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Characterization of Plant Antioxidative System in Response to Abiotic Stresses: A Focus on Heavy Metal Toxicity

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1. Introduction

During their life span, plants can be subjected to a number of abiotic stresses, like drought, temperature (both high and low), radiation, salinity, soil pH, heavy metals, lack of essential nutrients, air pollutants, etc. When affected by one, or a combination of abiotic stresses, a response is induced by changes in the plant metabolism, growth and general development.

Reactive Oxygen Species (ROS) are a natural consequence of the aerobic metabolism, and plants have mechanisms to deal with them in normal conditions, controlling the formation and removal rates. Under stress conditions, cell homeostasis is disrupted and ROS production can increase a lot putting a heavy burden on the those antioxidative mechanisms, some of which are activated in order to eliminate the excess ROS (Mittler et al., 2004).

Trace element contamination cause abiotic stress in plants and it can affect crop production and quality. Certain metals, like copper, are essential for plants, but at high concentrations (depending on plant species) can be considered toxic. Other elements like cadmium and arsenic (a metalloid), while not essential elements for plants, are widespread pollutants that are present in nature due to both natural and manmade activities.

Plants have developed different strategies to cope with these stresses. Some use an avoidance strategy to reduce trace element assimilation while others use internal defence mechanisms to cope with the increasing levels of the toxic species. Phytotoxic amounts of trace elements are known to affect several physiological processes and can cause oxidative stress. Plants have developed several trace element defence mechanisms, that allow them to grow despite the presence of variable concentrations of trace elements, but the threshold concentrations as well as the different response mechanisms strongly depend on plant species and on the type of metal. Metal toxicity can cause a redox imbalance and induce the increase of ROS concentration, activating the antioxidant defence mechanisms of plants (Sharma & Dietz, 2009). These mechanisms are very dependent on the metal and the plant but usually include the involvement of the ascorbate-glutathione cycle enzymes which is a major antioxidative defence mechanism, and of other antioxidant enzymes like catalase, peroxidases, and superoxide dismutase. Other non-enzymatic substances with reported antioxidant properties can also be involved in plant defence mechanisms, like ascorbate, glutathione, alkaloids, phenolic compounds, non-proteic amino-acids and carotenoids.

2. Oxidative stress and ROS production

ROS are produced by all aerobic organisms and are usually kept in balance by the antioxidative mechanisms that exist in all living beings. Because ROS have an important signalling role in plants (Foyer & Noctor, 2003; Vranova et al., 2002), their concentration must be carefully controlled through adequate pathways (Mittler, 2002). ROS can be formed during normal aerobic metabolic processes like photosynthesis and respiration and thus, the majority of ROS are produced in the mitochondria, chloroplast, peroxisomes, plasma membrane and apoplast (Ahmad et al., 2008; Moller, 2001). Other sources of ROS production are NADPH oxidases, amine oxidases and cell-wall peroxidases (Mittler, 2002).

Under certain stress conditions (like excess light, cold, heat, drought, heavy metals etc.) the production of ROS can exceed the capacity of the plant's defence mechanisms, an imbalance in intracellular ROS content is established and this results in oxidative stress (Gill & Tuteja, 2010). Thus, oxidative stress can be defined as the physiological changes resulting from the formation of excess quantities of reactive oxygen species (ROS) (Vangronsveld & Clijsters, 1994). This increase in ROS levels induces a metabolic response in the plant in order to eliminate them. This metabolic response is highly dependent on the plant species, plant growing state and the type and duration of the stress.

Heavy metals[1] are natural elements that are present at different concentrations throughout nature, but whose levels can increase and overtake the toxicity threshold of living beings due to both natural and anthropogenic causes (Sánchez, 2008). As plants must adapt (or die) to the conditions where they grow, the presence of heavy metals can induce oxidative stress and the activation of several defence factors in the plants (Prasad, 2004). It is important to understand how some plants can cope with high concentration of metals in order to produce crops able to grow on contaminated soils (Schröder et al., 2008), to help in environmental cleanup via phytoremediation (Adriano et al., 2004) and to breed plants with higher contents of essential nutrients (Zhao & McGrath, 2009).

2.1 Types of ROS

Molecular oxygen (O_2) is in itself a bi-radical[2], as it has two unpaired electrons that have parallel spins (Halliwell, 2006). The ground state of the oxygen molecule is the triplet oxygen ($^3O_2$ or ·O-O·), because this is an energetically more favourable state. Due to this electron configuration, it doesn't react easily with organic molecules that have paired electrons with opposite spins. Thus, in order for oxygen to react, it must be activated (Garg & Manchanda, 2009). If the oxygen molecule in its ground state absorbs sufficient energy, the spin of one of the unpaired electrons can be reversed forming singlet oxygen, that can readily react with organic molecules. This can happen during photosynthesis when an excess of light energy cannot be readily dissipated by the photosynthetic machinery (Foyer et al., 1994).

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[1] Although the term "heavy metal" can be misleading because it includes a whole range of substances some of which do not conform to the more usual chemical definitions of the term, we use it in this text in its environmental context, where it includes metals that are a cause of pollution concern. Other authors sometimes use the expression "trace elements" in this context, although this definition also has problems on its own.

[2] Radicals are usually highly unstable, and thus very reactive, species that have one or more unpaired electrons.

Another form of activation is by partial reduction adding one, two or three electrons giving rise to the superoxide radical, hydrogen peroxide and hydroxyl radical, respectively (Mittler, 2002). The complete reduction of oxygen (adding four electrons) results in water, which is the normal reduction of oxygen that occurs in the mitochondrial electron transport chain, catalyzed by cytochrome oxidase. As such, this type of activation can occur in metabolic pathways that involve an electron transport chain and can thus occur in several cell locations (Alscher et al., 2002).

In Table 1 we present the most important types of ROS. They can be free radicals or non-radicals.

Name	Structure	Type	Relative Reactivity
Singlet oxygen	$^1O_2$ (O-O:)	Radical	High
Superoxide	$O_2^{\bullet -}$ (·O-O:)	Radical	Medium
Hydrogen peroxide	H_2O_2 (H:O-O:H)	Non-radical	Low
Hydroxyl radical	$HO^{\bullet}$ (H:O·)	Radical	Very high

Table 1. Most important types of ROS

Singlet oxygen is mainly produced in the chloroplasts at photosystem II (Asada, 2006) but may also result from lipoxygenase activity and is a highly reactive species that can last for nearly 4 µs in water (Foyer et al., 1994). $^1O_2$ reactivity has as preferred target the conjugated double bonds present on polyunsaturated fatty acids (PUFAs) leaving a specific footprint in the cell (Moller et al. 2007) that can be followed by the detection of several aldehydes like malondialdehyde (MDA) formed by PUFA peroxidation.

The superoxide radical is mainly produced both in the chloroplasts (photosystems I and II) and mitochondria as sub products and in peroxisomes (del Rio et al., 2006; Moller et al., 2007; Rhoads et al., 2006), has a half-life of 2-4 µs and cannot cross phospholipid membranes (Garg & Manchanda, 2009) and so it is important that the cell has adequate *in situ* mechanism to scavenge this ROS. Superoxide dismutase can catalyse the conversion of this species into hydrogen peroxide. Superoxide radical can also be produced by NADPH oxidase in the plasma membrane (Moller et al. 2007).

Hydrogen peroxide is mainly produced in peroxisomes (del Rio et al., 2006) and also in mitochondria (Rhoads et al., 2006), and also results from the dismutation of superoxide. It is not a radical and can easily cross membranes diffusing across the cell and has a half-life of around 1 ms (Garg & Manchanda, 2009).

The hydroxyl radical, the most reactive of the species listed in Table 1, can be formed from hydrogen peroxide via Fenton and Fenton-like reactions (catalyzed by iron or other transition metals) and, unlike the previous two ROS mentioned, there are no known enzymatic systems able to degrade it (Freinbichler et al., 2011).

Although the superoxide radical and hydrogen peroxide are not as reactive as other species they are produced in large amounts in the cell and can initiate other reactions that lead to more dangerous species (Noctor & Foyer, 1998). In fact, superoxide radical can be converted by specific enzymes into hydrogen peroxide, and this can also be a problem as it cause the occurrence of Fenton reactions (Moller et al., 2007).

Heavy metals are known to induce oxidative stress increasing the ROS concentration. As an example, in figure 1 we present experimental results of work performed by the authors, of the effect of Cd and Cu in hydrogen peroxide content in roots of tobacco plants. As can be seen, there is a good correlation between Cu levels and H_2O_2 content, but the effect of Cd showed only a small non-significant increase. These metals, an essential and a non-essential, do seem to provoke different responses in the plant. The increase in hydrogen peroxide levels with metals is a frequently reported stress indicator (Khatun et al., 2008; Mobin & Khan, 2007).

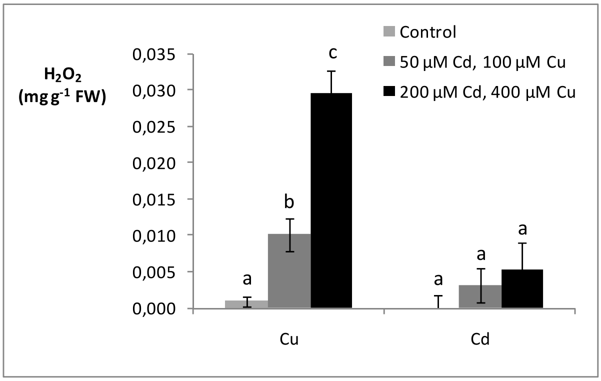


Fig. 1. Hydrogen peroxide content in *Nicotiana tabacum* L. plants grown in nutrient solution, with excess copper and cadmium.

2.2 ROS effect in different cellular components (lipids, DNA, proteins, carbohydrates)

When cell homeostasis is affected by a given stress, ROS production increases to the point where it can damage cellular components and ultimately lead to cell death. ROS can affect lipids, proteins, carbohydrates and DNA and the detailed mechanisms are well detailed in Moller et al. (2007).

Unsaturated fatty acids from lipid membranes are particularly susceptible to ROS oxidation, increasing membrane leakage. Lipid peroxidation occurs through a series of chain reactions that start when a ROS like the hydroxyl radical removes one hydrogen from a carbon from the fatty acid molecule (mainly at the unsaturation). An oxygen can then easily bond to that location forming a lipid peroxyl radical, that can continue and propagate the same kind of reactions (Gill & Tuteja, 2010).

Proteins can also suffer oxidation by ROS, causing certain enzymes to lose its catalytic function. One of the more susceptible targets in proteins are thiol groups the oxidation of which can lead to protein denaturation and loss of functional conformation (Moller et al., 2007). Also, protein oxidation leads to the production of carbonyl groups and to increased rate of proteolysis as the damaged proteins are targeted by proteolytic enzymes (Palma et al., 2002).

Changes in protein content and in protein profile can be found as a consequence of the stress induced by toxic metals. In figure 2, we show the effect of excess copper (50 µM) in the protein content of *Lupinus luteus* leaves, evidencing a significantly higher protein content after 11 days of excess copper, compared to control. In this work, lupin plants were grown in nutrient solution with the indicated Cu concentration. This protein increase could represent the positive balance from the inactivation of some proteins whereas other proteins are formed in relation to the defense response.

In figure 3, we present the protein profile of *Lupinus luteus* leaves after 11 days of exposition to different Cu concentrations. The protein profile showed some changes that can be related to the Cu concentration in nutrient solution. In fact, we found that some protein bands showed higher intensities (56.5 and 17.7 Da) while new protein bands were detected that were not present in control samples (28.5 and 14 Da). These new proteins could be related to the Cu defence mechanism of these plants.

DNA can also be attacked by ROS damaging nucleotide bases, causing mutations and genetic defects (Tuteja et al., 2001).

Both free carbohydrates and wall polysaccharides can react easily with the hydroxyl radical, and this can also be a defence mechanism if the radical reacts with these carbohydrates before damaging more biologically important molecules (Moller et al., 2007).

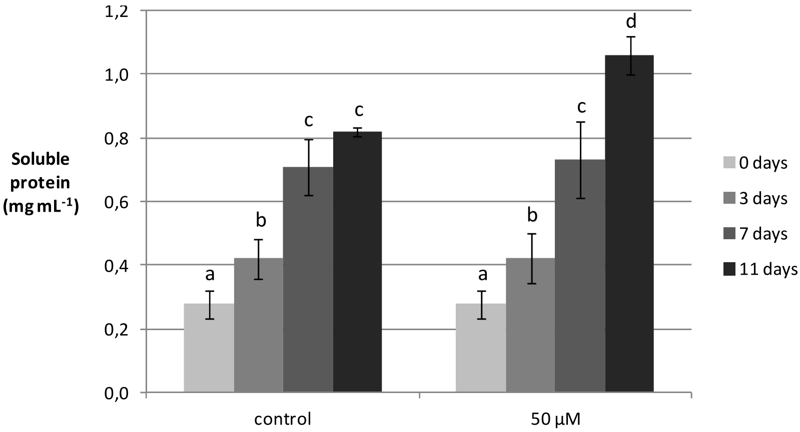


Fig. 2. Protein content of *Lupinus luteus* leaves grown in nutrient solution with 0.1 µM of Cu (control) and excess copper (50 µM) for up to 11 days.

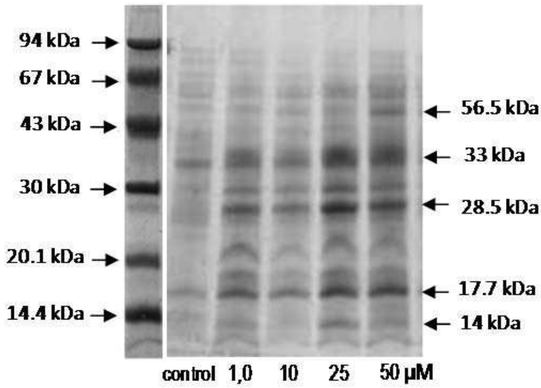


Fig. 3. SDS-PAGE protein profile of *Lupinus luteus* leaves after 11 days at different concentrations of copper. Electrophoresis was performed in a 12 % polyacrylamide gel stained with Commassie blue.

3. Antioxidant defence mechanisms

3.1 Enzymatic mechanisms

As was said before, enzymatic mechanisms and enzymes involved in specific metabolic pathways are one of the major antioxidative defence strategy of plant defence against excess ROS.

Superoxide dismutase (SOD, EC 1.15.1.1), catalyses the dismutation of superoxide molecules into hydrogen peroxide and oxygen (Alscher et al., 2002).

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \Rightarrow H_2O_2 + O_2$$

SOD has a metal cofactor and depending on the metal can be classified in three different groups, localized in different cell compartments: FeSOD (chloroplasts), MnSOD (mitochondria and peroxisomes), Cu/ZnSOD (chloroplast and cytosol). As SOD produces hydrogen peroxide that is subsequently converted to water by peroxidases and catalases, the activity of all these enzymes must be carefully balanced.

Catalase (CAT, EC 1.11.1.6) exists mainly in the peroxisomes and as during stress the number of these organelles increase, CAT can have an important role in H_2O_2 detoxification that can diffuse into the peroxisome from other cell locations where it is produced (Mittler, 2002). CAT catalyses the hydrogen peroxide breakdown to water:

$$H_2O_2 + H_2O_2 \Rightarrow 2H_2O + O_2$$

Peroxidases (EC 1.11.1) are a member of a large family of enzymes that are ubiquitous in the cell and have numerous roles in plant metabolism (Passardi et al., 2005), namely to remove hydrogen peroxide formed due to induced stress using different reductants. They have the general reaction:

$$H_2O_2 + R(OH)_2 \Rightarrow 2H_2O + RO_2$$

$R(OH)_2$ represents different electron donors: guaiacol peroxidase (GPOD, EC 1.11.1.7) uses mainly phenolic donors, ascorbate peroxidase (APX, EC 1.11.1.11) uses ascorbic acid and glutathione peroxidase (GPX, EC 1.11.1.9) uses glutathione.

Besides their role as a scavenger of hydrogen peroxide, cell-wall peroxidases are also involved in ROS formation, both as a defence against biotic stresses and as a signalling process against several stresses, leading to the activation of other defence mechanisms (Mika et al., 2004).

APX has a much higher affinity to H_2O_2 than CAT suggesting that they have different roles in the scavenging of this ROS, with APX being responsible for maintaining the low levels of hydrogen peroxide while CAT is responsible for the removal of its excess (Mittler, 2002).

The water-water cycle (Figure 4) occurs in chloroplasts and is a fundamental mechanism to avoid photooxidative damage (Rizhsky et al., 2003), using SOD and APX to scavenge the superoxide radical and hydrogen peroxide in the location where they are produced avoiding the deleterious effects of their reactivity with other cellular components (Asada, 1999; Shigeoka et al., 2002).

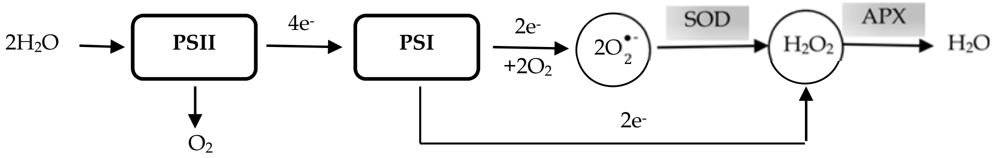


Fig. 4. The water-water cycle. PSI and PSII - Photosystems I and II, SOD - Superoxide dismutase, APX - ascorbate peroxidase.

The ascorbate-glutathione cycle (Figure 5) is an important group of reactions involved in ROS detoxification, as it converts hydrogen peroxide (formed as a consequence of an induced stress or via SOD action) and occurs in several cell compartments, like chloroplasts, cytosol, mitochondria, peroxisomes and apoplast. It uses APX and GPX as well as other enzymes like monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) that have a role in the regeneration of the reduced form of ascorbate and glutathione reductase (GR, EC 1.6.4.2), important to maintain the pool of reduced glutathione.

Other enzymes like heme oxygenase (HO, EC 1.14.99.3), that catalyzes the stereo specific cleavage of heme to biliverdin (Balestrasse et al., 2005) have been reported to have a role in plant defence mechanisms against oxidative stress.

3.2 Enzymatic responses to heavy metal stress

There has been extensive studies on the activities of the enzymes involved in ROS defence on plants subjected to heavy metal stress (Sharma & Dietz, 2009). Although different metals can cause oxidative stress, their mode of action is different. For example while copper, an essential element toxic at high concentrations, is involved in redox reactions, cadmium, which has no known biological function, cannot. However, they both can induce oxidative stress, through different mechanisms (Cuypers et al., 2010).

Of all the main enzymes involved in oxidative stress defence, like SOD, APX, CAT and peroxidases, published reports both describe an increase in its activity and a decrease (or no change), depending on plant species, plant organ, type of metal, duration of the treatment, plant age, and growing media (Gratão et al., 2005).

In table 2 we list some representative publications of this kind of studies, regarding determinations made at a single time of growth (time series studies show also changes along the time, further complicating the analysis). As can be seen, there is a huge variation between the behaviour of the enzymes involved in oxidative stress. In some situations the activity increases for lower concentrations of the metal and then decreases as the defence mechanism breaks down due to excessive concentration. This also shows that different components of the antioxidative defence system described above are active under different conditions. The increase in activity in a given enzyme can be a signal that that enzyme has been activated or its expression upregulated. On the other hand sometimes the effect of the metal can be so drastic that enzymes structures are being affected with a consequent decrease in activity. Several enzymes have metal cofactors so there could be a link between these enzymes expression and metal availability (Cohu & Pilon, 2007).

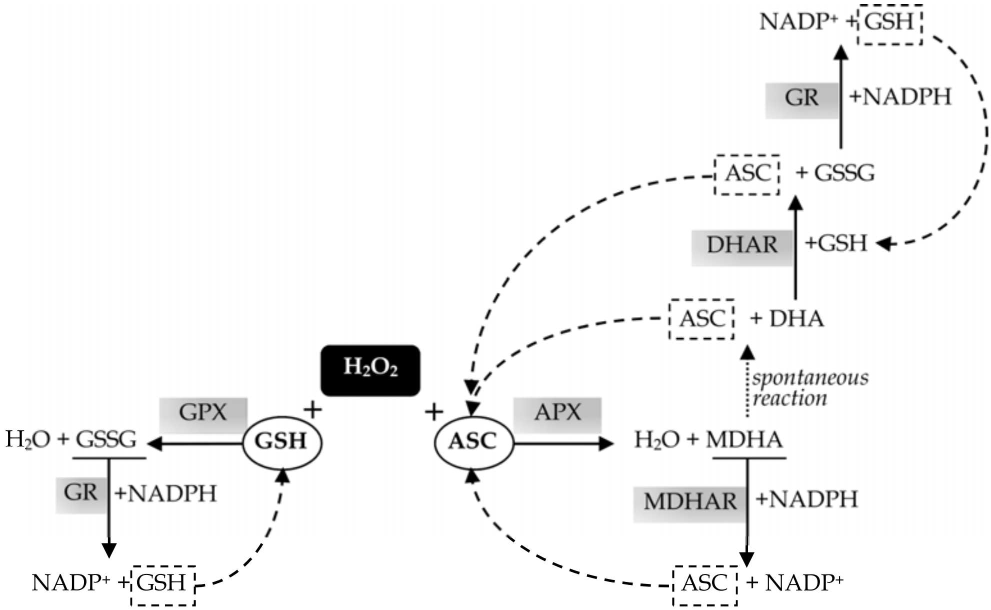


Fig. 5. The ascorbate-glutathione cycle. Non-enzymatic compounds: ASC - ascorbate, MDHA - monodehydroascorbate, DHA - dehydroascorbate, GSH - glutathione (reduced), GSSG - glutathione (oxidized). Enzymes (grey box): APX - ascorbate peroxidase, GPX - glutathione peroxidase, GR - glutathione reductase, MDHAR - monodehydroascorbate reductase, DHAR - dehydroascorbate reductase.

Consequently, enzymatic response can be complex to analyse. For example, in figure 6A we present the activity of guaiacol peroxidase in tomato roots growing for 3 days with 50 µM Cu in nutrient solution. As can be seen by the relative activity, no significant changes in POD activity were detected compared to control. However, the isoperoxidase profile (figure 6B) showed the appearance of new isoforms in tomato roots (C, D), while other isoforms showed less intensity (A, B). These results indicate that although enzymatic activity can be similar in control and stressed plants it is possible that some isoforms can be activated in response to excess copper.

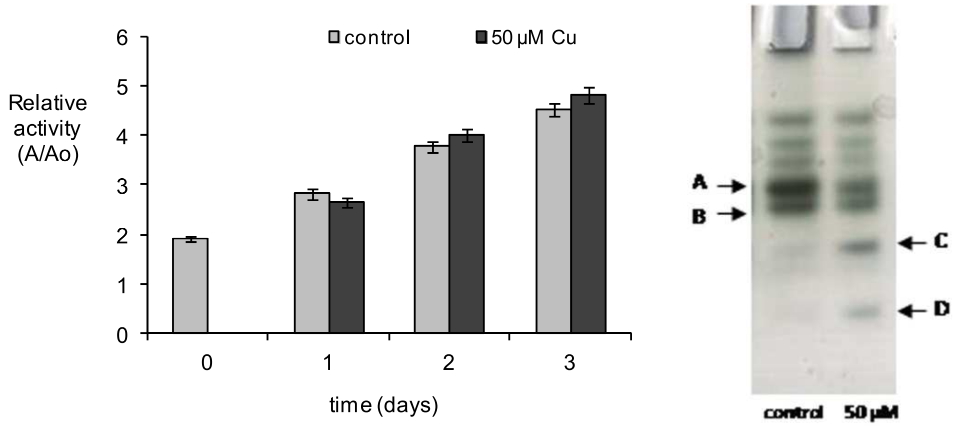
Polyphenol oxidase (PPO, EC 1.10.3.1) is an oxidoreductase that catalyzes the oxidation of phenols to quinones and its activity has been shown to increase under heavy metal stress and has thus been associated to some form of defence mechanism (Ali et al., 2006; L. L. Martins & Mourato, 2006). Kováčik et al. (2009) observed an increase in root PPO activity with Cu and Cd and concluded that the formation of polymerized phenols could be used to complex free metal ions. On the other hand PPO has also been associated to a catalase-like activity (Gerdemann et al., 2001), and could thus have a role in direct hydrogen peroxide removal.

Peroxiredoxins (PRX, EC 1.11.1.15) are ubiquitous antioxidant enzymes that participate in cellular redox homeostasis and reduce hydrogen peroxide to water. PRX levels have been shown to increase under several abiotic stresses suggesting a role in the defence mechanisms (Barranco-Medina et al., 2007).

Plant Species	Metal	Concentration	Organ	Enzyme	References
Arabidopsis thaliana	Cd	5, 10, 20 µM	leaves	APX ↑↓, GPOD ↑↓, SOD =, CAT =, GR ↓	(Smeets et al., 2008)
Brassica juncea	Cd	10, 25, 50 µM	leaves	APX ↓, GPOD ↓, CAT ↑, GR =	(Nouairi et al., 2009)
"	Cd	10, 30, 50, 100 µM	leaves	APX ↑↓, CAT ↓, GR ↑↓, GPX ↓, MDHAR ↑↓, DHAR ↑↓	(Markovska et al., 2009)
"	As	5, 25 µM	leaves	APX ↑, SOD ↑, CAT =	(Khan et al., 2009)
"	Cd	5, 15, 35 mg.kg^{-1}	leaves	GPOD ↑, APX ↑↓, CAT ↑↓	(Pinto et al., 2009)
Brassica napus	Cd	10, 25, 50 µM	leaves	APX ↓, GPOD ↑, CAT ↓, GR ↑↓	(Nouairi et al., 2009)
Cannabis sativa	Cd	25, 50, 100 mg.kg^{-1}	seedlings	GPOD ↑, SOD ↑, CAT =	(Shi et al., 2009)
Daucus carota	Cu	100, 200, 400, 800 mg.kg^{-1}	leaves	APX ↓↑, SOD ↑↓, CAT ↑↓	(Ke et al., 2007)
Elsholtzia splendens	Cu	25, 50, 100, 500 µM	leaves	APX ↑, GPOD ↓↑,SOD ↑, CAT ↑	(Peng et al., 2006)
Matricaria chamomilla	Cd	3, 60, 120 µM	leaves, roots	CAT ↑, GR ↑	(Kovácik & Backor, 2008)
"	Cu	3, 60, 120 µM	leaves, roots	CAT ↑, GR ↑	(Kovácik & Backor, 2008)
Nicotiana tabacum	Cd	10, 25, 50, 100 µM	young leaves old leaves	GPOD ↑, SOD ↓ GPOD ↑, SOD ↑	(Martins et al., 2011)
"	Cd	5, 15, 35 mg.kg^{-1}	leaves	GPOD =, APX ↓↑, CAT ↑	(Pinto et al., 2009)
Solanum nigrum	Cd	5, 15, 35 mg.kg^{-1}	leaves	GPOD ↑, APX ↑, CAT ↑	(Pinto et al., 2009)
Typha angustifolia	Cd	1 mM	leaves	GPOD ↑, APX =, GPX =, SOD ↑, CAT =	(Bah et al., 2011)
"	Cr	2 mM	leaves	GPOD ↑, APX =, GPX =, SOD ↑, CAT =	(Bah et al., 2011)
"	Pb	1 mM	leaves	GPOD ↑, APX =, GPX =, SOD =, CAT =	(Bah et al., 2011)

Plant Species	Metal	Concentration	Organ	Enzyme	References
Withania somnifera	Cu	10, 25, 50, 100, 200 µM	leaves	APX ↑↓, GPOD ↑, SOD ↓, CAT ↓, GR ↓, MDHAR ↑, DHAR ↓↑	(Khatun et al., 2008)
Zea mays	Cd	300, 600, 900 µM	leaves	APX ↑↓, GPOD ↑↓, SOD ↑, GR ↑↓	(Ekmekci et al., 2008)

Table 2. Changes in enzyme activities for several plants and metals. The symbols after the enzymes indicate if its activity increased (↑), decreased (↓), remained the same (=) or increased for lower concentrations and decreased for higher concentrations or vice-versa(↑↓ or ↓↑).



A B

Fig. 6. Guaiacol peroxidase activity of tomato roots for 3 days of 50 µM Cu in nutrient solution (A) and the correspondent isoperoxidase profile for plants collected after 3 days (B). Cu concentration in control plants was 0.1 µM.

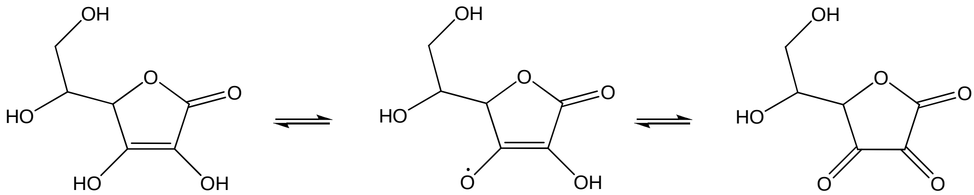
3.3 Non enzymatic mechanisms

Besides the enzymatic mechanisms described in the two previous chapters, there is a whole range of other substances that have been reported to be involved in antioxidative defence in plants. Some are well known and have been extensively studied (like the role of ascorbate and glutathione) while others are thought to be part of defence mechanisms but its role remains to be fully understood. While many studies report the increase in the concentration of some substance in relation to the induction of a stress, this type of correlation is not, by itself, conclusive enough to ascertain the effect of that substance in the plant metabolism.

Of course, when the levels of the compounds described in this chapter increase, most of the enzymes involved in the respective biosynthesis are also induced.

Several organic molecules have been reported to be able to form complexes with heavy metals, like phytochelatins (see section 3.4), organic acids and amino acids like proline. But exactly what kind of molecules complex the metals seem to be highly dependent not only on metal type and plant species but also on plant organ and compartment, as different complexes can be formed in the cytoplasm, the xylem and phloem, for example (Sharma & Dietz, 2006).

Ascorbate and glutathione are antioxidants that exist in relatively high concentration in cell compartments (Potters et al., 2002), and are involved in the ascorbate-glutathione cycle as described above being also a substrate for APX an enzyme important in H_2O_2 removal. Ascorbate (ASC) is an electron donor that can be oxidized to the radical monodehydroascorbate (MDHA) and this compound can then form dehydroascorbate (DHA) (Figure 7):

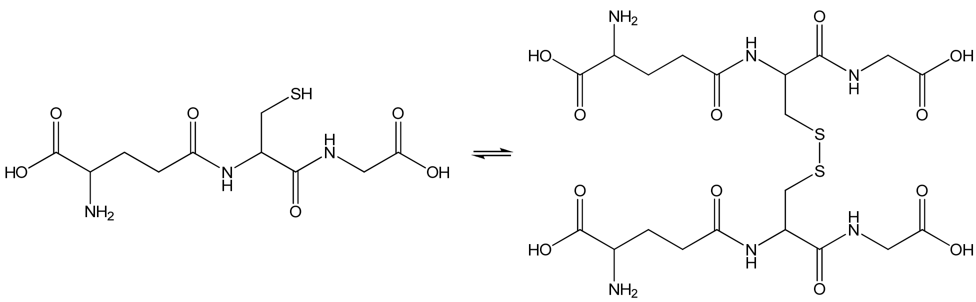


A B C

Fig. 7. Structure of ascorbate (A), monodehydroascorbate (B) and dehydroascorbate (C)

Besides its role as an enzyme substrate, ascorbate also reacts directly with singlet oxygen and superoxide and is important in the regeneration of α-tocopherol and certain carotenoids (Potters et al., 2002).

Glutathione (Figure 8) is a tripeptide (containing glutamate, cysteine and glycine) that can exist in two predominant forms: the reduced form (usually represented by GSH) and the oxidized form (usually represented by GSSG) (Noctor & Foyer, 1998). It is involved in the sulphur metabolism and in defence reactions against oxidative stress (Potters et al., 2002). It can also lead to the synthesis of phytochelatins that are important sequesters for certain heavy metals (Cobbett & Goldsbrough, 2002).



A B

Fig. 8. Structure of glutathione in its reduced (A) and oxidized forms (B)

Heat shock proteins (HSP) are proteins that not only showed increased expression under heat stress but are also molecular chaperones that protect other proteins from stress induced damage (Feder & Hofmann, 1999). HSP help proteins maintain or recover their native conformation, and remove potentially harmful polypeptides. HSP expression has been associated to high temperature, cold, drought, light, heavy metals, salt and ozone stresses (Timperio et al., 2008). Kochhar & Kochhar (2005) detected the induction of both high and low molecular weight HSPs in response to combined heat and cadmium stress. ROS also have a signaling role during a stress, in order to induce HSP production (Timperio et al., 2008).

Carotenoids have an important protective role during photosynthesis as these molecules can quench the excited states of chlorophyll in order to avoid the production of singlet oxygen. As a consequence, the carotenoid molecules become themselves excited but this is not a big problem as they don't have enough energy to form this ROS species (Taiz & Zeiger, 2002).

Terpenoids are a large class of organic compounds derived from the isoprene unit that could also have an antioxidative role in plants, although that is not yet clear (Grassmann et al., 2002).

Flavonoids are organic molecules with a structure similar to flavone (Figure 9), that have been shown to have a protective role against several stresses (Jaakola et al., 2004), both by themselves and in conjugation with peroxidases (Mika et al., 2004). Anthocyanins, a type of flavonoids (they are glucosides of anthocyanidins, Figure 9) present in the vacuoles, have an antioxidative capacity (Kahkonen & Heinonen, 2003) but its location in the cell prevents them to contact directly with ROS production sites, although its levels have been reported to increase under Cd stress (Mobin & Khan, 2007).

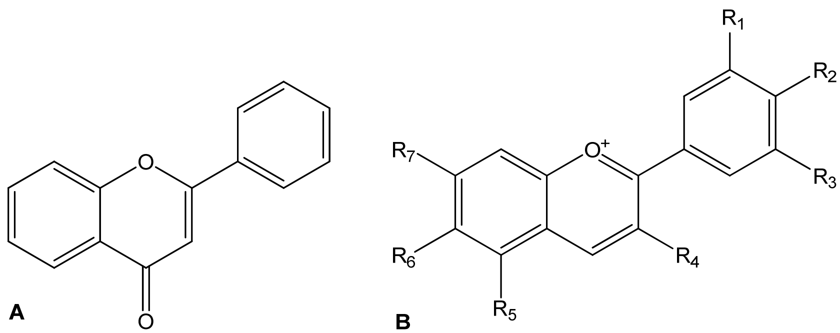


Fig. 9. Structure of flavone (A) and of anthocyanidins (B, where R_1 toR_7 can be H, OH or OCH_3 according to the exact type of anthocyanidin)

Thiols can play an important antioxidative role, protecting membrane lipids. Lipoic acid (Figure 10), both in its reduced and oxidized form, is reported to have antioxidative properties due to its direct scavenging of ROS. It is also able to chelate several metal ions that induces oxidative stress (Navari-Izzo et al., 2002) and thus can have an important role in cell protection.

Tocopherols are a class of compounds synthesized by photosynthetic organisms that have vitamin E activity. α-Tocopherol (Figure 11) is the most common form in leaves while γ-tocopherol is more common in roots (Abbasi et al., 2007). They have a role in ROS protection as they can quench singlet oxygen (Gill & Tuteja, 2010) and can act as an antioxidant and terminate chain reactions occurring during lipid peroxidation (Garg & Manchanda, 2009).

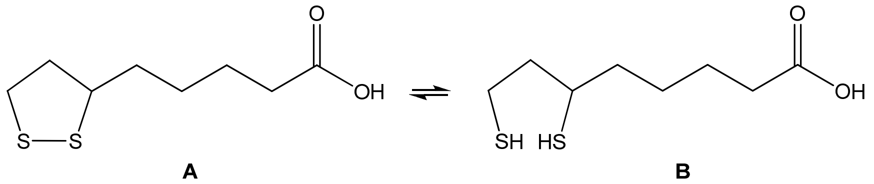


Fig. 10. Structure of lipoic acid in its oxidized (A) and reduced (B) forms.

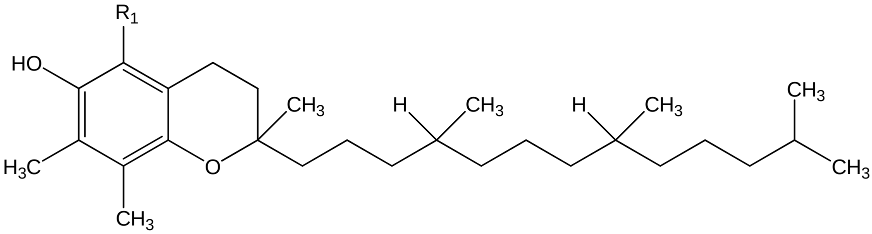


Fig. 11. Common structure of α-tocopherol (with R_1=CH_3) and of γ-tocopherol (with R_1=H).

Proline (an amino acid, shown in figure 12A), is a compatible solute that participate in the osmotic adjustment of plant cells being able to balance water stress. Proline has been reported to improve plant resistance to oxidative stress by scavenging ROS (namely by quenching singlet oxygen and hydroxyl radicals), increasing the activity of antioxidative enzymes and protecting them and maintaining redox homeostasis (Matysik et al., 2002), and they could also participate in signalling pathways that regulate stress related genes (Khedr et al., 2003). Although the concentration of proline has been shown to increase under heavy metal stress in certain plants (Martins et al., 2011), its exact role in heavy metal detoxification is unclear as, under certain conditions, it could be only an indirect response due to heavy-metal induced disturbances in plant water balance (Schat et al., 1997).

Histidine (figure 12B) is another amino acid that has been mainly linked to Ni hyperaccumulator plants (Sharma & Dietz, 2006).

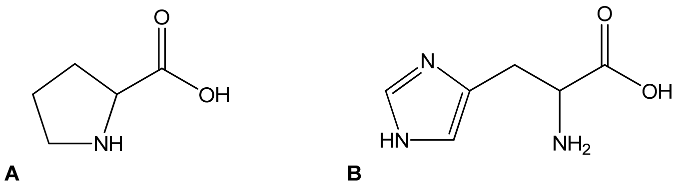


Fig. 12. Structure of proline (A) and histidine (B).

Glycine betaine (2-trimethylammonioacetate, figure 13A) is another compatible solute that is involved in plant resistance against abiotic stresses, namely salt stress (Banu et al., 2009). It helps not only in controlling water balance but can also help to maintain protein and membrane structure (F.-L. Zhang et al., 2008). Nicotianamine (figure 13B) is an amino acid that has a known role in heavy metal transport in plants (Stephan & Scholz, 1993), but recent findings have suggested an important role also in heavy metal tolerance namely in relation to hyper accumulating species (Sharma & Dietz, 2006). Mugineic acid (figure 13C) is a siderophore (that is, an iron-chelating compound) that promotes iron acquisition from the rhizosphere, but that may also participate in the distribution of other metals in the plant (Haydon & Cobbett, 2007). Meda et al. (2007) reports that phytosiderophores can alleviate Cd toxicity in maize.

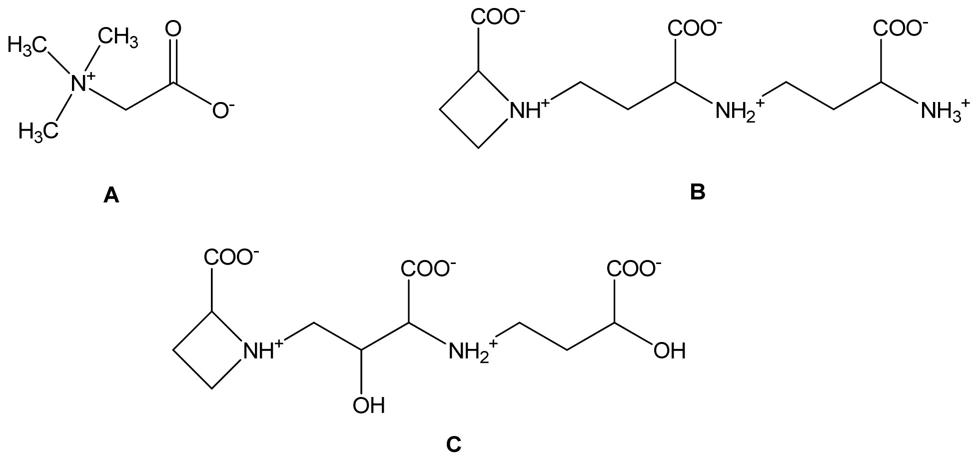


Fig. 13. Structure of glycine betaine (A), nicotianamine (B) and mugineic acid (C).

Polyamines are low molecular weight amines (figure 14) that have a role in plant growth and developmental processes (Kakkar & Sawhney, 2002). The concentration of some polyamines has been reported to increase under abiotic stress. Mascher (2002) showed that the concentration of putrescine, spermidine and spermine incresaed when red clover plants were subjected to As toxicity. However it is still not clear the exact role these compounds play in heavy metal defence, but a participation in the stabilization and protection of the membrane systems has been proposed (Sharma & Dietz, 2006).

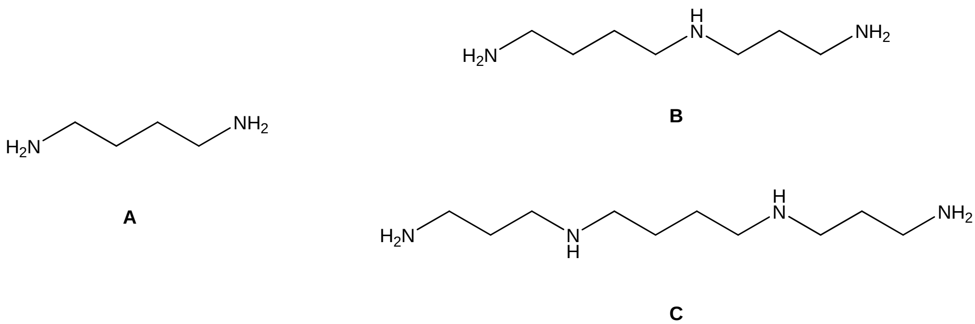
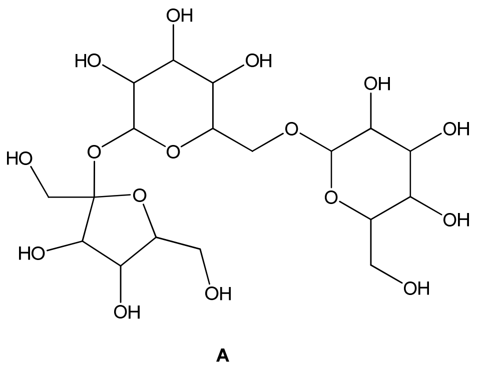


Fig. 14. Structure of several polyamines. A - putrescine (a di-amine); B - spermidine (a tri-amine); C - spermine (a tetra-amine)

Although soluble sugars have been linked to metabolic pathways that produce ROS, they can also have an important role in ROS scavenging mechanisms. Increased glucose levels can increase the production of NADPH (via the pentose-phosphate pathway), that is an important intermediate in the ascorbate-glutathione cycle (Couée et al., 2006) as NADPH is the primary electron donor that assures a intracellular reduction status. Both glucose and sucrose levels have been shown to increase in some plant species under certain abiotic stresses but it is not obvious that this happens due to a putative defence mechanism induction (Couée et al., 2006), although these sugars also participate in signalling mechanisms. Van den Ende and Valluru (2009) suggest that sucrose might have a protective role against stress due to its capacity to scavenge ROS. Other sugars like raffinose (Figure 15A) and fructans (which are fructose polymers) are also reported to have a protective role of membranes against several stresses, namely freezing and drought stress (Van den Ende & Valluru, 2009).

Trehalose (Figure 15B) is a non-reducing disaccharide that can also participate in the stabilization of membranes and protection of proteins under abiotic stresses (Luo et al., 2008). Although trehalose exists in numerous organisms (like bacteria, fungi and nematodes) it is not found widespread in plants, and when it is found is usually in very low concentrations (Wingler, 2002), but several studies have correlated the availability of this disaccharide (or the expression of the genes related to its synthesis) with stress responses. Almeida et al. (2005) found that transgenic tobacco plants over-expressing trehalose-6-phosphate synthase had higher resistance against different abiotic stresses (like drought and temperature). Nery et al. (2008) stated that trehalose participated in protecting cells against hydrogen peroxide, by preventing oxidation of both membranes and proteins.

Brassinosteroids are a family of polyhydroxysteroids that have been reported to modify the activity of antioxidant enzymes and the level of non-enzymatic compounds (like ascorbic acid and tocopherols), when plants are subjected to different abiotic stresses, but its effect is still poorly understood (Bajguz & Hayat, 2009). ROS also have a signalling role in hormone responses, like ABA, auxin and ethylene (Kwak et al., 2006).



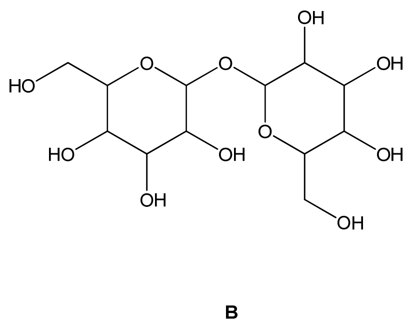


Fig. 15. Structure of raffinose (A) and trehalose (B)

3.4 Other non enzymatic substances involved in heavy metal tolerance

Several other substances, not described above, have been reported to be involved in the tolerance mechanisms against heavy metal tolerance. Metals are rarely available in the free ionic form in plants but are bound to different types of organic molecules. These include organic acids (like citric and maleic acid) and some amino acids (Haydon & Cobbett, 2007). In fact, organic acids, among other organic solutes of several types, can accumulate in leaves in stress conditions, such as water stress, with an important role on subcellular structures protection acting as osmolytes (Pinheiro et al., 2004). One possible effect of heavy metal stress is that plants restrict water uptake, thus indirectly inducing water stress.

Any changes in the concentrations of the intermediate organic acids can reflect an influence of a heavy metal in metabolic pathways as they are involved in primary plant metabolism such as cell respiration and the formation of ATP. Organic acids can have a detoxification role complexing metals, and reducing their availability to the plant, but this role is not yet fully understood (Hall, 2002).

The metabolism of several phenols has been reported to change under Cu and Cd stress indicating a putative role of these compounds in heavy metal detoxification by decreasing the presence of the free metal ions (Kováčik et al., 2009). Lignin accumulation has also been described as a consequence of Cu toxicity (Lequeux et al., 2010) but whether this is related to a heavy metal defence mechanism is still not clear.

Zorrig et al. (2010) suggested a transport role for citrate to translocate Cd from the roots to the shoots of lettuce plants while Panfili et al. (2009) also studied the effect of citrate in the uptake of Cd. Citrate and malate have been reported to be major ligands for Zn and Ni in several studies (Haydon & Cobbett, 2007).

Phytochelatins (PC) are small metal-binding peptides with the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (figure 16) where n ranges between 2 and 11 (Cobbett & Goldsbrough, 2002). Its synthesis is catalyzed by phytochelatin synthase using glutathione as a substrate (Clemens, 2006; Grill et al., 1989). They form complexes with metals like Cd (due to the high affinity metals have to the thiol group present in cysteine) and sequester them to the vacuoles and are thus an important mechanism that plants use to avoid heavy metal toxicity. Other peptides with a structure similar to phytochelatins have been identified, with the terminal Gly being replaced by other amino acids like serine, glutamic acid and glutamine in the case of iso-phytochelatins or alanine in homophytochelatins (Oven et al., 2002). All of these peptides have been reported to participate in the detoxification mechanisms of various plant species against several metals or metalloids, besides Cd, like As (Vázquez et al., 2009) and Pb (Z. C. Zhang et al., 2008).

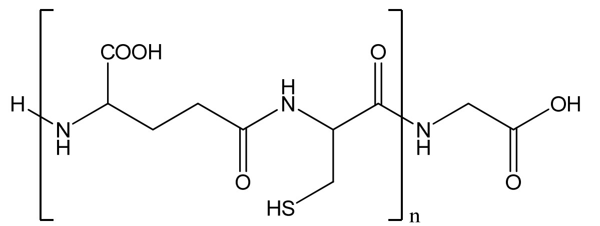


Fig. 16. General structure of phytochelatins (n ranges between 2 and 11).

Metallothioneins (MT) are other small peptides and in plants they contain typically between 60 to 85 amino acids (Freisinger, 2009), also containing cysteine in various amounts according to the type of MT. They have a different synthesis pathway from phytochelatins (Cobbett & Goldsbrough, 2002), and are thought to be important in metal complexation , but can also have other roles like ROS scavenging (Hassinen et al., 2011). MTs are reported to be much more important in Cu tolerance of certain plants than PCs (Mijovilovich et al., 2009).

4. Acknowledgments

The authors acknowledge the financial support of *Fundação para a Ciência e Tecnologia*, through its project PTDC/AGR-AAM/102821/2008 and the Research Unit Environmental Chemistry.

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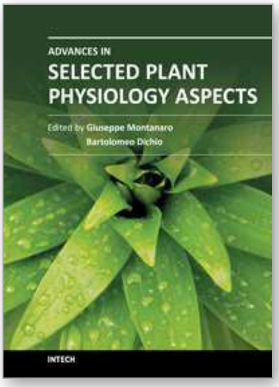

Advances in Selected Plant Physiology Aspects

Edited by Dr. Giuseppe Montanaro

ISBN 978-953-51-0557-2
Hard cover, 388 pages
Publisher InTech
Published online 25, April, 2012
Published in print edition April, 2012

The book provides general principles and new insights of some plant physiology aspects covering abiotic stress, plant water relations, mineral nutrition and reproduction. Plant response to reduced water availability and other abiotic stress (e.g. metals) have been analysed through changes in water absorption and transport mechanisms, as well as by molecular and genetic approach. A relatively new aspects of fruit nutrition are presented in order to provide the basis for the improvement of some fruit quality traits. The involvement of hormones, nutritional and proteomic plant profiles together with some structure/function of sexual components have also been addressed. Written by leading scientists from around the world it may serve as source of methods, theories, ideas and tools for students, researchers and experts in that areas of plant physiology.