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Changes in Nitrogen Metabolism in Leaves of Bean (*Phaseolus vulgaris* L. Cv Giza 6) in Response to Ozone

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Abstract

White bean (*Phaseolus vulgaris* L. cv. Giza 6) were exposed for 35 days (8 h d^{-1}) to 7, 60 and 100 ppb Ozone (O_3) in open- top-chambers were measured in leaves. The effect of chronic O_3 stress on characteristics of nitrogen metabolism. Protein content and enzyme activity of glutamine synthetase were both shown to decrease with increasing O_3 concentrations. The highest O_3, concentration (100 ppb) caused increases in the levels of spermidine arid total amino acids. This was associated with an increase of the concentrations of the individual amino acids glutamine, glutamate, alanine, threonine and, especially, asparagine. The concentration of glutamine was found to be higher at the intermediate O_3 level (60 ppb). Possible implications for carbohydrates metabolism and carbon partitioning are discussed.

Introduction

The pool size of free amino acids has been reported to increase during short term exposure of plants to high O_3 concentrations (200-1000 ppb) (Ito *et al*., 1985). The same observation was made after exposure of bush beans (*P. vulgaris* L. cv. Rintintin) to low levels during long-term fumigation (Bender *et al*., 1990b). Ito *et al*. (1986) detected an increase of the concentrations of the amides asparagine end glutamine in leaves of bean after exposure to O_3. Furthermore, the amounts of glycine and serine and also the nccrporation of [^{13}C] from [$^{13}CO_2$] into glycine and serine were found to be enhanced under O_3 treatment (Ito *et al*., 1985; Manderscheid *et al*., 1991), which indicate that the rate of photo respiration was elevated under O_3 exposure. This assumption is supported by the increase of the glutamine synthetase activity found after long-term O_3 fumigaticn of spruce trees (Bender *et al*., 1990a), because the major part of the activity of this enzyme is thought to be involved in the reassimilation of photo respiratory ammonia (Keys *et al*., 1978). Polyamines, another group of amino compounds, have been demonstrated to respond to a wide range of environmental stresses (Pierre and Queiroz, 1981). Recently, Bors *et al*. (1989) and Rowland-Bamford *et al*. (1989) have shown that feeding polyamines to plants reduces visible injury caused by O_3 treatment and that the polyamine level was increased during O_3 exposure. As underlaying mechanisms of polyamines are discussed to reduce O_3 effects by stabilization of membranes and by scavenging of O_3 derived free radical O_3 (Smith, 1985).

Bean (*Phaseolus vulgaris* L. cv. Giza 6) was chosen for this study because it has been reported, recently, to be sensitive to O_3 as the yield was decreased by about 30 percent due to exposure to ambient concentrations of about 100 ppb O_3 (Hassan, 1998b).

This study was conducted to investigate the response of nitrogen metabolism of Egyptian cultivar of white bean (*Phaseolus vulgaris* L, cv. Giza 6) to chronic low-level O_3 stress, with special emphasis on the parameters (glutamine synthetase, protein, amino acids and polyamines) that indicate an incorporation of N into organic compounds.

Materials and Methods

Plant culture and O_3 exposure: Bean plants (*Phaseolus vulgaris* cv. Giza 6) were sown in multipurpose compost in plastic pots in a heated glass house. Ten days after sowing, seedlings were thinned to one seedling per pot, and transferred to open-top chambers (OTCs). All plants were watered twice a week and there were no fertilizers or herbicides used.

Plants were exposed to either clean filtered-air (FA control, i.e., 7 ppb) or 60 and 100 ppb ozone 8 h d^{-1} (9:00-17:00 Egyptian local time) for 35 days in OTCS which have been previously described (Hassan, 1998b).

After 35 days of fumigation, fully expanded trifoliate leaves were harvested from all plants and immediately frozen and stored in liquid nitrogen until biochemical analysis

Amino acids and polyamine analyses: They were determined using HPLC according to Einarsson *et al*. (1983). The HPLC system consists of a varian Model 5500 Liquid Chromatograph connected with a flourecence monitor (Shimadzu Model RF-535) and autosampler (Model 9090) equibbed with a Valco injector provided with a 10 µL sample loop.

Sample preparation for amino acid analysis: Leaf tissue was extracted in 4 percent sulphosalicylic acid which contained β-DL-(2-thineyl) alanine as internal standard. The extract was kept on ice for 2 hours then neutralized with NaOH and then centrifuged for 20 min. at 15000 rpm, filtered and diluted with 100 mM borate as a buffer (pH 8.5). The extract was kept in liquid nitrogen and stored at 50°C in

a deep freezer and analyzed within the next couple of days. 25 µL of the extract was mixed with 25 µL 1.0 mM FMOC-Cl in acetone and extracted with 75 µL petane/ethylacetate (75/25 v/v).

Amino acids were separated using two elusion solvents were used; 15 mM sodium citrate and 10 mM tetraethylammonium chloride adjusted to pH 4 with concentrated H_3PO_4, at this pH value the two amides aspargin and glutamine were separated, (Manderscheid *et al.*, 1991). A reversed phase column (Amino Tag amino acid analysis column, Varian) was used and placed in a column oven at 30°C.

Leaf tissue was extracted in 5 percent perchloric acid which contained 1,6-diaminohexane as standard and the rest as described above for amino acids, with small modifications; 50 of the extract was mixed with 50 µL 0.5 mM FMOC in acetone and there was no extraction in pentane/ethylacetate.

For polyamine separation, the elusion solvents consisted of water and acetonitrile. A reversed phase column (Beckmann Ultrasphere ODSC18) was used at the same temperature as amino acids. Quantification was done by integration of the fluorescence chromatogram (excitation 260 nm and emission 310 nm). The content of each amino acid and polyamine was calculated on the basis of the internal standard (Manderscheid *et al.*, 1992).

Glutamine synthetase activity: Leaf tissue was homogenized in 100 mM Tris HCI buffer (pH 8.5) containing 0.5 mM Mg SO4, 0.5 mM EDTA, 1 mM cysteine and 10 mM dithioerythritol, centrifuged for 15 min at 20,000 rpm and the supernatant was used for enzyme assay as described by Wild and Manderscheid (1984).

Protein determination: Protein in the leaf was extracted and the crude extract was diluted with distilled water and the content was determined according to Hassan (1998a).

Results

Protein content and glutamine synthetase activity decrease by 10 and 25 percent, after exposure to 60 and 100 ppb O_3 respectively as compared with control treatment (Fig. 1). Fig. 2 shows that there was no significant ($p>0.05$) effect of on spermine whereas putrescine contents of leaves decreased by 47 percent after exposure to 60 ppb O_3 only. Moreover, spermidine content was significantly increased at the highest O_3 level (100 ppb) by 31 percent (Fig. 2).

Total amino acid content was increased after exposure to 100 ppb by about 60 percent, especially because of the increase of glutamate, asparagine and glutamine, which all together amounted to about 50 percent of the increase of total amino acid (Table 1). Contents of alanine, threonine, lysine, phenylalanine and isoleucine showed the same trend (Table 1). Glutamate concentration was the only parameter increased after exposure to 60 ppb O_3 (Table 1).

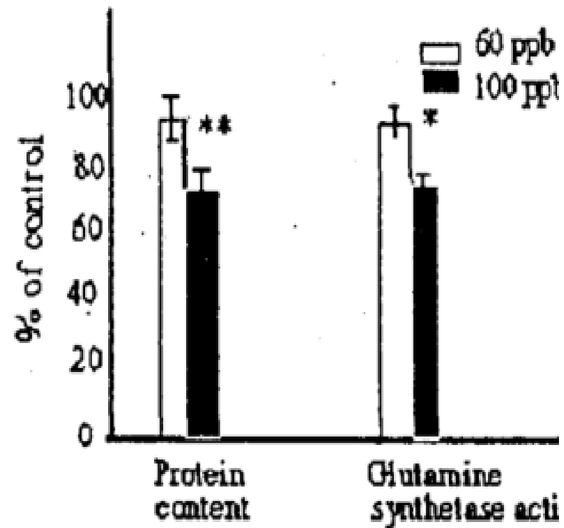

Fig. 1: Effects of ozone fumigation on soluble content and glutamine synthetase activity of bean. Bars represent ± SE (n = 8). * = $p \leq 0.05$; ** = $p \leq 0.01$

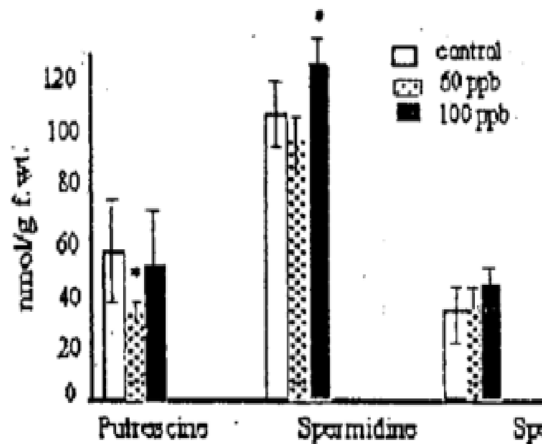

Fig. 2: Effect of ozone fumigation on polyarnine co Bars represent ± SE (n = 8). * = $p \leq 0.05$; ** = $p \leq 0.01$

Discussion

The concentrations of 0, used in the present study been reported to occur in Egyptian ambient air (WHO/UNEP, 1992; Hassan, 1999).

Table 1: Effect of O_3 exposure on amino acid contents of white bean (*Phaseolus vulgaris* L. cv Giza 6)

Amino Acid (μmol/g F.Wt.)	Control 10 ppb	Ozone 60 ppb	Ozone 100 ppb
Glutamic	1.19±0.34	2.09±0.49*	1.99+0.31*
Glutamine	0.26±0.09	0.25±0.04	0.73±0.23**
Aspartic	1.39±0.24	1.42±0.13	1.76±0.25
Asparagine	0.99±0.35	1.02±0.21	2.49±0.65*
Serine	1.35±0.23	1.42+0.18	1.90±0.55
Glycine	0.16±0.05	0.35 +0.07	0.55±0.15
Alanine	0.85±0.18	0.61±0.11	1.21±0.07*
Therionine	0.25±0.05	0.22±0.04	0.39±0.07**
Proline	1.89±0.31	1.60±0.28	2.17±0.21
Histidine	0.31±0.05	0.81 +0.25	1.12+0.55*
Lysine	0.16±0.05	0.18 +0.09	0.25±0.05*
Arginine	0.11±0.05	0.13±0.03	0.13±0.02
Phenylalanine	0.09±0.03	0.16±0.05	0.21±0.05*
Isoleucine	0.15±0.05	0.19±0.06	0.24±0.05*
Leucine	0.18±0.05	0.23±0.05	0.29±0.04
Valine	0.19±0.02	0.21± 0.02	0.25±0.05
Methionine	0.04±0.01	0.04±0.01	0.05±0.01
Tyrosine	0.05±0.01	0.05±0.01	0.06±0.01
Total amino acids.	9.56±0.98	10.97±0.85	15.4±1.56*

All figures are means±SE; * and **indicate significant difference at $p<0.05$ and $p<0.01$, respectively

Ozone-induced reductions in protein content and glutamine synthetase activity are due to accelerated leaf senescence, especially these parameters decline during senescence (Mori and Sodek, 1983; Reich and Lassoie, 1985; Lehnherr *et al*., 1988; Grandjean and Fuhrer, 1989; Manderscheid *et al*., 1991) and which is supported by the decrease of leaf biomass at the highest O_3 level (Hassan, 1998b).

The increase of spermidine content is in agreement with the results of (Rowland-Bamford *et al*., 1989) who reported an increase in spermidine content in barley leaves after exposure to O_3. This increase in polyamine content may act as to diminish the rat of ethylene big mthesis, which is thought to promote O_3 injury (Mehlhorn and Wellburn, 1987; Hassan *et al*., 1999). However, Pennazio and Roggero (1990) reported that ethylene biosynthesis is enhanced by adding spermididn and spermine exogenously to plants. Therefore, polyamines may scavenge free radicals and stabilize membranes when plants exposed to O_3 (Bors *et al*., 1989; Manderscheid *et al*., 1991, 1992).

Pollutant effects on total amino acids showed a common trend independent of the gaseous pollutant and plant species used: mostly the content of amino acids increased and especially there was an increase in the contents of amides as compared with control plants (Jager, 1977). Our study indicated that total amino acids and amides were increased after long-term exposure to O_3, particularly content of asparagine, and this contradicts results of Ito *et al*. (1986), as they could not find any effect on amino acids leaves after exposure of beans to 60 ppb O_3 for a week, and this may be due to short exposure period in their study. On contrast, Bender *et al*. (1991) reported a significant increase in the total amino acids after exposure to 60 ppb O_3 during the whole growing season. Serine and glycine contents, which are directly involved in photo respiratory N-cycle (Keys *et al*., 1978), tended to increase due to O_3 fumigation, but the variability in their concentrations was too high to indicate O_3 related changes. It has been reported that during plant senescence the decline in free amino acid concentrations proceeds parallel to the decline in protein content (De Kok and Graham, 1989; Sleciechowicz and Joy, 1989). However, this study indicated that the content of amino acids was the highest in the leaves of plants exposed to 100 ppb O_3, which contained a lower protein content than the control leaves. The possible explanation is that the rate of protein degradation is increased resulting in an enhancement of the amino acid pools and an overload of the N-export processes. During water stress, which is thought to he accompanied by an increase of photo respiration, similar changes in the amino acid content have been found in the present study as well as an increase in praline content (Bender *et al*., 1990b). Moreover, a striking increase in the content of asparagine was noticed (McMichael and Elmore, 1977). Madore and Grodzinski (1984) detected a corresponding pattern of changes of the content of amino acids in leaves after an enhancement of photo respiration. Moreover, they reported that the percentage of fixed carbon was allocated to starch, while a higher percentage appeared in amino acids and the export of these amino acids to sink oranges was increased. Ito *et al*. (1985, 1986) found an increase in the content of amino acids in leaves and roots of bush beans as well as an increase in photo respiration after exposure to O_3. These results are in good agreement

with the results of the present investigation, that O_3-induced alterations in the amino acid content may be a result of O_3 effects on photo respiration (Manderscheid *et al*., 1991). Amino-N from protein degradation in senescent leaves is fed into the photo respiratory N-cycle to synthesize amides via alanine (Joy, 1988). This pathway could provide a route for transfer of amino-N from protein degradation into amides for transport into fruiting structures. The appertaining pools of amino acids and amides was found to be increased after exposure to the highest O_3 treatment (100 ppb).

The effects of O_3 on nitrogen metabolism imply consequences for other physiological processes such as carbohydrate metabolism and carbon partitioning. Besides a reduction in net photosynthesis (Sasek and Richardson, 1989; Manderscheid *et al*., 1992) the increased demand of carbon for amino acid protein biosynthesis could contribute to the reduction in starch and sucrose content upon O_3 exposure (Paynter *et al*., 1991).

In conclusion, both acceleration of protein degradation rate and the increased rate of photo respiration resulted in the increase in amino acid contents in leaves of bean exposed to O_3. Moreover, during leaf senescence nitrogen from protein degradation is presumably converted via photo respiratory N-cycle into amides, which then are transported in the phloem. This warrant further investigation to assess whether O_3 affects nitrogen metabolism by the increase of photo respiration and/or by acceleration of leaf senescence and protein breakdown.

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