Ozone stress modulates amine oxidase and lipoxygenase expression in lentil (*Lens culinaris*) seedlings

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Abstract The effect of ozone stress on polyamine metabolism and membrane lipid peroxidation in lentil seedlings through the amine oxidase and lipoxygenase activity and expression has been investigated. Ozone is shown to control the expression of these enzymes at the transcriptional level, down-regulating the amine oxidase gene and up-regulating the lipoxygenase gene. The decrease of amine oxidase activity correlated with the increase of putrescine concentration in the ozone-treated plantlets, whereas the increase of lipoxygenase activity was paralleled by enhanced membrane lipid peroxidation. Finally, polyamines are shown to inhibit lipoxygenase activity in lentils.

Key words: Amine oxidase; Lipoxygenase; Polyamine; Hydroperoxide; Ozone

1. Introduction

Ozone (O_3) is an important constituent in photochemical air pollution and is known to affect plant growth and development through the induction of oxidative stress [1]. Among the biochemical responses of plants to ozone there are (i) changes of intracellular levels of radical scavengers [1] and antioxidant enzymes, such as superoxide dismutase and glutathione reductase [2]; (ii) induction of polyamine and ethylene biosynthetic pathways [3]; (iii) premature plant senescence, perhaps linked to membrane lipid peroxidation [2,4]. Plants of the Fabaceae family play an important role in agricultural production due to their ability of fixing atmospheric nitrogen. The legume lentil (*Lens culinaris*) is among the oldest domesticated crops. Recently, the first gene coding for a plant amine oxidase has been isolated from lentil seedlings [5], as well as the gene coding for lipoxygenase [6]. Lentil seedlings amine oxidase (LSAO, diamine:oxygen oxidoreductase [deaminating], EC 1.4.3.6) is a copper-containing homodimeric glycoenzyme which oxidizes primary amines producing the corresponding aldehydes, NH_3 and H_2O_2 [7]. LSAO is responsible for the catabolism of intracellular polyamines, purported natural substrates of the enzyme activity [8]. In legumes, amine oxidase is most active towards putrescine [9,10], whereas spermidine and spermine are oxidized to a much lower extent [11]. Little is known about the physiological role(s) of LSAO, though its activity has been shown to be involved in cell wall stiffening and lignification [12], plant growth regulation [13] and response to stress factors [14]. These roles might be played through the control of the intracellular level of polyamines. On the other hand, lipoxygenase (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron-containing dioxygenase which catalyzes the oxidation of unsaturated fatty acids with 1,4-*Z*,*Z*-pentadiene systems to *Z*,*E*-conjugated hydroperoxy acids [15]. Plant lipoxygenases play a role in growth and development, senescence and responses to wounding and pest attacks [16,17]. Interestingly, lipoxygenase activity has been implicated also in plant membrane deterioration [18,19], due to its ability to directly oxygenate biological membranes [20,21].

In this paper we investigated the effect of ozone on the activity and expression of LSAO and LOX in lentil seedlings, in relation to the effect of ozone on the metabolism of polyamines and membrane lipids. Moreover, the possible interaction between polyamines and lipoxygenase activity has been ascertained, because it has been proposed that polyamines might protect plants against ozone by inhibiting lipid peroxidation of membranes [22–24].

2. Materials and methods

Chemicals were of the purest analytical grade. GAR-AP and GAM-AP were from Bio-Rad. Digoxigenin (DIG) oligonucleotide Tailing kit, nylon membranes and DIG Luminescent Detection kit were from Boehringer Mannheim.

2.1. Plant material, stress induction and enzyme assays

Lentil (*Lens culinaris* L.) seeds were soaked in tap water for 4 h at room temperature, then they were germinated in a greenhouse for 5 days at 22°C, in the dark, and were watered daily with tap water. 5-Day-old, dark-grown lentil seedlings were subjected to ozone treatment as described [25], using an electrical discharge ozonizer (ROTAX) which affords an ozone flux of 5 ml/min. After ozone stress, seedlings (15 plantlets for each experimental point) were homogenized by Ultra Turrax at 4°C in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM PMSF. Homogenates were centrifuged 15 min at $4000 \times g$, the supernatants were collected and centrifuged twice for 15 min at $8000 \times g$. The final supernatants were used for enzyme assays and immunochemical determinations. Protein concentration was determined by the Bio-Rad assay [26], using BSA as a standard. LSAO and LOX activities were both assayed polarographically in 0.1 M sodium phosphate buffer, pH 7.0, using 1 mM putrescine [13] or 1.8 mM linoleic acid [25] as substrate, respectively.

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Abbreviations: BSA, bovine serum albumin; DIG-dUTP, digoxigenin-labeled deoxyuridine-triphosphate; GAM-AP, goat anti-mouse IgGs conjugated with alkaline phosphatase; GAR-AP, goat anti-rabbit IgGs conjugated with alkaline phosphatase; LOX, lipoxygenase; LSAO, lentil seedlings amine oxidase; PMSF, phenylmethylsulfonyl fluoride; RP-HPLC, reversed phase high performance liquid chromatography

This paper is dedicated to Prof. A. Ballio on the occasion of his 75th birthday.

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2.2. Enzyme-linked immunosorbent assay (ELISA)

The amount of LSAO or LOX in lentil seedlings was estimated by ELISA according to [25]. Anti-LSAO polyclonal antibodies, purified as described [27] and diluted 200-fold, or anti-LOX monoclonal antibodies [25] diluted 1000-fold, were used as primary antibodies. Anti-LSAO and anti-LOX immunoglobulins were reacted with GAR-AP or GAM-AP, respectively, both diluted 2000-fold. Color development of the alkaline phosphatase reaction was recorded at 405 nm. The A_{405} values of lentil homogenates were within the linearity range of calibration curves drawn by coating the ELISA plates with pure LSAO or LOX [13,25].

2.3. Dot-hybridization analysis

Isolation of total RNA from lentil seedlings, purification of poly-(A)$^+$RNA by messenger affinity paper chromatography and dot-hybridization analysis were performed as described [28]. Poly(A)$^+$RNAs from different samples (2 μg/dot) were separately hybridized with two different oligonucleotides, prepared with a Biosearch 8600 DNA Synthesizer: 5′-GCACCGCGTATTTGTGGATAAT-3′, specific for the LSAO mRNA [5], and 5′-TCGGTAACCAACTTGTGAGT-3′, specific for LOX mRNA [6]. The probes were labeled at their 3′-ends with terminal transferase in the presence of DIG-dUTP [28].

2.4. Polyamine analysis

Extraction of polyamines from plant homogenates and separation into free, conjugated soluble and conjugated insoluble fractions were performed according to Flores and Galston [29]. Extracts were then benzoylated for RP-HPLC analysis [29], performed using a HP1090 liquid chromatograph interfaced with a HP79994A analytical workstation and equipped with a 1040A diode array detector (Hewlett Packard). Benzoylated extracts were eluted isocratically through a CP-Spher C18 column (5 μm, 250×4.6 mm, Chrompack), using acetonitrile:water (52:48) at a flow rate of 1 ml/min. Samples were detected at 254 nm and their identity was ascertained by co-injection with authentic standards, treated as the unknown samples. The compounds were quantitated by peak area integration.

2.5. Membrane lipid analysis

A pool of membrane lipids was extracted from lentil seedlings [30] and separated into free fatty acids and esterified fatty acids by octadecyl solid-phase columns (J.T. Baker), as reported [20]. The amount of conjugated hydroperoxides present in each sample was calculated using the extinction coefficient at 235 nm of 25000 M^{-1} cm^{-1} [28].

Data reported in this paper are the mean (±S.D.) of three independent experiments. Statistical analysis, performed by the Student's *t*-test, showed that the difference between control and each treatment was significant at least at 5% level ($p < 0.05$), unless stated otherwise.

3. Results and discussion

5-Day-old, dark-grown lentil seedlings were chosen to study the effect of ozone stress on LSAO and LOX expression, because these plantlets show the highest levels of activity of both enzymes, as already reported [13,31]. In Fig. 1 it is shown that LSAO and LOX activities were inversely affected by ozone fumigation, the former decreasing and the latter increasing as a function of the duration of the treatment (Fig. 1A). Protein content and mRNA level of both enzymes followed the same trend as the specific activity, LSAO being reduced to 50% and LOX being increased to 200% of the control value after 6 h of treatment (Fig. 1B and C). Therefore, ozone regulates the expression of both LSAO and LOX genes at the level of transcription, suggesting that the role of these two enzymes in plant response to ozone might be critical. These results extend previous observations, which showed that ozone fumigation activates lipoxygenase in soybeans, though with different kinetics [25]. Therefore, LOX up-regulation might be of general relevance for plant response to ozone. This is the first report showing that LSAO expression is down-regulated by ozone, a finding which is noteworthy if one recalls that various stress factors (e.g. oxygen depletion, thermal injury, copper treatment) modulate LSAO activity without affecting gene expression [13,32].

Interestingly, the decrease of LSAO expression and activity upon ozone fumigation correlated with the increase in concentration of the enzyme substrate, putrescine, under the same conditions (Fig. 2). Putrescine was the most abundant polyamine in the cell extracts, followed by spermidine and sper-

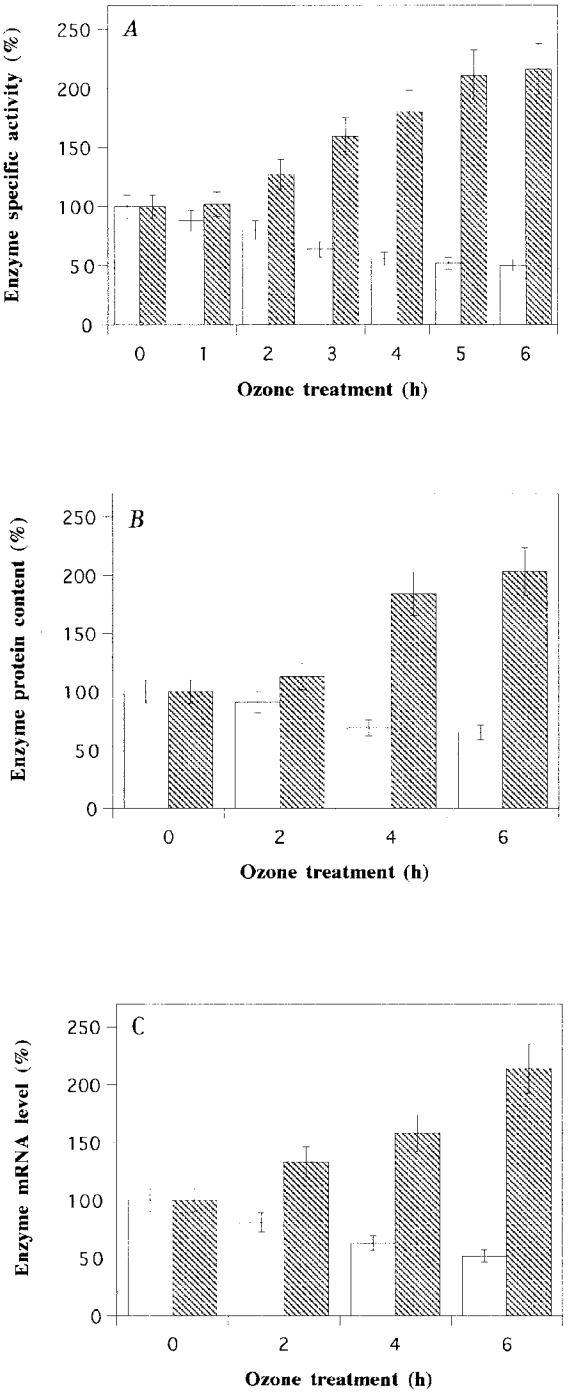

Fig. 1. Effect of ozone on LSAO (empty bars) and LOX (hatched bars) expression. (*A*) Specific activity (100% LSAO = 550 ± 50 nmol O_2 min^{-1} mg P^{-1}; 100% LOX = 1137 ± 110 nmol O_2 min^{-1} mg P^{-1}); (*B*) protein content; and (*C*) mRNA level of LSAO and LOX of 5-day-old, dark-grown lentil seedlings subjected to ozone fumigation.

mine (Fig. 2). Approximately 90% of the putrescine content was in the free and conjugated soluble forms, which are both substrates for the enzyme [33]. Ozone treatment significantly increased the amount of free and conjugated soluble putrescine, whereas the level of conjugated soluble spermidine increased only slightly and spermine was not affected at all (Fig. 2A and B). Finally, the amount of conjugated insoluble polyamines remained constant during the ozone treatment (Fig. 2C). These findings suggest that the correlation between down-regulation of LSAO expression and increase in free and conjugated soluble putrescine might be of a cause-effect type. Several factors, like chilling injury, UV-B radiations, salt and osmotic stress [19,24,34,35] are known to increase the intracellular level of free putrescine. Here, it is shown that also the conjugated soluble form of this polyamine follows the same trend as the free fraction (compare Fig. 2A and B). Ozone stress has been reported to stimulate the biosynthesis of putrescine in tobacco [3]. Here, it is suggested that ozone-induced putrescine accumulation might occur also by down-regulating LSAO, the main catabolic enzyme of putrescine. The fast accumulation of putrescine induced by O_3 might lead in turn to a slower conversion into the higher polyamines spermidine and spermine [36].

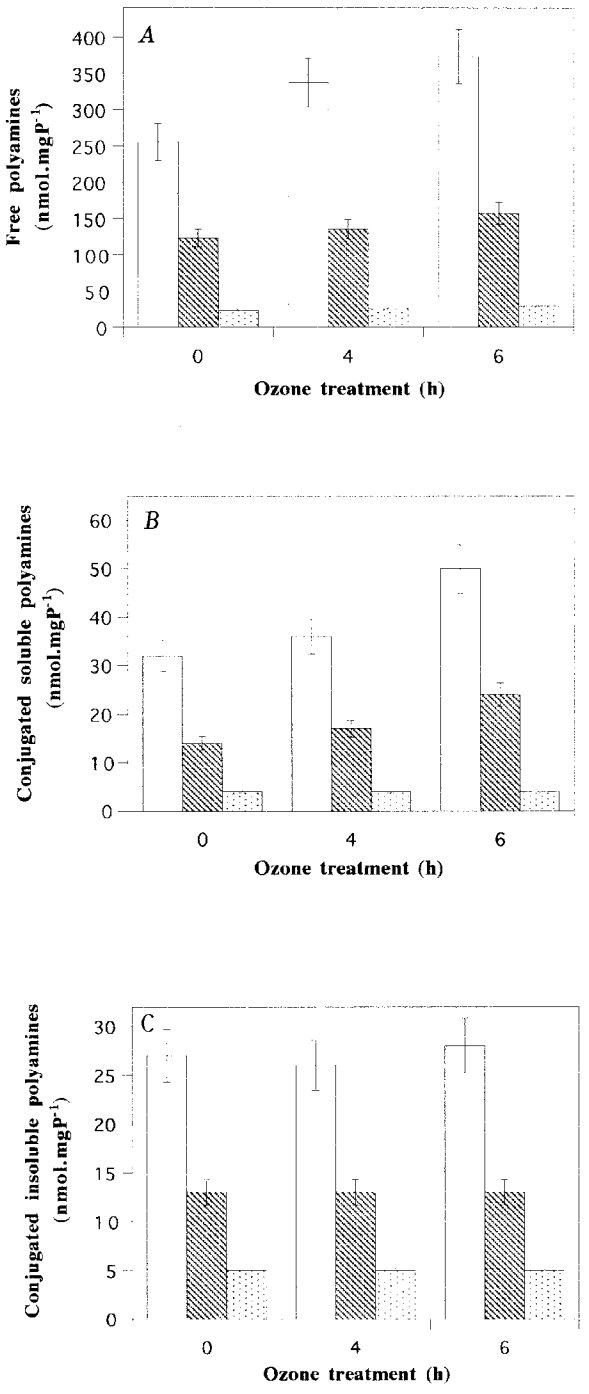

Fig. 2. Effect of ozone on polyamine metabolism. Putrescine (empty bars), spermidine (hatched bars) and spermine (dotted bars) were extracted from 5-day-old, dark-grown lentils after ozone treatment, then they were assayed as benzoylated derivatives by RP-HPLC in separate fractions: free (*A*), conjugated soluble (*B*) and conjugated insoluble (*C*) polyamines. Changes in conjugated insoluble putrescine, free and conjugated insoluble spermidine and all forms of spermine were not significant compared to the controls ($p > 0.05$).

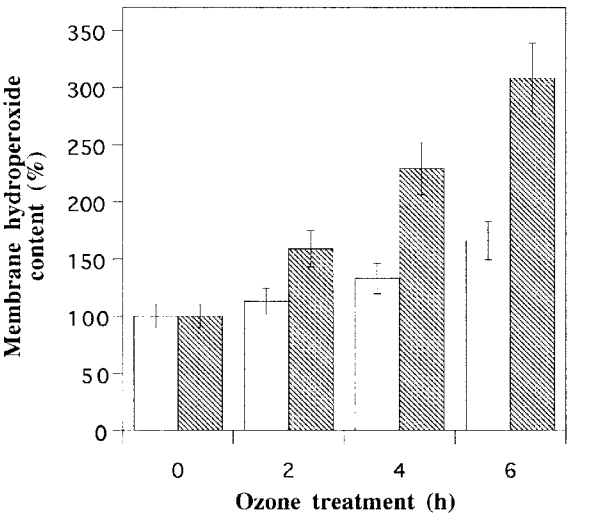

Fig. 3. Effect of ozone on membrane lipid peroxidation. The hydroperoxide content of the free fatty acids fraction (empty bars) and the esterified fatty acids fraction (hatched bars) of a membrane pool isolated from 5-day-old, dark-grown lentils exposed to ozone was quantitated and expressed as percentage of the control (100% of the free fatty acids fraction = 5.10 ± 0.60 nmol mg P^{-1}; 100% of the esterified fatty acids fraction = 13.33 ± 1.50 nmol mg P^{-1}).

In Fig. 3 it is shown that ozone stress stimulates lipid peroxidation of lentil membranes, thereby increasing the hydroperoxide content particularly in the esterified fatty acids frac-

Table 1
Inhibition of lentil lipoxygenase (LOX) activity by polyamines

Sample	LOX specific activity (nmol O_2 min^{-1} mg P^{-1})
Lentil homogenate	1137 ± 110 (100%)
Lentil homogenate+putrescine	965 ± 95 (85%)
Lentil homogenate+spermidine	739 ± 70 (65%)
Lentil homogenate+spermine	568 ± 60 (50%)

5-Day-old lentil seedlings were homogenized and assayed for LOX activity as described in Section 2, in the presence of different polyamines (2 mM final concentration). Specific activity values (± S.D.) were also expressed as percentage of the untreated control, arbitrarily set to 100 (in brackets).

tion. Interestingly, the lipid ester fraction of biological membranes is the target of LOX activity in vitro [21]. A role for polyamines as inhibitors of lipid peroxidation has been suggested [22–24], in particular a possible interaction between polyamines and lipoxygenase has been proposed to explain the anti-senescence effects of polyamines in osmotically-stressed oat leaves [19]. Thus, to ascertain whether polyamines could directly interact with LOX, lentil homogenates were incubated with different polyamines, in a concentration range (up to 2 mM) close to the intracellular levels [23]. In Table 1 it is shown that polyamines inhibit LOX activity and that the inhibitory power depends on the polyamine chain length: spermine > spermidine > putrescine. These findings suggest that the direct inhibition of LOX activity by polyamines could contribute to ozone-induced lipid peroxidation. It is tempting to suggest that ozone sensitivity, which is known to vary widely among plants, may result from a balance between the generation of toxic species (e.g. free radicals, alkyl radicals and peroxides) due to the activation of lipoxygenase, and the inhibition of LOX by polyamines to counteract the production of these reactive molecules.

In conclusion, this paper shows that ozone modulates LSAO and LOX expression, resulting in altered polyamine and membrane lipid metabolism. Therefore, the modulation of LSAO and LOX genes in legumes might provide plants with a better environmental stress tolerance.

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