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## Effect of Ozone and Simulated Acid Rain on Growth, Nitrogen Fixation and Peroxidase Activity in Faba Bean (*Vicia faba* L.) Plant

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**Abstract:** Faba beans (*Vicia faba* L. CV. Giza 400) were exposed to ozone (100 ppb) and simulated acid rain (SAR) at pH 3, separately and combined over 2-weeks period while grown in climate controlled growth chambers. Ozone fumigation ( $O_3$ ) exposure was for 5 h day<sup>-1</sup>, whereas acid rain exposure was for 2 h per week. The results showed that exposure to high  $O_3$  concentration (100 ppb) decreased the number and weight of root nodules, suppressed nitrogenase activity and strongly induced stomatal closure. Combination between high  $O_3$  concentration and acid rain generally caused more closure of the stomata than other treatments. Water content and leaf area were significantly reduced in high  $O_3$  treatment. Furthermore, high  $O_3$  concentration increased the shoot/root ratio by reducing root growth more than shoot growth. In contrast, low  $O_3$  concentration had no effect on these parameters. Acid rain, on the other hand, decreased root nodulation and  $N_2$ -fixation, but it ameliorated  $O_3$  induced injury. The reduced  $O_3$  injury induced in the presence of acid rain treatment was probably achieved by decreasing  $O_3$  uptake through stomatal closure. Furthermore, leaves treated with acid rain became greener than those of control and other treatments due to the increase in chlorophyll concentration. Peroxidase (POD) activity showed a positive response to ozone and acid rain treatments either combined or separately.

**Key words:** Faba beans, ozone, SAR, nitrogen fixation, POD

### Introduction

Because of human activities, elevated levels of tropospheric ozone occur chronically during much of the growing season (Taylor and Hannon, 1992). Thus, ozone, ubiquitous in industrial areas of the world, is considered one of the most phytotoxic pollutants (Krupa and Maning, 1988). This phytotoxic oxidant has been recognized as probably having the most detrimental effect on vegetation among widespread air pollutants (Reich, 1987). Therefore, exposure to ozone may severely damage plants, where ozone enters the leaves through stomata and diffuses in the intercellular spaces where in some plants not all it can be detoxified by the antioxidant system (Luwé *et al.*, 1993). An increase in peroxidase activity has been reported as an early response to ozone stress (Peters *et al.*, 1989 and Manes *et al.*, 1990) and may provide cells with resistance against the formation of  $H_2O_2$  which is formed when the plant is exposed to ozone and has frequently been used as a biochemical marker in monitoring stress induced by pollutants (Castillo and Greppin, 1986).

With regard to acid rain as one of air pollutants, Izuta *et al.* (1993) investigated the effect of simulated acid rain on fir seedlings and reported that severe damage, such as visible foliar injury and depression of growth, was observed in seedlings treated with rain of pH 2. However, it must be noted that in environment setting no air pollutant occurs in the absence of others such as acid rain,  $CO_2$ ,  $SO_2$  and  $NO_2$ . Air pollutants such as ozone and acid rain are common environmental stresses. Therefore, over the past few decades both ozone ( $O_3$ ) and acid rain separately have received much attention from the scientific and public communities, but there are few studies to date have examined the effects of acid rain and ozone interactions on plants, such studies are required to make more accurate predictions of future ecosystem responses to the changing environment. As with acid rain, much attention has been devoted to study the effects of elevated concentrations of  $O_3$  on plant chemistry, physiology and growth, often in combination with other environmental factors. Furthermore, limited investigations have shown that air pollution can affect root nodulation and nitrogen fixation in legume crops. Tingey and Blum (1973) found that  $Q_n$  decreased nodule number, nodule weight per plant in soybeans, which is positively correlated with  $N$ -fixing capacity and these reductions were reported to be indirect effects of ozone on the plant foliage.

The study had three objectives:

- To determine how growth and physiology of faba bean were influenced by the interaction between ozone and acid rain. Accordingly, the responses were examined by

measuring root nodules, nodule weight, nitrogenase (acetylene reduction) activity, shoot and root nitrogen concentrations and biomass production. Stomatal resistance, leaf water content, leaf area and chlorophyll content measurements were also used to investigate the effects of ozone and acid rain on stomata mechanism and some of the leaf characters.

- To determine whether leaching of metals from the leaves occurs when the faba bean plants were exposed to the acid rain and ozone.
- To determine the effect of these air pollutants either separately or in combination on peroxidase activity, which plays an important role in protecting plants against injury of oxidative stressors.

### Materials and Methods

**Plant material and culture:** Seeds of faba bean (*Vicia faba* L. CV. Giza 400) were surface sterilized in 95% ethanol for 3 min followed by 0.1 %  $HgCl_2$  for 2 min, then washed five times with sterile distilled water and germinated in plastic pots filled with sterilized quartz sand. After emergence, seedlings were transferred to others pots (two seedlings per pot) filled with sand-clay, 1:1 (by volume). On the day of transplanting, each seedling was inoculated with 1ml of suspension of *Rhizobium leguminosarum* strain. One week later, nutrient solution was applied. The nutrient components and concentrations ( $\mu M$ ) as described by Qifu and Murray (1993) were:  $KH_2PO_4$ , 20;  $K_2SO_4$ , 600;  $MgSO_4 \cdot 7H_2O$ , 200;  $CaCl_2 \cdot 2H_2O$ , 600;  $H_3BO_3$ , 5;  $Na_2MoO_4 \cdot 2H_2O$ , 0.03;  $ZnSO_4 \cdot 7H_2O$ , 0.75;  $MnSO_4 \cdot 7H_2O$ , 1.0;  $CoSO_4 \cdot 7H_2O$ , 0.2;  $CuSO_4 \cdot 5H_2O$ , 0.2;  $FeNaEDTA$ , 20.

All pots were maintained in a growth chamber (1.5 x 1.5 x 1.5m<sup>3</sup>) at day and night temperatures  $30 \pm 1$  and  $24 \pm 1$  °C respectively, with light/dark regimes of 16/8 h, light intensity was 560  $\mu mol m^{-2} s^{-1}$  (PAR) at the top of leaves supplied by fluorescent lamps and incandescent lamps (30% fluorescent wattage) and the relative humidity varied from 60 to 70%. When the plants were at 3 leaf stage, they were transferred to open top chambers (OTCs). This was located in the Botanic Garden, Faculty of Science, Alexandria University. These chambers were constructed of aluminum frame covered by PVC plastic. There were 15 pots per chamber. Plants were irrigated twice a week.

**$O_3$  exposure and simulated acid rain:** Plants were exposed to either clean filtered air (FA control, i.e.,  $O_3 = 7ppb$ ) or 100 ppb ozone 8 h day<sup>-1</sup> (7:00-15:00) for 15 days in OTCs, unless stated otherwise. Ozone was generated from dry air using an  $O_3$  generator (type BA

0203012 Wallace and Tiernan, Tonbridge, UK) and pumped to the chambers through a PVC pipe manifold and adjusted manually with solenoid box via in-line flow valves, the air was passed through a water trap to remove free radicals. Ozone was monitored with a UV photometer (Dasibi Model 1003-AH), with each of the chambers being monitored for 7 min in a 1-h cycle. Simulated acid rain at pH 3 was sprayed on the plants for once a week (3 h day<sup>-1</sup>). The artificial acid rain solution was prepared as described by Scalet *et al.* (1995). The acid rain concentrations (mg l<sup>-1</sup>) were: NH<sub>4</sub><sup>+</sup>, 0.73; NO<sub>3</sub><sup>-</sup>, 2.52; Mg<sup>+</sup>, 0.18; K<sup>+</sup>, 0.46; SO<sub>4</sub><sup>-2</sup>, 4.642; Na<sup>+</sup>, 0.54; Ca<sup>2+</sup>, 2.23; Cl<sup>-</sup>, 26.68. The pH was adjusted to an appropriate value using H<sub>2</sub>SO<sub>4</sub> and NaOH.

**Experimental design:** Four groups of pots (15 pots each) and each group formed an experimental set. Sets were put into 4 chambers (with open tops) and treated as follows:

Chamber 1: plants sprayed with acid rain at pH 3  
Chamber 2: plants treated with ozone fumigation (100 ppb)  
Chamber 3: plants sprayed with acid rain (pH 3) + ozone fumigation (100ppb).  
Chamber 4: plants were left as control (distilled water + clean filtered air).

Plants were harvested at the desired time. Three replicates were included per harvest. Nodules of similar size and appearance were carefully detached, rinsed with distilled water, dried with filter paper, weighed and kept on ice. Plants were used based on the daily inspection of the plant roots to establish the time when the first nodule became visible, to indicate the start of effective N<sub>2</sub>-fixation. Accordingly, the results obtained showed that the critical times of nodulation and nodule activity occurred in the period up to one month (Abdel Aal, 2001).

**Nitrogenase assay:** Nitrogenase (EC: 1.7.99.2) activity was determined by acetylene reduction. Plants were removed from pots and the sand was removed from the root. The root systems of each plant and nodulated root portions were placed in separate glass vials and sealed with serum caps. Assays were conducted by incubating the roots and nodule in 10% C<sub>2</sub>H<sub>4</sub>. Vials were then placed in a water bath maintained at 28 °C for 30 min to allow temperature equilibration. Samples of gas (2ml) were removed from the vials intervals at 10, 15 and 20 min and injected into a gas chromatograph (Perkin Elmer 8600) with a Porapak R column (Ligero *et al.*, 1986).

Nitrogenase activities were expressed as total nodule activity (TNA) per-plant and specific nodule activity (SNA) per-gram fresh weight of nodules. Thereafter, nodules were removed from the roots and their fresh weights were recorded.

**Stomatal and water content measurements:** Stomatal diffusive resistance was measured, using an automatic porometer (Delta T. Cambridge, UK). Ten of the fully expanded leaves from each treatment were measured.

Stomatal conductance was measured between 10.00 am and 3.00 pm, using a portable IRGA (Model ADC LCA2).

Water content was determined by measuring water loss from excised leaves which were treated according to the procedure of Mengel *et al.* (1987). Leaves were excised from plant. The cut surface of the excised leaves was immediately covered with silicon past. The leaves were kept in the dark in a desicator with silica gel. The change in weight of each leaves was recorded at intervals over 48 h and the decrease in weight was defined as the water loss. The decrease in weight was partly due to respiration loss which, however, appears to have been relatively small compared to the loss of water from the excised surface (Mengel *et al.*, 1989).

**Measurement of growth:** The growth of plant was evaluated by measurements of leaf area, plant height and root length. Plant heights were determined after 15 days of exposing the plants to ozone and acid rain. Measurements were taken from cotyledonary node to the uppermost node with a fully developed leaf on the

main stem. Selected plants were divided into shoots and roots and weighed for fresh weight and dried in a forced-air oven at 80 °C for 48 hr to obtain the dry weight. Leaf area was determined using a moving belt electronic planimeter (Delta T Devices, Burwell, UK.).

**Determination of protein and chlorophyll concentration:** Protein was extracted and the content was determined by the method of Bradford (1976), using bovine serum albumin as protein standard. Total chlorophyll content was estimated according to Vernon (1960) using spectrophotometer (LKB. UV-400. UK).

**Mineral analysis of the leaves:** Metal elements in the leaves were determined after the specific leaf weight determination of the samples. Dried leaves were ashed in a muffle furnace at 500 °C for 2hr. Ash of 10-15 mg was heated in 5 ml of 1% nitric acid at 80 °C for 2 h. After cooling, the solution was made up to volume 20 ml with redistilled water and the concentrations of ions were determined using atomic absorption (Spectrometer Perkin-Elmer. Ltd. UK).

**Peroxidase (PO) extraction and assay:** Leaves were harvested and immediately homogenized by a mortar and pestle, in an ice bath, with 5ml of 0.1 M K-phosphate buffer (pH 7) containing 0.5 M NaCl. Polyvinylpyrrolidone, (2ml) was added to remove soluble phenolic compounds. Homogenate was then centrifuged at 12,000 g for 10 min and supernatants used for enzymatic assay. Enzymatic activity was determined spectrophotometrically (spectrophotometer, LKB.UV-400, UK) according to Scalet *et al.* (1991) using guaiacol as a substrate. Peroxidase activity is reported as units of enzymatic activity (UE), which correspond to the change of absorbance, in 1 min, per gram of fresh weight.

**Statistical analysis:** Analysis of variance (ANOVA) was performed to partition the variance into the main effects and the interaction between the two factors (O<sub>3</sub> and acid rain). Data were subjected to Dunnet's T-test to find statistically the significant differences between treatments at P ≤ 0.05. The data shown are means ± SE (Scheffler, 1978 and Bishop, 1981)..

## Results and Discussion

**Nodulation and N<sub>2</sub> - fixation:** After 15 days of ozone (O<sub>3</sub>) and simulated acid rain (SAR) exposure, it was found that there was a significant effect of O<sub>3</sub> and acid rain and their interaction in regard to the measured parameters (Table 1). The fresh and dry weights as well as the number of nodules were decreased with O<sub>3</sub>. Furthermore, O<sub>3</sub> decreased the total and specific nodules activities. This might be caused by H<sub>2</sub>O<sub>2</sub> produced from decomposition of O<sub>3</sub> in water of the soil which is a very harmful molecule for the cell (Heath, 1987). Also, acid rain (pH 3), was found to suppress the root nodulation and nodules activities.

Antagonistic effects of acid rain against O<sub>3</sub> were observed in nodules number, nodules fresh weight and specific nodules activity, despite highly significant effect of O<sub>3</sub> alone (Table 1). Furthermore, plants treated with acid rain or O<sub>3</sub> were similarly delayed in nodulation. The appearance of the first nodules in the control plants occurred between 6-8 days. Nodulation was delayed for about 12-13 days when the plant was exposed to O<sub>3</sub> and acid rain separately or in combination. This result is consistent with that obtained by Abdel Aal (1995).

The results presented in Table 1 support the view that nitrogenase activity per gram of nodule (specific activity) is an important indicator of the efficiency of nitrogen assimilation, because the plants were not nitrogen fertilized in this study and the stress effects on root nodulation and nodule activity impaired nitrogen resources in the plants after the cotyledonary reserves were consumed. Thus, O<sub>3</sub> and acid rain significantly reduced the efficiency of N<sub>2</sub>-fixation process and presented a correlation between nitrogenase activity and O<sub>3</sub> or acid rain. About 52 %

Table 1: Effect of ozone and acid rain on the nodules number, nodules fresh weight, total nodules activity ( $\mu\text{mol C}_2\text{H}_4 \text{ plant}^{-1} \text{ min}^{-1}$ ) and specific nodules activity ( $\mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ fresh wt. min}^{-1}$ ) in *Vicia faba* plant

Treatments	Nodule number.	Nodule FW. (g)	Total nodule activity	Specific nodule activity
FA	44.8 ± 1.2a	0.75 ± 0.10a	162 ± 5.8a	404 ± 8.8a
O <sub>3</sub> (100 ppb)	26.3 ± 0.9c	0.49 ± 0.06c	084 ± 8.8c	210 ± 6.9a
SAR (pH 3)	35.6 ± 1.2b	0.63 ± 0.07b	128 ± 4.3b	303 ± 5.8b
SAR + O <sub>3</sub>	30.8 ± 1.0b	0.58 ± 0.06b	120 ± 2.2b	309 ± 7.9b

Data are means ± SE (n=10). Data within the same column followed by different letters are significantly different at P ≤ 0.05  
FA: filtered air, control  
SAR: simulated acid rain

Table 2: Percentage of injured leaves symptoms and stomatal conductance (g.)

Parameter	Treatment			
	FA	SAR	O <sub>3</sub>	O <sub>3</sub> + SAR
Number of injured leaves	0.32 ± 0.02a	0.48 ± 0.03b	1.70 ± 0.1c	1.28 ± 0.10c
Degree of injury	0.22 ± 0.01a	0.33 ± 0.02b	1.17 ± 0.2c	0.72 ± 0.08b
g <sub>s</sub> (mmol m <sup>-2</sup> s <sup>-1</sup> )	0.58 ± 0.02a	0.62 ± 0.01b	0.80 ± 0.2b	0.72 ± 0.05b

Data are mean + SE (n = 10). Data within the same column followed by different letters are significantly different at P ≤ 0.05

Table 3: Effect of ozone and acid rain treatments on the growth of shoots and roots, shoot / root ratio, plant height and root length of *Vicia faba* plant

Treatments	Shoot dry wt (g)	Root dry wt (g)	Whole plant (g)	Shoot/Root ratio	Plant height (cm)	Root length (cm)
FA	0.66 ± 0.08a	0.42 ± 0.04a	1.08 ± 0.10a	1.57	80.4 ± 4.4a	38.6 ± 24a
O <sub>3</sub> (100ppb)	0.46 ± 0.04b	0.30 ± 0.06b	0.76 ± 0.09b	1.53	66.4 ± 2.6c	26.6 ± 4.4b
SAR (pH3)	0.83 ± 0.08b	0.43 ± 0.01a	1.26 ± 0.12ab	2.02	89.9 ± 4.1b	37.9 ± 1.8a
SAR + O <sub>3</sub>	0.59 ± 0.05ab	0.33 ± 0.05ab	0.92 ± 0.08ab	1.69	73.6 ± 6.3b	29.5 ± 3.8b

Data are means ± SE (n = 10). Data within the same column followed by different letters are significantly different from control at P ≤ 0.05

Table 4: Effect of O<sub>3</sub> and acid rain treatments on the metal concentrations in *Vicia faba* leaves (mg g<sup>-1</sup> Dwt)

Treatments	Metal ions				
	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Al <sup>3+</sup>	Na <sup>+</sup>	K <sup>+</sup>
FA	08.46 ± 0.9a	12.42 ± 0.4a	2.38 ± 0.3a	0.220 ± 0.02a	0.202 ± 0.03a
O <sub>3</sub>	04.48 ± 0.2c	10.29 ± 1.9b	1.35 ± 0.5c	0.120 ± 0.06b	0.103 ± 0.02c
SAR	10.38 ± 0.6b	13.98 ± 0.8b	2.98 ± 0.8ab	0.302 ± 0.02c	0.256 ± 0.04ab
SAR + O <sub>3</sub>	08.22 ± 0.8a	10.08 ± 0.9b	2.14 ± 0.3a	0.196 ± 0.02ab	0.146 ± 0.01b

Data are means ± SE (n = 5). Data within the same column followed by different letters are significantly different at P ≤ 0.05

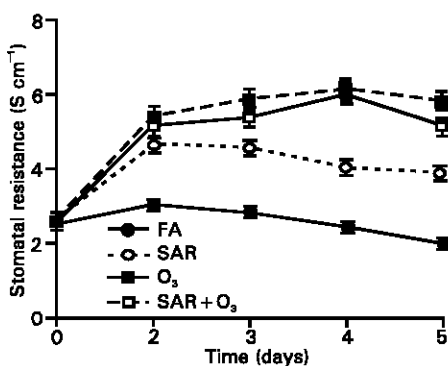


Fig. 1: Changes in stomatal resistance in leaves of *Vicia faba* plant during exposure to ozone and simulated acid

decrease in nitrogenase activity (Table 1) was associated with the presence of O<sub>3</sub>, this finding may be attributed to a direct effect of ozone on nitrogenase, but the percentage was reduced when O<sub>3</sub> and acid rain were applied together probably due to the antagonistic effects of these pollutants.

**Leaf characteristics:** Leaves injury symptoms appeared first on the lower epidermis. Injury appeared as chlorotic and necrotic spots

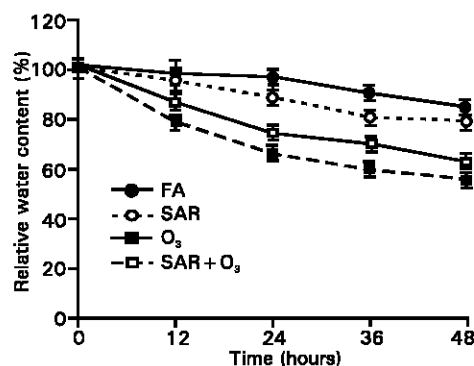


Fig. 2: Relative water content of leaves of *Vicia faba* during 48 h after treatment with ozone and (gs) stimulated acid rain

as reported by Hassan (1998). The percentage increase in the number of injured leaves and the degree of injury were higher following O<sub>3</sub> exposure than those treated with simulated rain only or combined with O<sub>3</sub> (Table 2) indicating that leaf senescence was accelerated by ozone (Reichenauer *et al.*, 1998). Moreover, O<sub>3</sub> caused a significant increase in g<sub>s</sub> than acid rain. The increased in stomatal conductance (g<sub>s</sub>) in response to O<sub>3</sub> can be considered as detrimental as it would increase O<sub>3</sub> flux and this may explain the greater sensitivity of the plants to O<sub>3</sub> as expressed in the number

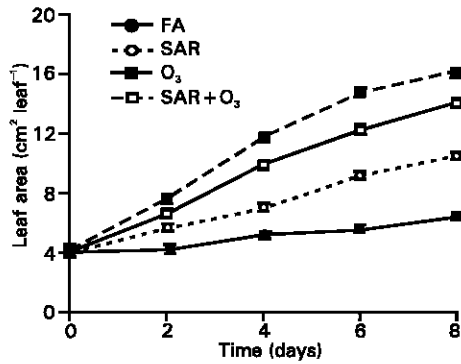


Fig. 3: Changes in the leaf area of *Vicia faba* plant during exposure to ozone and simulated acid rain

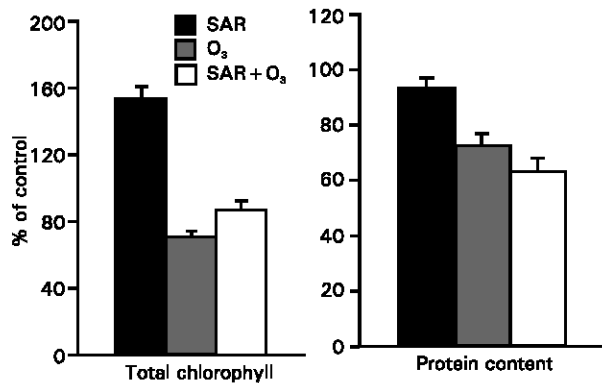


Fig. 4: Effect of ozone and acid rain on the total chlorophyll and soluble protein content in leaves of *Vicia faba* plant.

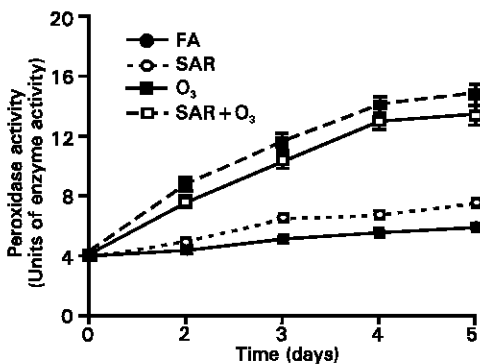


Fig. 5: Changes in the activity of peroxidase enzyme in leaves of *Vicia faba* during exposure to ozone and simulated acid rain. Values are mean  $\pm$  SE (n=10). FA (filtered air, control). SAR (simulated acid rain)

of injured leaves and the degree of injury on each leaf. In contrast, the decreased  $g_s$  in the presence of acid rain (Table 2) would reduce  $O_3$  flux and consequently increase the resistance to  $O_3$  induced injury.

Stomata are known to play an important role in mediating the response of plants to air pollutants by affecting ozone ( $O_3$ ) and acid rain uptake. However, stomatal response to  $O_3$  or to other air pollutants are very complex, because their effects depend on some factors such as air humidity, light intensity, physiological activity of tissue and pH value inside plant cells (Rudoff *et al.*, 1992 and

Qifu and Murray, 1993) in which stomatal responses are modified by these factors. In this study, measurements of stomatal resistance (Fig. 1) showed that  $O_3$  strongly induced stomatal closure in comparison to the control. Combination between  $O_3$  and acid rain generally caused more closure of the stomata. Stomatal closure appeared to be attributed to the low pH of the acid rain and collapse of guard cells of the leaves due to  $O_3$  effect. Therefore,  $O_3$  may enter the plant tissues through the cuticle.

Fig. 2 shows that water content of the excised leaf of plants treated with acid rain or  $O_3$  was decreased more than that of the control indicating that the loss of water could be a result of cuticular transpiration, since the wax structure of the leaf was damaged by treatment with simulated acid rain as reported by Mengel *et al.* (1989) and probably due to the reaction of  $O_3$  with the cell wall, plasma membrane causing leaf injuries (Antoinelli *et al.*, 1997). The larger water loss of the leaves damaged by  $O_3$  and acid rain than that of control should reflect the larger cuticular transpiration.

With regard to the leaf area, there was approximately linear increase in the leaf area with time for control plants and those exposed to acid rain (Fig. 3). Whereas, leaf area was significantly reduced in  $O_3$  treatment compared to control.

Another interesting feature, is that leaves treated with acid rain (Fig. 4) became greener than those of control. Accordingly, the total chlorophyll content was greater than the other treatments and even the control. This might be caused by the nitrogen absorbed by the canopy from acid rain (Igawa *et al.*, 1997). Whereas at high concentration of  $O_3$ , a decrease in the chlorophyll concentrations was observed which lead to chlorosis and necrosis. This might be due to the effect of  $O_3$  on the chlorophyll biosynthesis and / or through the interaction with chlorophyll binding protein, leading to destruction of chlorophyll or through the induced  $Fe^{2+}$  deficiency (Van-Assche and Clijsters, 1990). These results were consistent with those observed by Igawa *et al.* (1997). Protein content decreased by 28 per cent, after exposure to  $O_3$  as compared with control and acid rain treatments (Fig. 4) due to accelerated leaf senescence by  $O_3$  (Reich and Lassoie, 1985; Lehnher *et al.*, 1988; Grandjean and Fuhrer, 1989).

**Plant growth:** The response of whole-plant dry weight and plant height was significantly greater for those treated with acid rain compared to control (Table 3). The enhanced growth might be caused by the nitrogen and other elements taken by the plants from the elements of simulated acid rain (Dash, 1988) and probably because the plant can detoxify the toxic effect of acid rain indicating that biomass production is usually the last parameter to respond to environmental stressors after physiological and biochemical processes have been affected. Furthermore, shoot dry weight was significantly greater than control, whereas root dry weight was unaffected. As a result, exposure to acid rain, increased the ratio of shoot-to-root biomass. This result was in agreement with Whitmore *et al.* (1982), McLaughlin and McConathy (1983), Murray (1985) and Darrall (1989), who found that air pollutants can alter the pattern of assimilate allocation favoring shoot growth at the expense of root growth and as a result, ratio of shoot-to-root biomass can be increased.

However, this pattern was not found for plants treated with  $O_3$ , where elevated  $O_3$  caused reduction in whole plant dry weight (Table 3). This was most likely due to the  $O_3$ -induced decreases in photosynthesis and leaf area, which would result in a decreased whole-plant photosynthetic rate. In addition, increased leaf dark respiration in  $O_3$ -grown plants would result in further lowering whole plants net carbon balance. Accordingly, increased respiration and /or decreased photosynthesis have been reported as the probable causes of decreased growth due to  $O_3$  (Reich and Amundson, 1985). However, stressors and plant interactions may become even more complicated due to the influences of multiple environmental factors as mentioned before (Qifu and Murray, 1993).

**The metal element content:** The determined metal elements in the leaves are shown in Table 4. Compared with control, it was found that there were some differences in metal concentrations due to the effect of O<sub>3</sub>, where a significant decrease for these five elements in leaves treated with O<sub>3</sub> was observed. Such reductions in minerals may be due to reduced uptake, in combination with a greater mineral retention by other plant parts. (Sprugel *et al.*, 1980; Vandermeiren *et al.*, 1992), reported a similar results with wheat. In contrast, the effect of the acid rain treatment on the metal concentrations in the leaves was substantially and lead to the increase of metal elements concentrations, probably due to the metal ions taken by the leaves from the acid rain, although metal ions readily leached from leaves to stem flow.

**Peroxidase (PO) activity:** Peroxidase (PO) is involved in numerous physiological mechanisms and it is commonly reported that peroxidase activity is directly involved in plant response to ozone and other oxidative stressors. However, the extracellular peroxidase activity, involved in the reduction of H<sub>2</sub>O<sub>2</sub>, generated together with free radicals from O<sub>3</sub> degradation, has been found in leaves of bean (Peters *et al.*, 1989). The physiological roles of this enzymatic activity are complex and clearly not known or understood (Anderson *et al.*, 1986 and Lobarzewski, 1986). Evolution of PO activity was characterized by a distinct increase in all treatments but was clearly different in the treatment of high concentration of O<sub>3</sub>. Thus, the time course of PO activity measured in leaves of faba bean shows a highly significant increase in plants treated with O<sub>3</sub> (Fig. 5), indicating that there is a scavenging system towards H<sub>2</sub>O<sub>2</sub>, as was hypothesized for other plant species by Hyodo and Tanaka (1986) and Scalet *et al.* (1995). At the end of experiment the activity of PO was about 2.5 times higher than that of the control, although the activity of PO enzyme in untreated plants was slightly increased. The increase in PO activity in control plants was in agreement with the pattern of this enzymatic activity during leaf age which might be due to the different PO isoenzymes as reported by Barber (1980). As mentioned before O<sub>3</sub> decomposition in water can induce the formation of hydrogen peroxide (Heath, 1987), a very harmful molecule for the cell. The response of faba bean plant to combined ozone and acid rain treatments shows that the activity of peroxidase enzyme was stimulated, although acid rain as seen in Fig. 5 had little effect on the activity of PO enzyme. This means that the increase in PO activity is mainly due to the O<sub>3</sub> exposure.

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