Effect of Ozone Flux on Selected Structural and Antioxidant Characteristics of a Mountain Norway Spruce Forest

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Abstract

In the 32-year-old mountain Norway spruce (*Picea abies* (L.) H. Karst.) forest in Bily Kriz Experimental Station in the Beskydy Mountains (Czech Republic), the influence of ozone stomatal flux on anatomical structure of needle and protective function of carotenoids were studied. The study focuses on the enlargement of intercellular space in mesophyll parenchyma after ozone exposure of trees. A relationship was found between enlargement of intercellular area in mesophyll and accumulated Phytotoxic Ozone Dose above a threshold of 1 nmol m^{-2} s^{-1} (POD_1). A ratio between intercellular and total area in mesophyll has been introduced as IM index. Protective function of carotenoids and, respectively, the dependence of the concentration of these pigments in *P. abies* needles on POD_1 index was proved.

Keywords: POD_1; mesophyll, intercellular, β-carotene, lutein

Introduction

Oxidative stress is a stress factor which slows down vital functions in plants and has destructive influence on their cells, tissues, and organs, possibly leading to death. Oxidative damage in plants is caused by imbalance between production and degradation of active forms of oxygen (Piterková et al. 2005). Therefore, oxidative stress can be described as a phenomenon regulated by the ratio between oxidative and antioxidative activity which influences physiological processes in plants (Arora et al. 2002). Each disturbance of balance among the system which produces and degrades superoxide and other reactive products has always physiological consequence.

Active forms of oxygen have a key role in hypersensitive reaction (HR) for which induction of fast cell death is typical, resulting in release of reactive oxygen species (Dangl et al. 1996). Cell death caused by HR could be considered as a programmed cell death (Jabs et al. 1996, Naton et al. 1996, Lamb and Dixon 1997). Influence of oxidants on apoptosis is described in studies using *Arabidopsis thaliana* var. Landsberg *erecta*, soya, and tobacco BY-2 cell cultures (Levine et al. 1994, Desikan et al. 1998, Solomon et al. 1999, Houot et al. 2001). In small concentrations active forms of oxygen inhibit CO_2 fixation, inactivate enzymes of the Calvin Cycle, and oxidise flavonoids. To provide protection against hydrogen peroxide, chloroplast stroma contains various antioxidants, such as L-ascorbic acid, reduced glutathione, and peroxisomes containing components of catalase cycle (Osswald and Elstner 1986). Under *in situ* conditions, synergy effects of oxidative stressors have been observed. Ozone (O_3) is likely to be one of major causes of such damage. O_3 is produced in troposphere when primary pollutants, especially nitrogen oxides, are present in sunny days. Oxidizing influence of O_3 on economically important forest species has been studied by

numerous authors in different species, e.g. *Fagus sylvatica* L. (Matyssek et al. 2007, Löw et al. 2007), *Picea abies* (L.) Karst. (Luedemann et al. 2005, Wipfler et al. 2005), *Betula pendula* Roth (Oksanen et al. 2007), *Populus alba* L. (Hoshika et al. 2017), O_3 sensitive poplar in terms of leaves injuries *Populus deltoides* × *maximowiczii*, Eridano clone and O_3 tolerant *Populus* × *euramericana*, I-214 clone (Giacomo et al. 2010).

Ozone affects inner tissues of leaves (Vollenweider et al. 2013), with cell death and enlargement of intracellular spaces. Structural changes were observed and described by many other authors, such as Sutinen et al. (1990), Holopainen et al. (1992) and Kivimäenpää et al. (2004). Visible leaf injury is better correlated with O_3 flux than O_3 concentrations in poplar (Gerosa et al. 2009). Effects of O_3 (96 µg m^{-3} during growing season) on lutein to chlorophyll *a* ratio in needles of *Picea abies* growing in natural conditions in mountains were measured by Siefermann-Harms et al. (2004), observing a significant ratio increase. On the contrary, these authors found a decrease of α to β-carotene ratio in needles damaged by O_3, while the ratio in thylakoids remained unchanged. To our knowledge in literature there is no evidence about examining O_3 cumulative dose to carotenoid content in field conditions.

Based on several samplings for carotenoids content analyses and ozone modelling approaches, the objectives of this study are to assess the influence of stomatal ozone flux on anatomical structure of Norway spruce needles, and to evaluate the effects of stomatal ozone flux on carotenoid concentration in needles. For this study, we modelled stomatal ozone flux as by Emberson et al. (2000c) during the vegetation period of 2009. Then we calculated accumulated Phytotoxic Ozone Dose above a threshold of 1 nmol m^{-2} s^{-1} (POD_1) to assess the risk to Norway spruce trees. We test hypothesis that POD_1 effects the concentration of both lutein and β-carotene pigments.

Materials and Methods

Experimental site

The forest stand is located at the experimental research site Bily Kriz (the Beskydy Mountains, 49°33'N, 18°32'E, NE of the Czech Republic, 908 m a.s.l.) and it belong to CarboEurope-IP (www.carboeurope.org) and ICOS (www.icos-ri.eu) networks. The geological bedrock is formed by Mesozoic Godula sandstone (flysch type), with ferric podzols. The climate in this area is cool (annual mean air temperature is 6.7° C) and humid (annual mean relative air humidity is 80%), with high annual precipitation pattern (the mean for 2000–2009 is 1.374 mm). The forest stand (6.2 ha) consists of *Picea abies* (L.) H. Karst. (Norway spruce) (99% of total tree number) and *Abies alba* Mill. (silver fir) (1%) planted on the slope (11–16°) in 1981 by row planting of 4-year-old Norway spruce seedlings with S-SW orientation. In 2009, the stand density was 1,428 trees ha^{-1} (leaf area index is 9.5 m^2 m^{-2}), tree height and stem diameter at 1.3 m of height were 13.4 ± 0.1 m and 15.8 ± 0.2 cm, respectively, (means ± standard deviation). Measurements of meteorological parameters were conducted on 36-m tall experimental mast placed within the area of trees with mean tree height. O_3 concentrations at 5, 15 and 25 m above ground were measured by O_3 analysers ($O_3$41M, Environment S.A., Poissy, France). Snow cover was presented only outside of the period measured, during 1.1.-9.4.; 14.-27.10.; 11.-17.11.; 7.-24.12.; since 27.12. For details regarding the description of whole experimental site see Zapletal et al. (2011, 2012).

Modelling of total and stomatal flux

Modelling of total O_3 flux to Norway spruce stand was described in detail by Zapletal et al. (2011). Stomatal uptake of O_3 (go_3), which is inverse of stomatal resistance (R_{sto}) was calculated according to Emberson et al. (2000b):

$$go_3 = g_{max} \times g_{phen} \times max[g_{min}, (g_{light} \times g_{temp} \times g_{VPD} \times g_{SWP})] \quad (1)$$

where: g_{max} is the average maximum stomatal conductance of *Picea abies* to O_3 (mmol O_3 m^{-2} s^{-1}) expressed on total needle surface area; the parameters g_{phen}, g_{light}, g_{temp}, g_{VPD}, and g_{SWP} are expressed in relative terms between 0 and 1, and represent the modification of g_{max} due to phenological changes, light (µmol m^{-2} s^{-1}), air temperature (ºC), vapour pressure deficit (kPa), and soil water potential (MPa), respectively; g_{min} is the minimum go_3 that occurs during daylight hours. The values of vapour pressure deficit (VPD) were estimated according to Buck (1981). Soil moisture deficit (SMD) was estimated as a function of precipitation and daily mean surface temperature according to the principles of water budget (Mintz and Walker 1993). The soil physical characterization necessary to translate volumetric soil moisture deficit into soil water potential was based on the function in Milthorpe and Moorby (1974). Full details on the parameters and functions used to relate go_3 to environmental variables are given in Emberson et al. (2000a, 2000b) and Wieser and Emberson (2004). Stomatal O_3 flux (F_{sto}) was then calculated as (Cieslik 2004):

$$F_{sto} = \frac{R_c}{(R_a(z) + R_b + R_c)\, R_{sto}}\, c(z) \quad (2)$$

where $c(z)$ is O_3 concentration at a height z= 2 m above the canopy; R_a is the aerodynamic resistance, for the

turbulent layer; R_b is the laminar layer resistance for the quasi-laminar layer; and R_c is the surface or canopy resistance of the receptor. R_a was calculated from micrometeorological relations as suggested by Voldner et al. (1986) and Hicks et al. (1987). R_b was calculated from micrometeorological relations as suggested by Hicks et al. (1987). R_c was calculated using the following equation (Emberson et al. 2000c):

$$R_c = \left(\frac{LAI}{R_{sto}} + \frac{SAI}{R_{ext}} + \frac{1}{R_{inc} + R_{soil}} \right)^{-1} \quad (3)$$

where: R_{sto} is the specific needle stomatal resistance to O_3 uptake; R_{ext} is the resistance of the needles cuticle, branches and stem to uptake or destruction of O_3; R_{inc} is the specific canopy aerodynamic resistance to transport of O_3 towards the soil and lower parts of the canopy; R_{soil} is the soil resistance to destruction or absorption of O_3 at the ground surface; LAI is leaf area index; SAI is surface area index, including leaves and branches, value set to be equal to LAI in the growing season, otherwise set to 1 m^{-2} m^{-2} (Emberson et al. 2000c).

Ozone dose and exposure

O_3 exposure was calculated as AOT40 for daylight hours only (Fuhrer et al. 1997):

$$AOT40 = \Sigma[([O_3]_i - 40)]\Delta t \quad (4)$$
$$Glob.Rad \geq 50\ W/m^2$$

where the summation is made from May 6th to September 30th.

The accumulated stomatal flux with no threshold (POD_0) was calculated as follows (ICP Vegetation 2009):

$$POD_0 = \sum_{i=1}^{N} max(0, F_{sto,i})\ \Delta t \quad (5)$$

where the summation is made from May 6th to 13rd November.

The accumulated stomatal flux above a hourly threshold of 1 nmol m^{-2} s^{-1} (POD_1) was calculated as follows (ICP Vegetation 2009):

$$POD_1 = \sum_{i=1}^{N} max(0, F_{sto,i} - 1)\ \Delta t \quad (6)$$

where the summation is made from May 6th to 13rd November. Moreover, all the figures with including statistics were produced in MatLab (MathWorks, USA).

Sampling of needles

Two-year old sunny needles were taken from one sun-exposed part of the crown. The samples were collected from three levels above ground (8.6 – 12.7 m, 6.7 – 8.6 m, 4.5 - 6.7 m) with length of branches 1.5 – 2.5 m. The needles were collected four times in growing season 2009 (8th June, 19th July, 2nd November and 13rd November). At each sampling, 75 grams of needles were removed from 15 branches. Needles were collected between 10:00 – 12:00 of local time (GMT + 2 h) from selected trees of *Picea abies*. The specimen was selected as a representative sample within a homogenous forest stand. Needles with relevant damage caused by insects and endophytic fungi were excluded.

Analysis of anatomical parameters of needles

Immediately after sampling, the needles were put in a FAA fixation solution (1.82 M formaldehyde – 9.89 M ethanol – 0.87 M acetic acid) (Gebauer et al. 2011). Randomly selected needles were prepared for microscopic analysis: dehydration in ethanol series, transfer to xylene and paraffin embedding in a Shandon Citadel TM tissue processor – carousel type. The studied material was embedded in BIO-PLAST EXTRA at a temperature of 58 °C. Paraffin blocks were longitudinal-cut in three to four series in a HM 325 MICROM GmbH rotary microtome, the section thickness was 5-6 µm. After deparaffinization the samples were stained by the method of hematoxylin – eosin, polychrome methylene blue, Grocott's methenamine-silver nitrate stain and Gömöri stain. The stained preparations were mounted into enthellan with xylene. The analysis of the image was performed by ZEISS Axiostar Plus microscope connected to Olympus C5060 camera and multimedia PC. Photos of longitudinal sections of needles were taken with magnification 4× and 10×. The freeware software CERNOTA (Kalina and Slovák 2004) was used to analyse the images. The software counts the number of black pixels and the computed value is substituted into the calibration equation, which provides the area of an object coloured in black. Over 250 samples were analysed. We counted area of intercellular space, area of mesophyll and area of cells. Cell shrinking was analysed on a randomly selected area (1000 µm long). Tissue deformation was quantitatively defined by the ratio between the area of intercellular space and the total area of mesophyll (Bäck et al. 1999, IM index)

$$IM\ index = \frac{area\ of\ intercellular\ space}{area\ of\ intercellular\ space + area\ of\ cells} \quad (7)$$

Analysis of carotenoid concentration in needles

Samples for the analysis of photosynthetic pigments were fixed in liquid nitrogen and stored at -80 °C after the collection. One gram of the sample was homogenized in 100% acetone with $MgCO_3$ and sand. Samples were centrifuged at 6000 g min^{-1} and filtered. Carotenoids concentrations were determined in acetone extracts using HPLC gradient method (Pfeifhofer 1989).

Results

Environmental conditions during the sampling period

During the vegetation period from May to November 2009 the mean (±SD) air temperature was 11.7 ± 5.9 °C, relative air humidity was 84.5 ± 15.5%, global radiation was 202.9 ± 256.9 W m^{-2}, horizontal wind speed was 1.5 ± 1.3 m s^{-1} and O_3 concentration was 44.0 ± 11.6 nmol mol^{-1} at 15 m above ground. Precipitation sum was 704.4 mm. Minimal and maximal values were as follows: air temperature: -3.4 °C and 28.6 °C, and relative air humidity: 27.3% and 100%.

Indices of ozone exposure and uptake

Until the end of September, the ecosystem O_3 exposure, expressed in terms of AOT40 for daylight hours, of 11.6 ppm h (mmol mol^{-1}) was recorded. At the end of sampling period, POD_0 was 18 mmol m^{-2} PLA (per leaf area) and POD_1 was 15.6 mmol m^{-2} PLA (Figure 1).

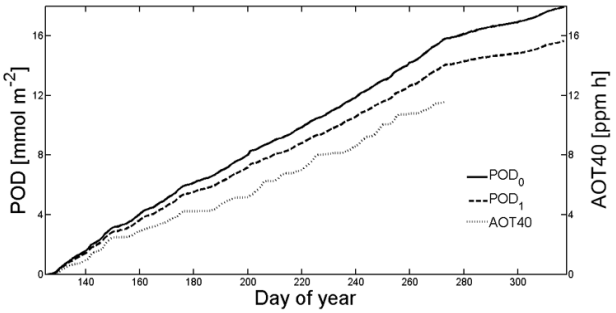

Figure 1. History of ozone exposure (AOT40) for the period May - September and history of Phytotoxic Ozone Dose (POD1 and POD0) for the period May-November 2009. Both POD_1 and POD_0 time periods were extended until 13rd November to cover all corresponding datasets

Relationship between stomatal ozone flux and IM index

Depending on growth rate of POD_1 (Figure 2), microscopic observations revealed a significant (p =2.32e-06) decrease in both the area and volume of mesophyll cells, manifested by protoplast deformation and resulting in cell wall shrinking (Figure 3). Average values of IM index varied between 0.41 (healthy looking tissue) and 0.49 (damaged tissue).

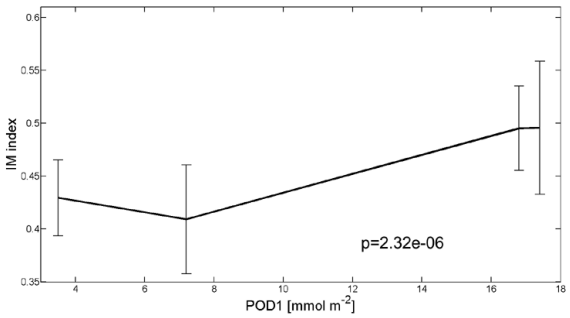

Figure 2. A relationship between POD_1 and the ratio of the area of intercellular space expressed as IM index. Vertical bars illustrate standard deviations

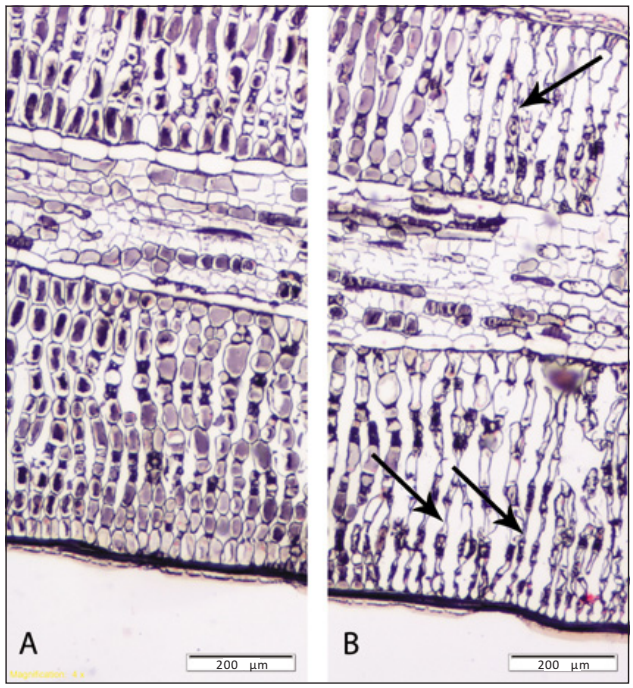

Figure 3. Anatomical longitudinal section of needle after oxidative stress (A – healthy cells, B – damaged cells). Damage of mesophyll cells is observed as zones of narrowing cell structures with examples shown with arrows. Stained by Grocott's methenamine-silver nitrate

Relationship between POD_1 and carotenoids

Concentrations of photosynthetic pigments with protective antioxidant function (β-carotene and lutein) were determined. Concentrations of carotenoids are primarily dependent on the date of sampling, which corresponds to increasing POD_1 index and higher O_3 exposure. Concentrations of β-carotene inside the needle tissue (Figure 4) is ranging from 0.078 to 0.092 mg g^{-1} DW, and concentrations of lutein (Figure 5) ranging from 0.278 to 0.392 mg g^{-1} DW., correlated with POD_1 ranging from 3.5 to 17.4 mmol m^{-2}. Concentration of lutein and β-carotene are significantly correlated with POD_1 (R^2 =0.88, p =6.61e-10; R^2 =0.86, p =2.32e-6) and with IM index (Figure 6).

Discussion and conclusions

Over just six months, the O_3 exposure (AOT40) exceeded by 131% the critical level of 5 ppm h suggested for forest protection (CLRTAP 2004), revealing a poten-

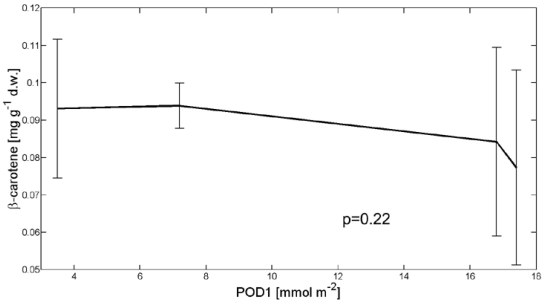

Figure 4. A relationship between concentration of β-carotene and POD_1. Vertical bars illustrate standard deviations

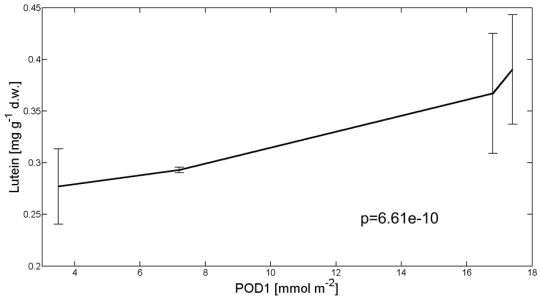

Figure 5. A relationship between concentration of lutein and POD_1. Vertical bars illustrate standard deviations

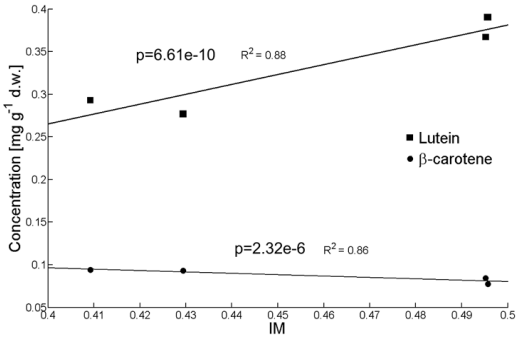

Figure 6. Relationship of IM and carotenoid concentration. Linear fitting is applied

tially O_3 hazard condition (Zapletal et al. 2012). The dose of O_3 in terms of POD_1 (15.6 mmol m^{-2} PLA) absorbed by the vegetation during the measuring period was above the critical flux level of 8 mmol m^{-2} (ICP Vegetation 2010, Zapletal et al. 2012). The POD_1 recorded at Bílý Kříž is similar to POD_1 (13.6-16.2 mmol m^{-2}) of *P. abies* forest located at the Tatra Mountains in the Western Carpathian Mountains (Bičárová et al. 2016). Moreover, Czech mountains regions are known to be polluted by O_3 as high as southern European regions or regions of high altitude (Hůnová et al. 2016), which correspond to $POD_{1.6}$ ranging between 13.4 and 22.3 mmol m^{-2} in *F. sylvatica* forest (Vlasáková-Matoušková and Hůnová 2015). According to Gerosa et al. (2009), critical cumulated flux levels related to visible leaf injury for *P. nigra* and *F. sylvatica* is ranging between 30 and 33 mmol m^{-2}. Therefore, it is clear that *P. abies* is more sensitive to smaller O_3 exposure than above mentioned tree species

Demonstration of leaf injury is related to relationship between enlargement of intercellular area in mesophyll and POD_1 index, which we found. Similarly, microscopic changes in the structure of needles induced by O_3 were observed by Sutinen et al. (1990) and Holopainen et al. (1992). Kivimäenpää et al. (2004) consider the specific symptoms of the O_3 damage in conifers such as decreased size of chloroplast. Furthermore, Vollenweider et al. (2013) observed massive cell wall thickening, which obstructed most of the intracellular space in mottles on needles, however intracellular space was not quantified.

We found significant (p =6.61e-10) positive increase of lutein concentration together with increasing O_3 dose and insignificant (p =0.2) negative for concentration of β-carotene. Relationship between concentration of carotenoids and concentration of O_3 has been studied on various plant species by many authors (Yamaji et al. 2003, Tausz et al. 2004, Calfapietra et al. 2008) with different conclusions. Haberer et al. (2007) and Herbinger et al. (2007) found similar changes of concentration of lutein on *F. sylvatica* after O_3 exposure. Increase of both lutein and β-carotene content were observed in tomato after one day of exposure to O_3, however the effect did not persist for other five days (Tzortzakis et al. 2007). Other experiments with *P. abies* seedlings showed unchanged β-carotene content at high O_3 concentration. Contrary to that, lutein content increased rapidly after 106 days of O_3 fumigation by 100 nmol mol^{-1} (Kronfuß et al. 1998), which we observed in similar manner. This suggests different behaviour of different carotenoids in response to high O_3 concentration treatment in controlled laboratory conditions. Here we specify the changes of lutein and β-carotene contents for POD_1 under field conditions; preventing so maximized stomatal O_3 uptake in chamber studies using high O_3 concentrations not relevant for field conditions (Massman et al. 2000) and thus exacerbating injury in plants.

We conclude that our hypothesis was confirmed: β-carotene and lutein exhibit concentration changes in needles depending on increasing ozone uptake expressed as POD_1 index. While lutein is increasing with higher ozone dose, β-carotene concentration is being reduced. That is due to influence of oxidative stress which might lead to transformation of carotenes to xanthophyll (Quinlan et al. 2012). Moreover, macroscopic observations revealed yellowing of the needles after ozone exposure, which is in consistency with increased lutein content while β-carotene is being used for antioxidant activities. Similar findings were published by Kim and DellaPenna (2006).

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References

Arora, A., Sairam, R. K. and Srivastava, G. C. 2002. Oxidative stress and antioxidative systems in plants. *Current Science* 82: 1227-1238.

Bäck, J., Vanderklein, D.W. and Topa, M.A. 1999. Effects of elevated ozone on CO_2 uptake and leaf structure in sugar maple under two light environments. *Plant, Cell and Environment* 22: 137-147.

Bičárová, S., Sitková, Z. and Pavlendová, H. 2016. Ozone phytotoxicity in the Western Carpathian Mountains in Slovakia. *Forestry Journal* 62: 77-88.

Buck, A.L. 1981. New equations for computing vapour pressure. *Journal of Applied Meteorology* 20: 1527-1532.

Calfapietra, C., Scarascia Mugnozza, G., Karnosky, D. F., Loreto, F. and Sharkey, T. D. 2008. Isoprene emission rates under elevated CO_2 and O_3 in two field-grown aspen clones differing in their sensitivity to O_3. *New Phytologist* 179: 55-61.

Cieslik, S. 2004. Ozone uptake by various surface types: a comparison between dose and exposure. *Atmospheric Environment* 38: 2409-2420.

CLRTAP, 2004. Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends. Umwelbundesamt, Berlin. 266 pp. Available online at: https://www.rivm.nl/media/documenten/cce/manual/Manual_UBA_Texte.pdf.

Dangl, J. L., Dietrich, R. A. and Richberg, M. H. 1996. Death don't have no mercy: cell death programs in plant-microbe interaction. *The Plant Cell* 8: 1793-1807.

Desikan, R., Reynolds, A., Hancock, J. J. and Neill, S. J. 1998. Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in Arabidopsis suspension cultures. *The Biochemical Journal* 330: 115-120.

Emberson, L.D., Wieser, G. and Ashmore, M.R. 2000a. Modelling of stomatal conductance and ozone flux of Norway spruce: comparison with field data. *Environmental Pollution* 109: 393-402.

Emberson, L.D., Ashmore, M.R., Cambridge, H., Simpson, D. and Tuovinen, J.P. 2000b. Modelling stomatal ozone flux across Europe. *Environmental Pollution* 109: 403-414.

Emberson, L.D., Simpson, D., Tuovinen, J.-P., Ashmore, M.R. and Cambridge, H.M. 2000c. Towards a Model of Ozone Deposition and Stomatal Uptake over Europe. EMEP MSC-W Note 6/2000, 57 pp.

Fuhrer, J., Skarby, L. and Ashmore, M.R. 1997. Critical levels for ozone effects on vegetation in Europe. *Environmental Pollution* 97: 91–106.

Gebauer, R., Volařík, D., Urban, J., Borja, I., Nagy, N. E., Eldhuset, T. D. and Krokene, P. 2011. Effect of thinning on anatomical adaptations of Norway spruce needles. *Tree Physiology* 31: 1103-1113.

Gerosa, G., Marzuoli, R., Desotgiu, R., Bussotti, F. and Ballarin-Denti, A. 2009. Validation of the stomatal flux approach for the assessment of ozone visible injury in young forest trees. Results from the TOP (transboundary ozone pollution) experiment at Curno, Italy. *Environmental Pollution* 157: 1497-1505.

Giacomo, B., Forino, L.M.C., Tagliasacchi, A.M., Bernardi, R. and Durante, M. 2010. Ozone damage and tolerance in leaves of two poplar genotypes. *Caryologia* 63 (4): 422-434.

Haberer, K., Herbinger, K., Alexou, M., Tausz, M. and Rennenberg, H. 2007. Antioxidative Defence of Old Growth Beech (*Fagus sylvatica*) under Double Ambient O_3 Concentrations in a Free-Air Exposure System. *Plant Biology* 9: 215-226.

Herbinger, K., Then, C., Haberer, K., Alexou, M., Löw, M., Remele, K., Rennenberg, H., Matyssek, R., Grill, D., Wieser, G. and Tausz, M. 2007. Gas Exchange and Antioxidative Compounds in Young Beech Trees under Free-Air Ozone Exposure and Comparisons to Adult Trees. Plant Biology 9: 288-297

Hicks, B.B., Baldocchi, D.D., Meyers, T.P., Hosker Jr. R.P. and Mat, D.R. 1987. A preliminary multiple resistance routine for deriving dry deposition velocities from measured quantities. *Water, Air, and Soil Pollution* 36: 311-330.

Holopainen, T., Anttonen, S., Wulff, A., Palomäki, V., Kärenlampi, 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 and Environment* 42: 365–398.

Hoshika, Y., Carrari, E., Zhang, L., Carriero, G., Pignatelli, S., Fasano, G., Materassi, A. and Paoletti, E. 2017. Testing a ratio of photosynthesis to O3 uptake as an index for assessing O3-induced foliar visible injury in poplar trees. *Environmental Science and Pollution Research* 1-12.

Houot, V., Etienne, P., Petitot, A. S., Barbier, S., Blein, J. P. and Suty, L. 2001. Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner. *Journal of Experimental Botany* 52: 1721-1730.

Hůnová, I., Stoklasová, P., Schovánková, J. and Kulasová, A. 2016. Spatial and temporal trends of ozone distribution in the Jizerské hory Mountains of the Czech Republic. *Environmental Science and Pollution Research* 23: 377-387.

ICP Vegetation 2009. Flux-based assessment of ozone effects for air pollution policy. ICP Vegetation Expert Panel Meeting, 9 – 12 November, 2009, JRC-Ispra, Italy.

ICP Vegetation 2010. Minutes of the 23rd Task Force Meeting. ICP Vegetation 23rd Task Force Meeting, 1–3 February, 2010, Tervuren, Belgium.

Jabs, T., Dietrich, R. and Dangl, J. L. 1996. Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide. *Science* 273: 1853-1856.

Kalina, J. and Slovák, V. 2004. The inexpensive tool for the determination of projected leaf area. *Ekológia (Bratislava)* 23: 163–167.

Kim, J. and Della Penna, D. 2006. Defining the primary route for lutein synthesis in plants: The role of Arabidopsis carotenoid β-ring hydroxylase CYP97A3. *Proceedings of the National Academy of Sciences of the United States of America* 103: 3474-3479.

Kivimäenpää, M., Jönsson, A. M., Stjernquist, I., Selldén, G. and Sutinen, S. 2004. The use of light and

electron microscopy to assess the impact of ozone on Norway spruce needles. *Environmental Pollution* 127: 441-453.

Kronfuß, G., Polle, A., Tausz, M., Havranek, M. M. and Wieser, G. 1998. Effects of ozone and mild drought stress on gas exchange, antioxidants and chloroplast pigments in current-year needles of young Norway spruce [*Picea abies* (L.) Karst.]. *Trees - Structure and Function* 12: 482-489.

Lamb, C. and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 251-275.

Levine, A., Tenhaken, R., Dixon, R. A. and Lamb, C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583-593.

Löw, M., Häberle, K. H., Warren, C. R. and Matyssek, R. 2007. O_3 flux-related responsiveness of photosynthesis, respiration, and stomatal conductance of adult *Fagus sylvatica* to experimentally enhanced free-air O_3 exposure. *Plant Biology* 9: 197-206.

Luedemann, G., Matyssek, R, Fleischmann, F. and Grams, T. E. 2005. Acclimation to ozone affects host/pathogen interaction and competitiveness for nitrogen in juvenile *Fagus sylvatica* and *Picea abies* trees infected with *Phytophthora citricola*. *Plant Biology* 7: 640-649.

Massman, W. J., Musselman, R. C. and Lefohn, A. S. 2000. A conceptual ozone dose-response model to develop a standard to protect vegetation. *Atmospheric Environment* 34: 745-759.

Matyssek, R., Bahnweg, G., Ceulemans, R., Fabian, P., Grill, D., Hanke, D. E., Kraigher, H., Osswald, W., Rennenberg, H., Sandermann, H., Tausz, M. and Wieser, G. 2007. Synopsis of the CASIROZ case study: carbon sink strength of *Fagus sylvatica* L. in a changing environment-experimental risk assessment of mitigation by chronic ozone impact. *Plant Biology* 9: 163-180.

Milthorpe, F.L. and Moorby, J. 1974. An Introduction to Crop Physiology. Cambridge University Press, London and New York. 260 pp.

Mintz, Y. and Walker, G.K. 1993. Global field of soil moisture and land surface evapotranspiration derived from observed precipitation and surface air temperature. *Journal of Applied Meteorology* 32: 1305-1334.

Naton, B., Hahlbrock, K. and Schmelzer, E. 1996. Correlation of rapid cell death with the metabolic changes in fungus-infected, cultured parsley cells. *Plant Physiology* 112: 433-444.

Oksanen, E., Kontunen-Soppela, S., Riikonen, J., Peltonen, P., Uddling, J. and Vapaavuori, E. 2007. Northern environment predisposes birches to ozone damage. *Plant Biology* 9: 191-196.

Osswald, W. F. and Elstner, E. F. 1986. Mechanismen der pathologischen Pigmentbleichung bei Pflanzen. *Berichte der Deutschen Botanischen Gesellschaft* 99: 341-365.

Pfeifhofer, H.W. 1989. Evidence of chlorophyll b and lack of lutein in *Neottia nidusavis* plastids. *Biochemie und Physiologie der Pflanzen* 184: 55–61.

Piterková, J., Tománková, K., Luhová, L., Petřivalský, M. and Peč, P. 2005. Oxidativní stres: lokalizace tvorby aktivních forem kyslíku a jejich degradace v rostlinném organismu [Oxidative stress: localisation of reactive oxygen species formation and degradation in plant tissue]. *Chemické listy* 99: 455-466 (in Czech with English abstract).

Quinlan, R.F., Shumskaya, M., Bradbury, L.M.T., Beltrán, J., Ma, C., Kennelly, E.J. and Wurtzel, E.T. 2012. Synergistic interactions between carotene ring hydroxylases drive lutein formation in plant carotenoid biosynthesis. *Plant Physiology* 160: 204-214.

Siefermann-Harms, D., Boxler-Baldoma, C., Wilpert, K. and Heumann, H.G. 2004. The rapid yellowing of spruce at a mountain site in the Central Black Forest (Germany). Combined effects of Mg deficiency and ozone on biochemical, physiological and structural properties of the chloroplasts. *Journal of Plant Physiology* 161: 423-437.

Solomon, M., Belenghi, B., Delledone, M., Menachem, E. and Levine, A. 1999. The involvement of cysteinproteases and protease inhibitor genes in the regulation of programmed cell death in plants. *The Plant Cell* 11: 431-443.

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

Tausz, M., Olszyk, D. M., Monschein, S. and Tingey, D. T. 2004. Combined effects of CO_2 and O_3 on antioxidative and photoprotective defence systems in needles of ponderosa pine. *Biologia plantarum* 48: 543-548.

Tzortzakis, N., Borland, A., Singleton, I. and Barnes, J. 2007. Impact of atmospheric ozone-enrichment on quality-related attributes of tomato fruit. *Postharvest Biology and Technology* 45: 317-325.

Vlasáková-Matoušková, L. and Hůnová, I. 2015. Stomatal ozone flux and visible leaf injury in native juvenile trees of *Fagus sylvatica* L.: a field study from the Jizerske hory Mts., the Czech Republic. *Environmental Science and Pollution Research* 22: 10034-10046.

Voldner, E.C., Barrie, L.A. and Sirois, A. 1986. A literature review of dry deposition of oxides of sulphur and nitrogen with emphasis on long-range transport modelling in North America. *Atmospheric Environment* 20: 2101-2123.

Vollenweider, P., Fenn, M. E., Menard, T., Günthardt-Goerg, M. and Bytnerowicz, A. 2013. Structural injury underlying mottling in ponderosa pine needles exposed to ambient ozone concentrations in the San Bernardino Mountains near Los Angeles, California. *Trees* 27: 895–911.

Wieser, G. and Emberson, L.D. 2004. Evaluation of the stomatal conductance formulation in the EMEP ozone deposition model for *Picea abies*. *Atmospheric Environment* 38: 2339-2348.

Wipfler, P., Seifert, T., Heerdt, C., Werner, H. and Pretzsch, H. 2005. Growth of adult Norway spruce (*Picea abies* [L.] Karst.) and European beech (*Fagus sylvatica* L.) under free-air ozone fumigation. *Plant Biology* 7: 611-618.

Yamaji, K., Julkunen-Tiitto, R., Rousi, M., Freiwald, V. and Oksanen, E. 2003. Ozone exposure over two growing seasons alters root-to-shoot ratio and chemical composition of birch (Betula pendula Roth). *Global Change Biology* 9: 1363-1377.

Zapletal, M., Cudlín, P., Chroust, P., Urban, O., Pokorný, R., Edwards-Jonášová, M., Czerný, R., Janouš, D., Taufarová, T., Večeřa, Z., Mikuška, P. and Paoletti, E. 2011. Ozone flux over a Norway spruce forest and correlation with Net Ecosystem Production. *Environmental Pollution* 159 (2011): 1024-1034.

Zapletal, M., Pretel, J., Chroust, P., Cudlín, P., Edwards-Jonášová, M., Urban, O., Pokorný, R., Czerný, R. and Hůnová, I. 2012. The influence of climate change on stomatal ozone flux to a mountain Norway spruce forest. *Environmental Pollution* 169 (2012): 267-273.