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Phytoremediation by poplar: polyphenols polyamines and oxidative damage in the response to heavy metals in *in vitro* and in hydroponic cultures.

Presentata dalla Dott.ssa Fiorella Tamanti

Tutor
Prof. ssa Patrizia Torrigiani

Co-Tutor:
Prof.ssa Stefania Biondi

Coordinator
Prof. Luca Corelli Grappadelli

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Abstract

Transition metals, such as copper (Cu), are essential for many physiological processes, but they become toxic at elevated concentrations and polluted soils need to be decontaminated. Nowadays, phytoremediation is viewed as an ecological alternative to the destructive physical remediation methods and many studies are focussed on soil clean-up by trees because of their rapid growth, extensive root system and easy propagation. Poplar is considered a good candidate for phytoremediation, but its tolerance to heavy metals has not been fully investigated yet. In the present work, two different culture systems (*in vitro* and aeroponic/hydroponic*)* and two different stress tolerant clones of *Populus alb*a (AL22 and Villafranca) were investigated for their total polyphenol and flavonoid content, individual phenolic compounds, polyamine, lipid peroxidation and hydrogen peroxide levels in response to Cu. In AL22 poplar plants cultured *in vitro* in the presence or absence of 50 μM Cu, total polyphenol and flavonoid content was higher in the leaves of treated samples than in controls but unaltered in the roots. Equally the same clone, grown under aeroponic growth conditions and hydroponically treated for 72 h with 100 μM Cu, displayed increased amount of polyphenols and flavonoids in the leaves, in particular chlorogenic acid and quercetin, and no differences in the roots. In exudates from treated roots total polyphenols and flavonoids, in particular catechin and epicatechin, were more abundant than in controls. Free and conjugated polyamine levels were measured and an increase in conjugated putrescine (Put) and spermidine (Spd) was found. In the other clone, Villafranca, treated with 100 μM Cu for 6, 24 and 72 h, a time course analysis showed that, in treated leaves, the pattern of polyphenol and flavonoid accumulationwas the same as in AL22; in Cu-treated roots these compounds decreased compared with controls while they increased in root exudates. Free polyamine levels rose at 24 and 72 h while only conjugated Put increased at 24 h. Cu-treated Villafranca plants exhibited a higher malondialdehyde (MDA) production than controls throughout experimental time, indicative of membrane lipid peroxidation and, therefore, oxidative stress. An *in vitro* experiment was carried to investigate the possible antioxidant effect of the polyamine spermidine (Spd). Exogenous Spd, supplied together with 100 μM Cu, reduced the accumulation of polyphenols and flavonoids, MDA and hydrogen peroxide induced by Cu. Results are discussed in the light of the differential response of the two clones and of the protective/antioxidant role exerted by phenolic compounds and polyamines.

Introduction

Phytoremediation

The word “Phytoremediation” comes from the Greek (phyto) = plant and the Latin (remedium) = remediating, and consists in de-polluting contaminated soils and waters by plants. It is defined as the use of plants to remove pollutants from the environment or to render them harmless (Salt et al., 1998) and it is thought as the effective alternative to the destructive physicochemical remediation methods practiced nowadays.

Pollutants can be subdivided in elemental (heavy metals and radionuclides) and organic pollutants (Meagher, 2000). This distinction is important because elemental pollutants are essentially immutable by any biological or physical process but the radionuclides, and the phytoremediation main goal consists in their extraction.

Instead, organic pollutants can be completely mineralized into relatively non-toxic constituents and the phytoremediation aim is their degradation.

We can divide phytoremediation in the following main categories:

- Phytoextraction: plants take up metals from the soil and concentrate them in their above-ground tissues (metal hyperaccumulator plants)
- Phytodegradation: plants degrade organic pollutants in their cellular metabolic processes
- Rhizodegradation: plants and associated microbes degrade organic pollutants in the rhizosphere
- Rhizofiltration: plant roots absorb elemental pollutants from water
- Phytostabilization: plants reduce the mobility and bioavailability of pollutants in the environment by immobilisation and preventing their migration
- Phytovolatilisation: volatilisation of pollutants into the atmosphere by transpiration

As far as phytoextraction is concerned, a plant is said to be a hyperaccumulator if it can accumulate in its tissues a large amount of pollutants. Most, but not all, hyperaccumulators are strictly endemic to metalliferous soils where they grow without showing toxicity symptoms. The minimum amount for a plant to be defined hyperaccumulator varies according to the pollutant involved (Baker, 1999) as shown in Fig.1.

Minimun amount	Pollutant
100 mg kg^{-1} dry weight	**Cd**
1.000 mg kg^{-1} dry weight	**Cu, Co, Cr, Ni, Pb**
10.000 mg kg^{-1} dry weight	**Zn, Mn**

Figure 1. Relation between minimum amount and pollutant in hyperaccumulator species

In spite of its hyperaccumulating potential, exploitation of metal uptake into plant biomass as a method of soil decontamination is limited because hyperaccumulator plants (e.g. Brassicaceae) have a small biomass and are mostly slow-growing. Besides, they usually hyperaccumulate only a specific pollutant whereas soils are often contaminated by several pollutants at the same time.

An alternative to the use of hyperaccumulators is the use of non-accumulator plants which instead are high biomass producers, such as fast-growing trees. Tree species are non-hyperaccumulator plants, but some of them can survive in metal-contaminated soils, showing stress-tolerance properties which could be improved by conventional breeding programmes or by genetic manipulation. Moreover, their large biomass allows trees to accumulate a total amount of pollutants higher than herbaceous plants and their roots penetrate more deeply and widely in the soil.

Among trees, the genera *Salix* and *Populus* seem to be the most efficient for phytoremediation. *Salix* has developed a large number of species and varieties which suggests a wide genetic variability within the genus, and the role of *Populus* as a model system among tree species has enormously increased by the recent sequencing of the *P. trichocarpa* genome (Pulford and Watson, 2003; Komives and Gullner, 2006).

Phytoremediation of organic pollutants

The main targets of phytoremediation of organic pollutants are polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), linear halogenated hydrocarbons such as trichloroethylene (TCE) and pesticides such as atrazine.

Polychlorinated biphenyls (PCBs) are a group of manufactured organic chemicals with 1 to 10 chlorine atoms attached to biphenyl which is a molecule composed of two benzene rings. They have been used widely as coolants and lubricants in transformers, capacitors, and other electrical equipments.

The main sources of PCBs are rubbish dumps containing transformers, capacitors and other PCB equipments and the incineration of municipal wastes which may produce dangerous by-products, such as hydrogen chloride (HCl) and dioxins (PCDDs and PCDFs).Because of their chemical stability, these compounds still remain in the environment, even if they were banned at the end of the 1970s in many countries.

PAHs are chemical compounds formed by several fused aromatic rings and are among the most widespread organic pollutants. They are formed during the incomplete combustion of coal, oil, gas, wood or other organic substances. They can also be found in substances such as crude oil and coal.

The most common linear halogenated hydrocarbon is trichloroethylene (TCE), a chlorinated hydrocarbon commonly used as an industrial solvent for a variety of organic materials. Most of the TCE is released into the atmosphere from industrial degreasing operations. Other significant sources of TCE emissions include paints and coatings, adhesive formulations and PVC production.

Pesticides are probably the most widespread contaminants in the environment and there are thousands different synthetic molecules which are used as pesticides in agriculture. Many of them does not contain only organic molecules, but also heavy metals such as copper, mercury and arsenic.

The main processes involved in phytoremediation of organic pollutants are rhizodegradation, phytodegradation and phytovolatilization.

1) Rhizodegradation

Organic pollutants can potentially be chemically degraded and mineralized into harmless biological compounds, and the complex physiology and biochemistry of plant roots play a significant role in phytoremediation.

Many bacteria living in the soil are able to degrade organic compounds and their degrading activity is widely enhanced by plants roots. Enhanced mineralization was found for atrazine by using 15 different plant species (Anderson and Coats, 1995) and some studies indicate that bacterial degradation of PAHs and PCBs in soil can be enhanced by plants (Donelly et al., 1994 and Fletcher, Hedge, 1995).

The mechanisms of the plant influence seems to be related to the production of mucilaginous materials and exudation of soluble organic compounds, such as flavonoids, organic acids, sugars etc, that creates a suitable physico-chemical environment for bacteria (Singer at al, 2003). Even if the chemistry of exudates is not yet well understood, they seem to play an important role in different processes such as the defence against pathogens and stimulation of degrading enzymes. Besides, these compounds may also be responsible for promoting vesicular-arbuscular mycorrhiza colonization (Travis et al., 2003).

2) Phytodegradation and phytovolatilization

Phytodegradation and phytovolatilization often occur in the same process.

Organic contaminants are taken up by the plant and then degraded by metabolic reactions. The intermediate or the final compounds can be incorporated into plant biomass or volatilized by transpiration.

An interesting example is the TCE degradation. Plants grown in polluted sites are known to extract TCE and efficiently transpire it , but some studies have shown that hybrid poplars (*Populus* sp.) actively take up TCE and degrade it to trichloroethanol, chlorinated acetates, and finally CO_2 (Gordon et al, 1998).

Another example concerns nitroaromatic compounds. A wide variety of plant species appear to be able to degrade TNT through multiple complex pathways and the final products of these plant degradation processes is CO_2 , and ammonium or nitrate

Phytoremediation of heavy metals

Heavy metals (HMs) can be divided into those that are essential but toxic at high concentrations (e.g. Zn, Cu, Mo), and those that are toxic anyway, such us Cd, Hg, As, Pb.

For some of these metals, the toxicity depends more on their state than on the metal in itself, and on their bioavailability. Mercury offers perhaps the best example of how the toxicity level can depend on the chemical form of the element. Mercury can be

introduced into the environment as Hg(0) from industrial sources or as Hg(II) from burning coal or volcanic activity, but the most toxic form is methylmercury (CH_3Hg) which alters genetic and enzyme systems and damages the nervous system. In aquatic environments the different Hg species are efficiently converted to methylmercury by anaerobic bacteria and end up in the food chain, as occurred at Minamata Bay (Japan) in 1956.

The main anthropic sources of metal pollution are industry, agriculture and combustion processes. Large amounts of fine dusts are discharged by iron and steel industries, and sludge coming from industrial processes contaminates soils and water resources.

In agriculture, fertilizers and pesticides are the main sources of heavy metals. Sewages contain Cu and Zn and natural phosphatic fertilizers, such as phosphorite and apatite, contain Cd, Ni, As and Cr. Inorganic fungicides such as copper sulphate and organic fungicides such as Ziram contain Cu and Zn, respectively.

The molecular mechanisms of heavy metal toxicity can be distinguished in (Shutzendubel and Polle, 2002):

- Production of reactive oxygen species by autoxidation and Fenton reaction. This reaction is typical for transition metals such as iron or copper
- Blocking of essential functional groups in biomolecules, especially enzymes with cysteine residues. This reaction has been mainly reported for non redox reactive heavy metals such as Cd and Hg
- Displacement of essential metals ions from biomolecules. Many enzymes contain metals in position important for their activity. The displacement of one metal by another normally leads to inhibition or loss of enzyme activity. This reaction occurs with different kinds of heavy metals.

In order to defend themselves from the injuries caused by heavy metals, plants have developed homeostatic mechanisms to maintain the concentration of essential elements within a physiological range and to reduce the negative effects of non-essential ones.The main processes of metal homeostasis are transport, chelation and sequestration, and the loss of one of them leads to hypersensitivity.

1) Transport

A comprehensive classification system for transmembrane molecular transporters has been developed and recently approved by the International Union of Biochemistry and Molecular Biology.

Transport systems are involved in numerous cellular processes: in particular they mediate the active extrusion of toxic substances and the uptake and the efflux of ionic species that must be maintained at concentrations that differ drastically from those in the external milieu.

Figure 2 shows the scheme of the currently recognized primary types of transporters found in nature.

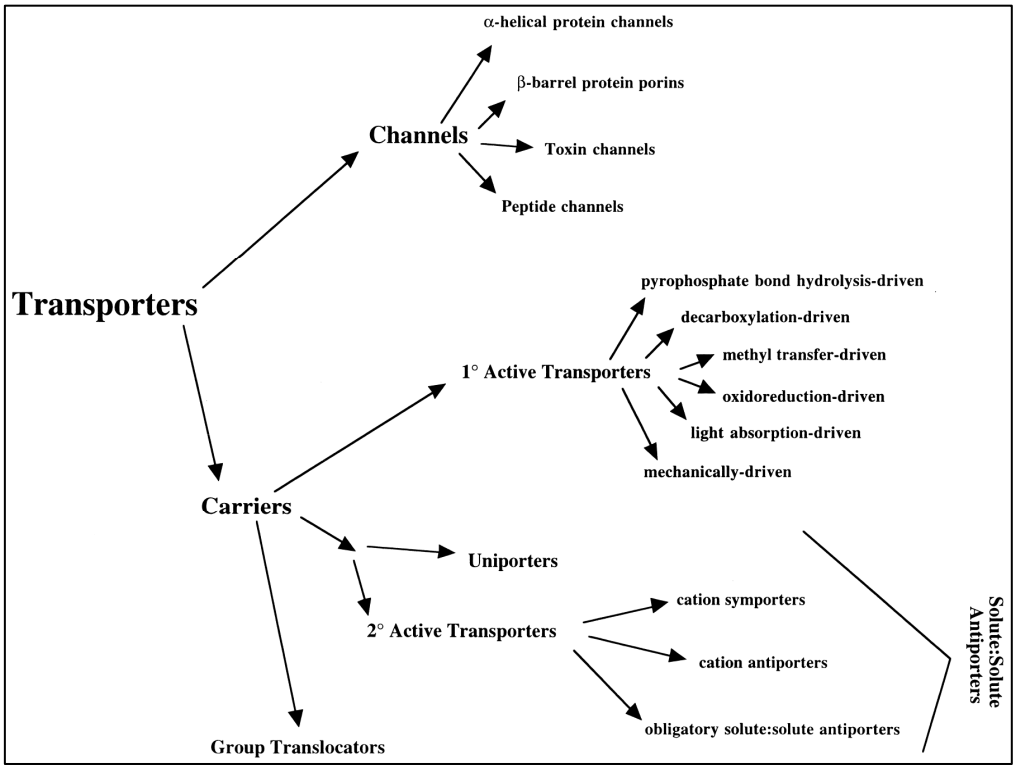


Figure 2. Scheme illustrating the currently recognized primary types of transporters found in nature

These proteins are initially divided into channels and carriers. Carriers, which are the proteins involved in metal transport, are subdivided into primary active carriers, secondary active carriers (including uniporters), and group translocators that modify their substrates during transport.

Primary active transport systems use a chemical source of energy (i.e, ATP) whereas secondary active transport is driven by ion and other solute electro-chemical gradients created by primary active transport systems (Saier, 2000).

Although research about the plant transport systems for elemental nutrients and pollutants is still in its first steps, some cell membrane transporters have been identified. The transporter families identified to date are the ZIP family, especially for Zn and Fe (but also for Cd and Mn), the Nramp family for Fe and Cd, the LCT family for Ca and Cd, and the Ctr family for Cu (Clemens, 2001).

2) Chelation

Because of their reactivity and limited solubility, metal ions must be constantly chelated once they are taken up into cytoplasm. In plants, the main classes of known chelators include:

- phytochelatins
- metallothioneins
- organic acids
- amino acids

Phytochelatins (PCs) are small non-ribosomally synthesized peptides whose general structure is $(\gamma\text{-glu-cys})_n\text{-gly}$ (n = 2-11) (Fig,3) that are synthesized from glutathione by phytochelatin synthase. PC synthesis is induced by exposure to a variety of metals.

Metallothioneins (MT) are a family of cysteine-rich, low molecular weight proteins, which in plants, are generally composed of 60–80 amino acids and

contain 9–16 cysteine residues (Fig.3). MTs have the capacity to bind both physiological and xenobiotic heavy metals via the thiol group of its cysteine residues.

MTs have been classified, based on the arrangement of their cysteine residues, into two classes:

Class I for vertebrates

Class II for plants, fungi and non-vertebrate animals

Recent studies suggest that MTs could be involved in regulating the cell redox status by directly scavenging oxygen radicals, in transferring of HMs to metalloproteins and in stress signalling (González-Guerrero et al, 2007).

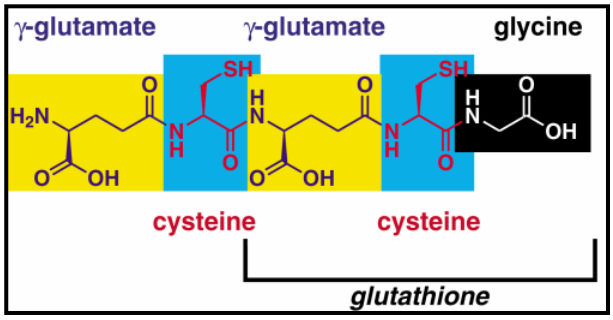
Many organic and amino acids are potential metal ligands because of the

reactivity of metal ions with S, N and O, but, with the exception of Al,

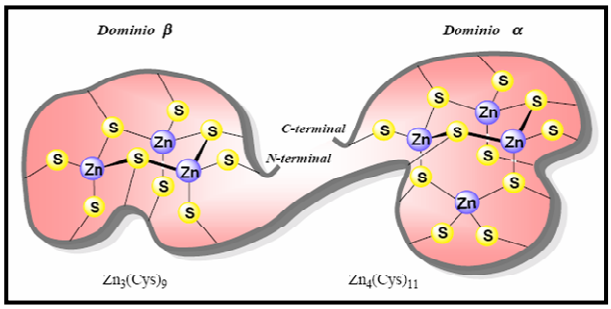
unequivocal evidence of their function in metal tolerance has not yet shown. The only example known to date concerns histidine (Fig. 3) whose levels reveals a linear relationship with Ni content in hyperaccumulator species.

A new family of soluble metal receptor proteins known as "metallochaperones" has been recently discovered and their involvement in intracellular Cu trafficking is now emerging (Fig. 4). The metallochaperone proteins do not protect the cell from metal toxicity, but they work in a "chaperone-like" manner, guiding and protecting by chelation the metal ion and facilitating partnerships with mitochondria, the Golgi apparatus and cytosolic superoxide dismutase (SOD1) enzyme (O'Halloran, Culotta, 2000). Metallochaperones are supposed to exist for other metals as well.

a)



b)



c)

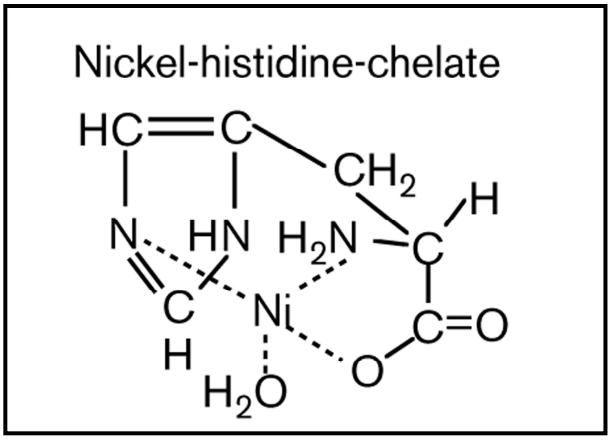


Figure 3. Structure of phytochelatins (a), metallothioneins (b) and nickel-histidine-chelate (c)

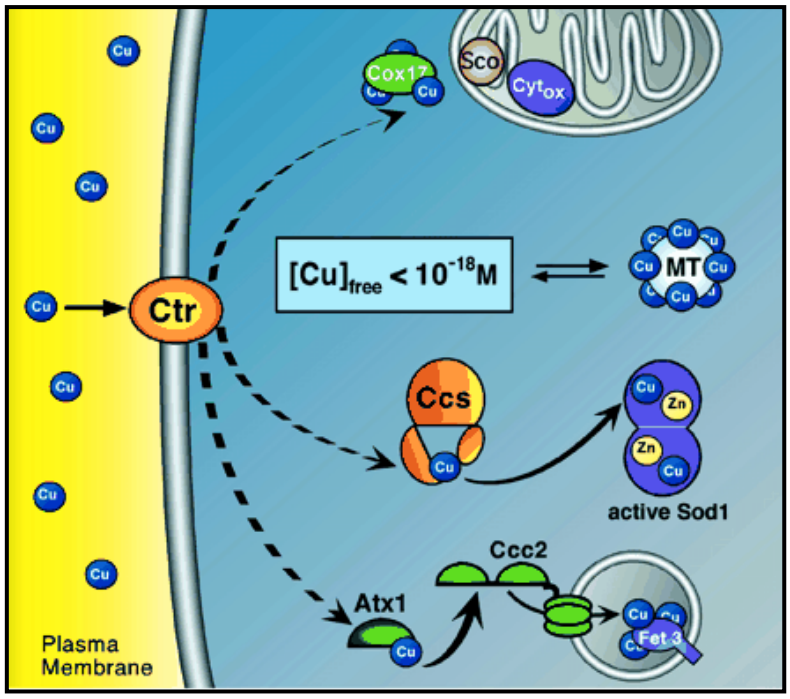


Figure 4. COX17 is a candidate metallochaperone involved in delivering Cu to mitochondria. ATX1 was found to specifically shuttle Cu to an intracellular Cu transporter, CCC2, located in the Golgi compartment. The target for CCS is the Cu- and Zn-requiring enzyme Cytosolic Superoxide Dismutase 1 (SOD1)

3) Sequestration

Excess metal ions have to be removed from the cytosol and this goal can be achieved by efflux or by compartmentalization. The main storage compartment for toxic compounds in plants are the vacuole and the main tonoplast transporters belong to the ABC-type transporter and CDF (Cation Diffusion Facilitator) families. The ABC transporter (ATP-binding cassette) is a superfamily, one of the largest families with representatives in all Phyla. These proteins are called ABC transporters because of the sequence and organization of their ATP-binding domain(s) and they are primary active transporters which utilize the energy originated from ATP hydrolysis.The CDF family is a ubiquitous family of primary active transporters which was found in several membranes, including the bacterial cell membrane, the vacuolar membrane of both plants and yeast, and the Golgi apparatus of animals.

Metal tolerance and homeostasis in trees

Trees are not hyperaccumulator species, but some of them can survive in metal-contaminated soils, showing tolerance traits, even if precise physiological mechanisms with a genetic basis are not yet characterized. Anyway, some studies

have shown that poplar genome contains at least six MT genes which seem to be involved in metal tolerance (Kohler et al, 2004)

Besides, various studies have shown that the acclimation of trees to their soil environment, soil fertility and mycorrhizal fungi may increase metal resistance.

Bioavailability of metals and their accumulation in tree tissues can vary according to the metal and to the site conditions such as the pH . As a matter of fact, treatments that significantly raise soil pH caused lower uptake of some metals.

The uptake also depends on the phenological phases: during the early vegetative growth there are relatively high contents of metals in plant tissues; a dilution of metal concentrations occurs during the vigorous growth until the flowering stage in which the minimum values for almost all elements are observed. Finally, senescence usually produces an increase of metal concentrations due to loss of fluids.

Trees species show different ability to translocate heavy metals from roots to shoots: generally, Pb, Cr and Cu are immobilized in roots, while Ni and Zn are more easily translocated in shoots.

Bark concentrations of heavy metals were found to be consistently greater than the concentrations in wood for *Salix* (Pulford, Watson 2003), but it was also found that there was little difference between bark and wood Cd concentrations in several fruit tree species (Korcak, 1989). Bark accumulation can play an important role in phytoremediation because these tissues can immobilise metals in a metabolically inactive compartment for a considerable period of time (Tlustos et al., 2006).

Poplars and willows: good candidates for use in phytoremediation

For the purpose of phytoremediation, the following plant characteristics are beneficial: ability to grow on nutrient-poor soil, a deep and/or wide-spreading root system, a fast rate of growth, and a metal-resistance trait (Punshon & Dickinson, 1997). In addition, coppice cultures and an economically viable secondary use (e.g., energy from biomass, pulp and paper industry) are desirable (Dutton & Humphreys, 2005). Trees, in particular *Populus* and *Salix* spp., have been shown to meet all of these requirements (Aronsson & Perttu, 2001; Di Baccio et al., 2003; Sebastiani et al., 2004; Soudek et al., 2004). At the same time, several studies have revealed a remarkable clonal variability in their ability to accumulate/tolerate HMs (Dos Santos Utmazian et al., 2007; Kopponen et al., 2001; Laureysens et al., 2004 and 2005; Zalesny et al., 2005).

The genus *Populus*

The genus *Populus* includes about 35 species of which only three are autochthonous in Europe, *P. alba*, *P. tremula* and *P. nigra* (Dickmann, 2001). *Populus* plants are fast-growing angiosperm trees and occupy a wide-ranging habitat.

The genus has a large genetic diversity, and can grow anywhere becoming 15–50 m tall, with trunks of up to 2.5 m diameter. The bark of young trees is smooth, from white to dark grey, often with conspicuous lenticels. The shoots are stout, with terminal buds. The leaves vary in shape from triangular to hearth-shaped or (rarely) lobed, with a long petiole; leaf size is very variable even on a single tree, typically with small leaves on young shoots and very large leaves on strong-old shoots.

The flowers are mostly dioecious (rarely monoecious) and appear in early spring before the leaves. They are long, drooping, sessile or pedunculate catkins produced from buds formed in the axils of the leaves of the previous year. The male flower has no calyx or corolla and comprise a group of 4–60 stamens inserted on a disk while anthers are oblong, purple or red. The female flower also has no calyx or corolla, and comprises a single-celled ovary seated in a cup-shaped disk. The style is short, with 2–4 stigmas, variously lobed, and the ovary host numerous ovules. The fruit is a two to four-valved dehiscent capsule, from green to reddish-brown, mature in mid summer, and contain numerous minute light brown seeds surrounded by tufts of long, soft, white hairs which aid wind dispersal.

In the past, *Populus* has been a subject of intensive research for the timber and paper industries, and recently it is also recognized as an important source of bioenergy crop.

The genetic variability of white and black poplar is mainly intra-population (Smulders *et al.*, 2008), and could be exploited for the selection of genotypes having interesting traits, such as tolerance to pollutants. Furthermore, the *Populus* genome is the first tree genome that has been completely sequenced and annotated, and has been publicly released by the Department of Energy Joint Genome Institute (Tuskan *et al.,* 2006). As a result of these attributes and of the possibility of using this genus in advanced silvicultural systems, *Populus* has been developed for bioenergy production, used for phytoremediation projects, and utilized for its role in carbon management/sequestration settings.

Role of mycorrhiza in phytoremediation

Soil microorganisms, such as ecto- (Karlinski *et al*., in press/2010) and endomycorrhiza, are also important in the recovery of polluted sites via phytoremediation because they can both modify metal bioavailability, and/or improve plant growth either by contributing to nutrient acquisition, by producing growth stimulating substances, and/or by conferring increased tolerance to stress by upregulation of foliar metallothionein and polyamine biosynthetic gene expression (Cicatelli *et al*.,)

An interesting option therefore consists in exploiting the synergistic effect of plants and microorganisms by a process called rhizoremediation or bio-augmentation (Kuiper *et a,.l* 2004; Lebeau *et al*., 2008).

Arbuscular mycorrhizal fungi (AMF) are biological constituents of the soil of most ecosystems where they can form associations with the roots of the vast majority of land plants. By increasing the exchange surface between plant and soil, AMF improve nutrient (especially phosphorus) uptake (Smith and Read, 1997). There is also increasing evidence that symbiotic fungi contribute to, or are responsible for, plant adaptation to stress, namely drought, HMs, disease, salinity, herbivory and pathogens (Rodriguez and Redman, 2008 and references therein; Lingua *et al*., 2002; Gamalero *et al*., 2009). In the case of HMs, results vary according to plant and fungal species, metal type and concentration (Takàcs *et al*., 2005; Bois *et al*., 2005; Todeschini *et al*., 2007; Lebeau *et al*., 2008), but, on the whole, microorganisms may also contribute to the efficiency of the phytoremediation effort (Hildebrandt *et al*., 2007).

The low amount of metals extracted by plants from the soil often depends on their low bio-availability and this rate is influenced by the soil characteristics such as pH and organic matter (Lebeau et al, 2008).

In a liquid medium plant roots can adsorb many elemental pollutants as well as nutrients, but in soils, these adsorption processes are less efficient than in liquid medium because root surfaces must compete for nutrients with diverse soil materials. Several studies have shown that plant-microbe interactions in rhizosphere, mainly with mycorrhiza and Plant Growth Promoting Rhizobacteria (PGPR), can increase the rate of metal extracted by plants.

Mycorrhizal fungi with a relevant role in phytoremediation are:

- Arbuscular Mycorrhizal Fungi (AMF)

- Ectomycorrhizal Fungi (EcM)
- Ericoid Mycorrhizal Fungi (ErM)

1) Arbuscular mycorrhizal fungi

AMF belong to the phylum Glomeromycota, and both paleobiological and molecular evidence indicate that theirs is an ancient symbiosis that originated at least 460 million years ago. They are endomycorrhizas whose hyphae enter into the cell and form symbiosis with more than 80% of vascular plants. AMF produce structures called arbuscules that colonize the root parenchyma. They may also produce vescicles containing lipids.

The development of AMF prior to root colonization, consists of three stages: spore germination, hyphal growth, host recognition and appressorium formation. Even if AMF are obligate symbionts, the germination of the spore does not depend on the plant and spores can germinated both *in vitro* and in soil. However, the rate of germination can be increased by host root exudates. Once inside the parenchyma the fungi forms arbuscules, which are the sites of exchange for phosphorus, carbon, water and other nutrients.

AMF are more frequent in plant communities with high biodiversity such as tropical rainforests and temperate grasslands where they have many potential host plants, but they are also found in desert environments, such as in a desert riparian forest in the arid region of Northwest China (Yang et al, 2008).

Towards HMs AMF employ strategies similar to those of its host and among these there is immobilization of metals by a compound secreted by the fungus, glomalin. Glomalin is an insoluble glycoprotein produced and secreted by AMF that binds HMs in the soil (Gonzalez-Chavez et al. 2004). AMF can also bind metals to chitin in the fungal cell wall, reducing the metal concentration in soil, or accumulate metals into fungal tissues.

Alternatively AMF can help phytoextraction, by enhancing plant uptake by increasing the interface between roots and soil and by increasing the biomass of plants, as result of improved nutrition (Gohre, Paszkowski, 2006).

The mechanisms involved depend on the metal and on the different type of mycorrhiza–plant association. Colonization by AMF can lead to increased uptake and subsequent accumulation of HMs in above-ground tissues of plants, but in several cases mycorrhizal colonization leads to accumulation of HMs in the roots

(phytostabilization). Finally, AMF colonization of the roots seem to have a significant impact on the expression of several plant genes coding for proteins presumably involved in HM tolerance/detoxification (Hildebrandt et al., 2007).

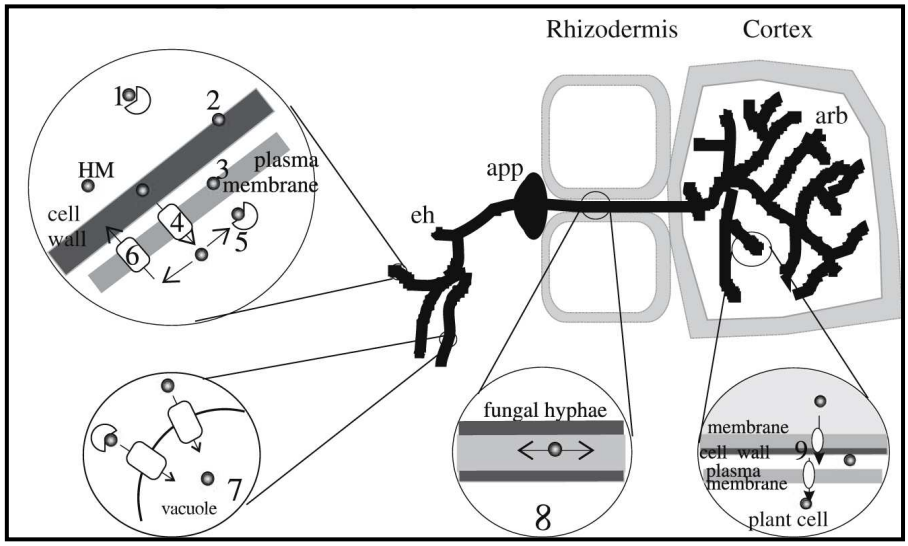


Figure 5. HM detoxification mechanisms of plants and AMF

Chelating agents secreted by the plant (e.g. organic acids) or by the fungus (e.g. glomalin) bind metals in soil. 2) Binding of HM to chitin of fungal cell wall. 3) The plasma membrane acts like a selective barrier. 4) Specific and non-specific metal transporters. 5) Chelates in the cytosol. 6) Export via specific or non-specific transporters. 7) Sequestration of HM in the vacuole of plant and fungal cell. 8) Transport of HM in the hyphae of the fungus. 9) Metal transit from fungus to plant cells.

2) Ectomycorrhizal fungi

The EcM associations are mainly formed by basidiomycetous or ascomycetous fungi. They change the morphology of the root system by forming a sheath covering the root tips. Outside the root, the fungal mycelium forms an extensive network in the soil, exploiting its nutrient sources.

The fungus gains carbon and other essential organic substances from the tree and in return helps the trees take up water, mineral salts and metabolites. It can also fight off parasites, predators such as nematodes and soil pathogens.

Whereas AMF can improve phytoextraction, EcM prevalently attenuate metal stress in their host plants, supporting plant survival. Heavy metals toxicity has been supposed to be the main reason for the “new-type forest decline” in Central Europe and North America and inoculation of EcM could play a crucial role in modifying the sensitivity of plants to metals.

Even if the mechanisms are still unclear, the four main metal exclusion mechanisms (Jentschke, Godboldb, 2000) are:

- Filtering of toxic metals in the hyphal sheath by adsorption (exclusion mechanism I)
- Restricted metal mobility in the fungal apoplast (exclusion mechanism II)
- Chelation by organic acids and other substances released by mycorrhizal fungi (exclusion mechanism III)
- Metal sorption on the external mycelium (exclusion mechanism IV)

Exclusion mechanism I: it was suggested that sorption of metals into fungal tissues and detoxification in fungal vacuoles reduce metal uptake into the host plant. Although there is no doubt that metal sorption on fungal structures does occur, the sorption properties of EcM and the mechanisms involved are non clear.

Exclusion mechanism II: metal sorption to fungal cells and metal uptake into host tissues might be affected by the degree of hydrophilicity of the fungal apoplast. Fungal hydrophobicity limited the apoplastic flux of water and of the metals dissolved in it.

Exclusion mechanism III: as mycorrhizal fungi produce different exudates capable of binding metals, it’s possible that organic compounds secreted by EcM are responsible for the amelioration of metal toxicity in mycorrhizal plants.

Exclusion mechanism IV: heavy metals are immobilized on external mycelium. The level of immobilization is supposed to depend both on the binding capacity of the mycelium and on the availability of metals in the rhizosphere.

Hypothetical exclusion mechanisms are synthesized in the Fig. 6.

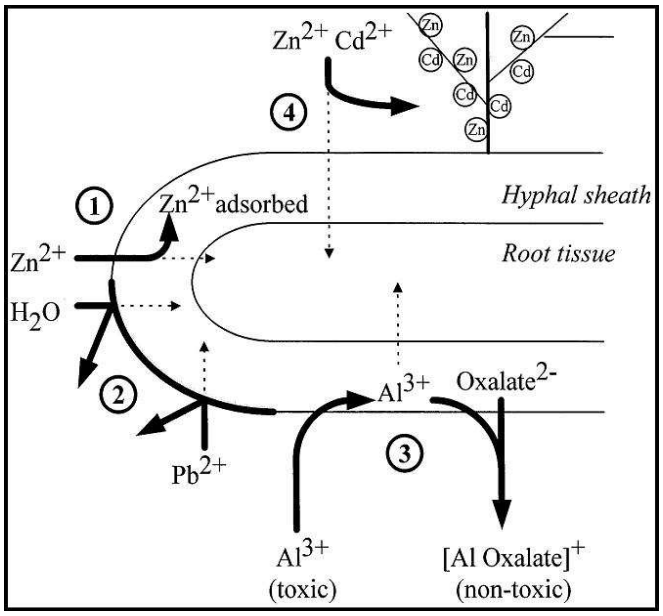


Figure 6. 1) Filtering of toxic metals in the hyphal sheath by adsorption. 2) The fungal sheath formed by a hydrophobic fungus provide a barrier to apoplastic transport of water and ions. 3) Chelation by organic acids and other substances released by EcM. 4) Metal sorption on the external mycelium

3) Ericoid mycorrhizal fungi

Ericoid mycorrhizal fungi (ErM) is a distinctive subtype of endomycorrhiza formed mostly by ascomycetous fungi, inhabiting fine roots of ericacae (Vosátka et al, 2006) and the symbiosis is characterized by a wide intra-cellular colonization. Their habitats are acid and nutrient poor soils, with high carbon/nitrogen ratio. Colonisation by ericoids is restricted to mature epidermal cells and the apical region of the roots, behind the growing meristem, remains uncolonised until the cells differentiate and mature.

ErM are facultative symbionts and they can be free-living in soil, and can also be cultured.

Although there are limited data about the effect of ErM in contaminated soils, some studies have pointed up that mycorrhizal colonization by ErM positively

affects the metal resistance. In general, mycorrhizal plants show lower content of heavy metals in shoot than non-mycorrhizal plants, but often the HM level in roots results increased.

The ability of plants to accumulate contaminants or to trigger exclusion mechanisms can be improved by mycorrhizal fungi, but their effectiveness depends strongly on different factors, among which the main important seem to be:

- Plant-fungus association
- Pre-inoculation
- Metal-tolerant genotypes
- Bacteria-fungi associations

The result of mycorrhizal symbiosis depends on both plant species or variety and mycorrhizal fungi, even if, generally, mycorrhizal plants are more efficient than non mycorrhizal plants in the acquisition of micronutrients.

Recent studies have shown that two poplar clones, Villafranca and Jean Pourtet, react in a different way to inoculation with the AMF *Glomus mosseae* (Lingua et al, 2007). Villafranca increased the Zn accumulation in leaves, in presence of G. mosseae, whereas Jean Pourtet had a better performance in its absence. Furthermore, *Glomus mosseae* and *G*. *deserticola* increased dry weight, shoot length, total N, P and K concentration and the quantity of chlorophyll in shoots of *Eucalyptus globulus* grown in soil contaminated with heavy metals, (Arriagada et al, 2007).

High levels of HMs inhibit spontaneous root colonization by mycorrhizal fungi, but pre-inoculation can ensure a mycorrhizal colonization.

In poplar (Lingua et al, 2007), high concentration (300 mg kg^{-1} soil) of Zn caused the total inhibition of spontaneous mycorrhiza colonization , but pre-inoculation with either *G. mosseae* or *G. intraradices* made the colonization level in Zn-treated plants comparable with that of plants grown without the metal, even if the amount of arbuscules was highly reduced.

Phenolic compounds

Biosynthesis

A phenolic compound can be defined as a substance which possesses an aromatic ring with one (phenol) or more (polyphenol) hydroxyl substituents, including functional derivatives (esters, methyl ethers, glycosides, etc).

Phenolic compunds are normally soluble in polar organic solvents, and methanol, ethanol, water and alcohol-water mixtures are most commonly used for extracting phenolic compounds for analytical purposes.

As a general rule, the term polyphenols is referred to all the secondary metabolites originating from either the shikimate/phenylpropanoid pathway (the central phenylpropanoid pathway) or acetate/malonate pathway, or both, producing monomeric and polymeric phenols which plays a very broad range of physiological roles in plants.

Biosynthetic pathways leading to flavonoids are well defined at both the biochemical and molecular genetic levels, and many of the enzymes have been characterized in different species. In the shikimate/phenylpropanoid pathway, phenylalanine ammonia lyase (PAL) catalyzes the deamination of phenylalanine to trans-cinnamate and subsequently the cinnamate is converted to 4-coumaroyl CoA by the cinnamate-4-hydroxylase and 4-coumaroyl CoA-ligase enzymes. 4-coumaroyl CoA probably represents the most important compound for phenylpropanoid biosynthesis in plants.

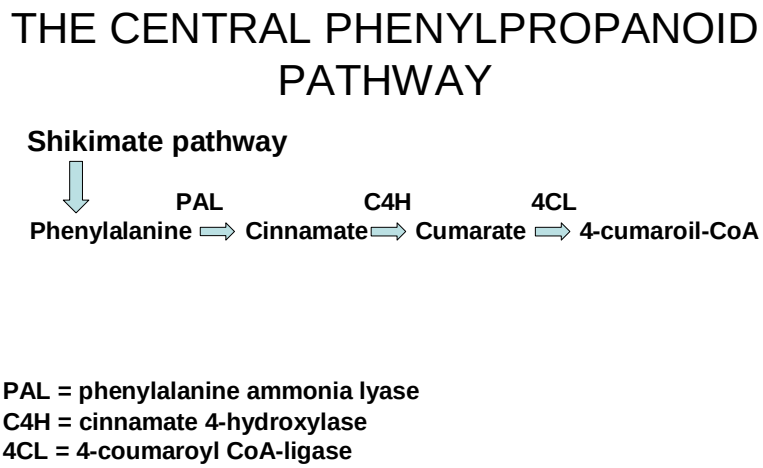


Figure 7. The central phenylpropanoid pathway

4-coumaroyl CoA is the main substrate for chalcone synthase (CHS), the enzyme which leads to the formation of flavonoids. CHS is a plant-specific polyketide synthase that converts 4-coumaroyl CoA into a variety of C6-C3-C6 products. A possible starting alternative is the deamination of tyrosine by tyrosine ammonia lyase (TAL), which leads to cinnamate.

Phenolic compounds represent one of the most common, wide and polyvalent groups of substances in plants and are involved in many biological functions such as growth, pigmentation, resistance to biotic and abiotic stress, etc.

Although phenolic compounds are found in the animal kingdom, it is in the plant kingdom and especially in the vascular plants that the full range of polyphenols is found.

The evolutionary significance of the greater range of phenolic compound in plants may be ascribed to the different ways that plants and animals have developed to protect themselves against predators, diseases, and abiotic stress factors.,

Animals have developed nervous and immune system as defence systems in response to environmental dangers; plants, that cannot escape from their biotic and abiotic stress agents, have developed a wide spectrum of chemicals to defend themselves from stressing situations.

Plant phenolics may be divided in two classes: preformed phenolics, that are synthesized during the normal development of plant tissues, and induced phenolics, that are synthesized by plants in response to physical injury, infection or when stressed by suitable elicitors such as heavy metals, salts, UV-irradiation, temperature, etc. (Lattanzio, 2003). Induced phenolics may also be constitutively synthesized but, additionally, their synthesis is often enhanced under biotic or abiotic stress.

Constitutive phenols

Plants mainly need phenolic compounds as sunscreen, as signal compounds, and for reproduction and growth.

Plants protect themselves from solar ultraviolet-B (UV-B) radiations (280–320 nm), which lead to mutagenesis and altered metabolism through the production of reactive oxygen species (ROS) by synthesizing phenolic compounds which act as sunscreen. This function seem to be especially performed by flavonoids, which have a high absorbance at 250-270 and 335-360 nm. Besides, several studies have shown that

tropical and high altitude plants have a higher amount of flavonoids than the temperate ones and that the activation of flavonoid biosynthetic genes is induced by UV radiation (Logemann et al., 2000). Another important role played by constitutive flavonoids is pigmentation. Pigmentation works as a visual signal for pollinators in flowers and, at a later stage, for animals eating the fruits and dispersing their seeds in the environment.

Phenols also act as signalling molecules in allelopathy. Some identified phenolic allelochemicals are: *p*-hydroxybenzoic acid and *p*-coumaric acid, present in leaves, quercetin, juglone and 2,4-dihydroxy-1,4(2H) benzoxazin-3-one (DIBOA), present in leaves, bark and root exudates, (-)-catechin and sorgoleone, found in the rhizosphere and root exudates (Weir et al., 2004).

Moreover, plant life depends on the ability of roots to communicate with microbes and create biological interactions. Specific phenolic compounds in root exudates play an important role in these interactions: for instance isoflavonoids and flavonoids present in the root exudates of many leguminous plants activate the *Rhizobium* genes responsible for the nodulation process and are supposed to be responsible for vesicular-arbuscular mycorrhiza colonization.

As far as the role of plant phenolics in plant growth is concerned, hydroxycinnamic acids, particularly *p*-coumaric acid and ferulic acid, are found in the wall-bound fraction and act as precursors for lignin biosynthesis. Cell wall-bound hydroxycinnamic acids are supposed to play a role in phototropism in higher plants (Turner et al., 1993).

Phenolic compounds in plant defence against pathogens

An appropriate response to attack by pest and pathogens can lead to tolerance or resistance mechanisms that enable the plant to survive. Resistance refers to mechanisms that inhibit or limit attack, while tolerance to physiological mechanisms that reduce or offset the consequences of the attack. For instance, resistance strategies include physical and/or chemical barriers and the limitation of the infection spread by localized cell death (hypersensitive response). Increasing the size of new leaves or improving nutrient uptake are typical tolerance strategies.

Most plants produce a wide range of secondary metabolites, which are toxic to pathogens and herbivores, either as part of their normal program of growth and development (constitutive or preformed) or in response to biotic stress (induced).

1) Preformed defences

Preformed antibiotic compounds such as phenolic and polyphenolic compounds are stored in plant cells as inactive forms but are readily converted into biologically active forms by plants in response to pathogen attack.

The distribution of preformed phenolics within plants is often tissue-specific and there is a tendency for many lipophilic compounds (e.g. flavone and flavonol methyl esters) to be located at the plant surface (leaf wax and bud exudates) or in the cytoplasmic fraction within the epidermal cells. In general, preformed phenolics are commonly sequestered in conjugated forms, usually as glycosides, in vacuoles or organelles (Beckman, 2000; Lattanzio et al., 2001).

Also tannins are quite potent antibiotics. In temperate trees, tannins and related phenolic compounds preserve heartwood from fungal decay and inhibit extracellular fungal enzymes. Lignans, a phenolic class of dimeric phenylpropanoid units, play a role in plant-fungus interactions. As in the case of tannins, their fungistatic action is expressed by the inhibition of the extracellular fungal enzymes, cellulase, polygalacturonase, glucosidase and laccase.

A relationship between lignification and disease resistance has been demonstrated in many experiments which showed that resistant plants accumulated lignins more rapidly than susceptible plants. Lignins are hypothesized to interfere with the enzymatic hydrolysis and mechanical penetration of plant tissue by fungal pathogens. Lignin precursors themselves might exert a toxic effect on pathogens by binding to fungal cell walls that make them more rigid and impermeable so that their growth and uptake of water and nutrients become more difficult.

2) Induced defences

The defence response induced after the pathogen attack requires a great use of all the cellular resources, including genetic reprogramming, because the expression of a large number of defence-related genes is essential to contrast pathogen attacks. Many defence-related genes encode proteins possessing antifungal and antimicrobial activities or enzymes that catalyse defence secondary metabolites, known as phytoalexins.

Phytoalexins are not detectable before infection, and are considered to inhibit the further development of most attacking pathogens of the species under consideration.

The molecules that signal plants to begin the process of phytoalexin synthesis are called elicitors. Nowadays the term “elicitor” refers to both biotic elicitors (such as complex carbohydrates from fungal and plant cell walls, and microbial enzymes) and abiotic elicitors (such as heavy metals, chloroform, detergents, exposure to UV light, etc).

The structures of phytoalexins are often unique at the family level: most phytoalexins produced by the Fabaceae (legumes) are isoflavonoids, while phytoalexins from the Vitaceae seem to consist of a restricted group of molecules belonging to the stilbene family.

Dicotyledonous species represent the majority of plants from which such compounds have been identified, but some monocotyledonous species, like rice, oat, sugarcane and sorghum, produce phenolic phytoalexins too.

Further defence genes encode regulatory proteins and other signalling molecules, important for defence signal transduction. By these molecules, locally induced defence responses generate a general resistance mechanism making uninfected parts of the plant less sensitive to further attack by pathogens, a phenomenon called systemic acquired resistance (SAR).

Salicylic acid is considered one of the key signalling molecules that activate plant defence responses against invading pathogens. Salicylic acid is synthesized from phenylalanine (phenylpropanoid metabolism), even if this pathway cannot explain all the amount of salicylic acid in plant cells, suggesting the presence of an alternative biosynthetic pathway. It has been shown that this simple phenolic metabolite is produced by plants locally, at the site of infection, but it has been also found in the phloem and in uninfected tissues. Preformed salicylates also show important proprieties against insect herbivores. A well-known example is represented by larvae of *Operophtera brumata* in *Salix* leaves. It has been observed that the levels of salicylates is negatively correlated with growth: larvae exposed to leaves rich in these compounds grew slowly and consumed less material, and this suggests that salicylates could be considered as anti-feedants for *O. brumata.*

Abiotic stress-induced phenolic compounds

Pests and pathogens are not the only cause of stress for plants: low or high temperature, low nutrients, HMs and other pollutants are as frequent as pathogen attacks.

A large number of stress-induced phenylpropanoids are derived from the flavonoid skeleton, which is synthesized via CHS by condensation of p-coumaroyl-coenzyme A (CoA) and three molecules of malonyl-CoA (Harborne, 1988).

Many phenylpropanoid compounds are induced in response to wounding or feeding by herbivores. lncreased levels of coumestrol and coumarin are toxic to potential herbivores, causing estrogenic and anticoagulant effects.

Chlorogenic acid, alkyl ferulate esters, and cell wall-bound phenolic esters may act directly as defence compounds or may serve as precursors for the synthesis of lignin, suberin, and other wound-induced polyphenolic barriers (Hahlbrock and Scheel, 1989; Bernards and Lewis, 1992).

Anthocyanins and flavones increase in response to excess visible light, and UV irradiation induces flavonoids (particularly kaempferol derivatives) and sinapate esters in *Arabidopsis* and isoflavonoids and psoralens in other species (Hahlbrock,1981; Li et al., 1993; Lois, 1994).

Nutritional stresses give rise to high concentrations of phenylpropanoids in roots or root exudates; for instance, low nitrogen induces flavonoid and isoflavonoid, whereas low iron levels can increase the release of phenolic acids, presumably to help solubilize metals and facilitate their uptake (Marschner, 1991).

It is known that in plants cultivated in the presence of heavy metals, phenolic compounds, such as hydroxycinnamic acids as well as flavonoids, accumulate and protect plants against metal toxicity (Dai et al., 2006). Thus, an increase of anthocyanins and sinapoyl derivatives was found in red cabbage seedlings cultivated under Cu stress (Posmyk et al. 2008). The protective function of polyphenols derives from the fact that they have a high tendency to chelate heavy metals. In particular, the hydroxyl and carbonyl groups of phenolics can strongly bind Cu and Fe. Moreover, the antioxidant activity of polyphenols is well established in different plants such as black tea (Turkmen et al., 2006), grapes and wines (Paixao et al., 2007) and currants (Chiou et al., 2007).

Phenolic compounds in poplar

Like other plants, *Populus* trees possess sophisticated systems to defend themselves against biotic and abiotic stresses. One of such defence strategies is based on the production and accumulation of a wide range of secondary metabolites and phenolic compounds are the most abundant class among them. Generally, these

shikimate-phenylpropanoid derivatives are classified into four categories: salicylate-derived phenolic glycosides, hydroxycinnamates and their derivatives, flavonoids, and condensed tannins (Chen et al., 2009)

1) . Salicylate-derived phenolic glycosides

Phenolic glycosides are prominently foliar and numerous structural forms have been identified from different species. In *P. tremuloides*, four structurally related phenolic glycosides, salicin, salicortin, tremuloidin and tremulacin, are well characterized. Those phenolic glycosides act as deterrents to a variety of herbivores, mainly insects, and provide protection against pathogens and abiotic stress, such as UV radiation. Phenolic glycosides are also related to CO_2 and ozone levels: elevated CO_2 did not affect salicortin, but increased tremulacin levels, whereas ozone reduced both salicortin and tremulacin levels (Kopper and Lindroth, 2003).

Salicylic acid derivates are also involved, as signalling molecules, in plant defence priming, which is defined as 'a physiological process by which a plant prepares itself for future attacks by herbivores and pathogens' (Frost et al., 2008).

The biosynthesis of these salicylate-derived phenolic glycosides in *Populus* is still poorly understood. These glycoconjugates are supposed to derive from precursors of salicylic acid biosynthesis (Dempsey et al., 1999). After being formed from salicylic acid or from the other biosynthetic precursors, salicin may then be benzoylated to form tremuloidin, esterified to form salicortin, or benzoylated and esterified to form tremulacin.

2) Hydroxycinnamates and their derivatives

Hydroxycinnamates and their derivatives constitute 2–8% of leaf dry weight in *Populus* and the other closely-related taxa, including *Salix* and *Betula* (Chen et al., 2009).

A variety of hydroxycinnamates have also been identified from *Populus* bud exudate. The early investigation in chemical composition of bud exudates of different *Populus* species led to the identification of up to 60 small molecules, among which, more than 70% are different types of hydroxycinnamates and their derivatives.

The principal phenylpropanoic acids in poplar are ferulic acid, isoferulic acid, *p*-coumaric acid, caffeic acid, vanillic acid, *p*-hydroxybenzoic acid, as well as their ester and aldehyde forms are formed by the shikimate/phenylpropanoid pathway (Fig.8).

Many of them are wall-bound phenolic esters or are utilized to synthesize lignin and suberin. Some of them, particularly chlorogenic acid, also act as UV-absorbing compounds to reduce the damage by excessive UV-B radiation.

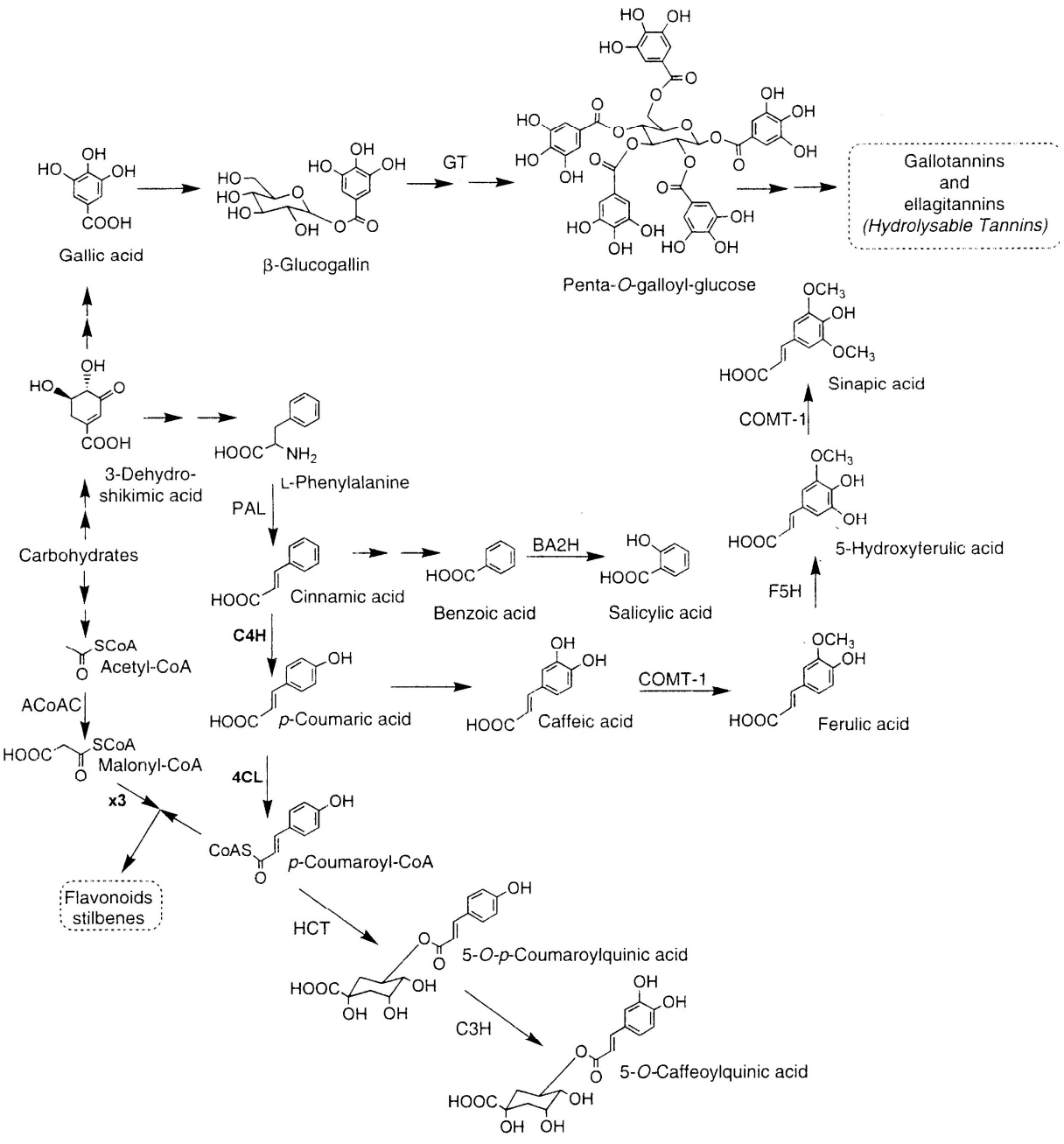


Figure 8. Schematic of the main pathways and key enzymes involved in the biosynthesis of salycilic acid and hydroxycinnamates. Enzyme abbreviatons: PAL, phenyalanine amminia-lyase; BA2H, benzoic acid 2-hydroxylase; CH4, cinnamate 4-hydroxylase; COMT-1, caffeic/hydroxyferulic acid O-methyltransferase; 4CL, p-cumarate:CoA ligase; F5H, ferulate 5-hydroxilase; GT, galloyltransferase; ACoAC, acetylCoA carboxylase

3) Flavonoids and condensed tannins

Populus species contain most of the major classes of flavonoids, including chalcones, dihydrochalcones, flavanones, flavones, dihydroflavonols (flavanonols), flavonols, flavan-3,4-diols, which are the precursors of anthocyanins and proanthocyanidins, and flavan-3-ols, the precursors of proanthocyanidins. The representative compounds found in *Populus* include chrysin, galagnin, pinocembrin, and quercetin derivatives (Chen et al., 2009).

Most flavanones and flavonols are methylated and methylation renders the molecule more lipophilic.

Like hydroxycinnamate derivatives, the occurrence and concentration of flavonoids vary among *Populus* species and clones. Proanthocyanidins, which are also called condensed tannins (CTs), are oligomers or polymers of flavonoid units (flavan-3-ols, also known as catechins; Fig. 9)

The study of condensed tannin synthesis provides evidence that they are important in defence against herbivores. A dihydroflavonol reductase (DFR), which is involved in condensed tannin synthesis, was isolated from trembling aspen. Both the expression of DFR and the concentrations of condensed tannins were significantly increased by insect treatment, suggesting that the induction of condensed tannins may be important for defence against herbivores in *Populus* (Peters and Constabel, 2002). CTs are also involved in *Populus* defence against microorganisms. When *P. trichocarpa* × *deltoides* was infected by *Melampsora medusae* (the leaf rust pathogen), genes encoding enzymes required for CT synthesis were enormously up-regulated. and, late in the infection, CT levels increased in infected leaves (Miranda *et al.,* 2007).

The rise of flavonoids and CTs induced by ozone suggests they are also involved in ozone defence as antioxidants.

Populus spp. are known to have allelopathic properties. For example, when wheat plants are grown under *Populus* plantations, their yield was significantly lower than crops grown under open field conditions. Allelopathy is often attributed to secondary metabolites produced by plants, such as flavonoids , but the exact identities of the responsible allelopathic compounds remain to be identified (Fig.10)

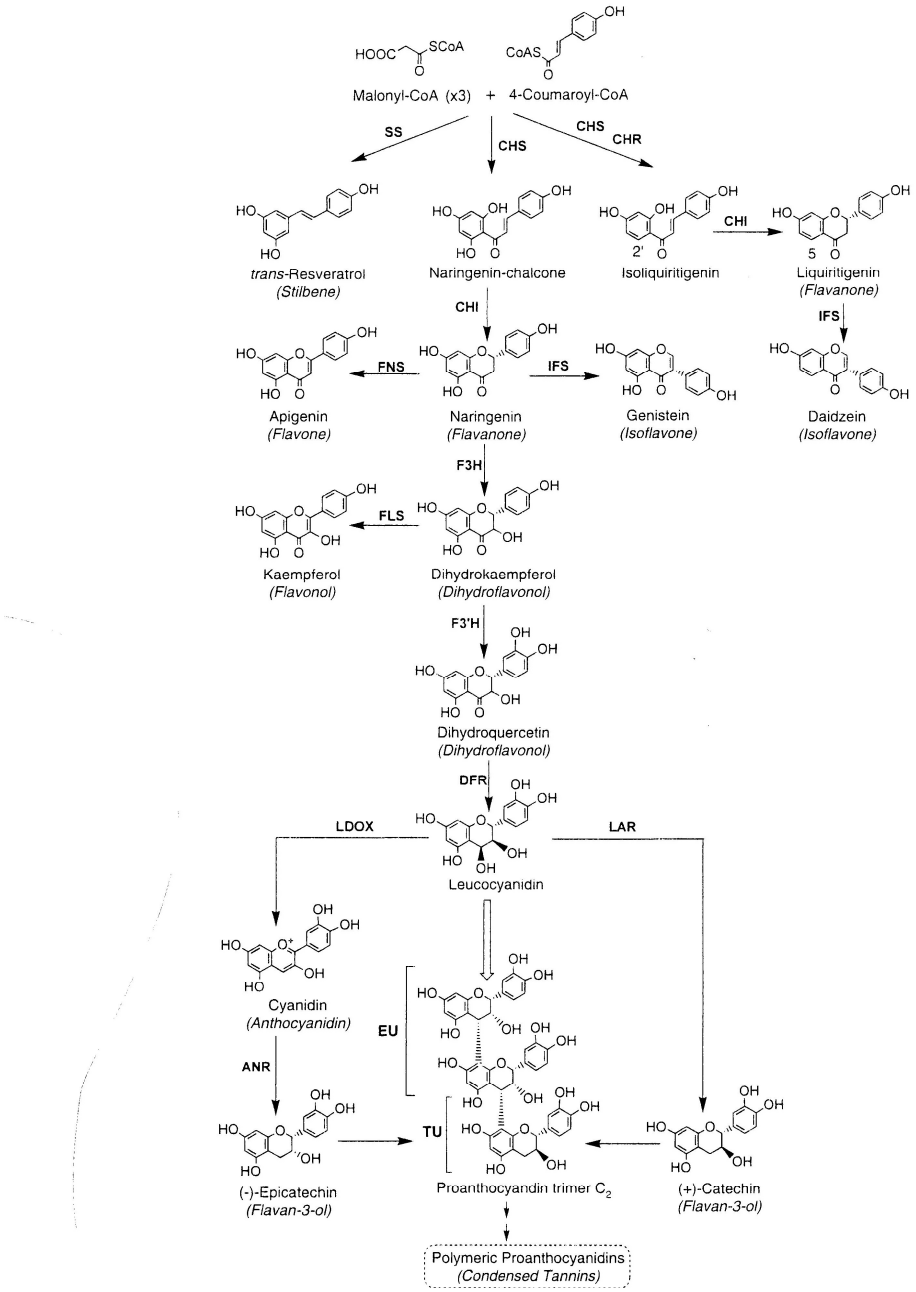


Figure 9. Main pathways and enzymes involved in the production of stilbenes and flavonoids. SS, stilbene synthase; CHS, calcone synthase; CHR, calcone reductase; CHI calcone isomerise; IFS, isoflavone synthase; FNS, flavone synthase; FLS, flavonol synthase, DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin 4-reductase; F3H, flavanone 3-hydroxylase; F3'H, flavonol 3'-hydroxylase; LAR, leucocyanidin 4-reductase; LDOX, leucocyanidin deoxygenase; ANR, anthocyanidin reductase

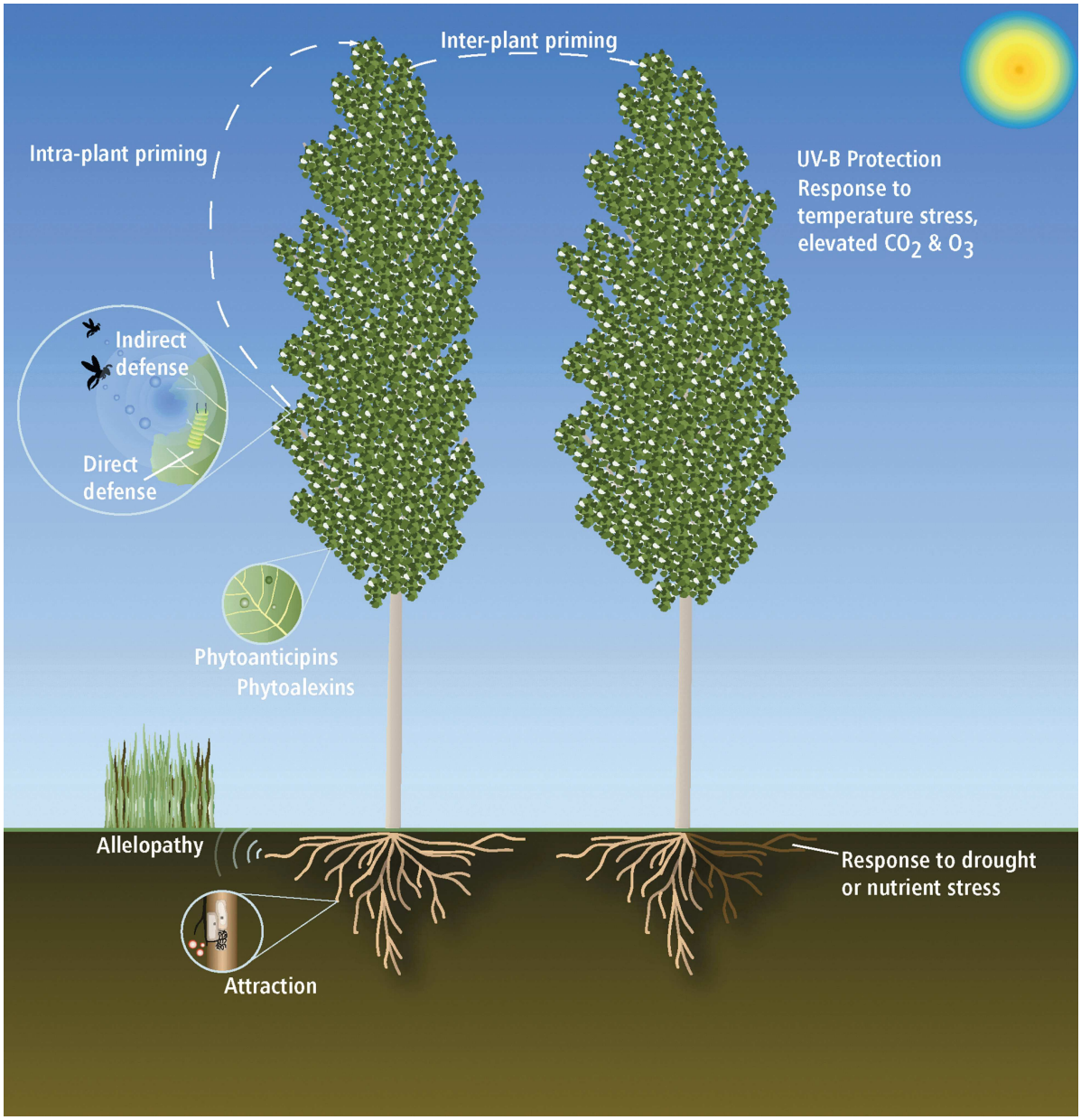


Fig.10. Functions of phenolic compounds in Populus-environment interactions

Polyamines

The aliphatic polyamines (PAs), of which putrescine (Put), spermidine (Spd) and spermine (Spm) are the most common, are considered as one of the oldest group of substances known in biochemistry and the tetramine Spm was discovered more than 300 years ago in human spermatozoa by Van Leeuwenhoek in 1678. Nowadays, PAs are believed to be ubiquitous in all cells of both eukaryotes and prokaryotes (Pegg and Michael 2009).

Their biological function was initially supposed to stabilize macromolecular structures because of their capability of binding different anionic macromolecules (DNA, RNA, chromatin and proteins). It was later confirmed that, in addition to this function, PAs act as regulatory molecules in many metabolic processes (Kusano, 2008).

It is also well-documented that PAs are present in all compartments of the plant cell, including the nucleus, which indicates their role in the control of diverse fundamental cellular processes (Kaur-Sawhney et al., 2003). Indeed , PAs have been implicated in cell division and differentiation processes, such as organogenesis, embryogenesis, floral initiation and development, leaf senescence, fruit development and ripening (Ziosi et al. 2006); moreover, they are involved in abiotic and biotic stress responses (Kusano, 2008; Alcazar et al., 2006). Also in plants, their biological activity is attributed to their cationic nature. PAs interact with negatively charged macromolecules, such as DNA, RNA, membrane phospholipids and anionic cell wall components; there is increasing evidence that they are also able to modulate the activity of some ion channels ((Kusano et al., 2007, Garufi et al., 2007).

Polyamines are low molecular weight aliphatic compounds, positively charged at physiological pH, having two primary terminal amino groups -NH_2, as shown from the following formulas:

1,3-diaminopropane $H_2N\text{-}(CH_2)_3\text{-}NH_2$

1,4-1,4-diaminobutane (putrescine) $H_2N\text{-}(CH_2)_4\text{-}NH_2$

1,5-pentanediamine (cadaverine) $H_2N\text{-}(CH_2)_5\text{-}NH_2$

1,8-diamino-4-azaoctane (spermidine) $H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH_2$

1,12-diamino-4,9-diazododecane (spermine)

$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH\text{-}(CH_2)_3\text{-}NH_2$

Availability of the complete genome sequence for *Arabidopsis* has facilitated the use of genomic approaches for identification and isolation of genes encoding PA biosynthetic enzymes (Liu et al., 2007). In *Arabidopsis*, there are six enzymes

responsible for PA biosynthesis encoding 10 genes: arginine decarboxylase (ADC)-encoding genes (ADC1 and ADC2 Watson et al., 1998), spermidine synthase (SPDS) (SPDS1 and SPDS2 Hanzawa et al., 2002), S-adenosylmethionine decarboxylase (SAMDC) (SAMDC1, SAMDC2, SAMDC3, SAMDC4 Franceschetti et al., 2001; Urano et al., 2003), and spermine synthase (SPMS1 and SPMS2). This complex metabolic pathway leads to the synthesis of free PAs. An additional route conjugates PAs to other molecules, most of them belonging to the phenolic compound class, leading to phenylamides (PhA, see next chapter).

Polyamine biosynthesis in plants

The biosynthesis of PAs starts with the formation of the diamine Put. The latter is formed from ornithine in a reaction catalysed by ornithine decarboxylase (ODC) or from arginine in a reaction catalysed by ADC (fig. 11).The ODC pathway is favoured in meristematic and dividing cells, while the ADC pathway predominates in mature tissues and in response to environmental stress (Flores, 1991).

The higher polyamines Spd and Spm are synthetized from Put by the enzymes SPDS and SPMS, respectively, which add aminopropyl groups coming from decarboxylated S-adenosylmethionine (dcSAM). DcSAM is synthesized from SAM by the action of SAMDC.

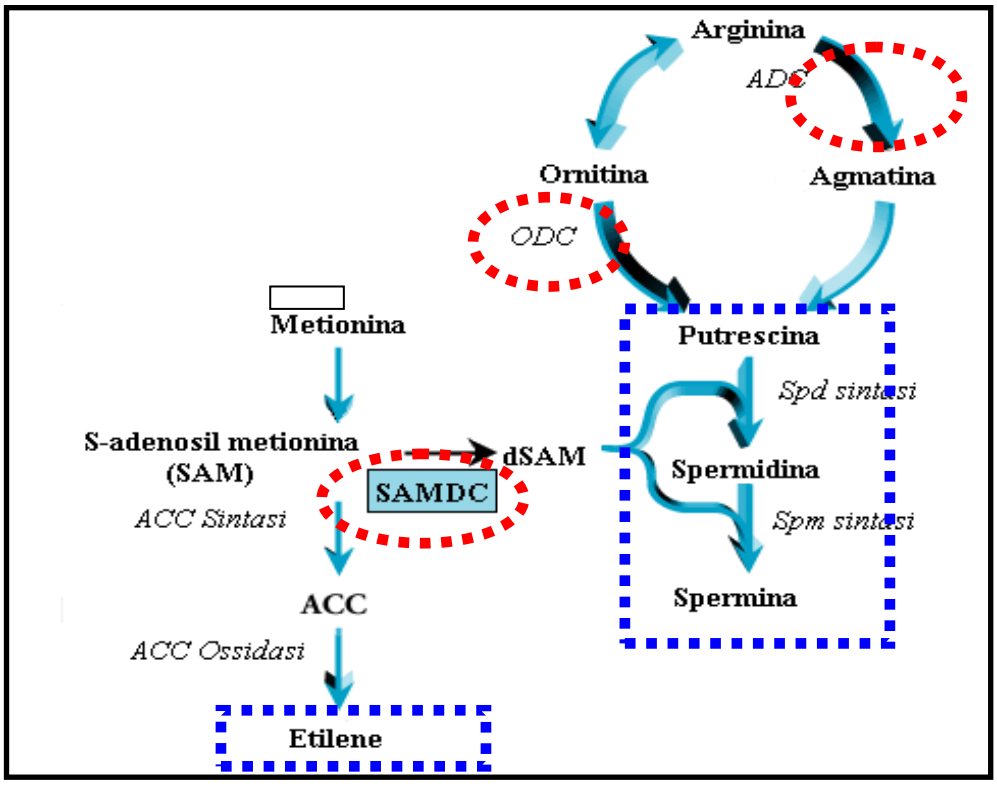


Figure 11. Polyamine metabolism and interaction with ethylene pathway.

As shown in Figure 11, PAs and ethylene share a common precursor (SAM), but polyamine metabolism also interacts with other metabolic routes, such as the proline and urea pathway (Fig.12) and tropane alkaloid biosynthesis (typical of the Solanaceae) via the formation of methylputrescine by putrescine methyltransferase.

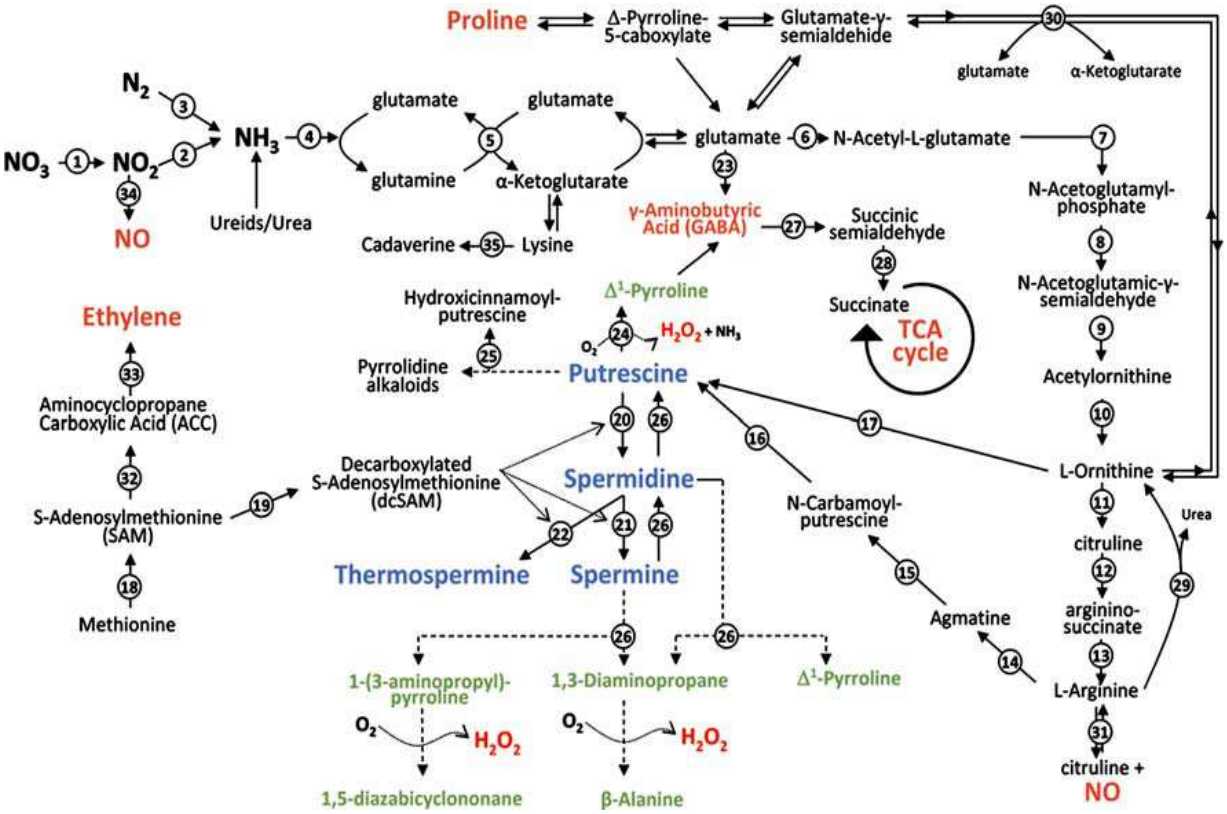


Figure12. Polyamine metabolism and interaction with other metabolic routes. Biosynthetic pathways for polyamines and related metabolites are indicated by continuous lines. Dashed lines show the formation of putrescine-derived alkaloids, polyamine conjugation and catabolic processes. Numbers refer to the following enzymes: 1 nitrate reductase, 2 nitrite reductase, 3 nitrogenase, 4 glutamine synthetase, 5 glutamate synthase, 6 glutamate N-acetyltransferase, 7 acetylglutamate kinase, 8 N-acetyl-γ-phosphate reductase, 9 acetylornithine transaminase, 10 acetylornithine deacetylase, 11 ornithine-carbamoyl transferase, 12 arginosuccinate synthase, 13 arginosuccinate lyase, 14arginine decarboxylase, 15 agmatine iminohydrolase, 16 N-carbamoylputrescine amidohydrolase, 17 ornithine decarboxylase, 18 SAM synthetase, 19 SAM decarboxylase, 20 spermidine synthase, 21 spermine synthase,22 thermospermine synthase, 23 glutamate decarboxylase, 24 diamine oxidase, 25 putrescine hydroxycinnamoyl transferase, 26 polyamine oxidase, 27 γ_aminobutyrate aminotransferase, 28 succinic semialdehyde dehydrogenase, 29 arginase, 30 ornithine aminotransferase, 31 nitric oxide synthase, 32 ACC synthase, 33 ACC oxidase, 34 nitrate reductase, 35 lysine decarboxylase. It has been shown recently that polyamine oxidase (26) is not only involved in the terminal catabolism of polyamines, but also in the back-conversion of spermine to spermidine and spermidine to putrescine.

PA catabolism occurs through the activity of diamine oxidases (DAO) and polyamine oxidases (PAO). DAOs are copper-containing enzymes that catalyse the oxidation of the diamines Put and Cad at the primary amino groups. The reaction products from Put are 4-aminobutanal, H_2O_2 and ammonia.

PAs and plant growth and development

In bacteria and animals, PAs are described as increasing cell growth and being essential for organ functionality. Although this is less clearly established in plants evidence is accumulating for the essential role of PAs in most aspects of plant growth and development (Takahashi, Kakehi, 2010).

An interesting example is the case of thermospermine. An *A. thaliana* mutant (*ACAULIS5*), initially though to be deficient in SPMS, shows a very severe dwarf phenotype and xylem over-proliferation (Hanzawa et al., 2000). It was later shown that the mutant phenotype is rescued by exogenous application of thermospermine, but not Spm (Kakehi et al., 2008). Thermospermine is a structural analogue of Spm, first discovered in thermophilic bacteria and now known to be present also in some plants. Thus, the Arabidopsis gene (*ACL5*) first regarded as encoding SPMS actually encodes thermospermine synthase, and it appears that is is essential for xylem differentiation and stem elongation growth (Takahashi, Kakehi, 2010).

On the other hand, Spd appears to be essential for plant embryogenesis because this PA is a precursor of deoxyhypusine which is essential for growth and proliferation of eukaryotic cells (Park, 2006).

It is becoming increasingly clear that PAs could also exert their biological activities through the crosstalk with almost all of the major plant hormones, and not only with ethylene. Indeed, expression of PA biosynthesis genes and PA levels are altered in response to treatment with cytokinins, auxins, abscisic acid (ABA) gibberellins, jasmonates and brassinosteroids (Hanzawa et al., 2002; Biondi et al., 2001; Urano et al., 2004; Imai et al., 2004a; Muñiz et al., 2008; Cui et al., 2010, Choudhary et al., 2010). The reverse is also true: whereas ABA and cytokinin biosynthesis is induced by PAs (Cuevas et al., 2009; Wang et al., 2009; Cui et al., 2010), ethylene and gibberellin production are downregulated (Alcázar et al., 2005; Hu et al., 2006). Accordingly, the interaction between different hormones might be mediated in part by PAs that would function as secondary messengers (Hanzawa et al., 2002).

Cyclin-dependent kinases (CYCD3 proteins), important regulators of cell cycle progression, have been identified as essential “sensors” for cytokinins and auxins (Dewitte et al., 2007); recent evidence suggests that they also function as sensors for Put, and that transcription activation of cell cycle genes seems to be an additional mode of action of Put to stimulate cell division and organogenesis (Stes et al., 2011).

As recently reported by Cui et al. (2010) the Arabidopsis mutant *bud2* displays altered root and shoot architecture, resulting from loss-of-function of a SAMDC gene (*SAMDC4*).The mutation of BUD2 results in hyposensitivity to auxin and hypersensitivity to cytokinin, suggesting that PAs may be involved in regulating plant sensitivity to these two essential hormones.

In addition, PAs have direct and indirect effects on plant development via other mechanisms, for example electrostatic binding to anionic macromolecules, including cell wall components (e.g. pectins), and interaction with ion channels and receptors, resulting in regulation of Ca^{++}, Na^{+} and K^{+} homeostasis, particularly during salt stress (Janicka-Russak et al., 2010)

The action of PAs on cation channels depends on their net positive charge with spermine > spermidine >> putrescine. Production of H_2O_2 upon PA catabolism is another level of regulation of plant growth and development. PAOs were shown to be developmentally regulated and associated to cell-wall strengthening, lignification and programmed cell death (Cona et al. 2006; Moschou et al., 2008). In plants, senescence of different organs can be delayed by PAs. Their role in developmental cell death is best documented in the case of the *Nicotiana tabacum* flower corolla (Della Mea et al., 2007).

Polyamines and plant responses to abiotic stresses

Many studies in different plant species have shown that PA accumulation occurs in response to several adverse environmental conditions, such as salinity, drought, hypoxia, ozone, extreme temperature, UV-B and UV-C, heavy metal toxicity, mechanical wounding and herbicide treatment (Alcázar et al., 2006; Groppa e Benavides, 2008). The physiological meaning of these responses remains not well understood; in particular, it is not clear if stress-induced PA levels contribute to the injury or are a protective response to abiotic stress.

Enhanced stress tolerance is always correlated with elevated levels of Put and/or Spd and Spm and either classical approaches, using exogenous PA application, or

more recent studies, using transgenic overexpressing or loss-of-function plants, seem to point at a protective role of PAs in plants (Alcázar et al., 2006; Kusano et al., 2008; Gill and Tuteja, 2010).

There are several reports which suggest that exogenous application of PAs may, in varying degrees, preserve plant cell membrane integrity, minimize growth inhibition caused by stress, modulate expression of osmotically responsive genes, reduce superoxide radical and H_2O_2 contents, reduce accumulation of Na^+ and Cl^- ions in different organs, and increase activities of antioxidant enzymes (Hussain et al., 2011). Results suggest that exogenous PA application acts as an elicitor of genes involved in abiotic stress responses (Gill and Tuteja, 2010). However, the protective effects of individual PAs are somewhat different. The reason for such a discrepancy may be due to differences in absorption, transport and utilization among plant species.

The results obtained from loss-of-function mutations in PA biosynthetic genes support the protective role of PAs in plant response to abiotic stress, as well. For example, mutants of *Arabidopsis* displaying reduced ADC activity are deficient in PA accumulation after acclimation to high NaCl concentrations and exhibit more sensitivity to salt stress (Kasinathan and Wingler, 2004).

There are also several works dealing with transgenic plants over-expressing PA biosynthetic genes and their response to different types of stress. For example, transgenic rice plants, expressing the *Datura stramonium* ADC gene, produced higher levels of Put under drought stress than wild type plants, which led to higher levels of Spd and Spm and improved drought tolerance (Capell et al., 2004).

PA biosynthesis is under complex metabolic control which is necessary for efficient regulation of cell metabolism. In *Arabidopsis*, differential expression of ADC genes has been reported under stress conditions. In fact, *ADC2* expression is strongly induced by several abiotic stresses like drought, high salinity, mechanical injury and potassium deficiency (Alcazar, 2006).

Generally, genome sequencing has allowed to characterize transcription responses of the polyamine biosynthetic pathway in *Arabidopsis* subjected to drought, salt and cold treatments (Fig.13).

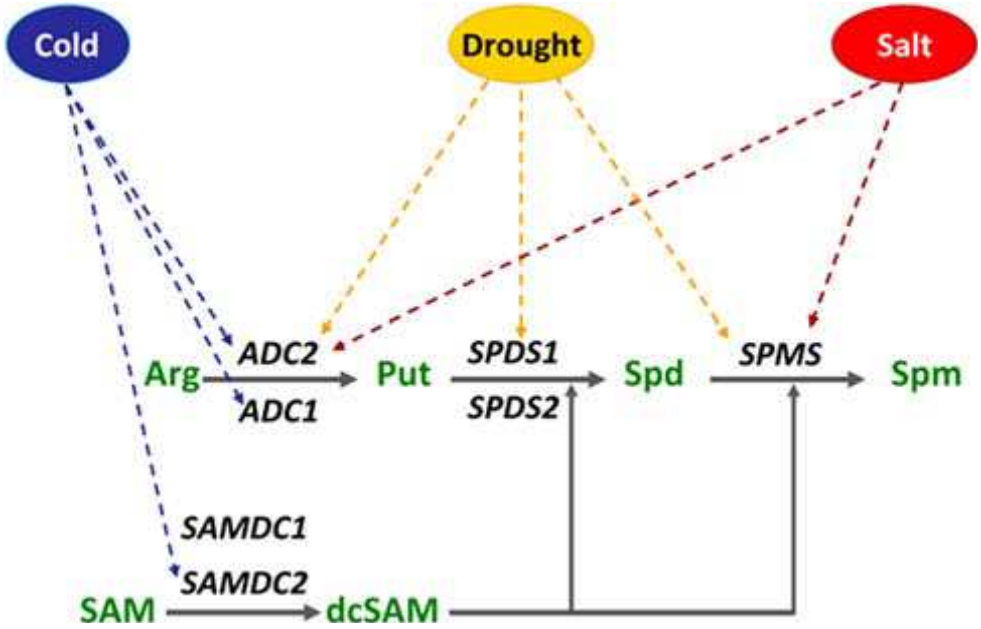


Figure 13 Effects of drought, salt and cold treatments on polyamine biosynthesis in Arabidopsis. Drought induces the expression of ADC2, SPDS1 and SPMS genes. The levels of Put increase, whilst Spd and Spm contents do not increase above basal levels (Alcázar et al. 2006). Salt treatment induces the expression of ADC2 and SPMS genes and results in increased Put and Spm levels (Urano et al. 2003). Cold induces the expression of ADC1, ADC2 and SAMDC2 genes. The levels of Put increase on cold treatment and this correlates with the induction of ADC genes. The content of Spd and Spm remain constant or even decrease in response to low temperature. The absence of correlation between enhanced SAMDC2 expression and the decrease of Spm levels has been suggested to be due to an increase in Spm catabolism (Cuevas et al. 2008).

Some recent studies indicated that PAs, because of their interaction with different metabolic routes, may act as cellular signals in hormonal pathways including abscisic acid (ABA) (Alcázar et al., 2010; Gill and Tuteja, 2010). Enhanced biosynthesis of ABA occurs in response to water deficit, but it has also been reported that Put, Spd and Spm regulate stomatal responses by reducing their aperture and inducing closure. It is known that some genes involved in PA biosynthesis are regulated by ABA, but, on the other hand, Put controls the levels of ABA in response to cold by modulating ABA biosynthesis at the transcriptional level (Cuevas et al. 2008; 2009). These results suggest that Put and ABA are integrated in a positive feedback loop, in

which ABA and Put reciprocally promote each other's biosynthesis in response to abiotic stress.

Under stress conditions, the level of PAs in plant organs could also be regulated by its catabolic pathway by generation of ROS (Cona et al., 2006). The PA catabolic process generates H_2O_2, which was often considered a toxic metabolite. Recently, however, H_2O_2 is considered a signal molecule which is able to spread from the site of production to neighbouring cells and tissues, activating the plant defence and stress responses.

Furthermore, PAs are reported to promote the production of nitric oxide (NO) in *Arabidopsis* (Tun et al. 2006). Both H_2O_2 and NO are involved in the regulation of stomatal movements in response to ABA, in such a way that NO generation depends on H_2O_2 production (Neill et al. 2008).

H_2O_2 from DAO-catalysed Put oxidation in guard cells has also been reported to increase the Ca^{2+} concentration in guard cells which modulates the stress signalling pathways controlling stress tolerance. This increase of cytosolic Ca^{2+} levels may result from apoplastic source but also from hydrolysis of phosphatidylinositol biphosphate (PIP2) to inositol trisphosphate (IP3) which releases Ca^{2+} from intracellular stores (Mahajan and Tuteja 2005). In guard cells, the increase in cytosolic Ca^{2+} may activate different ion channels and induce stomatal closure. Briefly, PAs appear to regulate stomatal closure by activating the biosynthesis of signalling molecules (H_2O_2 and NO) through different routes, and by acting synergistically with H_2O_2 in promoting ABA responses in guard cells (Fig.14)

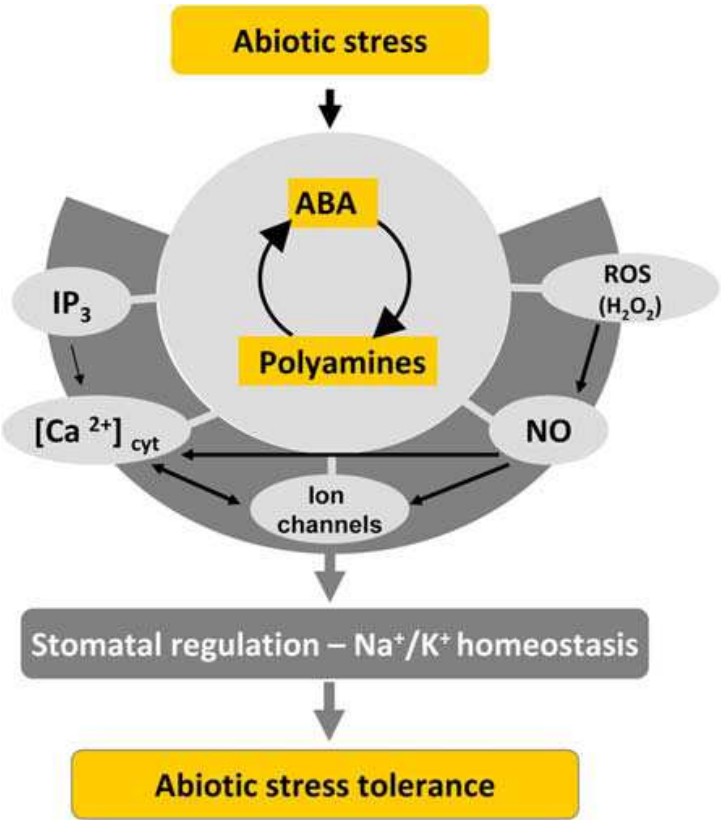


Figure 14. Simplified model for the integration of polyamines with ABA, ROS (H_2O_2), NO, Ca^{2+} homeostasis and ion channel signalling in the abiotic stress response (Alcazar et al., 2010)

PAs have also been investigated in the heavy metal stress response. Generally, in plants under heavy metal treatment, the endogenous PA levels increase, even if less compared with other abiotic stresses. A study on oat plants with six heavy metals (Cr, Co, Cu, Hg, Ni, Ag), has shown that the extent of increase depends on the metal (Wettlaufer et al., 1991).

Zn (less toxic) induces an early PA response in white poplar, especially in the presence of a high level of the metal while in Cu (more toxic)-treated shoots, the response in terms of both free and conjugated PA accumulation occurs after a more prolonged exposure (Franchin et al., 2007). In general, the less toxic metal stimulated an early PA response while the more toxic one did not, as also shown in soybean roots, where the less toxic/lower Cd concentration caused an increase in Put levels while with the higher one this increase did not occur (Balestrasse et al. 2005)

The molecular basis for the action of PAs in heavy metal stress has not been clarified, but there is evidence that they can act at several metabolic levels. Redox-active metals produce ROS and plants not only accumulate free PAs as scavengers of free radicals, but also produce their conjugated PAs (see below) which are more efficient antioxidants (Edreva et al., 2007). Evidence regarding the antioxidant role of PAs comes from the observation that exogenous application of PAs reduced H□O□ levels and malonyldialdehyde (MDA) content, and raised the antioxidant level in 15 day-old chickpea plants subjected to drought and cold stress for 4 days (Nayyar and Chander, 2004).

PAs have also been suggested to function as metal chelators (Lovaas 1996) and to facilitate metal ion compartmentation (Brueggemann et al. 1998).

PAs are also involved in inhibition of DNA oxidative degradation by ˙OH hydroxyl radicals as shown by an *in vitro* experiment. Total DNA from *Mesembryanthemum crystallinum* is degraded when incubated with an ˙OH generating system, and addition of Spm suppressed DNA damage (Kuznetsov and Shevyakova, 2007).

Polyamines and plant responses to biotic stresses

While our recent knowledge about PA metabolism in plants subjected to abiotic stresses has quickly increased, work on polyamine metabolism in plant-pathogen interactions is still relatively behind.

Nevertheless, PAs have long been known to be involved in the response to fungal (Asthir et al., 2004) and viral (Torrigiani et al., 1997) attacks.

In leaves, accumulation of PAs, particularly Spd, was found in compatible interactions between barley and *Puccinia hordei* (Greenland and Lewis, 1984). Polyamine accumulation has also been reported in leaves of barley following infection by powdery mildew fungus (*Blumeria graminis f. sp. hordei*) (Walters and Wylie, 1986; Walters et al., 1985).

In plants, programmed cell death (PCD) can occur following both pathogen attack and abiotic stress and there is substantial literature available indicating the involvement of PAs in apoptotic cell death in both animals and plants (Ha et al., 1997; Lindsay and Wallace, 1998). PA degradation seems to play an important role in apoptosis in plants. In fact, an increase in PA catabolism by DAO and PAO was observed in an incompatible interaction between barley and *Blumeria graminis* (Crowley and Walters, 2002). Similarly, it has been demonstrated (Yoda et al., 2003)

that cell death in tobacco plants infected by tobacco mosaic virus (TMV) is partially mediated by H□O□ production through PA catabolism.

Spm also seems to play an important role in the tobacco-TMV pathosystem. Tobacco *ZFT1* is a Spm-responsive gene involved in Spm-signalling pathway and tobacco plants over-expressing this gene are more resistant to TMV than control plants (Mitsuya et al., 2007). Thus, PAs in their free form are involved in the response to pathogens at different metabolic levels.

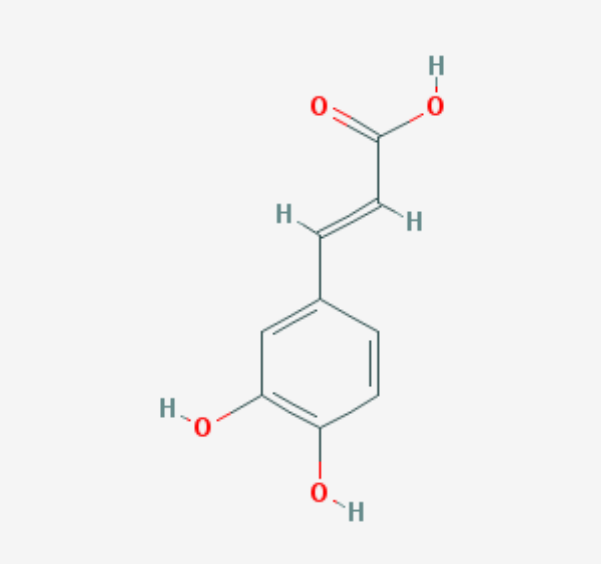
Phenylamides

Phenylamides (PhA), also termed phenolamides or hydroxycinnamic acid amides, are low-molecular weight products formed by the conjugation of hydroxycinnamic acids with polyamines (therefore also known as soluble conjugated PAs) or deaminated aromatic aminoacids through a covalent bound between carboxylic groups and amino groups (Fig.14).

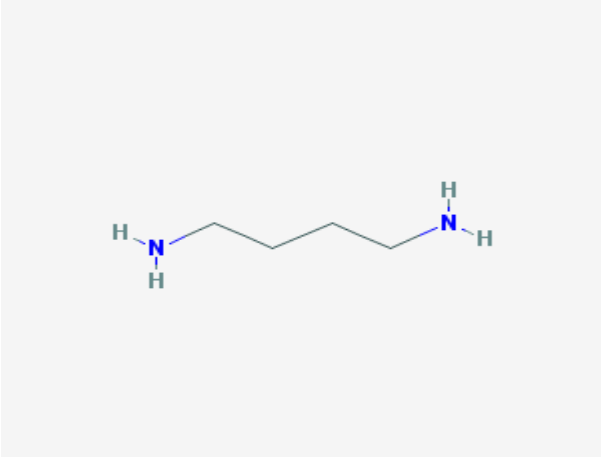
In plants, the most widely distributed hydroxycinnamic acids of PhA are caffeic, ferulic, and *p*-coumaric acids, while the aliphatic polyamines Put, Spd, and Spm, and the arylmonoamines tyramine and tryptamine are the predominant components of PhA (Martin-Tanguy, 1985).

Phenylamides, as conjugates of hydroxycinnamic acids and amines, combine the proprieties of either components which confer them a wide range of chemical and metabolic functions.

A)



B)



C)

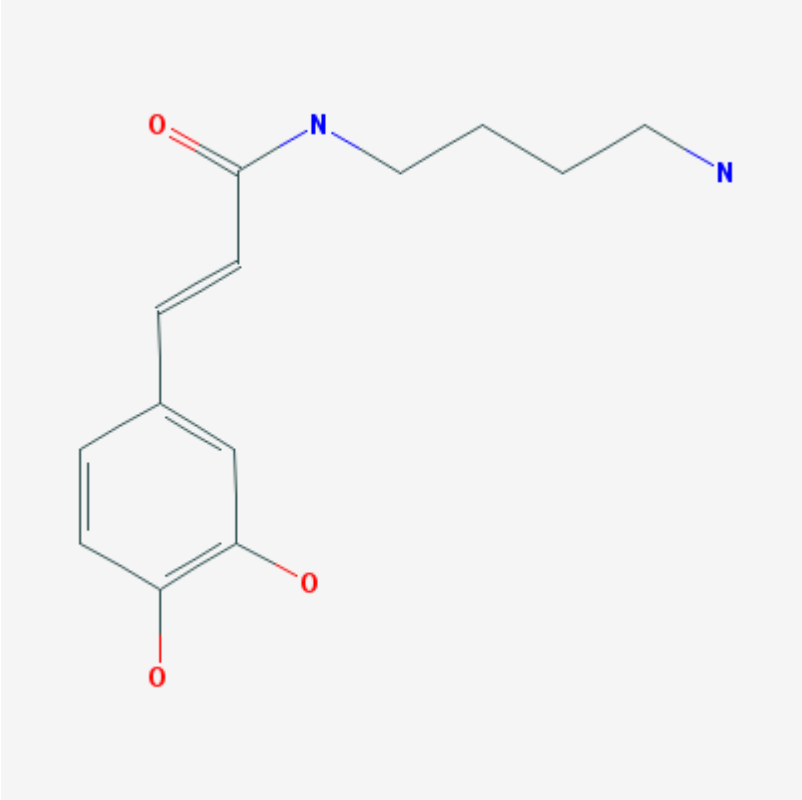


Fig.14. A) Caffeic acid, B) Putrescine, C) N-Caffeoylputrescine

Aliphatic PAs polyamines, carrying two terminal amino groups protonated, positively charged at physiological pH, can confer basic proprieties depending on the type of conjugate formed. Based on these preliminary remarks, we can classify PhA in two categories:

a) Basic. The basic character is determined by the availability of one protonated free amino group in their molecule. Monosubstituted aliphatic PAs, such as e feruloylputrescine, are referred to this class of PhA.

b) Neutral. Disubstituted aliphatic PAspolyamines, such as diferuloylputrescine, belong to this class and there are no free amino groups. Derivatives of arylmonoamines, such as *p*-coumaroyltryptamine and feruloyltyramine in which the only amino group is substituted, also behave as neutral compounds.

Basic PhA, because of their positive charge, act like organic cations forming electrostatic interactions with negatively charged residues in nucleic acids, proteins, membranes and cell walls, much as free PAs do (Martin-Tanguy, 1997).

The availability of one free amino group in the basic PhA, confers polar character and significant water solubility to the conjugates. The substitution of this group with

hydroxycinnamic acids in neutral PhA reduces polarity and water solubility, but enhances lipophilic proprieties.

The phenolic proprieties in all PhA conjugates provide interactions with compounds containing hydroxyl groups and mono- and polyhydroxyaliphatic compounds (alcohols, carbohydrates), as well as polymers, such as lignin and suberin, containing residues of aromatic alcohols.

The ability of both basic and neutral PhA to bind to polysaccharides, lignin, and suberin, can contribute to build-up a complex cross-linked network of different cell-wall polymers (Passardi and Penel, 2004). These events, leading to rigidification of the cell wall, are related to the decrease in wall extensibility and termination of cell elongation. They also enhance resistance of cell wall to physical, chemical, and enzymatic breakdown. Thus, PhA can be implicated in the molecular mechanisms of regulation of plant growth and stress responses.

The involvement of PhA in growth and development is well supported by different works. A correlation was found between the spatial distribution of PhA conjugates and the embryogenic capacity of leaf explants of *Solanum melongena* (Yadav and Rajam, 1997). Basic and especially neutral PhA, such as di-feruloylputrescine, di-feruloylspermidine, feruloyl tyramine have been detected in large amounts upon seed development and maturation in monocots like maize and rice (Martin-Tanguy, 1985; Bonneau et al., 1994).

PhA were also supposed to be a necessary component of the mobile signal to flower development (Tarenghi and Martin-Tanguy, 1995; Havelange et al., 1996). During floral initiation, PhA accumulate in the last initiated leaves and apices, but when the plant begins flowering PhA accumulate in the floral organs and disappear from the leaves.

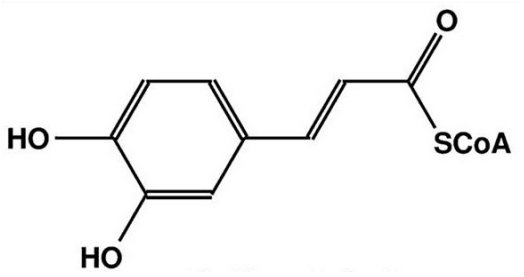
Conjugation can affect the binding and interactions of the parent compounds, influencing their proprieties and functions. Conjugation with aliphatic PAs can moderate the high cytotoxicity of free hydroxycinnamic acids because of the lower lipophilic character of the acids after the conjugation, minimizing the potential membrane damage. Conjugation could also moderate the competition between polyamines and Ca^{2+} for common sites on membranes and may also interfere with other polyamine functions, such as regulation of K^{+} channels and stomatal movements (White and Broadley, 2003).

1) Biosynthesis, transport and distribution of PhA

PhA are largely distributed, being found throughout the plant kingdom; they also occur in all plant organs, that is in roots, stems, leaves, flowers, as well as in tubers, fruits and seeds (Bregoli et al, 2002; Edreva, 2007). A large amount of PhA was detected in the reproductive organs in which they represent the main phenolic constituents. PhA are widely present in anthers and ovaries but not in calyx and corolla.

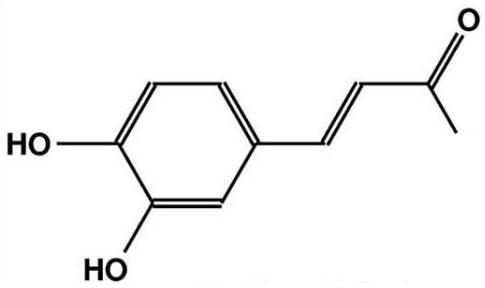
In the plant cell, PhA conjugates can occur in a soluble form, or bound to subcellular structures. Water-soluble basic PhA conjugates are abundantly present in the apoplastic fluid, but as cations they can be electrostatically bound to negatively charged sites in nuclei, membranes, and cell walls. Neutral PhA are located in cell walls bound to polysaccharides, lignin, and suberin.

The primary step in the biosynthesis of PhA is the activation, catalyzed by 4-coumarate CoA ligase, of hydroxycinnamic acid carboxylic groups by esterification with CoA. The ultimate biosynthetic event, i.e., the condensation of hydroxycinnamic CoA thioesters with PAs and arylmonoamines, is catalyzed by transferases specific to the amine substrate(Fig.15).



+NH□(CH□)4NH□

Caffeoyl – CoA **Putrescine**



NH-(CH□)4-NH□+CoA~SH

Caffeoylputrescine

Figure 15. Interaction of caffeoyl-CoA with putrescine yielding caffeoylputrescine

The biosynthesis of PhA appears to occur in the cytosol, but little is known about the transport mechanism from the cytosol to the cell wall. Probably, PhA are synthesized in the cytosol and sequestered to specific vesicles, which are then transported to the

plasma membrane to allow the deposition of PhA in the cell wall. A role for glutathione S-transferases as amide carrier proteins in the transport was proposed, in a similar way to the involvement of these enzymes in the transport of anthocyanins from cytosol to vacuoles (Facchini et al., 2002, Edwards et al., 2000).

The enzymatic control of the linking of PhA to the cell walls is poorly understood. A study about the incorporation of feruloyl esters into cell walls has shown that the primary step is the oxidation of feruloyl derivatives to 5,5'-dehydrodiferulate and the process is catalyzed by the peroxidase-H□O□ system. The dimer of ferulate is then attached to the cell wall by etheric bonding of hydroxyl group of ferulate with cell wall polysaccharides (Wallace and Fry, 1995). A similar pathway for binding PhA to cell walls can be supposed, because PhA are good substrates for peroxidase.

2) *Phenylamides in response to biotic stress*

Phenolamides are often described as bioactive compounds with antiviral, antibacterial, antifungal, insecticidal, deterrent or therapeutic activities (Bassard et al., 2010). The assumption that PhA can be important components in plant–pathogen interactions and resistance expression followed from experiments of Martin-Tanguy et al. (1976) with tobacco genotypes differentially reacting to TMV. Leaves of tobacco cv. Xanthi n.c., carrying the *NN* gene for TMV resistance, developed small local necrotic lesions (hypersensitive reaction, HR) and the virus remained confined in the lesions. A large amount of feruloylputrescine, diferuloylputrescine, and feruloyltyramine was found in the living cells around the lesions. On the other hand, in the susceptible (*nn*) cv. Samsun, the virus spreads systemically throughout the plant causing mosaic symptoms on the leaves and no accumulation of PhA was observed (Martin-Tanguy et al.,1976, 1981; Torrigiani et al. 1997).

PhA appear also to be involved in the resistance to fungal pathogens based on the formation of cell wall barrier structures. Feruloyl-3'-methoxytyramine, feruloyltyramine, and *p*-coumaroyltyramine were detected as fluorescing material in granular deposits and in cell walls of epidermal onion cells attacked by *Botrytis allii* (McLusky et al., 1999) and after *Phytophthora parasitica* elicitation in *Dianthus caryophyllus* (Ponchet et al.,1882).

3) *Phenylamides in response to abiotic stress*

In the early seventies, an induction of PA conjugates following abiotic stress was found for the first time (Deletang,1974). A remarkable accumulation of caffeoylputrescine, caffeoylspermidine and dicaffeoylspermidine was detected in leaves of tobacco plants deficient in K, Ca, Mg, and P, which did not contain PhA when grown under optimum nutrient conditions.

Later, PhA were reported to accumulate under different abiotic stress situations.

Experiments with bean plants subjected to high temperature stress, have shown an abundant accumulation of PAs and PhA (Edreva et al., 1998); caffeoylputrescine and feruloylputrescine were found in tobacco cell suspension cultures under sulphur-lacking conditions (Klapheck, 1883); in leaves of Oriental tobacco plants, formation of conjugates of Put and Spd with caffeic and ferulic acids was detected as a response to water excess stress (Edreva et al., 1995). More recently, over-accumulation of soluble conjugated PAs has been observed in poplar plants exposed to high concentrations of Zn and/or Cu (Franchin et al., 2007; Castiglione et al. 2009).

The mechanisms of the putative protective role of plant PhA against biotic and abiotic stresses remain largely obscure, but many experimental works support the idea they play a structural role, strengthening cell walls in response to pathogen attack (Hahlbrock e Scheel 1989), and that they act as antioxidants (Edreva et al. 2007).

Scavenging properties of PhA against hydroxyl, superoxide and hydroperoxyl free radicals were long ago demonstrated *in vitro* (Bors et al., 1989) but may also be operative *in vivo*. Higher antiradical activity of PhA, compared to the hydroxycinnamic acid parent components, was reported (Son and Lewis, 2002) and it could be supposed that the scavenging activity of the hydroxycinnamic acid moiety is strengthened by PA proprieties. For example, by forming a ternary complex with phospholipids and Fe3+, PAs decrease the Fe3+-catalyzed oxidation of Fe2+, thus limiting the oxidative damage caused by Fe2+ ions, initiators of chain free radical reactions and lipid peroxidation (Tadolini, 1989).

Oxidative stress and antioxidant defences

Oxigen

Atmospheric oxygen naturally exists as a diradical in its ground state as triplet oxygen. The high reactivity of atmospheric oxygen is due to its diradical state due to two unpaired electrons. This feature makes oxygen very unlikely to participate in reactions with organic molecules unless it is "activated". The requirement for activation occurs because the two unpaired electrons in oxygen have parallel spins. According to Pauli's exclusion principle, this precludes reactions with a divalent reductant, unless this reductant also has two unpaired electrons with parallel spin opposite to that of the oxygen, which is a very rare occurrence. Hence, oxygen is usually non-reactive to organic molecules which have paired electrons with opposite spins. This spin restriction means that the most common mechanisms of oxygen reduction in biochemical reactions are those involving transfer of only a single electron (monovalent reduction). Activation of oxygen may occur by absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or monovalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins (Fig. 16). This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction). Since paired electrons are common in organic molecules, singlet oxygen is much more reactive towards organic molecules than its triplet counterpart. A second mechanism of activation is by monovalent reduction of oxygen to form superoxide (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and finally water. The first step in the reduction of oxygen to superoxide is endothermic but subsequent reductions are exothermic. Superoxide can act as either an oxidant or a reductant; it can oxidise sulphur, ascorbic acid or NADPH; it can reduce cytochrome c and metal ions.

Triplet Oxygen (ground state)	·O-O·
Singlet Oxygen	O-O :
Superoxide	·O-O :
Perhydroxyl Radical	·O-O : H
Hydrogen Peroxide	H : O-O : H
Hydroxyl Radical	H : O ·
Hydroxyl Ion	H : O :
Water	H : O : H

Figure 16. *Nomenclature of the various forms of oxygen*

Oxidative damage

Fenton described in the late nineteenth century the oxidising potential of hydrogen peroxide mixed with ferrous salts. Forty years later, Haber and Weiss identified the hydroxyl radical as the oxidising species in these reactions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

In biological systems the availability of ferrous ions limits the rate of reaction, but the recycling of iron from the ferric to the ferrous form by a reducing agent and the presence of other metals can maintain an ongoing Fenton reaction; the latter leads to the generation of hydroxyl radicals which can initiate the oxidation of organic substrates as lipids, phosfolipid, proteins and DNA. The peroxidation of lipids involves three distinct steps: initiation, propagation and termination. The initiation reaction between an unsaturated fatty acid and the hydroxyl radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid:

$$\bullet OH + RH \rightarrow R\bullet + H_2O$$

In the propagation reaction, this resonance structure reacts with triplet oxygen, forming peroxy radicals:

$$\bullet R + O_2 \rightarrow ROO\bullet$$

The peroxy radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another free radical:

$$ROO\bullet + RH \rightarrow R\bullet + ROOH$$

In membrane lipids the peroxidation reaction is terminated when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals, such as those shown in reactions below:

$$R\bullet + R\bullet \rightarrow R - R$$

$$R\bullet + ROO\bullet \rightarrow ROOR$$

$$ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$$

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a peptide differ in their susceptibility to attack, and the various forms of activated oxygen differ in their potential reactivity. In spite of this complexity, generalizations can be made. Sulphur containing amino acids, and thiol groups, specifically, are very susceptible sites. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges. Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives.

Activated oxygen and agents that generate oxygen free radicals also induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects and the main cause of the damage is the oxidation of the sugar moiety by the hydroxyl radical.

Antioxidant defences

Generation of reactive oxygen species (ROS) is considered to be a primary event under a variety of stress conditions. Consequences of ROS formation depend on the intensity of stress and on the physicochemical conditions in the cell (i.e. antioxidant and redox status and pH). It has been generally accepted that active oxygen produced under stress is a detrimental factor, which causes gradual peroxidation of membrane lipid structures and produces antioxidant enzyme inactivation.

In order to survive, plants have evolved enzymatic and non-enzymatic defence mechanisms against oxidative stress. To control the level of ROS, plant tissues contain several ROS scavenging enzymes (Fig. 17) and a network of low molecular weight antioxidants, like ascorbate, glutathione, phenolic compounds, tocopherols and carotenoids.

Superoxide dismutase (SOD) was first isolated by Mann and Keilis (1938) and thought to be a copper storage protein. Subsequently, the enzyme was identified by a number of names, erythrocuprein, indophenol oxidase, and tetrazolium oxidase, until its catalytic function was discovered by McCord and Fridovitch (1969). SOD is now known to catalyse the dismutation of superoxide to hydrogen peroxide.

There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and the Ni type, which binds nickel; they catalize the dismutation of superoxide according to the two following half-reactions (M stands for metal):

$$M^{(n+1)+}\text{-SOD} + O_2^- \rightarrow M^{n+}\text{-SOD} + O_2$$

$$M^{n+}\text{-SOD} + O_2^- + 2H^+ \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2.$$

The hydrogen peroxide can be dismutated by catalase (CAT) into water and oxygen or can be utilized by glutathione peroxidase (GPX) or into the ascorbate-glutathione pathway (Asada 1999).

Glutathione (GSH) is a tripeptide (Glu-Cys-Gly) whose antioxidant function is facilitated by the sulphydryl group of cysteine (Rennenberg, 1982). On oxidation, the sulphur forms a thiyl radical that reacts with a second oxidised glutathione forming a disulphide bond (GSSG). GSH can function as an antioxidant in many ways. It can

react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore functions directly as a free radical scavenger.

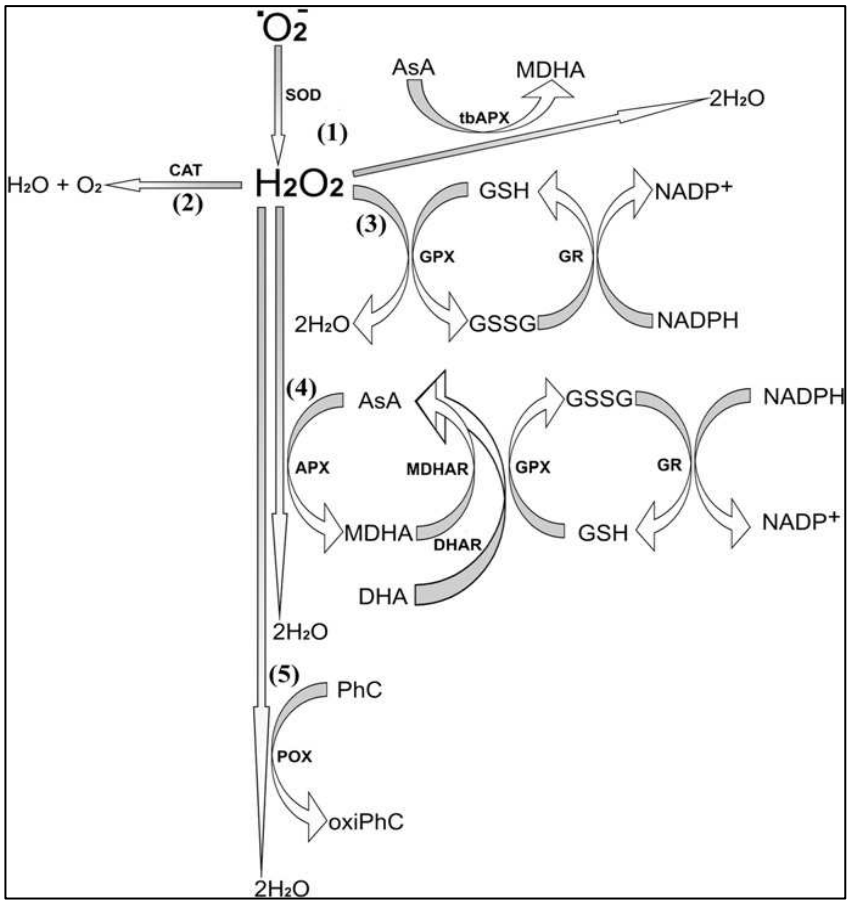


Figure 17. The main cellular pathways for ROS scaveging from plants. (1) superoxide dismutase, (2) catalase in peroxisomes; (3) glutathione peroxidation and its regenerating cycle; (4) ascorbate–glutathione (Halliwel–Asada) pathway in the stroma, cytosol, mitochondria and apoplast of plants; and (5) polyphenol oxidation (Dixon and Paiva 1996).

Aim of the thesis

Soil contamination by organic and inorganic pollutants of anthropogenic origin represent a serious challenge for the society. It has been shown that several plant species, mostly herbaceous, are able to alleviate soil pollution (phytoremediation) thanks to their ability to take up contaminants without substantial damage (ref). During the last decades it has been reported that also tree species may be used in phytoremediation; in particular some poplar, birch and willow species are suitable to this aim since they are capable of a rapid growth and naturally provided with tolerance to oxidative stress imposed by pollutants. Heavy metals (HM) are among the latter; they can be removed from the soil by the roots and neutralized inside plant organs (roots, stem or leaves) through a variety of biological mechanisms.

Recently, poplar has been studied extensively and a great clonal variability has been found with regard to HM stress tolerance (Castiglione et al., 2009). Results have been also reported concerning HM effects on the expression of many stress/defence-related genes(Cicatelli et al., 2010; 2011). In particular, it has been reported that HM-tolerant poplar clones, such as AL35, display higher foliar polyamine titers as compared to less tolerant clones, as also revealed by enhanced transcript levels of their biosynthetic enzymes (Cicatelli et al., 2009; 2010).

The aim of the thesis was to get a deeper insight into the physiological mechanisms that underlie plant tolerance to HM stress. Phenolic compounds and aliphatic polyamines are among the substances that respond to HM and protect plant tissues from HM-related oxidative damage (ref fenoli; Groppa et al.). Moreover, since root exudates are also involved in this physiological response and contribute to the detoxification process, changes in the phenolics content of root exudateswere also analyzed.

Two white poplar clones were adopted to investigate changes in phenolic compounds and polyamines induced by elevated/toxic Cu concentrations, and the antioxidant/protective effect of polyamines on HM stress. The experimental system is represented by *in vitro* and hydroponic cultures of micropropagated poplar plants of clone AL22 and Villafranca. The former was shown to tolerate high concentrations of Cu and Zn when grown on a polluted site exhibiting a 30% survival in the long-term and twice the foliar Cu concentration of most of the other clones (Castiglione et al.,

2010); the latter is a commercial clone and is also tolerant to HM (Lingua et al., 2008).

Initially an optimization of the substrate type and Cu concentration was carried out in order to establish the suitable culture conditions for poplar plants. Phenotypic aspects and physiological parameters were evaluated.After a preliminary trial aimed at testing various concentrations of $CuCl_2$added to the culture medium, a first experiment was performed in which 50 µM Cu was administered to micropropagated AL22 plants transferred to a semi-hydroponic culture on perlite; after8 days, morphological aspects, and total polyphenoland polyamine levels were evaluated in control and Cu-treated samples.

Given the modest results of this first experiment in terms of plant reaction/response to the Cu treatment, a second experiments followed in which AL22 plantswere exposed to a higher Cu concentration (100 µM). Micropropagated plants were transferred to a hydroponic culture for 72 h,. At the end of the experiment, the following were evaluated in Cu-treated and control samples:

a) Phenotypic observations
b) Leaf and root Cu concentrations by means of atomic absorption
c) Total phenol and flavonoid levels, and HPLC profiles in leaves, roots and root exudates
d) Free and conjugated polyamine titers in leaves and roots

In order to evaluate the variability of poplar clone tolerance to the metal, a further experiment was set up under hydroponic conditions using the same Cu concentration but a different poplar clone, Villafranca. In this case, the time-course changes in phenol and flavonoid levels and type, and in polyamine titers, as well as the oxidative damage (spectrophotometric analysis of lipid peroxidation and hydrogen peroxide production) imposed by the metal were determined.

Finally, 1 mM spermidine, together with 100 µM Cu, was administered to cultured in vitro AL22 plants with the aim to evaluate the possible protective/antioxidant potential of the polyamine. Also in this case phenolic profiles and polyamine concentrations were determined at the end of the experiment (72 h). Moreover, lipid peroxidation and hydrogen peroxide levels were measured in treated relative to control leaves

Results show that both phenolic compounds and polyamines respond to Cu-induced stress and confirm a role in tolerance for these compounds. Sd is also confirmed as stress responsive/protective molecule.

Materials and methods

Plant material

Two clones of white poplar (*Populus alba* L.) were utilized: the HM-tolerant AL22, a clone originating from a natural population growing along the banks of the Ticino river (Lombardia, Italy), which was selected during a field trial on an industrially contaminated soil containing high amounts of copper and zinc (Castiglione et al. 2009), and Villafranca, a commercial clone provided by CRA-Unità di Ricerca per le Produzioni Legnose Fuori Foresta, ex-Istituto di Sperimentazione per la Pioppicoltura, Casale Monferrato (AL). Clone Villafranca has been studied and partially characterized for its response to Cu and Zn (Franchin et al. 2007; Castiglione et al. 2007; Todeschini et al. 2007; Lingua et al. 2008), while AL22 has not been hitherto investigated.

Both clones were cultured *in vitro* by micropropagation; for some experiments plants were transferred *ex vitro* to hydroponic cultures.

3) In vitro cultures

Poplar plants were maintained *in vitro* by micropropagation technique under sterile conditions. The method is made up of two phases:

Multiplication phase: plant stems are cut to produce nodal explants about 10 mm in length with an axillary bud . The explant is then placed on medium A (Table 1) in ½-litre polypropylene vessels with a membrane on the lids (Microbox® EC02, Duchefa Biochemie B.V., Haarlem, The Netherlands).

Growth phase: after 4-5 weeks, shoots coming from axillary buds are cut and transplanted in larger vessels (Microbox® 140 mm height, 90 mm base diameter, Duchefa) containing 250 ml of medium B (Table 1) which allows shoot elongation and rooting so that they develop into new plants.

The cultures are incubated in a growth room at 24℃ , with 16 h daily exposure to light (intensity 50 µE m^{-2} s^{-1}).

Medium A (Rd11) :	**for 1 liter of medium**
	mg
Macro-salts WPM (Lloyd & McCown, 1991):	
NH_4NO_3	400
K_2SO_4	990
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
$Ca(NO_3)_2 \cdot 4H_2O$	556
$CaCl_2 \cdot 2H_2O$	96
Micro-salts MS (Murashige & Skoog, 1968):	
$MnSO_4 \cdot H_2O$	16.9
H_3BO_3	6.2
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
KI	0.83
Chelated Iron MS	
$FeSO_4 \cdot 7H_2O$	27.85
Na_2 EDTA	37.3
Organic compounds	
nicotinic acid	5
pyridoxine HCl	1
thiamine HCl	1
biotin	0.05
glycine	2
myo-inositol	100
casein	200
Ca pantothenate	1
folic acid	0.5
Sucrose	20 000

Medium B (WPM):	**for 1 litre of medium**
	mg
Macro-salts WPM:	
NH_4NO_3	400
K_2SO_4	990
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
$Ca(NO_3)_2 \cdot 4H_2O$	556

$CaCl_2 \cdot 2H_2O$	96
Micro-salts WPM	
$MnSO_4 \cdot H2O$	22.3
H_3BO_3	6.2
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.25
Chelated Iron MS	
$FeSO_4 \cdot 7H_2O$	27.85
Na_2 EDTA	37.3
Organic compounds	
nicotinic acid	0,5
pyridoxine HCl	0,5
thiamine HCl	1
glycine	2
myo-inositol	100
sucrose	20 000

Table 1. Chemical composition of medium A and medium B. The pH is adjusted to 5.7 and 3 g/l of gellifying agent (Phytagel, Sigma) are added.

After 4-5 weeks plants are ready (Fig. 18-foto in vitro) to be propagated again or to be subjected to a treatment or to be transferred in aeroponic/hydroponic system.

4) Aeroponic/hydroponic culture

Micropropagated plants were left to grow for about one month on medium B up to a height of about 10 cm and then transferred to a aeroponic system (GHE Aerofarm 5 sites and Nutriculture X-Stream Aeroponic Propagator 20 sites) consisting of a plastic reservoir covered with a lid in which perforated vessels (“baskets”) filled with expanded clay pellets are inserted (Fig.18). The plants were carefully transferred to the perforated vessels (one per vessel) and each one covered with a plastic container to maintain it under conditions of high humidity. In order to acclimate the plants gradually to the atmospheric humidity, the containers were slightly lifted every day until they could be removed completely. The box contained a nutrient solution (Table 2) which was sprayed on the roots for 15 min every hour by a small air pump.

The aeroponic system was placed in a growth chamber with a photoperiod of 16 h of light, and a temperature of 26℃

The system was set up and treatments were carried out at the Institute of Botany of the University of Urbino, with the collaboration of Prof. Valeria Scoccianti.

Figure18. Aeroponic growth system box

Compound	%
Total N	6.2
NH_4	0.4
NO_3	5.7
P_2O_5	2.1 (0,9% P)
K	6.4
K_2O	7.7
Ca	3.2
CaO	4.5
Mg	0.8
MgO	1.3
S	0.8
SO_3	2.0
B	0.007
Cu	0.001
Fe (DTPA)	0.021
Mn	0.014
Mo	0.002
Zn	0.007

Table 2. Chemical compounds in aeroponic nutritive solution

Treatments

3) Copper and spermidine treatments in vitro

Shoots in the early rooting phase were transferred from WPM medium to the same medium supplemented (or not) with 100 µM $CuCl_2$ and with or without 1 mM spermidine.

4) Copper treatments in hydroponic culture

After approximately one month in aeroponic culture, plants were transferred, without removing them from the baskets, to another container containing the same nutrient

solution added or not with 100 µM $CuCl_2$. Plants were harvested after 0, 6, 24 and 72 h, and samples of leaves and roots from 3-7 plants per treatment were pooled separately and frozen in liquid nitrogen and, in some cases, freeze dried.

Chlorophyll measurements

Leaves were homogenized on ice with 80% aqueous acetone or 100% methanol (0.1 g tissue/2 ml) and then centrifuged at 4,000 rpm for 10 min. The supernatant was collected and a second extraction was done. The combined supernatants were analyzed by spectrometer at 646 nm for the chlorophyll *b* and 663 nm for chlorophyll *a* (acetone) or 652nm or 665nm (methanol).

Chlorophyll was then calculated using the following equations (Lichtenthaler 1987):

For acetone:

$C_{a} = 12{,}25A_{663} - 2{,}79A_{645}$

$C_{b} = 21{,}50A_{646} – 5{,}10A_{663}$

$C_{a+b} = 7{,}15A_{663} + 18{,}71A_{645}$

For methanol:

$C_{a} = 16{,}72A_{665} – 9{,}16A_{65}$

$C_{b} = 34{,}09A_{652} – 15{,}28A_{665}$

$C_{a+b} = 1{,}44A_{665} + 24{,}93A_{652}$

Proline measurements

Tissue proline concentration was estimated following the method of Bates *et al.* (1973) with slight modifications. 50 mg of frozen seedling tissue (leaves, stems and roots separately) was crushed in 1.2 ml of 3% sulphosalicylic acid, and the homogenate centrifuged at 15.000 *g* at room temperature for 20 min. A 0.5-ml aliquot of the supernatant was made up to 1 ml with water, and to this 2 ml ninhydrin reagent [2.5% ninhydrin in glacial acetic acid-distilled water-85% orthophosphoric acid (6:3:1)] was added. The reaction mixtures were kept in a water bath at 90ºC for 1 h to develop the colour. Test tubes were then cooled in an ice-bath, and 2 ml toluene added to separate the chromophore. Absorbance of the toluene phase was read in a spectrophotometer at 525 nm, and proline concentration calculated by comparing sample absorbances with the standard proline curve.

Analysis of phenolic compounds

5) Extraction

Phenolic extraction was done following a protocol described by Chiou et al. (2007), with slight modifications. Fifty mg of each freeze-dried homogenized sample was mixed with 1 ml of methanol, placed in a sonicator bath for 15 minutes, and then left for 24 h under stirring at room temperature. The mixture was then centrifuged at 12,000 g for 15 min and the methanol extract was collected. The remaining residue was extracted four additional times with methanol (1 ml). For each of the latter four extractions the mixture was placed in a sonicator bath for 15 min and then stirred for 60 min. All extracts were combined, methanol was evaporated under reduced pressure, and the residue was re-suspended in 1 ml of methanol.

6) Determination of total polyphenol content (TPC)

The content of total phenolic compounds was determined by the Folin-Ciocalteu (FC) reagent (Singleton et al. 1974), using gallic acid as standard. For the preparation of the calibration curve, 5, 10, 20, 50 and 100 µg/µl aqueous gallic acid solutions were utilized. Fifty µl of standards and samples were mixed with 250 µl of FC reagent and 500 µl of water. The mixture was left to stand at room temperature for 10 min, and then 800 µl of 20% Na_2CO_3 solution were added. Samples were then incubated and stirred for two hours in dark conditions. The absorbances of the resulting blue complexes were measured at 680 nm using a Jasco (Tokyo, Japan) spectrophotometer, and the TPC was expressed as gallic acid equivalents (GAE).

7) Determination of total flavonoid content

The flavonoid content was spectrophotometrically determined according to Lamaison and Carnet (1990) method, which is based on the formation of a flavonoid-aluminium complex, having an absorbance at 430 nm. Rutin was used to make the calibration curve and for the preparation of the calibration curve, 5, 10, 20, 50 and 100 µg/µl aqueous rutin solutions were utilized.

Fifty µl of sample or standard were mixed with 1 ml of 2% aluminium chloride methanolic solution and left in incubation at room temperature for 15 min before measuring the absorbance with a spectrophotometer.Total flavonoids are expressed as rutin equivalents (RE).

8) HPLC analysis of phenolic compounds

Phenolic extracts were analysed by a Waters HPLC (Waters corp., Milford, MA, USA) system with a Photodiode Array Detector (Waters 2996) and a reverse-phase Supelcosil^{TM} LC-18 HPLC column (15 cm long, 4 mm internal diameter containing octadecyl silane particles of 5 µm diameter); following the methodology described by Andreotti *et al.* (2008).

Phenolic compound identification was carried out through a comparison of retention time values and UV spectra (detected between 210 and 560 nm wavelength) with authentic standards. Standards (dihydroxybenzoic acid, catechin, epicatechin, caffeic acid, ferulic acid, quercetin, naringenin, kaempferol and 6-Methoxyflavone as internal standard) for qualitative and quantitative determinations were purchased from Sigma-Aldrich (St Louis, MO, USA) and Extrasynthèse (Genay Cedex, France). Phenolic compound concentrations, expressed in mg g^{-1} dry weight (DW), were calculated from calibration curves obtained with the corresponding standards.

HPLC analysis of polyamines

Plant material (200 mg FW or 20 mg DW leaves or roots) was homogenized with 4% perchloric acid (PCA), kept for 1 h at 4°C, and centrifuged at 15,000 g for 30 min. Aliquots of the supernatants and standard solutions of Pu, Sd and Sm were derivatized with dansyl chloride essentially as described by Bregoli et al. (2002). Dansylated derivatives were extracted with toluene, taken to dryness and resuspended in acetonitrile. PAs were separated and quantified by HPLC (PU-980 Jasco, Tokyo, Japan) using a reverse phase C_{18} column (Spherisorb ODS2, 5-mm particle diameter, 4.6 x 250 mm, Waters, Wexford, Ireland) and a programmed acetonitrile:water step gradient, as previously described (Bregoli et al. 2002).

Aliquots of the supernatant and resuspended pellet were subjected to acid hydrolysis (6 N HCl overnight at 110°C) in order to release PAs from their PCA-soluble and PCA-insoluble conjugates, respectively; released PAs were derivatized and analysed as described above.

Determination of tissue Cu concentration

Leaves and roots of AL22 plants hydroponically grown on Cu-supplemented medium were carefully washed with distilled water, blotted dry, and then placed in an oven at 60°C for 3 days. Dry samples were ground to a powder. Approximately 0.5 g DW was

used for the determination of copper concentration in leaves and roots, separately. Samples were weighed and then digested in 10 ml concentrated HNO_3 in a CEM MARS 5 microwave digestor (Cologno al Serio, BG, Italy). The digested material was filtered on 45-mm filters, and then deionized water was added to a final volume of 100 ml. Metal concentration was assessed by means of a calibration curve, after measurement by Inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES) using an IRIS Advantage ICAP DUO HR series (Thermo Jarrell Ash, Franklin, MA, USA) spectrometer. Certified standards (BCR 062, 100, 129 and 145R, by the Institute for Reference Materials and Measurements, Ratieseweg, Belgium), with known element concentration, were analyzed with the samples in order to confirm the correctness of the procedure.

Malonyldialdehyde (MDA) determination

For the measurement of lipid peroxidation in leaves, the thiobarbituric acid (TBA) test, which determines MDA as an end product of lipid peroxidation (Heath and Packer, 1968) was used. Leaf material (500 mg)was homogenized in 5 ml 0.1% (w:v) TCA solution. The homogenate was centrifuged at 10 000x*g* for 20 min and 0.5 ml of the supernatant was added to 1 ml 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10 000x*g* for 5 min, and the absorbancy of supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

Hydrogen peroxide determination

Hydrogen peroxide levels were determined according to Sergiev et al. (1997).Leaf tissues (500 mg) were homogenized in ice bath with 5 ml 0.1% (w/v) TCA. The homogenate was centrifuged at12 000x*g* for 15 min and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI. The absorbancy of supernatant was read at 390 nm.The content of H_2O_2 was given on a standard curve.

Statistical analysis

All data represent the means (n=3-7) ± standard error (SE) and were analysed by analysis of variance (one way ANOVA) procedures using the SAS Statistical Software (SAS Institute, Cary, NC, USA). Means were separated, between controls and treatments, and among treatments, using Duncan's multiple range test at the 5% level.

Results

Optimization of culture medium and Cu concentration

In order to evaluate the suitable Cu concentration and substrate for *in vitro* culture studies, micropropagated AL22 poplar plants were grown in the presence of different concentrations of Cu (10, 50 e 100 µM) and types of substrate under semi-hydroponic conditions.

Initially, micropropagated plants were placed in vessels containing three different sand mixtures:

a) 45% coarse-grained and 55% fine-grained sand plus 100 ml of WPM liquid medium

b) 100% fine-grained sand plus 100 ml of WPM liquid medium

c) 100% medium-grained sand plus 100 ml of WPM liquid medium

and left to grow in the growth chamber. None of these conditions resulted suitable for adequate plant growth. In fact, plants didn't grow vigorously and appeared chlorotic nor developed a suitable root apparatus.

Therefore, micropropagated plants, grown on agarized WPM medium up to about 10 cm with well developed roots, were taken up and transferred into tubes containing liquid WPM medium. In this case plants wilted and also resulted unsuitable for the experiments.

Finally, rooted plants of about 10-cm height were transferred in vessels containing perlite in 80-ml WPM medium in the presence or absence of the various Cu concentrations, and left to grow in the culture chamber for 8 days (Fig. 19).

Figure 19. Four-week old AL22 poplar micropropagated plants cultured in a vessel containing perlite and WPM

Macroscopic evaluations were performed during plant growth and some biochemical parameters of toxicity were analysed.

1) Phenotypic evaluations

After 3 days in culture in the presence of either Cu concentrations, no phenotypic alterations were observed. On day 5, in plants treated with the lower Cu doses (10 and 50 µM), white small necrotic areas were observed (Fig. 20). With 100 µM Cu leaves appared wrinkled and epinastic at the end of culture (Fig. 20). On day 8, in all Cu treatments, red-colored adventitious roots developed on the stem (Fig. 21)

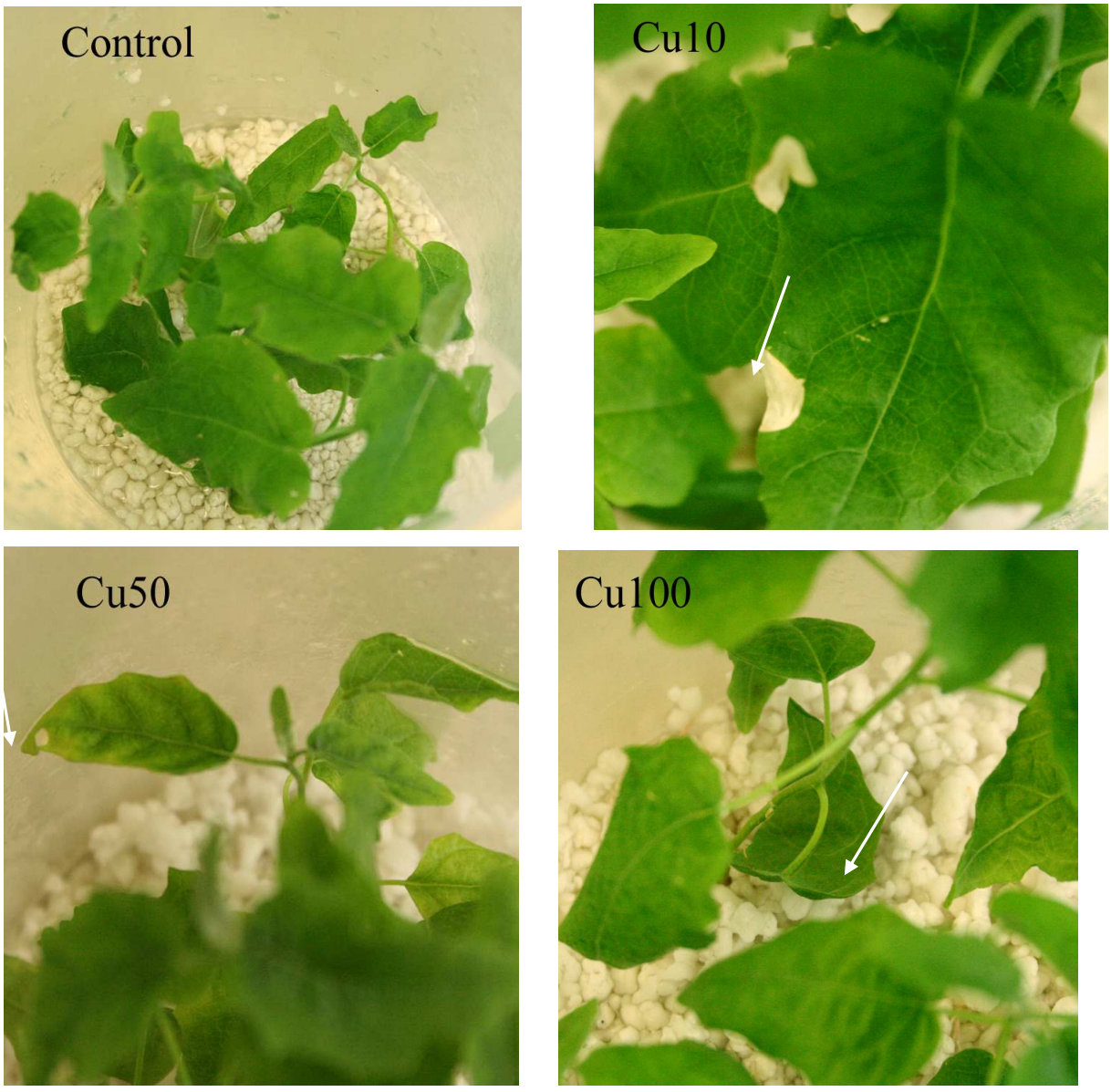


Figure 20. Leaf injury (arrows indicate white spots and epinastic wrinkled leaves) as observed in four-week old AL22 poplar plants cultured in vitro under different Cu concentrations. Numbers represent µM concentration of Cu

Figure 21. Red colored adventitious roots in four-week old AL22 poplar plants cultured in vitro in the presence of Cu

4) Chlorophyll and proline concentrations

Chlorophyll a and b concentrations were measured in control and treated leaves from three biological replicates on day 8. No significant differences in chlorophyll a content between control and treated plants were observed (Fig. 22A), whereas chlorophyll b concentration resulted significantly lower in leaves treated with 50 and 100 µM Cu (Fig. 22B).

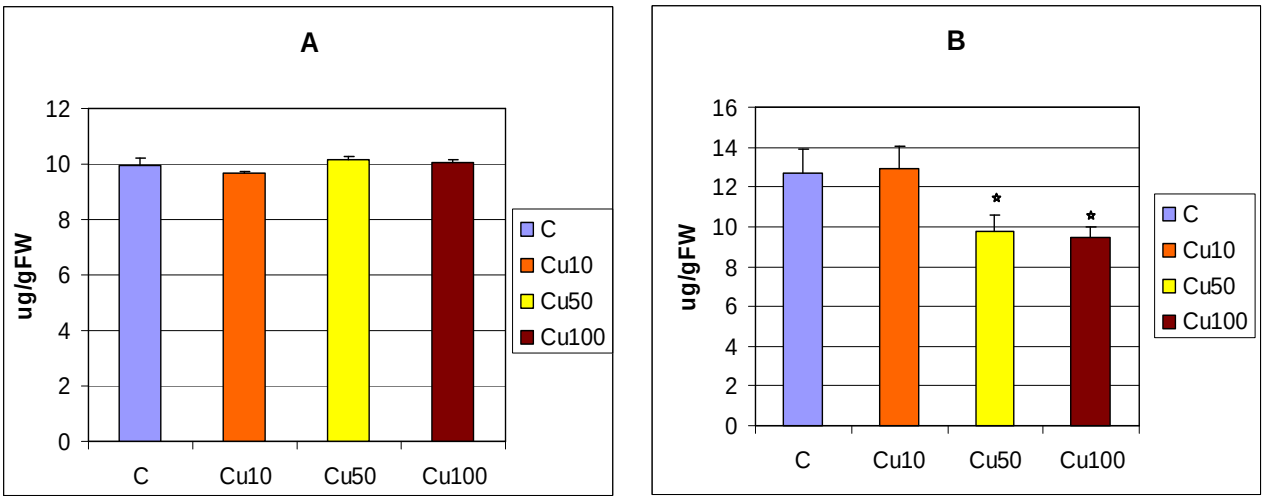


Figure 22. Chlorophyll a (A) and chlorophyll b (B) content in leaves from AL22 poplar plants cultured in vitro in the presence of different Cu concentration for 8 days. Asterisks indicate significant differences (P<0.05) relative to controls

Following all Cu treatments proline content was significantly lower relative to controls but no significant differences among Cu treatments were found (Fig. 23).

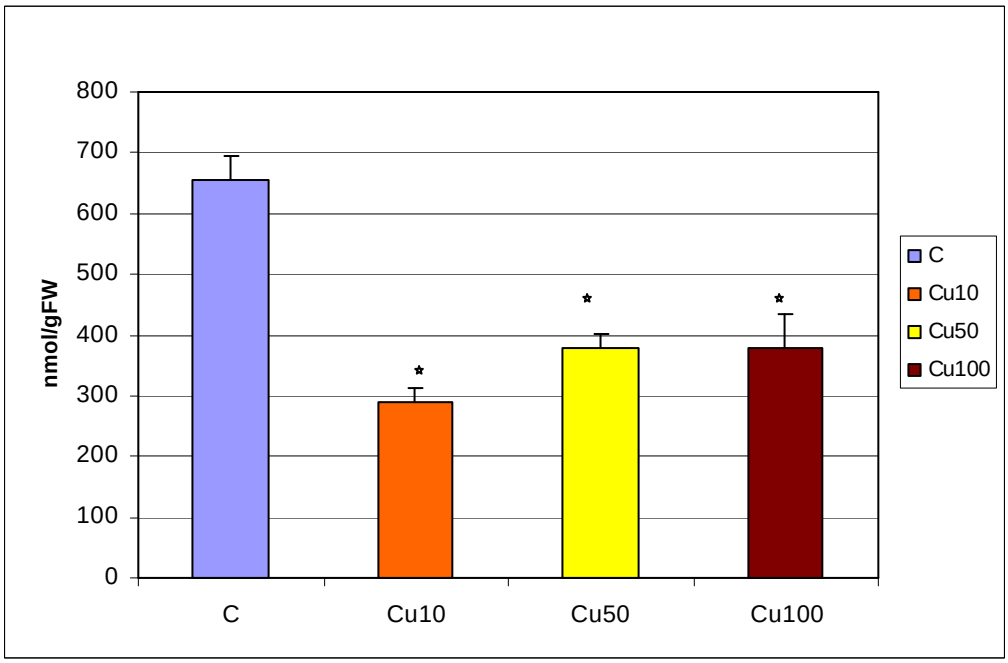


Figure 23. Proline content in leaves from AL22 poplar plants cultured in vitro in the absence (C) or in the presence of different Cu concentration for 8 days. Asterisks indicate significant differences (P<0.05) relative to controls

5) Polyphenolic profile

The HPLC phenolic profile of Cu-treated and control leaves showed differences in peak height with those from treated samples, already starting from 10 uM Cu, being higher than in controls (Fig. 24). Total peak areas were calculated and resulted about double in treated as compared with control leaves (Fig. 25).

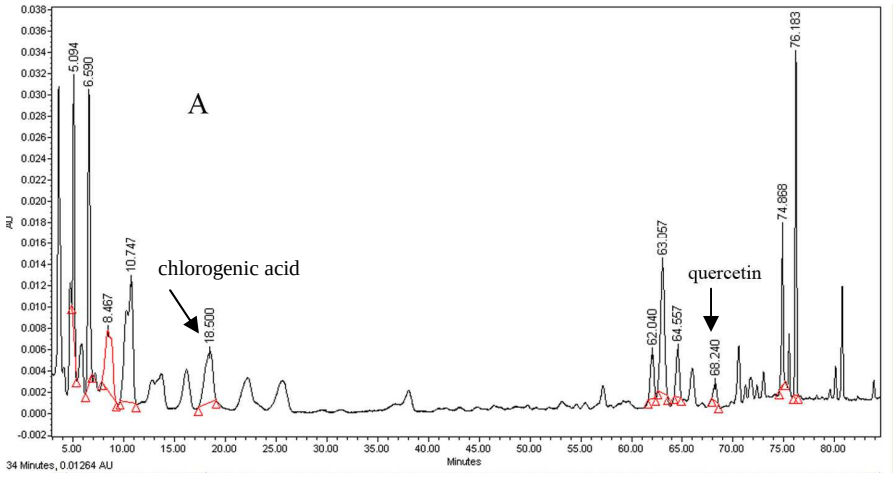


Figure 24. HPLC chromatogram in leaves from AL22 poplar plants cultured in vitro in the presence of 100 μM Cu concentration for 8 days

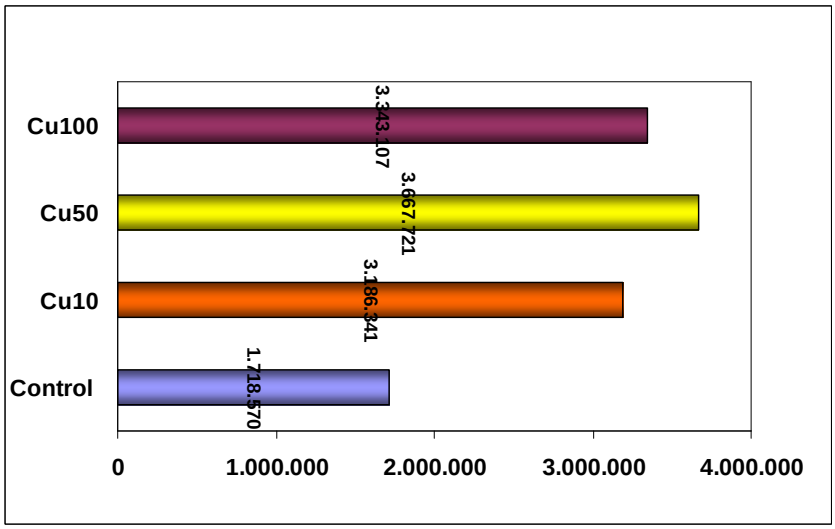


Figure 25. Total areas of phenolic peaks, as detected by HPLC, in leaves from AL22 poplar plants cultured in vitro in the absence (control) or in the presence of different Cu concentration for 8 days

On the basis of previous observations and results, 50 µM Cu concentrations and perlite as substrate was chosen for further experiments.

Response to copper in an *in vitro* culture system

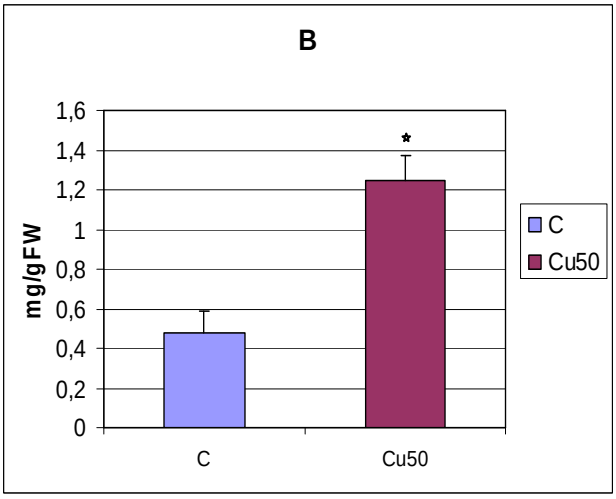
In this experiment micropropagated plants were transferred from agarized WPM medium to perlite, as described above, in the presence or not of 50 µM Cu for 10 days. At the end of this period phenotypic evaluations were conducted and total polyphenol and flavonoid amounts and polyamine concentrations were measured.

4) Phenotypic evaluations

No changes in plant growth or substantial morphological differences were observed between control and treated plants. Differently from the previous experiment, red roots developed in both control and treated samples and thus were not imputable to Cu stress.

5) Polyphenol and flavonoid amounts

Polyphenol and flavonoid concentrations were measured in four biological replicates, derived from 15 plants. In treated leaves polyphenol amount resulted slightly but significantly higher compared with controls (Fig. 26A) while flavonoid amount reached levels more than twice those of controls (Fig. 26B).



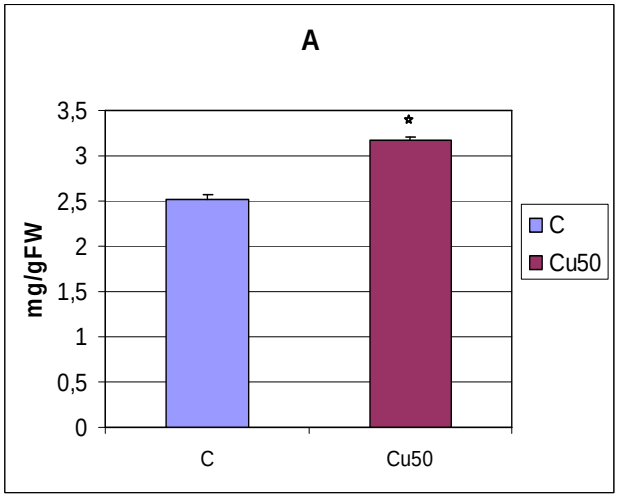
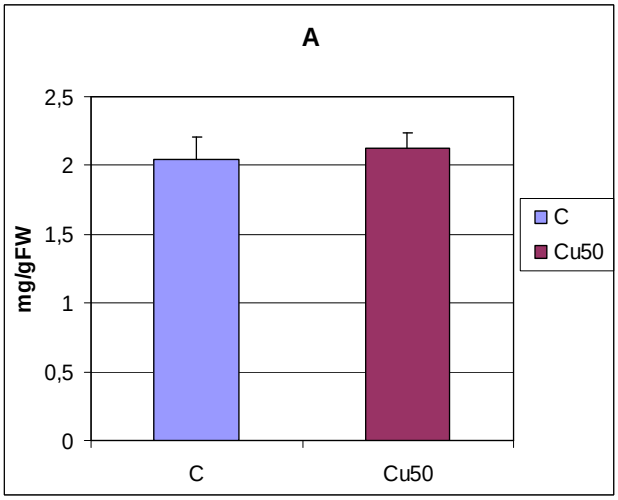


Figure 26. Total polyphenol (A) and flavonoid (B) contents in leaves from AL22 poplar plants cultured in vitro in the presence (Cu50) or absence (C) of 50 µM Cu for 8 days. Asterisks indicate significant differences (P<0.05) relative to controls

In the roots both polyphenol and flavonoid amounts were less abundant than in the leaves and not significantly different in controls relative to Cu-treated samples though there was a tendency to a lower flavonoid amount in control roots than in treated ones (Fig. 27).



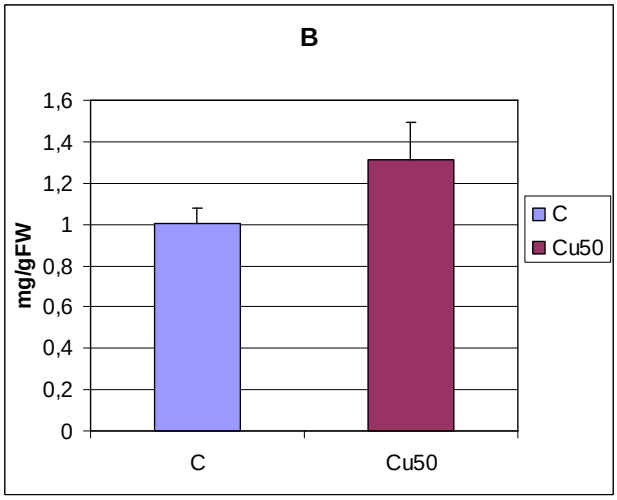


Figure 27. Total polyphenol (A) and flavonoid (B) contents in roots from AL22 poplar plants cultured in vitro in the presence (Cu50) or absence (C) of 50 µM Cu for 8 days

6) Polyamine levels

Free and PCA-soluble conjugated polyamines were measured in leaves from control and Cu-treated plants. In Cu-treated samples Put levels, but not those of Spd and Spm, were significantly lower than in controls (Fig. 28A). No significant differences in conjugated PAs were found between treated and control samples (Fig. 28B). Only traces of insoluble conjugated polyamines were detected (data not shown).

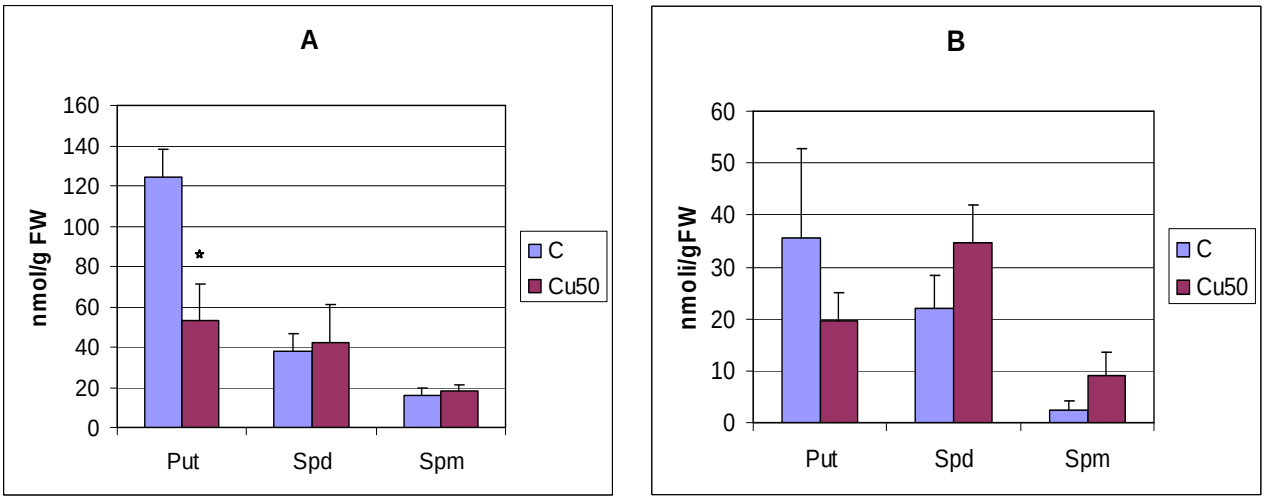


Figure 28. Free (A) and conjugated polyamine (B) contents in leaves from AL22 poplar plants cultured in vitro in the presence (Cu50) or absence (C) of 50 µM Cu for 8 days. Put, putrescine; Spd, spermidine; Spm, spermine. Asterisk indicates significant differences (P<0.05) relative to controls

Response to copper in aeroponic/hydroponic cultures

Due to the modest Cu response and plant biomass obtained with in vitro cultures this experiment was carried out using older plants with a well developed root apparatus grown under aeroponic conditions and a higher (100 µM) Cu concentration. Probably, in this way, plants were less stressed by the transfer and subsequently by Cu administration. In fact, plants were transferred from *in vitro* cultures on a agarized medium to the aeroponic system where they were left to grow for one month (Fig. 29); afterwards they were subjected to Cu treatment by immersion of the roots (hydroponic) in the same nutritive solution, containing or not Cu, for 6, 24 or 72 h.

Two experiments were performed using two different poplar clones, AL22 and Villafranca.

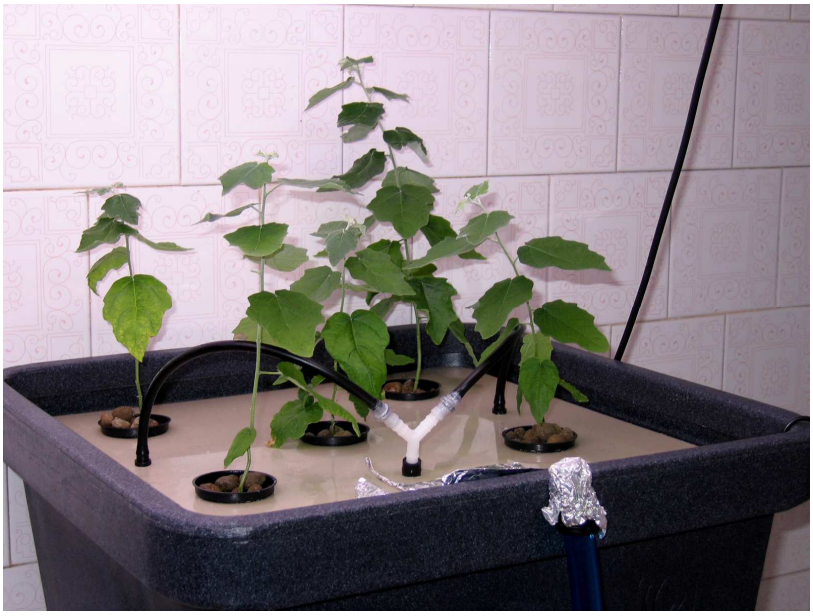


Figure 29. AL 22 poplar plants grown in an aeroponic culture system for one month

Poplar clone AL22

Plants were treated with Cu for 72 h and, at this time, all leaves and roots collected as well as root exudates were collected. Plants were subjected to macroscopic observations and leaves and roots were analyzed for Cu concentration, total phenol and flavonoid content, phenolic profiles and polyamine levels.

6) Phenotypic observations

No changes in growth rate were observed. In general, Cu-treated plants displayed wrinkled epinastic leaves compared with controls starting from about 72 h (Fig. 30); in particular, basal leaves appeared strongly wrinkled.

Figure 30. AL 22 poplar plants grown in an hydroponic culture system for 72 h in the absence (left) or in the presence (right) of 100 µM Cu

7) Cu concentration

Cu concentration was about twice in treated compared with control samples (Fig. 31A); in treated roots Cu was about 12-fold more concentrated than in controls (Fig. 31B).

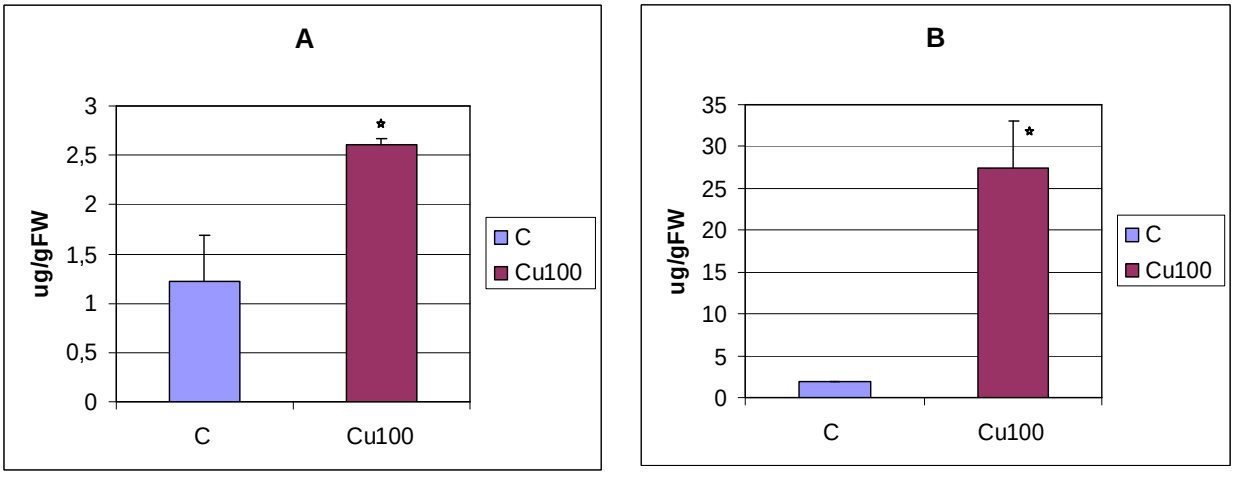


Figure 31. Copper content in leaves (A) and in roots (B) from AL22 poplar plants grown in an hydroponic culture system for 72 h in the absence (C) or in the presence (Cu100) of 100 µM Cu. Asterisk indicates significant differences (P<0.05) relative to controls

8) Total phenol and flavonoid contents

In Cu-treated leaves total phenol and flavonoid contents were significantly higher than in untreated leaves by about 40% and 80%, respectively (Fig. 32A and B).

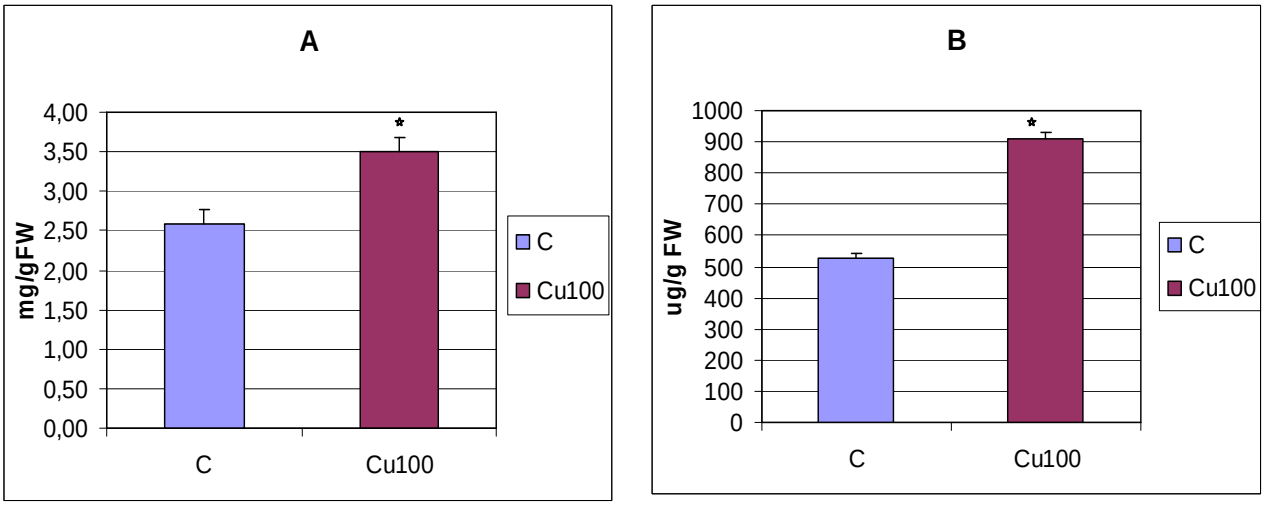


Figure 32. Total polyphenol (A) and flavonoid (B) contents in leaves from AL22 poplar plants cultured for 72 h in a hydroponic system in the presence or not (C) of 100 µM Cu concentration. Asterisk indicates significant differences (P<0.05) relative to controls

On the contrary, in treated roots, no significant differences in phenol and flavonoid content was found relative to controls (Fig. 33A and B).

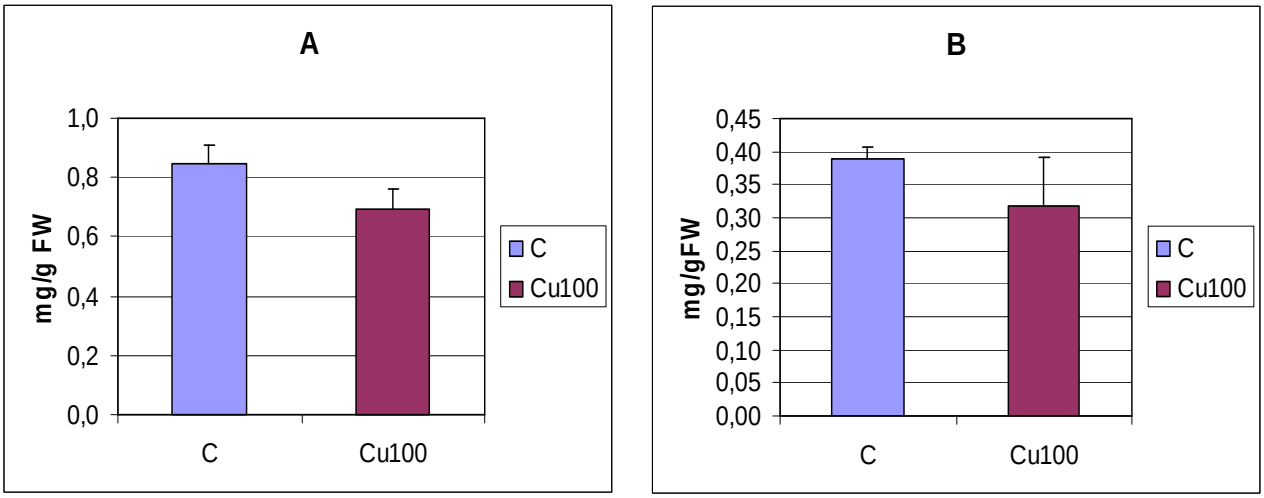


Fig.33. Total polyphenol (A) and flavonoid (B) contents in roots from AL22 poplar plants cultured for 72 h in a hydroponic system in the presence or not (C) of 100 µM Cu concentration

In root exudates, both polyphenol and flavonoid compounds were detected although in much lower amounts (ca. 100-fold less) than in plant tissues. Their concentration (expressed in term of root fresh weight) was considerably higher the external solution of Cu-stressed plants relative to controls (2.2-fold and 5.3-fold, respectively) (Fig. 34A and B).

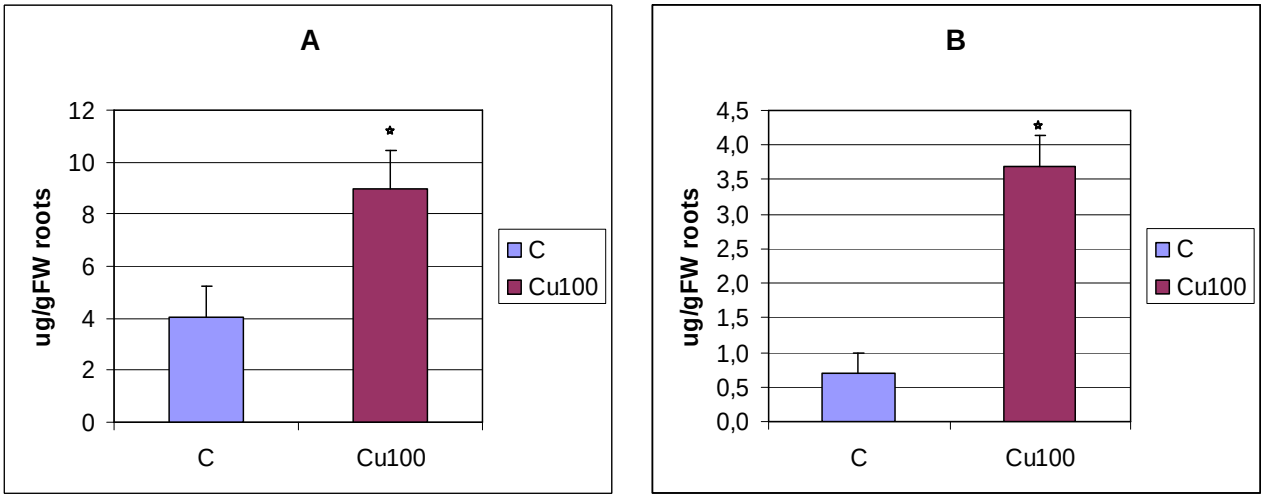


Figure 34. Total polyphenol (A) and flavonoid (B) contents in root exudates from AL22 poplar plants cultured for 72 h in a hydroponic system in the presence or not (C) of 100 µM Cu concentration

9) Phenolic profiles

In Figs 35, phenolic profiles of control (A) and Cu-treated (B) leaves are shown. The different scale of the two chromatograms is to take into consideration in order to avaluate results.

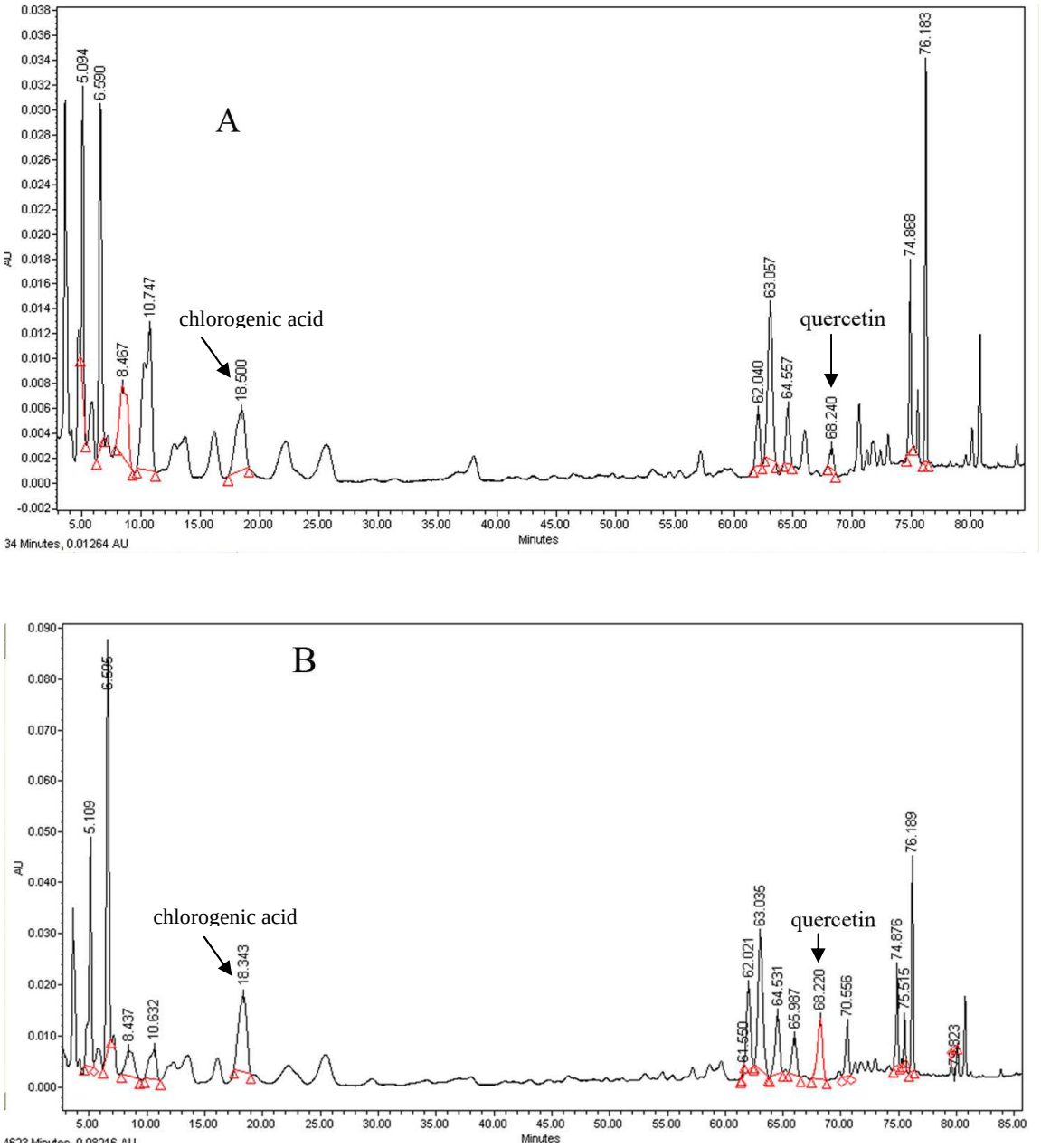


Figure 35. HPLC chromatographic profiles of phenolic compounds in control (A) and treated leaves (B) from AL22 plants cultured for 72 h in a hydroponic system in the presence or not (C) of 100 µM Cu concentration.

In leaves, two phenolic compounds were identified by comparison with standard retention times and UV-spectra: chlorogenic acid (class of cinnamic acids) and quercetin (class of flavonols) (Fig. 35). Results show that both chlorogenic acid and quercetin were more abundant (more than twice and almost 4-fold, respectively) in Cu-treated leaves compared with controls (Fig. 36A and B).

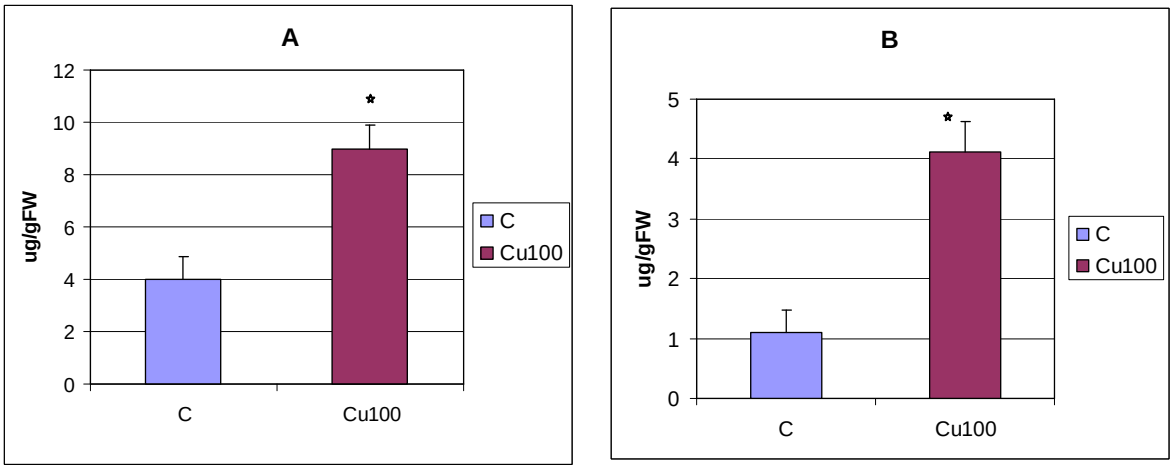


Figure 36. Chlorogenic acid (A) and quercetin (B) contents in leaves of AL22 poplar control and treated plants in a hydroponic system in the presence or not (C) of 100 µM Cu concentration. Asterisks indicate significant differences relative to controls at P<0.05

The phenolic profiles of control and treated roots are shown in Figs. 37A and B.

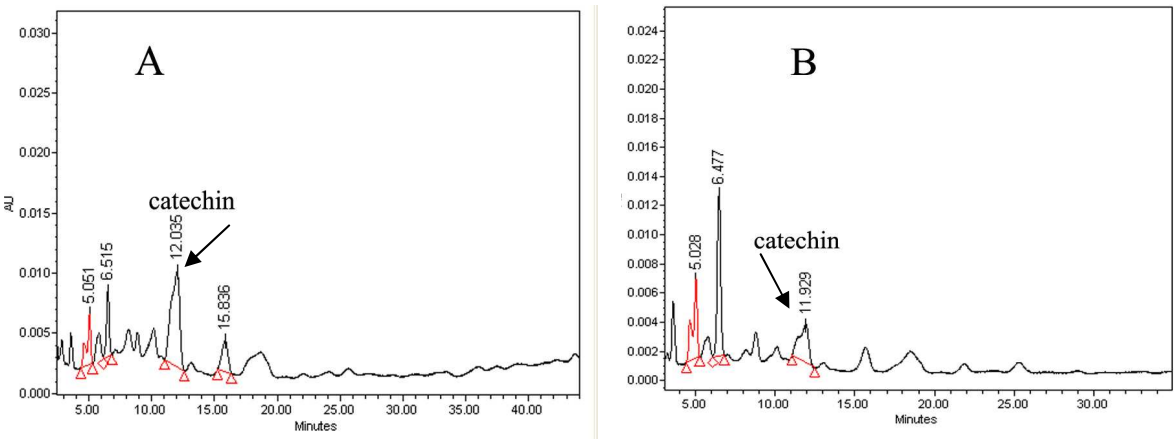


Figure 37. Phenolic chromatograms relative to control and treated roots from AL22 plants cultured in a hydroponic system in the presence or not (C) of 100 µM Cu concentration

Cathechin (class of flavan-3-ols) was the only identified compound and its amount in Cu-treated roots was less than half that of controls (Fig. 38). No other major changes in the HPLC profiles of control vs treated roots were observed.

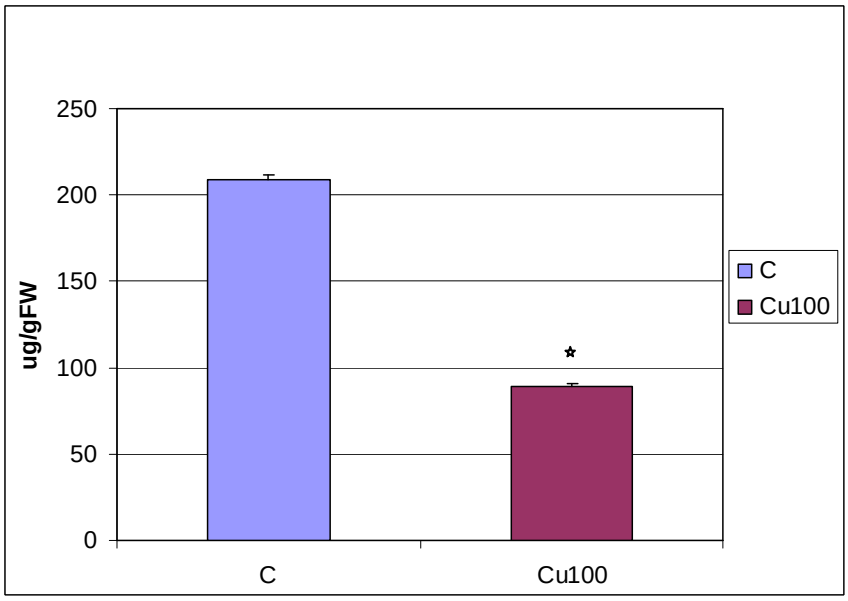


Figure 38. Catechin contents in roots of AL22 poplar control and treated plants cultured in a hydroponic system in the presence or not (C) of 100 µM Cu concentration. Asterisk indicates significant differences relative to controls at P<0.05

Phenolic profiles were also obtained for exudates from treated and control roots. While in control samples it was not possible to identify single phenolic compounds, in treated samples catechin and epicatechin (belonging to the flavan-3-ol class) were identified being the former about 4-fold more concentrated than the former (Fig. 39).

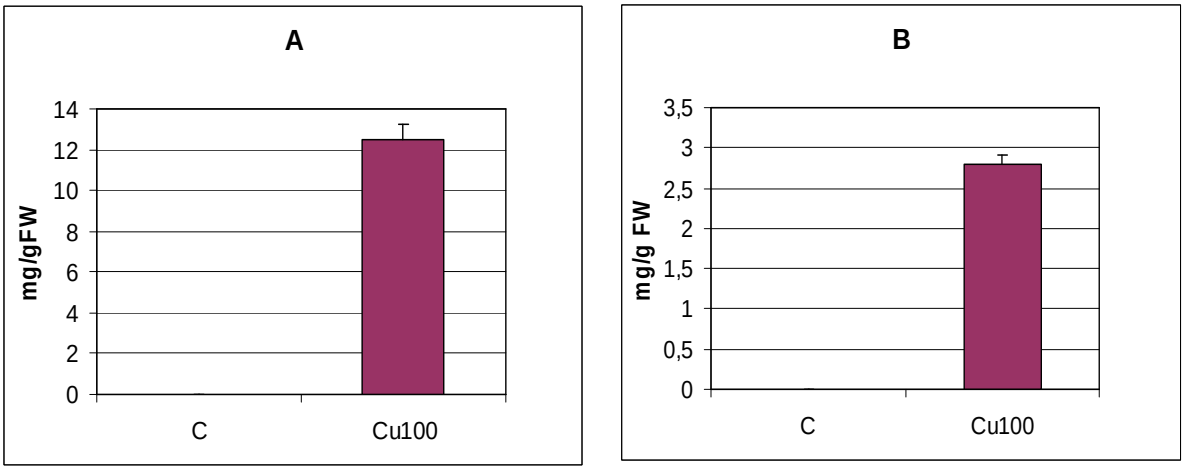


Figure 39. Catechin (A) and epicatechin (B) contents in root exudates of AL22 poplar control and treated plants cultured in a hydroponic system in the presence or not (C) of 100 µM Cu concentration

10) Polyamine levels

Free, PCA-soluble and PCA-insoluble polyamine levels were determined in leaves. No significant differences in free Put, Spd and Spm concentration were detected in controls and Cu-treated leaves; on the contrary, soluble conjugated Put and Spd amount was significantly higher (2.5-3-fold) in treated samples compared with controls (Fig. 40). Only traces of insoluble conjugated PAs were detected.

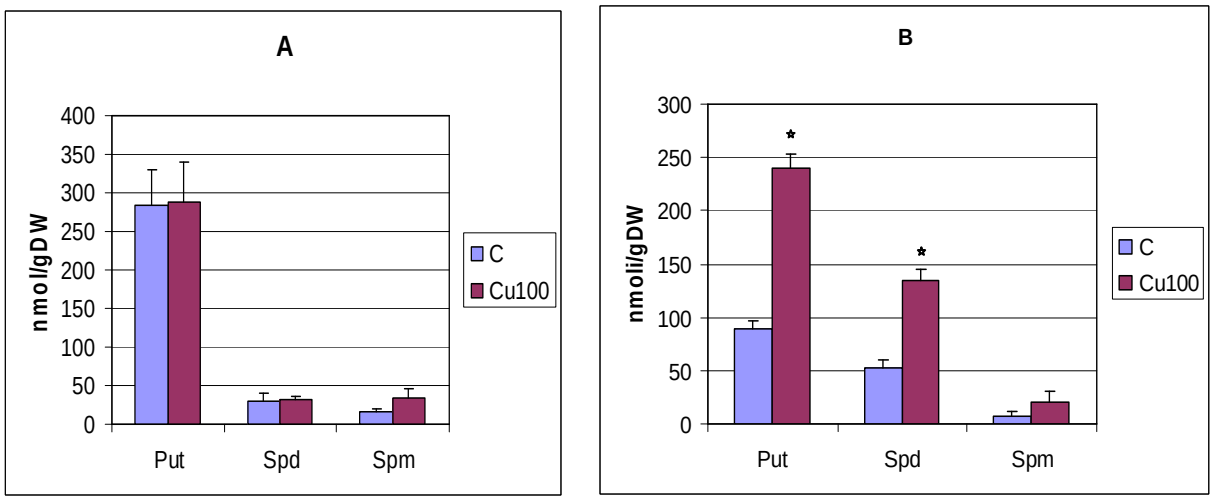


Figure 40. Free (A) and conjugated polyamine (B) contents in leaves from AL22 poplar plants cultured in a hydroponic system in the presence or not (C) of 100 μM Cu concentration. Put, putrescine; Spd, spermidine; Spm, spermine. Asterisks indicate significant differences relative to controls at P<0.05

Poplar clone Villafranca

Control and treated plants were hydroponically cultured for 72 h in the presence or not of 100 μM Cu; samples of leaves and roots were collected at 0, 6, 24 and 72 h. Root exudates were also collected at the same times. Plants were subjected to macroscopic observations and leaf and root samples were analyzed for total phenol and flavonoid content, phenolic profile and polyamine levels; moreover an MDA test was performed in order to test differences in oxidative damage.

6) Phenotypic observations

As already observed with AL22, Cu-treated plants displayed wrinkled epinastic leaves compared with controls starting from about 72 h; in particular, basal leaves

appeared strongly wrinkled. Moreover, treated leaves appeared strongly injured as deduced by the presence of white marginal necrotic areas (Fig.41)

Figure 41. Leaves from Cu-treated (left) and control (right) and Villafranca plants hydroponically cultured for 72 h

7) Total phenolic and flavonoid content

In Cu-treated leaves total phenolic content remained constant until 24 h and increased only at 72 h reaching values more than double compared with controls. Flavonoid content started to increase at 24 h (about 3-fold control levels) reaching at 72 h values about 5-fold higher than in controls (Fig. 42). In treated roots, as already observed in AL22, both polyphenol and flavonoid content significantly decreased compared with controls starting from 24 h and reached about 50% and 60%, respectively, of controls at 72 h (Fig. 43). In root exudates both classes of secondary metabolites were absent at the start of the experiment (0 h), and appeared – in very low amounts – after 6 h only in Cu-stressed plants (polyphenols) or in both control and treated samples (flavonoids). Starting from 6 h (falvonoids) or 24 h (polyphenols), both classes of compounds were significantly more abundant in root exudates of Cu-treated samples and increased gradually up to 72 h reaching in both cases values ca. 2-fold higher than in controls (Fig. 44).

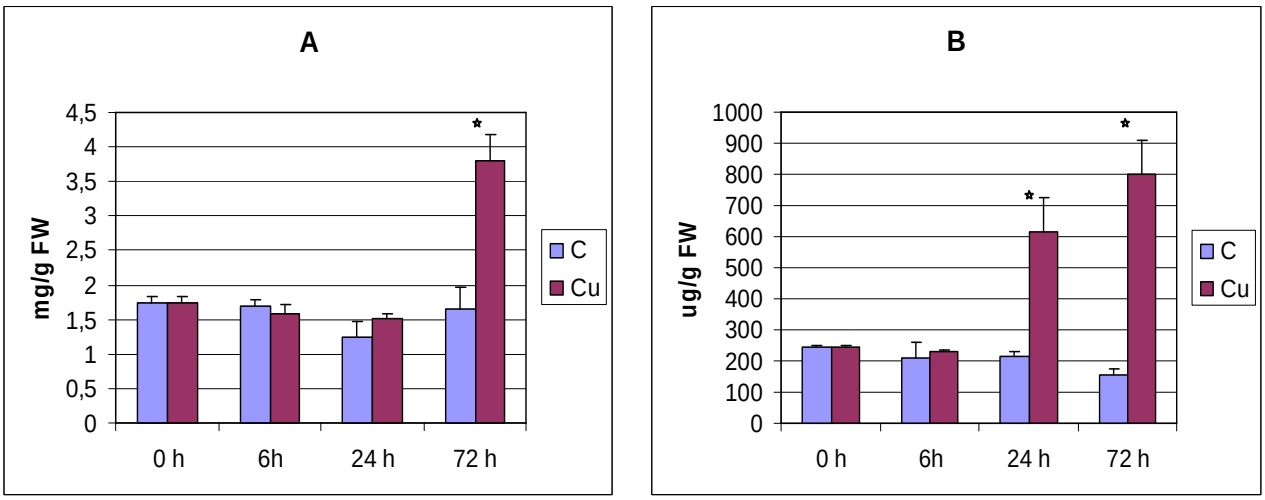


Figure 42. Total polyphenol (A) and flavonoid content (B) in the leaves of Villafranca poplar plants hydroponically cultured for 72 h in the presence (Cu) or in the absence (C) of 100 μM Cu. Asterisks indicate significant differences at P<0.05 relative to controls at each time point

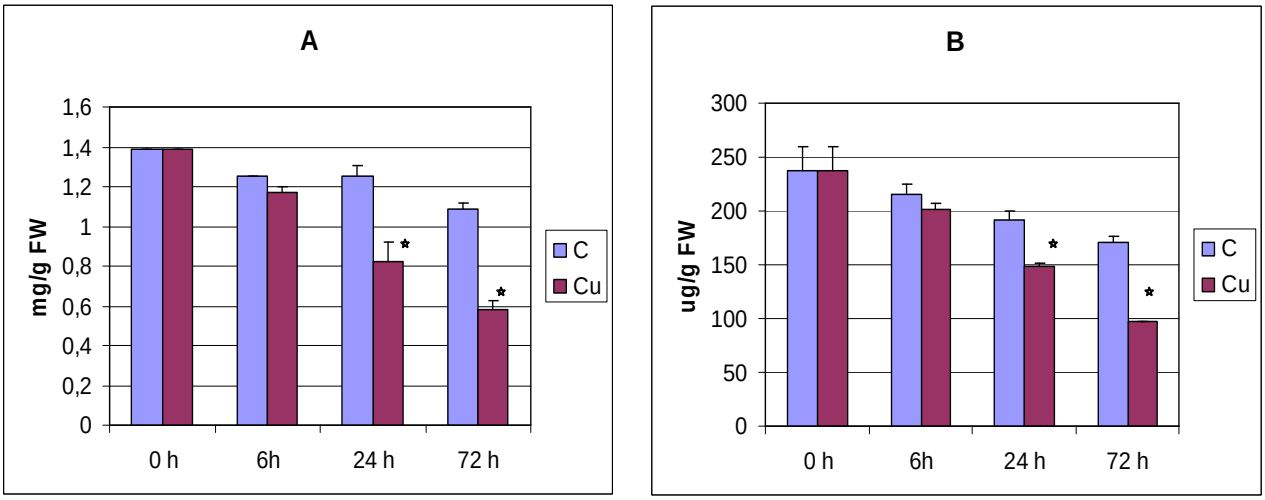


Figure 43. Total polyphenol and flavonoid content in the roots of Villafranca poplar plants hydroponically cultured for 72 h in the presence (Cu) or in the absence (C) of 100 μM Cu. Asterisks indicate significant differences at P<0.05 relative to controls at each time point

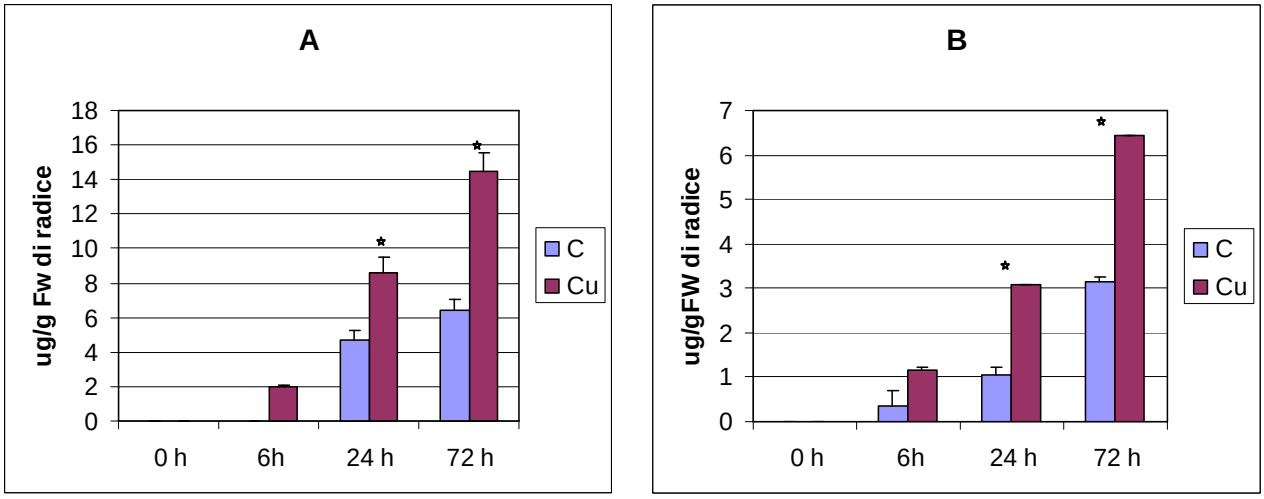


Figure 44. Total polyphenol and flavonoid content in root exudates of Villafranca poplar plants hydroponically cultured for 72 h in the presence (Cu) or in the absence (C) of 100 μM Cu. Asterisks indicate significant differences at P<0.05 relative to controls at each time point

8) *Phenolic profiles*

HPLC phenolic profiles were determined in leaves, roots and exudates at the different sampling times. In general, a number of phenolic compounds (xxxx) was detected (Fig. 45). Only few compounds displayed a change in concentration; these included chlorogenic acid in leaves and catechin in the roots. In Cu-treated leaves chlorogenic acid amount increased starting from 24 h reaching values more than double relative to controls at 72 h. On the other hand, in the roots, catechin content decreased at 24-72 h becoming less than half control amount (Fig. 46). In exudates from treated roots, few (unidentified) compounds were detected, which did not appeare in controls, but which varied among samples and times, so that it was impossible to establish a definite pattern.

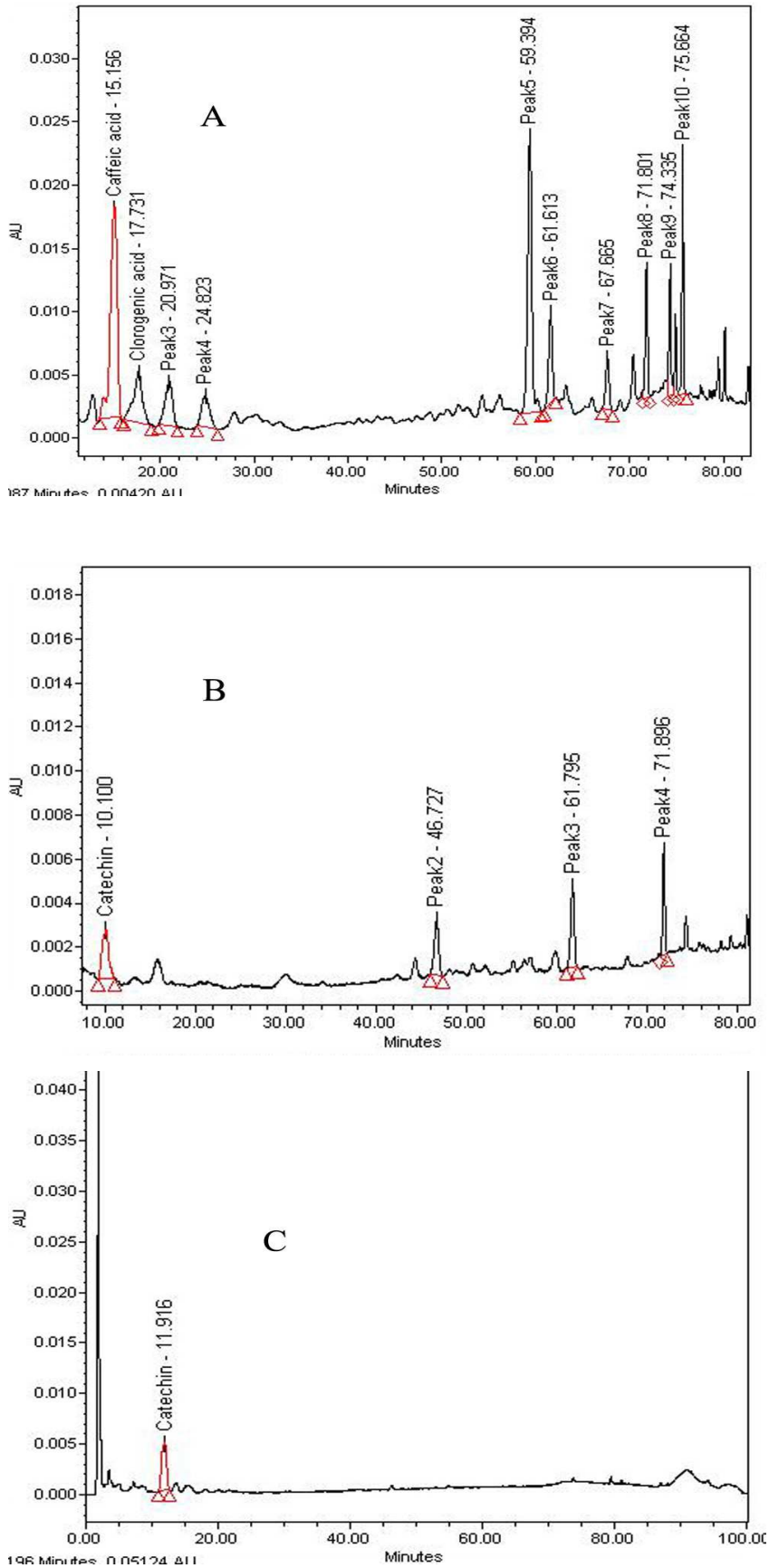


Figure 45 . Phenolic chromatograms relative to leaves (A), roots (B), exudates (C) of Villafranca poplar plants, hydroponically cultured for 72 h in the presence of 100 μM Cu

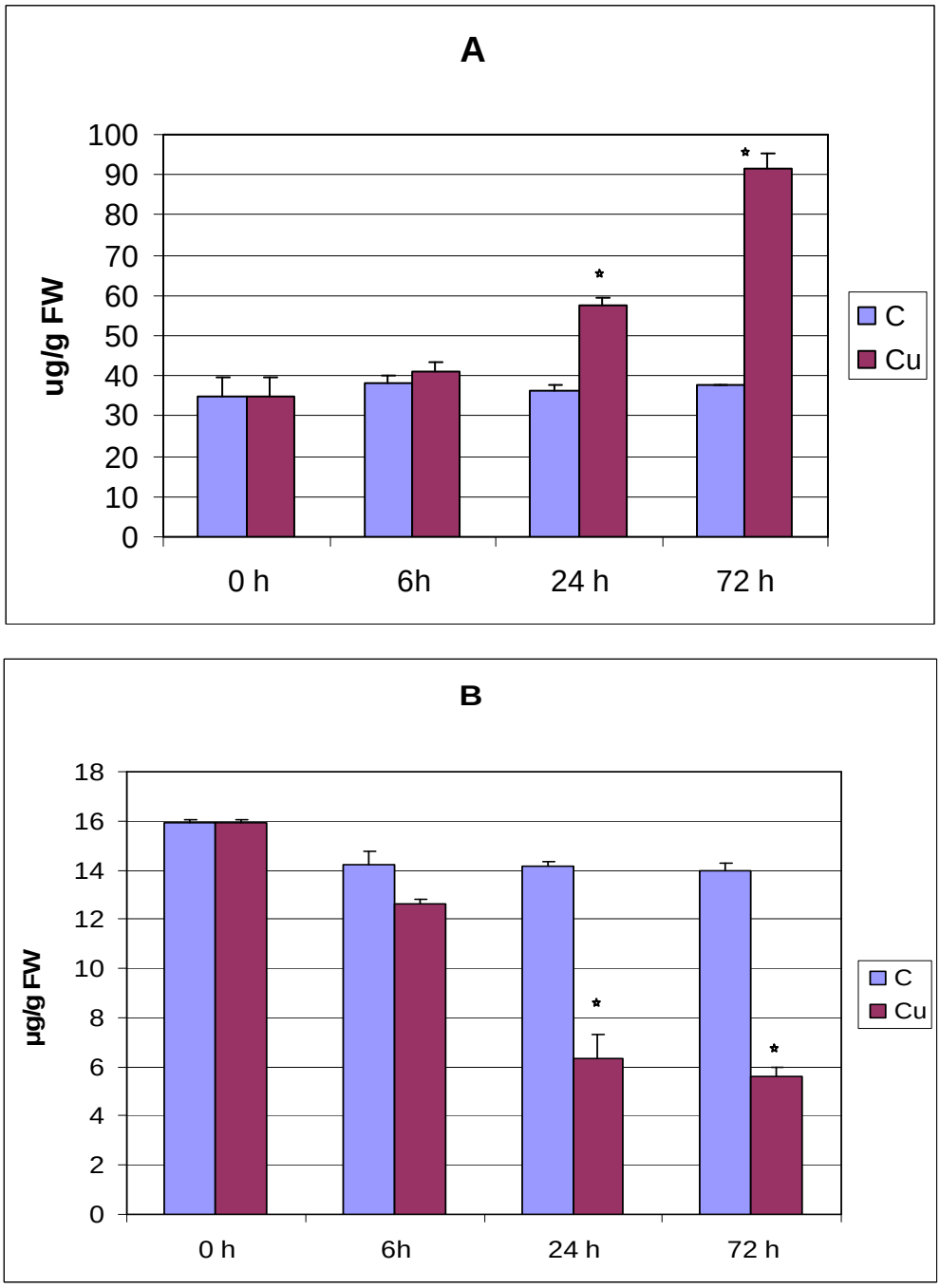


Figure 46. Chlorogenic acid (A) and catechin (B) content in the leaves of Villafranca poplar plants hydroponically cultured for 72 h in the presence (Cu) or in the absence (C) of 100 µM Cu. Asterisks indicate significant differences at P<0.05 relative to controls at each time point

9) Polyamine pattern

The time-course changes of free and conjugated polyamines were determined in control and Cu-treated leaves. In controls free Put levels decreased throughout culture time; free Spd amount increased at 24 h and then decreased at 72 h (Fig. 47A); Spm was not detected. In treated leaves free Put concentration also decreased with time but it was higher than in controls (by 25% at 24 h and more than double at 72 h); Spd amount was higher than in controls at 24-72 h. Conjugated polyamine levels did not display any significant difference except for Put concentration at 24 h which was significantly higher under Cu stress than in controls (Fig. 47B).

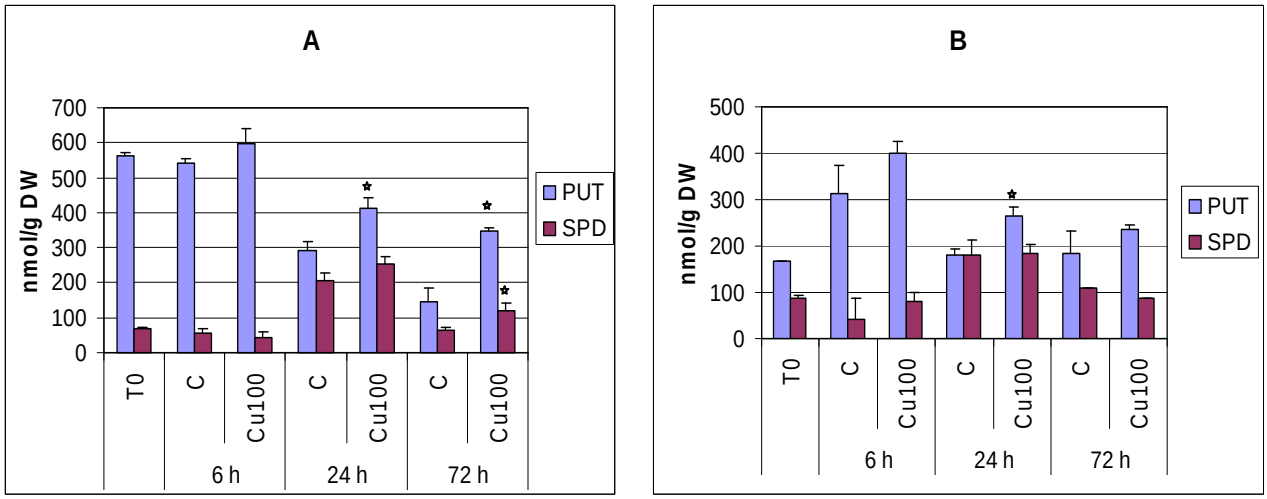


Figure 47. Free (A) and conjugated (B) polyamine content in the leaves of Villafranca poplar plants hydroponically cultured for 72 h in the presence (Cu100) or in the absence (C) of 100 μM Cu. Put, putrescine; Spd, spermidine. Asterisks indicate significant differences at P<0.05 relative to controls at each time point

10) MDA measurement

In control leaves, MDA concentration increased slightly with time (about two-fold from 0 to 72 h); in Cu-treated samples, the increase in MDA content over time was much sharper; in fact, MDA levels were significantly higher than in controls starting from 24 h, reaching concentrations twice those of controls at 72 h (Fig. 48).

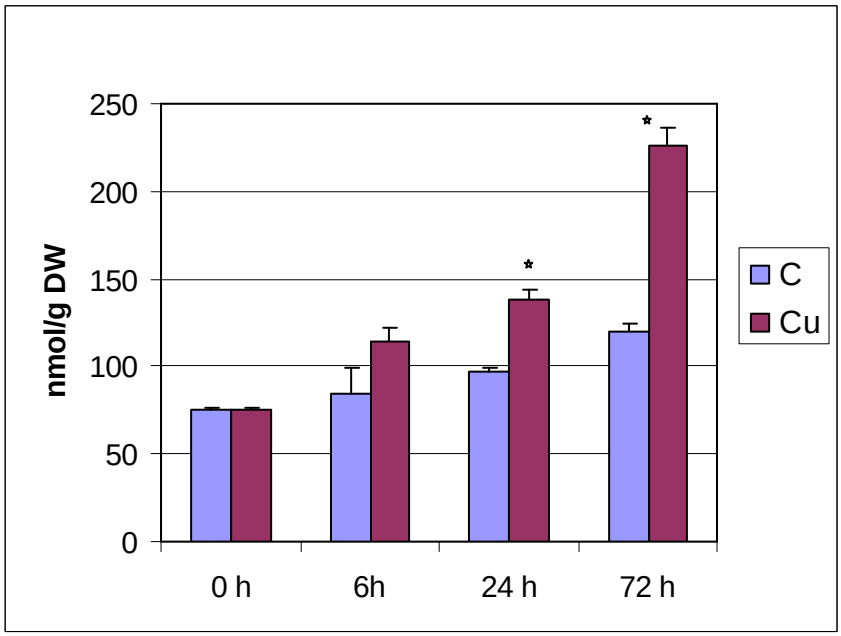


Figure 48. MDA content in the leaves of Villafranca poplar plants hydroponically cultured for 72 h in the presence (Cu) or in the absence (C) of 100 µM Cu. Asterisks indicate significant differences at P<0.05 relative to controls at each time point

Response to copper in the presence of spermidine in *in vitro* cultures

In this experiment micropropagated AL22 plants were transferred to perlite, as previously described, where they were exposed or not (controls) to 100 µM Cu or 100 µM Cu plus 1 mM Spd for 72 h. At zero time and at the end of experimental period, leaves were sampled and total polyphenol and flavonoid amounts and phenolic profiles were obtained. Moreover, MDA and hydrogen peroxide concentration were determined on the same samples to evaluate the level of oxidative stress.

4) Total polyphenol and flavonoid content

In controls, total phenol content increased slightly (+ 50%) at 72 h relative to zero time (T0). In Cu-treated leaves, total phenolics at 72 h rose markedly to 2.5-fold T0 levels, whereas in Cu+Spd-treated samples the increment was less pronounced (ca. 2-fold) and only about 30% more than controls (Fig. 49A). Total flavonoids in controls amount did not change during the considered period, but in Cu-treated samples they increased almost 2-fold, while in Cu+Spd-treated leaves the increment was only 40% (Fig. 49B).

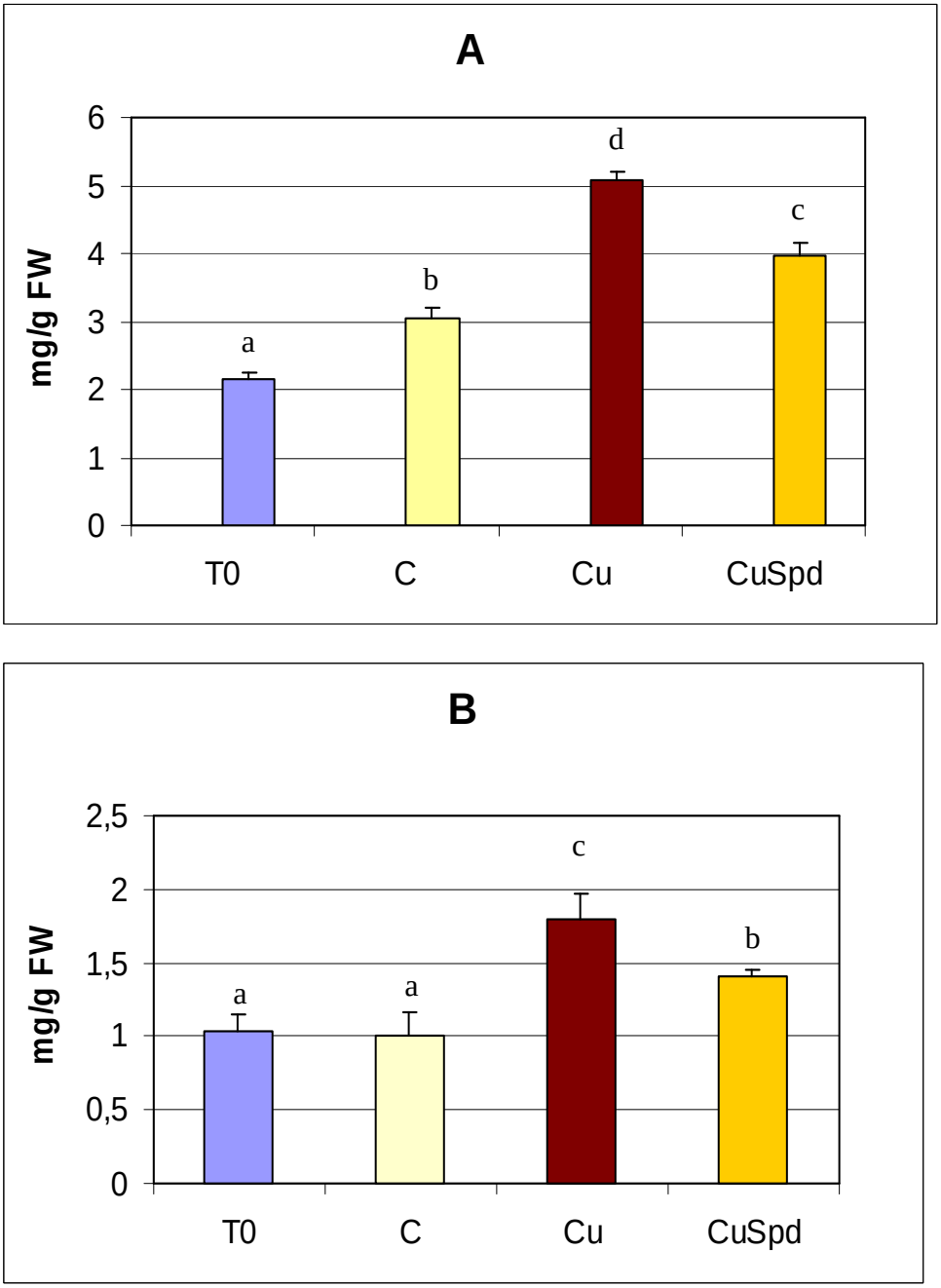


Figure 49. Total polyphenol (A) and flavonoid (B) content in the leaves of AL22 poplar plants cultured in vitro for 72 h in the absence (C) or in the presence of 100 μM Cu (Cu) or 100 μM Cu + 1 mM Spd (CuSpd). Different letters indicate significant differences among treatments at P<0.05

5) Phenolic profile

The HPLC chromatograms displayed numerous peaks including caffeic and chlorogenic acid (not shown). Only the latter changed in treated vs control samples. Thus, while in controls this compound increased very slightly (ca. 30%) during the 72-h experimental time, in Cu-treated samples it increased by about 2-fold relative to T0 while in Cu+Spd-treated samples the increase was less pronounced and resulted in a slightly (ca. 20%) higher concentration than in controls (Fig. 50).

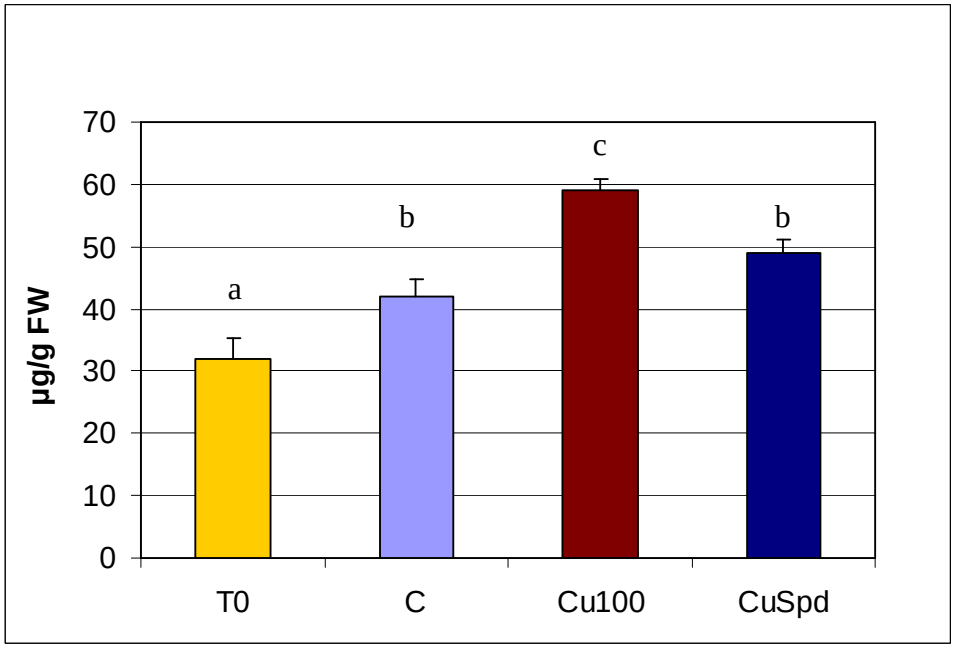
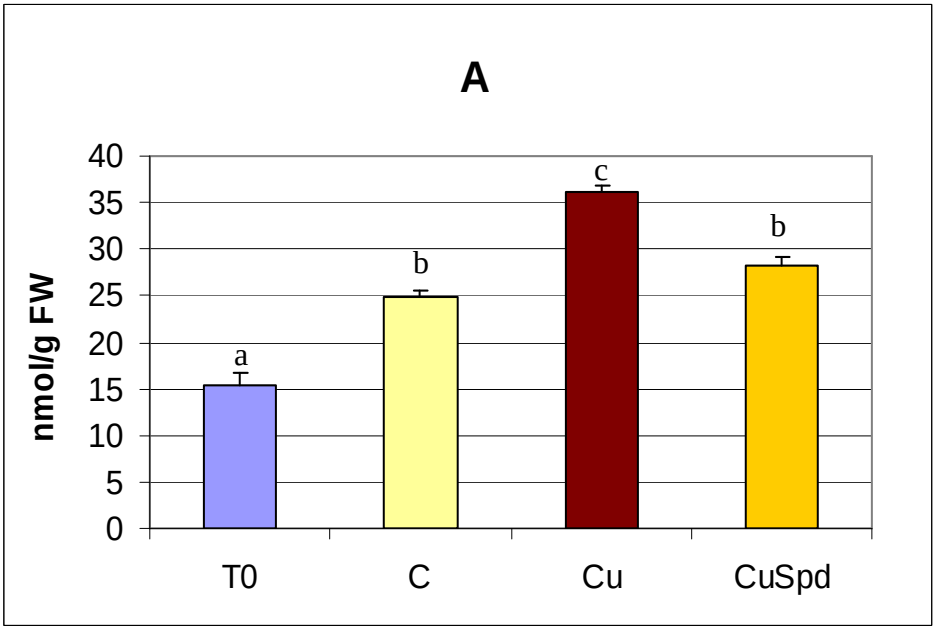


Figure 50. Chlorogenic acid content in the leaves of AL22 poplar plants cultured in vitro for 72 h in the absence (C) or in the presence of 100 µM Cu (Cu) or 100 µM Cu + 1 mM Spd (CuSpd). Different letters indicate significant differences among treatments at P<0.05

6) MDA and hydrogen peroxide measurement

In all samples MDA concentration rose at the end of the experimental time relative to T0. In Cu-treated samples, however, MDA concentration was about 40% higher than in controls, while in Cu+Spd-treated samples it was only about 10% (Fig. 51A). As far as hydrogen peroxide concentration is concerned, its pattern is similar to that of MDA: Cu-treated samples displayed almost twice the hydrogen peroxide concentration of controls; however, in the presence of Spd, levels returned to those of controls (Fig. 51B).



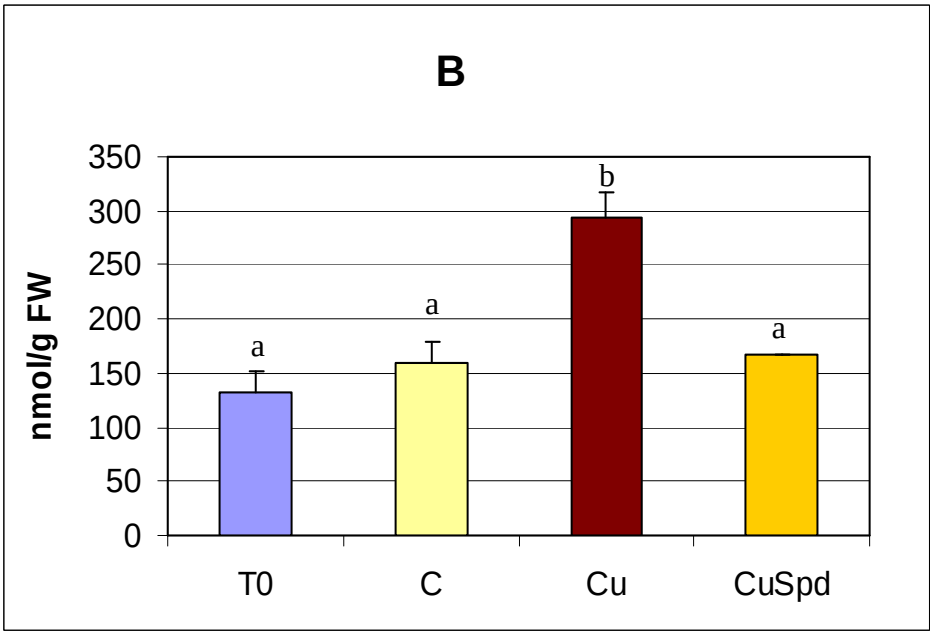


Figure 51. MDA (A) and H_2O_2 content in the leaves of AL22 poplar plants cultured in vitro for 72 h in the absence (C) or in the presence of 100 µM Cu (Cu) or 100 µM Cu + 1 mM Spd (CuSpd). Different letters indicate significant differences among treatments at P<0.05

Discussion

Cu induces toxicity symptoms and oxidative damage

The supplied Cu to poplar plants was accumulated in the leaves (2-fold) and in the roots where it reached levels 12-fold higher than in controls. It is known that Cu, differently from other HMs (zinc), is taken up by roots but hardly translocated to the stem and leaves (Kopponen et al.,, 2001; Todeschini et al., 2007). The extent of copper translocation is dose-dependent (Posmyk et al., 2009; Janas et al., 2010) and, in poplar, highly dependent on the clone due to the great genetic variability of this species (Castiglione et al., 2009; Posmyk et al., 2009).

Although growth reduction is a well known effect of the toxic impact of HMs, as reported in Cu-treated cabbage, lentil and rice seedlings (Posmyc et al., 2009; Janas et al., 2010; Ashan et al., 2007), no changes in the growth rate of Cu-treated poplar plants were presently observed. However, Cu induced visual toxicity symptoms at the higher concentrations in both culture systems and in both poplar clones, Villafranca and AL22. Symptoms mainly consisted in wrinkled leaves and a few necrotic areas. In Villafranca, however, these symptoms were more accentuated than in AL22 suggesting that the former clone is less tolerant to the metal than AL22. By contrast, severe Cu-induced damage was previously reported in Villafranca microcuttings (devoid of roots) cultured *in vitro* starting from day 2 in cultureet al.,: toxicity symptoms consisted of leaf chlorosis and browning up to necrosis, and inhibition of root formation with increasing Cu concentration (Franchin et al., 2007), indicating that the presence of a root apparatus is crucial in order to counteract Cu stress. In pot-grown poplar clones Villafranca and Jean Pourtet, although such toxicity symptoms were not observed, Cu induced a decrease in plant biomass production, leaf area, and shoot length, combined with a strong Cu accumulation in the root apparatus (Todeschini et al., 2007).

Concomitant with toxicity symptoms, in AL22 plants grown *in vitro* Cu negatively affected chlorophyll b content, in agreement with the known negative effects of HMs on photosynthetic pigments and photosystems (Prasad and Strzalka, 2002).

Moreover, proline concentration, which was expected to rise in response to metal stress, decreased in Cu-treated leaves. Accumulation of “compatible solutes”, such as glycinebetaine and proline, is one of the best known mechanisms for osmotic adjustment in plants exposed to salt or drought stress (Kavi Kishor et al., 2005). However, more recent studies suggest that proline is a “multifunctional amino acid” that can influence stress tolerance in multiple ways (Szabados and Savouré, 2010).

Indeed, proline, together with an array of other nitrogen-containing molecules, accumulates under HM stress (Sharma & Dietz, 2006). This is because, in addition to its role as an osmolyte, during exposure to abiotic stress, proline also acts as osmoprotectant, protecting subcellular structures and macromolecules by quenching ROS, as well as a signal molecule (Matysik et al., 2002). In accord with its putative role in cellular homeostasis, including redox balance and energy status, proline has been shown to reduce lipid peroxidation in algal cells exposed to HMs (Mehta & Gaur, 1999), and proline pretreatment alleviated Hg^{2+} toxicity in rice through its ROS scavenging capacity (Wang et al 2009). Recently, an increase in proline levels was observed in Cu-stressed carrot cultures (Szafranska et al., 2011) and radish seedling (Choudhary et al., 2010). In the present study, proline content was negatively affected by treatment with 100 μM Cu, suggesting that this amino acid does not contribute to the stress tolerance mechanism in this system.

In the cell Cu (II) is easily reduced to Cu (I). In this form it is unstable and tends to be oxidized, giving rise to Fenton-type reactions leading to the production of ROS, and thus to oxidative stress, when present in excess (Sgherri et al., 2003). Present data confirm that Cu induced oxidative stress in white poplar as evidenced by increased lipid peroxidation (both clones) and H_2O_2 production (AL22).

A common consequence of most biotic and abiotic stresses is that they result in an increased production of Active Oxygen Species (AOS) which include, not only free radicals such as superoxide and hydroxyl radicals, but also singlet oxygen and hydrogen peroxide. Superoxide radicals are the most abundant species, and their dismutation increases H_2O_2 production which is rapidly converted to hydroxyl radical which is a very dangerous oxygen form. These are very reactive and may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA injury leading to disruption of metabolic functions and loss of cell integrity. Consequently, tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins and MDA (Schützendübel and Polle 2002 JXB). Indeed, one of the first symptoms of oxidative stress in plant tissues is membrane lipid peroxidation, which results in MDA production. A fine tuning of H_2O_2 levels, is, however, necessary because this molecule is involved in important biosynthetic reactions and signal transduction pathways that contribute to plant defence (“oxidative burst”) and/or stress acclimation (Miller et al 2008).

Several studies have shown that exposure of plant tissues to toxic amounts of different HMs results in oxidative stress, and altered antioxidant enzyme activities (Schützendübel and Polle 2002; Groppa et al., 2008). In wheat leaves oxidative stress produced by HMs such as Cd^{2+} or Cu^{2+} was evidenced by an increase of thiobarbituric acid reactive substances (TBARS), i.e., enhanced lipid peroxidation, and altered enzyme activities (Groppa et al 2001, 2007). Results from a study to examine the effect of Al toxicity in barley suggested a correlation between Al uptake, Al-induced oxidative stress, and root growth inhibition (Tamas et al., 2006). Arsenate toxicity in red clover plants was also associated with oxidative stress as evidenced by increased superoxide dismutase (SOD) and peroxidase (POX) activities (Mascher et al., 2002), while in radish seedlings excess Cu (0.2 mM $CuSO_4$) enhanced activities of the antioxidant enzymes guaiacol POX, catalase (CAT), SOD and glutathione reductase (GR) (Choudhary et al 2010).

Phenolic compounds positively respond to Cu stress

Oxidative stress can be counteracted, and cells protected from HM-induced damage by activation of antioxidative systems. In red cabbage, SOD and POX activities increased in response to Cu suggesting their participation in the antioxidant system of this species (Posmyk et al 2009). Antioxidative enzymes and lipid peroxidation were studied in leaves and roots of two mangrove plants, *Kandelia candel* and *Bruguiera gymnorrhiza*, grown under control conditions or with different concentrations of Pb^{2+}, Cd^{2+}, and Hg^{2+} (Zhang et al., 2007). The increase in enzyme activities in HM-stressed *K. candel* and in lipid peroxidation in *B. gymnorrhiza* demonstrated that *K. candel* is more tolerant to HM than *B*. *gymnorrhiza*. The authors proposed that, for pollution monitoring purposes, POX activity and lipid peroxidation in roots and leaves may serve as biomarkers of HM stress.

In plants of Indian ginseng *(Withania somnifera*) an increase in lipoxygenase activity, H_2O_2 production and superoxide anions was reported in response to increasing concentrations (0-200 μM) of Cu; this was accompanied by enhanced activities of ascorbate peroxidase, glutathione-S-transferase and other antioxidant enzymes, but decreases in SOD, CAT and GR activities (Khatun et al., 2008). On the other hand, total phenolic content increased with increasing Cu concentration. In fact, in addition to the enzymatic antioxidative mechanism, antioxidant compounds (e.g. ascorbic acid, glutathione, phenylpropanoids) are also effective in mitigating oxidative stress.

Already at 10 μM Cu concentration, in AL22 plants grown *in vitro* on perlite, the amount of total phenolic compounds (PhC) increased substantially in the leaves, and this increase was maintained at the higher concentrations, reaching levels more than 2-fold higher relative to controls. In both culture systems, in fact, total PhC, and especially flavonoids (FLs), accumulated (up to 4-fold in Villafranca) in the leaves of samples treated with 100 μM Cu, and this increase was mainly due to specific compounds such as chlorogenic acid and the flavonol quercetin. The position of hydroxyl groups and other features in the chemical structure of FLs are important for their antioxidant and free radical scavenging activities. Quercetin is a potent antioxidant because it has the correct structural features for free radical scavenging activity.

There is evidence that in plants exposed to high concentrations of HMs, PhC accumulate and protect plants against their toxicity (Yamasaki et al., 1997). In *Lemna*, bilberry (*V. myrtillus*) and barley, total PhC levels increased in response to abiotic stress including HMs (Babu et al., 2003; Bialonska et al., 2007; Gunes et al., 2007). In cabbage, anthocyanin and sinapoyl esters accumulated under Cu stress (Posmyk et al., 2009), while in Cu-treated lentil seedlings total PhC increased or decreased depending on the cultivar (Jonas et al., 2009; 2010). In *Raphanus sativus* grown in hydroponic culture, lipid peroxidation and membrane damage occurred, and glutathione was oxidized with increasing Cu concentration (up to 15 μM), while phenolic acids (chlorogenic, vanillic, caffeic, ferulic, etc.) as well as total PhC levels increased with the intensification of the Cu treatment (Sgherri et al., 2003).

The accumulation of PhC, and in particular of FLs, is, therefore, the possible plant response to Cu-induced ROS production. PhC, in particular the hydroxyl and carbonyl groups, have a high tendency to chelate HMs. Indeed, phenylpropanoids are good chelators of all redox active transition metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}). Their metal-chelating capacity is well known and is considered a health-promoting factor for humans due to their ROS scavenging, antioxidant, and iron chelating properties (Korkina et al 2007).Their capacity to chelate HMs render them good candidates in preventing HM-induced stress also in plants (Lopes et al., 1999). Moreover, PhC are also free radical scavengers, and FLs (flavonols and flavanols) are most commonly known for their antioxidant activity *in vitro* (Lee et al., 2003; Silva et al 2007). Thus, in Cu-treated red cabbage, the increase in anthocyanin amount

was negatively correlated with membrane lipid peroxidation products (TBARS) in the tissue (Posmyk et al., 2009).

In contrast to these studies, total phenol content in Cu-stressed radish seedlings was significantly lower than control levels; however, treatments that mitigated Cu-induced growth inhibition and oxidative stress (i.e., exogenous application of epibrassinolide and/or putrescine) resulted in enhanced phenolics, suggesting that enhanced tolerance to Cu in radish seedlings was associated with increased amounts of these antioxidant compounds (Choudhary et al. 2011).

In contrast to the changes presently observed in leaves of poplar plants exposed to excess Cu, in the root of plants grown in both culture systems, total PhC and FL content did not change (AL22), despite Cu accumulation to levels 12-fold higher than in controls, or even decreased slightly (Villafranca). Therefore, while responding in a similar way to Cu stress, i.e., increased phenylpropanoid accumulation in leaves, and decreased or unchanged in roots, the response in the two white poplar clones differed insofar as the increase in foliar concentrations of both total PhC and FLs in Villafranca was much more pronounced, relative to controls, than in AL22, suggesting that the extent of over-accumulation of these compounds was inversely related to tolerance, and therefore a stress-related effect.

Phenolic compounds are secreted into root exudates

The increase in total PhC and total FLs in root exudates of Cu-stressed plants as compared with controls, as well as the appearance of abundant (mg/g root FW) flavan-3-ols, such as catechin and epicatechin, in root exudates of AL22 Cu-treated samples suggests that roots actually responded to Cu by increasing phenolic biosynthesis and accumulation, and then secreting them into the rhizosphere. These substances may have several roles when secreted into the rhizosphere. Catechin is considered a phytotoxin that when secreted into the soil is capable of displacing other plant species by triggering ROS generation, calcium signal transduction, genome-wide changes in gene expression, and ultimately death of the root system (Bais et al., 2003). The presence of catechin in root exudates might be interpreted as an allelopathic phenomenon.

More importantly, root exudates play an important role in rhizoremediation through their effect on metal bioavailability and on rhizosphere microorganisms. Plant roots continuously produce and secrete compounds into the rhizosphere. The Cu-binding

capacity of root exudates of several crops (wheat, rape, etc) was evaluated under hydroponic conditions showing that the different species displayed differing abilities to complex Cu (Dousset et al., 2001), and that the nature, as well as the amount, of organic compounds exuded by the roots was important in determining the extent of Cu chelation. In fact, root exudates influence the availability of nutrients – enhancing or reducing it – either directly by acidification, chelation or precipitation, or indirectly through their impact on microbial growth/activity (see below); thus, they affect the biological, physical and chemical properties of the rhizosphere.

The composition of root exudates is affected by the presence of metals in the substrate. A two-day exposure to Al, Cu and Zn stress induced exudation of organic acids from *Populus tremula* cuttings (Qin et al., 2007). The changes in sugar, organic acids, amino acids, and secondary metabolites in wheat root exudates induced by Al were studied, and results showed that Al stress affected exudate composition: some original secondary metabolites disappeared and other new secondary metabolites were released (Wang et al 2006). The results of another study on plants grown on a multiple metal-contaminated soil showed that the presence of metals stimulated the exudation of organic acids and phenolic acids as well as FLs (Quartacci et al., 2009). The authors suggested that root exudates played an important role in solubilising metals, thus favouring their uptake by roots and increasing the phytoextraction potential. In fact, in addition to synthetic chelants (e.g., EDTA, EDDS, Komarek et al 2007), molecules naturally exuded by plants, such as organic acids, have been used in chemically-assisted phytoextraction to enhance metal bioavailability and root uptake.

Plant performance also depends on the ability to communicate with microbes, and there is increasing evidence that root exudates initiate and modulate the dialogue between roots and soil-borne microorganisms (Badri et al., 2009). Furthermore, root exudates support the growth and activities of specific microorganisms, thus shaping the microbial communities present in their rhizosphere (Badri & Vivanco 2009;). These associations are often regulated by specific compounds. For example, isoflavonoids and FLs activate *Rhizobium* genes responsible for the nodulation process and vescicular arbuscular mycorrhiza colonization. In melon roots, a glycosylflavone (isovitexin) was reported to stimulate colonization by the arbuscular mycorrhizal fungus (AMF) *Glomus caledonium* (Akiyama et al 2002).

In a study on plant root growth of mulberry trees growing on a site contaminated with polyaromatic hydrocarbons (PAH), it was shown that the fine roots of these plants produced several flavonoid compounds known to support the growth of organisms capable of degrading aromatic soil contaminants (Olson et al., 2003). In fact, the metabolites released as exudates from living roots (and lysates from dead cells upon root death) are considered the chemical drivers of rhizosphere remediation (rhizoremediation). In support of this contention, Leigh et al., (2002) showed that flavones supported the growth of a PCB-degrading bacterium. Rhizosphere bacteria can also solubilize metals and hence promote their uptake by plant roots, while improving plant growth (Saravanan et al., 2007, Sheng and Xia 2006), and enhanced Cu solubility in soil in the presence of the soil bacterium *Elsholtzia splendens* has been reported (Chen et al., 2005). There is evidence suggesting that AMF can also improve the phytoextraction capacity of white poplar while improving its growth (Cicatelli et al 2010, 2011). Although the important role of secondary metabolites in the establishment of root-microbe interactions is well known (Fester et al., 1999), the relationship between HMs, root exudates, plant-microbe interactions, and phytoextraction/rhizoremediation requires further investigation. In the present work, the two white poplar clones behaved in a similar way in terms of phenylpropanoid secretion into the external medium, with two differences: a) the presence of detectable amounts of catechin and epicatechin in exudates of AL22 but not Villafranca, and b) a greater amount of total FLs in root exudates of both control and Cu-stressed Villafranca relative to AL22. The consequences of these differences in root exudate composition on rhizosphere microbial communities and/or the clone's phytoextraction/phytostabilization potential remains to be studied.

The polyamine response to copper

In poplar the PA response to Cu varied depending on the culture system, the timing and the clone. AL22 plants cultured *in vitro* respond to Cu with a decrease in Put levels. By contrast, in hydroponically grown Cu-treated plants PAs accumulated to higher levels relative to controls. Moreover, while in AL22 plants conjugated PAs levels rose following Cu treatment, in Villafranca the free forms were mainly accumulated.

In general, PA metabolism is up-regulated in response to stress including HMs even though a stress-specific pattern is not apparent (Bouchereau 1999; Groppa 2008

Alcazar et al., 2010). Moreover, while most authors measure free PA concentrations only few report changes in the levels of conjugated PAs. In general, free Put seems to be the most responsive PA to HM but the PA response varies depending upon metal type and concentration, and plant species. In oat and rice leaves Put levels increased following treatment with different metals (Wettlaufer et al., 1991; Lin and Cao 1999). Also in sunflower plants, Cu also induced free Put and Spm accumulation, and increased the activity of Put biosynthetic enzyme activity (Groppa et al., 2007). The latter authors suggested that the two amines could be considered useful markers of Cu stress, the more PA accumulation the more the stress. On the other hand, in carrot embryo cultures increased PA content was associated to the alleviation of Cu toxicity (Szafranska et al., 2011).

Previous work in poplar showed that in Villafranca shoots cultured *in vitro* in the presence of HMs, both free and conjugated PAs strongly and early (one day) accumulated in the presence of zinc while in the presence of Cu only free Put levels rose, revealing the incapacity of the plant to conjugate PAs to their phenolic partners in the presence of the metal (Franchin et al., 2007). Since the toxicity of Zn was much lower than that of Cu, as attested by visual toxicity symptoms and the lower ethylene production of Zn-treated shoots, the ability to early produce PAs, in particular the conjugated forms, was correlated with the capacity of the plant to protect itself from metal stress. The relevance of conjugated PAs was also revealed by results ofa field-screening trial for metal tolerance of several white and black poplar clones;in fact, the conjugated-to-free Put ratio increased strongly in tolerant plants grown on HM-contaminated soil, and a highly significant linear correlation between root Cu concentration and conjugated Put in leaves was observed in the most tolerant clones (Castiglione et al., 2009); in addition, the best performing clone, AL35, which accumulated the highest Cu amount in all plant organs, exhibited levels of conjugated Put which were up to 18-fold higher than in the other clones. In Cr(III)-treated celery seedlings, free and conjugated PA titres increased compared with controls, and this was associated to the amount of Cr(III) in plant tissues (Scoccianti et al., 2006). These observations lend support to the idea that the ability to conjugate PAs is relevant for metal tolerance. The incapacity of Villafranca to produce conjugated PAs in response to copper, as opposed to AL22 which produces them in abundance, may be related to its lower metal tolerance; this was also attested by the

more intense toxicity symptoms displayed by this clone, and by the differential response also in terms of phenolic compound accumulation.

Most conjugated PAs are hydroxycinnamic amides bridging PAs to phenolic metabolism (Edreva et al., 2007; Bassard et al., 2010). They are regarded as end-products of PA metabolism, or as storage forms of PAs and/or phenolics. The ability of conjugated PAs in contrasting metal toxicity may involve their ROS quenching and scavenging capacity (Bors et al., 1989; Velikova et al., 2007); moreover a role in detoxification of the accumulated phenolics cannot be excluded.

Spermidine counteracts Cu-imposed oxidative stress

In micropropagated AL22 poplar the protective effect of Spd against Cu toxicity was revealed by the extent of PhC accumulation, lipid peroxidation, and H_2O_2 levels in the presence of the triamine. Levels of PhC, in particular chlorogenic acid, MDA and H_2O_2 rose following exposure to Cu, reaching levels up to 2-fold those of controls. The addition of Spd significantly counteracted the rise observed in the presence of Cu alone, especially in the case of H_2O_2 that returned to control levels.

As stated above, Cu induces oxidative stress due to overproduction of ROS and Reactive Nitrogen Species (RNS; Szafranska et al., 2011; Zhang et al., 2008). Plants defend themselves against HMs through various antioxidative strategies, for instance by the production of low molecular mass compounds such as proline, PhC and PAs. The latter have been suggested to function as metal chelators (Lovaas 1996), as direct radical scavengers (Ha et al., 1998, Velikova et al., 2007) and by reducing oxidative damage by increasing the activity of antioxidant enzymes (Zhang et al., 2009). Several reports deal with the stress protective role of PAs. Elevation of Spd+Spm/Put ratio due to exogenous PA administration improved Cu tolerance in the leaves of *Nymphoides peltatum* (Wang et al., 2007 szafr). In Cd and Cu-treated sunflower and wheat leaf disks exogenous spermine (Spm), a tetramine whose biological activity is thought to be lesser than that of Spd (Imai et al., 2004), strongly reduced stress-induced ethylene production and the amount of TBARS produced following Cd and Cu treatments (Groppa et al., 2001; Groppa et al., 2003); the activities of antioxidant enzymes, such as GR and SOD, which decreased in the presence of Cu, were partly restored by Spd. In metal-stressed wheat leaf segments, Spm was able to reduce *in situ* H_2O_2 formation (Groppa et al., 2007). Upon exposure to Cu, exogenous Put application alleviated Cu-induced oxidative stress and

resulted in enhanced antioxidant capacity and free radical scavenging activity of *Raphanus sativus* seedlings (Choudhary et al., 2011a). Moreover, the positive effect exerted by exogenous epibrassinolide against Cr (VI) stress was accompanied by increased levels of endogenous Put and Spd (Choudhary et al., 2011b).

The antioxidant role of PAs was also shown under different stress conditions. In bean leaves subjected to acid rain, the increase in H_2O_2 and MDA amounts, and in POX and CAT activities was efficiently counteracted by both Spd and Spm (Velikova et al., 2000). In Virginia pine cultured *in vitro* under salt stress conditions, Put, Spd and Spm reduced salt-induced oxidative damage by increasing the activity of several antioxidant enzymes and decreasing lipid peroxidation (Tang and Newton 2005).

Also transgenic approaches corroborate the hypothesis that PAs efficiently counteract many different kinds of stress. Overexpression of PA biosynthetic enzymes leads to increased PA levels and this is associated to increased multi-stress tolerance (Alcazar et al., 2010). In particular, in *in vitro* cultured pear shoots, overexpression of an apple spermidine synthase (SPDS) gene leads to enhanced tolerance to NaCl, mannitol and Cu (Wen et al., 2008). In a long-term experiment, in the same transgenic shoots, the enhanced Spd levels correlated with enhanced HM tolerance by exerting antioxidant activity (Wen et al., 2010). Authors claim that genetic engineering would provide an effective method to create new germplasm with enhanced tolerance to a wide range of environmental stress.

The molecular basis for the action of PAs in alleviating stress has not been clarified yet but there is evidence that they can act directly at several metabolic levels. PAs may counteract lipid peroxidation possibly by binding to the negative charges of membrane phospholipids (Tadolini et al., 1984). They may also bind cations acting as metal chelators; Lovaas (1996) suggests that the antioxidant effect of PAs is due to a combination of their anion (phospholipids) and cation (Cu^{2+}, Fe^{2+})-binding properties, the latter efficiently preventing the generation of active oxygen species. PAs may facilitate metal ion compartmentation (Brueggemann et al., 1998) or directly scavenge free radicals (Drolet et al., 1986), stabilize membranes, for instance thylakoids, through lipid protection and avoidance of solute leakage (Besford et al., 1993). Borrell et al., (1997) report that treatment with Spd or Spm prevented chlorophyll loss and stabilized thylacoid membranes delaying senescence and suggest that inhibition of peroxidation may be one of the mechanisms responsible for the antisenescence effects of PAs. Finally, PAs can indirectly reduce ROS formation

by inhibiting NADPH oxidase activity with Spm>Spd>Put (Papadakis and Roubelakis-Angelakis 2005). The idea that Spd itself may be a stress-signaling regulator has been also put forward (Kasukabe et al., 2004).

Conclusions

In the present thesis it is shown that in white poplar, cultured *in vitro* or hydroponically, Cu toxicity, as revealed by leaf symptoms and oxidative damage, is associated to a response in terms of PhC, in particular FL, and of free and/or conjugated PAs; moreover, a specific PA, Spd, is able to counteract Cu-induced oxidative damage. The role of root exudates in counteracting environmental stress is bound to their effects on metal bioavailability and on rhizosphere microorganisms; the antioxidant role of Spd is mainly due to its cationic nature and to its ability to form conjugates. Spd may constitute a useful tool for plant protection against metal stress. The poplar response to Cu was differential in terms of PhC, especially in root exudates, and PAs in the two clones and appears associated to a different level of tolerance of Villafranca relative to AL22. This confirms that stress tolerance in poplar is genotype-specific.

References

Adriaensen K, van der Lelie D, Van Laere A, Vangronsveld J, Colpaert JV. A zinc-adapted fungus protects pines from zinc stress. New Phytol 2003 161: 549-555

Alcázar R, Marco F, Cuevas JC, Patrón M, Ferrando A, Carrasco P, Tiburcio AF, Altabella T. Involvement of polyamines in plant response to abiotic stress. Biotechnol Lett 2006 28:1867–1876

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Knocz C, Carrasco P, Tiburcio AF. Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta 2010 231:1237–49

Anderson, TA and Coats, JR. Screening rhizosphere soil sample for the ability to mineralize elevated concentrations of atrazine and metolachlor. J Env Sci Health 1995 B 30: 473 – 484

Andreotti C, Ravaglia D, Ragaini A, Costa G. Phenolic compounds in peach (*Prunus persica*) cultivars at harvest and during fruit maturation. Ann Appl Biol 2008;153: 11-23

Aronsson P, Perttu K. Willow vegetation filters for wastewater treatmentand soil remediation combined with biomass production. Forestry Chronicle 2001 77: 293–299.

Arriagada CA, Herrera MA, Ocampo JA. Beneficial effect of saprobe and arbuscular mycorrhizal fungi on growth of Eucalyptus globulus co-cultured with Glycine max in soil contaminated with heavy metals. J Environ Manage 2007 84: 93–99

Asada K. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 1999 50*:* 601–639

Asthir B, Spoor W, Duffus CM. Involvement of polyamines, diamine oxidase and polyamine oxidase in resistance of barley to *Blumeria graminis* f. sp. Hordei. Euphytica 2004;136:307–12.

Bagni N, Tassoni A. Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. Amino Acids 2001;20:301–17.

Baker AJ. Metal hyperaccumulator plants: a review of the biological resource for possible exploitation in the phytoremediation of metal-polluted soils. In

Phytoremediation of Contaminated Soil and Water. Edited by Terry N, Bañeulos GS. Boca Raton, Florida: CRC Press LLC 1999:85-107

Balestrasse KB, Gallego SM, Benavides MP, Tomaro ML. Polyamines and proline are affected by cadmium stress in nodules and roots of soybean plants. Plant Soil 2005; 270: 343–353

Bassard J.E, Ullmann P, Bernier F, Werck-Reichhart D. Phenolamides. Bridging polyamines to the phenolic metabolism. Phytochemistry 2010; 71: 1808–1824

Beckman CH. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiol Mol Plant Pathol 2000: 57: 101-110

Bernards, MA., and Lewls, N.G. Alkyl ferulates in wound healingpotato tubers. Phytochemistry 1992; 31, 3409-3412.

Besford R, Richardson C, Capell T, Tiburcio AF. Effect of polyamines on stabilization of molecular complexes in thylakoid membranes of osmotically-stressed oat leaves. Planta 1993; 89: 201–206

Bhadra R, Wayment DG, Hughes JB, Shanks JV. Confirmation of conjugation processes during TNT metabolism by axenic plant roots. Environ Sci Technol, 1999, 33:446-452.

Biondi S, Scaramagli S, Capitani F, Altamura MM, Torrigiani P. Methyl jasmonate upregulates biosynthetic gene expression, oxidation and conjugation of polyamines, and inhibits shoot formation in tobacco thin layers. J Exp Bot 2001; 52: 231–242

Bois G, Piche Y, Fung MYP, Khasa DP. Mycorrhizal inoculum potentials of pure reclamation materials and revegetated tailing sands from the Canadian oil sand industry. Mycorrhiza 2005;15:149-158.

Bonneau, L., Carre, M., Dreumont, C., Martin-Tanguy, J. Polyamine metabolism during seedling development in rice. Plant Growth Regul 1994:15, 83–92.

Borrell A, Carbonell L, Farras R, Puig-Parellada P, Tiburcio AF. Polyamines inhibit lipid peroxidation in senescing oat leaves. Physiol Plant 1997; 99, 385-390

Bors W, Langebartels C, Michel C, Sandermann H. Polyamines as radical scavengers and protectants against ozone damage, Phytochemistry 1989; 28:1589–1595.

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J. Polyamines and environmental challenges: recent developments, Plant Sci 1999; 140: 103–125.

Bregoli, A.M., Scaramagli, S., Costa, G., Sabatini, E., Ziosi, V., Biondi, S., Torrigiani, P. Peach (*Prunus persica* L.) 761 fruit ripening: aminoethoxyvinylglycine (AVG) and exogenous 762 polyamines affect ethylene emission and flesh firmness. Physiol Plant 2002; 114, 472–481

Brueggemann LI, Pottosin II, Scho¨nknecht G. Cytoplasmic polyamines block the fast-activating vacuolar cation channel. Plant J 1998; 16: 101–105

Capell T, Bassie L, Christou P. Modulation of the polyamine biosynthetic pathway intransgenic rice confers tolerance to drought stress. Proc Nat Acad Sci USA 2004; 101: 9909–14

Castiglione S. , Todeschini V, Franchin C, Torrigiani P, Gastaldi D, Cicatelli A, C. Rinaudo C, Berta G, Biondi S, G. Lingua G. Clonal differences in survival capacity, copper and zinc accumulation, and correlation with leaf polyamine levels in poplar: A large-scale field trial on heavily polluted soil. Environ Pollut 2009;157 2108–2117

Chen F, Chang-Jun Liu, Tschaplinski TJ, Zhao N. Genomics of Secondary Metabolism in Populus: Interactions with Biotic and Abiotic Environments. Crit Rev Plant Sci 2009; 28:375–392

Chiou A, Karathanos V T, Mylona A, Salta F N, Preventi F, Andrikopoulos N K. Currants (*Vitis vinifera* L.) content of simple phenolics and antioxidant activity. Food Chemistry 2007; 102: 516–522

Choudhary SP, Bhardwaj R, Gupta BD, Dutt P, Gupta RK, Biondi S, Kanwar M, Slathia S. Epibrassinolide induces changes in indole-3-acetic acid, abscisic acid, and polyamine concentrations, and enhances antioxidant potential of radish seedlings under copper stress. Physiol Plant 2010; 140: 280–296

Choudhary SP, Bhardwaj R, Gupta BD, Dutt P, Gupta RK, Kanwar M, Biondi S. Enhancing effects of 24-epibrassinolide and putrescine on the antioxidant capacity and free radical scavenging activity of *Raphanus sativus* seedlings under Cu ion stress. Acta Physiol Plant 2011a; 33:1319-1333

Choudhary SP, Kanwar M, Bhardwaj R, Gupta BD, Gupta RK Epibrassinolide ameliorates Cr (VI) stress via influencing the levels of indole-3-acetic acid, abscisic acid, polyamines and antioxidant system of radish seedlings. Chemosphere 2011b; 84: 592-600

Clemens S. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 2001, 212: 475-486

Cona A, Rea G, Angelini R, Federico R, Tavladoraki P. Functions of amine oxidases in plant development and defence. Trends Plant Sci 2006;11:80–8.

Crowley T, Walters DR. Polyamine metabolism in an incompatible interaction between barley and the powdery mildew fungus, Blumeria graminis f. sp. Hordei. J Phytopathol 2002;150(11–12):1–7

Cuevas JC, Lopez-Cobollo R, Alcázar R, Zarza X, Koncz C, Altabella T, Salinas J, Tiburcio AF, Ferrando A. Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiol 2008; 148:1094–1105

Cuevas JC, Lopez-Cobollo R, Alcazar R, Zarza X, Koncz C, Al tabella T, Salinas J, Tiburcio AF, Ferrando A. Putrescine as a signal to modulate the indispensable ABA increase under cold stress. PS&B 2009; 4:219–220

Cui X, Ge C, Wang R, Wang H, Chen W, Fu Z, Jiang X, Li J,Wang Y. The BUD2 mutation affects plant architecture through altering cytokinin and auxin responses in Arabidopsis. Cell Res 2010; 20: 576–586

Dai, L.-P., Xiong, Z.-T., Huang, Y., Li, M.-J. Cadmium-induced changes in pigments, total phenolics, and phenylalanine ammonialyase activity in fronds of Azolla imbricate. Environ Toxicol 2006; 21, 505–512.

Della Mea, M., De Filippis, F., Genovesi, V., Serafini Fracassini, D., Del Duca, S. The acropetal wave of developmental cell death of tobacco corolla is preceded by activation of transglutaminase in different cell compartments. Plant Physiol 2007; 144, 1211–1222.

Dempsey, D. A., Shah, J., and Klessig, D. F. Salicylic acid and disease resistance in plants. Crit Rev Plant Sci 1999; 18: 547–575

Dewitte W, Riou-Khamlichi C, Scofield S, Healy JMS, Jacqmard A, Kilby NJ, Murray JAH. Altered cell cycle distribution, hyperplasia, and inhibited differentiation in Arabidopsis caused by the D-type cyclin CYCD3. Plant Cell 2003; 15: 79–92

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V. Arabidopsis CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc Natl Acad Sci USA 2007; 104: 14537–14542

Di Baccio, D., Tognetti, R., Sebastiani, L., Vitagliano, C. Responses of *Populus deltoides* x *Populus nigra* (*Populus* x *euramericana*) clone I-214 to high zinc concentrations. New Phytol 2003; 159, 443–452.

Dixon R.A, Paiva N.L. Stress-Induced Phenylpropanoid Metabolism. Plant Cell 1995; 7, 1085-1097

Donelly PK, Hedge RS, Fletcher JS. Grown of PCB-degrading bacteria on compounds from photosynthetic plants. Chemosphere 1994, 28: 981 – 988

Dos Santos Utmazian M.N., Wieshammer G, Vega R, Wenzel WW. Hydroponic screening for metal resistance and accumulation of cadmium and zinc in twenty clones of willows and poplars. Environ Pollut 2007; 148, 155–165

Drolet G, Dumbroff EB, Legge R, Thompson JE. Radical scavenging properties of polyamines. Phytochemistry 1986; 25, 367-371

Dutton MV, Humphreys P. Assessing the potential of short rotation coppice (SRC) for cleanup of radionuclide-contaminated sites. Int J Phytoremediat 2005; 7, 279–293

Edreva A.M,. Velikova V.B, Tsonev T.D. Phenylamides in Plants. Russ J Plant Phys 2007, Vol. 54, No. 3, pp. 287–301

Edwards, R., Dixon, D.P., Walbot, V. Plant Glutathione *S*-Transferases: Enzymes with Multiple Functions in Sickness and in Health, Trends Plant Sci., 2000, vol. 5, pp. 193–198

Facchini PJ, Hagel J, Zulak KG. Hydroxycinnamic Acid Amide Metabolism: Physiology and Biochemistry. Can J Bot 2002; 80, 577–589

Fletcher JS, Hedge RS. Release of phenols by perennial plant roots and their potential importance in bioremediation. Chemosphere 1995; 31: 3009-16

Flores HE. Changes in polyamine metabolism in response to abiotic stress.In: Slocum, R.D., Flores, H.E. (Eds.), Biochemistry and Physiology of Polyamines in Plants. CRC Press, Boca Raton, 1991; pp. 213–226.

Franceschetti M, Hanfrey C, Scaramagli S, Torrigiani P, Bagni N, Burtin D, Michael AJ. Characterization of monocot and dicot plant S-adenosyl-L-methionine decarboxylasegene families including identification in the mRNA of a highly conserved pairof upstream overlapping open reading frames. Biochem J 2001; 53:403–9.

Franchin C, Fossati T, Pasquini E, Lingua G, Castiglione S, Torrigiani P, Biondi S. High concentrations of zinc and copper induce differential polyamine responses in micropropagated white poplar (*Populus alba*). Physiol Plant 2007; 130: 77–90

Frost CJ, Mescher MC, Dervinis C, Davis JM, Carlson JE, De Moraes CM. Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3-hexenyl acetate. New Phytol 2008; 180: 722– 734

Gamalero E, Berta G, Massa N, Glick BR, Lingua G. Interactions between Pseudomonas putida UW4 and Gigaspora rosea BEG9 and their consequences for the growth of cucumber under salt-stress conditions. J Appl Microbiol 2010;108:236-245.

Garufi A, Visconti S, Camoni L, Aducci P. Polyamines as physiological regulators of Interaction with the plant plasmamembrane H+ -ATPase. Plant Cell Physiol 2007; 48: 434–40

Gill S, Tuteja N. Polyamines and abiotic stress tolerance in plants. Plant Signal Behav 2010; 7: 5(1)

Göhre V, Paszkowski U. Contribution of the arbuscular mycorrhizal symbiosis to heavy metal Phytoremediation. Planta 2006; 223: 1115–1122

Gonzalez-Chavez MC, Carrillo-Gonzalez R, Wright SF, Nichols KA. The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. Environ Pollut 2004; 130:317–323

González-Guerrero M, Cano C, Azcón-Aguilar C, Ferrol N. GintMT1 encodes a functional metallothionein in Glomus intraradices that responds to oxidative stress. Mycorrhiza 2007; 17:327–335

Gordon M, Choe N, Duffy J, Ekuan G, Heilman P, Muiznieks I, Ruszaj M, Shurtleff BB, Strand S, Wilmoth J, Newman LA. Phytoremediation of trichloroethylene with hybrid poplars. Environ Health Perspect 1998; 106: 1001-1004

Groppa MD, Benavides MP. Polyamines and abiotic stress: recent advances. Amino Acids 2008; 34:35–45

Groppa MD, Tomaro ML, Benavides MP. Polyamines as protectors against cadmium or copper-induced oxidative damage in sunflower leaf discs. Plant Sci 2001; 161: 481–488

Groppa MD, Benavides MP, Tomaro ML. Polyamine metabolism in sunflower and wheat leaf discs under cadmium or copper stress. Plant Sci 2003; 164: 293–299

Greenland AJ, Lewis DH. Amines in barley leaves infected with brown rust and their possible relevance to formation of ‘green islands’. New Phytol 1984; 96: 283–91.

Ha HC, Woster PM, Jager JD, Casero RAJ. The role of polyamine catabolism in polyamineanalogue- induced programmed cell death. Proc Natl Acad Sci USA 1997; 94: 11557–62

Hahlbrock, K. Flavonoids. In Biochemistry of Plants, Vol. 7, P.K. Stumpf and E.E. Conn, eds (New York: Academic Press) 1981; pp. 425-456

Hahlbrock, K., and Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant MoI Biol 1989; 40, 347-369

Hanzawa Y, Imai A, Michael AJ, Komeda Y, Takahashi T. Characterization of the spermidine synthase-related gene family in Arabidopsis thaliana. FEBS Lett 2002; 527: 176–80

Hanzawa Y, Takahashi T, Michael AJ, Burtin, D., Long, D., Pineiro, M., Coupland,G., Komeda, Y. ACAULIS5, an Arabidopsis gene required for stem elongation, encodes a spermine synthase. EMBO J 2000; 19, 4248–4256

Harborne JB. The Flavonoids: Advances in Research since 1980. New York: Chapman and Hall, (1988) p. 125

Hauschild M.Z. Putrescine (1,4-diaminobutane) as an indicator of pollution-induced stress in higher plants: barley and rape stressed with Cr(III) e Cr(VI) Ecotox Environ Safety 1993; 26: 228-247

Havelange A, Lejeune P, Bernier, G., Kaur-Sawhney, R., Galston, A.W. Putrescine export from leaves in relation to floral transition in Sinapis alba. Physiol Plant 1996; 96, 59–65

Hildebrandt U, Regvar M, Bothe H. Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 2007; 68: 139–146

Hussain SS, Ali M, Maqbool Ahmad M, Siddique KHM. Polyamines: Natural and engineered abiotic and biotic stress tolerance in plants. Biotechnology Advances 2011; 29: 300–311

Imai A, Akiyama T, Kato T, Sato S, Tabata S, Yamamoto KT, Takahashi T. Spermine is not essential for survival of Arabidopsis. FEBS Lett 2004; 556: 148–152

Janicka-Russak M, Kabala K, Miodzinska E, Klobus G. The role of polyamines in the regulation of the plasma membrane and the tonoplast proton pumps under salt stress. J Plant Physiol 2010; 167, 261-269.

Jentschkea G, Godboldb DL. Metal toxicity and ectomycorrhizas. Physiol Plant 2000, 109: 107–116

Kakehi J, Kuwashiro Y, Niitsu M, Takahashi T. Thermospermine is required for stem elongation in Arabidopsis thaliana. Plant Cell Physiol. 2008; 49, 1342–1349.

Karliński L, Rudawska M, Kieliszewska-Rokicka B, Leski T. Relationship between genotype and soil environment during colonization of poplar roots by mycorrhizal and endophytic fungi. Mycorrhiza 2010; 20:315-324.

Kasinathan V, Wingler A. Effect of reduced arginine decarboxylase activity on salt tolerance and on polyamine formation during salt stress in *Arabidopsis thaliana*. Physiol Plant 2004; 121:101–107

Kasukabe Y, He L, Nada K, Misawa S, Ihara I, Tachibana S. Overexpression of spermidine synthase enhancestolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis* thaliana. Plant Cell Physiol 2004; 45: 712–722

Kaur-Sawhney R, Tiburcio AF, Altabella T, Galston A. Polyamines in plants: an overview. J Cell Mol Biol 2003; 2: 1-12

Kohler A, Blaudez D, Chalot M, Martin F. Cloning and expression of multiple metallothioneins from hybrid poplar. New Phytol 2004, 164 : 83–93

Komives T, Gullner G. Dendroremediation: the use of trees in cleaning up polluted soils. Focus on Biotechnology 2006; 9A: 23-31

Kopper BJ, Lindroth RL. Effects of elevated carbon dioxide and ozone on the phytochemistry of aspen and performance of an herbivore. Oecologia 2003; 134: 95–103

Korcak RF. Cadmium distribution in field-grown fruit trees. J Environ Qual 1989; 18: 519-23.

Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ. Rhizoremediation: a beneficial plant–microbe interaction. Mol Plant Microbe In 2004;17 :6-15

Kusano T, Yamaguchi K, Berberich T, Takahashi Y. The polyamine spermine rescues *Arabidopsis* from salinity and drought stresses. Plant Signal Behav 2007; 2: 250–1

Kusano T, Berberich T, Tateda C, Takahashi Y. Polyamines: essential factors for growth and survival. Planta 2008; 228: 367–381

Kuznetsov Vl, Shevyakova NI. Polyamines and stress tolerance of plants. Plant Stress 2007; 1: 50–71

Lamaison JLC, Carnet A. Teneurs en principaux flavonoids des fleurs del *Crataegus monogyna* Jacq et de *Crataegus laevigata* (Poiret D.C.) en function de la vegetation. Pharma Acta Helv 1990; 65: 315-320

Lattanzio, V. Advances in Phytochemistry, Imperato, F. (Ed.), ISBN 2003; 81- 271-0018-8. Research Signpost, Trivandrum, Kerala, India, p. 49

Lattanzio V, Di Venere D, Linsalata V, Bertolini P, Ippolito A, and Salerno M. Low temperature metabolism of apple phenolics and quiescence of *Phlyctaena vagabunda*. J Agric Food Chem 2001;49: 5817

Lattanzio V, Kroon PA, Quideau S, Treutter D. Plant phenolics – Secondary metabolites with diverse functions. In: Recent Advances in Polyphenol Research 2008; Vol. 1 (Daayf F and Lattanzio V, eds). Wiley-Blackwell, Chichester, Uk, pp 1-35

Laureysens I, Blust R, Temmerman L, Lemmens C, Ceulemans R. Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture: I. Seasonal variation in leaf, wood and bark concentrations. Environ Pollut 2004; 131, 485–494

Lebeau T, Braud A, Jézeéuel K. Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. Environ Pollut 2008, 153:497-522

Li J. Ou-Lee TA. Raba R. Amundson R.G. and Last RL. Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell 1993; 5, 171-179

Lin CH, Kao CH. Excess copper induces an accumulation of putrescine in rice leaves. Bot Bull Acad Sin 1999; 40, 213-218

Lindsay GS, Wallace HM. Changes in polyamine catabolism in HL-60 human promyelogenous leukaemic cells in response to etoposide induced apoptosis. Biochem J 1998; 337: 83–7.

Lingua G, D'Agostino G, Massa N, Antosiano M, Berta G. Mycorrhiza-induced differential response to a yellows disease in tomato*.* Mycorrhiza 2002*;*12*:* 191-198

Lingua G, Franchin C, Todeschini V, Castiglione S, Biondi S, Burlando B, Parravicini V, Torrigiani P, Berta G. Arbuscular mycorrhizal fungi differentially affect the response to high zinc concentrations of two registered poplar clones. Environ Pollut 2008, 153; 137-147

Logemann E, Tavernaro, A, Schulz, W, Somssich, I.E, and Hahlbrock K. UV light selectively coinduces supply pathways from primary metabolism and flavonoid secondary product formation in parsley. PNAS 2000; 97: 1903-1907

Lois, R. Accumulation of UV-absorbing flavonoids induced by UV-B radiation in *Arabidopsis thaliana* L. I. Mechanisms of UV resistance in *Arabidopsis*. Planta 1994; 194: 498-503.

Lovaas E. Antioxidant and metal-chelating effects of polyamines. In: Sies H (ed) Advances in Pharmacology. Antioxidants in Disease Mechanism and Therapy, 1996; Vol. 38, Academic Press, New York, pp 119–149

Mahajan S, Tuteja N. Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 2005; 444: 139–158

Marschner H. Root-induced changes in the availability of micronutrients in the rhizosphere. In Plant Roots, the Hidden Half, Y. Waisel, A. Eshel, and U. Kafkafi, eds (New York: Marcel Dekker, InC.) 1991; pp. 503-528

Martin C. and Martin-Tanguy J. Polyamines Gonjuguées et Limitation de l'Expansion Virale chez les Végétaux, C. R. Acad. Sci., Ser. 3*,* 1981; 292: 249–251.

Martin-Tanguy J, Martin C, Gallet M and Vernoy R Sur de Puissants. Inhibiteurs de Multiplication du Virus de la Mosaïque de Tabac, C. R. Acad. Sci., Ser. D 1976; 282: 2231–2234

Martin-TanguyJ. The occurrence and possible function of hydroxycinnamoy acid amides in plants. Plant Growth Regul 1985; 3: 381–399

Martin-Tanguy J. Conjugated polyamines and reproductive development: biochemical, molecular and physiological approaches. Physiol Plant 1997; 100: 675–688

McLusky SR, Bennett MH, Beale MH, Lewis MJ, Gaskin, P and Mansfield JW. Cell Wall Alterations and Localized Accumulation of Feruloyl-3'-Methoxytyramine in Onion Epidermis at Sites of Attempted Penetration by *Botrytis allii* Are Associated with ActinPolarization, Peroxidase Activity and Suppressionof Flavonoid Biosynthesis. Plant J 1999; 17: 523–534.

Meagher RB. Phytoremediation of toxic elemental and organic pollutants. Curr Opin Plant Biol 2000; 3: 153–162

Milton H. Saier Jr. A Functional-Phylogenetic Classification System for Transmembrane Solute Transporters. Microbiol Mol Biol R 2000; 64: 354-411

Miranda M, Ralph, SG, Mellway R, White R, Heath MC, Bohlmann J and Constabel C. P. The transcriptional response of hybrid poplar (*Populus trichocarpa* × *P. deltoides*) to infection by *Melampsora medusae* leaf rust involves induction of flavonoid pathway genes leading to the accumulation of proanthocyanidins. Mol. Plant Microbe In 2007; 20: 816–831

Mitsuya Y, Takahashi Y, Uehara Y, Berberich T, Miyazaki A, Takahashi H, Kusano T. Identification of a novel Cys2/His2-type zinc finger protein as a

component of a spermine-signaling pathway in tobacco. J Plant Physiol. 2007; 164: 785-93

Moschou PN, Paschalidis KA, Roubelakis-Angelakis KA. Plant polyamine catabolism: the state of the art. Plant Signal Behav. 2008; 3: 1061-1066

Muniz L, Minguet EG, Singh SK, Pesquet E, Vera-Sirera F, Moreau-Courtois CL, **Carbonell J, Bla´zquez MA, Tuominen H.** ACAULIS5 controls Arabidopsis xylem specification through the prevention of premature cell death. Development 2008; 135: 2573–2582

Nayyar H, Chander S. Protective effects of polyamines against oxidative stress induced by water and cold stress in chickpea. J Agron Crop Sci 2004;190: 355–65.

Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I. Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot 2008; 59: 165–176

O'Halloran TV, Cizewski Culotta V. Metallochaperones, an intracellular shuttle service for metal ions. J Biol Chem 2000; 275: 25057-25060

Paixao N, Perestrelo R, Marques J C, Jose´, Camara J S. Relationship between antioxidant capacity and total phenolic content of red, rose´ and white wines. Food Chem 2007; 105: 204–214

Papadakis AK, Roubelakis-Angelakis KA. Polyamines inhibit NADP oxidase-mediated superoxide generation and putrescine prevents programmed cell death induced by polyamine oxidase-generated hydrogen peroxide. Planta 2005; 220: 826-837

Park MH. The post-translational synthesis of a polyamine-dependent amino acid hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). J Biochem. 2006; 139: 161–169

Pegg AE, Michael A. Spermine synthase. Cell Mol Life Sci 2009; 67: 113–121

Peters DJ and Constabel CP. Molecular analysis of herbivore induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant J 2002; 32: 701–712

Ponchet M, Martin-Tanguy J, Andréoli C and Martin C. Apparition de Substances de Type Phénolamidelors de l'Interaction *Dianthus caryophyllus* L. var.*"Scania"*–*Phytophthora parasitica* Dastur. Agronomie 1982, 2: 37–44

Posmyk MM, Kontek R, Janas KM. Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotox Environ Safety 2009; 72 : 596–602

Pulford ID, Watson C. Phytoremediation of heavy metal-contaminated land by trees. Review. Environ Int 2003, 29: 529– 540

Punshon T, Dickinson NM. Acclimation of Salix to metal stress. New Phytol 1997; 137: 303–314.

Rodriguez R, Redman . More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J Exp Bot 2008; 59: 1109-1114

Salt DE, Smith RD, Raskin I. Phytoremediation. Ann Rev Plant Physiol Plant Mol Biol 1998; 49: 643-668

Scoccianti V, Crinelli R, Tirillini B, Mancinelli V, Speranza A. Uptake and toxicity of Cr(III) in celery seedlings. Chemosphere 2006; 64: 1695–1703

Sebastiani L, Scebba F, Tognetti R. Heavy metal accumulation and growth responses in poplar clones Eridano (*Populus deltoides* x *maximowiczii*) and I-214(*P. x euramericana*) exposed to industrial waste. Environ Exp Bot 2004; 52: 79–88

Shutzendubel A, Polle A. Plant responses to abiotic stress: heavy metal-indiced oxidative stress e protection by mycorrhization. J Exp Bot 2002; 53: 1351-1365

Singer AC, Crowley DE, Thompson IP. Secondary plant metabolites in phytoremediation and biotransformation. Review. Trends Biotechnol 2003; 21: 123-130

Smith SE, Read DJ. Mycorrhizal symbioses. 2nd edn. San Diego: Academic Press 1997.

Son S and Lewis BA. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure–activity relationship. J Agric Food Chem 2002; 50: 468–472

Soudek P, Tykva R, Vanek T. Laboratory analyses of Cs-137 uptake by sunflower, reed and poplar. Chemosphere 2004; 55: 1081–1087

Stes E, Biondi S, Holsters M, Vereecke D. Bacterial and plant signal integration via D3-type cyclins enhances symptom development in the *Arabidopsis thaliana—Rhodococcus fascians* interaction. Plant Physiol 2011; 156: 712-725

Szafranska K, Cvikrova M, Kowalska U, Gorecka K, Gorecki R, Martincova O, Janas KM. Influence of copper ions on growth, lipid peroxidation, and proline and

polyamine content in carrot rosettes obtained from anther culture. Acta Physiol Plant 2011; 33: 851-859

Tadolini B. Polyamine inhibition of lipoperoxidation. The influence of polyamines on iron oxidation in the presence of compounds mimicking phospholipids polar heads, Biochem J 1989; 249: 33–36

Tadolini B, Cabrini L, Landi L, Varani F, Pasquali P. Polyamine binding to phospholipid vesicles and inhibition of lipid peroxidation. Biochem Biophys Res Commun 1984; 122: 550-555

Takacs D, Radimszky L, Nemeth T. The arbuscular mycorrhizal status of poplar clones selected for phytoremediation of soils contaminated with heavy metals. Zeitschrift fur Naturforschung Series C 2005 ;60*:* 357-361

Taku Takahashi T, Kakehi JI. Polyamines: ubiquitous polycations with unique roles in growth and stress responses. Ann Bot 2010; 105: 1–6

Tang W, Newton RJ. Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in Virginia pine. Plant Growth Regul 2005; 46: 31-43

Tarenghi E, Martin-Tanguy J. Polyamides, floral induction and floral development of strawberry (*Fragaria ananassa duch*.). Plant Growth Regul 1995; 17: 157–165

Tlustos P, Pavliková D, Száková J, Fisherová Z, Balík J. Explotation of fast growing trees in metal remediation. Focus on Biotechnology 2006; 9A: 83–99

Todeschini V, Franchin C, Castiglione S, Burlando B, Biondi S**, Torrigiani P, Berta G, Lingua G.** Responses to copper of two registered poplar clones inoculated or not with arbuscular mycorrhizal fungi. Caryologia 2007*;* 60*:* 146-155*.*

Torrigiani P, Rabiti AL, Bortolotti C, Betti L, Marani F, Canova A, Bagni N. Polyamine synthesis and accumulation in the hypersensitive response to TMV in Nicotina tabacum. New Phytol 1997; 135: 467–73.

Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Floh EIS, Scherer GFE. Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant Cell Physiol 2006; 47: 346–354

Turkmen N, Sari F, Velioglu YS. Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. Food Chem 2006; 99: 835–841

Turner LB, Muller-Harvey I, McAllan AB. Phytochemistry 1993; 33: 791.

Tuskan GA et al. The genome of black cottonwood, *Populus trichocarpa* (Torr. and Gray). Science 2006; 313: 1596–1604

Urano K, Yoshiba Y, Nanjo T, Igarashi Y, Seki M, Sekiguchi F, Yamaguchi-Shinozaki K,Shinozaki K. Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. Plant Cell Environ 2003; 26: 1917–26

Urano K, Yoshiba Y, Nanjo T, Ito T, Yamaguchi-Shinozaki K, Shinozaki K. *Arabidopsis* stress-inducible gene for arginine decarboxylase AtADC2 is required for accumulation of putrescine in salt tolerance. Biochem Biophys Res Commun 2004; 313: 369–375

Velikova V, Yordanov I, Edreva A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Protective role of exogenous polyamines. Plant Sci 2000; 151: 59-66

Velikova VB, Edreva AM, Tsonev TD, Jones HG. Singlet oxygen quenching by phenylamides and their parent compounds. Z Naturforsch 2007; 62c: 833-838

Vosátka M, Rydlová J, Sudová R, Vohník M. Mycorrhizal fungi as helping in phytoremediation of degradated and contaminated soil. Focus on Biotechnology 2006, 9A: 83-99

Walker TS, Bais HP, Grotewold E, Vivanco JM. Root exudation and rhizosphere biology. Plant Physiol 2003, 132: 44-51

Wallace G and Fry SC. *In Vitro* Peroxidase-catalyzed oxidation of ferulic acid esters. Phytochemistry1995; 39: 1293–1299

Walters DR, Wylie MA. Polyamines in discrete regions of barley leaves infected with the powdery mildew fungus *Erysiphe graminis*. Physiol Plant 1986; 67: 630–3

Walters DR, Wilson PWF, Shuttleton MA. Relative changes in levels of polyamines and activities of biosynthetic enzymes in barley infected with powdery mildew fungus, *Erysiphe graminis* DC. Ex Merat f. sp. Hordei Marchal. New Phytol 1985; 101: 695–705

Watson MB, Emory KK, Piatak RM, Malmberg RL. Arginine decarboxylase (polyamine synthesis) mutants of *Arabidopsis* thaliana exhibit altered root growth. Plant J 1998; 13: 231–9

Weir T L, Park, SW, Vivanco J M. Biochemical and physiological mechanisms mediated by allelochemicals. Curr Opin Plant Biol 2004; 7: 472-479

Wettlaufer SH, Osmeloski J, Weinstein LH. Response of polyamines to heavy metal stress in oat seedlings. Env Toxicol Chem 1991; 10: 1083-1088

White, PI and Broadley MR. Calcium in Plants. Ann Bot 2003; 92: 487–511

Yang Y, Yaning Chen Y, Li W. Arbuscular mycorrhizal fungi infection in desert riparian forest and its environmental implications: A case study in the lower reach of Tarim River. Progress in Natural Science 2008; 18: 983–991

Yoda H, Hamaguchi R, Sano H. Induction of hypersensitive cell death by hydrogenperoxide produced through polyamine degradation in tobacco plants. Plant Physiol 2003; 132: 19731981

Zalesny Jr, RS, Bauer EO, Hall R., Zalesny JA, Kunzman, J, Rog CJ, Riemenschneider RE. Clonal variation in survival and growth of hybrid poplar and willow in an in situ trial on soils heavily contaminated with petroleum hydrocarbons. Int J Phytoremedia 2005; 7: 177–197

Zimmer D, Baum C, Leinweber P, Hrynkiewicz K, Meissner R. Associated bacteria increase the phytoestraction of cadmium and zinc from a metal-contaminated soil by mycorrhizal willows. In J Phytoremedia 2009; 11: 200–213

Ziosi V, Bregoli AM, Bonghi C, Fossati T, Biondi S, Costa G, Torrigiani P. Transcript levels of ethylene perception and biosynthesis genes as altered by putrescine, spermidine and aminoethoxyvinylglycine (AVG) during the course of ripening in peach fruit (*Prunus persica* L. Batsch). New Phytol 2006; 172: 229-238