DOI: 10.1007/s11099-014-0070-5 PHOTOSYNTHETICA 52 (4): 604-613, 2014

Photosynthesis of ozone-sensitive and -resistant *Phaseolus vulgaris* genotypes under ambient ozone and moderate heat stress

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Abstract

Physiological responses from sensitive (S156) and resistant (R123) genotypes of ozone bioindicator, snap bean, were investigated after exposing the plants to cumulative, phytotoxic ozone amounts. Daily course of gas-exchange parameters showed delayed stomatal response in S156 leaves to environmental changes comparing to the response of R123 leaves. Potential photosynthetic quantum conversion, Stern-Volmer nonphotochemical quenching (NPQ), and maximum photochemical efficiency of PSII (F_v/F_m) values changed differently in the two genotypes between the first and last measuring days. We concluded that the higher ozone sensitivity originated at least partly from inferior regenerating and/or antioxidant capacity. Experimental protocol proved to be determinant on chlorophyll fluorescence parameters: F_v/F_m and NPQ declined at midday, and only the sensitive leaves showed a slight increase in NPQ between 12 h and 16 h. We explained these results by moderately high temperatures and shade-adapted state of our experimental plants under substantial ozone stress. On the base of temperature dependence of minimal fluorescence yield (F_0), critical temperature proved to be higher than 32.7ºC for *Phaseolus vulgaris* under these conditions. We found a strong linear correlation between NPQ and nonphotochemical quenching of F_0, indicating that NPQ was determined mostly by energy-dependent quenching (q_E). The q_E is the light-harvesting complex located component of NPQ and depends on the amount of zeaxanthin molecules bound in PSII proteins. Thus, difference between daily courses of NPQ in the two genotypes was probably due to different ways of utilization of the zeaxanthin pool under the interactive effect of ozone and moderate heat stress.

Additional key words: AOT40; chlorophyll fluorescence; gas exchange; midday depression; nonphotochemical quenching; ozone sensitivity; snap bean; shaded conditions.

Introduction

Plants manifest their response to ozone exposition in short or long terms. Short-term effects are visible symptoms on leaves and fruits and impairment of photosynthetic processes. Long-term effects are decreasing biomass production and crop yield and premature senescence (Harmens *et al.* 2006). Ozone-induced deterioration of photosynthesis is connected with the injury of photosynthetic membranes, the lowered effectiveness of electron transport and carboxylation, and the changes in synthesis and activity of Rubisco (Pell *et al.* 1997). Membranes have been shown to be among the first targets of O_3, while changes in stomatal structure have been also mentioned among the early responses to ozone (Paoletti *et al.* 2007). These phenomena usually occur prior to visible symptoms. Lipid peroxidation and changes in membrane properties due to O_3 fumigation were also described by Calatayud *et al.* (2002a, b).

Visible symptoms in *P. vulgaris* have been shown to

Received 16 July 2013, *accepted* 18 April 2014.
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Abbreviations: ABA – abscisic acid; AOT40 – accumulated O_3 exposure over a threshold of 40 ppb; C_i – intercellular CO_2 concentration; E – transpiration; F_0 – minimal fluorescence yield of the dark-adapted state; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; LHCIIb – the major chlorophyll-protein complex of LHC associated with PSII; NPQ – Stern-Volmer nonphotochemical quenching; P_N – net photosynthetic rate; Q_A – primary quinone acceptor of PSII; q_E – energy-dependent component of NPQ; q_{F0} – nonphotochemical quenching of F_0; q_{NP} – nonphotochemical quenching coefficient; q_P – photochemical quenching coefficient; R123 – ozone-resistant bean genotype; RFd – potential photosynthetic quantum conversion; ROS – reactive oxygen species; S156 – ozone-sensitive bean genotype; T_c – critical temperature; T_l – leaf temperature; VPD – vapour pressure deficit; Φ_{PSII} – effective quantum yield of PSII.
Acknowledgements: This research has been supported by the Hungarian-Slovenian Intergovernmental Science and Technology Cooperation Programme (SI-2/08). This paper could not be written without the support of the Research Station of Cegléd of Fruit Research Institute, National Agricultural Research and Innovation Centre. Authors also thank the referees for providing constructive comments and suggestions. We would like to acknowledge Zoltán Nagy for revising the final version of this paper.

be preceded by cellular damages as a consequence of H_2O_2 deposits (Gerosa *et al.* 2009). The groups of cells with H_2O_2 deposits are destined to death, finally visualizing typical ozone symptoms (Faoro and Iriti 2005). Chlorophyll (Chl) concentration of O_3-exposed plants decreased and this change is explained either by reactive oxygen species (ROS)-induced Chl damage or by acclimation, which is an attempt to avoid excessive light absorption (Castagna *et al.* 2001).

Impairment of physiological processes is widely known to precede formation of visible symptoms (Pye 1988, Fredericksen *et al.* 1996, Guidi *et al.* 2001). Photosynthesis has been broadly studied when assessing plant responses to O_3 (Fiscus *et al.* 2005). Generally, ozone decreases carbon assimilation (Miller 1987, Pell *et al.* 1997, Morgan *et al.* 2003, He *et al.* 2007, Paoletti *et al.* 2007, Neufeld *et al.* 2012), F_v/F_m (Guidi *et al.* 1997, Grams *et al.* 1999, Leipner *et al.* 2001, Fiscus *et al.* 2005), potential photosynthetic quantum conversion (RFd) (Calatayud and Barreno 2001, Calatayud *et al.* 2002b), and carboxylation efficiency, and increases F_0 (Guidi *et al.* 2000, 2002; Leipner *et al.* 2001), NPQ (Calatayud and Barreno 2001), $(1 - q_P)$/NPQ, which is an estimate of photon excess and, therefore, of the susceptibility of PSII to high irradiance (Calatayud *et al.* 2002b). Ozone-exposed plants also raise dark respiration rate to suffice increased energy demand for maintenance and repair processes (Salvatori *et al.* 2013) in many studied crop and natural vegetation species. Ozone concentrations twice of ambient caused significant decline in both light and dark reactions of photosynthesis as expressed by F_v/F_m, NPQ, reduction state of Q_A, carboxylation efficiency, maximum carbon dioxide uptake rate (PC), and other fluorescence and leaf gas-exchange parameters in *Fagus sylvatica* (Grams *et al.* 1999). According to Schreiber *et al.* (1978), water splitting enzyme system was the first to be attacked followed by inhibition of electron transport between the photosystems; such an impairment of photosynthesis happened days before visible injuries appeared on *P. vulgaris* leaves.

Studies concerning stress-protective processes highlighted the role of xanthophyll cycle and the pigment molecules involved. When the plant is stressed by elevated temperatures, the interaction of xanthophyll cycle pigments and thylakoid membrane lipid phase decreases fluidity and increases thermostability of the membranes (Havaux 1998). As this process lowers susceptibility to lipid peroxidation, it is possibly included in a general protection against oxidative stress. Zeaxanthin is also described as a photoprotection of the thylakoid membranes under strong light (Havaux *et al.* 1991). Novel studies confirm that xanthophyll cycle is involved in ozone response. Heat dissipation caused by activation of xanthophyll cycle in ozone-exposed plants is shown in the study of Gerosa *et al.* (2009). Flowers *et al.* (2007) proved that photosynthetic electron transport is affected by the pollutant at levels of PSII function and xanthophyll cycle components.

Ozone response of *P. vulgaris* has been studied by several groups (Schreiber *et al.* 1978, Guidi *et al.* 1997, Leipner *et al.* 2001, Gerosa *et al.* 2009, Burkey *et al.* 2012). Measurement of Chl fluorescence is in turn shown to be an effective and simple method for detecting O_3 stress (Schreiber *et al.* 1978, Reiling and Davison 1992, Guidi *et al.* 1997). In our study, we used sensitive and resistant genotypes of snap bean grown under well watered and shaded conditions to measure daily course of Chl fluorescence and gas-exchange parameters to get closer to the explanation of difference in O_3 sensitivity.

Materials and methods

Plant material: Experimental plants were sensitive (S156) and resistant (R123) genotypes of *Phaseolus vulgaris* L. (bush bean, French dwarf bean) used as ozone bioindicator system (Burkey *et al.* 2005) in a Slovenian ozone biomonitoring experiment, a part of the Europe-wide biomonitoring experiment of the International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops (ICP-Vegetation). For measurements, 10 sensitive and 10 resistant plants were used. Plants were grown outside in 16-L pots filled with standard potting soil mixture, shaded by knitted agricultural nets, and regularly irrigated according to the ICP-Vegetation Ozone Experimental Protocol 2008 (ICP Vegetation 2008). Seeds were sown on 9 June, the exposure of seedlings to ozone started on 15 June. The flowering of both genotypes started on 15 July.

Experimental site: The experiment was conducted in Ljubljana, in the experimental field of Biotechnical Faculty, Department of Agronomy of University of Ljubljana.

Weather conditions: Meteorological data were recorded in the field by the meteorological station of Slovenian Environmental Agency. Here we present air temperature, vapour pressure deficit (VPD), PPFD, ozone concentration, and AOT40 (accumulated O_3 exposure over a threshold of 40 ppb) data (Table 1).

Chl fluorescence: Daily courses of Chl fluorescence parameters were measured on 22, 25, and 26 July, 2009. First day of this series of measurements was the 22 July and the last day was 26 July. Hourly measurements were made in three replicates on the central leaflet of the third trifoliate leaf from the top (the second fully expanded leaf). The same leaves of each plant were used for each measurement. In case of S156 genotype, both asymptomatic and symptomatic leaves (with 5–10% injury) were

investigated by using the asymptomatic parts of each leaf for measurement.

Chl fluorescence measurements were made with a *PAM-2000* fluorometer and the dark leaf-clip *DLC-8* (*Walz*, Effeltrich, Germany). Leaves were dark-adapted for at least 25 min prior to measurement. This period was long enough for the relaxation of the fast components of nonphotochemical quenching without influencing any of the slowly relaxing components.

Maximum photochemical efficiency (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}) were calculated as $(F_m - F_0)/F_m$ and $(F_m' - F_s)/F_m'$, respectively. The Stern-Volmer nonphotochemical quenching (NPQ) was evaluated as $(F_m - F_m')/F_m'$, and the nonphotochemical quenching of minimal fluorescence (q_{F0}) as $(F_0 - F_0')/F_0'$. Chl fluorescence decrease ratio (RFd), indicating the potential photosynthetic quantum conversion (Lichtenthaler and Buschmann 1987), or photosynthetic capacity of PSII (Lichtenthaler and Rinderle 1988), was determined as $(F_m - F_s)/F_s$.

Fluorescence induction measurements were implemented by using the same constant actinic light of *ca*. 300 μmol(photon) m^{-2} s^{-1} provided by the fluorometer instead of the actual incident illumination. When calculating the current NPQ, the actual (and not the daily maximal, *i.e*. predawn) F_m was used.

Gas exchange: Daily courses of different gas-exchange parameters were studied on the first measuring day. *LI-6400XT* portable photosynthesis system (*LI-COR Inc.*, Lincoln, Nebraska USA) was used for measurements on leaves identical in age and position to those used for fluorescence measurements. Carbon dioxide assimilation (P_N), transpiration (E), stomatal conductance (g_s), and inner CO_2 concentration (C_i) under the given environmental conditions were determined in five replicates for both genotypes.

Plant injury index: Following the ICP Vegetation experimental protocol the initial mean percentage ozone injury of genotypes was calculated according to Calatayud *et al.* (2007).

Statistics: All measurements were carried out at least in three replicates on both S156 and R123 genotypes during the hourly measurements between morning (8–9 h) and evening (20–21 h) hours. For statistical analysis of data we used *IBM SPSS Statistics 20* software. Repeated measures of analysis of variance (*ANOVA*) were applied to test the differences between results measured on resistant, asymptomatic, and symptomatic sensitive leaves. Significant differences (p>0.05) among the groups were tested by *Tukey*'s post-hoc multiple comparisons. Trends of physiological responses in time were checked by the statistics of regression analysis, while trends of different genotypes were compared by *ANCOVA*.

Results

Meteorological conditions and AOT40: During the time period from sowing to the time of measurements, temperature was relatively high (Fig. 1). In contrast to the sunny and hot first and fifth day, 25 July (fourth day of this experiment) was slightly rainy around noon accompanied by a drop in temperature. After the rain stopped, temperature climbed to 30ºC in the afternoon. The weather on the 23 and 24 of July (second and third day of the experiment) was very hot with high tropospheric ozone concentrations. The daily maximal ozone concentration and AOT40 were the highest on the first day, the lowest on the fourth day, and showed intermediate values on the fifth day (Table 1). On the intervening two days (23 and 24 July, second and third days of the experiment) these parameters were also high and medium, respectively. The cumulative AOT40 between the beginning of of the first and the end of the last day of this experiment was 1,378 ppb h, considered as a serious ozone load (Fig. 1).

Table 1. Daily maximum and median values of the ozone concentration, and the daily cumulative accumulated O_3 exposure over a threshold of 40 ppb (AOT40) values on one day before and during the measuring period in summer 2009. The days of physiological measurements are marked *in bold*.

Date	Day of the experiment	O_3 concentration [ppb]		AOT40 [ppb h]
		maximum	median	
July 21	0	73.1	45.3	249.6
July 22	**1**	**78.1**	**69.9**	**473.1**
July 23	2	66.2	54.5	301.2
July 24	3	69.9	38.9	235.1
July 25	**4**	**57.9**	**36.0**	**124.2**
July 26	**5**	**69.4**	**39.6**	**243.9**

Fluorescence: F_v/F_m did not show substantial changes during the day, but F_v/F_m values were slightly lower in the middle of the day compared to the values measured in the morning or in the evening hours. While on the first measuring day only the resistant plants showed this tendency (the slope of the afternoon F_v/F_m increase was significantly different from zero, p=0.011), sensitive ones showed the same tendency on the fifth day. Both the decrease before noon and the increase in the afternoon proved to be significant in case of both genotypes. During the fourth day, F_v/F_m of asymptomatic and symptomatic sensitive leaves presented a continual decline in both cases (Fig. 2).

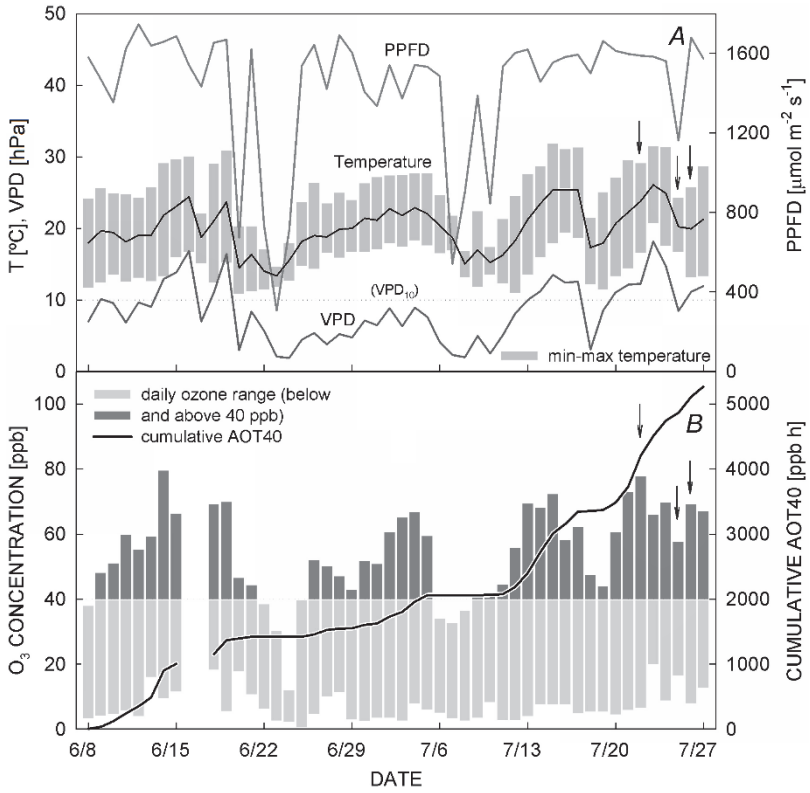

Fig. 1. (*A*) Minimum, maximum, and average air temperatures (T), PPFD, and vapour pressure deficit (VPD) values (VPD_{10} – 10 hPa VPD threshold), (*B*) tropospheric ozone concentration and cumulative AOT40 values (ozone concentrations over a phytotoxic threshold of 40 ppb) in Ljubljana, during the life cycle of our experimental plants between 8 June and 26 July, 2009. *Arrows* indicate the days of diurnal field measurements.

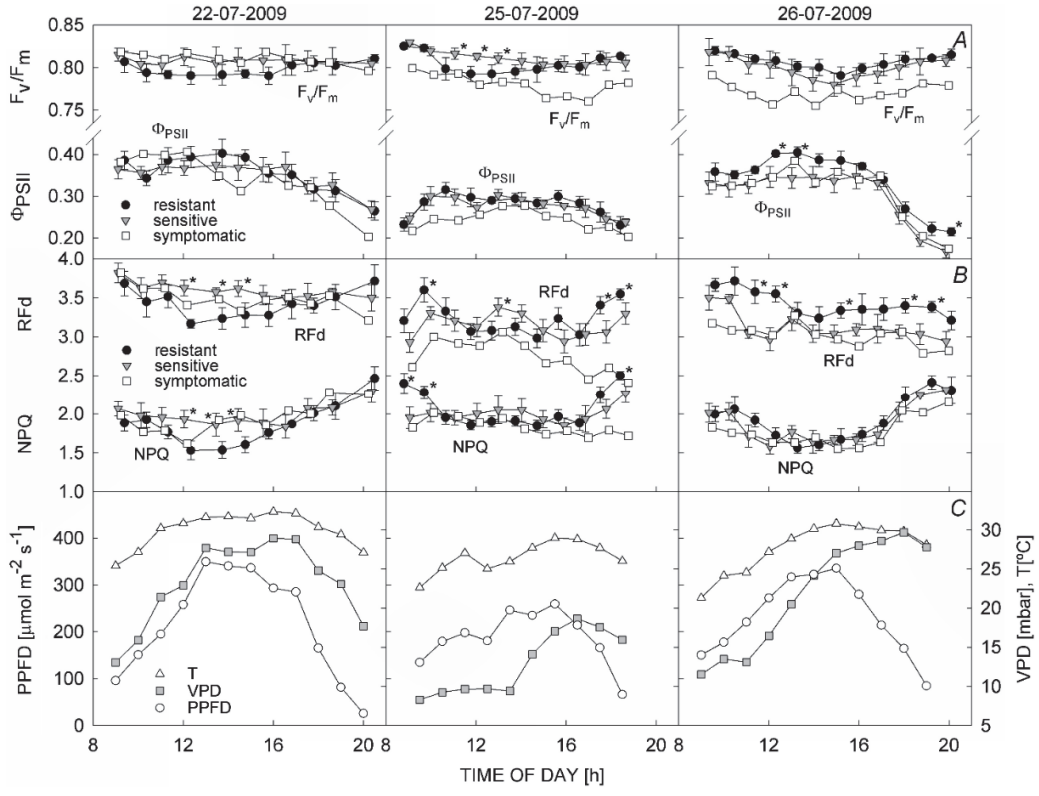

Fig. 2. (*A*) Daily courses of maximum photochemical efficiency of PSII (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}), (*B*) nonphotochemical quenching (NPQ) and potential photosynthetic quantum conversion (RFd) of ozone-resistant, -sensitive, and symptomatic sensitive bean plants. Each result represents the mean of three individual plants (± SD). (*C*) PPFD, vapour pressure deficit (VPD), and temperature (T) in Ljubljana on 22, 25, and 26 July, 2009, (on the 1st, 4th, and 5th days of the experiment). *Asterisks* indicate significant differences ($p<0.05$) between resistant and asymptomatic sensitive results.

On the first day of the experiment, which was the hottest day with the most significant phytotoxic ozone exposure, resistant genotype had the lowest daily average F_v/F_m. On the two subsequent measuring days (4th and 5th days of the experiment) this value gradually increased, and the resistant plants had already the highest F_v/F_m values on the 5th day. Average daily F_v/F_m of asymptomatic sensitive leaves did not change substantially, but that of the symptomatic leaves presented a significant decrease through the experiment. Regarding Φ_{PSII} neither genotypes nor measuring days differed. Average value of Φ_{PSII} was around 0.4 which was reduced by half in the late afternoon as a function of daily maximum temperature and/or ozone load. Within the range of registered air temperature, F_0 maintained high stability without any increase with rising temperature, whereas F_m decreased with temperature in both genotypes (Fig. 3). Only symptomatic leaves showed different F_m responses with significant decline and high variability at high temperatures; but this probably resulted from the adverse effect of ozone on Chl fluorescence.

Since we applied constant actinic light intensity during the whole fluorescence study, degree of NPQ did not run along with actual incident illumination. Apart from an initial change in the early hours, NPQ did not increase during the day. Moreover, it unusually decreased in resistant plants between the hours of late morning and early afternoon. This dynamics was also shown by sensitive plants on the last measuring day. Quenching of F_0 was closely correlated with NPQ of F_m, and this relationship proved to be genotype-independent (Fig. 4). Daily course of RFd was similar to NPQ, with somewhat lower midday values in the resistant genotype. When comparing RFd values of different days, they seemed to change together with F_v/F_m. Namely, it was lower in the resistant genotype on the first day of the experiment, but it

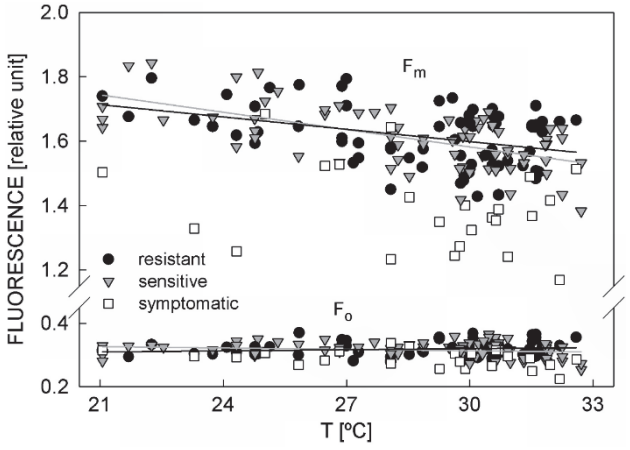

Fig. 3. Temperature (T) dependence of minimum (F_0) and maximum (F_m) chlorophyll fluorescence of resistant, asymptomatic, and symptomatic sensitive bean leaves. Data measured on the cloudy 25 of July (4th day) were omitted. Trends for symptomatic leaves were not fitted because these values were strongly affected by additional factors.

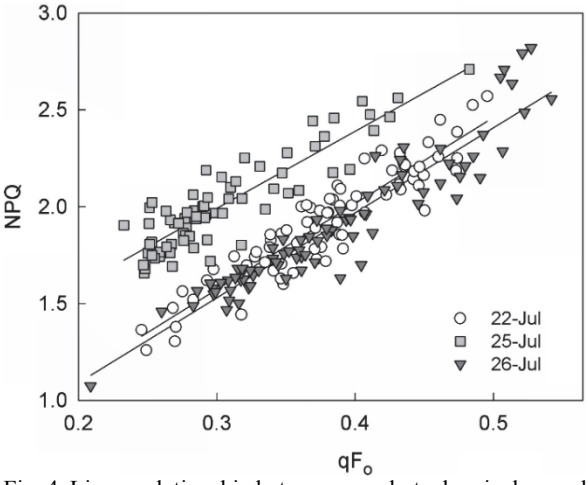

Fig. 4. Linear relationship between nonphotochemical quenching of F_0 (q_{F0}) and nonphotochemical quenching (NPQ) on 22 July (1st day, $r^2 = 0.857$), 25 July (4th day, $r^2 = 0.799$,) and 26 July (5th day, $r^2 = 0.890$). The slopes of trend lines are identical, while the intercept of the cloudy 25 July is significantly higher than the other two ($p<0.001$).

gradually increased from day to day, while it decreased in time in the sensitive genotype. This decrease was especially remarkable in symptomatic leaves on the fourth day.

Fig. 5 shows daily course of gas-exchange parameters on the first measuring day. Plant injury index was 0.16% for resistant plants and 2.96% for sensitive ones at the time of the experiment. According to the infrared gas analyser (IRGA) recorded environmental data during measurements under the shading net, light intensity, temperature, and VPD showed an even daily course without abrupt changes between data recordings (Fig. 2*C*). P_N changed along with the light intensity. Since experimental plants were continuously well watered, E changed according to VPD and T_l, without midday depression. While P_N of the two genotypes did not differ (Fig. 5*A*), E of the R123 was smaller than E of S156 and started to decrease from 14–15 h, while E of S156 declined only after 16 h (the average difference between the E of R123 and S156 was about 1.4 mmol m^{-2} s^{-1} in the late afternoon). This difference was also seen in the course of g_s which remained more or less steady in R123 between 8 h and 14 h before it started to drop, while in S156 it started to fall only after 17 h and showed higher values almost all day comparing to R123 (Fig. 5*B*).

We concluded that daily water loss of sensitive genotype was more significant, while both genotypes had similar P_N. As a consequence, water use was more efficient in the resistant than in the sensitive plants. Difference in C_i of the two genotypes in the afternoon implied that R123 maintained the same level of P_N in spite of its lower g_s values (Fig. 5*A*). As a consequence of higher E, T_l of sensitive plants was almost 1°C lower (0.6°C) after 16 h (Fig. 5*C*). In contrast to the first day, E and g_s of both genotypes did not show difference on the last measuring day (Fig. 6).

Discussion

In accordance with the protocol of the ICP-Vegetation, the experimental plants grew under shaded conditions. Consequently, the plants were not exposed to strong or even saturating irradiation [maximal daily light intensity peaked around 400 μmol(photon) m^{-2} s^{-1}], while air temperature was 1°C warmer than that in the open field which resulted in slightly increased VPD compared to open field conditions on sunny days. Daily maximal temperature on the first and fifth day exceeded 32°C, which can be considered as moderate heat stress.

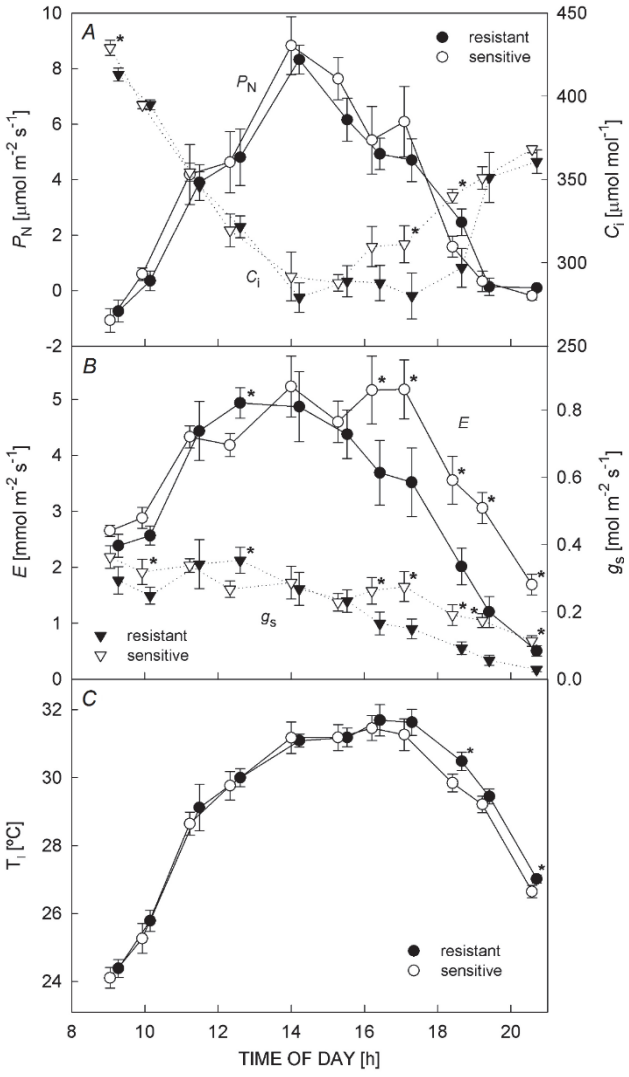

Fig. 5. (*A*) Daily course of net photosynthetic rate (P_N) and intercellular CO_2 concentration (C_i); (*B*) daily course of transpiration (E) and stomatal conductance (g_s); and (*C*) daily course of leaf temperature (T_l) of resistant and sensitive bean genotypes on the first day of the experiment. Each result represents the mean of data measured on five individual plants (± SD). *Asterisks* indicate significant differences (p<0.05) between resistant and sensitive plants.

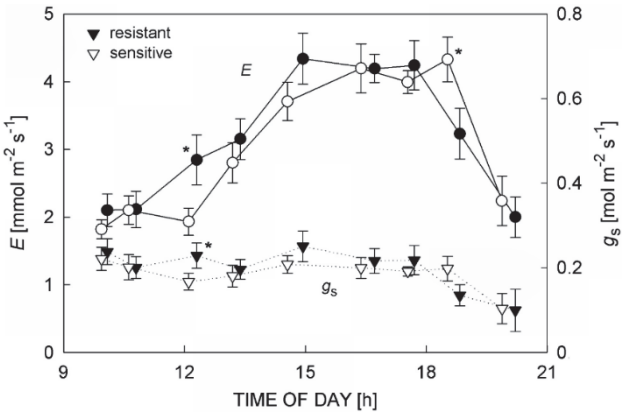

Fig. 6. Daily course of transpiration (E) and stomatal conductance (g_s) of resistant and sensitive bean genotypes on the last day of the experiment. Points represent means of data measured on five individual plants (± SD).

Transpiration, as a control process of leaf temperature, consistently followed the daily course of air temperature, however, it started to decrease earlier in the resistant plants due to reduced g_s. Stomatal conductance and transpiration were higher consequently, T_l remained somewhat lower in sensitive plants in the afternoon (Fig. 5). On the first day, both the maximal ozone concentration and the daily cumulative AOT40 were extremely high which could force stomatal closure in the R123 genotype. It is known that ozone induces the formation of ROS, which are natural components of the abscisic acid (ABA)-induced signal transduction pathway of stomatal closure in vascular plants (Zhang *et al.* 2001). Accordingly, induction of stomatal closure is possibly an early effect of phytotoxic ozone. If operation of this ABA-induced signal transduction pathway is incomplete or less responsive to ROS in the sensitive genotype than in the resistant one, initial doses of phytotoxic ozone is expected to trigger stomatal closure only in the resistant plants lowering the amount of O_3 absorbed by leaves. As a result, while E and g_s of the sensitive plants follow the diurnal course of air temperature alterations, these parameters decrease earlier in the resistant plants due to increasing O_3 stress. Therefore ozone uptake of the sensitive genotype may be larger and this extra quantity of O_3 leads to the overloading of the antioxidant system and induces oxidative damage. The arising free radicals induce a hypersensitive reaction (Apel and Hirt 2004) and the typical formation of necrotic symptoms. Despite the reduced g_s in the resistant genotype, P_N remained similar to the rate of the sensitive plants, which resulted in a smaller C_i than in the other group (Fig. 5). According to Fiscus *et al.* (2005), unaltered photosynthetic capacity along with stomatal closure also indicates the direct effect of O_3-generated H_2O_2 molecules on stomata.

Decline in F_v/F_m and growth of NPQ in the middle of sunny days are typical in leaves exposed to direct irradiation in the open field (*e.g.* Balaguer *et al.* 2002, Shirke and Pathre 2003). However, limited maximal light intensity can alter this typical course of both parameters, since shade-adapted plants have relatively low xanthophyll content (Thayer and Björkman 1990), therefore they are more sensitive to heat and photoinhibition (Demmig-Adams *et al.* 1995, Sun *et al.* 2007) and have lower antioxidant capacity (Sarry *et al.* 1994, Havaux and Niyogi 1999, Havaux *et al.* 2007) as compared to sun leaves.

The extent of NPQ is basically determined by the pH gradient across the thylakoid membrane (Peguero-Pina *et al.* 2013) which is primarily the function of light intensity. Therefore, if the actinic light is the actual incident illumination during diurnal fluorescence study, the daily course of NPQ follows the diurnal change in light intensity. However, if the intensity of actinic light is always the same, as it was in our experiment, the level of NPQ should be constant, and any change indicates some other, light independent effect on the NPQ forming processes. For example, marked effect of temperature transition was demonstrated on NPQ by Pollet *et al.* (2009). Under shaded light conditions [200 μmol(photon) m^{-2} s^{-1}] they investigated *Phalaenopsis* plants exposed to warm day/cool night conditions for 48 h in a greenhouse-climate chamber transfer experiment. For fluorescence kinetic studies they also applied constant actinic illumination. Daily pattern of fluorescence kinetics showed abrupt changes of NPQ and Φ_{PSII} upon transition from day (37.2°C in the greenhouse) to night (18.5°C in climate chamber) and *vice versa*. During the day, courses of Φ_{PSII} and NPQ were inversely related to air temperature, while F_v/F_m showed a light dependent response. In our experiment as well, time course of NPQ inversely followed temperature changes. Thus, the cause of observed midday decrease in NPQ could be also that the temperature rose above 30°C. Because the moderately high-temperature-induced increase in thylakoid membrane fluidity, ionic conductance, and lipid peroxidation can directly affect the rate of nonphotochemical quenching. In addition, elevated temperature was accompanied with very high ozone concentrations. Maximal daily temperature in our experiment was 32.7°C. On the basis of temperature dependence of F_0, this value is lower than the critical temperature (T_c) for *P. vulgaris* (Fig. 3). Critical temperature varies extensively among species and shows pronounced seasonal dependency (Weng and Lai 2005). Its value is defined as the temperature at which F_0 starts to increase sharply (Bilger *et al.* 1984). Although T_c is lower for shade-adapted plants, we did not experience any increase in the temperature dependency of F_0. However, F_m revealed a distinct continual decline between 21 and 33°C (Fig. 3). These findings are in accordance with the temperature dependent fluorescence yield results of Pospíšil *et al.* (1998).

We found an extremely strong, however, genotype-independent relationship between q_{F0} and NPQ (Fig. 4). This means that changes in NPQ basically resulted from the reduction of q_E, which is the predominant, xanthophyll cycle–related component of NPQ, and is located in the light-harvesting complex. Zhang *et al.* (2009) observed similar decrease in q_E as a result of moderate heat stress (40°C) in tobacco leaves. Besides the relatively high temperature, extremely high ozone concentration and AOT40 could also adversely affect the formation of NPQ, contributing to the experienced NPQ decline. Zeaxanthin has two other important complementary functions besides its role in the dissipation of excess energy protecting PSII against photoinhibition. It stabilizes thylakoid membranes by reducing lipid fluidity under high temperature stress (Subczynski *et al.* 1992, Havaux *et al.* 1996, Havaux 1998), and it can act as antioxidant preventing lipid peroxidation (Lim *et al.* 1992, Sarry *et al.* 1994, Havaux *et al.* 2007). Thus, ozone-induced oxidative stress can evoke complementary alteration in zeaxanthin utilization to the detriment of NPQ. Calatayud and Barreno (2004) also found the NPQ decrease under O_3 fumigation which they explained as an effort to maintain photochemical quenching in spite of its loss due to O_3-induced oxidative stress. They asserted that this defense reaction contributed to higher resistance to ozone in one of the studied lettuce varieties. Furthermore, they found a connection between photosynthetic pigment concentrations and NPQ decline.

In contrast to the resistant plants, neither decrease in NPQ nor early stomatal closure was observed in the sensitive plants on the first day. On the last measuring day, however, NPQ and F_v/F_m of asymptomatic sensitive leaves also declined by increasing temperature, and, in contrast to the first day, g_s of the two genotypes did not differ (Figs. 2*A,B*; 6). Retarded reaction of the sensitive genotype to growing temperature and cumulative AOT40 could be related to susceptibility to ozone in which delayed or minor utilization of zeaxanthin as an antioxidant may play a role. In conclusion, difference in physiological behaviour of both genotypes, which was recognizable on the first day, disappeared by the end of the experiment. This implies more severe oxidative damage in the sensitive plants probably because of delayed induction of protective processes. Remarkable operative perturbation and growing extension of visible symptoms in symptomatic leaves (data not shown) supported this conclusion.

F_v/F_m values did not change in lettuce plants exposed to ozone fumigation (Calatayud *et al.* 2002b). However, O_3-treated plants showed lower Φ_{PSII} (PSII efficiency) and q_P (fraction of open PSII centers) indicating the decline in quantum efficiency of noncyclic electron transport and in capacity for reoxidizing Q_A. Simultaneous increase in NPQ was observed in fumigated plants (which is in contrast to our findings). These facts may imply down-regulation of photosynthesis. RFd, indicating the potential quantum yield conversion (Lichtenthaler and Buschmann 1987), decreased with ozone fumigation, which is similar to the results of Ruth and Weisel (1993) and is in accord with our results, as RFd decreased with time in the

sensitive bean genotype. In our study, values of F_v/F_m ranged between 0.82 and 0.79 in the resistant and asymptomatic sensitive leaves which coincides with the typical interval for healthy plants. Maximum photochemical efficiency dropped below 0.8 only in the symptomatic leaves on the fifth day, with the lowest value of 0.75, which decline is attributable to severe ozone-induced damage in PSII photochemistry (Lorenzini *et al.* 1999, Calatayud and Barreno 2004, Degl'Innocenti *et al.* 2007). Accordingly, neither the temperature of 32°C, nor ozone loading had really adverse effect on the function of PSII in the resistant and asymptomatic, sensitive leaves. Moderately high temperature can induce state transition by the uncoupling of LHCIIb from the PSII core complex (Armond *et al.* 1980). This change causes a decline in the energy conversion efficiency of PSII (state-transition quenching, q_T), which could be the reason of the observed slight, transient decrease in F_v/F_m of the resistant plants on each day and in F_v/F_m of the asymptomatic, sensitive plants on the fifth measuring day (heat-induced state transition can take place even in dark and has a longer relaxation than the 25-min dark adaptation). However, on the base of q_{F0}-related NPQ decline, this small increase in q_T was negligible beside the q_E reduction caused by our assumed alteration in zeaxanthin distribution.

Substantial differences between the consecutively recorded daily courses were caused by the sharp rise in accumulated ozone exposure between 22 and 26 July (growth of cumulative AOT40 between the first hour of 22 and the last hour of 26 July was around 1,380 ppb h, Fig. 1). Effect of cumulative AOT40 was amplified by irrigation, as, under irrigated conditions, reduced stomatal closure leads to more significant stomatal ozone uptake. Gradually increasing impairment of photosynthetic processes in the sensitive leaves was traceable in changes in F_v/F_m, NPQ, and RFd parameters measured on the three different days. Since sensitive and resistant plots of F_v/F_m and RFd interchanged during the five-day measuring period (Fig. 2), difference between the two genotypes in these parameters was rather consequence than cause of their different ozone sensitivity.

The plants had been already exposed to significant ozone stress before the first measurements were done: cumulative AOT40 from the start of exposure to the first experimental day (during 37 days) was about 2,785 ppb h on the first measuring day. However, protective and regeneration processes could protect and, to some extent, regenerate the photosynthetic apparatus. Since the first experimental day (22 July) and the previous day were characterized by extremely high AOT40 values (Table 1), it seems reasonable to presume that this considerable ozone stress was perceptible on the photosynthetic activity of the resistant genotype as well. Different behaviour of the sensitive plants is possibly attributable to the insufficient regeneration processes. This theory is supported by the fact that the sensitive genotype showed further decrease in the abovementioned parameters on the fourth and fifth measuring days, when AOT40 values were somewhat lower (Table 1). The resistant plants were able to adapt to ozone and to regenerate photosynthetic processes, which was apparent from recovering RFd and F_v/F_m values. Since no considerable dissimilarity could be observed in either of the parameters, we conclude that poor efficiency of antioxidant and/or regenerating processes was probably the most important cause of ozone sensitivity.

In addition to excess energy dissipation and membrane rigidification, free zeaxanthin behaves mostly as antioxidant in the lipid phase of thylakoid membranes, where it can act as the scavenger of singlet oxygen and ROS as well as the terminator of peroxy-radical chain reactions (Lim *et al.* 1992, Sielewiesiuk *et al.* 1997). Thus, differently partitioned free and bound zeaxanthin fractions may cause difference in antioxidant capacity. Because of uncertainty in the methodology of fractionation, *in vivo* partitioning of zeaxanthin is presently unknown (Hieber *et al.* 2004). However, examination of the utilization of xanthophyll and proportion of different zeaxanthin fractions in leaves under the interacting effects of ozone and heat stress could lead to a broader understanding of the stress response and defense mechanisms of different plant species. In addition, such a method would possibly contribute to the revealing of the cause of difference in O_3 sensitivity among different plant genotypes.

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