

**UNIVERSITA' DEGLI STUDI DI PARMA
DOTTORATO DI RICERCA IN BIOTECNOLOGIE
XXIII° CICLO**

**FUNCTIONAL ANALYSIS OF POPLAR
(*Populus nigra* L. and *P. nigra* x *P. deltoids*)
DURING ENVIRONMENTAL
EXPOSURE TO METALS**

Coordinatore:

Chiar.mo Prof. Nelson Marmiroli

Relatore:

Chiar.mo Prof. Nelson Marmiroli

Tutor:

Dott.ssa Marta Marmiroli

Dott.ssa Giovanna Visioli

Dottorando:
Dott. Davide Imperiale

CONTENTS

1 GENERAL INTRODUCTION	4
1.1 HEAVY METALS POLLUTION.....	4
1.2 REMEDIATION TECHNOLOGIES AND PHYTOREMEDIATION	7
1.3 HYPERACCUMULATION	10
1.4 MECHANISM OF BIOACCUMULATION IN PLANTS.....	14
1.5 PHYTOREMEDIATION USING WOODY TREES.....	17
1.6 PROTEOMICS.....	21
1.7 PROTEIN SEPARATION TECHNIQUES	22
1.7.1 <i>Two-dimensional electrophoresis</i>	22
1.7.2 <i>Two-dimensional liquid chromatography</i>	27
1.7.3 <i>Liquid chromatography by "ProteomeLab PF 2D"</i>	27
1.7.4 <i>Advantages and disadvantages of 2D-gel electrophoresis vs 2D liquid chromatography</i>	28
1.8 PROTEIN IDENTIFICATION BY MASS SPECTROMETRY	30
1.8.1 <i>MALDI/TOF Mass spectrometry</i>	30
1.8.2 <i>Peptide mass fingerprinting (PMF)</i>	32
1.9 REFERENCES.....	34
2 FUNCTIONAL ANALYSIS OF POPLAR (<i>POPULUS NIGRA L.</i> AND <i>P. NIGRA X P. DELTOIDS</i>) DURING ENVIRONMENTAL EXPOSURE TO METALS	70
2.1 INTRODUCTION.....	70
2.1.1 <i>Poplar</i>	70
2.1.2 <i>Cadmium in the environment</i>	73
2.1.3 <i>Effects of cadmium toxicity in plants</i>	75
2.1.4 <i>Plants responses to Cd</i>	79
2.1.5 <i>Plant proteomics and cadmium</i>	80
2.1.6 <i>Objectives</i>	82
2.2 MATERIALS AND METHODS.....	83
2.2.1 <i>Plant Material</i>	83
2.2.2 <i>Hydroponic culture and treatments</i>	83
2.2.3 <i>Physiological measures and statistical analysis</i>	84
2.2.4 <i>Elemental analysis by Atomic Absorption Spectroscopy (AAS)</i>	84
2.2.5 <i>Scanning Electron Microscopy with microanalysis (SEM/EDX)</i>	85
2.2.6 <i>Protein Samples Preparation</i>	85
2.2.7 <i>Protein Quantification</i>	86
2.2.8 <i>PD10 desalting column</i>	86
2.2.9 <i>Two dimensional liquid chromatography "Proteome Lab PF2D"</i>	86
2.2.10 <i>MALDI-TOF Analysis</i>	88
2.2.11 <i>Software and statistical analysis</i>	89
2.3 RESULTS AND DISCUSSION	90
2.3.1 <i>Plant morphology and growth analysis</i>	90
2.3.2 <i>Mineral analysis</i>	94
2.3.3 <i>Scanning Electron Microscopy with microanalysis (SEM/EDX)</i>	98
2.3.4 <i>Proteomic Analysis</i>	110
2.3.5 <i>Proteins identification</i>	119
2.4 CONCLUSION	151
2.5 REFERENCES.....	157
3 ACKNOWLEDGEMENTS	180
4 LIST OF PAPERS	181

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 Heavy metals pollution

Heavy metals are commonly defined as elements with atomic number >20 and metallic properties (ductility, stability as cations, ligand specificity, conductivity, etc.). Metals are natural components in soil but the industrial revolution has accelerated drastically the pollution of the biosphere due to heavy metals mining and utilization. Soil pollution by metals differs from air or water pollution, because heavy metals persist in soil much longer than in other compartments of the biosphere (Lasat *et al.*, 2002).

Sources of heavy metal contamination in soils include point sources such as emission, effluents and solid discharge from industries, metals from smelting and mining, warfare and military training and vehicle exhaustion, and nonpoint sources such as soluble salt, disposal of industrial and municipal wastes, use of insecticides and pesticides in agriculture, and excessive use of fertilizers (McGrath *et al.*, 2001; Nriagu, 1979; Alloway *et al.*, 1995). For example, mine tailings rich in sulphide minerals may form acid mine drainage (AMD) through reaction with atmospheric oxygen and water, and AMD contains elevated levels of metals that could be harmful to animals and plants (Stoltz, 2004). Ground-transportation also causes metal contamination. Highway traffic, road maintenance, and de-icing operations generate continuous surface and ground water contamination. Tread ware, brake abrasion, and corrosion are well documented heavy metal sources associated with highway traffic (Ho and Tai, 1988; Fatoki, 1996; García and Millán, 1998; Sánchez Martín *et al.*, 2000). Heavy metal contaminants in roadside soils originate from engine and brake pad wear (Cd, Cu, and Ni) (Viklander, 1998); lubricants (Cd, Cu and Zn) (Birch and Scollen, 2003; Turer *et al.*, 2001); exhaust emissions, (Pb) (Gulson *et al.*, 1981; Al-Chalabi and Hawker, 2000; Sutherland *et al.*, 2003); and tire abrasion (Zn) (Smolders and Degryse, 2002). In the Table 1, are reported in detail the sources of contamination for each heavy metal.

Land and water pollution by heavy metals is a worldwide problem. Over recent decades, the annual worldwide release of heavy metals reached 22,000 t (metric ton) for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh *et al.*, 2003).

According to a report (ETCS 1998), in Western Europe about 1,400,000 sites were affected by heavy metals and/or organic pollutants (McGrath *et al.*, 2001). In the USA there are 600,000 fields which are contaminated with heavy metals and need reclamation. Coal mines have contaminated more than 19,000 km of US streams and rivers with heavy metals, acid mine drainage and polluted sediments. More than 100,000 ha of cropland, 55,000 ha of pasture and 50,000 ha of forest have been lost (Ragnarsdottir and Hawkins, 2005). The heavy metals pollution is also a great problem in

China, where one-sixth of total arable land has been polluted, and more than 40% has been degraded by erosion and desertification (Liu, 2006). Soil and water pollution is also high in India, Pakistan and Bangladesh, where small industries are spilling their untreated effluents in the drains, which spread near agricultural fields.

METALS	SOURCES	METALS	SOURCES
Pb	Mining and smelting of metalliferous ores, burning of leaded gasoline, municipal sewage, industrial wastes enriched in Pb, paints (<i>Gisbert et al., 2003; Seaward and Richardson, 1990</i>)	Hg	Volcano eruptions, forest fire, emissions from industries producing caustic soda, coal, peat and wood burning (<i>Lindqvist, 1991</i>)
Cu	Electroplating industry, smelting and refining, mining, biosolids (<i>Liu et al., 2005</i>)	Zn	Electroplating industry, smelting and refining, mining, biosolids (<i>Liu et al., 2005</i>)
Cd	Geogenic sources (<i>Baize, 1997</i>), anthropogenic activities (<i>Nriagu and Pacyna, 1988</i>), metal smelting and refining, fossil fuel burning, application of phosphate fertilizers, sewage sludge (<i>Alloway, 1995; Kabata-Pendias, 2001</i>)	As	Semiconductors, petroleum refining, wood preservatives, animal feed additives, coal power plants, herbicides, volcanoes, mining and smelting (<i>Nriagu, 1994; Walsh et al., 1979</i>)
Ni	Volcanic eruptions, land fill, forest fire, bubble bursting and gas exchange in ocean, weathering of soils and geological materials (<i>Knox et al., 1999</i>)	Se	Coal mining, oil refining, combustion of fossil fuels, glass manufacturing industry, chemical synthesis (e.g., varnish, pigment formulation)
Cr	Electroplating industry, sludge, solid waste, tanneries (<i>Knox et al., 1999</i>)		

Tab. 1: Different sources of heavy metals and metalloids (Lone, He, Stoffella, Yang, 2008).

As above-mentioned, heavy metals are present in soils and aqueous streams as natural components or as a result of human activity (Raskin *et al.*, 1994). Widespread low to medium pollution of agricultural land represents a specific problem and, in Europe, the polluted agricultural lands are likely to encompass several million of ha (Flathman and Lanza, 1998).

A European Union Council Directive (86/278/EEC, 1986) limited the values for concentrations of heavy metals in arable soils to 3 mg*kg⁻¹ for Cd, 140 mg*kg⁻¹ for Cu, 75 mg*kg⁻¹ for Ni, 300 mg*kg⁻¹ for Pb, 300 mg*kg⁻¹ for Zn, and 1.5 mg*kg⁻¹ for Hg (Grčman *et al.*, 2001). Besides, heavy metals are highly persistent in soils, with residence times in the order of thousands of years (McGrath, 1987). Unless remediation action is undertaken, the availability of arable land for cultivation will decrease because of stricter environmental laws limiting food production on contaminated lands (Grčman *et al.*, 2001), causing socioeconomic problems for the affected rural populations.

Excessive uptake of metals by plants may produce toxicity in human nutrition, and cause acute and chronic diseases. Heavy metals are toxic for humans mainly because they cause DNA damage, and their carcinogenic effects are probably caused by their mutagenic ability (Knasmuller *et al.*, 1998; Baudouin *et al.*, 2002). Exposure to high levels of these metals has been linked to adverse effects on human health and wildlife. Like with all toxic substances also for heavy metals there is an “acute”

exposure and a “chronic” exposure. The symptoms of the acute exposure differs from those of the chronic, in fact in the former case there is the formation of bleeding wounds and other skin lesions, breath impairment, severe neurotoxic syndroms and sudden death; in the latter case the development of cancer is more likely to occur. (Järup, 2003). Lead poisoning in children causes neurological damage leading to reduced intelligence, loss of short term memory, learning disabilities and coordination problems. The effects of arsenic include cardiovascular problems, skin cancer and other skin effects, peripheral neuropathy (WHO 1997) and kidney damage. Cadmium accumulates in the kidneys and is implicated in a range of kidney diseases and respiratory damages (WHO 1997). The principal health risks associated with mercury are damage to the nervous system, with such symptoms as uncontrollable shaking, muscle wasting, partial blindness, and deformities in children exposed in the womb (WHO 1997; www.atsdr.cdc.gov/spl/).

Some metals are essential for life because they provide cofactors for metalloproteins and enzymes. On the other hand, at high concentrations, metals can act in a deleterious manner by blocking essential functional groups in biomolecules (this reaction has been reported mainly for non-redox-reactive heavy metals such as Cd and Hg) (Schutzenhuber and Polle 2002), displacing essential metal ions, or modifying the active conformation of biological molecules (Collins and Stotzky, 1989).

Furthermore, high concentrations of heavy metals in soil can negatively affect crop growth, because metals interfere with metabolic functions in plants, including physiological and biochemical process, inhibition of photosynthesis, respiration and degeneration of main cell organelles, even leading to plants death (Schmidt, 2003; Schwartz *et al.*, 2003).

Metal toxicity for living organisms is known to involve oxidative and/or genotoxic mechanisms and plants protect themselves by controlling root metal uptake and transport (Briat and Lebrun, 1999). Inside plant cells, some proteins, such as ferritins and metallothioneins, and phytochelatins (glutathion-derived peptides) participate in excess metal storage and detoxification. Oxidative stress defense mechanisms also play an important role against metal toxicity in plants (Briat and Lebrun, 1999). Sanità di Toppi and Gabbrielli (1999) reviewed several mechanisms of plant response to cadmium, such as phytochelatin-based sequestration and compartmentation processes, additional defense mechanisms based on cell wall immobilization, plasma membrane exclusion, stress proteins, stress ethylene, peroxidases, metallothioneins, etc.

1.2 Remediation technologies and Phytoremediation

For above-mentioned reasons toxic metal contaminations needs an effective and affordable technological solution. In order to maintain good quality of soils and waters and to keep them free from contaminations, continuous efforts have been made to develop technologies that are easy to use, sustainable and economically feasible. Different approaches have been used and developed to mitigate the heavy metal polluted soils and waters. These may be classified into *physicochemical* and *biological* approaches.

The *physicochemical* approach includes chemical and physical techniques (McEldowney *et al.*, 1993) such as: excavation and burial of the soil at a waste site, fixation/inactivation and chemical processing of the soil to immobilize the metals, using acid solutions or proprietary leachants to desorb and leach the metals from soil followed by the return of clean soil residue to the site (Salt *et al.*, 1995), precipitation or flocculation followed by sedimentation , ion exchange, reverse osmosis and microfiltration (Raskin *at al.*, 1996). These approaches, although very fast, have some disadvantages: they are expensive (the costs of some of these are listed in Table 2), cause nuisance to people living near the area, and leave a barren environment. In fact they irreversibly affect soil properties, destroy biodiversity and may render the soil useless as a medium for plant growth. (Raskin *at al.*, 1997; McGrath *et al.*, 2001).

TREATMENT	Cost (\$/Ton)	Other factors
Vitrification	75 - 425	Long-term monitoring
Landfilling	100 - 500	Transport/excavation/monitoring
Chemical treatment	100 - 500	Recycling of contaminants
Elettrokinetics	20 - 200	monitoring

Tab. 2: Cost of traditional soil treatments (Glass, 1999).

The *biological* approach includes: (1) use of microorganism to detoxify the metals by valence transformation, extracellular chemical precipitation or volatilization and (2) use of any type of plants to extract, sequester, detoxify and decontaminate soil or water by inactivating metals or other organic compounds, in the rhizosphere or traslocating them in the aerial parts. This approach called **phytoremediation**, is considered as an highly promising technology, is cheaper then the physiochemical approaches, and more suitable for its positive impact on the public opinion (Garbisu and Alkorta, 2001; McGrath *et al.*, 2001; Raskin *et al.*, 1997). Phytoremediation is a green-technology that can be applied to both organic and inorganic pollutants present in the soil, water or

air (Salt *et al.*, 1998). The term ***phytoremediation*** (“*phyto*” meaning plant, and remediation from the Latin suffix “*remedium*” meaning to clean or restore) refers to a diverse collection of plant based technologies that use either naturally occurring, or genetically engineered, plants to clean contaminated environments (Cunningham *et al.*, 1997; Flathman and Lanza, 1998). Phytoremediation can be applied to remediate soils, liquid and gaseous substrates (Pilon-Smits, 2005). This bio-technology employs the naturally occurring processes by which plants and their rhizosphere microbial flora degrade and/or sequester organic and inorganic pollutants. Depending on their chemical properties organic pollutants may be degraded in the root zone of plants or taken up by the plant and subsequently degraded, sequestered or volatilized (Pilon-Smits, 2005). On the contrary, inorganic pollutants, as heavy metals, cannot be directly degraded (Nriagu, 1979) and therefore they can only be stabilized, sequestered and stored in plant tissues. However, the ability to accumulate heavy metals varies significantly between species and among cultivars within species, as different mechanisms of ion uptake are operative in each species, based on their genetic, morphological, physiological and anatomical characteristics.

Depending on the contaminants, the site conditions, then level of clean-up required, and the types of plants, phytoremediation technology can be used for containment or removal purposes (Thangavel and Subhuram, 2004). Five main different categories of phytoremediation have been identified including *phytoextraction*, *phytofiltration*, *phytostabilization*, *phytovolatilization* and *phytodegradation* (Fig. 1). Some plants may have one function whereas others can involve two or more functions of phytoremediation:

- *Phytoextraction* involves the use of plants to remove contaminants from soil and concentrate them in the harvestable parts of plants (Kumar *et al.*, 1995). The metal ions accumulated in the aerial parts that can be removed to dispose or burnt to recover metals.
- *Rhizofiltration* involves the plant roots or seedling for removal of metals from aqueous wastes. (Dushenkov *et al.*, 1995).
- In *Phytostabilization*, the plant roots absorb the pollutants from the soil and keep them in the rhizosphere, reduce the mobility and bioavailability of pollutants in the environment, rendering them harmless by preventing them from leaching. (Vangronsveld *et al.*, 1995; Smith and Bradshaw, 1972)
- *Phytovolatilization* involves the use of plants to volatilize pollutants from their foliage such as Se and Hg. (Burken and Schnoor, 1999; Banuelos *et al.*, 1997).
- *Phytodegradation* means the use of plants and associated microorganisms to degrade organic pollutants (Garbisu and Alkorta, 2001; Burken and Schnoor, 1997)

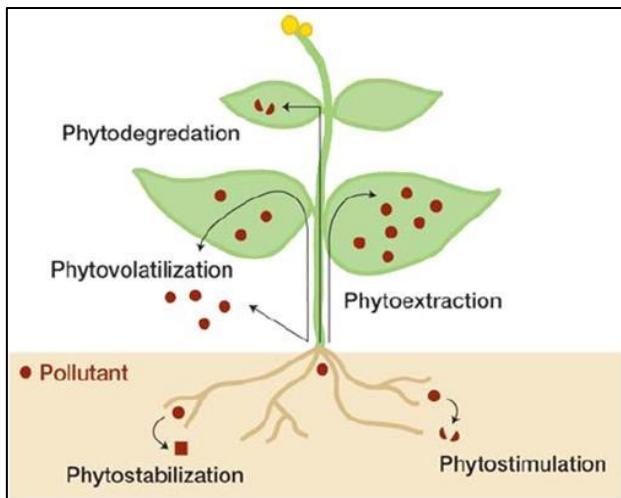


Fig 1: Categories of phytoremediation technologies: the pollutant (represented by red dots) can be stabilized or degraded in the rhizosphere, sequestered or degraded inside the plant tissue, or volatilized (Pilon-Smits, 2005).

The application of the phytoremediation technology is influenced by many aspects. Firstly, plants involved in phytoremediation processes must co-locate together with the pollutant. Secondly, phytoremediation is limited by the root depth, which should be long enough so that roots can reach the pollutant in the matrix. Aspects as the chemical/physical properties of soil, the toxicity level and also the climate are important for plant growth and consequently for their remediation performance. Moreover, depending on the biological processes, phytoremediation may be slower than the more established remediation methods like excavation, incineration or pump-and-treat systems: the flow-through phytoremediation systems and plant degradation of pollutants are rather fast (days or months), but soil cleanup via plant accumulation often takes years, limiting the applicability of these *in situ* techniques. Finally, pollutant bioavailability might limit the phytoremediation process: if only a fraction of the pollutant is bioavailable but the regulatory standard cleanup requires that all pollutant is removed, phytoremediation is not applicable by itself (Flechas and Latady, 2003).

Despite of these limitations, phytoremediation technology has gained popularity because of its low cost since biological processes are ultimately solar-driven. It has been estimated that phytoremediation is tenfold cheaper than engineering-based remediation methods, such as excavation, soil washing or burning and pump-and-treat systems (Glass, 1999). The low cost is also due to the fact that the phytoremediation is usually carried out *in situ*, reducing in addition the exposure of pollutants to humans. Last but not least, phytoremediation enjoys popularity with the public opinion as a “green clean” alternative to conventional remediation methods.

1.3 Hyperaccumulation

In addition to anthropologically polluted sites, many regions in the globe naturally present high concentrations of heavy metals in the soil and water. In these particular areas several plants have developed ability to tolerate and also accumulate high concentration of these heavy metals. In many ways these plants can be compared to solar driven pumps which can extract and concentrate several elements from their environment (Memon *et al.*, 2000). These plants exploit the natural propensity to take up heavy metals from soil and water because a lot of them (Fe, Zn, Cu, Mn, Mo, Se) at low concentration are essential nutrients for the plants. They are, in fact, co-factor of many enzyme and structural component of several proteins. The beneficial range of heavy metal concentration is extremely narrow and at high concentrations all the metals are toxic because they replace essential metals in pigments, denature enzymes, inactivate proteins or cause oxidative stress by formation of free radicals. Other heavy metals (Cd, Pb, Cr, Hg) have no known physiological activity and are important environmental pollutants, toxic at very low concentration (Lasat, 2002), but these few exceptional plants have evolved specific ability to tolerate and accumulate heavy metals at concentration toxic for the rest of the plants. Two interesting strategies have been developed by these plants to grow on contaminated and metalliferous soils (Baker and Walker, 1990). A first strategy is defined "metal exclusion": plants prevent the assumption and in particular the translocation of the metal in the aerial part; they are able to tolerate high concentration of heavy metals, these plants are not good for the phytoremediation because the large amount of the metals remain in the soil. The second strategy is defined "metal accumulation" these plant species can take up high quantities of metal, transfer effectively from roots to shoots and concentrate the metal in the above-ground parts (shoots and leaves): these plants are defined "**hyperaccumulators**" (Reeves and Baker, 2000). These hyperaccumulator plants are capable of accumulating potentially phytotoxic elements to concentrations more than 100 times than those found in nonaccumulators (Salt *et al.*, 1998; Chaney *et al.*, 1997; Raskin and Ensley, 2000), without symptoms of toxicity (Reeves and Brooks 1983; Baker and Brooks 1989; Baker *et al.* 1991; Entry *et al.* 1999). A plant is termed "**hyperaccumulator**" if it contains $1.000 \mu\text{g} \cdot \text{g}^{-1}$ (dry weight) or more of metal for Ni, Cu, Co, Cr or Pb, or $10.000 \mu\text{g} \cdot \text{g}^{-1}$ or more for Zn or Mn (Baker and Brooks, 1989). Hyperaccumulation is an active process that depends on an endogenous hypertolerance mechanisms to resist the cytotoxic levels of the accumulated metals (Salt, 2006). Heavy metal hyperaccumulators are not so common in terrestrial higher plants (only 0.2 % of all angiosperms) (Baker *et al.*, 2000). The first observation of plants containing high concentration of certain metals dates back to the 1865: F. Risso, a German botanist, observed that leaves of particular plant species growing in soils naturally enriched in Zn contained extraordinarily high levels of this element (up to 1.5 % of the shoot dry weight) (Sachs,

1865). Other two Italian botanists discovered that dried leaves of *Alyssum bertolonii*, grown on Nickel enriched ultramafic (serpentine) soils, contained ~ 1% Ni, more than 100 to 1000 times than others plants growing nearby (Minguzzi and Vergnano, 1948).

It has not yet been totally established what form of adaptive advantage may be conferred on plants by this capability. At least five principal hypotheses have been proposed to understand the ecological and evolutionary significance of the hyperaccumulation trait: (1) defence against herbivores and pathogens; (2) interference with neighbouring species (alleopathy); (3) increasing drought resistance; (4) inadvertent uptake while acquiring essential nutrient and (5) a corollary of the mechanism of metal tolerances (Boyd and Martens, 1995). The first hypothesis is presently favoured by several studies that have demonstrated the presence of high concentration of Zn or Ni in the hyperaccumulator plants as defensive role against herbivores (Martens and Boyd, 1994; Pollard and Baker, 1997; Pollard *et al.*, 2000; Davis and Boyd, 2000; Behmer *et al.*, 2005) and pathogens (Boyd, 2004; Ghaderian *et al.* 2000). Studies have also proven the other hypothesis that metal tolerance and hyperaccumulation were genetically independent traits (Macnair *et al.*, 1999; Pollard, 2000; Macnair, 2003): there are many plant species, in fact, that do not hyperaccumulate metal, but they are tolerant to high soil metal concentration, anyway hyperaccumulation implies mechanisms of metal tolerance and these two features may be genetically distinguishable in a hyperaccumulator species (Assuncao *et al.*, 2003a.; Bert *et al.*, 2003). There is also a third hypothesis that explain hyperaccumulation as a trade off between plant genome and plant phenotype (Maestri *et al.*, 2010).

To date, more than 400 metal hyperaccumulator species are known (Eapen and D'Souza, 2005), belonging to at least 45 plant families. A large part of these plants are Nickel-accumulator (about 80 % of them) or Zn accumulators (15 species) (Brooks, 1998), while only few species hyperaccumulate Cd. The most common are *Thlaspi* species, such as *T. caerulescens* J.Presl & C.Presl and *T. praecox* Schleich. ex DC. Syst. Nat., *A. Halleri* (L.) and *Alyssum bertolonii* Desv. J. Bot. Agric. (van de Mortel *et al.*, 2008). *Thlaspi* species are polymetallic hyperaccumulators known to accumulate high amounts of Zn, Cd, Ni and Pb (Mari *et al.*, 2006), whereas *A. halleri* is able to tolerate Zn, Cd and Pb and hyperaccumulates Zn and Cd (van Rossum *et al.*, 2004). *Sedum alfredi* Hance J. Bot. has been identified as a Zn hyperaccumulator, and only recently it has been confirmed to also hyperaccumulate Cd (Zhou and Qiu, 2005).

The idea of using plants to extract metals from contaminated soil was reintroduced and developed by Utsunamyia (1980) and Chaney (1983). The first field trial on Zn and Cd phytoextraction was conducted by Baker *et al.* (1991).

Exploitation of metal uptake into plant biomass as method of soil decontamination is limited by plant productivity and the concentrations of metals achieved (Baker *et al.*, 1991). For instance, *Thlaspi caerulescens* is a known Zn hyperaccumulator, but its use in the field is limited because individual plants are very small and slow growing (Ebbs and Kochian, 1997). The ideal plant species to remediate a heavy metal-contaminated soil would be a high biomass producing crop that can both tolerate and accumulate the contaminants of interest (Ebbs and Kochian, 1997). Such a combination may not be possible, there may have to be a trade-off between hyperaccumulation and lower biomass, and vice versa.

Hyperaccumulator plants can be directly employed as pollutant removers or can represent sources of genes to improve non-hyperaccumulator plants. Nevertheless, natural hyperaccumulators have generally low biomass and slow growth rate. These restrictions may be overcome by transferring the genetic potential responsible for hyperaccumulation from hyperaccumulator species to plants characterised by fast growth and high biomass production (Maestri *et al.*, 2011). For example, Indian mustard, *Brassica juncea* L., is a suitable target specie, because of its large biomass production, a relatively high metal uptake and accumulation and the already well established transformation technology. Differently to non-hyperaccumulator plants, which normally accumulate heavy metals in roots, hyperaccumulators are able to transport most of the absorbed metal to the shoots (Lasat *et al.*, 1998). Thus, a metal translocation from root to shoot through the xylem is a determinant for the hyperaccumulation phenotype. In fact, it has been recently demonstrated that the metal transporter HMA4, expressed at higher levels in the hyperaccumulator *A. halleri* if compared to the non-tolerant *A. thaliana*, is essential for the root-to-shoot transport (Hanikenne *et al.*, 2008). At molecular level, even amino- and organic acids have been proposed to play a role in heavy metal hyperaccumulation and tolerance (Sharma and Dietz, 2006). Typically, chelation of the metal ion, transport of metal or its complexation and subsequent compartmentalization in vacuoles are the processes where biotechnology can play a part in the enhancing the phytoremediation capacity of plants. For example, transferring a single gene involved in metal transport, such as HMA4, from *A. halleri* to *A. thaliana* has increased the shoot metal up-loading in this non-accumulator specie (Hanikenne *et al.*, 2008). Regarding metal-conjugates transport, plants overexpressing specific transport proteins, might acquire higher detoxification and compartmentalization of GS(glutathione)-Cd conjugates into the vacuoles (Krämer *et al.*, 2007). Transgenic *B. juncea* plants engineered to produce more glutathione and phytochelatins accumulated significantly more Cd than wild-type plants (Bennet *et al.*, 2003), while *A. thaliana* and tobacco plants overexpressing MT (metallothioneins) genes showed more Cd tolerance and accumulation (Eapen and D'Souza, 2005). Furthermore, Cd tolerance and accumulation is also

enhanced by overexpressing the γ -glutamylcysteine synthetase, an enzyme with an important role in controlling glutathione synthesis and therefore metal chelation (Zhu *et al.*, 1999). Another study revealed that the expression of the AtPCS1 gene, encoding phytochelatin synthase (PCS), increased Cd and As tolerance and accumulation in *B. juncea* (Gasic and Korban, 2007) and in tobacco plants (Pomponi *et al.*, 2006).

The comparison between hyperaccumulator and non-accumulator sister species suggests that the hyperaccumulating features could reside in sequence mutations, gene copy number and/or in different expression level of proteins that contribute to metal tolerance (Hanikenne *et al.*, 2008; Plaza *et al.*, 2007; Visioli *et al.*, 2010; Visioli and Marmiroli, 2012; Marmiroli M., 2004). These findings highlight that probably part of the genetic potential for metal detoxification is already present in most plant species and that small sequence changes, which influence both metal sensing and activation of appropriate responses, make the difference.

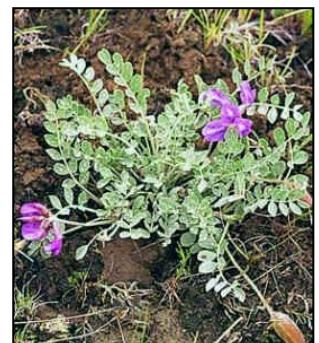


Fig. 2: Heavy metal hyperaccumulator species: *Thlaspi caerulescens* L., *Alyssum bertolonii* L., *Astragalus petersoni* L., *Sedum alfredi*, *Arabidopsis halleri* L., *Brassica juncea* L.

1.4 Mechanism of bioaccumulation in plants

The phytoavailability of metals in the soil is an important factor related to the mechanisms of bioaccumulation and the consequent possibility to use phytoremediation techniques. Heavy metals exist in colloidal, ionic, particulate and dissolved phase and they have high affinity for humic acids, clays and organic matter. The solubility of metals in soil is predominantly controlled by pH, concentration of metal, cation exchange capacity, organic matter content, oxidation state of the mineral components, and the redox potential of the system (Ghosh and Singh, 2005). In general pH seems to be the most important factor: under neutral or basic condition, typical of most soils, cationic metals are strongly adsorbed on the clay fractions. Study conducted by Naidu et al. (2003) focused their attention on the importance of the soil type to understand the phytotoxicity of the different metals: the bioavailability of elements such as Zn and Cd, in fact, is lower in clay soils than in sandy soils. The possibility to uptake heavy metals and the consequent capacity to bioaccumulate them is also increased by different strategies adopted by the plants: one of them is achieved by secreting phytosidophores into the rhizosphere to chelate and solubilize metals that are soil bound (Kinnarsley, 1993). After this process of mobilisation, a metal has to be captured by root cells and transferred into the organism. Inside the plant most metals are too insoluble to move freely within the vascular system, so they usually form carbonate, sulphate or phosphate precipitates being immobilized in apoplastic (extracellular) and symplastic (intra cellular) compartments (Raskin et al., 1997). Three processes govern the translocation of metal from the root into the xylem: sequestration of metals inside root cells, symplastic transport into the stele and release into the xylem. Metal ions can be actively transported into the xylem as free ions or as metal-chelate: nickel, for example, forms complexes with the amino acid histidine, in *A. halleri*, and is carried in the xylem (Kramer et al., 1996). Nicotinamine proteins also chelate metals and may facilitate their transport (Stephan et al., 1996; Von Wieren et al., 1999). The movement is generally a tightly controlled process mediated by membrane transport proteins (Ghosh and Singh, 2005) like ZIP (ZRT/IRT related proteins) proteins family and the NRAMP (Natural Resistance Associated Macrophage Proteins) proteins family. The first one has been shown to transport Zn^{2+} , Fe^{2+} , Cu^{2+} , Cd^{2+} , Co^{2+} , Mn^{2+} (Grotz et al., 1998; Pence et al., 2000; Lasat et al., 2000), the second one has been proven to transport Fe^{2+} , Cd^{2+} , Mn^{2+} (Curie et al., 2000; Goswami et al., 2001; Thomine et al., 2000). These transporters are not highly specific, they have high affinity for several metals but in many case also transport, with less affinity, other potentially dangerous metal ions like Pb^{2+} and Cd^{2+} . Large translocation of metals from roots to leaves and a high metal concentration in the xylem fluid are typical of the hyperaccumulators (Kramer et al., 1996). Essential metal ions enter into plant cells by an energy

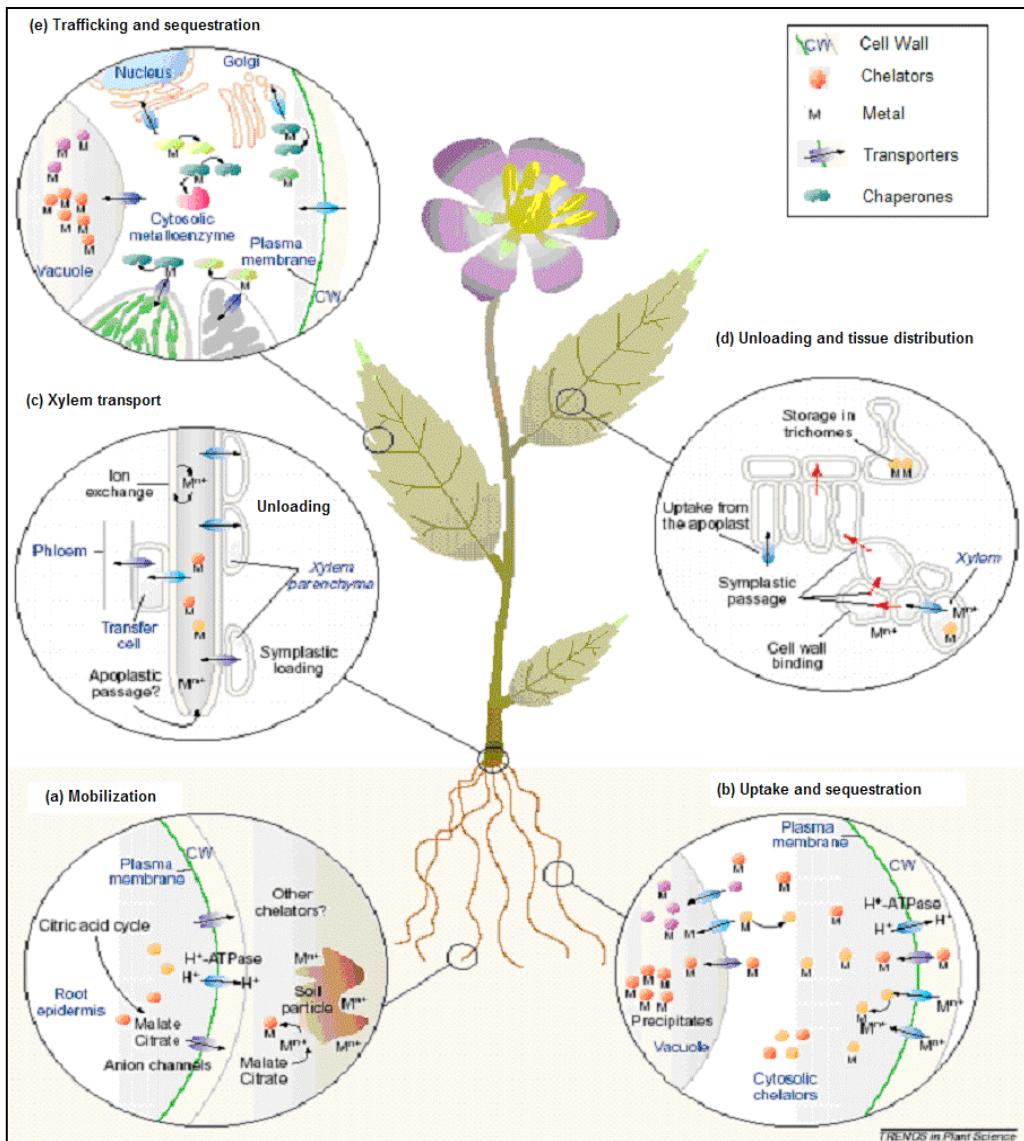


Fig. 3: Heavy metal uptake at plant level (Clemens *et al.*, 2002)

dependent saturable process via specific or generic metal ion carriers or channels, but non-essential and toxic heavy metals may compete for the same transmembrane carriers used by micronutrient heavy metals (Bubb and Lester, 1991). An example was provided by Crowley *et al.* (1991) who showed the competition between the essential Cu^{2+} and Zn^{2+} and the non essential Ni^{2+} and Cd^{2+} ions for the same transmembrane carrier by using kinetic data. Accumulation of large amounts of metals requires, also, the presence of high-capacity detoxification mechanisms, for example the sequestration in organelles like the vacuole that works as a buffer against excess amounts of metal in the cytosol. The synthesis of chelators or oligopeptide ligands like organic acids, amino acids, phytochelatins and metallothioneins, generally play an important role to avoid damage induced by the presence of high concentration of metals into the plant cells (Fig. 3) (Cobbett, 2000; Clemens *et al.*, 2002).

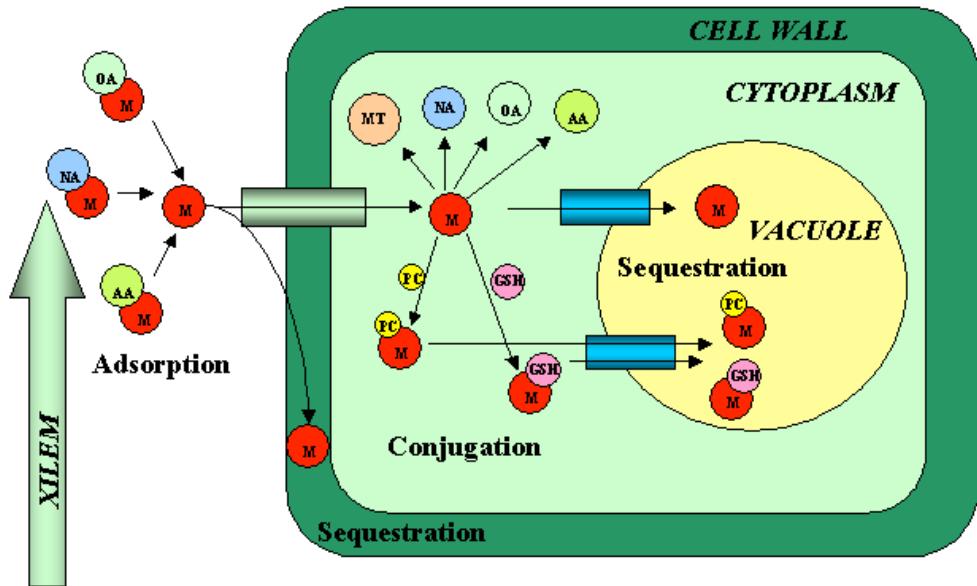


Fig. 4: metal response mechanisms for metal pollutants in plant cells generally involves conjugation followed by active sequestration in the vacuole and apoplast. Chelators shown are GSH: glutathione, MT: metallothioneins, NA: nicotinamine, OA: organic acids, PC: phytochelatins, AA: aminoacid. (Pirondini, 2007)

In conclusion we can summarize that the mechanisms adopted by hyperaccumulators works in different ways: plants may immobilise metals mostly in roots and stems or accumulate and store metals in non-toxic forms by chelation with proteins or bind potentially toxic metals to the cell walls molecules (cellulose and lignin) or in old leaves, away from sensitive sites (Memon *et al.*, 2001) (Fig. 4).

1.5 Phytoremediation using woody trees

The potential use of woody trees as a suitable vegetation cover for heavy metal-contaminated land has received increasing attention over the last twenty years (Aronsson and Perttu, 1994; Glimerveen, 1996; EPA, 1999, 2000). Trees have been suggested as a low-cost, sustainable and ecologically solution to the remediation of heavy metal-contaminated land (Dickinson, 2000), especially when it is uneconomic to use other treatments or there is no time pressure on the reuse of the land (Riddell-Black, 1994). Benefits can arise mainly from stabilisation of the soil or waste, although in some cases phytoextraction may be sufficient to provide clean up of the soil. Before these benefits can be realised, the trees must become established on a site. The physical and hydraulic conditions of a site are of primary importance to tree establishment. Phytostabilisation can result from either physical or chemical effects. Once the trees have become established, the vegetation cover can promote physical stabilisation of a substrate, especially on sloping ground. Moreover, on highly contaminated soils or on mining wastes, tree establishment may be inhibited by high concentrations of heavy metals. Under such conditions root immobilization, which would normally protect a plant, may not be able to prevent toxic amounts of metal being translocated to the aerial parts of the plant and stunting the growth. In less-contaminated soils, other factors may limit plant growth; such as macronutrient deficiencies (Pulford, 1991) and physical conditions, especially those properties leading to poor soil waterholding capacity, aeration and root penetration (Mullins, 1991). When trees are well fixed, long-term stability of the land surface can be achieved as the standing trees decrease erosion of the substrate by wind and water (Johnson *et al.*, 1992). Trees have massive root systems, which help to bind the soil (Stomp *et al.*, 1993), and the addition of litter to the surface quickly leads to an organic cover over the contaminated soil. In addition, transpiration of water by the trees reduces the overall flow of water down through the soil, thus, helping to reduce the amounts of heavy metals that are transferred to ground and surface waters. Phytostabilisation of a heavy metal contaminated substrate may also be achieved by causing chemical changes to specific metals, which result in their becoming less bioavailable. Chaney *et al.* (1997) identified two elements, Cr and Pb, which may be immobilised by a vegetation cover. They suggested that deep rooting plants could reduce the highly toxic Cr(VI) to Cr(III), which is much less soluble and, therefore, less bioavailable (James, 2001). Although no mechanism for this was suggested, organic products of root metabolism, or resulting from the accumulation of organic matter, could act as reducing agents. It is known that Cr tends to be held in plant roots, whether supplied as Cr(VI) or Cr(III) (Pulford *et al.*, 2001), which may also suggest reduction and immobilisation in the roots. Lead may be immobilised by the formation of the lead phosphate mineral chloropyromorphite in soils and within roots (Cotter-Howells *et al.*, 1994), which has been

shown to be formed in soils by *Agrostis capillaris* L. growing on lead/zinc mining wastes (Cotter-Howells and Caporn, 1996). For the purposes of phytoextraction, Punshon *et al.* (1996) suggested that the following characteristics were beneficial:

- a) ability to grow on nutrient-poor soil
- b) deep root system
- c) fast growth rate
- d) metal-resistance trait

In addition, an economically viable secondary use would be desirable. Trees have been shown to meet all of these requirements, the first three in particular. While a high metal content in agricultural crops is not desirable, and indeed is potentially dangerous, a higher metal content in trees is acceptable, as long as normal physiological activity is not affected (Labrecque *et al.*, 1995). Several studies have shown the potential of plants of genus *Populus* L. for site reclamation and partial decontamination, as several species and clones of the genus *Salix* L. take up relatively high levels of heavy metals (Riddell-Black, 1994; Watson *et al.*, 1999; Pulford *et al.*, 2002). Poplars (or cottonwoods) are being used throughout North America to clean up sites that contain heavy metals, pesticides, and landfill leachates. Poplars are well suited for phytoremediation because they can remove contaminants in several ways, including degrading them, confining them, or by acting as filters or traps (Isebrands and Karnosky, 2001). Poplar and willow are often grown in short rotation coppice cultures (SRC), intensively managed plantations for rotations shorter than 15 years (Dickmann and Stuart, 1983; Macpherson, 1995). Plant material is selected for high biomass production, high growth vigor, and disease resistance. Cultural management includes site preparation, high planting density, and coppicing (Dickmann and Stuart, 1983; Macpherson, 1995; Ledin and Willebrand, 1996). Coppicing refers to the cutting of a tree at the base of its trunk, resulting in the emergence of new shoots from the stump and/or roots (Blake, 1983). A coppice regime not only makes replanting of trees unnecessary for several rotations, but also results in a much higher biomass yield for several species (Sennerby-Forsse *et al.*, 1992; Macpherson, 1995). Studies on phytoextraction have mainly focused on metal hyperaccumulating plants, as they accumulate 100–1000-fold the levels normally accumulated in plants, with no adverse effects on their growth (Reeves *et al.*, 1999). In comparison with hyperaccumulators, trees tend to take up relatively small amounts of heavy metals, but they provide economic return from contaminated land through the production of reusable biomass. Moreover, SRC has many additional ecological benefits: a positive impact on biodiversity, nutrient capture and carbon sequestration (Gordon,

1975; Perttu, 1995). Wood from SRC has traditionally been seen as a resource for the paper and pulp industry. But, in light of the greenhouse effect and the depletion of fossil fuels, SRC is now seen as a source of energy, because of the possibility of carbon sequestration and the substitution of fossil fuels. In addition plants use the atmospheric CO₂ and produce oxygen. Furthermore, SRC on polluted land may reduce dust-blow, leaching and run-off of contaminated water (Watson *et al.*, 1999; Isebrands and Karnosky, 2001). Both biomass production and metal concentration should be taken into account when assessing the phytoextraction potential of a species or clone. Many studies have shown “toxic” metals to accumulate primarily in the root system; relatively high metal concentrations have also been found in leaves and bark (Rachwal *et al.*, 1992; Landberg and Greger, 1996; Pulford *et al.*, 2001; Thiry *et al.*, 2002). As the amount of bark of a stem depends on its diameter, the shoot diameter distribution and population dynamics of the species or clone might also be considered. Large clonal variations in the number of shoots per stool and in the diameter distribution have already been demonstrated in poplar SRC (Laureysens *et al.*, 2003) by repeated coppicing of the trees. Willow and poplar are considered best suited for this task because of their strong nature to coppice, their high capacity for metal uptake, and their high biomass production (Schnoor *et al.*, 1996; Greger and Landberg, 1999; Robinson *et al.*, 2000; Roselli *et al.*, 2003). *Salix* spp grow well and fast in soils with large quantity of water and can tolerate moderate chilling and altitudes, while poplars can tolerate moderately dry soils with discrete degree of salinity (Kuzminsky *et al.*, 1999). At the same time the high transpiration capacity of the salicaceae family allows some poplar clones to extract from soils organic xenobiotics which are moderately hydrophobic. Plant density is also an interesting characteristic of salicaceae which can be as high as 8-10,000 per ha, forming a dense and deep root system, useful to explore both superficial and deep ground contaminated soil. Moreover, salicaceae associate a very high biomass accumulation and transpiration rates with the ability of decontaminating soils from heavy metals (Robinson *et al.* 2000) and other contaminating substances such as hydrocarbon (Jordahl *et al.* 1997), herbicides (Gullner *et al.* 2001) and trichloroethylene (Newman *et al.* 1997). Furthermore, willows and poplars show a high intraspecific genetic polymorphism and a number of different genotypes could be available with a high degree of adaptability to a given climate and able to tolerate and uptake/degrade a given contaminant. For the genus *Populus*, for example, more than 30 species have been already classified, they all are diploids ($2n = 38$) and some species can be crossed. Hybrids are fertile and various pedigrees are also available. Further, the poplar genome of the species *P. trichocarpa* Torr. & A.Gray is small (only 4 times that of *Arabidopsis*, and 400 times smaller than that of *Pinus*) and is available (<http://genome.jgi-psf.org>). Bioinformatics is, thus, a powerful way to develop rapid analysis of genes involved in metabolic and physiological

mechanisms useful for phytoremediation. The genus Salix is a member of the Salicaceae plant family. There are 400 species of willows, with more than 200 listed hybrids (Newsholme, 1992). The majority of the genus Salix grow in lowland wetland habitats and have evolved a number of varieties and hybrids (Sommerville, 1992). The large number of species and hybrids of Salix suggest a wide genetic variability within the genus. The genus features many species of high productivity and invasive growth strategies (Punshon *et al.*, 1996). Many species, such as *S. caprea* L. and *S. cinerea* L., and the hybrid *S. viminalis* Balb. Fl. Taur., are known to colonise edaphically extreme soils (Dickinson *et al.*, 1994).

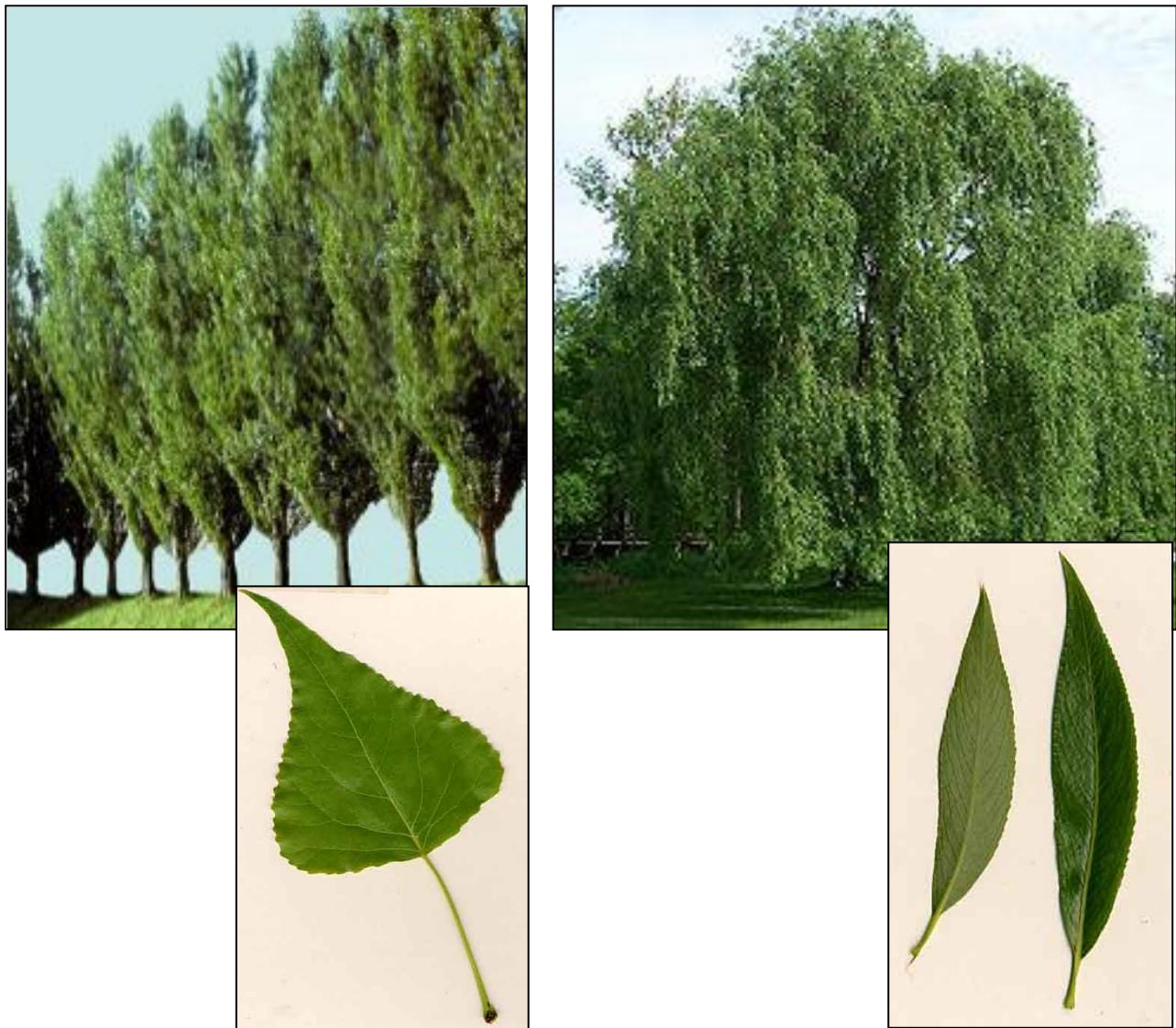


Fig. 5: Trees used for phytoremediation purpose: trees of the genus *Populus* L. and *Salix* L.

1.6 Proteomics

Proteome analysis is "the analysis of the entire PROTEin complement expressed by a genOME". (Wilkins *et al.*, 1996; Pennington *et al.*, 1997).

Proteomics can be defined as the systematic analysis of proteome, the protein complement of genome (Pandey and Mann, 2000; Phizicky *et al.*, 2003). This technology allows the global analysis of gene products in various tissues and physiological states of cells. With the completion of genome sequencing projects and the development of analytical methods for protein characterization, proteomics has become a major field of functional genomics (Park, 2003).

The essence of this "system biology" approach is that, for any given species, the space of possible biomolecules and their organization into pathways and processes is large but finite. In theory, therefore, the biological system operating in a species can be described comprehensively if a sufficient density of observation on all of the elements that constitute the system can be obtained. Proteomics is a particularly rich source of biological information because proteins are involved in almost biological activities and they also have diverse properties, which collectively contribute greatly to our understanding of biological system (Patterson and Aebersold, 2003). Many different technologies have been and are still being developed to collect the information contained in the properties of proteins (Patterson and Aebersold, 2003).

Many different extraction methods have been developed for protein extraction from different plant and animal tissues and a large number of separation techniques have been developed in order to decrease the complexity of the proteome.

1.7 Protein separation techniques

After carefully assessing the best method to extract the proteome from its medium, the second challenge is to extract information from the proteome, which generally starts by separating the proteins contained in the proteome. Some proteomes are relatively small, containing a few hundred proteins or less, while others contain thousands of proteins. One-dimensional (1D) separation techniques do not have the resolving power to separate complex mixtures. The combination, however, of orthogonal (independent) separation techniques can provide the required resolving power. Furthermore, it is important that the quantitative aspect of the proteome be conserved through the separation technique.

1.7.1 Two-dimensional electrophoresis

About 30 years ago, a technique called 2D gel electrophoresis was introduced, and this technique satisfied the resolving power requirement while conserving the quantitative aspect of the proteome. Two-dimensional gel electrophoresis has been the method of choice for the large-scale purification of proteins in proteomic studies. The 2D gel electrophoresis method can potentially separate several thousand proteins in a single experiment (Gorg *et al.*, 1988; Klose and Kobalz, 1995). Although the predictions for the number of genes in some genomes are high, it is generally believed that the number of genes expressed is, on average, between 5000 and 15,000 per cell type. However, these genes and post-translational modifications can lead to many forms of proteins, greatly increasing the complexity of the proteome.

Two-dimensional electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts out proteins according to two independent properties in two discrete steps: the first-dimension step, *isoelectric focusing (IEF)*, separates proteins according to their isoelectric points (pI); the second-dimension step, *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*, separates proteins according to their molecular weights (Mr, relative molecular weight). Each spot on the resulting two-dimensional gel corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained.

Two-dimensional electrophoresis was first introduced by P. H. O'Farrell and J. Klose in 1975. In the original technique, the first-dimension separation was performed in carrier ampholyte-containing polyacrylamide gels cast in narrow tubes. The power of 2-D electrophoresis as a biochemical separation technique has been recognized since its introduction.

- **IEF (Isoelectric focusing)** is an electrophoretic method that separates proteins according to their isoelectric points (pI). Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino and carboxyl-terminal. The isoelectric point (pI) is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. The presence of a pH gradient is critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero, with a specific velocity (Fig. 6). A protein with a positive net charge will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge will migrate toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is the focusing effect of IEF, which concentrates proteins at their pIs and allows proteins to be separated on the basis of very small charge differences. The resolution is determined by the slope of the pH gradient and the electric field strength. IEF is therefore performed at high voltages (typically in excess of 1000 V). When the proteins have reached their final positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically below 1 mA). IEF of a given sample in a given electrophoresis system is generally performed for a constant number of Volt-hours (Volt-hour (Vh) being the integral of the volts applied over the time). IEF performed under denaturing conditions gives the highest resolution and the cleanest results. Complete denaturation and solubilization is achieved with a mixture of urea and detergent, ensuring that each protein is present in only one configuration and aggregation and intermolecular interaction is minimized. The original method for first-dimension IEF depended on carrier ampholyte-generated pH gradients in polyacrylamide gel rods in tubes (O'Farrel, 1975; Klose, 1975). Carrier ampholytes are small, soluble, amphoteric molecules with a high buffering capacity near their pI. Commercial carrier ampholyte mixtures are comprised of hundreds of individual polymeric species with pIs spanning a specific pH range. When a voltage is applied across a carrier ampholyte mixture, the carrier ampholytes with the highest pI (and the most negative charge) move toward the anode and the carrier ampholytes with the lowest pI (and the most positive charge) move toward the cathode. The other carrier ampholytes align themselves between the extremes, according to their pIs, and buffer their environment to the corresponding pHs. The result is a continuous pH gradient. Although this basic method has been used in hundreds of 2-D electrophoresis studies, it has several limitations that have prevented its more widespread

application. As a result of the limitations and problems with carrier ampholyte pH gradients, immobilized pH gradients were developed (Bjellqvist *et al.*, 1982). Görg *et al.* (1985,1988) pioneered the development and use of IPG IEF for the first-dimension of 2-D electrophoresis. The techniques used today are largely based on the work of A. Görg and her colleagues. An immobilized pH gradient (IPG) is created by covalently incorporating a gradient of acidic and basic buffering groups into a polyacrylamide gel at the time it is cast. Immobilized pH gradients are formed using two solutions, one containing a relatively acidic mixture of acrylamide buffers and the other containing a relatively basic mixture. The concentrations of the various buffers in the two solutions define the range and shape of the pH gradient produced. Both solutions contain acrylamide monomers and catalysts. During polymerization, the acrylamide portion of the buffers copolymerize with the acrylamide and bisacrylamide monomers to form a polyacrylamide gel. For improved performance and simplified handling, the IPG gel is cast onto a plastic backing. The gel is then washed to remove catalysts and unpolymerized monomers, which could otherwise modify proteins and interfere with separation. Finally the gel is dried and cut into 3 mm-wide strips. The resulting IPG strips can be rehydrated with a rehydration solution containing the necessary components for first-dimension IEF. IEF is performed with the IPG strips placed horizontally on a flatbed electrophoresis unit.

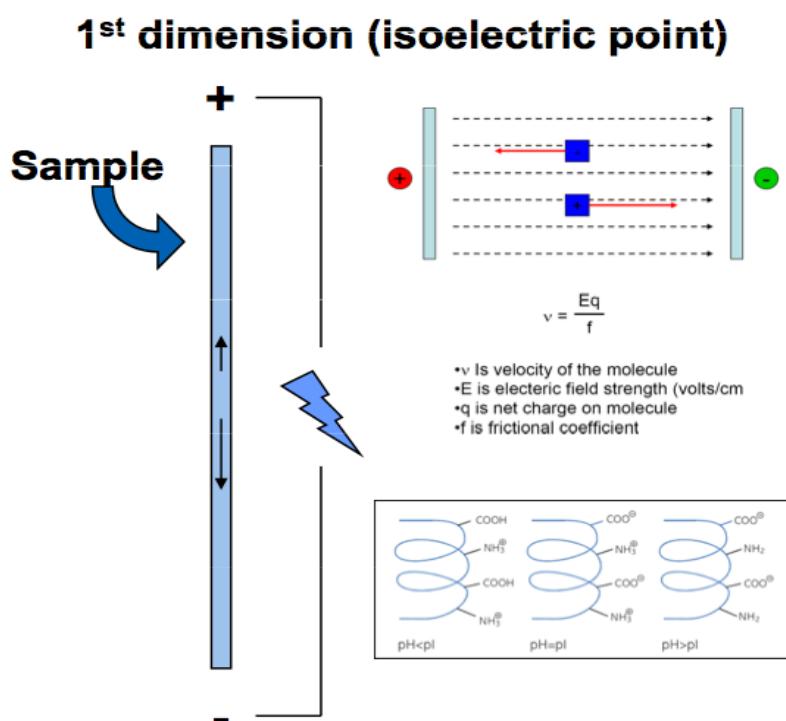


Fig. 6: principles of isoelectric focusing (IEF). Protein sample are charged into 3 mm-wide strips with a Immobilized pH gradients. Under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero.

- After IEF, the second-dimension separation can be performed on various flatbed or vertical systems. **SDS-PAGE (SDS-polyacrylamide gel electrophoresis)** is an electrophoretic method for separating polypeptides according to their molecular weights (Mr). The technique is performed in polyacrylamide gels containing sodium dodecyl sulfate (SDS) (Fig. 7). The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel. SDS is an anionic detergent, that, when in solution in water, forms globular micelles composed of 70–80 molecules with the dodecyl hydrocarbon moiety in the core and the sulfate head groups in the hydrophilic shell. SDS and proteins form complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments (Ibel *et al.*, 1990). The result of the necklace structure is that large amounts of SDS are incorporated in the SDS-protein complex in a ratio of approximately 1.4 g SDS/g protein. SDS masks the charge of the proteins themselves and the formed anionic complexes have a roughly constant net negative charge per unit mass. Besides SDS a reducing agent such as dithiothreitol (DTT) is also added to break any -S-S-linkages present in the proteins. When proteins are treated with both SDS and a reducing agent, the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular weight of the protein. In fact, there is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide complex. (Note: This linear relationship is only valid for a certain molecular weight range, which is determined by the polyacrylamide percentage). The most commonly used buffer system for second-dimension SDS-PAGE is the tris-glycine system. (Laemmli, 1970). This buffer system separates proteins at high pH, which confers the advantage of minimal protein aggregation and clean separation even at relatively heavy protein loads.

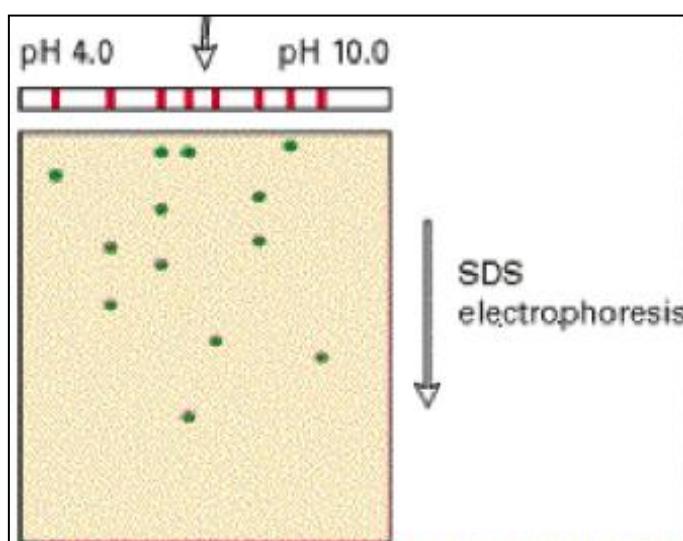


Fig. 7: SDS-PAGE (electrophoresis). After the first dimension, the IPG strip are charged horizontally on a SDS-polyacrylamide gel. Polypeptides are separated according to their molecular weights (Mr).

The result of this is a gel with proteins spread out on its surface. These proteins can then be detected by a variety of means, but the most commonly used stains are *silver* and *coomassie* staining. The silver binds to cysteine groups within the protein. The silver is darkened by exposure to ultra-violet light. The darkness of the silver can be related to the amount of silver and therefore the amount of protein at a given location on the gel. This measurement can only give approximate amounts, but is adequate for most purposes.

The two-dimensional gel electrophoresis application, however, has become significant only in the last few years as a result of a number of developments.

- The introduction of immobilized pH gradients and Immobiline™ reagents (Bjellqvist *et al.*, 1982) brought superior resolution and reproducibility to first-dimension IEF. Based on this concept, A. Görg and colleagues (Görg *et al.*, 1985; Görg *et al.*, 1988) developed the currently employed 2-D technique, where carrier ampholyte-generated pH gradients have been replaced with immobilized pH gradients and tube gels replaced with gels supported by a plastic backing.
- New mass spectrometry techniques have been developed that allow rapid identification and characterization of very small quantities of peptides and proteins extracted from single 2-D spots.
- More powerful, less expensive computers and software are now available, rendering thorough computerized evaluations of the highly complex 2-D patterns economically feasible.
- Data about entire genomes for a number of organisms are now available, allowing rapid identification of the gene encoding a protein separated by 2-D electrophoresis.
- The World Wide Web provides simple, direct access to spot pattern databases for the comparison of electrophoresis results and genome sequence databases for assignment of sequence information.

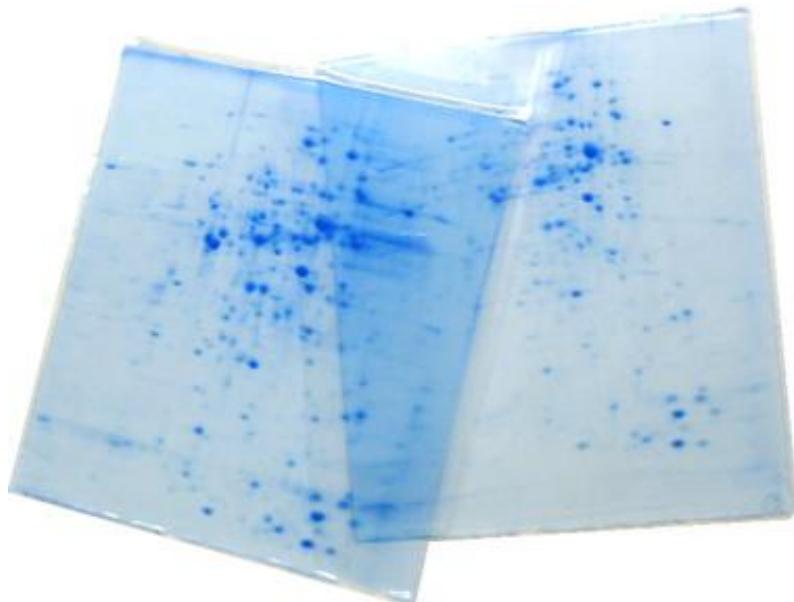


Fig. 8: 2D gels stained with Coomassie blue.

1.7.2 Two-dimensional liquid chromatography

Recently proteome analyses have also been performed in a “gel less” condition by using protein fractionation procedures based entirely on liquid chromatography (LC).

1.7.3 Liquid chromatography by “ProteomeLab PF 2D”

2-D LC, two dimensional liquid chromatography was performed by using ProteomeLab™ PF 2D instrument commercialized by Beckman Coulter (*Fullerton, CA, USA*) (Fig. 9).

- Proteins are separated in the first dimension by **HPCF (high-performance chromatofocusing)**, performed on an HPCF-1D column. With this technique, proteins bind to a strong anion exchanger followed by elution with a continuously decreasing pH gradient, generated on the column by two buffers. Up to 5 ml of sample are introduced with a manual injector into the column for the first dimension CF. The pH gradient affects the proteins net charge and their adsorption/desorption to the positively-charged matrix of the column, causing protein separation in the effluent. The proteins are eluted based on their isoelectric point (pI), measured for absorbance at 280 nm, and collected in a 96 deep-well plate by a fraction collector according to pre-determined pH decrements of 0.4 pH units during the gradient.
- The eluent from the 1st dimension was injected into the 2nd dimension, a **HPRP (high-performance reversed-phase chromatography)**. The 2nd dimension separation is based on protein hydrophobicity. HPRP is carried out in a C18 column. The mobile phase consists of 0.1 % TFA (Trifluoroacetic Acid) in water and 0.08 % TFA in Acetonitrile. During the run a gradient is created in the column by switching the flow from 0 % to 100 % of second buffer. The eluent from the second dimension is monitored by a second high performance UV/VIS detector at 214 nm, that provides a more universal and sensitive detection of proteins via peptide bonds. Fractions are immediately collected in Eppendorf tubes for MS analysis by using an automated fraction collector. Either after the 1st dimension and 2nd dimension, a specific software generates a graph (chromatogram) that shows the absorbance (280nm or 214nm) versus time; the height of each peak in the chromatogram is proportional to the concentration of that specific protein.



Fig. 9: ProteomeLab™ PF 2D instrument commercialized by Beckman Coulter (*Fullerton, CA, USA*)

1.7.4 Advantages and disadvantages of 2D-gel electrophoresis vs 2D liquid chromatography

Gels in the slab or tube format have been used primarily for the size-dependent separation of biological molecules, such as nucleic acids and proteins. The success of conventional 2D electrophoresis system is based on a series of inherent features that life scientists find very desirable or perhaps even essential. One is the easy preparative separation in slab and tube gel system. Most life scientists are separating proteins to recover them for further studies, so all the techniques to locate proteins and either excise them from gels or use them directly are desired. A second major advantage of gel system is that it is relatively easy to maintain the integrity of a separation while components are being subjected to further manipulation and once a protein zone has been located it is even possible to bring the protein back into solution for further analysis. The third major advantage of conventional slab and tube electrophoresis systems is the open nature of the format. The gel acts as both a fraction collector and transport medium, which, after a separation in one dimension, may be used to carry sample components into another separation dimension without disturbing the separation in the first dimension. A fourth advantage of conventional electrophoretic system is that they allow samples to be profiled, i.e., they provide a picture of everything that is present. Furthermore, it is a very popular method and used for a long time and then there is more bibliography than other used procedures and a great number of cellular fraction methods are available.

Although it is one of the most widely used separation techniques, slab gel electrophoresis generally suffers from long analysis time, low efficiencies and difficulties in detection (proteins can be poorly represented or not present in the gel) and complete automation. It is, in fact, a time consuming technique: it requires long times to prepare the polymerizations chamber (even if up to date commercial kits are available), to prepare the sample (in particular to find the best extraction and solubilization protocol), to polymerize the gel, to run the experiment, and to stain the final gel. Other limitations are the resistance of some proteins in this type of analysis: low abundance proteins, hydrophobic proteins, such as poorly soluble membrane proteins, proteins with extreme molecular weight, less than 10 kDa or greater than 200 kDa, as well as proteins with extreme isoelectric point, less than 4.0 or greater than 9.0. Finally, the need of high purification of samples, the limited sample loading capacity (0.1 – 3 mg) and the short dynamic range are further limitation of this technique (Corthals *et al.*, 2000; Gygi *et al.*, 2000; Cravatt *et al.*, 2000).

The main advantage, instead, of two-dimensional liquid chromatography is that crude protein extracts can be analysed after few purification steps thus achieving a higher level of sensibility and reproducibility than most of the chemical procedures, allowing a better comparison of protein

patterns (Agrawal *et al.*, 2005; Everberg *et al.*, 2004; Ferro *et al.*, 2000; Chivasa *et al.*, 2002; Okushima *et al.*, 2000; Borner *et al.*, 2003; Jung *et al.*, 2000; Lambert *et al.*, 2005) and allowing an high possibility of automation and interfacing with most of the mass analysis tools. Furthermore, it appears to be a less laborious procedure and no need for staining. However this technique also has limitations: chemistry of the instruments is incompatible with strong solubilisation buffer making difficult the choice of the right extraction method and the possibility that there are more than one protein in a single peak obtained with two-dimensional liquid chromatography analysis.

	2-D Gel Electrophoresis	2-D Liquid Chromatography
Strong points	<ul style="list-style-type: none"> ▪ small amount of material needed ▪ more bibliography ▪ sub cellular fraction methods available ▪ pI, Mw available after run 	<ul style="list-style-type: none"> ▪ Sensibility ▪ Reproducibility ▪ Automation ▪ No staining
Weak points	<ul style="list-style-type: none"> ▪ lower amount of proteins separated (0.1-3 mg) ▪ High purification of samples ▪ More laborious ▪ Reproducibility 	<ul style="list-style-type: none"> ▪ More proteins in one peak ▪ chemistry of the instruments is incompatible with strong solubilisation reagents ▪ No indication about Mr

Tab. 3: Comparison of the two protein separation techniques.

1.8 Protein Identification by Mass spectrometry

Mass spectrometry has been used in its different forms for the analysis of proteins. Over the years, the idea of using mass spectrometers to perform protein identification has evolved with the improvement in instrumentation performance and in the changes occurring in genomic databases. The introduction of effective matrix-assisted laser (Light Amplification by Stimulated Emission Radiation) desorption ionization and time-of-flight (MALDI-TOF) mass spectrometry and electrospray ionization (ESI) tandem mass spectrometry has revolutionized the field of mass spectrometry analysis. Manufacturers of these instruments have increased their efforts and their economic investments to produce more instruments for proteomic purposes (Figeyns, 2005).

1.8.1 MALDI/TOF Mass spectrometry

Several development in the field of mass spectrometry has allowed rapid and accurate mass measurements of analytes by a technique called *matrix-assisted laser desorption ionization* (MALDI) and *time of flight* (TOF) mass spectrometry. This technique allows the transfer of peptides from a solid state to the gas phase, while the TOF mass spectrometer rapidly separates peptides according to their m/z (mass/charge) ratio. Proteins isolated from 2D gel electrophoresis are digested with trypsin, desalting, and then spotted on a MALDI plate for co-crystallization with a saturated matrix solution. Alternatively, protein digests can be separated by HPLC (High Performance Liquid Chromatography) or 2D liquid chromatography and eluting peptides are digested with trypsin and deposited on a MALDI plate. The target plate is then introduced in the vacuum chamber of the mass spectrometer. A light pulse from a laser beam of a wavelength tuned to the absorbance of the matrix is focused onto a limited area of the spot. The rapid transfer of energy ejects an ionization plume of material from the plate surface and brings the peptides into the gas phase. The MALDI plate is biased to high voltages of +20 to +30kV, with respect to a grounded orifice. This induces the positively charged peptides to accelerate toward the orifice of the flight tube. The peptides are affected by the electric field during the time when they fly between the plate and the orifice. They reach the orifice with a velocity proportional to $(z/m)^{1/2}$. Once they pass through the orifice, the peptides have all the same kinetic energy but not the same velocity because of their different mass and the law of momentum conservation. Thus, molecules that have an identical charge will have a velocity inversely proportional to their masses. Generally, in MALDI the resulting peptide ions are singly charged. Once in the field-free region of the flight tube, the peptides fly through the tube only according to their initial velocity at the orifice. The vacuum in the flight tube is such that the likelihood of a collision with another molecule while in the flight tube is low on average 10^{-8} torr. Because the peptides are usually singly charged, they traverse the flight

tube according to their mass and hit the detector at different time intervals. The mass analyzer, triggered by the laser pulse, records the signal detected versus the time of flight, which can be readily transformed into m/z ratios if the mass spectrometer is properly calibrated. In principle, a longer tube allows a better separation of the different ions. Collisions with gas molecules in the path of the analytes would effectively destroy the separation. Therefore, the longer the path, the larger the vacuum requirement is imposed to maintain a path free of possible collisions. For this reason, the mass spectrometers are equipped with sufficient pumping to achieve even $10^{-9}/10^{-10}$ torr. A second factor that affects the resolution of the separation is the initial kinetic energy distribution due to the burst of analytes off the MALDI plate. The technique of delay extraction of the ion and the reflectron were added to the MALDI-TOF to improve the resolution, based on a more uniform kinetic energy. The delay extraction acts on the kinetic energy prior to the separation, while the reflectron acts on the kinetic energy distribution during the time of flight. The delay extraction is simply achieved by introducing a time delay prior to the high-voltage biasing of the MALDI plate. This allows the ions ejected from the plate to kinetically cool down providing improved resolution.

The reflectron allows the kinetic refocusing of the ions, which also results in better resolution. It is normal to obtain a peptide mass accuracy down to 10 to 50 ppm. The reflectron consist of hardware that is placed in the path of the ions. Therefore, the design of a MALDI-TOF equipped with a reflectron is very different than the conventional MALDI-TOF instrument. Furthermore, different designs have been fabricated; however, the principle remains the same. The reflectron is a set of ring lenses that are stacked together. Increasingly higher potentials are applied to the rings. In the time of flight each peptide is represented by a pocket of ions that travels with a small range of kinetic energy. The ions that are at the front edge of the pocket have a higher kinetic energy, while the ions at the trailing end of the pocket have a lower kinetic energy. The fast moving ions penetrate the reflectron first and start to feel the repulsing electric field imposed by the reflectron. Because of their high kinetic energy, they travel further into the reflectron before reaching a point of zero kinetic energy. Then they are reaccelerated by the repulsing field toward the entrance of the reflectron. Meanwhile, the other ions also enter the reflectron following the order of their decreasing kinetic energy. As a result, the slow moving ions are now at the front of the traveling pocket, while the fast moving ions stay behind. All the ions then travel back in the field-free region of the TOF tube. While traveling, the pockets of ions becomes focussed by the higher speed trailing ions catching up to the slower leading ions. The resolution achieved depends on the positioning of the detector at the focal point of the reflectron. The pocket of ions will not have time to focus if the detector is positioned too close to the reflectron. Also, the pocket of ions will start defocusing if the detector is positioned too far from the reflectron (Figeys, 2005).

1.8.2 Peptide mass fingerprinting (PMF)

The identification of proteins by MALDI-TOF mass spectrometry is generally achieved by measuring the m/z ratio of the peptides predominantly of charge +1. The combination of accurate peptide mass measurement with the availability of protein sequence databases forms the basis of protein identification by MALDI-TOF. Figure 10 shows the typical workflow used for the identification of proteins by peptide mass fingerprinting (PMF). After obtaining the list of the measured masses present in a tryptic digests, the known contaminants are deleted from this list. Thus, the mass list is then used to search proteins into a databases. Different software packages have been developed for the identification of proteins based on the accurate measurement of peptide masses. The simplest method for scoring is to add the number of peptide masses that match with the predicted masses for each entry in a protein database. The database entries are then ranked according to the number of hits. This forms the basis behind software such as **MS-Fit** (<http://prospector.ucsf.edu>) or **Mascot** (<http://www.matrixscience.com/>) (Pappin *et al.*, 1993). Mascot uses more information to make its decision on the score by taking into account the protein size and the relative abundance of peptides in the databases. Mascot further incorporates probability scoring for the probability that the match between the data and the entry in the database will be a random event. This score is calculated for every entry in the database. The identification is then established by ordering the proteins with a decreasing probability of being a random match. **ProFound** (<http://prowl.rochester.edu/>) (Zhang and Chait, 2000) uses a different approach based on Bayesian theory to rank the protein sequences in a database by their probability of occurrence. It is an expert system that uses detailed information about each protein sequence and empirical information about the distribution of proteolytic peptides that are included in the scoring scheme. Generally, all these software packages perform well when good-quality spectra are available. The ones that provide more advanced scoring schemes perform better when less information is available or when the quality of the MS spectra is reduced. Regardless of the software, the identification of the protein depends on the number of peptides observed, the accuracy of the measurement, and the size of the genome of the particular species. For small genomes, protein identification using the MALDI-TOF mass spectrometer is generally successful, but for large genomes the rate of success drops significantly using MALDI-TOF (Figeys, 2005).

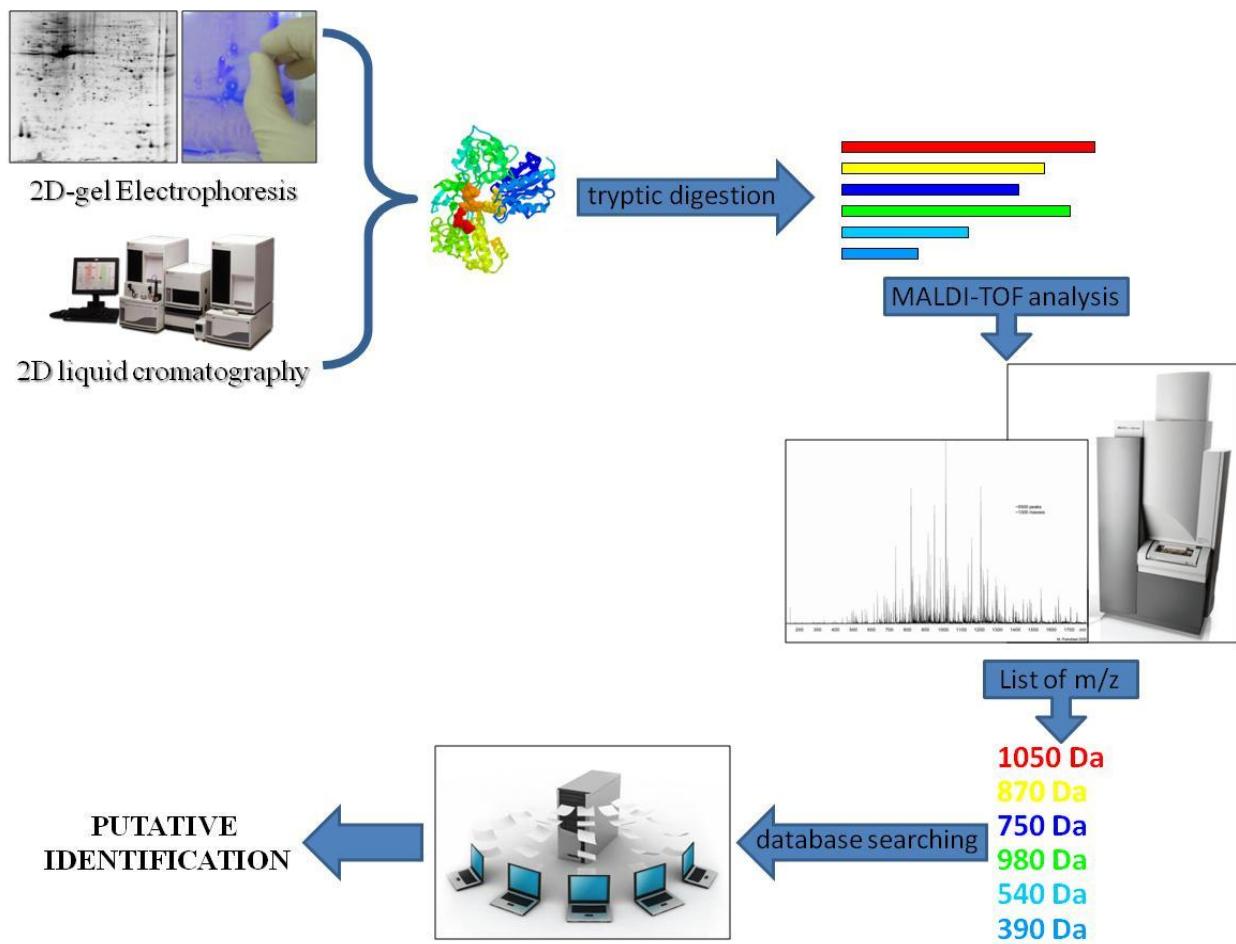


Fig. 10: Workflow for the identification of proteins based on MALDI-TOF and peptide mass fingerprinting. Entire extracted proteome is separate with 2D gel electrophoresis or 2D liquid chromatography. Protein of interest is digested with trypsin and the resulting peptide mixture is analyzed by MALDI-TOF mass spectrometry. The measured m/z ratios are then used to search protein/DNA databases leading to the identification of the protein.

1.9 References

- Abdel-Basset, R., Issa, A.A., Adam, M.S.**, (1995) Chlorophyllase activity: Effects of heavy metals and calcium. *Photosynthetica* 31, 421-425.
- Adriano, D.C.** (2001) Trace Elements in Terrestrial Environments. Biogeochemistry, Bioavailability, and Risks of Metals. *Springer-Verlag, New York*. 867 pp.
- Agrawal, G.K., Yonekura, M., Iwahashi, Y., Iwahashi, H., Rakwal, R.** (2005) System, trends and perspectives of proteomics in dicot plants Part I: Technologies in proteome establishment. *Journal of Chromatography B* 815, 109-123.
- Aidid, S.B., Okamoto, H.**, (1992) Effects of lead, cadmium and zinc on the electric membrane potential at the xylem:symplast interface and cell elongation of *Impatiens balsamina*. *Environmental and Experimental Botany* 32, 439–448.
- Alcantara, E., Romera, F.J., Canete, M., De La Guardia, M.D.** (1994) Effects of heavy metals on both induction and function of root Fe(III)reductase in Fe-deficient cucumber (*Cucumis sativus* L.) plants. *Journal of Experimental Botany* 45, 1893–1898.
- Al-Chalabi, A.S., Hawker, D.**, (2000) Distribution of vehicular lead in roadside soils of major roads of Brisbane, Australia. *Water, Air and Soil Pollution* 118, 299–310.
- Alloway, B.J.**, (1995) Soil processes and the behavior of metals. In: *Alloway, B.J., (Ed), Heavy metals in soils* (pp. 38–57). London: Blackie.
- Anderson C.W.N., Moreno F.N., Meech J.** (2005) A field demonstration of gold phytoextraction technology. *Minerals Engineering* 4:385 – 392.
- Araus, J.L., Amaro, T., Voltas, J., Nakkoul, H., Nachit, M.M.,** (1998) Chlorophyll fluorescence as a selection criterion for grain yield in durum wheat under Mediterranean conditions. *Field Crops Research* 55, 209-223.
- Arisi, A.C.M., Mocquot, B., Lagriffoul, A., Mench, M., Foyer, C.H., Jouanin, L.,** (2000) Responses to cadmium in leaves of transformed poplars overexpressing γ -glutamylcysteine synthetase. *Physiologia Plantarum* 109, 143-149.
- Arnon, D.I., Hoagland, D.R.,** (1940) Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrient. *Soil Science* 50, 463-483.
- Aronsson, P., Perttu, K.,** (1994) Willow vegetation filters for municipal wastewaters and sludges. A biological purification system. *Uppsala: Swedish University of Agricultural Sciences* p. 230.

Asada, K., Takahashi, M., (1987) Production and scavenging of active oxygen in photosynthesis. In: *Kyle, D.J., Osmond, C.B., Arntzen, C.D., (Eds.), Photoinhibition. Topics in Photosynthesis*, vol. 9. Elsevier, Amsterdam, pp. 227–287.

Assunção A.G.L., Da Costa Marins P., De Folter S., Vooijs R., Schat H., Aarts M.G.M. (2001) Elevated expression of metal transporter genes in three accessions of metal hyperaccumulator *Thlaspi caerulescens*. *Plant Cell and Environment* 24:217 – 226.

Assunção A.G.L., Schat H., Aarts M.G.M. (2003) *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* 159:411 – 419.

Assunção A.G.L., Ten Bookum W.M., Nelissen H.J.M., Vooijs R., Schat H., Ernst W.H.O. (2003) Acosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* 159:383 – 390.

Assunção A.G.L., Ten Bookum W.M., Nelissen H.J.M., Vooijs R., Schat H., Ernst W.H.O. (2003) Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* 159: 411 – 419.

Baker A.J.M., Brooks R.R. (1989) Terrestrial higher plants which hyperaccumulate metallic elements - a review of their distribution, ecology and phytochemistry. *Biorecovery* 1: 81 – 126.

Baker A.J.M., McGrath S.P., R.D., Reeves D.R., Smith J.A.C. (2000) Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils.

Baker A.J.M., Walker P.L. (1990) Ecophysiology of metal uptake by tolerant plants, heavy metal tolerance in plants. In: Shaw A.J., eds. Heavy metal tolerance in plants: evolutionary aspects. CRC Press, Coca Raton: 155-177

Baker, A.J.M., Brooks, R.R., (1989) Terrestrial higher plants which hyper accumulate metallic elements Review of their distribution, ecology, and phytochemistry. *Biorecovery*, 1, 81–126.

Baker, A.J.M., Ewart, K., Hendry, G.A.F., Thrope, P.C., Walker, P.L., (1990) The evolutionary basis of cadmium tolerance in higher plants. In: *4th International Conference on Environmental Contamination, Barcelona*, 23–29.

Baker, A.J.M., Reeves, R.D., McGrath, S. P. (1991) In situ decontamination of heavy metal polluted soils using crops of metal accumulating plants – A feasibility study. In *R.E. Hinchee & R.F. Olfenbuttel (Eds.), In-situ bioremediation* (pp. 539–544). Stoneham, M. A: Butterworth-Heinemann.

Bañuelos, G.S., Ajwa, H.A., (1999) Trace elements in soils and plants: an overview. *Journal of Environmental Science and Health Part A* 34 (4), 951–74.

Barbagallo, R.P., Oxborough, K., Pallett, K.E., Baker, N.R., (2003) Rapid, non-invasive screening for perturbations of metabolism and plant growth using chlorophyll fluorescence imaging. *Plant Physiology* 132, 485–493.

Barber, J., (1998) Photosystem two. *Biochimica Biophysica Acta* 1365, 269–277.

Barceló , J., Poschenrieder, C., Andreu, I., Gunsé, B., (1986) Cadmium-induced decrease of water stress resistance in bush bean plants (*Phaseolus vulgaris* L. cv. Contender). I. Effects of Cd on water potential, relative water content and cell wall elasticity. *Journal of Plant Physiology* 125, 17–25.

Barceló, J., Poschenrieder, C., (1990) Plant water relations as affected by heavy metal stress: a review. *Journal of Plant Nutrition* 13, 1–37.

Baron-Ayala, M., Arellano, J.B., Lopez, G.J., (1995) Copper and Photosystem II: A controversial relationship. *Physiologia Plantarum* 94, 174-180.

Baryla, A., Carrier, P., Franck, F., Coulomb, C., Sahut, C. Havaux, M., (2001) Leaf chlorosis in oilseed rape plants (*Brassica napus*) grown on cadmium-polluted soil: Causes and consequences for photosynthesis and growth. *Planta* 212, 696-709.

Baszynski, T., Tukendorf, A., Ruszkowska, M., Skorzynska, E., Maksymiec, W., (1988) Characteristic of the photosynthetic apparatus of copper non-tolerant spinach exposed to excess copper. *Journal of Plant Physiology* 132, 708-713.

Baudouin, C., Charveron, M., Tarrouse, R., Gall, Y., (2002) Environmental pollutants and skin cancer. *Cell Biology and Toxicology* 18, 341–348.

Becerril, J.M., Munoz-Rueda, A., Aparicio-Tejo, P., Gonzales-Murua, C., (1988) The effects of cadmium and lead on photosynthetic electron transport in clover and lucerne. *Plant Physiology and Biochemistry* 26, 357-363.

Behmer S. T., Lloyd C. M., Raubenheimer D., Stewart-Clark J., Knight J., Harper F. A., Smith J. A. C. (2005) Metal hyperaccumulation in plants: mechanisms of defence against insect herbivores. *Functional Ecology* 19:55 -66.

Bennett LE, Burkhead JL, Hale KL, Terry N, Pilon M, Pilon-Smits EHA (2003) Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. *Journal of Environmental Quality* 32:432-440.

Berger, S., Benediktyova, Z., Matous, K., Benfig, K., Mueller, M.J., Medbal, L., Roitsch, T., (2007) Visualization of dynamics of plant-pathogen interaction by novel combination of chlorophyll fluorescence imaging and statistical analysis: differential effects of virulent and

avirulent strains of *P. syringae* and of oxylipins on *A. thaliana*. *Journal of Experimental Botany* 58, 797–806.

Berger, S., Papadopoulos, M., Schreiber, U., Kaiser, W., Roitsch, T., (2004) Complex regulation of gene expression, photosynthesis and sugar levels by pathogen infection in tomato. *Physiologia Plantarum* 122, 419–428.

Bert V., Meerts P., Saumitou-Laprade P., Salis P., Gruber W., Verbruggen N. (2003) Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* 249: 9 – 18.

Bilger, W., Björkman, O., (1991) Temperature dependence of violaxanthin deepoxidation and non-photochemical fluorescence quenching in intact leaves of *Gossypium-hirsutum* L. and *Malvaviriflora*. L. *Planta* 184, 226–234.

Birch, G.E., Scollen, A., (2003) Heavy metals in road dust, gully pots and parkland soils in a highly urbanised subcatchment of Port Jackson, Australia. *Australian Journal of Soil Research* 41, 1329–1342.

Bjellqvist B., Ek K., Righetti P.G., Gianazza E., Görg A., Westermeier R., Postel W. (1982) Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. *Journal of Biochemical and Biophysical Methods* 6, 317–339.

Blake, T.J., (1983) Coppice systems for short-rotation intensive forestry: the influence of cultural, seasonal and plant factors. *Australian Forest Research* 13, 279–291.

Boddi, B., Oravecz, A.R., Lehoczki, E., (1995) Effect of cadmium on organization and photoreduction of protochlorophyllide in dark-grown leaves and etioplast inner membrane preparations of wheat. *Photosynthetica* 31, 411-420.

Bohn, H.L., McNeal, B.L., O'Conner, G.A., (1985) Soil Chemistry. *Wiley Interscience, Wiley, NY*

Booth, B., (2005) The added danger of counterfeit cigarettes. *Environmental Science and Technology* 39, 34A.

Boyd R. S. and Martens S. N. (1995) The raison d'être for metal hyperaccumulation by plants. In: The Vegetation of Ultramafic (Serpentine) soils, Proceeding of the 1st International Conference on Serpentine Ecology. *Intercept Ltd., Andover, Hampshire* 279 - 289.

Boyd R.S. (2004) Ecology of metal hyperaccumulation. *New phytologist* 162:563 - 567.

Boyd R.S., Jaffre' T. (2001) Phytoenrichment of soil Ni content by *Sebertia acuminata* in New Caledonia and the concept of the concept allelopathy. *South African Journal of Science* 97:535 – 538.

Borner, G.H.H., Lilley, K.S., Stevens, T.J., Dupree, P. (2003) Identification of Glycosylphosphatidylinositol-Anchored Proteins in Arabidopsis. A Proteomic and Genomic Analysis. *Plant Physiology* 132:568-577.

Brady K.U., Kruckeberg A.R., Bradshaw H.D.Jr. (2005) Evolutionary ecology of plant adaptation to serpentine soils. *Annual Review of Ecology, Evolution and Systematics*. 36:243 – 266.

Briat, J.F., Lebrun, M., (1999) Plant responses to metal toxicity. *Comptes Rendus de l'Académie des Sciences-Series III Sciences de la Vie*. 322, 43–54.

Bro, E., Meyer, S., Genty, B., (1996) Heterogeneity of leaf CO₂ assimilation during photosynthetic induction. *Plant Cell Environment* 19, 1349–1358.

Brooks R.R. (1998) Geobotany and hyperaccumulators. In: Brooks R.R., eds. Plants that hyperaccumulate heavy metals. *Wallingford, UK, CAB International*: 55 – 94

Bubb J.M., Lester J.N. (1991) The impact of heavy metals on lowland rivers and the implication for man and environment. *Science of Total Environment* 100:207 – 233.

Burken, J.G., Schnoor, J.L., (1999) Distribution and volatilisation of organic compounds following uptake by hybrid poplar trees. *International Journal of Phytoremediation* 1, 139–51.

Burken, J.G., Schnoor, J.L., (1997) Uptake and metabolism of atrazine by poplar trees. *Environmental Science and Technology* 31,1399– 406.

Butler, W.L., (1978) Energy distribution in the photochemical apparatus of photosynthesis. *Annual Review of Plant Physiology* 29, 345–378.

Ceulemans, R., Scarascia-Mugnozza, G., Wiard, B.M., Braatne, J.H., Hinckley, T.M., Stettler, R.F., Isebrands, J.G., Heilman, P.E., (1992) Production physiology and morphology of *Populus* species and their hybrids grown under short rotation. I. Clonal comparisons of 4-year growth and phenology. *Canadian Journal of Forest Research* 22, 1937-1948.

Chaerle, L., Leinonen, I., Jones, H.G., Van Der Straeten, D., (2007) Monitoring and screening plant populations with combined thermal and chlorophyll fluorescence imaging. *Journal of experimental Botany* 58, 773–784.

Chaerle, L., Van Der Straeten, D., (2000) Imaging techniques and the early detection of plant stress. *Trends in Plant Science* 5, 495–501.

Chaerle, L., Van Der Straeten, D., (2001) Seeing is believing: imaging techniques to monitor plant health. *Biochimica et Biophysica Acta* 1519, 153–166.

Chaney, R.L., (1983) Plant uptake of inorganic waste constituents. In J.F. Parr, P.B. Marsh, & J. M. Kla (Eds.), *Land treatment of hazardous wastes* (pp. 50–76). Park Ridge, NJ: Noyes Data Corp.

Chaney, R.L., Malik, K.M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., (1997) Phytoremediation of soil metals. *Current Opinion in Biotechnology* 8, 279 – 84.

Chen, A., Komives, E.A., Schroeder, J.I. (2006) An improved grafting technique for mature arabidopsis plants demonstrates long-distance shoot-to-root transport of phytochelatins in arabidopsis. *Plant Physiology* 141, 108–120.

Chen, J., Goldsbrough, P.B. (1999) Increased activity of γ -glutamylcysteine synthetase in tomato cells selected for cadmium tolerance. *Plant Physiology* 106, 233–239.

Cheng, C.X., Jeon, Y.A., Lee, C.H. (1999) Relative contribution of photochemical and onphotochemical routes to excitation energy in rice and barley illuminated at a chilling temperature, *Physiologia Plantarum* 107, 881–889.

Chivasa, S., Ndimba, B.K., Simon, W.J., Robertson, D., Yu, X.L., Knox, J.P., Bolwell, P., Slabas, A.R. (2002) Proteomic analysis of the Arabidopsis thaliana cell wall. *Electrophoresis*, 23, pp. 1754–1765

Chugh, L.K., Sawhney, S.K., (1999) Photosynthetic activities of *Pisum sativum* seedlings grown in the presence of cadmium. *Plant Physiology and Biochemistry* 37, 297-303.

Ciscato, M., Valcke, R., (1998) Chlorophyll fluorescence imaging of heavy metal treated plants. In: Garab G, ed. *Photosynthesis: mechanisms and effects*, Vol. IV. Dordrecht, the Netherlands: Kluwer Academic Publishers, 2661–2663.

Clemens S., Palmgren M.G., Kraemer U. (2002) A long way ahead: understanding and engineering plant metal accumulation. *Trends Plant Science* 7:309 – 315.

Clemens, S. (2001) Molecular mechanism of plant metal tolerance and homeostasis. *Planta*, 212, 475-486.

Clemens S (2006) Evolution and function of phytochelatin synthases. *Journal of Plant Physiology* 163, 319–332.

Clijsters, H., Van Assche, F. (1985) Inhibition of photosynthesis by heavy metals. *Photosynthesis Research* 7, 32-40.

Cobbett C.S. (2000) Phytochelatins and their role in heavy metals detoxification. *Plant physiology* 123:825 – 832.

Collins, Y.E., Stotzky, G. (1989) Factors affecting the toxicity of heavy metals to microbes. In: *Beveridge, T.J., and Doyle, R.J., (Eds) Metal Ions and Bacteria* (pp 31–90). Wiley, Toronto, Canada.

Condon, A.G., Richards, R.A., Rebetzke, G.J., Farquhar, G.D., (2002) Improving intrinsic water-use efficiency and crop yield. *Crop Science* 42, 122–131.

Connolly Erin L., Fett Janette P., Guerinot Mary Lou (2002) Expression of the IRT1 Metal Transporter Is Controlled by Metals at the Levels of Transcript and Protein Accumulation. *The Plant Cell*, Vol. 14, 1347-1357.

Cordero, B., Lodeiro, P., Herrero, R., Esteban Sastre de Vicente, M., (2004) Biosorption of cadmium by *Fucus spiralis*. *Environmental Chemistry* 1, 180–187.

Corthals, G. L.; Wasinger, V. C.; Hochstrasser, D. F.; Sanchez, J. C. (2000) The dynamic range of protein expression: a challenge for proteomic research. *Electrophoresis* 21, 1104-1115

Cosio C, DeSantis L, Frey B, Diallo S, Keller C. (2005) Distribution of cadmium in leaves of *Thlaspi caerulescens*. *Journal of Experimental Botany* 56: 765–775.

Cosio, C., Vollenweider, P., Keller, C., (2006) Localization and effects of cadmium in leaves of tolerant willows (*Salix viminalis* L.). Part I. Macrolocalization and phytotoxic effects of cadmium. *Environmental and Experimental Botany* 58, 64–74.

Costa, G., Morel, J.L., (1994) Water relations, gas exchange and amino acid content in Cd-treated lettuce. *Plant Physiology and Biochemistry* 32, 561–570.

Cotter-Howells, J.D., Caporn, S., (1996) Remediation of contaminated land by formation of heavy metals phosphates. *Applied Geochemistry* 11, 335–42.

Cotter-Howells, J.D., Champness, P.E., Charnock, J.M., Pattrick, R.A.D. (1994) Identification of pyromorphite in mine-waste contaminated soils by ATEM and EXAFS. *European Journal of Soil Science* 45, 393–402.

Cravatt, B. F.; Sorensen, E. J. (2000) Chemical strategies for the global analysis of protein function. *Current Opinion in Chemical Biology* 4, 663-668.

Crowley D.E., Wang Y.C., Reid C.P.P. Szansiszlo P.J. (1991) Mechanism of iron acquisitionfrom siderophoresy microrganisms and plants. *Plant Soil* 130:179 – 198.

Cunningham, S.D., Ow, D.W., (1996) Promises and prospects of phytoremediation. *Plant Physiology*, 110, 715-719.

Cunningham, S.D., Shann, J.R., Crowley, D.E., Anderson, T.A. (1997) Phytoremediation of contaminated water and soil. In *E. L. Kruger, T. A. Anderson, & J. R. Coats (Eds.), Phytoremediation of soil and water contaminants. ACS Symposium series 664* (pp. 2–19). Washington, DC: American Chemical Society.

Curie C., Alonso J.M., Le Jean M., Ecker R., Briat J.F. (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochemical Journal* 347:749 – 755.

DalCorso Giovanni, Farinati Silvia, Maistri Silvia, Furini Antonella (2008) How Plants Cope with Cadmium: Staking All on Metabolism and Gene Expression. *Journal of Integrative Plant Biology*, 50 (10): 1268–1280

Daley, P.F., (1995) Chlorophyll fluorescence analysis and imaging in plant stress and disease. *Canadian Journal of Plant Pathology* 17, 167–173.

Dannehl, H., Herbik, A., Godde, D., (1995) Stress-induced degradation of the photosynthetic apparatus is accompanied by changes in thylakoid protein turnover and phosphorylation. *Physiologia Plantarum* 93, 179–186.

Das, P., Samantaray, S., Rout, G.R., (1997) Studies on cadmium toxicity in plants: a review. *Environmental Pollution* 98, 29–36.

Davis M. A., Boyd R. S. (2000) Dynamics of Ni-based defence and organic defences in the Ni hyperaccumulator, *Streptanthus polygaloides* (Brassicaceae). *New Phytologist* 146:211 – 217.

Davis M.A., Boyd R.S., Cane J.H. (2001) Host-switching does not circumvent the Ni-based defense of the Ni hyperaccumulator. *Streptanthus polygaloides* (Brassicaceae). *South African Journal of Science* 97:554 – 557.

De Filippis, L.F., Hampp, R., Ziegler, H., (1981) The effects of sublethal concentrations of zinc, cadmium and mercury on *Euglena* - Growth and pigments. *Zeitschrift für Pflanzenphysiologie* 101, 37-47.

De Knecht, J.A.P., Koevoets, P.L.M., Verkleij, J.A.C., Ernst, W.H.O. (1992) Evidence against a role for phytochelatins in naturally selected increased cadmium tolerance in *Silene vulgaris* (Moench) Garke. *New Phytologist* 122, 681–688.

De Vos, C.H.R., Vonk, M.J., Vooijs, R., Schat, H. (1992) Glutathione depletion due to copperinduced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. *Plant Physiology* 98, 853–858.

Demmig-Adams, B., Adams III, W., Barker, D., Logan, B., Bowing, D., Verhoeven, A. (1996) Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. *Physiologia Plantarum* 98, 253–264.

Deng, H., Ye, Z.H., Wong, M.H., (2004) Accumulation of lead, zinc, copper and cadmium by 12 wetland plant species thriving in metal-contaminated sites in China. *Environmental Pollution*, 132, 29-40.

Di Cagno, R., Guidi, L., Stefani, A., Soldatini, G.F., (1999) Effects of cadmium on growth of *Helianthus annuus* seedlings: physiological aspects. *New Phytologist* 144, 65-71.

Dickinson, N.M., (2000) Strategies for sustainable woodland on contaminated soils. *Chemosphere* 41, 259–63.

Dickinson, N.M., Lepp, N.W., (1997) Metals and trees: impacts, responses to exposure and exploitation of resistance traits. In: Prost, R. (Ed.), *Contaminated soils: the 3rd International Conference on the Biogeochemistry of Trace Elements*. Paris: INRA pp. 247–254.

Dickinson, N.M., Pulford, I.D., (2005) Cadmium phytoextraction using short-rotation coppice *Salix*: the evidence trail. *Environment International* 31, 609-613.

Dickinson, N.M., Punshon, T., Hodgkinson, R.B., Lepp, N.W. (1994) Metal tolerance and accumulation in willows. In: Aronsson P, Perttu K, editors. *Willow vegetation filters for municipal wastewaters and sludges. A biological purification system*. Uppsala: Swedish University of Agricultural Sciences 121– 27.

Dickmann, D.I., Stuart, K.W., (1983) The Culture of Poplars in Eastern North America. *Michigan State University Publications. Michigan State University, East Lansing, Michigan*.

Dos Santos Utmaian, M.N. Wieshammer, G., Vega, R., Wenzel, W.W., (2007) Hydroponic screening for metal resistance and accumulation of cadmium and zinc in twenty clones of willows and poplars. *Environmental Pollution* 148, 155-165.

Dushenkov, V., Kumar, P.B.A.N., Motto, H., Raskin, I., (1995) Rhizofiltration: the use of plants to remove heavy metals from aqueous streams. *Environmental Science and Technology* 29, 1239–45.

Eapen S, D'Souza SF (2005). Prospects of genetic engineering of plants for phytoremediation of toxic metals. *Biotechnology Advances* 23:97-114.

Ebbs, S.D., Kochian, L.V., (1997) Toxicity of zinc and copper to *Brassica* species: implications for phytoremediation. *Journal of Environmental Quality* 26, 776–81.

Eckstein, J., Beyschlag, W., Mott, K.A., Ryel, R.J., (1996) Changes in photon flux can induce stomatal patchiness. *Plant Cell Environment* 19, 1066–1074.

Ederli L., Reale L., Ferranti F., Pasqualini S. (2004) Responses induced by high concentration of cadmium in *Phragmites australis* roots. *Physiologia Plantarum* 121, 66-74.

Entry, J.A., Watrud, L.S., Reeves, M., (1999) Accumulation of ^{137}Cs and ^{90}Sr from contaminated soil by three grass species inoculated with mycorrhizal fungi. *Environmental Pollution*, 104, 449–457.

EPA. Introduction to phytoremediation. (2000) Washington: U.S. Environmental Protection Agency. EPA/600/R-99/107.

EPA. Phytoremediation resource guide. (1999) Washington: U.S. Environmental Protection Agency. EPA 542-B-99-003.

Escarre' J., Lefebvre C., Gruber W., Leblanc M., Lepart J., Riviere Y., Delay B. (2000) Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implication for phytoremediation. *New phytologist* 145:429 – 437.

ETCS (European Topic Centre Soil) (1998) Topic report—Contaminated sites. European Environment Agency, Copenhagen, Denmark

European Community (1986) On the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Council Directive 86/278/EEC. In: EC Official Journal L181. European Community, Brussels, Belgium, 4.7.1986

Everberg H., Sivars U., Emanuelsson C., Persson C., Englund A.K., Haneskog,L., Lipniunas P., Jornten-Karlsson M., Tjerneld F., (2004) Protein pre-fractionation in detergent–polymer aqueous two-phase systems for facilitated proteomic studies of membrane proteins. *Journal of Chromatography A Volume 1029, Issues 1–2, 113–124*

Farmer, R.E., Jr. (1996) Genecology of *Populus*. In: Stettler, R.F., Bradshaw, H.D., Jr., Heilman, P.E., Hinckley, T.M., (Eds.), *Biology of Populus and its implications for management and conservation*. NRC Research Press, Ottawa, pp. 33-55.

Fatoki, O.S., (1996) Trace zinc and copper concentration in roadside surface soils and vegetation: A measurement of local atmospheric pollution in Alice, South Africa. *Environmental Interpretation* 22, 759–762.

Ferro, M., Seigneurin-Berny, D., Rolland, N., Chapel, A., Salvi, D., Garin, J., Joyard, J. (2000) Organic solvent extraction as a versatile procedure to identify hydrophobic chloroplast membrane proteins. *Electrophoresis*, 21:3517-3526.

Fischerova, Z., Tlustos, P., Szakova, J., Sichorova, K., (2006) A comparison of phytoremediation capability of selected plant species for given trace elements. *Environmental Pollution* 144, 93-100.

Figeys Daniel (2005) Proteomics: the basic overview. In: *Industrial Proteomics: Applications for Biotechnology and Pharmaceuticals*, edited by Daniel Figeys ISBN 0-471-45714-0 by John Wiley & Sons, Inc.

Flathman, P.E., Lanza, G.R., (1998) Phytoremediation: current views on an emerging green technology. *Journal of Soil Contamination* 7, 415–432.

Flechas FW, Latady M (2003). Regulatory evaluation and acceptance issues for phytotechnology projects. *Advances in Biochemical Engineering/Biotechnology* 78:172-185.

Gallego, S.M., Benavides, M.P., Tomaro, M.L., (1996) Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Plant Science* 121, 151–159.

Garbisu, C., Alkorta, I., (1997) Bioremediation: principles and future. *Journal of Clean Technology Environmental Toxicology and Occupational Medicine* 6, 1–16.

García, R., Millán, E., (1998) Assessment of Cd, Pb and Zn contamination in roadside soils and grasses from Gipuzkoa (Spain). *Chemosphere* 37, 1615–1625.

Gasic K, Korban SS (2007) Transgenic Indian mustard (*Brassica juncea*) plants expressing an *Arabidopsis* phytochelatin synthase (AtPCS1) exhibit enhanced As and Cd tolerance. *Plant Molecular Biology* 64:361-369.

Gawel, J.E., Trick, C.G., Morel, F.M.M., (2001) Phytochelatins are bioindicators of atmospheric metal exposure via direct foliar uptake in trees near Sudbury, Ontario, Canada. *Environmental Science Technology* 35, 2108 –2113.

GE Healthcare (2010) 2-D Electrophoresis: principles and methods. *Elanders*

Genty, B., Briantais, J.M., Baker, N.R., (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990, 87–92.

Genty, B., Meyer, S., (1995) Quantitative mapping of leaf photosynthesis using chlorophyll fluorescence imaging. *Australian Journal of Plant Physiology* 22, 277–284.

Ghaderian Y. S. M., Lyon A. J. E., Baker A. J. M. (2000) Seedling mortality of metal hyperaccumulator plants resulting from damping off by *Pythium* spp. *New Phytologist* 146:219 – 224.

Ghosh M, Singh S.P. (2005) A review on phytoremediation of heavy metals and utilization of its product. *Applied ecology and environmental research* 3: 1 – 18

Ghosh, M., Singh, S.P., (2005) A comparative study of cadmium phytoextraction by accumulator and weed species. *Environmental Pollution*, 133, 365–371.

Gielen, B., De Boeck, H.J., Lemmens, C.M.H.M., Valcke, R., Nijs, I., Ceulemans, R., (2005) Grassland species will not necessarily benefit from future elevated air temperatures: a chlorophyll fluorescence approach to study autumn physiology. *Physiologia Plantarum* 125, 52–63.

Gilmore, A.M., Hazlett, T.L., Debrunner, P.G., Govindjee, (1996) Comparative time-resolved photosystem II chlorophyll a fluorescence analyses reveal distinctive differences between photoinhibitory reaction center damage and xanthophyll cycle dependent energy dissipation. *Photochemistry and Photobiology* 64, 552–563.

Glass D.J. (1999) Economic potential of phytoremediation. In *Raskin, BD Ensley, John Wiley & Sons, eds I Phytoremediation of toxic metals: using plants to clean up the environment, Inc, New York, NY: 15 – 31*

Glass, D.J., (1999) U.S. and international markets for phytoremediation, 1999–2000 (p. 266). Needham, MA: D. Glass Associates.

Glass, D.J., (2000) Economic potential of phytoremediation. In: *Raskin, I., Ensley, B.D. (Eds.), Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment. John Wiley & Sons, Inc., New York, USA, pp. 15–31.*

Glimmerveen, I., (1996) Heavy metals and trees. *Edinburgh: Institute of Chartered Foresters p. 206.*

Gordon, J.C., (1975) The productive potential of woody plants. *Iowa State Journal of Research* 49, 267–274.

Görg, A., Postel, W., Günther, S., Weser, J. (1985) Improved horizontal two-dimensional electrophoresis with hybrid isoelectric focusing in immobilized pH gradients in the first dimension and laying-on transfer to the second dimension. *Electrophoresis* 6, 599–604

Görg, A., Postel, W., Günther, S. (1988) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 9, 531–546

Görg, A., Boguth, G., Obermaier, C., Posch, A., Weiss, W. (1995) Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt): The state of the art and the controversy of vertical versus horizontal systems. *Electrophoresis* 16, 1079–1086.

Goswami T., Bhattacharjee A., Babal P., Searle S., Moore E., Li M., Blackwell J.M. (2001) Natural resistance-associated macrophage protein 1 is an H⁺/bivalent cation antiporter. *Biochemical Journal* 354:511 – 519.

Govindjee (1995) Sixty-three years since Kautsky: Chlorophyll a fluorescence. *Australian Journal of Plant Physiology* 22, 131–160.

Govindjee, Nedbal, L., (2000) The chlorophyll fluorescence imaging and its application in plant science and technology. *Seeing is believing. Photosynthetica* 38, 481–482.

Granel, T., Robinson, B., Mills, T., Clothier, B., Green, S., Fung, L., (2002) Cadmium accumulation by willow clones used for soil conservation, stock fodder, and phytoremediation. *Australian Journal of Soil Research* 40, 1331–1337.

Grčman, H., Velikonja-Bolta, Š., Vodnik, D., Kos, B., Leštan, D., (2001) EDTA enhanced heavy metal phytoextraction: metal accumulation, leaching and toxicity. *Plant and Soil* 235, 105–114.

Greger, M. and Landberg, T., (1999) Use of willow in phytoextraction. *International Journal of Phytoremediation* 1, pp. 115–123.

Greger, M., Ogren, E., (1991) Direct and indirect effects of Cd²⁺ on photosynthesis in sugar beet (*Beta vulgaris*). *Physiologia Plantarum* 83, 129-135.

Grillet L. (2006) A phylogenetic approach to study heavy metal tolerance and accumulation in *Thlaspi caerulescens*. *Thesis. EED Plant Production 2005-2006. Amsterdam Vrije Universiteit, Amsterdam, The Netherlands*

Grotz N., Fox T., Connolly E., Park W., Guerinot M.L., Eide D. (1998) Identification of a family of zinc transporters genes from *Arabidopsis* that respond to zinc deficiency. *PNAS* 95: 7220-7224

Gullner, G., Kömives, T., Rennenberg, H., (2001) Enhanced tolerance of transgenic poplar plants overexpressing g-glutamylcysteine synthetase towards chloroacetanilide herbicides. *Journal of Experimental Botany* 52, 971-979.

Gulson, B.L., Tiller, K.G., Mizon, K.J., Merry, R.H., (1981) Use of lead isotopes in soils to identify the source of lead contamination near Adelaide, South Australia. *American Chemical Society, 15(6), 691–696.*

Gussarson, M., (1994) Cadmium-induced alterations in nutrient composition and growth of *Betula pendula* seedlings: the significance of fine roots as primary target for cadmium toxicity. *Journal of Plant Nutrition.* 17, 2151–2163.

Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold R. (2000) Evaluation of two dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci U.S.A.* 97, 9390-9395

Hagemeyer, J., Breckle, S.W., (1996) Growth under trace element stress. - in: *Y. Waisel, A. Eshel, U. Kafkafi (eds.): Plant Roots: The Hidden Half. 2nd Ed., M. Dekker, New York: pp. 415-433.*

Hagemeyer, J., Heppel, T., Breckle, S.W. (1994) Effects of Cd and Zn on the development of annual xylem rings of young Norway spruce (*Picea abies*) plants. *Trees* 8: pp. 223-227.

Hagemeyer, J., Waisel, Y., (1989) Influence of NaCl, Cd(NO₃)₂ and air humidity on transpiration of *Tamarix aphylla*. *Physiologia Plantarum* 75, 280-284.

Hall, J.L., (2002) Cellular mechanism for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, 53, 1-11.

Hendry, G.A.F., Baker, A.J.M., Ewart, C.F., (1992) Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus*. *Acta Botanica Neerlandica* 41, 271–281.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U (2008) Evolution of metal hyperaccumulation required cis regulatory changes and triplication of HMA4. *Nature* 453: 391-395.

Hernandez, L.E., Carpeta-Ruiz, R., Garate, A., (1996) Alterations in the mineral nutrition of pea seedlings exposed to cadmium. *Journal of Plant Nutrition* 19, 1581–1598.

Ho, Y.B., Tai, K.M., (1988) Elevated levels of lead and other metals in roadside soil and grass and their use to monitor aerial metal depositions in Hong Kong. *Environmental Pollution* 49, 37–51.

Hogue, C., (2004) Metallic refinement. EPA is working to ensure that the unique properties of metals are part of risk assessments. *Chemical and Engineering News* 82 (32), 23–24.

Horton, P., Ruban, A.V., Walters, R.G., 1996. Regulation of light harvesting in green plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 47, 655-694.

Howden, R., Goldsbrough, P.B., Andersen, C.R., Cobbett, C.S., (1995) Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiology* 107, 1059–1066 .

Hughes, J. B., Shanks, J., Vanderford, M., Lauritzen, J., Bhadra, R., (1997) Transformation of TNT by aquatic plants and plant tissue cultures. *Environmental Science & Technology* 31, 266–271.

Ibel, K., May, R.P., Kirschner, K., Szadkowski, H., Mascher, E., Lundahl, P. (1990) Protein-decorated micelle structure of sodium-dodecyl-sulfate protein complexes as determined by neutron scattering. *European Journal of Biochemistry* 190, 311–318.

Isebrands, J.G., Karnosky, D.F., (2001) Environmental benefits of poplar culture. In *Poplar Culture in North America. Part A, Chapter 6. Eds. D.E. Dickmann, J.G. Isebrands, J.E.*

Eckenwalder and J. Richardson. NRC Research Press, National Research Council of Canada, Ottawa, ON, pp 207–218.

James, B.R., (2001) Remediation-by-reduction strategies for chromate-contaminated soils. *Environmental Geochemistry Health 23, 175–9.*

Järup Lars (2003) Hazards of heavy metal contamination. *British Medical Bulletin 68(1): 167-182.*

Johnson, M.S., Cooke, J.A., Stevenson, J.K.W., (1992) Revegetation of metalliferous wastes and land after metal mining. In: *Hester RE, Harrison RM, editors. Mining and its environmental impact. London: Royal Society of Chemistry p. 31–47.*

Jones, R., Lapp, T., Wallace, D., (1993) Locating and Estimating Air Emissions from Sources of Cadmium and Cadmium Compounds, *Prepared by Midwest Research Institute for the U.S. Environmental Protection Agency, Office of Air and Radiation, Report EPA-453/R-93-040, September 1993.*

Jordahl, J.L., Foster, L., Schnoor, J.L., Alvarez, P.J.J., (1997) Effect of hybrid poplar trees on microbial populations important to hazardous waste bioremediation. *Environmental Toxicology and Chemistry 16, 1318–1321.*

Joshi, M.K., Mohanty, P., (2004) Chlorophyll *a* fluorescence as a probe of heavy metal ion toxicity in plants. In: *Papageorgiou, G.C., Govindjee (Eds.), Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Advances in photosynthesis and respiration vol 19. Springer, Dordrecht, The Netherland, pp.637-661.*

Jung, E., Heller, M., Sanchez, J.C., Hochstrasser, D., (2000) Proteomics meets cell biology: The establishment of subcellular proteomes. *Electrophoresis, Volume 21, Issue 16, pages 3369–3377.*

Kabata-Pendias A., Pendias H. (1989) Trace elements in the soil and plants. *1st eds. Florida CRC Press.*

Kahle, H., (1993) Response of roots of trees to heavy metals. *Environmental and Experimental Botany 33, 99–119.*

Kamnev, A.A., Lelie, Daniel van der, (2000) Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. *Bioscience Report 20 (4), 239.*

Keltjens, W.G., van Beusichem, M.L., (1998) Phytochelatins as biomarkers for heavy metal stress in maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.): combined effects of copper and cadmium. *Plant and Soil, 203, 119-126.*

Kessler, A., Brand, M.D., (1995) The mechanism of the stimulation of state 4 respiration by cadmium in potato tuber (*Solanum tuberosum*) mitochondria. *Plant Physiology and Biochemistry* 33, 519–528.

Kinnersley A.M. (1993) The role of phytochelates in plant growth and productivity. *Plant growth regulation* 12:207 – 217

Klose, J. (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutation in mammals. *Humangenetik* 26, 231–243.

Knasmuller, S., Gottmann, E., Steinkellner, H., Fomin, A., Pickl, C., Paschke, A., (1998) Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays. *Mutation Research*, 420, 37–48.

Kobayashi H., Quoreshi A.M., Uemora S., Koike T. (2005) Differences in growth characteristics and dynamics of elements absorbed in seedlings of three spruce species raised on serpentine soil in northern Japan. *Ann Bot* 95: 661-672

Kowalewska, G., Hoffmann, S.K., (1989) Identification of the copper porphyrin complex formed in cultures of blue-green alga *Anabaena variabilis*. *Acta Physiologia Plantarum* 11,39-50.

Kramer U., Cotter-Howells J.D., Charnock J.M., Baker A.J.M., Smith J.A.C. (1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature* 379: 635 – 638.

Krämer U, Talke IN, Hanikenne M (2007). Transition metal transport. *FEBS Lett.* 581:2263-2272.

Krause, G.H., Weis, E., (1991) Chlorophyll fluorescence and photosynthesis: The basics. *Annual Review of Plant Physiology and Plant Molecular Biology* 42, 313-349.

Kruckeberg A.R. (2002) The influence of lithology on plant life. In: Geology and Plant Life: the effects of landforms and rock type on plants. *Seattle/London: Univ. Wash. Press.*: 160 – 181

Krupa, Z., (1988) Cadmium-induced changes in the composition and structure of the lightharvesting complex II in radish cotyledons. *Physiologia Plantarum* 73, 518–524.

Krupa, Z., (1999) Cadmium against higher plant photosynthesis - a variety of effects and where do they possibly come from? *Zeitschrift für Naturforschung* 54c, 723-729.

Krupa, Z., Baranowska, M., Orzol, D., (1996) Can anthocyanins be considered as heavy metal stress indicator in higher plants? *Acta Physiologia Plantarum* 18, 147-151.

Krupa, Z., Baszynski, T., (1995) Some aspects of heavy metal toxicity towards photosynthetic apparatus-direct and indirect effects on light and dark reactions. *Acta Physiologia Plantarum* 17, 177-190.

Krupa, Z., Oquist, G., Huner, N.P.A., (1992) The influence of cadmium on primary photosystem-II photochemistry in bean as revealed by chlorophyll a fluorescence – A preliminary study. *Acta Physiologia Plantarum* 14, 71-76.

Krupa, Z., Oquist, G., Huner, N.P.A., (1993) The effects of cadmium on photosynthesis of *Phaseolus vulgaris* - a fluorescence analysis. *Physiologia Plantarum* 88, 626-630.

Kumar, P.B.A.N., Dushenkov, V., Motto, H., Rasakin, I., (1995) Phytoextraction: the use of plants to remove heavy metals from soils. *Environmental Science and Technology* 29, 1232–38.

Küpper, H., Kroneck, P.M.H., (2005) Heavy metal uptake by plants and cyanobacteria. In: *Sigel A, Sigel H, Sigel RKO, eds. Metal ions in biological systems*. New York, NY, USA: Marcel Dekker Inc, 97–142.

Küpper, H., Küpper, F., Spiller, M., (1998) In situ detection of heavy metal substituted chlorophylls in water plants. *Photosynthesis Research* 58, 123-133.

Kuzminsky, E., Sabatti, M., Scarascia Mugnozza, G.E., (1999) Growth and physiology of different poplar genotypes under saline stress. In: *Proceedings of IIInd international poplar symposium, vol 1, Orléans, France*, pp 55.

Kuzovkina, Y. A., Knee, M., Quigley, M.F., (2004) Cadmium and copper uptake and translocation in five willow (*Salix* L.) species. *International Journal of Phytoremediation* 6, 269-287.

Kuzovkina, Y. A., Quigley, M.F., (2005) Willows beyond wetlands: uses of *Salix* L. species for environmental projects. *Water, Air, and Soil Pollution*, 162, 183–204.

Labrecque, M., Teodorescu, T.I., Daigle, S., (1995) Effect of wastewater sludge on growth and heavy metal bioaccumulation of two *Salix* species. *Plant Soil* 171, 303–16.

Lambert J-P., Ethier M., Smith J.C., Figeys D. (2005) Proteomics: from gel based to gel free. *Analytical Chemistry*, Jun 15;77(12):3771-87.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Landberg, T., Greger, M., (1996) Differences in uptake and tolerance to heavy metals in *Salix* from unpolluted and polluted areas. *Applied Geochemistry* 11, 175–180.

Landberg, T., Greger, M., (2004) No phytochelatin (PC2 and PC3) detected in *Salix viminalis*. *Physiologia Plantarum* 121, 481–487.

Larsson, E.H., Bornman, J.F., Asp, H., (1998) Influence of UV-B radiation and Cd²⁺ on chlorophyll fluorescence, growth and nutrient content in *Brassica napus*. *Journal of Experimental Botany* 49, 1031–1039.

Lasat M.M. (2002) Phytoextraction of Toxic Metals: a review of Biological Mechanisms *Journal of Environmental Quality* 31, 109–120.

Lasat MM, Baker A, Kochian LV (1998) Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiology* 118: 875-883

Lasat M.M., Pence N.S., Garvin D.F., Ebbs S.D., Kochian L.V. (2000) Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* 342: 71 – 79.

Laureysens I., Blust R., De Temmerman L., Lemmens C., Ceulemans R., (2004) Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture: I. Seasonal variation in leaf, wood and bark concentrations. *Environmental Pollution*, 131, 485–494.

Laureysens, I., Bogaert, J., Blust, R., Ceulemans, R., (2004) Biomass production of 17 poplar clones in a short-rotation coppice culture on a waste disposal site and its relation to soil characteristics. *Forest Ecology and Management*, 187, 295–309.

Laureysens, I., Deraedt, W., Indeherberge, T., Ceulemans, R., (2003) Population dynamics in a six-year old coppice culture of poplar. I. Clonal differences in stool mortality, shoot dynamics and shoot diameter distribution in relation to biomass production. *Biomass and Bioenergy* 24, 81–95.

Ledin, S., Willebrand, E., (1996) Handbook on How to Grow Short Rotation Forests. Department of Short Rotation Forestry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Lepp, N.W., (1996) Uptake, mobility and loci of concentrations of heavy metals in trees. In: Glimmerveen, I. (Ed.), *Heavy metals and trees. Proceedings of a Discussion Meeting, Glasgow. Institute of Chartered Foresters, Edinburgh*, pp. 68–82.

Li Y.J., Dankher, O.P., Carreira, L., Smith, A.P., Meagher, R.B., (2006) The shoot-specific expression of gamma-glutamylcysteine synthetase directs the long-distance transport of thiol-peptides to roots conferring tolerance to mercury and arsenic. *Plant Physiology* 141, 288–298.

Lichtenthaler, H.K., Miehé, J.A., (1997) Fluorescence imaging as a diagnostic tool for plant stress. *Trends in Plant Science* 2, 316–320.

Linger, P., Ostwald, A., Haensler, J., (2005) *Cannabis sativa* L. growing on heavy metal contaminated soil: growth, cadmium uptake and photosynthesis. *Biologia Plantarum* 49, 567-576.

Liu Xingmei, Wu Jianjun, Xu Jianming (2006) Characterizing the risk assessment of heavy metals and sampling uncertainty analysis in paddy field by geostatistics and GIS. *Environmental Pollution Volume 141, Issue 2, May 2006, Pages 257-264.*

Liphadzi, M.S., Kirkham, M.B., (2006) Chelate-assisted heavy metal removal by sunflower to improve soil with sludge. *Journal of Crop Improvement 16, 153–172.*

Lloyd-Thomas D.H. (1995) Heavy metal hyperaccumulation by *Thlaspi caerulescens*. *J. & C. Presl. PhD Thesis, University of Sheffileld, UK*

Lombi E., Zhao F.J., Dunham S.J., McGrath S.P. (2000) Cadmium accumulation in population of *Thlaspi caerulescens* and *Thlaspi goesingense*. *New Phytolgist 145: 11 – 20.*

Lone Mohammad Iqbal, He Zhen-li, Stoffella Peter J., Yang Xiao-e (2008) Phytoremediation of heavy metal polluted soils and water: Progresses and perspectives. *Journal of Zhejiang University - Science B Volume 9, Number 3, 210-220.*

Lösch, R., Köhl, K.I., (1999) Plant respiration under the influence of heavy metals. In: *Prasad, M.N.V., Hagemeyer, J. (Eds.), Heavy Metal Stress in Plants. Springer, Heidelberg, pp. 139-156.*

Lozano-Rodríguez, E., Hernandez, L.E., Bonay, P., Carpena-Ruiz, R.O., (1997) Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. *Journal of Experimental Botany 306, 123–128.*

Lugon-Moulin, N., Zhang, M., Gadani, F., Rossi, L., Koller, D., Krauss, M., Wagner, G.J., (2004) Critical review of the science and options for reducing cadmium in tobacco (*Nicotiana tabacum* L.) and other plants. *Advances in Agronomy 83, 111–180.*

Lunáčková, L., Masarovičová, E., Králová, K., Streško, V., (2003) Response of fast growing woody plants from family Salicaceae to cadmium treatment. *Bulletin of Environmental Contamination and Toxicology 70, 576-585.*

Lunáčková, L., Šottníková, A., Masarovičová, E., Lux, A., Streško, V., (2003/4) Comparison of cadmium effect on willow and poplar in response to different cultivation conditions. *Biologia Plantarum 47, 403-411.*

Lux, A., Šottníková, A., Opatrná, J., Greger, M., (2004) Differences in structure of adventitious roots in *Salix* clones with contrasting characteristics of cadmium accumulation and sensitivity. *Physiologia Plantarum, 120, 537-545.*

Macnair M. R. (2003) The hyperaccumulation of metals by plants. *Advances in Botanical Research* 40: 63 - 105.

Macnair M.R., Bert V., Huitson S.B., Saumitou-Laprade P., Petit D. (1999) Zinc tollerance and hyperaccumulation are genetically independent characters. *Proceeding of the Royal Society of London, Series B* 266: 2175 - 2179.

Macpherson, G., (1995) Home-grown Energy from Short-rotation Coppice. *Farming Press Books, Ipswich, UK.*

Maestri E., Marmiroli M., Visioli G., Marmiroli N. (2010) Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment. *Environmental and Experimental Botany* 68, 1–13

Maestri Elena, Marmiroli Nelson (2011) Transgenic Plants for Phytoremediation. *International Journal of Phytoremediation Volume 13, Supplement 1, 2011 Special Issue: 6th International Phytotechnologies Conference St. Louis, MO, 2009*

Marmiroli M., Gonnelli C., Maestri E., Gabbrielli R., Marmiroli N. (2004) Localisation of nickel and mineral nutrients Ca, K, Fe, Mg with scanning electron microscopy microanalysis in tissues of the nickel-hyperaccumulator *Alyssum bertolonii* Desv. and the non-accumulator *Alyssum montanum* L. *Plant Biosystems*, 138:231-243.

Maksymiec, W., Baszynski, T., (1999) The role of Ca^{2+} ions in modulating changes in bean plants by an excess of Cu^{2+} ions. Chlorophyll fluorescence measurements. *Physiologia Plantarum* 105, 62-568.

Maksymiec, W., Bednara, J., Baszynski, T., (1995) Responses of runner bean plants to excess copper as a function of plant growth stages: Effects on morphology and structure of primary leaves and their chloroplast ultrastructure. *Photosynthetica* 31, 427-435.

Marchiol, L., Sacco, P., Assolati, S., Zerbi, G. (2004) Reclamation of polluted soil: phytoremediation potential of crop-related *Brassica* species. *Water, Air, and Soil Pollution*, 158, 345-356.

Mari S, Gendre D, Pianelli K, Ouerdane L, Lobinski R, Briat JF, Lebrun M, Czernic P (2006). Root-to-shoot long-distance circulation of nicotianamine and nicotianamine-nickel chelates in the metal hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* 57: 4111-4122.

Marmiroli Marta, Pietrini Fabrizio, Maestri Elena, Zacchini Massimo, Marmiroli Nelson, Massacci Angelo (2011) Growth, physiological and molecular traits in Salicaceae trees investigated for phytoremediation of heavy metals and organics. *Tree Physiology*.

Marmiroli Marta, Visioli Giovanna, Maestri Elena, Marmiroli Nelson (2011) Correlating SNP Genotype with the Phenotypic Response to Exposure to Cadmium in *Populus spp.* *Environmental Science & Technology* 45, 4497–4505.

Marschner, H., (1995) Mineral Nutrition of Higher Plants. *Academic Press. Harcourt Brace and Company Publishers, London.*

Martens S. N., Boyd R. S. (1994) The ecological significance of nickel hyperaccumulation - a plant chemical defense. *Oecologia* 98: 379 – 384.

Mäser Pascal, Thomine Sébastien, Schroeder Julian I., Ward John M., Hirschi Kendal, Sze Heven, Talke Ina N., Amtmann Anna, Maathuis Frans J.M., Sanders Dale, Harper Jeff F., Tchieu Jason, Gribkov Michael, Persans Michael W., Salt David E., Kim Sun A, Guerinot Mary Lou (2001) Phylogenetic Relationships within Cation Transporter Families of Arabidopsis. *Plant Physiology*, vol. 126 no. 4 1646-1667.

Mattina, M.J.I., Lannucci-Berger, W., Musante, C., White, J.C., (2003) Concurrent plant uptake of heavy metals and persistent organic pollutants from soil. *Environmental Pollution*, 124, 375-378.

Maxted, A.P., Black, C.R., West, H.M., Crout, N.M.J., McGrath, S.P., Young, S.D., (2007) Phytoextraction of cadmium and zinc by *Salix* from soil historically amended with sewage sludge. *Plant and Soil* 290, 157-172.

Maxwell, K., Johnson, G.N., (2000) Chlorophyll fluorescence: a practical guide. *Journal of Experimental Botany* 51, 659–668.

McEldowney, S., Hardman, D.J., & Waite, S., (1993) Treatment technologies. In *S. McEldowney, D.J. Hardman, & S. Waite (Eds.), Pollution, ecology and biotreatment* (pp.48–58). Singapore: Longman Singapore Publishers Pvt. Ltd.

McGrath S.P., (1987) Long-term studies of metal transfers following applications of sewage sludge. In: *Coughtrey, P.J., Martin, M.H. and Unsworth, M.H., (Eds) Pollutant Transport and Fate in Ecosystems. Special Publication No. 6 of the British Ecological Society* (pp 301–317). Blackwell Scientific, Oxford, UK

McGrath SP, Zhao FJ, Lombi E (2001) Plant and rhizosphere process involved in phytoremediation of metal-contaminated soils. *Plant Soil* 232: 207-214.

McLaughlin, M.J., Bell, M.J., Wright, G.C., Cozens, G.D., (2000) Uptake and partitioning of cadmium by cultivars of peanut (*Arachis hypogaea* L.). *Plant Soil* 222, 51–58.

Meharg, A.A., (1994) Integrated tolerance mechanisms: Constitutive and adaptive plants responses to elevated metal concentrations in the environment. *Plant Cell Environment* 17, 989-993.

Memon A. R., Aktoprakligil D., Ozdemir A., Vertii A.(2001) Heavy metal accumulation and detoxification mechanisms in Plants. *Turkish Journal of Botany* 25:111 – 121.

Meyer, S., Genty, B., (1998) Mapping intercellular CO₂ mole fraction (Ci) on *Rosa rubiginosa* leaves fed with abscisic acid by using chlorophyll fluorescence imaging. *Plant Physiology* 116, 947–957.

Meyer, S., Genty, B., (1999) Heterogeneous inhibition of photosynthesis over the leaf surface of *Rosa rubiginosa* L. during water stress and abscisic acid treatment: induction of a metabolic component by limitation of CO₂ diffusion. *Planta* 210, 126–131.

Miller S.P. & Cumming J.R. (2000) Effects of serpentine soil factors on Virginia pine (*Pinus virginiana*). *Tree Physiology* 20: 1129 – 1135

Mills, T.M., Robinson, B.H., Green, S., Clothier, B., Fung, L.E., Hurst, S., (2000) Difference in Cd uptake and distribution within poplar and willow species. (In: *Proceedings of the 42nd Annual Conference and Expo of the New Zealand Water and Waste Association, Rotorua, New Zealand*).

Minguzzi C., Vergnano O. (1948) Il contenuto in Nichel nelle ceneri di *Alyssum bertolonii*. *Desv. Mem Soc. Tosc. Sci Nat Ser A* 55: 49 – 77

Molas J. (2002) Changes of chloroplast ultrastructure and total chlorophyll concentration in cabbage leaves caused by excess of organic Ni (II) complexes. *Environmental and Experimental Botany* 47: 115 – 126

Molas, J., (1997) Changes in morphological and anatomical structure of cabbage (*Brassica oleracea* L) outer leaves and in ultrastructure of their chloroplasts caused by an in vitro excess of nickel. *Photosynthetica* 34: 513-522.

Mott, K.A., (1995) Effects of patchy stomatal closure on gas exchange measurements following abscisic acid treatment. *Plant Cell Environment* 18, 1291–1300.

Müller, P., Li, X.P., Niyogi, K.K., (2001) Non-photochemical quenching: a response to excess light energy. *Plant Physiology* 125, 1558–1566.

Mullins, C.E., (1991) Physical properties of soils in urban areas. In: *Bullock P, Gregory PJ, editors. Soils in the urban environment. Oxford: Blackwell p. 87– 118.*

Naidu R.; Olivier D.; McConnell S. (2003). Heavy metal phytotoxicity in soils. In: *Langley A., Gilbey M. and Kennedy B eds., Proceeding of the fifth national workshop on the assessment of site contamination National Environment Protection Council service corporation Adelaide S. A. 1 – 10*
NJ DEP. (1996) Soil cleanup criteria. Proposed cleanup standards for contaminated sites, 1st eds, New Jersey department of environmental protection. NJ, NJAC 7: 26D

Newman, L.A., Strand, S.E., Choe, N., Duffy, J., Ekuan, G., (1997) Uptake and biotransformation of trichloroethylene by hybrid poplars. *Environmental Science and Technology* 31, 1062–1067.

Newsholme, C., (1992) Willows: the genus *Salix*. *London: Batsford*.

Nriagu JO (1979). Global inventory of natural and anthropogenic emissions of trace metals to the atmosphere. *Nature* 279:409-411.

Oberlunder, R., Roth, V., (1978) In: Ross, Sheila M. (Ed.), *Toxic Metals in Soil–Plant Systems*. John Wiley and Sons, New York.

Okushima, Y., Koizumi, N., Kusano, T., Sano, H., (2000) Secreted proteins of tobacco cultured BY2 cells: identification of a new member of pathogenesis-related proteins. *Plant Molecular Biology Volume 42, Number 3*, 479-488.

Omasa, K., Saji, H., Youssefian, S., Kondo, N., (2002) Air Pollution and Plant Biotechnology. *Prospects for Phytomonitoring and Phytoremediation*. pp. 155–178, 287–359. Springer, Tokyo.

Osmond, C.B., Daley, P.F., Badger, M.R., Lüttge, U., (1998) Chlorophyll fluorescence quenching during photosynthetic induction in leaves of *Abutilon striatum* Dicks. Infected with Abutilon mosaic virus, observed with a field-portable imaging system. *Botanica Acta* 111, 390–397.

Oxborough, K., Baker, N.R., (1997) An instrument capable of imaging chlorophyll a fluorescence from intact leaves at very low irradiance and at cellular and subcellular levels. *Plant, Cell and Environment* 20, 1473–1483.

Padmavathiamma, P.K., Li, L.Y., (2007) Phytoremediation technology: hyper-accumulation metals in plants. *Water, Air, and Soil Pollution*, 184, 105-126.

Page, A.L., Bingham, F.T., Chang, A.C., (1982) Cadmium. In: Lepp, N.W. (Ed.), *Effect of Trace Metals on Plant Function. In Effect of Heavy Metal Pollution on Plants*, vol. 1. Applied Science Publishers, Englewood, NJ, pp. 77–109.

Pandey, A. and Mann, M. (2000) Proteomics to study genes and genomes. *Nature* 405, 837-846.

Park Ohkmae K. (2003) Proteomic Studies in Plants. *Journal of Biochemistry and Molecular Biology*, Vol. 37, No. 1, January 2004, pp. 133-138

Patterson, S. D. and Aebersold, R. H. (2003) Proteomics: the first decade and beyond. *Nat. Genet. Suppl.* 33, 311-323.

Pence N.S., Larsen P.B., Ebbs S.D., Letham D.L.D., Lasat M.M., Garvin D.F., Eide D. and Kochian L.V. (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *PNAS* 97: 4956 – 4960

Pennington, S.R., Wilkins, M.R., Hochstrasser, D.F., Dunn, M.J. (1997) Proteome analysis: From protein characterization to biological function. *Trends in Cell Biology* 7, 168–173.

Perfus-Barbeoch L., Leonhardt N., Vavasseur A., Forestier C. (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *Plant Journal* 32, 539-548.

Perttu, K.L., (1995) Ecological, biological balances and conservation. *Biomass and Bioenergy* 9, 107–116.

Perttu, K.L., (1999) Environmental and hygienic aspects of willow coppice in Sweden. *Biomass and Bioenergy*, 16, 291-297.

Phizicky, E., Bastiaens, P. I., Zhu, H., Snyder, M. and Fields, S. (2003) Protein analysis on a proteomic scale. *Nature* 422, 208-215.

Pichorner, H., Korori, S.A.A., Thur, A., Ebermann, R., (1993) The two and the four electron transfer to molecular oxygenmediated by plant peroxidase in the presence of thiols. In: *Welinder, K.G., Rasmussen, S.K., Penel, C., Greppin, H.* (Eds.), *Plant Peroxidases: Biochemistry and Physiology*. University of Geneva, pp. 131–136.

Pietrini, F., Iannelli, M.A., Montanari, R., Bianconi, D., Massacci. A., (2005) Cadmium interaction with thiols and photosynthesis in higher plants. In: *Hemantaranjan, A.* (Ed.), *Advances in Plant Physiology*. Scientific Publishers (India), Jodhpur, pp. 313-326.

Pietrini, F., Iannelli, M.A., Pasqualini, S., Massacci, A., (2003) Interaction of cadmium with glutathione and photosynthesis in developing leaves and chloroplasts of *Phragmites australis* (Cav.) Trin. ex strudel. *Plant Physiology* 133, 829-837.

Pilipović, A., Nikolić, N., Orlović, S., Petrović, N., Krstić, B., (2005) Cadmium phytoextraction potential of poplar clones (*Populus* spp.). *Zeitschrift für Naturforschung* 60c, 247-251.

Pilon-Smits E (2005) Phytoremediation. *Annual Review of Plant Biology* 56:15-39.

Pirondini A. (2007) Genomic and proteomic analysis of natural variation for metal hyperaccumulation in model plants and its relevance for phytoremediation. *PhD Thesis, University of Parma, Italy*

Pirondini A., Visioli G., Malcevschi A., Marmiroli N. (2006) A 2-D liquid-phase chromatography for protomi analysis in plant tissue. *Journal of Chromatography B* 833: 91 – 100.

Plaza S, Tearall KL, Zhao FJ, Buchner P, McGrath SP, Hawkesford MJ (2007). Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* 58: 1717-1728.

Pollard A.J. (2000) Metal hyperaccumulation: a model system for coevolutionary studies. *New Phytologist* 146: 179 -181

Pollard A.J., Baker A.J.M. (1997) Deterrence of herbivory by zinc hyperaccumulation in *Thlaspi caerulescens* (Brassicaceae). *New Phytologist* 135: 655 - 658.

Pollard A.J., Dandridge K.L., Jhee E.M. (2000) Ecological genetics and evolution of trace element hyperaccumulation in plants. In: Terry N. Bañuelos G.S. eds *Phytoremediation of Contaminated Soil and Water USA*, FL-Boca-Raton: CRC Press 251 – 264

Pollard A.J., Powell K.D., Harper F.A., Smith J.A.C. (2002) The genetic basis of metal hyperaccumulation in plants. *Critical Reviews in Plant Sciences* 21:539 – 566.

Polle A, Schutzendubel A (2003) Heavy metal signalling in plants: linking cellular and organismic responses. In: Hirt H, Shinozaki K, eds. *Plant Responses to Abiotic Stress*. Springer-Verlag, Berlin-Heidelberg. pp. 187–215.

Pomponi M, Censi V, Di Girolamo V, De Paolis A, Sanità di Toppi L, Aromolo R, Costantino P, Cardarelli M (2006). Overexpression of *Arabidopsis* phytochelatins synthase in tobacco plants enhances Cd²⁺ tolerance and accumulation but not translocation to the shoot. *Planta* 223: 180-190.

Prasad, M.N. (2003) Phytoremediation of Metal Polluted Ecosystems: Hype for Commercialization. *Russian Journal of Plant Physiology* 50, 764–780.

Prasad, M.N.V., Hagemeyer, J. (1999.) Heavy metal stress in plants: from molecules to ecosystems. Berlin, Germany: Springer.

Pulford, I.D. (1991) Nutrient provision and cycling in soils in urban areas. In: Bullock P, Gregory PJ, editors. *Soils in the urban environment*. Oxford: Blackwell p. 119– 38.

Pulford, I.D., Riddell-Black, D., Stewart, C. (2002) Heavy metal uptake by willow clones from sewage sludge-treated soil: the potential for phytoremediation. *International Journal Phytoremediation* 4, 59–72.

Pulford, I.D., Watson, C. (2003) Phytoremediation of heavy metal-contaminated land by trees - a review. *Environment International* 29, 529-540.

Pulford, I.D., Watson, C., McGregor, S.D. (2001) Uptake of chromium by trees: prospects for phytoremediation. *Environmental Geochemistry Health* 23, 307– 11.

Punshon, T., Dickinson, N.M. (1997) Mobilisation of heavy metals using short rotation coppice. *Aspects of Applied Biology* 49, 285–292.

Punshon, T., Dickinson, N.M. (1999) Heavy metal resistance and accumulation characteristics in willows. *International Journal of Phytoremediation* 1, 361 – 385.

Punshon, T., Dickinson, N.M., Lepp, N.W. (1996) The potential of *Salix* clones for bioremediating metal polluted soil. In: Glimmerveen I, editor. *Heavy metals and trees. Proceedings of a Discussion Meeting, Glasgow*. Edinburgh: Institute of Chartered Foresters p. 93–104.

Rachwal, L., De Temmerman, L.O., Istan, J.R. (1992) Differences in the accumulation of heavy metals in poplar clones of various susceptibility to air pollution. *Arboretum Kórnickie* 37, 101–111.

Ragnarsdottir K. V., Hawkins D. (2005) Trace metals in soils and their relationship with scrapie occurrence. *Geochimica et Cosmochimica Acta Supplement*, Vol. 69, Issue 10, Supplement 1, Goldschmidt Conference Abstracts 2005., p.A196.

Ralph, P.J., Burchett, M.D. (1998) Photosynthetic response of *Halophila ovalis* to heavy metal stress. *Environmental Pollution* 103, 91-101.

Raskin I., Smith R.D., Salt D.E. (1997) Phytoremediation of metals: using of plants to remove pollutants from environment. *Current Opinion in Biotechnology* 8(2): 221 – 226.

Raskin, I., Ensley, B.D. (2000) Phytoremediation of toxic metals using plants to clean up the environment. New York: Wiley; 2000. p. 316.

Raskin, I., Kumar, P.B.A.N., Dushenkov, S., Salt, D.E., (1994) Bioconcentration of heavy metals by plants. *Current Opinion in Biotechnology* 5, 285–290.

Raskin, I., Smith, R.D., Salt, D.E., (1997) Phytoremediation of metals: Using plants to remove pollutants from the environment. *Current Opinion in Biotechnology*, 2, 221-226.

Rauser W.E. (1999) Structure and function of metal chelators produced by plants: the case for organic acids. Amino acids, phytin, and metallothioneins. *Cell Biochemistry and Biophysics* 31, 19-48.

Rauser, W.E., Muwly, P., (1995) Retention of cadmium in roots of maize seedlings. Role of complexation by phytochelatins and related thiol peptides. *Plant Physiology*, 109, 195-202.

Rea P.A.; Li Z.S., Lu Y.P., Drozdowicz Y.M., Martinoia E. (1998) From vacuolar GS-X pumps to multispecific ABC transporters. *Annual Review of Plant Biology* 49: 727 – 760

Reese, R.N., White, C.A., Winge, D.R., (1992) Cadmium-sulfide crystallites in Cd-(gEC) nG peptide complexes from tomato. *Plant Physiology* 98, 225–229.

Reeves R.D., Baker A.J.M. (2000) Phytoremediation of toxic metals: using plants to clean up the environment. In: *Raskin I. & Ensley B.D., eds. Metal accumulating plants. New York* 193 – 229

Reeves R.D., Schwartz C., Morel J.L., Edmoundson J. (2001) Distribution and metal accumulating behaviour of *Thlaspi caerulescens* and associated metallophytes in France. *International Journal of Phytoremediation* 3: 1 – 28.

Reeves, R.D., Baker, A.J.M., Borhidi, A., Berazain, R., (1999) Nickel hyperaccumulation in the serpentine flora of Cuba. *Annals of Botany* 83, 29–38.

Reeves, R.D., Brooks, R.R., (1983) Hyperaccumulation of lead and zinc by two metallophytes from a mining area of Central Europe. *Environmental Pollution Series A*, 31, 277–287.

Riddell-Black D. (1994) Heavy metal uptake by fast growing willow species. In: *Aronsson P, Perttu K, editors. Willow vegetation filters for municipal wastewaters and sludges. A biological purification system. Uppsala: Swedish University of Agricultural Sciences* p.145– 51.

Riesen, O., Feller, U., (2005) Redistribution of nickel, cobalt, manganese, zinc and cadmium via the phloem in young and in maturing wheat. *Journal of Plant Nutrition* 28, 421–430.

Rigola D., Fiers M., Vurro E., Aarts M.G.M. (2006) The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses many species-specific genes, as identified by comparative expressed sequence tag analysis. *New phytologist* 170: 753 – 766.

Riley R.G., Zachara J.M., Wobber F.J. (1992) Chemical contaminants on DOE lands and selection of contaminated mixtures for subsurface science research. *US-DOE Off, Energy Res Subsur Sci Prog, Washington DC*

Robinson, B.H., Mills, T.M., Green, S., Chancerel, B., Clothier, B., Fung, L.E., Hurst, S., McIvor, I., (2005) Trace element accumulation by poplars and willows used for stock fodder. *New Zeland Journal of Agriculture Research*, 48, 489-497.

Robinson, B.H., Mills, T.M., Petit, D., Fung, L.E., Green, S.R. and Clothier, B.E., (2000) Natural and induced cadmium-accumulation in poplar and willow: implications for phytoremediation. *Plant Soil* 227, 301–306.

Rockwood, D.L., Naidu, C.V., Carter, D.R., Rahmani, M., Spriggs, T.A., Lin, C., Alker, G.R., Isebrands, J.G., Segrest, S.A., (2004) Short-rotation woody crops and phytoremediation: Opportunities for agroforestry? *Agroforestry Systems*, 61, 51-63.

Rohácek, K., (2002) Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and natural relationships. *Photosynthetica* 40, 13–29.

Roosens N., Verbruggen N., Meerts P., Ximenez de Embun P., Smith J.A.C. (2003) Variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *Thlaspi caerulescens* from Western Europe. *Plant Cell & Environment* 26: 1657 – 1672

Roselli, W., Keller, C. and Boschi, K., (2003) Phytoextraction capacity of trees growing on a metal contaminated soil. *Plant Soil* 256, 265–272.

Rustichelli C. (2008) Green biotechnologies: from genomic to proteomic approaches. *PhD Thesis, University of Parma, Italy*

Sachs J. (1865) Handbuch der Experimental-Physiologie der Pflanzen. Handbuch der Physiologischen Botanik: 153 – 154.

Salin, M.L., (1988) Toxic oxygen species and protective systems of the chloroplasts. *Physiologia Plantarum* 72, 681–689.

Salt, D.E., Smith, R.D., Raskin, I., (1998) Phytoremediation. *Annual Review of Plant Physiology* 49, 643–68.

Sánchez Martín, M.J., Sánchez Camazano, M., Lorenzo, L.F., (2000) Cadmium and lead contents in suburban and urban soils from two medium-sized cities of Spain: Influence of traffic intensity. *Bulletin of Environmental Contamination and Toxicology* 64, 250–257.

Sandmann, G., Boger, P., (1980) Copper-mediated lipid peroxidation processes in photosynthetic membranes. *Plant Physiology* 66, 797-800.

Sanità di Toppi, L., Gabbrielli, R., (1999) Responses to cadmium in higher plants. *Environmental and Experimental Botany* 41, 105–130.

Sanità di Toppi, L., Lambardi, M., Pazzagli, L., Cappugi, G., Durante, M., Gabbrielli, R., (1998) Response to cadmium in carrot in vitro plants and cell suspension cultures. *Plant Science* 137, 119–129.

Scarascia-Mugnozza, G., Ceulemans, R., Heilman, P.E., Isebrands, J.G., Stettler, R.F., Hinckley, T.M., (1997) Production physiology and morphology of *Populus* species and their hybrids grown under short rotation. II. Biomass components and harvest index of hybrid and parental species clones. *Canadian Journal of Forest Research* 27, 285-294.

Schmidt U (2003). Enhancing phytoremediation: the effect of chemical soil manipulation on mobility, plant accumulation , and leaching of heavy metals. *Journal of Environmental Quality* 32:1939-1954.

Schnoor, J.L., (2002) Phytoremediation of Soil and Groundwater. *Technology Evaluation Report TE 02-01, Ground-Water Remediation Technologies Analysis Center: Pittsburgh, PA.*

Schnoor, J.L., Erickson, L.E., Pierzynski, G.M., Just, C., (1996) Metal soil pollution and vegetative remediation. *Great Plains/Rocky Mountain HSRC, USA.*

Schreiber, U., Bilger, W., (1987) Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. In: *Tenhunen, J.D., Catarino, F.M., Lange, O.L., Oechel, W.D., (Eds.), Plant Response to Stress. Springer, Heidelberg*, pp. 27-53.

Schreiber, U., Schliwa, U., Bilger, W., (1986) Continuous recording of photochemical and nonphotochemical quenching with a new type of modulation fluorometer. *Photosynthesis Research 10*, 51-62.

Schützendübel, A., Polle, A., (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by micorrhization. *Journal of Experimental Botany 53*, 1351–1365.

Schwartz C, Echevarria G, Morel JL (2003). Phytoextraction of cadmium with *Thlaspi caerulescens*. *Plant soil.* 249: 27-35.

Sennerby-Forsse, L., Ferm, A., Kauppi, A., (1992) Coppicing ability and sustainability. In: *Mitchell, C.P., Ford-Robertson, J.B., Hinckley, T., Sennerby-Forsse, L. (Eds.), Ecophysiology of Short Rotation Forest Crops. Elsevier Applied Science, Oxford*, pp. 146–184.

Seregin, I.V., Ivanov, V.B., (2001) Physiological aspects of cadmium and lead toxic effects on higher plants. *Russian Journal of Plant Physiology 48*, 523-544.

Sharma SS, Dietz KJ (2006). The significance of amino acids and amino acid derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany 57*: 711-726.

Shaw, B.P., Rout, N.P., (1998) Age-dependent responses of *Phaseolus aureus* Roxb to inorganic salts of mercury and cadmium. *Acta Physiologia Plantarum 20*, 85-90.

Sheoran, I.S., Singal, H.R., Singh, R., (1990) Effect of cadmium and nickel on photosynthesis and the enzymes of photosynthetic carbon reduction cycle in pigeonpea (*Cajanus cajan* L.). *Photosynthesis Research 23*, 345-351.

Sheoran, I.S., Singh, R., (1993) Effect of Heavy Metals on Photosynthesis in Higher Plants. In: *Abrol, Y.P., Mohanty, P., (Eds.), Photosynthesis: Photoreactions to Plant Productivity. New Delhi, Bombay: Oxford and IBH*, pp. 453–468.

Shukla, U.C., Singh, J., Joshi, P.C., Kakkar, P., (2003) Effect of bioaccumulation of cadmium on biomass productivity, essential trace elements, chlorophyll biosynthesis, and macromolecules of wheat seedlings. *Biological Trace Elements Research* 92, 257–273.

Siedlecka, A., Baszynsky, T., (1993) Inhibition of electron flow around photosystem I in chloroplasts of cadmium-treated maize plants is due to cadmium-induced iron deficiency. *Physiologia Plantarum* 87, 199–202.

Siedlecka, A., Krupa, Z., (1996) Interaction between cadmium and iron and its effects on photosynthetic capacity of primary leaves of *Phaseolus vulgaris*. *Plant Physiology and Biochemistry* 34, 833–841.

Siedlecka, A., Krupa, Z., Samuelsson, G., Öquist, G., Gardeström, P., (1997) Primary carbon metabolism in *Phaseolus vulgaris* plants under Cd/Fe interaction. *Plant Physiology and Biochemistry* 35, 951-957.

Singh, O.V., Labana, S., Pandey, G., Budhiraja, R., Jain, R.K., (2003) Phytoremediation: an overview of metallicion decontamination from soil. *Applied Microbiology and Biotechnology*, 61, 405–412.

Skorzynska, E., Baszynski, T., (1995) Photochemical activity of primary leaves in cadmium stressed *Phaseolus coccineus* depends on their growth stages. *Acta Societatis Botanicorum Poloniae* 64, 273-279.

Skorzynska, E., Baszynski, T., (1997) Differences in sensitivity of the photosynthetic apparatus in Cd-stressed runner bean plants in relation to their age. *Plant Science* 128, 11-21.

Skorzynska, E., Bednara, J., Baszynski, T., (1995) Some aspects of runner bean plant response to cadmium at different stages of the primary leaf growth. *Acta Societatis Botanicorum Poloniae* 64, 165-170

Skorzynska, E., Urbanik-Sypniewska, T., Russa, R., Baszynski, T., (1991) Galactolipase activity of chloroplasts in cadmium treated runner bean plants. *Journal of Plant Physiology* 138, 453-459.

Smeets, K., Ruytinx, J., Semanea, B., Van Belleghemc, F., Remansa, T., Van Sanden, S., Vangronsveld, J., Cuypers, A., (2008) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environmental and Experimental Botany* 63, 1–8.

Smith RAH, Bradshaw AD (1972) Stabilization of toxic mine wastes by the use of tolerant plant populations. *Trans. Inst. Min. Metall.*

Smolders, E., Degryse, F., (2002) Fate and effect of zincfrom tire debris in soil. *Environmental Science and Technology* 36, 3706–3710.

Somashekaraiah, B.V., Padmaja, K., Prasad, A.R.K., (1992) Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*): involvement of lipid peroxides in chlorophyll degradation. *Physiologia Plantarum* 85, 85–89.

Sommerville, A.H.C., (1992) Willows in the environment. *Proceedings of the Royal Society of Edinburgh Sect B* 98, 215–44.

Šottníková, A., Lunáčková, L., Masarovičová, E., Lux, A., Streško, V., (2003) Changes in the rooting and growth of willow and poplars induced by cadmium. *Biologia Plantarum* 46, 129–131.

Soukupová, J., Smatanova, S., Nedbal, L., Jegorov, A., (2003) Plant response to destruxins visualized by imaging of chlorophyll fluorescence. *Physiologia Plantarum* 118, 399–405.

Steffens, J.C., (1990) The heavy metal-binding peptides of plants. *Annual Review of Plant Physiology and Molecular Biology* 41, 553–575.

Stephan U.W., Schmidke I., Stephan V.W., Scholz G. (1996) The nicotinamine molecules is made-to-measure for complexation of metal micronutrients in plants. *Biometals* 9: 84 – 90

Stephens, W.E., Calder, A., (2005) Source and health implications of high toxic metal concentrations in illicit tobacco products. *Environmental Science and Technology* 39, 479–488.

Stiborova, M., (1988) Cd²⁺ ions affect the quaternary structure of ribulose-1,5-bisphosphate carboxylase from barley leaves. *Biochemie und Physiologie der Pflanzen* 183, 371-378.

Stiborova, M., Doubravova, M., Brezinova, A., Friedrich, A., (1986) Effect of heavy metal ions on growth and biochemical characteristics of photosynthesis of barley (*Hordeum vulgare* L.). *Photosynthetica* 20, 418-425.

Stiborova, M., Doubravova, M., Leblova, S. (1986) A comparative study of the effect of heavy metal ions on ribulose-1,5-biphosphate carboxylase and phosphoenolpyruvate carboxylase. *Biochemie und Physiologie der Pflanzen* 181, 373-379.

Stobart, A.K., Griffiths, W.T., Ameen-Bukhari, I., Sherwood, R.P., (1985) The effect of Cd2+ on biosynthesis of chlorophyll in leaves of barley. *Physiologia Plantarum* 63, 293-298.

Stoltz, E., (2004) Phytostabilisation: use of wet plants to treat mine tailings. *Doctoral thesis, Department of Botany, Stockholm University*.

Stomp, A.M., Han, K.H., Wilbert, S., Gordon, M.P. (1993) Genetic improvement of tree species for remediation of hazardous wastes. *In Vitro Cellular & Developmental Biology –Plant* 29, 227–32.

Strasdeit, H., Duhme, A.K., Kneer, R., Zenk, M.H., Hermes, C., Nolting, H.F., (1991) Evidence for discrete Cd (SCys) 4 units in cadmium phytochelatin complexes from EXAFS spectroscopy. *Journal of the Chemical Society. Chemical communications* 91, 1129–1130.

Sutherland, R.A., Day, J.P., Bussen, J.O., (2003) Lead concentrations, isotope ratios and source apportionment in road deposited sediments, Honolulu, Oahu, Hawaii. *Water, Air and Soil Pollution*, 142, 165–186.

Terashima, I., (1992) Anatomy of non-uniform leaf photosynthesis. *Photosynthesis Research* 31, 195–212.

Terry N., Banuelos G., eds. Pytoremediation of contaminated soils and water. *Boca Raton, FL, USA: CRC press LLC*: 171 -188

Thangavel, P., Subhuram, C.V., (2004) Phytoextraction – Role of hyper accumulators in metal contaminated soils. *Proceedings of the Indian National Science Academy. Part B*, 70(1), 109–130.

Thiry, Y., Goor, F., Riesen, T., (2002) The true distribution and accumulation of radiocaesium in stem of Scots pine (*Pinus sylvestris L.*). *Journal of Environmental Radioactivity* 58, 243–259.

Thomine S., Wang R., Ward J.M., Crawford N.M., Schroeder J.I. (2000). Cadmium and iron transport by members of a plant transporter gene family in *Arabidopsis* with omology to NRAMP genes. *PNAS* 97: 4991 – 4996

Tukendorf, A., Skorzynska, E., Baszynski, T., (1997) Homophytochelatin accumulation in Cd-treated runner bean plants is related to their age. *Plant Science* 129, 21-28.

Turer, D., Maynard, J.B., Sansalone, J. J., (2001) Heavy metal contamination in soils of urban highways: Comparison between runoff and soil concentrations at Cincinnati, Ohio. *Water, Air, and Soil Pollution* 132, 293–314.

Unterbrunner, R., Puschenreiter, M., Sommer, P., Wieshammer, G., Tlustos, P., Zupan, M., Wenzel, W.W., (2007) Heavy metal accumulation in tree growing on contaminated sites in Central Europe. *Environmental Pollution* 148, 107-114.

Ushijima T., Kaneko A., Yamamoto T. (2004) Magnesium excess - mediated chlorosis in leaves of cucumber grown on soils of granodiorite origin under a plastic house. *Journal of the Japanese Society for Horticultural Science* 73: 476 – 483

Utsunamyia, T., (1980) *Japanese patent application no. 55-72959.*

Van Assche, F., Clijsters, H., (1990) Effects of metals on enzyme activity in plants. *Plant Cell and Environment* 13, 195-206.

Vandecasteele, B., De Vos, B., Tack, F.M.G., (2002) Cadmium and zinc uptake by volunteer willow species and elder rooting in polluted dredged sediment disposal sites. *Science of the Total Environment* 299, 191-205.

Vandecasteele, B., Lauriks, R., De Vos, B., Tack, F.M.G., (2003) Cd and Zn concentration in hybrid poplar foliage and leaf beetles grown on polluted sediment-derived soils. *Environmental Monitoring and Assessment* 89, 263-283.

Van de Mortel JE, Schat H, Moerland PD, Ver Loren van Themaat E, van der Ent S, Blankestijn H, Ghandilyan A, Tsatsiani S, Aarts MG (2008). Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* 31: 301-324.

Vangronsveld, J., Clijsters, H., (1994) Toxic effects of metals. In: Farago M E (ed) *Plants and the chemical elements*, pp. 149-175. VCH Weinheim

Vangronsveld, J., van Assche, F., Clijsters, H., (1995) Reclamation of a bare industrial area contaminated by non-ferrous metals: in situ metal immobilization and revegetation. *Environmental Pollution* 87, 51–59.

Van Rossum F, Bonnin I, Fenart S, Pauwels M, Petit D, Saumitou-Laprade P (2004). Spatial genetic structure within a metallophilous population of *Arabidopsis halleri*, a clonal, self-incompatible and heavy-metal-tolerant species. *Molecular Ecology* 13: 2959-2967.

Vassilev A., Yordanov I., (1997) Reductive analysis of factors limiting growth of Cd-exposed plants: A review. *Bulgarian Journal of Plant Physiology* 23, 114–133.

Vassilev, A., Tsonev, T., Yordanov, I., (1998) Physiological response of barley plants (*Hordeum vulgare*) to cadmium contamination in soil during ontogenesis. *Environmental Pollution* 103, 287–293.

Viklander, M., (1998) Particle size distribution and metal content in street sediments. *Journal of Environmental Engineering* 124, 761–766.

Visioli G., Pirondini A., Malcevschi A., Marmiroli N. (2010) Comparison of Protein Variations in *Thlaspi Caerulescens* Populations from Metalliferous and Non-Metalliferous Soils. *International Journal of Phytoremediation Volume 12, Issue 8, 2010*

Visioli G., Marmiroli N. (2012) Proteomics of Plant Hyperaccumulators. *Metal Toxicity in Plants: Perception, Signaling and Remediation* 2012, 165-18.

Vögeli-Lange, R., Wagner, G.J., (1990) Subcellular localization of cadmium and cadmiumbinding peptides in tobacco leaves. Implications of transport function for cadmium-binding peptides. *Plant Physiology* 92, 1086–1093.

Vollenweider, P., Cosio, C., Gunthardt-Goerg, M.S., Keller, C., (2006) Localization and effects of cadmium in leaves of a tolerant (*Salix viminalis* L.). Part II. Microlocalization and cellular effects of cadmium. *Environmental and Experimental Botany*, 58, 25–40.

Von Caemmerer, S., Farquhar, G.D., (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153, 376–387.

Von Wieren N., Klair S., Bansal S., Briat J.F., Khodr H., Sihori T. (1999) Nicotinamine chelates both FeIII and FeII. Implication for metal transport in plants. *Plant Physiology* 119: 1107 – 1114

Wagner, G.J., (1993) Accumulation of cadmium in crop plants and its consequences to human health. *Advances in Agronomy* 51, 173–212.

Watson, C., Pulford, I.D., Riddell-Black, D., (1999) Heavy metal toxicity responses of two willow (*Salix*) varieties grown hydroponically: development of a tolerance screening test. *Environmental Geochemistry Health* 21, 359– 64.

Watson, C., Pulford, I.D., Riddell-Black, D., (2003) Screening of willow species for resistance to heavy metals: comparison of performance in a hydroponics system and field trials. *International Journal of Phytoremediation*, 5, 351-365.

Weissenhorn, I., Leyval, C., Berthelin, J., (1995) Bioavailability of heavy metals and abundance of arbuscular mycorrhiza in a soil polluted by atmosphere deposition from a smelter. *Biology and Fertility of Soils* 19, 22–28.

Wellburn, A.R., (1994) The spectral determination of Chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology* 144, 307-313.

Whittaker R.H. (1954) The ecology of serpentine soils: a symposium. *I. Introduction. Ecology*. 35: 258 – 259

WHO (1997) Health and environment in sustainable development. *Geneva: WHO*

Wilkins, D.A., (1978) The measurement of tolerance to edaphic factors by means of root growth. *New Phytologist*, 80, 623-633.

Wilkins, M.R., Pasquali, C., Appel, R.D., Ou, K., Golaz, O., Sanchez. J.-C., Yan, J.X., Gooley, A.A., Hughes, G., Humphrey-Smith, I., Williams, K.L., Hochstrasser, D.F. (1996) From

proteins to proteomes: Large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *BioTechnology* 14, 61–65.

Wu L. (1990) Colonisation and establishment of plants in contaminated sites. In: *Shaw, A.J. (Ed.), Heavy metal tolerance in plants: Evolutionary Aspects. CRC Press, Boca Raton*, pp. 269-284.

Yang X., Baligar V.C., Martens D.C., Clark R.B. (1996) Plant tolerance to nickel toxicity. Nickel effects on influx and transport of mineral in four plant species. *Journal of Plant Nutrition* 19 265 – 279

Yeung, A.T., Hsu, C.N., (2005) Electrokinetic remediation of cadmiumcontaminated clay. *Journal of Environmental Engineering* 131, 298–304.

Zarcinas, B.A., Ishak, C.F., McLaughlin, M.J., Cozens, G., (2004) Heavy metals in soils and crops in southeast Asia. *Peninsular Malaysia. Environmental Geochemistry and Health* 26, 343– 357.

Zayed, A., Gowthaman, S., Terry, N., (1998) Phytoaccumulation of trace elements by wetlands plants: I. Duckweed. *Journal of Environmental Quality*, 27, 715-721.

Zhao, F.J., Hamon, R.E., Lombi, E., McLaughlin, M.J., and McGrath, S.P. (2002) Characteristics of cadmium uptake in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* 53, 535-543.

Zhou W, Qiu B (2005). Effects of cadmium hyperaccumulation on physiological characteristics of *Sedum alfredii Hance (Crassulaceae)*. *Plant Science* 169: 737-745.

Zhu YL, Pilon-Smits EHA, Tarun SA, Weber SU, Jouanin L, Terry N (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ -glutamylcysteine synthetase. *Plant Physiology* 121: 1169-1177.

Zhuang, P., Yang, Q.W., Wang, H.B., Shu, W.S., (2007) Phytoextraction of heavy metals by eight plant species in the field. *Water, Air, and Soil Pollution* 184, 235-242.

CHAPTER 2

2 FUNCTIONAL ANALYSIS OF POPLAR (*Populus nigra* L. and *P. nigra x P. deltoids*) DURING ENVIRONMENTAL EXPOSURE TO METALS

2.1 Introduction

2.1.1 Poplar

Considering that forest trees are the dominant life form in many ecosystems and constitute approximately 25% of the volume of all industrial materials, the importance of the presence of these plants is fundamental. For this reason the economic and ecological importance of forest trees provide the impetus for developing model systems to study tree biology.

Many aspects of tree biology are common to all plants, and hence can be studied in very tractable model species such as *Arabidopsis thaliana* L. Heynh., but some unique facets of tree anatomy and physiology must be investigated in trees themselves. Despite the fact that other forest trees have been suggested as model systems, including *Salix* (willow), *Eucalyptus*, and *Pinus* (pine), *Populus* remain a widely accepted model in forest tree biology.

Populus is a member (like *Arabidopsis*) of the clade ‘eurosid I’ in the Angiosperm Phylogeny Group (APG) system (APG, 2003) and more precisely of the family *Salicaceae* in the order *Malpighiales*. Poplars are widely distributed across the Northern Hemisphere within a wide range of climates, from the tropics to latitudes almost reaching the Arctic Circle (Dickmann *et al.*, 2001).

Populus is a genus of trees which includes the cottonwoods, poplars, and aspens, all of which are sometimes termed poplars. Various classifications have been suggested. The species are organised in sections but their classification is not simple and never complete because the members of a section, chosen on morphological and reproductive characters, can hybridize with each other very easily (Rajora and Zsuffa, 1984; Cervera *et al.*, 2005).

For this reason the poplar research offers a rich source of variation in tree morphology, anatomy, physiology, phenology, and response to biotic and abiotic stress as well as prominent genetic polymorphisms in local populations. Poplars are dioecious, wind-pollinated, are capable of rapidly invading disturbed sites as the dynamic environment of riverine floodplains, where they form a key component of riparian forests (Braatne *et al.*, 1996). The ease with which most materials can be vegetatively propagated is one of poplar’s premier assets. All poplars, in fact, have the capacity to reproduce asexually, mostly by sprouting from the root collar of killed trees or from abscised or broken branches that become embedded in the soil. Rapid growth is the hallmark of poplars. As a consequence of the rapid juvenile growth of poplars, it is possible to measure short-term responses

to biotic (for example, disease) and abiotic (for example, drought, elevated CO₂, ozone) factors. The poplar normally became 40 m in height in less than 20 years. The large physical size of poplars, coupled with the well-understood movement of carbon assimilates and cotransported systemic wound signals between source and sink leaves (Davis *et al.*, 1991) is an advantage over small plant models such as *Arabidopsis thaliana* in studies involving systemic signal movement.

The *Populus* genus has several attributes that have led to its emergence as the model system for tree molecular biology (Bradshaw *et al.*, 2000; Wullschleger *et al.*, 2002; Bhalerao *et al.*, 2003):

- i)* modest genome size (the haploid genome size is similar to rice and 40 times smaller than pine tree), facilitating the gene isolation, sequencing and functional study
- ii)* rapid juvenile growth (3-10 months);
- iii)* easy vegetative propagation (preferred to the use of seeds that are small, with a limited lifespan and high heterozygosity ratio);
- iv)* simple regeneration;
- v)* high-throughput transformation;
- vi)* phylogenomic proximity to well-studied angiosperms such as *Arabidopsis thaliana*;
- vii)* high ratio of genetic to physical map distance important for gene identification (Bradshaw *et al.*, 2000);

In addition to their value for biotechnology, members of the genus *Populus* show a range of important ecological properties such as: *i)* posses a wide ecological diversity (ca. 30 species from desert riparian to subalpine areas) that underline an abundant genetic and adaptive variation (Eckenwalder 1996), *ii)* may live for a long time (25-30 years old), *iii)* can be utilized for the production of paper or energy thanks to their high biomass.

In terms of ecological benefits the genus *Populus* provides *i)* carbon sequestration, *ii)* nutrient cycling, *iii)* bioremediation and *iv)* biofiltration (Brunner *et al.*, 2004).

The weaknesses of poplar as a model system, instead, are: *i)* poplars are deciduous, *ii)* like willows, many poplars have very strong and invasive root systems, *iii)* poplar has a modest commercial importance, *iv)* poplar is grown commercially on a wide geographic scale but, from a corporate viewpoint, poplars are much less important than pines and other softwoods or eucalypts, *v)* most poplars will not flower earlier than when 4 years of age, and many will take twice that long; the long generation interval is an impediment to practical breeding and selection and the development of informative pedigrees, *vi)* poplars are dioecious, so self-pollinations cannot be done (with a very few hermaphroditic exceptions). Today, poplar is cultivated worldwide in plantations for pulp, paper, excelsior (packing material), engineered wood products (for example, oriented strand board), lumber and energy.

Plants from the *Populus* genus, have become the model tree species for biological research for molecular genetics, still information on poplar genetics is not abundant. The poplar genome consists of some 35000 genes distributed in 19 chromosomes ($2n= 38$) and more than 500 million base pairs (Mb). Up to now the complete sequencing of the genome of *Populus trichocarpa Nisqually 1* Torr. & A.Gray is the most exhaustive among all the genetics studies on these species, and allows new approaches to tree biology (Brunner *et al.*, 2004). Poplar has a small haploid genome (4 times larger than the genome of Arabidopsis and 40 times smaller than the genomes of conifers) that simplifies gene cloning, Southern blotting, and other standard molecular genetics techniques. Another advantage that make *Populus* an attractive target for map-based (positional) cloning of genes is the physical/genetic distance ratio (close to 200 kb/centiMorgan), almost identical to Arabidopsis (Bradshaw *et al.*, 2000). Unlike the pines and other gymnosperms, poplars diverged relatively recently from other angiosperms, such as Arabidopsis, which serve as models for integrating genetics into the study of plant biology.

For all these reasons, poplars have been proven effective in the phytoremediation of environmental pollution (Flathman and Lanza, 1998; Eapen and D'Souza, 2005; Bhalerao *et al.*, 2003), more specifically for phytoextraction, and as bioindicators for pollution in the atmosphere (Jepsen, 1994).

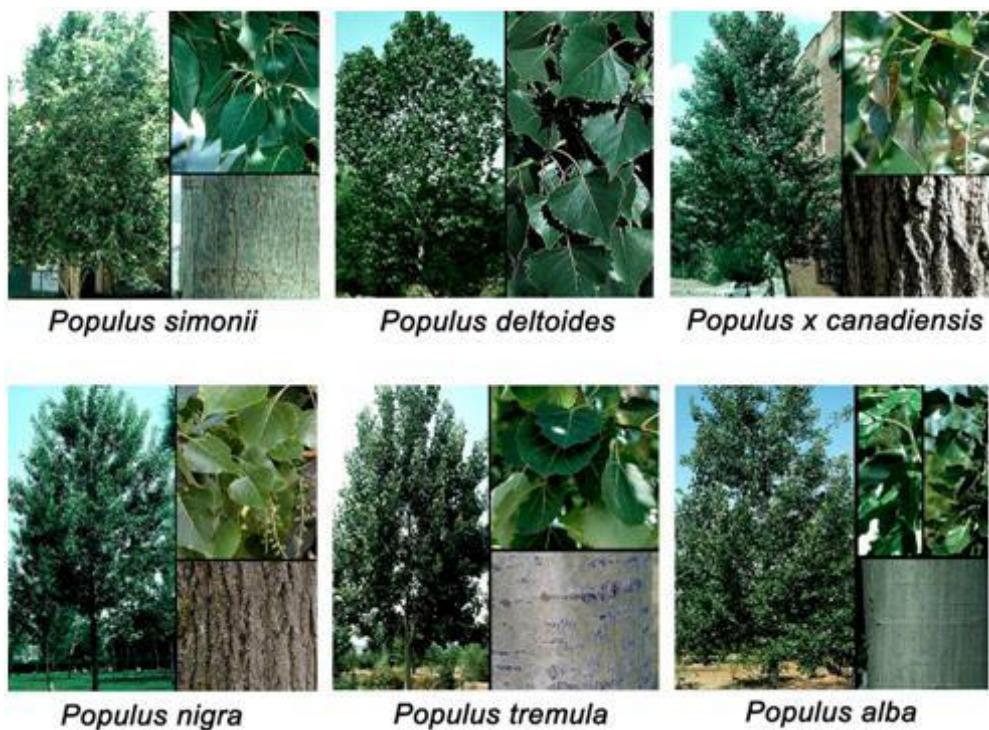


Fig. 11: Some examples of *Populus* species: *Populus simonii* Carrière, *Populus deltoides* W.Bartram ex Marshall, *Populus x canadiensis*, *Pupulus Nigra* L., *Populus tremula* L., *Populus alba* L.

2.1.2 Cadmium in the environment

The Cadmium element (Cd) is a silvery grey metallic element having the atomic number 48 and atomic weight 112.411g, the solid state is the standard state (at 298K). Cadmium is a soft and bluish-white metal. The name derives from the greek “Kademia” that indicate zinc oxides, in fact it is similar in many respects to zinc and is often found in minerals associated to zinc. Cadmium is a heavy metal naturally present in soil; it is non-essential and highly toxic to most organisms, having toxicity 2 to 20 times higher than many other heavy metals (Vassilev *et al.*, 1998). It is the fourth most toxic metal to vascular plants (Jones *et al.*, 1993; Oberlunder and Roth, 1978). It is placed in seventh position in the top ten priority hazardous substances list as provided by the American Agency for Toxic Substance and Disease Registry (Kamnev and Lelie, 2000), and therefore is considered a very serious pollutant. Cadmium is one of the metals under scrutiny by the U.S. Environmental Protection Agency (EPA) (Hogue, 2004), and contamination from it occurs in more than 8% of hazardous waste sites in the United States (Yeung and Hsu, 2005).

At low concentrations, it is toxic to microorganisms, plants and animals (Sanità di Toppi *et al.*, 1999; Benavides *et al.*, 2005). Total cadmium levels exceeding $8 \text{ mg} \cdot \text{kg}^{-1}$, or soluble (bioavailable) levels exceeding $0.001 \text{ mg} \cdot \text{kg}^{-1}$, are considered toxic to plants (Kabata-Pendius *et al.*, 1992; Bohn *et al.*, 1985).

In nature, Cd is present at concentrations of 0.1 – 0.5 mg/kg, but in contaminated sites, it may reach concentrations as high as 150 mg/kg. Since Greeks and Romans Cd sulphide based minerals have been used as pigments and also the brilliant red in Van-Gogh paintings is Cd sulphide. Cadmium is a widespread element in the environment, mainly from anthropogenic sources. More recently, anthropogenic pathways by which cadmium enters the environment are through industrial waste from processes such as electroplating, manufacturing of plastics, mining, paint pigments, alloy preparation, and batteries that contain cadmium (Adriano, 2001; Cordero *et al.*, 2004). Household appliances, automobiles and trucks, agricultural implements, airplane parts, industrial tools, hand tools, and fasteners of all kinds (e.g., nuts, bolts, screws, nails) are commonly cadmium coated. Cadmium is also used for luminescent dials, in photography, rubber curing, and as fungicides (Adriano, 2001). Tobacco concentrates cadmium, leading to human exposure to this carcinogenic metal through smoking (Lugon-Moulin *et al.*, 2004). Heavy metals enter soils through addition of sludge, composts, or fertilizers. Even with the strictest source control, domestic sewage sludge contains heavy metals because they are present in items washed down drains or toilets. Cadmium is given off from rubber when car tires run over streets, and after a rain, the cadmium is washed into sewage systems where it collects in the sludge. Composted sludge can contain high levels of cadmium. Phosphate fertilizers are contaminated with cadmium. People who smoke are exposed to

increased concentrations of cadmium. The most likely origin of the excess cadmium is from heavy applications of cheap, contaminated phosphate fertilizers (Booth, 2005; Stephens *et al.*, 2005).

In human, Cd intake is normally by ingestion or inhalation and the majority of ingested Cd coming from contaminated foods (meat and plants) and water (Dudka *et al.*, 1999); furthermore, the high solubility of Cd causes rapid distribution in the environment, where its high availability to plants can move up through the entire food chain (Schutzendubel *et al.*, 2002). Indeed, the primary risk pathway associated with cadmium contaminated soils has been identified as the soil–plant–human pathway and the consumption of crops or byproducts grown on these soils leads to its biomagnification in the food chain (Page *et al.*, 1982).

Cadmium accumulates in animals, especially in the kidney, liver, and reproductive organs. Sheep in New Zealand are allowed to graze only a short period of time on pasturelands that have elevated cadmium concentrations due to repeated applications of cadmium-rich superphosphate fertilizer (Granel *et al.*, 2002). The meat then has cadmium levels that are allowed in export. Elevated levels of cadmium in humans can cause kidney damage, and low levels of cadmium in the diet are linked to renal dysfunction. Other diseases associated with cadmium exposure are pulmonary emphysema and the notorious Itai–Itai (“ouch–ouch”) disease (Yeung *et al.*, 2005). It results in painful bone demineralization (osteoporosis), because cadmium replaces calcium in the bones. Cadmium poisoning has occurred worldwide.

2.1.3 Effects of cadmium toxicity in plants

Cadmium is considered a non-nutrient element, since it has no known function in plant development and life. In higher plants, Cd negatively affects both plant growth and development, resulting in stunting growth and eventually plant death. The critical tissue concentration, at which the metal injury, plant physiology, is in the range of 8 to 12 mg/kg dry mass (Balsberg *et al.*, 1989). The base of Cd toxicity is still not completely understood, but it might be due to its high affinity for sulphhydryl groups (Schützendübel *et al.*, 2002). Cd binds to sulphhydryl groups of structural proteins and enzymes and leads to protein misfolding, inhibition of enzyme activity and/or interference with the redox enzyme regulation (Hall, 2002; Meharg, 1994). Another toxicity mechanism is due to the chemical similarity between Cd²⁺ and functional ions situated in active sites of enzymes and signaling components. Consequently, Cd²⁺ ions can interfere with homeostatic pathways for essential metal ions (Roth *et al.*, 2006) and the displacing of divalent cations, such as Zn and Fe, from proteins could cause the release of “free” ions, which might trigger oxidative injuries (Polle and Schützendübel, 2002).

Moreover heavy metal ions induce specific indirect responses in plants which vary according to the concentration of metal ions, length of exposure and the developmental stage of the plants, external environmental conditions, and depend also on the plant species. For example, hyperaccumulators or tolerant plants may activate cellular mechanisms that weaken the impairment due to Cd stress. When relatively aged plants were treated with heavy metals, an increased damage was observed (Skorzynska and Baszynski, 1995). The threshold of metal ion toxicity seems to be specific to the metal, the plant and also the age at which it is applied. Even the specificity of heavy metal toxicity appears to be dependent on the growth stage of the plant. A root growth study in metal treated plants revealed that Hg was more toxic than Cd during germination, whereas Cd was more toxic during the seedling stage (Shaw and Rout, 1998).

Cadmium may enter plants via the root system. Several transporter families have been identified as playing a potential role in the uptake, among them members of the ZIP family of metal transporters (ZRT1/IRT1-like proteins) (Connolly *et al.*, 2002), calcium channels (Perfus-Barbeoch *et al.*, 2002) or members of the Nramp family (Maser *et al.*, 2001), which are normally responsible for the uptake of Zn²⁺, Fe²⁺, Ca²⁺ or Mn²⁺. Cadmium ions are taken up as “opportunistic hitchhikers” by ion transporter/channels with a low specificity (Clemens *et al.*, 2006).

Root tip damage is, together with decrease of root elongation rate, collapsing of root hairs or decrease of their number, decrease biomass, increase or decrease of lateral root formation, differentiation of numerous root hairs, one of the main morphological and structural effects caused by cadmium (Hagemeyer and Breckle, 1996). Inhibition of root extension growth can be the result

of interference with cell division or with cell elongation. However, the result of stress depends not only on its cause, but also on its intensity (Hagemeyer and Breckle, 1996). For example the dry mass production of roots was increased by low soil concentration of cadmium or zinc. But, at higher concentration of both metals, root growth was strongly inhibited (Hagemeyer et al. 1994).

The most evident symptoms of Cd toxicity are leaf crumple and chlorosis and stomatal closure (Clemens, 2006). Chlorosis is due to changes in Fe:Zn ratio caused by cadmium (Root *et al.*, 1975) and to the negative effects on chlorophyll metabolism (Chaffei *et al.*, 2004). In fact, Cd damages the photosynthetic apparatus, particularly the two photosystems and causes a decrease in chlorophyll and carotenoid content (Sanità di Toppi and Gabbielli, 1999). Reduction of chlorophyll content and of other pigments, such as carotenoids, is a common symptom of heavy metal toxicity (Molas, 1997; Baron *et al.*, 1995; Krupa and Baszynski, 1995). In fact, the extent of loss of pigments is demonstrated to be a simple and reliable indicator of heavy metal toxicity in higher plants (Krupa *et al.*, 1996). The decrease in chlorophyll content is contributed by both the inhibition of its biosynthesis and the induction of its degradation (Abdel-Basset *et al.*, 1995; Molas, 1997). Furthermore, cadmium can substitute Mg in the chlorophyll molecules, but lacks photochemical properties leading to the breakdown of the photosynthetic pathway (Kowalewska and Hoffmann, 1989; Kupper *et al.*, 1998; DalCorso *et al.*, 2008), and in a general disintegration of chloroplast membrane ultrastructure (Baszynski *et al.*, 1988; Krupa and Baszynski, 1995; Maksymiec *et al.*, 1995; Molas, 1997). The stomatal closure can be driven by *abscisic acid* (ABA) induced Ca^{2+} accumulation in the cytosol of the guard cells. The increase in cytosolic free Ca^{2+} promotes opening of plasma membrane anion and K^+ exit through channels, hence water follows ions and turgor is lost, with stomatal pore closure (MacRobbie and Kurup, 2007). Being chemically similar to Ca ions, Cd probably enters guard cells through Ca^{2+} channels and once in the cytosol, it mimics Ca^{2+} activity (Perfus-Barbeoch *et al.* 2002). The stomatal movements are not directly affected by cadmium, but rather are by the strong interference of cadmium with movements of K^+ , Ca^{2+} and abscisic acid in the guard cells (Barceló *et al.*, 1986). All together, stomatal closure, damages of photosynthetic machinery and interference with pigment synthesis, cause a downfall of the photosynthetic efficiency. Therefore cadmium induces inhibition of photosynthesis, as well by interfering with different step in the Calvin cycle, as by acting on photosystem II or by interfering with RuBisCO activation (Prasad *et al.*, 1995). Moreover, cadmium inhibits enzymes involved in CO_2 fixation, decreasing carbon assimilation (Perfus-Barbeoch *et al.* 2002). Cd exposure inhibits the activity of metabolic enzymes such as *glucose-6-phosphate dehydrogenase*, *glutamate dehydrogenase*, *malic enzyme*, *isocitrate dehydrogenase*, *Rubisco* and *carbonic anhydrase* (Sanità di Toppi and Gabbielli 1999). Cadmium effects on the *Rubisco* (ribulose bisphosphate carboxylase

oxygenase) may result from the substitution of metal ions at the Mg²⁺ site in the enzyme-CO₂ complex, or by reaction with the SH groups of the enzyme (Clijsters and van Assche, 1985; Stiborova *et al.* 1986). All different abiotic stresses lead to major alterations in carbohydrate metabolism (DalCorso *et al.*, 2008). In fact, sugar signaling pathways play an important role in plant growth and development during abiotic stress by regulating carbohydrate metabolism (Gupta *et al.*, 2005). An accumulation of various sugars, among them raffinose family oligosaccharides (RFOs) may also occur during abiotic stresses; it has been proposed that these molecules may play a role in osmotic adjustments or protection of cell (Taji *et al.*, 2002).

Cadmium, at the root level can interfere with the uptake and metabolism of other nutrients such as Ca, Mg, K and P (Benavides *et al.* 2005). For example, cadmium inhibits the *Fe(III) reductase* in roots and leads to a Fe(II) deficiency (Alcantara *et al.*, 1994). In different plant species Cd alters the activity of different enzymes involved in nitrogen metabolism (Nussbaum *et al.*, 1988; Boussama *et al.*, 1999). At the root level, the reduction of nitrate absorption may be due to the inhibition of the transpiration. Moreover, both the *nitrate reductase* and *nitrite reductase* activity in roots and leaves are affected (Chaffei *et al.*, 2004; Hernandez *et al.*, 1996) as well as nitrate transport from roots to shoots (Sanità di Toppi and Gabbrielli, 1999) leading to a reduced nitrate assimilation by the plant. Cadmium compromises the activity of the *glutamine* and *glutamate synthetase*, responsible for the incorporation of ammonium molecules into the carbon skeleton (Chaffei *et al.*, 2004). On the other hand, the activity of the *glutamate dehydrogenase (GDH)* is enhanced during Cd-stress (Boussama *et al.*, 1999). Because high activity of GDH enzyme has been related with pathogen response and senescence induction (Osugi and Madu 1996; Masclaux *et al.* 2000) it has been hypothesized that Cd induces senescence-like symptoms, leading to nitrogen mobilization and nitrogen storage mechanisms (Chaffei *et al.*, 2004). It was shown that Cd induces peroxisome-senescence in leaves activating the *glyoxylate cycle* enzymes, *malate synthase* and *isocitrate lyase*, as well as *peroxisomal peptidases*, the latter being well-known as leaf senescence-associated factors (Chaffei *et al.*, 2004; DalCorso *et al.*, 2008).

Regarding sulfur metabolism, exposure to Cd induces an increase in the amount of thiol compounds, because it causes a decrease in the activity of enzymes involved in the sulfate assimilation pathway (Astolfi *et al.*, 2004).

Furthermore, cadmium inhibits the oxidative mitochondrial phosphorylation, probably by increasing the passive permeability to H⁺ of the mitochondrial inner membrane (Kessler and Brand, 1995).

Also, cadmium was found to induce oxidative stress (Hendry *et al.*, 1992; Somashekaraiah *et al.*, 1992), but it does not seem to act directly on the production of oxygen reactive species (ROS)

(Salin, 1988). In fact cadmium acts indirectly and induces oxidative stress affecting the activity of several antioxidant metabolic systems, enzymatic (i.e., superoxide dismutase, catalase) and nonenzymatic (i.e., ascorbate, glutathione) and triggering H₂O₂ and O⁻² (reactive oxygen species, ROS) over-accumulation (Romero-Puertas et al. 2004). Oxidative stress and its effects are common responses in plants facing environmental constraints, like drought, heat and cold. It is still not clear if the over-production of ROS during Cd treatment is the cause of redox cellular imbalance or if this is a specific stress mechanism activated by the plant cell to cope with the heavy metal ions (Romero-Puertas et al., 2004; DalCorso et al., 2008).

A secondary effect due to the accumulation of ROS in the cell compartments is the alteration of the signaling mediated by H₂O₂ and other oxygen species that can play a role as signal molecule in triggering the induction of defense mechanisms against both biotic and abiotic stresses, such as temperature changes and drought (Sharma et al., 1996; Dat et al., 2000) and pathogen attack (Thordal-Christensen et al., 1997; Bestwick et al., 1998).

Furthermore, Cd interferes with plasma membrane ion transporters and ATPase (Sanità di Toppi and Gabbrielli, 1999) disturbing ions and metabolites movement and accumulation and leads to a lower utilization of ATP, NADPH, and to an higher thylakoid proton-gradient, resulting in a lower photochemical yield (Krupa et al., 1992; Krupa et al., 1993).

Cadmium treatment of plants can induce lipid peroxidation in photosynthetic membranes (Sandmann and Boger, 1980), hydrolysis of membrane lipids and release of free fatty acids (Skorzynska et al., 1991). Cadmium treatment increased lipid peroxidation in pea plants (Lozano-Rodríguez et al., 1997), whereas no peroxidation was noticed in cadmium-exposed plants and hairy roots of *Daucus carota* L. (Sanità di Toppi et al., 1998).

Cd²⁺ ions can hamper the activity of Zn-finger transcription factors, substituting Zn ions and consequently interfering with transcription mechanisms (Sanità di Toppi and Gabbrielli, 1999). Similarly, Cd²⁺ replace Ca²⁺ ions in calmodulin proteins, causing the perturbation of intracellular calcium level and altering the calcium dependent signaling (Ghelis et al. 2000; Perfus-Barbeoch et al. 2002).

Furthermore, in different plant species, cytotoxicity of Cd exposure appears as chromosomal aberrations and inhibition of mitotic processes with consequent altered cell cycle and division (Benavides et al., 2005).

2.1.4 Plants responses to Cd

Plants have evolved a complex network of mechanisms to minimize the damages from exposure to cadmium and other non essential metal ions. First, to avoid Cd toxicity, plants developed strategies of exclusion of the heavy metal ion. Plants can produce exudates such as malate or citrate that binds metal ions in the soil matrix excluding them from root absorption (Delhaize and Ryan, 1995). Second, the cell wall and extracellular carbohydrates (callose, mucilage) can play a significant role in immobilizing toxic ions and preventing their uptake into the cytosol (Sanità di Toppi and Gabbrielli, 1999).

Furthermore, plants can react to cadmium contamination producing chelating compounds (phytochelatins and/or metallothioneins) involved in the detoxification and compartmentalization of the toxic cadmium ions in specific cellular compartments, cytosol or vacuole (Cobbet *et al.*, 2000). Cadmium can induce the synthesis of small metal-binding peptides defined as phytochelatins (PCs). Phytochelatins, with a general structure of (γ -Glu-Cys) n -Gly ($n=2-11$) are able to chelate and sequester cadmium ions in the cytosol or vacuole (Sanità di Toppi *et al.*, 1999; Cobbett and Goldsbrough, 2002). Due to the presence of the thiolic groups of Cys, PCs chelate Cd and form several complexes with molecular weight of about 2500 or 3600 Da, protecting the cytosol from free Cd ions (Cobbett, 2000). Glutathione is necessary for PCs synthesis, which is catalyzed by the cytosolic *PCs synthetase* (PCS). It has been shown that PCS is constitutively expressed and post-translationally activated by heavy metals (Cobbett and Goldsbrough, 2002). After synthesis, PCs bind the heavy metal ions and transport them as complexes into the vacuole (Clemens 2006) where they eventually form high-molecular-weight (HMW) complexes. Several studies demonstrated that in *Arabidopsis thaliana* the transport of HMW complexes across the tonoplast is mediated by ATP-binding cassette (ABC) transporters (Cobbett and Goldsbrough, 2002).

The depletion of glutathione (GSH) by chelation to cadmium, (and incorporation into phytochelatins) is the most direct effect of cadmium on the oxidative balance in cells, and it has been proposed that this depletion of GSH is one of the main causes for oxidative stress and reactive oxygen species (ROS) generation induced by cadmium (Clemens *et al.*, 2006; Sanità di Toppi *et al.*, 1999). In fact, glutathione, together with ascorbate, takes part in the Halliwell–Asada pathway for detoxification of ROS (May *et al.*, 1998).

Metallothioneins (MTs) are cysteine-rich peptides, able to bind metal ions. MTs are products of mRNA translation, induced in response to heavy metal stress (Cobbett and Goldsbrough, 2002). MT proteins in vertebrates are characterized by a stretch of 20 Cys residues highly conserved (Cherian and Chan 1993; DalCorso *et al.*, 2008), MT genes are expressed in all tissues.

Under metal stress, an accumulation of proline has also been reported; it has been proposed that this accumulation is a consequence of a metal-induced water deficit (Schat *et al.*, 1997).

Cadmium produces indirectly the presence of ROS which trigger the biosynthesis of enzymes that protect the cell against oxidative stress such as *catalase* and *superoxide dismutase* (Schutzendubel *et al.*, 2001; Romero-Puertas *et al.*, 2004). *Catalase* (CAT) represents a key enzyme for the defense responses against oxidative stress. It is expressed in presence of cadmium contamination in peroxisomes and catalyzes the H₂O₂ breakdown (Buchanan *et al.*, 2000). Also *superoxide dismutase* (SOD) enzyme plays a role in protecting cells against ROS accumulation, SOD activity was induced after prolonged Cd treatment (Dong *et al.*, 2006; Lin *et al.*, 2007).

Moreover, as for other abiotic stresses, Cd resistance involves the synthesis of stress-related proteins and signal molecules (heat shock proteins, salicylic and abscisic acids, ethylene) (Sanità di Toppi and Gabbrielli, 1999).

2.1.5 Plant proteomics and cadmium

In recent years proteomics has become an essential tool to investigate plant physiological response to environmental stimuli (Rossignol, 2001; Chen and Harmon, 2006; Roberts, 2002; Heazlewood and Millar, 2003; Canovas *et al.*, 2004; Rose *et al.*, 2004; Rossignol *et al.*, 2006). An essential aspect of the plant proteomic analysis is the “comparative proteomics”, which is utilized mainly for the study of proteomic profiles of individuals under different treatments (Ingle *et al.*, 2005). However, so far the study of the proteomic variability has been restricted to several model species such as *Orzya sativa* and *Arabidopsis thaliana* (Chevalier *et al.*, 2004). Numerous proteomic studies have been carried out in plants facing abiotic stressing conditions, like cold and ozone exposure (Bohler *et al.*, 2007; Renaut *et al.*, 2006). Studies have been scarce in the field of heavy metal exposure; only a few recent findings on the effects of metal ions, and cadmium in particular, on the proteome of plants have been published (Aina *et al.*, 2007; Sarry *et al.*, 2006). Results of these analyses show the importance of transporter proteins in roots, the effect on primary metabolism, most noticeably the importance of the sulfur assimilation and metabolism in roots, as well as phytochelatin and glutathione synthesis (Aina *et al.*, 2007; Sarry *et al.*, 2006).

In rice plants treated with different cadmium concentrations, changes in root proteome showed the regulation of several transporter proteins such as Nramp1 (Aina *et al.*, 2007). In the same study, from a physiological point of view, cadmium induced senescence, since authors observed an upregulation of proteins involved in degradation of oxidatively modified proteins from the ubiquitin/proteasome pathway. Similarly, in *Arabidopsis thaliana* cell culture exposed to different

concentrations of Cd, carbon, nitrogen and sulfur metabolisms showed activation by the upregulation of several key proteins (Sarry *et al.*, 2006). Most notably, among the upregulated proteins, several enzymes were involved in the biosynthesis of glutamate, cysteine and glycine. These three molecules are precursors required for the formation of glutathione and phytochelatins, key components for the cellular detoxification of cadmium (Sarry *et al.*, 2006). In *Arabidopsis* roots treated with 10 µM Cd during 24 hours, an accumulation of phytochelatins could be observed (Roth *et al.*, 2006). Proteomic changes revealed by this study included an upregulation of ATP-sulfurylase, catalyzing the first step in sulfur assimilation by activation of sulfate. As phytochelatin and glutathione synthesis represent important sinks for sulfur, this upregulation correlates with the importance of the sulfur metabolism for the detoxification of Cd (Roth *et al.*, 2006).

In cadmium-exposed poplar plants (*Populus tremula* L.), during 14 days of exposure, changes in several metabolic pathways could be highlighted, most noticeably a repression of important reactive oxygen detoxifying proteins, as well as a deleterious effect on photosynthesis and depletion of carbon fixation (Kieffer *et al.*, 2008; Kieffer *et al.*, 2009).

In other poplar clones (*Populus tremula* L. x *Populus Alba* L.), during 60 days of cadmium exposure, Durand *et al.* (2010) showed that Cd toxicity principally impaired photosynthesis and primary metabolism. Consequently, primary growth was reduced. The resulting loss of photosynthates in all plant tissues, like cambium, partly accounts for the decreased activity of these tissues. Thus, cambial proteome changes resulted in part from a systemic toxicity, and in part from the cadmium present inside the tissue. Their proteomics data showed contrasted responses to Cd between leaf and cambial zone (Durand *et al.*, 2010).

In recent study, cuttings of *Populus Nigra* L. were grown hydroponically and treated with 50 µM CdSO₄ for three weeks. Proteomic analysis was performed using protein separation by 2D liquid chromatography and showed twenty proteins differently abundant in treated poplar plants. These proteins were related to different functional classes: uptake, proteolysis, stress related proteins, metabolism, regulatory protein and glutathione pathway (Visioli *et al.*, 2010).

2.1.6 Objectives

Poplar clones, due to their fast growth, genetic and physiological characteristics, are natural candidates for the remediation of contaminated soils. The characterisation of several *Salicaceae* clones for the effectiveness to tolerate and bioconcentrate metals could be interesting in specifying the potentiality of these plants to remediate polluted soils.

In this work, firstly, three clones of the genus *Populus* (58-861, *Poli*, A4A) were investigated in relation to their response to contamination by cadmium, in order to understand and quantify the differences between the clones in terms of strength, cadmium tolerance, accumulation capacity and translocation of the contaminant to the aerial parts and tissue localization. The evaluation of phytoremediation potential of these clones has been performed combining all chemical, biochemical, physiological and growth parameters.

Furthermore, this work was undertaken to explore the potential of proteomics to investigate the response induced in the three different poplar clones by Cd treatments under uniform environmental conditions using hydroponic cultures. The proteomic profile obtained by the not treated and treated samples were compared using a two dimensional liquid chromatography technique (Pirondini *et al.*, 2006) to understand the molecular basis underlying the different responses.

Physiological and proteomic data obtained from the study of *P. nigra* and *P. nigra x deltoides* grown in hydroponics will be discussed. These fundamental aspects should form the principles to be followed in plant screening for phytoremediation selection, and the data obtained could be useful for improving Cd phytoremediation strategies through the application of this ecologically and economically important tree species.

2.2 Materials and Methods

2.2.1 Plant Material

Three clones of *Populus* spp. were chosen for this work: *Populus nigra* L. (clones **58-861** and **Poli**) and *P. euramericana*, hybrid *P. nigra* x *P. deltoides* (**A4A**), which showed different Cd tolerance and capacity and Cd uptake, accumulation and translocation (Zacchini *et al.*, 2009). Furthermore these clones were analyzed for there SNPs (single nucleotide polymorphisms) for the major genes involved in Cd tolerance and it was found that *Poli* and *58-861* were more closely related than the hybrid *A4A*, which diverged significatively from the first two clones. (Mermiroli *et al.*, 2010). These clones were chosen from the collection maintained by Institute of Agro-environmental and Forset Biology, National Research Council, Monterotondo Scalo, Rome, Italy. Cuttings of three poplar clones were obtained and were placed into pots with ideal soil (*VigorPlant Italia S.r.l.*, *Fombio, Lodi*).

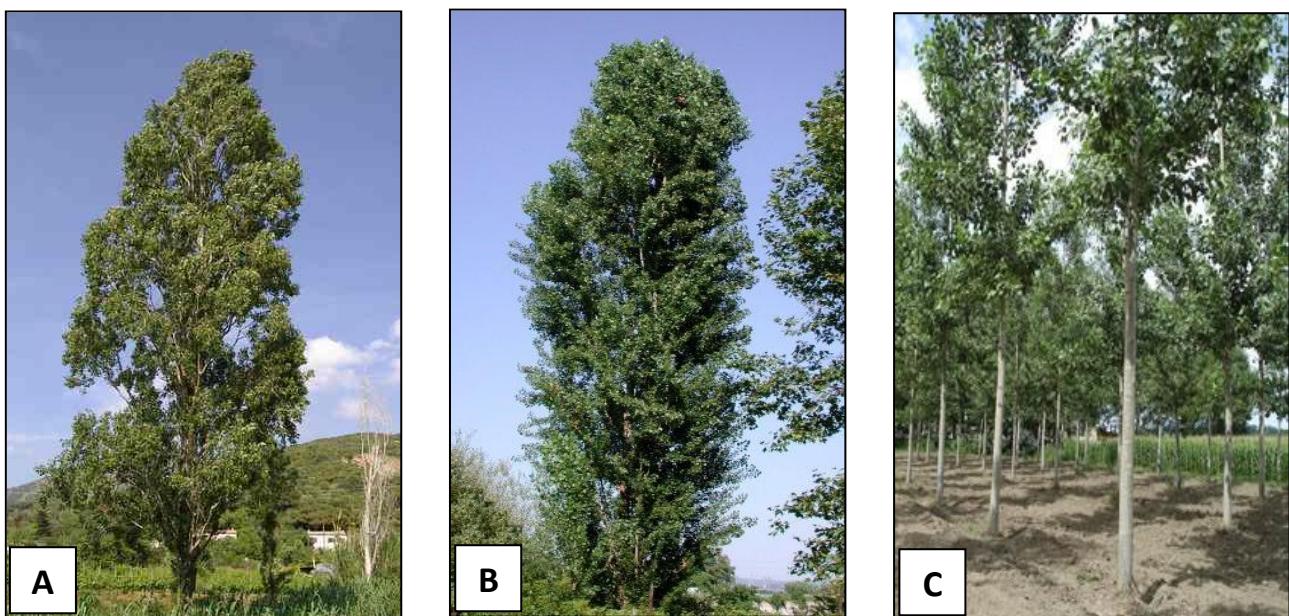


Fig. 12: Three selected Poplar clones: Poli (A) and 58-861 (B) are *Populus nigra* L., A4A (C) is *P.nigra* x *P.deltoides*.

2.2.2 Hydroponic culture and treatments

Cuttings of three poplar clones were grown, to allow the growth of the roots, in hydroponic conditions (Half strength Hoagland's solution) for 2 weeks in a controlled climate chamber equipped with metal halide lamps providing a photon flux density of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 14h at 25°C ; during the 10 h dark period the temperature was 22°C ; the relative humidity was 60-70%. The Half strength Hoagland's solution was composed by 3 mM KNO_3 , 2 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 0.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mM $\text{Fe}(\text{Na})\text{EDTA}$, 1 μM KCl , 25 μM H_3BO_3 , 2 μM

$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (*J.T. Baker, Deventer, Holland*), 2 mM MES (*Sigma-aldrich, St. Louis, MO, USA*), and adjusted to pH 5.5 with 1M KOH (*J.T. Baker, Deventer, Holland*).

The volume of the solution was controlled and kept at the same level adding fresh Half strength Hoagland's solution, to compensate the high transpiration rates of these plants. The pH also was kept constant.

Five rooted cuttings for each clones were treated with 0 μM CdSO_4 (control) and 20 μM CdSO_4 (*J.T. Baker, Deventer, Holland*) for 48 hours (short term treatment) and for 14 days (long term treatment). The nutrient solution were replaced every week to prevent depletion of metals and nutrients and to expose plants to a constant metal concentration.

An amount of 5g of leaves and 2g of roots of all the plants (treated and untreated) were sampled, frozen in liquid nitrogen and stored at -80°C before protein extractions. Pools of roots, in control and treated conditions, were harvested and washed with 0.05 M calcium chloride (*J.T. Baker, Deventer, Holland*) for 30 minutes in slow agitation, as a desorption step to remove only the metal adsorbed to the root surface.

2.2.3 Physiological measures and statistical analysis

Different morphophysiological parameters were analyzed: number of leaves, total leaf area and elongation of the main root. The variations of these physiological parameters were measured for all clones in all conditions, and differences between control and treated plant were analyzed in a Student's t-test for a statistical evaluation.

All one way ANOVA analyses were performed using "SPSS Statistics 17.0" and were considered significant at $p < 0.05$. Data were tested for homogeneity of variance (F-max) and comparison of means using the Tukey test.

2.2.4 Elemental analysis by Atomic Absorption Spectroscopy (AAS)

Pools of roots, stems and leaves of three clones, in control and treated conditions, were dried out for three days at 70° C ; 300 mg of each dried sample was digested in 10 ml of 69% [v/v] HNO_3 (*J.T. Baker, Deventer, Holland*) in 0.5 l glass cylinder at 120° C for 25 min and 250°C for 15min in a heating digestor (*DK20 Velp Scientifica S.r.l., Usmate MB, Italy*) and then 7 ml MilliQ water (*J.T. Baker, Deventer, Holland*) was added. The Cd concentration was determined using a flame atomic absorption spectrometer (AAS) (*Perkin Elmer 1100B, Waltham, Massachusetts USA*).

2.2.5 Scanning Electron Microscopy with microanalysis (SEM/EDX)

All the biological replicates were harvested, and utilised for elements quantification and localization through SEM/EDX. Leaves were removed from the stem at the nodal intersection, while stems and roots were cut into 1 mm thick transverse sections and then dried at room. The dried sections were positioned on a microscope slide and covered with colloidal graphite to allow electrons conductivity. The material was prepared following methods described elsewhere (Marmiroli M. *et al*, 2004). Three types of analyses were performed on each sample: morphological analyses, elements quantification, and elements mapping, using a Jeol 6400 Scanning electron microscope (*Jeol, Osaka, Japan*) with an Oxford X-ray detector (*Oxford Instruments, Oxford, UK*), supported by INCA software. The operating parameters were set as follows: working distance: 14 mm; electron beam energy: 20KeV; dead time in X-ray data acquisition: between 15% and 25%. For elemental mapping resolution was standardized by the program and the number of frames set at 3000 (acquisition time of 2 hours for each map). The magnification varied from 100 times up to 700 times for specific tissues. Linescan analysis where performed in order to identify the pattern of elemental distribution and to highlight elements following the same pattern. The samples analysed where from the short term period experiments because the material from the long term experiments where too damaged to perform the sectioning with the scalpel.

2.2.6 Protein Samples Preparation

Crude protein extracts of leaves of three poplar clones were obtained by using MgSO₄-based extraction buffer (Pirondini *et al.*, 2006). A total amounts of 3,5 g of frozen leaves from each plant and treatment were finely ground in liquid nitrogen with a mortar and pestle with addition of sand of SiO₂ (*Sigma-aldrich, St. Louis, MO, USA*), to favour breakage of the cell walls. The fine powder was resuspended in 50 mM Tris [tris (hydroxymethyl) aminomethane] HCl pH 7.8, 10 mM MgSO₄, 0.1 % [v/v] β mercaptoethanol and 0.1 % [v/v] Protease Inhibitor Cocktail (*Sigma-aldrich, St. Louis, MO, USA*). The crude mixture was sonicated for 10 minutes at 35kHz (*Transsonic T460, Elma*) and then the solution was placed in ice for 40 minutes. After another step of sonication, the sample was centrifuged in a precooled rotor spin at 16000×g for 5 min at 4°C. The pellet, containing the larger cellular residues and SiO₂, was discarded; the supernatant was finally centrifuged at 16.000 x g for 30 min at 4 °C. The upper phase was stored at -20° C for the following steps. Leaf samples from each treatments were extracted in triplicate for crude total proteins mixtures.

2.2.7 Protein Quantification

Protein quantification was performed by using Quick Start Bradford Protein Assay (*BioRad, Hercules, CA, USA*). The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) (Compton and Jones, 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Absorbance max at 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (Absorbance max at 595 nm) (Reisner *et al.* 1975, Fazekes de St. Groth *et al.* 1963, Sedmack and Grossberg, 1977). It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer.

Each samples was analyzed with a UV-visible spectrophotometer (*Uvikon 931, Kontron Instruments*) at 595 nm with different dilutions of BSA (Bovine Serum Albumin) (*Sigma-aldrich, St. Louis, MO, USA*), from a standard of 2 mg/ml, in the range of 100 – 2.000 µg/ml. The BSA dilutions and sample dilutions were prepared in the suitable buffer (Start buffer) for the next two dimensional liquid chromatography.

2.2.8 PD10 desalting column

The plant protein extracts were desalted and equilibrated to the column environment by PD-10 Desalting Workmade disposable columns (*GE-Healthcare Biosciences, Uppsala, Sweden*) containing prepacked Sephadex G-25 Medium with exclusion limit of 5000 Da. Column equilibration was performed by using approximately 25 ml of CF start buffer (*Beckman Coulter, Fullerton, CA, USA*) and the sample was then eluted with 3.5 ml of CF start buffer. The capacity of the system allows the loading of up to 2.5 ml of sample, with a range of loading capacity between 0.5 mg and 5 mg of protein sample.

2.2.9 Two dimensional liquid chromatography "Proteome Lab PF2D"

3 mg of each total protein extract were separated by two dimensional liquid chromatography (2-D LC). The separation was performed by using ProteomeLab™ PF 2D instrument commercialized by Beckman Coulter (*Fullerton, CA, USA*). HPCF-1D column (250 × 2.1 mm internal diameter, 300 Å pore size) and HPRP C18 column (4.66 mm length × 3.3 mm internal diameter, 1.5 µm particle size) were commercialized by Beckman Coulter (*Fullerton, CA, USA*). Proteins were separated in the first dimension by high-performance chromatofocusing (HPCF), performed on an HPCF-1D

column. With this technique, proteins bonded to a strong anion exchanger followed by elution with a continuously decreasing pH (8.5 to 4.0) gradient. The pH gradient was generated on the column by two buffers: Start Buffer (SB) (*Beckman Coulter, Fullerton, CA, USA*) and Eluent Buffer (EB) (*Beckman Coulter, Fullerton, CA, USA*). The calibration of both buffers was an important step: SB and EB were sonicated for 5 minutes and then their pH was adjusted to 8.5 and 4.0 respectively using either a saturated solution (50 mg/ml) of iminodiacetic acidic (*Sigma-aldrich, St. Louis, MO, USA*) if the buffer was too basic or 1M NH₄OH (*J.T. Baker, Deventer, Holland*) if the buffer was too acidic. The column was first equilibrated to the initial pH 8.5 using CF Start buffer at a flow rate of 0.2 ml/min for 3 hours. After this step, 5 ml of sample were introduced with a manual injector into the column for the first dimension CF. Twenty minutes after sample injection, the first dimension pump switches to the CF Eluent buffer (pH 4) at a flow rate of 0.2 ml/min. The interaction of the column filling with the CF Eluent buffer produced a gradually decreasing pH gradient that travelled through the column as a retained front. The pH gradient affected the proteins net charge and their adsorption/desorption to the positively-charged matrix of the column, causing protein separation in the effluent. The pH of the mobile phase was monitored on-line by a post-detector pH flow cell. The proteins were eluted based on their isoelectric point (pI), measured for absorbance at 280 nm, and collected in a 96 deep-well plate by a fraction collector according to pre-determined pH decrements of 0.4 pH units during the gradient, or in 1 ml volumes when the pH did not change. At the 115th min the most acidic proteins were recovered by washing the column with 1M NaCl 30% n-propanol [v/v] for 15 min. The column was finally washed with water for 45 min, therefore the CF separation took of total of approximately 185 min.

The eluent from the 1st dimension was injected into the 2nd dimension, a high-performance reversed-phase chromatography (HPRP). The 2nd dimension separation was based on protein hydrophobicity. HPRP was carried out in a C18 column. The mobile phase consisted of A: 0.1 % TFA (Trifluoroacetic Acid) (*J.T. Baker, Deventer, Holland*) in water and B: 0.08 % TFA in Acetonitrile (*J.T. Baker, Deventer, Holland*). Separation was performed at 0.75 ml/min with an increasing gradient of B. During the first 2 min 100 % of solvent A was pumped into the column; in the next 35 min the gradient was created in the column by switching the flow from 0 % to 100 % solvent B; this is followed by 100 % B for 4 min and 100 % A for 9 min. In order to obtain a better resolution, the separation was done at 50° C. The eluent from the second dimension was monitored by a second high performance UV/VIS detector at 214 nm, that provided a more universal and sensitive detection of proteins via peptide bonds. Fractions were immediately collected in Eppendorf tubes for MS analysis by using an automated fraction collector.

Either after the 1st dimension and 2nd dimension, software generated a graph (chromatogram) that shows the absorbance (280nm or 214nm) versus time; the height of each peak in the chromatogram is proportional to the concentration of that specific protein.

2.2.10 MALDI-TOF Analysis

Mass Spectrometry analyses were performed by using MALDI-LR in TOF/MS mode instrument commercialised by Micromass Waters Corporation (*Milford, Massachusetts, USA*).

Eluted fractions were evaporated to a final individual volume of 10 μl , using a Speed Vac Concentrator 5301 (*Eppendorf AG, Barkhausenweg, Hamburg, Germany*). Protein digestion was performed by incubating each fraction in 25 mM NH₄HCO₃ and 2 mM DTT (DL-Dithiothreitol) in a water bath at 60°C for 1 hr. The alkylation of the reduced sulphhydryl groups was carried on by adding 1 mM Iodoacetamide, at 25°C, for 30 min in the dark, and then 10 μL of Trypsin (125 $\mu\text{g}/\text{mL}$) in 50 mM NH₄HCO₃ was added. Digestion was carried out putting the fractions at 37°C for 24 hours. The digested samples were then purified and concentrated with a ZipTipC18 using the procedure recommended by the manufacturer (Millipore Corporation, Billerica, MA, USA). Then 1 μL of each purified peptide was spotted directly onto a stainless steel MALDI target plate with 1 μL of a saturated solution of α -cyano-4-hydroxycinnamic acid in 0.1% TFA:ACN (2:1, v/v). The solution was allowed to dry at room temperature and a spot was produced. Positively charged ions were analyzed in reflectron mode. External calibration was performed by using ProteoMassTM Peptide & protein MALDI/MS calibration kit (*Sigma St. Louis, MO, USA*). In particular calibration peptide fragments were: angiotensin II (human) [m/z] 1,046,542 ACTH fragment 18-39 (Adrenocorticotropic Hormone human) [m/z] 2,465,198, Insulin oxidized B chain (bovine) [m/z] 3,494, 651. The spectra were obtained by random scanning of the sample surface with an ablation LASER. Typically 100 shots were averaged to improve the signal to-noise ratio. All spectra were analyzed using the MassLynx 4.0 software (*Micromass Waters Corporation, Milford, MA, USA*). Three technical replicates from each spectrum were analyzed by MS, and only peptides common to all of the resolved spectra were considered for protein identification.

2.2.11 Software and statistical analysis

ProteoVue software (*Eprogen, Darien, IL, USA*) was utilised to convert chromatographic intensities from the 2-D LC of each pH fraction into a band intensity format. This produced a highly detailed map with the dimensions of hydrophobicity and *pI*. The 2-D LC maps could be viewed in several coloured formats where the colour intensity was proportional to the relative intensity of each chromatographic peak.

DeltaVue software (*Eprogen, Darien, IL, USA*) was utilised for the differential analysis of corresponding fractions from two different sample sets. This software compared chromatogram peaks corresponding to the same protein in the two samples, allowing quantification by a subtraction analysis. A differential map was achieved by point-to-point subtraction and it is viewed between the two original sample sets.

Peptide mass fingerprinting analysis was carried out with the **Mascot** program (<http://www.matrixscience.com>). Proteins were identified by searching against Swiss-Prot database of Viridiplantae (Green Plants). The following parameters were used for database search: mass accuracy below 100 ppm, maximum of one missed cleavages by trypsin, carbamidomethylation of cysteine as fixed modifications, oxidation of methionine as variable modifications. The search was based on the monoisotopic masses of the peptides. For mass-spectrometry (MS) analyses, three technical replicates for each spectrum were performed. For proteins identification, only the peptides that were common to all the resolved spectra were considered. Subsequently the amino acid sequence of each identified protein was processed by tBLASTn search in poplar Expressed Sequence Tags (ESTs) database on PopulusDB (<http://www.populus.db.umu.se>).

Heat maps was carried out with the **TreeView** (software version 1.60, November 2002, Michael Eisen; *Copyright@ 1998-9 by Stanford University, Copyright@ 2000-2 by University of California*) The software evidences the comparison of proteins abundance between control and treated samples.

2.3 Results and Discussion

Poplar clones, due to their fast growth, genetic and physiological characteristics, are natural candidates for the remediation of contaminated soils. In this work, three clones of the genus *Populus* were investigated in relation to their response to contamination by cadmium in order to understand and quantify the differences between the clones in terms of strength, cadmium tolerance, accumulation capacity and translocation of the contaminant to the aerial parts, and in order to understand the molecular basis underlying the different responses. These fundamental aspects should form the principles to be followed in plant screening for phytoremediation selection.

2.3.1 Plant morphology and growth analysis

Metal tolerance, and the protection of the integrity and functionality of the physiological process is an essential property for a plant to be utilized for phytoremediation (Pietrini *et al.*, 2003).

Therefore, in this work, a first visual analysis was performed to evaluate possible signs of metal stress on the leaves surface of the treated plants. Previous studies have shown that on leaves surface of poplar clones treated with Cadmium it is possible to see, after seven days, the presence of chlorotic areas and small necrotic spots, typical pinpoint necrosis, near the main leaf vein. After an extend treatment period the chlorotic and necrotic areas increased in number and extension, and in some cases necrotic lesions appeared. (Kieffer *et al.*, 2008).

In our study, three clones did not show any particular sign on leaves surface after the short period treatment (48 hours); some leaves showed only a slight wilting. Instead, after the long period treatment (14 days), the clones 58-861 and *Poli* showed more severe symptoms on leaves surface, with larger chlorotic areas, necrotic areas near the leaf veins, and some necrotic lesions. These signs were quantified in percentage of leaves number that showed at least one lesion (Kieffer *et al.*, 2008).

Clones of the type *Poli*, after the long period treatment with 20 µM CdSO₄, showed chlorotic areas and small necrotic spots near the main leaf vein in 92% of leaves (Fig.13). Instead 75% of leaves of the clone 58-861 clones, after the long period treatment with 20 µM CdSO₄, showed large chlorotic and necrotic areas larger than those presented by *Poli* (Fig. 14). In plants of the clone A4A, only 6% of the leaves were affected by small chlorotic areas (Fig. 15).

Damage exerted by cadmium at leaf level is an important aspect to evaluate when screening for plants used in phytoremediation. In fact, an efficient photosynthetic apparatus allows plants to

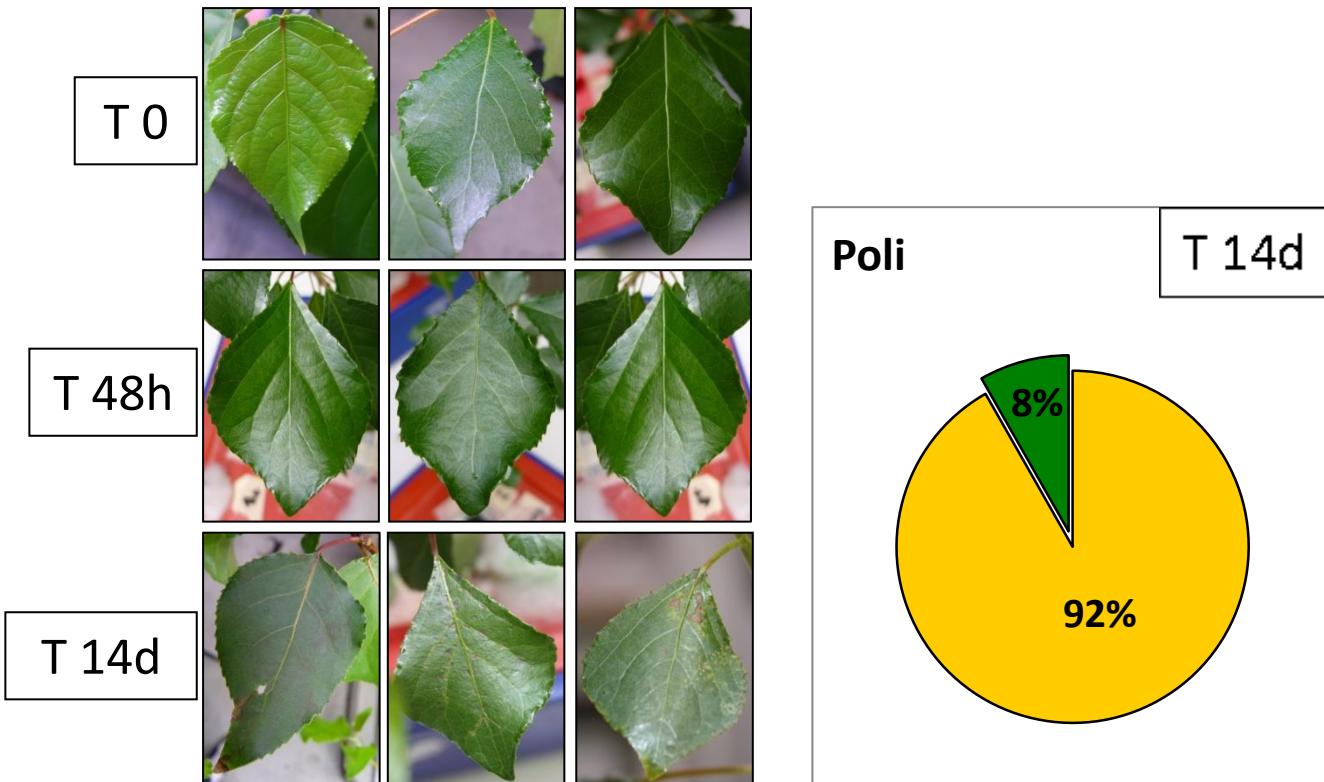


Fig. 13: Leaves of the clones Poli before treatment (T 0), after the short period treatment with 20 μM CdSO₄ (T 48h) and after the long period treatment with 20 μM CdSO₄ (T 14d). This clone showed chlorotic areas, small necrotic spots near the main leaf vein and some foliar injuries. 92% of leaves of treated plants showed at least one lesion after the long period treatment (yellow portion in the pie chart).

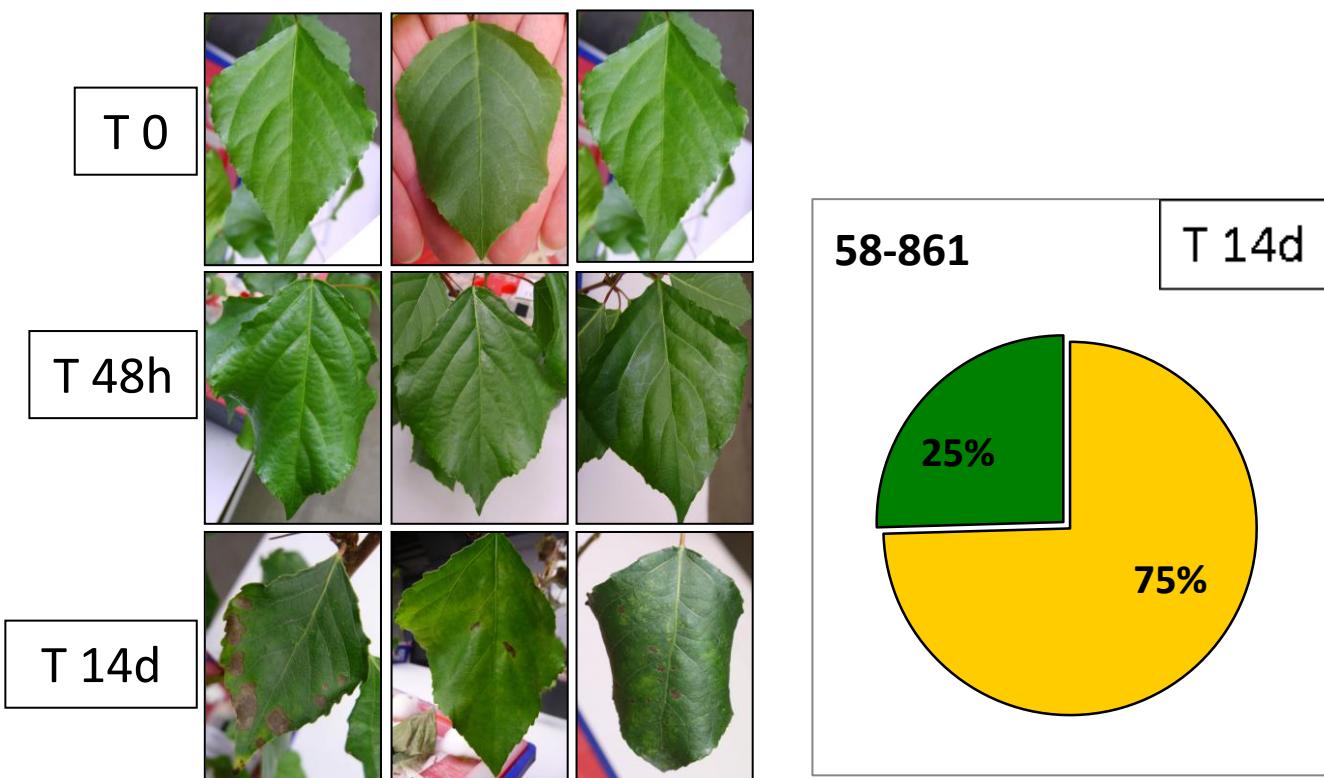


Fig. 14: Leaves of the clones 58-861 before treatment (T 0), after the short period treatment with 20 μM CdSO₄ (T 48h) and after the long period treatment with 20 μM CdSO₄ (T 14d). This clone showed chlorotic areas and large necrotic spots near the main leaf vein. 75% of leaves of treated plants showed at least one lesion after the long period treatment (yellow portion in the pie chart).

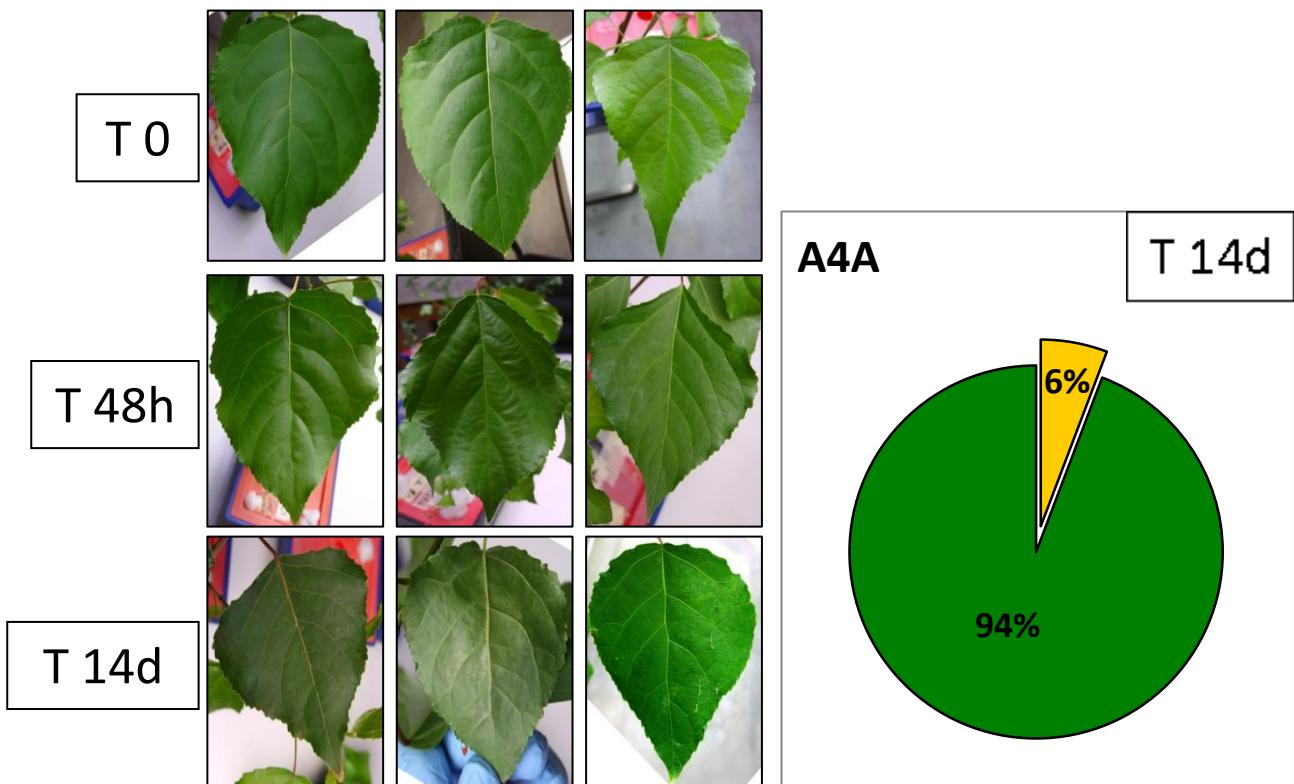


Fig. 15: Leaves of the clones A4A before treatment (T 0), after the short period treatment with 20 μM CdSO₄ (T 48h) and after the long period treatment with 20 μM CdSO₄ (T 14d). This clone did not show necrotic spots and only 6% of the leaves were affected by small chlorotic areas (yellow portion in the pie chart).

maintain an effective transpiration flux that drives metals from roots to aerial parts. The increase in leaves number and total leaf area are sensitive parameters for cadmium presence in the growth medium (Zacchini *et al.*, 2009). In leaves cadmium can represent a toxic agent, and it can destroy thylacoidal membranes and reduce enzyme activities, and depleting photosynthesis (Baceril *et al.*, 1988; Pietrini *et al.*, 2009). In the present work, the effect of cadmium on leaves of poplar clones was evaluated by measuring the total leaf area and the increase in leaves number per plant.

Figure 16 shows the increase of leaves number of three poplar clones subjected to 48 hours or 14 days of cadmium treatment, in comparison with the non treated samples, in a hydroponic experiment. Differences in the increase of these parameters between controls and treated plants were analyzed with the T-Student test for a statistical evaluation.

None of the poplar clones seem to be affect by cadmium presence after the short term treatment; but the long period treatment seem to inhibit the increase of leaves number. The clones *Poli* and 58-861 revealed a dramatic reduction in the increase of leaves number after the long term treatment.

Poli and 58-861 showed a reduction in the increase of total leaf area (Fig. 17), caused by cadmium exposure, already after the short term treatment, and a dramatic reduction after the long term treatment.

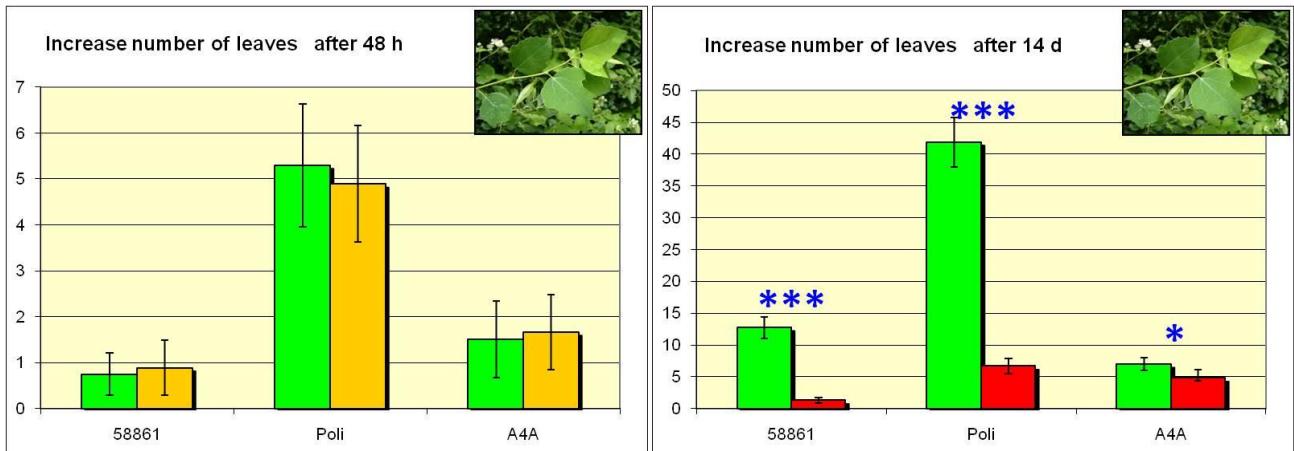


Fig. 16: The Increase of leaves number in cuttings of three poplar clones grown in hydroponic culture with 0 μM CdSO_4 ,control (green bars), and 20 μM CdSO_4 treated (yellow bars for short term treatment and red bars for long term treatment). *;Observed significance levels (P values) from T Student Test ***<0,001 - **<0,01 - *<0,05

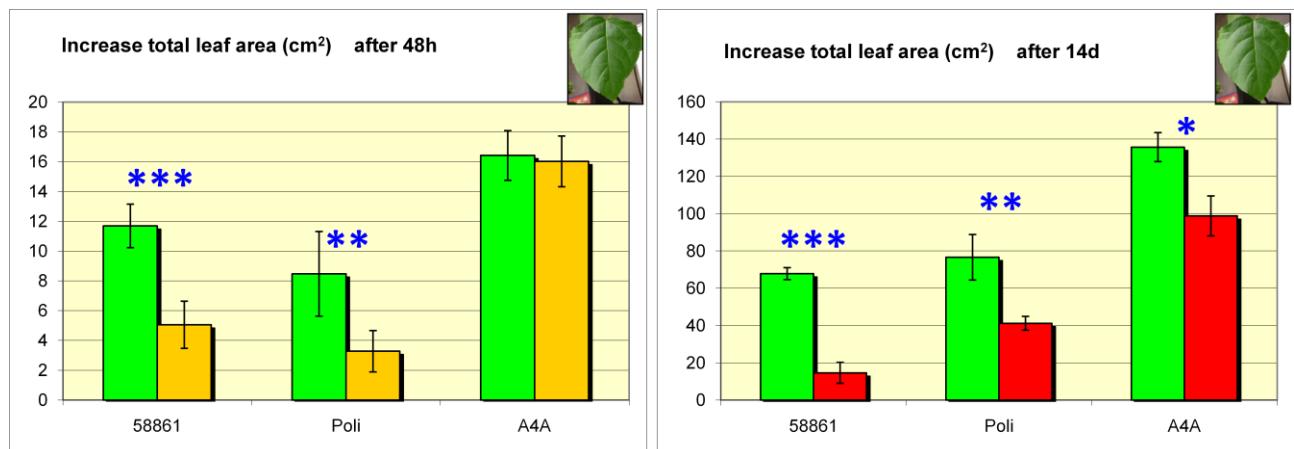


Fig. 17: The increase of total leaf area in cuttings of three poplar clones grown in hydroponic culture with 0 μM CdSO_4 ,control (green bars), and 20 μM CdSO_4 treated (orange bars for short term treatment and red bars for long term treatment). *;Observed significance levels (P values) from T Student Test ***<0,001 - **<0,01 - *<0,05

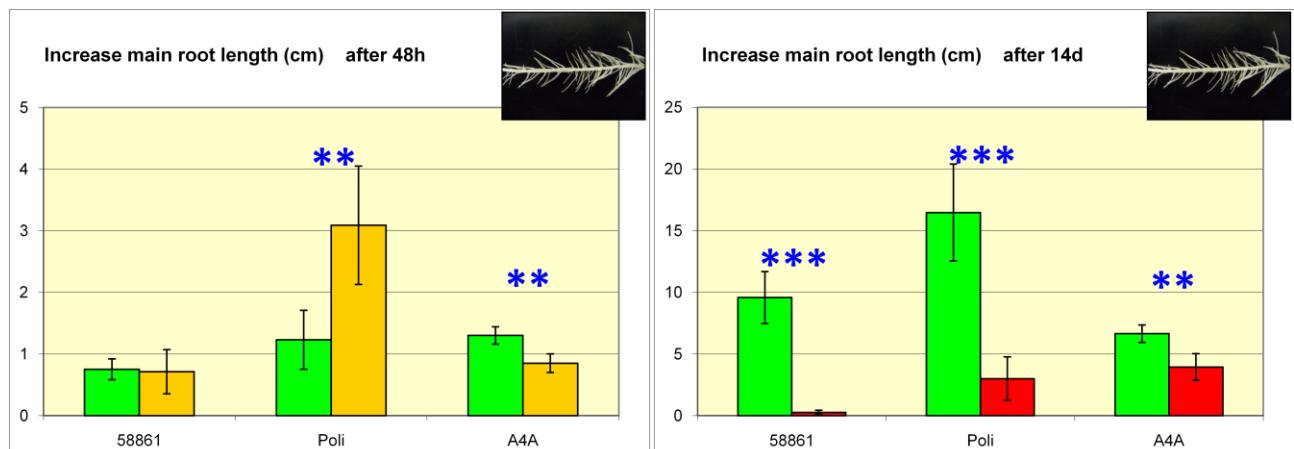


Fig. 18: The increase of main root length in cuttings of three poplar clones grown in hydroponic culture with 0 μM CdSO_4 ,control (green bars), and 20 μM CdSO_4 treated (orange bars for short term treatment and red bars for long term treatment). *;Observed significance levels (P values) from T Student Test ***<0,001 - **<0,01 - *<0,05

The clone *A4A* did not show statistically significant differences after 48 hours treatment, and less inhibition than the other clones under the long term metal treatment.

The root system plays a key role in the interaction between contaminants and plant; thus, another parameter analyzed in this work has been main root elongation (Fig.18). The main root length in each plant was affected by cadmium treatment. There were no differences in the increase of main root length in the *58-861* clone between control and treated after 48 hours treatment. On the contrary, *A4A* showed a reduction in the increase of main root length, caused by cadmium exposure, already after the short term treatment. The clone *Poli* showed an interesting behavior: in fact, these cuttings showed a rise in the increase of main root length in treated plants after the short term treatment. Several studies have shown how some plant species increase the roots lenght under heavy metals exposure, probably to reach deeper soil layers, not subject to contamination (Arduini *et al.*, 2006).

For each poplar clones, there was a dramatic reduction in the increase of main root length after the long term treatment (Fig.18). Furthermore, all clones showed growth inhibitions of lateral roots when exposed to cadmium, in accordance with previous studies (Dos Santos *et al.*, 2007).

2.3.2 Mineral analysis

In figure 19 the cadmium concentrations detected in roots, stems and leaves of poplar cuttings, exposed for 48 hours and 14 days to 20 μM cadmium sulphate, are reported. Cadmium concentrations in the untreated control were generally below or near the instrument detection limit and there were therefore not shown in the figures.

Tolerance at root level represented the first step in metal absorption and loading into the xylem vessels (Massacci *et al.*, 2009). A considerable concentration of cadmium was found in the roots of each poplar clone. *P.Nigra* clones *58-861* showed the highest metal root concentration either after short and long treatment; *P.Nigra Poli* clones showed the lowest cadmium root concentration, while *A4A* showed an intermediate concentration (Fig. 19A).

On the contrary, the highest Cd concentration in stems was detected in clone *A4A*, both after the short and the long term treatment. There are no statistically significant differences in cadmium concentration in the stem of the other clones after the short term treatment (Fig. 19B). After the long term treatment *Poli* clones showed a cadmium concentration in stems higher than *58-861* cadmium concentration. These data are in accordance with literature (Marmiroli *et al.*, 2011), where the same clone *A4A* accumulated more Cd in stems than did either *Poli* or *58-861*, after a treatment with 50 μM CdSO_4 for three weeks.

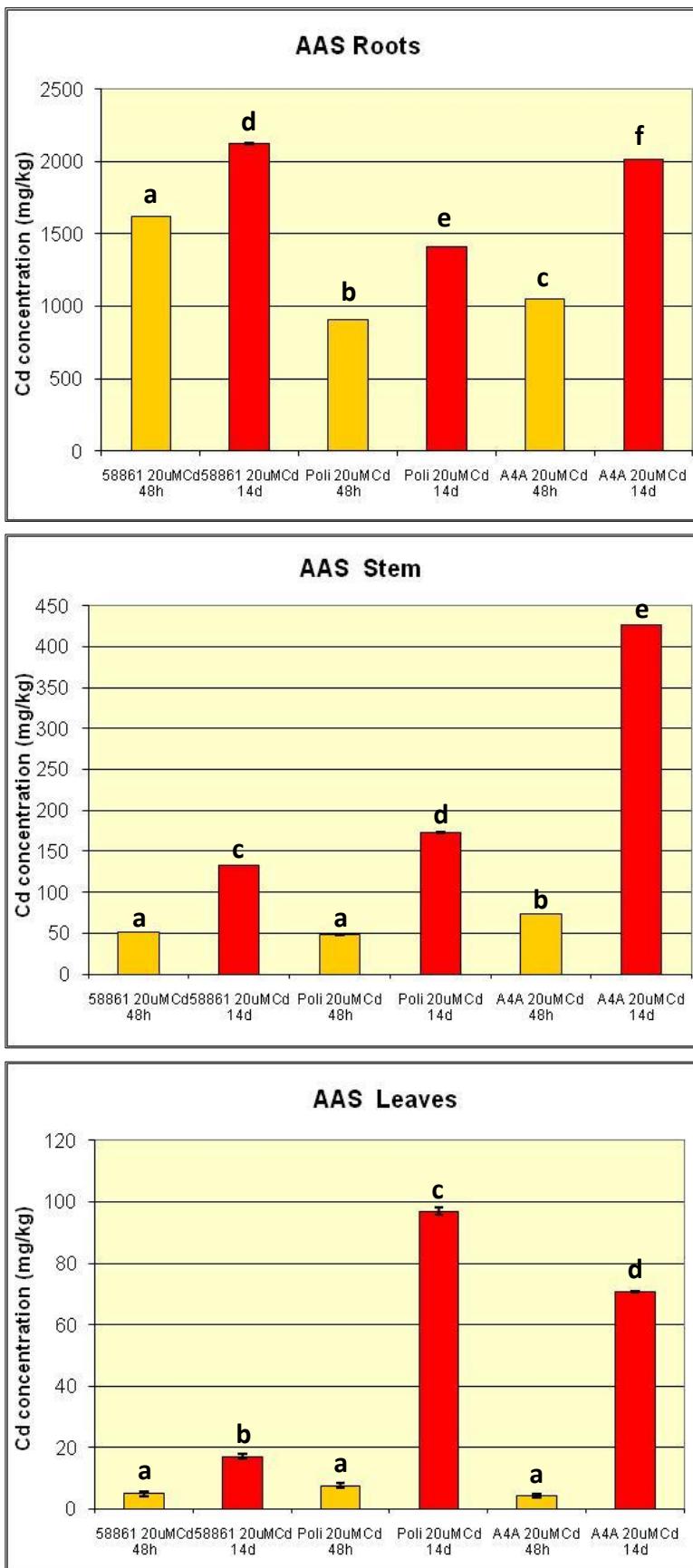


Fig. 19: Cadmium concentration detected in stems (A), leaves (B) and roots (C) of three poplar clones grown in hydroponic culture with 20 μM CdSO_4 (orange bars for short term treatment and red bars for long term treatment). Metal concentration in control cuttings was below the threshold of detection. Letter a-f: Observed significance levels (P values) from T Student Test; different letters have significant differences with $p<0,05$.

Notwithstanding the similar low cadmium concentration in 58-861 and *Poli* stems, in the leaves of the latter cadmium was higher than the former, after the long term treatment. A4A showed the intermediate concentration. The concentration of cadmium in the leaves of all the three clones was very low and not statistically different after the short term treatment. (Fig. 19C). These data are in accordance with Marmiroli *et al.* (2011), showing that clone *Poli* accumulated more Cd in leaves than did either A4A or 58-861, after a treatment with 50 µM CdSO₄ for three weeks.

However, data reported in literature about cadmium concentrations and allocation within organs are often contradictory. This is due, probably, to the different experimental conditions adopted. In fact, metal concentrations, type and length of metal exposure, type of substrate, can modify plant metal availability, and type of poplar clone used can modify metal concentrations within the different organs.

In this context the **bio-concentration factor (BCF)** can give further valuable information regarding the capability for these *Salicaceae* to extract metals from a contaminated matrix. So, according to Zayed *et al.*, 1998, to evaluate the capability of poplar clones to extract and accumulate cadmium in the plant, the bio-concentration factor (BCF) was calculated as the ratio between the cadmium concentration in the harvested plant material and the cadmium concentration in the solution (Fig. 20). In the figure 20 the BCF for the three poplar clones, referring to the root system and the aerial part of the plant, is reported. The highest root BCF was calculated in clones 58-861 and the lowest in *Poli*. The aerial parts BCF indicates A4A as the poplar clones with the greatest capability to accumulate cadmium in leaf and stem tissues, especially after the long term treatment.

The capability of poplar clones to accumulate cadmium in the above ground tissues was further confirmed by calculating the **Translocation Factor (T_f)**, that indicated the percentage of the absorbed metal that reached the aerial part of the plant in respect to that present in the roots (Fig. 21). Translocation factors for the clone A4A and *Poli* were the highest, while the lowest was found in clone 58-861.

Thus, from our experimental data it is possible to assume that *P.Nigra* clones 58-861, having a large concentration of cadmium in roots (>1500mg/kg) associated with a small concentration of cadmium in aerial parts with a leaf:root ratios < 1, can be considered a “root accumulator”, a plant species with high metal uptake but inefficient transport to shoots (Dos Santos *et al.*, 2007). These assumptions are in accordance with literature (Masacci *et al.*, 2009), where clone 58-861 showed the higher bio-concentration factor for root and lower for aerial part than BCF of ten poplar clones tested with cadmium contamination. Furthermore, it showed the lower translocation factor than T_f of other ten clones.

On the other hand, the clone A4A, having a large concentration of cadmium in roots ($>1500\text{mg/kg}$) associated with a moderate concentration in aerial parts, can be considered a “good translocator”, also indicated by its highest value of translocation factor (Fig.21) and bio-concentration factor of the aerial parts (Fig.20).

P.Nigra clone Poli showed also a high translocation factor, similar to A4A, and a moderate bio-concentration factor in the aerial parts, but it showed the smallest cadmium concentration in roots than all the other two clones, and a low uptake from substrate. Moreover, it showed higher cadmium concentration in leaves than the other two clones, which could be correlated with the presence of several signs of stress on the leaves surface after the long term treatment (Fig.13).

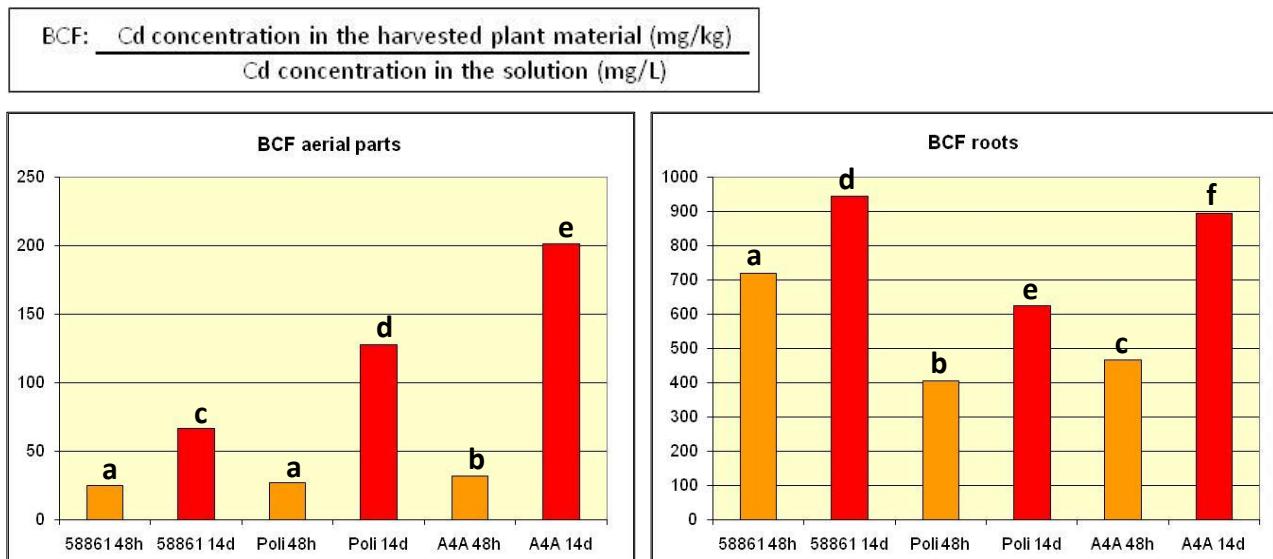


Fig. 20: Bio-concentration factor (BCF) in aerial part and roots of the three poplar clones grown in hydroponic culture with $20 \mu\text{M CdSO}_4$ treated (orange bars for short term treatment and red bars for long term treatment). Letter a-f: Observed significance levels (P values) from T Student Test; different letters have significant differences with $p<0,05$.

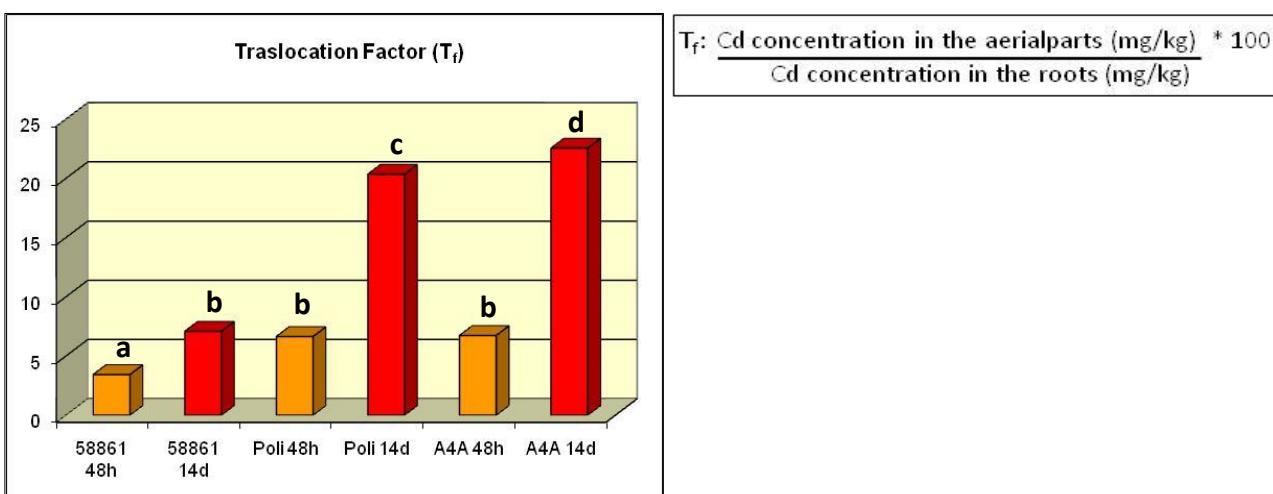


Fig. 21: Translocation factor (T_f) calculated after short term treatment (orange bars) and long term treatment (red bars) of three poplar clones grown in hydroponic culture in the presence of $20 \mu\text{M CdSO}_4$. Letter a-d: Observed significance levels (P values) from T Student Test; different letters have significant differences with $p<0,05$.

2.3.3 Scanning Electron Microscopy with microanalysis (SEM/EDX)

All the biological replicates were harvested, and utilised for elements quantification and localization through SEM/EDX. The operating parameters were set as follows: working distance: 14 mm; electron beam energy: 20KeV; dead time in X-ray data acquisition: between 15% and 25%. In the figure 22, it is possible to see an example of the spectrum acquired from a section (within the purple square in Fig. 22A) of the *Poli* clone stem.

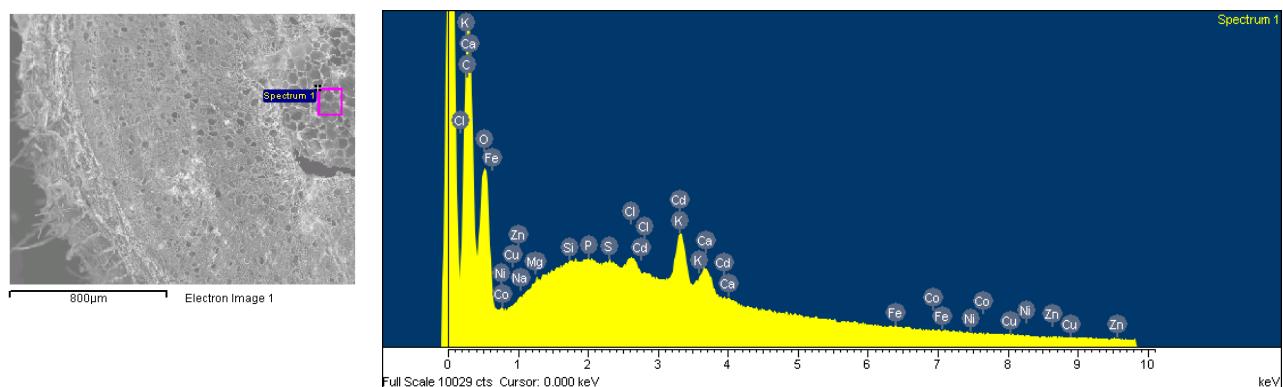


Fig. 22: **A:** SEM image of the horizontal section of the stem of the clone *Poli* magnified 50x, working energy: 20KeV, working distance: 14 mm. **B:** X-ray emission spectrum acquired from the section within the purple square.

In general to perform the elemental mapping a whole section is scanned at the same time continuously for a period of time varying from two to four hours even though longer maps are possible (10-12 hours). The time length of a map is expressed in “frames”, usually 50 frames last about 45 minutes. The spectrum acquired is a total spectrum of the whole image and is converted by the software INCA into distribution dot-maps for each element. In the figure 22B it is possible to identify the main element present in the sample: Ca, K, P, S, Cl, Mg, Na, Fe, Co, Zn, Ni, Cu, Cd. Our treatments were based on cadmium which has the L β 2 X-ray emission line at 3,528 KeV, which is very close to the calcium K α 1 and K α 2 emission line at 3,6 KeV. Since the quantity of Ca is always very high in poplar samples there could be a superimposition of K α 1 and K α 2 peaks creating a large peak that can simulate the presence of Cd, as illustrated in figure 23 (in fact, in Figure 22B the peaks of Ca and Cd appear as superimposed).

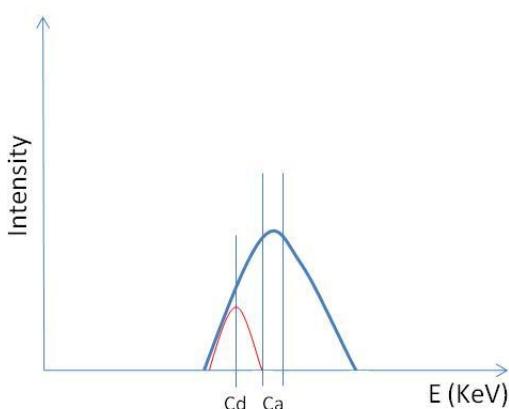


Fig. 23: schematization of peaks superimposition convolution due to calcium abundance in respect to Cadmium abundance.

A further problem that can arise from this phenomenon is that Cd is detected even if it is not present. Therefore in our dot-maps we counterchecked the effective presence of cadmium either through linescan or “map-mix”.

Cadmium content in control plants was generally below or near the instrument detection limit and there were therefore not shown in the figures.

2.3.3.1 ROOTS AFTER THE SHORT TERM TREATMENT

The integrity of root system involved in Cd uptake, coupled with the capacity of translocation to shoots, represents an important factor in screening clones for phytoremediation (Shen *et al.* 1997; Cocozza *et al.* 2011). In this study, internal and external characteristics of primary and secondary roots were not strongly affected by cadmium treatment, despite the fact that metal was found dispersed throughout all root tissues. Indeed, no cell jerk or tissues necrosis was observed in roots of these three poplar clones after the short term treatment.

A remarkable Cd accumulation was identified in root levels, showing clone-specific Cd thresholds. In figure 24 are reported examples of dot-maps of a set of specimens of root cross sections from the clone **Poli** treated for 48 hours at $20\mu\text{M}$ CdSO_4 . The shades of grey represent the concentration of each element: the brighter areas are the spots with higher concentrations, the darker areas are those with lower concentrations of elements respectively reported under each map.

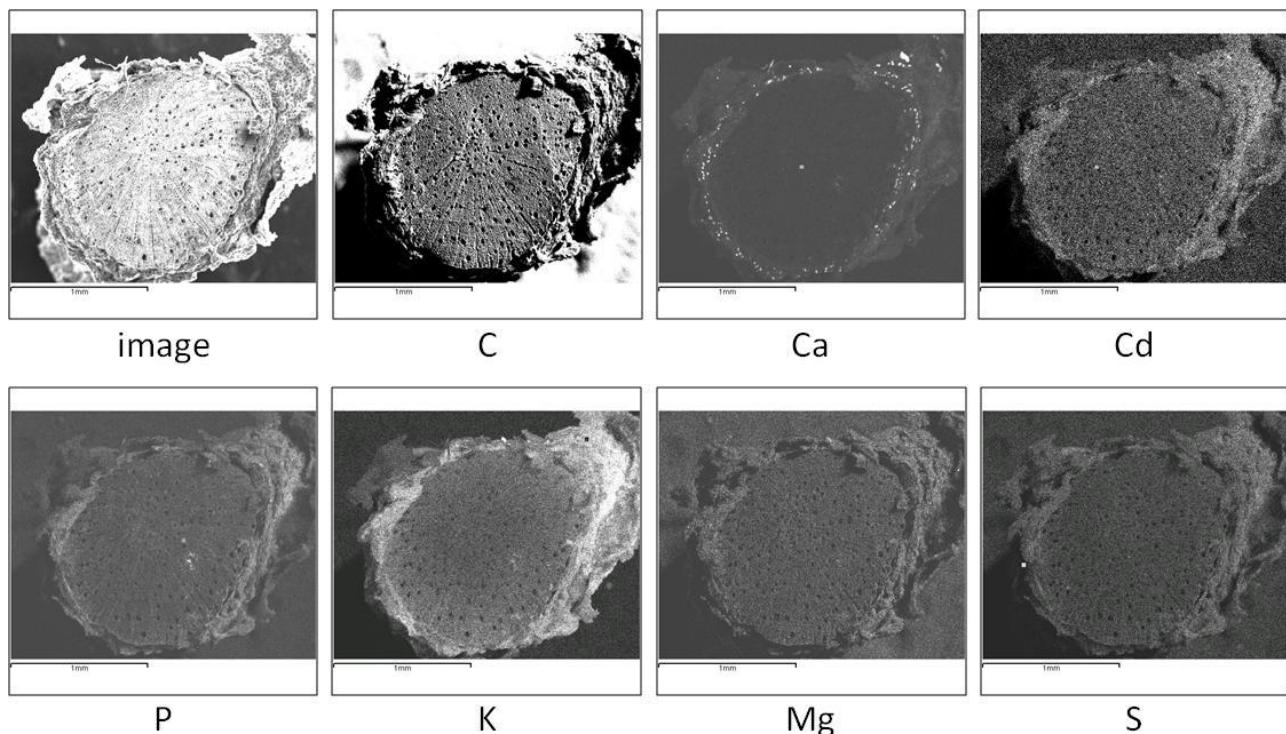


Fig. 24: Dot-maps of a root section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 55x; 1130 frames; working energy: 20 KeV; working distance: 14mm.

In the figure 25 is reported an example of the *linescan* for calcium, cadmium and potassium and the transect is represented both on the maps and on a separate graph. In the latter we can observe that the trend followed by cadmium and calcium are not totally superimposed. This means that there is a factual presence of cadmium in the samples not due to a “ghost” of the calcium spectrum.

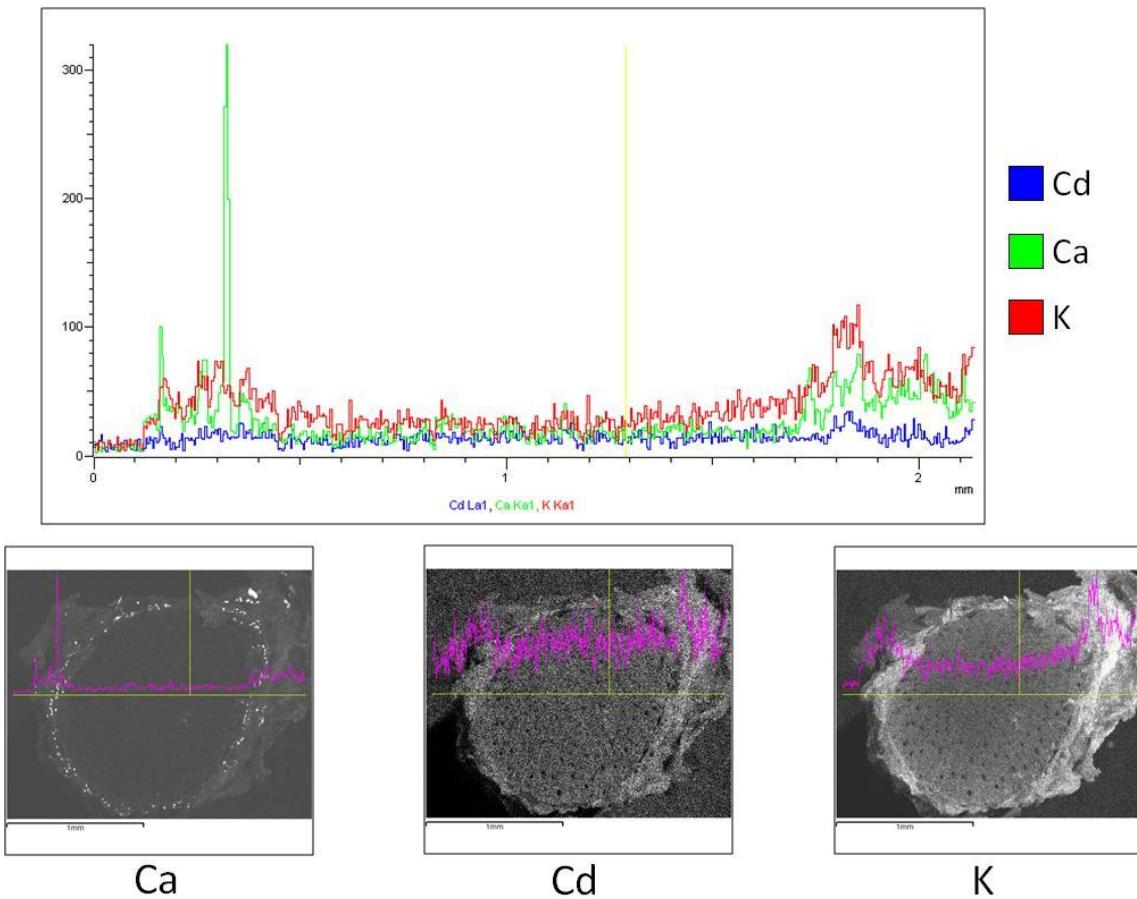


Fig. 25: Linescan of a root section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours. Graphic representation of the trend along the yellow transect across the sample for cadmium (blue), calcium (green) and potassium (red).
B: Dot-maps of stem section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours, representing the distribution of Ca, Cd and K. Magnification: 55x; 1130 frames; working energy: 20

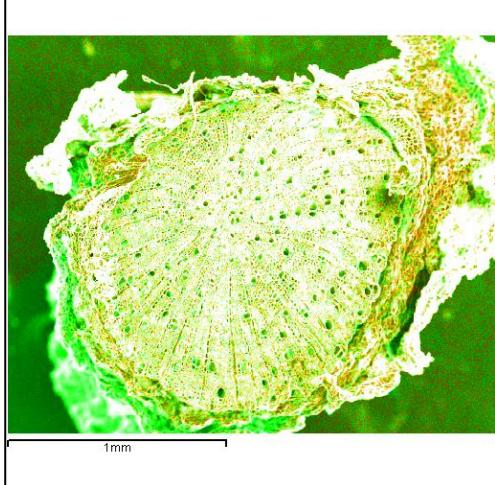


Fig. 26: Dot-maps “mix” of a root section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours. Orange shades is for cadmium and light green shades is for calcium.
Magnification: 55x; 1130 frames; working energy: 20 KeV; working distance: 14mm.

In roots of the *Poli* clone (Fig. 24-25) Cd was concentrated especially in the periderm and in parenchyma cells of cortex. As shown both in the “mix” dot-map and in the line-scans (Fig. 26-25) cadmium and calcium were complementary localized especially in lignified and suberified tissues. The nutrients K, P, Mg and S are evenly distributed in the whole root section with different intensities because of their different concentration (Fig. 24).

Compared with other important ions, the distribution of cadmium within the *Poli* root, was similar to that of calcium and potassium (Fig. 25) and was preferentially localized in the surroundings of central cylinder. Many studies have shown that the main site of Cd accumulation in roots is the apoplast, particularly cell walls (Wójcik *et al.*, 2005). Khan *et al.* (1984) and Lozano-Rodríguez *et al.* (1997) found that in maize roots the metal was mainly detected in cell walls of cortex parenchyma, endodermis and pericycle, while much smaller amounts were found in protoplasts.

In figure 27 are reported dot-maps of a set of specimens of root cross sections from the clone **58-861** treated for 48 hours at 20 μ M CdSO₄. In this clone, an higher cadmium contents than in *Poli* was found in the roots cross sections, and this is in agreement with data obtained from elemental analysis by atomic absorption spectroscopy (see section 2.3.2, Fig. 19A). Furthermore, 58-861 clone showed an unaltered root length under excess of cadmium after 48 hours treatment, (see section 2.3.1). Also, in roots of the 58-861 clone Cd was concentrated especially in the periderm and in parenchyma cells of cortex, and it was not detected in high percentage in internal tissues and central pith.

In figure 28 are reported dot-maps of a set of specimens of root cross sections from the clone **A4A** treated for 48 hours at 20 μ M CdSO₄. In this clone the roots showed a similar concentration of Cd in respect to the roots of *Poli* (Fig. 24), after the short term treatment. Also the cadmium distribution pattern within the root tissues of the two clones was similar, mainly localised in the periderm and in the cortex.

Whereas, 58-861, A4A and *Poli* had high Cd contents in the cortex, Cd was not detected in high percentage in internal tissues, central pith, being important for identifying any anatomical barrier to metal movement, as obstruction to the distribution of elements from the epidermis to the central pith. This, in general, could give control over mineral uptake to the plant, but can also represent a tolerance strategy in phytoremediation systems. On the other hand, differential Cd localization in cross sections could suggest clone-specific metal mobility through internal root tissues. Other than exclusion strategy, internal detoxification is also an important strategy in metal tolerance, and these clones may have evolved different strategies for metal tolerance (Castaglione *et al.* 2010; Cocozza *et al.* 2010).

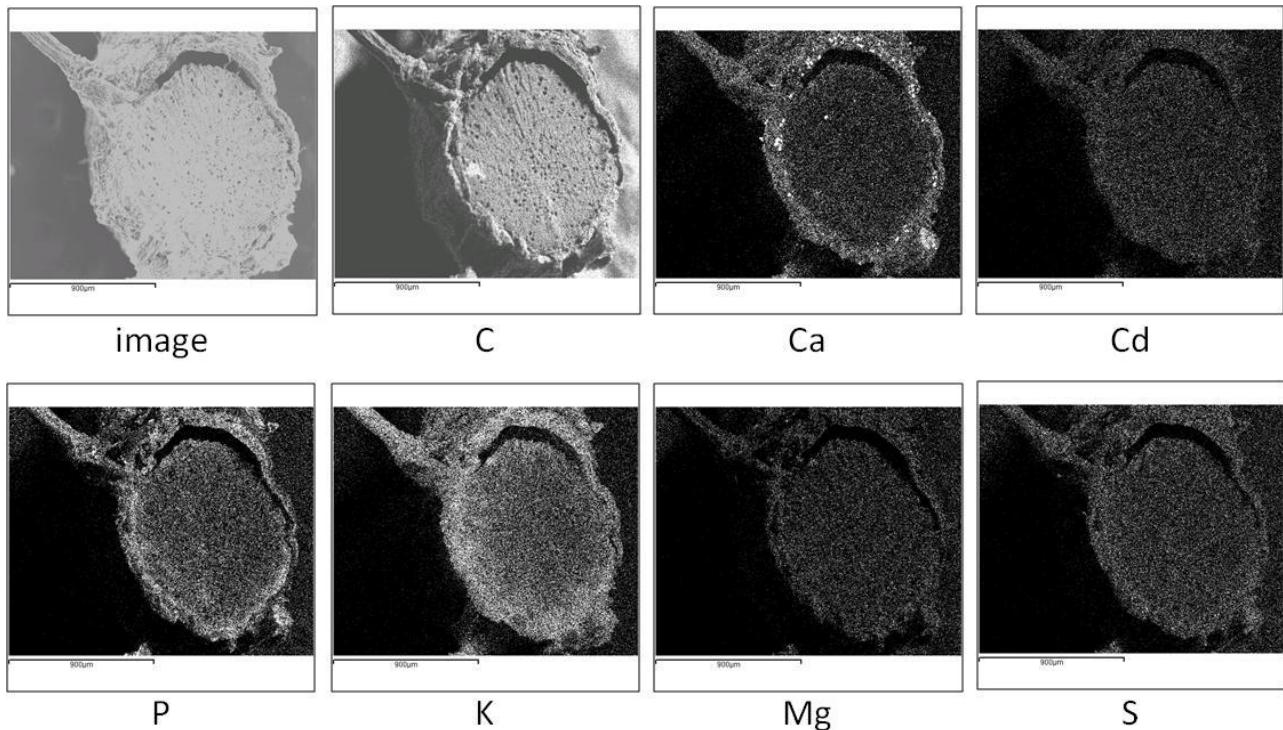


Fig. 27: Dot-maps of a root section of 58-861 clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 65x; 220 frames; working energy: 20 KeV; working distance: 14mm.

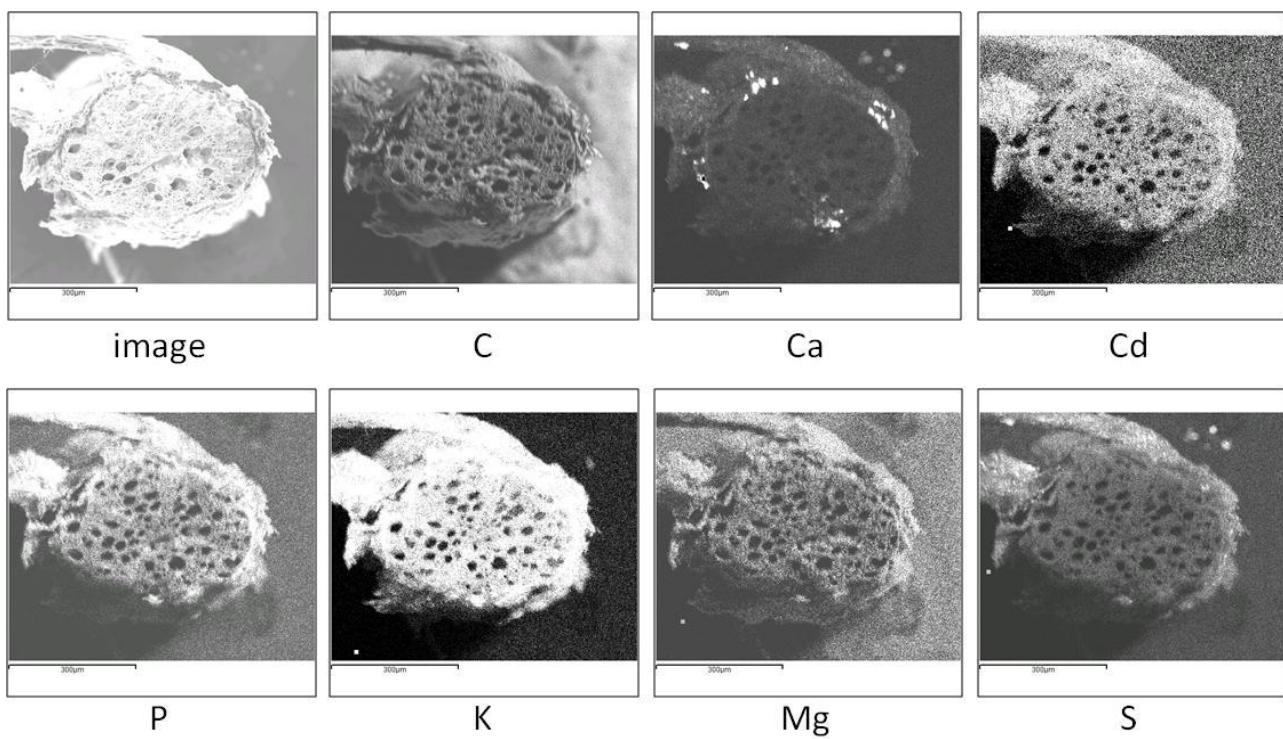


Fig. 28: Dot-maps of a root section of A4A clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 170x; 240 frames; working energy: 20 KeV; working distance: 14mm.

A partial root barrier may facilitate while a tight barrier may obstruct the uptake of water, ions and nutrients at the basal root zones (Soukup *et al.* 2007, Deng *et al.* 2004), therefore, these mechanisms could be likewise adopted by plants to control the metal and toxic elements distribution in roots (Cocozza *et al.* 2011). In this contest, the Caspary strip could be an obligatory passage to filter the aqueous absorbed solution, from the cortex to the central pith in roots (Haynes, 1980), thus limiting Cd distribution crossways the root.

A relationship was found between Cd and Ca contents (and also Mg), suggesting that the accumulation of Cd could interfere with macronutrient absorption, while these clones cope with metal stress. In fact, there is no specific transporter for selective Cd uptake, but the metal could also permeate through Ca^{2+} or Mg^{2+} channels, being both divalent cations (Hinkle *et al.* 1992, Perfus-Barbeoch *et al.* 2002). The distribution of Ca in roots was more affected by Cd, in *Poli* and in 58-861 clones, and was less affected for the A4A clone, and this results on Ca distribution in roots might certificate efficient transport Ca-channels under excess Cd and/or metal concentration below critical threshold. Commonly, high concentrations of heavy metals may block plant water channels and also affect ion channels and transport of osmotically active elements, which provide the driving force for water transport (Eckert *et al.* 1999). However a component of apoplastic rather than symplastic Cd transport can not be overlooked.

2.3.3.2 STEMS AFTER THE SHORT TERM TREATMENT

An amount of Cd was found also at stem level, in each poplar clone considered.

In figure 29 are reported dot-maps of a set of specimens of stem cross sections from the clone *Poli* treated for 48 hours at $20\mu\text{M}$ CdSO_4 . In the figure 30 is reported an example of the *linescan* for calcium, cadmium and potassium and are represented both on the maps and on a separate graph. From all these analysis it was possible to observe that in the stems of the clone *Poli* after the short term treatment, cadmium was more localized in epidermis and schlerenchyma, but it was also present in the vessels (phloem and xylem) and pith (Fig. 29-30). Other macro-nutrients such as Ca and K were localized both on the outer tissues and in the central part of the stem. Especially calcium and cadmium were more concentrated in the lignified tissues. Furthermore, cadmium was more concentrated in old stem epidermis and schlerenchyma, while the young stems did not show an high Cd accumulation.

In figure 31 are reported dot-maps of a set of specimens of stem cross sections from the clone **58-861** treated for 48 hours at $20\mu\text{M}$ CdSO_4 . The amount of cadmium accumulated and its metal distribution through the cross sections of 58-861 stems seems to be very similar to that accumulated

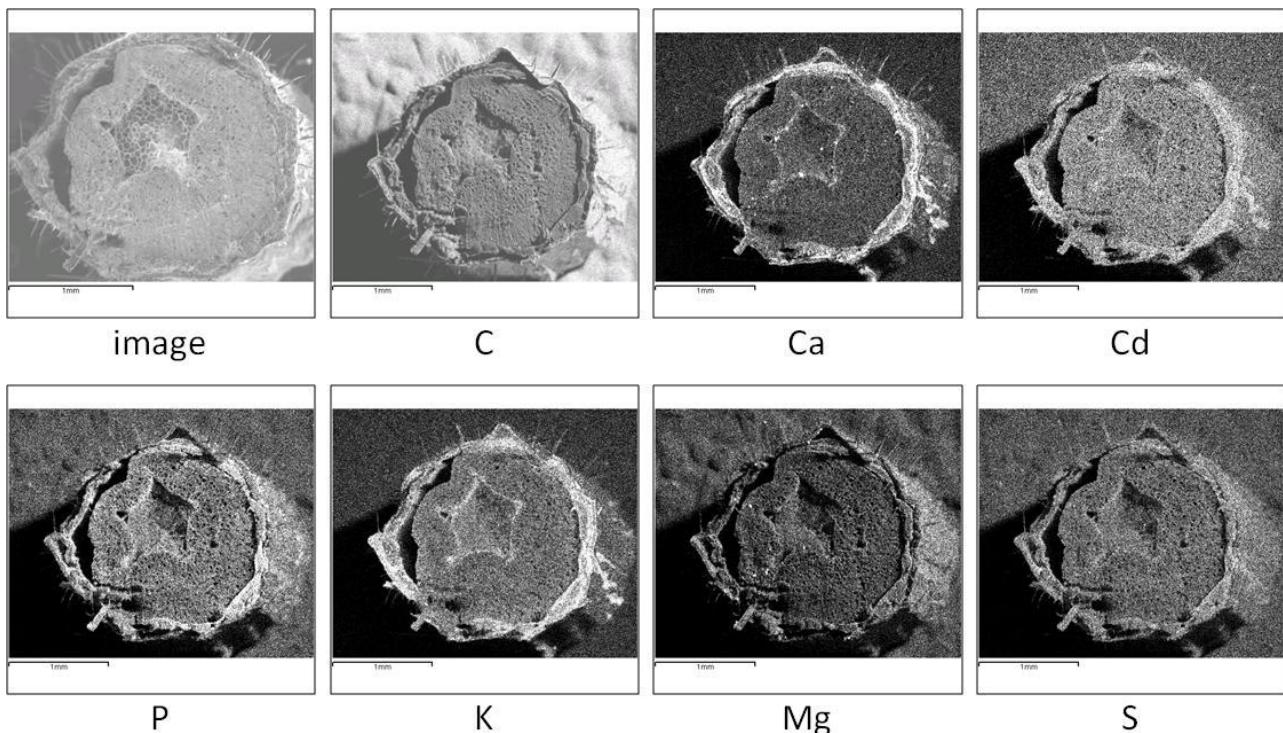


Fig. 29: Dot-maps of a stem section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 40x; 80 frames; working energy: 20 KeV; working distance: 14mm.

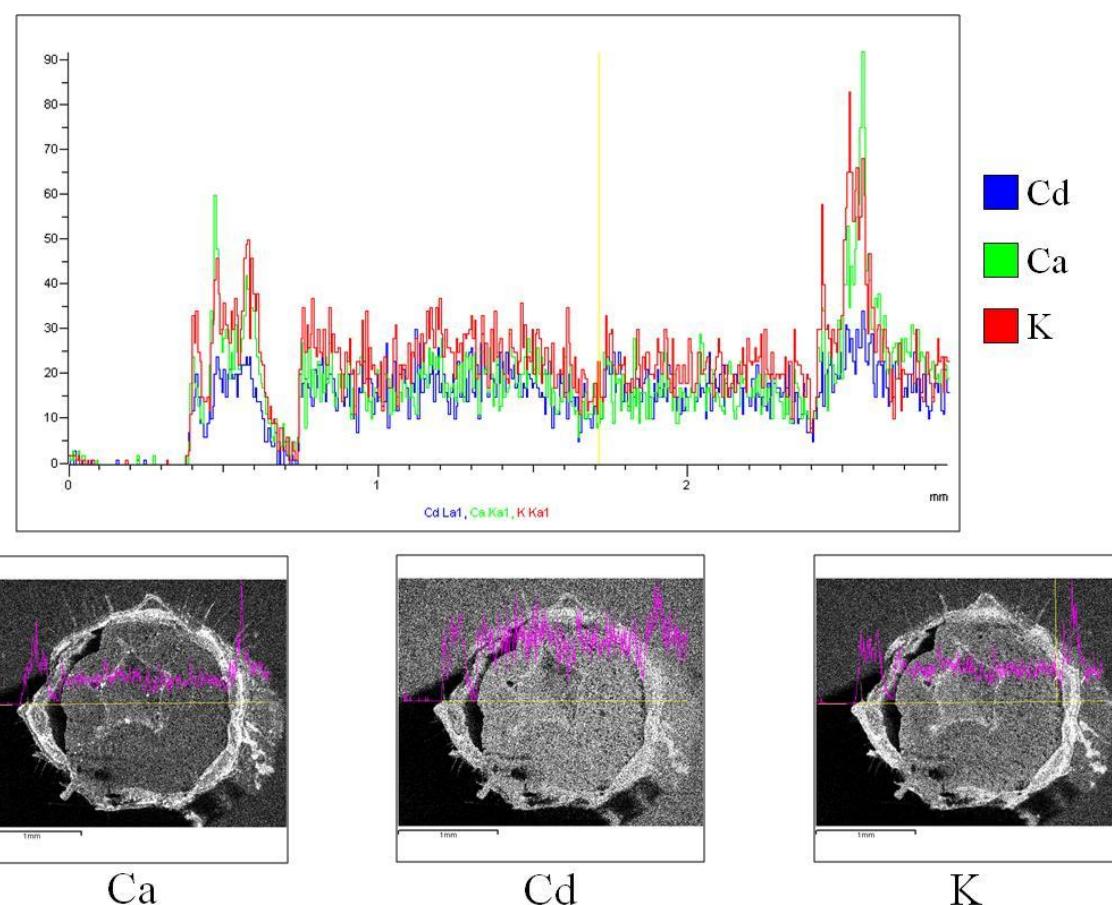


Fig. 30: **A:** Linescan of a stem section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours. Graphic representation of the trend along the yellow line across the sample for cadmium (blue), calcium (green) and potassium (red).
B: Dot-maps of stem section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours, representing the distribution of Ca, Cd and K. Magnification: 40x; 80 frames; working energy: 20 KeV; working distance: 14mm

from the *Poli* clone. This results from SEM/EDX are in accordance with those from the chemical analyses AAS (see section 2.3.2, Fig. 19B) in which no statistically significant differences in cadmium concentration were found, in the stem of the *Poli* and 58-861 clones after the short term treatment.

In figure 32 are reported dot-maps of a set of specimens of stem cross sections from the clone **A4A** treated for 48 hours at 20 μ M CdSO₄. On the contrary, in A4A stems the Cd concentration was higher than in other two clones and both old and new stems store Cd in their tissues, especially in chlorenchyma, parenchyma and epidermis, however also the pith and vascular bundles showed Cd presence. The changes in the external tissue structure were most extreme in clone A4A. This results from SEM/EDX are in accordance with those from the chemical analyses AAS (see section 2.3.2), in which the highest Cd concentration in stems was detected in clone A4A, both after the short and the long term treatment (Fig. 19B).

Also in the stems of each considered clones, cadmium and calcium were complementary localized especially in lignified and suberified tissues of the epidermis and chlorenchyma (Fig. 29, 31, 32).

Marmiroli *et al.* (2011) also showed a pronounced accumulation of Cd in the stems of the same clone A4A treated with 50 μ M of CdSO₄ for three weeks, associated with an increased production of sclerenchymatous tissue (Marmiroli *et al.*, 2011).

Heavy metal stress has been repeatedly shown to increase the production of secondary metabolites and to activate stress response pathways which modify cell wall structure (Maksymiec, 2007), and in particular lead to its lignifications (van de Mortel *et al.*, 2006). The distribution of cadmium in stem tissues can be explained, by several studies that showed the ability of lignified tissues to adsorb and chelate toxic metals (Crist *et al.*, 2002; Marmiroli *et al.*, 2004; Marmiroli *et al.*, 2011), and furthermore, because parenchyma is composed of living cells with thin walls, large intracellular spaces and large vacuoles. In fact, plants can react to cadmium toxicity by the accumulation of glutathione, phytochelatines and metallothioneins, in order to sequester toxic cadmium ions in the vacuole (Cobbet *et al.*, 2000).

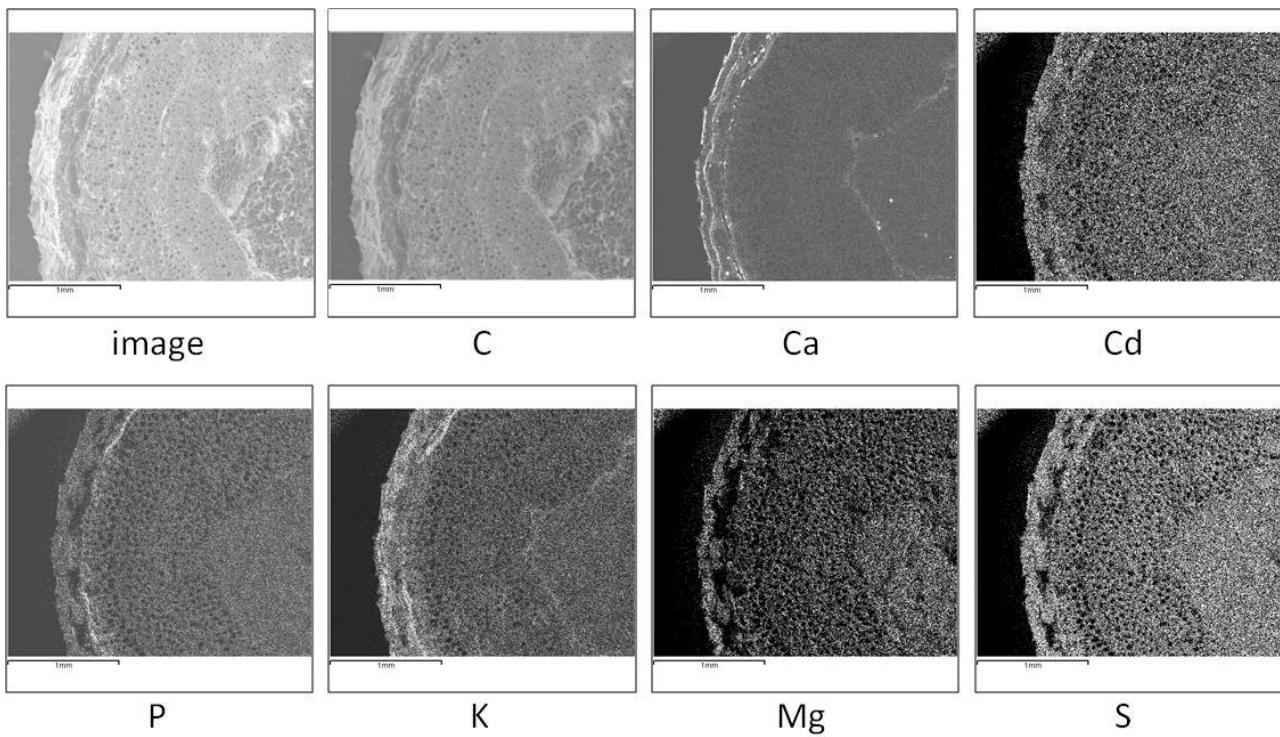


Fig. 31: Dot-maps of a stem section of 58-861 clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 85x; 420 frames; working energy: 20 KeV; working distance: 14mm.

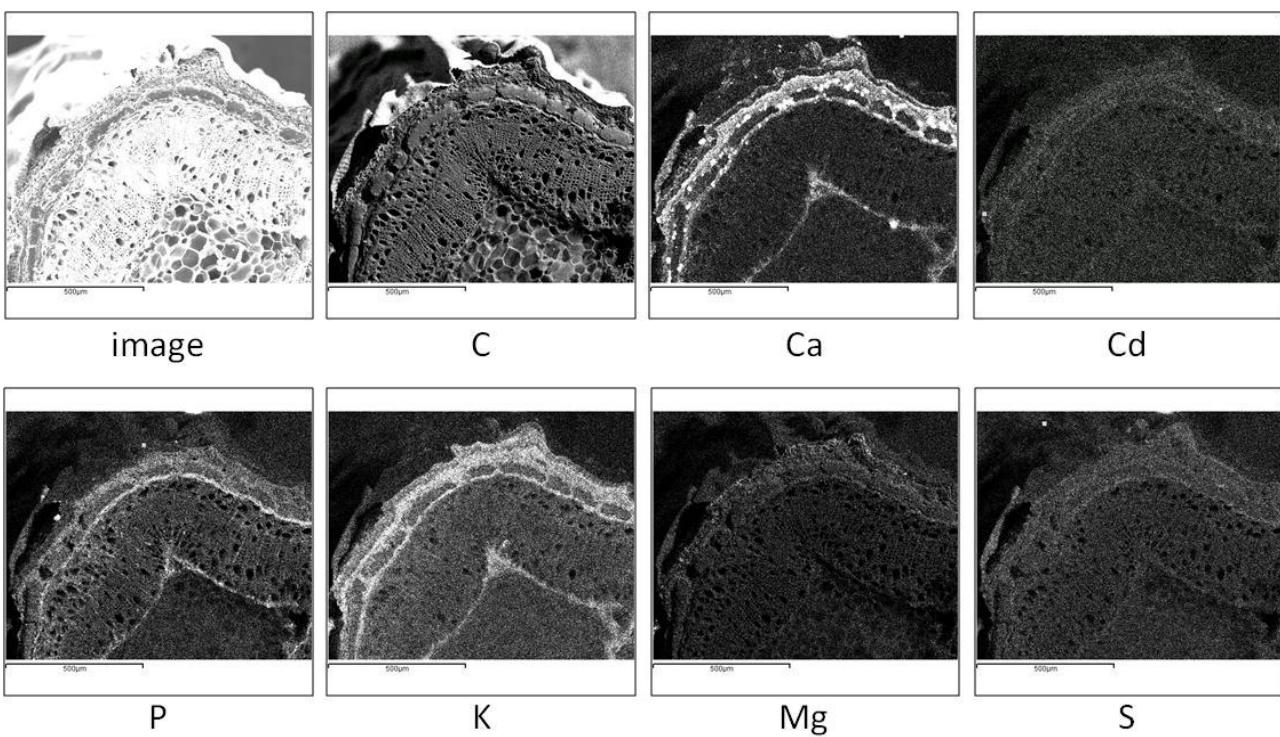


Fig. 32: Dot-maps of a stem section of A4A clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 110x; 270 frames; working energy: 20 KeV; working distance: 14mm.

2.3.3.3 LEAVES AFTER THE SHORT TERM TREATMENT

Leaves samples were harvested from control and treated plants, and utilised for elements quantification and localization through SEM/EDX.

The SEM analysis showed that the leaves of plants exposed to Cd from each of the three clones carried a deep layer of cuticular wax (Fig. 33); in some cases, trichomes developed in the midrib area. In contrast, the leaves of nontreated plants produced almost no wax and were devoid of trichomes on their upper surfaces. We can observe that leaves surface of each clone were covered with a layer of wax, which make it difficult to perform a good x-ray analysis. However it is apparent that calcium and cadmium are localized within the whole leaf, and particularly the metal occurred in mesophyll cells surrounding the vascular cylinder (Fig. 34, 35). Moreover cadmium concentration was similar in leaves of each clones. This results were in accordance with those from the chemical analyses AAS (see section 2.3.2), in which the Cd concentration in leaves was not significantly different between the three clones, after the short term treatment (Fig. 19C).

Previous studies showed that in leaves of *Arabidopsis thaliana*, cadmium is mostly located in the trichomes with the highest amounts of metal encountered in the strip of the trunk, in the end of the branches, and in the small bumps of the trichome surface. The metal is even more concentrated in the outer parts of this strip, suggesting that it is associated to the cell wall or cuticle (Isaure *et al.*, 2006). This suggests that trichomes are the major compartment of Cd accumulation in the leaves. Trichomes are also enriched with Ca while P, S, Cl and K are more concentrated in the leaf tissue than in the trichomes (Isaure *et al.*, 2006). Sequestration of various metals in trichomes has been observed in numerous plants, including hyperaccumulating species: zinc and cadmium in *Arabidopsis halleri* (Zhao *et al.*, 2000), nickel in *Alyssum murale* (Broadhurst *et al.*, 2004), and non-hyperaccumulating species: cadmium in *Brassica juncea* (Salt *et al.*, 1995).

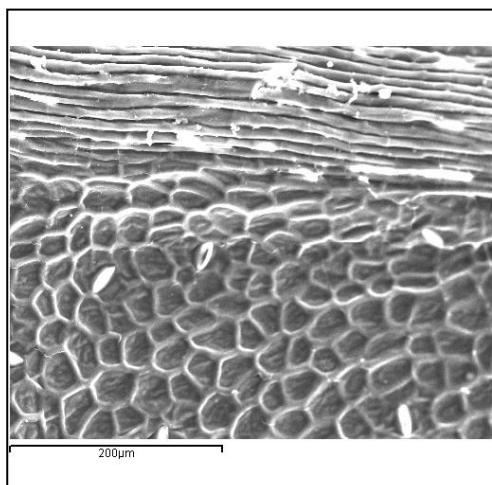


Fig. 33: SEM image of a leaf section of *Poli* clone treated with 20µM CdSO₄ for 48 hours.
Magnification: 220x; 270 frames; working energy: 20 KeV; working distance: 14mm.

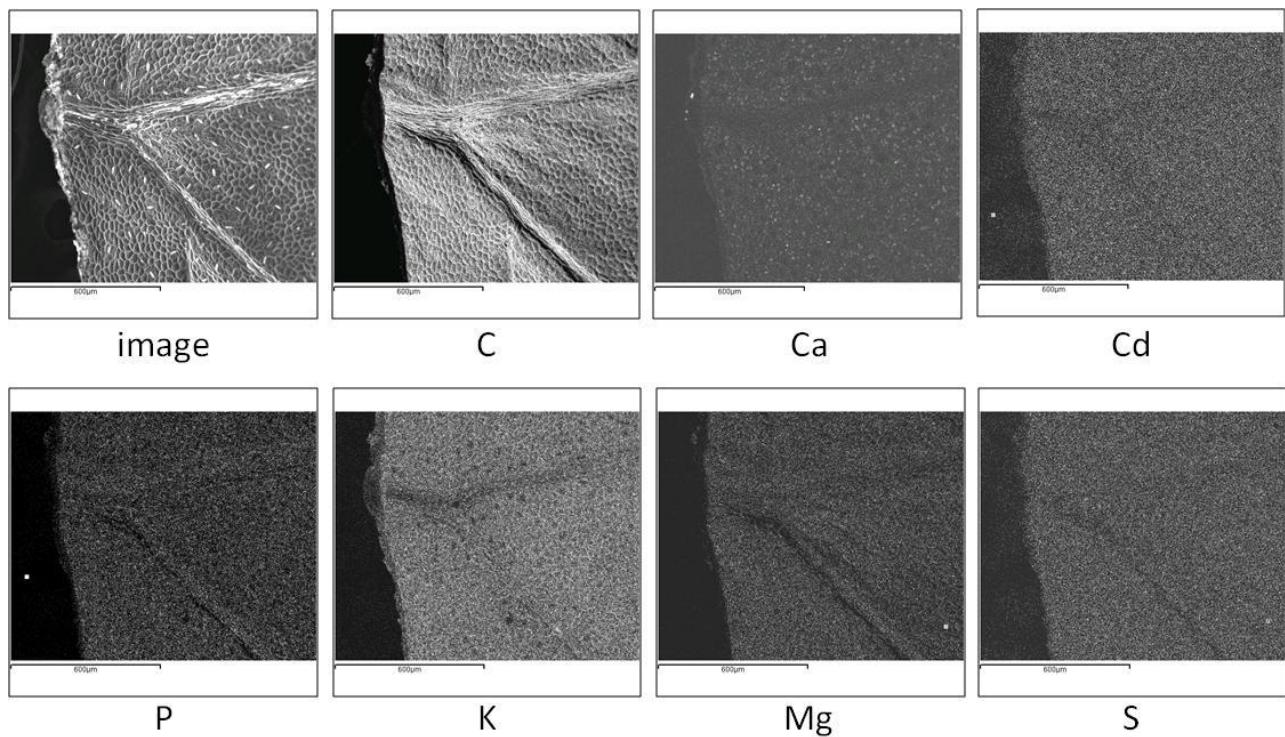


Fig. 34: Dot-maps of a leaf section of *Poli* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 100x; 218 frames; working energy: 20 KeV; working distance: 14mm.

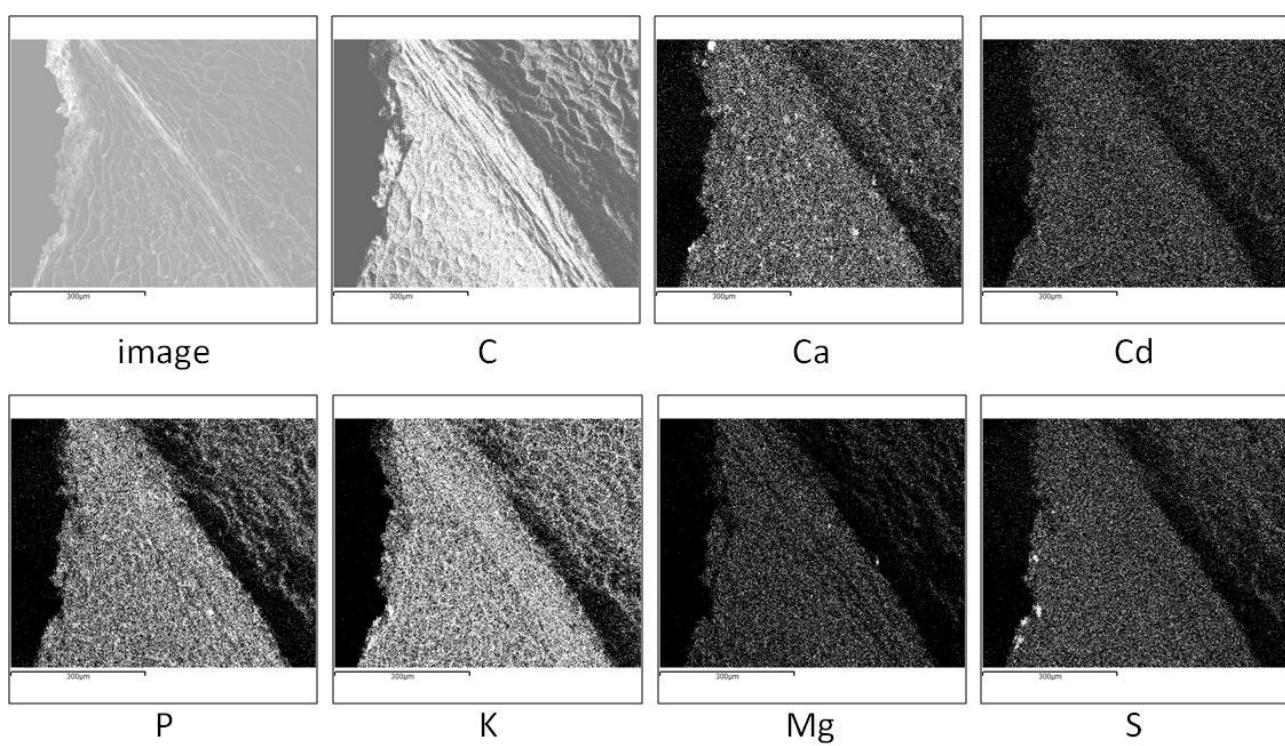


Fig. 35: Dot-maps of a leaf section of *A4A* clone treated with $20\mu\text{M}$ CdSO_4 for 48 hours.
Magnification: 100x; 221 frames; working energy: 20 KeV; working distance: 14mm.

In the hyperaccumulating plants *Arabidopsis halleri*, although trichomes present the highest metal concentrations, mesophyll cells are the major storage sites for metal in leaves (Kupper *et al.*, 2000; Sarret *et al.*, 2002). On the contrary, Isaure *et al.* (2006) found that the leaf epidermis and mesophyll of *Arabisopsis thaliana* contained only traces of cadmium, and trichomes were likely the major storage compartment for cadmium. The sequestration of Cd in these trichomes might be a way to protect the metabolically active cells from metal toxicity.

In *Thlaspi caerulescens* leaves, cadmium was detected in granules localized in vacuoles of spongy and palisade mesophyll cells and of some epidermal cells. Cd was also found in cells of lower epidermis in direct neighbourhood of stomata as well as in some cells of upper epidermis. The metal occurred in mesophyll cells surrounding the vascular cylinder. The detected metal was always present in dark deposits within vacuoles of the observed cells, but it was not found in other compartments of protoplast, in cell walls and cuticle layers on the leaf surface (Wójcik *et al.*, 2005). The presence of S at Cd localization sites encouraged us to examine phytochelatin (thiol peptides) accumulation induced by Cd (Wójcik *et al.*, 2005). Specific Cd localization in mesophyll cells lying on the way of water migration from vascular cylinder to epidermis and stomata distinctly indicates involvement of transpiration in metal translocation in the leaves. Some authors suggest that in plant leaves, similarly as in roots, the capacity to bind Cd in the call wall has a protective action against the deleterious effect of Cd by reducing the amounts of cytosolic Cd (Lozano-Rodríguez *et al.*, 1997; Ramos *et al.*, 2002; Zornoza *et al.*, 2002).

2.3.3.4 ROOTS, STEMS AND LEAVES AFTER THE LONG TERM TREATMENT

Samples of roots, stems and leaves were harvested and collected also from poplar cuttings grown in hydroponics with 20µM CdSO₄ for 14 days, to perform elements quantification and localization through SEM/EDX. The intense damage caused by the Cd treatment the cross sections were not profitably observable under the scanning microscope due to their uneven surface.

2.3.4 Proteomic Analysis

Total proteins from leaves were extracted following the protocol in Pirondini *et al.* (2006) (see materials and methods, 2.2.6). Total proteins were extracted from leaves of each clone in control and treated conditions after the short term treatment and in control and treated conditions after the long term treatment. Leaf protein total extracts were quantified using Bradford assay (Materials and Methods, 2.2.7) in order to evaluate the exact protein concentration of each extract, and purified by PD-10 Column (*GE-Healthcare Biosciences, Uppsala, Sweden*) (materials and methods, 2.2.8).

Proteins from each extract were subjected to 2D-LC separation by using ProteomeLab™ PF-2D (*Beckman Coulter, USA*) (Material and Methods, 2.2.9). High-performance chromatofocusing (HPCF) produces liquid pH fractions as the 1st dimension separation, followed by high-performance reversed-phase (HPPR) of each of the pH fractions as the 2nd dimension.

In figures 36 and 37, 1st dimension chromatograms were reported showing the absorbance at 280nm versus time; the height of each peak in the chromatogram is proportional to the protein concentration. All 1st dimension chromatograms are quite similar to each other (coming from different clones of the same plant species) and, in each chromatogram was evident the presence of three great protein groups: basic proteins, proteins of the gradient and acid proteins.

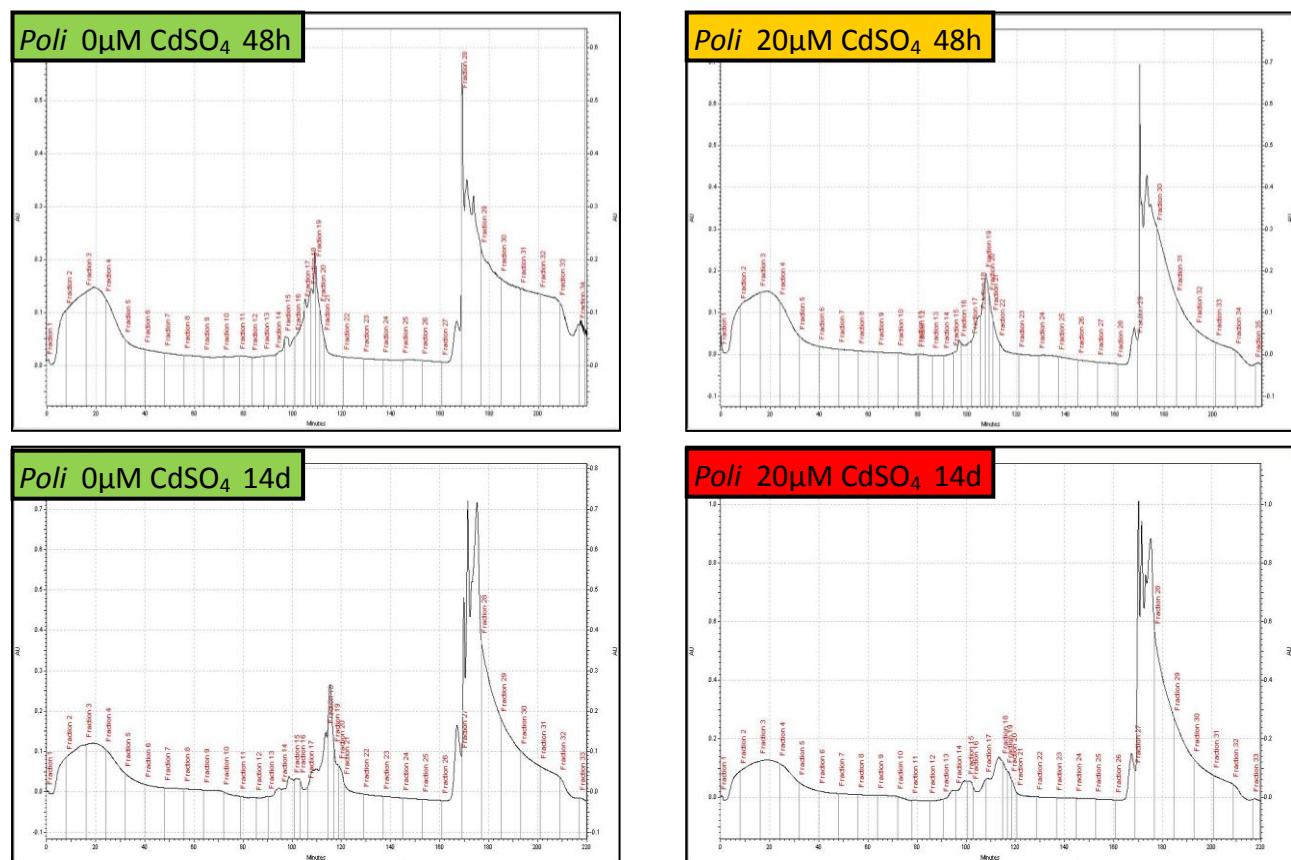


Fig. 36: 1st dimension chromatograms of 3mg of total leaf protein extract from *Poli* clone cuttings grown in hydroponic culture with 0 µM CdSO₄, control (green), and 20 µM CdSO₄ treated (orange for short term treatment and red for long term treatment). The y-axis displays the absorbance at 280nm. The x-axis displays the increase of pH during time.

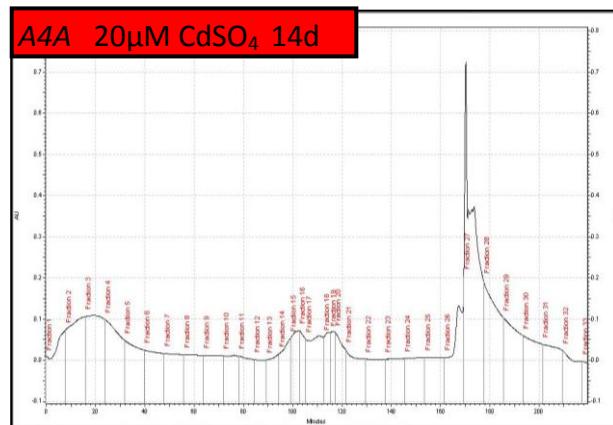
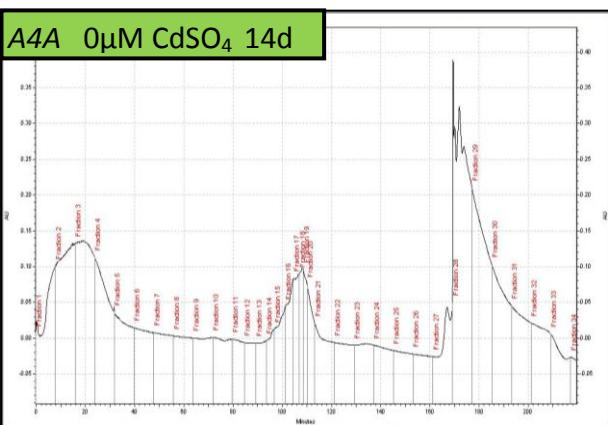
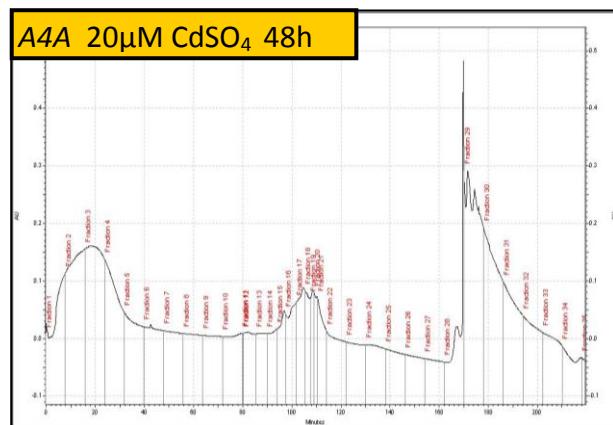
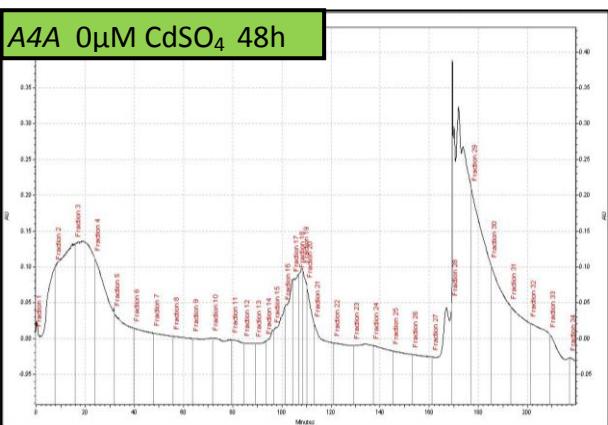
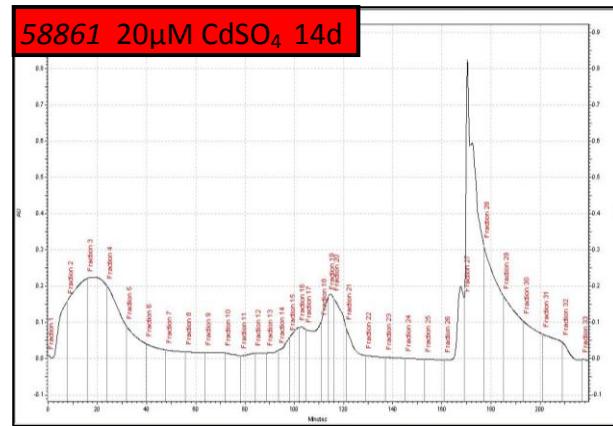
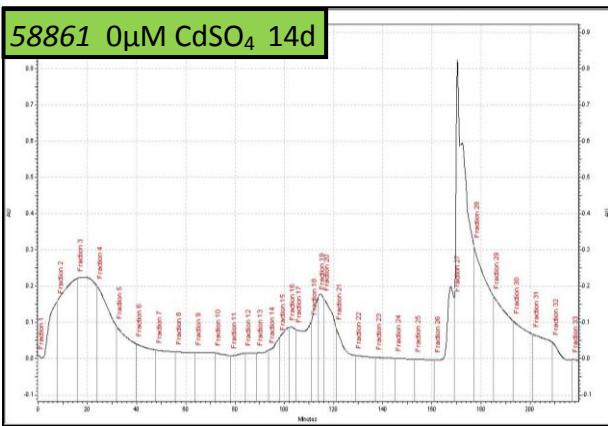
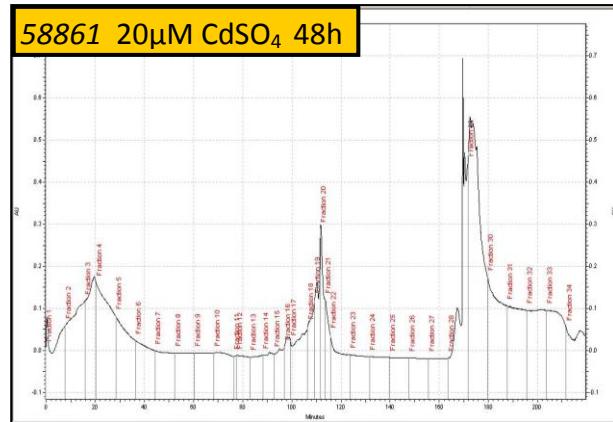
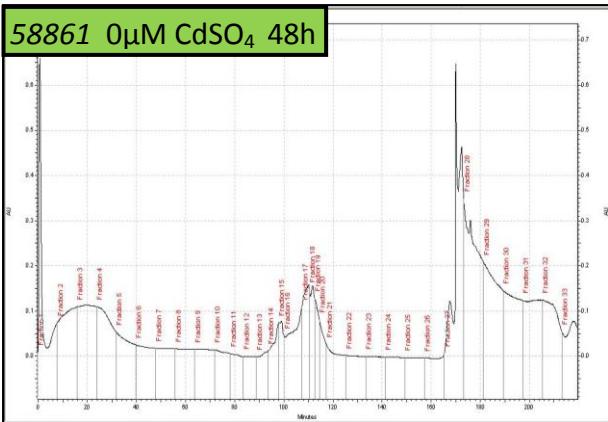


Fig. 37: 1st dimension chromatograms of 3 mg of total leaf protein extract of cuttings from the clone 58-861 and A4A grown in hydroponic culture with 0 µM CdSO₄, control (green), and 20 µM CdSO₄ treated (orange for short term treatment and red for long term treatment). The y-axis displays the absorbance at 280nm. The x-axis displays the increase of pH during time.

Each liquid fraction obtained from high-performance chromatofocusing was subjected to high-performance reversed-phase (HPRP) as the 2nd dimension. Software generated 2nd dimension chromatograms that show the absorbance at 214 nm versus retention time; the height of each peak in the chromatogram is proportional to the abundance of the corresponding protein.

The Proteovue software (*Eprogen, Darien, IL, USA*) converts all 2nd dimension chromatograms of one sample into easily visualized 2-D maps, "virtual gels" (an example is shown in figure. 38).

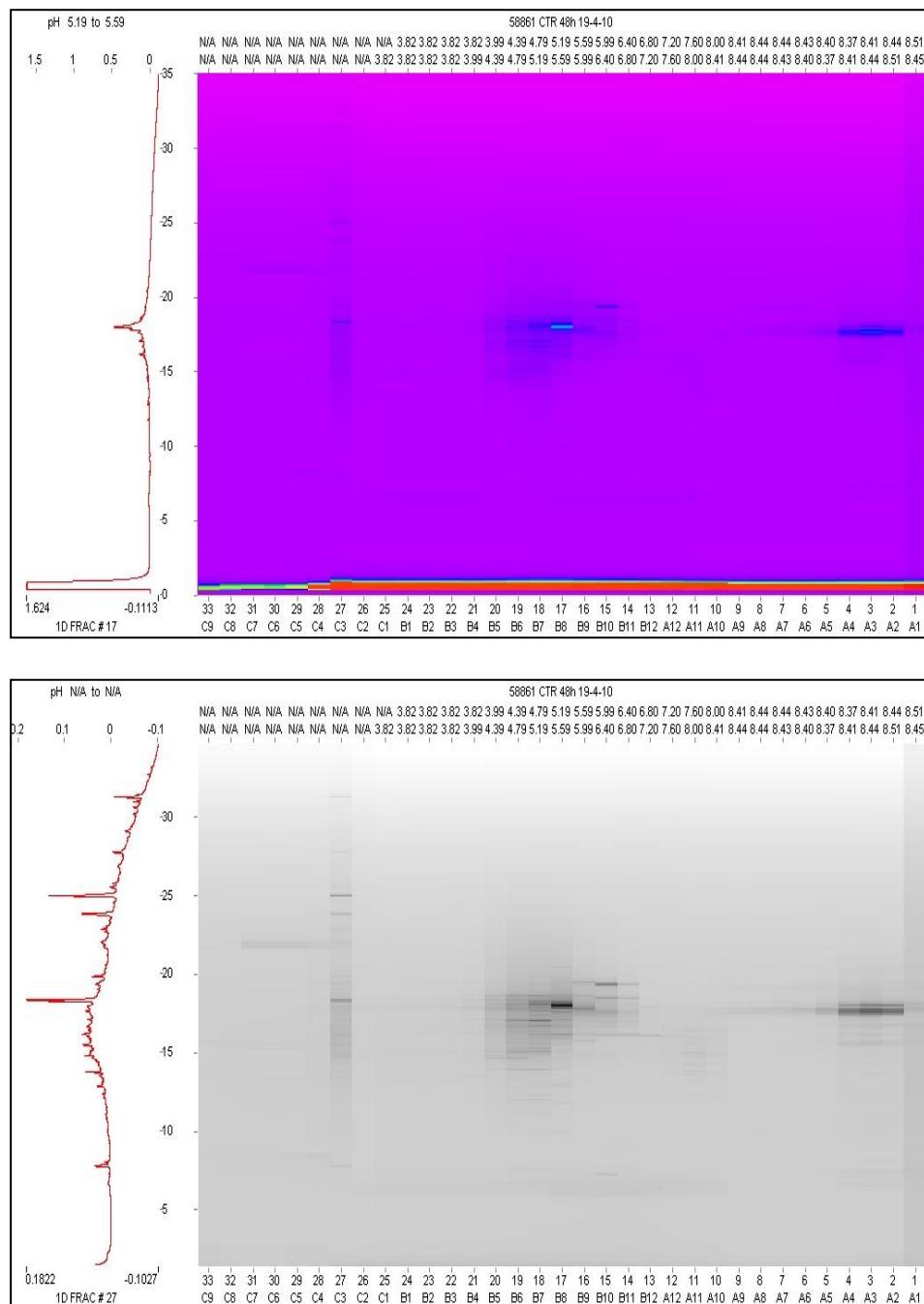


Fig. 38: ProteoVue 2-D expression map of a protein leaf extract of 58-861 clone cuttings grown in hydroponic culture with 0 μM CdSO₄ (Control). The x-axis is in isoelectric point (pl) units from 4.0 to 8.0. The y-axis displays increasing hydrophobicity. The colour scale of the bands represents the relative intensity of each band by UV detection at 214 nm. The chromatogram of a single lane (lane 17 and 27) is evidenced on the left. The two figures are two representations of the same map used different colours.

For each clone the protein profile of the treated ($20 \mu\text{M}$ of CdSO_4) and untreated ($0 \mu\text{M}$ of CdSO_4) samples were analysed. A total of 600 resolved proteins were detected for each treatment and the data were visualized on twelve different “virtual gels” by using the ProteoVue software (*Eprogen, Darien, IL, USA*) (an example is shown in figure. 38).

The comparison of protein profiles between 0 (control) and $20 \mu\text{M}$ of CdSO_4 (treatment) in each poplar clone was performed by DeltaVue software (*Eprogen, Darien, IL, USA*) (an example is shown in figure. 39). This software generates “differential maps” in which the bands corresponding to protein peaks have different shades of colours, with different intensity, according to their abundance in the treated sample compared to the control sample.

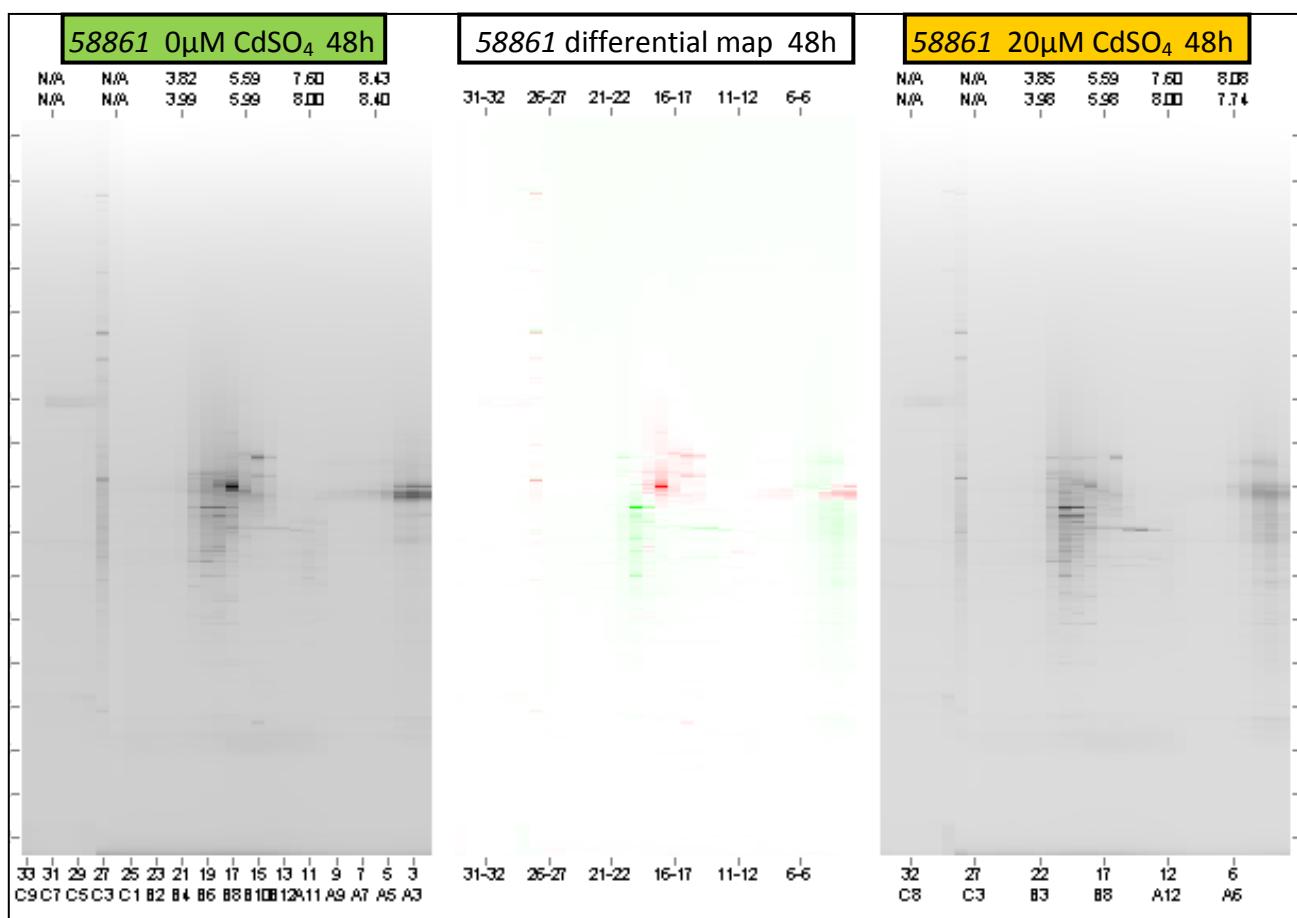


Fig. 39: 2-D differential maps of 58-861 clone leaf protein extraction after short term treatment. The 2-D virtual gel of control plants ($0 \mu\text{M}$ CdSO_4) is shown on the left and the 2-D virtual gel of treated plants ($20 \mu\text{M}$ CdSO_4) is shown on the right. The central lane is a differential map of the protein expression of the control vs $20 \mu\text{M}$ CdSO_4 protein samples. Proteins that showed an higher concentration in control sample are in shades of red, proteins that showed an higher concentration in treated sample are in shades of green.

In detail, this software compares peaks corresponding to the same protein in the 0 and $20 \mu\text{M}$ of CdSO_4 treatments, allowing a quantification by subtractive analysis (an example is shown in figure. 40). For each pair of peaks a value L/R is obtained which represents the ratio between the

absorbance value of that peak in the control sample and the absorbance value of the same peak in the treated sample, and vice versa (R/L) (upper table in figure 40).

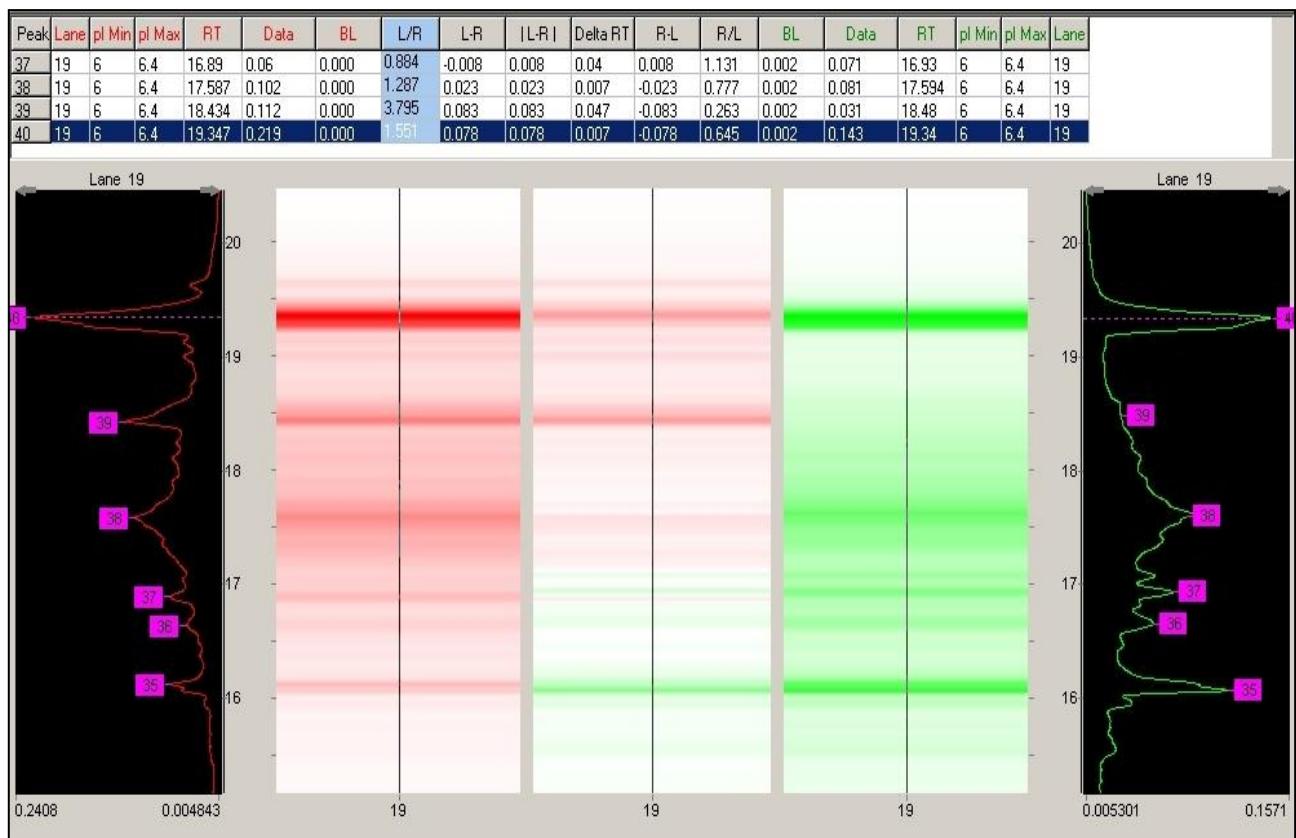


Fig. 40: 2-D differential maps (central lane) of 58-861 clone leaf protein extraction after short term treatment (19th pH fraction). The protein pattern of control plants ($0 \mu\text{M} \text{CdSO}_4$) is shown in shades of red and the protein pattern of treated plants ($20 \mu\text{M} \text{CdSO}_4$) is shown in shades of green. The central lane is a differential map of the protein expression of the control vs $20 \mu\text{M} \text{CdSO}_4$ protein samples. The yellow squares correspond to the proteins which show a level of differential abundance more than 1,5 times respectively in the control and in the treated condition. In the upper table there are quantitative data from each peaks obtained by subtracting the area of the peak in the red lane from the corresponding peak area in the green lane (L/R) and vice versa (R/L).

In order to obtain a graphical representation of how the entire proteome of the three clones was affected by cadmium presence, we performed a scatter diagram of the absorbance values, measured by UV detection at 214 nm, of all isolated peaks from the proteomic analysis of untreated sample (X-axis) and treated sample (Y-axis) after the short (Fig. 41A) and the long term cadmium treatment (Fig. 41B). All the peaks with similar absorbance values, from the differential analysis between treated and untreated samples, were represented as points close to the 45° bisect line; all the peaks with different absorbance values, and therefore more or less abundant in treated samples, are represented as points distant from the 45° bisect line (Fig. 41).

The clone A4A seemed to be less affected by the short term cadmium treatment because the points representing its differential analysis, in the Scatter diagram, are closer to the bisect line, while the

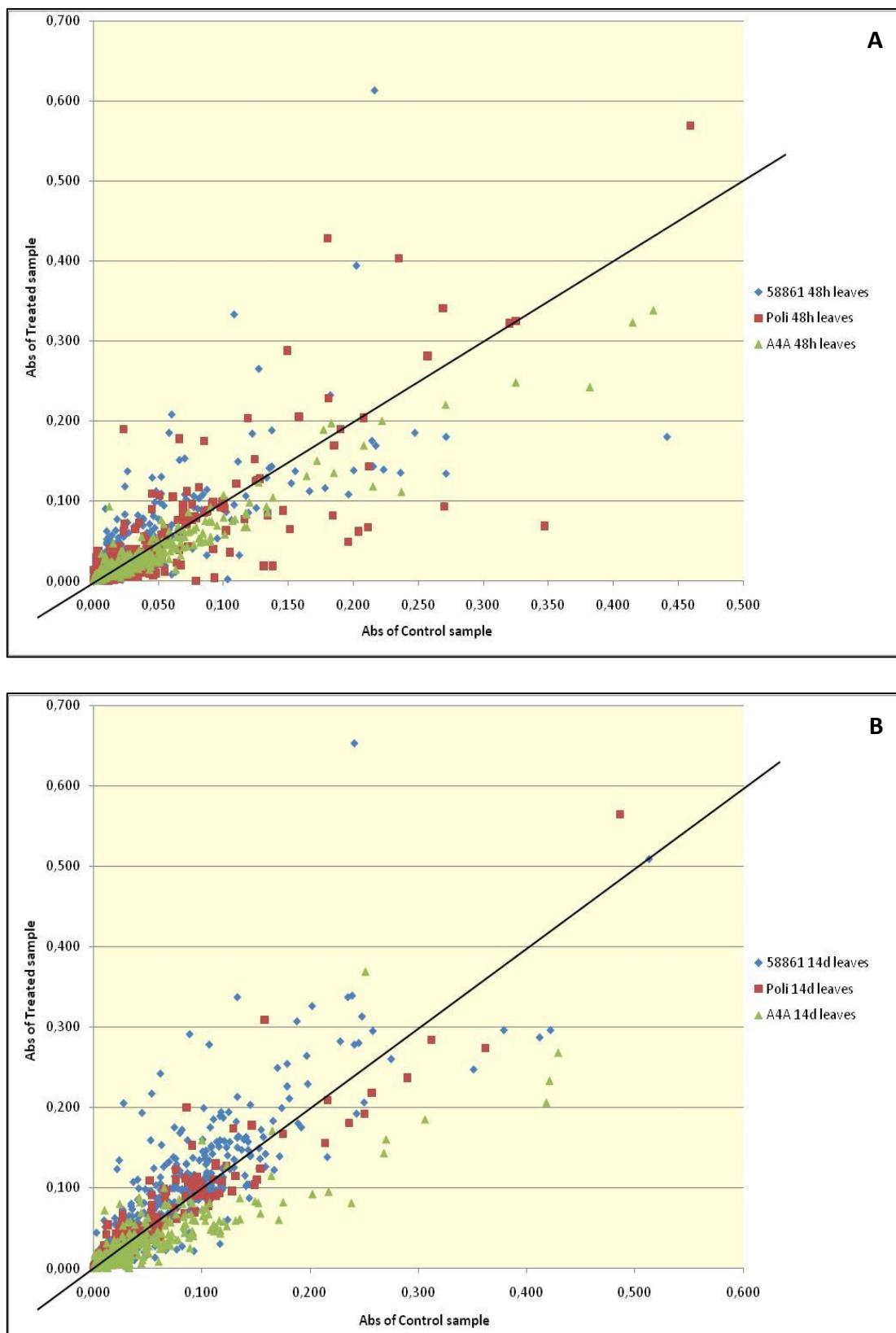


Fig. 41: Scatter diagram of the absorbance values measured by UV detection at 214 nm of all isolated peaks from the proteomic analysis of untreated sample (X-axis) and treated sample (Y-axis). The upper diagram refers to proteomic analysis after short term treatment (48h) and the lower diagram refers to proteomic analysis after long term treatment (14d). The symbols according to the legend represent three different poplar clones. All the peaks with similar absorbance values, from the differential analysis between treated and untreated samples, are represented as points close to the 45° bisect line.

points for the other clones are more scattered, hence they have been more affected by the short term cadmium treatment (Fig. 41A).

On the contrary, after the long term treatment, clones 58-861 and *Poli* seemed to be less affected by cadmium treatment than the short term treatment and clone *A4A* seems to be more affected by cadmium long term treatment (Fig. 41B).

For each of the six comparisons a list of the 600 R/L ratios was obtained. In order to assess which of these values were significant, the logarithm (\log_{10}) of these values was calculated and the results were grouped into frequency categories; all values were distributed according to a Gaussian curve, and then, the values that were higher than the addition between average and double standard deviation ($\mu+2\sigma$) and less than the subtraction between average and double standard deviation ($\mu-2\sigma$), were the values considered statistically significant ($p<0,05$) and chosen for subsequent analysis (Fig.42). In all cases analyzed, these values considered statistically significant, corresponded to proteins over abundant or under-abundant, more than three times, in treated sample compared to control sample.

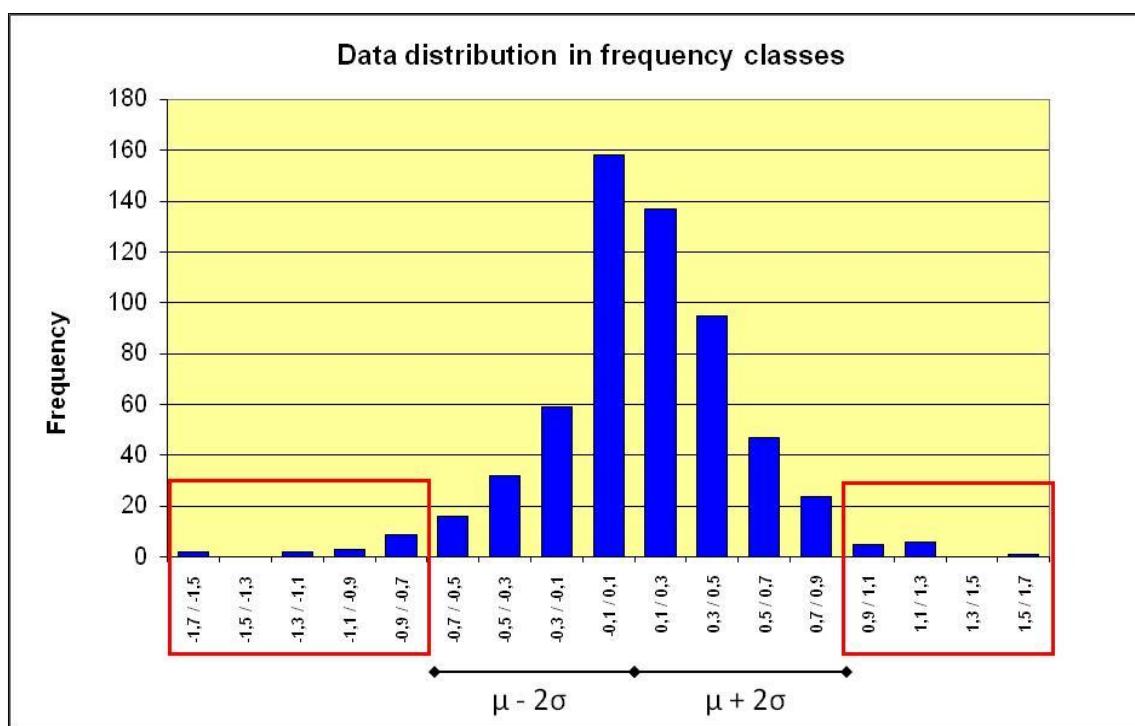


Fig. 42: Distribution in frequency classes of logarithm of all ratios R/L obtained with DeltaVue software in the comparison between control sample and treated sample after short term treatment for the clone 58-861. Significant values contained into two tails of the normal distribution are highlighted in red squares.

SHORT TERM TREATMENT (48h)

Figure 43, shows how many proteins are more than 2.5 times abundant in the treated condition compared to the untreated condition and vice versa, in each clone after the short term treatment. Both poplar clones *58-861* and *Poli* had more differentially abundant proteins between control and treated samples than the clone *A4A*. Therefore, the leaf proteome of clone *A4A* seemed to be less affected by the short term cadmium treatment than the other two clones.

With regard to the *P. Nigra* clone *58-861* the DeltaVue software identified 175 proteins displaying a more than 2.5-fold increased or decreased in response to the short term cadmium treatment; 122 of these proteins had an increase or decrease from 2.5-fold to 5-fold, 38 from 5-fold to 10-fold and 15 more than 10-fold (Fig.43). With regard to the other *P. Nigra* clone *Poli* the DeltaVue software identified 168 proteins more than 2.5-fold increased or decreased in response to short term cadmium treatment; 118 of these proteins had an increase or decrease from 2.5-fold to 5-fold, 36 from 5-fold to 10-fold and 14 more than 10-fold (Fig.43). Therefore, the leaf proteome of the last two poplar clones appeared to be influenced in a similar way by exposure to cadmium for 48 hours. But, in the clone *Poli*, most of these proteins were more abundant in control plants compared to plants exposed to the metal. On the contrary, in the clone *58-861*, most of these proteins were abundant in treated plants compared to control plants. This important difference could be caused by different physiological response of the two clones after short-term treatment and by their different translocation capacities shown by previous analysis (Fig. 21 ,Par. 2.3.2). With regard to the clone *A4A*, the DeltaVue software identified only 39 proteins with an increase/decrease more than 2.5-fold in response to the short term cadmium treatment; 32 of these proteins from 2.5-fold to 5-fold increased or decreased, 6 from 5-fold to 10-fold and only 1 more than 10-fold (Fig.43).

SHORT TERM TREATMENT			
	2.5 - 5 fold	5-10 fold	>10 fold
58-861 0 µM CdSO ₄	34	11	5
58-861 20 µM CdSO ₄	88	27	10
TOT.	122	38	15
Poli 0 µM CdSO ₄	100	26	8
Poli 20 µM CdSO ₄	18	10	6
TOT.	118	36	14
A4A 0 µM CdSO ₄	19	2	0
A4A 20 µM CdSO ₄	13	4	1
TOT.	32	6	1

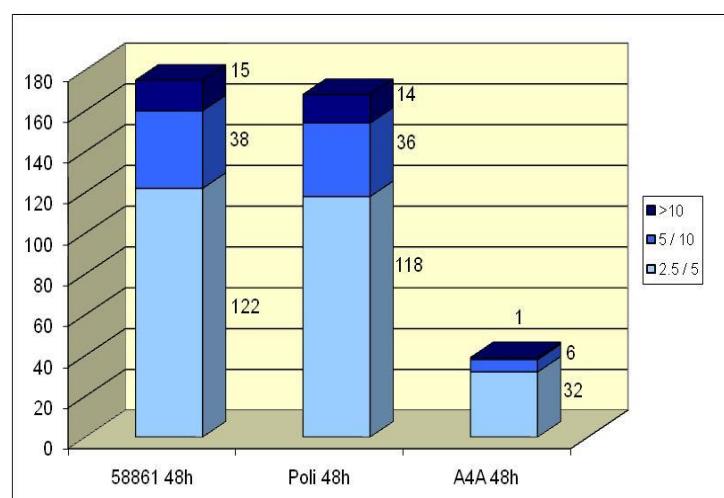


Fig. 43: Abundance rate of the proteins in leaves. Number of proteins 2.5-fold abundant or more in the control condition ($0 \mu\text{M CdSO}_4$) related to treated condition ($20 \mu\text{M CdSO}_4$) and vice versa, after the short term treatment. The graph on the right shows the number proteins more or less abundant than 2.5-5 times (in light blue), than 5-10 times (blue) or more than 10 times (dark blue), in the treated samples compared to the control samples.

LONG TERM TREATMENT (14d)

The figure 44, shows how many proteins are more abundant than 2.5 times in the treated conditions compared to the untreated condition and vice versa, after the long term treatment. Both poplar clones 58-861 and *Poli* had less differentially abundant proteins between control and treated samples than clone *A4A*.

The last one showed 116 proteins increased/decreased more than 2.5-fold in response to the long term cadmium treatment; 97 of these proteins from 2.5-fold to 5-fold, 12 from 5-fold to 10-fold and 7 more than 10-fold (Fig.44).

With regard to the clone *Poli*, the DeltaVue software identified 32 proteins increased/decreased more than 2.5-fold in response to short term cadmium treatment; 31 of these proteins from 2.5-fold to 5-fold, only 1 from 5-fold to 10-fold and no proteins were more or less abundant than 10-fold in control plants compared to treated plants (Fig.44).

With regard to the clone 58-861, the DeltaVue software identified 38 proteins increased/decreased more than 2.5-fold in response to short term cadmium treatment; 32 of these proteins from 2.5-fold to 5-fold, 5 from 5-fold to 10-fold and only 1 more than 10-fold (Fig 44).

Therefore, the leaf proteome of clone *A4A* seemed to be more affected by the long term cadmium treatment than the other two clones.

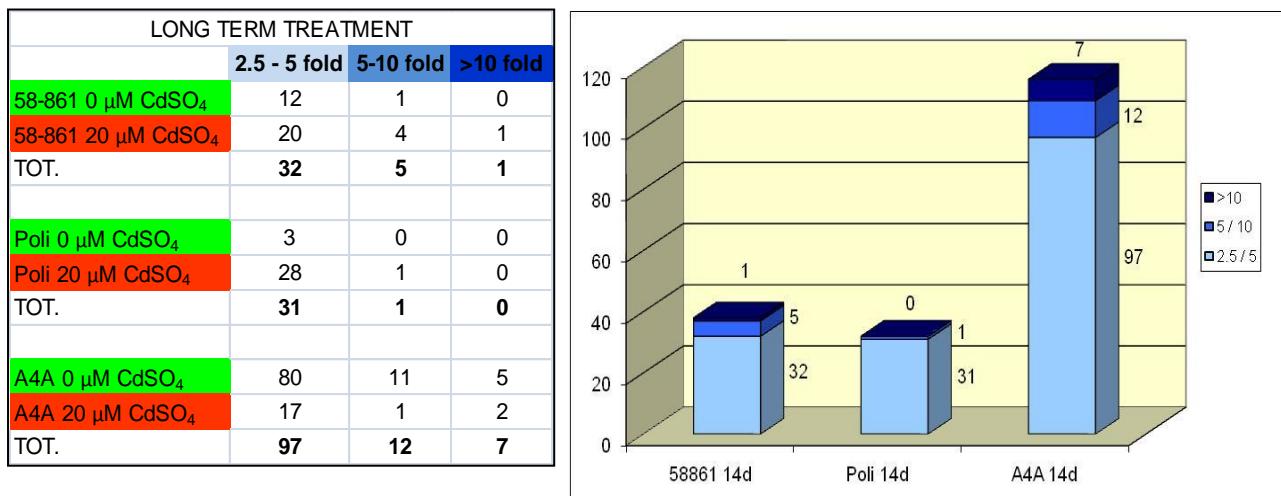


Fig. 44: Abundance rate of the proteins in leaves. Number of proteins 2.5-fold abundant or more in the control condition (0 µM CdSO₄) related to treated condition (20 µM CdSO₄) and vice versa, after the long term treatment. The graph on the right shows the number of proteins more or less abundant than 2.5-5 times (in light blue), than 5-10 times (blue) or more than 10 times (dark blue), in the treated samples compared to the control samples.

2.3.5 Proteins identification

Some of the isolated proteins were subjected to identification by mass spectrometry (MS), in order to understand the molecular basis underlying the different responses to cadmium contamination.

The MALDI Spectra obtained (Fig 45) by MALDI-LR in TOF/MS mode and in “reflectron” mode (materials and methods, 2.2.11) were used to enter the Swiss-Prot database to gain information on the biological identity of the proteins whose peaks have been obtained. The Mass spectrometry combined with “in silico” analysis provided identity information on the putative proteins represented by the peaks including their exact protein mass (Mr) and theoretical isoelectric point (pI).

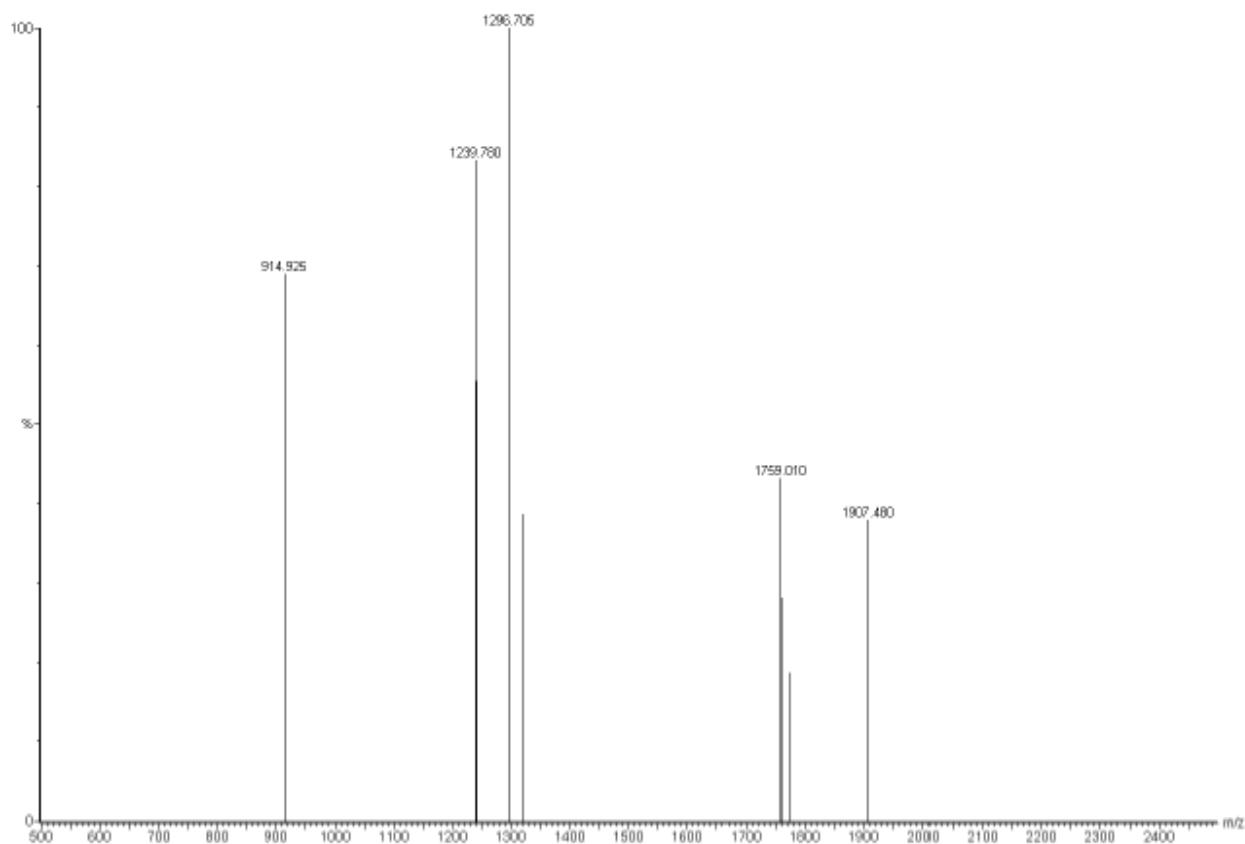


Fig. 45: Mass Spectrum of one identified protein.

For some of the proteins the experimental isoelectric point (pI_{app}) value showed only a partial correspondence with the theoretical pI value, but for most of the proteins identified the correspondence was acceptable. Similar inconsistencies have been described by other authors (Soldi *et al.*, 2005) and have been attributed to the fact that in liquid chromatography systems the pI_{app} is influenced also by the concentrations of ions present in the liquid and adsorbed phase and by the binding of a small part of the charged protein to the ion exchanger (A.S. Essander *et al.*, 2005; X. Kang *et al.*, 2003).

Subsequently, the amino acid sequence of the identified protein was blasted against the *Populus trichocarpa* EST database (<http://www.populus.db.umu.se>) to find a match with the putative protein identified through Mascot research.

Tables 4, 5, 6a, 6b and 7 show the putative identifications by “PMF” (Peptide Mass Fingerprinting) made by MALDI TOF/MS analysis of some isolated protein after differential proteomic analysis between control and treated plants of the three poplar clones (*Poli*, 58-861, A4A) for the short (Tab. 4, 5) and the long term treatments (Tab. 6a, 6b, 7).

Tables 4, 6a and 6b contain together the identified proteins for the clones *Poli* and 58-861 because the differential proteomic analysis of these clones showed that many proteins were similarly more or less abundant in treated plants compared with control plants of these two clones, although with different ratios.

For each putative identification step the parameters reported are: fraction and peak number as given by *Proteovue* Software after 2D-LC, putative protein identification and accession number of the closest match in the Swiss-Prot database by Mascot research, ratio L/R if the peaks were more abundant in the control samples or ratio R/L if the peaks were more abundant in the treated samples, organism, predicted mass value and theoretical pI of the closest match in the Swiss-Prot database, score and percentage of coverage of the matching peptide sequence tags resulting by MASCOT algorithm, matches and coverage identified by tBLASTn search in *poplar* EST database (PopulusDB, website: <http://www.populus.db.umu.se>).

2.3.5.1 *Poli* AND 58-861 CLONES AFTER THE SHORT TERM TREATMENT (Tab. 4)

Proteomic data showed that several proteins involved in plant defense, stress response and oxidative stress response were more-abundant in *P.Nigra Poli* and 58-861 clones treated for 48 hours with cadmium.

PLANT DEFENCE – STRESS RESPONSE

Protein 354 (**Probable disease resistance protein At1g63360**), 551 (**Disease resistance protein RPM1**) and 584 (**Probable protein phosphatase 2C 59**) are three disease resistance proteins that modulates defense responses to pathogenic bacteria and pathogenic attacks. The last one promotes salicylic acid (SA) accumulation and it is known as this acid is essential for expression of multiple modes of plant disease resistance (Delaney *et al.*, 1994). This upregulation of pathogens related protein could indicate that similar signalling pathways are shared between biotic stress response and heavy metals exposure. The protein 567 (**17.5 kDa class I heat shock protein**) belongs to the small heat shock proteins (HSP20) family, involved in stress response (Nagao *et al.*, 1985).

frac/peak ^a	clone	Protein name (SWISS PROT database) ^b	L/R ^c	R/L ^c	Accession n. ^d	Organism ^e	Mw(kDa) ^f	pl ^g	score ^h	cov. ⁱ	EST poplar ^j	cov. ^k
PLANT DEFENCE - STRESS RESPONSE												
21 - 354	<i>both</i>	Probable disease resistance protein At1g63360			4,54	DR120_ARATH	Arabidopsis Thaliana	102439	6,87	27	27%	T106G09
31 - 551	<i>both</i>	Disease resistance protein RPM1			8,10	RPM1_ARATH	Arabidopsis Thaliana	107556	8,58	38	8%	R013C06
31 - 567	<i>both</i>	17.5 kDa class I heat shock protein			2,90	HSP13_SOYBN	Soybean	17535	5,33	34	18%	P070F06
32 - 584	<i>both</i>	Protein phosphatase 2C_59			36,36	P2C59_ARATH	Arabidopsis Thaliana	33341	4,67	27	16%	N007D06
OXIDATIVE STRESS RESPONSE												
17 - 253	<i>58-861</i>	Putative poly [ADP-ribosel] polymerase 3			3,36	PARP3_MEDTR	Medicago Truncatula	91074	5,20	37	7%	F116F28.5pR
28 - 582	<i>both</i>	Peroxidase 5			3,73	PER5_ARATH	Arabidopsis thaliana	35007	8,24	50	38%	
PHOTOSYNTHESIS												
20 - 423	<i>Poli</i>	Phosphoenolpyruvate carboxylase 4			6,40	CAPP4_ARATH	Arabidopsis Thaliana	117254	6,68	35	6%	FL_GENBANK92
21 - 346	<i>58-861</i>	Cytochrome c6, chloroplastic			9,30	CYC6_ARATH	Arabidopsis Thaliana	19540	6,73	23	13%	S077A06
33 - 661	<i>both</i>	Photosystem I reaction center subunit IV B, chloroplastic			10,40	PSAEB_NCSY	Nicotiana sylvestris	15215	9,74	20	13%	V040B07
CARBOHYDRATE METABOLISM												
20 - 424	<i>Poli</i>	Ribulose bisphosphate carboxylase large chain			3,09	RBL_PELAN	Pellaea andromedifolia	49149	6,39	35	12%	R040F02
23 - 477	<i>58-861</i>	Ribulose bisphosphate carboxylase small chain SSU40A			5,30	RBS3_LEMGI	Lemma gibba	20074	8,24	50	38%	M116D05
23 - 489	<i>both</i>	Ribulose bisphosphate carboxylase small chain SSU1			5,77	RBS1_LEMGI	Lemma gibba	19795	8,24	49	32%	M116D05
GLYCOLYSIS												
20 - 340	<i>58-861</i>	Enolase 1			5,88	ENO1_MAIZE	Zea mays	48262	5,20	34	18%	R005C03
PROTEOLYSIS												
32 - 578	<i>58-861</i>	U-box domain-containing protein			15,29	PUB48_ARATH	Arabidopsis Thaliana	51330	6,18	43	12%	/
PROTEIN FOLDING												
23 - 387	<i>both</i>	Chaperonin HSP60, mitochondrial			13,36	CH60_SOLTU	Solanum tuberosum	4237	8,48	34	75%	P069C06
MOLECULAR TRANSPORTERS												
20 - 321	<i>both</i>	Magnesium transporter MRS2_E			38,63	MRS2E_ORYSI	Oryza sativa	46543	5,06	38	11%	/
LIGNIN and ISOPRENOID BIOSYNTHESIS												
32 - 585	<i>58-861</i>	Pentatricopeptide repeat-containing protein At4g14850			4,64	PR512_ARATH	Arabidopsis Thaliana	77037	8,13	42	11%	/
LIPID SYNTHESIS												
20 - 341	<i>both</i>	AcyI-[acyl-carrier-protein] desaturase			7,43	STAD_CARTI	Carthamus tinctorius	45197	6,85	38	16%	V033E12
MISCELLANEOUS												
7 - 64	<i>58-861</i>	Pentatricopeptide repeat-containing protein At1g28020			43,57	PPR61_ARATH	Arabidopsis Thaliana	65610	8,54	49	14%	RD33F12
												35%

Table 4: Proteins separated by 2D-LC and identified by MALDI-TOF/MS and “*in silico*” analysis for 58-861 and *Poli* clones after the short term treatment. ^afraction and progressive peak number as given by 2D-LC, ^bputative protein identification and ^daccession number for the closest match in the Swiss-Prot database, ^cL/R ratio or R/L ratio, ^eorganism, ^fpredicted mass value and ^gpl of the closest match in the database, ^hscore and ⁱpercentage of coverage of the matching peptide sequence tags resulting by MASCOT algorithm, ^jmatches and ^kpercentage of coverage identified by tBLASTn search in *poplar* EST database *PopulusDB*.

frac/peak ^a	Protein name (SWISS PROT database) ^b	L/R ^c	R/L ^c	Accession n. ^d	Organism ^e	Mw(kDa) ^f	pI ^g	score ^h	cov. ⁱ	EST poplar ^j	cov. ^k
PLANT DEFENCE - STRESS RESPONSE											
2 - 10	Levopimaradiene synthase, chloroplastic	2,776	TPSD1_PNTA	Pinus taeda	98178	5,52	40	6%	N032C12	38%	
3 - 22	Putative disease resistance protein RGA3	3,05	RGA3_SOLBU	Solanum bulbocastanum	114864	6	48	9%	V044B09	44%	
21 - 269	Pathogenesis-related protein 1	2,85	PR1_ASPOF	Asparagus officinalis	17025	6,5	35	20%	R035F11	37%	
OXIDATIVE STRESS RESPONSE											
3 - 20	Protein SRG1	8,11	SRG1_ARATH	Arabidopsis Thaliana	41184	5,3	41	21%	P04H04	60%	
25 - 514	Ferritin-2, chloroplastic	3,33	FR12_ARATH	Arabidopsis thaliana	28360	5,53	25	14%	M128D09	72%	
27 - 551	Monothiol glutaredoxin-S17	2,81	GRS17_ARATH	Arabidopsis thaliana	53424	5,01	64	17%	UK105TE10	70%	
PHOTOSYNTHESIS											
3 - 18	Phosphoenolpyruvate carboxylase 4	5,61	CAPP4_ARATH	Arabidopsis Thaliana	117254	6,68	42	7%	X018E01	87%	
3 - 21	Light-independent protochlorophyllide reductase	4,90	CHLN_CHLRE	Chlamydomonas reinhardtii	61571	8,3	44	13%	/	/	
24 - 491	Photosystem I reaction center subunit II-2, chloroplastic	3,01	PSAD2_ARATH	Arabidopsis thaliana	22350	9,78	42	21%	S033A09	74%	
CARBOHYDRATE METABOLISM											
9 - 108	Alpha-1,4 glucan phosphorylase L isozyme	2,90	PHSL_VICFA	Vicia faba	114079	5,63	46	7%	UA53DF08_3pR	87%	
25 - 515	Beta-fructofuranosidase, insoluble isoenzyme 6	3,46	INV6_ORYSJ	Oryza sativa	65159	5,14	52	11%	Q001H02	57%	
PROTEOLYSIS											
13 - 160	SKP1-like protein 19	3,14	ASK19_ARATH	Arabidopsis thaliana	22872	4,44	43	29%	A056P33_3pR	56%	
25 - 513	Nicastrin	3,42	NICA_ARATH	Arabidopsis thaliana	73645	6,04	45	8%	Q048C06	42%	
ATP SYNTHESIS coupled PROTON TRANSPORT											
17 - 272	V-type proton ATPase catalytic subunit A	2,89	VATA_BETVU	Beta vulgaris	68790	5,1	48	12%	/	/	
21 - 369	ATP synthase subunit beta	2,75	ATPB_NEPOL	Nephroselmis olivacea	52389	5,23	41	14%	K024F84	78%	
10 - 119	GDP-mannose 4,6 dehydratase 1	3,39	GMD1_ARATH	Arabidopsis thaliana	40939	6,61	52	14%	V041B08	82%	
TRANSCRIPTION and TRANSLATION											
25 - 512	50S ribosomal protein L21, chloroplastic	4,34	RK21_PSIINU	Psilotum nudum	13801	10,3	30	16%	F06GP38_3pR	37%	
AMINOACID BIOSYNTHESIS											
8 - 102	Glutamate synthase 1 [NADH]	3,50	GLUT1_ARATH	Arabidopsis Thaliana	243741	5,9	44	4%	UA52DPB04	62%	
20 - 341	Histidinol dehydrogenase	3,04	HISX_ARATH	Arabidopsis Thaliana	50747	5,8	57	13%	F094P29	89%	
21 - 372	Cysteine synthase, chloroplastic/chromoplastic	3,09	CYSKP_ARATH	Arabidopsis thaliana	41925	8,13	44	13%	T085E08	75%	
22 - 420	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 1	4,64	ARD1_ORYSI	Oryza sativa	23855	5,07	42	22%	P063G10	73%	
NITROGEN and SULFUR METABOLISM											
28 - 567	Molybdopterin synthase catalytic subunit	6,02	MOC2B_ORYSI	Nuphar advena	21022	5,5	43	17%	R002D05	64%	
MISCELLANEOUS											
7 - 73	Probable alpha,alpha-trehalose-phosphate synthase	5,47	TPS4_ARATH	Arabidopsis thaliana	90330	6,09	37	8%	X020E03	69%	
34 - 718	Probable protein phosphatase 2C 24	4,32	P2C24_ARATH	Arabidopsis thaliana	41241	5,34	30	8%	F029P39_3pR	67%	

Table 5: Proteins separated by 2D-LC and identified by MALDI-TOF/MS and “*in silico*” analysis for the A4A clone after the short term treatment. ^afraction and progressive peak number as given by 2D-LC, ^bputative protein identification and ^daccession number for the closest match in the Swiss-Prot database, ^cL/R ratio or R/L ratio, ^eorganism, ^fpredicted mass value and ^gpI of the closest match in the database, ^hscore and ⁱpercentage of coverage of the matching peptide sequence tags resulting by MASCOT algorithm, ^jmatches and ^kcoverage identified by tBLASTn search in *poplar* EST database *PopulusDB*.

frac/peak ^a	clone	Protein name (SWISS PROT database) ^b	L/R ^c	R/L ^c	Accession n. ^d	Organism ^e	Mw(kDa) ^f	pI ^g	score ^h	cov. ⁱ	EST.poplar ^j	cov. ^k
		PLANT DEFENCE - STRESS RESPONSE										
7 - 54	<i>Poli</i>	17.5 kDa class I heat shock protein	2.87	HSP13_SOYBN	Soybean	17535	5.33	34	18%	P070F06	82%	
7 - 87	both	Putative defensin-like protein 230	2.98	DF230_ARATH	Arabidopsis thaliana	10726	8.64	40	24%	V01TA12	28%	
12 - 224	58-861	Putative late blight resistance protein homolog R1A-3	3.90	R1A3_SOLDE	Solanum demissum	89645	6.43	40	9%	I051PB2.5pR	33%	
27 - 512	58-861	Heat stress transcription factor A4b	3.12	HFAB_ORYSU	Oryza sativa subsp. Jap.	49528	5.26	34	12%	T064E07	44%	
27 - 515	58-861	ABC transporter B family member 19	2.80	AB19B_ARATH	Arabidopsis thaliana	137215	8.38	46	5%	X017B03	63%	
		OXIDATIVE STRESS RESPONSE										
2 - 111	58-861	Peroxidase 5	5.60	PER5_ARATH	Arabidopsis thaliana	35007	8.24	50	38%	FL_GENBANK92	49%	
8 - 98	both	1-Cys peroxiredoxin	3.26	REHY_MEDTR	Medicago truncatula	24569	6.08	60	25%	S067D06	78%	
		PHOTOSYNTHESIS										
7 - 88	58-861	Light-independent protoclorophyllide reductase subunit N	3.88	CHLN_CHLAT	Chlorokybus atmophyticus	54247	5.70	37	16%	/	/	
15 - 261	58-861	Photosystem I reaction center subunit IVB, chloroplastic	3.39	PSAEB_NICSY	Nicotiana sylvestris	15215	9.74	20	13%	V040E07	71%	
24 - 409	both	Light-independent protoclorophyllide reductase subunit N	2.80	CHLN_CHLRF	Chlamydomonas reinhardtii	61571	8.30	62	21%	/	/	
		CARBOHYDRATE METABOLISM										
21 - 339	58-861	Ribulose bisphosphate carboxylase/oxygenase activase A	2.73	RCAA_HORVU	Hordeum vulgare	51383	8.04	34	13%	UL98TD06	92%	
15 - 260	58-861	Ribulose bisphosphate carboxylase large chain (Fragment)	4.44	RBL_PELAN	Pellelea andromedifolia	49149	6.39	35	12%	R040F02	52%	
15 - 262	58-861	Ribulose bisphosphate carboxylase small chain SSU40A	3.87	RBS3LEMGI	Lemna gibba	20074	8.24	50	38%	M116D05	70%	
24 - 414	<i>Poli</i>	Ribulose bisphosphate carboxylase small chain, chloroplastic	2.50	RBS_MUSAC	Musa acuminate	20839	9.23	31	27%	V049D08	71%	
24 - 415	<i>Poli</i>	Ribulose bisphosphate carboxylase small chain SSU1	2.80	RBS1LEMGI	Lemna gibba	19795	8.24	49	32%	M116D05	72%	
25 - 433	58-861	Ribulose bisphosphate carboxylase small chain, chloroplastic	2.70	RBS_HEVBR	Hevea brasiliensis	21049	8.70	45	25%	S062F04	79%	
		GLYCOLYSIS										
1 - 4	<i>Poli</i>	Bifunctional enolase 2/transcriptional activator	3.63	ENO2_ARATH	Arabidopsis thaliana	47719	5.38	42	21%	T084D02	90%	
17 - 253	<i>Poli</i>	Sucrose-phosphatase 1	2.80	SPPI_MAIZE	Zea mays	47583	5.48	26	13%	V02B11	78%	
17 - 247	<i>Poli</i>	Glucose-1-phosphate adenylyltransferase small subunit	3.18	GLGS_ARATH	Arabidopsis thaliana	56957	6.13	27	9%	V041D08	80%	
18 - 286	<i>Poli</i>	L-type lectin-domain containing receptor kinase VII.1	2.55	LRK71_ARATH	Arabidopsis thaliana	76930	6.21	51	14%	UR108TC05	47%	
		PROTEOLYSIS										
13 - 229	both	Proteasome subunit alpha type-3	2.7	PSA3_ORYSU	Oryza sativa subsp. Jap.	27506	5.75	32	22%	Q003A09	86%	
		NUCLEOTIDES and ATP SYNTHESIS, PROTON TRANSPORT										
11 - 198	both	Nucleoside diphosphate kinase 3	2.65	NDK3_SPIOL	Spinacia olaracea	17107	8.12	38	30%	S013B10	82%	
17 - 249	<i>Poli</i>	ATP synthase subunit beta, chloroplastic	2.80	ATPB_TRAFO	Trachycarpus fortunei	53644	5.39	47	14%	K024P84	79%	
15 - 270	58-861	Adenylosuccinate synthetase 1, chloroplastic	7.28	PURA1_PHYPAA	Physcomitrella patens	56101	6.25	34	9%	G069P38	67%	

Table 6a: Proteins separated by 2D-LC and identified by MALDI-TOF/MS and “*in silico*” analysis for 58-861 and *Poli* clones after the long term treatment. ^afraction and progressive peak number as given by 2D-LC, ^bputative protein identification and ^daccession number for the closest match in the Swiss-Prot database, ^cL/R ratio or R/L ratio, ^eorganism, ^fpredicted mass value and ^gpI of the closest match in the database, ^hscore and ⁱpercentage of coverage of the matching peptide sequence tags resulting by MASCOT algorithm, ^jmatches and ^kcoverage identified by tBLASTn search in *poplar* EST database *PopulusDB*.

frac/peak ^a	clone	Protein name (SWISS PROT database) ^b	L/R ^c	R/L ^c	Accession n. ^d	Organism ^e	Mw(kDa) ^f	pI ^g	score ^h	cov. ⁱ	EST.poplar ^j	cov. ^k
TRANSCRIPTION and TRANSLATION												
8 - 76	<i>both</i>	Pentatricopeptide repeat-containing protein At2g45350	3,40	PP202_ARATH	Arabidopsis thaliana	70052	6,78	39	10%	T02G12	40%	
18 - 273	<i>Poli</i>	40S ribosomal protein S10-1	2,57	RS101_ARATH	Arabidopsis thaliana	19549	9,69	47	33%	P005G10	82%	
19 - 324	58-861	30S ribosomal protein S15, chloroplastic	2,72	RR15_AETCO	Aethionema cordifolium	10735	11,25	30	30%	/	/	
22 - 375	58-861	30S ribosomal protein S3, chloroplastic	2,69	RR3_EUCGG	Eucalyptus globulus	25081	9,97	51	26%	P034F11	87%	
11 - 202	58-861	ASCORBATE METABOLISM	4,29	IDND_VITV1	Vitis vinifera	40181	6,66	39	18%	X044G12	80%	
MOLECULAR TRANSPORTERS												
7 - 67	<i>Poli</i>	Potassium channel AKT2	2,56	AKT2_ORYSJ	Oryza sativa subsp. Jap.	95408	6,64	38	10%	FL_GENBANK35	61%	
13 - 155	<i>Poli</i>	Magnesium transporter MR32-E	2,50	MRS2E_ORYSJ	Oryza sativa subsp. Indica	46543	5,06	34	11%	/	/	
16 - 233	<i>Poli</i>	Aquaporin NIP2-1	2,64	NIP21_ARATH	Arabidopsis thaliana	30846	6,78	19	14%	M104C11	58%	
24 - 413	<i>Poli</i>	Protein TOC75, chloroplastic	2,90	TOCT5_PEA	Pisum sativum	88728	7,01	53	14%	R068A04	74%	
CELL DIVISION												
17 - 248	<i>both</i>	Auxin-responsive protein IAA6	3,37	IAA6_ORYSJ	Oryza sativa subsp. Jap.	36912	7,64	42	21%	/	/	
19 - 304	<i>Poli</i>	Auxin transport protein BIG	2,50	BIG_ORYSJ	Oryza sativa subsp. Jap.	560057	5,71	65	4%	UA51CP09	8%	
13 - 241	58-861	Phytoinsulin receptor 1	4,03	PSKR1_DAUCA	Daucus carota	113112	6,14	35	8%	UB651DFB11_3pR	49%	
7 - 82	58-861	Cyclin-dependent kinase inhibitor 1	15,41	KRP1_ORYSJ	Oryza sativa subsp. Jap.	27293	9,93	43	24%	S012D05	35%	
12 - 223	58-861	Cell wall	2,21	EXB13_ORYSJ	Oryza sativa subsp. Jap.	24653	5,55	46	22%	P018B12	40%	
MISCELLANEOUS												
7 - 68	<i>Poli</i>	Histone H1	2,59	H1_MAIZE	Zea mays	25333	10,71	33	13%	M03H01	51%	
7 - 71	<i>Poli</i>	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 1	3,40	MTN1_ARATH	Arabidopsis thaliana	28547	4,65	31	20%	Q018H09	71%	
17 - 254	<i>Poli</i>	Ethylene receptor 2	2,85	ETR2_PELHQ	Pelargonium hortorum	83528	6,53	40	11%	F1_G106P76Y	68%	
11 - 199	58-861	Probable protein phosphatase 2C 28	2,71	P2C28_ARATH	Arabidopsis thaliana	26460	5,71	42	20%	S075D07	57%	
16 - 230	<i>Poli</i>	Cysteine-rich receptor-like protein kinase 37	2,55	CRK37_ARATH	Arabidopsis thaliana	74191	7,15	41	11%	X042D07	66%	
27 - 514	<i>both</i>	Pentatricopeptide repeat-containing protein At1g71210	5,48	PP113_ARATH	Arabidopsis thaliana	101735	8,42	40	11%	/	/	

Table 6b: Proteins separated by 2D-LC and identified by MALDI-TOF/MS and “*in silico*” analysis for 58-861 and *Poli* clones after the long term treatment. ^afraction and progressive peak number as given by 2D-LC, ^bputative protein identification and ^daccession number for the closest match in the Swiss-Prot database, ^cL/R ratio or R/L ratio, ^eorganism, ^fpredicted mass value and ^gpI of the closest match in the database, ^hscore and ⁱpercentage of coverage of the matching peptide sequence tags resulting by MASCOT algorithm, ^jmatches and ^kcoverage identified by tBLASTn search in *poplar* EST database *PopulusDB*.

frac./peak ^a	Protein name (SWISS PROT database) ^b	L/R ^c	R/L ^c	Accession n. ^d	Organism ^e	Mw(kDa) ^f	pI ^g	score ^h	cov. ⁱ	EST poplar ^j	cov. ^k	
PLANT DEFENCE - STRESS RESPONSE												
4 - 37	UDP-glycosyltransferase 84A2			4,50	U84A2_ARATH	Arabidopsis thaliana	56334	6,45	39	8%	T095H09	60%
7 - 61	Putative defensin-like protein 2/70			4,43	DF270_ARATH	Arabidopsis thaliana	8579	8,73	41	27%	/	/
3 - 27	Metallothionein-like protein 1			4,25	MT1_MMGJU	Mimulus guttatus	8027	4,55	32	16%	R062D01	67%
6 - 53	Putative defensin-like protein 2/11			3,89	DF211_ARATH	Arabidopsis thaliana	10205	9,72	34	25%	/	/
OXIDATIVE STRESS RESPONSE												
8 - 102	1-Cys peroxiredoxin			3,26	REHY_MEDTR	Medicago truncatula	24569	6,08	60	25%	S067D06	76%
14 - 180	Monothiol glutaredoxin-S5			3,20	GRX55_ORYSJ	Oryza sativa subsp. Jap.	15249	6,84	30	14%	X018F01	44%
PHOTOSYNTHESIS												
7 - 62	Protochlorophyllide reductase iron-sulfur ATP-binding protein			3,22	CHLL_OLTIV	Oltmannsiellopsis viridis	34504	4,78	37	10%	/	/
PROTEOLYSIS												
33 - 653	U-box domain-containing protein 51			7,51	PUB51_ARATH	Arabidopsis thaliana	90566	6,30	41	8%	N002D09	42%
ATP SYNTHESIS PROTON TRANSPORT												
4 - 32	Protein ycf2			11,82	YCF2_PNTTH	Pinus thunbergii	245820	9,46	45	3%	T058A10	52%
TRANSCRIPTION and TRANSLATION												
5 - 43	Elongation factor 2			3,15	EF2_PARKE	Parachlorella kessleri	94853	5,84	46	7%	R019D08	83%
6 - 47	50S ribosomal protein L16, chloroplastic			2,67	RK16_MANES	Manihot esculenta	15347	11,84	34	28%	T035D08	94%
7 - 63	50S ribosomal protein L1, chloroplastic			16,51	RK1_ARATH	Arabidopsis thaliana	37780	9,30	32	10%	N064A08	64%
5 - 46	DNA-directed RNA polymerase subunit beta			17,68	RPOB_ACOC1	Acorus calamus	121626	8,34	37	4%	A029P57_3pR	40%
7 - 59	Protein CUP-SHAPED COTYLEDON			75,22	NAC31_ARATH	Arabidopsis thaliana	38222	0,25	35	6%	X018H04	52%
33 - 651	Maturase K			3,57	MATK_LEMNN	Lemna minuta	60771	9,51	48	9%	/	/
AMINOACID BIOSYNTHESIS												
33 - 649	Homocysteine S-methyltransferase 4			4,60	HMT4_MAIZE	Zea mays	36836	5,39	31	9%	X020A10	62%
LIPID SYNTHESIS												
19 - 338	Acetyl-coenzyme A carboxylase carboxyl transferase sub. Alpha			6,50	ACCA_ARATH	Arabidopsis thaliana	85367	5,60	40	8%	A057P54_3pR	41%
31 - 610	Putative linoleate 9-S-lipoxygenase 3			2,87	LOX3_ORYSJ	Oryza sativa subsp. Jap.	96305	5,30	40	5%	S027D10	63%
MOLECULAR TRANSPORTERS												
7 - 60	Vacuolar protein sorting-associated protein 22 homolog			6,67	VP221_ARATH	Arabidopsis thaliana	28634	9,00	29	6%	/	/
7 - 69	Magnesium transporter MRS2-I			8,77	MRS21_ORYSJ	Oryza sativa subsp. Jap.	42334	4,92	33	14%	/	/
CELL WALL												
6 - 54	Expansin-A14			2,58	EXP14_ARATH	Arabidopsis thaliana	28172	9,69	32	7%	N042D11	78%
14 - 184	Probable pectinesterase 53			3,02	PEE53_ARATH	Arabidopsis thaliana	42913	9,20	25	9%	F131RF5_3pR	72%
14 - 189	45 kDa cell wall protein			3,43	CWP09_PHAVU	Phaseolus vulgaris	1634	8,59	29	80%	/	/
MISCELLANEOUS												
4 - 39	Probable protein phosphatase 2C 57			51,44	P2C57_ORYSJ	Oryza sativa subsp. Jap.	40100	5,32	49	17%	UR108TF11	56%
12 - 148	VaIy-tRNA synthetase			7,67	SYV_ARATH	Arabidopsis thaliana	126816	6,69	38	5%	F061P70_3pR	53%

Table 7: Proteins separated by 2D-LC and identified by MALDI-TOF/MS and “*in silico*” analysis for the A4A clone after the long term treatment. ^afraction and progressive peak number as given by 2D-LC, ^bputative protein identification and ^daccession number for the closest match in the Swiss-Prot database, ^cL/R ratio or R/L ratio, ^eorganism, ^fpredicted mass value and ^gpI of the closest match in the database, ^hscore and ⁱpercentage of coverage of the matching peptide sequence tags resulting by MASCOT algorithm, ^jmatches and ^kcoverage identified by tBLASTn search in *poplar* EST database *PopulusDB*.

OXIDATIVE STRESS RESPONSE

The proteins 253 (**Putative poly [ADP-ribose] polymerase 3**) and 582 (**Peroxidase 5**) were more-abundant in treated plants and they are considered involved in response to oxidative stress. The former is involved in the base excision repair (BER) pathway, by catalyzing the poly(ADP-ribosylation) of a limited number of acceptor proteins involved in chromatin architecture and in DNA metabolism; this modification follows DNA damages and appears as an obligatory step in a detection/signaling pathway leading to the reparation of DNA strand breaks (Lamb *et al.*, 2011). The latter is a peroxidase responsible in removal of H₂O₂, oxidation of toxic reductants, biosynthesis of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding and pathogen attack (Tognolli *et al.*, 2002). In many proteomic studies were found peroxidases to be more abundant in poplars exposed to cadmium contamination (Kieffer *et al.*, 2008; Kieffer *et al.*, 2009).

PHOTOSYNTHESIS

Furthermore, in this study, it appeared that cadmium had a negative effect on the light phase of the photosynthesis through the downregulation of the expression of proteins in the electron transport chain. The proteins 423 (**Phosphoenolpyruvate carboxylase 4**), 346 (**Cytochrome c6**), 661 (**Photosystem I reaction center subunit IV B**), in fact, were less-abundant in treated plants and are involved in the photosynthesis and electron transport chain.

CARBOHYDRATE METABOLISM and GLYCOLYSIS

The carbon metabolism, carbon fixation, calvin cycle and glycolysis showed also numerous key proteins less abundant in conditions of Cd treatment, including several **ribulose bisphosphate carboxylases**, large and small chain (proteins 424, 477, 489). The protein 340 (**Enolase 1**) with phosphopyruvate hydratase activity is also less abundant suggesting that carbon fixation and glycolysis might be less efficient under Cd treatments.

PROTEOLYSIS

Increase abundance of proteolysis related proteins in leaves could be observed for several types of peptidases and proteasome-related proteins throughout the experiment. For example, one protein of this functional class we identified was the protein 578 (**U-box domain-containing protein**) involved in the ubiquitination process.

PROTEIN FOLDING

Several chaperone proteins also showed an increase in abundance; these were mitochondrial heat shock proteins (387, **Chaperonin HSP60**). HSP60 and HSP70 proteins showed an increase in abundance in response to various abiotic stresses, they can prevent aggregation and they assist in refolding of non-native proteins under both normal and stress conditions (Wang *et al.*, 2004; Kieffer

et al., 2009). Various studies have shown an increase in HSPs expression under heavy metal treatments (Hall JL., 2002).

MOLECULAR TRANSPORTERS

The protein 321 (**Magnesium transporter MRS2-E**) showed a decrease in abundance after cadmium short term treatment. This protein is the major transport protein for Mg⁺ uptake into mitochondria and the expression of hMrs2 is essential for the maintenance of respiratory complex I and cell viability (Piskacek., 2008). In plants, Cd can be taken up by roots through the same plasma membrane transporters as those used for other cations such as calcium (Ca), iron (Fe) and zinc (Zn) (Korshunova *et al.*, 1999; Clemens, 2006; Nakanishi *et al.*, 2006; Lux *et al.*, 2011), therefore a reduction of the abundance of cellular magnesium transporters can be explained as an attempt to reduce uptake of the toxic ions by a simple down-regulation of the transport activity. However, recent studies have shown that Mg deficiency also promoted an increase in the iron (Fe) concentration in Cd-treated plants, because high Fe concentrations have previously been reported to prevent the harmful effects of Cd (Hermans *et al.*, 2011).

LIGNIN and ISOPRENOID BIOSYNTHESIS

The protein 585 (**Pentatricopeptide repeat-containing protein At4g14850**) showed an increase in abundance in treated plants. This protein acts as a regulatory factor to promote the isoprenoid biosynthesis. Isoprenoids or terpenes are the major components of resins and essential oils of plants. As already seen above, protein 582 (**Peroxidase 5**) was more-abundant in treated plants, and it is not only involved in response to oxidative stress and removal of H₂O₂, but also it is responsible for the biosynthesis of lignin and it is involved in the process of suberization. Peroxidases are inducible by heavy metal stress and are involved in the regulation of growth and a number of biochemical pathways. The peroxidase protein is considered as a putative wall rigidification enzyme (Cosgrove *et al.*, 1997; Quiroga *et al.*, 2007) because peroxidase activity reduces growth rate (in fact *Poli* and 58-861 clones showed a decrease of growth rate, see section 2.3.1) and it is directly involved in the synthesis of lignin precursors and their cross-linking (Verma *et al.*, 2008).

Previous studies have shown how plant tissues that accumulated large amounts of heavy metals showed higher rates of suberisation and lignifications (Choi *et al.*, 1994). A lignification of cell walls have been described in roots and leaves of cadmium-treated plants, probably resulting from elevated oxidative stress and peroxide content (Cunha *et al.*, 2008; Vollenweider *et al.*, 2006). Heavy metal stress has been repeatedly shown to increase the production of secondary metabolites and to activate stress response pathways which modify cell wall structure (Maksymiec, 2007), and in particular lead to its lignifications (van de Mortel *et al.*, 2006). Furthermore, this experimental data was in accordance with the data from SEM analysis (2.3.5, Scanning Electron Microscopy with

microanalysis) in which the results showed that Cd was present mainly within lignified and suberified tissues. Considering the short term experiment, we may argue whether Cd induced the synthesis of lignin or whether the presence of already lignifies tissues acted as a sink for the contaminant. However, in recent years, some progress has been made determining the ability of lignified material to adsorb and chelate toxic metals (Crist *et al.*, 2002; Marmiroli *et al.*, 2004).

LIPID SYNTHESIS

Proteins involved in lipid synthesis were less-abundant in short treated plants. The protein 341 (**Acyl-[acyl-carrier-protein] desaturase**) was 8-fold less-abundant in treated plants compared to control plants. This protein converts stearoyl-ACP to oleoyl-ACP by introduction of a cis double bond between carbons Delta(9) and Delta(10) of the acyl chain.

2.3.5.2 A4A CLONE AFTER THE SHORT TERM TREATMENT (Tab. 5)

Proteomic data showed that several proteins involved in plant defense, stress response and oxidative stress response were also more-abundant in the hybrid clone A4A treated for 48 hours with cadmium.

PLANT DEFENCE – STRESS RESPONSE

Protein 10 (**Levopimaradiene synthase**), 22 (**Putative disease resistance protein RGA3**) and 269 (**Pathogenesis-related protein 1**) were more than 3-fold abundant in the cadmium treated plants. The last two proteins are involved in defense response to biotic stimuli, and the **Putative disease resistance protein RGA3** is a resistance protein protecting the plant against pathogens that contain an appropriate avirulence protein via a direct or indirect interaction with this avirulence protein; that triggers a defense system which restricts the pathogen growth (Song *et al.*, 2003). Protein 10 (**Levopimaradiene synthase**) is involved in defensive oleoresin formation in conifers in response to insect attack or other injuries and it is involved in diterpene (C₂₀) olefins biosynthesis; it is a bifunctional enzyme that catalyzes two sequential cyclizations of geranylgeranyl diphosphate (GGPP) to levopimaradiene. Levopimaradiene is the major products of the enzyme followed by abietadiene, neoabietadiene and palustradiene (Ro and Bohlmann, 2006).

OXIDATIVE STRESS RESPONSE

Proteins 20 (**Protein SRG1**) and 551 (**Monothiol glutaredoxin-S17**) are involved in cell redox homeostasis and were more-abundant in treated plants. The protein 514 (**Ferritin-2**), on the contrary, was more than 3-fold less-abundant in treated plants. This protein is involved in response to oxidative stress, but its function is to store iron in a soluble, non-toxic, readily available form.

Infact, it may be plausible that in high concentrations of cadmium, iron ions are less available and therefore is no longer required a protein whose function is to store excess of iron.

CARBOHYDRATE METABOLISM

As above, for the *Poli* and *58-861* clones, it appeared that cadmium had a negative effect on carbohydrate metabolism after the short term treatment also for the clone *A4A*. In fact, Proteins 108 (**Alpha-1,4 glucan phosphorylase L isozyme**) and 515 (**Beta-fructofuranosidase**) are enzymes with a key-role in the carbohydrates metabolic process (Buchner *et al.*, 1995; Cho *et al.*, 2005), and in our study, were less-abundant in treated plants.

PHOTOSYNTHESIS

On the contrary, cadmium short term treatment did not seem to have a negative effect on photosynthesis and tricarboxylic acid cycle in *A4A* clone. Proteins 18 (**Phosphoenolpyruvate carboxylase 4**), 21 (**Light-independent protochlorophyllide reductase**) and 491 (**Photosystem I reaction center subunit II-2**) were more than 3-fold abundant in the cadmium treated plants.

PROTEOLYSIS

Several proteins involved in proteolysis and ubiquitination processes also showed a decrease in abundance, such as the protein 160 (**SKP1-like protein 19**) and the protein 513 (**Nicastrin**). The former protein is involved in ubiquitination and subsequent proteasomal degradation of target proteins (Zhao *et al.*, 2003), the latter is a putative subunit of the gamma-secretase complex, an endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch (Yu *et al.*, 2000). If taken as a stress marker, these data, together with the trend showed by the proteins related to photosynthesis, would confirm the observed morphological changes in the aerial parts: up to 48 hours, toxicity symptoms were not clearly visible in clone *A4A*. Furthermore, while *Poli* and *58-861* showed a reduction in the increase of total leaf area between control plants and treated plants (Fig. 17 in section 2.3.1: Plant morphology and growth analysis), caused by short term cadmium exposure, the clone *A4A* did not show differences in the increase of total leaf area.

AMINOACIDS BIOSYNTHESIS

A great number of previous studies have shown how, upon exposure to heavy metals, plants often synthesize a set of various metabolites that accumulate in concentrations within the millimolar range, in particular specific amino acids, small amino acids chains such as glutathione and phytochelatins (PC), and also many amines, such as spermine, spermidine, putrescine, nicotianamine and mugineic acid (Cobbett CS., 2000; Shanti and Dietz, 2006; Rauser WE., 1995). These findings indicate that the association between amino acids and metals could be important particularly because metals complexed with amino acids are considerably more stable than when

complexed with carboxylic acids (Homer *et al.*, 1997). In this study, several proteins involved in aminoacids biosynthesis showed an increase in abundance in treated plants. The protein 341 (**Histidinol dehydrogenase**) is involved in histidine biosynthetic process, and in UV and cadmium stress response. Several plants, such as *Alyssum lesbiacum* and *Brassica juncea*, were demonstrated to be Ni-hyperaccumulators. The Ni-hyperaccumulation traits have been demonstrated to be specifically linked to the ability in free histidine production in some species such as *Arabidopsis halleri* (Kramer *et al.*, 1996); whereas the exposure to Ni resulted in a large increase in histidine concentration in xylem sap in hyperaccumulating plants, such a response was lacking in the non accumulator plants. Thus, Ni-hyperaccumulation relies for some species on histidine-dependent root-to-shoot translocation of heavy metal (Kerkeb and Kramer., 2003). This could explain the experimental evidence that the clone A4A showed higher root-shoot translocation factor than the 58-861 clone, already after short term treatments. Furthermore, another multi-metals hyperaccumulator, *Thlaspi goesingense* exhibited increased His concentrations when compared with other non hyperaccumulators *Thlaspi* species (Persans *et al.*, 1999). Besides Ni, Salt *et al.*, (1999) identified Zn-His complexes in the roots of Zn-hyperaccumulator *Thlaspi caerulescens*. Histidine levels have also been demonstrated to correlate with tolerance to Ni and Cd of the yeast *Saccharomyces cerevisiae* (Pearce and Sherman, 1999). Probably, this clone can produce a certain amount of free histidine to increase the xylematic translocation of Cd to the shoots.

The protein 102 (**Glutamate synthase 1**) is involved in glutamate biosynthesis, and it is required for non-photorespiratory ammonium assimilation, and it is over-expressed in response to cadmium ion. Bhatia *et al.* (2005) showed how in the metals hyperaccumulator *Stackhousia tryonii* the total amino acid concentrations slightly decreased from 22 to 18 mM and the proportion of Gln declined from 48% to 22 mol% while Ala, Asp, and Glu increased. Glu, Asp and Ala may contribute to complexation of heavy metals in the xylem (Bhatia *et al.*, 2005). Several studies have found that a great number of glutamate and glutamine synthase were over-expressed in poplar plants exposed to cadmium contamination (Kieffer *et al.*, 2008; Kieffer *et al.*, 2009; Durand *et al.*, 2010).

The protein 372 (**Cysteine synthase, chloroplastic/chromoplastic**) is involved in cysteine biosynthetic process, and in response to cadmium stress. Cysteine contents increased by factors of 4.5 and 3.8 in cadmium tolerant and cadmium sensitive ecotypes of *Silene vulgaris*, respectively, in response to metal stress that caused similar inhibition of root growth (Harmens *et al.*, 1993). Cysteine is required for methionine and glutathione/phytochelatin synthesis, and, therefore, is a central metabolite in antioxidant defence and metal sequestration. Genetically increased capacity for metal-induced Cys synthesis was shown to support survival of *Arabidopsis thaliana* under acute Cd stress (Dominguez-Solis *et al.*, 2004). Therefore an upregulation of the GSH synthesis pathway, by

the means of glutamate and cysteine biosynthesis, could be an attempt to rebalance a depletion of GSH by Cd, and by this way limit oxidative stress and ROS generation, and also detoxify directly Cd by chelating it (Kieffer *et al.*, 2008).

The protein 420 (**1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 1**) is involved in methionine biosynthetic process, and catalyzes the formation of formate and 2-keto-4-methylthiobutyrate (KMTB) from 1,2-dihydroxy-3-keto-5-methylthiopentene (DHK-MTPene). It may play a role in recycling S-adenosylmethionine (SAM) for ethylene biosynthesis, which is competing with polyamines biosynthesis pathway for SAM substrate. This result could be of interest in our case: in fact, studies carried out over 20 years ago and recent ones showed that heavy metals stress induce ethylene synthesis in plants (Sandmann and Boger, 1980; Matto *et al.* 1986; Gora and Clijsters, 1989; Yamauchi and Peng, 1995; Blechert *et al.* 1995; Vassiliev *et al.*, 2004). In addition, it is known that also polyamines have a role in response to environmental stresses. Polyamines are ubiquitous in all organisms and they influence a variety of growth and development processes in plants and have been suggested to be a class of plant growth regulators (Evans and Malmberg, 1989; Slocum and Flores, 1991; Kakkar and Sawhney, 2002). The levels of polyamines and the activities of their biosynthetic enzymes in plants increase under environmental stresses (Evans and Malmberg, 1989), but a specific role of polyamine in plants under metal stress is not yet known. However, there is a strong possibility that they can effectively stabilize and protect the membrane systems against the toxic effects of metal ions particularly the redox active metals (Sharma and Dietz, 2006). Therefore, the enzyme **1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 1**, was more than 4-fold abundant in the cadmium treated plants, and may play a key role in the balance between the ethylene biosynthesis pathway and the polyamines biosynthesis pathway.

NITROGEN and SULFUR METABOLISM

Nitrogen metabolism is affected by cadmium treatment, as it has been shown by studies on tomato plants (Chaffei *et al.*, 2004), and on poplar cuttings (Kieffer *et al.*, 2009; Durand *et al.*, 2010; Kieffer *et al.*, 2008). A significant decrease in enzyme activity of nitrate reductase, nitrite reductase and glutamine synthetase was observed by Kieffer *et al.* (2009) in poplar plants (*Populus tremula L.*) exposed to cadmium contamination for 14 days. In our study, the protein 567 (**Molybdopterin synthase catalytic subunit**) was more than 6-fold less-abundant in the cadmium treated plants. This subunit is the catalytic part of the molybdopterin synthase complex, which catalyzes the conversion of precursor Z into molybdopterin. Molybdopterin is an important cofactor of enzymes such as nitrate reductase, sulfite oxidase and xanthine oxidase (Mendel, 1997). Hence, we may

argue that these enzymes, under deficiency of the cofactor, decrease their enzymatic activity, with a net decline in nitrate and sulfate cell content.

ATP SYNTHESIS coupled with PROTON TRANSPORT

Recent studies have shown, in poplar clones, a decrease in abundance in protein involved in ATP synthesis coupled with proton transport, after short term treatments with Cd (3 days) (Kieffer *et al.*, 2008). This is in accordance with our results, in which the protein 272 (**V-type proton ATPase catalytic subunit A**) and the protein 369 (**ATP synthase subunit beta**), were more than 2-fold less-abundant in the cadmium treated plants. The former is a vacuolar ATPase and is responsible for acidifying a variety of intracellular compartments in eukaryotic cells; the latter produces ATP from ADP in the presence of a proton gradient across the cell membrane.

TRANSCRIPTION and TRANSLATION

Cadmium stress, also, induce a decrease in abundance in protein involved in transcription and translation mechanisms, such as many ribosomal proteins, elongation factors and transcription factors (Durand *et al.*, 2010; Kieffer *et al.*, 2008). In this study, the protein 512 (**50S ribosomal protein L21, chloroplastic**) was more than 4-fold less-abundant in the cadmium treated plants.

Lastly, among other identified proteins, we found that the protein 718 (**Protein phosphatase 2C 24**) were more than 4-fold less-abundant in the cadmium treated plants. This protein is a serine/threonine phosphatase and requires Mg²⁺ or Mn²⁺ to dephosphorylate the target molecule. The phosphorylation and dephosphorylation of regulatory proteins and enzymes is a general mechanism to transmit signals from the extracellular environment to the interior of the cell. Protein phosphatases, by opposing the action of the protein kinases, provide modulation and reversibility of the phosphoregulatory mechanism. In eukaryotes, **Protein phosphatase 2C** (PP2C) has been implicated in the negative regulation of protein kinase cascades that are activated as a result of stress. For instance, human PP2C catalyses the dephosphorylation and subsequent inactivation of the AMP-activated protein kinase (AMPK) (Moore *et al.*, 1991). AMPK is a central component of a protein kinase cascade which is activated by cellular stresses, especially those which deplete ATP levels (Corton *et al.*, 1994). In plants, members of the family PP2Cs, such as ABI1 and ABI2, are involved in ABA signal transduction. Abscisic acid is a plant hormone crucial to mediate the plant responses to environmental stresses (Chandeler *et al.*, 1994).

2.3.5.3 *Poli* AND 58-861 CLONES AFTER THE LONG TERM TREATMENT (Tab. 6a-6b)

PLANT DEFENCE – STRESS RESPONSE

Even after the longer term treatment, proteomic data showed that several proteins involved in plant defense and stress response were more-abundant in P.Nigra *Poli* and 58-861 clones treated with cadmium. Protein 54 (**17.5 kDa class I heat shock protein**) and 512 (**Heat stress transcription factor A-4b**) were more abundant in treated plants, they are two heat shock proteins involved in stress response. Furthermore, the latter is a transcriptional regulator factor that specifically binds DNA of heat shock promoter elements (HSE) (Baniwal *et al.*, 2004). The protein 87 (**Putative defensin-like protein 230**) and 224 (**Putative late blight resistance protein homolog R1A-3**) were more than 3-fold abundant in the cadmium treated plants, and they are involved in defense response to biotic stress, their role is to destroy the cells of the pathogen organisms. The latter confers resistance to late blight (*Phytophthora infestans*) races carrying the avirulence gene Avr1. Resistance proteins guard the plant against pathogens that contain an appropriate avirulence protein via an indirect interaction with this avirulence protein. That triggers a defense system including the hypersensitive response, which reduces the pathogen growth. (Kwaung *et al.*, 2005). The protein 515 (**ABC transporter B family member 19**) was also more abundant in 58-861 and *Poli* treated plants. This protein is an auxin efflux transporter that acts as a negative regulator of light signaling to promote hypocotyl elongation; it also mediates the accumulation of chlorophyll and anthocyanin, as well as the expression of genes in response to light, participates in auxin efflux and thus regulates the polar auxin basipetal transport (from auxin-producing leaves to auxin-sensitive tissues) (Martinoia *et al.*, 2002). Thus, an over-abundance of this protein could inhibit the hypocotyls elongation and it could explain the data shown in the growth analysis (2.3.1, Plant morphology and growth analysis) in which the clones *Poli* and 58-861 showed a reduction in the increase of leaves number between control plants and treated plants (Fig. 16 in section 2.3.1), caused by long term cadmium exposure.

OXIDATIVE STRESS RESPONSE

The treated plants continued to be subjected to oxidative stress also after the long term treatment. This is in accordance with Kieffer *et al.* (2009). Proteins involved in oxidative stress response were more abundant in treated plants. The protein 11 (**Peroxidase 5**) was more than 3-fold abundant in the cadmium short term treated plants and became more than 5-fold abundant in the treated plants after the long term treatment. The protein is a peroxidase responsible in the removal of H₂O₂, oxidation of toxic reductants, biosynthesis of lignin, suberization, auxin catabolism and response to environmental stresses (Tognolli *et al.*, 2002). In many proteomic studies on poplar peroxidase were found to be more abundant in plants exposed to cadmium contamination (Kieffer *et al.*, 2008;

Kieffer *et al.*, 2009). The protein 98 (**1-Cys peroxiredoxin**) was also more abundant in treated plants, and it is an antioxidant protein that seems to contribute to the inhibition of germination during stress. Durand *et al.* (2010) found that **1-Cys peroxiredoxin** were more abundant in poplar plants (*Populus tremula L. x Populus Alba L.*) exposed to cadmium contamination for 60 days.

PHOTOSYNTHESIS

Furthermore, in this study, it appeared that cadmium continued to have a negative effect on the light phase of photosynthesis by downregulating the expression of proteins of the electron transport chain. Proteins 88 and 209 (**Light-independent protochlorophyllide reductase subunit N**) and 261 (**Photosystem I reaction center subunit IV B, chloroplastic**) were less-abundant in treated plants and are involved in chlorophyll biosynthetic process and photosynthesis. Especially in leaves, cadmium has a negative impact on electron transport in chloroplasts by damaging photosystems I and II (Sanita di Toppi *et al.*, 1999). Furthermore, in recent study on spinach, photosystem I was described as more sensitive to Cd²⁺ (Fagioni *et al.*, 2009). Kieffer *et al.* (2008) found many subunit of photosystem I and II less abundant in poplars (*Populus tremula L.*) exposed for 14 days to cadmium contamination.

CARBOHYDRATE METABOLISM

The carbon metabolism and carbon fixation showed also numerous key proteins less abundant in cadmium stress conditions, including several **Ribulose-1,5-bisphosphate carboxylase oxygenase** large and small chain (proteins 260, 262, 414, 415, 433). Recent proteomic studies found that several large and small subunit of **Ribulose-1,5-bisphosphate carboxylase oxygenase** were less abundant in poplars (*Populus tremula L.*) exposed for 14 days to cadmium contamination (Kieffer *et al.*, 2008; Kieffer *et al.*, 2009; Durand *et al.* 2010). The proteins 339 (**Ribulose bisphosphate carboxylase/oxygenase activase A**) was also less abundant in treated plants and is responsible in the activation of RuBisCO involves the ATP-dependent carboxylation of the epsilon-amino group of lysine leading to a carbamate structure. Together the decrease observed for these two groups of proteins (RuBisCO isoforms and RuBisCO activases) indicate the lowering of the efficiency of CO₂ fixation. Other authors observed similar results in cadmium-treated rice leaves: RuBisCO activases and RuBisCO small and large chain subunits were less abundant (Hajduch *et al.*, 2001).

GLYCOLYSIS

If we analyze the L/R ratios, we found that the impact of cadmium treatment on protein abundance from photosynthesis, calvin cycle and carbohydrate metabolism is becoming less pronounced with increasing esposure time to cadmium. Consequently, after the “alarm” phase and stabilization of the short-time stress response, an important increase in abundance of proteins related to the glycolysis and the TCA cycle indicated that cadmium-exposed plants increased the energy supply, by

activating mitochondrial respiration. Because of the inhibition of growth induced by the cadmium treatment in the plants, photosynthetic products were no more important because the developmental process had been slowed down. These carbohydrates could rather be stored as more complex sugars, or (simultaneously) injected directly into mitochondrial respiration to compensate the higher energy needs of cells to counteract the cadmium stress effects (Kieffer *et al.*, 2008).

So far, the proteomic data in this study are in agreement with this hypothesis. Several key step enzymes from the glycolysis as well as from the TCA cycle showed an increase in abundance. The protein 4 (**Bifunctional enolase 2/transcriptional activator**) was more abundant in treated plants and it is involved in glycolysis. It is a multifunctional enzyme that acts as an enolase involved in the metabolism and as a positive regulator of cold-responsive gene transcription. The protein 247 (**Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic**) was also more abundant, and is involved in glycogen biosynthetic process. This protein plays a role in the synthesis of starch, it catalyzes the synthesis of the activated glycosyl donor, ADP-glucose from Glc-1-P and ATP.

The protein 253 (**Sucrose-phosphatase 1**) showed an increase in abundance after the long term Cd treatment. The protein is involved in sucrose biosynthetic process and catalyzes the final step of sucrose synthesis. Previous studies have shown an increase in sucrose phosphate synthase (SPS) and sucrose phosphatase (SPP) transcript levels, showing the initial increase in sucrose that occurs during stress acclimation in plants (Guy *et al.*, 2008). In fact, most sugars are known to improve osmoprotection during stress; sugars can have the same role in metal stress (Jouve *et al.*, 2004). Moreover several hexoses, sucrose and more complex carbohydrates as raffinose, showed an important increase in content in plants exposed to metals contamination, indicating that photoassimilates were less available for development and growth, but rather accumulated as free soluble sugars. This accumulation could also be beneficial to stressed plant cells, as these compounds can act as osmoregulants and osmoprotectants (Kieffer *et al.*, 2009). This may also explain the reason why protein 286 (**L-type lectin-domain containing receptor kinase VII.1**) was more abundant in treated plants. In fact this membrane protein is involved in sugar binding. Several different classes of plant lectins serve a diverse array of functions. The most prominent of these include participation in plant defense against predators and pathogens and involvement in symbiotic interactions between host plants and symbiotic microbes (De Holff *et al.*, 2009).

PROTEOLYSIS

Cadmium continued to have an effect in promoting proteolysis as a stress and senescence marker. In our study, the proteins 229 (**Proteasome subunit alpha type-3**) showed an increase in abundance in treated plants. The protein is involved in the ubiquitin-dependent proteins catabolic process. The

proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. Several studies showed that proteins involved in degradation of oxidatively modified proteins from the ubiquitin/proteasome pathway showed an upregulation under cadmium contamination, indicating that Cd stress-induced senescence (Aina *et al.*, 2007). In particular, Kieffer *et al.* (2008) found that one proteasome subunit alpha type 5 were more abundant in poplar plants (*Populus tremula L.*) exposed to 20µM CdSO₄ contamination for 14 days.

NUCLEOTIDES and ATP SYNTHESIS - PROTON TRANSPORT

In poplar plants exposed to cadmium contamination, during a first short term phase in which proteins involved in ATP synthesis coupled with proton transport showed a decrease in abundance, after a long period of 14 days they showed an increase in abundance (Kieffer *et al.*, 2008). We found that protein 249 (**ATP synthase subunit beta, chloroplastic**) showed an increase in abundance in treated plants and is involved in the synthesis of ATP: this protein produces ATP from ADP in the presence of a proton gradient across the membrane.

Proteins 198 (**Nucleoside diphosphate kinase 3**) and 270 (**Adenylosuccinate synthetase 1, chloroplastic**) showed an increase in abundance in treated plants, they are involved in the synthesis of all nucleoside triphosphates other than ATP and in purine nucleotide biosynthetic process. Adenylosuccinate synthetase plays an important role in the “*de novo*” pathway and in the salvage pathway of purine nucleotide biosynthesis, catalyzes the first step in the biosynthesis of AMP from IMP, and plays a key role in the biogenesis of sulphide-containing phytochelatin complex (Juang *et al.* 1993). Phytochelatins (PCs) are metal-chelating peptides produced in plants in response to heavy metal exposure and generally play an important role in avoiding damage induced by the presence of high concentration of metal ions (Cobbet, 2000). It has also been found that purine biosynthetic genes, particularly genes codifying for adenylosuccinate synthetase, are required for cadmium tolerance in *Schizosaccharomyces pombe* (Speiser *et al.*, 1992).

TRANSCRIPTION and TRANSLATION

After the long term treatment, cadmium stress seemed to induce an increase in abundance in proteins involved in transcription and translation mechanisms, such as many ribosomal subunits: proteins 273 (**40S ribosomal protein S10-1**), 324 (**30S ribosomal protein S15, chloroplastic**), 375 (**30S ribosomal protein S3, chloroplastic**). The protein 375 (**Pentatricopeptide repeat-containing protein At2g45350**) was more than 3-fold abundant in the cadmium treated plants, and plays a major role in chloroplast RNA editing. This result is not in agreement with several previous studies, in which one 30S ribosomal protein was less abundant in poplar plants (*Populus tremula L.*)

exposed to 20 μ M CdSO₄ contamination for 14 days (Kieffer *et al.*, 2008), and one 60S ribosomal protein was less abundant in poplar plants (*Populus tremula* L. x *Populus Alba* L.) exposed to cadmium contamination for 60 days (Durand *et al.*, 2010).

ASCORBATE METABOLISM

One of the plants mechanisms to react to cadmium toxicity is the accumulation of glutathione (GSH), ascorbate and phytochelatines, in order to sequester toxic ions in the cytosol and vacuole (Cobbet *et al.*, 2000). The depletion of glutathione by chelation to cadmium, (and incorporation into phytochelatins) is the most direct effect of cadmium on the oxidative balance in cells, and it has been proposed that this depletion of GSH is one of the main causes for oxidative stress and reactive oxygen species (ROS) generation induced by cadmium (Clemens *et al.*, 2006; Sanità di Toppi *et al.*, 1999). Glutathione, together with ascorbate, takes part in the Halliwell–Asada pathway for detoxification of ROS (May *et al.*, 1998). Some authors also linked a depletion of reduced glutathione during plant–pathogen reaction and the subsequent up-regulation of several typical stress-related proteins, specifically PR-proteins, glutathione peroxidases and glutathione S-transferases (May *et al.*, 1998; Mauch *et al.*, 1993). Several studies observed a decrease in glutathione reductase and ascorbate peroxidase activity in response to cadmium stress (Lyubenova *et al.*, 2007; Aravind *et al.*, 2005). In rice plants, superoxide dismutase, ascorbate peroxidase and glutathione reductase showed a reduced activity in response to cadmium treatment (Chien *et al.*, 2001). Finally, during a study on partially purified enzyme extracts from *Calystegia sepium* L., a direct inhibitory effect on glutathione reductase activity *in vitro* could be observed.

In this study the protein 202 (**L-idonate 5-dehydrogenase**) was more than 4-fold abundant in the long term cadmium treated plants. The protein is a dehydrogenase involved in the catabolism of ascorbate.

MOLECULAR TRANSPORTERS

During the long term treatment, cadmium produced significant changes in the abundance of several molecular transporters. For example, the protein 155 (**Magnesium transporter MRS2-E**) was more than 38-fold less-abundant in the cadmium short term treated plants and became less than 3-fold less-abundant in the treated plants after long term treatment. This protein is the major transport protein for Mg⁺ uptake into mitochondria (Piskacek., 2008).

The protein 233 (**Aquaporin NIP2-1**) was more abundant in treated plants. Aquaporins are channel proteins that facilitate the transport of water and small neutral molecules, including gases, across cell membranes of most of the living organisms (Maurel *et al.*, 2009). Aquaporin subgroup II (NIP2) proteins on the other hand form glyceroporins with an exceedingly low water permeability (Cabello-Hurtado *et al.*, 2004; Wallace *et al.*, 2005), and also transport other uncharged substrates,

including urea (Wallace *et al.*, 2005; Klebl *et al.*, 2003) as well as metalloid nutrients such as boron (Takano *et al.*, 2006) and silicon (Ma *et al.*, 2006). Overall, these observations suggest that NIPs are likely to be involved in transport functions other than water flux (Choi *et al.*, 2007). Recent studies have shown that these aquaporins facilitate also the bi-directional diffusion of As and other metalloids across membranes in *Arabidopsis thaliana* plants (Bienert *et al.*, 2007). Previous studies found that another aquaporin (NIP12) was more abundant in the same poplar clone plants (*P.nigra Poli*) exposed to 50µM CdSO₄ contamination for 14 days (Visioli *et al.*, 2010).

Also the protein 413 (**Protein TOC75**) was more abundant in treated plants; it has a role in mediating the insertion of proteins targeted to the outer membrane of chloroplasts and forms the voltage-dependent preprotein translocation channels (hydrophilic beta barrel) of the TOC complex in the chloroplastic outer membrane; it is also required for the import of protein precursors into chloroplasts (Bauer *et al.*, 2000). This protein is important to assembly members of the P_{1B}-adenosine triphosphatase (ATPase) transporter family (HMAs), involved in heavy metal transport. Particularly HMA1 is referred as a potential cadmium/zinc-transporting ATPase (HMA1) and belongs to the cation transport ATPases family (E1-E2 ATPases, subfamily IB) (Gravot *et al.*, 2004; Yu-Young *et al.*, 2009).

The protein 67 (**Potassium channel AKT2**), was more abundant in treated plants. It is an inward-rectifying potassium channel, with open or closed conformations in response to the voltage difference across the membrane, it activates the channel by hyperpolarization. A stimulated potassium channel is in keeping with previous results showing a significant alteration of potassium homeostasis under Cd stress (Damerval *et al.*, 1986). The [K⁺] in leaves increased 2.3-fold compared with control plants. Only few reports on the regulation of mineral homeostasis under Cd stress are available in the literature. A recently published paper indicated no change in K⁺ and Mg²⁺ content in leaves of Cd-tolerant mungbean (*Vigna radiate* L.) under Cd exposure (Wahid *et al.*, 2008). Neither the mobilization of K⁺ in leaves in response to Cd nor the implication of a potassium channel were reported so far in literature. However, also in a recent proteomic study, Durand *et al.* (2010) found that **potassium channel subunits** were more abundant in poplar plants (*Populus tremula* L. x *Populus Alba* L.) exposed to cadmium contamination for 60 days.

CELL DIVISION

After the long term treatment, cadmium seemed to influence the auxin (indole-3-acetic acid, IAA) production in poplar cuttings, and as a result of this also cell division, differentiation and elongation. In fact, it is known that cadmium inhibited cell division and altered the chromosomes structure. The inhibition of cell proliferation, shown by the low mitotic index, was proportional to the concentration and time of exposure to cadmium (Das *et al.*, 1997). Furthermore, other

deleterious effects of heavy metal stress in plants may be the stimulation of some enzymatic activities that limit cell growth and, consequently, accelerate tissue ageing (Hasenstein *et al.*, 1988; Schützendübel *et al.*, 2001). At cellular level, increase of indole-3-acetic acid (IAA) oxidase activity, acting on auxin catabolism by oxidative decarboxylation (Gazaryan *et al.*, 1996), may control the endogenous rate of this growth phytohormone (Goldberg *et al.*, 1986) and, thereby, reduce cell growth. Increase in activity of IAA oxidase and reduction in cell growth associated to auxin catabolism have been observed in other metal-stressed plants (Hasenstein *et al.*, 1988). An accumulation of insoluble phenols, as lignin, in secondary cell wall have been reported in plants exposed to heavy metal and could be associated to an increase in activity of lignifying peroxidases (Pandolfini *et al.*, 1992; Schützendübel *et al.*, 2001). Lignification decreases the cell-wall plasticity and, therefore, reduces the cell growth (Schützendübel *et al.*, 2002). It seems that the reduction in leaves growth in plants stressed with 20 µM of Cd might be associated to an increase in the activity of IAA oxidase and of enzymes implicated in lignification (Chaoui and El Ferjani, 2005). For the most deleterious treatments (100 µM of CdSO₄), the growth reduction could also be related to an increase in auxin degradation and in lignification, together with depletion in the antioxidant systems (Chaoui and El Ferjani, 2005). It appears that the auxin degradation would be a resultant contribution of the enhanced IAA oxidase activity from all cell fractions, while the covalently bound cell wall peroxidases seem to be the only contributor to the stimulation of lignification pathway. All these observations taken together point to a possible induction of leaf ageing after exposure to Cd.

In this work, the protein 304 (**Auxin transport protein BIG**) was more than 2-fold less abundant in treated plants. It is a transport protein required for auxin efflux and polar auxin transport (PAT) influencing auxin-mediated developmental responses (e.g. cell elongation, apical dominance, lateral root production, inflorescence architecture, general growth and development), and it is plausible that it would be less abundant in conditions of low auxins.

On the other hand, protein 248 (**Auxin-responsive protein IAA6**) was more abundant in treated plants. These proteins are short-lived transcriptional factors for early auxin response genes at low auxin concentrations. In other studies, Durand *et al.* (2010) and Kieffer *et al.*, (2008) found that **auxin-binding protein ABP19** was more than 2-fold less-abundant in poplar plants exposed to cadmium contamination.

The protein 82 (**Cyclin-dependent kinase inhibitor 1**) was more than 15-fold more abundant in treated plants and it is responsible for the inhibition of cellular proliferation. May be the important intermediate by which p53/TP53 mediates its role as an inhibitor of cellular proliferation in response to DNA damage. Binds to and inhibits cyclin-dependent kinase activity, preventing

phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression. Functions in the nuclear localization and assembly of cyclin D-CDK4 complex and promotes its kinase activity towards RB1. At higher stoichiometric ratios, inhibits the kinase activity of the cyclin D-CDK4 complex (Fischer *et al.*, 2003; Dai *et al.*, 2003). Recent studies have shown that the cadmium-sensitive *Arabidopsis thaliana* mutant (cad2-1) was also shown to be defective in Cyclin-dependent kinase inhibitor (CKI) and in the γ -glutamylcysteine synthetase (γ -GCS) gene in *Arabidopsis thaliana* plants (Cobbet *et al.*, 1998). Cyclin-dependent kinase inhibitor (CKI) activation also could account for the G1 block induced by depletion of intracellular glutathione which occurred in cadmium stress conditions (Vernoux *et al.*, 2000).

All these experimental evidences, taken together, could explain the observed morphological changes in the aerial parts after the long term treatment: *P.Nigra Poli* and 58-861 clones showed a significant reduction in the increase of total leaf area between control plants and treated plants, and a significant reduction in the increase of leaves number (Fig. 16 and Fig. 17 in section 2.3.1: Plant morphology and growth analysis).

However, two proteins seemed to contradict this behavior. The protein 241 (**Phytosulfokine receptor 1**) was more abundant in treated plants and it is a phytosulfokine receptor with a serine/threonine-protein kinase activity that regulates, in response to phytosulfokine binding, a signaling cascade involved in plant cell differentiation, organogenesis and somatic embryogenesis. Matsubayashi *et al.* (2006), showed that the disruption of *Arabidopsis thaliana* phytosulfokine receptor gene affects cellular longevity and potential for growth.

CELL WALL

The protein 223 (**Expansin-B13**) was also more abundant in treated plants and it may cause loosening and extension of plant cell walls by disrupting non-covalent bonding between cellulose microfibrils and matrix glucans. However, some studies have shown that an increase of metal-ion concentration results in the activation of wall-loosening enzymes (Moustacas *et al.*, 1991).

MISCELLANEOUS

Among other indentified proteins, was found the protein 199 (**Protein phosphatase 2C 28**) that was more than 2-fold abundant in the cadmium treated plants. In plants, proteins phosphatase 2C (PP2C) has been implicated as regulator of protein kinase cascades that are activated as a result of stress. As seen above, in the differential proteomic analysis for the clone A4A after the short term treatment, another protein phosphatase 2C (24) was found to be less abundant in treated plants (see pag. 69). It is possible that this protein could be a negative regulator of protein kinase cascades that are activated as a result of stress, and another protein phosphatase 2C (28), could operate in different ways. For instance, another PP2C acts as a negative regulator of a plant mitogen-activated

protein kinase (MAPK) pathway (Rodriguez *et al.*, 1998). Thus, the plant PP2Cs family work as regulators of various signal transduction pathways, and it is plausible to expect that some member of the family can be over-abundant while other might be under-abundant, at the same time, in plants under metal stress conditions.

Proteins 71 (**5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 1**), and 254 (**Ethylene receptor 2**) were more than 2-fold more abundant in the cadmium treated plants and are both involved in the ethylene synthesis pathway. The former is an enzyme of the methionine cycle that catalyzes the irreversible cleavage of the glycosidic bond in 5'-methylthioadenosine (MTA) to adenine and 5'-methylthioribose, contributes to the maintenance of AdoMet homeostasis and is required to sustain high rates of ethylene synthesis. The latter may act early in the ethylene signal transduction pathway, possibly as an ethylene receptor, or as a regulator of the pathway (Stepanova *et al.*, 2000). This results could be interesting: in fact, many studies showed that heavy metals stress (Sandmann and Boger, 1980; Matto *et al.*, 1986; Gora and Clijsters, 1989; Yamauchi and Peng, 1995) in particular cadmium stress (Blechert *et al.*, 1995; Vassiliev *et al.*, 2004) induce ethylene synthesis in plants. The high level of ethylene, resulting from inhibition of the photosystems, can increase senescence processes observed usually in Cu-treated plants at the final growth stages or after a long exposure to the excess metal (Maksymiec *et al.* 1995; Maksymiec and Baszynski, 1996). Ethylene in stress conditions can also increase the rigidity of cell walls through intensification of its lignification process followed by growth inhibition (Enyedi *et al.*, 1992). Cu and Cd stimulated ethylene production via increase of ACC synthase activity and the over-expression of its codifying genes (Pell *et al.* 1997). More recently, it has been observed that increase in ethylene content (after Cu and Zn exposure) lead to lipoxygenase activity increase (Gora and Clijsters, 1989). This phenomenon indicates that heavy metals can induce the jasmonate pathway and/or lipoxygenase mediated reactive oxygen species (ROS) formation, the more so, that exogenous JA also increases ethylene concentration (Kruzmane *et al.* 2002), especially by stimulating the activity of ACC synthase and oxidase (Saniewski *et al.* 2003).

2.3.5.4 A4A CLONE AFTER THE LONG TERM TREATMENT (Tab. 7)

PLANT DEFENCE – STRESS RESPONSE

After the longer term treatment, proteomic data showed that several proteins involved in plant defense and stress response were more-abundant in the hybrid clones A4A (*P. nigra x P. Deltoids*) treated with cadmium. Proteins 61 (**Putative defensin-like protein 270**) and 53 (**Putative defensin-like protein 211**) were more than 3-fold abundant in treated plants, and they are two

proteins involved in defense response to biotic stress, defense response to fungi and in killing the cells of the pathogen organisms.

As already discussed (see section 2.1.3), plants react to cadmium toxicity by the accumulation of glutathione, phytochelatines and metallothioneins, in order to sequester toxic cadmium ions in the cytosol or vacuole (Cobbet *et al.*, 2000). In this study, the protein 27 (**Metallothionein-like protein 1**) was more than 4-fold abundant in treated plants.

The protein 37 (**UDP-glycosyltransferase 84A2**) was also more than 4-fold abundant in treated plants and it is a sinapate glucosyltransferase (SGT) required for the biosynthesis of the glucose ester sinapoylglucose and subsequently sinapoylmalate and sinapoylcholine. It can glucosylate the phytotoxic xenobiotic compound 2,4,5-trichlorophenol (TCP). The UGT gene superfamily, in fact, in higher plants is thought to encode for enzymes that glucosylate a broad array of aglycones, including plant hormones, all major classes of plant secondary metabolites, and xenobiotics such as herbicides (Vogt *et al.*, 2000). Glycosylation regulates many properties of the aglycones, such as their bioactivity, their solubility and their transport properties within the cell and throughout the plant (Ross *et al.*, 2001).

OXIDATIVE STRESS RESPONSE

Cadmium continued to cause oxidative stress also after the long term treatment in A4A clone. Two proteins involved in oxidative stress response were more abundant in treated plants. The protein 102 (**1-Cys peroxiredoxin**) is an antioxidant protein that seems to contribute to the inhibition of germination during stress. Durand *et al.* (2010) found that **1-Cys peroxiredoxin** were more abundant in poplar plants (*Populus tremula* L. x *Populus Alba* L.) exposed to cadmium contamination for 60 days.

The proteins 180 (**Monothiol glutaredoxin-S5**) continued to be more abundant in treated plants even after the long term treatment. It may only reduce GSH-thiol disulfides, but not protein disulfides, and so it is required for cell redox homeostasis (Herrero *et al.*, 2006).

PHOTOSYNTHESIS

On the contrary, cadmium long term treatment did not seem to have a negative effect on photosynthesis or tricarboxylic acid cycle, carbohydrate metabolism and glycolysis in A4A clone. Rather was found the protein 62 (**Protochlorophyllide reductase iron-sulfur ATP-binding protein**) involved in chlorophyll biosynthetic process and photosynthesis, more abundant in treated plants. This protein uses Mg-ATP and reduced ferredoxin to reduce ring D of protochlorophyllide to form chlorophyllide a, in a light-independent reaction. The expression of CHLL gene, encoding the regulatory subunit of the protochlorophyllide reductase, is regulated by the redox state of the chloroplast (Rubinelli *et al.* 2002). In the same study, Rubinelli *et al.*, (2002) shown that results a

small increase in CHLL transcript abundance can also result from oxidizing conditions such as Cd exposure, in *Chlamydomonas*. Also another Light-independent protochlorophyllide reductase was more abundant in treated plants in the same clone, after cadmium short term treatment (see section 2.3.4.2).

PROTEOLYSIS

Some proteins involved in proteolysis activity continued to be less-abundant in A4A treated plants. The protein 653 (**U-box domain-containing protein 51**) was more than 7-fold less-abundant in treated plants. On the contrary, the same protein was found to be more abundant in 58-861 treated plants (see section 2.3.4.1). This different pattern of proteolysis related proteins may be one explanation of the differences occurred in signs of stress on the leaves surface of the treated plants. In fact, after the long period treatment, clones of the type *Poli* and 58-861, showed many chlorotic areas and small necrotic spots near the main leaf vein of leaves. Instead the leaves of the clone A4A, were less affected by chlorotic areas and necrotic spots (Fig. 13, 14, 15 in the section 2.3.1).

ATP SYNTHESIS - PROTON TRANSPORT

In poplar plants exposed to cadmium contamination, after a first phase in which protein involved in ATP synthesis and decomposition showed a decrease in abundance, after 14 days of cadmium exposure they showed an increase in abundance (Kieffer *et al.*, 2008). In this study, the protein 32 (**Protein ycf2**) was more abundant in treated plants, and is an ATPase of unknown function (Huang *et al.*, 2010). Its presence in a non-photosynthetic plant (*Epifagus virginiana*) and experiments in tobacco indicate that it has an essential function which is probably not related to photosynthesis (Drescher *et al.*, 2000).

AMINOACID BIOSYNTHESIS

Unlike the short-term treatment in which cadmium seemed to cause an increase in abundance in treated plants of proteins involved in aminoacids biosynthesis (see section 2.3.4.2), long-term cadmium treatment did not seem to cause any changes in abundance of these proteins. Only protein 649 (**Homocysteine S-methyltransferase 4**) was more than 4-fold less abundant in treated plants. It catalyzes methyl transfer from S-methylmethionine (SMM) to adenosyl-L-homocysteine (AdoMet). This result is not in agreement with a previous study, in which a transcriptomic analysis in the aquatic *Blastocladiella emersonii*, shown an induction of gene HMT for Homocysteine S-methyltransferase in response to cadmium contamination (Georg *et al.*, 2007).

TRANSCRIPTION and TRANSLATION

As seen previously for 58-861 and *Poli* clones (see section 2.3.4.3), after the long term treatment, cadmium stress seemed to induce, also in A4A clone, an increase in abundance in protein involved in transcription and translation mechanisms, such as two ribosomal subunits: proteins 47 (**50S**

ribosomal protein L16, chloroplastic) and 63 (**50S ribosomal protein L1, chloroplastic**). The protein 43 (**Elongation factor 2**) was more than 3-fold more abundant in treated plants. This protein catalyzes the GTP-dependent ribosomal translocation step during translation elongation. During this step, the ribosome changes from the pre-translocational (PRE) to the post-translocational (POST) state as the newly formed A-site-bound peptidyl-tRNA and P-site-bound deacylated tRNA move to the P and E sites, respectively. Catalyzes the coordinated movement of the two tRNA molecules, the mRNA and conformational changes in the ribosome (Robinson *et al.*, 1974). Contrariwise, Durand *et al.* (2010) found one translation elongation factor EF-G and two elongation factors 1 less abundant in poplar plants (*Populus tremula* L. x *Populus Alba* L.) exposed to cadmium contamination for 60 days, but they found in the same study another elongation factor 1 more abundant in treated plants.

In this study, however, was found other three proteins less abundant in treated plants and involved in transcription and translation mechanisms. The protein 46 (**DNA-directed RNA polymerase subunit beta**) was more than 17-fold less abundant in treated plants and is a DNA-dependent RNA polymerase that catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates. For a long time, it is known that cadmium affects protein synthesis and induces a reduction of RNA-polymerase activity in rat liver (Hidalgo *et al.*, 1976).

Furthermore the protein 59 (**Protein cup-shaped cotyledon**) was more than 75-fold less abundant in treated plants. This is a transcription activator involved in molecular mechanisms regulating shoot apical meristem (SAM) formation during embryogenesis and organ separation. It is required for axillary meristem initiation and separation of the meristem from the main stem. May act as an inhibitor of cell division. (Takada *et al.*, 2001). In one recent study on *Zea mays*, the gene codifying for Protein cup-shaped cotyledon was described as repressed in a particular Al-tolerant maize genotype (Cat100-6) under Al stress condition, and over-expressed in another Al-sensitive genotype (S1587-17) (Cançado *et al.*, 2009).

The protein 651 (**Maturase K**) was also less abundant in treated plants and usually probably it assists in splicing other chloroplast group II introns.

MOLECULAR TRANSPORTERS

The protein protein 69 (**Magnesium transporter MRS2-I**) is membrane transporter that may mediate the influx of magnesium. It showed a decrease in abundance after the cadmium long term treatment. The same magnesium transporter was found to be less abundant also in 58-861 and *Poli* clones after both the short and long term treatment (see section 2.3.4.1 and 2.3.4.3).

On the contrary, the protein 60 (**Vacuolar protein sorting-associated protein 22 homolog**) was more than 6-fold abundant in treated plants. This protein is a component of the endosomal sorting

complex required for transport II (ESCRT-II), which is required for multivesicular body (MVB) formation and sorting of endosomal cargo proteins into MVBs. Many previous evidences have shown that various stress condition (e.g. metal contaminations, waterlogging, salt, biotic stress) may promote the formation of multivesicular body (Zhou *et al.*, 2011; Eleftheriou *et al.*, 1991; Barhoumi *et al.*, 2007; Stoyanova *et al.*, 1997); this could promote an increase in toxic ions storage in vacuole by chelating them with glutathione, phytochelatines and metallothioneins (Cobbet *et al.*, 2000).

LIPID SYNTHESIS

One important consequence of oxidative stress is lipid peroxidation, producing toxic intermediate chain-length aldehydes. Toxic aldehydes can be eliminated to their respective carboxylic acids through the activity of aldehyde dehydrogenases, a diverse protein family (Chien *et al.*, 2001). Kieffer *et al.* (2008), in fact, found that enzymes involved in lipid peroxidation (aldehyde dehydrogenase 1 precursor and mitochondrial aldehyde dehydrogenase) increased their expression in poplar plants (*Populus tremula L.*) exposed to 20 μ M CdSO₄ contamination for 14 days.

In this study, two proteins involved in lipogenesis were less abundant in treated plants. The protein 338 (**Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha**) is a component of the acetyl coenzyme A carboxylase (ACC) complex. First, biotin carboxylase catalyzes the carboxylation of biotin on its carrier protein (BCCP) and then the CO₂ group is transferred by the carboxyltransferase to acetyl-CoA to form malonyl-CoA. However, since ACC is chronologically the first enzyme involved in fatty acid biosynthesis, it is considered as the rate-limiting enzyme of this process (Rollin *et al.*, 2003; Wakil *et al.*, 1983). A recent study in *Anguilla anguilla* showed that Cd exposure triggered a down-regulation of the gene encoding the ACC subunits (Pierron *et al.*, 2007). Other previous studies found that acetyl-coenzyme A carboxylase was more than 3-fold less abundant in poplar plants exposed to 50 μ M CdSO₄ contamination for 14 days (Visioli *et al.*, 2010).

The protein 610 (**Putative linoleate 9S-lipoxygenase 3**) was also less abundant in treated plants, and it is a plant lipoxygenase involved in a number of diverse aspects of plant physiology including growth and development, pest resistance, senescence and responses to wounding. It catalyzes the hydroperoxidation of lipids containing a cis,cis-1,4-pentadiene structure.

CELL WALL

Some studies have shown that an increase of metal-ion concentration results in the activation of wall-loosening enzymes (Moustacas *et al.*, 1991). In this work, three proteins involved in loosening and extension of plant cells were more abundant in treated plants after the long term treatment. The protein 54 (**Expansin-A14**) causes loosening and extension of plant cell walls by disrupting non-covalent bonding between cellulose microfibrils and matrix glucans. The protein 184 (**Probable**

pectinesterase 53) acts in the modification of cell walls via demethylesterification of cell wall pectin. Recent studies have shown that the exposure to cadmium-contaminated soils increases the concentration of a pectinesterase in *Poa annua* plants (Aina *et al.* 2010). The protein 189 is a **45 kDa cell wall protein** that plays a mechanochemical and regulatory roles in plants development (Cassab, 1998).

2.3.5.5 HEAT MAPS and PIE-CHARTS

Heat-map were produced using the TreeView software, for the poplar clones treated for 48 hours with 20 μ M CdSO₄. The image evidenced the comparison of proteins abundance between control and treated samples. In shades of red were represented the proteins more abundant in the control than in the treated plants; hence less abundant in the treated samples. In shades of green were represented the proteins more abundant in the treated than in the control plants; hence less abundant in the control samples. In black were evidenced the proteins whose abundance in the plants did not change between control and Cd treatment (Fig. 46, 47).

The pie charts, in figure 48, visualized the differently abundant functional protein classes, already commented, according to their percentage in each clone for each treatment. These diagrams provided us a global and more clear idea of how cadmium affected several metabolic and functional processes of the poplar plant.

Pie charts and heat maps are two statistical visualisation of the relative abundance of each protein in each clone according to the presence or absence of Cd and to the time of exposure. The main difference among the two types of graphic aspects is that in the pie charts were evidenced for each clone the abundance of proteins according to the time of exposure, whilst in the heat maps the three clones are compared among each other and with the control, according to the time of exposure. Furthermore, in the heat maps it is possible to visualise the proteins that do not change in abundance between controls and treated samples (in black, Fig. 46, 47).

The cadmium short term treatment (48h) influenced the abundance of proteins involved in stress response and plant defense, oxidative stress response, proteolysis pathway, carbohydrate metabolism and photosynthesis process in each poplar clone (Fig 48A, 48B). While proteins involved in stress response, plant defense and oxidative stress response were over-abundant in the treated plants in respect to the control plants, proteins involved in carbohydrate metabolism were under-abundant in the treated plants (Fig. 46). On the contrary, proteins involved in photosynthesis process and proteolysis pathway had an opposite behavior: in hybrid clone A4A proteins involved in photosynthesis process were over-abundant in treated plants and proteins involved in proteolysis

pathway were under-abundant in treated in respect to the control plants (Fig. 46). In the clones *Poli* and *58-861* proteins involved in photosynthesis process were under-abundant in treated plants and proteins involved proteolysis pathway were more-abundant in treated plants (Fig. 46).

The short metal stress influenced the abundance of proteins involved in ATP synthesis and proton transport, transcription and translation mechanisms, aminoacid biosynthesis and nitrogen metabolism only in hybrid clone *A4A* (Fig 48B). On the contrary, cadmium influenced the abundance of proteins involved in glycolysis pathway, protein folding, lignin biosynthesis, lipid synthesis and molecular transport in the clones *Poli* and *58-861* (Fig 48A).

The cadmium long term treatment (14d) continued to influence the abundance of proteins involved in stress response and plant defense, oxidative stress response, proteolysis, photosynthesis process in each poplar clone, and also the abundance of proteins involved in ATP synthesis and proton transport, transcription and translation mechanisms, molecular transport and cell wall construction (Fig 48C, 48D). While proteins involved in stress response, plant defense, oxidative stress response, ATP synthesis and proton transport, molecular transport and cell wall construction were more abundant in the treated plants than in the control plants in each clone (Fig. 47), proteins involved in photosynthesis process and proteolysis pathway had an opposite behavior: in hybrid clone *A4A* proteins involved in photosynthesis process were over-abundant in treated plants and proteins involved proteolysis pathway were under-abundant in treated than in the control plants (Fig. 47). In the clones *Poli* and *58-861* proteins involved in photosynthesis process were under-abundant in treated plants and proteins involved proteolysis pathway were more-abundant in treated plants (Fig. 47).

The long metal stress influenced the abundance of proteins involved in aminoacid biosynthesis and lipid synthesis only in the hybrid clone *A4A* (Fig 48D). On the contrary, cadmium long exposure influenced the abundance of proteins involved in carbohydrate metabolism, glycolysis pathway, cell division and ascorbate metabolism in the clones *Poli* and *58-861* (Fig 48C).

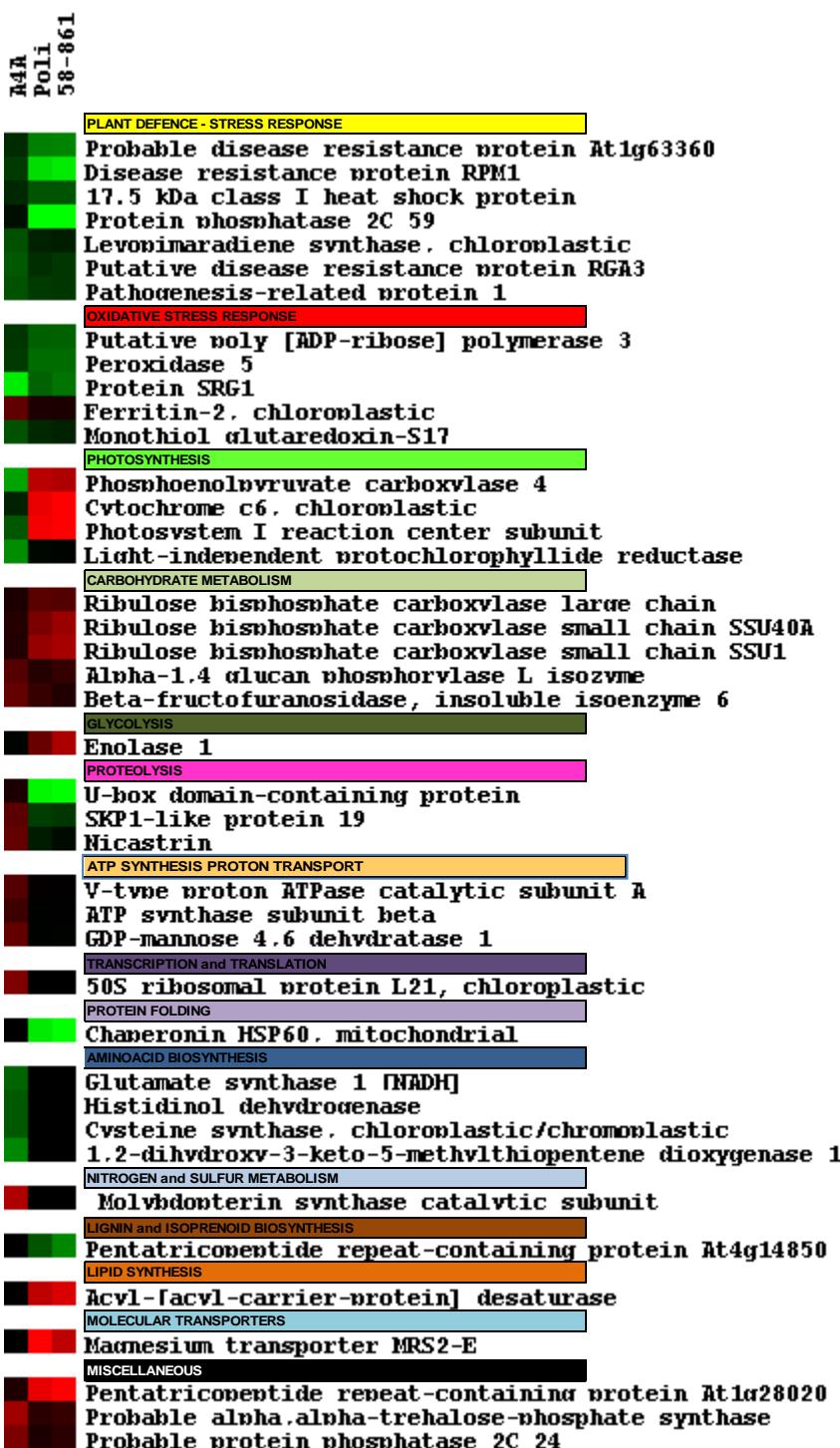


Fig. 46: Heat-map produced using the TreeView software, for the three clones treated for 48 hours with 20µM CdSO₄. The image evidences the comparison of proteins abundance between control and treated samples. In shades of red are represented the proteins more abundant in the control than in the treated plants; hence less abundant in the treated samples. In shades of green are represented the proteins more abundant in the treated than in the control plants; hence less abundant in the control samples. In black are evidenced the proteins whose abundance in the plants did not change between control and Cd treatment.

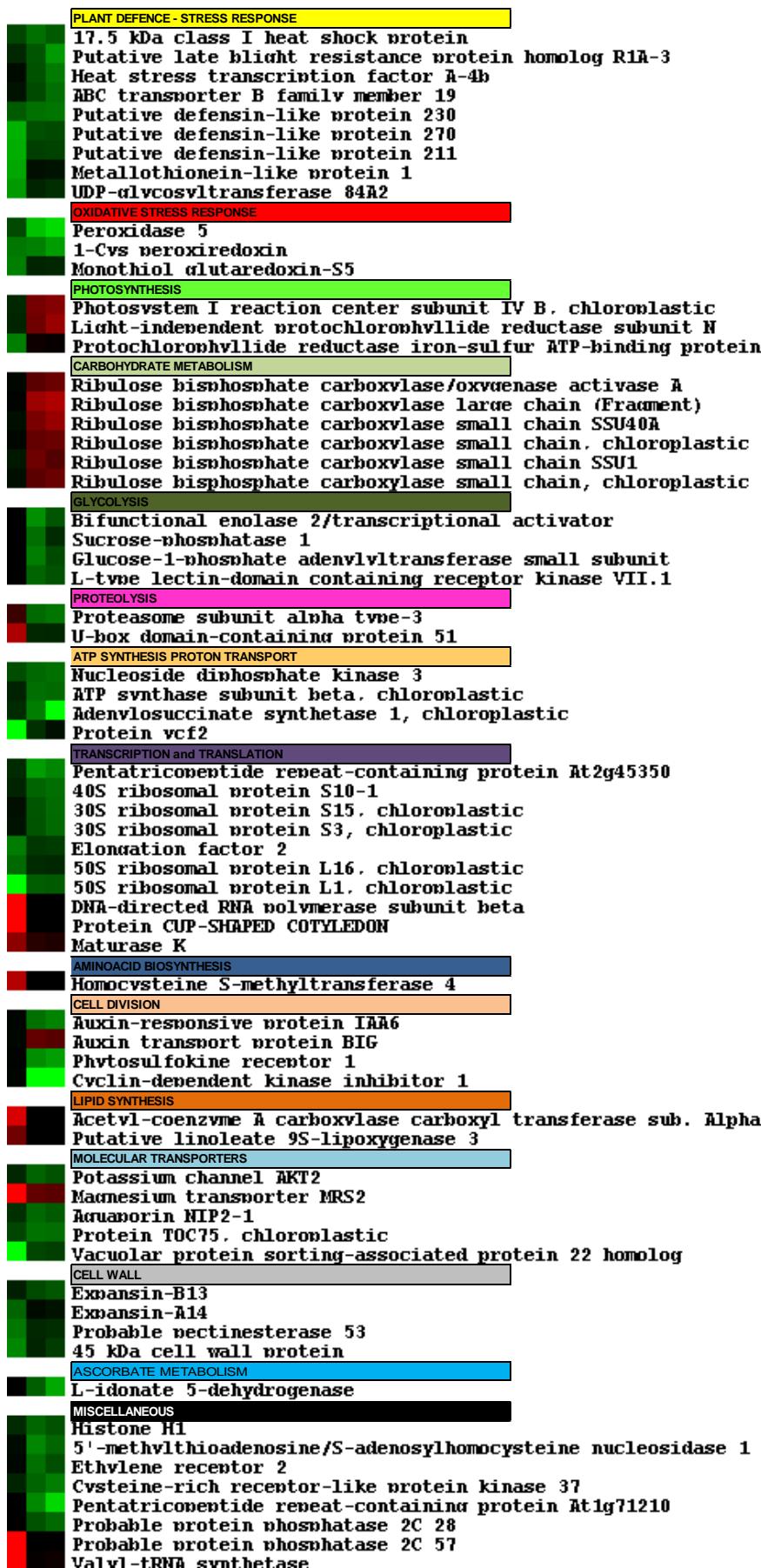


Fig. 47: Heat-map produced using the TreeView software, for the three clones treated for 14days with 20 μ M CdSO₄. The image evidences the comparison of proteins abundance between control and treated samples. In shades of red are represented the proteins more abundant in the control than in the treated plants; hence less abundant in the treated samples. In shades of green are represented the proteins more abundant in the treated than in the control plants; hence less abundant in the control samples. In black are evidenced the proteins whose abundance in the plants did not change between control and Cd treatment.

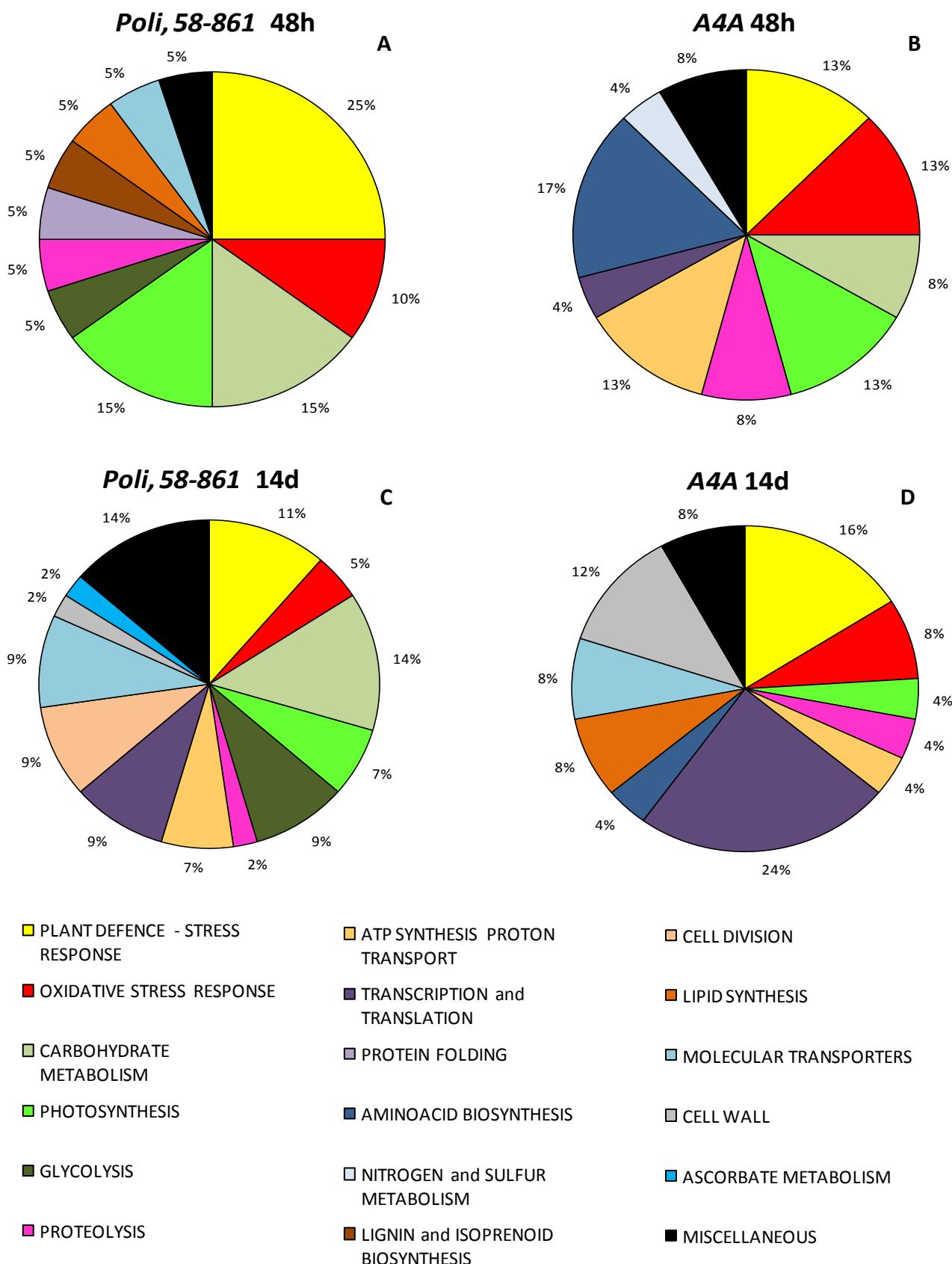


Fig. 48: Pie charts of all the protein classes identified through the proteomic approach. The diagrams evidence with different colors according to the legend below, the percentage of the differently abundant proteins for each clone and treatment. **A:** total proteins pool for the clones Poli and 58-861 after the short term treatment (48h); **B:** total proteins pool for the clone A4A the short term treatment (48h); **C:** total proteins pool for the clones Poli and 58-861 after the long term treatment (14d); **D:** total proteins pool for the clone A4A the long term treatment (14d).

2.4 Conclusion

Health problems caused by environmental contamination are a growing concern worldwide. Proper environmental management is the key to avoid one fourth of all preventable illnesses, which are directly caused by environmental factors. The environment influences our health in many ways: through exposures to physical, chemical and biological risk factors, and through related changes in our behaviour in response to those factors. Population growth and the associated development pressure are increasing the difficulties associated with sustainability of public health practices and policies. Water contamination, airborne contaminants, bioaccumulative contaminants in the food chain, and environmental threats to public health require the deployment of joint efforts and of scientific knowledge and know-how to develop new solutions. Understanding environmental and ecological problems is a prerequisite to protect public health (Rustichelli, 2008).

Plants could be a suitable solution to solve some environmental pollution problems. Plants have always been the answer for human needs, as food, materials, fibres but also as drugs. In the last 40 years the importance of plants for the survival of the globe, as an interwoven woof of ecosystems, has been definitively determined.

Thus we studied how a complete organism responded to environmental stimuli in the form mainly of polluting molecules and elements. In fact, metals in soil may constitute a significant environmental risk. Conventional methods for removing metals from contaminated areas are usually laborious and expansive. The first important step in any reclamation activity is the identification of the pollutants and in which concentrations they are present in the soil, water and/or air, in order to establish the best solution. We focussed on heavy metals pollution, in particularly on the problem of Cd. The application of plant-based technologies to remediate metals pollution is often quite attractive because it is economical and has a low impact on the site and on its surrounding environments. The identification of the problem is followed by the search of an ecological solution, which requires the knowledge of plant bio molecular mechanisms in presence of the heavy metals, specifically concerning cadmium.

Populus species were taken into consideration for their usefulness for phytoremediation purposes, for their economical interest due to high biomass, fast growth, high adaptability to different soils and good accumulation ability for cadmium. Poplar genetic variability is often found to be very low within natural population (Breyne *et al.*, 1999) but the genes that contribute to variation in complex traits are difficult to identify (Glazier *et al.*, 2002) even if *Populus* has evidenced a high variability. The characterisation of several *Salicaceae* clones for the effectiveness to tolerate and bioconcentrate metals could be interesting in specifying the potentiality of these plants to remediate polluted soils.

In this work, three clones of the *Populus* spp. (clones **58-861** and **Poli** of *Populus nigra* L. and hybrid **A4A** *P. nigra* x *P. deltoids*) were investigated in relation to their response to contamination by cadmium, in order to understand and quantify the differences between the clones in terms of cadmium tolerance, accumulation capacity and translocation of the contaminant to the aerial parts. The evaluation of the phytoremediation potential of these clones has been performed combining chemical, biochemical and physiological parameters.

Cuttings for each clones were treated with 20 µM of CdSO₄ for 48 hours and for 14 days in order to evaluate the short and long term effects of the cadmium contamination.

Cadmium influenced root apparatus of poplar clones already after the short term treatment. The accumulation and distribution of Cd in roots were important traits for the evaluation and screening of these clones under Cd treatment. After 48 hours of metal treatment, the clones *Poli* and *A4A* accumulated in roots a similar cadmium concentration (Fig. 19A), less than that accumulated by the clone *58-861*. But they showed different behaviors: in fact, while the radical growth of the clone *A4A* was negatively affected already after 48 hours treatment and continued to be constant after 14 days treatment, the root growth of the clone *Poli* seemed stimulated by the presence of the contaminant after the short term treatment and returned to be inhibited after the long-term treatment (Fig. 18). However, after the long-term treatment, roots of *A4A* accumulated more cadmium than the roots of *Poli*. In both clones, either after the short and the long-term treatments, roots accumulated less metal than roots of the clone *58-861*. Thus, it is possible to assume that *P.Nigra* clone *58-861*, having a large concentration of cadmium in roots (>1500mg/kg) associated with a small concentration of cadmium in aerial parts, can be considered a “root accumulator”, a plant species with high metal uptake but inefficient transport to shoots (Dos Santos *et al.*, 2007), either after the short or the long-term treatment, the clone stabilizes the metal within the root system. Moreover, the root apparatus of this clone was not physiologically affected by cadmium short-term treatment (Fig. 18), though it was affected by cadmium long-term treatment. Clone *58-861* could keep a large and widely resistant root system absorbing high metal contents, and thus had a great potential applications in large-scale field phytoremediation projects.

Cadmium concentrations in leaves of each clone were similarly low after the 48 hours treatment (Fig. 19C). Cadmium contamination did not influence drastically the foliage of the three poplar clones. In fact, the leaves of each clone did not show any stress signs (chlorotic areas and necrotic spots) after the cadmium short-term treatment, nor was the leaf number influenced by the treatment. Thus, cadmium did not affect immediately the apical meristems and the growth of young leaves (Fig. 16). On the contrary, the short-term cadmium treatment inhibited the increase of total leaf area of *Poli* and *58-861* clones, suggesting severe impacts on their photosynthetic capacity (Fig. 17).

This observation was confirmed by results of proteomic analysis on the two clones: cadmium induced a decrease in the concentration of proteins involved in photosynthesis process (*Phosphoenolpyruvate carboxylases*, *Cytochromes c6*, *Photosystem I reaction center subunits*) (Tab. 4; Fig. 46). Oppositely, the increase of total leaf area of A4A clones was not affected by the short-term cadmium treatment (Fig. 17). Moreover, in this clone, the short-term cadmium treatment induced an increase in the concentration of proteins involved in the photosynthesis pathway (*phosphoenolpyruvate carboxylases*, *Light-independent protochlorophyllide reductases*, *Photosystem reaction center subunits*) (Tab. 5; Fig 46).

The clones *Poli* and 58-861 showed also a dramatic reduction in the increase of total leaf area (Fig. 17), caused by long-term cadmium treatment, and the clone A4A showed less inhibition than the other clones under the long term treatment. According to the data acquired with the proteomic analysis of *Poli* and 58-861 clones, cadmium continued to induce a decrease in the concentration of proteins involved in photosynthesis process (*Light-independent protochlorophyllide reductases*, *Photosystem I reaction center subunits*) during the long term treatment (Tab. 6A; Fig 47). In clone A4A, cadmium did not induce any changes in the concentration of proteins involved in photosynthesis process. This was also confirmed by the presence of many stress signs (chlorotic areas and necrotic spots) on leaves surface of *Poli* and 58-861 clones, after cadmium long-term treatment (Fig. 13, 14).

The clones A4A and *Poli* resulted “good translocator”, having a huge concentration of cadmium in roots (>1500mg/kg) associated with a moderate concentration in aerial parts, greater than cadmium concentration in 58-861 aerial part (Fig.20). But, while the clone A4A concentrated more cadmium in the stems compared to the clone *Poli*, after both short and long-term treatments, *Poli* concentrated more cadmium in leaves. This could be a cause or a consequence for which the clone A4A has a widen and more lignified stem, and the clone *Poli* showed more symptoms of metal stress on the leaves surface, after 14 days of treatment (Fig. 14).

In conclusion, *P.Nigra* clones 58-861, can be considered a “root accumulator”, because reacted to cadmium contamination limiting the translocation in the aerial parts.

P.Nigra clones *Poli*, although, was not a “root accumulator” limiting the radical uptake, but could be considered a “good translocator” because most of the contaminant was translocated in leaves.

The hybrid A4A, colud be considered a moderate “root accumulator” and a “good translocator”, because most of the contaminant is translocated in stems.

In conclusion, this work showed that a significant variability for a high translocation of absorbed Cd to woody parts existed among the considered poplar clones as well as a certain variability for reduced accumulation both in roots and in leaves. The strategy to select the plant using these

phenotypic traits might be useful for phytoremediation purposes and allow to find even more suitable plant material if large local collections were to be explored.

Furthermore, this work was undertaken to explore the potential of proteomics to investigate the response induced in the three different poplar clones by Cd treatments under uniform environmental conditions using hydroponic cultures. The proteomic profile obtained by the not treated and treated samples were compared using a two dimensional liquid chromatography technique (Pirondini *et al.*, 2006) to understand the molecular basis underlying the different responses.

Thus, the growth analysis, evidenced that the clone *A4A* was less influenced by cadmium short-term treatment than the other two clones. This was also confirmed by the proteomic analysis. In fact, the clone *A4A* showed less proteins differentially abundant in the short-term treated plant (39) than other two clones, *Poli* (168) and 58-861 (175) (Fig. 43). Cadmium contamination quickly affected clones *Poli* and 58-861, inducing a short-term response and a major change in the protein pool. In each clone, short-term cadmium treatment induced, in the treated plants, an increase in the concentration of proteins related to plant defense (*pathogenesis-related proteins, disease resistance proteins, protein phosphatases*) and stress response (*heat shock proteins*), and also an increase in the concentration of proteins related to the oxidative stress response (*peroxidases, peroxiredoxins, thioredoxins*) (Tab. 4-5; Fig 46, 48A). The short-term cadmium treatment induced, in the treated plants of each clone, a downfall of the carbohydrate metabolism with a decrease in the concentration of proteins involved in this pathway (*ribulose bisphosphate carboxylase subunits, alpha-1,4glucan phosphorylases, beta-fructofuranosidases*) (Tab. 4-5; Fig 46, 48A). But, the clone *A4A* displayed a short-term response to cadmium by inducing an increase in photosynthetic capacity in order to compensate the downfall of the carbohydrate metabolism. Thus in this clone, short-term cadmium treatment induced an increase in the concentration of proteins involved in photosynthesis pathway (*phosphoenolpyruvate carboxylases, Light-independent protochlorophyllide reductases, Photosystem reaction center subunits*) (Tab. 5; Fig 46, 48B). Furthermore short-term cadmium treatment induced, in the treated *A4A* plants, an increase in the concentration of proteins involved in aminoacids biosynthesis (*Glutamate and cysteine synthases, Histidinol dehydrogenase*) (Tab. 5; Fig 46, 48B) in order to produce more free aminoacids to chelate the metal ions because metals complexed with amino acids are considerably more stable and less toxic (Homer *et al.*, 1997). Both these findings may explain why the growth parameters for the clone *A4A* were not affected by the short-term cadmium treatment (Fig. 16-17).

On the contrary, the short-term cadmium treatment induced, in the treated plants of *Poli* and 58-861 clones, in addition to the downfall of the carbohydrate metabolism even the breakdown of

photosynthetic and glycolytic capacity. Infact, cadmium induced a decrease in the concentration of proteins involved in these two pathway (*Phosphoenolpyruvate carboxylases*, *Cytochromes c6*, *Photosystem I reaction center subunits* and *enolases*) (Tab. 4; Fig 46, 48A). This downfall of all the energy pathways could explain the strong negative impact of short-term cadmium treatment on the total leaf area of these two clones (Fig.17) and the subsequent appearing of stress signs on leaves surface (chlorotic areas and small necrotic spots). However, an attempt to counteract cadmium contamination could be observed since there has been an increase in the concentration of proteins involved in lignin and isoprenoid biosynthesis (*Pentatricopeptide repeat-containing protein*) (Fig. 46). This experimental data was in accordance with the data from SEM analysis (2.3.5, Scanning Electron Microscopy with microanalysis) in which the results showed that Cd was present mainly within lignified and suberified tissues. Considering the short term experiment, we may speculate about the fact that Cd induced the synthesis of lignin or the presence of already lignifies tissues acted as a sink for the contaminant.

From proteomic analysis it was possible to understand that clone *A4A* is more affected by cadmium long-term treatment than the other two clones. In fact, the clone *A4A* showed more proteins differentially abundant in the long-term treated plants (116) than other two clones, *Poli* (32) and 58-861 (38) (Fig. 44). Thus, after an initial strong impact of the presence of cadmium, *Poli* and 58-861 clones appeared to undergo an fast adaptation. On the contrary, *A4A* clone seemed react with a delayed response to the cadmium contamination. In each clone, after the long-term treatment, the contamination continued to cause an increase in the concentration of proteins related to plant defense (*defensin-like proteins*, *UDP-glycosyltransferases*) and stress response (*heat shock proteins*, *heat stress transcription factors*), and also an increase in the concentration of proteins related to the oxidative stress response (*peroxidases*, *peroxiredoxins*, *monothiol glutaredoxins*) (Tab. 6a-7; Fig 47, 48C, 48D). The long-term cadmium treatment induced, in the treated plants of *Poli* and 58-861 clones, a downfall of the carbohydrate metabolism and photosynthesis, but the impact of cadmium treatment on protein abundance from photosynthesis, calvin cycle and carbohydrate metabolism became less pronounced with increasing esposure time. Infact, cadmium induced a decrease in the concentration of proteins involved in these two pathways (*Ribulose bisphosphate carboxylase/oxygenase activases*, *Ribulose bisphosphate carboxylase subunits*, *Light-independent protochlorophyllide reductases*, *Photosystem I reaction center subunits*) (Tab 6a; Fig 47, 48C). Consequently, after the initial “alarm” phase and stabilization of the short-time stress response, an important increase in abundance of proteins related to the glycolysis and the TCA cycle indicated that cadmium-exposed plants increased the energy supply by activating mitochondrial respiration. Infact, in the clones *Poli* and 58-861 clone, cadmium induced an increase

in the concentration of proteins involved in glycolysis (*enolases*, *Sucrose-phosphatases*, *Glucose-1-phosphate adenylyltransferase subunits*) (Tab. 6a; Fig 47, 48C).

Overall these studies underlined the importance to study plants genome and proteome in relation to possible solution of pollution problems. In particular the proteomic data obtained in the different poplar clones, evidenced a correlation between uptake, translocation and tolerance mechanisms toward cadmium involving different classes of proteins. Further analyses on specific proteins might provide an inside view of plant response to heavy metals in order to develop possible phytotechnologies applications for heavy metal decontamination.

There still are some aspects of these experiment to be considered having room for improvement and follow up. For example hydroponic screenings bear inherent limitations to reflect real-world conditions, the most tolerant clones identified here will be tested for metal tolerance and accumulation when grown in soils.

These fundamental aspects should form the principles to be followed in plant screening for selecting plant material for phytoremediation, and the data obtained could be useful for improving Cd phytoremediation strategies through the application of this ecologically and economically important tree species such as poplars.

Furthermore, the results of this study demonstrated the importance of applying multiple methodologies for a fuller understanding of both physiologic and cellular response to heavy-metal exposure in plants. Only through synergic studies it is possible to gain the insight needed to make predictive statements regarding the effect of metal toxicity on different plants, and more specifically on different poplar clones, as in our case. This integrated study using a global proteomic approach to study stress response in cadmium poplar plants, together with physiological measurements and biochemical analyses, leads to a better understanding of some of the molecular mechanisms at the basis of cadmium stress response, such as the long and the short term physiological and proteomic reaction to cadmium.

2.5 References

- Adriano D.C.**, (2001) Trace Elements in Terrestrial Environments. Biogeochemistry, Bioavailability, and Risks of Metals. *Springer-Verlag, New York*. 867 pp.
- Alcantara, E., Romera, F.J., Canete, M., De La Guardia, M.D.**, (1994) Effects of heavy metals on both induction and function of root Fe(III)reductase in Fe-deficient cucumber (*Cucumis sativus L.*) plants. *Journal of Experimental Botany* 45, 1893–1898.
- Aina R., Asero R., Ghiani1 A., Marconi G., Albertini E., Citterio S.** (2010) Exposure to cadmium-contaminated soils increases allergenicity of *Poa annua* L. pollen. *Allergy* 65 1313–1321.
- Aina R., Labra M., Fumagalli P., Vannini C.** (2007) Thiolpeptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots. *Environmental and Experimental Botany* 59, 381–392.
- Aina, R.; Labra, M.; Fumagalli, P.; Vannini, C.; Marsoni, M.; Cucchi,U.; Bracale, M.; Sgorbati, S.; Citterio, S.** (2007) Thiol-peptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots. *Environmental and Experimental Botany* 59 (3), 381–392.
- Aravind P, Prasad MNV.** (2005) Modulation of cadmium-induced oxidative stress in *Ceratophyllum demersum* by zinc involves ascorbate–glutathione cycle and glutathione metabolism. *Plant Physiology and Biochemistry* 43:107–16.
- Arduini Iduna, Godbold Douglas L, Onnis Antonino** (2006) Cadmium and copper change root growth and morphology of *Pinus pinea* and *Pinus pinaster* seedlings. *Physiologia Plantarum Volume 92, Issue 4, pages 675–680.*
- Astolfi S, Zuchi S, Passera C** (2004) Role of sulphur availability on cadmium-induced changes of nitrogen and sulphur metabolism in maize (*Zea mays L.*) leaves. *Journal of Plant Physiology* 161, 795–802.
- Balsberg Pahlsson AM** (1989) Toxicity of heavy metals (Zn, Cu, Cd,Pb) to vascular plants. *Water Air Soil Pollution* 47, 287–319.
- Baniwal Sanjeev Kumar, Bharti Kapil, Chan Kwan Yu, Fauth Markus, Ganguli Arnab, Kotak Sachin, Mishra Shravan Kumar, Nover Lutz, Port Markus, Scharf Klaus-Dieter, Trip Joanna, Weber Christian, Zielinski Dirk, Von Koskull-Doring Pascall** (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. *Journal of Biosciences* 29(4), December 2004, 471–487.
- Barceló , J., Poschenrieder, C., Andreu, I., Gunsé, B.,** (1986) Cadmium-induced decrease of water stress resistance in bush bean plants (*Phaseolus vulgaris L. cv. Contender*). I. Effects of Cd on

water potential, relative water content and cell wall elasticity. *Journal of Plant Physiology* 125, 17–25.

Barhoumi Z., Djebali W. (2007) Salt impact on photosynthesis and leaf ultrastructure of Aeluropus littoralis. *Journal of Plant Research* 120: 529-537, 2007.

Baron-Ayala, M., Arellano, J.B., Lopez, G.J. (1995) Copper and Photosystem II: A controversial relationship. *Physiologia Plantarum* 94, 174-180.

Baszynski, T., Tukendorf, A., Ruszkowska, M., Skorzynska, E., Maksymiec, W., (1988) Characteristic of the photosynthetic apparatus of copper non-tolerant spinach exposed to excess copper. *Journal of Plant Physiology* 132, 708-713.

Bauer Jorg, Chen Kunhua, Hiltbunner Andreas, Wehrli Ernst, Eugster Monika, Schnell Danny, Kessler Felix (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* 403, 203-207, 2000

Benavides MP, Gallego SM, Tomaro ML (2005) Cadmium toxicity in plants. *Braz. Journal of Plant Physiology* 17: 21-34.

Bestwick CS, Brown IR, Mansfield JW (1998) Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a non-host hypersensitive reaction in lettuce. *Plant Physiology* 118, 1067–1078.

Bhalerao R, Nilsson O, Sandberg G. (2003) Out of the woods: forest biotechnology enters the genomic era. *Current Opinion in Biotechnology* 14: 206–213.

Bhatia NP, Walsh KB, Baker AJM. (2005) Detection and quantification of ligands involved in nickel detoxification in the herbaceous Ni hyperaccumulator Stackhousia tryonii Bailey. *Journal of Experimental Botany* 56, 1343–1349.

Blechert S., Brodschelm W., Holder S., Kammerer L., Kutchan T., Mueller M., Xia Z-Q, Zenk M. (1995) The octadecenoic pathway: signal molecule for regulation of secondary pathways. *PNAS USA* 92:4099–4105.

Bohler, S.; Bagard, M.; Oufir, M.; Planchon, S.; Hoffmann, L.; Jolivet, Y.; Hausman, J.-F.; Dizengremel, P.; Renaut, J. (2007) A DIGE analysis of developing poplar leaves subjected to ozone reveals major changes in carbon metabolism. *Proteomics* 2007, 7 (10), 1584–1599.

Bohn H.L., McNeal B.L., O'Conner G.A., (1985) Soil Chemistry. *Wiley Interscience*, Wiley, NY.

Booth, B., (2005) The added danger of counterfeit cigarettes. *Environmental Science and Technology* 39, 34A.

Boussama N, Ouariti O, Suzuki A, Ghorbel MH (1999) Cd-stress on nitrogen assimilation. *Journal of Plant Physiology* 155, 310–317.

Braatne, J.H., Rood, S.B., Heilman, P.E., In: Stettler RF, Bradshaw HD, Jr., Heilman PE, Hinckley TM, (1996) Biology of Populus and its implications for management and conservation, Ottawa: NRC Research Press, 1996, p 57–85.

Bradshaw H. D., Jr., Ceulemans Reinhart, Davis John, Stettler Reinhard (2000) Emerging Model Systems in Plant Biology: Poplar (*Populus*) as a Model Forest Tree. *Journal of Plant Growth Regulation* 19:306–313

Broadhurst C.L., Chaney R.L., Angle J.S., Maugel T.K., Erbe E.F., Murphy C.A. (2004) Simultaneous hyperaccumulation of nickel, manganese, and calcium in *Alyssum* leaf trichomes, *Environmental Science & Technology* 38 5797–5802.

Buchanan BB, Gruisse W, Jones RL (2000) Biochemistry and Molecular Biology of Plants. *American Society of Plant Physiologists, Rockville, Maryland.*

Buchner Peter, Borisjuk Ljudmilla, Wobus Ulrich (1995) Glucan phosphorylases in *Vicia faba* L.: cloning, structural analysis and expression patterns of cytosolic and plastidic forms in relation to starch. *Planta* (1996) 199:64–73

Cabello-Hurtado F., Ramos, J. (2004) Isolation and functional analysis of the glycerol permease activity of two new nodulin-like intrinsic proteins from salt stressed roots of the halophyte *Atriplex nummularia*. *Plant Science* 166, 633–640.

Cançado G. M. A., Nogueira F. T. S., Camargo S. R., Drummond R. D., Jorge R. A., Menossi M. (2008) Gene expression profiling in maize roots under aluminum stress. *Biologia Plantarum* 52 (3): 475–485.

Canovas F.M., Dumas-Gaudot E., Recorbet G., Jorrin J., Mock H.P., Rossignol M. (2004) Plant proteome analysis. *Proteomics* 4: 285 – 298.

Cassab Gladys I. (1998) Plant cell wall proteins. *Annual Review of Plant Biology and Plant Molecular Biology* 49:281–309

Castiglione S., Todeschini V., Franchin C., Torrigiani P., Gastaldi D., Cicatelli A., Rinaudo C., Berta G., Biondi S., Lingua G. (2009). 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. *Environmental Pollution* 157: 2108–2117.

Cervera M. T., Storme V., Soto A., Ivens B., Van Montagu M., Rajora O. P., Boerjan W. (2005) Intraspecific and interspecific genetic and phylogenetic relationships in the genus *Populus* based on AFLP markers. *Theoretical and Applied Genetics* 111: 1440–1456.

Chaffei C., Pageau K., Suzuki A., Gouia H., Ghorbel MH., Masclaux-Daubresse C. (2004) Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. *Plant Cell Physiolg* 2004;45:1681–93.

Chandler PM., Robertson M. (1994) Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annual Review of Plant Biology and Plant Molecular Biology* 45: 113–141 (1994).

Chaoui Abdelilah, El Ferjani Ezzedine (2005) Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum L.*) seedlings. *C. R. Biologies* 328 (2005) 23–31.

Chen S., Harmon A.C. (2006) Advances in plant proteomics. *Proteomics* 20: 5504 – 5516.

Cherian GM, Chan HM (1993) Biological functions of metallothioneins: a review. In: *Suzuki KT, Imura N, Kimura M, eds. Metallothionein III. Birkhauser, Basel.* pp. 87–109.

Chevalier F., Martin O., Rofidal V., Devauchelle A.D., Barreau S., Sommerer N., Rossignol M. (2004) Proteomic investigation of natural variation between *Arabidopsis* ecotypes. *Proteomics* 4: 1372 - 1381

Chien HF., Wang JW., Lin CC., Kao CH. (2001) Cadmium toxicity of rice leaves is mediated through lipid peroxidation. *Plant Growth Regulation* 33:205–13.

Choi Doml, Bostock Ruchard M., Avdiushko Sergei, Hildebrand David F. (1994) Lipid-derived signals that discriminate wound- and pathogenresponsive isoprenoid pathways in plants:- Methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum L.* *Proc. Natl. Acad. Sci. USA Vol. 91, pp. 2329-2333, March 1994 Plant Biology.*

Choi Won-Gyu, Roberts Daniel M. (2007) *Arabidopsis NIP2;1*, a Major Intrinsic Protein Transporter of Lactic Acid Induced by Anoxic Stress. *Journal of Biological Chemistry* 282, 33, 24209–24218.

Clemens S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* 2006;88:1707–19.

Clemens S. (2006) Evolution and function of phytochelatin synthases. *Journal of Plant Physiology.* 163, 319–332.

Clijsters, H., Van Assche, F. (1985) Inhibition of photosynthesis by heavy metals. *Photosynthesis Research* 7, 32-40.

Cobbett CS. (2000) Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology* 123, 825–833.

Cobbett CS, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology and Plant Molecular Biology* 53, 159–182.

Cobbett C.S., May M.J., Howden R., Rolls B. (1998). The glutathione-deficient, cadmium-sensitive mutant, cad2-1, of *Arabidopsis thaliana* is deficient in g-glutamylcysteine synthetase. *Plant Journal* 16, 73–78.

Cocozza C., Maiuro L., Tognetti R. (2011) Mapping Cadmium distribution in roots of Salicaceae through scanning electron microscopy with x-ray microanalysis. *iForest* (2011) 4: 113-120.

Cocozza C., Minnoci A., Tognetti R., Iori V., Zacchini M., Scarascia Mugnozza G. (2008) Distribution and concentration of cadmium in root tissue of *Populus alba* determined by scanning electron microscopy and energy-dispersive x-ray microanalysis. *iForest* (2008) 1: 96-103.

Compton Steve J., G. Jones Clive (1985) Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry Volume 151, Issue 2, December, Pages 369-374*

Connolly EL, Fett JP, Guerinot ML (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell* 14:1347–57.

Cordero B., Lodeiro P., Herrero R., Esteban Sastre de Vicente M., (2004) Biosorption of cadmium by *Fucus spiralis*. *Environmental Chemistry* 1, 180–187.

Corton JM, Gillespie JG, Hardie DG (1994) Role of the AMPactivated protein kinase in the cellular stress response. *Current Biology* 4: 315–324.

Cosgrove, D. J. (1997) Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. *Plant Cell*, 9, 1031–1041.

Cosio Claudia, Vollenweider Pierre, Keller Catherine (2006) Localization and effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.) I. Macrolocalization and phytotoxic effects of cadmium. *Environmental and Experimental Botany* 58 (2006) 64–74.

Crist Rayh, Martin J. Robert, Crist Delanson R. (2002) Heavy Metal Uptake by Lignin: Comparison of Biotic Ligand Models with an Ion-Exchange Process. *Environmental Science & Technology* 36, 1485-1490.

Cunha, K. P. V.; Nascimento, C. W. A.; Mendonça Pimentel, R. M.; Ferreira, C. P. (2008) Cellular localization of cadmium and structural changes in maize plants grown on a cadmium contaminated soil with and without liming. *Journal of Hazardous Materials* 160 (1), 228–234.

Dai Yun, Grant Steven (2003) Cyclin-dependent kinase inhibitors. *Current Opinion in Pharmacology Volume 3, Issue 4, August 2003, Pages 362-370.*

DalCorso Giovanni, Farinati Silvia, Maistri Silvia, Furini Antonella (2008) How Plants Cope with Cadmium: Staking All on Metabolism and Gene Expression. *Journal of Integrative Plant Biology, 50 (10): 1268–1280*

Damerval C., De Vienne D., Zivy M., Thiellement H. (1986) Technical improvement in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. *Electrophoresis 1986, 7, 52–54.*

Das P., Samantaray S., Rout G. R. (1997) Studies on cadmium toxicity in plants: a review. *Environmental Pollution, Vol. 98, No. 1, pp. 29-36.*

Dat JF, Vandenabeele S, Vranová E, Van Montagu M, Inzé D, Van Breusegem F (2000) Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences 57, 779–795.*

Davis JM, Gordon MP, Smit BA (1991). Assimilate movement dictates remote sites of wound-induced gene-expression in poplar leaves. *PNAS USA 88, 2393–2396.*

Deng H, Ye ZH, Wong MH (2004). Accumulation of lead, zinc, copper and cadmium by 12 wetland plant species thriving in metal-contaminated sites in China. *Environmental Pollution 132: 29-40.*

De Holff Peter L., Brill Laurence M., Hirsch Ann M. (2009) Plant lectins: the ties that bind in root symbiosis and plant defense. *Molecular Genetics & Genomics 282:1–15*

De Knecht JA, Van Dillen M, Koevoets PLM, Schat H, Verkleij JAC, Ernst WHO. (1994) Phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*. *Plant Physiology 104, 255–261.*

Delaney Terrence P., Uknes Scott, Vernooij Bernard, Friedrich Leslie, Weymann Kris, Negrotto David, Gaffney Thomas, Gut-Rella Manuela, Kessmann Helmut, Ward Eric, Ryals John (1994) A Central Role of Salicylic Acid in Plant Disease Resistance. *Science Vol. 266 no. 5188 pp. 1247-125.*

Delhaize EP, Ryan R (1995) Aluminium toxicity and tolerance in plants. *Plant Physiology 107, 315–321.*

Dickmann, Isebrands, Eckenwalder, Richardson (2001) Poplar culture in North America. *NRC-CNRC.*

Dominguez-Solis JR, Lopez-Martin MC, Ager MC, Ynsa MD, Romero LC, Gotor C. (2004) Increased cysteine availability is essential for cadmium tolerance and accumulation in *Arabidopsis thaliana*. *Plant Biotechnology Journal* 2, 469–476.

Dong J, Wu F, Zhang G (2006) Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). *Chemosphere* 64, 1659–1666.

Dos Santos Utmaian, M. N.; Wieshammer, G.; Vega, R.; Wenzel, W. W. (2007) Hydroponic screening for metal resistance and accumulation of cadmium and zinc in twenty clones of willows and poplars. *Environmental Pollution* 148 (1), 155–165.

Drescher Anja, Ruf Stephanie, Calsa Jr Tercilio, Carrer Helaine, Bock Ralph (2000) The two largest chloroplast genome-encoded open reading frames of higher plants are essential genes. *The Plant Journal Volume 22, Issue 2, pages 97–104.*

Dudkaa S., Millera W. P. (2008) Accumulation of potentially toxic elements in plants and their transfer to human food chain. *Journal of Environmental Science and Health, Volume 34, Issue 4*

Durand Thomas C., Sergeant Kjell, Planchon Sebastien, Carpin Sabine, Label Philippe, Morabito Domenico, Hausman Jean-Francois, Renaut Jenny (2010) Acute metal stress in *Populus tremula* X *P. alba* (717-1B4 genotype): Leaf and cambial proteome changes induced by cadmium²⁺. *Proteomics* 2010, 10, 349–368.

Eckert M, Biela A, Siefritz F, Kaldenhoff R (1999). New aspects of plant aquaporin regulation and specificity. *Journal of Experiment Botany* 50: 1541-1545.

Eleftheriou E.P., Tsekos I. (1991) Fluoride effects on leaf cell ultrastructure of olive trees growing in the vicinity of the aluminium factory of Greece. *Trees* 5: 83-89, 1991.

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Signal molecules in systemic plant resistance to pathogens and pests. *Cell* 70:879–886.

Essader A.S., Cargile B.J., Bundy J.L., Stepheson J.L. Jr, (2005) A comparison of immobilized pH gradient isoelectric focusing and strong-cation-exchange chromatography as a first dimension in shotgun proteomics. *Proteomics* 5 (2005) 24-34.

Evans PT, Malmberg RL. (1989) Do polyamines have a role in plant development? *Annual Reviews of Plant Physiology and Plant Molecular Biology* 40, 235–269.

Fagioni M., D'Amici G. M., Timperio A. M., Zolla L. (2009) Proteomic analysis of multiprotein complexes in the thylakoid membrane upon cadmium treatment. *Journal of Plant Protection Research* 8, 310–326.

Fischer PM, Endicott J, Meijer L. (2003) Cyclin-dependent kinase inhibitors. *Progress in Cell Cycle Research* 5:235-48.

French, C. J.; Dickinson, N. M.; Putwain, P. D. (2006) Woody biomass phytoremediation of contaminated brownfield land. *Environmental Pollution* 141, 387–395.

Gang Yu, Masaki Nishimura, Shigeki Arawaka, Diane Levitan, Lili Zhang, Anurag Tandon, You-Qiang Song, Ekaterina Rogova, Fusheng Chen, Toshitaka Kawarai, Agnes Supala, Lyne Levesque, Haung Yu, Dun-Sheng Yang, Erin Holmes, Paul Milman, Yan Liang, Dong Mei Zhang, Dong Hong Xu, Christine Sato, Evgeny Rogoza, Marsha Smith, Christopher Janus, Yanni Zhang, Ruedi Aebersold, Lindsay Farrerk, Sandro Sorbi, Amalia Bruni, Paul Fraser, Peter St George-Hyslop (2000) Nicastatin modulates presenilin-mediated notch/glp-1 signal transduction and β APP processing. *Nature*, Vol. 40

Gazaryan I.G., Lagrimini L.M., Ashby A.G., Thorneley N.F. (1996) Mechanism of indole-3-acetic acid oxidation by plant peroxidases: anaerobic stopped-flow spectrophotometric studies on horseradish and tobacco peroxidases, *Biochemical Journal* 313, 841–847.

Ghelis T, Dellis O, Jeannette E, Bardat F, Miginiac E, Sott B (2000) Abscisic acid plasmalemma perception triggers a calcium influx essential for RAB18 gene expression in *Arabidopsis thaliana* suspension cells. *FEBS Letters* 483, 67–70.

Georg Raphaela C., Gomes Suely L. (2007) Transcriptome Analysis in Response to Heat Shock and Cadmium in the Aquatic Fungus *Blastocladiella emersonii*. *Eukaryotic Cell*, J, p. 1053–1062.

Goldberg R., Imbert A., Liberman M., Prat R. (1986) Relationships between peroxidasic activities and cell wall plasticity, in: *H. Greppin, C. Penel, T. Gaspar (Eds.), Molecular and Physiological Aspects of Plant Peroxidases, University of Geneva Press, Geneva, 1986*, pp. 209–220.

Gora L, Clijsters H (1989) Effect of copper and zinc on the ethylene metabolism in *Phaseolus vulgaris* L. In: Clijsters H (eds) Biochemical and physiological aspects of ethylene production in lower and higher plants, Kluwer, Dordrecht, pp 219–228.

Gora L, Clijsters H (1989) Effect of copper and zinc on the ethylene metabolism in *Phaseolus vulgaris* L. In: *Clijsters H (eds) Biochemical and physiological aspects of ethylene production in lower and higher plants, Kluwer, Dordrecht*, pp 219–228.

Gravot Antoine, Lieutaud Aurelie, Verret Frederic, Auroy Pascaline, Vavasseur Alain, Richaud Pierre (2004) AtHMA3, a plant P1B-ATPase, functions as a Cd/Pb transporter in yeast. *FEBS Letters* 561 (2004) 22-28.

Granel T., Robinson B., Mills T., Clothier B., Green S., Fung L., (2002) Cadmium accumulation by willow clones used for soil conservation, stock fodder, and phytoremediation. *Australian Journal of Soil Research* 40, 1331–1337.

Gupta, A. K.; Kaur, N. (2005) Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *Journal of Biosciences* 30 (5), 761–776.

Guy Charles, Kaplana Fatma, Kopkac Joachim, Selbigc Joachim, Hinchac Dirk K. (2008) Metabolomics of temperature stress. *Physiologia Plantarum* 132: 220–235.

Hajduch, M.; Rakwal, R.; Agrawal, G. K.; Yonekura, M.; Petrova, A. (2001) High-resolution two-dimensional electrophoresis separation of proteins from metal-stressed rice (*Oryza sativa*) leaves: Drastic reductions/ fragmentation of ribulose-1,5-bisphosphate carboxylase/ oxygenase and induction of stress-related proteins. *Electrophoresis*, 22 (13), 2824–2831.

Hall JL., Ang W., Vinocur B., Shoseyov O., Altman A. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science* 9: 244-52.

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* 53, 1–11.

Harmens H, Den Hartog PR, Ten Bookum WM, Verkleij JAC. (1993) Increased zinc tolerance in *Silene vulgaris* (Moench.) Garcke is not due to increased production of phytochelatins. *Plant Physiology* 103, 1305–1309.

Hartley-Whitaker J, Ainsworth G, Vooijs R, Ten Bookum W, Schat H, Meharg AA. (2001) Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiology* 126, 299–306.

Hasenstein H.K., Evens M.L., Stinemetz C.L., Moore R., Fondren W.M., Koon E.C., Higby M.A., Smucker A.J.M, (1988) Comparative effectiveness of metal ions in inducing curvature of primary roots of *Zea mays*. *Plant Physiology* 86, 885–889.

Haynes RJ (1980) Ion exchange properties of roots and ionic interactions within the root apoplasm: their role in ion accumulation by plants. *Botanical Review* 46: 75-99.

Heazlewood J.L., Millar A.H. (2003) Integrated plant proteomics - putting the green genomes to work. *Functional Plant Biology* 30: 471 - 482

Hendry, G.A.F., Baker, A.J.M., Ewart, C.F., (1992) Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus*. *Acta Botanica Neerlandica* 41, 271–281.

Hermans Christian, Chen Jiugeng, Coppens Frederik, Inze Dirk, Verbruggen Nathalie (2011) Low magnesium status in plants enhances tolerance to cadmium exposure. *New Phytologist* 192: 428–436.

Hernandez, L.E., Carpeta-Ruiz, R., Garate, A., (1996) Alterations in the mineral nutrition of pea seedlings exposed to cadmium. *Journal of Plant Nutrition* 19, 1581–1598.

Herrero E., de la Torre-Ruiz M. A. (2007) Monothiol glutaredoxins: a common domain for multiple functions. *Cellular and Molecular Life Sciences* 64, 1518 – 1530.

Hidalgo HA, Koppa V, Bryan SE (1976) Effect of cadmium on RNA-polymerase and protein synthesis in rat liver. *FEBS Letters Apr 15;64(1):159-62.*

Hinkle PM, Shanshala ED, Nelson EJ (1992). Measurement of intracellular cadmium with fluorescent dyes. Further evidence for the role of calcium channels in cadmium uptake. *Journal of Biological Chemistry* 267: 25553-25557.

Homer FA, Reeves RD, Brooks RR. (1997) The possible involvement of amino acids in nickel chelation in some nickelaccumulating plants. *Current Topics in Phytochemistry* 14, 31–33.

Hogue, C., (2004) Metallic refinement. EPA is working to ensure that the unique properties of metals are part of risk assessments. *Chemical and Engineering News* 82 (32), 23–24.

Isaure Marie-Pierre, Fayard Barbara, Sarret Géraldine, Pairis Sébastien, Bourguignon Jacques (2006) Localization and chemical forms of cadmium in plant samples by combining analytical electron microscopy and X-ray spectromicroscopy. *Spectrochimica Acta Part B* 61 1242–1252

Jones, R., Lapp, T., Wallace, D., (1993) Locating and Estimating Air Emissions from Sources of Cadmium and Cadmium Compounds. *Prepared by Midwest Research Institute for the U.S. Environmental Protection Agency, Office of Air and Radiation, Report EPA-453/R- 93-040, September 1993.*

Jouve L., Hoffmann L., Hausman J.-F. (2004) Polyamine, carbohydrate, and proline content changes during salt stress exposure of aspen (*Populus tremula L.*): involvement of oxidation and osmoregulation metabolism. *Plant Biology* 6, 74–80.

Juang R.H., McCue K.F., Ow D.W. (1993) Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate in nitro. *Archives of Biochemistry and Biophysics* 304, 392-401.

Jung-II Cho, Sang-Kyu Lee, Seho Ko, He-Kyung Kim, Sung-Hoon Jun, Youn-Hyung Lee, Seong Hee Bhoo, Kwang-Woong Lee, Gynheung An, Tae-Ryong Hahn, Jong-Seong Jeon

(1995) Molecular cloning and expression analysis of the cell-wall invertase gene family in rice (*Oryza sativa L.*). *Plant Cell Reports* 24: 225–236

Kabata-Pendius, A., Pendius, H., (1992) Trace Elements in Soils and Plants. *CRC Press Inc., Boca Raton, Florida.*

Kakkar RK, Sawhney VP. (2002) Polyamine research in plants: a changing perspective. *Physiologia Plantarum* 116, 281–292.

Kamnev A.A., Van der Lelie Daniel, (2000). Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. *Bioscience Report* 20 (4), 239.

Kang Xuezen, Frey Douglas D., (2003) High-performance cation-exchange chromatofocusing of proteins. *Journal of Chromatography A* 991, 117-228.

Kerkeb L, Kramer U. (2003) The role of free histidine in xylem loading of nickel in *Alyssum lesiacum* and *Brassica juncea*. *Plant Physiology* 131, 716–724.

Kessler, A., Brand, M.D., (1995) The mechanism of the stimulation of state 4 respiration by cadmium in potato tuber (*Solanum tuberosum*) mitochondria. *Plant Physiology and Biochemistry* 33, 519–528.

Khan DH, Duckett JG, Frankland B, Kirkham JB (1984). An X-ray microanalytical study of the distribution of cadmium in roots of *Zea mays L.* *Journal of Plant Physiology* 115: 19-28.

Kieffer Pol, Dommes Jacques, Hoffmann Lucien, Hausman Jean-Francois, Renaut Jenny (2008) Quantitative changes in protein expression of cadmium-exposed poplar plants. *Proteomics* 8, 2514–2530.

Kieffer Pol, Planchon Sebastien, Oufir Mouhssin, Ziebel Johanna, Dommes Jacques, Hoffmann Lucien, Hausman Jean-Francois, Renaut Jenny (2008) Combining Proteomics and Metabolite Analyses To Unravel Cadmium Stress-Response in Poplar Leaves. *Journal of Proteome Research* 8, 400–417.

Kieffer Pol, Schröder Peter, Dommes Jacques, Hoffmann Lucien, Renaut Jenny, Hausman Jean-François (2009) Proteomic and enzymatic response of poplar to cadmium stress. *Journal of Proteomics* 72, 379-396.

Kim Yu-Young, Choi1 Hyunju, Segami Shoji, Cho Hyung-Taeg, Martinoia Enrico, Maeshima Masayoshi, Lee Youngsook (2009) AtHMA1 contributes to the detoxification of excess Zn(II) in Arabidopsis. *The Plant Journal* 58, 737–753.

Klebl F., Wolf M., Sauer N. (2003) A defect in the yeast plasma membrane urea transporter Dur3p is complemented by CpNIP1, a Nod26-like protein from zucchini (*Cucurbita pepo* L.), and by *Arabidopsis thaliana* delta-TIP or gamma-TIP. *FEBS Letters* 547, 69–74

Kramer U., Cotter-Howells J.D., Charnock J.M., Baker A.J.M., Smith J.A.C. (1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature* 379: 635 – 638

Krupa, Z., Baranowska, M., Orzol, D., (1996) Can anthocyanins be considered as heavy metal stress indicator in higher plants? *Acta Physiologia Plantarum* 18, 147-151.

Krupa, Z., Baszynski, T., (1995) Some aspects of heavy metal toxicity towards photosynthetic apparatus-direct and indirect effects on light and dark reactions. *Acta Physiologia Plantarum* 17, 177-190.

Krupa, Z., Oquist, G., Huner, N.P.A., (1992) The influence of cadmium on primary photosystem-II photochemistry in bean as revealed by chlorophyll a fluorescence – A preliminary study. *Acta Physiologia Plantarum* 14, 71-76.

Krupa, Z., Oquist, G., Huner, N.P.A., (1993) The effects of cadmium on photosynthesis of *Phaseolus vulgaris* - a fluorescence analysis. *Physiologia Plantarum* 88, 626-630.

Kruzmane D, Jankevica L, Ievinsh G (2002) Effect of regurgitant from *Leptinotarsa decemlineata* on wound responses in *Solanum tuberosum* and *Phaseolus vulgaris*. *Physiologia Plantarum* 115:577–584.

Kuang Hanhui, Wei Fusheng, Marano Maria Rosa, Wirtz Uwe, Wang Xiaoxue, Liu Jia, Shum Wai Pun, Zaborsky Jennifer, Tallon Luke J., Rensink Willem, Lobst Stacey, Zhang Peifen, Tornqvist Carl-Erik, Tek Ahmet, Bamberg John, Helgeson John, Fry William, You Frank, Luo Ming-Cheng, Jiang Jiming, Buell C. Robin, Baker Barbara (2005) The R1 resistance gene cluster contains three groups of independently evolving, type I R1 homologues and shows substantial structural variation among haplotypes of *Solanum demissum*. *The Plant Journal* 44, 37–51

Kupper H., Lombi E., Zhao F.J., McGrath S.P. (2000) Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* 212 75–84.

Lamb Rebecca S., Citarelli Matteo, Teotia Sachin (2011) Functions of the poly(ADP-ribose) polymerase superfamily in plants. *Cellular and Molecular Life Sciences* 69, 2, 175-189.

Laureysens, I.; Blust, R.; De Temmerman, L.; Lemmens, C.; Ceulemans, R. (2004) Clonal variation in heavy metal accumulation and biomass production in poplar coppice culture: I. Seasonal variation in leaf, wood and bark concentrations. *Environmental Pollution*, 131 (3), 485–494.

Laureysens, I.; De Temmerman, L.; Hastir, T.; Van Gysel, M.; Ceulemans, R. (2005) Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture. II. Vertical distribution and phytoextraction potential. *Environmental Pollution*, 133 (3), 541–551.

Lin R, Wang X, Luo Y, Du W, Guo H, Yin D (2007) Effects of soil cadmium on growth, oxidative stress and antioxidant system in wheat seedlings (*Triticum aestivum L.*). *Chemosphere* 69, 89–98.

Ling Huang Jin, Ling Sun Gui, Da-Ming Zhang (2010) Molecular evolution and phylogeny of the angiosperm ycf2 gene. *Journal of Systematics and Evolution* 48, 4, 240–248.

Lyubenova L, Götz C, Golan-Goldhirsh A, Schröder P. (2007) Direct effect of Cd on glutathione S-transferase and glutathione reductase from *Calystegia sepium*. *International Journal of Phytoremediation* 9:465–73.

Lozano-Rodríguez E, Hernandez LE, Bonay P, Carpeta-Ruiz RO (1997). Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. *Journal of Experimental Botany* 48: 123-128.

Lugon-Moulin N., Zhang M., Gadani F., Rossi L., Koller D., Krauss M., Wagner G.J., (2004) Critical review of the science and options for reducing cadmium in tobacco (*Nicotiana tabacum L.*) and other plants. *Advances in Agronomy* 83, 111–180.

Lunackova L., Masarovicova E., Kralova K., Stresko V. (2003) Response of Fast Growing Woody Plants from Family Salicaceae to Cadmium Treatment. *Bulletin of Environmental Contamination and Toxicology* 70:576–585

Ma J. F., Tamai K., Yamaji N., Mitani N., Konishi S., Katsuhara M., Ishiguro M., Murata Y., Yano M. (2006) A silicon transporter in rice. *Nature* 440, 688–691

MacRobbie EA, Kurup S (2007) Signalling mechanisms in the regulation of vacuolar ion release in guard cells. *New Phytologist* 175, 630–640.

Maestri Elena, Marmiroli Marta, Vissioli Giovanna, Marmiroli Nelson (2010) Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment. *Environmental and Experimental Botany* 68, 1–13

Maestri Elena, Marmiroli Nelson (2011) Transgenic Plants for Phytoremediation. *International Journal of Phytoremediation Volume 13, Supplement 1, 2011 Special Issue: 6th International Phytotechnologies Conference St. Louis, MO, 2009*

Maksymiec W. (2007) Signaling responses in plants to heavy metal stress. *Acta Physiologiae Plantarum* 29, 177–187.

Maksymiec W, Baszyński T (1996) Chlorophyll fluorescence in primary leaves of excess Cu-treated runner bean plants depends on their growth stages and the duration of Cuaction. *Journal of Plant Physiology* 149:196–200.

Marmiroli M., Gonnelli C., Maestri E., Gabbrielli R., Marmiroli N., (2004) Localisation of nickel and mineral nutrients Ca, K, Fe,Mg with scanning electron microscopy microanalysis in tissues of the nickel-hyperaccumulator *Alyssum bertolonii* Desv. and the non-accumulator *Alyssum montanum* L. *Plant Biosystems*, 138, 231–243.

Marmiroli Marta, Pietrini Fabrizio, Maestri Elena, Zacchini Massimo, Marmiroli Nelson, Massacci Angelo (2011) Growth, physiological and molecular traits in Salicaceae trees investigated for phytoremediation of heavy metals and organics. *Tree Physiology*.

Marmiroli Marta, Visioli Giovanna, Maestri Elena, Marmiroli Nelson (2011) Correlating SNP Genotype with the Phenotypic Response to Exposure to Cadmium in *Populus spp.* *Environmental Science & Technology* 45, 4497–4505.

Martinoia Enrico, Klein Markus, Geisler Markus, Bovet Lucien, Forestier Cyrille, Kolikisaoglu Uner, Muller-Rober Bernd, Schulz Burkhard (2002) Multifunctionality of plant ABC transporters – more than just detoxifiers. *Planta* 214: 345-355.

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, SzeH, et al. (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiology* 126:1646–67.

Masclaux C, Valadier M, Brugière N, Morot-Gaudry J, Hirel B (2000) Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. *Planta* 211, 510–518.

Matsubayashi Yoshikatsu, Ogawa Mari, Kihara Hitomi, Niwa Masaaki, Sakagami Youji (2006) Disruption and Overexpression of Arabidopsis Phytosulfokine Receptor Gene Affects Cellular Longevity and Potential for Growth. *Plant Physiology* 142, 45–53.

Mauch F, Dudler R. (1993) Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiology* 102:1193–201.

Maurel Christophe, Santoni Véronique, Luu Doan-Trung, Wudick Michael M, Verdoucq Lionel (2009) The cellular dynamics of plant aquaporin expression and functions. *Current Opinion in Plant Biology* 12, 6, 690-698.

May MJ, Vernoux T, Leaver C, Van Montagu M, Inze D. (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. *Journal of Experimental Botany*; 49:649–67

Meharg, A.A. (1994) Integrated tolerance mechanisms: Constitutive and adaptive plants responses to elevated metal concentrations in the environment. *Plant, Cell & Environment* 17, 989-993.

Mendel Ralf R. (1997) Molybdenum cofactor of higher plants: biosynthesis and molecular biology. *Planta* 203: 399±405.

Molas, J. (1997) Changes in morphological and anatomical structure of cabbage (*Brassica oleracea* L) outer leaves and in ultrastructure of their chloroplasts caused by an in vitro excess of nickel. *Photosynthetica* 34: 513-522.

Moore F, Weekes J, Hardie DG (1991) Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. *European Journal of Biochemistry* 199: 691–697.

Moustacas Anne-Marie, Nari Joannes, Borel Maguy, Noat Georges, Ricard Jacques (1991) Pectin methylesterase, metal ions and plant cell-wall extension. The role of metal ions in plant cell-wall extension. *Biochemical Journal* 279, 351-354.

Nagao R.T., Czarnecka E., Gurley B., Schöffl F., Key J.L. (1984) Genes for low-molecular-weight heat shock proteins of soybeans: sequence analysis of a multigene family. *Molecular and Cellular Biology*, 5, 12 3417-3428.

Nussbaum S, Schmutz D, Brunold C (1988) Regulation of assimilatory sulfate reduction by cadmium in *Zea mays* L. *Plant Physiology* 88, 1407–1410.

Oberlander, R., Roth, V., (1978) In: Ross, Sheila M. (Ed.), Toxic Metals in Soil–Plant Systems. *John Wiley and Sons, New York*.

Osuji GO, Madu WC (1996) Ammonium ion salvage by glutamate dehydrogenase during defense response in maize. *Phytochemistry* 42, 1491–1498.

Page A.L., Bingham F.T., Chang A.C., (1982) Cadmium. In: Lepp, N.W. (Ed.), Effect of Trace Metals on Plant Function. In Effect of Heavy Metal Pollution on Plants, vol. 1. *Applied Science Publishers, Englewood, NJ*, pp. 77–109.

Pandolfini T., Gabbrielli R., Comparini C. (1992) Nickel toxicity and peroxidase activity in seedlings of *Triticum aestivum* L. *Plant, Cell & Environment* 15, 719–725.

Pearce DA, Sherman F. (1999) Toxicity of copper, cobalt, and nickel salts is dependent on histidine metabolism in the yeast *Saccharomyces cerevisiae*. *Journal of Bacteriology* 181, 4774–4779.

Pell EJ, Schlagnahaufer CD, Artega RN (1997) Ozone-induced oxidative stress: mechanisms of action and reaction. *Plant Physiology* 100:264–273.

Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C. (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *The Plant Journal* 32:539–48.

Persans MW, Yan X, Patnoe JM, Kramer U, Salt DE. (1999) Molecular dissection of the role of histidine in Ni hyperaccumulation in *Thlaspi goesingense*. *Plant Physiology* 121, 1117–1126.

Pierron Fabien, Baudrimont Magalie, Bossy Angelique, Bourdineaud Jean-Paul, Brethes Daniel, Elie Pierre, Massabuau Jean-Charles (2007) Impairment of lipid storage by cadmium in the European eel (*Anguilla anguilla*). *Aquatic Toxicology* 81, 304–311

Pietrini Fabrizio, Zacchini Massimo, Iori Valentina, Pietrosanti Lucia, Bianconi Daniele, Massacci Angelo (2010) Screening of Poplar Clones for Cadmium Phytoremediation Using Photosynthesis, Biomass and Cadmium Content Analyses. *International Journal of Phytoremediation*, 12:105–120.

Pirondini Andrea, Visioli Giovanna, Malcevschi Aliosha, Marmiroli Nelson (2006) A 2-D liquid-phase chromatography for proteomic analysis in plant tissues. *Journal of Chromatography B*, 833, 91–100

Piskacek Martin, Zotova Ludmila, Zsurka Gábor, Schweyen Rudolf J. (2008) Conditional knockdown of hMRS2 results in loss of mitochondrial Mg⁺ uptake and cell death. *Trends in Plant Science* 9: 244-52.

Prasad, M. N. V. (1995) Cadmium toxicity and tolerance in vascular plants. *Environmental and Experimental Botany* 35 (4), 525–545.

Pulford, I. D.; Watson, C. (2003) Phytoremediation of heavy metalcontaminated land by trees review. *Environment International* 29, 529–540.

Quiroga, M.; Guerrero, C.; Botella, M. A.; Barcelo, A.; Amaya, I.; Medina, M. I.; Alonso, F. J. S.; de Forchetti, M.; Tigier, H.; Valpuesta, V. (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. *Plant Physiology* 122, 1119–1127.

Rajora OP, Zsuffa L (1984) Interspecific crossability and its relation to the taxonomy of the genus *Populus* L. In: Proceedings of the joint meeting of the working parties S2-02-10 poplar provenances and S2-03-07 breeding poplar, XVII session of the international poplar commission, October 1–4, 1984. *National Research Council, Ottawa*, pp 33–45

Ramos I., Esteban E., Lucena J.J., Gárate A., (2002) Cadmium uptake and subcellular distribution in plants of *Lactuca* sp. Cd–Mn interaction. *Plant Science* 162, 761–767.

Rauser WE. (1995) Phytochelatins and related peptides. Structure, biosynthesis, and function. *Plant Physiology* 109, 1141–1149.

Reisner A.H., Nemes Patty, Bucholtz Carolyn (1975) The use of Coomassie Brilliant Blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Analytical Biochemistry* 64, 2, 509-516.

Renaut, J.; Hausman, J. F.; Wisniewski, M. E. (2006) Proteomics and low temperature studies: bridging the gap between gene expression and metabolism. *Plant Physiology* 126 (1), 97–109.

Robinson Elizabeth A., Henriksen Ole, Maxwell Elizabeth S. (1974) Elongation Factor 2: Aminoacid sequence at the site of adenosine diphosphate ribosylation. *Journal of Biological Chemistry*, 249, 5088-5093.

Roberts J.K. (2002) Proteomics and a future generation of plant molecular biologists. *Plant Molecular Biology* 48: 143 – 154.

Robinson, B. H.; Mills, T. M.; Petit, D.; Fung, L. E.; Green, S. R.; Clothier, B. E. (2000) Natural and induced cadmium-accumulation in poplar and willow: Implications for phytoremediation. *Plant Soil* 227 (1-2), 301–306.

Ro Dae-Kyun, Bohlmann Jorg (2006) Diterpene resin acid biosynthesis in loblolly pine (*Pinus taeda*): functional characterization of abietadiene/levopimaradiene synthase (PtTPS-LAS) cDNA and subcellular targeting of PtTPS-LAS and abietadienol/abietadienal oxidase (PtAO, CYP720B1) *Phytochemistry* 67, 1572–1578.

Rodriguez Pedro L. (1998) Protein phosphatase 2C (PP2C) function in higher plants. *Plant Molecular Biology* 38: 919–927.

Rollin X., Medale F., Gutieres S., Blanc D., Kaushik S.J., (2003) Shortand long-term nutritional modulation of acetyl-CoA carboxylase activity in selected tissues of rainbow trout (*Oncorhynchus mykiss*). *British Journal of Nutrition* 89, 803–811.

Romero-Puertas, M. C.; Rodriguez-Serrano, M.; Corpas, F. J.; Gomez, M.; Del Rio, L. A.; Sandalio, L. M. (2004) Cadmium-induced subcellular accumulation of O₂•- and H₂O₂ in pea leaves. *Plant, Cell & Environment* 27 (9), 1122–1134.

Rose J. K.C., Bashir S., Giovannoni J. J., Jahn M.M., Subramanian Saravanan R., (2004) TECHNIQUES FOR MOLECULAR ANALYSIS Tackling the plant proteome: practical approaches, hurdles and experimental tools. *The Plant Journal* 39:715–733.

Ross Joe, Li Yi, Lim Eng-Kiat, Bowles Dianna J. (2001) Higher plant glycosyltransferases. *Genome Biology*, 2(2):reviews3004.1–3004.6.

Rossignol M. (2001) Analysis of the plant proteome. *Current Opinion in Biotechnology* 12: 131 – 134.

Rossignol M., Peltier J.B., Mock H.P., Matros A., Maldonado A.M., Jorrín J.V. (2006) Plant proteome analysis: a 2004–2006 update. *Proteomics* 20:5529 – 5548.

Roth, U.; von Roepenack-Lahaye, E.; Clemens, S. (2006) Proteome changes in *Arabidopsis thaliana* roots upon exposure to Cd²⁺. *Journal of Experimental Botany* 57 (15), 4003–4013.

Rubinelli Peter, Siripornadulsil Surasak, Gao-Rubinelli Fei, T. Sayre Richard (2002) Cadmium-and iron-stress-inducible gene expression in the green alga *Chlamydomonas reinhardtii*: evidence for H43 protein function in iron assimilation. *Planta* 215:1–13.

Rustichelli C. (2008) Green biotechnologies: from genomic to proteomic approaches. *PhD Thesis, University of Parma, Italy*

Salin, M.L., (1988) Toxic oxygen species and protective systems of the chloroplasts. *Physiologia Plantarum* 72, 681–689.

Salt DE, Prince RC, Baker AJM, Raskin I, Pickering IJ. (1999) Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environmental Science and Technology* 33, 713–717.

Salt D.E., Prince R.C., Pickering I.J., Raskin I. (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiology* 109 1427–1433.

Salt, D. E.; Smith, R. D.; Raskin, I. Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology* 49 (1), 643–668.

Sandmann G, Boger P (1980) Copper-mediated lipid peroxidation processes in photosynthetic membranes. *Plant Physiology* 66:797–800.

Saniewski M, Ueda J, Miyamoto K, Urbanek H (2003) Interaction between ethylene and other plant hormones in regulation of plant growth and development in natural conditions and under abiotic and biotic stresses. In: *Vendrell M, Klee H, Pech JC, Romojaro F (eds) Biology and Biotechnology of the Plant Hormone Ethylene III*, IOS Press, pp 263–270.

Sanità di Toppi, L., Lambardi, M., Pazzagli, L., Cappugi, G., Durante, M., Gabbrielli, R., (1998) Response to cadmium in carrot in vitro plants and cell suspension cultures. *Plant Science* 137, 119–129.

Sanità di Toppi L, Gabbrielli R. (1999) Response to cadmium in higher plants. *Environmental and Experimental Botany* 41:105–30.

Sarret G., Saumitou-Laprade P., Bert V., Proux O., Hazemann J.L., Traverse A., Marcus M.A.M., Manceau A., (2002) Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* 130 1815–1826

Sarry, J.-E.; Kuhn, L.; Ducruix, C.; Lafaye, A.; Junot, C.; Hugouvieux, V.; Jourdain, A.; Bastien, O.; Fievet, J. B.; Vailhen, D.; Amekraz, B.; Moulin, C.; Ezan, E.; Garin, J.; Bourguignon, J. (2006) The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. *Proteomics* 6 (7), 2180–2198.

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J, Bleeker PM (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany* 53, 2381–2392.

Schat Henk., Sharma Shanti S, Vooijs Riet (1997) Heavy metal-induced accumulation of free proline in a metal-tolerant and a nontolerant ecotype of *Silene vulgaris*. *Physiologia Plantarum* 101, 3, 477–482.

Schmoger ME, Oven M, Grill E. (2000) Detoxification of arsenic by phytochelatins in plants. *Plant Physiology* 122, 793–801.

Schützendübel A., Polle A. (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* 53, 1351–1365.

Schützendübel A., Schwanz P., Teichmann T., Gross K., Langenfeld-Heyser R., Godbold O.L., Polle A. (2001) Cadmium induced changes in antioxidative systems, H₂O₂ content and differentiation in pine (*Pinus sylvestris*) roots. *Plant Physiology* 127, 887–892.

Shanti S. Sharma and Karl-Josef Dietz (2006) The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany* 57, 4, 711–726.

Sharma Y, Leòn J, Raskin I, Davis KR (1996) Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defence-related transcripts and induced resistance. *PNAS* 93, 5099–5104.

Shen ZG, Zhao FJ, McGrath SP (1997). Uptake and transport of zinc in the hyperaccumulator *Thlaspi caerulescens* and the non hyperaccumulator *Thlaspi ochroleucum*. *Plant, Cell & Environment* 20: 898-906.

Shinobu Takada, Ken-ichiro Hibara, Tetsuya Ishida, Masao Tasaka (2001) The cup-shaped cotyledon1 gene of *Arabidopsis* regulates shoot apical meristem formation. *Development* 128, 1127-1135.

Skorzynska, E., Urbanik-Sypniewska, T., Russa, R., Baszynski, T., (1991) Galactolipase activity of chloroplasts in cadmium treated runner bean plants. *Journal of Plant Physiology* 138, 453-459.

Slocum RD, Flores HE. (1991) Biochemistry and physiology of polyamines in plants. Boca Raton: CRC Press.

Soldi M., Sarto C., Valsecchi C., Magni F., Proserpio V., Ticozzi D., Mocarelli P. (2005) Proteome profile of human urine with two-dimensional liquid phase fractionation. *Proteomics* 5 2641-2647

Somashekaraiah, B.V., Padmaja, K., Prasad, A.R.K., (1992) Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*): involvement of lipid peroxides in chlorophyll degradation. *Physiologia Plantarum* 85, 85–89.

Song Junqi, Bradeen James M., Naess S. Kristine, Raasch John A., Wielgus Susan M., Haberlach Geraldine T., Liu Jia, Kuang Hanhui, Austin-Phillips Sandra, Buell C. Robin, Helgeson John P., Jiang Jiming (2003) Gene RB cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight. *PNAS* 100, 108 (46).

Soukup A., Armstrong W., Schreiber L., Franke R., Votruba O. (2007) Apoplastic barriers to radial oxygen loss and solute penetration: a chemical and functional comparison of the exodermis of two wetland species, *Phragmites australis* and *Glyceria maxima*. *New Phytologist* 173: 264-278.

Speiser D M, Ortiz D. F., Kreppel L., Scheel G, McDonald G, Ow D. W. (1992) Purine Biosynthetic Genes Are Required for Cadmium Tolerance in *Schizosaccharomyces pombe*. *Molecular and cellular biology* 5301-5310.

Stepanova AN, Ecker JR (2000) Ethylene signaling: from mutants to molecules. *Current Opinion in Plant Biology* 3:353–360.

Stephens, W.E., Calder, A., (2005) Source and health implications of high toxic metal concentrations in illicit tobacco products. *Environmental Science and Technology* 39, 479–488.

Stiborova, M., Doubravova, M., Brezinova, A., Friedrich, A., (1986) Effect of heavy metal ions on growth and biochemical characteristics of photosynthesis of barley (*Hordeum vulgare L.*). *Photosynthetica* 20, 418-425.

Stoyanova D.P., Tchakalova, F.S. (1997) Cadmium-induced ultrastructural changes in chloroplasts of the leaves and stems parenchyma in *Myrophyllum spicatum* L. *Photosynthetica* 34: 241-248.

Taji, T.; Ohsumi, C.; Iuchi, S.; Seki, M.; Kasuga, M.; Kobayashi, M.; Yamaguchi-Shinozaki, K.; Shinozaki, K. (2002) Important roles of droughtand cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *The Plant Journal* 29 (4), 417–426.

Takano J., Wada M., Ludewig U., Schaaf G., von Wieren N., Fujiwara T. (2006) The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *Plant Cell* 18, 1498–1509

Thordal-Christensen H, Zang Z, Wei Y, Collinge DB (1997) Subcellular localization of H₂O₂ accumulation in papillae and hypersensitive response during the barley powdery mildew interaction. *The Plant Journal* 11, 1187–1194.

Tognolli Michael, Penel Claude, Greppin Hubert, Simon Patrice (2002) Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. *Gene* 288, 129–138

Unterbrunner, R.; Puschenreiter, M.; Sommer, P.; Wieshammer, G.; Tlustos, P.; Zupan, M.; Wenzel, W. W. (2007) Heavy metal accumulation in trees growing on contaminated sites in Central Europe. *Environmental Pollution* 148 (1), 107–114.

Van deMortel J. E., AlmarVillanueva L., Schat H., Kwekkeboom J., Coughlan S., Moerland P. D., Ver Loren van Themaat E., Koornneef M., Aarts M.G. M. (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* 142, 1127–1147.

Vassiliev A, Lidon F, Da Graca M, Yordanov I (2004) Cadmium-induced changes in chloroplast lipids and photosystem activities in barley plants. *Plant Biology* 48:153–156.

Vassilev, A., Tsonev, T., Yordanov, I. (1998) Physiological response of barley plants (*Hordeum vulgare*) to cadmium contamination in soil during ontogenesis. *Environmental Pollution* 103, 287–293.

Verma, K.; Shekhawat, G. S.; Sharma, A.; Mehta, S. K.; Sharma, V. (2008) Cadmium induced oxidative stress and changes in soluble and ionically bound cell wall peroxidase activities in roots of seedling and 3-4 leaf stage plants of *Brassica juncea* (L.) Czern. *Plant Cell Reports* 27, 1261–1269.

Vernoux Teva, Wilson Robert C., Seeley Kevin A., Reichheld Jean-Philippe, Muroy Sandra, Brown Spencer, Maughan Spencer C., Cobbett Christopher S., Van Montagu Marc, Inzé Dirk, May Mike J., Sung Zinmay R. (2000) The root meristemless1/cadmium sensitive2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. *The Plant Cell* 12, 97–109.

Visioli Giovanna, Marmiroli Marta, Marmiroli Nelson (2010) Two-Dimensional Liquid Chromatography Technique Coupled with Mass Spectrometry Analysis to Compare the Proteomic Response to Cadmium Stress in Plants. *Journal of Biomedicine and Biotechnology Volume 2010*.

Vogt T, Jones P (2000) Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. *Trends in Plant Science 5:380-386.*

Vollenweider P., Cosio C., Gunthardt-Georg M. S., Keller C. (2006) Localization and effects of cadmium in leaves of a cadmiumtolerant willow (*Salix viminalis* L.): Part II Microlocalization and cellular effects of cadmium. *Environmental and Experimental Botany 58 (1-3), 25–40.*

Wahid A., Ghani,A., Javed F. (2008) Effect of cadmium on photosynthesis, nutrition and growth of mungbean. *Agronomy for Sustainable Development 28, 273–280.*

Wakil, S.J., Stoops, J.K., Joshi, V.C., (1983) Fatty acids synthesis and its regulation. *Annual Review of Biochemistry 52, 537–579.*

Wallace, I. S., Roberts, D. M. (2005) Distinct transport selectivity of two structural subclasses of the nodulin-like intrinsic protein family of plant aquaglyceroporin channels. *Biochemistry 44, 16826–16834*

Wang W., Vinocur B., Shoseyov O., Altman A. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science 9: 244-52.*

Wójcik M, Vangronsveld J, D'Haen J, Tukiendorf A (2005) Cadmium tolerance in *Thlaspi caerulescens*. II. Localization of cadmium in *Thlaspi caerulescens*. *Environmental and Experimental Botany 53:163-171.*

Wullschleger Stan D., Jansson Stefan, Taylor Gail (2002) Genomics and Forest Biology:Populus Emerges as the Perennial Favorite. *The Plant Cell 14, 2651.*

Yamauchi M, Peng XX (1995) Iron toxicity and stress-induced ethylene production in rice leaves. *Plant Soil 173:21–28.*

Yeung A.T., Hsu C.N., (2005) Electrokinetic remediation of cadmiumcontaminated clay. *Journal of Environmental Engineering 131, 298–304.*

Zacchini Massimo, Pietrini Fabrizio, Scarascia Mugnozza Giuseppe, Iori Valentina, Pietrosanti Lucia, Massacci Angelo (2009) Metal Tolerance, Accumulation and Translocation in Poplar and Willow Clones Treated with Cadmium in Hydroponics. *Water, Air, & Soil Pollution 197:23–34*

Zarcinas, B.A., Ishak, C.F., McLaughlin, M.J., Cozens, G., (2004) Heavy metals in soils and crops in southeast Asia. 1. Peninsular Malaysia. *Environmental Geochemistry and Health* 26, 343–357.

Zhao Dazhong, Ni Weimin, Feng Baomin, Han Tianfu, G. Petrasek Megan, Ma Hong (2003) Members of the Arabidopsis-SKP1-like Gene Family Exhibit a Variety of Expression Patterns and May Play Diverse Roles in Arabidopsis. *Plant Physiology*, 133, 203–217.

Zhao F., Lombi E., Breedon T., McGrath S., (2000) Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. *Plant, Cell & Environment* 23 507–514.

Zhou J., Wan S.W., Li G., Qin P. (2011) Ultrastructure changes of seedlings of *Kosteletzkya virginica* under waterlogging conditions. *Biologia Plantarum* 55(3):493-498.

Zornoza P., Vázquez S., Esteban E., Fernández-Pascual M., Carpeta R., (2002) Cadmium-stress in nodulated white lupin: strategies to avoid toxicity. *Plant Physiology and Biochemistry* 40, 1003–1009.

3 ACKNOWLEDGEMENTS

This research was supported by funding to Prof. Nelson Marmiroli and Dr. Marta Marmiroli (Department of Environmental Sciences, Division of Genetics and Environmental Biotechnology, University of Parma) from PRIN “Identificazione di descrittori analitici e molecolari per la capacità di decontaminazione in pioppo”.

The author acknowledge the kind assistance of Dr. Marta Marmiroli (Department of Environmental Sciences, Division of Genetics and Environmental Biotechnology, University of Parma) for SEM/EDX analysis.

The author acknowledge the kind assistance of Dr. Aliosha Malcevschi (Department of Environmental Sciences, Division of Genetics and Environmental Biotechnology, University of Parma) for protein MS analysis and CIM (Interdepartment Measure Centre, Parma, Italy) for access to MALDI-TOF-MS facilities.

We are grateful to the Institute of Agro-environmental and Forset Biology, National Research Council (Monterotondo Scalo, Rome, Italy) for providing us with the original poplar cuttings.

4 LIST OF PAPERS

The scientific production carried out by Dr. Davide Imperiale in the last three years led to the publication of the following conference proceedings:

- COST Action FA0603 WG1 Meeting: Technical aspect inherent to plant proteomics.

“Classical and Novel Approaches in Plant Proteomics”

(Viterbo, Italy, 5/6 May 2009)

Molecular response of plants to heavy metals: a proteomic approach

Visioli G., Marmiroli M., Pirondini A., Imperiale D., Marmiroli N.

Department of Environmental Sciences, Division of Genetics and Environmental Biotechnologies, University of Parma,
Via G.P. Usberti 33/A, 43100 Parma, Italy

Objectives

Plants tolerate excess of heavy metals through mechanisms acting at two different levels: metal exclusion in the root or metal accumulation followed by compartmentalization or sequestration of metal ions, useful for phytoremediation. In particular some plant species growing on metal-rich soils can accumulate high quantity in leaves. Hyperaccumulating plants have been described for Ni, Cd, Zn, Se, As whereas accumulation capacity towards other metals have not been clearly demonstrated. However, the practical application of hyperaccumulators to phytoremediation is made difficult by their little biomass and slow growth rates. It is therefore necessary to understand the biological mechanisms at the basis of hyperaccumulation, and to analyze variability within and among species in order to apply in phytoremediation, plants considered to be more important from an economical point of view, and with higher biomass.

In this research we focused on two different plants species: *i*) the natural Ni hyperaccumulating *Thlaspi caerulescens* (J&C. Presl.) and *ii*) Poplar which is not defined as hyperaccumulator but it is capable to accumulate high amount of heavy metals in shoots. In particular we utilised a proteomic profiling approach to identify possible protein biomarkers in a *T. caerulescens* population subjected or not to Ni treatment and in a poplar clone subjected or not to Cd treatment.

Methods

Shoots crude protein extracts were analysed by using a 2D liquid chromatography technique. Complex protein chromatograms obtained from each protein sample were analysed with ProteoVue software (Eprogen, Darien, IL, USA). Proteins were separated in the first dimension by isoelectric point (pI) with an high-performance chromatofocusing, (HPCF). The proteins were eluted based on their pI, measured for absorbance at 280 nm, collected according to pre-determined pH decrements of 0.3 pH units. In the second dimension proteins were separated by hydrophobicity with an high-resolution, reversed-phase, chromatography (HPRP). Fractions were immediately collected for MS analysis.

Results

Among the 500 polypeptides detected in shoot tissues, few were found to change their abundance, in the two conditions analysed, in both the two species. In particular, in *T. caerulescens* only some proteins involved in transport and metal chelation resulted over-expressed in the treated samples. Interestingly in the condition of absence of Ni many proteins involved in sulphur metabolism, protection against reactive oxygen species or stress response were over-expressed. The results correlated to physiological data seemed to evidence a better physiological performance of this species in the condition of Ni treatment. In the poplar clone under Cd stress treatment, proteins implicated in protection against oxidative damage and in sulphur metabolism showed a slight increase. The possible role of some of the proteins identified in Cd accumulation and tolerance will be discussed.

- *XIX Congresso S.I.E. Società Italiana di Ecologia: Dalle vette alpine alle profondità marine
(Bolzano, Italy, 15/18 September 2009)*

Risposta genetica e fisiologica in diversi cloni di pioppo sotto stress da cadmio

Marmiroli M., Visioli G., Imperiale D., Maestri E., Marmiroli N.

Dip. Scienze Ambientali, Sezione Biotecnologie Genetica, Università degli Studi di Parma

In questo studio si sono analizzati 11 cloni di pioppo per determinare le variazioni di singolo nucleotide (SNPs) indotte a seguito di trattamento con Cd solfato, in condizioni idroponiche. Sette geni coinvolti nell'omeostasi del Cd (“geni candidati”) e altri due fino ad ora ritenuti non rilevanti per tale processo (geni “non-candidati”) sono stati analizzati rispetto alla omologa sequenza nel genoma di *P. trichocarpa* Torr. & A. Gray depositata nel sito web JGI. I polimorfismi sono stati tradotti in variazioni amminoacidiche per ogni gene e per ogni clone sul pool dei geni considerati. Tali dati hanno consentito di determinare la distanza genetica tra i vari cloni, l’analisi delle coordinate principali (PCA) ha consentito di determinare la percentuale di variabilità entro cloni spiegabile dai polimorfismi dei “geni candidati”. Si è inoltre prodotta una matrice di correlazione tra i dati genetici e quelli fisiologici relativi alla risposta al Cd per determinare quali geni, e in quali cloni, erano correlati a variazioni di biomassa, area fogliare, lunghezza radicale e concentrazione interna alla piana di Cd. Analisi al SEM/EDX su quattro cloni di particolare interesse hanno evidenziato zone di accumulo di Cd e di aumentata lignificazione e suberificazione possibilmente indotte dallo stress da Cd. Alcuni cloni hanno mostrato un alto livello di polimorfismi in tutti i “geni candidati” anche se la loro risposta al Cd era di tipo diverso, maggiore tolleranza alternata a maggiore accumulo di Cd.

- *ItPA 2010: 5th Annual National Conference (Firenze, Italy, 9/12 June 2010)*

2D-LC as a suitable technique for comparative proteomics of different plant species in response to metal contaminants

Giovanna Visioli, Davide Imperiale, Marta Marmiroli, Elena Maestri and Nelson Marmiroli

Division of Genetic and Environmental Biotechnologies, Department of Environmental Sciences, University of Parma, Parma, Italy

In many plant species subjected to metal stress a network of proteins and biochemical cascades that may lead to a controlled homeostasis of metals has been recently identified. There is no protein technology that can be as sensitive as transcripts microarray analyses, thus it is necessary to combine different protein separation techniques to enrich proteomic data. In this work we tested a 2D-LC system for its ability to identify proteins regulated by excess of metals in different plant species. The use of liquid protein mapping instead of gel mapping, to scan many samples for important or landmark proteins has some advantages: *i*) the possibility to load protein crude extracts, avoiding purification steps and limiting the risk to lose less abundant proteins in the sample; *ii*) the higher amount of proteins that is loaded in a single run (up to 10 mg of protein extract), could reveal also less abundant proteins with regulatory functions; *iii*) working with liquid protein fractions is more suitable for a direct identification by MS techniques; *iv*) the automation of the system allows for a reduction of contaminations and for a better reproducibility of the results. Moreover important steps to pay attention for in a 2D-LC analyses were: *i*) the freshness of the protein sample and *ii*) the reproducible pH gradients performed by the exchange buffers. Protein extracted from different plant species and tissues (*Arabidopsis thaliana*; *Thlaspi caerulescens*; lichens, poplar) treated or not with different metals and metalloids such as Cs, Ni, Cd were analysed by 2D-LC methodology and data obtained enriched the information present in literature on 2D-gels giving a more comprehensive analysis of the plants proteome in the tested conditions.

- 7th International Phytotechnologies Conference: Phytotechnologies in the 21st century: Challenges after Copenhagen 2009. Remediation – Energy – Health – Sustainability.
(Parma, Italy, 26/29 September 2010)

Correlation between habitat and molecular features in the Ni hyperaccumulator *Thlaspi caerulescens* Monte Prinzera population.

Giovanna Visioli, Davide Imperiale, Simone Vincenzi, Andrea Pirondini, Nelson Marmiroli

Department of Environmental Sciences, University of Parma, Italy

In the context of soil pollution, an important aspect is the treatment and management of sites contaminated by metals such as Ni, Cr, Co, Pb, Zn, Cd, Cu, Mn. In natural conditions many of these elements are present at high rates in ophiolitic soils which are widespread in the Italian territory. Thus the ophiolite territories represent natural sites in which heavy metals are concentrated and the vegetation growing in these sites, which are usually rare endemic plant species, have been forced to adapt to a series of ecological factors among which a peculiar physical-chemical composition of the soil (Baker et al., 2010; Maestri et al., 2010). Monte Prinzera (MP) is one of the ophiolitic Italian territories, situated in the Tosco-Emilian Appennin and it is a Natural Reserve. The physical-chemical characteristics of this site allowed for the selection of some plant species tightly linked to these substrates, with specific adaptive traits, such as tolerance and accumulation of metals. In particular a metallophilous population of *Thlaspi caerulescens* is found in this site and studied and classified as a Ni hyperaccumulator population. The absence of this plant nearby, in non-ophiolitic soils, and its growth and reproductive traits render *Thlaspi caerulescens* MP an interesting model to study the adaptation to extreme environments.

By studying *Thlaspi caerulescens* population of MP, the presence of different morpho-types were observed in sub-sites which showed differences in the geo-morphology. A multivariate statistical analysis performed on morphological traits such as *i*) number of leaves of the rosettes; *ii*) dimension of leaves of the rosettes; *iii*) height of stalk; *iv*) number of inflorescences; *v*) weight of seeds; *vi*) dimension of seeds evidenced the significance of this phenotypic variability. Environmental soil parameters such as *i*) pH; *ii*) soil organic content; *iii*) metal content such as Ni, Fe, Co, Zn, Mg; Mn, Ca, K; *iv*) water content were also analysed in correlation with plants' growth behaviour. Plant phenotypic variability was analysed also at molecular level by a comparative high-throughput analysis of protein variations. Differences in protein abundance were observed between plants in the different sub-sites evidencing a strong phenotypic plasticity of these plants in the adaptation to specific micro-environments. In conclusion, in this work, the combination of phenotypic and molecular descriptors showed to be a useful approach for a better characterization of the biodiversity in a species adapted to grow on metalliferous soils.

Key words: *Thlaspi caerulescens*; serpentine soil; phenotypic plasticity; proteomic markers

- 7th International Phytotechnologies Conference: Phytotechnologies in the 21st century: Challenges after Copenhagen 2009. Remediation – Energy – Health – Sustainability.
(Parma, Italy, 26/29 September 2010)

Proteomic Analysis in Poplar Clones Treated with Cadmium

Davide Imperiale*, Marta Marmiroli, Elena Maestri, Giovanna Visioli, Nelson Marmiroli

Department of Environmental Sciences, Division of Genetics and Environmental Biotechnologies, University of Parma, Italy

Dispersal of trace elements, metals and organics is relevant for environmental contamination and for fresh water utilization. Plant species have been used to decrease or remove the contaminants in polluted sites and in particular, tolerant and hyperaccumulator species seem promising for removal of metals in contaminated soils. The practical application of natural hyperaccumulators in phytoremediation is difficult because of their small biomass at maturity and reduced growth rates. For these reasons the metal uptake capacity of plants with higher biomass, such as trees belonging to Salicaceae family like poplar and willow, has been investigated. Laboratory and field trials have found that different clones of the genus *Populus* have different tolerance and metal uptake in contaminated environments.

In this work we studied the behavior of three Italian selected poplar clones: *P.nigra* (clones 58-861 and Poli) and a hybrid *P.nigra* x *deltoides* (A4A), which showed different Cd tolerance and capacity and Cd uptake, accumulation and translocation (1).

The modifications occurring in the proteome of the three clones subjected to Cd treatments were investigated by comparing also the physiological behaviour of the clones.

Rooted cuttings of three poplar clones were grown in hydroponic cultures with 0 µM CdSO₄ (control) and 20 µM CdSO₄ for 48h (short term treatment).

(i) Different physiological parameters were analyzed: total leaf area, stem growth and elongation of the roots. Metal uptake and root to shoot translocation were observed by mineral analysis by Atomic Absorption Spectroscopy (ASS). Metal compartmentalization in leaves, roots and stems were analyzed by Scanning Electron Microscopy with microanalysis (SEM/EDX).

(ii) Proteomic analysis was performed on crude protein extracts, obtained from leaves and roots. Proteins with different isoelectric point (pI) and hydrophobicity were separated by a 2D liquid chromatography technique (ProteomeLab PF2D, Beckman) (2).

Qualitative and quantitative differences between protein profiles of treated and untreated samples were evidenced by DeltaVue Software (Eprogen). Proteins differently expressed in various conditions will be further characterized by MALDI-TOF/MS to infer on their possible role in metal response.

The results will be discussed with the aim to understand the different processes at the basis of accumulation and tolerance of metal by plant species.

Key words: Poplar, cadmium, proteomics, phytoremediation, 2D-LC technique

- 2° Workshop C.I.M. Centro Interdipartimentale Misure: "Scienza al CIM: la parola ai giovani"
(Parma, Italy, 11 November 2011)

CHARACTERIZATION OF POPLAR DIFFERENTIAL EXPRESSED PROTEINS IN RESPONSE TO CADMIUM CONTAMINATION BY MEANS OF MALDI-TOF SPECTROMETRY

IMPERIALE D., MARMIROLI M., MALCEVSCHI A., MAESTRI E., VISIOLI G., MARMIROLI N.

Department of Environmental Sciences, Division of Genetics and Environmental Biotechnologies, University of Parma,
Viale Usberti 11/A, 43124 Parma (Italy)

Key words: Proteomics, MALDI-TOF, Poplar, cadmium.

In order to understand the different processes at the basis of accumulation and tolerance of metal in plants, rooted cuttings of three poplar clones were grown in hydroponics culture with 0 µM (control) and 20 µM CdSO₄ for 48h (short term treatment) and 14 days (long term treatment). Proteomic analysis has been performed on crude protein extracts, obtained from leaves. Proteins with different isoelectric point (pI) and hydrophobicity have been separated by a 2D liquid chromatography technique (ProteomeLab PF2D, Beckman), and an analysis using DeltaVue Software (Eprogen) has been carried out on qualitative/quantitative differentially expressed bands between treated and untreated samples. Proteins whose expression was statistically different in response to various experimental conditions are being currently identified by MALDI-TOF/MS to infer their possible role in the plant metabolism response to metals.