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Review

Phenolic Compounds and Their Antioxidant Activity in Plants Growing under Heavy Metal Stress

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Abstract

As a consequence of industrial development, the environment is increasingly polluted with heavy metals. Plants possess homeostatic mechanisms that allow them to keep correct concentrations of essential metal ions in cellular compartments and to minimize the damaging effects of an excess of nonessential ones. One of their adverse effects on plants is the generation of harmful active oxygen species, leading to oxidative stress. Besides the well-studied antioxidant systems consisting of low-molecular antioxidants and specific enzymes, recent works have begun to highlight the potential role of flavonoids, phenylopropanoids and phenolic acids as effective antioxidants. During heavy metal stress phenolic compounds can act as metal chelators and on the other hand phenolics can directly scavenge molecular species of active oxygen.

Phenolics, especially flavonoids and phenylopropanoids, are oxidized by peroxidase, and act in H_2O_2-scavenging, phenolic/ASC/POX system. Their antioxidant action resides mainly in their chemical structure. There is some evidence of induction of phenolic metabolism in plants as a response to multiple stresses (including heavy metal stress).

Keywords: H_2O_2-scavenging, polyphenols, ascorbic acid, peroxidase, soil pollution

Abbreviations: APX - ascorbate peroxidase, ASC - ascorbic acid, CAT - catalase, DHA - dehydroascorbic acid, cDHAR - cytosolic dehydroacscorbate reductase, GR - glutathione reductase, GSH - glutathione, GSSG - glutathione disulfide, MDA - monodehydroascorbate, MDAR - monodehydroascorbate reductase, POX - peroxidase, ROS - reactive oxygen species, SOD - superoxide dismutase

Heavy Metals Toxicity

Heavy metals are defined as that group of elements that have specific weights higher than about 5g/cm^3. A number of them (Co, Fe, Mn, Mo, Ni, Zn, Cu) are essential micronutrients and are required for normal growth and take part in redox reactions, electron transfers and other important metabolic processes in plants. Metals which are considered nonessential (Pb, Cd, Cr, Hg etc.) are potentially highly toxic for plants [1- 3]. Large areas of land are contaminated with heavy metals (the main group of inorganic contaminants) resulting from urban activities, agricultural practices and industry [4, 5]. Excessive concentrations of trace elements (Cd, Co, Cr, Hg, Mn, Ni, Pb and Zn) are toxic and lead to growth inhibition, decrease in biomass and death of the plant [6]. Heavy metals inhibit physiological processes such as respiration, photosynthesis, cell elongation, plant-water relationship, N-metabolism and mineral nutrition [7].

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Some external mechanisms that limit the uptake of metals by roots can help plants tolerate a certain amount of toxic metal in soil. One of them is the formation of non-toxic metal-ligand chelates in rhizosphere involving organic acids and other substances exuded from roots. Heavy metals tolerance is enhanced by the action of mycorrhizae [4, 8]. Metals can be transported via an apoplastic system and immobilized in cell walls [7]. Some plants (*Anthyllis vulnenaria* and *Biscutella leavigata*) can transport the excess of metals to aging organs and leaves and remove them seasonally [9]. Toxic metals become a real threat to plants mainly when they reach the cytosol of the cell. Therefore, the ability of root cells to control the transport of heavy metals via membranes determines their tolerance by plants [8]. They can be immediately complexed, inactivated and transformed into a physiologically tolerable form via action of phytochelatins and sequestred in cell vacuoles [6, 8]. In many cases plants resistant to heavy metal stress have lower nutritional requirements and specific mineral (cadmium, potassium and phosphorus) and water economies to cope with this stress [8, 9].

Remarkably resistant plants (and organisms that constitute the plant's rhizosphere) are involved in phytoremediation (consisting of phytoextraction, rhizofiltration and phytostabilization) of metal polluted sites [1]. Such plants are uncommon and according to Khan et al. [4], about 400 hyperaccumulator species (plants able to accumulate huge amounts of heavy metals in their tissues) [8] have been identified. According to the chemical and physical properties of heavy metals we can divide their harmful action into:

a) generation of ROS (*reactive oxygen species*) by autooxidation and Fenton reaction,
b) blocking of essential functional groups in biomolecules: proteins (by the inactivation of the SH-groups in enzymes active centers) and polynucleotides [8, 11],
c) substitution of essential metal ions by other incorrect ones [3].

Generation of ROS under Heavy Metal Stress

Aerobic organisms are exposed to ROS (***r**eactive **o**xygen **s**pecies*) formation. These incomplete reduced oxygen species are toxic by-products, generated at low levels in non-stressed plant cells in chloroplasts and mitochondria, and also by cytoplasmic, membrane-bound or exocellular enzymes involved in redox reactions (especially photosynthetic electron transport processes and respiration). Extra amounts of ROS occur under stressful conditions such as pathogen attacks, wounding, herbivore feeding, UV light, heavy metals and others [12, 13].

Several metabolic processes may use ROS in a good way. Some of the ROS are involved in lignin formation in cell walls. They participate in an oxidative burst and act not only as direct protectants against invading pathogens, but also as signals activating further reactions (HR-hypersensitive response or phytoalexin biosynthesis) [13, 14].

Generally a plant's cells try to keep the concentration of ROS at the possible low level because they are more reactive than molecular oxygen (O_2) [13], and they react with almost every organic constituent of the living cell. The high reactivity of ROS is based on the specificity of their electronic configuration.

ROSs are known to damage cellular membranes by inducing lipid peroxidation [2]. They also can damage DNA, proteins, lipids and chlorophyll [15]. The most popular ROS are $\cdot O_2^-$ -*superoxide radical*, H_2O_2 -*hydrogen peroxide*, and $\cdot OH$ -*hydroxyl radical* originating from one, two or three electron transfers to dioxygen (O_2).

Under physiological conditions $\cdot O_2^-$ is not very reactive against the biomolecules of the cell and in aqueous solutions at neutral or slithly acidic pH disproportionates to H_2O_2 and O_2.

H_2O_2 is relatively stable and not very reactive, electrically neutral ROS, but is very dangerous because it can pass through cellular membranes and reaches cell compartments far from the site of its formation [13].

Dietz et al. [16] and Sahw et al. [17] have reported that heavy metals induce oxidative stress in cells and tissues in the following ways:

a) they transfer electrons directly in single-electron reactions, which generate free radicals. The so-called transition metals (Fe, Cu, Mn, etc.), which have unpaired electrons in their orbitals, accept and donate single electrons, thus promoting monoelectron transfers to O_2 and generally ROS interconversion and oxireduction phenomena,
b) metals disturb metabolic pathways, especially in the thylakoid membrane, which also results in increased formation of free radicals and reactive oxygen species,
c) in addition, heavy metals mainly inactivate the antioxidant enzymes (peroxidases, catalases, superoxide dismutases) responsible for free radical detoxification, although peroxidases also may be activated due to metal stress,
d) finally, heavy metal accumulation results in the depletion of low molecular weight antioxidants, such as glutathione, which is consumed under phytochelate formation.

H_2O_2 in the presence of $\cdot O_2^-$ can generate highly reactive $\cdot OH$ *hydroxyl radicals* via the metal-catalyzed Haber-Wiess reaction (scheme 1.) thus the scavenging of H_2O_2 in cells is critical to avoid oxidative damage [14, 19]. In the presence of redox active transition metals such as Cu^+ and Fe^{2+}, H_2O_2 can be converted to $\cdot OH$ molecule in a metal-catalyzed reaction via the Fenton reaction [11, 13] (scheme 1.).

Other metals (e.g. Hg^{2+}) not belonging to transient metals cannot replace cuprum and iron in Fenton's reaction, but such ions can inhibit the activities of antioxidative enzymes, especially glutathione reductase and lead consequently to accumulation of ROS [11].

Cadmium causes oxidative stress probably through indirect mechanisms such as interaction with the antioxida-

tive defence, disruption of the electron transport chain or induction of lipid peroxidation. The activation of lipoxygenase, an enzyme that stimulates lipid peroxidation, has been reported after cadmium exposure [20].

Scheme 1:

Haber-Wiess reaction

$$H_2O_2 + \cdot O_2^- \rightarrow \cdot OH + OH^- + O_2$$

Fenton reaction

$$H_2O_2 + Fe^{2+}(Cu^+) \rightarrow Fe^{3+}(Cu^{2+}) + \cdot OH + OH^-$$

$$O_2 + Fe^{3+}(Cu^{2+}) \rightarrow Fe^{2+}(Cu^+) + O_2$$

Plant's Defense Systems

Plant damage occurs when the capacity of antioxidant processes and detoxification mechanisms are lower than the amount of ROS production. Aerobic organisms have developed complex systems protecting them from ROS, consisting of several enzymes and antioxidants. Those mechanisms can slow down or even stop the oxidation of biomolecules and block the process of oxidative chain reactions [21]. The most important are low-molecular antioxidants such as ascorbic acid, glutathione, thiols, α-tocopherol and protective pigments such as carotenoids [2, 14, 22]. Non-enzymatic scavengers are essential in the protection of cellular components from most ROS, but they cannot cope with reducing radicals such as superoxide or metastable hydroperoxides [23]. The most important antioxidant enzymes are: superoxide dismutase (SOD EC 1.15.1.1), catalase (CAT EC 1.11.1.6), ascorbate peroxidase (APX EC 1.11.1.11), monodehydroascorbate reductase (MDAR EC 1.1.5.4), dehydroacscorbate reductase (DHAR EC 1.8.5.1) and glutathione reductase (GR EC 1.6.4.2). At least four of them participate in a highly developed detoxification system named the ascorbate-glutathione cycle (Halliwell-Asada cycle) [15, 24-27].

APX uses ascorbic acid as a reductant in the first step of the ascorbate-glutathione cycle. This is the most important peroxidase in H_2O_2 detoxification operating both in cytosol and chloroplasts [15, 28]. *Apx* gene expression is rapidly induced by various stress conditions [14].

Phenolic Compounds and Their Functions

All plants produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites are phenolic compounds. Phenolics are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. They are mainly synthetized from cinnamic acid, which is formed from phenylalanine by the action of L-phenyloalanine ammonia-lyase PAL (EC 4.3.1.5), the branch point enzyme between primary (shikimate pathway) and secondary (phenylopropanoid) metabolism [30]. The significance of this route can be supported by the fact that, in normal growth conditions, 20% of carbon fixed by plants flows through this pathway [12] (Fig.1.) Phenols are divided into several different groups, distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton (simple phe-

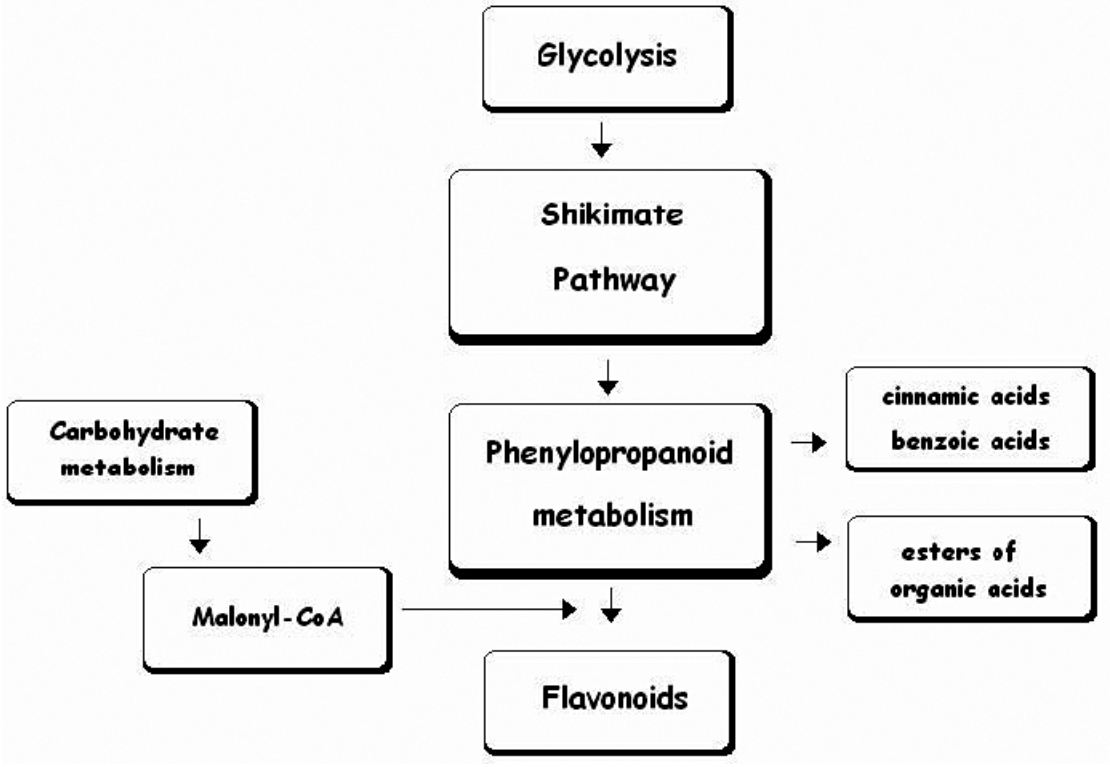

Fig. 1. Biosynthesis pathways leading to formation of main groups of phenolic compounds (according to [29], changed).

nols, benzoic acids, phenylopropanoids and flavonoids) [23, 31-33]. Phenolics have various functions in plants. An enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions [12, 18, 35, 36]. The synthesis of isoflavones and some other flavonoids is induced when plants are infected or injured [34, 37], or under low temperatures and low nutrient conditions [18, 37]. Most of them have antimicrobial activity. Plants accumulate UV-absorbing flavonoids and other phenolic compounds mainly in vacuoles of epidermal cells, to prevent the penetration of UV-B into the deeper tissues of the plant [38]. Flavonoids secreted from roots of legumina activate genes of root nodule bacteria [39].

The induction of phenolic compound biosynthesis was observed in wheat in response to nickel toxicity [12] and in maize in response to aluminium [39]. *Phaseolus vulgaris* exposed to Cd^{2+} acccumulate soluble and insoluble phenolics and *Phyllantus tenellus* leaves contain more phenolics than control plants after being sprayed with copper sulphate [12].

An increase of phenolics correlated to the increase in activity of enzymes involved in phenolic compounds metabolism was reported, suggesting *de novo* synthesis of phenolics under heavy metal stress. In contrast, some evidence indicates that the increase in flavonoid concentration is mainly the result of conjugate hydrolysis and not due to *de novo* biosynthesis [40]. Increase in soluble phenolics such as intermediates in lignin biosynthesis can reflect the typical anatomical change induced by stressors: increase in cell wall endurance and the creation of physical barriers preventing calls against harmful action of heavy metals [12]. In recent years there has been a growing interest in antioxidant properties of phenolic compounds.

Antioxidant Action of Phenols

The conception of antioxidant action of phenolic compounds is not novel [41]. There have been many reports of induced accumulation of phenolic compounds and peroxidase activity in plants treated with high concentrations of metals.

Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper [10]. The roots of many plants exposed to heavy metals exude high levels of phenolics [39]. They may inactivate iron ions by chelating and additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of ROS [42, 43]. Tannin-rich plants such as tea, which are tolerant to Mn excess, are protected by the direct chelatation of Mn. Direct chelation, or binding to polyphenols, was observed with methanol extracts of rhizome polyphenols from *Nymphaeae* for Cr, Pb and Hg [44]. According to Morgan et al. [45] this general chelating ability of phenolic compounds is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule.

There is another mechanism underlying their antioxidant ability. Metal ions decompose lipid hydroperoxide (LOOH) by the hemolytic cleavage of the O-O bond and give lipid alkoxyl radicals, which initiate free radical chain oxidation. Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical. This activity depends on the structure of the molecules, and the number and position of the hydroxyl group in the molecules [46].

Arora et al. [47] show that phenolics (especially flavonoids) are able to alter peroxidation kinetics by modifying the lipid packing order. They stabilize membranes by decreasing membrane fluidity (in a concentration-dependent manner) and hinder the diffusion of free radicals and restrict peroxidative reaction [48, 47]. According to Verstraeten et al. [49], in addition to known protein-binding capacity of flavanols and procyanidins, they can interact with membrane phospholipids through hydrogen bonding to the polar head groups of phospholipids. As a consequence, these compounds can be accumulated at the membranes' surface, both outside and inside the cells. Through this kind of interaction, as they suggest, selected flavonoids help maintain membranes' integrity by preventing the access of deleterious molecules to the hydrophobic region of the bilayer, including those that can affect membrane rheology and those that induce oxidative damage to the membrane components.

On the other hand, *in vitro* studies have shown that flavonoids can directly scavenge molecular species of active oxygen: $\cdot O_2^-$ -*superoxide*, H_2O_2 -*hydrogen peroxide*, $\cdot OH$ -*hydroxyl radical,* $^1O_2$ -*singlet oxygen* or *peroxyl radical*. Their antioxidant action resides mainly in their ability to donate electrons or hydrogen atoms [4, 14, 42, 50]. Polyphenols posess ideal structural chemistry for this acitvity and have been shown to be more effective *in vitro* than vitamins E and C on molar basis [43].

As described by Bors et al. [41] there are three structural features that are important determinants for the antioxidant potential of flavonoids:

a) the *orto* 3',4'-dihydroxy structure in the B ring (e.g. in catechin, quercetin);
b) the 2,3-double bond in conjunction with the 4-oxo group in the C ring (which allows conjunction between the A and B ring, or electron delocalization;
c) the presence of a 3- OH group in C ring and a 5-OH group in the A ring.

Among them the 3-OH group is the most significant determinant of electron-donating activity. The glycosilated flavonoids lose their activity in comparison with aglycones [19, 51]. The hydrogen peroxide-dependent oxidation of flavonols has been observed *in situ* in epidermal strips of leaves of *Vicia faba* [52], *Tradescantia virginiana* [53] and in mesophyll cells of *V. faba* [52].

Plants contain two major types of peroxidases, which can be divided into two groups: peroxidases (APX) which use ASC as the preferential electron donor and others, which use phenolics. APX is mainly localized in chloro-

plasts, cytosol and peroxisomes and its function is to scavenge the H_2O_2 which is formed in these organelles (Fig. 2) [56-59]. In these compartments, ascorbate is oxidized to the MDA (monodehydroascorbate) radical by APX to detoxify H_2O_2. MDA is a radical with a short lifetime that, if not rapidly reduced disproportionates to ascorbate and DHA (dehydroascorbic acid) which is reduced to ascorbate by (GSH)-dependent glutathione reductase (DHAR) [28, 55]. MDA radical can be reduced to ascorbate by non-enzymatic reaction of ferrodoxin (Fd) or by NAD(P)-dependent enzymatic reaction of MDAR (monodehydroascorbate reductase) [56]. Some works indicate that high concentrations of heavy metals can inhibit action of APX [24].

Peroxidases which use phenolics can be divided into soluble and cell wall-bound, apoplastic POXs and vacuolar ones. Cell wall-bound POXs have traditionally been considerated to participate in lignin monomers oxidation, providing oxidized substrates for lignin formations and other physiological processes [12, 34, 60, 61, 62, 63]. According to Rai et al. [3], peroxidase which participate in lignin biosynthesis and might built up a physical barrier against poisoning of heavy metals is important in cadimium toxicity, wounding and pathogen response. Soluble, apoplastic POXs can scavenge H_2O_2, cooperating with phenolics and ASC [34]. It has been proposed that phytophenolics, especially flavonols and phenylopropanoids of vacuoles and the apoplast, can detoxify H_2O_2 as electron donors for phenol peroxidases (guaiacol peroxidases) localized in these compartments, which results in the formation of respective phenoxyl radicals [19, 34, 54].

This first step of antioxidant action is catalyzed by peroxidases (reaction 1). Phytophenolics can be regenerated from phenoxyl radicals by non–enzymatic reaction with ascorbate (reaction 2) inhibiting the formation of degraded products [19, 21, 55]. If monodehydroascorbate radical is formed in vacuoles the radicals are disproportionated to ascorbate and DHA (reaction 3) which can be transported to cytoplasm and is reduced there by DHAR [34]. The transport of both ASC and DHA across tonoplast [64] and between symplast and apoplast has been observed [65]. According to Sakihama et al. [50] it's possible that MDA reductase could act as phenoxyl radical reductase in the apoplast to regenerate the redox status of phenols.

A scheme of four reactions is:

$$2FlavOH + H_2O_2 \rightarrow 2FlavO^{\cdot} + 2H_2O \quad (1)$$

$$2FlavO^{\cdot} + ASC \rightarrow 2FlavOH + 2MDA^{\cdot} \quad (2)$$

$$MDA^{\cdot} + MDA^{\cdot} \rightarrow ASC + DHA \quad (3)$$

$$H_2O_2 + ASC \rightarrow 2H_2O + DHA \quad (4)$$

where reaction 4 is the sum of 1,2 and 3.

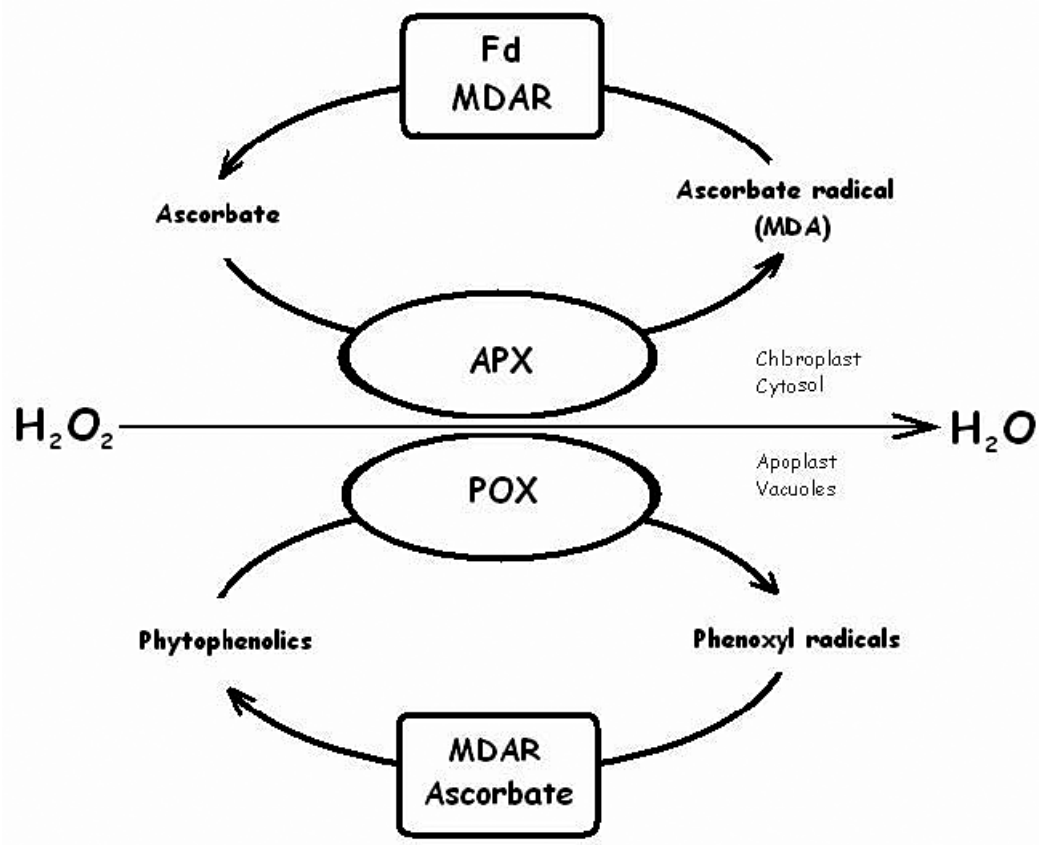

Fig. 2. Similarity between APX action (in chloroplast and cytosol) and POX action (in apoplast and vacuole). According to Sakihama et al. [50], it's possible that MDA reductase could act as phenoxyl radical reductase in the apoplast to regenerate redox status of phenols. POX uses phenolics as substrates to detoxify H_2O_2 (drawn according to [55], changed).

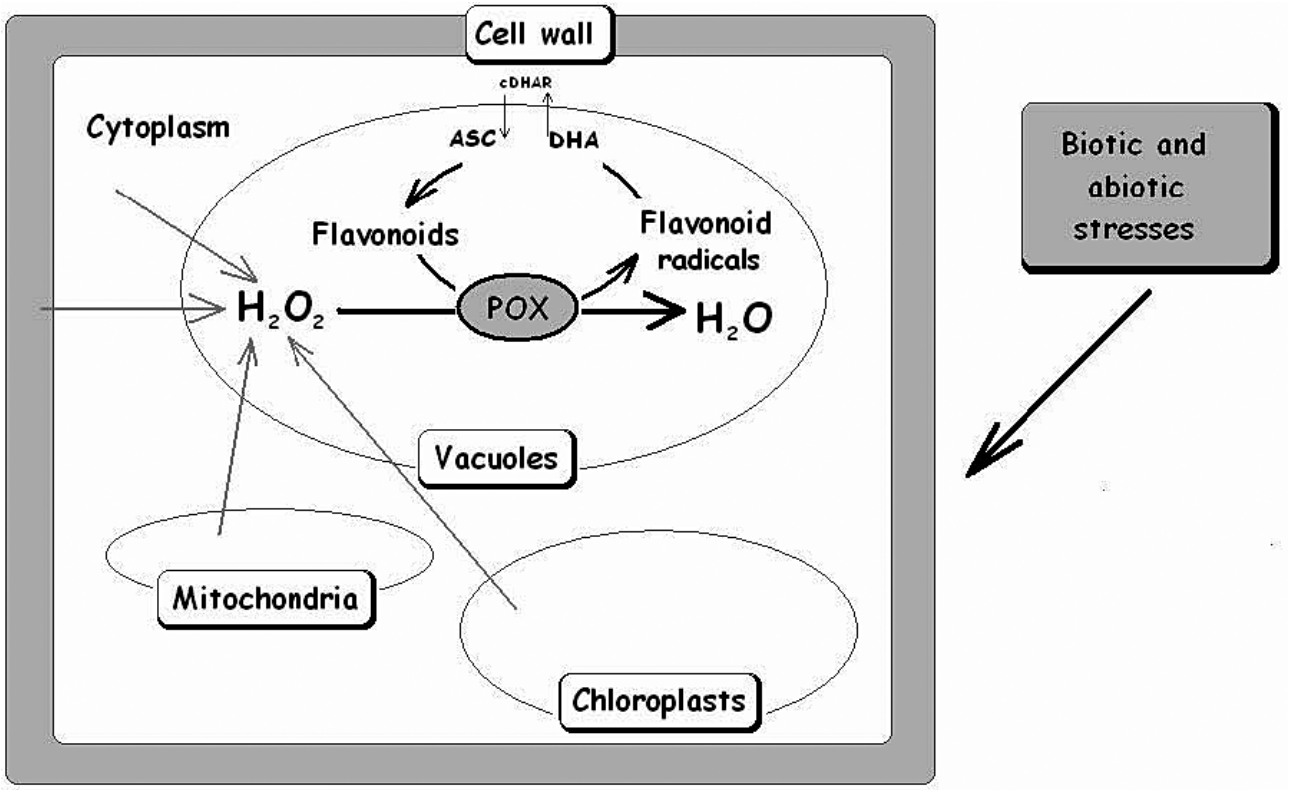

Fig. 3. H_2O_2 can pass through cell membranes and reach cell compartments far from the site of its formation. If the concentration of phenolics and ascorbate can be present at mM order in vacuoles, the ascorbate/phenolics/POX system can efficiently reduce H_2O_2 without any accumulation of oxidized phenolic products. It has been proposed that the vacuoles and apoplast can function as sinks of H_2O_2 in plant cells, which allow the delocalized detoxification mechanism against H_2O_2 produced in other compartments during stress and development (according to [19, 21, 22, 54], changed).

It's proper to mention that although the reduced forms of phenolic compounds act as antioxidants, the oxidized ones (phenoxyl radicals) may exert cytotoxic, pro-oxidant activity when the lifetime of the radicals is prolonged by effectors of spin-stabilization [55, 67]. This is true also for other natural antioxidants like vitamin C, vitamin E and carotenoids [66].

Under normal physiological conditions these radicals usually do not show harmful action because they are unstable and are rapidly changed to non-radical products. In fact sometimes they act in a good way as prooxidants, *o*-dihydroxy phenolics show anti-herbivore activity under certain condition [68]. But in general phenoxyl radicals are toxic to living systems because of their ability to initiate free-radical chain reactions in the membrane and their propensity to cross-link with a variety of molecules [55]. It has also been reported that prooxidant activity can be elicited by metal ions. Metal ions may influence the nature of plant phenolics *in vivo* by altering the lifetime of phenoxyl radicals. The toxicity of metals such as Al^{3+}, Cd^{2+} and Zn^{2+} retained in the root apoplast could be explained in this way [67].

If there is a deficit of ASC, toxic, brown polymerization products of flavonoids, may be generated irreversibly [19, 54, 69].

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