



Study of the histological and physiological effects of ozone on
bioindicator plant species

The main points of the thesis

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Gödöllő

2015

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1 AIMS AND SCOPE

Tropospheric ozone jeopardizes natural as well as cultivated plant communities. It directly and indirectly enhances greenhouse effect: O₃ behaves as a greenhouse gas and contributes to the elevation of tropospheric CO₂ levels by decreasing dry matter production of plant communities. As a highly effective oxidant, it has an adverse effect on living organisms and causes considerable economic losses for the affected countries by reducing yield of cultivated crops. The quantity of tropospheric ozone has continuously been increasing in the last decades. Background values doubled since the beginning of industrialization. However, the frequently occurring ozone episodes – when ozone concentration of an area remains above 50-60 ppb for several days – are the most hazardous for plant organisms. Consequently, indication and quantification of phytotoxic ozone and the determination of its histological and physiological effects on plants is very important in economical as well as in environmental regards.

Our aim with the present study was to find the answer to the following questions:

1. Is there any kind of histological difference between O₃ sensitive and resistant genotypes which is in connection with their different ozone sensitivity or physiological traits?
2. What stands in the background of weaker stress resistance and lower effectiveness of O₃ sensitive genotypes?
3. How does elevated CO₂ influence the ozone response of sensitive and resistant genotypes?
4. Do the photosynthetic efficiencies of S156 (sensitive) and R123 (resistant) genotypes of *Phaseolus vulgaris* differ? If they do, what are the reasons why? Is this difference a cause or a consequence of ozone sensitivity?
5. Which are the most useful parameters for ozone bioindication that can be measured on bioindicator species?

2 MATERIALS AND METHODS

2.1 *Experimental sites*

Most of the measurements were made in the Botanical Garden of Szent István University in Gödöllő. Measurements made on the experimental plants of a European Biomonitoring Programme were performed in the experimental area of Agronomic Department of University of Ljubljana. According to the protocol of the ICP-Vegetation, experimental plants were grown under shading net, where the measurements were performed as well.

2.2 *Plant material*

Studies were made on R123 (resistant) and S156 (sensitive) genotypes of *Phaseolus vulgaris* L.; and on NC-R (resistant) and NC-S (sensitive) genotypes of *Trifolium repens* L..

2.3 *Meteorological data*

Data were gathered from the meteorological station of Institute of Botany and Ecophysiology of Szent István University placed in the Botanical Garden of the University in Gödöllő, and from the meteorological station of the Agronomic Department of University of Ljubljana. Ozone concentrations were defined as hourly average values (ppb), cumulative ozone concentrations were defined as the sum of hourly average values for certain time periods expressed in total cumulative ozone and/or cumulative AOT40 (accumulated amount of ozone over the threshold value of 40 ppb) values (ppbh, ppmh). For the evaluation of our results, we used the hourly average values of temperature, vapour pressure deficit (VPD) and photosynthetic photon flux density (PPFD).

2.4 *Histological examinations*

Histological examinations were made by the comparison of the samples derived from the different genotypes of both bioindicator species. Samplings were made in two seasons: once in winter, from mature leaves, and once in summer, from young, mature and old leaves. In winter, plants were grown in greenhouse. The plants were continuously well watered in both seasons. Parameters examined on epidermal imprints and cross sections were cell sizes, thickness of different tissues, and the ratio (f_{ias}) and gas conductance (g_{ias}) of intercellular spaces.

2.5 OTC experiment

Several accessions of both bioindicator species were examined during two subsequent vegetation periods. Plants were grown under three different treatments: in OTCs (Open Top Chamber) with elevated (700 ppm) CO₂ level, in OTCs with ambient air circulation, and in field plots. Parameters measured on bush bean (*Phaseolus vulgaris*) were number and S/R ratio (the ratio of the value measured on sensitive and that measured on resistant plants) of leaves and pods, dry weight and S/R ratio of dry weight of pods. On white clover (*Trifolium repens*) plants we determined number and S/R ratio of number of flowers, dry weight and S/R ratio of dry weight of aboveground biomass. Parameters determined on both bioindicator species were stomatal conductance (g_s), Fv/Fm (maximum photochemical efficiency of PSII) and RFd (potential photosynthetic quantum conversion) values and their S/R ratio and the extent of visible injuries on the plant canopies. The connections between the measured parameters and cumulative ozone values or the number of days between measurements; and the connection between certain parameters were tested by correlation analysis.

2.6 Case study on the experimental plants of a European biomonitoring program placed in Ljubljana

Measurements were made on S156 and R123 genotypes of *P. vulgaris*. Daily courses of fluorescence induction parameters (Fv/Fm, RFd, NPQ (non-photochemical quenching), ΦPSII (effective quantum yield of PSII) and qFo (non-photochemical quenching of Fo)) were measured on three warm summer days, while daily courses of transpiration and stomatal conductance were measured on two of the three days.

3 RESULTS AND DISCUSSION

3.1 Histological comparison of ozone sensitive and resistant plant genotypes

3.1.1 Comparison of the two genotypes of *Phaseolus vulgaris*

Epidermal cells of S156 genotypes were smaller than that of R123 genotype among winter, and the firstly developed (old) summer leaves. However, epidermal cells on mature summer leaves of the sensitive genotype were larger than that of the resistant genotype. This means that the genetically coded cell size was smaller in S156 than in R123. This difference was typical when the plants grew under low ozone concentrations, as it was peculiar to the winter season, and to the firstly developed (old) summer leaves. Among leaves developed after exposure to higher cumulative ozone dose (mature leaves at the time of sampling) and the

consequent phenotypical changes, S123 genotype had larger epidermal cells as compared to R123 genotype.

Size of guard cells and size and openness of stomata on mature leaves were typically higher in S156 plants, while those of old leaves were typically higher in R123 plants which imply to that environmental stress and climatic conditions adversely affected the different genotypes. This was supported by the fact that latter developed sensitive leaves had larger, while latter developed resistant leaves had smaller stomata as compared to earlier developed leaves. Based on these observations, natural metabolic pathways of stress response were impeded in S156 genotype, as in spite of exposure to environmental stress, their expansive cell growth was intense, which is peculiar to plants growing under favourable environmental conditions. Larger epidermal cells resulted in less stoma per unit leaf area.

Although g_s of the two genotypes did not significantly differ, higher C_i in S156 in winter could be due to their somewhat higher g_s , since no difference between the photosynthetic rates of the genotypes was measurable. Similarly, in spite of significantly lower CO_2 assimilation rate of S156 plants in summer, C_i of the two genotypes did not differ, which was caused by the somewhat higher g_s values of S156 genotype. Higher g_s of the sensitive genotype was attributable to the higher number of stomata, the more open stomata and the larger total area of stomatal apertures in winter, while it was attributable to the larger stomata and stomatal apertures in summer. According to the measurements made in summer, young and old sensitive leaves had more open stomata as compared to the resistant leaves.

The differences measured on the cross sections of winter samples of the different genotypes did not appear on the summer samples. In winter, sensitive leaves developed thicker layers of parenchyma tissues, among summer mature leaves however, resistant ones had thicker mesophyll layer. During the development of summer leaves of resistant plants, thickness and ratio of the different parenchyma layers and the surface of mesophyll cells showed remarkable changes. The latter developed leaves (mature leaves) grew thicker palisade layer as compared to the leaves developed at an earlier ontogenetic stage (old leaves). This could be the sign of growing photosynthetic capacity of resistant plants in summer. In winter however, S156 genotype had significantly thicker palisade layer as compared to R123 genotype. Thinner palisade layer of sensitive genotype could play a role in ozone sensitivity. However, this difference appeared only in summer samples, which means that a phenotypical change in histological traits of the two genotypes occurred first under the meteorological conditions of the summer season. We explained the different phenotypical changes of the two genotypes in summer by the defective light adaptation of S156 genotype. Old resistant plants had higher f_{ias}

and g_{ias} values. This could be a naturally occurring histological change during ontogenesis, which was not observable in sensitive plants.

A lot of parameters describing cell sizes or tissue thicknesses differed in winter between the two genotypes. This supported the existence of genetically coded differences in several histological traits. However, these differences were contrasting in summer as compared to the winter samples. We assumed that these phenotypical changes in summer were due to the effect of altered and stressing environmental conditions. In winter, a lot of parameter showed significantly smaller values in resistant comparing to the sensitive plants. Smaller values of histological parameters in sensitive leaves in summer could be due to cell decay and consequent shrinking of tissues. At the same time, because of enhanced oxidative stress and enhanced need of energy for defensive and repair processes, sensitive plants had less energy for increasing cell and tissue sizes.

3.1.2 Comparison of the two genotypes of *Trifolium repens*

The NC-R genotype had generally more stomata and epidermal cells. However, size of stomata and guard cells were higher in NC-R genotype in winter, while in summer, NC-S genotype had larger stomata and guard cells comparing to NC-R genotype. This phenomenon implied to the dissimilar effects of ozone on the proliferation and growth of cells of the different genotypes. Only resistant plants had smaller stomata and stomatal aperture – on the lower epidermis – in summer than in winter. The development of smaller stomata on newly developed leaves is a possible defence reaction against air pollution. In our experiment, this phenomenon was peculiar to the resistant plants, thus different stomatal size could be a determinant factor of different ozone sensitivity of the two genotypes. Latter developed (mature) resistant leaves had thicker epidermis comparing to the earlier developed (old) leaves, which could also be a defence reaction as part of the air pollutants invade in the leaves through the epidermis. In summer, higher stomatal conductance of NC-S genotype was due to the larger stomata as compared to the NC-R genotype, while in winter, NC-R genotype had larger stomata as compared to NC-S. Higher stomatal size was due to larger guard cells resulting in longer stomatal apertures and going together with larger epidermal cells and lower densities of epidermal cells and stomata.

Gas conductance of intercellular spaces (g_{ias}) in old leaves of NC-S genotype was higher than that of the old NC-R leaves. This could also be in connection with the ozone sensitivity of the NC-S genotype. Ratio of intercellular air spaces (f_{ias}) of old sensitive leaves were higher than that of mature sensitive leaves, while f_{ias} of resistant leaves remained unchanged. This

implied to that mesophyll tissues of sensitive leaves were attacked by ozone while increasing the ratio of intercellular spaces inside the leaf. In sensitive plants, g_{ias} of old (and injured) leaves was higher than that of the young leaves.

Among young leaves in summer, thickness of sensitive and resistant leaves was similar. Among mature leaves, resistant leaves were thicker, although still not significantly. Among old leaves however, resistant ones were significantly thicker as compared to the sensitive leaves. Old (and injured) sensitive leaves had significantly thinner spongy parenchyma layer and smaller volume of mesophyll cells comparing to the mature, still uninjured sensitive leaves. Thus, the cells in old sensitive white clover leaves "shrunk", especially in the spongy parenchyma, and the leaf tissues attenuated (unlike in resistant leaves). These phenomena can be explained by the early senescence of NC-S leaves.

3.1.3 Comparison of histological traits of the two bioindicator species

Principal component analysis performed on the data measured on cross sections gave similar results in the two studied species. In both species, total variance was explained mostly by the thickness and cell sizes of mesophyll, especially spongy mesophyll tissue, the ratio of the two mesophyll layers, and the size, ratio and gas conductance of the intercellular spaces.

Several parameters showed significant difference between different genotypes in winter, when the plants were not exposed to stressing environmental conditions. This means that there are several genetically coded differences between the two genotypes of both species. Sensitive genotypes generally showed higher values of the parameters measured on cross sections in winter as compared to resistant genotypes. The differences measurable in winter were shaded or reversed in summer, when ozone load, excessive light or heat stress could affect the development of plants. In summer, sensitive leaves had generally higher values of parameters related to stomatal size. Thus, seasonally varying environmental factors had substantially dissimilar effect on the phenotypic appearance of the different genotypes of both species. Reactions of each genotype to changes in environmental factors showed many similarities in the two studied species. It was typical of the sensitive genotypes of both species that they could not properly exploit the benefits of favourable light conditions in summer. Sensitive leaves did not show the thickening that is peculiar to light adapted leaves under summer climatic conditions. This implied to the disorder of the perception of and response to environmental factors in sensitive plants. Delayed or missing stress response could be revealed by comparing the alterations in stomatal and epidermal cell sizes during ontogenesis of the different

genotypes of both species, and by comparing the alterations in epidermal thickness during ontogenesis of NC-S and NC-R genotypes of *T. repens*.

S 156 genotype of *P. vulgaris* and NC-S genotype of *T. repens* were produced by artificial selection to showing ozone symptoms. This artificial selection aims the selection of most O₃ sensitive, consequently the weakest individuals from a population. During this process, the genetic variability decreased because of inbreeding depression. Arising from homozygosity, sensitive genotypes might have lower phenotypic plasticity, which means that their adaptability to environmental effects and oxidative stress is weaker than that of their resistant genotype-pairs. These causes might stand in the background of maladaptation of sensitive genotypes.

Numerous additional histological, biochemical and genetic examinations should be accomplished for deciding which of the above mentioned factors is the mostly determinant in the different behaviour of sensitive and resistant genotypes. These interdependent phenomena most likely act in concert to give the observed characteristic histological differences between the different genotypes.

3.2 Evaluation of the results of the OTC experiment

3.2.1 Visible injuries

Although elevated CO₂ level inhibited the development of visible injuries, visible injuries on the leaves of sensitive bush bean showed the strongest correlation with ozone load under elevated CO₂. In control OTC plots, other factors were more determinant in the development and the final ratio of visible injuries in the plant canopy. This was probably due to the elevated temperature and dry microclimate of the control OTC, without the effect of elevated CO₂ level. Even the decrement in the percent of visible injuries was observable during the most intensive growth period in control OTC.

Surprisingly, no connection could be found between tropospheric ozone load and visible injuries developing on white clover leaves. This was partly due to the continual and fast growth of the canopy during the vegetation period. As a consequence, the detectability as well as the ratio of visible injuries inside the total canopy decreased. Another explanation for the lack of the connection between the extent of injuries and ozone load is the acclimation of the mother plant to the increasing ozone stress. This theory is supported by the fact that the number of the studied accession negatively correlated with the extent of visible injuries. Consequently, every time after harvesting, the newly developed canopy showed less injury comparing to the formerly developed canopy. This meant that an acclimation process of the mother plant took

place. Correlation coefficient of this connection was -0,908 under elevated CO₂. This means that elevated CO₂ level can contribute to the decrement of ozone sensitivity of a plant species in the long term. Considering this acclimation, it seems reasonable to use seedling plants as bioindicators whose life cycle ends with harvesting.

3.2.2 Vegetative and generative growth

Temperature elevation effect of OTCs and the resulting longer plant life cycle were apparent from the changes of dry weight of pods of bush bean under the different treatments. Growing conditions and day length were generally rather determinant on vegetative and generative growth of bush bean as compared to ozone concentrations. However, differences between genotypes were affected by cumulative ozone pollution. Under elevated CO₂, differences between genotypes grew larger as a function of ozone load.

According to the results of the correlation analysis, correlations of ozone load with the extent of visible injuries (positive) and S/R ratio of number of pods (negative) of bush bean, and with S/R ratio of number of flowers of white clover (negative) were strongest under elevated CO₂ level. Sensitive plants could not as effectively exploit the available energy sources as resistant plants could. Growth rate of leaf number of sensitive plants already started to decrease at the tenth week after sowing in the control OTC. Resistant plants grew more pods as compared to sensitive plants after exposure to extremely high ozone concentrations for a longer time period in 2009. Thus, R123 genotype was remarkably more effective in vegetative as well as in generative growth beside high tropospheric ozone concentrations, especially in the control OTC and under elevated CO₂ level.

Induction of leaf fall by exposure to ozone was more considerable in S156 comparing to R123 plants. Consequently, early leaf fall of a certain plant genotype possibly implies to its ozone sensitivity. While percent of visible injuries correlated not only with ozone load but with the number of days spent after sowing, decrease in leaf number of sensitive plants showed significant correlation only with cumulative ozone values. Thus, evolution of visible injuries could be due to the temporal change of a factor different from ozone, while the decrement of leaf number solely indicated ozone exposure. However, S/R ratio of number of leaves gives a more objective approach and also correlates only with ozone load, and not with the number of days spent after sowing. Therefore, S/R ratio of number of leaves is such a good or even better parameter for the estimation of the quantity of phytotoxic ozone pollution than the extent of visible injuries. This is apparent from the close and significant negative connection of S/R ratio of number of leaves and ozone values. According to the results measured on bush bean,

elevated CO₂ level contributed to the ozone dependent evolution of visible symptoms and decrement of S/R ratio of number of pods, but it impeded the O₃ dependent decrease of number of leaves.

In spite of that aboveground biomass of white clover was mostly determined by the number of flowers, dry weight was generally higher in sensitive, while number of flowers was generally higher in resistant plants. This means that vegetative growth was more determinative in NC-S, while generative development was more determinative in NC-R genotype. Number of flowers of sensitive plants exceeded that of resistant plants only in the field plot, when cumulative ozone values were low. Under elevated CO₂ level, dry weight of resistant plants exceeded that of sensitive plants only once.

3.2.3 *Physiological traits*

According to our data, g_s of both species were rather determined by developmental stage and chamber effect than either by elevated CO₂ level or by O₃ load. However, elevated CO₂ level alleviated the effect of OTCs on g_s of white clover. In 2009, elevated CO₂ level also impeded the evolution of extreme differences between g_s of the sensitive and resistant genotypes which is an effect of tropospheric ozone burden. Elevated CO₂ promoted stomatal closure of sensitive plants in summer, thus g_s S/R never rose above 1.5 under elevated CO₂ level, unlike under the OTC control and field treatments. In this regard, elevated CO₂ counteracted the physiological ozone response. It was also apparent from our measurements that not only stomatal closure was deficient in S156 genotype, but stomatal opening as well. Cumulative exposure to tropospheric ozone largely determined g_s S/R of white clover. This ratio continually grew with time and with ozone load. We concluded that if measured under identical conditions, S/R ratio of the stomatal conductance of the white clover genotypes could give reliable information about the quantity of tropospheric ozone load. According to our results, excessive stomatal opening of sensitive white clover plants was not the cause but the consequence of their ozone sensitivity. Thus, ozone sensitivity of NC-S genotype is probably not directly derived from its higher stomatal conductance. The close correlation between the extent of visible injuries on S156 plants and cumulative ozone values under elevated CO₂ level was not in connection with their stomatal operation either.

In field plots, photosynthetic efficiency of S156 plants was often higher than that of R123 plants. However, after exposure to high cumulative ozone load, both Fv/Fm and RFd values were lower in sensitive than in resistant plants. Life cycle was generally longer in OTCs because of the chamber climate, but elevated CO₂ further retarded senescence and enhanced

stress resistance of the experimental plants as it was apparent from the values of fluorescence induction parameters.

Earlier senescence of sensitive white clover plants comparing to resistant plants is apparent from the low S/R ratio of Fv/Fm values of the autumn measurements. Microclimate of the OTCs acted as a stress factor without the effect of elevated CO₂ level (in control OTC). This adverse effect revealed itself from the enhanced decrement of RFd and g_s values of white clover. Drying effect of air circulation could enhance stomatal closure which is confirmed by the highest g_s values measured in field plots, which significantly differed from g_s measured in control OTCs. This phenomenon was observable on both species. Lower g_s sometimes alleviated ozone effects in OTCs.

Unlike R123 plants, stomata of S156 plants became more and more closed towards the end of ontogeny. Also photosynthetic efficiency of S156 plants became lower with time, which implies to the the lower stress resistance of this genotype. However, changes in photosynthetic processes were definitely connected with ozone stress, while g_s was highly affected by other environmental factors.

Values of Fv/Fm, which indicate the efficiency of energy conversion inside PSII, did not react as sensibly to either of the emerging stress factors (ozone load, chamber effect, heat etc.) as RFd values did. RFd values indicate potential photosynthetic quantum conversion, which is in connection with other processes of the light phase and with CO₂ fixation. RFd values of sensitive genotypes of both species closely correlated with ozone load. Decrement of fluorescence induction parameters in sensitive plants as a function of cumulative ozone pollution confirms that sensitive plants were exposed to more considerable physiological stress comparing to the resistant plants. In addition, it was apparent from our data that impairment of photosynthetic processes certainly took place as a result of ozone stress and that it was independent from the emergence of visible injuries.

3.3 Daily courses of fluorescence induction parameters in the two genotypes of *Phaseolus vulgaris*

During the five day long experimental period we made considerable deductions from the daily courses as well as from changes between different measuring days. If the plants are exposed to direct radiation Fv/Fm typically decrease and NPQ increase in the middle of the day. Since the measurements were implemented under shading net in our experiment, the plants were not exposed to direct radiation, no photoinhibition occurred, and Fv/Fm hardly decreased in the middle of the day. Since the plants grew under shading net and became shade adapted,

they were more susceptible to elevated temperature, which explains that NPQ reversely changed with temperature during the day. Temperature raised above 30°C in the middle of the day which presumably enhanced the fluidity of thylakoid membranes which resulted in increased ion conductance. These phenomena directly influence the degree of non-photochemical quenching. In addition, high temperature values were attended by high ozone concentrations. Since zeaxanthin, which is strongly responsible for non-photochemical quenching also plays a role as an antioxidant, oxidative stress causes NPQ decrease. Consequently, high ozone concentrations contributed to the decrease of NPQ. During our experiment, the highest measurable temperature was 32.7°C, which is, according to the temperature dependence of F_o , lower than the critical temperature for *P. vulgaris*. However, F_m (maximal fluorescence) continuously decreased between 21°C and 33°C. Energy-dependent quenching (q_E) is the most important among the three components of NPQ (q_E , q_T , q_I). Both *P. vulgaris* genotypes showed strong correlation between qF_o and NPQ. Consequently q_E , which is in connection with the xanthophyll cycle, was the most determinant in modifying the values of NPQ.

Sensitive plants did not show middle-day NPQ decrease, neither early stomatal closure on the first day of the experiment. On the last day however, F_v/F_m and NPQ values, as well as g_s of sensitive plants changed similarly to that of resistant plants, decreasing with increasing temperature and ozone exposure. This retarded reaction of sensitive plants to high temperature and cumulative AOT40 values is likely in connection with their ozone sensitivity. Thus, differences in the behaviour of the two bush bean genotypes that was observable on the first day disappeared by the last measuring day. Namely, defence reactions that could be experienced only on resistant plants on the first day also started to function in sensitive plants on the third measuring day. We concluded that enhanced oxidative damage of sensitive plants was caused by the delayed activation of their defence reactions. The cause of the difference between the daily courses of the consecutive measuring days was the dramatic growth of cumulative ozone load during the five day long experimental period. In this period, cumulative AOT40 was 1380 ppbh. The accordant growing impairment of photosynthetic processes was evident from the changes of F_v/F_m , NPQ and Rf_d values between the consecutive measuring days. Curves of daily courses of F_v/F_m and Rf_d values of sensitive and resistant genotype interchanged during the five day long measuring period, from which we concluded that the dissimilarities in these parameters were rather the consequences than the causes of enhanced ozone sensitivity of the sensitive genotype.

The plants had already been exposed to considerable ozone burden before the first day of measurements. However, defence and recovery processes probably repaired photosynthetic processes for some extent. Extremely high AOT40 values on the first measuring day and on the previous two days probably largely affected the physiological operation of the resistant plants as well. The following different behaviour of the two genotypes indicates the insufficiency of defence or regenerative processes in the sensitive genotype. This is supported by the further decrement of Fv/Fm and RFd values in sensitive plants on the following two measuring days, while AOT40 values were lower. Contrastingly, Fv/Fm and RFd of resistant plants were continuously growing, which implied to the adaptation to environmental conditions and to the recovery of photosynthetic processes of this genotype. Besides dissipating energy and reducing membrane fluidity in thylakoid membranes, zeaxanthin plays a role as an antioxidant. Consequently, partitioning of zeaxanthin molecules affects the antioxidant capacity of the plants. Minor or delayed utilization of zeaxanthin molecules in antioxidative processes provides a plausible explanation for ozone sensitivity.

3.4 *New scientific results*

- Several histological parameters showed significant difference between sensitive and resistant genotypes of both studied species in winter, when levels of ozone and other environmental stressors were low. However, seasonally changing environmental factors such as high light intensity, heat and ozone stress dissimilarly affected phenotypic appearance of the different genotypes. Most conspicuously, summer leaves of sensitive genotypes did not show the light-adapted thickening.

- In summer, resistant genotypes developed smaller stomata, which was in connection with their lower stomatal conductance and acted as a natural defence reaction against the permeation of ozone. Among the measured histological parameters, larger stomata, larger guard cells and stomatal apertures in *P. vulgaris* S156, while larger stomata and guard cells in *T. repens* NC-S were responsible for higher stomatal conductance of the sensitive genotypes.

- The latter developed (mature) leaves of NC-R genotype of *T. repens* developed thicker epidermis as compared to the earlier developed (old) leaves. This form of defence against the permeation of ozone was also absent from the NC-S genotype.

- The microclimatic conditions prevailing in OTCs lengthen the life cycle of our experimental plants and reduced their stomatal conductance. Detrimental effect of the chambers was obvious from the enhanced reduction of RFd values in sensitive genotypes of both species and from the reduction of S/R ratio of number of leaves of *P. vulgaris* under

OTC treatments. Values of S/R ratio of number of flowers and dry weight revealed that chamber effect decreased the vitality of sensitive *T. repens* genotype comparing to the resistant genotype in the long term.

- Growth conditions and developmental stage were rather determinant on the vegetative and generative growth of *P. vulgaris* and on the stomatal conductance of both species than ozone load.

- The sensitive genotype of the perennial *T. repens* species acclimated to tropospheric ozone in the long term. As a consequence of this acclimation process, the extent of ozone induced visible injuries decreased from accession to accession.

- Elevated CO₂ level promoted the acclimation of *T. repens* to O₃ pollution in the long term. Thus, we concluded that elevated CO₂ level can alleviate ozone sensitivity of a plant species as regards the appearance of visible injuries.

- Elevated CO₂ reduced the difference between stomatal conductance of the different genotypes of both species, between the dry weights of NC-S and NC-R genotypes, and between number of leaves of S156 and R123 genotypes. In addition, elevated CO₂ level reduced the development of visible injuries, delayed senescence and alleviated the chamber effect.

- By contrast, elevated CO₂ level enhanced specific ozone response regarding the extent of visible injuries, the S/R ratio of number of pods on *P. vulgaris* and the S/R ratio of number of flowers of *T. repens*. These parameters showed the strongest correlation with cumulative ozone under elevated CO₂ level. In these regards, elevated CO₂ enhanced the bioindicative values of the studied bioindicator species.

- S/R ratio of stomatal conductance of *T. repens* genotypes and S/R ratio of number of leaves of *P. vulgaris* genotypes correlated best with ozone load. RFd values of both sensitive genotypes showed strong correlation with cumulative ozone. Consequently, these are the most appropriate parameters for the bioindication of ozone.

- Although the appearance of visible injuries on NC-S plants indicated the presence of phytotoxic ozone, we did not find relationship between the extent of visible injuries on *T. repens* and the degree of ozone pollution.

- Most of the parameters showed stronger correlation with total cumulative ozone than with cumulative AOT40 values. However, extent of visible injuries on S156 genotype correlated only with cumulative AOT40. Minor physiological impairments observable before the appearance of visible injuries were related to total cumulative ozone.

4 CONCLUSIONS AND PROPOSITIONS

Based on our measurements, several genetically coded histological differences exist between the different genotypes of both studied species. Gene activity measurements and the comparison of genes coding parameters differing in winter samples of the different genotypes would be necessary to confirm this theory.

Our experimental results from both species confirmed that different behaviour of the different genotypes was in connection with the insufficient function of defence or recovery processes in the sensitive genotypes. This insufficient or delayed activity might stand in the background of ozone sensitivity, increased oxidative damage and accelerated senescence of the sensitive genotypes.

We experienced lacking stress response in sensitive plants when comparing the changes in stomatal and epidermal cell sizes and epidermal thickness of the different genotypes: Decrement of stomatal size and epidermal thickening took place only in resistant plants. These phenomena could be the components of a natural defence system against the pollutant. We observed delayed stress response during the daily course measurements of fluorescence-induction parameters. Based on the results of this experiment increased ozone sensitivity of S156 plants is partly due to the lower availability of zeaxanthin molecules for antioxidative processes in this genotype comparing to the R123 genotype. For verifying this theory, the differently localized fractions of zeaxanthin inside the thylakoid membranes should be revealed. To our present knowledge however, this kind of fractionation of the constituents of the thylakoid membrane is unresolved.

Insufficient stress response of sensitive plants might be in connection with the defective perception of and reaction to different environmental signals. This deficiency could result in the lack of the typical light adapted changes in the leaf structure of sensitive genotypes of both species. Different adaptability of the sensitive genotypes could be based on the mode of their production. S156 and NC-S genotypes were produced by artificial selection to displaying visible ozone injuries. During this process, sensitive populations possibly underwent inbreeding depression. As a consequence, sensitive genotypes could have lower phenotypic plasticity, accordingly lower adaptability to environmental effects and lower effectiveness in exploiting the available energy sources. This theory could also be verified only by genetic examinations or by direct adaptability tests under fully controlled environmental conditions.

From the results of the OTC experiment and the short series of fluorescence induction measurements we concluded that dissimilar F_v/F_m and RF_d values of the different genotypes

were rather the consequences than the causes of the increased ozone sensitivity of the sensitive genotypes.

Based on the relationship between the extent of visible injuries and ozone load, *P. vulgaris* proved to be the more effective bioindicator as compared to *T. repens*. In addition, RFd value proved to be the more effective parameter for the detection of stress exposure as compared to Fv/Fm. We set up a range of the studied parameters according to their bioindicative value. In *P. vulgaris*: 1) S/R ratio of number of leaves and the extent of visible injuries on the canopy of the sensitive plants. These two were identically effective in bioindication. 2) S/R ratio of number of pods. This parameter showed the strongest correlation with cumulative ozone values, however rarely measurable thus less informative. In our experiment, only one parameter measured on *T. repens* could be used in bioindication, and it was the S/R ratio of stomatal conductance. Since g_s S/R of *T. repens* gave the best quantitative relationship with ozone burden, we suggest the development of a biomonitoring system based on simultaneous stomatal conductance measurements of NC-S and NC-R genotypes. Stomatal conductance reacts very sensitively to several internal and external factors. These effects can hardly be controlled in field, but they can be eliminated by simultaneous g_s measurements on the different genotypes and by the determination of g_s S/R during the examination of ozone response. We also suggest the consideration of the development of a biomonitoring method based on the determination of S/R ratio of number of leaves on the two *P. vulgaris* genotypes. This method would provide a more objective and exact approach comparing to the determination of the extent of visible injuries, while facilitating a more frequent sampling comparing to the measurements of dry weights of pods. Also, it seems reasonable to perform trials for the determination of ozone load by measuring RFd values. For the exact development of these methodologies, fully controlled climatic chamber experiments with artificial ozone exposure should be implemented.

5 LIST OF THE RELEVANT PUBLICATIONS

1. Full-text scientific publications published in peer reviewed journals

Publications in English with IF (according to the WEB OF SCIENCE):

Villányi, V., Déri, H., Péli, E.R., Csintalan, Z. (2013): Differences in histological and physiological traits of ozone sensitive and resistant bean strains *Central European Journal of Biology* 8(4):386-397

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