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Oxidative Stress, the Paradigm of Ozone Toxicity in Plants and Animals

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Abstract Tropospheric ozone represents a relevant atmospheric pollutant, because of its strong oxidizing potential. The risk for animal (human) and plant health, at molecular and cellular level, arises from the oxidative damage to lipids, proteins and nucleic acids, depending on the dose. Therefore, ozone concentration and exposure time determine the chronic or acute toxicity and, consequently, the severity of injury at biochemical and physiological level. In living organisms, reactive oxygen species (ROS), directly or indirectly derived from ozone exposure, are scavenged by enzymatic and non-enzymatic antioxidant defensive mechanisms, overall deputed to preserve cell structures and biomacromolecules from the oxidative damage. These defences are essentially those also involved in detoxifying the ROS inevitably produced by the metabolism of organisms living in oxygenic atmosphere.

Keywords Atmospheric pollution · Tropospheric ozone · Reactive oxygen species · Antioxidant defences

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1 Introduction

In metabolism of aerobic organisms, oxygen is essential for providing them with energy through the combustion of nutritive substrates. Because oxygen is the combustive agent, this controlled combustion is an oxidation, i.e. an aerobic respiration. However, this catabolic route unavoidably and continuously leads to the production of partially reduced oxygen intermediates, more reactive than molecular oxygen in its ground state, including both radical and nonradical forms, collectively termed as reactive oxygen species (ROS) (Halliwell 2006) (Fig. 1). In mitochondria of animal and plant cells, leakage of electrons, from their transport chain, leads to the single-electron reduction of oxygen, with the consequent formation of superoxide anion (O_2^-) (Möller 2001). Moreover, plant cell experienced an additional source of oxygen by-products in chloroplasts, the site of photosynthesis, i.e. the inorganic carbon fixation (Asada 2006).

Because of their high reactivity, uncontrolled ROS production may cause injury to biomacromolecules, if the homeostasis of the oxidation/reduction (redox) state is not preserved (Fig. 2). In other words, a disturbance in the prooxidant–antioxidant balance, in favour of the former, may lead to an oxidative stress (Foyer and Noctor 2005). In order to overcome this side-effect of aerobic life, organisms evolved sophisticated strategies, collectively termed antioxidant defences, to counteract the imbalance of the cellular redox state and keep the

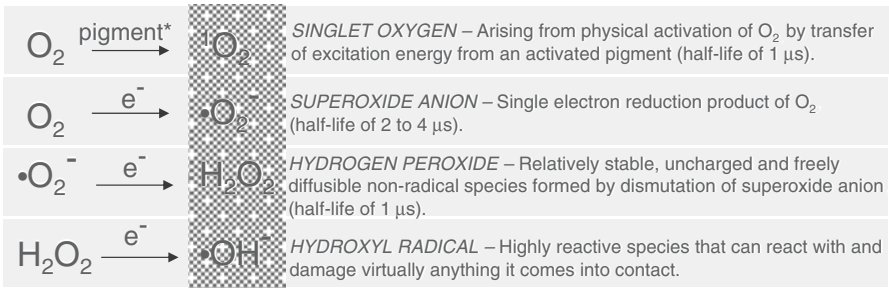

Fig. 1 Reactive Oxygen Species (ROS). Radical and non-radical intermediates deriving from single-electron reduction of molecular oxygen and derivatives

ROS concentration under the cytotoxic threshold (Yu 1994).

In eukaryotic cells, damaging prooxidant shift may be the consequence of either an antioxidant defence deficiency or enhanced pro-oxidant environment, due to a plethora of external adverse stimuli (Fig. 2). Both these conditions may imbalance the pro-oxidant vs. antioxidant ratio, thus rising the intracellular levels of ROS (Jones 2006). External factors that detrimentally affect aerobic organisms include diseases, extreme environmental conditions, xenobiotics and pollutants (Gechev et al. 2006). Thus, apart from the aforementioned accidental sources of ROS under physiological steady-state conditions, namely respiration and photosynthesis in addition to photorespiration and fatty acid β-oxidation, different sites of deliberate ROS production exist (Yu 1994) (Fig. 3). In animals, during inflammation and immune response, the activated phagocytic white cells (neutrophils, macrophages, monocytes) generates $\cdot O_2^-$ by a NADPH oxidase, then transformed in other ROS (mainly hydrogen peroxide and hydroxyl radical) involved in direct toxicity towards microbes, in a process known as respiratory burst (Dahlgren and Karlsson 1999). In the same manner, in plants, oxidative burst occurs as an early defence response. A plasma membrane located NADPH oxidase, sharing homology with its mammalian counterpart, produces $\cdot O_2^-$, in turn transformed in other ROS, contributing to create an hostile apoplastic (extracellular) environment for the pathogens (Wojtaszek 1997). Furthermore, significant amounts of ROS are generated during the metabolism of xenobiotic, by the cytochrome P-450 oxidase detoxifying system, and as consequence of exposition to environmental pollutants (Schuler 1996). Finally, physical activation of O_2 may occur by transfer of excitation energy from a photoactivated pigment, such as an excited chlorophyll molecule, to oxygen. The latter absorbs sufficient energy to invert the spin of one electron, thus forming the singlet oxygen ($^1O_2$), a highly diffusible ROS capable of reacting with organic molecules with paired electrons (Halliwell 2006).

The focus of this survey is to compare the effect of ozone pollution in plants and animals (humans), emphasizing on the pathophysiology of ROS and the cellular antioxidant defensive mechanisms in charge of counteracting the detrimental effect of oxidative stress.

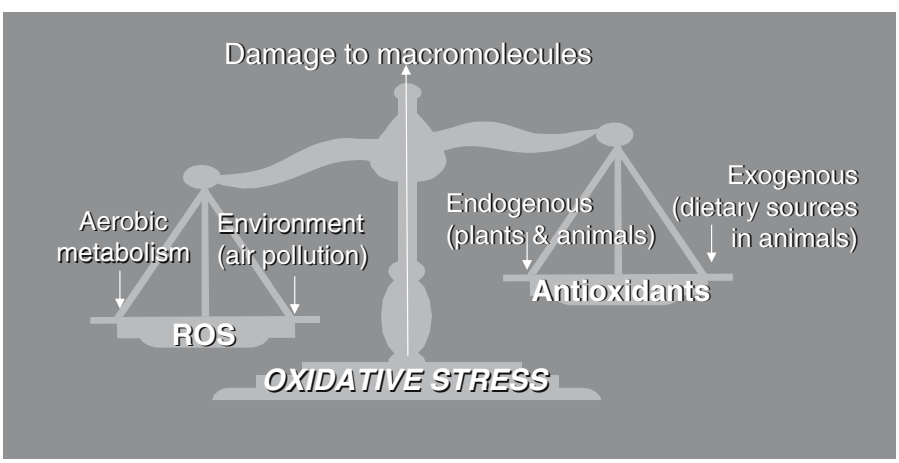

Fig. 2 Homeostasis of the cell oxidation/reduction state. The disturbance in the prooxidant–antioxidant balance may lead to an oxidative stress, harmful for biomacromolecules

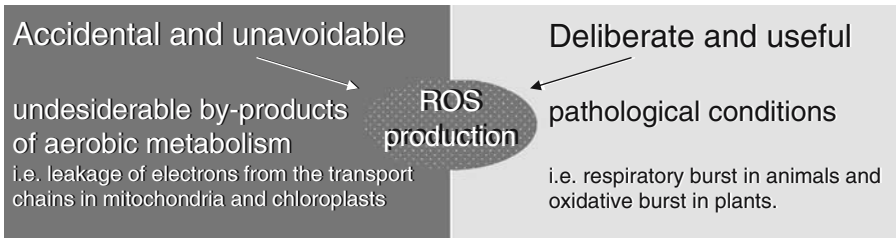

Fig. 3 Sources of reactive oxygen species (ROS) in eukaryotic cells. Routes of ROS production may be both accidental, as by-products of aerobic metabolism, and deliberate, as a defence mechanism. Both this traits are conserved in plants and animals

2 Atmospheric Pollution and Troposheric Ozone

Exposition to gaseous atmospheric pollutants, such as ozone (O_3), may perturb the equilibrium between production and scavenging of ROS, within animal and plant tissues (Fig. 2). Indeed, in the atmosphere, two different pools of O_3 exist, the beneficial and detrimental one. In the stratosphere (the higher atmosphere, ranging approximately from 15 to 40 km in altitude), the ozone layer absorbs the harmful UV-B and UV-C radiations, thus screening the living organisms (Dutsch 1978, Kerr and McElroy 1993). In the past decades, emission of ozone-depleting chemicals led to the reduction of the ozone shield (commonly referred to as ozone hole) against UV radiation, worsening its harmful effects to animals and plants (Platt and Hönninger 2003). Otherwise, in the troposphere (the lower part of the atmosphere, approximately from the Earth surface to 10–12 km in altitude), the layer where the climatic conditions originate and temperature decreases with elevation, ozone is regarded as a pollutant (Logan 1985).

Tropospheric ozone is an oxidant constituent of the photochemical smog. It is a secondary pollutant produced through reactions between primary pollutants, those emitted directly into the air (mainly nitric oxides, sulphur oxides, carbon oxides and hydrocarbons), and sunlight (Crutzen and Lelieveld 2001). Hence, ozone is produced on bright sunny days over areas with intense primary pollution, due mainly to vehicle exhausts, fossil fuel burning and industrial processes, in the so-called photochemical cycle (Fowler et al 1999; Kley et al. 1999). Meteorological conditions can move ozone, or its precursors, from these areas towards less polluted ones, such as rural zones, with detrimental effects on natural and cultivated plant species. Again, meteorological conditions may exacerbate the rate of ozone formation, particularly atmospheric inversion, a restricted air circulation associated to a warmer air layer above a cooler one (Baumbach and Vogt 2003). Additionally, ozone is a greenhouse gas, although it plays a minor role in regulating the air temperature and contributing to the warming effect (Wang et al. 1995).

3 Ozone Chemistry and Toxicity in Biological Systems

From the Greek *ozein* (to smell), ozone, the triatomic allotropic form of oxygen, is a colourless gas with a slightly sweet, water melon-like odour (odour threshold between 0.0076 and 0.036 ppm). Because of its strong oxidizing potential (+2.07 eV), O_3 is a powerful oxidizing agent capable of reacting with virtually any biomacromolecule, including lipids, proteins, nucleic acids and carbohydrates, although it is neither a radical species nor a ROS (Mustafa 1990; Kelly et al. 1995). Ozone is considered too reactive to penetrate far into tissues, so that only a minor amount of the pollutant is believed to pass unreacted through a membrane and nothing through a cell (Pryor 1992). Furthermore, its toxicity can be greatly enhanced by the spontaneous hydroxyl radical (−OH) generation in aqueous solution, strongly accelerated by traces of $Fe^{2+}$and favoured at alkaline pH, although occurring even at physiological pH (Pryor 1994).

In cell membrane, polyunsaturated fatty acids represent the primary target for ozone, stimulating lipid peroxidation and impairing membrane fluidity (Fig. 4). The chemistry of O_3-induced lipid peroxidation, known as Criegee ozonation pathway, involves ozonolysis of alkenes of polyunsaturated chains, i.e. the electrophilic O_3 addition across the carbon–carbon double bonds, to give the Criegee ozonide (Criegee 1957). Afterwards, ozonide decomposes, under suitable conditions, to form organic radicals, aldehydes and peroxides. In further steps,

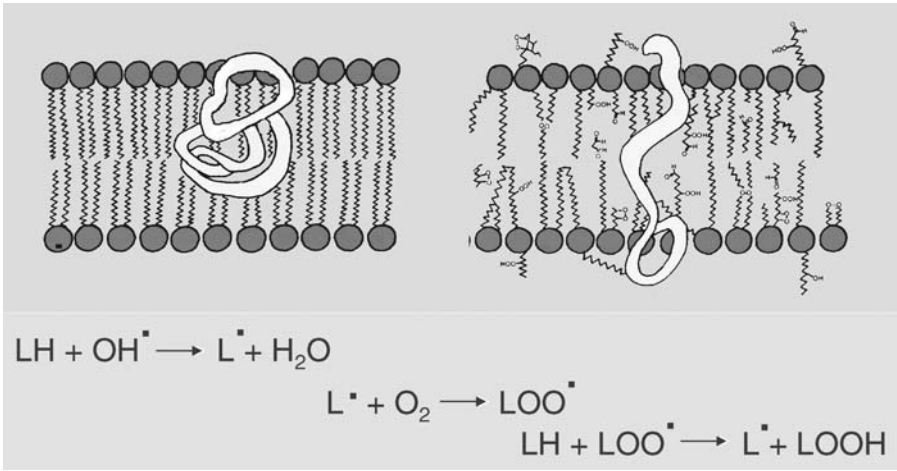

Fig. 4 Lipid peroxidation. In cell membrane, polyunsatured fatty acids (PUFAs) represent the primary target of ozone toxicity, with the consequent alteration of the bilayer structure and function. Lipid peroxidation is a chain reaction starting by the extraction of a H atom from PUFA due to ROS and forming a fatty acid radical (L·); the latter reacts with O_2 to give a lipid peroxyl radical (LOO·) and, finally, a lipid hydroperoxide (LOOH)

H_2O_2 can react with transition metals (Cu or Fe), according to Fenton or Haber–Weiss reactions, to form other ROS (Pryor et al. 1991; Pryor 1993).

As a result of the ozone-induced oxidation, modification of proteins also occurs, both in their structure and activity. The pollutant directly, or through highly reactive free radical mediated reactions, oxidizes the amino acidic residues, mainly of tyrosine, tryptophan, cysteine, methionine and histidine (Mudd et al. 1969). In particular, it reacts with the exposed sulphydryl groups to form disulphides bridges, and with tryptophan to give protein ozonides, in turn generating protein hydroperoxides and hydrogen peroxide (Freeman and Mudd 1981). Tyrosine residues can be cross-linked too, after the oxidation of their −OH groups, to give *O*,*O*'-dityrosine (Ignatenko et al. 1984). DNA damage can be produced as well, as shown by the increased activity of poly(ADP-ribose) synthetase, a chromatin-bound enzyme promoting damaged DNA repair (Hussain et al. 1985).

4 Ozone at the Interface

Which are the major biosurfaces exposed to ozone, in aerobic organisms? In the main, it is possible to distinguish between external and internal epithelial tissues, or biosurfaces. The former include the epidermal tissues and the respective overlapped protective layers, both in plants and animals, whereas the latter consist of the apoplast in plants and respiratory tract in animals, although apoplast, belonging to leaf parenchyma, cannot be regarded as an epithelium (Cross et al. 1998a).

External Interface In land plants, epigeous organs (stem and leaves, exclusive of roots), are outwardly covered by cuticle and epidermis. Above the cuticle, there are glandular and non-glandular trichomes, uni- or pluricellular appendages originated from epidermal cells. Cuticle is a gas- and water-impermeable layer, with the function of protecting the above ground parts of plants from desiccation. In turn, cuticle is composed by epicuticular waxes and cutin, a complex polymeric compound consisting of esterified hydroxyfatty acids bound to phenylpropanoidic units (Kerstiens and Lendzian 1989; Müller and Riederer 2005). Therefore, the gas exchange between the air and mesophyll (the internal leaf parenchyma) is make possible through small pores (stomata) scattered both on the adaxial (upper) and abaxial (lower) surfaces of leaf lamina. These openings can be regulated, that is opened or closed, by the action of specialized epidermal cells, known as guard cells, according to the water status of the soil–plant–air continuum and an array of ecophysiological and meteorological conditions (Tingey and Hogsett 1985; Blatt 2000).

In animals, tegument outwardly includes the stratum corneum, epidermis and dermis. The outermost layer, the stratum corneum, prevents water evaporation, preserving skin hydration. It is composed of dead cells (corneocytes) embedded in a lipid-rich intercellular matrix, arranged in several layers and continuously replaced by new cells from the stratum germinativum. Epidermis is a non vascularized tissue, whose main constituent cells are keratinocytes and melanocytes. Dermis is the connective layer beneath the epidermis, harbouring nerve endings, blood vessels, hair follicles, sweat and sebaceous glands. Eventually, the lowermost

layer, in animal skin, is the hypodermis, or subcutaneous adipose layer (Rawlings 2006).

Internal Interface In plant tissues, apoplast includes both the intercellular spaces and cell walls. It represents the non living continuum outside the plasma membrane, functioning as a free diffusional space involved in cell-to-cell communication. All gaseous molecules exchanged between the plant and its environment (CO_2, O_2, water vapour, O_3 and other atmospheric pollutants) diffuse through the apoplast, as well as ions, molecular signals, antioxidants, hormones, mineral nutrients, xenobiotics absorbed by roots and, lastly, molecules involved in plant defence mechanisms against biotic and abiotic stresses (Felle et al. 2005).

In land vertebrates, respiratory system is the structure responsible for ventilation, i.e. gas exchange, although it shares a number of non-ventilatory functions, such as thermal regulation and humidification. Respiratory system includes two main tracts: the conducting zone (upper respiratory tract) and respiratory zone (lower respiratory tract). The conducting airways serve as air conductors from the external environment to the distal gas exchange area. They begin with the nasal and oral cavities, opening through the pharynx and larynx into the trachea, belonging to the lower respiratory zone. Inside the thoracic cavity, where lungs are placed, the trachea divides to form two bronchi (the right main bronchus and left main bronchus), several time branched to form smaller bronchioles. In turn, the terminal bronchioles lead to the respiratory bronchioles, alveolar ducts and sacs, where most of the gas exchange occurs (Blundell 2006).

5 Ozone Effects on Plant Health

Ozone enters the plant through the stomata, placed onto the adaxial and abaxial leaf epidermis. Within the substomatal cavity and leaf parenchyma, the pollutant encounters its first interface, the apoplast, and, only successively, it and/or its by-products come into contact with the mesophyll cells (Cross et al. 2002).

Plants are exposed to either acute and chronic ozone doses, according to the gas concentration and exposure time. An acute exposure consists of relatively high ozone concentration (>80 ppb, or 80 nL/L, or 160 $\mu g/m^3$) for a few consecutive hours to days, whereas a chronic exposure involves a relatively low gas concentration (<40 ppb, or 40 nL/L, or 80 $\mu g/m^3$) for the entire life of a plant, with intermittent episodes of high concentration, either periodically or accidentally (Krupa et al. 2000; Mauzerall and Wang 2001). In every instance, symptoms of injury appear typically in the leaves, being, in the main and according to leaf morphology, unifacial and visible in the adaxial (upper) surface of leaf blade. The most common symptoms due to chronic injury include chlorosis (yellowing due to the chlorophyll breakdown, often distributed in spots over the leaf) and bronzing (red-brown pigmentation caused by phenylpropanoid accumulation), while lesions attributable to acute exposures are much more diversified. In broad-leaved plants, they include bleaching (small unpigmented necrotic spots), flecking (small brown necrotic areas fading to grey or white), stippling (small punctuate spots, white, black or red in colour) and tipburn (dying tips, firstly reddish, later turning brown) specially in conifers (Bergmann et al. 1999; Krupa et al. 2000).

Despite the above mentioned visible injury, in particular conditions and geographical areas, plants may experience the ozone stress without manifesting any leaf symptoms (Paoletti 2006), thus suffering of the so-called invisible damages (Fig. 5) (Faoro and Iriti 2005). The latter contribute collectively to create a complex multifactorial syndrome affecting plant growth and yield. Pathophysiological conditions related to the ozone-induced invisible damages include the stomatal function impairment, the decrease of photosynthetic activity and the senescence induction, resulting in dysfunction of transpiration and water use efficiency, reduction of dry matter production, detrimental effects on flowering and pollen tube extension, and yield losses (Reddy et al. 1994; Pell et al. 1994; Torsethaugen et al. 1999; Black et al. 2000). Although the cellular and molecular targets of ozone injury are not yet completely established, it is well known that ozone impairs the activity of the major enzyme involved in CO_2 assimilation, the ribulose-1,5-bisphosphate carboxylase/oxydase (Rubisco), in addition to promote stomatal closure and alter the structural integrity of thylakoid membranes within chloroplasts (Fig. 6), with a consequent reduction of net photosynthesis rate (Enyedi et al. 1992; Violini et al. 1992).

Intriguingly, although ozone does not possibly exert a direct toxicity towards the pathogens, it can modify, either beneficial or detrimental, the plant response to biotic stresses, through an array of not-

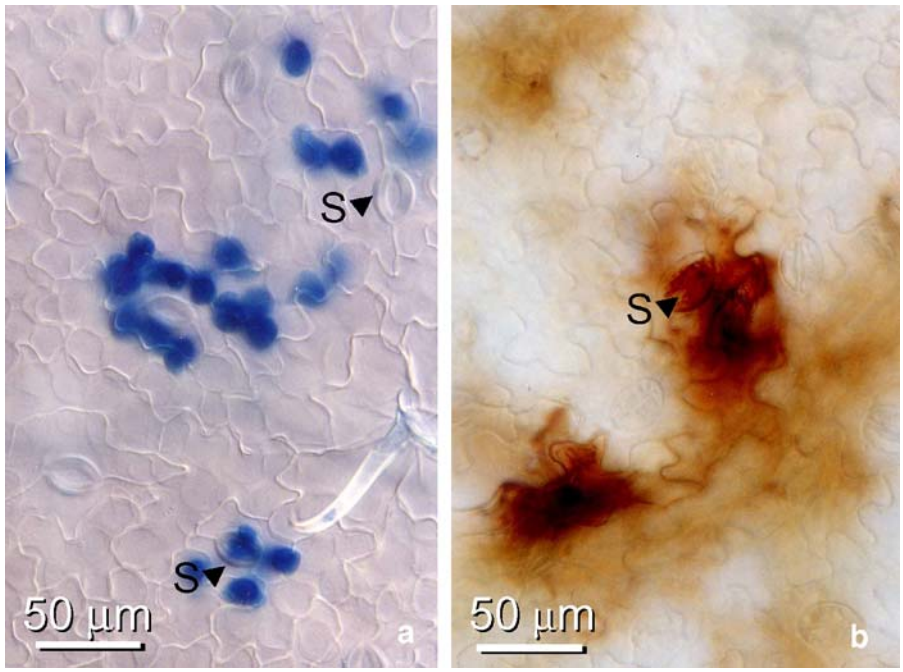

Fig. 5 Microscopic symptoms induced by ozone on bean leaf cells. Some mesophyll cells around stomata (*S*) are stained in blue by Evans' (**a**) after experimental acute ozone exposure (150 ppb per 2 h), indicating that they are dead; the same cells also show extensive H_2O_2 deposits, stained in brown by 3-3'-diaminobenzidine (**b**), as a consequence of an oxidative burst

well understood host-mediated mechanisms which can impair disease progression. Thus, ozone may function as an elicitor of plant immune response, priming the host defence mechanisms against infections (Sandermann et al. 1998). In plants, immunity is generally triggered by an oxidative burst, frequently associated with a hypersensitive response (HR), a form of programmed cell death (PCD) generated at the site of the attempted pathogen penetration (Iriti et al. 2006). ROS produced (directly or indirectly) by ozone within the cells may elicit HR-like phenomena as well, providing that they do not exceed the cytotoxic threshold (Fig 5). When this happens, necrotic cell death occurs, with lesion propagation

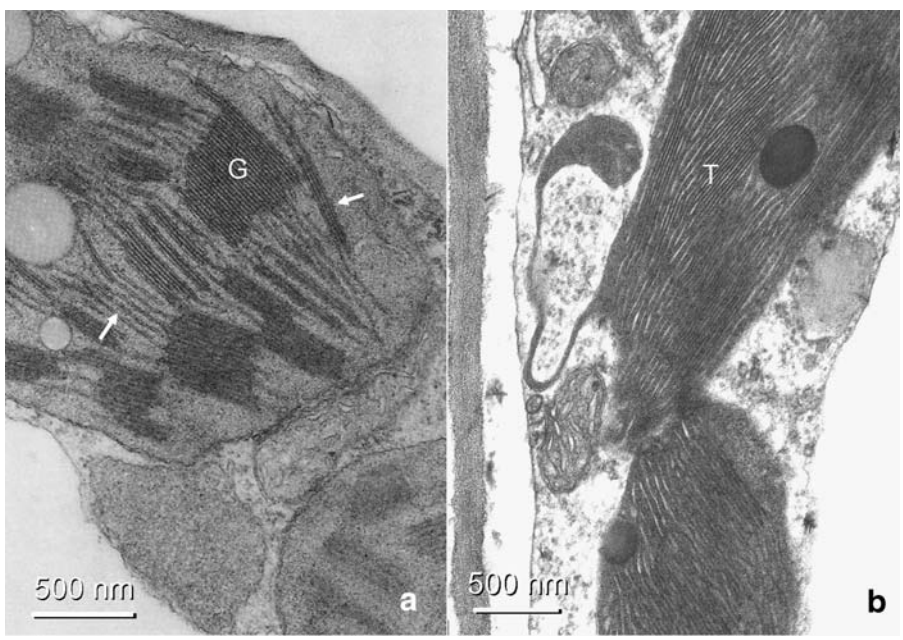

Fig. 6 Damages to bean chloroplasts by chronic exposure to ozone. (**a**) In plants grown in a filtered open top chamber (OTC), thylakoid membranes (*arrow*) are normally organized in grana (*G*), while, in plants exposed in a non-filtered OTC (**b**), thylakoids (*T*) are highly disorganized and their membranes appear abnormally proliferated, possibly as a result of functional adaptation

and formation of visible symptoms (Dat et al. 2000). The cytotoxic threshold not only depends on the dose (ambient pollutant concentration per plant exposure time), but foremost on endogenous factors, primarily the pool of cellular antioxidant defences, able to dampen the harmful ROS potential (Rao and Davis 2001; Langebartels et al. 2002).

Many of the molecular, biochemical and physiological responses at the basis of the above exemplified processes can be further emphasized in terms of interaction among different hormonal signalling pathways. The main phytohormones regulating the plant armamentarium against either biotic and abiotic stresses, ozone included, are salicylic acid (SA), jasmonic acid (JA) and ethylene. The phenylpropanoid SA, in addition to be the one of the main compounds involved in HR and plant systemic acquired immunity (or systemic acquired resistance, SAR), has been reported to orchestrate the cell chances in shifting between ozone-induced HR-like and necrotic/toxic cell death, depending on its concentration (Rao and Davis 1999). Therefore, a low level of tissue SA activates only a weak antioxidant defence, impairing PCD initiation in lieu of toxic cell death, while a higher concentration is required for the ozone-induced HR-like reactions, by upregulating the cellular antioxidant systems (Rao et al. 2000a). Furthermore, ozone-activated SA pathway may prime the host defences against pathogens, such as the synthesis of antimicrobial compounds (phytoalexins), in a phenomenon known as 'cross induction' (Sharma et al. 1996). Ozone exposure stimulates JA biosynthesis, in plant, as well (Rao et al. 2000b). Jasmonic acid arises from linole(n)ic acid via the octadecanoid pathway, similar to the eicosanoid one, leading to arachidonic acid derivatives (such as prostaglandins) in animal tissues. These compounds, collectively termed as oxylipins, i.e. oxygenated lipids, require phospholipase activity to release polyunsatured fatty acids (PUFAs) from plasma membrane and to make them available for (lipo)oxygenase activity (Creelman and Mullet 1997). It has been reported a trade off between JA and SA signalling pathways, that means, in other words, a JA-mediated attenuation of the oxidative burst, prevention of tissue SA accumulation in high amount and almost completely abolition of the ozone-induced cell death (Koch et al. 2000). Finally, ethylene is a plant hormone involved in plant growth, development, senescence and stress response. In acute ozone exposures, stress ethylene production is closely linked to lesion propagation while in chronic exposures is responsible for premature senescence, that is very detrimental for crop productivity (Mehlhorn et al. 1991). Its biosynthesis is positively influenced by SA, whereas a mutually antagonistic interaction occurs with JA (Rao et al. 2002; Tuominen et al. 2004).

6 Ozone Effects on Animal Health

In humans and animal models, O_3 can adversely affect the respiratory tract, causing both chronic and acute health effects. The pollutant produces a plethora of injures either at physiological and cellular level, depending on the exposure conditions (Bromberg and Koren 1995). Besides, as a component of air pollution, it has been associated with increased mortality rates due to cardiovascular and respiratory diseases, as well as shortening of life expectancy (Brunekreef 1997). Ozone-related cardiovascular morbidity may result from primary alterations in cardiovascular function or pulmonary vascular integrity, due to the release of cytokines, hormones, inflammation mediators, oxidative by-products and/or from secondary effects due to cardiopulmonary dysfunction, for which an already compromised cardiovascular system might be unable to compensate (Aris et al. 1993; Mudway and Kelly 2000; van der Vliet and Cross 2000).

Exposure under 80 ppb for 8 h a day is considered not harmful for public health, at least by the U.S.A. Environmental Protection Agency (EPA) that sets this precautionary limit. However, to attain this standard, the 3-year average of the fourth-highest daily maximum 8-h average ozone concentrations, measured at each monitor within an area over each year, must not exceed 80 ppb (http://www.epa.gov/air/criteria.html).

Acute exposures, ranging from 80 to 200 ppb and lasting from 5 min to 6 h, induce an array of pulmonary responses, including reversible decrease in respiratory function, that can be observed within the first few hours after the start of the exposure and may persist for many hours or days after the exposure cessation (Lippmann and Schlesinger 2000). Chronic health effects, due to repetitive daily or intermittent exposures over several days or weeks, can prolong or exacerbate the transient effects on the baseline respiratory function parameters or alter the lung structure, as a result of the cumulative damage and/or side effect of functional adaptive responses (Christian et al. 1998; Frank et al. 2001).

Additionally, different responses to O_3 occur in different population subgroups. Ozone risk groups include, besides children and senior citizens, people with: (1) severe pulmonary diseases, such as asthma or chronic obstructive pulmonary disease; (2) increased respiratory rate, such as outdoor workers and athletes, especially during the warmer months; (3) cardiovascular diseases (Höppe et al. 1995).

In humans, typical symptoms caused by O_3 inhalation consist of rapid and superficial breathing (tachypnea), accompanied by substernal (tracheal) irritation exacerbated by attempted deep inspiration and cough. Besides the increased respiratory frequency, as proved by reduced inspiratory capacity, functional spirometric parameters able to quantify decrements in respiratory function are: (1) forced vital capacity (FVC); (2) forced expiratory volume in 1s (FEV_1); (3) forced expiratory flow rate between 25 and 75% of vital capacity ($FEF_{25–75}$) (Weinmann et al. 1995, Balmes et al. 1996; McDonnell et al. 1999).

The loss of ability to take in a deep breath, the inspiratory chest discomfort and reduction of vital capacity following O_3 exposure mostly depend on neurally-mediated involuntary inhibition of inspiration, due to the stimulation of afferent vagal C-fibers. Opioid-mediated bronchial C-fibers are nociceptive unmyelinated sensory fibers, whose neurons increase their firing rate during the O_3 exposure (Coleridge et al. 1993; Passannante et al. 1998; Krishna et al. 1997; Ho and Lee 1998). Furthermore, release of neuropeptides, such as tachykinins and neurokinins, from the nerve endings, mediates this neural pathway by interaction with neurokinin receptor in cells of airway tissues. Additionally, neuropetides released from the sensory nerve terminals, such as substance P, a tachykinin synthesised in the jugular/nodose ganglia, mediate bronchial hyper-responsiveness, increasing smooth-muscle tone, inducing oedema, mucus hypersecretion (mucorrhea), local inflammation and, finally, broncoconstriction (Hazbun et al. 1993; Tepper et al. 1993; Koto et al. 1995). Moreover, bronchial responsiveness to inhaled allergens may be improved in atopic asthmatic patients after O_3 exposure (Sarnat and Holguin 2007).

Airway inflammation includes release of mediators, increased vascular permeability and cellular chemotaxis, as attested by the bronchoalveolar lavage fluid (BALF) and bronchial mucosa biopsy (Krishna et al. 1997; DeLorme et al. 2002; Mudway and Kelly 2004).

The inflammatory effects due to O_3 inhalation consist of: (1) elevated levels of vascular endothelial adhesion molecules, mediators (chemokines and cytokines) modulating neutrophil recruitment into the lung (Fig. 7) and soluble markers (eicosanoids, such as prostaglandins PGE_2 and PGF_2) of inflammation in BALF (Takahashi et al. 1995; Jörres et al. 2000; Bayram et al. 2001; Bosson et al. 2003); (2) increased epithelial and vascular permeability (Kehrl et al. 1987); (3) neutrophil (neutrophilia) and lymphocyte infiltration into the airway mucosa (Fig. 7) (Schelegle et al. 1991). Thus, the release of vascular endothelial adhesion molecules, from the endothelial cells of bronchial submucosal capillaries (such as ICAM-1), and neutrophil attractants, from the airway epithelial cells (such as interleukin-6, interleukin-8 and granulocyte-macrophage colony-stimulating factor) directs the adhesion of neutrophils to the airway microvasculature and their subsequent migration into and across the epithelium (Fig. 7) onto the airway surface (Takahashi et al. 1995; Jörres et al. 2000; Bayram et al. 2001; Bosson et al. 2003). Furthermore, oxylipins (eicosanoids) arise from cell membrane phospholipids, as a result of arachidonate metabolism via cyclooxygenase/lipooxygenase pathway (McKinnon et al. 1993). In addition, these responses may be associated with an increase in total proteins, albumin, fibronectin, fibrinogen (a marker of plasma transudation) and lactate dehydrogenase, indicative of cellular damage, epithelial and vascular leakage and increased permeability (Devlin et al. 1991, 1994).

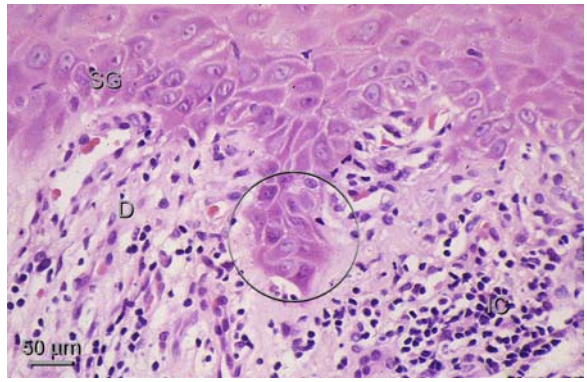

Fig. 7 ROS-induced injury in human oral respiratory epithelium (Eosin-hematoxylin staining). Morphological alterations (*encircled*) in basal cells of *stratum germinativum* (*SG*), showing nuclear derangement and cytoplasm shrinkage; see the inflammatory cell infiltration (*IC*) in dermal tissue (*D*)

Histopathological alterations due to O_3 inhalation occur at all levels of the respiratory tract, including all the cells lining the respiratory tract and tight junctions between epithelial cells (Barr et al. 1988; Cho et al. 1999). Particularly, O_3-induced histopathological alterations progress from the proximal airways towards the distal structures of the lung (distal airways, bronchiole-alveolar duct junctions and proximal alveolar regions) (Boorman et al. 1980). Ciliated cells and type I pneumocytes, the predominant cell types in the airway and alveolar epithelium, respectively, appear to be especially vulnerable to O_3, showing loss of cilia and necrotic cell death. Therefore, alteration in the rate of mucociliary particle clearance may take place (Hyde et al. 1992, 1999). Hypertrophy of Clara cells (non-ciliated bronchiolar cells secreting the extracellular lining fluid) and degranulation of secretory cells (mucous and serous cells) of submucosal glands have been reported too (Royce and Plopper 1997; Sterner-Kock et al. 2000).

Ultimately, susceptibility to respiratory infections may be enhanced, after O_3 inhalation, due to the inability of the phagocytic cells to destroy the bacteria, although O_3 exposure triggers the inflammatory infiltration of the lung by macrophage and polymorphonuclear leukocytes (Gilmour et al. 1993).

7 Tolerance Mechanisms in Plants and Animals

The strategies deputed to counteract the injuries caused by oxidative stress show an impressive array of similarities, in plants and animals, at least at biochemical and cellular level. Nonetheless, even before to activate their own tolerance mechanisms, living organisms can prevent the ozone exposure. Consequently, plants can close the stomata in order to avoid the pollutant uptake, compatibly with gas exchange (transpiration and CO_2 assimilation) (Nali et al. 2004), whereas animals, not being able to cease the ventilation, can escape towards less polluted sites (Cross et al. 2002).

Any compound capable of quenching ROS, without itself undergoing conversion to a destructive radical species, can be considered as an antioxidant. In general, antioxidant defences include both enzymatic and nonenzymatic scavenging systems (Fig. 8). The former include ascorbic acid (vitamin C, AA), glutathione (GSH), *N*-acetyl cysteine (NAC), tocopherols (vitamin E), carotenoids, phenylpropanoids, polyamines and indoles, whereas detoxifying enzymes are, mainly, superoxide dismutases (SODs), catalases (CATs), ascorbate peroxidases (APXs) and glutathione peroxidases (GPXs). Moreover, specific sites exist where ROS detoxification occurs, namely apoplast and mesophyll cells, in plant tissues, respiratory tract lining fluid (RTLF) and epithelial cells (RTECs), in animal tissues (Fig. 8) (Cross et al. 1998a, b).

Nonenzymatic Scavengers Ascorbic acid is the most abundant hydrophilic antioxidant in plants, reaching levels up to 10% of the soluble carbohydrate content of cells (Smirnoff and Pallanca 1995). Its synthesis arises from glucose and, probably, occurs in cytosol. AA is present in all tissues, with the exception of dormant seeds, and in all subcellular compartments, where it directly detoxifies ROS. The highest AA concentrations are found in chloroplastic and cytosolic compartments (up to 50 mM), whereas vacuolar concentrations are relatively low (0.6 mM). In apoplast, its concentration, ranging from 0.15 to 2 mM, is pivotal for the plant tolerance against atmospheric pollutants, thus representing the first line of defence against O_3 (Luwe et al. 1993; Rautenkranz et al. 1994; Foyer and Lelandais 1996). Additionally, AA may play a role as indirect antioxidant, providing reducing power to (ascorbate) peroxidases and repairing the tocopheroxyl radical of vitamin E, thereby restoring its antioxidant activity (Padh 1990).

The same of AA, GSH is ubiquitously distributed in plant cell. This tripeptide thiol (γ-glutamylcysteinyl glycine) is synthesized, in cytosol and chloroplast, from glutamate, cysteine and glycine, in reactions catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase (Alscher 1989). Chloroplastic GSH concentrations range from 1 to 4.5 mM, whereas, in apoplast, it is found at very low concentrations (Foyer and Halliwell 1976; Law et al. 1983). It can directly detoxify ROS, besides being indirectly involved in enzymatic scavenging, as reducing power of GPXs, and in recycling the ascorbate pool, through the sequence of enzymatic reactions collectively named as Halliwell–Asada or ascorbate–glutathione cycle (Fig. 9) (Nakano and Asada 1981; Eshdat et al. 1997). Moreover, it can be the substrate of glutathione S-transferases (GSH), as well as the precursor of phytochelatins, involved in

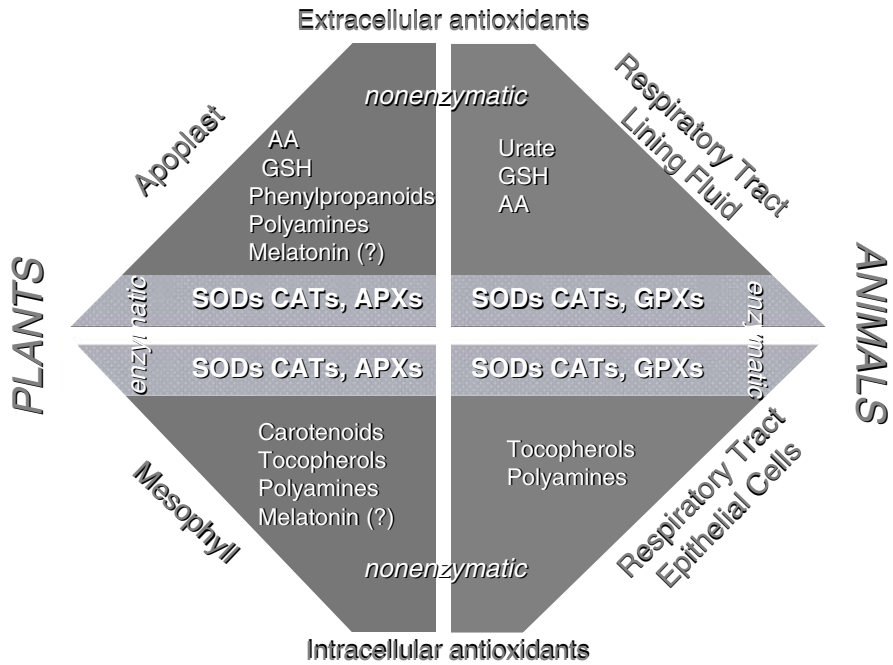

Fig. 8 Antioxidant defences in plants and animals. Both extracelluar and intracellular ROS scavengers are reported and further subdivided into nonenzymatic and enzymatic; (*AA*, ascorbic acid; *GSH*, glutathione, *SODs*, superoxide dismutases; *CATs*, catalases; *APXs*, ascorbate peroxidases; *GPXs*, glutathione peroxidases)

detoxification of xenobiotics and heavy metals, respectively (Rauser 1995; Marrs 1996). In any case it must be pointed out that GSH has a response time slower than AA.

Phenylpropanoids are phenylalanine derivatives, consisting of an array of plant secondary metabolites with a broad spectrum antioxidant activities. Major groups of phenylpropanoids include phenolic acids, lignans, stilbenes (i.e. resveratrol), flavonoids (including anthocyanins) and proanthocyanidins (or condensed tannins). In particular, polyphenols, a term indicating collectively flavonoids, stilbenes and condensed tannins, are soluble compounds which may be stored in the vacuole or secreted in the apoplastic space (Iriti and Faoro 2004).

Carotenoids are highly unsaturated tetraterpenes with an extensive double-bond system. Because of their lipophilic properties, in plants, these compounds confer protection to thylakoidal membranes from lipid peroxidation, thereby preserving the integrity of photosynthetic apparatus (Della Penna and Pogson 2006).

Lipid-soluble tocopherols derive from the prenylation of homogentisic acid, a tyrosine derivative via 4-hydroxyphenyl-piruvate intermediate, thus involving either isoprenoid and shikimate/chorismate (or aromatic amino acid) pathway, the same routes involved in carotenoids and phenylalanine biosynthesis, respectively. α-Tocopherol, or vitamin E, is the most abundant tocopherol of the four forms found in plants (α-, β-, γ- and δ-tocopherols), functioning as a ROS scavengers in cell membranes, the same as carotenoids (Della Penna and Pogson 2006). In addition, α-tocopherol protects lipid bilayers by eliminating the peroxyl radical, in combination with GSH, and by scavenging singlet oxygen (Kanofsky and Simall 1990).

Melatonin content, in plant tissues, has been associated to ozone tolerance of some species, because of its strong antioxidant activity (Dubbels et al. 1995). Melatonin is an indoleamine derived from the essential aromatic amino acid tryptophan, for long thought to be a neurohormone synthesized and secreted exclusively by the vertebrate pineal gland (Iriti and Faoro 2006). Interestingly, the currant tomato (*Lycopersicon pimpinellifolium*), highly sensitive to ozone injury, has a very lower melatonin content than the more tolerant *Lycopersicon esculentum* species (Dubbels et al. 1995).

Polyamines are biologically active amines occurring in virtually all prokaryotic and eukaryotic cells, and arising from free, non proteinogenic amino acid ornithine (Tabor and Tabor 1984). Polyamines, particularly putrescine, can interact with senescence processes, limiting the action of lipoxygenase and phospholipase D (Borrell et al. 1997). In plants, ozone exposure improves arginine decarboxylase (ADC), a key enzyme in polyamine biosynthesis (Rowland-Bamford et al. 1989). Free and conjugated polyamines increase ozone tolerance with

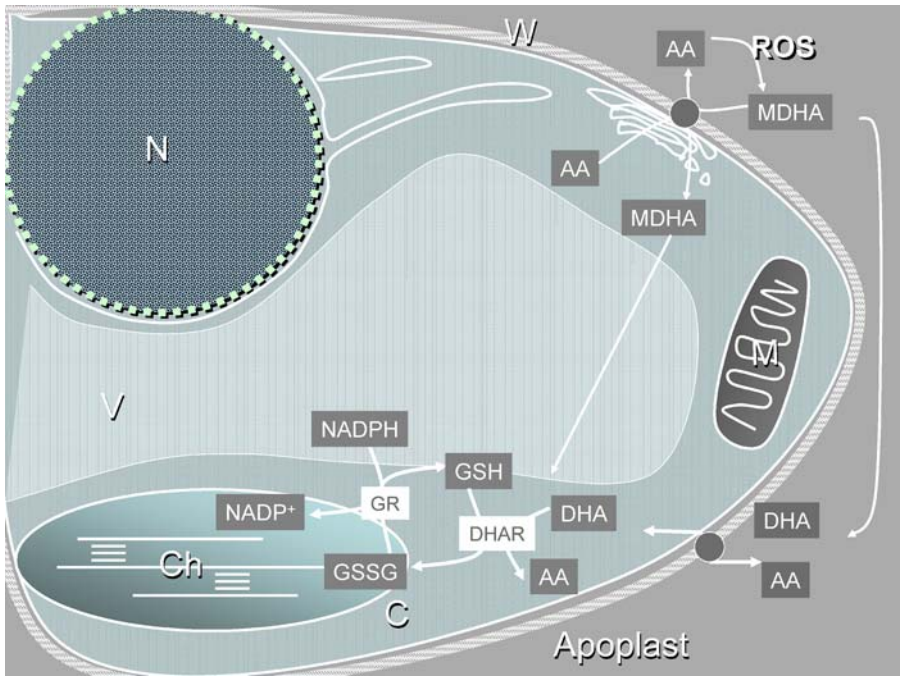

Fig. 9 Ascorbate–glutathione (Halliwel–Asada) cycle. Intracellular and extracellular (apoplastic) ROS are reduced by ascorbate (*AA*) with the formation of monodehydroascorbate (*MDHA*) and dehydroascorbate (*DHA*). The latter is reduced to AA by dehydroascorbate reductase (*DHAR*), through the reducing potential of glutathione (*GSH*) that is oxidized to glutathione disulfide (GSSG). GSSG is then reduced by glutathione reductase (*GR*) to GSH. In this way AA and GSH are continuously recycled (*N*, nucleous, *C*, cytoplasm; *Ch*, chloroplast; *M*, mitochondrion; *W*, cell wall; *V*, vacoule)

two different mechanisms: by inhibiting the ethylene biosynthesis and by direct ROS scavenging, respectively (Bors et al. 1989; Ormrod and Beckerson 1986). Ethylene and polyamines share the same biosynthetic precursor, *S*-adenosyl methionine (SAM) and, thereby, they mutually inhibit their own biosynthesis. This metabolic shift to ethylene or polyamine biosynthesis can improve ozone susceptibility or tolerance, respectively, due to the correlation between the stress ethylene production and visible ozone injury (Langebartels et al. 1991). Furthermore, apoplastic polyamines can form conjugates with hydroxycinnamates, phenolic acid derivatives, effective in ROS detoxification (Bouchereau et al. 1999).

In animals, O_3 enters through the respiratory tract and imposes an oxidative burden on the lung. Respiratory tract lining fluid (RTLF) represents the first interface between the underlying epithelial cells (RTECs) and the external environment, and, accordingly, antioxidants secreted by RTECs in RTLF are the first line of defence against inhaled oxidant pollutants (Langford et al. 1995; Mudway and Kelly 2000; van der Vliet and Cross 2000). RTLF is a two-layer structure, comprising a lower aqueous sol-phase and an upper mucus gel-phase. The lower, sol-phase of RTLF bathes the RTECs, whereas the upper, gel-phase entraps micro-organisms and large particles from the airstream. Furthermore, the thickness of the epithelial lining fluid varies along the respiratory tract: it is of 1–10 μm, in the upper airways, whereas, in the distal bronchoalveolar regions, the RTLF is only 0.2–0.5 μm thick (Quinton 1979; Duneclift et al. 1997; Widdicombe 1997). In general, uric acid, a water-soluble oxidized purine base, is by far the most prevalent antioxidant in RTLF, in amounts that, in nasal cavity, approximate those of plasma (100–400 vs. 100–500 μM) (Peden et al. 1990; Housley et al. 1995, van der Vliet et al. 1999). Urate directly quenches ROS, by donating an electron and forming a resonance-stabilized radical (Bruce et al. 1981), and prevents GSH and AA oxidation by chelating transition metal ions (Davies et al. 1986). GSH concentration, especially in bronchoalveolar RTFL, considerably exceeds that reported in plasma, ranging from 100 to 500 μM (Rahman et al. 1999; van der Vliet et al. 1999). GSH precursor NAC, initially developed as a mucolytic agent, is a cysteine donor which increases GSH level in lung tissues, thus accounting for its critical role in

protecting lung from the oxidative stress (Meyer et al. 1995; Kluchová and Tkáčová 2006). Additionally, both nasal and bronchoalveolar RTLF contain significant levels of AA, comparable to those found in plasma (van der Vliet et al. 1999), whereas vitamin E is present in RTLF at relatively low concentration (Rustow et al. 1993). Therefore, considerable variability exists among different lung anatomical compartments, where RTLF is equipped with an elaborate antioxidant network, according to their variable demand for detoxifying systems.

Ozone inhalation strongly stimulates ornithine decarboxylase (ODC) activity in the lung, a key enzyme of polyamine route, reflecting *de novo* biosynthesis of polyamines. In RTECs, polyamines can directly quench ROS and protect DNA from oxidative damage (Hoet and Nemery 2000).

Enzymatic Scavengers In animals and plants, enzymatic detoxification of ROS requires the orchestrated action of several enzymes, located both in cell compartments and extracellular space. SODs catalyze the one-electron dismutation of $\cdot O_2^-$ into H_2O_2 and molecular oxygen. They are classified, according to their metal cofactor, as isozymes containing copper/zinc (Cu/Zn), iron (Fe) or manganese (Mn). In turn, H_2O_2 can be reduced to H_2O by CATs, APXs and GPXs, although with different mechanisms. CATs, found mainly in peroxisomes, are porphyrin-containing enzymes which catalyze the two-electron dismutation of H_2O_2 into water and oxygen. APXs and GPXs scavenge H_2O_2, by using directly AA and GSH as reducing agents, respectively. Oxidized AA and GSH, dehydroascorbate (DHA) and glutathione disulfide (GSSG) respectively, are then re-reduced by means of the Halliwell–Asada cycle and glutathione reductase (GR), respectively (Fig. 9) (Noctor and Foyer 1998; van der Vliet and Cross 2000; Comhair and Erzurum 2002; Apel and Hirt 2004).

In animals, RTECs synthesize cytosolic Cu/Zn SODs and mitochondrial Mn SODs, cytosolic and membrane-associated GPXs and CATs. Two forms of GPXs exist: selenium (Se)-dependent and Se-independent, found in cytosol and mitochondria. Additionally, these cells can secrete, in the RTLF, extracellular isoforms of those enzymes (Comhair and Erzurum 2002; Kinnula and Crapo 2003). In plants, Cu/Zn SODs are localized in chloroplasts, either in stroma and thylakoids, cytosol and apoplast, Mn SODs in mitochondria and Fe SODs, absent in animals, still in chloroplasts. APXs are almost completely restricted to plant cells, where, like SODs, they have been reported in many subcellular compartments: cytosol, chloroplasts, mitochondria, peroxisomes and apoplast (Bowler et al. 1994; Willekens et al. 1995).

8 Conclusion

In spite of the huge biological diversity, plants and animals share some common traits in response to ozone injury, due to their eukaryotic cellular organization. Nevertheless, some structures and molecules are more vulnerable than others to oxidative stress, because of the ROS chemistry in the different biological systems. Some tolerance mechanisms, at biochemical level, have also been conserved during the evolution, between the two kingdoms. Otherwise, plants and animals differ in the availability of nonenzymatic antioxidants. The evolutionary success of autotrophic sessile organisms relied on their ability of synthesizing a plethora of primary and secondary metabolites, included antioxidants, enabling them to tolerate any adverse environmental, biotic and nutritional condition. Conversely, several scavengers in RTLF are of dietary origin. Hence, animals, being unable of synthesizing vitamin A, E and C, depend from the dietary intake of plant foodstuffs, for their survival (Kelly et al. 2003; Kelly 2004) (Fig. 2). Intriguingly, it has been suggested that the loss of uricase, the enzyme which degrades uric acid to allontoin, around the Paleolithic era, coupled with the loss of the ability to synthesize AA, thus resulting in the replacement of AA by uric acid as the major endogenous water-soluble antioxidant in human biological fluids (Cutler 1984). In fact, primates and guinea pigs are the only animals unable to synthesize AA, since they lack *L*-gulono-1,4-lactone oxidase, the enzyme involved in the last step of its biosynthesis (Domitrović 2006). Furthermore, the lack in aromatic amino acid biosynthesis impedes animals of synthesizing polyphenols and indoleamines (Iriti and Faoro 2004, 2006).

A link between dietary antioxidant micronutrients and lung function has been recently emphasised, especially in those respiratory diseases where ROS toxicity is involved, such as asthma, emphysema and cystic fibrosis (Samet et al. 2001; Romieu et al.

2002). Likewise, plant treatment with antioxidants, antiozonants (i.e. ethylenediurea EDU) or resistance inducers able to modulate oxidative burst, such as functional analogues of SA (i.e. benzothiadiazole, BTH), may counteract the ozone injury, thus improving the plant tolerance (Iriti et al. 2003; Manning et al. 2003, Carrasco-Rodriguez et al. 2005; Hassan 2006).

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