Phyton (Austria) Special issue: "D. Grill"	**Vol. 45**	Fasc. 3	(279)-(293)	1.9.2005

Changes of the Redox Status of Glutathione and Ascorbate in Leaves and Apoplast of *Phaseolus vulgaris* Cultivars under Ozone Stress

By

H. BRÄUNING[1)], E. PAHLICH[2)], C. MÜLLER[1)] & H.-J. JÄGER[1)]

K e y w o r d s : *Phaseolus vulgaris* cv. 'Tenderette' and 'Oregon', ozone stress, redox status, glutathione, ascorbate, apoplast, whole leaf.

S u m m a r y

BRÄUNING H., PAHLICH E., MÜLLER C. & JÄGER H.-J. 2005. Changes of the redox status of glutathione and ascorbate in leaves and apoplast of *Phaseolus vulgaris* cultivars under ozone stress. - Phyton (Horn, Austria) 45 (3): (279)-(293).

The bean cultivars 'Tenderette' and 'Oregon' were treated in open top chambers with 30 or 50 ppb ozone which was added to ambient air during daylight. The development of apoplastic radicals and of the antioxidative capacity was determined in the leaves with the main interest in the development of the redox status of ascorbate and glutathione in the apoplast fluid and in the total leaf extract. We show that despite large individual changes in concentration the redox ratios of ascorbate, dehydroascrobate, gluthatione and oxidised glutathtione were more or less constant. Over the course of the study the ratios experienced only moderate shifts and stayed at the reduced states. The cultivars 'Tenderette' and 'Oregon' are considered to show a difference in ozone sensitivity. Both cultivars show typical symptoms of ozone damage that occur in the more ozone tolerant cultivar 'Tenderette' at a time shift of 10 days. A relationship between the differences in tolerance towards ozone and the development of the redox status of the antioxidatives could not be established.

I n t r o d u c t i o n

Tropospheric ozone (O_3) poses a critical threat and a challenging problem to present and future world food, fiber and timber production and conservation of natural communities, including their species diversity (KRUPA & al. 2001). The air

[1)] Department of Plant Ecology, University Giessen, Heinrich Buff-Ring 26 - 32 (IFZ), D 35392 Giessen; e-mail: hans-juergen.jaeger@bot2.bio.uni-giessen.de

[2)] Department of Plant Physiology, University Giessen, Heinrich Buff-Ring 54 - 62, D 35392 Giessen; e-mail: edwin.pahlich@bot3.bio.uni-giessen.de

pollutant causes foliar injury and reduces growth and yield in many agronomic and horticultural crops, deciduous trees, and conifers (HECK & al. 1988, JÄGER & al. 1992, KRUPA & al. 1998). Ozone is deposited from the atmosphere onto plant canopies by diffusion and enters the leaf through stomata. The ozone flux to the surface of an individual leaf is affected by the laminar boundary resistance, which is a function of leaf properties and wind speed (GRÜNHAGE & JÄGER 2001). Ozone uptake essentially takes place via the stomata of the leaves or needles and results from the concentration gradient between the ambient air and the leaf interior. Studies on isolated cuticles showed that these are not very permeable to ozone (KERSTIENS & al. 1992), thus this route of uptake is negligible. Therefore stomatal conductance and stomatal transport resistance govern ozone uptake, subsequent effects in the plant interior, and differences in sensitivity among cultivars and species.

Ozone causes negative effects on a number of plant processes, including photosynthesis, dry matter production, flowering, rate of senescence, and yield (KRUPA 1997). The reactions of a plant to ozone pollution are based on the sequence of biochemical and physiological processes that can ultimately lead to damage. While many physiological and biochemical functions necessary for growth and reproduction are impaired by O_3, the specific cellular sites that undergo damage are largely unknown (WINNER 1994, SRIVASTAVA 1999, HEATH 1999). The fundamental mechanisms of ozone proceed at cellular and subcellular activity sites and are characterised by the low solubility of the gas and its highly oxidizing properties. Ozone decomposes in the intercellular spaces forming radicals and other highly reactive oxygen species (ROS) including H_2O_2 (cf. MUDD 1996). The plant antioxidant system, which scavenges naturally occuring reactive oxygen compounds, could function as a primary mechanism to alleviate the oxidative burden resulting from O_3 exposure. In the course of evolution, plant cells have developed protective systems against activated oxygen species (SALIN 1987, LLOYD 1999). The most important of these antioxidants include metabolites such as ascorbate, glutathione, α - tocopherol, polyamines, and a number of enzymes such as ascorbate peroxidase, glutathione reductase, superoxide dismutase and catalase. Recent studies on the effect of ozone on extracellular antioxidants have given rise to speculation that the first important defense mechanisms against O_3 or its secondary products take place as soon as it appears in the apoplast, i.e. before ozone or its products reach the plasmalemma (HEATH & TAYLOR 1997, DIETZ 1997). No clear correlation has been found to date between differing species sensitivity and cultivar sensitivity to ozone and the contents or activities of antioxidants (CHANWAY & RUNECKLES 1984, BECKER & al. 1989). Transgenic plants containing overexpressed (intracellular) antioxidant enzymes such as glutathione reductase or superoxide dismutase have also, to date, exhibited virtually no greater tolerance to ozone than their respective wild types (PITCHER & al. 1991, CREISSEN & al. 1994, CREISSEN & al. 1999, PITCHER & ZILINSKAS 1996, TORSETHAUGEN & al. 1997).

In this paper we report on the scavenger patterns of ascorbate and glutathione (apoplast, total leaf) in two bean cultivars that differ in ozone sensitivity.

Material and Methods

Plant material

The experiments have been performed with *Phaseolus vulgaris* var. 'Tenderette' and 'Oregon' which according to their phenotypic responses are considered to be ozone tolerant and sensitive respectively (BURKEY & al. 2000). Individual seedlings were grown in a soil - compost mix (1:3) and watered weekly with a modified Knop solution (BRÄUNING 2002). The first and second leaves of 3 individual plants were harvested in the morning and analyzed as batches in three replicates.

Ozone treatment

The ozone treatment of plants occurred in open-top chambers (FANGMEIER & al. 1992). Controls were aerated with external air and for the ozone treatment 30 or 50 ppb ozone were added to the external air. The ozone concentration was monitored in 7.5 min. intervals.

Determination of ascorbate and glutathione

The ascorbate and glutathione redox states were determined in apoplasts and whole leaf extracts respectively. Ascorbate was extracted from frozen leaf material (shock frozen in liquid nitrogen, stored at -80°C) in 0.1 N HCl + 10% PVP (1:10, w/w) and ground at 4°C according to the method of OKAMURA 1980. Leaves for glutathione determinations were ground in 8% (w/v) sulfosalicylic acid +10% PVP at 4°C according to O'KANE & al. 1996. The homogenates were filtered through cheese cloth and afterwards clarified by centrifugation.

Ascorbate in the apoplast was determined in a mixture of apoplastic fluid and 0.1 N HCl (1:2, v/v), while the respective mixture for glutathione determination contained 8% salicylic acid (w/v) at a ratio of 1:2 (v/v). The mixtures were shock frozen in liquid nitrogen and analyzed for ascorbate and glutathione as described above for total leaf extracts.

The antioxidant capacity in crude extracts

The antioxidant capacity of leaf extracts was determined with the 1.1-Diphenyl-2-Picrylhydrazine method (LØVAAS & OLSEN 1998) (DPPH assay). Methanolic extracts of the plant material (1:5 w/w) were clarified by centrifugation. The dye in its radical form oxidizes the total amount of the scavengers of a leaf. The process can be followed by measuring the decrease of the absorbance at 514 nm in a spectrophotometer ($\varepsilon = 22 * 10^3 * mM^{-1} * cm^2$).

Measurement of apoplastic radicals/oxidants (ROS)

Apoplastic oxidants were determined through oxidation of the impermeable dye phenol red (SCHRAUDNER & al. 1998). Disks of freshly harvested leaves were vacuum infiltrated three times for 1 min with a solution of 0.14 mM phenol red in 10 mM MES-KOH, pH 6.5 and incubated for 90 min. The reaction was stopped by addition of 1M NaOH (20 µl per ml of fluid) to the apoplast fluid. The oxidized phenol red was determined at 610 nm ($\varepsilon = 12.5 * 10^3 * mM^{-1} * cm^2$).

Preparation of apoplast fluid

Apoplast fluid was extracted according to HUSTED & SCHJOERRING 1995. Leaf stripes (1 cm) were vacuum infiltrated in solutions of 0.28 M Sorbitol at 100 mbar (3 re-runs lasting 3 min. each). The infiltrated stripes were mechanically stabilized in aluminium foil and placed into centrifuge tubes. After centrifugation for 20 min at 2000 * g the fluid was collected and stored in Eppendorf vials.

Scaling of the redox fractions of ascorbate and glutathione

The redox balance of the ascorbate moiety is given by equation a) the scaled form of it is shown in equation b).

a) ASC + DHA = ASCo

b) ASC/ASCo + DHA/ASCo = 1

The reduced fraction of ascorbate (ASC/ASCo) is expressed as ASC/(ASC + DHA) by some authors.

The redox balance of the glutathione moiety is given in equation c) and the scaled form in equation d).

c) GSH + 2GSSG = Go

d) GSH/Go + 2GSSG/Go = 1

The reduced fraction of GSH is GSH/Go or GSH/(GSH + 2GSSG).

(Note: For balancing the redox capacity of the glutathione couple it must be considered that GSSG has the potential to carry two H instead of one in GSH).

Results and Discussion

After having past the stomata ozone is expected to react primarily with water phases of the apoplast, the cell wall and of the intercellular cavities (reviewed in ROSHCHINA & ROSHCHINA 2003, PLÖCHL & al. 2000). Superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) singulette oxygen and especially H_2O_2 are formed (ROS) (ROSHCHINA & ROSHCHINA 2003). In addition ozone is trapped by components of the extracellular surface of the plasmalemma with -S-S- and lipid double bonds as main targets (MUDD 1996, MACH & GREENBERG 2004). The secondary reaction product H_2O_2 formed by ozonolyses of water and of unsaturated fatty acids (ROSHCHINA & ROSHCHINA 2003) is believed to travel significant distances from its place of formation and is viewed as a main trigger of antioxidative responses (SCHRAUDNER & al. 1998).

Apoplast radicals

In this investigation long-term responses on the redox status of the apoplast and the whole leaf scavengers glutathione and ascorbate were studied in ozone treated *Phaseolus* cultivars 'Tenderette' and 'Oregon'. Figs. 1A and B show the effect of application of 50 ppb ozone during day time on the appearance of radicals in apoplasts and in leaves. The oxidative potential of the apoplast fluid was nearly identical in controls of both plant cultivars and remained more or less unchanged during the 14 days measuring period. In ozone treated plants the apoplast radicals increased in both cultivars. It can be observed that after a lag period of 2 days the apoplast radicals peaked in 'Tenderette' to nearly 3 times the

amount as observed in the control and later decreased to a value which was nearly twice of the control level (Fig. 1A). In 'Oregon' however, the radicals increased with the onset of the ozone treatment, peaked after 5 days to more than the 4 fold amount of the controls and declined to 3.5 to 4 times the control level (Fig. 1B).

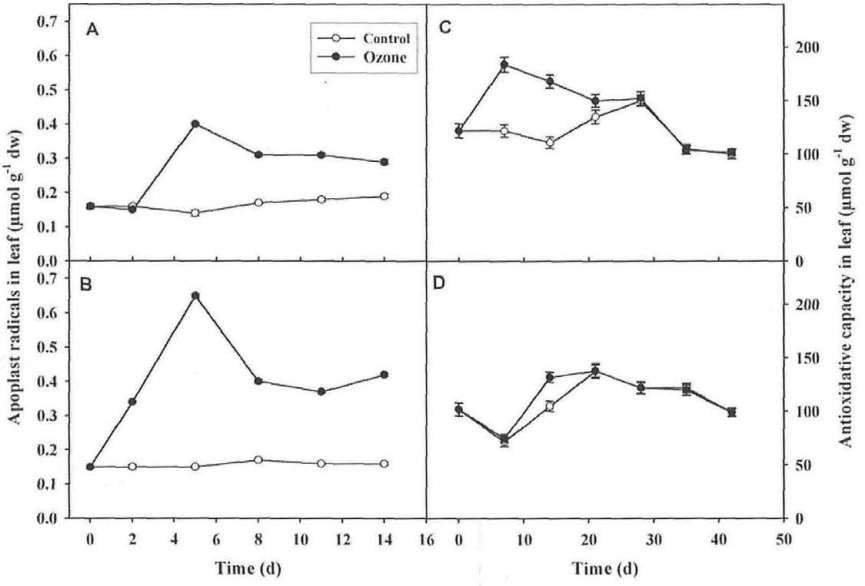

Fig. 1. Apoplast radicals (A,B) and antioxidative capacity (C,D) in bean leaves of the cultivars 'Tenderette' (A,C) and 'Oregon' (B,D). Plants were exposed to ambient air (controls) or exposed to 50 ppb ozone added to the ambient air during the daily photoperiod.

Both cultivars responded with an "overshoot - like" accumulation profile of apoplast radicals which then approached towards a steady level well above that of the controls (Fig. 1A and B). The effect is more pronounced in 'Oregon' which is considered to be more sensitive than 'Tenderette'. Similar response profiles were reported from ozone sensitive and tolerant tobacco lines (SCHRAUDNER & al. 1998) which were gained under higher ozone burdens (150 nl l^{-1}) and on a shorter exposure time of five hours. Under a permanent ozone burden during the photoperiod the plants obviously establish new steady-states between radical formation and detoxification. These new states probably are manifested in a permanently increased radical level in our experiments. 'Overshoots' have been interpreted as 'cost of adaptation' (BURTON 1939) and characterize transitions from an old to a new steady state under changed signal conditions (VON BERTALANFFY & al. 1977). Continuous transition profiles from established steady states of a starting condition

(control) into those of stressed plants might occur. But most likely discontinuous or even step or jump-like transitions may be more typical phenomena in stress physiology (PAHLICH 1995, MALDONADO & PAHLICH 1997). The question must be asked under which conditions short time response courses can give adequate insights into mechanisms of adaptative rearrangements. Overshoots have not been discussed as possible explanations for early stress responses though this phenomenon might confuse the explanation of short term responses to external noxes. From the fairly smooth long time profiles in Figure 1 it can not be deduced that short-term effects are missing which might superimpose these basic response courses.

Leaf antioxidative capacity

It is discussed that the antioxidative potential of the apoplast is mainly a function of the regenerative machinery of the cytosol (PLÖCHL & al. 2000) and not of the apoplast. Therefore the antioxidative capacity of the extracts of total leaves of the cultivars of beans has been measured. The basic level of antioxidants (Fig. 1C and D) was somewhat higher in the tolerant cultivar 'Tenderette' than in 'Oregon' and showed an increased response following the beginning of the ozone treatment. This sudden supply of increased antioxidants could explain the lag effect in the ROS development as shown in Fig. 1A. From the seventh day onwards the antioxidative potential decreased continuously and finally followed the course of the controls after 28 days of growth (Fig. 1C).

The antioxidative response in leaves of 'Oregon' is obviously different (Fig. 1D). The antioxidative capacity resides on a lower level of 100 µmol in 'Oregon' instead of 120 µmol in 'Tenderette'. After an initial drop of the antioxidative capacity in controls and in ozone treated beans the level increased slightly in both cases until day 21 and than levelled off to the starting value of about 100 µmol g^{-1} dw.

In both cases the antioxidative capacity was much higher in the total leaf in comparison to the oxidant level in the apoplast. This observation supports the view (PLÖCHL & al. 2000) that the antioxidative potential of the apoplast is supplied by an export of scavengers from the cytosol. If this is true then the ratios of the export to import fluxes of reduction equivalents and of the oxidized fractions of the redox moieties at the plasmalemma are also determinants of the redox ratios of the antioxidants in the apoplast.

It should be recognized that the methods for measuring the apoplastic ROS and the antioxidative capacity in leaves are determinations of sums of radicals and scavengers. All oxidative molecules (ROS) derived from reactions of ozone must be expected to contribute to the bleaching of the dye though H_2O_2 is thought to be the main contributor (SCHRAUDNER & al. 1998). From the same reason it is questionable whether schemes considering particular 'ozone sensors' at the outer surface of the plasmalemma really delineate the most probable concept (ROSHCHINA & ROSHCHINA 2003). Besides H_2O_2 as ozone derived signal one could expect signals of degradation products of lipids (GRECHKIN 1998) which have assigned signal functions in plants (GRECHKIN & TARCHEVSKY 1999). Multiple "causes" derived from different ROS and their secondary products induce most

likely multiple "oxidative stress responses". Ozone toxicity in plants can hardly be expected to follow a "monocausal" signal cascade.

Ascorbate of the apoplast

Ascorbate is thought to be the major apoplastic antioxidant. The total ascorbate content (ascorbate reduced + dehydroascorbate) of the bean apoplasts is shown in Fig. 2 A and B. In 'Tenderette' (Fig. 2A) a nearly 7 fold increase in total ascorbate was observed during the first 14 days of ozone treatment. After this period the content levelled off but remained about two-fold higher than the content of the control.

The development of total ascorbate was quite different in the apoplasts of the cultivar 'Oregon' (Fig. 2B). In control plants the ascorbate content was only slightly increased as compared to the starting concentrations. After three weeks of growth its content started to decrease. In ozone treated plants instead the ascorbate content decreased from the onset of the treatment and finally dropped down to a level of about 70% beyond the starting content.

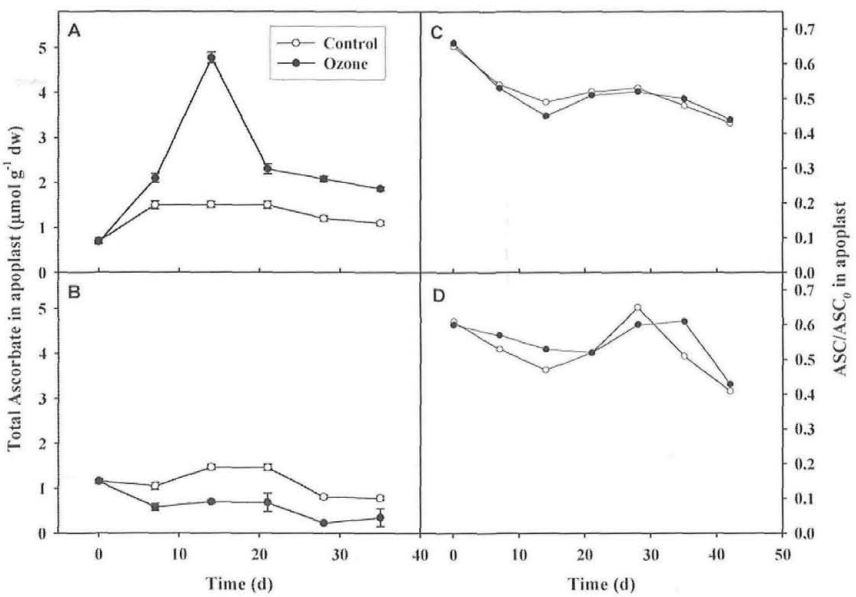

Fig. 2. Total ascorbate content (ascorbate + dehydroascorbate) (A,B) and the ascorbate redox status (C,D) in the apoplast fluid of bean leaves of the cultivars 'Tenderette' (A,C) and 'Oregon' (B,D). Plants were exposed to ambient air (controls) or exposed to 30 ppb ozone added to the ambient air during the daily photoperiod.

Ascorbate redox state of the apoplast

The reduced fraction of ascorbate of the apoplasts is shown in Fig. 2 C and D. Interestingly this reduced fraction was almost identical in comparison of controls and of the ozone treated cultivars 'Tenderette' and 'Oregon' (Fig. 2C and D). The curve of the reduced fraction of ascorbate has the form of a flat wave in both cultivars with a downward bended tendency over the 42-days growth period.

The different shapes of the time courses of total ascorbate and the concomitant profile of the reduced fraction seems to be contradictory. While the amount of total ascorbate in the apoplast changed drastically during progressing time it was observed that the reduced fraction of ascorbate was fluctuating without dramatic changes of its redox state. A reasonable change of the contents of the total ascorbate was accompanied by respective movements of the reduced ascorbate. (Fig. 2C and D). There must be mechanisms which very efficiently equilibrated the oxidized and reduced forms of ascorbate thus stabilizing the reduced fraction ASC/ASCo. But the reduced level of about 0.6 to 0.4 (a range which is also described in different bean cultivars (BURKEY & al. 2003) represents a fairly oxidized state of the ascorbate moiety (see later) and indicates that an effective regeneration of the reduced fraction is missing in this compartment. The changes of ascorbate in the apoplast are not easy to correlate with the acute oxidative burden of ozone which was kept constant during the photoperiod during the entire observation period.

Ascorbate of the leaf

There were differences of the movements of the ascorbate moieties in whole leaf extracts as compared to the apoplast (Fig. 3 A and B). In general there were no significant differences in the long-term profiles of total ascorbate between the controls and the ozone treated cultivars 'Tenderette' and 'Oregon'. The amounts of total ascorbate in both cultivars ranged between 600 and 450 $\mu mol\ g^{-1}$ dw. This amount is about 500 to 600 fold higher than that in the apoplast. An ozone dependent increase of the total ascorbate could not be identified. But both profiles showed that there was a step-like decrease from an upper level to a lower plateau of the total ascorbate pools. The point of inflection is found after 12 days of growth in 'Tenderette' and about three days earlier in 'Oregon'. Whether this step marked a developmental transition between two growth phases of the plants or was metabolically caused is not clear. This transition was also found and even more pronounced for total glutathione (see glutathione).

Redox state of ascorbate in leaves

The reduced fraction of ascorbate was fairly stable in whole leaf extracts with only small changes (Fig. 3C and D). As in whole leaves there were hardly any differences between the time courses of the controls and the ozone treated plants in both cultivars (Fig. 3C and D). However a major difference was identified in the absolute levels of the reduced fractions as compared to the apoplast. The absolute amounts of reduced ascorbate in leaves of 'Tenderette' and 'Oregon' are well above the starting levels of the apoplast, i.e. 0.9 instead of ~ 0.6 respectively and

stayed on a higher reduced state over the whole time of observation, i.e. not below 0.75 after 20 days. The difference of 0.25 to 0.35 relative units might be related to the permanent ozone detoxification in the apoplast. This remarkable differences between the cytoplasmic and apoplastic ascorbate pools could well be overcome via appropriate export / import fluxes of reduced ascorbate and dehydroascorbate respectively. We did not measure flux rates over the plasmalemma and therefore any discussion about flux ratios and their contribution to the establishment of the apoplast redox state remains speculative at the moment.

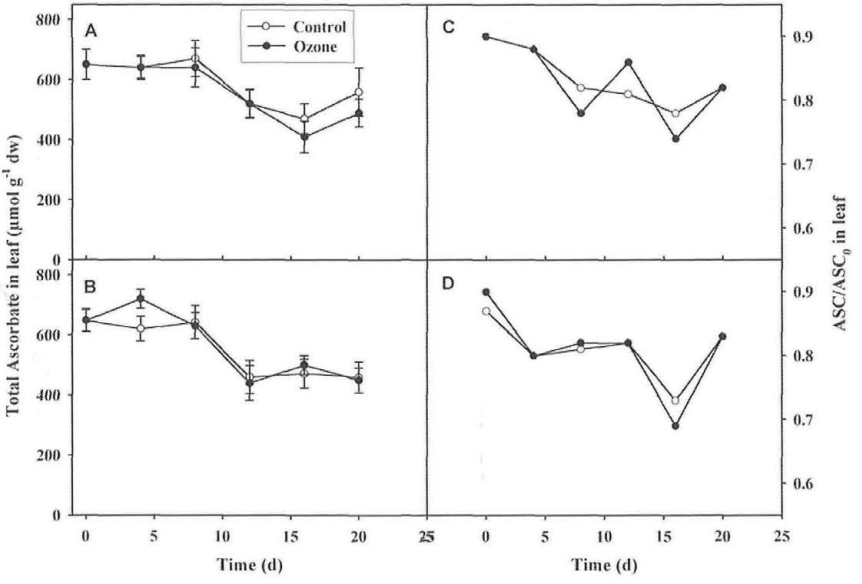

Fig. 3. Total ascorbate (A,B) and the ascorbate redox status (C,D) in bean leaves of the cultivars 'Tenderette' (A,C) and 'Oregon' (B,D). Plants were exposed to ambient air (controls) or exposed to 30 ppb ozone added to the ambient air during the daily photoperiod.

The highly reduced state of ascorbate in leaves are in accordance with the function of the glutathione ascorbate regenerating cycles (FOYER & HALLIWELL 1976). With the help of flux modelling of this system it was shown (PAHLICH, MÜLLER & JÄGER, in progresss) that in the presence of any reductant (GSH) in the cytosol the ascorbate fraction can be kept close to 100% via the irreversible and highly active reaction of the dehydroascorbate reductase. It might be argued that ASC/ASCo fractions which highly deviate from unity in particular cell compartments must lack an efficient ASC regenerative potential which is comparable to the Halliwell Asada mechanism.

Glutathione in the apoplast

The time dependent development of the pool of the total glutathione in apoplasts on the µmolar level followed in both bean cultivars different time courses (Fig. 4A and B). A fairly stable level of total glutathione of about 0.06 µmol g^{-1} dw is measured in the controls of 'Tenderette' over a period of 35 days. In 'Oregon' instead we found a decrease in pool size from 0.085 to 0.02 µmol g^{-1} dw (Fig. 4B). At the same time the total glutathione pool in ozone treated plants followed different time courses in both cultivars. It was increasing from 0.065 to a final level of 0.15 µmol g^{-1} dw after 35 days in 'Tenderette' (Fig. 4A). In 'Oregon' instead the total glutathione was always below the control level and changed between 0.08 and 0.01 µmol g^{-1} dw (Fig. 4 B).

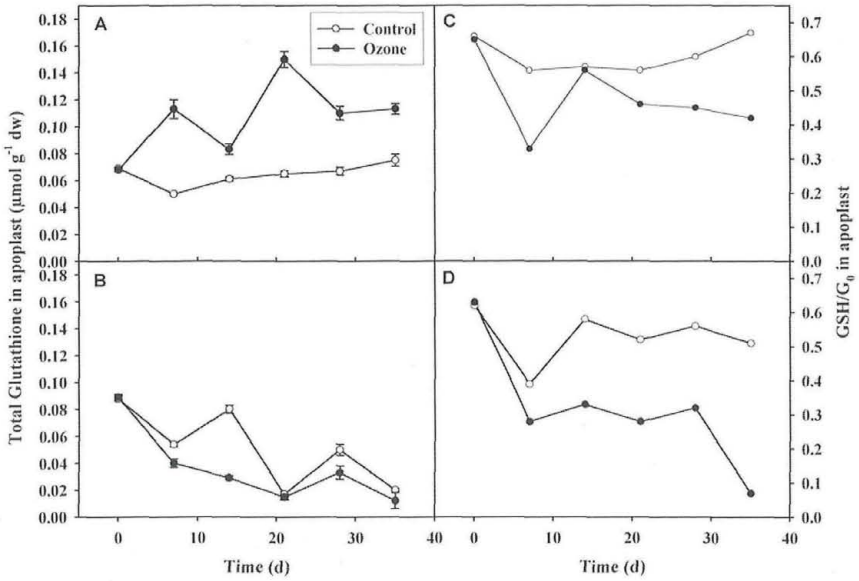

Fig. 4. Total glutathione content (glutathione + 2 glutathione disulfide) (A,B) and glutathione redox status (C,D) in the apoplast of beans leaves of the cultivars 'Tenderette' (A,C) and 'Oregon' (B,D). Plants were exposed to ambient air (controls) or exposed to 30 ppb ozone added to the ambient air during the daily photoperiod.

Redox state of glutathione in the apoplast

The initial fraction of the reduced glutathione in apoplasts was about 0.65 in both cultivars 'Tenderette' and 'Oregon' respectively (Fig. 4C and D). The control levels in 'Tenderette' (Fig. 4C) follow a smooth time course and ranged between 0.65 and 0.58. Despite of slightly increasing concentration of total glutathione decreased the reduced fraction under the influence of ozone in this

cultivar. After passing an inflection point on day 7 the level of the fraction of reduced glutathione increased again and dropped from 0.6 to about 0.4 in 'Tenderette' (Fig. 4C). In 'Oregon' the apoplast fraction of reduced glutathione fluctuated with a downward tendency. In ozone treated plants the reduced glutathione dropped from 0.6 to a plateau level of about 0.3 and finally decreased to a value lower than 0.1 (Fig. 4D).

Glutathione in leaves

The time courses of total glutathione and of the fraction of the reduced glutathione in leaf extracts can hardly be correlated with the apoplastic moieties. In both cultivars the total glutathione passed a threshold from a higher to a lower level (Fig. 5A and B). In 'Tenderette' the high plateau (~10 µmol g^{-1} dw) was shifted to a low plateau with a point of inflection at day 12. A similar shift was observed in 'Oregon' at day 8. Ozone treatment provided insight regarding a basic difference between both cultivars. The point of inflection was shifted from day 12 in 'Tenderette' to day 26 (Fig 5A). In 'Oregon' instead both time courses followed nearly an identical track with the point of inflection on day 8 (Fig. 5B).

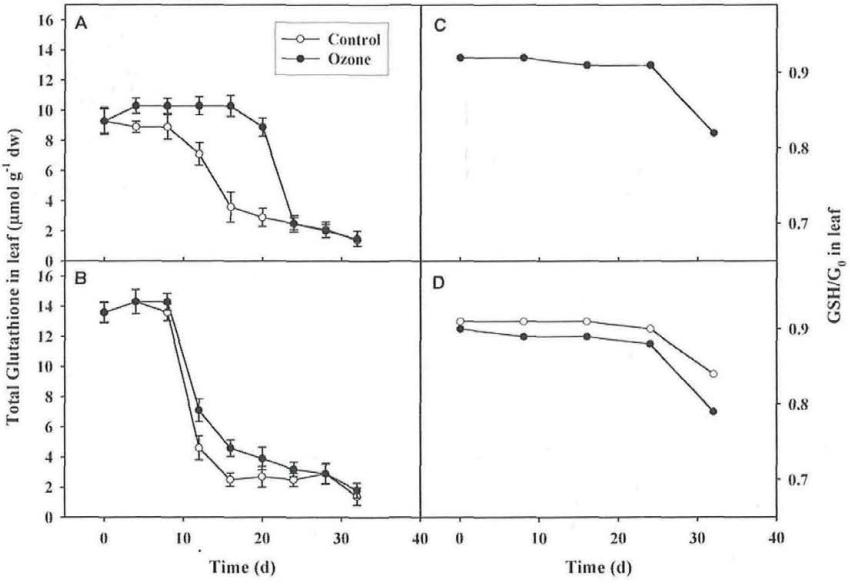

Fig. 5. Total glutathione content (glutathione + 2 glutathione disulfide) (A,B) and glutathione redox status (C,D) in bean leaves of the cultivars 'Tenderette' (A,C) and 'Oregon' (B,D). Plants were exposed to ambient air (controls) or exposed to 30 ppb ozone added to the ambient air during the daily photoperiod (note: the values for the control and ozone treatment of 'Tenderette' in C were indistinguishable).

Redox state of glutathione in leaves

Despite of the conspicuous time courses of the total glutathione the fraction of the reduced glutathione stayed at stable values close to 0.9 (Fig. 5C and D). The reduced fractions in 'Tenderette' are indistinguishable between controls and ozone treated pants. The data also underline reported findings that glutathione of the apoplast was on the level of ~ 1% as compared to the control (ZECHMANN & al. 2005). In comparison to the ascorbate content in leaves (Fig. 3) the glutathione remained on a 40 to 70 fold lower level in both cultivars. The same is true in the apoplast where the ascorbate level was 10 to 30 fold higher than the level of the glutathione. The underlying concentration differences definitively qualify the ascorbate as the superior detoxificant especially if both scavengers are present in a common compartment. The question arises whether glutathione plays a significant role in direct antioxidative defence. The priority of ascorbate over glutathione results simply from the statistics of random targeting (i.e. the initial event for molecule interactions/reactions) of the oxidant by a scavenger, irrespective whether direct chemical or enzymatic detoxification occurred. But despite of this it is questionable whether absolute amounts of antioxidants can be taken as evidence for their antioxidative efficiency. Reaction cycles like the Halliwell Asada antioxidative system can be efficient also when low intermediate concentrations are combined with high catalytic potentials of the entire cycle system. In cycle reactions it is difficult to identify a 'bottle neck' reaction which could be assigned as flux generator of the system. Therefore changes of particular enzymes of cycle systems are not very informative per se in respect to predictions of underlying catalytic potentials.

Our experiments showed that with respect to the reductants glutathione and ascorbate some differences existed between 'Tenderette' and 'Oregon' which were found in the controls as well as in the ozone treated plants. However, these differences did not give rise for a straight forward argument of a correlation between the contents of the scavengers and stress tolerance. Ozone injuries occur in both lines. In 'Oregon' chlorotic leaves and stipplings were already noticed on day 12. This phenomenon occurred in 'Tenderette' 10 days later. There probably exists an intimate interference between changes of the scavengers which arise from developmental traits, ozone directed changes of developmental traits and differentially modulated scavenger pools as direct responses to ozone stress. It is hypothesized that ozone sensitivity or tolerance is a subordinate effect of structural leaf developments (BARTH & CONKLIN 2003, OKSANEN & al. 2004). There is no doubt that particular structures are assigned with particular modes of biochemical detoxification potentials and responses. In open systems this structure - function interrelationship is a crucial point because it determines an 'initial or starting condition' and defines which particular response to externally applied noxes can occur during future development (PAHLICH 1993).

The conspicuous stability of the redox balances of ascorbate and glutathione should be regarded as important. Even seemingly dramatic differences of the concentrations of GSH and GSSG can turn out to be very stable (HERBINGER & al. 1999) in respect to the redox state. From modelling experiments it can be

deduced that the glutathione - ascorbate cycle system (FOYER & HALLIWELL 1976) as designed in wheat roots (MALDONADO & PAHLICH 1997) is such a stabilizing element which endows homeostasis to glutathione and ascorbate (PAHLICH, MÜLLER & JÄGER, in progress). This regenerative redox system can keep the reduced fraction of ascorbate close to 100% as a result of the irreversible reaction of the glutathione - dehydroascorbate reductase. The reduced glutathione is predicted to be close to 90% under comparable conditions. The latter percentage results from the fact that the mass action ratio of the glutathione - reductase is under control of the equilibrium constant of this reaction. The lowered reduced fractions of glutathione and ascorbate in the apoplast might be taken as evidence that the transfer reactions across the plasmalemma are not effective enough to export or re-import the reduced and oxidized scavengers. If a regenerative system exists in the apoplast it most likely has a quite different design than the cytosolic counterpart.

References

BARTH C. & CONKLIN P. 2003. The lower cell density of leaf parenchyma in the *Arabidopsis thaliana* mutant lcd1-1 is associated with increased sensitivity to ozone and virulent *Pseudomonas syringae*. - Plant Journal 35(2): 206-218.

BECKER K., SAURER M., EGGER A. & FUHRER J. 1989. Sensitivity of white clover to ambient ozone in Switzerland. - New Phytologist 112: 235-243.

BRÄUNING H. 2002. Ozoneffekte auf unterschiedlich tolerante *Phaseolus vulgaris* Varietäten - das Ascorbat - Glutathion - System und die apoplastische Stressantwort. - Dissertation, Justus-Liebig-Universität, Giessen.

BURKEY K.O., EASON G. & FISCUS E. 2003. Factors that affect leaf extracellular ascorbic acid content and redox status. - Physiologia Plantarum 117: 51-57.

— , WEI C., EASON G.,GOSH P. & FENNER G.P. 2000. Antioxidant metabolite levels in ozone-sensitive and tolerant genotypes of snap bean. - Physiologia Plantarum 110: 195-200.

BURTON A.C. 1939. The properties of the steady state compared to those of equilibrium as shown in characteristic biological behavior. - Journal Cellular and Comparative Physiology 14: 327-349.

CHANWAY C.P. & RUNECKLES V.C. 1984. The role of superoxide dismutase in the susceptibility of bean leaves to ozone injury. - Canadian Journal of Botany 62: 236-240.

CREISSEN G.P., BROADBENT P., KULAR B., REYNOLDS H., WELLBURN A.R. & MULLINEAUX P.M. 1994. Manipulation of glutathione reductase in transgenic plants: implications for plants' responses to environmental stress. - Proceedings Royal Society Edinburgh Sect. B. 102: 167-175.

— , FIRMIN J., FRYER M., KULAR B., LEYLAND N., REYNOLDS H., PASTORI G., WELLBURN F., BAKER N., WELLBURN A.R. & MULLINEAUX P.M. 1999. Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. - Plant Cell 11: 1277-1291.

DIETZ K.J. 1997. Functions and responses of the leaf apoplast under stress. - Progress in Botany 58: 221-254.

FANGMEIER A., STEIN W. & JÄGER H.-J. 1992. Advantages of an open-top chamber plant exposure system to assess the impact of atmospheric trace gases on vegetation. - Angewandte Botanik 66: 97-105.

FOYER C.H. & HALLIWELL B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. - Planta 133: 21-25.

GRECHKIN A.N. 1998. Recent development in biochemistry of the plant lipoxygenase pathway. - Progress in Lipid Research 37 (5): 317-352.

— & TARCHEVSKY I.A. 1999. The lipoxygenase signaling system. - Russian Journal of Plant Physiology 46: 114-123.

GRÜNHAGE L. & JÄGER H.-J. 2001. Austausch von Stoffen zwischen Atmosphäre und Biosphäre. - In: GUDERIAN R. (Hrsg.), Handbuch der Umweltveränderungen und Ökotoxikologie. Bd. II: Terrestrische Ökosysteme, pp. 227-272. - Springer, Berlin.

HEATH R.L. 1999. Biochemical processes in an ecosystem: how should they be measured? - Water, Air, and Soil Pollution 116: 279-298.

— & TAYLOR G.E. 1997. Physiological processes and plant responses to ozone exposure. - In: SANDERMANN H., WELLBURN A.R. & HEATH R.L. (Eds.), Forest decline and ozone, pp. 317-368. - Springer, Berlin.

HECK W.W., TAYLOR O.G. & TINGEY D.T. 1988. Assessment of crop loss from air pollutants. - Elsevier Applied Science, London, New York.

HERBINGER K., TAUSZ M. & GRILL D. 1999. Are winter adaptive changes in glutathione and chlorophyll contents in spruce needles triggered by low temperatures or short days? - Phyton 39 (3): 171-174.

HUSTED S.U. & SCHJOERRING J.K. 1995. Apoplastic pH and ammonium concentration in leaves of *Brassica napus* L. - Plant Physiology 109: 1453-1460.

JÄGER H.-J., UNSWORTH M., DE TEMMERMAN L. & MATHY P. 1992. Effects of air pollution on agricultural crops in Europe. - Air pollution research report No. 46, CEC-Commission of the European Communities, Directorate - General for Science, Research and Development, Brussels.

KERSTIENS G.R., FEDERHOLZNER R. & LENDZIAN K.J. 1992. Dry deposition and cuticular uptake of pollutant gases. - Agriculture, Ecosystems and Environment 42: 239-253.

KRUPA S.V. 1997. Air pollution, people, and plants. - APS Press, St. Paul, Minnesota.

— , TONNEIJCK A.E.G. & MANNING W.J. 1998. Ozone. - In: FLAGLER R.B. (Ed.), Recognition of air pollution injury to vegetation, pp. 2/1-2/28. - Air & Waste Management Association, Pittsburgh.

— , MCGRATH M.T., ANDERSEN C.P., BOOKER F.L., BURKEY K.O., CHAPPELKA A.H., CHEVONE B.I., PELL E.J. & ZILINKSAS B.A. 2001. Ambient ozone and plant health. - Plant Disease 85: 4-12.

LLOYD D. 1999. How to avoid oxygen. - Science 286: 249.

LØVAAS E. & OLSEN J.E. 1998. No induction of polyamines and radical scavenging antioxidants in *Nicotiana tabaccum* exposed to iron excess, as investigated by DPPH assay and differential spectroscopy. - Journal Plant Physiology 153: 401-408.

MACH J.M. & GREENBERG J.T. 2004. Free radicals and oxidative stress. - In: NOODEN L. (Ed.), Plant cell death processes, pp. 203-212. - Elsevier Academic Press, Amsterdam.

MALDONADO C. & PAHLICH E. 1997. Stability and autoregulation of the cytoplasmic glutathione redox system in wheat cultivars under mild water deficit. - Russian Journal of Plant Physiology 44 (4): 448 -453.

MUDD J.B. 1996. Biochemical basis for the toxicity of ozone. - In: YUNUS M. & IQBAL M. (Eds.), Plant response to air pollution, pp. 267-283. - Wiley and Sons, Chicester.

O'KANE D., GILL V., BOYD P. & BURDON R. 1996. Chilling, oxidative stress and antioxidant responses in *Arabidopsis thaliana* callus. - Planta 198: 371-377.

OKAMURA M. 1980. An improved method for determination of l-ascorbic acid and l-dehydroascorbic acid in blood plasma. - Clinical Chimica Acta 103: 259-268.

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(3): 791-799.

PAHLICH E. 1993. Larcher's definition of stress: a valuable principle for metabolic adaptability research. - Brasilian Journal of Plant Physiology 5 (2): 209-216.

— 1995. Plant stress. The adaptive potential of dynamic systems. - In: BEHNKE H.-D., LÜTTGE U., ESSER K., KADEREIT J.W. & RUNGE M. (Eds.) Progress in Botany, pp. 165-200. - Springer Verlag Berlin, Heidelberg, New York, London, Paris, Tokyo, Hong Kong, Barcelona, Budapest.

PITCHER L.H. & ZILINSKAS B.A. 1996. Overexpression of copper/zinc superoxide dismutase in the cytosol of transgenic tobacco confers partial resistance to ozone - induced foliar necrosis. - Plant Physiology 110: 583-588.

— , BRENNAN E.G., HURLEY A., DEMSNUIV P., TEPPERMAN J.M. & ZILINSKAS B.A. 1991. Overproduction of petunia chloroplastic copper/zinc superoxide dismutase does not confer ozone tolerance in transgenic tobacco. - Plant Physiology 97: 452-455.

PLÖCHL M., LYONS T., OLLERENSHAW J. & BARNES J. 2000. Simulating ozone detoxification in the leaf apoplast through the direct reaction with ascorbate. - Planta 210 (3): 454-467.

ROSHCHINA V.V. & ROSHCHINA V.V. 2003. Ozone and plant cell, pp. 7-26, 27-53. - Kluwer Academic Publishers, Dordrecht, Boston, London.

SALIN M.L. 1987. Toxic oxygen species and protective systems of the chloroplast. - Physiologia Plantarum 72: 681-689.

SCHRAUDNER M., MOEDER W., WIESE C., CAMP W.,VAN INZE D., LANGEBARTELS CH.& SANDERMANN H. 1998. Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. - The Plant Journal 235 (16): 235-245.

SRIVASTAVA H.S. 1999. Biochemical defence mechanisms of plants to increased levels of ozone and other atmospheric pollutants. - Current Science 76: 525-533.

TORSETHAUGEN G., PITCHER L.H., ZILINSKAS B.A. & PELL E.J. 1997. Overproduction of ascorbate peroxidase in the tobacco chloroplast does not provide protection against ozone. - Plant Physiology 114: 529-537.

VON BERTALANFFY L., BEIER, W. & LAUE R. 1977. Biophysik des Fließgleichgewichts, pp. 33-34. - Vieweg, Braunschweig.

WINNER W.E. 1994. Mechanistic analysis of plant responses to air pollution. - Ecological Applications 4: 651-661.

ZECHMANN B., ZELLNIG G. & MÜLLER M. 2005. Changes in the subcellular distribution of glutathione during virus infection in *Cucurbita pepo* (L.). - Plant Biology 7(1): 49-57.