean (ppb)	O_3 daily max. (ppb)	AOT40 (ppm h)	O_3 mean (ppb)	O_3 daily max. (ppb)
Ambient	5.4	39.8	52.6	13.4	41.5	54.1
Control	8.1	35.9	49.1	10.3	38.5	50.9
O_3+	16.5	53.4	87.8	21.9	53.7	70.7
O_3++	26.7	63.1	98.6	38.4	65.6	87

Measurements of chlorophyll fluorescence and content

No statistically significant differences in maximum quantum yield of primary photochemistry or chlorophyll content were found among the O_3 treatments. In 2010 only, F_v/F_m was significantly higher in July than in May (Table 2), and chlorophyll content was significantly higher at the end of the season. The average chlorophyll content was also higher in 2010 than in 2009 (Table 2).

Table 2. Maximum PSII quantum yield of primary photochemistry (Fv/Fm) ± SE (n=18) and chlorophyll content in SPAD units ± SE (n=23-24) of the current-year needles, during the 2009 and 2010 growing seasons for the three ozone treatments. Letters next to numbers indicate statistical differences at $p<0.05$. Statistical analyses were conducted independently for each year. n.a. = no data available.

	2009			2010			
Fv/Fm	25th July	19th August	11th October	29th May	5th July	25th August	28th October
Control	$0.757^{n.s.}$ (±0.008)	$0.789^{n.s.}$ (±0.013)	$0.772^{n.s.}$ (±0.019)	0.537^{a} (±0.039)	0.713^{b} (±0.024)	n.a.	n.a.
O_3+	$0.730^{n.s.}$ (±0.014)	$0.778^{n.s.}$ (±0.013)	$0.769^{n.s.}$ (±0.018)	0.602^{a} (±0.024)	0.689^{b} (±0.032)	n.a.	n.a.
O_3++	$0.749^{n.s.}$ (±0.02)	$0.775^{n.s.}$ (±0.01)	$0.813^{n.s.}$ (±0.013)	0.519^{a} (±0.042)	0.757^{b} (±0.020)	n.a.	n.a.
Chlorophyll content (SPAD units)							
Control	25.58^{A} (±3.29)	26.57^{A} (±3.29)	41.10^{B} (±5.28)	19.71^{a} (±3.10)	36.37^{b} (±4.40)	70.10^{c} (±2.91)	61.87^{c} (±3.76)
O_3+	23.60^{A} (±3.36)	20.35^{A} (±1.90)	35.35^{B} (±3.95)	21.70^{a} (±3.06)	41.27^{b} (±4.51)	70.80^{c} (±3.04)	66.03^{c} (±3.24)
O_3++	26.22^{A} (±3.48)	20.65^{A} (±2.49)	36.66^{B} (±3.72)	18.80^{a} (±3.26)	37.74^{b} (±3.74)	72.70^{c} (±3.36)	62.25^{c} (±5.09)

Measurements of gas exchange

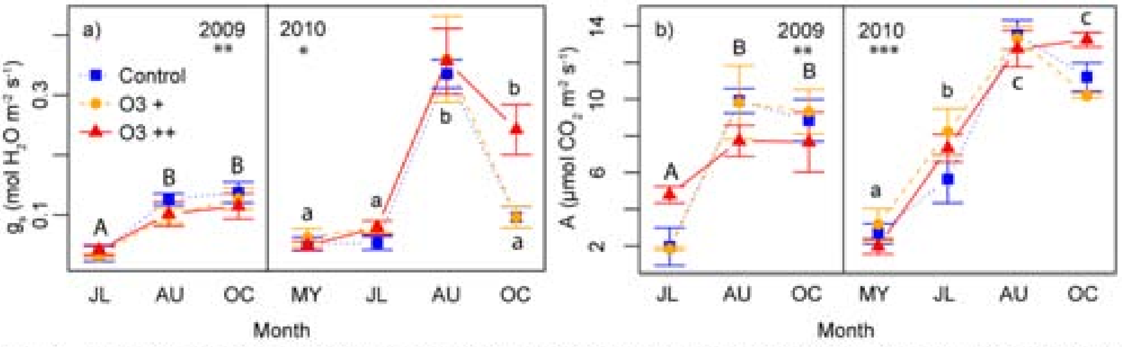


Fig. 1. a) Mean stomatal conductance (mol H_2O m-2 s-1) ± SE and b) net photosynthetic rate (µ mol CO_2 m-2 s-1) ± SE over time in the control, O_3+ and O_3++ treatments on July 26th (JL), August 24th (AU) and October 11th (OC) 2009 and May 23rd (MY), July 6th (JL), August 25th (AU) and October 27th (OC) 2010. Letters indicate significant differences at * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Statistical analyses were conducted independently for each year. n = 7-9.

Neither stomatal conductances (g_s) nor photosynthetic activity (A) showed statistically significant differences among O_3 treatments, except at the end of the experiment (October 2010) when g_s and A in the O_3++ treatment were higher than in the other two treatments. Both g_s and A were higher in August and October than in May or July, for both years (Fig. 1).

Visible injury

Typical visible O_3 injury was expressed as tiny and diffuse greenish mottles centred on stomatal lines on the sides of needles exposed to light (Fig. 2). Both the number of trees showing visible O_3 injury and the intensity of the injury increased with increasing exposure to O_3 (Fig. 3). The percentage of affected trees was 8.3% in the control and 54.2% and 78.2% in the O_3+ and O_3++ treatments, respectively. Similarly, the intensity of injury was very low (around score 0: no injury present), low (around score 1: 1-5% of the surface affected), and moderate (around score 2: 6-50 % of the surface affected) in the control, O_3+, and O_3++ treatments, respectively (Fig. 3).

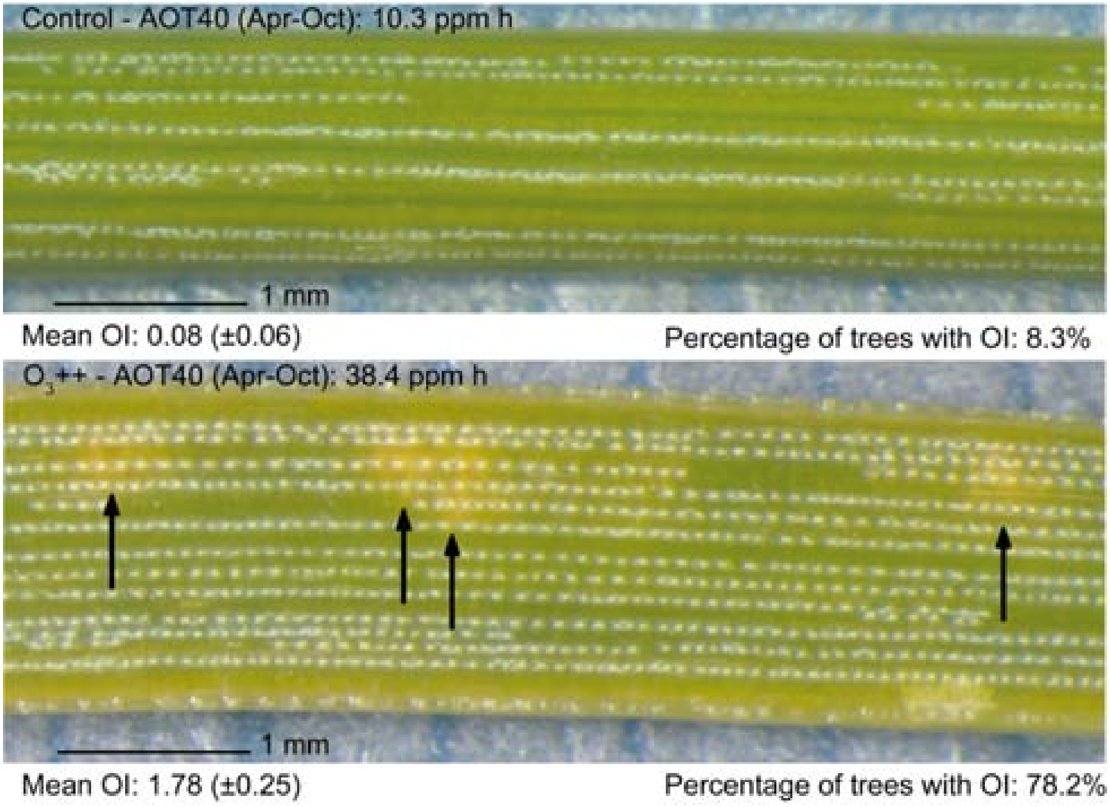


Fig. 2. Current-year needles at the end of the 2nd growing season from two different saplings grown under control and O_3++ treatments. Mean ozone injury (OI) ± SE and percentage of trees showing ozone visible injury for each treatment. Arrows show ozone visible injury after ozone fumigation was applied for one growing season.

The occurrence and intensity of biotic injury did not differ between O_3 treatments. One-year-old needles, however, showed a higher presence and intensity of biotic injury than needles of the current year (100%, 100%, and 91.3% of the trees from control, O_3+, and O_3++ treatments, respectively, showed biotic injury on the C+1 needles) (Fig. 3). The frost-injury index showed no statistically significant differences among O_3 treatments or age of needles (Fig. 3).

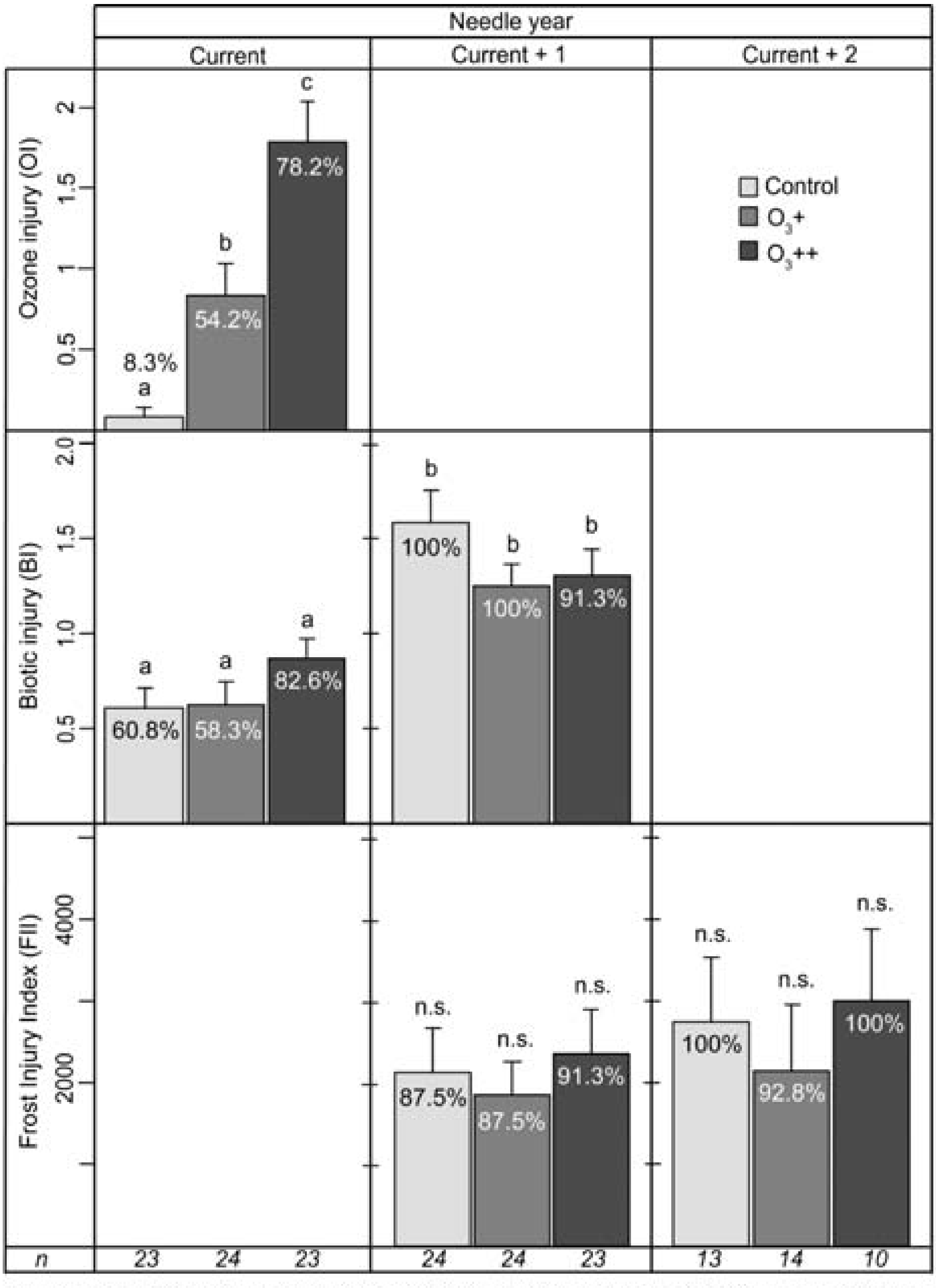


Fig. 3. Mean ozone injury (OI), mean biotic injury (BI) and mean frost injury index (FII) ± SE at the end of the 2nd growing season in the control, O_3+ and O_3++ treatments. Letters indicate significant differences at $p<0.05$. Percentages within each column refer to percentage of trees showing BI, OI and FII for each O_3 treatment and needle age. n is the total number of assessed trees retaining needles of the specified age for each treatment.

Measurements of growth

Saplings of *P. uncinata* showed an increase of the stem log-size over time, with no significant differences between O_3 treatments (Fig. 4). Nonetheless, both treatments of elevated levels of O_3 had reduced dry biomass in comparison to the control treatment, although only reductions in the root biomass were statistically significant (Table 3, Fig. 5). The ratio S/R (shoot biomass/root biomass) in control saplings was 2.47. This S/R ratio was significantly higher, ca. 22%, in the O_3 treatments. No significant differences in the stem-weight or needle-weight ratios were found among the O_3 treatments (Table 3).

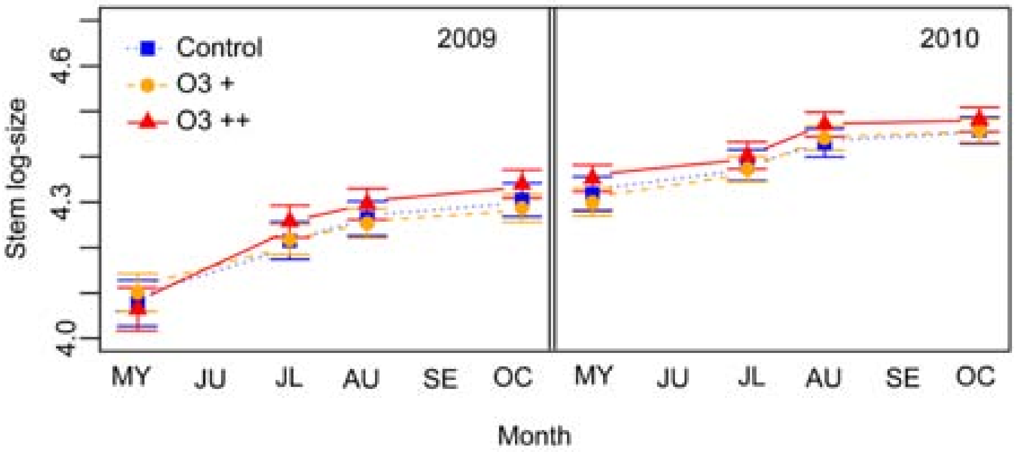


Fig. 4. Growth of saplings expressed as mean stem log-size (Stem log-size = d^2h, d = diameter in mm, h = height in mm) ± SE for the three O_3 treatments during the two growing seasons of 2009 and 2010 at Alp Flix. Measurements were conducted on May 7th (MY), July 16th (JL), August 19th (AU), October 9th (OC) 2009 and May 22nd (MY), July 5th (JL), August 24th (AU) and October 26th (OC) 2010. n = 23-24.

5.4. DISCUSSION

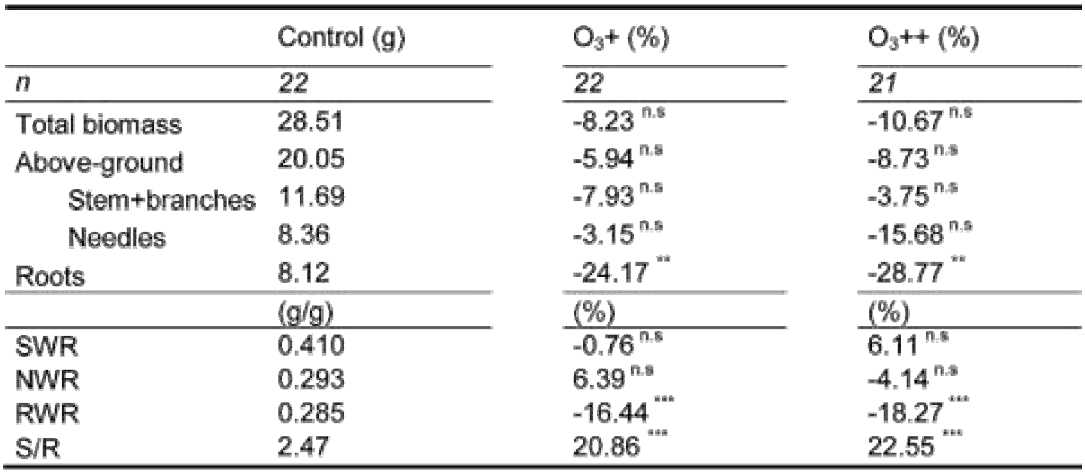
Experimental and field climatic conditions and similar ozone exposures

Weather conditions and ozone exposures at Alp Flix during 2009 and 2010 were comparable to the conditions usually found in the Pyrenees. Average climatic conditions at Alp Flix are slightly more humid (very similar annual precipitation but higher precipitation and lower temperatures in summer) than in the Pyrenees. A higher availability of water, however, might be compensated by a lower capacity of the saplings to acquire water due to the limitation of rooting depth to the height of the soil in the 12-litre pots. On the other hand, the lower average temperatures recorded at Alp

Flix compared to the Pyrenees (2ºC lower) might result in a shorter growing period, which could lead to a lower uptake of ozone than in the Pyrenees under similar conditions. The uptake of ozone at Alp Flix may thus be underestimated compared to that in the Pyrenees. A potential overestimate for the uptake of ozone due to favourable wetter conditions at Alp Flix or a potential underestimate due to a shorter growing season, compared to the Pyrenees, though, are assumptions that need further investigation, based on the findings from this study.

The AOT40 (April-September) measured at 1224 m a.s.l. in the Catalan Pyrenees present values in the same range as the O_3+ treatment (1994-2009 average is 17.4 ppm h, approaching 23.3 ppm h in 2003 (unpublished data)). Nevertheless, higher values of AOT40 are expected to occur at higher altitudes in the Pyrenees. In the Central Catalan Pyrenees, annual mean concentrations of ozone at 2300 m a.s.l. are 58% higher than at 1040 m a.s.l. (Díaz-de-Quijano et al., 2009), which suggests that values of AOT40 can be expected to be higher at higher altitudes. In fact, the average annual concentration of O_3 from 2004 to 2008 at 2300 m a.s.l. in the Central Catalan Pyrenees was 56 ppb, which is very similar to the mean concentrations of O_3 in the O_3+ treatment (53 ppb). We can thus conclude that the values of AOT40 in the O_3++ treatment would be comparable to those found at the upper elevational limit of *P. uncinata* (2300 m a.s.l) in the Eastern Pyrenees, whereas the values of AOT40 in the O_3+ treatment would be lower than those expected at the lower distributional limit of this species (1500 -1700 m a.s.l.).

Table 3. Total above ground, stem plus branches, needles and root dry biomass of *P. uncinata* saplings expressed in grams for the control and as percentage relative to the control for the O_3+ and O_3++ treatments. Stem plus branches dry weight ratio (SWR), needles dry weight ratio (NWR) and roots dry weight ratio (RWR) relative to the whole dry plant weight and shoot/root ratio (S/R) are presented for the control and percentage increase or decrease are presented for O_3+ and O_3++ treatments. Linear mixed effects model results: n.s. = not significant; significant at ** p<0.01, *** p<0.001.



	Control (g)	O_3+ (%)	O_3++ (%)
n	*22*	*22*	*21*
Total biomass	28.51	-8.23 $^{n.s}$	-10.67 $^{n.s}$
Above-ground	20.05	-5.94 $^{n.s}$	-8.73 $^{n.s}$
Stem+branches	11.69	-7.93 $^{n.s}$	-3.75 $^{n.s}$
Needles	8.36	-3.15 $^{n.s}$	-15.68 $^{n.s}$
Roots	8.12	-24.17 **	-28.77 **
	(g/g)	(%)	(%)
SWR	0.410	-0.76 $^{n.s}$	6.11 $^{n.s}$
NWR	0.293	6.39 $^{n.s}$	-4.14 $^{n.s}$
RWR	0.285	-16.44 ***	-18.27 ***
S/R	2.47	20.86 ***	22.55 ***

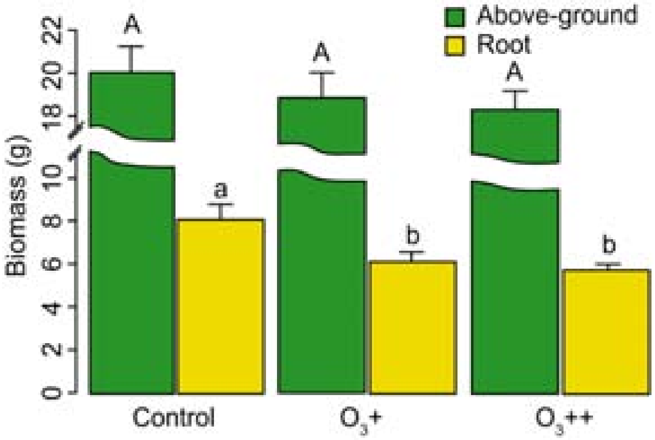


Fig. 5. Dry weight of above ground biomass and root biomass for the three treatments after two years of fumigation. Lower case letters indicate significant differences at $p<0.001$. n = 21-22.

Absence of significant changes in chlorophyll fluorescence and content

The low values of F_v/F_m observed in May 2010 can be explained by low temperatures and the presence of snow until early spring. Photosystem II (PSII) efficiency has been found to decrease in winter (Oliveira and Peñuelas, 2004; Verhoeven et al., 2009), decreasing with low air and soil temperatures (Ogaya et al., 2011; Repo et al., 2004). In this case, increased concentrations of O_3 did not seem to influence the maximum quantum yield of primary photochemistry, but which does not imply the absence of O_3 effects. Likewise, in a previous study, O_3 symptomatic and asymptomatic trees did not differ in the kinetics of chlorophyll fluorescence in needles of the current year (Patterson and Rundel, 1995).

Chlorophyll content in needles was expected to decrease in the O_3 treatments as a result of degradation typical of accelerated cell senescence (Elvira et al., 1998; Zheng et al., 2002). Chlorophyll content, however, did not significantly vary among O_3 treatments. Identical results were found in Scots pine after a three-year experiment with O_3 fumigation and were attributed to the lower g_s typical of coniferous species (Broadmeadow and Jackson, 2000). The high density of needle tissue in mountain pine could also explain why we found no differences in chlorophyll content, since leaf thickness and tissue density should increase tolerance to O_3 (Bussotti et al., 2005). The increase in chlorophyll content over the season could be due to foliar development to maturity, while higher chlorophyll content in the second year could be the result of acclimation to the conditions of the pots.

Higher A and g_s only at the end of the second growing period

The process of acclimation to the conditions of the pots and/or to better environmental conditions (e.g. higher availability of water) may have contributed to the higher g_s and A in 2010. Even though the monthly average accumulated precipitation in summer at Alp Flix is nearly three times the average in the Eastern Pyrenees, the uptake of water by *P.uncinata* saplings in pots was probably hindered by a restricted development of roots. The higher precipitation in 2010 could have thus favoured the growth of saplings during the second growing season.

A and g_s did not significantly differ among O_3 treatments until the end of the second growing season, where A and g_s were higher in the O_3++ treatment. Even though a decline in A and g_s is generally reported in trees fumigated with ozone (see review by Wittig et al., (2007)), Eamus et al. (1990) observed higher A in trees that had been exposed to O_3 (daylight average of 76 µg m^{-3}) the previous year than those that had been kept in clean air (charcoal-filtered air). Higher A in trees fumigated with O_3 has also been observed during fumigation or immediately afterwards (Taylor et al., 1986). In fact, increased A occurs in response to oxidative stress, since it enables plants to respond to their need of metabolites for the processes of detoxification and repair (Liska et al., 2004; Retuerto et al., 2004).

Significant visible ozone injury

As expected, the occurrence and intensity of visible O_3 injury (OI) correlated positively with exposures to O_3. Visible ozone injury on needles (OI) may have started to develop at the end of the growing season, even though negative effects may have started to develop on a microscopic level earlier. Bussotti et al. (2007) detected the onset of foliar injury at the end of the summer, most likely due to the reduction in the capacity of leaves to detoxify after being exposed to elevated levels of O_3 for the entire summer. In fact, the late onset of symptoms of foliar ozone injury in other tree species has been related to a high density of leaf tissue (Bussotti et al., 2007; Gerosa et al., 2003), as would be the case for the tissues of *P. uncinata* needles (unpublished data). High densities of leaf tissue imply lower intercellular space and increased resistance to diffusion of gases into the tissues (Gerosa et al., 2003), whereas low densities of leaf tissue favour the diffusion of gases into the mesophyll and entail a reduced capacity of detoxification linked to enzymes of the cell wall (Wieser et al., 2002).

Biotic injury tended to increase with exposure to O_3. The increased demand for carbon for the processes of detoxification and repair induced by ozone might reduce the availability of resources to fight pathogenic infections (Andersen, 2003). The higher intensity and occurrence of biotic injury in one-year-old needles in comparison to needles of the current year seem to result from longer periods of exposure to potential biotic damage.

Frost injury showed no tendency dependent on O_3 treatment or age of needles, even though O_3 increases the risk of frost damage by both reducing cold hardiness and increasing predisposition to dehardening when favourable temperatures occur during winter (Eamus and Murray, 1991). Longer experiments would have been needed to better determine any possible relationships between O_3 and frost injury.

Reduced growth of roots

No significant reductions in the growth of stems were observed in saplings in the O_3+ and O_3++ treatments relative to the control during this experiment. Similarly, differences in stem log-size among O_3 treatments were also not found in Aleppo pine after 36 months of increased exposure to O_3 (Inclán et al., 2011). Other studies, though, have reported reductions in the growth of stems in response to ozone, due to season-long exposures (Matyssek et al., 2010). Reductions in the rate of growth were found in Aleppo pine after four years of repeated exposure to elevated levels of O_3 (Barnes et al., 2000).

The significant reductions in root biomass and root/shoot biomass observed under elevated exposures to O_3 have also been reported for other species (Andersen, 2003). Ozone is known to produce a decline in the availability of carbon in leaves due to a reduction in the assimilation of carbon caused by the impairment of stomatal function and a decrease in Rubisco activity and leaf longevity, and to an increase in demand for carbon in the shoots for the processes of repair and the synthesis of antioxidants. In our study, the assimilation of carbon did not noticeably decrease, which suggests a possible decline in the availability of carbon due to an increased demand for carbon for detoxification and repair. In any case, a reduction in the availability of carbon in leaves can alter a plant's carbon balance, decreasing the allocation of carbon to roots and shoot and leading to an altered root/shoot ratio (Andersen, 2003; Matyssek et al., 2008). Since the growth of roots usually depends on the products of photosynthesis (Van Den Driessche, 1991), a decrease in the allocation of carbon to roots can have very rapid and significant consequences on their growth. Seedlings exposed to O_3 may thus be especially susceptible to other stressors, such as drought or nutrient deficiency, since a reduction in the growth of roots will hinder the uptake of water and nutrients (Vollsnes et al., 2010). Moreover, a reduction in the growth of roots in tree species might reduce resistance to windthrow (Broadmeadow and Jackson, 2000).

In our study, effects on roots occurred before any changes in above-ground biomass could be detected. During most of the experiment, when no significant effects among ozone treatments were found for the measured variables, a diminution in the allocation of carbon to roots may already be occurring as a result of an increased demand for carbon in needles for the processes of repair. At the end of the experiment, the ozone-induced increase of g_s and A without reductions in chlorophyll content and PSII efficiency may indicate an investment of the assimilated carbon towards the pathways for the synthesis of antioxidants. This production of antioxidants would prevent ozone damage and would entail a decrease in the allocation of carbon to the roots. In fact, the synthesis of antioxidants and other secondary compounds for avoiding ozone damage implies a much higher metabolic cost than that incurred by the processes of repair (Tingey, 1991), which could explain the increase of A at the end of the second growing season. In several previous studies, ozone-induced changes in the root system, such as a reduction in the growth of roots and metabolic changes in the root tips, have been observed before any noticeable changes in the above-ground biomass, most likely because shoots have immediate access to carbon for the processes of repair (Andersen, 2003; Miller et al., 1997). Thus, although above-ground responses to O_3 might not be detectable, below-ground responses may have already begun, may be larger in magnitude, and may have larger long-term effects on productivity and the carbon, nutrient, and water cycles of forest ecosystems (Andersen, 2003; Laurence and Andersen, 2003).

5.5. CONCLUSIONS

To the best of our knowledge, our results quantify for the first time ozone-induced injury in *P. uncinata*. Moreover, the high ambient concentrations of O_3 in the Pyrenees can be expected to considerably reduce the growth of roots even in the absence of detectable above-ground effects on growth. Reductions in the growth of roots result in reduced productivity and imply long-term effects on the nutrient, carbon, and water cycles of the ecosystem. Despite the caution with which results on potted saplings should be translated into natural forest ecosystems, these mechanistic findings contribute to a better understanding of the processes of decline observed in the Mountain pine forests of the Pyrenees. Our results nevertheless demonstrate that ozone does have a negative effect on *P. uncinata* and provide a needed basis for the further investigation of forest decline in the Catalan Pyrenees. Intensive, long-term studies of sap-flow are urgently needed and recommended to answer open questions of possible interactions between elevated exposure to ozone and frost hardiness, the actual uptake of ozone into plants, and the possible effects of climate change (such as drought) on the water balance of mature trees.

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CONCLUSIONS

The main conclusions of this thesis are the following:

1) The O_3 concentrations in the overall Catalan Pyrenees have greatly exceeded the critical levels for the protection of forests and semi-natural vegetation set by the UNECE's CLRTAP and the long-term objective and target value for the protection of vegetation set by the European Directive 2008/50/EC from 1994 to 2009, and they show a general increasing trend over time with a slight decrease during the last 3 years. Hence, plant life in this mountainous range is at risk of damage by O_3, specifically at high-altitude sites, where exceedances of these thresholds are greater.

2) Ozone concentrations in the Central Catalan Pyrenees were found to significantly increase along an altitudinal gradient from 35 ppb_v at 1040 m a.s.l. to 56 ppb_v at 2300 m a.s.l. (on average for 2004-2007 period) and annual means at the highest site (2300 m a.s.l.) ranged between 38 and 67 ppbv (38-74 ppbv during the warm period). On average, O_3 concentrations were 35-38% greater during the warm period and had a characteristic pattern with minimum values in the early morning, a rise during the morning and a decline overnight, that was less marked the higher the altitude. The analysis of meteorological variables and NO_x values suggested that the O_3 mainly originated from urban areas and was transported to high mountain sites, remaining aloft in absence of NO.

3) The severity of O_3 visible injury in the two altitudinal and O_3 gradient in the Central Catalan Pyrenees were found to increase with increasing annual mean $[O_3]$ when summer P/PET was above a threshold of 0.96, whereas in drier conditions, higher $[O_3]$ did not yield higher O_3 visible injury. Crown defoliation and tree mortality mean values of 20.4-66.4% and 0.6-29.6%, respectively, depending on the sites, pointed to an increased poor tree vitality in comparison with previous works and were positively correlated with the accumulated O_3 exposure during the last 5 years and with variables referring to soil water availability, which pointed to be result of a possible greater O_3 uptake. Ozone might be part of the cause of the observed crown defoliation and tree mortality although further research is definitely needed to determine the extent of contribution of the multiple other acting stress factors.

4) At the macroscopic level, the O_3-induced visible injury found in *Pinus uncinata* stands from the Central Catalan Pyrenees had the form of tiny and diffuse green-yellowish mottles located around the stomata lines on the light exposed side of the needles. At the microscopic level, the injury was characterized by chloroplasts reduction in size with stroma electron dense -being more pronounced in the outer mesophyll cell layers-, increased gradient of oxidation of cell content from the inner to the outer mesophyll, cell wall thickenings and wart-like protrusions, accumulation of antioxidants, and accelerated cell senescence processes. The observation of the same typology of mottles in a free-air O_3 fumigation experiment with *Pinus uncinata* saplings and the fact that most of the cytochemical markers observed below mottles in our symptomatic samples had already been observed to be induced by O_3 under controlled conditions in other tree species, confirm that the observed mottles in field mature individuals of *Pinus uncinata* are most probably caused by the effect of O_3.

5) Increased exposures to O_3 on saplings of *Pinus uncinata* after a two-year experiment with O_3 fumigation led to a higher occurrence and intensity of visible injury from O_3 and a 24-29% reduction of root biomass, which may render trees more susceptible to other stresses such as drought. The high ambient concentrations of O_3 in the Pyrenees can be expected to considerably reduce the growth of roots even in the absence of detectable above-ground effects on growth. Reductions in the growth of roots result in reduced productivity and imply long-term effects on the nutrient, carbon, and water cycles of the ecosystem. Despite the caution with which results on potted saplings should be translated into natural forest ecosystems, these mechanistic findings contribute to a better understanding of the processes of decline observed in the *Pinus uncinata* forests of the Pyrenees. Our results nevertheless demonstrate that O_3 does have a negative effect on *P. uncinata* and provide a needed basis for the further investigation of forest decline in the Catalan Pyrenees.

REFERENCES

Aamlid, D., Torseth, K., Venn, K., Stuanes, A.O., Solberg, S., Hylen, G., Christophersen, N., Framstad, E., 2000. Changes of forest health in Norwegian boreal forests during 15 years. Forest Ecology and Management 127, 103-118.

Ainsworth, E.A., 2008. Rice production in a changing climate: a meta-analysis of responses to elevated carbon dioxide and elevated ozone concentration. Global Change Biology 14, 1642-1650.

Alonso, R., Bytnerowicz, A., 2003. Monitoring and modelling of ozone status and effects in the Sierra Nevada: a comparison with studies in North America and Europe, in: Bytnerowicz, A., Arbaugh, M., Alonso, R. (Eds.), Ozone air pollution in the Sierra Nevada: distribution and effects on forests. Elsevier, pp. 371-389.

Alscher, R.G., Amthor, J.S., 1988. The physiology of freeradical scavenging: Maintenance and repair processes, in: Schulte-Hostede, S., Darral, N.M., Blank, L.W., Wellburn, A.R. (Eds.), Air pollution and plant metabolism Elsevier Applied Science, London, U.K pp. 94-115.

Amiot, M.J., Tacchini, M., Aubert, S., Nicolas, J., 1992. Phenolic composition and browning susceptibility of various apple cultivars at maturity. Journal of Food Sciences 57, 958-962.

Andersen, C.P., 2003. Source-sink balance and carbon allocation below ground in plants exposed to ozone. New Phytologist 157, 213-228.

Aneja, V.P., Businger, S., Li, Z., Claiborn, C.S., Murthy, A., 1991. Ozone Climatology at High Elevations in the Southern Appalachians. Journal of Geophysical Research-Atmospheres 96, 1007-1021.

Aneja, V.P., Claiborn, C.S., Li, Z., Murthy, A., 1994a. Trends, Seasonal-Variations, and Analysis of High-Elevation Surface Nitric-Acid, Ozone, and Hydrogen-Peroxide. Atmospheric Environment 28, 1781-1790.

Aneja, V.P., Li, Z., Das, M., 1994b. Ozone Case-Studies at High-Elevation in the Eastern United-States. Chemosphere 29, 1711-1733.

Anfossi, D., Sandroni, S., 1994a. Surface Ozone at Mid Latitudes in the Past Century. Nuovo Cimento Della Societa Italiana Di Fisica C-Geophysics and Space Physics 17, 199-208.

Anfossi, D., Sandroni, S., 1994b. Surface ozone at mid latitudes in the past century. Il Nuovo Cimento C 17, 199-208.

Anttonen, S., Herranen, J., Peura, P., Karenlampi, L., 1995. Fatty-Acids and Ultrastructure of Ozone-Exposed Aleppo Pine (Pinus-Halepensis Mill) Needles. Environmental Pollution 87, 235-242.

Anttonen, S., Karenlampi, L., 1996. Slightly elevated ozone exposure causes cell structural changes in needles and roots of Scots pine. Trees-Structure and Function 10, 207-217.

Anttonen, S., Kittila, M., Karenlampi, L., 1998. Impacts of ozone on Aleppo pine needles: Visible symptoms, starch concentrations and stomatal responses. Chemosphere 36, 663-668.

Anttonen, S., Sutinen, M.-L., Heagle, A.S., 1996. Ultrastructure and some plasma membrane characteristics of ozone-exposed loblolly pine needles. Physiologia Plantarum 98, 309-319.

Arbaugh, M.J., Bytnerowicz, A., Grulke, N., Fenn, M.E., Poth, M., Temple, P., Miller, P., 2003. Photochemical smog effects in mixed conifer forests along a natural gradient of ozone and nitrogen deposition in the San Bernardino Mountains. Environment International 29, 401-406.

Arbaugh, M.J., Miller, P.R., Carroll, J.J., Takemoto, B., Procter, T., 1998. Relationships of ozone exposure to pine injury in the Sierra Nevada and San Bernardino Mountains of California, USA. Environmental Pollution 101, 291-301.

Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell and Environment 28, 949-964.

Àvila, A., Molowny-Horas, R., Gimeno, B.S., Penuelas, J., 2010. Analysis of Decadal Time Series in Wet N Concentrations at Five Rural Sites in NE Spain. Water Air and Soil Pollution 207, 123-138.

Barbo, D.N., Chappelka, A.H., Somers, G.L., Miller-Goodman, M.S., Stolte, K., 1998. Diversity of an early successional plant community as influenced by ozone. New Phytologist 138, 653-662.

Barnes, J., Gimeno, B., Davison, A., Bussotti, F., Velissariou, D., Gerant, D., 1999. Air pollution impacts on Mediterranean pine forests, in: Ne‘eman, G., Traband, L. (Eds.), Ecology, biogeography and management of *Pinus halepensis* and *P. brutia* forest ecosystems in the Mediterranean Basin. Backhuys Publishers, Leiden, pp. 1-14.

Bartholomay, G.A., Eckert, R.T., Smith, K.T., 1997. Reductions in tree-ring widths of white pine following ozone exposure at Acadia National Park, Maine, USA. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 27, 361-368.

Bassin, S., Volk, M., Fuhrer, J., 2007a. Factors affecting the ozone sensitivity of temperate European grasslands: An overview. Environmental Pollution 146, 678-691.

Bassin, S., Volk, M., Suter, M., Buchmann, N., Fuhrer, J., 2007b. Nitrogen deposition but not ozone affects productivity and community composition of subalpine grassland after 3 yr of treatment. New Phytologist 175, 523-534.

Bergmann, E., Bender, J., Weigel, H.J., 1995. Growth responses and foliar sensitivities of native herbaceous species to ozone exposures. Water Air and Soil Pollution 85, 1437-1442.

Beven, K., Kirkby, M.J., 1979. A physically based variable contributing area model of basin hydrology. Hydrological Science Bulletin 24, 43-69.

Black, V.J., Black, C.R., Roberts, J.A., Stewart, C.A., 2000. Impact of ozone on the reproductive development of plants. New Phytologist 147, 421-447.

Bortier, K., Ceulemans, R., de Temmerman, L., 2000. Effects of tropospheric ozone on woody plants, in: Agrawal, S.B., Agrawal, M. (Eds.), Environmental Pollution and Plant Responses. Lewis Publishers, New York.

Bosac, C., Roberts, J.A., Black, V.J., Black, C.R., 1994. Impact of O_3 and SO_2 on Reproductive Development in Oilseed Rape (*Brassica napus* L) .2. Reproductive Site Losses. New Phytologist 126, 71-79.

Bréhélin, C., Kessler, F., van Wijk, K.J., 2007. Plastoglobuies: versatile lipoprotein particles in plastids. Trends in Plant Science 12, 260-266.

Broadmeadow, M.S.J., Jackson, S.B., 2000. Growth responses of *Quercus petraea, Fraxinus excelsior* and *Pinus sylvestris* to elevated carbon dioxide, ozone and water supply. New Phytologist 146, 437-451.

Brönnimann, S., Buchmann, B., Wanner, H., 2002. Trends in near-surface ozone concentrations in Switzerland: the 1990s. Atmospheric Environment 36, 2841-2852.

Burriel, J.A., Gràcia, C., Ibàñez, J.J., 2004. Inventari Ecològic i Forestal de Catalunya. CREAF, Barcelona.

Bussotti, F., Desotgiu, R., Cascio, C., Strasser, R.J., Gerosa, G., Marzuoli, R., 2007. Photosynthesis responses to ozone in young trees of three species with different sensitivities, in a 2-year open-top chamber experiment (Curno, Italy). Physiologia Plantarum 130, 122-135.

Bussotti, F., Pancrazi, M., Matteucci, G., Gerosa, G., 2005. Leaf morphology and chemistry in *Fagus sylvatica* (beech) trees as affected by site factors and ozone: results from CONECOFOR permanent monitoring plots in Italy. Tree Physiology 25, 211-219.

Bytnerowicz, A., Arbaugh, M.J., Schilling, S., Fraczek, W., Alexander, D., Dawson, P., 2007a. Air pollution distribution patterns in the San Bernardino Mountains of southern California: a 40-year perspective. TheScientificWorldJOURNAL 7, 98-109.

Bytnerowicz, A., Godzik, B., Grodzinska, K., Fraczek, W., Musselman, R., Manning, W., Badea, O., Popescu, F., Fleischer, P., 2004. Ambient ozone in forests of the Central and Eastern European mountains. Environmental Pollution 130, 5-16.

Bytnerowicz, A., Manning, W.J., Grosjean, D., Chmielewski, W., Dmuchowski, W., Grodzinska, K., Godzik, B., 1993. Detecting ozone and demonstrating its phytotoxicity in forested areas of Poland: A pilot study. Environmental Pollution 80, 301-305.

Bytnerowicz, A., Omasa, K., Paoletti, E., 2007b. Integrated effects of air pollution and climate change on forests: A northern hemisphere perspective. Environmental Pollution 147, 438-445.

Caird, M.A., Richards, J.H., Donovan, L.A., 2007. Nighttime stomatal conductance and transpiration in C-3 and C-4 plants. Plant Physiology 143, 4-10.

Calatayud, V., Cerveró, J., Calvo, E., García-Breijo, F.-J., Reig-Armiñana, J., Sanz, M.J., 2011. Responses of evergreen and deciduous *Quercus* species to enhanced ozone levels. Environmental Pollution 159, 55-63.

Calatayud, V., Sanz, M.J., Calvo, E., Cervero, J., Ansel, W., Klumpp, A., 2007. Ozone biomonitoring with Bel-W3 tobacco plants in the city of Valencia (Spain). Water Air and Soil Pollution 183, 283-291.

Carnicer, J., Coll, M., Ninyerola, M., Pons, X., Sanchez, G., Penuelas, J., 2011. Widespread crown condition decline, food web disruption, and amplified tree mortality with increased climate change-type drought. Proceedings of the National Academy of Sciences of the United States of America 108, 1474-1478.

Carreras, J., Carrillo, E., Masalles, R.M., Ninot, J.M., Soriano, I., Vigo, J., 1996. Delimitation of the supra-forest zone in the Catalan Pyrenees. Bulletin de la Societe Linneenne de Provence 47, 27-36.

Chevalier, A., 2007. Influence of altitude on ozone levels and variability in the lower troposphere: a ground-based study for western Europe over the period 2001-2004 in: Gheusi F (Gheusi, F., Delmas R (Delmas, R.), Ordonez C (Ordonez, C.), Sarrat C (Sarrat, C.), Zbinden R (Zbinden, R.), Thouret V (Thouret, V.), Athier G (Athier, G.), Cousin JM (Cousin, J.-M.) (Ed.). ATMOSPHERIC CHEMISTRY AND PHYSICS pp. 4311-4326.

Cocheo, V., Boaretto, C., Sacco, P., 1996. High uptake rate radial diffusive sampler suitable for both solvent and thermal desorption. American Industrial Hygiene Association Journal 57, 897-904.

Cooper, S.M., Peterson, D.L., 2000. Spatial distribution of tropospheric ozone in western Washington, USA. Environmental Pollution 107, 339-347.

Coyle, M., Smith, R.I., Stedman, J.R., Weston, K.J., Fowler, D., 2002. Quantifying the spatial distribution of surface ozone concentration in the UK. Atmospheric Environment 36, 1013-1024.

Cvitas, T., Kezele, N., Klasinc, L., Sorgo, G., 2006. AOT40 as preliminary indicator for ozone induced forest injury on Mountain Medvednica near Zagreb. Periodicum Biologorum 108, 639-641.

Daley, M.J., Phillips, N.G., 2006. Interspecific variation in nighttime transpiration and stomatal conductance in a mixed New England deciduous forest. Tree Physiology 26, 411-419.

Dalstein, L., Vollenweider, P., Vas, N., Günthardt-Goerg, M.S., 2002. L'ozone et les conifères du Sud-Est de la France. Association Forêt méditerranéenne, Marseille, FRANCE.

Dann, M.S., Pell, E.J., 1989. Decline of Activity and Quantity of Ribulose Bisphosphate Carboxylase Oxygenase and Net Photosynthesis in Ozone-Treated Potato Foliage. Plant Physiology 91, 427-432.

Dawson, T.E., Burgess, S.S.O., Tu, K.P., Oliveira, R.S., Santiago, L.S., Fisher, J.B., Simonin, K.A., Ambrose, A.R., 2007. Nighttime transpiration in woody plants from contrasting ecosystems. Tree Physiology 27, 561-575.

de Bauer, M.d.L., Hernandez-Tejeda, T., 2007. A review of ozone-induced effects on the forests of central Mexico. Environmental Pollution 147, 446-453.

De Bauer, M.D.L.D.L.I., Tejeda, T.H., Manning, W.J., 1985. Ozone Causes Needle Injury and Tree Decline in Pinus-Hartwegii at High Altitudes in the Mountains Around Mexico City Mexico. Journal of the Air Pollution Control Association 35, 838.

De Leeuw, F., 2000. Trends in ground level ozone concentrations in the European Union. Environmental Science and Policy 3, 189-199.

De Vries, W., Klap, J.M., Erisman, J.W., 2000. Effects of environmental stress on forest crown condition in Europe. Part I: Hypotheses and approach to the study. Water Air and Soil Pollution 119, 317-333.

Denman, K., Brasseur, G., Chidthaisong, A., Ciais, P., Cox, P., Dickinson, R., Hauglustaine, D.A., Heinze, C., Holland, E., Jacob, D., Lohmann, U., Ramachandran, S., Leite da Silva Dias, P., Wofsy, S., Zhang, X., 2007. Couplings Between Changes in the Climate System and Biogeochemistry, in: Solomon, S., Quin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., Tignor, M., Miller, H. (Eds.), Climate Change 2007:

The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. International Panel on Climate Change, Cambridge, United Kingdon, pp. 500-588.

Dentener, F., Stevenson, D., Ellingsen, K., van Noije, T., Schultz, M., Amann, M., Atherton, C., Bell, N., Bergmann, D., Bey, I., Bouwman, L., Butler, T., Cofala, J., Collins, B., Drevet, J., Doherty, R., Eickhout, B., Eskes, H., Fiore, A., Gauss, M., Hauglustaine, D., Horowitz, L., Isaksen, I.S.A., Josse, B., Lawrence, M., Krol, M., Lamarque, J.F., Montanaro, V., Muller, J.F., Peuch, V.H., Pitari, G., Pyle, J., Rast, S., Rodriguez, J., Sanderson, M., Savage, N.H., Shindell, D., Strahan, S., Szopa, S., Sudo, K., Van Dingenen, R., Wild, O., Zeng, G., 2006. The global atmospheric environment for the next generation. Environmental Science & Technology 40, 3586-3594.

Díaz-de-Quijano, M., Peñuelas, J., Ribas, À., 2009. Increasing interannual and altitudinal ozone mixing ratios in the Catalan Pyrenees. Atmospheric Environment 43, 6049-6057.

Díaz-de-Quijano, M., Peñuelas, J., Ribas, À., 2012a. Trends of AOT40 at three sites in the Catalan Pyrenees over the last 16 years. Journal of Atmospheric Chemistry DOI 10.1007/s10874-012-9222-9.

Díaz-de-Quijano, M., Schaub, M., Bassin, S., Volk, M., Peñuelas, J., 2012b. Ozone visible symptoms and reduced root biomass in the subalpine species Pinus uncinata after two years of free-air ozone fumigation. Environmental Pollution, doi:10.1016/j.envpol.2012.1002.1011.

Directive, E., 2002. Directive 2002/3/EC of the European Parliament and of the Council of 12 Februray 2002 relating to ozone in ambient air.

. Official Journal of the European Communities, pp. 0014–0030.

Directive, E., 2008. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. Official Journal of the European Comunities, pp. 0001-0044.

Dizengremel, P., 2001. Effects of ozone on the carbon metabolism of forest trees. Plant Physiology and Biochemistry 39, 729-742.

Dobbertin, M., 2005. Tree growth as indicator of tree vitality and of tree reaction to environmental stress: a review. European Journal of Forest Research 124, 319-333.

Dobbertin, M., Brang, P., 2001. Crown defoliation improves tree mortality models. Forest Ecology and Management 141, 271-284.

Drobyshev, I., Linderson, H., Sonesson, K., 2007. Relationship between crown condition and tree diameter growth in southern Swedish oaks. Environmental Monitoring and Assessment 128, 61-73.

Duenas, C., Fernandez, M.C., Canete, S., Carretero, J., Liger, E., 2002. Assessment of ozone variations and meteorological effects in an urban area in the Mediterranean Coast. Science of the Total Environment 299, 97-113.

Duncan, B.N., Martin, R.V., Staudt, A.C., Yevich, R., Logan, J.A., 2003. Interannual and seasonal variability of biomass burning emissions constrained by satellite observations. Journal of Geophysical Research-Atmospheres 108.

Duriscoe, D., Stolte, K., Pronos, J., 1996. History of ozone injury monitoring methods and the development of a recommended protocol, in: Miller, P.R., Stolte, K.W., Duriscoe, D.M. (Eds.), Evaluating ozone air pollution effects on pines in the western United States. Pacific Southwest Research Station, Forest Service, US Department of Agriculture, Albany, CA, pp. 19-28.

Eamus, D., Barnes, J.D., Mortensen, L., Ropoulsen, H., Davison, A.W., 1990. Persistent Stimulation of CO_2 Assimilation and Stomatal Conductance by Summer Ozone Fumigation in Norway Spruce. Environmental Pollution 63, 365-379.

Eamus, D., Murray, M., 1991. Photosynthetic and Stomatal Conductance Responses of Norway Spruce and Beech to Ozone, Acid Mist and Frost - a Conceptual-Model. Environmental Pollution 72, 23-44.

Eckmullner, O., Sterba, H., 2000. Crown condition, needle mass, and sapwood area relationships of Norway spruce (Picea abies). Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 30, 1646-1654.

EEA, 2007. Air Pollution in Europe 1990–2004. EEA Report No2/2007. European Environment Agency, Copenhagen.

EEA, 2009. NEC Directive status report 2008, Reporting by the Member States under Directive 2001/81/EC of the European Parliament and of the Council of 23 October 2001 on national emission ceilings for certain atmospheric pollutants. Annex 1: Member State country profiles. EEA, Luxembourg.

EEA, 2011. NEC Directive status report 2010, Reporting by the Member States under Directive 2001/81/EC of the European Parliament and of the Council of 23 October 2001 on national emission ceilings for certain atmospheric pollutants. European Environment Agency, Luxembourg.

Elvira, S., Alonso, R., Castillo, F.J., Gimeno, B.S., 1998. On the response of pigments and antioxidants of *Pinus halepensis* seedlings to Mediterranean climatic factors and long term ozone exposure. New Phytologist 138, 419-432.

Elvira, S., Alonso, R., Inclan, R., Bermejo, V., Castillo, F.J., Gimeno, B.S., 1995. Ozone effects on Aleppo pine seedlings (Pinus halepensis Mill.) grown in open-top chambers. Water, Air, and Soil Pollution 85, 1387-1392.

Emberson, L.D., Ashmore, M.R., Cambridge, H.M., Simpson, D., Tuovinen, J.P., 2000. Modelling stomatal ozone flux across Europe. Environmental Pollution 109, 403-413.

Evans, L.S., Adamski, J.H., Renfro, J.R., 1996. Relationships between cellular injury, visible injury of leaves, and ozone exposure levels for several dicotyledonous plant species at Great Smoky Mountains National Park. Environmental and Experimental Botany 36, 229-237.

Feng, Z., Kobayashi, K., Ainsworth, E.A., 2008a. Impact of elevated ozone concentration on growth, physiology, and yield of wheat (Triticum aestivum L.): a meta-analysis. Global Change Biology 14, 2696-2708.

Feng, Z.Z., Kobayashi, K., Ainsworth, E.A., 2008b. Impact of elevated ozone concentration on growth, physiology, and yield of wheat (Triticum aestivum L.): a meta-analysis. Global Change Biology 14, 2696-2708.

Ferretti, M., Fagnano, M., Amoriello, T., Badiani, M., Ballarin-Denti, A., Buffoni, A., Bussotti, F., Castagna, A., Cieslik, S., Costantini, A., De Marco, A., Gerosa, G., Lorenzini, G., Manes, F., Merola, G., Nali, C., Paoletti, E., Petriccione, B., Racalbuto, S., Rana, G., Ranieri, A., Tagliaferri, A., Vialetto, G., Vitale, M., 2007. Measuring, modelling and testing ozone exposure, flux and effects on vegetation in southern European conditions - What does not work? A review from Italy. Environmental Pollution 146, doi:10.1016/j.envpol.2006.1005.1012.

Fiala, J., Cernikovsky, L., de Leeuw, F., Kurfuerst, P., 2003. Air pollution by ozone in Europe in Summer 2003. Overview of exceedances of EC ozone threshold values during the summer season April-August 2003 and comparisons with previous years, Report to the European Comission by the European Environment Agency, European Topic Centre on Air and Climate Change. European Environment Agency, Copenhagen.

Finlayson-Pitts, B.J., Pitts, J.N., 2000. Chemistry of the Upper and Lower Atmosphere. Academic Press, San Diego.

Finlayson-Pitts, B.J., Pitts, J.N., Jr., 1997. Tropospheric air pollution: Ozone, airborne toxics, polycyclic aromatic hydrocarbons, and particles. Science (Washington D C) 276, 1045-1052.

Fiscus, E.L., Booker, F.L., Burkey, K.O., 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant Cell and Environment 28, 997-1011.

Fiscus, E.L., Reid, C.D., Miller, J.E., Heagle, A.S., 1997. Elevated CO_2 reduces O_3 flux and O_3-induced yield losses in soybeans: Possible implications for elevated CO_2 studies. Journal of Experimental Botany 48, 307-313.

Flagler, R.B., Chappelka, A.H., 1995. Growth response of southern pines to acidic precipitations and ozone, in: Fox, S., Mickley, R.A. (Eds.), Impact of Air Pollutants on Southern Pine Forests. Ecological Studies, Springer, Berlin, pp. 388-424.

Fluckiger, W., Braun, S., Hiltbrunner, E., 2002. Effects of air pollution on biotic stress, in: Bell, J.N.B., Treshow, M. (Eds.), Air pollution and plant life. John Wiley, Chichester, UK, pp. 379-406.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., 2007. Changes in atmospheric constituents and in radiative forcing, in: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., Tignor, M., Miller, H. (Eds.), Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK, pp. 129-234.

Fowler, D., Amann, M., Anderson, R., Ashmore, M.R., Cox, P., Depledge, M., Derwent, D., Grennfelt, P., Hewitt, N., Hov, O., Jenkin, M., Kelly, F., Liss, P., Pilling, M., Pyle, J., Slingo, J., Stevenson, D., 2008. Ground-level ozone in the 21st century: future trends, impacts and policy implications. The Royal Society, London, p. 148.

Fowler, D., Cape, J.N., Coyle, M., Flechard, C., Kuylenstierna, J., Hicks, K., Derwent, D., Johnson, C., Stevenson, D., 1999. The global exposure of forests to air pollutants. Water Air and Soil Pollution 116, 5-32.

Foyer, C.H., Lelandais, M., Kunert, K.J., 1994. Photooxidative Stress in Plants. Physiologia Plantarum 92, 696-717.

Fuhrer, J., 2009. Ozone risk for crops and pastures in present and future climates. Naturwissenschaften 96, 173-194.

Fuhrer, J., Booker, F., 2003. Ecological issues related to ozone: agricultural issues. Environment International 29, 141-154.

Galiano, L., Martinez-Vilalta, J., Lloret, F., 2010. Drought-Induced Multifactor Decline of Scots Pine in the Pyrenees and Potential Vegetation Change by the Expansion of Co-occurring Oak Species. Ecosystems 13, 978-991.

Gangoiti, G., Millan, M.M., Salvador, R., Mantilla, E., 2001. Long-range transport and re-circulation of pollutants in the western Mediterranean during the project Regional Cycles of Air Pollution in the West-Central Mediterranean Area. Atmospheric Environment 35, 6267-6276.

Gerosa, G., Finco, A., Mereu, S., Vitale, M., Manes, F., Denti, A.B., 2009. Comparison of seasonal variations of ozone exposure and fluxes in a Mediterranean Holm oak forest between the exceptionally dry 2003 and the following year. Environmental Pollution 157, 1737-1744.

Gerosa, G., Marzuoli, R., Bussotti, F., Pancrazi, M., Ballarin-Denti, A., 2003. Ozone sensitivity of Fagus sylvatica and Fraxinus excelsior young trees in relation to leaf structure and foliar ozone uptake. Environmental Pollution 125, 91-98.

Gettelman, A., Holton, J.R., Rosenlof, K.H., 1997. Mass fluxes of O-3, CH4, N2O and CF2Cl2 in the lower stratosphere calculated from observational data. Journal of Geophysical Research-Atmospheres 102, 19149-19159.

Gheusi, F., Ravetta, F., Delbarre, H., Tsamalis, C., Chevalier-Rosso, A., Leroy, C., Augustin, P., Delmas, R., Ancellet, G., Athier, G., Bouchou, P., Campistron, B., Cousin, J.M., Fourmentin, M., Meyerfeld, Y., 2011. Pic 2005, a field campaign to investigate low-tropospheric ozone variability in the Pyrenees. Atmospheric Research 101, 640-665.

Ghude, S.D., Kulkarni, P.S., Beig, G., Jain, S.L., Arya, B.C., 2010. Global distribution of tropospheric ozone and its precursors: a view from space. International Journal of Remote Sensing 31, 485-495.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Elvira, S., 2004. Growth response to ozone of annual species from Mediterranean pastures. Environmental Pollution 132, 297-306.

Gimeno, B.S., Penuelas, J., Porcuna, J.L., Reinert, R.A., 1995. Biomonitoring ozone phytotoxicity in eastern Spain. Water Air and Soil Pollution 85, 1521-1526.

Gimeno, B.S., Velissariou, D., Barnes, J.D., Inclán, R., Peña, J.M., Davison, A., 1992. Daños visibles por ozono en acículas de *Pinus halepensis* Mill. en Grecia y España. Ecología 6, 131-134.

Gonçalves, M., Jiménez-Guerrero, P., Baldasano, J.M., 2009. Contribution of atmospheric processes affecting the dynamics of air pollution in South-Western Europe during a typical summertime photochemical episode. Atmospheric Chemistry and Physics 9, 849-864.

Grantz, D.A., Farrar, J.F., 2000. Ozone inhibits phloem loading from a transport pool: compartmental efflux analysis in *Pima cotton*. Australian Journal of Plant Physiology 27, 859-868.

Gravano, E., Bussotti, F., Strasser, R.J., Schaub, M., Novak, K., Skelly, J., Tani, C., 2004. Ozone symptoms in leaves of woody plants in open-top chambers: ultrastructural and physiological characteristics. Physiologia Plantarum 121, 620-633.

Grayson, R., Western, A., Wilson, D., Young, R., McMahon, T., Woods, R., Duncan, M., Blöschl, G., 1999. Measurements and interpretation of soil moisture for hydrological applications, Water 99: Joint Congress, Brisbane, pp. 5-9.

Guderian, R., Tingey, D.T., Rabe, R., 1985. Effects of photochemical oxidants on plants, in: Guderian, R. (Ed.), Air pollution by photochemical oxidants. Springer Verlag, Berlin, pp. 129-334.

Günthardt-Goerg, M.S., 1996. Different responses to ozone of tobacco, poplar, birch, and alder. Journal of Plant Physiology 148, 207-214.

Günthardt-Goerg, M.S., McQuattie, C.J., Scheidegger, C., Rhiner, C., Matyssek, R., 1997. Ozone-induced cytochemical and ultrastructural changes in leaf mesophyll cell walls. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 27, 453-463.

Günthardt-Goerg, M.S., Vollenweider, P., 2007. Linking stress with macroscopic and microscopic leaf response in trees: New diagnostic perspectives. Environmental Pollution 147, 467-488.

Hayes, F., Jones, M.L.M., Mills, G., Ashmore, M., 2007. Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. Environmental Pollution 146, 754-762.

Heagle, A.S., Miller, J.E., Booker, F.L., Pursley, W.A., 1999. Ozone stress, carbon dioxide enrichment, and nitrogen fertility interactions in cotton. Crop Science 39, 731-741.

Heath, R., Taylor, G., 1997. Physiolgical processes and plant responses to ozone exposure, in: Sanderman, H., Wellbum, A., Heath, R. (Eds.), Ozone and Forest Decline: A Comparison of Controlled Chamber and Field Experiments. Ecol. Studies. Springer-Verlag, Berlin, pp. 317-368.

Heath, R.L., 2008. Modification of the biochemical pathways of plants induced by ozone: What are the varied routes to change? Environmental Pollution 155, 453-463.

Heggestad, H.E., 1991. Origin of Bel-W3, Bel-C and Bel-B Tobacco Varieties and Their Use as Indicators of Ozone. Environmental Pollution 74, 264-291.

Hermle, S., Vollenweider, P., Günthardt-Goerg, M.S., McQuattie, C.J., Matyssek, R., 2007. Leaf responsiveness of Populus tremula and Salix viminalis to soil contaminated with heavy metals and acidic rainwater. Tree Physiology 27, 1517-1531.

Holopainen, T., Anttonen, S., Palomaki, V., Kainulainen, P., Holopainen, J.K., 1996. Needle ultrastructure and starch content in Scots pine and Norway spruce after ozone fumigation. Canadian Journal of Botany 74, 67-76.

Holopainen, T., Anttonen, S., Wulff, A., Palomaki, V., Karenlampi, L., 1992. Comparative evaluation of the effects of gaseous pollutants, acidic deposition and mineral deficiencies: structural changes in the cells of forest plants. Agriculture Ecosystems & Environment 42, 365-398.

IDESCAT, 2011. Institut d'Estadística de Catalunya. http://www.idescat.cat.

Illa, E., Carrillo, E., Ninot, J.M., 2006. Patterns of plant traits in Pyrenean alpine vegetation. Flora 201, 528-546.

Inclán, R., Gimeno, B.S., Peñuelas, J., Gerant, D., Quejido, A., 2011. Carbon Isotope Composition, Macronutrient Concentrations, and Carboxylating Enzymes in Relation to the Growth of Pinus halepensis Mill. When Subject to Ozone Stress. Water Air and Soil Pollution 214, 587-598.

INE, 2011. Instituto Nacional de Estadística. http://www.ine.es/.

Innes, J.L., 1998. An assessment of the use of crown structure for the determination of the health of beech (Fagus sylvatica). Forestry 71, 113-130.

IPCC, 2007a. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change in: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., Tignor, M., Miller, H. (Eds.). Cambridge University Press, Cambridge, UK and New York, USA, p. 996.

IPCC, 2007b. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, in: Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and, Miller, H.L. (Eds.). Cambridge University Press, Cambridge, UK and New York, NY, USA, p. 996.

Isaksen, I.S.A., Zerefos, C., Kourtidis, K., Meleti, C., Dalsøren, S.B., Sundet, J.K., Grini, A., Zanis, P., Balis, D., 2005. Tropospheric ozone changes at unpolluted and semipolluted regions induced by stratospheric ozone changes. J. Geophys. Res. 110, D02302.

Islam, K.R., Mulchi, C.L., Ali, A.A., 2000. Interactions of tropospheric CO_2 and O_3 enrichments and moisture variations on microbial biomass and respiration in soil. Global Change Biology 6, 255-265.

Jaffe, D., Ray, J., 2007. Increase in surface ozone at rural sites in the western US. Atmospheric Environment 41, 5452-5463.

Jimenez-Guerrero, P., Jorba, O., Baidasanoa, J.M., Gasso, S., 2008. The use of a modelling system as a tool for air quality management: Annual high-resolution simulations and evaluation. Science of the Total Environment 390, 323-340.

Jonson, J.E., Simpson, D., Fagerli, H., Solberg, S., 2006. Can we explain the trends in European ozone levels? Atmospheric Chemistry and Physics 6, 51-66.

Kalabokas, P.D., Viras, L.G., Bartzis, J.G., Repapis, C.C., 2000. Mediterranean rural ozone characteristics around the urban area of Athens. Atmospheric Environment 34, 5199-5208.

Karlsson, P.E., Uddling, J., Braum, S., Broadmeadow, M., Elvira, S., Sánchez-Gimeno, G., Le Thiec, D., Oksanen, E., Vandermeiren, K., Wilkinson, M., Emberson, L., 2003. New critical levels for ozone impact on trees based on AOT40 and leaf cumulated uptake of ozone, in: Kalsson, P.E., Selldén, G., Pleijel, H. (Eds.), Establishing ozone critical levels. IVL Swedish Environmental Research Institute, Gothenburg, pp. 236-250.

Kaufmann, M.R., 1976. Stomatal Response of Engelmann Spruce to Humidity, Light, and Water Stress. Plant Physiology 57, 898-901.

Kim, J.S., Chappelka, A.H., Miller-Goodman, M.S., 1998. Decomposition of blackberry and broomsedge bluestem as influenced by ozone. Journal of Environmental Quality 27, 953-960.

Kivimäenpää, M., Sellden, G., Sutinen, S., 2005. Ozone-induced changes in the chloroplast structure of conifer needles, and their use in ozone diagnostics. Environmental Pollution 137, 466-475.

Kivimäenpää, M., Sutinen, S., Calatayud, V., Jose Sanz, M., 2010. Visible and microscopic needle alterations of mature Aleppo pine (Pinus halepensis) trees growing on an ozone gradient in eastern Spain. Tree Physiology 30, 541-554.

Kivimäenpää, M., Sutinen, S., Karlsson, P.E., Sellden, G., 2003. Cell structural changes in the needles of Norway spruce exposed to long-term ozone and drought. Annals of Botany 92, 779-793.

Klumpp, A., Ansel, W., Klumpp, G., Belluzzo, N., Calatayud, V., Chaplin, N., Garrec, J.P., Gutsche, H.J., Hayes, M., Hentze, H.W., Kambezidis, H., Laurent, O., Penuelas, J., Rasmussen, S., Ribas, A., Ro-Poulsen, H., Rossi, S., Sanz, M.J., Shang, H., Sifakis, N., Vergne, P., 2002. EuroBionet: A Pan-European biomonitoring network for urban air quality assessment. Environmental Science and Pollution Research 9, 199-203.

Klumpp, A., Ansel, W., Klumpp, G., Calatayud, V., Garrec, J.P., He, S., Penuelas, J., Ribas, A., Ro-Poulsen, H., Rasmussen, S., Sanz, M.J., Vergne, P., 2006. Ozone pollution and ozone biomonitoring in European cities. Part I: Ozone concentrations and cumulative exposure indices at urban and suburban sites. Atmospheric Environment 40, 7963-7974.

Koch, K.E., 1996. Carbohydrate-modulated gene expression in plants. Annual Review of Plant Physiology and Plant Molecular Biology 47, 509-540.

Kraus, T.E.C., Dahlgren, R.A., Zasoski, R.J., 2003. Tannins in nutrient dynamics of forest ecosystems - a review. Plant and Soil 256, 41-66.

Krupa, S.V., Legge, A.H., 2000. Passive sampling of ambient, gaseous air pollutants: an assessment from an ecological perspective. Environmental Pollution 107, 31-45.

Krupa, S.V., Tonneijck, A., Manning, W., 1998. Ozone, in: Flagler, R.B. (Ed.), Recognition of Air Pollution Injury to Vegetation: A Pictorial Atlas. Air & Waste Management Association, Pittsburgh, PA., pp. 2-1 to 2-28.

Landmann, G., Bonneau, M., 1995. Forest decline and atmospheric deposition effects in the French mountains. Springer.

Landolt, W., GunthardtGoerg, M.S., Pfenninger, I., Einig, W., Hampp, R., Maurer, S., Matyssek, R., 1997. Effect of fertilization on ozone-induced changes in the metabolism of birch (*Betula pendula*) leaves. New Phytologist 137, 389-397.

Larsen, J.B., Yang, W., Vontiedemann, A., 1990. Effects of Ozone on Gas-Exchange, Frost-Resistance, Flushing and Growth of Different Provenances of European Silver Fir (Abies Alba Mill). European Journal of Forest Pathology 20, 211-218.

Laurence, J.A., Andersen, C.P., 2003. Ozone and natural systems: understanding exposure, response and risk. Environment International 29, 155-160.

Lefhon, A.S., 1992. Surface Level Ozone Exposures and Their Effects on Vegetation. Lewis Publishers, Chelsea.

Lefohn, A.S., Jackson, W., Shadwick, D.S., Knudsen, H.P., 1997. Effect of surface ozone exposures on vegetation grown in the Southern Appalachian Mountains: Identification of possible areas of concern. Atmospheric Environment 31, 1695-1708.

Lelieveld, J., Berresheim, H., Borrmann, S., Crutzen, P.J., Dentener, F.J., Fischer, H., Feichter, J., Flatau, P.J., Heland, J., Holzinger, R., Korrmann, R., Lawrence, M.G., Levin, Z., Markowicz, K.M., Mihalopoulos, N., Minikin, A., Ramanathan, V., de Reus, M., Roelofs, G.J., Scheeren, H.A., Sciare, J., Schlager, H., Schultz, M., Siegmund, P., Steil, B., Stephanou, E.G., Stier, P., Traub, M., Warneke, C., Williams, J., Ziereis, H., 2002. Global air pollution crossroads over the Mediterranean. Science 298, 794-799.

Liska, A.J., Shevchenko, A., Pick, U., Katz, A., 2004. Enhanced photosynthesis and redox energy production contribute to salinity tolerance in *Dunaliella* as revealed by homology-based proteomics. Plant Physiology 136, 2806-2817.

Lucas, P.W., Diggle, P.J., 1997. The use of longitudinal data analysis to study the multi-seasonal growth responses of Norway and Sitka spruce to summer exposure to ozone: implications for the determination of critical levels. New Phytologist 137, 315-323.

Manninen, S., Sorjamaa, R., Kurki, S., Pirttiniemi, N., Huttunen, S., 2003a. Ozone affects Scots pine phenology and growth. Air Pollution, Global Change and Forests in the New Millennium 3, 231-246.

Manninen, S., Sorjamaa, R., Kurki, S., Pirttiniemi, N., Huttunen, S., 2003b. Ozone affects Scots pine phenology and growth, Air Pollution, Global Change and Forests in the New Millennium. Elsevier Science Bv, Amsterdam, pp. 231-246.

Manning, W.J., Godzik, B., Musselman, R., 2002. Potential bioindicator plant species for ambient ozone in forested mountain areas of central Europe. Environmental Pollution 119, 283-290.

Marenco, A., 1986. Variation of CO and ozone in the troposphere: evidence of ozone chemistry. Atmospheric Environment 20, 911-918.

Marenco, A., Gouget, H., Nedelec, P., Pages, J.P., Karcher, F., 1994. Evidence of a Long-Term Increase in Tropospheric Ozone from Pic Du Midi Data Series - Consequences - Positive Radiative Forcing. Journal of Geophysical Research-Atmospheres 99, 16617-16632.

Martín-Vide, J., 1992. El Clima, in: Carreras, C. (Ed.), Geografia general dels Països Catalans. Enciclopèdia Catalana, Barcelona.

Marzuoli, R., Gerosa, G., Desotgiu, R., Bussotti, F., Ballarin-Denti, A., 2009. Ozone fluxes and foliar injury development in the ozone-sensitive poplar clone Oxford (Populus maximowiczii x Populus berolinensis): a dose-response analysis. Tree physiology 29, 67-76.

Matyssek, R., Bytnerowicz, A., Karlsson, P.E., Paoletti, E., Sanz, M., Schaub, M., Wieser, G., 2007. Promoting the O(3) flux concept for European forest trees. Environmental Pollution 146, 587-607.

Matyssek, R., Gunthardtgoerg, M.S., Maurer, S., Keller, T., 1995. Nighttime Exposure to Ozone Reduces Whole-Plant Production in Betula-Pendula. Tree Physiology 15, 159-165.

Matyssek, R., Karnosky, D.F., Wieser, G., Percy, K., Oksanen, E., Grams, T.E.E., Kubiske, M., Hanke, D., Pretzsch, H., 2010. Advances in understanding ozone impact on forest trees: Messages from novel phytotron and free-air fumigation studies. Environmental Pollution 158, 1990-2006.

Matyssek, R., Sandermann, H., Wieser, G., Booker, F., Cieslik, S., Musselman, R., Ernst, D., 2008. The challenge of making ozone risk assessment for forest trees more mechanistic. Environmental Pollution 156, 567-582.

Maurer, S., Matyssek, R., GunthardtGoerg, M.S., Landolt, W., Einig, W., 1997. Nutrition and the ozone sensitivity of birch (Betula pendula) .1. Responses at the leaf level. Trees-Structure and Function 12, 1-10.

Mereu, S., Gerosa, G., Finco, A., Fusaro, L., Muys, B., Manes, F., 2009. Improved sapflow methodology reveals considerable night-time ozone uptake by Mediterranean species. Biogeosciences 6, 3151-3162.

Millán, M.M., Mantilla, E., Salvador, R., Carratala, A., Sanz, M.J., Alonso, L., Gangoiti, G., Navazo, M., 2000. Ozone cycles in the western Mediterranean basin: Interpretation of monitoring data in complex coastal terrain. Journal of Applied Meteorology 39, 487-508.

Miller, J.D., Arteca, R.N., Pell, E.J., 1999. Senescence-associated gene expression during ozone-induced leaf senescence in Arabidopsis. Plant Physiology 120, 1015-1023.

Miller, J.E., Shafer, S.R., Schoeneberger, M.M., Pursley, W.A., Horton, S.J., Davey, C.B., 1997. Influence of a mycorrhizal fungus and/or rhizobium on growth and biomass partitioning of subterranean clover exposed to ozone. Water Air and Soil Pollution 96, 233-248.

Miller, P., McBride, J., 1999. Oxidant Air Pollution Impacts in the Montane Forests of Southern California: the San Bernadino Case Study. Springer-Verlag, New York, USA.

Mudd, J.B., Mc Manus, T.T., Ongun, A., 1971. Inhibition of Lipid Metabolism in Chloroplasts by Ozone, in: Englund, H.M., Beerr, W.T. (Eds.), Proceedings of the 2nd International Clean Air Congress. Academic Press, New York, pp. 256-260.

Naja, M., Lal, S., Chand, D., 2003. Diurnal and seasonal variabilities in surface ozone at a high altitude site Mt Abu (24.6 degrees N, 72.7 degrees E, 1680 m asl) in India. Atmospheric Environment 37, 4205-4215.

Nali, C., Pucciariello, C., Lorenzini, G., 2002a. Mapping ozone critical levels for vegetation in Central Italy. Water Air and Soil Pollution 141, 337-347.

Nali, C., Pucciariello, C., Lorenzini, G., 2002b. Ozone distribution in central Italy and its effect on crop productivity. Agriculture Ecosystems & Environment 90, 277-289.

Ninot, J.M., Carrillo, E., Font, X., Carreras, J., Ferré, A., Masalles, R.M., Soriano, I., Vigo, J., 2007. Altitude zonation in the Pyrenees. A geobotanic interpretation. Phytocoenologia 37, 371-398.

Novak, K., Schaub, M., Fuhrer, J., Skelly, J.M., Frey, B., Kraeuchi, N., 2008. Ozone effects on visible foliar injury and growth of Fagus sylvatica and Viburnum lantana seedlings grown in monoculture or in mixture. Environmental and Experimental Botany 62, 212-220.

Novak, K., Skelly, J.M., Schaub, M., Kräuchi, N., Hug, C., Landolt, W., Bleuler, P., 2003. Ozone air pollution and foliar injury development on native plants of Switzerland. Environmental Pollution 125, 41-52.

Nunn, A.J., Kozovits, A.R., Reiter, I.M., Heerdt, C., Leuchner, M., Luetz, C., Liu, X., Loew, M., Winkler, J.B., Grams, T.E.E., Haeberle, K.H., Werner, H., Fabian, P., Rennenberg, H., Matyssek, R., 2005a. Comparison of ozone uptake and sensitivity between a phytotron study with young beech and a field experiment with adult beech (Fagus sylvatica). Environmental Pollution 137, 494-506.

Nunn, A.J., Kozovits, A.R., Reiter, I.M., Heerdt, C., Leuchner, M., Lutz, C., Liu, X., Low, M., Winkler, J.B., Grams, T.E.E., Haberle, K.H., Werner, H., Fabian, P., Rennenberg, H., Matyssek, R., 2005b. Comparison of ozone uptake and sensitivity between a phytotron study with young beech and a field experiment with adult beech (Fagus sylvatica). Environmental Pollution 137, 494-506.

O'Loughlin, E.M., 1986. Predictions of surface saturation zones in natural catchments by topographic analysis. Water Resources Research 22, 794-804.

Ogaya, R., Penuelas, J., Asensio, D., Llusia, J., 2011. Chlorophyll fluorescence responses to temperature and water availability in two co-dominant Mediterranean shrub and tree species in a long-term field experiment simulating climate change. Environmental and Experimental Botany 71, 123-127.

Oksanen, E., Haikio, E., Sober, J., Karnosky, D.F., 2004. Ozone-induced H_2O_2 accumulation in field-grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity. New Phytologist 161, 791-799.

Oliveira, G., Peñuelas, J., 2004. Effects of winter cold stress on photosynthesis and photochemical efficiency of PSII of the Mediterranean Cistus albidus L. and Quercus ilex L. Plant Ecology 175, 179-191.

Ollinger, S.V., Aber, J.D., Reich, P.B., 1997. Simulating ozone effects on forest productivity: Interactions among leaf-, canopy-, and stand-level processes. Ecological Applications 7, 1237-1251.

Olsen, M.A., Schoeberl, M.R., Douglass, A.R., 2004. Stratosphere-troposphere exchange of mass and ozone. Journal of Geophysical Research-Atmospheres 109.

Olsen, S.C., McLinden, C.A., Prather, M.J., 2001. Stratospheric N(2)O-NO(y) system: Testing uncertainties in a three-dimensional framework. Journal of Geophysical Research-Atmospheres 106, 28771-28784.

Olszyk, D.M., Johnson, M.G., Phillips, D.L., Seidler, R.J., Tingey, D.T., Watrud, L.S., 2001. Interactive effects of CO_2 and O_3 on a ponderosa pine plant/litter/soil mesocosm. Environmental Pollution 115, 447-462.

Pääkkönen, E., Günthardt-Goerg, M.S., Holopainen, T., 1998. Responses of leaf processes in a sensitive birch (Betula pendula Roth) clone to ozone combined with drought. Annals of Botany 82, 49-59.

Panek, J.A., Goldstein, A.H., 2001. Response of stomatal conductance to drought in ponderosa pine: implications for carbon and ozone uptake. Tree Physiology 21, 337-344.

Paoletti, E., Contran, N., Bernasconi, P., Gunthardt-Goerg, M.S., Vollenweider, P., 2010. Erratum to "Structural and physiological responses to ozone in Manna ash (Fraxinus ornus L.) leaves of seedlings and mature trees under controlled and ambient conditions". The Science of the total environment 408, 2014-2024.

Paoletti, E., De Marco, A., Racalbuto, S., 2007. Why should we calculate complex indices of ozone exposure? Results from Mediterranean background sites. Environmental monitoring and assessment 128, 19-30.

Patterson, M.C., Samuelson, L., Somers, G., Mays, A., 2000. Environmental control of stomatal conductance in forest trees of the Great Smoky Mountains National Park. Environmental Pollution 110, 225-233.

Patterson, M.T., Rundel, P.W., 1995. Stand characteristics of ozone-stressed populations of *Pinus jeffreyi* (Pinaceae): Extent, development, and physiological consequences of visible injury. American Journal of Botany 82, 150-158.

Pell, E.J., Landry, L.G., Eckardt, N.A., Glick, R.E., 1994. Air pollution and Rubisco: Effects and implications, Plant responses to the gaseous environment: Molecular, metabolic and physiological aspects, pp. 239-253.

Pell, E.J., Schlagnhaufer, C.D., Arteca, R.N., 1997. Ozone-induced oxidative stress: Mechanisms of action and reaction. Physiologia Plantarum 100, 264-273.

Penuelas, J., Inoue, Y., 1999. Reflectance indices indicative of changes in water and pigment contents of peanut and wheat leaves. Photosynthetica 36, 355-360.

Peñuelas, J., Ribas-Carbo, M., González-Meler, M., Azcón-Bieto, J., 1994. Water status, photosynthetic pigments, C/N ratios and respiration rates of stika spruce seedlings exposed to 70 ppbv ozone for a summer. Environmental and Experimental Botany 34, 443-449.

Peterson, D.L., Bowers, D., Brace, S., 1999. Tropospheric ozone in the Nisqually River drainage, Mount Rainier National Park. Northwest Science 73, 241-254.

Pleijel, H., 2000. Ground-level ozone. A problem largely ignored in southern Europe, air pollution and climate series, in: Rain, S.S.o.A. (Ed.), Göteburg, Sweden, p. 26.

Prather, M.J., Dentener, F., Derwent, R., Dlugokencky, E., Holland, E., Isaksen, I., Katima, J., Kirchhoff, V., Matson, P., Midgley, M., Wang, M., 2001. Atmospheric chemistry and greenhouse gases, in: Houghton, J., Ding, Y., Griggs, D., Noguer, M., van der Linden, P., Dai, X., Maskell, K., Johnson, C. (Eds.), Climate Change 2001: The Scientific Basis. Contribution of working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change. Intergovernmental Panel on Climate Change, Cambridge, pp. 239-287.

Pretzsch, H., Dieler, J., Matyssek, R., Wipfler, P., 2010. Tree and stand growth of mature Norway spruce and European beech under long-term ozone fumigation. Environmental Pollution 158, 1061-1070.

Rennenberg, H., Herschbach, C., Polle, A., 1996. Consequences of air pollution on shoot-root interactions. Journal of Plant Physiology 148, 296-301.

Repo, T., Leinonen, I., Ryyppo, A., Finer, L., 2004. The effect of soil temperature on the bud phenology, chlorophyll fluorescence, carbohydrate content and cold hardiness of Norway spruce seedlings. Physiologia Plantarum 121, 93-100.

Retuerto, R., Fernandez-Lema, B., Rodriguez, R., Obeso, J.R., 2004. Increased photosynthetic performance in holly trees infested by scale insects. Functional Ecology 18, 664-669.

Ribas, A., Peñuelas, J., 2000. Effects of ethylene diurea as a protective antiozonant on beans (*Phaseolus vulgaris* cv Lit) exposed to different tropospheric ozone doses in Catalonia (NE Spain). Water Air and Soil Pollution 117, 263-271.

Ribas, A., Peñuelas, J., 2003. Biomonitoring of tropospheric ozone phytotoxicity in rural Catalonia. Atmospheric Environment 37, 63-71.

Ribas, A., Peñuelas, J., 2004. Temporal patterns of surface ozone levels in different habitats of the North Western Mediterranean basin. Atmospheric Environment 38, 985-992.

Ribas, À., Peñuelas, J., 2006. Surface ozone mixing ratio increase with altitude in a transect in the Catalan Pyrenees. Atmospheric Environment 40, 7308-7315.

Ribas, À., Peñuelas, J., Elvira, S., Gimeno, B.S., 2005. Ozone exposure induces the activation of leaf senescence-related processes and morphological and growth changes in seedlings of Mediterranian tree species. Environmental Pollution 134, 291-300.

Ro-Poulsen, H., Mikkelsen, T.N., Hovmand, M.F., Hummelsehoj, P., Jensen, N.O., 1998. Ozone deposition in relation to canopy physiology in a mixed conifer forest in Denmark. Chemosphere 36, 669-674.

Saliba, M., Ellul, R., Camilleri, L., Gusten, H., 2008. A 10-year study of background surface ozone concentrations on the island of Gozo in the Central Mediterranean. Journal of Atmospheric Chemistry 60, 117-135.

Sanz, M.J., Calatayud, V., Calvo, E., 2000. Spatial pattern of ozone injury in Aleppo pine related to air pollution dynamics in a coastal-mountain region of eastern Spain. Environmental Pollution 108, 239-247.

Sanz, M.J., Calatayud, V., Sánchez-Peña, G., 2007. Measures of ozone concentrations using passive sampling in forests of South Western Europe. Environmental Pollution 145, 620-628.

Sanz, M.J., Millán, M.M., 2000. Ozone in the Mediterranean region: evidence of injury to vegetation, in: Innes, J., Oleksyn, J. (Eds.), Forest Dynamics in Heavily Polluted Regions. CAB International 2000, pp. 165-192.

Schaub, M., Calatayud, V., Ferretti, M., Brunialti, G., Lövblad, G., Krause, G., Sanz, M.J., 2010. Monitoring of Ozone Injury. Manual Part X., Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. UNECE ICP Forests Programme Co-ordinating Centre, Hamburg.

Schaub, M., Emberson, L., Büker, P., Kräuchi, N., 2007. Preliminary results of modeled ozone uptake for *Fagus sylvatica* L. trees at selected EU/UN-ECE intensive monitoring plots. Environmental Pollution 145, 636-643.

Schaub, M., Skelly, J.M., Steiner, K.C., Davis, D.D., Pennypacker, S.P., Zhang, J., Ferdinand, J.A., Savage, J.E., Stevenson, R.E., 2003. Physiological and foliar injury responses of *Prunus serotina, Fraxinus americana,* and *Acer rubrum* seedlings to varying soil moisture and ozone. Environmental Pollution 124, 307-320.

Schoene, K., Franz, J.T., Masuch, G., 2004. The effect of ozone on pollen development in *Lolium perenne* L. Environmental Pollution 131, 347-354.

Schraudner, M., Langebartels, C., Sandermann, H., 1997. Changes in the biochemical status of plant cells induced by the environmental pollutant ozone. Physiologia Plantarum 100, 274-280.

Schroeder, H., Yocum, D., 2006. European institutions for controlling chemical air pollution: An analysis of CLRTAP-European Union interplay, Chemicals as Intentional and Accidental Global Environmental Threats, pp. 321-336.

Seinfield, J.H., Pandis, S.N., 2006. Atmospheric chemistry and physics. From air pollution to climate change. John Wiley & Sons, Hoboken, New Jersey.

Shindell, D.T., Faluvegi, G., Koch, D.M., Schmidt, G.A., Unger, N., Bauer, S.E., 2009. Improved Attribution of Climate Forcing to Emissions. Science 326, 716-718.

Sicard, P., Dalstein-Richier, L., Vas, N., 2011. Annual and seasonal trends of ambient ozone concentration and its impact on forest vegetation in Mercantour National Park (South-eastern France) over the 2000-2008 period. Environmental Pollution 159, 351-362.

Simpson, D., Ashmore, M.R., Emberson, L., Tuovinen, J.P., 2007. A comparison of two different approaches for mapping potential ozone damage to vegetation. A model study. Environmental Pollution 146, 715-725.

Skarby, L., Ro-Poulsen, H., Wellburn, F.A.M., Sheppard, L.J., 1998. Impacts of ozone on forests: a European perspective. New Phytologist 139, 109-122.

Skelly, J.M., Chappelka, A.H., Laurence, J.A., Frederickson, T.S., 1997. Ozone and its known and potential effects on forests in the eastern United States, in: Sandermann, H., Wellburn, H.R., Heath, R.I. (Eds.), Forest Decline and Ozone. Springer-Verlag, Berlin, pp. 69-93.

Skelly, J.M., Ferdinand, J.A., Savage, J.E., Mulik, J.D., 2001. A 13-week comparison of passive and continuous ozone monitors at forested sites in north-central Pennsylvania. Journal of the Air & Waste Management Association 51, 1280-1287.

SMC, 2010. Climatologia comarca a comarca, in: Sostenibilitat, D.d.T.i. (Ed.), p. 60.

Stevenson, D.S., Dentener, F.J., Schultz, M.G., Ellingsen, K., van Noije, T.P.C., Wild, O., Zeng, G., Amann, M., Atherton, C.S., Bell, N., Bergmann, D.J., Bey, I., Butler, T., Cofala, J., Collins, W.J., Derwent, R.G., Doherty, R.M., Drevet, J., Eskes, H.J., Fiore, A.M., Gauss, M., Hauglustaine, D.A., Horowitz, L.W., Isaksen, I.S.A., Krol, M.C., Lamarque, J.F., Lawrence, M.G., Montanaro, V., Muller, J.F., Pitari, G., Prather, M.J., Pyle, J.A., Rast, S., Rodriguez, J.M., Sanderson, M.G., Savage, N.H., Shindell, D.T., Strahan, S.E., Sudo, K., Szopa, S., 2006. Multimodel ensemble simulations of present-day and near-future tropospheric ozone. Journal of Geophysical Research-Atmospheres 111.

Stewart, C.A., Black, V.J., Black, C.R., Roberts, J.A., 1996. Direct effects of ozone on the reproductive development of *Brassica* species. Journal of Plant Physiology 148, 172-178.

Sutinen, S., 1987. Ultrastructure of Mesophyll-Cells of Spruce Needles Exposed to O-3 Alone and Together with So2. European Journal of Forest Pathology 17, 362-368.

Sutinen, S., 1990. Structural Changes in Needle Tissue of Spruce Trees from a Damaged Stand in Southern Finland. Scandinavian Journal of Forest Research 5, 403-412.

Sutinen, S., Koivisto, L., 1995. Microscopic structure of conifer needles as a diagnostic tool in the field, in: Munawar, M., Hänninen, O., Roy, S., Munawar, N., Kärenlampi, L., Brown, D. (Eds.), Bioindicators of Environmental Health. SPB Academic Publishing, Amsterdam, pp. 73-81.

Sutinen, S., Skärby, L., Wallin, G., Sellden, G., 1990. Long-term exposure of Norway Spruce, *Picea abies* (L) Karst, to ozone in open-top chambers II. Effects on the ultrastructure of the needles. New Phytologist 115, 345-355.

Taylor, G.E., Norby, R.J., McLaughlin, S.B., Johnson, A.H., Turner, R.S., 1986. Carbon-Dioxide Assimilation and Growth of Red Spruce (*Picea rubens* Sarg) Seedlings in Response to Ozone, Precipitation Chemistry, and Soil Type. Oecologia 70, 163-171.

Tingey, D.T., Andersen, C.P. , 1991. The physiological basis of differential plant sensitivity to changes in atmospheric quality, in: Taylor, G.E.J., Pitekla, L.F., Clegg, M.T. (Ed.), Ecological genetics and air pollution. Springer-Verlag, New York, USA, pp. 209-235.

Tomlinson, H., Rich, S., 1969. Relating Lipid Content and Fatty Acid Synthesis to Ozone Injury of Tobacco Leaves. Phytopathology 59, 1284-&.

Tranquillini, W., 1979. Physiological Ecology of the Alpine Timberline. Springer, Berlin.

UNECE, 2010. Manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. Convention on Long-range Transboundary Air Pollution. http://www.icpmapping.org.

UNECE/CLRTAP, 1999. Gothenburg Protocol to Abate Acidification, Eutrophication and Ground-level Ozone

UNECE/CLRTAP, 2006. Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. Part II: Visual assessment of crown condition. International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests. http://icp-forests.net/.

Utriainen, J., Holopainen, T., 2000. Impact of increased springtime O_3 exposure on Scots pine (*Pinus sylvestris*) seedlings in central Finland. Environmental Pollution 109, 479-487.

Utriainen, J., Holopainen, T., 2001. Nitrogen availability modifies the ozone responses of Scots pine seedlings exposed in an open-field system. Tree Physiology 21, 1205-1213.

Utriainen, J., Janhunen, S., Helmisaari, H.S., Holopainen, T., 2000. Biomass allocation, needle structural characteristics and nutrient composition in Scots pine seedlings exposed to elevated CO2 and O-3 concentrations. Trees-Structure and Function 14, 475-484.

Van Den Driessche, R., 1991. New Root Growth of Douglas-Fir Seedlings at Low Carbon Dioxide Concentration. Tree Physiology 8, 289-296.

VanderHeyden, D., Skelly, J., Innes, J., Hug, C., Zhang, J., Landolt, W., Bleuler, P., 2001. Ozone exposure thresholds and foliar injury on forest plants in Switzerland. Environmental Pollution 111, 321-331.

Verhoeven, A., Osmolak, A., Morales, P., Crow, J., 2009. Seasonal changes in abundance and phosphorylation status of photosynthetic proteins in eastern white pine and balsam fir. Tree Physiology 29, 361-374.

Viezee, W., Johnson, W.B., Singh, H.B., 1983. Stratospheric Ozone in the Lower Troposphere .2. Assessment of Downward Flux and Ground-Level Impact. Atmospheric Environment 17, 1979-1993.

Vigo, J., 2009. L'alta muntanya catalana. Flora i vegetació., 2nd revised ed, Barcelona.

Vingarzan, R., 2004. A review of surface ozone background levels and trends. Atmospheric Environment 38, 3431-3442.

Volk, M., Geissmann, M., Blatter, A., Contat, F., Fuhrer, J., 2003. Design and performance of a free-air exposure system to study long-term effects of ozone on grasslands. Atmospheric Environment 37, 1341-1350.

Vollenweider, P., Günthardt-Georg, M.S., 2006. Diagnosis of abiotic and biotic stress factors using the visible symptoms in foliage (vol 137, pg 455, 2005). Environmental Pollution 140, 561-571.

Vollenweider, P., Ottiger, M., Gunthardt-Goerg, M.S., 2003. Validation of leaf ozone symptoms in natural vegetation using microscopical methods. Environmental Pollution 124, 101-118.

Vollsnes, A.V., Kruse, O.M.O., Eriksen, A.B., Oxaal, U., Futsaether, C.M., 2010. In vivo root growth dynamics of ozone exposed *Trifolium subterraneum*. Environmental and Experimental Botany 69, 183-188.

Volz, A., Kley, D., 1988. Evaluation of the Montsouris series of ozone measurements made in the nineteenth century. Nature 332, 240-242.

Wager, D.J., Baker, F.A., 2003. Potential effects of ozone, climate, and spruce budworm on Douglas-fir growth in the Wasatch Mountains. Canadian Journal of Forest Research 33, 910-921.

Waldner, P., Schaub, M., Pannatier, E.G., Schmitt, M., Thimonier, A., Walthert, L., 2007. Atmospheric deposition and ozone levels in Swiss forests: Are critical values exceeded? Environmental Monitoring and Assessment 128, 5-17.

Wellbum, A., 1994. Air Pollution and Climate Change, The Biological Impact. Longman Scientific and Technical, Essex, England.

Wellburn, F.A.M., Wellburn, A.R., 1994. Atmospheric ozone affects carbohydrate allocation and winter hardiness of *Pinus halepensis* (Mill.). Journal of Experimental Botany 45, 607-614.

Whitfield, C.P., Davison, A.W., Ashenden, T.W., 1998. The effects of nutrient limitation on the response of *Plantago major* to ozone. New Phytologist 140, 219-230.

Wieser, G., 1999. Evaluation of the impact of ozone on conifers in the Alps: A case study on spruce, pine and larch in the Austrian Alps. Phyton (Horn) 39, 241-252.

Wieser, G., Hasler, R., Gotz, B., Koch, W., Havranek, W.M., 2000. Role of climate, crown position, tree age and altitude in calculated ozone flux into needles of Picea abies and Pinus cembra: a synthesis. Environmental Pollution 109, 415-422.

Wieser, G., Manning, W.J., Tausz, M., Bytnerowicz, A., 2006. Evidence for potential impacts of ozone on Pinus cembra L. at mountain sites in Europe: An overview. Environmental Pollution 139, 53-58.

Wieser, G., Tegischer, K., Tausz, M., Haberle, K.H., Grams, T.E.E., Matyssek, R., 2002. Age effects on Norway spruce (Picea abies) susceptibility to ozone uptake: a novel approach relating stress avoidance to defense. Tree Physiology 22, 583-590.

Wild, O., 2007. Modelling the global tropospheric ozone budget: exploring the variability in current models. Atmospheric Chemistry and Physics 7, 2643-2660.

Wittig, V.E., Ainsworth, E.A., Long, S.P., 2007. To what extent do current and projected increases in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review of the last 3 decades of experiments. Plant Cell and Environment 30, 1150-1162.

Wu, S., Mickley, L.J., Jacob, D.J., Logan, J.A., Yantosca, R.M., Rind, D., 2007. Why are there large differences between models in global budgets of tropospheric ozone? Journal of Geophysical Research-Atmospheres 112.

Wulff, A., Anttonen, S., Heller, W., Sandermann, H., Karenlampi, L., 1996. Ozone-sensitivity of Scots pine and Norway spruce from northern and local origin to long-term open-field fumigation in central Finland. Environmental and Experimental Botany 36, 209-&.

XVPCA, 2001. Implantació de la nova legislació europea sobre avaluació i gestió de la qualitat de l'aire a Catalunya. Document 1: Delimitació de Zones de Qualitat de l'aire. Departament de Medi Ambient.

Zheng, Y., Shimizu, H., Barnes, J.D., 2002. Limitations to CO_2 assimilation in ozone-exposed leaves of *Plantago major*. New Phytologist 155, 67-78.

Zierl, B., 2002. Relations between crown condition and ozone and its dependence on environmental factors. Environmental Pollution 119, 55-68.

Ziomas, I.C., 1998. The Mediterranean Campaign of Photochemical Tracers Transport and chemical evolution (MEDCAPHOT-TRACE): An outline. Atmospheric Environment 32, 2045-2053.