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Benzothiadiazole-Induced Resistance Modulates Ozone Tolerance

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The effects of ozone on bean plants pretreated with the SAR activator benzothiadiazole (BTH) have been investigated after fumigations with an acute dose of the pollutant (200 nL·L^{-1} for 4 h), carried out at different times from BTH application. BTH pretreatment induced opposite effects on bean susceptibility to O_3, depending on the time elapsed before fumigation. When this time was only 1−2 days, bean plants were more susceptible to O_3 than untreated controls, showing rapid and extensive cell death in both palisade and spongy mesophyll. These damages appeared to be closely correlated with the amount and localization of H_2O_2 in the leaf tissues. In BTH-pretreated, but not fumigated, plants, H_2O_2 accumulation occurred in the cell walls and no dead cells were detected, whereas O_3 fumigation of untreated plants produced H_2O_2 accumulation also inside some palisade mesophyll cells, causing their death. When BTH pretreatments were carried out 5−7 days before fumigation, plants appeared to be more tolerant to O_3 compared to untreated controls. Under these conditions, no visible symptoms of phytotoxicity were observed for at least 2 weeks after fumigation and no H_2O_2 accumulation was detected. Biochemical assays showed a significant increase in the ascorbate (AA) level, taking place from the fifth to the seventh day after BTH treatment and unaffected by O_3 when given at these times. Ascorbate peroxidase (APX) activity appeared to decrease during the first 2 days after BTH treatment, and the decrease was somewhat enhanced by fumigation. On the contrary, guaiacol peroxidase (GuPX) activity was found to steadily increase up to the fifth day after BTH treatment but showed a bimodal trend upon fumigation. These results suggest that, during the first 1−2 days after BTH application, the H_2O_2 level is enhanced by O_3 over a critical threshold for cell viability. However, in the absence of the pollutant, H_2O_2 decreases in the following days under the effect of AA accumulation and increased GuPX activity. As GuPX is known to promote cell wall lignification and protein cross-linking, these effects would protect plasmalemma from O_3 irreversible damage, provided the priming by BTH has been fully developed.

KEYWORDS: French bean; BTH; SAR; H_2O_2; cell death

INTRODUCTION

In contrast to protective stratospheric ozone (O_3), which shields biologically harmful solar ultraviolet (UV) radiation from reaching the earth's surface, tropospheric O_3 is a widespread phytotoxic secondary air pollutant (*1*). This allotropic oxygen form is one of the predominant components of smog produced via oxygen-mediated photolysis of anthropogenic ozone precursors nitrogen oxides and hydrocarbons (*2*). However, increasing ozone concentrations in the troposphere are not restricted to areas of intense urbanization and industrialization; it is often more pronounced in rural areas, where O_3 formation is usually favored on sunny days (*3*).

Morphological, physiological, and biochemical responses to O_3 can vary with plant species and the dose and time of exposure. Acute exposure for relatively small periods (150−300 nL·L^{-1} for 4−6 h) rapidly causes severe alterations, such as necrotic lesions and senescence (*4*). Typical symptoms are silvering and small reddish necrotic lesions. In contrast, chronic exposure at a lower level (<100 nL·L^{-1} for days to months) does not cause visible injury, although plant productivity and biomass are significantly affected as a consequence of photosynthesis inhibition (*5*, *6*).

Because of its strong oxidizing potential (+2.07 V), O_3 first reacts with oxidizable constituents of the apoplast, where it may spontaneously generate reactive oxygen species (ROS). In fact,

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it has been shown to degrade in aqueous solution with production of hydroxyl radicals ($^{\bullet}OH$), which is strongly accelerated by traces of Fe^{2+} (*7*). Moreover, in an aqueous extract of plant intercellular fluid, O_3 was found to generate singlet oxygen, probably by reaction with ascorbic acid (*8*). As soon as O_3 comes into contact with the cell wall, it can react with several compounds typically sensitive to its action (phenolics, double bonds, and glycoproteins), giving rise to other ROS and resulting in cell injury. This is expected to become particularly severe when O_3 reaches the plasmalemma, where lipid peroxidation may take place by ozonation of polyunsaturated chains. The reaction is thought to produce aldehydes, 1 mol of H_2O_2 per mole of consumed O_3, and organic radicals (*9*).

Besides altering the physiochemical properties of plasmalemma (*10*) and other membranes, O_3 affects Ca^{2+} and H^+/K^+ fluxes and deeply changes chloroplast structure (*11*, *12*). The decreasing stomatal conductance and the damages to photosynthetic machinery cause a reduction in net photosynthesis rate (*5*).

Plant resistance to O_3 includes its avoidance, that is, physical exclusion of the pollutant from cellular targets by stomatal closure, or effect tolerance, that is, the capacity to intercept and detoxify O_3 and its byproducts (*13*, *14*). The aqueous cellular matrix is known to contain many compounds that can act as antioxidants and whose activity and/or concentration may determine the severity of O_3 injury. These include either nonenzymatic scavengers, such as ascorbate (AA), glutathione, α-tocopherol, polyamines, and phenolics (flavonoids), or several detoxifying enzymes [glutathione *S*-methyl transferase, superoxide dismutase, catalases, peroxidases, ascorbate peroxidase, glutathione reductase, dehydroascorbate (DHA) reductase, and monodehydroascorbate reductase of the Halliwell−Asada cycle (*15*−*20*)]. All of them are normally utilized to neutralize ROS produced in many metabolic processes, such as the electron transport chains in chloroplasts and mitochondria (*21*). With regard to bean plants, O_3 tolerance was recently found to be associated with genetic capacity to maintain extracellular high ratios of AA/[AA + DHA] under O_3 stress (*22*).

A new prospective in the control of pathogens is triggered by chemical resistance activators, which stimulate active plant defense mechanisms. Among them, one of the most interesting is benzothiadiazole (BTH, Bion), which induces a broad-spectrum, long-lasting systemic resistance in many plant species (*23*) against different pathogens (listed in ref *24*). Moreover, due to its low or nil toxicity to plants, animals, and the environment (*25*), its utilization in crop protection is becoming a reality.

BTH is a salicylic acid (SA) functional analogue that inhibits catalases (CAT) and ascorbate peroxidases (APX), the two major H_2O_2 scavenger enzymes, increasing the H_2O_2 pool in treated tissues (*26*). Consequently, it enhances anionic peroxidase activity involved in cell wall straightening (*24*).

We have previously demonstrated that the BTH-induced resistance is correlated with H_2O_2 accumulation in the apoplast of treated leaf tissues. We have also shown that, despite the extensive H_2O_2 deposits found in the epidermal cells, no cell death was induced (*24*).

As BTH-treated plants, with their increased H_2O_2 pool, can be subject to ozone pollution in the open field, an intriguing question arises: what are the combined effects of the two ROS-producing compounds on plants?

In the attempt to answer this question, we have carried out a number of experiments showing that the interaction between the pollutant and the resistance activator strictly depends on the number of BTH treatments and the time elapsed between treatment and O_3 fumigation. When this time interval was short (up to 2 days), plant susceptibility to ozone increased significantly; on the contrary, when 5−7 days elapsed between treatment and O_3 fumigation, plants became more tolerant to the pollutant with respect to untreated control.

MATERIALS AND METHODS

Plant Materials and Treatments. *Phaseolus vulgaris* plants, cv. Borlotto nano lingua di fuoco (BLF), were sown in 12 cm pots and grown in a greenhouse at 24 ± 2 °C, relative humidity 60 ± 5%, and 16 h/8 h light/dark period; 10−12 days after sowing, when the primary leaves were almost completely expanded, plants were sprayed with a water suspension of BTH [benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester] (trade name Bion, Syngenta) at the concentration of 0.3 mM, prepared from a wettable formulation containing 50% (w/w) reactive ingredient. Control plants were sprayed with a water suspension of wettable powder alone. Either single or multiple treatments were carried out as specified in the results.

Ozone Fumigations. Ozone fumigation was performed in air-conditioned chambers (0.48 m^3), with charcoal-filtered air, 26 ± 1 °C, relative humidity 70 ± 5%, fluence rate 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Ozone was generated by electric discharge passing pure oxygen through a Fischer ozone generator 500 MM (Fischer Labor und Verfahrenstechnik GmbH, Meckenheim, Germany). Ozone concentration in the fumigation chambers was continuously monitored with a photometric O_3 analyzer model 400 (Advanced Pollution Instrumentation Inc. API, San Diego, CA) operating on the principle of UV absorption and interfaced with a personal computer. Control plants were kept in charcoal-filtered air chambers under the same conditions. Plants from the greenhouse were preadapted to the chamber conditions for 48 h, and half of them were exposed to 200 $nL \cdot L^{-1}$ of ozone for 4 h; the untreated ones were used as a control.

Histochemistry and Cytochemistry. To detect H_2O_2 accumulation sites in vivo, a method published first by Thordal-Christensen and coworkers (*27*) and modified by Faoro et al. (*28*) was used. Briefly, primary leaves were detached with a razor blade 1 cm above the base of the petiole and immediately placed in a beaker containing 1 $mg \cdot mL^{-1}$ 3,3′-diaminobenzidine (DAB)−HCl, adjusted to pH 5.6 with NaOH, and incubated in a growth chamber for 8 h in the dark. Leaves were kept in vertical position with 5 mm of the basal part dipped into the DAB solution. After DAB uptake, 5 mm^2 samples were cut from these leaves, cleared in 96% boiling ethanol, and examined with a light microscope. H_2O_2 was visualized as a reddish-brown coloration. As negative control, DAB solution was supplemented with 10 mM ascorbic acid.

Evans blue staining, to asses cell death, was carried out by boiling leaf tissues for 1 min in a mixture of phenol, lactic acid, glycerol, and distilled water containing 20 $mg \cdot mL^{-1}$ Evans blue (1:1:1:1), prepared immediately before use. Tissues were then clarified overnight in a solution of 2.5 $g \cdot mL^{-1}$ chloral hydrate in water (*29*). Dead cells stained blue, whereas the undamaged ones appeared unstained. All samples were examined with an Olympus BX50 light microscope (Olympus) equipped with differential interference contrast (DIC) and epipolarization filters.

Electron Microscopy. Tissue fragments (1 mm^2) were excised from treated and untreated leaves and incubated in a freshly prepared 5 mM cerium chloride ($CeCl_3$), in 100 mM Hepes, pH 5.2, or in buffer alone, as control, for 1 h (*30*). Tissues were then fixed in 1.25% (v/v) glutaraldehyde and 1.25% (w/v) paraformaldehyde in 50 mM sodium cacodylate (CAB), pH 7.2, for 1 h, washed for 15 min in CAB, and postfixed for 1 h in 1% osmium tetraoxide in CAB. Samples were dehydrated in ethanol and embedded in Spurr resin. Ultrathin sections were examined without further staining with a JEOL 100SX transmission electron microscope (TEM, JEOL). For conventional ultrastructural observations, the same protocol was adopted, with $CeCl_3$ incubation omitted.

Biochemical Assays. For biochemical analysis, leaves were excised from treated and/or fumigated plants, as well as from untreated controls,

Table 1. Symptom Evaluation at Different Times after O_3 Fumigation (200 nL·L^{-1} for 4 h) of Plants Primed by BTH Treatment

treatment before O_3 fumigation	12 h	1–2 days	4–5 days
none	symptomless	small chlorotic spots	necrotic spots
BTH 1 or 2 days before	chlorotic spots	chlorotic lesions	large necrotic lesions (3–7 mm)
BTH 5 or 7 days before	symptomless	symptomless	symptomless
BTH 2, 5, or 7 days before	extensive silvering	extensive silvering	reddish necrotic lesions (2–5 mm)

rinsed in distilled water, and immediately frozen in liquid nitrogen, powdered in a precooled pestle and mortar, and stored in liquid nitrogen until analysis.

AA and DHA were extracted in 2% metaphosphoric acid (w/v). The homogenate was centrifuged at 4000*g* for 10 min (4 °C). The supernatant, neutralized to pH 5.6 with 1.25 M K_2CO_3, was used for the determination (*31*). The assay was performed at 25 °C in 100 mM sodium phosphate, pH 5.6, by complete oxidation of AA to DHA by 1 unit·μL^{-1} of ascorbate oxidase (EC 1.10.3.3. from *Cucurbita* sp., Sigma Chemical Co.). In a second assay DHA was reduced to AA by adding dithiothreitol (DTT) to a final concentration of 0.1 mM (*32*). It was used a coefficient for AA at 265 nm of 13.4 mM^{-1}·cm^{-1}.

Ascorbate peroxidase (APX) and guaiacol peroxidase (GuPX) were extracted in 66 mM potassium phosphate buffer, pH 7.0, 1% insoluble PVPP (w/v), and 100 mM KCl, filtered on Miracloth, and centrifuged at 10000*g* for 10 min (4 °C). The resulting supernatant fluid was immediately analyzed for APX and GuPX activity and protein content.

APX was determined in a reaction medium containing 100 mM Hepes−KOH, pH 7.0, 0.1 mM sodium ascorbate, and 100 μM H_2O_2. The enzyme activity was measured by following the oxidation of AA, monitored with a Beckman DU 50 spectrophotometer at 265 nm, and calculated from the ascorbate extinction coefficient of 13.4 mM^{-1}·cm^{-1} (*33*).

GuPX was determined in a reaction medium containing 66 mM postassium phosphate, pH 6.0, 2 mM guaiacol, and 30 mM H_2O_2. The enzyme activity was monitored with a Beckman DU 50 spectrophotometer at 470 nm (ϵ = 26.6 mM^{-1}·cm^{-1}) (*33*, *34*). The protein content was measured according to the method of Bradford (*35*) with γ-globulin as a standard.

Presentation of Results. Each experiment was repeated at least three times. As the repeated experiments showed similar tendencies, the results of one representative experiment are presented in the case of histo-cytochemical studies. For biochemical assays, the statistical variation of the results is specified in the figure legend.

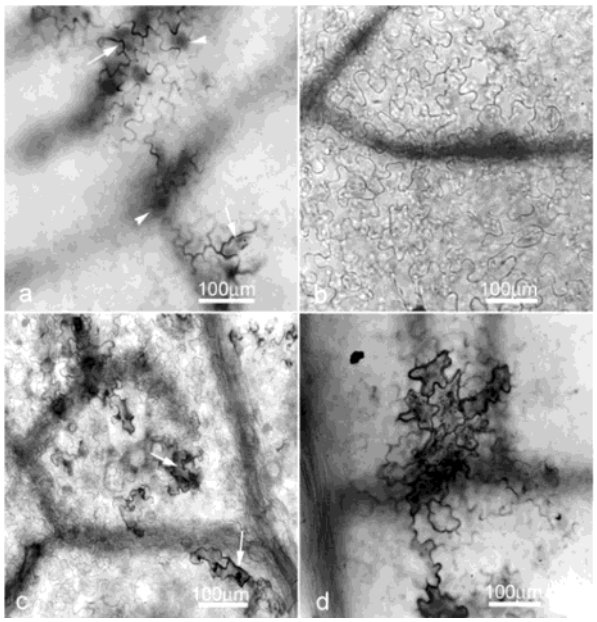

Figure 1. Localization of H_2O_2 in bean leaves with the DAB uptake method: (a) single BTH treatment 12 h before sampling [H_2O_2 accumulation is visible as dark brown precipitates of polymerized DAB on the adaxial epidermal cell walls (arrows), particularly those around stomata (arrowheads)]; (b) untreated control with no DAB precipitates; (c) leaf only fumigated and collected soon after fumigation [H_2O_2 accumulation is mainly restricted to the adaxial epidermis and is also present inside the cells (arrows)]; (d) leaf fumigated a day after BTH treatment and collected soon after fumigation (H_2O_2 accumulation is intense and diffused in numerous adjacent cells).

RESULTS

BTH Treatments and O_3 Fumigations. BTH treatments were carried out at the concentration of 0.3 mM that is normally used to protect beans from rust and does not cause any apparent phytotoxic effect in the treated tissues (*24*). Under these conditions bean plants accumulate H_2O_2 for up to 1−2 days after treatment, and this ROS is no longer detectable after 1 week (*24*). Therefore, plants were O_3 fumigated at either 1, 2, 5, or 7 days after BTH spraying with the aim to check the O_3 effect at different levels of the oxidative burst. In some experiments, the level of H_2O_2 in the tissues was further enhanced by three successive applications 7, 5, and 2 days before fumigation, respectively. Even in these cases no phytotoxic effects were observed upon BTH applications.

Acute O_3 exposure was performed at 200 nL·L^{-1} for 4 h, simulating an extreme condition of pollutant concentration that can be reached sometimes in the field in very hot and sunny summer days. Under these conditions, untreated control plants showed very small chlorotic spots on the adaxial leaf surface within 2 days from fumigation. Some of the chlorotic spots evolved into small necrotic lesions after a further 2−3 days.

In BTH-treated plants, fumigated 1−2 days after the treatment, chlorotic spots appeared usually within 12 h and increased in size in the following 12−24 h, to develop into large necrotic lesions over the next 2−3 days (**Table 1**).

BTH-treated plants, fumigated 5−7 days after the treatment, did not show any apparent symptoms, even 2 weeks later. In contrast, plants treated three times and fumigated after 2 days from the last treatment appeared to be heavily damaged, with extensive silvering that became visible in a few hours and evolved into typical reddish necrotic lesions within 2−3 days (**Table 1**).

H_2O_2 Detection. H_2O_2 localization with the DAB uptake method showed that 0.3 mM BTH induced accumulation of this compound in leaf tissues within 12 h from a single treatment (**Figure 1a**) or up to 2 days after triple treatment (not shown). H_2O_2, revealed as dark brown precipitates of polymerized DAB, was localized in the wall of many epidermal cells, particularly those around stomata, in both the adaxial and abaxial leaf surfaces (**Figure 1a**). These precipitates were lacking in untreated and not fumigated plants (**Figure 1b**). A similar H_2O_2 localization was found in untreated, but O_3-fumigated, plants within 12 h from fumigation. However, in this case H_2O_2 deposits were mainly restricted to the adaxial epidermis and were slightly heavier (**Figure 1c**) than those induced by BTH in the same time course. Usually cells showing precipitates on

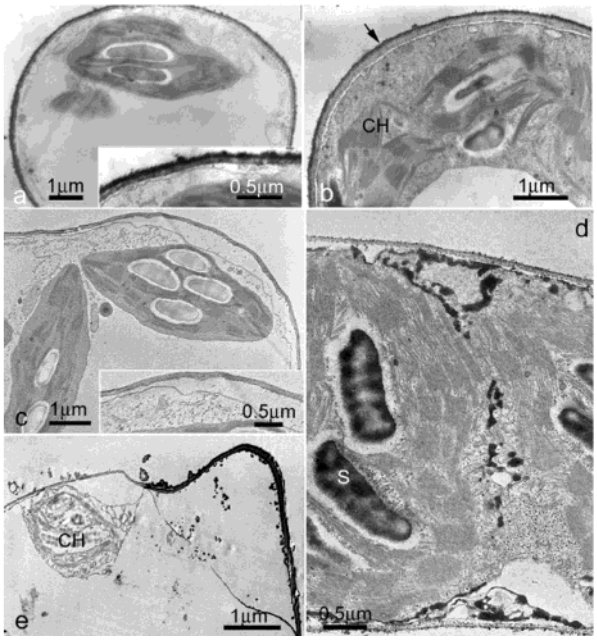

Figure 2. TEM localization of H_2O_2 with cerium chloride, ultrathin sections of mesophyll cells: (a) BTH-treated sample, 12 h after treatment [cerium black precipitates are restricted to the external wall layers (see the enlarged inset) of an apparently undamaged cell]; (b) O_3-treated tissue, 12 h after fumigation, showing similar precipitates (arrow) as in (a) and a chloroplast (CH) ultrastructure slightly altered; (c) another O_3-fumigated sample, negative control of cerium staining [note the absence of precipitates (enlarged inset) in a slightly plasmolyzed cell]; (d) cell of palisade tissue fumigated 2 days from BTH application and showing cerium precipitates on cell membranes [note that chloroplasts are swollen and contain dark starch grains (S)]; (e) same sample as in (d) showing a plasmolyzed cell of the spongy tissue with heavily stained wall and a deranged chloroplast (CH).

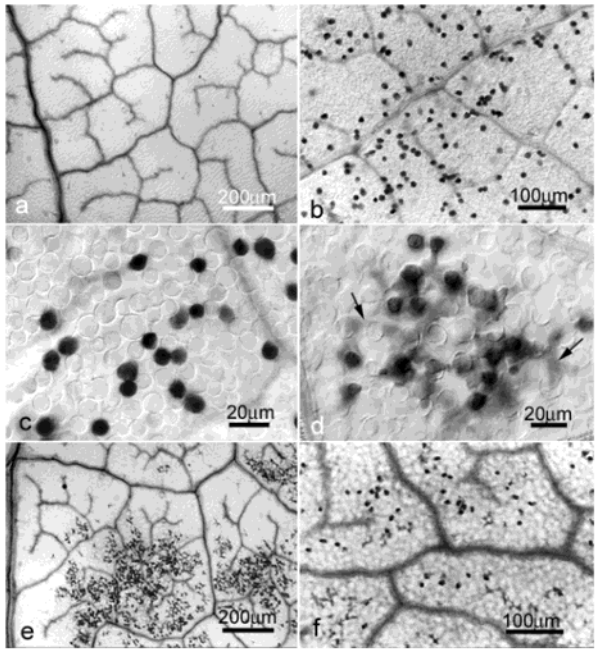

Figure 3. Localization of dead cells with Evans blue staining: (a) leaf treated with 0.3 mM BTH, 2 days before sampling (no dead cells are present); (b) leaf stained 12 h after O_3 fumigation [numerous dead cells (dark blue), uniformly scattered through the tissue, are visible, before the appearance of macroscopic symptoms]; (c) leaf stained soon at the end of O_3 fumigation (dead cells are already present and only localized in the palisade mesophyll); (d) BTH-treated leaf fumigated with O_3 a day after treatment [dead cells are present both in the palisade and in the spongy mesophyll (underfocus, arrows) and are already visible at the end of fumigation]; (e) same treatment as in (d) but the leaf was sampled 2 days after fumigation (the number of dead cells is now massive); (f) leaf fumigated 7 days after BTH treatment and showing a small number of dead cells.

the wall were even dark, suggesting that they were not alive anymore (**Figure 1c**).

Approximately 1 day after either BTH treatment or O_3 fumigation of untreated plants, deposits were rare and became no longer detectable in the following days (not shown).

Leaf tissues fumigated 1 day after BTH treatment showed heavier DAB deposits with respect to those only treated or fumigated (**Figure 1d**). In this case, dark brown precipitates were visible soon after fumigation and were present also inside some cells (**Figure 1d**).

With the more sensitive $CeCl_3$ technique, and by examining ultrathin cross sections of the leaf lamina, it was possible to detect black precipitates not only in the epidermal cell walls but also in the external wall layers of mesophyll cells of either BTH-treated (**Figure 2a**) or O_3-fumigated (**Figure 2b**) plants. As these precipitates were present only in cerium-treated tissues but not in the control (**Figure 2c**), they can be regarded as the reaction product of cerium with H_2O_2 deposits. In treated plants, fumigated within 2 days from BTH application, deposits were more intense and often localized on cell membrane, or even inside the cell of palisade tissue (**Figure 2d**). Some of these cells showed a different degree of plasmolysation that was usually correlated with the extent of H_2O_2 accumulation. Cells with heavily stained walls were completely plasmolyzed and showed deranged organelles (**Figure 2e**), indicating that cell death had already occurred.

When fumigation was carried out 5−7 days from the treatment, H_2O_2 accumulation was rare and restricted to a very thin external wall layer of epidermal and mesophyll cells (not shown).

Cell Death. Evans blue staining showed that no dead cells occurred in tissues treated with 0.3 mM BTH (**Figure 3a**), even in the case of triple treatment and up to a week of observation. On the contrary, O_3 acute exposure revealed Evans positive cells (**Figure 3b**) before the occurrence of macroscopic symptoms on the leaf. Soon after fumigation, some dead cells were already present in the palisade mesophyll (**Figure 3c**). They were uniformly scattered through the tissue with a preferential localization nearby stomata. The number of dead cells increased significantly in the following 1−2 days, and a few of them belonged to the spongy mesophyll.

Plants exposed to the pollutant 1−2 days after BTH treatment showed a considerably higher number of dead cells than those only fumigated. In this case, dead cells were equally localized both in the palisade and in the spongy mesophyll (**Figure 3d**) and were already present at the end of fumigation. Their number became massive 1 day later (**Figure 3e**); thus, they were easily identifiable even by the naked eye in the stained tissue samples.

Plants fumigated 5−7 days after BTH treatment showed a reduced number of dead cells (**Figure 3f**), even when compared with those only fumigated (**Figure 3b**). Moreover, dead cells were not present at the end of fumigation but at least 1−2 days later.

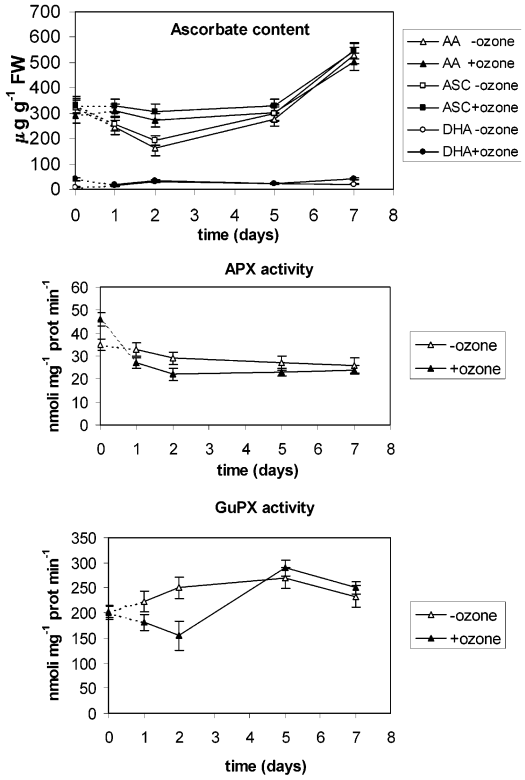

Figure 4. Effect of O_3 fumigation on ascorbate (AA), dehydroascorbate (DHA), and AA + DHA (ASC) levels and on ascorbate peroxidase (APX) and guaiacol peroxidase (GuPX) activities in BTH-treated plants. Time values represent the days elapsed between BTH treatment and O_3 fumigation. Data at time 0 are referred to untreated BTH controls. The values represent the means of three replicates; SD values averaged 7% for AA, 5% for APX, and 9% for GuPX and did not exceed 12, 9, or 15% of the mean, respectively.

Biochemical Assays. All data in this section refer to leaf samples collected at the end of the fumigation. Data at time 0 after BTH treatment actually refer to untreated and only fumigated plants (**Figure 4**).

BTH treatment induced a time-dependent variation of AA concentration in the leaf tissues. Within 2 days from treatment, AA concentration quickly dropped to half of the initial level and then gradually rose to the seventh day. In plants treated with BTH and fumigated, AA concentration in the tissues did not change significantly when fumigation was carried out up to the fifth day after treatment, but rose to a similar level as in tissues treated with BTH on the seventh day. A similar trend was observed for total ascorbate (ASC).

BTH negatively affected APX activity, which steadily decreased during a 7 day time course, whereas the combined effect of BTH and O_3 was different, depending on the time elapsed between treatment and fumigation. When the pollutant was applied 2 days after BTH, APX activity further decreased, whereas no significant difference was found when plants were fumigated 7 days after treatment. As expected, O_3 enhanced APX activity in plants only fumigated (see time 0 in **Figure 4**).

BTH progressively enhanced total GuPX activity with a maximum at 5 days, whereas the combined effect of BTH and O_3 again was different in regard to the time elapsed between treatment and fumigation. Within 2 days from the treatment O_3 greatly reduced GuPX activity. However, at 5 days this activity was found even higher than in tissues only treated with BTH and remained higher in the following days.

DISCUSSION

The extensive research on resistance induced by chemicals has not taken into account the possible interference by ambient ozone, the most dangerous summer air pollutant. O_3 enhances premature leaf senescence, stimulates respiration rate, and inhibits photosynthesis and shifts in resource partitioning. All of these responses ultimately lead to reduced plant growth and, consequently, depressed crop yields and harvest quality (*3*, *5*).

The only interaction between agrochemicals and O_3 reported to date considered herbicides and fungicides (*36*−*38*). It was demonstrated that pretreatment with the herbicide chloramben increased the severity of O_3 damage in both tolerant and sensitive tobacco cultivars, because both compounds inhibit photosynthesis and lead to ROS generation (*36*−*39*). Moreover, in *Conyza bonariensis* no cross-tolerance was observed between nonselective contact herbicide paraquat and pollutant (*40*).

O_3 exposure enhanced sugarbeet tolerance to the herbicide phenmedipham by improving the activity of the endogenous antioxidant enzymes (*37*). On the contrary, some fungicides were reported to be effective in reducing O_3 injury in several crops (*41*, *42*). This result is reminiscent of O_3-induced cross-tolerance to pathogens, where the eliciting or damaging effect depends on the pollutant concentration and the phytotoxic threshold (*43*).

In the present study bean plants have been shown to become more susceptible to O_3 if treated with BTH up to 2 days before fumigation. This can be explained by the high H_2O_2 level detected in the tissues in this time course as a consequence of the drop in APX activity caused by the combined effect of BTH and O_3 and the enhanced ROS production due to the latter. Very likely, H_2O_2 concentration rises over the phytotoxic threshold in many cells, including those of spongy tissues that are not usually damaged by O_3. This leads to a massive cell death, as shown by Evans blue staining and the subsequent appearance of necrotic lesions.

The reduction of APX activity due to BTH has already been reported, together with the inhibition of catalase (*26*). Considering that on the basis of our and other authors' evidence, APX activity is enhanced by the exposure to O_3 alone (*44*); its drop as a consequence of the combined effect of O_3 and BTH is even more significant.

As far as GuPX is concerned, it is known that its activity is enhanced by BTH (*24*), and this was confirmed here. However, when plants were fumigated 2 days after BTH treatment, GuPX activity was significantly reduced, possibly because of the pH increase in the apoplast due to O_3 (*45*) or the direct effect of O_3 on this enzyme. Interestingly, this activity steadily increased in the following days to a higher value than that due to BTH alone, very likely as a consequence of the high accumulation in the first 2 days of H_2O_2, which is the oxygen donor for this enzyme. H_2O_2 is in fact utilized by GuPX, the enhanced activity of which leads to cell wall lignification and hydroxyproline-rich glycoprotein (HPRG) cross-linking (*46*, *47*). This, in turn, diminishes cell wall permeability and protects plasmalemma from O_3 damage. Moreover, the striking increase of AA concentration at 5−7 days after BTH treatment further prevents O_3 deleterious effects. In fact, in the apoplast, AA reacts directly with O_3 and represents a first line of defense against this pollutant and its reactive products (*14*, *22*, *32*).

Despite this, the redox state of ascorbate, expressed by the ratio AA/ASC, remains constantly close to 1, apparently unaffected by O_3 fumigations. This differs remarkably from the observations of Pasqualini et al. (*48*) in the apoplast of tobacco leaves during the first hours following O_3 fumigation. However, it must be noted that, because of difficulties encountered in

isolating the intercellular fluid, our biochemical assays were carried out on the whole leaf extract, which probably masks the apoplastic fraction. The above-mentioned difficulties may be a consequence of plasmolysis caused by the acute O_3 exposure.

All of these events together would explain the increased tolerance to O_3 observed in plants fumigated after 5−7 days from BTH treatment. The increased tolerance is also confirmed by the small number of dead cells found with Evans blue in the leaf tissues and the consequent lack of visible symptoms. In this regard, Evans blue staining combined with the in vivo DAB reaction has proved to be a powerful technique to detect early ozone damage in the tissues, before the appearance of macroscopic symptoms.

In conclusion, these data indicate that BTH treatments can prevent ozone injury when carried out at least 5 days before exposure to an acute O_3 level of 200 $nL \cdot L^{-1}$ for 4 h, whereas in shorter times they could amplify pollutant damage. This would make difficult a practical field application of this compound with O_3 protective aims when high levels of the pollutant are expected in a few days.

ABBREVIATIONS USED

AA, ascorbic acid; DHA, dehydroascorbic acid; ASC, AA + DHA; APX, ascorbate peroxidase; DAB, 3,3′-diaminobenzidine; GuPX, guaiacol peroxidase; ROS, reactive oxygen species.

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