

Chapter 4

Uptake and Effect of Heavy Metals on the Plant Detoxification Cascade in the Presence and Absence of Organic Pollutants

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4.1 Introduction: Which Metals are “Heavy”?

Among the 110 elements present in the periodic table of the elements, 69 possess metallic properties. Seven of the elements in the Earth’s crust are also metals.

The term “metals” refers to elements with very good electrical conductance (this property declines with decreasing temperature) and that exhibit an electrical resistance that is proportional to the absolute temperature. Amongst these, heavy metals are metals that have a density of 4.5 g cm^{-3} or more. The other metals are referred to as light metals ($<4.5 \text{ g cm}^{-3}$).

Heavy metals are widely spread and can be found in various background concentrations in all environmental compartments. Their availabilities depend on the geology and geomorphology of the given ecological–geochemical system, and they can also be affected by anthropogenic activities.

4.2 Classification of Metals

Metals can be classified according to the HSAB (hard and soft acids and bases) concept.



Hard acceptors prefer to bind to hard donors whereas soft acceptors prefer to bind to soft donors to form stable compounds (Shaw et al. 2004). This is the so-called HSAB concept, which is very common in nature: some metals occur in the Earth’s crust as ores of oxide and carbonate, whereas other metals occur as sulfides. This is because hard acids will form strong bonds with hard bases, and conversely softer acids prefer soft bases.

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For example, minerals containing Al, Ca or Mg along with oxygen and CO₃ are hard acids, while minerals with Hg or Pb and sulfur are soft acids.

4.3 Metal Toxicity

The relative toxicity of a heavy metal depends on its availability, which is determined by the properties of the soil and the plant species of interest. Once taken up by the plant, heavy metals interact with different cell components and result in disturbances to the normal metabolic processes. This can cause cell injuries and in some cases the death of the organism (Shaw et al. 2004).

The toxicity occurs because either the biological functions of the enzymes are blocked or essential metal ions in the biomolecules are replaced with nonfunctional ions (Shaw et al. 2004), so that the catalytic properties of the biomolecules change or are even lost.

According to Nieboer and Richardson (1980), metals have different ligand binding preferences, which is why they can be classified into three groups:

- Class A metals prefer ligands with available oxygen
- Class B metals bind to ligands containing sulfur or nitrogen
- Class C metals have binding properties that are intermediate between those of classes A and B.

Nieboer and Richardson (1980) hypothesise that a preference for specific ligands will lead to identical effects in different organisms.

Several enzymes rely on the presence of certain metals at their active centres; if these metal ions are shifted in position or displaced by other metals with same size and charge, the activity of the enzyme is inhibited. For example, zinc, which is crucial to the activities of several metal enzymes, can be displaced by cadmium, which is just below it in the periodic table. Despite of the chemical similarities between cadmium and zinc, enzymes that should contain zinc are not able to perform catalysis in an appropriate manner when they contain cadmium instead (Shaw et al. 2004).

The heavy metals with the highest toxicity are those included in class B of Nieboer and Richardson's system (those that bind to ligands containing sulfur and nitrogen). They also exhibit a wide spectrum of toxic mechanisms. No class B metal has ever been found to occur naturally in any enzyme. These metals bond most effectively with SH groups such as cysteine, and with nitrogen-containing groups such as lysine and the active centres of enzymes (Shaw et al. 2004).

4.4 Mixed Pollution with Organic Pollutants

Organic pollutants generate a great deal of interest in many countries due to environmental problems connected to the controlled or uncontrolled emission of organic chemicals into the environment (Schröder and Collins 2002). Frequently, these

organic chemical contaminations are accompanied by various heavy metals, yielding a complex pollution mix. Plants growing on sites that are contaminated in such a manner have many problems to contend with (Schröder et al. 2009). Again, uptake is governed by the properties of the soil, the physicochemistry of the pollutants in the mixture, and by plant-specific features. We may expect that the plants assimilate the damage as single or multiple stress signals; in any case, they must take remedial action very rapidly.

4.5 Sources of Environmental Contamination

Most known heavy metal contaminations are not natural; they have been generated by human activities. Such activities result in the transportation of heavy metals into the environment via particulates in the air, dissolution in contaminated water, or as lump ore deposits. The twentieth century, with its continuously increasing progress in industrial production and the exploitation of resources, was also a period in which the distribution of anthropogenic pollutants in the soil, ground and surface waters, and in the atmosphere increased greatly (Nriagu 1979).

Contaminants are substances that can endanger the environment, humans, animals, plants, soil or water (Hoffmann 1998). Even though the quantities of such contaminants have been reduced across much of Europe during the last decade, they are still a current major problem in many countries. The main sources of contaminants are:

- Critical spot sources, like fuelling stations, oil or metal manufacturers, military garrisons and shooting ranges, mining industry and processing ashes, open pit mining and tailings, municipal and industrial waste depots, and ore depot leaching
- Diffuse sources like burning fossil fuels, pesticides, phosphate fertilisers and communal waste waters (Kabata-Pendias and Pendias 1989).

These sources provide pathways for the release of not only organic contaminants but also heavy metals, which can be highly toxic in very small quantities (Memon et al. 2001; Memon and Schröder 2009).

Heavy metal contaminations of geogenic origin (Shaw et al. 2004) are found in areas where metalliferous veins reach close to the surface, and so metals can be washed out by rain or surface water. This can typically be seen for nickel and arsenic. However, such areas are relatively scarce in comparison to those contaminated by human activities (Pilon-Smiths 2005).

Soil and water contamination with anthropogenic burdens are a general environmental problem for which effective and affordable solutions are urgently required (Memon et al. 2001).

Most of the more frequently used technological methods, like dig and dump, soil restoration, storage or isolation of the contaminated surfaces, are very sophisticated and expensive and so are out of the reach of most of the communities with such pollution problems.

Interestingly, most contaminated sites are populated by plant species that can exist and survive undisturbed on metal-enriched soils (Memon et al. 2001; Memon and Schröder 2009). Some of these endemic plant species have attracted attention because of their high heavy metal accumulation capacities. Even more interestingly from the viewpoint of biomass productivity, some species that do not take up the metal ions and hence are classified as metal avoiders have been found. Such species obviously own detoxification mechanisms that can soften or repair the negative effects of the metals, or transform them in a chemical form that is unable to cause stress. This type of plant can be used for specific heavy metal removal from soils and waters, a task that is part of phytoremediation. The aim of phytoremediation is to remove contaminants from the environment with help of different plant species in a cost-effective, sustainable and environmentally compatible manner.

4.6 Uptake Mechanisms for Metal and Organic Xenobiotics in Plants

Hot spots for heavy metal contamination of plants are the air, water, soil and sediments, and plants make use of the opportunity to enrich themselves in metals when their growth capacities allow this (Greger 2004). Whereas higher plants can take up metals from the air via shoots and leaves, entry via roots and rhizomes from the soil substrate predominates. Of course, heavy metals can be much more concentrated in soils than in water (Förstner 1979).

In any case, metal uptake through leaves and roots depends on the concentration of the metal in the medium. However, uptake does not increase linearly with the concentration of the metal in the medium. This is because metals are often present under bound conditions. The uptake efficiency is highest at lower concentrations, because the low metal concentration also minimises competition between the metal ions at the absorption (uptake) surface (Greger et al. 1991). The larger the root surface area available, the more effective the uptake of the metal ions. Competition for the metal ions between plants at the same location can also occur, reducing uptake efficiency (Marschner 1995).

4.6.1 Factors that Influence Metal Uptake

There are different pathways associated with the entry of dissolved substances into plant cells. The cytosol is a barrier between the vacuole and the outside of the plant cell that offers high resistance to the passage of any solution that includes salts and bases (Nultsch 2001). Plants have a natural tendency to take up metals, and their passage into plant cells will probably be hampered by this barrier. The effectiveness of the metal uptake is highly dependent on the availability, which in turn depends on

many factors such as pH and content of organic matter in the soil. Solubilised metal ions enter the root via either extracellular (apoplastic) or intracellular (symplastic) pathways. The apoplast is the extracellular space into which water molecules and dissolved low molecular mass substances will diffuse. On the other hand, the symplastic compartment consists of a continuum of cells connected via plasmodesmata.

The apoplast plays an important role in the binding, transport and distribution of ions and in cellular responses to environmental stress, contributing to the total elemental content of the roots.

This space comprises about 10–25% of the capillary space of the rhizodermis and the cortex cell walls. The ions flow with the water taken up by the apoplast into free spaces, where some of them will diffuse and some of them will bind to the carboxyl groups on the cell walls or the negatively charged groups of the proteins. The specifics of this internal dissemination depend on the metal and the plant (Greger 2004). Wierzbicka (1998) reported that most of the lead taken up by *Allium cepa* remains bound in the apoplast.

The ions can reach the endodermis, which is the beginning of the “internal space”, by travelling along this waterway (Nultsch 2001). To get into the xylem, the ions must pass through the endodermis and the Casparian strip. The Casparian strip (Fig. 4.1) is a waterproof lipophilic surface coating in the radial cylinder of the endodermal cells of the root that consists of suberic substances and lignin. Its role is to block the passage of soluble minerals and water from the internal symplast through the cell walls (predominantly the cells in the central cylinder).

Metal uptake generally occurs in young roots without developed Casparian strips (Marschner 1995). It is not clear how metals pass through the older parts of the root.

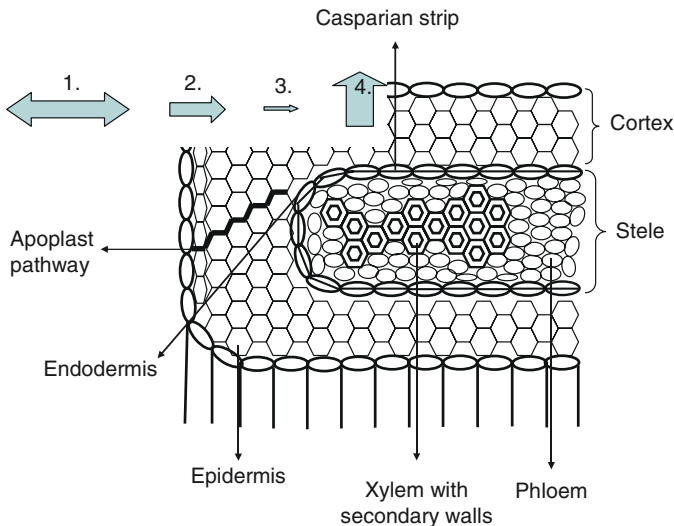


Fig. 4.1 Root uptake of solutes. 1, Free diffusion in the rhizosphere; 2, apoplastic diffusion in apparent free space and Donnan free space of the rhizodermis and parenchyma; 3, transfer to the symplast in the endodermis; 4, transpiration-stream-driven transport to the shoot

Metal uptake from the soil solution is selective and depends on specific or genetic metal ion carriers or channels located in the plasma membrane. It starts with the influx of the individual ions into the “apparent free space” (AFS) (Nultsch 2001).

Most metal ions enter the cells via an energy-dependent saturable process. Carrier systems transport cations into plant root cells. Nonessential heavy metals can compete for the same transmembrane carriers used by essential heavy metals. Heavy metals are transported acropetally to the roots.

Root uptake and transport of organic xenobiotics is determined by the so-called root concentration factor (RCF) (Schröder and Collins 2002). The RCF is heavily dependent on $\log K_{o/w}$ (i.e. the lipophilicity of the compound under consideration), and this seems to be governed by the absorptive properties of the root bark. Compounds with $\log K_{o/w} < 1$ cannot penetrate the lipid-containing root epidermis, while compounds with $\log K_{o/w} > 2$ become increasingly retained by the lipid in the root epidermis and the mucilage surrounding the root because of their enhanced hydrophobicities (Schröder and Collins 2002).

Compounds with a $\log K_{o/w}$ of about 2 are only transported in the transpiration stream, while those with a $\log K_{o/w}$ of about 1 are mobile in both phloem and xylem, although these are probably the only metabolites that enter the phloem. For compounds with $\log K_{o/w}$ 1.0–3.5, metabolism may occur in the leaf and stem tissue (Schröder and Collins 2002).

Once they are taken up by the roots, both organic xenobiotics and metals can be stored in underground tissues or exported to the shoot. Transport into the shoot involves loading in the xylem sap and translocation to the aerial parts.

In this case, the Casparian strip is the barrier that limits the entry of both xenobiotics and metals into the xylem. Dissociated molecules and ions are transferred relatively easy, whereas substances with higher lipophilicities or strong binding capacities are usually retained. Inside the root stele, transfer to xylem vessels follows the laws of accelerated diffusion in the water stream moving towards the plant shoot. Xylem cell walls have a high cation exchange capacity. The metal chelate complexes reduce the interior concentration of metals in the xylem and facilitate metal transfer into the transpiration stream. Organic acids (especially citrate) as well as amino acids are the main metal chelators in the xylem.

Marschner (1995) reported that the metal uptake increases when the pH increases. The opposite happens in soils. He supposed that there is a competition between the hydrogen ions and the metal ions in the root growth area.

Experiments with aquatic plants have shown that increasing the salt content decreases Cd, Cu and Zn uptake because metal- Cl_x complexes are formed (Greger et al. 1995). These types of complexes are not appropriate for plant uptake. The opposite can be expected in sediment systems containing salt; the cadmium uptake in such a system will be significantly higher. In the case of a high salt concentration, an exchange reaction occurs between sodium ions and cadmium ions bound to colloids in the soil colloids, leading to higher cadmium concentrations in the plant (Greger et al. 1995).

Most of the metal enters the plant via the roots after metal–root contact occurs. In the case of diffuse uptake, metals migrate along their concentration gradients together with other ions to the root and across the cortex tissue. Nearby, ion mass

flow can occur along the gradient in the water potential, which is held at a high level by transpiration, and this leads to enrichment in the shoot (Marschner 1995).

If the root uptake is high and the concentration of the element in the soil is low, the uptake of the element will be limited by diffusion.

4.6.2 *Metal Transport in Shoots*

The transport of heavy metals in phloem can be complicated because ions can easily be coupled to the phloem of living cells. Cadmium for example can be found in the stipule and in the leaf stalk of pea after the leaves have been treated, but it is not transported further (Greger et al. 1993). Stephan and Scholz (1993) suppose that nicotinamide, which is a metal chelator, influences the content of heavy metals in the phloem. Aquatic plants transport heavy metals in both vessel types. If the osmotic potential around the roots increases, the basipetal transport of zinc and cadmium will also increase; in contrast, the acropetal transport was overbalanced when the leaves were treated with osmotica (Greger 2004).

The plasma membrane acts like a barrier to toxic elements and inhibits uncontrolled uptake into the cell lumen. Metals are taken up in the form of cations and cation transport systems may be used by designated elements. Zinc is transported by specific zinc transporters (Lasat et al. 2000), and copper passes through the membranes via the ATP-dependent copper outflow (Knauer et al. 1997). Light and temperature affect the uptake of cadmium and lead (Hu et al. 1996; Chawla et al. 1991; Hooda and Alloway 1993). An increase in biomass production promotes the uptake of such elements. Accumulation in plants is reduced with dilution, which is affected by growth (Ekvall and Greger 2003).

Costa and Morel (1994) hypothesise that 30% of cadmium is taken up passively by plants; the rest passes through the membrane actively via specific H⁺-ATPases. It is not yet known whether these ATPases are identical to the MDR-like tonoplast carriers responsible for the sequestration of xenobiotic glutathione conjugates. In the cytoplasm, the metal binds to negatively charged macromolecules, or to parts of bigger cell structures. Biomolecules, for example phytochelatins, can spontaneously form complexes with the cadmium; using specific transporters, these complexes can pass through the tonoplast and reach the vacuole, where the cadmium separates and complexes with organic acids. The free phytochelatin molecules exit the vacuole and again become available to act as binding partners for metals in the cytosol. The cleavage of the phytochelatin–cadmium complexes happens spontaneously under the low-pH regime of the vacuole (Steffens 1990).

4.7 What Causes Oxidative Stress?

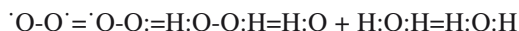
Oxygen is essential not only for energy metabolism and respiration; it also plays a role in degenerative processes (Marx 1987). It is a biradical, which means that it has two unpaired electrons with parallel spins (see Table 4.1). While oxygen does have unpaired electrons, which would normally make it rather reactive, the fact that

Table 4.1 Nomenclature of the different oxygen species

Species	Formula
Triplet oxygen (ground state)	$\cdot\text{O}-\text{O}\cdot$
Singlet oxygen	$\text{O}=\text{O}$:
Superoxide	$\cdot\text{O}-\text{O}$:
Perhydroxide radical	$\cdot\text{O}-\text{O}-\text{H}$
Hydrogen peroxide	$\text{H}-\text{O}-\text{O}-\text{H}$
Hydroxy radical	$\text{H}-\text{O}\cdot$
Hydroxide ion	$\text{H}:\text{O}:$
Water	$\text{H}:\text{O}:\text{H}$

these electrons have parallel spins (known as the “triplet state”) actually makes it difficult for oxygen to participate in reactions with organic molecules. Such reactions become easier if the biradical is activated. Activation can occur if enough energy is absorbed by triplet oxygen to “flip” the spin of one of its unpaired electrons (i.e. making the spins antiparallel – the “singlet state”). Singlet oxygen is much more reactive towards organic molecules than triplet oxygen.

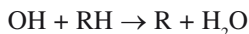
Another way to activate oxygen is through the stepwise monovalent reduction of oxygen to superoxide, superoxide to hydrogen peroxide, and hydrogen peroxide to hydroxide radical. The final product of this series of reductions is water:



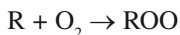
Hydrogen peroxide is a very important metabolite, because it can diffuse across membranes and is not dispersed in cells. Peroxidase enzymes (POX) use hydrogen peroxide as a substrate in oxidative reactions, primarily during the complex synthesis of organic molecules.

4.7.1 Oxidative Damage to Lipids

Lipid peroxidation proceeds via three phases: activation, distribution and cleavage. The activation of one unsaturated fatty acid (linoleate) by one hydrogen radical results in the cleavage of one H^+ atom from the methyl vinyl group of the fatty acid:



During this reaction, the resonance structure will react with triplet oxygen, which (as discussed above) is a biradical with two unpaired electrons. These unpaired electrons make it easy for triplet oxygen to react with other radicals. This reaction produces a peroxide radical:

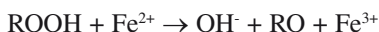


This peroxide radical assimilates a hydrogen atom from a second fatty acid, resulting in the formation of a lipid hydroxide. The free carbon centre can then participate in the secondary assimilation of hydrogen:



The main reason for the high reactivity of hydroxide radicals in any given lipid system is their ability to initiate a chain reaction at even very low concentrations.

Iron is a well-known catalyst of lipid oxidation, and it is also a critical reactant in the production of OH^- via the classical Fenton reaction, leading to the formation of reactive alcohol radicals:



This is the reason for the propagation of the chain reaction in the absence of iron. The end products of the reduction of ROOH are ethylene and ethane, which derive from alkyl chains that are set free during lipid peroxidation.

4.7.2 *Oxidative Damage to Proteins*

Any oxidative stress that reaches the cytosol can lead to the disruption of lipid bilayers, changes in conductivity, disturbances to proton gradients, and to higher functional protein sensitivity in general. Amino acids vary considerably in terms of their binding and reactivity with radicals.

In particular, metal-containing amino acids and the thiol groups of the proteins are very sensitive. Activated oxygen can extract a hydrogen atom (H^+) from cysteine to form a thiol radical, which can then bond with other cysteines via a disulfide bond. Alternatively, oxygen can bond with methionine residues, so that methionine–sulfur derivatives are formed. The oxidation of iron–sulfur centres via superoxide disturbs enzymatic function (Gardner and Fridovich 1991). When several amino acids are modified by radicals, the protein is oxidised.

4.7.3 *Where are the Products of Activated Oxygen Formed?*

The reduction of oxygen to its products – superoxide, hydrogen peroxide and hydroxide radicals – is the principal oxygen activation mechanism in most biological systems (McKersie 1996). The formation of singlet oxygen in the photosystems of higher plants is their main source of radicals (Wagner 2006). Oxygen radicals are produced either as end-products of chemical reactions in order to activate certain metabolic pathways, or as an early sign of chemical or environmental stress. Plant cells will also produce active oxygen during interactions with potential pathogens (Baker and Orlandi 1995). Active oxygen species, including superoxide, hydrogen peroxide and hydroxide radicals, can potentially affect other cell processes that occur during plant–pathogen interactions.

Active oxygen formed as a response to a pathogen or elicitor probably exerts direct antimicrobial effects before it induces other defence mechanisms, such as lignin production, lipid peroxidation and oversensitive reactions (Baker and Orlandi 1995).

However, in order to protect the cell from damage, reactive oxygen is deactivated in several cell organelles.

- Chloroplasts

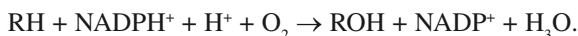
Elstner (1991) described chloroplasts as a source of activated oxygen. In photosystem I, oxygen can be reduced via the Mehler reaction. The monovalent reduction of oxygen is affected in cases of NADP⁺ limitation, which can occur if the Calvin cycle cannot oxidise NADPH⁺ as rapidly as photosystem I delivers electrons. Furthermore, photoactivated chlorophyll may transfer its energy to the reactive centre of the photosystem but electron transport may be inhibited. This has been observed in the presence of xenobiotics or herbicides, and we can expect that heavy metals will escalate this effect. Damage could be caused to the membrane transport system, to stomata movement and to nutrient acquisition. Photosystem II can fail during water cleavage and release triplet instead of normal oxygen. Last but not least, photorespiration is a fast oxidation mechanism in the chloroplast. Although RuBisCO favours CO₂ as substrate, oxygenation of RuBisCO occurs frequently, producing glycolate and glycerate. This usually happens when oxygen levels are high; i.e. when stomata are closed to prevent excessive water loss.

- Mitochondria

The largest proportion of the oxygen is consumed in cells as a substrate for cytochrome oxidase in mitochondria. Four electrons are transferred to each oxygen molecule, yielding water as a product of this reduction. Some mitochondria will also produce hydrogen peroxide and O₂ in the absence of NADH⁺ to reduce the oxygen (Loschen et al. 1973, 1974). It is thought that Fe-S proteins and NADH⁺ dehydrogenase are also potential sources for superoxide and hydrogen peroxide formation (Turrens et al. 1982).

- Endoplasmic Reticulum

The endoplasmic reticulum contains cytochrome P450 monooxygenases. The reaction catalysed by cytochrome P₄₅₀ is:



Hence, superoxide can be produced by the electron-dependent NAD(P)H⁺, which includes P₄₅₀. After the reduction of the substrate (RH) and the addition of triplet oxygen, the complex P₄₅₀-ROOH is obtained. This can be degraded to P₄₅₀-RH by splitting off superoxide (Winston and Cederbaum 1983).

- Peroxisomes

NAD(P)H⁺ oxidase activity was detected in the plasmalemma of peroxisomes. In roots, NAD(P)H⁺ oxidase reduces Fe³⁺ to Fe²⁺ for iron transport. Disturbances to the function of this enzyme lead to superoxide formation (Cakmak and Marschner 1988).

Pathogens and elicitors, injuries, heat stress or xenobiotics can also stimulate superoxide formation via NADPH oxidase activation. It is thought that these reactions

cause cascades of signals in plant cells that prevent them from physical, chemical and biological stress. This could lead to hypersensitive responses and cell death (Doke et al. 1991).

- Cell Walls

Cell walls have recently been found to be regions of active metabolism and oxygen activation. This can happen during defensive reactions against pathogens or the degradation of xenobiotics. NADH⁺, for example, is formed by extracellular malate dehydrogenase in cell walls, where it is used in hydrogen peroxide formation, probably by the NADH⁺ oxidase of the plasmalemma (Vianello and Macri 1991). Again, this can initiate programmed cell death.

4.7.4 Defence Mechanisms Against Oxidative Stress

4.7.4.1 Superoxide Dismutase (SOD) (EC 1.15.1.11)

Superoxide dismutase was first insulated by Mann and Keilin (1938), who thought that the protein was responsible for the storage of copper. Its catalytic functions were described years later by McCord and Fridovich (1969). Since then, superoxide dismutases (Fig. 4.2) have been shown to act as catalysts for the dismutation of superoxide to hydrogen peroxide and oxygen:



Because superoxide dismutase is found in all aerobic organisms, it is generally thought to play a central role as a defence mechanism against oxidative stress (Beyer et al. 1991; Bowler et al. 1992; Scandalias 1993). A recent publication

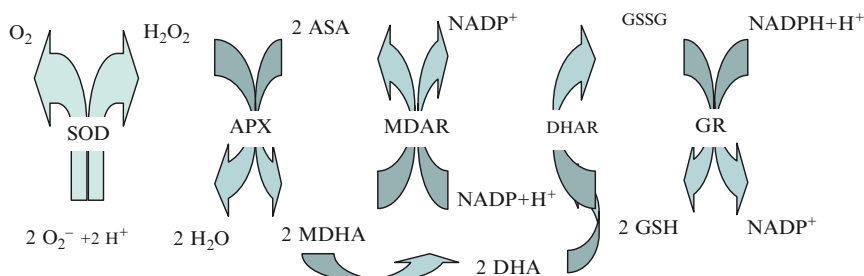


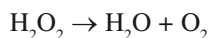
Fig. 4.2 Halliwell–Asada cycle (modified from Foyer et al. 1993). Superoxide and hydrogen peroxide detoxification with the consumption of ascorbate, and recovery of ascorbate at the expense of glutathione and NADPH. ASA, ascorbate; APX, ascorbate peroxidase; DHA, dehydroascorbate reductase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; MDHA, monodehydroascorbate radical; MDAR, monodehydroascorbate reductase; SOD, superoxide dismutase

hypothesises that the level of superoxide dismutase activity may be proportional to the degree of environmental and xenobiotic stress in the cell (McKersie 1996). Three different types of superoxide dismutase are known, and they are classified according to their metal cofactors: Mn-SOD in mitochondria, Fe-SOD in chloroplasts, and CuZn-SOD in both chloroplasts and the cytosol.

The prokaryotic and eukaryotic cells of some algae possess only Mn-SOD and Fe-SOD isoenzymes, leading to the assumption that these are very old forms of superoxide dismutase.

4.7.4.2 Catalase (1.11.1.6)

Catalase mediates the dismutation of hydrogen peroxide formed by SOD and other sources:



This enzyme is present in all eukaryotic organisms and is primarily responsible for the breakdown of hydrogen peroxide formed in peroxisomes during the oxidation of fatty acids (Fig. 4.3). All forms of this enzyme are tetrameric. Catalase is highly photosensitive (Hertwig et al. 1992).

4.7.4.3 Ascorbic Acid

L-Ascorbic acid (vitamin C) is an omnipresent antioxidant in plants. Green leaves contain ascorbic acid and chlorophyll in equimolar concentrations. Ascorbic acid plays a very important role in many physiological processes such as growth, differentiation and metabolism (Foyer 1993). Ascorbate is also important to our discussion, as it reduces the damage caused by free radicals.

Ascorbic acid is synthesised from D-glucose and acts as an oxidant in cytosol and chloroplasts. Ascorbate binds with superoxide, hydrogen peroxide or radicals of tocopherol to yield monodehydroascorbic acid or dehydroascorbic acid. These reduced forms are then recycled to ascorbic acid, a process catalysed by

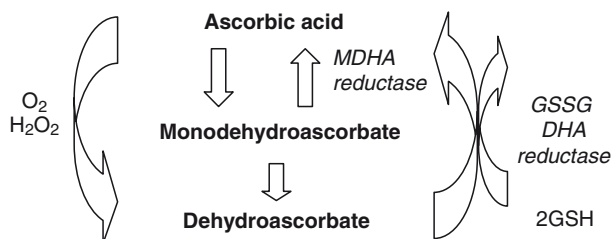


Fig. 4.3 Synthesis and degradation of ascorbic acid

monodehydroascorbate reductase (EC 1.6.5.4) and dehydroascorbate reductase (EC 1.8.5.1) using NAD(P)H⁺ and GSH as sources of electrons. The dehydroascorbate can also be metabolised to tartrate or oxalate (Fig. 4.3).

4.7.4.4 Peroxidase (EC 1.11....)

The class of enzymes known as peroxidases detoxify reactive oxygen species (ROS). For many of these enzymes the optimal substrate is hydrogen peroxide (H₂O₂), but others are more active with organic hydroperoxides, such as lipid peroxides. Peroxidases can contain a heme cofactor at their active sites, or redox-active cysteine or selenocysteine residues. The nature of the electron donor is very dependent on the structure of the enzyme. Peroxidases reduce H₂O₂ with the aid of an electron donor to water. Some peroxidases are crucial to the processes of lignification (Hess 1991) and pathogen defence (Messner and Schröder 1999).

4.7.4.5 Glutathione Reductase (EC 1.6.4.2)

Glutathione reductase (Fig. 4.3) is a regenerative enzyme. With NADPH+H⁺ as cosubstrate, it maintains the content of reduced glutathione in the cell at a constant level (Kiefer 2002). This means that it contributes indirectly to the detoxification of reactive oxygen. The NADPH needed for the reduction is again provided by photosynthesis, which confirms that the detoxification of ROS in the Halliwell–Asada cycle is a light-dependent process (Kiefer 2002).

Overall, in the Halliwell–Asada cycle, toxic hydrogen peroxide is reduced by glutathione reductase and GSH and subsequently by glutathione peroxidase to water. The oxidised glutathione dimer GSSG is also reduced via glutathione reductase (via the consumption of NADPH as the reductant) to GSH. This provides glutathione for various detoxification processes. Recently, we have been able to demonstrate that GR is strongly inhibited by heavy metals in vitro (Lyubenova et al. 2007). This finding indicates that the Halliwell–Asada cycle can undergo detrimental changes if its initial reactions are corrupted and free heavy metal ions flood the cytosol.

4.7.4.6 Glutathione

Glutathione (GSH) is the tripeptide Glu-Cys-Gly, which functions as an antioxidant due to the sulfhydryl group of the cysteine (Meister 1988). Glutathione is a ubiquitous molecule that performs many functions in chloroplasts and the cytosol. It is present in all of the cells of higher plants in millimolar concentrations. The glutathione level is highest under conditions of high light intensity. At the subcellular level, its concentration in chloroplasts is higher than that in the cytosol. Glutathione can function as antioxidant in different ways (Fig. 4.3). It reacts chemically with

the singlet oxygen of the superoxide and the hydroxy radical and scavenges other free radicals. On the other hand, glutathione recycles ascorbic acid from its oxidised to its reduced form with the aid of dehydroascorbate reductase (Loewus 1988).

Glutathione is also involved in the transport of reduced sulfur from the leaves to the roots (Meister 1988), and in xenobiotic detoxification, where it acts as a co-substrate for glutathione *S*-transferases.

Additionally, glutathione acts as a substrate in the formation of phytochelatins, γ -glutamylcysteine oligomers, which chelate heavy metals in plants (see below).

4.7.4.7 Phytochelatins

To avoid the toxic effects of heavy metals, plants have developed mechanisms to deactivate and scavenge metal ions that penetrate into the cytosol. Phytochelatins (PCs), which possess the structure $(\text{NH}_3)^-\gamma\text{-Glu-Cys-}\gamma\text{-Glu-Cys-}\gamma\text{-Glu-Cys-Gly-COO}^-_{(n)}$ (Grill et al. 1985), act as chelators of Cd^{2+} and other heavy metals in such mechanisms. The length of the PC chain varies between $n=2$ to $n=11$ ($\gamma\text{-Glu-Cys}$) $_n\text{-Gly}$ (Gekeler et al. 1989). Ions of cadmium have been reported to bind the most strongly to phytochelatins *in vivo*. The Cd-PC complex adopts the structure shown in Fig. 4.4. The dots in Fig. 4.4 represent uncoordinated carboxyl groups. They influence the transport of the PCs through the tonoplast (Strasdeit et al. 1991). The synthesis of these heavy metal-phytochelatin complexes is a vital metabolic process in higher plants. The resulting depletion of glutathione in the cytosol is compensated for by the induction of sulfur assimilation and glutathione biosynthesis (Rüegsegger et al. 1990; Rüegsegger and Brunold 1992).

The synthesis of phytochelatins is catalysed by the enzyme γ -glutamylcysteine dipeptidyl transpeptidase, also known as phytochelatin synthase (EC 2.3.2.15). This enzyme catalyses the initial reaction and couples $\gamma\text{-Glu-Cys-Gly}$ to $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, which then results in $(\gamma\text{-Glu-Cys})_{n-1}\text{-Gly-Gly}$. This reaction is strongly induced by heavy metals. A direct correlation between the properties of the respective metal and the efficacy at inducing the action the PC enzyme has been reported,

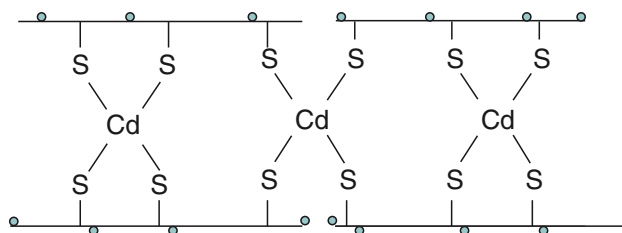


Fig. 4.4 Structure of the $[\text{Cd}_3(\text{Pc}_n)]$ complex. The blue dots represent uncoordinated carboxyl groups. The positions of these groups depend strongly on the negative electric charge (Strasdeit et al. 1991)

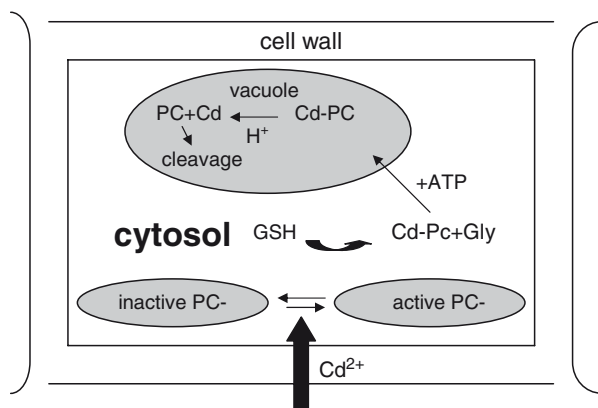


Fig. 4.5 Cd²⁺ ions penetrate into the cell and activate the synthesis of phytochelatin. Phytochelatin is synthesised from glutathione. The Cd-PC complex is actively transported into the vacuole. The metal is stored there in a different form (i.e. complexed with organic acids), while the phytochelatin is degraded and recycled to the cytosol (Zenk 1996). Hyperaccumulation in vacuoles may be the basis for a practical application, i.e. phytomining (Baker and Brooks, 1989)

according to the following sequence: Cd > Ag > Pb > Cu > Hg > Zn > Sn > Au > As > In > Tl > Ge > Bi > Ga.

Besides detoxification, phytochelatin also contribute to the regulation of metal occurrence and homeostasis in plant cells (Fig. 4.5). Metal ions like Cu and Zn play distinct roles in catalytic proteins or structural elements. Hence, phytochelatin play a double role: on the one hand they complex, detoxify and store metal ions in the vacuole, and on the other they guide essential metals to newly synthesised apoenzymes and facilitate contact (Thumann et al. 1991).

4.7.4.8 Carotenoids

Carotenoids are members of the C₄₀ isoprenoid and tetraterpene groups of plant metabolites. They are mainly localised in plastids with and without photosynthetic functions. In chloroplasts, carotenoids act as light-scavenging accessory pigments, as well as inactivators of reactive oxygen species, stimulating energy dissipation within light-harvesting proteins by nonphotochemical quenching. Hence, carotenoids contribute to detoxification by reacting with the products of lipid hydroperoxidation to prevent chain reactions (Burton and Ingold 1984), reacting with triplet or excited chlorophyll molecules to inhibit the formation of singlet oxygen, and via the release of excess energy as heat through the xanthophyll cycle (Mathis and Kleo 1973; McKersie 1996). The xanthophyll cycle involves the enzymatic removal of epoxy groups from xanthophylls to create so-called de-epoxidised xanthophylls.

4.7.5 *Other Detoxification Mechanisms in Plants*

When organic contaminants are present at a given site along with heavy metals, other problems arise. Current research has identified the need to study other detoxification mechanisms too (Lyubenova et al. 2007). Shimabukuro was first to describe a three-phase cascade responsible for the metabolism of herbicides and organic xenobiotics that involved (1) activation of the xenobiotics, (2) detoxification and (3) excretion, a process analogous to animal hepatic metabolism (Schröder and Collins 2002). Activation may be catalysed by esterases, P450 monooxygenases (in membrane fractions of cells) and peroxidases (cytosolic). The second phase is detoxification *sensu stricto*, and is catalysed by glutathione and glycosyl transferases. It makes the compound under consideration less toxic through substitution and conjugation via reactions with sugars, amino acids and glutathione, which can be transferred to the activated xenobiotic according to the structure of the molecule and its active site (Schröder and Collins 2002). Available hydroxyl groups, amine groups, thiol functional groups and carboxylic acid functions on a given molecule usually trigger glycosyl transfer (Schröder and Collins 2002). When conjugated double bonds, halogen or nitro functions are present in a molecule, glutathione conjugation catalysed by glutathione *S*-transferases is the predominant reaction (Coleman et al. 1997). In this phase, reactions such as cleavage, rearrangement and secondary conjugation are also performed. The last phase can be split in two; the first part of this phase involves membrane transport and storage in the vacuole, whereas the second part includes final cell wall binding reactions or excretion (Theodolou 2000; Schröder 2006).

4.7.5.1 **Glutathione *S*-Transferases**

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) were first described in animals, where they catalyse the conjugation of pharmaceuticals with the tripeptide glutathione (Booth et al. 1961). Years later, they were also described in plants, where they were found to conjugate atrazine with GSH in maize (Frear and Swanson 1970). This process of conjugation leads to the cleavage of electrophilic groups from xenobiotics, and is considered to be true detoxification. Plant GSTs are found in the cytosol and in membranes (microsomal GST). Both groups include homodimeric (they have two identical subunits) or heterodimeric (different subunits) enzymes with subunit sizes ranging from 23 to 30 kDa (Schröder 2001).

GST holoenzymes possess two independent catalytic domains to create conjugates from glutathione and electrophilic substances. Each of these domains consists of a G site for GSH binding and an H site for the binding of the xenobiotic (herbicide).

The cytosolic GSTs are classified into six classes: phi (F), tau (U), theta (T), zeta (Z), lambda (L) and the dehydroascorbate reductase (DHAR) (Edwards and Dixon 2005). The phi and tau GSTs represent the largest groups, and they are plant specific.

The other classes are also present in the animal kingdom. Each of the GST classes has been found to play an important role:

- Phi: stress response, metabolism of plant hormones, drought stress
- Tau: biotic and abiotic stress, herbicide detoxification via GSH addition
- Theta: peroxidase activity
- Zeta: isomerase activity
- Lambda: reductase activity
- DHAR: ascorbate reduction.

The soluble GSTFs, GSTTs, GSTUs and GSTZs are polypeptides around 25 kDa in size that associate with other subunits of each class and form homodimers (Schröder and Collins 2002). Marrs (1996) mentions that a group of type III GSTs (due to inconsistencies in the nomenclature of animal and plant GSTs, type III GSTs were later identified as tau GSTs) regulate heat, heavy metal and pathogen stress. A list of the herbicides conjugated by plant GST is presented by Schröder (Schröder and Collins 2002), while a list of herbicide and heavy metals is presented by Lyubenova et al. (2009). Like glutathione reductase, GST enzymes are also inhibited *in vitro* in the presence of heavy metals, albeit at higher concentrations (Lyubenova et al. 2007). It is not yet clear whether this holds true for all isoforms or only for distinct GST classes, but it is an important finding in the context of mixed pollution.

4.7.5.2 Mixed Pollution

Contamination is an important threat to European soils, aside from loss of fertility, deterioration of the soil structure and increases in pathogens. Especially considering demographic and ecological trends, soil contamination impacts on water and food production, exerting heavy effects on ecosystems and human life. For EU countries, the estimated number of potentially contaminated sites is almost three million (EEA 2007). Around 80,000 sites have been cleaned up in the last 30 years, but treatment is urgently needed for roughly 250,000 sites in EEA member countries. Mineral oil (38%), heavy metals (37%) and PAHs (13%) are the most common soil contaminants. Contaminated soils are frequently treated as waste to be disposed of rather than as a valuable resource to be cleaned and reused. The problem is that remediation must be effective at both reducing or controlling health or environmental risks associated with the particular mixture of pollutants and also preserving and improving soil quality and function, and all at an affordable cost. Phytoremediation provides a variety of remediation techniques associated with plants and microbes that involve treatment strategies for contaminant degradation, accumulation or immobilisation. The use of plants offers efficient and environmentally friendly solutions for cleaning up contaminated sites and water, as well as food safety and the development of renewable energy sources, all of which will ultimately contribute to sustainable land use (Vangronsveld et al. 2000).

4.8 Conclusion

Most known heavy metal contaminations have been generated by human activities, and the relative heavy metal toxicity depends on its availability, which is determined by the properties of the soil and the plant species of interest. Once taken up by the plant, heavy metals interact with different cell components and disturb normal metabolic processes. The antioxidative system protects cells from immediate damage, but if exposure persists or critical doses are exceeded, the antioxidant store will be used up and so stress will occur. Antioxidative stress leads to the induction of numerous enzymes, but may also result in suppression. The latter effect is crucial under the conditions of mixed pollution, when plants must fight chemicals with various modes of action. Here, the occurrence of certain heavy metals can be detrimental to the detoxification of organic xenobiotics that would easily be detoxified if they were present alone.

Recent phytotreatments have used plants without characterising them properly beforehand. Selecting species that grew on certain local soils or in given regions was taken to be a sufficient selection parameter. However, species-specific differences seem to exist between the regulation of primary defence enzymes like SOD, catalase and peroxidases, and other species prefer to induce glutathione-dependent enzymes. As long as the pollutant mix encountered is simple and dominated by heavy metals, the defences of the plant may be sufficient. When the pollution contains heavy metals and organic xenobiotics at the same time, part of the plant's detoxification capacity – the utilisation of glutathione-conjugating reactions at the very least – is withdrawn from the heavy metal front to serve other purposes. In fact, glutathione *S*-transferases show strong reactions in stressed plants or in the presence of heavy metals. We have described in this chapter how pollution with heavy metals will interfere with both plant oxidative stress defence and the ability of plants to conjugate organic xenobiotics. Despite species-dependent differences, general reactions seem to include oxidative stress and the induction of antioxidative enzymes. Several processes seem to depend on the direct binding of heavy metals to enzyme proteins, but effects on transcription are also observed. Xenobiotic metabolism is induced at high heavy metal concentrations, when plant stress is elevated. It is becoming clear that plants intended for the phytoremediation of complex pollution mixtures must be selected according to three major issues: uptake/accumulation capacity, antioxidative stress management, and their detoxification/binding properties in relation to both trace elements and the organic xenobiotics.

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