

Physiological disturbances promoted by ozone in five cultivars of *Phaseolus vulgaris* L.

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Received: 17 November 2008; Accepted: 16 March 2010.

ABSTRACT

Bean seedlings of Fepagro 26, Guapo Brilhante, Iraí, Macotaço and US Pinto 111 cultivars were submitted to treatments with or without addition of ozone to the ambient air, in order to evaluate the effects of exposure on photosynthesis, relative electrolyte leakage, foliar abscission and biomass of the seedlings. Exposure to ozone caused significant decreases in the net assimilation of all cultivars except Iraí. It also caused a significant increase in electrolyte leakage from the Pinto cultivar, but only when AOT₄₀ was the highest. It also produced significant anticipation in the time of foliar abscission in the Pinto, Fepagro and Guapo cultivars. The variability observed in the biomass measurements reflected the limitations to perform long-term controlled-environment studies, one of the major challengers yet to be overcome in order to obtain more conclusive data on damages induced on crop species resulting from tropospheric ozone enrichment.

Key words: photosynthesis, relative electrolyte leakage, foliar abscission, biomass.

RESUMO

Distúrbios fisiológicos promovidos pelo ozônio em cinco cultivares de *Phaseolus vulgaris* L. Plântulas de feijão das cultivares Fepagro 26, Guapo Brilhante, Iraí, Macotaço e US pinto 111 foram submetidas aos tratamentos com e sem adição de ozônio ao ar ambiente, com o objetivo de avaliar os efeitos da exposição sobre a fotossíntese, permeabilidade relativa a eletrólitos, abscisão foliar e biomassa das plântulas. A exposição ao ozônio causou decréscimos significativos na assimilação líquida de todas as cultivares, exceto na Iraí. Causou também um aumento significativo na permeabilidade relativa a eletrólitos da cultivar Pinto, mas apenas quando a AOT₄₀ foi a mais elevada. Além disso, produziu uma antecipação significativa no tempo de abscisão foliar nas cultivares Pinto, Fepagro e Guapo. A variabilidade observada nas medidas de biomassa total, refletiu as limitações para se executar estudos de longo prazo em condições ambientais controladas, um dos principais desafios ainda por ser superado antes que se possa obter dados mais conclusivos sobre os danos em espécies cultivadas resultantes do enriquecimento do ozônio topográfico.

Palavras-chave: fotossíntese, permeabilidade relativa a eletrólitos, abscisão foliar, biomassa.

Abbreviations: A, Instantaneous net assimilation per unit leaf area; A_{\max} , Maximum assimilation; AOT_{40} , Hourly accumulative O_3 concentration over the 40 ppbv threshold; ATP, Adenosine triphosphate; C_i/C_a , Ratio of the concentration of CO_2 in the leaf intercellular air space and the bulk CO_2 ; CO_2 , Carbon dioxide; E, Transpiration per unit leaf area; ce, Carboxylation efficiency; ROS, Reactive oxygen species g_s , Stomatal conductance to CO_2 diffusion; J_{\max} , Maximum rate of electron transport; Ls, Stomatal limitation; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; O_3 , Ozone; RuBP, Ribulose-1,5-biphosphate; $V_{c\max}$, Maximum rate of carboxylation of rubisco; REL, Relative electrolyte leakage; WUE, Water use efficiency.

INTRODUCTION

Ozone (O_3) is present in low concentrations throughout the atmosphere and in the stratosphere is beneficial, since its O_3 layer prevents potentially damaging ultraviolet light from reaching the Earth's surface. However, when O_3 is continuously formed in the troposphere it becomes an air pollutant with harmful effects on a wide variety of plants, including native vegetation and important crops. In the last few decades, anthropic activities have induced significant increases in tropospheric ozone concentrations on a global scale (Bray et al., 2000). The majority of ground level O_3 formation occurs when precursor gases such as nitrogen oxides (NOx), carbon monoxide (CO) and several volatile organic compounds, react in the atmosphere in the presence of sunlight. Industrial emissions, motor vehicle exhaust, and chemical solvents are the major anthropogenic sources of these precursors. Ozone, which is part of the group of photochemical oxidants, has a harmful effect on vegetation and animals because of its high oxidative power (Halliwell and Gutteridge, 2007), thus intensifying the formation of reactive oxygen species (ROS) in the cells (Bray et al., 2000).

In human beings, O_3 or its derived free radicals are associated with the increased incidence of lung diseases; irritated eyes, nose and throat; nausea; headache and prematurely aged skin (Klumpp et al., 2001). In plants several physiological and biochemical processes associated with carbon assimilation and, thus, with vigor and productivity, are adversely impacted by O_3 , and some evidences suggest that this pollutant could be more damaging to vegetation than all other air pollutants together (Elagöz and Manning, 2005).

According to Heggstad (1991) and Nali et al. (1998), sensitivity to O_3 differs among species and even among cultivars and ecotypes, but the mechanisms involved in this differential susceptibility has not yet been well understood. Moreover, it would be very useful to define the characteristics of the plants that can be used as indicators of their sensitivity potential to O_3 exposure, but the key parameter is not yet known (Ribas et al., 2005). The factors that determine plant

sensitivity or tolerance have not yet been clarified, but it seems clear that they involve physiological, anatomical, biochemical and environmental aspects (Alonso et al., 2001). Therefore, the ranking of relative resistance to ozone often varies with the criterion used for its determination (Pearson et al., 1996).

Among the few articles that concern sensitivity to O_3 for native and naturalized species, most are based, for the short term, on observing the development of visible symptoms, and for the long term, on the effects on growth and reproductive effort (Bungener et al., 1999). However, these parameters are not always easy to interpret, since they are influenced by several other environmental factors or take a long time to evaluate. Furthermore, they must be considered evidence of a physiological and/or biochemical response to the pollutant (Scebba et al., 2006) and even so, visible damage does not necessarily indicate sensitivity in terms of growth reduction. Nonetheless, a close relationship has been shown between the appearance of some symptoms and different degrees of susceptibility to the pollutant (Guidi et al., 1998).

Of all physiological processes affected by O_3 , photosynthesis appears to be particularly sensitive and reductions in this process may occur even before appearance or in the absence of any visible symptoms (Guidi et al., 1997). According to Pell et al. (1994), the photosynthetic process may be affected by the occurrence of damage involving stomatal movements, the collection of light or the biochemical stage of CO_2 fixation. The total photosynthetic capacity of the plant may also be reduced by loss of leaves (accelerated senescence, and/or reduced production), and by the increased respiratory rate to perform functions such as repairing cell constituents, or by the increased production of certain compounds (ATP and reductant compounds) needed for detoxification and defense (Mooney and Winner, 1988). On the other hand, the biomass responses are part of the ozone-induced physiological changes, and thus they can be used as a valuable criterion to classify the sensitivity of species/cultivars to this pollutant (Ribas et al., 2005). Finally, relative electrolyte

leakage is a precious tool to evaluate the lipid damage that so commonly occurs under oxidative stress conditions.

Based on the above, and taking into account that the current critical levels of O₃ to protect agricultural crops, natural vegetation and forest species against adverse effects are based on dose-response relations derived mainly from experiments with open-top chambers (Grünhage et al., 2001), a plant gasification system was built with an ozone -enriched atmosphere. The main objective of this work was to evaluate the effects of ozone exposure on five bean (*Phaseolus vulgaris* L.) cultivars, four of them developed by FEPAGRO North Coast (located in Rio Grande do Sul, Brazil), and one that is known to be ozone sensitive, US Pinto 111 (Arndt et al., 1987), using for this purpose photosynthetic parameters, relative electrolyte leakage, foliar abscission and total biomass of the seedling.

MATERIALS AND METHODS

The chambers: Based on the model developed by Mandl et al. (1973) and Heagle et al. (1973), and improved by Aidar et al. (2002), two open-top chambers were built, 90 cm in diameter by 106 cm high. In one of them an ozonizer was connected (OZ Engenharia, model GHR150B, Porto Alegre, Brazil), equipped with three O₃ production cells, corona effect, with a nominal production of 85 mg.h⁻¹ each.

Cultivation conditions: The bean seeds (*Phaseolus vulgaris* L.) of the Fepagro 26, Guapo Brilhante, Macotaço, Irai and US Pinto 111 cultivars were sown in plastic 2-liter bottles, cut at a height of 20 cm, with a perforated base and involved with brown paper. The culture substrate used was prepared with a volume proportion of 2:1:1 of vermiculite (medium), sand (medium and washed), and peat, respectively, which was previously homogenized. In each vase three seeds were placed and before the beginning of exposure the excess seedlings were removed leaving only a single seedling per vase.

Until the time of exposure, the seedlings were kept on a bench at the Center of Ecology of the Federal University of Rio Grande do Sul (UFRGS), in the municipality of Porto Alegre – RS, under a sombrite-type mesh (cutting 30% radiation) and in the unpolluted ambient air. The vases were irrigated daily, and one day before the beginning of exposure, each vase received 100 mL of fertilizer solution (4 g L⁻¹ of Vitaplan Nutriverde 13-13-15 + Micronutrients - Nutriplan Products Company, Paraná, Brazil).

Fumigation: Seven fumigation events (experiments) were performed in all, the first four of them during the period from

December 2006 to March 2007, and the last three during the period from September to December 2007. In all of them, eight days after sowing (stage V₂, according to Elagöz and Manning, 2005), the seedlings were submitted to treatments with and without the addition of ozone to the ambient air inside the two open-top chambers, next to the Center of Ecology at UFRGS, in a fenced-in area of 9 m² lined with sombrite-type mesh (cutting 30% radiation), in the open air. The period of exposure was always one week, with daily fumigation from 1000 to 1600 h.

During exposure each chamber was irrigated daily with five liters of water, and in each of the 7 experiments performed, 5 seedlings/treatment/cultivar were used, a total of 50 seedlings per experiment, i.e. 25 seedlings per chamber. The distribution of the plants within each chamber was done by drawing lots, but the position of each plant was not changed during the period of exposure. However, it should be emphasized that there was rotation of the chambers, i.e. in each lot of the experiment, the ozonized and non-ozonized chamber were alternated.

Ozone concentration inside the chambers was monitored by the iodometric method (APHA, 1992). Gases were sampled in a fresh washer of the impinger type, containing 75 mL of absorbent solution of KI 2% with the help of a gas sampler (LaMotte, model BD, Chestertown, USA) at a flow of 1.5 L min⁻¹. The sampling point was located in the central position of the chambers in relation to their diameters, and at the height of the unifoliated primary leaves. The concentrations at each exposure and in each chamber can be viewed in table 1. In order to compare the ozone doses fumigated in each lot of the experiment, the AOT₄₀ (AOT – Accumulative Exposure over a Threshold) criterion was adopted, which is simply the hourly accumulative O₃ concentration over the 40 ppbv threshold (expressed in ppb h) (Martins and Rodrigues, 2001).

Table 1. Ozone Concentrations in Each of the Experiments in the Fumigation Chamber

Experiment	Hourly accumulative O ₃ concentration over the 40 ppbv threshold AOT ₄₀	Hours of exposure above 40 ppbv h	Maximum concentration of O ₃
	ppb h		ppbv
1	154	18.0	59
2	209	7.5	150
3	153	23.7	52
4	587	18.0	118
5	304	6.0	91
6	147	5.4	67
7	212	24.0	60

*In the control chamber, the presence of O₃ was only found in experiments 2 (20 ppbv) and 7 (25 ppbv).

Photosynthetic parameters: The response curves to CO₂ (A/Ci curves), performed after the end of the first, fourth and fifth experiments were obtained by using an infrared gas analyzer (CIRAS 2, PPSystems, Hitchin, UK), with a cuvette measuring 4.5 cm², photosynthetically active radiation of 800 μmol.m⁻².s⁻¹, chamber temperature at 25°C and humidity adjusted to 60% of relative ambient air humidity. The measurements were performed during the period from 0800 to 1700 h, always on the unifoliated primary leaves, in a room at the Center of Ecology at UFRGS, with controlled temperature between 24-26°C. In addition, care was taken to measure always in pairs, i.e, exposed and non-exposed seedlings of a same cultivar were measured sequentially.

Based on the data extracted from the A/Ci curves, the following parameters were estimated: instantaneous net assimilation (A); maximum assimilation (A_{max}); carboxylation efficiency (ce); maximum rate of carboxylation of rubisco (V_{cmax}); maximum rate of electron transport (J_{max}); stomatal limitation (L_s); stomatal conductance (g_s); transpiration (E); CO₂ ratio of the leaf intercellular air space and ambient CO₂ (C_i/C_a); water use efficiency (WUE) and respiration.

To obtain instantaneous net assimilation, stomatal conductance, transpiration and C_i/C_a ratio, the first point on the A/Ci curve of each plant (C_a of approximately 350 μmol.m⁻².s⁻¹) was used.

The estimation of maximum photosynthetic assimilation and carboxylation efficiency was obtained from the adjustment of curve A/Ci to the equation proposed by Prado and Moraes (1997).

Maximum rate of carboxylation of rubisco, maximum rate of electron transport and respiration were estimated from the A/Ci curves with the help of the Photosynthesis Assistant program, version 1.1.2 (Parsons and Ogston, 1998), which is based on the von Caemmerer and Farquhar equations (1981).

The stomatal limitation was calculated from the response curves to CO₂ using the equation developed by Farquhar and Sharkey (1982), modified by T. D. Sharkey.

Finally, the water use efficiency in each plant was calculated from the ratio between net photosynthetic assimilation and the transpiration of the first point on curve A/Ci, where the CO₂ concentration is close to ambient.

Relative electrolyte leakage: In order to evaluate the relative electrolyte leakage, measured after the end of the first,

fourth and fifth experiment, 10 disks of 6.7 mm in diameter each were used, extracted from the unifoliated primary leaves, washed and incubated in three times distilled water. After 24 h incubation at ambient temperature, the electrical conductivity of the solution was measured in a conductivity meter (WTW, model LF197). The disks were then frozen in liquid nitrogen and then immersed in three times distilled water for over 24h, after which the electrical conductivity was measured again. The relative electrolyte leakage (REL) was calculated according to the following equation:

$$REL (\%) = \left(\frac{C_1}{C_1 + C_2} \right) \cdot 100$$

Where: C₁ = conductivity measured during the first 24 h,

C₂ = conductivity measured during the following 24h.

Foliar abscission: Foliar abscission, evaluated in the third and seventh experiment, was calculated from the average of the number of days after appearing, that the two unifoliated primary leaves took to fall off.

Dry mass accumulation: The total biomass of each seedling, both in the second and the sixth experiment, was washed in running water, dried in an oven for seven days at 60°C and weighed on a semi-analytic scale (Sartorius, 2006 MP).

Statistical analysis: The data obtained were analyzed in the Sigmapstat SPSS Institute Inc., version 2.03) statistical program, using the t-Test. When the data was not homogeneous as to variances and normality of distribution (required to apply this analysis), the Mann-Whitney non-parametric test was performed. The significance criteria adopted were values of α ≤ 0.05 and α ≤ 0.10 was considered the tendency.

RESULTS AND DISCUSSION

Photosynthetic parameters: The photosynthetic parameters were evaluated in the first, fourth and fifth experiment. In the first, that had an AOT₄₀ of approximately 154 ppb h, exposure to ozone caused a significant reduction in the instantaneous net assimilation of Guapo, Fepagro and Macotaço cultivars (p = 0.005; 0.038 and 0.046, respectively) (Figure 1A). In the fourth experiment, with an AOT₄₀ of about 587 ppb h, the exposure to ozone did not cause significant reductions in the instantaneous net assimilation of any cultivar,

although a tendency was found for cultivars Fepagro and Guapo ($p = 0.076$ and 0.090 , respectively) (Figure 1B), and an anticipation of the senescence of primary leaves occurred in treatment with O_3 , which culminated in foliar abscission, even before the A/Ci curves were measured in four of the five seedlings of the Pinto cultivar and in a seedling of Iraí, Fepagro and Macotoço cultivars. In the fifth experiment with an AOT_{40} of approximately 304 ppb h, there was a significant reduction in the instantaneous net assimilation of Fepagro and Pinto cultivars and a tendency to reduction in the Iraí cultivar ($p = 0.004$, 0.006 and 0.084 , respectively) (Figure 1C).

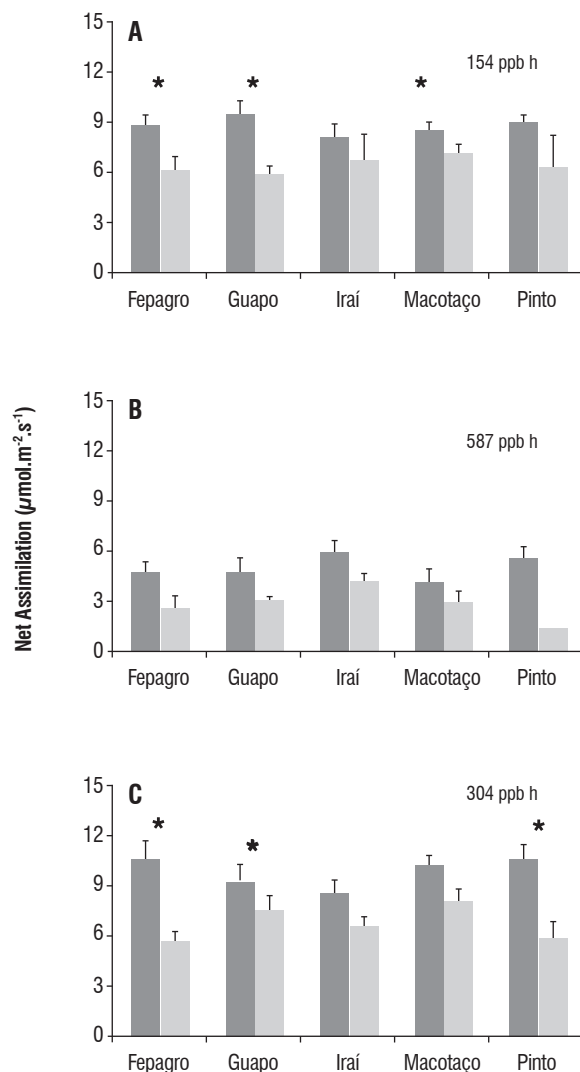


Figure 1. Instantaneous net assimilation of bean seedlings of five different cultivars exposed (light grey column) or not (grey column) to an ozone enriched atmosphere. Experiments 1 (A), 4 (B) and 5 (C). The asterisks indicate significant differences at 5% level of significance between treatments. The data are the mean \pm SE. The value in the upper right corner of each chart indicates AOT_{40} of the exposed plants.

The mean instantaneous net assimilation values of all cultivars, both for plants exposed and for the controls, were above those found by Guidi et al. (2000) in their experiment with beans of the Pinto cultivar, which found assimilation values of $5 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ for the plants in the control treatment and $3.64 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ for plants exposed to ozone (150 ppb for 2 hours). The exception was experiment 4, which had values that were generally a bit lower, although very similar to those of Guidi et al. (2000).

Knowing that the instantaneous net photosynthesis suffers a strong effect from the environment, the low photosynthesis values found in experiment 4, which was performed in March 2007, may, at least in part, be accounted for by the high temperatures associated with low light intensity. The low light intensity during this lot of the experiment occurred due to less obliqueness of the sunrays, which was worsened by the existence of a building that blocked the light during most of the day, because of the season at the time.

In Fepagro cultivar, instantaneous net assimilation was reduced by exposure to O_3 in experiment 1 (154 ppb h of O_3) and 5 (304 ppb h of O_3), while in experiment 4 (587 ppb h of O_3) there was only a tendency to reduction in this parameter. In the first experiment, this reduction was caused by the limitation in the carboxylation process (significant reduction in the maximum rate of carboxylation of rubisco – $40.120 \pm 7.011 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the control treatment against $25.260 \pm 7.385 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants – and a tendency to reduction in the carboxylation efficiency – $0.148 \pm 0.064 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the control treatment against $0.080 \pm 0.035 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants) and by the availability of RuBP (tendency for reduction in the maximum rate of electron transport – $70.760 \pm 13.737 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $52.060 \pm 7.385 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants). Although the stomatal conductance and transpiration did not present significant differences among treatments, there was a significant reduction in the stomatal limitation ($10.546 \pm 1.972\%$ in the control treatment against $7.138 \pm 2.430\%$ in the exposed plants) and, consequently, in the water use efficiency ($6.661 \pm 0.928 \mu\text{mol.mmol}^{-1}$ in the control treatment against $4.623 \pm 1.084 \mu\text{mol.mmol}^{-1}$ in the exposed plants), while the Ci/Ca ratio increased significantly (0.170 ± 0.049 in the control treatment against 0.642 ± 0.056 in the exposed plants). On the other hand the maximum assimilation showed a tendency to reduction ($12.856 \pm 1.547 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the control

treatment against $10.252 \pm 2.537 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants), because of the limitations in the carboxylation process and the availability of RuBP. In the fifth experiment, the significant reduction in instantaneous net assimilation was accompanied by a significant reduction in the stomatal conductance ($174.000 \pm 15.149 \text{ mmol.m}^{-2}.\text{s}^{-1}$ in the control treatment against $78.800 \pm 30.277 \text{ mmol.m}^{-2}.\text{s}^{-1}$ in the exposed plants), which caused a significant reduction in transpiration ($2.790 \pm 0.158 \text{ mmol.m}^{-2}.\text{s}^{-1}$ in the control treatment against $1.616 \pm 0.463 \text{ mmol.m}^{-2}.\text{s}^{-1}$ in the exposed plants). However, the stomatal limitation had a tendency to reduction in the exposed plants ($14.048 \pm 5.141\%$ in the control treatment against $8.894 \pm 3.422\%$ in the exposed plants), which indicates that the CO_2 entering the leaves was not the limiting factor. Besides this, the Ci/Ca ratio and respiration did not present differences among treatments, which supports the idea that the limitation of the photosynthesis is not at the CO_2 influx but in the tendency to reduce the maximum rate of electron transport. In the fourth experiment, although AOT_{40} was higher, there was only a tendency to reduction in the instantaneous net assimilation of this cultivar, caused by a significant reduction in carboxylation efficiency ($0.125 \pm 0.068 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $0.036 \pm 0.020 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants). The maximum carboxylation velocity did not present a significant difference or tendency to difference among the treatment in this experiment.

In Guapo cultivar, instantaneous net assimilation was significantly reduced only in experiment 1. In the fourth experiment there was only a tendency to the reduction in assimilation without changes in the other parameters evaluated, and in the fifth, not even the instantaneous net assimilation was changed by exposure to ozone. The reduction of net assimilation in experiment 1 was caused by the limitation in the carboxylation process (significant reduction in the maximum rate of carboxylation of rubisco – $67.280 \pm 35.688 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the control treatment against $28.580 \pm 7.374 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants – and of carboxylation efficiency – $0.340 \pm 0.400 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $0.090 \pm 0.027 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants) and by the the availability of RuBP (tendency to reduction in the maximum rate of electron transport – $88.100 \pm 25.106 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $61.960 \pm 14.622 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the plants exposed). Although stomatal conductance and transpiration did not present significant differences among the treatments, there was a tendency to reduction in the stomatal limitation ($13.847 \pm 4.433\%$ in control treatment against $8.845 \pm 2.369\%$ in the exposed

plants) and a significant reduction in the water use efficiency ($8.473 \pm 2.138 \mu\text{mol.mmol}^{-1}$ in control treatment against $5.617 \pm 0.855 \mu\text{mol.mmol}^{-1}$ in exposed plants), while the Ci/Ca ratio increased significantly (0.408 ± 0.121 in control treatment against 0.584 ± 0.063 in the exposed plants).

In Iraí cultivar instantaneous net assimilation was not significantly reduced in any of the three experiments performed, and the single parameters that were significantly altered were dark respiration in experiment 4, which increased in the plants exposed to ozone ($0.820 \pm 0.288 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $1.232 \pm 0.097 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the plants exposed), and maximum assimilation in experiment 5, which was reduced in the plants exposed ($20.205 \pm 5.069 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $13.931 \pm 3.256 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in exposed plants).

In Macotaço cultivar the instantaneous net assimilation was significantly reduced only in experiment 1, and this was caused by a tendency to reducing carboxylation efficiency, ($0.222 \pm 0.078 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $0.135 \pm 0.035 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in plants exposed) without concomitant changes in the maximum carboxylation velocity.

Finally, the Pinto cultivar did not present a significant difference in the instantaneous net assimilation and in any other photosynthetic parameter evaluated in experiment 1, which had the lowest AOT_{40} . With an intermediate AOT_{40} (experiment 5), the instantaneous net assimilation was significantly reduced and with a higher concentration (experiment 4) there was foliar abscission in 4 of the 5 seedlings (exposed) even before the assimilation was measured, and a significant increase was only found in the relative electrolyte leakage since no statistical analysis could be performed. The reduction of net assimilation in experiment 5 can be attributed to a limitation in the carboxylation process (significant reduction in maximum rate of carboxylation of rubisco – $49.560 \pm 10.769 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $25.900 \pm 11.195 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants – and to the tendency to reduction in carboxylation efficiency – $0.127 \pm 0.053 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the control treatment against $0.066 \pm 0.028 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants) and by the availability of RuBP (significant reduction in the maximum rate of electron transport – $103.220 \pm 23.550 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in control treatment against $54.360 \pm 17.371 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the exposed plants). Although stomatal conductance has presented a tendency to reduction ($187.600 \pm 30.851 \text{ mmol.m}^{-2}.\text{s}^{-1}$ in control treatment against $131.600 \pm$

52.219 mmol.m⁻².s⁻¹ in the exposed plants), which reflected a tendency to concomitant reduction in transpiration (2.932 ± 0.277 mmol.m⁻².s⁻¹ in control treatment against 2.260 ± 0.645 mmol.m⁻².s⁻¹ in the exposed plants), there was a significant reduction in the stomatal limitation (14.363 ± 3.688% in control treatment against 7.320 ± 2.686% in exposed plants), and in water use efficiency (3.594 ± 0.393 μmol.mmol⁻¹ in control treatment against 3.643 ± 0.735 μmol.mmol⁻¹ in exposed plants). The significant reduction in the stomatal limitation mentioned above, the tendency to an increase in the Ci/Ca ratio (0.619 ± 0.074 in the control treatment against 0.706 ± 0.060 in the exposed plants) and significant reduction in respiration (1.786 ± 0.422 μmol.m⁻².s⁻¹ in control treatment against 1.184 ± 0.401 μmol.m⁻².s⁻¹ in exposed plants) indicate that the influx of CO₂ into the leaf was not the limiting factor of photosynthesis.

The stomatal limitation reduction in the Fepagro cultivar (experiment 1) and in the Pinto cultivar (experiment 5), and the tendency to reduction of the stomatal limitation in the Fepagro cultivar (experiment 5) and in the Guapo cultivar (experiment 1) is directly related to the limitation in the carboxylation process (amount and activity of rubisco), since it will generate a smaller difference between the net assimilation of Ci₃₅₀ and Ca₃₅₀ in the plants exposed. On the other hand, the reduction in the efficiency of water use for these same cultivars and experiments (except the Fepagro cultivar – experiment 5, which did not present changes in water use efficiency when exposed to ozone) was the consequence of a significant reduction in the photosynthesis without concomitant reduction in transpiration. In the Pinto cultivar (experiment 5), although there was a tendency to reduction of transpiration, it did not occur in the same proportion as the decrease in assimilation, which justifies the significant reduction in water use efficiency for this cultivar.

The significant increase in the Ci/Ca ratio in Fepagro (experiment 1) and Guapo cultivar (experiment 1) and the tendency to increase this same ratio in Pinto cultivar (experiment 5) are consequences of the significant reduction in the maximum rate of carboxylation of rubisco, and the reduction (Guapo cultivar) or tendency to reduction (Fepagro and Pinto cultivars) of the carboxylation efficiency which, in turn, cause a reduction in CO₂ use, making it accumulate in the leaves, and thus allowing to even cause stomatal closing. That is why the significant reduction in stomatal conductance in Fepagro cultivar (experiment 5) and the tendency to reduction in

Pinto cultivar (experiment 5) did not cause increased stomatal limitation. As to dark respiration, this cannot be involved in the increased Ci/Ca ratio, since the concentration of CO₂ respiration is very small compared to what is assimilated, and also because no significant difference was found between the treatments for this parameter in Fepagro and Guapo cultivars (experiment 1), and in Pinto cultivar (experiment 5) there was a significant reduction in respiration.

The reduction in net assimilation is often the first detectable symptom observed in plants treated with ozone (Reichenauer et al., 1998). Among the parameters most associated with this decrease in the present study are maximum rate of carboxylation of rubisco, which presented significant reduction in Fepagro (experiment 1), Guapo (experiment 1) and Pinto (experiment 5), and carboxylation efficiency which presented a significant reduction in Fepagro cultivar (experiment 4) and Guapo cultivar (experiment 1) and the tendencies to reduction in Fepagro cultivar (experiment 1), Macotaço cultivar (experiment 1) and Pinto cultivar (experiment 5). Both maximum rate of carboxylation of rubisco, and carboxylation efficiency reflect the activity of rubisco in vivo (Rogers and Humphries 2000), and carboxylation reductions were considered the main factors responsible for impairment in photosynthesis by Pell et al. (1994) and Guidi et al. (2001).

Guidi et al. (2000), working with bean plants of Pinto cultivar, also found results that agree with the idea that the primary target of ozone are the enzymes involved in the Calvin cycle and, particularly, rubisco activity. Rubisco inhibition induces less use of NADPH and ATP, and this consequently causes a reduction in the activity of the electron transportation system, and an increased proportion of closed reaction centers of Photosystem II. Closed reaction system, in turn, are in a greater state of reduction in QA (Quinone A), which may increase plant susceptibility to photoinhibition.

In this study, the maximum rate of electron transport, which presented a significant reduction only in Pinto cultivar (experiment 5), but which had a tendency to reduction in Fepagro cultivar (experiment 1 and 5) and in Guapo (experiment 1), also appears to have contributed to the reductions found in net assimilation. Another reason for reduction in electron transportation is mentioned by Calatayud et al. (2003), who say that ozone can alter photochemical reactions of photosynthesis, reducing the electron transportation rate between the two photosystems. In this way, photosynthesis inhibition may be

the result of reduced ATP and NADPH availability (Calatayud et al., 2004). Besides, glutathione, which is acknowledged as the central component of antioxidant defense in most aerobic organisms (Foyer et al., 1997), requires NADPH both for its synthesis and for its reduction. Thus again, the photosynthesis decline observed may be the result of less NADPH available for carbon reduction (Gupta et al., 1991). Calatayud et al. (2002) mention decreased electron transportation as one of the mechanisms responsible for reducing the photosynthesis process in *Lactuca sativa* L.

On the other hand, stomatal conductance presented a significant reduction only in Fepagro cultivar (experiment 5) and a reduction tendency only in Pinto cultivar (experiment 5), but these reductions were not related to decreased assimilation. Reductions in stomatal conductance caused by exposure to ozone were also found by Guidi et al. (2000) in the Pinto cultivar of *P. vulgaris* plants. Studies indicate that stomatal conductance may increase or diminish in response to damage caused by ozone, depending on the genotype, ozone concentration and sensitivity of the guard cells (Robinson et al., 1998). According to McAinsh et al. (2002) a rapid decline in stomatal opening, and therefore in stomatal conductance, is commonly observed in the presence of ozone during a short period of exposure, while long duration exposure to ozone slows down stomatal responses.

Noormets et al. (2001) and Zheng et al. (2002) reported that the decreases in rubisco content and activity, rather than the electron transportation and regenerative capacity of ribulose-1,5-bisphosphate (RuBP), are the primary factors responsible for the deleterious effects of ozone in CO_2 assimilation. In addition to the effects on rubisco and RuBP regeneration, another factor which may potentially affect the assimilation of CO_2 is the decreased stomatal opening (Zheng et al., 2002). However the decreased stomatal opening may be a reflection of maintaining the leaf intercellular concentration of CO_2 caused by the inhibition of the carboxylation process. Therefore, according to Farage and Long (1999) the low stomatal conductance may be only partially responsible for the decreased rate of photosynthesis through the limitation of CO_2 influx.

Relative electrolyte leakage: Concerning the relative electrolyte leakage, O_3 -exposure to 154 ppb h (experiment 1) did not produce a change in the relative electrolyte leakage in any cultivar (Figure 2A). When AOT_{40} was raised to 304 ppb

h (experiment 5) there was a tendency to an increase in Pinto cultivar (Figure 2C) and, finally, when exposure became 587 ppb h, there was a significant increase in Pinto cultivar and a tendency to an increase in Macotoço cultivar (Figure 2B). In the plants exposed to ozone, the electrolyte leakage is one of the first symptoms of damage to the membrane and results from oxidation of proteins of the sulphhydryl group and lipids from the membrane (Guidi et al., 1999). Changes in membrane functioning affect a large range of cell processes, through loss of metabolites and loss of compartmentalization, and may lead to increased tissue senescence (Weber et al., 1994).

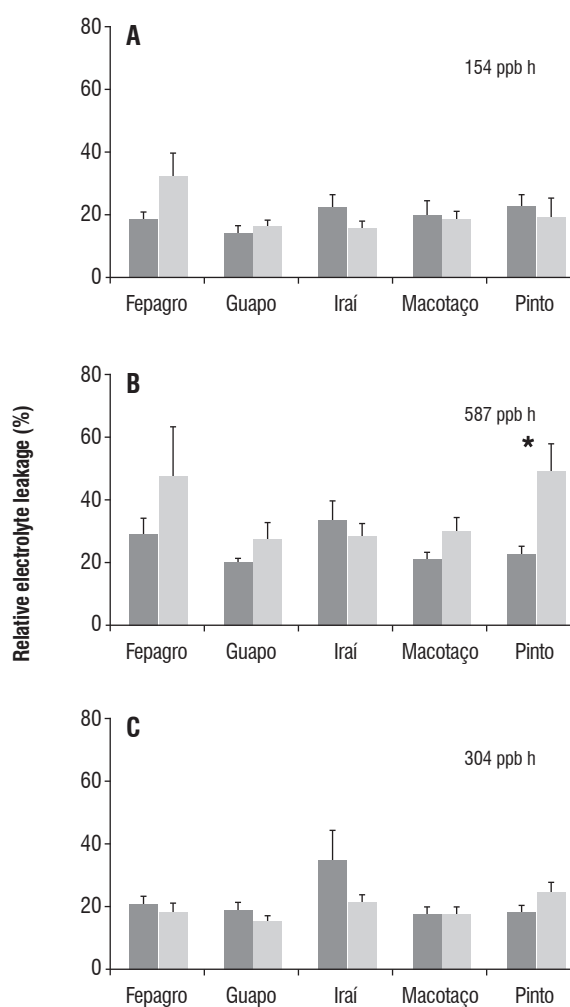


Figure 2. Relative electrolyte leakage of bean seedlings of five different cultivars, exposed (light grey column) or not (grey column) to the ozone enriched atmosphere. Experiments 1 (A), 4 (B) and 5 (C). The asterisks indicate significant differences at 5% level of significance between treatments. The data are the mean \pm SE. The value in the upper right corner of each chart indicates AOT_{40} of the exposed plants.

Foliar Abscission: Foliar abscission was monitored in the third and seventh experiment. In the third, which had an AOT₄₀ of approximately 153 ppb h, ozone exposure anticipated the abscission of the primary leaves of Pinto cultivar on average by 11 days ($p = <0.001$), and of Fepagro cultivar by 7 days ($p = 0.004$), besides presenting a tendency to a 5-day anticipation in Guapo cultivar ($p = 0.090$) (Figure 3A). However, under an AOT₄₀ of about 212 ppb h (seventh experiment) Guapo cultivar was the only one which had a significant anticipation in abscission (an average of 3 days, with $p = 0.004$), and Fepagro cultivar presented a strong tendency to anticipation of approximately 4 days ($p = 0.051$) (Figure 3B).

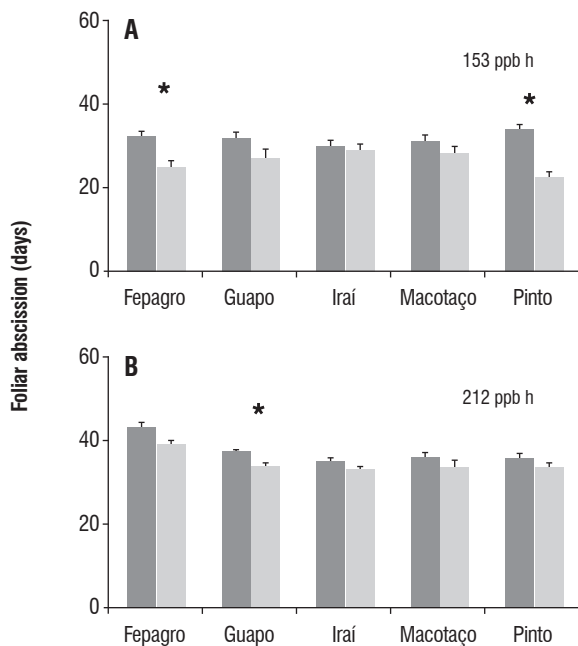


Figure 3. Foliar abscission of bean seedlings of five different cultivars exposed (light grey column) or not (grey column) to the ozone enriched atmosphere. Experiments 3 (A) and 7 (B). The asterisks indicate significant differences at 5 % level of significance between treatments. The data are the mean \pm SE. The value in the upper right corner of each chart indicates AOT₄₀ of the exposed plants.

Under physiological conditions, senescence is a highly regulated process, involving chlorophyll degradation, photosynthetic decline, protein degradation and lipid peroxidation, culminating in the remobilization of nutrients and foliar abscission (Ribas et al., 2005). The acceleration of senescence has been widely reported as one of the characteristic processes of exposure to O₃ (Ribas et al., 2005; Ranford and Reiling, 2007). It can be anticipated by changes in the functioning of the membrane, which affect a great variety of cell processes by

metabolite losses and the compartmentalization caused by lipid peroxidation from the membrane (Weber et al., 1994; Calatayud et al., 2003). However, it is not clear whether the acceleration of senescence is due to the induction of the normal process, i.e., to a type of programmed cell death as part of the hypersensitivity response, or the change in carbon budget, favoring respiration in detriment of net assimilation. Since in this study there were practically no changes in respiration and in the relative electrolyte leakage, and since there were few drops in assimilation, possibly senescence in response to oxidative stress is not related directly to the adverse effect on photosynthesis.

Total biomass of the seedling: The accumulation of total dry mass of the plant was evaluated in the second and sixth experiment. In the second, which had an AOT₄₀ of approximately 209 ppb h, ozone treatment caused a tendency to reduction in this parameter, only in the Iraí ($p = 0.056$) and Fepagro ($p = 0.084$) cultivars (Figure 4A). In the sixth experiment, a slight increase of the total biomass of Iraí cultivar seedlings ($p = 0.098$) was observed at exposure to approximately 147 ppb h (Figure 4B).

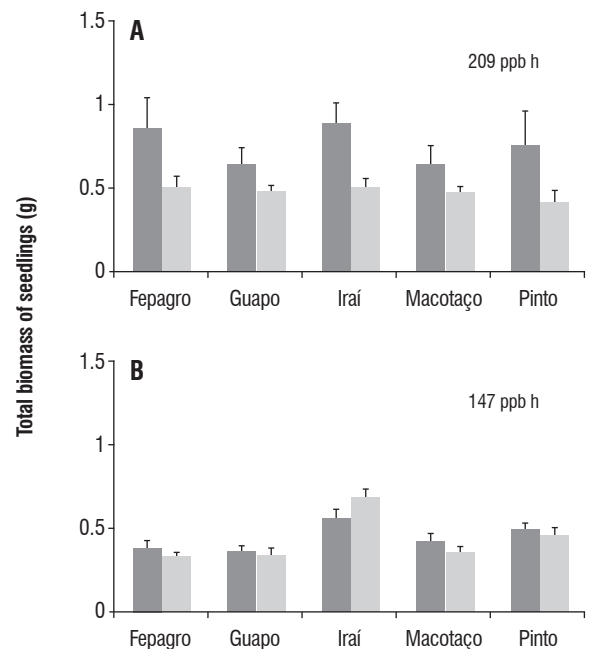


Figure 4. Total biomass of bean seedlings of five different cultivars exposed (light grey column) or not (grey column) to the ozone enriched atmosphere. Experiments 2 (A) and 6 (B). The asterisks indicate significant differences at 5 % level of significance between treatments. The vertical bars indicate the standard error of the mean. In the upper right corner of each chart is the AOT₄₀ of the plants exposed.

Although ozone and its reaction products are known to reduce photosynthesis and change the carbohydrate allocation, causing a reduced accumulation of biomass (Rennenberg et al., 1996), this generally occurs over the longer term, as suggested by Davison and Barnes (1998).

In fact, it is noted that the results were not very stable, and a larger dose of ozone did not always damage the plants. This may be partly explained because the response of the plants to ozone is more strongly correlated with the cumulative influx of ozone through the stomata, than with the ozone concentration in the environment, which will depend on soil humidity, atmospheric vapor pressure deficit, wind velocity and air temperature, which influence the degree of stomatal opening (Fuhrer et al., 1992). Besides, it is known that although it was not measured, there was variation in the light intensity intercepted by the seedlings in every experiment depending on the season, and this was lower, especially in experiment 4, thus rendering less clear the differences between the control and exposed seedlings. It was also observed that on a day with bright sun the response to ozone was more marked than on cloudy days. This is because, besides speeding up the metabolism, high intensity light conditions intensify the production of ROS, which occur naturally during the photosynthetic process. In this sense, Guidi et al. (2000) evaluated the combined effects of high irradiance and ozone on the photosynthesis of primary bean leaves of Pinto cultivar, and concluded that the high light intensity may change the reaction of leaves to ozone treatment by reducing the thylakoidal electron transport rate and quantum productivity.

CONCLUSIONS

This study presents an overview of how five different bean cultivars respond to ozone exposure. It was noted that the response of photosynthetic parameters of bean seedlings to ozone exposure showed similar patterns in all cultivars, although they exhibited slightly different sensitivities.

In general, ozone shows a harmful effect on the instantaneous net assimilation sometimes yet affecting maximum assimilation. The reductions in both these parameters showed strong correlation with limitation in the carboxylation process (decrease in maximum rate of carboxylation of rubisco and carboxylation efficiency) and also with the decrease in maximum rate of electron transport. The limitation in the

carboxylation process resulted in turn in a lower demand by CO_2 , which caused often an enhancement of Ci/Ca ratio, with few exceptions in which the Ci/Ca ratio remained unchanged. This buildup of leaf CO_2 may lead a decrease in stomatal conductance and even transpiration although in our study both these parameters sometimes remain unchanged despite significantly enhance in Ci/Ca ratio. In addition, the buildup of CO_2 leads to a decrease in stomatal limitation. Finally, even that cases in which there are some reduction in transpiration, the water use efficiency usually is reduced in plants exposed to ozone due the decrease in transpiration not be in the same proportion as the reduction in net assimilation.

Regarding the relative electrolyte leakage, relatively high doses of ozone (AOT_{40}) were necessary to induce some increase in membrane ion leakage and even so it was not observed in all cultivars. Notwithstanding, the foliar abscission was anticipated in most cultivars with relatively low doses of ozone.

Despite the obvious harmful effects on the crop cultivation, we cannot make clear predictions of anticipated damages on the *Phaseolus vulgaris* species resulting from tropospheric ozone enrichment. The limitations to perform long-term controlled-environment studies, as discussed earlier, are one of the greatest challenges yet to be overcome in order to obtain well-replicated and more conclusive experiments to be performed in the field.

Acknowledgments: The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Copesul for their financial help, and also OZ Engenharia for donating the ozonizer, as well as the support from engineers Sepé Tiarajú Fernandes dos Santos from Copesul and Fábio Rahmeier from OZ Engenharia.

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